EVALUATION OF THE GROWTH AND SURVIVAL OF PROBIOTIC MICROORGANISMS IN COMMERCIAL BIO-YOGURT

EVALUATION OF THE GROWTH AND SURVIVAL OF PROBIOTIC MICROORGANISMS IN COMMERCIAL BIO-YOGURT

by

ANALIE HATTINGH (neè LOURENS)

Submitted in fulfilment of the requirements for the degree of

PHILOSOPHIAE DOCTOR

in the

Faculty of Natural Sciences and Agriculture, Department of Microbiology and Biochemistry

University of the Orange Free State, Bloemfontein

November 2000

Supervisor: **Prof. B.C. Viljoen**

Every calling is great when greatly pursued.

Oliver Wendell Holmes

Dedicated to my parents,

Fanie & Corrie Lourens, and my husband, Pieter Hattingh.

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude and appreciation to the following persons and institutions for their contributions to the succesful completion of this study:

All the recognition and praise to the Father, Son and Holy Spirit, who enabled me to do and complete this study;

Prof. B.C. Viljoen, Department of Microbiology and Biochemistry, University of the Free State, for his able guidance during the study, constructive criticism of the manuscript and for working in the Food Biotechnology laboratory during the study;

Prof. P.J. Jooste, Department Animal Products and Food, ARC, Irene, for his guidance during the study and constructive criticism of the manuscript;

Judit Tornai Lehocski, Head of the Department, University of Food Science and Horticulture, Budapest, Hungary, for permitting me to work in their laboratory for one month during this study.

Gabor Peter, University of Food Science and Horticulture, Budapest Hungary, for his able guidance and assistance on the yeast identification.

Mr. Piet Botes, for his able technical assistance with the gas chromatographic analysis;

The National Research Foundation (NRF), for financial assistance;

My family and friends, for all their interest and encouragement;

My husband Pieter Hattingh for his care, patients and love during the study;

Finally to my parents, to whom I dedicate this thesis as a token of appreciation for the opportunity of a study career.

LIST OF ABBREVIATIONS

Anon. :	Anonymous (author)
AB:	live acidophilus and bifidobacteria culture
Ac-MRS:	Acidified MRS
cfu:	colony forming units
CRM:	Callichia et al's resuspension medium
d:	day(s)
Fig(s). :	Figure(s)
g :	gram
h:	hour
hrs:	hours
DI:	distilled
LAB:	Lactic acid bacteria
min:	minute(s)
ml:	milliliter
M-MRS:	Maltose-MRS
μm:	micrometer
w/v:	weight per volume

LIST OF CHAPTERS ACCEPTED AND SUBMITTED FOR PUBLICATION:

Chapter 2:

Lourens, A. and Viljoen, B.C. (2000) Yogurt as probiotic carrier food – A review. *International Dairy Research* - accepted.

Chapter 4:

Lourens, A., Viljoen, B.C. and Jooste, P.J. (2000) Level of probiotic bacteria in South African commercial bio-yogurt. *South African Food and Beverage manufacturing Review.* **27**(2), 31 – 33.

Chapter 5:

Lourens, A., Viljoen, B.C. and Jooste, P.J. (2000) Survival of probiotic bacteria in South African commercial bio-yogurt *South African Journal of Science.* – accepted.

Chapter 6:

Lourens, A. and Viljoen, B.C. (2000) Growth and survival of a probiotic yeast in dairy products. *Food Research International* – accepted.

Chapter 7:

Lourens, A. and Viljoen, B.C. (2000) Growth and survival of dairy-associated yeasts in yogurt and yogurt-related products. *International Journal of Food Microbiology* – accepted.

Chapter 8:

Lourens, A. and Viljoen, B.C. (2000) Enhancement of the viability of probiotic bacteria in bio-yogurt: the effect of *Debaryomyces hansenii* and *Yarrowia lipolytica. International Journal of Food Microbiology* – submitted.

Chapter 9:

Lourens, A. and Viljoen, B.C. (2000) Enhancement of *Bifidobacteria* by neokestose in bio-yogurt. *International Journal of Food Microbiology* - submitted.

TABLE OF CONTENTS

CHAPTER 1

INTRODUCTION

For centuries it has been recognised that diet and health are inextricably linked. Metchnikoff and Tissier were the first to prescribe 'bacteriotherapy' in 1908, embracing the proper treatment of diseases by the ingestion of live probiotic microorganisms (Hugh and Hoover, 1991). These probiotics, by definition, are mono- or mixed cultures of live microorganisms which beneficially affect the host by serving the purpose of regulating the microbial colonisation in the digestive tract (e.g. as dried cells or as fermented products) (Huis in't Veld and Havenaar, 1991). In relation to fermented dairy products, probiotic starter cultures add an extra nutritional-physiological value including a range of metabolites, partly degraded product constituents, various inhibitors, stimulants, enzymes and coenzymes leading to the increase of nutritional value, antioxidant properties as well as therapeutic and health effects (Jakobsen and Narvhus, 1996). The subsequent inclusion of these microbial populations with probiotic properties, has led to the innovation of value-added food products, which have the potential to improve health and reduce risk of some important diseases. The microorganisms predominantly associated with the optimum balance in microbial populations in the digestive system, are lactobacilli and bifidobacteria.

The most active area of functional food development has been the application of probiotics to yogurt, commonly referred to as bio-yogurt. Strains of *Lactobacillus acidophilus* and *Bifidobacterium* species are used in addition to the traditional yogurt bacteria, *Streptococcus thermophilus* and *Lactobacillus bulgaricus* during the production of bio-yogurt. Adequate numbers of viable cells, namely the 'therapeutic minimum', need to be consumed for transfer of

the 'probiotic effect' to consumers. The suggested minimum level for probiotic bacteria in yogurt is more than a million cells per ml (10^6 cfu/ml) (Rybka and Kailasapathy, 1995). Poor survival of probiotic species in yogurt during retail storage, however, is a major constraint in the advancement of new fermented dairy products (Gilliland and Speck, 1977; Hull and Roberts, 1984; Klaver et al., 1993; Rybka and Kailasapathy, 1995; Nigswonger et al., 1996).

Consequently, it has been considered relevant to study the levels and survival rates of probiotic bacteria incorporated in commercial South African bio-yogurt. In order to study the incidence of the probiotic bacteria in the presence of the conventional starter cultures, it is imperative to standardize enumeration methods for microbial analyses. Accordingly, existing media proposed for the selective enumeration of starter cultures employed in the manufacture of bio-yogurt are compared and evaluated.

Subsequently, the levels of viable cell numbers of probiotic bacteria present in commercial South African AB-yogurt could be determined. Different commercial brands of AB-yogurt are obtained from different supermarket outlets. The results obtained from the yogurts are statistically compared based on the incidence and the maintenance of probiotic bacteria with respect to the 'therapeutic minimum'.

Growth and survival of probiotic bacterial cultures in commercial AB-yogurt and the influence of temperature abuse on the viability of the cultures are established by obtaining bio-yogurt from different manufacturers and storage at normal and elevated temperatures.

Out of these results attempts are, therefore, made to enhance growth and survival of probiotics in dairy products such as the addition of yeast and prebiotics to bio-yogurt.

The poor survival of probiotic bacteria is mainly due to the low pH of the yogurt. The ability of yeasts to utilise organic acids and thereby increasing the pH of the yogurt may create a more favourable environment for probiotic bacteria growth. The application of yeasts in association with lactic acid bacteria in various fermented dairy products, like acidophilus-yeast milk, kefir, laban, etc. has been implemented successfully (Subramanian and Shankar, 1985).

Firstly the possibility of growing a probiotic yeast in association with probiotic bacteria resulting in the stimulation of growth of the probiotic cultures is investigated. In this study the probiotic yeast species, *S. boulardii*, is added to yogurt simultaneously with the conventional yogurt starter cultures and probiotic cultures.

In order to further study the effect of yeast growth in yogurt on the progression of probiotic bacteria, it is imperative to assess the ability of yeast isolates to grow and survive in yogurt. Accordingly, the growth of several dairy associated yeasts in association with probiotic bacteria are investigated with the intention to stimulate the growth of the probiotic organisms and to assure their survival.

Based on the previous study *Yarrowia lipolytica* and *Debaryomyces hansenii* is incorporated into bio-yogurt and the possible influence of *Yarrowia lipolytica* and *Debaryomyces hansenii* on the growth and survival of probiotics in bio-yogurt is examined.

The addition of prebiotics should encourage the growth and survival of the probiotic bacteria, due to a more readily available and specific substrate for utilisation, as well as the individual advantages that each should offer (Fooks et al., 1999). Therefore in the last research chapter, the possible enhancement of viability of *Bifidobacteria* is assessed in commercial AB-yogurt fortified with 1%, 2% and 3% neokestose.

The main objectives of the present study will, therefore, be to:

1. Construct an adequate review of the literature explaining, in essence, the concept of 'therapeutic minimum' levels and the importance of the survival of probiotic microorganisms in food products;

2. Standardize methods for enumeration and identification of *L. acidophilus*, *B. bifidum*, and *S. thermophilus* and *L. bulgaricus* to eventually monitor their levels and survival in commercial South African AB-yogurts;

3. Establish the levels of probiotic bacteria in South African AB-yogurts with respect to the 'therapeutic minimum';

4. Determine the survival of AB culture and yogurt organisms in commercial yogurt;

5. Evaluate the growth and survival of the probiotic yeast, *Saccharomyces boulardii*, in bio-yogurt;

6. Evaluate the growth and survival of dairy associated yeasts in yogurt and yogurt-related products;

7. Study the effect of incorporation of *Debaryomyces hansenii* and *Yarrowia lipolytica* on the growth and survival of probiotic bacteria in bio-yogurt;

8. Enhance the growth and survival of bifidobacteria through the addition of a prebiotic, neokestose, to bio-yogurt.

REFERENCES

Fooks, L.J., Fuller, R. and Gibson, G.R. (1999) Prebiotcs, probiotcs and human gut microbiology. *International Dairy Journal.* **9**, 53 - 61.

Gilliland, S.E. and Speck, M.L. (1977) Instability of *Lactobacillus acidophilus* in yogurt. *Journal of Dairy Science.* **60**, 1394 - 1398.

Hughes, D.B. and Hoover, D.G. (1991) Bifidobacteria: Their potential for use in American dairy products. *Food Technology.* **45**(4), 74 - 83.

Huis in't Veld, J.H.J. and Havenaar, R. (1991) *Journal of Chemical Technology and Biotechnology.* **51**, 562 - 567.

Hull, R.R. and Roberts, A.V. (1984) Differential enumeration of *Lactobacillus acidophilus* in yogurt. *The Australian Journal of Dairy Technology.* **39**, 160 - 163.

Jakobsen, M. & Narvhus, J. (1996) Yeasts and their beneficial and negative effects on the quality of dairy products. *International Dairy Journal.* **6**, 75 - 768.

Klaver, F.A.M.; Kingma, F. and Weerkamp, A.H. (1993) Growth and survival of bifidobacteria in milk. *Netherlands Milk Dairy Journal.* **47**, 151 -164.

Nighswonger, B.D.; Brashears, M.M. and Gilliland, S.E. (1996) Viability of *Lactobacillus acidophilus* and *Lactobacillus casei* in Fermented Milk Products During Refrigerated Storage. *Journal of Dairy Science.* **79**, 212 - 219.

Rybka, S. and Kailasapathy, K (1995) The survival of culture bacteria in fresh and freeze-dried AB yoghurts. *The Australian Journal of Dairy Technology.* **50** (2), 51 - 57.

Subramanian, P. & Shankar, P.A. (1985) Commensalistic interaction between *L. acidophilus* and lactose-fermenting yeast in the preparation of acidophilus-yeast. *Cultured dairy product Journal*. **Nov**, 17 - 26.

CHAPTER 2

YOGURT AS PROBIOTIC CARRIER FOOD – A REVIEW

ABSTRACT

This paper reviews the history of the development of probiotics and provides a comprehensive overview on the potential health effects on the human gastro intestine. The paper also briefly reviews the concepts of prebiotics and synbiotics. Furthermore, the application of probiotics to yogurt commonly referred to as bio-yogurt and the effectiveness of yogurt as probiotic carrier food are discussed. In essence, the concept of 'therapeutic minimum' levels according to literature are explained, and the importance of the survival of probiotic microorganisms in food products. The production of bio-yogurt, regulatory requirements of a probiotic organism, technical considerations for incorporating probiotic microorganisms into yogurt and starter culture technology are also reviewed. Media for differential enumeration of probiotic and yogurt organisms is presented. The typical poor growth of probiotic organisms in yogurt is highlighted, and factors affecting the survival of probiotic species in yogurt during retail storage. Use of growth factors and efforts to establish optimum manufacture and environmental conditions for their growth are also reviewed.

1. INTRODUCTION

Interest in the role of probiotics for human health goes back at least as far as 1908 when Metchnikoff suggested that man should consume milk fermented with lactobacilli to prolong life (Hugh and Hoover, 1991; O' Sullivan et al., 1992). It is only recently, however, that the interrelationship between intestinal microorganisms and the health benefits deriving from it are beginning to be understood. At present it is generally recognised that an optimum 'balance' in microbial population in our digestive tract is associated with good nutrition and health (Rybka and Kailasapathy, 1995). The microorganisms primarily associated with this balance are lactobacilli and bifidobacteria. Factors that negatively influence the interaction between intestinal microorganisms, such as stress and diet, lead to detrimental effects in health. Increasing evidence indicates that consumption of 'probiotic' microorganisms can help maintain such a favourable microbial profile and results in several therapeutic benefits. In recent years probiotic bacteria have increasingly been incorporated into foods as dietary adjuncts. One of the most popular dairy products for the delivery of viable *Lactobacillus acidophilus* and *Bifidobacterium bifidum* cells is bio-yogurt. Adequate numbers of viable cells, namely the 'therapeutic minimum'-need to be consumed regularly for transfer of the 'probiotic' effect to consumers. Consumption should be more than 100g per day of bio-yogurt containing more than 10^6 cfu/ml (Rybka and Kailasapathy, 1995). Survival of these bacteria during shelf life and until consumption is therefore an important consideration.

2. BACKGROUND ON PROBIOTICS

2.1. History

The history recording the beneficial properties of live microbial food supplements such as fermented milks dates back many centuries. Their use in treatment of body ailments has been mentioned even in Biblical scriptures. Known scientists in early ages, such as Hippocrates and others considered fermented milk not only a food product but a medicine as well. They prescribed sour milks for curing disorders of the stomach and intestines (Oberman, 1985).

At the beginning of the twentieth century, the Russian bacteriologist Eli Metchnikoff (Pasteur Institute, France) was the first to give a scientific explanation for the beneficial effects of lactic acid bacteria present in fermented milk (Hugh and Hoover, 1991; O' Sullivan et al., 1992). He attributed the good health and longevity of the Bulgarians to their consumption of large amounts of fermented milk, called *yahourth.* In 1908 he postulated his ' longevity-without-aging' theory. The principle of his theory was that the lactic acid bacteria resulted in the displacement of toxin producing bacteria normally present in the intestine resulting in prolonged life. Metchnikoff explained that owing to lactic acid and other products produced by lactic acid bacteria in sour milks, the growth and toxicity of anaerobic, spore-forming bacteria in the large intestine are inhibited.

Almost at the same time, in 1899, Tissier (Pasteur Institute, France) isolated bifidobacteria from the stools of breast-fed infants and found that they were a predominant component of the intestinal flora in humans (Ishibashi and Shimamura, 1993). Tissier recommended the administration of bifidobacteria to infants suffering from diarrhea, 'believing' that the bifidobacteria would displace putrefactive bacteria responsible for gastric upsets, while re-establishing themselves as the dominant intestinal microorganisms (O'Sullivan

et al., 1992).

Studies on the use of lactic cultures in foods continued throughout the century. Many reports since then have yielded variable results with regard to the benefits of consuming probiotic foods. Earlier work dealt with the use of fermented milk to treat intestinal infections. More recent studies have focused on other aspects of health benefits that might be derived from these organisms, as well as strain selectivity to ensure survival of these bacteria in the gastrointestinal tract and the carrier food.

2.2. Definition of 'probiotics'

The word 'probiotic', derived from the Greek language, means 'for life' (Fuller, 1989) and has had many definitions in the past. Definitions such as 'substances produced by protozoa that stimulate the growth of another' or 'organisms and substances that have a beneficial effect on the host animal by contributing to its intestinal microbial balance' were used. These general definitions were unsatisfactory because 'substances' include chemicals such as antibiotics. The definition of probiotics has since then been expanded to stress the importance of live cells as an essential component of an effective probiotic. Most recently Huis in't Veld and Havenaar (1991) broadened the definition of probiotics as being 'a mono- or mixed culture of live microorganisms which, applied to man or animal (e.g. as dried cells or as a fermented product), beneficially effects the host by improving the properties of the indigenous microflora. This definition implies that probiotic products, for example bio-yogurt, contain live microorganisms and improve the health status of the host by exerting beneficial effects in the gastrointestinal tract.

2.3. Human gastrointestinal ecology

The total mucosal surface area of the adult human gastrointestinal tract is up to $300m^2$, making it the largest body area interacting with the environment

(Collins et al, 1998). The intestinal tract constitutes a complex ecosystem of microorganisms; more than 400 bacterial species have been identified in the faeces of a single subject (Finegold et al., 1977). The bacterial population in the large intestine is very high and reaches maximum counts of 10^{12} cfu/g of gut contents. In comparison to other regions of the gastrointestinal tract, the human large intestine is a complex, heavily populated and diverse microbial ecosystem (Fooks et al, 1999). In the small intestine the bacterial content is considerably lower, 10^4 to10^8 cfu/g, while in the stomach only 10^1 to 10^2 cfu/g are found due to the low pH (Hoier, 1992).

Considerable changes in the intestinal microflora occur from the day a baby is born until he or she becomes an adult. Benno et al. (1984) and others studied the development of intestinal microflora in newborn babies and the changes occurring with age. The intestine of a newborn infant is devoid of intestinal flora, but immediately after birth colonisation by many bacteria begins. Within one to two days, coliforms, enterococci, clostridia and lactobacilli are detected in the faeces; within three to four days, bifidobacteria appear and become predominant around the fifth day. The coliforms and other bacteria are restricted and decrease in response to the increase of bifidobacteria (Fig. 2.1.). Bifidobacteria counts of 10^{10} to 10^{11}cfu/g faeces are common in breast-fed infants (Modler et al., 1990) representing 25% of the intestinal bacteria. Lactococci, enterococci and coliforms represent less than 1% of the intestinal population, and normally Bacteroides, clostridia and other organisms are absent (Rasic, 1983). Bottle-fed babies normally have 1-log count less of bifidobacteria (10^9 – 10^{10}/g) present in their faecal samples than breast-fed babies (Braun, 1981), and there is a tendency for bottle-fed babies to have higher levels of enterobacteriaceae, streptococci, and other putrefactive bacteria (Yuhara et al., 1983). This suggests that breast-fed infants are more resistant to infections than bottle-fed infants due to antibacterial substances produced by bifidobacteria.

With weaning and ageing of the human being, gradual changes in the intestinal flora profile occur. The proportion of bifidobacteria declines to represent the third most common genus in the gastrointestinal tract; Gram negative rods belonging to the *Bacteroides fragilis* group predominates at 86% of the total flora in the adult gut, followed by *Eubacterium* (Finegold et al, 1977; Fooks et al, 1999). In addition, infant type bifidobacteria, *B.bifidum*, are replaced with adult type bifidobacteria, *B. longum* and *B. adolescentis*. This change in profile may be facilitated by the intake of bifidogenic factors (Modler et al, 1990). The adult type flora is rather stable but during the middle and again at an older age the intestinal flora changes again. Bifidobacteria decrease even further while certain kinds of harmful bacteria increase (Benno et al, 1984). For example, a dramatic decrease in the number of bifidobacteria and an increase in *Clostridium perfringens*, causes diarrhoea in elderly persons (Hoier, 1992).

The complex composition of the intestinal flora is relatively stable in healthy human beings. Any disturbance in this balance results in changes in the intestinal flora, which consequently allows undesirable microorganisms to dominate in the intestine and as a result leads to a number of clinical disorders, including cancer, inflammatory disease, ulcerative colitis, whilst making the host more susceptible to infections by transient enteropathogens like *Salmonella, Campylobacter, Escherichia coli* and *Listeria* (Fooks et al, 1999). Maintenance of the intestinal "balance" appears to be increasingly difficult as lifestyles change. Changes in the intestinal flora are not only due to ageing but also by extrinsic factors e.g. stress, diet, drugs, bacterial contamination and constipation (Hoier, 1992). However, this "balance" can be maintained through increased predominance of bacteria such as lactobacilli and bifidobacteria in the gut.

Interestingly, in 1987, Mitsuoka proposed a hypothetical scheme in which he illustrates the interrelationship between intestinal bacteria and human health (Fig. 2.2.) (Ishibashi and Shimamura, 1993). The intestinal bacteria were

classified into three categories, namely harmful, beneficial, or neutral with respect to human health. Among the beneficial bacteria are *Bifidobacterium* and *Lactobacilli.* Harmful bacteria are *Escherichia coli*, *Clostridium*, *Proteus* and types of *Bacteroides*. These bacteria produce a variety of harmful substances, such as amines, indole, hydrogen sulfide, or phenols, from food components and cause certain intestinal problems. These bacteria could also occasionally be potentially pathogenic (Ishibashi and Shimamura, 1993).

2.4. Therapeutic value

The claimed beneficial effects from consumption of fermented milks were once a very debatable issue. Research conducted since the turn of the century has however, enhanced the understanding of the resulting therapeutic effects and it is currently widely recognized as wholesome. The consumption of probiotic products is helpful in maintaining good health, restoring body vigour, and in combating intestinal and other disease orders (Mital and Garg, 1992). A list of the main therapeutic benefits attributed to consumption of probiotics is indicated in Table 2.1. Most scientific papers refer to research using *L.acidophilus* and *Bifidobacterium* species as dietary cultures.

2.4.1. Control of intestinal infections

Probiotic bacteria such as bifidobacteria and lactobacilli possess antimicrobial properties (Hugh and Hoover, 1991). Both *L.acidophilus* and *B.bifidum* have been shown to be inhibitory towards many of the commonly known food borne pathogens (Gilliland and Speck, 1977a; Gilliland, 1979; Lim et al., 1993; Rasic and Kurmann, 1983, Sandine, 1979). Several studies indicated the preventative control of intestinal infections through administering milk cultured with *L.acidophilus* or *B.bifidum* or both (Rasic and Kurmann, 1983, Gorbach et al, 1987).

Mechanisms for the inhibition of pathogens ascribed to lactobacilli and bifidobacteria include:

- the production of inhibitory/antimicrobial substances such as: organic acids, hydrogen peroxide, bacteriocins, antibiotics and deconjugated bile acids
- their acting as competitive antagonists i.e. competition for adhesion sites and nutrients
- stimulation of the immune system

Production of organic acids by the probiotics lowers the pH and alters the oxidation-reduction potential in the intestine, resulting in antimicrobial action. Combined with the limited oxygen content in the intestine, organic acids inhibit especially pathogenic Gram-negative bacteria types e.g. coliform bacteria (Sandine, 1979). Bifidobacteria produce both lactic and acetic acids, but higher amounts of acetic acid are produced which exhibits a stronger antagonistic effect against Gram-negative bacteria than lactic acid (Rasic, 1983).

Probiotic microorganisms may prevent harmful bacterial colonisation of a habitat by competing more effectively than an invading strain for essential nutrients or adhesion sites or by making the local environment unfavourable for the growth of the invader by producing antibacterial substances (Sandine, 1979, Gurr, 1987). Regular consumption of probiotic bacteria may induce an improved immunological response in humans (Rasic, 1983).

2.4.2. Reducing lactose intolerance

The inability to digest lactose adequately by certain people is due to the absence of β-D-galactosidase in the human intestine and this leads to various degrees of abdominal discomfort (Kim and Gilliland, 1983). Lactic acid bacteria used as starter cultures in milk and fermentation, and probiotic bacteria such as *L. acidophilus* and *B. bifidum* produce β-D-galactosidase. This enzyme

hydrolyses lactose, which results in increased tolerance for dairy products (Kim and Gilliland, 1983). This utilisation is ascribed to intra-intestinal digestion by β-D-galactosidase.

Kim and Gilliland (1983) investigated the effect of *L. acidophilus* as a dietary adjunct in milk to aid lactose digestion in humans. They found that improved digestion of lactose was not caused by hydrolysis of the lactose prior to consumption, indicating that the beneficial effect must have occurred in the digestive tract after consumption of milk containing *L. acidophilus*. The continued utilisation of lactose within the gastrointestinal tract depends on the survival of the lactobacilli in that environment.

2.4.3. Reduction in serum cholesterol levels

There are claims that consumption of fermented milk significantly reduces serum cholesterol (Gilliland et al. 1985, Gilliland, 1989, Mann and Spoerry, 1974). For hypercholesterolemic individuals, significant reductions in plasma cholesterol levels are associated with a significant reduction in the risk of heart attacks.

The principal site of cholesterol metabolism is the liver, although appreciable amounts are formed in the intestines. Claims are strong that certain *Lactobacillus acidophilus* strains and some bifidobacteria species are able to lower cholesterol levels within the intestine. Cholesterol co-precipitates with deconjugated bile salts as the pH declines as a consequence of lactic acid production by the lactic acid bacteria (Marshall, 1996). The role that bifidobacteria cultures may play in lowering serum cholesterol is not yet understood. In rat models, serum cholesterol was lowered by feeding of bifidobacteria in a mechanism that may involve HMG-CoA reductase (Homma, 1988). In this respect Gilliland (1989) reports on various experiments that conclude that a factor is produced in the fermented milk that inhibits cholesterol synthesis in the body.

Another theory is that *L. acidophilus* deconjugates bile acids into free acids, which are excreted more rapidly from the intestinal tract than are conjugated bile acids. As free bile salts are excreted from the body, the synthesis of new bile acids from cholesterol can reduce the total cholesterol concentration in the body (Gilliland and Speck, 1977b). A third hypothesis is that reduction of cholesterol may also be due to a co-precipitation of cholesterol with deconjugated bile salts at lower pH values as a result of lactic acid production by the bacteria (Kailasapathy and Rybka, 1997).

Deconjugation of bile acids can result in the formation of cytotoxic secondary bile salts (Marshall, 1996). The net effect of the probiotic activity towards cholesterol control is therefore questionable.

2.4.4. Anticarcinogenic activity

The anti-tumour action of probiotics is attributed to the inhibition of carcinogens and/or procarcinogens, inhibition of bacteria that convert procarcinogens to carcinogens (Gilliland, 1989; Gorbach et al, 1987), activation of the host's immune system (Rasic, 1983) and/or reduction of the intestinal pH to reduce microbial activity.

Kailasapathy and Rybka (1997) reported on several animal studies confirming that the intake of yogurt and fermented milks containing probiotic bacteria inhibited tumour formation and proliferation.

3. PREBIOTICS AND SYNBIOTICS

3.1. Prebiotics

Bacterial growth and survival in the gut require a sources of carbon and nitrogen. These carbohydrates must survive hydrolyses in the upper intestine

to be available for fermentation in the large intestine. They are mainly starch, non-starch polysaccharides, sugar alcohols, unabsorbed sugars, synthetic carbohydrates, and oligosaccharides such as fructo-oligosaccharides, lactulose, raffinose, stachyose and inulin oligomers, and are used as 'prebiotics' or bifidogenic factors. In definition "*A prebiotic is a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, that can improve host health*" (Gibson and Roberfroid, 1995). Prebiotics are therefore complex sugars that cannot be metabolised directly by humans but serve as a carbohydrate source for intestinal flora.

Criteria which allow the classification of a food ingredient as a prebiotic, include (Fooks et al, 1999):

1) It must be neither hydrolysed, nor absorbed in the upper part of the gastro-intestinal tract.
2) Potentially beneficial bacteria in the colon must ferment it.
3) Alter the composition of the colonic microbiota towards a healthier composition.
4) Preferably, induce effects which are beneficial to host health.

Many oligosaccharides have been shown to have prebiotic properties. The oligosaccharides produced in greatest quantity are isomalto-, fructo-, and galacto-oligosaccharides, lactulose, lactosucrose, cyclodextrins, coupling sugars, and palatinose. Non-digestible oligosaccharides (NDOs) have been introduced as functional food ingredients with additional nutritional value during the last few decades. Commercially, they are produced as a powder or syrup. About half the total production is used in beverages. Other major uses of prebiotics are in milk powders, confectionery and dairy desserts (Kaplan and Hutkins, 2000).

3.2. Bifidogenic factors

Prebiotics are used to supplement human diets and support the growth of bifidobacteria in the intestine hence the name 'bifidogenic factors' (Modler et al., 1990). Bifidogenic prebiotics are often complex carbohydrates such as fructo-, xylo- and galacto-oligosaccharides. Prebiotics are able to alter the composition of the human gut flora towards a predomination of bifidobacteria (Fooks et al., 1999).

3.3. Growth factors

The term "growth factors" needs clarification. In contrast to prebiotics, growth factors are compounds that promote the growth of probiotic organisms *in vitro* but cannot be delivered to the large bowel or cecum to selectively promote proliferation of probiotic bacteria (Modler, 1994). Examples of biological compounds identified as growth factors for bifidobacteria and lactobacilli include threonine, cysteine, peptone, maltose, dextrin, casein hydrolysates, tomato juice, etc. The application of these compounds will be discussed in later paragraphs.

3.4 Synbiotics

Synbiotics is where probiotics and prebiotics are used in combination. The end result should be improved survival of the probiotic, which has a readily available and specific substrate for its fermentation, as well as the individual advantages that each should offer (Fooks et al, 1999). Some of these products, as indicated in Table 2.2, in addition to the probiotic also contain inulin or oligofructose as 'bifidogenic factors', therefore, called synbiotics. While bifidobacteria are difficult to propagate in food due to oxygen sensitivity and low acid tolerance, the addition of prebiotics to dairy foods may lead to promising results to ensure the presence of high numbers of bifidobacteria during normal shelf life of the dairy products (Modler et al., 1990).

4. DIFFERENTIAL ENUMERATION OF PROBIOTIC AND TRADITIONAL YOGURT BACTERIA IN DAIRY PRODUCTS

The need exists for simple and reliable methods for routine enumeration of both *Bifidobacterium* sp. and *L. acidophilus* to determine the initial counts of the probiotic bacteria after manufacture of the product, and also to ascertain the viability of the probiotic cells during refrigerated storage and in the product distribution chain. Monitoring the level and survival of *L. acidophilus* and *Bifidobacterium* species in probiotic yogurt has often been neglected in the past due to unavailability of suitable selective media to enumerate these species (Kailasapathy and Rybka, 1997).

Culture media for the enumeration of starter bacteria in bio-yogurt can be divided into three groups: (a) general media that will give an overall total colony count without differentiating between different genera or species, e.g. MRS medium (de Man, Rogosa, Sharpe, 1960) which supports good growth of 'lactic acid bacteria' in general, (b) media formulated to selectively grow each genus, e.g. NNLP agar (neomycin-nalidix acid-lithium chloride-paramomycin agar) for isolating *B. bifidum* (Laroia and Martin, 1991b) or M17 for *S. thermophilus* (Terzaghi and Sandine, 1975) and (c) differentiating media that permit the enumeration of all four bacterial types found in bio-yogurt as visually distinguishable colonies on the same plate, e.g. TPPYPB agar (tryptone-proteose-peptose- yeast extract with Prussian blue agar) (Teraguchi et al., 1978).

4.1. Yogurt starter bacteria

The standard media accepted by the International Dairy Federation for differential enumeration of the yogurt species, *L. bulgaricus* and *S. thermophilus*, are MRS and M17 agar, respectively (IDF bulletin, 1983). Agar media allowing the simultaneous enumeration of *S. thermophilus* and *L.*

bulgaricus are LAB (Lactic acid bacteria) agar (Davis et al., 1971), TPPY (Tryptose-proteose-peptose-yeast extract agar) agar (Bracquart, 1981) and Lee's medium (Lee et al., 1974). See Table 2.1.

4.2. *L. acidophilus*, *Bifidobacterium* species and yogurt starter bacteria in bio-yogurt.

Most media have proven unsatisfactory for specific differentiation between *L.acidophilus* and *L.bulgaricus* from bio-yogurt (Charteris et al., 1997). Media proposed for differential enumeration of *L.acidophilus* are listed in Table 2.1.

Media for the specific enumeration of *Bifidobacterium* species are also listed in Table 2. These media usually contain substances which lower the redox potential (for example cysteine, cystine, ascorbic acid, or sodium sulphite), or selective agents (antibiotics, a single carbon source, propionic acids and lithium chloride) to inhibit the growth of lactic acid bacteria (Charteris et al., 1997), and are frequently fortified with horse or sheep blood (Rasic, 1990). The incubation conditions are generally anaerobic at 37°C. Media proposed for the differential enumeration of *Bifidobacterium* species from water, and human and animal faeces, such as TPPY (Bracquart, 1981) have been modified to TPPYPB (Teraguchi et al., 1978) to selectively enumerate *Bifidobacterium* from dairy products. TOS agar (transgalactosylated oligosaccharides as sole carbohydrate source) (Wijsman et al., 1989) is used for selective enumeration of bifidobacteria in mixed populations with *Lactobacillus* and *Streptococcus* species. Wijsman et al. (1989) modified the TOS agar to improve its selectivity by including neomycin sulphate, nalidix acid, lithium chloride and paramomycin sulphate (NNLP agar). Scardovi (1986) reported that one selective medium is not appropriate for all species of bifidobacteria. Lankaputra et al. (1996) proposed seven different media that could be used for selective enumeration of six strains of *L. acidophilus* and nine strains of *Bifidobacterium* species.

MRS-maltose and NNLP agars are the media of choice of Chr. Hansen's Laboratorium for differential enumeration of *Lactobacillus acidophilus* and *Bifidobacterium bifidum*, respectively (Anon., 1994; Anon., 1997).

Recently, 'Bif' agar (Pacher and Kneifel, 1996) has been formulated. It is a MRS-based medium with L-cysteine HCL and selective (antibiotics) ingredients. It enables the enumeration of bifidobacteria in commercial fermented milk and yogurt, and together with acidified-MRS, X-Glu and M17 agars it was proposed for complete analysis of probiotic bacteria from bio-yogurt.

5. APPLICATION OF PROBIOTIC MICROORGANISMS IN FUNCTIONAL FOODS

Consumption of probiotic bacteria via food products is an ideal way to re-establish the intestinal microflora balance.

For a culture to be considered a valuable candidate for use as a dietary adjunct and to exert a positive influence, it must conform to certain requirements (Martin and Chou, 1992; Collins et al, 1998). The culture must be a normal inhabitant of the human intestinal tract, survive passage through the upper digestive tract in large numbers, be capable of filling an ecological niche, and have beneficial effects when in the intestine (Gilliland, 1989). In order to survive, the strain must be resistant to bile salts present in the lower intestine, gastric conditions (pH 1-4), enzymes present in the intestine (lysozyme) and toxic metabolites produced during digestion (Hoier, 1992). The bacteria used in traditional yogurt fermentation, *Lactobacillus bulgaricus* and *Streptococcus thermophilus*, do not belong to the indigenous intestinal flora, are not bile acid resistant and do not survive passage through the gut (Gilliland, 1979). These traditional yogurt bacteria may nevertheless have positive effects as a result of fermentation metabolites, either by an inhibitory action towards pathogens or improvement of lactose digestion (Hoier, 1992).

The probiotic culture must multiply to reach high cell counts in the fermented product and possess a high acid tolerance to ensure high viable cell numbers during storage. The selected strains must be able to ferment milk relatively quickly, either alone or in combination with other strains.

The possibility of influencing the composition of the intestinal flora by consuming probiotic bacteria partly depends on the dose level. It is generally recognised that $10^8 - 10^9$ bacteria are necessary at the time of consumption (Speck, 1978). Therefore the probiotic culture must remain viable in the food carrier up to consumption.

A number of food bioproducts have been employed or are in the process of being developed to enhance their usage as delivery vehicles of probiotic cells fed to humans. Approximately 80 bifid-containing products are estimated to be on the world market (Hughes and Hoover, 1991). Most of these products are of dairy origin and include fresh milk (Klaver et al., 1993), fermented milk (Tamime et al., 1995; Mital and Garg, 1992), beverages, cheese (Gomes et al., 1995; Dinakar and Mistry, 1994; Roy et al., 1995), cottage cheese (Blanchette et al., 1995), powdered milk, cookies, health foods, ice cream (Hekmat and McMohan, 1992), and dairy desserts (Laroia and Martin, 1991a). Some examples of probiotic products seen on the world market are indicated in Table 2.3.

6. YOGURT AS PROBIOTIC CARRIER FOOD

Since the renewed interest in probiotics, different types of products were proposed as carrier foods for probiotic microorganisms by which consumers can take in large amounts of probiotic cells for the therapeutic effect. Yogurt has long been recognised as a product with many desirable effects for consumers, and it is also important that most consumers consider yogurt to be 'healthy'. In recent years, there has been a significant increase in the popularity of yogurt (Hamann and Marth, 1983) as a food product,

accentuating the relevance of incorporating *L. acidophilus* and *B. bifidum* into yogurt to add extra nutritional-physiological value. The conventional yogurt starter bacteria, *Lactobacillus bulgaricus* and *Streptococcus thermophilus*, lack the ability to survive passage through the intestinal tract and consequently do not play a role in the human gut (Gilliland, 1979). However, Gurr (1987) speculated that the ingestion of live traditional yogurt cultures and their metabolites may influence the enzymic activities of other organisms in the gut in ways that may be beneficial to health.

6.1. Yogurt production

Yogurt is a fermented milk product that has been prepared traditionally by allowing milk to sour at 40-45°C. Modern yogurt production is a well-controlled process that utilises ingredients of milk, milk powder, sugar, fruit, flavours, colouring, emulsifiers, stabilisers, and specific pure cultures of lactic acid bacteria (*Streptococcus thermophilus* and *Lactobacillus bulgaricus)* to conduct the fermentation process. The basic process of yogurt production is outlined in Fig.3.

Yogurt is prepared by heat treating whole or skim milk (80°C – 90°C for 30 –60 min) and then cooling the milk to around 40°C-45°C. The yogurt starter culture (*S. thermophilus* and *L. bulgaricus*) is added at a level of 2 % by volume and incubated at 43°C for 3 – 4 hrs, followed by cooling to 4°C (Tamime and Robinson, 1985).

S. thermophilus and *L. bulgaricus* exhibit a symbiotic relationship during the processing of yogurt, with the ratio between the species changing constantly (Radke-Mitchell and Sandine, 1984). During fermentation, *S. thermophilus* grows quickly at first, utilizing essential amino acids produced by *L. bulgaricus*. *S. thermophilus*, in return, produces lactic acid, which reduces the pH to an optimal level for growth of *L. bulgaricus*. The lactic acid produced, and lesser amounts of formic acid stimulate the growth of *L. bulgaricus*. The

streptococci are inhibited at pH values of 4.2-4.4, whereas lactobacilli tolerate pH values in the range of 3.5-3.8. After approximately 3 h of fermentation, the numbers of the two organisms should be equal. With longer fermentation, the growth rate of *S. thermophilus* declines while *L. bulgaricus* continues to reduce the pH by producing excessive amounts of lactic acid. The pH of commercial yogurt is usually in the range of 3.7 to 4.3 (Hamann and Marth, 1983). *S. thermophilus* produces diacetyl, which gives yogurt its creamy or buttery flavour, whereas *L. bulgaricus* produces acetaldehyde responsible for the characteristic sharp flavour (Davis et al., 1971).

6.2. Fermentation products of yogurt

During the production of yogurt, changes to the milk constituents are attributed to fermentation, and the ingredients added during manufacturing. Changes induced during fermentation, include the fermentative action of the inoculated starter cultures, the secretion of nutritional and chemical substances by the microorganisms, as well as the presence of the microorganisms and their associated enzymes (Gurr, 1987). Fermentation affects the carbohydrate, protein, and vitamin components as well as production of flavour compounds, particularly acetaldehyde.

The primary role of lactic acid bacteria is to utilize lactose as a substrate and convert it into lactic acid during fermentation of milk. Lactose is taken up as the free sugar and split with β-galactosidase to glucose and galactose. The glucose is rapidly metabolized to lactic acid. About 3% of the lactose is converted, giving about 1,5% galactose and 1% lactic acid. Some lactose remains, the exact amount depending on the degree of fortification. Most yogurts can be expected to have about 5% lactose (Deeth, 1984). Both glucose and galactase are metabolized simultaneously, via the glycolytic and D-tagatose 6-phosphate pathways, respectively (Thomas and Crow, 1984). In addition galactose can also be further metabolized by enzymes of the Leloir pathway (Hutkins et al., 1985). Since the lactic acid present in yogurt is

produced from the glucose moiety of lactose rather than the galactose moiety, galactose accumulates in fermented milk products. Free galactose can later be utilized by *Streptococcus thermophilus* or *Lactobacillus bulgaricus*. This indicates that sparing galactose is utilized while lactose is still present and continues after lactose exhaustion. This suggests that the enzymes for galactose metabolism are present, but at low activity (Thomas and Crow, 1984). Compared to milk, the lactose concentration in yogurt is lower, provided that no milk powder was added, while the concentration of galactose present is higher. Fruit yogurt contains 9-12% of additional carbohydrates in the form of sucrose, glucose and fructose (Renner, 1983).

During fermentation the bacteria produce proteases and peptidases which act on milk proteins and cause increases in peptides and free amino acids. The heat treatment (85 – 90°C for 30 min) also causes changes in the proteins, denaturing the whey proteins and producing some peptides and amino acids (Tamime and Robinson, 1985). The total amino acid content of yogurt does not differ substantially from milk but the free amino acid content is higher due to proteolytic activity of microorganisms (Rasic and Kurmann, 1983). The protein content of protein-enriched yogurt (addition of milk powder) is increased to 4-5%, whereas normal yogurt exhibits an average protein content of 3% (Renner, 1983). In total, the soluble non-protein nitrogen content in yogurt is about 50% higher than in the original milk mix (Deeth, 1984).

The microbial inoculum has a substantial influence on the vitamin content of yogurt. While some bacteria require B vitamins, particularly B_{12}, for growth, several others synthesise certain vitamins such as folic acid and niacin during fermentation. Fermentation has little effect on the mineral content of milk and therefore the total mineral content remains unaltered in the yogurt (Gurr, 1987). Yogurt is, however, a rich source of minerals, particularly if fortified.

Fermentation has little effect on the fat component. Very little hydrolysis occurs as the starter bacteria are only weak lipase producers (Deeth, 1984).

In summary, the concentrations of lactic acid, galactose, free amino acids and fatty acids increase as a result of fermentation while lactose concentration decreases. Addition of ingredients mainly increases the protein and sugar content.

7. BIO-YOGURT

In recent years some yogurt products have been reformulated to include live strains of *Lactobacillus acidophilus* and species of *Bifidobacterium* (known as AB-cultures) in addition to the conventional yogurt organisms, *S. thermophilus* and *L. bulgaricus*. Bio-yogurt, is therefore, yogurt that contains live probiotic microorganisms, the presence of which may give rise to claimed beneficial health effects.

7.1. Production of AB-yogurt

For the production of AB-yogurt, similar processing procedures to traditional yogurt are applied with the exception of the incorporation of live probiotic starter cultures. The probiotic culture can be added prior to fermentation simultaneously with the conventional yogurt cultures or after fermentation to the cooled (4°C) product before packaging. Heat-treated, homogenised milk with an increased protein content (3.6-3.8%) is inoculated with the separate cultures of *L. acidophilus* and bifidobacteria as well as the conventional starter culture at either 45°C or 37°C. Chr. Hansens recommend that if freeze-dried DVS cultures are used, 25g of each culture are added to 1000 L milk. Owing to the relatively slow growth of *L. acidophilus* and bifidobactera in milk the fermentation time must be extended to around 14 – 16 hours at 37 – 40°C (Anon., 1994).

7.2. Regulatory requirements for starter cultures in bio-yogurt

Bio-yogurt, containing *L. acidophilus* and *B. bifidum* (AB-yogurt), is a potential vehicle by which consumers can take in probiotic cells. To achieve the optimal potential therapeutic effects, the number of probiotic organisms in a probiotic product should meet a suggested minimum of $>10^6$ cfu/ml (Kurmann and Rasic, 1991). Other authors stipulate $>10^7$ and 10^8 cfu/ml as satisfactory levels (Davis et al., 1971; Kailasapathy and Rybka, 1997). This criterion is referred to as the 'therapeutic minimum' in literature (Davis et al., 1971, Rybka and Kailasapathy, 1995). One should aim to consume 10^8 live probiotic cells per day. Regular consumption of 400-500g/week of AB-yogurt, containing 10^6 viable cells per ml would provide these numbers (Tamime et al., 1995).

Ishibashi and Shimamura (1993) reported that the Fermented Milks and Lactic acid Bacteria Beverages Association of Japan has developed a standard which requires a minimum of 10^7 viable bifidobacteria cells per ml to be present in fresh dairy products. The criteria developed by the National Yogurt Association (NYA) of the United States specifies 10^8 cfu/g of lactic acid bacteria at the time of manufacture, as a prerequisite to use the NYA 'Live and Active Culture' logo on the containers of products (Kailasapathy and Rybka, 1997). The Australian Food Standards Code regulation, requires that the lactic acid cultures used in the yogurt fermentation must be present in a viable form in the final product, the populations are not specified. At the same time, attainment of pH 4.5 or below is also legally required to prevent the growth of any pathogenic contaminants (Micanel et al., 1997).

It has been claimed that only dairy products with viable microorganisms have beneficical health effects. However, in the case of lactose tolerance, treatment of acute gastro-enteritis and treatment of candidiases, probiotics used showed the same beneficial effect in viable and non-viable form. Ouwehand and Salminen (1998) give an overview on this.

8. LEVEL AND SURVIVAL OF *L. ACIDOPHILUS* AND BIFIDOBACTERIA IN BIO-YOGURT

L. acidophilus and *B. bifidum* have to retain viability and activity in the food carrier to meet the suggested 'therapeutic minimum' at the time of consumption (Playne, 1994). It is essential that products sold with any health claims meet this criterion. Viability of probiotic bacteria in products over a long shelf life at refrigeration temperature is reported to be unsatisfactory (Rybka and Kailasapathy, 1995; Dave and Shah, 1997a).

8.1. Factors affecting the viability of *L. acidophilus* and bifidobacteria species in dairy bio-products

Fermented milk bio-products containing *Lactobacillus* and *Bifidobacterium* cultures are a microbiologically sensitive group of products. Incorporation of these bacteria into the food chain can be difficult. Bifidobacteria in particular usually exhibit weak growth in milk and require an anaerobic environment (Rasic, 1990), a low redox potential (Klaver et al., 1990) and the addition of bifidogenic factors to achieve the desired levels of growth (von Hunger, 1986; Modler, 1994; Klaver et al., 1990).

The survival of probiotic bacteria in fermented dairy bio-products depends on such varied factors as the strains used, interaction between species present, culture conditions, chemical composition of the fermentation medium (e.g. carbohydrate source), final acidity, milk solids content, availability of nutrients, growth promoters and inhibitors, concentration of sugars (osmotic pressure), dissolved oxygen (especially for *Bifidobacterium* sp.), level of inoculation, incubation temperature, fermentation time and storage temperature (Hamman and Marth, 1983; Young and Nelson, 1978; Kneifel et al., 1993).

8.1.1. Yogurt acidity

According to Klaver et al. (1993), one of the most constraining drawbacks associated with the use of dietary cultures in fermented milk products is the lack of acid tolerance of some species and strains. When the lactic acid content increases, pH levels correspondingly decrease during fermentation. 'Over-acidification' or 'post-production acidification' is due to the decrease in pH after fermentation and during storage at refrigerated temperature. Excessive acidification is mainly due to the uncontrollable growth of strains of *L. bulgaricus* at low pH values and refrigerated temperatures. The 'over-acidification' can be prevented to a limited extent by applying 'good manufacturing practice' and by using cultures with reduced 'over-acidification' behaviour (Kneifel et al., 1993).

The survival of microorganisms is affected by low pH of the environment. Hood and Zottola (1988) reported that *L. acidophilus* (strain BG2FO4) showed a rapid decline in numbers at pH 2.0, but at pH 4.0 the number of viable cells did not decrease significantly. These results were confirmed by Lankaputhra and Shah (1995), who concluded that six strains of *L. acidophilus* studied, survived well at pH 3.0 or above and the viable counts remained above 10^7 cfu/ml after 3h incubation. Playne (1994), however, reported that *L. acidophilus* does not grow well below pH 4.0.

It has been reported that *L. acidophilus*, survives better than the traditional yogurt culture organisms, *L. bulgaricus* and *S. thermophilus*, in yogurt under acidic conditions (Shah and Jelen, 1990; Hood and Zottola, 1988). Lankaputhra and Shah (1995) concluded that *L. acidophilus* is also more tolerant to acidic conditions than *B. bifidum*.

The pH of yogurt may decline to a level as low as 3.6 (Lankaputhra et al., 1996), which may result in the inhibition of growth of bifidobacteria since their growth is retarded below pH 5.0 (Bergey's Manual, 1974; Gilliland, 1979). Martin and Chou (1992) reported that a pH of 5.5–5.6 was determined as being

the minimum pH for survival of some species/strains of bifidobacteria. However, acid tolerance of *Bifidobacterium* is strain-specific. Lankaputhra and Shah (1995) studied the survival of nine strains of *Bifidobacterium* spp. in acidic conditions (pH 1.5-3.0) and concluded that *B. longum* and *B. pseudolongum* survived better in acidic conditions than *B. bifidum.* The growth of *B. bifidum* was retarded below pH 5.0. More recently Reilly and Gilliland (1999) evaluated four strains of *Bifidobacterium longum* surivial as related to pH during growth and found that one of the strains, *B. longum* S9, was more stable than the others regardless of pH during growth.

Overall, most strains of bifidobacteria are sensitive to pH values below 4.6. Therefore, for practical application, a pH value of the final product must be maintained above 4.6 to prevent the decline of bifidobacteria populations (Tamime and Robinson, 1985; Modler et al., 1990; Laroia and Martin, 1991a).

8.1.2. Species/strains

Viability of both *Lactobacillus* and *Bifidobacterium* species diminishes markedly during refrigerated storage at low pH levels (Gilliland and Lara, 1988; Klaver et al., 1990; Hugh and Hoover, 1995; Shah et al., 1995). Consequently, careful strain selection and monitoring are necessary to ensure high quality fermented bioproducts. The main requirement in selecting bifidobacteria for use in a yogurt product, is the ability to grow in milk. Utilising different strains of *L.acidophilus* and different yogurt cultures, indicated that some strains competed better and remained viable in yogurt up to 28 days of storage at 7°C.

It is important for the culture supplier that culture strains can be produced on a large-scale in commercial production. Strains selected as DVS (direct vat set) cultures, need to be concentrated reaching populations of $10^{10} - 10^{11}$ cfu/g to guarantee the desired performance in commercial manufacturing of fermented milk bio-products (Hoier, 1992).

Strain variation contributed to differences observed in different survival studies (Nighswonger et al., 1996).

8.1.3. Co-culture and species interaction

The composition of the species participating in the fermentation has been found to affect the survival of *L. acidophilus* and *Bifidobacterium* species. A potential growth medium, such as bio-yogurt, contains metabolic products secreted by other microorganisms, which influence the viability of *L. acidophilus* and *B. bifidum* (Gilliland and Speck, 1977c). Dave and Shah (1997a) have reported that the inhibition of bifidobacteria was not due to organic acids or hydrogen peroxide. Therefore, inhibition of this organism was presumed to be due to antagonism effects among starter bacteria.
Dave and Shah (1997b) found that the bacteriocin, Acidophilicin LA-1, produced by *L. acidophilus* was active against seven strains of *L. bulgaricus*, one strain each of *L. casei* , *L. helveticus* and *L. jugurti*, but not against other LAB.

In a study conducted by Gilliland and Speck (1977c), *L. acidophilus* added to yogurt decreased in numbers during refrigerated storage. Substances produced by *L. bulgaricus* caused this instability. Hydrogen peroxide produced during the manufacture and storage of yogurt appeared to be the main substance responsible for the antagonism of *L. bulgaricus* towards *L. acidophilus* since added catalase reduced the antagonism. Hull et al. (1984) referred to the dramatic loss in viability of *L. acidophilus* as 'acidophilus death'. *L.acidophilus* failed to survive in commercial yogurt when high populations of *L. bulgaricus* were present (Rybka, 1994). In the survey by Rybka (1994), the presence of *L. bulgaricus* was also found to be the main detrimental factor responsible for *L. acidophilus* and *Bifidobacterium* spp. mortality. When *L. bulgaricus* was excluded from fermentation, the decrease in pH was significantly reduced during storage. *L. bulgaricus* causes 'over-acidification' during manufacture and storage. This can be prevented by using modified or

ABT-yogurt starter cultures (fermented with *L. acidophilus*, *B. bifidum* and *S. thermophilus*) (Kim et al., 1993).

Synergistic growth-promoting effects between *L. acidophilus* and *B. bifidum* are known to occur (Kneifel et al., 1993). While co-inoculation with yogurt organisms suppressed the growth of the bifidobacteria, subsequent storage in the presence of the yogurt cultures reduced the decline in numbers (Samona and Robinson, 1994).

B. bifidum is dependent on other lactic acid bacteria to ensure its growth. Out of 17 bifidobacteria strains grown in pure milk, 15 failed to survive (Klaver et al. 1993). Since these strains lack proteolytic activity, they could be grown by adding casein hydrolysates or by co-culturing with proteolytic species such as lactobacilli, e.g. *L. acidophilus*. Therefore, *L. acidophilus* strains live in excellent symbiosis with bifidobacteria providing the necessary growth stimulants (Hansen, 1985). The two species are used in a certain ratio, for example 700-800 million acidophilus bacteria/ml and 400-500 million bifidobacteria/ml in the production of AB-yogurt (Hansen, 1985). The growth rate of *L. acidophilus* is not affected by *B. bifidum*, but the growth of *B. bifidum* is suppressed unless the initial inoculum is in the ratio of 10^4:10^3 (*B. bifidum*:*L. acidophilus*) (Rasic and Kurmann, 1983).

S. thermophilus acts as an oxygen scavenger in bioyogurt and is therefore beneficial to the growth of *Bifidobacterium* spp. (Shankar and Davies, 1976; Ishibashi and Shimamura, 1993).

8.1.4. Inoculation practice

The common practice in bio-yogurt production is to use premixed, 'direct vat set' (DVS) cultures of *L. delbrueckii* subsp. *bulgaricus*, *S. thermophilus*, *L. acidophilus* and *Bifidobacterium* spp. The *L. acidophilus* and *Bifidobacterium* spp. can also be grown separately before incorporation into the bioyogurt, to

ensure a desirable level of probiotic culture in the final retail product (Kailasapathy and Rybka, 1997). Modler and Villa-Garcia (1993) reported that the ideal procedure is to grow the *Bifidobacterium* spp. separately, followed by washing out of free metabolites and the transfer of the cells to the yogurt base. Hull et al. (1984) observed that *L. acidophilus* had improved yogurt stability during refrigerated storage if added at the same time as the traditional yogurt cultures and allowing growth during the fermentation process. *L.acidophilus* added after yogurt manufacture died off rapidly and the survival rate after 7 days storage at 5°C was less than 1%. These findings were supported by Gilliland and Speck (1977c). Death of cells of *L. acidophilus* was attributed to the effects of hydrogen peroxide produced in the yogurt. Better survival of *L.acidophilus* was obtained due to increased tolerance to hydrogen peroxide when *L.acidophilus* and yogurt cultures were grown simultaneously. Apparently, the *L. acidophilus* cultures developed the ability to split hydrogen peroxide.

Inoculum size of probiotic bacteria is an important key factor to ensure sufficient viable cells in the final food product. According to Samona and Robinson (1994) the presence of yogurt cultures restricted the growth of bifidobacteria, but they have little impact on the long-term viability of an existing culture. Therefore, it is imperative that AB-yogurt manufacturers ensure that at least one million viable cells of *Bifidobacterium* species/g are present at the end of fermentation. If the required criterion is met, the number of probiotic bacteria should remain stable throughout the anticipated shelf-life (Samona and Robinson, 1994). However, increased inoculum in the study of Dave and Shah, 1997a) did not improve viability of bifidobacteria in yogurt.

Growth and progression of *Bifidobacterium* species in yogurt are suppressed due to different rates of multiplication of bacteria strains present during fermentation. The inability of *Bifidobacterium* to progress in a mixed culture is considered a major cultivation problem (Schuler-Malyoth and Muller, 1968).

Incubation temperature is also an important factor related to inoculation practice. Usually, yogurt is fermented at 43°C (the optimal temperature for lactic acid production by starter cultures), however, the optimum temperature for growth of *Bifidobacterium* is 37°C. Consequently, lower incubation temperatures (37°C – 40°C) will favor the growth rate and survival of probiotic species (Kneifel et al., 1993).

If a higher inoculation percentage of *S.thermophilus* and *L.bulgaricus* is used during AB-yogurt fermentation, these cultures will dominate the fermentation and result in lower populations of *L.acidophilus* and *B.bifidum* in the final product (Anon., 1994).

8.1.5. Dissolved oxygen

Since *Bifidobacterium* is strictly anaerobic, oxygen toxicity is an important and critical problem. Milk with a low initial oxygen content should be used to obtain the low redox potential required in the early phase of incubation to guarantee growth of bifidobacteria (Klaver et al., 1993).

During yogurt production, oxygen easily penetrates and dissolves in milk. Oxygen also permeates through packages during storage. To avoid the oxygen problem, it has been suggested to inoculate *S. thermophilus* and *Bifidobacterium* simultaneously during fermentation (Ishibashi and Shimamura, 1993). *S. thermophilus* has a high oxygen utilisation ability, which results in the depletion of dissolved oxygen in yogurt and an enhancement in the viability of bifidobacteria.

8.1.6. Storage conditions

The temperature of storage of fermented probiotic products is important for the viability of probiotic microorganisms. Low temperature restricts the growth of *L. bulgaricus* and consequently also over-acidification (Kneifel et al., 1993).

Most studies showed that higher survival rates of lactic acid bacteria were obtained at lower storage temperatures (Gilliland and Lara, 1988; Foschino et al., 1996).

Bifidobacteria are substantially less tolerant to low temperature storage when compared to *L.acidophilus* (Hughes and Hoover, 1995).

8.2. Improvement in the survival of *L. acidophilus* and *Bifidobacterium* species in dairy bio-yogurt.

The poor survival of *L. acidophilus* and *Bifidobacterium* species mentioned previously, can be improved by means of modification and control of the manufacturing process and storage conditions, and by better selection of probiotic starter cultures.

8.2.1. Prevention of over-acidification

Over-acidification can be prevented by controlling pH (>5) (Varnam and Sutherland, 1994), applying 'heat shock' (58°C for 5 min) to yogurt (Marshall, 1992), lowering storage temperature to less than 3-4°C and improving the buffering capacity of yogurt by the addition of whey protein concentrate (Kailasapathy and Rybka, 1997).

8.2.2. Modification of incubation temperature and inoculum size

A lower incubation temperature of 37°C favours the growth of bifidobacteria (Kneifel et al., 1993).

Using a high level of inoculum, will ensure a high cell count at the end of the incubation and survival of the probiotic bacteria during storage until consumption (Samona and Robinson, 1994). An inoculum level of 10-20% is recommended by Varnam and Sutherland (1994). Rasic and Kurmann (1983)

recommended the use of a freeze-dried DVS culture. Concentrated starter cultures (liquid, frozen or dried), should contain a minimum of 5 x 10^9 cfu/g, and unconcentrated starter cultures a population of 1 x 10^8 cfu/g (IDF, International Standard 149, 1996).

8.2.3. *Selection of starter cultures*

Proper selection of acid tolerant strains of AB-cultures capable of progression in low pH yogurt will ensure better survival of the organisms in the bio-yogurt (Martin and Chou, 1992). Using ABT-cultures (*L. acidophilus*, *B. bifidum* and *S. thermophilus*), and the exclusion of *L. bulgaricus* from fermentation, will eliminate antagonistic effects by hydrogen peroxide against AB cultures (Rybka, 1994). *S. thermophilus* acting as an oxygen scavenger, creates an anaerobic environment and may enhance growth and survival of *Bifidobacterium* when used together in starter cultures (Shankar and Davies, 1976; Rybka, 1994).

8.2.4. *Addition of growth promoting substances*

A number of substances are known to improve the growth of probiotic bacteria. Supplementation of milk with a combination of casitone, casein hydrolysate and fructose stimulate the growth of *L. acidophilus* (Saxena et al., 1994). Whey protein concentrate, tomato juice and papaya pulp also stimulate the growth of *L. acidophilus* (Babu et al., 1992; Kailasapathy and Supriadi, 1996)). The stimulation in growth is incurred due to an enhanced availability of simple sugars, mainly glucose and fructose, and minerals (i.e. magnesium and manganese) which are growth promoters for *L. acidophilus* (Ahmed and Mital, 1990). Growth of *L.acidophilus* is also enhanced by acetate (Marshall, 1991).

Dave and Shah (1998) investigated the effects of cysteine, acid hydrolysates, tryptone, whey protein concentrate and whey protein on the viability of yogurt

and probiotic bacteria in yogurt. Addition of each of these supplements, except whey powder, improved the viability of bifidobacteria to a variable extent in the yogurt made with ABT (*Lactbobacillus acidophilus*, bifidobacteria, *Streptococcus thermophilus*) starter culture. The nitrogen source in the form of peptides and amino acids probably improved viability of the bifidobacteria. Addition of vitamins, dextrin and maltose stimulate the growth of bifidobacteria species in milk, while sucrose and iron salts have little effect. The survival of *B. longum* in milk can be improved by the addition of 0.01% baker's yeast (Kailasapathy and Rybka, 1997). Use of ascorbic acid as an oxygen scaveger (Dave and Shah, 1997a) also did not improve viability of bifidobacteria in bio-yogurt.

Addition of prebiotics such as oligosaccharides to food is mainly to allow the preferential growth of probiotic organisms in the colon, as these substances are not utilized by other intestinal bacteria, and thereby can improve host health (Gibson and Roberfroid, 1995). Synbiotics is where probiotics and prebiotics are used in combination to manage microflora (Fooks et al., 1999). These oligosaccharides may have the potential for incorporating into bio-yogurt to enhance the numbers of bifidobacteria not only in the colon but also during shelf-life in the product.

When yogurt bacterial cells were ruptured to release their intracellular β-galactosidase and reduce their viable counts to improve the viability of probiotic bacteria (Shah and Lankaputhra, 1997), bifidobacteria counts were 2 log cycles higher after fermentation, viability remained above 10^6 cfug^{-1} during storage and the yogurt contained less hydrogen peroxide. β-galactosidase hydrolyse lactose in milk to galactose and glucose which could be used by *L. acidophilus* and *Bifidobacterium* spp. Rupturing also reduced viable count of the yogurt bacteria and thus the amount of hydrogen peroxide produced by these bacteria.

Added oligosaccharides in probiotic products have been used satisfactorily to increase the survival of probiotic organisms in the human intestine. It can also

be applied to ensure better survival of *L. acidophilus* and *B. bifidum* in the AB-yogurt (Kailasapathy and Rybka, 1997).

8.2.5. Micro-encapsulation

Micro-encapsulation (protective coating of microorganisms) technology may provide protection to acid sensitive probiotic organisms. Coating materials available includes gelatin, vegetable gums, modified starch, dextrin, non-gelling protein and butterfat (Modler et al, 1990).

9. CONCLUSIONS

Although the concept of probiotics has been around since biblical times, we are still at an early stage in the development of consistently effective probiotics for human application. In an increasingly health-conscious society the market for probiotic containing products shows a substantial increase in popularity recently. However, scientific approaches to establishing the functional benefits of probiotic foods are still a complicated case. Evidence from *in vitro* studies suggests beneficial effects, but considerable progress has not yet been made in both effects on host health and mechanisms of action. Also whether specifically viable microorganisms are necessary for health benefits, needs clarification.

The typical poor growth of these probiotic species is highlighted, therefore investigation of bifidogenic- and growth factors, and efforts to establish optimum environmental conditions for their growth are critical, in addition to effects of the type of foods and storage conditions on microbial survival.

Criteria for the selection of effective microbial strains for a probiotic affect have to be established. Further development of probiotic products is also dependent on the availability of strains which will fulfill this strict criteria of a probiotic. New species and more specific strains of probiotic bacteria are constantly being

identified. Genetic modifications are continuously applied to improve fermentation efficiency and shelf life of probiotic bacteria. However, the safety of these modified bacteria should be considered.

Incorporation of probiotics into other food commodities for example cheese is promising and should be intensively investigated.

One issue has become obvious from probiotic research in recent years; while for centuries it has been recognized that diet and health are inextricably linked, it is now quite clear that the gut microflora is an essential component of the healthy human and is essential for optimal resistance to disease. However, consumers should become aware and educated about the potential health benefits of probiotic/prebiotic dairy foods. In some countries, inclusion of probiotic cultures is almost taken for granted while in South Africa, the presence of live cultures in yogurt still seems to be nothing more than an important marketing tool.

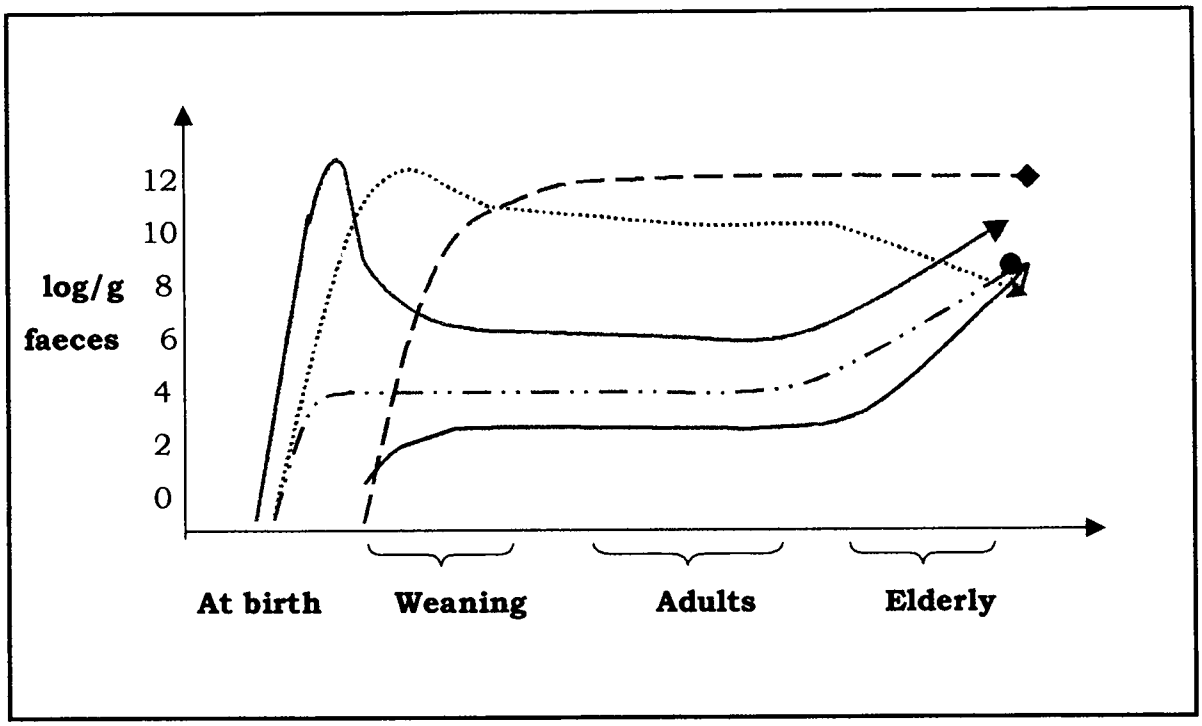


Fig. 2.1 Changes of intestinal flora with age (Mitsuoka, 1982).

Welch's Bacilli (*C. perfringens*)

Coliform bacteria and enterococci

Bifidobacteria

Lactobacilli

Bacteroides, eubacteria and anaerobic streptococci

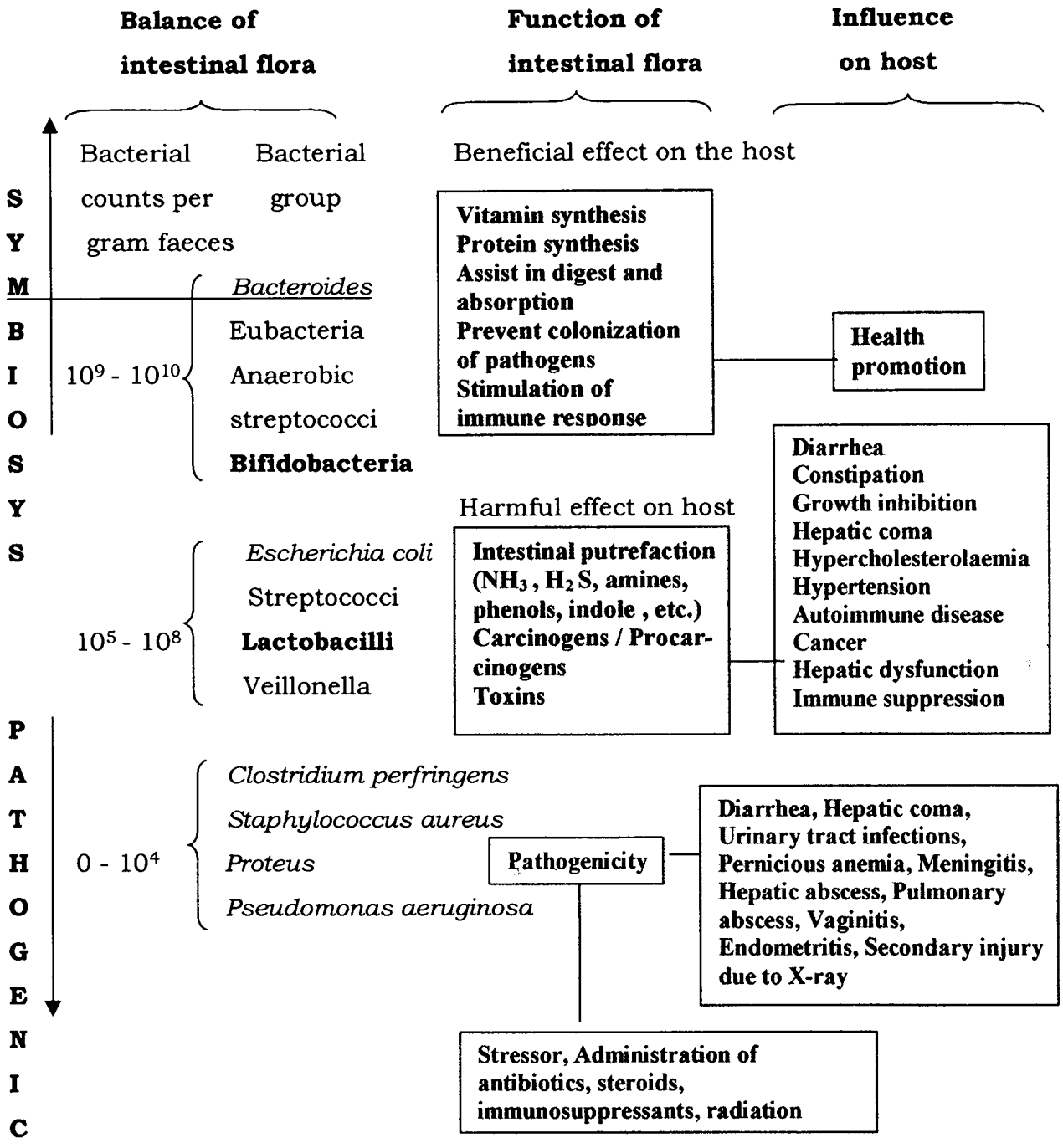


Fig. 2.2 The interrelationship between intestinal bacteria and human health as proposed by Mitsuoka (Ishibashi and Shimamura, 1993).

Table 2.1 Claimed beneficial effects and therapeutic application of probiotic bacteria in humans (Fuller, 1989).

Beneficial effects:
Maintenance of normal intestinal microflora
Enhancement of the immune system
Reduction of lactose-intolerance
Reduction of serum cholesterol levels
Anticarcinogenic activity
Improved nutritional value of foods
Therapeutic applications:
Prevention of urogenital infection
Alleviation of constipation
Protection against traveller's diarrhoea
Prevention of infantile diarrhoea
Reduction of antibiotic-induced diarrhoea
Prevention of hypercholesterolaemia
Protection against colon/bladder cancer
Prevention of osteoporosis

Table 2.2 Differential media for enumeration of *L.acidophilus* and *Bifidobacterium* species in the presence of yogurt starter bacteria.

Bacterial group	**Agar medium**	**Reference**
Lactobacillus acidophilus	MRS-maltose (de Man,Rogosa,Sharpe)	Hull and Roberts, 1984 Coker and Martley, 1982
	EC (Esculin-cellobiose)	Von Hunger, 1986
	TPPY (Tryptose-proteose-peptone yeast extract)	Bracquart, 1981
	LBSO (Lactobacillus selective agar with oxgall)	Gilliland and Speck, 1977d
	PCA (Agar plate count method)	Collins, 1978
	X-Glu	Kneifel and Pacher, 1993
	MNA + salicin (minimal nutrient agar)	Lankaputhra and Shah, 1996
Bifidobacterium	RCPB (Reinforced clostridial agar with Prussian blue)	Van der Wiel-Korstanje and Winkler, 1970
	M17	Terzaghi and Sandine, 1975
	NNLP (Neomycin-nalidix acid-lithium chloride paromomycin)	Laroia and Martin, 1991 Modler and Villa-Garcia, 1993

	Modified NNLP	Teraguchi et al, 1978
	X-α-gal (5-bromo-4chloro—3-indolyl-α-galactoside)	Chevalier et al., 1991
	YN-6	Resnick and Levin, 1981
	YN-17	Mara and Oragni, 1983
	TOS (Transgalactosylated Oligo saccharide)	Wijsman et al., 1989
	L-arabinose	Wijsman et al., 1989
	TOS-NNLP	Wijsman et al., 1989
	Modified Columbia	Beerens, 1990
	LP (Lithium chloride-sodium propionate)	Lapierre et al., 1992
	BL-OG (Blood glucose liver + oxgall + gentamicin	Lim et al., 1995
	BIM-25 (*Bifidobacterium* iodoacetate medium 25)	Munoa and Pares, 1988
	PSM (Petuely's selective medium)	Tanaka and Mutai, 1980
	Modified HBSA	Arany et al., 1995
	'Bif' (*Bifidobacterium*)	Pacher and Kneifel, 1996
Both *L.acidophilus* and *B.bifidum*	HHD (Homofermentative Heterofermentative differential)	McDonald et al., 1987

	Modified HHD	Zúñiga et al., 1993
	LB	IDF, 1993
	Modified TPPY	Ghoddusi and Robinson, 1996
L.bulgaricus	RCA pH5.5 (Reinforced clostridial agar)	Johns et al., 1978
	Acidified-MRS	IDF, 1983
S.thermophilus	M17	IDF, 1983
	β-Glycerophosphate	Shankar and Davies, 1977
	PCA (Plate count agar) with 10% milk	Johns et al., 1978
Both *L.bulgaricus* and *S.thermophilus*	TPPY	Braqquart, 1981
	Lee's	Lee et al., 1974
	LAB	Davis et al., 1971

Table 2.3. Some examples of probiotic dairy products available on the world market.

Product	**Country**	**Culture**	**Prebiotic additive**	**Reference**
AB milk products	Denmark	A + B		Tamime et al, 1995
Acidophilus bifidus yogurt	Germany	A+B + Yogurt culture		Tamime et al, 1995
BA 'Bifidus active'	France	*B. longum* + Yogurt culture		Tamime et al, 1995
Bifidus milk	Germany	*B. bifidum* or *B. longum*		Tamime et al, 1995
Bifidus yogurt	Many countries	*B. bifidum* or *B. longum* + Yogurt culture		Tamime et al, 1995
Bifighurt	Germany	*B. longum* + *S. thermophilus*		Tamime et al, 1995
Bifilak(c)t	USSR	A+B		Tamime et al, 1995
Biobest	Germany	*B. bifidum* or *B. longum* + Yogurt culture		Tamime et al, 1995
Biokys (=Femilact)	Chechoslovakia	A+B + *Pediococcus acidilactici*	Contains 'biogerm' grain	Tamime et al, 1995
Biomild	Germany	A+B		Tamime et al, 1995
Mil-Mil	Japan	A+B *B. breve*		Tamime et al, 1995

Bioghurt	Germany	A+B + *S. thermophilus*		Tamime et al, 1995
Cultura	Denmark Norway	A+B		Hoier, 1992
Philus	Sweden	A+B+ *S.thermophilus*		Hoier, 1992
BA live	United Kingdom	A+B+ Yogurt culture		Hoier, 1992
A-38	Denmark	A+B+ Mesophilic LD-culture		Hoier, 1992
Acidophilus milk	Sweden	A+B+ Mesophilic LD-culture		Hoier, 1992
Kyr	Italy	A+B+ Yogurt culture		Hoier, 1992
Ofilus	France	A+B+ *S.thermophilus*		Hoier, 1992
BIO	France	A+B+ Yogurt culture		Hoier, 1992
Biogarde	Germany	A+B+ *S.thermophilus*		Hoier, 1992
ABC Ferment	Germany	A+B+ *L.casei*	Inulin	Holzapfel et al., 1997
AKTIFIT plus	Switzerland	A+B+ *L.casei* GG+ *S.thermophilus*	Oligofructose	Holzapfel et al., 1997
Symbalance	Switzerland	A+B+ *L.reuteri*+ *L.casei*	Inulin	Holzapfel et al., 1997

Mona fysig	Netherlands	*L.acidophilus*	Inulin	Holzapfel et al., 1997
Actimell	Germany	*L.casei*		Holzapfel et al., 1997
LC-1	Germany	*L.acidophilus*		Holzapfel et al., 1997
LA-7 plus	Bauer	A+B	Oligofructose	Holzapfel et al., 1997
Vifit	Germany	*L.casei* GG	Oligofructose	Holzapfel et al., 1997
Primo	Germany	BactoLab cultures		Holzapfel et al., 1997
Zabady	Egypt	*B.bifidum*+ Yogurt culture		Kebary, 1996

A: *L. acidophilus* **B: Bifidobacteria** **Yogurt culture: *S. thermophilus* and *L. bulgaricus***

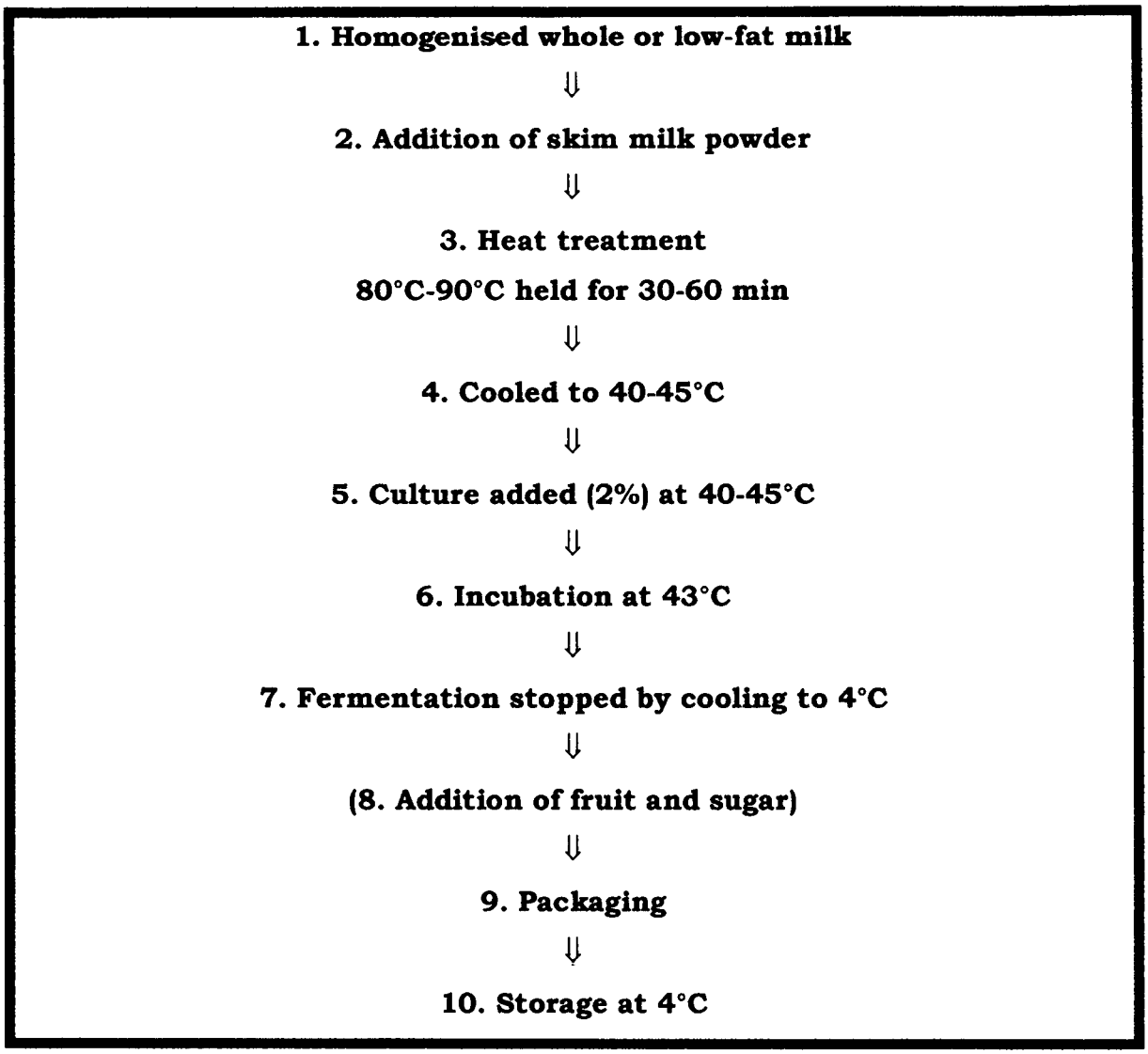


Fig. 2.3. A schematic presentation of the production of yogurt (Tamime and Robinson, 1985).

REFERENCES:

Ahmed, B. and Mital B.K. (1990) Effects of magnesium and manganese ions on the growth of *Lactobacillus acidophilus*. *Journal of food Technology*. **27**(4), 228 - 229.

Anon. (1997) *Lactobacillus acidophilus*, *Lactobacillus casei* and *Bifidobacteria* in milk products made with nu-trish cultures. *CHR. Hansen*. Guideline. Count of bacteria, 1 - 5.

Anon. (1994) Method of analysis for identification of *L. acidophilus* and *Bifidobacteria* (A medium for differentiating between *L. acidophilus*, *Bifidobacteria* and *S. termophilus* in ABT products) Chr.Hansen's Laboratorium, Copenhagen, Denmark.

Arany, C.B., Hackney, C.R., Duncan, S.E., Kator, H., Webster, J., Pierson, M., Boling, J.W. and Eigel, W.N. (1995) Improved recovery of stressed *Bifidobacterium* from water and frozen yoghurt. *Journal of Food Protection*. **58**(10), 1142 - 1146.

Babu, V., Mital, B.K. and Garg, S.K. (1992) Effect of tomato juice addition on the growth and activity of *Lactobacillus acidophilus*. *International Journal of Food Microbiology*. **17**, 67 - 70.

Beerens, H. (1990) An elective and selective isolation medium for *Bifidobacterium* species. *Letters of Applied Microbiology*. **11**(3), 155 - 157.

Benno, Y., Sawada, K. and Mitsuoka, T. (1984) The intestinal microflora of infants: composition of fecal flora in breast-fed and bottle-fed infants. *Microbiology and Immunology*. **28**(9), 975 - 986.

Bergey's Manual of Determinative Bacteriology (1974) Eighth edition. Edited by Buchanan, R.E. and Gibbons, N.E. The Williams and Wilkins Company, Baltimore, pp. 490-6, 503-4, 576-83, 669-71.

Blanchette, L., D. Roy and S.F. Gauthier. (1995) Production of cultured Cottage Cheese Dressing by Bifidobacteria. *Journal of Dairy Science.* **78**, 1421 - 1429.

Bracquart, P. (1981) An agar medium for the differential enumeration of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* in yoghurt. *Journal of Applied Bacteriology.* **51**(2), 303 - 305.

Braun, O.H. (1981) Effect of consumption of human and other formulas on intestinal bacterial flora in infants. In: Gastroenterology and Nutrition Infancy. Edited by Lebenthal, E., Raven Press, New York, pp. 247 - 251.

Charteris, W.P, Kelly, P.M., Morelli, L. Collins, J.K. (1997) Selective detection, enumeration and identification of potentially probiotic *Lactobacillus* and *Bifidobacterium* species in mixed bacterial populations. *International Journal of Food Microbiology.* **35**, 1 - 7.

Chevalier, P., Roy, D. and Savoie, L. (1991) X-α-Gal-based medium for simultaneous enumeration of bifidobacteria and lactic acid bacteria in milk. *Journal of Microbiological Methods* **13**, 75 - 83.

Coker, C.J. and Martley, F.G. (1982) Selective enumeration of thermophilic *Lactobacilli* in association with *Streptococcus thermophilus. New Zealand Journal of Dairy Science and Technology.* **17**, 269 - 272.

Collins, E.B. (1978) Enumeration of *L. acidophilus* with the agar plate count. *Journal of Food Protection.* **41**(6), 439 - 442.

Collins, J.K., Thornton, G. and Sullivan, G.O. (1998) Selection of probiotic strains for human Applications. *International Dairy Journal.* **8**, 487 – 490.

Dave, R.I. and Shah, N.P. (1997a) Viability of Yoghurt and Probiotic Bacteria in Yoghurts Made form Commercial Starter Cultures. *International Dairy Journal.* **7**, 31 – 41.

Dave, R.I. and Shah, N.P. (1997b) Characteristics of Bacteriocin Produced by *Lactobacillus acidophilus* LA-1. *International Dairy Journal.* **7**, 707 – 715.

Dave, R.I. and Shah, N.P. (1998) Ingredient Supplementation Effects on Viability of Probiotic Bacteria in Yogurt. *Journal of Dairy Science.* **81**, 2804 - 2816.

Davis, J.G., Ashton, T.R. and McCaskill, M. (1971) Enumeration and viability of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* in yogurt. *Dairy Industries.* **36**, 569 - 573.

Deeth, H.C. (1984) Yogurt and cultured products. *The Australian Journal of Dairy Technology.* **September**, 111 – 113.

De Man, J.C., Rogosa, M. and Sharpe M. E. (1960) *Applied Bacteriology.* **23**, 130 - 135.

Dinakar, P. and V.V. Mistry. (1994) Growth and Viability of *Bifidobacterium bifidum* in Cheddar Cheese. *Journal of Dairy Science.* **77**(10), 2854 - 2864.

Fooks, L.J., Fuller,R. and Gibson, G.R. (1999) Prebiotcs, probiotcs and human gut microbiology. *International Dairy Journal*, **9**, 53 - 61.

Finegold, S.M., Sutter, V.L., Sugihara, P.T., Elder, H.A., Lehmann, S.M. and Phillips, R.L. (1977) Fecal microbial flora in seventh day adventist populations and control subjects. *The American Journal of Clinical Nutrition.* **30**, 1781 - 1792.

Fuller, R. (1989) Probiotics in man and animals. *Jounal of Applied Bacteriology* **66**, 365 - 378.

Gibson, G.R. and Roberfroid, M.B. 1995) Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. *Journal of Nutrition.* **125**, 1401 – 1412.

Ghoddusi, H.B. and Robinson, R.K. (1996) Enumeration of starter cultures in fermented milks. *Journal of Dairy Research* **63**, 151 - 158.

Gibson, G.R. and Roberfroid, M.B. (1995) Dietary modulation of the human colonic microbiotia : introducing the concept of prebiotics. *Journal of Nutrition.* **125**, 1401 - 1412.

Gilliland, S.E. (1979) Beneficial inter-relationships between certain microorganisms and humans: Candidate microorganisms for use as dietary adjuncts. *Journal of Food Protection* **42**, 164 - 167.

Gilliland, S.E. (1989) Acidophilus milk products: a review of potential benefits to consumers. *Journal of Dairy Science.* **72**, 2483 - 2494.

Gilliland, S.E. and Lara, R.C. (1988) Influence of storage at Freezing and Subsequent Refrigerator temperature on β-Galactosidase Activity of

Lactobacillus acidophilus. Applied. Environmental Microbiology. **45** (4), 898 - 902.

Gilliland, S.E. and Speck, M.L. (1977a) Antagonistic action of *Lactobacillus acidophilus* toward intestinal and food-borne pathogens in associative cultures. *Journal of Food Protection.* **40**(12), 820 - 823.

Gilliland, S.E. and Speck, M.L. (1977b) Deconjugation of bile acids by intestinal lactobacilli. *Applied Environmental Microbiology.* **33**(1), 15 - 18.

Gilliland, S.E. and Speck, M.L. (1977c) Instability of *Lactobacillus acidophilus* in yogurt. *Journal of Dairy Scienc.e* **60**, 1394 - 1398.

Gilliland, S.E. and Speck, M.L. (1977d) Enumeration and identity of lactobacilli in dietary products. *Journal of Food Protection.* **40**(11), 760 - 762.

Gilliland, S.E., Nelson, C.R. and Maxwell, C. (1985) Assimilation of cholesterol by *Lactobacillus acidophilus. Applied and Environmental Microbiology.* **49(**49), 377 - 385.

Gomes, A.M.P., Malcata, F.X.; Klaver, A.M. and Grande, H.J. (1995) Incorporation and survival of *Bifidobacterium* sp. strain Bo and *Lactobacillus acidophilus* strain Ki in a cheese product. *Netherlands Milk and Dairy Journal.* **49**, 71 - 95.

Gomes, A.M.P. and Malcata, F.X. (1999) *Bifidobacterium* spp. and *Lactobacillus acidophilus*: biological, biochemical and therapeutical properties relevant for use as probiotics. *Trends in Food Science & Technology.* **10**, 139 – 157.

Gorbach, S.L. Chang, T.W. and Goldin, B. (1987) Successful treatment of relapsing *Clostridium difficile* colitis with *Lactobacillus* GG. The Lancet. **2**(8574), 1519.

Gurr, M.I. (1987) Nutritional aspects of fermented milk products. In: *Milk – The vital force.* Proceedings of the XXII International Dairy Congress, The Hogue. Edited by the Organizing Committee of the XXII International Dairy Congress. D. Reidel Publishing Company, Dordrect, pp. 641 - 655.

Hansen, R. (1985) Bifidobacteria have come to stay. Reprint from: *North European Dairy Journal.* **3**, 1 - 6.

Hamann, W.T. and Marth, E.H. (1983) Survival of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* in commercial and experimental yogurts. *Journal of Food protection,* **47**(10), 781 - 786.

Hekmat, S. and McMahon, D.J. (1992) Survival of *Lactobacillus acidophilus* and *Bifidobacterium bifidum* in Ice Cream for Use as a Probiotic Food. *Journal of Dairy Science.* **75**, 1415 - 1422.

Hoier, E. (1992) Use of probiotic starter cultures in dairy products. *Food Australia.* **44**(9), 418 - 420.

Holzapfel, W., Schillinger, U., Du Tiot, M. and Dicks, L. (1997) Systematics lactic acid bacteria with reference to modern phenotypic and genomic methods. *Microecology and Therapy.* **10**, 1 - 10.

Homma, N. (1988) Bifidobacteria as a resistance factor in human beings. *Bifidobacteria Microflora.* **7**(1), 35.

Hood, S.K. and Zottola, E.A. (1988) Effect of low pH on the ability of *Lactobacillus acidophilus* to survive and adhere to human intestinal cells. *Journal of Food Science.* **53**(5), 1514 - 1516.

Hughes, D.B. and Hoover, D.G. (1991) Bifidobacteria: Their potential for use in American dairy products. *Food Technology.* **45**(4), 74 - 83.

Hughes, D.B. and Hoover, D.G. (1995) Viability and enzymatic activity of bifidobacteria in milk. *Journal of Dairy Science.* **78**, 268 - 276.

Huis in't Veld, J.H.J. and Havenaar, R. (1991) *Journal of Chemical Technology and Biotechnology.* **51**, 562 - 567.

Hull, R.R. and Roberts, A.V. (1984) Differential enumeration of *Lactobacillus acidophilus* in yogurt. *The Australian Journal of Dairy Technology.* **39**, 160 - 163.

Hull, R.R., Roberts, A.V. and Mayes, J.J. (1984) Survival of *Lactobacillus acidophilus* in Yoghurt. *The Australian Journal of Dairy Technology.* **39**(4), 164 - 166.

von Hunger, W. (1986) Asculin-Cellobiose agar für die Keimzahlbestimmung von *Lactobacillus acidophilus. Milchwissenschaft* **41**(5), 283 - 285.

Hutkins, R., Moris, H.A., and McKay, L.L. (1985) Galactose transport in *Streptococcus thermophilus. Applied and Environmental Microbiology.* **50**(4), 772 - 776.

IDF (1983) Yoghurt: enumeration of characteristic microorganisms-colony count technique at 37°C. IDF Standard 117 International Dairy Federation. Brussels.

IDF (1993) Preparation of a standard for enumeration of *L. acidophilus* in fermented and non-fermented milks. *IDF Questionaire* 1293E. International Dairy Federation, Brussels.

IDF, International Standard 149, (1996) Dairy starter cultures of lactic acid bacteria-standard of identity.

Ishibashi, N. and Shimamura, S. (1993) Bifidobacteria: Research and development in Japan. *Food Technology.* **47**(6), 126, 129 - 134.

Iwana, H., Masuda, H., Fujisawa, T., Suzuki, H. and Mitsuoka, T. (1993) Isolation and identification of *Bifidobacterium* spp. in commercial yogurts sold in Europe. *Bifidobacteria Microflora* b(1), 39 - 45.

Johns, F.E., Gordon, J.F. and Shapton, N. (1978) The separation from yoghurt cultures of *lactobacilli* and *Streptococci* using reinforced clostridial agar at pH 5.5 and Plate count agar incorporating milk. *Journal of the Society of Dairy Technology.* **31**(4), 209 - 212.

Kailasapathy, K. and Rybka, S. (1997) *L.acidophilus* and *Bifidobacterium* spp. – their therapeutic potential and survival in yogurt. *The Australian Journal of Dairy Technology.* **52**, 28 - 35.

Kailasapathy, K. and Supriadi, D. (1996) Effect of whey protein concentrate on the survival of *Lactobacillus acidophilus* in lactose hydrolysed yoghurt during refrigerated storage. *Milchwissenschaft.* **51**(10), 565 - 567.

Kaplan, H. and Hutkins, R.W. (2000) Fermentation of Fructooligosaccharides by Lactic Acid Bacteria and Bifidobacteria. *Applied and Environmental Microbiology.* **66**(6), 2682 – 2684.

Kebary, K.M.K. (1996) Viability of *Bifidobacterium bifidum* and its effect on quality of frozen Zabady. *Food Research International.* **29**(5-6), 431 - 437.

Kim, H.S. and Gilliland, S. (1983.) *Lactobacillus acidophilus* as a dietary adjunct for milk to aid lactose digestion in humans. *Journal of Dairy Science.* **66**, 959 - 966.

Kim, E.R., Lee, K.W., Park, Y.H. and Kwak, H.S. (1993) The study of lactic acid bacteria in yogurt during delivery and storage. *Korean Journal of Dairy Science.* **14**(3), 260 - 268.

Klaver, F.A.M.; F. Kingma and Bolle A.C. (1990) Growth relationships between bifidobacteria and lactobacilli in milk. *Voedingsmiddelentechnologie.* **23**(9), 13 - 16.

Klaver, F.A.M.; Kingma, F. and Weerkamp, A.H. (1993) Growth and survival of bifidobacteria in milk. *Netherlands Milk Dairy Journal.* **47**, 151 - 164.

Kneifel, W. and Pacher, B. (1993) An X-Glu based agar medium for enumeration of *Lactobacillus acidophilus* in yoghurt-related milk products. *International Dairy Journal.* **3**(3), 277 - 291.

Kneifel, W.; Jaros, D. and Erhard, F. (1993) Microflora and acidification properties of yogurt and yogurt-related products fermented with commercially available starter cultures. *International Journal of Food Microbiology.* **18**, 179 - 189.

Kurmann, J.A. and Rasic, J.L. (1991) The health potential of products containing bifidobacteria. In: *Therapeutic Properties of Fermented Milks.* Edited by R. K. Robinson. Elsevier Applied Sciences, London, pp. 117 - 158.

Lankaputhra, W.E.V. and Shah, N.P. (1995) Survival of *Lactobacillus acidophilus* and *Bifidobacterium* species in the presence of acid and bile salts. *Cultured Dairy Products Journal.* **30**(3), 113 - 118.

Lankaputhra, W.E.V., Shah, N.P. and Britz, M.L. (1996) Evaluation of media for selective enumeration of *Lactobacillus acidophilus* and *Bifidobacterium* species. *Food Australia.* **48**(3), 113 - 118.

Lapierre, L., Underland, P. and Cox, L.J. (1992) Lithium chloride-sodium propionate agar for the enumeration of *bifidobacteria* in fermented dairy products. *Journal of Dairy Science.* **75**(5), 1192 - 1196.

Laroia, S. and Martin, J.H. (1991a) Effect of pH on Survival of *Bifidobacterium bifidum* and *Lactobacillus acidophilus* in Frozen Fermented Dairy Desserts. *Cultured Dairy Products Journal.* **Nov.**, 13 - 21.

Laroia, S. and Martin, J.H. (1991b) Methods for enumerating bifidobacteria. *Cultured Dairy Products Journal.* **5**, 32 - 33.

Lee, S.Y., Vedamuthu, E.R., Washam, C.J. and Reinbold, G.W. (1974) An agar medium for the differential enumeration of yoghurt starter bacteria. *Journal of Milk Food Technology.* **37**(5), 272 - 276.

Lim, K.S., Huh, C.S., Baek, Y.J. (1993) Antimicrobial Susceptibility of Bifidobacteria. *Journal of Dairy Science.* **76**(8), 2168 - 2174.

Lim, K.S., Huh, C.S., Baek, Y.J. and Kim, H.U. (1995) A selective enumeration medium for bifidobacteria in fermented dairy products. *Journal of Dairy Science.* **78**(10), 2108 - 2112.

Mann, S.V. and Spoerry, Y. (1974) Studies of a surfactant and cholesterolemia in the Maasai. American Journal of Clinical Nutrition. **27**, 464 - 470.

Mara, D.D. and Oragui, J.I. (1983) Sorbitol-fermenting *bifidobacteria* as specific indicators of feacal pollution. *Journal of Applied Bacteriology.* **55**(3), 349 - 357.

Marshall, V.M. (1991) Gut-derived organisms for milk fermentations. Is Probiotics facts of fiction? *Journal of Chemistry, Technology and Biotechnology.* **51** (4), 548 - 553.

Marshall, V.M. (1992) Inoculated ecosystems in a milk environment. *Journal of Applied Bacteriology.* Symposium supplement. **73**, 127S- 135S.

Marshall, V.M. (1996) Bioyogurt: how healthy? *Dairy Industries International.* **61**(1), 28 - 29.

Martin, J.H. and Chou, K.M. (1992) Selection of Bifidobacteria for Use as Dietary Adjuncts in Cultured Dairy Foods: 1 - Tolerance to pH of Yogurt. *Cultured Dairy Products Journal.* **27**(4), 21 - 26.

McDonald, L.C., McFeeters, R.F., Daeschel, M.A. and Fleming, H.P. (1987) A Differential Medium for the Enumeration of Homofermentative and Heterofermentative Lactic Acid Bacteria. *Applied and Environmental Microbiology* **53** (6), 1382 - 1384.

McFarland, L. and Bernasconi, P. (1993) *Saccharomyces boulardii*: A Review of an innovative biotherapeutic agent. *Microbial ecology in health and disease.* **6**, 157 - 171.

Micanel, N., I.N. Haynes and Playne, M.J. (1997) Viability of probiotic cultures in commercial Australian yogurts. *Australian Journal of Dairy Technology.* **52**, 24 - 27.

Mital, B.K. and Garg, S.K. (1992) Acidophilus milk products: manufacture and therapeutics. *Food Reviews International.* **8**(3), 347 - 389.

Mitsuoka, T. (1982) Recent trends in research on intestinal flora. *Bifidobacteria Microflora.* **1**, 13 - 24.

Modler, H.W. (1994) Bifidogenic factors-sources, metabolism and applications. *International Dairy Journal.* **4**(5), 383 - 407.

Modler, H.W., McKellar, R.C. and Yaguchi, M. (1990) Bifidobacteria and bifidogenic factors. *Canadian Institute of Food Science and Technology Journal.* **23**, 29 - 41.

Modler, H.W. and Villa-Garcia, L. (1993) The growth of *Bifidobacterium longum* in a whey based medium and viability of this organism in frozen yogurt with low and high levels of developed acidity. *Cultured Dairy Products Journal.* **28** (1), 4 - 8.

Munoa, I.J. and Pares, R. (1988) Selective medium for isolation and enumeration of *Bifidobacterium* species. *Applied and Environmental Microbiology.* **54**(7), 1715 - 1718.

Nelson, D.P., and Mato. (1970) Bacterial flora associated with the human gastrointestinal mucosa. *Gastroenterology.* **58**, 56 - 61.

Nighswonger, B.D.; Brashears, M.M. and Gilliland, S.E. (1996) Viability of *Lactobacillus acidophilus* and *Lactobacillus casei* in Fermented Milk

Products During Refrigerated Storage. *Journal of Dairy Science.* **79**, 212 - 219.

Oberman, H. (1985) Fermented milks. In: *Microbiology of fermented foods.* Edited by Wood, B.J.B. Volume 1.Elsevier Applied Science Publishers. London and New York, pp. 167 - 186.

O' Sullivan, M.G., Thornton, G., O' Sullivan, G.C. and Collins, J.K. (1992) Probiotic bacteria: myth or reality? *Trends in Food Science and Technology.* **3**, 309 - 314.

Ouwehand, A.C. and Salminen, S.J. (1998) The Health Effects of Cultured Milk Products with Viable and Non-viable Bacteria. *International Dairy Journal* **8**, 749 – 758.

Pacher, B. and Kneifel, W. (1996) Development of a culture medium for the detection and enumeration of bifidobacteria in fermented milk products. *International Dairy Journal.* **6**(1), 43 - 64.

Playne, M. (1994) Probiotic foods. *Food Australia.* **46**(8), 362.

Radke-Mitchell, L. and Sandine, W.E. (1984) Associative growth and differential enumeration of *Streptococcus thermophilus* and *Lactobacillus bulgaricus*: A review. *Journal of Food Protection.* **47**, 245 - 248.

Rasic, J.L. (1983) The role of dairy foods containing bifido and acidophilus bacteria in nutrition and health? *North European Dairy Journal.* **4**, 1 - 5.

Rasic, J.L. (1990) Culture media for detection and enumeration of bifidobacteria in fermented milk products. *IDF Bulletin.* **252**, 22 - 48.

Rasic, J.L. and Kurmann, J.A. (1983) *Bifidobacteria and their role.* Basel: Birkhauser Verlag, pp. 12-13, 21, 42-50, 102 - 133, 144 - 158.

Reilly, S.S. and Gilliland, S.E. (1999). *Bifidobacteirum longum* Survival During Frozen and Refrigerated Storage as Related to pH during Growth.*Journal of Food Science.* **64** (4), 714 – 718.

Renner, E. (1983) Microbiological aspects of cultured milk products in the diet. In: Milk and Dairy Products in Human Nutrition, *Volkswirtschaftlicher, Verlag,* München.

Resnick, I.G. and Levin, M.A. (1981) Quantitative Procedure for Enumeration of Bifidobacteria. *Applied and Environmental Microbiology* **42** (3), 427 - 432.

Roy, D.; Desjardins, M. and Mondou, F. (1995) Selection of bifidobacteria for use under cheese-making conditions. *Milchwissenschaft.* **50** (3), 139 - 142.

Rybka, S. (1994) The enumeration of *Lactobacillus, Streptococcus* and *Bifidobacterium* species in yogurt. B.Sc Dissertation, University of New South Wales, Sydney.

Rybka, S. and Kailasapathy, K. (1995) The survival of culture bacteria in fresh and freeze-dried AB yoghurts. *The Australian Journal of Dairy Technology.* **50** (2), 51 - 57.

Sandine, W.E. (1979) Role of *Lactobacillus* in the intestinal tract. *Journal of Food Protection.* **42**, 295.

Samona, A. and Robinson, R.K. (1994) Effect of yogurt cultures on the survival of bifidobacteria in fermented milks. *Journal of the Society of Dairy Technology.* **47** (2), 58 - 60.

Samona, A.; Robinson, R.K. and Marakis, S. (1996) Acid production by bifidobacteria and yoghurt bacteria during fermentation and storage of milk. *Food Microbiology.* **13**, 275 - 280.

Saxena, S.N.; Mital, B.K. and Garg, S.K. (1994) Effect of casitone and fructose on the growth of *Lactobacillus acidophilus* and its survival during storage. *International Journal of Food Microbiology.* **21**, 271 - 276.

Scardovi, V. (1986) Genus Bifidiobacterium. In: *Bergey's Manual of Systematic Bacteriology.* Edited by Sneath, P.H., Nair, N.S., Sharpe, M.E. and Holt, J.G. Volume 2. Williams and Wilkens. Baltimore, pp. 1418 - 1434.

Schuler- Malyoth, R.R.A. and Muller, F. (1968) The micro-organisms of the bifidus group (*Lactobacillus* and bifidus). II-Technology of bifidus culture in dairy plant. *Milchwissenschaft* **23** (9), 554 - 558.

Shah, N.P. and Lankaputhra, E.V. (1997). Improving Viability of *Lactobacillus acidophilus* and *Bifidobacterium* spp. In Yogurt. *International Dairy Journal* **7**, 349 - 356.

Shah, N.P.; Lankaputhra, W.E.V.; Britz, M.L. and Kyle, W.S.A. (1995) Survival of *Lactobacillus acidophilus* and *Bifidobacterium bifidum* in commercial yoghurt during refrigerated storage. *International Dairy Journal.* **5**, 515 - 521.

Shah, N. and Jelen, P. (1990) Survival of Lactic Acid Bacteria and Their Lactases under Acidic Conditions. *Journal of Food Science.* **55** (2), 506 - 509.

Shankar, P. and Davies, F. (1976) Associative bacterial growth in yogurt starters, initial observation on stimulatory factors. *Journal of the Society of Dairy Technology.* **30**(1), 31 - 32.

Shankar, P.A. and Davies, F.L. (1977) A note on the suppression of *Lactobacillus bulgaricus* in media containing beta-glycerophophate and application of such media to selective isolation of *Streptococcus thermophilus* from yoghurt. *Journal of Society of Dairy Technology.* **30**(1), 28 - 30.

Speck, M.L. (1978) Enumeration of viable *Lactobacillus acidophilus* organisms in dairy products. *Journal of Food Protection.* **41**(2), 135 - 137.

Surawicz, C.M.; Elmer, G.W.; Speelman, P. McFarland, L.V.; Chinn, J. and van Belle, G. (1989) Prevention of antibiotic-associated diarrhea by *Saccharamyces boulardii*: A prospective study. *Gastroenterology.* **96**, 981- 8.

Tanaka, R. and Mutai, M. (1980) Improved medium for selective enumeration of bifidobacterium. *Applied Environmental Microbiology.* **40**(5), 866 869.

Tamime, A.Y. and Robinson, R.K. (1985) Yogurt: Science and Technology. Pergamon Press, Oxford.

Tamime, A.Y., Marshall, V.M.E. and Robinson, R.K. (1995) Microbiological and technological aspects of milks fermented by bifidobacteria. *Journal of Dairy Research.* **62**, 151- 187.

Teraguchi, S., Uehara, M., Ogasa, K. and Mitsuoka, T. (1978) Enumeration of Bifidobacteria in Dairy Products. *Japanese Journal of Bacteriology.* **33**, 753 - 761.

Terzaghi, B.E. and Sandine, W.E. (1975) Improved medium for lactic streptococci and their bacteriophages. *Applied Microbiology.* **29**(6), 807-813.

Thomas, T.D. & Crow, L.V., (1984) Selection of galactose-fermenting*Streptococcus thermophilus* in lactose-limited chemostat cultures. *Applied and Environmental microbial.* **48**(1), 186 – 191.

Van der Wiel-Korstanje, J.A.A. and Winkler, J.C. (1970) Medium for differential count of the anaerobic flora in human faeces. *Applied Microbiology.* **20**(1), 168 - 169.

Varnam, A.H. and Sutherland, J.P. (1994) In: *Milk and milk products.* Chapman and Hall, London, pp. 347 - 380.

Vaughan-Martini, A. and Martini, A. (1998) Descriptions of teleomorphic ascomycetous genera and species. In: *The Yeasts, A Taxonomic Study.* Fourth edition. Edited by Kurtzman, C.P. and Fell, J.W. Elsevier. pp. 361 - 363.

Wijsman, M.R., Hereijgers, J.L.P.M. and de Groote, J.M.F.H. (1989) Selective enumeration of bifidobacteria in fermented dairy products. *Netherlands Milk Dairy Journal.* **43**, 395 - 405.

Young, J. (1996) A perspective on functional foods. *Food Science and Technology Today.* **10**(1), 18 – 21.

Young, C.K. and Nelson, F.E. (1978) Survival of *Lactobacillus acidophilus* in "Sweet Acidophilus Milk" during Refrigerated Storage. *Journal of Food Protection.* **41** (4), 248 - 250.

Yuhara, T., Isojima, S., Tsuchiya, F., and Mitsuoka,T. (1983) On the intestinal flora of bottle-fed infants. *Bifidobacteria Microflora.* **2,** 33 - 39.

Zúñiga, M., Pardo, I. and Ferrer, S. (1993) An improved medium for distinguishing between homofermentative and heterofermentative lactic acid bacteria. *International Journal of Food Microbiology.* **18**, 37- 42.

CHAPTER 3

EVALUATION OF MEDIA FOR SELECTIVE ENUMERATION OF PROBIOTIC YOGURT CULTURES IN COMMERCIAL BIO-YOGURT

ABSTRACT

Different media were evaluated to determine their suitability for selective enumeration of the probiotic bacteria *Lactobacillus acidophilus* and *Bifidobacterium bifidum*, and the conventional starter organisms *L. bulgaricus* and *Streptococcus thermophilus* employed for the manufacture of bio-yogurt. The media evaluated included TPPYPB agar, RCPB agar, MNA + salicin agar, L-arabinose agar, NNLP agar, maltose-MRS agar, Acidified MRS agar and M17 agar. The media recommended by Chr. Hansen's Laboratory were included for comparison reasons and proved to be the most suitable for selective enumeration. NNLP agar for *B. bifidum*, maltose-MRS for *L. acidophilus*, M17 for *S. thermophilus* and Acidified-MRS for *L. bulgaricus* were finally selected to examine counts and bacterial types in commercial AB-yogurt. Pure cultures and commercial AB-yogurts were used in deciding on suitable methods to enumerate probiotic and yogurt starter cultures.

INTRODUCTION

In recent years, the probiotic bacterial cultures *Lactobacillus acidophilus* and *Bifidobacterium bifidum* (AB-culture) have been used increasingly in the processing of conventional yogurt, resulting in bio-yogurt with excellent therapeutic value. However, for these dietary cultures to be of any therapeutic value, they should be viable and meet the suggested 'therapeutic minimum' at the time of consumption (Playne, 1994). For this reason, changes in the numbers of viable bacterial cells during the storage period and the level at the time of consumption should be known.

Monitoring the level and survival of *L. acidophilus* and *Bifidobacterium* species in probiotic yogurt has often been neglected in the past due to the unavailability of suitable selective media for the enumeration of these species (Kailasapathy and Rybka, 1997). As a result, several probiotic related dairy products reach the consumer with insufficient numbers of viable probiotic cells. Differential enumeration of *L. acidophilus* and *Bifidobacterium* species in yogurt proved to be inaccurate owing to the presence of similar bacteria, *S. thermophilus* and *L. bulgaricus,* used for the manufacturing of yogurt.

Media that have been proposed for the differential enumeration of starter cultures of a typical bio-yogurt are listed in Table 2.2. Differential enumeration of the probiotic bacteria, *L. acidophilus* and *B. bifidum*, and the yogurt bacteria, *L. bulgaricus* and *S. thermophilus*, was achieved by two different procedures. The first comprised a single medium, either TPPYPB (Ghoddusi and Robinson, 1996) or RCPB (Onggo and Fleet, 1993), allowing the simultaneous growth of different genera. Differentiation of the four constitutive bacterial strains was obtained based on colony morphology and colour. Alternatively, each species is counted separately on a selective medium. M17 agar (Tergazhi and Sandine, 1975) for *S.thermophilus*, acidified-MRS agar (IDF, 1996) for *L. bulgaricus* and Maltose-MRS agar (Hull and Roberts, 1984) and MNA+ Salicin (Lankaputhra and Shah, 1996) for *L. acidophilus* were proposed. For the isolation of *B.*

bifidum, NNLP agar (Laroia and Martin, 1991) and L-arabinose agar (Wijsman et al., 1989) were proposed. NNLP agar, M-MRS, M17 agar and Acidified-MRS are considered as the most suitable media for differential enumeration of the constitutive genera in AB-yogurt by Chr. Hansen's Laboratory (Anon, 1994; Anon., 1997).

In this study we, therefore, endeavoured to standardise enumeration methods and to assure the proper identification of the probiotic cultures, *L. acidophilus* and *B. bifidum* as well as the conventional starter cultures for yogurt, *S. thermophilus* and *L. bulgaricus*. Once standardised methods are established, the incidence, survival and interaction of the species in commercial South African AB-yogurt will be monitored.

MATERIALS AND METHODS

Pure cultures

Individual and mixed freeze-dried cultures of the probiotic strains *B. bifidum* and *L. acidophilus*, as well as AB-yogurt starter cultures, containing mixed populations of *B. bifidum*, *L. acidophilus*, *S. thermophilus* and *L. bulgaricus*, were used. Pure cultures of *L. acidophilus* and *B. bifidum*, and *S. thermophilus* and *L. bulgaricus* were obtained from Darleon (South Africa) representing CHR. Hansen's "nu-trish" culture range.

Commercial cultures

Samples of commercial plain AB-yogurt and AB-yogurt bulk starters were obtained from a local yogurt manufacturer. These yogurts were manufactured with starter cultures obtained from CHR. Hansen's, and contained probiotic AB cultures (*B. bifidum* and *L. acidophilus*) in addition to the yogurt starter cultures (*S. thermophilus* and *L. bulgaricus*).

Media preparation

TPPYPB agar (Ghoddusi and Robinson, 1996)

TPPYPB agar was prepared by adding 0.03% Prussian Blue (Ferry-III-chrome cyanide; Aldrich) to TPPY agar (Bracquart, 1981). The two dyes, Eriochrome Black-T and Prussian Blue were suspended in water and dissolved by heating before adding to the rest of the ingredients. The bacteriological agar (Merck) was added last. The pH of the medium was adjusted to 6.8 with 1N NaOH, and sterilised by autoclaving at 121°C for 20 min.

RCPB (Reinforced Clostridial Prussian Blue) agar (Onggo and Fleet, 1993)

Reinforced Clostridial agar (RCA) was supplemented with the dye, Prussian Blue, to obtain Reinforced Clostridial Prussian Blue (RCPB) agar as described by Onggo and Fleet (1993). The dehydrated commercial RCA (Oxoid CM151) was supplemented with 0.03% (w/v) of Prussian Blue in powder form (Aldrich). A modification to the commercial RCA formula included the omission of horse blood (Oxoid Manual, 1990). The supplemented commercial dehydrated RCA was prepared according to the manufacturer's instructions. The pH of RCPB agar was adjusted to 5.0 (Rybka, 1994) and sterilised by autoclaving for 20 min.

L-arabinose agar (Wijsman et al., 1989)

L-arabinose agar was prepared and sterilised as described for TOS agar (Wijsman et al., 1989), except for the replacement of the TOS component by 100 ml of a 20% L-arabinose (Merck, Darmstadt) solution.

MNA + Salicin (Lankaputhra and Shah, 1996)

The media was prepared and sterilised as described by Lankaputhra and Shah (1996).

Maltose-MRS (deMan Rogosa and Sharpe) (Hull and Roberts, 1984) (pH6.9)

M-MRS (Table 3.1) was prepared using MRS-IM as the base medium (Anon., 1997). A 20% (w/v) maltose solution was prepared by filter sterilising (0,45 μm) and aseptically added to MRS-IM base medium previously autoclaved and cooled to 47°C ± 1°C (100ml to 1000ml).

NNLP agar (Laroia and Martin, 1991) (pH 6.9)

NNLP was prepared according to the CHR. Hansen method (Anon., 1994; Anon., 1997). NNLP agar was supplemented with L-cysteine hydrocloride (Solution C) which lowers the oxidation-reduction potential of the medium and enhances anaerobic growth of the bifidobacteria (Teraguchi et al., 1978). MRS-IM is the basal medium, the composition being given in Table 3.1.

Solution A: NNLP antibiotic solution (Anon., 1994)

0.200g	neomycin-sulphate (Sigma N1876),
0.030g	nalidix acid (Sigma N8878),
6.000g	lithium chloride (Merck)
0.250g	paramomycin sulphate (Sigma P9297).

The compounds of solution A were suspended in 100ml DI water in a 100ml-graduated flask and the pH adjusted to 7.2-7.5 with 0.1 N NaOH. The solution was filter sterilised (0.45 μm) and kept at refrigerated temperature (Anon., 1994).

Solution B: 20% (w/v) glucose solution (Anon., 1997)

10g glucose (Merck) was suspended in 100ml DI water and filter sterilised (0.45 μm).

Solution C: 10% (w/v) L-cysteine HCL solution (Anon., 1997)

10g L-cysteine hydrocloride (BDH laboratories, 3705553M) was suspended in 100ml DI water and sterilised by autoclaving. It has a shelf life of maximum two weeks at refrigeration temperature.

50ml of solution A, 100ml of solution B and 5ml of solution C were added to 1000ml sterile MRS-IM agar, previously cooled to 47°C ± 1°C, before use.

M17 agar (pH7.1-7.2) (Tergazhi and Sandine, 1975)

Dehydrated M17 agar (Oxoid, Basingstoke, CM 785) was prepared according to the manufacture's instructions. The pH was adjusted to 7.1 – 7.2 at 25°C with 0.1 N NaOH.

Acidified MRS-agar (pH 5.4) (IDF, 1996)

Dehydrated MRS agar (Oxoid CM 361) was prepared according to the manufacturer's instructions. Prior to sterilisation glacial acetic acid was used to adjust the pH of the medium to 5.4 at 25°C.

TPY broth (Bergey's Manual, 1974)

The broth was prepared according to the manufacturer's instructions.

Cultivation and enumeration procedures

The freeze-dried pure cultures were aseptically transferred into tubes containing sterile MRS broth (Oxoid CM359) and incubated at 37°C for 8-12h (until growth was evident). The cultures were streaked on MRS agar (Oxoid CM359) and incubated at 37°C for 24-48h. *B. bifidum* colonies were transferred to sterile TPY broth and incubated for 24 hrs at 37°C. To determine growth and selectivity of the pure cultures on each media, typical colonies grown on the MRS agar plates were streaked out on all the media. Colony isolates from the different media and TPY broth were Gram stained and microscopically examined to confirm their characteristic cell morphology (Bergey's Manual, 1974).

Serial dilutions of the cultures in MRS-broth, bulk starter and yogurt were prepared as required in sterile peptone buffer solution. Duplicate plates of

each medium were spread plated and incubated at the recommended temperature under aerobic or anaerobic conditions (Table 3.2). Anaerobic conditions were obtained by using an anaerobic jar and anaerobic kit from Oxoid (Basingstoke). An anaerobic indicator (Oxoid) was enclosed in the jar on all the occasions.

Identification procedures

Colony isolates were identified using an identification scheme (Fig. 3.1). The catalase test, gram stain procedure, fermentation tests and their reagents were performed as described in Harrigan and McCance (1976). Cell morphology characteristics for lactobacilli and bifidobacteria as presented in the 8th edition of Bergey's Manual of Determinative Bacteriology (1974) were used as basis for the microscopical identification of isolates.

RESULTS AND DISCUSSION

Microscopic examination of cell morphology of pure strains

The *Lactobacillus* species were differentiated based on characteristic differences in cell morphology. Both *Lactobacillus* species appeared as rods. *L. bulgaricus* cells were typically straight, slender and long rods with sharp ends, and exhibited internal granulation with the gram stain (Fig. 3.2 a). *L. acidophilus* is characterised by thicker rods, sometimes bent, with round ends (Fig. 3.2 b).

B. bifidum cells also appeared as rods. Bifurcated Y and V forms were characteristically observed in 24h old culture in TPY broth (Fig. 3.2 c). The morphology of bifidobacteria is influenced by nutritional conditions and on sub-culturing (Bergey's Manual, 1974). *Bifidobacterium* is gram-positive but often stained irregularly.

S. thermophilus subsp. *thermophilus* had characteristic spherical to ovoid cells, in pairs to long chains (Fig. 3.2 d).

Media

TPPYPB agar

TPPYPB agar allows the visible separation of all four of the organisms found in a typical bio-yogurt, namely *L. acidophilus*, *B. bifidum*, *L. bulgaricus* and *S. thermophilus* (Ghoddusi and Robinson, 1996).

Differentiation of pure cultures on TPPYPB agar:

All the pure bacterial species grew on the TPPYPB agar. Each species developed characteristic colony morphology as described in literature (Fig. 3.3 a-d). *L. acidophilus* produced typical large pale blue colonies surrounded by a wide royal blue zone on TPPYPB agar (Fig. 3.3 a). *B. bifidum* produced white colonies with no zone (Fig. 3.3b). *L. bulgaricus* produced shiny white colonies smaller than *L. acidophilus*, surrounded by a wide royal blue zone (Fig. 3.3 c). *S. thermophilus* was distinguished as pale blue colonies surrounded by a narrow light blue zone (Fig. 3.3 d).

Differentiation of commercial AB-yogurt cultures on TPPYPB agar:

Typical colony formation of *L. acidophilus*, *B. bifidum*, *S. thermophilus* and *L. bulgaricus* incorporated as a mixed population in commercial AB-yogurt, could not be obtained on the agar medium. Therefore, limitations existed when using TPPYPB agar for general enumeration purposes. Microscopical examination of these colonies indicated mixed bacterial cells consisting of all the different species. Even at higher dilutions, when smaller numbers of colonies were visible on the plates, differentiation between the species by typical colony formation proved to be difficult due to the presence of contaminating bacterial

species present in high numbers in the yogurt. When individual organisms were present in low numbers, typical colony formation and differentiation were inhibited by groups present at higher numbers. Consequently, proper enumeration of all four species present in the AB-yogurt using TPPYPB agar as a single medium, proved to be inadequate. The inadequacy of using TPPYPB agar as a single medium for the isolation of the different species is in contrast with results obtained by Ghoddusi and Robinson (1996). They did, however, caution that the medium needs modification to cope with the specific cultures in a given setting due to strain differences.

RCPB agar

While *L. acidophilus* failed to grow on RCPB agar, pure cultures of *B. bifidum*, *L. bulgaricus* and *S. thermophilus* were distinguishable when grown this medium and it proved to be suitable for limited enumeration purposes. Poor differentiation and enumeration results, however, were observed when applied to sampling of commercial AB-yogurt. *B. bifidum*, *L. bulgaricus* and *S. thermophilus* colonies could only poorly be distinguished on the medium. Microscopic examination of the colonies, revealed mixed populations.

MNA + Salicin agar

According to literature, MNA+Salicin agar is specific for the differential enumeration of *L. acidophilus* in bio-yogurt. Pure cultures of *L. acidophilus*, as well as *B. bifidum*, *L. bulgaricus* and *S. thermophilus*, however, were able to grow on MNA + Salicin agar proving the inadequacy of the medium as a selective medium (Table. 3.3). Accordingly, *L. acidophilus* present in commercial bio-yogurt, could not be enumerated using MNA + Salicin in this study.

L-Arabinose agar

Pure cultures of *B. bifidum* showed growth on this agar (Table 3.3), whereas the cultures isolated from commercial yogurt, showed no growth. However, L-arabinose agar is considered specific for the selection of *B. longum*. The *Bifidobacterium* species generally associated with commercial yogurt, is *B. bifidum*.

M-MRS, NNLP, M17 and Ac-MRS agar

M-MRS, NNLP, M17 and Ac-MRS agar were recommended by Chr. Hansens and were therefore incorporated in this study for comparative reasons. The results are indicated in Table 3.4.

M-MRS agar

Pure cultures of *L. acidophilus* grew on the M-MRS plates, whereas the remaining species could not. Furthermore, use of the M-MRS agar also resulted in the differentiation of *L. acidophilus* from *B. bifidum*, *L. bulgaricus* and *S. thermophilus* obtained from commercial AB-yogurt. The selectivity is based on the utilisation and growth of *L.acidophilus* on maltose as a carbohydrate source, whereas the conventional yogurt starter cultures and bifidobacteria were unable to utilize this carbon source.

The use of bile salts as a selective inhibitor was proposed for the recovery of viable *L. acidophilus* organisms from sweet acidophilus milk (Collins, 1978; Speck, 1978). Maltose-MRS media, however, gave higher cell recoveries compared to lactobacillus selection agar containing bile salts, and proved most suitable for enumeration of *L. acidophilus* in Australian yoghurt (Hull and Roberts, 1984).

NNLP agar

The NNLP antibiotic solution suppresses the growth of lactobacilli and *S. thermophilus* (Anon., 1994). Colonies isolated from the NNLP plates were microscopically examined and the cells were recognised as being typical of *Bifidobacterium* species. Pure cultures of *S. thermophilus* showed very weak growth on the NNLP plates. Comparative investigations with commercial AB-yogurt indicated similar results. Colonies were enumerated more effectively after 5 days of incubation than after 3 days as recommended (Anon., 1994).

According to the literature only 50-60% of *Bifidobacteria* species are recovered on NNLP agar when compared to the anaerobic growth response on MRS agar (Anon., 1994; Micanel et al., 1997). Consequently, NNLP agar could not be regarded as a suitable enumeration medium for *Bifidobacteria.* Shah et al. (1995), however, argued that despite the restriction of growth of *B. infantis* and *B. adolescentis,* the medium is suitable for the enumeration of *B. bifidum* claimed to be present in commercial AB-yogurt.

M17 agar

M17 agar was found to be selective solely for the isolation and enumeration of *S. thermophilus*, preventing the growth of lactobacilli due to high levels of the buffering agent β-glycerophosphate (Shankar and Davies, 1977). Aerobic incubation prevented the growth of the anaerobic bifidobacteria.

Ac-MRS

Ac-MRS was successful for the selective isolation and enumeration of *Lactobacillus bulgaricus.* Small colonies representing *L. acidophilus,* however, were observed on Ac-MRS plates when inoculated with pure cultures of the

species. The ability of *L. acidophilus* to grow on Ac-MRS agar may be a limiting factor when using the media for the enumeration of *L. bulgaricus* populations.

Based on the results obtained in this study, which included pure cultures and commercial yogurts, we concluded that the media recommended Chr. Hansen's Laboratory suited the best for differentiation and enumeration of the yogurt cultures. Accordingly, these media will be used to determine the level and survival of the yogurt cultures in commercial AB-yogurt.

Table 3.1 Preparation* of MRS-IM agar (Anon., 1997).

Compound	Quantity
Tryptone (Oxoid L42)	10g
Yeast extract (Difco)	5g
Tween 80 (Merck)	1ml
K_2HPO_4 (MandB laboratories)	2.6g
Sodium acetate $3H_2O$ (AnalaR)	5g
Di-Ammonium hydrogen citrate (Merck)	2g
$MgSO_2.7H_2O$ (Merck)	0.2g
$Mn_2SO_4.H_2O$ (AnalaR)	0.05g
Agar (Oxoid L11)	13g
Distilled (DI) water	1000ml

*The MRS-IM agar is sterilised by autoclaving at 121°C for 15 min. The pH is adjusted to 6.9 ± 0.1 after sterilisation with 0.1 N NaOH at 25°C.

Table 3.2 Incubation temperature, time and conditions for the different media*.

Medium	Temperature (°C)	Time (days)	Condition
TPPYPB agar	42	3	Anaerobic
RCPB	37	3	Anaerobic
MNA + Salicin	37	3	Aerobic
L-arabinose	37	3	Anaerobic
M-MRS	37	3	Anaerobic
NNLP	37	3	Anaerobic
M17	37	3	Aerobic
Ac-MRS	37	3	Anaerobic

* For full names of and references to the media, see text.

Table 3.3 Reaction of pure and commercial cultures of bio-yogurt on MNA+Salicin and L-arabinose agar.

	MNA+SALICIN		L-ARABINOSE	
	Pure cultures	Commercial AB-yogurt culture	Pure cultures	Commercial AB-yogurt culture
L.a	+	-	-	-
B.b	+	-	+	-
L.b	+	-	-	-
S.t	+	-	-	-

+ = Growth, - = No growth

L.a = *Lactobacillus acidophilus; B.b = Bifidiobacterium bifidum; L.b = Lactobacillus bulgaricus; S.t = Streptococcus thermophilus*

Table 3.4 Colony counts of mixed cultures (AB-yogurt bulk starter) and growth of pure cultures of *L. acidophilus*, *B. bifidum*, *S. thermophilus* and *L. bulgaricus* on differential media recommended by Chr. Hansens (Anon., 1997).

Medium	**Bulk starter Culture**	**Pure cultures**			
	Cfu/ml	***L.acidophilus***	***B.bifidum***	***S.thermophilus***	***L.bulgaricus***
M-MRS	$1.8x10^8$	+	-	-	-
NNLP	$1.9x10^7$	-	+	W	-
M17	$1.4x10^9$	-	-	+	-
Ac-MRS	$5.0x10^7$	W	-	-	+

+ = growth, - = no growth, w = weak growth

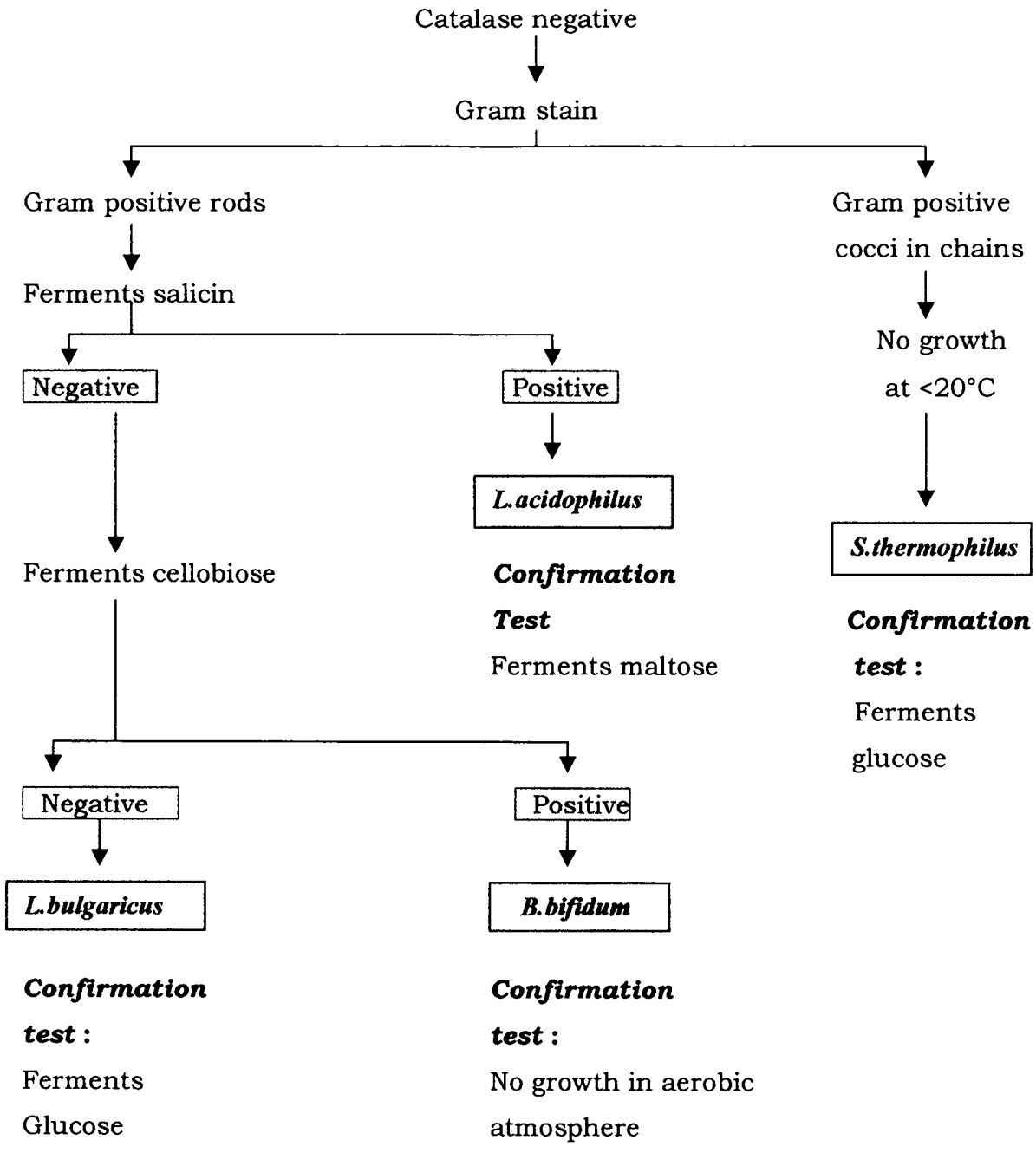
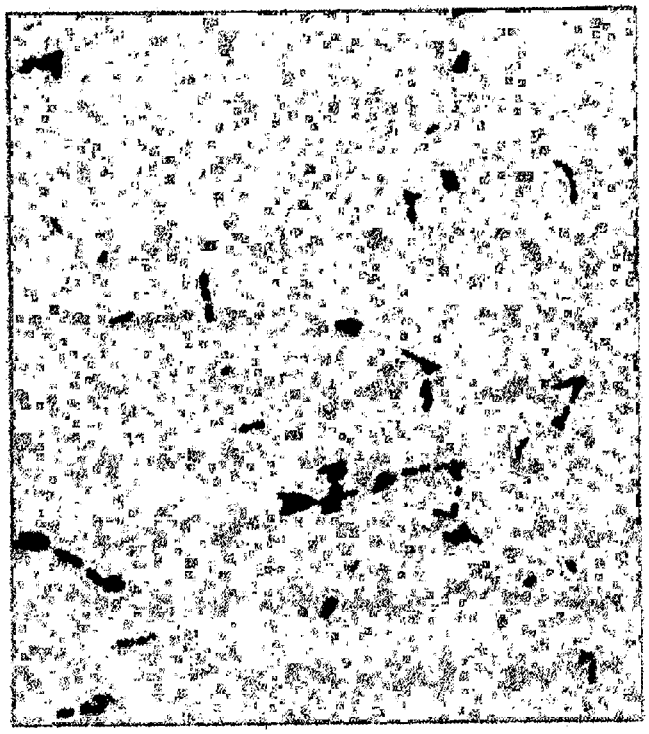
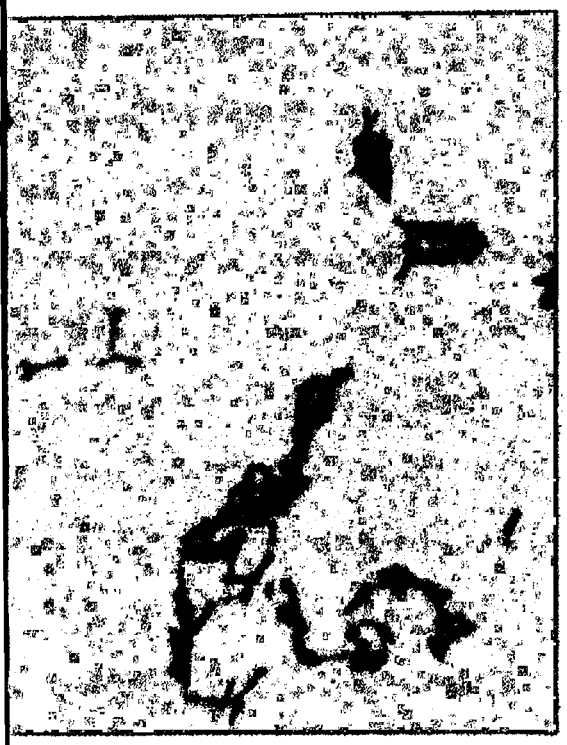


Fig. 3.1. Scheme used for the identification of bacterial isolates

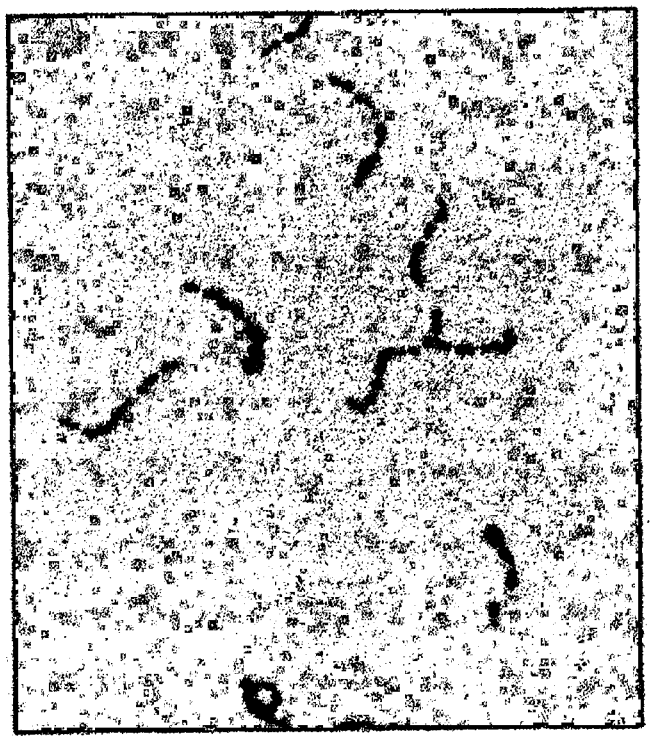
Lactobacillus bulgaricus



(b) *Lactobacillus acidophilus*

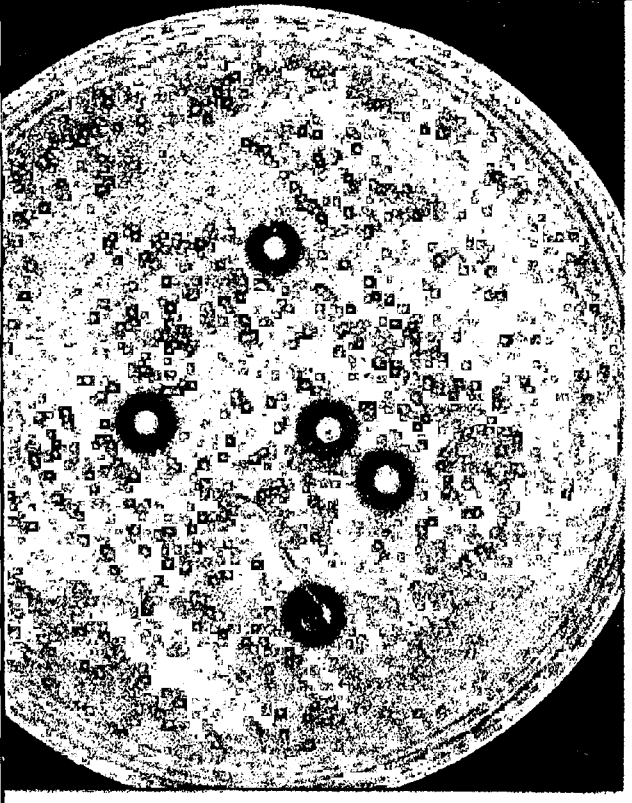


Bifidobacterium bifidum



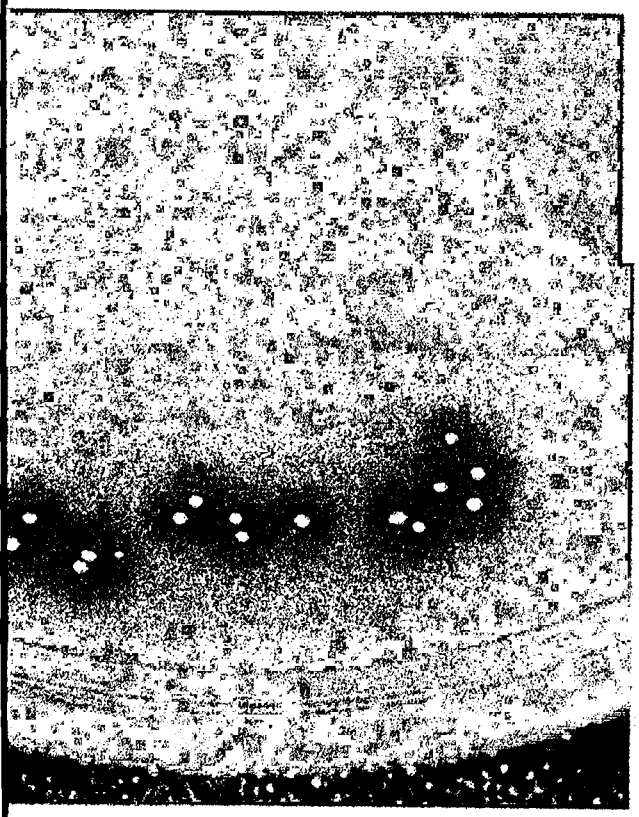
(d) *Streptococcus thermophilus*

3.2 (a - d). Gram stain to illustrate cellular morphology (magnified 1000X)

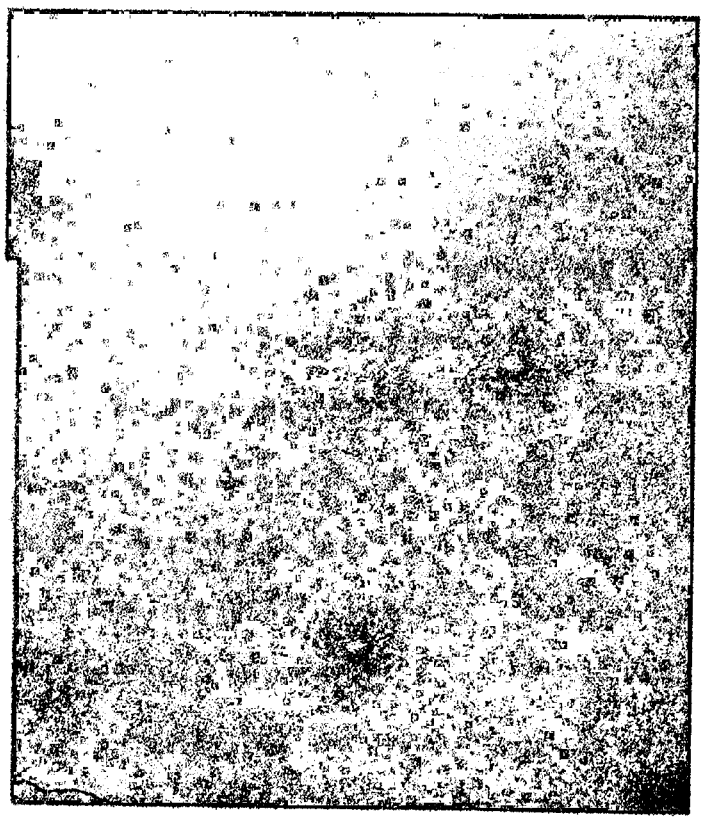


L. acidophilus colonies

(b) *B. bifidum* colonies



L. bulgaricus colonies



(d) *S. thermophilus* colonies

3.3 (a – d) Typical colony formation of pure cultures of bio-yogurt bacteria on TPPYPB agar medium.

REFERENCES:

Anon. (1997) *Lactobacillus acidophilus*, *Lactobacillus casei* and *Bifidobacteria* in milk products made with nu-trish cultures. *CHR. Hansen.* Guideline. Count of bacteria, 1-5.

Anon. (1994) Method of analysis for identification of *L. acidophilus* and *Bifidobacteria* (A medium for differentiating between *L. acidophilus*, *Bifidobacteria* and *S. termophilus* in ABT products) Chr.Hansen's Laboratory, Copenhagen, Denmark.

Bergey's Manual of Determinative Bacteriology (1974) Eighth edition. Edited by Buchanan, R.E. and Gibbons, N.E. The Williams and Wilkins Company, Baltimore. 490-6, 503-4, 576-83, 669-71.

Bracquart, P. (1981) An agar medium for the differential enumeration of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* in yoghurt. *Journal of Applied Bacteriology.* **51**(2), 303-305.

Collins, E.B. (1978) Enumeration of *L. acidophilus* with the agar plate count. *Journal of Food Protection.* **41**(6), 439-442.

De Man, J.C., Rogosa, M. and Sharpe M. E. (1960) *Applied Bacteriology.* **23** 130-135.

Ghoddusi, H.B. and Robinson, R.K. (1996) Enumeration of starter cultures in fermented milks. *Journal of Dairy Research* **63**, 151-158.

Harrigan, W.F. and McCance, M.E. (1976) Laboratory methods in food and dairy microbiology. 2nd Edition. Academic Press, London.

Hull, R.R. and Roberts, A.V. (1984) Differential enumeration of *Lactobacillus acidophilus* in yogurt. *The Australian Journal of Dairy Technology.* **39,** 160 - 163.

IDF, International Standard 149, (1996) Dairy starter cultures of lactic acid bacteria-standard of identity.

Kailasapathy, K. and Rybka, S. (1997) *L.acidophilus* and *Bifidobacterium* spp. – their therapeutic potential and survival in yogurt. *The Australian Journal of Dairy Technology.* **52,** 28 - 35.

Lankaputhra, W.E.V. and Shah, N.P. (1996) A simple method for selective enumeration of *Lactobacillus acidophilus* in yogurt supplemented with *L. acidophilus* and *Bifidobacterium* species. *Milchwissenschaft* **51**(8), 446 - 450.

Laroia, S. and Martin, J.H. (1991) Methods for enumerating bifidobacteria. *Cultured Dairy Products Journal.* **5**, 32 - 33.

Micanel, N., I.N. Haynes and Playne, M.J. (1997) Viability of probiotic cultures in commercial Australian yogurts. *Australian Journal of Dairy Technology.* **52** 24 - 27.

Onggo, I. and Fleet, G.H. (1993) Media for the isolation and enumeration of lactic acid bacteria from yoghurts. *Australian Journal of Dairy Technology.* **48**(11), 89 - 92.

Oxoid Manual, The. (1990) 6th edition. Compiled by Bridson, E.Y. Alphaprint, Alton, Hants.

Playne, M. (1994) Probiotic foods. *Food Australia.* **46**(8), 362.

Rybka, S. (1994) The enumeration of *Lactobacillus*, *Streptococcus* and *Bifidobacterium* species in yogurt. B.Sc Dissertation, University of New South Wales, Sydney.

Shah, N.P.; Lankaputhra, W.E.V.; Britz, M.L. and Kyle, W.S.A. (1995) Survival of *Lactobacillus acidophilus* and *Bifidobacterium bifidum* in commercial yoghurt during refrigerated storage. *International Dairy Journal.* **5,** 515 - 521.

Shankar, P.A. and Davies, F.L. (1977) A note on the suppression of *Lactobacillus bulgaricus* in media containing beta-glycerophophate and application of such media to selective isolation of *Streptococcus thermophilus* from yoghurt. *Journal of Society of Dairy Technology.* **30**(1), 28 - 30.

Speck, M.L. (1978) Enumeration of viable *Lactobacillus acidophilus* organisms in dairy products. *Journal of Food Protection.* **41**(2), 135 - 137.

Teraguchi, S., Uehara, M., Ogasa, K. and Mitsuoka, T. (1978) Enumeration of Bifidobacteria in Dairy Products. *Japanese Journal of Bacteriology.* **33**, 753 - 761.

Terzaghi, B.E. and Sandine, W.E. (1975) Improved medium for lactic streptococci and their bacteriophages. *Applied Microbiology.* **29**(6), 807 - 813.

Wijsman, M.R., Hereijgers, J.L.P.M. and de Groote, J.M.F.H. (1989) Selective enumeration of bifidobacteria in fermented dairy products. *Netherlands Milk Dairy Journal.* **43**, 395 - 405.

CHAPTER 4

LEVELS OF PROBIOTIC BACTERIA IN SOUTH AFRICAN COMMERCIAL BIO-YOGURT

ABSTRACT

It is essential that bio-yogurt meet the criteria of a minimum of 10^6 cfu/ml of probiotic bacteria until the expiry date to have potential therapeutic advantages for the consumer. Samples of three South African brands of commercial AB-yogurt were obtained from supermarket outlets and enumeration studies of viable probiotic cultures, *Lactobacillus acidophilus* and *Bifidobacterium bifidum,* as well as conventional yogurt starter cultures, *Streptococcus thermophilus* and *Lactobacillus bulgaricus*, were carried out. The pH and product (shelf) temperatures were also measured on every sampling occasion. All three product brands of plain yogurt contained 10^6 - 10^7 viable cells of *L. acidophilus*, whereas all the products contained $<10^4$ viable cells of *B. bifidum*. *S. thermophilus* and *L. bulgaricus* counts were in the range of 10^8 and 10^6 -10^7 respectively. *L. acidophilus* counts in the entire flavoured yogurt complied with the suggested minimum level of 10^6 cells/ml for probiotic cultures in dairy products. The *B. bifidum* counts did not meet the proposed criteria in any of the yogurt brands or flavours. The low numbers of *B. bifidum* suggests that these organisms either could not attain the required levels during manufacture or were incapable of maintaining these levels during storage. *L. acidophilus* and *B. bifidum* counts were predominantly lower in plain yogurt compared to flavoured yogurt. Average pH values between the different brands ranged from 3.9 to 4.1. The shelf temperatures varied from -1° to 7°C, while diversity in temperature control was also experienced in the positions on the shelf.

INTRODUCTION

AB yogurt, containing *Lactobacillus acidophilus* and *Bifidiobacterium bifidum*, is a potential vehicle by which consumers can take in probiotic cells. To achieve the optimal potential therapeutic effects, the minimum number of viable cells in a probiotic product should be $>10^6$ cfu/ml, the so called - "therapeutic minimum"- (Davis et al., 1971; Kurmann and Rasic, 1991; Rybka and Kailasapathy, 1995). Consequently, one should strive to consume 10^8 live cells of *L.acidophilus* and *B.bifidum* per day. Regular consumption of 400 - 500g/week of AB yogurt, containing 10^6 viable cells/ml AB yogurt, would supply the efficient amount (Thamime et al., 1995).

Furthermore, it is imperative that bio-yogurt, claiming "with live AB cultures", meet the criteria of 10^6 cfu/ml viable cells of probiotic bacteria at the expiry date. If the bio-yogurt does not comply with the required criteria, it can be considered misleading to the consumers and limited health properties are conferred.

Viability and activity of the probiotic bacteria are therefore important considerations since the bacteria have to survive for the duration of the shelf life with resulting therapeutic effects. Studies have shown, however, that *L. acidophilus* and *B. bifidum* are unstable in yogurt (Hull and Roberts, 1984; Rybka and Kailasapathy, 1995; Klaver et al., 1993). Accordingly, probiotic organisms often fail to survive in yogurt, resulting in reduced levels and activity. Limited numbers of lactobacilli were found in products, sold as sources of *L. acidophilus*. Of seven products, supposed to contain lactobacilli, *L. acidophilus* was present in only three (Gilliland and Speck, 1977). More recently, Kailasapathy and Rybka (1997) reported that several brands of bio-yogurt sold in Australian supermarkets, contained very low numbers of *B. bifidum*. The *L. acidophilus* count was also low in some brands. Of eight commercial yogurt samples sold in London claiming the presence of viable bifidobacteria, only five were found to contain viable bifidobacteria cells at

populations >10^6 /ml, while the remaining three contained no bifidobacteria (Iwana et al., 1993). Modler and Villa –Garcia (1993) reported on the absence of bifidobacteria in several bio-yogurt products in North America due to the high acid conditions. The presence of *L. bulgaricus,* contributing to acid production, was found to be mainly responsible for the inhibition of *L. acidophilus* and *Bifidobacterium.*

Consequently, there was a need to establish the level of probiotic bacteria in South African AB-yogurt. This study reports on the viable cell numbers of probiotic bacteria found in commercial South African AB-yogurt. In addition, yogurts obtained from different manufacturers were statistically compared with regard to the incidence of probiotic bacteria. The maintenance of the 'therapeutic minimum' was also determined.

MATERIALS AND METHODS

Yogurt samples

Duplicate batches, each consisting of six commercial yogurts of the same brand, flavour and batch dates, were obtained from a supermarket outlet. Sampling of batches was repeated with three different manufacturer brands and the entire sampling replicated to include two different flavours. In total 72 commercial yogurt products comprising flavoured (apricot) and plain yogurt, were tested. Normal retail packs (175ml) were taken from the shelf, immediately placed in insulated cool-boxes and transported to the laboratories for microbiological and chemical analysis. Once in the laboratory, all the samples were stored at refrigeration temperature (4°C). Products were analysed within 24 hrs of sampling.

The age of the yogurt samples ranged from 6 to 15 days according to the manufacturing date. All the products claimed to contain "live AB" cultures

(viable acidophilus and bifidobacteria cultures) in addition to conventional yogurt cultures.

Chemical analysis

The pH values were determined using a HANNA Microprocessor pH meter (model Hi 9321).

Media preparation

M-MRS agar, NNLP agar, M17 agar and Ac-MRS agar were prepared as described in Chapter 3, and used for the differential enumeration of *L. acidophilus*, *B. bifidum*, *S. thermophilus* and *L. bulgaricus* respectively.

Sample preparation: resuspension and enumeration

The contents of each yogurt container were uniformly mixed, and a 5.0ml sample was aseptically withdrawn, resuspended in 45 ml of Callichia et al. (1993) resuspension medium (CRM) and shaken vigorously to break up the rod- and coccus chains.

The resuspension and dilution medium described by Callichia et al. (1993) consisted of 4.5g KH_2PO_4 (M and B laboratories), 6g of Na_2HPO_4 (Merck), 0.4g of agar (Oxoid) and 1 ml 10% Antifoam B silicone Emulsion (Dow Corning) suspended in distilled water (1000ml). For dilution purposes, the medium was adjusted to pH 7.0 +/- 0.1 and then autoclaved at 121°C for 30 minutes.

Yogurt suspensions were serially diluted as required in CRM. One ml of each of the appropriate dilutions was transferred into duplicate sterile disposable Petri dishes. The time elapsing between the preparation of the dilutions and the pouring never exceeded 15 min as recommended (Callichia et al., 1993). Molten agar of the appropriate media was then poured into the plates,

thoroughly swirled and allowed to solidify. A control plate of each media was also poured and incubated as described to ensure that the media were sterile.

Incubation

The solidified pour plates of M-MRS agar and NNLP agar were incubated anaerobically in anaerobic jars (Oxoid) at 37°C for 4 and 5 days respectively. The anaerobic atmosphere was generated by Anaerocult A blocks (Merck). An anaerobic indicator (Merck) was included in the jar. M17 agar plates and Ac-MRS plates were incubated aerobically at 37°C for 3 days.

Recording and expression of results

Plates with colonies between 25 and 300 were counted with the aid of a colony counter and recorded as $\log_{10}$ colony forming units (cfu) per ml yogurt. The results are presented in Table 4.1 and Figs. 4.1 and 4.2. Results are the means of all available replicates within a batch.

Statistical analysis

All results were tabulated and statistically analysed employing the Analysis of Variance (ANOVA). Means were compared by using the Student-Neumann-Keuls multiple comparison test. A significant F-value of $p \leq 0.05$ was employed (Schefler, 1979).

Confirmation of identity

Three colonies were randomly isolated from the highest dilution of countable plates on M17, Ac-MRS, M-MRS and NNLP agar plates representing each brand at sampling occasions, gram stained and microscopically examined. The identity of the cultures was based on the phenotypic characteristics as

presented in the 8th edition of *Bergey's Manual of Determinative Bacteriology* (1974).

RESULTS AND DISCUSSION

Level

The average *L. acidophilus* count in the apricot yogurts of all three manufacture's brands was 10^7 cfu/ml, whereas the counts in plain yogurt ranged between 10^6 to 10^7 cfu/ml. *B. bifidum* populations in apricot yogurt had mean counts of 10^3 cfu/ml yogurt. The average *B. bifidum* counts within the plain yogurt for all the brands were significantly less ($p \leq 0.05$), namely 10^1 cfu/ml. Some yogurt samples exhibited counts as low as <10 cfu/ml. The counts observed regarding *B. bifidum* present in the plain yogurt, were on average one log unit lower than the counts obtained from the flavoured yogurt. The better survival of *B. bifidum* in flavoured yogurt is ascribed to the higher sucrose level.

The predominance of *L. acidophilus* species compared to *B. bifidum* is clearly indicated in Figs. 4.1a and b. The major difference in the counts is attributed to the better survival rate of *L. acidophilus* under acidic conditions (Shah and Jelen, 1990). According to Shah and Jelen (1990), *L. acidophilus* strains are capable of surviving better under acidic conditions compared to the traditional yogurt cultures (*L. bulgaricus* and *S. thermophilus*) in yogurt (Shah and Jelen, 1990). The final pH of the yogurt therefore has the most profound effect on the survival of bifidobacteria (Hekmat and McMahon, 1992; Kailasapathy and Rybka, 1997). Furthermore, since bifidobacteria are strict anaerobes, dissolved oxygen present in the yogurt also restricted their growth and survival.

S. thermophilus was the most dominant species with counts ranging between 10^7 to 10^8 cfu/ml in both the flavoured and plain yogurt. The predominance of *S. thermophilus* in yogurt is consistent with the studies of Kneifel et al. (1993),

who reported that almost 80% of the yogurts studied revealed higher counts of cocci than rods. High levels of *S. thermophilus* in combination with sufficient levels of AB cultures resulted in the successful progression of all three species when grown in association. The positive association is probably due to the oxygen-scavenger effect induced by *S. thermophilus* species and thereby stimulating the growth of *B. bifidum* (Ishibashi and Shimamura, 1993).

The average *L. bulgaricus* counts in apricot and plain yogurt were very high, in the range of 10^6 to 10^7 cfu/ml, if one takes into consideration that Chr. Hansens (Anon., 1997) advises manufacturers to use ABT (*L. acidophilus, B. bifidum, S. thermophilus*) DVS yogurt cultures without *L. bulgaricus*.

The exclusion of *L. bulgaricus* will result in maintaining high populations of *L.acidophilus* and *B. bifidum*. Mixed cultures containing *L. acidophilus* and *L. bulgaricus* may produce a fermented product with improved flavour and texture, but the numbers of viable cells of *L. acidophilus* may be reduced due to overgrowth by *L. bulgaricus*. Furthermore, *L. bulgaricus* produces lactic acid, leading to a decrease in pH value of less than 4.0 in the yogurt which inhibits the growth of *B. bifidum*. Gilliland and Speck (1977) reported that milk cultured with *L. bulgaricus* was antagonistic against *L. acidophilus* species causing loss in viability. Apparently, the instability of *L. acidophilus* was caused by hydrogen peroxide produced by *L. bulgaricus*. Therefore, a significant increase ($p \leq 0.05$) in the survival rates of *B. bifidum* and *L. acidophilus* were observed in the flavoured yogurt when lower numbers of *L. bulgaricus* cells were present. The presence of various fruits in yogurt, however, had little effect on the survival of the yogurt starter bacteria (Hamman and Marth, 1983).

Statistical comparisons

Statistical comparisons showed significant differences between brands, batches, and flavours (Table 4.1). The *L. acidophilus* count of apricot yogurt in

the different brands did not differ significantly ($p \geq 0.05$), except for one batch. *B. bifidum* counts differed significantly ($p \leq 0.05$) between brands as well as between batches. Similar results were obtained for *S. thermophilus* and *L. bulgaricus*.

pH and shelf temperature

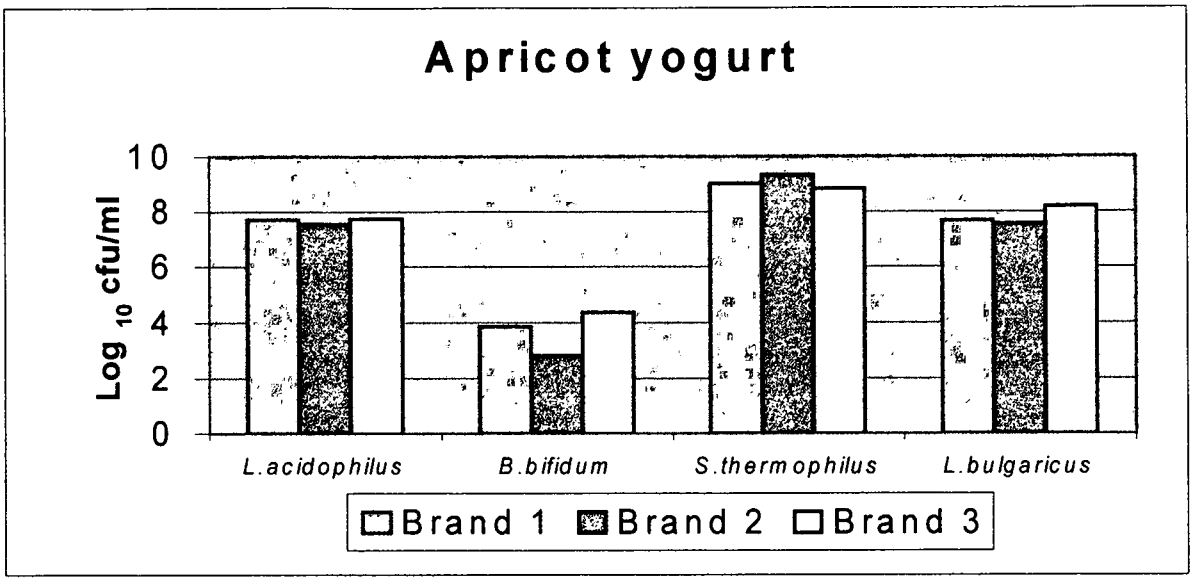
The pH of the yogurt was measured on every sampling occasion, being an important factor for the survival of the probiotic bacteria. Viable cell populations of *L. acidophilus* and *B. bifidum* decreased when grown at low pH levels. The average pH level between brands differed by at least 0.16 units (Table 4.2). The traditional yogurt starter bacteria proved to be active at refrigerated temperatures, producing low amounts of lactic acid due to the utilization of lactose resulting in a pH decrease.

The shelf temperatures were in the range of –1 to 0.5°C, and 4°C and 7°C at the different supermarket outlets. It was found that the temperature of the yogurts at the front of the shelf differed from those at the back by an average of 5°C. According to Kneifel et al. (1993), storage temperature substantially influenced the production of lactic acid, related to the growth and survival of the starter cultures at higher temperatures. The storage temperature furthermore, plays an important role in the control of excessive growth of *L. bulgaricus* responsible for over-acidification of the products (Kneifel et al., 1993).

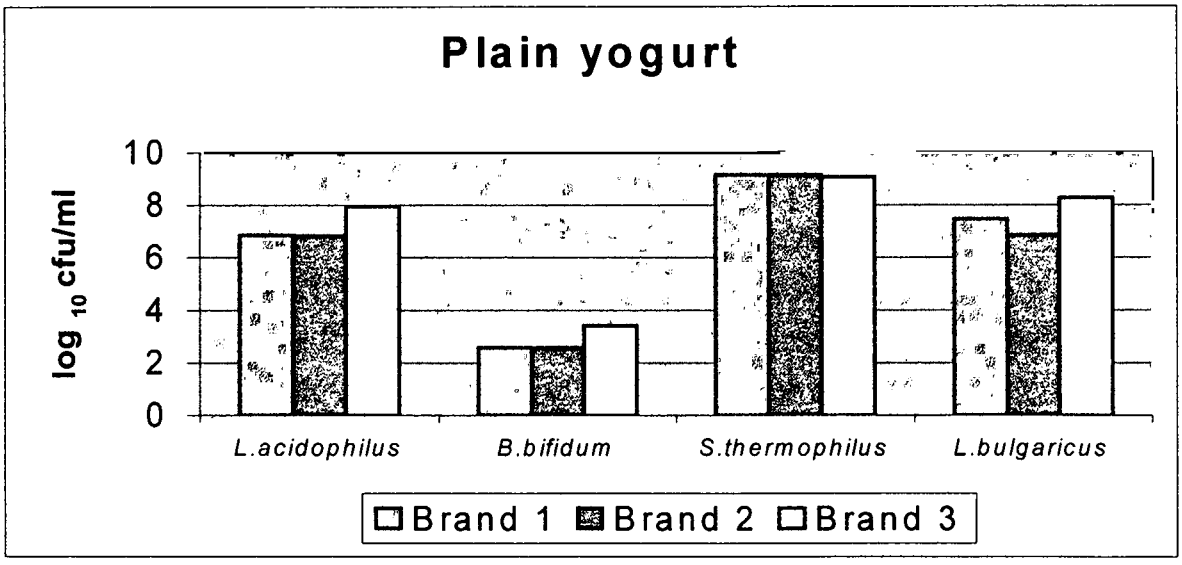
Conclusions

As mentioned previously, for the AB-yogurt to have any therapeutic effect, the consumer should consume a 100ml serving of AB-yogurt containing $>10^6$ viable cells of *L. acidophilus* and *B. bifidum* per day. Based on the results obtained in this study, however, the AB-yogurts examined complied with the criteria regarding the number of viable cells of *L. acidophilus*, but the

consumer would not have received sufficient numbers of *B. bifidum* cells at the time of consumption. The low population numbers of *B. bifidum* is attributed to the high numbers of *L. bulgaricus,* responsible for the decline in pH and as a result inhibited the growth of *B. bifidum.*



a)



b)

* **Means of 10 determinations**

Fig. 4.1 Level (log 10 cfu/ml*) of *L. acidophilus*, *B. bifidum*, *S. thermophilus* and *L. bulgaricus* in three brand types of commercial a) plain and b) apricot AB-yogurt.

Table 4.1 Statistical comparison of colony count data (log_{10} cfu/ml).

Strains	**Brand 1**				**Brand 2**				**Brand 3**		
	Batch 1		Batch 2		Batch 1		Batch 2		Batch 1		Batc
	Apricot	Plain	Apricot	Plain	Apricot	Plain	Apricot	Plain	Apricot	Plain	Apricot
L. acidophilus	**E**	**B**	**E**	**F**	**E**	**C**	**D**	**A**	**E**	**E**	**E**
	*7.81	6.85	7.62	8.15	7.67	7.06	7.46	6.65	7.83	7.79	7.69
	± 9.09	± 0.08	± 0.14	± 0.13	± 0.11	± 0.13	± 0.01	± 0.21	± 0.10	± 0.09	± 0.06
B. bifidum	**E**	**B**	**D**	**A**	**D**	**C**	**C**	**A**	**E**	**F**	**F**
	4.19	2.66	3.55	2.24	3.59	3.02	3.05	2.09	4.08	4.69	4.62
	± 0.15	± 0.06	± 0.23	± 0.02	± 0.07	± 0.05	± 0.14	± 0.12	± 0.07	±0.16	±0.04
S.thermophilus	**AF**	**BCG**	**BC**	**BCF**	**D**	**AE**	**BC**	**DG**	**AH**	**CFH**	**AE**
	8.84	9.17	9.13	9.07	9.49	8.87	9.19	9.36	8.82	9.05	8.85
	0.12	± 0.07	± 0.22	± 0.08	± 0.16	± 0.06	± 0.11	± 0.14	± 0.08	±0.15	± 0.16
L. bulgaricus	**D**	**A**	**C**	**E**	**CD**	**B**	**C**	**A**	**E**	**E**	**E**
	7.83	6.70	7.50	8.12	7.68	7.06	7.45	6.56	8.27	8.15	8.12
	± 0.09	± 0.28	± 0.17	± 0.22	± 0.13	± 0.24	± 0.04	± 0.21	± 0.03	± 0.11	± 0.03

* **Means with different superscripts differ significantly ($p \leq 0.05$).**

Table 4.2 Mean pH of plain and apricot flavoured yogurt of different yogurt brands.

Product	**Brand 1**	**Brand 2**	**Brand 3**
Apricot Batch 1	4.10	4.35	4.83
Apricot Batch 2	4.05	4.05	4.2
Plain Batch 1	4.00	4.00	3.93
Plain Batch 2	3.83	3.86	3.58
	4.00[a]	**4.06[b]**	**3.90[c]**

a,b,c: means

REFERENCES:

Anon. (1997) *Lactobacillus acidophilus, Lactobacillus casei* and *Bifidobacteria* in milk products made with nu-trish cultures. *CHR. Hansen.* Guideline. Count of bacteria, 1-5.

Bergey's Manual of Determinative Bacteriology (1974) Eighth edition. Edited by Buchanan, R.E. and Gibbons, N.E. The Williams and Wilkins Company, Baltimore. 490-6, 503-4, 576-83, 669-71.

Callichia, M.L., Wang, C.I.E., Nomura, T., Yotsuzuka, F. and Osato, D.W. (1993) Selective enumeration of *Bifidobacterium bifidum, Enterococcus*

faecium, and streptomycin-resistant *Lactobacillus acidophilus* from a mixed probiotic product. *Journal of Food Protection* **56** (11), 954-957.

Davis, J.G., Ashton, T.R. and McCaskill, M. (1971) Enumeration and viability of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* in yogurt. *Dairy Industries.* **36**, 569-573.

Gilliland, S.E. and Speck, M.L. (1977) Instability of *Lactobacillus acidophilus* in yogurt. *Journal of Dairy Science* **60,** 1394-1398.

Hamann, W.T. and Marth, E.H. (1983) Survival of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* in commercial and experimental yogurts. *Journal of Food protection.* **47**(10), 781-786.

Hekmat, S. and McMahon, D.J. (1992) Survival of *Lactobacillus acidophilus* and *Bifidobacterium bifidum* in Ice Cream for Use as a Probiotic Food. *Journal of Dairy Science.* **75,** 1415-1422.

Hull, R.R. and Roberts, A.V. (1984) Differential enumeration of *Lactobacillus acidophilus* in yogurt. *The Australian Journal of Dairy Technology.* **39,** 160-163.

Ishibashi, N. and Shimamura, S. (1993) Bifidobacteria: Research and development in Japan. *Food Technology.* **47**(6), 126, 129-134.

Iwana, H., Masuda, H., Fujisawa, T., Suzuki, H. and Mitsuoka, T. (1993) Isolation and identification of *Bifidobacterium* spp. in commercial yogurts sold in Europe. *Bifidobacteria Microflora.* **12**(1), 39-45.

Kailasapathy, K. and Rybka, S. (1997) *L.acidophilus* and *Bifidobacterium* spp. – their therapeutic potential and survival in yogurt. *The Australian Journal of Dairy Technology.* **52,** 28-35.

Klaver, F.A.M., Kingma, F. and Weerkamp, A.H. (1993) Growth and survival of bifidobacteria in milk. *Netherlands Milk Dairy Journal.* **47,** 151-164.

Kneifel, W., Jaros, D. and Erhard, F. (1993) Microflora and acidification properties of yogurt and yogurt-related products fermented with commercially available starter cultures. *International Journal of Food Microbiology.* **18,** 179-189.

Kurmann, J.A. and Rasic, J.L. (1991) The health potential of products containing bifidobacteria. In: *Therapeutic Properties of Fermented Milks.* Edited by R. K. Robinson. Elsevier Applied Sciences, London, 117-158.

Modler, H.W. and Villa-Garcia, L. (1993) The growth of *Bifidobacterium longum* in a whey based medium and viability of this organism in frozen yogurt with low and high levels of developed acidity. *Cultured Dairy Products Journal.* **28** (1) 4-8.

Rybka, S. and Kailasapathy, K. (1995) The survival of culture bacteria in fresh and freeze-dried AB yoghurts. *The Australian Journal of Dairy Technology.* **50** (2) 51-57.

Schefler, W.C. (1979) In: Statistics for the Biological Sciences, 2nd Edition. Addison-Wesley, London.

Shah, N. and Jelen, P. (1990) Survival of Lactic Acid Bacteria and Their Lactases under Acidic Conditions. *Journal of Food Science.* **55** (2), 506-509.

Tamime, A.Y., Marshall, V.M.E. and Robinson, R.K. (1995) Microbiological and technological aspects of milks fermented by bifidobacteria. *Journal of Dairy Research.* **62** 151-187.

CHAPTER 5

SURVIVAL OF PROBIOTIC BACTERIA IN SOUTH AFRICAN COMMERCIAL BIO-YOGURT

ABSTRACT

Flavoured yogurt from three manufacturers of commercial AB-yogurt was obtained directly after processing. Samples were stored at 4°C and 10°C and enumeration of viable *Lactobacillus acidophilus* and *Bifidobacterium bifidum*, as well as *Streptococcus thermophilus* and *Lactobacillus bulgaricus*, and determination of pH were carried out at three-day intervals until the expiry date. At 4°C, counts of *S. thermophilus* were generally the highest in all products, with initial counts higher than 10^8 cfu/ml and decreasing between 1 and 2 log cycles during the storage period. Initial levels of *L. bulgaricus* in most products were higher than 10^7 cfu/ml and final levels more than 10^6 cfu/ml. Most of the products contained more than 10^6 cfu/ml *L. acidophilus* counts on day 1, but only 50% contained more than 10^6 cfu/ml on the expiry date. The initial counts of *B. bifidum* in all the products were less than 10^6 cfu/ml. The effect of storage at 10°C had little effect on viability of all organisms despite changes in pH. Initial pH values ranged from 3.45 – 4.32 and these declined to 3.28 – 4.19 after 31 days of storage.

INTRODUCTION

Yogurt containing *Lactobacillus acidophilus* and *Bifidobacterium bifidum* is rapidly gaining popularity on the world market (Shah et al., 1995). As a result, several brands of yogurt sold in South Africa have recently been introduced containing *L. acidophilus* and *B. bifidum* (live AB culture). The beneficial effects of consuming large numbers of viable cells (>10^6cfu/ml) of *L. acidophilus* and *B. bifidum* have been well established (Rybka and Kailasapathy, 1995). According to literature, these probiotic cultures control gastrointestinal disorders and infections, and restore the normal intestinal microflora which may have been destroyed by antibiotic administration (Ishibashi and Shimamura, 1993). With regard to yogurt, the minimum number of viable *L. acidophilus* and *B. bifidum* cells present, should exceed counts of 10^6cfu/ml (Robinson, 1987) to have any therapeutic effect. Consequently, it is important to maintain viability of these organisms until the products are consumed in order to ensure any health aspects.

Studies have shown, however, that *L. acidophilus* and *B. bifidum* are unstable in yogurt. Shah et al. (1995) examined five brands of Australian commercial yogurt, obtained directly from processors, for viable *L. acidophilus* and *B. bifidum* at three-day intervals consecutively for five weeks. Three of the five brands exhibited $10^7 - 10^8$ /g of viable cells of *L. acidophilus*, whereas the other two contained <10^5cfu/g. The initial count of *B. bifidum* was 10^6-10^7 cfu/g in two of the five products, while the viable numbers were <10^3/g in the other three products. All the products showed a constant decline in the viable count of *L. acidophilus* and *B. bifidum* during storage.

Survival of *L. acidophilus* and *B. bifidum* is mainly affected by low pH of the environment. Although *L. acidophilus* survives better than yogurt culture organisms (*Lactobacillus delbrueckii* spp. *bulgaricus* and *Streptococcus salivarius* spp. *thermophilus*), a rapid decrease in their numbers has been observed under acidic conditions (Hood and Zottola, 1988; Shah and Jelen,

1990). Growth of *L. acidophilus* is inhibited below pH 4.0 (Playne, 1994). *B. bifidum* is less acid-tolerant compared to *L. acidophilus* (Lankaputhra and Shah, 1995) and its growth is retarded at pH values below 5.0 (Bergey's Manual, 1974). Martin and Chou (1992) reported that a pH of 5.5–5.6 was established as the minimum pH for survival of some species/strains of bifidobacteria.

Storage conditions also affect the viability of *L. acidophilus* and *B. bifidum* in bio-yogurt. Low temperature restricts the growth of *L. bulgaricus* and consequently also prevents over-acidification of the yogurt, which promotes higher survival of *L. acidophilus* and *B. bifidum* (Kneifel et al., 1993). Most studies showed that higher survival rates of probiotic bacteria were obtained at lower storage temperatures (Gilliland and Lara, 1988; Foschino et al., 1996). However, bifidobacteria are substantially less tolerant to very low temperature storage compared to *L.acidophilus* (Hughes and Hoover, 1995).

This study reports on the growth and survival of probiotic bacterial cultures in commercial AB-yogurt and examines the influence of temperature abuse on the viability of the cultures.

MATERIALS AND METHODS

Yogurt samples

AB-yogurts were obtained from three manufacturers of commercial AB-yogurt directly after production. Samples of low-fat apricot flavoured fruit yogurt were supplied in 175 ml sealed retail tubs. The yogurt samples were transported in cooler-boxes on ice (temperatures less than 7°C). All the yogurt products included, claimed to contain 'live acidophilus and bifidobacterium cultures' and the expiry date was 30-31 days after manufacturing.

Bacterial enumeration and growth

The experimental yogurts were incubated at either 4°C or 10°C for up to 31 days. During incubation, samples (two retail tubs from each manufacturer) were taken every three days and analysed for viable lactic acid bacteria counts and pH. Yogurt samples were prepared for microbial analysis by uniformly mixing the contents of the containers and aseptically withdrawing 10 ml of each. The yogurt samples were subjected to serial decimal dilutions in Callichia's resuspension medium (CRM) (Callichia et al., 1993) and agitated by means of a vortex mixer. The appropriate dilutions were pour plated in duplicate using the appropriate media. Results were expressed as the means of four repetitions. M-MRS agar (Hull and Roberts, 1984) was used for the differential enumeration of *L. acidophilus*. Enumeration of *B. bifidum* was carried out using NNLP agar (Laroia and Martin, 1991) enriched with L-cysteine hydrochloride. *S. thermophilus* was enumerated using M17 agar (Tergazhi and Sandine, 1975) and *L. bulgaricus* using Ac-MRS (IDF, 1996). These media are recommended by Chr. Hansen's laboratories and were found to be the most suitable media for enumeration of the yogurt organisms in commercial AB-yogurt (see chapter 3). The media were prepared as described in Chapter 3.

Anaerobic incubation was carried out in anaerobic jars (Oxoid) at 37°C for four days for M-MRS agar plates and five days for NNLP agar plates. The anaerobic atmosphere was provided using Anaerocult A blocks (Merck). An anaerobic indicator (Merck) was included in the jar. M17 agar plates and Ac-MRS agar plates were incubated aerobically for three and four days respectively at 37°C.

The pH values were determined using a HANNA Microprocessor pH meter (model Hi 9321).

RESULTS AND DISCUSSION

Survival of AB-yogurt organisms and pH changes during storage at 4°C.

The enumeration results obtained are presented in Figs. 5.1 to 5.3. The differences between the initial and final levels of counts and pH's of the different product brands are listed in Table 5.1.

L. acidophilus, B. bifidum, L. bulgaricus and *S. thermophilus,* present in all the brands, remained viable at 4°C, but their numbers generally decreased. The viable cell numbers of *L. acidophilus* in brand 1, initially increased by one log cycle reaching a maximum population of 8.7 log $_{10}$ cfu/ml units in 7 days. Although the counts varied between days 7 and 19, a definite declining tendency set in after 19 days reaching a minimum cell population of 6.41 log-units. In total, the cell counts decreased by 1.21 log-units during incubation over a period of 31 days. The viability of *L. acidophilus* in brand 2 decreased gradually by one log cycle during the first three weeks from an initial level of 8.6 to 7.6 log $_{10}$ cfu/ml, followed by a rapid decline through 3 log cycles to 4.7 log $_{10}$ cfu/ml. A total decrease in viable cell numbers of 3.90 log-units was observed. The rapid decrease in cell numbers is related to 'acidophilus death' as reported by Gilliland and Speck (1977a), attributed to hydrogen peroxide produced by *L. bulgaricus* during storage at low temperatures. The rapid decrease in cell numbers corresponded with a decline in pH from 3.45 to 3.28 at the time. Brand 3 maintained a consistent level of *L. acidophilus* of more than 6.9 log $_{10}$ cfu/ml over the entire period. Co-ordinately, the pH also remained stable at pH 3.5 in the yogurt. Hood and Zottola (1988) also found no significant reduction in the number of viable *L. acidophilus* cells at a pH level of 4 in a similar study. However, Playne (1994) reported that the growth of *L. acidophilus* is inhibited at a pH level below 4.0.

The initial *B. bifidum* counts in all the yogurt products were less than 10^6 cfu/ml. Brand 3 represented the lowest cell count of *B. bifidum.* Cell counts of

less than 2 log $_{10}$ cfu/ml units were observed. Samona and Robinson (1994) reported that the use of high cell inocula, will ensure a high bifidobacteria cell count at the end of incubation and the survival of the probiotic bacterium during storage until consumption. In brand 1 and 2, however, a decreasing tendency in *B. bifidum* levels was obtained. The levels of *B. bifidum* in brand 1, showed a decline from 5.2 to 4.9 log $_{10}$ cfu/ml, whereas a total decline in viable cell numbers in brand 2 of 0.90 log-units were observed. In contrast, *B. bifidum* populations in brand 3 showed an increase in growth reaching a maximum of log 2.23 cfu/ml.

The initial pH of the different yogurt product brands was in the range of 3.45-3.75 and declined during the storage period by 0.05-0.17 (Table 5.1). The low pH values of the yogurts resulted in the inhibition of growth of *B. bifidum* since their growth is retarded below pH 5.0 (Bergey's Manual, 1974; Gilliland, 1979). Martin and Chou (1992) reported that a pH of 5.5–5.6 was determined as the minimum pH for survival of some species/strains of bifidobacteria. However, acid tolerance of *Bifidobacterium* is strain-specific.

Growth of *S. thermophilus* in brand 1 increased initially reaching a maximum population of log 9.3 cfu/ml, and thereafter declined gradually until a minimum of log 7.97 cfu/ml was obtained after 31days. Viability of *S. thermophilus* in brand 2 declined sharply after 19 days, whereas, the cell densities in brand 3 remained stable over the entire incubation period at 4°C. The levels of viable cells remained high, consistently exceeding counts of 9.0 log $_{10}$ cfu/ml.

S. thermophilus clearly predominated in all the yogurts incubated at 4°C , yielded the highest cell numbers, surviving at levels in excess of 8.9 log $_{10}$ cfu/ml up to 31 days. The high incidence of *S. thermophilus* confirmed the domination of *S. thermophilus* during storage, as indicated in literature (Anon., 1994). The high frequency of occurrences of *S. thermophilus* is beneficial to the growth of *Bifidobacterium* species, since the organism acts as an oxygen

scavenger in bio-yogurt and consequently stimulates the growth of bifidiobacteria (Shankar and Davies, 1976; Ishibashi and Shimamura, 1993).

L. bulgaricus in brand 1, progressed rapidly in 7 days, with counts reaching a maximum of 8.7 log $_{10}$ cfu/ml. Thereafter, a gradual decline was observed, with a lowest count of 6.3 log $_{10}$ cfu/ml after 31 days. *L. bulgaricus* exhibited a weak survival rate in brand 2, and slowly died off from an initial cell count of 8.6 to 5.5 log $_{10}$ cfu/ml. The final cell level of *L. bulgaricus* in brand 3 was the same as the initial level (7.0 log $_{10}$ cfu/ml).

According to literature, *S. thermophilus* and *L. bulgaricus* are active at refrigeration temperatures and produce small amounts of lactic acid by fermentation of lactose resulting in a pH decrease (Laroia and Martin, 1991; Hekmat and McMahon, 1992). The final pH of yogurt can affect the growth and viability of *L. acidophilus* and *B. bifidum* (Hekmat and McMahon, 1992). It has been found that acid production after incubation (over-acidification), affected cell viability of *L. acidophilus* and *B. bifidum* (Ishibashi and Shimamura, 1993).

Survival of AB-yogurt organisms and pH change during storage at 10°C.

Results obtained with the yogurts incubated at 10°C are presented in Figs. 5.4 to 5.6, and Table 5.1.

The decrease in viability of all organisms at 10°C was similar to results obtained at 4°C. This corresponds with results obtained by Shah et al. (1995) who reported that storage at 10°C had little bearing on the survival of most cultures. According to Hamman and Marth (1983), cell numbers of yogurt bacteria decreased to a larger extent when stored at 10°C than at 5°C.

The 'therapeutic minimum' requires more than 10^6 cfu/ml of the probiotic organisms in a product. Some of the freshly prepared products examined in this study would be suitable in this regard with respect to *L. acidophilus*, at

any time during the shelf-life period (Table 5.1; for example yogurt products of brand 1). The level of *L.acidophilus* in brand 2, however, declined to less than 10^6 cfu/ml at the end of storage. None of the products investigated, contained the required level of *B. bifidum* at any stage of the shelflife. All the yogurts had low levels of *B. bifidum* initially, and these levels continued to decrease during the period of incubation.

The bacteria most likely died off in the yogurt during storage due to post-acidification by yogurt culture organisms (pH change 0.01 to 0.33). Several other factors may be responsible for reduced viability of these organisms and these include hydrogen peroxide produced by the yoghurt culture organisms, oxygen level in the product, a low initial inoculum level, strains used, interaction between species present, culture conditions, chemical composition of the fermentation medium, final acidity, water activity, availability of nutrients, growth promoters and inhibitors, concentration of sugars (osmotic pressure), dissolved oxygen (especially for *Bifidobacteium* sp.), incubation temperature, fermentation time and storage temperature (Hamman and Marth, 1983; Young and Nelson, 1978; Kneifel et al., 1993).

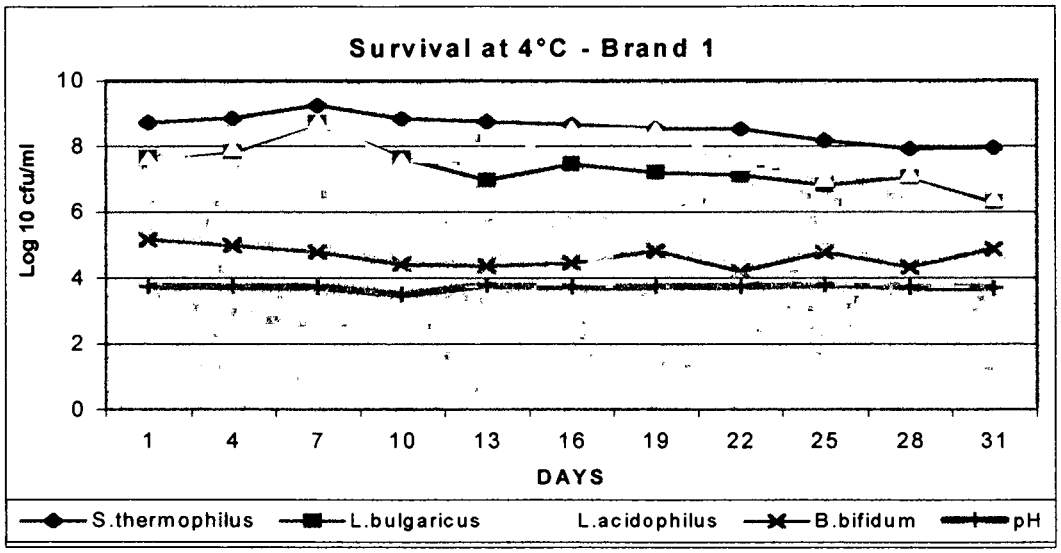


Fig. 5.1 Changes in viable counts of AB-yogurt organisms in commercial brand 1 during storage at 4°C until expiry date

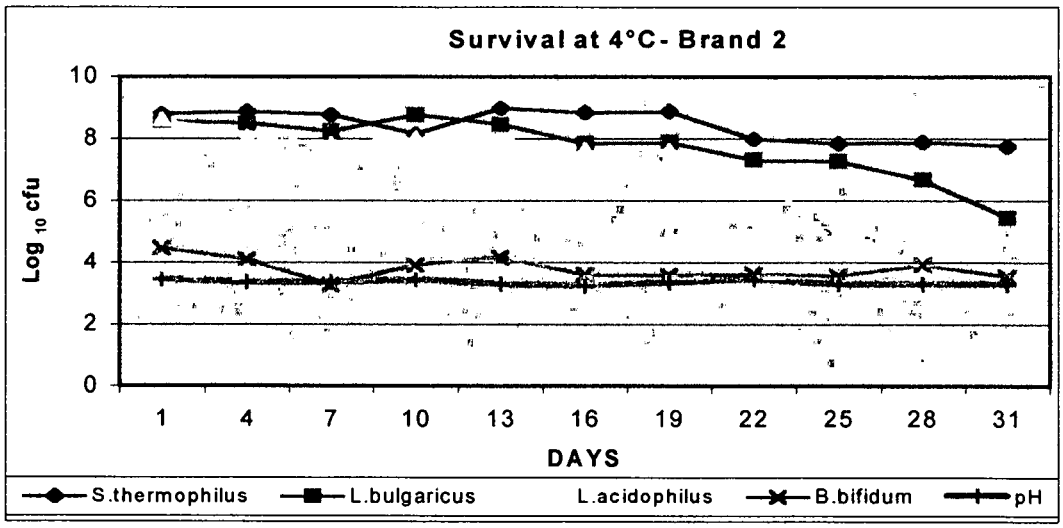


Fig. 5.2 Changes in viable counts of AB-yogurt organisms in commercial brand 2 during storage at 4°C until expiry date

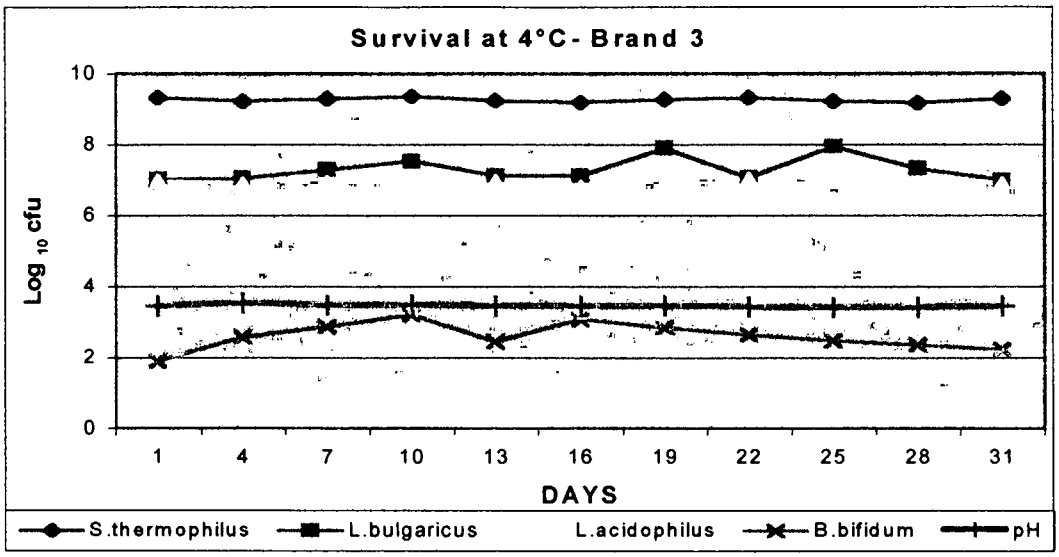


Fig. 5.3 Changes in viable counts of AB-yogurt organisms in commercial brand 3 during storage at 4°C until expiry date

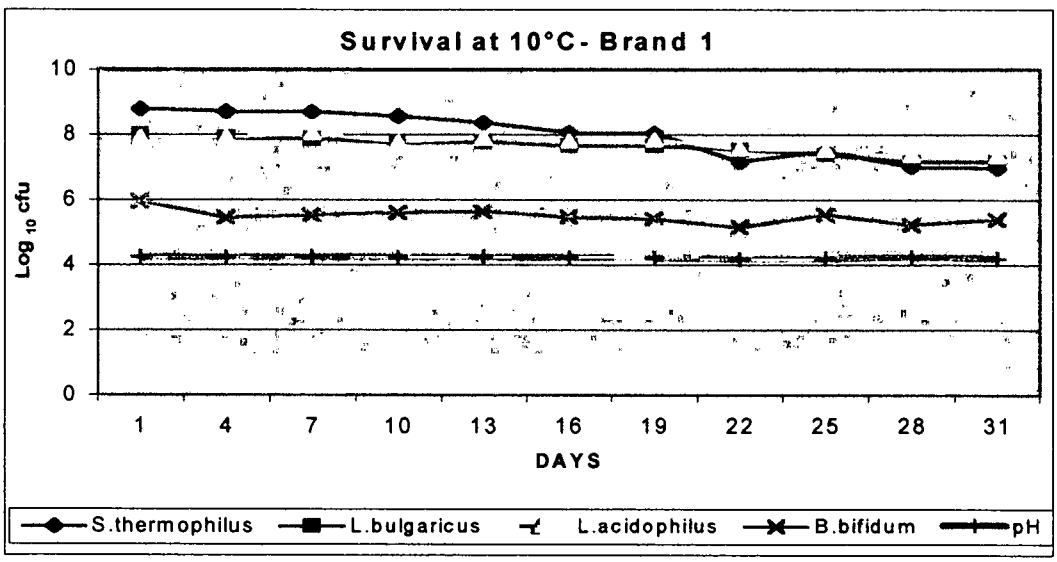


Fig. 5.4 Changes in viable counts of AB-yogurt organisms in commercial brand 1 during storage at 10°C until expiry date

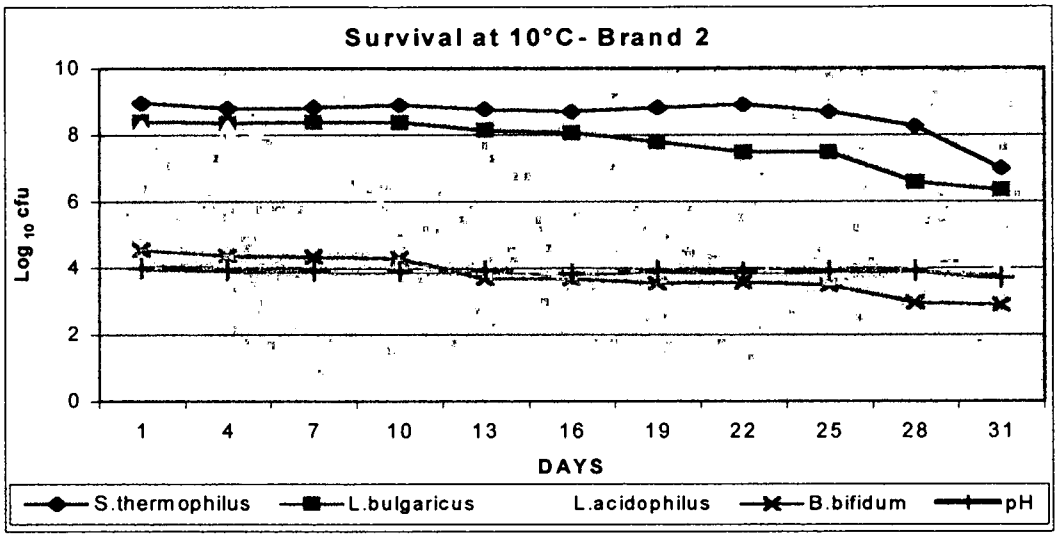


Fig. 5.5 Changes in viable counts of AB-yogurt organisms in commercial brand 2 during storage at 10°C until expiry date

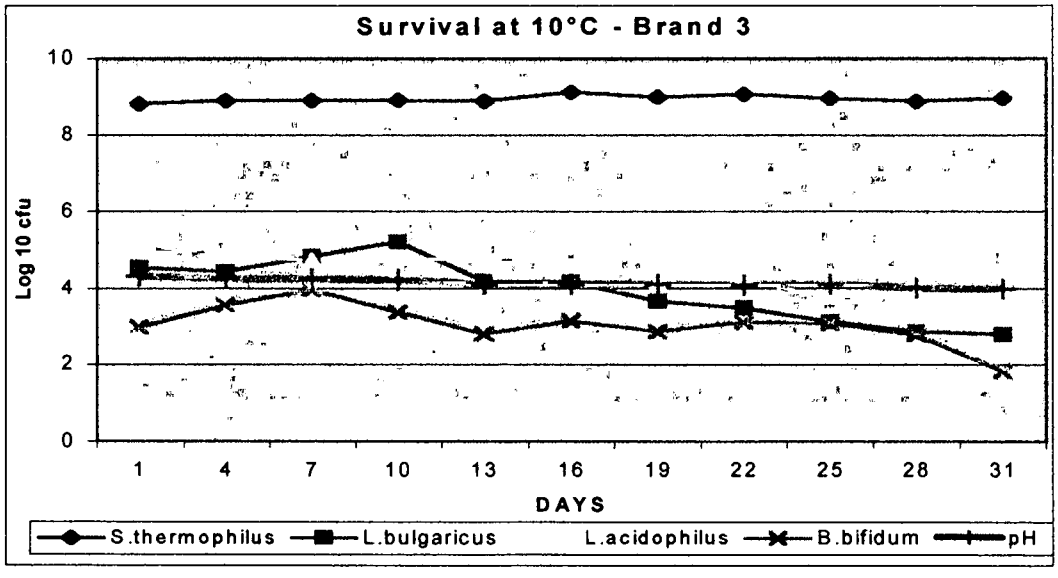


Fig. 5.6 Changes in viable counts of AB-yogurt organisms in commercial brand 3 during storage at 10°C until expiry date

Table 5.1 Cell counts (log 10 cfu/ml) of probiotic and yogurt starter bacteria and pH of yogurt after manufacturing and at expiry date.

	Cultures	4°C			10°C		
		Day 1	**Day 31**	**D**	**Day 1**	**Day 31**	**D**
Brand 1	*L.acidophilus*	7.62	6.41	1.21	7.93	7.36	0.57
	B.bifidum	5.18	4.89	0.29	5.97	5.41	0.56
	S.thermophilus	8.74	7.97	0.77	8.79	6.98	1.81
	L.bulgaricus	7.67	6.3	1.37	8.0	7.17	0.83
	pH	3.75	3.70	**0.05**	4.25	4.19	**0.06**
Brand 2	*L.acidophilus*	8.63	4.73	3.90	8.24	4.19	4.05
	B.bifidum	4.48	3.56	0.92	4.56	2.90	1.66
	S.thermophilus	8.8	7.76	1.16	8.98	7.0	1.98
	L.bulgaricus	8.58	5.45	3.13	8.41	6.35	2.06
	pH	3.45	3.28	**0.17**	4.00	3.72	**0.28**
Brand 3	*L.acidophilus*	6.99	6.9	0.09	5.08	3.74	1.34
	B.bifidum	1.92	2.23	+0.31	2.99	1.85	1.14
	S.thermophilus	9.34	9.3	0.04	8.83	8.97	+0.14
	L.bulgaricus	7.07	7.0	0.07	4.54	2.81	1.73
	PH	3.45	3.46	**+0.01**	4.32	3.99	**0.33**

D difference between initial and final level of organism (log 10 cfu/ml) and of pH.

REFERENCES:

Anon. (1994) Method of analysis for identification of *L. acidophilus* and *Bifidobacteria* (A medium for differentiating between *L. acidophilus*, *Bifidobacteria* and *S. termophilus* in ABT products) Chr.Hansen's Laboratorium, Copenhagen, Denmark.

Bergey's Manual of Determinative Bacteriology (1974) Eighth edition. Edited by Buchanan, R.E. and Gibbons, N.E. The Williams and Wilkins Company, Baltimore. 490-6, 503-4, 576-83, 669-71.

Callichia, M.L., Wang, C.I.E., Nomura, T., Yotsuzuka, F. and Osato, D.W. (1993) Selective enumeration of *Bifidobacterium bifidum*, *Enterococcus faecium*, and streptomycin-resistant *Lactobacillus acidophilus* from a mixed probiotic product. *Journal of Food Protection.* **56** (11), 954-957.

Foschino, R., E. Fiori and Galli, A. (1996) Survival and residual activity of *Lactobacillus acidophilus* frozen cultures under different conditions. *Journal of. Dairy Research.* **63,** 295-303.

Gilliland, S.E. (1979) Beneficial inter-relationships between certain microorganisms and humans: Candidate microorganisms for use as dietary adjuncts. *Journal of Food Protection.* **42,** 164-167.

Gilliland, S.E. and Lara, R.C. (1988) Influence of storage at Freezing and Subsequent Refrigerator temperature on β-Galactosidase Activity of *Lactobacillus acidophilus. Applied. Environmental Microbiology.* **45** (4), 898-902.

Gilliland, S.E. and Speck, M.L. (1977) Antagonistic action of *Lactobacillus acidophilus* toward intestinal and food-borne pathogens in associative cultures. *Journal of Food Protection.* **40**(12), 820-823.

Hamann, W.T. and Marth, E.H. (1983) Survival of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* in commercial and experimental yogurts. *Journal of Food protection.* **47**(10), 781-786.

Hekmat, S. and McMahon, D.J. (1992) Survival of *Lactobacillus acidophilus* and *Bifidobacterium bifidum* in Ice Cream for Use as a Probiotic Food. *Journal of Dairy Science.* **75,** 1415-1422.

Hood, S.K. and Zottola, E.A. (1988) Effect of low pH on the ability of *Lactobacillus acidophilus* to survive and adhere to human intestinal cells. *Journal of Food Science.* **53**(5), 1514-1516.

Hughes, D.B. and Hoover, D.G. (1995) Viability and enzymatic activity of bifidobacteria in milk. *Journal of Dairy Science.* **78**, 268-276.

Hull, R.R. and Roberts, A.V. (1984) Differential enumeration of *Lactobacillus acidophilus* in yogurt. *The Australian Journal of Dairy Technology.* **39**, 160-163.

IDF, International Standard 149, (1996) Dairy starter cultures of lactic acid bacteria-standard of identity.

Ishibashi, N. and Shimamura, S. (1993) Bifidobacteria: Research and development in Japan. *Food Technology.* **47**(6), 126, 129-134.

Kneifel, W., Jaros, D. and Erhard, F. (1993) Microflora and acidification properties of yogurt and yogurt-related products fermented with commercially available starter cultures. *International Journal of Food Microbiology.* **18,** 179-189.

Lankaputhra, W.E.V. and Shah, N.P. (1995) Survival of *Lactobacillus acidophilus* and *Bifidobacterium* species in the presence of acid and bile salts. *Cultured Dairy Products Journal.* **30**(3), 113-118.

Laroia, S. and Martin, J.H. (1991a) Effect of pH on Survival of *Bifidobacterium bifidum* and *Lactobacillus acidophilus* in Frozen Fermented Dairy Desserts. *Cultured Dairy Products Journal.* Nov., 13-21.

Martin, J.H. and Chou, K.M. (1992) Selection of Bifidobacteria for Use as Dietary Adjuncts in Cultured Dairy Foods: 1 - Tolerance to pH of Yogurt. *Cultured Dairy Products Journal.* **27**(4), 21-26.

Playne, M. (1994) Probiotic foods. *Food Australia.* **46**(8), 362.

Robinson, R.K. (1987) Survival of *Lactobacillus acidophilus* in Fermented Products. *South African Journal of Dairy Science.* **19** (1), 25-27.

Rybka, S. and Kailasapathy, K. (1995) The survival of culture bacteria in fresh and freeze-dried AB yoghurts. *The Australian Journal of Dairy Technology.* **50** (2) 51-57.

Samona, A. and Robinson, R.K. (1994) Effect of yogurt cultures on the survival of bifidobacteria in fermented milks. *Journal of the Society of Dairy Technology.* **47** (2), 58-60.

Shankar, P. and Davies, F. (1976) Associative bacterial growth in yogurt starters, initial observation on stimulatory factors. *Journal of the Society of Dairy Technology.* **30**(1), 31-32.

Shah, N. and Jelen, P. (1990) Survival of Lactic Acid Bacteria and Their Lactases under Acidic Conditions. *Journal of Food Science.* **55** (2), 506-509.

Shah, N.P.; Lankaputhra, W.E.V.; Britz, M.L. and Kyle, W.S.A. (1995) Survival of *Lactobacillus acidophilus* and *Bifidobacterium bifidum* in commercial yoghurt during refrigerated storage. *International Dairy Journal.* **5,** 515-521.

Terzaghi, B.E. and Sandine, W.E. (1975) Improved medium for lactic streptococci and their bacteriophages. *Applied Microbiology.* **29**(6), 807-813.

Young, C.K. and Nelson, F.E. (1978) Survival of *Lactobacillus acidophilus* in "Sweet Acidophilus Milk" during Refrigerated Storage. *Journal of Food Protection.* **41** (4), 248-250.

CHAPTER 6

GROWTH AND SURVIVAL OF A PROBIOTIC YEAST IN DAIRY PRODUCTS

ABSTRACT

Poor survival of probiotic bacteria in yogurt has been recorded. Growth of a probiotic yeast, *Saccharomyces boulardii*, in association with the bio-yogurt microflora, by incorporating the yeast into commercial bio-yogurt, has been suggested to stimulate the growth of the probiotic organisms and to assure their survival during shelflife. Therefore the ability of growth and survival of the probiotic yeast itself in diary products, namely bio-yogurt, sterile yogurt and UHT milk were investigated. *S. boulardii* was incorporated into the dairy products and stored at 4 °C over a four-week period. pH, lactose utilisation and the production of organic acids were monitored at each interval. Based on the results in this study, the probiotic yeast species, *S. boulardii*, has the ability to grow in bio-yogurt reaching maximum counts exceeding 10^7 cfu/g. The number of yeast populations was substantially higher in the fruit based yogurt, mainly due to the presence of proportions of sucrose and fructose derived from the fruit. Despite the inability of *S. boulardii to* utilize lactose, the yeast species utilized available organic acids, galactose and glucose derived from bacterial metabolism of the milk lactose, as well as possible free fatty acids or free amino acids present in the dairy products. Excessive gas and alcohol production by the yeast species proved, however, to be major constraints for incorporating this yeast into bio-yogurt.

INTRODUCTION

Probiotic organisms are increasingly incorporated into food as dietary adjuncts to help maintain a healthy microbial gastrointestinal balance, with possible resulting benefits for the human health (Rasic, 1983). Such microorganisms predominantly incorporated into fermented dairy products are the probiotic bacteria lactobacilli and bifidobacteria. So far the active use of yeasts as dietary adjuncts for humans has been limited, despite the occurrences of yeasts as an integral part of the microflora of many dairy related products (Fleet & Mian, 1987). This may indicate that the potential of yeasts for incorporation into dairy products as probiotic agents has been overlooked (Jakobsen & Narvhus, 1996). Therefore, yeasts must be included in the search for finding new starter cultures for fermented milk products and in introducing cultures with the desired characteristics (Jakobsen & Narvhus, 1996).

Historically, yeast as a probioticum has been linked with livestock feed, especially the genus *Saccharomyces* (Jakobsen & Narvhus, 1996). *S. boulardii*, a non-pathogenic yeast, was first isolated from lychee fruit in Indochina in the 1950's and since has been used both as a preventative and therapeutic agent for the treatment of a variety of diarrhoeal diseases (McFarland & Bernasconi, 1993; Surawicz et al., 1989). Several specific antagonistic interactions have been reported between culture yeasts like *S. cerevisiae* and enteric pathogens e.g. enteropathogenic *Esherichia coli*, *Shigella* and *Salmonella* as reviewed by Gedek (1991). It appears that *S. cerevisiae* can survive passage through the intestinal tract, which further accentuates the possible use of yeasts as probiotics (Gedek, 1991).

Although yeasts are not involved in the fermentation of yogurt, they are frequently associated with the spoilage of the final product. It is not uncommon to find yeast populations of 10^3 cells/g or more in retail samples of either plain or fruit yogurts (Fleet, 1990) and if conditions permit, yeast populations exceeding 10^7 cfu/ml are feasible. Little consideration has been

given to the growth kinetics and biochemical basis of yeast growth in milk and yogurt (Roostita & Fleet, 1996). The frequent occurrences of yeast in dairy related products indicate the ability of yeasts to survive and metabolise milk constituents. Yeasts might develop in milk as secondary flora, after yogurt culture bacterial growth. Lactic acid bacteria (LAB) ferment about 35% of the lactose in milk through hydrolysis to glucose and galactose. Only the glucose is changed into lactic acid, while the galactose moiety is released into the extracellular environment (Goodenough & Klein, 1976). The high concentration of galactose present (about 1%) was the main reason for the growth of galactose positive and non-fermenting yeasts in yogurt in the study by Giudici et al. (1996). Furthermore, the low pH of yogurt and the ability of yeasts to utilise organic acids create a selective environment for yeast growth (Fleet & Mian, 1987). For this reason, bacteria may help to establish the growth of yeasts in dairy products. The commensalistic association between yeasts and lactic cid bacteria is also observe in kefir, acidophilus milk, and many food fermented products like sorghum beer, kenkey, lambic-beer, etc. (Subramanian and Shankar, 1985; Deak and Beuchat, 1996; Borregaard and Arneborg, 1998). Yeasts also add to the association by stimulating the growth of the lactic acid bacteria due to the excretion of growth factors and metabolites. Consequently, the presence of yeasts in yogurt may also stimulate the growth of pro-biotic microorganisms.

Milk products that include yeast in their starter culture are acidophilus-yeast milk (Lang & Lang, 1975), kefir, koumiss and laban. These are produced by fermentation of milk by a mixture of lactic acid bacteria, yeasts and other bacteria, such as acetic acid bacteria. In a comparative study of the formation of antibiotic substances in acidophilus-yeast milk, the titre of the antibiotic was higher than in milk fermented by yeast or acidophilus bacteria alone. It was contended that the yeasts and acidophilus bacteria mutually stimulate each other's viability (Skorodumava, 1958). The possibility of producing acidophilus milk with a mixed culture of *Lactobacillus acidophilus* and the lactose fermenting yeasts *Kluyveromyces marxianus* and *Candida*

pseudotropicalis has been described by Subramanian and Shankar (1983). *L. acidophilus* growth was stimulated in the presence of the yeasts. Also, *Bacillus cereus* and *Escherichia coli* failed to survive in acidophilus-yeast milk. Graham (1943), found that *Lactobacillus bulgaricus* usually died in 2-3 weeks in pure culture, but survived many months when grown with different yeasts. In a study by Soulides (1955) a number of non-lactose fermenting yeasts belonging to the genus Torulopsis (now called *Candida*) were isolated from yogurt. Growth of these yeasts in milk with strains of *Streptococcus thermophilus* and *L. bulgaricus* resulted in synergistic peptonization followed by a gradual decrease in acidity. As a result of the associative growth between yeasts and the bacteria, the viability of the yogurt bacterial strains was maintained in milk for 5 to 8 months.

It is essential that products sold with any health indication meet the criterion of minimum a million cells per ml at the expiry date, as the minimum therapeutic dosage per day is considered to be 10^8 to 10^9 cfu (Kurmann & Rasic, 1991; Robinson, 1987) corresponding to an intake of 100g product containing 10^6 to10^7 cfu/ml per day. However, it has been reported (Shah et al., 1995) that *L. acidophiluss* and *B. bifidum*, present in bio-yogurt, are unstable. Their poor survival in yogurt is attributed to the low pH of the environment and low acid-tolerance. Since yeasts have the ability to utilise organic acids, thereby increasing the pH of the environment, growth of a probiotic yeast in association with probiotic bacteria has been suggested.

This study reports on the ability of *S. boulardii* to grow in bio-yogurt and other dairy products in order to further investigate the effect on the survival of the AB bacteria during shelflife. This was determined by inoculating a pure culture of *S. boulardii* into commercial AB-plain yogurt, fruit flavoured yogurt, as well as sterile milk and sterile sweetened yogurt for comparison and control reasons. In addition, the yeast survival and progression in fermented vs. non-fermented, flavoured vs. non-flavoured dairy products, are compared.

MATERIALS AND METHODS

Yeast culture

A pure culture of *S. boulardii* was obtained from the American Type Culture Collection (ATCC 74012). The yeast culture was maintained on slants of YM (yeast-extract, malt-extract) agar (Wickerham, 1951) and stored at 5°C. When required, the yeast culture was activated by transferring to YM agar plates, incubated at 25°C for 48 h and checked for purity before use in experiments.

Physiological examination of the pure culture

Assimilation of the carbohydrate sources, lactose, citric acid, lactic acid and succinic acid, was performed by means of the auxanographic method (Kreger van Rij, 1984). Fermentation of lactose was examined by inoculation of *S. boulardii* into fermentation media with Durham tubes. Proteolytic activity (casein digestion) was determined according to the methods of Ahearn et al. (1968). Screening for lipase production was performed by means of agar plates containing olive oil as carbon source and Rhodamine B, pH 7.0 (Kouker & Jeager, 1987).

Samples of dairy products

Growth and survival experiments were conducted in two different batches of:

a) 500 ml Plain AB-yogurt,
b) 500ml Fruit cocktail AB-yogurt,
c) 500ml Sterile yogurt and
d) 500ml UHT treated milk, respectively.

The AB-yoghurt samples, namely plain (natural) yogurt and 'fruit cocktail' flavoured yogurt, were obtained from a local yogurt manufacturer immediately after production. Sterile UHT milk and sterile yogurt (ultra high temperature

treated after fermentation) were purchased from a supermarket outlet. Samples were transported in a cooler box on ice and refrigerated upon arrival in the laboratory. Samples were inoculated within 12 h of receipt.

Inoculation

Yeast cells of *S. boulardii* were grown in YNB (yeast nitrogen base, Oxoid, Basingstoke) broth on a shake machine at 25°C to late stationary phase (48h). These yeast cells were harvested by centrifugation at 14 000 *g* for 10 minutes. The supernatant was decanted and the yeast cells weighed. Appropriate aliquots of sterile water were added to obtain yeast cell suspensions of 1%, 2% and 3%. Yeast cells per ml suspension were predetermined with a Klett Sumerson. This was plotted against yeast concentration (1, 2 and 3%) to determine the percentage (w/v) inoculation size necessary to obtain an inoculation level of 10^6 cells/ml in the experimental dairy products.

All the dairy products were aseptically dispensed into sterilised Schott bottles (2l), and then inoculated simultaneously at approximately the same level of 10^6 yeast culture/ml dairy product by using a inoculation level of 2,5% (weight cells per volume product). The products were uniformly mixed. The inoculated live AB-yogurt were aseptically transferred into 175ml retail tubs containing the same amount of product, and heat-sealed with aluminium lids. Inoculated samples were stored at 5°C.

Microbiological analyses

Microbial analyses were performed on all the products directly after inoculation on the same day to determine the inoculation level of each product (Day 1), and thereafter every 24 hrs for the first 5 days and then consecutively at three-day intervals for 29 days. From each dairy product at each sampling interval, one sample of 10ml were aseptically withdrawn after vigorous shaking, dispensed into 90 ml 0.1% sterile peptone water and diluted for plate

counting. Aliquots (0.1 ml) of the dilutions were spread plated in duplicate on YM agar (Wickerham, 1951) plates. Inoculated plates were aerobically incubated at 25°C for 5 days. After incubation, yeast colonies between 30 and 300 were counted and the results expressed as yeast count per ml (cfu/ml) of the product. The data presented are the means of results obtained from duplicate plates of the samples analysed in log $_{10}$ cfu/ml.

pH determination

The pH of each sample at every sampling occasion was measured using a HI 9321 Microprocessor pH meter (HANNA Instruments)

Chemical analysis

For chemical analysis, at each sampling occasion, 2ml samples were taken and stored in Eppendorf tubes at –18°C until needed. The samples were clarified by centrifugation at 14000 for 2 min and the supernatant ultra filtrated by using Whatman filter discs (0.45μm) prior to chromatographic analysis of the alcohol, sugar and organic acids content of the dairy product.

Alcohol and sugar

Sugar and alcohol concentrations were measured by means of a WATERS HPLC system with a Biorad-aminex C42 Column and Refractive index detector. The concentrations of individual sugars and alcohol were calculated by reference to 1.0%, (w/v) standard solutions (Sigma, USA).

Organic acids determination

The concentration of organic acids was measured by a HPLC (WATERS HPLC system with a Biorad-aminex C42 Column and Refractive index detector). Identification of organic acids and quantification of their concentrations were

conducted by comparison of unknown peak positions with 1.0%, (w/v) standard solutions (Sigma, USA).

RESULTS AND DISCUSSION

Physiological properties of S. boulardii

Results confirmed the inability of *S. boulardii* to assimilate or ferment the milk sugar, lactose. Positive growth, however, was obtained on galactose. The species also failed to utilize some of the organic acids, namely citric- and succinic acid but gave positive growth on lactic acid. No proteolytic and lipolytic activity was detected.

Growth and survival of S. boulardii in dairy products

The general cell population of *S. boulardii* in plain yogurt and UHT yogurt remained virtually the same, with several fluctuations (Fig. 6.1) over the storage period. The mean count of *S. boulardii* in fruit yogurt showed an increase from an initial cell population of 7.7 $\log_{10}$ cfu/ml to 8.1 $\log_{10}$ cfu/ml over the storage period. This increase is attributed to the more easily fermented sugars, sucrose and fructose, derived from the added fruit. The presence of high concentrations of sucrose in fruit flavoured yogurt, which acted as a fermentable growth substrate, explained the predominance of *S. cerevisiae* in the study of Fleet and Mian (1987).

In the UHT milk, the cell population of *S. boulardii* survived and increased slightly from 8.15 $\log_{10}$ cfu/ml to 8.5 $\log_{10}$ cfu/ml over the storage period at 5°C. Unlike the growth in fruit yogurt, it is more difficult to explain the growth of *S. boulardii* in the UHT milk in terms of its metabolic utilisation of milk constituents. According to literature, *S. boulardii* does not ferment or assimilate lactose (Kreger van Rij, 1984), but it did produce small amounts of lactic acid and alcohol (Table 6.3d) suggesting carbohydrate metabolism. In a survey by Fleet and Mian (1987), they showed that *Saccharomyces cerevisiae*,

could reach counts as high as 10^8 –10^9 cells/ml when grown in UHT-treated milk. Growth could not be explained by their ability to utilise milk lactose, protein or fat, since *S. cerevisiae* lacks these abilities (Roostita & Fleet, 1996). Presumably, other milk components were used, such as small amounts of free amino acids and fatty acids or trace amounts of galactose and glucose present in milk (Fleet & Mian, 1987; Roostita & Fleet, 1996). UHT treatment may hydrolyse a small portion of the lactose, partially explaining the carbohydrate metabolism in UHT milk (Berg, H.E., 1993).

Utilisation of sugars; production of alcohol and gas.

Saccharomyces boulardii was examined for changes that occurred during growth in different dairy products. The data are presented in Fig. 6.2 a-d.

The decrease in lactose content in plain yogurt from 4.5-4.0% suggests that the sugar was utilised by lactic acid bacteria (LAB) present in the product during storage. The galactose initially present (0.68%) was apparently utilised by *S. boulardii* corresponding with its survival in plain yogurt (Fig. 6.1). *S. boulardii* is not able to ferment or assimilate lactose, but it utilises galactose (Vaughan-Martini & Martini, 1998). According to literature, high proportions of galactose are always present in yogurt derived from the breakdown of lactose by the LAB (Goodenough & Klein, 1976; Giudici et al., 1996). No glucose was detected.

The lactose content of fruit yogurt decreased (4.4%-4.1%), again it is attributed to the growth of LAB resulting in the increase in galactose during the later stage. The growth of *S. boulardii* in fruit yogurt at 5°C correlated with a decrease in the concentrations of glucose and fructose and an excessive production of alcohol of 5.9% (Table. 6.3b and Fig. 6.2b).

The lactose content of the UHT yogurt (sweetened and flavoured) remained stable due to the absence of LAB in UHT products and the inability of the yeast

species to utilise milk lactose (Fig. 6.2c). The survival of *S. boulardii* corresponds with a decrease in concentrations of glucose and fructose added to the product (sweetened and flavoured) and high alcohol production (4.03%) (Table 6.3c). Despite the complete utilisation of the glucose and fructose present in the UHT yogurt, poor growth of the yeast species was observed (Fig. 6.1). Lactose was not utilised during the growth of *S. boulardii* in UHT milk (Fig. 6.2d). Small proportions of galactose, however, were observed.

The higher percentages of alcohol produced in the fruit yogurt and UHT (sweetened and flavored) yogurt are attributed to the presence of fermented sugars derived from the added fruit, as well as added sucrose for sweetening. In such yogurts, non-lactose fermenting yeast species causes alcoholic fermentation and consequently resulting in CO_2 production (Goodenough & Klein, 1976). The progression of the yeast species being present at high levels in fruit yogurt and UHT yogurt was responsible for excessive gas production during fermentation. Gas formation stopped at the 5^{th} and 11^{th} day of yeast growth in UHT yogurt and fruit yogurt respectively (results not shown) due to the depletion of fermented sugars available.

Despite the production of organic acids, the pH of the different yeast inoculated dairy products remained stable over the storage period. This may be due to the utilisation of the organic acids by *S. boulardii*.

Changes in the concentrations of organic acids

The main organic acid present in the fermented dairy products before yeast growth was lactic acid (1.57 – 1.98%). This was followed by lesser amounts of citric acid (0.46 – 0.55%) and succinic acid (0.025 – 0.050%) (Table 6.3a-c). No succinic acid was detected in fruit yogurt (Fig. 6.3b). In the UHT milk, however, citric acid represented the highest amounts (0.55%), followed by lesser amounts of lactic acid (0.07%) and succinic acid (0.026%).

The slight increase in the lactic acid contents observed in plain-and fruit yogurt (Table 6.3a and b) is attributed to the presence of LAB utilizing the lactose and generating lactic acid. Although *S. boulardii* is able to utilize lactic acid as a carbon source, it is unlikely that this substrate could be assimilated under the anaerobic conditions that exist in yogurts (Suriyarachchi & Fleet, 1981). The lactic acid contents in the UHT yogurt (Table 6.3c) and UHT milk (Table 6.3d) remained stable due to the absence of LAB. In general, the citric- and succinic acid contents exhibited an increase in all the dairy products. This may be attributed to the inability of the yeast species to utilize the acids, the growth of LAB, or it may arise from the yeast metabolism of glucose and sucrose.

Conclusions

In this study we have shown that the yeast species *S. boulardii* is capable of utilising the yogurt constituents as growth substrates and maintaining cell counts exceeding 10^6 cfu/ml. Therefore, the application of the yeast species as a probiotic microorganism seems promising. The production of excessive amounts of alcohol and gas formation, however, is major constraints in implementing the yeast species into standard yogurt.

Table. 6.2 Changes in pH of dairy product samples inoculated with *S. boulardii.*

Days	UHT milk	UHT yogurt	Plain yogurt	Fruit yogurt
1	6.55	4.23	4.30	4.16
2	6.55	4.23	4.30	4.16
3	6.45	4.07	4.26	4.10
4	6.35	4.10	4.24	4.08
5	6.51	4.08	4.22	4.06
8	6.45	4.11	4.23	4.11
11	6.60	4.11	4.22	4.10
14	6.55	4.11	4.23	4.11
17	6.55	4.23	4.19	4.13
20	6.56	4.13	4.27	4.11
23	6.56	4.15	4.17	4.11
26	6.60	4.17	4.20	4.10
29	6.59	4.16	4.18	4.12

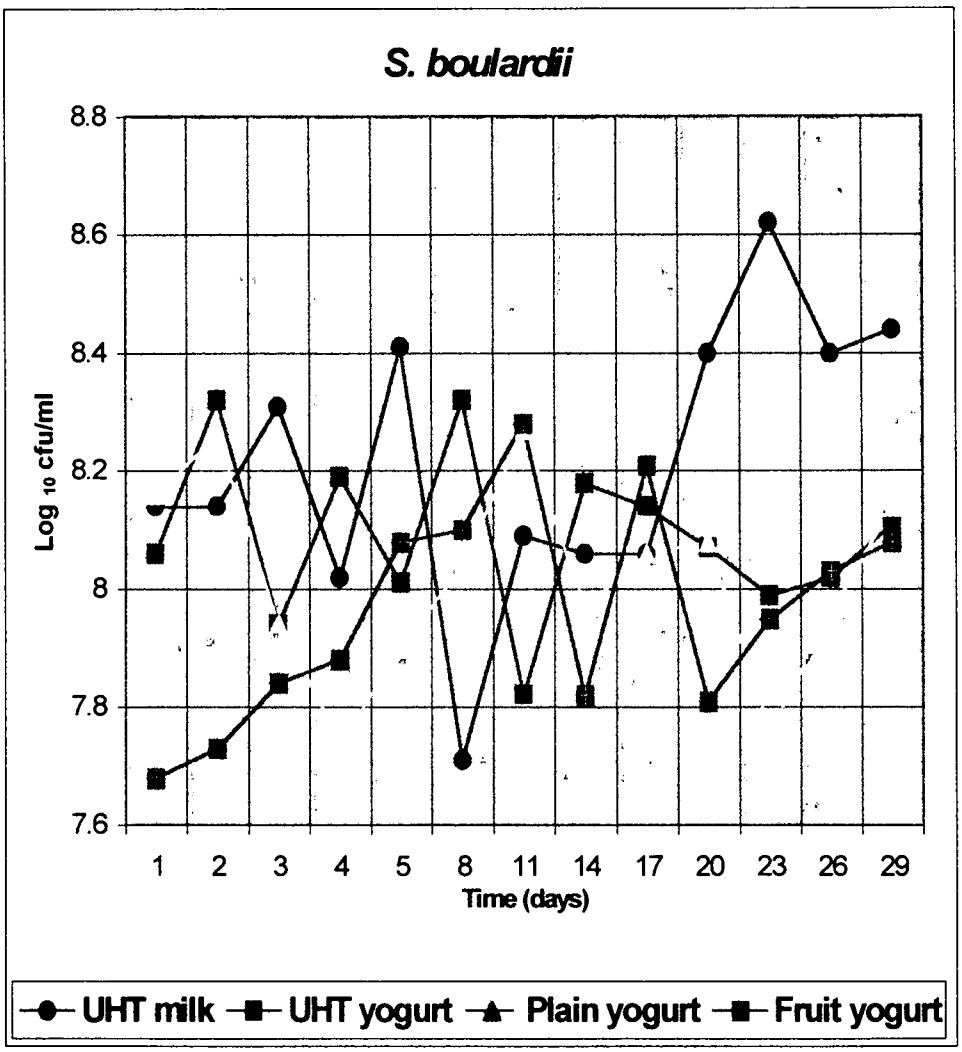
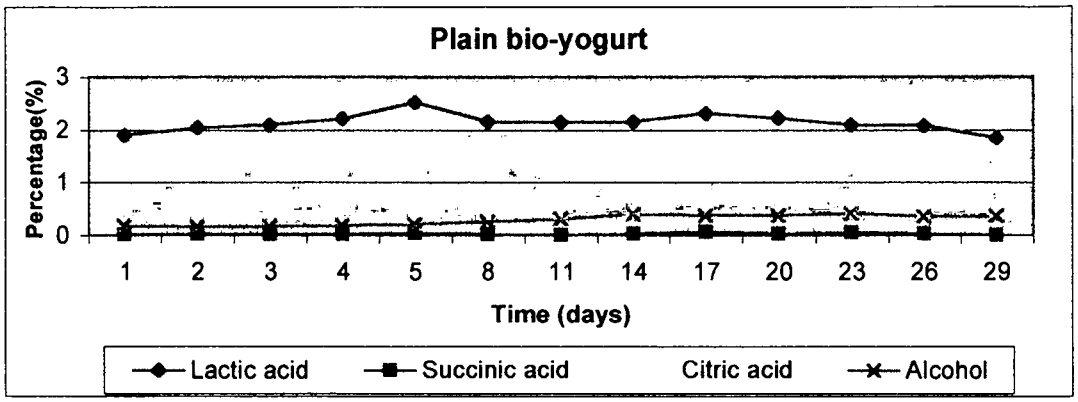
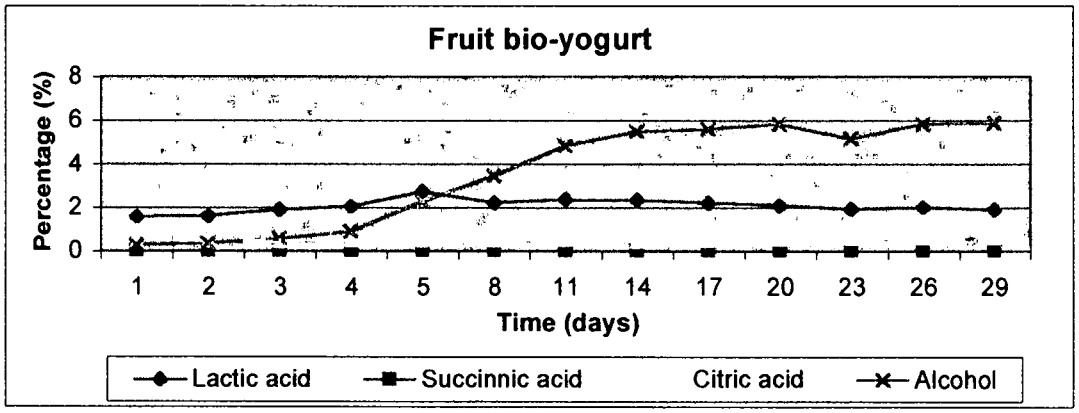


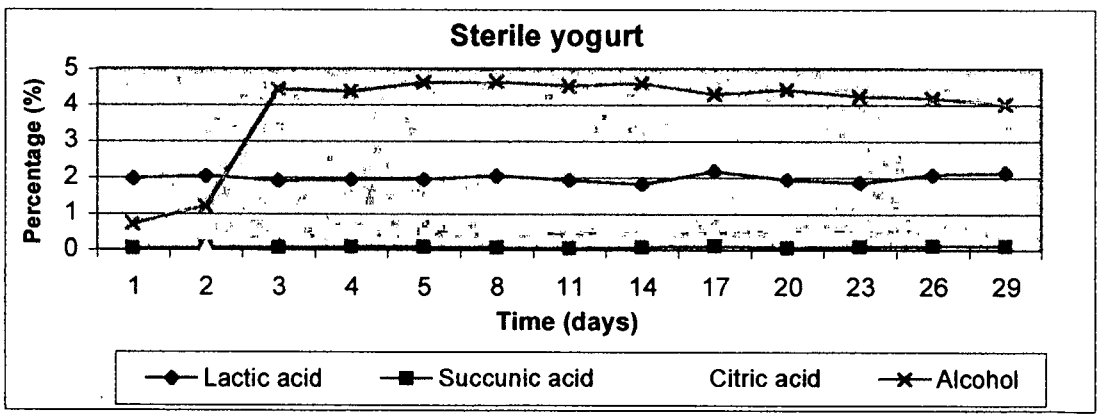
Fig. 6.1. Growth of *S. boulardii* in different dairy products stored at 5°C for 4 weeks.


Plain bio-yogurt
Percentage(%)
3
2
1
0
1 2 3 4 5 8 11 14 17 20 23 26 29
Time (days)
Lactic acid
Succinic acid
Citric acid
Alcohol

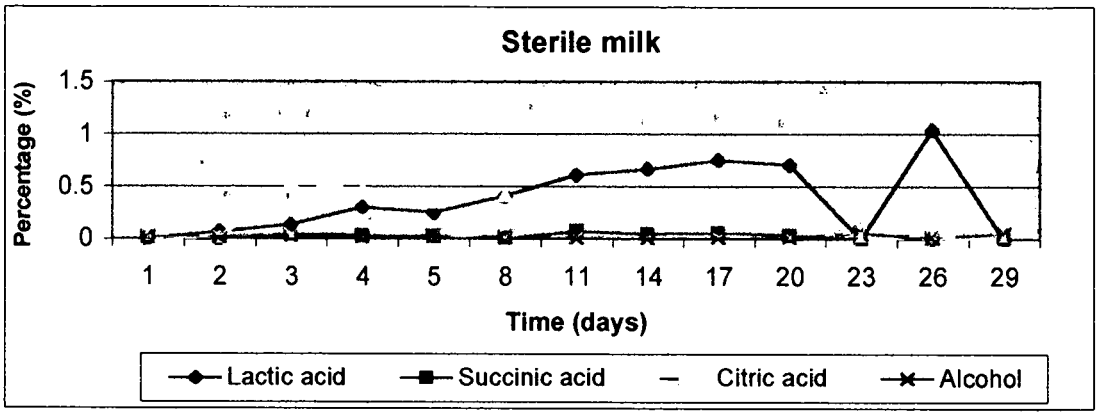
a)


Fruit bio-yogurt
Percentage (%)
8
6
4
2
0
1 2 3 4 5 8 11 14 17 20 23 26 29
Time (days)
Lactic acid
Succinnic acid
Citric acid
Alcohol

b)

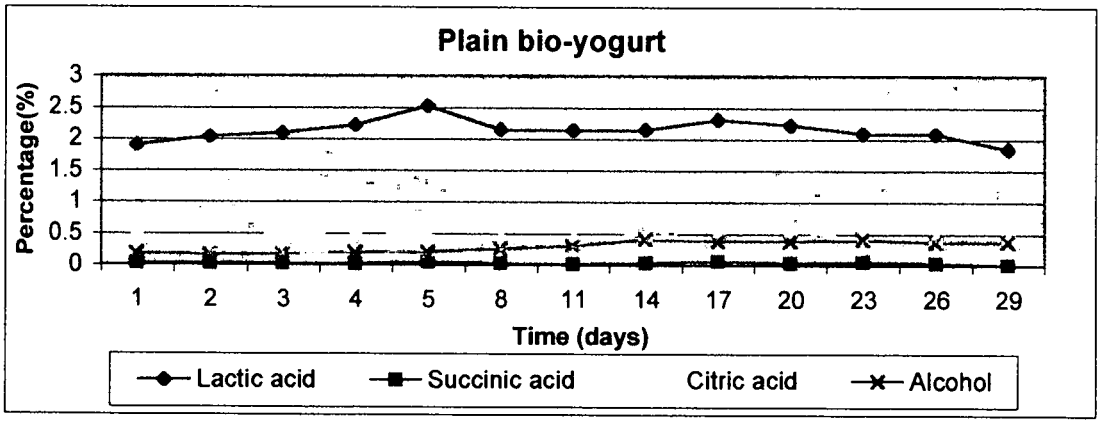

Sterile yogurt
Percentage (%)
5
4
3
2
1
0
1 2 3 4 5 8 11 14 17 20 23 26 29
Time (days)
Lactic acid
Succunic acid
Citric acid
Alcohol

c)

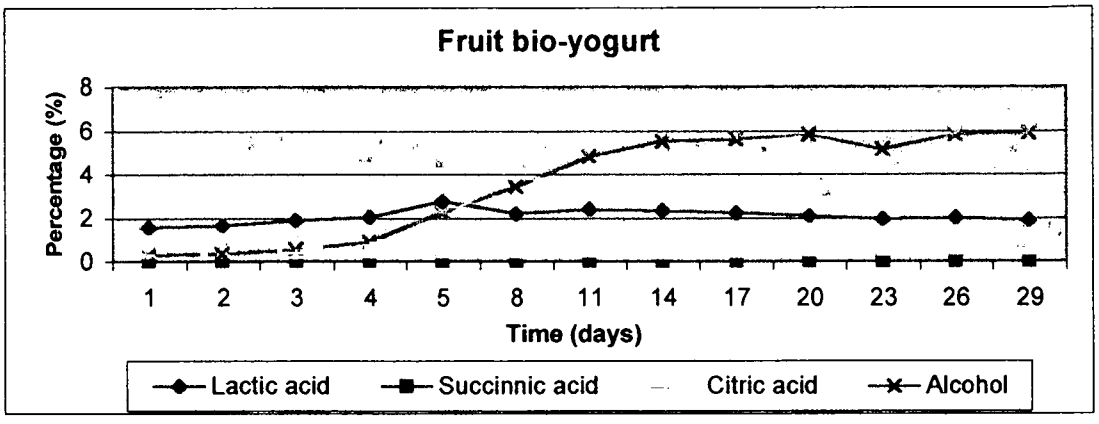


d)

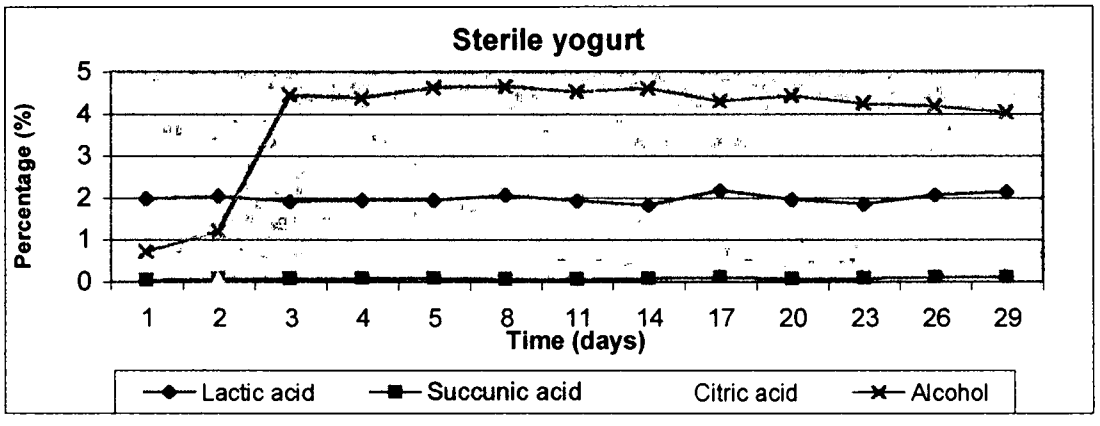
Fig. 6.2a-d. Changes in the concentrations of sugars during the growth of *S. boulardii* in a) plain yogurt; b) fruit yogurt; c) UHT yogurt and d) UHT milk



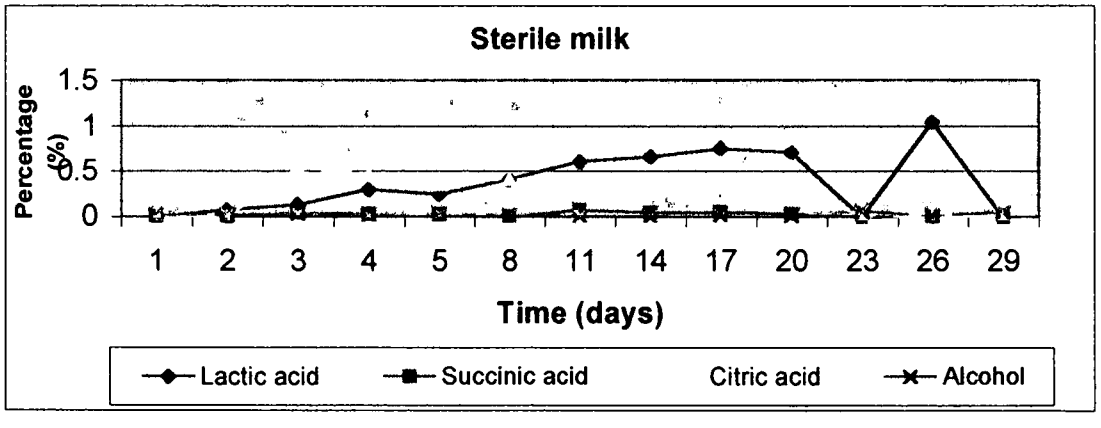
a)



b)



c)



d)

Fig. 3a-d. Percentage (%) organic acids and alcohol present during the growth of *S. boulardii* in a) plain yogurt; b) fruit yogurt; c) UHT yogurt and d) UHT milk.

REFERENCES

Ahearn, D.G., Meyers, S.P. & Nichols, R.A. (1968). Extracellular proteinase of yeasts and yeast-like fungi. *Applied Microbiology.* **16**, 1379-1374.

Berg, H.E. (1993). Reactions of lactose during heat treatment of milk: a quantative study. Ph.D. thesis. CIP-gegevens Koniklijke Bibiotheek, Den Haag.

Deâk, T. and Beuchat, L.R. (1996) Handbook of Food Spoilage. New York, CRC Press.

Fleet, G.H. (1990). Yeasts in dairy products - A review. *Journal of Applied Bacteriology.* **68**, 199-211.

Fleet, G.H. & Mian, M.A. (1987). The occurrence and growth of yeasts in dairy products. *International Journal of Food Microbiology.* **4**, 145-155.

Gedek, B.R. (1991). Regulierung der darmflora über die nahrung. *Zbl. Hyg.* **191**, 277-301.

Giudici, P., Masini, G. & Caggia, C. (1996). The role of galactose fermenting yeast in plain yogurt spoilage. *Annali di Microbiologia ed Enzimologia.* **46**, 11-19.

Goodenough, E.L. & Klein, D.H. (1976). Qualitative and quantitative changes in carbohydrates during the manufacture of yogurt. *Journal of Dairy Science.* **59**, 45-47.

Jakobsen, M. & Narvhus, J. (1996). Yeasts and their beneficial and negative effects on the quality of dairy products. *International Dairy Journal.* **6**, 755-768.

Kouker, G. & Jeager, R.K. (1987). Specific and sensitive plate assay for bacterial lipases. *Applied and Environmental Microbiology.* **53**, 211-213.

Kreger van Rij, N.J.W. (Ed.) (1984). In: The Yeasts – A Taxonomic study, 3rd ed. Elsevier, Amsterdam.

Kurmann, J.A. and Rasic, J.L. (1991) The health potential of products containing bifidobacteria. In: *Therapeutic Properties of Fermented Milks.* Edited by R. K. Robinson. Elsevier Applied Sciences, London, 117-158.

Lang, F. & Lang, A. (1975). Acidophilus milk products: little known cultured milks of great potential. *The Milk Industry.* **77**, 4-6.

Lues, J.F.R., Viljoen, B.C., Miller, M. & Prior, B.A. (1993). Interaction of non-culture microbial flora on dough fermentation. *Food Microbiology,* **10**, 205-213.

McFarland, L. & Bernasconi, P. (1993). *Saccharomyces boulardii*: A Review of an innovative biotherapeutic agent. *Microbial Ecology in Health and Disease.* **6**, 157-171.

Rasic, J.L. (1983). The role of dairy foods containing bifido and acidophilus bacteria in nutrition and health? *North European Dairy Journal.* **4**, 1-5.

Robinson, R.K. (1987). Survival of *Lactobacillus acidophilus* in Fermented Products. *South African Journal of Dairy Science.* **19**, 25-27.

Roostita, R. & Fleet, G.H. (1996). Growth of yeasts in milk and associated changes to milk composition. *International Journal of Food Microbiology.* **26,** 268-276.

Shah, N.P.; Lankaputhra, W.E.V.; Britz, M.L. & Kyle, W.S.A. (1995). Survival of *Lactobacillus acidophilus* and *Bifidobacterium bifidum* in commercial yoghurt during refrigerated storage. *International Dairy Journal.* **5,** 515-521.

Subramanian, P. & Shankar, P.A. (1985). Commensalistic interaction between *L. acidophilus* and lactose-fermenting yeast in the preparation of acidophilus-yeast. *Cultured dairy product Journal* . **Nov**, 17 - 26.

Surawicz, C.M.; Elmer, G.W.; Speelman, P. McFarland, L.V.; Chinn, J. & van Belle, G. (1989) Prevention of antibiotic-associated diarrhea by *Saccharamyces boulardii*: A prospective study. *Gastroenterology.* **96** 981-8.

Suriyarachchi, V.R. & Fleet, G.H. (1981) Occurrence and growth of yeasts in yogurts. Applied and Environmental Microbiology. **42**, 574-579.

Vaughan-Martini, A. & Martini, A. (1998). Descriptions of teleomorphic ascomycetous genera and species. In: *The Yeasts, A Taxonomic Study.* Fourth edition. Edited by Kurtzman, C.P. and Fell, J.W. Elsevier. p. 361-363.

Wickerham, L.J. (1951) Taxonomy of yeasts. *US. Department of Agr. Tech. Bulletin* 1029.

CHAPTER 7

GROWTH AND SURVIVAL OF DAIRY ASSOCIATED YEASTS IN YOGURT AND YOGURT-RELATED PRODUCTS

ABSTRACT

The poor survival of probiotic bacteria is mainly due to the low pH of the yogurt. The ability of yeasts to utilise organic acids and thereby increasing the pH of the yogurt may create a more favourable environment for probiotic bacteria growth. Therefore growth of yeasts in association with probiotic bacteria has been suggested with the intention to stimulate the growth of the probiotic organisms and to assure their survival. Four dairy-associated yeasts, *Debaryomyces hansenii*, *Kluyveromyces marxianus*, *Yarrowia lipolytica* and *Issatchenkia orientalis* were isolated from yogurts and subsequently inoculated into yogurt and related dairy products during processing. The incidence and growth of the yeasts were monitored over a four-week period, the normal time accepted as the shelf-life of yogurt. pH, sugar utilisation and the production of organic acids were determined on a regular basis during the shelf-life to evaluate the possible contribution of the yeasts towards the products. Based on the results obtained, the yeast species were able to progress in bio-yogurt reaching maximum counts exceeding 10^7 cfu/g. Despite the inability of some species to utilise lactose, the yeast species utilised available organic acids, galactose and glucose derived from bacterial metabolism of the milk lactose, as well as possible free fatty acids or free amino acids present in the dairy products. Excessive gas and alcohol production initiated by some yeast species proved, however, to be major constraints.

INTRODUCTION

In order to achieve optimal potential therapeutic effects from a probiotic product, the minimum number of viable probiotic cells present should exceed counts of 10^6cfu/ml (Robinson, 1987). Consequently, it is important to maintain the viability and activity of these organisms until the products are consumed. With regard to probiotic (AB) yogurt, studies have shown, however, that *Lactobacillus acidophilus* and *Bifidiobacterium bifidum* are unstable in yogurt (Rybka and Kailasapathy, 1995).

The poor survival of these probiotic microorganisms is attributed to the low pH prevailing in the yogurt environment (Shah and Jelen, 1990). The low pH of yogurt, however, creates a selective environment stimulating yeast growth (Suriyarachchi and Fleet, 1981). Furthermore, those yeasts able to metabolise organic acids produced by the lactic acid bacteria and galactose derived from the breakdown of lactose (Fleet and Mian, 1987), growth are amplified, enabling these yeasts to establish them as part of the microbial community.

A commensalistic association between yeasts and lactic acid bacteria is also observed in kefir, acidophilus milk, and many fermented food products like sorghum beer, kenkey, lambic-beer, etc. (Deak and Beuchat, 1996). Milk products that include yeasts as part of the starter culture comprises acidophilus-yeast milk (Lang and Lang, 1975), kefir, koumiss and laban. The possibility of producing acidophilus milk with a mixed culture of *Lactobacillus acidophilus* and the lactose fermenting yeasts *Kluyveromyces marxianus* and *Candida pseudotropicalis* has been described by Subramanian and Shankar (1985). In a study by Soulides (1955) a number of on-lactose fermenting yeasts belonging to the genus *Torulopsis* (now called *Candida*) were isolated from yogurt. Growth of these yeasts in milk with strains of *Streptococcus thermophilus* and *L. bulgaricus* resulted in synergistic peptonization followed by

a gradual decrease in acidity. The decrease in acidity was attributed to assimilation of lactic acid. As a result of the associative growth between the yeasts and the bacteria, the viability of the yogurt bacterial strains was maintained in milk for 5 to 8 months.

Lactic acid bacteria present in Dahi were found to live longer when associated with a non-lactose fermenting yeast due to the prevention of accumulation of toxic quantities of acid (Graham, 1943). Presumably, the yeast utilises the lactic acid produced by the bacteria, thereby increasing the pH of the product (Subramanian and Shankar, 1983). Yeasts also add to the association by stimulating the growth of the lactic acid bacteria due to the excretion of growth factors and metabolites. Similar interactive results were reported by Borregaard and Arneborg (1998) during the study of interactions between *Lactococcus* and *Issatchenkia* in milk fermentations. Consequently, the presence of yeasts in yogurt may also stimulate the growth of pro-biotic microorganisms by increasing the pH.

In order to study the effect of yeast growth in yogurt and the simultaneous progression of probiotic bacteria, it is imperative to assess the ability of yeast isolates to grow and survive in yogurt. Accordingly, yeast strains were isolated from commercial yogurt, assuming that the isolates already adapted to the immediate environment. Four predominant yeast strains, *Kluyveromyces marxianus*, *Issatchenkia orientalis*, *Debaryomyces hansenii* and *Yarrowia lipolytica*, commonly associated with yogurt (Deak and Beuchat, 1996) were selected and inoculated into plain yogurt, fruit flavoured yogurt, sterile milk and pasteurised sweetened yogurt. The yeasts' survival and progression in fermented and non-fermented flavoured and non-flavoured dairy products are compared.

MATERIALS AND METHODS

Yeast cultures.

Yeasts were isolated from commercial yogurt and identified according to the conventional methods described by Kreger- van Rij (1984). *Debaryomyces hansenii, Kluyveromyces marxianus, Yarrowia lipolytica* and *Issatchenkia orientalis* were selected for inoculation into the experimental yogurt. The yeast cultures were maintained on slants of YM (yeast-extract, malt-extract) agar (Wickerham, 1951) and stored at 5°C. When required, the yeast cultures were activated by transferring to YM agar plates, incubated at 25°C for 48 h and checked for purity before use in experiments.

Dairy product samples

AB-yogurt samples, namely plain (natural) yogurt and 'fruit cocktail' flavoured yogurt, were obtained from a local yogurt manufacturer within 1 d after production. Sterile UHT milk and pasteurised yogurt (heat treated after fermentation) were purchased from a supermarket outlet. Interference from natural contaminants and LAB (lactic acid bacteria) was avoided by using UHT milk and pasteurised yogurt. Samples were transported in a cooler box on ice and refrigerated upon arrival in the laboratory. Samples were inoculated within 12 h of receipt.

Inoculation

The different yeast cultures were grown in YNB (yeast nitrogen base, Oxoid, Basingstoke) broth to late stationary phase. These yeast cells were harvested by centrifugation at 14 000 *g* for 10 minutes. The supernatant was decanted and the yeast cells weighed. Appropriate aliquots of sterile water were added to

the cells to obtain different percentages yeast cells. Yeast cells per ml suspension were predetermined with a Klett Sumerson to determine the percentage (w/v) inoculation size necessary to obtain an initial inoculation level of approximately 10^6 cells/ml in the experimental dairy products.

The plain AB-yogurt, fruit cocktail yogurt, sterile milk and pasteurised yogurt samples were aseptically dispensed into sterilised Schott bottles (1l). All the dairy samples were then inoculated with the yeast culture at a level of 2% (weight cells per volume product) to obtain an inoculation level of approximately 10^6 cells/ml in the experimental dairy products. The inoculated dairy samples were uniformly mixed and stored at 5°C.

Microbiological analyses

Microbial analyses were performed every 24 h for 5 days and then consecutively at three-day intervals for 32 days. From each dairy product, 10ml samples were aseptically withdrawn, dispensed into 90 ml 0.1% sterile peptone water and further diluted for plate counting. Aliquots (0.1 ml) of the dilutions were spread plated in duplicate on YM agar (Wickerham, 1951) plates. Inoculated plates were aerobically incubated at 25°C for 5 days. After incubation, yeast colonies between 30 and 300 per dilution were counted and the results expressed as yeast count per ml (cfu/ml) of the product. The data presented are the means of results obtained on duplicate plates and expressed in $\log_{10}$ cfu/ml.

pH determination

The pH of each sample at every sampling occasion was measured using a HI 9321 Microprocessor pH meter (HANNA Instruments).

Chemical analysis

For chemical analysis, at each sampling occasion, samples (2ml) were taken and stored in Eppendorf tubes at −18°C until needed. The samples were clarified by centrifugation at 14000 for 2 min and the supernatant ultra-filtrated by using Whatman filter discs (0.45μm) prior to chromatographic analysis.

Alcohol and sugar

Sugar and alcohol concentrations were determined by means of a WATERS HPLC system with a Biorad-aminex C42 Column and Refractive index detector. The concentrations of individual sugars and alcohol were calculated by reference to standard solutions (1.0%, w/v).

Organic acids

The concentration of organic acids was measured by a HPLC (WATERS HPLC system with a Biorad-aminex C42 Column and Refractive index detector). Identification of organic acids and quantification of their concentrations were conducted by comparison of unknown peak positions with standard solutions (1.0%, w/v).

RESULTS AND DISCUSSION

Growth and survival of yeasts in dairy products

Kluyveromyces marxianus, Yarrowia lipolytica, Issatchenkia orientalis and *Debaryomyces hansenii* grew in yogurt and related products, and survived sufficiently during a 32 day period of growth (Fig. 7.1) to prevent excessive

decreases in pH. All the yeasts growth rates remained stable or increased when inoculated into UHT milk, whereas *D. hansenii* and *Y. lipolytica* cell densities decreased when grown in plain yogurt, fruit yogurt, and pasteurised yogurt over the same period. *I. orientalis* and *K. marxianus* cell densities, however, remained constant or increased in all the products. The lactose fermenting ability (Gancedo and Serrano, 1989) and the utilization of citric and succinic acids (Roostita and Fleet, 1996) by *K. marxianus* explain its growth in dairy products, whereas the proteolytic and lipolytic activities, and the utilization of organic acids contribute to the growth of *Y. lipolytica* and *D. hansenii* (Ratledge and Tan, 1990; Ogrydziak, 1993). It is, however, more difficult to explain the growth of *I. orientalis* in milk, since the species lack the ability to utilise lactose and galactose, but being capable to utilise glucose and lactic acids (Borregaard and Arneborg, 1998). According to Rosenthal (1991), milk contains trace amounts of glucose and galactose and therefore the first could contribute to the growth of the species.

Although the chemical composition of milk will support the growth of yeasts (Fleet, 1990; Deak, 1991), the faster growing psychrotropic bacteria restricted their growth (Cousin, 1982; Roostita and Fleet, 1996). The increase in growth rate of the individual yeasts when grown in sterilized milk, therefore, may be attributed to the lack of competitive microorganisms present. The maximum population of the yeasts in UHT-treated milk exceeded counts of 10^7 cfu/ml.

Changes in the concentrations of sugars, organic acids, alcohol and pH.

The growth of *K. marxianus* in all the yogurt and milk correlated with a decrease in the lactose contents and increases in galactose, excreted due to lactose hydrolysis, and alcohol, attributed to lactose fermentation (Table 7.1). The incomplete utilisation of lactose may be attributed to the growth at low temperatures (5°C). The glucose content also increased in the UHT-treated

milk due to lactose hydrolysis. The excretion of the lactose hydrolysis products in milk was also reported by Carvalho and Spencer (1990) and Roostita and Fleet, 1996). The increase in the lactic and citric acid contents in the UHT milk is also regarded as secondary products of lactose fermentation (Roostita and Fleet, 1996). These values were higher during the middle stages of growth (results not shown), but were utilised in the later stages. Similarly, despite initial accumulation of glucose and galactose contents during the growth of *K. marxianus* in the yogurts, the glucose produced was utilised during the later stages, whereas the galactose contents continued to increase.

Lactose was not utilised during the growth of either *I. orientalis, D. hansenii* or *Y. lipolytica* in milk or yogurts. These results agree with the fact that the species are unable to utilise lactose as carbon and energy source (Kreger-van Rij, 1984). Consequently, no production of glucose or galactose was observed. Varying galactose results (slightly higher) obtained when the species were grown in fruit yogurt and plain yogurt, are attributed to the presence of lactic acid bacteria still viable at the later stages. This correlated with the decrease in galactose concentration when *D. hansenii* was grown in pasteurised yogurt in the absence of lactic acid bacteria. Furthermore, the galactose concentration initially produced by the lactic acid bacteria before pasteurisation in the pasteurised yogurt, was not utilised by *I. orientalis* or *Y. lipolytica.* According to Barnett et al. (1990), both species lack the ability to utilise galactose.

The main carbohydrate in the fruit and pasteurised yogurts was sucrose (Table 7.1). Growth of *D. hansenii* in these products was characterised by partial utilisation of the sugar whereas *K. marxianus* utilised less, and *I. orientalis* and *Y. lipolytica* failed to utilise the sugar. According to Barnett et al. (1990), the latter two species lack the ability to utilise sucrose. Again, the slight decrease in the sucrose concentrations when *I. orientalis* and *Y. lipolytica* were grown in the fruit yogurt might be attributed to the growth of lactic acid bacteria, since

no utilisation of sucrose was observed when these two species were grown in pasteurised yogurt.

Small amounts of alcohol (< 0.06%) were found in fruit and plain yogurt after the growth of *I. orientalis,* mainly due to partial utilisation of glucose. The species, however, produced a significant amount of alcohol (1.00%) when grown in pasteurised yogurt. The increase in the amount of alcohol correlated with the total depletion of glucose. High amounts of alcohol were found when *K. marxianus,* capable of fermenting lactose, was grown in UHT-treated milk (1.49%) and pasteurised yogurt (1.24%). The amount of alcohol produced was significantly reduced when the species was grown in fruit (0.40%) and plain (0.07%) yogurt. No alcohol was produced when either *D. hansenii* or *Y. lipolytica* was grown in any of the dairy products. According to Barnett et al. (1990), *Y. lipolytica* is a non-fermenting yeast species, whereas *D. hansenii* shows only weak fermentation.

In contrast to previous findings, we detected higher amounts of lactic acid compared to citric acid (Roostita and Fleet, 1996) in UHT-treated milk before yeast growth. Growth of *K. marxianus* in the UHT treated milk was characterised by initial accumulation of lactic acid due to lactose fermentation (results not shown), but it was utilised at the later stages resulting in a small nett increase. Growth of *I. orientalis, D. hansenii* and *Y. lipolytica,* in UHT-treated milk was characterised by the partial utilisation of lactic acid. These results agree with the fact that the species are able to utilise lactic acid (Barnett et al., 1990) as carbon and energy sources. Although the species are also capable to utilise citric acid (Barnett et al., 1990), only minor or no changes in the concentration of citric acid were observed. Similar lactic and citric acid utilisation patterns were observed when the yeast species were grown in pasteurised yogurt (Table 7.1), although higher concentrations were initially present after lactic acid bacterial growth in the yogurt. When the yeast

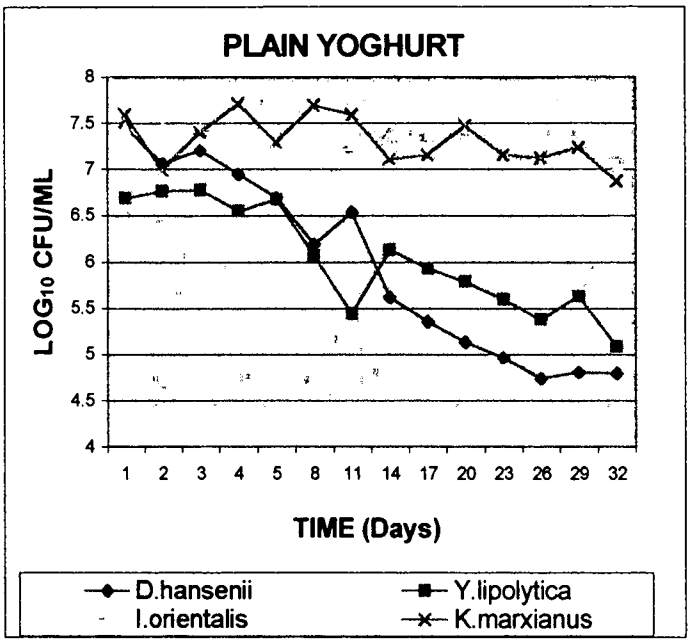
species were grown in fruit yogurt, constant increases in lactic acid concentrations were obtained. The increases in the concentration of lactic acid might be attributed to the presence of viable lactic acid bacteria still present in the yogurt. Interesting to note, however, is the decrease in lactic acid concentration when the yeasts were grown in plain yogurt, except for *K. marxianus*, despite the presence of viable lactic acid bacteria. A possible explanation for the decreases in lactic acid concentrations in the plain yogurt may be due to the absence of sucrose, which was found in the fruit yogurt, and consequently the yeasts utilised the lactic acid as carbon and energy source.

Due to the utilisation of organic acids by the individual yeasts when grown in pasteurised and plain yogurt, significant increases in pH values were obtained (Table 7.2). The increases in the organic acid concentrations when the individual yeasts were grown in fruit yogurt correlated with decreased pH values. Despite the decreased pH values in fruit yogurt, the nett decrease (0.08) in pH values was significantly less when compared with normal yogurt processing procedures (> 0.3) stored at 5°C using only lactic acid bacteria (Rosenthal, 1991).

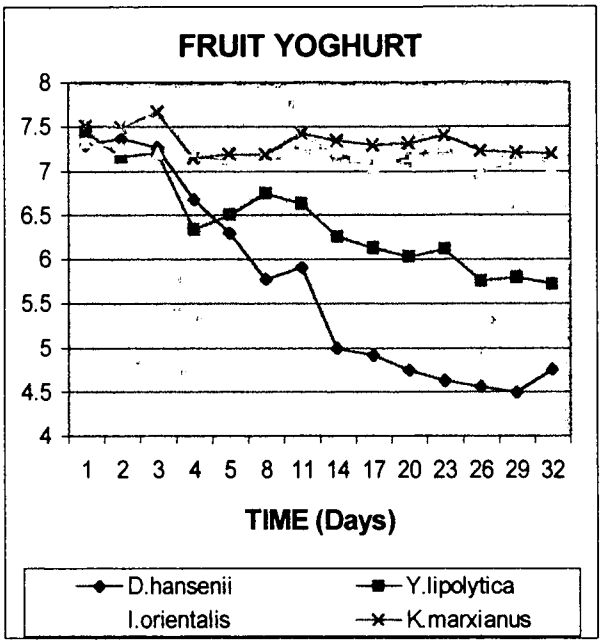
CONCLUSION

In this study we have shown that the yeast isolates, *K. marxianus, D. hansenii, Y. lipolytica* and *I. orientalis* were able to survive in the yogurts, utilising the yogurt constituents as growth substrates during the 32 day storage period, and sufficiently contribute to the retention or enhancing of the pH values. Despite better survival and growth rates obtained when *K. marxianus* and *I. orientalis* were inoculated into the yogurts, the production of alcohol and gas formation is major constraints in implementing these yeast species into yogurt. The inclusion of *Y. lipolytica* and *D.hansenii* in AB yogurts, therefore, seems

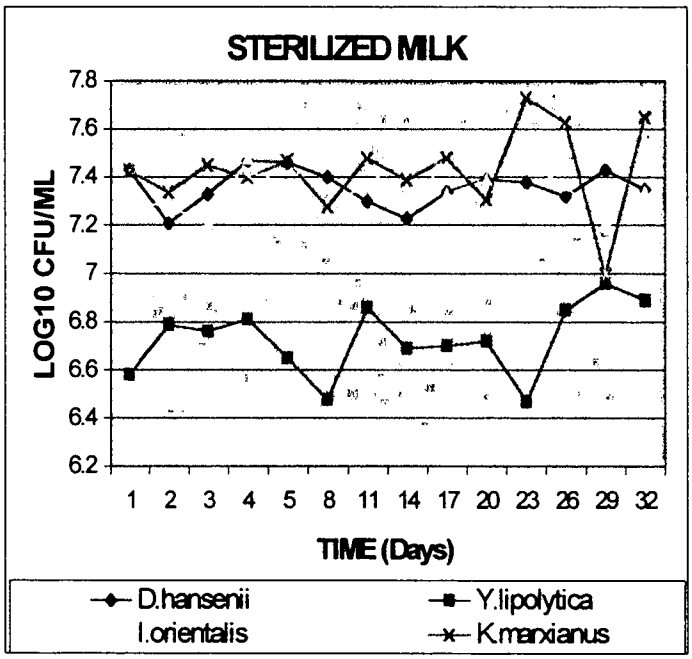
the most promising in controlling the pH to assure the viability of the probiotic microorganisms. More studies, however, are needed to determine the positive or detrimental interaction between the yeasts and the probiotic cultures as well as the traditional starter cultures when grown in association in yogurt. In addition, the contribution of the yeasts to taste, texture and odors in the yogurt, also need to be evaluated.



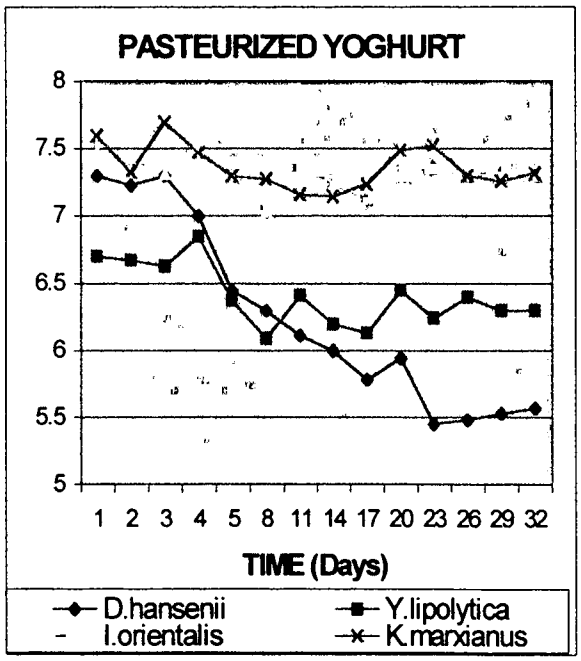
a)



b)



c)



d)

Fig. 7.1a-d Growth and survival of *D. hansenii, Y. lipolytica, I. orientalis* and *K. marxianus* in a) plain yogurt, b) fruit yogurt, c) sterilised milk and d) pasteurised yogurt at 5°C.

Table 7.I. Concentration of sugar, organic acids, alcohol and pH initially and after the growth of yeasts at 32 days storage.

	Initial conc. (%) (day 1)	**Final concentration (%) in UHT treated milk after yeast growth (day 32).**			
		K. marxianus	*I. orientalis*	*D. hansenii*	*Y. lipolytica*
Lactose	3.5	0.70	3.00	3.5	3.29
Glucose	0.0	0.33	0.0	0.0	0.0
Galactose	0.03	0.55	0.0	0.0	0.0
Sucrose	0.0	0.0	0.0	0.0	0.0
Lactic acid	0.21	0.20	0.13	0.19	0.18
Citric acid	0.07	0.13	0.07	0.05	0.05
Alcohol	0.0	1.49	0.0	0.0	0.03
pH	6.56	5.73	6.52	6.47	6.29

	Initial conc. (%) (day 1)	Final concentration (%) in pasteurised yogurt after yeast growth (day 32)			
		K. marxianus	*I. orientalis*	*D. hansenii*	*Y. lipolytica*
Lactose	2.66	2.36	2.61	2.63	2.66
Glucose	0.86	0.0	0.0	0.32	0.35
Galactose	1.45	1.49	1.43	0.73	1.41
Sucrose	5.89	5.32	5.82	4.90	5.84
Lactic acid	0.77	0.91	0.24	0.77	0.76
Citric acid	0.30	0.37	0.12	0.29	0.28
Alcohol	0.0	1.24	1.00	0.01	0.0
pH	3.92	3.97	3.98	4.01	4.01

	Initial conc. (%) (day 1)	Final concentration (%) in fruit yogurt after yeast growth (day 32)			
		K. marxianus	*I. orientalis*	*D. hansenii*	*Y. lipolytica*
Lactose	3.13	2.87	3.13	3.12	3.10
Glucose	1.02	0.72	0.52	1.12	1.10
Galactose	1.30	1.45	1.31	1.42	1.39

Sucrose	4.48	4.35	4.15	3.74	4.26
Lactic acid	1.06	1.30	1.56	1.25	1.46
Citric acid	0.36	0.33	0.38	0.37	0.29
Alcohol	0.0	0.40	0.05	0.0	0.01
PH	4.07	3.99	3.99	3.99	3.97
	Initial conc. (%) (day 1)	**Final concentration (%) in plain yogurt after yeast growth (day 32)**			
		K. marxianus	*I. orientalis*	*D. hansenii*	*Y. lipolytica*
Lactose	3.75	3.40	3.73	3.74	3.76
Glucose	0.0	0.0	0.0	0.0	0.0
Galactose	0.90	0.92	0.96	0.92	0.95
Sucrose	0.0	0.0	0.0	0.0	0.0
Lactic acid	1.82	2.04	0.30	1.79	0.32
Citric acid	0.34	0.43	0.08	0.34	0.11
Alcohol	0.0	0.07	0.01	0.0	0.0
PH	4.19	4.25	4.26	4.24	4.25

* **Results are the means of duplicate results.**

Table 7.2. Changes in pH of dairy product samples inoculated with *D. hansenii* (D.h), *Y. lipolytica* (Y.l), *I. orientalis* (I.o) and *K. marxianus* (K.m) during storage at 4°C for 4 weeks.

Time	Sterilized milk				Pasteurized yogurt				Fruit yogurt				Plain yogurt			
	D.h	**Y.l**	**I.o**	**Km**	**D.h**	**Y.l**	**I.o**	**Km**	**D.h**	**Y.l**	**I.o**	**Km**	**D.h**	**Y.l**	**I.o**	**Km**
1	6.56	6.56	6.56	6.56	3.92	3.92	3.92	3.92	4.07	4.07	4.07	4.07	4.19	4.19	4.19	4.19
2	6.50	6.51	6.53	6.06	3.95	3.97	3.92	3.92	4.01	4.06	3.97	3.94	4.16	4.19	4.14	4.14
3	6.55	6.55	6.53	5.97	3.96	3.99	3.93	3.91	3.99	4.06	3.94	3.91	4.10	4.11	4.11	4.13
4	6.54	6.54	6.53	5.85	3.87	3.91	3.83	3.80	3.99	4.02	3.99	3.96	4.12	4.16	4.08	4.08
5	6.56	6.53	6.56	5.81	3.89	3.98	3.81	3.75	3.84	3.83	3.85	3.84	4.16	4.14	4.12	4.11
8	6.49	6.46	6.48	5.77	3.95	3.98	3.93	3.93	3.99	4.01	3.98	3.96	4.19	4.19	4.19	4.19
11	6.55	6.47	6.52	5.91	4.02	3.99	3.98	3.95	3.98	4.08	3.94	3.89	4.06	4.06	4.08	4.07
14	6.48	6.41	6.50	6.08	3.91	3.93	3.88	3.87	3.89	3.89	3.87	3.85	4.10	4.10	4.10	4.10
17	6.32	6.23	6.30	5.65	3.80	3.80	3.77	3.75	3.77	3.79	3.75	3.73	4.00	4.00	4.01	4.00
20	6.25	6.13	6.26	5.54	3.75	3.74	3.70	3.67	3.69	3.68	3.65	3.64	3.94	3.95	3.94	3.94
23	6.40	6.27	6.48	5.79	3.92	3.91	3.96	3.94	3.88	3.87	9.89	3.89	4.12	4.13	4.15	4.22
26	6.51	6.35	6.52	5.77	4.02	4.03	3.99	3.99	3.98	3.96	3.96	3.96	4.23	4.23	4.24	4.19
29	6.45	6.34	6.47	5.74	3.97	3.98	3.94	3.93	3.92	3.93	3.92	3.91	4.16	4.18	4.19	4.18
32	6.47	6.29	6.52	5.73	4.01	4.01	3.98	3.97	3.99	3.97	3.99	3.99	4.24	4.25	4.26	4.25
D	**-.09**	**-.27**	**-.04**	**-.83**	**+.09**	**+.09**	**+.06**	**+.05**	**-.08**	**-.10**	**-.08**	**-.08**	**+.05**	**+.06**	**+.07**	**+.06**

D = difference between initial and final level of pH, + = increase in pH, - = decrease in pH

REFERENCES

Barnett, J.A., Payne, R.W. and Yarrow, D. (1990) Yeasts: Characteristics and Identification, 2nd edition. Cambridge University Press, Cambridge.

Borregaard, E. and Arneborg, N. (1998) Interactions between *Lactococcus lactis* subs. *lactis* and *Issatchenkia orientalis* at milk fermentation. *Food Technology and Biotechnology.* **36**, 75- 78.

Cousin, M.A. (1982) The presence and activity of psychrotrophic microorganisms in milk and dairy products: A review. *Journal of Food Protection.* **45**, 172-207.

Carvalho, S.M. and Spencer, M.I. (1990) Modes of lactose uptake in the yeast species *Kluyveromyces marxianus. Antonie van Leeuwenhoek.* **57**, 77-81.

Deàk, T. (1991) Foodborne yeasts. *Advances in Applied Microbiology.* **36**, 180-277.

Deàk, T. and Beuchat, L.R. (1996) Handbook of Food Spoilage. New York, CRC Press.

Fleet, G.H. and Mian, M.A. (1987) The occurrence and growth of yeasts in dairy products. *International Journal of Food Microbiology.* **4**, 145-155.

Fleet, G. (1990) Yeasts in dairy products – A review. *Journal of Applied. Bacteriology.* **68**, 199-211.

Gancedo, C. and Serrano, R. (1989) Energy-yielding metabolism. In: Rose, A.H., Harrison, J.S. (Eds.), The Yeasts: Metabolism and Physiology of Yeasts, Vol 3, Academic Press, London, pp. 205-257.

Graham, V.E. (1943) The maintenance of viability in *Lactobacillus bulgaricus* cultures by growth in association with certain yeasts. *Journal of Bacteriology.* **45**, 51.

Kreger van Rij, N.J.W. (Ed.) (1984) In: The Yeasts – A Taxonomic study, 3rd ed. Elsevier, Amsterdam.

Lang, F. and Lang, A. (1975) Acidophilus milk products: little known cultured milks of great potential. *The Milk Industry.* **77**, 4-6.

Ogrydziak, D.M. (1993) Yeast extracellular proteases. *Crit. Rev. Biotechnol.* **13**, 1-55.

Ratledge, C., Tan, K.H., 1990. Oils and fats: Production, degradation and utilisation by yeasts. In: Verachert, H., De Mot, R. (Ed.), Yeast Biotechnology and Biocatalysis, Marcel Dekker Inc., New York, pp. 225-245.

Robinson, R.K. (1987) Survival of *Lactobacillus acidophilus* in Fermented Products. *South African Journal of Dairy Science.* **19** (1), 25-27.

Roostita, R. and Fleet, G.H. (1996) Growth of yeasts in milk and associated changes to milk composition. *International Journal of Food Microbiology.* **26**, 268-276.

Rosenthal, I. (1991). Milk and dairy Products: Properties and Processing, VCH

Publishers Inc., Weinheim, New York.

Rybka, S. and Kailasapathy, K. (1995) The survival of culture bacteria in fresh and freeze-dried AB yoghurts. *Australian Journal of Dairy Technology.* **50**(2) 51-57.

Shah, N. and Jelen, P. (1990). Survival of Lactic Acid Bacteria and Their Lactases under Acidic Conditions. *Journal of Food Science.* **55**(2), 506-509.

Soulides, D.A. (1955) A synergism between yogurt bacteria and yeasts and the effect of their association upon the viability of the bacteria. *Applied Microbiology.* **3**, 129-131.

Subramanian, P. and Shankar, P.A. (1985) Commensalistic interaction between *L. acidophilus* and lactose-fermenting yeast in the preparation of acidophilus-yeast. *Cultured dairy product Journal.* **Nov**, 17 - 26.

Suriyarachchi, V.R. and Fleet, G.H. (1981) Occurrence and growth of yeasts in yogurts. *Applied & Environmental Microbiology.* **42**, 574-579.

Wickerham, L.J. (1951) Taxonomy of yeasts. *US Department of Agricultural Technology Bulletin* 1029.

CHAPTER 8

ENHANCEMENT OF THE VIABILITY OF PROBIOTIC BACTERIA IN BIO-YOGURT: THE EFFECT OF *DEBARYOMYCES HANSENII* AND *YARROWIA LIPOLYTICA*

ABSTRACT

The ability of *Yarrowia lipolytica* and *Debaryomyces hansenii* to improve viability of probiotic bacteria was investigated by inoculation of pure cultures of the species into commercial AB-yogurt directly after manufacture (10^5–10^6 cfu/ml) and monitoring viable counts during storage at 5°C. In the second part of the study *D. hansenii* were inoculated before fermentation with the ABT-starter at a lower level (10^2–10^3 cfu/ml). Inoculation with the yeast resulted in stabilization of bifidobacteria counts in general. Improved survival of bifidobacteria was obtained in plain yogurt (0.2 log unit decrease) with *D. hansenii* added before manufacture. In contrast, bifidobacteria survived better in fruit yogurt with *D. hansenii* addition after manufacture (remained at the same level). A rapid decrease in the viability of *L. acidophilus* was observed after 2 weeks storage, indicating a possible antagonistic action from the yeasts against *L.acidophilus*. The decrease in *L. acidophilus* counts, however, was less in fruit yogurt inoculated with *D. hansenii* before manufacture. Apparently, both yeasts species encouraged better survival of the streptococci; counts of streptococci remained above 10^8 cfu/ml until the end of storage. The initial pH values of plain and fruit yogurt were 4.31 and 4.26 respectively. Consequently, due to the enhanced survival of the streptococci, an enhanced decline in pH was obtained. The sensory quality of the yeast-inoculated yogurt was still acceptable at expiry date.

INTRODUCTION

Probiotic bacteria such as *Lactobacillus acidophilus* and *Bifidobacterium* species are increasingly employed in the starter culture of many dairy products, especially in the most popular cultured milk product yogurt. These probiotic bacteria are believed to, when applied in for example yogurt, exhibit a beneficial effect on the health of the human host upon ingestion (Huis in't Veld & Havenaar, 1991). Consumption should be more than 100g per day of bio-yogurt containing more than 10^6 probiotic cfu/ml in order to have any therapeutic effect (Robinson, 1987; Kurmann & Rasic, 1991; Rybka & Kailasapathy, 1995). Therefore probiotic bacteria have to remain viable and active during the shelflife of yogurt to maintain these levels. However, surveys have shown poor survival of probiotic bacteria in bio-yogurts (Shah et al., 1995; Hull et al., 1984; Lourens et al., 2000).

The frequent occurrences of yeasts in dairy related products indicate the ability of yeasts to survive and metabolise milk constituents (Fleet, 1990). Yeasts might develop in yogurt as secondary flora, after yogurt starter culture growth. Lactic acid bacteria (LAB) ferment the lactose in milk through hydrolysis to glucose and galactose. Only the glucose is changed into lactic acid, whereas the galactose moiety is released into the extracellular environment (Goodenough & Klein, 1976). The high concentration of galactose present (about 1%) was the main reason for the growth of galactose positive and non-fermenting yeasts in yogurt in the study by Giudici et al. (1996). Furthermore, the low pH of yogurt and the ability of yeasts to utilise organic acids create a selective environment for yeast growth (Fleet & Mian, 1987).

In a study by Soulides (1955), a number of non-lactose fermenting yeasts belonging to the genus *Torulopsis* (now called *Candida*) were isolated from yogurt. Growth of these yeasts in milk with strains of *Streptococcus*

thermophilus and *L. bulgaricus* resulted in synergistic peptonization followed by a gradual decrease in acidity. The decrease in acidity was attributed to the assimilation of lactic acid. As a result of the associative growth between the yeasts and the bacteria, the viability of the yogurt bacterial strains was maintained in milk for 5 to 8 months. The possibility of producing acidophilus milk with a mixed culture of *Lactobacillus acidophilus* and the lactose fermenting yeasts, *Kluyveromyces marxianus* and *Candida pseudotropicalis,* has been described by Subramanian and Shankar (1983). *L. acidophilus* growth was stimulated in the presence of the yeasts. Also, *Bacillus cereus* and *Escherichia coli* failed to survive in acidophilus-yeast milk. In a more recent study by Rada (1997), *Kluyveromyces marxianus* significantly prolonged the survival of bifidobacteria in milk at 4°C.

Yeasts add to the association by stimulating the growth of the lactic acid bacteria due to the excretion of growth factors and metabolites. Similar interactive results were reported by Borregaard and Arneborg (1998) during the study of interactions between *Lactococcus* and *Issatchenkia* in milk fermentations.

Other milk products that include yeasts as part of the starter culture and where a commensalistic association between yeasts and lactic acid bacteria exists, comprise acidophilus-yeast milk (Lang & Lang, 1975), koumiss, laban, Dahi, (Graham, 1943), Kefir (Leroi & Pidoux, 1993a,b) as well as cheese (Fernandez Del Pozo et al., 1988). Other fermented food products include sorghum beer, kenkey, lambic-beer, etc. (Deak & Beuchat, 1996) and even Champagne (Lemaresquier, 1987).

Therefore the aim of this study was to examine the possible influence of *Yarrowia lipolytica* and *Debaryomyces hansenii* on the growth and survival of probiotics in bio-yogurt. *Y. lipolytica* and *D.hansenii* were selected based on a

previous study where the incidence and growth of four dairy-associated yeasts, *Debaryomyces hansenii, Kluyveromyces marxianus, Yarrowia lipolytica* and *Issatchenkia orientalis*, in bio-yogurt were monitored (Lourens and Viljoen, 2000). *Y. lipolytica* and *D.hansenii* were the only species that did not produce gas or alcohol in the yogurt or little at all. *Y. lipolytica* and *D.hansenii* frequently occur in dairy products, linked to their nutritional requirements, growth at low temperature, low pH values, low water activities and high salt concentrations (Davenport, 1980; Seiler & Busse, 1990; Roostita & Fleet, 1996; Welthagen & Viljoen, 1999).

The positive or detrimental interaction between the yeasts and the probiotic cultures, bifidobacteria and *L. acidophilus*, as well as the traditional yogurt culture, *S. thermophilus*, when grown in association in yogurt were investigated. In addition, the affect of time of yeast inoculation, and inoculum size of the yeast as part of the starter culture were compared.

The sensory effect contributed by the yeasts to taste, texture and odors in the yogurt, were also evaluated.

MATERIALS AND METHODS

Yeast cultures

Yarrowia lipolytica and *Debaryomyces hansenii* were selected as starter cultures for the inoculation of yogurt samples. These strains were isolated from commercial yogurt and identified according to the conventional methods described by Kreger- van Rij (1984). The yeast cultures were maintained on slants of YM (yeast-extract, malt-extract) agar (Wickerham, 1951) and stored at 5°C. When required, the yeast cultures were activated by transferring to YM

agar plates, incubated at 25°C for 48 h and checked for purity before use in experiments.

Yogurt samples and yogurt preparation

Growth experiments were conducted in plain (natural) AB-yogurt and fruit flavoured AB-yogurt obtained from a local yogurt manufacturer immediately after processing and cooling to 4°C. The commercial AB-yogurts were manufactured using freeze-dried direct vat set (DVS) ABT (*Streptococcus thermophilus, Lactobacillus acidophilus* and *Bifidobacterium bifidum*) starter cultures from Chr. Hansen's (Denmark). Retail samples (500 ml's size) were transported in a cooler box on ice and refrigerated upon arrival in the laboratory. The fresh yogurt samples were inoculated with yeast cultures within 4 h after receipt.

In addition, a commercial yogurt mix prior to processing, immediately after LAB starter inoculation (2 x 1L) and cooled to < 7°C, and fruit pulp concentrate from the same manufacturer were also obtained and transported to the laboratory on ice.

Inoculation

Yarrowia lipolytica and *Debaryomyces hansenii* cultures were cultured in YNB (yeast nitrogen base, Oxoid, Basingstoke) broth to late stationary phase (48h) after which the yeast cells were harvested by centrifugation (14 000 *g*, 4°C for 10 minutes). The supernatant was decanted and the yeast cells weighed. Appropriate aliquots of sterile water were added to the cells to obtain different concentrations of yeast cells. A Klett Sumerson was used to predetermine the number of yeast cells per ml suspension of each percentage yeast cells. Based on these data we were able to predetermine the percentage (w/v) inoculation

size necessary to obtain an initial inoculation level of approximately 10^6 cells/ml in the experimental dairy products.

The manufactured set yogurt samples in the retail cups was each aseptically inoculated simultaneously with the yeast species. Two samples of plain AB-yogurt were each inoculated with 2% (weight cells per volume product) of the *Yarrowia lipolytica* culture, and two inoculated with *Debaryomyces hansenii* yeast culture, and uniformly mixed. Fruit AB-yogurts were inoculated similarly. Plain and fruit yogurt control samples (not inoculated) were used to determine possible natural yeast contamination in the yogurt. The inoculated dairy samples and controls were stored at 5°C.

The yogurt milk mix was heated to 32°C before inoculating with 1% *Debaryomyces hansenii* yeast culture, and then uniformly mixed. Incubation proceeded at 32°C until the pH reached 4.6 after 10 – 12 h (according to the manufacturer's instructions), followed by cooling to 5°C overnight. The final pH after cooling ranged from 4.1 – 4.3. One part of this set yogurt was flavored and sweetened with fruit pulp concentrate to 15 – 16°Brix. The finished plain and fruit yogurts were immediately sampled microbiologically (day 1) and then stored at 5°C for 30 days.

Microbiological analyses

Analyses were performed immediately after inoculation (day 1), thereafter every 24 h for the next 2 days, consecutively at three-day intervals until the 15th day, and then at five-day intervals until the 30th day (expiry date). In total 10 analyses were conducted during storage. Control samples were analysed on a regular basis from day 1 to day 30.

Yogurt samples (5ml) were aseptically withdrawn and diluted (1:10) in Callichia et al. (1993) resuspension medium (CRM) and shaken vigorously. CRM

contains L-cystein HCL, which lowers the redox potential, for improved recovery of anaerobic *Bifidobacteria.* Subsequent serial dilutions were prepared as required in CRM. The time elapsing between the preparation of the dilutions and the pouring never exceeded 15 min as recommended (Callichia et al., 1993).

Fruit pulp was analysed to determine possible natural yeast contamination in the yogurt by spread plating aliquots on Chloramphenicol agar (Merck).

Selective media

The yeast counts were enumerated on Chloramphenical agar (Merck) using the spread plate technique. Plates were incubated aerobically at 25°C for 5 days.

M17 agar (Tergazhi & Sandine, 1975), NNLP agar (Laroia & Martin, 1991) and Maltose-MRS agar (M-MRS) (Hull & Roberts, 1984) were prepared as described in Chapter 3, and used for differential enumeration of *S. thermophilus, B. bifidum* and *L. acidophilus* respectively. Pour plate technique was used. The solidified plates of M-MRS agar and NNLP agar were incubated anaerobically in anaerobic jars (Oxoid) at 37°C for 4 and 5 days respectively. The anaerobic atmosphere was generated by Anaerocult A blocks (Merck). An anaerobic indicator (Merck) was included in the jar. M17 agar plates and Ac-MRS plates were incubated aerobically at 37°C for 3 days.

pH determination

The pH values of the yogurt samples were measured on every sampling occasion. Values were measured at ~ 15°C using a HI 9321 Microprocessor pH meter (HANNA Instruments) after calibrating with fresh pH 4.0. and 7.0 standard buffers.

RESULTS AND DISCUSSION

Growth and survival – yeast inoculation after manufacturing

The viable counts of the lactic acid bacteria and yeasts in fruit and plain yogurt are presented in Figs. 8.1a and b, and Figs. 8.2a and b. For all the yogurt samples artificially inoculated with either *D. hansenii* or *Y. lipolytica* at moderate-level ($10^5 - 10^6$), the trend of all microbial counts was very similar at 5°C storage over 30 days.

The initial level of *L. acidophilus* at ~8.5 log $_{10}$ cfu/ml remained stable in all the yogurt products until day 15 and then decreased rapidly through 4 log cycles to a final level of ~4 log $_{10}$ cfu/ml in three of the four yeast inoculated products; while decreasing through 2 log cycles in fruit yogurt inoculated with *D. hansenii* to 6.68 log $_{10}$ cfu/ml. Canganella et al. (1998) also found that the number of lactobacilli in yeast-inoculated yogurt (*Kluyveromyces marxianus* = $10^5 - 10^6$ cfu/ml) was stable for 2-3 weeks but then diminished rapidly. The loss of viability in the number of lactobacilli was attributed to the growth of yeasts, since in the control without yeasts, a limited decline in the number of lactobacilli was observed. This indicates a possible antagonistic action from the yeasts against *L.acidophilus*. However, *L. acidophilus* growth was stimulated in the presence of the yeasts (*Kluyveromyces marxianus* and *Candida pseudotropicalis*) in acidophilus milk (Subramanian and Shankar, 1983).

Consequently, due to the decline in viable numbers, the relative level of *L. acidophilus* did not meet the required minimum of 10^6 probiotic cells per ml in the yogurt until the of the storage period. In a previous survival study (of the same brand), *L.acidophilus* cell counts decreased in total by only 1.21 log units during storage at 5°C over a period of 31 days compared with the decrease of 4

and 2 log units in the yeast-inoculated yogurts in this study (Lourens et al., 2000). Therefore, the effect of dairy associated yeasts on lactobacilli species should be further investigated.

The initial *B. bifidum* count of the commercial yogurt directly after processing was less than 10^6 cells/ml, thus the required minimum probiotic levels could not be maintained during manufacture. A decreasing tendency in viable *B. bifidum* levels was obtained in fruit yogurt and *Y. lipolytica* (0.5 log), plain yogurt and *Y. lipolytica,* as well as the plain yogurt with *D. hansenii* (>1 log). In contrast, the relative *B. bifidum* population in fruit yogurt inoculated with *D. hansenii* increased with 0.4 log units until day 15 and then decreased to the same initial level at the end of storage (4.89 $\log_{10}$ cfu/ml). *B. bifidum* counts in the same brand of yogurt (not inoculated with yeast) showed a decline from 5.2 to 4.9 $\log_{10}$ cfu/ml previously (Lourens & Viljoen, 2000). In the latter study the initial *B. bifidum* counts in all the yogurt products were also less than 10^6 cfu/ml. Use of, and maintaining a higher cell inoculum by the manufacturers during manufacture, will ensure a higher viable bifidobacteria cell count at the end of incubation, and the survival of the probiotic bacterium during refrigerated storage until consumption (Samona & Robinson, 1994).

S. thermophilus grew well in all the yogurts; counts of streptococci remained above 10^8 cfu/ml from the second day until the end of storage. Viable counts increased in both the inoculated plain yogurts (1 log cycle) and the fruit yogurt with *D. hansenii* (0.5 log units), whereas the level of *S. thermophilus* remained stable in the fruit yogurt inoculated with *Y. lipolytica.* The increase in viable counts of the streptococci is in contrast with counts usually observed in traditional yogurt. According to Hamann and Marth (1984), a typical survival curve for the yogurt microorganisms is represented by an initial increase, reaching a maximum, followed by a decrease as observed in the control curve

(results not shown). Apparently, the yeasts encouraged better survival of the streptococci.

Although, the viable cell densities of *D. hansenii* and *Y. lipolytica* decreased during storage in plain yogurt and fruit yogurt, the yeasts species survived sufficiently during a 30 day period of growth in yogurt to primarily encourage the growth of the streptococci. Consequently, due to the enhanced survival of the streptococci, an enhanced decline in pH was obtained (Table 8.1) in . The growth and survival of the yeasts were unaffected by the type of yogurt. The proteolytic and lipolytic activities, and the utilization of organic acids contribute to the growth of *Y. lipolytica* and *D. hansenii* (Ratledge & Tan, 1990; Ogrydziak, 1993) since these yeast species are unable to utilize lactose as carbon and energy source (Kreger-van Rij, 1984). However, the faster growing psychrotropic bacteria restricted their growth (Cousin, 1982; Roostita & Fleet, 1996).

The main carbohydrate in the fruit yogurt is sucrose. According to Barnett et al. (1990), *Y. lipolytica* lacks the ability to utilise sucrose, while growth of *D. hansenii* in fruit yogurt can be ascribed to partial utilisation of sucrose.

No wild yeasts were initially present in the control yogurts without addition of yeasts as starter cultures. After 30 days, 280 cfu yeasts/ml were detected in fruit yogurt and 2 moulds/ml in the plain yogurt. The fruit pulp concentrate was clear from yeast contamination prior to processing. (Data not shown).

Changes in pH in yogurts during refrigerated storage

The changes in pH values during a 30-day storage at 5°C of the yogurts inoculated with yeasts and the controls without the addition of yeasts are

shown in Table 8.1. All the yogurts showed a decrease in pH. The initial pH values of plain and fruit yogurt were 4.31 and 4.26 respectively when fresh at day 1. Interesting to note, however, is the higher initial pH of this yogurt compared with the initial pH of experimental yogurt in previous studies (Lourens et al., 2000). This corresponds with the fact that the manufacturers now make use of an ABT starter where *L.bulgaricus* is excluded. *L. bulgaricus* produces hydrogen peroxide which is antagonistic against *L.acidophilus*, and lactic acid after fermentation and during storage at refrigerated temperature which causes 'post-production acidification'. Exclusion of *L. bulgaricus* in ABT-yogurt resulted in maintaining high populations of *L. acidophilus* and *B. bifidum* (Kim et al., 1993; Rybka, 1994).

Although a gradual decrease in the pH of all the yogurts was observed throughout the storage period, no excessive decline in the pH of the different yeast inoculated dairy products was observed compared with previous experimental findings. This may be due to the exclusion of *L. bulgaricus*, but also to the utilization of organic acids and synergistic peptonization of proteins by *D. hansenii* and *Y. lipolytica* (Soulides, 1955; Fleet, 1990).

In fruit yogurt with the addition of *Y. lipolytica* the nett decrease (0.11) in pH values was significantly less when compared with control fruit yogurt (0.18) stored at 5°C with only lactic acid bacteria present (Rosenthal, 1991). The nett decrease (0.18) in pH values of plain yogurt and *Y. lipolytica,* and of the control plain yogurt was the same. In the case where *D. hansenii* was inoculated into yogurt, the decrease in pH values of fruit yogurt (0.27) and plain yogurt (0.22) was more than the control yogurts (0.18 – 0.19). The moderate difference in pH values may be attributed to a faster declining curve of *D. hansenii* compared to *Y. lipolytica* which consequently also resulted in less utilization of organic acids.

Growth and survival – inoculation before fermentation with starter cultures

When a culture of *D.* hansenii was added as part of the starter culture before yogurt manufacture at the time of starter addition, the yeasts survived significantly better (Figs. 8.3 a and b). *D.hansenii* inoculated in plain yogurt, multiplied almost 3 fold during manufacture and reached a maximum viability count exceeding 10^7 cfu/ml after 30 days. When the same culture was inoculated in fruit yogurt, however, a moderate decrease (0.6 log units) in viability was observed. These data showed a marked increase in viability of *D. hansenii* cells compared with results obtained when the culture was added after manufacture resulting in decreased viability of 2.5 log units in fruit yogurt and 2.4 log units in plain yogurt. Onset of loss of viability of *D. hansenii* in fruit yogurt occurred after 2 days of manufacture and progressed at a similar rate until the end at 30 days. *D. hansenii* inoculated in plain yogurt, gradually increased from 2 days after manufacture until day 30. The significant increase in the number of cells of the yeast in plain yogurt was unexpected, since limited carbohydrates for yeast growth is available.

Improved survival of bifidobacteria was also observed in plain yogurt (0.2 log unit decrease) with *D. hansenii* added before manufacture compared to the addition after manufacture (1 log unit decrease). In contrast, bifidobacteria survived better in fruit yogurt with *D. hansenii* addition after manufacture (remained at the same level). Bifidobacteria present in the fruit yogurt inoculated with the yeast culture before manufacture increased with 0.5 log units until day 11, followed by a decrease of 2 log cycles to day 30.

L. acidophilus decreased significantly in plain yogurt with *D. hansenii* added before manufacture (3.2 log) as was also observed in yogurt with yeast added

after manufacture. *L. acidophilus* survived better in fruit yogurt inoculated with *D. hansenii* before manufacture (decrease of 0.3 log $_{10}$ cfu/ml).

In contrast with the increased numbers of *S thermophilus* in yogurt inoculated with *D.hansenii* species after manufacture, declining curves were obtained when the yeast was inoculated before manufacture. *S. thermophilus* counts increased for 15 days after manufacture in fruit yogurt, but rapidly decreased beyond to levels less than 10^7 cfu/ml after 30 days. In plain yogurt, its numbers remained stable until day 25, followed by a decrease of 1 log unit until day 30. The decline in the numbers of streptococci at the end of refrigerated storage, resulted in a moderately higher pH at the end of storage (results not shown).

Sensory evaluation

No alcohol was produced when either *D. hansenii* or *Y. lipolytica* was grown in any of the dairy products in a previous study (Lourens and Viljoen, 2000). According to Barnett et al. (1990), *Y. lipolytica* is a non-fermenting yeast species, whereas *D. hansenii* shows only weak fermentation. However, in the fruit yogurt inoculated with *Y. lipolytica*, minor gas was produced possibly due to the utilization of sucrose by contaminating yeasts (280 cfu/ml on day 30).

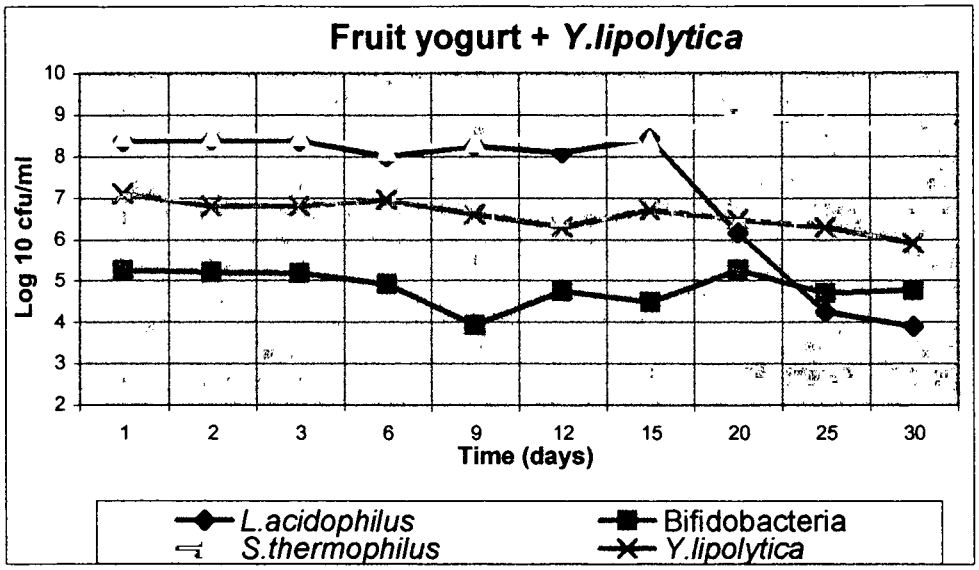
The organoleptic and textural properties of the different products were evaluated. A rancid flavor was detected in the plain and fruit yogurt inoculated with *Y. lipolytica* possibly due to strong lipolysis by this yeast (Fleet, 1990). These yogurts also had a fluidy consistency due to proteolysis. The yogurt products inoculated with *D. hansenii* had no difference in taste or consistency compared to the yogurt controls even at the high yeast inoculum levels.

The possibility of producing yogurt with these characteristics is not far fetched since products such as kefir, koumiss and laban are all acidic, slightly alcoholic, liquid to semi liquid and effervescent, and consumed as a beverage (Oberman, 1985).

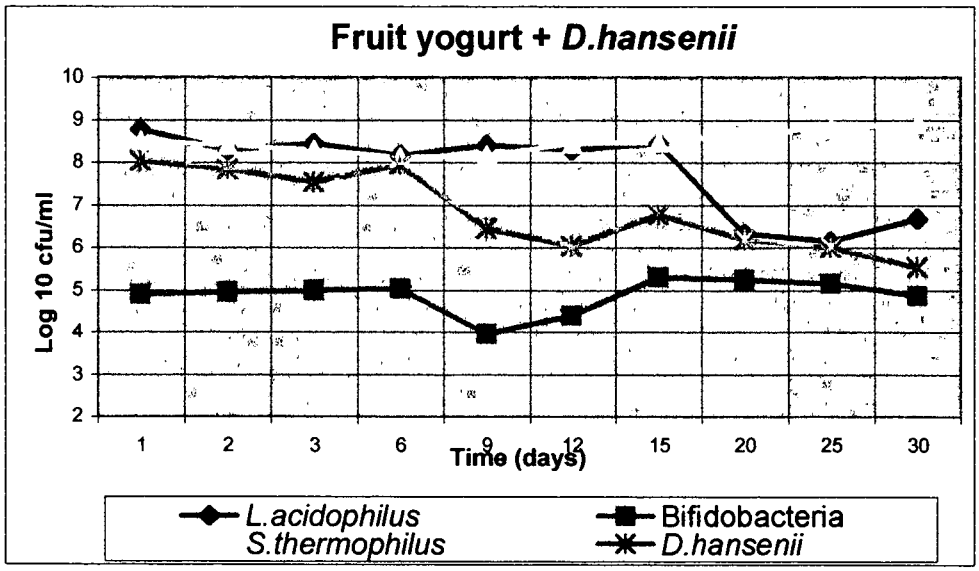
CONCLUSIONS

The yeast isolates, *D. hansenii* and *Y. lipolytica* were able to survive in the yogurts, utilising the yogurt constituents as growth substrates during the 30 day storage period, and sufficiently contribute to the retention or enhancing of the pH values.

Fruit yogurt, inoculated with *D. hansenii* is the most promising yeast-inoculated yogurt product, resulting in the stabilization of bifidobacteria counts during the refrigerated storage period. The decrease in the viability of *L. acidophilus* cells proved to be a constraint, and further investigation is necessary. The elevated number of streptococci at the end of the storage period was unexpected, but the enhanced numbers may contribute to improved taste. Even at the high yeast inoculum level, the overall quality of the yogurt was still acceptable and had a pleasant taste compared to the control yogurt after 30 days storage. Inoculation of yogurt with yeast to improve viability of probiotic bacteria in bio-yogurt, therefore, seems promising.

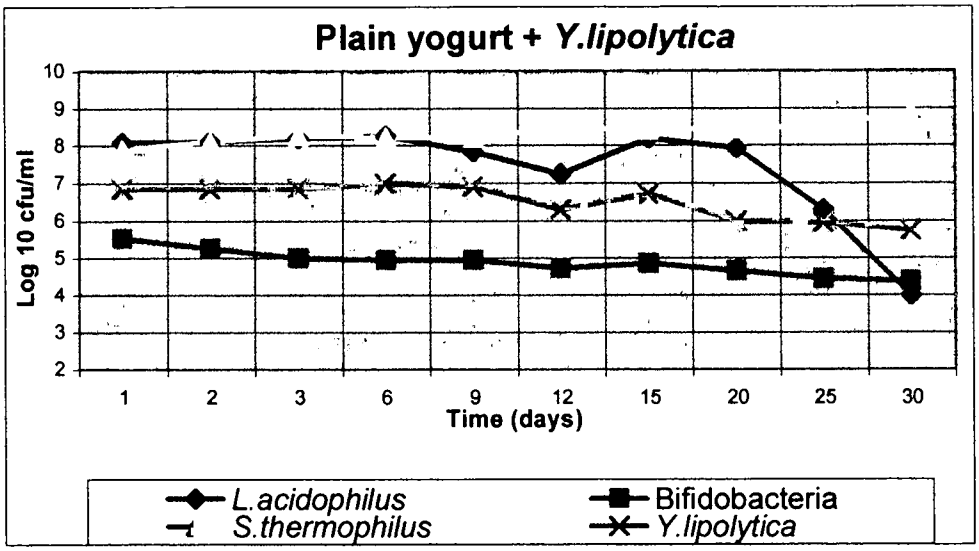


a)

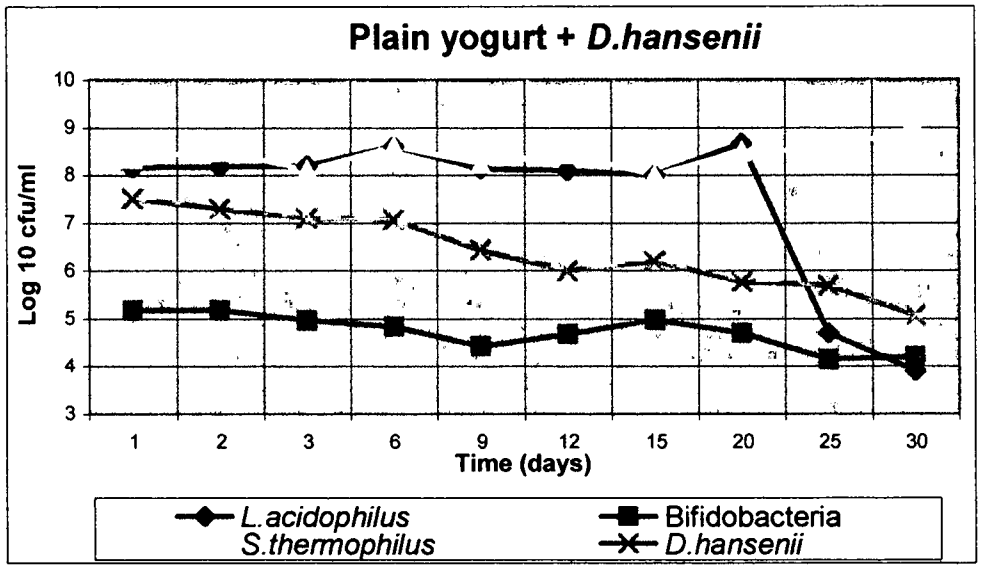


b)

Figs. 8.1a-b Growth and survival of LAB and a) *Y. lipolytica* b) and *D. hansenii*, inoculated after manufacture, in fruit yogurt at 5°C during 30 days storage.

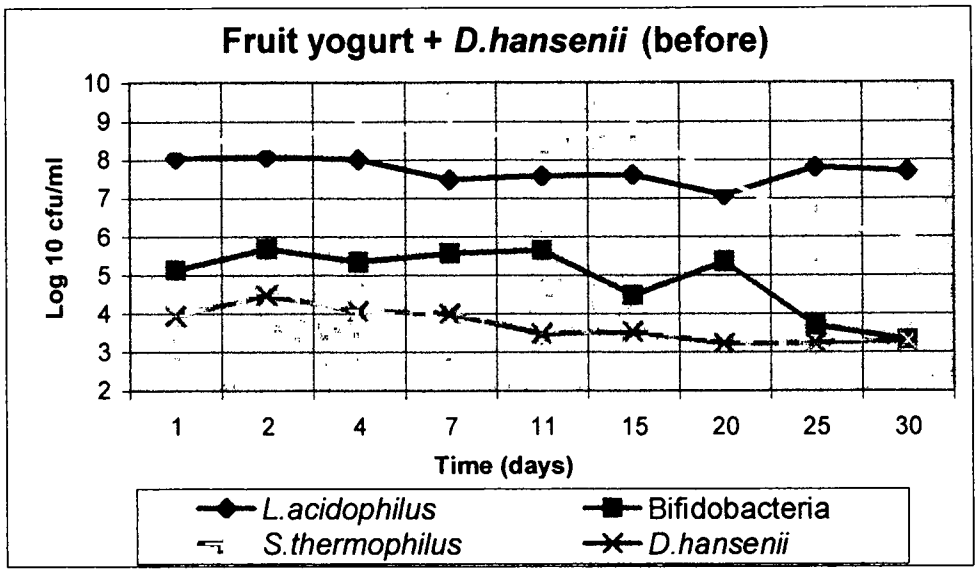


c)

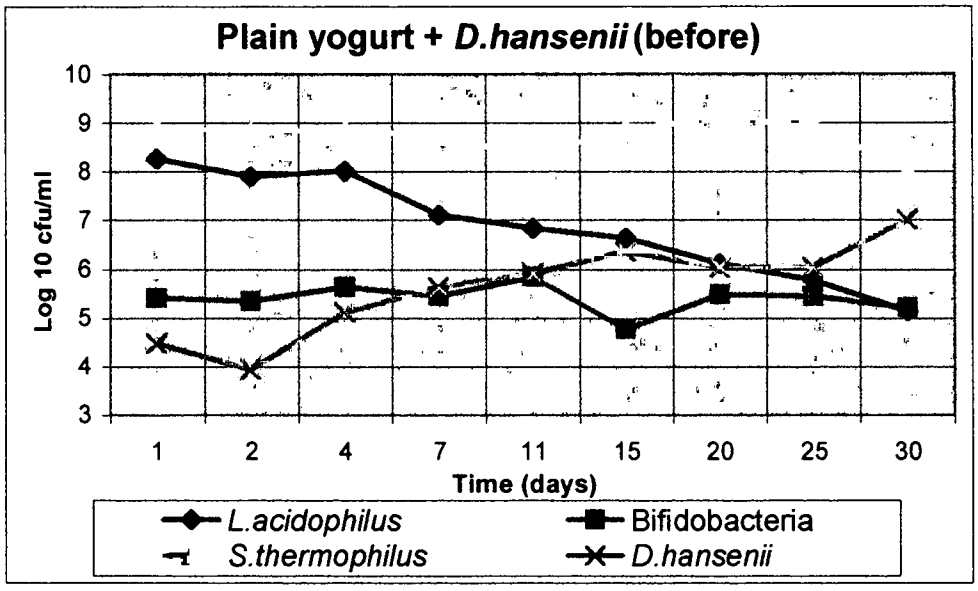


d)

Figs. 8.2a-b. Growth and survival of LAB, and a) *Y. lipolytica* and b) *D. hansenii*, inoculated after manufacture, in fruit yogurt at 5°C during 30 days storage.



a)



b)

Figs. 8.3a-b. Growth and survival of LAB and *D. hansenii*, inoculated before fermentation, into a) fruit yogurt and b) plain yogurt at 5°C during 30 days storage.

Table 8.1. Changes in pH of dairy product samples inoculated with *D. hansenii* (D.h) or *Y. lipolytica* (Y.l) after manufacture and of their controls during storage.

	Fruit yogurt			***Plain yogurt***		
	Control	**+ Y.l**	**+ D.h**	**Control**	**+ Y.l**	**+ D.h**
1	4.26	4.27	4.26	4.31	4.33	4.34
2	4.23	4.27	4.25	4.32	4.29	4.30
3	4.22	4.22	4.21	4.31	4.29	4.26
6	4.23	4.20	4.20	4.29	4.26	4.25
9	4.16	4.20	4.21	4.25	4.23	4.26
12	4.17	4.18	4.18	4.24	4.23	4.21
15	4.19	4.21	4.18	4.29	4.25	4.22
20	4.06	4.07	4.05	4.13	4.12	4.09
25	4.04	4.06	4.02	4.12	4.10	4.07
30	4.05	4.10	4.05	4.14	4.13	4.09
36	4.07	4.16	3.99	4.13	4.15	4.12
D	**0.19**	**0.11**	**0.27**	**0.18**	**0.18**	**0.22**

D difference between initial and final level of pH

REFERENCES

Bankole, M.O. & Okagbue, R.N. (1992) Properties of "Nono", a Nigerian Fermented Milk Food. *Ecology of Food and Nutrition.* **27**, 145 – 149.

Borregaard, E., Arneborg, N. (1998) Interactions between *Lactococcus lactis* subs. *lactis* and *Issatchenkia orientalis* at milk fermentation. *Food Technology and Biotechnology.* **36**, 75- 78.

Callichia, M.L., Wang, C.I.E., Nomura, T., Yotsuzuka, F. and Osato, D.W. (1993) Selective enumeration of *Bifidobacterium bifidum*, *Enterococcus faecium*, and streptomycin-resistant *Lactobacillus acidophilus* from a mixed probiotic product. *Journal of Food Protection.* **56** (11), 954-957.

Davenport, R.R., (1980) Cold-tolerant yeasts and yeast-like organisms. In: Skinner, R.A., Passmore, S.M., Davenport, R.R. (eds.), Biology and Activities of Yeasts. Academic Press, London, pp. 215 – 228.

Deàk, T. and Beuchat, L.R. (1996) Handbook of Food Spoilage. New York, CRC Press.

Fernandez Del Pozo, B., Gaya, P. Medina, M., Rodriguez-Martin, M.A. and Nunez, M (1988) Changes in chemical and rheological characteristics of La Serena ewes' milk cheese during ripening. Journal of Dairy Research. **55**, 457 – 465.

Fleet, G. (1990) Yeasts in dairy products – A review. *Journal of Applied Bacteriology.* **68**, 199-211.

Fleet, G.H., Mian, M.A. (1987) The occurrence and growth of yeasts in dairy products. *International Journal of Food Microbiology.* **4**, 145-155.

Giudici, P., Masini, G. & Caggia, C. (1996) The role of galactose fermenting yeast in plain yogurt spoilage. *Annali di Microbiologia ed Enzimologia.* **46**, 11-19.

Gomes, A.M.P. and Malcata, F.X. (1999) *Bifidobacterium* spp. and *Lactobacillus acidophilus* : biological, biochemical and therapeutical properties relevant for use as probiotics. *Trends in Food Science & Technology* **10**, 139 – 157.

Goodenough, E.L. & Klein, D.H. (1976) Qualitative and quantitative changes in carbohydrates during the manufacture of yogurt. *Journal of Dairy Science.* **59,** 45-47.

Graham, V.E. (1943) The maintenance of viability in *Lactobacillus bulgaricus* cultures by growth in association with certain yeasts. *Journal of Bacteriology.* **45**, 51.

Huis in't Veld, J.H.J. and Havenaar, R. (1991) *Journal of Chemical Technology and Biotechnology.* **51**, 562-567.

Hull, R.R. and Roberts, A.V. (1984) Differential enumeration of *Lactobacillus acidophilus* in yogurt. *The Australian Journal of Dairy Technology.* **39**, 160-163.

Hull, R.R., Roberts, A.V., Mayes, J.J. (1984) Survival of *Lactobacullus acidophilus* in yogurt. *Australian Journal of Dairy Technology.* **39**, 164 – 166.

Kreger van Rij, N.J.W. (Ed.), 1984. In: The Yeasts – A Taxonomic study, 3rd ed. Elsevier, Amsterdam.

Kurmann, J.A. and Rasic, J.L. (1991) The health potential of products containing bifidobacteria. In: *Therapeutic Properties of Fermented Milks.* Edited by R. K. Robinson. Elsevier Applied Sciences, London, 117-158.

Lang, F., Lang, A. (1975) Acidophilus milk products: little known cultured milks of great potential. *The Milk Industry.* **77**, 4-6.

Lankaputhra, W.E.V. and Shah, N.P. (1995a) Investigation of factors affecting viability of *Lactobacillus acidophilus* and *Bifidobacterium* in yogurt. 24th International Dairy Congress. Melbourne, Australia, Sept. 18 – 22, 1994.

Lankaputhra, W.E.V. and Shah, N.P. (1995b) Survival of *Lactobacillus acidophilus* and *Bifidobacterium* species in the presence of acid and bile salts. *Cultured Dairy Products Journal.* **30**(3), 113-118.

Laroia, S. and Martin, J.H. (1991) Effect of pH on Survival of *Bifidobacterium bifidum* and *Lactobacillus acidophilus* in Frozen Fermented Dairy Desserts. *Cultured Dairy Products Journal.* **Nov.**, 13-21.

Lemaresquier, H. (1987) Inter-relationships between strains of *Saccharomyces cerevisiae* from the Champagne area and lactic acid bacteria. *Letters in Applied Microbiology.* **4**, 91 – 94.

Leroi, F. & Pidoux, M. (1993) Detection of interactions between yeasts and lactic acid bacteria isolated form sugary kefir grains. *Journal of Applied Bacteriology.* **74**, 48 – 53.

Leroi, F. & Pidoux, M. (1993) Characterization of interactions between *Lactobacillus hilgardii* and *Saccharomyces florentinus* isolated form sugary kefir grains. *Journal of Applied Bacteriology.* **74**, 54 - 60.

Lourens, A and Viljoen, B.C. (2000) Growth and survival of dairy associated yeasts in yogurt and yogurt-related products. *International Journal of Food Microbiology.* **In print**.

Oberman, H. (1985) Fermented milks. In: *Microbiology of fermented foods.* Edited by Wood, B.J.B. Volume 1.Elsevier Applied Science Publishers. London and New York, pp. 167-186.

Ogrydziak, D.M. (1993) Yeast extracellular proteases. *Crit. Rev. Biotechnology.* **13**, 1-55.

Ratledge, C., Tan, K.H. (1990) Oils and fats: Production, degradation and utilisation by yeasts. In: Verachert, H., De Mot, R. (Ed.), Yeast Biotechnology and Biocatalysis, Marcel Dekker Inc., New York, pp. 225-245.

Robinson, R.K., 1987. Survival of *Lactobacillus acidophilus* in Fermented Products. *South African Journal of Dairy Science.* **19**(1), 25-27.

Roostita, R. & Fleet, G.H. (1996) The occurrence and growth of yeasts in Camembert and Blue-veined cheeses. *International Journal of Food Microbiology.* **28**, 393 –404.

Rybka, S., Kailasapathy, K., (1995) The survival of culture bacteria in fresh and freeze-dried AB yoghurts. *Australian Journal of Dairy Technology.* **50**(2) 51-57.

Seiler, H & Busse, M. (1990) The yeasts of cheese brines. *International Journal of Food Microbiology.* **11**, 289 – 304.

Shah, N. and Jelen, P. (1990) Survival of Lactic Acid Bacteria and Their Lactases under Acidic Conditions. *Journal of Food Science.* **55** (2), 506-509.

Shah, N.P.; Lankaputhra, W.E.V.; Britz, M.L. and Kyle, W.S.A. (1995) Survival of *Lactobacillus acidophilus* and *Bifidobacterium bifidum* in commercial yoghurt during refrigerated storage. *International Dairy Journal.* **5,** 515-521.

Soulides, D.A. (1955) A synergism between yogurt bacteria and yeasts and the effect of their association upon the viability of the bacteria. *Applied Microbiology.* **3**, 129-131.

Subramanian, P., Shankar, P.A. (1985) Commensalistic interaction between *L. acidophilus* and lactose-fermenting yeast in the preparation of acidophilus-yeast. *Cultured dairy product Journal* . **Nov**, 17 - 26.

Terzaghi, B.E. and Sandine, W.E. (1975) Improved medium for lactic streptococci and their bacteriophages. *Applied Microbiology.* **29**(6), 807-813.

Welthagen, J.J. & Viljoen, B.C. (1999) The isolation and identification of yeasts

obtained during the manufacture and ripening of Cheddar cheese *Food Microbiology*. **16**, 63 – 73.

Wickerham, L.J. (1951) Taxonomy of yeasts. *US. Department of Agricultural Technology Bulletin* 1029.

CHAPTER 9

ENHANCEMENT OF *BIFIDOBACTERIA* BY NEOKESTOSE IN BIO-YOGURT.

ABSTRACT

Possible enhancement of viability of *Bifidobacteria* was assessed in commercial AB-yogurt fortified with 1%, 2% and 3% neokestose. The growth of *L. acidophilus* and *S. thermophilus* was also monitored over the storage period of 30 days at 5°C. Utilization of neokestose at the end of the storage period were assessed using high performance liquid chromatography (HPLC). The viable bifidobacteria counts remained significantly higher in all the yogurts when compared to traditional yogurts without the addition of neokestose. *L. acidophilus* reduction in viable counts did not exceed a 30% reduction; therefore neokestose also had a better survival effect on *L. acidophilus*. The addition of neokestose had no affect on the survival of *S. thermophilus*.

INTRODUCTION

Due to the beneficial activities of Bifidobacterium, interest enhanced in the inclusion of the bacterium in a variety of products, such as bio-yogurt containing AB-cultures (*L. acidophilus* and bifidobacteria) are now produced and consumed worldwide. Consumption of such probiotic products is considered to impart many potential health benefits. These include control of intestinal infections, stabilizing intestinal microflora, control of serum cholesterol level, prevention of cancer and enhanced immunity (Laroia and Martin, 1990; Modler et al., 1990).

However, despite the importance of viability of the probiotic bacteria, surveys have shown that bifidobacteria survive poorly in yogurt which results in insufficient therapeutic levels ($>10^6$ cells/ml) of the probiotic organism in the bio-yogurt (Rybka, 1994; Tamime et al., 1995; Rybka and Kailasapathy, 1995; Shah et al., 1995; Micanel et al., 1997). Many attempts have, therefore, been made to enhance growth and survival of bifidobacteria in dairy products such as the addition of growth factors (Modler, 1994; Kailasapathy and Rybka, 1997; Dave and Shah, 1998), manipulating the manufacturing and storage conditions (Gardini et al., 1999), better strain and starter selection (Kneifel et al., 1993; Dave and Shah, 1997; Collins et al., 1998), improved starter culture technology (Foschino et al., 1996; Gardiner et al., 2000), co-culturing (Samona and Robinson, 1994), lowering redox potential (Klaver et al., 1993), micro-encapsulation (Modler et al., 1990), and addition of prebiotics (Young, 1996). When probiotics and prebiotics are used in combination it is called synbiotics (Fooks et al., 1999). In the latter case, the live probiotic bacteria growth are stimulated by the inclusion of a specific substrate for growth, for example fructo-oligosaccharides (FOS) with *Bifidobacterium* strains. Consequently, the addition of prebiotics should encourage the growth and survival of the probiotic bacteria, due to a more readily available and specific substrate for utilisation, as well as the individual advantages that each should offer (Fooks et al., 1999).

Interest in fructo-oligosaccharides has increased in recent years due to its successful application as prebiotics to enhance the population of *Bifidobacterium* sp. in the large intestine (Wang and Gibson, 1993; Bouchnic et al., 1996; Campbell et al., 1997; Kaplan and Hutkins, 2000). It is believed that bifidobacteria have β-fructosidase acitivity selective for β-1-2-glycosidic bonds present in fructo-oligosaccharides (McKellar and Modler, 1989). Inclusion of these non-nutritive sweeteners in the diet has shown to encourage the growth of bifidobacteria (Hidaka et al., 1986). Fermented milk products containing probiotics with added galacto-oligosaccharides are commercially available in Japan as well as in Europe (Sako et al., 1999). "Fyos" is a typical fermented milk drink combining the probiotic *Lactobacillus casei* with the prebiotic inulin and oligofructose obtained from chicory (Young, 1996).

Consequently, the objective of this investigation was to study the effects of a prebiotic on the growth and survival of bifidobacteria in bio-yogurt. The fructo-oligosaccharide, neokestose, was produced in our laboratory during growth of *Phaffia rhodozyma* on sucrose (Kilian, et al., 1996) and it was shown to exhibit potential bifidogenic effects (Kritzinger, 1999) Neokestose is a trisaccharide consisting of one glucose molecule and two fructose molecules (Kilian, et al., 1996). Commercial plain and fruit AB-yogurt were fortified with 1%, 2% and 3% neokestose, and growth and survival of bifidobacteria as well as *L.acidophilus* and *S. thermophilus* were monitored during storage at 4°C over 30 days.

MATERIALS AND METHODS

Yogurt sample preparation

Commercial plain and fruit bio-yogurt, manufactured using an ABT DVS (direct vat set) starter culture (*Streptococcus thermophilus, Lactobacillus acidophilus* and *Bifidobacterium bifidum*) from Chr. Hansen's, was obtained from a local yogurt manufacturer immediately after production. Samples were

transported to the laboratory in 500 ml retail cups in a cooler box on ice. Upon arrival in the laboratory all the dairy samples were aseptically dispensed into sterilised 1L Schott bottles. Samples were individually fortified with either 1%, 2% or 5% neokestose in crystal form, uniformly mixed and stored at 5°C. Experiments were conducted in duplicate.

Microbiological analyses

Analyses were performed immediately after the addition of neokestose (day 1), thereafter every 24 h for the next 2 days, consecutively at three-day intervals until the 15th day, and then at five-day intervals until the 30th day (expiry date). In total 10 analyses were conducted during storage.

Yogurt samples (5 ml) were aseptically withdrawn and diluted 1:10 in Callichia resuspension medium (CRM) (Callichia et al., 1993) and shaken vigorously. CRM contains L-cystein HCL, which lowers the redox potential, for improved recovery of anaerobic *Bifidobacteria.* Subsequent serial dilutions were prepared as required in CRM. The time elapsing between the preparation of the dilutions and the pouring never exceeded 15 min as recommended (Callichia et al., 1993).

Recording and expression of results

Plates with colonies between 25 and 300 were counted with the aid of a colony counter and recorded as $\log_{10}$ colony forming units (cfu) per ml yogurt. The results presented in Figs. 9.1a – c and Figs. 9.2a - c are the means of duplicate yogurt samples, each again sampled in duplicate.

Selective media

M17 agar (Tergazhi and Sandine, 1975), NNLP agar (Laroia and Martin, 1991) and Maltose-MRS agar (M-MRS) (Hull and Roberts, 1984) were prepared as

described, and used for differential enumeration of *S. thermophilus*, *B. bifidum* and *L. acidophilus* respectively. The pour plate technique was used. The solidified plates of M-MRS agar and NNLP agar were incubated anaerobically in anaerobic jars (Oxoid, Basingstoke) at 37°C for 4 and 5 days respectively. The anaerobic atmosphere was generated by Anaerocult A blocks (Merck, Darmstadt). An anaerobic indicator (Merck) was included in the jar. M17 agar plates were incubated aerobically at 37°C for 3 days.

RESULTS AND DISCUSSION

Changes in the viable counts of bifidobacteria, *L. acidophilus* and *S. thermophilus* during storage of plain yogurt and fruit yogurt at 5C°, fortified with 3 different levels of neokestose, are presented in Figs. 9.1a – c and Figs 9.2a – c respectively. Traditionally, the yogurt manufacturer also included *L. delbrueckii* spp. *bulgaricus* as part of the starter culture, but due to its enhanced acid production causing "post production acidification" and as a consequence the affect on the growth and viability of *L. acidophilus* and *B. bifidum* (Kim et al., 1993; Rybka, 1994; Lourens et al., 2000a) it is currently omitted to assure better survival ratios of the probiotic microorganisms. The application of ABT starter cultures, with the exclusion of *L. bulgaricus*, resulted in moderately enhanced pH values at the end of processing. In general, the pH values never declined to values lower than 4.1 (results not shown) after being initially present at values of 4.2 – 4.3.

Plain yogurt with neokestose

Changes in the viable counts of bifidobacteria, fortified with 1, 2 and 3% neokestose, in plain yogurt are given in Fig. 9.1a. In all three cases, the initial counts of bifidobacteria directly after processing exceeded 10^5 cfu/ml. Yogurts fortified with 1 and 3% neokestose showed an initial increase in the number of bifidobacteria followed by a rapid decline in viable numbers after four days for the 3% yogurt whereas a gradual decline was observed within the yogurts

fortified with 1%. Increased viable counts of bifidobacteria, however, were obtained in the yogurts with 3% neokestose after seven days reaching a maximum after 25 days. Yogurts with 5% neokestose, showed a gradual decrease in numbers from day 1. The viable *B. bifidum* counts in all the plain yogurts, in general decreased with at least one log unit from day 1 to day 30, mainly attributed to the rapid decline in numbers after the 25th day. Despite the decrease after 25 days, the viable bifidobacteria counts remained significantly higher in all the yogurts when compared to traditional yogurts without the addition of neokestose (Lourens et al., 2000b; Shah et al., 1995).

Fig. 9.1b shows the viable counts of *L. acidophilus* in the plain yogurt fortified with different ratios of neokestose and monitored for 30 days. All the yogurts contained viable counts of *L. acidophilus exceeding* 10^7 cfu/g initially. The organisms survived well in all the yogurts, showing an increase in viable numbers within two days. *L. acidophilus* counts in yogurt fortified with 1% neokestose continued to increase gradually for about two weeks followed by a sharp increase after 15 days reaching a maximum count exceeding 7.8 log units after 20 days. The sharp increase was followed by a rapid decline in viable *L. acidophilus* numbers after three weeks. A similar trend was also observed in yogurt fortified with 3% neokestose. In contrast, in yogurt fortified with 5% neokestose, viability of *L. acidophilus* was lost after two days, showing a reduction of almost 50%. An increase in the number of organisms, however, was obtained after 11 days but a lower maximum count was evident compared to yogurts fortified with 1 and 3% neokestose.

Despite fluctuations in the number of viable *L. acidophilus* species during the storage period, the final reduction in viable counts, from fresh to 30 days old, did not exceed a 30% reduction. In general, *L. acidophilus* survived better with the addition of neokestose when compared with the control (high reduction, results not shown), prepared under similar conditions without neokestose. The increased survival and viability of the *L. acidophilus* in the current study, compared with yogurt previously prepared (Lourens et al., 2000b) was

attributed to the exclusion of *L. bulgaricus*. According to literature (Gilliland and Speck, 1977; Hull et al., 1984; Shah et al., 1995), hydrogen peroxide produced by *L. bulgaricus*, is antagonistic to acidophilus organisms resulting in a lost of viability.

The survival of *S. thermophilus* in plain yogurt fortified with neokestose is indicated in Fig. 9.1c. The addition of neokestose had no affect on the survival of *S. thermophilus*. As shown, the initial counts when fresh at day 1 exceeded 10^8 cfu/g, and the yogurt with the addition of 5% neokestose followed the "typical" curve for populations applied as starters (Hamann and Marth, 1983); an initial increase in the numbers directly after manufacture followed by a decrease during refrigerated storage. The survival rates of *S. thermophilus* in yogurts fortified with 1 and 3% neokestose were similar, started to decrease from the onset of the storage period. The reduction in viable cells, however, was less severe compared with the yogurt fortified with 5% neokestose.

Fruit yogurt with neokestose

Significant differences in the viability of *B. bifidum* and *L. acidophilus* present in fruit yogurt fortified with neokestose were observed when compared with plain yogurt fortified with neokestose (Figs. 9.2a and b). In contrast, no significant difference in the viability of *S. thermophilus* in fruit or plain yogurt was detected (Fig. 9.2c). Despite the inclusion of neokestose in this study, similar findings were obtained by Hamann and Marth (1983) in traditional yogurts, indicated that the presence of fruit has no effect on the shapes of the viability curves and the maximum numbers of the organism.

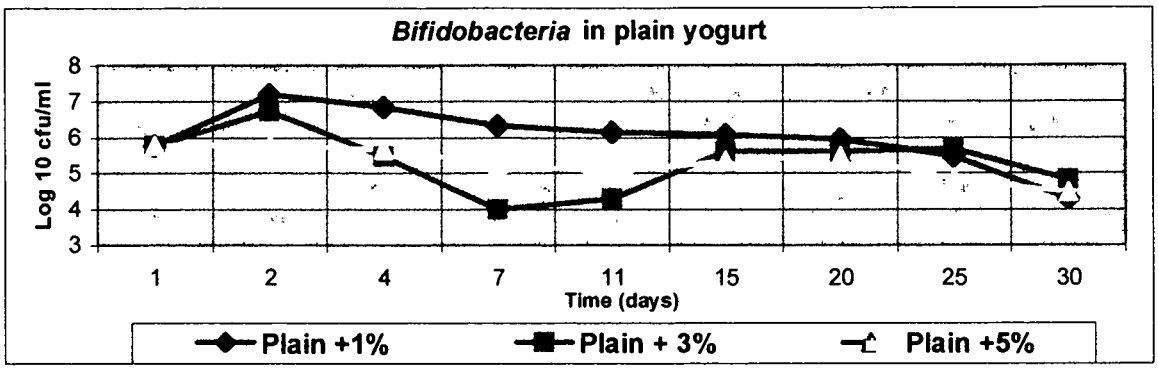
As shown in Fig. 9.2a, *B. bifidum* populations in fruit yogurt with the addition of neokestose at all levels, initially being present in the fresh yogurt at counts exceeded 10^4 cfu/ml yogurt. In all three cases, the number of bifidobacteria rapidly increased after day 1 until day 4. The bifidobacteria in yogurt fortified with 5% neokestose continued to increase reaching a maximum count higher

than 10^6 cfu/ml after 11 days. A sharp decline in the number of viable cells, however, was observed after 11 days and was followed by a continuous decline until 30 days. In the yogurt fortified with 1 and 3% neokestose, the number of viable cells of *Bifidobacteria* remained high, showing a gradual increase from fresh to 30 days old. Viable counts exceeding 10^5 cfu/ml were frequently encountered during the refrigerated storage period. Enhanced survival rates of bifidobacteria in fruit flavored yogurt compared to plain yogurt was also observed in earlier survival studies (Lourens et al., 2000b) and was ascribed to the higher sucrose level. The enhanced viability of bifidobacteria cells in fruit yogurt, fortified with neokestose, as shown in Fig. 9.2a clearly indicated on a better survival rate when compared with results in plain yogurt (Fig. 9.1a) and fruit yogurt without the addition of neokestose (results not shown). The addition of neokestose therefore encouraged a better survival of bifidobacteria species in fruit yogurt. The enhanced growth patterns of bifidobacteria corresponded with the decrease in the amount of neokestose, suggesting possible utilization by these organisms. Wang and Gibson (1993) and Hopkins et al. (1998) reported that bifidobacteria utilize fructo-oligosaccharides better than glucose. In the study by Kritzinger (1999), *L. salivarius* growth on neokestose was characterized, by limited substrate utilization and low biomass yield. Therefore, one can assume that *L. acidophilus* do not utilize neokestose to the same extend as bifidobacteria.

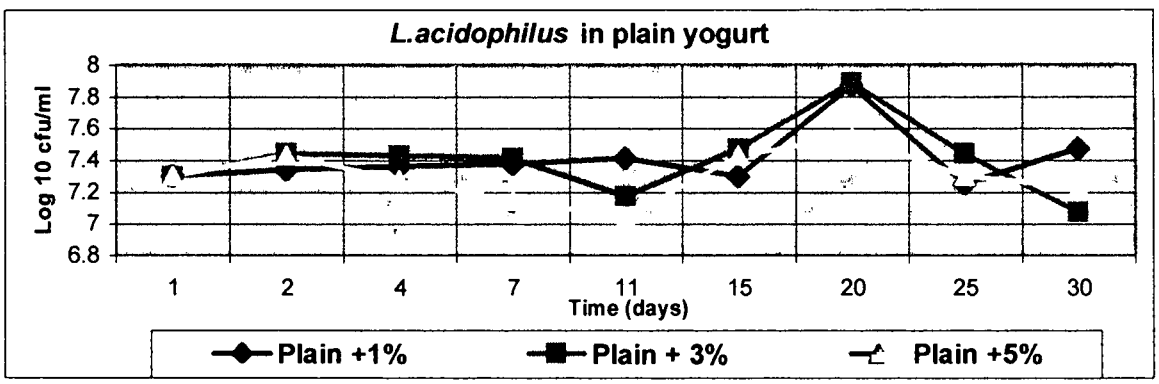
In all the fruit yogurt products, the viable cells of *L. acidophilus* remained high until day 30 (Fig. 9.1b). Initial counts of fresh yogurt at day 1, were almost 7.8 log units. Viable counts in the yogurt fortified with 3 and 5% neokestose showed a gradual increase of 0.6 log units reaching a maximum of 8.4 log units after 30 days. Yogurt with 1% neokestose, showed a gradual decline of 0.1 log units in the number of viable cells of *L. acidophilus* from the onset until day 20 after a moderate increase was observed. In general, however, *L. acidophilus* showed enhanced viability levels in yogurts fortified with neokestose when compared with traditional yogurt without neokestose (results not shown) and with the inclusion of *L. bulgaricus* (Lourens et al., 2000b).

CONCLUSIONS

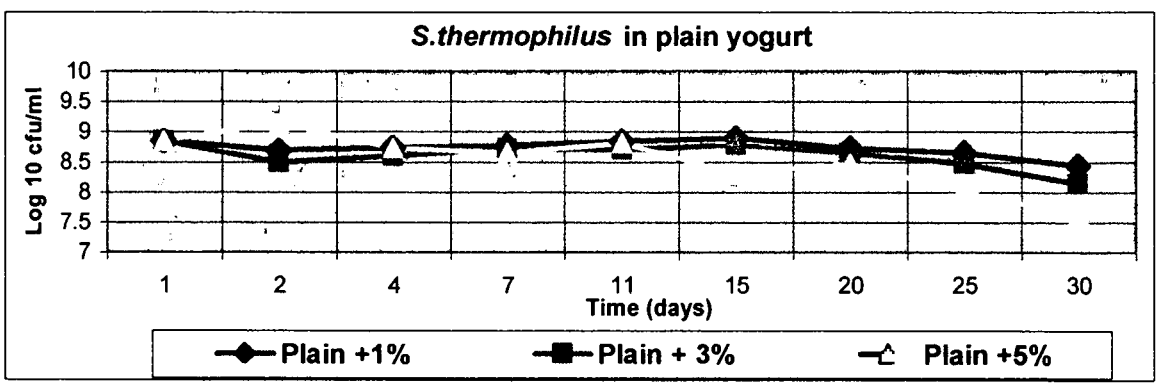
The bifidobacteria did not decrease extensively in any of the yogurt products at any of the neokestose levels compared with the decrease in viable counts of bifidobacteria in traditional yogurt during previous studies (Lourens et al., 2000b). In fact, an increase in viable cells of bifidobacteria was observed in fruit yogurt with the addition of neokestose. Hence, better survival of bifidobacteria was obtained during the refrigerated storage period. However, despite the assurance of enhanced survival rates of bifidobacterium in bio-yogurt with the addition of neokestose, the therapeutic level could not be attained. Consequently, a higher inoculum level is necessary to assure reportedly beneficial numbers (10^6 cfu/ml) throughout the normal shelf life of yogurt (Samona and Robinson, 1994). Enhanced viability of bifidobacteria was observed in fruit yogurt compared to plain yogurt. *L. acidophilus* also exhibited improved viability levels with the addition of neokestose, and in fruit yogurts, whereas the addition of fruit or neokestose had little effect on the viability of *S. thermophilus*.



a)

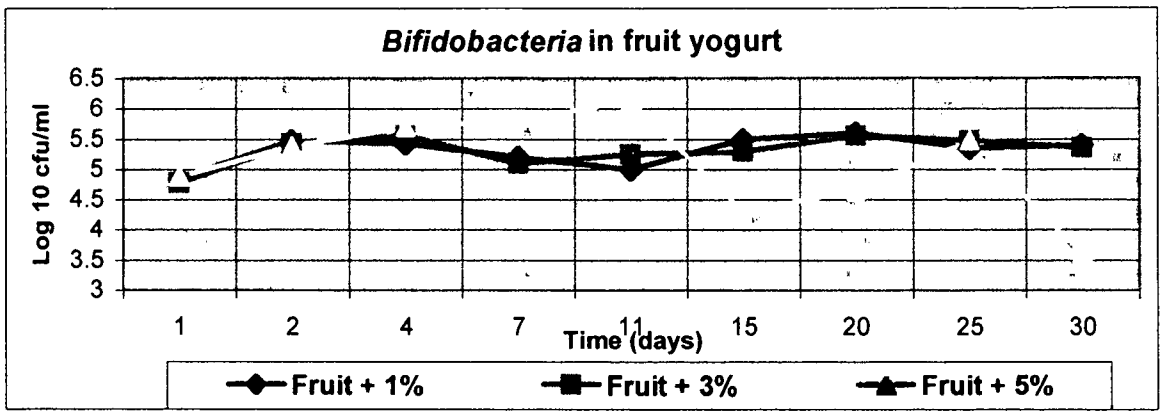


b)

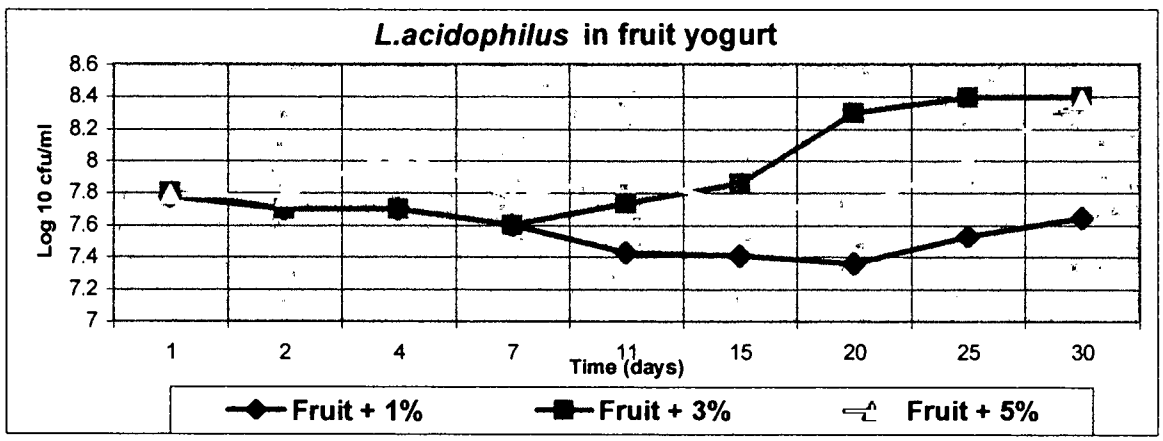


c)

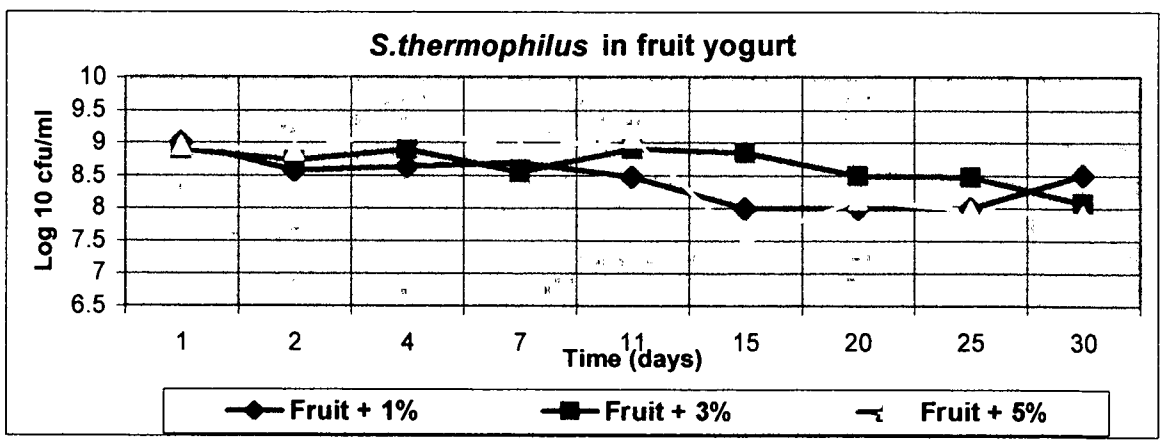
Figs. 9.1a–c Survival of a) bifidobacteria, b) *L.acidophilus* and c) *S.thermophilus* in plain yogurt with 1, 2 and 3% neokestose stored at 4°C for 30d.



a)



b)



a)

Figs. 9.2a-c Survival of a) bifidobacteria, b) *L.acidophilus* and c) *S.thermophilus* in fruit yogurt with 1, 2 and 3% neokestose stored at 4°C for 30d.

REFERENCES

Bouchnic, Y., Flourie, B., Riottot, M., Bisetti, N., Gailing, M., Guibert, A., Bornet, F. and Rambaud, J. (1996) Effects of fructo-oligosaccharides ingestion on faecal bifidobacteria and selected metabolic indexes of colon carcinogenesis in healthy humans. *Nutrition Cancer.* **26**, 21 – 29.

Callichia, M.L., Wang, C.I.E., Nomura, T., Yotsuzuka, F. and Osato, D.W. (1993) Selective enumeration of *Bifidobacterium bifidum*, *Enterococcus faecium*, and streptomycin-resistant *Lactobacillus acidophilus* from a mixed probiotic product. *Journal of Food Protection.* **56** (11), 954-957.

Campbell, J.M., Fahey, G.C. and Wolf, B.W. (1997) Selected indigestible oligosaccharides affect large bowel mass, cecal and fecal short-chain fatty acids, pH and microflora in rats. *Journal of Nutrition.* **127**, 130 – 136.

Collins, J.K., Thorton, G. and Sullivan, G.O. (1998) Selection of Probiotic strains for Human applications. *International Dairy Journal.* **8,** 487 – 490.

Dave, R.I. and Shah, N.P. (1997) Viability of Yoghurt and Probiotic Bacteria in Yoghurts Made form Commercial Starter Cultures. *International Dairy Journal.* **7**, 31 – 41.

Dave, R.I. and Shah, N.P. (1998) Ingredient Supplementation Effects on Viability of Probiotic Bacteria in Yogurt. *Journal of Dairy Science* . **81**, 2804-2816.

Fooks, L.J., Fuller, R. & Gibson, G. R. (1999) Prebiotics, probiotics and human gut microbiology. *International Dairy Journal.* **9**, 53 – 61.

Foschino, R., Fiori, E. and Galli, A. (1996) Survival and residual activity of *Lactobacillus acidophilus* in frozen cultures under different conditions. *Journal of Dairy Research.* **63**, 295 – 303.

Gardiner, G. E., O'Sullivan, E., Kelly, J., Auty, M.A.E., Fitsgerald, G.F., Collins, J.K., Ross, R.P. and Stanton, C. (2000) Comparative survival rate of human derived probiotic *Lactobacillus paracasei* and *L. salivarius* strains during heat treatment and spray drying. *Applied and Environmental Microbiology.* **66**(6), 2605 – 2612.

Gardini, F., Lanciotti, R., Guerzoni, M. E. and Torriani, S. (1999). Evaluation of aroma production and survival of *Streptococcus thermophilus, Lactobacillus delbrueckii* subsp. *bulgaricus* and *Lactobacillus acidophilus* in fermented milks. *International Dairy Journal.* **9**, 125 – 134.

Gilliland, S.E. and Speck, M.L. (1977c) Instability of *Lactobacillus acidophilus* in yogurt. *Journal of Dairy Science.* **60**, 1394 - 1398.

Hamann, W.T. and Marth, E.H. (1983) Survival of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* in commercial and experimental yogurts. *Journal of Food protection.* **47**(10), 781-786.

Hidaka, H., Eida, T., Takizawa, T., Tokunaga, T. and Tashiero, Y. (1986). Effects of fructo-oligosaccharides on intestinal flora and human health. *Bifidobacteria Microflora.* **5**, 37 – 50.

Hopkins, M.J., Cummings, J.H. and Macfarlane, G.T. (1998) Inter-species differences in maximum specific growth rates and cell yields of bifidobacteria cultured on oligosaccharides and other simple carbohydrates sources. *Journal of Applied Microbiology.* **85**, 381 – 386.

Hull, R.R. and Roberts, A.V. (1984). Differential enumeration of *Lactobacillus*

acidophilus in yogurt. *The Australian Journal of Dairy Technology.* **39**, 160-163.

Hull, R.R., Roberts, A.V. and Mayes, J.J. (1984) Survival of *Lactobacillus acidophilus* in Yoghurt. *The Australian Journal of Dairy Technology.* **39**(4), 164 - 166.

Kim, E.R., Lee, K.W., Park, Y.H. and Kwak, H.S. (1993) The study of lactic acid bacteria in yogurt during delivery and storage. *Korean Journal of Dairy Science.* **14**(3), 260-268.

Kailasapathy, K. and Rybka, S. (1997) *L.acidophilus* and *Bifidobacterium* spp. – their therapeutic potential and survival in yogurt. *The Australian Journal of Dairy Technology.* **52**, 28-35.

Kaplan, H. & Hutkins, R.W. (2000) Fermentation of Fructooligosaccharides by Lactic acid bacteria and Bifidobacteria. *Applied and Environmental Microbiology.* **66** (6), 2682 – 4.

Kilian, S.G., Sutherland, F.W.C., Meyer. P.S. and du Preez, J.C. (1996) Transport-limited sucrose utilisation and Neokestose production by *Phaffia rhodozyma. Biotechnology Letters.* **18**, 975 – 980.

Klaver, F.A.M.; Kingma, F. and Weerkamp, A.H. (1993) Growth and survival of bifidobacteria in milk. *Netherlands Milk Dairy Journal.* **47**, 151-164.

Kneifel, W., Jaros, D. and Erhard, F. (1993) Microflora and acidification properties of yogurt and yogurt-related products fermented with commercially available starter cultures. *International Journal of Food Microbiology.* **18**, 179-189.

Kritzinger, S.M. (1999) The production of Neokestose by *Phaffia rhodozyma* and its bifidogenic properties. *M.Sc thesis*, University of the Orange Free State, South Africa.

Lourens, A, Viljoen B.C and Jooste, P.J. (2000a) Levels of probiotic bacteria in South African commercial bio-yoghurt. *South African Food and Beverage manufacturing Review.* **27**(2), 31 – 33.

Lourens, A, Viljoen, B.C. and Jooste, P.J. (2000b) Survival of probiotic bacteria in South African commercial bio-yogurt. *South African Journal of Science* – **In press**.

Laroia, S. & Martin, J.H., (1990) Bifidobacteria as Possible Dietary Adjuncts in Cultured Dairy Products - A Review. *Cultured Dairy Products Journal.* **25**: 18 – 22.

Laroia, S. and Martin, J.H. (1991) Methods for enumerating bifidobacteria. *Cultured Dairy Products Journal.* **5**, 32 - 33.

McKellar, R.C. and Modler, H.W. (1989) Metabolism of fructo-oligosaccharides by *Bifidobacterium* spp. *Applied Microbiology and Biotechnology.* **31**, 537 – 541.

Micanel, N., Haynes, I.N. and Playne, M.J. (1997) Viability of probiotic cultures in commercial Australian yogurts. *The Australian Journal of Dairy Technology.* **52**, 24 – 27.

Modler, H.W. (1994) Bifidogenic factors-sources, metabolism and applications. *International Dairy Journal.* **4**(5), 383-407.

Modler, H.W., McKellar, R.C. and Yaguchi, M. (1990) Bifidobacteria and bifidogenic factors. *Canadian Institute of Food Science and Technology Journal.* **23**, 29-41.

Rybka, S. (1994) The enumeration of *Lactobacillus*, *Streptococcus* and *Bifidobacterium* species in yogurt. *B.Sc Dissertation*, University of New South Wales, Sydney.

Rybka, S. and Kailasapathy, K. (1995) The survival of culture bacteria in fresh and freeze-dried AB yoghurts. *The Australian Journal of Dairy Technology.* **50** (2) 51-57.

Samona, A. and Robinson, R.K. (1994) Effect of yogurt cultures on the survival of bifidobacteria in fermented milks. *Journal of the Society of Dairy Technology.* **47** (2), 58-60.

Shah, N.P.; Lankaputhra, W.E.V.; Britz, M.L. and Kyle, W.S.A. (1995) Survival of *Lactobacillus acidophilus* and *Bifidobacterium bifidum* in commercial yoghurt during refrigerated storage. *International Dairy Journal.* **5**, 515-521.

Sako, T, Matsumoto, K. & Tanaka, R. (1999) Recent progress on research and applications of non-digestible galacto-oligosaccharides. *International Dairy Journal.* **9**, 69 – 80.

Samona and Robinson, (1994) Effect of yogurt cultures on the survival of bifidobacteria in fermented milks. *Journal of the Society of Dairy Technology.* **47**(2), 58 – 60.

Tamime, A.Y., Marshall, V.M.E. and Robinson, R.K. (1995) Microbiological and technological aspects of milks fermented by bifidobacteria. *Journal of Dairy Research.* **62**, 151-187.

Terzaghi, B.E. and Sandine, W.E. (1975) Improved medium for lactic streptococci and their bacteriophages. *Applied Microbiology.* **29**(6), 807 - 813.

Shah, N.P., Lankaputhra, W.E.V.; Britz, M.L. and Kyle, W.S.A. (1995) Survival of *Lactobacillus acidophilus* and *Bifidobacterium bifidum* in commercial yoghurt during refrigerated storage. *International Dairy Journal.* **5**, 515-521.

Wang, X. and Gibson, G.R. (1993) Effect of the *in vitro* fermentation of oligofructose and inulin by bacteria growing in the human large intestine. *Journal of Applied Bacteriology.* **75**, 373 – 380.

Young, J. (1996) A perspective on functional foods. *Food Science and Technology Today* **10** (1), 18 – 21.

CHAPTER 10

GENERAL DISCUSSION AND CONCLUSIONS

Cultures used in fermented products are usually chosen on the basis of some technological characteristics rather than their suitability for the gastrointestinal ecosystem and health-promoting attributes. *Lactobacillus bulgaricus* and *Streptococcus thermophilus*, traditionally used in yogurt manufacture, have poor resistance to acid and bile salts. Conscious of this fact, many yogurt manufacturers now also include *Lactobacillus acidophilus* and *Bifidobacterium* spp. supposedly to be more resistant and contributing to the health aspects. Several authors, however, reported on the poor survival of the probiotic cultures in yogurt (Rybka and Kailasapathy, 1995; Klaver et al., 1993; Nigswonger et al., 1996). Consequently, the poor survival of probiotic cultures in yogurt obtained in studies conducted, accentuates the importance to investigate similar implications in South African commercial bio-yogurt and the possible use of yeasts as probiotics.

10.1 Evaluation of media for selective enumeration of probiotic yogurt cultures in commercial bio-yogurt

The need exists for simple and reliable methods for routine enumeration of both *Bifidobacterium* sp. and *L. acidophilus* to determine the initial counts of the probiotic bacteria after manufacture of the product, and also to ascertain the viability of the probiotic cells during refrigerated storage and in the product distribution chain.

Numerous advancements in methods for the enumeration of probiotic cultures in the presence of traditional yogurt starter cultures were developed, however,

in recent years. A survey was conducted by including eight different selective media in order to evaluate the feasibility of these media in the enumeration of probiotic cultures as well as traditional yogurt starter cultures. The media recommended by Chr. Hansen's laboratory were also included for comparison reasons. Pure cultures and commercial AB-yogurt samples provided the basis for selecting suitable methods to enumerate probiotic and yogurt starter cultures. The media proposed by Chr. Hansen's laboratory proved to be the most suitable for the enumeration of the different cultures based on microscopic evaluation of individual cultures.

Based on the results obtained we selected the following media for sampling of commercial AB-yogurt: NNLP agar for *B. bifidum*, maltose-MRS for *L. acidophilus*, M17 for *S. thermophilus* and Acidified-MRS for *L. bulgaricus*.

10.2 Levels of probiotic bacteria in South African commercial bio-yogurt

It is essential that bio-yogurts meet the criteria of a minimum of 10^6 cfu/ml of probiotic bacteria until the expiry date to induce any potential therapeutic advantages for the consumer (Kurmann and Rasic, 1991; Rybka and Kailasapathy, 1995). Due to the lack of survival of probiotic cultures in yogurts, these criteria are seldom met. In this study we evaluated samples of three South African manufacturers of commercial AB-yogurt obtained from supermarket outlets based on enumeration studies of viable probiotic cultures, *Lactobacillus acidophilus* and *Bifidobacterium bifidum*, as well as conventional yogurt starter cultures, *Streptococcus thermophilus* and *Lactobacillus bulgaricus*.

Based on the results obtained, the AB-yogurts examined comply with the criteria regarding the number of viable cells of *L. acidophilus*, but the consumer would not have received sufficient numbers of *B. bifidum* cells at the time of consumption. The low population numbers of *B. bifidum* are attributed to the high numbers of *L. bulgaricus*, responsible for the decline in pH and as a

result inhibited the growth of *B. bifidum*. Although significant differences ($p \leq 0.05$) in the number of probiotic bacteria were observed among manufacturers and between batches of the same manufacturer, no significant difference ($p \geq 0.05$) was obtained within each batch comprising ten samples and using the same selective medium. This clearly indicates on the adequacy of implementing the selected media for the enumeration of the cultures.

10.3 Survival of probiotic bacteria in South African commercial bio-yogurt

The poor survival of *L. acidophilus* and *B. bifidum* in yogurt is mainly attributed to their lack of resistance against low pH values present in the environment (Playne, 1994). Although *L. acidophilus* is considered to be more resistant against acidic conditions compared to the traditional yogurt culture organisms (*Lactobacillus delbrueckii* spp. *bulgaricus* and *Streptococcus salivarius* spp. *thermophilus*), its number of viable cells in yogurt decreases during storage (Shah and Jelen, 1990). *B. bifidum* is even less acid-tolerant compared to *L. acidophilus* (Lankaputhra and Shah, 1995) resulting in a reduced survival rate in yogurt. The poor survival of the probiotic cultures, therefore, contributes to bio-yogurt products with insufficient numbers of viable probiotic cells. As a result, the consumer will not receive the supposed beneficial therapeutic or health effects.

In this study we monitored the changes in viable cell counts of the probiotic cultures and starter cultures present in yogurt at frequent intervals from d 1 until the expiry date at d 31 stored at 4°C and 10°C. *S. thermophilus* predominated in all yogurt samples stored at both temperatures, while the number of viable cells of *B. bifidum* was always the lowest. *B.bifidum* never exceeded counts of 10^6 cfu/g in any of the samples and a constant decline in its numbers was observed. *L. acidophilus,* despite maintaining in general counts higher than 10^6 cfu/g in the yogurt samples, also exhibited a substantial decrease in its numbers during storage. The storage of the yogurt

samples at different temperatures had little effect on the viability of the organisms.

10.4 Growth and survival of a probiotic yeast in dairy products

The ability of the probiotic yeast species, *Saccharomyces boulardii*, to survive passage through the intestinal tract (Gedek, 1991), the poor survival of the probiotic cultures in yogurt, and numerous microbial interactions in fermented dairy products involving yeasts (Fleet, 1990) emphasize the possible role of positive interactions between yeasts and starter cultures. Consequently, the probiotic yeast species was incorporated into commercial bio-yogurt containing the normal starter cultures as well as the probiotic cultures. We monitored all the changes in chemical compositions, pH, alcohol, and the utilization of lactose and relevant organic acids at intervals from d 1 to d 31 when grown in UHT milk, UHT yogurt, plain yogurt and fruit yogurt stored at refrigerated temperatures. In addition, we also monitored the proliferation of the yeast species.

Based on the results in this study, the probiotic yeast species, *S. boulardii*, has the ability to grow in bio-yogurt reaching maximum counts exceeding 10^7 cfu/g. The number of yeast populations was substantially higher in the fruit based yogurt, mainly due to the presence of proportions of sucrose and fructose derived from the fruit. Despite the inability of *S. boulardii* to utilize lactose, the main carbohydrate available in dairy products, the yeast species utilized available organic acids, galactose and glucose derived from bacterial metabolism of the milk lactose, as well as possible free fatty acids or free amino acids present in the dairy products. Excessive gas and alcohol production by the yeast species proved, however, to be major constraints.

10.5 Growth and survival of dairy associated yeasts in yogurt and yogurt-related products

It is a well-known fact that yeasts utilises the lactic acid produced by the LAB, thereby increasing the pH of the product (Subramanian and Shankar, 1983). Yeasts also add to the association by stimulating the growth of the LAB due to the excretion of growth factors and metabolites. Consequently, the presence of yeasts in yogurt may also stimulate the growth of pro-biotic microorganisms by increasing the pH.

The yeast strains, *Kluyveromyces marxianus*, *Issatchenkia orientalis*, *Debaryomyces hansenii* and *Yarrowia lipolytica*, commonly associated with yogurt (Deak and Beuchat, 1996) were isolated from commercial yogurt, identified and inoculated into plain AB-yogurt, fruit flavoured AB-yogurt, as well as sterile milk and pasteurised sweetened yogurt. The incidence and growth of the yeasts were monitored over a four-week period (until the expiry date) of the yogurt. The yeasts survival and progression in fermented and non-fermented, flavoured and non-flavoured dairy products were compared. pH, sugar utilisation and the production of organic acids were determined on a regular basis during the shelf-life to evaluate the possible contribution of the yeasts towards the products.

Based on the results obtained, the yeast species were able to progress in bio-yogurt reaching maximum counts exceeding 10^7 cfu/g. Despite the inability of some species to utilise lactose, the yeast species utilised available organic acids, galactose and glucose derived from bacterial metabolism of the milk lactose. The growth of the yeasts was also encouraged by possible free fatty acids or free amino acids present in the dairy products. Consequently, due to the utilization of organic acids, the yeasts thereby sufficiently contributed to the retention or enhancing of the pH values. Despite better survival and growth rates of the pro-biotic microorganisms obtained when *K. marxianus* and *I. orientalis* were inoculated into the yogurts, the production of excessive gas and

alcohol were major constraints in implementing these yeast species into yogurt. The inclusion of *Y. lipolytica* and *D.hansenii* in AB yogurts, therefore, seemed the most promising in controlling the pH to assure the viability of the pro-biotic microorganisms.

10.6 Enhancement of the viability of probiotic bacteria in bio-yogurt: the effect of *Debaryomyces hansenii* and *Yarrowia lipolytica.*

In chapter 8, the interaction and possible positive influence of *Yarrowia lipolytica* and *Debaryomyces hansenii* on the growth and survival of the probiotic cultures, bifidobacteria and *L. acidophilus*, as well as the traditional yogurt culture, *S. thermophilus*, when grown in association in bio-yogurt were investigated. Pure cultures of the yeast species were inoculated at moderate levels ($10^5 - 10^6$ cfu/ml) into commercial AB-yogurt directly after manufacture and refrigerated at 5°C for 30 days. In the second part of the study, *D.hansenii* was added at a lower level ($10^2 - 10^3$ cfu/ml) jointly with the ABT-starter into commercial AB-yogurt before manufacture to compare the effects on viability of probiotic bacteria.

Viable bifidobacteria counts remained virtually the same in the bio-yogurt inoculated with the yeast cultures during the refrigerated storage period. Compared to the control (yogurt without the addition of yeast), improved survival of bifidobacteria was obtained in plain yogurt (0.2 log unit decrease) with *D. hansenii* added before manufacture while bifidobacteria survived better in fruit yogurt with *D. hansenii* addition after manufacture (remained at the same level).

A considerable decrease in *L. acidophilus* occurred after 2 weeks storage (2-4 log cycles) in the yeast-inoculated bio-yogurt. The same effect was observed in the bio-yogurt inoculated with *D. hansenii* before fermentation, except in the case of fruit yogurt with *D. hansenii* (decrease of 0.3 $\log_{10}$ cfu/ml). The

interaction between dairy associated yeasts and lactobacilli species should be further investigated.

Addition of the yeast after manufacture primarily encouraged the growth of streptococci which had an influence on the pH of the environment. Counts of streptococci remained above 10^8 cfu/ml from the second day until the end of storage. This is in contrast with counts usually observed in traditional yogurt (Hamann and Marth, 1983). Consequently due to the enhanced survival of the streptococci, an enhanced decline in pH was obtained due to the production of lactic acid. In contrast, declining curves were obtained when the yeast was inoculated before manufacture. *S. thermophilus* rapidly decreased after 15 days to levels < 10^7 cfu/ml after 30 days. In plain yogurt, its numbers remained stable until day 25, followed by a decrease of 1 log unit until day 30. The decline in the numbers of streptococci at the end of refrigerated storage resulted in a moderately higher pH at the end of storage (results not shown).

A gradual decrease in the pH of all the bio-yogurt products was observed. In the case where *D. hansenii* was inoculated into yogurt, the decrease in pH values of fruit yogurt (0.27) and plain yogurt (0.22) was more than the control yogurts (0.18 – 0.19). The moderate difference in pH values may be attributed to a faster declining curve of *D. hansenii* compared to *Y. lipolytica* which consequently also resulted in less utilization of organic acids. The enhanced growth of the streptococci resulted in an enhanced decline in pH of products with *D. hansenii*. However, an excessive decline in pH was prevented, due to the utilization of organic acids and synergistic peptonization of proteins by *D. hansenii* and *Y. lipolytica* (Fleet, 1990). Also, the fact that manufacturers now make use of an ABT starter where *L.bulgaricus* is excluded resulted in a higher pH initially (4.26-4.31) and during storage.

The overall quality of the yogurt was still acceptable and had a pleasant taste compared to the control yogurt after 30 days storage, even at the higher yeast

inoculum level. Inoculation of yogurt with yeast to improve viability of probiotic bacteria in bio-yogurt seems promising.

10.7 Enhancement of *bifidobacteria* by neokestose in bio-yogurt.

Many attempts were made to enhance the growth and survival of bifidobacteria in probiotic dairy products, among such is the addition of prebiotics (Young, 1996). Fructo-oligosaccharides have in recent years increasingly been applied in various foods as prebiotics to enhance the population of *Bifidobacterium* sp. in the large intestine (Wang and Gibson, 1993; Kaplan and Hutkins, 2000). The addition of prebiotics to AB-yogurt should therefore encourage the growth and survival of the probiotic bacteria, due to a more readily available and specific substrate for utilisation, as well as the individual advantages that each could offer (Fooks et al., 1999).

Consequently, the objective of this study was to determine the effects of the prebiotic, neokestose, on the growth and survival of bifidobacteria in bio-yogurt. Commercial plain and fruit AB-yogurt were fortified with 1%, 2% and 3% neokestose, and growth and survival of bifidobacteria as well as *L.acidophilus* and *S. thermophilus* were monitored during storage at 4°C over 30 days.

The viable bifidobacteria counts remained significantly higher in all the yogurts when compared to traditional yogurts without the addition of neokestose. *L. acidophilus* reduction in viable counts did not exceed a 30% reduction; therefore neokestose also had a better survival effect on *L. acidophilus*. The addition of neokestose had no affect on the survival of *S. thermophilus*.

7.8 Future research

Based on the results obtained in this study, we recommended the following for future research:

a) Further studies of the inclusion of different yeast species as part of the starter culture for bio-yogurts.

b) The development of new yeast containing probiotic dairy products with main emphasis on the technological properties of yeasts such as aroma formation, lipolytic and proteolytic activities, positive microbial interactions and inhibitory effects against spoilage organisms.

c) More extensive studies on the biochemical and physiological growth kinetics of yeasts in yogurt.

d) The possible antagonistic effect of yeasts against *L. acidophilus* should be further investigated.

e) Inoculation levels of probiotic starter cultures should be monitored to ensure sufficient viable cells at the expiry date.

f) The influence of preservatives, if permitted, on the survival of probiotic cultures in yogurt.

REFERENCES

Deàk, T. and Beuchat, L.R. (1996) Handbook of Food Spoilage. New York, CRC Press.

Fleet, G.H. (1990) Yeasts in dairy products - A review. *Journal of Applied Bacteriology.* **68**, 199-211.

Fooks, L.J., Fuller, R. and Gibson, G. R. (1999) Prebiotics, probiotics and human gut microbiology. *International Dairy Journal.* **9**, 53 – 61.

Gedek, B.R. (1991) Regulierung der darmflora über die nahrung. *Zbl. Hyg.* **191**, 277-301.

Hamann, W.T. and Marth, E.H. (1983) Survival of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* in commercial and experimental yogurts. *Journal of Food protection* **47**(10), 781-786.

Kaplan, H. & Hutkins, R.W. (2000) Fermentation of Fructooligosaccharides by Lactic acid bacteria and Bifidobacteria. *Applied and Environmental Microbiology.* **66**(6), 2682 – 4.

Klaver, F.A.M.; Kingma, F. and Weerkamp, A.H. (1993) Growth and survival of bifidobacteria in milk. *Netherlands Milk Dairy Journal.* **47**, 151-164.

Kurmann, J.A. and Rasic, J.L. (1991) The health potential of products containing bifidobacteria. In: *Therapeutic Properties of Fermented Milks.* Edited by R. K. Robinson. Elsevier Applied Sciences, London, 117-158.

Lankaputhra, W.E.V. and Shah, N.P. (1995) Survival of *Lactobacillus acidophilus* and *Bifidobacterium* species in the presence of acid and bile salts. *Cultured Dairy Products Journal.* **30**(3), 113-118.

Nighswonger, B.D.; Brashears, M.M. and Gilliland, S.E. (1996) Viability of *Lactobacillus acidophilus* and *Lactobacillus casei* in Fermented Milk Products During Refrigerated Storage. *Journal of Dairy Science.* **79**, 212-219.

Playne, M. (1994) Probiotic foods. *Food Australia.* **46**(8), 362.

Rybka, S. and Kailasapathy, K. (1995) The survival of culture bacteria in fresh and freeze-dried AB yoghurts. *The Australian Journal of Dairy Technology.* **50**(2) 51-57.

Shah, N. and Jelen, P. (1990) Survival of Lactic Acid Bacteria and Their Lactases under Acidic Conditions. *Journal of Food Science.* **55**(2), 506-509.

Subramanian, P. and Shankar, P.A. (1983) A note on lactose fermenting yeasts in milk products. *Journal of Food Science and Technology.* **20**, 181-183.

Young, J. (1996) A perspective on functional foods. *Food Science and Technology Today* **10**(1), 18 – 21.

Wang, X. and Gibson, G.R. (1993) Effect of the *in vitro* fermentation of oligofructose and inulin by bacteria growing in the human large intestine. *Journal of Applied Bacteriology.* **75**, 373 – 380.

CHAPTER 11

SUMMARY

A review of the literature highlighting the importance of the 'therapeutic minimum' and the survival of the probiotic bacteria in fermented milk bio-products is given in Chapter 2. Special reference is made to the historical background of probiotics, its therapeutic value and the survival through passage in the gastrointestinal tract. In addition, technology of bio-yogurt, factors affecting the survival of probiotic bacteria in yogurt, and the media for the differential enumeration of these microorganisms in dairy products are discussed.

In Chapter 3 existing media proposed for the selective enumeration of starter cultures employed in the manufacture of bio-yogurt are compared and evaluated. It is essential for comparison reasons to standardize enumeration methods for microbial analyses in order to study the incidence of the probiotic bacteria in the presence of the conventional starter cultures. The media proposed by Chr. Hansen's laboratory proved to be the most suitable for the enumeration of the different cultures.

It is essential that bio-yogurts meet the criteria of a minimum of 10^6 cfu/ml of probiotic bacteria until the expiry date to induce any potential therapeutic advantages for the consumer. Consequently, in Chapter 4 we evaluated samples of AB-yogurt obtained from supermarket outlets statistically based on the enumeration of viable probiotic cultures, *Lactobacillus acidophilus* and *Bifidobacterium bifidum,* as well as conventional yogurt starter cultures, *Streptococcus thermophilus* and *Lactobacillus bulgaricus* and the maintenance with respect to the 'therapeutic minimum'. Based on the data obtained, the

AB-yogurts examined comply with the criteria regarding the number of viable cells of *L. acidophilus*, but the consumer would not have received sufficient numbers of *B. bifidum* cells at the time of consumption.

In Chapter 5, we monitored the survival of viable cells of the probiotic cultures and starter cultures present in bio-yogurt at frequent intervals from day 1 until the expiry date at day 31 stored at 4°C and 10°C. *B.bifidum* never exceeded counts of 10^6 cfu/g in any of the samples and a constant decline in its numbers was observed. *L. acidophilus,* despite maintaining counts higher than 10^6 cfu/g in the yogurt samples, also exhibited a substantial decrease in its numbers during storage.

Due to the poor survival of probiotic cultures in yogurt, we incorporated a probiotic yeast species, *S. boulardii* as part of the starter culture in Chapter 6 and monitored its progression and survival in yogurt and milk products. Despite good growth and the survival of the yeast species until the expiry date, excessive gas and alcohol production proved, however, to be major constraints.

In order to further study the effect of yeast growth on the survival of probiotic bacteria in bio-yogurt, pure cultures of *Kluyveromyces marxianus, Issatchenkia orientalis, Debaryomyces hansenii* and *Yarrowia lipolytica* were inoculated into commercial AB-yogurt, sterile milk and pasteurised sweetened yogurt in Chapter 7. The yeast species were able to progress in the bio-yogurt reaching maximum counts exceeding 10^7 cfu/g. Despite the inability of some species to utilise lactose, the yeast species utilised available organic acids, galactose and glucose derived from bacterial metabolism of the milk lactose, as well as possible free fatty acids or free amino acids present in the dairy products and thereby sufficiently contributed to the retention or enhancing of the pH values. The production of excessive gas and alcohol was major constraints in implementing *Kluyveromyces marxianus, Issatchenkia orientalis.* The inclusion of *Y. lipolytica* and *D.hansenii* in AB-yogurts, therefore, seemed the most

promising in controlling the pH to assure the viability of the pro-biotic microorganisms.

In Chapter 8, *Y. lipolytica* and *D.hansenii* cultures were inoculated into commercial plain and fruit AB-yogurt at moderate (10^5 –10^6 cfu/ml) and low level (10^2 – 10^3 cfu/ml), directly after manufacture and with the ABT-starter before fermentation, to compare the effects that the yeast will have on viability of probiotic bacteria. Viable bifidobacteria counts remained virtually the same in the bio-yogurt inoculated with the yeast cultures during the refrigerated storage period. A rapid decrease in *L. acidophilus* occurred after 2 weeks storage (2-4 log cycles) in the yeast-inoculated bio-yogurt suggesting possible antagonistic action of the yeast against *L. acidophilus*. Addition of the yeast primarily encouraged the growth of streptococci, which had an influence on the pH of the yogurt environment. A gradual decrease in the pH of all the bio-yogurt products was observed. pH was affected by enhanced growth of streptococci, utilization of organic acids by the yeasts and the fact that *L. bulgaricus* was excluded form the yogurt starter culture. The yogurt inoculated with *D. hansenii* was still acceptable and had a pleasant taste compared to the control yogurt after 30 days storage. Inclusion of yeast as part of the starter culture for bio-yogurts seems promising.

In Chapter 9, possible enhancement of the growth and survival of *Bifidobacteria* in bio-yogurt by the addition of a prebiotic was investigated. Commercial AB-yogurt was fortified with 1, 2 and 3% of the fructo-oligosaccharide, neokestose, and growth and survival of bifidobacteria as well as *L.acidophilus* and *S. thermophilus* were monitored during storage at 5°C over 30 days. With the addition of neokestose the viable bifidobacteria count remained significantly higher in all the yogurts when compared to traditional yogurts without the addition of neokestose. *L. acidophilus* reduction in viable counts did not exceed a 30% reduction; therefore neokestose also had a better survival effect on *L. acidophilus*. The addition of neokestose had no affect on the survival of *S. thermophilus*.