INTRODUCTION

1.1. Background

The practice of consuming foods containing live bacteria is one way to provide both nutrition and health. Such foods enable to increase the numbers of advantageous bacteria called 'Probiotics' in the intestinal tract. The evidence of probiotics conferring health benefits on humans has put into motion the commercial development of products containing them [1]. Probiotic foods comprise 60-70 % of the whole functional food market [2]. Indeed the global market of probiotics is more than 28 billion US dollars. Advancements in developing probiotic foods and their health benefits are presented in this Chapter.

1.2. Probiotic definition

'Probiotics' is the subject of evolving definitions as more research is undertaken in this field. The term "probiotic" is derived from the Greek meaning 'for life', and was first coined by Lilly and Stillwell in 1965 to describe substances produced by one microorganism which stimulates the growth of other organisms. Subsequently, the definition was expanded to include organisms and substances which improve intestinal microbial balance [3].

Probiotics are "live microbial food ingredients that beneficially affect the health of consumers by improving their intestinal microflora balance when ingested live in sufficient numbers" [4]. Fuller's descriptions give emphasis to viability of probiotic and limitation to the intestinal tract. Schrezenmeir and de Vrese [5] proposed a new definition for probiotic as " a preparation of or a product containing viable, defined microorganisms in sufficient numbers, which alter the microflora (by implantation or colonisation) in a compartment of the host and by that exert beneficial health effects in this host".

Regardless of the numerous versions, the most used and widely acknowledged definition by scientific community describes probiotics as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" [6]. Probiotic bacteria is able to survive in the gastric environment as well as exposure to bile and pancreatic juice in the upper small intestine to exert beneficial effects in the lower small

intestine and the colon, however, there are persuasive data on beneficial immunological effects also from dead cells[7].

1.3. Probiotic microorganisms

Probiotics primarily belong to the genera *Lactobacillus* and *Bifidobacterium*, however, other microorganisms including *Propionibacteria, Leuconostoc, Pediococci, Enterococci* and *Escherichia coli* have also been considered as probiotic cultures. A summary of potential probiotic species is provided in **Table 1.1.**

Table 1.1. List of most commonly used species of lactic acid bacteria in probiotic preparations [8]

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

1.3.1. The genus *Lactobacillus*

Lactobacilli are Gram-positive, non-spore forming rods or coccobacilli, catalase-negative, facultative anaerobes, sometimes microaerophilic and often nonmotile. As chemoorganotrophic organisms, they are extremely fastidious and require rich nutritional media to grow. Members of the genus *Lactobacillus* are strictly fermentative and may be either homofermentative, producing mainly lactic acid from glucose, or heterofermentative, producing lactic acid, CO_2, ethanol, and/or acetic acid [9]. Lactobacilli

are taxonomically classified into three groups: obligately homofermentative, facultatively heterofermentative, and obligately heterofermentative [10]. At present, the genus *Lactobacillus* comprises 100 validly recognised species [11].

Lactobacillus species are widely found in environments such as animal and vegetable food products, respiratory, gastrointestinal and genital tracts of humans and [11]. The ability of lactobacilli to produce lactic acid and other organic acids, as well as flavour compounds, results in the transformation of raw material to a wide variety of new food products, in particular fermented vegetable and dairy products. Furthermore, their ability to lower the pH of the environment and to produce some inhibitory compounds *e.g*. organic acids and bacteriocins causes them to exert an antagonistic action toward harmful microorganisms such as *Escherichia coli*, *Salmonella* sp. and *Helicobacter pylori*. The role of lactobacilli in intestinal ecosystems has received much attention with respect to their beneficial health effect on humans and animals, especially in regards to ingestion of lactobacilli as probiotics [12]. Some commercial probiotic *Lactobacillus* strains and their clinical benefits are presented in **Table 1.2**.

1.3.2. The genus *Bifidobacterium*

In 1900, for the first time, *Bifidobacteria,* members of the lactic acid bacteria group were isolated from the intestinal layer of breast fed infants and found that they are the predominant component of the intestinal microflora [13]. Only eight species within the genus *Bifidobacterium* have been considered as probiotics (**Table 1.1**)[8]. Bifidobacteria are characterised as Gram-positive, catalase negative, polymorphic branched rods, non-motile and non-sporeforming anaerobic heterofermentatives (Dellaglio & Felis, 2005). The morphology of bifidobacteria depends on the strain/species as well as cultural conditions used [14]. Clinical benefits of two commonly available commercial probiotic bifidobacteria are presented in **Table 1.2.**

1.3.3. The genus *Propionibacterium*

The genus *Propionibacterium* presently comprises of eleven recognised species [15] and organisms are characterised by their ability to produce copious amounts of propionic acid and acetic acid and often small amounts of carbon dioxide during growth. The species of this genus are characterised as irregularly staining Gram-positive, usually catalase

Table 1.2. *In vivo* studies addressing the beneficial health effects of some commercial probiotic *Lactobacillus* strain [16].

Probiotic strain (Owner of the strain)	**Health effects**
L. rhamnosus GG (Valio Ltd, Finland)	Prevention of atopic eczema/dermatitis, suppression of allergic airway inflammation/asthma, suppression of some symptoms of IBS, healing gastric ulcer, prevention/treatment of rotavirus diarrhoea, prevention of antibiotic associated diarrhoea, treatment of *Clostridium difficile*- associated diarrhoea
L. reuteri (BioGaia AB, Sweden)	Prevention of enteric colonisation by *Candida* in preterm newborns; reduction of functional abdominal pain in children; improvement of intestinal comfort in cystic fibrosis patients; improvement of symptoms of rotavirus gastroenteritis ; reduction of symptoms of atopic eczema in children; decreasing antibiotic-associated diarrhoea; control of *H. pylori* infection; improvement of oral health
L. plantarum 299v (Probi AB, Sweden)	Improvement of *Clostridium difficile*-associated diarrhoea; treatment of IBS; reduction of cardiovascular disease risk factors; inhibition of *Escherichia coli*-induced intestinal permeability; reduction of pathogenic bacteria in the oropharynx of intubated patients
L. casei Shirota (Yakult Honsha Co. Ltd., Japan)	Improving stool consistency, constipation and bowel movements, modulating natural killer (NK) cell activity in subjects with low NK cell activity, modulating the immune response in allergic rhinitis, reducing risk of bladder cancer and colorectal tumors
Bifidobacterium animalis subsp *lactis* BB-12® (Chr. Hansen A/S, Hørsholm, Denmark)	Alleviating symptoms of atopic eczema, decreasing frequency and duration of diarrhea, increasing fecal secretory IgA levels in infants, reducing the incidence of respiratory infections in infants
B. lactis HN019 (marketed as DR10 by Fonterra, New Zealand, and HOWARU Bifido by Danisco, USA)	Decrease in number of iron-deficient preschoolers, confer desired changes in the intestinal microflora of elderly human subjects, enhancement of immunity in the elderly, reduces the severity of *Escherichia coli* O157: H7 infection, Enhances resistance to oral *Salmonella typhimurium* infection, prevention of morbidity in preschoolers, immunomodulatory effect on fetal immune parameters and breast milk

positive, diphtheroid, pleomorphic rods that may bifurcate or even branch; they are nonsporeforming, nonmotile and facultative anaerobes, but variable aerotolerant [15]. The propionibacteria are comprised of two principal groups: (a) the "classical propionibacteria" that have been mainly isolated from dairy products, especially cheese, and (b) "Cutaneous propionibacteria" or non-dairy strains which are found in spoilt and fermenting fruits, silage and soil, human skin, mouth, the female genital tract, and faeces [15] and have been identified to be pathogens and cause diseases including endophthalmitis, brain abscesses, meningitis, arthritis, osteomyelitis, endocarditis and infections of the central nervous system [17]. Some classical *Propionibacterium* species such as *P. freudenreichii* and *P. jensenii* have been considered as potential probiotic microorganisms with health benefits [16].

1.3.4. *Saccharomyces boulardii*

Saccharomyces boulardii is considered a non-pathogenic, mesophilic and non-colonising baker's yeast, which is morphologically and physiologically related to brewer's yeast (*S. cerevisiae)* but differs from *S. cerevisiae* in some genotypic characteristics [18]. *S. boulardi* is known as a unique microorganism that can survive GI tract transit, proliferate in the gut and exert many beneficial health effects on humans and animals [19].

1.4. Selection criteria for probiotics

Many criteria have been considered by several researchers as desirable properties for potential probiotic strains [20]. Probiotics must fulfil a number of safety, functional and technological properties and characteristics to be used in probiotic food products (**Table 1.3**).

1.5. Health benefits of probiotics

Beneficial health effects of probiotics are strain specific [22,23]. Some of the health benefits claimed include prevention and/or treatment of infections, irritable bowel syndrome (IBS), chronic gut disorder such as inflammatory bowel disease (IBD) and colon cancer, coronary heart disease (CHD), recurrent vaginal thrush, skin problems and food allergy, alleviation of lactose intolerance, treatment of different diarrhoeal diseases, lowering serum cholesterol and triglyceride levels, modulation of the immune system,

enhancement of mineral bioavailability, chemopreventative effects, improvement of constipation, improvement of dermatitis and liver disease.

Table 1.3. Selection criteria of probiotic organisms for human use [21]

Safety	• Non-pathogenic and not associated with diseases *e.g.* infective endocarditis or GI disorders • Non-inflammatory promoting • Not able to deconjugate or dehydroxylate bile salts • Not able to carry transmissible antibiotic resistance genes • Not having clinical side effects
Functional	• Resistant to low pH, gastric juice, bile acid and pancreatic juice • Adhesion to the intestinal cells and colonisation of the human gut • Modulation of immune system • Antagonistic against pathogens via competition for adhesion sites and production of antimicrobial metabolites • Antimutagenic and anticarcinogenic properties • Potential for the delivery of recombinant proteins and peptides to the human GI tract
Technological	• Reasonable sensory properties • Phage resistant • Viability during production and storage of the product

1.5.1. Lipid modulation

Ingestion of probiotics has been proposed to be an effective way in lowering serum lipid levels including cholesterol and triglycerides [24]. Possible mechanisms for hypocholesterolaemic effect of probiotics are as follows:

a. Direct cholesterol assimilation by some probiotic bacteria in the presence of bile acids and under anaerobic conditions and thus making it unavailable for absorption into the blood.
b. Enzymatic deconjugation of bile salts by probiotic bile-salt hydrolase (BSH) activity resulting in free (deconjugated) bile salts which are less soluble and may be excreted more likely from the intestinal tract than conjugated bile salts. Faecal loss of bile salts should result in a higher demand for cholesterol as a precursor for the synthesis of new bile salts (in the liver) and therefore may lower serum cholesterol concentrations.
c. Fermentation of food-derived indigestible carbohydrates in the human gut that results in an increased production of short-chain fatty acids (SCFA) which can decrease blood

levels either by preventing hepatic cholesterol synthesis, or by redistributing cholesterol from plasma to the liver.

d. Cholesterol binding to bacterial cell walls

1.5.2. Modulation of the immune system

Possible stimulation of an immune response by probiotic bacteria may explain the potential therapeutic and prophylactic applications of such cultures in the treatment of infections and carcinogenesis [25]. Probiotic cultures have been shown to stimulate both non-specific (innate) and specific (adaptive) immunity [26]. It has been documented that administration of probiotics enhanced lymphocyte proliferation, increased serum levels of IgG and IgM, enhanced gut mucosal IgA-secreting cells, and stimulated production of different types of interleukin and interferon in immune cells.

1.5.3. Prevention/treatment of infections

Many authors have recently shown that probiotics prevent and/or treat some intestinal and urogenital infections and so may be useful as alternatives to antibiotics that have given an increase in the incidence of microbial antibiotic resistance. It has been reported that some probiotic bacteria such as *L. paracasei* and *L. rhamnosus* and *B. animalis* subsp *lactis* Bb12 can prevent adhesion of pathogens like *E. coli, Listeria monocytogenes, C. difficile, Salmonella enterica* serovar Typhimurium and *Enterobacter sakazakii in vitro* [27].

Probiotic bacteria can prevent infections by mechanisms which include competition for nutrients, secretion of antimicrobial substances (bacteriocins, hydrogen peroxide, carbon dioxide and diacetyl), reduction of pH, blocking of adhesion sites, attenuation of virulence, blocking of toxin receptor sites, immune stimulation, and suppression of toxin production [28]. *L. rhamnosus* GG, has been shown to be effective in the treatment of rotavirus diarrhoea and gastrointestinal disease caused by *Salmonella, Shigella* and *E. coli* in human trials [28] and Traveller's diarrhoea [29]. Other probiotic strains such as *L. casei* shirota, *B. infantis, B. breve* and *S. thermophilus* have also been found to be effective in the prevention/treatment of diarrhoea in children [30]. It has been reported that probiotics such as *L. acidophilus, L. reuteri, L. casei* shirota and some other LAB can inhibit *Helicobacter pylori* [31] which is an important agent in peptic ulcer disease.

1.5.4. Amelioration of lactose maldigestion

One of the health benefits of probiotics is alleviation of lactose maldigestion symptoms [32]. Oral supplementation with *L. reuteri and L. acidophilus* improved lactose maldigestion symptoms in lactose intolerant patients [33].

1.5.5. Management of allergy

The possible mechanisms of probiotic therapy include the normalisation of intestinal permeability and improving gut microecology, improvement of the intestine's immunological barrier functions, especially through intestinal immunoglobulin A (IgA) responses, improvement of intestinal inflammatory responses, and balanced control of proinflammatory and anti-inflammatory cytokines [34]. It has been reported that oral administration of an extensively hydrolysed whey formula supplemented with *B. animalis* subsp *lactis* Bb12 or *L. rhamnosus* GG significantly alleviated the clinical symptoms of atopic dermatitis [34], a common allergic skin condition which results in dry, itchy, inflamed skin patches. *L. rhamnosus* GG prevented incidence of early atopic eczema in infants [35]. Recent *in vivo* studies using murine models have shown that probiotics such as *L. rhamnosus* GG and *L. reuteri* can prevent experimental asthma development and reduce airway hyperresponsiveness in mice [36].

1.5.6. Prevention of cancer

It has been proposed that probiotics have anti-cancer effects. There are some potential mechanisms for anti-carcinogenic effect of probiotics [37] including:

a. Binding, blocking or deactivation of carcinogen/procarcinogen, thereby preventing the induction of DNA damage and genotoxic injury as an early event in the process of carcinogensis.

b. Decreasing levels of certain colonic bacterial enzymes (β-glucuronidase, nitroreductase, azoreductase and dehydroxylase) that produce carcinogens and co-carcinogens (including secondary bile acids) or convert procarcinogens to carcinogens through controlling the growth of fecal bacteria.

c. Altering intestinal bacterial activity and bile acid solubility by lowering the intestinal pH.

d. Immuno-stimulating effect.

e. Decreasing the colonic transit time, thereby removing faecal carcinogens more rapidly.

Some human trials have shown that oral consumption of probiotic strains such as *L. acidophilus, L. rhamnosus* GG and *Bifidobacterium* spp. generally reduced activity levels of glucuronidase and nitroreductase, but there are fewer reports on influence of probiotics on decreasing azoreductase levels [38]. A study with colon cancer patients revealed that consumption of fermented milk containing *L. acidophilus* decreased two risk markers for colon cancer including soluble faecal bile acid levels and colonic bacterial enzymes [39]. McIntosh *et al.* [40] studied the effect of oral administration of Lactic Acid Bacteria (LAB) on development of tumours in intestine of rats challenged with a carcinogen, DMH. They found that a commercial probiotic culture, *L. acidophilus* LAFTI® L10 was more effective than other LAB. Also, it has been shown that *L. casei* prevented the recurrence of superficial bladder cancer in humans [41]. The results of a study conducted by Tomita *et al.* [42] indicated that *L. casei* treated rats with bladder cancer induced by *N*-butyl-*N* (4-hydroxybutyl) nitrosamine (BBN) had lower tumor volume than control group. It has been shown that consumption of *L. casei* Shirota reduced the risk of bladder cancer [43] and prevented development of colorectal tumours [44].

It has been reported that several LAB can inhibit growth of microorganisms which can convert pro-carcinogenic substances to active carcinogens [45]. Gourama & Bullerman [46] found that *L. casei* subsp *Pseudoplantarum* inhibited biosynthesis of potential carcinigens, aflatoxins B1 and G1 by *Aspergillus flavus* subsp *parasiticus.* Also a human trial showed that administration of a fermented dairy product containing *L. acidophilus* reduced mutagenic activity in the faeces and urine through absorption of cooked/fried food mutagens [39].

Biffi *et al.* [47] reported that fermented milks containing *B. infantis, B. bifidum, B. animalis, L. acidophilus* and *L. paracasei* inhibit growth of the breast cancer cell line. *B. infantis* and *L. acidophilus* showed the highest inhibition among the strains. *In vitro* and *in vivo* studies have demonstrated the anti-tumour effects of *L. casei* LC9018 [48]. Potential probiotic propionibacteria have also been shown to bind a variety of carcinogens including mycotoxins [49], cyanotoxins [50], dietary lectins [51] and some heavy metals [50]. Antimutagenic properties of some dairy propionibacteria also have been reported [52]. Moreover, it has been demonstrated that *P. freudenreichii* and *P. acidipropionici* induce

apoptosis in colorectal carcinoma cells via production of short chain fatty acids including propionate and acetate [53].

1.6. Probiotic products

The many health benefits associated with probiotic bacteria as outlined above, have led to probiotics increasingly being incorporated into food products in order to develop "functional foods" which are defined as "foods claimed to have a positive effect on health" [54]. The first products of probiotics as functional food ingredients were different types of yogurts but nowadays, a wide range of probiotic products is available in the market including pharmaceuticals, different kinds of dairy products, probiotic drinks, dried fruits, baby foods or confectioneries [55].

Probiotic products can be made in three ways [56]:

a. Fermented probiotic products: probiotic culture is inoculated into the food product and allowed to ferment the food and provide flavours and organoleptic changes to it.
b. Non-fermented probiotic products: probiotics are added to the final product in suitable levels, with no opportunity for culture growth and fermentation.
c. Dietary supplements: probiotic cultures are utilised as concentrated and dried cells in the form of powders, capsules, or tablets.

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 colonise at this specific location [57]. Besides this, the probiotic strain must be tolerated by the immune system and not provoke the formation of antibodies against the probiotic strain. On the other hand, the probiotic strain can act as an adjuvant and stimulate the immune system against pathogenic microorganisms. 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 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 [57].

Factors affecting the quality of the probiotic product include [21]:

a. The ability of the probiotic product in delivering viable probiotic bacteria with desired health benefits at a suitable level to the consumer until the time of consumption

b. Strain selection regarding its reaction to the matrix/components of the targeted food
c. Sensory properties of the product
d. Packaging materials
e. Storage condition of the probiotic food

In order for the beneficial health effects of probiotics to be realised, regular consumption of high levels of probiotic bacteria is necessary. It has been suggested that minimum cell counts of viable bacteria should be more than 10^6 CFU per gram or millilitre of the probiotic product [58]. Saxelin *et al.* [29] showed that the minimum dietary intake of *L. rhamnosus* GG (in either freeze-dried powder or gelatine capsules) needed for recovery in the faeces of human subjects was 10^{10} CFU/day. Defining a specific effective number of probiotic microorganisms depends on the type of strain and delivery system used [54].

1.6.1. Dairy products

During the past few decades, probiotic bacteria have been increasingly exploited in commercial dairy products such as fermented milk and yoghurt. Dairy products are considered to be desirable food systems for the delivery of probiotics to humans. The high buffering capacity of dairy foods protects the probiotic bacteria against high acid levels in the stomach and supports viability of these microorganisms [59]. In addition, health promoting effects of probiotics are added to the healthful properties (vitamins, minerals and protein) of dairy products and make a healthy functional food [60].

(a) Probiotic yoghurt

Yoghurt has been considered as a healthy product with various desirable effects for consumers. In recent years, the production and marketing of probiotic yoghurts and other fermented milk products has increased significantly throughout the world. It is recommended that one or both of the conventional yoghurt starter cultures (*L. bulgaricus* and *Streptococcus thermophilus*) is used in order to manufacture a probiotic yoghurt with desirable flavour and texture [61].

(b) Probiotic ice cream

It has been shown that ice cream could be used as a suitable food vehicle for delivery of probiotics to human diet without any unfavourable effect on sensory properties

of the final product [62] using *L. acidophillus* and *B. bifidum* to make a probiotic ice cream. In another study, Hagen and Narvhus [63] produced a probiotic ice cream using four probiotic strains including *L. acidophilus*, *L. reuteri*, *L. rhamnosus* GG and *B. bifidum*. Their results indicated that viable counts of the mentioned probiotic bacteria remained above 10^6 CFU/g over 52 weeks of storage at -20ºC. All the ice cream samples obtained high scores in the sensory evaluation. In another trial, the viability of probiotic strains (*B. longum* and *B. lactis*) used for manufacturing a probiotic ice cream as well as sensory acceptance of the final product was evaluated during 15 weeks of frozen storage at -18ºC. The results indicated high levels of viable counts (>10^6 CFU/g) and acceptable organoleptic properties [64].

(c) Probiotic cheese

Cheese has certain advantages over other fermented dairy products (such as fermented milk and yoghurt) as a carrier of probiotics because of its higher pH, more stable matrix, higher fat content and higher buffering capacity. These unique characteristics support the long-term survival of probiotic bacteria and protect them during passage through the GI tract [65]. The successful production of probiotic cheeses relies on probiotic organisms remaining viable during ripening and shelf-life without adversely affecting cheese flavour, texture, composition and other sensory properties [66]. In one study, *B. bifidum* was incorporated into Cheddar cheese. The viability of this strain remained at 2.0×10^7 CFU/g for up to six months with no adverse effect on the sensory characteristics [67]. Gomes *et al*. [68] made a probiotic Gouda cheese using bifidobacteria in combination with *L. acidophilus* strain. After nine weeks of ripening, cheese flavour was significantly affected by the bifidobacteria possibly because of acetic acid production. It has been reported that *B. bifidum*, *B. longum* and *B. infantis* incorporated into a traditional soft rindless Italian cheese (Crescenza cheese) survived at levels of 10^8, 10^7 and 10^5 CFU/g respectively for two weeks after cheese making [69]. O'Riordan & Fitzgerald [70] studied the survival of different bifidobacteria species (*B. longum*, *B. breve*, *B. catenulatum*, *B. bifidum*, *B. angulatum*, and *B. infantis*) in cottage cheese after two weeks storage at 4ºC. Their results revealed that viability of bifidobacteria is strain dependant and *B. bifidum* showed the best survival. Kourkoutas *et al*. [71] produced a probiotic cheese using immobilized *L. casei* on apple and pear pieces. They concluded that fruit pieces can

support viability of the probiotic cells during 71 days of ripening at 4 to 6ºC and also have acceptable sensory properties.

1.6.2. Probiotic fruit and vegetable juice

For several years, most of the probiotic products in the market have been in the form of fermented milk and dairy products. In recent years, fruit juice has been used as an ideal medium for carrying probiotics [72]. One reason is that the residence time in the stomach of fruit juice is short, so that the bacteria are not exposed for too long to the unfavourable acidic conditions of the stomach. Also, fruit juice is a good source of nutrients such as vitamins, minerals, dietary fibres and phytochemicals (*e.g*. polyphenols and carotenoids). Furthermore, fruit juice is considered as a healthy and refreshing product that is pleasing to a large percentage of the consumers. It also suits consumers who have allergy to milk products, are lactose intolerant or have no desire to eat dairy foods. Fruit juices are also vegan compliant, cholesterol free and soy free and suitable to specific categories of people [73]. Some examples of the most common commercial probiotic fruit drinks are presented in **Table 1.4**.

(a) Fermented fruit/vegetable juice-based probiotics beverages

A number of studies have been done on fermented probiotic fruit or vegetable juice. Yoon *et al*. [74] produced a tomato juice fermented by four probiotic cultures (*L. acidophilus* LA39, *L. plantarum* C3, *L. casei* A4, and *L. delbrueckii* D7) with viable numbers of the cultures ranging from 10^6-10^8 CFU/ml after one month of refrigeration at 4ºC. Yoon *et al*. [75] examined the suitability of red beets as a substrate for producing probiotic beet juice by the above four probiotic strains. The results showed that with the exception of *L. acidophilus*, the viability of all other cultures remained at levels greater than 10^6 CFU/mL after 4 weeks of refrigerated storage. Yoon *et al*, [76] found that both *L. plantarum* and *Lb. delbrueckii* could survive in fermented cabbage juice during four weeks storage at 4ºC, whereas *L. casei* completely lost its viability in the cabbage juice due to low pH and high acidity after 2 weeks of refrigerated storage.

(b) Non-fermented fruit/vegetable juice-based probiotic drinks

For producing the probiotic fruit/vegetable juice, juice is pasteurised and probiotic culture (10^{10} - 10^{11} CFU per litre of beverage) is added to cooled juice (< 6°C). The juice is

then packed in suitable containers and stored at refrigeration temperature. Consumers can recognize a sensory difference between probiotic orange juices and conventional ones and prefer the organoleptic characteristics of conventional juices [72]. Age and gender are important factors in the acceptance of probiotic fruit juice [77]. Luckow *et al*. [78] observed that tropical fruit juices including pineapple, mango and passionfruit can mask "off-flavours" in orange juice containing *L. paracasei* ssp. *Paracasei.*

1.7. Probiotic survival in food matrixes

The factors that affect the viability of probiotics in a food matrix during processing and storage include pH, oxygen levels, temperature, and presence of competing microorganisms and inhibitors [79]. Since the probiotic food should contain viable probiotic cultures at suitable levels at the time of consumption, using some techniques for improving stability of probiotic strains in food systems is of great importance [21]. These methods include stress adaptation, microencapsulation, and inclusion of prebiotics and modulation of packaging conditions**.**

1.7.1. Stress adaptation

Probiotic organisms are exposed to various stressful conditions (heating, cooling, oxidative stress, low pH, osmotic conditions, bile salts, starvation, etc.) in their natural habitats and during industrial processes, storage and passage through gastro-intestinal tract [80]. Exposure to sub-lethal stresses may enhance the resistance of the cultures to subsequent stressful conditions [54]. Park *et al*. [81] reported that acid adaptation (at pH 5.2 for 2 h) improved survival of the *B. breve* in different stressful conditions (2-5 pH, 0.2-1.0 % bile and 100–1000 ppm H_2O_2). Results of a study conducted by Broadbent *et al*. (1997) revealed that heat shock pre-treatment (50ºC), considerably enhanced the ability of exponential phase *L. acidophilus* to tolerate subsequent high temperature (63ºC). In another study, it was shown that log phase *L. acidophilus* subjected to acid stress (pH 3.8-6.0) was capable of withstanding lower pH values (Lorca *et al.*, 1998). Schmidt & Zink [82] reported the presence of a heat shock gene for some *Bifidobacterium* spp. (*B. longum* strains NCC481, NCC490 and NCC585, *B. adolescentis* NCC251, and *B. breve* NCC298.). However it was induced on the transcriptional level only in *B. longum* NCC481 and *B. adolescentis* NCC251 by rising temperatures. They observed that log phase of *B.adolescentis* exposed to a sub-lethal heat stress (45ºC and 47ºC) or sub-lethal salt stress

Table 1.4. Examples of commercial fruit juice-based probiotic drinks [73]

Country	Brand	Fruit juice Composition	Juice content %	Probiotic strain
Sweden	Pro Viva	Strawberry, Blackcurrant, Bluberry, Rosehip	≈ 20	*L. plantarum* vv
The UK	SHOT	Raspberry, blackcurrant and grape	?	*L. plantarum* 299v
Finland/ Sweden	Gefilus/ Gfilac	Whey drink with Apricot and Peach juice	17	*L. rhamnosus GG*
Finland/ Sweden	Gefilus/ Gfilac	Orange/ Peach juice + prebiotic + Vit. C	60	*L. rhamnosus* GG
Finland/ Sweden	Gefilus/ Gfilac	Pineapple and Carrot + Ca++ + β-caroten	50 and 10	*L. rhamnosus GG*
Finland/ Sweden	Gefilus/ Gfilac	Apple and grape	100	*L. rhamnosus GG*
Norway	Biola	Orange-Mango	> 95	*L. rhamnosus* GG
Norway	Biola	Apple-Pear	> 95	*L. rhamnosus GG*
Sweden/ Finland	Rela	Orange	?	*L. reuteri*
The UK	"Its Alive"	Peach-Banana	?	*B. lactis*
Japan	Bikkle	Fruit (?) + whey mineral + Prebiotics + Dietary fibres	?	*Bifidobacterium* spp.
Germany	Pianola	Orange juice	?	*L. casei*
USA	GoodBelly	Pomegranate-Blackberry, Cranberry-Watermelon, Mango, Blueberry-Acai, Strawberry or Lemon Ginger	100	*L. plantarum* 299v
Ireland	Dawn	Orange juice	100	*B. animalis* subsp. *Lactis*

(1.5 and 2.0% NaCl) showed a considerably risen resistance to lethal temperature of 55ºC. Furthermore, pre-treatment of the mentioned strain with 0.1% bile salts led to a noticeable protection against higher bile salts concentrations (0.3% and 0.4%). Lorca & de Valdez [83] found that *L. acidophilus* grown in uncontrolled pH fermentation (final pH 4.5) showed more resistance to acid stress as well as other different stress conditions (including ethanol, hydrogen peroxide, freezing and freeze drying) than the cells grown in controlled pH conditions (pH 6.0). Desmond *et al*. [84] demonstrated that exposure of probiotic *L. paracasei* to sub-lethal temperature (52ºC for 15 min.) resulted in 300 and 700 fold protection against lethal temperature of 60ºC in MRS medium and skim milk, respectively. Sub-lethally heat treated and salt adapted *L. paracasei* showed 18 and 16 fold greater survival respectively during spray drying at outlet high temperature (95-105ºC) compared to non-treated cells. It has been reported that pre-treatment of *L. rhamnosus* with heat (50ºC) or salt (0.6 M NaCl) resulted in a marked viability improvement of powdered form of the strain during storage at 30ºC (Prasad *et al.*, 2003). Saarela *et al*. [85] examined the viability improvement of *Lactobacillus* and *Bifidobacterium* strains sub-lethally treated with acid and heat (3.0-4.0 pH and 47ºC for 30 min-60 min) in subsequent lethal conditions (pH 2.5, 1.5 % bile and 55ºC for 1-3 h). They found that stress adaptation enhanced the viability of lactobacillus strains more than that of bifidobacteria at both laboratory and fermentor scale.

1.7.2. Microencapsulation

Microencapsulation is defined as a technology of including sensitive ingredients (solid, liquid or gaseous) within several matrices since the ingredients are entrapped or completely surrounded by the protective matrices [86]. Early on, microencapsulation was mainly used to mask off-flavors of food ingredients and for conversion of liquids to solids. Encapsulation helps in the physical separation of sensitive viable cells from the external adverse environment thus improving the viability of cells [87]. Methods of microencapsulating probiotics include spray drying, freeze drying, extrusion, coacervation, chemical methods using Ca-Alginate, k-carrageenan, gums (xanthan, Arabic, etc.), starch, etc. The size of the microspheres usually ranges between 0.2 to 5000 µm. The purpose of microencapsulation is not just a protection through physical barrier for long term storage but also a controlled release of the functional probiotics passing through the stomach to effectively reach the intestines [88]. Among the different techniques of microencapsulation,

spray drying and coacervation are considered by Chavarri et al [89] as the cheapest techniques, eventhough the former is rarely applied because of thermal cell inactivation and the scale up of latter is quite arduous.The stability and activity of microcapsules in gastro-intestinal system is dependent on several factors like pH of the core and the gut, particle size, chemicals present in the microencapsulating material and enzymes present in the gut. Some studies on encapsulation of probiotic microorganisms by different methods are summarised in **Table 1.5**.

Sub-lethal thermal shock of 50 °C- 52.5 °C to *Lactobacillus acidophilus* NRRL B-4495 and *Lactobacillus rhamnosus* NRRL B-442 during microencapsulation in raspberry juice through spray drying improved percentage of cell survival [90]. Microencapsulation of *L. reuteri* DSM 17938 in alginate by vibrating technology was found to produce stable probiotic microcapsules with improved survivality during storage and exposure to gastrointestinal and osmotic stress conditions. Whey protein isolate (WPI) containing matrix is reported to have potential to deliver live *Lactobacillus rhamnosus* GG in spray dried microencapsulates in low pH beverage (apple juice) even after storage at 25°C for 5 weeks due to the ability of WPI to create a buffered microenvironment within the hydrated colloid particle surrounding the embedded probiotic, thus isolating the bacteria from the stresses of the low pH external environment [91].

1.7.2.1. Wall Materials

The encapsulation efficiency and the microsphere stability are greatly dependent on the encapsulating material known as wall material. Ideally the wall material should be water soluble since most spray drying suspensions are water based and possess good mechanical strength, compatibility with the core materials, emulsification properties and film forming and low viscous properties [92]. Biopolymers, natural gums (acacia, k-carrageenan, alginates, etc), low molecular weight carbohydrates and proteins (whey protein, gelatin, etc.) are generally considered as good wall materials [92]. This of course varies from strain to strain, however the carriers (like Arabic gum, inulin, FOS, maltodextrin, polydextrose, skim milk powder, soy milk protein, etc.) in the suspension may have a significant effect on the viability [93, 94]. Since these wall materials contain prebiotic sources, when mixed with probiotics the produced powders can be considered as synbiotics (Roberfroid, 1998). Single and double chitosan coated alginate beads of

Lactobacillus plantarum were able to have more than 5.5 log CFU/mL of the cells in pomegranate juice after 4 weeks of storage at 4^{o}C whereas free cells died under similar conditions [95]. Nualkaekul et al [96] coated alginate beads containing cells of *Lactobacillus plantarum* and *Bifidobacterium longum* with chitosan, gelatin, and glucomannan as wall materials to study the cell survivality in pomegranate and cranberry juice. The study revealed that chitosan coated beads increased cell survival the most in pomegranate juice during 6 weeks storage.

(a) Maltodextrin

Maltodextrin is a white granular hygroscopic powder usually soluble in water that is obtained by partial hydrolysis of starch. The Dextrose equivalent usually varies between 4 and 20. Maltodextrins act as osmotically inactive bulking compounds and increase the cell space thus strengthening the glassy matrix [97]. Proteins (soy protein and whey protein) are often used as adjuvants along with maltodextrin at a defined ratio, to improve the microencapsulation of selected microorganisms. High DE maltodextrins help in preventing lipid oxidation by forming a strong barrier. Caking, crystallization and collapse was observed when low molecular weight carbohydrates were used because of their low glass transition temperature (Tg) which requires very low temperature for spray drying. Research suggests that spray drying process is unsuitable for sugar and acid rich foods due to their stickiness to the spray drying chamber caused by their low Tg [97]. Maltodextrins are metabolized by membrane bound glucosidases unlike simple sugars. Glucoamylase acts on the alpha 1,4 glucan link which is the non reducing end of maltodextrin. The end products have a high amount of organic acids [98] lowering the pH which is detrimental to the pathogens in the gut. In addition, maltodextrin has a protective effect during the reconstitution of the probiotic powder before its usage [99].

(b) Pectins

Pectins are soluble dietary fibers with natural emulsifying property when present in a suspension due to their high acetyl content. Pectin was also found to have microencapsulating property due to the protein residues present in the chain [115, 116]. Sugar beet pectin was used to microencapsulate lipophilic food successfully and it was also observed that there was no interference in the spray drying [118]. So, in summary the wall material should offer good emulsification and solubility, good rheological property,

chemically non-reactive to the core, and an ability to hold and protect the core during severe drying. Considering all the above factors and their manipulations, spray drying is a form of art rather than a science [119].

1.7.3. Spray Drying of Probiotics

Sprays drying of yogurt to preserve Lactobacillus and dairy starter cultures have been long investigated [120]. Though spray drying processing cost is low, there are difficulties like low survival rates of the probiotics, poor rehydration properties of the resulting powders etc. Fermented rice drink *lao-chao* was successfully spray dried with *L. acidophilus* and *B. longum* strains and the higher the temperature, the better was the microspheres' uniformity with a high cell count [121]. Spray drying at lower outlet temperatures gave a better survival rate in studies performed using different microencapsulating materials like cellulose acetate phthalate, starch and its derivatives (maltodextrin), acacia gum, etc. [122]. Trehalose-monosodium glutamate supplemented medium also proved to be a good encapsulating material for preventing cell damage of probiotics (*L. rhamnosus*) and also ensuring longer storage [123].

Spray drying outlet temperature is mainly responsible for the inactivation of the viable cells which is dependent on inlet temperatures, air flow and feed rate, suspension composition and nozzle droplet size [124]. High viability was achieved at lower outlet temperatures during spray drying [125] and high temperatures reduced the viability of the lactobacilli irrespective of cell load [126]. An outlet temperature of 70°C gave a maximum yield of 97% where as an outlet temperature of 120°C gave 0% survival in spray drying studies of *Lb. paracasei* NFBC 338 [120].

Denaturation or melting of DNA is the common cause for cell death at temperatures above $90^{o}C$ [127]. Inclusion of thermo-protectants and microencapsulating agents like maltodextrin and starch showed better resistance towards high temperatures. When fresh cultured cells and spray dried cells were grown in the presence of 5% NaCl, spray dried culture showed a reduction in viability. This reduced viability in presence of NaCl is accounted to extensive cell membrane damage and the stress during spray drying. But few properties like bacteriocin production, cell wall adherence, acid and bile tolerance (to an extent) were unaffected even after being subjected to high temperatures [120].

Table 1.5. Encapsulation of probiotic microorganisms by different methods

Microorganism	Method	Support material	Application	Reference
L. reuteri	Emulsion or extrusion	Alginate	Dry fermented sausage	[100]
L. acidophilus and *B. lactis*	Emulsion	Calcium-induced alginate-starch	Yoghurt	[101]
L. acidophilus, B. bifidum and *L. casei*	Extrusion	Chitosan coated alginate beads	Stirred yoghurt	[102]
B. longum and *B. infantis*	Spray drying	Gelatin, starch, skim milk and Arabic gum	-	[103]
B. longum	Spray drying	Whey protein	yoghurt	[104]
L. acidophilus	Extrusion	Alginate	-	
B. longum and *B. infantis*	Spray drying	Gelatin, starch, skim milk and Arabic gum	-	[105]
B. longum	Gel beads/emulsion	κ-carrageenan	Stirred yoghurt	[105]
L. acidophilus and *B. infantis*	Gel beads/emulsion	Alginate/starch	Ice cream	[106]
L. acidophilus and . infantis	Gel beads/emulsion	Alginate/starch	Cheddar cheese	[106]
Bif. breve, Bif. longum and Lb. acidophilus	Emulsion/spray drying	Milk fat/whey protein	-	[104]
Lb. acidophilus and Bif. lactis	Spray drying	Cellulose acetate phthalate	-	[107]
B. longum, B. bifidum, B. infantis, B. breve and B. adolescentis	Gel beads/emulsion	Alginate	Milk	[108]

Microorganism	Method	Support material	Application	Reference
Bifidobacterum PL1	Spray drying	Modified waxy maize starch	-	[109]
B. lactis and L. acidophilus	Gel beads/extrusion	Alginate	-	[110]
L. acidophilus and Bifidobactreium spp.	Freeze drying	Alginate	Frozen fermented dairy dessert	[111]
L. acidophilus and B. infantis	Gel beads/emulsion	Alginate/starch	Yoghurt	[112]
B. bifidum	Freeze drying	κ-carrageenan	Cheddar cheese	[67]
B. longum and *B. infantis*	Spray drying	Gelatin, starch, skim milk and Arabic gum	-	[113]
Lb. acidophilus	Extrusion	alginate plus prebiotics (Hi-maize starch, Raftiline and Raftilose) coated with different coating materials (chitosan, poly-L-lysine, and Alginate)	Yoghurt	[114]
L. acidophilus and *B. lactis*	Emulsion	Calcium-induced alginate-starch	Yoghurt	[115]
L. plantarum MTCC5422	Freeze drying	Fructooligosaccharides/ denatured whey protein	Noodle	[116]

1.7.4. Sub-lethal temperature shock treatment- causes and effects

Exposure of bacteria to a temperature slightly above the optimal growth temperature induces tolerance and adaptation strategies during subsequent stress events [128]. Different sub-lethal stresses studied so far include thermal, acid, salt, osmotic, high pressure, peroxides, UV, etc [129]. Usually a temperature rise of 10°C above its optimal growth temperature leads to shock [127]. Most commonly studied stress adaptation is thermal stress. During the stationary phase, cells develop resistance mechanisms against adverse conditions caused by nutrient depletion and carbon source starvation. In studies conducted by Teixeira et al [127], the mixture was inoculated with the probiotic culture before spray drying and incubated for 30 min with constant stirring for the microbial adaptation. More than 50% survival was seen when *L. rhamnosus GG* was spray dried during the stationary phase of its growth [130].

Cell membrane seems to play a vital role in any type of stress (acid, bile, osmotic, etc.) though characterizing the membrane proteins of individual strains is technologically unfeasible. The stress resistance proteins are produced mainly during the sub-lethal exposure prior to drying [127]. Stress conditions provoke the substrate depletion as some pathways may be hindered during starvation irrespective of the concentration of the actual substrate present in extra cellular medium. This may lead to changes in cellular physiology by diminishing size or formation of spores (*Lactobacilli* are non-sporing though). But there is only limited evidence proving the physiological response of the microbial cells towards stress in *Lactobacillus*. Sub-lethal temperature is usually around 53°C for *L. acidophilus* while the lethal temperature is 60°C. Heat treatments gave the best viability after sub-lethal stress followed by salt, peroxides, and bile, maintaining high viability after spray drying in *L. paracasei* [84]. However, spray drying during the exponential phase also gave significantly high recovery (83%) of probiotic cells [131]. Thermo tolerance in *L. bulgaricus* was induced effectively in the log phase of the growth cycle at 52°C for 20 min at constant agitation while in the latter growth, cells in the stationary phase were more thermotolerant than log phase bacteria [132]. During storage in their dried form, following stress adaptation, mid log phase stressed cells showed better survival up to 14 weeks [133]. Studies show that acid resistance genes cross reacted with heat shock proteins which could be a possible explanation of cross tolerance [134]. Heating at a temperature of 64°C and lower resulted in damage to the cytoplasmic membrane while above 65°C it caused a permanent damage

(due to denaturation) to cell wall and cytoplasmic proteins. Increase in tolerance of *L. bulgaricus* towards other stress inductions like antibiotic resistance, high salt and pH concentration was observed when subjected to sub-lethal effect of heat stress below 64°C [127]. Heating menstrum also has an effect on thermo-tolerance induction where a complex media is believed to give better adaptation to the bacterial cells rather than a simple media due to the presence of proteins. Thermal sub-lethal treatment can increase the survival rate of *Lactobacilli* remarkably (between 16-18 folds depending on the adaptation media) during and following spray drying [14]. Viability of *Lactobacillus reuteri* DSM 20016 starin in red fruit juice was improved on starin adaptation to thermal abuse at 25^{o}C (24-48 h) before storage at 4^{o}C.

1.7.5. Spray drying of fruit juices

Fruit juice powders are easy to store, handle and transport, offering stable natural aroma for a longer time and versatile in use. Fruit powders with moisture content less than 4% can be used to make toffees, flavor toppings, instant-mix drink powders, etc [135]. Fruit juices have a low glass transition temperature due to the low molecular weight of the sugars present which increases the problem of stickiness during processing and handling. Thermoplasticity and hygroscopicity (ability to absorb moisture from high relative humidity surrounding) of fruit juice might pose problems during the spray drying causing them to adhere to the chamber wall due to their stickiness, clogging and caking [136]. Glass transition temperature (Tg) refers to the transformation temperature for transition from liquid to glass occurring during rapid cooling. Tg is usually lower than the melting temperature of the substance [137]. Inclusion of additives, like maltodextrin increases Tg and hence reduces problems of stickiness and agglomeration by increasing the operating temperature [135].

Recovery is one of the main indicators of a successful spray drying process. Product recovery is dependent on several factors like the viscosity of the liquid to be spray dried, solid content (dissolved and suspended solids), additives (maltodextrins, soy proteins, starches, etc) added. Juice stickiness can be reduced by either increasing the drying temperature or by adding anti-caking/non sticking additives. But the usage of additives might increase the cost of the process. Over all, higher inlet temperature, higher maltodextrin concentration with lower DE gave a higher rehydration, low hygroscopicity

and hence low caking of powders from pulps like orange and tomato [138]. Combination of maltodextrin and gum Arabic (10% each) showed better survival rate under refrigerated storage of probiotic cashew apple powder [139].

1.7.5.1. Cell recovery after spray drying

The physicochemical properties of the rehydration media (pH, solutes in the media, temperature etc) as well as the conditions of rehydration also affect the viability and resuscitation of the injured encapsulated cells. Slow rehydration and higher temperatures were preferred for a better viability [140]. Increasing the rehydration media temperature increases the viability after spray drying linearly until a certain temperature. Injured cells normally have an extended lag phase and hence their growth cycle is longer than the regular ones [141] although this is strain dependent. Reduced droplet size has also been shown to reduce the thermal inactivation. Electron microscopy is usually employed to study the obtained microcapsules. Spray dried capsules are smaller than freeze dried particles and a better survival during storage is expected.

1.7.6. Storage and shelf life

Probiotics are very sensitive to environmental stresses like heat, oxygen, humidity, etc., and hence special protection is needed to maintain viability as high as 10^6-10^8 CFU/mL. Packaging materials play a significant role in maintaining stable viable counts of the cells. Different authors have suggested different packaging materials like glass, metal pouch etc. Several factors like oxygen permeability, temperature, light, humidity, etc, need to be taken into account and are strain dependent. Due to the absence of electron transport chain during storage and catalase enzyme, the free oxygen from atmosphere is converted to peroxides during storage which is detrimental to the probiotic. An increase in relative humidity can lead to problems like caking and agglomeration. Glass bottle storage of spray dried microencapsulated (spray dried) probiotics had a high shelf life (>6 log CFU/g) of more than 40 days at low temperatures (4^oC) [142]. Inclusion of desiccants can improve storage at 25^oC (at least 25 days) irrespective of the coating material used, while survival in glass bottles was higher than in PET bottles maintained under same conditions [142].

Autocatalytic lipid oxidation and non enzymatic browning, enzymatic reaction, starch retrogradation, degradation of enzymes and vitamins are influenced by water

activity (a_w). There is usually no microbial proliferation seen between the range of 0.2 to 0.5. Water activity between 0.2-0.6 was suggested for stable maintenance of probiotics in spray dried milk powders [130]. Although a_w >0.5 is detrimental to the cell viability due to lipid peroxidation and outgrowth of unwanted microorganisms. It is essentially the bound water on the cell membranes, which affects the death rate of cell during drying as it stabilizes the proteins and cell membranes [94]. Moisture content and water activity have to be carefully monitored to ensure quality during long term storage. Spray dried powder of cashew apple with *L.casei* NRRLB-442 and 20% maltodextrin lost microbial viability at 28 days when stored at 25^{o}C but showed higher viability until 35 days at 4^{o}C [139].

1.8. Prebiotics

"Prebiotics are non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, thus improving host health" [143]. Modulation of the colonic microflora, enhancing resistance to pathogens, reducing the risk of colon cancer, heart disease, obesity, diabetes and digestive tract disorders, enhancing mineral bioavailability and adsorption, and lipid modulation are some possible beneficial health effects of prebiotics [23].

Prebiotics comprise disaccharides (such as lactolose and lactitol), oligosaccharides [such as fructooligosaccharides (FOSs) and transgalactooligosaccharides (TOSs)], soybean oligosaccharides (mainly trisaccharide raffinose and the tetrasaccharide stachyose), lactosucrose, xylooligosaccharides and polysaccharides (such as resistant starch). The most intensive studies have focused on FOSs and TOSs. Prebiotics are not degraded or absorbed in the stomach or in the small intestine and reach the colon (largely intact) where they are fermented by the gut bacteria (specially bifidobacteria and lactobacilli), to short-chain fatty acids (SCFA) (mainly acetate) and other metabolites (*e.g*., lactate). It has been suggested by many studies that consumption of prebiotics combined with probiotic bacteria as synbiotics may enhance the beneficial effect of each of them. Probiotic strain, Lactobacillus plantarum PCS26 was found to utilise the inulin in Jerusalem artichoke juice converting to fructose and the culture attained 10^{10} CFU/mL in just 12 h of diauxic growth [144]. Addition of prebiotics during microencapsulation by extrusion technique provided protection to probiotics and also enhanced their growth in simulated digestive system and in apple juice [88]. The effect on probiotics is influenced by the type and concentration of

prebiotics. The authors also observed that galactooligosaccharides had the better effect on probiotic compare to inulin. On the other hand, resistant starch showed no protective effect on probiotic cells in apple juice when microencapsulated with *Lactobacillus rhamnosus* GG [91].

1.9. Effect of fruit juice as carrier matrix on probiotic survival and functional performances

1.9.1. Survival

As stated earlier it has been suggested that the minimum level of viable bacteria should be 10^6 CFU per gram or millilitre of the probiotic product or 10^8 CFU per day at the consumption point [58]. It is therefore important that viability and activity of the probiotic remains optimal throughout the anticipated shelf life of the products [56]. Previous research has shown that survivability of probiotics in fruit juice/drink is genus, species and strain dependent [21]. Furthermore, the type of fruit juice, intrinsic parameters such as pH and the presence of particular compounds (*e.g.* benzoic acid or lactones), as well as extrinsic factors such as storage temperature, storage duration, packaging material, and dissolved oxygen level, have all been considered as decisive factors in determining the survivability of probiotics in fruit juice [21].

Higher viability of 5 strains of *Lactobacillus* and one *Bifidobacterium* was reported in orange juice (pH 3.65) and pineapple juice (pH 3.40) than in cranberry juice (pH 2.50). Loss of viability of the probiotics occurred more slowly in cranberry juices with higher adjusted pH (pH 4.50 and 5.50) than lower pH values (pH 2.50 and 3.50). Moreover, different probiotic strains showed different survival rates in the same fruit juice over the storage time (Sheehan *et al.*, 2007). In another study, storage stability of 9 *Lactobacillus* strains (*L. acidophilus* LB2, LB3 and LB45, *L. brevis* LB6, *L. rhamnosus* LB11 and LB24, *L. fermentum* LB32, *L. plantarum* LB42 and *L. reuteri* LB38) was investigated in a commercial fruit drink (pH 4.2) containing a mixture of fruit juice concentrates, purees and dairy ingredients over a period of 80 days at 4°C. Viability of *L. rhamnosus* LB11 and LB24, *L. reuteri* LB38, *L. plantarum* LB42 and *L. acidophilus* LB45 was maintained throughout the entire storage period in the drink, reducing by less than one order of

magnitude across the 80 days of storage, whereas viability of *L. acidophilus* LB2, LB3 declined more than 5 logarithmic cycles over the same period [21].

Oligofructose can be used as a sucrose substitute and prebiotic in probiotic apple juice having *Lactobacillus paracasei* spp paracasei as the probiotic culture with acceptable sensory profile [145]. Addition of prebiotic enhanced probiotic growth in the juice.

1.9.2. Acid and bile tolerance

To be effective in exerting their health promoting benefits for the host, probiotic microorganisms must adequately survive harsh environmental conditions encountered during gastro-intestinal passage, and then persist in the intestine [146]. The strong acidic environment of the stomach as well as the proteolytic activity of pepsin act as a natural, highly protective barrier against harmful microorganisms ingested through the consumption of food and drink. Exposure to hostile conditions of stomach also can result in viability losses of probiotics ingested [99]. While the normal internal pH of the human stomach ranges from 2.5 to 3.5 [147], this value can vary depending on the nature and composition of food and drinks ingested. Another important factor is the residence time of food entering the stomach, which depends largely on its physico-chemical properties. For example liquids, which pass through the stomach more rapidly than solids, may take less than 20 min to leave the stomach while a mixed meal can remain in the stomach up to 4 h.

Subsequently, probiotics confront with bile salts and pancreatin in the intestine which are further challenges to the viability of probiotics[99]. Primary role of bile in digestion is the emulsification and solubilisation of lipids. This property is mediated through the amphipathic nature of bile salts. In fact, bile salts act as a detergent, lowering the surface tension of dietary fats and breaking them down into tiny droplets, thus increasing the surface area for lipase activity. In the same way, bile salts may lethally damage bacteria via interaction with membrane lipids [99].

Moreover, it has been shown that the food matrix can influence the ability of probiotics to survive the gastro-intestinal environment, and that incorporation into carrier matrices such as milk, fermented milk, cheese, soymilk and meat may enhance the ability of probiotic bacteria to survive gastrointestinal passage [85]. It has also been speculated that due to the short gastro-intestinal transit time of fruit juices, inclusion in such carriers may

reduce exposure of probiotics to the harsh GI environment, and thereby enhance their effectiveness [148].

Saarela *et al*. [85] reported that the acid and bile tolerance of freeze-dried *B. animalis* subsp *lactis* E-2010 (Bb12) included in milk was significantly higher than that in a commercial fruit drink (pH 3.7, a blend of orange, grape and passion fruit). Champagne and Gardner [54] showed that 35 days refrigerated storage of *L. acidophilus* LB3, *L. rhamnosus* LB11, *L. reuteri* LB38 and *L. plantarum* LB42 included separately in commercial fruit beverages (a blend of 10 fruit juices and purees, pH 4.2) impaired their survival when exposed to simulated gastric juice (pH 2.0) as compared to the fresh cultures. The same study also revealed that 35 days storage of the probiotics in the fruit juice did not affect their tolerance to bile salts (0.3%) or pancreatin. Addition of prebiotics during microencapsulation of probiotics increases the resistance of these organisms to low pH and the presence of bile salt in simulated digestive system, resulting in higher number of cells than without prebiotics (control). The presence of galactooligosaccharides (0.3%) during microencapsulation of *L. acidophilus* and *L. casei* had a protective effect with only 3.1 and 2.9 logs reduction, respectively, after incubation in simulated gastric juice (pH 1.55), followed by simulated intestinal juice containing 0.6% bile salt ([88], because prebiotic compounds provide carbon and nitrogen sources for the growth of probiotic bacteria [149].

The lack of enzymes in the stomach to digest β glucan and the high stability of β glucan at low pH range helped β glucan encapsulated *Lactobacillus* species to maintain significant cell viability in high acidic gastric conditions (Shah et al., 2016). Fructooligosaccharide and β glucan [116] in encapsulates offered resistance to cell wall degradation by preventing the encapsulated cells from interaction with the bile salt.

1.9.3. Adhesion

It has been recognised that in order to exert health promoting properties on the host, probiotic micro-organisms need to survive in sufficiently high number and colonise the gastrointestinal tract. A prerequisite for intestinal colonisation is adherence to intestinal epithelial mucosa. Adhesion to intestinal epithelial mucosa is one of the main criteria by which a microorganism can be selected as a probiotic [20]. Bacterial adhesion to intestinal

epithelial mucosa is a complicated process, mediated through multiple surface biophysical and biochemical properties of both bacteria and epithelial mucosa such as passive forces, electrostatic interactions, hydrophobicity, steric forces and most importantly specific cellular surface components.

The ability of potential probiotics to adhere to intestinal epithelial mucosa could be evaluated using *in vivo* and *in vitro* assays [150]. Availability and ethical issues however hamper the widespread use of animal models or human/animal intestinal-derived biopsy samples] [151]. A number of *in vitro* models have been developed to evaluate the bacterial adhesion to intestinal mucosa [150]. Even though *in vitro* assays cannot mimic the complexities of *in vivo* conditions completely, various well controlled experimental conditions could be applied to demonstrate the adhesion ability of potential probiotics. Moreover a large number of potential probiotics could be screened using *in vitro* models [151]. Tissue cultures of intestinal epithelial cell lines Caco-2 and HT-29 are most extensively used *in vitro* models of assessment of adhesion ability of microorganisms. Moreover, since the entire intestine is lined by a thin layer of mucus produced by the epithelial cells, the ability of probiotic candidates to adhere to the intestinal mucosa *in vitro* is tested by performing adhesion assay to intestinal mucus [150].

Adhesion to intestinal epithelial mucosa by probiotics depends on many factors such as bacterial strain, bacterial concentration, probiotic formulation (combination), composition of bacterial growth medium, cell culture and co-culture medium, pH of co-culture medium, bacterial growth stage, intestinal cell culture growth conditions, incubation time, host specificity, the intestine section, digestion and composition of gut microbiota [26].

It is also likely that delivery vehicle matrices affect adhesion characteristics of probiotics, however to date, little is known about the effect of food matrices on adhesion ability of probiotics [22]. In order to more closely simulate *in vivo* conditions of bacterial adhesion to intestinal mucosa, it has been recommended that microorganisms are exposed to the food matrix before adhesion assay [150]. Study on the effect of food matrix on the adhesion ability of probiotics, to our knowledge, is only limited to the work of Ouwehand *et al.* [150], in which pre-treatment of probiotics with milk was shown to significantly

decrease the adhesion of probiotics to intestinal mucus glycoproteins compared with the control (HEPES-Hanks' buffer, pH 7.4).

1.10. Objectives of the present investigation

Most of the work done on probiotic fruit juices are on fruits grown in the temperate region. There are a large number of fruits that grow in tropical regions like in the North-East region of India. Fruits such as litchi, guava, banana, pineapple and orange have great potential to serve as carriers of probiotic bacteria. The ability of probiotic strains to confer probiotic properties in the juices of these fruits need to be ascertained. As cell stability and viability in such juices are strain specific, the ability of selected probiotic strains of *Lactobacillus* to retain probiotics property needs to be confirmed. The impact of prebiotics in the microcapsules on the quality of probiotic juice is also dependent on the specific strain used. Such addition also influences physical properties of microcapsules like size, capsule morphology, solubility, hygroscopicity etc [88]. The efficacy of the bacteria employed in fruits from tropical regions must be tested for their resistance to gastric digestion in order to provide the desirable health benefits. The heterogeneous nature of the tropical fruit juices and selection of cultures or strain(s) with potential probiotic properties are the major constraints. Hence, there is enormous scope for further research in this area. The changes in phenolics and flavonoids in the fruit juices on probiotication of these fruit juices have not been studied. The use of prebiotics in spray drying of probiotics and fruit juice on the viability of probiotic cultures during processing and storage need to be explored.

This study was therefore undertaken to make probiotic juices from four fruits of Assam, namely, litchi, guava, pineapple and orange using specific *Lactobacillus* strains that have not been reported in probiotication of such juices. There is limited literature available regarding changes in phytochemical and antioxidant properties of fortified probiotic juices of these fruits. The effect of the microencapsulation with different prebiotics and in-vitro stability in gastric environment that have not been reported were also studied. The study covered the following objectives.

1. To select potential strains of *Lactobacillus* and suitable fruit juice for probiotication based on cell viability and stability of the fermented juice during storage

2. To study the changes in physicochemical and functional properties of probiotic juices during refrigerated storage
3. To optimize the process conditions for development of probiotic fruit juice powders by spray drying technique
4. To study the stability and viability of spray dried *Lactobacillus plantarum* in probiotic litchi juice with varied coating materials
5. To study the stability and viability of spray dried probiotic litchi juice powder during storage and in simulated gastric environment

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