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Bloemfontein

OPTIMIZATION OF PROBIOTICS IN DAIRY PRODUCTS

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by

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"If you give God the right to yourself, He will make a holy experiment out of you. God's experiments always succeed"

Oswald Chambers

Dedicated to my husband,
Paul Jansen van Rensburg, and my parents, Kobus and Susan Uys

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LIST OF ABBREVIATIONS

A_f	Accuracy factor
ALTS	Veterinary experts for food and food hygiene
ANOVA	Analysis of variance
BA	Basal agar medium
BA-R	Basal agar with rhamnose
BA-RV	Basal agar with rhamnose, vancomycin
CCD	Central composite design
cfu	colony forming units
EC	Esculin-cellobiose agar
EOC	Ease of counting
FDA	Food and Drug Administration
FOSHU	Foods for Specified Health Use
g	gram
GIT	Gastro intestinal tract
GLM	General liner model
GRAS	Generally Regarded As Safe
h	hour (s)
HOWARU	‘How are U? ‘
HPLC	High performance liquid chromatography
IDF	International Dairy Federation
ISO	International organization of standards

L	liter (s)
LAB	Lactic acid bacteria
LAR	*Lactobacillus rhamnosus*
LC	*Lactobacillus casei* agar
M	molar
min	minute (s)
ml	milliliter (s)
mm	millimeter (s)
mLBS	modified *Lactobacillus* agar
MRS	deMan Rogosa Sharpe medium
MRS-M	MRS with maltose
MRS-V	MRS with vancomycin
MRS +++	MRS medium supplemented with cysteine + lithium chloride + sodium propionate
n	number of observations
nm	nano-meter (s)
NNLP	Neomycin sulphate, nalidixic acid, lithium chloride and Paromomycin
NSLAB	Non-starter lactic acid bacteria
OD_{690}	Optical density measurement at a wavelength of 690nm
R^2	Coefficient of determination
RS	Response surface
RSREG	Response surface regression
rpm	rotations per minute

SAS	Statistical analysis system
SEP	Standard error of prediction
B_f	Bias factor
sqrt	square root
T^0	Time (0) of observation
U	Unit (s)
WC	Wilkens-Chalgren
µm	micro-meter (s)
µl	micro-liter (s)
μ_{max}	maximum specific growth rate
^oC	degrees Celsius

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CHAPTER 1

Literature review

1.1. INTRODUCTION

Lactic acid bacteria (LAB) have been associated with health promoting effects from as far back as 1907. It was then that Nobel Prize Winner, Eli Metchnikoff, postulated that LAB could restore the balance of intestinal flora and subsequently improving health and thereby prolonging life. He postulated that man should consume certain types of dairy products, mainly fermented milk products, and preserved material containing large numbers of lactic acid producing bacteria. This theory gave birth to the concept of 'probiotic' or "avant la lettre" which has since became popular by scientist and consumers (Huis in't Veld *et al*., 1998; Lourens-Hattingh and Viljoen, 2001; Mercenier *et al*., 2002; Young, 1996). Consumer awareness of the link between microorganisms and health became increasingly dominant during the past decade, resulting in a world wide increase in sales of probiotic containing food products (also referred to as functional foods) (O'Sullivan *et al*., 1992).

The most active area of functional food development has been the application of probiotics to yoghurt, commonly referred to as bio-yoghurt (Gilliland and Speck, 1977; Hull *et al*., 1984). Numerous studies have also shown that Cheddar cheese may offer certain advantages as a delivery system for live probiotics to the gastro-intestinal tract (GIT) (Dinikar and Mistry, 1994; McBrearty *et al*., 2001; Stanton *et al*., 1998). The successful incorporation of probiotics into cheese would expand the probiotic food range and could be of considerable economic importance for the dairy industry.

The bacteria mainly used as probiotics, include strains of the genera lactobacilli and bifidobacteria, which are part of the natural flora of the human GIT. A balanced flora in the GIT is known to be conducive to good health with increasing evidence that specific strains of probiotic bacteria have special properties that can help maintain such a healthy digestive system.

Adequate amounts of viable cells, referred to as the ‘therapeutic minimum’, need to be consumed in order to transfer beneficial effects to the consumer. It is therefore generally accepted in literature that probiotic bacteria are only effective when present in amounts larger than 10^6 cfu (colony forming units) /ml or /g and that an amount of 100g per product needs to be consumed on a daily basis in order to maintain continuous beneficial effects. Poor survival of probiotic species, however, is a major constraint in the advancement and development of new products to expand the probiotic food range (Dave and Shah, 1997; Klaver *et al*., 1993; Lourens and Viljoen, 2002; Rybka and Kailasapathy, 1995). Consequently, it has been considered relevant to study the levels and survival of probiotic bacteria incorporated into fermented dairy products. Not only is this done to ensure product credibility but also to prevent consumers from being mislead by inaccurate product labelling information. In order to study the presence of probiotic species in functional food products, it is imperative to standardise enumeration methods for microbial analysis. However, implementing existing media for selective enumeration of probiotic microorganisms proofed to be troublesome due to strain and species selectivity.

It is evident that the lack of standardised systems and methods regarding enumeration, identification and health claims of probiotics, are causing world wide variation and disharmony in research and routine quality control systems within the industry. The implementation of a probiotic legislative system would not only be of benefit to the industry, but also to the consumer who determines the future development of these products.

1.2. BACKGROUND ON PROBIOTIC MICROORGANISMS

1.2.1. History and present situation

Much of the scientific interest in the beneficial role of live microbial food supplements (such as fermented dairy products) dates back many centuries and has previously been well documented (Bibel, 1982; Fuller, 1992). Their medicinal value has been reported in pre-biblical times where it was used in the treatment of body ailments. Hippocrates, amongst other known scientists, also considered its value as a medicine instead of as a traditional food source. Sour milk was then prescribed for curing stomach and intestinal disorders (Oberman, 1985). Although a definition for probiotics was not established until 1965, the concept was worked on by Elie Metchnikoff (Pasteur Institute France) from the beginning of the 19th century. He believed for a very long time that the complex microbial population in the colon was having adverse effects on the host by the so-called 'autointoxication' process, giving rise to his 'longevity-without-aging' theory in 1908. In this theory, Metchnikoff suggested that the long healthy lives of Bulgarian people were due to their consumption of large amounts of fermented milk products. As such, he was the first to suggest the ingestion of fermenting bacilli (Lactobacilli) to decrease 'putrefaction' and toxic microbial activities present in the intestine. In doing so, he established that bacteria are not necessarily detrimental to man but, on the contrary, play an important role in their general well-being (Fooks *et al*., 1999; Lourens-Hattingh and Viljoen, 2001; Mercenier *et al*., 2002; O'Sullivan *et al*., 1992). It was during that time, in 1899, that Tissier (Pasteur institute, France) isolated bifidobacteria from stools of breast-fed infants and suggested administration of these bacteria to infants suffering from diarrhoea (Ishibashi and Shimamura, 1993; O'Sullivan *et al*., 1992). Tissier also believed that bifidobacteria would re-establish themselves as the dominant species in the intestines by displacing the putrefactive bacteria responsible for gastric upsets.

Clinical and epidemiological studies done on lactic acid- and bifidobacteria ever since, continue to illustrate their beneficial role in health (Huis in't Veld *et al*., 1998). Related research in the past primarily focused on the abilities of fermented milk to treat intestinal disorders whereas at current the focus is turned to the development of functional foods with additional health benefits that may be derived from these organisms, as well as strain selectivity to ensure the survival of these bacteria in the GIT and the carrier food.

1.2.2. Definitions

Derived from two Greek words, 'for life' (Fuller, 1989), probiotics have had many definitions due to the rapid evolution of the functional food field. The term was first used by Lilly and Stillwell in 1965, describing substances produced by protozoan, affecting and or stimulating the growth of one another (O'Sullivan *et al*., 1992). These generalised definitions, however, were still unsatisfactory since the word 'substances' include chemical supplements such as antibiotics. Fuller (1989) revised the definition of probiotics to 'a live microbial feed supplement beneficially affecting the host animal by improving its intestinal microbial balance', which stressed the importance of live cells as an effective probiotic component. Huis in't Veld and Havenaar (1991) expanded this definition to probiotics being 'mono- or mixed cultures of live organisms which, when applied to man or animal (eg. as freeze-dried cells /or in a fermented form), beneficially affects the host by improving the properties of the indigenous microflora'. The definition implies that; by containing live microrganisms in the format described above, a probiotic product (i.e. bio-yoghurt) can improve the health status of the consumer by exerting beneficial effects in the GIT (O'Sullivan *et al*., 1992). This definition, popularised by Fuller (1989), was redefined by an Expert Committee as 'living microorganisms, which upon ingestion in certain numbers, exert health benefits beyond inherent general nutrition' (Guarner and Shaafsma, 1998). Despite all these proposed definitions, none have universal acceptance.

Points referred to in numerous “probiotic” discussions relate to the site of activity (e.g. oral cavity, upper and lower GIT), viability of the probiotic strain (cells dead or alive upon digestion), cell concentrations needed for exerting beneficial probiotic effects, use of mono-or mixed cultures, form of intake, carrier products (i.e. dairy products), food supplements, pharmaceutical preparations (e.g. powders, tablets), and its beneficial functionality beyond supplying the basic nutritional needs. Relating to the functionality of proposed probiotic products, discussions are also conducted by focusing on characteristics such as adhesion, translocation, etc. Lack of techniques for determining the presence and efficacy of possible strains together with the fact that many scientists claimed probiotic benefits relating more to prevention rather than therapy, makes a reliable definition difficult. The above being the main cause of ongoing discussions (Mercenier *et al*., 2002).

1.2.3. Human gastrointestinal ecology and well-being

Apart from the respiratory tract, the GIT (250m^2) constitutes the largest body surface area. In addition to the large amounts of food that passes through this canal, these surfaces are continuously challenged with chemicals (i.e. pharmaceutical preparations like antibiotics) and possible pathogenic organisms. Even more, they are the target of several disturbances induced by the lifestyle and food of the ‘Western world’ (Mercenier *et al*., 2002). Historically, there has always been an interest in modulating the composition of gut flora allowing a more favourable balance of bacteria to reside in the gut (Marteau *et al*., 2001). Today the complexity of the constituting GIT flora is very well recognised. Colonization with intestinal microflora begins at birth and continues throughout life, leading to a very rich flora of more than 400 different species (Finegold *et al*., 1977). There is great variability between the composition of intestinal flora in the stomach, small intestine and large intestine (colon).

A maximum bacterial count of 10^{12} cfu/g is reached in the colon, however the numbers decline reaching the small intestines (10^4 – 10^8 cfu/g) and to values lower than 10^3 cfu/g in the stomach due to the lower pH (Hoier, 1992). Although the complex composition of the intestinal flora remains relatively constant during life, extrinsic factors like stress, diet, drugs, environmental conditions etc., tend to disrupt the balance and allow undesirable microorganisms to establish in the intestine. As a result, the disrupted balance leads to a number of clinical disorders, whilst making the host more susceptible to infections by transient enteropathogens like *Salmonella*, *Campylobacter*, *Escherichia coli* and *Listeria* (Fooks *et al.*, 1999). It should be emphasised that this intestinal balance can only be maintained through increased predominance of bacteria such as lactobacilli and bifidobacteria.

Analysis of the intestinal microflora, though, is still in its infancy and knowledge about this ecosystem will increase significantly due to the recent developments on molecular level. In 1987, Mitsuoka proposed a hypothetical scheme in which he illustrates the intricate interrelationship between intestinal bacteria and human health (Fig.1) (Lourens-Hattingh and Viljoen, 2001). In this scheme intestinal bacteria are categorized into three categories; *(1)* harmful, *(2)* beneficial and *(3)* neutral bacteria. Bifidobacteria and lactobacilli are categorised amongst those exerting beneficial effects to the host.

1.2.4. Selection criteria for probiotics

Despite the increasing market trend, there are still certain requirements that must be met before a probiotic culture can be used as a food adjunct with proven beneficial effect (Collins *et al*., 1998; Martin and Chou, 1992). Currently, there is no concrete basis for the conclusive and optimal selection of probiotic bacteria, however, certain criteria have been established (Havenaar *et al.*, 1992). These can be divided into three main categories:

General microbiological criteria

- The organism must be safe to use (i.e. non-pathogenic) / GRAS- status.
- It should survive initial attacks of the human defence system (saliva, gastric and bile juice).
- The microbes should presumably be of human origin.
- Genetically stable strains.

Technological effects

- The organisms must be culturable on an industrial scale.
- A suitable carrier for fermenting substance (i.e. milk) should be available.
- The final product should have an acceptable shelf-life and sensory attributes, including colour, taste, aroma and texture.

Proven functional effects

- Stimulation of the immune response,
- promotion of colonization, and
- resistance of functional effects on the intestinal flora (i.e. modification of bacterial counts and /or their metabolic activity).

1.2.5. Therapeutic effects attributed to probiotic microorganisms

The original idea with probiotics has always been to change the composition of the normal intestinal microflora from a potentially harmful composition towards a microflora population that would be beneficial for the host. Research conducted since the turn of the century, however, has enhanced our understanding of the resulting therapeutic effects. It is obvious that by avoiding colonisation by pathogens and as a result reduce the risk of overgrowth of potential pathogenic bacteria, will be beneficial to the host. However, in some cases too much emphasis is placed on the change in microflora composition without considering the actual health benefits.

1.2.6. Therapeutic value

The criterion in literature generally referred to as the 'therapeutic minimum' (Davis *et al*., 1971; Rybka and Kailasapathy, 1995) dates back to Speck (1978) who proposed that probiotic bacteria must be present in numbers ranging from 10^8 – 10^9 cfu/g to have a positive influence on the intestinal microflora. The definition by Fuller (1989), redefined by Guarner and Shaafsma (1998), however still outlines the requirements that the microorganisms must be alive, not pasteurised or otherwise inactivated. It has been claimed that only dairy products with viable microorganisms have beneficial health effects. However, in the case of lactose intolerance and the treatment of acute gastro-enteritis and candiases, the use of probiotics showed the same beneficial affect whether the cells were viable or non-viable (Ouwehand and Salminen, 1998). More critical than the concentration of the probiotic bacteria in the food, however, is the daily intake of probiotics in order to obtain a therapeutic effect. Despite the lack of defining specific numbers or concentrations, it is generally believed that a minimum of 10^6 cfu/g probiotic product (s) needs to be ingested on a daily basis (Ouwehand *et al*., 2002). It is thus imperative that the probiotics should remain viable in the food carrier up until consumption. Various authors believe that at least 10^8 - 10^9 viable cells/g, which can be achieved with a daily consumption of at least 100g of product (s) containing between 10^6 and 10^7 viable cells.g^{-1}, is required and this has been suggested as the minimum intake required to provide a therapeutic effect (Blanchette *et al*., 1995; Gomes and Malcata, 1999).

This standard, however, appears to be adapted to provide bacterial concentrations that are technologically attainable and cost-effective rather than to achieve a specific health effect in humans (Roy, 2001). Currently, data pertaining to specific health benefits attributed to probiotic microorganisms is insufficient. Gilliland (2001) stated that this is especially the case in the United Sates where health claims regarding probiotic organisms are associated with dairy products.

Before a health claim can be made, clinical trials have to be carried out in order to establish whether the benefits originate from the presence of a particular probiotic strain or not.

1.2.7. Clinical trials

Various clinical studies have indicated beneficial effects caused by probiotic activity. A tentative list of therapeutic benefits attributed to the consumption of probiotics is detailed in Table 1. Though each of these effects have been supported by increasing evidence resulting from various *in vitro* and animal studies, the effects must also be supported by a number of human intervention trials, performed as a randomised double-blind placebo-controlled (traditional pharmacological) approach (Mercenier *et al*., 2002). Strains used in these studies belong to different microbial species, but predominantly include lactic acid bacteria (LAB) and bifidobacteria. Some of the clinical trials conducted to date included the following:

- **Improvement of lactose intolerance**

The inability of certain people to adequately digest lactose into its component sugars, glucose and galactose, is due to the absence of the β-galactosidase enzyme in the human intestine. The clinical importance is most predominant in young children leading to various degrees of abdominal discomfort, acidic diarrhoea, cramps and flatulence (Kim and Gilliland, 1983). Some LAB applied as starter cultures (*Lactobacillus acidopilus* and *Bifidobacterium bifidum*) in fermented dairy products are however, capable of producing this enzyme. Consequently, the presence of this enzyme leads to the hydrolyzation of lactose, resulting in increased tolerance for dairy products (Kim and Gilliland, 1983; Martini *et al*., 1991; Mercenier *et al*., 2002). Optimal effect and continuous utilisation of lactose are guaranteed through the continuous intake and establishment of live lactase containing bacteria.

- **Antibiotic Associated Diarrhoea (AAD)**

Approximately 20% of all individuals treated with antibiotics will develop antibiotic associated diarrhoea since the intestinal microflora responsible for natural resistance, are disrupted. Many probiotic preparations have been tested against the effects of AAD (Mercenier *et al*., 2002) with good effects, though more studies are needed using controlled strains and conditions.

- **Gastroenteritis**

Gastroenteritis, the main and most common cause of diarrhoea, can be viral, bacterial or parasitic of origin. Although a spontaneous recovery is possible within a few days by taking oral rehydration solutions, the use of probiotics could be considered from a preventative rather than therapeutic point of view (Elmer *et al*., 1996; Saavedra, 1995). One of the first studies conducted by Watkins and Miller (1983) illustrated that animals initially fed with *L. acidophilus* prior to challenge with pathogens, survived much better than those first challenged with the pathogen. Furthermore, continued feeding of *L. acidophilus* to animals exposed to pathogens was the best form of treatment.

- **Bacterial overgrowth**

Some studies indicated that a mild overgrowth of negative bacteria could be treated with lactobacilli (Attar *et al*., 1999; Mercenier *et al*., 2002). Irradiation of the abdomen, causing diarrhoea, has also been treated with probiotic administration (Salminen *et al*., 1988).

- **Inflammatory Bowl Disease (IBD) / Irritable Bowl Syndrome (IBS)**

The cause for this complex disease is not known, though it is believed that microbial, genetic and environmental factors, especially stress and poor diet are involved (Hendrickson *et al*., 2002; Mercenier *et al*., 2002). Due to its complexity, the application of probiotics should be studied with care, with special attention given to the fact that strain specific properties may be required for specific categories of patients. Cocktails of probiotic strains applied at specific doses may be developed for individual usage. A need also exists for a more mechanistic type of research, which is very important for effective selection of the most suitable strain for each specific patient and their condition (Mercenier *et al*., 2002).

- **Allergy reduction**

In recent years, the general occurrence of atopic (allergy-causing) diseases has progressively increased in Western societies where the hygiene hypothesis has not been abandoned (Mercenier *et al*., 2002). This hypothesis implies that the rapid increase in atopy is related to the minimised exposure to microbes at early stages in life, consequently, lowering the number of infection (Strachan, 1989). The preventative potential of probiotics has been demonstrated in a double-blind, placebo-controlled study conducted by Kalliomaki *et al*. (2001). Probiotics were administered pre- and pos-nattily for a period of six months to children highly susceptible to these diseases. A reduction up to 50% in occurrence in atopic diseases was achieved when compared to infants receiving placebo.

- **Colon cancer**

Colorectal cancer is diverse and diets have been indicated as main causative agents for this disease (Greenwald *et al*., 2001).

Diets including those high in meat and fat, and/or low in fibre, have been implicated in the changes that take place in the intestinal microflora. An increase in *Bacteriodes* and *Clostridium* levels and a decrease in levels of the beneficial *Bifidobacterium* were observed (Benno *et al*., 1991). These changes in the intestinal microbial population are due to the increase in faecal enzymatic activity. Faecal enzymes like ß-glucuronidase, azoreductase, urease, nitroreductase and glycoholic acid reductase, convert pro-carcinogens into carcinogens and may contribute to the development of colon cancer. It has been observed that through the consumption of certain selected probiotic lactobacilli, the amount of faecal enzymes is significantly reduced (Huis in't Veld *et al*., 1998; Ouwehand *et al*., 2002; Saarela *et al*., 2000). Kailasapathy and Rybka (1997) confirmed that the intake of fermented milk products containing probiotic bacteria, inhibit tumour formation and proliferation in animal studies. Whether it actually contributes to reduce the risk of cancer, remains unknown. Most, but not all epidemiological studies suggested that regular intake of fermented dairy products are related to reducing the risk of obtaining certain types of colon cancer (Hirayama and Rafter, 2000).

- **Control of serum cholesterol**

Hypercholesterolemia has been linked with increased risk of coronary heart disease, one of today's leading causes of death. The principle site for cholesterol metabolism is in the liver, although significant amounts are also formed within the intestines, making the use of probiotics very attractive. Claims based on numerous studies indicated that certain strains of *L. acidophilus* and some *Bifidobacterium* sp. are able to lower intestinal cholesterol levels. Several laboratories have investigated the relation between cholesterol and LAB consumption and speculated that *L. acidophilus* could remove cholesterol from laboratory media in the presence of bile. Klaver and Van der Meer (1993) however, argued that this was due to a bile salt–deconjugating activity.

A few human related studies also indicated the lowering of serum cholesterol levels during the consumption of yoghurt and fermented milk (Mercenier *et al*., 2002). Andersson *et al*. (1995) suggested that bile flow was indeed stimulated by regular consumption of fermented milk. These preliminary reports are most often not properly controlled and therefore do not promote the use of selected probiotic strains for this purpose.

1.3. APPLICATION OF PROBIOTICS

The potential for probiotic cultures to provide health and nutritional benefits to the consumer, was once a very debatable issue. Research done since the turn of the century has led to a better understanding and a considerable increase in the functional food market. In order to maintain the link between recognised good health and probiotic microorganisms, it has been suggested that the manipulation of the composition and metabolic activity of the intestinal microflora is necessary. This is done by introducing live bacteria or stimulating certain beneficial population groups.

1.3.1. Factors affecting the viability of probiotic species in bio-products

- **Product acidity**

One of the most compelling drawbacks associated with the use of probiotic cultures in the fermented milk industry are the reduced lack of acid tolerance of some of the species and strains (Klaver *et al*., 1993; Lourens and Viljoen, 2002). When the lactic acid content increases during fermentation, the pH levels correspondingly decreases, affecting the direct environment and thus also the viability of beneficial bacteria. According to Hood and Zotolla (1988) *L. acidophilus* grows and survives better at pH 4.0 than at pH 2.0. Martin and Chou (1992) reported that a pH range of 5.5 – 5.6 was the minimum pH for survival of bifidobacteria, whereas a pH lower than 4.6 and higher than 8.0 would not support growth of certain species/strains. Overall, it would appear that tolerance to low pH for extended periods is not a common trait amongst *Bifidobacterium* strains. It was however illustrated by Crittenden *et al*. (2001) that *Bifidobacterium lactis* Bp-12 survived well in gastro-intestinal models and has been demonstrated to survive intestinal transit in humans (Fukushima *et al*., 1997; Hove *et al*., 1994; Mattila-Sandholm *et al*., 1999).

A study conducted by Maus and Ingham (2003) illustrated that the acid-tolerance of *B. lactis* increased significantly when the pH of the growth medium was decreased from 6.0 to a value of 5.2. It should however be emphasised that survival of bifidobacteria at the pH values of fermented dairy products and gastric fluid varies dramatically and that stress-responses (i.e. lowered pH) can be species-dependent (Maus and Ingham, 2003). In practical application, the pH value of the final product must be maintained above 4.6 in order to prevent a rapid decline in bifidobaterial populations (Laroia and Martin, 1991a,b; Modler *et al*., 1990; Tamime and Robinson, 1985).

- **Species/strains**

Various species of both lactobacilli and bifidobacteria diminish markedly during refrigerated storage at low pH levels, while others succeed to maintain their viability and even tend to increase in population. Another strain selective property of bifidobacteria is the ability to grow in milk. Many strains are unable to grow in milk due to lack of protease activity. Careful strain selection and monitoring are therefore necessary for high quality fermented bio-products (Gilliland and Lara, 1988; Hughes and Hoover, 1995; Klaver *et al*., 1990, 1993; Shah *et al*., 1995).

- **Co-culture and species interaction (s)**

The composition of the species participating in fermentation within the same carrier food has been found to affect the survival and the growth of *L. acidophilus* and *B. bifidum* and subsequently, the quality of the probiotic product. The starter cultures used might improve the growth conditions of the probiotic cultures by producing substances favourable to their growth. Most strains of *Bifidobacterium* lack proteolytic activity (Klaver *et al*., 1993), therefore *L. acidophilus* lives in excellent symbiosis with bifidobacteria providing the necessary stimulants for growth (Hansen, 1985).

Gomes *et al*. (1998) reported that the growth rate and acidification by *B. lactis* are enhanced when co-cultured with *L. acidophilus*. Aerobic microorganisms act as oxygen scavengers and therefore creating a favourable growth environment for the anaerobic *Bifidobacterium* species (Ishibashi and Shimamura, 1993; Shankar and Davies, 1976; Van den Tempel *et al*., 2002). It should however be emphasized that in order to select suitable starter cultures for co-culturing with probiotic bacteria, the negative impacts should also be taken into consideration. In doing so, the most appropriate starter-probiotic interaction could be achieved for improved product functionality.

- **Inoculation size**

An important factor in ensuring a sufficient amount of viable cells within the final product is the inoculum size of probiotic bacteria. It is therefore essential that the manufactures of probiotic products ensure that at least one million viable cells/g are present at the end of fermentation (Samona and Robinson, 1994) and more than 10^6 cfu/ml of viable probiotic cells at the time of consumption (Dave and Shah, 1997; Robinson, 1987; Rybka and Kailasapathy, 1995). It has been indicated that using a high inoculum level of probiotic organisms will ensure a high cell count at the end of the incubation period as well as sufficient survival during storage (Samona and Robinson, 1994). According to Dave and Shah (1997), however, an increased inoculum size did not improve the viability of bifidobacteria in yoghurt. Varnam and Sutherland (1994) recommended an initial inoculum level of 10-20%. The numbers required may vary from species to species and even among strains within the same species.

- **Temperature**

Kneifel *et al*. (1993) reported that storage temperature substantially influences lactic acid production, relative to the growth and survival of starter cultures at high temperatures.

Furthermore, the storage temperature plays an important role in the control of excessive growth of microorganisms responsible for over-acidification of the products (Kneifel *et al*., 1993). According to Hughes and Hoover (1995), bifidobacteria are less tolerant to low storage temperatures when compared to *L. acidophilus*. Gomes *et al*. (1998) illustrated that pure cultures of *B. lactis* exhibited no statistically significant loss of viability in milk when stored at temperatures ranging from 5-15^{o}C, however, when co-cultured with *L. acidophilus*, *B. lactis* was significantly less tolerant to increasing storage temperatures. Crittenden *et al*. (2001) also suggested that elevated temperatures used during yoghurt manufacture did not adversely affect the growth and survival of certain *B. lactis* isolates.

- **Dissolved oxygen**

Bifidobacteria are considered to be strict anaerobic intestinal bacteria, which are unable to grow at the surface of agar plates in the presence of air (Meile *et al*., 1997). Oxygen toxicity is, thus, an important and critical problem for most of the *Bifidobacterium* species (Klaver *et al*., 1993). During production of fermented milk products, oxygen easily penetrates and dissolves into milk (Ishibashi and Shimamura, 1993). In order to overcome this obstacle, it has been proposed that bifidobacteria be introduced at a later stage during the cheese making process (Dinikar and Mistry, 1994). During storage, however, oxygen also permeates through the packaging material. To avoid the problem of oxygen toxicity, the simultaneous inoculation of microorganisms with high oxygen utilisation ability and *Bifidobacterium* species (Ishibashi and Shimamura, 1993) or the use of selected strains that are more oxygen tolerant, has been suggested. The degree of oxygen tolerance, however, depends on the species and culture medium and even on the morphology of the stains, such as whether they are branched or not (Boylston, 2004). The recently identified *B. lactis* strain was able to tolerate elevated oxygen concentrations of above 5% and proof to be a promising candidate for incorporation into fermented dairy products (Meile *et al*., 1997).

1.4. EXPANSION OF THE PROBIOTIC FOOD RANGE: APPLICATION OF PROBIOTIC CULTURES INTO CHEESE

To date, the most popular food delivery systems have been fermented milk products, such as bio-yoghurts and fermented milk, as well as unfermented milk with added cultures (Bourlioux and Pochart, 1988; Fernandes *et al*., 1987; Sanders *et al*., 1996). A small number of researchers and companies have endeavoured to expand the probiotic product range by manufacturing cheeses that are high in viable probiotic cultures. The incorporation of the health promoting cultures into cheese would only result in functional foods if the culture (s) are able to maintain viability during ripening and if the added culture (s) do not adversely affect the composition, texture or sensory criteria of the products (Stanton *et al*., 1998). In doing so, it is compulsory to understand the growth characteristics of the probiotic cultures in question so that the processing conditions can be manipulated to optimize their survival. Gomes *et al*. (1995) used bifidobacteria in combination with *L. acidophilus* strain Ki as the starters in Gouda cheese manufacture. Both the species survived very well and their application in Gouda cheese was suggested. After nine weeks of ripening, however, a significant defect in the cheese flavour was detected, probably due to the production of acetic acid by bifidobacteria. A study conducted by Blanchette *et al*. (1996), illustrated that cottage cheese do not support a high viability of *Bifidobacterium infantis* that had been introduced during manufacturing. A large decline of viability was observed after 15 days of storage at normal shelf-life temperature (4^0C) of the product. In addition, consumers showed preference to the control cheese over the model cheese with added bifidobacteria. *Lactobacillus reuteri, L. rhamnosus, L. acidophilus and B. bifidum* cultures were used for the production of a soft, fresh cheese (Nayra *et al*., 2002). The organisms remained above the therapeutic minimum for 2 months. Different combinations of *Bifidobacterium* and *Lactobacillus* species showed satisfactory viability in Argentinean Fresco cheese during storage of 60 days (Vinderola *et al*., 2000).

1.5. CHEDDAR CHEESE AS PROBIOTIC CARRIER FOOD

Cheddar cheese, as a delivery system for live probiotics into the GIT of humans, has certain advantages over the systems used to date. Having a higher pH (4.8 – 5.6) than the most probiotic carrier foods, it may provide a more stable environment to support the long-term survival of probiotic organisms (Van den Tempel *et al*., 2002). The matrix and the high fat content of the cheese may offer protection to the fragile organisms during passage through the GIT (Stanton *et al*., 1998). Dinikar and Mistry, (1994) reported that the oxygen toxicity problem may be overcome by introducing bifidobacteria at a later stage of cheese making, such as milling or salting. Furthermore, the metabolism of the microorganisms within the cheese results in an almost anaerobic environment within a few weeks of ripening, favouring the growth and survival of bifidobacteria and other anaerobic microorganisms (Van den Tempel *et al*., 2002).

Bifidobacterium bifidum was successfully incorporated into Cheddar cheese as a starter adjunct (Dinakar and Mistry, 1994). The strain survived well in the cheese and retained viability of approximately 2 x 10^7 cfu/g of cheese after a 6 month ripening period, without adversely affecting the flavour, texture and /or the appearance of the cheese. Stanton *et al*. (1998) as well as Gardiner *et al*. (1998) illustrated that Cheddar cheese can be an effective vehicle for the delivery of some *L. paracasei* strains to the consumer without any negative impact on the cheese quality, aroma, flavour and/or texture. McBrearty *et al*. (2001) also demonstrated Cheddar cheese to be a suitable carrier food for the delivery of some commercially available strains of probiotic bifidobacteria to the consumer. *Lactobcillus paracasei* NFBC 338 Rif ® remained highly viable during a 3 month ripening period of Cheddar cheese, without affecting the cheese quality (Gardiner *et al*., 2002). This again suggests that Cheddar cheese could provide a suitable environment for the maintenance of probiotic organisms at high levels over long periods of time.

Throughout the world, cheeses are consumed on a regular daily basis, making it an excellent delivery system for beneficial probiotic organisms (Boylston *et al*., 2004). The possibility of manufacturing a probiotic cheese with little or no alteration to the cheese making technology, would make the development of probiotic cheese very attractive for commercial exploitation. It would expand the probiotic product range, with cheese industries benefiting from marketing advantages such as value-added probiotic containing cheeses.

1.6. ENUMERATION OF PROBIOTIC MICROORGANISMS IN FUNCTIONAL FOODS

Not only are there challenges regarding the viability of probiotic bacteria, there are also similar challenges related to its enumeration. The ability to accurately enumerate specific probiotic species in the presence of other LAB is crucial in assessing the health benefits and determining whether the products will provide therapeutic effects. As expected, variations in probiotic response currently encountered within the industry, are mainly due to factors affecting the physiological conditions of the host or the quality of the probiotic product itself. Administration of probiotic microorganisms at levels too low to be effective, improper identification of used strains, and the failure to validate counts of microorganisms in test products, have all contributed to difficulties in the industry regarding interpreting results. The general presumption made is that the viability of probiotic bacteria is a reasonable measure of probiotic activity, for it usefully indicates the numbers of cells present. This is certainly a defensible assumption for situations where probiotic viability is not required for probiotic activity. This includes the digestion of lactose and some immune system modulation activities (Boylston *et al*., 2004). The fact still remains that probiotic products are standardized based on viable counts and is therefore the factor to consider in the product's functionality. It is obvious that despite the progress made over the last decade, large gaps still exist in our experimental set-ups, directly affecting industrial quality control systems.

1.6.1. Enumeration media

Currently, the lack of standardised methods for monitoring levels of probiotic bacteria in dairy products has caused difficulties in routine quality control and in the establishment and monitoring of official administration levels (Vinderola and Reinheimer, 1999).

Consequently, the introduction of rapid and reliable techniques for identification and enumeration of probiotic species, alone or together with other starter lactic acid bacteria (sLAB) has become essential in the dairy industry. Charteris *et al.* (1997) emphasized that quality programs in the research, development, production and validation of health benefits of probiotic products, require microbiological procedures for the detection, identification and differential enumeration of probiotic microorganisms. These methods are needed for routine control of initial levels of probiotic bacteria after manufacture and to predict the storage period these organisms can withstand in order to remain viable within the product distribution chain.

Several culture media have been proposed for the isolation and differential / selective enumeration of bifidobacteria and lactobacilli species in fermented dairy products. Table 2 illustrates various media proposed in literature for the differential enumeration of *L. acidophilus* and the specific enumeration of *Bifidobacterium* species. Media for culturing these organisms can be divided into three groups: *(a)* general media, e.g. MRS medium (deMan *et al*., 1960) for an overall total colony count without differentiating between different genera or species, *(b)* selective growth media, allowing selective growth of a particular genus, i.e. NNLP agar (comprising neomycin-nalidixic acid-lithium chloride-paramycin agar) for isolating *B. bifidum* (Laroia and Martin, 1991b) and M17 agar for *Streptococcus thermophilus* (Terzaghi and Sandine, 1975), and *(c)* differential media permitting the enumeration of various species on the same media (Teraguchi *et al*., 1978). The range of different culture media used for the detection and enumeration of probiotic bacteria, however, indicates that there is no standard culture medium (Roy, 2001). The difficulties associated with the detection and enumeration, are caused by the strain specificity of results, the simultaneous use of different species in the product and differences found in cell recovery or colony differentiation.

The simultaneous presence of several species in fermented food products can make it difficult to achieve a differential or a selective colony count of each individual species, for there is an evident lack of resolution necessary for differentiation (Boylston *et al*., 2004).

1.6.2. Selectivity and strain specificity

A growing concern however, is that selective media containing selective agents (i.e. antibiotics, bile etc.) may also restrict the growth of *L. acidophilus* and *Bifidobacterium* species. Starter cultures generally used in the dairy industry include *S. thermopilus, B. bifidus* and *B. lactis* species. When enumerating *Bifidobacterium* sp. from yoghurt, the selective agents added to increase the selectivity of the medium, tend to affect the actual viable cell counts of the microorganism within the product. Wijsman *et al*. (1989) observed that the same mixture of antibiotics in NNLP agar inhibits the colony formation of bifidobacteria completely, whereas relative lower concentrations (up to 30%) had no effect. This indeed, suggests a need to countercheck the efficacy of this medium. Literature and studies conducted by the International Dairy Federation (IDF), indicate that the media for isolation and enumeration of *Bifdobacteruim* spp. are very strain specific (IDF Bulletin 340, Group E140; Roy, 2001). Although, the standard medium accepted by the IDF is NNLP agar (IDF, International Standard 149A, 1991), the contrary was illustrated in a study conducted by Group E104. A wide variety of routinely used laboratory media, including NNLP, were compared. Statistical analysis of the results illustrated that no medium, including NNLP agar, appeared to be valid due to the great variability of the strains on the given media. Despite being regarded as an internationally recognised standard medium for the enumeration and isolation of bifidobacteria, numerous discrepancies regarding its application still prevails. These include the following: preliminary trails illustrating that the recovery of some of the bifidobacteria strains was too low, long incubation periods, very time-consuming preparation, complex to prepare as it contains 24 ingredients, and therefore a very expensive medium.

Based on these criteria, NNLP agar was not included in a comparative study done by Bonaparte (1997) and, to date, still hinders its routine use for enumeration purposes. The inhibitory effect caused by antibiotics, was also mentioned by various other authors (Lim *et al*., 1995; Pacher and Kneifel, 1996). Studies conducted by Group E104 illustrated that MRS+++ agar (MRS agar + L-cysteine + LiCl + Na-propionate) retained an almost full recovery, rendering it a more suitable medium for enumeration of bifidobacteria (IDF Bulletin 340, Group E104). Conflicting proposals are also present in the enumeration of *L. acidophilus*. According to Chr. Hansen's Laboratory, MRS-Maltose is the preferred medium for differential enumeration of *L. acidophilus* (Anon, 1994). On the other hand, Ingham (1999) suggested that modified *Lactobacillus* (mLBS) medium is the ideal medium with a potential industrial application. Industrial applications in routine enumeration, however, require a medium to be relatively inexpensive, convenient to use and obtain, should offer a good cell recovery, and for which validated scientific standards exist.

Apparent distinctions exist between countries world wide regarding methods and media to be used for identification and enumeration of probiotic species in dairy products. The final selection of media, however, still depends on the type of food, species and/or strains used, inhibitory components present in selective media as well as the nature of the other competing genera.

1.7. PROBIOTIC REGULATIONS

The future of products containing probiotics strongly relies on the regulatory framework within which they are to be marketed. Currently, one of the main problems in the industry is the lack of standardised legislation regarding product labelling, health claims, enumeration etc. of the species used as probiotics. An acceptable regulatory system allows products to comply with the limits set within the law, but also addresses consumer needs as well as targeting the acceptability by the general public. Standard and valid product labelling protect the consumer from information that could potentially be misleading and incorrect. Consequently, confusion regarding claims, particularly health claims, is addressed and minimised. In an ISO Bulletin report (July 2000), it was stated that since trade handling, and especially application of milk and milk products are carried out on a world wide scale, standards preferentially need to be developed on an international level. Standard methods are not only used for quality compliance of milk products to legal requirements, but are also for the development of independent, valid and reliable methods. Reliable international standards for methods of analysis and sampling are the tools that assure quality control. The implementation of quality systems within the processing chain or via official authorities must be reliable in order to guarantee consumer confidence in the products. A “World wide agreement on methods means worldwide quality systems” (ISO Bulletin, February 2003).

1.7.1. World perspectives and regulatory platforms

No country other than Japan, has such a progressive approach to functional foods. Possessing an affirmed legal status and specific labelling benefits, numerous functional foods (FOSHU; Foods for Specified Health Use) are currently on the Japanese market. On the other hand, despite a high corporate interest for functional foods in the United States (US), there is no legal definition for it.

Product success can be attributed to efficacy, safety and by targeting specific health issues (i.e. cancer, obesity, high cholesterol, etc.) that the American consumers are concerned about. Apart from the US and Japan, Europe also has an existing trend for these foods. Grijspaardt-Vink (1996) identified health and convenience as the two most important trends in the European market. An increasing trend in the functional food market has also been apparent in South Africa. It is however, increasingly evident that this country is, with regards to probiotic legislation, one step ahead compared to the rest of the world. A brief review on current legislation and regulatory systems in various countries and states are discussed in the following sections.

- **South Africa**

To date, South Africa is the only country with a specified legislative system in place. Though in a draft format and still open for discussion (to scientists, authority representatives of food surveillance, and economy and consumer associations), South Africa has a head start when compared to other countries. This country has established an acceptable definition for the term ‘probiotic’ meaning; “live organisms indigenous to the human intestinal tract, which, when consumed in adequate numbers, beneficially affect the health and functioning of the host’s intestinal tract by modulating mucosal and systematic immunity as well as improving the nutritional and microbial balance and are therefore considered a dietary adjuvant and which are used in nutritional supplements for their therapeutic properties or added to foodstuffs for their prophylactic and health enhancing properties” (Regulations on probiotics in South Africa: An abstract from the new draft, 2004). Various aspects regarding prerequisites for health claims, identification of genus, species and strains, screening for safety of strains, efficacy assessments and other general information are discussed within this draft. For the purpose of this review, however, these will not be discussed.

- **The European Union (EU)**

The European regulatory environment specific for LAB (e.g. *Lactobacillus* and *Bifidobacterium* spp.) is probably one of the most diverse, due to the legislative variety existing amongst its Member States and the fact that it is only gradually being harmonised. Having a collaborative character where different partners from various sectors (such as the food industry, official control laboratories, private and official research organizations, universities, consumer related organizations and legislators) work together, makes Europe difficult for the establishment of a satisfactory regulatory system. There is still no formal definition for functional foods at European Commission (EC) level, although it is generally understood that the term encompasses the day-to-day food consumed as part of a normal diet and not food supplements (Gibson *et al*., 2000). Probiotics fall within a grey area between food and medicines in many European countries. The Veterinary experts for food and food hygiene (ALTS) working group, initiated a working group (Probiotic cultures of microorganisms in food) in Germany (1997). This working group, represented by scientists, representatives of the authorities for food surveillance, economy and consumer's associations, attempts to find solutions for discrepancies and questions arising from probiotic containing products. Apart from the above, guidelines from yet another working group, FAO/WHO (Guidelines for the evaluation of probiotics in food, 2002), are also taken into account. The opinions of these working groups are the current guidelines followed, though not regarded as specific legislation.

- **The United States of America (USA)**

The USA still has a long way to go concerning probiotic regulations. No legal definition for functional foods exits, in fact, the USA has not defined a concrete definition for regulatory purposes. It should, however, also be emphasised that from a legislative point of view, there is no explicit recognition of any health benefits proposed by probiotics.

Though it is generally believed that no legislation regarding probiotics exists, the US Food and Drug Administration (FDA) is the current regulatory system who manages all probiotic and related issues (i.e. health claims etc.). The FDA approves and classifies food and food supplements into the categories summarised in Table 3. At present there is still confusion in this classification system, for there is no clear distinction as to whether probiotics are considered to be a food supplement, a dietary food or a medicinal food.

- **Japan**

Japan is considered to be the 'home' of the functional food concept and is in the unique position of having a regulatory program in place for the explicit approval of functional foods. A FOSHU food product is defined as food which is 'expected to have certain health benefits and has been licensed to bear a label to that effect' (Shinohara, 1995). Thereby, permission is granted to put a health claim on a food package along with a nationally known symbol (Fig. 2). To be labelled as suitable for specified health use, foods must go through an approval procedure. The Ministry of Health and Welfare hands out this authority upon the approval of a scientific dossier which fully substantiates the claim(s) made. The procedure though, has been under review ever since and is considered to be too complex for full effectiveness. This type of legislation is beneficial to both the producer, who has to discriminate between research-supported products and products without, and the consumer who subsequently is protected against non-approved claims.

1.7.2. Regulations regarding administration levels of probiotic organisms

- **South Africa**

The current South African draft (2004) on the legislation of probiotics, states that the preferred standardised method for analysis is that depicted by the IDF.

According to the IDF, the number of viable cells expressed as colony forming units per gram (cfu/g) must meet the minimum specifications claimed by the starter culture manufacturer and/or supplier. Concentrated starter cultures (liquid, frozen or dried) should contain a minimum of 5 x 10^9 cfu/g whereas unconcentrated starter cultures, a minimum of 1 x 10^8 cfu/g viable bacteria are required (IDF, International Standard 149A, 1997). The standards and conditions accompanying the application of probiotics in South Africa are illustrated in Table 4.

- **The European Union (EU)**

Though no legislation regarding probiotics exists in Europe at present, there is a publication of a working group, which acts as guidelines regarding this matter in Germany (Germany - Publication of a working group on probiotics, May 2003). With regard to the use of probiotics in food and the desired positive effect on humans, the importance of the minimum cell count (‘therapeutic minimum’) in probiotic products is well recognised, however, no standardised amounts have definitely been determined. Clinical studies usually propose a certain quantity of viable microorganisms to be administered daily, including the amount per kg weight of the particular patient. At present, it does not seem reasonable to determine a defined ‘therapeutic minimum’ since meaningful clinical data are not available. Experience in clinical studies illustrated that even *in vitro,* the physiological effects of the metabolic activities of the bacteria are relevant and measurable only at values above 10^6 cfu/g of a product. (Germany- publication of a working group on probiotics, May 2003). For most products, depending on the amount consumed and its shelf-life, it is necessary to be consumed on a regular basis. It was estimated that an approximate daily amount of 10^8 – 10^9 cfu/g need to be obtained to elicit a ‘probiotic effect’ (Germany-publication of a working group on probiotics, May 2003).

- **The United States of America (USA)**

No formal probiotic legislation exists in the USA. Guidelines from the joint FAO/WHO working group are currently in practice, though no mention of exact 'therapeutic minimum' standards are present. The compositional standards for milk and milk products, developed under the protection of the Codex Alimentarius Commission (Joint FAO/WHO Food Standards Programme) strongly rely on standards for methods of analysis and sampling provided by the IDF. Therefore, standards regarding the 'therapeutic minimum' in the USA correspond with standards mentioned by the IDF (1997).

- **Japan**

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/ml to be present in fresh dairy products. To go through the approval process in obtaining a license, FOSHU food manufacturers must compile scientific data (including appropriate level of intake) on the food or food compound of interest to be submitted to the Ministry of Health and Welfare (Berner and O'Donnell, 1998).

1.8. CONCLUSION

In this climate of increasing consumer awareness that diet and health are inseparable, research on probiotics remains a more fascinating challenge than ever. Despite all the current scientific problems, many researchers in this field are gradually accepting the idea that probiotics will help a high number of patients / people in the future. It is however, evident that the regulatory processes are trailing behind with respect to the vast development of new products, in some countries to a higher extend than others. The South African legislation, still in its early developing stages, seems to be the most effective, being one step ahead compared to regulatory systems of the world. Differences in standard policies regarding enumeration and identification media, health claims etc. in various countries world wide are causing disharmony, affecting the future of the probiotic market. In reviewing media for the enumeration and identification of *Bifidobacterium* sp., it seems that NNLP is not as good a medium as initially anticipated. MRS+++ media, instead, would be recommended, for it is less expensive, less complex, and less time consuming with an evident higher recovery rate. In the study conducted on the enumeration and identification of *L. acidophilus,* contrasting ideas prevail. In conclusion, I would recommend that in order to develop a universal, validated and standardised system of analysis, the responsibility lies with the producers of probiotic products. Thorough clinical studies should be conducted on each possible strain and in the process developing the best possible medium for its specific isolation. A detailed description of the microbial content, as well as its specific isolation media must be associated with each product. Thereby, eliminating problems that are currently arising during routine analysis in research laboratories within the industry. Whether legislative systems will be implemented accordingly, still remains to be seen.

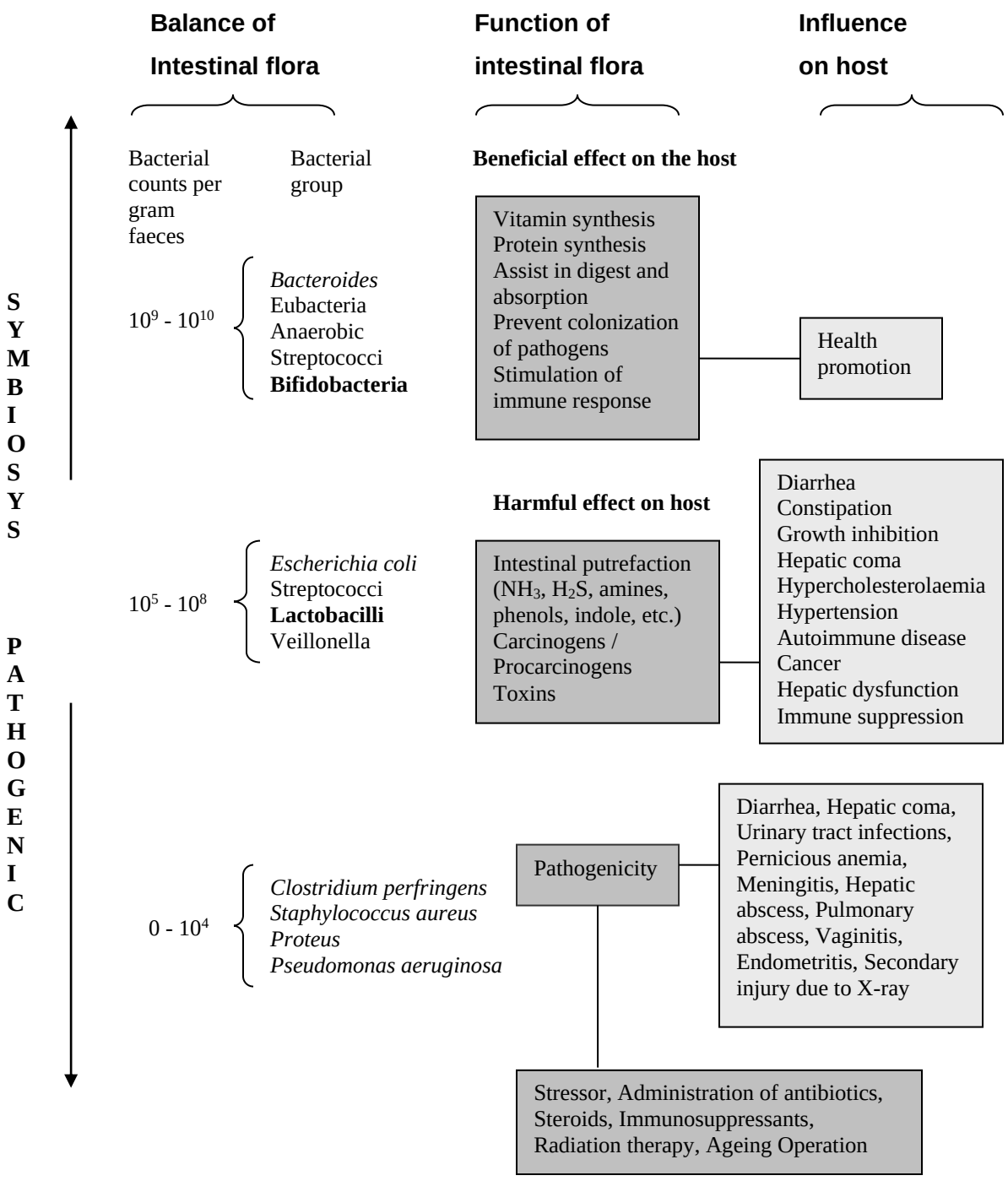

Fig.1. The interrelationship between intestinal bacteria and human health as proposed by Mitsuoka (Ishibashi and Shimamura, 1993).

Table 1. Beneficial health effects attributed to lactic acid bacteria (LAB) (adapted from Mercenier *et al*., 2002).

Health benefit :	**Proposed mechanism (s)**
• Alleviation of lactose intolerance :	
	- Bacterial β-galactosidase acts on lactose
• Positive influence on intestinal flora :	
	- Lactobacilli influence activity of overgrowth flora, decreasing toxic metabolite production
	- Antibacterial characteristics (Antimicrobial substances-organic acids, hydrogen peroxide, bacteriocins, antibiotics and decojugated bile salts, antagonistic reactions)
• Prevention of intestinal infections :	
	- Adjuvant effect increasing antibody production
	- Stimulation of the systematic or secretory immune response
	- Competitive exclusion
	- Alteration of intestinal conditions to be less favorable for pathogenicity (pH, short chain fatty acids, bacteriocins)
	- Alteration of toxin binding sites
	- Gut flora alteration
	- Adherence to intestinal mucosa, preventing pathogen adherence
	- Competition for nutrients
• Improvement of the immune system :	
	- Strengthening of non-specific defense against infection
	- Increased phagocytic activity of white blood cells
	- Increased serum IgA after attenuated *Salmonella typhimurium* challenge
	- Increase in IgA production
	- Proliferation of intra-epithelial lympocytes
	- Adjuvant effect in antigen-specific immune responses
	- Regulation of the Th1/Th2 balance, induction of cytokine synthesis
• Reduction of inflammatory or allergic reactions :	
	- Restoration of the homeostasis of the immune system
	- Regulation of cytikine synthesis
	- Prevention of antigen translocation into blood stream
• Anti-colon cancer effect	
	- Mutagen binding
	- Carcinogen deactivation
	- Alteration of activity of colonic microbes
	- Immune response
	- Influence on secondary bile salt concentration

(Table 1.) contd. …

- Blood, lipids, heart disease :
  - Assimilation of cholesterol
  - Alteration of activity bile salt hydrolase enzyme
  - Antioxidative effect
- Antihypertensive effect :
  - Peptidase action on milk results in antihypertensive (angiotensin converting enzyme inhibitors)
  - Cell wall components acts as angiotensin converting inhibitors
- Urogenital infections :
  - Adhesion to urinary and vaginal tract cells
  - Competitive exclusion
  - Inhibitor production (H_2O_2, biosurfactants)
- Infections caused by *Campylobacter pylori* :
  - Competitive exclusion
  - Lactic acid production
  - Decreased urease activity of *H. pylori* in humans after administration of a supernatant of a *Lactobacillus* culture
- Regulation of gut motility (constipation)
- Feeling of well-being

Table 2. Selective and/or differential media for the enumeration of *L. acidophilus* and *Bifidobacterium* sp. (adapted from Lourens-Hattingh and Viljoen, 2001).

Bacterial Group	Agar medium	Reference
L. acidophilus	LBSO (*Lactobacillus* Selective agar with Oxgall)	Gilliland and Speck (1997)
	PCA (Plate Count Agar)	Collins (1978)
	TPPY (Tryptose-Proteose-Peptone Yeast extract)	Bracquart (1981)
	MRS (deMan, Rogosa, and Sharp)	Coker and Martley (1982)
	MRS-Maltose	Hull and Roberts (1984)
	EC (Esculin-Cellobiose)	Von Hunger (1986)
	X-Glu	Kneifel and Pacher (1993)
	MNA + Salicin (Minimal Nutrient Agar)	Lankaputhara and Shah (1996)
	MLBS (LBS agar + tomato juice and acetic acid)	Ingham (1999)
Bifidobacterium	RCPB (Reinforced Clostridial agar with Prussian Blue)	Van der Wiel-Korstanje and Winkler (1970)
	M17	Terzaghi and Sandine (1975)
	Modified NNLP	Teraguchi *et al*. (1978).
	PSM (Petuely's Selective Medium)	Tanaka and Mutai (1980)
	YN-6	Resnick and Levin (1981)
	YN-17	Mara and Oragui (1983)
	TPY agar	Scardovi (1986)
	TOS (Transgalactosylated Oligo Saccharide)	Wijsman *et al*. (1989).
	L-Arabinose	Wijsman *et al*. (1989)
	TOS-NNLP	Wijsman *et al*. (1989)
	Modified Columbia	Beerens (1990)
	X- Ÿ -gal (5-bromo-4-chloor-3-indolyl-α -galactoside)	Chevalier *et al*. (1991)
	NPNL medium	IDF(1991)
	LP (Lithium chloride-sodium Propionate)	Lapierre *et al*. (1992)
	LP-MRS	Vinderola and Reinheimer (1999)
	BL-OG (Blood glucose liver + Oxgall + Gentamicin)	Lim *et al*. (1995)
	Modified HBSA	Arany *et al*. (1995)
	Rogosa agar	Tamine *et al.* (1995)
	Bif' (*Bifidobacterium*)	Pacher and Kneifel (1996)
	RB (Raffinose-Bifido)	Hartemink *et al*. (1996)
	DP (Columbia agar + Propionic acid + Diclxacillin)	Roy (2001)
	Raf 5.1	Roy *et al.* (1997)
	MBIM (Modified *Bifidobacterium* Iodoacetate Medium)	Ingham (1999)
	BFM (*Bifidobacterium* Medium)	Nebra and Blanch (1999)

(Table 2.) contd. …

	Modified TPY agar	Rada and Petr (2000)
	Wilkins-Chalgren (WC) agar	Rada and Petr (2000)
	Wilkins-Chalgren (WC) agar + Neomycin	Rada and Koc (2001)
	Wilkens-Chalgren (WC) agar + Mupirocin	Rada and Koc (2000)
	Tomato juice agar	Rada and Petr (2000)
	Modified MRS	Rada and Petr (2000)
	RCM (Reinforced Clostridial Medium)	Rada and Petr (2000)
	NNLP	Roy (2001), Hartemink *et al*. (1999)
	MRS-NNLP / MRS+++	Tharmaraj and Shah (2003)
	AMRS (Acidified MRS)	Leuschner *et al*. (2003)
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)

Fig. 2. The Food for Specified Health Use (FOSHU) label in Japan (Mercenier *et al*., 2002).

Table 3. FDA-defined categories of foods and dietary supplements (Berner and O'Donnell, 1998).

Regulatory Category	Brief Definition
Conventional Foods	Foods, beverages, and chewing gum not defined by one of the following categories.
Foods for special dietary use	To supply particular dietary needs existing due to age, physiological conditions, food allergy, underweight and overweight.
Medicinal Foods	For specific dietary management of disease or condition for which there are distinctive nutritional requirements; to be used under doctor supervision during active or ongoing treatment for the condition.
Dietary supplements	A product intended to supplement the diet (must be labeled as dietary supplement); includes but not limited to vitamins, minerals, herbs, other botanical amino acids.

Table 4. Standards and conditions for application of probiotics in South Africa (Modified from the Regulations on probiotics in South Africa: An abstract from the new draft, 1 July, 2004)

Permissible Information	Conditions
Regarding foods for individuals older than 1 year	
When ingested on a regular basis as part of a prudent, balanced diet, probiotics improve the microbial balance in the human intestines and the functioning of the digestive tract. By inhibiting the growth of harmful (pathogenic) microorganisms, assisting in the digestion of lactose, normalizing bowel movement and stimulating the functions of the human immune system, they significantly improve general health.	The viable count of probiotic bacteria shall exceed 1 x 10^8 cfu per 100 ml of foodstuff at the end of the shelf-life period. Only live, selected strains with premarket approval for their confirmed probiotic properties shall be permitted in accordance with the requirements of Regulation 63. The following species do no need premarket approval: - *Lactobacillus acidophilus* - *Lactobacillus casei* - *Lactobacillus rhamnosus* - *Bifidobacterium bifidum* - *Bifidobacterium longum* - *Bifidobacterium lactis* - *Lactococcus lactis*
Regarding foods and formula for infants younger than 1 year	
	The probiotic bacterial count should exceed 10^8 cfu/ single serving of foodstuff at the end of the shelf-life period. Permitted strains are: - Live *Bifidobacterium infantis* - or *Bifidobacterium longum* only.

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CHAPTER 2

Statistical analysis of enumeration media for probiotic adjunct *Lactobacillus rhamnosus* HN001 (DR20^{TM}) in dairy products

ABSTRACT

Nine bacteriological media (and combinations thereof) were evaluated to assess their suitability and to selectively enumerate *Lactobacillus rhamnosus* strain HN001 (DR20^{TM}), in the presence of other lactic acid starter bacteria, from a commercially produced South African matured Cheddar cheese. Bacteriological media evaluated included pH-modified MRS agar, MRS-vancomycin agar, sugar-based (such as rhamnose, esculin) media, and *L. casei* (LC) agar under aerobic and anaerobic incubation conditions. The suitability of the media was statistically compared based on significant differences in recovery between the different media (ANOVA, NCSS, 2004) as well as the interaction between the media and the availability of oxygen (Turkey-Kramer multiple comparison test, NCSS, 2004), compared to the non-selective MRS agar applied as reference medium. MRS-V and BA-R(20%)V agar were ranked superior based on qualitative and quantitative results compared to other evaluated media. MRS-vancomycin agar and aerobic incubation conditions at 43^{o}C for 48h proved to be the most selective medium to enumerate *L. rhamnosus* in the presence of other cheese starter cultures. Superiority was attributed to distinctive individual bacterial colonies and the inhibition of starter lactic acid bacteria.

(**Keywords** : selective media, *Lactobacillus rhamnosus*, Cheddar cheese, enumeration)

2.1. INTRODUCTION

The past decade has witnessed a strong expansion of the probiotic market and, in parallel to that, an increase in the number of research projects, addressing fundamental and applied aspects of probiotics. Probiotics can be defined as "living microorganisms, which upon ingestion in certain numbers, exert health benefits beyond inherent general nutrition" (Guarner and Shaafsma, 1998). A number of probiotic organisms incorporated in dairy products include *Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus rhamnosus, Bifidobacterium* sp. and *Propionibacterium* (Tharmaraj and Shah, 2003). Normal practise in manufacturing these probiotic products (i.e. yoghurt) is to incorporate the slow growing probiotic cultures with the yoghurt starter cultures (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*). The lactic acid bacterial starter cultures grow rapidly contributing to the fermentation, however, they are unable to withstand the gastric passage or to colonize in the gut (Shah and Jelen, 1990) and are therefore unlikely to provide any therapeutic benefits.

Providing health benefits to the consumer, probiotics should be present in a minimum concentration ('therapeutic minimum') of 10^6 cfu/g of product (Ouwehand *et al*., 2002; Shah, 2000). Standards regarding the 'therapeutic minimum' of probiotic bacteria in fermented milk products have been introduced by several food organizations world wide (Bibiloni *et al*., 2001; IDF, 1992). This standard, however, appears to have been adapted to provide bacterial concentrations that are technologically attainable and which are cost-effective rather than to achieve a specific health effect to the consumer (Roy, 2001). It therefore seems reasonable to assume that beneficial effects posed by probiotic bacteria can only be expected when viable cells are ingested. An important parameter in monitoring the official levels of viable probiotics, in assessing product quality is the ability to count probiotic bacteria differentially.

Enumeration of a single probiotic culture, using a selective medium where the cultured dairy product contains only a specific culture, poses negligible problems. However, most cheeses and other cultured dairy products also contain starter lactic acid bacteria (sLAB) (Lactococci, lactobacilli, streptococci etc.) (Boylston *et al*., 2004), which have similar growth characteristics and as a result complicating differentiation. In order to assess viability and survival of probiotics it is important to have an accurate, working method for the selective enumeration of these bacteria.

At present, survival estimates of probiotic bacteria in dairy products are conflicting. Various studies done on selective and/or differential media have reported low counts of probiotic bacteria (Anonymous, 1999; Shah *et al*., 1995; Shah *et al*., 2000), while others have encountered satisfactory results (Shin *et al*., 2000; Lourens *et al*., 2000). Variable counts have also been reported elsewhere (Dave and Shah, 1996; Pacher and Kneifel, 1996; Vinderola and Reinheimer, 1999; Vinderola *et al*., 2000). These contrasting reports, however, pose the likelihood of over and/or underestimation of probiotic bacterial counts, misleading the consumer. Consequently, dairy product manufacturers are very interested in techniques that can provide reliable counts of probiotic bacteria in their products. The range of different culture media used for the detection and enumeration of probiotic bacteria in fermented foods, indicates that there is no standard culture medium (Roy, 2001). Not all the proposed culture media give satisfactory results when either selectively / differentially enumerating a specific probiotic species in the presence of sLAB (Dave and Shah, 1996; Ghoddusi and Robinson, 1996; Samona and Robinson, 1991). Several media for the selective enumeration of *Bifidobacterium* sp. and *L. acidophilus* have previously been reported (Dave and Shah, 1996; Hull and Roberts, 1984; Hunger, 1986; Lankaputhra and Shah, 1996; Laroia and Martin, 1991; Shah, 1997, 2000; Wijsman *et al*., 1989). Only a few reports have described selective enumeration of *Lactobacillus casei* in the presence of other probiotic and yoghurt bacteria (Chapagne *et al*., 1997; Ravula and Shah, 1998).

Champagne *et al.* (1997) studied the selective enumeration of *L. casei* from probiotic products, based on a 15ºC incubation temperature for 14 days. However, an incubation period of 14 days may not be practical for the dairy industry if the population of *L. casei* is to be known within a short time. Ravula and Shah (1998) developed a medium, known as *L. casei* (LC) agar, for selective enumeration of this particular species. Talwalkar and Kailasapathy (2004) concluded that LC agar offers good selectivity and provides reliable counts of *L. casei* in fermented yoghurt products. Selective enumeration methods for *L. rhamnosus* have not been studied extensively, though it has been reported that MRS-vancomycin agar is suitable for the enumeration of *L. rhamnosus* in the presence of other probiotics and lactic acid starter bacteria (Tharmaraj and Shah, 2003).

The aim of this study, therefore, was to develop and evaluate the suitability of the various selective media used in population studies of probiotic bacteria in fermented dairy products in order to provide reliable counts of *L. rhamnosus*. The different selective media were assessed based on literature evidence of suitability for demonstrating a single colony type, whereas the differential media were examined for their ability to easily distinguish colonies of probiotic bacteria.

2.2. MATERIALS AND METHODS

2.2.1. Bacterial cultures and propagation

Lactobacillus rhamnosus HN001 (DR20^{TM}) / HOWARU Rhamnosus®, was obtained from Visbyvac DIP (Danisco, Cultor Niebüll GmbH, Denmark). Cheese starter cultures, RAO24 (*L. lactis* ssp. *lactis, L. lactis* ssp. *cremoris, S. salivarius* ssp. *thermophilus)*, were obtained from Texel Group Rhône (Dangé Saiut, Romain, France). All cultures were in the form of concentrated freeze-dried pellets, routinely stored at -4^{o}C and tested for purity using Gram stains. The freeze-dried bacterial pellets were anaerobically activated in 10-ml MRS (deMan, Rogosa, Sharpe) broth (Merck, Darmstadt, Germany) at 37^{o}C for a period of 6h. The culture mixture was subsequently transferred to an MRS (deMan, Rogosa, Sharpe) agar plate, incubated anaerobically at 37^{o}C until a dense 48h bacterial smear culture was observed. Anaerobic conditions were ensured and maintained throughout by means of gas generating kits (Merck, Darmstadt, Germany).

2.2.2. Media preparation

***Bacteriological diluent* :** Bacteriological diluent was prepared by dissolving 20g of bacteriological peptone (Merck, Darmstadt, Germany) in 1L of distilled water. The pH was adjusted (Cyberscan 500, Eutech Instruments, Singapore) to 7.0 ± 0.2 and aliquots of 9-ml prepared, followed by autoclaving at 121^{o}C for 15 min.

Media* : *MRS agar (reference), pH-modified (pH 5.20) MRS agar and MRS-vancomycin agar. MRS agar was prepared according to the manufacturers instructions. The pH of the agar was adjusted to 5.20 using 1.0 M HCl to obtain the pH-modified agar and autoclaved at 121^{o}C for 15min.

To prepare MRS-vancomycin *(MRS-V)* agar, 2-ml of membrane filtered sterile 0.05% (w/v) vancomycin (Sigma-Aldrig, Castle Hill, Australia) was aseptically transferred to 1L of MRS agar prior to pouring the agar medium.

Basal agar (BA-rhamnose agar, BA-rhamnose + vancomycin agar). Basal agar (BA) was prepared (composition: Tryptone, 10 $g.l^{-1}$; Lab Lemco powder, 10 $g.l^{-1}$; Yeast extract, 5 $g.l^{-1}$; Tween 80, 1 $g.l^{-1}$; K_2HPO_4, 2.6 $g.l^{-1}$; Sodium acetate, 5 $g.l^{-1}$; Tri-ammonium citrate, 2 $g.l^{-1}$; $MgSO_4.7H_2O$, 0.2 $g.l^{-1}$; $MnSO_4.4H_2O$, 0.05 $g.l^{-1}$; Bacteriological agar, 12 $g.l^{-1}$) and autoclaved at 121^oC for 15 min. To prepare BA supplemented with rhamnose (BA-R) (10%, 20%), 10-ml of sterile membrane filtered stock solutions of rhamnose (10%, 20%) were aseptically added to 90-ml of BA (final concentrations: 1%, 2% respectively) just before pouring the agar medium. In addition, for the preparation of BA supplemented with rhamnose and vancomycin (BA-RV), sterile membrane filtered vancomycin (0.05%) was aseptically added to the respective agar mediums prepared as described above.

Esculin-Cellobiose (EC) agar was prepared using the method described by Hunger (1986) whereas ***Lactobacillus casei (LC) agar*** was prepared using the method described by Ravula and Shah (1998).

Media (15-20ml) were poured into 90-mm Petri dishes and stored at room temperature for 24 - 48h to facilitate removal of excess surface water (plates for aerobic use). In addition, media plates for anaerobic enumeration purposes were stored in an anaerobic chamber to facilitate the removal of excess oxygen. Selective properties of all the respective media are illustrated in Table 1. Inoculated plates were incubated in triplicate at 43^oC for 48h under anaerobic incubation conditions.

2.2.3. Experimental procedure

In preparation of the inoculum, a pre-inoculum was prepared by inoculating 100-ml of MRS broth, with the 48h bacterial smear culture and incubated anaerobically at 37ºC for 10h. A 10-ml aliquot / 10% from the pre-inoculum was then aseptically transferred to 90-ml MRS broth in preparation of the final inoculum. The inoculum was incubated at 37ºC for a period of 5h until mid- to late-exponential phase was reached. All incubations were performed statically within an anaerobic chamber (Forma Scientific, Anaerobic Systems). The target inoculum level was 10% (v/v) / $\geq 10^6$ colony forming units per milliliter (cfu/ml) in order to comply to set limits of the 'therapeutic minimum'.

The active, homogenised cell suspension was serially diluted (10^{-1} – 10^{-7}) in 20% (w/v) bacteriological peptone diluent. Enumeration was carried out using the spread plate technique. All media plates were incubated aerobically as well as anaerobically at 43ºC for 48h, except for LC agar plates, which were incubated at 27ºC for 72h. Anaerobic conditions were ensured and maintained throughout by means of gas generating kits (Merck, Darmstadt, Germany). Plates containing 25 - 250 colonies were counted and recorded as cfu/ml of the culture. Colonies ≥0.5 mm in diameter were considered as developing colonies and included for enumeration purposes. Media generating the highest recovery of *L. rhamnosus* (pure culture) were selected for further studies to determine whether it can conclusively distinguish between *L. rhamnosus* in the presence of other lactic acid starter cultures (mixed culture). The mixed culture comprised of probiotic, *L. rhamnosus,* and cheese starter cultures (*L. lactis* ssp. *lactis, L. lactis* ssp. *cremoris, S. salivarius* ssp. *thermophilus*). All experiments and analysis were repeated at least three times. The results presented are the means of at least three replicates.

2.2.4. Statistical analysis

Means of viable bacterial counts (cfu/ml), standard deviations (Table 2) and ease of counting (EOC) colonies (Table 3) were indicated for each media in both experimental procedures (pure culture, mixed cultures respective). Only media where bacterial enumeration of *L. rhamnosus* was successful and/or easy were subjected to further statistical analysis. Analysis of variance (ANOVA) was carried out to determine whether significant differences exist between the different mediums (NCSS, 2004). The interaction between media and the availability of oxygen was further investigated by means of the Turkey-Kramer multiple comparison test ($\alpha = 0.05$) (NCSS, 2004).

2.3. RESULTS AND DISCUSSION

Table 2 shows the means of the viable counts of *L. rhamnosus* obtained from utilizing different selective media containing different inhibitory substances namely vancomycin and hydrochloric acid, and different carbohydrates namely rhamnose, ribose, cellobiose, and esculin. MRS, originally proposed by deMan, Rogosa, and Sharpe (deMan *et al*., 1960) was implemented as medium of reference in this study as well as in various other enumeration studies (Hartemink *et al*., 1997; Ingham, 1999; Leuschner *et al*., 2003; Shah, 2000; Temmerman *et al*., 2003; Vinderola and Reinheimer, 2000; Vinderola *et al*., 2002). This well-known rich, slightly acidic (pH 6.5), non-selective medium was especially formulated for the general growth and enumeration of *Lactobacillus* species. Lactobacilli are highly acid-resistant species, with growth being possible at an initial pH of 5.0 (Hammes and Vogel, 1995), while growth of bifidobacteria, enterococci, and streptococci are not inhibited at such low pH levels (Devriese and Pot, 1995; Hardie and Whiley, 1995; Sgorbati *et al*., 1995).

Counts obtained on MRS-Ref in general showed no significant differences ($p>0.001$) compared to counts obtained on the different selective media. Counts, however obtained on MRS-Ref were slightly lower under aerobic conditions, whereas the opposite, of slightly higher counts were observed under anaerobic conditions. Statistical analysis performed on the enumeration data only illustrated a significant difference ($p<0.001$) between MRS-Ref and MRS-V under aerobic conditions and BA-R(20%)V under anaerobic conditions (Table 2). When the pH of MRS agar was reduced to 5.20, *L. rhamnosus* (which formed 1–2 mm smooth, white colonies), showed good growth under aerobic conditions with a relative higher recovery compared to that of MRS-Ref agar. The mean count obtained under anaerobic conditions were considerably lower, and differed significantly ($p<0.001$) from those under aerobic conditions (Table 2).

The ability of *L. rhamnosus* to ferment carbohydrates; esculin, cellobiose, ribose and rhamnose (Kandler and Weiss, 1986), was used as an approach to develop a medium for the selective enumeration of this organism in fermented dairy products containing sLAB (present in cheese, yoghurts), probiotic bacteria and NSLAB. Esculin-cellobiose (EC) agar (Hunger, 1986) enables the selective and quantitative determination of *L. acidophilus* in fermented dairy products, though there is no clear distinction between the colony morphology of *L. acidophilus* and *L. casei-rhamnosus*. In this study EC agar supported satisfactory growth of *L. rhamnosus* (Table 2) resulting in colonies with a diameter of 1.5 mm. This result corroborates with data obtained by Hunger (1986) (Fig. 2). At present, no enumeration studies exist of *L. rhamnosus* on LC agar, although good recovery, comparable to that of other selective media evaluated, was obtained (Table 2). Round, smooth colonies that are 0.5-1.0 mm in diameter and transparent-white-green in colour were observed (Fig. 3).

Vancomycin is widely used, relatively inexpensive and easily obtainable. It has previously been applied for the selective isolation of lactobacilli from brewery products (Simpson *et al*., 1988). Several authors have since confirmed this finding since (Billot-Klein *et al*., 1994; Handwerger *et al*., 1994). Hamilton-Miller and Shah (1998) concluded that vancomycin susceptibility can be used as an aid to differentiate between two commonly isolated species, namely *L. acidophilus* and *L. rhamnosus.* The latter species is highly resistant towards vancomycin, whereas *L. acidophilus* is sensitive to even the lowest concentrations (Hartemink *et al*., 1997). Vancomycin is however, not useful as a selective agent in dairy products containing *Leuconostoc* or *Pediococcus* species, as both species are vancomycin resistant (Bille *et* al., 1992; Swenson *et al*., 1990; Tsakri and Maniatis, 1991). *L. delbrueckii*, enterococci, streptococci and bifidobacteria species are also susceptible to vancomycin (Green *et al*., 1991; Hamilton-Miller and Shah, 1998; Lim *et al*., 1993; Potgieter *et al*., 1992), although it is known that small percentages of enterococci are vancomycin resistant.

Hartemink et al. (1997) successfully isolated various lactobacilli species from dairy products in the presence of streptococci and bifidobacteria, using a new selective medium (LAMVAB) containing vancomycin. During a more recent study by Tharmaraj and Shah, (2003), MRS agar supplemented with vancomycin, proved to be satisfactory and reliable for isolating *L. rhamnosus* from a mixture of bacteria. Therefore, they concluded that MRS-V agar at 43ºC under anaerobic incubation supports the growth of only *L. rhamnosus.* No other cultures tested, including *L. delbrueckii* ssp. *bulgaricu*s, *S. thermophilus*, *L. casei*, *L. acidophilus*, *B. lactis* and *Propionibacterium* grew in this medium.

In this study, the incorporation of 0.05% vancomycin into MRS agar, supported reliable growth of *L. rhamnosus* in pure- and mixed cultures. MRS-V and Basal agar (BA) supplemented with 0.05% vancomycin and 20% rhamnose (BA-R(20%)V), at 43ºC under aerobic incubation conditions, are regarded the superior of the media tested, generating the highest viable counts (Table 2). Though, BA-R(20%)V performed slightly better quantitatively when comparing viable counts (cfu/ml), MRS-V agar was still regarded as superior. MRS-V generated qualitatively enlarged colonies (2.0-3.0mm, smooth, cream/white) making their enumeration easier compared to that in BA-R(20%)V (1.5-2.0mm, smooth, cream/white) (Fig. 1).

Despite the fact that the enumeration of *L. rhamnosus* was effortless on all media evaluated, MRS-V and BA-R(20%)V agar were selected for further evaluation, as both media were responsible for enlarged colony formation and slightly higher counts under aerobic conditions (Table 3). Both media successfully suppressed the growth of cheese starter cultures (RAO24; *L. lactis* ssp. *lactis, L. lactis* ssp. *cremoris, S. salivarius* ssp. *thermophilus*) when co-inoculated, while supporting reliable growth of only *L. rhamnosus.* The selectivity of the media was not influenced by the dilution of the sample, thus only one type of colony could be seen on all dilution series (10^{-1} – 10^{-7}).

Statistical analysis proved no significant difference existed between the two selective media under aerobic and anaerobic conditions, though a statistical difference ($p<0.001$) in microbial numbers was observed when incubated at different conditions. Aerobic incubation conditions at 43^{o}C proved to be the most suitable condition under which *L. rhamnosus* can utilise MRS-V and/or BA-R(20%)V, either in/or without the presence of sLAB. (Table 2 and 3).

2.4. CONCLUSION

In this study, nine bacteriological media were evaluated under different incubation conditions for their suitability to recover and enumerate the probiotic organism *L. rhamnosus* in pure cultures as well as in fermented dairy products containing sLAB. The evaluation was based on carbohydrate / sugar fermentation patterns, use of inhibitory substances (namely acids and antibiotics), and different incubation conditions (namely aerobic and anaerobic).

For industrial purposes, the enumeration methods for probiotic bacteria need to be rapid, convenient and economical. MRS medium suits this description the best, though, generated the lowest recovery when comparing viable counts (cfu/ml) to that of other evaluated media. Therefore, contradicting the reliability of this medium generally regarded and implemented as reference medium in growth studies of Lactobacilli species. Vancomycin which is widely used, relatively inexpensive and easily obtainable was suitable for the recovery of *L. rhamnosus*. MRS-V and BA-R(20%)V agar were ranked superior based on qualitative and quantitative results compared to the other media. Aerobic incubation conditions at 43^{o}C proved to be the most suitable condition under which *L. rhamnosus* can utilize MRS-V and/or BA-R(20%)V, either in/or without the presence of sLAB. Superiority was attributed to distinctive individual bacterial colonies and the inhibition of sLAB.

Table 1. Media used for enumeration of *Lactobacillus rhamnosus.*

Medium	Base[a]	Selectivity based on	Reference
MRS 5.2	MRS	pH 5.2	Tharmaraj and Shah (2003)
MRS-V	MRS	Vancomycin (0.05%, w/v)	Tharmaraj and Shah (2003)
BA-R	BA	Rhamnose (10%, 20%, w/v)	
BA-RV	BA	Rhamnose (10%, 20%, w/v) Vancomycin (0.05%, w/v)	
EC agar	MRS	Cellobiose (2%, w/v), Esculin (0.1%, w/v), Chlorophenol-rot (0.2%, w/v)	Hunger (1986)
LC agar	MRS	Ribose (1%, w/v), incubation temperature (27^{o}C)	Ravula and Shah (1998)

[a] MRS: deMan, Rogosa, Sharpe, BA: Basal agar

Table 2. Viable counts (log_{10} cfu/ml) of *Lactobacillus rhamnosus* (pure cultures[b]) enumerated on several selective media (aerobic, anaerobic incubation, 43°C, 48h).

Recovery media	Mean[a] viable counts (log_{10} cfu/ml)	
	Pure[b] cultures	
	Aerobic incubation	Anaerobic incubation
MRS-Ref	8.71	8.62
S[d]	0.1[bfh]	0.09[bd]
MRS 5.2	8.99	8.54
S[d]	0.17[cdefg]	0.17[ab]
♦ MRS-V	9.15	8.59
S[d]	0.1[gijk]	0.03[bc]
BA-R(10%)	8.96	8.47
S[d]	0.09[cdefg]	0.05[ab]
BA-R(10%)V	9.03	8.15
S[d]	0.04[efi]	0.32[a]
BA-R(20%)	9.03	8.42
S[d]	0.1[defhk]	0.26[ab]
♦ BA-R(20%)V	9.23	8.39
S[d]	0.05[hijk]	0.17[ab]
LC-Agar	9.1	8.46
S[d]	0.02[fj]	0.06[ab]
EC-Agar	9.07	8.65
S[d]	0.04[fj]	0.08[be]

MRS-Ref—(deMan, Rogosa, Sharpe) agar used as reference medium for enumeration of Lactobacilli in general; MRS 5.2—(deMan, Rogosa Sharpe) agar with modified pH (5.2); MRS-V—(deMan, Rogosa, Sharpe) agar supplemented with 0.05% (w/v) vancomycin; BA-R and/or BA-RV—Basal Agar (BA) agar medium supplemented with 10, 20% (v/v) rhamnose and 0.05% (w/v) vancomycin respectively; LC Agar—*Lactobacillus casei* agar; EC Agar—Esculin-Cellobiose agar

[a] Mean counts of *L. rhamnosus* recovered on each media in triplicate; means with different superscripts in the same row differ significantly ($p<0.001$); [b] Test culture only containing probiotic strain *L. rhamnosus*;

S[d] Standard deviation of the mean; ♦ — Mediums selected for further analysis.

Table 3. Media performance of the enumeration of *Lactobacillus rhamnosus* (mixed cultures [c]) in the presence of starter lactic acid bacteria (sLAB) (RAO24) (aerobic, anaerobic incubation, 43°C, 48h).

Recovery media	**Mean[a] viable counts (log_{10} cfu/ml)**		
	Aerobic incubation	**Anaerobic incubation**	**Colony morphology**
MRS-V	9.16	8.36	2.0 - 3.0mm, smooth, cream/white
S[d]	0.11[b]	0.32[a]	
EOC	***	***	
BA-R(20%)V	9.13	8.56	1.5 - 2.0mm, smooth, cream/white
S[d]	0.05[b]	0.06[a]	
EOC	***	***	

MRS-V—(deMan, Rogosa Sharpe) agar supplemented with 0.05% vancomycine;

BA-R(20%)V—Basal Agar (BA) medium supplemented with 20% rhamnose and 0.05% vancomycine

[a] Mean counts of *L. rhamnosus* recovered on each media in triplicate; means with different superscripts in the same row differ significantly ($p<0.001$)

[c] Test culture containing a cocktail mixture of *L. rhamnosus* and starter lactic acid bacteria RAO24

S[d] Standard deviation of the mean

EOC—Ease of counting colonies;

***— Enumeration successful, only one type of colony, i.e. obtained the best results.

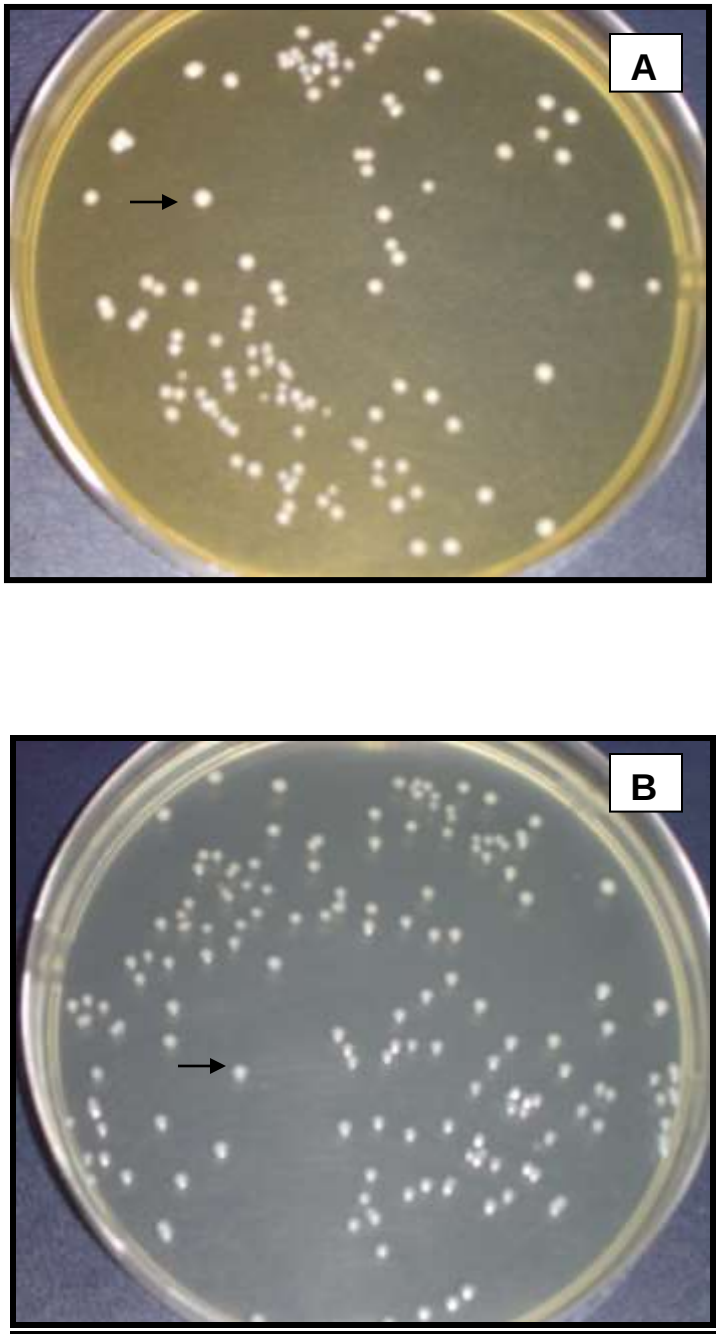

Fig. 1. Colony morphology of *Lactobacillus rhamnosus* observed on (**A**) MRS-V and (**B**) BA-R(20%)V on 10^{-7} dilution (aerobic incubation, 43ºC, 48h).

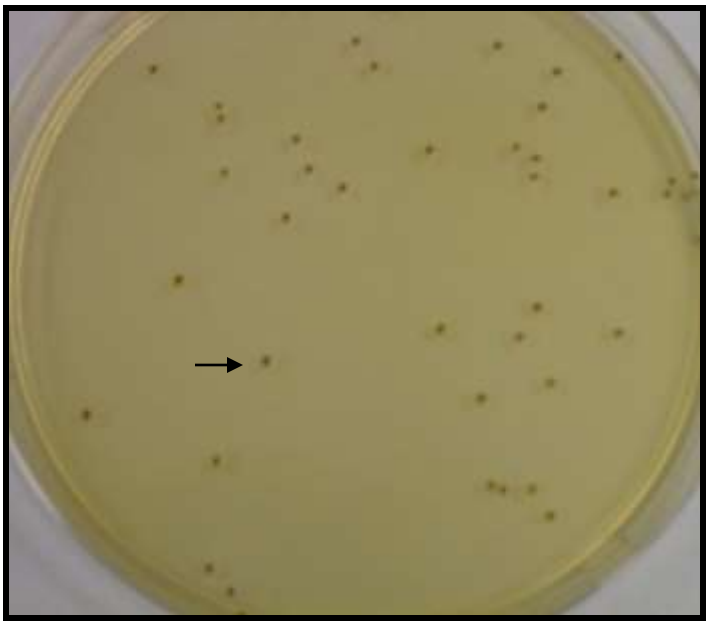

Fig. 2. Colony morphology of *Lactobacillus rhamnosus* observed on EC agar on 10^{-7} dilution (anaerobic incubation, 43ºC, 48h).

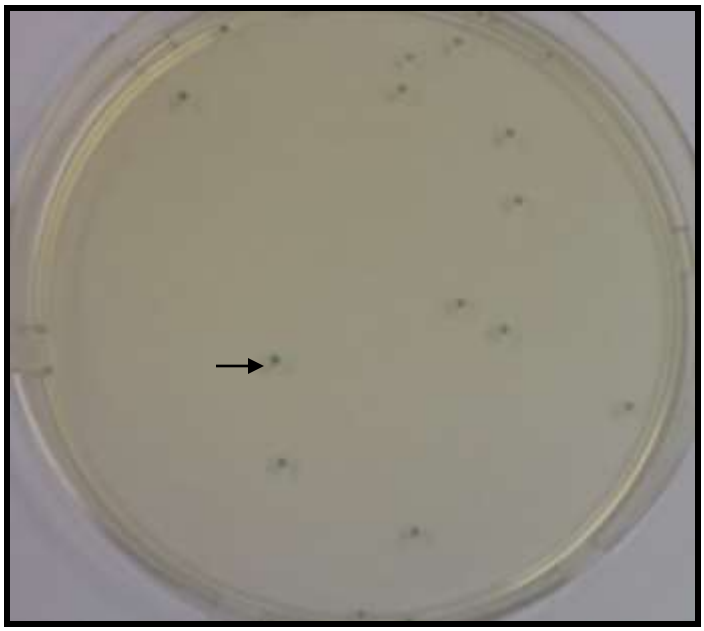

Fig. 3. Colony morphology of *Lactobacillus rhamnosus* observed on LC agar on 10^{-7} dilution (anaerobic incubation, 27ºC, 72h).

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CHAPTER 3

The combined effects of pH, lactic acid, NaCl and temperature on the growth of probiotic adjunct *Lactobacillus rhamnosus* HN001 (DR20™) in a Cheddar cheese environment

ABSTRACT

A factorial design was used to measure the effects and interactions of four kinetic parameters; pH (5.2, 5.7, 6.2), lactic acid (0, 1, 2%), salt (NaCl) (0, 1, 2%) and incubation temperatures (10, 30, 37^{o}C) on the anaerobic growth of *Lactobacillus rhamnosus* strain HN001 (DR20^{TM}) in MRS (deMan, Rogosa, Sharpe) broth. The Response Surface (RS) model was developed using Response Surface Analysis and provided reliable estimates of the parameters studied, with R^2-value of 90.7%, a bias factor of 1.09, and accuracy factor of 1.26 in anaerobic conditions. The SEP statistic resulted in a value of 18.21%. This model provides a means for rapidly estimating how the probiotic bacterium is likely to respond to any combination of the four variables within the specified ranges, and is a valuable tool in enabling its application for shelf-life estimation within a food product.

(**Keywords**: *Lactobacillus rhamnosus*, factorial design, Response Surface model)

3.1. INTRODUCTION

In addition to starter cultures, probiotic bacteria are now more often included in fermented dairy products (i.e. yoghurts, fermented milks, cheeses etc.) with the aim to contribute to the health and well-being of consumers. The bacteria used as probiotics are predominantly selected from the genera *Lactobacillus* and *Bifidobacterium*, both of which form part of the normal human intestinal microbiota (Crittenden *et al*., 2001). To elicit beneficial effects, the bacteria must be viable at the time of consumption and should maintain their viability through out the gastrointestinal tract (GIT) (Blanchette *et al*., 1995; Robinson and Samona, 1992). Recommendations for the minimum suggested level of probiotics in food to attain viability are quite variable, however, it is suggested that consumption should be ≥100g of probiotic product per day, containing more than 10^6 cfu/ml viable cells (Ouwerhand *et al*., 2002; Rybka and Kailasapathy, 1995).

To successfully develop cheeses and other dairy products containing *Lactobacillus* species it is important to understand the growth characteristics thereof. Lactobacilli generally grow at pH 5.0, with an initial optimum growth pH between 5.5 and 6.2. Growth is ceased / inhibited when pH 4.0–3.6 is reached (Kandler and Weiss, 1986). The initial growth temperature ranges from 2^{o}C - 53^{o}C, while optimum growth occurs at 30^{o}C – 40^{o}C. *Lactobacillus rhamnosus* are distinguished from other lactobacilli species in that they are able to withstand temperatures as high as 45^{o}C and as low as 15^{o}C (Kandler and Weiss, 1986). The most important properties, however, for commercial utilization of a probiotic starter culture should include; rapid growth and acidification of the carrier food, and good acid, bile salt and oxygen tolerance (Gomes *et al*., 1998; Gomes and Malcata, 1998). *Lactobacillus rhamnosus* is classified as a heterofermentative, facultative anaerobe, producing L (+)-lactic acid and ethanol under anaerobic conditions (Narayanan *et al*., 2004).

The acidification and enzymatic processes accompanying the growth, introduces the key flavor, texture, and preservative qualities to the fermented foods (Klaenhammer, 2002). Being an organism that occupies important niches in the GIT of humans and other warm-blooded animals, *L. rhamnosus* are considered one of the few species of Lactobacilli that have been used as probiotic organisms in functional foods (Dekker and Lubbers, 2002; Prasad *et al*., 2003). *Lactobacillus rhamnosus* strain HN001 (also known as DR20^{TM}) not only has flavor enhancing and probiotic attributes, but also has the ability to withstand acid and bile (Dekker and Lubbers, 2002).

Microbial growth kinetics, i.e. that of probiotic bacteria, largely depend on the presence of intrinsic and extrinsic factors like; dissolved oxygen, pH, and growth promoters and inhibitors (i.e. NaCl), within the food matrix (McDonald and Sun, 1999). The level of inoculation, incubation and storage temperatures, and fermentation time also plays a critical role (Gomes and Malcata, 1999; Modler *et al*., 1990; Rybka and Kailasapathy, 1997). To be successful, manufacturers must consider the effects of the microenvironment of the food during processing and storage to ensure that the concentration of probiotic species at the time of consumption provides a therapeutic dose to consumers. It has been reported that by manipulating the manufacturing and storage conditions of these products, the survival and viability of lactic acid bacteria (LAB) and bifidobacteria have shown a dramatic increase, thus, optimizing their growth and survival (Rybka and Kailasapathy, 1997). Predictive microbiology is an important tool in the food industry to predict the behavior of microorganisms. Although the application of mathematical modeling techniques (i.e. growth predictive models) to the growth and survival of microorganisms in foods has not received wide attention until the 1980’s (Ross and McMeekin, 1994), it has become increasingly accepted as informative tools that assist in rapid and cost-effective assessments and predictions of microbial growth for product development, risk assessment and education purposes (Zurera-Cosano *et al*., 2004b).

Developing predictive models, however, requires a large amount of growth data. The time-consuming nature of traditional plate-count techniques has prompted a need for swifter and more convenient data-collection methods, which subsequently, represent considerable saving in effort as well as resources (Cole, 1991). A proposed alternative is based on absorbance measurements (Begot *et al*., 1996; Dalgaard *et al.*, 1994). Predictive models derived from automated optical density data are reliable, generally validate well against models based on traditional methods, and provide favorable estimations of microbial responses (Dalgaard *et al*., 1997; Dalgaard and Koustsoumanis, 2001; Nebrink *et al*., 1999; Neumeyer *et al*., 1997). Over the past few years, much effort has been directed towards developing models describing combined effects of environmental factors on microbial growth in foods (Buchanan, 1993; McClure *et al*., 1994; Ross *et al*., 2000; Ross *et al*., 2003; Sutherland *et al*., 1995; Zurera-Cosano *et al*., 2004a). The relationships between the combination of factors and the growth curve parameters are most frequently described using Response Surface Methodology (Devlieghere *et al*., 1998).

At environmental conditions similar to those during manufacturing, ripening and/or storage processes of cheese, the present work has been conducted with the purpose of investigating the combined effects of pH, lactic acid, salt (NaCl), and incubation temperature on the growth of probiotic culture *L. rhamnosus* HN001 (DR20^{TM}) in MRS (deMan, Rogosa, Sharpe) laboratory broth. Given the lack of mathematical models for *L. rhamnosus* in current scientific literature, the aim of the present work was to attempt to define the boundaries of growth for all factors, and subsequently construct a dynamic model. Using the Response Surface Methodology, a polynomial model was developed for predicting the growth of *L. rhamnosus* HN001 (DR20^{TM}) as a function of pH, lactic acid, salt (NaCl), and incubation temperature.

3.2. MATERIALS AND METHODS

3.2.1. Culture and maintenance

Lactobacillus rhamnosus HN001 (DR20^{TM}) / HOWARU Rhamnosus® was supplied in the form of concentrated freeze-dried pellets by Visbyvac DIP (Danisco, Cultor Niebüll GmbH, Denmark) and routinely stored at -4^{o}C. Prior to the commencement of each experiment, the freeze-dried bacterial pellets were activated in 10-ml of MRS broth at 37^{o}C for a period of 6h under anaerobic incubation conditions. The culture mixture was subsequently transferred to a MRS agar plate, incubated anaerobically at 37^{o}C until a dense 48h bacterial smear culture was observed. Anaerobic conditions were ensured and maintained throughout by means of gas generating kits (Merck, Darmstadt, Germany).

3.2.2. Experimental design

A traditional problem solving approach, the matrix method (Figure 1), was used, laying out all the possible combinations of the particular variables. (The range of conditions selected, corresponds to that of cheese microenvironments during manufacturing, ripening and/or storage processes). Data from the large, full factorial experimental design, investigating the effects of the four primary variables; pH, lactic acid, salt (NaCl), and temperature on the growth of *L. rhamnosus* HN001 (DR20^{TM}) were used. The effects of pH (5.2, 5.7, 6.2), lactic acid (0, 1, 2%), salt (NaCl) (0, 1, 2%) and incubation temperatures (10, 30, 37^{o}C) on the growth of the culture included in the study were investigated, resulting in 81 (3x3x3x3) combinational treatments with three replicates for each treatment. A total, thus, of 243 combinational treatments were performed on the organism (81 x 3).

3.2.3. Experimental preparations

Laboratory media

Enumeration. MRS (deMan, Rogosa, Sharpe) agar (Merck, Darmstadt, Germany) was prepared according to the manufacturer's instructions and autoclaved at 121ºC for 15 min. The agar plates were prepared aseptically within a laminar flow cabinet by adding 15-ml of the sterile medium to 90-mm diameter Petri dishes.

***Growth*.** MRS (deMan, Rogosa, Sharpe) broth (Merck, Darmstadt, Germany) was prepared according to manufacturer's instructions. Growth media was modified to have the combinational pH (5.2, 5.7, 6.2), lactic acid (0 - 2%, v/v; BDH Ltd. Prod. 10138, Poole, UK), and salt (NaCl) concentrations (0 - 2%, w/v; Saarchem, Gauteng, SA) mentioned in the experimental design. Modification was carried out by the addition of crystalline NaCl and adjustment of pH using 1 M HCl (Merck, Darmstadt, Germany) and 1 M NaOH (Merck, Darmstadt, Germany). All media were autoclaved at 121ºC for 15 min.

Culture and preparation of inocula

For preparation of the final inocula, a pre-inoculum was prepared by inoculating 100-ml of anaerobic MRS broth, with the 48h bacterial smear culture and incubated anaerobically at 37ºC for 10h. An aliquot of 10-ml / 10% from the pre-inoculum were then aseptically transferred to 90-ml anaerobic MRS broth in preparation of the inoculum. The inoculum was incubated at 37ºC for a period of 5h. All incubations were performed statically within an anaerobic chamber (Forma Scientific, Anaerobic Systems). The target inoculum level chosen for each combinational treatment in the study was 10% / $\geq 10^6$ cfu/ml at time 0 (T_0) of observation, in order to comply to set limits of the 'therapeutic minimum'. All cultivations of combinational treatments were repeated in triplicate (81 x 3).

3.2.4. Experimental procedures

***Enumeration*.** A preliminary enumeration study was performed prior to the commencement of each experimental run. This was done in order to determine whether the inoculum level used (T_0) was in accordance with the set 'therapeutic minimum' standard. The methodology followed was the same as that described above. An inoculum level of 10% active growing culture cells were used to inoculate 45-ml MRS broth to give a total volume of 50-ml. Upon inoculation (T_0), the homogenized medium was serially diluted (10^{-1} – 10^{-7}) using 2% ($20g.l^{-1}$) buffered peptone water (Biolab, Gauteng, SA). By implementing the spread-plate technique, 0.1-ml of each dilution was spread (x3) onto the appropriate plates in order to determine the population based on viable plate counts. All incubations were performed within an anaerobic chamber (Forma Scientific, Anaerobic Systems) at 37^{o}C for 48h. Plates containing typical white colonies in the range of 25 – 250 and visible with the aid of a Darkfield Quebec Colony Counter (American Optical Co., Buffalo, USA) were counted and recorded as colony forming units per milliliter (cfu/ml). The recorded cfu/ml was plotted against incubation time (results not shown). The procedure was carried out hourly until the organism had entered the stationary phase.

Growth. A preliminary growth study was performed (x 3) under normal cultivation conditions in order to determine the harvest time (early stationary phase) as well as to monitor the activity of the freeze-dried culture throughout the duration of the experiment. A 10% inoculum (as above) were inoculated into 45-ml MRS broth to a final volume of 50-ml and incubated statically at 37^{o}C within an anaerobic chamber (Forma Scientific, Anaerobic Systems). To simplify detection of growth, turbidimetric methods were used. Samples were aseptically extracted every hour until the stationary phase has been reached. Growth was monitored colorimetrically using a Photolab 56 Photometer (WTW GmbH, Weilheim, Germany) at an absorbance of 690nm (OD_{690}).

Absorbance values (OD_{690}) were plotted against incubation time (*h*) to construct the relevant growth curves (results not shown), resulting in the estimation of the time required for the organism to enter the stationary phase. This experiment was regarded as the control and was simultaneously performed with each combinational treatment. All combinational treatments were performed in triplicate (81 x 3) at the three different temperatures by implementing the same methodology as with the control.

3.2.5. Modeling

Modeling was carried out in two stages; *(1)* characterizing the response variable of growth (fitting growth curves) and then *(2)* modeling the variable with respect to the environmental conditions used.

Data generation / curve fitting

The first stage involved fitting the obtained bacterial growth responses (growth curves). The maximum specific growth rate (μ_{max}) of *L. rhamnosus* HN001 (DR20^{TM}) subjected to various environmental combinational treatments, were calculated with the help of the Solver function (Microsoft Excel, 1998). The linear relationship (exponential growth phase) between optical density (OD_{690}) and incubation time (*h*) were considered and subsequently, estimating the maximum specific growth rates (μ_{max}) by means of linear regression.

Statistical analysis and model development

The second stage of modeling generally concentrated on describing the variation of the response variable (μ_{max}) with respect to the environmental conditions used. In order to homogenize the variance associated with the kinetic parameters / estimates, and to establish normality, the square-root of the growth rate [sqrt(μ_{max})] was modeled as a function of the conditions used.

Prior to the model development process, a series of supplemental analysis were performed. The response variable [sqrt(μ_{max})] was subjected to Multiple linear Regression, Stepwise Regression Analysis (STATISTICA, StatSoft Inc., version 7, 2005) as well as Analysis of Variance (ANOVA), using the SAS GLM Procedure (SAS, 9.1.) and STATISTICA software (StatSoft Inc., 7, 2005) respectively. The effect of the four independent variables i.e. pH, lactic acid, salt (NaCl), and temperature on the growth of *L. rhamnosus* HN001 (DR20^{TM}) was modeled using the SAS RSREG Procedure (SAS, 9.1). This procedure analyzes the fitted response surface to determine the factor level of optimum response and performs an additional Ridge Analysis to search for the region of optimum response. The aptness of the model was determined by calculating the value (s) of R^2 (coefficient of determination), for it gives the fraction of the variation of the response explained by the model (Nebrink *et al*., 1999).

Evaluation of model performance and validation

To evaluate the fitting and prediction accuracy of the Response Surface (RS) model, the following evaluation criteria were employed; *(1)* Root-Mean-Squares Error (***RMSE***); *(2)* Standard Error of Prediction (***SEP***) (Hervás *et al*., 2001); *(3)* Bias factor ($\boldsymbol{B_f}$) (a multiplicative factor by which the model, on average, over- or under-predicts the response time of bacterial growth); and *(4)* the Accuracy factor ($\boldsymbol{A_f}$) (indicates the spread of the results concerning the prediction) (Ross, 1996).

$$\boldsymbol{RMSE} = \text{sqrt}\left[\ \Sigma\ (\mu max_{\text{Obs}} - \mu max_{\text{Pred}})^{2} / n\ \right] \qquad (1)$$

$$\boldsymbol{\%\ SEP} = (100 / mean\ \mu max_{\text{Obs}}) * (\text{sqrt}\left[\Sigma\ (\mu max_{\text{Obs}} - \mu max_{\text{Pred}})^{2} / n\ \right]) \qquad (2)$$

Bias factor (B_f) = $10^{(\Sigma \log (\mu max_{Pred} / \mu max_{Obs}) / n)}$ *(3)*

Accuracy factor (A_f) = $10^{(\Sigma | \log (\mu max_{Pred} / \mu max_{Obs}) | / n)}$ *(4)*

where;

μmax_{Obs} : observed square root of maximum specific growth rate values

μmax_{Pred} : predicted square root of maximum specific growth rate values

mean μmax_{Obs} : mean of the observed square root of maximum specific growth rate values

n : number of observations / comparisons used in calculation

3.3. RESULTS AND DISCUSSION

In this study, optical density measurements were applied to determine the growth of *L. rhamnosus* HN001 (DR20^{TM}) in the laboratory medium at environmental conditions similar to the microenvironment in cheese during manufacturing, ripening and/or storage processes. This method is characterized by its rapidity and simplicity, where the use of the plate count method would be laborious and require long periods of incubation. Dalgaard *et al*. (1994, 1997) and Neumeyer *et al*. (1997) found in their studies that optical density measurements might be used reliably for estimation of bacterial growth rates, instead of viable count measurements. The conclusion is that absorbance measurements may be used for model development and which is in accordance with studies conducted by Begot *et al*. (1996), Dalgaard *et al*. (1997) and Neumeyer *et al*. (1997). Although *L. rhamnosus* HN001 (DR20^{TM}) is a widely used probiotic starter culture in dairy products, no studies, on the combined effects of pH, lactic acid, salt (NaCl), and temperature, have been carried out to date.

The combined effects of pH, lactic acid, salt (NaCl), and temperature on the growth of L. rhamnosus HN001 (DR20^{TM}) and model development

The series of supplemental Regression Analysis were performed in order to identify and determine which of the primary independent variables has the greatest influence on the development and growth of *L. rhamnosus*. Regression analysis, analyzes the relationship between one variable and another set of variables. The relationship is then expressed as an equation that predicts a response variable (dependant variable) from a function of independent variables and parameters. The parameters are adjusted so that a measure of fit is optimized (SAS/STAT User's Guide, 1990).

Analysis of Variance (ANOVA), using the SAS General Linear Model (GLM) Procedure (SAS, 9.1) (based on type III Sum of Squares), was used as a means of evaluating the relative importance of the variables and their interactions (Table 1.). The majority of the microorganism's response could be attributed to three of the primary variables; i.e. temperature, pH, and salt (NaCl) (p<0.0001). Relatively little effect was associated with the interaction terms, suggesting that the primary variables acted mainly independently of each other. The only exception to this was the significant interaction between temperature and NaCl (i.e. Temp*NaCl) (p<0.0001). The significance of this cross product term can be explained by both significant effects attributed to primary variables; NaCl and temperature, and are therefore not regarded as significant. It has been reported in various other studies (Masoud and Jakobsen, 2004; Zurera-Cosano *et al*., 2004b) that all non-significant factors and their interactions, should be eliminated during the modeling process and only the factors that had significantly affected the model, remained in the equation. In this study, however, all the non-significant factors and their interactions (Table 1 and 2) were regarded as valuable and subsequently included in the model equation (*Equation 5*), for they contributed to the overall goodness of fit (R^2) of the model. The combined effects of NaCl & Temperature, pH & Temperature, and pH & NaCl are shown in Figures 2, 3 and 4, respectively. It seems from Figure 2 that the [sqrt(μ_{max})]–values increase with increasing temperature and decreasing NaCl concentrations, with temperature having a much more pronounced effect. Not only increasing temperatures, but also higher temperatures, will cause the [sqrt(μ_{max})]-values to increase with increasing pH values, however, with a much less pronounced effect than observed with temperature (Fig. 3). Figure 4 represents a summary of the interaction between pH and NaCl, supporting the above observations.

During the model development process, the independent variables was fitted to a response surface in order to determine which estimated values of the independent variables would lead to an optimized response (i.e. generating a maximum μ_{max}).

(When each independent variable is measured at three or more values, as in the case of this study, a quadratic response surface can be estimated by means of the SAS RSREG Procedure (SAS/STAT, User's Guide, 1990)). This type of analysis is also useful if the optimum response is not found to be within the experimental region. The resulting RS model was elaborated following various different mathematical transformations such as the use of logarithms and square-roots of the response variable. (The equations that produced the best fit and prediction accuracy, were selected). The function chosen to model the kinetic parameters was a second degree polynomial of the form;

$$y = a + b_1t - b_2p - b_3s - b_4L - b_5t^2 + b_6pt + b_7p^2 - b_8st + b_9sp + b_{10}s^2 - b_{11}Lt + b_{12}Lp + b_{13}Ls - b_{14}L^2 \quad (5)$$

where; y is the response variable [sqrt (μ_{max})] i.e. the parameter to be modeled; a, $b_{1,2...14}$ are unknown parameters to be estimated; and regressor variables (independent variables): p, pH (5.2, 5.7, 6.2); L, lactic acid (% v/v); s, NaCl (% w/v); t, temperature (^oC). Table 2 illustrates the parameter estimates derived from the Response Surface Analysis, and which is subsequently applicable to the above equation.

The predicted optimal response, mentioned above, can be located from the estimated response surface, providing, that the surface is shaped like a 'hill' or a 'valley' (RSREG Procedure, Canonical Analysis, SAS 9.1). In the case of this particular study, though, the estimated surface (s) was more complicated (Fig. 2, 3, and 4), with no evident 'hill' or 'valley'. The optimal / stationary point (s) on the surface(s) (Fig. 2, 3 and 4) resulted in a saddle point which, i.e. produces a minimum value when certain variables increase / decrease, and simultaneously produces a maximum value when the rest of the variables tend to increase / decrease.

Because the Canonical Analysis resulted in a saddle point, the estimated surface does not have a unique optimum (globally, no maximum or minimum value was present), which may indicate that the 'predicted optimum' response is far from the region of experimentation. A resulting Ridge Analysis (RSEG Procedure, SAS 9.1), performed to analyze the shape of the surface, however, indicated the direction (s) in which further experiments should be conducted. At this saddle point the largest eigenvalue of 0.04 shows that the 'valley' orientation is less curved than the 'hill' orientation, with the smallest eigenvalue of -0.05. The coefficients of the associated eigenvectors (i.e. Temp, pH, NaCl, and Lactic acid (L_A_)) show that the 'valley' is more alligned with NaCl (0.93) and the 'hill' with Temp (0.92), while pH and L_A virtually stay constant (Table 4). Ridge analysis indicated that maximum yields (fastest growth rate) (i.e. highest estimates response = 0.893611) will result from high temperatures (35^{o}C), a pH value of 5.78, and salt (NaCl) concentrations of 0.51%, while lactic acid (L_A) concentrations is kept constant at a value of 1%; since this effect is not significant (as illustrated with ANOVA) (Table 5). Following the tendencies of the factor values, further experimentation is required. It might be best to focus on temperatures higher than 37^{o}C, salt (NaCl) concentrations varying between 0 and 1%, while lactic acid (L_A) concentrations and pH is kept constant at values of 1% and 5.7, respectively. The standard error of the estimated responses should however not be too large, which in this case, is satisfactory, for the 'ridge-movement' is still within the data range.

Model evaluation and validation

The R^2 statistic is often used as an overall measure of the fit attained and measures the fraction of the variation about the mean that is explained by the fitted model (Sutherland *et al*., 1995). A value of one indicates that the statistical model explains all of the variability in the data, whereas, the contrary being true for a value of zero. A practical rule of thumb for evaluating R^2, states that it should be at least 0.75 (75%) or greater (Haaland, 1989).

The polynomial equation (*Equation 5*) produced a high value for the multiple regression coefficient (R^2) and indicates that approximately 90.7% (R^2 = 0.9071) of the total variance of the responses can be explained by the model. In addition, the four primary variables produced low values for the *RMSE* (0.1023) and *SEP* (18.2%) statistics, supporting the above and subsequently indicating a good model fit, with good predictive power in the described broth system.

It is, however, compulsory for developed models to undergo validation in real situations (McDonald and Sun, 1999) before they are implemented to aid in food systems. Validation involves the comparison of model predictions to experimental observations, other than that used for model development (i.e. data generated by other laboratories and/or different media / food substrates) (McClure *et al*., 1994). Validation studies should, therefore demonstrate that microorganisms behave in similar ways, both in laboratory as well as in real food systems. It has been stated in literature that model validation can be carried out by reference to published results (Blackburn *et al*., 1997), however, many modelers use laboratory media to develop and validate models under static conditions (Hudson and Mott, 1993; Walls *et al*., 1996). The latter approach was implemented in this study. Some deviations from predictions have been reported by using this method, but do not necessarily imply that a model is defective (McMeekin *et al*., 1997). In practice, the issue is not necessarily how well the model fits the data, but the accuracy with which it mimics the microbial responses (Jones *et al*., 1994). Because little / no data is available in literature on the combined effects of pH, lactic acid, salt (NaCl), and temperature on the growth of *L. rhamnosus* HN001 (DR20^{TM}) in foods, validating the model data posed great difficulty. The two primary tools subsequently used to measure the model performance were the bias- (B_f) and accuracy factors (A_f) (*Equation 3, 4* respectively). Table 3 demonstrates, in detail, the calculation of these factors, and can be interpreted as quantitative summaries of the type of plot shown in Fig. 5.

The bias factor value of 1.09 not only indicates that the model tend to be 'fail-dangerous' because it predicts longer growth rates than observed, but also that the predictions exceed the observations, on average, by 9%. Conversely, a bias factor less than one, indicates that a model is in general 'fail-safe', but a factor of 0.5 indicates a poor model that is overly conservative because it predicts growth rates, on average, half of that actually observed (Ross, 1996). Perfect agreement between predicted and observed values will lead to unity, where $B_f = 1 = A_f$ (Ross, 1996), however, Ross *et al*. (2000) consider an A_f value to be acceptable with an increase of up to 0.15 (15%) for each variable included in the model. Therefore, in our study, with four primary variables (i.e. pH, lactic acid, salt (NaCl), and temperature) we should expect to have an A_f value of up to 1.6. Based on the accuracy factor value of 1.26, the predictions are on average, within 26% of the observation; and still within the predicted range proposed by Ross *et al*. (2000).

Figure 6 is the resulting residual plot after fitting the estimated RS model (*Equation 5*) to the experimental data and enables the fit of the model to the data to be visualized. The predicted values on the plot are calculated from the model, whereas the observed values are the actual values of the response. If the model successfully predicts the responses, the predicted data points versus the observed data points will lie on/or in close proximity to the line passing through the origin. This is also were the R^2 statistic is a criterion of. The observations and predictions are expressed as the square root of the relative rate in order to homogenize the variance in the data (Fig. 6). The poorer predictions (residual points further from line of origin) may simply be a consequence of the inherently greater variability in responses as the conditions become less favourable for growth. The residual points in this region may represent the extremes of the distribution of possible response times and may likely be corrected in future models by increasing the number of variable combinations in this region (i.e. Central Composite Designs (CCD)).

Additional information about CCD is stated elsewhere (Box *et al.*, 1978; Cheroutre-Vialette and Lebert, 2000). An additional means of assessment, according to Ross (1996), was carried out to verify the bias factor, for it may fail to reveal some forms of systematic bias (i.e. systematic over-prediction in one region of the response surface may be balanced by systematic under-prediction in another region). Examination of the signs of the residuals (Fig. 5 and 6), confirmed that there is no evidence of systematic over- or under prediction patterns as a function of the response time. The bias factor can, thus, be regarded as a reliable measure.

3.4. CONCLUSION

Predictive modeling may contribute to a better understanding of and control of microbial processes, and help to clarify in which manner, and to which degree, the food environment will interfere with the functionality of the strains used. The experimental system used in this study, which is characterized by its simplicity, illustrated good validity for developing models to predict growth of *L. rhamnosus*. The Response Surface model reported here gave an acceptable description of growth for this lactobacilli strain which is used as an adjunct probiotic starter culture under environmental conditions, which in terms of pH, lactic acid, salt (NaCl), and temperature, are similar to conditions during that of manufacturing, ripening and/or storage processes of cheese. In addition, it also enables the prediction of the combined effects of the four primary variables on the growth kinetics of *L. rhamnosus* strain HN001 (DR20^{TM}) in MRS culture broth. In order to show, however, that this model is valid for growth of *L. rhamnosus* in foods, additional studies of the growth of this organism in food are required. Future research, focusing on Central Composite Designs (CCD) is also required, not only to optimize the model performance and to subsequently minimize variance associate with the kinetic parameters, but also to estimate optimum responses within the experimenting range.

Fig. 1. The matrix-method – a possible approach to experimentation. Effective, but insufficient due to many measurements (Haaland, 1989).

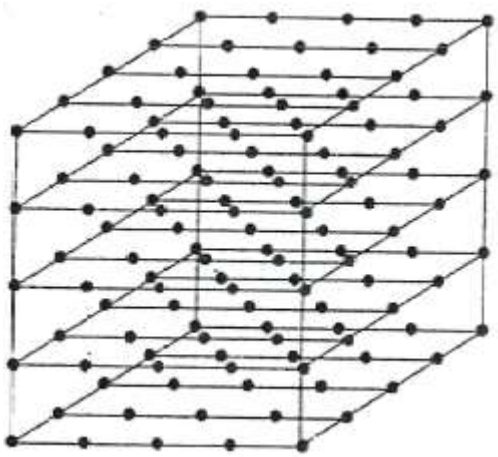


Table 1. F-values for independent primary variables and their cross products resulting from the General Linear Model (GLM) Procedure (SAS, 9.1).

Source	DF	SS	Mean Square	F-value	Pr > F
Temp	2	18.88546224	9.44273112	798.27	<.0001 *
pH	2	0.28200283	0.14100142	11.92	<.0001 *
Temp*pH	4	0.09795955	0.02448989	2.07	0.0868
NaCl	2	1.89111654	0.94555827	79.94	<.0001 *
Temp*NaCl	4	0.33119057	0.08279764	7	<.0001 *
pH*NaCl	4	0.01946915	0.00486729	0.41	0.8002
Temp*pH*NaCl	8	0.0719908	0.00899882	0.76	0.6378
L_A	2	0.07626165	0.03813083	3.22	0.0422
Temp*L_A	4	0.0321369	0.00803423	0.68	0.6073
pH*L_A	4	0.00651009	0.00162752	0.14	0.9682
Temp*pH*L_A	8	0.02657104	0.00332138	0.28	0.9716
NaCl*L_A	4	0.06351214	0.01587804	1.34	0.2563
Temp*NaCl*L_A	8	0.16853939	0.02106742	1.78	0.0838

F-values are based on Type III Sum of Squares (SS) (SAS, 9.1)

* $P < 0.001$; parameters with a significance influence on the growth of *L. rhamnosus*

Root Mean Square Error (***RMSE***) : 0.108761

$\boldsymbol{R^2}$: 0.928464 (± 92.8%)

Table 2. Parameter estimates derived from the results of the Response Surface Analysis (RSREG Procedure, SAS, 9.1) of the response variable [sqrt (μ_{max})], subjected to various environmental conditions (i.e. pH, lactic acid, salt (NaCl), and temperature) for *Lactobacillus rhamnosus* strain HN001 (DR20™) in MRS Broth.

Parameter	Estimate	t-value	Pr > | t |
Intercept	0.983553	0.54	0.5919
Temp	0.019956	2.17	0.031
pH	-0.371749	-0.58	0.5652
NaCl	-0.188462	-1.63	0.1047
L_A	-0.029564	-0.26	0.7988
Temp*Temp	-0.000217	-2.05	0.0416
pH*Temp	0.003654	2.57	0.0108
pH*pH	0.029552	0.52	0.6036
NaCl*Temp	-0.004041	-5.78	<.0001
NaCl*pH	0.01839	0.93	0.3535
NaCl*NaCl	0.026975	1.86	0.0639
L_A*Temp	-0.00048	-0.68	0.4952
L_A*pH	0.012198	0.61	0.54
L_A*NaCl	0.018041	1.85	0.0658
L_A*L_A	-0.028765	-2	0.0468

R² : 0.9071 (± 90.7%)

Root Mean Square Error (***RMSE***) : 0.1023

%SEP : 18

Table 3. Demonstration of the calculation of bias (B_f) and accuracy (A_f) factors (from Ross, 1996).

Food Type	Variables: Temperature (ºC)	Variables: Water Activity	Observed GT (h)	Predicted GT (h)	Pred/Obs	log (Pred/Obs)	Absolute value
Smoked Salmon	12.5	0.965	11.50	17.4	1.52	0.18	0.18
Smoked Salmon	17.5	0.965	4.05	3.89	0.96	-0.02	0.02
Smoked Salmon	22.5	0.975	1.65	1.52	0.92	-0.04	0.04
Smoked Salmon	25.0	0.955	1.90	1.34	0.71	-0.15	0.15
Smoked Salmon	27.5	0.975	0.73	0.84	1.15	0.06	0.06
Smoked Salmon	32.5	0.965	0.57	0.58	1.02	0.01	0.01
Smoked Salmon	35.0	0.955	0.50	0.53	1.06	0.03	0.03
	Mean					0.01	0.07
	Bias factor (= antilog$_{10}$ 0.01)						**1.02**
	Accuracy factor (= antilog$_{10}$ 0.07)						**1.17**

Table 4. Resulting coefficients associated with eigenvectors from Ridge Analysis (RSREG Procedure, SAS 9.1) of *Lactobacillus rhamnosus* strain HN001 (DR20™).

	Eigenvectors			
Eigen values	**Temp**	**pH**	**NaCl**	**L_A**
0.038113	-0.330130	0.021263	[v*] 0.932832	0.142779
0.010490	0.212330	0.973906	0.042550	0.067911
-0.030329	0.005323	-0.063528	-0.147733	0.986970
-0.052242	[h*] 0.919729	-0.216837	0.325866	0.029859

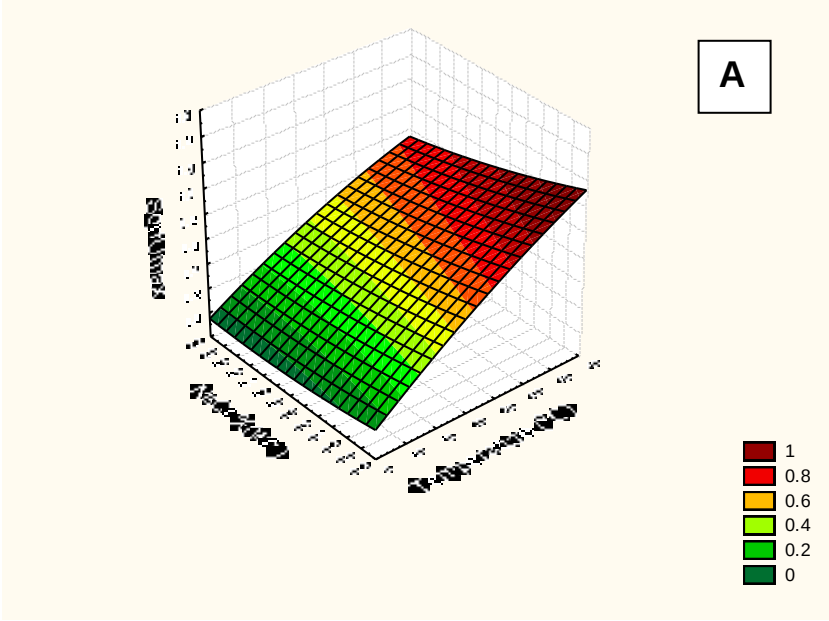
[v*] Eigenvector coefficient illustrating 'valley' alignment with salt (NaCl)

[h*] Eigenvector coefficient illustrating 'hill' alignment with Temperature

Table 5. Estimated ridge of maximum response for response variable [sqrt(μ_{max})] from Ridge Analysis (RSREG Procedure, SAS 9.1).

			Factor Values			
Coded Radius	**Estimated Response**	**Standard Error**	**Temp**	**pH**	**NaCl**	**L_A**
0.0	0.529540	0.020986	23.500000	5.700000	1.000000	1.000000
0.1	0.566389	0.020691	24.773507	5.705856	0.969117	0.996808
0.2	0.602980	0.020233	26.025348	5.712370	0.934066	0.993062
0.3	0.639373	0.019642	27.253265	5.719493	0.894840	0.988750
0.4	0.675629	0.018962	28.455275	5.727169	0.851487	0.983869
0.5	0.711811	0.018252	29.629744	5.735329	0.804103	0.978420
0.6	0.747977	0.017587	30.775443	5.743902	0.752831	0.972415
0.7	0.784185	0.017059	31.891571	5.752815	0.697852	0.965868
0.8	0.820493	0.016770	32.977753	5.761994	0.639379	0.958801
0.9	0.856951	0.016826	34.034013	5.771366	0.577643	0.951237
1.0	0.893611 *	0.017315	35.060728 *	5.780866 *	0.512885 *	0.943206

* Estimated factor values for maximum response yield



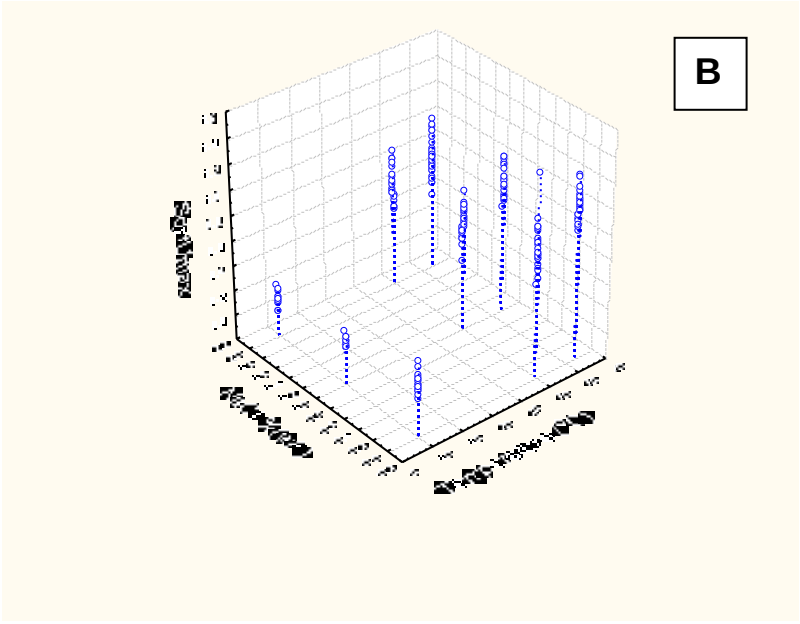


Fig. 2. (**A**) Three dimensional surface- and (**B**) scatter plots of the combined effects of NaCl % (w/v) and temperature (^{o}C) on the growth [sqrt (u_{max})] of *Lactobacillus rhamnosus* strain HN001 (DR20^{TM}).

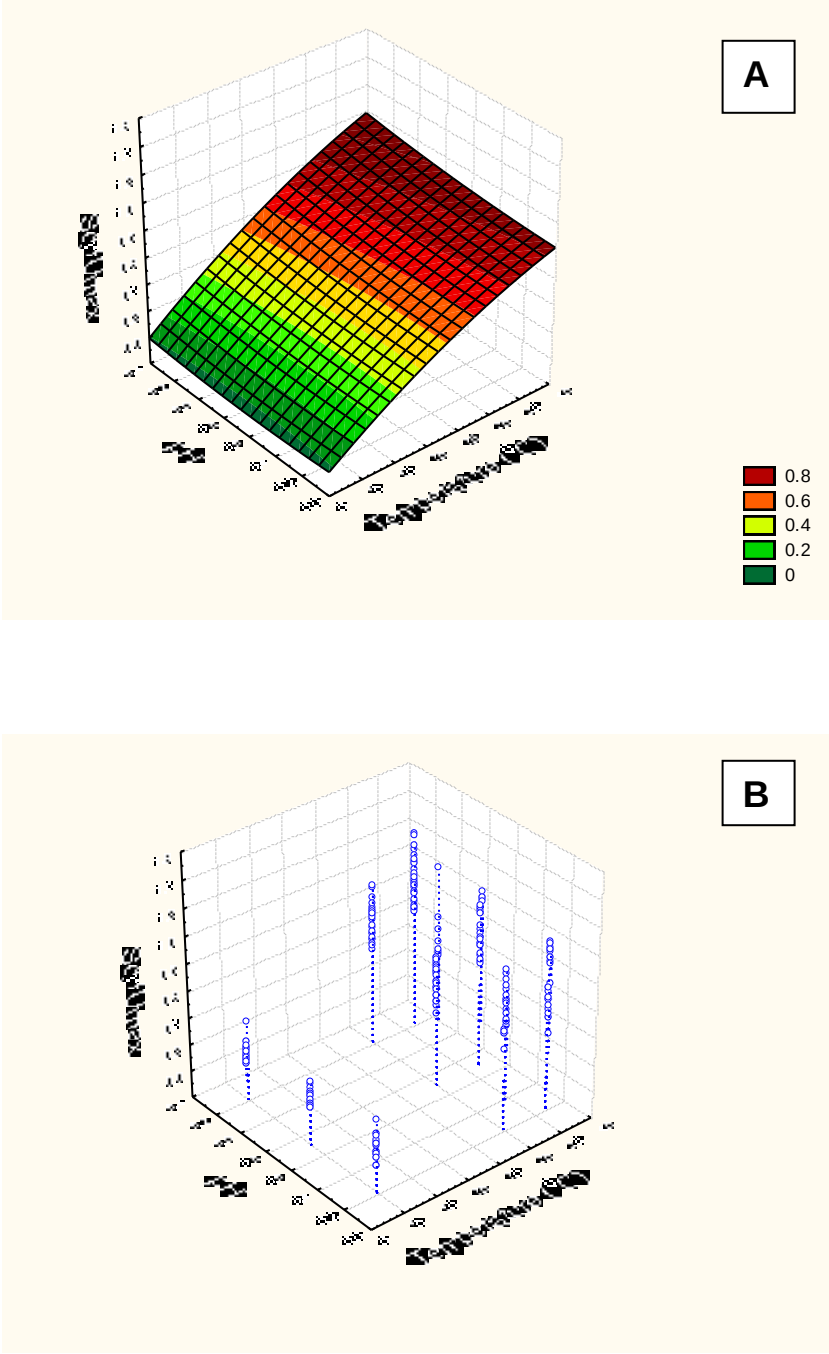


Fig. 3. (**A**) Three dimensional surface- and (**B**) scatter plots of the combined effects of pH and temperature (ºC) on the growth [sqrt (u_{max})] of *Lactobacillus rhamnosus* strain HN001 (DR20^{TM}).

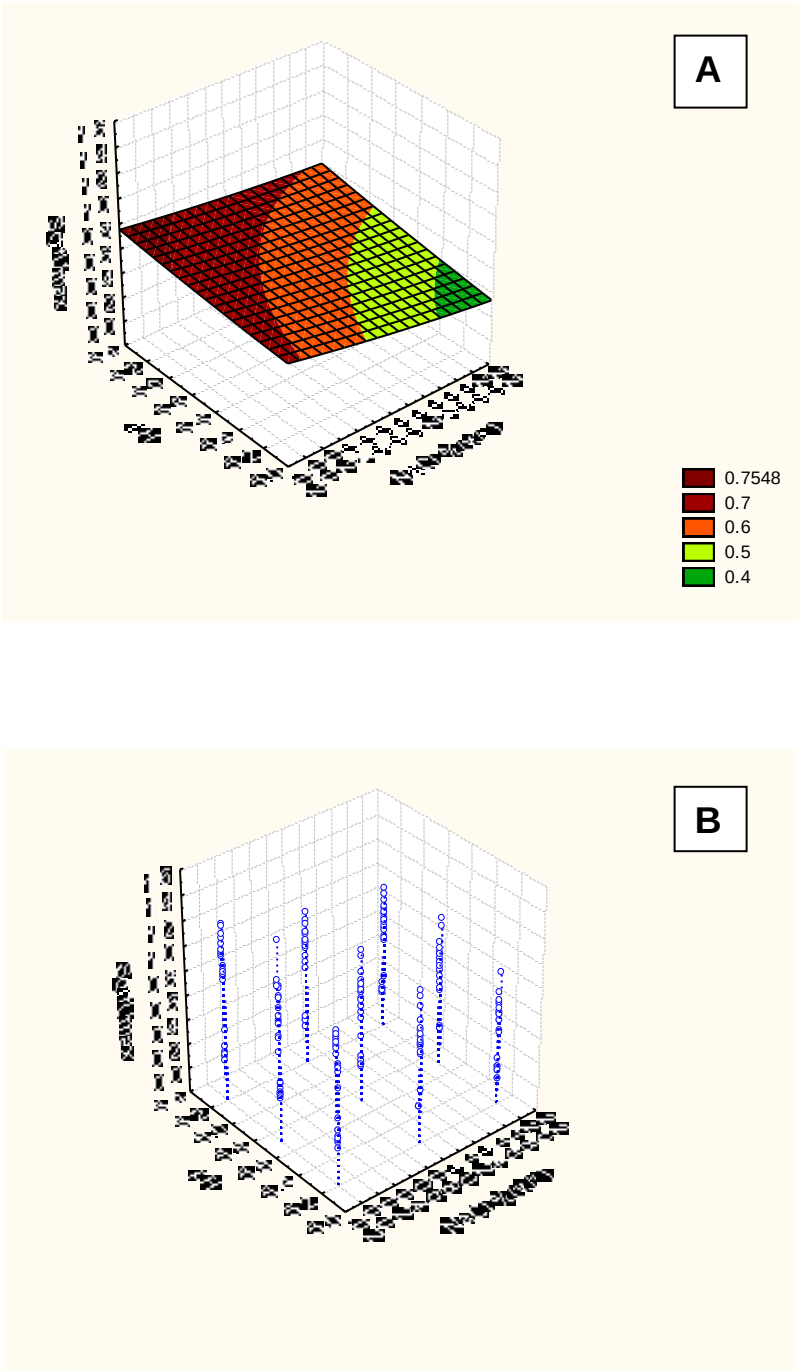


Fig. 4. (**A**) Three dimensional surface- and (**B**) scatter plots of the combined effects of pH and NaCl % (w/v) on the growth [sqrt (u_{max})] of *Lactobacillus rhamnosus* strain HN001 (DR20^{TM}).

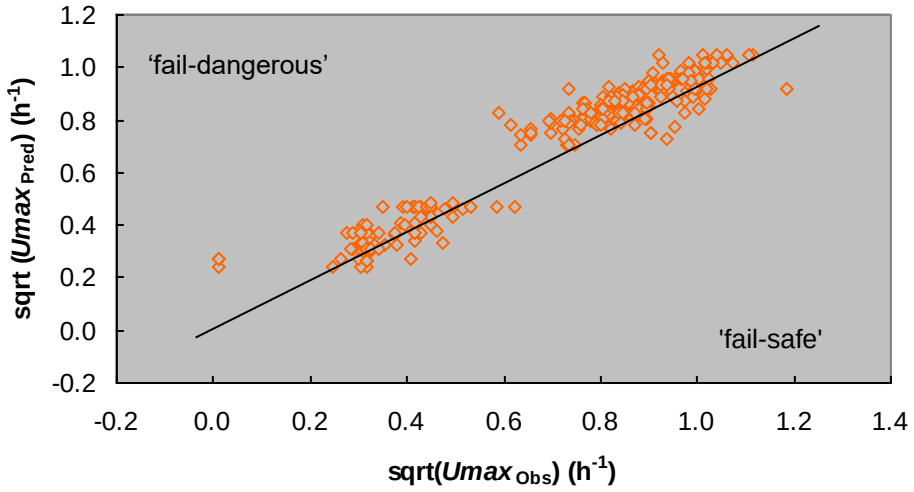


Fig. 5. Predicted growth rates of the RS model for *Lactobacillus rhamnosus* strain HN001 (DR20™) compared to observed experimental data. The diagonal line is the line of identity. Points above this line represents predictions which are longer than the observed growth rates and are thus 'fail-dangerous'. Conversely, points below the line of identity are 'fail-safe' predictions. (B_f = 1.09; A_f = 1.26).

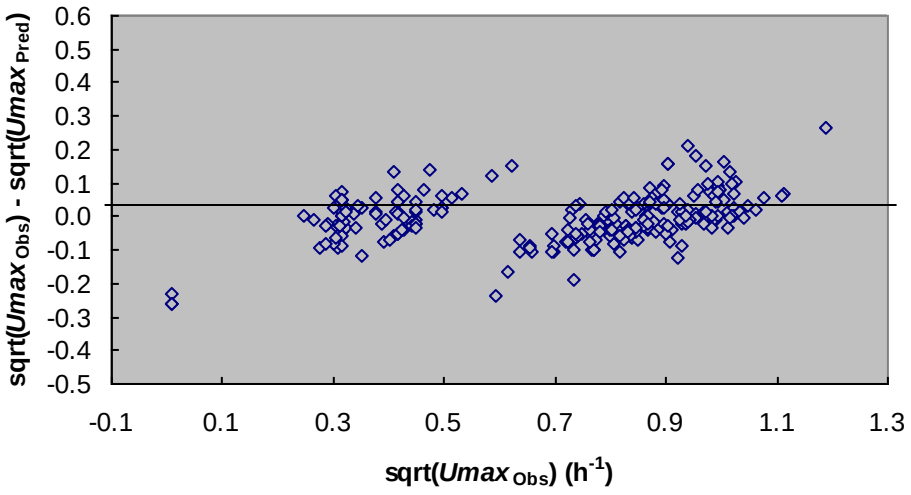


Fig. 6. Residual plot of the predictions made by the estimated RS model in Eq. 5. The observations and predictions are expressed as square root of relative rate to test the assumption that the square root prediction homogenizes the variance in the data.

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CHAPTER 4

Microbial development within matured Cheddar cheese containing *Lactobacillus rhamnosus* HN001 (DR20^{tm}) as an adjunt probiotic culture

ABSTRACT

The health benefits of probiotic-containing products, the so-called functional foods, are becoming a key factor affecting consumer choice, and therefore the existing limited range of such products needs to be expanded. A limited number of studies have demonstrated different cheeses being able to support the variability of certain Bifidobacteria and *Lactobacillus* strains. Consequently, in this study *Lactobacillus rhamnosus* strain HN001 (DR20^{TM}) was incorporated into a South African matured Cheddar cheese. This was done without any alteration of the cheesemaking technology, which make the system attractive for commercial exploitation. The viability of this organism during long-term ripening and storage as well as their effect on cheese flavor and texture were determined. The chemical properties of the cheese were also determined. *L. rhamnosus* strain HN001 (DR20^{TM}) sustained high viability for at least 114 days of ripening, while still remaining above the 'therapeutic minimum'. No substantial differences in the normal aerobic microflora of the cheese were detected. Chemical properties suggest that *L. rhamnosus* maintained a high metabolic activity during the storage process without any adverse impact on organoleptic properties of the cheese. This adjunct possibly provides control over contaminating secondary flora and limiting flavor defects while delivering a health culture to the consumer.

(**Keywords** : *Lactobacillus rhamnosus*, Cheddar cheese)

4.1. INTRODUCTION

Over the last few decades, some strains of lactic acid bacteria (LAB) belonging to bifidobacteria and lactobacilli have been introduced in food products for consumption, with the aim to improve health. These organisms are called probiotic bacteria and are defined as a “mono- or mixed cultures of live microorganisms which when applied to man or animal (e.g. as dried cells or fermented product), beneficially effect the host by improving the properties of the indigenous microflora (Huis int’ Veld and Havenaar, 1991). Numerous health affects have been attributed to the regular consumption of certain LAB, and are reviewed in detail elsewhere (Gilliland and Speck, 1977; Kim and Gillland, 1983; Lee and Salminen, 1995; Richardson, 1996; Sandine, 1979; Marshall, 1996). Despite the controversy surrounding the ‘therapeutic minimum’, it is generally believed that a minimum of 10^6 colony forming units per gram (cfu/g) probiotic product (s) needs to be ingested on a daily basis (Ouwehand *et al*., 2002) and should be viable at the time of consumption in order to be effective (Dave and Shah, 1997; Robinson, 1987; Rybka and Kailasapathy, 1995).

To date, the most important food delivery system for these cultures have been freshly fermented dairy foods, such as yoghurt, cultured- and buttermilk, and powder preparations (Bourlioux and Pochart, 1988; Fernandes *et al*., 1987; Hoover, 1993; Ishibashi and Shimamura, 1993; Sanders *et al*., 1996). By addressing the growing consumer needs and expanding the limited probiotic food range, researchers and industries have attempted to develop cheeses with probiotic bacteria as adjuncts. Most of the work on probiotic cheese production has been carried out with bifidobacteria alone, or mixed with cultures of bifidobacteria and *Lactobacillus acidophilus*, but *Lactobacillus casei* and *Lactobacillus rhamnosus* have rarely or never been used. Limited studies have been published about cheese as carrier of live probiotic organisms.

The published studies dealt with the incorporation of probiotics into commercial cheeses like Gouda, Cheddar, semi-hard goat cheese and Feta cheese (Daigle *et al*., 1999; Dinakar and Mistry, 1994; Ghoddussi and Robinson, 1996; Gomes and Malcata, 1998; Gomes *et al*., 1995; Stanton *et al*., 1998). From the above it could be concluded that Cheddar cheese is suitable as a potential probiotic 'functional food' with a number of probiotic strains demonstrating the capability to survive the manufacture and ripening processes of cheese. It should however be emphasized that the survival of probiotic strains is strain dependent, as many probiotic strains do not perform well in the cheese environment (Gardiner *et al*., 1998).

Cheddar cheese, as the food system, harbors the probiotic and has an important role in determining and maintaining viability. In this respect, cheese may offer certain advantages compared to other delivery systems used to date (Boylston *et al*., 2004; Gardiner *et al*., 1998; Ross *et al*., 2002; Stanton *et al*., 1998). Since cheese has higher pH values (4.8 – 5.6) than the more traditional probiotic foods (i.e. yoghurts and fermented milks) (pH range 3.7 – 4.3), it may provide a more stable milieu to support the long-term survival of probiotic organisms. In addition, a more solid consistency and a higher fat content of the cheese may offer protection to the organisms during passage through the gastro-intestinal tract (GIT) (Gardiner *et al*., 1998; Ross *et al*., 2002; Stanton *et al*., 1998). Cheese also has a considerably higher buffering capacity compared to that of yoghurt (Gardiner *et al*., 1999).

Although the application of non-starter lactic acid bacteria (NSLAB) adjuncts in Cheddar cheese is not a novelty (Lane and Hammer, 1935), there has recently been an increased interest in their use (Broome *et al*., 1990; Crow *et al*., 2001; Fox *et al*., 1998; Lynch *et al*., 1996; McSweeney *et al*., 1994; Puchades *et al*., 1989; Stanton *et al*., 1998).

NSLAB are mesophillic lactobacilli and pediococci, which form a significant portion of the microbial flora of most cheese varieties during ripening. They are not part of the normal starter flora, do not grow well in milk (Cogan *et al*., 1997), and do not contribute to acid production in the cheese vat (Beresford *et al*., 2001). The deliberate incorporation of NSLAB as adjuncts to cheese milk have been reported to improve and accelerate Cheddar cheese flavor and texture development (Broadbent *et al*., 2002; Broome *et al*., 1990; Drake *et al*., 1996; Lynch *et al*., 1996; Madkor *et al*., 2000; Marilly and Casey, 2004; McSweeney *et al*., 1994), although in some cases have been responsible for some flavor defects (Lee *et al*., 1990; Puchades *et al*., 1989). A positive result, thus, in terms of flavor improvement, is however heavily dependant on the particular strain used with the starter (Stanton *et al*., 1998).

L. rhamnosus is one of the two most common NSLAB species found in New Zealand Cheddar cheese, the other being *Lactobacillus paracasei* (Crow *et al*., 2001, 2002). *L. rhamnosus,* designated HN001, is an ideal candidate for an adjunct that can be used during cheese manufacture to reduce contaminant adventitious secondary microflora, accelerate cheese ripening, and improve cheese flavor (Dekker and Lubbers, 2002). The strain was selected because it possesses many of the ‘prerequisite’ probiotic criteria namely, being of intestinal origin and therefore able to adhere to human intestinal epithelial cells, non-pathogenic, and bile and acid tolerant (Gopal *et al*., 2001; Prasad *et al*., 1998,1999; Price *et al*., 2001; Sheih *et al*., 2001; Zhou *et al*., 2000a, b, 2001). Moreover, the probiotic efficacy of this strain has previously been demonstrated in clinical trials (Gill *et al*., 2000, 2001; Jaya *et al*., 1998; Tannock *et al*., 2000). This health adjunct, when used in different cheese types, predominates over the adventitious NSLAB and delivers at least 10^6 - 10^7 cfu of HN001/g cheese, during a 12 month ripening period (Crow *et al*., 2002).

Consequently, the objectives of this study was to explore the possibility of deliberately introducing *Lactobacillus rhamnosus* HN001 (DR20^{TM}) as a NSLAB adjunct into a South African commercial matured Cheddar cheese and to study viability during long-term ripening and storage. The effect of this organism on cheese flavor and texture, as well as the chemical properties of the cheese was determined. Such incorporation could contribute to a more diverse range of probiotic products, offering a probiotic cheese with better nutritional and physiological properties.

4.2. MATERIALS AND METHODS

4.2.1. Bacterial test strains

Fifty units of the RAO22 lactic acid starter strains (*Lactococcus lactis* ssp. *lactis, Lactococcus lactis* ssp. *cremoris, Streptococcus salivarius* ssp. *thermophilus)* (Rhodia Foods, France) were used as the starter culture for the production of matured Cheddar cheese. *Lactobacillus rhamnosus* HN001 (DR20^{TM}) / HOWARU Rhamnosus®, in the form of freeze-dried probiotic starter concentrates (20U), was obtained from Visbyvac DIP (Danisco, Cultor Niebüll GmbH, Denmark) and were added to the pasteurized milk in the cheese vat immediately after starter culture inoculation, during processing. The same RAO22 lactic acid starter cultures were also applied for the production of a traditional matured Cheddar cheese without any adjuncts from the same milk and under the same manufacturing conditions serving as a control cheese.

4.2.2. Cheddar cheese manufacture

Pasteurized cows milk (600 L) was used for the manufacture of mature Cheddar cheese at a pilot cheese plant in the Free Sate, South Africa. The procedure for cheese making was carried out as described by Kosikowski (1970a).

4.2.3. Sampling description

Sampling was done under aseptic conditions at selected points during the processing of Cheddar cheese – from the cheese vat, before renneting, at the end of the renneting process, before and after the cutting process, during and after cheddaring and after the salting process. The cheese was kept under controlled ripening conditions (10 - 12^{o}C) and sampled randomly by means of a sterilized borer at regular intervals. Similar samples were collected from the model cheese and the control cheese.

4.2.4. Sampling procedure

Duplicate cheese samples were prepared for microbial analysis on each occasion by opening the cheese aseptically and cutting portions using a sterilized borer. For each sample, 10g of cheese were aseptically weighed and added to 90-ml of 2% (20 $g.l^{-1}$) sterile phosphate buffer into Whirl Pak bags (Nasco, USA) and homogenized for 2 min using a Colworth 400 Stomacher (London, UK). The samples were subjected to further serial decimal dilutions in 9-ml sterile phosphate buffer and agitated by means of a vortex mixer. For the starter bacteria, the appropriate dilutions (10^{-1} – 10^{-7}) were plated in duplicate by the spread plate technique onto MRS (deMan, Rogosa, Sharpe) agar plates (Merck, Darmstadt, Germany), elective for LAB, and incubated under aerobic conditions for 48hrs at 37^{O}C. For the probiotic bacteria, appropriate dilutions (10^{-1} – 10^{-7}) were spread plated onto selective MRS-V media (Tharmaraj and Shah, 2003), highly selective for *L. rhamnosus* species, and incubated anaerobically for 48h at 43^{o}C. Anaerobic incubation conditions were ensured and maintained using anaerobic jars and gas generating kits (Merck, Darmstadt, Germany).

4.2.5. Sample analysis

Plates containing 25 to 250 colonies on the highest dilution (or the highest number if below 25) were counted and recorded as colony forming units per gram (cfu/g) of cheese. Colonies ≥ 0.5 mm in diameter were considered as developing colonies and included for enumeration purposes. The results are the means of duplicate plate samples originated from duplicate cheese samples manufactured on three occasions.

4.2.6. Physical and chemical analysis

The production / utilization of sugars (i.e. lactose, galactose and glucose) and organic acids (i.e. lactic acid, acetic acid and citric acid) were determined by HPLC (Bouzas *et al*., 1991). Sampling for the chemical analysis was performed as described for the microbial samples. On each sampling occasion, an additional 10g of cheese were weighed into 10-ml of distilled water in Whirl Pak bags (Nasco, U.S.A.), and homogenized for 2 min using a Colworth 400 Stomacher (London, UK) for chemical analysis. The slurry was transferred to Eppendorf tubes with the addition of three drops of 30% H_2SO_4 and centrifuged (model info) at 5000 rpm for 5 min. The supernatant was then filtered through a 0.45µm membrane filter (Millipore, Bedford, MA) into Eppendorf vials and stored at 4-5^{o}C for less than 1h (Roy *et al*., 1997), and injected (20-µl) accordingly for analysis. Sugar contents were measured by means of a Waters HPLC system with a biorad-aminex C42 Column and Refractive index detector (Bouzas *et al*., 1991). The organic acid content was measured by means of a HPLC system equipped with a variable wavelength detector set at 220nm. A biorad-aminex 87H Column with a 0.01N H_2SO_4 at 0.6 ml/min eluent was used (Bouzas *et al*., 1991). The pH was measured at 24^{O}C with a Cyberscan 500 (Eutech Instruments, Singapore) according to the method described by Kosikowski (1970b).

4.2.7. Sensory analysis

The sensory quality of the model and control cheeses was judged by a panel of experts in the field of cheese evaluation, based on standard protocol including openness, texture and taste at various intervals during the ripening period. Both cheeses were evaluated after 2 months and 3 months and the results compared.

4.3. RESULTS AND DISCUSSION

4.3.1. Changes in microbial populations

It is generally believed that during Cheddar cheese manufacture, the starter lactococci grow rapidly, reaching maximum levels of approximately 10^8 – 10^9 cfu/g at salting (Stanton *et al*., 1998). The LAB counts in our model cheese, however, increased during the manufacturing of the cheese from 7.9 x 10^6 cfu/g to a value of 3.98 x 10^7 cfu/g after the salting process (Fig.12). A continued proliferation was observed throughout the ripening period with an increase of more than one logarithmic cycle, reaching maximum viable numbers of 1.15 x 10^9 cfu/g after a ripening period of only 8 days. This was followed by a gradual decrease in numbers to reach a value of 5.01 x 10^6 cfu/g after 114 days of ripening (Fig.12). The profile based on the viability of the LAB associated with the model cheese corresponds with the profile observed in the control cheese (Fig.12). The presence of the probiotic culture therefore did not affect the normal aerobic microflora of the cheese, although *L. rhamnosus* exhibits antibacterial activity (Dekker and Lubbers, 2002; Gopal *et al*., 2001; Saxelin, 1997).

It has been reported that probiotic *L. paracasei* strains remained viable in Cheddar cheese from initial numbers of 10^7 cfu/g to reach levels exceeding that of 10^8 cfu/g after three months of ripening, without any negative impact on the cheese quality, aroma, flavour and/ or texture. Viability was sustained at this level for the remainder of the ripening period of eight months (Gardiner *et al*., 1998; Gardiner *et al*., 2002; Stanton *et al*., 1998). Gomes *et al*. (1995) and Vinderola *et al*. (2000) observed a decrease in numbers of some *L. acidophilus* strains during cheese ripening, although the final numbers were still above the ‘therapeutic minimum’ after a 60 day ripening period.

Little is known regarding the incorporation of *L. rhamnosus* as probiotic adjunct in cheese. Sondini *et al*. (2002) reported that although *L. rhamosus* grow weakly in milk, the strain exhibits a remarkable stability during storage when compared to *L. acidophilus*.

L. rhamnosus HN001 (DR20^{TM}) grew and sustained a high viability in the cheese during the ripening period. The viable cell numbers of the organism decreased with one log cycle during the manufacturing, from 1.35 x 10^8 cfu/g in the milk to a value of 1.0 x 10^7 cfu/g after salting of the curd (Fig. 1). This was followed by an increase of two log units during the maturation until a maximum count of 1.02 x 10^9 cfu/g was reached after only eight days of maturation. This initial increase in cell numbers was followed by a gradual decrease to a value of 1.4 x 10^7 cfu/g by the sell by date (77 days of maturation), consequently, still complying with the set limits of the 'therapeutic minimum'. This level of viability was sustained up until the consumer expiry date (84 days of ripening), reaching levels of 8.9 x 10^7 cfu/g of cheese. The cell numbers only decreased to a value of 5.01 x 10^6 cfu/g after 114 days of ripening (Fig. 1). *L. rhamnosus* could therefore survive for at least three months in matured Cheddar cheese at a level higher than 10^6 cfu/g and consequently satisfy the criteria for a probiotic food product.

4.3.2. Physical and chemical analysis

During the processing of matured Cheddar cheese, pH levels, as expected, continued to decrease from an initial value of 6.55 in the raw milk to a value of 5.52 after salting of the curd (Table 1, Fig.2). This decrease in the pH value can be attributed to the production of organic acids by the mesophillic lactococci applied as starters, as well as by *L. rhamnosus* applied as probiotic adjunct (Kandler and Weiss, 1986). After the decline, the pH remained relatively constant throughout the maturation process and reached a value of 5.39 after 114 days. In the control cheese a pH of 5.40 was reached after salting of the curd followed by a decrease to a value of 5.27 after 70 days of ripening (Table 1).

The pH of the control cheese remained constant for the remaining period of ripening, reaching a final value of 5.28 after 114 days. Therefore, the pH of the model cheese remained in the same range compared to the control cheese throughout the ripening period, being in close proximity to the normal pH range for Cheddar cheese (~4.8 - 5.2) (Fox *et al*., 1998). This suggests that no detrimental acid development occurred within the probiotic-inoculated model cheese.

Lactose is the major sugar present in dairy products (Scott, 1981), being a dissacharide that is hydrolyzed to glucose and galactose before entering the catabolic pathway for hexoses (Marilley and Casey, 2004; Schlegal, 1993). All the lactose in the milk, hydrolyzed to glucose and galactose, was depleted during the cheddaring process (Table1, Fig.2 [Addendum]). This corresponds with the findings of Thomas and Pearce (1981) who observed that lactose in cheese is utilized within the first week of ripening. *L. rhamnosus* is able to utilize lactose (Kandler and Weiss, 1986; Østlie *et al*., 2003; Saarela *et al*., 2003) and may add to an enhanced depletion of lactose compared to the control cheese (Table 1). Only residual galactose was detected, which confirmed that glucose was the preferred microbial substrate utilized, derived from lactose hydrolysis. (Table 1; Fig.3 [Addendum]). The galactose content present in the cheese initially accumulated, ranging from a value of 0.05% after cutting to 0.49% after salting of the curd. The utilization of galactose was initiated at this stage, since the lactose was depleted. Accordingly a decrease in galactose concentration was observed, reaching a value of 0.36% after 8 days of ripening. Despite a marginal increase in galactose content at the beginning of ripening, the sugar was depleted after 70 days of ripening (Table 1; Fig.3 [Addendum]). *L. rhamnosus* has the ability to utilize galactose (Kandler and Weiss, 1986) and probably attributed to the depletion thereof. Traces of galactose were still found at the end of ripening in the model as well as in the control cheese.

The production of lactic, and citric acids correlated with the utilization of lactose and proliferation of the lactic acid bacterial starters during the processing period. The lactic acid content in the model cheese increased gradually from a percentage of 0.01% in the raw milk to a percentage of 0.90% after the salting process (Table 1; Fig.2 [Addendum]) resulting to a concentration of 0.61% after 114 days of ripening. This was remarkably lower than the 1.68% observed in the control cheese at that time. Despite the differences in lactic acid contents present in the control and model cheese, pH values in the cheeses remain similar. The citric acid content present in both cheeses remained similar, varying between 0.09% and 0.38% during the ripening period (Table 1; Fig.2 [Addendum]). Citric acid metabolism by *Lactobacillus* species is not well documented, although different aspects of it have been addressed in several studies. De Figueroa *et al*. (1996) reported that *L. rhamnosus* ATCC 7409 could utilize citrate as an energy source and that diauxic growth occurred on glucose plus citrate, with citrate not being used until glucose was exhausted. However, it has been reported that citrate fermentations by representative strains of *L. paracasei* and *L. rhamnosus* are not considered a negative property for adjuncts (Crow *et al*., 2001).

4.3.3. Sensory analysis

Cheddar cheeses incorporated with probiotic bacteria, *L. paracasei* (Gardiner *et al*., 1998; Stanton *et al*., 1998) and *Bifidobacterium bifidum* (Dinakar and Mistry, 1994), were found to cause adverse effects on sensory criteria while others (Broome *et al*., 1990; McSweeney *et al*., 1994; Lynch *et al*., 1996) found that probiotic adjuncts improved the flavor of Cheddar cheese. In this study matured Cheddar cheese incorporated with a probiotic starter adjunct, *L. rhamnosus,* developed an enhanced, mature and intense flavor profile, comparable to that of the control cheese. Corresponding to these findings, Gardiner *et al*. (1998) indicated that the addition of probiotic Lactobacilli had no adverse effects on the sensory criteria of cheese.

This could be attributed to the ability of *L. rhamnosus* to provide control over contaminant secondary flora which usually are responsible for flavor defects in cheese (Crow *et al*., 2001).

4.4. CONCLUSION

Cheese provides an attractive food delivery system for delivery of probiotic bio-cultures to the human GIT. A limited number of studies, however, have demonstrated different cheeses being able to support the viability of certain probiotic strains. The incorporation of the probiotic adjunct, *Lactobacillus rhamnosus* HN001 (DR20^{TM}) into matured Cheddar cheese, as conducted in this study, can be achieved without any alteration to the cheese making technology, making this system attractive for commercial exploitation. The microbiological data presented here, suggest that *L. rhamnosus* sustained high viability for at least 114 days of ripening, remaining above the 'therapeutic minimum'. No substantial differences in the normal aerobic microflora of the cheese were detected. In addition, observations regarding the production of organic acids and sugar metabolism, suggest that *L. rhamnosus* maintained its metabolic activity during the storage process without any impact on organoleptic properties of the cheese. This adjunct probably provides control over contaminating secondary flora, limiting flavor defects and delivers a health culture to the consumer. This study consequently illustrates that South African commercial matured Cheddar cheese has the potential to be an affective carrier system for the delivery of *L. rhamnosus* to the consumer. Such an expansion of the probiotic food range may offer a big marketing advantage to cheese industries in South-Africa and may result in major economic advantages.

Table 1. Analytical data of matured Cheddar cheese during processing and ripening over a period of 114 days.

Sampling time	pH	pH (Control)	Sugars				Organic Acids					
			Model		Control		Model			Control		
			Lactose (%)	Galactose (%)	Lactose (%)	Galactose (%)	Lactic acid (%)	Acetic acid (%)	Citric acid (%)	Lactic acid (%)	Acetic acid (%)	Citric acid (%)
Cheese vat (Start)	6.55	6.62	4.55	0.00	4.72	0.00	0.01	0.00	0.16	0.58	0.00	0.15
After starter-Stirring (Start)	6.51	6.61	4.16	0.05	4.78	0.00	0.06	0.00	0.15	0.59	0.00	0.15
After adding probiotic (LAR)	6.50	6.59	4.07	0.00	4.83	0.00	0.18	0.00	0.18	0.60	0.00	0.15
End probiotic mix	6.50	6.59	3.87	0.07	4.84	0.05	0.14	0.00	0.16	0.65	0.00	0.15
Renneting (Start)	6.49	6.59	3.67	0.05	4.85	0.09	0.20	0.00	0.17	0.69	0.00	0.15
Renneting (End)-Cutting (Start)	6.46	6.57	3.45	0.06	4.69	0.17	0.25	0.00	0.17	0.62	0.00	0.15
Cutting (End)	6.46	6.53	2.88	0.05	1.03	0.57	0.29	0.00	0.15	1.60	0.00	0.21
Drainage-Stirring (End)	6.23	6.00	2.61	0.06	1.05	0.97	0.31	0.00	0.14	1.24	0.00	0.20
Cutting-Stirring (End)	6.10	5.74	0.44	0.51	1.06	1.29	0.33	0.00	0.13	1.22	0.00	0.20
Turning (End)	5.90	5.48	0.08	0.54	1.06	1.61	0.29	0.00	0.09	1.20	0.00	0.19
Curd after salting	5.52	5.40	0.05	0.49	1.00	1.42	0.90	0.00	0.35	1.17	0.00	0.19
Pressing (End) - 5days	5.65	5.27	0.20	0.39	0.00	1.35	0.30	0.00	0.18	1.19	0.00	0.19
8 days	5.33	5.26	0.01	0.36	0.00	1.19	0.32	0.00	0.22	1.21	0.00	0.18
15 days	5.60	5.26	0.02	0.52	0.00	1.00	0.36	0.00	0.27	1.29	0.00	0.17
33 days	5.47	5.29	0.01	0.59	0.00	0.56	0.44	0.00	0.30	1.31	0.00	0.16
36 days	5.56	5.28	0.02	0.45	0.00	0.53	0.35	0.00	0.09	1.45	0.00	0.17
57 days	5.44	5.27	0.00	0.33	0.00	0.49	0.43	0.00	0.25	1.58	0.00	0.18
70 days	5.31	5.26	0.00	0.03	0.00	0.32	0.50	0.00	0.36	1.59	0.00	0.18
77 days (Sell by)	5.30	5.27	0.00	0.05	0.00	0.25	0.49	0.00	0.38	1.61	0.00	0.19
84 days (Exp./BB)	5.20	5.27	0.00	0.10	0.00	0.13	0.42	0.00	0.28	1.64	0.00	0.20
97 days	5.30	5.27	0.00	0.07	0.00	0.12	0.52	0.00	0.28	1.66	0.00	0.21
114 days	5.39	5.28	0.00	0.09	0.00	0.11	0.61	0.00	0.28	1.68	0.00	0.21

* Data are the means of 3 repetitions

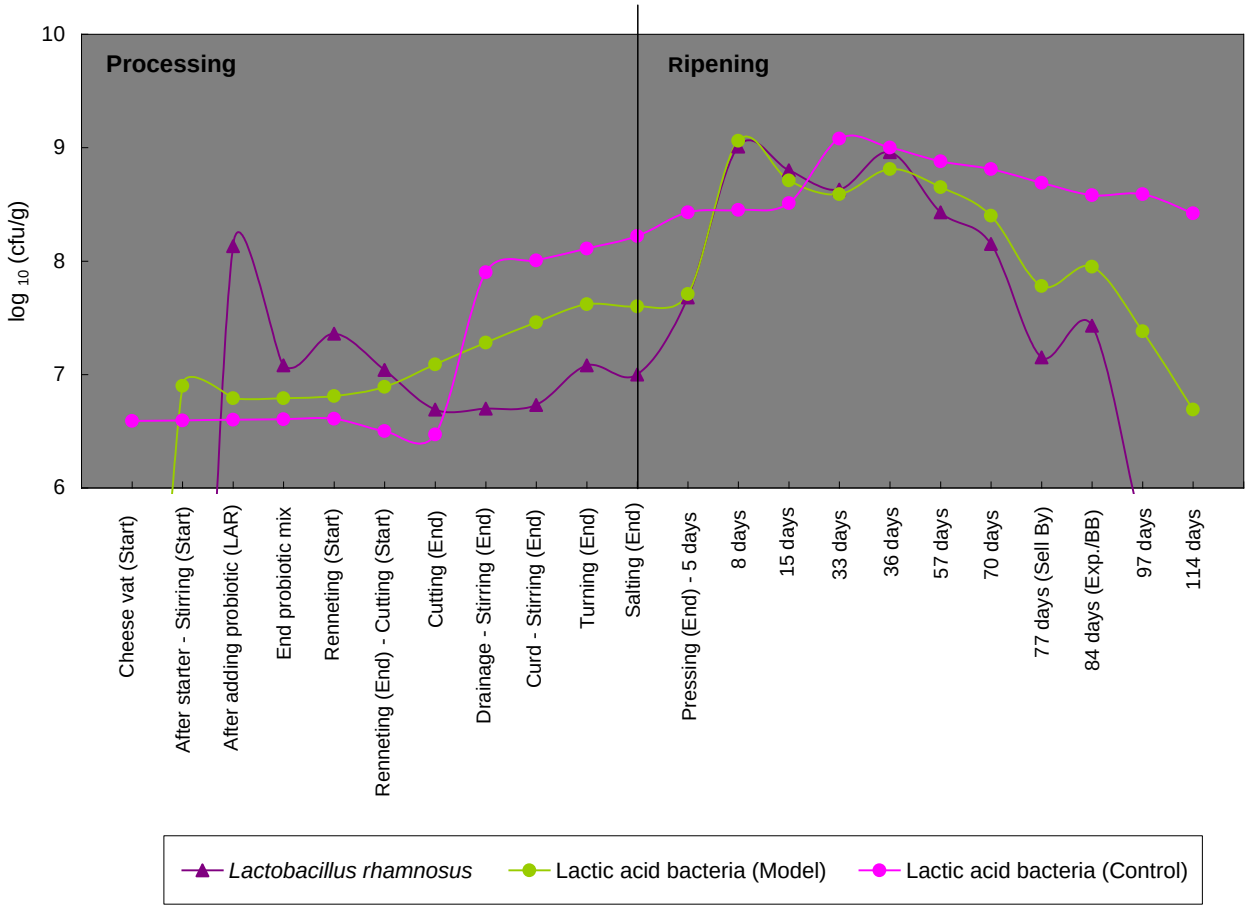

Fig. 1. Changes in the counts of lactic acid bacteria (control), lactic acid bacteria (model) and *Lactobacillus rhamnosus* during processing and ripening of matured Cheddar cheese.

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ADDENDUM

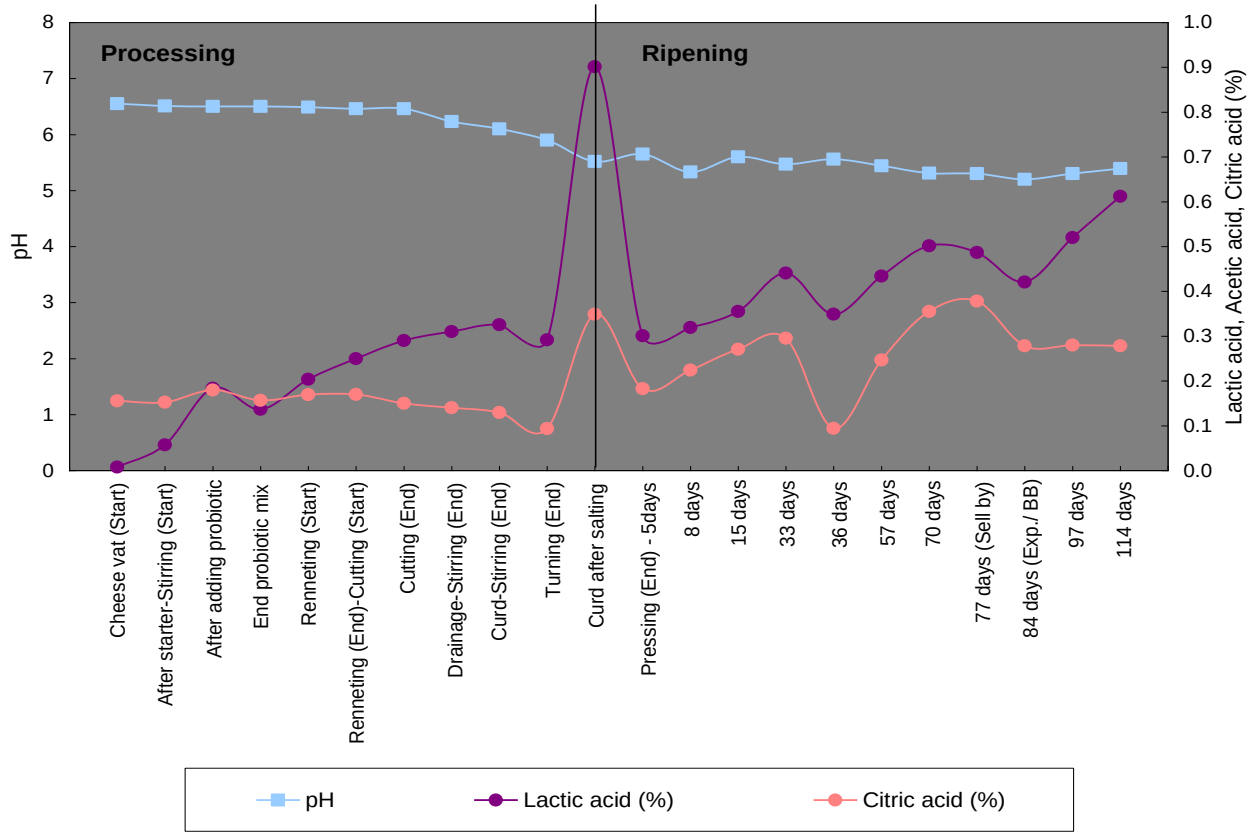

Fig. 2. Changes in the pH and organic acid concentrations during processing and ripening of matured Cheddar cheese with the incorporation of *Lactobacillus rhamnosus* as probiotic adjunct.

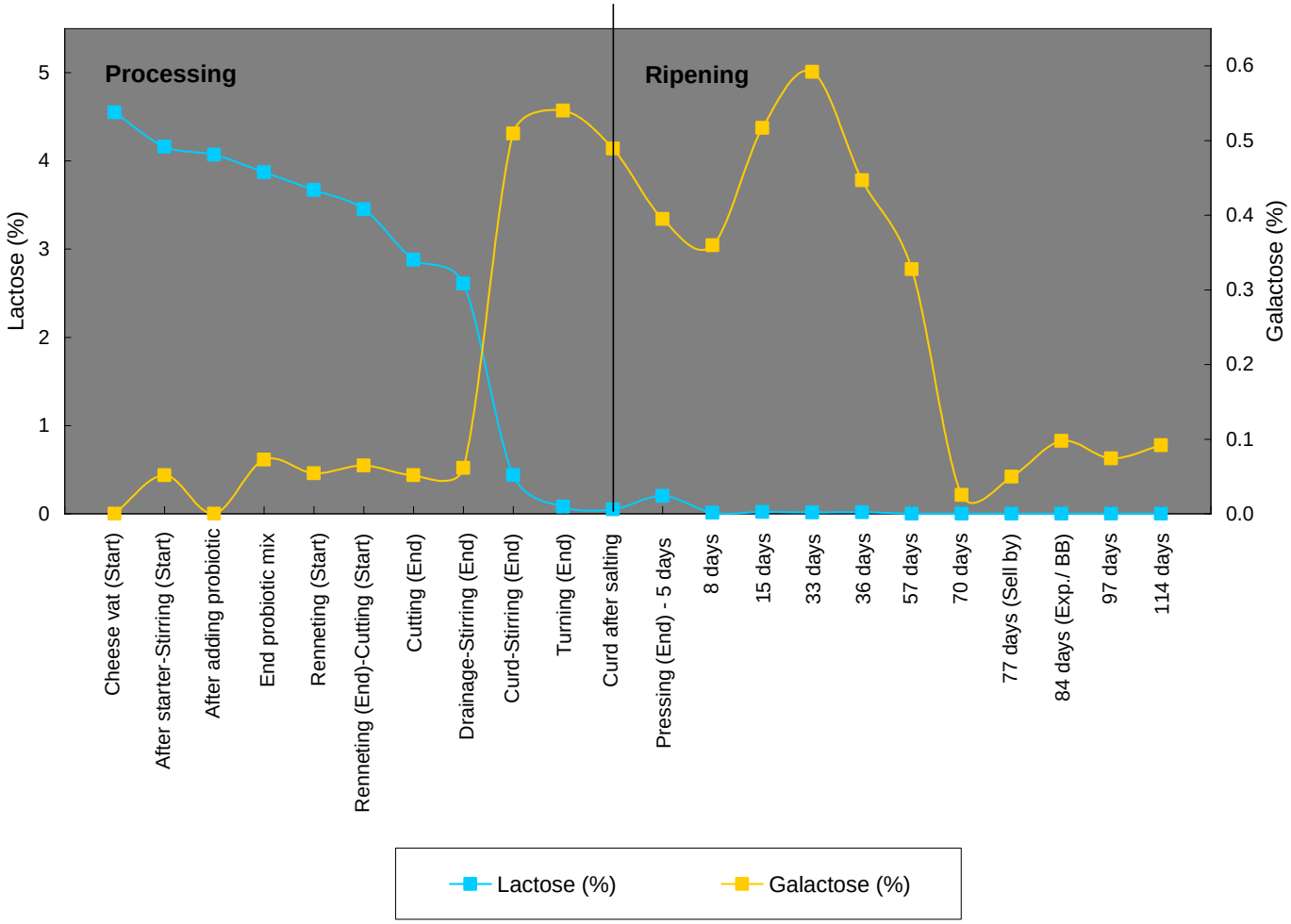

Fig. 3. Changes in the sugar concentrations during processing and ripening of matured Cheddar cheese with the incorporation of *Lactobacillus rhamnosus* as probiotic adjunct.

CHAPTER 5

General discussion and conclusion

Consumers are becoming increasingly concerned about nutrition and health properties, and therefore a health promoting niche was established with the introduction of functional foods. Probiotics, as part of the functional food product range, are a mono- or mixed culture of live microorganisms which, when administered to either human or animal, beneficially affect the host by improving the properties of the indigenous microflora (Huis in't Veld and Havenaar, 1991). These beneficial effects can only be elicited when viable cells ($\geq 10^6$cfu/g) are ingested, however the poor survival of probiotic species is a major constraint in the development of new products for the expansion of the probiotic food range (Dave and Shah, 1997; Klaver *et al*., 1993; Lourens and Viljoen, 2002; Rybka and Kailasapathy, 1995). The estimated survival of probiotic bacteria in products is based on the ability of the enumeration media to provide reliable cell counts, though, numerous studies have illustrated contrasting reports (Pacher and Kneifel, 1996; Vinderola *et al*., 2000; Vinderola and Reinheimer, 1999). Consequently, product manufacturers are very interested in techniques and enumeration media that can provide reliable counts of probiotic bacteria in their products.

For successfully developing probiotic containing products, it is important to understand the growth characteristics of the probiotic strains introduced into the food product. The growth kinetics of microorganisms, i.e. that of probiotic bacteria, largely depend on the presence of intrinsic and extrinsic factors like; dissolved oxygen, pH, growth promoters and inhibitors (i.e. NaCl), within the food matrix (McDonald and Sun, 1999). The level of inoculation, incubation and storage temperatures as well as fermentation time plays an equally important role (Gomes and Malcata, 1999; Modler *et al*., 1990; Rybka and Kailasapathy, 1997). Predictive modeling may contribute to a better understanding of, and control of microbial processes, and help to clarify in which manner, and to which extend, the food environment will interfere with the functionality of the strains used.

Yoghurt and fermented milks have received considerable attention as carriers of live probiotic cultures. With the growing consumer awareness between the link of maintaining good health and a balanced and varied diet, a demand for new food products with proven health claims was pursued. Cheddar cheese may offer certain advantages as a carrier system for live probiotics to the gastro-intestinal tract of humans. If probiotic cheese can be produced with little or no alteration to the cheese making technology, it would make the development of probiotic cheeses attractive for commercial exploitation. The successful incorporation of probiotic cultures into cheese will also expand the current limited probiotic food range, whereas, the cheese industries will benefit from a marketing advantage such as value-added probiotic containing cheeses.

1. Statistical analysis of enumeration media for probiotic adjunct *Lactobacillus rhamnosus* HN001 (DR20^{TM}) in dairy products

In order to provide health benefits to the consumer, probiotics should be present in a minimum concentration ('therapeutic minimum') of 10^6cfu/g of product (Ouwehand *et al*., 2002; Shah, 2000) and is therefore reasonable to assume that these effects can only be elicited when viable cells are ingested. The accuracy of the survival of probiotic bacteria in products is based on the ability of the enumeration media to provide reliable cell counts, however, numerous studies have illustrated contrasting reports (Dave and Shah, 1997; Lourens *et al*., 2000; Shin *et al*., 2000; Vinderola *et al*., 2000; Vinderola and Reinheimer, 1999). These variable counts pose the likelihood of over and/or underestimation of probiotic bacterial counts, misleading the consumer. In addition, the range of different culture media used for the detection and enumeration of probiotics in fermented foods, also indicates that there is no standard culture medium (Roy, 2001). It has therefore, become necessary to evaluate these media for their reliability and suitability for identification and enumeration purposes.

The objectives of this study were to evaluate nine different bacteriological media and to assess their suitability to selectively enumerate *Lactobacillus rhamnosus* strain HN001 (DR20^{TM}), in the presence of other lactic acid starter bacteria, from a commercially produced South African matured Cheddar cheese. The suitability of the media was statistically compared based on significant differences in recovery between the different mediums (ANOVA, NCSS, 2004) as well as the interaction between the media and the availability of oxygen (Turkey-Kramer multiple comparison test, NCSS, 2004), compared to the non-selective MRS agar applied as reference medium. MRS-vancomycin (MRS-V) and basal agar medium supplemented with 20% rhamnose and 0.05% vancomycin (BA-R(20%)V) were ranked superior based on qualitative and quantitative results, compared to other evaluated media. MRS-V and aerobic incubation conditions at 43^{o}C for 48h proved to be the most selective medium to enumerate *L. rhamnosus* in the presence of other cheese starter cultures.

2. The combined effects of pH, lactic acid, NaCl and temperature on the growth of probiotic adjunct *Lactobacillus rhamnosus* HN001 (DR20^{TM}) in a Cheddar cheese environment

In addition to starter cultures, probiotic bacteria are now more often included in fermented dairy products (i.e. yoghurts, fermented milks, cheeses etc.) with the aim to contribute to the health and well-being of consumers. However, in order to successfully develop these products, it is important to understand the growth characteristics thereof. The growth kinetics of microorganisms, i.e. that of probiotic bacteria largely depend on the presence of intrinsic and extrinsic factors like; dissolved oxygen, pH, and growth promoters and inhibitors (i.e. NaCl), within the food matrix (McDonald and Sun, 1999). The level of inoculation, incubation and storage temperatures, and fermentation time also plays a critical role (Gomes and Malcata, 1999; Modler *et al*., 1990; Rybka and Kailasapathy, 1997).

To be successful, manufacturers should consider the effects of the microenvironment of the food product, during processing and storage processes, in order to ensure that the concentration of probiotic species at the time of consumption provides a therapeutic dose to consumers. It has been reported that by manipulating the manufacturing and storage conditions of these products, the survival and viability of lactic acid bacteria (LAB) and bifidobacteria have shown a dramatic increase, thus, optimizing their growth and survival (Rybka and Kailasapathy, 1997). Predictive microbiology is an important tool in the food industry to predict the behavior of microorganisms (Zurera-Cosano *et al*., 2004b).

The objectives of this study were to study the combined effects of environmental conditions; pH (5.2, 5.7, 6.2), lactic acid (0, 1, 2%), salt (NaCl) (0, 1, 2%) and incubation temperatures (10, 30, 37^{o}C) on the anaerobic growth of *Lactobacillus rhamnosus* strain HN001 (DR20^{TM}) in MRS culture broth. This was done in an attempt to define the boundaries of growth for all factors, and subsequently construct a dynamic predictive model. Using Response Surface Methodology, a polynomial model, with good predictive power, was developed, explaining approximately 90.7% (R^2 = 0.9071) of the total variance of the responses. This model provides a means for rapidly estimating how the probiotic bacterium is likely to respond to any combination of the four variables within the specified ranges, and is a valuable tool in enabling its application for shelf-life estimation of a food product

3. Microbial development within matured Cheddar cheese containing *Lactobacillus rhamnosus* HN001 (DR20^{TM}) as an adjunct probiotic culture

Beneficial effects of probiotic bacteria have been well established (Rybka and Kailasapathy, 1995) and pobiotic-containing foods are becoming a key factor affecting consumer choice.

To date, the most popular food delivery system for these cultures has been freshly fermented dairy foods (Hoover, 1993; Ishibashi and Shimamura, 1993; Sanders *et al*., 1996). Cheddar cheese may offer certain advantages as carrier system for live probiotic cultures. The higher pH, the matrix, a more solid consistency, and higher fat content of cheese may offer protection to the organism and support their long-term survival (Gardiner *et al*., 1998; Ross *et al*., 2002; Stanton *et al*., 1998). The deliberate incorporation of non-starter lactic acid bacteria (NSLAB) as adjuncts to cheese milk have been reported to improve and accelerate Cheddar cheese flavor and texture development (Broadbent *et al*., 2002; Madkor *et al*., 2000; Marilly and Casey, 2004), although in some cases have been responsible for some flavor defects (Lee *et al*., 1990). Such an expansion of the probiotic product range may offer a big marketing advantage to cheese industries in South Africa.

The objectives of this study were to explore the possibility of introducing the probiotic NSLAB, *Lactobacillus rhamnosus* strain HN001 (DR20^{TM}) (Dekker and Lubbers, 2002), into a South African commercial Cheddar cheese in order to study their viability during long-term ripening and storage. The effect of these two organisms on cheese flavor and texture, as well as the chemical properties of the cheese, were determined. This was done without alteration of the cheesemaking technology, which makes the system attractive for commercial exploitation. *L. rhamnosus* remained highly viability for at least 114 days of ripening, while still remaining above the ‘therapeutic minimum’. The normal aerobic microflora of the cheese was not substantially affected, while *L. rhamnosus* sustained a high metabolic activity during the storage process, without adversely affecting organoleptic properties of the cheese. This adjunct probably provides control over contaminating secondary flora and limiting flavor defects while subsequently delivering a beneficial health culture to the consumer.

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CHAPTER 6

Summary

A literature review regarding the properties of probiotic bacteria were discussed including their therapeutic value, the survival in bio-products and the expansion of the probiotic food range. Cheese offers certain advantages as delivery system for live probiotic bacteria to the human gastro-intestinal tract. Furthermore, the enumeration of probiotics in functional foods was discussed, with special reference made to enumeration media. Probiotic regulations were also discussed, with an in-dept look at regulatory platforms world wide as well as regulations regarding administration levels.

Normal practise in the manufacturing of functional food products, is to incorporate the slow growing probiotic cultures, containing beneficial effects, with the fast growing lactic acid bacterial starter cultures, which aid in the speed of the fermentation process. An important parameter in monitoring official levels of viable probiotics, is the ability to count probiotic bacteria differentially, however great controversy exists regarding administration levels as well as standard enumeration media. Nine bacteriological media were subsequently evaluated to assess their suitability and to selectively enumerate *L. rhamnosus* strain HN001 (DR20^{TM}), in the presence of other lactic acid starter bacteria in South African matured Cheddar cheese. Differences in recovery between the different media as well as the interaction between the media and the availability of oxygen were investigated. MRS-V and BA-R(20%)V agar were ranked as the superior media, while MRS-V agar and aerobic incubation conditions at 43^{o}C for 48h proved to be the most selective medium to enumerate *L. rhamnosus* in the presence of other cheese starter cultures.

Predictive modeling may contribute to a better understanding of and control of microbial processes, and help to clarify in which manner, and to which degree, the food environment will interfere with the functionality of the probiotic strains used.

At environmental conditions similar to those during manufacturing, ripening and/or storage processes of cheese, the combined effects of pH, lactic acid, salt (NaCl), and incubation temperatures on the growth of probiotic culture *L. rhamnosus* HN001 (DR20^{TM}) were investigated in MRS laboratory broth. The developed Response Surface (RS) model provided reliable estimates of the parameters studied and explains approximately 90.7% of the variance within the model data. This model provides a means for rapidly estimating how the probiotic bacterium is likely to respond to any combination of the four variables within the specified ranges and is a valuable tool in enabling its application for shelf-life estimation within a food product. Future research, focusing on Central Composite Designs (CCD) is required in order to optimize the model performance, to subsequently minimize variance associate with the kinetic parameters, but also to estimate optimum responses within the experimenting range.

The health benefits of probiotic-containing products are becoming a key factor, affecting consumer choice and therefore the existing limited range of such products needs to be expanded. Cheese may offer certain advantages as carrier system for live probiotic organisms to the human gastro-intestinal tract. The possibility of introducing *Lactobacillus rhamnosus* strain HN001 (DR20^{TM}) into South African matured Cheddar cheese was explored. The long-term ripening and storage of the organism, the effect on cheese flavor and texture as well as the chemical properties of the cheese were determined. *L. rhamnosus* strain HN001 (DR20^{TM}) remained highly viable for at least 114 days of ripening, while still satisfying the criteria for probiotic foods. The viability profile of the lactic acid starter bacteria was not substantially affected and a normal, good cheese texture, flavor and appearance were retained. This probiotic adjunct possibly provides control over contaminating secondary flora and limiting flavor defects while delivering a health culture to the consumer.

(**Keywords**: *Lactobacillus rhamnosus*, selective enumeration media, predictive modeling, Response Surface model)