IDENTIFICATION OF PROBIOTIC MICROBES FROM SOUTH AFRICAN PRODUCTS USING PCR-BASED DGGE ANALYSES

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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously, in its entirety or in part, submitted it at any other university for a degree.

ABSTRACT

The regular consumption of probiotics is becoming a recognized trend in the food industry due to several reported health benefits. A probiotic is defined as a live microbial feed supplement that beneficially affects the host animal by improving its intestinal microbial balance. A wide variety of probiotic food products are available on the South African market and comprise an assortment of fermented milks, as well as lyophilized preparations in tablet or capsule form.

Strains of *Lactobacillus acidophilus* and *Bifidobacterium* species are mostly used as probiotic microbes in the industry due to their health enhancing effect. The survival of sensitive probiotic microbial species in food matrices are influenced by various factors such as oxygen concentration, pH levels and manufacturing and storage conditions. These should be considered and monitored as the South African food and health regulations stipulate that probiotic microbes should be present at a concentration of 10^6 cfu.ml^{-1} in order to exert a beneficial effect. Some health benefits are also correlated to specific microbial species and strains and these factors have resulted in the need for the rapid and accurate identification of probiotic microbes present in food products.

The probiotic microbes present in probiotic yoghurts and supplements have in the past been identified using traditional methods such as growth on selective media, morphological, physiological and biochemical characteristics. However, even some of the most sophisticated cultural-dependant techniques are not always sufficient for the identification and classification of especially *Bifidobacterium*, as well as closely related *Lactobacillus* species. Molecular techniques are more often employed for the rapid and accurate detection, identification and characterization of microbial species present in food products.

The aim of this study was to detect and identify the probiotic species present in various commercial South African yoghurts and lyophilized preparations using PCR-based DGGE analysis. A 200 bp fragment of the V2-V3 region of the 16S rRNA gene was amplified and the PCR fragments were resolved by DGGE. The unique fingerprints obtained for each product were compared to two reference markers A and B in order to identify the bands present. The results obtained were verified by species-specific PCR, as well as sequence analyses of bands that could not be identified when compared to the reference markers.

Only 54.5% of the South African probiotic yoghurts that were tested did contain all the microbial species as were mentioned on the labels of these products, compared to merely one third (33.3%) of the lyophilized probiotic food supplements. Some *Bifidobacterium* species were incorrectly identified according to some product labels, while other products contained various microbes that were not mentioned on the label. Sequence analysis confirmed the presence of a potential pathogenic *Streptococcus* species in one of the yoghurt products and in some instances the probiotic species claimed on the labels were non-scientific and misleading.

The data obtained in this study showed that the various South African probiotic products tested were of poor quality and did not conform to the South African regulations. PCR-based DGGE analysis proofed to be a valuable approach for the rapid and accurate detection and identification of the microbial species present in South African probiotic products. This could help with future implementation of quality control procedures in order to ensure a reliable and safe probiotic product to the consumer.

UITTREKSEL

Die gereelde inname van probiotiese produkte is besig om 'n erkende tendens in die voedselindustrie te word, as gevolg van verskeie gesondheidsvoordele wat daaraan gekoppel word. 'n Probiotika word gedefinieer as 'n voedingsaanvulling wat uit lewendige mikrobes bestaan en wat 'n voordelige effek op mens of dier het deur 'n optimale mikrobiese balans in die ingewande te handhaaf. 'n Wye verskeidenheid probiotiese voedselprodukte is tans beskikbaar op die Suid-Afrikaanse mark. Hierdie bestaan hoofsaaklik uit verskeie gefermenteerde melkprodukte asook 'n reeks tablette en kapsules wat probiotiese mikrobes in gevriesdroogde vorm bevat.

Lactobacillus acidophilus tipes en *Bifidobacterium* spesies word die algemeenste in die voedselindustrie gebruik aangesien hierdie spesifieke mikrobes bekend is om goeie gesondheid te bevorder. Die oorlewing van sensitiewe probiotiese mikrobiese spesies in voedsel matrikse word beïnvloed deur faktore soos suurstof konsentrasie, pH-vlakke en vervaardigings- en opbergings kondisies. Hierdie faktore moet in aanmerking geneem word en verkieslik gemonitor word aangesien die Suid-Afrikaanse voedsel en gesondheids regulasies stipuleer dat probiotiese mikrobes teen 'n konsentrasie van 10^6 kolonie vormende eenhede per ml teenwoordig moet wees om 'n voordelige effek te toon. Sommige gesondheidsvoordele word direk gekoppel aan spesifieke mikrobiese spesies en spesie-tipes. Hierdie faktore het gelei tot 'n groot aanvraag na vinnige en akkurate metodes vir die identifikasie van probioties mikrobes in voedselprodukte.

Die probiotiese mikrobes teenwoordig in probiotiese joghurts en ook die gevriesdroogde vorms in tablette en kapsules, was al geïdentifiseer deur gebruik te maak van tradisionele metodes soos groei op selektiewe media, morfologiese, fisiologiese en biochemiese eienskappe. Selfs van die mees gesofistikeerde kultuur-afhanklike tegnieke is egter nie altyd voldoende vir die identifikasie en klassifikasie van veral *Bifidobacterium* en na-verwante *Lactobacillus* spesies nie. Molekulêre metodes word dikwels aangewend vir die vinnige en akkurate deteksie, identifikasie en karakterisering van mikrobes teenwoordig in voedselprodukte.

Die doel van hierdie studie was om die probiotiese mikrobes teenwoordig in verskeie Suid-Afrikaanse joghurts en gevriesdroogde aanvullings, te identifiseer deur gebruik te maak van polimerase kettingreaksie (PKR)-gebaseerde denaturerende gradiënt jelelektroforese (DGGE) analise. 'n PKR fragment van 200 bp van die V2-V3 gedeelte van die 16S ribosomale RNS (rRNS) geen is geamplifiseer, en die PKR fragmente is geskei met behulp van DGGE. Die unieke vingerafdrukke wat verkry is vir elke produk is teen twee verwysings merkers A en B vegelyk om die bande teenwoordig in die profiele te identifiseer. Die resultate is bevestig deur spesies-spesifieke PKR en ook deur die ketting volgordes van die DNS fragmente te bepaal wat nie geïdentifiseer kon word deur vergelyking met die verwysings merkers nie.

Slegs 54.5% van die Suid-Afrikaanse probiotiese joghurts wat getoets is het al die mikrobiese spesies bevat soos aangedui was op die etikette van hierdie produkte, teenoor slegs 'n derde (33.3%) van die gevriesdroogde voedingsaanvullings. Sekere *Bifidobacterium* spesies is verkeerd geïdentifiseer op sommige van die produk etikette, terwyl ander produkte verskeie mikrobes bevat het wat nie op die etiket aangedui was nie. 'n Potensiële patogeniese *Streptococcus* spesie is in een van die joghurt produkte gevind soos bevestig deur DNS kettingvolgorde bepalings. In sommige gevalle was die probiotiese spesienaam wat aangedui is op die etiket onwetenskaplik en misleidend.

Die resultate wat uit hierdie studie verkry is dui aan dat die Suid-Afrikaanse probiotiese produkte wat getoets is van 'n swak gehalte is en nie aan die Suid-Afrikaanse regulasies voldoen nie. Daar is getoon dat PKR-gebaseerde DGGE analise 'n waardevolle tegniek kan wees vir die akkurate deteksie en identifisering van die mikrobiese spesies teenwoordig in probiotiese produkte. Dit kan help met die toekomstige implementering van kwaliteitskontrolerings prosedures om 'n mikrobiologiese betroubare en veilige produk aan die verbruiker te verseker.

dedicated to my parents

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Language and style used in this thesis are in accordance with the requirements of the *International Journal of Food Science and Technology*. This thesis represents a compilation of manuscripts where each chapter is an individual entity and some repetition between chapters has, therefore, been unavoidable.

CHAPTER 1

INTRODUCTION

The maintenance and improvement of human health by the consumption of specific food commodities is becoming a well known phenomenon. This awareness has led to an increasing interest in probiotic food products. A probiotic is generally defined as a live microbial feed supplement that beneficially affects the host by improving the intestinal microbial balance (Fuller, 1989). Health benefits that are commonly associated with the regular consumption of probiotics include the improvement of lactose intolerance (Kim & Gilliland, 1983; Marteau *et al.*, 1990), the reduction of cholesterol levels (Gilliland, 1990; Akalin *et al.*, 1997) and the control of intestinal infections (Saavedra *et al.*, 1994; McFarland *et al.*, 1995).

A wide variety of probiotic products and supplements are commercially available on the South African market, either in lyophilized form or as fermented foods. World-wide efforts are being made to incorporate probiotic microbes into food products other than fermented milks. Cheese (Dinakar & Mistry, 1994; Blanchette *et al.*, 1996; Gomes & Malcata, 1998), ice cream (Modler *et al.*, 1990a; Hekmat & McMahon, 1992) and dried fruit (Betoret *et al.*, 2003) are examples of foods that are currently available or are being investigated as suitable carrier foods for probiotic microbes.

South African health and food regulations stipulate that the label of probiotic foods should indicate the full scientific name of the microbial species present in the product (Anon., 2002). This is important as it is mostly accepted that different species from the same genus may have different beneficial properties (Salminen *et al.*, 1998) and that probiotic properties are strain-specific (Prasad *et al.*, 1998; Sanders, 1999). The species or strains of probiotic microbes used in foods are further considered important due to the fact that some *Bifidobacterium* species or strains are more acid- and oxygen-tolerant (Modler *et al.*, 1990b), thereby increasing their survival in food environments.

World-wide safety concerns have arisen from reports indicating the presence of microbial species in probiotic products that were not listed on the label (Fasoli *et al.*, 2003; Temmerman *et al.*, 2003), as well as the presence of potentially pathogenic species in probiotic foods (Hamilton-Miller *et al.*, 1999).

Due to this safety awareness and expanding interest in probiotics by the general public, there is an increased demand for the rapid and accurate detection and identification of probiotic microbes.

Various selective cultural media have been proposed for the detection of probiotic bacteria. However, even the most sophisticated traditional isolation and identification techniques are not always effective for the identification of closely related isolates (Yaeshima *et al.*, 1996; Holzapfel *et al.*, 1997; O'Sullivan, 1999). The preparation of cultural media is also labour intensive (Matsuki *et al.*, 2002) and in some cases it is difficult, if not impossible to achieve complex nutritional and environmental conditions in a laboratory (Tannock, 2002). Due to fastidious requirements for anaerobiosis as well as complex nutritional interactions between microbes the problem arises that some viable microbes may be non-cultivable on laboratory culture media (Tannock, 2002).

Advances in molecular techniques have led to various improvements in the field of microbial detection and identification (Cocolin *et al.*, 2004) and are often implemented to establish microbial diversity in complex food samples (Gonzalez *et al.,* 2003; Fasoli *et al.*, 2003). The objective of this study was to identify the different probiotic microbes present in various South African products by PCR-based DGGE fingerprinting combined with species-specific PCR detection.

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CHAPTER 2

LITERATURE REVIEW

A. BACKGROUND

A wide variety of products and supplements containing viable microbes with probiotic properties are commercially available on the South African market, either in lyophilised form or as fermented food commodities. Strains of *Lactobacillus acidophilus* and *Lactobacillus casei* have the longest history of application due to their health benefits (Holzapfel *et al.*, 1998). Currently various *Lactobacillus* spp., as well as *Bifidobacterium* spp. are used in commercial probiotic products and strains of *Lb. acidophilus* and *Bifidobacteria* spp. are collectively known as the AB-cultures. Other lactic acid bacteria as well as a few other genera are also currently used in probiotic products (Table 1). The major probiotic microbes used in these foods include the lactic acid-excreting bacteria such as lactobacilli, lactococci, streptococci or bifidobacteria although some yeasts are also used. Monocultures or mixed cultures, containing up to nine different species, can be used in probiotic products (Gibson & Fuller, 2000). The non-lactic acid bacteria are seldom used in food commodities but are rather administered as lyophilised or encapsulated pharmaceutical preparations (Holzapfel *et al.*, 1998).

The most frequently produced commercial probiotic products are of dairy origin. Japan produces and markets more than 50 different dairy products containing viable probiotic cultures. Similar trends are observed in other European countries such as France, Germany and Sweden where probiotic products account for at least 25% of all fermented milk products. Approximately 80 different bifido-containing products are available world wide (Hoier, 1992). Probiotic food products available on the world market are summarized in Table 2.

Besides the fermented milk products (Mital & Garg, 1992; Tamime *et al.*, 1995), cheese (Dinakar & Mistry, 1994; Blanchette *et al.*, 1996; Gomes & Malcata, 1998), ice cream (Modler *et al.*, 1990a; Hekmat & McMahon, 1992), fermented soya milk (Valdez & de Giori, 1993), soya yoghurt (Murti *et al.*, 1992) and dried fruit (Betoret *et al.*, 2003) are currently available or is being investigated as suitable carrier food products for probiotic cultures.

Table 1. Probiotic microbes (Holzapfel *et al.*, 1998).

***Lactobacillus* spp.**	***Bifidobacterium* spp.**	**Other LAB[a]**	**Non-LAB**
Lb. acidophilus	*B. adolescentis*	*Enterococcus faecalis*[b]	*Bacillus cereus* ('toyoi')[b]
Lb. casei	*B. animalis*	*Enterococcus faecium*	*Escherichia coli* ('Nissle 1917')
Lb. crispatus	*B. bifidum*	*Lactococcus lactis*	*Propionibacterium freudenreichii*[b]
Lb. gallinarum[a]	*B. breve*	*Leuconostoc mesenteroides*	*Saccharomyces cerevisiae* (boulardii)
Lb. gasseri	*B. infantis*	*Pediococcus acidilactici*	
Lb. johnsonii	*B. lactis*	*Sporolactobacillus inulinus*[b]	
Lb. paracasei	*B. longum*	*Streptococcus thermophilus*	
Lb. plantarum			
Lb. reuteri			
Lb. rhamnosus			

[a]Lactic acid bacteria

[b]Mainly used in animal feed

Table 2. Examples of probiotic foods available world-wide.

Product	Country of Origin	Probiotics	References
AB milk products	Denmark	*Lb. acidophilus* *B. bifidum*	Tamime *et al.*, 1995
Acidophilus bifidus yoghurt	Germany	Yoghurt culture[a] *Lb. acidophilus* *B. bifidum* or *B. longum*	Tamime *et al.*, 1995
Acidophilus milk	Several countries	*Lb. acidophilus*	Gomes & Malcata,1999
Acidophilus yoghurt	Several countries	*Lb. acidophilus* Yoghurt culture[a]	Gomes & Malcata,1999
Acidophilus yeast milk	USSR	Yeast *Lb. acidophilus*	Mital & Garg, 1992
ABC ferment	Germany	*Lb. acidophilus* Bifidobacteria *Lb. casei*	Holzapfel *et al.*, 1997
A-38	Denmark	*Lb. acidophilus*	Mital & Garg, 1992
Akult	Japan	*Lb. acidophilus* *B. bifidum* *B. breve* *Lb. casei* subsp. *casei*	Gomes & Malcata,1999
Bifidus milk	Germany	*B. bifidum* or *B. longum*	Tamime *et al.*, 1995
Bifidus yoghurt	Several countries	*B. bifidum* or *B. longum* Yoghurt culture[a]	Tamime *et al.*, 1995
Bifighurt	Germany	*B. longum* *Str. thermophilus*	Tamime *et al.*, 1995
Bifilakt	USSR	*Lactobacillus* spp. *Bifidobacterium* spp.	Tamime *et al.*, 1995
Biogarde	Germany	*Lb. acidophilus* *B. bifidum* *Str. thermophilus*	Mital & Garg, 1992

Table 2. (continued).

Product	Country of Origin	Probiotics	References
Biogurt	Germany	*Lb. acidophilus* *Str. thermophilus*	Mital & Garg, 1992
Biokys	Czech Republic	*Lb. acidophilus* *B. bifidum* *Pediococcus acidilactici*	Mital & Garg, 1992
Cultura-AB	Denmark	*Lb. acidophilus* *B. bifidum*	Mital & Garg, 1992
Gefilac	Finland	*Lb. rhamnosus*	Du Toit, 1998
Gaio	Denmark	*Enterococcus faecium* *Str. thermophilus*	Du Toit, 1998
Mil-Mil	Japan	*Lb. acidophilus* *B. bifidum* *B. breve*	Tamime *et al.*, 1995
Miru-Miru	Japan	*Lb. casei* *Lb. acidophilus*	Du Toit, 1998
Ofilus	France	*Str. thermophilus* *Lb. acidophilus* *B. bifidum* or *Lactococcus lactis* subsp.*cremoris* *Lb. acidophilus* *B. bifidum*	Tamime *et al.*, 1995
Proghurt	Chile	*Lactococcus lactis* biovar. *diacetylactis* *Lactococcus lactis* subsp. *cremoris* *Lb. acidophilus* *B. bifidum*	Tamime *et al.*, 1995
Yakult	Japan	*Lb. casei*	O'Sullivan *et al.*, 1992
Zabady	Egypt	*B. bifidum* Yoghurt culture[a]	Kebary, 1996

[a]Yoghurt culture: *Streptococcus thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus*

Some of the probiotic products may also contain bifidogenic factors that are defined as compounds, usually of carbohydrate nature, that survive direct metabolism by the host and reach the large intestine where they are preferentially metabolised by bifidobacteria as a source of energy. Bifidogenic factors may fall under the relatively new concept of prebiotics that are defined as non-digestible food ingredients that selectively stimulates the growth and/or activity of one or a limited number of bacteria in the colon and, thereby, beneficially influencing the health of the host (Gibson & Roberfroid, 1995). Due to the sensitivity of bifidobacteria to oxygen and due to their low acid tolerance it is difficult to maintain viability of these species in dairy products and the application of bifidogenic factors together with probiotics can help to encourage growth and the presence of high microbial numbers during normal shelf-life conditions (Modler *et al.*, 1990b). Oligosaccharides (Yamada *et al.*, 1993; Tomomatsu, 1994), fructo-oligosaccharides such as inulin and oligofructose (Gibson & Wang, 1994a) and lactulose (Modler *et al.*, 1990b; Crittenden, 1999) are examples of compounds that serve as bifidogenic factors.

B. HISTORY

The history of live microbial feed supplements goes back thousands of years and it is most likely that chance contamination and favourable environmental and climatic conditions played the key roles in the development of many traditional soured milks and cultured dairy products. These products that are still widely consumed were often in the past used therapeutically before the existence of bacteria was acknowledged. Scientists such as Hippocrates prescribed milk for curing disorders of the stomach and intestines (Öberman & Libudzisz, 1998). Even though there have been doubts regarding the health benefits of these cultured dairy products their effect in the prevention of the spoilage of milk indisputably had a beneficial effect on the nutritional status of the community (Fuller, 1992).

It was not until the beginning of the twentieth century that the bacteriologist, Eli Metchnikoff (Pasteur Institute), gave a scientific rationale for the beneficial effects of yoghurt bacteria (Hughes & Hoover, 1991; Fuller, 1992; O'Sullivan *et al.*, 1992). He noted that the Bulgarian peasants, who consumed a large volume of

soured milk, lived to an old age and he contributed the long life of these peasants to their yoghurt intake. Metchnikoff's work can therefore be regarded as the birth of probiotics (Fuller, 1992).

C. DEFINITION OF PROBIOTICS

The word probiotics is derived from the Greek meaning 'for life' and the definition of probiotics has evolved over the years. The most accepted definition is that of Fuller (1989) who stated that a probiotic is 'a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance'. This version emphasizes the need for the supplement to be composed of viable microbes. The definition was broadened in the last decade by Havenaar & Huis in't Veld (1992) who defined probiotics as 'viable microbes that exhibit a beneficial effect on the health of the host upon ingestion, by improving the properties of its indigenous microflora'. This definition did not restrict probiotic activity to the microbial populations in the gut but included the possibility of its beneficial effect on other microbial communities such as those in the respiratory tract and on the skin (Shortt, 1999). Probiotics, therefore, aim to produce a beneficial effect on health by the intake of live microbes such as those found in traditional fermented dairy products, other foods, powders, tablets, liquid suspensions and lyophilized forms in capsules (Gibson & Fuller, 2000).

D. HUMAN GASTROINTESTINAL MICROBIAL ECOLOGY

The gastrointestinal tract of vertebrate animals encompasses a variety of habitats, encouraging colonization of a range of different microbes. These habitats are diverse and include the liquid fraction of the gut contents, the surfaces of particulate material in the digesta, the mucus secreted by the epithelial cells lining the tract and then the epithelial cells themselves. The diversity and complexity of these habitats are also reflected in the at least 400 types of bacteria that have been isolated from the faeces of humans (Tannock, 1992).

Bacterial numbers and populations in the human gastrointestinal tract vary between the stomach, small intestine and the colon. Bacterial numbers range from 10^4 to 10^6-10^7 per millilitre of faeces from the small intestine to the ileum

(Fooks *et al.*, 1999), while bacterial cell numbers in the human large intestine are approximately 10^{11}-10^{12} per gram of faeces. In comparison to other regions in the gastrointestinal tract the large intestine is an intricate and heavily populated ecosystem (Cummings & Mcfarlane, 1991).

Several hundred bacterial species are thought to be present in the large intestine of which *Bacteroides fragilis* are the most predominant culturable bacteria. Other bacteria that persist together with these Gram-negative rods are Gram-positive rods and cocci such as bifidobacteria, clostridia, peptococci, streptococci, eubacteria, lactobacilli, peptostreptococci, ruminococci, enterococci, coliforms, methanogens, sulphate-reducing bacteria and acetogens. Regardless of the great variety of bacteria present in the gut, it is considered that some of the inhabitants of the gastrointestinal tract have not been identified (Fooks *et al.*, 1999).

The microbial population that inhabits the human intestinal tract adapts to its surroundings and consequently forms a very stable ecosystem with each species occupying a niche. This very gentle balance of the bacteria needs to be maintained in order for the intestine to function optimally. However, many external factors contribute to an undesirable shift in the microbial balance from potentially beneficial and health promoting bacteria such as lactobacilli and bifidobacteria towards potentially harmful and pathogenic bacteria. These unwanted bacteria includes clostridia, sulphate-reducers and certain *Bacteroides* species of which some may result in health disorders such as cancer, inflammatory disease and ulcerative colitis (Fooks *et al.*, 1999).

In 1978, Mitsuoka proposed a hypothetical scheme that illustrated the relationship between the intestinal bacteria and human health (Fig. 1). The beneficial bifidobacteria and lactobacilli were said to contribute to digestion, immunity promotion and inhibition of pathogens. On the other hand the harmful bacteria produced substances such as amines, indole, hydrogen sulphide and phenols from food components leading to various intestinal infections (Ishibashi & Shimamura, 1993).

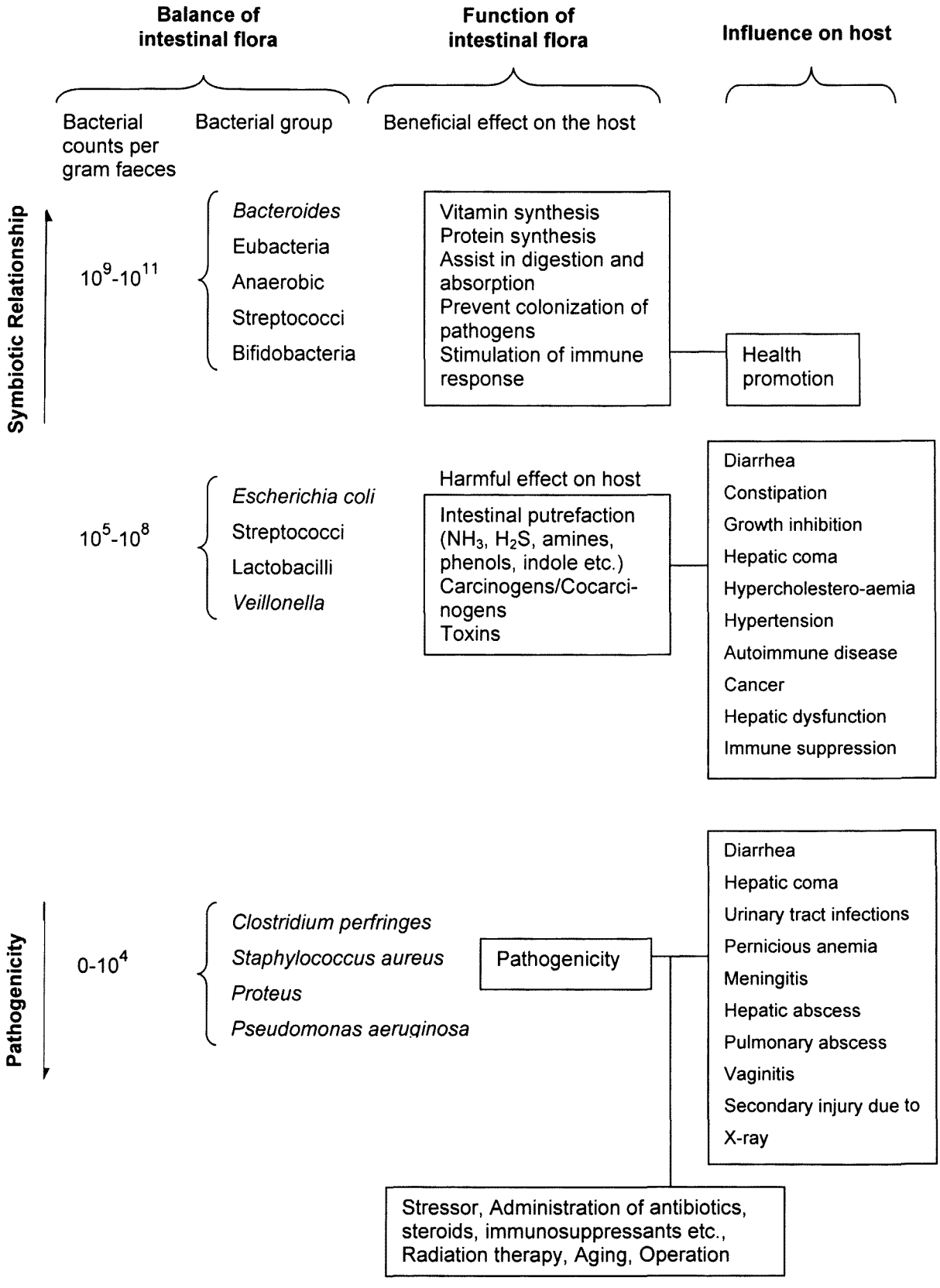

Figure 1. Interrelationships between intestinal bacteria and human health as proposed by Mitsuoka (1978).

E. THERAPEUTIC EFFECT OF PROBIOTICS

Nutritional and health aspects of probiotic foods have received a lot of attention in the literature (Gurr, 1987; Gilliland, 1990; Marteau & Rambaud, 1993; Gomes & Malcata, 1999). Despite the many studies done on the beneficial health aspects of probiotic bacteria, the results are variable and in some cases even inconsistent. Worldwide research efforts are, however, attempting to establish the health aspects of probiotics and the precise doses of probiotics required to ensure a health promoting effect on human or animal subjects.

Control of intestinal infections

The intestinal epithelium and the normal intestinal microbial populations represent a barrier against the movement of pathogenic bacteria, antigens and other invasive substances from the gut lumen to the blood. Factors such as dietary antigens, pathogens, chemicals or radiation may affect either the normal microflora or the intestinal epithelial cells, leading to defects in the barrier mechanism (Salminen *et al.*, 1998).

Several studies have documented the use of probiotic bacteria to treat intestinal disorders such as acute rotavirus diarrhoea in children, food allergies and colonic disorders (Salminen *et al.*, 1996). Antibiotic associated diarrhoea (AAD) is commonly treated with the administration of probiotics. *Saccharomyces boulardii* given in combination with antibiotics as compared to antibiotics alone is effective in reducing AAD (Surawicz *et al.*, 1989; McFarland *et al.*, 1995). Other trials have shown that recurrence of *Clostridium difficile* related infections could significantly be reduced by the administration of *Saccharomyces boulardii* together with antibiotic treatment (McFarland *et al.*, 1994). Overgrowth of *Candida* in the gut is also a frequent consequence of antibiotic use and studies in hamsters have shown that the gut microbial population is involved in suppression of *Candida albicans* (Kennedy & Volz, 1985). A human trial indicated the effective reduction in *Candida* occurring in faeces by the administration of milk containing *Lb. acidophilus* and *Bifidobacterium* (Tomoda *et al.*, 1983). Probiotics containing Bifidobacteria are also effective against antibiotic associated diarrhoea, clostridial spores (Colombel *et al.*, 1987) and childhood forms of diarrhoea (Saavedra *et al.*, 1994).

The mechanisms by which these favourable clinical responses are achieved are not fully understood. Possible mechanisms of action may include the potential of human probiotic bacteria to inhibit the cell association and cell entry of human entero-pathogens in the gut (Bernet *et al.*, 1993), the capacity to prevent pathogen adherence or pathogen activation by the production of inhibitory metabolites such as organics acids (lactic- and acetic acid), hydrogen peroxide and bacteriocins (Gibson & Wang, 1994b; Fujiwara *et al.*, 1997) and the uptake of ferrous iron, making it unavailable to pathogenic microbes. Probiotics may also influence bacterial enzyme activity and subsequently influence the gut mucosal permeability (Salminen *et al.*, 1996).

Alleviation of lactose intolerance

Lactose maldigestion or lactose intolerance results from a deficiency in the enzyme, β-galactosidase (lactase), which is responsible for the metabolism of the milk carbohydrate, lactose. When lactose intolerant individuals consume milk or lactose-containing products, they may experience abdominal pain, bloating, flatulence and diarrhoea (Kim & Gilliland, 1983). Lactose maldigestion also manifests itself by the presence of breath hydrogen and is derived from the fermentation of lactose in the large intestine. This phenomenon is then used as a quantitative measure for the intensity of lactose intolerance (Ouwehand & Salminen, 1998). Yoghurt has been reported to be well tolerated by lactose-deficient individuals and this has been attributed to the presence of bacterial β-galactosidase in the viable yoghurt starter culture. During the fermentation of milk, the lactobacilli present produce the enzyme β-galactosidase which hydrolyses milk lactose to glucose and galactose. Kim & Gilliland (1983) noted that the administration of fermented acidophilus milk markedly decreased the breath level of hydrogen in lactose-intolerant individuals when compared to the high levels after consumption of unfermented milk. The observation that oro-caecal transit time was significantly longer in subjects consuming yoghurt or pasteurised yoghurt than when consuming milk show that consumption of pasteurised yoghurt causes a delay in the maximum breath hydrogen excretion as apposed to milk consumption (Marteau *et al.*, 1990). These results indicate a clear improvement in intestinal absorption of lactose in yoghurt as compared to unfermented milk. Less

diarrhoea, flatulence and abdominal distension was also noted in individuals eating yoghurt when compared with those that ingested a similar amount of lactose from milk or water solutions. The evidence for the beneficial health effect of probiotics in allowing lactose-intolerant individuals to consume food products containing lactose is among the most convincing of all the health claims for probiotics (Goldin & Gorbach, 1992).

Treatment of hypercholesterolaemia

High serum cholesterol levels have been associated with an increased risk of heart disease in humans. There are currently a number of drugs available to lower plasma cholesterol, but non-pharmacological agents that could accomplish this reduction would be favourable. A number of studies have been conducted to determine whether probiotics could aid in lowering cholesterol levels (Goldin & Gorbach, 1992). This effect may be due to the presence of organic acids such as uric, orotic and hydroxymethylglutaric acids, which inhibits cholesterol synthesis (Fernandes *et al.*, 1987). Some animal studies were successful in attributing cholesterol-lowering properties to *Lb. acidophilus* administered in dairy products to pigs (Gilliland, 1990), weanling rats (Grunewald, 1982) and mice (Akalin *et al.*, 1997). The data obtained from animal studies cannot be extrapolated to humans since there are differences in cholesterol metabolism between humans and animals. Data from human studies did not use smaller dose volumes, and the lack of controls and the use of ill-defined strains further impoverish these results. While the mechanism by which fermented dairy products may reduce serum cholesterol levels is still a matter of dispute it is an established fact that cholesterol and bile salt metabolism are closely linked. Gilliland *et al.* (1985) showed that *Lb. acidophilus* is able to utilise cholesterol in growth media by assimilation and precipitation with deconjugated bile salts under acidic conditions. Bile salts may be deconjugated by the enzyme bile salt hydrolase, typical of some gut bacteria. The free bile salts are excreted more readily and may thus contribute to reducing cholesterol levels (Chikai *et al.*, 1987). However, this hypothesis is disputed and is not supported by studies done on the passive absorption kinetics of free bile acids in the gastro-intestinal tract (Holzapfel *et al.*, 1998).

Potential antitumour activity

The colonic bacteria are involved in colonic carcinogenesis (Marteau & Rambaud, 1993) by the production of the enzymes β-glucuronidase, β-glucosidase, nitroreductase and urease which are involved in the conversion of procarcinogens into carcinogens. Humans consuming probiotic bacteria had a general reduction in the microbial enzyme activities that are responsible for the activation of procarcinogens. The consumption of *Lb. acidophilus* by healthy volunteers resulted in a significant decrease in β-glucuronidase, nitroreductase and azoreuctase activities (Goldin & Gorbach, 1992). Goldin & Gorbach (1984) also found that the consumption of milk containing viable *Lb. acidophilus* (2×10^6 cells.ml^{-1}) exhibited a 2 to 4-fold decrease in the activity of these enzymes.

Nutritional benefits

The nutritional benefits of probiotics have mostly been studied in products fermented with lactobacilli and bifidobacteria. Fermented milks are characterised by a lower lactose concentration and higher concentrations of free amino acids than non-fermented milks. Although lactobacilli require B vitamins for growth, it was found that *Lb. acidophilus* and bifidobacteria can synthesize folic acid, niacin, thiamine, riboflavin, pyridoxine and vitamin K (Tamime *et al.*, 1995). Bifidobacteria are also unique in that the lactic acid that they produce is in the L(+) form. This form is easily metabolized by infants while D(-) lactic acid, produced by *Lb. acidophilus* may cause metabolic acidosis during the first year of development (Modler *et al.*, 1990b). Furthermore, fermented dairy products are good sources of especially calcium, phosphorous, magnesium and zinc in humans (Gurr, 1987).

F. MICROBIAL SELECTION OF PROBIOTICS

Substantiated health claims regarding probiotic bacteria must be supported by the knowledge of which strains of bacteria can be used and from what sources they can be obtained. Using stringent guidelines it will be possible to select probiotic microbes that will exert a positive effect on human health (Collins *et al.*, 1998). Various studies have been conducted to establish the characteristics of effective

probiotics and criteria for their selection (Kurmann & Rasic, 1991; Mattila-Sandholm *et al.*, 2002).

Preferably, probiotic strains should be from human origin as only human strains can adhere and colonise the human gastrointestinal tract, which is the first step in promoting resistance to colonisation by pathogens (Huis in't Veld *et al.*, 1994). One of the most important characteristics to establish regarding a probiotic strain is that it must be non-pathogenic and should posses GRAS (Generally Regarded As Safe) status (Collins *et al.*, 1998). There is general agreement that the consumption of probiotics, even in dosages as high as 10^{12} cfu.d^{-1} must fail to exhibit any toxicity (Holzapfel *et al.*, 1998).

To survive transit through the gastrointestinal tract, a probiotic strain must be able to tolerate a low pH and high concentrations of conjugated and deconjugated bile acids. The probiotic strain must be tolerated by the immune system and should not provoke the formation of antibodies (Collins *et al.*, 1998). Antimicrobial production and antagonistic activity against pathogens such as *Helicobacter pylori*, *Salmonella* spp., *Listeria monocytogenes* and *Clostridium difficile* are desirable characteristics (Mattila-Sandholm *et al.*, 2002). Furthermore, it is important for the strains to maintain viability during processing and storage of the product. Strain survival will mainly depend on factors such as the final product pH, the presence of other microbes, the storage temperature and the presence or absence of microbial inhibitors in the substrate. Exploitation of modern biotechnological improvements in culture production, preservation and storage should help in maintaining high numbers of probiotic bacteria in products (Collins *et al.*, 1998).

G. GENERAL CHARACTERISTICS OF PROBIOTIC MICROBES

Genus *Lactobacillus*

Lactobacilli are generally characterised as Gram-positive, non-motile, non-sporeforming microbes. Their cell morphology varies from long and slender rods to short, often coryneform coccobacilli. They are strictly fermentative and microaerophilic microbes with their growth usually enhanced by anaerobiosis or reduced oxygen pressure. Lactobacilli are also catalase negative and have complex nutritional requirements for amino acids, peptides, nucleic acid

derivatives, vitamins, salts, fatty acids and fermentable carbohydrates (Kandler & Weiss, 1986). Lactobacilli are found in a wide range of habitats including the mucosal membranes of humans and animals, on plants or material form plant origin and in manure, sewage and fermenting and spoiling foods (Hammes & Vogel, 1995).

Lactobacillus acidophilus is the *Lactobacillus* species most commonly suggested for use in probiotic food products (Gomes & Malcata, 1999). This *Lactobacillus* species is the most prominent species in the intestine and is believed to exert a beneficial effect on human and animal health. *Lactobacillus acidophilus* is homofermentative and converts lactose to DL-lactic acid (Kandler & Weiss, 1986). *Lactobacillus acidophilus* may grow at 45°C, but optimum growth occurs at 35° – 40°C and at an optimum pH of 5.5 - 6.0 (Gomes & Malcata, 1999).

Comprehensive genetic studies have shown that what was believed to be *Lb. acidophilus* can now be divided into six DNA-DNA homology groups at the species level (Fujisawa *et al.,* 1992, Pot *et al.*, 1994). These include *Lb. acidophilus, Lb. crispatus, Lb. amylovorus, Lb. gallinarum, Lb. gasseri* and *Lb. johnsonii* and these species cannot be differentiated by simple phenotypic assays. *Lb. helveticus* is very closely related to *Lb. acidophilus* with respect to DNA-DNA homology, biochemical features and 16S rRNA sequence (Hammes & Vogel, 1995).

Genus *Bifidobacterium*

Bifidobacteria are rod-shaped, non gas-producing, anaerobic microbes with bifid morphology and are present in the faeces of breast-fed infants (Sgorbati *et al.*, 1995). Bifidobacteria are generally characterized as Gram-positive, non-spore forming, non-motile and catalase negative anaerobes. Bifidobacteria are anaerobic and do not develop in synthetic media under aerobic conditions, although the sensitivity to oxygen may vary among different strains and species. Glucose is exclusively metabolised by the fructose-6-phosphate pathway (Fig. 2) which is also known as the bifid pathway (Scardovi, 1986). The key enzyme of the glycolitic fermentation, fructose-6-phosphate phosphoketolase, serves as a taxonomic character in the identification of the genus although it does not allow for the identification of specific species (Sgorbati *et al.*, 1995).

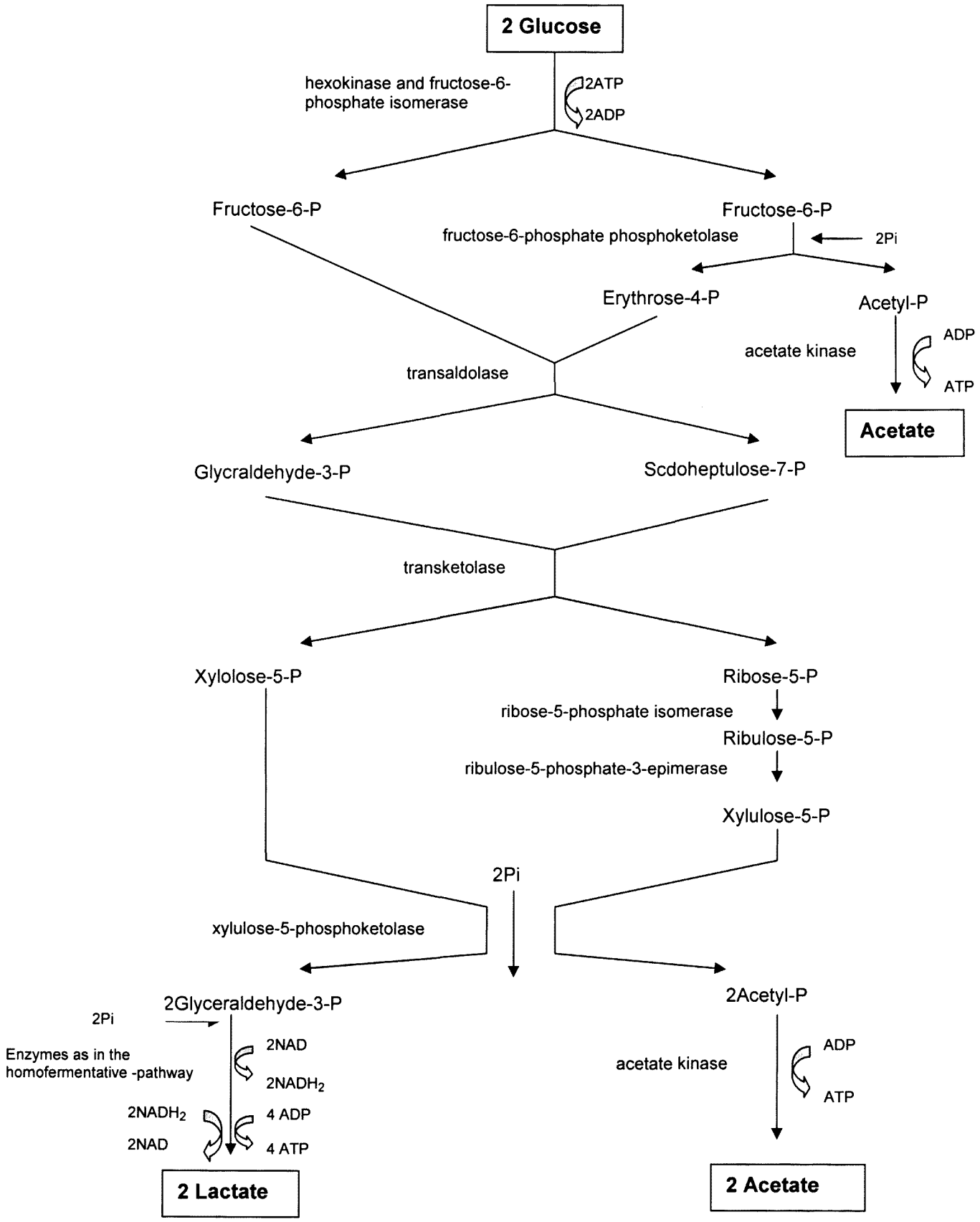

Figure 2. Formation of acetate and lactate from glucose by the bifidum pathway (Gomes & Malcata, 1999

Thirty-one species of bifidobacteria have been identified of which 11 have been isolated from human faeces (Tannock, 1999). In the manufacturing of probiotic fermented milk products *B. bifidum* is the species most often used, followed by *B. longum* and *B. breve*. *Bifidobacterium infantis* is often used in pharmaceutical preparations, usually in conjunction with other lactic acid bacteria. The bifidobacteria added to fermented products are usually used in combination with lactic acid bacteria due to their slow acid production (Kurmann & Rasic, 1991). The optimum growth temperature of bifidobacteria is 37° – 41°C and the optimum pH for growth of these microbes is 6.5 – 7.0. Growth is inhibited at pH 4.5 – 5.0 and at pH 8.0 – 8.5 (Scardovi, 1986).

H. ISOLATION AND IDENTIFICATION OF PROBIOTIC AND TRADITIONAL YOGHURT BACTERIA IN PROBIOTIC PRODUCTS

Few methods are available for the accurate enumeration of probiotic bacteria from yoghurts and other products (Vinderola & Reinheimer, 1999). A recent report of a joint FAO/WHO working group on drafting guidelines for the evaluation of probiotics in foods recommended that the information on the label of a probiotic product should give the genus, species and strain designation of the particular probiotic cultures. The label should also indicate the minimum viable numbers of each probiotic strain at the end of the shelf-life (Anon., 2002a). South African regulations stipulate that the viable count of probiotic bacteria should exceed 1×10^8 colony forming units per serving (100ml) (Anon., 2002b). At present the numbers of AB-cultures or *Lb. acidophilus* and *Bifidobacterium* spp. in probiotic products are especially difficult to determine for manufacturing and regulatory purposes (Rybka & Kailasapathy, 1996) and it is difficult to distinguish between closely related probiotic cultures. Consumer concerns have increased since studies have confirmed that the presence of certain microbes (e.g. *Enterococcus faecium*) in probiotic products is not given on the label and that some probiotic species are incorrectly identified (Fasoli *et al.*, 2003; Temmerman *et al.*, 2003). The need, therefore, exists for simple and reliable methods for the routine enumeration of both *Bifidobacterium* spp. and *Lb. acidophilus* to determine the initial counts of the probiotic bacteria after the manufacture of the products and also to assure cell viability during refrigerated storage and product distribution

(Kailasapathy & Rybka, 1997). The application of molecular techniques for the rapid and accurate identification of lactobacilli and bifidobacteria could help to characterise microbial populations from complex ecosystems (Tannock, 1999).

Development of differential culture media

The standard media accepted by the International Dairy Federation for differential enumeration of the yoghurt starter cultures, *Lactobacillus bulgaricus* and *Streptococcus thermophilus* are De Man, Rogosa and Sharpe medium and M17 agar, respectively (IDF, 1997). Other media used for the enumeration of these microbes are given in Table 3.

Although there are several proposed selective media for the isolation of *Lb. acidophilus* and *Bifidobacterium* spp. very few media allows the simultaneous enumeration of these bacteria in the presence of the yoghurt cultures, *Str. thermophilus* and *Lb. bulgaricus* (Vinderola & Reinheimer, 1999). Rybka & Kailasapathy (1996) described a procedure for the isolation and enumeration of *Lb. delbrueckii* ssp. *bulgaricus*, *Str. thermophilus*, *Lb. acidophilus* and *Bifidobacterium* species from yoghurt by making use of three different culture media. Media proposed for the selective enumeration of AB cultures are summarized in Table 3.

Most selective culture media do, however, have the disadvantages of not being absolutely selective and also of toxicity against certain strains within the genus. A further limitation of culture media is the inability of selecting for non-culturable bacteria (O'Sullivan, 1999). Due to the generalization about the probiotic performance of species and insufficient scientific evidence, it should be assumed that probiotic properties are strain-specific (Sanders, 1999). Even the most sophisticated classical culture dependent techniques are not always sufficient for the identification of closely related isolates and are labour intensive (O'Sullivan, 1999). This has led to a great demand for rapid strain-specific identification and detection techniques (Sanders, 1999). The advent of various molecular techniques has increased the ability for rapid, accurate and reliable detection of closely related and unknown microbial species (O'Sullivan, 1999).

Table 3. Proposed differential media for the enumeration of *Lactobacillus acidophilus* and *Bifidobacterium* species.

Microbial Species	Growth Medium	Reference
Lb. acidophilus	MRS broth	De Man *et al.*, 1960
	LBSO (Lactobacillus Selection Agar + 0.15% Oxgall)	Gilliland & Speck, 1977
	PCA (agar plate count method)	Collins, 1978
	Maltose-MRS	Hull & Roberts, 1984
	Cellobiose Esculin Agar	Von Hunger, 1986
	Oxygen-reducing membrane fraction	Burford, 1989
	Modified Brigg's Agar	Calicchia *et al.*, 1993
	MNA + salicin (minimal nutrient agar)	Lankaputhra & Shah, 1996
	T-MRS	Vinderola & Reinheimer, 1999
	Bile MRS	Vinderola & Reinheimer, 1999
Bifidobacterium spp.	BIM-25	Munoa & Pares, 1988
	Oxygen-reducing membrane fraction	Burford, 1989
	Lithium Chloride-Sodium Propionate Agar	Lapierre *et al.*, 1992
	Modified VF-Bouillon Agar	Calicchia *et al.*, 1993
	BL-OG (Blood-glucose-liver agar + oxgall + gentamicin)	Lim *et al.*, 1995
	RCPBpH5	Rybka & Kailasapathy, 1996
	Bif (*Bifidobacterium*)	Pacher & Kneifel, 1996
	LP-MRS	Vinderola & Reinheimer, 1999
Both *Lb. acidophilus* and *Bifidobacterium* spp.	Modified TPPY	Ghoddusi &Robinson, 1996
	M-17 agar RCPBpH5 agar M-MRS	Rybka & Kailasapathy, 1996
Lb. bulgaricus	Acidified-MRS medium	DeMan *et al.*, 1960
	RCPBpH5 agar	Rybka & Kailasapathy, 1996
Str. thermophilus	M-17	Jordona *et al.*, 1992
Both *Lb. bulgaricus* and *Str. thermophilus*	Lactic Acid Bacteria Agar	Davis *et al.*, 1971
	TPPY (tryptose-proteose-peptone yeast extract)	Braquart, 1981
	Lee's medium	Lee *et al.*, 1974
	RCPB (reinforced clostridial prussian blue agar)	Ghoddusi & Robinson, 1996
	SM (skim milk) agar	Vinderola & Reinheimer, 1999

Plasmids

Certain metabolic and physiological characteristics of lactic acid bacteria are encoded by plasmids. The majority of plasmids detected in lactobacilli are cryptic, meaning that it has no known, associated phenotype. However, in certain strains N-acetyl-D-glucosamine fermentation, proteolysis, lactose metabolism, maltose utilization, cysteine uptake, bacteriocin production or antibiotic resistance are encoded by plasmid-borne genes (Tannock *et al.*, 1990; Duffner & O'Connell, 1995; Reid *et al.*, 1996). Plasmid-derived DNA probes have been used to identify *Lb. fermentum* in the porcine stomach (Tannock *et al.*, 1992) and biotin-labeled DNA probes were used to detect closely related lactobacilli in the forestomach of mice (Tannock, 1989). Plasmid profiles may, however, only have significance when used in combination with other techniques, since plasmids may be unstable (Du Toit, 1998).

Ribotyping

In order to obtain a ribotype of an organism, the organism must first be cultured to obtain enough cells for the isolation of DNA. Total DNA is cut into multiple fragments using restriction enzymes. The restricted fragments are then separated by agarose gel electrophoresis and hybridised with a probe targeted to either the 16S, 23S or 5S rRNA genes (O'Sullivan, 1999). Following probe detection the restriction bands are visualised and the distinct pattern of the band sizes then represents a characteristic restriction fragment length polymorphism (RFLP) fingerprint. This technique is reproducible and it has been used in the analysis of the human intestinal microbes (McCartney *et al.*, 1996; Kimura *et al.*, 1997). Some of the currently available rRNA-gene-targeted oligonucleotide probes for the identification of potentially probiotic lactic acid bacteria (LAB) are summarised in Table 4. Most of these probes are species specific and they are especially useful for the identification of LAB that cannot be differentiated reliably by simple phenotypic tests or of LAB that show unusual growth requirements.

Pot *et al.* (1993) demonstrated the reliable and fast identification and classification of *Lb. acidophilus, Lb. gasseri* and *Lb. johnsonii* by making use of SDS-PAGE and rRNA-gene-targeted oligonucleutide probe hybridization. The reverse dot blot hybridisation method described by Ehrmann *et al.* (1994) is a useful method for the direct identification of LAB from fermented foods. The use of

Table 4. Oligonucleotide probes for the identification of probiotic lactobacilli, enterococci and bifidobacteria.

Microbial Species	Probe Sequence (5' - 3')	Target DNA	Reference
Lb. acidophilus	AGCGAGCUGAACCAACAGAUUC	16S rRNA	Hensiek *et al.*, 1992
Lb. acidophilus	TCTTTCGATGCATCCACA	23S rRNA	Roy *et al.*, 2000
Lb. amylovorus	GTAAATCTGTTGGTTCCGC	16S rRNA	Roy *et al.*, 2000
Lb. brevis	TGTTGAAATCAGTGCAAG	16S rRNA	Vogel *et al.*, 1994
Lb. casei/rhamnosus	GCAGGCAATACACTGATG	23S rRNA	Hertel *et al.*, 1993
Lb. casei/paracasei/ rhamnosus	CTGATGTGTACTGGGTTC	23S rRNA	Hertel *et al.*, 1993
Lb. collinoides	AGCACTTCATTTAACGGG	16S rRNA	Schleifer *et al.*, 1995
Lb. crispatus	CAATCTCTTGGCTAGCAC	23S rRNA	Ehrmann *et al.*, 1994
Lb. curvatus	ATGATAATACCCGACTAA	23S rRNA	Hertel *et al.*, 1991
Lb. delbrueckii	AAGGATAGCATGTCTGCA	23S rRNA	Hertel *et al.*, 1993
Lb. farciminis	CTCGCTGCTAACTTAAGTC	16S rRNA	Vogel *et al.*, 1994
Lb. fermentum	GCGACCCCCCTCAATCAGG	16S rRNA	Vogel *et al.*, 1994
Lb. fermentum	AACGCGUUGGCCCAAUUGAUUG	16S rRNA	Hensiek *et al.*, 1992
Lb. gasseri	TCCTTTGATATGCATCCA	23S rRNA	Roy *et al.*, 2000
Lb. helveticus	ACTTACCTACATCCACAG	23S rRNA	Roy *et al.*, 2000
Lb. johnsonii	ATAATATATGCATCCACAG	23S rRNA	Roy *et al.*, 2000

Table 4. (continued).

Microbial Species	Probe Sequence (5' – 3')	Target DNA	Reference
Lb. paracasei	CACTGACAAGCAATACAC	23s rRNA	Hertel *et al.*, 1993
Lb. plantarum	AACGAACUAAUGGUAUUGAUUGG	16S rRNA	Hensiek *et al.*, 1992
Lb. plantarum/pentosus	ATCTAGTGGTAACAGTTG	23S rRNA	Hertel *et al.*, 1991
Lb. plantarum/pentosus/ paraplantarum	PyrDFE gene	DNA	Bringel et al., 1996
Lb. reuteri	GATCCATCGTCAATCAGG	16S rRNA	Vogel *et al.*, 1994
Lb. ruminis	AACGAGGCUUUCUUUCACCGAA	16S rRNA	Hensiek *et al.*, 1992
Enterococcus faecalis	GGTGTTGTTAGCATTTGG	23S rRNA	Beimfohr *et al.*, 1993
Enterococcus faecium	CACACAATCGTAACATCC	23S rRNA	Beimfohr *et al.*, 1993
S. thermophilus	CATGCCTTCGCTTACGCT	23S rRNA	Beimfohr *et al.*, 1993
Genus *Bifidobacterium*	CATCCGGCATTACCACCC	16S rRNA	Langendijk *et al.*, 1995
Genus *Bifidobacterium*	CCACCGTTACACCGGGAA	16S rRNA	Langendijk *et al.*, 1995
Genus *Bifidobacterium*	CCGGTTTTCAGGGATCC	16S rRNA	Langendijk *et al.*, 1995
B. adolescentis	GCTCCCAGTCAAAAGCG	16S rRNA	Yamamoto *et al.*, 1992
B. bifidum	GCAGGCTCCGATCCGA	16S rRNA	Yamamoto *et al.*, 1992
B. breve	AAGGTACACTCAACACA	16S rRNA	Yamamoto *et al.*, 1992
B. infantis	TCACGCTTGCTCCCCGATA	16S rRNA	Yamamoto *et al.*, 1992
B. longum	TCTCGCTTGCTCCCCGATA	16S rRNA	Yamamoto *et al.*, 1992

fluorescently labelled oligonucleotide probes as described by Beimfohr *et al.* (1993) makes it possible to detect lactococci, streptococci and enterococci in raw milk samples within one day.

Pulse field gel electrophoresis

A discrete number of DNA fragments can be generated by digesting the isolated genome with rare cutting restriction enzymes, which generally have an 8 or 6 bp recognition site and which may statistically be rare for the particular genome. The restriction endonucleases *XbaI* and *SpeI* are often used and will cut the genome infrequently and generate between 10 and 30 DNA fragments ranging from 20 to 400 kb (McBrearty *et al.*, 2000). Pulse field gel electrophoresis (PFGE) can be used to migrate these very large DNA fragments through an agarose gel. The resulting RFLPs are highly characteristic of the particular organism (O'Sullivan, 1999). McCartney *et al.* (1996) and Kimura *et al.* (1997) used this technique to monitor the prevalence of lactobacilli and bifidobacteria in human faecal samples.

Polymerase chain reaction

The polymerase chain reaction (PCR) allows the rapid amplification of a specific DNA sequence and is considered to be one of the most useful molecular techniques of our time (O'Sullivan, 1999). The technique allows the rapid and specific identification of *Bifidobacterium* and *Str. thermophilus* strains from the faeces of human subjects (Matsuki *et al.*, 1998; Brigidi *et al.*, 2003). Species specific primers were designed by Drake *et al.* (1996) for differential amplification of DNA from *Lb. casei*, *Lb. delbrueckii*, *Lb. helveticus* and *Lb. acidophilus* from dairy products. Walter *et al.* (2000) designed 11 species-specific PCR primer pairs for the detection and identification of gastrointestinal *Lactobacillus* species. Species-specific PCR was also effectively implemented by Torriani *et al.* (1999) for the rapid differentiation between the closely related *Lb. delbrueckii* subsp. *bulgaricus* and *Lb. delbrueckii* subsp. *lactis* species. Brandt & Alatossava (2003) have developed strain-specific primers for the identification of three probiotic *Lb. rhamnosus* strains.

Advantages of PCR analyses include high throughput, specificity and sensitivity (McBrearty *et al.*, 2000). A disadvantage of the technique is that prior

sequence knowledge is required and that it is technically challenging to design optimum reaction conditions (O'Sullivan, 1999). Furthermore, this technique cannot be used to discriminate between live and dead cells and this could lead to false positive results. This problem could be overcome by various pre-treatment methods including cell purification, concentration and culturing methods (Van der Vossen & Hofstra, 1996). These enrichment steps may be time-consuming and are not desirable when rapid identification must be made (Salzano *et al.*, 1995).

Denaturing gradient gel electrophoresis

The denaturing gradient gel electrophoresis (DGGE) technique can separate DNA fragments of the same size, but with different base-pair sequences, by electrophoresis through a linearly increasing gradient of denaturants. It is based on the melting of the DNA fragments at specific denaturing points and the subsequent transition of the helical molecule to a partially melted molecule. This result in a halt in the migration of the molecule and the difference in melting temperatures are based on small sequence variations (Muyzer *et al.*, 1993). Urea and formamide are generally used to form the denaturing gradient (O'Sullivan, 1999). The DGGE technique was successfully used to differentiate between *Lactobacillus* species present in the gastrointestinal tract (Walter *et al.*, 2000). The use of molecular techniques in food microbiology have resulted in various improvements especially in the field of microbial detection and identification (Cocolin *et al.*, 2004) and are often implemented to establish microbial diversity in various samples (Gonzalez *et al.*, 2003). Cocolin *et al.* (2004) developed a PCR-DGGE protocol for the detection and differentiation between different *Clostridium* species in cheeses with late-blowing symptoms. Fasoli *et al.* (2003) found PCR combined with DGGE to be an appropriate culture-independent approach for the rapid detection of predominant species in mixed probiotic cultures found in probiotic foods.

I. CONCLUSION

Probiotic products represent a strong growth area within the functional food group. In 1997 probiotic yoghurts and milks accounted for 65% of the European functional foods market, valued at US$ 889 million. Given the worldwide concern over

antibiotic resistance, natural alternatives such as probiotics for the inhibition of pathogens are receiving more attention (Stanton *et al.*, 2001). The increased interest of the consumer to maintain optimum health through a healthy diet is another factor for the expanding interest in probiotic foods (Saarela *et al.*, 2000).

Some of the very first bacteria that were used in probiotic products include *Lb. acidophilus* and *Lb. casei*. The number of microbial species used as probiotics and the types of probiotic food products available on the world market has rapidly increased. Fermented milks of various kinds are still the main vehicle for probiotic administration. However, the minimum number of viable microbial cells that should be present in a probiotic product has been the subject of much discussion, but 10^6 - 10^8 cfu.ml^{-1} is usually recommended (Robinson, 1987; Kurman & Rasic, 1991; Anon., 2002b). Advances in micro-encapsulation techniques have shown to increase the survival of probiotic bacteria in food products by up to 80 - 95% (Krasaekoopt *et al.*, 2003).

There are few methods available for the accurate enumeration of the probiotic bacteria from food products. The need therefore exists for simple and reliable methods for routine enumeration of especially *Bifidobacterium* spp. and *Lb. acidophilus*. This is then also important for quality control of products and to monitor fermentation processes and subsequent shifts in microbial populations. Molecular techniques such as PCR and DGGE are highly specific and could serve as accurate and reliable tools for the detection of probiotic cultures from foods. These molecular techniques can help to simplify the food labelling process in the future by providing the correct information to the consumer regarding the probiotic content.

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CHAPTER 3

IDENTIFICATION OF PROBIOTIC MICROBES IN SOUTH AFRICAN PRODUCTS USING PCR-BASED DGGE ANALYSIS

Abstract

Probiotic microbes in commercial yoghurts and other food products are currently identified by traditional methods such as growth on selective media, morphological and biochemical characteristics. In this study PCR-based DGGE analysis was used for the rapid and accurate identification of probiotic microbes from South African yoghurts and lyophilized preparations in capsule and tablet form. To identify the microbes present in these products the DGGE profiles obtained were compared to two reference markers (A and B) composed of five lactobacilli and seven *Bifidobacterium* species, respectively. The results obtained were confirmed by species-specific PCR, as well as sequence analyses of unknown bands not present in the reference markers. It was found that only 54.5% of the probiotic yoghurts contained the microbes stated on the label compared to only a third (33.3%) of the lyophilized probiotic products. Some *Bifidobacterium* species were incorrectly identified and various microbes were detected that were not listed on the label. Sequence analyses confirmed the presence of *Streptococcus* spp. other than the yoghurt starter, *Str. thermophilus*, in some of these products and in some instances label information was vague and non-scientific. PCR-based DGGE analyses proofed to be a valuable culture-independent approach for the rapid and specific identification of the microbial species present in South African probiotic products.

Introduction

A wide variety of probiotic products and supplements are commercially available on the South African market, either in lyophilized form or as fermented food commodities. A probiotic is generally defined as a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial

balance (Fuller, 1989). Various health benefits are related to the regular consumption of viable probiotic microbes and these include the improvement of lactose intolerance (Kim & Gilliland, 1983; Marteau *et al.*, 1990), the reduction of cholesterol levels (Gilliland, 1990; Akalin *et al.*, 1997) and the control of intestinal infections (Saavedra *et al.*, 1994; McFarland *et al.*, 1995).

Strains of *Lactobacillus acidophilus* and *Lb. casei* have the longest history of application as probiotic cultures (Holzapfel *et al.*, 1998). Currently various *Lactobacillus* spp., as well as *Bifidobacterium* spp. are used in commercial probiotic products. *Lactobacillus acidophilus* strains and *Bifidobacteria* spp. are collectively known as AB-cultures and this term is used to indicate the presence of these health-promoting microbes in yoghurts and other fermented milk products.

South African health and food regulations stipulate that the label of probiotic foods should indicate the viable count of bacteria per gram of the product at the end of the stipulated shelf-life, as well as the full scientific name of the microbial species present in the product (Anon., 2002). It is mostly accepted that different species from the same genus may have different beneficial properties (Salminen *et al.*, 1998) and it has been proposed that it should be assumed that probiotic properties are strain-specific (Sanders, 1999). Reports indicating the presence of microbial species in probiotic products that were not listed on the label (Fasoli *et al.*, 2003; Temmerman *et al.*, 2003), as well as the presence of potentially pathogenic species in probiotic foods (Hamilton-Miller *et al.*, 1999) have led to serious safety concerns.

Various selective culture media have been proposed for the detection of probiotic bacteria, but even the most sophisticated classical cultural dependent techniques are not always effective for the identification of closely related isolates (Yaeshima *et al.*, 1996; Holzapfel *et al.*, 1997; O'Sullivan, 1999). The preparation of selective culture media is labour intensive and has the limitation of not being absolutely selective. A further disadvantage is the fact that not all microbes can be cultured on growth media, making it impossible to isolate and identify a significant number of microbial species (O'Sullivan, 1999). This has led to an increased demand for rapid species and strain-specific identification and detection techniques (Sanders, 1999).

The use of molecular techniques in food microbiology has offered various improvements in the field of microbial detection and identification (Cocolin *et al.*,

2004). These are often implemented to establish microbial diversity in complex food samples (Gonzalez *et al.*, 2003). The aim of this study was to identify the different probiotic microbes present in various South African products by PCR-based DGGE analysis combined with species-specific PCR detection.

Materials and methods

Probiotic products

A total of 20 different commercially available South African probiotic products were collected and analysed. These products included 11 different yoghurt products, eight probiotic lyophilized preparations in tablet and capsule form and one baby milk formula. The probiotic yoghurts were purchased from local supermarkets, while the lyophilized products were obtained from pharmacies. All these products were tested prior to the use-by date as was indicated on the labels of the products.

DNA isolation

DNA was isolated according to the modified method of Van Elsas *et al.* (1997) from the 20 South African probiotic products. For the yoghurt samples, 1 ml of a sterile saline solution (0.85% (m/v) NaCl) was added to 1 ml yoghurt prior to DNA isolation. This suspension was mixed and centrifuged at 5 900 × g for 10 min. For the baby formula, tablets and capsules 1 g of the product was dissolved in 10 ml of a sterile saline solution and 1 ml of the suspension was centrifuged at 5 900 × g for 10 min to recover the cells.

After centrifugation, 0.6 g sterile glass beads (0.2 - 0.3 mm diameter) (Sigma), 800 µl phosphate buffer (1 part 120 mM NaH_2PO_4 to 9 parts 120 mM Na_2HPO_4; pH 8), 700 µl phenol (Saarchem) and 100 µl 20% (m/v) sodium dodecyl sulphate (SDS) (Merck) were added to the harvested cells. The bead/cell mixture was then vortexed for 2 min and incubated in a water bath at 60°C for 20 min. This step was repeated twice before the samples were centrifuged at 1 500 × g for 5 min. The aqueous phase was collected and the proteins were extracted with 600 µl phenol (pH 4.3) (Saarchem), followed by an extraction with 600 µl phenol/chloroform/isoamylalcohol (25:24:1) until the interphase was clean. The

DNA was then precipitated with 0.1 × volume 3 M sodium acetate (NaAc) (pH 5.5) (Saarchem) and 0.6 × volume isopropanol (Saarchem) on ice for 60 min. This mixture was centrifuged for 10 min at 15 000 × g and the pellet was washed with 200 µl 70% (v/v) ethanol (Merck). The supernatant was removed and the pellet was air-dried for 20 min after which it was re-dissolved in 100 µl TE (10mM Tris, 1 mM EDTA; pH8).

For the flavoured yoghurts, a pre-treatment step was incorporated prior to DNA isolation (Drake *et al.*, 1996). This pre-treatment step was included to improve the DNA extraction and to remove substances that could inhibit the PCR reaction. The method included the addition of 2 ml 25% (m/v) ammonium hydroxide (Pal Chemicals), 2 ml 99.9% (v/v) ethanol (Merck), 4 ml petroleum ether (Saarchem) and 200 µl 10% (m/v) SDS to each 10 ml food sample. This mixture was vortexed and another 2 ml petroleum ether was added after which the solution was centrifuged at 7 500 × g for 15 min. The supernatant was discarded and the pellet was used for DNA extraction as previously described.

DNA amplification

The PCR amplification of approximately 200 base pairs (bp) of the V2 - V3 variable region of the 16S rRNA gene was obtained using the primers HDA1-GC (5' CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAC TCC TAC GGG AGG CAG CAG T 3') (GC-clamp sequence underlined) and HDA2 (5' GTA TTA CCG CGG CTG CTG GCA 3') (Walter *et al.*, 2000). This PCR reaction was performed in a total reaction volume of 40 µl containing 0.5 µM of each of the primers, 200 µM of each dNTP (Promega), 2.5 mM $MgCl_2$ (Promega), 0.5 U of *Taq* DNA Polymerase (Promega), 1 × PCR buffer (Promega) and 2 µl of the isolated DNA.

The PCR products were generated using an initial denaturation step of 4 min at 94°C followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 56°C for 30 sec and elongation at 72°C for 60 sec. A final chain elongation at 72°C for 8 min was done (Fasoli *et al.*, 2003). PCR reactions were performed in an Eppendorf Mastercycler Personal. All the PCR amplification products were analysed on 1% (m/v) agarose gels containing ethidium bromide in 0.5 × TBE

electrophoresis buffer and the separated fragments were visualised under UV light (Vilber Lourmat).

Denaturing gradient gel electrophoresis (DGGE)

The PCR fragments were separated by DGGE using the Biorad DCode™ Universal Mutation Detection System (Bio-Rad Laboratories, USA). Separation of the PCR amplicons was obtained by the direct application of 35 µl of the PCR products onto 8% (m/v) polyacrylamide gels in 0.5 × TAE buffer containing a linear denaturant gradient of between 30 and 70%. The 100% denaturing solution contained 40% (v/v) formamide (Saarchem) and 7.0 M urea (Merck). Electrophoresis was performed with a constant voltage of 130 V at 60°C for 5 h, the gel was stained with ethidium bromide for 30 min and the fragments were visualised under UV light (Vilber Lourmat).

The DNA from pure probiotic cultures were obtained from Prof. Sandra Torriani (University of the Studies of Verona, Italy) and were used for the amplification of two DGGE reference markers (A and B) (Fasoli *et al.*, 2003). Marker A was composed by mixing equal amounts of amplicons obtained from five *Lactobacillus* species, as well as *Streptococcus thermophilus* and *Enterococcus faecium*, while marker B contained the amplicons from seven *Bifidobacterium* species (Table 1).

DGGE detection limit

A standard curve of the pure culture, *B. longum* (DSM 20219), was prepared using the bacterial cell concentration (cfu/ml) against the optical density at 500 nm. An accurate 10-fold dilution series (10^6 to 10^2) of *B. longum* was then prepared. DNA was extracted from 1 ml of each dilution, containing this reference species. In order to determine the detection limit of the DGGE-based technique, the DNA obtained from each dilution was evaluated by PCR-based DGGE using the previously described protocols.

Table 1. Bacterial species present in the DGGE markers (Fasoli *et al.*, 2003).

Marker A	**Marker B**
Lactobacillus acidophilus NCFB 1748	*Bifidobacterium adolescentis* DSM 20083
Lb. casei ATCC 334	*B. animalis* LMG 10508
Lb. delbrueckii ssp. *bulgaricus* ATCC 11842	*B. bifidum* LMG 11041
Lb. delbrueckii spp. *lactis* NCFB 1438	*B. breve* LMG 11042
Lb. plantarum ATCC 14917	*B. infantis* LMG 11046
Streptococcus thermophilus DSM 20617	*B. lactis* DSM 10140
Enterococcus faecium ATCC 19434	*B. longum* LMG 10497

Sequencing analysis

Some DGGE bands could not be identified when compared to bands present in markers A and B. These unknown bands were selected and isolated from the acrylamide gels and the DNA was directly re-amplified with the primers HDA1 (without the GC-clamp) and HDA2 (Walter *et al.*, 2000). These PCR products were purified by using the SigmaSpin Post-Reaction Purification Columns (Sigma) as specified by the manufacturers. The purified PCR fragments were sequenced using the ABI PRISM 377 DNA Sequencer (Perkin Elmer) at the DNA Sequencing Facility at Stellenbosch University. The sequences obtained were identified by comparing them to recognized sequences available in Genbank using the BLAST algorithm (Altschul *et al.*, 1997).

Species-specific PCR

Species-specific PCR was used to detect the following microbial species: *Lactobacillus acidophilus* (Walter *et al.*, 2000); *Lb. bulgaricus* (Torriani *et al.*, 1999); *Lb. rhamnosus* (Berthier *et al.*, 2001); *Bifidobacterium adolescentis; B. bifidum* and *B. longum* (Matsuki *et al.*, 1999) (Table 2). The pure cultures *Lb. acidophilus* DSM 20079, *Lb. bulgaricus* ATCC 11842, *B. adolescentis* DSM 20083, *B. bifidum* DSM 20456 and *B. longum* DSM 20219 were used to optimize the PCR conditions. All 20 probiotic products were tested for the presence of these microbes in order to substantiate the results obtained by the PCR-based DGGE analyses. The PCR protocol for the detection of *B. adolescentis*, *B. bifidum* and *B. longum* differed from that described by Matsuki *et al.* (1999). For these *Bifidobacterium* species the reaction mixture (20 µl) consisted of 1 × PCR buffer (Promega), 100 µM of each dNTP (Promega), 1.5 µM (for the *B. bifidum* specific PCR) or 0.5 µM (for the *B. longum* and *B. adolescentis* specific PCRs) of each primer, 1.5 mM $MgCl_2$ (Promega), 0.5 U *Taq* DNA polymerase (Promega) and 1 µl DNA. All the PCR reactions were performed in an Eppendorf Mastercycler Personal and the PCR amplification products were analysed on a 1% (m/v) agarose gel containing ethidium bromide in 0.5 × TBE electrophoresis buffer. The separated DNA fragments were visualised under UV light (Vilber Lourmat).

Table 2. PCR conditions and primer pairs used for the species-specific identification of probiotic microbes present in food products.

Microbes	Primers	Conditions for PCR amplification	Number of Cycles	Amplicon Size (bp)	Reference
Lb. acidophilus	Aci 16SI/16SII	92°C, 2 min	1	800	Walter *et al.*, 2000
		95°C, 30 sec	30		
		62°C, 30 sec	1		
		72°C, 30 sec	1		
		72°C, 1 min	1		
Lb. bulgaricus	LB1/LBB1	94°C, 2 min	1	1065	Torriani *et al.*, 1999
		94°C, 45 sec	35		
		58°C, 30 sec	1		
		72°C, 30 sec	1		
		72°C, 10 min	1		
Lb. rhamnosus	16reverse/rhamnosus16S	94°C, 5 min	1	350 and 185	Berthier *et al.*, 2001
		94°C, 1 min	35		
		53°C, 30 sec	1		
		72°C, 1 min	1		
		72°C, 5 min	1		
B. adolescentis	BiADO-1/BiADO-2	94°C, 1 min	1	279	Matsuki *et al.*, 1999
B. bifidum	BiBIF-1/BiBIF-2	94°C, 30 sec	35	278	
		55°C, 30 sec	1		
B. longum	BiLON-1/BiLON-2	72°C, 4 min	1	831	
		72°C, 10 min	1		

Results and discussion

Detection limit and reproducibility of PCR-based DGGE

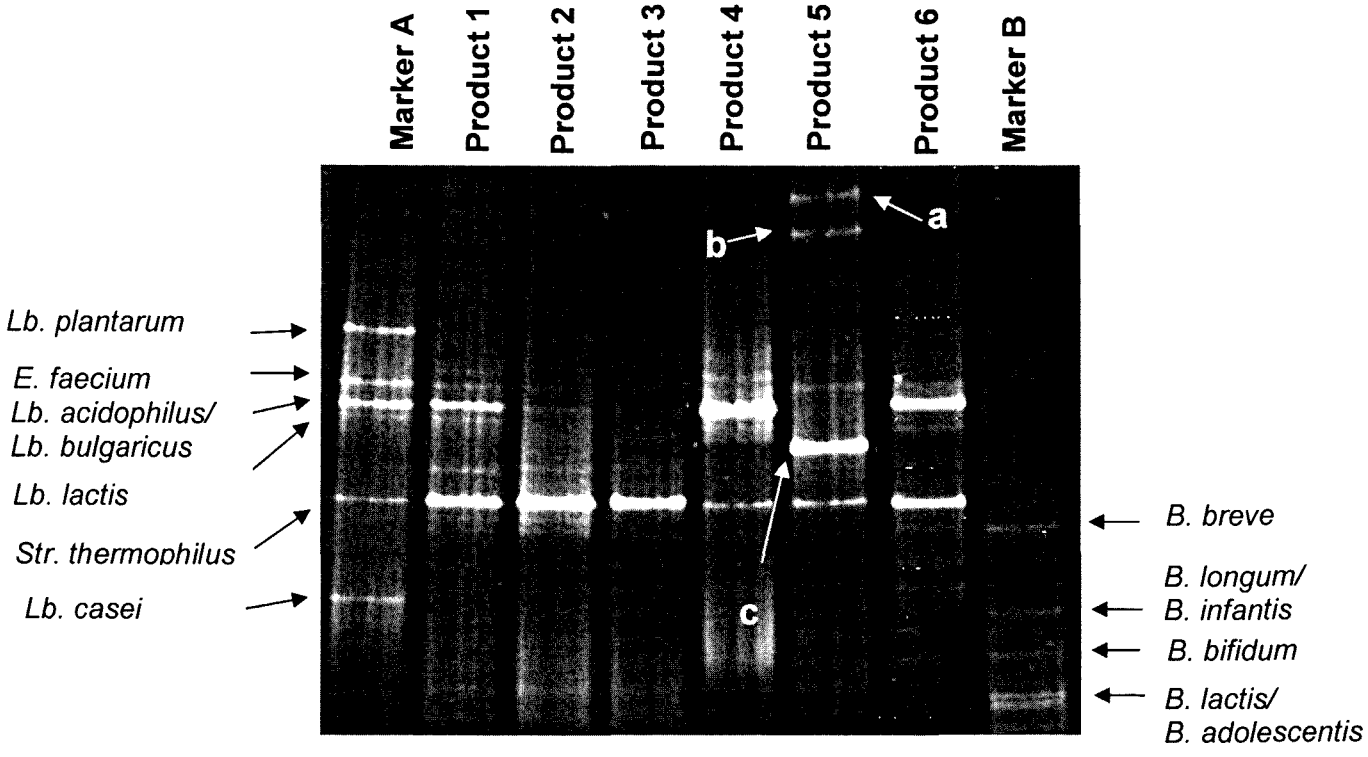
A detection limit indicated the lowest bacterial concentration at which bands could be visualized by DGGE. *Bifidobacterium longum* was used as reference strain and this species could be detected at a minimum concentration of 10^5 cfu.ml^{-1}. These values correlated well with the detection limit (10^7) observed by Fasoli *et al.* (2003) for the detection of probiotics from yoghurt products. It is stipulated that a probiotic food product should contain viable probiotic microbes at a concentration of 10^6 cfu.ml^{-1} food product at the end of the shelf-life period to have a beneficial health effect (Robinson, 1987; Ouwehand & Salminen, 1998; Anon., 2002). A probiotic concentration below this detection limit is therefore considered as insufficient to exert a positive effect on human health.

Repeated DNA isolation from the same probiotic sample, as well as from different batches of the same probiotic yoghurts resulted in reproducible DGGE fingerprints. Repetitive species-specific PCR reactions were performed using the same DNA and this resulted in reproducible results with excellent correlation to the DGGE fingerprints obtained.

DGGE analyses of the probiotic yoghurts

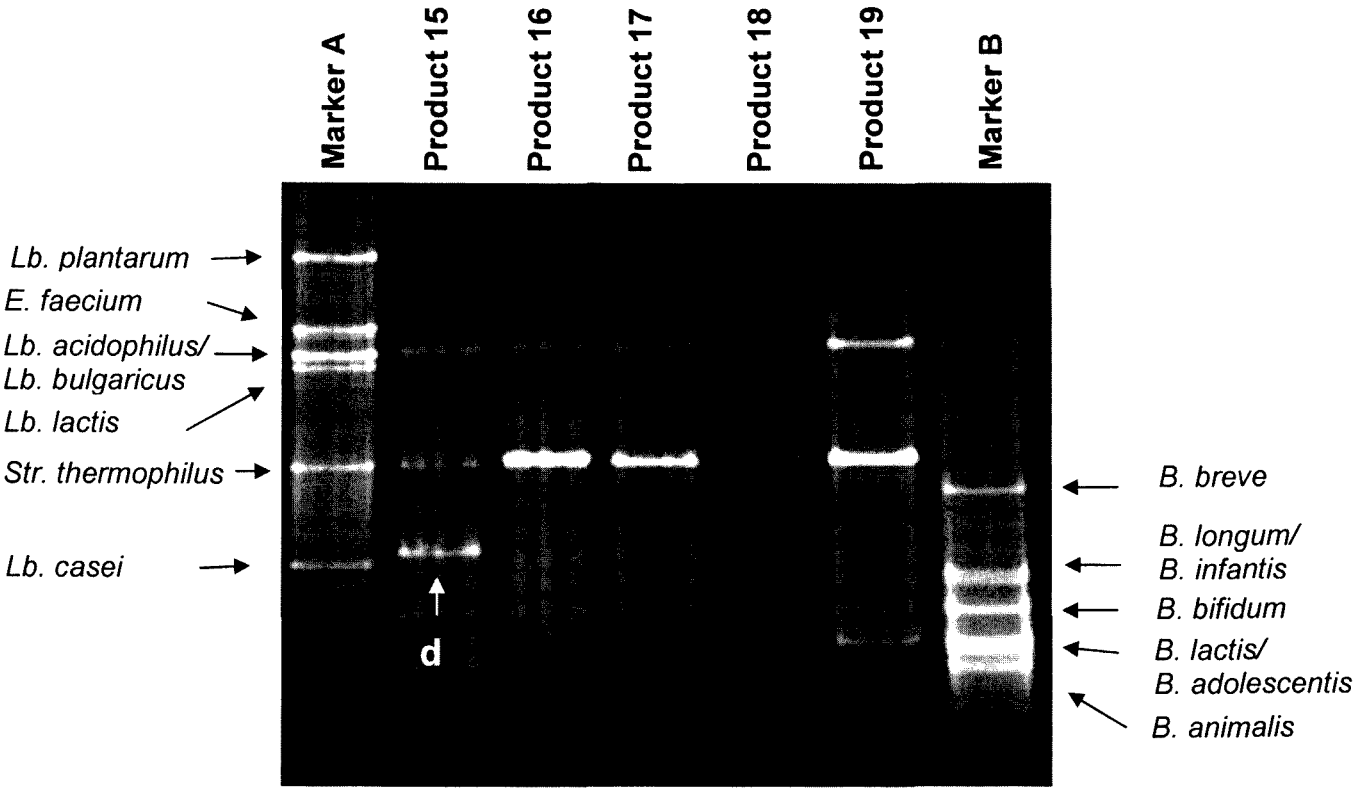
The expected 200 bp PCR fragments were successfully amplified from the probiotic products. The DGGE fingerprints obtained by PCR-based DGGE analyses of the 11 probiotic yoghurt products are shown in Figs. 1 and 2.

Each band present in the DGGE fingerprint represents a different microbial species in the probiotic product (Muyzer *et al.*, 1993; Fasoli *et al.*, 2003). It was found that Products 1, 2, 4, 6, 16 and 19 (55% of the probiotic yoghurts tested) did contain all the probiotic types of microbes that were specified on the product label, although none of these products listed all the probiotic bacteria to species level (Table 3). Products 3, 5, 15, 17 and 18 did not contain the exact microbial species as indicated on the label.



a, b, c – DGGE bands identified using DNA sequencing.

Figure 1. PCR-based DGGE fingerprints of the microbial population present in different South African probiotic yoghurts. The label information of each product is given in Table 3.



d – DGGE band identified using DNA sequencing.

Figure 2. PCR-based DGGE fingerprints of the microbial population present in different South African probiotic yoghurts. The label information of each product is given in Table 3.

Table 3. Product label information of the different South African probiotic yoghurts and the identifications obtained by PCR-based DGGE and species-specific PCR.

Product	Microbes declared on the label[a]	cfu.g^{-1} declared[a]	Microbes detected	PCR-DGGE[b]	Specific PCR[b]
1	"Original Live AB Cultures"	Not declared	*Lb. acidophilus*	+	+
			B. bifidum	-	+
			Str. thermophilus	+	nd
2	"acidophillus bifidobacterium"	Not declared	*Lb. acidophilus*	+	+
			B. bifidum	-	+
			Str. thermophilus	+	nd
3	"Live and Active AB Cultures"	Not declared	*Lb. acidophilus*	+	+
			B. bifidum	-	-
			Str. thermophilus	+	nd
4	"Lb. acidophilus Lb. bulgaricus Str. thermophilus Bifidobacterium spp."	Not declared	*Lb. acidophilus*	+	+
			Lb. bulgaricus	+	+
			Str. thermophilus	+	nd
			B. bifidum	+	+
5	"Selected cultures including AB cultures"	Not declared	*Lb. acidophilus*	-	-
			B. bifidum	-	-
			Str. thermophilus	+	nd
			Streptococcus spp.	s	nd
6	"Live AB Cultures"	Not declared	*Lb. acidophilus*	+	+
			B. bifidum	+	+
			Str. thermophilus	+	nd
15	"Live probiotic AB cultures"	Not declared	*Lb. acidophilus*	+	+
			Str. thermophilus	+	nd
			Soya DNA	s	nd
16	"Lb. acidophilus(La-5) Bifidobacterium(Bb-12)"	1 000 000/ml	*Lb. acidophilus*	+	+
			B. bifidum	-	+
		1 000 000/ml	*Str. thermophilus*	+	nd
17	"Lb. acidophilus Bifidobacterium"	1.2×10^8 viable cfu	*Lb. acidophilus*	+	+
			Str. thermophilus	+	nd
18	"Bifidobacterium"	1 000 000/ml	*Str. thermophilus*	+	nd
			B. adolescentis	-	-
			B. bifidum	-	-
			B. longum	-	-
19	"Lb. acidophilus(La-5) Bifidobacterium(Bb-12)"	1 000 000/ml	*Lb. acidophilus*	+	+
			B. bifidum	-	+
			B. lactis	+	+
			Str. thermophilus	+	nd

[a] Description exactly as given on the label.

[b] **+** = detected; **-** = not detected; **nd** = not determined; **s** = confirmed by sequence comparisons.

The data showed that all the yoghurt products that were tested did contain the normal yoghurt starter culture, *Str. thermophilus*, and *Lb. acidophilus* was detected in all the products that claimed it on the labels (Products 1, 2, 3, 4, 6, 15, 16, 17 and 19) with the exception of Product 5. *Lactobacillus bulgaricus*, as indicated on the label, was detected in Product 4. This was confirmed by PCR detection with specific primers (Table 2) as the DGGE technique (30-70% DGGE denaturant gradient) could not clearly distinguish between the two bands representing *Lb. acidophilus* and *Lb. bulgaricus*.

The presence of *Bifidobacterium* was declared on the labels of all the yoghurt products examined, although not one product indicated the full species names, as stipulated by the South African regulations (Anon., 2002). *Bifidobacterium bifidum* was the *Bifidobacterium* species most detected in the products and was found to be present in Products 1, 2, 4, 6, 16 and 19. No *Bifidobacterium* species could be detected in Products 3, 5, 15, 17 and 18 using DGGE and species-specific PCR. This may be due to a concentration below the detection limit or due to the fact that none of these bacteria were present in the yoghurt product. Again it should be noted that a concentration below the detection limit is not considered suitable in providing a beneficial effect and is therefore not up to standard (Anon., 2002).

Consistency was found between the species detected by DGGE and those detected using species-specific PCR, except for *B. bifidum*. In some instances the bands corresponding to this species were not visualized in the DGGE profile even though the specific PCR gave a positive result (Table 3; Figs. 1 and 2). This is probably due to a lower detection limit of the PCR technique used.

The DGGE technique as used in this study could not distinguish between *B. adolescentis* and *B. lactis* and a species-specific PCR for *B. adolescentis* was used to confirm the DGGE results when a positive band for *B. adolescentis*/ *B. lactis* were obtained. Based on this the *Bifidobacterium* species present in Product 19 was identified as *B. lactis*.

Product 18 was subjected to a pre-treatment step prior to a repeated DNA isolation and this resulted in a much better and brighter DGGE fingerprint than shown in Fig. 2. This was due to an improved DNA extraction from the flavoured yoghurt product and the removal of compounds that could inhibit the PCR

reaction. Despite the pre-treatment of the DNA, no *Bifidobacterium* could be detected in this product by PCR-based DGGE or species-specific PCR.

Three bands, (a, b, c in Fig.1) were observed in Product 5 that could not be identified with the reference markers A and B. Sequencing results of the isolated bands (a and b) identified both these as *Str. thermophilus* (97% homology; 167 out of 172 bases) even though these bands did not correlate with the *Str. thermophilus* strain represented in reference marker A. The appearance of more than one band for a single species is usually due to the presence of multiple, heterogeneous rRNA operons (Cilia *et al.*, 1996; Nubel *et al.*, 1996; Rainy *et al.*, 1996).

The dominant band c (Product 5; Fig. 1) was grouped as either *Str. equinus* or *Str. bovis* (99% homology; 170 out of 172 bases). The DNA sequence data of the 200 bp fragments of these two species were compared and found to be identical and it was therefore not possible to identify the DGGE band as either *Str. bovis* or *Str. equinus*. *Streptococcus bovis* is known to be the cause of a variety of diseases (Whitehead & Cotta, 2000) and although *Str. equinus* has not been described as a human pathogen, cases of illness resulting from this species have been reported (Sechi *et al.*, 2000). Neither of these two species, therefore, is desirable in any food product.

The unidentified band d (Fig. 2) in Product 15 was identified as *Glycine max* (soya) (94% homology; 141 out of 150 bases) and although the label indicated that it was a soya-based yoghurt, the detection of DNA other than the targeted microbial region was not expected.

The product label indicated that potassium sorbate was used as a preservative in Products 3, 4 and 15 even though future South African regulations will stipulate that a probiotic claim may not be made on foodstuffs that contain any preservative other than pimaricin (Anon., 2002). Sorbic acid and its salts are generally added to food products as preservatives and are effective against moulds and yeasts. Research over the years has indicated that sorbates may also be highly effective against a wide range of bacteria (Kabara & Eklund, 1991). The resistance of *Bifidobacterium* to potassium sorbate is however not certain and this may explain the absence or low concentration (not detected by PCR-based DGGE and specific PCR) of *Bifidobacterium* species found in Products 3 and 15. Pimaricin or natamycin, on the other hand has a pronounced effect against yeasts and moulds but exhibits no antibacterial activity (Kabara & Eklund, 1991).

DGGE analyses of the lyophilized products

The probiotic species and concentration, as stated on the original product label, varied greatly between the nine lyophilized preparations that were examined in this study. The label information in some instances was vague and did not indicate the full scientific name of the probiotic microbes as stipulated by the International Code of Nomenclature (Anon., 2002) (Table 4). The DGGE fingerprints of the nine lyophilized products are presented in Fig. 3. It was found that only one third of the lyophilized products examined did contain the probiotic microbial composition specified on the product label (Products 7, 10 and 20) although the label of Product 20 did not specify the *Bifidobacterium* species used in the product.

Product 7 did contain *Lb. acidophilus* as was stated on the label. This product label however, also claims that this probiotic microbe is "killed" and consequently the probiotic benefits of this product are unclear as it is generally suggested that probiotic bacteria should be viable to exert a positive effect.

Streptococcus thermophilus and *B. lactis* were detected in Product 8 as were declared on the label, as well as *Lb. casei* and *Lb. acidophilus* which were not declared. The unknown band e as shown in Fig. 3 and found present in Product 8 was sequenced and identified as *Lb. delbrueckii* (90% homology; 125 out of 139 bases). Product 8, therefore, contained more microbes than had been listed on the label.

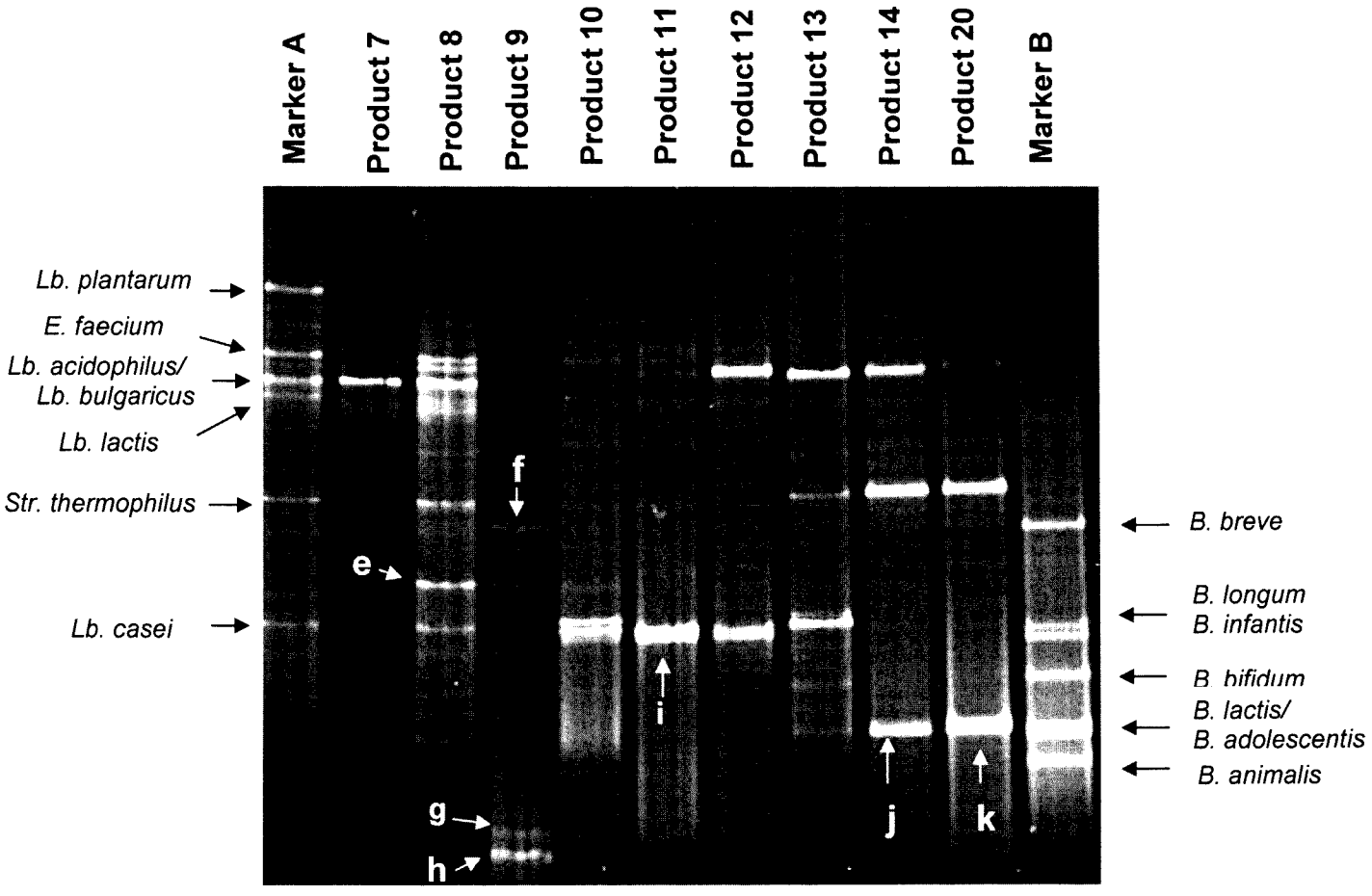
Three distinct bands (f, g, h in Fig. 3) were observed in the DGGE profile of Product 9. The unspecific label information ("lactic acid bacillus") did not give any indication as to which microbe or microbes could be expected in this product. DNA sequencing data of these bands identified all three bands as *Bacillus coagulans* (93% homology; 131 out of 140 bases). These unexpected results were verified by examination of the morphology of this microbe by Gram staining, as well as the presence of catalase. Although *B. coagulans* has been used as a probiotic in animal feed (Cavazzoni & Adami, 1993; Adami & Cavazzoni, 1999), studies have shown that the vegetative cells of this genus cannot survive the aerobic and acidic conditions found in the gastro-intestinal tract. *Bacillus* spores are more resistant and therefore considered most effective in probiotic preparations in providing a health-enhancing effect (Spinosa *et al.*, 2000). The use of *Bacillus* in probiotic preparations for human consumption is controversial

Table 4. Label information of the nine South African lyophilized products and the results obtained by PCR-based DGGE and species-specific PCR.

Product	Microbes declared on the label[a]	cfu.g^{-1} declared[a]	Microbes detected	PCR-DGGE[b]	Specific PCR[b]
7	"Lyophilizate of killed *Lb. acidophilus*"	5,10^9 per capsule	*Lb. acidophilus*	+	+
8	*"Str. thermophilus B. lactis"*	10^6 cfu/g	*Str. thermophilus* *B. lactis* *Lb. acidophilus* *Lb. casei spp.* *Lb. delbrueckii spp.*	+ + + + s	nd + + nd nd
9	"Lactic acid bacillus"	40 × 10^6	*Bacillus coagulans*	s	nd
10	"B. longum Lb. rhamnosus"	10 × 10^7	*B. longum* *Lb. rhamnosus*	+ +	+ +
11	*"B. infantis"*	One billion viable cells	*B. infantis* *B. longum*	- +/s	nd +
12	*"B. longum B. bifidum Lb. acidophilus"*	One billion viable cells	*B. longum* *B. bifidum* *Lb. acidophilus*	+ - +	+ - +
13	"Lb. Rhamnosus Delbrueckii subsp. Bulgaricus Str. Thermophilus"	Not declared	*Lb. rhamnosus* *Lb. bulgaricus* *Str. thermophilus* *B. bifidum* *B. lactis*	+ + + + +	+ + nd + +
14	"Lb. acidophilus Lb. bulgaricus Bifidobacteria animalis Str. thermophilus"	3.5 billion viable cells	*Lb. acidophilus* *Lb. bulgaricus* *B. animalis* *Str. thermophilus* *B. lactis*	+ + - + +/s	+ + nd nd nd
20	"Lb. acidophilus (La-5) Bifidobacterium (Bb-12) Str. thermophilus"	Not declared	*Lb. acidophilus* *B. lactis* *Str. thermophilus*	+ +/s +	+ nd nd

[a] Description exactly as given on the label.

[b] **+** = detected; **-** = not detected; **nd** = not determined; **s** = confirmed by sequence comparisons.



e, f, g, h, i, j, k – DGGE bands identified using DNA sequencing.

Figure 3. PCR-based DGGE fingerprints of the microbial population present in different South African lyophilized preparations. The label information of each product is given in Table 4.

and South African regulations stipulate that microbes other than those approved in the regulation should be submitted to the Director-General of the Department of Health prior to market appearance (Anon., 2002).

Product 10 was found to contain both the microbes *B. longum* and *Lb. rhamnosus* as declared on the label of the product. As the DGGE technique could not distinguish between strains of *Lb. casei* and *Lb. rhamnosus*, a specific PCR assay was required to confirm the presence of *Lb. rhamnosus* (Table 2). This product did contain all the probiotic microbes claimed on the label.

Bifidobacterium infantis, as claimed on the label of Product 11 could not be detected in this product. However, *B. longum* was found to be present even though it was not declared. This presence of *B. longum* was confirmed by species-specific PCR and sequencing of the selected band i (Fig. 3) (93% homology; 122 out of 131 bases). These results are of importance as it is stipulated that no baby food supplement (Product 11), for children under the age of 12 months, may contain any probiotic bacteria other than *B. infantis* and *B. longum* (Anon., 2002). Although this product is within the legal stipulations regarding the probiotic microbial species which may be present in baby products, it does not conform to the regulations in providing the correct species information on the label.

Only two bands were observed in the DGGE-profile of Product 12. These bands were identified as *Lb. acidophilus* and *B. longum* as claimed on the label. No *B. bifidum* could be detected in this product by DGGE, as well as with species-specific PCR, although the presence of this probiotic was stated on the label.

In addition to the three probiotic bacteria claimed on the label of Product 13 (*Lb. rhamnosus, Lb. bulgaricus* and *Str. thermophilus*), this product also contained the species *B. bifidum* and *B. lactis*.

Product 14 was found to contain *Lb. acidophilus*, *Lb. bulgaricus* and *Str. thermophilus* as was indicated on the label. In addition, *B. lactis* was identified and this was confirmed by the sequence data of band j (Fig. 3). No *B. animalis* was found in this product.

The DGGE profiles of Product 20 revealed the presence of three microbes that were identified as *Lb. acidophilus*, *Str. thermophilus* and the *Bifidobacterium* species *B. lactis* (band k in Fig. 3) (94% homology; 148 out of 157 bases). These

microbes were all declared on the label of the product although the *Bifidobacterium* species name was not indicated.

Conclusions

The results obtained in this study show a general lack of accuracy in the identification and naming of probiotic microbes as presented on the labels of various South African probiotic containing products. This is not allowed according to the South African health and food regulations (Anon., 2002) as safety and probiotic health-related effects cannot be guaranteed. This further result in misleading information being relayed to the consumer regarding the health benefits of the product. A lack in efficient technological and manufacturing practices in the industry may result in the contamination of starter cultures and products. Effective in-process monitoring and quality control of the end product may have an improved effect on the quality of the probiotic products but this does not appear to be done. It seems that manufacturers of probiotic supplements are either not aware of the regulations relating to probiotics or simply do not abide by these rules. The strict monitoring by regulating authorities and independent parties, as well as the assignment of penalties, may persuade manufacturers to improve the quality of their products. Improvements in labelling of the products should be easy to achieve.

The findings indicate that PCR-based DGGE can effectively be used as a rapid and accurate culture-independent approach for the identification of the microbial composition of probiotic products. The study did not focus on, or determine the number of viable colony forming units (cfu) present in the probiotic food products. It has previously been shown that non-viable probiotic microbes can also have health benefits when present in recommended concentrations (Ouwehand & Salminen, 1998). The DGGE technique used in this study was rather used as a rapid and accurate method to determine whether the probiotic microbes indicated on the label were present in the product above a concentration of 10^5 cfu.ml^{-1}. All of the bands present in the DGGE profiles could be identified to species level, by comparing them to two reference markers. Sequence analyses and species-specific PCR are sufficient for the verification of the DGGE results.

This application could, therefore, serve as a valuable tool for the microbiological quality control of probiotic preparations in order to provide a reliable and safe product to the South African consumer.

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CHAPTER 4

GENERAL DISCUSSION AND CONCLUSIONS

A wide variety of probiotic products and supplements are commercially available on the South African market. These include an assortment of fermented milk products and lyophilized preparations in capsule or tablet form. The word probiotic is derived from the Greek meaning 'for life' and a probiotic is generally defined as a live microbial feed supplement which beneficially affects the host by improving its intestinal microbial balance (Fuller, 1989). The regular consumption of viable probiotic microbes is associated with various health benefits (Gurr, 1987; Gilliland, 1990; Gomes & Malcata, 1999).

Probiotic microbes in commercial products are commonly identified by traditional methods such as growth on selective media, morphological and biochemical characteristics. However, these identification methods are time-consuming and labour intensive and sometimes fail to accurately characterize bacteria to species level (Yaeshima *et al.*, 1996; Holzapfel *et al.*, 1997; Matsuki *et al.*, 2002). This is important as health enhancing effects of probiotic bacteria are reported to be species (Salminen *et al.*, 1998) and strain (Prasad *et al.*, 1998; Sanders, 1999) specific. Some probiotic bacteria, especially *Bifidobacterium*, have complex nutritional requirements and grow under strict anaerobic conditions, therefore making it difficult to cultivate on selective media (Matsuki *et al.*, 2002). Due to the abovementioned factors a great demand for rapid and sensitive techniques has arisen to identify probiotic microbes present in probiotic foods. Some molecular techniques are more commonly being used as rapid and accurate methods for the identification and characterization of microbes from various environments (Prasad *et al.*, 1998; Satokari *et al.*, 2001). These cultural independent techniques have the advantages of not requiring anaerobic conditions and of being very specific and sensitive (Matsuki *et al.*, 2002).

In this study the probiotic microbial consortium present in various South African products were identified using PCR-based DGGE fingerprinting. DNA was isolated from 11 different South African yoghurts and from eight different probiotic lyophilized preparations, as well as one baby milk formula. The V2-V3 region of

the 16S rRNA gene was amplified using the primers HDA1 and HDA2 (Walter *et al.*, 2000) and the PCR fragments obtained were separated using PCR-based DGGE. Unique DGGE fingerprints were observed for each probiotic product and the bands representing different probiotic bacteria were identified by comparison to two reference markers A and B. The results obtained were verified by species-specific PCR and sequence analysis of selected and unidentified bands. The PCR-based DGGE and specific PCR techniques were found to be reproducible, resulting in the same band patterns with repeated DNA isolation within a sample and between different batches of the same sample.

It was found that only five out of the 11 probiotic yoghurts tested (54.5%) did contain all the probiotic microbes as indicated on the label. The probiotic, *Lactobacillus acidophilus* was found to be present in all the products that claimed to contain it, except for product 5. Although all of these products declared the presence of *Bifidobacterium*, none of the product labels identified the *Bifidobacterium* to species level. *Bifidobacterium bifidum* was the *Bifidobacterium* species mostly detected in the products and *B. lactis* was found in one product. No *Bifidobacterium* could be detected in 45.4% of the yoghurt products, either by PCR-based DGGE or species-specific PCR. A detection limit of 10^6 cfu.ml^{-1} was established for the DGGE technique and it should be noted that a probiotic microbial concentration below this limit is not considered to be sufficient in establishing a health enhancing effect (Robinson, 1987; Anon., 2002).

A potential pathogenic microbe (either *Streptococcus bovis* or *Str. equinus*) was detected in one of the yoghurt products by sequence comparison. Even though sequence data within the V2-V3 region of the 16s rRNA gene could not distinguish between the two species, neither one of these microbes are desirable in any food product as both have been known to cause disease (Sechi *et al.*, 2000; Whitehead & Cotta, 2000).

The preservative potassium sorbate was found to be present in three of the probiotic yoghurts as indicated on the product labels. In future, South African regulations will not allow a probiotic claim to be made on a food product if the product contains any preservative other than pimaricin (Anon., 2002). The preservative used may have had a negative influence on the probiotic microbes

and could explain the absence or low concentration of *Bifidobacterium* in two of these products.

Only a third (33%) of all the South African probiotic lyophilized products tested contained the exact microbial species as specified on the product label. Some of these products contained more microbes than listed on the label and in most instances *Bifidobacterium* spp. other than those mentioned on the label, were detected. *Bacillus coagulans* was detected and identified in one of the products, although this microbe is not known to be used in human probiotic food preparations. Studies have shown that this genus is incapable of exerting a positive probiotic effect as vegetative cells but is more efficient as spores (Spinosa *et al.*, 2000). The label information of this product was misleading as it claimed to contain "lactic acid bacillus".

Concluding Remarks

From the data obtained it is clear that PCR-based DGGE combined with species-specific PCR can be used as a rapid and accurate cultural-independent technique for the detection and identification of the microbial species in probiotic products. The results obtained show a poor standard of label information and probiotic species content for most of the South African probiotic products tested. It is evident that there are serious problems in quality control and labelling of probiotic products commercially available in South Africa. Similar reports have highlighted the poor quality of various probiotic supplements available to the public world-wide (Hamilton-Miller *et al.*, 1999; Hoa *et al.*, 2000; Fasoli *et al.*, 2003; Temmerman *et al.*, 2003). This is not only of concern as the consumer is misled regarding the health effect of the product but it also undermines efforts to prove the efficacy of probiotic supplements (Hamilton-Miller & Shah, 2001). The strict implementation of existing regulations and legislations should be able to avert shortcomings in the microbiological quality and labelling of probiotic products.

PCR-based DGGE could serve as a valuable method for the quality control and evaluation of probiotic products in order to ensure a microbiological safe and reliable product to the consumer. It is also useful for the detection and identification of undesirable microbes, as well as potential pathogens.

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