

# **CHAPTER 2**

## **Viability of probiotic cultures from yoghurt samples randomly selected from South African retail stores**

## 2.1 ABSTRACT

Incorporation of probiotics into dairy fermented products is now a well known practice. It has developed due to an increase in the number of studies reporting human health benefits coupled with regular intake of these vital microorganisms. However, several studies using both culture-dependent and -independent methods for detection and identification of the probiotic microbes, reported not only a problem with survival and stability of these microbes, but also in some cases their absence in products that claim to contain them. The aims of this study were to determine viability of cultures in commercial South African probiotic yoghurts and to determine if product labels specified the probiotic cultures and their levels in colony forming units (cfu) by the end of the shelf life. Eleven yoghurt samples randomly collected from retail stores were serially diluted in sterile ¼ strength Ringer's solution after thawing at room temperature. Appropriate dilutions were pour-plated in triplicate on de Man, Rogosa and Sharpe (MRS) + maltose agar and MRS agar supplemented with 0.05 % cysteine hydrochloride for lactobacilli and bifidobacteria, respectively. All plates were incubated at 37 °C for 72 h in anaerobic jars with Anaerocult C for lactobacilli and Anaerocult A for bifidobacteria. All samples showed presence of high numbers of *Lactobacillus* while only six (54.5 %) of the samples showed presence of bifidobacteria. None of the samples adequately identified probiotic organisms on their labels to species level, while only two (18 %) specified the numbers of viable bacteria as cfu/ml. Generally, there was a problem of stability of probiotic bifidobacteria cultures in products and therefore a need for development of a method that will improve survival and hence shelf life of probiotic products.

Keywords: Probiotics, Bifidobacteria, *Lactobacillus*, yoghurt,

## 2.2 INTRODUCTION

The normal intestinal microflora is the first line of defence against infections. The microflora assist in absorption of nutrients from food, and it is therefore vital for good health (Holzapfel and Schillinger, 2002). The normal microflora is not permanently stable. It can be changed by a number of factors that reduce numbers of viable microbes and thus rendering it deficient as an infection barrier. These factors include among others starvation, poor diet, use of antibiotics, stress, travelling and ageing (Havenaar and Huisint’Veld, 1992; Mitsuoka, 1996; Fooks et al., 1999).

It was observed by Mechnikoff in 1907 that Bulgarian peasants were healthy and lived longer due to their consumption of yoghurt. This led the scientific world to look at the link between the intestinal microflora and the prevention of human diseases (Oliveira et al., 2001; Schrezenmeir and de Vrese, 2001; Teitelbaum and Walker, 2002). The intentional introduction of strains of beneficial bacteria in order to starve off pathogenic microbes has since been adopted (Mombelli and Gismodo, 2002). Various *Lactobacillus* spp. and *Bifidobacterium* spp. collectively known as AB cultures are incorporated into commercial probiotic products such as yoghurts and other fermented foods (Theunissen et al., 2005). These probiotic bacteria are becoming more and more popular as their contribution to good health is reported. These reports also increased people’s knowledge on benefits of probiotics, leading to acceptance of these cultures (Desmond et al., 2002).

Probiotic microorganisms have been shown to help in the treatment of several diseases such as diarrhoea (Fooks et al., 1999 ; Marteau et al., 2001 ; Teitelbaum and Walker, 2002), cancer (Fooks et al., 1999; Macfarlane and Cummings, 1999; Marteau et al., 2001; Miguel, 2001; Schrezenmeir and de Vrese, 2001), coronary heart disease (Fooks et al., 1999; Schrezenmeir and de Vrese, 2001), modulating immune responses (Holzapfel et al., 1998; Macfarlane and Cummings, 1999) and alleviating the symptoms of lactose intolerance (Fooks et al., 1999; Miguel, 2001; Elliott and Teversham 2004).

It is very important that probiotic cultures retain their viability in sufficient numbers for them to confer health benefits on the host (Salminen et al., 1996; Holzapfel et al., 1998; Macfarlane and Cummings, 1999; Miguel, 2001; Teitelbaum and Walker, 2002). Thus, actions of beneficial bacteria, and not just their presence in fermented milk products, are responsible for positive effects (Schrezenmier and de Vrese, 2001; Teitelbaum and Walker, 2002). Dairy food products incorporating bifidobacteria are now well established in the market (Doleyres and Lacroix, 2005). However, it has been documented that not all fermented milk products available in retail stores, actually contain viable beneficial bacteria. It has been found that labels do not clearly state the contents and probiotic effects of the product, nor do the labels state the actual number of viable microorganisms that can be expected in the product. Labels generally have an exceptionally poor correlation with the actual content (Elliott and Teversham, 2004; Huff, 2004).

In the year 2004, the South African probiotic industry was reported as being worth R45 million per annum. This was equal to over a million doses taken per year with 30 000 doses of probiotics taken daily. With probiotics being such a significant industry, it is very essential that these products deliver what they should to the consumers. The main objectives of this study were therefore to:

- Check clarity of product labels with regard to proper scientific names (genus, species) of incorporated probiotic microorganisms, and levels (cfu/g or cfu/ml) at the end of shelf life, as required
- Enumerate viable *Lactobacillus* and *Bifidobacteria* present in probiotic yoghurts using conventional plating techniques
- Compare levels of viable cultures obtained in the recent study with those claimed on product labels.

## 2.3 MATERIALS AND METHODS

### 2.3.1 Sample collection and storage

Yoghurt samples were collected at random from various retail stores. Frozen and drinking yoghurts of various flavours from different manufacturers were collected. The samples were stored at 4 °C before analysis. All analyses were carried out within 24 h of sampling. The samples were left at room temperature for 30 min before analysis.

### 2.3.2 Bacterial enumeration

1 ml of each yoghurt sample was suspended in 9 ml of sterile ¼ strength Ringer's solution. Ten fold serial dilutions of the resulting suspension were prepared in sterile ¼ strength Ringer's solution to obtain a suitable dilution. For viable *Bifidobacteria* counts, 0.1 ml of the appropriate dilutions was pour plated on MRS-agar supplemented with 0.05 % cysteine-hydrochloride. For viable *Lactobacillus* counts, 0.1 ml of the appropriate dilutions was pour plated on MRS-agar. All the dilutions were plated out in triplicate. Plates were incubated in anaerobic jars with Anaerocult A gas-packs at 37 °C for 72 h. Anaerobic conditions were verified using an anaerobic indicator, Anaerotest strips (Merck). After incubation, the visible colonies were counted and expressed as colony-forming units per milliliter (cfu/ml), representing the number of viable *Bifidobacteria* and *Lactobacillus* present in each yoghurt sample.

## 2.4 RESULTS AND DISCUSSION

It is stipulated in the SA health and food draft regulations that labels of probiotic products should indicate viable counts of bacteria per gram of product at the end of shelf life and also give full scientific name of the probiotic species present in the product (Anonymous, 2002). Earlier reports have already highlighted the importance of documentation of viability, colony counts and species identification of organisms in probiotic products

(Marcon, 1997). These reports also indicated the inaccuracy of labelling of some probiotic products. Results of the current study on labels of probiotic products support this finding.

**Table 2.1: Information from South African probiotic yoghurts and counts obtained using conventional plate counts**

Product	Microorganisms claimed on the label	Counts claimed on the label	<i>Bifidobacteria</i>	<i>Lactobacillus</i>
			counted	counted
cfu/ml				
1	“Live and active NitriPlus cultures”	NS	NG	1.51 x 10 <sup>9</sup>
2	“Yoghurt cultures”	NS	3.80 x 10 <sup>8</sup>	1.16 x 10 <sup>9</sup>
3	“HOWARU Bifido probiotic cultures”	1 x 10 <sup>6</sup>	8.10 x 10 <sup>7</sup>	5.33 x 10 <sup>9</sup>
4	“HOWARU Bifido probiotic cultures”	1 x 10 <sup>6</sup>	NG	5.00 x 10 <sup>9</sup>
5	“Contains live AB cultures”	NS	1.89 x 10 <sup>10</sup>	2.39 x 10 <sup>9</sup>
6	“AB cultures”	NS	2.09 x 10 <sup>8</sup>	1.02 x 10 <sup>10</sup>
7	“Live AB cultures”	NS	NG	1.52 x 10 <sup>10</sup>
8	“Abkhasian unique live cultures”	NS	1.41 x 10 <sup>7</sup>	2.18 x 10 <sup>9</sup>
9	“Live AB cultures/ AB yoghurt cultures”	NS	1.21 x 10 <sup>8</sup>	9.46 x 10 <sup>8</sup>
10	“Live AB cultures”	NS	NG	1.93 x 10 <sup>9</sup>
11	“Live Probiotic AB cultures”	NS	NG	5.40 x 10 <sup>9</sup>

**cfu= colony forming units, NS = Not specified, NG = No growth**

Information from the labels of products and the results of the counts obtained in this study are summarized in Table 2.1. This study found that information given on labels of all eleven yoghurt samples collected was insufficient. None of the samples packaging gave proper species identification for the probiotic incorporated i.e. none of the products gave full names (identification) genus and species, of the incorporated cultures, while nine of the samples did not specify microbial counts. Only two of the analyzed products

(3 and 4) (Table 2.1) indicated numbers of organisms present. The label indicated that the products contained about  $1 \times 10^6$  cfu/ml. Both products were from the same manufacturer. Even though the manufacturer in this case tried to comply with the requirements, the information given was still not complete. Firstly, it was not clear whether the number given was referring to the counts of all the cultures present in the product, or whether each of the organisms was present at the levels indicated. Secondly, it was not explicit whether the levels stated on the product label were those present or added during manufacturing or whether it was the numbers of bacteria that will be available at the end of shelf life. This leaves out very important information that should be given to consumers as it is desired that products contain sufficient numbers of viable probiotic bacteria to confer health benefits at the time of use and not only at the time of manufacture (Reid, 2003). Results of the current study correlated with findings by Theunissen et al. (2005) who found that there was a general inaccuracy in identification and naming of probiotics presented on SA probiotic products.

All the analyzed samples contained high numbers of viable *Lactobacillus*. The levels of lactobacilli in products ranged between  $9.46 \times 10^8$  cfu/ml to  $1.52 \times 10^{10}$  cfu/ml (Table 2.1). Bifidobacteria were detected from only six of the products tested. The counts of bifidobacteria were however lower than those of lactobacilli in all the samples. Results indicated that survival of lactobacilli was generally better than that of bifidobacteria. Bifidobacteria are reported to require an anaerobic environment and a neutral pH to survive and maintain levels greater than  $10^6$  cfu/ml that is adjudged as the requirement to provide therapeutic benefits (Boylston et al., 2004). Schillinger (1999) also found that lactobacilli remained at high levels until their “best before use” period indicating relative stability of cultures. The results of this study correlated with observations from other studies (Micanel et al., 1997, Shah et al., 2000, Vinderola et al., 2000, Huff, 2004, Elliot and Teversham, 2004).

Elliot and Teversham (2004) analysed nine South African probiotic products using both culture dependent and independent methods. Their study found that four of the products did not show presence of either lactobacilli or bifidobacteria. In the recent study it was

found that all products that contained both lactobacilli ( $9.46 \times 10^8$  -  $1.52 \times 10^{10}$  cfu/ml) and bifidobacteria ( $1.41 \times 10^7$  -  $3.80 \times 10^8$  cfu/ml), contained them in numbers high enough to confer beneficial effects in the host (Table 2.1). The numbers of viable bifidobacteria and lactobacilli from all the products containing them were greater than the suggested minimum as required by SA legislation and international standards. However, in the study by Elliot and Teversham (2004) it was found that only three of the five products that contained viable cultures had sufficient bacteria for probiotic effect. On the other hand, Theunissen and Witthuhn (2004) also analyzed 20 different South African probiotic products for their probiotic content. These researchers found that 55 % of the products contained all cultures claimed on their labels but no bifidobacteria could be detected from 45 % of the products. All these studies showed poor correlation between labels and contents of SA probiotic products, and problem of culture viability in the food products, especially bifidobacteria. The problem of viability is however not encountered in SA products only but has been found by researchers in probiotic products from elsewhere in the world.

Micanel et al. (1997) investigated the viability of cultures in four Australian commercial probiotic yoghurts over the shelf life of the products, with shelf life expectancy (use-by date) in each instance varying between 5 and 6 weeks. The numbers of *Lactobacillus acidophilus* in the products varied widely. One product retained levels of  $>10^7$ , another  $>10^6$  cfu/g. The third product reduced slowly, but maintained levels of  $>10^5$  cfu/g while no viable organisms ( $<10^3$  cfu/g) were detected in the fourth product. Of the three products that claimed to contain bifidobacteria, one maintained high levels of  $>10^6$  cfu/g, another showed a steep decline in numbers from  $1.5 \times 10^5$  to  $<10^3$  cfu/g within 2 weeks of manufacture while no viable cultures were detected in the third.

A randomised double blind study by Huff (2004) on North American products purchased from six different retail stores in the lower British Columbia mainland, to assess whether commercially prepared probiotic products claiming to contain *Lactobacillus*, contained viable organisms as claimed by their manufacturers, found similar problems as observed in the recent study. None of the products matched their labelled microbiological

specifications qualitatively and quantitatively. Of the ten products tested, two did not show any growth, four did not grow the *Lactobacillus* species listed on their labels while the remaining four contained species not listed on labels. It was found that commercially available over the counter products were inaccurately labelled and that some of the products tested contained dead bacteria only.

The results indicated that consumers all over the world are buying products that are not what their manufacturers claim them to be. Death of probiotic bacteria in products is due to a number of factors including H<sub>2</sub>O<sub>2</sub> produced by starter bacteria, oxygen content, pH, storage environment and concentration of metabolites such as lactic and acetic acids (Lourens-Hattingh and Viljoen, 2002; Talwalkar and Kailaspathy, 2003; Akalin et al., 2004). When H<sub>2</sub>O<sub>2</sub> is present in cells it inhibits metabolism of sugar by bifidobacteria, through blockage of the key enzyme, fructose-6-phosphofruktokinase (Talwalkar and Kailaspathy, 2003). Post acidification or over-acidification occurs during refrigerated storage resulting in a decrease in pH (Lourens-Hattingh and Viljoen, 2002). This often reduces the pH of yoghurt during storage to levels as low as 3.6. This is said to be the most important factor in bifidobacteria mortality, with storage temperature having a secondary effect (Kailaspathy and Rybka, 1997). It was found that uncontrollable growth of strains of *Lactobacillus delbrueckii* subsp. *bulgaricus* (Kailaspathy and Rybka, 1997; Lourens-Hattingh and Viljoen, 2002) at refrigerated temperatures leads to accumulation of D-lactic acid in the product. Bifidobacteria are susceptible to acids and oxygen, consequently, their counts in yoghurts supplemented with these organisms may easily decrease during storage (Kailaspathy and Rybka, 1997). The increase in titratable acidity or decrease in pH during the storage of yoghurt could be attributed to residual fermentation (Dave and Shah, 1997). As bifidobacteria are strict anaerobes, oxygen adversely affects their growth or viability (Kailaspathy and Rybka, 1997; Talwalkar and Kailaspathy, 2003). They are more vulnerable to the deleterious effects of oxygen than *L. acidophilus*. Presence of oxygen results in accumulation of oxygenic metabolites such as superoxide anion (O<sub>2</sub><sup>-</sup>), hydroxyl radicals (OH<sup>•</sup>) and H<sub>2</sub>O<sub>2</sub>. The oxygenic metabolites are toxic to bacterial cells and their toxic effects damage these cells and finally result in their death (Talwalkar and Kailaspathy, 2004; Talwalkar et al., 2004). Bifidobacteria

can also undergo physiological changes due to exposure to oxygen, whereby their cells become longer than usual, with rough surfaces and require extended incubation before they adapt and start growing in culture media (Talwalkar and Kailasapathy, 2004). Oxygen can be introduced into yoghurts at various steps during and after production. During the production process, the mixing and agitation that occurs at steps such as homogenization, cooling and starter culture inoculation can incorporate atmospheric oxygen into the product. After production, atmospheric oxygen can enter into the product during filling and packaging. Oxygen also diffuses into the product through the packaging material during storage (Talwalkar and Kailasapathy, 2004; Talwalkar et al., 2004).

## **2.5 CONCLUSIONS**

Most of the South African probiotic yoghurts available in retail stores were not properly labeled i.e. did not specify probiotic levels in (cfu/g or cfu/ml) or bacterial names with genus and species, of cultures present. There was a poor correlation between the actual contents of most of the analysed South African probiotic products available in retail stores and their label claims. Lactobacilli generally survived better in probiotic yoghurts than bifidobacteria. The problem of survival of bifidobacteria in products needs serious attention. To ensure that needs of consumers are met, research into methods that would increase the survival of probiotic cultures in products, especially bifidobacteria, must be undertaken.

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