# CHAPTER 1

# LITERATURE REVIEW

## 1.1 Normal intestinal microflora

The indigenous microflora of the gastrointestinal (GI) tract in new-born humans and animals does not appear spontaneously. The foetus is sterile but it becomes contaminated with different microorganisms during birth. These microorganisms are then selected over time as changes occur in the GI tract. The different microorganisms inhabit different parts of the GI tract and then become characteristic of that particular habitat. Most of the microorganisms are thus eliminated such that at the end the GI tract of infants is dominated by lactic acid bacteria and coliforms. Further changes in the population of the microflora occur during weaning ending with the majority of the microbes being obligate anaerobes (Berg, 1996).

The microorganisms become distributed throughout the GI tract. Different parts of the GI tract become colonized by various populations of microorganisms. The stomach has a less dense microbial population which contains less than $10^3$ cfu/ml of contents. Its population is dominated by aerobic Gram positive organisms. The small intestine separates the less populated stomach from the densely populated colon. The microflora of the small intestine is similar to that of the stomach but has higher numbers of microorganisms at the concentration of $10^3$-$10^4$ cfu/ml (Berg, 1996; Richardson, 1996). The most densely populated part of the GI tract (and of the whole body) is the large intestine (colon) (Berg, 1996; Richardson, 1996; Taylor et al., 1999). In humans the intestinal contents contain about $10^{11}$ to $10^{12}$ bacteria per gram of stool. At least 500 species of anaerobic and facultative microbes, consisting among others several species of lactobacilli and bifidobacteria, are found in the contents (Percival, 1997; Wolfson, 1999; Losada and Olleros, 2002). Lactobacilli population comprising of about 60 species is found mainly in the small intestine while bifidobacteria are found in the colon. The *Lactobacillus* genus includes *L. acidophilus*, *L. planturum*, *L. casei* and *L. rhamnosus* while bifidobacteria include *B. longum*, *B. bifidum* and *B. infantis* (Wolfson, 1999). The microflora depends on dietary residues in the GI tract for their metabolism and has the potential to influence processes in the colon (Taylor et al., 1999).

The microorganisms in the intestines can be either beneficial or detrimental. This division is dependent on whether the various enzymes produced by these microorganisms perform functions that harm or support the host. The overall health of an individual depends on the balance between the beneficial and detrimental effects of the intestinal microflora (Percival, 1997). The major physiological functions of the gut microflora may be summarised as follows (Holzapfel and Schillinger, 2002):

- barrier function
- immune system stimulation
- maintenance of mucosa nutrition and circulation
- production of nutrient/improved bioavailability
- stimulation of bowel motility

The importance of indigenous microflora as a natural resistance factor against potential pathogenic microorganisms was originally recognised in the 19th century by Metchnikoff. However, research interest on the use of bacteria for therapy disappeared in the 1940's and then re-emerged around the 1960's due to increased interest of people in natural ways of promoting health. The demand for natural products such as fermented foods and bacterial cultures for alleviating diarrhoea has stimulated the industry to conduct research into new products (Havenaar and Huis int'Veld, 1992).

The indigenous microflora can be influenced by factors both in the internal and external environments, resulting in an imbalance between beneficial and detrimental microbes. External factors include lack of food and water, travelling, use of antibiotics, and drugs for treatment of tumours and radiation (Havenaar and Huis int'Veld, 1992; Luchansky et al., 1999). Other factors include peristalsis disorders, surgical operations of the stomach or small intestine, liver or kidney diseases, anaemia, immune disorders, emotional stress, poor diet and ageing ((Mitsuoka, 1996; Luchansky et al., 1999). Loss of indigenous microflora due to disturbances by one or more of these factors favour predominance of microflora by harmful bacteria ((Mitsuoka, 1996; Luchansky et al., 1999) and also result in availability of empty adhesion sites on the intestinal epithelium. These empty sites may be occupied by any organisms including transient pathogenic microorganisms.

Occupation of the empty adhesion sites by potentially pathogenic transient organisms may lead to an outbreak of an infectious disease (Havenaar and Huis int'Veld, 1992).

The balance between the detrimental and beneficial microflora may be restored to normal by a well balanced diet or by oral administration of bacteria. Strains of lactobacilli and bifidobacteria isolated from the intestines i.e. members of the indigenous microflora, can be administered to produce beneficial or health benefits (Mitsuoka, 1996). These bacteria prevent the growth of putrefactive flora through competitive inhibition, which can be by acidification of the medium, production of proteins with antibiotic activity and other hostile substances (Adams and Moss, 2000; Losada and Olleros, 2002). Their metabolic activities generate B vitamins, accompanied by proteolytic, lipolytic and β-galactosidase activity which improve tolerance to lactose (Adams and Moss, 2000; Losada and Olleros, 2002). They also improve digestion and hence absorption of different nutrients (Adams and Moss, 2000). Other benefits include improvement in disorders such as hepatic encephalopathy, stomatitis, vaginitis, intestinal infection, tumorigenesis and an increase in immune response (Losada and Olleros, 2002). These bacteria used for restoration of normal indigenous microflora are termed probiotics.

## 1. 2 Probiotics

Probiotics have been defined differently by various researchers. The definition has been modified with increasing knowledge in the field as researchers understand how probiotics function. Probiotics were defined as live microbial cultures fed by mouth and surviving transit through the large intestine where they colonise the system (Frost and Sullivan, 2000; Saarela et al., 2000; Matilla-Sandholm et al., 2002; Betoret et al., 2003). Schrezenmeir and de Vrese (2001) defined the term probiotic as a preparation of or a product containing viable, defined microorganisms in sufficient numbers, which alter the microflora by implantation or colonization, in a compartment of the host and by that, exert beneficial effects on host health. The World Health Organization and Food and Agriculture Organization of the United Nations (FAO/WHO, 2001) agreed on the

definition of probiotics as; "live microorganisms which, when administered in adequate amounts confer a health benefit on the host" (Leahy et al., 2005).

Probiotic food cultures have become popular due to appreciation of their contribution to good health (Desmond et al., 2002). In probiotic therapy, these beneficial microorganisms are ingested and thereby introduced to the intestinal microflora intentionally. This results in high numbers of beneficial bacteria to participate in competition for nutrients with and starving off harmful bacteria (Mombelli and Gismondo, 2000). The probiotics take part in a number of positive health promoting activities in human physiology (Chen and Yao, 2002).

The beneficial effects of the ingested probiotic bacteria are provided by those organisms that adhere to the intestinal epithelium (Salminen et al., 1998). The presence and adherence of probiotics to the mucous membrane of the intestines build up a strong natural biological barrier for many pathogenic bacteria (Chen and Yao, 2002). Adhesion is therefore regarded as the first step to colonization. Adhesion to the epithelium can be specific, involving adhesion of bacteria and receptor molecules on the epithelial cells, or non-specific, based on physicochemical factors. There are strains of lactic acid bacteria, both of human and dairy origin, which can effectively adhere to human cell lines (Salminen et al., 1998).

A specific microorganism has to fulfil a number of specific properties for it to be regarded as a probiotic strain. These properties are dependent on its specific purpose and on the location on which the specific property has to be expressed. The most important characteristic of probiotics include among others the following (Havenaar and Huis int'Veld, 1992):

- Survival in environmental conditions on the location where it must be active
- Proliferation and/or colonisation on the location where it is active
- No immune reaction against the probiotic strain i.e. the host must be immuno-tolerant to the probiotic

- No pathogenic, toxic, allergic, mutagenic or carcinogenic reaction by the probiotic strain itself, its fermentation products or its cell components after death of the bacteria
- Genetically stable, no plasmid transfer
- Easy and reproducible production

Lactobacilli were the first and largest group of microorganisms to be regarded as probiotics (Wolfson, 1999; Mombelli and Gismondo, 2000). They include *Lb. acidophilus*, *Lb. casei*, *Lb. delbrueckii* ssp. *bulgaricus*, *Lb. reuteri*, *Lb. brevis*, *Lb. cellobiosus*, *Lb. curvatus*, *Lb. fermentum*, and *Lb. planturum*, and Gram positive cocci such as *Lactococcus lactis* ssp. *cremoris*, *Streptococcus thermophilus*, *Enterococcus faecium*, *Streptococcus diacetylactis* and *Streptococcus intermedius*. (Krasaekoopt et al., 2003). Today probiotics include other lactic acid bacteria such as Bifidobacteria, Enterococci, *Leuconostoc* and Pediococci. Bifidobacteria occur naturally in the human intestines, with *Bifidobacterium infantis* being the first to inhabit the intestines of newborns. Other microbes used in probiotics but not related to lactic acid bacteria are yeasts (*Saccharomyces cereviciae*, *Saccharomyces boulardii*), filamentous fungi (*Aspergillus oryzae*) and some spore forming bacilli (Wolfson, 1999; Mombelli and Gismondo, 2000; Fuller, 2003)

Probiotic bacteria beneficially affect the individual by improving the properties of the indigenous microflora and its microintestinal balance (Frost and Sullivan, 2000; Saarela et al., 2000; Matilla-Sandholm et al., 2002; Betoret et al., 2003). They compete with disease causing bacteria for villi attachment sites and nutrients (Chen and Yao, 2002). Probiotic bacterial cultures encourage growth of beneficial microorganisms and crowd out potentially harmful bacteria thereby reinforcing the body's natural defence mechanisms (Saarela et al., 2000). They provide specific health benefits by modifying gut microflora, strengthening gut mucosal barrier, e.g. adherence of probiotics to the intestinal mucosa with capacity to prevent pathogen adherence, pathogen inactivation, modification of dietary proteins by intestinal microflora, modification of bacterial enzyme activity, and influence on gut mucosal permeability, and regulation of the immune system (Salminen et al., 1998; Betoret et al., 2003; Krasaekoopt et al., 2003).

Their probiotic effect is accredited to their production of metabolic by-products such as acid, hydrogen peroxide, bacteriocins, e.g. lactocidin, and acidophilin that manifest antibiotic properties and inhibit the growth of a wide spectrum of pathogens and/or potential pathogens such as *Escherichia coli*, *Klebsiella*, *Enterobacter*, *Pseudomonas*, *Salmonella*, *Serratia* and *Bacteroides* (Chen and Yao, 2002; Krasaekoopt et al., 2003). Lactic acid bacteria inhibit growth of pathogenic microorganisms by producing short chain fatty acids such as acetic, propionic, butyric as well as lactic and formic acids which reduces intestinal pH. Lactic acid produced by bifidobacteria in substantial amounts has antimicrobial activity against yeasts, moulds and bacteria (Percival, 1997; Adams and Moss, 2000). These species are also active in reducing the faecal activity of enzymes implicated in the production of genotoxic metabolites such as beta glucoronidase and glycolic acid hydroxilase (Collins and Hall, 1984; Mombelli and Gismondo, 2000). Probiotic organisms produce enzymes that help in digestion of proteins, fats and lactose (Frost and Sullivan, 2000). They also produce β-galactosidase, an enzyme that aid lactose intolerant individuals with breaking down or digestion of lactose (Krasaekoopt et al., 2003).

Commercially, viable probiotic strains are incorporated into fermented food products or are supplied as freeze-dried supplements or pharmaceutical preparations (Holzapfel and Schillinger, 2002). The basic requirement for probiotics is that products should contain sufficient numbers of microorganisms up to the expiry date (Fasoli et al., 2003). Thus, probiotics must contain specific strains and maintain certain numbers of live cells for them to produce health benefits in the host (Mattila-Sandholm et al., 2002). Different countries have decided on the minimum number of viable cells required in the probiotic product for it to be beneficial. In Australia, a minimum viable count of $10^6$ organisms per gram should be available in fermented milk products at the end of the shelf life (Wahlqvist, 2002). However, according to Krasaekoopt et al. (2003), there are no specifications as to how many probiotics should be available in Australian fermented products. The same minimum count ($10^6$ organisms per gram) was approved by countries of MERCOSUR which includes Argentina, Paraguay, Brazil and Uruguay

(Krasaekoopt et al., 2003). In products containing multiple probiotic organisms, at least a million of each of them per gram should be present to produce required beneficial effects (Wahlqvist, 2002). In Japan, a minimum of $10^7$ viable cells per millilitre of fresh dairy product is required. The South African legislation states that functional foods containing probiotic bacteria must deliver 1 x $10^8$ bacterial cells per day. A daily intake of $10^9$ to $10^{10}$ cfu viable cells is considered the minimum dose shown to have positive effects on host health (Fasoli et al., 2003). This could be achieved by consuming 100 g of a product containing between $10^6$ and $10^7$ viable cells $g^{-1}$ daily (Boylston et al., 2004).

Retention of viability presents a major marketing and technological challenge for application of probiotic cultures in functional foods (Desmond et al., 2002; Mattila-Sandholm et al., 2002). Many active cultures die during manufacturing, storage or transport of the finished product (Siuta-Cruce and Goulet, 2001) and also during the passage to the intestine (Sakai et al., 1987; Siuta-Cruce and Goulet, 2001; Park et al., 2002). Thus, the majority die even before the consumer receives any of the health benefits (Siuta-Cruce and Goulet, 2001). A serious problem of shelf instability had been encountered with dried cultures. Refrigerated products also have short lives due to negative effects of low temperature and formation of crystals on bacterial cells. The numbers of viable bacteria continually decrease with time during refrigerated storage (Porubcan et al., 1975). Market surveys have revealed much lower counts in the products even before the expiry date (Talwalkar et al., 2001). Shelf life for probiotics is thus unpredictable; hence, the industry has had difficulty backing up label claims. (Siuta-Cruce and Goulet, 2001). Excesses of 50 to 200 % cells have been incorporated into products in an attempt to make-up for cells that die during storage. For example, in tablets containing dry cells, where the tablets are labelled as containing a certain minimum count of active cells per tablet, to be safe, the manufacturer must incorporate an excess of cells at the time the tablets are manufactured, thereby assuring that the labelling will remain accurate while the product is in stock by the retailers. This practice increases the cost and makes the use instructions inaccurate (Porubcan et al., 1975).

Probiotics, after surviving food processing, are then exposed to conditions prevailing in the stomach and small intestine before they reach their site which is the colon (Siuta-Cruce and Goulet, 2001; Hansen et al., 2002; Lian et al., 2002). The microbes may die during their transit through the upper intestinal tract to the colon and therefore they may not be able to colonize the colon (Talwakar et al., 2001). They must therefore survive gastric acidity and bile salts which they encounter during their passage through the GI tract (Sakai et al., 1987; Siuta-Cruce and Goulet, 2001; Hansen et al., 2002; Lian et al., 2002). Their survival in the GI tract depends on the strain and species-specific resistance to low pH (pH values ranging from 1.3 to 3.0) in gastric juice and to bile salts found in the small intestine (Hansen et al., 2002; Lian et al., 2002).

Probiotic bacteria can only perform when they find adequate environmental conditions and when they are protected against stresses (e.g extreme temperatures, high pressure, shear forces) they encounter during their production at the industry level or in the gastrointestinal tract (gastric acids and bile salts) (Siuta-Cruce and Goulet, 2001). Factors affecting viability during storage such as temperature, moisture, light and air should also be taken into consideration (Percival, 1997; Mattila-Sandholm et al., 2002). Oxygen toxicity is another major problem in the survival of probiotic bacteria in dairy foods. High levels of oxygen in the product are detrimental to the availability of these anaerobic bacteria (Talwakar et al., 2001).

Manufacturers of probiotics are facing the challenge that they should produce probiotic cultures that can survive for long periods in the products, and are resistant to acidity in the upper intestinal tract so that they can reach the colon in high numbers to colonize the epithelium. Probiotic cultures should therefore be produced in a way that will protect these sensitive bacteria from unfavourable interactions with detrimental factors (Siuta-Cruce and Goulet, 2001).

## 1.2.1 Bifidobacteria

Bifidobacteria are Gram positive, fermentative, strictly anaerobic rods, often Y-shaped or clubbed at the ends (Bergy, 1974; Mombelli and Gismondo, 2000). They are mostly of human origin (Mombelli and Gismondo, 2000). The most direct and definitive criterion for assigning bacterial strains to genus *Bifidobacterium* is to demonstrate presence of fructose-6-phosphate in cellular extracts (Berg, 1974; Orban and Patterson, 2000). Freshly isolated strains appear either as uniform rods or branched rods with Y and V forms and club or spatulate. The morphology of bifidobacteria cells is influenced by nutritional conditions. They are non-acid fast, non-spore forming and non-motile. They are saccharoclastic, i.e. they produce lactic and acetic acids without generation of $CO_2$ (gas is not produced during fermentation). Glucose is primarily fermented to acetic acid and L (+) lactic acid in the molar ratio of 3:2. Bifidobacteria are generally anaerobic but they differ in their tolerance to oxygen in the presence of $CO_2$. The G + C content vary from 57.2 to 64.5 % (Bergy, 1974).

Bifidobacteria have probiotic properties and therefore stimulating their levels in the colon may result in enhancement of immune system functions, improvement of digestion and absorption of essential nutrients and the synthesis of vitamins (Theuer and Cool, 1998). They are used in different conditions such as diarrhoea, for immune stimulation, as antimutagens and anticholesterol agents. Enzymes produced by bifidobacteria assist in the deconjugation of bile acid, catabolism of carbohydrates and synthesis of vitamins. They are used *in vivo*, especially in children, to restore the immune defence. They are mainly administered in combination in food (Mombelli and Gismondo, 2000). Their anaerobic nature however causes handling to be troublesome, resulting in their death during dehydration and plating (Fasoli et al., 2003).

Some of the most common *Bifidobacterium* species incorporated into probiotic products are discussed briefly below:

1.2.1.1 *Bifidobacterium bifidum*

They are Gram-positive rods highly variable in appearance. Under anaerobic conditions they form circular, convex or lens shaped colonies, whitish but not transparent, with smooth to mucoid soft surfaces. They give a final pH of 4.0 to 4.8 when grown anaerobically in glucose broth. *B. bifidum* does not hydrolyse gelatin, does not produce hydrogen sulphide and cannot produce ammonia from arginine. They require organic nitrogen to grow in the presence of fermentable carbohydrate. Their optimum temperature for growth is between 36-38 $^{o}$C. They have variably limited growth at 23-25 $^{o}$C and cannot grow at or below 20 $^{o}$C or at 45 $^{o}$C. Their optimum initial pH is between 6 and 7, with little or no growth at pH 5.5 or less. Strains of human and animal origin are non-pathogenic. They are found in the alimentary tracts and stools of breast fed infants and adults (Bergy, 1974).

*B. bifidum* is predominant in the intestinal tracts of breastfed infants though it is also present in adults. It was hypothesized that the reduced susceptibility of breastfed infants to infection when compared to bottle-fed infants, was due to the presence of *B. bifidum* in breastfed infants. *B. bifidum* inhibits the growth of competing pathogenic bacteria such as *E. coli*, *Shigella* and *Salmonella typhi*, through production of organic acids, leading to an increased hydrogen ion concentration in the growth medium. It also helps in the breakdown of complex carbohydrates, fat and proteins during digestion. They produce depolymerising enzymes that break the larger molecules down into smaller components that the body can utilise efficiently. These organisms have a detoxifying effect. For example, when *B. bifidum* was administered to 20 liver disease patients, a reduction in blood ammonia, free serum phenol and free amino nitrogen in the treated patients, was observed. The investigators attributed the positive effects to the *Bifidobacterium bifidum* bacteria (Nutraceutix, 2001).

1.2.1.2 *Bifidobacterium longum*

*Bifidobacterium longum* cells are long, curved, club shaped, swollen or dumb-bell shaped rods which may be bifurcated.  Unlike *B. bifidum* which is Gram-positive, they are Gram-variable.  They form convex to pulvinate colonies that are soft, moist, shiny or slimy.  They ferment glucose to produce acetic acid and L(+)- lactic acid with no production of gas.  They are also anaerobic, grow at 36-38 $^{o}$C but not at 46.5 $^{o}$C and 20 $^{o}$C.  *B. longum* has been isolated from faeces of infants and adults, and from the intestine of rats, guinea pigs and calves.  It is present in adults throughout life (Boylston et al., 2004).

Beneficial effects of *B. longum* have been investigated in a number of clinical trials.  It was indicated through studies that *B. longum* helps in digestion, due to its ability to degrade complex carbohydrates and improve levels of by-products of digestion such as ammonia and beta-glucuronidase.  *B. longum* has also been used for prevention of antibiotic induced diarrhoea.  Its antioxidative capabilities have been also shown in recent studies.  *B. longum* cells and its cell extracts decreased levels of free radicals and increased inhibition of oxide cytotoxicity by 90 %.  They chelate metal ions, especially copper, and scavenge reactive oxygen species such as hydrogen peroxide (Nutraceutix, 2001).

1.2.1.3 *Bifidobacterium adolescentis*

They are short, curved, occasionally bifurcated and anaerobic rods.  They produce acetic and L(+)-lactic acid during glucose metabolism.  They have an inducible gluconate fermentation pathway in which acid and gas ($CO_2$) are produced.  They grow optimally at temperatures between 35-37 $^{o}$C, with no growth at 46.5 $^{o}$C and 20 $^{o}$C.  *B. adolescentis* have been isolated from faeces, appendix, dental carries and vagina of human adults and infants (Bergy, 1974).

1.2.1.4 *Bifidobacteriun infantis*

*B. infantis* cells are small, thin and spherical or bubble shaped, often containing central granules. Their growth conditions are similar to those of *B. bifidum* and *B. adolescentis*. They are predominant in the faeces of breast-fed infants (Bergy, 1974).

1.2.1.5 *Bifidobacterium breve*

Their cells are short, slender or thick, often club shaped rods, with or without bifurcation. Colonies are convex to pulvinate, smooth or undulating surface 2-3 cm in diameter, and have a soft consistency. They ferment glucose to produce acetic and L(+)-lactic acid. Gas is not produced during fermentation. They too, cannot grow at 46.5 $^{\circ}$C and 20 $^{\circ}$C. They have been isolated from infant faeces and from the vagina (Bergy, 1974).

**1.3 Prebiotics**

Prebiotics are non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon (Roberfroid, 1998; Theuer et al., 1998; Young, 1998; Femia et al., 2002). They change the intestinal microflora, favouring growth of potentially health-promoting bacteria, especially lactobacilli and bifidobacteria such that their numbers are predominant (Roberfroid, 1998). This subsequently lead to predominant numbers of stimulated endogenous bacteria in faeces as well (Femia et al., 2002; Losada and Olleros, 2002). They provide a beneficial effect through the selective stimulation of the growth or activity of a single species of bacteria which already resides in the colon, thereby improving a person's health. Prebiotics also modulate lipid metabolism through fermentation. The selective stimulation of growth of bifidobacteria by prebiotics is characterized by a substantial decrease in numbers of potentially pathogenic bacteria (Losada and Olleros, 2002).

Carbohydrates are used to promote growth of beneficial bacteria. These carbohydrates used for promotion of growth of lactic acid producing bacteria are called bifidogenic oligosaccharides (Farmer, 2002). Bifidogenic oligosaccharides include fructooligosaccharides (FOS) (inulin), glucooligosaccharides (GOS), other long chain oligosaccharides polymers of fructose and/or glucose and the trisaccharide, raffinose. They are not readily digested by pathogenic bacteria (Annika et al., 2002; Farmer, 2002; Femia et al., 2002).

Non-digestible carbohydrates are found in many fruits, vegetables (e.g. artichoke, garlic, leek and onion) and cereals (Femia et al., 2002; Losada and Olleros, 2002). They are a mixture of sugar chains formed by a glucose molecule and molecules of fructose joined together. Taking foods containing prebiotic oligosaccharides is not enough for modulation of gut flora as they are present in only small concentrations in these foods. Instead, prebiotics are extracted from these foods and transferred into more commonly ingested foodstuffs like biscuits and other carbohydrate based materials (Taylor et al., 1999). These natural compounds can also be manufactured economically using the β-D-fructofuranosidase enzyme or with fructosyltransferase, which joins the additional fructose molecules by means of transfructosylation mechanisms. Enzymes such as α-amylase, saccharase and maltase do not digest these oligosaccharides, especially in humans (Losada and Olleros, 2002).

This material reaches the colon, where it may be fermented completely or partially, or remain unfermented. None of the molecules of fructose and glucose that form inulin and oligofructose appear in portal blood. These materials are quantitatively fermented by colonic microflora. Fermentation of prebiotics by colonic microflora leads to the selective stimulation of the growth of bifidobacteria population (Flamm et al., 2001). In the GI tract bifidogenic oligosaccharides are metabolized exclusively by the indigenous bifidobacteria and lactobacillus and not by detrimental microorganisms such as *Clostridia*, *Staphylococcus*, *Salmonella* and *Escherichia coli.* The use of bifidogenic oligosaccharides together with lactic acid bacteria allows these beneficial, probiotic bacteria to grow and then out-compete any undesirable, pathogenic microorganisms

within the GI tract. Bifidogenic oligosaccharides increase the level of nutrient supplementation and enhance nutrient solubility (Farmer, 2002). Prebiotics unlike probiotics, are not living organisms, and therefore they do not have survival problems both in the products and the gut (Frost and Sullivan, 2000).

## 1. 3.1 Non digestible oligosaccharides (NDO's)

Oligosaccharides are available on the market as crystalline powders or 75% (w/v) syrups. The choice of oligosaccharides is affected by several factors such as regulatory considerations, the effects on the food's physiochemical properties and the stability of the oligosaccharide at differing pH and temperatures which may lead to hydrolysis of the NDO. Other factors that to be taken into consideration are the effects of the NDO on the human physiology, health claims that can be made for different NDO's as well as the cost of the NDO. NDOs have a number of physiological effects in humans. They are used as fat replacers because they are low in calories. They have low cariogenicity preventing erosion of teeth and bone, and they act as a form of dietary fibre. However their excess levels can cause symptoms such as flatulence, bloating and diarrhoea. This may be caused by a change in osmotic potential or due to excessive fermentation. Undesirable effects only occur when very high doses of NDOs are ingested. This is advantageous as it allows a relatively broad "therapeutic window", i.e. the dose above the minimal effective level (Holzapfel and Schillinger, 2002).

## 1. 3.2 Fructooligosaccharides(FOS)

FOS are oligosaccharides composed of a molecule of glucose and one to three molecules of fructose. They are polymers of β-D-fructosyl units having short length chains with a degree of polymerisation of up to 9 (oligofructose) to medium length chains with a degree of polymerisation of up to 60 (inulin). They occur naturally in many kinds of plants such as onions, asparagus roots, tubers of Jerusalem artichoke and wheat, but also in banana, beer, burdock, Chinese chives, garlic, graminae (fodder grass), honey, oat, pine, rye, chicory, stone leak and even bacteria and yeast (Ziemer and Gibson, 1998;

Bengmark et al., 2001). Short chain FOS are a mixture of oligosaccharides consisting of glucose linked to fructose units by β (1-2)- glycosidic bonds. They are not efficiently digested in the human small intestine but are fermented in the colon by colonic microflora (Bouhnik et al., 1999). Their monomers are joined by specific linkages that resist breakage by mammalian enzymes (Ziemer and Gibson, 1998). Most species of bifidobacteria are among the limited range of microorganisms able to ferment FOS. Bifidobacteria have relatively high activity of β-fructosidase that is selective for β(1-2)-glycosidic bonds present in these oligosaccharides. Fructose, a product of FOS hydrolysis, serves as an efficient growth substrate for the bifidobacteria pathway of hexose fermentation, which is almost exclusively carried out by bifidobacteria (Bouhnik et al., 1999). The presence of oligofructose and its fermentation products *in vitro* result in an increase in the number and metabolic activity of bifidobacteria (Theuer and Cool, 1998). Reports have indicated their beneficial effects on serum cholesterol and triglyceride levels, and blood pressure in elderly patients with hyperlipidemia (Bengmark et al., 2001).

FOS represents a selective nutrient for beneficial microorganisms and therefore has the potential to increase the effectiveness of current probiotic products. Scientific studies in Japan indicated that consumption of FOS shifts the balance of microflora in the intestine towards greater populations of bifidobacteria and other beneficial microorganisms even in the absence of probiotics in diet (Losada and Olleros, 2002).

They are produced commercially using two different manufacturing techniques that produce slightly different end products. During the first method FOS are enzymatically synthesised from sucrose using transfructosylases and in the second method inulin is derived from chicory. The polysaccharide is then subjected to a controlled enzyme hydrolysis (Frost and Sullivan, 2000). The two FOS produced are of similar structure and approximately equal size and hence there is a little difference in their prebiotic functionality. Studies involving humans and animals have established the effectiveness and safety of FOS. They have no genotoxic, carcinogenic or toxicological effects. FOS not only provide health benefits, they also contribute texture and body to the foods that

contain them (Frost and Sullivan, 2000). However, they are slightly laxative and produce flatulence when taken in high doses (Losada and Olleros, 2002).

## 1. 3.3 Galactooligosaccharides (GOS)

They are synthesised from lactose syrup using the enzyme β-galactosidase (Frost and Sullivan, 2000; Gibson, 2004). GOS are neither hydrolysed nor absorbed in the human intestine and act as a substrate for bifidobacteria (Frost and Sullivan, 2000).

## 1. 3.4 Soy oligosaccharide

They are extracted directly from soybean whey. Bifidogenecity of soy oligosaccharides has been confirmed in humans (Frost and Sullivan, 2000).

## 1. 3.5 Cereals

Cereal, nuts, legumes and oil seeds are sources of dietary fibre. Phytic acid is a hexaphosphorylated sugar and constitutes up to 1-5 % weight of these foods. Phytic acid chelates iron thereby inhibiting production of reactive oxygen species such as the hydroxyl radical. This may account for some of the protective effects of dietary fibre (Taylor et al., 1999). Cereals can also be used as fermentable substrates for the growth of probiotic organisms (Charalampopoulos et al., 2002). Fermentation of dietary fibre by colonic bacteria leads to the production of short chain fatty acids (Taylor et al., 1999; Ridlon et al., 2006).

Butyrate is one of the short chain fatty acids produced by fermentation of dietary fibre. *In vitro* tests and animal models have shown that butyrate increases the proliferation and differentiation of colonic cells. Apoptosis (programmed cell death) in human colonic tumour cell lines was also shown *in vitro* to be induced by butyrate (Taylor et al., 1999). Carcinogens such as nitrosamines are neutralized by butyric acid produced by some probiotic bacteria (Kailasapathy and Chin, 2000). The fermentation of fibre to short

chain fatty acids by colonic bacteria lowers the gut pH which may then reduce the conversion of primary bile acids to more toxic secondary bile acids (Taylor et al., 1999). Primary bile salts stimulate digestion and absorption of lipids and lipid-soluble vitamins in the intestines. However, when transformed to secondary bile salts they cause GI diseases such as colon cancer and gallstones (Ridlon et al., 2006).

Additionally, they can be used as sources of non-digestible carbohydrates that promote several beneficial physiological effects and also selectively stimulate the growth of lactobacilli and bifidobacteria present in the colon, thus acting as prebiotics. They contain water-soluble fibre, such as glucan and arabinoxylan, oligosaccharides, such as GOS and FOS and resistant starch, all of which have prebiotic properties. Starch can also be used as a coat for encapsulating probiotics in order to improve their stability during storage and enhance their viability during their passage through the adverse conditions of the GI tract (Charalampopoulos et al., 2002).

## 1. 4 Synbiotics

Synbiotics are products that contain both a probiotic and a prebiotic (Holzapfel and Schillinger, 2002; Touhy et al., 2003). They are defined as a mixture of a probiotic and a prebiotic that beneficially affects the host by improving the survival and establishment of live microbial dietary supplements in the GI tract, by selectively stimulating the growth and/or by activating the metabolism of one or a limited number of health promoting bacteria and thus improving the host welfare (Kailasapathy and Chin, 2000; Touhy et al., 2003). Thus, synbiotics could improve the survival of the probiotic organism by providing specific substrate to the probiotic organism for its fermentation (Gallaher and Khil, 1999). Synbiotic supplements available include combinations of bifidobacteria and FOS, *Lactobacillus* GG and inulin and bifidobacteria, lactobacilli and FOS or inulin. Fermented milks contain both live beneficial bacteria (probiotics) and fermentation products that may positively stimulate the intestinal microflora (prebiotics). They are therefore referred to as synbiotics (http://www.invista.com/health/nutrition/biotics/synbiot.htm). Research has indicated the

potential of fermented milks in alleviating the risk of diseases such as colon cancer (Kieβling et al., 2002; Saikali et al., 2004).  Synbiotics have antimicrobial, anticarcinogenic, immunomodulatory, antidiarrhoeal, antiallergenic, hypolipidemic, and hypoglycaemic activities.  They may also have activity in improving mineral absorption and balance and may have antiosteoporotic activity (Gallaher and Khil, 1999; http://www.invista.com/health/nutrition/biotics/synbiot.htm).

## 1. 5 Application of probiotics in gastrointestinal dysfunctions associated with gut microflora imbalance

### 1. 5.1 Lactose indigestion

Lactose intolerance is the inability to hydrolyze lactose caused by lack of the enzyme β-galactosidase.  This enzyme is found in large quantities at the tip of the villi of the small intestines (Salminen et al., 1998a; Vesa, 2000).  It hydrolyzes lactose into glucose and galactose which are absorbed in the small intestine (Adams and Moss, 2000).  Lactose intolerant individuals cannot digest a lot of lactose but only small amounts are metabolized by intestinal microbes (Salminen et al., 1998a).  The undigested lactose passes to the colon where it is attacked by lactose fermenting organisms residing in the colon (Adams and Moss, 2000).  Fermentation of lactose in the large intestine leads to production of hydrogen in breath (Mombelli and Gismondo, 2000).  Incompletely absorbed lactose causes watery diarrhoea and large amounts of water may lead to dysfunctions of intestinal microflora (Salminen et al., 1998a).

Several strains of probiotics alleviate symptoms of lactose intolerance by providing lactase (β-galactosidase) to the intestine and stomach where lactose is degraded (Dairy Council of California, 2003).  Probiotic strains produce β-galactosidase which breaks down lactose thereby improving tolerance to lactose (Fooks et al., 1999).  Hydrogen in breath is used for diagnosis of lactose maldigestion (Vesa et al., 2000) or as an indicator of bacterial metabolism of lactose (Mombelli and Gismondo, 2000) whereby the amount of hydrogen in breath is equal to that of lactose not digested (Vesa et al., 2000).  It has

been shown that hydrogen production is lower in subjects treated with fermented milk than in subjects with non-fermented milk. The low hydrogen content in those consuming fermented milk indicated that most of their lactose was metabolised (Mombelli and Gismondo, 2000).

## 1. 5.2 Constipation

Constipation is a disorder of motor activity of the large bowel characterized by bowel movements that are less frequent than normal (Salminen et al., 1998b). It is mainly caused by inappropriate diets, such as those low in fibre or non-starch polysaccharides. The symptoms of constipation are pain during defecation, abnormal swelling and incomplete emptying of colon contents (Salminen et al., 1998a). Total gut transit time in constipated individuals is generally longer than in non-constipated individuals, and stools are hard and difficult to pass (Salminen et al., 1998b). The link between intestinal microflora and constipation suggests that probiotics may be used for treatment and prevention of constipation.

In a study by Ouwenhand et al. (2002), twenty-eight constipated elderly subjects were enrolled in an open parallel study. The subjects were divided into three groups receiving juice, juice supplemented with *Lactobacillus reuteri* and the other supplemented with *Lactobacillus rhamnosus* and *Propionibacterium freudenreichii*. The results showed a 24 % increase in defecation frequency in subjects receiving the probiotic supplemented juice. Probiotics were then suggested to improve intestinal motility and reduce fecal enzyme activity.

## 1. 5.3 Antibiotic associated and rotaviral diarrhoea

Antibiotics cause diarrhoea in about 20 % of patients taking them. This is because antibiotics are non-selective, killing both pathogenic microbes and beneficial GI tract microflora (Tuohy et al., 2003). The antibiotic treatment shifts the equilibrium of the normal gut flora in favour of detrimental microorganisms (BergogneBérézin, 2000).

Reduction of beneficial microorganisms disturbs processes such as fermentation (BergogneBérézin, 2000) and compromises colonization resistance (BergogneBérézin, 2000; Tuohy et al., 2003), favouring growth of pathogens like *Clostridium difficile* and *Klebsiella oxytoca* (Tuohy et al., 2003). AAD results in poor digestion of carbohydrates, metabolized by colonic bacteria as an energy source. The normal production of lactic acid and short chain fatty acids (acetate, butyrate, and propionate) by anaerobic flora is decreased. Viable microorganisms (probiotics) can be used instead of conventional antibiotics to control GI disorders since they can restore the indigenous microflora (BergogneBérézin, 2000). Probiotics are seen as an important tool for improving health and nutrition of people in developing countries (Goldin, 1998).

AAD is usually caused by *Clostridium difficile*. Administration of probiotic *L. rhamnosus* GG either as powder or as a fermented product to children aged 4-45 months showed a significant reduction in duration of the diarrhoea. In a separate trial, administration of *Bifidobacterium* spp. and *Streptococcus salivarius* subsp. *thermophilus* as probiotic treatment resulted in reduction of the incidence of diarrhoea in the probiotic group after 17 months when compared to control group without treatment (Fuller, 2003). Recently, Kotowska et al. (2005) showed that *Saccharomyces boulardii* reduces the risk of AAD in children.

The common cause of diarrhoea in children is rotavirus infection. The intestinal mucosa is disturbed by infection, resulting in loss of microvilli and a decrease in the villus crypt ratio. Gut permeability increases, causing an increase in the absorption of macromolecules (Salminen et al., 1998a). Administration of *Lactobacillus rhamnosus* GG (LGG) resulted in significant reduction in the incidence of rotaviral diarrhoea compared with standard pasteurised yoghurt or placebo in paediatric populations. Additionally, a study of traveller's diarrhoea among 245 subjects who travelled to a developing nation for 1-3 weeks showed that the risk of developing diarrhoea on any given travel day was 7.4 % in the placebo group compared with 3.9 % in the LGG group. LGG provided a protection rate of 47 % against traveller's diarrhoea (Goldin, 1998; Gorbach, 2000).

Treatment and prevention of AAD by use of probiotics is one the most researched fields of probiotics in clinical applications and results thereof have been documented (BergogneBérézin, 2000; Marteau et al., 2001, McNaught and MacFie, 2001; Cremonini et al., 2002). The findings reported in these studies demonstrate that probiotics are effective in treating AAD in adults and rotaviral diarrhoeal disease in young children.

## 1. 5.4 Crohn's disease

Crohn's disease is an inflammatory disease of the GI tract occurring most commonly in the small intestine and the colon. It is caused by immunological disturbances, influence of agents such as bacteria or viruses that enter the lumen and activate the immune system, or inheritance. Crohn's disease is associated with destruction of the barrier function (Salminen et al., 1998a). Probiotics have been used in trials to treat this disease. *Lactobacillus* GG was administered orally for 10 d to sufferers of the disease. There was an increase in Immunoglobulin A (IgA) specific antibody secreting cells (sASC) to dietary B-lactoglobulin and casein. IgA is an antibody found in external secretions like saliva, tears, bile, urine and nasal, tracheobronchial, intestinal and cervical fluids. It is used to characterize the immunoglobulin patterns of these secretions. The potential of probiotic bacteria to increase gut IgA and thereby promote the gut immunological barrier has been indicated (Salminen et al., 1998b). IgA plays a very important function in local immunity and in creating a barrier against bacterial and viral infections (Fukushima et al., 1998).

**1.5.5 Other application of probiotics**

1.5.5.1 Food allergy

Food allergy is an immunologically mediated adverse reaction against dietary antigens. It is prevalent in infants because of the immaturity of their immune systems and the gastrointestinal barrier (Salminen et al., 1998a). An example of food allergy is that of infants with allergic reactions to milk due to casein. Some lactobacilli degrade casein into smaller peptides and amino acids. Studies showed that hydrolysis of different casein proteins by *Lactobacillus* GG decreased proliferation of mitogen induced human lymphocytes compared to non-treated caseins. Probiotics may exert a beneficial effect on allergic reaction by improving mucosal barrier function (Dairy Council of California, 2003). The supplementation of *Lactobacillus* GG to infants with atopic eczema after elimination of cow milk from their diet showed a significant reduction in the duration and intensity of atopic dermatitis. This may be due to reductions in intestinal inflammation and hypersensitivity reaction (Mombelli and Gismondo, 2000).

1.5.5.2 Atopic dermatitis

Atopic dermatitis is a common chronologically relapsing skin disorder affecting infants and children (Salminen et al., 1998a). It affects people who come from families with hay fever and asthma (hereditary). Individuals suffering from the disease develop rash on the skin and in addition have thickened itchy skin on the front of elbows, back of knees and on cheeks. Isolauri et al. (2000) investigated the effect of probiotics in infants who showed symptoms of atopic dermatitis during exclusive breastfeeding (i.e. no infant formula was given to these infants). The SCORAD score which measures the extent and severity of the disease was 16 in these infants. The infants were divided into groups and weaned to whey formula containing either *Bifidobacterium lactis* Bb-12 or *Lactobacillus* LGG. Skin conditions of infants receiving probiotic supplemented formula were improved when compared to infants receiving formula without probiotic cultures. The

SCORAD score was reduced to 0 in the bifidobacteria group and to 1 in the lactobacillus group.

The effect of *Lactobacillus* GG on atopic dermatitis was investigated in another study by Kalliomaki et al. (2001). *Lactobacillus* GG was given to expectant mothers whose relatives suffered from the disease and to their infants for 6 months after birth. Atopic eczema occurred twice in infants who together with their mothers received a placebo than in those receiving the probiotic.

Atopic dermatitis in pregnant and nursing mothers was prevented by consumption of probiotics. Children with this disease have high levels of allergic IgE antibodies. These levels were reduced in breast-fed infants whose mothers were fed *Lactobacillus*. Incidences of the diseases were fewer in these infants than in those whose mothers received a placebo. Atopic dermatitis occurred three times more commonly in the later group than in infants whose mothers received a probiotic (Mirkin, 2002). Reduction in the duration and severity of the disease may be due to both reduced intestinal inflammation and hypersensitivity reaction (Mombelli and Gismondo, 2000). The results of all the above mentioned studies indicated that probiotics may be used for the prevention of atopic disease.

1.5.5.3 Cholesterol and heart disease

Probiotics have the ability to lower levels of cholesterol in serum, contributing to the prevention of cardiovascular disease. In one study, men with high serum cholesterol levels were given a drink containing live lactobacilli and the control group was given a drink with no live lactobacilli. There was a 7.3 % reduction in total cholesterol and a 9.6 % fall in low-density lipoprotein (LDL) cholesterol in patients who received lactobacilli. No change in blood lipids, glucose or fibrinogen was observed in the control group (Proviva, 2002). LDL carries cholesterol to various tissues throughout the body. It is also referred to as "bad" cholesterol because high levels of LDL correlate most directly with coronary heart disease. Thus, the lower the levels of LDL in blood, the lower the

risk of heart disease or stroke. High density lipoprotein (HDL) carries excess cholesterol and probably other phospholipids and proteins, to the liver for "repackaging" or excretion in the bile. Higher levels of HDL are protective against coronary artery disease and as such HDL is referred to as "good" cholesterol.

Oral administration of *Lactobacillus johnsonii* and *L. reuterii* decreased serum cholesterol in pigs and rats (Mombelli and Gismondo, 2000). Probiotics probably interfere with cholesterol or produce metabolites that affect the system levels of blood lipids (Fooks et al., 1999). For example, reduction in cholesterol levels may be attributed to deconjugation of bile salts by hydrolase, an enzyme produced by some lactobacilli strains (Mombelli and Gismondo, 2000).

1.5.5.4 Cancer

Probiotics have powerful anticarcinogenic features that are active against certain tumors (Chen and Yao, 2002). Several studies indicated that probiotics in diet reduce the risk of cancer (Sanders, 1999). Clinical trials in humans showed anticarcinogenic effects of *Bifidobacteriun bifidum* and *Lactobacillus acidophilus* (Fooks, 1999). Production of short chain fatty acids in the colon during fermentation by colonic microflora is the main process that prevents colorectal cancer (Holzapfel and Schillinger, 2002). Probiotic strains also reduce levels of some colonic enzymes such as glucoronidase, β-glucoronidase nitroreductase, azoreductase (Fooks et al., 1999; Adams and Moss, 2000; Gorbach, 2000; Chen and Yao, 2002) and glycoholic acid hydrolase (Chen and Yao, 2002). These enzymes convert procarcinogens to carcinogens such as nitrosamine or secondary bile acids (Chen and Yao, 2002). Low levels of these enzymes therefore decrease chances of cancer development in the colon (Kasper, 1998; Gorbach, 2000).

A synbiotic containing *B. longum* and inulin was shown to reduce risks related to tumors. Animal models also indicated that lyophilised *B. longum* suppressed the development of azoxymethane induced aberrant cryptic foci formation in rat tumours. How exactly

probiotics produce antitumor action is unknown but a few possible mechanisms in addition to those mentioned above, were proposed (Fooks et al., 1999):

- reducing intestinal pH, thereby altering microflora activity

- altering colonic transit time to remove faecal mutagens more effectively

- stimulation of the immune system

## 1. 6 Shelf life stability of probiotics

A number of studies have indicated that probiotic cultures do not survive satisfactorily in most products. Nevertheless, some researchers and companies claim that their products are stable and have acceptable shelf lives. A few of these are described.

Universal Preservation Technologies have preserved probiotic cultures such as *Lactobacillus acidophilus*, *Lactococcus lactis* and *Bordetella*, at temperatures above 50 $^o$C. Bacteria are preserved using specially developed fermentation and drying protocols and protectants in a process termed Vitrilife preservation. In this process bacteria are reported to be produced as a dry product that can be reconstituted with an aqueous solution. It is also reported that bacteria preserved using this process can survive at 37$^o$C for 30 d with no loss of viable cells (Universal Technologies, 2000).

Achour et al. (2001) preserved *Lactococcus* starter cultures by freeze-drying. Cells are harvested by centrifugation and then washed with a saline solution. The sample is supplemented with $CaCO_3$, then glycerol or 5% saccharose. The product has a half life of 7 d at 25 $^o$C and 43 d at 4 $^o$C. Worthington et al. (2001) claim a shelf life of 6 -12 months for flavoured fruit yoghurt. The product is stabilized by adjusting the pH to optimum for cultures used and then frozen in the presence of gas and then blended with a low moisture food. The frozen mixture is then extruded into a bar or other intended form, freeze-dried and then packaged.

Kafanani and Mize, (2002) produced a milk composition containing probiotics in amounts sufficient to benefit the consumer's health. The composition is ultrapasteurized, cooled to about 20-30 $^{o}$C and inoculated with a probiotic culture that has been prepared under aseptic conditions. The resulting milk composition is ready to use, has an extended shelf life and contains sufficient probiotics (at least $10^8$ cfu/ml) to be beneficial to the consumer even after more than 90 days. The milk is filled into containers which are sealed with a sterile closure under aseptic conditions. The containers are flushed under aseptic conditions with sterile gas, typically nitrogen to remove oxygen from the container just before sealing.

Nutraceutix uses technology called cryotabletting. The process employs liquid nitrogen to reduce heat during tabletting, resulting in significantly less loss and a more potent tablet. The process involves every step of the manufacturing process, including careful strain selection, and step-by-step monitoring of the starter culture growth, fermentation, freeze-drying, blending, tabletting and bottling process. LiveBac $^{TM}$ process produces probiotic tablets that do not require refrigeration and that are stable at room temperature for more than a year. (Nutraceutix, 2002).

Though longer shelf lives have been claimed, surveys on probiotic products still reveal that stability of cultures is a problem. As such, researchers are using different approaches to develop methods that will successfully preserve viability of probiotic cultures.

## 1.7 Moving towards improving shelf life of probiotics

### 1.7.1 Cell immobilisation

Immobilization is the physical entrapment of microbial cells in or on a polymer matrix. Although immobilized cells are separated from the medium containing substrates and products, exchange of substrates, products and inhibitors between the two still occurs. The microenvironment within which the immobilized cells exist differs from that of free cells (Ramakrishna and Prakasham, 1999).

When cells are immobilized inside a matrix, the matrix provides protection for cells against harsh environmental conditions such as pH, temperature, organic solvents, water molecules and poison (Bryers, 1990; Park and Chang, 2000. Productivity of lactic acid bacteria during fermentation may be improved through this process. When cells are artificially immobilized they do not grow, and this is advantageous as it minimizes the chances of contamination normally associated with growing cells (Bryers, 1990).

Methods of cell immobilization include cross-linking, entrapment (Tanaka and Kawamoto, 1999, Ramakrishna and Prakasham, 1999), adsorption, covalent binding and encapsulation (Ramakrishna and Prakasham, 1999). These methods are classified based on the mode of attachment of cells which can be mechanical, chemical or ionic. Mechanically immobilized cells are localized by means of physical barriers, chemically immobilized cells by covalent bonds formed among cells or between cells and the solid phase. Electrostatic and van der Waals or London forces are used in ionic immobilization (Phillips and Poon, 1988). Each method has its own advantages and disadvantages.

1.7.1.1 Entrapment method

This is the most commonly used method for immobilization of cells. It involves incorporation of the cells within a network of a polymeric material such as carbohydrate, protein, and organic or inorganic synthetic polymers (Phillips and Poon, 1988). Cells are entrapped using cellulose, its derivatives, gel-like extractions from seaweeds such as agar, alginate and carrageenan, and pectin from skins of citrus fruits (Phillips and Poon, 1988; Picot and Lacroix, 2004). The entrapped cells remain stable as the polysaccharides used are similar to the physical environment found in microbial cells (Phillips and Poon, 1988).

Cellulose and its derivatives are insoluble in water, but dissolve in polar aprotic organic solvents such as dimethyl formamide, acetone, and dimethylsulphoxide. Therefore when microbial cells are entrapped using cellulose, they are first added to a solution containing

an aprotic solvent. The solution is then passed into water where it is drawn as fibres or formed into beads and membranes containing the immobilized microorganism. κ-carrageenan is used in conjunction with a hardening agent gluteraldehyde, giving high carrier stability and long microbial half-lives. κ-carrageenan provide favourable conditions for viable microorganisms. Polyacrylamide is the most commonly used synthetic polymer. It is soluble in water and is normally cross-linked to the co-polymer N, N'-methylenebisacrylamide to produce a polymer with lattice like structure better suited to cell immobilization. (Phillips and Poon, 1988).

However, this method has a number of disadvantages. Alginate beads are sensitive to acid and it was reported that they shrink and lose mechanical strength during lactic fermentation. The formation of κ- carrageenan beads requires potassium ions, which could damage bacterial cells during fermentation. Potassium ions are involved in the maintenance of electrolyte balance in body fluids and should therefore not be taken in large amounts in diet (Sun and Griffiths, 2000). Agar is unstable towards high temperature and calcium alginate is unstable in the presence of chelating agents such as phosphate salts (Phillips and Poon, 1988). The technologies for production of gel beads present serious problems for large-scale production such as low production capacity, large bead diameters for the droplet extrusion methods, large size distribution for the emulsion techniques and transfer from organic solvents. Also in some countries addition of these polysaccharides to fermented milks is not allowed (Picot and Lacroix, 2004).

1.7.1.2 Covalent attachment

In this method, covalent bonds are formed between cells and the polymer lattice, or among the cells themselves to form a mat. There is contact between cells and chemical reagents and this normally leads to death of cells (Phillips and Poon, 1988). Loss of viability of immobilized cells is a disadvantage especially when live cells are needed.

1.7.1.3 Ionic attachment

It involves formation of electrostatic forces such as hydrogen bonding, coordinate binding, Van der Waals and dispersion forces.  Microbial cells can be immobilized by ionic attachment either by flocculation or adsorption.  The most commonly method used is flocculation which is usually used in fixed bed reactors (Phillips and Poon, 1988).

**1.7.2 Microencapsulation**

Microencapsulation is a process whereby sensitive actives such as microbial cells are enclosed within a protective coat (Vasishtha, 2003).  Encapsulation reduces cell loss by separating bacterial cells from the adverse environment (Sultana et al., 2000).  The protective coat reduces cell loss and injury by blocking reactive components such as atmospheric moisture, oxygen and acids (Kim et al., 1988; Reid, 2002; Krasaekoopt et al., 2003; Vasishtha, 2003,).  The coat also protects cells from high temperature and pressure, attack by bacteriophages, negative effects of freezing and freeze-drying (Krasaekoopt et al., 2003).  The sensitive active or the core material can be retained within a coat until it is released at a particular targeted location (Finch, 1993; Vasishtha, 2003).  Targeted release in case of probiotics can be obtained by use of coating materials made from sugars that do not allow hydration until the probiotics reach the alkaline pH of the colon (Reid, 2002; Krasaekoopt et al., 2003).  Encapsulation allows cells to tolerate acidity better (Siuta-Cruise and Goulet, 2001).  This process can increase shelf life of cultures by slowing down the rate of viability loss at room temperature (Kim et al., 1988) and of food products by alleviating problems encountered during processing (Finch, 1993; Vasishtha, 2003).  Through this process, manufacturing of new products, protection of the environment from poisonous products and masking of unpleasant tastes of some nutrients, are made possible (Finch, 1993; Vasishtha, 2003).

It has been found that lactic acid bacteria enclosed within solid fat microcapsules retain all their activity or vitality (Krasaekoopt et al., 2003).  The technique has been applied to strains of probiotics used in food applications including *Lactobacillus acidophilus*,

*Lactobacillus rhamnosus* and *Bifidobacterium longum*. *Pediococcus acidilactici* and *Enterococcus faecium* were incorporated in animal feeds (Siuta-Cruce and Goulet, 2001).

It is difficult to commercialize encapsulated product as only coats made from FDA-approved GRAS (generally recognized as safe) materials will be accepted. The GRAS shell material must stabilize the core material, must not react negatively with the active ingredient rendering it inactive, and should release at the target site. The production process must be able to produce a stable product with desired morphology, and large scale production must be cost effective (Vasishtha, 2003).

Different methods of encapsulation are classified either as physical or chemical (Versic, 1988; Vasishtha, 2003). Physical methods include use of commercially available equipment to create and stabilize the capsules (Vasishtha, 2003). Examples include spray coating, annular jet, spinning disk, spray cooling, spray drying and spray chilling (Versic, 1988), extrusion coating, fluidized bed coating, liposome entrapment, coarcervation, inclusion complexation, centrifugal extrusion and rotational suspension separation (Vasishtha, 2003). Chemical methods are water-in-oil and oil-in-water (complex coacervation) preparations (Versic, 1988). They apply ionic chemistry to create the microspheres in batch reactors (Vasishtha, 2003). Two methods of encapsulation commonly used are the extrusion technique and emulsion technique (Krasaekoopt et al., 2003). The release of the core from the coat can be either site specific, stage specific or signalled by changes in pH, temperature, irradiation or osmotic shock (Gibbs et al., 1999; Vasishtha, 2003).

1.7.2.1 Extrusion

In this technique, a hydrocolloid solution is prepared, microorganisms are added to it to form a suspension, and then the suspension is extruded as droplets through a syringe needle into a hardening solution or setting bath. The size of the beads depends on the diameter of the needle, and the concentration and viscosity of sodium alginate, while the shape depends on the distance of free fall from the needle into the hardening solution.

The supporting material commonly used is alginate, extracted from various species of algae. To form beads, a cell suspension is mixed with a sodium solution, the mixture dripped into a solution containing a multivalent cation. The multivalent cation used is usually $Ca^{2+}$ in the form of $CaCl_2$. The droplets form gel spheres which entrap the cells in a network of ionically cross-linked alginate. The size of beads decreases with an increase in the concentration and viscosity of sodium alginate (Krasaekoopt et al., 2003).

1.7.2.2 Emulsion

In this technique a small volume of cell polymer suspension is mixed with a large volume of vegetable oil e.g. soybean oil, sunflower oil, canola oil or corn oil. The mixture is then homogenized to form a water-in-oil emulsion. Once the emulsion is formed, the water-soluble polymer is cross-linked to form tiny gel particles within the oil phase. A $CaCl_2$ solution is then added to the homogenized mixture to break the emulsion and form a gel. The beads are harvested by filtration. The size of beads is controlled by the speed of agitation, and can vary between 25 μm and 2 mm. This technique had been used to encapsulate lactic acid bacteria for both batch and continuous fermentation. A number of supporting materials can be used. They include a mixture of κ-carrageenan and locust bean gum, cellulose acetate phthalate, alginate, chitosan and gelatin (Krasaekoopt et al., 2003). The disadvantage of emulsion the technique is that it is difficult to produce large quantities of beads and to remove oil from them (Stormo and Crawford, 1992).

The advantages and disadvantages of extrusion and emulsion encapsulation techniques are tabulated in Table 1.1. (Krasaekoopt et al., 2003).

**Table 1.1:** Positive and negative features of extrusion and emulsion techniques

|  | Extrusion | Emulsion |
| --- | --- | --- |
| Technological feasibility | Difficult to scale up | Easy to scale up |
| Cost | Low | High |
| Simplicity | High | Low |
| Survival of microorganisms | 80 to 95% | 80 to 95% |
| Size of beads | 2 to 5 mm | 25 µm to 2mm |

The main disadvantage of these methods is use of water and other solvents. The sensitive active, specifically probiotics, need protection from moisture as it is unfavourable for their survival. Also, use of solvents is no longer favoured due to concerns with their impact on the environment, and due to their high costs (Sihvonen et al., 1999). Therefore, a method that will exclude use of water and solvents will be more favourable, both to the encapsulated active and the environment.

1.7.2.3 Spray drying

Spray drying is a process in which an aqueous solution containing the sensitive active core material and solution of the wall material are atomised into hot air (Finch, 1993; Reineccius, 1988). The process involves three basic steps: preparation of a dispersion or emulsion to be processed; homogenization of the dispersion; and atomization and introduction of the mass into the drying chamber under controlled temperature and inflow conditions (Niro Inc, 2004). Products of spray-drying can be in powder, granulate or agglomerate form. Heat sensitive foods and pharmaceuticals are among products dried using this method (Rattes and Oliveira, 2004).

Spray drying has a number of advantages. Equipment used is readily available, the process is relatively cheap, it is compatible with a variety of carrier materials, large quantities of microcapsules can be produced, volatile substances can be easily retained and its products are stable (Reineccius, 1988; Picot and Lacroix, 2004). Like many other methods, it has problems too. Its main disadvantage is loss of viability of cells. Reduction in cell viability after spray drying was suggested to be a result of shear by atomizing air pressure, heating inside the atomizer, dehydration and thermal inactivation. These problems limit the application of spray-drying specifically for encapsulation of sensitive probiotic bacteria such as *Bifidobacterium* spp. Reduction in viability can be lessened by lowering the outlet air temperature which is the main cause of cell death. However, this approach cannot be applied to small spray dryers which are unable to achieve complete and satisfactory drying of suspensions at outlet air temperatures below $80^{o}$C (Picot and Lacroix, 2004).

**1.7.3 Freeze drying of probiotics**

Freeze-drying involves the removal of water from frozen cell suspension by sublimation under reduced pressure (Malik, 1990). Sublimation is the process whereby water is removed as water vapour directly from ice, without passing through the liquid state (Klamathbluegreen, 2003). Freeze drying is well suited for preservation of sensitive biological material because freezing slows or stops most chemical reactions. The process occurs under vacuum and in the absence of oxygen which make it impossible for oxidative reactions to occur. It is regarded as the gold standard of drying methods where the preservation of biological activity, flavour, aroma and/or chemistry is important (Klamathbluegreen, 2003).

Freeze-drying is a convenient method for the preservation and long term storage of a wide variety of microorganisms. Special precautions are needed for the preservation of microorganisms sensitive to desiccation, light, oxygen, osmotic pressure, surface tension and other factors. Effective protective agents, for example skim milk and meso inositol, honey or glutamate or raffinose are used to suspend cells to be freeze dried in order to

protect them against freezing and drying injuries. Along with the protective agents mentioned, anaerobic bacteria which are sensitive to aerobic freeze drying can be preserved using activated charcoal (5 % w/v) in the suspending media (Malik, 1990).

## 1.8 Supercritical fluids

A supercritical fluid is a substance that, at temperatures and pressures greater than its critical temperature and pressure, is a gas-like, compressible fluid that takes the shape of its container and fills it. The critical temperature is the temperature at the critical point and is the temperature above which a substance cannot exist as a liquid at any pressure. The critical pressure is the pressure at the critical point and is the pressure that will cause liquefaction of a gas at the critical temperature (Demirbaş, 2001). The supercritical state is when the temperature and pressure of a substance are raised over these critical values. In this state, the distinction between the liquid and gas phases disappears and the fluid can no longer be liquefied by raising the pressure nor gas be formed on increasing the temperature (Sihvonen et al., 1999).

A supercritical fluid has liquid like densities (0.1 – 1 g/ml) and solvating power, although it is not a liquid (Demirbaş, 2001). Physicochemical properties such as density, diffusivity, dielectric constant and viscosity can be easily controlled by changing pressure and temperature without crossing phase boundaries (Sihvonen et al., 1999). Supercritical fluids are compressible, and small pressure changes produce significant changes in their density and in their ability to dissolve compounds (Demirbaş, 2001). A supercritical fluid has a higher diffusion coefficient, lower viscosity and surface tension than a liquid solvent, and this translates into more favourable mass transfer (Sihvonen et al., 1999; Demirbaş, 2001).

The expense of organic solvents, environmental factors and the requirements of extra pure products by the medical and food industries have increased the need to develop new processing techniques (Sihvonen et al., 1999). The most widely used compound in pharmaceutical, nutraceutical and food applications is carbon dioxide though there are a

number of compounds e.g. ethane, propane, acetone, etc. (Demirbaş, 2001) that can be used as fluids in supercritical techniques. Carbon dioxide is a good solvent as it is environmentally benign (Hénon et al., 1999), non-toxic, non-flammable, inexpensive (Hénon et al., 1999; Sihvonen et al., 1999; Demirbaş, 2001), non corrosive (Demirbaş, 2001), easy to remove from product (Sihvonen et al., 1999) and easily recyclable (Hénon et al., 1999). Its use is also justified by its wide availability and relatively low critical temperature and pressure (31 $^{o}$C and 72±1 bar) (Hénon et al., 1999; Sihvonen et al., 1999; Demirbaş, 2001) and heat of vaporization at 294 K is only 0.1512 MJ/kg (Demirbaş, 2001). As the pressure and temperature is varied, $CO_2$ assumes a density and polarity range similar to a solvent strength of pentane to benzene (Demirbaş, 2001). Supercritical $CO_2$ is a non-polar solvent and can therefore not be used for dissolving polar molecules (Sihvonen et al., 1999). Though the non-polar nature of $CO_2$ limits its solubility of polar reactants, its combination of liquid like density and gas like viscosity and diffusivity leads to high reaction rate and easy recovery of products (Sarrade et al., 2003). Although $CO_2$ has no dipole moment, it does have a quadropole moment, which allows for specific interactions with some molecular groups such as carboxyl, ether and ester groups, leading to increased compatibility of molecules containing such groups with supercritical $CO_2$. Supercritical fluids have been widely used in extraction and recovery of high value compounds. Experience accumulated in recent years on the use of supercritical fluids and their processes have indicated that it is possible to explore and envision their uses beyond the common practice of extraction (Sarrade et al., 2003). Supercritical fluid technologies can also be applied in making new innovative products. One of the very promising areas of research is microencapsulation of drug molecules, which are used for controlled drug release in the human body (Sihvonen et al., 1999).

Development of an encapsulation technology that overcomes the problems encountered using the current technologies would enable the protection and preservation of sensitive substances, improved viability, effectiveness and shelf life. The main objective of this research is therefore to investigate the suitability of the novel method of encapsulation based on the formation of an interpolymer complex in supercritical carbon dioxide, for the encapsulation of probiotics for food and pharmaceuticals applications.

## 1.9 Methods for detection of probiotic cultures

The methods used for detection of viable probiotic cells include conventional plate counts (culture dependent) and molecular techniques (culture-independent). Though traditional plate counting techniques are generally criticized due to the possibility of underestimation of numbers as a result of clumping of cells (Lahtinen et al., 2006) and unsuitability (inappropriateness) of media for growing of viable but non-culturable cells (Lahtinen et al., 2006; Veal et al., 2000), there is no method that can replace this yet though a number of methods are being tried. New methods include molecular based techniques such as quantitative real-time polymerase chain reaction (PCR), fluorescent in situ hybridization (FISH) and LIVE/DEAD *BacLight* bacterial viability kits (Veal et al., 2000; Boulos et al., 1999). All these methods have their own disadvantages. For example, L/D kits and real time PCR are based on bacterial DNA which is not only present in live cells but can also be retained by dead cells in significant amounts. Both PCR and FISH are not independent as they require determination of a standard curve which is determined most of the times using standard plate counts. PCR requires expensive reagents which cannot be afforded by everyone in the industry. Detection limits for PCR and FISH are relatively high, being about $10^4$ cells/ml and $10^6$ cells/ ml, respectively. FISH is based on detection of 16s rRNA whose presence is not a direct proof of metabolic activity but rather an indication of potential viability (Biggerstaff, 2006). A recent study by Lahtinen et al., 2006 indicated the limitation of real-time PCR and FISH with regards to viability whereby counts of bacteria decreased but PCR and FISH results remained higher over the experimental period. The authors indicated that results showed that degradation of DNA had not occurred and rRNA levels remained high enough for the cells to still be detected. The intensity of rRNA in dead cells may still be strong enough for visually counting (detection) though it is expected to decrease upon cell death. Thus, the RNA content of the cell detected by fluorescent probes cannot be regarded as reliable indicator of cellular viability (Vives-Rego et al., 2000). Therefore, for the purposes of this study, detection of viable cells was mainly done using conventional plate counts on selective media.

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