Probiotics: Recent Understandings and Biomedical Applications

Jamila K Adam[a], Bharti Odhav[b] and K Suresh Babu Naidu[a*]
[a]Department of Biomedical and Clinical Technology, Durban University of Technology, Durban-4000, South Africa
[b]Department of Biotechnology and Food Technology, Durban University of Technology, Durban-4000, South Africa
*For Correspondence - dr.naidu@hotmail.com

Abstract

Probiotics are defined as "microbial food supplements" with beneficial effects on the consumers. Several aspects including safety, functional and technological characteristics, have to be taken into consideration in the selection process of probiotic microorganisms. Our Knowledge about probiotics and their interactions with the host has grown ever since Metchnikoff's theory of longevity and proven mechanism of action on probiotics published elsewhere in medical literature. Certainly, now there is enough clinical evidence to support claimed health attributes to selected strains of *Lactobacillus* and *Bifidobacterium* spp. The aim of this review article is to summarise selection criteria of probiotics, technological challenges for probiotic formulations, safety assessment and potential applications of probiotics for health care professionals and common man.

Keywords: Probiotics, *Lactobacillus, Bifidobacterium, Bacillus*, Biomedical Applications

1. Introduction

Probiotics have been defined as living microorganisms which upon ingestion in adequate numbers exert positive health effects beyond inherent basic nutrition (22). There health benefits include improvement of the normal microflora, prevention of infectious diseases (39), food allergies (14), reduction of serum cholesterol (42), anticarcinogenic activity (15, 27), stabilization of gut mucosal barrier (21), immune adjuvant properties (17), alleviation of intestinal bowel disease symptoms and improvement in the digestion of lactose intolerance hosts (25). For decades probiotics have been used in fermented dairy products such as yogurts and fermented milks and the technologies to incorporate these organisms into fresh, refrigerated dairy products is now a mature science. The continuing emergence of clinical evidence for benefits to consumers and the subsequent marketing power of these ingredients have now seen probiotics become the fastest growing category of functional food (FF) ingredients (63). Presently, probiotics play a vital role in promoting health for humans and are also used as therapeutic, prophylactic and growth supplements in animal production (1, 53, 54, 58, 74). From birth till death, all animals are exposed and colonized by a vast, complex and vibrant group of microorganisms that have important effects on immune functions, nutrient process and a broad range of other host activities (36). These microorganisms can mediate the critical balance between health and disease; provide therapeutics for animal and human inflammatory disorders on the basis of novel biological principles (26).

The most widely researched and used species belong to *Lactobacilli* and *bifidobacteria* (60, 75). *Pediococcus acidilactici* has a wide range of potential benefits of probiotic properties which are still being studied. Though it is being used as probiotic supplements in treating constipation, diarrhea, relieving stress, enhancing immune response among birds and small animals, human trials are still limited. *Pediococcus acidilactici* is also known to prevent colonization of the small intestine by pathogens like *Shigella*, *Salmonella*, *Clostridium difficile* and *Escherichia coli* among small animals. *Pediococcus acidilactici* in conjunction with *Saccharomyces boulardii*is found to stimulate humoral immune response to produce higher Eimeria-specific antibodies while also reducing the number of oocysts shed by possible competitive inhibition and pediocin production which inhibit pathogenic bacteria and other gram-positive spoilage. Other commonly studied probiotics include the spore forming *Bacillus* spp. Cutting (10) have given a critical review of *Bacillus* probiotics and products for human use.

Probiotics have created considerable interest in the scientific community, and this has resulted in a very substantial increase in published research. The preponderance of the publications reported positive results for probiotic therapy as an important adjunct in the treatment of a host of pathologic processes (9, 71). The aim of the present paper was to review the recent understandings, biotechnological applications and various parameters in considering a microorganism for potential probiotic applications.

2. Selecting Probiotic Microorganism: Fundamental aspects : According to FAO/WHO guidelines it is necessary to identify the microorganism to species/strain level given that the evidence suggests that the probiotic effects are strain specific (45). It has been recommended to employ a combination of phenotypic and genetic techniques to accomplish the identification, classification, and typing of microorganism. For the nomenclature of bacteria, scientifically recognized names must be employed and it is recommended to deposit the strains in an internationally recognized culture collection. Further characterization of strains must be undertaken taking into account the "functional" or probiotic aspects and safety assessment. In addition, even if these genera have a long history of safe consumption in traditionally fermented products and several species have been awarded as "General Recognised As Safe" (GRAS) status by the American Food and Drug Association or a qualified presumption of safety (QPS) consideration by the European Food Safety Authority (EFSA), some characteristics (Fig. 1) must be studied to ensure the safety of the novel *Llactobacilli* and *Bifidobacteria* strains. Several of the *in vitro* tests can be correlated with *in vivo* studies with animal models, but probiotics for human use must be validated with human studies covering both safety (Phase 1 trials) and efficacy (Phase 2 trials) aspects. Phase 2 studies should be designed as double-blind, randomized, and placebo-controlled to measure the efficacy of the probiotic strain compared with a placebo and also to determine possible adverse effects (22). Briefly, the critical parameters to be considered for probiotic candidate are discussed.

2.1 Isolation and Selection criteria : Currently, probiotics are being used successfully to improve the quality of feed provided to domestic animals. The resulting benefits of effective administration of probiotics in feed to cattle, pigs, and chickens include enhanced general health, faster growth

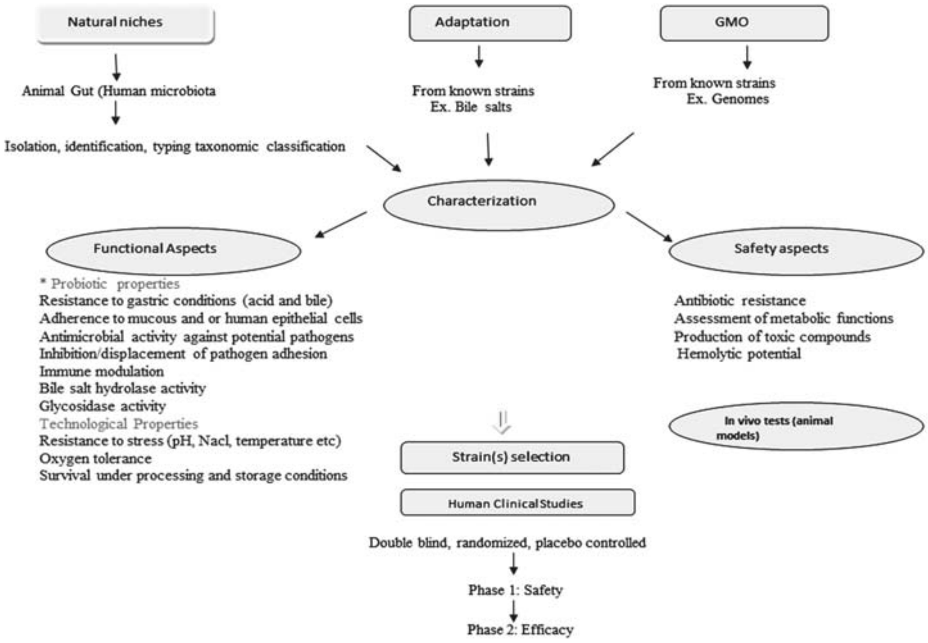

Fig. 1. Procedure for the isolation and characterization of novel strains with putative probiotic Status (43)

rates as a result of improved nutrition, enhanced immunity and increased production of milk and eggs. Some of the microorganisms most commonly used to promote animal health and nutrition include strains of the *Lactobacillus*, *Bifidobacterium*, *Bacillus*, *Streptococcus*, *Pediococcus*, *Enterococcus* genera and yeast of the *Saccharomyces*, *Aspergillus*, and *Torulopsis* genera (71). In the development of probiotic foods intended for human consumption,strains of lactic acid bacteria, such as *Lactobacillus*, *Bifidobacterium*, and *Streptococcus*, have been used most commonly, primarily because of the perception that they are desirable members of the intestinal microflora (28) (Table 1). In addition, these bacteria have traditionally been used in the manufacture of fermented dairy products and have GRAS status. However, some of the probiotic isolates currently used in the dairy food industry are not of human origin and therefore do not meet the criteria outlined for the selection of acceptable probiotic microorganisms, hence thorough characterization of strains must be undertaken taking into account the functional or probiotic aspects and safety assessment. *In vitro* tests, some of them summarized in Fig. 1. are useful to gain knowledge of both strains and mechanisms of the probiotic effect. The selection criteria for a microorganism to be used as 'probiotic' include the following

1. Safety
2. Detailed definition and typing
3. Absence of pathogenic characteristics (including production of enterotoxins and cytotoxins, enteroinvasivity, pathogenic adhesion, hemolysis, serum resistance, serum pathogenicity, presence of genes of antibiotic resistance)
4. Resistance to gastric acid and to bile
5. Ability to adhere to intestinal epithelium
6. Ability to colonize the colon
7. Clinically proven beneficial health effects
8. Ability to withstand into food stuff at high cell counts and remain viable through the shelf-life of the product (23)

Table 1. The most commonly used species in probiotic preparations

Lactobacillus Sp.	*Bifidobacterium* Sp.	*Enterococcus* Sp.	*Streptococcus* Sp.	*Bacillus* Sp.	*Pediococcus* Sp.
L. acidophilus	*B. bifidum*	*Ent. faecalis*	*S. cremoris*	*B. subtilis*	*Pediococcus acidilactici*
L. casei	*B. adolescentis*	*Ent. faecium*	*S. salivarius*	*B. licheniformis*	
L. delbrueckii ssp. (bulgaricus)	*B. animalis*		*S. diacetylactis*	*B.polyferminticus*	
L. cellobiosus	*B. infantis*		*S. intermedius*	*B.coagulans*	
L. curvatus	*B. thermophilum*			*B. laterosporus*	
L. fermentum	*B. longum*			*B.polymyxa*	
L. lactis				*B.pumilus*	
L. plantarum				*B.clausii*	
L. reuteri				*B. cereus var toyoi*	
L. brevis					

The use of human origin probiotic and its administration in living form are only relative criteria. *Saccharomyces boulardii*, the probiotic character of which has been sufficiently proven has no human origin and hence it may probably more suitable to name it as a "biotherapeutic agent" simulataneously. Anti-inflammatory effects of probiotics in experimental colitis may be mediated by microbial components such as peptidoglucan, lipopolysaccharide, and non methylated DNA (57, 53). Much attention is paid to detail typing of probiotics. DNA-DNA hybridization or sequencing DNA regions encoding species-specific areas of 16S rRNA are routinely used molecular techniques to test species classification. These techniques are combined with specific cultivation methods for verifying the microbial phenotype.

Genomic analysis is of principal importance for the detailed knowledge of individual probiotics. The first probiotic to be sequenced of whole genome was *Lactococcus lactis* subsp. IL1403 (3). This was followed by the complete genomic analysis of *Lactobacillus plantarum* (35), *Bifidobacterium longum* (68), and *Escherichia coli* (26). Nevertheless, genomically non- defined microorganisms (e.g. *L. casei Shirota, L. reuteri, Streptococcus salivarius* subsp. thermophilus, and non pathogenic *E. coli* O83: K24: H1) or even mixtures of microbes are also used as probiotics. Genomic analysis is indispensable for predicting the effects of individual probiotics as well as for studying the relationship between probiotics and prebiotics and life conditions of the intestinal microflora. The final goal of these studies is to create a global genome bank of intestinal prokaryotes (30).

2.2 Molecular tools for assessment of Probiotic microorganism : Interest of the research community in the study of microbial ecosystem at molecular level is expanding. The Human Microbiome Project (HMP) has recently been launched by the National Institutes of Health (NIH) with the mission of generating resources enabling comprehensive characterization of the human microbiota and analysis of its role in human health and disease. In Europe, the MetaHIT (Metagenomics of the Human Intestinal Tract) project funded by the European Commission is investigating the role of the gut microbiota in obesity and inflammatory bowel diseases (48). Topics surrounding the gut microbiota and its intrinsic relations with health and disease also interest the food and pharmaceutical industries (10).

Broadly, several different methodologies are in use to assess intestinal microbiota which fall in two distinct categories: culture- dependent and culture independent methods (Fig. 2). Culture-dependent methods aim both qualitatively and quantitatively and has been traditionally carried out by cultivation of feces and reviewed in detail by Lee and Salminen (44). One of the most widely applied approaches deals with the use of 16S rRNA and its encoding genes as target molecules. The 16S rDNA gene contains highly conserved regions, present in all bacteria, and highly variable ones that are specific for certain microbes. Specific polymerase chain reaction (PCR) primers and probes can thus be designed based on these variable regions to detectcertain species or groups of bacteria. These culture-independent approaches include16S rRNA measurements, PCR amplification with specific primers of 16S rDNA extracted from faecal or mucosal samples, universal or group 16S rDNA PCR amplification followed by cloning and sequencing, Temperature gradient gel electrophoresis (TGGE), denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP) analysis, fluorescence *in situ* hybridization (FISH), real-time quantitative PCR (RT-PCR), and oligonucleotide-microarrays. In more recent years metagenomic and metaproteomic approaches have also been applied to the intestinal microbiota assessment.

2.3 Technological Challenges for Probiotic formulations: The development of new novel food products and their delivery in acceptable form turns out to be challenging, as it has to fulfill the consumer's expectancy for products that are simultaneously relish and healthy (15). Probiotic

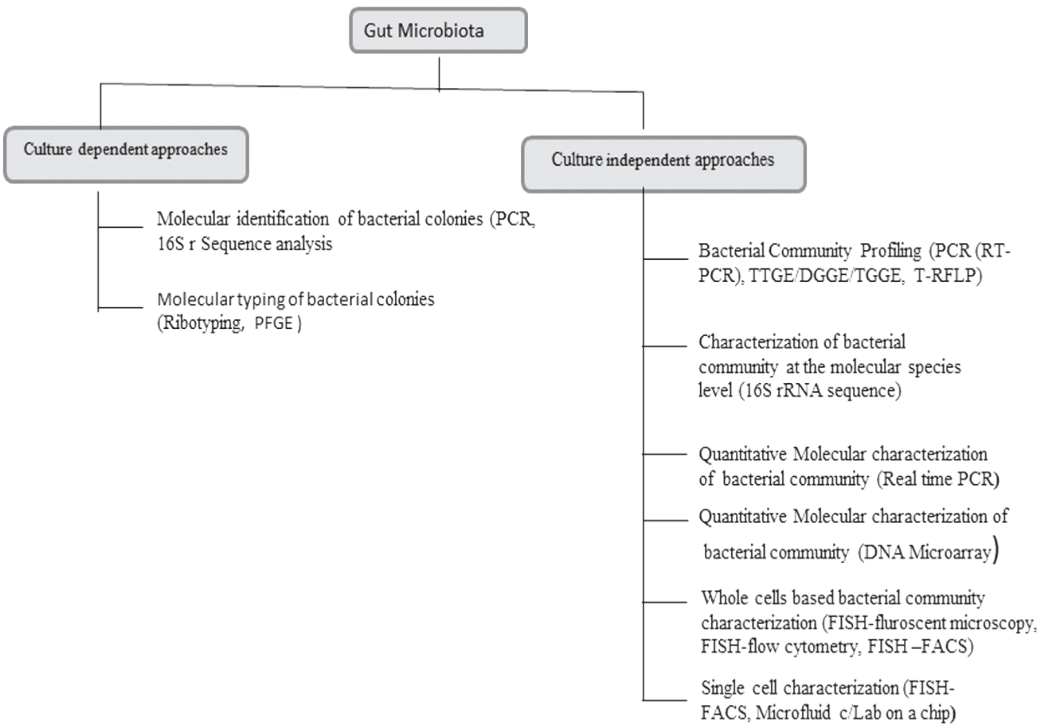

Fig. 2. Molecular tools to investigate the gut Microbiota for probiotic properties (10)

Vol. 6 (1) 1-14 January 2011, ISSN 0973-8916 (Print), 2230-7303 (Online)

food is defined as a food product that contains viable probiotic microorganisms in sufficient populations incorporated in a suitable matrix (29, 61). This means that their viability and metabolic activity must be maintained in all the steps of the food processing operation, from their production stage to ingestion by the consumer, and also that they must be able to survive in the gastrointestinal tract (63). The information available concerning the concentration of probiotic microorganisms needed for biological effects leads to the conclusion that it will vary as a function of the strain and the health effect desired (12). Nevertheless, populations of 10^6–10^7 CFU/g in the final product are established as therapeutic quantities of probiotic cultures in processed foods (72).

From a technological stand point, there are many challenges in the development of probiotic containing food product such as selection of strain(s), inoculum preparation, survival during processing, viability and functionality during storage, access the viable counts of the probiotic strain(s) (particularly when multiple probiotic strains are added along with starter cultures) and management of effects on sensory properties (11). Processing of microbial systems for functional food (FF) is also dependent on the composition and processing history of the raw material used as substrate, the viability and productivity of the starter cultures applied, processing and storage conditions of the final food products. The viability and activity of probiotic cultures are affected during all steps involved in a delivery process through the exposure to different stress factors (74). In general, probiotics are extremely susceptible to environmental conditions such as water activity, redox potential (presence of oxygen), temperature, and acidity (70). Different stress factors associated with development of probiotic formula during processing are summarized in table 2. The reader is suggested to consult Girgis *et al*. (28) for in-depth review of this subject. Most conventional food processing efforts are aimed towards the reduction or inactivation of unwanted microbial populations. This is achieved by thermal processing (i.e. blanching, pasteurization, sterilization) using water, steam, electrical, light or microwave energy as well as ultrasound or hot air as a means for heat transfer

Table 2. Different stress factors affecting viability of probiotic during processing (74)

Processing step	Stress factor
Production of probiotic preparations	Presence of organic acids during cultivation Concentration-high osmotic pressure, low water activity, higher concentration of particular ion Temperature freezing, vacuum and spray drying Prolonged storage Oxygen exposure
Production of probiotic containing product	Nutrient depletion, strain antagonism, increased acidity, positive redox potential, presence of antimicrobial compounds (Hydrogen peroxide and bacteriocin), storage temperature.
Gastrointestinal transit	Gastric acid, bile salt, microbial antagonism.

(31). Ambient temperature processes include the use of anti-microbials, irradiation, high hydrostatic pressure treatment, high intensity electric fields or light pulses as processing tools, as well as combination processes such as the use of the hurdle technology concept (30). In addition, modified atmosphere packaging and cold or frozen storage is designed to reduce or control microbial growth. Another way of achieving this is to use competitive microbial flora (including probiotic organisms) thus mimicking the situation in the human gut where the competitive microflora also needs to be constantly maintained, improved or re-established. On the other hand, fermentation which is a process to improve the digestibility, quality, safety and physico-chemical properties of the raw material and primarily aimed to produce probiotics and FF or food ingredients can be counterproductive to the viability of microorganisms because it requires maximum productivity of microorganisms which can lead to poor microbial viabilities in the fermented product. Consequently, challenges to retain and optimize microbial viability and at the same time for improving productivity are reviewed by Knorr (41).

Safety of Probiotics : There is abundant experimental evidence to support the health benefits of probiotics, including improvement of the intestinal microbial balance by antimicrobial activity, alleviation of lactose intolerance symptoms, prevention of food allergies, enhancement of immune potency, antitumorigenicactivities, antioxidative and antiatherogenic effects, and a hypo-cholesterolemic property (49, 52) (Table 3).

However, in recent years, many species of the genera *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Enterococcus*, and *Bifidobacterium* have been isolated from infected lesionsin patients with such conditions as bacterial endocarditis and blood stream infections (26, 46, 47). This is a clear indication of probiotic translocation from gastrointestinal tract to extra intestinal sites which can be measured by the recovery of viable bacteria from lymph nodes, spleen, liver, blood stream and other tissues. This phenomenon is observed due to defective intestinal barrier, immuno-suppression or prematurity. Translocation may result in the transfer of bacteria to other organs, thereby potentially causing bacteremia, septicemia, and multiple organ failure. The ability of microorganisms to translocate, survive, and proliferate in extra intestinal tissues involves complex interactions between the host defense mechanisms and the bacteria's ability to invade host tissues; however, the precise mechanisms involved remain unknown (56).

Bacteria of the indigenous microflora are not normally found in the mesenteric lymph nodes, spleen, liver, or blood of healthy animals. The host's immune defenses normally eliminate indigenous bacteria that would translocate across the mucosal epithelium. Thus, most of the studies in which probiotics were administered at high dosages to healthy subjects found an absence of probiotic translocation. Indeed, probiotics rarely cause severe disease in healthy subjects, even when probiotic bacteria translocate from the gastrointestinal tract. Despite these reports of an absence of translocation of probiotics in healthy subjects, *Lactobacilli* or *Enterococci* have been identified as the strains translocating most commonly into the mesenteric lymph nodes of healthy pathogen-free mice (4) and in immunocompromised patients (64). Although *Lactobacilli* are usually considered contaminants in blood cultures, they have been identified in some clinical reports as causal agents of dentalcaries, infectious endocarditis, urinary

Vol. 6 (1) 1-14 January 2011, ISSN 0973-8916 (Print), 2230-7303 (Online)

Table 3. Beneficial health effects of specific probiotics (43)

Microflora	Mode of action	Reference
Bifidobacteria species	Reduced incidence of neonatal necrotizing enterocolitis	(52)
	Treatment of rotavirus diarrhea, balancing of intestinal microflora, treatment of viral diarrhea	(37)
Enterococcus faecium	Decreased duration of acute diarrhoea from gastroenteritis	(38)
	Probiotic assessment of *Enterococcus faecalis* CP58 isolated during an *in vitro* screening of lactic acid bacteria	(49)
Lactobacillus strains	Administration of multiple organisms, predominantly *Lactobacillus* strains shown to be effective in ameliorating pouchitis	(74)
	Lactose digestion improved, decreased diarrhoea and symptoms of intolerance in lactose intolerant individuals, children with diarrhoea, and individuals with short-bowel syndrome	(38)
	Microbial interference therapy – the use of nonpathogenic bacteria to eliminate pathogens and as an adjunct to antibiotics	(6)
	Improved mucosal immune function, mucin secretion and prevention of disease	(47)
	Vaccine adjuvant, adherence to human intestinal cells, balancing of intestinal microflora	(5)
Saccharomyces boulardii	Prevention of traveler's diarrhea, prevention and treatment of *C. difficile* diarrhea Shortened the duration of acute gastroenteritis	(7, 38)
Bacillus Sp.	Heat stable oral vaccine delivery, prophylactics and prevention of gastrointestinal infections	(58)
Pediococcus	Enhanced immune responses against infectious coccidioidal diseases	(44)

tract infections, corioamniotitis, endometritis, meningitis and intra-abdominal, liver and spleen abscesses (34). Commonly, these infections can be correlated with previous illnesses (recent surgery, transplants, valvulopathy, diabetes mellitus, AIDS and cancer) with either immunosuppressive therapy or antibiotic treatment, which could promote the development or the selection of the microorganism.

Despite these findings, most studies conducted with healthy subjects have not reported severe disease caused by probiotics even when they do translocate from the gastrointestinal tract. The reasons for this remain unclear and several theories have been proposed. One possibility may be that probiotics are more susceptible to intracellular killing by macrophages upon translocation, since phagocytes are known to exhibit a protective effect during the induction of infective endocarditis caused by gram-positive bacteria (17). The long history of consumption, available epidemiological data, clinical trials, acute toxicity studies that have been conducted all suggest that the *Lactobacillus* sp. commonly occurring in fermented foods and used in current probiotics are safe. It is likely, however, that expansion in this area and the introduction of new probiotic strains will have to take safety aspects into more detailed consideration, particularly should those strains be genetically modified or derived from animals.

Applications

Fermented dairy products enriched with probiotic bacteria have developed into one of the most successful categories of functional foods. They gave rise to the creation of a completely new category of probiotic products like the daily-dose drinks in small bottles, yoghurt, ice creams, milk based desserts, powdered milk for infants, butter, may onnaise, cheese, products in the form of capsules or fermented food of vegetable origin. It has been estimated that there were approximately 70 probiotic-containing products marketed in the world (65), the list is continuously expanding. Moreover, probiotic products containing *Bacillus* species have been in market for at least 50 years with the Italian product known as Enterogermina®registered 1958 in Italy as an OTC medicinal supplement (9). Of the species that have been most extensively examined are *B. subtilis, B. clausii, B. cereus, B. coagulans* and *B. licheniformis*. Spores being heat-stable have a number of advantages over other non-spore formers such as *Lactobacillus* spp., namely, that the product can be stored at room temperature in a desiccated form without any deleterious effect on viability. A second advantage is that the spore is capable of surviving the low pH of the gastric barrier (2) which is a limitation for all species of *Lactobacillus* (72).

Conclusion

The global market for functional foods is growing at a very fast rate and probiotic products represent a potential growth area. Intense research efforts are under way to develop products into which probiotic organisms such as *Lactobacillus* and *Bifidobacterium* species are incorporated. The long term exploitation of probiotics would depend on scientifically proven clinical evidence of health benefit, of consumer expectation and of effective marketing strategies (66).Today, probiotics are used to prevent and treat a wide variety of conditions. The evidence is strongest in support of their use for gastrointestinal disorders including diarrhea, pouchitis, inflammatory bowel disease, traveller’s diarrhea, allergy, antibiotic associated diarrhea and *Clostridium difficle* infection (32). Many consider probiotics to be Complementary or alternative medicine (CAM). The use of CAM is increasing rapidly in United States and recent survey found that 36% of all adults used some form of CAM and US $ 37- $ 47 billion is spent annually Interestingly, probiotic therapy and CAM are widely accepted and used frequently by children and adolescents attending gastroenterology clinics (16).

One approach that should be encouraged for health effects is the concept of Synbiotic (use of prebiotic and probiotics), use of genetically engineered recombinant micro-organisms and in combination with food enzymes and antibiotics. There are many probiotic products in the market place and most have supporting evidence behind the advertized health claims (9). However, there is increasing demand for technology for probiotic formulation which can be prepared in viable manner on large scale at low operational cost. Probiotics should not be treated as "elixir of life" but can be incorporated into a balanced diet to exercise good health. Here we hope to have provided an important insight into the recent developments of probiotics and its potential in future to serve in food industry.

Acknowledgements

We extend our sincere acknowledgement to Prof. S. Mayo, Research Management Division, Durban University of Technology, South Africa for awarding Post Doctoral fellowship to Dr. K. Suresh Babu Naidu.

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