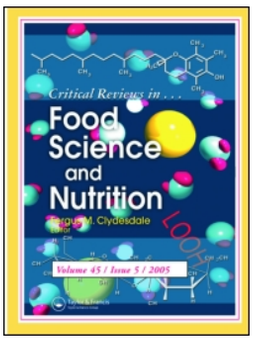

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Probiotics, prebiotics, and microencapsulation: A review

Loveleen Kaur Sarao and M. Arora

Department of Microbiology, College of Basic Sciences and Humanities, Punjab Agricultural University, Ludhiana, Punjab, India

ABSTRACT

The development of a suitable technology for the production of probiotics is a key research for industrial production, which should take into account the viability and the stability of the organisms involved. Microbial criteria, stress tolerance during processing, and storage of the product constitute the basis for the production of probiotics. Generally, the bacteria belonging to the genera Lactobacillus and Bifidobacterium have been used as probiotics. Based on their positive qualities, probiotic bacteria are widely used in the production of food. Interest in the incorporation of the probiotic bacteria into other products apart from dairy products has been increasing and represents a great challenge. The recognition of dose delivery systems for probiotic bacteria has also resulted in research efforts aimed at developing probiotic food outside the dairy sector. Producing probiotic juices has been considered more in the recent years, due to an increased concern in personal health of consumers. This review focuses on probiotics, prebiotics, and the microencapsulation of living cells.

KEYWORDS

Probiotic; prebiotic; microencapsulation; LAB; functional food

Introduction

Over the past decade, functional food also called as healthy food has become increasingly important with positive impact on both world health and international trade. At the same time, economic benefits of functional food grow in both developing and industrialized countries (Yang, 2008). The production of functional food is being recognized as the number one food biotechnology industry as changing trends in population demography, consumer affluence, increased education, life expectancy, and improved health care give rise to rapidly emerging diet and health conscious clientele (Dillard and German, 2000).

The term functional food was first introduced in Japan in mid-1980s and refers to the processed food containing ingredients that aid specific bodily functions in addition to being nutritious (Swinbanks and O' Brien, 1993). According to "The Provision for Functional Foods Administration," which was promulgated by the Ministry of Health (MOH) in March 1996 (MOH, 1996), a functional food is defined as a food that has special health benefits. It is suitable for the consumption by special group of people and has the function of regulating human body functions and not used for therapeutic purposes. Later, in July 2005, "The Guideline of Registration for Functional Foods" was promulgated by the China State Food and Drug Administration (SFDA) and the definition of a functional food was extended as follows, "health food means that a food that has special health functions or is able to supply vitamins or minerals. It is suitable for consumption by specific group of people and has the function of regulating human body functions and it will not cause any harm whether acute or sub-acute or chronic" (SFDA, 2005).

Functional food ingredients include probiotics, prebiotics, vitamins, and minerals and are found in diverse products such as fermented milk, yoghurt, sports drinks, baby foods, sugar-free confectionary, and chewing gum (Khan and Ansari, 2007). The origin of cultured dairy products dates back to the dawn of civilization. In the recent years, there has been an upsurge in the research of probiotics, as well as growing commercial interest in the probiotic food concept (Senok et al., 2005). This research has resulted in significant advances in our understanding and ability to characterize specific probiotic organisms. Probiotic food constitute a sizeable part of the functional food market (Stanon et al., 2001). It will continue to grow at an exponential rate with the potential for market growth estimated at a staggering US$120 million per month (Anonymous, 2001). Quality control is a concern with commercially available probiotics due to the lack of regulatory oversight.

The term probiotic was first used in 1965, by Lily and Stillwell to describe substances secreted by one organism which stimulate the growth of another (Gupta and Garg, 2009). A probiotic is defined as a viable microbial dietary supplement that beneficially affects the host through its effects in the intestinal tract (Mc Farland, 2000; Salaminen et al., 2001). This definition, however, was initially intended for use with animal feed products. Two main genera of gram positive bacteria *Lactobacillus* and *Bifidobacterium* are used extensively as probiotics (Holzapfel et al., 2001). However, other genera such as *Escherichia, Enterococcus, Streptococcus*, and *Saccharomyces* have also been marketed as probiotic. Viable lactic acid bacteria (LAB) of probiotic food have several scientifically established and clinically proved health effects, such as reduction and prevention of

CONTACT Loveleen Kaur Sarao micro.loveleen@gmail.com, loveleensarao@gmail.com Department of Microbiology, College of Basic Sciences and Humanities, Punjab Agricultural University, Ludhiana-141004, Punjab, India.

diarrhea of different origin, improvement of the intestinal microbial balance by antimicrobial activity, alleviation of lactose intolerance symptoms, prevention of food allergy, enhancement of immune potency, and anti-tumor activities (Anderson et al., 2001).

A probiotic strain should withstand the manufacturing process without the loss of viability or negative effect on the sensory properties of the food product. The strain and the claimed properties should maintain stability in the food product during processing and also during subsequent storage (Saarela et al., 2000). For human nutrition, probiotics are defined as "live microbial food supplements or components of bacteria which have been shown to have beneficial effects on human health" (Salminen et al., 1998). A large number of viable organisms are required in order to exert a probiotic effect in the food product. It is postulated that an active probiotic food should contain at least 10^5 cfu/g and the food should be consumed in order to achieve a beneficial effect (Lee and Salminen, 1995). Probiotic food are a group of health promoting, so-called functional food, with large commercial interest and growing market shares (Parvez et al., 2006). In general, their health benefits are based on the presence of selected viable strains of LAB, which when taken in up in adequate amounts, confer a health benefit on the host (FAO/WHO, 2001).

Probiotic bacteria beneficially affect human health by improving the gut microbiota balance and the defenses against pathogens. Additional benefits attributed to probiotics are the stimulation of the immune system, blood cholesterol reduction, vitamin synthesis, anti-carcinogenesis and anti-bacterial activities. Other important criteria to determine the efficacy and the success of the product containing probiotics are the acceptance of the product by the consumer and the survival of probiotics microorganisms during its production (Heenan et al., 2004). In general, the food industry has applied the recommended level of 10^6 cfu/g at the time of consumption for *Lactobacillus acidophilus, Bifidobacteria*, and other probiotic bacteria (Boylston et al., 2004).

A prebiotic is defined as "non-absorbable food component that beneficially stimulate one or more of the gut-beneficial microbe groups and thus has a positive effect on human health" (Gibson and Roberfroid, 1995). The most commonly used prebiotics are carbohydrate substrates (e.g., dietary fiber) with the ability to promote the components of the normal intestinal microflora which may provide a health benefit to the host. This group involves certain dietary components resistant to digestive enzymes hydrolysis and that are not absorbed in the upper portion of gastrointestinal tract including the small bowel. These compounds need to get to the large bowel, where the microbiota is located and stimulate the growth of some beneficial microorganisms in the gut (Roberfroid, 2002).

"Synbiotics" is the word coined for the combined administration of specific probiotics with prebiotics to provide definite health benefits by synergistic action. A synbiotic is a product containing probiotic and prebiotic in which the prebiotic compound specifically favors the probiotic compound (Schrezenmeir and de Vrese, 2001).

Probiotic bacteria are used widely in producing food based on their positive qualities. The common probiotics that have been extensively studied and found in the market are dairy products such as yoghurt and cheese. Latest studies reveal that other novel probiotics such as fruit juices, cereals, and chocolate etc. are better and superior carriers for the delivery of probiotics. Producing probiotic juices have been considered more in recent years. Very less work has been done on synbiotic foods. Hence, there is a need to develop diverse probiotic and synbiotic foods, which can be used as nutrient supplements to promote health. Moreover, many reports also indicate the poor survival of probiotics in functional foods. The stability and viability of the probiotic cultures can be increased by a recent technology known as micro-encapsulation. Extensive research however is required to be conducted on the efficacy of microencapsulation to deliver probiotics for their controlled and targeted release in the gastrointestinal tract.

The concept of probiotics, prebiotics, synbiotics and microencapsulation

2.1. The history of probiotics and its subsequent development

Fermentation of milk is one of the oldest medical sciences, which dates back to 2500 BC. The consumption of yogurt is believed to help in maintenance of overall health (Chopra and Doiphode, 2002). A scientific explanation of the beneficial effects of LAB present in fermented milk was first provided in 1907 by the Nobel Prize winning Russian physiologist, Eli Metchnikoff, who stated that the dependence of the intestinal microbes on the food makes it possible to adopt measures to modify the flora in our bodies and to replace the harmful microbes by useful microbes (Holzapfel and Schillinger, 2002). Early scientists recommended fermented milk for its nutritional and medicinal properties, so the use of live flora to enhance the human health is over thousands of years. In 1900 and before Metchnikoff and for the first time, bifidobacteria, members of the LAB group were isolated from the intestinal layer of breast-fed infants and found that they are the predominant component of the intestinal microflora (O'Sullivan et al., 1992; Ishibashi and Shimamura, 1993; Harmsen-Hermie et al., 2000; Fooks and Gibson, 2002).

Work done in the earlier part of the last century concentrated on the use of fermented milk with probiotics to take care of intestinal infections. Recent studies have focused on the survival of these bacteria in the gastrointestinal tract and the carrier food to have their beneficial effect on the host (Lourens-Hattingh and Viljoen, 2001; Roy, 2005). Moreover, there has been more interest in using probiotics instead of antibiotics (Isolauri, 2001; Gibson and Rastall, 2004). However, the use of the term "Probiotic" was done in the 1970s and it was derived from the Greek term meaning "for life." However, a lot of scientific studies have been done about the beneficial effect of probiotics, which suggest their high use in the fermented products market.

2.2. Definition of the term "probiotics"

One manner in which modulation of the gut microbiota composition has been attempted is through the use of live microbial dietary additions, as probiotics. The word "probiotic" is translated from the Greek word meaning "for life." An early definition was given by Parker (1974) as "Organisms and substances

which contribute to intestinal microbial balance." However, this was subsequently refined by Fuller (1989) as "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance." This latter version is the most widely used definition and has gained widespread scientific acceptability. A probiotic would therefore incorporate living micro-organisms into diet, which are beneficial for gut health.

Probiotics have a long history. In fact, the first records of intake of bacterial drinks by humans are over 2000 years old. However, at the beginning of this century probiotics were first put onto a scientific basis by the work of Metchnikoff at the Pasteur Institute in Paris. Metchnikoff (1907) observed longevity in Bulgarian peasants and associated this with their elevated intake of soured milks. During these studies, he hypothesized that the normal gut microflora could exert adverse effects on the host and that consumption of certain bacteria could reverse this effect. Metchnikoff refined the treatment by using pure cultures of what is now called *Lactobacillus delbruckeii* subsp. *bulgaricus*, which, with *Streptococcus salivarius* subsp. *thermophilus*, is used to ferment milk in the production of traditional yoghurt. Subsequent research has been directed toward the use of intestinal isolates of bacteria as probiotics (Fernandes et al., 1987). Over the years, many species of micro-organisms have been used. They mainly consist of lactic acid producing bacteria (lactobacilli, streptococci, enterococci, lactococci, bifidobacteria) but also *Bacillus* spp. and fungi such as *Saccharomyces* spp. and *Aspergillus* spp.

Despite the very widespread use of probiotics, the approach may have some difficulties. The bacteria used are usually anaerobic and do not survive the extremes of temperature. To be effective, probiotic must be amenable to the preparation in a viable form at a large scale. During use and under storage, the probiotic should remain viable and stable and be able to survive in the intestinal ecosystem and the host animal should gain beneficially from harboring the probiotic (Desai, 2008). It is therefore proposed that the exogenous bacteria reach the intestine in an intact and viable form and establish therein and exert their advantageous properties. In order to do so, microbes must overcome a number of physical and chemical barriers in the gastrointestinal tract. These include gastric acidity and bile acid secretion. Moreover, on reaching the colon the probiotics may be in some sort of stressed state that would probably compromise chances of survival.

2.3. The probiotic bacteria

Probiotic bacteria are defined as "live microorganisms, which, when administered in adequate amounts, confer a health benefit on the host" (FAO/WHO, 2002). A wide range of commercial probiotic products is available, which contain different probiotic strains that elicit varying health-beneficial effects. The majority of the probiotic bacteria in use today (Table 1) belong to the genera *Lactobacillus* and *Bifidobacterium* (Marco et al., 2006; Minocha, 2009), but products containing strains from other genera such as *Propionibacterium, Enterococcus*, and *Escherichia* are also available (Ouwehand et al., 2002).

Table 1. The most commonly used species of lactic acid bacteria in probiotic preparations (Parvez et al., 2006).

Lactobacillus sp.	*Bifidobacterium sp.*	*Enterococcus sp.*	*Streptococcus sp.*
L. acidophilus	*B. bifidum*	*Ent.faecalis*	*S. cremoris*
L. casei	*B. adolescentis*	*Ent.faecium*	*S. salivarius*
L. delbrueckii ssp. (bulgaricus)	*B. animalis*		*S. diacetylactis*
L. cellobiosus	*B. infantis*		*S. intermedius*
L. curvatus	*B. thermophilum*		
L. fermentum	*B. longum*		
L. lactis			
L. plantarum			
L. reuteri			
L. brevis			

2.3.1. Characteristics of probiotics

Most of the probiotics are related to the *Lactobacillus* and *Bifidobacterium* genera (Bezkorovainy et al., 1997; Salminen and von Wright, 1998b; Sanders, 2003; Guarner et al., 2005). However, to consider the use of different strain as probiotics it should be a normal inhabitant of a healthy intestinal tract, survive the upper digestive tract and capable of surviving and growing in the intestine (acid and bile resistant), safe for human consumption, should produce antimicrobial substances like bacteriocins and have the ability of adherence to human intestinal lining and colonization (Guarner and Schaafsma, 1998; Morelli, 2000; Guarner et al., 2005).

LAB have a good safety record as compared to the probiotics and are rarely involved in disease. The most commonly used of these probiotics are *Lactobacillus* spp., *Bifidobacterium* spp. and *Lactococcus* spp. Therefore, these organisms have been accorded the GRAS—Generally Recognized as Safe status (Salminen and von Wright, 1998a).

Probiotics-criteria (Harish and Varghese, 2006)

For organisms to be considered as probiotics, the following criteria need to be fulfilled:

- It should be isolated from the same species as its intended host
- It should have a demonstrable beneficial effect on the host
- It should be non-pathogenic
- It should be able to survive the transit through the gastrointestinal tract
- On storage, large number of viable bacteria must be able to survive prolonged periods

2.3.2. The concept of functional food and probiotic bacteria

The growing understanding of the relationship between diet and health has increased the demand for food with specific benefits beyond their basic nutrition such as improving the health and well being of humans. This food is called as "Functional Food." However, functional food has been defined as one, which provides a specific health benefit over and above its normal nutritional status (Gibson and Rastall, 2004). Moreover, the functional food must remain as food (not capsules, etc.) and they must also reveal their effects in amount that can usually be expected to be consumed in the diet. It has been suggested that food will be used as functional when it has shown beneficial effect on one or more targets in the body and that beside their

nutritional effects such as well-being and health of the host (Isolauri et al., 2002).

The old generation of functional food indicates the use of supplements to the food to increase their nutrition and health effects such as vitamins and micronutrient. However, in the new concept of functional food there is interest in the gastrointestinal interactions (Salminen et al., 1998), that led for more interest in the dominated organisms in the gastrointestinal tract (indigenous microflora) which has found to beneficially effect human health, which are known as probiotic bacteria. Therefore, the use of probiotic micro-flora is one of the most promising areas for the development of functional foods in the recent studies (Gibson and Roberfroid, 1999) because of what probiotics has established a great benefit to human health.

Bifidobacteria are the most dominated organisms in the gastrointestinal tract and their viability and metabolic activity has shown beneficial effects on the health of the gastrointestinal tract (Gibson and Roberfroid, 1995). This always relates to the presence of a suitable environment and nutrients, which are very important for the viability and activity of bifidobacteria and for it to use it in the bowel as carbon and energy source, these compounds are referred to as "bifidogenic factors" (O'Sullivan, 1996). At present, probiotic products and especially probiotic dairy foods are marketed successfully all over the world because of their acceptance by the consumers and the awareness about their positive aspect for the health benefits.

2.4. The required properties for probiotics to be effective in nutritional and therapeutic settings

A probiotic can be used exogenously or endogenously to enhance nutritional status and/or the health of the host. In the case of exogenous use, microorganisms are most commonly used to ferment various foods and by this process can preserve and make nutrients bioavailable (Desai, 2008). In addition, microorganisms can metabolize sugars, such as lactose in yoghurt, making this food more acceptable for the consumption by individuals suffering from lactose intolerance. However, the most interesting properties that probiotics acting exogenously can have are the production of substances that may be antibiotics, anticarcinogens, or have other pharmaceutical properties.

The properties required for exogenously derived benefits from probiotics are the ability to grow in the food or the media in which the organism is placed and the specific metabolic properties which result in the potential beneficial effects mentioned above. The selection of organisms that can be helpful therapeutically and nutritionally would be based on specific properties that are desired. This can be achieved by either classical biological selection techniques or genetic engineering (Desai, 2008). Probiotics that are ingested by the host and exert their favorable properties by virtue of residing in the gastrointestinal tract must have certain properties in order to exert an effect.

2.4.1. The requirements for probiotics

It is important for the probiotic strain to survive the location, where it is presumed to be active. For a longer and perhaps higher activity, it is necessary that the strain can proliferate and colonize at this specific location (Desai, 2008). Probably only host-specific microbial strains are able to compete with the indigenous microflora and to colonize the niches. Besides this, the probiotic strain must be tolerated by the immune system and not provoke the formation of antibodies against the probiotic strain. So, the host must be immuno-tolerant to the probiotic. On the other hand, the probiotic strain can act as an adjuvant and stimulate the immune system against pathogenic microorganisms. It goes without saying that a probiotic has to be harmless to the host and there must be no local or general pathogenic, allergic or mutagenic/carcinogenic reactions provoked by the microorganism itself, its fermentation products, or its cell components after decrease of the bacteria.

For the maintenance of its favorable properties the strain must be genetically stable. For the production of probiotics it is important that the microorganisms multiply rapidly and densely on relatively cheap nutrients and that they remain viable during processing and storage (Desai, 2008). Besides the specific beneficial property, these general requirements must be considered in developing new probiotics and also for determining the scientific value of a claimed probiotic. A number of these requirements can be screened during *in vitro* experiments. Drawing up of a decision-tree can be done for the minimal requirements which can be tested *in vitro*, such as culture conditions and viability of the probiotic strains during processing and storage, sensitivity to low pH values, gastric juice, bile, pancreas, intestinal juice, and intestinal or respiratory mucus adherence to isolated cells or cell cultures and interactions with other (pathogenic) microorganisms. If these *in vitro* experiments are successful, further research can be performed during *in vivo* experiments in animals or humans.

Requirements of probiotics (Salminen and von Wright, 1998b) that are important for their use in humans are as follows:

- Survival of the environmental conditions on the location where it must be active
- Proliferation and/or colonization on the location where it is active
- No immune reaction against the probiotic strain
- No pathogenic, toxic, allergic, mutagenic, or carcinogenic reaction by the probiotic strain itself, its fermentation products or its cell components after decrease of the bacteria
- Genetically stable, no plasmid transfer
- Easy and reproducible production
- Viable during processing and storage

Probiotic foods are becoming increasingly popular. A number of health benefits have been claimed for *Bifidobacterium* sp. as depicted in Fig. 1. Therefore, inclusion of these organisms in the diet is considered to be important in maintaining good health (Champagne et al., 1994). Probiotics have anticarcinogenic properties, a specific probiotic effect, which is of three types:

- elimination of procarcinogens
- modulation of procarcinogenic enzymes
- tumor suppression.

Furthermore, the consumption of these organisms is an ideal method to re-establish the balance in the intestinal flora after antibiotic treatment (Gibson et al., 1995). There is a growing agreement relating to the beneficial aspects of specific dairy products such as fermented milk and yoghurt and of bacterial cultures that ferment the dairy products in human and animal

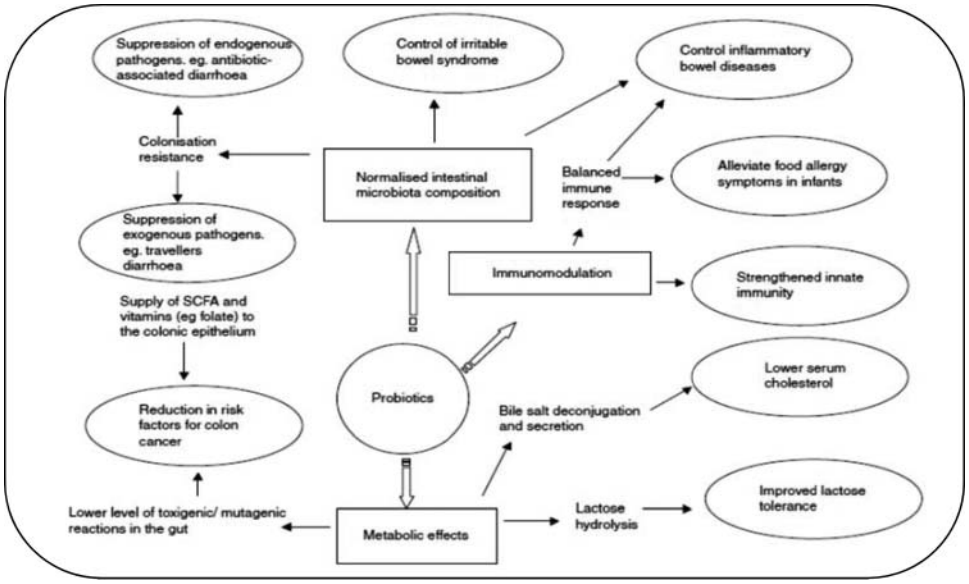

Figure 1. Various health benefits of probiotics (Parvez et al., 2006).

nutrition. Experimental and epidemiological studies provide evidence that fermented milk and bacterial cultures that are routinely used to ferment the milk reduce the risk of certain types of cancer and inhibit the growth of certain tumors and tumor cells.

Many health promoting effects have been attributed to certain *Bifidobacterium* sp. These include reduction of ammonia levels, stimulation of the immune system, and alleviation of lactose intolerance and prevention of gastrointestinal disorders (O'Sullivan, 1996). Several probiotic bacteria have been introduced in the market and the range of products in which probiotic bacteria are added is increasing. However, many of the prophylactic and therapeutic properties of these foods containing bifidobacteria are a matter of speculation, because there are inherent difficulties in obtaining definitive evidence for proposed effects of ingesting bifidobacteria.

2.4.2. Viability of the probiotics

Microorganisms introduced orally have to, at least, transiently survive in the stomach and small intestine. Although this appears to be a rather minimal requirement, many bacteria including the yoghurt-producing bacteria *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* often do not survive to reach the lower small intestine (Desai, 2008). The reason for this appears to be low pH of the stomach. In fasting individuals, the pH of the stomach is between 1.0 and 2.0 and most microorganisms, including lactobacilli, can only survive from 30 seconds to several minutes under these conditions. Therefore, in order for a probiotic to be effective, even the selection of strains that can survive in acid at pH 3.0 for sometime would have to be introduced in a buffered system such as milk, yoghurt, or other food.

2.4.3. Tolerance of acid and bile

One of the most important criteria for the selection of probiotic organisms is their ability to survive in the acidic environment of the product and in the stomach, where the pH can reach as low as 1.5. Similarly, the organisms must be able to survive in the bile concentrations encountered in the intestine. Lankaputhra and Shah (1995) showed that, among several strains of *L. acidophilus* and *Bifidobacterium* sp. studied, only a few strains survived under the acidic conditions and bile concentrations normally encountered in fermented products and in the gastrointestinal tract, respectively. Therefore, it cannot be generalized that all probiotic strains are acid and bile tolerant. Lankaputhra and Shah (1995) showed that *Bifidobacterium longum* survives better in acidic conditions and is able to tolerate a bile concentration as high as 4%. Acid and bile tolerance is strain dependent and care should be taken to select strains based on these attributes.

2.4.4. The antimicrobial properties

As mentioned previously, the intestinal microflora is a complex ecosystem. Introducing new organisms into this highly competitive environment is difficult (Fuller, 1992). Thus, organisms that can produce a product that will inhibit the growth or kill existing organisms in the intestinal milieu have a distinct advantage. The bactericidal and bacteriostatic activity of the growth media filtrates and sonicates from the bacterial cells of prospective probiotics should be tested in well-plates against a wide variety of pathogens. The ability of probiotics to establish in the gastrointestinal tract will be enhanced by their ability to eliminate competitors.

2.4.5. The anticarcinogenic properties

In the last two decades, the number of people suffering from colon cancer has been gradually increasing, particularly in industrialized countries. Studies by Goldin and Gorbach (1992) have indicated that diet and antibiotics can lower the generation of carcinogens in the colon and reduce chemically induced tumors. These effects appear to be mediated through the intestinal microflora. Additional studies have shown that the introduction of *L. acidophilus* into the diet lowered the incidence of

chemically induced colon tumors in rats (Goldin et al., 1992). A possible mechanism for these anticancer effects relies on inhibiting intestinal bacterial enzymes that convert procarcinogens to more proximal carcinogens. This technique can be expanded in the future by testing probiotics for their ability to inhibit the growth of organisms normally found in the flora that have high activities of enzymes such as β-glucuronidase, nitroreductase, azoreductase, and β-glycosidase or the capability for nitrososation. The ability of probiotics to deactivate fecal mutagens can also be used as a marker to introduce organisms that lower cancer risk.

2.4.6. The phenomenon of antagonism among bacteria

Bifidobacteria produce acetic and lactic acids in a molar ratio of 3:2. *Lactobacillus acidophilus* and *L. casei* produce lactic acid as the main end product of fermentation. In addition to lactic and acetic acids, probiotic organisms produce other acids, such as hippuric and citric acid. LAB also produce hydrogen peroxide, diacetyl, and bacteriocin as antimicrobial substances. These inhibitory substances create antagonistic environments for foodborne pathogens and spoilage organisms. Yoghurt bacteria are reported to produce bacteriocin against probiotic bacteria and vice versa (Dave and Shah, 1997).

2.4.7. The adherence of probiotic bacteria

It is not clear if adhesion to the intestinal epithelium is essential for the persistence of a probiotic in the human intestinal tract. However, adhesion seems to be a property that enhances long-term survival. The ability of microorganisms to adhere to epithelial cells is to a large extent species specific, although this may be relative (Desai, 2008). Screening of organisms for their ability to survive in the human gastrointestinal tract is not difficult. The selection of human bacterial isolates will enhance the possibility of finding organisms that will survive. The isolates can then be tested by administering orally between 10^9 and 10^{11} viable organism in a single dose with an appropriate buffering agent and the bacterial counts of the specific organism are then measured in the feces over a several week period. This technique is most successful if the natural flora does not contain the organism being tested or only in small numbers. The first question of transient survival can be established in 48–96 hours.

The evaluation of the ability of the organism to permanently establish in the gastrointestinal tract, by proliferation, can be established by continuous appearance in the feces over several weeks to several months. The fecal counts should exceed 10^6 g^{-1} of feces. The application of this screen for selecting probiotics should be encouraged. There are several tests for determining if a prospective probiotic can bind to intestinal epithelium (Desai, 2008). Radiolabeling the microorganisms with an amino acid and then counting for adhering radioactivity in either ileal cells recovered from ileostroma effluent or from buccal cells obtained by gently scraping the inside of the cheek are effective methods. Good adhesion properties should enhance the possibility of long-term survival of the organism in the intestinal tract by countering the peristaltic action of the intestine.

2.4.8. Resistance of colonization

The indigenous microflora on body surfaces inhibit the colonization of non-indigenous microorganisms. Nevertheless, in some cases (potential) pathogenic microorganisms are able to penetrate and/or colonize these body surfaces due to a massive attack of the pathogens or to a (temporarily) reduced colonization resistance (Desai, 2008). In different studies on humans and animals, beneficial microorganisms are used to improve the colonization resistance on body surfaces, such as gastrointestinal, the urogenital, and the respiratory tract.

2.4.9. Immunological enhancement

In recent years, there have been several reports indicating that lactobacilli used in dairy products can enhance the immune response of the host. Organisms that have been identified as having this property are *Bifidobacterium longum, L. acidophilus, L. casei* subsp. *rhamnosum* and *L. helveticus*. The prospective probiotics, in the appropriate settings (anticancer or infection resistance), should be tested for enhancement of the immunological response (Desai, 2008). The measurements that should be considered are lymphocyte proliferation, interleukin 1, 2 and 6, tumor necrosis factor, prostaglandin E production and serum total protein, albumin, globulin, and gamma interferon.

2.4.10. Hormone production

The possibility of genetically engineering strains of bacteria that can produce substances such as insulin, androgens, estrogens, growth hormone, or cholesterol-lowering compounds, just to mention a few is intriguing. The ability to produce in situ over a long period of time drugs or hormones that are constantly required by individuals suffering from various diseases (i.e. diabetes and hypercholesteremia) is of particular interest (Desai, 2008). There are problems to this approach, however; e.g. control of production and contamination of normal individuals with the organism. Establishing the maximum achievable production level of the organism in the gut and thereby setting an upper limit on dose may solve the first problem. The contamination problem may be more difficult to solve, although antibiotic sensitivity can be introduced into the strains, so that the organism could be rapidly eliminated if a normal individual is infected with a specifically designed probiotic. This idea may have too many regulatory problems associated with it, however, it is still something that may have potential use in human disease regulation.

2.4.11. Reduction of cholesterol

Experiments by Gilliland et al. (1984) have shown that dietary elevation of plasma cholesterol levels in pigs can be prevented by the introduction of a *L. acidophilus* strain that is bile resistant and assimilates cholesterol. These findings were supported by research conducted by Pereira and Gibson (2002) who demonstrated that probiotic strains were able to assimilate cholesterol in the presence of bile into their cellular membranes. Results, however, were influenced greatly by the bacterial growth stage and inoculum used as resting cells did not interact with cholesterol.

2.5. The common properties of probiotic bacteria

2.5.1. The important properties of probiotic bacteria

To be suitable for probiotic use, a bacterial strain should have certain characteristics. It should survive the passage through the GIT and thus be resistant to GIT conditions, including acidic pH and bile acids (Fuller, 1989; Bezkorovainy, 2001). The safety of the strain must be evident (Fuller, 1989; EFSA, 2008) and strains of human origin are usually preferred to achieve host-specific probiotic effects (Ouwehand et al., 2002). The ability to adhere to intestinal mucosa is a desired property of a probiotic because close contact and prolonged colonization may intensify the favorable effects of the probiotics (Ouwehand et al., 2002).

Good technological properties are also important for a strain to be used in a commercial probiotic product, the strain should be suitable for large-scale cultivation, remain viable and stable under storage and should not confer an unpleasant taste on the product (Saarela et al., 2000) and it should impart beneficial effects to the host (Fuller, 1989).

2.5.2. The probiotic mechanisms

Most of the proven health-effects' probiotics elicit are provided in the gastro-intestinal tract (GIT). The probiotic mechanisms of action in the GIT can be roughly divided into luminal, mucosal, and submucosal effects (Sherman et al., 2009). The basic luminal effect (an effect which appears in gut lumen) of probiotics is the improvement of intestinal microbial balance. The human GIT is colonized by a myriad of microbes, whose balanced composition and activity are essential for human health (Round and Mazmanian, 2009). Eating probiotics maintains or promotes the GIT homeostasis and probiotics have been found to stimulate the growth of indigenous beneficial gut microbes such as bifidobacteria and inhibit the growth of pathogenic or opportunistic pathogenic microbes (Ohashi and Ushida, 2009; Ouwehand et al., 2002; Sherman et al., 2009).

The examples of stimulatory effects are reported in a study by Benno et al. (1996), who showed that the consumption of probiotic *Lactobacillus rhamnosus* GG increased the number of fecal Bifidobacteria and in a study by Sui et al. (2002) in which ingestion of probiotic *Lactobacillus acidophilus* NCFM changed the colonic lactobacilli composition. Pathogenic or opportunistic pathogenic microbes are inhibited by antibacterial products such as bacteriocins and lactic acid, as shown in several in vitro studies. For example, probiotic *L. rhamnosus* GG inhibited the growth of pathogenic *Salmonella enterica* by producing lactic acid and other secreted antimicrobial molecules (Marianelli et al., 2010) and several bacteriocins produced by LAB have been shown to have antimicrobial activity against the gastric pathogen, *Helicobacter pylori* (Kim et al., 2003).

Probiotics can also adhere to the gut and prevent pathogens from occupying this living space which is called colonization resistance (Gueimonde et al., 2007; Saxelin et al., 2005; Sherman et al., 2009). Probiotic *Streptococcus thermophilus* ATCC 19258 and *L. acidophilus* ATCC 4356 have been shown to interfere with the adhesion and invasion of enteroinvasive *Escherichia coli* in human intestinal epithelial cells in vitro (Resta-Lenert and Barrett, 2003).

The mucosal effects of probiotics include the enhancement of host mucin production, which improves the ability of the mucus layer to act as an antibacterial shield (Mack et al., 2003; Sherman et al., 2009). Some probiotics, such as *E. coli* Nissle 1917 and a few *Lactobacillus* strains, induce antimicrobial peptide (e.g., defensin) production in the host and thus help the host strengthen its innate defence mechanisms (Wehkamp et al., 2004; Schlee et al., 2008). Probiotics have also been shown to enhance the integrity of the host intestinal barrier, treatment of human colonic cells with *L. rhamnosus* GG prevented injuries in the epithelial cell barrier that are induced by enterohemorrhagic *E. coli* (Johnson-Henry et al., 2008) and *L. acidophilus* LB protected human colonic cells from aspirin-induced damage in tight junctions (Montalto et al., 2004).

Submucosal effects include the effects of probiotics on the host immune system. Probiotics have been shown to improve the intestine's immunological barrier functions and alleviate the intestinal inflammatory response by mechanisms that include diverse effects on immune activation, cytokine production, immunomodulation, and inflammation (Delcenserie et al., 2008; Wells, 2011).

2.5.3. Beneficial effects of probiotics on health

The potential health effects of probiotics have been studied for several diseases and conditions using a variety of different strains and varying results have been achieved. The best proven health benefit for several probiotic strains is the reduction of the risk of diarrhea (e.g., antibiotic-associated and traveller's diarrhea) and the shortening of diarrheal episodes (Ouwehand et al., 2002; Saxelin et al., 2005; Hickson et al., 2007; Minocha, 2009; Weichselbaum, 2009). A meta-analysis of 34 blinded, randomized, placebo-controlled trials studying the effect of different probiotics (mainly *Lactobacillus* strains) in the prevention of acute diarrhea showed that probiotics significantly reduced antibiotic-associated diarrhea by 52% and acute diarrhea of various causes by 34% (Sazawal et al., 2006). Other diseases of the gut may also be alleviated with probiotics.

A meta-analysis of 20 randomized, controlled, blinded trials showed that probiotic use may be associated with an improvement in irritable bowel syndrome symptoms compared to placebo (McFarland and Dublin, 2008). The use of probiotics may be related to the relief of constipation (Koebnick et al., 2003; Weichselbaum, 2009) and lactose intolerance (Vesa et al., 2000). Probiotics may increase host immune defenses and thus decrease the frequency or duration of infections like the common cold (de Vrese et al., 2005; Weizman et al., 2005; Minocha, 2009; Weichselbaum, 2009). In a double-blind, randomized trial, the ingestion of *Lactobacillus gasseri* PA 16/8, *Bifidobacterium longum* SP 07/3 and *Bifidobacterium bifidum* MF 20/5 shortened the duration of common cold episodes in healthy adults (de Vrese et al., 2005) and among children in day-care centers, the intake of *Lactobacillus reuteri* ATCC 55730 decreased the number of days with fever (Weizman et al., 2005).

Probiotics have also been shown to be helpful in preventing allergic disorders (Kalliomaki et al., 2001; Ouwehand et al., 2002; Saxelin et al., 2005; Minocha, 2009) in children with allergic rhinitis, the consumption of *Lactobacillus casei* DN-114 001-containing fermented milk lowered the annual number of

rhinitis episodes (Giovannini et al., 2007) and the intake of *L. casei* Shirota was shown to modulate immune responses of adults suffering from seasonal allergic rhinitis (Ivory et al., 2008). To achieve the desired health effects of a probiotic, the correct dosage is required to deliver a sufficient amount of live probiotics to the GIT, as demonstrated by Whorwell et al. (2006). They reported that a dose of 1×10^8 cfu/ml of *Bifidobacterium infantis* 35624 significantly reduced the symptoms of irritable bowel syndrome, but doses of 1×10^6 and 1×10^{10} cfu/ml were not significantly different from placebo.

Probiotics have to be taken regularly because probiotics usually do not colonize the GIT permanently (Marco et al., 2006; Ohashi and Ushida, 2009; Weichselbaum, 2009). It also has to be emphasized that every probiotic strain has its own specific effects and the research results for one strain can never be directly applied to other strains. In addition, there may be differences in responses between individuals (Ohashi and Ushida, 2009). Moreover, the form in which the probiotic is ingested is also important, as has been shown with probiotic *Propionibacterium* and *Bifidobacterium* strains, which showed higher fecal counts when they were administered as capsules or yogurt than when they were consumed in cheese (Saxelin et al., 2005). The number of food and other dietary adjuncts products containing live *Bifidobacterium* and *Lactobacillus* bacteria has significantly increased over the last 20 years due in part to the beneficial effects these probiotic organisms are believed to provide (Laroia and Martin, 1991). Although research is ongoing, the available evidence indicates that ingestion of probiotic bacteria may promote desirable changes in the gastrointestinal tract of humans (Kaplan and Hutkins, 2000).

2.6. Prebiotics

There is currently much interest in the concept of actively improving the host health by managing the colonic microflora. Traditionally, this has been attempted by using probiotics. An alternative approach is the consumption of food ingredients known as prebiotics (Rycroft et al., 2001). The term "prebiotic" was first coined by Gibson and Roberfroid (1995) Prebiotic is defined as "a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, that can improve the host health." The function of prebiotics is to basically stimulate existing metabolisms in the colon (Coussement, 1996). Prebiotics are carbohydrates of relatively short chain length (Cummings et al., 2001). Additionally carbohydrates that have escaped digestion in the upper gastrointestinal tract form the predominant substrates for bacterial growth in the colon (Roberfroid, 2001).

Present evidence concerning the two most studied prebiotics, fructooligosaccharides and inulin, is consistent with their resisting digestion by gastric juice and pancreatic enzymes in vivo. In the large intestine, prebiotics, in addition to their selective effects on bifidobacteria and lactobacilli, influence many aspects of bowel function through fermentation (Campbell et al., 1997). Short-chain fatty acids are a major product of prebiotic breakdown, but as yet, no characteristic pattern of fermentation has been identified. Through stimulation of bacterial growth and fermentation, prebiotics affect bowel habit and are mildly laxative (Cummings et al., 2001). Thus, the prebiotic approach advocates administration of non-viable entities and therefore overcomes survival problems in the upper gastrointestinal tract. The prebiotic concept considers that many potentially health-promoting micro-organisms, such as bifidobacteria and lactobacilli, are already resident in the human colon.

To be an effective prebiotic, an ingredient must neither be hydrolyzed nor absorbed in the upper part of the gastrointestinal tract, have a selective fermentation such that the composition of the large intestinal microbiota is altered toward a healthier composition. Prebiotics may have many advantages over probiotics. This is first related to survivability problems. These include maintenance of viability in the product, gastric acidity, bile salts, pancreatic enzymes and proteins, competition for colonization sites and nutrients with the resident gastrointestinal flora.

Oligosaccharides are carbohydrates with a low degree of polymerization (DP) and therefore have a low molecular weight. They have been variously defined as including anything from 2 to 20 monosaccharide units. The main categories of nondigestible oligosaccharide presently available or in development as food ingredients include carbohydrates in which the monosaccharide unit is fructose, galactose, glucose, and/or xylose. Non-digestible oligosaccharides are readily water soluble and exhibit some sweetness, but solubility decreases with longer chain length. Furthermore, due to being undigested in the colon, they have caloric value but due to colonic fermentation, they have an energy contribution to food of about 1.5 kcal/g, similar to soluble fiber (Roberfroid et al., 1993).

2.6.1. Some of the most common prebiotics

2.6.1.1. Oligosaccharides. Oligosaccharides are a group of short chain nondigestible polysaccharides consisting of between 2 and 20 (approximately) saccharide units, which may be linear or branched and occur in a wide variety of foods (Shin et al., 2000). Fructooligosaccharides are widely distributed in plants such as onion, asparagus, chicory, Jerusalem artichoke, garlic, wheat and oat as well as soybean asparagus and also made by the action of fructosyl transferase on sucrose. Oligosaccharides can be commercially produced through the hydrolysis of polysaccharides (e.g. dietary fibers, starch) or through enzymatic transfer reactions from lower molecular weight sugars.

An array of oligosaccharides has been tested using various in vitro methods, animal models, and human clinical trials for their prebiotics effect on probiotic bacteria. Significant increases of bifidobacteria populations in feces after consumption of fructooligosacccharides after relatively short periods of time have been reported (Desai, 2008). They are not hydrolyzed by the human digestive enzymes, but are utilized by intestinal bacteria such as bifidobacteria (Kaplan and Hutkins, 2000), the *Bacteroides fragilis* group, peptostreptococcaceae and klebsiellae.

2.6.1.2. Inulin. Inulin is a blend of fructan chains found widely distributed in nature as plant storage carbohydrates and is present in more than 36,000 plant species. Chemically, inulin is a polydisperse β-(2,1) fructan. The fructose units in the

mixture of linear fructose polymers and oligomers are each linked by β-(2,1) bonds. A glucose molecule typically resides at the end of each fructose chain and is linked by an α-(1, 2) bond, similar to sucrose. Chain lengths of these chicory fructans range from 2 to 60 with an average degree of polymerization of 10 (Flickinger et al., 2003). The majority of inulin commercially available today is extracted from chicory roots. In vitro they selectively stimulate the growth of *Bifidobacterium* (Gibson et al., 1995).

2.6.1.3. Fructooligosaccharides (FOS). Inulin and oligofructose have a specific chemical structure which our digestive enzymes cannot hydrolyze. Both substances are metabolized as dietary fibers in our body. Inulin and oligofructose also show beneficial dietary fiber effects, such as a relief of constipation, increased stool volume and an increased fecal acidity (Coussement, 1996). Inulin and oligofructose belong to a group of carbohydrates known as nondigestible oligosaccharides (NDO), which are commonly consumed in a standard Western diet. Chemically speaking, inulin is a mixture of poly and oligosaccharides of which almost all have the chemical structure GFn (G = glucose, F = fructose and n = number of fructose units linked to one another). The maximum amount of fructoses in inulin from chicory is about 60. The links between the molecules are of a very special type, the β(2-1) form, which makes these molecules indigestible for all higher animals.

Inulin-type fructans contain both GFn (α D glucosyl- (β D fructosyl) n-1- D fructoside) and FFn (β D fructosyl-(β D fructosyl)n-1- D fructoside) molecules, with the number of fructose units varying from two to more than 70 units. The structural relatives of inulin, fructooligosaccharides (FOS, a lower molecular weight version) are well documented oligosaccharides with regard to their effect on intestinal bifidobacteria and are considered important prebiotics. Inulin naturally occurs in thousands of different plants with garlic, onion, asparagus, chicory, artichoke, and wheat being especially rich. The two different types of fructooligosaccharides are common. First, inulin extracted from chicory roots can be hydrolyzed under controlled conditions by the enzyme inulinase to produce short-chain FOS represented as Glu-α1-2(β-D-Fru 1-2)n where n = 2–9. Another FOS product known as "neosugar" or "meioligo" is a mixture of three oligosaccharides of different lengths, i.e. 1-ketose (Glu-Fru2) and 1F-β-fructosylnystose (Glu-Fru4). The mixture is enzymatically synthesized from sucrose by the transfructosylation action of β-fructosidase from the fungus *Aspergillus niger*.

It is accepted that FOS are not degraded or absorbed in the upper human gastrointestinal tract. As such, they enter the colon intact where they are susceptible to metabolism by the resident microbiota (Hidaka et al., 1986). The β configuration of anomeric C2 in fructose monomers is thought to make FOS resistant to hydrolysis by human digestive enzymes which display a high degree of specificity for glycosidic linkages (Gibson et al., 2000). In pure culture, most species of bifidobacteria are adept at the utilization of inulin-type fructans. Many other bacteria are also capable of metabolizing these substrates including *Klebsiella pneumoniae, Staphylococcus aureus* and *S. epidermis, Enterococcus faecalis* and *E. faecium, Bacteroides vulgatus, B. thetaiotaomicron, B. ovatus* and *B. fragilis, Lactobacillus acidophilus* and *Clostridium* sp. In mixed batch and chemostat culture studies, it has been demonstrated that both inulin and its hydrolysate selectively stimulated the growth of bifidobacteria, which at the end of the incubation period, become numerically predominant.

The prebiotic nature of these substrates was indicated by batch culture studies where fecal slurries were incubated with FOS, starch, polydextrose, fructose, and pectin for 12 hours (Desai, 2008) reporting the greatest increase in bifidobacteria with the FOS. Continuous culture systems inoculated with fecal slurries were later used to investigate the fermentation. In accordance with the studies conducted previously, bifidobacteria and to a lesser extent lactobacilli preferred FOS to glucose. Three-stage chemostats (gut models) confirmed an enhanced proliferation of bifidobacteria by FOS in conditions resembling the proximal colon.

2.6.1.4. Isomalto-oligosaccharides. Isomalto-oligosaccharides exist in fermented foods such as miso, soy, sauce, sake, and honey. The effect of isomalto-oligosaccharides on human fecal flora was also studied. Bifidobacteria and the *Bacteroides fragilis* group were able to utilize isomalto-oligosaccharides, but *Escherichia coli* and other bacteria were not. After the administration of 13.5 g of isomalto-oligosaccharides per day for two weeks to healthy adults, bifidobacteria remarkably increased (Kohmoto et al., 1988).

2.6.2. Consumer utilization of prebiotics

Japan is at the forefront in the development and use of prebiotics due to the influence of the food industry regulatory system. This allows some degree of health claim for a product. Prebiotics have been incorporated into many functional foods and drinks in Japan. These products include, soft drinks, candies, biscuit, frozen yoghurt, table-top sweetener, LAB drink, coffee drink and custard desserts (Desai, 2008). The oligosaccharides used in the application of these "foods for specified health use" (FOSHU) in Japan include: fructo-oligosaccharides, lactulose, lactosucrose, xylo-oligosaccharides, isomalto-oligosaccharides, soybean oligosaccharides, and transgalacto-oligosaccharides.

The incorporation of prebiotic into foodstuffs in Europe is significantly less advanced than in Japan. However, the potential market of a dietary ingredient with a carbohydrate base is enormous. This includes yoghurts, cereals, confectionary, biscuits, cakes, sauces, powdered drinks, pasta, snack foods, processed vegetables, rice, cereal bars, breads, infant formula foods, and fruit juices among others (Desai, 2008). Specific examples of prebiotic containing foods that already exist include *Frutex, Actilife, Symbalance, Aviva, LactoPro+, b2* and *Kinder-Flakes*. Although these particular products are based on fructo-oligosaccharides, new biotechnological capabilities promise to expand the prebiotic market exponentially. In addition, the main European manufacturers of prebiotics (e.g. Sensus, Orafti and Solvay) all produce prebiotic supplements to be consumed on a daily basis.

2.6.3. Selection of the suitable prebiotic

One of the biggest constraints in the development of prebiotics is the limited knowledge of these molecules. At the current time, there is little information on noncarcinogenic properties, vehicles, and characteristics. The use of prereduced,

anerobically sterilized media or an anerobic chamber is essential. Serial dilutions of specimens may be prepared in an anerobic chamber or, if the pre-reduced anerobically sterilized medium is used, in tubes gassed with oxygen-free gas. Plates or roll tubes of pre-reduced media are inoculated from these dilution series. This enables an estimate to be made of the numbers of bacteria present by counting colonies. However, in feces and with the contents of the large intestine the situation is complicated by the large number of bacterial species present (Desai, 2008). It is easy to obtain a total viable count but very laborious to obtain counts of the identifiable bacterial species. There is little information available on the efficacy and selectivity of these molecules. A useful prebiotic would have the following properties:

- Have low dosage forms
- Have a low calorific value
- Have multifunctional properties
- Easily incorporated into food
- Exert good preservative and drying action
- Target the distal colon.

2.7. Synbiotics

The term *synbiotic* is used when a product contains both probiotics and prebiotics (Jürgen and Michael, 2001). As the word alludes to synergism, this term should be reserved for products in which the prebiotic compound selectively favors the probiotic compound. However, one might argue that synergism is attained in vivo by ingestion of lactobacilli on one hand and promotion of indigenous bifidobacteria on the other hand.

The effect of synbiotics, i.e. probiotics and prebiotics mixture, on the gut microbial ecology and digestive enzyme activities in rats was investigated by Suh-Ching et al. (2005). Forty-eight SD rats weighing about 280 g were used in this study. Rats were divided into three groups according to the contents of probiotics and prebiotics mixture in the feed as control, low and high dose groups. The duration of the experiment was eight weeks compared with the control group, the fecal *Lactobacillus* and *Bifidobacterium* counts were significantly increased and the fecal Coliform organism counts were markedly reduced in the low and high dose groups. Concerning the digestive enzyme activity of jejunum, only lactase activity increased in low dose group. However, significant increase of lipase, lactase, sucrose, and isomaltase activities were observed in high dose group. Intake of low and high dosages of probiotics and prebiotics mixture significantly improved the ecosystem of the intestinal tract by increasing the probiotics population and digestive enzyme activities in rats.

2.8. Synergistic interactions among inulin, fructooligosaccharides (fos), and probiotics

The synbiotic concept has been widely used by European dairy drink and yogurt manufacturers (Niness, 1999). It reflects the synergistic relationship between the beneficial bacteria and their selective substrates to stimulate their growth when they survive passing through the stomach to the large intestines to establish their predominance (Rao, 2002). Inulin and fructooligosaccharides have their stimulating effect because of their ability to be fermented by bifidobacteria and LAB in vivo (Gibson et al., 1995) and in vitro (Kaplan and Hutkins, 2000; Perrin et al., 2001).

However, recent studies have determined that the ability of bifidobacteria to metabolize fructo-oligosaccharides and inulin is a species-dependent feature and only to a small extent a strain-dependent one related to their enzyme content (Bielecka et al., 2002; Bielecka and Biedrzycka, 2004). For example β-fructofuranosidase from *B. adolescentis* G1 has a unique substrate specificity to fructooligomers rather than inulin (Muramatsu et al., 1994; Van der Meulen et al., 2004) and the same applies for *B. bifidum* strain (Hartemink and Rombouts, 1997). On the other hand, strains of *B. longum* and *B. animalis* were able to hydrolyze the wide range of oligosaccharides with a high degree of polymerization (DP) including FOS with degree of polymiraztion 2–4 and inulin having a DP $>$ 8 (Van Laere et al., 1997; Bruno et al., 2002).

The highest viable number of bifidobacteria ($3.59–2.25 \times 10^7$ cfu/g) was obtained in the product containing *B. animalis* and FOS and was greatest with hi-amylose corn starch (Bruno et al., 2002). Viability of *B. longum* in yogurt containing FOS remained above 10^6 cfu/g for up to 21 days (Akalin et al., 2004). However, *Bifidobacterium* is significantly less tolerant to low temperature storage in milk than *L. acidophilus* (Hughes and Hoover, 1995). *Bifidobacterium lactis* has been reclassified as a subspecies of *Bifidobacterium animalis* (Meile et al., 1997).

Commercial probiotic strain *Bifidobacterium animalis ssp. lactis* Bb12 were classified with a distinctive tolerance to heat and oxygen (Simpson et al., 2005) and was isolated from over 15 dairy products in Europe as the main *Bifidobacterium* strains (Iwana et al., 1993; Meile et al., 1997; Klein et al., 1998; Gueimonde et al., 2004). *Bifidobacterium lactis* possesses the required enzyme, β-glucosidase (Martinez-villaluenge and Gomez, 2007) and β-fructofuranosidase (Janer et al., 2004a) to utilize oligosaccharides (Gopal et al., 2003) and to cleave the related β -(2,6) linked fructans containing substrate (Semjonoves et al., 2004) in MRS media and fermented milk which enhanced its growth and metabolic activity.

2.9. The safety aspects of probiotics

Probiotics are generally defined as microorganisms that, when consumed, generally confer a health benefit on humans (David, 2008). There is considerable interest in probiotics for a variety of medical conditions and millions of people around the world consume probiotics daily for perceived health benefits. *Lactobacilli, Bifidobacteria, and Lactococci* have generally been regarded as safe. There are three theoretical concerns regarding the safety of probiotics, first, the occurrence of disease, such as bacteremia or endocarditis, second, toxic or metabolic effects on the gastrointestinal tract; and third, the transfer of antibiotic resistance in the gastrointestinal flora. The evidence for safety of the use of or the study of probiotics was examined. Although there are rare cases of bacteremia or fungemia related to the use of probiotics, epidemiologic evidence suggests no population increase in risk on the basis of usage data. There have been many controlled clinical trials on the use of probiotics that demonstrate safe use. The use of probiotics in clinical trials should be accompanied by the use of a data-safety monitoring

board and by knowledge of the antimicrobial susceptibilities of the organism used.

Lactobacilli have a long history of safe use in foods and dairy products (Hammes and Tichaczek, 1994). There is a natural association of lactobacilli with human flora and lactobacilli are found in animals as well as plants. LAB have traditionally been used in fermented milks and by different societies around the world for the treatment of intestinal disturbances, especially in children. Rarely, lactic acid bacilli will cause infection in humans, which has manifested as either bacteremia or endocarditis, particularly in immunocompromised hosts (Salminen et al., 2002). Lactobacilli fall into the category of organisms classified as "generally regarded as safe" (Cunningham Rundles et al., 2000). Organisms that are generally regarded as safe include lactobacilli, lactococci, *Bifidobacterium and* yeast. There are other probiotic organisms, such as *Enterococcus, Bacillus* and other spore-forming bacteria, as well as streptococci that are not generally regarded as safe but have been used as probiotics.

2.10. The lactic acid bacteria

2.10.1. The history pertaining to lactic acid bacteria

Lactic acid fermentation is an old invention. Many different cultures in various parts of the world have used fermentation to improve the storage qualities and nutritive value of perishable foods such as milk, vegetables, meat, fish, and cereals. The organisms that produce this type of fermentation, LAB, have had an important role in preserving food. In developed world, LAB are mainly associated with fermented dairy products such as cheese, buttermilk, and yogurt (Desai, 2008). The use of dairy starter cultures has become an industry during this century. The concept of the group name "lactic acid bacteria" was created for bacteria causing fermentation and coagulation of milk and defines as those which produce lactic acid from lactose. The family name Lactobacteriaceae was applied by Orla-Jensen (1919) to a physiological group of bacteria producing lactic acid alone or acetic and lactic acids, alcohol and carbon dioxide. Today, LAB are regarded as synonymous by and large with the family Lactobacteriaceae (Breed et al., 1957).

LAB are a group of gram-positive bacteria united by a constellation of morphological, metabolic, and physiological characteristics. They are non-sporing, carbohydrate-fermenting lactic acid producers, acid tolerant of non-aerobic habitat, and catalase negative. Typically they are non-motile and do not reduce nitrite. They are subdivided into four genera *Streptococcus, Leuconstoc, Pediococcus, and Lactobacillus*. As per the recent taxonomic revisions, it has been suggested that the LAB group could be comprised of genera *Aerococcus, Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, Streptococcus, Tetragenococcus, and Vagococcus*. Originally, bifidobacteria were included in the genus *Lactobacillus* and the organism was referred to as *Lactobacillus bifidus*.

Although the classification of LAB into different genera is mainly based on the characteristics used by Orla-Jensen (1919), however, confusion was still prevalent when the monograph of Orla-Jensen (1919) appeared. This work has had a large impact on the systematic of LAB and although revised to some extent, it is still valid and the basis of classification remarkably unchanged. The classification of LAB into different genera is largely based on morphology, mode of glucose fermentation, growth at different temperatures and configuration of the lactic acid produced, ability to grow at high salt concentrations, and acid or alkaline tolerance. Even some of the newly described genera of LAB, additional characteristics such as fatty acid composition and motility are used as the basis of classification.

In the present, an increasing number of health food and so-called functional foods as well as pharmaceutical preparation are promoted with health claims based on the characteristics of certain strains of LAB. Most of these strains, however, have not been thoroughly studied and consequently the claims are not well substantiated. Moreover, health benefits are judged mainly using subjective criteria. Additionally the specific bacterial strains used in the studies are often poorly identified. Most information about the health effects of LAB is thus subjective.

The term LAB was used synonymously with "milk souring organisms." Important progress in the classification of these bacteria was made when the similarity between milk-souring bacteria and other lactic-acid producing bacteria of other habitats was recognized (Axelsson, 1993). LAB are generally associated with habitats rich in nutrients, such as various food products (milk, meat, vegetables), but some are also members of the normal flora of the mouth, intestine, and vagina of mammals. The genera that, in most respects, fit the general description of the typical LAB are (as they appear in the latest *Bergey's Manual* from 1986) *Aerococcus, Lactobacillus, Leuconostoc, Pediococcus*, and *Streptococcus*. The genera *Lactobacillus, Leuconostoc* and *Pediococcus* have largely remained unchanged, but some rod-shaped LAB, previously included in *Lactobacillus*, is now forming the genus *Carnobacterium* (Collins et al., 1987).

2.10.2. Lactobacillus as potential probiotic bacteria

Lactobacilli colonize the gastrointestinal and urinary tracts of humans, making them an integral part of the microbial flora (Slover and Danziger, 2008). However, in certain circumstances, they can cause disease. Although lactobacilli are often misidentified as streptococci, it is highly likely that these infections will be attributed to them due to current improvements in laboratory techniques.

A recent resurgence in interesting natural products has caused an increased focus on the use of probiotics. Many of these probiotic formulations contain *Lactobacillus* species. Although there have been reports of lactobacilli providing benefit in infectious diarrhea, the use of these probiotic products in immunosuppressed or critically ill patients is not advised, since these populations are at increased risk of developing infections due to lactobacilli.

Study of *Lactobacillus* as probiotic bacteria was carried out by Nowroozi et al. (2004). Because of inhibitory effect, selected probiotic lactobacilli may be used as biological preservative, so, the aim of the study was to present some data on *lactobacillus* as probiotic bacteria. LAB were isolated from sausage. Each isolate of *lactobacillus* species was identified by biochemical tests and comparing their sugar fermentation pattern. Antibacterial activities were done by an agar spot, well diffusion, and blank disk method. Enzyme sensitivity of supernatant fluid and concentrated cell free culture after treatment with α-amylase, lysozyme, and trypsin was determined. The isolated bacteria were

L. plantarum, L. delbruekii, L. acidophilus, L. brevis. The isolated bacteria had strong activity against indicator strains. The antibacterial activity was stable at 100°C for 10 minutes and at 56°C for 30 minutes, but activity was lost after autoclaving. The maximum production of plantaricin was obtained at 25–30°C at pH 6.5. As lactobacilli that used to process sausage fermentation are producing antimicrobial activity with heat stability bacteriocin, so these bacteria may be considered to be a healthy probiotic diet. Lactobacilli originally isolated from meat products are the best candidates as probiotic bacteria to improve the microbiological safety of these foods.

The efficacy of a probiotic drink containing Lactobacillus for the prevention of any diarrhea associated with antibiotic use and that caused by Clostridium difficile in 135 hospital patients (mean age 74) taking antibiotics was investigated by Hickson et al. (2007). Exclusions included diarrhea on admission, bowel pathology that could result in diarrhea, antibiotic use in the previous four weeks, severe illness, immunosuppression, bowel surgery, artificial heart valves, and history of rheumatic heart disease or infective endocarditis. Consumption of a 100 g (97 ml) drink containing Lactobacillus casei, L bulgaricus and Streptococcus thermophilus twice a day during a course of antibiotics and for one week after the course finished. The placebo group received a long life sterile milkshake. Primary outcome was the occurrence of antibiotic associated diarrhea and the secondary outcome was the presence of C difficile toxin and diarrhea. About 7/57 (12%) of the probiotic group developed diarrhea associated with antibiotic use compared with 19/56 (34%) in the placebo group. The absolute risk reduction was 21.6% (6.6 to 36.6%) and the number needed to treat was 5 (3 to 15). No one in the probiotic group and 9/53 (17%) in the placebo group had diarrhea caused by C difficile. The absolute risk reduction was 17% (7to 27%) and the number needed to treat was 6 (4 to 14). Consumption of a probiotic drink containing L casei, L bulgaricus and S thermophilus can reduce the incidence of antibiotic associated diarrhea and C difficile associated diarrhoea. This has the potential to decrease morbidity, healthcare costs, and mortality if used routinely in patients aged over 50.

Standard therapies for antibiotic-associated diarrhea (AAD) and *Clostridium difficile* associated diarrhea (CDAD) have limited efficacy (Xing et al., 2010). Probiotic prophylaxis is a promising alternative for reduction of AAD and CDAD incidence. In this single-center, randomized, double-blind, placebo-controlled dose-ranging study, 255 adult inpatients were randomized to one of the three groups, two probiotic capsules per day (Pro-2, $n = 86$), one probiotic capsule and one placebo capsule per day (Pro-1, $n = 85$), or two placebo capsules per day ($n = 84$). Each probiotic capsule contained 50 billion cfu of live organisms (*Lactobacillus acidophilus* CL1285® + *Lactobacillus casei LBC80R*® Bio-K + CL1285). Probiotic prophylaxis began within 36 h of initial antibiotic administration, continued for 5 days after the last antibiotic dose and patients were followed for an additional 21 days. Pro-2 (15.5%) had a lower AAD incidence versus Pro-1 (28.2%). Each probiotic group had a lower AAD incidence versus placebo (44.1%). In patients who acquired AAD, Pro-2 (2.8 days) and Pro-1 (4.1 days) had shorter symptom duration versus placebo (6.4 days). Similarly, Pro-2 (1.2%) had a lower CDAD incidence vs. Pro-1 (9.4%). Each treatment group had a lower CDAD incidence vs. placebo (23.8%). Gastrointestinal symptoms were less common in the treatment groups vs. placebo and in Pro-2 versus Pro-1. The proprietary probiotic blend used in this study was well tolerated and effective for reducing risk of AAD and in particular, CDAD in hospitalized patients on antibiotics. A dose-ranging effect was shown with 100 billion cfu, yielding superior outcomes and fewer gastrointestinal events compared to 50 billion cfu.

2.10.3. Probiotic Lactobacillus casei and Lactobacillus acidophilus

Optimization of probiotic *Lactobacillus casei* ATCC 334 production using date powder as carbon source was carried out by Sharav et al. (2012). This study was conducted to optimize culture conditions for economic production of a probiotic bacterium, *Lactobacillus casei* ATCC 334, in which palm date powder was applied for the first time as a low-cost main carbon source. The effect of 11 factors on bacterial growth was investigated using the Taguchi experimental design and three factors including palm date powder, tryptone and agitation rate were found to be the most significant parameters. The optimum conditions including date powder concentration, 38 g/L, tryptone concentration, 30 g/L and an agitation rate of 320 rpm were determined by response surface methodology of Box–Behnken. A third-order polynomial model was suggested to predict the design space following which the predicted values were validated experimentally. The maximum log value of the viable cells in the optimized alternative medium was 9.97 at 24 h of incubation which was comparable to that obtained in the complex and expensive MRS medium.

The researches on probiotic bacteria in food systems have proved a low viability during preservation, difficulty of their colonization and survival in vivo, fact that diminishes the beneficial activity on the customers' health (Aida et al., 2011). The main purpose of this study was the improvement of the behavior and functionality of probiotic bacteria *Lactobacillus casei spp. paracasei* in fermenting milk by adding oat bran (*Avena sativa*) and buckwheat flour (*Fagopyrum esculentum*), vegetal substrate rich in bioactive compounds. The best results regarding probiotic functionality and stability were obtained by adding 5% and 9% oat bran or buckwheat flour, respectively, in fermentative medium. The adding of the vegetal substrate during fermentation also increases the resistance of probiotic bacteria in simulated gastric juice during 90 min of incubation.

Salmonella enteritidis infection has received attention during recent years owing to its high prevalence worldwide. In the study conducted by Jain et al. (2008), the protective effect of probiotic dahi (curd) supplemented with *Lactobacillus acidophilus* and *L. casei* against *Salmonella enteritidis* infection in mice was investigated. Seven days pre-feeding with probiotic dahi significantly increased anti-*S. enteritidis* sIgA (secretary IgA) antibodies and lymphocyte proliferation in *S. enteritidis* infected mice. IL-2, IL-6 and IFNγ production were significantly increased in supernatant of cultured splenocytes collected from mice pre-fed with probiotic dahi, while IL-4 levels were not changed significantly. Moreover, activities of β-galactosidase and β-glucuronidase and counts of *S. enteritidis* in intestine, liver and spleen were decreased, whereas total lactobacilli in feces were increased in mice pre-fed with probiotic

dahi. Pre-feeding of probiotic dahi for seven days was more effective than two days pre-feeding. Thus, the results indicate that, pre-feeding with probiotic dahi ameliorated *S. enteritidis* infection by stimulating specific and non-specific immune response. Above all, it lowered colonization of gastrointestinal tract as well as translocation of *S. enteritidis*.

Determination of the efficacy of triple therapy supplemented with a specially designed fermented milk product containing specific probiotic *Lactobacillus casei* (*L. casei*) DN-114 001 strain on *Helicobacter pylori* eradication in children was investigated by Sýkora et al. (2005). *Lactobacillus* species possess in vitro activity against *H. pylori*. There are no consistent data on the impact of eradication therapy supplemented with probiotics on *H. pylori* cure rates in childhood in vivo. In a multicenter, prospective, randomized, double-blind controlled study, 86 symptomatic *H. pylori*-positive children were randomized either to receive the control treatment of omeprazole, amoxicillin and clarithromycin (OAC) for 7 days or the test treatment of omeprazole, amoxicillin and clarithromycin for 7 days supplemented with fermented milk (Actimel) containing *L. casei* DN-114 001 (OAC-LC), for 14 days. The status of *H. pylori* was assessed at four weeks following therapy using two noninvasive tests. Intention-to-treat (ITT) based eradication rates for the OAC-LC group were 84.6% (95% CI, 71.2–95.5%) and 91.6% (95% CI, 76.9–98.2%) by per-protocol (PP) analysis. Eradication in the OAC group was 57.5% (95% CI, 42.2–72.3%) in the ITT set and 61.3% (95% CI, 44.4–75.0%) in the PP group. Eradication success was higher in the OAC-LC group compared with the OAC group in both ITT and PP analysis. Primary resistance for clarithromycin could be determined in 21.2%. Side effects were infrequent. Drug compliance was good throughout the study. Supplementation with fermented milk, containing live special probiotic *L. casei* DN-114 001, confers an enhanced therapeutic benefit on *H. pylori* eradication in children with gastritis on triple therapy.

2.11. Probiotic juices

Probiotic bacteria are widely used in producing food based on their positive qualities. Interest in the incorporation of the probiotic bacteria into other products apart from the dairy products has been increasing and represents a great challenge. The recognition of dose delivery systems for the probiotic bacteria has also resulted in research efforts aimed at developing probiotic foods outside the dairy sector. Production of probiotic juices has been given more consideration in the recent years. Fruit juice containing the probiotics has been recently developed due to an increased concern in the personal health of the consumers.

In an investigation carried out by Ravinder et al. (2012) two *Lactobacillus* isolates, viz. *L. plantarum* and *L. acidophilus*, were observed to be able not only to survive but to utilize fruit juices for their cell synthesis, as indicated by a decrease in fruit sugar and pH and increase in acidity. *Lactobacillus acidophilus* was found to consume the sugar at a faster rate than *L. plantarum*, although the fall in sugar and pH and increase in acidity was faster during the first 24 h and became a little slower during the next 48 h, which could be due to the accumulation of too much acid during the initial 24 h of fermentation. Still, both cultures were found to be able to survive in fermented juices with high acidity and low pH. Therefore, they concluded that such probiotic-fortified fruit juices could certainly be exploited as a medium for the delivery of probiotics and could be used as a functional healthy beverage to promote better health and nutrition of the population, especially for those who are allergic or intolerant to milk-based products.

In a study conducted by Mohammad et al. (2012) production of healthful probiotic drinks with apple and orange concentrates was assessed (brix 11 and 15). Milk and glucose, maltose and lactose were applied as growth supplements. After being produced, the products were incubated to let *Lactobacillus acidophilus*, and *Bifdobacterium bifidum* grow. The samples' acidity and pH were measured as well as the number of microbes was counted using "direct microscopic count" method. Raising the juices' brix resulted in acidity elevation which was not an appropriate situation for bacteria to grow. Adding milk to these products made up a more suitable situation for bacterial growth than the one without concentrate but resulted in lower shelf life period. Sugars were not effective on bacterial growth but glucose and lactose had positive effects on increasing shelf life period. The results of the questionnaire were analyzed, indicating no significant difference between odors, tastes, and colors of the samples ($p < 0.05$). The samples were analyzed for acidity and *Lactobacillus acidophilus* and *Bifidobacterium bifidum* numbers by non-parametric statistical test, "manviteni" and "croscal valis" tests. It was conceived that more acidity in brix 15 than 11 was due to the orange concentrate influence on acidity in comparison with apple concentrate with no effects.

Probiotic beverage production from cashewapple juice fermented with *Lactobacillus casei* was carried out by Ana et al. (2011). They optimized the conditions of *Lactobacillus casei* NRRL B-442 cultivation in cashewapple juice, as well as, determined the proper inoculum amount and fermentation time. Moreover, the survivability ability of the probiotic bacteria in cashewapple juice during refrigerated storage (4°C) for 42 days was investigated. The optimum conditions for probiotic cashewapple juice production were initial pH 6.4, fermentation temperature of 30°C, inoculation level of 7.48 log cfu/ml (*L. casei*) and 16 h of fermentation process. It was observed that the *L. casei* grew during the refrigerated storage. Viable cell counts were higher than 8.00 log cfu/ ml throughout the storage period (42 days). The values of lightness, yellowness, and total color change increased and the values of redness reduced along the fermentation and refrigerated storage periods. The fermented juice with *L. casei* is a good and healthy alternative functional food containing probiotics. Cashewapple juice showed to be as efficient as dairy products for *L. casei* growth.

Although the survival of probitoics in fruit juices, which were nominated to be a good probiotic carrier, could be improved by using microencapsulation technique in alginate bead coated with chitosan, the satisfactoriness of the consumer must be considered. Consumer assessment and sensory evaluation of this product were performed by consumers in Thailand and by using descriptive analysis, respectively (Krasaekoopt and Kitsawad, 2010). Four hundred consumers from Bangkok and the suburbs of Bangkok were served with orange and grape juices containing probiotic beads together with questionnaire

in order to determine the consumer demographic, buying behavior and consumer acceptance. Most consumers bought fruit juice due to its taste (9.6) and nutritional value (8.9). However, the addition of probiotic beads influenced the sensory quality of the product by increasing the swallowing difficulty and remaining particles of the products. The majority of consumers accepted orange and grape juices containing probiotic beads (82.3 and 84.3%, respectively), giving scores of texture and overall preferences as 6.6 and 6.7 for orange juice and 6.8 and 6.9 for grape juice. Application of probiotic beads also increased turbidity of grape juice. Moreover, more than 86% of the participants were willing to try and purchase the product, reflecting existence of a potential market for fruit juice containing probiotic beads.

Improving the stability of probiotic bacteria in model fruit juices using vitamins and antioxidants was investigated by Shah et al. (2010). This study examined the survival of probiotic bacteria in a model fruit juice system. Three different strains of probiotic bacteria were used in this study: HOWARU *Lactobacillus rhamnosus* HN001, HOWARU *Bifidobacterium lactis* HN001, and *Lactobacillus paracasei* LPC 37. The probiotic bacteria were inoculated into model juice with various vitamins and antioxidants, namely white grape seed extract, green tea extract, vitamin B2, vitamin B3, vitamin B6, vitamin C, and vitamin E. The model juice without any additives was used as a control. Their viability was assessed on a weekly basis using plate count method. The model juice was made with sucrose, sodium citrate, citric acid powder, and distilled water and was pasteurized before use. Their findings showed that probiotic bacteria did not survive well in the harsh environment of the model fruit juice. However, the model juice containing vitamin C, grape extract and green tea extract showed better survival of probiotic bacteria. The model juice containing grape seed extract, green tea extract, and vitamin C had the same initial population of 8.32 log cfu/ ml and at the end of the six-week storage period it had an average viability of 4.29 log cfu/ ml, 7.41 log cfu/ ml, and 6.44 log cfu/ ml, respectively. Juices containing all other ingredients tested had viable counts of <10 cfu/ ml at the end of the six-week storage period.

Survival of Free and Microencapsulated Probiotic Bacteria in Orange and Apple Juices was done by Ding and Shah (2008). They investigated the survival of free and microencapsulated probiotic bacteria in orange and apple juices. Eight different strains of probiotic bacteria were used in this study including *Lactobacillus rhamnosus, Bifidobacterium longum, L. salivarius, L. plantarum, L. acidophilus, L. paracasei, B. lactis* type Bi-04, and *B. lactis type* Bi-07. The free or microencapsulated probiotic bacteria were inoculated into orange and apple juices and their viability was assessed on a weekly basis for up to six weeks. The °Brix, malic acid concentration and pH were also monitored. Encapsulated probiotic bacteria survived in fruit juices throughout the six weeks of storage, whereas free probiotic bacteria lost their viability within five weeks. In general, fruit juices containing microencapsulated probiotic bacteria were more stable than those containing free probiotic organisms.

Physico-chemical analysis of celery and beetroot juices with and without pulp, fermented with a bifidobacteria culture was studied by Moraru et al. (2007). The pH, acidity (as lactic acid), fermentative and reducing sugars were analyzed during 48 h of fermentation. Also, the evolution of bacteria number was assayed in order to achieve a level appropriate for a probiotic product. During fermentation, the sugars from the celery juice are rapidly consumed and the acidity is 4.2 times higher and in beetroot juice, the process of fermentation is slower (small slope) and the lactic acid accumulation is only 2.7 times. Although the level of acidity is different, the pH values are similar. This could be explained by the different type of acids present in two juices or by the existence in the beetroot juice of a higher level of nitrogenous compounds, which act as buffers. The bacterial count demonstrated that only after 36–48 h of fermentation a level of 10^7–10^8 cells is attained, values which are characteristic to a probiotic product. The fermented beetroot juice has a pleasant taste, while the celery juice had a pronounced sour taste and required to be corrected.

Probioticated watermelon juice was produced using four strains of lactobacilli namely *Lactobacillus casei, L. acidophillus, L. fermentum*, and *L. plantarum* by Fazeli et al. (2007). The watermelon juice was pasteurized for 30 min at 63°C and was inoculated with a 24 h culture of individual lactobacilli and incubated at 37°C. All of the lactobacilli were capable of growing in watermelon juice and reached a cell density of 10^8 cfu/ml after 48 h incubation at 37°C. Overnight culture of *S. typhi*murium was added to probioticated watermelon juice and reduction of the viable cells were assayed, on bismuth sulfite agar medium for 24 h. Antimicrobial activities of the lactobacilli cells against the test strain of *Salmonella* were also determined by measuring the diameter of growth inhibition zone in agar spot test. All of the lactobacilli could inhibit the growth of *S. typhimurium* with *L. casei* being the most potent. *Salmonella typhimurium* was totally eradicated in probioticated watermelon juice after 2–6 h. The probioticated watermelon juices could differ in their antagonistic activities against *Salmonella* which could be due to the metabolite secreted by the LAB specially a type of organic acid.

Probiotication of tomato Juice by LAB was undertaken to determine the suitability of tomato juice as a raw material for production of probiotic juice by four LAB namely *Latobacillus acidophilus* LA39, *Lactobacillus plantarum* C3, *Lactobacillus casei* A4 and *Lactobacillus delbrueckii* D7 by Yoon et al. (2004). Tomato juice was inoculated with a 24-hour-old culture and incubated at 30°C. Changes in pH, acidity, sugar content, and viable cell counts during fermentation under controlled conditions were measured. The lactic acid cultures reduced the pH to 4.1 or below and increased the acidity to 0.65% or higher and the viable cell counts (cfu) reached nearly $1.0–9.0 \times 10^9$/ml after 72 h fermentation. The viable cell counts of the four LAB in the fermented tomato juice ranged from 10^6 to 10^8 cfu/ml after four weeks of cold storage at 4°C. Probiotic tomato juice could serve as a health beverage for vegetarians or consumers who are allergic to dairy products.

Survival of probiotic bacteria, *Lactobacillus casei* 01 and *Lactobacillus acidophilus* TISTR 450, in commercial fruit juices during low temperature storage was investigated to assure that these bacteria could remain above the therapeutic level in the product that could provide the benefit to the consumer (Krasaekoopt et al., 2003). The probiotic cells both as free cells and microencapsulated cells in alginate beads coated with

chitosan were added as 10% into five commercial fruit juices as grape, pineapple, apple, tangerine, and red orange. The products were kept at 4°C for four weeks. The survival of encapsulated probiotic bacteria was higher than free cells by approximately 4 logs after four-week storage. In addition, the number of microencapsulated probiotic bacteria was above the therapeutic minimum level (10^7 cfu/ml) throughout the storage. The viabilities of microencapsulated probiotic bacteria among various types of fruit juices were not significantly different ($p > 0.5$). Moreover, there were no changes in acidity of fruit juices during storage.

2.12. Synbiotic juices

Studies on Anti Diarrhoeal Activity of Synbiotic Plum Juice were carried out by Sheela and Suganya (2012). This study evaluated that effect of prebiotic food containing oligosaccharide to enhance the growth and activity of probiotic strains. Plums juice probioticated using different strains of probiotics are *Lactobacillus kefiranofaciens, Candida kefir* and *Saccharomyces boluradii*. To select a suitable prebiotics like inulin for the development of synbiotic plum juice and for food preservation. Synbiotic plum juice tested for antibacterial activity against diarrhea causing pathogen such as *Esherichia coli, Staphylococcus aureus, Salmonella paratyphi A, Shigella dysenteriae, Vibrio cholerae*. Analysis of identified compound from synbiotic plums juice was done using GC-MS.

2.13. Microencapsulation of living cells

2.13.1. The meaning of the term "encapsulation" and its purpose

In order to entrap any substance in a material for producing particles having a diameter of a few nanometers to a few millimeters, encapsulation is carried out. Encapsulation is a physiochemical or mechanical process (Chen and Chen, 2007). The substance which is encapsulated is referred to as the core material. The term "coating" or "shell" is used for the matrix in which the core material is dispersed. Food grade carrier material is used in case the encapsulated product is to be employed in the food industry. The carrier material is such that it is able to form a barrier in order to protect the encapsulated substance. The encapsulated bioactive components can be employed for serving various purposes in the food industry such as masking flavors, colors and odors, controlling oxidative reactions, extending the shelf life, enabling sustained and controlled release. The encapsulation of the probiotic cells is usually carried out to protect of the probiotic living cells against and unfavorable or adverse environment (Champagne and Kailasapathy, 2008; Zuidam and Shimoni, 2009).

There are different types of encapsulates but the two most common types are the reservoir type and the matrix type. In the former type, a shell is present around the core material and therefore, the term "capsule" is also used for this type. In the latter type, the active agent is dispersed over the carrier material which can also be found on the surface. When these two types combine, another type of encapsulate results in which the active agent is recovered by a coating (Zuidam and Shimoni, 2009). Encapsulation ultimately imparts a structure, allowing the creation of new function or innovative systems for probiotic products (Poncelet et al., 1993). The evolution of the technology involving the encapsulation of the probiotic living cells took place from the biotechnology industry employing the immobilized cell culture technique. When encapsulating the probiotics, two types of problems arise, one is owing to their size (generally having a diameter between 1 and 5 mm), which immediately excludes nanotechnologies. The second difficulty faced while encapsulating probiotics is to keep them in a living/viable state which is determined by the selection of the appropriate technology for microencapsulation (Champagne and Fustier, 2007a; Zuidam and Shimoni, 2009).

In order to encapsulate the probiotic cells, different technologies can be used. The microencapsules produced by different technologies exhibit variation in size. Microencapsules having broad size range (0.2–5000 mm) are produced by employing the technique of emulsification. Small-sized microencapsules (not less than 300 mm) are produced by employing the method of extrusion. The proclaimed probiotic health benefits are strongly based on the ability of the microorganism to survive as well as multiply in the host. As per the study conducted by De Vos et al. (2010), certain factors namely, the concentration of lactic acid and acetic acid, reduction in pH, the presence of hydrogen peroxide and high content of oxygen result in reduction in viability of the probiotics in dairy products such as yoghurt and frozen desserts. The survival rate of microorganisms in dairy products as well as the GI tract can be enhanced by employing encapsulation (Krasaekoopt et al., 2003; Picot and Lacroix, 2004). Besides the protection of the probiotic cell against the unfavorable environment, another major aim of encapsulating the probiotic cells is to allow their release in the intestine in a viable and metaboloically active state (Picot and Lacroix, 2004). The physico-chemical properties of the microencapsules determine the viability of the probiotic cells. According to Chen and Chen (2007), the indispensible parameters which need to be taken care of are the bacterial strains, concentration of the coating material, the initial cell count, the particle size, etc. In order to maintain the integrity of the microencapsules in the food matrix as well as in the GI tract's upper part, they need to be water insoluble. The properties of the particles should be such that they allow the liberation of the cells during the intestinal phase in a progressive manner (Picot and Lacroix, 2004; Ding and Shah, 2007).

Generally, the encapsulation technology is held in three different stages. In the initial stage, the bioactive component is incorporated in either a solid or a liquid matrix. The incorporation will comprise of a dissolution or dispersion in the matrix in the case of a liquid core, while it will be agglomeration or an adsorption in the case of a solid core. In the second step, the dispersion of the liquid matrix is carried out besides the pulverization of a solution on the solid matrix. The last step comprises of stabilization by a process which could be physical (evaporation, solidification, and coalescence), chemical (polymerization), or physicochemical (gelification) as reported by Poncelet and Dreffier (2007). Microparticles can also be obtained using liposome, molecular inclusion coacervation, co-crystallization, etc. (Champagne and Kailasapathy, 2008). Owing to their high cost and large size of the bacteria these techniques find limited use.

2.13.2. Microencapsulation of probiotic bacteria

Eight strains of probiotic bacteria, including *Lactobacillus rhamnosus, Bifidobacterium longum, L. salivarius, L. plantarum, L. acidophilus, L. paracasei, B. lactis* type Bl-O4 and *B. lactis* type Bi-07, were studied for their acid, bile, and heat tolerance (Ding and Shah, 2007). Microencapsulation in alginate matrix was used to enhance survival of the bacteria in acid and bile as well as a brief exposure to heat. Free probiotic organisms were used as a control. The acid tolerance of probiotic organisms was tested using HCl in MRS broth over a two-hour incubation period. Bile tolerance was tested using two types of bile salts, oxgall and taurocholic acid, over an eight-hour incubation period. Heat tolerance was tested by exposing the probiotic organisms to 65°C for up to one hour. Results indicated microencapsulated probiotic bacteria survived better ($p < 0.05$) than free probiotic bacteria in MRS containing HCl. When free probiotic bacteria were exposed to oxgall, viability was reduced by 6.51 log cfu/ml, whereas only 3.36 log cfu/ml was lost in microencapsulated strains. At 30 min of heat treatment, microencapsulated probiotic bacteria survived with an average loss of only 4.17 log cfu/ml, compared to 6.74 log cfu/ml loss with free probiotic bacteria. However, after one hour of heating both free and microencapsulated probiotic strains showed similar losses in viability. Overall microencapsulation improved the survival of probiotic bacteria when exposed to acidic conditions, bile salts, and mild heat treatment.

2.13.3. Materials employed for the encapsulation of probiotics

2.13.3.1. Alginate. Alginate is a naturally derived polysaccharide which is extracted from various species of algae. It is composed of β-D-mannuronic and α-L-guluronic acids. Based on the source of alginate, the composition of the polymer chain is different in amount and in the sequential distribution. These factors determine the functional properties of alginate which is used as a supporting material. In the encapsulation of cells, the hydrogels of alginate are used extensively (Rowley et al., 1999). According to the study of Krasaekoopt et al. (2003), calcium alginate, owing to it being non-toxic, biocompatible, cheap and simple, is the preferred material for the encapsulation of the probiotic cells. The use of alginate as the encapsulating material has certain disadvantages as well. The main challenge posed is that the alginate beads are sensitive to the acidic environment (Mortazavian et al., 2008). This makes the resistance of the microparticles under the conditions of stomach doubtful. In addition to this, the scaling up of the process using the alginate is quite difficult. Besides these pitfalls, the obtained microparticles are quite porous, which is undesirable as the objective of encapsulation is protecting the cells from the harsh environmental conditions (Gouin, 2004). According to Krasaekoopt et al. (2003), these problems can be overcome by using a mixture of alginate and some other polymer compound. The method of coating the capsule using another compound or the use of various additives for applying structural modification of the alginate may be put to use. One of the commonly used methods is mixing starch with alginate (Sultana et al., 2000; Sun and Griffiths, 2000; Truelstrup-Hansen et al., 2002; Krasaekoopt et al., 2003). An improvement in probiotic encapsulation effectiveness has been reported by employing the use of this method.

2.13.3.2. k-Carrageenan. One of the natural polymer which is used in the food industry is k-Carrageenan. A temperature range of 40–50°C is required for using k-Carrageenan. The cells are added to the polymer solution at this temperature. The gelation occurs when the mixture is brought to room temperature (Krasaekoopt et al., 2003). The addition of potassium ions stabilizes the formed microparticles. According to Dinakar and Mistry (1994), the probiotic bacterial cells are kept in a viable state when encapsulated in k-carrageenan. Chen and Chen (2007) reported that the gels obtained by this method are unable to withstand stress and are brittle.

2.13.3.3. Gellan gum and xanthan gum. Gellan gum is a microbial polysaccharide, which is derived from *Pseudomonas elodea*. It consists of a repeating unit of four monomers, namely glucose, glucuronic acid, glucose, and rhamnose (Chen and Chen, 2007). Encapsulation of probiotic cells was carried out by using a mixture of xanthan–gelan gum (Sultana et al., 2000; Sun and Griffiths, 2000). As compared to the use of alginate for encapsulation, high resistance toward acid conditions is exhibited by the use of combination of xanthan–gelan gum.

2.13.3.4. Chitosan. Chitosan is a linear polysaccharide which is made up of glucosamine units. In the presence of anions and polyanions, the constituent units can polymerize by formation of a cross-link. A good method of delivering viable bacterial cells to the colon is encapsulation of the probiotic cells using a coating of alginate and chitosan as this provides protection in the simulated GI conditions (Chávarri et al., 2010). The use of chitosan exhibits some disadvantages. Groboillot et al. (1993) reported inhibitory effects of chitosan on LAB.Mortazavian et al. (2008) reported that encapsulation using chitosan does not exhibit appreciable efficiency for enhancing the viability of the cells. Chitosan is put to use more as a coat rather than as a capsule.

2.13.3.5. Starch. Starch is a very common polysaccharide. It is composed of a large number of glucose units joined together by glucosidic bonds. The main constituent units of starch are amylose, a linear polymer of D-glucopyranose joined by α-1-4 glucosidic bond and amylopectin, a branched polymer of glucose joined by α-1-4 glucosidic bond and α-1-6 glycosidic bond for ramification (Sajilata et al., 2006). The starch which is not digested in the small intestine by pancreatic enzymes such as amylases is known as resistant starch. This type of starch can reach the colon, where it is fermented (Sajilata et al., 2006; Anal and Singh, 2007). A good enteric delivery characteristic is provided by this type of specificity. This leads to a better release of the bacterial cells in the large intestine. With its prebiotic functionality, the use of resistant starch can be made in the large intestine by the probiotic bacteria (Mortazavian et al., 2008). For the adherence of the probiotic cells to the starch granules, the resistant starch serves as an ideal surface (Anal and Singh, 2007). It was reported by Crittenden et al. (2001) that the probiotic delivery to the intestine in a viable and metabolically active state can be enhanced by this way.

2.13.3.6. Gelatin. Gelatin is a gum which can make a thermoreversible gel. This, either alone or in combination with some other compounds has been used for encapsulation of probiotic cells. It can make an excellent combination with anionic polysaccharides, e.g., gellan gum, owing to its amphoteric nature. As they both repel each other due to carrying a net negative charge, there hydrocolloids are miscible at a pH higher than 6 (Krasaekoopt et al., 2003; Anal and Singh, 2007). On adjusting the pH below the isoelectric point, gelatin take up a net positive charge, leading to the formation of a strong interaction with the gellan gum which is carrying a negative charge.

2.13.3.7. Cellulose acetate phthalate. In the simulated GI condition good protection is provided to the probiotic bacteria when encapsulated in cellulose acetate phthalate (Fávaro-Trindade and Grosso, 2002). Cellulose acetate phthalate is used for controlling drug release in the intestine as it has a safe nature as reported by Mortazavian et al. (2008). This encapsulating material does not solubilize at acidic pH i.e. below 5 but at a pH above 6, it solubilizes. This is one of the main advantages of using this encapsulating material.

2.13.3.8. Milk proteins. For the probiotic cells, the milk proteins serve as a natural vehicle. Livney (2010) reported that they can be used as a delivery system based on their structural and physicochemical properties. Heidebach et al. (2009a, 2009b) encapsulated probiotic cells in these proteins based on their specificity of possessing excellent gelation properties. Owing to their biocompatibility, using milk proteins for encapsulation is an attractive method. The results of these studies are quite promising (Livney, 2010).

2.13.4. Emulsification method for encapsulation

This method is of two types as discussed below.

2.13.4.1. Emulsification and ionic gelification. Emulsification is a chemical method for encapsulating the living probiotic cells. The hydrocolloids such as alginate, pectin, and carrageenan are put to use for encapsulation. The relationship between the continuous and discontinuous phases constitutes the basis of this method. An emulsifier and a surfactant are required for carrying out encapsulation in an emulsion. Then, a solidifying agent such as calcium chloride is added to the emulsion (Chen and Chen, 2007; Kailasapathy, 2009; De Vos et al., 2010). The production of the microcapsules of the desired size is achieved by this method by variation of agitation speed and the water/oil ratio.

Chen and Chen (2007) reported that the emulsion technique is easy to scale-up and gives a high survival rate of the bacteria. Capsules having a small diameter are obtained by this method. Large size range and shape provided by this method constitutes the major drawback of this method. Additional protection can be provided to the cells and enhanced organoleptic properties may be achieved by introducing the gel beads into a solution of second polymer for creating a layer of coating (Kailasapathy, 2009).

2.13.4.2. Emulsification and enzymatic gelification. In some countries, the use of coatings such as alginate, gellan-gum, xanthan, k-carrageenan etc. for encapsulation is not permitted for dairy products (Picot and Lacroix, 2004). This is one of the problems posed by the classical encapsulation technique. According to Heidebach et al. (2009a, 2009b) the alternative to this method is the use of milk proteins in which the encapsulation of the probiotic cells can be done by employing the method of enzymatic induced gelation. The milk proteins serve as a natural vehicle for the probiotic membrane. They are also reported to possess excellent gelation properties (Kailasapathy 2002; Livney, 2010). The productivity in fermentation can be improved by employing interfacial polymerization for carrying out the encapsulation of the microorganisms.

2.13.5. Atomization method for encapsulation

This method is also of two types as discussed below.

2.13.5.1. Spray drying method. In this method, first a solution comprising of the probiotic living cells and the dissolved polymer matrix is prepared. The commonly used polymer matrices are gum arabic and starches as during the process of drying, they tend to form microparticles which are spherical in shape (Chen and Chen, 2007; Kailasapathy, 2009; De Vos et al., 2010). An additional layer of coating on the spray dried capsules can be done to protect them from the stomach's acidic environment and also to reduce the adverse effects of the bile salts (Semyonov et al., 2010). This technique has both advantages and disadvantages. One of the main advantages of spray drying is that it is rapid and the cost of the technology is comparatively cheap. This technique is suitable for industrial application and it is highly reproducible as well. One of the major disadvantage of this method is that a high temperature is used which is not favorable for the survival of the bacteria. Another drawback of this technique is that it has small field of application. The addition of protective agents such as trehalose (a thermoprotectant) to the media before drying can be done for enhancing the probiotic survival. The viability of the cultures during drying and storage can be enhanced by using granular starch, while the probiotic viability during storage can be enhanced by the use of soluble fiber.

2.13.5.2. Spray freeze drying method. The steps common to freeze-drying and spray drying are put together in spray freeze drying. The probiotic cells to be spray freeze dried are taken in a solution form and atomized into a cold vapor phase of cryogenic liquid e.g. liquid nitrogen. A dispersion of frozen droplets is produced as a result of this. Then, with the help of a freeze dryer, the frozen droplets are dried (Wang et al., 2006; Kailasapathy, 2009; Semyonov et al., 2010; De Vos et al., 2010). An additional shell coating can be provided to the capsules for protecting them from the adverse environmental condition (Semyonov et al., 2010). This method has various benefits such as providing controlled size, larger specific surface area as compared to spray-dried capsules. The input of high energy and long time of processing are the drawbacks of this method (Zuidam and Shimoni, 2009). As compared to spray drying, this method is 30–50 times more costly.

2.13.6. Encapsulation by coating and agglomeration

In the process of spray coating, the core material should be in a solid form. A specially designed vessel is used for keeping it in motion (Champagne and Fustier, 2007a; De Vos et al., 2010). This method though difficult to master is easy to scale up and owing to this advantage this method is commonly used for encapsulation of probiotics for nutraceuticals. This method is specifically used for obtaining multilayer coatings.

A Danish Korean company dedicated to the probiotics is the "Cell Biotech". A dual coating technology for LAB has been developed and patented by it, which are marketed under the brand name " Duaolac." Soy peptides are used for the first coating while cellulose and gum are used for the second coating. A pH-dependant release mechanism is the basis of this method which protects the cells against the acidic environment of the stomach and release the coating in the pH-neutral environment of the intestines. Increased probiotic viability during the processing shelf life and while passing through the GI tract have been observed by employing the use of this methodology.

Products containing bacteria and particularly probiotic products are produced by a Canadian company constituted of Institute Rosell and Lal'food. A technology "Probiocap" for microencapsulation has been developed and patented by them (Durand and Panes, 2003). The freeze dried LAB are coated with fatty acids in this process. The adverse effects of gastric acidity, temperature, and compression are resisted by the strains encapsulated by this method. Numerous new products and supplements can be developed by employing this method.

2.13.7. Encapsulation by extrusion method

Extrusion comprises a physical method of encapsulating the probiotic cells. It is a simple and cheap method which consists of a gentle operation which does not damage the probiotic cells and gives high probiotic viability (Krasaekoopt et al., 2003). It employs the use of hydrocolloids such as alginate and carrageenan for encapsulation. The solution containing the probiotic cells is projected through a nozzle at a high pressure for carrying out microencapsulation. This method does not involve deleterious solvents and can be performed under erobic and anerobic conditions. The major disadvantage of this method is that it is difficult to use in large scale productions owing to the slow formation of the microbeads. The term "prilling" is used, in case the droplet formation occurs in a controlled manner, unlike spray drying. Generally, this is achieved by the pulsation or the vibration of the jet nozzle. Kailasapathy (2002) reported another common technique for droplet formation which uses the coaxial flow or an electrostatic field.

2.13.8. Encapsulated probiotics in food products

During storage of the food products and their passage through the digestive tract, microencapsulation serves as an important means for the survival of the probiotic cells. As per Champagne and Fustier (2007a), the sensory properties of the food products containing the microcapsules should not be adversely affected. Truelstrup-Hansen et al. (2002) reported that it is desirable to obtain a size below 100 mm in order to prevent negative sensorial impacts of microcapsules in the food products.

2.13.8.1. Recent developments in the research sector

2.13.8.1.1. Yoghurt. There are many reports which say that the therapeutic value of yoghurt increases on the addition of probiotic living cells to it (Chen and Chen, 2007; Weichselbaum, 2009) even though owing to its low pH (4.2–4.6), the level of probiotic viability in it is low. Several researchers have employed the use of probiotic cells for incorporating into yoghurt. The use of encapsulated probiotic bacteria has been reported to enhance survival in several studies. Kailasapathy (2009) suggested that the incorporation of the yoghurt could be done with probiotic cells without making many modifications from the traditional process. Initially, the mixtures of gellan–xanthan gum were used for obtaining encapsulated probiotic beads. This served as a method to enhance survival in the acidic environments (Sun and Griffiths, 2000). Probiotic survival seems to be enhanced by the addition of encapsulated probiotic cells in the set yoghurt. Adhikari et al. (2000) reported that the bacteria have low metabolic activity and there is poor acetic acid production. A sour sensory attribute is imparted by this compound to yogurt and the lack of it is considered as a defect.

Adhikari et al. (2003) incorporated stirred yoghurt with encapsulated bifidobacteria. A grainy texture was reported by the consumers in these yogurts (size range of particles was about 22–50 mm). The microencapsulation protected the probiotic bacteria in yoghurt but the sensory quality was affected which for consumer acceptance posed a problem. Prebiotic ingredients such as resistant starch or cryoprotectants such as glycerol could be used for encapsulating probiotic cells for enhancing their viability (Sultana et al., 2000; Capela et al., 2006). Sultana et al. (2000) reported that this method increases probiotic survival in the product but not under simulated GI conditions.

Godward and Kailasapathy (2003c) described the method of co-encapsulation for enhancing the probiotic viability which involves the encapsulation of two different probiotic bacterial strains together. On comparing the survival of free and encapsulated cells, the encapsulation and co-encapsulation were observed to enhance the survival and in particular, freeze-dried cells after encapsulation survived better in the yogurt. Therefore, if the cells are encapsulated, yogurt can serve as a good probiotic carrier. They also reported that the protected bacteria in a viable state at the time of consumption can survive through the GI tract and reach the intestine in a viable state.

The use of raftilose, a prebiotic has been reported in several studies for co-encapsulating two probiotic strains for stimulating the bacterial growth and providing protection against adverse environmental conditions to the cells. This concept of co-encapsulation concept leads to an increase of functional food efficiency owing to the synergy between the probiotic and prebiotic components. Employing prebiotic ingredients is also a factor to improve the viability of the probiotic cells. Anjani et al. (2004) demonstrated that coating the capsule with chitosan imparts greater protection to probiotic cells as compared to the alginate when their survival in considered yogurt and in the simulated conditions of the GI passage.

Bifidobacteria was encapsulated using whey proteins and it was reported that this method could be used for delivering the viable probiotic cells incorporated into the fermented dairy

products into the gastrointestinal tract. High temperature is required, while employing the spray drying technology, hence, considering the strain properties for heat resistance is an important factor (Picot and Lacroix, 2004). It was reported that the properties of yoghurt such as appearance, color, flavor, taste, and acidity were not affected by the incorporation of capsules containing probiotic cells (Kailasapathy, 2006). On the contrary, the textural properties such as smoothness were affected by incorporating probiotic cells and the consumers also reported grittiness in yogurts. However, it was reported that the post-acidification process during the storage was slow on the addition of probiotic cultures to yoghurt.

A correlation between post-storage pH and the survival of probiotic bacteria was shown by Kailasapathy et al. (2008). The presence of the fruit pulp was reported to have a negative impact on it but it was found that even after a shelf life of 35 days, the yoghurts contained the probiotic bacteria in the recommended levels. Urbanska et al. (2007) reported that the delivery of a high number of bacteria to the desired targets in the gastrointestinal tract was possible by employing the method of microencapsulation. It was also reported that the cell therapy using the capsules containing the probiotic cells administered orally seemed beneficial.

Picot and Lacroix (2004) reported two important parameters which affected the encapsulation yield significantly. These were the technology employed for encapsulating the cells and the strains of the probiotics. It was reported that when oxygen is present in the medium and the microenvironment has a lower oxygen level, the microencapsulation has a positive effect on the probiotic cells (Talwalkar and Kailasapathy, 2004a). Differences in the viability of the cells in yoghurt in different studies may be because of the various oxygen sensitivities between the strains or to different oxygen levels (Champagne and Fustier, 2007b). The type of coating material (e.g. chitosan) or the addition of starch in alginate core which improves the viability of the cells could constitute another reason for this variability. Talwalkar and Kailasapathy (2004b) reported that the microencapsulation of the probiotic cells for the addition into yogurts seem to avoid losses of oxygen-sensitive strains more than protecting the cells against acidic environment.

I nutshell, it can be said that the consumer very clearly detects the addition of encapsulated probiotic cells into yogurts. Such effects on the sensory parameters however can be made desirable, by making the consumer aware in advance about the presence of the particles.

2.13.8.1.2. Cheese. The use of encapsulated probiotic cells have been reported in several studies. Majority of such studies are related to Cheddar cheese. The composition of the cheddar cheese is not affected by probiotic incorporation and the addition of bifidobacteria in it is advantageous as the bacteria can stay alive for at least six months. This can be attributed to the relatively high pH of about pH 5.5 of Cheddar cheese. Owing to which it acts as a good carrier of probiotic cells. Several authors have also made the observation that incorporation of bifidobacteria into Cheddar cheese does not negatively impact cheese quality in aroma, flavor, and texture (Stanton et al., 1998). Protection is provided to the probiotic bacteria against enzymatic degradation and acidic environment of the GI tract because of its appreciable buffering capacity and relatively high content of fat (Gardiner et al., 1998; Stanton et al., 1998).

Bifidobacterium bifidum was immobilized with an emulsification technique and the resultant gel beads were frozen and lyophilized (Dinakar and Mistry, 1994). The viability of the bifidobacteria in the cheese was not affected by the non-uniform addition of the immobilized cells in the cheese. The cells were found to be viable until 24 weeks and no adverse effect was reported on the flavor and the flavor intensity, texture, and appearance of the cheese, etc. These results could be attributed to the lack of metabolism of bifidobacteria as it requires substrates such as lactose for the production of acetic acid and lactic acid which were not available here. The growth of bifidobacteria generally occurs at 37°C but it is inhibited at low temperature of ripening i.e. between 6 and 7°C, though the viability of the bacteria is retained.

Gardiner et al. (1999) reported that the addition of *E. faecium* strain to the cheese may have a positive impact on its qualities. Prior to their incorporation into the Cheddar cheese, the probiotic cells may be encapsulated using the method of spray-drying as well. Gardiner et al. (2002) reported that the spray dried culture was stable for seven weeks both at room temperature and during refrigeration. In comparison to the traditional methods like freezing or freeze-drying, the benefits of this method are that it is cost effective and applicable to large-scale production. However, when considering cheese ripening, seven weeks does not seem to be sufficient. Codex Alimentarius defines five weeks as the minimum ripening time. The overall ripening time is over five weeks for developing cheese aroma. If this is not so, it may seem that microencapsulation is not required for enhancing the probiotic viability in Cheddar cheese, rather it is required for increasing the probiotic viability in the case of fresh cheese. Kailasapathy (2002) reported that microencapsulation is a good way of improving the probiotic viability owing to the low pH value of the product.

Godward and Kailasapathy (2003a) reported that the Cheddar cheese is an ideal carrier for probiotics but the survival of probiotics is not enhanced by microencapsulation. It was observed that the release of the acids produced by the bacteria is inhibited by the physical barriers and cell death results due to their accumulation in the surroundings of the bacteria. The reports of Dinakar and Mistry (1994) were contradictory to this. Several probiotic strains were employed and during the ripening phase, they may not react in a similar manner.

Gobetti et al. (1998) incorporated the encapsulated probiotic bacterial cells in Crescenza cheese. It is a soft cheese having its origin in Italy and requiring a short ripening period. Here, probiotic incorporation in the cheese did not require any change in flavor, appearance and microbial and physico-chemical properties. Kasar cheese was added two probiotic strains (*L. acidophilus* and *B. Bifidum*) after encapsulating them using extrusion or emulsification technology. The final product in both the cases had similar bacterial counts, proteolysis, and organoleptical properties of the final product. Microencapsulation was reported to be an appreciable method for enhancing the probiotic viability in Kasar cheese (Özer et al., 2008).

L. acidophilus and *B. Bifidum* were incorporated in the white-brined cheese by Özer et al. (2009). Despite the fact that microencapsulation induced the formation of acetaldehyde and

diacetyl, the sensory properties of the cheese were not affected by the addition of the encapsulated probiotic cells. A method of continuous production of pre-fermented milk was given by Prevost and Divies (1987) in which the entrapment of the cells was done in Ca alginate particles. Constant characteristics were observed in the final product which was not so in the industrial level batch-wise processing. Besides this, there was a reduction in the incubation time by 50% as compared to the normal fermentation using the starter culture. On an industrial scale, Sodini et al. (1997) gave the confirmation for the feasibility of a continuous milk pre-fermentation process. He also reported that this process could be automated easily.

2.13.8.1.3. Frozen dairy desserts. The properties of the frozen dairy desserts such as high acidity, freeze injury, high osmotic pressure, and exposure to the incorporated air during freezing poses difficulties in incorporating the probiotic microbial cells into them. Chen and Chen (2007) reported that using the encapsulated cells for incorporation into such desserts could help in overcoming these problems producing health benefits and creating a useful market. As compared to the free cells, entrapping the lactobacilli in the Ca-alginate provides a higher survival rate of about 40% when freezing ice cream (Sheu and Marshall, 1993; Sheu et al., 1993). The incorporation of the probiotic cells in ice cream in different states was studied by Godward and Kailasapathy (2003b). As a matter of fact, the cells could be free, freshly encapsulated, encapsulated, and freeze-dried and finally co-encapsulated and freeze-dried. It was reported that the free cells had a better survival as compared to the encapsulated cells. The survival of the freshly encapsulated probiotic cells was found to be higher when compared to cells which were freeze dried after encapsulation. The survival of both *L. acidophilus* and *B. bifidum* was reported to increase when they were co-encapsulated. Air incorporation into the ice cream is not affected by the addition of the probiotics.

Other studies report that the survival of encapsulated probiotic bacteria and the free cells does not differ significantly. Kailasapathy and Sultana (2003) reported that the free cells in ice cream may be protected due to the high total solids in it making ice cream a suitable food for delivering probiotic living cells to the consumer. Homayouni et al. (2008) also reported a similar observation of high rate of total solid encountered in ice cream and resistant starch providing further protection for probiotics. The viability of the probiotics in ice cream is enhanced by encapsulation and there was no effect on the products' sensory parameters. After three months of storage, Homayouni et al. (2008) reported the number of viable probiotic cells between 10^8 and 10^9 cfu/g while the International Dairy Federation (IDF) recommended a viable number of 10^7 cfu/g in food product at the time of consumption.

2.13.8.1.4. Other food products. The problem of lactose intolerance has directed the attention toward the development of non dairy food carriers for probiotics as most of the probiotic products available today are dairy based (Ranadheera et al., 2010). Researchers are trying to identify new food carriers. An example can be quoted here of good quality mayonnaise which was obtained by incorporating bifidobacteria in the encapsulated form. Protection is provided to bifidobacteria against the bactericidal effects of vinegar by using calcium alginate. In addition to this, the other advantages were mentioned by Khalil and Mansour (1998) of using the encapsulated probiotic cells such as growth inhibitors of yeasts for over 10 weeks. This property may be attributed to the antibacterial effect of the probiotics.

Beverages and soft foods containing viable encapsulated probiotic cells were obtained by McMaster et al. (2005) having a range size of 20–2200 mm by an extrusion method. Tsen et al. (2004) carried out the fermentation of banana media by using encapsulated probiotic cells and the product obtained is proposed to be a synbiotic. The fermentation of tomato juice using the encapsulated probiotic cells has also been demonstrated. The survival of the probiotic cells in unfavorable pH encountered in tomato juice is made possible by employing microencapsulation. It has been reported that the sensory quality of the product has been improved on the addition of encapsulated cells as compared to free cells (An-Erl King et al., 2007; Tsen et al., 2008).

Muthukumarasamy and Holley (2006, 2007) carried out the investigation for determining the use of microencapsulation for protecting the cells in meat products. The sensory properties of the dry fermented sausage were not affected by the incorporation of encapsulated probiotic cells into them. Moreover, the viability of encapsulated probiotic cells was improved compared to free cells. Besides this, it was reported that the probiotics could lower *E. coli* O157:H7 in number but microencapsulation lowered this potential. Maillard and Landuyt (2008) investigated the incorporation of probiotic cells encapsulated by spray-coating technology in chocolate. They reported a three times higher probiotic viability in the small intestine when incorporated in chocolate as compared to that in dairy product. This probiotic chocolate process was shifted to a larger scale but the biggest problem posed here was of obtaining a process which is compatible with probiotic survival as high temperatures are required in the process.

Another investigation involving incorporation of the encapsulated probiotic cells in chocolate was carried out by Possemiers et al. (2010). It was reported that the incorporation of encapsulated probiotic strains into chocolate could serve as a good means of protecting them from the conditions of environmental stress. Lahtinen et al. (2007) reported that the lipid fraction of cocoa butter was protective for bifidobacteria. Carrying out the encapsulation of the probiotic cells in the whey protein gel particles could offer protection during processing and storage. They may also help in extending the food applications to biscuits, vegetable, and frozen cranberry juice, etc.

A protective effect is imparted to the probiotics by proteins. The process of microencapsulation is helpful as it creates a microenvironment suitable for the survival of the cells against adverse conditions such as acidic pH. An alternative to microencapsulation using alginate-type gels or spray-coatings is employing the use of protein-based technology to encapsulate probiotic cells.

2.13.8.1.5. Importance of food carriers for probiotics. The growth and survival of the probiotics can be affected by several product factors namely the concentration of proteins, sugar, and fat and the pH (Ranadheera et al., 2010). The

encapsulation of the probiotic cells was done under the same conditions, but the encapsulated cells were introduced into different food matrices, e.g. Cheddar cheese, ice cream and yoghurt obtaining different results in each case. The observations made were that encapsulation is not a necessity for the survival of the cells in the case of Cheddar cheese and ice cream but while considering yoghurt, it was important. Hence, this laid stress on the importance of the food carrier for the probiotic survival. As per the study of Sharp et al. (2008), both Cheddar cheese and yogurt are able to protect probiotic cells by providing a suitable environment while manufacturing as well as during storage. Cheddar cheese, however, was observed to be better as compared to yoghurt for delivering the probiotics as the cells were able to resist the low pH in the stomach in a better way.

The investigation of abilities of different prebiotic fibers for protecting the stability and viability of the probiotic strains of *L. rhamnosus* proved quite important in context of probiotic encapsulation. Saarela et al. (2006) reported this sort of protection while freeze-drying, storing in the freeze-dried state and after formulation into the apple juice and breakfast cereals coated with chocolate. As per Gibson and Roberfroid (1995), the fibers are considered to be prebiotics as they constitute a component which cannot be digested and the probiotic viability is stimulated by them. The fact that the optimal carrier for probiotic might depend, in part on how it is eventually used was shown by this investigation.

Apple wedges were dipped in a solution containing probiotic cells by Roble et al. (2010). The product obtained was acceptable both in terms of its quality and the quantity of the probiotics in order to impart a beneficial effect. It was also reported that employing encapsulation could enhance the resistance of the probiotics. Klayraung et al. (2009) discussed the importance of choosing the matrix. The production of a tablet with lyophilized bacterial cells was shown by them. The survival of the probiotics in the stomach is affected by the matrix. An example can be quoted here of sodium alginate allowing higher cell survival in simulated GI conditions. The advantages of this technique as its ease of treatment, nontoxic nature, and low cost were demonstrated by Ross et al. (2008). Several different trials have been carried out for innovating in terms of food matrices for incorporating the probiotic cells.

2.13.8.2. Recent developments in the industrial field. In the last few years, several food products having the probiotic cells in the encapsulated form have been introduced to the consumers. Barry Callebaut, in the year 2007, developed a process to produce chocolate containing encapsulated probiotic cells with the Probiocap technology in partnership with Lal'food. According to them, the addition of encapsulated probiotic cells to the chocolate did not influence the taste of the chocolate, its texture, or the mouth feel. Consuming 13.5 g of this probiotic chocolate seemed to be good enough for ensuring the balance of the intestinal microflora.

Balchem Encapsulates and Institute Rosell developed a stabilized form of encapsulated probiotics after two years of their collaboration. Encapsulated probiotic cells have been incorporated into yogurt-covered raisins, nutrient bars, chocolate bars, and tablets by the Institute Rosell (Siuta-Cruce and Goulet, 2001). According to the available sources, the clinical testing has revealed "an unprecedented 100% delivery rate." A high recovery rate was shown by the tests carried out on chocolate bars. Probio-Tec capsules are distributed by the Chr Hansen for dietary supplement, infant formula, and pharmaceutical industry. They, along with the Kerry Group in Ireland, have developed the first probiotic orange juice termed "Dawn" for the Irish market. As per the reports of these companies, the probiotic cells remain viable throughout the shelf-life of the product. To cope up with the harsh conditions in juices, the use of the encapsulated probiotic cells could be advantageous.

The products such as probiotic chocolate and Probio'Stick have been introduced by the Institut Rosell and Lal'food with the Probiocap technology. The product Probio'Stick is an orodispersible powder comprising of the probiotic strains *Bifidobacterium* and *Lactobacillus*. Diop et al. (2008) reported that this product allows a reduction of physical symptoms related to stress such as nausea, vomiting, and abdominal pain. DSM Food Specialities also used chocolate for producing a bar named "Attune," which was launched in 2007 January in the United States of America. The prebiotic ingredient inlulin is also contained in it which supports the healthy functioning of the digestive system. The innovative product line of Attune is found in the refrigerated yogurt section and advertising of this product highlights more input in calcium, fiber with less sugar than that found in the majority of the yogurts.

In Latin America, one of the top players in Central American ice cream industry, Chr Hansen and Dos Pinos, have developed a probiotic ice cream. It is an innovative yogurt ice cream having a number of health benefits. Unilever, in the 1999, had launched a probiotic ice cream but way back then, the consumers were unwilling to accept this kind of an innovation. Hence, it is quite important to consider the expectations of consumers in creating new food products containing the probiotics. Lee and Heo (2000) described that in Korea, yogurts containing the LAB in the encapsulated form are available in the market under the brand name Doctor-Capsule (Bingrae Co.Kyunggi-do).

A range of products containing the encapsulated probiotic cells are marketed by Jinta Capsule Technology. To mention a few, yogurt containing encapsulated bifidobacteria and bifina-constipation a pharmaceutical product which contains encapsulated bifidobacteria available in a capsule form. The majority of the products containing encapsulated probiotic cells are available in a tablet/capsule form e.g. Forever Active Probiotic, Probiotic 7, Multi-probiotic or in the form of a powder, e.g., PureBaby Probiotic, ThreeLac^{TM}. The ThreeLac^{TM} proposed by GHT^{TM} Global Health Trax is a powder which contains the encapsulated probiotic cells. This powder has to be sprinkled into the mouth. These probiotic cells get through the unfavorable stomach acids to reach the intestine owing to the encapsulation technology employed.

Innovance Probiotiques, a nutraceutical having probiotic cells encapsulated by the Probiocap technology is marketed by Ysonut Laboratories. A mixture of three probiotic strains produced by Institut Rosell is used in this product. Cerbios-Pharma SA distributes the Cernivet LBC ME10 containing the encapsulated probiotic strain *E. faecium* SF68, is a pelletable microbial feed employed for the stabilization of intestinal microflora. This product is registered for use in animal food,

such as for chicken and pig fattening. At refrigerated storage, the shelf life of Cernivet LBC ME10 is 24 months. An American company, Nutraceutix, provides probiotic cells in a number of different forms ranging from bulk powder to capsules and advanced tablets. The Bio-tract tablets are a patented formula which provides protection for probiotic living cells against the adverse conditions of low pH stomach. Geneflora^{TM} was propsed by America's BioPlus Corporation, which is a synbiotic comprising of encapsulated *Lactobacillus sporogenes* as a probiotic and a fructo-oligosaccharide as a prebiotic. EA Pharma marketed the capsules which contain cranberry (Granio + reducys). These present recognized effects on urinary disorders such as cystitis. The product contains encapsulated probiotic cells having positive effects on urinary flora. The constituting ingredients make this product quite helpful in prevention of cystitis. In the present times, the probiotic cells in the encapsulated form are essentially introduced in the nutraceutical products. Efforts are being put in for the development of novel food as a suitable carrier for the bacteria.

2.13.8.3. The expected future trends related to probiotics and microencapsulation. Several companies have explored the application of microencapsulation for improving the resistance of the probiotic cells in the gastro-intestinal tract besides enhancing the viability of the bacterial strains in the food products. The most commonly used encapsulating agents employed in these studies are the natural biopolymer such as alginate, gellan gum, or κ-carrageenan. The results at the laboratory scale are appreciable but the scaling up poses difficulties. An example can be quoted here of extrusion methods, where reports have been obtained of large particle size and low production capacities, while large-sized dispersions are obtained in the emulsion method. Picot and Lacroix (2004) reported another hurdle which is posed is that in some European nations, there is prohibition on the addition of certain polysaccharides in dairy products.

In the supermarkets, the majority of the probiotic foods are kept in the refrigerated areas as the probiotic bacteria are heat sensitive and can be destroyed on being exposed to heat. This provides the biggest advantage to the dairy industry in context of probiotic foods. Research efforts are being directed toward the expansion of the currently available categories of food. On an industrial scale, there are several hurdles in the application of microencapsulation. A persistent effort must be put in for the enhancement of the properties of the microcapsules and overcoming the technological challenges besides taking into consideration the consumer acceptance of these novel foods.

These days, the incorporation of the encapsulated probiotic cells can be done in a number of foods and as per the development of the technology, a wide variety of functionalities can be achieved by microencapsulation. Now, the probiotics can be found in cereals and chocolates as well, besides the dairy products. Reducing the particle size is one of the biggest challenges while encapsulating the probiotics as the sensorial and textural properties may be negatively affected because of improper size. Generally, the method of choice in the case of laboratory applications is emulsification but owing to several reasons, this method has certain drawbacks for application in the food sector. The product's textural and organoleptic characteristics are negatively affected by the residual oil present on the surface of the capsule. The residual oil, emulsifier, or surfactant may be toxic for probiotic cells and it also interferes with the incorporation of the capsules in the food products. Both, an incompatible food matrix owing to the low pH or the presence of the competing microbes and the unsuitable storage conditions, hinder the incorporation of the probiotic cells into a wide variety of food products.

The use of prebiotics and fiber as protectant for probiotic cells has been gaining considerable interest since the last couple of years. To cater to the expectations of the consumers related to the healthy and beneficial food products, developing novel functional foods is a big challenge. It is the time for the researchers to provide a cost effective technology for the industrial scale. Efforts are being made since the last decade for achieving this goal. The future research should focus on the optimization of the use of the probiotic cells in the encapsulated form keeping in view the factors such as safety and ecological production. In nut shell, it can be said that the increasing demands of the consumers will strengthen the probiotic market. Owing to the proper documentation of the beneficial effects of probiotics, there will be a marked rise in the consumer requirements related to such food and beverage products.

2.14. Theoretical adverse risks associated with probiotics

It was reported by Hammes and Tichaczek (1994) that there are certain theoretical adverse risks associated with the use of probiotics in humans. The potential for transmigration and the fact that colonization with probiotics may affect the gastrointestinal physiology and function negatively constitute these risks. These include metabolic and physiological effects as well besides the immunological ones which could be both localized and generalized. In addition to these, there is a tendency for the transfer of antibiotic resistance from commensal or probiotic bacteria to other bacteria or potential pathogens within the gastrointestinal tract.

2.15. Genetically engineered probiotics

The Genetic modification of the probiotics has been undertaken to increase certain physiological or immunological properties within the organism and to use the probiotic as a mucosal delivery system or a vaccine vector (David, 2008). Till now, the use of these genetically engineered products has been quite limited, but the steps for monitoring the safety of the probiotics should be followed for employing the use of any engineered strains introduced into human studies. Some caution must be employed when assessing safety of any genetically engineered product.

2.16. Steps to monitor safety of probiotics

Worldwide, the probiotics are being introduced and extensively employed. This calls for monitoring the safety of probiotics making the population-based surveillance for the isolation of probiotic bacteria from patients with infection important. As the introduction and use of the probiotics is increasing globally, conducting population based surveillance is quite important in

order to isolate the probiotic organisms from the infected individuals. Prior to employing any strain for clinical trials, its susceptibility profile should be studied. As per David (2008), molecular methods should be employed to compare the clinically isolated strain with the probiotic strain. It is important to conduct active surveillance for cases of infection and the possibility of adverse effects associated with the use of probiotic strain. Despite the fact that a certain amount of caution is quite important in every trial involving the probiotics, toxicity aspect should also be given full consideration. The risk benefit and the possibility and the level of toxicity should be assessed in every case. The individuals who are aged, immunocompromised, having short bowel syndrome, having central venous catheters, having a cardiac valve disease and the premature infants might be warranted about the use of probiotics (David, 2008). As a matter of fact, the presence of any of these above mentioned factors may not necessarily preclude a clinical trial. Each investigation should be assessed individually with the appropriate involvement of a human investigation review committee and a data safety monitoring committee, as well as specific hypotheses to be tested and surveillance for bloodstream infection with the probiotic strain. Preferably, there should be population-based surveillance for *Lactobacillus* bacteremia, including the use of a reference laboratory and molecular confirmation.

3. Conclusion

Generally, the bacteria belonging to the genera *Lactobacillus* and *Bifidobacterium* have been used as probiotics. Probiotic bacteria beneficially affect human health by improving the gut microbiota balance and the defenses against pathogens. Additional benefits attributed to probiotics are the stimulation of the immune system, blood cholesterol reduction, vitamin synthesis, anti-carcinogenesis, and anti- bacterial activities. Other important criteria to determine the efficacy and the success of the product containing probiotics are the acceptance of the product by the consumer and the survival of probiotics microorganisms during its production. A probiotic strain should withstand the manufacturing process without the loss of viability or negative effect on the sensory properties of the food product. The strain and the claimed properties should maintain stability in the food product during processing and also during subsequent storage. For human nutrition, probiotics are defined as "live microbial food supplements or components of bacteria which have been shown to have beneficial effects on human health." A large number of viable organisms are required in order to exert a probiotic effect in the food product. It is postulated that an active probiotic food should contain at least 10^5 cfu/g and the food should be consumed in order to achieve a beneficial effect.

A prebiotic is defined as "non-absorbable food component that beneficially stimulate one or more of the gut-beneficial microbe groups and thus has a positive effect on human health." "Synbiotics" is the word coined for the combined administration of specific probiotics with prebiotics to provide definite health benefits by synergistic action. Probiotic bacteria are used widely in producing foods based on their positive qualities. The common probiotics that have been extensively studied and found in the market are dairy products such as yoghurt and cheese. Latest studies reveal that other novel probiotics such as fruit juices, cereals, chocolate etc. are better and superior carriers for the delivery of probiotics. Producing probiotic juices have been considered more in recent years. Very less work has been done on synbiotic food. Hence, there is a need to develop diverse probiotic and synbiotic food, which can be used as nutrient supplements to promote health. Moreover, many reports also indicate the poor survival of probiotics in functional food. The stability and viability of the probiotic cultures can be increased by a recent technology known as microencapsulation. Extensive research however is required to be conducted on the efficacy of micro-encapsulation to deliver probiotics for their controlled and targeted release in the gastrointestinal tract.

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