

**IMPROVEMENT IN VIABILITY OF *LACTOBACILLUS*
ACIDOPHILUS IN YOGURT BY STRESS ADAPTATION**

A Thesis Submitted
for the degree of

DOCTOR OF PHILOSOPHY

by

SANDRA MCKECHNIE

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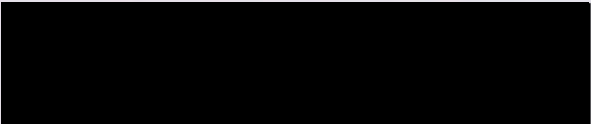
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DECLARATION

I, Sandra McKechnie, declare that the PhD thesis entitled 'Improvement in viability of *Lactobacillus acidophilus* in yogurt by stress adaptation' is no more than 100,000 words in length, exclusive of tables, figures, appendices, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work.



Sandra McKechnie

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SUMMARY

There have been many claims about the potential health benefits provided by the consumption of probiotic organisms such as *Lactobacillus acidophilus* including alleviation of symptoms of lactose intolerance, reduction of serum cholesterol levels and faecal enzyme activity, stimulation of the immune system and the control of gastrointestinal infections *via* pathogen interference. With the advent of widespread antibiotic resistance, scientists have focused on the potential of probiotic supplements as an alternative to classic antibiotic treatments for gastrointestinal infections.

Dairy products such as yogurt are consumed world-wide and are a popular vehicle for consumption of probiotic organisms. In order to maintain therapeutic value, probiotic organisms must remain viable during production and storage of yogurt and during passage through the gastrointestinal tract, in order to colonise the human colon. Recent studies have shown that the probiotic organism, *Lb. acidophilus*, loses viability in yogurt and some strains are sensitive to the acid and bile salts commonly encountered during transit through the gastrointestinal tract.

The main factor claimed to be responsible for the loss of viability of probiotic organisms during storage is the decrease in intracellular pH caused by accumulation of organic acids in the yogurt. This leads to inactivation of essential metabolic pathways, deprivation of ATP to the cell, and eventually, cell death. A strategy to improve the viability of *Lb. acidophilus* in yogurt is to adapt the organism to the harsh environments that normally cause loss in viability. This has been achieved by culturing cells in an environment that allows them to prepare for normally lethal conditions. Acquired acid tolerance was previously reported for *Lb. acidophilus* following cell conditioning at moderate pH followed by a challenge in normally lethal pH conditions. The primary objective of the work in this thesis was to design a protocol for preparing probiotic cultures for yogurt manufacture that would improve their viability during long-term refrigerated storage.

Candidate strains were selected using a polyphasic approach to characterize, differentiate and identify strains originally purchased as *Lb. acidophilus*. No single strain proved superior in terms of acid tolerance, bile tolerance and survival during six weeks of refrigerated storage in a milk-based medium. Carbohydrate fermentation analyses were useful for pre-identification of the strains used in this study, with strains being confirmed as members of the *Lb. acidophilus* complex of species. Following pulsed-field gel electrophoresis of *Sma*I digested genomic DNA, most strains were clustered into one group, sharing > 70% similarity with the *Lb. acidophilus* type strain VUP 12,006, whilst VUP 12,028 shared only 24% similarity with the type strain. The difference in DNA fingerprints was explained following sequence analysis of the V1 and V3 regions of 16S rRNA of the *Lb. acidophilus* complex. Whilst strain VUP 12,028 was re-classified as a member of the *Lactobacillus johnsonii* species, the remaining strains were confirmed as members of the *Lb. acidophilus* species. Antibiotic susceptibility tests provided differentiation of the *Lb. acidophilus* strains. *Lb. acidophilus* strain VUP 12,015 and *Lb. johnsonii* strain VUP 12,028 were selected for further studies based on their poor viability in a milk-based medium during long-term storage and their high survival rates at pH 2.5. For comparison, *Lb. acidophilus* strain VUP 12,005 was selected for its ability to maintain viability above the recommended level during long-term storage.

Lb. acidophilus strains VUP 12,005 and VUP 12,015 and *Lb. johnsonii* strain VUP 12,028 were grown to exponential phase in flasks containing MRS broth at optimal (pH 5.8, 39°C) conditions. The cells were then collected by centrifugation and resuspended into MRS broth previously adjusted with lactic acid to pH 5.8 (control), 4.5 or 4.0 and incubated at 39°C for one hour (conditioning phase). Following incubation at moderate pH, cells were resuspended in MRS broth (39°C) previously adjusted to pH 3.5 or pH 3.0 using lactic acid and incubated for five hours. Percentage survival was determined hourly by comparison of viable counts with the initial counts. In general, cells incubated at pH 5.8 (control) during the cell conditioning period were sensitive to pH challenge at 3.5 or 3.0, whilst cells that were conditioned in MRS broth at pH 4.5 or 4.0 demonstrated higher survival rates compared to the control. The level of acquired pH tolerance was dependent on the conditioning pH (pH 4.5 or 4.0) and time, the challenge pH (pH 3.5 or 3.0) and the strain being tested. The greatest improvement in survival was observed for *Lb.*

acidophilus strain VUP 12,015 after incubation at pH 3.5 for five hours following conditioning at pH 4.5, with 63% survival compared to 1.2% for cells incubated at pH 5.8 during the cell conditioning period. In contrast, cells of *Lb. johnsonii* strain VUP 12,028 conditioned at pH 4.5 for one hour showed the greatest adaptive ability during incubation at pH 3.0 for one hour, with 66% survival compared to 1.6% for cells incubated at pH 5.8 during the conditioning period. For *Lb. acidophilus* strain VUP 12,005, conditioning cells at pH 4.0 resulted in 44% survival after five hours at pH 3.5, compared to 0.09% survival for cells incubated at pH 5.8 during the conditioning period.

Strains are normally sensitive to 60°C, 10 mM hydrogen peroxide, 20% ethanol and 3M sodium chloride. However, incubation of exponential phase cells for one hour in MRS broth at pH 4.5 improved survival during these challenges. Inhibiting protein synthesis during cell conditioning at pH 4.5 reduced the survival rates during challenge. This suggests that *de novo* protein synthesis contributes to cross-resistance. These findings have relevance for cells in environments where they can be exposed to combinations of stress conditions, such as those found during the production and storage of yogurt.

Many papers have reported that when cells are placed under stress, there is an increase in the production of certain proteins that are produced at levels higher than observed in normal cells. Results from ³⁵S- methionine/cysteine radiolabelling experiments showed that as the adaptation pH decreased, so too did the growth rates of the organism and number of proteins produced during cell conditioning. Down-regulation of a 60 kDa protein was observed using 1-D SDS-PAGE analysis following conditioning at pH 4.5, 4.0 and 5.8 to 4.5 for strain VUP 12,005. In addition, the repression of 123 kDa and 40.5 kDa proteins was observed for strain VUP 12,015 following conditioning at pH 4.5. Further analysis is required to determine the role, if any, of these proteins in the acid tolerance response.

Long-term survival of cells conditioned in MRS broth at moderate pH, followed by yogurt simulation trials or small-scale yogurt production was strain dependent. Conditioning exponential phase cells using lactic acid or cell filtrates enhanced survival of strains VUP 12,015 and VUP 12,028, but not strain VUP 12,005 during

long-term refrigerated storage following simulation of yogurt production. The greatest improvement in survival was observed by conditioning strain VUP 12,015 in MRS broth containing 100% cell filtrate, with 14% survival following simulated yogurt production and seven weeks of refrigerated storage compared to 0.4% survival for cells that were incubated at pH 5.8 during the conditioning period. Survival was also enhanced for strains VUP 12,015 and VUP 12,028 during refrigerated storage in yogurt produced with the starter cultures *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*, following conditioning at pH 4.5. However, these improvements were only observed during the first three weeks of refrigerated storage.

The stress resistance properties of *Lb. acidophilus* and *Lb. johnsonii* discovered now could form the basis for the design of alternative procedures for the efficient and low cost maintenance, production, storage and application of probiotic yogurts. An understanding of the underlying molecular mechanisms may provide targets for specific manipulation to promote growth and survival of probiotic organisms in adverse conditions. Improving the viability of probiotic organisms in yogurt increases the chance of establishment in the intestinal tract, and consequently provides increased benefits to the consumer.

PUBLICATIONS

Conference Abstracts:

McKechnie, S., Shah, N., and Britz, M.L. (1997). A comparison of techniques for the identification of nine strains of *Lactobacillus acidophilus*. Proceedings of the 30th National Convention of the Australian Institute of Food Science and Technology, Perth, Western Australia.

McKechnie, S., Britz, M., and Shah, N. (1998). Strategies to improve the viability of *Lactobacillus acidophilus* in probiotic yogurts: responses to heat and acid stress. Proceedings of the American Dairy Science Association Conference, Denver, Colorado.

McKechnie, S., Britz, M., and Shah, N. (1998). Improving the viability of *Lactobacillus acidophilus* in yogurt by stress adaptation. Proceedings of the 25th International Dairy Federation Congress, Aarhus, Denmark.

McKechnie, S., Britz, M., and Shah, N. (1999). Improving the viability of *Lactobacillus acidophilus* in probiotic yogurts by stress adaptation. World Congress, Australian Institute of Food Science and Technology, Sydney, Australia.

McKechnie, S, Shah, N.P., and Britz, M.L. (2000). Characterisation and differentiation of *Lactobacillus acidophilus* strains for use as probiotics. Proceedings of the American Dairy Science Association Conference, USA.

LIST OF ABBREVIATIONS

A ₆₀₀	Absorbance at 600 nm
ACH	Acid casein hydrolysate
ANGIS	Australian National Genomic Information Service
ATP	Adenosine tri-phosphate
ATR	Acid tolerance response
BLIS	Bacteriocin-like inhibitory substances
bp	Base pairs
BSA	Bovine serum albumin
cfu	Colony forming units
cm	Centimetre
CSCC	CSIRO culture collection
CSIRO	Commonwealth Scientific and Industrial Research Organisation
D	Day
°C	Degrees Celsius
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dpm	Disintegrations per minute
EDTA	Ethylene Diamine Tetra Acetic acid
g	Gram
GC	Gas chromatography
h	Hour
HPLC	High performance liquid chromatography
IDF	International Dairy Federation
IFN	Interferon

IgA	Immunoglobulin class A
IL	Interleukin
kb	Kilobases
kDa	KiloDalton
l	Litre
M	Molar
mA	Milliamps
Mb	Megabase
mg	Milligram
μg	Microgram
μl	Microlitre
μm	Micrometre
MIC	Minimum inhibitory concentration
min	Minute
ml	Millilitre
mm	Millimetre
mM	Millimolar
mPa	Milli Pascals
MRS	deMan, Rogosa and Sharpe
MW	Molecular weight
NFDM	Non-fat dried milk
ng	Nanogram
NGYEC	Non-fat dry milk supplemented with glucose (2% w/v), yeast extract (1% w/v), and L-cysteine hydrochloride (0.05% w/v)
nm	Nanometres

OD	Optical density
%	Percentage
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
pmol	Picomoles
RAPD	Random amplified polymorphic DNA
RCA	Reinforced clostridial agar
rDNA	Ribosomal deoxyribonucleic acid
RFLP	Restriction fragment length polymorphism
RNase	Ribonuclease
rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
SD	Standard deviation
SDS-PAGE	Sodium-dodecyl sulphate poly-acrylamide gel electrophoresis
sec	Second
ssp.	Subspecies
ST agar	<i>Streptococcus thermophilus</i> agar
TNF	Tumour necrosis factor
Tris	Tris (hydroxymethyl) aminomethane
U	Unit
UV	Ultra violet
v	Volume
v/v	Volume per volume
w/v	Weight per volume

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.0 INTRODUCTION

In view of the rapid development of widespread antibiotic resistance in recent years, it seems increasingly important to find alternatives to classic antibiotic treatments for gastrointestinal infections. Probiotics, defined as 'microbial food ingredients that are beneficial to health' (Salminen *et al.*, 1998), have been suggested as one such alternative, with many reports claiming potential health benefits following consumption of these organisms. Potential benefits include the ability to alleviate symptoms of lactose intolerance (Labayen *et al.*, 2001), lower serum cholesterol (Gilliland *et al.*, 1985), reduce blood pressure, lower faecal enzyme activity (Salminen *et al.*, 1996), reduce diarrheal incidence and duration (Marts and Longo, 1994), stimulate the immune system (Kitasawa *et al.*, 1994; Salminen *et al.*, 1996; Mattila-Sandholm *et al.*, 1999), control infections *via* pathogen exclusion and antagonism and suppress tumors and protect against colon/bladder cancer by maintaining the normal ecological balance of the gut flora (Sandine, 1979; Salminen *et al.*, 1996; Scheinbach, 1998; Ouwehand *et al.*, 1999; Holzapfel *et al.*, 1998; Gomes and Malcata, 1999; Marteau, 2002).

In order to maintain therapeutic value, probiotic organisms must maintain viability and activity in the carrier food and during passage through the gastrointestinal tract. It is essential to meet the criterion of a minimum of 10^7 cfu/ml at the expiry date, as the minimum therapeutic dosage per day is considered to be 10^8 to 10^9 (Kurmman and Rasic, 1991) corresponding to an intake of 100 g of product containing 10^6 to 10^7 cfu/ml. Furthermore, the selected strains must produce a final product with good taste and acceptable body and texture, since only by satisfying the demands of the consumer can the food industry succeed in promoting the consumption of functional probiotic products in the future.

In many countries, regulatory levels of probiotic bacteria in dairy products have been established. Generally, products are required to contain at least ten million live lactic acid bacteria per millilitre at the time of sale (Roberts and Maust, 1995; Shortt, 1999). Many studies have reported that some strains of *Lactobacillus acidophilus* lose viability in yogurt and are sensitive to the acid and bile salts commonly encountered during transit through the gastrointestinal tract (Walker and Gilliland, 1993; Buck and Gilliland, 1994; Lankaputhra and Shah, 1995; Shah *et al.*, 1995; Nighswonger *et al.*, 1996; Micanel *et al.*, 1997; Rybka and Fleet 1997; Schillinger, 1999; Vinderola *et al.*, 2000; Oliveira *et al.*, 2002). The main factor for loss of viability of *Lb. acidophilus* during storage has been attributed to the low pH of the medium due to accumulation of organic acids during growth and fermentation (Hood and Zottola, 1988, Shah and Jelen, 1990; Hughes and Hoover, 1991; Lankaputhra and Shah, 1995; Dave and Shah, 1997a). Other factors that may affect viability include the storage temperature, the absence of nutrients (Vinderola *et al.* 2000) and the production of hydrogen peroxide (Hull *et al.*, 1984). There are several points for intervention that may result in improved viability of probiotics in dairy products, including strain selection, conditions of growth prior to inoculation and the conditions encountered during storage. Attempts to improve viability have been made through the selection of acid and bile resistant strains, use of oxygen impermeable containers (Dave and Shah, 1997b), two-step fermentation and micro-encapsulation (Carcoba and Rodriguez, 2000; Sultana *et al.*, 2000), incorporation of micronutrients such as peptides and amino acids or by sonication of yogurt bacteria to release β -galactosidase (Shah and Lankaputhra, 1997).

The general aim of the project reported in this thesis was to investigate the physiological factors that may influence the survival of *Lb. acidophilus* in dairy products and to develop a protocol for probiotic culture preparation that would improve their survival during refrigerated storage in yogurt. In Chapter 3, commercially acquired microorganisms were screened for their ability to survive bile and acid concentrations normally encountered in the gastrointestinal tract and also for their ability to survive during long-term storage in a yogurt-like environment. Selected isolates were differentiated and identified using a polyphasic approach that encompassed a variety of biochemical, physiological and molecular typing techniques. The results reported in Chapter 4 describe the further characterization of

the commercially available isolates in terms of their sensitivity to various temperatures and pH values, and these parameters were used to design a protocol for conditioning cells to survive normally lethal pH environments. The results presented in Chapter 5 demonstrate the acquired acid tolerance response from a physiological perspective, including *de novo* protein synthesis and the ability of pH pre-conditioning to enhance the survival of cells exposed to a variety of challenge conditions, including temperature, hydrogen peroxide, ethanol, to determine whether cross-protection to stresses occurred. The ability of pre-conditioned cells to survive during yogurt production and refrigerated storage was examined in Chapter 6, as well as the inhibitory activity of starter cultures against probiotic strains.

1.1 CONCEPT OF PROBIOTICS

1.1.1 A historical perspective

The importance of lactobacilli for human health and longevity was first hypothesised in '*The Prolongation of Life*', published in 1907 by Elie Metchnikoff, the Director of the Pasteur Institute and Nobel Prize winner for the discovery of phagocytosis (Metchnikoff, 1907). Metchnikoff considered the intestinal flora as detrimental rather than beneficial for well-being and suggested that beneficial effects might only be expected by their replacement with the yogurt bacteria *Lactobacillus bulgaricus* and *Streptococcus thermophilus* (Holzapfel *et al.*, 1998). Around 1906, Cohendy reported 'a decrease in the number of putrefactive products in subjects administered milk soured with *Lb. bulgaricus*' and Tissier reported 'clinical benefits from modulating the flora in infants with intestinal infections' (Tissier, 1906). These findings were followed by a study in the early 1920s, with Cheplin and Rettger reporting that *Lb. acidophilus* milk had therapeutic benefits, including the ability to aid digestion (Cheplin and Rettger, 1922). The early 1930s saw Shirota develop his first product containing *Lactobacillus casei* Shirota strain, which was the basis for the establishment of the Yalkult Central Institute for Microbiological Research (Holzapfel *et al.*, 1998). By the 1950s, yogurts were often used to re-establish the balance of the gut flora upset by antibiotics and to alleviate or prevent conditions such as diarrhoea, constipation, ulcerative colitis and dermatitis (Newman, 1915; Cheplin and Rettger, 1922; Hawley *et al.*, 1959).

1.1.2 Definition of Probiotics

The definition for probiotics has gradually changed with the emergence of a better understanding of the mechanisms by which they influence human health. The word probiotic is derived from the Greek word meaning 'for life' (Shortt, 1999). Lilly and Stillwell (1965) first used the word 'probiotic' to describe 'substances secreted by one protozoan to stimulate the growth of another'. Almost a decade later, the term 'probiotic' had evolved to describe 'animal feed supplements including organisms and substances that had a beneficial effect on an animal by contributing to its intestinal flora balance' (Parker, 1974). Parker's definition included the use of antibiotics to promote the growth of farm animals. In 1989, Fuller proposed one of the most popular definitions for probiotics: 'Probiotics are live microbial feed supplements, which beneficially affect the host animal by improving its intestinal microbial balance.' This definition stressed the importance of viable cells as a component of an effective probiotic and excluded the use of antibiotics. In 1991, Huis in't Veld and Havenaar re-defined the term 'probiotics' to include human consumption: 'a mono- or mixed-culture of live microorganisms which when applied to man or animal affects beneficially the host by improving properties of the indigenous microflora'. An EC-supported group of European scientists suggested that probiotics for use in human nutrition are best defined as 'microbial food ingredients that are beneficial to health' (Salminen *et al.*, 1998). This definition takes into account results from research that demonstrated non-microflora mediated probiotic effects, *e.g.* probiotic effects on immune parameters.

In 1999, Salminen published a new definition, which incorporated not only probiotic cells, but also, components of these cells: 'Probiotics are microbial cell preparations or components of microbial cells that have a beneficial effect on the health and well-being of the host' (Salminen *et al.*, 1999). More recently, Schrezenmeir and de Vrese (2001) published a new definition: 'a preparation of or a product containing viable, defined micro-organisms in sufficient numbers, able to alter the microflora, by implantation or colonisation, in a compartment of the host and by that, exert beneficial effects on the host health'. Regardless of the definition, all share the common theme that probiotic organisms contribute to the health of the consumer.

1.1.3 Types of cultures used for developing probiotic products

The ability of lactic acid bacteria to inhibit food spoilage and demonstrate the potential to contribute to the health of the host has found application in a range of conventional food fermentation processes and has helped to increase the availability, safety and variety of foods (Malik *et al.*, 1995). Lactic acid bacteria have been used to manufacture a variety of products, including fermented milks, cheeses and meats, beer, wine, pickles, sourdough bread, silage, medicines and several biologically active substances (Stiles and Holzapfel, 1997).

Lyophilised probiotic preparations as a vehicle for consumption of probiotic organisms are available, although it is argued that these preparations are unfavourable, since the organisms are required to reactivate and multiply in the intestinal tract, which is a challenging environment (Mikolajcik and Hamdan, 1975). Dairy products are perfect vehicles for consumption of probiotic organisms, since 78% of calcium intake in the human diet is derived from dairy products (Kitts and Yuan, 1992). Yogurt is a fermented milk product obtained through the lactic acid fermentation of milk by the starter cultures *Str. thermophilus* and *Lb. bulgaricus*. (Matalon and Sandine, 1986). Starter cultures are, by definition, used in order to change the sensory properties of the food (Lucke, 2000).

An increase in demand for probiotic products has seen widespread automation throughout the dairy industry to achieve higher quantities at reduced costs (Daly *et al.*, 1998). The usual practice is to incorporate the yogurt cultures *Str. thermophilus* and *Lb. bulgaricus* along with probiotic cultures, as the probiotic cultures grow slowly in milk during product manufacture (Hoier, 1992; Saxelin *et al.*, 1999).

The addition of the starter cultures *Str. thermophilus* and *Lb. bulgaricus* during production of probiotic yogurts is desirable because:

- a) the concentration of free amino acids is higher when yogurts are made with the starter cultures *Str. thermophilus* and *Lb. bulgaricus* as the probiotic cultures are weakly proteolytic (Matalon and Sandine, 1986);

- b) extracellular exopolysaccharides are produced by *Str. thermophilus* and *Lb. bulgaricus*, which act to viscosify, stabilize and give texture to yogurt, which decreases its susceptibility to syneresis (Van den Berg *et al.*, 1995);
- c) the bacterial end-products, lactic acid and acetaldehyde, are generally considered the predominant flavor compounds in yogurts made with *Str. thermophilus* and *Lb. bulgaricus* (Matalon and Sandine, 1986; Sinha *et al.*, 1989);
- d) more rapid acid development is observed in mixed cultures of *Lb. bulgaricus* and cultures *Str. thermophilus* than in single strain cultures, due to their symbiotic relationship (Morichi, 1991);
- e) *Lb. bulgaricus* hydrolyses alpha- and beta-caseins (Abraham *et al.*, 1993) which stimulate the growth of *Str. thermophilus* by providing free amino acids. In return, the *Str. thermophilus* enhances the growth of *Lb. bulgaricus* by production of formate and carbon dioxide (Matalon and Sandine, 1986; Sinha *et al.*, 1989; Shah, 2000).

It is possible to make yogurt with probiotic organisms as sole starter cultures, although the longer incubation and product quality would be sacrificed in the process (Sodini *et al.*, 2002).

One disadvantage of using the starter culture *Lb. bulgaricus* is that it produces lactic acid during refrigerated storage, a process known as post-acidification. Post-acidification can cause defects in yogurt such as syneresis, reduction in viable counts and accumulation of lactic acid in the medium (Gardini *et al.*, 1999).

To overcome the loss of viability of probiotic organisms through post-acidification, yogurts made without the presence of *Lb. bulgaricus* (called ABT yogurts) are manufactured. ABT starter cultures increase the fermentation time significantly (Sodini *et al.*, 2002), because there is no symbiosis without the *Lb. bulgaricus* culture. These yogurts may require the addition of micronutrients, peptides and amino acids for reducing the fermentation time and for improving the viability of probiotic bacteria (Dave and Shah, 1997b; Sodini *et al.*, 2002). However, the addition of nutrients is not permitted in many countries and some countries have legislation

stating that ‘the fermented product can be called “yogurt” only if the bacteria growing in milk are *Str. thermophilus* and *Lb. bulgaricus*’ (FAO, 1992; Ministero Della Sanita, 1972). Therefore the ABT starters are considered undesirable, given the rigid controls in modern yogurt manufacturing.

The most common probiotic bacteria that are included in fermented milks are representatives of *Lb. acidophilus*, *Lb. bulgaricus*, *Lb. casei*, *Bifidobacterium bifidum*, *Bifidobacterium longum*, *Bifidobacterium infantis*, *Bifidobacterium adolescentis* and *Bifidobacterium breve* (Driessen and De Boer, 1989; Shah *et al.*, 1995; Klaenhammer and Kullen, 1999). Some food companies add new probiotic organisms to yogurt, such as *Lactobacillus reuteri*, *Lactobacillus amylovorus*, *Lactobacillus gasseri*, *Lactobacillus johnsonii*, *Lactobacillus crispatus*, *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, *Lactobacillus fermentum*, *Lactobacillus helveticus*, *Lactobacillus gallinarum*, *Lactobacillus salivarius*, *Lactobacillus paracasei*, *Bifidobacterium animalis*, *Enterococcus faecium* and the yeast *Saccharomyces boulardii* (Holzapfel *et al.*, 1998; Klein *et al.*, 1998; Gismondo *et al.*, 1999; Shah, 2000, Molin, 2001).

1.1.4 Overview of probiotic products in the market

Probiotic organisms can be consumed in a number of forms, including milk products, dairy desserts and freeze-dried preparations, but the nature of dairy products such as yogurt offers a protective environment for lactic acid bacteria during gastric passage (Conway *et al.*, 1987; Clark *et al.*, 1993; Kos *et al.*, 2000; Fernandez *et al.*, 2003). In addition, the buffering capacity of yogurt enhances adherence in the digestive tract (Conway *et al.*, 1987).

Turkey produces a wide variety of yogurt products, ranging from standard and stirred yogurts, concentrated yogurt and cooked yogurt (Akin and Rice, 1994). In Japan and Europe, probiotic-supplemented dairy foods and drinks continue to be a fast-growing industry, numbering over 70 products containing *Lb. acidophilus* and *Bif. bifidum* including sour cream, buttermilk, yoghurt, milk powder and frozen dairy desserts (Klupsch, 1983; Ishibashi and Shimamura, 1993). Fermented milk drinks have been available in Japan since 1919 and the market consumes approximately 110 million

litres per year (Shortt, 1999). One such product, a citrus-flavored drink containing *L. casei* (Yakult), sold one million bottles per day in Japan and twenty-three million bottles world-wide (Ichikawa, 1994) and the product continues to thrive today. Between 1985 and 2004, the consumption of yogurt containing probiotic organisms in Australia has almost doubled; from 3.0 to 5.9 Kg per person per year (Dairy Australia, 2005). Table 1.1 provides examples of probiotic products currently available on the world-wide market.

1.1.5 Health benefits of probiotics

Gastrointestinal disturbances are caused by a number of factors, including (a) motility and pH of the host gastrointestinal tract, (b) adhesive ability of microbes, (c) nutritional deficiencies, (d) microbial interactions such as synergy and antagonism and (e) diet (Holzapfel *et al.*, 1998; Klaenhammer and Kullen, 1999).

Probiotic organisms such as *Lb. acidophilus* are believed to impart a number of health benefits to the consumer (Table 1.2), including the ability to alleviate symptoms of lactose intolerance (Labayen *et al.*, 2001), lower serum cholesterol (Gilliland *et al.*, 1985), reduce blood pressure, lower faecal enzyme activity (Salminen *et al.*, 1996), reduce diarrhoeal incidence and duration (Marts and Longo, 1994), stimulate the immune system (Kitasawa *et al.*, 1994; Salminen, 1996; Mattila-Sandholm *et al.*, 1999), control infections *via* pathogen interference, exclusion and antagonism (Lee *et al.*, 2000), suppress tumors and protect against colon/bladder cancer by maintaining the normal ecological balance of the gut flora (Sandine, 1979; Fuller, 1989; Salminen *et al.*, 1996; Holzapfel *et al.*, 1998; Scheinbach, 1998; Gomes and Malcata, 1999; Ouwehand *et al.*, 1999; Marteau, 2002).

In addition to therapeutic benefits, various nutritional benefits have also been reported (nutraceuticals). Nutritional benefits following consumption of probiotic organisms include their role in enhancing the bioavailability of calcium, zinc, iron, manganese, copper and phosphorus (McDonough *et al.*, 1983), increasing digestibility of protein in yogurt (Breslaw and Kleyn, 1973) and synthesis of vitamins in yogurt such as folic acid, niacin, thiamine, riboflavin, pyridoxine and vitamin K, which are slowly

Table 1.1. Examples of probiotic products available on the worldwide market.

Product Name/Country	Organisms ^a	Reference
Biogurt/Australia	L.A, S.T.	Hull <i>et al.</i> (1984)
Natural Bio/ Germany	Natural Bifidus	Fuller (1993)
Cultura/Denmark	L.A, B.S.	Hoier (1992)
Philus/Sweden	L.A, S.T, B.S.	As above
BA live/UK	L.A, L.B., S.T, B.S.	As above
Kyr/Itlay	L.A, L.B., S.T, B.S.	As above
Ofilus/France	L.A., S.T, B.S.	As above
BIO/France	L.A, L.B., S.T, B.S.	As above
Biogarde/Germany	L.A, L.B., S.T, B.B.	Kurmann and Rasic (1988)
Bifigurt /Germany	S.T., B.S.	Kurmann and Rasic (1988)
A-B yogurt /France	L.A., B.S.	Hoier (1992)
B-Active/France	L.A, L.B., S.T, B.S.	As above
Yoplus/Australia	L.A, L.B., S.T, B.S.	As above
Bifidus Yogurt/Japan	B.L.	Shortt (1999)
Yakult/Japan	L. C., B.Br.	As above
Actimel Orange/ Germany	L. C.	Scheinbach (1998)
ProCult3/Germany	B. L.	As above
Symbalance/ Switzerland	L.R., L.A., L.C.	As above
Fysiq /Netherlands	L.A	As above
Gaio/Denmark	S.T., E.F.	As above

^a L.A. = *L. acidophilus*, L.B. = *L. bulgaricus*, L.C. = *L. casei*, S.T. = *S. thermophilus*, B.S. = *Bifidobacterium sp.*, B.B. = *B. bifidum*, B.L. = *B. longum*, B.Br. = *B. breve*, E.F. = *E. faecium*, L.R. = *L. reuteri*.

Table 1.2 Potential health and nutritional benefits of functional foods prepared with probiotic bacteria (Gomes and Malcata, 1999; Ouwehand *et al.*, 1999).

Beneficial effect	Possible causes and mechanisms
Improved digestibility	Partial breakdown of proteins, fats and carbohydrates
Improved nutritional value	Higher levels of B vitamins and certain free amino acids
Improved lactose utilisation	Reduced lactose in product and further availability of lactase
Antagonistic action towards enteric pathogens	Disorders, such as functional diarrhoea, mucous colitis, ulcerated colitis, diverticulitis and antibiotic colitis controlled by acidity, microbial inhibitors and prevention of pathogen adhesion or pathogen activation
Colonisation in gut	Survival in gastric acid, resistance to lysozyme and low surface tension of intestine, adherence to intestinal mucosa, multiplication in the intestinal tract, immune modulation
Anticarcinogenic effect	Conversion of potential pre-carcinogens into less harmful compounds Reduction in carcinogen-promoting enzymes and stimulation of the immune system
Hypocholesterolemic action	Production of inhibitors of cholesterol synthesis Use of cholesterol by assimilation and precipitation with deconjugated bile salts
Immune modulation	Enhancement of macrophage formation, stimulation of production of suppressor cells and gamma-interferon

absorbed by the body (Deeth and Tamime, 1981; Rasic and Kurmann, 1983; Tamime *et al.*, 1995; Hugenholtz and Smid, 2002).

Alleviation of lactose intolerance following the consumption of probiotic organisms is probably the most established health claim. People with lactose intolerance are unable to digest significant amounts of lactose because of a genetic deficiency that decreases production of the lactase enzyme. Common symptoms include abdominal pain and bloating, excessive flatulence and diarrhoea following the ingestion of foods containing lactose. A number of studies provide evidence that the addition of certain starter cultures to milk products, even with high initial levels of lactose, allows them to be consumed by lactose intolerant individuals without experiencing the usual symptoms (Kolars *et al.*, 1984; Saviano *et al.*, 1984; Lin *et al.*, 1991; Martini *et al.*, 1991a; Martini *et al.*, 1991b; Labayen *et al.*, 2001). However, Lin *et al.*, (1991) and Sanders, (1993a,b) reported that consuming *Lb. acidophilus* supplemented milk products did not diminish intolerance to lactose.

Studies on the potential of lactic acid bacteria to alleviate diarrhoea have yielded positive results (Bellomo *et al.*, 1980; Isolauri *et al.*, 1991; Saavedra *et al.*, 1994; Shornikova *et al.*, 1997). Shortening of the duration of rotavirus diarrhoea using *Lactobacillus* GG is perhaps the best-documented probiotic effect, as demonstrated in a recent multi-centre study conducted in Europe (Guandalini, 1998).

Niedzielin *et al.* (2001) reported a decrease in the symptoms associated with irritable bowel syndrome following consumption of *Lb. plantarum* preparations, whilst prophylactic feeding of *Lactobacillus sporogenes* has also shown a preventative effect on the incidence and duration of acute rotavirus diarrhoea (Chandra, 2002). These effects have been attributed to an altered intestinal microflora and stimulation of the immune system (Ouwehand *et al.*, 1999).

There are a few studies on the prevention of travellers' diarrhoea and these show positive outcomes for *Lactobacillus* GG and a combination of *Lb. acidophilus* La-5 with *Bifidobacterium lactis* Bb-12 (Black *et al.*, 1989; Oksanen *et al.*, 1990), but there are few studies using defined strains on acute diarrhoea (Ouwehand *et al.*, 1999). In a

placebo-controlled, double-blind study conducted by Salminen and Deighton (1992), the occurrence of travellers diarrhoea decreased by 39.5%, whilst in a similar study by Black *et al.*, (1989), the incidence was reduced by 38%.

Reports that probiotic organisms have hypocholesterolemic activity are controversial. When bile acids are deconjugated they lose their solubility and emulsifying properties and, in this form, do not promote lipid absorption and digestion. It has long been recognised that lactobacilli can deconjugate bile acids *in vitro* (Klaenhammer, 1995; Kimoto *et al.*, 2002; Pereira and Gibson, 2002). Studies demonstrating the ability of lactic acid bacteria to deconjugate bile salts have been performed *in vivo* (Gilliland and Speck, 1977a; Gilliland *et al.*, 1985; Gilliland, 1990; Klaver and van der Meer, 1993; Walker and Gilliland, 1993; Takahashi and Morotomi, 1994). Although some animal models have proven successful for the ability to decrease serum cholesterol levels using lactic acid bacteria (Gilliland *et al.*, 1985; Chikai *et al.*, 1987), studies in humans have not been as successful (Lin *et al.*, 1989; Agerbaik *et al.*, 1995). Ashar and Prajapati (2000) used acidophilus milk to demonstrate its ability to lower serum cholesterol levels in humans that were in different categories of cholesterol status. Overall, intake of acidophilus milk was only effective with subjects categorized as a group with lipidemic status of borderline elevated cholesterol level (2.0 to 2.2 g/L) group.

Probiotic organisms are thought to be able to decrease certain fecal bacterial enzymes that may act as carcinogens. Reports have shown the reduction in enzyme activities (beta-glucuronidase, azoreductase and nitroreductase) following consumption of milk supplemented with *Lb. acidophilus* (Goldin and Gorbach, 1984; Goldin *et al.*, 1992) or *Lb. casei* (Ling *et al.*, 1994) to humans. This resulted in a decrease in the activity of carcinogen-activating microbial enzymes and may have a beneficial effect in the colon, the urinary tract and the bladder. Recent studies have shown that the cytoplasm fractions of *Bif. longum* and *Lactococcus lactis* ssp. *lactis* can inhibit proliferation of both human adenocarcinoma and human gastric cancer cell lines (Kim *et al.*, 2002). The evidence is encouraging, although there is no direct experimental evidence for cancer suppression in humans as a result of consumption of lactic acid bacteria.

Several studies have shown that selected probiotic strains have the ability to up-regulate the immune response (Perdigon *et al.*, 1986; Halpern *et al.*, 1991; Isolauri, 1999; Matar *et al.*, 2001; Cross *et al.*, 2002; Morita *et al.*, 2002). Immune system stimulation has been demonstrated following administration of lactobacilli to mice. *Lb. bulgaricus* (Nishimura-Uemura, *et al.*, 2003) and *Lb. rhamnosus* (Gill and Rutherford, 2001), stimulated macrophage activity and Perdigon *et al.* (1986) observed the up-regulation of IgA. The administration of yogurt to humans was found to increase interferon production by cytotoxic T cells (Halpern *et al.*, 1991). In another study, mice fed with *Lb. rhamnosus* HN001 during antigen sensitization produced higher levels of lymphocyte derived IFN-gamma and the interleukins IL-4 and IL-5 in comparison to control animals (Cross *et al.*, 2002). Perdigon *et al.* (2002) observed cytokine secretion in the gut of BALB/c mice fed with various lactic acid bacteria including *Lb. acidophilus*, *Lb. casei*, *Lb. bulgaricus* and *Str. thermophilus*, including the stimulation of TNF- α and γ , and production of various interleukins. Responses were found to be strain dependent.

Immune system stimulation by LAB would be beneficial in the case of an individual who demonstrated an insufficient immune response following infection or vaccination. However, in circumstances where the immune system is over-stimulated, such as allergic reactions, further induction of the immune response would not be advantageous (Pelto *et al.*, 1998). In the healthy host, it is uncertain if an increased immune response is of value and, in any case, would be difficult to observe (Spanhaak *et al.*, 1998). No reports of increases in allergic reactions or any reduction in immune function upon the use of probiotics have been published. Thus far, there are no known negative effects from the modulation of the immune system by lactic acid bacteria.

Bacteriocins produced by lactobacilli have been studied extensively over the past two decades and there is a wealth of knowledge on the ability of certain lactic acid bacteria to inhibit growth of other species. Bacteriocins generally exert their anti-microbial action by interfering with the cell membrane of target organisms, either by hindering cell wall development or causing pore formation, resulting in cell death (O'Sullivan *et al.*, 2002). Bacteriocin-producing LAB have the potential to improve

the safety and quality of various foods. Barefoot and Klaenhammer (1983) reported that 63% of 52 *Lb. acidophilus* strains produced bacteriocins. Subsequently, some of those *Lb. acidophilus* strains have been re-classified by DNA homology into the newly recognised species *Lb. gasseri* and *Lb. johnsonii* (Fujisawa *et al.*, 1992). The bacteriocins produced were active against closely related lactobacilli and also *Ent. faecalis*. Anand *et al.* (1984) reported that *Lb. acidophilus* causes inhibition of *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhimurium* and *Clostridium perfringens* whilst Carrasco *et al.* (2002) reported that 27 strains of lactic acid bacteria isolated from commercial Argentinian cheeses produced bacteriocins that were inhibitory towards *Listeria monocytogenes*, *Pseudomonas* sp., *E. coli*, *Salmonella enteritidis*, *Shigella* sp. and *Vibrio cholerae*. *Lb. bulgaricus* produces bacteriocins that are active against several Gram positive and negative bacteria including *Staphylococcus* sp., *Sarcina*, *Pseudomonas*, *Eschericia* and *Serratia* species. Dahiya and Speck (1968) reported that *L. lactis* and *Lb. bulgaricus* inhibited growth of *S. aureus* due to the production of hydrogen peroxide. *L. lactis* has also been found to inhibit the growth of *L. monocytogenes* in alfalfa sprout broth (Palmai and Buchanan, 2002). *L. casei* inhibits the growth of *Helicobacter pylori* *in vitro* and the closely related *Lactobacillus paracasei* subsp. *paracasei* strain was found to inhibit *E. coli* (Caridi, 2002).

Bacteriocins of lactic acid bacteria include nisin A, lactacin, pediocin and leucocin (O'Sullivan *et al.*, 2002; Kim *et al.*, 2003). Another bacteriocin, plantaricin, produced by *Lb. plantarum* strain 423 is bactericidal for many Gram-positive foodborne pathogens and spoilage bacteria including *Listeria*, *Staphylococcus*, *Pediococcus* and *Lactobacillus* species (Van Reenan *et al.*, 2002). Although there is potential for bacteriocin-producing strains to provide health benefits to humans, their benefits *in vivo* have yet to be determined (Van Reenan *et al.*, 2002).

Beyond these traditional roles, probiotic bacteria are also being investigated as live delivery vehicles that can potentially be used to express vaccine components, enzymes, or antimicrobials within targeted locations of the gastrointestinal tract, or at other mucosal surfaces in the body (Klaenhammer, 1998; Havenith *et al.*, 2002; Scheppeler *et al.*, 2002; Seegers, 2002).

Although promising, many of the health benefits attributed to the ingestion of probiotic organisms remain scientifically unsubstantiated *in vivo* (O'Sullivan *et al.*, 1992; Shanahan, 2002), thus specific health claims can rarely be made (Sanders, 1993a,b). A lot of information has been gathered about the possible health benefits of particular probiotic organisms using animal models, but extrapolation to humans is difficult. A major problem of assessing the true benefit of probiotics is the inability to perform certain types of trials in humans as performed with animals. Obviously it would be unethical to try to show that lactic acid bacteria can protect against experimentally induced tumours in humans as has been done for rats. Translating animal results to humans must be done with care because there are significant differences between them and it cannot be assumed that the same probiotic is equally suitable for all individuals. In addition, confirmation of the identity, viability and activity of probiotic strains needs to be done before administering probiotics to humans (Sanders, 1993a).

For healthy individuals, the main market for probiotic products, the benefits of probiotics are unproven. Protective effects are difficult to measure in the absence of specific targets. Studies that are performed in humans are hard to analyse due to a lack of understanding of the complex gastrointestinal environment and failure to identify the thousands of potential species that impact the microecology of the gastrointestinal tract (Sanders, 1993a). To have any therapeutic value, *Lb. acidophilus* must maintain viability during storage until the product has been consumed and then must survive transit through the gastrointestinal tract to colonise the human gut. There are doubts as to the success of colonisation of probiotic bacteria in the compromised individual. Even in the 'unbalanced' gastrointestinal tract, overgrowth by certain indigenous bacteria or exogenously introduced organisms probably limits colonisation by any newly introduced probiotic. This will also depend on the speed of the reconstitution and length of the lag phase of probiotic organisms in the gastrointestinal tract (Berg, 1998). Results of feeding trials of *Lb. acidophilus* have suggested that it does not permanently colonise the gastrointestinal tract, as it could not be detected two weeks after feeding trials (Crowell, 1998; Alander *et al.*, 1999; Klaenhammer and Kullen, 1999). These studies support statements that

‘probiotics are not likely to colonise the gastrointestinal tract permanently’ and ‘require continuous delivery to maintain their presence’ (Tannock, 1999). A requirement for long-term and continuous administration is certainly a desirable criterion for culture manufacturers and functional food industries developing vehicles to deliver probiotics.

Lactobacillus are mostly known for their perceived health benefits, however, some endogenous lactic acid bacteria have been involved in rare cases of septicemia and endocarditis (Oakey *et al.*, 1995; Asahara *et al.*, 2003). In 1994, a European Union-sponsored workshop was organised by the Lactic Acid Bacteria Industrial Platform, which concluded that ‘the overall risk of lactic acid bacteria infection is very low’. Only one case of infection has been linked to the consumption of probiotics (De Vrese and Schrezenmeir, 2002) and bacteremia-associated strains *Lb. rhamnosus* and *Lb. casei* were reported to be moderately infective in a rabbit infective endocarditis model (Asahara *et al.*, 2003). In the same study, probiotic strains *Lb. acidophilus* ATCC 4356, *Lb. gasseri* and *Lb. casei* Shirota strain did not show pathogenic potential and Salminen *et al.* (2002) reported that ‘human consumption of *Lb. rhamnosus* GG did not lead to an increase in bacteremia’.

Regardless of this, ‘industrial interest in developing probiotics and probiotic-functional foods is thriving, driven largely by the market potential for foods that target general health and well being’ (Sanders *et al.*, 1998).

1.2 IDENTIFICATION OF *LACTOBACILLUS* SPECIES

Identification of *Lb. acidophilus* has become an important topic in the last decade for determining the presence, activity and viability of probiotic bacteria in the human gastrointestinal tract and probiotic products.

Lb. acidophilus was first isolated from infant faeces by Moro in 1900, and he named it “*Bacillus acidophilus*”. In 1929, Holland included “*Bacillus acidophilus*” in the genus *Lactobacillus* (Holzapfel *et al.*, 1998) (Table 1.3). Subsequently, this species

Table 1.3. List of species of *Lactobacillus* (adapted from Gomes and Malcata, 1999 and Holzapfel *et al.*, 1998).

<i>L. acetotolerans</i>	<i>L. homohiochii</i>
<i>L. acidophilus</i> ^a	<i>L. intestinalis</i>
<i>L. agilis</i>	<i>L. jensenii</i> ^a
<i>L. alimentarius</i>	<i>L. johnsonii</i>
<i>L. amylophilus</i>	<i>L. kandleri</i>
<i>L. amylovorus</i>	<i>L. kefir</i>
<i>L. avarius</i>	<i>L. kefiranoformis</i>
<i>L. bif fermentans</i>	<i>L. malefermentans</i>
<i>L. brevis</i> ^a	<i>L. mali</i>
<i>L. buchneri</i> ^a	<i>L. minor</i>
<i>L. casei</i> subsp. <i>casei</i> ^a	<i>L. murinus</i>
<i>L. collinoides</i>	<i>L. oris</i> ^a
<i>L. confusus</i>	<i>L. parabuchneri</i> ^a
<i>L. coryniformis</i>	<i>L. paracasei</i> ^a
<i>L. crispatus</i> ^a	<i>L. perntosus</i>
<i>L. curvatus</i>	<i>L. pontis</i>
<i>L. delbrueckii</i>	<i>L. plantarum</i> ^a
<i>L. farciminis</i>	<i>L. reuteri</i> ^a
<i>L. fermentum</i> ^a	<i>L. rhamnosus</i> ^a
<i>L. fructivorans</i>	<i>L. ruminis</i>
<i>L. fructosus</i>	<i>L. sake</i>
<i>L. gallinarum</i>	<i>L. salivarius</i> ^a
<i>L. gasseri</i> ^a	<i>L. sanfrancisco</i>
<i>L. graminis</i>	<i>L. sharpeae</i>
<i>L. halotolerans</i>	<i>L. suebicus</i>
<i>L. hamsteri</i>	<i>L. vaccinofermentans</i>
<i>L. helveticus</i>	<i>L. vaginalis</i> ^a

^a Species isolated from human sources

was studied using phenotypic characteristics such as sugar fermentation, colony morphology, Gram stain, the ability to grow at 15°C and 45°C, serological properties, formation of lactic acid dehydrogenase, G + C content of DNA and DNA-DNA hybridization (Fujisawa *et al.*, 1992; Nighswonger *et al.*, 1996).

In 1980, the *Lb. acidophilus* group was recognised to be quite heterogeneous (Johnson *et al.*, 1980; Lauer *et al.*, 1980) and was separated into two main DNA homology groups (A1-A4 and B1-B2). Group A consisted of *Lb. acidophilus sensu stricto* (A1), *Lb. crispatus* (A2), *Lb. amylovorus* (A3), and *Lb. gallinarum* (A4), whilst group B consisted of *Lb. gasseri* (B1) and *Lb. johnsonii* (B2). The division into two major clusters was confirmed by ribotyping (Ryu *et al.*, 2001). Johnson *et al.* (1980) referred to these six homology groups collectively as the *Lb. acidophilus* group, which have consequently been divided into six distinct species now including *Lb. acidophilus* (Moro, 1900; Hansen and Moquot, 1970), *Lb. amylovorus* (Nakamura, 1981), *Lb. crispatus* (Brygoo and Aladame, 1953; Cato *et al.*, 1983), *Lb. gallinarum* (Fujisawa *et al.*, 1992), *Lb. gasseri* (Lauer and Kandler, 1980) and *Lb. johnsonii* (Fujisawa *et al.*, 1992). Exact identification, with the aid of genetic methods, reveals that the different species in the *Lb. acidophilus* complex can be distinguished by habitat and host, with *Lb. acidophilus*, *Lb. crispatus*, *Lb. gasseri* and *Lb. johnsonii* found in human faeces (Mitsuoka, 1992); *Lb. crispatus*, *Lb. gallinarum* and *Lb. johnsonii* found in the chicken cloaca (Miyamoto *et al.*, 2000) and *Lb. crispatus* and *Lb. gasseri* found in the human vagina (Song *et al.*, 1999; Boris and Barbes, 2000).

Lactobacilli are characterised as Gram-positive, non-spore-forming and non-flagellated rods (Gomes and Malcata, 1999). Lactobacilli produce lactic acid from hexoses and they obtain energy *via* substrate-level phosphorylation, since they lack functional electron transport chains. The lactose may enter the cell using either a lactose carrier or lactose permease, and once inside the cell, lactose is hydrolysed by β -galactosidase to glucose and galactose (Varnam, 1993; Caplice and Fitzgerald, 1999). The glucose is metabolised *via* the Embden-Meyerhof-Parnas pathway with lactic acid as essentially the sole end product (Gomes and Malcata, 1999). The yield of lactic acid is 1.8 mol/mol glucose, accompanied by minor amounts of other compounds (Gomes and Malcata, 1999). Lactobacilli are aciduric or acidophilic,

producing pH 4 in foods containing a fermentable carbohydrate (Fujisawa *et al.*, 1992).

A member of the lactobacilli group, *Lb. acidophilus*, is typically 0.6 to 0.9 μm in width and 1.5 to 6.0 μm in length. They are non-motile, intolerant to salt, and do not contain cytochromes and are therefore benzidine negative. In addition, *Lb. acidophilus* are microaerophilic, so surface growth on solid media is generally enhanced by anaerobiosis or reduced oxygen pressure and 5-10% CO_2 . Most strains of *Lb. acidophilus* can ferment amygdalin, cellobiose, fructose, galactose, glucose, lactose, maltose, mannose, salicin, sucrose, trehalose and aesculine (Gomes and Malcata, 1999).

Growth of *Lb. acidophilus* may occur between 15 to 45°C, but optimum growth occurs within 35 to 40°C. It is generally well accepted that lactobacilli species grow up to a maximum of pH 7.2 (Stiles and Holzapfel, 1997), with the optimum pH for growth ranging from 5.5 to 6.0 (Gomes and Malcata, 1999). Cells have a tolerance to >0.8% Oxygall when grown on MRS-lactobacillus agar. They require low oxygen tension, fermentable carbohydrates, protein and protein breakdown products, a number of vitamins of the B-complex, nucleic acid derivatives, unsaturated free fatty acids, and minerals such as magnesium, manganese and iron for their growth (Gomes and Malcata, 1999). Varying media components can alter colonial morphology between rough and smooth (McDonald and Frazier, 1951). Although *Lb. acidophilus* is well defined, difficulties are often encountered in allocating newly isolated strains to each of the species of the *Lb. acidophilus* complex.

1.2.1 Misclassification of probiotic organisms

Bacterial systematics is a continually evolving science and organisms used in foods are often re-classified into new species. For example, a prominent strain that was patented by Gorbach and Goldin as *Lb. acidophilus* (Gorbach and Goldin, 1989), then reported as *Lb. casei* (Isolauri *et al.*, 1995) has now been re-classified as *Lb. rhamnosus* (Arvola *et al.*, 1999).

Characterisation based solely on phenotypic traits is problematic for identification of the closely related species of the *Lb. acidophilus* complex. One of the problems in identifying members of the lactic acid bacteria is the transfer (*via* plasmids) of some characteristics between various strains of some species. Another challenge is posed by organisms that display similar phenotypic characteristics. For example, in 1992, Fujisawa *et al.* (1992) reported that sugar fermentation patterns did not allow differentiation between *Lb. acidophilus* and the phenotypically very similar *Lb. johnsonii*, *Lb. crispatus*, *Lb. gasseri*, *Lb. amylovorus* and *Lb. gallinarum*. These results were supported by Gancheva *et al.* (1999), Ryu *et al.* (2001) and Yeung *et al.* (2002), who found that species identified as *Lb. acidophilus* actually belonged to closely related members of the *Lb. acidophilus* complex.

A survey in the laboratory of Klaenhammer showed that of six strains provided as '*Lb. acidophilus*' by culture manufacturers, two were *Lb. acidophilus*, and the remaining strains were identified as either *Lb. johnsonii*, *Lb. gasseri* or *Lb. crispatus* (Sanders *et al.*, 1996). Schillinger (1999) also reported that some strains had been misclassified by their investigators. DNA homology analysis revealed three strains designated as *Lb. acidophilus* were actually *Lb. johnsonii* and another was *Lb. crispatus*. Strains designated as *Lb. casei* were found to be members of three separate species: *Lb. casei*, *Lb. paracasei* and *Lb. rhamnosus*. Schillinger (1999) analysed the identity of 26 *Lactobacillus* strains isolated from various mild yoghurts and novel type probiotic dairy products using the DNA-DNA hybridization technique. The species present in those products was found to be *Lb. acidophilus*, *Lb. johnsonii*, *Lb. crispatus*, *Lb. casei*, *Lb. paracasei* and *Lb. rhamnosus*.

The inferior differentiation using phenotypic methods may explain why most commercial *Lb. acidophilus* strains in fact belong to other *Lactobacillus* species. On the other hand, manufacturers may favor using *Lb. acidophilus* on the label as it is generally more recognized by consumers.

Incorrect designations of the starter cultures by the manufacturers are still common and species names used often do not correspond to nomenclature. For instance, the

name “*Lactobacillus bifidus*” can still be found on the packaging of some yogurts instead of *Bif. bifidum*, which is not a *Lactobacillus* species (Schillinger, 1999).

Misidentification of probiotic bacteria is common since it is often difficult to speciate bacteria without using modern genetic techniques (O’Brien *et al.*, 1999). With the broader use of 16S rDNA gene sequencing, it is now generally accepted that ‘species delineation may be based on 3% sequence difference between a new species and its closest relative, providing phenotypic techniques allow for differentiation from previously identified species’ (McCartney, 2002). It is therefore important for both manufacturers and regulatory bodies to keep up to date with changes in taxonomy and the methodologies used to differentiate and classify species more accurately.

1.2.2 Techniques for identifying probiotic organisms

In recent years, there has been a shift towards molecular-based typing methods for identification of unknown isolates. At the species level there are several reports on specific PCR and other DNA-based identification systems for lactobacilli, mainly targeting ribosomal genes (Du Plessis and Dicks, 1995; Schleifer *et al.*, 1995; Drake *et al.*, 1996; Tilsala-Timisjarvi and Alatossava, 1997; Roy *et al.*, 2000; Walker *et al.*, 2000; Guarneri *et al.*, 2001; Heilig *et al.*, 2002; Ogier *et al.*, 2002; Sui *et al.*, 2002; Yeung *et al.*, 2002) and ribosomal intergenic regions (Tannock *et al.*, 1990; Tilsala-Timisjarvi and Alatossava, 1997).

These systems are not sensitive enough to differentiate bacteria below the species level. A number of strain identification techniques have been applied to probiotic species, including plasmid profiling (Tannock *et al.*, 1990), lectin typing (Annuk *et al.*, 2001) and protein profiling (Dykes and von-Holy, 1993; Hertel *et al.*, 1993; Du Plessis and Dicks, 1995; Vandamme *et al.*, 1996; Corsetti *et al.*, 2003). Various DNA-based methods such as restriction fragment length polymorphisms (RFLP), pulsed-field gel electrophoresis (PFGE), ribotyping, PCR amplification of repetitive bacterial DNA elements and random amplified polymorphic DNA (RAPD) techniques have been used for identification and differentiation of *Lactobacillus* strains (Rodtong and Tannock, 1993; Roussel *et al.*, 1993; Du Plessis and Dicks,

1995; Sanders *et al.*, 1996; Walker *et al.*, 1996; Tilsala-Timisjarvi and Alatossava, 1997; Gevers *et al.*, 2001; Corsetti *et al.*, 2003). The discriminatory power of these methods may vary among various bacterial species and some of these methods are laborious to use for large numbers of complex bacterial samples (Brandt *et al.*, 2001).

1.2.2.1 Poly-acrylamide gel electrophoresis

Poly-acrylamide gel electrophoresis of sodium dodecyl sulphate denatured whole-cell proteins (SDS-PAGE) has been used to classify and type *Lactobacillus* species (Dykes and von-Holy, 1993; Hertel *et al.*, 1993; Gancheva *et al.*, 1999; Corsetti *et al.*, 2003; Sanchez *et al.*, 2003). This technique can be used to measure relationships among isolates by allowing comparisons to be made on a large number of proteins, which represent a significant portion of the genes encoded by the genome. Proteins of bacterial strains that are closely related display similar electrophoretic patterns (Kerstens and De Ley, 1975). Gancheva *et al.* (1999) reported that SDS-PAGE did not allow differentiation of *Lb. gasseri* from *Lb. johnsonii* and *Lb. amylovorus* from *Lb. gallinarum*, respectively. RAPD analysis allowed for the separation of these strains into discrete groups.

A substantial agreement has been observed between results obtained from DNA-DNA hybridisation experiments, RAPD and whole cell protein electrophoresis within several Gram positive and negative genera including *Lb. acidophilus*, *Lb. crispatus*, *Lb. amylovorus*, *Lb. gallinarum*, *Lb. gasseri* and *Lb. johnsonii* (Dykes and von-Holy, 1993; Du Plessis and Dicks, 1995; Vandamme *et al.*, 1996; Gancheva *et al.*, 1999). Corsetti *et al.* (2003) and De Angelis *et al.* (2001b) have reported that SDS-PAGE was more effective than RAPD analysis for assigning strains of a species to the same group.

1.2.2.2 Pulsed field gel electrophoresis (PFGE)

Molecular methods such as PFGE have been used in preference to traditional biochemical techniques as they provide a more discriminatory analysis for strain typing of bacteria (Farber, 1996). PFGE involves isolating, purifying and digesting chromosomal DNA before resolving the DNA restriction fragments using standard

pulsed-field gel electrophoresis equipment. The DNA restriction patterns produced can be used to determine relatedness between strains belonging to the same species and to group strains within a species (McCartney, 2002).

PFGE has been applied to strain typing of members of the *Lb. acidophilus* complex (Rousell *et al.*, 1993; Chevallier *et al.*, 1994; Daniel, 1995; Lortal *et al.*, 1997; Klaenhammer, 1998; Klein *et al.*, 1998; Vincent *et al.*, 1998; El-Osta *et al.*, 2002; Ventura and Zink, 2002) and various lactic acid bacteria including: *Lc. lactis* (Tanskanen *et al.*, 1990), *Lb. gasseri* (El-Osta *et al.*, 2002), *Leuconostoc oenos* (Kelly *et al.*, 1993), *Lb. plantarum* (Daniel, 1995), *Str. thermophilus* (Boutrou *et al.*, 1995), *Lactobacillus sake* (Bjorkroth *et al.*, 1996), *Lb. helveticus* (Lortal *et al.*, 1997), *Propionibacterium* (Gautier *et al.*, 1996), *Bif. breve* (Bourget *et al.*, 1993), *Lb. casei* (Ferrero *et al.*, 1996) and *Lb. johnsonii* (Ventura and Zink, 2002).

1.2.2.3 16S ribosomal RNA gene sequencing

Bacterial 16S rRNA gene sequences are perfect targets for classification studies because of their high copy number and their sequence homology patterns. 16S ribosomal RNA gene sequencing involves extraction of nucleic acids, amplification of genes encoding 16S rRNA, and comparison of rRNA gene restriction patterns with control strains in identification databases (Guarneri *et al.*, 2001; Delley and Germond, 2002; Lyhs *et al.*, 2002; Ogier *et al.*, 2002; Roy *et al.*, 2000; Fasoli *et al.*, 2003).

Many authors have identified species of the *Lb. acidophilus* complex using DNA probes, species-specific primers, or by sequencing the variable regions of the 16S rRNA gene and aligning these sequences with those of the type strains. The accuracy of 16S rRNA sequencing is highly dependant on the selection of probes/primers used to amplify regions of the 16S rRNA gene. Ribosomal RNA targeted oligonucleotide probes have been used to identify *Lb. acidophilus* (Sui *et al.*, 2002), *Lb. gasseri*, *Lb. johnsonii*, *Lb. crispatus* and *Lb. amylovorus* (Roy *et al.*, 2000). However, the oligonucleotide probe designed for *Lb. acidophilus* has been shown to hybridize with nucleic acids of type strains of *Lb. gallinarum* and *Lb. helveticus* (Roy *et al.*, 2000). These species are closely related phylogenetically, with less than 3% difference in the

16S ribosomal sequence of these organisms (Drake *et al.*, 1996). In addition, 16S rRNA gene sequence analysis showed that a strain previously identified as *Lb. acidophilus* based on phenotypic criteria clustered with both the *Lb. acidophilus* and *Lb. lactis* group (Dewhirst *et al.*, 1999).

The success of this technique also relies on the completeness and accuracy of the databases used for sequence comparison. Yeung *et al.* (2002) reported that ‘all strains speciated as *Lb. crispatus* by GenBank were identified as *Lb. acidophilus* by the Ribosomal Database Project’. In addition, ‘some databases did not represent the most current nomenclatures’ (Yeung *et al.*, 2002).

The majority of probes/primers have been designed to recognise two main variable regions of the *Lb. acidophilus* complex, the V1 region (Hensiek *et al.*, 1992; Schleifer *et al.*, 1995; Kullen *et al.*, 2000; Horie *et al.*, 2002; Tarnberg *et al.*, 2002; Ventura and Zink, 2002) and the V3 region (Sui *et al.*, 2002; Tarnberg *et al.*, 2002). The V1 region is located just downstream of the 5’ end of the 16S rRNA gene and consists of approximately 50 base pairs of sequence. The second region, V3 (a + b), is located about 1,000 base pairs downstream of the 5’ of the 16S rRNA gene and is approximately 50 base pairs in length.

The taxonomy and physiology of probiotic lactic acid bacteria can only be fully understood by using a polyphasic taxonomy approach combining morphological, biochemical and physiological characteristics with molecular based phenotypic and genomic techniques.

1.3 SELECTION CRITERIA OF PROBIOTICS

1.3.1 Survival aspects during consumption

A characteristic of special importance when selecting strains for probiotic use is the ability of strains to survive transit through the gastrointestinal tract. Undoubtedly, probiotic organisms will vary in their ability to survive the harsh physico-chemical conditions present in the gastrointesinal tract, including the gastric acid, bile secretions and competition with resident flora.

After the more or less neutral pH of the oral cavity, the low pH of the stomach (ranging from 1.0 to 3.5) is harmful to most microbes. The population averages 10^3 bacteria per gram, and is dominated by Gram-positive bacteria such as streptococci and lactobacilli, and by yeasts. The duodenum also represents a hostile environment and contains relatively low numbers of transient microbes due to the aggressive nature of the intestinal fluids and the relatively short transit time of one to two hours (Holzapfel *et al.*, 1998). During digestion, bile (made up of cholic and chenodeoxycholic acids) enters the small intestine and the stomach. Bile concentrations range from 0.5 to 2.0% in the first hour of digestion and the levels may decrease during the second hour (Davenport, 1977). The regions immediately following the stomach have the highest acidity, with the pH of these areas falling to as low as 1.5 (during fasting), and after a meal the pH may rise to between pH 4 to 5.

A number of different *in vitro* assays have been described to identify acid resistant strains, ranging from exposure of strains to pH-adjusted phosphate buffered medium (PBS) (Conway *et al.*, 1987; Charalampopoulos *et al.*, 2003), incubation in gastric contents (Mayara-Makinen *et al.*, 1983) and the use of a dynamic model of the stomach (Berrada *et al.*, 1991; Marteau *et al.*, 1997; Fernandez *et al.*, 2003). Walker and Gilliland (1993) reported that there was considerable variation between cultures in their ability to grow in the presence of bile, to deconjugate sodium taurocholate, and to assimilate cholesterol.

Although it is difficult to compare results between individual studies, in general, acid and bile tolerance was found to be a strain specific characteristic (Gilliland and Speck, 1977a; Gilliland *et al.*, 1984; Conway *et al.*, 1987; Berrada *et al.*, 1991; Clark *et al.*, 1993; Walker and Gilliland, 1993; Buck and Gilliland, 1994; Lankaputhra and Shah, 1995; Gupta *et al.*, 1996; Godward *et al.*, 2000). *Lb. acidophilus* strains generally survived well above pH 3.0 (Conway *et al.*, 1987; Lankaputhra and Shah, 1995) and at bile concentrations varying from one to three percent bile (Overdahl and Zottola, 1991; Sanders *et al.*, 1996).

A higher proportion of strains of human origin were found to be tolerant to both low pH and high bile in comparison to strains of dairy origin (Prasad *et al.*, 1998).

However, in many of these studies, species identity was confirmed using a series of phenotypic marker tests. Therefore, observed differences in bile tolerance between strains may be a result of misidentification of species; different species within the *Lb. acidophilus* complex once identified as *Lb. acidophilus*.

1.3.2 Selection of probiotic strains: additional requirements

By the late 1950s, Gordon and colleagues noted that successful *Lactobacillus* therapy depended on certain criteria: being of human origin (if intended for human use), strains being non-pathogenic and that a large number of viable cells were required for rapid establishment (Gordon *et al.*, 1957). Probiotic organisms should also be able to survive gastric passage (Hoier, 1992); adhere to mucosal surfaces; be safe for food and medical use; have clinical validation and published health benefits; and display good technological properties (Gilliland, 1979; Ouwehand *et al.*, 1999). Collins *et al.* (1998) compiled a list of 12 important criteria for selecting potential probiotic strains. Essentially, these criteria suggest the ‘strains must be safe, viable and metabolically active within the gastrointestinal tract in order to exert a beneficial impact on the host’.

Following accurate identification and evidence confirming health benefits to the consumer, a demonstration of the ability to maintain the desired characteristic during culture preparation, storage, delivery and also when the organism reaches the intestinal tract is necessary.

One of the most important characteristics of a probiotic strain is that it should possess GRAS (Generally Regarded As Safe) status. Collins *et al.* (1998) stated that ‘The long history of human exposure and consumption of probiotic products has led to the reasonable conclusion that they are generally regarded as safe’. For this reason, in the USA, manufacturers are required to self-declare GRAS status by notifying the appropriate authority. ‘The notification should outline the basis of the determination of GRAS status, either through scientific procedures or common use in food, and must provide reasonable certainty that the substance is not harmful under the intended conditions of use’ (Feord, 2002).

Another important consideration is the probiotic must be non-pathogenic. Lactobacilli are rarely responsible for infection in humans, nor are probiotics products based on these organisms (Borriello *et al.*, 2003). Lactic acid bacteria have been administered to patients with Crohn's disease, diarrhoea or Acquired Immune Deficiency Syndrome (AIDS), and to premature children, allergic infants, healthy children and the elderly without any adverse effects being reported (O'Brien *et al.*, 1999). There have, however, been a number of reports of human infections caused by lactic acid bacteria, although the source of the infection was the commensal lactic acid bacteria flora rather than probiotic bacteria and the patient had some underlying disease or predisposing condition (Adams, 1999).

The use of new strains, particularly if the strains have been genetically modified or have been derived from animals, will likely require more detailed evidence before use (Adams, 1999). The key criteria for the safety assessment of genetically modified microorganisms include: the safety of the gene product and its effect on the product and the properties of the microorganism, specificity of expression of the novel genetic material, the potential for gene transfer to occur in the gut, and the ability of the organism to survive and colonise in the human gut (Feord, 2002).

It is important that strains do not carry transferable resistance determinants or have the ability to facilitate plasmid transfer (Danielsen and Wind, 2003). 'The presence of resistance genes and the transfer of plasmids and conjugative transposons to and from lactic acid bacteria' have been reported as reviewed by Teuber *et al.* (1999). In 1997, a report by the Scientific Committee for Animal Nutrition recommended to the European Union Commission that 'the absence of transferable resistance genes should be an important pre-requisite for approval of starter cultures and probiotic organisms' (European Commission, 2001). In addition, probiotic organisms should not carry intrinsic resistance to antibiotics that would prevent treatment of rare probiotic infection (Borriello *et al.*, 2003).

Strains intended for dairy products must efficiently ferment milk, either alone or in combination with other strains. The culture should also have an acceptable stability during the preparation process including centrifugation, pellet freezing or freeze-

drying, and consecutive storage and distribution (Hoier, 1992; Collins *et al.*, 1998; Klaenhammer and Kullen, 1999). Furthermore, probiotic organisms must remain viable and active in the carrier matrix before consumption (Gilliland, 1979). It is necessary to meet the criterion of minimum of 10^6 cfu/ml at the expiry date, as the minimum therapeutic dosage per day is considered to be 10^8 to 10^9 (Kurmann and Rasic, 1991) corresponding to an intake of 100 g product containing 10^6 to 10^7 cfu/ml. The selected strains must multiply to high cell counts in the fermented product and possess a high acid tolerance so as to maintain a high viable cell count during storage. Further, the selected strains must produce a final product with good taste and acceptable body and texture, since only by satisfying the demands of the consumer can the food industry succeed in promoting the consumption of functional probiotic products in the future (Klaenhammer and Kullen, 1999).

In addition, selected strains must be capable of survival, proliferation and metabolic activity at the target site, be able to compete with the resident microflora, and be resistant to bacteriocins, acid and other antimicrobials produced by them (Holzapfel *et al.*, 1998; Klaenhammer and Kullen, 1999). Thus the ability to produce effective antimicrobial substances and antibacterial factors that are antagonistic for potentially pathogenic microorganisms becomes an important consideration for selecting strains for dietary adjuncts.

Despite all potential weaknesses of *in vitro* models, they do appear to show promise for the adhesive abilities of probiotic organisms *in vivo* (Johansson *et al.*, 1993; Mattila-Sandholm *et al.*, 1999; Matijasic *et al.*, 2003). Lactic acid bacteria adhere specifically to cells of the species from which they were isolated (Morishita *et al.*, 1971; Wesney and Tannock, 1979; Barrow *et al.*, 1980; Gilliland *et al.*, 1980) and the ability to adhere is a strain dependent characteristic (Conway *et al.*, 1987; Klaenhammer, 1995; Kimoto *et al.*, 1999; Plant and Conway, 2002). Therefore it is important that strains selected be of human origin (Savage, 1977; Gilliland, 1979; Tannock, 1995).

One of the major scientific challenges for the selection of the best species or strains for use as probiotics is understanding the mechanisms by which probiotics exert their beneficial effects *in vivo* (Vaughn *et al.*, 1999). One consistent feature of probiotic research is the variability of experimental results. However, too many publications demonstrate the beneficial effects of probiotics to simply reject the probiotic concept. Therefore, we need a greater understanding of our indigenous microflora and the basic ecological principles operating in the gastrointestinal tract to establish a scientific basis for the probiotic concept (Holzapfel *et al.*, 1998).

1.4 REGULATORY ASPECTS OF PROBIOTIC PRODUCTS

‘While consumers are readily adopting these developing concepts of nutrition, the legislative environment is slowly adapting to encompass all potential applications of these products’ (Feord, 2002). The Australian Food Standards Code (Standard H8) states that yogurt must have a pH of less than or equal to 4.5 and be prepared with *Str. thermophilus* and *Lb. bulgaricus* or other suitable lactic acid bacteria. There are no specifications regarding the numbers of viable probiotic organisms in the products (Sibraa, 1993), however advisory standards for starter culture bacteria stipulate bacterial counts of greater than 10 million colony forming units per gram (cfu/g) as satisfactory (Davies and Ashton, 1971), whereas the suggested minimum level for probiotic bacteria in yogurt is one million colony forming units per gram (Kurmann and Rasic, 1991). These standards were probably introduced to provide bacterial concentrations that are technologically attainable and cost-effective rather than to achieve a specific health effect in humans (Sanders *et al.*, 1996).

The International Dairy Federation (1997) defined fermented milk as ‘a milk product fermented by the action of specific microorganisms resulting in reduction of pH and coagulation. These specific microorganisms shall be viable, active and abundant in the product to the date of minimum durability.’ Furthermore, in many countries, including Japan, America and France, regulatory levels of probiotic bacteria in dairy products have been established (Reins, 1984; Ishibashi and Shimamura, 1993; Roberts and Maust, 1995; Shortt, 1999). Generally, products are required to contain at least ten million live lactic acid bacteria per millilitre at the time of sale in order to place a

‘Live and Active Culture’ logo on the product packaging (Roberts and Maust, 1995; Shortt, 1999).

‘Within Europe, there has never been a harmonised authorisation process for placing microorganisms into foods, or using them as processing aids in the manufacture of a variety of products’ (Feord, 2002). Where a microorganism is used as an ingredient, it is labelled on the final food product. However, some countries classify microorganisms as processing aids and there is no requirement to indicate their use on the label of the final food (Feord, 2002).

Probiotic dairy products have been placed on the market with rather vague claims regarding therapeutic effects. Claims include: ‘beneficial *Bif. longum* and *Lb. acidophilus* have long been associated with good health...’; ‘...helps to maintain the balance of your digestive system’; ‘...Medical evidence suggests that these cultures can have a beneficial effect on the digestive system...’ (Fuller, 1993).

In Japan, 13 probiotic species have been authorised under Foods for Specific Health Use (Feord, 2002), whilst Sweden, the Netherlands, Belgium and the United Kingdom all follow ‘Codes of Practice’ when generating evidence to substantiate health claims. Feord (2002) reported that ‘the FDA recognises three types of health claim that can be made for foods and dietary supplements. These are:

- a) NLEA (Nutrition Labelling and Education Act) authorised health claims.
This is based on extensive review of the scientific literature (Feord, 2002),
- b) Health claims based on authoritative statements (from a scientific body of the US Government or the National Academy of Sciences), as defined in the 1997 Food and Drug Administration Modernization Act (FDAMA) (Feord, 2002),

- c) Qualified health claims, based on the weight of scientific evidence, as a result of the US Court of Appeals for the DC Circuit 1999 decision in the case of *Pearson v. Shalala* (Feord, 2002).

In most countries, including Australia, it is not acceptable to print health claims on the packaging of foods (Hoier, 1992; Feord, 2002). For example, in Canada, the Food and Drug Act Regulations prevent manufacturers from making health claims in the marketing of a food product; even if a manufacturer has a health claim that is known to be true, the claim cannot be used legally in this country. This issue is the subject of strong debate between scientists, authorities and industry, in order to try to reach some agreement on claim acceptability and substantiation (Feord, 2002).

As scientific knowledge and biotechnologic proficiency advance at an accelerating pace, the requirement for informed legislation is essential (Dunne and Shanahan, 2002). In the absence of such legislation, the recommendations of the voluntary codes are extremely important. However, until legislation is passed, it is likely that certain countries will continue to work along voluntary lines, while other countries, including Australia, forbid all types of health claims.

1.5 VIABILITY OF *LB. ACIDOPHILUS* IN FERMENTED PRODUCTS

To maintain confidence in probiotic products, it is important to demonstrate good survival of the bacteria in consumer products during their shelf life. In addition, an agreeable taste and attractive texture are imperative for all dairy products irrespective of the health message of the product (Saxelin *et al.*, 1999).

Studies (Hull *et al.*, 1984b; Shah *et al.*, 1995; Dave and Shah, 1997c; Micanel, *et al.*, 1997; Rybka and Fleet 1997) that analysed the survival of *Lb. acidophilus* in a variety of yogurt products have shown that *Lb. acidophilus* is unstable in yogurt and exhibited a constant decline in the viable count during storage, particularly after 20 days of storage. Vinderola *et al.* (2000) and Lourens-Hattingh and Viljoen (2002)

reported that after four weeks of refrigerated storage, all products contained lower than the suggested levels of 10^6 cfu/g of viable *Lb. acidophilus*. Other studies have found that viability of *Lb. acidophilus* in yogurt is a strain dependant characteristic (Nighswonger *et al.*, 1996 Rybka and Fleet, 1997; Schillinger, 1999), with some strains of *Lb. acidophilus* surviving at the recommended quantity of 10^6 cfu/g of yogurt over the storage period (Oliveira *et al.*, 2002). Rybka and Fleet (1997) studied the viability of *Lb. acidophilus* in 50 commercial Australian yogurts and found that only 24% of samples contained $>10^6$ cfu/g of *Lb. acidophilus* (Rybka and Fleet, 1997).

When added to set yogurt after its manufacture, *Lb. acidophilus* rapidly lost viability (90 to 99 %) within three to five days and completely disappeared within seven days (Hull *et al.*, 1984). When the organism was added with the yogurt culture organisms during manufacture, loss of viability still occurred but survival improved (Gilliland and Speck, 1977b; Schioppa *et al.*, 1981; Hull *et al.*, 1984b).

Lb. acidophilus maintained viability above the recommended level in other products, including fermented milks (Mijacevic *et al.*, 2001), cultured soy milks (Wang *et al.*, 2002), oat-based and non-dairy products (Martensson *et al.*, 2002), but failed to maintain viability in coleslaw during refrigerated storage (Rodgers and Odongo, 2002). These results cannot be compared to viability studies in yogurt products, as these products were assayed over shorter periods and at different storage temperatures.

Differences in survival of *Lb. acidophilus* between studies may be attributed to differences in the culture medium and conditions and also differences in the starter cultures selected for yogurt production, as these may produce bacteriocins against the *Lb. acidophilus* (Young and Nelson, 1978; Gilliland and Lara, 1988; Kurmann and Rasic, 1991; Costello, 1993). Also, differences observed could be a result of misidentification of probiotic organisms due to the use of biochemical assays to differentiate between the species formerly belonging to the *Lb. acidophilus* group (Antonio *et al.*, 1999).

1.5.1 Reasons for loss of viability during manufacture and storage

‘The incompatibilities between the natural growth environment and the conditions prevalent in industrial fermentation place the bacterium under a severe stress. The end of fermentation imposes a hostile environment, involving low pH, absence of nutrients, unfavorable osmotic conditions and low temperature’ (McDonald *et al.*, 1990).

Factors which may affect the viability of probiotic organisms in yogurt are the selected strains (Vinderola *et al.*, 2000), interaction between the species present, culture conditions, production of hydrogen peroxide due to bacterial metabolism (Hull *et al.*, 1984; Dave and Shah, 1997a,b), final acidity of the product (Lourens-Hattingh and Viljoen, 2002), the concentrations of lactic acid and acetic acids, altered oxidation-reduction potential and the production of bacteriocins (Young and Nelson, 1978; Gilliland and Lara, 1988; Kurmann and Rasic, 1991; Costello, 1993). The viability also depends on the availability of nutrients, growth promoters and inhibitors (sodium chloride and hydrogen peroxide), concentration of sugars (osmotic pressure), dissolved oxygen and oxygen permeation through the package, inoculation level and incubation, and fermentation and storage temperature (Gilliland and Lara, 1988; Dave and Shah, 1997a; Vinderola *et al.*, 2000). However, the main factor for loss of viability of *Lb. acidophilus* has been attributed to a decrease in the pH of the medium and accumulation of organic acids as a result of growth and fermentation (Hood and Zottola, 1988, Shah and Jelen, 1990; Hughes and Hoover, 1991; Lankaputhra and Shah, 1995; Dave and Shah, 1997a; Lourens-Hattingh and Viljoen, 2002).

The yogurt cultures *Str. thermophilus* and *Lb. bulgaricus* are active even during refrigerated storage and produce small amounts of lactic acid as a result of lactose fermentation. This leads to a pH decrease from 4.5 to 5 to between 3.76 to 4.35 during storage, depending on the starter cultures used and the level of inoculum (Hull *et al.*, 1984; Sinha *et al.*, 1989; Nighswonger *et al.*, 1996; Dave and Shah, 1997a).

‘As the yogurt acidifies, the proportion of undissociated acid increases, making more organic acid available to penetrate the cell membrane. After the associated form of

the acid is inside the cell, the more alkaline intracellular environment will cause dissociation of the weak acid and acidification of the cytoplasm' (Baik *et al.*, 1996). This leads to the inhibition of nutrient transport, as a result of the collapse of the proton motive force (Baik *et al.*, 1996). 'The primary mechanism of pH maintenance in microorganisms is dependent upon the expulsion of protons from the cytoplasm by H^+ -ATPases at the expense of ATP' (McDonald *et al.*, 1990). Therefore, organic acids in the medium ultimately contribute to suppression of growth because of a decrease in availability of energy for processes other than maintenance of pH homeostasis.

Hydrogen peroxide (H_2O_2) or oxygen (O_2) may damage the bacteria that produce them by reacting with cell components or by giving rise to other potentially toxic agents (Gregory and Fridovich, 1974). Dave and Shah (1997c) found that oxygen content affected the viable counts of *Lb. acidophilus*, with cultures stored in glass bottles maintaining higher counts compared to those stored in plastic cups.

Storage temperature may be a catalyst for decreased viability of probiotic organisms, only because at higher temperatures, increased growth leads to higher final acidity and production of hydrogen peroxide (Matalon and Sandine, 1986).

The viability of *Lb. acidophilus* is also dependent on the associative yogurt organism, that is, whether *Lb. bulgaricus* is present in the culture mix (Gilliland and Speck, 1977; Dave and Shah, 1997; Rybka and Fleet 1997). Rybka and Fleet (1997) reported that yogurts containing *Lb. bulgaricus* were below pH 4.0 and tended to contain probiotic organisms at a rate of less than 10^6 per gram. Dave and Shah (1997a) also found that the counts were highest for *Lb. acidophilus* in products devoid of *Lb. bulgaricus*, with viable counts remaining at satisfactory levels for 35 days. Yogurts containing *Lb. bulgaricus* demonstrated lower survival rates of *Lb. acidophilus*, with the majority at around 10^4 cfu/g after 35 days of storage. Similarly, Gilliland and Speck, (1977b) reported that products containing high counts of *Lb. acidophilus* had low counts of *Lb. bulgaricus*, suggesting that *Lb. bulgaricus* has an impact on the viability of *Lb. acidophilus* due to production of D(-) lactic acid and production of hydrogen peroxide (Gilliland and Speck, 1977b; Dave and Shah, 1997b).

1.5.2 Strategies to improve viability of probiotic organisms

Due to the quantities of probiotic products being produced on a regular basis, small improvements in survival of probiotic organisms will have an important impact on the dairy industry, in regards to fermentation parameters as well as the storage stability and activity of probiotic organisms during consumption.

Viability of probiotic bacteria can be improved by appropriate selection of acid and bile resistant strains, use of oxygen impermeable containers (Dave and Shah, 1997b), two-step fermentation, micro-encapsulation (Carcoba and Rodriguez, 2000; Sultana *et al.*, 2000), incorporation of micronutrients such as peptides and amino acids or by sonication of yogurt bacteria (Shah and Lankaputhra, 1997).

Shah and Lankaputhra (1997) prepared yogurts using ruptured starter cultures *Str. thermophilus* and *Lb. bulgaricus* and whole probiotic cells. The viability of probiotic bacteria was then compared to yogurts made with unruptured starter cultures and probiotic organisms. The yogurt bacterial cells were ruptured to release their intracellular β -galactosidase enzyme and reduce their viable counts to improve the viability of probiotic bacteria. Viable counts of probiotic bacteria after fermentation were two log cycles higher in yogurt made with ruptured yogurt bacteria and whole cells of probiotic bacteria and their viability after six weeks of storage remained above the recommended level of 10^6 cfu/g. Yogurt made with ruptured yogurt bacteria contained less hydrogen peroxide during fermentation. The β -galactosidase released upon sonication could be used to hydrolyse a portion of lactose in milk and the products of lactose hydrolysis, glucose and galactose, could be used by organisms such as *Lb. acidophilus* and bifidobacteria. The limitation of this process is that the fermentation time of yogurts made with ruptured cells was two hours longer than that using unruptured starter cultures. These incubation times would not meet the manufacturer's requirements of production efficiency.

Vinderola *et al.* (2000) evaluated the carbonation of pasteurised milk as a method for improving the survival of *Lb. acidophilus*. Their results showed that the viability of *Lb. acidophilus* over 49 days of cold storage in fermented milks was dependent on the

presence of other probiotic organisms, since the viability of *Lb. acidophilus* was higher in yogurts containing only *Str. thermophilus* rather than *Str. thermophilus* and *Bif. bifidum*. Milks with *Bif. bifidum* generally had lower manufacture times (to pH 5.0) than milks with *Lb. acidophilus* and *Str. thermophilus* only. Carbonated milk products decreased to pH 5.0 quicker than non-acidified milks.

Manipulating the conditions in the manufacture and storage of yogurt can increase the survival of lactic acid bacteria. For example, terminating fermentation at a higher pH (pH >5) (Varnam and Sutherland, 1994), lowering the storage temperature to less than 3 to 4°C (Sakai *et al.*, 1987), adding retardants that prevent pH change during refrigerated storage (Supraidi *et al.*, 1994), application of hydrostatic pressure (200 to 300 mPa for 10 min at room temperature) to prevent post-acidification (Tanaka and Hatanaka, 1992), heat shock of yogurt at 58°C for five minutes to prevent post-acidification (Marshall, 1992) and adjusting the level of inoculation of organisms (Varnam and Sutherland, 1994) have all been found to enhance the survival of lactic acid bacteria in yogurt.

Nutritional supplements have been added to yogurts to improve the survival of probiotic organisms during storage. Supplements include glucose, fructose, magnesium and manganese (Ahmed and Mital, 1990), casitone, casein hydrolysate (Sodini *et al.*, 2002) and fructose (Saxena *et al.*, 1994), acetate (Marshall, 1991), ascorbic acid (Dave and Shah, 1997c), cysteine, whey powder, whey protein concentrate, acid casein hydrolysate or tryptone (Dave and Shah, 1998; Sodini *et al.*, 2002). Dave and Shah (1997c) used ascorbic acid as an oxygen scavenger for the improved viability of probiotic cultures in yogurt. They found that ascorbic acid was effective as an oxygen scavenger, but only for up to 10-15 days when products were stored in plastic cups. The addition of ascorbic acid did not seem to have an effect on hydrogen peroxide production. The addition of ascorbic acid decreased the counts of *Str. thermophilus*, but increased the counts of *Lb. bulgaricus* and *Lb. acidophilus*. With *Lb. acidophilus*, controls not containing ascorbic acid maintained counts between 10^4 to 10^5 cfu/gram at day 35, whilst yogurts containing ascorbic acid had counts of *Lb. acidophilus* at 10^6 /gram (Dave and Shah, 1997c).

Dave and Shah (1998) repeated these experiments using cysteine, whey powder, whey protein concentrate, acid casein hydrolysate and tryptone. Addition of cysteine and acid caseine hydrolysate during the yogurt making process improved the viability of *Lb. acidophilus* after 35 days of storage. Those samples having higher protein content were found to have less post-acidification, probably due to increased buffering action of milk proteins and differences in the ratio of starter cultures to probiotic cultures.

A similar study was performed by Sodini *et al.* (2002). The fermentation times for pure cultures of *Lb. acidophilus*, *Str. thermophilus* and *Lb. bulgaricus* to reach pH 5.0 was 11.4 hours. However, the addition of either casein hydrolysates or milk proteins decreased the fermentation time. The addition of nutrients to yogurt products is accepted in certain countries, but these nutrients often affect yogurt flavour.

Genetic improvement of starter strains and probiotic cultures has been suggested as a potential approach for enhancing their performance, but the nature of genetic variation has an impact on the safety assessment and legal implications in different countries (Mollet, 1999; Aureli and Franciosa, 2002). Strains undergoing any genetic manipulation must be regarded as 'engineered' and therefore assessed for safety (Mollet, 1999; Aureli and Franciosa, 2002). From a safety and food legislative viewpoint, it is necessary to determine the extent and nature of the genetic modifications introduced. Complete characterisation of the DNA rearrangement and of the final product, in order to establish the homogeneity with the parental strain is required (Aureli and Franciosa, 2002). An example is the trans-species genetic modification involving the transfer of good texturing properties of selected exopolysaccharide (EPS) producing strains to a non-EPS producer which is industrially or organoleptically more interesting as a starter strain (Mollet, 1999). Other examples include the enhanced acid and flavour (diacetyl and acetoin) producing properties of *Lac. lactis* ssp. *diacetylactis* following ultraviolet light-induced mutation (Kuila and Ranganathan, 1978); the re-routing of the pathway producing D-lactate in *Lb. johnsonii* to L-lactate (Vaughn *et al.*, 1999) and ultraviolet irradiation to improve the survival of *Lb. gasseri* in the presence of normally lethal levels of sodium chloride and sodium nitrite.

1.6 STRESS ADAPTATION: A NEW APPROACH FOR IMPROVING THE VIABILITY OF PROBIOTIC ORGANISMS IN YOGURT

Lactic acid bacteria regularly experience life-threatening stresses during the processing and consumption of dairy products. In response to changes in the environment, bacteria often undergo physiological alterations, which are generally referred to as stress responses. Some bacteria are also known to demonstrate an adaptive response; that is, when cells are exposed to moderate levels of stress, they acquire increased tolerance when subsequently exposed to extreme levels of the same stress. Adaptation to mild changes in the environment may allow bacteria not only to survive, but also to prosper when confronted with conditions that may ordinarily be lethal. Since lactic acid bacteria are routinely used in dairy fermentation, studies of adaptive physiology may facilitate efforts to improve bacterial activity and function in fermented dairy foods.

1.6.1 Adaptation resulting in improved viability of bacteria in other products

List. monocytogenes is capable of withstanding low pH after initial exposure to sub-lethal acidic conditions (Kroll and Patchett, 1992; Davis *et al.*, 1996; O'Driscoll *et al.*, 1997; Faleiro *et al.*, 2003; Ferreira *et al.*, 2003). Maximal acid resistance to hydrochloric acid was seen when the organism was exposed to pH 5.0 for one hour prior to challenge at pH 3.0 (Davis *et al.*, 1996; O'Driscoll *et al.*, 1997). *List. monocytogenes* was also shown to exhibit a significant adaptive acid tolerance response following one hour exposure to mild acid (pH 5.5), which is capable of protecting cells from severe acid stress at pH 3.5 (O'Driscoll *et al.*, 1996; Faleiro *et al.*, 2003).

In an attempt to determine the industrial significance of the Acid Tolerance Response (ATR), Gahan *et al.* (1996) examined the survival of adapted and non-adapted cells in a variety of acidic foods. Acid adaptation enhanced the survival of *List. monocytogenes* in acidified dairy products, including cottage cheese, yogurt and whole-fat cheddar cheese. Acid-adapted *List. monocytogenes* cultures also

demonstrated increased survival during active milk fermentation by a lactic acid culture and greatly improved survival in low-pH foods such as orange juice and salad dressing containing acids other than lactic acid. However, in foods with marginally higher pH, such as mozzarella cheese, a commercial cottage cheese, or low-fat cheddar cheese, acid adaptation did not appear to enhance survival. Collectively, the data suggest that ATR mechanisms, whether constitutive or induced, can greatly influence the survival of *List. monocytogenes* in low-pH food environments.

The ability of cells to adapt to both weak and strong acids following acid adaptation is particularly relevant when the nature of *List. monocytogenes* is considered, in that the most likely acid that the organism will encounter in yogurt is weak lactic acid whereas the initial barrier to a successful survival is presented by hydrochloric acid in the stomach. *List. monocytogenes* has also been found to adapt to high hydrostatic pressure following cold shock, with viable counts 100-fold higher than exponentially growing cells at 37°C (Wemekamp-Kamphuis *et al.*, 2002). In addition, *List. monocytogenes* developed osmotolerance at 20% sodium chloride after prior incubation in 3.5% sodium chloride (Faleiro *et al.*, 2003). These findings may affect the efficiency of preservation techniques in the food industry (Hill *et al.*, 2002).

Leyer *et al.* (1995) showed that *E. coli* O157:H7 can be adapted to low pH by culturing for one to two doublings at pH 5.0. This process increased the resistance to lactic acid and these cells survived better than their non-adapted counterparts during sausage fermentation and show enhanced survival in shredded dry salami (pH 5) and apple cider (pH 3.4).

Jan *et al.* (2001) showed that transient exposure of the cheese starter culture, *Pseudomonas freudenreichii*, to pH 5.0 for 60 minutes, afforded protection toward acid challenge at pH 2.0 with cell division during the thirty minute pH challenge compared to 4.3% survival for the control or unadapted culture. *Ps. freudenreichii* have also shown adaptive responses to bile salts (1.0 g/litre) if cells are first pre-treated with a moderate concentration of bile salts (0.2 g/litre) (Leverrier *et al.*, 2003).

1.6.2 The acid tolerance response in enteric bacteria

Acid tolerance has been extensively studied in enteric bacteria, where it may be an important factor towards enhanced pathogenicity (Foster and Hall, 1991). A variety of organisms including *Sal. typhimurium*, *Sal. enteritidis* and *E. coli* have been found to adapt to normally lethal acidic environments after being exposed initially to a moderate acid shock (Foster, 1991, 1993; Foster and Hall, 1990, 1991; Leyer and Johnson, 1992, 1997; Humphrey *et al.*, 1993; Leyer *et al.*, 1995; Lin *et al.*, 1995; Fratamico, 2003). This adaptation process increases the survival rate of these organisms in harsh conditions as compared to those that had been shifted directly into lethal acidic conditions.

Lin *et al.* (1995) compared the extreme acid survival of *Sal. typhimurium*, *Shigella flexneri* and *E. coli*, all members of the family *Enterobacteriaceae*. *Sal. typhimurium* and *E. coli*, but not *Sh. flexneri*, expressed low-pH inducible log-phase and stationary phase acid tolerance response systems that protected cells at pH 3.0.

Sal. typhimurium prefers neutral-pH environments but can adapt to survive in challenging environments such as the stomach, where significant fluctuations in hydrogen ion concentrations lead to pH values well below 5.0. This process, termed the acid tolerance response, involves culturing cells at mild pH (pH 5.5 to 6.0) before subsequent challenge at low pH (3.3). Cells cultured at mild pH were 100 to 1,000 times more resistant to extreme low pH compared to cells transferred directly from optimal (7.7) to low pH conditions (Foster and Hall, 1990; Foster, 1991, 1993). *Sal. enteritidis* also exhibits an adaptive acid tolerance response. Cells grown in Lemco broth at pH 4.0 for one hour (using hydrochloric acid) were more resistant to an acid challenge at pH 2.5 than cells transferred directly from pH 7 to pH 2.5 (Humphrey *et al.*, 1993).

Similarly, Goodson and Rowbury (1989) reported that *E. coli* grown initially at pH 5 survived exposure to normally lethal acid challenge of pH 3.5 (hydrochloric acid adjusted) compared to cells grown initially at pH 7.0. Samelis *et al.* (2002) reported that *E. coli* cells exposed to an acidic mix of lactic/acetic acid maintained viability at a higher rate during meat decontamination than did non-adapted populations, whilst de

Jonge *et al.* (2003) reported that acid adaptation of *E. coli* O157 can take place during passage of the human stomach, without pre-conditioning at moderate pH values, as acid sensitive cultures became acid resistant when incubated in an environment with a changing pH. Cheng *et al.* (2003) subjected three strains of *E. coli* O157:H7 to pH 5.0 for 1 to 6 hours prior to acid challenge at pH 3.0, 4.0 or 5.0 (acidified using hydrochloric acid). All strains exhibited adaptive acid tolerance responses, although the degree of adaptation was dependant on the strain, adaptation time and pH of the challenge.

1.6.3 Stress responses in Lactic Acid Bacteria

It is now well established that lactic acid bacteria, like other bacteria, have evolved stress-alert systems and defenses against stress that permit them to resist harsh conditions and sudden environmental changes. Adaptive responses can be induced readily in lactic acid bacteria by transferring cells to moderate growth conditions for the time required for biomass doubling (Hartke *et al.*, 1996; Lorca *et al.*, 1998; Oberg *et al.*, 1998; Baati *et al.*, 2000; Duwat and *et al.*, 2000; Lorca and Valdez, 2001a; Kim *et al.*, 2002; Cotter and Hill, 2003).

Hartke *et al.* (1996) found a dramatic increase in survival of *Lac. lactis* to a severe acid stress (pH 3.9) was obtained by pre-exposing the cells for 30 minutes to mild acid conditions at pH 5.5. Kim *et al.* (1999) reported that, in agreeance with other species, stationary phase cultures of *Lac. lactis* ssp. *lactis* and *Lac. lactis* ssp. *diacetylactis* are naturally tolerant to acid, bile-salt and freezing stresses compared to their log-phase counterparts (Davis *et al.*, 1996; O'Driscoll *et al.*, 1997).

There have been several reports on the adaptive responses of probiotic bacteria to a range of stresses. Oberg *et al.* (1998) found that heat shock improved the ability of log phase *Lb. acidophilus* NCFM, *Lb. casei* LC301 and *Lb. helveticus* LH212 cells to withstand 20 minutes at 50°C, 42°C and 52°C respectively, with 27-fold, 5-fold and 11-fold improvements in survival.

Similarly, Kim *et al.* (2002) found that if *Lb. acidophilus* cells were first exposed to moderate levels of bile, heat or sodium chloride, their survival rates improved significantly compared to cells directly challenged, with 100-fold improvement for bile and heat stress and a 28% improvement for sodium chloride challenge.

Baati *et al.* (2000) reported that a slow cooling rate and pre-freezing brought about an increase in cell resistance and preservation of the physiological characteristics of *Lb. acidophilus* at -80°C , with 75% of cells surviving compared to 43% of cultures transferred directly to -80°C .

Recent reports have shown that *Lb. acidophilus* cultures are also capable of adapting to low pH conditions following transfer into moderate pH-adjusted medium during exponential growth. Lorca and Valdez (2001b) showed that *Lb. acidophilus* cultures grown without pH control were tolerant to pH 3.0, whilst cultures grown in pH controlled medium (pH 6.0) were sensitive to acid challenge.

1.6.4 Inducing cross-protection following cell conditioning

The ability of one stress condition to provide protection against another stress is referred to as cross-protection. The stress responses to acid have also been found to either fully or partially protect the cell against other forms of stress (Foster and Hall, 1990; Leyer and Johnson, 1993; Foster and Spector, 1995; Koga *et al.*, 1999; Laplace *et al.*, 1999; Kim *et al.*, 2001; Begley *et al.*, 2002). Leyer and Johnson, (1993) reported that acid-adapted *Sal. typhimurium* had an increased tolerance towards various stresses including heat, salt, an activated lactoperoxidase system, and the surface-active agents crystal violet and polymyxin B. Wang and Doyle (1998) found that *E. coli* cells that underwent heat shock at 48°C for 10 minutes survived pH 2.5 in minimal glucose medium 10 to 100 times greater than untreated cells.

Cross-protection has also been observed for *List. monocytogenes*, with cells adapted at pH 5.5 for one hour demonstrating increased tolerance toward thermal stress, crystal violet (Foster and Spector, 1995; Koga *et al.*, 1999), osmotic stress, ethanol (Foster and Spector, 1995), bile and deoxycholic acid (Koga *et al.*, 1999). *List.*

monocytogenes subjected to a short osmotic shock of 0.5, 1.0 or 1.5 mol l⁻¹ sodium chloride for five minutes showed an increase in thermotolerance (Jorgensen *et al.*, 1995), whilst another study using *List. monocytogenes* reported that prior adaptation of exponential phase cells using sub-lethal levels of bile acids, pH, temperature, salt or sodium dodecyl sulfate stress significantly enhanced bile resistance (Begley *et al.*, 2002).

Reports have also extended to the lactic acid bacteria group. Subjecting *Lac. lactis* cells to irradiation resulted in improved survival against acid, ethanol, hydrogen peroxide or heat stress (Hartke *et al.*, 1996). In a selection procedure for thermoresistant mutants of the thermosensitive *Lac. lactis* recA strain, a number of disrupted genes appeared to be the same as those found during selection for acid resistant mutants (Rallu *et al.*, 1996; Hartke *et al.*, 1997). These results suggest a link between the heat shock response and acid resistance of *Lac. lactis*.

Murga *et al.* (2001) reported that *Lb. acidophilus* CRL 640 was less sensitive to freeze-thawing when it was grown at 25°C, with 33% cell death compared to 87% cell death for cells grown at 37°C. In addition, cells grown at 25°C were less sensitive to sodium chloride. Kim *et al.* (2001) reported that *Lb. acidophilus* that were pre-exposed to bile stress were significantly more resistant to heat stress, but not to sodium chloride stress, whilst cells pre-exposed to sodium chloride stress were significantly more resistant to bile and heat stresses. In contrast, pre-exposure to heat stress did not increase the resistance against either bile or sodium chloride stress.

Laplace *et al.* (1999) reported that for the lactic acid bacterium commonly found in fermenting apple juice, *Lactobacillus collinoides*, cross-resistance to ethanol and acid shock could be induced following pre-treatment at 30°C. However, exposing cells to acid pre-treatment did not afford protection against ethanol or heat shock. Induction of tolerance was maximal with stresses of a homologous nature.

In another study using probiotic organisms, Prasad *et al.* (2003) reported that *Lb. rhamnosus* cultures pre-stressed with either heat (50°C) or salt (0.6M) showed

significant improvement in viability compared with the non-stressed control culture after storage at 30°C in the dried form.

1.7 ANALYSIS OF THE ADAPTIVE RESPONSE

1.7.1 Stationary versus exponential phase

Bacterial growth is a self-limiting process. When the medium can no longer provide the necessary nutrients (Hartke *et al.*, 1995), or if a substance inhibitory for growth is produced, bacterial division ceases and cultures enter the stationary growth phase. It is generally accepted that stationary phase is the most common state of bacterial cells in nature. Many authors have reported that survival in extreme conditions is affected by factors such as growth phase (Foster and Hall, 1991; Hall *et al.*, 1995; Hartke *et al.*, 1994, 1995; Davis *et al.*, 1996; O'Driscoll *et al.*, 1996; Lorca *et al.*, 1998; Kim *et al.*, 1999; Giard *et al.*, 2001; Kim *et al.*, 2001; Begley *et al.*, 2002; Leverrier *et al.*, 2003).

The best examples of multiple stress resistance are that found in the stationary phase of growth. Stationary phase cultures of *Lac. lactis* (Hartke *et al.*, 1994, 1995, 1996) and *Lb. acidophilus* (Lorca *et al.*, 1998; Kim *et al.*, 2001) are highly resistant to acid, bile-salt and temperature stresses compared to their log-phase counterparts. Lorca and de Valdez (2001b) reported that stationary phase cells of *Lb. acidophilus* showed 10 to 1,000 times more resistant to ethanol, hydrogen peroxide, freezing and lyophilization compared to cells grown in pH 6.0 controlled medium. However, stationary phase did not afford protection to cells during a challenge with heat or osmotic stress.

List. monocytogenes cultures acquired acid resistance upon entry into the stationary phase, while exponential phase cultures require adaptation to induce acid tolerance (Davis *et al.*, 1996; O'Driscoll *et al.*, 1996; Begley *et al.*, 2002). Stationary phase cells of *List. monocytogenes* L028 were naturally tolerant to concentrations of bovine, porcine and human bile acids well in excess of those encountered *in vivo*, whilst exponential phase cells were sensitive to unconjugated bile acids, prior to adaptation at sublethal levels of bile acids (Begley *et al.*, 2002).

Lorca and de Valdez (1999, 2001b) reported two main differences between the adaptive responses that are initiated during exponential phase versus the stationary phase-associated stress response. The first is that 'the adaptive response usually involves the induction of specific groups of genes designed to cope with a specific stress condition, whilst the stationary phase-associated stress response involves the induction of numerous regulons designed to cope with numerous stress conditions'. The second difference is that 'unlike the adaptive response, the stationary phase-associated response does not require any exposure to stress to develop the response'. This implies that the regulatory mechanisms underlying the induction of the two systems could be quite different, although some of the gene products produced by the two systems are the same.

1.7.2 Production of proteins during the stress response

Many papers have reported that when cultures are exposed to cellular stress (temperature, pH, cold shock, salt) there is an upshift in the production of certain proteins that are produced over the level of naïve cells (Lindquist, 1986; Foster, 1991, 1993; Foster and Hall, 1991; Kunji *et al.*, 1993; Hall *et al.*, 1995; Hartke *et al.*, 1994, 1995, 1996; Davis *et al.*, 1996; Broadbent *et al.*, 1998; Lorca and Valdez, 1998; Oberg *et al.*, 1998; Wang and Doyle, 1998; Sanders *et al.*, 1999; Baati *et al.*, 2000; De Angelis *et al.*, 2001; Jan *et al.*, 2001; Lorca and de Valdez, 2001b; Leverrier *et al.*, 2003; Prasad *et al.*, 2003).

These proteins probably act to prevent or repair cellular damage caused by the stressor. While some 'stress proteins' are induced under a variety of conditions (universal stress proteins), others are induced in response to a specific stress (Lorca and de Valdez, 2001a,b; Champomier-Verges *et al.*, 2002). Transiently produced proteins confer only short-term protection to the organism, whilst constitutive protein production is associated with extended survival periods (Heyde and Portalier, 1990; Foster, 1991, 1993; Leyer and Johnson, 1993). Foster (1993) found that several acid shock proteins important for pH 3.3 acid tolerance are only transiently produced. Their disappearance after 30 to 40 minutes of pH 4.4 acid shock coincides with an

inability to survive subsequent pH 3.3 acid challenge. Jan *et al.* (2001) also reported that acid-induced proteins (pH 5.0) have an early, transient, late or permanent nature.

The term 'molecular chaperone' originally described the 'supporting and supervisory function' of the GroE and Hsp families in preserving the structure of proteins and facilitating folding under otherwise inhospitable conditions' (Buchner, 1996). These proteins minimise irreversible unfolding reactions by preventing unspecific aggregation during refolding, stabilising proteins against inactivation and refolding proteins with an incorrect conformation (Salotra *et al.*, 1995; Buchner, 1996; Ellis and Hartl, 1996). Among the most abundant bacterial proteins under typical conditions, the GroES and GroEL chaperone proteins are greatly induced by any form of cellular stress that leads to protein denaturation (Hightower, 1991).

The general importance of these chaperones is demonstrated by the observation that in *E. coli*, GroEL and GroES are required for growth at all temperatures (Fayet *et al.*, 1989; Walker *et al.*, 1999). Mutations in either gene led to a pleiotropic phenotype, particularly at higher temperatures, resulting from impaired synthesis of RNA and proteins. In addition to its role in protein folding and assembly, GroEL was shown to participate in a complex capable of protecting mRNA from nuclease degradation, suggesting an additional role as an RNA chaperone (Georgellis *et al.*, 1995).

'The chaperones GroES and GroEL are typically arranged as an operon, and their translation products are assembled into single or double heptameric rings, respectively' (Georgopoulos and Welch, 1993). Langer *et al.* (1992) reported that 'in the presence of nucleotides, GroES forms a 1:1 complex with GroEL, which binds the protein substrate, possibly in its central cavity'.

Additional proteins elucidated in the acid tolerance response in *Sal. typhimurium* include the outer membrane proteins such as OmpC and OmpF (Foster and Hall, 1990; Leyer and Johnson, 1993; Foster and Spector, 1995), the F₀F₁ protein unit that forms a pore through which protons can be pumped out of the cell as part of the inducible pH homeostasis system (Foster and Hall, 1991) and GrpE proteins which

have been identified in *Lb. acidophilus* and *Lac. lactis* in response to heat shock (Sanders *et al.*, 1999).

Proteins produced as part of the stress response have been found to be homologous to the highly conserved chaperone proteins DnaK and GroE of *E. coli* including *Listeria mesenteroides*, *Lac. lactis* subsp. *lactis* (Hartke *et al.*, 1996; Kim *et al.*, 1998; Sanders *et al.*, 1999), *Lb. acidophilus*, *Lb. helveticus* (Kim *et al.*, 1998; Oberg *et al.*, 1998), *L. casei* (Oberg *et al.*, 1998), *Lactobacillus sanfranciscensis* CB1 (De Angelis *et al.*, 2001a); *En. faecalis* (Giard *et al.*, 2001); *Lb. rhamnosus* (Prasad *et al.*, 2003); *Pseudomonas freudenreichii* (Jan *et al.*, 2001); *Shewanella putrefaciens* (Leblanc *et al.*, 2003) and *Bacillus subtilis* (Kim *et al.*, 1998). Homologs of the heat shock protein, DnaJ, have also been detected in *Lb. acidophilus*, *Lb. casei*, and *Lb. helveticus*, (Oberg *et al.*, 1998) but expression of this protein was not stimulated by heat shock in any of the *Lactobacillus* species (Broadbent *et al.*, 1997; Oberg *et al.*, 1998; Sanders *et al.*, 1999).

The factors involved in stress responses are highly conserved (Champomier-Verges *et al.*, 2002), suggesting that different organisms respond to stress in similar ways. Proteins induced after heat shock, including the chaperonin class proteins DnaK and GroEL, have shown homology with proteins produced during acid shock (Foster, 1993; Hartke *et al.*, 1996; Broadbent *et al.*, 1997; Oberg *et al.*, 1998; Sanders *et al.*, 1999). Nevertheless, the response to stress has been shown to depend on the phase of growth at the time of stress. For example, an acid shock in stationary phase *Sal. typhimurium* cells triggers the synthesis of some 15 proteins, of which only four are in common with the exponential phase acid induced cells (Hall *et al.*, 1995).

There are conflicting reports as to whether *de novo* protein synthesis is necessary for adaptation to normally lethal conditions. Many authors have reported inhibition of adaptation by rifampicin or chloramphenicol in a complex medium (Foster and Hall, 1990; Davis *et al.*, 1996; Hartke *et al.*, 1996; Rallu *et al.*, 1996; O'Driscoll *et al.*, 1997; Wang and Doyle, 1998; Koga *et al.*, 1999; De Angelis *et al.*, 2001a) and by lack of adaptation by cells suspended in phosphate buffer at pH 5 (Ma *et al.*, 1997) whilst Lorca and de Valdez (2001b) and Jan *et al.* (2001) reported that cells adapted in the presence of chloramphenicol still improved in viability over the level of naïve

cells, but not the extent of those without protein synthesis inhibition, suggesting that the stress response is not entirely dependent of *de novo* protein synthesis. In contrast, Begley *et al.* (2002) showed that adaptation of *List. monocytogenes* to 0.3% bile was not dependent on *de novo* protein synthesis during the pre-conditioning period.

The specific modulation of messenger RNA stability appears to be the main mechanism by which bacteria cope with environmental stress. RpoS, the alternative sigma factor of RNA polymerase, is reported to be the global regulator of gene expression during cellular stress (Leverrier *et al.*, 2003). RpoS initiates the binding of RNA polymerase to promoters of selected genes, initiating transcription. 'The RpoS-induced gene products are directly involved in the stationary-phase-associated stress response or they themselves induce other genes, which in turn are involved in the stress response' (Kim *et al.*, 2001). The RpoS system is responsible for the induction of at least another eight acid shock proteins (Lee *et al.*, 1995; Bearson *et al.*, 1997). A potential function of the σ^S -dependant system may be to provide protection against organic acids (Foster, 1993; Foster and Spector, 1995; Bearson *et al.*, 1997; Castanie-Cornet *et al.*, 1999).

Although RpoS is important in the stationary-phase associated stress response, it is not the sole inducer of transcription. New evidence shows that there are other regulatory molecules, which operate independent of the RpoS system. One regulator of the σ^S -independent acid tolerance response is *phoP* (Foster and Spector, 1995). This system has been shown to sense external Mg^{2+} and also protect against low pH. Several genes have been associated with acid tolerance. The ferric uptake regulator (*fur*), in the presence of excess intracellular Fe^{2+} , inhibits the expression of iron-regulated genes, which affects the metabolism of iron. *Fur* also controls the expression of several acid shock proteins, via iron-dependant activation (Bearson *et al.*, 1997). The genes *atrD* and *ent* have similar effects on iron metabolism (Foster and Spector, 1995). Other genes with demonstrable effects on acid tolerance include the *polA* and *ada* genes involved in DNA repair (Foster and Spector, 1995); *icd*, which increases the internal buffer; *fabF*, involved in fatty acid synthesis, the cAMP receptor gene *crp*; *atp*, which interferes with proton pumping (Foster and Spector, 1995; Bearson *et al.*, 1997; Sanders *et al.*, 1999) and *gadE*, which regulates that acid

response in *E. coli* and *List. monocytogenes* (Begley *et al.*, 2002; Hommais *et al.*, 2004). *Sal. typhimurium* mutants which are acid sensitive include *atp*, *fur*, *phoP*, *atrB*, *atrC*, *atrF* and *atrG* (Wilmes-Riesenberg *et al.*, 1996).

1.7.3 pH homeostasis systems

The ability of lactic acid bacteria to maintain their intracellular pH is an essential function. Not only do most lactic acid bacteria grow more slowly at low pH, but acid damage and loss of cell viability may also occur. Fermentation end-products exert their harmful effects by acidifying the cytoplasm below the permissible pH. Many microorganisms are able to maintain their intracellular pH even when the pH of the external medium decreases (pH homeostasis). Ultimately, however, the internal pH also begins to decline and the difference between the internal and external pH will approach zero, resulting in loss of cell viability (Nannen and Hutkins, 1991; Hutkins and Nannen, 1993).

pH homeostasis is particularly important for lactic acid bacteria used as starter cultures because these obligate acid-producing bacteria must cope with low pH environments during growth in milk. Cook and Russell (1994) reported that 'weak acids in their uncharged, protonated forms can diffuse across the cell membrane and dissociate inside the cell, lowering internal pH in the process'. According to Foster and Hall (1991) 'acid induced death is the direct result of lowered internal pH and not due to external damage to the cell'. Several authors have reported that internal acidification reduces the activity of acid-sensitive enzymes and damages proteins and DNA (Kashket, 1984; Hutkins and Nannen, 1993; Gutche *et al.*, 2002). Inhibition of starter cultures by lactic acid and low pH prevents overacidification of the finished product. However, maintenance of culture viability under acidic conditions is desired and necessary. Probiotic cultures, added to yogurt for therapeutic value, must remain viable during storage at low pH and must survive transit through the acidic environment of the stomach.

The most important homeostatic system in fermentative bacteria is the membrane-bound, proton-translocating ATPase, which can either synthesize ATP using protons or extrude protons out of the cell *via* ATP hydrolysis (Mitchell, 1973; Schneider and

Altendorf, 1987; Foster and Hall, 1990; Foster and Hall, 1991; Russell, 1992). This reaction requires energy because expulsion of protons from a relatively alkaline environment into an acidic environment requires movement of protons against a concentration gradient (Mitchell, 1973, Bond and Russell, 1996). Mutants affected in the ATPase activity were obtained in various lactic acid bacteria. All of them exhibited growth defects at low pH (Suzuki *et al.*, 1988; Yokota *et al.*, 1995; Yamamoto *et al.*, 1996; Tourdot-Marechal *et al.*, 1999). The stimulation of the F_0F_1 -ATPase activity at low pH may result from (i) the stimulation of transcription of the *atp* locus as reported for *Lb. acidophilus* (Kullen *et al.*, 1999) and/or (ii) the stabilisation of the subunits that constitute the enzyme as observed in *Ent. hirae* (Arikado *et al.*, 1999).

H^+ ATPase complexes consist of two main portions; a hydrophilic, peripheral membrane protein called F_1 and the hydrophobic F_0 , which is integrated within the membrane (Papa *et al.*, 1984; Schneider and Altendorf, 1987; Foster and Hall, 1991; Deckers-Hebesstreit and Altendorf, 1992). The *atp* loci contain all the genes encoding the subunits of the F_0 - F_1 complex (Martin-Galiano *et al.*, 2001; Quivey *et al.*, 2001). The F_1 protein consists of five subunits, whilst the transmembrane F_0 consists of three subunits and mediates the translocation of H^+ across the membrane (Papa *et al.*, 1984; Schneider and Altendorf, 1987; Foster and Hall, 1991; Deckers-Hebesstreit and Altendorf, 1992). The passage of the proton through F_0 is regulated by the F_1 portion. Protons passing into the cell through F_0 will generate ATP *via* F_1 whereas exiting protons will expend an ATP (Kobayashi and Anraku, 1972). Therefore, the F_0F_1 complex is responsible for maintaining the internal pH by pumping protons out of the cell.

For several organisms that inhabit the gastrointestinal tract, the F_1F_0 -ATPase is an important element in the response and tolerance to low pH (Kobayashi *et al.*, 1984, 1986; Foster and Hall, 1991; Jordan *et al.*, 1999; Lorca and Valdez, 2001a). Both the expression level and the activity of this protein complex are increased in response to a decrease in cytoplasmic pH (Kobayashi *et al.*, 1984, 1986; Foster and Hall, 1991;

Nannen and Hutkins, 1991; Lorca and Valdez, 2001a; Sakamoto *et al.*, 2002). Foster and Hall (1991) reported that adaptation enables the cells to better maintain internal pH. *Sal. typhimurium* cells that underwent an adaptation process survived strong acid conditions better than unadapted cells (Foster and Hall, 1990, 1991). This indicates that the acid tolerance response produces an inducible homeostatic function that protects cells over and above the normal constitutive pH homeostasis system. The fermentative bacteria *Enterococcus hirae* and *Streptococcus faecalis* also maintain their cytoplasmic pH using proton-translocating ATPase (Kobayashi *et al.*, 1984, 1986).

Foster and Hall (1991) reported that normal constitutive pH homeostasis that occurred down to external pH of 4.0 did not depend upon the synthesis of new proteins, as evidenced by the maintenance of this system in the presence of protein synthesis inhibitors. In contrast, protein synthesis was essential for pH homeostasis when the external pH was below 4.0. Perhaps a protein is induced at pH values below 4.0 that interacts with pre-existing H^+ -translocating ATPase complexes, rendering them more active or more stable under extremely acidic conditions (Foster and Hall, 1991; Hutkins and Nannen, 1993). Mutants of *Sal. typhimurium* that lack ATPase are acid sensitive (Foster and Hall, 1991; Hutkins and Nannen, 1993).

Kullen and Klaenhammer (1999) investigated the influence of low pH on inducible gene expression in *Lb. acidophilus* using differential display. Log phase cells that were exposed to pH 3.5 produced a 0.7 kb mRNA that had sequence homology with bacterial *atpBEF* genes of the *atp* operon. These genes encode the various subunits of the F_1F_0 -ATPase. An increase in *atp*-mRNA induced by low pH was accompanied by an increase in the activity of the enzyme in membrane extracts. The structure and genetic organisation indicated that the H^+ -ATPase of *Lb. acidophilus* is a typical F_1F_0 -type ATPase, suggesting that it may function in the ATP-dependent extrusion of protons and maintenance of cytoplasmic pH.

1.7.4 Arginine deiminase system

In fermentative organisms, the production of acid end-products and their subsequent accumulation by cells results in the breakdown of pH homeostasis (Baronofsky *et al.*,

1984). To combat this problem, *Ps. aeruginosa* and a variety of streptococci including *Streptococcus sanguis*, *Streptococcus rattus*, and *Str. faecium* have evolved mechanisms that convert acidic products to neutral compounds to prevent the toxic effect of weak acids in the medium.

‘The arginine deiminase system provides a source of ATP derived from catabolism of arginine to ornithine, CO₂ and NH₃ in a variety of organisms. The resulting NH₃ reacts with H⁺ and helps to alkalize the environment whilst the ATP enables extrusion of cytoplasmic protons by the F₀F₁-ATPase’ (Marquis *et al.*, 1987). The factors involved in arginine deiminase regulation are a combination of arginine availability, energy depletion, catabolite repression and oxygenation, rather than low pH (Cunin *et al.*, 1986; Zuniga *et al.*, 1998; Champomier Verges *et al.*, 1999).

The arginine deiminase system promoted acid tolerance in a variety of lactic acid bacteria. The ammonia produced from arginine at low pH protected the cells against acid damage until the environmental pH value was sufficient to allow for the re-establishment of a difference in pH (Δ pH) across the cell membrane (Marquis *et al.*, 1987; Casiano-Colon and Marquis, 1988). This system was found to protect *Str. rattus* (Casiano-Colon and Marquis, 1988); streptococci, *Ps. aeruginosa*, *Lac. lactis* (Marquis *et al.*, 1987) and *Sal. typhimurium* (Bearson *et al.*, 1997).

The three main enzymes of the arginine deiminase system are: arginine deiminase, ornithine carbamoyltransferase and carbamate kinase. They catalyse the reaction: arginine + H₂O₂ + ADP + Pi → ornithine + CO₂ + NH₃ + ATP. These enzymes are active at low pH (2 to 3) in several *Streptococcus* species (Casiano-Colon and Marquis, 1988), suggesting they are responsible for maintaining the survival of organisms at low pH as a major provider of ATP for the growth. Casiano-Colon and Marquis (1988) suggested that ‘the capacity of cells to carry out glycolysis depends mainly on the inherent acid stabilities of the arginolytic enzymes rather than on the abilities of the cells to maintain a pH difference across the cell membrane in acidified environments’.

1.7.5 Microscopic changes following adaptation

Another response to stress has been the colonial and microscopic variation on solid media following response to altered growth conditions (Klaenhammer and Kleeman, 1981). This has been apparent in numerous *Lactobacillus* species, including *Lb. acidophilus*, *Lb. bulgaricus*, *Lb. helveticus* and *Lb. casei* (Klaenhammer and Kleeman, 1981). Growth conditions that have been found to affect the colony morphology of the lactobacilli include the presence or absence of oxygen, temperature of incubation, pH and components of the growth medium such as acetate (McDonald and Frazier, 1951), salt (Anonymous, 1968), fatty acid esters (Rogosa and Mitchell, 1950) and antibiotics with surfactant properties (Anonymous, 1968).

In Gram stains of rough colonies, a assortment of long and short rods in long chains and filaments was observed, often in tangled masses of cells whereas the smooth colonies contained only individual short rods (Hadley *et al.*, 1930; Klaenhammer and Kleeman, 1981). The smooth colonies were more resistant to 0.15% bile than rough colonies. *Lb. acidophilus* strains may be heterogeneous during growth and this may explain differences reported in various phenotypic studies.

Many authors reported that filamentous growth (observed in rough colonies) of the lactobacilli results from growth conditions that are not supportive of proper cell multiplication (Jeener and Jeener, 1952; Deibel *et al.*, 1956; Holden and Holman, 1957; Kojima *et al.*, 1970). Morichi (1991) suggested that the intracellular concentration of purine nucleotides limits RNA synthesis and the depressed RNA synthesis of these cells may result in cell elongation caused by defective septum formation.

In another study, Jorgensen *et al.* (1995) subjected *List. monocytogenes* to a short osmotic shock of 0.5, 1.0 or 1.5 mol l⁻¹ sodium chloride for five minutes which increased the thermotolerance of this species. Phase-contrast microscopy revealed cellular changes, with cell elongation following osmotic shock and septa formation after a 'downshift' to normal osmotic conditions.

Jan *et al.* (2001) reported that *Ps. freudenreichii* formed 'pleomorphic rods around 1.22 μm at pH 7.0, whilst at pH 2.0, cells were segmented and contained bleb-like structures on their surface.' Cells that had undergone adaptation at pH 5.0 prior to challenge at pH 2.0 appeared similar to cells at pH 7.0 using scanning electron microscopy. Leverrier *et al.* (2003) also reported morphological changes following challenge with bile salts. Incubation of *Ps. freudenreichii* S141 in bile salts (0.2g/L) enhanced survival during a subsequent lethal challenge (1.0 g/L bile salts) compared to non-adapted cells. Challenge with bile salts led to drastic morphological changes, consistent with intracellular material leakage, for non-adapted cells but not for pre-exposed ones.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Purchase of materials

Chemicals, reagents, enzymes, solvents and microbiological media were purchased from Ajax Chemicals (NSW, Australia), Amersham Biosciences UK Ltd (Buckinghamshire, England), Amyl Media Pty. Ltd. (Victoria, Australia), Auspep Pty. Ltd., (Victoria, Australia), BDH Laboratory Supplies (Poole, England), Bio-Rad Laboratories (CA, USA), Boehringer-Mannheim (Germany), Gibco BRL (Rockville, MD), ICN Biochemicals (Research Product Division, CA, USA), ICN Biochemicals (Radiochemicals Division, CA, USA), Life Technologies Ltd. Pty. (Victoria, Australia), Merck Pty. Ltd. (Victoria, Australia), Oxoid Unipath Ltd. (Hampshire, England); Roche Molecular Systems, Inc., (New Jersey, USA), Progen Industries Ltd. (NSW, Australia), and Sigma Chemical Company (MO, USA). All chemicals, solvents and reagents were of analytical grade or higher. Further details regarding the source of reagents have been provided in cases where the source of the reagent is important to the experimental outcome.

2.1.2 Bacterial strains

The starter cultures *Str. thermophilus* and *Lb. bulgaricus* and various strains of the probiotic bacterium *Lb. acidophilus*, were used in this study as shown in Table 2.1.

2.2 GENERAL METHODS

Fine chemicals were weighed using a Mettler AE200 analytical balance; large quantities were weighed using a Mettler PJ 3600 Delta Range balance (Denver Instrument Company, USA). All media and stock solutions were prepared using ultra-pure distilled

Table 2.1. Source of bacterial strains used in this study.

Microorganism	Culture Collection Number ¹	Alternate Codes	Source
<i>Lactobacillus acidophilus</i>	VUP 12, 002	CSCC 2404	CSIRO ²
<i>Lactobacillus acidophilus</i>	VUP 12, 012	CSCC 2406	CSIRO
<i>Lactobacillus acidophilus</i>	VUP 12, 005	CH5, CSCC 2412	Christian Hansen ³
<i>Lactobacillus acidophilus</i>	VUP 12, 006	CH6, CSCC 2413	Christian Hansen
<i>Lactobacillus acidophilus</i>	VUP 12, 015	CSCC 2414	CSIRO
<i>Lactobacillus acidophilus</i>	VUP 12, 007	CSCC 2415	CSIRO
<i>Lactobacillus acidophilus</i>	VUP 12, 016	ATTC 53103, CSCC 2422	ATCC 53103 ⁴
<i>Lactobacillus acidophilus</i>	VUP 12, 025	BDLA1	Gist-Brocades ⁵
<i>Lactobacillus acidophilus</i>	VUP 12, 027	MOLA2	Gist-Brocades
<i>Lactobacillus acidophilus</i>	VUP 12, 028	OLA2	Gist-Brocades
<i>Streptococcus thermophilus</i>	--	CSCC 2010	CSIRO
<i>Lactobacillus delbrueckii</i> ssp. <i>bulgaricus</i>	--	CSCC 2515	CSIRO
<i>S. thermophilus</i> - <i>L. bulgaricus</i> 217 co-culture	--		Gist-Brocades

¹Victoria University of Technology, CBFT Culture Collection, Werribee, Australia.²Commonwealth Scientific and Industrial Research Organisation, Division of Food Science and Technology, Highett, Australia.³Christian Hansen Pty. Ltd., Horsholm, Denmark.⁴American Type Culture Collection, USA.⁵Gist-Brocades Australia Pty. Ltd., Moorebank, Australia.

water produced using a Millipore Milli Q ultra pure water system (Millipore, North America). The pH of the media and stock solutions was measured using a calibrated Orion 410 A pH meter (Boston, USA). Solutions were stored at room temperature unless otherwise specified. Optical densities were measured using a Novaspec II UV/Visible spectrophotometer (Pharmacia Biotech). Centrifugation was performed with a Beckman J2-HS centrifuge for volumes 50 ml or greater, a Sorvall RT centrifuge for volumes between 2 to 50 ml, or an Eppendorf Centrifuge 5415C for volumes of 1.5 ml or less. Media and stock solutions were either sterilised by autoclaving at 121°C for 15 minutes or filter sterilised using 0.22 µm disposable filters (Sartorius Minisart, Germany) unless otherwise noted. All microbiological work was performed in a laminar flow workstation (Gelman Sciences, HWS Series). All hazardous chemicals were handled in a Smoothflow safety control fume cupboard (E & I Lewis Plastics, Victoria, Australia).

2.3 STOCK SOLUTIONS

Ammonium Persulphate (10%, w/v): Ammonium persulphate (0.1 g) was dissolved in one ml of distilled water immediately before use.

Bile Extract (10%, w/v): Bile salts (10 g) (Amyl Media) were dissolved in 100 ml of distilled water. The solution was filter sterilised and stored at room temperature until used.

Bovine Serum Albumin (BSA): A stock solution of BSA (5% w/v) was prepared by dissolving in 0.45M NaH₂PO₄ to achieve a concentration of 100 µg/ml BSA. The BSA solution was stored at 4°C.

Bromocresol Purple (0.6%, w/v): Bromocresol purple (6.0 g) was dissolved in one litre of distilled water and filter sterilised.

Coomassie Blue Fixing Solution: Fixing solution was prepared by mixing ethanol (400 ml) and acetic acid (100 ml) with 500 ml distilled water.

Coomassie Blue Staining Solution: Staining solution was composed of Coomassie Brilliant Blue R (1.5 g), 95% ethanol (105 ml) and acetic acid-glacial (30 ml). The volume was made up to 300 ml using distilled water and the solution was stored at room temperature.

Copper Sulphate (5%, w/v): CuSO_4 (50 g) was dissolved in 800 ml of distilled water. The volume was adjusted to one litre and the solution was stored at 4°C.

Cysteine Hydrochloride (0.05%, w/v): L-Cysteine hydrochloride (5.0 g) was dissolved in 80 ml of water and adjusted to a final volume of 100 ml. The solution was adjusted to pH 7.0, filter sterilised and stored at room temperature.

Destaining Solution: Methanol (350 ml), trichloroacetic acid (130 g) and sulphosalicylic acid (35 g) were dissolved in one litre of distilled water and stored at room temperature for up to four weeks.

Di-sodium β -glycero-phosphate (19%, w/v): A stock solution of β -glycero-phosphate was prepared by dissolving 19 g in 80 ml of distilled water prior to adjusting the volume to 100 ml. The solution was prepared and filter sterilised immediately before use.

DNase I (10 mg/ml): DNase I (0.01 g) was dissolved in one ml distilled deionised water and stored at 4°C.

EC Lysis Solution: EC lysis solution (Cantor *et al.*, 1988) was composed of 6 mM Tris (hydroxymethyl) methylammonium chloride (Tris-Cl), 1 M NaCl, 100 mM EDTA (pH 7.5), 0.2% deoxycholate, 1% sarkosyl, 10 mg/ml lysozyme and 20 $\mu\text{g/ml}$ DNase-free RNase. The EC lysis solution was prepared immediately before use by combining stock solutions of the constituents with the appropriate amount of water.

ESP (pH 9.0): The composition of ESP (Cantor *et al.*, 1988) was 0.5 M EDTA (pH 9.0), 1% sarkosyl and 1 mg/ml proteinase K. ESP was prepared immediately prior to use by adding stock solutions of the respective constituents to the appropriate volume of distilled water.

Ethidium Bromide: Ethidium bromide (1 g) was dissolved in 100 ml of H₂O. The solution was stored in the dark at 4°C.

Ethylene-Diamine-Tetra-Acetic-Acid (EDTA) (0.5 M, pH 7.5, 8.0 and 9.0): Disodium EDTA.2H₂O (186.1 g) was dissolved in 800 ml of water. The pH was adjusted appropriately using 5 M NaOH and the volume made up to one litre. The stock solutions were autoclaved and stored at room temperature.

Folin-Ciocalteu reagent (10% v/v): Folin Ciocalteu reagent (10 ml) was added to 90 ml of distilled deionised water. The solution was prepared immediately before use.

Hydrochloric Acid (1 M): Hydrochloric acid (86.2 ml) was added to 900 ml of water. The stock solution was adjusted to a final volume of one litre and stored at room temperature.

Loading Buffer: Bromophenol Blue (25 mg) and sucrose (4 g) were dissolved in five ml of sterile distilled deionised water and the volume was adjusted to 10 ml. The solution was aliquoted into one ml lots prior to storage at – 20°C.

Lowry Reagent A: Reagent A (Lowry *et al.*, 1951) consisted of 2% Na₂CO₃ in 0.1 M NaOH. The solution was stored at room temperature.

Lowry Reagent B: Reagent B (Lowry *et al.*, 1951) consisted of 0.5% CuSO₄.5H₂O in 1% Na-K-tartrate. The pH was adjusted to 9.0 and the solution was stored at 4°C.

Lowry Reagent C: Reagent C (Lowry *et al.*, 1951) was prepared by mixing Lowry reagent A with Lowry reagent B at a ratio of 50:1. Lowry reagent C was prepared immediately before use.

Lysozyme Solution (0.5 mg/ml): Lysozyme (0.5 mg) was dissolved in sterile distilled water (1 ml) and stored at 4°C.

MgCl₂ (1 M): Magnesium chloride (203.3 g) was dissolved in 400 ml of distilled water. The volume was adjusted to 500 ml using distilled water and the solution was stored at room temperature.

Peptone Water (0.15%, w/v): Dehydrated peptone water (Oxoid) (1.5 g) was added per litre of distilled water. The mix was autoclaved and stored at room temperature.

Phosphate Buffer (pH 7.0): Na₂HPO₄ (17.3 g) and NaH₂PO₄ (9.36 g) were dissolved in one litre of distilled water. The solution was adjusted to pH 7.0 using 1N HCl, autoclaved and stored at room temperature.

Phosphate Buffered Saline (PBS): NaCl (8.0 g), KCl (0.2 g), Na₂PO₄ (1.44 g), KH₂PO₄ (0.24 g) were added to 800 ml of distilled water. The solution was adjusted to pH 7.4 using 1N HCl and the volume made up to one litre. The solution was autoclaved and stored at room temperature.

PIV Solution: (Cantor *et al.*, 1988). PIV consisted of 10 mM Tris Cl (pH 7.6) and 1 M NaCl. The solution was made by mixing 25 ml of 0.2 M Tris-Cl and 100 ml 5 M NaCl to 375 ml of distilled water. The mixture was sterilised by autoclaving and stored at 4°C.

Potassium-sodium-tartrate (1% w/v): KNaC₄H₄O₆·4H₂O (1.0 g) was mixed with 100 ml distilled water and stored at 4°C.

PPO (0.5%, w/v): 2,5-Diphenyloxazole (0.5 g) was dissolved in 100 ml of toluene and stored at room temperature.

Sample Buffer (2X): 1.25 M pH 6.8 Tris-Cl (2.5 ml), sodium dodecyl sulphate (1.0 g), 2-mercaptoethanol (2.5 ml), 87% glycerol (5.8 ml), bromophenol blue (5.0 mg) were dissolved in 10 ml of distilled water. The volume was adjusted to 25 ml distilled water and aliquoted into one ml lots prior to storage at – 20°C.

Silver Staining Developing Solution: Developing solution was prepared by dissolving 6.25 g of sodium carbonate in 250 ml of distilled water containing 25 μ l of formaldehyde.

Silver Staining Fixing Solution: Fixing solution was prepared by mixing ethanol (400 ml) and acetic acid (100 ml) with 500 ml distilled water.

Silver Staining Incubation Solution: Incubation solution was prepared by dissolving sodium acetate (10.25 g) and sodium thiosulfate (0.5 g) in ethanol (75 ml), and glutardialdehyde (1.3 ml). The volume was made up to 250 ml using distilled water.

Silver Staining Silver Solution: Silver solution was prepared immediately prior to use by dissolving silver nitrate (0.25 g) and formaldehyde (50 μ l) in 250 ml distilled water.

Silver Staining Stop Solution: Stop solution was prepared by mixing EDTA- $\text{Na}_2\cdot 2\text{H}_2\text{O}$ (3.65 g) with 250 ml distilled water.

Sodium Carbonate (1 M): Sodium carbonate (105.99 g) was dissolved in 800 ml of distilled water. The volume was adjusted to one litre and the solution was autoclaved prior to storage at room temperature.

Sodium Chloride (0.9% w/v): NaCl (4.5 g) was added to 500 ml distilled water. The mixture was autoclaved and stored at room temperature.

Sodium Chloride (5 M): Sodium chloride (292.2 g) was dissolved in 800 ml of distilled water. The volume was adjusted to one litre and the solution was autoclaved and stored at room temperature.

Sodium Dodecyl Sulphate (SDS) (10%, w/v): Electrophoresis grade SDS (10 g) was dissolved in 85 ml of distilled water. The volume was adjusted to 100 ml and the stock solution was stored at room temperature.

Sodium Hydroxide (5 M): Sodium hydroxide (200 g) was dissolved in 800 ml of distilled water. The volume was adjusted to one litre and stored at room temperature.

Sucrose Solution (50%, w/v): Sucrose (50 g) was dissolved in 80 ml of distilled water. The volume was adjusted to 100 ml and the mixture was autoclaved at 110°C for 10 minutes and stored at room temperature.

Sulphuric Acid (5N): Analytical grade H₂SO₄ (24.51 ml) was added to distilled water (25.48 ml) and stored at room temperature.

T1 Buffer (pH 8.0): The composition of T1 buffer was 4 mg lysozyme, 20 mM Tris-Cl (pH 8.0), 2 mM EDTA and 1% Triton X-100. The stock solution was stored at room temperature.

TBE Buffer: TBE buffer (Sambrook *et al.*, 1989) was prepared by dissolving Tris base (54 g), boric acid (27.5 g) and 0.5 M EDTA (20 ml) in one litre of distilled water. The pH was adjusted to 8.0, autoclaved and stored at 4°C until required.

TE1 Buffer (pH 8.0): TE1 buffer consisted of 10 mM Tris (pH 8.0) and 1 mM EDTA. The solution was made by mixing the stock solutions of the respective constituents, adding the appropriate amount of distilled water and autoclaving before storage at 4°C.

TEPMSF: Phenylmethanesulfonyl flouride, α -toluenesulfonyl flouride (0.0175 g) was added to isopropanol (1 ml) and heated for 1-2 minutes at 60°C to dissolve. TE buffer (9 ml) was added to the mixture and the sample was reheated at 60°C for 30 minutes until dissolved. The mixture was prepared immediately before use.

Trichloroacetic Acid (TCA) (20%, v/v): 100% TCA (20 ml) was mixed with 80 ml distilled water and stored at room temperature.

Tris-Cl (0.05 M, pH 8.0): Tris-Cl (1.5 g) was dissolved in 150 ml of distilled water. The pH was adjusted to 8.0 using 5 M HCl and the volume was made up to 250 ml with distilled water before storage at 4°C.

Tris-Cl (1.25 M, pH 6.8): Tris-Cl (37.8 g) was dissolved in 150 ml of distilled water. The pH was adjusted to 6.8 using 5 M HCl and the volume was made up to 250 ml with distilled water before storage at 4°C.

Tris-Cl (1.875 M, pH 8.8): Tris-Cl (56.8 g) was dissolved in 150 ml of distilled water. The pH was adjusted to 8.8 using 5 M HCl and the volume was made up to 250 ml with distilled water before storage at 4°C.

Tris-Cl (2 M, pH 7.6): Tris-Cl (158 g) was dissolved in 500 ml of distilled water. The pH was adjusted using 5 M HCl prior to sterilisation and storage at room temperature.

Tris-Cl (200 mM, pH 9.0): Tris-Cl (1.58 g) was added to 45 ml distilled water. The pH was adjusted to 9.0 and the volume made up to 50 ml using distilled water.

Tris-glycine buffer (10X, pH 8.3): Glycine (144.2 g), Tris-Cl (30.3 g) and SDS (10 g) were dissolved in one litre of distilled water. The buffer was stored at room temperature.

2.4 MEDIA COMPOSITION AND PREPARATION

2.4.1 Basal medium for sugar fermentation studies (Gupta *et al.*, 1996)

Beef extract (3.0 g) and peptone (5.0 g) were added to one litre of distilled water and the mixture was autoclaved. The appropriate sugar was added at the time of testing at 1% along with bromocresol purple dye at 0.05%.

2.4.2 Glycerol storage broth

Glycerol storage broth contained 50% (v/v) glycerol in Nutrient Broth (Oxoid).

2.4.3 M17 Agar (Terzaghi and Sandine, 1975)

M17 broth (37.25 g) and bacteriological agar (12 g) were added to one litre of distilled water. After autoclaving, a filter-sterilised preparation of lactose was added to the cooled medium to give a final concentration of 1% prior to pouring plates.

2.4.4 MRS broth and MRS agar (De Man *et al.*, 1960)

Dehydrated MRS broth (52 g) (Oxoid) was dissolved in one litre of distilled water. For MRS agar, twelve grams of bacteriological agar was added to the MRS broth. To obtain pH modified MRS broth or MRS agar, 1.0 M HCl or 1.0 M lactic acid was used to adjust the pH. The pH of the media was measured using a calibrated Orion 410 A pH meter (Boston, USA). The media was sterilised by autoclaving. To obtain pH-buffered MRS broth, β -glycero-phosphate was added at a rate of 1.9% w/v after the media was sterilised.

2.4.5 MRS-maltose, MRS-raffinose (Hull and Roberts, 1984a) and MRS-sorbitol agar (Dave and Shah, 1996)

Dehydrated MRS-Maltose agar (58 g) (Amyl) and SP437 Amyl media MRS supplement (40 ml) were mixed with 960 ml of distilled water before sterilisation. To prepare MRS-raffinose and MRS-sorbitol agars, MRS basal medium was prepared without dextrose. After autoclaving the media, filter-sterilized solutions of raffinose or d-sorbitol were added to the mixture at a final concentration of 1.0% prior to pouring plates.

2.4.6 MRS-oxygall agar (Micanel *et al.*, 1997)

Neutralised liver digest (20.0 g) (Oxoid, Australia) was added to one litre of MRS broth. Twelve grams of bacteriological agar was then added and the media was sterilised by autoclaving.

2.4.7 Non-Fat Dry Milk (NFDM, 10%)

Non-fat dry milk (100 g) was dissolved in one litre of distilled water and media was sterilised by autoclaving.

2.4.8 NGYEC Broth (Lankaputhra and Shah, 1995)

NGYEC was prepared with the following ingredients (per litre): non-fat dry milk (120 g), glucose (20 g) and yeast extract (10 g). The mixture was autoclaved and cooled prior to the addition of filter-sterilised L-cysteine-HCl at a rate of 0.05% (w/v).

2.4.9 Nutrient Broth (Oxoid, 1990)

Nutrient Broth (25 g) was dissolved in one litre of distilled water. The medium (10 ml) was dispensed into McCartney bottles prior to autoclaving and storage at room temperature.

2.4.10 Plate Count Agar (PCA) with 10% skim milk and 0.0005% bromocresol purple (Johns *et al.*, 1978)

Plate count agar (22.5 g) and non-fat dry milk (100 g) were added to one litre of distilled water. Bromocresol purple (100 µl of a 5% stock solution) was added to the media prior to sterilisation by autoclaving.

2.4.11 Reinforced Clostridial Agar (RCA) (pH 5.3) (Johns *et al.*, 1978)

RCA (52.5 g) was dissolved in one litre of distilled water. The mixture was adjusted to pH 5.3 using 1 M HCl prior to sterilisation by autoclaving.

2.4.12 *Streptococcus thermophilus* agar (ST) (Dave and Shah, 1996)

Tryptone (10 g), sucrose (10 g), yeast extract (5 g) and K₂HPO₄ (2 g) were dissolved in one litre of distilled water. The pH was adjusted to 6.8 ± 0.1 and six ml of 0.5% bromocresol purple was added. Bacteriological agar (12 g) was added to the medium before sterilisation.

2.5 MICROBIOLOGICAL METHODS

2.5.1 Preparation and storage of *Lb. acidophilus* strains

Cultures were assessed for purity before being prepared for storage. Freeze-dried cultures were resuspended in twenty millilitres of MRS broth or 10% NFDM in sterile McCartney bottles and incubated at 37°C overnight in a standing incubator. After overnight incubation, *Lactobacillus* cultures were streaked onto MRS agar and the plates were placed immediately into Oxoid anaerobic jars along with an AnaeroGen anaerobic kit (Oxoid, Australia) and incubated at 37°C for 72 hours. The anaerobic kit reduces the oxygen level to <1% within 30 minutes and creates a carbon dioxide level between 9 and 13% (Oxoid). *Str. thermophilus* cultures were streaked onto ST agar and the plates

were incubated aerobically at 37°C for 24 hours. Agar plates containing pure cultures were selected and several colonies were removed using sterile swabs and added to 1 to 2 ml of Glycerol storage broth or Protect cryoprotectant medium containing Protect beads (Technical Service Consultants, Heywood, UK). After five minutes, the excess cryoprotectant medium was removed and tubes were placed at either -20°C or -80°C. Working stocks (stored at -20°C) were replaced with -80°C stock when required. When the -80°C supply was depleted, freeze dried cultures were re-inoculated into MRS broth and new batches of Protect beads were prepared.

2.5.2 Preparation of inocula

When required, cultures maintained on Protect beads and stored at -20°C were inoculated into either MRS broth or 10% NFDM by transferring one protect bead into 10 ml of the appropriate media. Following overnight incubation in a standing incubator at 37°C, cultures were transferred to fresh media at a rate of one percent and re-incubated at 37°C overnight. This process was repeated two to three times to prepare inoculum for experimentation.

2.5.3 Enumerating yogurt starter cultures and *Lb. acidophilus* strains

Viable counts were performed to determine survival of cultures under various conditions. Samples (one millilitre of liquid culture or one gram of yogurt) were taken at appropriate time points and serially diluted (10^1 to 10^7) using nine millilitres of 0.15% sterile peptone water. The pour-plate technique was the primary procedure for enumerating bacteria. This was performed by adding one millilitre of diluted culture to the centre of a sterile labelled Petri dish followed by the addition of cooled molten agar. The spread plate technique was used on several occasions and was performed by spreading 100 µl of diluted culture onto the surface of an agar plate. All viable counts were performed in duplicate. Plates containing either *Lb. acidophilus* strains or *Lb. bulgaricus* CCCC 2515 were incubated anaerobically at 37°C and 43°C respectively. Plates were incubated for 72 hours using Oxoid anaerobic jars and AnaeroGen anaerobic kits (Oxoid, Australia). Plates containing *Str. thermophilus* cultures were incubated aerobically at 37°C for 24 hours. Following incubation, plates containing 25 to 250 colonies were enumerated and recorded as log colony-forming units (cfu) per gram or millilitre of yogurt or liquid culture, respectively.

2.5.4 Selecting media for enumeration of *Lb. acidophilus*, *Lb. bulgaricus* and *Str. thermophilus* from yogurt

The pour, spread and streak plating techniques were used in conjunction with a range of selective media for the selective enumeration or detection of yogurt starter cultures and *Lb. acidophilus* from milk-based media. Streak plates were prepared using pure *Lb. acidophilus* cultures and a *Str. thermophilus*/*Lb. bulgaricus* 217 mix (Gist-Brocades). For pour plates and spread plates, cultures were mixed and grown overnight at 37°C in 12% NGYEC and added to plates according to section 2.5.3. The following were used for selective enumeration:

- a) *Str. thermophilus*: plate count agar with 10% skim milk and 0.0005% bromocresol purple (PCA), M17 agar and *Str. thermophilus* agar (ST).
- b) *Lb. bulgaricus*: reinforced clostridial agar (RCA, pH 5.3) and De Mann, Rogosa and Sharpe agar (MRS, pH 5.3).
- c) *Lb. acidophilus*: MRS-maltose, MRS-sorbitol, MRS-raffinose and MRS-oxygall agar.

2.5.5 Preparation of yogurt

Set yogurt was prepared according to the method outlined in Figure 2.1. Homogenised and pasteurised milk supplemented with 2% non-fat dry milk was heated to 85°C for 30 minutes and cooled to 43°C prior to the addition of yogurt starter cultures and probiotic bacteria. The yogurt starter cultures *Str. thermophilus* CSCC 2010 and *Lb. bulgaricus* CSCC 2515 were prepared for yogurt production by sequential transfer in 10% NFDM according to section 2.5.2. These cultures were used for yogurt preparation by their

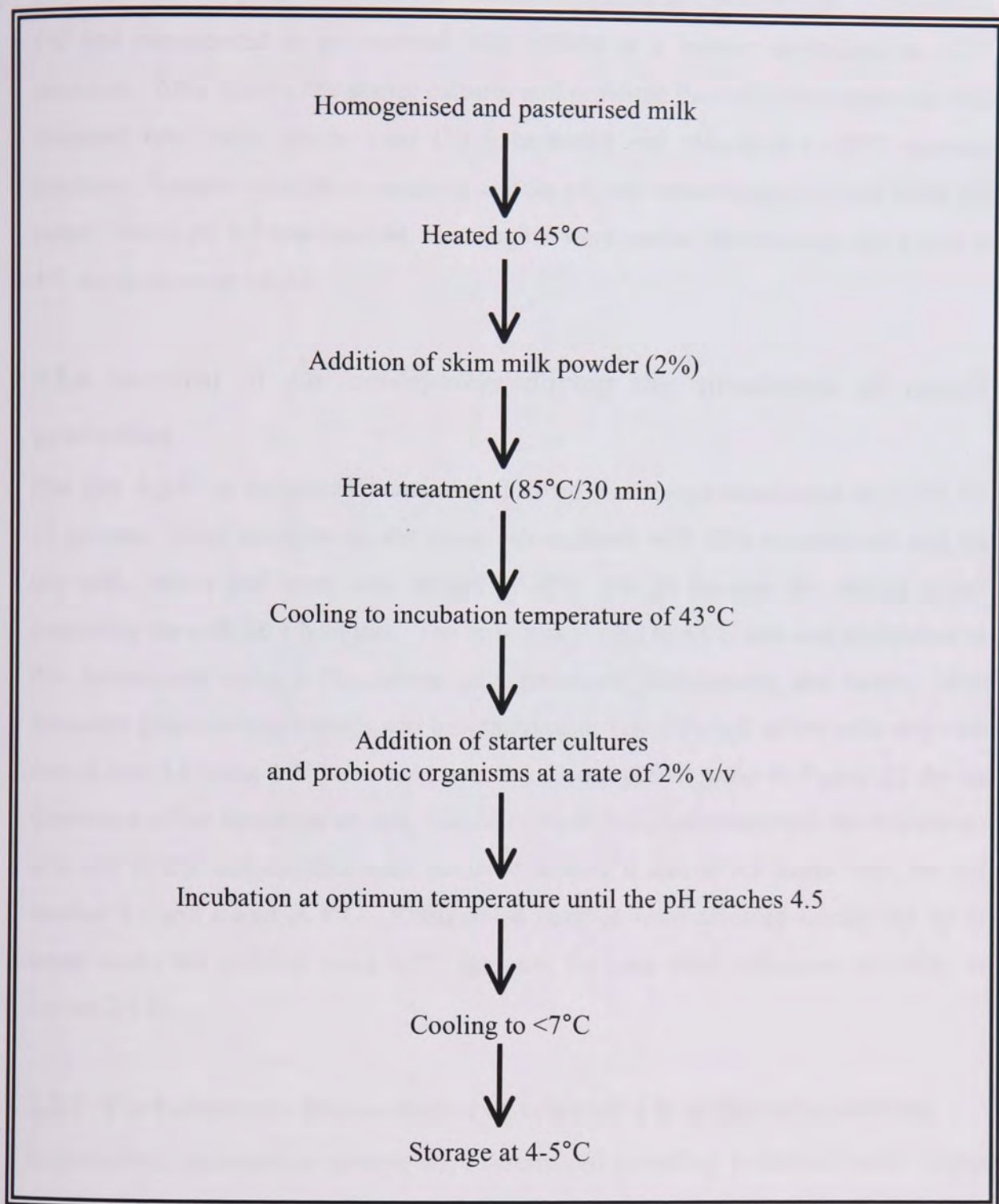


Figure 2.1. Flow diagram for the preparation of yogurt

addition at a rate of 2% v/v. Probiotic bacteria were prepared according to section 2.5.13 and the cell pellet was collected *via* centrifugation at 4,000 rpm for 15 minutes at 4°C and resuspended in pre-warmed 10% NFDM at a volume equivalent to a 2% inoculum. After mixing the starter cultures and probiotic bacteria, the yogurt mix was aliquoted into sterile plastic cups (50 g capacity) and placed in a 43°C standing incubator. Samples were taken regularly and the pH was tested using an Orion 410A pH meter. When pH 4.5 was reached, the samples were cooled immediately and stored at 4°C for up to seven weeks.

2.5.6 Survival of *Lb. acidophilus* during the simulation of yogurt production

One litre Applicon fermentors containing distilled water were autoclaved at 121°C for 15 minutes. After sterilisation, the water was replaced with 12% reconstituted non-fat dry milk, which had been heat treated at 85°C for 30 minutes by placing a pot containing the milk on a heat mat. The milk was cooled to 43°C and was maintained at this temperature using a Checktemp microprocessor thermometer and heater. The fermentor programming system was programmed to lower the pH of the milk stepwise from 6.3 to 4.5 using 1 N lactic acid over a 3.5 hour period (refer to Figure 2.2 for an illustration of the fermentor set up). Liquid cultures were inoculated into the fermentors at a rate of 2% and samples were removed at time 0 and at 3.5 hours once the pH reached 4.5 and stored at 4°C. Refrigerated samples were assessed weekly for up to seven weeks for viability using MRS agar and the pour plate technique according to section 2.5.3.

2.5.7 Carbohydrate fermentation of selected *Lb. acidophilus* strains

Carbohydrate fermentation patterns were determined according to the method of Gupta *et al.* (1996). The carbohydrates used are listed in Bergey's Manual of Systematic Bacteriology (Kandler and Weiss, 1986, p. 1227). Briefly, basal fermentation media containing the appropriate sugar was prepared according to section 2.4.1. Bacterial inoculum (10%) was added to the fermentation tubes prior to their incubation at 37°C for 24 hours. A positive result was recorded after a colour change of the medium from purple to yellow. A negative result was recorded if no colour change was observed in the tubes. Positive controls were prepared by inoculating cultures into basal medium



Figure 2.2. Fermentor system used to simulate yogurt production.

containing glucose, and negative controls were prepared by inoculating cultures into basal medium devoid of a carbohydrate substrate.

2.5.8 Antibiotic sensitivity of selected *Lb. acidophilus* strains

Antibiotic sensitivity tests were performed using the agar well diffusion technique (Tagg *et al.*, 1976). Unless otherwise stated, stock solutions of the antibiotics were prepared at 1 mg/ml in sterile distilled water and filter sterilised using a 0.45 µm filter (Sartorius Minisart) prior to refrigerated storage. Chloramphenicol and rifampicin were prepared in methanol, and tetracycline was prepared in 70% ethanol and stored at -20°C. Prior to their use, antibiotics were diluted in sterile distilled water at concentrations ranging from 1 to 1000 µg/ml. *Lb. acidophilus* cultures were prepared in MRS broth according to section 2.5.2. After overnight incubation, *Lb. acidophilus* cultures (100 µl) were spread onto the surface of MRS agar in duplicate prior to the preparation of seven millimetre wells using a bore cutter. One hundred microlitres of the appropriate antibiotic at concentrations between 0 to 1,000 µg/ml were added to the wells and plates were incubated at 37°C for 72 hours using Oxoid anaerobic jars and AnaeroGen anaerobic kits (Oxoid, Australia). The antibiotics tested were: amoxicillin, ampicillin, chloramphenicol, clindamycin, erythromycin, gentamycin, kanamycin, lincomycin, methicillin, neomycin, piperacillin, rifampicin, spectinomycin, tetracycline and vancomycin. The lowest inhibitory concentration (MIC) of each antibiotic was defined as the lowest concentration required to inhibit growth of the organism and was determined by measuring the zones of clearance around the wells.

2.5.9 Assessment of survival of *Lb. acidophilus* in the presence of acid and bile

The acid and bile tolerance of each strain was assessed according to the method of Lankaputhra and Shah (1995). Briefly, aliquots of log-phase cultures were inoculated into 12% NGYEC broth at a rate of 10% and incubated for 18 hours at 37°C in a standing incubator. Acid tolerance was assessed by adjusting the pH of the 18-hour cultures from 6.0 to 3.5, 2.5 or 1.5 with hydrochloric acid. Bile tolerance was assessed by adjusting the pH of the 18-hour culture to 4.5 before adding bile salts (Amyl Media) at 0 or 1.5%. All *Lb. acidophilus* cultures were incubated for three hours at 37°C and

sampled every hour for viable counts using MRS agar and the spread plate technique (refer to section 2.5.3). A benchmark of 80% survival was used to define acid and bile resistant isolates (Prasad *et al.*, 1998).

2.5.10 Inhibitory activity of yogurt starter cultures against *Lb. acidophilus*

The well diffusion technique was an inhibitory assay of filtered supernatants of *Str. thermophilus*, *Lb. bulgaricus* and *Lb. acidophilus* against the indicator strains *Str. thermophilus*, *Lb. bulgaricus* and *Lb. acidophilus* (Tagg *et al.*, 1976). Media (20 ml) seeded with the indicator organism at a rate of one percent was poured into sterile Petri dishes prior to the preparation of seven millimetre wells using a sterilised bore cutter. Filtered supernatants were obtained from cultures grown overnight in MRS broth or 12% NFDM following centrifugation at 3,800 rpm and filter sterilisation with a 0.45 μm filter. Cell-free extracts were added into the agar wells and allowed to diffuse for two hours at 37°C. Three sets of plates were prepared (in duplicate) and contained supernatants that were either left untreated, neutralised to pH 6.0 or neutralised to pH 6.0 then treated with 0.05 to 0.1 $\mu\text{g/ml}$ of catalase. A control plate was also prepared that tested the effect of MRS broth (pH 6.0) or MRS broth containing 0.1 $\mu\text{g/ml}$ of catalase (without culture) on the indicator strains. Plates were incubated at room temperature for two hours to allow for diffusion of the supernatant and then incubated for two days according to section 2.5.3. Inhibitory activity of the starter cultures and *Lb. acidophilus* strains was assessed by measuring the zones of inhibition using a millimetre rule, with average zone sizes recorded.

2.5.11 Determination of pH and temperature parameters for conditioning cells

The specific growth rates of *Lb. acidophilus* strain VUP 12,005 were determined at different pH and temperature values, and the maximum growth rates measured from the optical density readings. To determine growth traits at temperature values ranging from 30°C to 50°C, *Lb. acidophilus* strain VUP 12,005 was inoculated into flasks containing MRS broth, which were subsequently incubated for 12 hours in temperature-controlled water baths. The MRS broth was maintained at pH 6.0 using 1.9% w/v β -glycero-

phosphate buffer, which maintained the pH for up to 12 hours (data not shown). To determine growth traits at pH values ranging from pH 3.5 to 6.5, one litre Applicon fermentors containing pre-warmed MRS broth (37°C) at various pH values (adjusted using hydrochloric acid or lactic acid) were inoculated with *Lb. acidophilus* strain VUP 12,005 to an initial optical density of 0.1 at 600 nm. The rotor speed was maintained at 150 rpm and the pH was maintained by the computer controller system using 5N sodium hydroxide. Samples were taken every hour and the optical density at 600 nm was measured to determine the specific growth rates of the organism at various pH and temperature values. The specific growth rate (μ) was determined by the slope of a semi-logarithmic plot of A_{600} versus time, and was used to determine the optimum conditions for growth of *Lb. acidophilus* strains.

In addition, strains were grown to mid-exponential phase (0.5 OD at 600 nm) at optimal conditions (39°C, pH 5.8) before being subjected to a rapid shift in pH using lactic acid or hydrochloric acid (pH 5.8, 5.0, 4.5 or 4.0) or a rapid shift in temperature (39°C, 45°C, 50°C or 55°C). The impact on growth was monitored by spectrophotometry (at 600 nm) and viable counts using the pour plate technique outlined in section 2.5.3. Samples were taken prior to a shift in temperature or pH. Following a rapid shift in temperature and pH, samples were taken at 15 minutes, 30 minutes and hourly for five hours to determine changes in growth rates and viability of cells. Determination of sub-optimum conditions for conditioning cells was based on the shift in growth rate and viability of cells at various pH and temperature values (Rallu *et al.*, 1996).

To select a medium for conditioning cells, the generation time of *Lb. acidophilus* strain VUP 12,005 was compared in 10% non-fat dry milk and MRS broth (pH 6.2). *Lb. acidophilus* cultures were prepared according to section 2.5.2 and then inoculated into flasks containing 10% NFDM or MRS broth (pH 6.2) pre-warmed to 39°C to achieve an optical density of 0.3 at 600 nm. Flasks were placed in a 39°C waterbath throughout the experiment. Samples were taken hourly and viable counts were performed to monitor growth rates using the pour plate technique and MRS agar as per section 2.5.3. Optical densities were monitored every hour using a spectrophotometer set at 600 nm until the optical density of the MRS inoculated culture reached 1.0, then the experiment was terminated.

2.5.12 Determining optimal times for conditioning *Lb. acidophilus* cultures

Optimal conditioning times were determined according to the method of Rallu *et al.* (1996). *Lb. acidophilus* strain VUP 12,005 was prepared according to section 2.5.2 using MRS broth. Inocula were transferred into flasks containing fresh, pre-warmed, MRS broth (pH 5.8) to give an optical density (600 nm) of 0.3. Cultures were incubated in a 39°C waterbath for one doubling time (optical density of 0.6), which represented exponential phase of growth. Exponential-phase cells were then centrifuged at 4,000 rpm at 4°C for 15 minutes and the supernatant discarded. Cell pellets were resuspended in MRS broth adjusted to pH 5.8 (control cells), pH 4.5 or pH 4.0 (test cells) using lactic acid (Refer to section 2.4.4). Cells were incubated for 0, 15, 30, 60, 90 or 120 minutes in a 39°C waterbath, followed by centrifugation at 4,000 rpm for 15 minutes at 4°C. Cell pellets were re-suspended in MRS broth adjusted to pH 3.5 using lactic acid to give an optical density of 1.0 at 600 nm. Samples were taken immediately after re-suspension (time 0) and every hour for a five-hour period. Viable counts were performed on diluted cultures using MRS agar and the pour plate technique according to section 2.5.3. After incubation at 37°C for three days, colonies were counted and the percentage survival was calculated by dividing the number of cfu/ml at various times by cfu/ml at time zero.

2.5.13 Standard protocol for conditioning cells

Conditioning protocols were based on techniques described for *L. lactis* (Rallu *et al.*, 1996) and for a variety of oral lactic acid bacteria (Ma *et al.*, 1997). *Lb. acidophilus* cultures were prepared according to section 2.5.2 (Figure 2.3) using MRS broth. Inocula were transferred into flasks containing fresh, pre-warmed, MRS broth (pH 5.8) to give an optical density (600 nm) of 0.3. Cultures were incubated in a 39°C waterbath for one doubling time (optical density of 0.6), which represented exponential phase of growth (Appendix 1). Exponential-phase cells were then centrifuged at 4,000 rpm at 4°C for 15 minutes and the supernatant discarded. Cells pellets were re-suspended in pre-warmed

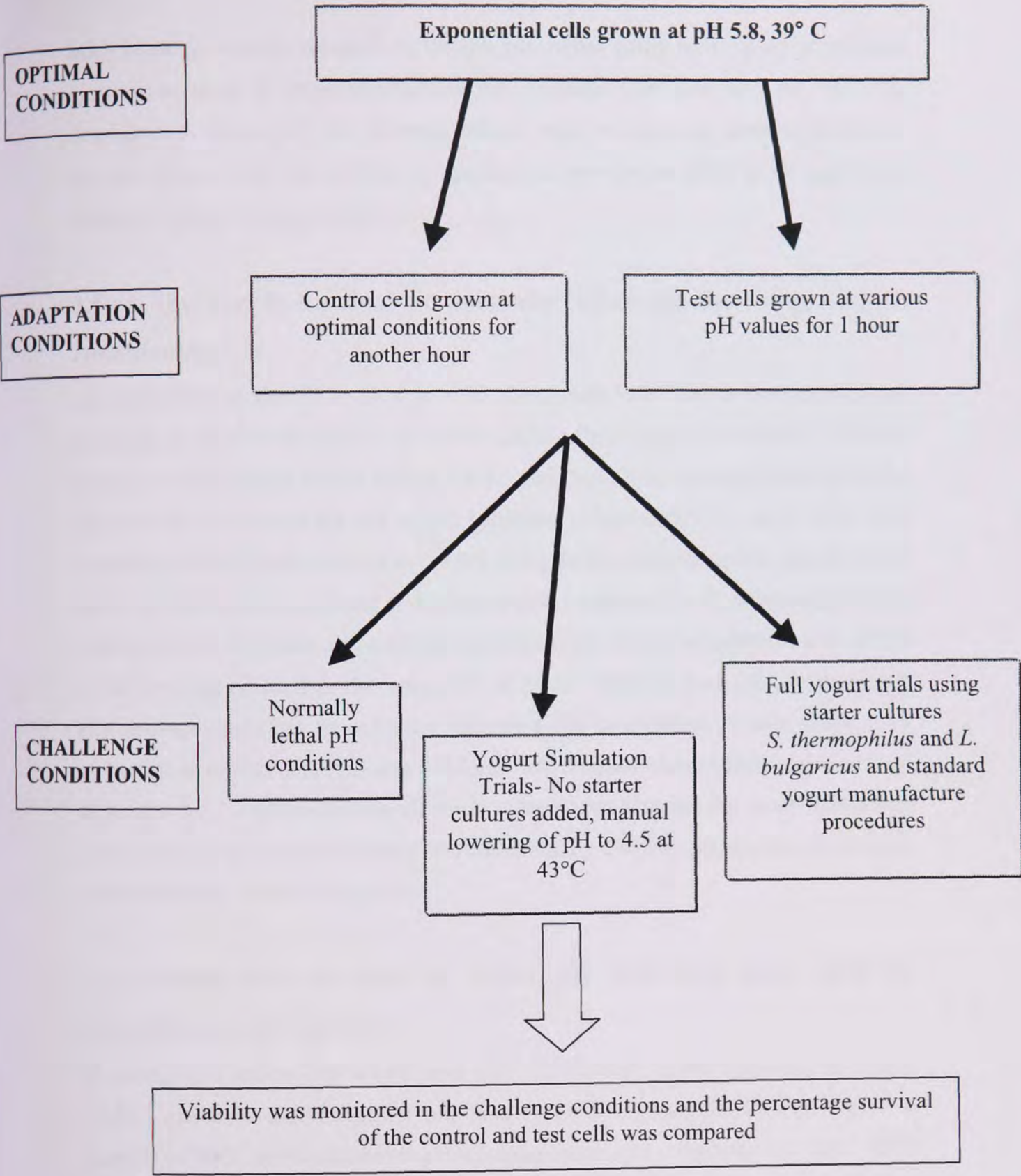


Figure 2.3 Flow diagram for the stress adaptation and challenge of cells

MRS broth (previously adjusted to various pH values using lactic acid) at volumes identical to those at pre-centrifugation and incubated for one hour at 39°C (as determined in section 2.5.12). Control cultures were treated in an identical fashion to the test culture, with cell pellets resuspended in pre-warmed MRS broth previously adjusted to pH 5.8 during conditioning.

2.5.14 Short-term survival at normally lethal pH following culture conditioning

Lb. acidophilus strains VUP 12,005, VUP 12,015 and VUP 12,028 were conditioned according to the method outlined in section 2.5.13. Cells were conditioned at 39°C for one hour at pH 5.0 or 4.5 (acid stress), pH 4.0 (acid shock) or step-wise lowering of the pH over the hour from pH 5.8 to 4.5 (stepwise conditioning). Control cells were incubated in MRS broth adjusted to pH 5.8 (using lactic acid) during this period. After one-hour, cells were centrifuged at 4,000 rpm for 15 minutes at 4°C and resuspended in either pH 3.5 or 3.0 lactic acid adjusted-MRS broth (to achieve an optical density of 1.0 at 600 nm) and returned to the water bath at 39°C. Samples were taken immediately after re-suspension (time 0) and every hour for a five-hour period. Viable counts were performed on diluted cultures using MRS agar and the pour plate technique as described in section 2.5.3. After incubation, colonies were counted and the mean percentage survival from triplicate experiments was calculated by dividing the number of cfu/ml at various times by cfu/ml at time zero.

2.5.15 Short-term survival at lethal pH following lactic acid or hydrochloric acid exposure

Lb. acidophilus strains VUP 12,005 and VUP 12,015 were treated according to section 2.5.13. Cells were conditioned for one hour at 39°C in either MRS broth at pH 5.8 (control) or MRS broths adjusted to pH 4.5 using lactic acid or hydrochloric acid. After one-hour, all samples were centrifuged at 4,000 rpm at 4°C for 15 minutes and resuspended in MRS broth at pH 3.5, where lactic acid was used to adjust the pH, then returned to the water bath (39°C). Samples were taken hourly and analysed for viable counts using MRS agar (pH 5.8) and the pour plate technique according to the protocol outlined in section 2.5.3. After incubation, colonies were counted and the percentage

survival was calculated by dividing the number of cfu/ml at various times by cfu/ml at time zero.

2.5.16 Detection of cross-protective responses following conditioning of exponential phase *Lb. acidophilus* cultures

Cross-protection studies were performed to determine whether exposure of cells to moderate pH during exponential phase protected cells against a variety of other challenges. Exponential phase cells of *Lb. acidophilus* strain VUP 12,015 were conditioned at pH 4.5 for one hour as described in section 2.5.13. Control cells were incubated at pH 5.8 during this period. Cultures were prepared in duplicate, with one set containing 200 µg/ml chloramphenicol (determined in section 2.5.8), which was added just prior to conditioning. After incubation for one hour at 39°C, all samples cells were centrifuged at 4,000 rpm for 15 minutes at 4°C and those containing chloramphenicol were washed to remove excess antibiotic. Cells were then exposed to a variety of challenges as previously determined for *Lb. acidophilus* (Lorca and de Valdez, 1999), including:

- a) pH 3.0 hydrochloric-acid-adjusted MRS broth at 39°C for four hours,
- b) pH 3.5 lactic-acid-adjusted MRS broth at 39°C for five hours,
- c) 3M NaCl in MRS broth at 39°C for 24h,
- d) 20% ethanol (v/v) in MRS broth at 39°C for 30 min,
- e) 10 mM hydrogen peroxide in MRS broth at 39°C for 60 min,
- f) MRS broth (pH 5.8) stored at -20°C for 24 h, or
- g) MRS broth (pH 5.8) stored at 60°C for 30 min.

Prior to and following challenge, cell viability was assessed using MRS agar and the pour plate technique according to methods described in section 2.5.3. Following incubation at 37°C for three days, colonies were counted and the percentage survival was calculated by dividing the number of cfu/ml at various times by cfu/ml at time zero.

2.5.17 Conditioning *Lb. acidophilus* cultures using fermentation end-products

Lb. acidophilus strains VUP 12,005 and VUP 12,015 and *L. johnsonii* strain VUP 12,028 were conditioned using cell filtrates containing fermentation end-products to determine whether exposure to these products during exponential phase of growth would improve survival during production and refrigerated storage of yogurt. Cell filtrates were used to condition cells in an attempt to introduce them to products that may cause loss of viability during refrigerated storage in yogurt. The cell-free filtrates were prepared by inoculating a 2% mono-culture of the strain being tested into MRS broth and incubating the culture at 37°C overnight (according to section 2.5.2). Cultures were then centrifuged at 4,000 rpm for 10 minutes at 4°C to collect the supernatant, which was then diluted to 50 or 75% of its original concentration (v/v) using fresh MRS broth and stored at 4°C until required. *Lb. acidophilus* VUP 12,005 and VUP 12,015 and *L. johnsonii* strain VUP 12,028 were conditioned using 50, 75 or 100% of the cell filtrate preparations for one hour at 39°C according to section 2.5.13. Control cells were incubated in fresh MRS broth (pH 5.8, 39°C) during this period. After one hour, cultures were centrifuged and resuspended in 20 ml of NFDM prior to challenge during simulated yogurt production, which is outlined in section 2.5.6.

2.5.18 Yogurt trials following conditioning of *Lb. acidophilus* cultures

Lb. acidophilus strains VUP 12,005 and VUP 12,015 and *L. johnsonii* strain VUP 12,028 were conditioned according to the method outlined in section 2.5.13. Briefly, cultures were grown to exponential phase in MRS broth at 39°C (pH 5.8) prior to centrifugation and re-incubation for one hour under the following conditions: pH 5.0 or 4.5 (acid stress), pH 4.0 (acid shock) or step-wise lowering of the pH from 5.8 to 4.5 (stepwise adaptation). Control cells were held at pH 5.8 during this period. After the one hour incubation period, cells were centrifuged and re-suspended in pre-warmed 10% Non-Fat Dry Milk (NFDM). This suspension was added along with the primary inoculation of *Str. thermophilus* and *Lb. bulgaricus* 217 for yogurt production according to section 2.5.5.

Survival of each strain was monitored throughout the fermentation process and during seven weeks of refrigerated storage according to methods outlined in section 2.5.3.

After incubation at 37°C for three days, colonies were counted and the percentage survival calculated to determine whether prior exposure of cells to low pH improved viability following yogurt preparation.

Conditioning of cultures using 10% NFDM instead of MRS broth as the culture medium was also performed in parallel to experiments described above. Volumes of medium and inoculum were identical to experiments performed using MRS broth. In contrast, cultures were not centrifuged after conditioning in 10% NFDM. Instead cells were grown to exponential phase, the pH of the milk was adjusted to pH 5.0 or 4.5 (acid stress), pH 4.0 (acid shock) or step-wise lowering of the pH from 5.8 to 4.5 (stepwise adaptation) and cells were incubated at 39°C for one hour (as described above) before being used as an inoculum for yogurt preparation according to section 2.5.5.

2.6 BIOCHEMICAL AND MOLECULAR BIOLOGICAL METHODS

2.6.1 Poly-acrylamide gel electrophoresis (PAGE) of sodium dodecyl sulphate (SDS) denatured cell proteins

2.6.1.1 Protein extraction

Samples were collected after one hour of conditioning (refer to section 2.5.13) or after preparation of inocula (refer to section 2.5.2), optical density values were determined, samples were centrifuged at 4,000 rpm for 15 min at 4°C and cells were washed once in an equivalent amount of 0.05 M Tris-Cl (pH 8.0) buffer. Cells were concentrated in the appropriate amount of 0.05 M Tris-Cl (pH 8.0) to give an optical density value equivalent to 20 at a wavelength of 600 nm. Cells were disrupted by application of temperature, homogenisation or sonication methods. Samples disrupted by heat were placed in Eppendorf tubes with double strength sample buffer in a 1:1 ratio and heated at 100°C for five minutes. For homogenisation, whole cell extracts were obtained by mixing two parts of the sample to one part of 0.45-0.50 mm glass beads (Braun, Melsungen AG, Germany) in ten ml centrifuge tubes. Cells were ruptured by homogenisation for 30 sec in a Kaiser-Braun homogeniser (Melsungen, Germany) and the cell debris removed by centrifugation for two to three min at 4,000 rpm.

Alternatively, whole cell suspensions were placed in Eppendorf tubes and sonicated for 6 x 5 seconds on ice using a Branson Sonifier 450 with a tapered three millimetre micro tip (ultra high intensity) and an output setting of five (Branson Ultrasonics Corporation, Eagle Road, Danbury, CT, USA). Samples of cell extract obtained by homogenisation or sonication methods were mixed in a 1:1 ratio with double strength sample buffer and boiled for 30 sec prior to loading samples on the gel.

2.6.1.2 Determination of protein levels in bacterial extracts

A modified Lowry protein assay (Lowry *et al.*, 1951) was used to determine the amount of protein in cell-free extracts (prepared in section 2.6.1.1) prior to their separation on PAGE gels. Standards containing 0 to 100 µg of bovine serum albumin in a total sample volume of 0.5 ml were prepared in duplicate and used to create a calibration curve. In addition, samples were diluted 1:9 using double distilled water and 50 µl was added to labelled tubes. Lowry reagent C (0.5 ml) was prepared by adding Lowry reagents A + B in a ratio of 50:1. Lowry reagent C was added to the tubes, which were then vortexed and incubated at 37°C for 10 minutes prior to the addition of 1.5 ml of 10% v/v Folin-Ciocalteu's reagent (BDH Laboratory Supplies, Poole, England). The tubes were then incubated at 52°C for 20 minutes, cooled at room temperature and the absorbance measured at 680 nm. The concentration of protein was determined from a linear standard curve plotted for the amount of protein standard versus the absorbance of the standards at 680 nm.

2.6.1.3 SDS-PAGE

Discontinuous SDS-PAGE was performed according to the procedure described initially by Laemmli (1970), with some modification. Samples prepared according to section 2.6.1.1 and 15 to 25 µl were loaded onto a 4% acrylamide stacking gel (0.125M Tris-Cl, pH 6.8) over a gradient of 7 to 17% acrylamide (0.375M Tris-Cl, pH 8.8). The electrophoresis buffer was 25mM Tris-Cl-0.192 M glycine (pH 8.3). The final concentration of SDS in both gels and in the electrode buffer was 0.1%. Electrophoresis was performed using a Protean II apparatus (BioRad Laboratories, CA, USA) at 10°C with a constant current of 40 mA until the bromophenol blue dye front reached the bottom of the gel. Molecular weight marker polypeptides (one microlitre) ranging in

size from 6.5 to 200 kDa (Unstained SDS-PAGE Standards-Broad Range; Bio-Rad) were run in parallel with each gel.

2.6.1.4 Protein Staining Techniques

a) Coomassie Blue Staining

Protein gels were stained according to manufacturers instructions (Progen Industries Ltd., Ipswich, Australia). Briefly, protein gels were placed in fixing solution for one hour before being placed in Coomassie blue staining solution for at least one hour. Gels were rinsed in water for 2 x 10 minutes and then placed in de-staining solution until the bands were visible. Gels were destained further in water before being photographed according to section 2.6.5.

b) Silver Staining (Sambrook et al., 1989)

Protein gels were placed into fixing solution for 30 minutes followed by incubation solution for 30 minutes. The gel was washed at least three times in distilled water before being placed in silver solution for 40 minutes. The gel was then placed in developing solution until the bands were visible and then transferred immediately into stop solution for 5 to 10 minutes to stop the reaction. Excess stop solution was washed from the gel by sequential washes in distilled water before the gel was preserved in 10% glycerol. Gels were photographed according to section 2.6.5.

c) SYPRO Orange Staining

SYPRO Orange protein stain (Biorad-Rad Laboratories, Hercules, CA; 10 μ l) was added to 7.5% acetic acid (50 ml). The gel was incubated in SYPRO Orange staining solution for 30 minutes with gentle agitation at room temperature and then rinsed with 7.5% acetic acid for one minute to remove excess stain according to the manufacturer's instructions. Gels were photographed according to section 2.6.5.

2.6.1.5 Protein Gel Analysis

Comparisons of bands were made using an LKB Bromma Ultrascan XL Enhanced Laser Densitometer and GELSCAN XL 2.1 Software (Sweden). Alternatively, gels were photographed using the LAS 1000 plus luminescent imaging system and bands were quantified using an Image Gauge 3.122 image analyser. For similarity calculations

between protein profiles, the peak position data obtained by densitometry and visual comparisons were used to calculate the Dice coefficient of similarity (S_D) represented by the following formula:

$$S_{D(A,B)} = 2n_{(A,B)} / (n_A + n_B)$$

Where $n_{(A,B)}$ is the number of matched bands occupied by both strains A and B, and n_A , n_B the number of bands occupied by strains A and B respectively (Boutrou *et al.*, 1995).

2.6.2 Incorporation of ^{35}S -labelled cysteine/methionine into proteins following a shift of exponential phase cells into various pH values

2.6.2.1 Conditioning period

Radiolabelling was performed to detect the synthesis and repression of protein synthesis during cell conditioning. Conditioning of cells was performed according to section 2.5.13. Briefly, *Lb. acidophilus* strains VUP 12,005, VUP 12,015 and VUP 12,028 were grown to exponential phase prior to centrifugation and re-suspension in pre-warmed MRS broth pH-adjusted to either 4.5, 4.0 or lowered stepwise from 5.8 to 4.5 over the one hour incubation period at 39°C. Control cells were grown at optimal conditions of pH 5.8 at 39°C throughout the experiment. Radiolabel, 100 $\mu\text{Ci/ml}$ of ^{35}S -labelled cysteine/methionine (^{35}S - cys/met) mixture (specific activity 1500 Ci.mmol^{-1} , ICN Biomedicals, Australia) was added to all samples during the conditioning period (Hartke *et al.*, 1996; Broadbent *et al.*, 1997). In addition, 200 $\mu\text{g/ml}$ chloramphenicol (determined in section 2.5.8) was added to one set of samples that were conditioned at pH 4.5.

2.6.2.2 Extracting Proteins

After the one-hour conditioning period, cells were cooled on ice, centrifuged at 3,000 rpm for five minutes at 4°C and washed with ice-cold phosphate buffered saline to remove excess radiolabel. Sample buffer was added to the cell pellet to achieve an optical density of 20.0 at 600 nm and proteins were extracted by sonication using methods outlined in section 2.6.1.1.

2.6.2.3 Estimating total amounts of radiolabel incorporated

Quadruplicate aliquots (10 µl) of each cell lysate were transferred into Eppendorf tubes. Ice-cold TCA (20% w/v) was added to the tubes and mixed thoroughly before leaving on ice for 30 minutes. Each precipitate was filtered under vacuum using a three-piece filter funnel unit (Whatman, England) containing 21 mm GC 50-glass fiber filter paper (Advantec, Australia). The filter paper was washed twice with two millilitre ice-cold 20% TCA followed by 2 x 2 ml washes with ice-cold acetone. Filters were divided into two sets and two µl of ³⁵S-cys/met stock of known activity was added to one set of filters (standard filters), whilst the remaining filters were labelled sample filters. After all of the filters had dried, they were placed in scintillation vials (Crown Scientific, Vic., Australia) and covered with 2 ml of PPO (2,5 diphenyloxazole) scintillation solution. Scintillation vials were placed in a Wallac 1410 liquid scintillation counter (Pharmacia Biotech) and counts were obtained over 60 seconds using the cpm (counts per minute) counting mode. Total incorporation of radiolabel into newly synthesized proteins using scintillation counting was determined using the following formula (refer to Appendix 2 for a sample calculation):

$$\text{Counting Efficiency} = \frac{(\text{cpm of standard} + \text{sample}) - (\text{cpm of sample})}{\text{known dpm of standard}} \times 100$$

From the counting efficiency and the cpm values for each sample, the dpm for each sample was determined using the following formula:

$$\text{Dpm (sample)} = \text{cpm of sample} \times \frac{100}{\text{counting efficiency}}$$

2.6.2.4 Identifying stress-induced proteins

Proteins were separated using 12% poly-acrylamide gels loaded with equal amounts of protein samples and Protean II Minigel system according to the methods outlined in section 2.6.1.3. Gels were run in duplicate. One gel was stained with SYPRO orange (refer to section 2.6.1.4) and scanned using Image Gauge 3.1 software to ensure that protein loading was equivalent (refer to section 2.6.1.5). The duplicate gel was placed on Whatman number one paper and dried at 80°C for three hours using a Dry gel SE

1160 slab gel dryer (Hoefer Scientific Instruments, San Francisco, USA) and a Bio-Rad Hydro Tech Vacuum Pump (Bio-Rad Laboratories, CA, USA). Dried gels were exposed on Kodak X-OMAT AR X-ray film for 2 to 3 days in a 35 x 43 cm X-ray cassette. The X-ray film was developed using a manual X-ray fixing bath and developer (AGFA-GEVAERT, Australia). Autoradiography images were then scanned using LAS 1000 and analysed using Image Gauge 3.1 software (refer to section 2.6.1.5) to determine the intensity of bands and thus the level of synthesis or repression of proteins during acid adaptation.

2.6.3 Pulsed-field gel electrophoresis of genomic DNA (PFGE)

2.6.3.1 Preparation of genomic DNA

Chromosomal DNA was isolated according to the method described by Lin and Johnson (1995), with slight modification. *Lb. acidophilus* strains VUP 12,016, 2402, VUP 12,012, VUP 12,005, VUP 12,006, VUP 12,015, VUP 12,007, VUP 12,027, VUP 12,028, VUP 12,025 and *Lb. bulgaricus* strain CSCC 2515 were grown at 37°C overnight in MRS broth according to section 2.5.2, harvested by centrifugation and washed several times with PIV solution. The suspension was treated for 10 min at 50°C to inactivate DNase activity and mixed with 1% low melting temperature chromosomal grade agarose (BioRad Laboratories). The mixture was cast in a BioRad gel mould and allowed to set at 4°C.

The cells were lysed in EC lysis solution at 37°C overnight, followed by overnight treatment with proteinase K (0.5 M EDTA, pH 9.0, 1% sarkosyl, 1 mg/ml proteinase K) at 45°C. The agarose blocks were then treated for three hours with phenylmethylsulfonyl fluoride in TE buffer (10 mM Tris chloride, pH 8.0, 1 mM EDTA). The blocks were washed four times with TE buffer at 45°C and stored at 4°C until further use.

2.6.3.2 DNA digestion with endonucleases

Half of an agarose block was prepared for each restriction digest using a sterile scalpel blade. The blocks were placed in fresh TE buffer and warmed at 45°C before undergoing digestion using a restriction digest containing one of the following enzymes:

IceuI, *BstXI*, *NotI*, *DrdI*, *SfiI*, *SpeI*, *KpnI*, *PvuII*, *BamHI*, *MspI*, *Eco91I*, *NheI*, *HpaII*, *BclII*, or *XbaI*. (New England Biolabs, MA, USA), according to the manufacturers instructions. The digested blocks were washed several times in TE buffer at 45°C to stop the reaction.

2.6.3.3 Pulsed-field gel electrophoresis

Agarose blocks containing digested DNA were loaded in 1% DNA-grade agarose gels and electrophoresis was performed in a Pharmacia Gene Navigator with circulating 0.5 x TBE buffer at 12°C. High molecular weight bands, greater than 48.5 Kb in size were separated using a five second pulse time for 10 hours, followed by a 10 second pulse time for 15 hours at 170 volts. DNA bands less than 48.5 Kb in size were separated using a 1.5 second pulse time for 10 hours, followed by a 2.5 second pulse for 10 hours at 170 volts (this thesis). The gels were stained with ethidium bromide solution (final concentration 1 µg/ml), viewed by UV transillumination and photographed as per section 2.6.5. The sizes of DNA fragments were estimated by comparison with high, medium and low range molecular weight markers (bacteriophage λ DNA concatamers; New England Biolabs, MA, USA) which were run with each set of samples. The Dice coefficient of similarity was used to determine the relatedness between the restriction patterns obtained (Dice, 1945).

2.6.4 DNA sequencing of the 16S rRNA gene

2.6.4.1 DNA preparation for PCR

DNA from *Lb. acidophilus* strains VUP 12,016, VUP 12,002, VUP 12,012, VUP 12,005, VUP 12,006, VUP 12,015, VUP 12,007, VUP 12,027, VUP 12,025 and VUP 12,028 were extracted from pre-prepared Pulsed-Field Gel Electrophoresis (PFGE) blocks using a Progen Bandpure DNA purification kit (Progen Industries Ltd., Ipswich, Australia). Briefly, three volumes of NaI binding buffer containing 0.1 volumes of TBE assist were added to an Eppendorf tube containing a pre-prepared PFGE block. BANDPURE silica matrix suspension (5 µL) was added to the Eppendorf containing the PFGE block and incubated at room temperature for five minutes with frequent gentle mixing. The tube was centrifuged at 15,000 rpm for 30 seconds, and the matrix pellet

was washed several times with 500 μ L of cold ethanol wash solution. After the final wash, the DNA was eluted in TE buffer at 55°C for five minutes and the supernatant was collected after centrifugation for 30 seconds (as per manufacturer's instructions). DNA from each strain was prepared in duplicate.

2.6.4.2 Determining DNA concentration

DNA concentrations were determined using the spectrophotometric method as described by Sambrook *et al.* (1989). Extracted nucleic acid samples (section 2.6.4.1) or synthesized oligonucleotides were diluted 1:50 (for DNA) or 1:100 (for oligonucleotides) in distilled water and absorbance readings were recorded at wavelengths of 260 nm and 280 nm. The A_{260}/A_{280} ratio provided an estimation of the purity of the samples. Nucleic acid concentration was determined according to the equation: 1 A_{260} unit (1 cm light path) = 50 μ g/ml solution for double-stranded DNA and 33 μ g/ml solution for single stranded oligonucleotides.

2.6.4.3 Primer design

The 16S rRNA gene of approximately 1.4 Kb were amplified using a combination of the forward primers: C75 (5'-GAGAGTTTGATYCTGGCTCAG^{3'}) (Dewhirst *et al.*, 1999) and HE1 (5'-AGCAGATCGCATGATCAGCT^{3'}) (Drake *et al.*, 1996) and the reverse primers SM1 (5'-TACAACGAGGAGCAAGCCTG^{3'}), SM3 (5'-GCACGAAGCTGGAATCGCTA^{3'}), and SM4 (5'-GTAATCGCGGATCAGCACGC^{3'}) (this thesis). The reverse primers were designed using the GenBank database (Australian National Genomic Information Services- ANGIS) and the multiple sequence alignment of species within the *Lb. acidophilus* complex. Primers were designed within the conserved regions of the 16S rRNA sequences.

2.6.4.4 Amplification of 16S rDNA regions using primers

The 16S rRNA cistrons of one DNA strand were amplified using a combination of forward and reverse primers. PCR was performed in thin-walled tubes with a Perkin Elmer PTC-100 thermocycler. DNA template (10 ng) was added to a reaction mix (final volume, 25 μ L) containing 20 pmol of each primer, 200 μ M deoxynucleoside triphosphates, 2.5 U of AmpliTaq DNA Polymerase (Perkin Elmer), 1.5 mM $MgCl_2$, and

10 mM Tris-HCl, pH 8.3, 50 mM KCl. Samples were preheated at 94°C for 2 minutes, and this was followed by amplification in which the following conditions were used: denaturation at 94°C for 15 sec, annealing at 60°C for 1 min, and elongation at 72°C for 1.5 min. A total of 30 cycles were performed before the final elongation step at 72°C for seven minutes. Amplification of the 16S rDNA regions was performed in duplicate.

2.6.4.5 Visualising DNA product using agarose gel electrophoresis

Electrophoresis of DNA was normally performed with horizontal 0.8 % (w/v) agarose slab gels measuring 10.5 x 8 cm (Gel Electrophoresis Apparatus GNA-100, Pharmacia, Sweden). Electrophoresis was carried out using 1 x TBE buffer and running at 80 volts until the dye front ran near to the bottom of the gel. DNA samples were prepared by aliquoting (5 µl) PCR product into an Eppendorf tube and adding loading buffer (0.5 µl).

Lambda DNA digested with *Eco* RI and *Hind* III (Progen) containing 11 fragments (21.2, 5.1, 5.0, 4.3, 3.5, 2.0, 1.9, 1.6, 1.4, 0.9, 0.8, 0.6, 0.1 kb) was used as a molecular marker for the genomic DNA. Alternatively, a 100 base pair DNA Ladder (GeneRuler Plus, MBI Fermentas) containing 14 DNA fragments (3000, 2000, 1500, 1200, 1031, 900, 800, 700, 600, 500, 400, 300, 200, 100 bp) was used as the size marker for PCR products. The DNA samples were visualised by the addition of ethidium bromide staining solution (0.5 µg/ml) to the gel. The size of DNA fragments was estimated after visualisation and photography of the gel under short-wavelength UV light ($\lambda = 302$ nm) (LKB 2011 Macrovue transilluminator, section 2.6.5) by comparing the migration distance of the sample DNA with DNA markers.

2.6.4.6 PCR product purification

PCR products obtained from amplification of 16S rDNA regions (section 2.6.4.4) were purified using Microspin S-300 HR columns (Pharmacia-Biotech). Microspin columns were vortexed briefly and centrifuged at 3,000 rpm for one minute to re-suspend the Sephacryl HR resin in pre-equilibrated TE buffer. PCR products were added to the top of the resin and the column was centrifuged at 3,000 rpm for two minutes. The purified sample was collected in the support tube and stored at -20°C until required.

2.6.4.7 Sequencing PCR products

Preparation of the templates for automated sequencing was performed using a BigDye terminator cycle sequencing kit (Perkin-Elmer). The reaction consisted of 8 µl BigDye terminator, 40 ng PCR product (determined visually using a 100 base pair marker containing a 10 ng/µl indicator band, Promega), 10 pmol/µl primer, either C75 (5'GAGAGTTTGATYCTGGCTCAG3') (Dewhirst *et al.*, 1999)) or SM4 (5'GTAATCGCGGATCAGCACGC3') (this thesis), and distilled deionised water (total volume = 16 µl). Cycle sequencing was performed under the following conditions: denaturation at 96°C for 30 sec, annealing at 50°C for 15 sec, extension at 60°C for four min. A total of 25 cycles were performed.

2.6.4.8 Precipitation of PCR products

Sequencing products were precipitated using the sodium acetate/ethanol precipitation technique (Personal communication, Department of Microbiology, Monash University, sequence@med.monash.edu.au). A 1.5 ml microcentrifuge tube was prepared for each sequencing reaction and contained 2.0 µl of 3 M sodium acetate (pH 4.6) and 50 µl of 95% ethanol. After the addition of samples, tubes were vortexed and left at room temperature for 15 minutes to precipitate the extension products. The tubes were then spun in a microfuge for 30 minutes at 12,000 x g and the supernatant was aspirated with a pipette. The pellet was rinsed with 250 µl of 70% ethanol and the tubes were re-centrifuged for five minutes at 12,000 x g. The supernatant was aspirated from the tube and the tubes left in the laminar flow with the lids open for 15 to 30 minutes to completely dry the pellet. Samples were submitted for automated sequencing to the Department of Microbiology, Monash University (joint VUT-Monash University facility).

2.6.4.9 Analysis of DNA Sequences

DNA sequence data were analysed using the GenBank database similarity search program, BLASTn, which was accessed through the Internet at <http://mellangis.org.au>.

2.6.5 Photography of DNA gels, protein gels and agarose plates

Photographs were taken with a Polaroid MP4 Land Camera using either black and white positive (667) or positive/negative film (665) (Polaroid Polapan 667, Polaroid Corporation, MA, USA). Details of camera settings are outlined in Table 2.2.

For photographs prepared using positive/negative film, negatives were immediately placed in water to remove the film lining and then washed in 18% Na₂SO₃. Negatives were washed in Kodak Photoflow 600 solution (1/600 dilution in distilled water) for 10 seconds and dried.

Table 2.2. Photography using the Polaroid MP4 Land Camera

Sample	Stain used for visualisation	Film	Shutter Speed	Apperture	Other details
Protein	Silver	667+	1/125 sec	32	Lightbox
Protein	Coomassie	667+	1/30 sec	32	Lightbox Orange Filter
Protein	Sypro Orange	667+	1/2 sec	5.6	Transilluminator 302 nm [#] Orange Filter
DNA	Ethidium Bromide	665 +/-	45 sec	5.6	Transilluminator 302 nm
		667 +	1-1/8 sec	5.6	Orange Filter
Agarose Plates	N/A	667+	1/125 sec	8	Side lights Orange filter Plates placed on dark surface

N/A = Not applicable

[#] = Transilluminator 302 nm (LKB 2011 Macrovue).

CHAPTER 3

DIFFERENTIATION, IDENTIFICATION AND CHARACTERISATION OF PROBIOTIC STRAINS

3.1 INTRODUCTION

In order to exert health-benefits to the consumer, it is essential that probiotic strains survive the inhospitable environment of the human gastrointestinal tract. To achieve this, selected strains must withstand the low pH of the stomach and also secretion of bile acids in the upper intestinal tract. It is also crucial to verify the safety of those strains used in products intended for human consumption. This requires their definitive taxonomic characterisation.

Identification of probiotic cultures has become an important topic in the last decade for determining the presence, activity, and viability of probiotic bacteria in the human gastrointestinal tract and probiotic products. Traditionally, these species were studied by phenotypic characteristics such as sugar fermentation, colony morphology, Gram reaction and growth traits at various temperatures (Fujisawa *et al.*, 1992; Nighswonger *et al.*, 1996). However, phenotypic characterisation is inadequate for identification of closely related species, like those in the *Lb. acidophilus* complex, that occupy similar environments and are likely to play similar functional roles. Fujisawa *et al.* (1992) reported that sugar fermentation patterns did not allow a differentiation between *Lb. acidophilus* and the phenotypically very similar *Lb. johnsonii*, *Lb. crispatus*, *Lb. gasseri*, *Lb. amylovorus* and *Lb. gallinarum*.

A number of current strain identification and tracking techniques have been applied to probiotic lactobacilli species. At the species level there are several reports on specific PCR and other DNA-based identification systems for lactobacilli that target ribosomal genes (Du Plessis and Dicks, 1995; Schleifer *et al.*, 1995; Drake *et al.*, 1996; Tilsala-Timisjarvi and Alatossava, 1997; Roy *et al.*, 2000; Walter *et al.*, 2000; Guarneri *et al.*, 2001; Heilig *et al.*, 2002; Ogier *et al.*, 2002; Sui *et al.*, 2002; Yeung *et al.*, 2002) and ribosomal intergenic regions (Tannock *et al.*, 1990; Tilsala-Timisjarvi and Alatossava,

1997). These systems are not sensitive enough to differentiate bacteria below the species level.

A variety of strain identification techniques have been applied to probiotic species, including plasmid profiling (Tannock *et al.*, 1990), lectin typing (Annuk *et al.*, 2001) and protein profiling (Dykes and von-Holy, 1993; Hertel *et al.*, 1993; Du Plessis and Dicks, 1995; Vandamme *et al.*, 1996; Corsetti *et al.*, 2003). Various DNA-based methods such as restriction fragment length polymorphisms (RFLP), pulsed-field gel electrophoresis (PFGE), ribotyping, PCR amplification of repetitive bacterial DNA elements and random amplified polymorphic DNA (RAPD) technique have been used for identification of *Lactobacillus* strains (Rodtong and Tannock, 1993; Roussel *et al.*, 1993; Du Plessis and Dicks, 1995; Sanders *et al.*, 1996; Walker *et al.*, 1996; Tilsala-Timisjarvi and Alatossava, 1997; Gevers *et al.*, 2001; Corsetti *et al.*, 2003).

The introduction of these techniques has seen the re-classification of some organisms used in foods, as identification based on phenotypic studies are often contradictory to those based on genomic identification techniques (Gilliland and Speck, 1977b; Busse *et al.*, 1996; Sanders *et al.*, 1996; Holzapfel *et al.*, 1998; Klein *et al.*, 1998; Schillinger, 1999).

A polyphasic approach, as suggested by Vandamme *et al.* (1996) was used in this chapter for the identification and characterisation of *Lactobacillus* strains. The techniques encompass phenotypic (sugar fermentation analysis, antibiotics sensitivity, acid and bile tolerance), chemotaxonomic (whole cell protein profiles), genotypic (PFGE) and phylogenetic (16S rDNA sequence) analysis to both characterize and identify strains purchased as *Lb. acidophilus*.

The general aim of research reported in this chapter was to select candidate strains for their potential use as probiotic cultures. In particular, the specific aims were to:

- a) differentiate strains based on their sugar fermentation patterns, antibiograms, protein profiles and DNA restriction patterns,
- b) confirm the identify of selected strains using 16S rRNA analysis, and

- c) characterise strains according to their relative acid and bile tolerance and their ability to survive storage in yogurt-simulating conditions.

3.2 DIFFERENTIATION OF ISOLATES

3.2.1 Carbohydrate fermentation tests

Ten strains of *Lb. acidophilus* (presumptive identification) and one strain of *Lb. bulgaricus* were tested for their ability to ferment twenty sugar substrates as a sole carbon source (according to section 2.5.7). A positive result was recorded following a visible colour change of the fermentation broth from purple to yellow. Results conformed to those published in Bergey's Manual (Kandler and Weiss, 1986), with the exception of strains VUP 12,015 and VUP 12,025, which were unable to ferment galactose and amygdalin, respectively (Table 3.1). Nevertheless, these strains still conformed to the 90% similarity necessary for classification as *Lb. acidophilus* (Kandler and Weiss, 1986).

3.2.2 Antibiotic sensitivity tests

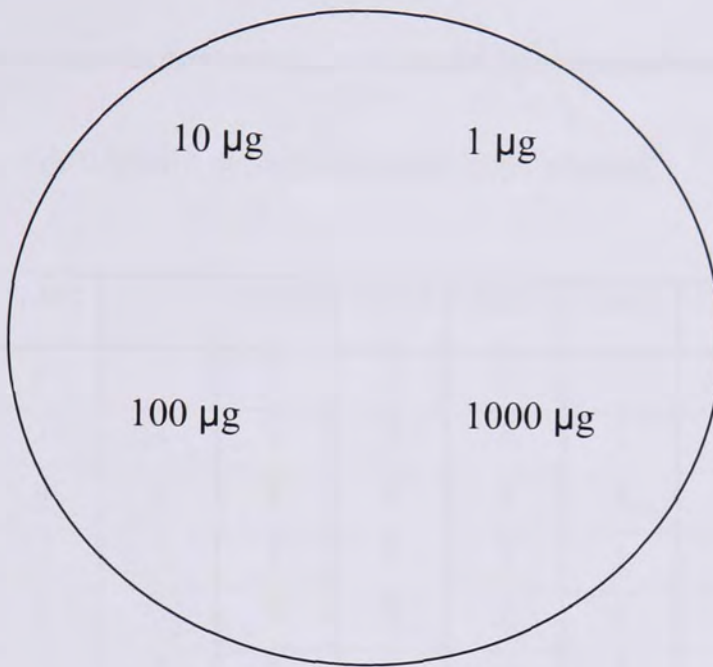
Antibiotic sensitivity tests were performed to determine whether strains could be differentiated based on their antibiotic susceptibility patterns. The agar well diffusion assay (described in section 2.5.8) was used to test the sensitivity of strains (initially identified as *Lb. acidophilus*) to 1 to 1,000 µg/ml of selected antibiotics. The agar well diffusion technique was an appropriate method for determining the minimum inhibitory concentration of each antibiotic, since clear zones of inhibition were present following antibiotic exposure (Figure 3.1). Results showed that strains could be differentiated based on their antibiotic sensitivity patterns (Table 3.2). Overall, no two antibiotic sensitivity patterns were alike, particularly with the antibiotics clindamycin, lincomycin and rifampicin, which showed variation between strains. However, the antibiotics ampicillin, gentamycin, kanamycin, methicillin, neomycin, piperacillin, streptomycin, and tetracycline could not be used to differentiate strains, since antibiotic sensitivity patterns of the strains were almost identical using these antibiotics.

Table 3.1 Utilisation of various sugars as a sole carbon source by *Lb. acidophilus*.

Fermentation of sugars by strains ^a :										
Sugar	12,002	12,012	12,005	12,006	12,015	12,007	12,027	12,028	12,025	12,016
Amygdalin	+	+	+	+	+	+	+	+	-	+
Arabinose	-	-	-	-	-	-	-	-	-	-
Cellobiose	+	+	+	+	+	+	+	+	+	+
Esculin	+	+	+	+	+	+	+	+	+	+
Fructose	+	+	+	+	+	+	+	+	+	+
Galactose	+	+	+	+	-	+	+	+	+	+
Glucose	+	+	+	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+	+	+	+
Mannitol	-	-	-	-	-	-	-	-	-	-
Mannose	+	+	+	+	+	+	+	+	+	+
Melezitose	-	-	-	-	-	-	-	-	-	-
Melibiose	+	+	+	-	-	+	-	-	+	-
Raffinose	+	+	+	+	-	+	+	+	+	+
Rhamnose	-	-	-	-	-	-	-	-	-	-
Ribose	-	-	-	-	-	-	-	-	-	-
Salicin	+	+	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+	+	+
Trehalose	+	+	+	-	+	+	+	+	+	+
Xylose	-	-	-	-	-	-	-	-	-	-

^aUtilisation of tested substrate signified as acid production (+), minor acid production (+/-) or no acid production (-). Strain VUP 12,016 = *Lb. acidophilus* Type strain.

a)



b)

Spectinomycin

Piperacillin

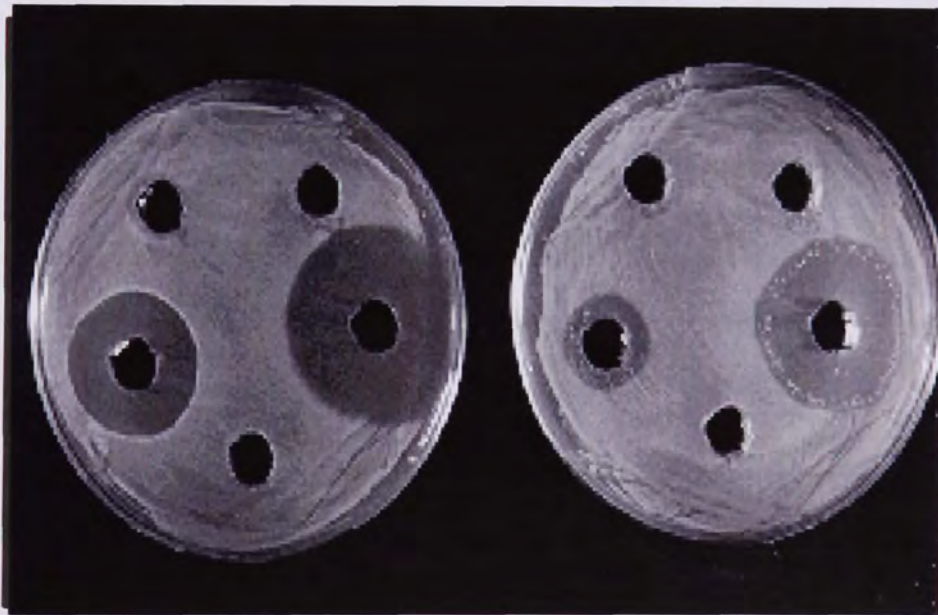


Figure 3.1. a) Antibiotic concentration in wells shown in Figure b. b) Antibiotic resistance patterns of *Lb. acidophilus* strain VUP 12,002 exposed to a concentration of 0 to 1,000 µg/ml of either spectinomycin or piperacillin.

Table 3.2. Antibiotic sensitivity patterns of *Lb. acidophilus* strains.

Antibiotic sensitivity pattern of strains ^a :									
Antibiotic	12,002	12,012	12,005	12,006	12,015	12,007	12,027	12,028	12,025
Amoxicillin	1	1	1	2	1	1	2	2	2
Ampicillin	1	2	2	2	2	2	2	2	2
Chloramphenicol	4	4	4	4	3	4	3	3	4
Clindamycin	3	3	1	3	4	1	3	2	3
Erythromycin	2	3	3	3	2	2	2	2	2
Gentamycin	4	4	3	4	4	4	4	4	4
Kanamycin	4	4	4	4	4	4	4	4	4
Lincomycin	4	4	2	4	4	2	3	3	4
Methicillin	3	3	3	3	3	3	3	3	3
Neomycin	4	4	4	4	4	4	4	4	4
Piperacillin	3	3	2	3	3	3	3	3	3
Rifampicin	2	2	3	3	3	2	3	1	3
Spectinomycin	3	4	4	3	4	4	4	4	3
Streptomycin	4	4	3	4	4	4	4	4	3
Tetracycline	3	3	3	3	3	3	3	3	3
Vancomycin	2	2	2	2	2	3	2	3	3

^a Tabulated values represent the Minimum Inhibitory Concentration (MIC) of each antibiotic in $\mu\text{g/ml}$. 1 = MIC $\leq 1 \mu\text{g/ml}$, 2 = MIC $\leq 10 \mu\text{g/ml}$, 3 = MIC $\leq 100 \mu\text{g/ml}$, 4 = MIC $\geq 1000 \mu\text{g/ml}$.

3.2.3 Pulsed-Field Gel Electrophoresis

Chromosomal DNA of ten strains initially identified as *Lb. acidophilus* was characterised by DNA restriction patterns using low-frequency cleavage endonucleases and pulsed-field gel electrophoresis (section 2.6.3). Of the restriction enzymes tested, digestion with *Sma*I (5' CCC↓ GGG 3') showed the best profiles, generating nineteen to twenty-two fragments ranging in size from 8 to 230 kb. These fragments were separated using two sets of running conditions. The first, for separation of high molecular weight bands greater than 48.5 kb in size, (Figure 3.2) was a five second pulse time for 10 hours, followed by a 10 second pulse for 15 hours. The second, for low molecular weight bands below 48.5 kb in size (Figure 3.3), was a 1.5 second pulse time for 10 hours followed by 2.5 second pulse for 10 hours. Other enzymes tested, including *Ice*uI, *Bst*xI, *Not*I, *Drd*I, *Sfi*I, *Spe*I, *Kpn*I, *Pvu*II, *Bam*HI, *Msp*I, *Eco*91I, *Nhe*I, *Hpa*II and *Bcl*II produced too many or too few fragments (data not shown).

The size of each DNA fragment was calculated on the basis of the electrophoretic mobility in relation to the mobility's of standards with known molecular masses (Table 3.3). This data was used to calculate the genome size of each strain. No fragments were considered as doublets due to their uniform intensity and therefore were not taken into account twice to estimate the genomic sizes obtained. The genome sizes estimated as the sum of the restriction fragments of the *Sma*I digests for *Lb. acidophilus* were between 1.5 to 2.0 Mb (Table 3.3), with most strains clustering at 1.5 Mb. The *Sma*I digestion patterns of strains VUP 12,016/VUP 12,015 and VUP 12,012/VUP 12,006/VUP 12,025 were indistinguishable and hence their genome sizes were found to be identical.

DNA patterns were compared to the type strain (VUP 12,016) and Dice coefficients of similarity were calculated on the basis of the percentage of the co-migrating fragments (Dice, 1945). Dice coefficients for *Lb. acidophilus* strains ranged from 0.24 (24% similarity, strain VUP 12,028) to 1.0 (100% similarity, strain VUP 12,015) when compared to the type strain VUP 12,016 (Table 3.4), with most strains clustering at a level of >80% similarity.

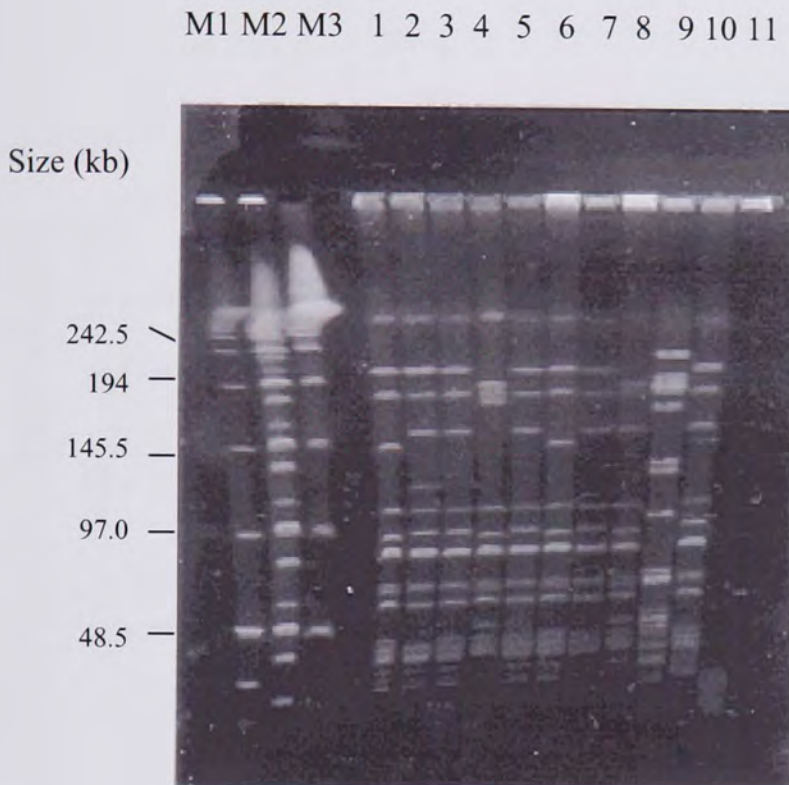


Figure 3.2. PFGE patterns of *Sma I* digests of genomic DNA. High molecular weight band separation. Lanes: M1, Low-Range Marker; M2, Mid-Range Marker and M3, Lambda Ladder Markers; 1, *Lb. acidophilus* VUP 12,016 (Type strain); 2, VUP 12,002; 3, VUP 12,012; 4, VUP 12,006; 5, VUP 12,015; 6, VUP 12,015; 7, VUP 12,007; 8, VUP 12,027; 9, VUP 12,028; 10, VUP 12,025; 11, *Lb. delbrueckii* ssp. *bulgaricus* CSCC 2515.

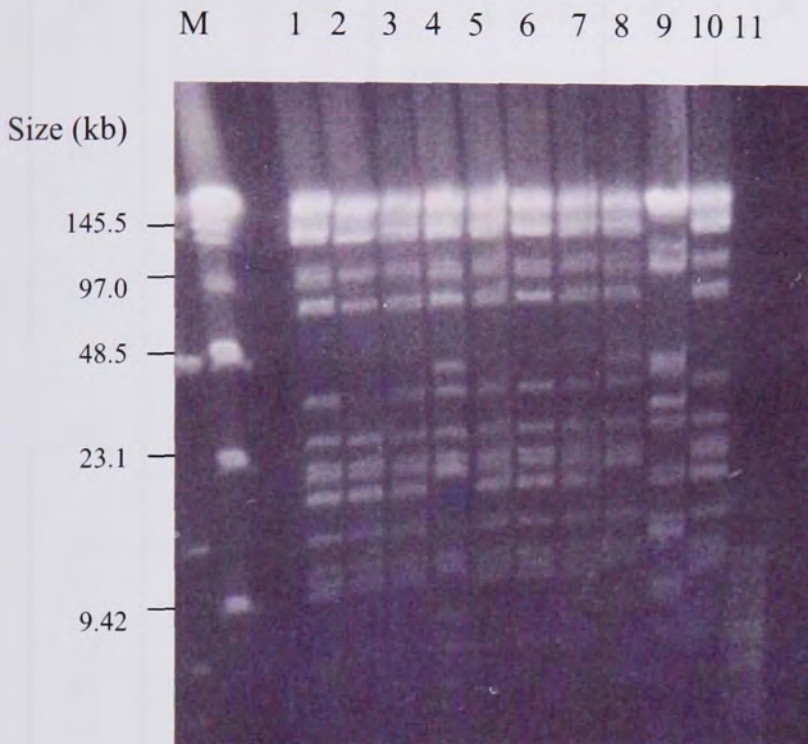


Figure 3.3. PFGE patterns of *Sma I* digests of genomic DNA. Low molecular weight band separation. Lanes: M, Low-Range Marker; 1, *Lb. acidophilus* VUP 12,016 (Type strain); 2, VUP 12,002; 3, VUP 12,012; 4, VUP 12,006; 5, VUP 12,015; 6, VUP 12,015; 7, VUP 12,007; 8, VUP 12,027; 9, VUP 12,028; 10, VUP 12,025; 11, *Lb. delbrueckii* ssp. *bulgaricus* CSCC 2515.

Lb. bulgaricus, when digested with *Sma*I, produced numerous fragments that were below 15 kb in size. Therefore there was no resemblance to fingerprints produced by the *Lb. acidophilus* species, and the genome size could not be calculated. Digestion of *Lb. bulgaricus* genomic DNA using *Xba*I was more appropriate for optimal separation of DNA fragments (Figure 3.4).

3.2.4 SDS-PAGE

Analysis of cell proteins was assessed according to section 2.6.1. SDS-PAGE was a relatively simple and reproducible procedure for the differentiation of *Lactobacillus* strains, which yielded protein fingerprints with 51 to 66 bands (Figure 3.5). Dice coefficients of the denistometry tracing of the gels removed the subjectivity of the procedure and confirmed the visual observations. All protein profiles from the *Lactobacillus* species were similar to that of the type strain, with Dice coefficients ranging from 0.82 to 0.88, whilst *Lb. bulgaricus* had a similarity index of 0.78 when compared to the *Lb. acidophilus* type strain (Table 3.4).

3.3 IDENTIFICATION OF PROBIOTIC STRAINS USING 16S rRNA GENE SEQUENCING

3.3.1 Construction of primers

Ribosomal RNA sequencing was performed to confirm the identity of selected strains, including type strain *Lb. acidophilus* VUP 12,016 according to section 2.6.4. Since the strains were initially identified as *Lb. acidophilus*, the forward primers HE1 and C75 were selected and prepared according to Drake *et al.* (1996) and Dewhirst *et al.* (1999) respectively.

Reverse primers were designed for specific binding to a conserved region of the genomes of strains from the *Lb. acidophilus* complex based on a multiple sequence alignment of the 16SrRNA gene sequences of *Lb. gasseri* (GenBank Accession Code AF243142-AF243173), *Lb. johnsonii* (GenBank Accession Code LBARR16SAZ, LAAJ2515), *Lb. cripatus* (GenBank Accession Code AF243141-AF243175), *Lb. amylovorus* (GenBank Accession Code LAM241720, LAM241737, LAM241754, AF133264), *Lb. acidophilus* (GenBank Accession Code LBARR16SA, LAC241775, LAC16SRNA) and *Lb. gallinarum* (GenBank Accession Code LGA242968,

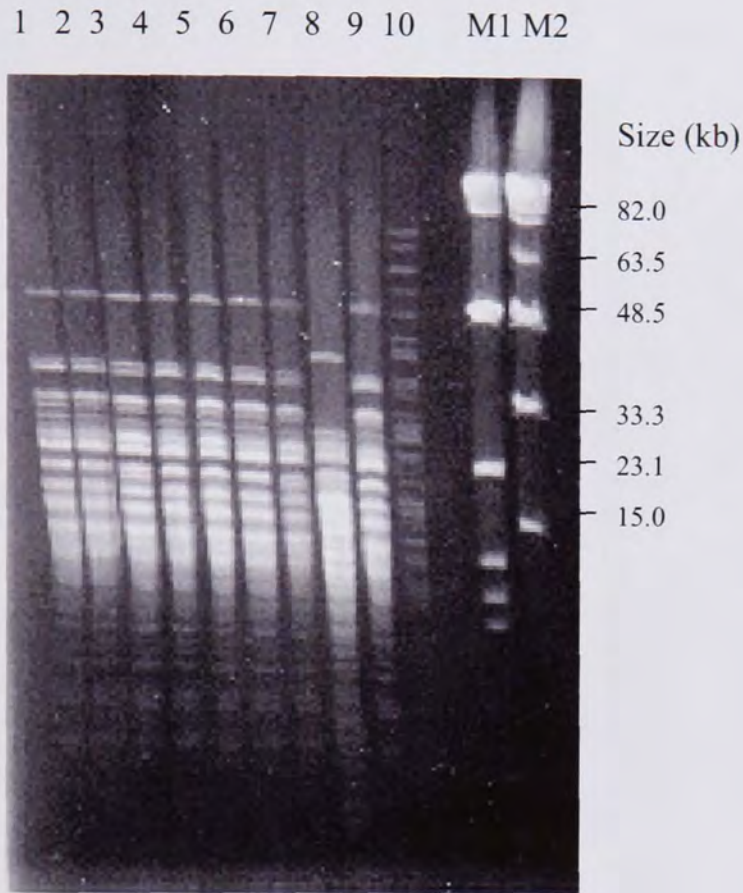


Figure 3.4. PFGE patterns of *Xba I* digests of genomic DNA. Restriction fragment separation with pulse times at 1.5sec/10h and 2.5sec/10h. Lanes: 1, VUP 12,002; 2, VUP 12,012; 3, VUP 12,005; 4, VUP 12,006; 5, VUP 12,015; 6, VUP 12,007; 7, VUP 12,027; 8, VUP 12,028; 9, VUP 12,025; 10, *Lb. delbrueckii* ssp. *bulgaricus* CSCC 2515; M1, Low-Range Marker; M2, Mid-Range Marker.

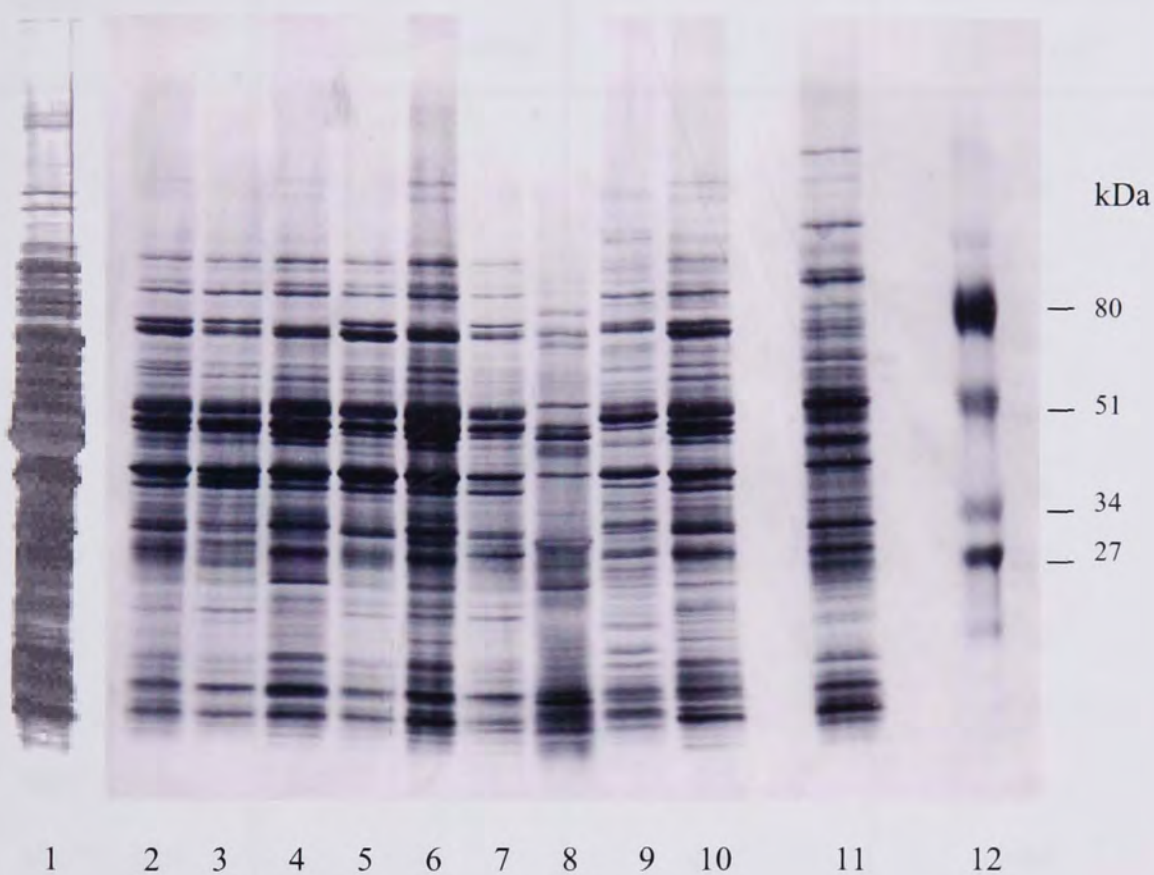


Figure 3.5. SDS-PAGE patterns of cell protein extracts from ten *Lb. acidophilus* strains and one strain of *Lb. delbrueckii* ssp. *bulgaricus*. Lanes: 1, *Lb. acidophilus* VUP 12,016 (Type strain); 2, VUP 12,002; 3, VUP 12,012; 4, VUP 12,005; 5, VUP 12,006; 6, VUP 12,015; 7, VUP 12,007; 8, VUP 12,027; 9, VUP 12,028; 10, VUP 12,025; 11, *Lb. delbrueckii* ssp. *bulgaricus* CSCC 2515; 12, Low Range Marker.

Table 3.4. Genome size estimation and Dice coefficients for pulsed-field gel electrophoresis and poly-acrylamide gel electrophoresis.

Strain number	Genome size estimation ^{a, c} (Mb)	Dice coefficient PFGE ^c	Dice coefficient SDS-PAGE ^c
VUP 12,016 ^b	1.52	1.00	1.00
VUP 12,002	1.56	0.82	0.86
VUP 12,012	1.47	0.87	0.86
VUP 12,005	1.59	0.73	0.89
VUP 12,006	1.47	0.87	0.88
VUP 12,015	1.52	1.00	0.85
VUP 12,007	1.62	0.87	0.83
VUP 12,027	1.57	0.76	0.82
VUP 12,028	1.96	0.24	0.86
VUP 12,025	1.47	0.87	0.84
CSCC 2515	NT ^d	0.10	0.78

^a Sum of the sizes of individual fragments.

^b Type strain.

^c Figures represent the average of duplicate runs. The standard deviation for all parameters did not exceed 0.08 for genome size estimations and 0.10 for the Dice coefficients of PFGE and SDS-PAGE results.

^d NT = Not tested.

LG16RRNA) obtained from the ANGIS (Table 3.5). This was performed using a multiple comparison program called 'Pileup' and a clear picture of the conservative region among these sequences was generated using the 'Prettybox' program. When the 16S rRNA gene of *Lactobacillus* strain VUP 12,028 was amplified using combinations of these primer sets, all sets produced PCR products of expected size (Figure 3.6) ranging from 1089 to 1382 base pairs. From this data, primer set C75-SM4 was selected for sequencing reactions, generating a PCR product of 1382 base pairs.

3.3.2 PCR Amplification of 16S rRNA Genes

Genomic DNA of all ten strains was amplified using the primer combination C75-SM4 according to section 2.6.4.4. All strains produced a PCR amplification product of approximately 1.4 kb, however, the length of the 16S rRNA gene sequences containing good sequence data ranged from 1200 to 1266 base pairs (Figure 3.7 - 3.9). With the exception of strain VUP 12,028, all sequences of the 16S rRNA region were identical. Consequently, a representative strain, VUP 12,016, and strain VUP 12,028 were selected for further analysis.

Many authors have identified species of the *Lb. acidophilus* complex using DNA probes, species-specific primers, or by sequencing the variable regions of the 16S rRNA gene and aligning these sequences with those of the type strains. The majority of probes/primers have been designed to recognise two main variable regions of the *Lb. acidophilus* complex, the V1 region (Hensiek *et al.*, 1992; Schleifer *et al.*, 1995; Tilsala-Timisjarvi and Alatossava, 1997; Kullen *et al.*, 2000; Horie *et al.*, 2002; Tarnberg *et al.*, 2002; Ventura and Zink, 2002) and the V3 region (Sui *et al.*, 2002; Tarnberg *et al.*, 2002). The V1 region is located just downstream of the 5' end of the 16S rRNA gene and consists of approximately 50 base pairs of sequence. The second region, V3 (a + b), is located about 1,000 base pairs downstream of the 5' of the 16S rRNA gene and is made up of approximately 50 base pairs (Figure 3.10). The 'FIND PATTERN' program in ANGIS was used to determine whether these signature sequences were present in strains VUP 12,016 and VUP 12,028 and to determine the sequence homology to the six species of the *Lb. acidophilus* complex (Table 3.6). The sequence variations of the V1 region allowed the establishment of the representative

Table 3.5. List of primers used in the amplification of 16S rRNA genes of lactobacillus species.

Primer	Sequence (5'....3')	Length (bases)	^c T _m (°C)
C75 ^a	GAGAGTTTGATYCTGGCTCAG	21	60
HE1 ^a	AGCAGATCGCATGATCAGCT	20	60
SM1 ^b	TACAACGAGGAGCAAGCCTG	20	62
SM3 ^b	GCACGAAGCTGGAATCGCTA	20	62
SM4 ^b	GTAATCGCGGATCAGCACGC	20	64

^a Primers C75 and HE1 were used as forward primers.

^b Primers SM1, SM3 and SM4 were used as reverse primers.

^c The annealing temperature (T_m) was calculated by applying the formula 4 x number of GC bases + 2 x number of AT bases to each primer. For example, primer C75 has 10 x G and C bases and 10 x A and T bases, therefore 4 x 10 + 2 x 10 = 60°C annealing temperature.

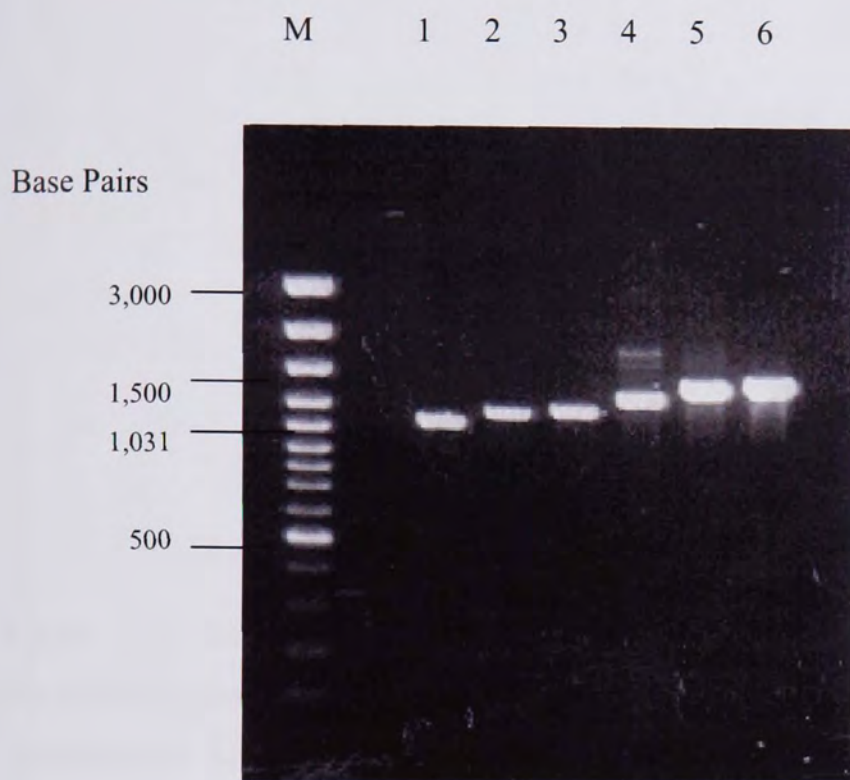


Figure 3.6. PCR products of strain VUP 12,028 using a range of primers. Lanes: 1, 100 base pair DNA Ladder Plus; 2, Primers HE1/SM1; 3, HE1/SM3; 4, HE1/SM4; 5, C75/SM1; 6, C75/SM3; 7, C75/SM4.

Figure 3.7. Sequence obtained by amplification of the 16SrDNA gene of the representative strain VUP 12,016 using the primers C75 and SM4. A PCR product of approximately 1.4 kb was amplified from strain VUP 12,016 (page 108 – 109).


```

      10      20      30      40      50      60
...|...|...|...|...|...|...|...|...|...|...|
CATGCAAGTCGAGCGAGCTGAACCAACAGATTCACTTCGGTGATGACGTTGGGAACGCGA

      70      80      90     100     110     120
...|...|...|...|...|...|...|...|...|...|...|
GCGGCGGATGGGTGAGTAACACGTGGGGAACCTGCCCCATAGTCTGGGATACCACTTGA

      130     140     150     160     170     180
...|...|...|...|...|...|...|...|...|...|...|
AACAGGTGCTAATACCGGATAAGAAAGCAGATCGCATGATCAGCTTATAAAAGGCGGCGT

      190     200     210     220     230     240
...|...|...|...|...|...|...|...|...|...|...|
AAGCTGTCGCTATGGGATGGCCCCGCGGTGCATTAGCTAGTTGGTAGGGTAACGGCCTAC

      250     260     270     280     290     300
...|...|...|...|...|...|...|...|...|...|...|
CAAGGCAATGATGCATAGCCGAGTTGAGAGACTGATCGGCCACATTGGGACTGAGACACG

      310     320     330     340     350     360
...|...|...|...|...|...|...|...|...|...|...|
GCCCAAACCTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATG

      370     380     390     400     410     420
...|...|...|...|...|...|...|...|...|...|...|
GAGCAACGCCGCGTGAGTGAAGAAGGTTTTTCGGATCGTAAAGCTCTGTTGTTGGTGAAGA

      430     440     450     460     470     480
...|...|...|...|...|...|...|...|...|...|...|
AGGATAGAGGTAGTAACCTGGCCTTTATTTGACGGTAATCAACCAGAAAGTCACGGCTAAC

      490     500     510     520     530     540
...|...|...|...|...|...|...|...|...|...|...|
TACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGATTTATTGGGCGT

      550     560     570     580     590     600
...|...|...|...|...|...|...|...|...|...|...|
AAAGCGAGCGCAGGCGGAAGAATAAGTCTGATGTGAAAGCCCTCGGCTTAACCGAGGAAC

      610     620     630     640     650     660
...|...|...|...|...|...|...|...|...|...|...|
TGCATCGGAAACTGTTTTTCTTGAGTGCAGAAGAGGAGAGTGGAAGTCCATGTGTAGCGG

      670     680     690     700     710     720
...|...|...|...|...|...|...|...|...|...|...|
TGAATGCGTAGATATATGGAAGAACACCAAGTGGCGAAGGCGGCTCTCTGGTCTGCAACT

      730     740     750     760     770     780
...|...|...|...|...|...|...|...|...|...|...|
GACGCTGAGGCTCGAAAGCATGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCATGCC

      790     800     810     820     830     840
...|...|...|...|...|...|...|...|...|...|...|
GTAAACGATGAGTGCTAAGTGTTGGGAGGTTTCCGCCTCTCAGTGCTGCAGCTAACGCAT

      850     860     870     880     890     900
...|...|...|...|...|...|...|...|...|...|...|
TAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGC

```


910 920 930 940 950 960
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
CCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTC

970 980 990 1000 1010 1020
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
TTGACATCTAGTGCAATCCGTAGAGATACGGAGTTCCCTTCGGGGACACTAAGACAGGTG

1030 1040 1050 1060 1070 1080
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
GTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTAAAGTCCCGCAACGAGCGCA

1090 1100 1110 1120 1130 1140
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
ACCCTTGTCATTAGTTGCCAGCATTAAAGTTGGGCACTCTAATGAGACTGCCGGTGACAAA

1150 1160 1170 1180 1190 1200
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
CCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGCCCTTATGACCTGGGCTACACACG

1210 1220 1230 1240 1250
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
TGCTACAATGGACAGTACAACGAGGAGCAAGCCTGCGAAGGCAAGCGAATCTCTTAAAG

Figure 3.8. Sequence obtained by amplification of the 16SrDNA gene of strain VUP 12,028 using the primers C75 and SM4. A PCR product of approximately 1.4 kb was amplified from strain VUP 12,028 (pages 111 - 112).


```

      10      20      30      40      50      60
.....|.....|.....|.....|.....|.....|.....|.....|
CATGCAAGTCGAGCGAGCTTGCCTAGATGATTTTAGTGCTTGCACTAAATGAACTAGAT

      70      80      90     100     110     120
.....|.....|.....|.....|.....|.....|.....|.....|
ACAAGCGAGCGGCGGACGGGTGAGTAACACGTGGGTAACTGCCCAAGAGACTGGGATAA

      130     140     150     160     170     180
.....|.....|.....|.....|.....|.....|.....|.....|
CACCTGGAAACAGATGCTAATACCGGATAACAACACTAGACGCATGTCTAGAGTTTGAAA

      190     200     210     220     230     240
.....|.....|.....|.....|.....|.....|.....|.....|
GATGGTTCCTGCTATCACTCTTGGATGGACCTGCGGTGCATTAGCTAGTTGGTAAGGTAAC

      250     260     270     280     290     300
.....|.....|.....|.....|.....|.....|.....|.....|
GGCTTACCAAGGCAATGATGCATAGCCGAGTTGAGAGACTGATCGGCCACATTGGGACTG

      310     320     330     340     350     360
.....|.....|.....|.....|.....|.....|.....|.....|
AGACACGGCCCAAACCTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAG

      370     380     390     400     410     420
.....|.....|.....|.....|.....|.....|.....|.....|
TCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAGCTCTGTTGGTA

      430     440     450     460     470     480
.....|.....|.....|.....|.....|.....|.....|.....|
GTGAAGAAAGATAGAGGTAGTAAGTGGCCTTTATTTGACGGTAATTACTTAGAAAGTCAC

      490     500     510     520     530     540
.....|.....|.....|.....|.....|.....|.....|.....|
GGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTAT

      550     560     570     580     590     600
.....|.....|.....|.....|.....|.....|.....|.....|
TGGGCGTAAAGCGAGTGCAGGCGGTTCAATAAGTCTGATGTGAAAGCCTTCGGCTCAACC

      610     620     630     640     650     660
.....|.....|.....|.....|.....|.....|.....|.....|
GGAGAATTGCATCAGAACTGTTGAACTTGAGTGCAGAAGAGGAGAGTGGAACTCCATGT

      670     680     690     700     710     720
.....|.....|.....|.....|.....|.....|.....|.....|
GTAGCGGTGGAATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTCTCTGGTC

      730     740     750     760     770     780
.....|.....|.....|.....|.....|.....|.....|.....|
TGCAACTGACGCTGAGGCTCGAAAGCATGGGTAGCGAACAGGATTAGATACCCTGGTAGT

      790     800     810     820     830     840
.....|.....|.....|.....|.....|.....|.....|.....|
CCATGCCGTAAACGATGAGTGCTAAGTGTTGGGAGGTTTCCGCCTCTCAGTGCTGCAGCT

      850     860     870     880     890     900
.....|.....|.....|.....|.....|.....|.....|.....|
AACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGA

```


910 920 930 940 950 960
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
CGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTA

970 980 990 1000 1010 1020
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
CCAGGTCCTTGACATCCAGTGCAAACCTAAGAGATTAGGTGTTCCCTTCGGGGACGCTGAG

1030 1040 1050 1060 1070 1080
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
ACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCTGTGAGATGTTGGGTTAAGTCCCGCAAC

1090 1100 1110 1120 1130 1140
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
GAGCGCAACCCTTGTCATTAGTTGCCATCATTAAAGTTGGGCACTCTAATGAGACTGCCGG

1150 1160 1170 1180 1190 1200
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
TGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGCCCTTATGACCTGGGCT

1210 1220 1230 1240 1250 1260
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
ACACACGTGCTACAATGGACGGTACAACGAGAAGCGAACCTGCGAAGGCAAGCGGATCTC

.....|
TTAAAG

Figure 3.9. Similarity of 16SrRNA gene sequences for strains VUP 12,016, VUP 12,002, VUP 12,012, VUP 12,005, VUP 12,006, VUP 12,015, VUP 12,007, VUP 12,027, VUP 12,025 and VUP 12,028 using BLASTN similarity search (pages 114-116). The degree of similarity is shown by the black highlighted section throughout the sequence output. The BLASTn similarity search was accessed from ANGIS at <http://mellangis.org.au>.

VUPI2,016 CATGCAAGTCGAGCGAGCTGAACCAACAGATTC ACTTCGGT GATGACGTTGGGAAC GCGAGCGCGGATGGG
 VUPI2,002 CATGCAAGTCGAGCGAGCTGAACCAACAGATTC ACTTCGGT GATGACGTTGGGAAC GCGAGCGCGGATGGG
 VUPI2,012 ----- AGCGAGCGCGGATGGG
 VUPI2,005 CATGCAAGTCGAGCGAGCTGAACCAACAGATTC ACTTCGGT GATGACGTTGGGAAC GCGAGCGCGGATGGG
 VUPI2,006 CATGCAAGTCGAGCGAGCTGAACCAACAGATTC ACTTCGGT GATGACGTTGGGAAC GCGAGCGCGGATGGG
 VUPI2,015 CATGCAAGTCGAGCGAGCTGAACCAACAGATTC ACTTCGGT GATGACGTTGGGAAC GCGAGCGCGGATGGG
 VUPI2,007 CATGCAAGTCGAGCGAGCTGAACCAACAGATTC ACTTCGGT GATGACGTTGGGAAC GCGAGCGCGGATGGG
 VUPI2,027 CATGCAAGTCGAGCGAGCTGAACCAACAGATTC ACTTCGGT GATGACGTTGGGAAC GCGAGCGCGGATGGG
 VUPI2,025 CATGCAAGTCGAGCGAGCTGAACCAACAGATTC ACTTCGGT GATGACGTTGGGAAC GCGAGCGCGGATGGG
 VUPI2,028 CATGCAAGTCGAGCGAGCTTGCCTAGATGATT TTAGTGCTT GCACTAAATGAACTAGATACAA GCGAGCGCGGATGGG

VUPI2,016 TGAGTAACACGTGGGGAACCTGCCCCATAGTCTGGGATACCACTTGGAACAGGTGCTAATACCGGATAAGAAAGCAGAT
 VUPI2,002 TGAGTAACACGTGGGGAACCTGCCCCATAGTCTGGGATACCACTTGGAACAGGTGCTAATACCGGATAAGAAAGCAGAT
 VUPI2,012 TGAGTAACACGTGGGGAACCTGCCCCATAGTCTGGGATACCACTTGGAACAGGTGCTAATACCGGATAAGAAAGCAGAT
 VUPI2,005 TGAGTAACACGTGGGGAACCTGCCCCATAGTCTGGGATACCACTTGGAACAGGTGCTAATACCGGATAAGAAAGCAGAT
 VUPI2,006 TGAGTAACACGTGGGGAACCTGCCCCATAGTCTGGGATACCACTTGGAACAGGTGCTAATACCGGATAAGAAAGCAGAT
 VUPI2,015 TGAGTAACACGTGGGGAACCTGCCCCATAGTCTGGGATACCACTTGGAACAGGTGCTAATACCGGATAAGAAAGCAGAT
 VUPI2,007 TGAGTAACACGTGGGGAACCTGCCCCATAGTCTGGGATACCACTTGGAACAGGTGCTAATACCGGATAAGAAAGCAGAT
 VUPI2,027 TGAGTAACACGTGGGGAACCTGCCCCATAGTCTGGGATACCACTTGGAACAGGTGCTAATACCGGATAAGAAAGCAGAT
 VUPI2,025 TGAGTAACACGTGGGGAACCTGCCCCATAGTCTGGGATACCACTTGGAACAGGTGCTAATACCGGATAAGAAAGCAGAT
 VUPI2,028 TGAGTAACACGTGGGTAACCTGCCAAGAGACTGGGATAACACTGGAACAGATGCTAATACCGGATAACCACTAGTA

VUPI2,016 CGCATGATCAGCTTATAAAAGGCGGCGTAAGCTGTGCTATGGGATGGCCCCGCGGTGCATTAGCTAGTTGGTAGGGTAA
 VUPI2,002 CGCATGATCAGCTTATAAAAGGCGGCGTAAGCTGTGCTATGGGATGGCCCCGCGGTGCATTAGCTAGTTGGTAGGGTAA
 VUPI2,012 CGCATGATCAGCTTATAAAAGGCGGCGTAAGCTGTGCTATGGGATGGCCCCGCGGTGCATTAGCTAGTTGGTAGGGTAA
 VUPI2,005 CGCATGATCAGCTTATAAAAGGCGGCGTAAGCTGTGCTATGGGATGGCCCCGCGGTGCATTAGCTAGTTGGTAGGGTAA
 VUPI2,006 CGCATGATCAGCTTATAAAAGGCGGCGTAAGCTGTGCTATGGGATGGCCCCGCGGTGCATTAGCTAGTTGGTAGGGTAA
 VUPI2,015 CGCATGATCAGCTTATAAAAGGCGGCGTAAGCTGTGCTATGGGATGGCCCCGCGGTGCATTAGCTAGTTGGTAGGGTAA
 VUPI2,007 CGCATGATCAGCTTATAAAAGGCGGCGTAAGCTGTGCTATGGGATGGCCCCGCGGTGCATTAGCTAGTTGGTAGGGTAA
 VUPI2,027 CGCATGATCAGCTTATAAAAGGCGGCGTAAGCTGTGCTATGGGATGGCCCCGCGGTGCATTAGCTAGTTGGTAGGGTAA
 VUPI2,025 CGCATGATCAGCTTATAAAAGGCGGCGTAAGCTGTGCTATGGGATGGCCCCGCGGTGCATTAGCTAGTTGGTAGGGTAA
 VUPI2,028 CGCATGCTCTAGAGTTTGAAAGATGGT-TCTGCTATCACTCTTGGATGGACCTGCGGTGCATTAGCTAGTTGGTAGGGTAA

VUPI2,016 CGGCCTACCAAGGCAATGATGCATAGCCGAGTTGAGAGACTGATCGGCCACATTGGGACTGAGACACGGCCCCAAACTCCT
 VUPI2,002 CGGCCTACCAAGGCAATGATGCATAGCCGAGTTGAGAGACTGATCGGCCACATTGGGACTGAGACACGGCCCCAAACTCCT
 VUPI2,012 CGGCCTACCAAGGCAATGATGCATAGCCGAGTTGAGAGACTGATCGGCCACATTGGGACTGAGACACGGCCCCAAACTCCT
 VUPI2,005 CGGCCTACCAAGGCAATGATGCATAGCCGAGTTGAGAGACTGATCGGCCACATTGGGACTGAGACACGGCCCCAAACTCCT
 VUPI2,006 CGGCCTACCAAGGCAATGATGCATAGCCGAGTTGAGAGACTGATCGGCCACATTGGGACTGAGACACGGCCCCAAACTCCT
 VUPI2,015 CGGCCTACCAAGGCAATGATGCATAGCCGAGTTGAGAGACTGATCGGCCACATTGGGACTGAGACACGGCCCCAAACTCCT
 VUPI2,007 CGGCCTACCAAGGCAATGATGCATAGCCGAGTTGAGAGACTGATCGGCCACATTGGGACTGAGACACGGCCCCAAACTCCT
 VUPI2,027 CGGCCTACCAAGGCAATGATGCATAGCCGAGTTGAGAGACTGATCGGCCACATTGGGACTGAGACACGGCCCCAAACTCCT
 VUPI2,025 CGGCCTACCAAGGCAATGATGCATAGCCGAGTTGAGAGACTGATCGGCCACATTGGGACTGAGACACGGCCCCAAACTCCT
 VUPI2,028 CGGCCTACCAAGGCAATGATGCATAGCCGAGTTGAGAGACTGATCGGCCACATTGGGACTGAGACACGGCCCCAAACTCCT

VUPI2,016 ACGGGAGGCGAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGTTTTTCG
 VUPI2,002 ACGGGAGGCGAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGTTTTTCG
 VUPI2,012 ACGGGAGGCGAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGTTTTTCG
 VUPI2,005 ACGGGAGGCGAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGTTTTTCG
 VUPI2,006 ACGGGAGGCGAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGTTTTTCG
 VUPI2,015 ACGGGAGGCGAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGTTTTTCG
 VUPI2,007 ACGGGAGGCGAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGTTTTTCG
 VUPI2,027 ACGGGAGGCGAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGTTTTTCG
 VUPI2,025 ACGGGAGGCGAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGTTTTTCG
 VUPI2,028 ACGGGAGGCGAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGTTTTTCG

VUPI2,016 GATCGTAAAGCTCTGTTGTTGGTGAAGAAGGATAGAGGTAGTAAGTGGCCTTTATTTGACGGTAATCAACCAGAAAGTCA
 VUPI2,002 GATCGTAAAGCTCTGTTGTTGGTGAAGAAGGATAGAGGTAGTAAGTGGCCTTTATTTGACGGTAATCAACCAGAAAGTCA
 VUPI2,012 GATCGTAAAGCTCTGTTGTTGGTGAAGAAGGATAGAGGTAGTAAGTGGCCTTTATTTGACGGTAATCAACCAGAAAGTCA
 VUPI2,005 GATCGTAAAGCTCTGTTGTTGGTGAAGAAGGATAGAGGTAGTAAGTGGCCTTTATTTGACGGTAATCAACCAGAAAGTCA
 VUPI2,006 GATCGTAAAGCTCTGTTGTTGGTGAAGAAGGATAGAGGTAGTAAGTGGCCTTTATTTGACGGTAATCAACCAGAAAGTCA
 VUPI2,015 GATCGTAAAGCTCTGTTGTTGGTGAAGAAGGATAGAGGTAGTAAGTGGCCTTTATTTGACGGTAATCAACCAGAAAGTCA
 VUPI2,007 GATCGTAAAGCTCTGTTGTTGGTGAAGAAGGATAGAGGTAGTAAGTGGCCTTTATTTGACGGTAATCAACCAGAAAGTCA
 VUPI2,027 GATCGTAAAGCTCTGTTGTTGGTGAAGAAGGATAGAGGTAGTAAGTGGCCTTTATTTGACGGTAATCAACCAGAAAGTCA
 VUPI2,025 GATCGTAAAGCTCTGTTGTTGGTGAAGAAGGATAGAGGTAGTAAGTGGCCTTTATTTGACGGTAATCAACCAGAAAGTCA
 VUPI2,028 GCTCGTAAAGCTCTGTTGTTGTTGAAGAAGGATAGAGGTAGTAAGTGGCCTTTATTTGACGGTAATCACTTAGAAAGTCA

490 500 510 520 530 540 550 560
 VUPI2,016 CGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTATTGGGCGTAAAGCGAGCGCA
 VUPI2,002 CGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTATTGGGCGTAAAGCGAGCGCA
 VUPI2,012 CGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTATTGGGCGTAAAGCGAGCGCA
 VUPI2,005 CGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTATTGGGCGTAAAGCGAGCGCA
 VUPI2,006 CGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTATTGGGCGTAAAGCGAGCGCA
 VUPI2,015 CGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTATTGGGCGTAAAGCGAGCGCA
 VUPI2,007 CGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTATTGGGCGTAAAGCGAGCGCA
 VUPI2,027 CGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTATTGGGCGTAAAGCGAGCGCA
 VUPI2,025 CGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTATTGGGCGTAAAGCGAGCGCA
 VUPI2,028 CGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTATTGGGCGTAAAGCGAGCGCA

570 580 590 600 610 620 630 640
 VUPI2,016 GGCGGAAGAATAAGTCTGATGTGAAAGCCCTCGGCTTAACCGAGGAAGTGCATCGGAAACTGTTTTCTTGAGTGCAGAA
 VUPI2,002 GGCGGAAGAATAAGTCTGATGTGAAAGCCCTCGGCTTAACCGAGGAAGTGCATCGGAAACTGTTTTCTTGAGTGCAGAA
 VUPI2,012 GGCGGAAGAATAAGTCTGATGTGAAAGCCCTCGGCTTAACCGAGGAAGTGCATCGGAAACTGTTTTCTTGAGTGCAGAA
 VUPI2,005 GGCGGAAGAATAAGTCTGATGTGAAAGCCCTCGGCTTAACCGAGGAAGTGCATCGGAAACTGTTTTCTTGAGTGCAGAA
 VUPI2,006 GGCGGAAGAATAAGTCTGATGTGAAAGCCCTCGGCTTAACCGAGGAAGTGCATCGGAAACTGTTTTCTTGAGTGCAGAA
 VUPI2,015 GGCGGAAGAATAAGTCTGATGTGAAAGCCCTCGGCTTAACCGAGGAAGTGCATCGGAAACTGTTTTCTTGAGTGCAGAA
 VUPI2,007 GGCGGAAGAATAAGTCTGATGTGAAAGCCCTCGGCTTAACCGAGGAAGTGCATCGGAAACTGTTTTCTTGAGTGCAGAA
 VUPI2,027 GGCGGAAGAATAAGTCTGATGTGAAAGCCCTCGGCTTAACCGAGGAAGTGCATCGGAAACTGTTTTCTTGAGTGCAGAA
 VUPI2,025 GGCGGAAGAATAAGTCTGATGTGAAAGCCCTCGGCTTAACCGAGGAAGTGCATCGGAAACTGTTTTCTTGAGTGCAGAA
 VUPI2,028 GGCGGTTCAATAAGTCTGATGTGAAAGCCCTCGGCTTAACCGAGGAAGTGCATCGGAAACTGTTGAACCTTGAGTGCAGAA

650 660 670 680 690 700 710 720
 VUPI2,016 GAGGAGAGTGGAACTCCATGTGTAGCGGTGGAATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTCTCTGGT
 VUPI2,002 GAGGAGAGTGGAACTCCATGTGTAGCGGTGGAATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTCTCTGGT
 VUPI2,012 GAGGAGAGTGGAACTCCATGTGTAGCGGTGGAATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTCTCTGGT
 VUPI2,005 GAGGAGAGTGGAACTCCATGTGTAGCGGTGGAATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTCTCTGGT
 VUPI2,006 GAGGAGAGTGGAACTCCATGTGTAGCGGTGGAATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTCTCTGGT
 VUPI2,015 GAGGAGAGTGGAACTCCATGTGTAGCGGTGGAATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTCTCTGGT
 VUPI2,007 GAGGAGAGTGGAACTCCATGTGTAGCGGTGGAATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTCTCTGGT
 VUPI2,027 GAGGAGAGTGGAACTCCATGTGTAGCGGTGGAATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTCTCTGGT
 VUPI2,025 GAGGAGAGTGGAACTCCATGTGTAGCGGTGGAATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTCTCTGGT
 VUPI2,028 GAGGAGAGTGGAACTCCATGTGTAGCGGTGGAATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTCTCTGGT

730 740 750 760 770 780 790 800
 VUPI2,016 CTGCAACTGACGCTGAGGCTCGAAAGCATGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAACGATGAG
 VUPI2,002 CTGCAACTGACGCTGAGGCTCGAAAGCATGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAACGATGAG
 VUPI2,012 CTGCAACTGACGCTGAGGCTCGAAAGCATGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAACGATGAG
 VUPI2,005 CTGCAACTGACGCTGAGGCTCGAAAGCATGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAACGATGAG
 VUPI2,006 CTGCAACTGACGCTGAGGCTCGAAAGCATGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAACGATGAG
 VUPI2,015 CTGCAACTGACGCTGAGGCTCGAAAGCATGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAACGATGAG
 VUPI2,007 CTGCAACTGACGCTGAGGCTCGAAAGCATGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAACGATGAG
 VUPI2,027 CTGCAACTGACGCTGAGGCTCGAAAGCATGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAACGATGAG
 VUPI2,025 CTGCAACTGACGCTGAGGCTCGAAAGCATGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAACGATGAG
 VUPI2,028 CTGCAACTGACGCTGAGGCTCGAAAGCATGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAACGATGAG

810 820 830 840 850 860 870 880
 VUPI2,016 TGCTAAGTGTTGGGAGGTTTCCGCCTCTCAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAG
 VUPI2,002 TGCTAAGTGTTGGGAGGTTTCCGCCTCTCAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAG
 VUPI2,012 TGCTAAGTGTTGGGAGGTTTCCGCCTCTCAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAG
 VUPI2,005 TGCTAAGTGTTGGGAGGTTTCCGCCTCTCAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAG
 VUPI2,006 TGCTAAGTGTTGGGAGGTTTCCGCCTCTCAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAG
 VUPI2,015 TGCTAAGTGTTGGGAGGTTTCCGCCTCTCAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAG
 VUPI2,007 TGCTAAGTGTTGGGAGGTTTCCGCCTCTCAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAG
 VUPI2,027 TGCTAAGTGTTGGGAGGTTTCCGCCTCTCAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAG
 VUPI2,025 TGCTAAGTGTTGGGAGGTTTCCGCCTCTCAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAG
 VUPI2,028 TGCTAAGTGTTGGGAGGTTTCCGCCTCTCAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAG

890 900 910 920 930 940 950 960
 VUPI2,016 GTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAAGCGGTGGAGCATGTGGTTTAATTCTGAAGCAACGCGAAGAACCCTT
 VUPI2,002 GTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAAGCGGTGGAGCATGTGGTTTAATTCTGAAGCAACGCGAAGAACCCTT
 VUPI2,012 GTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAAGCGGTGGAGCATGTGGTTTAATTCTGAAGCAACGCGAAGAACCCTT
 VUPI2,005 GTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAAGCGGTGGAGCATGTGGTTTAATTCTGAAGCAACGCGAAGAACCCTT
 VUPI2,006 GTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAAGCGGTGGAGCATGTGGTTTAATTCTGAAGCAACGCGAAGAACCCTT
 VUPI2,015 GTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAAGCGGTGGAGCATGTGGTTTAATTCTGAAGCAACGCGAAGAACCCTT
 VUPI2,007 GTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAAGCGGTGGAGCATGTGGTTTAATTCTGAAGCAACGCGAAGAACCCTT
 VUPI2,027 GTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAAGCGGTGGAGCATGTGGTTTAATTCTGAAGCAACGCGAAGAACCCTT
 VUPI2,025 GTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAAGCGGTGGAGCATGTGGTTTAATTCTGAAGCAACGCGAAGAACCCTT
 VUPI2,028 GTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAAGCGGTGGAGCATGTGGTTTAATTCTGAAGCAACGCGAAGAACCCTT

	970	980	990	1000	1010	1020	1030	1040
VUPI2,016	ACCAGGCTCTGACATCTAGTGCAATCCGTAGAGATACGGAGTTC	CCCTTCGGGGACACTAAGACAGGTGGTGCATGGCTGT						
VUPI2,002	ACCAGGCTCTGACATCTAGTGCAATCCGTAGAGATACGGAGTTC	CCCTTCGGGGACACTAAGACAGGTGGTGCATGGCTGT						
VUPI2,012	ACCAGGCTCTGACATCTAGTGCAATCCGTAGAGATACGGAGTTC	CCCTTCGGGGACACTAAGACAGGTGGTGCATGGCTGT						
VUPI2,005	ACCAGGCTCTGACATCTAGTGCAATCCGTAGAGATACGGAGTTC	CCCTTCGGGGACACTAAGACAGGTGGTGCATGGCTGT						
VUPI2,006	ACCAGGCTCTGACATCTAGTGCAATCCGTAGAGATACGGAGTTC	CCCTTCGGGGACACTAAGACAGGTGGTGCATGGCTGT						
VUPI2,015	ACCAGGCTCTGACATCTAGTGCAATCCGTAGAGATACGGAGTTC	CCCTTCGGGGACACTAAGACAGGTGGTGCATGGCTGT						
VUPI2,007	ACCAGGCTCTGACATCTAGTGCAATCCGTAGAGATACGGAGTTC	CCCTTCGGGGACACTAAGACAGGTGGTGCATGGCTGT						
VUPI2,027	ACCAGGCTCTGACATCTAGTGCAATCCGTAGAGATACGGAGTTC	CCCTTCGGGGACACTAAGACAGGTGGTGCATGGCTGT						
VUPI2,025	ACCAGGCTCTGACATCTAGTGCAATCCGTAGAGATACGGAGTTC	CCCTTCGGGGACACTAAGACAGGTGGTGCATGGCTGT						
VUPI2,028	ACCAGGCTCTGACATCAGTGCAAAACCTAAGAGATTAGGTTTCC	CTTCGGGGACACTGAGACAGGTGGTGCATGGCTGT						

	1050	1060	1070	1080	1090	1100	1110	1120
VUPI2,016	CGTCAGCTCGTGTCTGAGATGTTGGGTAAAGTCCCGCAACGAGCG	CAACCCCTTGTCATTAGTTGCCAGCATTAAAGTTGG						
VUPI2,002	CGTCAGCTCGTGTCTGAGATGTTGGGTAAAGTCCCGCAACGAGCG	CAACCCCTTGTCATTAGTTGCCAGCATTAAAGTTGG						
VUPI2,012	CGTCAGCTCGTGTCTGAGATGTTGGGTAAAGTCCCGCAACGAGCG	CAACCCCTTGTCATTAGTTGCCAGCATTAAAGTTGG						
VUPI2,005	CGTCAGCTCGTGTCTGAGATGTTGGGTAAAGTCCCGCAACGAGCG	CAACCCCTTGTCATTAGTTGCCAGCATTAAAGTTGG						
VUPI2,006	CGTCAGCTCGTGTCTGAGATGTTGGGTAAAGTCCCGCAACGAGCG	CAACCCCTTGTCATTAGTTGCCAGCATTAAAGTTGG						
VUPI2,015	CGTCAGCTCGTGTCTGAGATGTTGGGTAAAGTCCCGCAACGAGCG	CAACCCCTTGTCATTAGTTGCCAGCATTAAAGTTGG						
VUPI2,007	CGTCAGCTCGTGTCTGAGATGTTGGGTAAAGTCCCGCAACGAGCG	CAACCCCTTGTCATTAGTTGCCAGCATTAAAGTTGG						
VUPI2,027	CGTCAGCTCGTGTCTGAGATGTTGGGTAAAGTCCCGCAACGAGCG	CAACCCCTTGTCATTAGTTGCCAGCATTAAAGTTGG						
VUPI2,025	CGTCAGCTCGTGTCTGAGATGTTGGGTAAAGTCCCGCAACGAGCG	CAACCCCTTGTCATTAGTTGCCAGCATTAAAGTTGG						
VUPI2,028	CGTCAGCTCGTGTCTGAGATGTTGGGTAAAGTCCCGCAACGAGCG	CAACCCCTTGTCATTAGTTGCCAGCATTAAAGTTGG						

	1130	1140	1150	1160	1170	1180	1190	1200
VUPI2,016	GCACTCTAATGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGAT	GACGTCAAGTCATCATGCCCCCTTATGACCTGGGC						
VUPI2,002	GCACTCTAATGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGAT	GACGTCAAGTCATCATGCCCCCTTATGACCTGGGC						
VUPI2,012	GCACTCTAATGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGAT	GACGTCAAGTCATCATGCCCCCTTATGACCTGGGC						
VUPI2,005	GCACTCTAATGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGAT	GACGTCAAGTCATCATGCCCCCTTATGACCTGGGC						
VUPI2,006	GCACTCTAATGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGAT	GACGTCAAGTCATCATGCCCCCTTATGACCTGGGC						
VUPI2,015	GCACTCTAATGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGAT	GACGTCAAGTCATCATGCCCCCTTATGACCTGGGC						
VUPI2,007	GCACTCTAATGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGAT	GACGTCAAGTCATCATGCCCCCTTATGACCTGGGC						
VUPI2,027	GCACTCTAATGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGAT	GACGTCAAGTCATCATGCCCCCTTATGACCTGGGC						
VUPI2,025	GCACTCTAATGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGAT	GACGTCAAGTCATCATGCCCCCTTATGACCTGGGC						
VUPI2,028	GCACTCTAATGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGAT	GACGTCAAGTCATCATGCCCCCTTATGACCTGGGC						

	1210	1220	1230	1240	1250	1260
VUPI2,016	TACACACGTGCTACAATGGACAGTACAACGAGGAGCAAGCCTGCGA	AAGGCAAGCGAATCTCTTAAAG				
VUPI2,002	TACACACGTGCTACAATGGACAGTACAACGAGGAGCAAGCCTGCGA	AAGGCAAGCGAATCTCTTAAAG				
VUPI2,012	TACACACGTGCTACAATGGACAGTACAACGAGGAGCAAGCCTGCGA	AAGGCAAGCGAATCTCTTAAAG				
VUPI2,005	TACACACGTGCTACAATGGACAGTACAACGAGGAGCAAGCCTGCGA	AAGGCAAGCGAATCTCTTAAAG				
VUPI2,006	TACACACGTGCTACAATGGACAGTACAACGAGGAGCAAGCCTGCGA	AAGGCAAGCGAATCTCTTAAAG				
VUPI2,015	TACACACGTGCTACAATGGACAGTACAACGAGGAGCAAGCCTGCGA	AAGGCAAGCGAATCTCTTAAAG				
VUPI2,007	TACACACGTGCTACAATGGACAGTACAACGAGGAGCAAGCCTGCGA	AAGGCAAGCGAATCTCTTAAAG				
VUPI2,027	TACACACGTGCTACAATGGACAGTACAACGAGGAGCAAGCCTGCGA	AAGGCAAGCGAATCTCTTAAAG				
VUPI2,025	TACACACGTGCTACAATGGACAGTACAACGAGGAGCAAGCCTGCGA	AAGGCAAGCGAATCTCTTAAAG				
VUPI2,028	TACACACGTGCTACAATGGACAGTACAACGAGGAGCAAGCCTGCGA	AAGGCAAGCGAATCTCTTAAAG				

Figure 3.10. Alignment of the rRNA signature sequences used to identify members of the *Lb. acidophilus* complex

Sequence specificity	Sequence (5'-3') ^a			
	V1 Region ^b			
<i>L. acidophilus</i>	GAGCGAGCTGAACCAACAGATTC	ACTTCGGT	GATGACGTTGGGAA	CGCGAGCGG
<i>L. amylovorus</i>	GAGCGAGCGGAACCAACAGATTT	ACTTCGGT	AATGACGTTGGGAA	AGCGAGCGG
<i>L. crispatus</i>	GAGCGAGCGGAACCAACAGATTT	ACTTCGGT	AATGACGTTAGGAA	AGCGAGCGG
<i>L. gallinarum</i>	GAGCGAGCAGAACCAGCAGATTT	ACTTCGGT	AATGACGCTGGGGA	CGCGAGCGG
<i>L. gasseri</i>	GAGCGAGCTTGCCTAGATGAATTTGGTGCTTGCACCAGATGAAACTAGATACAAGCGATCGG			
<i>L. johnsonii</i>	GAGCGAGCTTGCCTAGATGATTTTAGTGCTTGCCTAAATGAAACTAGATACAAGCGAGCGG			
	V3 region ^c (a + b)			
<i>L. acidophilus</i>	AGGTCTTGACATCTAGTGCAATCCG-----	GTCTTAGTGTCCCCGAAGGGAACTC		
<i>L. amylovorus</i>	AGGTCTTGACATCTAGTGCAATCTG-----	GTCTTAGCGTCCCCGAAGGGAACTC		
<i>L. crispatus</i>	AGGTCTTGACATCTAGTGCCATTG-----	GTCTTAGCGTCCCCGAAGGGAACTT		
<i>L. gallinarum</i>	AGGTCTTGACATCTAGTGCCATCCT-----	GTCTTAGCGTCCCCGAAGGGAACTC		
<i>L. gasseri</i>	AGGTCTTGACATCCAGTGCAAACCT-----	GTCTCAGCGTCCCCGAAGGGAAACAC		
<i>L. johnsonii</i>	AGGTCTTGACATCCAGTGCAAACCT-----	GTCTCAGCGTCCCCGAAGGGAAACAC		

^a Shading represents nucleotides that are identical for that position. Dashes (-) represent gaps in the sequences introduced during alignment.

^b Sequences obtained from Kullen *et al.*, 2000.

^c Sequences obtained from Tarnberg *et al.*, 2002.

strains into their respective species, whilst the V3 region was used to support this designation, as it did not allow for differentiation of all six species of the *Lb. acidophilus* complex. For example, *Lb. johnsonii* and *Lb. gasseri* shared the same signature sequences in both the V3a and V3b regions, whilst *Lb. amylovorus* and *Lb. gallinarum* were homologous in the V3b region (Figure 3.10). These results showed that strain VUP 12,016 aligns perfectly with all three variable regions of the *Lb. acidophilus* type strains and shared 50.0 to 92.6% sequence identity at the V1 region for the remaining species of the *Lb. acidophilus* complex. Strain VUP 12,028 aligned perfectly with regions V1 and V3 of the *Lb. johnsonii* type strains and shared a sequence identity of 53.2 to 91.9% for the V1 region of the remaining species of the *Lb. acidophilus* complex (Table 3.6).

Submitting the sequences to ANGIS using the BLASTn similarity search tool confirmed the species assignments that were based on the V1 and V3 regions, with strain VUP 12,016 showing 98.5 % homology to the *Lb. acidophilus* species and strain VUP 12,028 showing 99% homology to the *Lb. johnsonii* species included in the GenBank database (Table 3.7).

3.4 CHARACTERISATION OF PROBIOTIC STRAINS

3.4.1 General morphological characteristics

Cells of the strains tested from MRS agar plates were non-motile, non-spore-forming, Gram positive rods occurring singly, in pairs and sometimes in short chains. Surface colonies on MRS agar plates (incubated at 37°C for 72 hours) were 0.5 to 2.0 mm in diameter, circular to slightly irregular, convex, cream in colour and shiny.

3.4.2 Survival of cultures exposed to low pH and bile salts

Tolerance to acid and bile was performed to select strains that would have the greatest chance of survival during transit through the gastrointestinal tract. Bile tolerance was assessed by monitoring the survival of 18 hour cultures grown in 12% NGYEC (pH 4.5) and supplemented with 0 or 1.5% bile salts (according to section 2.5.9). Tolerance to 1.5% bile was assessed over a three hour period, as Davenport (1977) states that 'this is the maximum time period that the organisms are likely to be exposed to high bile concentrations'. Strains not challenged with bile demonstrated

Table 3.6. Similarity of 16SrRNA signature gene sequences of the six species of the *Lactobacillus acidophilus* complex to strains VUP 12,016 and VUP 12,028.

Species/Variable Region	Strain Number	% Similarity to strain:	
		VUP 12,016	VUP 12,028
<i>Lb. acidophilus</i> V1	^a ATCC 4356	100	53.2
<i>Lb. amylovorus</i> V1	ATCC 33620	92.6	56.5
<i>Lb. crispatus</i> V1	ATCC 33820	88.9	59.7
<i>Lb. gallinarum</i> V1	ATCC 33199	88.9	58.1
<i>Lb. gasseri</i> V1	ATCC 33323	50.0	91.9
<i>Lb. johnsonii</i> V1	ATCC 333200	53.2	100
<i>Lb. acidophilus</i> V3a	^b CCUG 5917	100	88.0
<i>Lb. amylovorus</i> V3a	CCUG 27201	96.0	84.0
<i>Lb. crispatus</i> V3a	CCUG 30722	88.0	76.0
<i>Lb. gallinarum</i> V3a	CCUG 30724	92.0	88.0
<i>Lb. gasseri</i> / <i>Lb. johnsonii</i> V3a	CCUG 31451 / 30725	88.0	100
<i>Lb. acidophilus</i> V3b	CCUG 5917	100	88.0
<i>Lb. amylovorus</i> / <i>Lb. gallinarum</i> V3b	CCUG 27201 / 30724	96.0	92.0
<i>Lb. crispatus</i> V3b	CCUG 30722	92.0	88.0
<i>Lb. gasseri</i> / <i>Lb. johnsonii</i> V3b	CCUG 31451 / 30725	88.0	100

^aATCC= American Type Culture Collection.

^bCCUG= Culture Collection, University of Goteborg.

Table 3.7. Comparison of the similarity of the 16S r RNA genes of strain VUP 12,016 and VUP 12,028 with species of the *Lb. acidophilus* complex using a BLASTn search run through ANGIS.

Species	% Similarity to strain:	
	VUP 12,016	VUP 12,028
<i>Lb. acidophilus</i>	98.5	94.5
<i>Lb. amylovorus</i>	96.5	93.5
<i>Lb. crispatus</i>	97.5	95
<i>Lb. gallinarum</i>	91.5	93
<i>Lb. gasseri</i>	95.5	98.5
<i>Lb. johnsonii</i>	94.5	99

cell division during the three hour incubation period (data not shown). Most strains were tolerant to 1.5% bile for the three hour incubation period tested (Figure 3.11), with strains VUP 12,006, VUP 12,015 and VUP 12,025 multiplying during this time and the remaining strains maintaining 50.0 to 86% survival.

Acid tolerance was assessed by adjusting the pH of 18 hour cultures grown in 12% NGYEC to pH 3.5, 2.5 or 1.5 using hydrochloric acid and monitoring survival over a three hour period (according to section 2.5.9). Strains varied in their ability to survive acid challenge. A pH challenge of 3.5 did not prevent cell division in strains VUP 12,002, VUP 12,005, VUP 12,006, VUP 12,028 and VUP 12,025, whilst strains VUP 12,015 and VUP 12,027 maintained 78% and 66% survival respectively (Figure 3.12). Cells challenged at pH 2.5 varied in their ability to survive during the three-hour incubation period. For example, strains VUP 12,015 (79%) and VUP 12,028 (61%) were tolerant and strains VUP 12,002 (0.02%) and VUP 12,005 (1%) were sensitive to low pH environments (Figure 3.13). At pH 1.5, cell viability rapidly decreased for all strains tested, with less than 1% of cells surviving the three hour challenge period (Figure 3.14). The acid tolerance of strains VUP 12,012 and VUP 12,007 has been previously reported by Lankaputhra and Shah (1995).

3.4.3 Determination of viability in yogurt simulation trials

The viability of nine strains was assessed over a six-week period using preparation methods similar to standard yogurt manufacture, except for the absence of the yogurt starter cultures (refer to section 2.5.6). Viability over this period was strain dependant (Figure 3.15), with strain 2415 showing the highest percentage survival (16%), with less than one log drop in viability during the six week storage period, whilst strains VUP 12,015 and VUP 12,028 showed the lowest survival rates with a decrease of more than two log values in viability and only 0.23 and 0.19% of cells surviving six weeks of refrigerated storage. Overall, most strains fell below 10^6 viable cells per gram of yogurt after four weeks of refrigerated storage, with only strain VUP 12,005, VUP 12,007 and VUP 12,027 surviving at this level after six weeks of refrigerated storage.

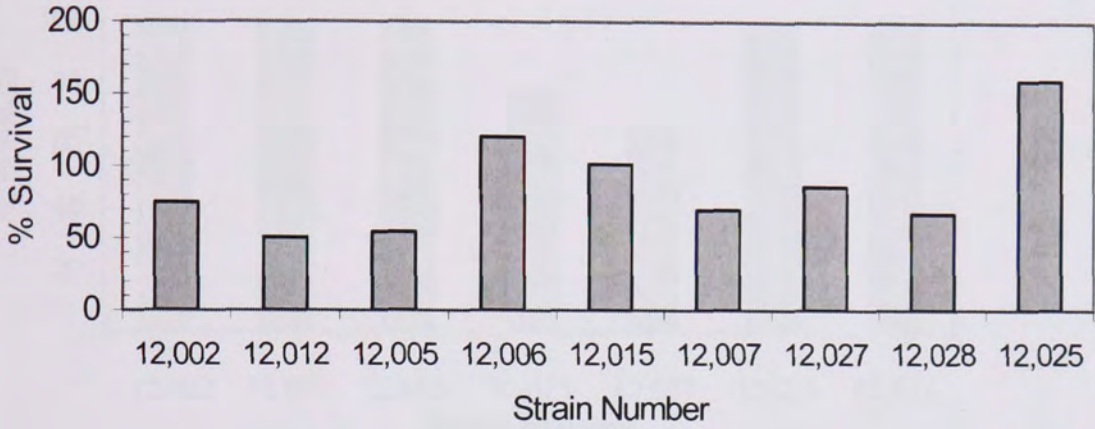


Figure 3.11. Percentage survival of nine *Lb. acidophilus* strains following exposure to 1.5% bile for 3 hours in 12% NGYEC broth. Results represent average of duplicate experiments, with less than 5% variation.

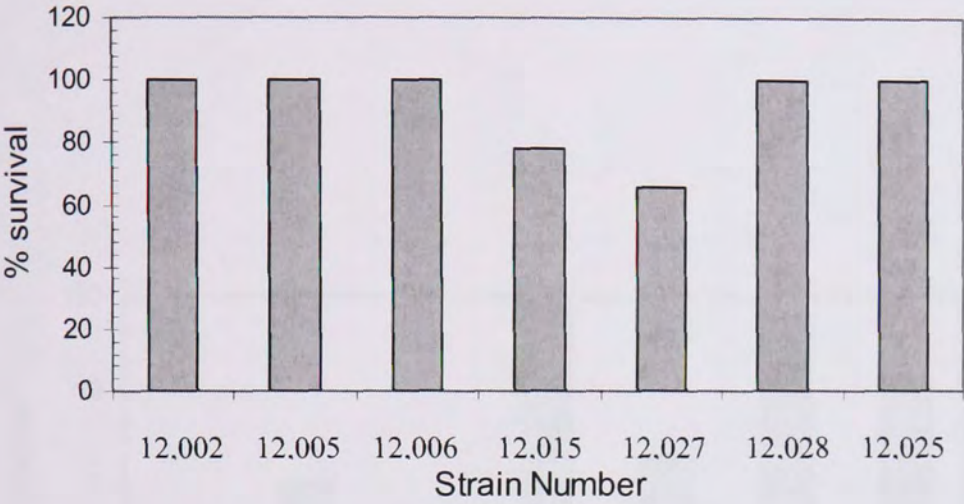


Figure 3.12. Percentage survival of seven *Lb. acidophilus* strains following incubation for three hours in 12% NGYEC (37°C) adjusted to pH 3.5 using hydrochloric acid. Results represent average of duplicate experiments, with less than 5% variation.

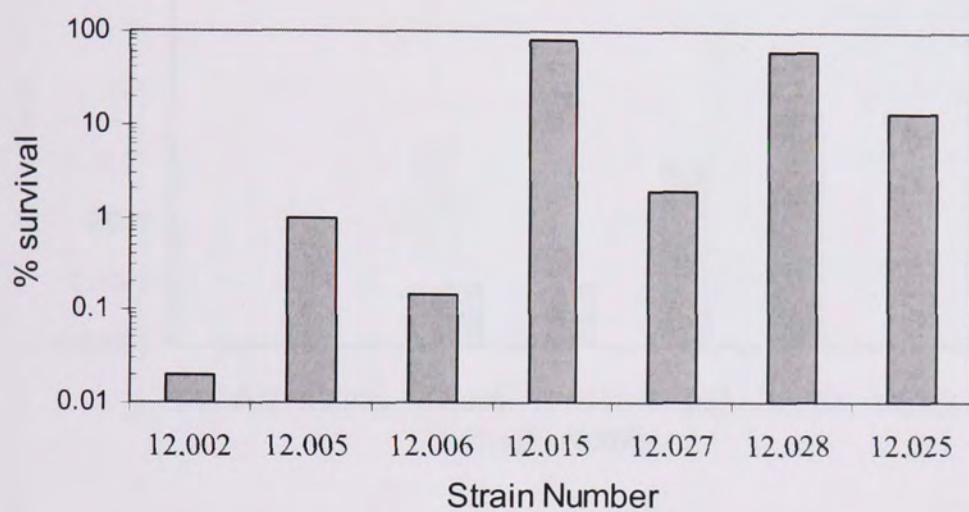


Figure 3.13. Percentage survival of seven *Lb. acidophilus* strains following exposure to pH 2.5 for 3 hours. Results represent average of duplicate experiments, with less than 5% variation.

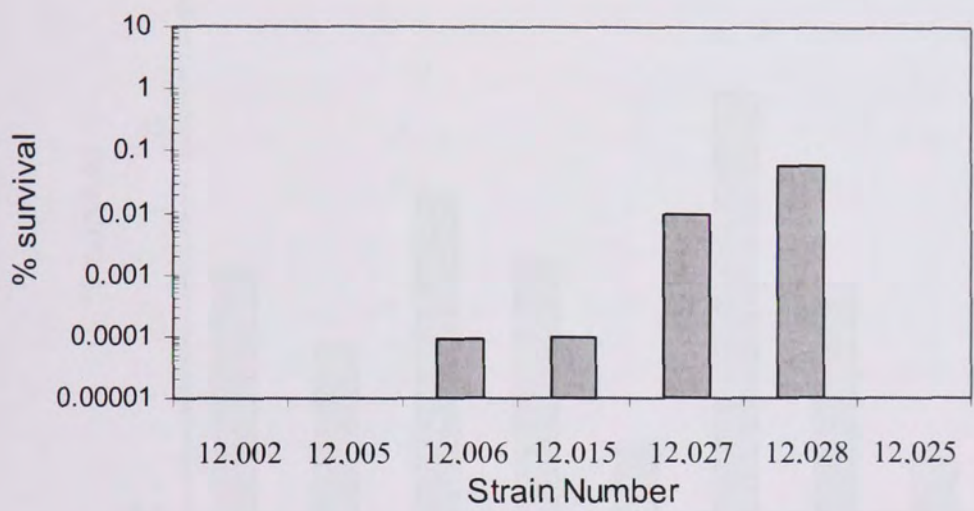


Figure 3.14. Percentage survival of seven *Lb. acidophilus* strains following exposure to pH 1.5 for 3 hours. Results represent average of duplicate experiments, with less than 5% variation.

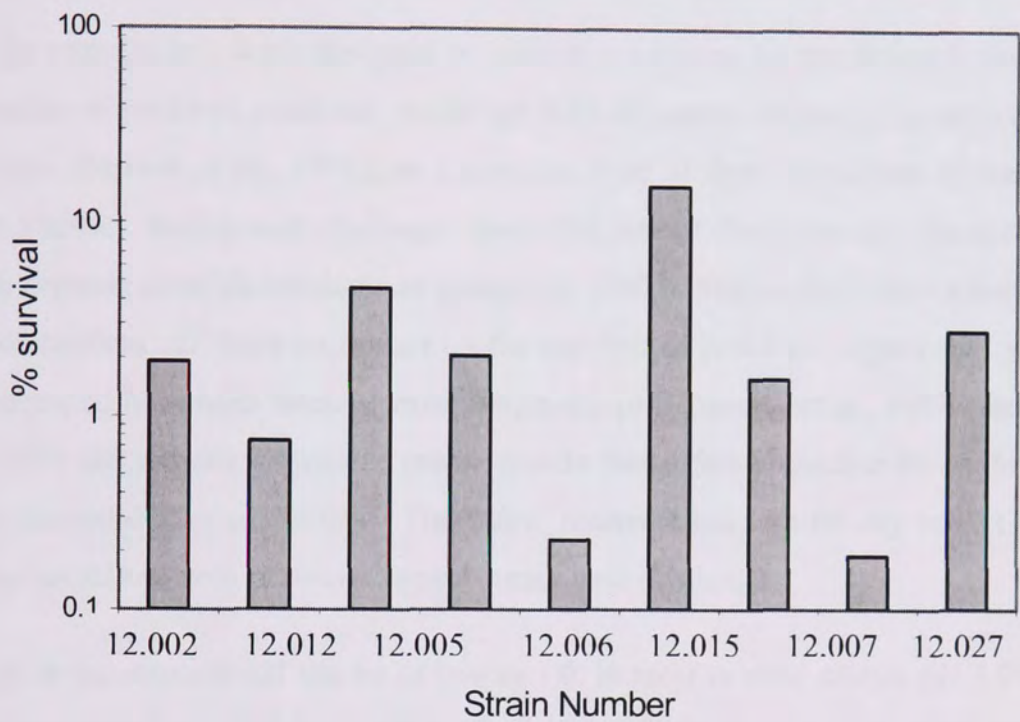


Figure 3.15. Percentage survival of nine bile-tolerant strains after six weeks of refrigerated storage in 10% NFDM following yogurt simulation trials (according to section 2.5.6). Figures represent the average of duplicate experiments with <5% variation

3.5 DISCUSSION

3.5.1 Characterisation of probiotic strains

Ten strains showing cellular morphology characteristic of that reported for *Lb. acidophilus* (Kandler and Weiss, 1986; Prasad *et al.*, 1998) were tested for their ability to survive in conditions normally found during transit through the gastrointestinal tract, including acid and bile tolerance, and also for their survival in a milk-based medium during refrigerated storage over a six-week period.

Acid tolerance studies were designed to mimic conditions in the stomach during consumption of probiotic products. Although 80% of gastric emptying occurs within 90 minutes (Berrada *et al.*, 1991), an incubation time of three hours was chosen to monitor viability during acid challenge, since the rate of food passage through the stomach depends upon its composition (Gangong, 1987). Studies have shown that the choice of medium can have an impact on the survival of probiotic organisms. Both milk and yogurt have been found to raise the gastric pH (Conway *et al.*, 1987; Martini *et al.*, 1987) and provide a buffered carrier matrix that offers protection for probiotic cultures (Fernandez *et al.*, 2003). Therefore, reconstituted non-fat dry milk (10% w/v) was inoculated with probiotic strains during acid challenge.

Although in the stomach pH can be as low as 1.0, in most *in vitro* assays, pH 3.0 has been preferred (Hood and Zottola, 1988; Gupta *et al.*, 1996; Prasad *et al.*, 1998; Chou and Weimer, 1999). This is due to the fact that a substantial decrease in viability of strains is observed at pH 2.0 or below (Gupta *et al.*, 1996; Lankaputhra and Shah, 1995; Hood and Zottola, 1988; Prasad *et al.*, 1998). Similar observations were recorded for strains exposed to pH 1.5 over the three-hour incubation period, with less than 1% of cells surviving acid challenge.

Wide variations with respect to acid tolerance of *Lb. acidophilus* strains have been observed in the literature. This is, in part, due to the variety of parameters used to determine tolerance, including the incubation medium (MRS broth, phosphate buffered saline, milk), pH challenge (pH 5.0 to 1.5), incubation times (1.5 to 3 hours) and the strains being tested (Berrada *et al.*, 1991; Gilliland *et al.*, 1984; Lankaputhra and Shah, 1995; Walker and Gilliland, 1993; Gupta *et al.*, 1996). In agreement with most studies, strains showed resistance at or above pH 3.0 (Hood and Zottola, 1988;

Prasad *et al.*, 1998; Chou and Weimer, 1999; Gupta *et al.*, 1996) and demonstrated strain specific variations in their ability to survive a challenge at pH 2.5, with survival rates ranging from 9.0×10^{-5} to 1 %.

Prasad *et al.* (1998) used a threshold of 80% survival to select for bile resistant strains, whilst other papers have defined bile tolerance as growth of the organism at various percentages of bile rather than survival (Gilliland *et al.*, 1984; Overdahl and Zottola, 1991; Walker and Gilliland, 1993). Interpreting the results presented in this chapter using these standards would lead to the conclusion that strains VUP 12,006, VUP 12,015, VUP 12,027 and VUP 12,025 are resistant to bile, with strains VUP 12,006, VUP 12,015 and VUP 12,025 were able to grow in the presence of 1.5% bile. Since the mean intestinal bile concentration is believed to be 0.3% (Gilliland *et al.*, 1984) most of the strains tested would be expected to survive transit through the upper intestinal tract.

With the aim of selecting strains for further studies, the viability of the lactobacillus cultures was assessed over a six-week period during refrigerated storage in a milk-based medium. To provide therapeutic value, advisory standards recommend that probiotic strains maintain viability of no less than one million cells per gram of yogurt during storage (Kurmann and Rasic, 1991). Results demonstrated that all strains are able to maintain viability above the suggested threshold for the first four weeks of refrigerated storage (data not shown), but most fail to meet these standards beyond this point. Strains VUP 12,015 and VUP 12,028 were targeted for further studies, based on their poor performance, with only 0.23% and 0.19% of cells surviving after six weeks of refrigerated storage. Interestingly, these strains maintained the highest survival rates during acid tolerance studies at pH 2.5. In contrast, strain VUP 12,005 was selected for its ability to maintain viability above the recommended level during six weeks of refrigerated storage and the observation that strain VUP 12,005 barely survived a challenge of pH 2.5 for 3 hours at 37°C, with less than 1% of cells surviving.

3.5.2 Differentiation and identification of isolates

In line with the literature, variable fermentation patterns were observed for the sugars melibiose, raffinose and trehalose for the 10 strains tested here. These sugars have often been the focus of differentiation of the species of the *Lb. acidophilus* complex (Kandler and Weiss, 1986; Fujisawa *et al.*, 1992; Stiles and Holzapfel, 1997; Klein *et al.*, 1998). Table 3.8 summarizes the findings of these authors and this demonstrates the overlap reported previously for the *Lb. acidophilus* complex members. For example, the fermentation of raffinose by *Lb. amylovorus* has been reported as negative (Kandler and Weiss, 1986; Klein *et al.*, 1988), positive (Stiles and Holzapfel, 1997) and variable (Fujisawa *et al.*, 1992). In another example, Johnson *et al.* (1980, 1987) reported that *Lb. acidophilus* does not ferment mannitol, whereas Gupta *et al.* (1996) reported that three out of six *Lb. acidophilus* strains utilized mannitol. Overall, the results for carbohydrate utilisation suggest that strains are members of the *Lb. acidophilus* complex of species, however, differentiation between *Lb. acidophilus* and the phenotypically very similar *Lb. johnsonii*, *Lb. crispatus*, *Lb. gasseri*, *Lb. amylovorus* and *Lb. gallinarum* is not possible using sugar fermentation analysis. However, the variable results reported with the sugars melibiose, raffinose and trehalose could be used to differentiate strains that have been previously identified as one of the six species of the *Lb. acidophilus* complex.

Antibiotic susceptibility has become an important part of selecting probiotic organisms. It has been suggested that antibiotic-challenged environments can lead to the production of dairy products containing antibiotic-resistant lactic acid bacteria (Huys *et al.*, 2002). This is disturbing when you consider the ability of probiotic organisms to survive and grow in a variety of food matrices as well as in the human colon. It has also been suggested that certain lactic acid bacteria can act as reservoirs of transferable antibiotic resistance genes (Teuber *et al.*, 1999; Gevers *et al.*, 2000). In this regard, routine antibiotic susceptibility testing of food-associated organisms is advisable for confirming the safety of probiotic organisms and also as a guide for specific antimicrobial therapy (Charteris *et al.*, 2001; Danielson *et al.*, 2003).

A variety of techniques have been reported for antimicrobial susceptibility testing of lactic acid bacteria in food isolates, including disc diffusion (Orberg and Sandine,

Table 3.8. Comparison of fermentation patterns previously reported for the *Lb. acidophilus* group

Carbohydrate ^a												
Species	Melibiose				Raffinose				Trehalose			
	A ^b	B	C	D	A	B	C	D	A	B	C	D
<i>Lb. acidophilus</i>	v	-	-	v	v	v	v	+	v	+	+	v
<i>Lb. amylovorus</i>	-	v	v	-	-	v	+	-	+	v	+	+
<i>Lb. crispatus</i>	-	v	+	+	-	+	+	+	-	v	-	+
<i>Lb. gallinarum</i>	n	+	+	+	n	+	+	+	n	-	-	-
<i>Lb. gasseri</i>	v	-	-	-	v	-	-	v	v	+	+	+
<i>Lb. johnsonii</i>	n	v	v	+	n	v	v	+	n	v	+	+

^a v = variable results, - = no fermentation, + = fermentation, n = no result available.

^b Data reported by: A = Kandler and Weiss, 1986; B = by Fujisawa *et al.*, 1992; C = Stiles and Holzapfel, 1997; D = Klein *et al.*, 1998.

1985), the E-test (Katla *et al.*, 2001), broth dilution (Perreten *et al.*, 1998) and agar dilution (Butaye *et al.*, 2000). The agar well diffusion assay, when used to determine antibiotic sensitivity patterns, is an ideal tool for differentiation of probiotic strains. As described in other studies (Vescovo *et al.*, 1982; Charteris *et al.*, 1998a,b; Huys *et al.*, 2002) the use of MRS medium allowed optimal growth of the test organisms and also provided a suitable gel matrix to allow reproducible and uniform diffusion of the antibiotic agent.

The antibiotics, clindamycin, lincomycin and rifampicin were particularly important for differentiating strains, as sensitivity to these antibiotics was a strain specific characteristic. The concentrations of antibiotics selected for this study were not sufficient to determine the exact inhibitory concentration of each antibiotic, but allowed for the strains to be classified as sensitive or resistant to a particular antibiotic. In general, the patterns of antibiotic sensitivity/resistance that emerged were similar to those previously reported for the *Lb. acidophilus* complex of species (Charteris *et al.*, 2001; Danileson *et al.*, 2003).

DNA fingerprinting by PFGE, allowing the comparison of large restriction fragments, has been successfully applied to strain typing of various lactic acid bacteria including *Lac. lactis* (Tanskanen *et al.*, 1990), *Lb. oenos* (Kelly *et al.*, 1993), the *Lb. acidophilus* group (Roussel *et al.*, 1993), *Lb. plantarum* (Daniel, 1995; Chevallier *et al.*, 1994), *Lb. sake* (Bjorkroth *et al.*, 1996) and *Str. thermophilus* (Boutrou *et al.*, 1995). The results show that PFGE was an ideal method for determining relationships between strains of a given species. *Sma*I was shown to be a suitable enzyme for typing the strains tested. This was not surprising, since similar conclusions have been drawn for closely related species that have a similar G + C content, including *Lb. acidophilus* (Klein *et al.*, 1998), *Lb. helveticus* (Lortal *et al.*, 1997), *List. monocytogenes* (Moore and Datta, 1994), *Lb. casei* (Ferrero *et al.*, 1996), *Str. thermophilus* (Boutrou *et al.*, 1995) and *Lac. lactis* subsp. *lactis* and subsp. *cremoris* (Tanskanen *et al.*, 1990).

The genome sizes estimated as the sum of the restriction fragments of the *Sma*I digests were between 1.47 to 1.96 Mb, with most strains clustering at 1.5 Mb. This is

lower than the 1.85 Mb previously reported for *Lb. acidophilus* (Roussel *et al.*, 1993). Chromosome size has been found to vary between strains of the same species and between studies that adopt different running conditions and restriction enzymes. For example, the genome size of *Lb. gallinarum* was calculated at 1.45 Mb after digestion with *ApaI* and 1.95 Mb after digestion with *SmaI* using PFGE (Klein *et al.*, 1998). In another example, Adlerbeth *et al.* (1996) and Daniel (1995) reported that *Lb. plantarum* had a genome size of 2.7 to 2.9 Mb, whilst Chevallier *et al.* (1993) reported a genome size of 3.3 Mb. These differences in genome size have often been explained by the presence of undigested plasmids that migrate differently to linear DNA molecules of the same molecular weight (Stahl *et al.*, 1990; Tanskanen *et al.*, 1990). However, lactobacilli generally harbour few, if any, plasmids (Roussel *et al.*, 1993). Another explanation is that fragments of the same molecular weight could have migrated the same distance and been counted as single bands rather than doublets (Bourget *et al.*, 1993).

The *SmaI* digestion patterns of strains VUP 12,016/VUP 12,015 and VUP 12,012/VUP 12,006/VUP 12,025 were indistinguishable and their genome sizes were found to be identical. This suggests that these strains share similarity in their overall genomic organisation. According to Tenover *et al.* (1995) such results would suggest that these strains are identical. However, additional enzymes and fragmentation analyses would be required to confirm identities or to detect minor differences. Phenotypic techniques performed in this study formed the basis of differentiation of strains that appeared identical following PFGE (Table 3.9). A combination of these techniques allowed for the differentiation of the isolates VUP 12,012, VUP 12,006 and VUP 12,025. Differentiation of strains VUP 12,015 and VUP 12,016 was achieved by comparing carbohydrate fermentation profiles. Strain VUP 12,015 cannot ferment galactose or raffinose, whilst strain VUP 12,016 can utilize both sugars.

Calculating the Dice coefficient was an efficient method for determining the relationship between the reference strain and the probiotic strains provided for this study. Dice coefficients for *Lactobacillus* strains ranged from 0.24 (24% similarity, strain VUP 12,028) to 1.0 (100% similarity, strain VUP 12,015) when compared to

Table 3.9. Differentiation of strains displaying identical PFGE restriction patterns

Differentiation of strains:			
Characteristic	VUP 12,012	VUP 12,006	VUP 12,025
Antibiotic sensitivity			
Amoxicillin	1	2	2
Erythromycin	3	3	2
Piperacillin	3	3	2
Rifampicin	2	3	3
Spectinomycin	4	3	3
Streptomycin	4	4	3
Vancomycin	2	2	3
Sugar fermentation			
Melibiose	+	-	+
Trehalose	+	-	+
Bile tolerance			
(1.5%, 3 hours)	50.5%	100%	100%
Acid tolerance			
(pH 2.5, 3 hours)	0.02%	0.15%	13.8%
Survival in milk			
(4°C, 6 weeks)	0.75%	2.07%	2.79%

the type strain VUP 12,016, with most strains clustering at a level of >80% similarity. Several reports have shown large differences between banding patterns between members of the same species. Lortal *et al.*, (1997) showed that the Dice coefficients of similarity of *Lb. helveticus* strains analysed by PFGE ranged from 26-100%.

Strain VUP 12,028 provided a unique restriction pattern when digested with *Sma*I and analysed using the PFGE technique. Carbohydrate fermentation patterns mimicked those published in Bergey's Manual, and the protein profile was 86% similar to the reference strain, which suggests that strain VUP 12,028 is of the *Lactobacillus* species. However, the discriminatory power of the PFGE technique is too low to perform speciation of the isolates (Roussel *et al.*, 1993; Bourget *et al.*, 1993; Chevallier *et al.*, 1994; Boutrou *et al.*, 1995; Bjorkroth *et al.*, 1996; Busse *et al.*, 1996; Drake *et al.*, 1996; Ferrero *et al.*, 1996; Descheemaeker *et al.*, 1997; Klein *et al.*, 1998; Sanders *et al.*, 1996), therefore an additional technique, such as ribosomal sequencing, would be required to determine the level of relatedness of strain VUP 12,028 to the other strains tested.

SDS-PAGE was assessed as a tool to differentiate strains purchased as *Lb. acidophilus* and to determine their similarity to a *Lb. acidophilus* type strain. All protein profiles from the probiotic species were similar to that of the type strain, with Dice coefficients ranging from 0.82 to 0.88. Normally, these results would suggest that these strains all belong to the *Lb. acidophilus* species, as similarities of >80% have been reported as acceptable for species designations (Dykes and Von Holy, 1993; Howe *et al.*, 1995). However, Klein *et al.*, (1998) claimed that it was difficult to distinguish between closely related species of the *Lb. acidophilus* complex using analysis of the whole protein spectrum. Instead, analysis of the major proteins resulted in the production of two main homology groups. The species *Lb. acidophilus*, *Lb. crispatus*, *Lb. amylovorus* and *Lb. gallinarum* (group A) had a relationship of 75.9%, whilst group B, containing *Lb. gasseri* and *Lb. johnsonii*, clustered at a level of 72.9%. When groups A and B were compared to each other, they shared 68.2% similarity. The results presented in this thesis demonstrate the failure of SDS-PAGE as a technique to discriminate between strains of the *Lb.*

acidophilus species, taking into account the level of variation between replicates of each strain.

16S rRNA gene sequencing was performed to formally identify strains purchased originally as *Lb. acidophilus*. Following *Sma*I digestion of whole chromosomal DNA and PFGE, most strains clustered into one group, sharing > 70% similarity with the *Lb. acidophilus* type strain VUP 12,016. In contrast, strain VUP 12,028 shared only 24% resemblance with the type strain. Thus, the aim was to determine whether strain VUP 12,028 was a member of the *Lb. acidophilus* species.

As previously reported (Hensiek *et al.*, 1992; Schleifer *et al.*, 1995; Tilsala-Timisjarvi and Alatossava, 1997; Kullen *et al.*, 2000; Horie *et al.*, 2002; Tarnberg *et al.*, 2002; Ventura and Zink, 2002; Yeung *et al.*, 2002) analysis of the V1 region of the 16S rRNA gene is a practical tool for differentiation the ten strains purchased as *Lb. acidophilus*. The obtained sequences could be clustered into two main groups clearly corresponding to *Lb. acidophilus* (nine strains) and *Lb. johnsonii* (one strain). The 16S rRNA region of the nine *Lb. acidophilus* sequences was identical, thus this technique did not provide discrimination below the species level. A comparison of these sequences with those of the V1 region of the *Lb. acidophilus* complex (Kullen *et al.*, 2000) demonstrated that *Lb. acidophilus* strain VUP 12,016 was closely aligned with *Lb. amylovorus*, *Lb. crispatus* and *Lb. gallinarum*, sharing greater than 89% similarity. In contrast, *Lb. johnsonii* strain VUP 12,028 was closely aligned with *Lb. gasseri*, sharing greater than 90% similarity. This observation is consistent with the placement of these strains into the DNA homology groups designated by Johnson *et al.* (1980) and Lauer *et al.* (1980b).

Analysis of the V3 region (Tarnberg *et al.*, 2002) of the *Lb. acidophilus* complex could not be used to discriminate between *Lb. gasseri* and *Lb. johnsonii* or *Lb. amylovorus* and *Lb. gallinarum*. However, the V3 region did confirm that strain VUP 12,016 is a member of the *Lb. acidophilus* species, sharing an identical V3 region with the *Lb. acidophilus* type strain CCUG 5917. In addition, the V3 region of the *Lb. gasseri*-*Lb. johnsonii* combination matched strain VUP 12,028. Therefore, the V3 region can be used in combination with the V1 region to confirm the results obtained with the V1 assignments.

Sequence data is predominantly analysed by submitting each sequence to ANGIS (GenBank) and using the BLASTn tool to search for sequences bearing similarity. Those sequences that show more than 95-97% homology with existing GenBank database entries are assigned to that species (Vandamme *et al.*, 1996; Pryde *et al.*, 1999). Submitting the sequences to ANGIS using the BLASTn similarity search tool confirmed the species assignments that were based on the V1 and V3 regions, with strain VUP 12,016 showing 98.5 % homology to the *Lb. acidophilus* species and strain VUP 12,028 showing 99% homology to the *Lb. johnsonii* species included in the GenBank database. However, sending the whole sequence (1200 base pairs) to BLASTn was not as discriminatory as was analysing the individual 50 base pair variable regions (Refer to table 3.7), with only 1% difference between some of the species of the *Lb. acidophilus* complex. Therefore, it is suggested that sequences be analysed by comparison with the published variable regions.

Additional probes and species-specific primers that did not form part of the V1 or V3 regions were also tested for their ability to hybridize with strains VUP 12,016 and VUP 12,028. This was possible using the 'FindPatterns' tool accessed through ANGIS. The *Lb. acidophilus* species-specific probe (L.ACRDR) published by Sui *et al.*, (2002), matched perfectly with strain VUP 12,016. Interestingly, the *Lb. crispatus* species-specific primer, LCRI16S, published by Horie *et al.*, (2002), also paired with strain VUP 12,016, but this primer also bound to *Lb. acidophilus* JCM 1034 (Horie *et al.*, 2002), suggesting that this primer cannot be used for species-specific designations.

These results demonstrated that the species pre-identified as *Lb. acidophilus* strains VUP 12,002, VUP 12,012, VUP 12,005, VUP 12,006, VUP 12,015, VUP 12,007, VUP 12,027 and VUP 12,025 were confirmed as *Lb. acidophilus* species whilst strain VUP 12,028 was re-classified as *Lb. johnsonii*. These results are consistent with the differences observed in PFGE patterns for strain VUP 12,028 relative to the *Lb. acidophilus* species. Together, 16S rRNA and PFGE were ideal for both identifying the isolates of members of a particular species and differentiating them into separate strains.

Originally identified as *Lb. acidophilus*, *Lb. johnsonii* strain LA1 has been reported as an ideal candidate as a probiotic strain (Holzapfel *et al.*, 2001). Strain LA1 is a representative of the microbial flora in the human gastrointestinal tract. It has been given GRAS (generally regarded as safe) status, is resistant to acid and bile, can produce bacteriocins and has the ability to colonize the human gut. *Lb. johnsonii* LA1 also demonstrates a high survival rate in dairy products, with viability between 7.1 to 8.0 log cfu/g of yogurt. Therefore, strain VUP 12,028 may satisfy the requirements as a novel probiotic strain if it shows similar properties to LA1.

3.6 CONCLUDING REMARKS

A polyphasic approach was used in this chapter to characterise, differentiate and identify strains originally purchased as *Lb. acidophilus*.

Conventional microbiological identification, based on physiological and biochemical characteristics, were useful for differentiating strain biotypes. Carbohydrate fermentation patterns indicated that the strains were members of the *Lb. acidophilus* complex of species, whilst the antibiotics clindamycin, lincomycin and rifampicin increased the depth of the hierarchical line through strain typing of these species.

PFGE of *Sma*I restriction fragments is a valuable method for determining relationships between these strains: the majority clustered into one group, sharing >70% similarity with the *Lb. acidophilus* type strain VUP 12,016, whilst strain VUP 12,028 shared only 24% resemblance with the type strain. The *Sma*I digestion patterns of strains VUP 12,016/VUP 12,015 and VUP 12,012/VUP 12,006/VUP 12,025 were indistinguishable using PFGE, but these strains could be differentiated on the basis of other phenotypic traits.

16S rRNA gene sequencing could be used to identify the strains purchased originally as *Lb. acidophilus*. Analysis of the V1 and V3 regions of the *Lb. acidophilus* complex demonstrated that strains VUP 12,002, VUP 12,012, VUP 12,005, VUP 12,006, VUP 12,015, VUP 12,007, VUP 12,027 and VUP 12,025 were confirmed as *Lb. acidophilus*, whilst strain VUP 12,028 was re-classified as *Lb. johnsonii*.

No single strain proved superior in terms of acid tolerance, bile tolerance and survival during long-term storage. *Lb. acidophilus* strain VUP 12,015 and *Lb. johnsonii* strain VUP 12,028 were selected for further studies based on their poor viability in a milk-based medium during long-term storage and their high survival rates at pH 2.5. For comparison, *Lb. acidophilus* strain VUP 12,005 was selected for its ability to maintain viability above the recommended level during long-term storage.

CHAPTER 4

SHORT-TERM IMPROVEMENT IN SURVIVAL OF PROBIOTIC STRAINS IN NORMALLY LETHAL ACIDIC CONDITIONS

4.1 INTRODUCTION

Lactic acid bacteria periodically experience life-threatening stresses during the processing and storage of dairy products. In response to changes in the environment, bacteria often undergo physiological changes, which are generally referred to as the stress response. Some bacteria are also known to demonstrate an adaptive response, that is, when cells are exposed to moderate levels of stress, they acquire increased tolerance when subsequently exposed to extreme levels of the same stress (Foster, 1991, 1993; Foster and Hall, 1990, 1991; Leyer *et al.*, 1995; Gahan *et al.*, 1996; Baati *et al.*, 2000; Lorca and de Valdez, 2001b; Kim *et al.*, 2002).

Since lactic acid bacteria are routinely used in dairy fermentation, studies of adaptive physiology may facilitate efforts to improve bacterial activity and function in fermented dairy foods. The ability of cells to adapt to both weak and strong acids following acid adaptation is particularly relevant when the nature of probiotic organisms are considered, in that the most likely acid that the organism will encounter in yogurt is weak lactic acid whereas the initial barrier to successful survival in the stomach is hydrochloric acid, a strong acid.

The results reported in this chapter are concerned with determining the growth parameters for a protocol aimed at improving the survival of probiotic strains during refrigerated storage in yogurt. Strains VUP 12,005, VUP 12,015 and VUP 12,028 were selected as representatives for these studies based on their traits as described in the previous chapter. This chapter describes studies on the kinetics of the selected strains at various pH and temperature values. These studies were undertaken to establish optimal, moderate and lethal pH and temperature conditions of growth, to produce base-line data for investigating stress and adaptive responses.

The general aim of the research reported in this chapter was to investigate the adaptive acid tolerance response in selected strains. In particular, the specific aims were to:

- a) determine the suitability of MRS medium to support growth of the selected strains,
- b) determine optimum, moderate and lethal growth parameters for strains of the *Lb. acidophilus* complex,
- c) determine the most appropriate acidulant for conditioning selected strains,
- d) determine the most appropriate time for conditioning of selected strains at various pH values,
- e) determine whether exposing cells to moderate pH conditions during growth will protect them during short-term lethal pH challenge.

4.2 GROWTH KINETICS AT VARIOUS PH AND TEMPERATURE VALUES

4.2.1 Selection of media for growth kinetics studies

The generation time of *Lb. acidophilus* strain VUP 12,005 was determined in 10% non-fat dry milk (NFDM) and MRS broth (pH 6.2) according to section 2.5.11. Briefly, cultures were inoculated into fresh MRS broth or reconstituted 10% NFDM and incubated at 39°C until cells reached stationary phase. Samples were taken hourly and assessed for viable counts and the mean of duplicate experiments was used to calculate the generation time of cells. *Lb. acidophilus* strain VUP 12,005 grew faster in MRS broth than in 10% non-fat dry milk. Generation times were 40 minutes in MRS broth and 65 minutes for 10% non-fat dry milk, therefore, MRS broth was the medium selected for further studies.

4.2.2 Optimum growth rates at a range of pH and temperature values

The specific growth rates of *Lb. acidophilus* strain VUP 12,005 were determined in MRS medium in 1L Applicon fermentors at a range of pH and temperature values, and the maximum growth rates were measured from optical density readings (Refer to section 2.5.11). The specific growth rates (μ) were determined by the slope of a semi-

logarithmic plot of A_{600} against time. The temperature and pH of incubation had an impact on the specific growth rate: strain VUP 12,005 was able to grow between pH 4.0 to 6.5 in MRS broth adjusted using hydrochloric acid, but not at pH 3.5 (Figure 4.1) at 37°C. In addition, cells were able to grow in MRS broth (pH 6.0) at 30 to 45°C but not at 50°C or above (Figure 4.2). Maximum growth rates were observed between pH 5.5 to 6.0 and 39°C.

4.2.3 Determination of temperature and pH values for conditioning protocols

Sub-lethal (moderate) pH values were defined by growing cells to exponential phase in one litre Applicon fermentors containing MRS broth (pH 5.8, 39°C) before adjusting the pH to 5.8, 5.0, 4.5 or 4.0 using either lactic acid or hydrochloric acid as the acidulant. Cells were monitored for the rate of growth and viability, during this period as described in section 2.5.11. These experiments were performed in duplicate using both hydrochloric acid and lactic acid to determine whether the choice of acid used to adjust the pH influenced the outcome. Figure 4.3 shows the viability of strain VUP 12,005 following shifts from pH 5.8 to 5.8, 5.0, 4.5 and 4.0. The data shows that the growth traits were similar regardless of which acid was used to lower the pH. The growth rates at pH 5.8 and 5.0 were similar, but at pH 4.5 the rate of growth decreased. Growth ceased after adjusting cultures to pH 4.0 and after five hours of incubation at pH 4.0 (using hydrochloric acid to adjust pH) the cell viability was reduced by 36% whereas using lactic acid as the acidulant, a 28% reduction in cell viability was observed.

Lowering the pH of the exponential phase cultures was likely to have consequences for the growth rate of the culture. The extent of these effects on growth rate was assessed by directly measuring growth during this period. The pH of a series of exponential phase cultures was adjusted to either pH 5.8, 5.0 4.5 or 4.0 with hydrochloric acid. The growth rate of each culture was measured by recording the OD_{600} over the ensuing hour. Results indicate that as the pH decreased, so too did the growth rate of the culture, with rates (μ) at 1.09, 0.84, 0.74 and 0.46 h^{-1} following one hour at pH 5.8, 5.0, 4.5 or 4.0 respectively. Experiments were repeated using lactic acid to lower the pH showed similar results with growth rates at 1.15, 0.78, 0.38 and 0.16 h^{-1} following one hour at pH 5.8, 5.0, 4.5 or 4.0 respectively.

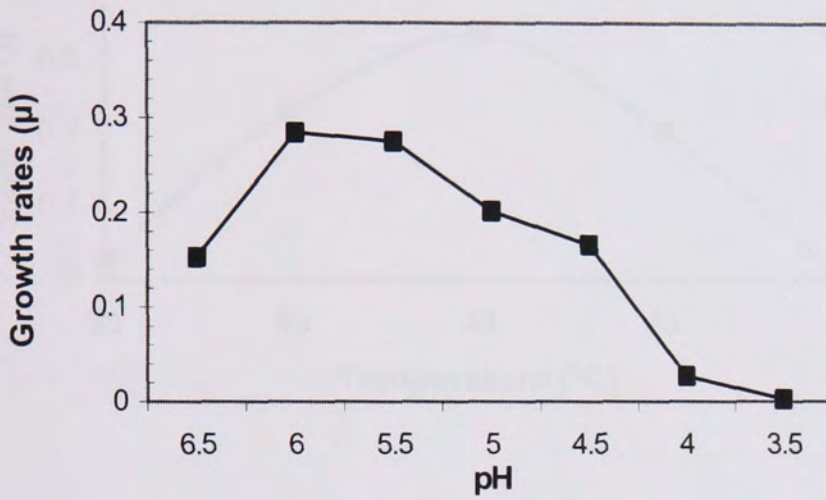
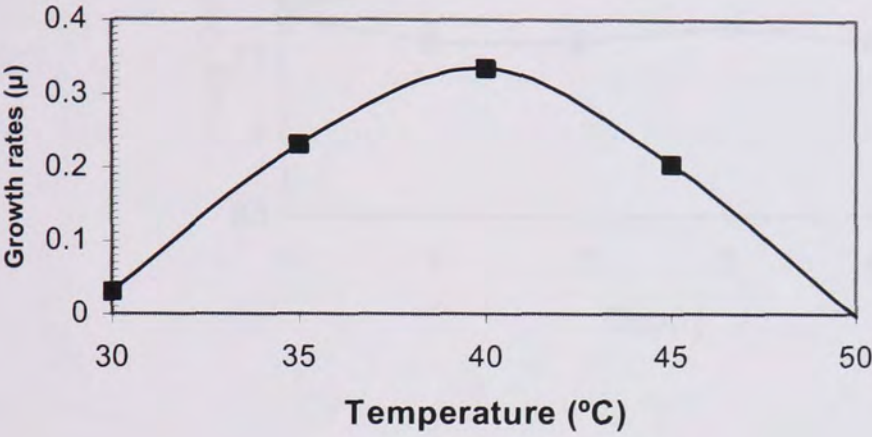


Figure 4.1. Growth rates (μ) of strain *Lb. acidophilus* strain VUP 12,005 grown in fermentors containing pH MRS broth (37°C) at various pH values. Results represent the mean of two separate experiments.

a)



c)

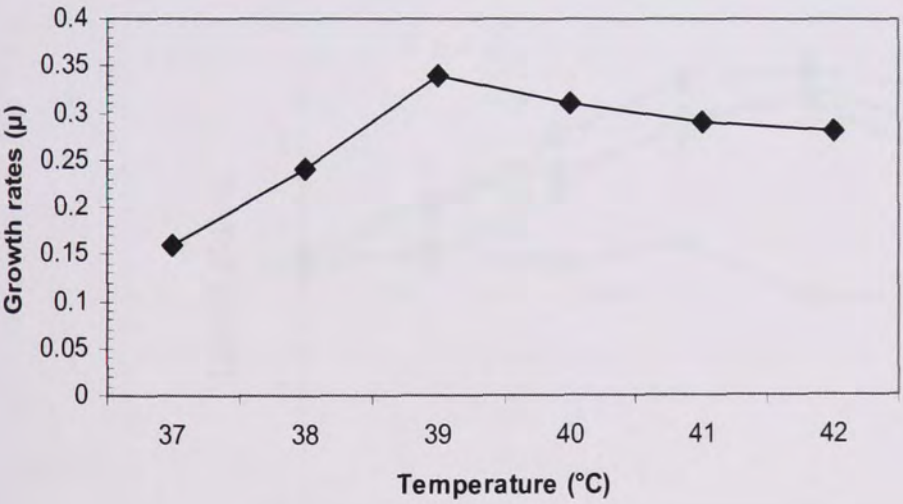
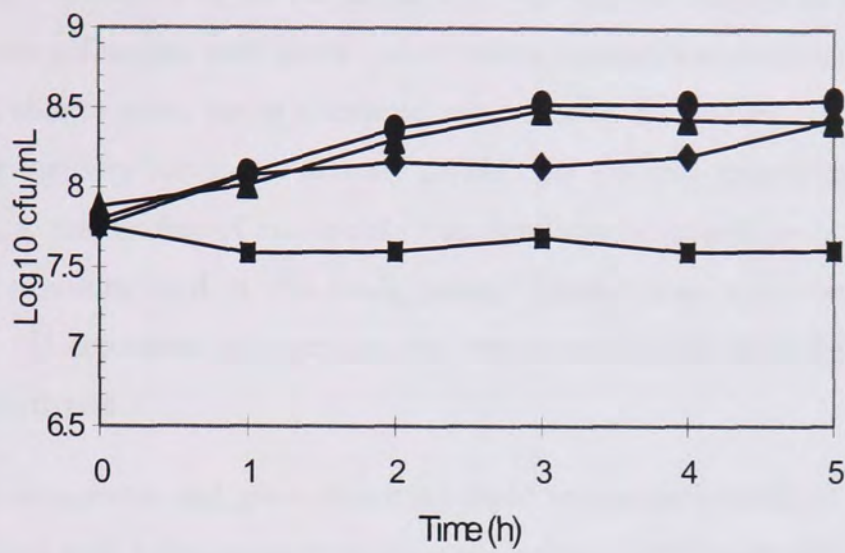


Figure 4.2. Growth rates (μ/h^{-1}) of strain *Lb. acidophilus* strain VUP 12,005 inoculated into flasks containing pH buffered MRS broth (pH 6.0) at temperatures between (a) 30°C to 50°C and (b) 37°C to 42°C. Results represent the mean of two separate experiments.

a)



b)

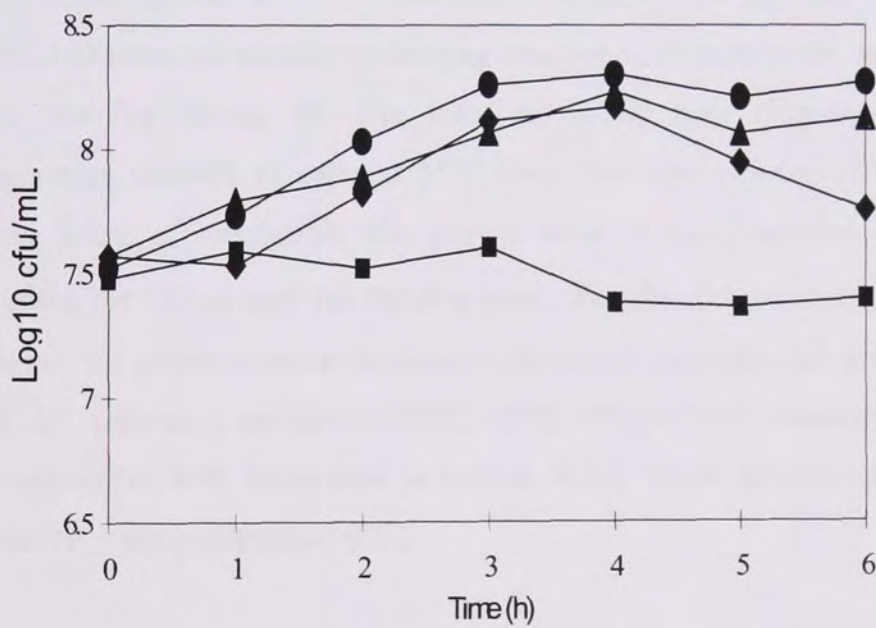


Figure 4.3. Viable counts of *Lb. acidophilus* strain VUP 12,005 grown to exponential phase (39°C, pH 5.8) in fermentors containing MRS broth followed by the adjustment of pH to either pH 5.8 (●), 5.0 (▲), 4.5 (◆) or 4.0 (■) using a) hydrochloric acid or b) lactic acid.

From the results, pH 4.5 and pH 4.0 were selected for conditioning cells prior to exposure to lethal pH conditions at pH 3.5 or pH 3.0. pH 4.5 was defined as 'acid stress' and pH 4.0 was defined as 'acid shock'. Acid stress represents an environment where cells are still able to grow, but at a reduced rate, whereas for acid shock, cells are able to maintain viability but do not actively divide. pH 3.5 was selected as the lethal pH condition, as the number of viable cells was significantly reduced at this pH over the period of exposure used in this study period. These values were used to determine whether pH-dependent changes in acid resistance simply reflected the effects of pH on growth rate.

The ability of cells to survive and grow following rapid temperature up-shifts was determined by growing cells to exponential phase in pH buffered MRS broth (pH 5.8) and rapidly shifting the temperature to 39°C, 45°C, 50°C or 55°C before monitoring viable counts and growth rates (refer to section 2.5.11). Figure 4.4 shows that cells grew optimally at 39°C, with a steady increase in viable counts seen over the incubation period. Cells grown at 50°C maintained viability for the first hour followed by a 65% reduction in viability following two hours of incubation, and a 98% reduction in viability during the five hour incubation time (Figure 4.4). Following a temperature up-shift of cells to 55°C, less than one percent of cells survived after three hours of incubation. The growth rates of these cultures were measured by recording the OD₆₀₀ over the ensuing hour. Results showed that as the temperature increased, the growth rates of the culture decreased with rates (μ) at 0.86, 0.56, 0.48 and 0.07 h⁻¹ following one hour at 39°C, 45°C, 50°C or 55°C respectively. These results are consistent with those seen in section 4.2.2, where growth ceased shortly after incubation at temperatures >50°C.

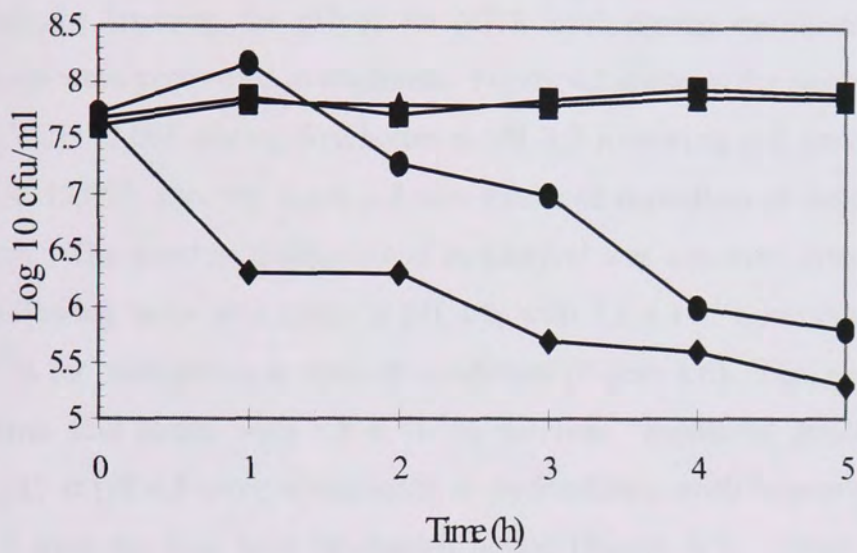


Figure 4.4. Viable counts of *Lb. acidophilus* strain VUP 12,005 grown to exponential phase in flasks containing MRS broth (pH 5.8, 39°C) prior to incubation at 39°C (■), 45°C (▲), 50°C (●), 55°C (◆). Figures represent the mean of duplicate experiments.

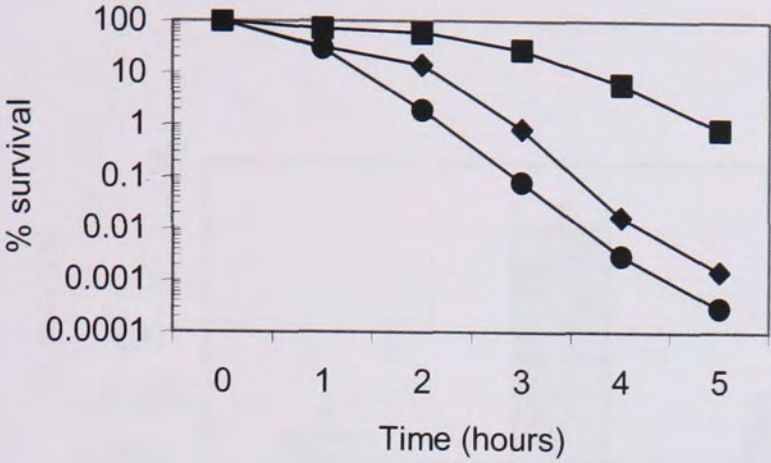
4.3 EFFECT OF LACTIC ACID AND HYDROCHLORIC ACID ON THE SURVIVAL OF PROBIOTIC STRAINS UNDER NORMALLY LETHAL ACIDIC CONDITIONS

Short-term survival of strains VUP 12,005 and VUP 12,015 was monitored in MRS broth at pH 3.5 (39°C) for five hours following conditioning of cells for one hour using lactic acid or hydrochloric acid-adjusted MRS broth at pH 4.5 (as described in section 2.5.15). This experiment was performed to determine which acid was most appropriate for lowering the pH of the MRS broth during conditioning protocols. Experiments were performed in duplicate. Figure 4.5 presents the percentage survival of strain VUP 12,005 during five hours at pH 3.5 following cell conditioning. For strain VUP 12,005, survival at pH 3.5 was enhanced regardless of the method of cell preparation. The greatest improvement in survival was observed after five hours at pH 3.5 following lactic acid stress at pH 4.5, with $8.6 \times 10^{-1}\%$ survival compared to $3.0 \times 10^{-4}\%$ for cells grown at optimal conditions (Figure 4.6). This was followed by hydrochloric acid stress, with $1.5 \times 10^{-3}\%$ survival. Similarly, conditioning strain VUP 12,015 at pH 4.5 using either lactic or hydrochloric acids improved the survival at pH 3.5 over the five hour incubation period (Figure 4.5). After five hours of incubation, 12.8% of unadapted (control) cells survived compared to 47.8% and 32.4% of cells conditioned using lactic or hydrochloric acids, respectively (Figure 4.6). Therefore, lactic acid was used to lower the pH of the medium in conditioning protocols.

4.4 SURVIVAL OF *LB. ACIDOPHILUS* VUP 12,015 UNDER NORMAL LETHAL ACIDIC CONDITIONS FOLLOWING CONDITIONING AT MODERATE PH FOR 0 TO 120 MINUTES

The preceding data shows how conditioning cells at a moderate pH during exponential growth improves survival at normally lethal pH levels. The next step was to determine the optimum time for conditioning cells to obtain greatest survival during acid challenge at a low pH. *Lb. acidophilus* strain VUP 12,015 was grown to exponential phase under optimal conditions (39°C, pH 5.8) before cells were collected and resuspended in pH-adjusted MRS broth (using lactic acid) and incubated for

a)



b)

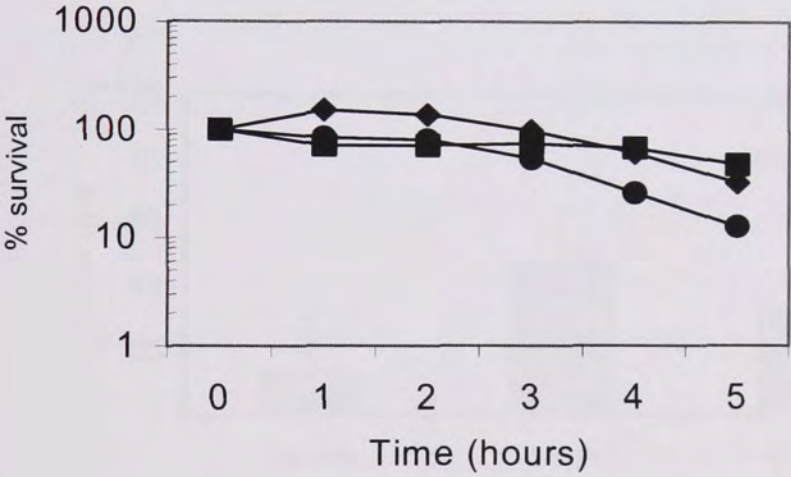
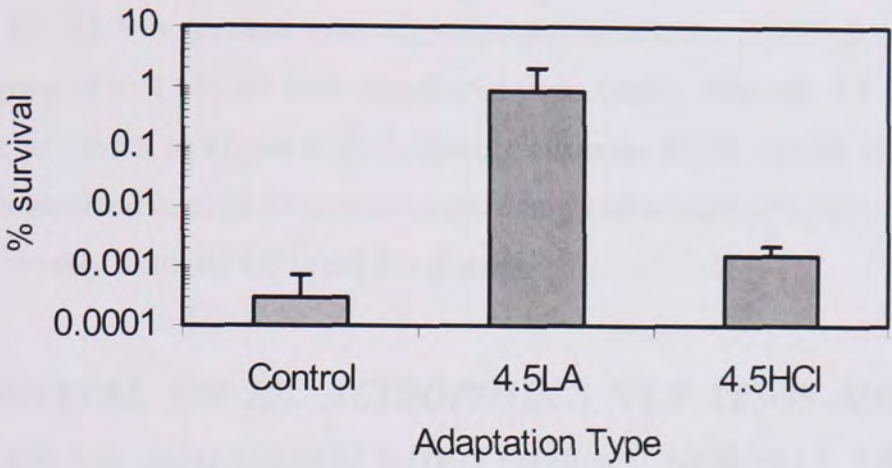


Figure 4.5. Percentage survival of *Lb. acidophilus* strain a) VUP 12,005 and b) VUP 12,015 during five hours of incubation in MRS broth at pH 3.5 (39°C) following conditioning at pH 5.8 (●) or pH 4.5 in MRS broth adjusted using lactic acid (■) or hydrochloric acid (◆).

a)



b)

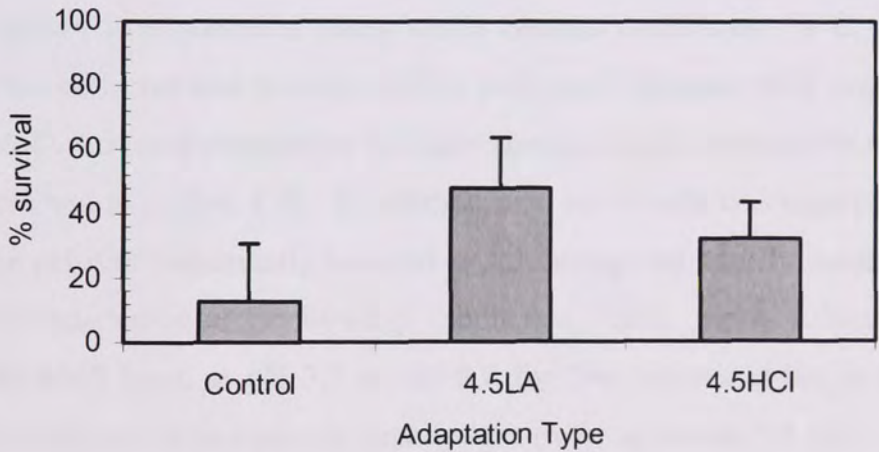


Figure 4.6. Comparison of survival of *Lb. acidophilus* strain a) VUP 12,005 and b) VUP 12,015 at five hours incubation in MRS broth at pH 3.5 (39°C) following conditioning at pH 5.8 (Control) or pH 4.5 in MRS broth adjusted using lactic acid (4.5LA) or hydrochloric acid (4.5HCl). Figures represent the mean and standard deviation of duplicate experiments.

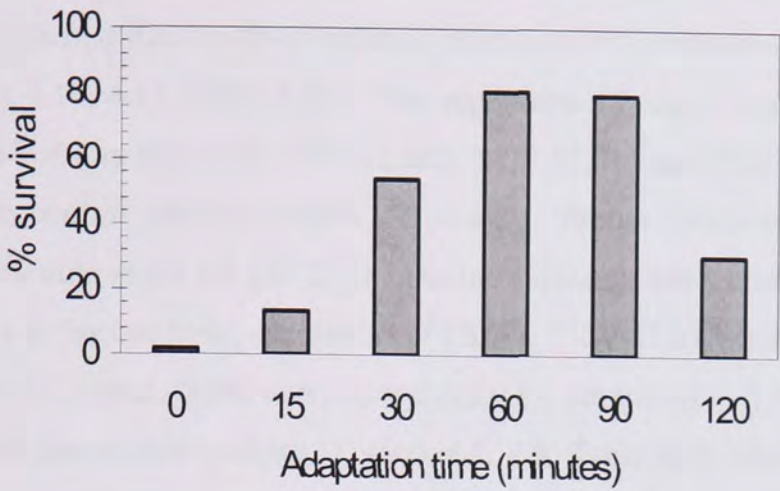
0, 15, 30, 60 90 or 120 minutes. Following incubation, cells were collected and resuspended in MRS broth at pH 3.5 for four hours and the percentage survival was determined (refer to section 2.5.13). The results showed that survival improved following incubation at either pH 4.5 or 4.0 at all the times tested (Figure 4.7). Survival at pH 3.5 was greatest following one-hour incubation at pH 4.5, with 81% survival compared to 1.6% for cells transferred immediately from pH 5.8 to pH 3.5 (represented as time 0 in Figure 4.7). Following exposure to pH 4.0 for 30 minutes, 78% of cells survived at pH 3.5 for four hours, compared to 0.05% for cells that were transferred directly from pH 5.8 to pH 3.5 (Figure 4.7).

4.5 SURVIVAL OF *LB. ACIDOPHILUS* VUP 12,005 AND VUP 12,015 AND *LB. JOHNSONII* VUP 12,028 AT NORMAL LETHAL ACIDIC CONDITIONS FOLLOWING CONDITIONING AT MODERATE PH

Lb. acidophilus strains VUP 12,005 and VUP 12,015 and *Lb. johnsonii* strain VUP 12,028 were grown to exponential phase under optimal conditions (39°C, pH 5.8) before cells were collected and re-suspended in lactic acid-adjusted MRS broth at pH 5.8 (non-adapted), 4.5 (acid stressed) or 4.0 (acid shocked) and incubated for one hour at 39°C (determined in section 4.4). In addition, one set of cells was resuspended in pH 5.8 and the pH was sequentially lowered to 4.5 during one hour of incubation at 39°C (stepwise-adaptation). Following incubation, cells were collected and resuspended in MRS broth at pH 3.5 or pH 3.0 for five hours and the percentage survival was determined from triplicate experiments (refer to section 2.5.14).

The sensitivity of strains to pH challenge is strain dependent. Within 60 minutes at pH 3.0, the viability of strain VUP 12,005 and VUP 12,028 declined to 1% of the original population, whereas strain VUP 12,015 declined to 25% during this period (Figures 4.8, 4.9; Table 4.1). However, if cells are introduced to moderate pH prior to acid challenge, there is a marked effect on cell survival. Cultures conditioned in MRS broth at pH 4.5 for one hour (39°C) demonstrated the greatest level of resistance to acid lethality (pH 3.5). After five hours of incubation in MRS broth at pH 3.5 (39°C), strains VUP 12,005, VUP 12,015 and VUP 12,028 conditioned at pH 4.5 maintained

a)



b)

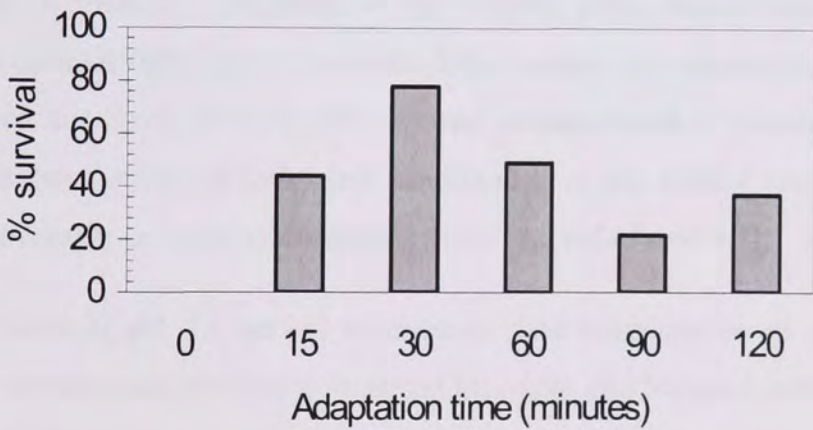


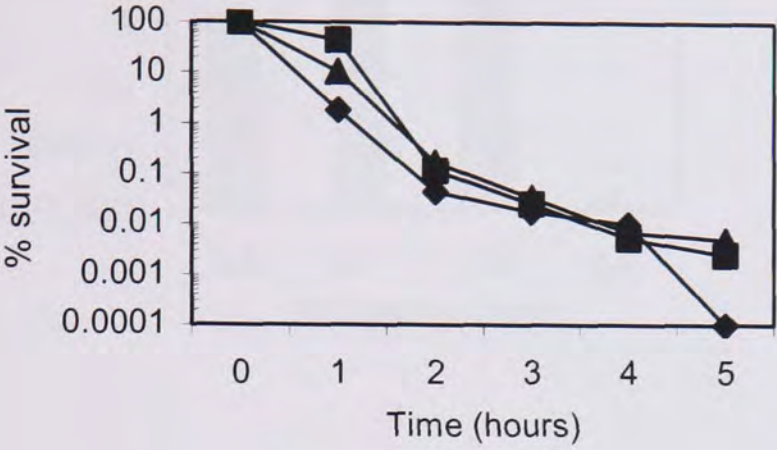
Figure 4.7. Percentage survival of exponential cells of *Lb. acidophilus* strain VUP 12,015 in MRS broth adjusted to pH 3.5 for four hours following conditioning in lactic acid adjusted MRS broth (39°C) at a) pH 4.5 or b) pH 4.0 from 0 to 120 minutes. Time 0 represents the control which was transferred to fresh MRS broth at pH 5.8 during conditioning prior to challenge at pH 3.5 for 4 hours. Figures represent the mean of duplicate experiments.

2.3%, 63% and 57% of their original viability, respectively. This was compared to 0.09%, 1.18% and 8% survival of control cultures VUP 12,005, VUP 12,015 and VUP 12,028 transferred directly from optimal (pH 5.8, 39°C) to challenge conditions (pH 3.5) (Figure 4.10, 4.11, Table 4.1). This represents a 2-log, 1-log and 0.71-log improvement in viability for strains VUP 12,005, VUP 12,015 and VUP 12,028 at pH 3.5 compared to control cultures (Tables 4.2 – 4.4). Similar trends were observed following conditioning at pH 4.5 (39°C, 1h) and incubation in MRS broth (previously adjusted to pH 3.0) for one hour. Strains VUP 12,005, VUP 12,015 and VUP 12,028 maintained 44%, 95% and 65.9% survival respectively, compared to 1.8%, 23% and 1.6% survival for the control cultures (Figures 4.8, 4.9, Table 4.1), which represents 1.3-log, 0.53-log and 1-log improvements in viable counts for strains VUP 12,005, VUP 12,015 and VUP 12,028 at pH 3.0 compared to the control cultures (Tables 4.5 – 4.7).

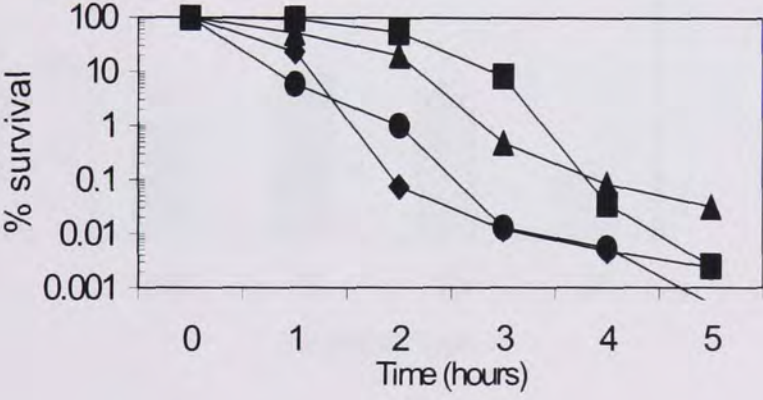
Conditioning cells to pH 4.5 lactic acid-adjusted MRS broth resulted in the greatest improvements in viability compared to the control cells which were transferred directly from optimal (pH 5.8) to normally lethal acidic pH values (pH 3.5 and pH 3.0). However, for strain VUP 12,028, optimal enhancement of viability at pH 3.5 over five hours was observed following conditioning at pH 4.0 for one hour, with a 7.5-fold improvement in viability compared to the control (Table 4.1).

Conditioning cells at pH 4.0 served to enhance acid tolerance in all strains tested compared to cultures not previously exposed to acidic conditions (control culture at pH 5.8). After five hours of incubation at pH 3.5, strains VUP 12,005, VUP 12,015 and VUP 12,028 previously conditioned at pH 4.0 maintained 3%, 45% and 60% of their original viability respectively. This was compared to 0.09%, 1.2% and 8% survival of cultures VUP 12,005, VUP 12,015 and VUP 12,028 that were transferred directly from optimal (pH 5.8) to challenge conditions (pH 3.5) (Figure 4.10, 4.11; Table 4.1). Similar trends were observed following conditioning at pH 4.0 and incubation at pH 3.0 for one hour, with strains VUP 12,005, VUP 12,015 and VUP 12,028 maintaining 11%, 53%, and 37% survival respectively, compared to 1.8%, 23% and 1.6% survival of the control cultures (Figure 4.9, Table 4.1).

a)



b)



c)

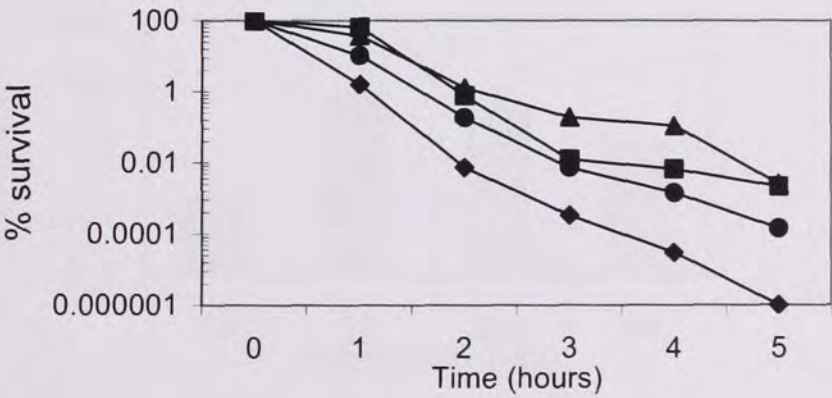
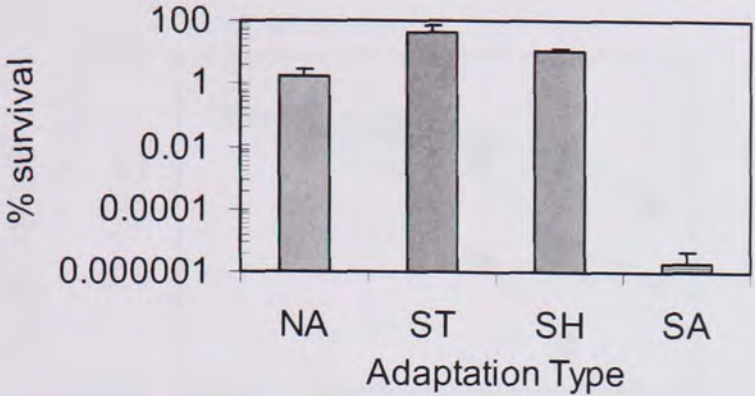
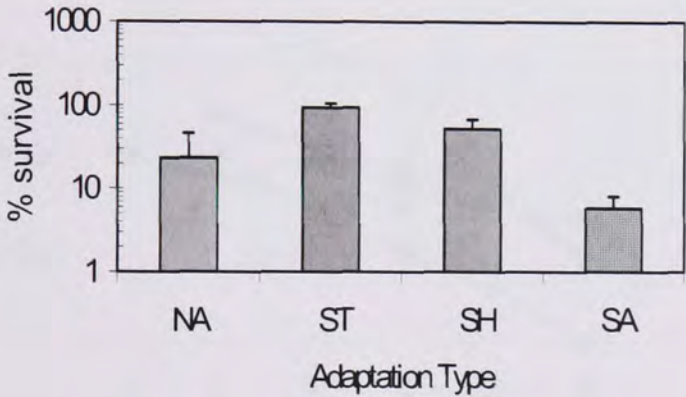


Figure 4.8. Percentage survival of *Lb. acidophilus* strain a) VUP 12,005, b) VUP 12,015 and c) *Lb. johnsonii* strain VUP 12,028 during five hours of incubation in MRS broth (39°C) adjusted to pH 3.0 using lactic acid, following conditioning for one hour in MRS broth at pH 5.8 (♦), pH 4.5 (■), pH 4.0 (▲) or pH 5.8 to 4.5 (●) at 39°C. Results represent mean values obtained from triplicate experiments.

a)



b)



c)

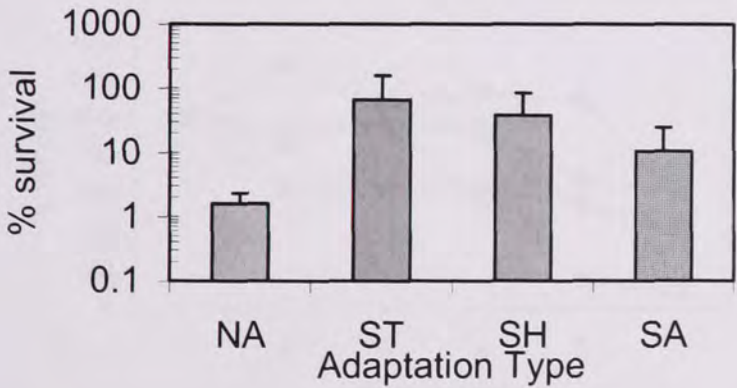
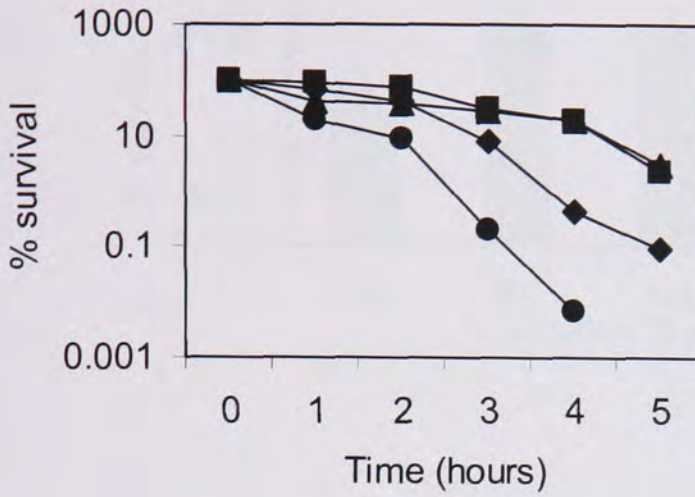
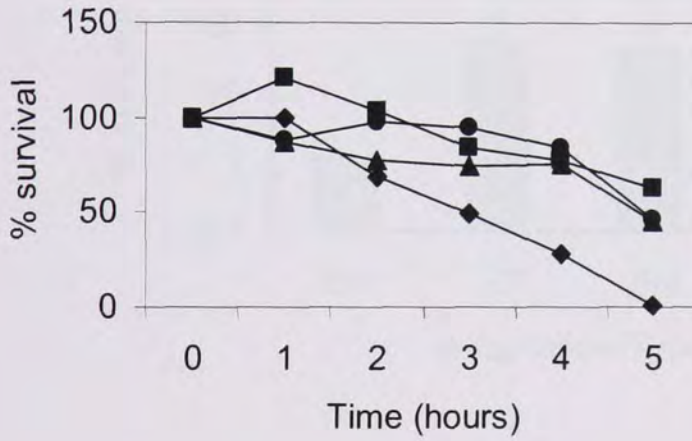


Figure 4.9. Percentage survival of *Lb. acidophilus* strain a) VUP 12,005, b) VUP 12,015 and c) *Lb. johnsonii* strain VUP 12,028 at one hour of incubation in MRS broth (39°C) adjusted to pH 3.0 using lactic acid, following conditioning for one hour in MRS broth at pH 5.8 (NA, non-adapted), pH 4.5 (ST, acid stressed), pH 4.0 (SH, acid shocked) or pH 5.8 to 4.5 (SA, stepwise adapted) at 39°C. Results represent mean values obtained from triplicate experiments.

a)



b)



c)

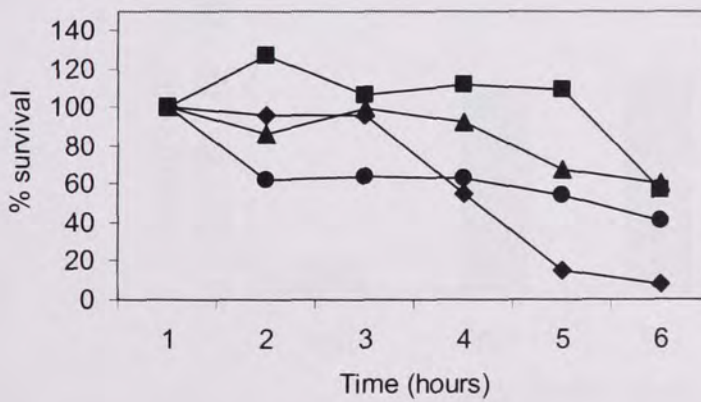
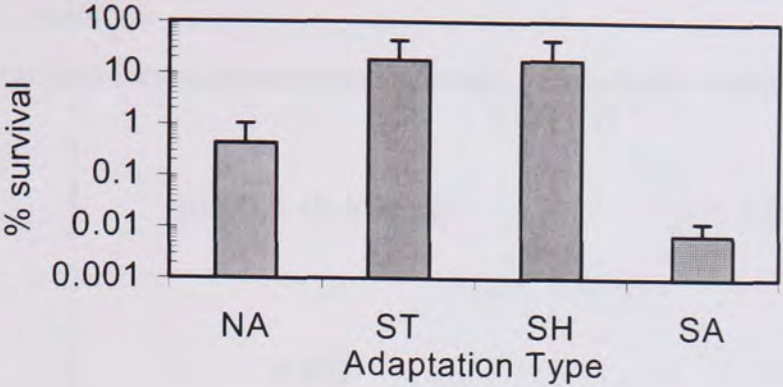
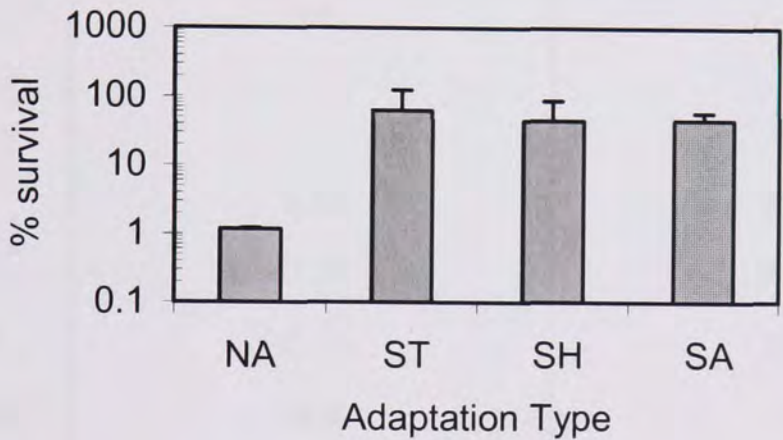


Figure 4.10. Percentage survival of *Lb. acidophilus* strain a) VUP 12,005, b) VUP 12,015 and c) *Lb. johnsonii* strain VUP 12,028 during five hours of incubation in MRS broth (39°C) adjusted to pH 3.5 using lactic acid, following conditioning for one hour in MRS broth at pH 5.8 (◆), pH 4.5 (■), pH 4.0 (▲) or pH 5.8 (●) to 4.5 at 39°C. Results represent mean values obtained from triplicate experiments.

a)



b)



c)

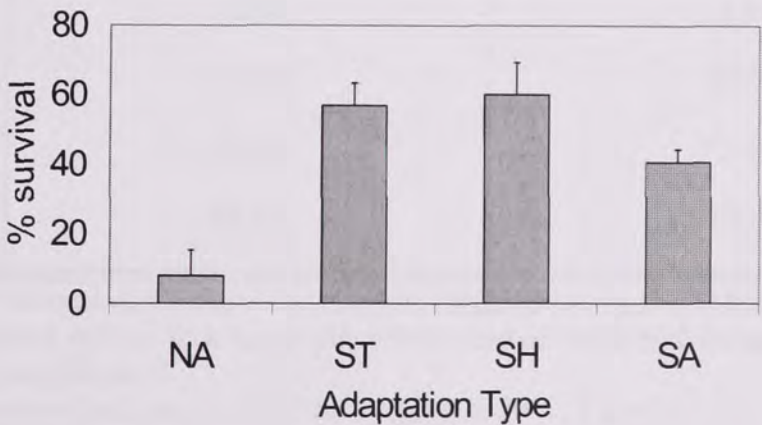


Figure 4.11. Percentage survival of *Lb. acidophilus* strain a) VUP 12,005, b) VUP 12,015 and c) *L. johnsonii* strain VUP 12,028 at five hours of incubation in MRS broth (39°C) adjusted to pH 3.5 using lactic acid, following conditioning for one hour in MRS broth at pH 5.8 (NA, non-adapted), pH 4.5 (ST, acid stressed), pH 4.0 (SH, acid shocked) or pH 5.8 to 4.5 (SA, stepwise adapted) at 39°C. Results represent mean values obtained from triplicate experiments.

Table 4.1. Summary of percentage survival following conditioning and inoculation into various pH conditions.

Strain/ conditioning protocol	Test pH ^a	
	pH 3.5 (5 hours)	pH 3.0 (1 hour)
VUP 12,005		
Non-adapted ^b	0.093	1.78
Acid stressed ^c	2.31	44
Acid shocked ^d	3.07	10.6
Stepwise adapted ^e	0.0	2 x 10 ⁻⁶
VUP 12,015		
Non-adapted ^b	1.18	23.38
Acid stressed ^c	62.88	94.46
Acid shocked ^d	45.28	52.77
Stepwise adapted ^e	46.0	5.85
VUP 12,028		
Non-adapted ^b	7.97	1.6
Acid stressed ^c	56.96	65.9
Acid shocked ^d	60.15	37.45
Stepwise adapted ^e	40.57	10.4

^a Figures represent the average of duplicate experiments. Highlighted figures indicate the best conditions for conditioning cells to their target pH, where survival has improved compared to cells that have not been conditioned.

^b Non-adapted cultures were incubated in MRS broth (pH 5.8, 39°C, 1 hour) during conditioning.

^c Acid-stressed cultures were incubated in MRS broth (pH 4.5, 39°C, 1 hour) during conditioning.

^d Acid-shocked cultures were incubated in MRS broth (pH 4.0, 39°C, 1 hour) during conditioning.

^e Stepwise-adapted cultures were incubated in MRS broth which was adjusted from pH 5.8 to 4.5 (39°C) during the one hour conditioning period.

Table 4.2. Influence on the growth pH during conditioning on the recovery of *Lb. acidophilus* VUP 12,005 during five hours of incubation in MRS broth adjusted to pH 3.5 using lactic acid.

Time (hours)	pH values ^a			
	5.8	4.5	4.0	5.8 – 4.5
0	8.46	8.55	8.46	8.52
1	8.25	8.49	8.07	7.81
2	8.04	8.42	8.13	7.48
3	7.33	7.96	8.00	5.83
4	6.05	7.74	7.80	4.39
5	5.37	6.84	7.03	0

^a Exponential phase cultures grown in MRS broth (39°C, pH 5.8) were conditioned at either pH 5.8, 4.5, 4.0 or 5.8-4.5 for one hour (39°C) prior to their incubation in fresh MRS broth (39°C) adjusted to pH 3.5 using lactic acid. Figures represent mean log₁₀ colony forming units per ml of culture. Experiments were performed in triplicate.

Table 4.3. Influence on the growth pH during conditioning on the recovery of *Lb. acidophilus* VUP 12,015 during five hours of incubation in MRS broth adjusted to pH 3.5 using lactic acid.

Time (hours)	pH values			
	5.8	4.5	4.0	5.8 – 4.5
0	8.41	8.17	8.29	8.28
1	8.36	8.26	8.25	8.23
2	8.19	8.19	8.19	8.27
3	7.97	8.09	8.18	8.26
4	7.73	8.05	8.19	8.21
5	7.12	7.95	7.98	7.94

^a Exponential phase cultures grown in MRS broth (39°C, pH 5.8) were conditioned at either pH 5.8, 4.5, 4.0 or 5.8-4.5 for one hour (39°C) prior to their incubation in fresh MRS broth (39°C) adjusted to pH 3.5 using lactic acid. Figures represent mean log₁₀ colony forming units per ml of culture. Experiments were performed in triplicate.

Table 4.4. Influence on the growth pH during conditioning on the recovery of *Lb. johnsonii* VUP 12,028 during five hours of incubation in MRS broth adjusted to pH 3.5 using lactic acid.

Time (hours)	pH values			
	5.8	4.5	4.0	5.8 – 4.5
0	8.80	8.43	8.42	8.71
1	8.77	8.50	8.34	8.49
2	8.74	8.49	8.39	8.51
3	8.53	8.52	8.44	8.50
4	8.15	8.39	8.25	8.37
5	7.83	8.17	8.17	8.33

^a Exponential phase cultures grown in MRS broth (39°C, pH 5.8) were conditioned at either pH 5.8, 4.5, 4.0 or 5.8-4.5 for one hour (39°C) prior to their incubation in fresh MRS broth (39°C) adjusted to pH 3.5 using lactic acid. Figures represent mean log₁₀ colony forming units per ml of culture. Experiments were performed in triplicate.

Table 4.5. Influence on the growth pH during conditioning on the recovery of *Lb. acidophilus* VUP 12,005 during five hours of incubation in MRS broth adjusted to pH 3.0 using lactic acid.

Time (hours)	pH values			
	5.8	4.5	4.0	5.8 – 4.5
0	8.41	8.32	8.34	8.31
1	6.65	7.92	7.35	0.69
2	5.09	5.40	5.57	0
3	4.70	4.71	4.80	0
4	4.52	4.02	4.02	0
5	2.30	3.69	4.00	0

^a Exponential phase cultures grown in MRS broth (39°C, pH 5.8) were conditioned at either pH 5.8, 4.5, 4.0 or 5.8-4.5 for one hour (39°C) prior to their incubation in fresh MRS broth (39°C) adjusted to pH 3.0 using lactic acid. Figures represent mean log₁₀ colony forming units per ml of culture. Experiments were performed in triplicate.

Table 4.6. Influence on the growth pH during conditioning on the recovery of *Lb. acidophilus* VUP 12,015 during five hours of incubation in MRS broth adjusted to pH 3.0 using lactic acid.

Time (hours)	pH values			
	5.8	4.5	4.0	5.8 – 4.5
0	8.21	8.06	8.16	8.12
1	7.66	8.04	7.88	6.89
2	5.07	7.79	7.45	6.14
3	4.40	6.96	5.84	4.27
4	4.00	4.65	5.13	4.55
5	3.69	3.63	4.67	2.84

^a Exponential phase cultures grown in MRS broth (39°C, pH 5.8) were conditioned at either pH 5.8, 4.5, 4.0 or 5.8-4.5 for one hour (39°C) prior to their incubation in fresh MRS broth (39°C) adjusted to pH 3.0 using lactic acid. Figures represent mean log₁₀ colony forming units per ml of culture. Experiments were performed in triplicate.

Table 4.7. Influence on the growth pH during conditioning on the recovery of *Lb. johnsonii* VUP 12,028 during five hours of incubation in MRS broth adjusted to pH 3.0 using lactic acid.

Time (hours)	pH values			
	5.8	4.5	4.0	5.8 – 4.5
0	8.64	8.37	8.25	8.47
1	6.81	7.53	7.18	7.19
2	4.55	5.61	5.51	5.42
3	3.30	3.82	4.66	4.07
4	2.30	3.95	4.33	3.30
5	0.69	3.28	2.77	2.29

^a Exponential phase cultures grown in MRS broth (39°C, pH 5.8) were conditioned at either pH 5.8, 4.5, 4.0 or 5.8-4.5 for one hour (39°C) prior to their incubation in fresh MRS broth (39°C) adjusted to pH 3.0 using lactic acid. Figures represent mean log₁₀ colony forming units per ml of culture. Experiments were performed in triplicate.

Cells of strain VUP 12,005 were more sensitive to a lethal challenge of pH 3.5 or 3.0 following sequential lowering of the pH from 5.8 to 4.5 during the conditioning period (stepwise adaptation) compared to cells maintained at pH 5.8 during conditioning (control cells), with counts decreasing from 8-log to less than 1-log over the incubation period (Table 4.2, 4.5).

Stepwise-adaptation was detrimental to strain VUP 12,015 following challenge at pH 3.0, with viable counts less than that of the control after one-hour of incubation (Table 4.6). In contrast, stepwise adaptation enhanced survival of strain VUP 12,015 during challenge at pH 3.5 for five hours, with a 1-log improvement in viability compared to the control (Table 4.3).

Survival of *Lb. johnsonii* strain VUP 12,028 was enhanced following stepwise adaptation and challenge at either pH 3.5 and 3.0. Survival improved 5-fold at pH 3.5 (Table 4.4) and 6-fold at pH 3.0 (Table 4.7) compared to the control culture, which represents a 0.5-log improvement in survival at both pH challenges.

4.6 DISCUSSION

4.6.1 Selection of media for growth kinetics studies

The generation time of *Lb. acidophilus* strain VUP 12,005 was compared in 10% non-fat dry milk and a complex nutrient broth formulation, MRS (pH 6.2) to determine the performance and functionality of these media for supporting growth of selected strains. In line with the results of Chou and Weimer (1999), strain VUP 12,005 grew faster in MRS broth than in 10% NFDM, suggesting that MRS broth satisfies the growth requirements of strain VUP 12,005. MRS broth is more adaptable than milk as a growth medium, as cell populations cannot be harvested easily from milk-based media without prior solubilisation or hydrolysis of the milk proteins, therefore turbidity measurements, as a measure of cell growth, become complicated. MRS broth was therefore used in later studies related to viability and growth of selected strains at different temperature and pH values.

4.6.2 Optimum growth rates at a range of pH and temperature values

The use of turbidity measurements to analyse the growth in liquid cultures, as described in Gahan *et al.* (1996), was successful for determining growth parameters of *Lb. acidophilus* VUP 12,005. Both temperature and pH of incubation had an impact on the specific growth rate of *Lb. acidophilus* VUP 12,005, which was able to grow between pH 6.5 to 4.0 but not at pH 3.5. In addition, cells were able to grow at temperatures between 30 to 45°C but not at 50°C or above. Maximum growth rates were observed between pH 5.5 to 6.0 ($\mu = 0.28$, 39°C) and 39°C ($\mu = 0.271$, pH 5.8). Strain VUP 12,005 displayed growth profiles within the published range of growth parameters (Gomes and Malcata, 1999), which state that ‘growth of *Lb. acidophilus* may occur at as high a temperature as 45°C, but optimum growth occurs within 35 to 40°C, with an optimum pH lying at 5.5 to 6.0’. These values were then used as the standard growth parameters for closely related strains of the *Lb. acidophilus* complex cultured in MRS.

4.6.3 Temperature and pH values for adaptation experiments

Moderate and lethal pH values were defined by growing cells to exponential phase in MRS broth (pH 5.8, 39°C) before transferring cells to a variety of pH environments. Exponential phase of growth was selected because this is where cells experience maximum sensitivity to acidic environments (O’Driscoll *et al.*, 1996; Lorca *et al.*, 1998). As the culture proceeds into the stationary phase, cells become more tolerant to challenging conditions (Arnold and Kaspar, 1995; Baik *et al.*, 1996; Kim *et al.*, 1999; Hartke *et al.*, 1994; Davis *et al.*, 1996).

These experiments were performed using both hydrochloric acid and lactic acid, as probiotic organisms are faced with challenges from both acidulants during passage through the gastrointestinal tract and during storage in yogurt. The results showed that *Lb. acidophilus* strain VUP 12,005 displayed similar growth traits in MRS broth, regardless of whether lactic acid or hydrochloric acid was used to lower the pH.

From the results, pH 4.5 and pH 4.0 were selected for conditioning cells prior to exposure to lethal pH at pH 3.5 or pH 3.0. pH 4.5 was defined as ‘acid stress’ and pH

4.0 was defined as 'acid shock'. Acid stress represents an environment where cells are still able to grow, but at a reduced rate, whereas for acid shock, cells are able to maintain viability but do not actively divide. pH 3.5 was selected as the lethal pH condition, as the number of viable cells was significantly reduced at this pH over the period of exposure selected for the study period. These values were used to determine whether pH-dependent changes in acid resistance simply reflected the effects of pH on growth rate. Many researchers have used a similar approach for selecting the optimum, sub-optimum and lethal conditions for studying the adaptive responses of lactic acid bacteria (Rallu *et al.*, 1996; Lorca *et al.*, 1998; Kim *et al.*, 2001).

4.6.4 Effect of lactic acid and hydrochloric acid on the survival of probiotic strains under normally lethal acidic conditions

Lb. acidophilus strains VUP 12,005 and VUP 12,015 were exposed to pH 4.5 MRS broth (adjusted with either lactic or hydrochloric acid) and the percentage survival was assessed during five hours of incubation at pH 3.5 (adjusted using lactic acid). Using lactic acid or hydrochloric acid to adjust the pH of the MRS broth to 4.5 during conditioning resulted in improved survival compared to cells held at pH 5.8 during the conditioning period (control). Although lactic acid was more efficient than hydrochloric acid at inducing a protective response, protective mechanisms were nevertheless induced by hydrochloric acid that served to protect cells against lactic acid challenge (pH 3.5). Similar findings were reported by Leyer and Johnson (1992): *Sal. typhimurium* was adapted to acid by exposure to hydrochloric acid at pH 5.8 for one to two doublings. Acid-adapted cells had increased resistance to inactivation by organic acids commonly present in cheese, including lactic, propionic and acetic acids. These results are particularly relevant when the functional nature of probiotic organisms is considered. These organisms are not only required to survive lactic acid induced reductions in pH during refrigerated storage in yogurt products, but also the low pH of the stomach (caused by hydrochloric acid) during consumption.

The observation that lactic acid was more efficient than hydrochloric acid at inducing survival mechanisms may be a reflection of the lower level of dissociation of lactic acid, which has been reported to cause a more extensive decrease in the internal pH than hydrochloric acid (O'Driscoll *et al.*, 1997). Alternatively, 'the lactic acid might

affect pH homeostasis through a mechanism which is not solely dependent upon the undissociated acid molecule or the hydrogen ion concentration' (McDonald *et al.* 1990). The latter is supported by the findings that *Sal. typhimurium* cells adapted with lactic acid showed a different protein profile compared to cells adapted with hydrochloric acid (O'Driscoll *et al.*, 1997). In addition, the optimal pH for adaptation varied from pH 5.5 for lactic acid and pH 5.0 for hydrochloric acid for *Sal. typhimurium*.

4.6.5 Determination of optimum conditioning times

To date, there have been several reports which demonstrate acquired acid tolerance of bacterial species after exposure to moderate pH levels (Leyer *et al.*, 1995; Gahan *et al.*, 1996; Foster, 1993; Foster and Hall, 1990, 1991; Davis *et al.*, 1996; O'Driscoll *et al.*, 1997; Hartke *et al.*, 1996; Kim *et al.*, 1999). The data presented here shows that *Lb. acidophilus* VUP 12,015 is also capable of acquiring acid tolerance in batch culture during exponential phase growth, when the cultures are transferred to acidified media during growth at a non-lethal acid pH.

Several reports have claimed that the effectiveness of the acid tolerance response appears to be critically dependant upon two principal factors: (i) the pH of the medium and (ii) the duration of incubation during conditioning of cells (Davis *et al.*, 1996; Lorca *et al.*, 1998b). To this end, cultures were grown to exponential phase and conditioned in MRS broth adjusted to pH 4.5 or 4.0 using lactic acid for 0, 15, 30, 60, 90 or 120 minutes prior to challenge in MRS broth at pH 3.5. Viable plate counts were performed immediately before acid challenge (elapsed time 0) and after four hours at pH 3.5. Survival improved following incubation at all incubation times tested. Following conditioning at pH 4.5, the acquisition of tolerance occurred gradually over the incubation period. Optimum tolerance at pH 3.5 was observed following conditioning for one-hour at pH 4.5 and after 30 minutes at pH 4.0. This indicates that a decrease in pH during growth contributes to a high level of acid resistance and that the incubation pH and time for conditioning cells appears to be essential for optimising acid tolerance. Since acid stress at pH 4.5 provided the best improvements in survival, a one-hour conditioning period was selected for further studies on the acquired acid tolerance response. A conditioning time of one hour has been reported for the analysis of adaptive responses of several lactic acid bacteria to

bile, sodium chloride (Kim *et al.*, 2001) and low pH (Lorca *et al.*, 1998; Laplace *et al.*, 1999).

The protective effect of conditioning cells at moderate pH is unlikely to be entirely a growth-rate-related phenomenon. Conditioning cells at pH 4.5 or 4.0 induced similar levels of acid resistance, despite a reduced growth rate at pH 4.0. These results conflict with Belli and Marquis (1991) who reported that cells grown at a faster growth rate during conditioning showed somewhat greater adaptive ability. They also reported that division of cells was essential for acquired acid tolerance. Results reported in this thesis show that survival of strain VUP 12,015 improved after 15 minutes at pH 4.5 and 4.0, which was insufficient time for cell division. These results may suggest that different mechanisms may be induced as the pH is lowered from 4.5 to 4.0. In fact, the rapid increase in acid resistance at pH 4.0 may indicate that the response is occurring at the physiological level, through the increased activity of enzymes already present in the cells. On the contrary, the more gradual acquisition of acid tolerance at pH 4.5 may indicate that major changes in cellular composition are occurring, representing a total cellular response at the genetic level.

Lactic acid was used to lower the pH in these experiments because it is directly related to loss of viability in dairy products (Hood and Zottola, 1988; Hughes and Hoover, 1991; Lourens-Hattingh and Viljoen, 2002). In addition, lactic acid was shown to have more of an effect on the cells than hydrochloric acid, since it is a weak organic acid that is not charged at low pH and can easily pass through the cell membrane in the protonated form. At cytoplasmic pH, lactic acid dissociates and thus poses a stronger threat to cells at a given extracellular pH (Cook and Russell, 1994).

Many authors have reported that lactic acid is more effective than acetic acid (McDonald *et al.*, 1990; Leyer and Johnson, 1992), propionic acid (Leyer and Johnson, 1992) or hydrochloric acid (O'Driscoll *et al.*, 1997) for lowering the internal pH of organisms, even though the undissociated concentration of the other acids may be higher. For example, the intracellular pH of *Lac. lactis* cells in suspension was slightly reduced (from 7 to 6) when the extracellular pH was reduced with HCl (from

6.75 to 5) but decreased linearly (from 7 to 5.25) with the extracellular pH when that was adjusted with lactic acid (Cook and Russell, 1994).

Like other studies (Hartke *et al.*, 1996; Lorca *et al.*, 1998a,b; Palmfeldt and Hahn-Hagerdal, 2000; Lorca and Valdez, 2001a,b), MRS broth and agar were used for conditioning and enumerating cells. It is possible that damaged cells may not have been recovered using this medium, however, it was important that the viable counts represent those populations having the greatest chance of survival during refrigerated storage in dairy products and transit through the gastrointestinal tract.

4.6.6 Comparison of acquired acid tolerance in probiotic strains

Though many organisms are able to develop acid tolerance, the degree of tolerance varies between bacterial species and strains (Arnold and Kaspar, 1995; Leyer *et al.*, 1995; Lin *et al.*, 1995). Rallu *et al.* (1996) showed that viability was significantly improved in normally lethal pH conditions for *Lac. lactis* subsp. *lactis* following acid stress, but not for *Lac. lactis* subsp. *cremoris* strains.

Exponential phase cells from probiotic strains VUP 12,005, VUP 12,015 and VUP 12,028, conditioned at a variety of pH values (4.5, 4.0) in MRS broth, show strain specific characteristics during challenge at pH 3.5 and pH 3.0. *Lb. acidophilus* VUP 12,015 showed the greatest adaptive ability at pH 3.5 after conditioning cells at pH 4.5 in lactic acid adjusted MRS broth, with a 53-fold improvement in survival. In contrast, at pH 3.0, acid stressed cells of *Lb. johnsonii* VUP 12,028 showed the greatest adaptive ability, with a 41-fold improvement in survival. The most appropriate pH for conditioning cells was dependent on the pH of the challenge. For example, for *Lb. acidophilus* strain VUP 12,005, acquired acid tolerance was greatest at pH 3.5 following acid shock (pH 4.0) and at pH 3.0 following acid stress (pH 4.5). This suggests that different mechanisms may be responsible for the acquired tolerance at pH 3.5 versus pH 3.0.

In addition to conditioning cells at pH 4.5 and pH 4.0, stepwise lowering of the pH from 5.8 to 4.5 was introduced (stepwise adaptation). Baati *et al.* (2000) reported that stepwise lowering of the temperature during conditioning was effective for enhancing the survival of bacterial cultures at -80°C . Stepwise adaptation was designed to mimic the natural stepwise decrease in pH that is more likely to be encountered by

this organism during yogurt production. Stepwise adaptation served to enhance the viability of *Lb. johnsonii* VUP 12,028 at both of the challenge pHs, although the degree of survival was not as pronounced as observed following acid stress or acid shock. For *Lb. acidophilus* VUP 12,005, stepwise adapted cells behaved similarly to the control. A higher sensitivity of the cells conditioned during stepwise adaptation before transfer to lethal pH environments may be related to a fraction of the population undergoing division, since the pH transitions during conditioning mimicked the normal growth process.

A detailed comparison of the results from adaptation studies described here with reports for other *Lactobacillus* species is difficult due to differences in the methodology used, such as exponential versus stationary phase cells (Baati *et al.*, 2000; Lorca *et al.*, 1998; Lorca and Valdez, 2001a) and different adaptive and challenge conditions (Lorca and Valdez, 1998, 2001b; Baati *et al.*, 2000; Kim *et al.*, 2001; Laplace *et al.*, 2001). However, in line with the literature, *Lb. acidophilus* VUP 12,005 and VUP 12,015 and *Lb. johnsonii* VUP 12,028 displayed the ability to adapt to normally lethal pH values (pH 3.5 or less) after first being exposed to moderately low pH values (pH 4.5 to pH 4.0).

4.7 CONCLUDING REMARKS

There are several reports which demonstrate acquired acid tolerance of food spoilage or enteric organisms after exposure to moderate pH levels (Leyer *et al.*, 1995; Gahan *et al.*, 1996; Foster, 1993; Foster and Hall, 1990, 1991; Davis *et al.*, 1996; O'Driscoll *et al.*, 1997; Hartke *et al.*, 1996; Kim *et al.*, 1999). The results in this chapter demonstrated the ability of selected strains to acquire acid tolerance to normally lethal acidic pH conditions following growth at a non-lethal acid pH. The acquired acid tolerance response was optimally induced between pH 4.5 and 4.0, using lactic acid as the acidulant. The level of acquired tolerance depended on the pH of the media during conditioning, the conditioning time and the strain being tested. The data indicates that the protective effect of conditioning cells at moderately low pH is unlikely to be entirely a growth-rate-related phenomenon, as cell division was not essential for acquired acid tolerance.

These results supported further investigation into the use of conditioning protocols as a model system to improve the survival of probiotic cultures in yogurt during long-term refrigerated storage, the results of which are presented in Chapter 6.

2.1 INTRODUCTION

It is well known that the taste and texture have a great influence on the acceptance and consumption of food. The taste and texture of food are determined by the chemical and physical properties of the food. The taste and texture of food are determined by the chemical and physical properties of the food. The taste and texture of food are determined by the chemical and physical properties of the food.

Many papers have reported the effect of the taste and texture of food on the acceptance and consumption of food. The taste and texture of food are determined by the chemical and physical properties of the food. The taste and texture of food are determined by the chemical and physical properties of the food. The taste and texture of food are determined by the chemical and physical properties of the food.

From the above reports it is evident that the taste and texture of food have a great influence on the acceptance and consumption of food. The taste and texture of food are determined by the chemical and physical properties of the food. The taste and texture of food are determined by the chemical and physical properties of the food. The taste and texture of food are determined by the chemical and physical properties of the food.

CHAPTER 5

ANALYSIS OF THE ADAPTIVE ACID TOLERANCE RESPONSE

5.1 INTRODUCTION

It is now well established that lactic acid bacteria have evolved stress-alert systems and defences against stress that permit them to resist harsh conditions and sudden environmental changes. Results reported in chapter four demonstrated that adaptive responses are induced in strains of *Lb. acidophilus* and *Lb. johnsonii* upon transfer of cells to moderately low pH conditions. These responses protected cells subsequently transferred to normally lethal pH environments.

Many papers have reported that when cells are placed under stress, such as temperature and pH extremes, there is an increase in the production of specific proteins above the level observed in optimal environments. Some of these stress-induced proteins are highly conserved across genera (Parsell *et al.*, 1994), as different organisms respond to stress in similar ways. Proteins induced during acid shock include the chaperone class proteins DnaK and GroEL (Foster, 1993; Hartke *et al.*, 1996; Broadbent *et al.*, 1997; Kim *et al.*, 1998; Oberg *et al.*, 1998; Sanders *et al.*, 1999; Jan *et al.*, 2001; Leblanc *et al.*, 2003; Prasad *et al.*, 2003), the outer membrane proteins OmpC and OmpF produced in *Sal. typhimurium* (Foster and Hall, 1990; Leyer and Johnson, 1993; Foster and Spector, 1995) and the F₀F₁ protein unit that facilitates the extrusion of protons from the cell (Foster and Hall, 1991).

There are conflicting reports as to whether *de novo* protein synthesis is necessary for adaptation to normally lethal conditions. Some authors suggest that the addition of a protein inhibitor during conditioning prevents adaptation (Foster and Hall, 1990; Davis *et al.*, 1996; Hartke *et al.*, 1996; Rallu *et al.*, 1996; O'Driscoll *et al.*, 1997; Wang and Doyle, 1998; Koga *et al.*, 1999; De Angelis *et al.*, 2001b). In contrast, others have reported that cells adapted in the presence of chloramphenicol still show improved viability relative to control cells, but the improvements are not as great as those cells that are allowed to perform protein synthesis during adaptation (Jan *et al.*,

2001; Lorca and Valdez, 2001b). This suggests that the stress responses in some organisms are not entirely dependent on *de novo* protein synthesis.

The responses induced by one stressor have also been found to either fully or partially protect cells against other forms of stress (Foster and Hall, 1990; Leyer and Johnson, 1993; Foster and Spector, 1995; Koga *et al.*, 1999; Laplace *et al.*, 1999; Kim *et al.*, 2001). Murga *et al.* (2001) reported that *Lb. acidophilus* CRL 640 was less sensitive to freeze-thawing and sodium chloride when it was grown at 25°C. Kim *et al.* (2001) reported that *Lb. acidophilus* cells pre-exposed to bile stress were significantly more resistant against heat stress, but not against sodium chloride stress, whilst cells pre-exposed to sodium chloride stress were significantly more resistant to bile and heat stress.

The general aim of this chapter was to analyse the response of *Lb. acidophilus* VUP 12,005, VUP 12,015 and *Lb. johnsonii* VUP 12,028 to moderately low pH levels.

The specific aims were to:

- a) determine whether the response to acid stress would also afford cross-protection of cells in a variety of challenge environments
- b) determine whether the adaptive acid tolerance response required *de novo* protein synthesis.

5.2 DETERMINING THE CONCENTRATION OF CHLORAMPHENICOL REQUIRED TO INHIBIT GROWTH AND PROTEIN SYNTHESIS DURING CONDITIONING PROTOCOLS

Lb. acidophilus strain VUP 12,015 was assessed for its sensitivity to various concentrations of the antibiotic chloramphenicol following conditioning for one hour in MRS broth adjusted to pH 5.8 or 4.5 (39°C) using lactic acid. Antibiotic sensitivity was tested by streaking *Lb. acidophilus* strain VUP 12,015 onto MRS agar containing 0 to 2000 µg/ml of chloramphenicol according to section 2.5.8 and observing plates for growth following incubation. Chloramphenicol, when added to the MRS agar at concentrations ≥ 20 µg/ml, was efficient at inhibiting cell division. In addition, samples that contained 200 µg/ml chloramphenicol during cell conditioning

in MRS broth at pH 5.8 and pH 4.5 demonstrated confluent growth on antibiotic-free MRS agar (Table 5.1).

In addition, 100 μCi of ^{35}S -labelled cysteine-methionine mixture was added to the MRS broth during cell conditioning and autoradiographs were prepared from SDS-PAGE gels (as described in section 2.6.2) to determine whether protein synthesis was inhibited by the presence of 200 $\mu\text{g/ml}$ chloramphenicol during cell conditioning. The data presented in Figure 5.1 demonstrates the effectiveness of 200 $\mu\text{g/ml}$ chloramphenicol for inhibition of protein synthesis during cell conditioning, with a 95% decrease in the incorporation of radiolabel in the presence of chloramphenicol (Table 5.2).

5.3 THE EFFECT OF CELL CONDITIONING ON THE SURVIVAL OF STRAIN VUP 12,015 DURING A VARIETY OF CHALLENGES

Cross-protection studies were performed to determine whether the adaptive response observed in Chapter 3 following cell conditioning at moderate pH would also protect cells in a variety of other challenge conditions. *Lb. acidophilus* VUP 12,015 was conditioned for one hour in MRS broth (39°C) adjusted to pH 4.5 or pH 5.8 (control) using lactic acid before being challenged in an assortment of conditions previously defined for *Lb. acidophilus* by Lorca and de Valdez in 1999 (refer to section 2.5.16). Conditioning cells for one hour in MRS broth adjusted to pH 4.5 using lactic acid improved survival in most challenge conditions, compared to control cells, which were incubated at pH 5.8 during this period (Figure 5.2, Table 5.3a). The greatest improvements in survival were observed following cell conditioning at pH 4.5 and challenge in MRS broth containing 3M sodium chloride for 24 hours, 20% ethanol for 20 minutes or lactic acid (pH 3.5) for five hours (Figure 5.2). A 2-log improvement in survival following these challenges was observed, compared to the control (Table 5.3a). Slight improvements in viability were observed during challenge in MRS broth held at 60°C for 30 minutes or MRS broth containing 10 mM hydrogen peroxide for one hour, with 0.78 and 0.12 log improvements, respectively (Table 5.3a). In contrast, conditioning cells at pH 4.5 was detrimental to their survival at -20°C over 24 hours, with a 26% decrease in survival compared to the control (Table 5.3a).

Cross-protection studies were repeated in the presence of the protein synthesis inhibitor, chloramphenicol (200 µg/ml), in an effort to determine whether protein synthesis was necessary for the protection of cells during challenge following conditioning. Chloramphenicol was added to exponential phase cultures in MRS broth (39°C) during the period of cell conditioning at pH 5.8 (unadapted) or pH 4.5 (adapted). Following conditioning, cell suspensions were centrifuged and cells were re-suspended in fresh MRS broth which was prepared for a variety of challenges. In general, inhibiting protein synthesis during cell conditioning had an impact on the ability of cells to survive challenge conditions (Table 5.3b). Inhibiting protein synthesis during cell conditioning resulted in a half-log decrease during challenge in MRS broth containing 3M sodium chloride or 20% ethanol compared to unadapted (control) cells (Table 5.3b). In contrast, despite the presence of growth-inhibiting levels of chloramphenicol during adaptation, cell survival improved during challenge at pH 3.5, pH 3.0, 10mM hydrogen peroxide, and 60°C compared to unadapted (control) cells (Table 5.3b). Nonetheless, allowing *de novo* protein synthesis to continue during cell conditioning provided optimal improvements in survival (Figure 5.2).

5.4 COMPARISON OF PROTEIN EXTRACTION TECHNIQUES FOR THE OPTIMIZATION OF PROTEIN PROFILING AND RADIOLABELLING EXPERIMENTS

Lb. acidophilus VUP 12,015 was grown to exponential phase in MRS broth at optimal conditions (pH 5.8, 39°C) in the presence of the radiolabel ³⁵S- labelled cysteine-methionine mixture (100 µCi). Proteins were extracted by boiling samples for five minutes or by sonication for 30 seconds (refer to section 2.6.1.1) and separated using SDS-PAGE (refer to section 2.6.1.3). Protein gels were stained using Sypro Orange (as per section 2.6.1.4) and quantified according to section 2.6.1.5. The amount of radiolabel that was incorporated into the cell was calculated according to section 2.6.2.3. The results reveal that sonication was the most efficient method for extraction of proteins, with a 160% increase in the amount of protein detected and an

Table 5.1. Effect of different concentrations of chloramphenicol (Cm) on the growth of *Lb. acidophilus* VUP 12,015 on MRS agar supplemented with 0 to 2000 µg/ml chloramphenicol.

Antibiotic Concentration (µg/ml)	^a Conditioning parameters:			
	pH 5.8 Control	pH 4.5 Lactic acid	pH 5.8 + Cm	pH 4.5 + Cm
0	+++	+++	+++	+++
2	++	++	++	++
20	-	-	-	-
200	-	-	-	-
2000	-	-	-	-

^a +++ = confluent growth; ++ = moderate growth; - = no growth. Cells were tested for antibiotic sensitivity following conditioning for one hour in MRS broth lactic acid adjusted to pH 5.8 or 4.5 (39°C) with or without the addition of 200 µg/ml chloramphenicol. Antibiotic sensitivity was tested by streaking *Lb. acidophilus* VUP 12,015 onto MRS agar containing 0 to 2000 µg/ml of chloramphenicol according to section 2.5.8).

Lane 1 2 3 4



Figure 5.1. Autoradiograph displaying the impact of chloramphenicol on ^{35}S -uptake for *Lb. acidophilus* VUP 12,015 during conditioning at pH 5.8 or pH 4.5. Cells were grown for one hour in MRS broth (39°C) at pH 5.8 in the presence (Lane 3) or absence (Lane 1) of 200 $\mu\text{g}/\text{ml}$ chloramphenicol. Alternatively, cells were conditioned in MRS broth (39°C) at pH 4.5 for one hour in the presence (Lane 4) or absence (Lane 2) of 200 $\mu\text{g}/\text{ml}$ chloramphenicol.

Table 5.2. The effect of cell conditioning and protein synthesis inhibition on the incorporation of ^{35}S radiolabel and induction of proteins of *Lb. acidophilus* strain VUP 12,015.

Cell Preparation ^a	Incorporation of Radiolabel (dpm) ^b	Percentage Incorporation ^c (%)	Standard Deviation (%)
Control	133,262	100	0
Adapted pH 4.5	45,450	31.8	14
Control + Cm	8,561	6.1	1.8
Adapted pH 4.5 + Cm	6,956	4.8	2.4

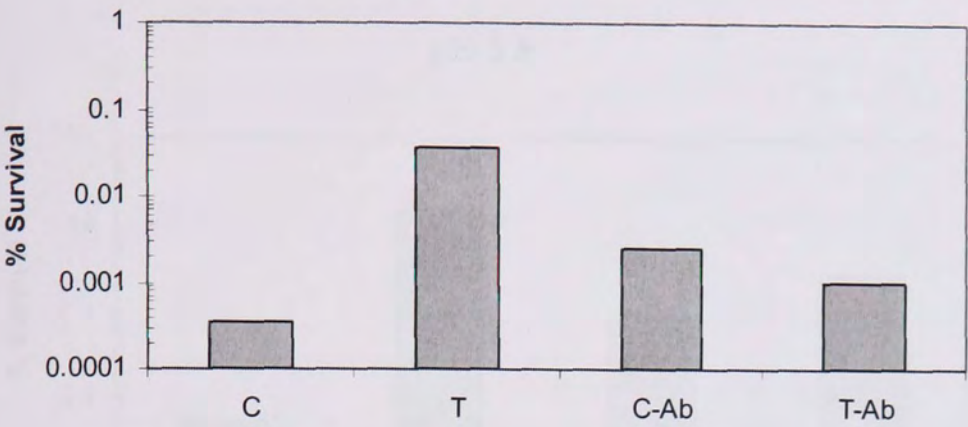
^a *Lb. acidophilus* strain VUP 12,015 was grown to exponential phase prior to conditioning at either pH 5.8 (control) or pH 4.5 (test) in the presence or absence of the protein synthesis inhibitor, chloramphenicol (200 $\mu\text{g}/\text{ml}$).

^b Incorporation of ^{35}S -cysteine-methionine radiolabel measured in disintegrations per minute (dpm). Results represent the average of quadruplicate results.

^c Percentage incorporation of ^{35}S into proteins relative to the control (refer to section 2.6.2).

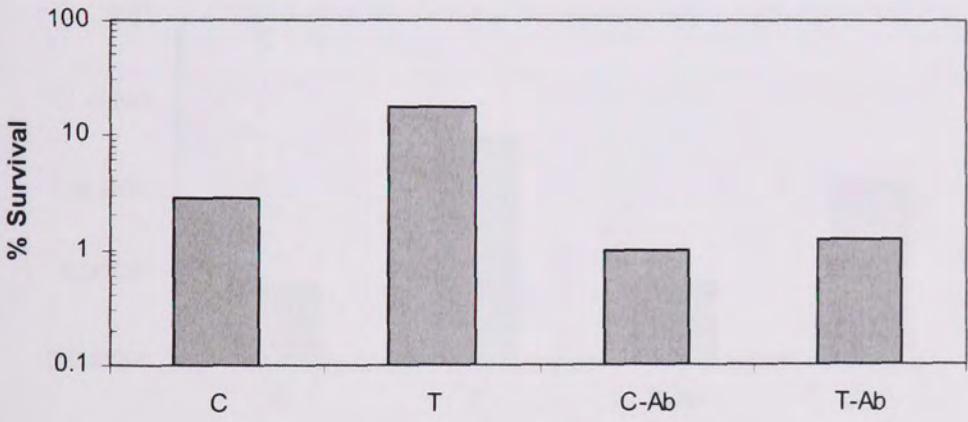
a)

3M Sodium Chloride

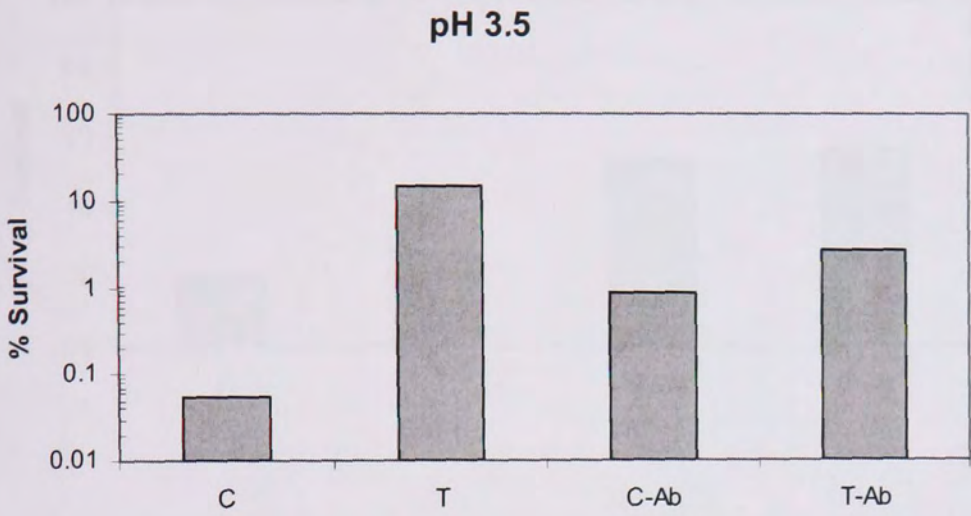


b)

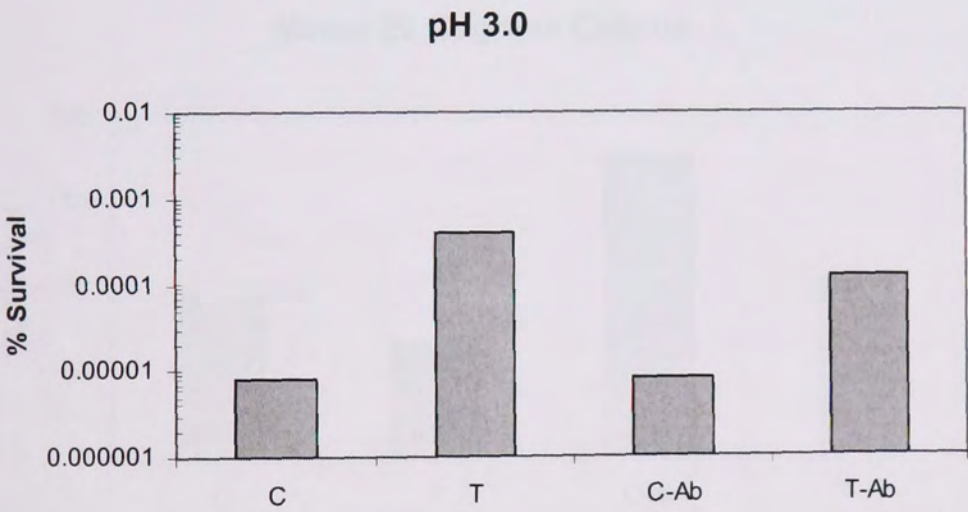
60 Degrees Celcius



c)

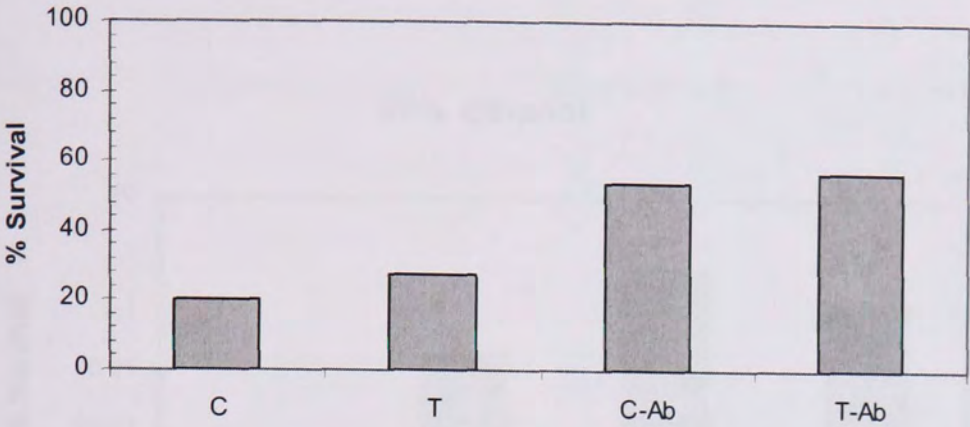


d)



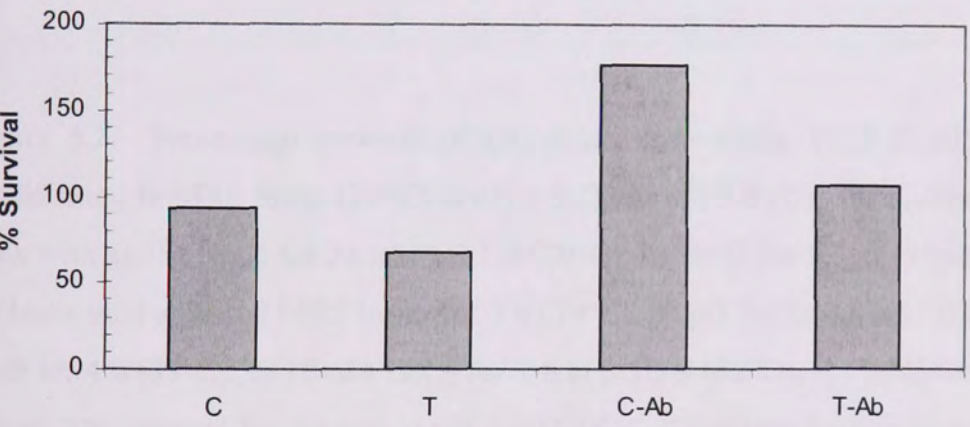
e)

10 mM Hydrogen Peroxide



f)

Minus 20 Degrees Celcius



g)

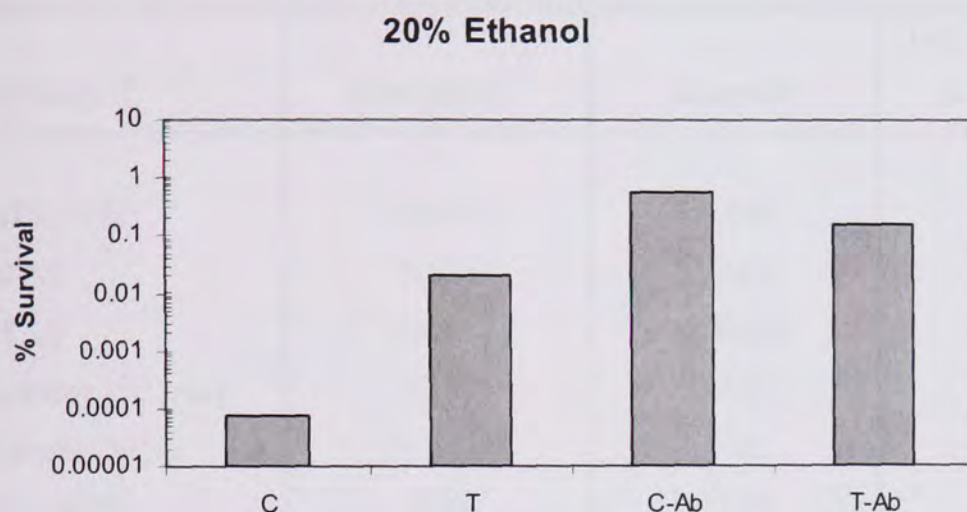


Figure 5.2. Percentage survival of *Lb. acidophilus* strain VUP 12,015 following conditioning in MRS broth (39°C) at pH 4.5 (T) or pH 5.8 (C) and challenge in MRS broth with a) 3M NaCl for 24 h at pH 5.8 (39°C); b) 60°C for 30 min at pH 5.8; c) pH 3.5 lactic acid adjusted MRS broth for 5 h (39°C); d) pH 3.0 lactic acid adjusted MRS broth for 4 h (39°C); e) 10mM H₂O₂ for 1 h at pH 5.8 (39°C); f) -20°C for 24 h at pH 5.8; g) 20% ethanol for 30 min at pH 5.8 (39°C). Conditioning was also repeated in MRS broth (39°C) in the presence of 200 µg/mL chloramphenicol at pH 5.8 (C-Ab) or 4.5 (T-Ab) prior to challenge according to section 2.5.16. Results represent the average of duplicate experiments, where the standard deviation was less than 5%.

Table 5.3. The influence of conditioning exponential phase cells in MRS broth at pH 4.5 (adapted) or at pH 5.8 (unadapted) without (a) or with (b) the presence of a protein synthesis inhibitor on the percentage survival of *Lb. acidophilus* VUP 12,015 during a variety of challenges.

a)

Challenge ^a	Unadapted ^b	Adapted ^c	Log ₁₀ difference in survival ^d
NaCl (3M)	0.00035	0.038	+ 2.04
pH 3.5	0.056	14.81	+ 2.42
pH 3.0	8 x 10 ⁻⁶	0.00039	+ 1.69
Peroxide (10 mM)	20.23	27.37	+ 0.12
Ethanol (20%)	7.6 x 10 ⁻⁵	0.02	+ 2.41
Heat (60°C)	2.85	17.47	+ 0.78
Freeze (-20°C)	93.33	67.11	0.14

b)

Challenge ^a	Unadapted ^e + Cm	Adapted ^f + Cm	Log ₁₀ difference in survival ^d
NaCl (3M)	0.00258	0.00104	0.41
pH 3.5	0.88	2.62	+ 0.47
pH 3.0	8.3 x 10 ⁻⁶	0.00012	+ 1.18
Peroxide (10 mM)	54.29	56.79	+ 0.03
Ethanol (20%)	0.55	0.16	0.54
Heat (60°C)	0.99	1.24	+ 0.09
Freeze (-20°C)	176.47	106.38	0.28

^a Challenge conditions: NaCl, 3M NaCl/24 h; pH 3.5 (lactic acid/5 h); pH 3.0 (lactic acid/4 h); Peroxide (10mM H₂O₂/1 h); Ethanol (20%/30 min); Heat (60°C/30 min); Freeze (-20°C / 24 h).

^b Unadapted cells were incubated in MRS broth at pH 5.8, 39°C prior to challenge.

^c Adapted cells were conditioned in MRS broth at pH 4.5, 39°C prior to challenge.

^d Positive values represent enhanced survival following adaptation compared to unadapted cells.

^e Adapted cells were conditioned in the presence of 200µg/mL chloramphenicol at pH 4.5, 39°C prior to challenge

^f Positive values represent enhanced survival following adaptation compared to unadapted cells.

increase of 2.3×10^4 dpm in the detection of radiolabel compared to proteins extracted by boiling (Table 5.4).

5.5 PROTEIN PROFILING FOR THE DETECTION OF PROTEIN INDUCTION DURING CELL CONDITIONING

One-dimensional SDS-PAGE was performed on *Lb. acidophilus* strains VUP 12,005, VUP 12,015 and *Lb. johnsonii* VUP 12,028 according to section 2.6.1 following cell conditioning in MRS broth adjusted to pH 5.8 (control), pH 4.5, pH 4.0 or pH 5.8 to 4.5 for one hour at 39°C (refer to section 2.5.13). This technique was selected to determine whether specific proteins were induced or repressed during acid adaptation. Twelve percent polyacrylamide gels provided protein separation, with up to 35 proteins per fingerprint, ranging from 10 to 174 kDa.

For *Lb. acidophilus* strain VUP 12,005, a 60 kDa protein was down-regulated compared to the control cells (pH 5.8, 39°C) following conditioning in lactic acid-adjusted MRS broth at pH 4.5, 4.0 or 5.8 to 4.5 for a one hour period (Figure 5.3). Similarly, lowering the pH of the MRS broth during cell conditioning of *Lb. acidophilus* strain VUP 12,015 at pH 4.5, resulted in the repression of 123 and 40.5 kDa proteins (Figure 5.4). For *Lb. johnsonii* strain VUP 12,028, the expression of proteins ranging from 10 to 175 kDa following cell conditioning was similar to that of the control cells (Figure 5.5).

5.6 ³⁵S-RADIOLABELLING OF *DE NOVO* PROTEINS DURING CELL CONDITIONING

The next step was to confirm the involvement of proteins specifically produced or repressed during conditioning at moderate pH values. A radiolabelled amino acid marker, ³⁵S-cysteine-methionine, was chosen to tag newly synthesized proteins during acid adaptation, which could be detected using X-ray films.

Lb. acidophilus strains VUP 12,005 and VUP 12,015 and *Lb. johnsonii* strain VUP 12,028 were grown in MRS broth at optimal conditions (pH 5.8, 39°C) to exponential phase prior to their collection and re-suspension in fresh MRS broth which had

Table 5.4. Comparison of protein extraction techniques on the quantity of proteins and the amount of incorporation of radiolabel used.

Protein Preparation	Band Intensity (a.u.) ^a	Incorporation of Radiolabel (dpm) ^b	Staining technique
Sonicated (30 sec)	6.65	111033	Sypro Orange
Boiled (5 minutes)	2.56	87665	Sypro Orange

^a Quantification of protein was determined by scanning gels using the LAS 1000 plus, Luminescent Image Analyser and Image Gauge 3.122 Software and expressed in arbitrary units (a.u.).

^b Incorporation of radiolabel was determined as disintegrations per minute (dpm).

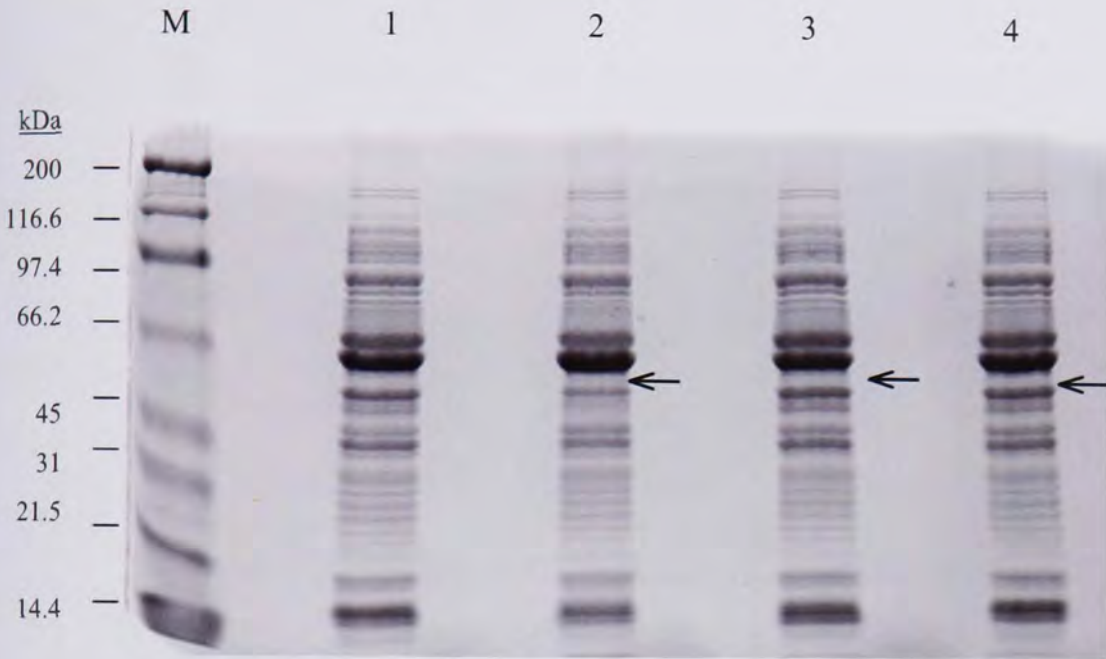


Figure 5.3. One dimensional SDS-PAGE gels from *Lb. acidophilus* VUP 12,005 following one hour of conditioning in lactic acid adjusted MRS broth (39°C) at 1) pH 5.8, 2) pH 4.5, 3) pH 4.0 or 4) pH 5.8 – 4.5. M, Broad Range Marker. Protein samples were prepared by sonication for 30 seconds (as per section 2.6.1). Equivalent amounts of protein were loaded per well. Induction or repression of specific proteins was determined by measuring the ratio of each band with the total proteins expressed. The arrows identify proteins that have been repressed during the conditioning period, compared to the control.

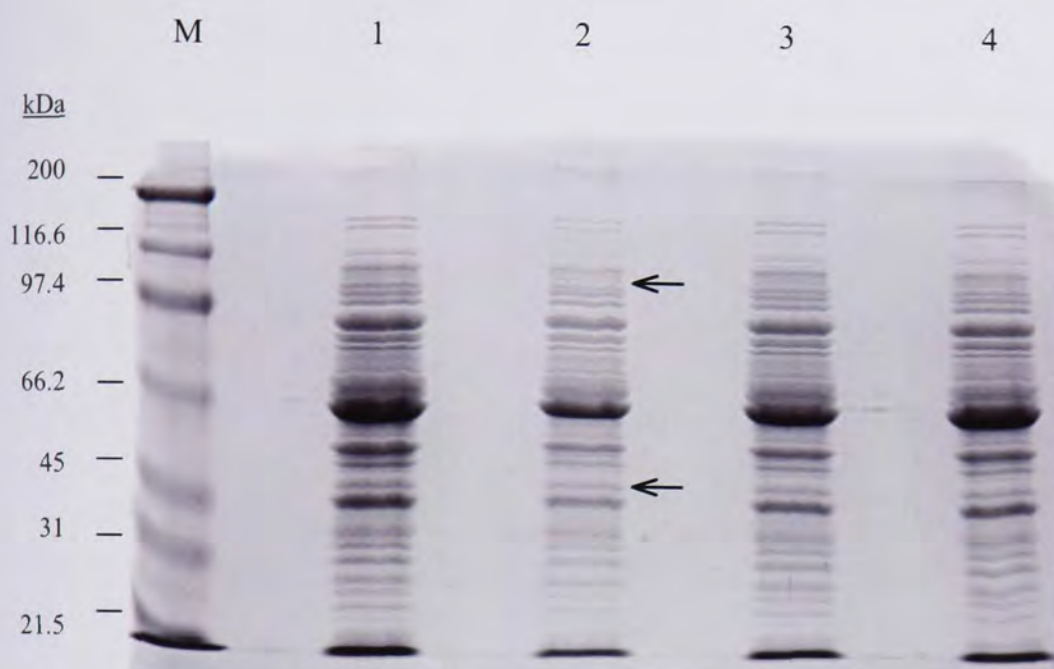


Figure 5.4. One dimensional SDS-PAGE gels from *Lb. acidophilus* VUP 12,015 following one hour of conditioning in lactic acid adjusted MRS broth (39°C) at 1) pH 5.8, 2) pH 4.5, 3) pH 4.0 or 4) pH 5.8 – 4.5. M, Broad Range Marker. Protein samples were prepared by sonication for 30 seconds (as per section 2.6.1). Equivalent amounts of protein were loaded per well. Induction or repression of specific proteins was determined by measuring the ratio of each band with the total proteins expressed. The arrows identify proteins that have been repressed during the conditioning period, compared to the control.

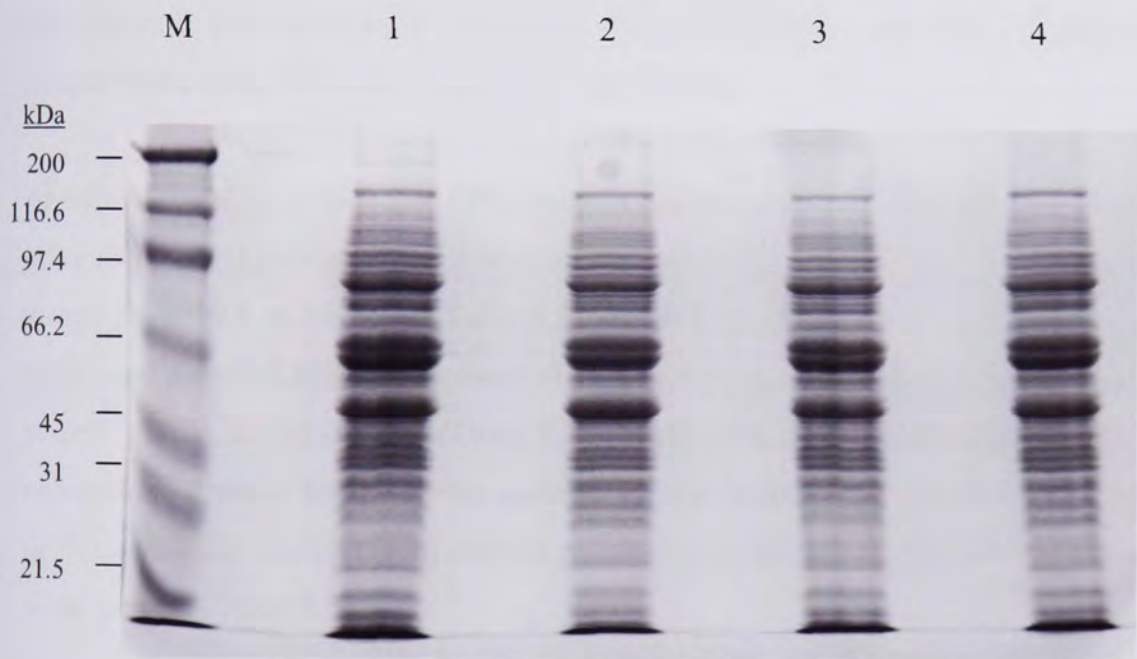


Figure 5.5. One dimensional SDS-PAGE gels from *Lb. johnsonii* VUP 12,028 following one hour of conditioning in lactic acid adjusted MRS broth (39°C) at 1) pH 5.8, 2) pH 4.5, 3) pH 4.0 or 4) pH 5.8 – 4.5. M, Broad Range Marker. Protein samples were prepared by sonication for 30 seconds (as per section 2.6.1). Equivalent amounts of protein were loaded per well. Induction or repression of specific proteins was determined by measuring the ratio of each band with the total proteins expressed.

previously been adjusted to pH 5.8 (control), pH 4.5, pH 4.0, or pH 5.8 to 4.5 using lactic acid (according to section 2.5.13). Cells were incubated for one hour at 39°C in the presence of 100 µCi of ^{35}S -labelled cysteine-methionine mixture (specific activity 1500 Ci.mmol⁻¹) and proteins were extracted *via* sonication for 30 seconds (refer to section 2.6.1.1). The total amounts of radiolabel incorporated into cells during conditioning were determined according to section 2.6.2.3 and stress induced proteins were identified by loading equivalent amounts of protein onto 12% polyacrylamide gels (according to section 2.6.1.3) followed by the development of autoradiography images using X-ray film according to section 2.6.2.4.

At optimal conditions (pH 5.8, 39°C) protein synthesis was uninhibited, with up to 28 proteins being expressed at detectable levels during this period, ranging in molecular weight from 10.8 to 174 kDa (Table 5.5, Figure 5.6). Cells that were conditioned in lactic acid adjusted MRS broth from pH 5.8 to 4.5 over the hour produced the same protein profile as the control (Table 5.5, Figure 5.6), although the overall level of expression of these proteins was reduced for *Lb. acidophilus* strains VUP 12,005 (29%), and VUP 12,015 (76%) and *Lb. johnsonii* strain VUP 12,028 (30%) compared to the control (Table 5.6).

Conditioning cells in lactic acid-adjusted MRS broth at pH 4.5 for one hour resulted in a reduction in the number and level of expression of proteins for *Lb. acidophilus* strain VUP 12,005 and *Lb. johnsonii* strain VUP 12,028 compared to the control (Figure 5.6). *Lb. acidophilus* strain VUP 12,005 produced 20 detectable proteins at pH 4.5 (Table 5.5), with 72% expression (Table 5.6), whilst *Lb. johnsonii* strain VUP 12,028 produced 19 detectable proteins (Table 5.5) at 6.54% expression compared to the control (Table 5.6). In contrast, *Lb. acidophilus* strain VUP 12,015 produced the same number of proteins as the control during conditioning at pH 4.5 (Table 5.5), albeit at a reduced level of expression (31%) (Table 5.6).

Conditioning cells in MRS broth adjusted to pH 4.0 (using lactic acid) reduced the number of proteins produced by all strains tested (Table 5.5, Figure 5.6). *Lb. acidophilus* strain VUP 12,005 produced two detectable proteins at pH 4.0 (14 kDa, 35 kDa), with 2% expression relative to the control at pH 5.8, whilst *Lb. acidophilus*

Table 5.5. Impact of cell conditioning on the number and molecular weight of proteins produced determined from ^{35}S -cysteine/methionine radiolabelling experiments.

Cell Preparation/ Strain Number ^a	Number of Proteins ^b	Molecular weight of proteins produced during cell pre-conditioning (kDa) ^c
VUP 12,005		
Control	28	165, 160, 140, 130, 120, 115, 110, 99, 92, 83, 79, 76, 66, 60, 54, 48, 45, 37, 35, 32, 28.5, 28, 25, 22, 20.5, 15, 12, 10.8
pH 4.5	20	130, 110, 99, 92, 83, 79, 76, 66, 60, 54, 48, 45, 37, 35, 32, 28.5, 28, 22, 20.5, 15, 12, 10.8
pH 4.0	2	35, 14
pH 5.8 – 4.5	26	165, 160, 140, 130, 120, 115, 110, 99, 92, 83, 79, 76, 66, 60, 54, 48, 45, 37, 35, 32, 28.5, 28, 25, 22, 20.5, 15, 12, 10.8
VUP 12,015		
Control	27	174, 165, 144, 131.5, 123, 117, 106, 98, 92.5, 86, 81, 78.5, 68.5, 58.5, 52, 47.5, 43, 40.5, 37.5, 34.8, 32.3, 28, 25.3, 24, 22, 20.8, 19
pH 4.5	27	174, 165, 144, 131.5, 123, 117, 106, 98, 92.5, 86, 81, 78.5, 68.5, 58.5, 52, 47.5, 43, 40.5, 37.5, 34.8, 32.3, 28, 25.3, 24, 22, 20.8, 19
pH 4.0	1	39
pH 5.8 – 4.5	27	174, 165, 144, 131.5, 123, 117, 106, 98, 92.5, 86, 81, 78.5, 68.5, 58.5, 52, 47.5, 43, 40.5, 37.5, 34.8, 32.3, 28, 25.3, 24, 22, 20.8, 19
VUP 12,028		
Control	27	150, 135, 127, 115, 112, 108, 105, 96, 89, 84, 78, 74, 70, 66, 59, 50, 45, 40, 38, 35, 32, 30, 28, 26.5, 25.5, 20, 18
pH 4.5	19	150, 127, 115, 112, 108, 105, 96, 70, 66, 59, 50, 45, 40, 38, 32, 30, 25.5, 20, 18
pH 4.0	11	108, 70, 66, 59, 50, 45, 38, 32, 30, 25.5, 20, 18
pH 5.8 – 4.5	27	150, 135, 127, 115, 112, 108, 105, 96, 89, 84, 78, 74, 70, 66, 59, 50, 45, 40, 38, 35, 32, 30, 28, 26.5, 25.5, 20, 18

^a *Lb. acidophilus* VUP 12,005 and VUP 12,015 and *Lb. johnsonii* VUP 12,028 were grown to exponential phase prior to conditioning at either pH 5.8 (control), pH 4.5, pH 4.0 or pH 5.8 to 4.5 for one hour at 39°C. ^b Figures represent total number of detectable proteins produced during cell conditioning. ^c Molecular weight of proteins measured against standard curve of molecular weight versus distance traveled. Figures represent the average of duplicate results.

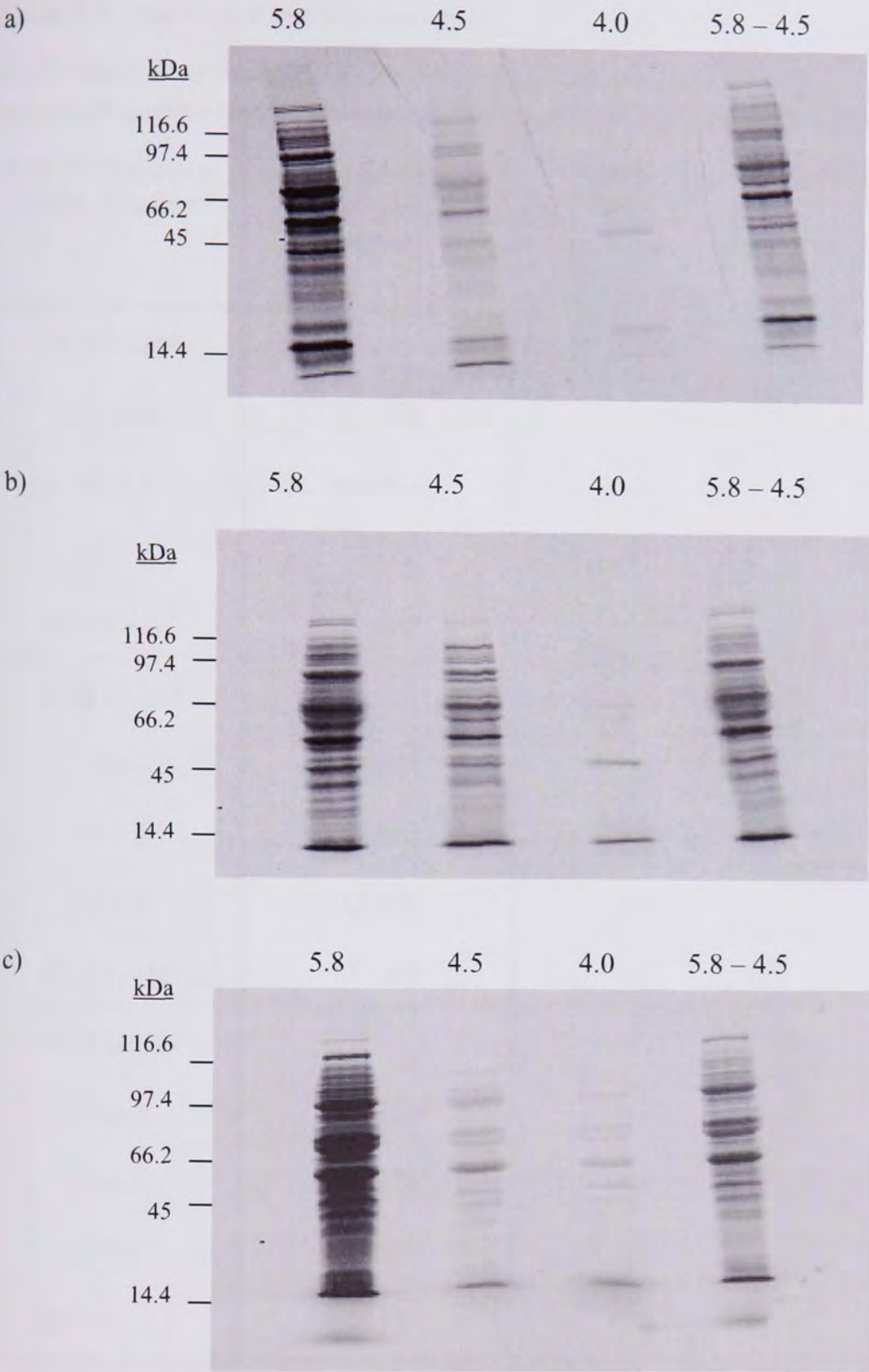


Figure 5.6. Autoradiographs displaying the uptake of ^{35}S -methionine/cysteine radiolabel during conditioning for one hour in MRS broth (39°C) at pH 5.8, 4.5, 4.0 or 5.8-4.5 for *Lb. acidophilus* strains a) VUP 12,005 and b) VUP 12,015 and c) *Lb. johnsonii* strain VUP 12,028 (refer to section 2.6.2). An equivalent amount of protein was loaded in each well.

Table 5.6. Percentage induction of protein synthesis during conditioning at a variety of pH values as determined by ^{35}S incorporation into new proteins.

Cell Preparation/ Strain Number ^a	Incorporation of Radiolabel (dpm) ^b	Percentage Induction ^c (%)	Standard Deviation ^d (%)
VUP 12,005			
Control	155,489	100	-
pH 4.5	112,511	72.4	2.51
pH 4.0	3,343	2.2	0.22
pH 5.8 – 4.5	45,807	29.5	3.61
VUP 12,015			
Control	193,078	100	-
pH 4.5	61,340	31.8	14.03
pH 4.0	12,608	6.5	0.75
pH 5.8 – 4.5	147,608	76.5	2.74
VUP 12,028			
Control	518,029	100	-
pH 4.5	33,879	6.5	1.45
pH 4.0	10,153	2.0	0.008
pH 5.8 – 4.5	156,548	30.2	2.05

^a*Lb. acidophilus* VUP 12,005 and VUP 12,015 and *Lb. johnsonii* strain VUP 12,028 were grown to exponential phase prior to conditioning at either pH 5.8 (control), pH 4.5, pH 4.0 or pH 5.8 to 4.5 for one hour at 39°C.

^b Incorporation of radiolabel was measured in disintegrations per minute (dpm); results represent the range of dpm in three separate experiments.

^c Percentage induction of protein synthesis during conditioning calculated by dividing the dpm of test sample by the control dpm

^d Standard deviation represents the deviation of percentage induction between replicates

strain VUP 12,015 and *Lb. johnsonii* strain VUP 12,028 produced one (30 kDa) and eleven (18, 20, 25.5, 30, 32, 45, 50, 59, 66, 70, 108 kDa) detectable proteins respectively, corresponding to a 6.5% and 1.9% expression compared to the control (Figure 5.6, Table 5.6).

5.7 DISCUSSION

5.7.1 Inducing cross-protection following cell conditioning

Cross-protection has been observed in a number of species including *List. monocytogenes* (Foster and Spector, 1995; Jorgensen *et al.*, 1995; O'Driscoll *et al.*, 1996; Koga *et al.*, 1999; Begley *et al.*, 2002), *Sal. typhimurium* (Leyer and Johnson, 1993), *Lac. lactis* (Hartke *et al.*, 1994, 1996; Duwat *et al.*, 1997), *E. coli* (Wang and Doyle, 1998), *Lb. rhamnosus* (Prasad *et al.*, 2003) *Lb. johnsonii* (Walker *et al.*, 1999) and *Lb. collonoides* (Laplace *et al.*, 1999).

Although stationary phase cells are naturally tolerant to a variety of challenges (cross-protection), logarithmic phase cells often require an adaptive step to improve their survival (Hartke *et al.*, 1994, 1995, 1996, Lorca *et al.*, 1998b; Kim *et al.*, 2001; Lorca and de Valdez, 1999, 2001b; Davis *et al.*, 1996; O'Driscoll *et al.*, 1996).

Lorca and Valdez (2001b), reported that *Lb. acidophilus* cells cultured without pH control, where the final pH was pH 4.5 (~10 hours), were more resistant to a challenge at pH 3.0 for one hour compared to cells cultured at a controlled pH (pH 6.0). In addition, these cells were more resistant to freezing at -20°C for 24 hours, 20% v/v ethanol during 30 minutes, 3M NaCl for 24 hours, 10 mM hydrogen peroxide for 60 minutes and heating at 60°C for 60 minutes. Thus, the transition of cells from exponential to stationary phase induced tolerance to a variety of challenges. Results reported in this chapter demonstrate that tolerance to these challenges can also be induced in *Lb. acidophilus* during exponential phase by culturing cells for a doubling time at pH 4.5 prior to challenge.

Conditioning exponential phase cells to lactic acid in MRS broth at pH 4.5 was essential to their survival during challenge, since incubating cells at pH 5.8 failed to protect cells during these challenges. This pre-requisite for activation by protons

demonstrates that the induction of tolerance in growing cells is different from that of stationary-phase cells, which need no activation by acid pH (Lorca and Valdez, 2001).

Kim *et al.* (2001) also reported that conditioning exponential-phase cells of *Lb. acidophilus* strain LA1-1 using one type of stress could enhance the survival during another type of stress. Cells that were pre-exposed to 0.05% bile stress for one hour were more tolerant to 0.5% bile and incubation at 60°C, but not against 18% w/v sodium chloride compared to cultures grown in bile-free media before challenge. In addition, cells that were pre-exposed to 2% sodium chloride for one hour were slightly more tolerant to 18% sodium chloride and significantly more tolerant to challenge with 0.5% bile and incubation at 60°C than cells grown in sodium chloride-free media. In contrast, pre-exposing cells to 53°C for 30 minutes enhanced their survival at 60°C, but not during challenge with sodium chloride or bile salts.

These findings have relevance for cells in environments where they can be exposed to combinations of stress conditions. Probiotic cultures, such as *Lb. acidophilus*, are subjected to a variety of challenges during production and storage of yogurt. These include hydrogen peroxide, low pH, absence of nutrients, unfavourable osmotic conditions, and temperature variations between fermentation and storage (Vinderola *et al.*, 2000).

5.7.2 Influence of protein synthesis on cross-protection

To investigate whether protein synthesis was required for the acquisition of tolerance during challenge, chloramphenicol (200 µg/ml) was included in the growth medium during the exposure to sub-lethal acid conditions (pH 4.5). This concentration of chloramphenicol was sufficient to inhibit protein synthesis and cause cessation of growth, but not cell death, consistent with the bacteriostatic properties of chloramphenicol, since confluent growth was observed for cells exposed to 200 µg/ml and then re-inoculated onto antibiotic-free MRS medium. Also, radiolabelling chloramphenicol-treated cells using ³⁵S-labelled cysteine-methionine mixture and subsequent SDS-PAGE analysis of labelled proteins revealed that chloramphenicol treated cells exhibited minimal labelling of proteins. When this protein synthesis inhibitor was included in the growth medium during conditioning, the protective

effect conferred during 3M NaCl and 20% ethanol challenges was lost, with a half-log decrease in viable counts during these challenge conditions compared to the control cells (Table 5.3).

The requirement for protein synthesis during the induction process has also been reported for *List. monocytogenes* (Davis *et al.*, 1996; O'Driscoll *et al.*, 1996), *Lac. lactis* (Rallu *et al.*, 1996), *Str. faecalis* (Kobayashi *et al.*, 1986), *Sal. typhimurium* (Foster, 1991, 1993; Foster and Hall, 1990), *E. coli* (O'Hara and Glenn, 1994; Wang and Doyle, 1998), *A. hydrophilia* (Karem *et al.*, 1994) and various oral lactic acid bacteria (Ma *et al.*, 1997). For example, Wang and Doyle (1998) found that *E. coli* cells that underwent heat shock at 48°C for 10 minutes survived pH 2.5 in minimal glucose medium at rates 10 to 100 times greater than untreated cells. This process required the synthesis of proteins, since the addition of chloramphenicol inhibited survival.

In contrast, despite the presence of chloramphenicol during adaptation, cell survival improved during challenge at pH 3.5, pH 3.0, 10mM hydrogen peroxide and 60°C following exposure to sub-lethal acid conditions, compared to the control. Similar observations have been reported for other organisms including *Ps. freudenreichii* (Jan *et al.*, 2001), *Lb. acidophilus* (Lorca and de Valdez, 2001b), *Lac. lactis* (Hartke *et al.*, 1996; Rallu *et al.*, 1996) and *S. enteritidis* (Humphrey *et al.*, 1993). This suggests that there are alternative mechanisms, which are independent of *de novo* protein synthesis, that protect the cell during challenge. Perhaps there is a change in the membrane lipid profile or an increase in the enzyme activity of proteins that are already present in the cell? Lorca and de Valdez (2001) reported that exposing *Lb. acidophilus* CRL 639 to pH 4.2, in the presence of chloramphenicol, served to protect cells subsequently challenged at pH 3.0. To evaluate if this homeostatic response was mediated by the membrane bound F_1F_0 ATPase, an F_0F_1 ATPase inhibitor, N,N'-dicyclohexylcarbodiimide (DCCD) was added to the medium. The addition of DCCD to the medium during exposure at a sub-lethal pH of 4.2 prevented an acid tolerance response.

Humphrey *et al.* (1993) reported that 'the importance of the protein synthesis dependent and independent processes is governed by the challenge pH following

habituation'. There were no differences in acid resistance between cells of *Sal. enteritidis* habituated at pH 4 in either the presence or absence of chloramphenicol and challenged at pH 2.8. However, cells challenged at pH values below 2.8 in the presence of a protein synthesis inhibitor showed progressively less acid resistance as the pH of the challenge broth was lowered. At pH 2.5, such cells were significantly more acid-sensitive than organisms where protein synthesis had not been inhibited, although they still survived better than control cultures grown at pH 7.0. Therefore, a direct relationship exists between protein synthesis dependency and the intensity of the challenge following conditioning in the presence of chloramphenicol.

Differences observed in protein synthesis dependency may also be a reflection of the stage that the protein synthesis inhibitor was added to the culture. Adding chloramphenicol after shifting *E. coli* to an alkaline pH indicates that full acid sensitivity induction depends on protein synthesis for the first 3 to 5 minutes, after this though, the response did not seem to be dependent on protein synthesis (Rowbury *et al.*, 1993). Therefore induction for the first three to five minutes was sufficient to protect cells with the existing complement of proteins.

In addition, adding inhibitory concentrations of chloramphenicol to the medium prior to challenge at -20°C and 20% ethanol, increased survival rates of *Lb. acidophilus* VUP 12,015, without the requirement for adaptation at a sub-lethal pH. The lower survival rates of cells grown in a chloramphenicol-free environment may be a result of a higher fraction of cells undergoing cell division, rendering them more sensitive to challenge in MRS medium at -20°C or containing 20% ethanol.

The observation that cells exposed to one form of stress can adapt to a variety of other forms of stress suggests that there is an overlap between the control circuits regulating responses to these stresses. In 1996, Hartke *et al.* reported that nine of the 33 proteins that were induced at low pH by *Lac. lactis* were also induced by heat shock and in 1997, Duwat *et al.* reported homology between genes responsible for thermoresistance in *Lac. lactis* with those involved in acid resistance (Duwat *et al.*, 1997). These results suggest a link between the heat shock response and acid resistance of *Lac. lactis*.

5.7.3 Protein profiling for the detection of protein induction during cell conditioning

One-dimensional SDS-PAGE was performed on exponential phase cells of *Lb. acidophilus* strains VUP 12,005, VUP 12,015 and *Lb. johnsonii* strain VUP 12,028 following conditioning in MRS broth adjusted with lactic acid to pH 4.5, pH 4.0 or pH 5.8 to 4.5 and incubated for one hour at 39°C to determine whether specific proteins were induced or repressed during conditioning.

Induction or repression of proteins following conditioning was determined by comparing the proportions of proteins at each pH relative to the control cells, which were incubated at pH 5.8 during the conditioning period. Preliminary data suggests that for *Lb. acidophilus* VUP 12,005, a 60 kDa protein was repressed at all conditioning pHs compared to cells incubated at pH 5.8 during conditioning (control). For *Lb. acidophilus* VUP 12,015, 123 and 40.5 kDa proteins were repressed following conditioning at pH 4.5 compared to the control. N-terminal sequencing of these proteins could determine their homology with previously identified stress proteins, which would be the next step in deducing their function. 1-D gel electrophoresis using the Protean mini-gel system did not provide sufficient separation of the complex protein profiles. Therefore, alternative techniques such as 2-D gel electrophoresis are necessary to provide a clearer picture of protein expression during the conditioning period.

Many authors have reported the induction of 60 to 70 kDa proteins by lactic acid bacteria in response to changes in the pH or temperature of the culture medium, including *Lac. lactis* (Hartke *et al.*, 1996; Champomier-Verges *et al.*, 2002), *Lb. bulgaricus* (Champomier-Verges *et al.*, 2002), *Lb. helveticus* (Oberg *et al.* 1998; Broadbent *et al.* 1998) and *Lb. rhamnosus* (Prasad *et al.*, 2003). These proteins, induced non-specifically by several stresses, have been found to be homologs of the highly conserved chaperone proteins GroEL and DnaK, which are involved in DNA or protein repair (Champomier-Verges *et al.*, 2002). Other proteins found to contribute to the acid tolerance response in lactic acid bacteria include the ATPase (Hartke *et al.*, 1996; Champomier-Verges *et al.*, 2002; Sakamoto *et al.*, 2002) and

GrpE (De Angelis *et al.*, 2001a). The role of these proteins, if any, during acid adaptation in the strains studied here is yet to be determined.

Using ^{35}S -labelled cysteine-methionine mixture to tag newly synthesized proteins was an effective method for tracking the overall expression of proteins during conditioning at low pH. At optimal conditions (pH 5.8, 39°C) up to 28 proteins were expressed over the ensuing hour, ranging in molecular weight from 10.8 to 174 kDa. Cells that underwent conditioning in MRS broth at pH 5.8-4.5 produced similar numbers of proteins to cells incubated at pH 5.8 (control) during this period. The results presented in Chapter 4 demonstrated that conditioning cells at pH 5.8 to 4.5 was not as effective at improving the viability of cells during pH challenge as was subjecting cells to acid stress (pH 4.5) or acid shock (pH 4.0) (refer to table 4.3), possibly because cells behaved similarly to the controls in relation to protein synthesis.

The induction or repression of proteins reported in this thesis was analysed following conditioning in MRS broth for one hour at 39°C. Foster (1993) found that following acid shock at pH 4.3, most of the acid shock induced proteins reached maximum levels by 20 minutes and remained at this level without change even after 60 minutes. However, 14 proteins were only transiently induced, disappearing after 20 minutes of acid shock. Jan *et al.* (2001) also observed that acid induced proteins have either an early, transient, late or permanent nature. Therefore, the analysis of protein expression may have excluded proteins that are transiently produced, and disappear before the end of the conditioning period. If time permitted it may have been of value to observe protein induction or repression over several time-points during the one-hour induction period to determine whether any proteins are transiently produced.

In general, as the conditioning pH decreased, so too did the rate of growth and the number of proteins synthesized during the conditioning period. Conditioning cells at pH 4.5 or 4.0 was dominated by the repression of a large number of the proteins compared to the control cells. Similar findings were reported by Heyde and Portalier (1990), who observed that in *E. coli*, protein synthesis decreased abruptly after a drastic acid shift. The reduction in protein synthesis reported in this thesis coincided

with a decrease in growth rates (μ) following a downshift from pH 5.8 (1.15 h^{-1}) to pH 4.5 (0.38 h^{-1}) or pH 4.0 (0.16 h^{-1}) for strain VUP 12,005 (section 4.2.3). The higher sensitivity of control cells compared to cells conditioned at lower pH values may, in part, be a result of a higher percentage of cells undergoing cell division, rendering cells more sensitive to challenge. Lorca and de Valdez (1999) reported that there is some evidence that the sensitivity of cells to cold shock increases during cell division, thus the higher sensitivity of the cells grown at optimal conditions might be related to a fraction of the population undergoing division.

In addition, cells may protect themselves at low pH by using physiologic processes that form part of the pH homeostasis system. Quivey *et al.* (1995) and Ma *et al.* (1997), reported that *Str. mutans* strain GS-5 cells adapted at pH 5.0 for one doubling time showed reduced permeability to protons compared to unadapted cells. This excretion of protons has been shown by Bender *et al.* (1986) to be related directly to levels of F^+ -ATPase activity.

The proton-translocating ATPase was also found to play an important role in acid tolerance response of *Sal. typhimurium* (Foster and Hall, 1990; Foster, 1991, 1993), *Ent. hirae* and *Str. faecalis* (Kobayashi *et al.*, 1984, 1986; Arikado *et al.*, 1999). Mutants that lack ATPase activity were acid sensitive (Suzuki *et al.*, 1988; Foster and Hall, 1991; Hutkins and Nannen, 1993; Yokota *et al.*, 1995; Yamamoto *et al.*, 1996; Tourdot-Marechal *et al.*, 1999). Perhaps a protein is induced during adaptation that interacts with pre-existing H^+ -translocating ATPase complexes, rendering them more active or more stable under extremely acidic conditions.

The role of ATPase in the acid tolerance response was also demonstrated for *Lb. acidophilus*. Using differential display, Kullen and Klaenhammer reported the induction of a cDNA product which showed sequence similarity to bacterial *atpBEF* genes of the *atp* operon, whose genes encode the various subunits of the F_1F_0 -ATPase. An increase in *atp* mRNA induced by low pH was accompanied by an increase in the activity of the ATPase enzyme in membrane extracts. The similarity to streptococcal ATPases and the acid inducibility of this operon suggest that it may function in the ATP-dependent extrusion of protons and maintenance of cytoplasmic pH (Kullen and Klaenhammer, 1999). Further studies are required to determine the

role of the proton translocating ATPase during conditioning of *Lb. acidophilus* strain VUP 12,005 and VUP 12,015 and *Lb. johnsonii* strain VUP 12,028 at moderate pH.

5.8 CONCLUDING REMARKS

There are several reports which demonstrate the ability of stress responses from one stressor to protect cells during exposure to other forms of stress (Foster and Hall, 1990; Leyer and Johnson, 1993; Foster and Spector, 1995; Koga *et al.*, 1999; Laplace *et al.*, 1999; Kim *et al.*, 2001). The results in this chapter show that tolerance to a variety of challenges can also be induced in *Lb. acidophilus* during exponential phase by culturing cells for a doubling time at pH 4.5 prior to challenge. These findings have relevance for cells in environments where they can be exposed to combinations of stress conditions, such as those found during the production and storage of yogurt.

There are conflicting reports as to whether *de novo* protein synthesis is necessary for adaptation to normally inhospitable environments. The acquired tolerance of *Lb. acidophilus* strain VUP 12,015, conditioned at pH 4.5 and subsequently challenged in 3M NaCl or 20% ethanol, was protein synthesis dependent. In contrast, despite the presence of chloramphenicol during adaptation, cell survival improved during challenge at pH 3.5, pH 3.0, 10mM hydrogen peroxide and 60°C compared to control cells following conditioning at pH 4.5. Nonetheless, allowing *de novo* protein synthesis to continue during cell conditioning provided optimal improvements in survival. This suggests that *de novo* protein synthesis contributes to cross-resistance; however, other mechanisms such as the pH homeostasis system may also play a part in acquired tolerance responses.

Many papers have reported that when cells are placed under stress, there is an increase in the production of certain proteins that are produced over the level of cells grown at optimal conditions. The results here showed that, in general, as the pH decreased, so too did the number of proteins produced. 123 kDa, 60 kDa and 40.5 kDa proteins were down-regulated compared to cells grown in optimal conditions during conditioning. Sequencing of these proteins to determine their homology with previously identified stress proteins would be the next step in deducing their function, although their induction does not necessarily ascertain their function in the acid stress response.

CHAPTER 6

VIABILITY OF PROBIOTIC CULTURES DURING LONG-TERM STORAGE FOLLOWING CONDITIONING

6.1 INTRODUCTION

To maintain confidence in probiotic products, it is important to demonstrate survival of the bacteria during their shelf life. Studies which analysed the survival of *Lb. acidophilus* in a variety of yogurt products have shown that *Lb. acidophilus* is unstable in yogurt and exhibited a constant decline in the viable count during storage, particularly after 20 days (Hull *et al.*, 1984; Shah *et al.*, 1995; Dave and Shah, 1997c; Micanel, *et al.*, 1997; Rybka and Fleet 1997). Vinderola *et al.* (2000) and Lourens and Viljoen (2002) reported that after four weeks of refrigerated storage, all products contained lower than the acceptable level of 10^6 cfu/g of viable *Lb. acidophilus*. Other studies have found that viability of *Lb. acidophilus* in yogurt is a strain dependant characteristic (Nighswonger *et al.*, 1996; Rybka and Fleet, 1997; Schillinger, 1999), with some strains of *Lb. acidophilus* surviving at the recommended quantity of 10^6 cfu/g of yogurt over the storage period (Oliveira *et al.*, 2002). Rybka and Fleet (1997) studied the viability of *Lb. acidophilus* in 50 commercial Australian yogurts and found that only 24% of samples contained $>10^6$ cfu/g of *Lb. acidophilus*.

Variations in survival of *Lb. acidophilus* between studies may be attributed to differences in the culture medium and conditions used, or differences in the starter cultures selected for yogurt production, as these may produce bacteriocins against the *Lb. acidophilus* strains (Young and Nelson, 1978; Gilliland and Lara, 1988; Kurmann and Rasic, 1991; Costello, 1993).

The differential enumeration of *Lb. acidophilus* in yogurt is difficult owing to the presence of biochemically and physiologically similar microbes, used traditionally in yogurt manufacturing, namely, *Lb. bulgaricus* and *Str. thermophilus*. Selective media containing ingredients such as bile acids and antibiotics (Calicchia *et al.*, 1993) have been

used for selective enumeration of probiotic and yogurt organisms, but the organisms are restricted in this environment and the counts may not be representative of that in the product (Lankaputhra *et al.*, 1996). A number of differential plating methodologies have been developed which seek to detect and enumerate these bacterial groups selectively in bio-products. Differences in oxygen tolerance, nutritional requirements, antibiotic susceptibility and colony morphology constitute the bases of differentiation using these methods (Charteris *et al.*, 1997).

Studies have shown that exposing bacterial cells to moderate pH values during exponential phase of growth can improve the survival of organisms during storage of dairy products (Gahan *et al.*, 1996). The viability of *List. monocytogenes* was enhanced in acidified dairy products, including cottage cheese, yogurt and whole-fat cheddar cheese. Acid adapted *List. monocytogenes* cultures also demonstrated increased survival during active milk fermentation by a lactic acid culture and greatly improved survival in low-pH foods such as orange juice and salad dressing containing acids other than lactic acid (Gahan *et al.*, 1996).

Leyer *et al.* (1995) showed that *E. coli* O157:H7 can be adapted to low pH by culturing for one to two doublings at pH 5.0. This process increased the resistance to lactic acid and these cells survived better than their non-adapted counterparts during sausage fermentation and show enhanced survival in shredded dry salami (pH 5) and apple cider (pH 3.4). Leyer and Johnson (1992) found that acid adapted cells of *Sal. typhimurium* survived better than non-adapted cells during milk fermentation by a lactic acid culture. Acid adapted cells also showed enhanced survival over a period of two months in cheddar, Swiss and mozzarella cheeses kept at 5°C.

The general aim of this chapter of research was to determine whether exposure of probiotic cultures to moderate pH values prior to yogurt production would afford protection of the organism in yogurt during storage. The specific aims were to:

- a) determine the most appropriate media for selective enumeration of probiotic and starter cultures,

- b) determine whether exposure of cultures to moderate pH conditions will improve its survival in a yogurt-like environment and during full-scale yogurt production and,
- c) determine whether yogurt starter cultures have inhibitory activity against probiotic cultures.

6.2 SURVIVAL OF PROBIOTIC STRAINS IN A YOGURT-LIKE ENVIRONMENT DURING REFRIGERATED STORAGE FOLLOWING CONDITIONING OF CELLS

6.2.1 Conditioning using lactic acid

Lb. acidophilus VUP 12,005 and VUP 12,015 and *Lb. johnsonii* VUP 12,028 were conditioned in MRS broth (39°C) lactic acid-adjusted to pH 5.0, 4.5 or 4.0 for one hour according to section 2.5.13. Following conditioning, the cells were inoculated at a rate of 2% into 1L Applicon fermentors containing 10% NFDM (43°C), which were programmed to lower the pH from 6.0 to 4.5 over a 3.5 hour period using lactic acid to simulate yogurt production. Percentage survival relative to initial counts was calculated to determine whether conditioning cells would result in protection during long-term storage compared to the control, which was incubated at pH 5.8 during the conditioning period.

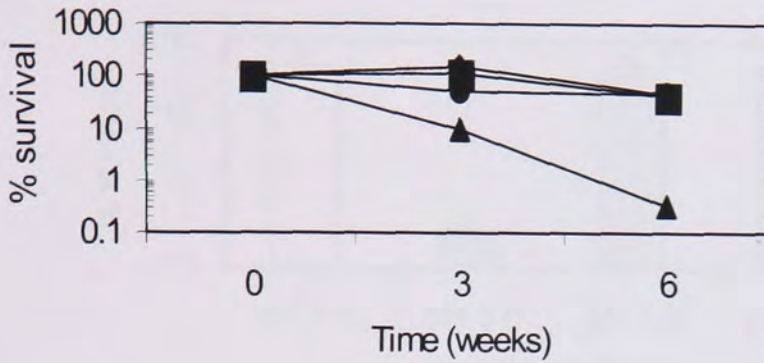
Results showed that survival following exposure to moderate pH levels (pH 5.0, 4.5, 4.0) was strain dependent (Table 6.1, Figure 6.1). Conditioning *Lb. acidophilus* VUP 12,005 at pH 4.0 or 4.5 for one hour resulted in cell division during the first three weeks of refrigerated storage in yogurt, with an 8% and 55% increase in cell viability at week three, respectively, compared to a 47% decrease in viability for the control cells. Between weeks three and six, cells that were conditioned at either pH 4.5 (46%) or 4.0 (42%) behaved similarly to the control (47%) (Figure 6.2), whereas, cells exposed to pH 5.0 during the conditioning period decreased to less than 1% of the original cell count, with 3×10^{-1} % survival.

Table 6.1. Summary of the percentage survival and viable counts of strain *Lb. acidophilus* strain a) VUP 12,005, b) VUP 12,015 and c) *Lb. johnsonii* strain VUP 12,028 at 6 weeks of refrigerated storage relative to initial cell numbers (week 0) following conditioning in lactic acid adjusted MRS broth (39°C) at pH 5.8 (control), pH 5.0, 4.5, or 4.0 and subsequent inoculation into milk prior to yogurt simulation or small-scale yogurt production (according to section 2.5.5).

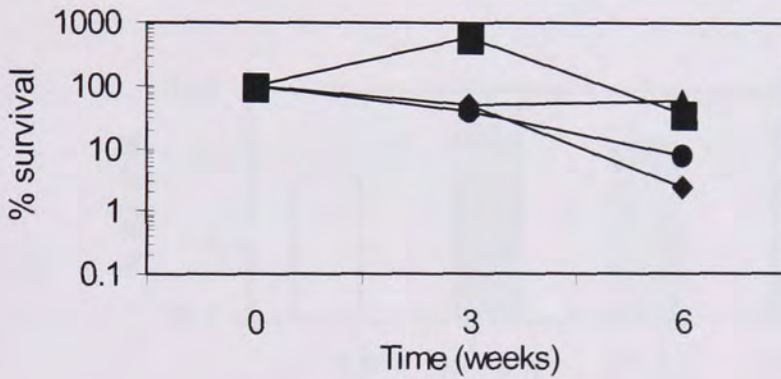
Strain tested / conditioning pH	Yogurt simulation trials		Full yogurt trials	
	% Survival	log ₁₀	% Survival	log ₁₀
VUP 12,005^a				
Control (pH 5.8)	47.23	8.14	17.44	7.49
pH 5.0	0.33	5.52	9.46	7.02
pH 4.5	45.73	7.52	5.36	6.84
pH 4.0	42.07	7.95	1.56	6.46
VUP 12,015^a				
Control (pH 5.8)	7.42	6.88	16.42	7.22
pH 5.0	55.21	8.49	2.46	6.77
pH 4.5	20.39	6.01	3.44	5.92
pH 4.0	36.39	7.39	14.89	7.34
VUP 12,028^a				
Control (pH 5.8)	28.02	7.48	82.41	8.03
pH 5.0	110.1	7.43	0.65	6.0
pH 4.5	117.08	7.66	1.31	6.0
pH 4.0	8.59	6.41	25	7.0

^aFigures represent the average of results from separate experiments. Highlighted figures indicate the best parameters for conditioning cells to their target pH, where survival improved compared to cells that have not been conditioned.

a)



b)



c)

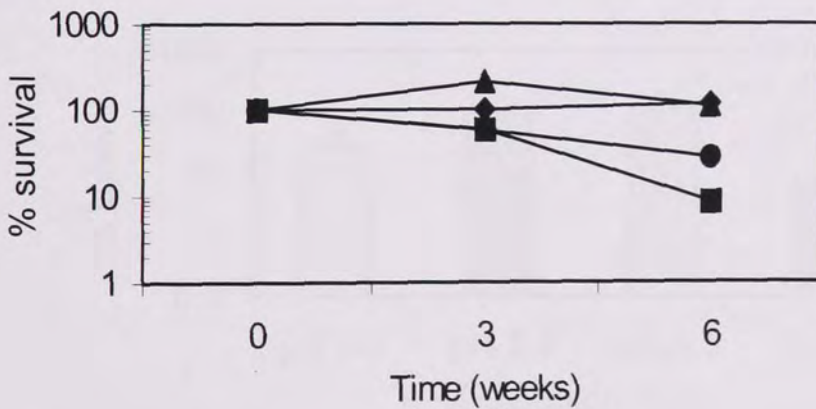
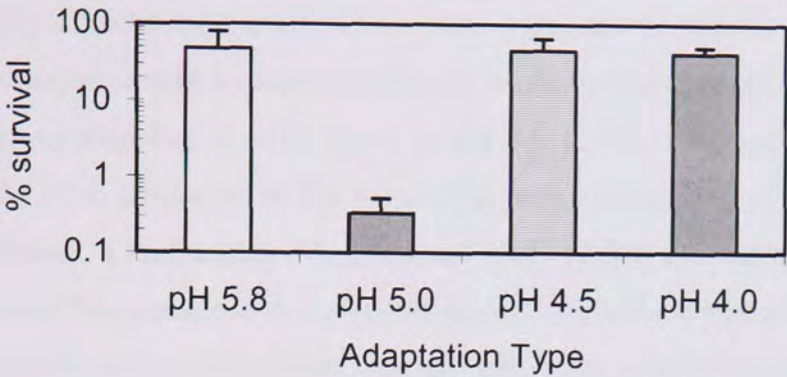
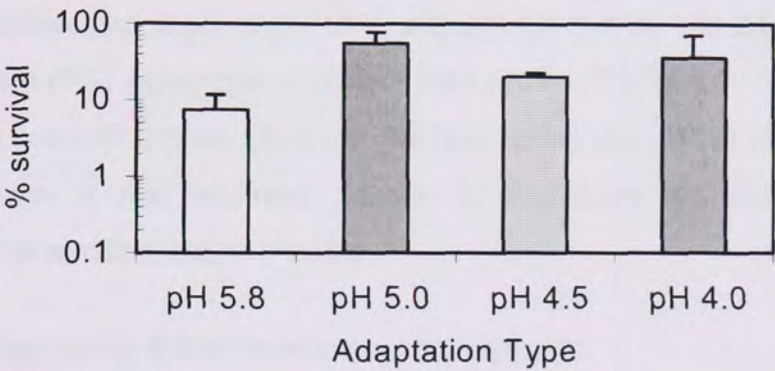


Figure 6.1. Percentage survival of strain *Lb. acidophilus* strain a) VUP 12,005, b) VUP 12,015 and c) *Lb. johnsonii* strain VUP 12,028 at 0, 3 and 6 weeks of refrigerated storage relative to initial cell numbers (week 0) following conditioning in lactic acid adjusted MRS broth (39°C) at pH 5.8 (●) (control), pH 5.0 (▲), 4.5 (◆), 4.0 (■) and simulation of yogurt production.

a)



b)



c)

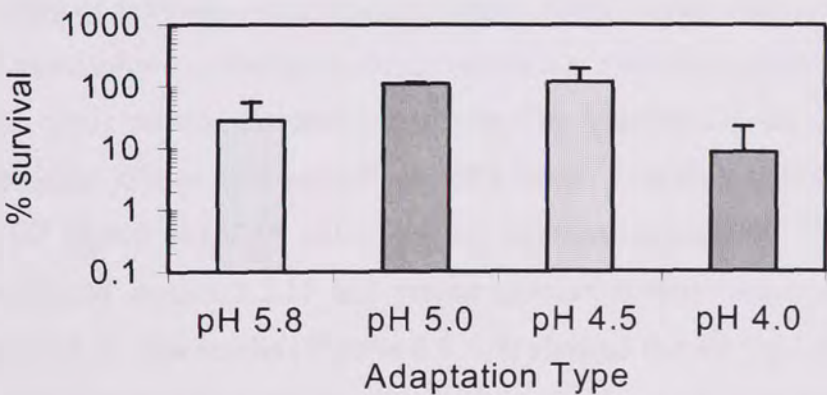


Figure 6.2. Percentage survival of strain *Lb. acidophilus* strain a) VUP 12,005, b) VUP 12,015 and c) *Lb. johnsonii* strain VUP 12,028 at 6 weeks of refrigerated storage relative to initial cell numbers (week 0) following conditioning in lactic acid adjusted MRS broth (39°C) at pH 5.8 (control), pH 5.0, 4.5, 4.0 and simulation of yogurt production. Figures represent the average and standard deviation of duplicate experiments.

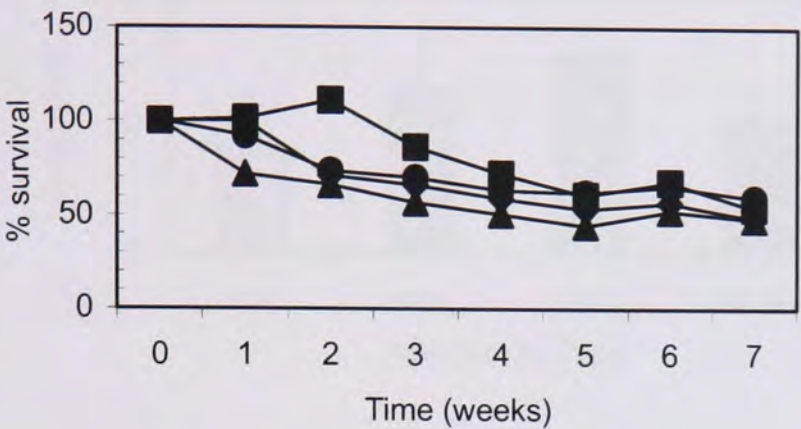
In contrast, improvements in survival were observed during six weeks of refrigerated storage for *Lb. acidophilus* VUP 12,015 and *Lb. johnsonii* VUP 12,028 following conditioning and yogurt simulation trials (Table 6.1). For *Lb. acidophilus* strain VUP 12,015, the greatest improvement in survival after six weeks of refrigerated storage was observed following conditioning in MRS broth at pH 5.0 (55%), followed by pH 4.0 (36%), then pH 4.5 (20%) compared to 7% for control cells incubated at pH 5.8 during the conditioning period. Conditioning *Lb. johnsonii* VUP 12,028 at moderate pH, not only enhanced survival, but permitted cell division during long-term refrigerated storage. Survival was enhanced during refrigerated storage following conditioning at pH 5.0 (214%), pH 4.5 (100%), but not following conditioning at pH 4.0 (58.0%) compared to control cells (58%). Similarly, survival was enhanced at six weeks of refrigerated storage following conditioning at pH 5.0 (110%) and pH 4.5 (117%) but not following conditioning at pH 4.0 (9%) compared to control cells (28%). (Table 6.1, Figure 6.2). Despite the variation in survival rates, all of the probiotic strains survived at rates greater than 10^6 cfu/g, which is the minimum amount of organisms recommended for consumption to provide potential health benefits.

6.2.2 Conditioning using fermentation end-products

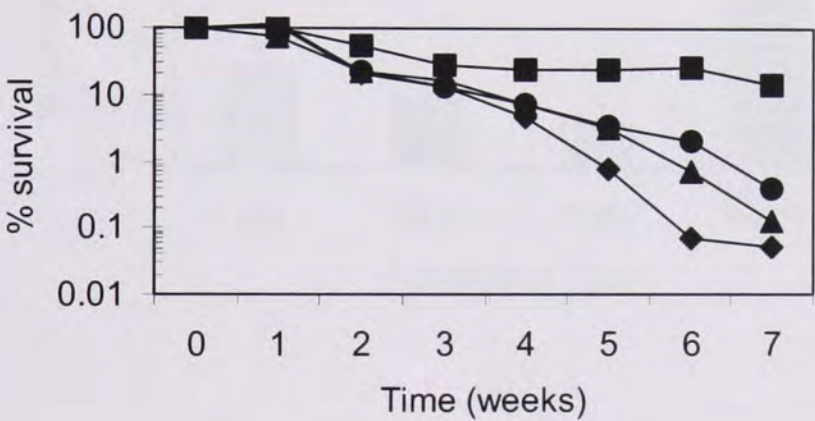
Cell-free filtrates (end-products) were used instead of lactic acid to condition probiotic strains prior to yogurt simulation trials in an effort to mimic the conditions that normally cause loss of viability of probiotic organisms in yogurt. Cell filtrates were prepared by inoculating a 2% monoculture of the strain being tested into MRS broth and incubating overnight at 37°C. Cells were removed *via* centrifugation followed by the dilution of filtrates (100%) to either 75% or 50% using fresh MRS broth. Exponential phase cells of *Lb. acidophilus* VUP 12,005 and VUP 12,015 and *Lb. johnsonii* strain VUP 12,028 were conditioned according to section 2.5.17 and yogurt simulation trials were carried out according to section 2.5.6. The results (Figures 6.3, 6.4) showed that survival after seven weeks of refrigerated storage following conditioning with fermentation end-products was strain dependent.

Lb. acidophilus strain VUP 12,005 cells exposed to 100% (pH 3.9) end-product during conditioning underwent cell division for the first two weeks of refrigerated storage in

a)



b)



c)

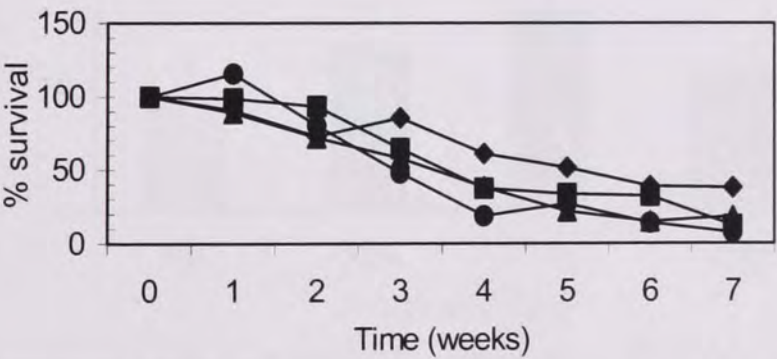
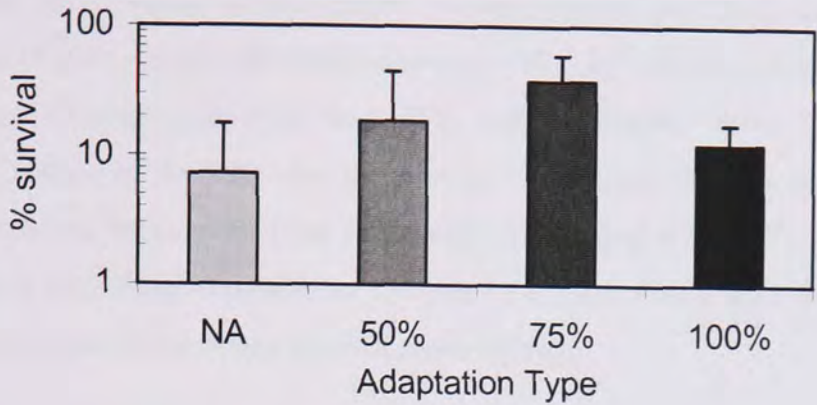
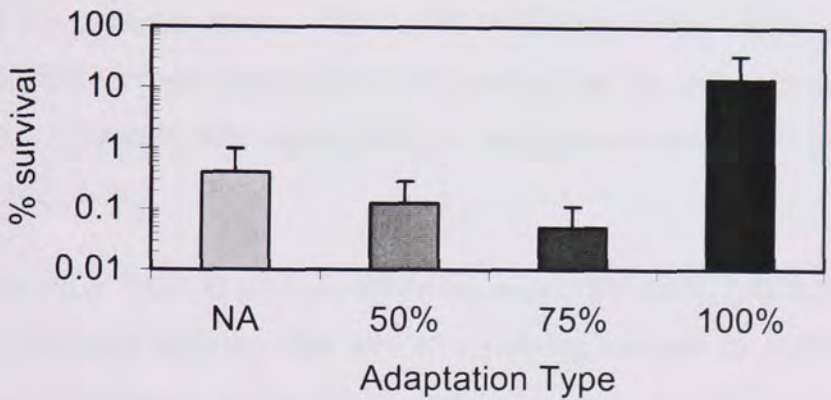


Figure 6.3. Percentage survival of strain *Lb. acidophilus* strain a) VUP 12,005, b) VUP 12,015 and c) *Lb. johnsonii* strain VUP 12,028 for 7 weeks of refrigerated storage relative to initial cell numbers (week 0) following conditioning in MRS broth (39°C) containing 0% (●), 50% (▲), 75% (◆), or 100% (■) cell-free filtrates (prepared according to section 2.5.17) and simulation of yogurt production (refer to section 2.5.6).

a)



b)



c)

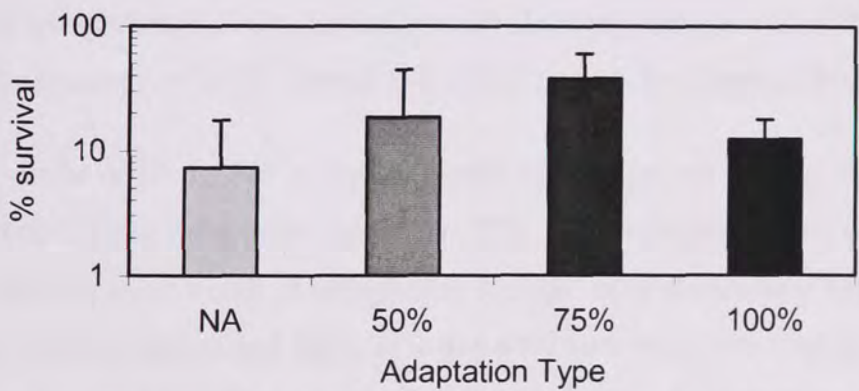


Figure 6.4. Percentage survival of strain *Lb. acidophilus* strain a) VUP 12,005, b) VUP 12,015 and c) *Lb. johnsonii* strain VUP 12,028 at 7 weeks of refrigerated storage relative to initial cell numbers (week 0) following conditioning in MRS broth (39°C) containing 0% (NA), 50%, 75%, or 100% cell-free filtrates (prepared according to section 2.5.17) and simulation of yogurt production (refer to section 2.5.6). Figures represent the average and standard deviation of duplicate experiments.

10% NFDM (pH 4.5), with initial counts of 8.5×10^7 cfu/ml increasing to 9.2×10^7 cells per ml. After three weeks of refrigerated storage, counts decreased to 86% of their original value (Figure 6.3a), with viable counts at 7.0×10^7 cfu/ml, whilst survival rates following conditioning with 50% and 75% culture filtrates were 57% and 66% respectively, compared to 70% for the control. Between four to seven weeks of refrigerated storage, the survival rates following conditioning with 100% culture filtrates were consistent with those reported for the control (Figure 6.4a), with viable counts of 4.0×10^7 cfu/ml after seven weeks of refrigerated storage.

Conditioning using either 50% (pH 4.4) or 75% (pH 4.0) was unsuccessful at improving the survival of probiotic strain VUP 12,005 following seven weeks of refrigerated storage, with 48% survival compared to 59% survival for the control or non-conditioned cells (pH 5.8). However, after seven weeks of refrigerated storage, all cell counts were greater than 10^6 cfu/g.

For *Lb. acidophilus* VUP 12,015, conditioning cells with 100% (pH 4.2) fermentation end-product improved survival with almost a two-log increase in viable counts after seven weeks of refrigerated storage, with viable counts at 5.8×10^6 cfu/ml compared to 1.4×10^5 cfu/ml for control cells. This represented 14% survival compared to $4 \times 10^{-1}\%$ for the control. In contrast, conditioning cells using 50% (pH 4.7) or 75% (pH 4.4) fermentation end-product failed to improve survival during storage, with 0.1% and 0.05% survival of cells compared to the control (pH 5.8) (Figure 6.3b, Figure 6.4b).

Survival of strain VUP 12,028 at seven weeks of refrigerated storage was enhanced following conditioning with 50% (pH 4.3), 75% (pH 4.0) and 100% (pH 3.9) cell filtrates. Following three weeks of refrigerated storage, cells conditioned with 50%, 75% or 100% cell filtrates maintained 58%, 85% and 64% survival of the original population, respectively, compared to 48% survival of the control cells. In comparison, after seven weeks of refrigerated storage, cells maintained 19%, 38% and 12% survival of the original population, respectively, compared to 7% survival of the control cells. The greatest improvement in survival was observed following conditioning using 75%

fermentation end-product, with 38% of the original population surviving after seven weeks refrigerated storage compared to 7% of the control or unadapted cells, with counts of 2.9×10^6 cfu/ml compared to 4.4×10^5 for the control cells (conditioned at pH 5.8) (Figures 6.3c, 6.4c).

6.3 MEDIA TRIAL FOR SELECTIVE ENUMERATION OF PROBIOTIC AND STARTER CULTURES

The combination of probiotic (*Lb. acidophilus*, *Lb. johnsonii*) and starter cultures (*Str. thermophilus*, *Lb. bulgaricus*) into one yogurt product presents a microbiological challenge for differential enumeration. Various media were tested by streaking pure cultures onto a range of media and evaluating plating efficiencies following incubation according to section 2.5.4. Streaking a pure culture of *Str. thermophilus* onto ST agar produced well-developed colonies after 24 h of incubation at 37°C under aerobic conditions (Tables 6.2, 6.3). In contrast, growth was not observed on ST agar streaked with *Lb. acidophilus* VUP 12,005 and VUP 12,015, *Lb. johnsonii* VUP 12,028 or *Lb. bulgaricus* grown under the same conditions (Tables 6.2, 6.3). Gram staining several colonies from a mixed culture (probiotic and starter cultures) grown on ST agar produced only Gram positive cocci (Table 6.4), which are characteristic of the *Str. thermophilus* species (Kandler and Weiss, 1986). Whilst PCA and M17 agar supported the growth of pure cultures of *Str. thermophilus*, they also supported the growth of the *Lactobacillus* species, albeit at a reduced level (Table 6.2) and Gram staining of mixed cultures showed not only Gram positive cocci, but also Gram positive rods (Table 6.4). Streaking pure cultures of *Str. thermophilus* onto RCA or MRS agars and incubating plates at 37°C for three days under anaerobic conditions inhibited the growth of *Str. thermophilus* (Table 6.2).

Streaking pure cultures of probiotic and starter cultures onto RCA agar supported the confluent growth of *Lb. bulgaricus* and limited growth of all probiotic cultures (Table 6.2), as evidenced by observing colonies 2 to 5 mm in diameter for pure cultures of *Lb. bulgaricus* compared to non-definable pinpoint colonies for the probiotic strains (Table

Table 6.2. Evaluation of media for the selective enumeration of *Lb. acidophilus*, *Lb. johnsonii*, *Str. thermophilus* and *Lb. delbrueckii* ssp. *bulgaricus* during co-culture in yogurt.

^a Pure cultures of strain:					
Media Type ^b	VUP 12,005	VUP 12,015	VUP 12,028	CSCC 2010	CSCC 2515
ST	0	0	0	2	0
PCA	0.25	0.5	0.5	2	0.25
M17	0.5	0.5	0.5	1	0.5
RCA (pH 5.3)	0.25	0.25	0.25	0	3
MRS (pH 5.3)	2.5	2.5	3	0	3
MRS-Maltose	3	3	3	0	2.5
MRS-Sorbitol	0.25	0.25	0.25	0.125	0.125
MRS-Raffinose	2	0	3	0	0
MRS-Oxygall	3	3	3	0	3

^a *Lb. acidophilus* strains VUP 12,005 and VUP 12,015, *Lb. johnsonii* strain VUP 12,028, *Str. thermophilus* strain CSCC 2010 and *Lb. delbrueckii* ssp. *bulgaricus* strain CSCC 2515 were used in this study. Active cultures were inoculated into various media using the streak plate technique (according to section 2.5.3) and the degree of growth was ranked according to the scale 0 (no growth), 1, (minimal growth), 2 (medium growth), 3 (confluent growth).

^b ST, *Str. thermophilus* agar (Dave and Shah, 1996); PCA, Plate Count Agar (Johns *et al.*, 1978); M17 agar (Charteris *et al.*, 1997); RCA, Reinforced Clostridial Agar (Johns *et al.*, 1978); MRS (pH 5.3) agar, MRS-sorbitol agar (Dave and Shah, 1996); MRS-maltose agar, MRS-raffinose agar (Hull and Roberts, 1984); MRS-oxygall agar (Micanel *et al.*, 1997).

Table 6.3. Colony morphology of *Lb. acidophilus*, *Lb. johnsonii*, *Str. thermophilus* and *Lb. delbrueckii* ssp. *bulgaricus* from pure cultures streaked onto various selective media.

"Strain tested:				
Media Type ^b	VUP 12,005	VUP 12,015	VUP 12,028	ST/LB Mix
ST	No growth	No growth	No growth	Circular, shiny, raised, cream-pink colonies, 1-2mm diameter
PCA	Circular, transparent colonies, 0.5 – 1 mm diameter	Circular, transparent colonies, 0.5 – 1 mm diameter	Circular, dull, cream colonies, 1-2 mm diameter	Circular, cream, inverted colonies with clear perimeter, 1mm
M17	Circular, dull, transparent colonies, 0.5 – 1 mm diameter	Circular, shiny, cream colonies, 0.5 mm diameter	Circular, solid, dull, cream colonies, 1 mm diameter	Circular, cream, solid, shiny, 1-2mm diameter
RCA (pH 5.3)	Pinpoint colonies, not definable	Pinpoint colonies, not definable	Pinpoint colonies, not definable	Irregular, shiny, cream colonies 2-5mm diameter
MRS (pH 5.3)	Circular, solid, cream, dull colonies, 1-2 mm diameter	Circular, cream, dull colonies with irregular border, 1-3mm diameter	Circular/irregular, solid cream, dull, flat, 2mm diameter	Circular, shiny, cream, 2-4mm diameter
MRS-Maltose	Circular, shiny, white centre, off-white perimeter, 0.5-2mm diameter	Circular, shiny, off-white, 1-3mm diameter	Circular, white, shiny, raised, clear perimeter, 2-3 mm diameter	Circular, shiny, off-white, 0.5-2mm diameter
MRS-Sorbitol	Pinpoint colonies, circular, clear	Pinpoint colonies, circular, clear	Circular, cream, dull, 0.5 mm diameter	Pinpoint colonies circular, clear
MRS-Raffinose	Circular, cream, shiny, raised, 0.5 mm diameter	Pinpoint colonies, circular, clear	Circular, dull, solid, cream, 1-2mm diameter	Pinpoint colonies circular, clear
MRS-Oxygall	Circular, 1-2mm, cream, shiny, 1-3mm	Circular, 1-2mm, cream, shiny, 1-3mm	Circular-irregular shape, cream, dull, inverted centre, 2-5 mm diameter	Circular, raised, shiny, cream-yellow colour, 2-5mm diameter

^a *Lb. acidophilus* strains VUP 12,005 and VUP 12,015, *Lb. johnsonii* strain VUP 12,028, *Str. thermophilus* strain CSCC 2010 and *Lb. delbrueckii* ssp. *bulgaricus* strain CSCC 2515 were used in this study. Active cultures were streaked onto various media (according to section 2.5.3) and colony morphology was recorded.

^b Media as listed in Table 6.2.

Table 6.4. Gram stain reaction of colonies selected from media inoculated with mixed cultures of *Lb. acidophilus* VUP 12,005 and VUP 12,015, *Lb. johnsonii* VUP 12,028, *Str. thermophilus* and *Lb. delbrueckii* ssp. *bulgaricus*.

Gram Reaction,^{a,b} <i>Lb. bulgaricus</i>/Str. <i>thermophilus</i> plus strain:			
Media Type^c	VUP 12,005	VUP 12,015	VUP 12,028
ST	A	A	A
PCA	A, C	A, B	A, B, C
M17	A, C	A	C
RCA (pH 5.3)	D	B, D	B, D
MRS (pH 5.3)	B, E	B, E	B, D
MRS-Maltose	C, E	C, E	C, E
MRS-Sorbitol	D	F	F
MRS-Raffinose	C	F	C
MRS-Oxygall	B, C	B, C	B, C

^a *Lb. acidophilus* strains VUP 12,005 and VUP 12,015 and *Lb. johnsonii* VUP 12,028 were grown in combination with the starter cultures *Str. thermophilus* strain CSCC 2010 and *Lb. delbrueckii* ssp. *bulgaricus* strain CSCC 2515 in 12% NGYEC broth at 37°C overnight. Mixed cultures were streaked onto various media (according to section 2.5.4) and a range of colonies were selected for Gram staining.

^b Notation represent Gram reactions A) Gram positive cocci in chains; B) Gram positive rods, long, thin, in singles and chains; C) Short, thick, Gram positive rods in chains and pairs; D) Gram positive rods, short, curled; E) Gram positive rods, small, thin, singles; F) Nothing observed on slide.

^c Media as listed in Table 6.2.

6.3). MRS agar (pH 5.3) supported the growth of both probiotic and *Lb. bulgaricus* cultures (Tables 6.2, 6.3) and Gram positive rods with variable morphology were detected by Gram staining several colonies from a mixed culture containing both probiotic and starter cultures grown on MRS agar (pH 5.3).

MRS-Maltose and MRS-Oxygall were successful in culturing both probiotic and *Lb. bulgaricus* cultures (Table 6.2), presenting Gram positive rods with two distinct morphologies on plates prepared with mixed cultures (Table 6.3). In contrast, MRS-Sorbitol did not encourage growth of any of the cultures tested (Table 6.2) and MRS-Raffinose successfully selected for *Lb. acidophilus* strain VUP 12,005 and *Lb. johnsonii* VUP 12,028, whilst inhibiting the growth of *Lb. acidophilus* strain VUP 12,015, *Lb. bulgaricus* and *Str. thermophilus* (Table 6.2, 6.4).

6.4 SURVIVAL OF PROBIOTIC STRAINS IN A YOGURT DURING REFRIGERATED STORAGE FOLLOWING EXPOSURE OF CELLS TO MODERATE PH VALUES

6.4.1 Yogurt trials following conditioning of probiotic strains using lactic acid

Exponential phase cells of *Lb. acidophilus* strains VUP 12,005 and VUP 12,015 and *Lb. johnsonii* strain VUP 12,028 were conditioned in MRS broth adjusted to pH 5.0, 4.5, or 4.0 using lactic acid and incubated at 39°C for one hour (as per section 2.5.13) and the viability was monitored for six weeks following small-scale yogurt production (refer to section 2.5.5). For *Lb. acidophilus* VUP 12,005, conditioning cells at pH 5.0, 4.5 or 4.0 enhanced cell viability during the first three weeks of refrigerated storage, with 102%, 36% and 75% survival, respectively, compared to cells incubated at pH 5.8 during the conditioning period (control), which displayed 33% survival after three weeks of refrigerated storage. Following six weeks of refrigerated storage, the survival of the control or unadapted cells of *Lb. acidophilus* strain VUP 12,005 was greater than cells

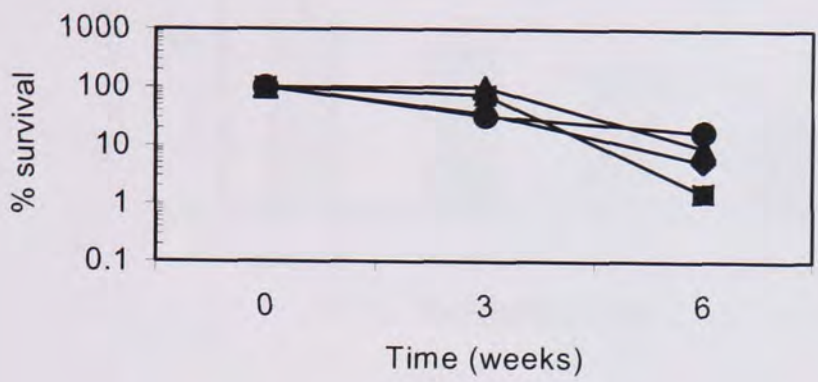
conditioned at moderate pH prior to yogurt production (Figure 6.5, 6.6), with cell counts at 3.1×10^7 cfu/g of yogurt.

For *Lb. acidophilus* VUP 12,015, conditioning cells at pH 4.5 for one hour resulted in cell division during the first three weeks of refrigerated storage, with a 13% increase in cell numbers (113% survival) compared to 45% survival for cells incubated at pH 5.8 during the conditioning period (control). However, between three to six weeks of refrigerated storage, survival of cells conditioned at pH 4.5 decreased rapidly; with only 3% (8.3×10^5 cfu/g) of cells surviving six weeks of refrigerated storage compared to 16% (1.66×10^7 cfu/g) survival for the control cells. Conditioning cells at pH 5.0 or 4.0 did not serve to enhance viability during refrigerated storage in yogurt (Figure 6.5b, 6.6b), with 2% and 15% survival rates, respectively, after six weeks of refrigerated storage compared to 16% survival for the control (Table 6.1).

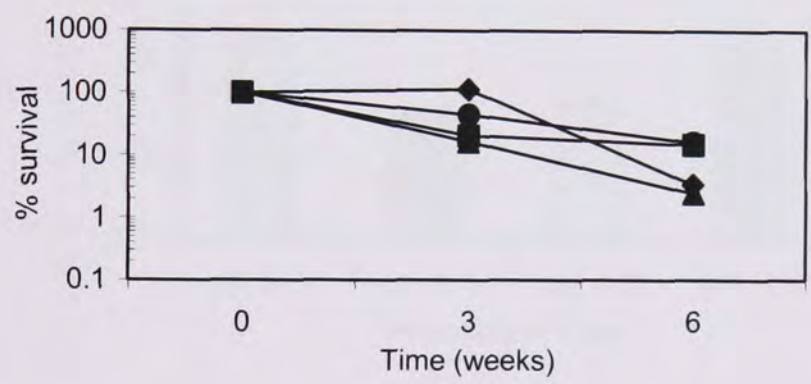
Conditioning *Lb. johnsonii* strain VUP 12,028 in MRS broth (39°C) at moderate pH for one hour was detrimental to its survival following production and refrigerated storage of yogurt for six weeks. Cells incubated in MRS broth at pH 5.8 during the conditioning period (control) maintained 82% survival after six weeks of refrigerated storage, compared to $6.4 \times 10^{-1}\%$, 1% and 25% of cells conditioned at pH 5.0, 4.5 or 4.0, respectively.

The starter cultures *Lb. bulgaricus* strain CSCC 2515 and *Str. thermophilus* strain CSCC 2010 were selected because of their high extracellular β -galactosidase activity (Lankaputhra and Shah, unpublished data). *Str. thermophilus* counts decreased progressively during refrigerated storage, with counts 2.2×10^8 at week zero, 4.7×10^6 at week three and 9.0×10^4 at week six (Figure 6.7). In contrast, less than a $\log_{10}$ decrease was observed *Lb. bulgaricus* counts over the six week storage period, with 3.1×10^8 at week zero and 4.2×10^7 at week six (Figure 6.7).

a)



b)



c)

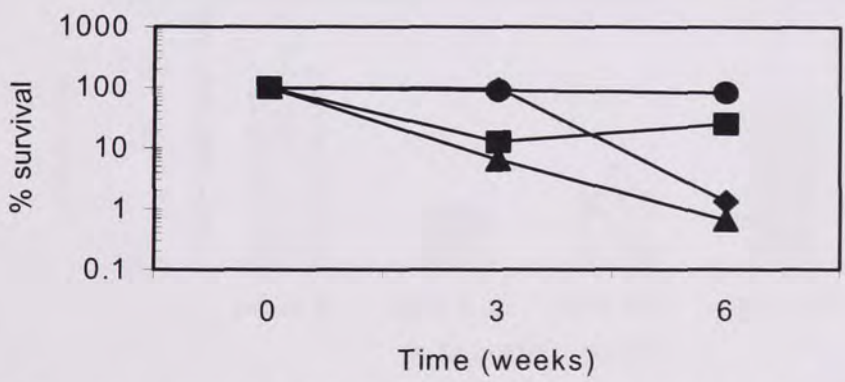
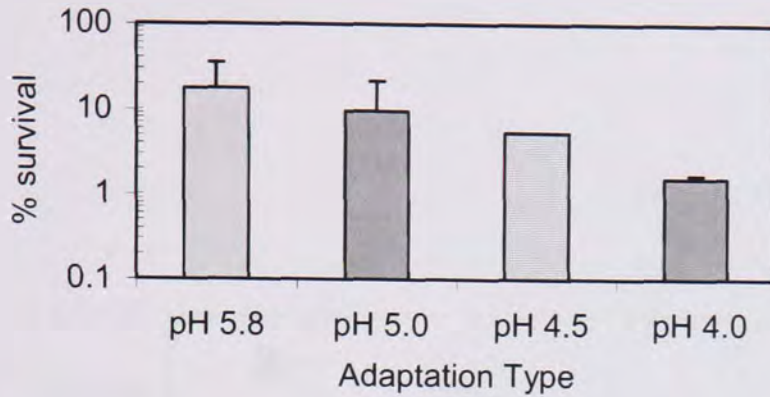
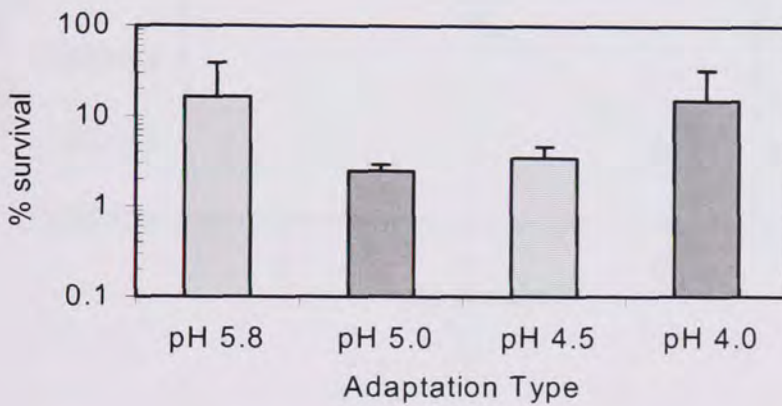


Figure 6.5. Percentage survival of strain *Lb. acidophilus* strain a) VUP 12,005, b) VUP 12,015 and c) *Lb. johnsonii* strain VUP 12,028 at 0, 3 and 6 weeks of refrigerated storage relative to initial cell numbers (week 0) following conditioning in lactic acid adjusted MRS broth (39°C) at pH 5.8 (●) (control), pH 5.0 (▲), 4.5 (◆), 4.0 (■) and subsequent inoculation into milk prior to small-scale yogurt production (according to section 2.5.5). Viable counts were determined by subtracting the *Lb. bulgaricus* counts on RCA agar from the total counts of *Lb. bulgaricus* and probiotic strains on MRS-oxygall agar.

a)



b)



c)

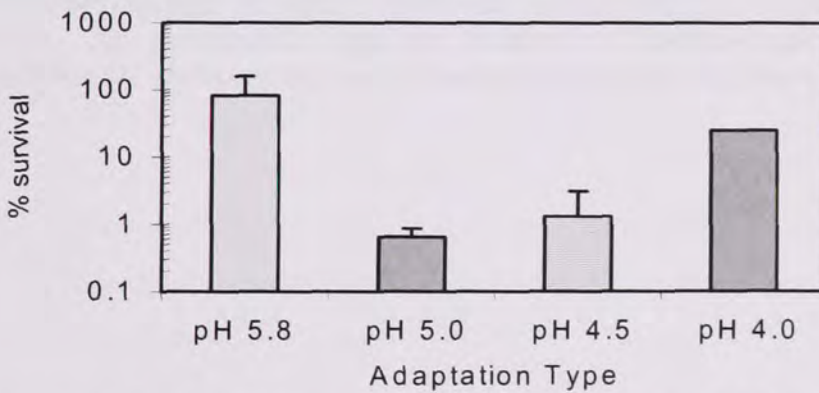


Figure 6.6. Percentage survival of strain *Lb. acidophilus* strain a) VUP 12,005, b) VUP 12,015 and c) *Lb. johnsonii* strain VUP 12,028 at 6 weeks of refrigerated storage relative to initial cell numbers (week 0) following conditioning in lactic acid adjusted MRS broth (39°C) at pH 5.8 (control), pH 5.0, 4.5, or 4.0 and subsequent inoculation into milk prior to small-scale yogurt production (according to section 2.5.5). Viable counts were determined by subtracting the *Lb. bulgaricus* counts on RCA agar from the total counts of *Lb. bulgaricus* and probiotic strains on MRS-oxygall agar. Figures represent the average and standard deviation of duplicate experiments.

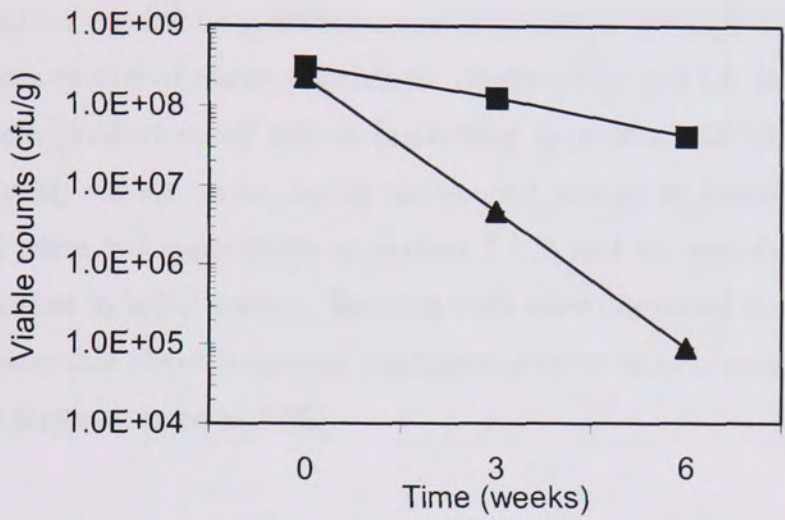


Figure 6.7. Viability of *Lb. bulgaricus* (■) and *Str. thermophilus* (▲) cultures at 0, 3 and 6 weeks of refrigerated storage in yogurt containing conditioned *Lb. acidophilus* or *Lb. johnsonii* cultures. *Str. thermophilus* agar and Reinforced Clostridial agar were used to selectively enumerate *Str. thermophilus* and *Lb. bulgaricus* cultures, respectively.

6.4.2 Yogurt trials following conditioning of probiotic strains in 10% non-fat dry milk versus MRS broth

Lb. acidophilus VUP 12,015 and *Lb. johnsonii* VUP 12,028 were grown to exponential phase at optimal conditions (pH 5.8, 39°C) then conditioned in pH 4.5 lactic acid adjusted MRS broth or 10% NFDM (pre-warmed to 39°C) for one hour according to section 2.5.13. Following conditioning, cells were re-suspended in 10% NFDM and, along with the primary inoculation of starter cultures *Str. thermophilus* and *Lb. bulgaricus*, used for the small-scale production of yogurt (according to section 2.5.18). Viability was monitored weekly for six weeks during refrigerated storage of yogurt using MRS agar and the pour plate technique (refer to section 2.5.3) and the percentage survival was calculated relative to initial counts. Survival rates were compared to samples that were grown to exponential phase at optimal conditions prior to re-suspension into fresh MRS broth at pH 5.8 (pre-warmed to 39°C).

For *Lb. acidophilus* strain VUP 12,015, conditioning at pH 4.5 in MRS broth was detrimental to the survival of cells during six weeks of refrigerated storage compared to cells that were incubated at pH 5.8 during the conditioning period (control). Control cells incubated in MRS broth (pH 5.8) during the incubation period maintained 33% survival in yogurt following three weeks of refrigerated storage and at six weeks, the percentage survival relative to the initial counts declined to 10%, which represented a population of 1.5×10^6 cfu/g of yogurt. In comparison, cells conditioned in MRS broth at pH 4.5 for one hour maintained 30% survival following three weeks of refrigerated storage in yogurt (Figure 6.8), which declined to 3% survival after six weeks of refrigerated storage, with a final cell count of 1.0×10^6 cfu/g of yogurt (Figure 6.9).

Conditioning *Lb. acidophilus* strain VUP 12,015 at pH 4.5 in 10% NFDM displayed similar trends to cells that were incubated at pH 5.8 during the conditioning period (control). Control cells incubated at pH 5.8 in 10% NFDM during the conditioning period demonstrated cell division for the first three weeks of refrigerated storage in yogurt, with a 49% increase in viability, followed by a decline in cell populations to 73%

of the original population after six weeks of refrigerated storage. Initial cell counts of 1.5×10^7 (week zero) increased to 2.2×10^7 during the first three weeks of refrigerated storage before declining to 1.1×10^7 after six weeks of refrigerated storage. In comparison, cells conditioned in lactic acid-adjusted 10% NFDM at pH 4.5 for one hour maintained 99% survival at three weeks, followed by a decline to 66% survival at six weeks of refrigerated storage in yogurt. This represented a decrease in initial viable counts from 2.5×10^7 cfu/g to 2.5×10^7 cfu/g at week three, followed by a further decline to 1.7×10^7 cfu/g at six weeks of refrigerated storage.

For *Lb. johnsonii* strain VUP 12,028, conditioning cells at pH 4.5 in MRS broth or 10% NFDM improved the survival of cells during the first three weeks of refrigerated storage in yogurt compared to cells incubated at pH 5.8 during the conditioning period (Control). However, at six weeks of refrigerated storage, cells conditioned at pH 4.5 displayed lower survival rates than the control. Control cells incubated in MRS broth (pH 5.8) during the conditioning period underwent cell division for the first three weeks of refrigerated storage, with a 58% increase in viability compared to initial counts, which represented an increase in cell counts from 1.9×10^7 to 3.0×10^7 cfu/g of yogurt. At six weeks, the survival rates decreased to 43%, which represented cell counts of 8.2×10^6 cfu/g of yogurt. In comparison, cells conditioned in MRS broth at pH 4.5 also underwent cell division for the first three weeks of refrigerated storage, with a 186% increase in cell viability compared to initial counts. This represented an increase in cell counts from 2.7×10^7 to 7.8×10^7 cfu/g of yogurt. At six weeks, the survival rates decreased to 10.8%, which represented cell counts of 2.9×10^6 cfu/g of yogurt (Figure 6.9).

Conditioning *Lb. johnsonii* strain VUP 12,028 at pH 4.5 in 10% NFDM displayed similar trends to cells conditioned at pH 4.5 in MRS broth, with cell conditioning being detrimental to the survival of cells during six weeks of refrigerated storage compared to cells that were incubated at pH 5.8 during the conditioning period (Control). Control cells incubated at pH 5.8 in 10% NFDM during the conditioning period demonstrated cell division for the first three weeks of refrigerated storage in yogurt, with a 678% increase

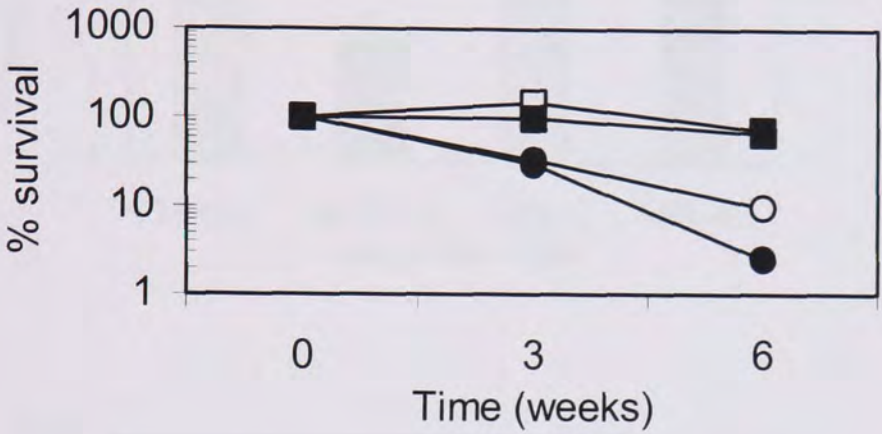
in viable counts, followed by 515% decline in the cell population after six weeks of refrigerated storage. Initial cell counts of 7.5×10^6 (week zero) increased to 5.8×10^7 during the first three weeks of refrigerated storage before declining to 2.0×10^7 after six weeks of refrigerated storage. In comparison, cells conditioned in lactic acid-adjusted 10% NFDM at pH 4.5 for one hour increased by 697% at three weeks, followed by a decline to 155% survival at six weeks of refrigerated storage in yogurt. This represented a decrease in initial viable counts from 1.0×10^7 cfu/g to 8.0×10^7 cfu/g at week three, followed by a further decline to 1.6×10^7 cfu/g at six weeks of refrigerated storage (Figures 6.8, 6.9).

Overall, conditioning cells at pH 4.5 was detrimental to their survival during six weeks of refrigerated storage in yogurt. In addition, preparing cells in 10% non-fat dry milk prior to yogurt trials resulted in higher cell populations during refrigerated storage of yogurt than cells prepared in MRS broth (Figure 6.8, 6.9).

6.5 INHIBITORY ACTIVITY ASSAYS OF YOGURT STARTER CULTURES AGAINST PROBIOTIC CULTURES

Agar well diffusion assays were performed to determine whether the yogurt starter cultures *Lb. bulgaricus* and *Str. thermophilus* inhibited the survival of the probiotic strains *Lb. acidophilus* strains VUP 12,005 and VUP 12,015 and *Lb. johnsonii* VUP 12,028 following production and refrigerated storage of yogurt. Culture filtrates of the starter and probiotic cultures were prepared by inoculating cultures in MRS broth or 10% NFDM, followed by incubation at 37°C for 18 hours. Cells were removed via centrifugation and filtration. Culture filtrates were added to wells prepared in MRS agar plates seeded with the indicator organism and following incubation of plates at 37°C for two days, plates were observed for zones of inhibition according to section 2.5.10.

a)



b)

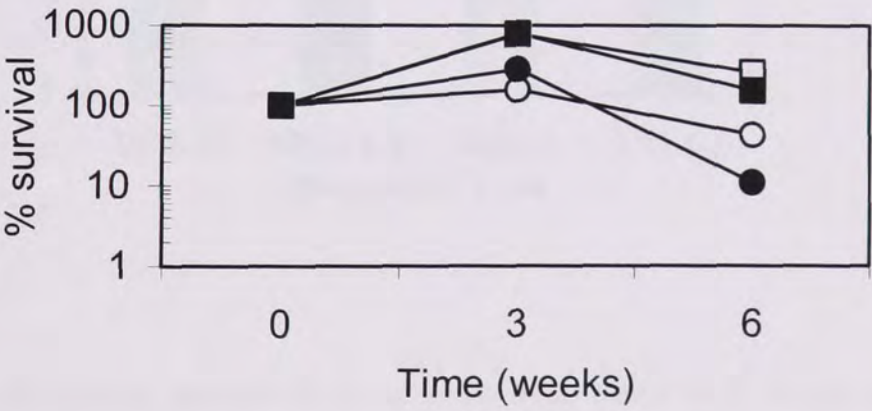
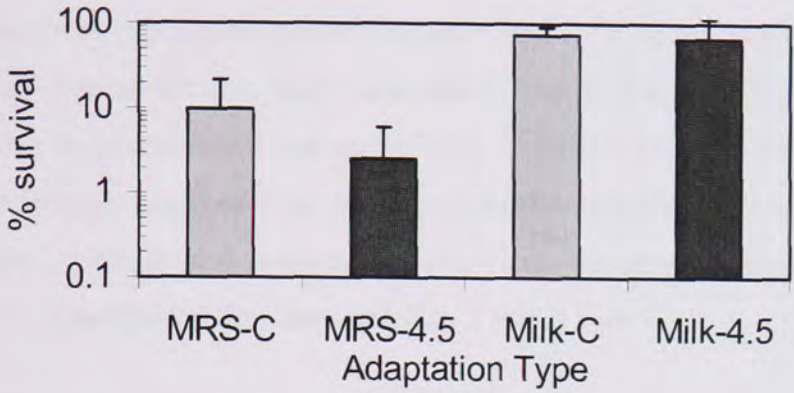


Figure 6.8. Percentage survival of a) *Lb. acidophilus* strain VUP 12,015 and b) *Lb. johnsonii* strain VUP 12,028 at 0, 3 and 6 weeks of refrigerated storage in yogurt (according to section 2.5.5) relative to initial cell numbers (week 0) following conditioning of exponential phase cells in lactic acid adjusted MRS broth (39°C) at pH 5.8 (○) (control) or pH 4.5 (●) or 10% NFDM adjusted with lactic acid to pH 5.8 (□) or pH 4.5 (■).

a)



b)

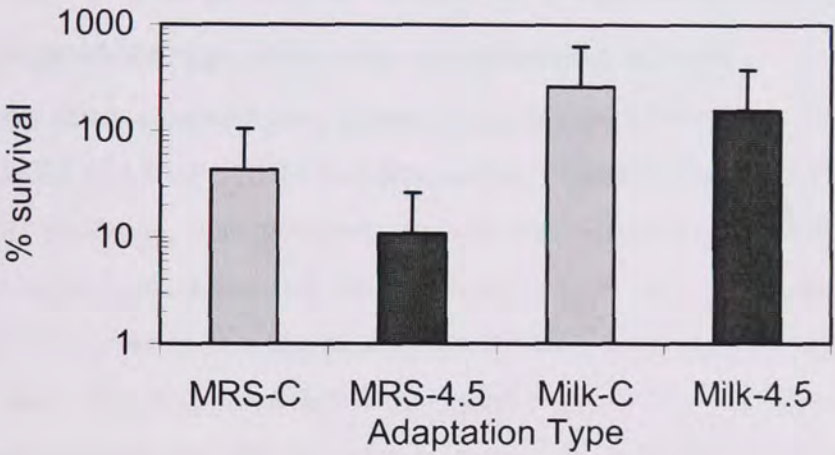


Figure 6.9. Percentage survival of a) *Lb. acidophilus* strain VUP 12,015 and b) *Lb. johnsonii* strain VUP 12,028 at 6 weeks of refrigerated storage in yogurt (according to section 2.5.5) relative to initial cell numbers (week 0) following conditioning of exponential phase cells in lactic acid adjusted MRS broth (39°C) at pH 5.8 (MRS-C) (control) or pH 4.5 (MRS-4.5) or 10% NFDM adjusted with lactic acid to pH 5.8 (Milk-C) or pH 4.5 (Milk-4.5). Figures represent the average and standard deviation of duplicate experiments.

Results showed that growth of probiotic strains was uninhibited by culture filtrates from either *Lb. bulgaricus* or *Str. thermophilus*. This result was consistent whether the culture filtrates were prepared in MRS broth (Table 6.5) or 10% non-fat dry milk (Table 6.6). In contrast, zones of inhibition of up to 14 millimetres were observed when *Str. thermophilus* was exposed to culture filtrates prepared from *Lb. bulgaricus* (Figure 6.10). When these culture filtrates were neutralised with sodium hydroxide, growth of *Str. thermophilus* was uninhibited and no zones of inhibition were observed. However, when the culture filtrates obtained from *Str. thermophilus* and *Lb. bulgaricus* were neutralised with sodium hydroxide and also treated with 0.1 mg catalase, zones of inhibition of 10 to 11 mm were observed for *Str. thermophilus* (Table 6.5, 6.6).

6.6 DISCUSSION

6.6.1 Survival of probiotic strains in a yogurt-like environment during refrigerated storage following conditioning of cells

In chapter three, a conditioning protocol was designed for probiotic strains VUP 12,005, VUP 12,015 and VUP 12,028 that demonstrated improved survival rates during normally lethal pH challenge. This protocol involved conditioning exponential phase cells in MRS broth at pH 4.5, pH 4.0 or pH 5.8-4.5 (using lactic acid to adjust the pH) for a doubling time at 39°C prior to re-suspension in fresh MRS broth adjusted to pH 3.5 or 3.0 using lactic acid. Survival rates following conditioning were dependent on the pH of the challenge medium and the strain being tested. In general, conditioning cells enhanced survival at normally lethal pH.

In this chapter, *Lb. acidophilus* and *Lb. johnsonii* cells conditioned in lactic acid adjusted MRS broth were assessed for their ability to improve in survival during long-term refrigerated storage following in a yogurt-simulated environment. Yogurt simulation protocols were designed based on conditions of standard yogurt manufacture. Hull *et al.* (1984) reported that the pH reached 4.5 after three to four hours of incubation at 43°C, therefore, the pH of heat-treated milk was sequentially lowered from 6.2 to 4.5 during 3.5 hours at 43°C to mimic that of normal yogurt production.

Table 6.5. Inhibition of *Lb. acidophilus*, *Lb. bulgaricus*, and *Str. thermophilus* cultures by filtered supernatants of *Lb. acidophilus*, *Lb. bulgaricus*, and *Str. thermophilus* grown in MRS broth using the agar well diffusion assay (section 2.5.10).

Cell Supernatant Type ^b	^a Culture type :				
	LA VUP 12,005	LA VUP 12,015	LJ VUP 12,028	ST C SCC 2010	LB C SCC 2515
Control	^c				
i.	-	-	-	-	-
ii.	-	-	-	-	-
iii.	-	-	-	-	-
LA					
i.	-	-	-	UC	-
ii.	-	-	-	-	-
iii.	-	-	-	-	-
ST					
i.	-	-	-	UC	-
ii.	-	-	-	-	-
iii.	-	-	-	-	-
LB					
i.	-	-	-	++++	-
ii.	-	-	-	-	-
iii.	-	-	-	-	-
ST + LB					
i.	-	-	-	++++	-
ii.	-	-	-	UC	-
iii.	-	-	-	++	-
ST + LB + LA					
i.	-	-	-	++++	-
ii.	-	-	-	-	-
iii.	-	-	-	-	-

^a *Lb. acidophilus* strains VUP 12,005 (LA VUP 12,005) and VUP 12,015 (LA VUP 12,015), *Lb. johnsonii* strain VUP 12,028 (LJ VUP 12,028), *Str. thermophilus* strain C SCC 2010 (ST) and *Lb. delbrueckii* ssp. *bulgaricus* strain C SCC 2515 (LB) were used in this study.

^b i) Untreated supernatant, ii) supernatant neutralised to pH 6.0 using 5M NaOH, iii) supernatant neutralised to pH 6.0 and treated with 0.1 mg/ml catalase.

^c Figures represent the average of duplicate experiments. - = no inhibition, + = 1-9mm, ++ = 10 -11mm, +++ = 12 - 13mm, >14mm = +++, UC = unclear inhibition.

Table 6.6. Inhibition of *Lb. acidophilus*, *Lb. bulgaricus*, and *Str. thermophilus* cultures by filtered supernatants of *Lb. acidophilus*, *Lb. bulgaricus*, and *Str. thermophilus* grown in 10% NFDM using the agar well diffusion assay (section 2.5.10).

Cell Supernatant Type ^b	^a Culture type:				
	LA VUP	LA VUP	LJ VUP	ST CSCC	LB CSCC
	12,005	12,015	12,028	2010	2515
Control	^c				
i.	-	-	-	-	-
ii.	-	-	-	-	-
iii.	-	-	-	-	-
LA					
i.	-	-	-	-	-
ii.	-	-	-	-	-
iii.	-	-	-	-	-
ST					
i.	-	-	-	-	-
ii.	-	-	-	-	-
iii.	-	-	-	-	-
LB					
i.	-	-	-	++++	-
ii.	-	-	-	-	-
iii.	-	-	-	-	-
ST + LB					
i.	-	-	-	++++	-
ii.	-	-	-	-	-
iii.	-	-	-	++	-
ST + LB + LA					
i.	-	-	-	++++	-
ii.	-	-	-	-	-
iii.	-	-	-	-	-

^a *Lb. acidophilus* strains VUP 12,005 (LA VUP 12,005) and VUP 12,015 (LA VUP 12,015), *Lb. johnsonii* strain VUP 12,028 (LJ VUP 12,028), *Str. thermophilus* strain CSCC 2010 (ST) and *Lb. delbrueckii* ssp. *bulgaricus* strain CSCC 2515 (LB) were used in this study.

^b i) Untreated supernatant, ii) supernatant neutralised to pH 6.0 using 5M NaOH, iii) supernatant neutralised to pH 6.0 and treated with 0.1 mg/ml catalase.

^c Figures represent the average of duplicate experiments. - = no inhibition, + = 1-9mm, ++ = 10 -11mm, +++ = 12 - 13mm, >14mm = +++++, UC = unclear inhibition.

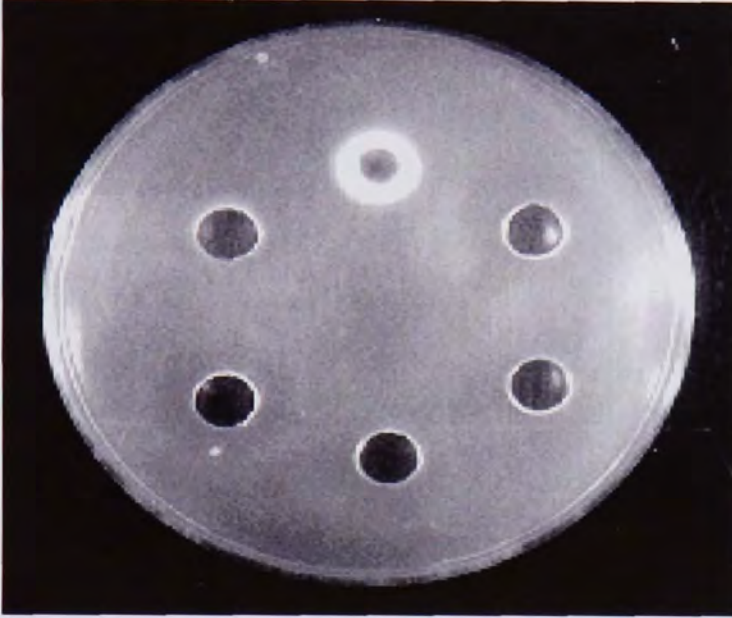


Figure 6.10. Agar well diffusion assay of the *Str. thermophilus* indicator strain seeded onto MRS agar versus culture filtrates of: 1, 10% NFDM; 2, *Lb. acidophilus*; 3, *Str. thermophilus*; 4, *Lb. delbrueckii* ssp. *bulgaricus*; 5, *Str. thermophilus*/*Lb. delbrueckii* ssp. *bulgaricus* mix; 6, *Lb. acidophilus* / *Str. thermophilus*/ *Lb. delbrueckii* ssp. *bulgaricus* mix grown in 10% NFDM (refer to section 2.5.10).

In agreement with the findings presented in chapter three, conditioning cells at moderate pH in lactic acid adjusted MRS broth for one hour prior to the simulated production of yogurt altered the survival rates of probiotic strains during six weeks of refrigerated storage. The success of conditioning protocols was dependent on the pH of the challenge environment and the strain being tested. For example, conditioning *Lb. acidophilus* strain VUP 12,015 or *Lb. johnsonii* strain VUP 12,028 cells in MRS broth adjusted to pH 5.0 or pH 4.5 prior to yogurt simulation trials enhanced the ability of strains to survive six weeks of refrigerated storage compared to cells that were incubated at pH 5.8 during the conditioning period (control). In addition, conditioning cells in MRS broth at pH 4.0 enhanced the survival of *Lb. acidophilus* strain VUP 12,015, but not *Lb. johnsonii* strain VUP 12,028, whereas conditioning *Lb. acidophilus* strain VUP 12,005 at moderate pH was detrimental to survival during six weeks of refrigerated storage. These results demonstrate the ability of some probiotic strains to adapt to normally challenging conditions if first conditioned at moderate pH during exponential phase of growth.

In addition to the inhibitory effect of lactic acid, cell filtrates obtained from overnight cultures may contain additional products that can invoke an adaptive response. Conditioning probiotic strains using cell filtrates produced similar trends to cells conditioned using lactic acid adjusted MRS broth, with improvements in percentage survival for strain VUP 12,015 following conditioning using 100% cell filtrates (pH 4.22) and for strain VUP 12,028 following conditioning using 50% (pH 4.32) and 100% (pH 3.87) cell filtrates. Conditioning strain VUP 12,005 using cell filtrates did not improve survival during refrigerated storage in a yogurt-like environment, which is consistent with the lack of improvement in survival of strain VUP 12,005 following conditioning using lactic acid adjusted MRS broth. The percentage survival of cells incubated at pH 5.8 during the conditioning period was consistent with survival rates observed for the control during protocols involving lactic acid stress.

These experiments were performed with cell filtrates produced by probiotic organisms only. However, the presence of the starter culture *Lb. bulgaricus* has been reported to have an impact on the survival of probiotic cultures in yogurt (Gilliland and Speck, 1977b; Dave and Shah, 1997a,c; Rybka and Fleet 1997; Gardini *et al.*, 1999; Lourens-

Hattingh and Viljoen, 2002). Therefore, it may have been more realistic to produce a fermentation mix that included cell filtrates from the starter culture organisms, *Str. thermophilus* and *Lb. bulgaricus*, as these organisms may excrete products that initiate cell death for probiotic organisms during refrigerated storage in yogurt.

Due to the variable responses between probiotic strains, the conditioning of cells using cell filtrates is not a reliable method for cell preparation during industrial yogurt manufacture. Not only would every probiotic strain have to be tested to find the optimal conditions for conditioning, it would be difficult to reproduce the cell filtrate mix for these organisms. However, it would be of benefit to classify the individual components of the cell filtrate, in order to explain differences in survival between cells conditioned using cell filtrates versus those conditioned using lactic acid adjusted MRS broth. For example, the survival of *Lb. johnsonii* strain VUP 12,028 was enhanced following conditioning using culture filtrates at 75%, with a final pH of 4.0, but not following conditioning with MRS broth adjusted to pH 4.0 using lactic acid compared to cells incubated in pH 5.8 MRS broth (control).

6.6.2 Media trial for selective enumeration of probiotic and starter cultures

Various media were screened to select the most appropriate for selective enumeration of probiotic and starter cultures. The ability to enumerate cells is important from a legislative perspective, with many countries introducing standards related to the types and numbers of organisms included in probiotic products (Feord, 2002). A number of differential plating methodologies have been developed which seek to selectively detect and enumerate probiotic organisms in yogurts (Johns *et al.*, 1978; Hull and Roberts, 1984; Dave and Shah, 1996; Micanel *et al.*, 1997; Rybka and Fleet, 1997). Differences in oxygen, pH and bile tolerance, nutritional requirements, antibiotic susceptibility, and colony morphology constitute the basis of differentiation in these methods (Calicchia *et al.*, 1993; Charteris *et al.*, 1997). In many of these media types, counts may not be representative of that in the product, as the selective ingredients in the media may not provide the optimum conditions for growth. In addition, the differential enumeration of

probiotic organisms in yogurt is a challenge, due to the presence of similar microbes, which are used traditionally in yogurt manufacturing, namely, *Lb. bulgaricus* and *Str. thermophilus*. Therefore, the first step in the selection of appropriate growth medium was to assess the growth of pure strains of probiotic cultures using various media before they are adopted for enumeration purposes in the presence of yogurt cultures.

The results show that no single media type was sufficient for the isolation and differentiation of probiotic and starter cultures. This is because the organisms have different temperature and oxygen requirements for growth. For *Str. thermophilus* strain CSCC 2010, ST agar was deemed most appropriate as selective media, with confluent growth of *Str. thermophilus* and no growth of either *Lb. acidophilus* or *Lb. bulgaricus* strains after 24 hours of incubation. The incubation time was based on results published by Dave and Shah (1996) who reported that '*Lb. acidophilus* failed to grow on ST agar plates within 24 h of incubation under aerobic conditions, however, colonies formed after 72 h of incubation'.

PCA and M17 were effective for culturing *Str. thermophilus* strain CSCC 2010, but these media also supported the limited growth of *Lactobacillus* cultures and the colony morphologies did not permit the differentiation of strains tested. In addition, *Str. thermophilus* counts were higher on ST agar than on PCA or M17 agar, which was also reported by Dave and Shah (1996).

RCA and MRS agar (pH 5.3) have been used for selectively isolating *Lb. bulgaricus* from yogurts (Johns *et al.*, 1978; Onggo and Fleet, 1993; Dave and Shah, 1996). In line with the literature, RCA produced the highest recovery of *Lb. bulgaricus*, with limited growth of all probiotic cultures and no growth of *Str. thermophilus*. Differentiation of colonies was possible using RCA agar, although the *Lb. bulgaricus* colonies varied in size. MRS (pH 5.3) had confluent growth of both *Lb. acidophilus* and *Lb. bulgaricus* cultures and colonies of these organisms could not be differentiated. RCA agar was selected as the medium of choice for the enumeration of *Lb. bulgaricus* strain CSCC 2515.

Pure probiotic and starter cultures were also tested on MRS media supplemented with oxygall, sorbitol or raffinose as the sole carbon source. Dave and Shah (1996) reported that *Lb. bulgaricus* did not grow on MRS-oxygall, maltose, or sorbitol agars. However, results reported here indicated that MRS-maltose and MRS-oxygall were successful for culturing both probiotic and *Lb. bulgaricus* cultures, suggesting that *Lb. bulgaricus* strain CSCC 2010 could use substrates that the published strain could not. MRS-Sorbitol did not encourage growth of either the probiotic or starter cultures whilst MRS-Raffinose encouraged the growth of probiotic strains VUP 12,005 and VUP 12,028, but not VUP 12,015. This is not surprising, given the negative fermentation of raffinose by VUP 12,015 in carbohydrate fermentation studies (Table 3.1). These results indicated that selection of the appropriate media for differentiation of probiotic and starter cultures is strain dependent and pure probiotic and starter cultures need to be screened for their ability to grow on various media for selective differentiation.

Many authors have reported the success of MRS-maltose for differential enumeration of probiotic organisms (Charteris *et al.*, 1997; Sharma and Prasad, 1986; Rybka and Kailasapathy, 1996; Lankaputhra *et al.*, 1996; Hull and Roberts, 1984; Dave and Shah, 1996). The use of this media is based on the assumption that *Lb. bulgaricus* will not ferment maltose (Hull and Roberts, 1984). However, there are conflicting reports on the proportion of maltose fermenting lactobacilli found in yoghurt and yoghurt starters, ranging from 1 in 103 isolates, 103 in 125 (Hull and Roberts, 1984) to 40 in 47 (Coker and Martley, 1982). In addition, some old cultures of *Lb. bulgaricus* have been found to acquire the ability to ferment maltose (Wheater, 1955). Our results indicate that *Lb. bulgaricus* CSCC 2515 was able to use maltose as a growth substrate in MRS media.

Based on these results, probiotic strains were enumerated using two techniques. Firstly, to calculate populations of strains VUP 12,005, VUP 12,015 and VUP 12,028, *Lb. bulgaricus* counts determined from RCA media were deducted from total counts obtained using MRS-oxygall agar or MRS-maltose (strain VUP 12,015). Secondly, viable counts of strains VUP 12,005 and VUP 12,028 were performed using MRS-raffinose agar.

6.6.3 Survival of probiotic strains in a yogurt during refrigerated storage following exposure of cells to moderate pH values

Conditioning exponential phase cells for one hour in MRS broth (39°C) adjusted to moderate pH (using lactic acid) resulted in lower survival rates following six weeks of refrigerated storage in yogurt compared to the control cells, which were incubated at pH 5.8 during the conditioning period. These results suggest that the adaptive responses that served to protect cells during challenge at normally lethal pH (Chapter 3) and during simulation of yogurt production (section 6.2.1) did not enhance their survival during small-scale yogurt production after six weeks of refrigerated storage compared to cells that were incubated at pH 5.8 during the conditioning period.

In contrast, similar trends were observed for the survival of probiotic strains during the first three weeks of refrigerated storage following conditioning and simulation of yogurt production compared to small-scale yogurt production, with conditioning at moderate pH improving the survival during the production and storage of yogurt. For example, following conditioning in MRS broth at pH 4.5 for one hour followed by three weeks of refrigerated storage in yogurt, *Lb. acidophilus* strain VUP 12,005 and VUP 12,015 and *Lb. johnsonii* strain VUP 12,028 demonstrated 36%, 113% and 97% survival respectively, compared to 33%, 45% and 89% for non-adapted cells incubated at pH 5.8 during the conditioning period. In addition, the survival of *Lb. acidophilus* strain was enhanced during three weeks of refrigerated storage in yogurt following conditioning at pH 5.0 (102%) and pH 4.0 (75%) compared to the unadapted control (33%).

It is likely that the presence of starter cultures *Str. thermophilus* and *Lb. bulgaricus* had an impact on the survival of probiotic cells following production of yogurt, as conditioning of strains VUP 12,015 and VUP 12,028 in MRS broth at moderate pH improved their survival following yogurt simulation and six weeks of refrigerated storage, but not following small-scale production of yogurt.

Schillinger (1999) reported that most of the probiotic products containing acceptable counts of probiotic cultures did not contain *Lb. bulgaricus*, whereas *Str. thermophilus* was detected in high numbers in all yogurts. Results show that *Str. thermophilus* counts

decreased progressively during refrigerated storage, with counts 2.2×10^8 at week zero, 4.7×10^6 at week three and 9.0×10^4 at week six. In contrast, less than a $\log_{10}$ decrease was observed *Lb. bulgaricus* counts over the six week storage period, with 3.1×10^8 at week zero and 4.2×10^7 at week six.

The apparent lack of adaptation of conditioned cells following yogurt production and six weeks of refrigerated storage may be due to the fact that, following conditioning, cells were inoculated in milk prior to yogurt manufacture, where the pH was close to optimal for the probiotic strains. Therefore, responses induced during conditioning to protect cells during acid challenge were no longer required and the cells may have reverted to their 'normal' physiology. Belli and Marquis (1991) found that cells switched from pH 7.0 to 5.0 adapted after one generation, shown by a decline in the minimum glycolytic pH and an increase in the ATPase activity of *S. mutans*. However, when the pH was switched back to 7.0, cells underwent de-adaptation, shown by a slow increase in the minimum pH for glycolysis and a decrease in ATPase activity. De-adaptation was reported to occur over a number of generations and appears to reflect not genetic selection but a progressive physiologic process (Belli and Marquis, 1991). Foster (1993) reported that key acid tolerance proteins are labile unless promptly engaged in their protective roles at a severely acid pH. This may suggest that proteins produced during conditioning protocols are transient and are down regulated because they are not required at optimal pH conditions. Alternatively, conditioning protocols may have invoked a response that was not appropriate for improving the survival of strains during six weeks of refrigerated storage in yogurt.

It may be more advantageous to inoculate conditioned strains at a lower pH during yogurt manufacture. Leyer and Johnson (1992) found that acid-adapted cells of *Str. typhimurium* survived better than non-adapted cells during milk fermentation if they added the cultures to the fermenting milk when it had reached pH 5.1.

Vinderola *et al.* (2000) reported that viability in acidified milk was different to that demonstrated in yogurt, thus other physico-chemical factors may reduce or increase the

inhibitory power of lactic acid against probiotic cultures. Such factors may include the interaction between the yogurt starter and probiotic cultures, production of hydrogen peroxide (Hull *et al.*, 1984; Dave and Shah, 1997), production of acetic acids (Charalampopoulos *et al.*, 2002), final acidity of the product (Lourens-Hattingh and Viljoen, 2002) and the production of bacteriocins (Young and Nelson, 1978; Gilliland and Lara, 1988; Kurmann and Rasic, 1991; Costello, 1993; Bertoni, *et al.*, 1994). The viability also depends on the availability of nutrients, concentration of sugars, dissolved oxygen and oxygen permeation through the package, inoculation level and incubation, and fermentation and storage temperature (Gilliland and Lara, 1988; Dave and Shah, 1997a; Vinderola *et al.*, 2000). These factors may account for differences in viability observed in yogurt simulation trials versus full yogurt trials.

6.6.4 Yogurt trials following conditioning of probiotic strains in 10% non-fat dry milk versus MRS broth

Lb. acidophilus strain VUP 12,015 and *Lb. johnsonii* strain VUP 12,028 were grown to exponential phase at optimal conditions (pH 5.8, 39°C) prior to being conditioned at pH 4.5 in milk (reconstituted 10% non-fat dry milk) or MRS broth. Following conditioning, cells were re-suspended in 10% NFDM and, along with the primary inoculation of starter cultures *Str. thermophilus* and *Lb. bulgaricus*, used for the small-scale production of yogurt. The viability was monitored for six weeks following refrigerated storage of the yogurt. This experiment was performed to compare the behavior of probiotic strains in milk compared to MRS broth, since conditioning studies had been performed essentially in MRS broth, which is not an ideal medium to prepare cells for large-scale yogurt manufacture. In addition, cells grown in rich media in the laboratory probably do not accurately represent the physiology of cells found in a yogurt-manufacturing environment.

In general, conditioning of cells in either medium did not enhance viability of probiotic strains VUP 12,015 and VUP 12,028 compared to the control cells following six weeks of refrigerated storage. These results support the observation that conditioning cells using lactic acid does not enhance survival following small-scale yogurt production.

Interestingly, preparing cells in 10% non-fat dry milk prior to yogurt trials resulted in higher survival rates during refrigerated storage compared to initial counts than cells prepared in MRS broth. This may be a reflection of differences in the conditioning protocols in 10% NFDM versus MRS broth. Cells conditioned in MRS broth were centrifuged prior to conditioning and preparation for yogurt production, whereas, conditioning cells in 10% NFDM involved the manual lowering of the pH from optimal to moderate pH conditions. Therefore, the pH was not as tightly controlled for cells conditioned in 10% NFDM. Alternatively, the buffering capacity of the milk medium, differences in the composition of nutrients, differences in survival mechanisms or a reduction in the growth rate in the milk medium compared to the MRS broth may be responsible for higher survival rates of cells conditioned in 10% NFDM. Further studies could analyse the survival rates of probiotic cultures in yogurt following conditioning in MRS broth medium without pH control.

6.6.5 Inhibitory activity assays of yogurt starter cultures against probiotic cultures

During growth in fermented products, dairy starters including lactobacilli and streptococci have been reported to produce inhibitory metabolites such as organic acids, hydrogen peroxide and bacteriocins (Barefoot and Nettles, 1993). The production of such metabolites has been linked with the inhibition of growth of various organisms. For example, Barefoot *et al.* (1994) reported that the bacteriocin Lactacin B produced by *Lb. acidophilus* strain N2 is active against closely related lactobacilli, including *Lb. bulgaricus*. Agar well-diffusion assays were performed to determine whether there was any interference of yogurt starter cultures on the ability of probiotic cultures to survive in full yogurt trials. Culture filtrates were prepared by growing cultures in both MRS broth or 10% reconstituted skim milk as Shahani *et al.* (1976) reported that milk or its constituents were essential as nutrients in the medium for the organism to produce inhibitory compounds. Results reveal that growth of probiotic strains was uninhibited by culture filtrates from either *Lb. bulgaricus* or *Str. thermophilus*, regardless of whether the culture filtrates were prepared in MRS broth or 10% non-fat dry milk. This suggests that the fermentation end-products produced following 18 hours of incubation of the starter

cultures did not contain inhibitory products against probiotic strains. Similar findings were reported by Joseph *et al.* (1998), with seven strains of *Lb. acidophilus* displaying resistance to seven strains of *Str. thermophilus* and five strains of *Lb. bulgaricus*, as determined by with the absence of zones of inhibition on MRS agar following the agar well diffusion technique.

The production of hydrogen peroxide by *Lactobacillus* species is reported to be a mechanism for competitive antagonism of other organisms (Dahiya and Speck, 1968; Collins and Aramaki, 1980; Barefoot and Klaenhammer, 1983). The amount of production of hydrogen peroxide by *Lactobacillus* species is strain dependent (Collins and Aramaki, 1980) and reports show that maximum inhibitory activity is observed after five days of refrigerated storage (Collins and Aramaki, 1980). Culture filtrates were prepared by growing cultures overnight at 37°C. These preparation conditions may not have allowed enough time for production of metabolites at inhibitory concentrations.

In contrast, zones of inhibition were observed when *Str. thermophilus* was exposed to culture filtrates prepared from either probiotic cultures or *Lb. bulgaricus*. The inhibitory zones produced by supernatants of probiotic cultures were not as clear as those produced for *Lb. bulgaricus* and when these culture filtrates were neutralised with sodium hydroxide, growth of *Str. thermophilus* was uninhibited. This suggests that inhibition observed in initial experiments was largely due to low pH produced by fermentation end-products such as lactic acid. In contrast, when the culture filtrates obtained from a combination of *Str. thermophilus* and *Lb. bulgaricus* were neutralised with sodium hydroxide and also treated with 0.1 mg catalase, zones of inhibition of 10 to 11 mm were observed. This may indicate the presence of a bacteriocin-like inhibitory substance (BLIS) produced only when these two organisms are co-cultured. Further studies may involve the isolation and identification of this BLIS using poly-acrylamide gel electrophoresis, followed by N-terminal sequencing and comparison with published sequences for the confirmation of its identity.

6.7 CONCLUDING REMARKS

Exposing cells of *List. monocytogenes* and *Sal. typhimurium* to moderate pH values during exponential phase of growth can improve their survival during storage in dairy products (Leyer and Johnson, 1992; Gahan *et al.*, 1996). Results demonstrated that conditioning cells using lactic acid or culture filtrates enhanced survival of strains VUP 12,015 and VUP 12,028 during long-term refrigerated storage following simulation of yogurt production, but not following small-scale yogurt production using the starter cultures *Str. thermophilus* and *Lb. bulgaricus*.

Many factors have been reported to affect the viability of probiotic cultures in yogurt, including the interaction between the yogurt starter and probiotic cultures (Hull *et al.*, 1984; Dave and Shah, 1997a), and the production of bacteriocins (Young and Nelson, 1978; Gilliland and Lara, 1988; Kurmann and Rasic, 1991; Costello, 1993; Bertoni, *et al.*, 1994). Results showed that growth of probiotic strains was uninhibited by culture filtrates from either *Lb. bulgaricus* or *Str. thermophilus*, regardless of whether the supernatants were prepared in MRS broth or 10% non-fat dry milk. However, culture filtrates were prepared by growing cultures overnight at 37°C, which may not have allowed sufficient time for production of metabolites at inhibitory concentrations.

Overall, the results support further investigation into the potential for conditioning protocols that enhance the survival of probiotic strains in yogurt.

CHAPTER 7

CONCLUSIONS AND RECOMMENDATIONS

7.1 CONCLUSIONS

7.1.1 Differentiation, identification and characterisation of probiotic strains

Strains originally identified as *Lb. acidophilus* were screened for their ability to survive conditions equivalent to those found during transit through the gastrointestinal tract. Strains demonstrated the ability to tolerate 1.5% bile over three hours, whilst cells challenged at pH 2.5 showed variable survival patterns. Strains VUP 12,015 and VUP 12,028 showed the highest degree of tolerance to pH 2.5.

Further selection of strains was achieved by monitoring survival rates during six weeks of refrigerated storage in a milk-based medium. With the exception of strain VUP 12,005, VUP 12,007 and VUP 12,027, viability decreased below the minimum therapeutic dosage of 10^6 cells per gram of yogurt, with strain VUP 12,015 and VUP 12,028 showing the lowest survival rates.

Conventional microbiological identification, based on physiological and biochemical characteristics, was useful for pre-identification of strains used in this study. Carbohydrate fermentation patterns indicated that the strains were members of the *Lb. acidophilus* complex of species, whilst the antibiotics clindamycin, lincomycin and rifampicin increased the depth of differentiation of the strains tested by producing strain specific sensitivity patterns.

PFGE of *Sma*I restriction fragments is a valuable method for determining relationships between the probiotic strains. Most strains clustered into one group, sharing >70% similarity with the *Lb. acidophilus* type strain VUP 12,016, whilst strain VUP 12,028 shared only 24% resemblance with the type strain. The *Sma*I digestion patterns of strains VUP 12,016/VUP 12,015 and VUP 12,012/VUP 12,006/VUP 12,025 were indistinguishable using PFGE, but could be differentiated based on the results of other phenotypic techniques.

Identification of the nine probiotic strains was more precisely conducted using 16S rRNA gene sequence analysis. Strains purchased originally as *Lb. acidophilus* were formally identified by analysis of the V1 and V3 regions of the *Lb. acidophilus* complex. Based on this technique, strains VUP 12,002, VUP 12,012, VUP 12,005, VUP 12,006, VUP 12,015, VUP 12,007, VUP 12,027 and VUP 12,025 were confirmed as *Lb. acidophilus*, whilst strain VUP 12,028 was re-classified as *Lb. johnsonii*.

No single strain proved superior in terms of acid tolerance, bile tolerance and survival during long-term storage. *Lb. acidophilus* strain VUP 12,015 and *Lb. johnsonii* strain VUP 12,028 were selected for further studies based on their poor viability in a milk-based medium during long-term storage and their high survival rates at pH 2.5. For comparison, *Lb. acidophilus* strain VUP 12,005 was selected for its ability to maintain viability above the recommended level during long-term storage.

7.1.2 Short-term improvement in survival of probiotic strains in normally lethal acidic conditions

The optimal, moderate and lethal pH and temperature conditions were determined for representative strain VUP 12,005, with the aim of defining parameters for conditioning protocols. *Lb. acidophilus* strain VUP 12,005 grew over a range of pH 6.5 to pH 4.0 and between 30°C to 45°C. Cells grew optimally in pH 5.5 to 6.0 MRS broth maintained at 39°C.

Lb. acidophilus strain VUP 12,005 and VUP 12,015 and *Lb. johnsonii* strain VUP 12,028 display an acquired acid tolerance to normally lethal acidic pH conditions during growth at a non-lethal acid pH. The acquired acid tolerance response is optimally induced between pH 4.5 and 4.0, and the level of acquired tolerance is dependent on the pH of the culture, the conditioning time, and the strain being tested.

Transferring *Lb. acidophilus* VUP 12,015 to pH 4.5 for one hour provided maximum tolerance to challenge, with a 10-fold improvement in viability after five hours of incubation at pH 3.5. These results support further investigation into the use of

conditioning as a model system to improve the survival of probiotic cultures in yogurt during long-term refrigerated storage.

7.1.3 Analysis of the adaptive acid-tolerance response

Lactic acid was more efficient than hydrochloric acid at inducing survival mechanisms of *Lb. acidophilus* strains VUP 12,005 and VUP 12,015 during challenge at pH 3.5. Higher survival rates, as induced by lactic acid compared to hydrochloric acid, may be a result of the higher level of dissociation of lactic acid, which has been reported to cause a more extensive decrease in the internal pH than hydrochloric acid. Alternatively, lactic acid may induce a unique response system compared to hydrochloric acid.

Tolerance to a variety of challenges (60°C, hydrogen peroxide, ethanol, sodium chloride) can be induced in *Lb. acidophilus* during exponential growth by culturing cells for a doubling time at pH 4.5 prior to challenge. These findings have relevance for cells in environments where they can be exposed to combinations of stress conditions, such as those found during the production and storage of yogurt.

The acquired tolerance of *Lb. acidophilus* strain VUP 12,015, conditioned at pH 4.5 and subsequently challenged in 3M NaCl or 20% ethanol, was protein synthesis dependent, since the addition of a protein synthesis inhibitor prevented adaptation. In contrast, despite the presence of chloramphenicol during adaptation, cell survival improved during challenge at pH 3.5, pH 3.0, 10mM hydrogen peroxide and 60°C compared to control cells following conditioning at pH 4.5. Nonetheless, allowing *de novo* protein synthesis to continue during cell conditioning provided optimal improvements in survival. This suggests that *de novo* protein synthesis contributes to cross-resistance; however, other mechanisms such as the pH homeostasis system may also play a part in acquired tolerance responses.

A decrease in the pH during conditioning is dominated by the repression of a large number of proteins and a decline in the growth rates of the organism. In particular, the repression of a 60 kDa protein following conditioning of strain VUP 12,005 at moderate pH and a 123 kDa and 40.5 kDa protein following conditioning of strain VUP 12,015 at pH 4.5, below that expressed by the control, was observed. These

proteins may play a role in the protection of cells during challenge. Improvements in survival following adaptation may also result from a higher sensitivity of control cells compared to cells conditioned at lower pH values, due to a higher percentage of cells undergoing cell division.

7.1.4 Viability of probiotic cultures during long-term storage following conditioning

Survival mechanisms induced following conditioning at moderate pH not only improved survival of strains during short term challenge at low pH, but also during extended storage in conditions that simulated yogurt production. Conditioning cells using lactic acid enhanced the survival of strains VUP 12,015 and VUP 12,028, but not strain VUP 12,005 during six weeks of refrigerated storage in a yogurt-like environment. The greatest improvement in survival during refrigerated storage, a 7.4 fold increase, was observed following conditioning of *Lb. acidophilus* VUP 12,015 at pH 5.0 for one hour.

Cell filtrates were prepared by growing probiotic cultures at optimal conditions in MRS broth for 18 hours. Using cell filtrates as an alternative to lactic acid for conditioning cells enhanced the survival of strains VUP 12,015 and VUP 12,028, but not strain VUP 12,005 during long-term refrigerated storage in a yogurt-like environment.

Due to variations in growth requirements, no single medium could be used to isolate the starter cultures *Str. thermophilus*, *Lb. bulgaricus* and the probiotic cultures *Lb. acidophilus* and *Lb. johnsonii*. Studies on the colony morphology, Gram reaction and degree of growth of probiotic and starter cultures on a range of media provided valuable information that enabled the selection ST agar, RCA agar, MRS-Oxygall, MRS-Raffinose or MRS-Maltose agar for the enumeration of starter and probiotic cultures in yogurt.

Conditioning cells using lactic acid or cell filtrates did not enhance the survival of strains following small-scale yogurt production using the starter cultures *Str.*

thermophilus and *Lb. bulgaricus*. Therefore, the presence of the starter cultures had a negative impact on the viability of the probiotic cultures during refrigerated storage.

Overall, the results support further investigation into the potential for conditioning protocols that enhance the survival of probiotic strains in yogurt.

7.2 RECOMMENDATIONS FOR FURTHER WORK

7.2.1 Differentiation of probiotic strains using phenotypic procedures

In chapter three, cells were characterised and differentiated using a variety of techniques including carbohydrate fermentation analysis, antibiotic sensitivity tests and pulsed-field gel electrophoresis. Whilst these results provided valuable information, a comparison of these results with those from other strains representing the *Lb. acidophilus* complex including *Lb. crispatus*, *Lb. johnsonii*, *Lb. gasseri*, *Lb. amylovorus*, *Lb. gallinarum*, would provide a broader view of the potential of these techniques for identifying strains within the *Lb. acidophilus* complex.

7.2.2 Analysis of the adaptive acid tolerance response

Genetic regulation

Analyses of adaptive responses are advancing towards examination of gene expression at the transcriptional level using Microarray analysis or *via* more traditional techniques involving PCR amplification and sequencing, followed by Northern blot analysis (Walker *et al.*, 1999; Broadbent *et al.*, 1998). The identification of stress-related genes will reveal targets for specific manipulation, to develop tools to screen for tolerant and sensitive strains and to evaluate the fitness of a culture for use in probiotic products.

Protein synthesis

Although results from one-dimensional gel electrophoresis provided valuable information, analysis of protein induction using two-dimensional gel electrophoresis, as has been reported for several *Lactobacillus* species (Broadbent *et al.*, 1997; Oberg *et al.*, 1998; Laplace *et al.*, 1999; Prasad *et al.*, 2003) may provide a more in depth

analysis of the induction or repression of specific proteins during conditioning at moderate pH values.

The repression of 123, 60 and 40.5 kDa proteins during conditioning of probiotic strains at moderate pH for one hour may be associated with improvements in survival observed during challenge. To elucidate the role of these proteins in the adaptive acid tolerance response, purification, followed by N-terminal sequencing of the proteins is necessary to determine their homology with previously identified stress proteins. In addition, analysis of protein induction or repression at several time points during the one hour conditioning period may highlight the transient production of proteins that disappear before the conditioning period is complete.

Regulation of intracellular pH

Lactic acid was more efficient than hydrochloric acid at inducing survival mechanisms of *Lb. acidophilus* strains VUP 12,005 and VUP 12,015 during challenge at pH 3.5 (Chapter 5). The higher survival rates induced by lactic acid may be a result of the higher level of dissociation of lactic acid, which has been reported to cause a more extensive decrease in the internal pH than hydrochloric acid. These results, coupled with the observation that adaptive responses inducing cross-protection were not entirely dependent on protein synthesis, suggest that, in addition to protein synthesis, cells may protect themselves at low pH by using physiologic processes that form part of the pH homeostasis system, such as an increase in F₁-F₀ ATPase activity following transfer of *Lb. acidophilus* CRL 639 to pH 4.2 for 15 minutes (Lorca and de Valdez, 2001a).

Cell morphology

Analysis of microscopic changes following a conditioning step at moderate pH values may provide an indication of the adaptive ability of probiotic species. Jan *et al.* (2001) reported that cells that had undergone adaptation at pH 5.0 prior to challenge at pH 2.0 displayed a similar morphology to cells at pH 7.0, whilst cells transferred from pH 7.0 to pH 2.0 changed from pleomorphic rods to segmented rods with 'bleb-like' structures on their surface. Therefore, examination of the cell morphology post-adaptation may provide an initial screening procedure for assessing the potential of probiotic species to adapt to conditions that are normally detrimental to survival.

Stability of adapted culture

Results presented in Chapter 4 demonstrate that cells are able to 'adapt' to adverse conditions following their incubation in moderate pH environments. However, the responses to external stress may be transiently expressed and when cells are cultured in optimal conditions, they may revert back to their 'sensitive' phenotype. Prior to large-scale yogurt production, it is necessary to determine whether 'adapted' cells will revert back to their normal phenotype upon transfer to normal media/growth conditions.

Effect on therapeutic properties

Preliminary studies on the cellular response to conditioning at moderate pH were reported in Chapter 5. Protein profiling and protein radiolabelling showed the induction and repression of specific proteins during conditioning at moderate pH. Cell conditioning at pH 4.5 also improved the survival of strains during challenge at normally lethal concentrations of hydrogen peroxide, ethanol, sodium chloride and temperature conditions. Therefore, it is necessary to screen strains following conditioning at moderate pH and prior to their consumption to ensure that they have maintained their therapeutic properties, such as bacteriocin production, β -galactosidase activity, the ability to lower serum cholesterol levels etc.

7.2.3 Factors affecting survival of probiotic cultures following small-scale yogurt production

Production of inhibitory substances

The potential of the starter cultures *Str. thermophilus* and *Lb. bulgaricus* to produce inhibitory products that reduce the viability of probiotic organisms during refrigerated storage in yogurt was explored in Chapter 6. Cell filtrates from each culture were prepared following overnight incubation in MRS broth or milk medium and the agar well diffusion assay was performed. Results showed that the starter cultures did not produce inhibitory products against the probiotic cultures. However, overnight incubation of starter cultures may not be adequate time to generate products at concentrations necessary to inhibit growth of probiotic cultures. Therefore, these

experiments could be repeated using cell filtrates that have been prepared over extended periods, providing a more accurate reflection of the relationship between starter and probiotic cultures in yogurt during refrigerated storage.

Adaptation using fermentation end-products

Using cell-free supernatants for conditioning cells enhanced the survival of strains VUP 12,015 and VUP 12,028 during refrigerated storage in a milk-based medium but not following small-scale yogurt production. Cell-free supernatants were prepared by growing probiotic cultures in batch culture in MRS broth for 18 hours. Preparing cell-free supernatants using a combination of starter cultures and probiotic cultures may provide a more realistic challenge during conditioning, since it is evident that the presence of the starter cultures during small-scale yogurt production has an impact on the survival of probiotic strains.

Changing the parameters for yogurt production

Survival of probiotic cultures in a yogurt-simulated environment can be improved if cells are first adapted at a moderate pH. However, this protective effect is reduced when cells are challenged with the small-scale production of yogurt using the starter cultures *Str. thermophilus* and *Lb. bulgaricus*. Previous reports have shown that the viability of probiotic organisms can be affected by the choice of starter cultures (Vinderola *et al.*, 2000). Counts of the probiotic *Lb. acidophilus* are generally higher in products that do not contain *Lb. bulgaricus* (Gilliland and Speck, 1977; Dave and Shah, 1997a; Rybka and Fleet, 1997). Therefore, the preparation of yogurts using various combinations of starter cultures, with and without *Lb. bulgaricus*, would facilitate efforts to improve the survival of conditioned probiotic species in yogurt during refrigerated storage. In addition, starter cultures could be selected following pre-screening for their ability to produce inhibitory products that reduce the viability of probiotic organisms during refrigerated storage in yogurt.

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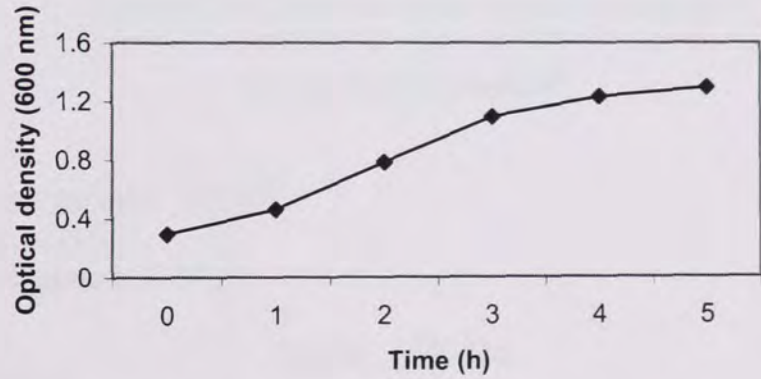
APPENDIX A

Determination of growth phase of *Lb. acidophilus* strain VUP 12,005 during the standard protocol for conditioning cells (refer to section 2.5.13).

Table A.1. Optical density and viable counts of *Lb. acidophilus* strain VUP 12,005 inoculated into pre-warmed MRS broth (as per section 2.5.13).

Time (hours)	Optical Density (600 nm)	Viable counts (x 10 ⁸ cfu/ml)
0	0.294	0.2
1	0.463	0.6
2	0.785	1.8
3	1.095	3.6
4	1.230	5.7
5	1.294	6.0

a)



b)

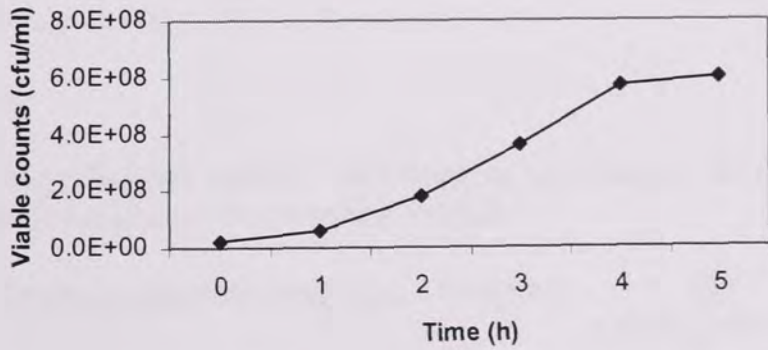


Figure A.1. Optical density (a) and viable counts (b) of *Lb. acidophilus* VUP 12,005 inoculated from an overnight culture into flasks containing MRS broth at 39° C.

APPENDIX B

Determining the activity in disintegrations per minute (dpm) of ^{35}S -labelled cysteine-methionine during protein synthesis of *Lb. acidophilus* strains.

^{35}S standard activity:

Standard activity is based on 5 μl of a 1:1000 dilution of ^{35}S at 98% activity.

1 μCi = 2.22×10^6 dpm at 100 % activity.

At 98% activity: $2.22 \times 10^6 \times 0.98 = 2.17 \times 10^6$ dpm/ μCi

Original Stock: 10.5 mCi/ml = 10.5 $\mu\text{Ci}/\mu\text{l}$.

At 1:1000 dilution of the original stock = 0.0105 $\mu\text{Ci}/\mu\text{l}$

Therefore 5 μl ^{35}S = 0.0525 μCi

2.17×10^6 dpm/ μCi (at 98% activity) $\times$ 0.0525 μCi

Known dpm of standard = 113,925 dpm

Counter Efficiency:

$$\text{Counting Efficiency} = \frac{(\text{cpm of standard} + \text{sample}) - (\text{cpm of sample})}{\text{known dpm of standard}} \times 100$$

For example:

cpm of standard + sample: 461907

cpm of sample: 362524

known dpm of standard: 113925

$$\text{Counting Efficiency} = \frac{461907 - 362524}{113925} \times 100$$

Counting efficiency = 87%

Sample activity:

From the counting efficiency and the cpm values for each sample, the dpm for each sample was determined using the following formula

$$\text{Dpm (sample)} = \text{average cpm of sample} \times \frac{100}{\text{counting efficiency}}$$

For example:

Average cpm of sample: 362524

Counting efficiency: 87%

$$\text{Dpm of sample} = 362524 \times \frac{100}{87} = \text{Sample activity: 416694 dpm.}$$