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Probiotic functional foods: Survival of probiotics during processing and storage

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ABSTRACT

Probiotic foods are reported to provide several health benefits, as they help in maintaining a good balance and composition of intestinal flora, and increase the resistance against invasion of pathogens. The demand of probiotic functional foods is growing rapidly due to increased awareness of consumers about the impact of food on health. Development of foods with adequate doses of probiotics at the time of consumption is a challenge, because several factors during processing and storage affect the viability of probiotic organisms. The presence of probiotics in food products may also adversely affect their quality and sensory properties. Several attempts have been made during the last few decades to improve the viability of probiotics in different food products during their production until the time of consumption. Major emphasis has been given to protect the microorganisms with the help of encapsulation technique, by addition of different protectants, and by alteration of processing and storage conditions. This contribution provides an overview of probiotic foods, factors responsible for survival of probiotics, and advance technologies used to stabilize their viability during processing and storage.

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1. Introduction

Probiotics are defined as live microorganisms when administered in adequate amounts confer a health benefit on the host (FAO/WHO, 2001). Elie Metchnikoff hypothesized the concept of probiotics around the year 1900, when he noticed that the long, healthy lives of Bulgarian peasants were the result of their consumption of fermented milk products. Later on it was found that yogurt contained the organisms necessary to protect the intestine from the damaging effects of other harmful bacteria. Different microorganisms have been used thereafter as probiotics in the last century for their ability to prevent and cure diseases (Lee, Nomoto, Salminen, & Gorbach, 1999). Probiotic microorganisms are usually available as culture concentrates in dried or deep-freeze form to be added to a food for industrial or home uses. These may be consumed either as food products (fermented or non-fermented) or as dietary supplements (products in powder, capsule or tablet forms).

Consumption of probiotic cells through food products is the most popular approach at present. Most of the probiotic food products are categorized as functional foods, and represent a significant part of it. The demand of probiotic functional foods is growing rapidly due to increased awareness of consumers. The global market for functional foods and beverages has grown from $33 billion in 2000 to $176.7 billion in 2013 that accounts for 5% of the overall food market, and is the driving growth for the food industry as a whole (Granato, Branco, Cruz, Faria, & Nazzaro, 2010; Hennessy, 2013). It has been estimated that probiotic foods comprise between 60% and 70% of the total functional food market (Holzapfel, 2006; Kołozyn-Krajewskaa & Dolatowski, 2012; Stanton et al., 2001).

Significant success has been achieved during the past few decades in development of dairy products containing probiotic

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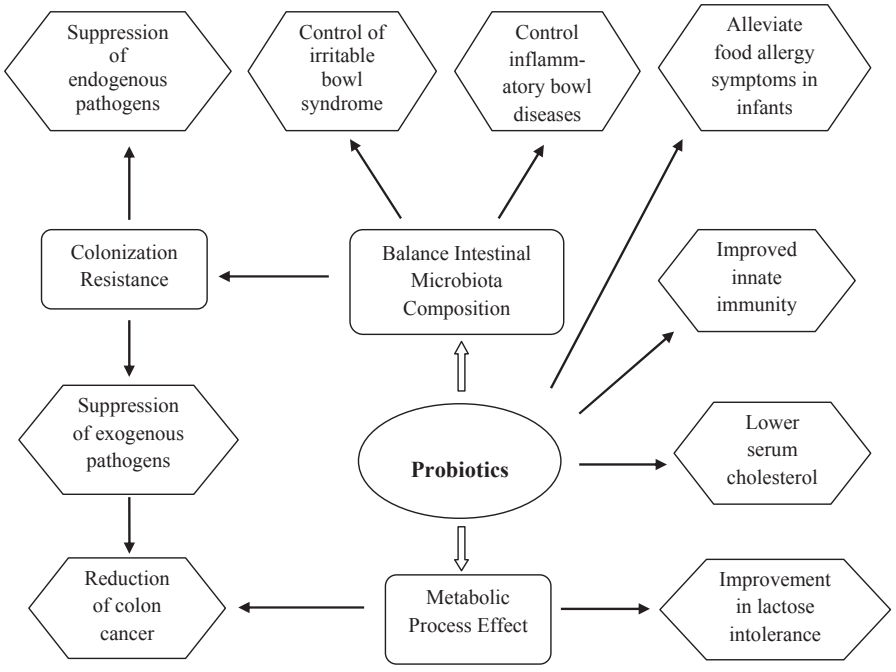

Fig. 1 – Probiotics consumption and health benefits (*Adapted from* Parvez, Malik, Ah Kang, & Kim, 2006).

bacteria, such as fermented milks, ice cream, various types of cheese, baby food, milk powder, frozen dairy desserts, whey-based beverages, sour cream, buttermilk, normal and flavored liquid milk (Mohammadi & Mortazavian, 2011). However, keeping in mind the high prevalence of lactose intolerance, different non-dairy probiotic products such as vegetarian-based products, cereal-based products, fruit juices, soya-based products, oat-based desserts, confectionary products, breakfast cereals and baby foods have been developed in recent years (Anekella & Orsat, 2013; Chen & Mustapha, 2012; Granato et al., 2010; Gupta & Abu-Ghannam, 2012; Lee & Salminen, 1995; Mortazavian, Khosrokhvar, Rastegar, & Mortazaei, 2010; Noorbakhsh, Yaghmaee, & Durance, 2013; Rivera-Espinoza & Gallardo-Navarro, 2010).

The probiotic foods should be safe and must contain the appropriate probiotic organisms in sufficient numbers at the time of consumption. Therefore, the probiotic strains selected should be suitable for large-scale industrial production with the ability to survive and retain their functionality during production and storage as frozen or dried cultures. It must survive during the food processing operations, and also in the food products into which they are finally formulated. The purpose of this contribution is to provide an overview of probiotic foods and factors responsible for survival of probiotic microorganisms, and recent technological advances in maintaining their viability during processing, packaging and storage.

2. Probiotic microorganisms in food

2.1. *Beneficial health effects of probiotics*

Probiotics provide a number of health benefits mainly through maintenance of normal intestinal microflora, protection against gastrointestinal pathogens (D'Aimmo, Modesto, & Biavati, 2007; Lourens-Hattingh & Viljoen, 2001), enhancement of the immune system (Gilliland, 1990), reduction of serum cholesterol level and blood pressure (Rasic, 2003), anti-carcinogenic activity (Rasic, 2003), improved utilization of nutrients and improved nutritional value of food (Lourens-Hattingh & Viljoen, 2001) (Fig. 1). Therapeutic applications of probiotics include prevention of infantile diarrhea, urinogenital diseases, osteoporosis, food allergy and atopic diseases; reduction of antibody-induced diarrhea; alleviation of constipation and hypercholesterolemia; control of inflammatory bowel diseases; and protection against colon and bladder cancer (Lourens-Hattingh & Viljoen, 2001; Mattila-Sandholm et al., 2002; Salminen, 1996; Venturi et al., 1999).

There are several evidences supporting potential clinical applications of probiotics in the prevention and treatment of gastrointestinal, urinogenital tracts and respiratory diseases (Gardiner et al., 2002). Mann and Spoerry (1974) discovered that blood serum cholesterol levels reduced significantly by drinking yogurt fermented with wild strains of Lactobacillus sp. Harrison, Peat, and de Heese (1975) reported decreased levels of serum cholesterol by consuming infant formula added with cells of *Lactobacillus acidophilus*. Similarly, Gilliland (1990) and Gill and Guarner (2004) showed control of serum cholesterol levels in adult human experiments.

It is hypothesized that these benefits may result from the growth and action of the probiotics during the manufacturing of cultured foods, while some may result from the growth and action of certain species of probiotics in the intestinal tract (Rasic, 2003). Stanton et al. (2005) stated that the claimed health place benefits of fermented functional foods are either due to probiotic effect (through the interaction of ingested live microorganisms with the host), or indirectly due to biogenic effect

(as a result of ingestion of microbial metabolites produced during the fermentation process).

2.2. *Commercially used microorganisms for probiotic foods*

Though a wide variety of genera and species of microorganisms are considered as potential probiotics (Holzapfel, Haberer, Snel, Schillinger, & Huisin't Veld, 1998; Shah & Ravula, 2004), the one used commercially in probiotic foods are predominantly bacteria from the genera Lactobacillus and Bifidobacterium (Tables 1 and 2). The primary reason being both these genera have a long history of safe use and are considered as GRAS (generally recognized as safe). Lactobacillus and Bifidobacterium species are also dominant inhabitants in the human intestine (*Lactobacillus* in the small intestine and Bifidobacterium in the large intestine). However, species belonging to the genera Lactococcus, Enterococcus, Saccharomyces and Propionibacterium yeasts (e.g. *Saccharomyces cerevisiae* and *Saccharomyces boulardii*) and filamentous fungi (e.g. *Aspergillus oryzae*) are also used as probiotics due to their health-promoting effects (Rivera-Espinoza & Gallardo-Navarro, 2010; Vinderola & Reinheimer, 2003).

2.3. *Selection of probiotics*

Selecting the suitable probiotic strains in adequate dose is the first requirement for developing a probiotic food product. Viability during processing operations and storage, survival during intestinal transit, and potential health benefits on consumers are the primary criteria for selecting suitable strains of probiotic bacterial species (Talwalkar & Kailasapathy, 2004; Ventura & Perozzi, 2011).

The survival of bacteria against different detrimental factors during processing and product development is species and strain specific (Tamime, Saarela, Sondergaard, Mistry, & Shah, 2005). In terms of robustness of probiotic organisms, Lactobacilli are generally stronger than Bifidobacteria (Mättö, Alakomi, Vaari, Virkajärvi, & Saarela, 2006; Ross, Desmond, Fitzgerald, & Stanton, 2005). Found naturally in traditional fermented foods, Lactobacilli are more resistant to low pH and have adaptation to milk and other food substrates. A large number of probiotic Lactobacillus species are therefore technologically suitable for food applications compared to Bifidobacteria (Lee & Salminen, 2009).

Numerous criteria have been recognized and suggested for selection of suitable probiotic organisms (Mattila-Sandholm et al., 2002; Ouwehand, Kirjavainen, Shortt, & Salminen, 1999; Reid, 1999). Table 3 depicts some of the technological and physiological characteristics of probiotic strains as desirable criteria for selection of probiotics in commercial applications.

Table 1 – Commonly used species of lactic acid bacteria in probiotic preparations.

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

Table 2 – List of probiotic strains used in commercial applications.

Source/product	Strain
Chr. Hansen	*L. acidophilus* LA1/LA5
	L. delbrueckii ssp. bulgaricus Lb12
	L. paracasei CRL431
	B. animalis ssp. lactis Bb12
Danisco	*L. acidophilus* NCFMs
	L. acidophilus La
	L. paracasei Lpc
	B. lactis HOWARUTM/Bl
DSM Food Specialties	*L. acidophilus* LAFTIs L10
	B. lactis LAFTIs B94
	L. paracasei LAFTIs L26
Nestle	*L. johnsonii* La1
Snow Brand Milk Products Co. Ltd.	*L. acidophilus* SBT-20621
	B. longum SBT-29281
Institute Rosell	*L. rhamnosus* R0011
	L. acidophilus R0052
Yakult	*L. casei* Shirota
	B. breve strain Yaku
Foneterra	*B. lactis* HN019 (DR10)
	L. rhamnosus HN001 (DR20)
Probi AB	*L. plantarum* 299V
	L. rhamnosus 271
Danone	*L. casei* Immunitas
	B. animalis DN173010 (Bioactiva)
Essum AB	*L. rhamnosus* LB21
	Lactococcus lactis L1A
Biogaia	*L. reuteri* SD2112
Morinaga Milk Industry Co. Ltd.	*B. longum* BB536
Lacteol Laboratory	*L. acidophilus* LB
Medipharm	*L. paracasei* F19

Source: Holm, 2003; Shah, 2004.

2.4. *Doses of probiotics*

The intended health benefits of probiotics can only be obtained when the food contains the required minimum viable microorganism count at the time of consumption. The food industry in general has adopted the minimum recommended level of 10^6 CFU ml^{-1} at the time of consumption (Boylston, Vinderola, Ghoddusi, & Reinheimer, 2004; Kailasapathy & Rybka, 1997). US FDA has also recommended that the minimum probiotic count in a probiotic food should be at least 10^6 CFU ml^{-1}. Depending on the amount ingested and taking into account the effect of storage on probiotic viability, a daily intake of 10^8–10^9 probiotic microorganisms is essential to achieve probiotic action in the human organism (Knorr, 1998). It has also been

stated that probiotic products should be consumed regularly with an approximate amount of 100 g/day in order to deliver about 10^9 viable cells into the intestine (Karimi, Mortazavian, & Cruz, 2011).

2.5. *Development of probiotic foods*

More than 500 probiotic food products have been introduced in the global market during last couple of decades (Anonymous, 2013; Sveje, 2007), and the list is continuously expanding. Probiotic food products made out of fermentation of cereals, fruits and vegetables (juices, snacks, cut fruits) and meat products (hams, loins, sausages) are receiving attention from the scientific world as well as consumers (Gupta & Abu-Ghannam, 2012; Kołozyn-Krajewskaa & Dolatowski, 2012; Rouhi, Sohrabvandi, & Mortazavian, 2013; Rößle, Auty, Brunton, Gormley, & Butler, 2010). Cheese and cheese-based dips (Ong, Henriksson, & Shah, 2006; Tharmaraj & Shah, 2004), mayonnaise (Khalil & Mansour, 1998), edible spreads (Charteris, Kelly, Morelli, & Collins, 2002) and meat-based products (Arihara et al., 1998; Kołozyn-Krajewskaa & Dolatowski, 2012) are few examples of probiotic foods developed in recent past. Probiotic organisms are also available commercially in milk, sour milk, fruit juices, ice cream, single shots and oat-based products.

During the development of probiotic foods, the probiotic cultures are artificially introduced into the food. Most of the culture preparations are available commercially in highly concentrated form, and most of them are prepared for DVS (direct vat) applications (Kailasapathy, 2013) either as highly concentrated frozen cultures or in the form of freeze-dried powders. Use of these concentrated DVS cultures by food manufacturers is common as it is difficult to propagate probiotic microorganisms at the production site. Frozen cultures contain more than 10^{10} CFU g^{-1}, whereas freeze-dried cultures typically contain more than 10^{11} CFU g^{-1} (Oberman & Libudzisz, 1998).

Table 3 – Desirable criteria for the selection of probiotics in commercial applications.

General	Property
Safety criteria	Pathogenicity and infectivity
	Origin
	Virulence factors (toxicity, metabolic activity and intrinsic properties, i.e., antibiotic resistance)
Technological criteria	Genetically stable strains
	Desired viability during processing and storage
	Good sensory properties
	Large-scale production
	Phage resistance
Functional criteria	Tolerance to gastric acid
	Bile tolerance
	Adhesion to mucosal surface
Physiological criteria	Immunomodulation
	Antagonistic activity
	Cholesterol metabolism
	Lactose metabolism
	Antimutagenic and anticarcinogenic properties

Source: Shah, 2006; Morelli, 2007.

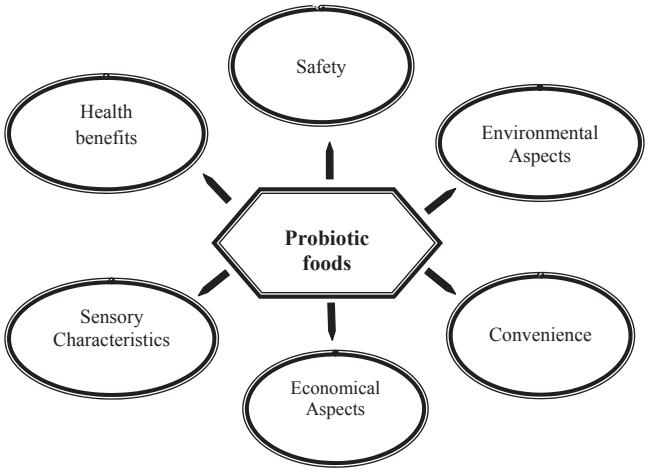

Fig. 2 – Qualitative aspects of probiotic food products.

The taste and aroma of the food product may be altered by addition of probiotics due to production of different metabolic components such as acetic acid produced by Bifidobacterium spp. during fermentation and over storage period. The presence of the probiotic culture in food product, therefore, should not adversely affect product quality or sensory properties (Mohammadi & Mortazavian, 2011; Stanton et al., 2003). The technological properties associated with the incorporation of probiotic strains into food products are presented in Fig. 2 (Ross et al., 2005). The packaging materials used and the storage conditions under which the products are stored are important for the quality of products containing probiotics.

3. Survival of probiotics during processing and storage

The medicinal efficacy of probiotic food products depends upon the number of viable and active cells per gram or milliliter of the products at the moment of consumption (Korbekandi, Mortazavian, & Iravani, 2011). It is therefore essential to ensure a high survival rate of the probiotics during production as well as over the product shelf life in order to maintain consumer confidence in probiotic products (Cruz et al., 2010; Saxelin et al., 1999).

A number of attempts have been made to improve the viability of probiotics in different food products during their production until the time of consumption. Many factors were found to influence the viability of probiotic microorganisms in food products during production, processing and storage (Fig. 3). The identified factors include food parameters (pH, titratable acidity, molecular oxygen, water activity, presence of salt, sugar and chemicals like hydrogen peroxide, bacteriocins, artificial flavoring and coloring agents); processing parameters (heat treatment, incubation temperature, cooling rate of the product,

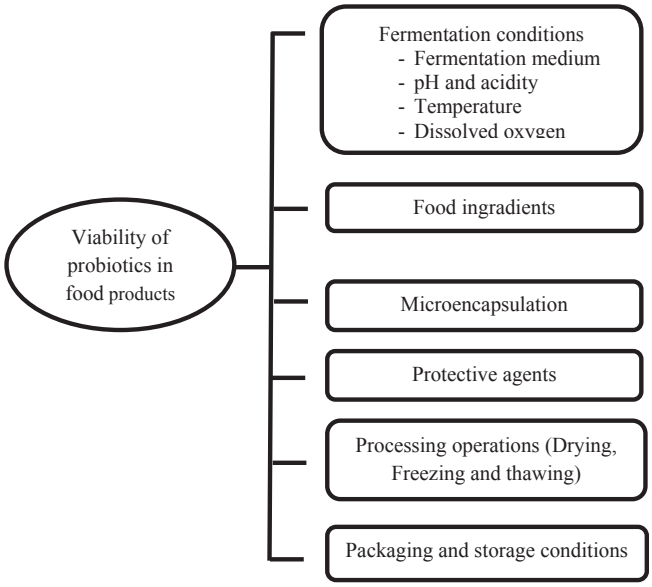

Fig. 3 – Important factors affecting viability of probiotics.

packaging materials and storage methods, and scale of production); and microbiological parameters (strains of probiotics, rate and proportion of inoculation).

3.1. *Factors affecting survival of probiotics during processing*

3.1.1. *Fermentation conditions*

Fermentation temperature is one of the important factors affecting viability of probiotic microorganisms and other qualitative parameters of probiotic fermented products. The favorable temperature for growth of most probiotics is in the range of 37–43 °C (Boylston et al., 2004; Korbekandi et al., 2011; Lee & Salminen, 2009). Though certain species like *L. acidophilus* can grow at temperatures as high as 45 °C, but the optimum growth occurs within 40–42 °C. Temperatures above 45–50 °C during processing are detrimental to probiotic survival. The exposure time has to be shorter at higher temperature in order to save the probiotics. It is advisable to add probiotics during downstream of heating/cooking/pasteurization processes in food manufacturing (Lee & Salminen, 2009).

Exposure to oxygen during fermentation plays a major role in loss of viability of oxygen sensitive bacteria (Gaudreau, Champagne, Remondetto, Bazinet, & Subirade, 2013). Several methods have been used to decrease oxygen content during fermentation. The most important one is accomplishing fermentation under vacuum (Cruz, Faria, & Van Dender, 2007).

The resistance of probiotic bacteria to heat stress can be increased by mild heat treatment prior to their use. Application of nonlethal heat shock allows bacteria to tolerate a second heat stress higher in intensity and it has been found that the heat adaptation increases the thermal tolerance of Lactobacilli (Teixeira, Castro, & Kirby, 1994). This may benefit industrial fermentation processes requiring bacteria with enhanced thermal tolerance. Research has revealed that heat adaptation of live microorganisms prior to heat stress has positive effect to improve the thermal tolerance of Lactococci and Lactobacilli by up to 300-fold compared to the untreated parent strains (Desmond, Stanton, Fitzgerald, Collins, & Ross, 2001).

3.1.2. *Freezing and thawing operations*

Probiotic microorganisms can survive a longer duration in frozen products. However, the cell membranes of probiotics get damaged during freezing process due to mechanical stresses of the ice crystals formed in the external medium or inside the cells, thereby causing fatal injury to them. The solutes condensate in the extracellular/intracellular media and the cells get dehydrated during freezing. As a result, the vital metabolic activities of the cells are reduced or interrupted (Akin, Akin, & Kirmaci, 2007). The rate of freezing affects cell survival, as larger ice crystals produced by slow freezing cause greater damage to the cells and rapid freezing helps in better maintenance of the microorganisms in the product (Fowler & Toner, 2005; Gill, 2006; Mohammadi, Mortazavian, Khosrokhavar, & Cruz, 2011).

Mortality also takes place during thawing of the frozen products due to exposure of the microbial cells to osmotic effects as well as to the high concentrations of detrimental factors such as hydrogen ions, organic acids, oxygen and other poisoning components in melting media (Jay, Loessner, & Golden, 2005).

3.1.3. *Drying*

Probiotic foods are sometimes dried in order to increase their shelf life at ambient temperature and to reduce the cost of frozen storage. Drying also stabilizes probiotics for their ease of storage, handling, transport and subsequent use in functional food applications. Drying probiotic foods is a challenge as it causes severe loss in viability of probiotics (Santivarangkna, Kulozik, & Foerst, 2006).

Hot air drying, freeze drying, spray drying and vacuum drying are the common methods used for drying of food products. Spray drying is the most common and economical method for drying of liquid foods. However, spray drying process leads to a loss of viability as the probiotic cells are subjected to very high temperature, mechanical shearing, dehydration and osmotic pressure. Freeze drying, on the other hand, maintains the viability of the probiotic cells but is a costlier process. More recently, fluidized bed drying and radiant energy vacuum drying technique has successfully been tried for improving ambient air stability of probiotics (Nag & Das, 2013; Noorbakhsh et al., 2013). Fluidized bed drying was able to retain 2.5 log CFU g^{-1} higher viability of *Lactobacillus casei* CRL 431 over freeze dried samples after 52 weeks of storage at 25 °C. When fluidized bed drying of osmotically stressed probiotic cells was carried out, further improvement of 0.83 log CFU g^{-1} was observed compared to the unstressed cells (Nag & Das, 2013).

Freeze drying: Probiotic powders are being manufactured by freeze-drying technique for decades. In freeze drying, the cells are first frozen at temperatures as low as –190 °C and then dried by sublimation under high vacuum. The drying is carried out in three phases: freezing, primary drying, and secondary drying. High probiotic survival rates are usually achieved in freeze-dried powders as the processing conditions associated with freeze drying are milder compared to other methods (Wang, Yu, & Chou, 2004). Freeze drying also preserves the morphology of probiotic microcapsules (Chen & Mustapha, 2012).

Cellular inactivation during freeze drying occurs mostly at the freezing step (Tsvetkov & Brankova, 1983). It has been reported that the higher the surface area of the cell, the higher the membrane damage owing to extracellular ice crystal formation during freezing. Hence, small spherical cells such as enterococci are more resistant to freezing and survive better than larger rod shaped Lactobacilli during freeze drying (Fonseca, Beal, & Corrieu, 2000).

Removal of bound water from bacterial cells during drying leads to damage of surface proteins, cell wall and the cell membrane. Consequently, water removal during desiccation can lead to destabilization of the structural integrity of these cellular components, resulting in loss or impairment of function (Brennan, Wanismail, Johnson, & Ray, 1986). It has been proposed that the lipid fraction of the cell membrane is the primary target area for damage during drying, where lipid peroxidation may occur (Brennan et al., 1986). Therefore, approaches must be focused toward minimization of damage to these cellular components during desiccation of probiotics. The freeze-drying parameters as well as physico-chemical parameters of the food formulation are critical for bacterial survival. Hence, there is a need to optimize the drying process parameters, independent of the strain (Bergenholtz, Wessman, Wuttke, & Håkansson, 2012).

Spray drying: Spray drying is an alternative inexpensive method to freeze drying, yielding higher production rates (Zamora, Carretero, & Pares, 2006). The spray-drying process involves the injection of liquid food in atomized form into a hot drying medium at temperatures up to 200 °C. The products with probiotics are therefore exposed to very high temperatures for a short time, which can be harmful to the live bacterial cells. In addition to the heat stress, bacterial cells also encounter other stresses as mentioned in freeze drying (Brennan et al., 1986). The cytoplasmic membranes as well as cell wall, DNA and RNA are mostly affected by stresses during spray drying, thereby resulting in loss of or reduced metabolic activities (Crowe et al., 1988; Teixeira, Castro, Mohacsi-Farkas, & Kirby, 1997). Spray drying leads to increased cell permeability by affecting the cell membrane, causing loss of intracellular components from the cell into the surrounding environment.

A number of studies have been conducted on spray drying of a variety of probiotics and reported on their performance. Gardiner et al. (2002) have achieved a survival rate of more than 80% during spray drying of a resistant variant of *Lactobacillus paracasei* NFBC 338 in reconstituted skim milk (RSM) at outlet temperatures of 85–90 °C. Ananta and Knorr (2003) reported a survival rate of greater than 60% for *L. rhamnosus* GG under similar conditions of outlet temperature. Kim and Bhowmik (1990) and Gardiner et al. (2000) highlighted the outlet/inlet air temperature as an important parameter affecting bacterial survival. They reported that the numbers of different bacterial species (*Streptococcus salivarius* subsp. *thermophilus*, *L. debrueckii* subsp. *bulgaricus*, *L. paracasei* NFBC 338 and *L. salivarius* UCC 118) decreased with increasing outlet or inlet air temperatures and atomizing air pressure. Thus viability can be improved by reducing the outlet and inlet air temperatures during spray drying, but the final moisture content of the powder and its quality are also influenced by the drying air temperature. Zayed and Roos (2004) stated that a moisture content of 3.5% is preferred for shelf-stableproducts.

Mild heat treatment (52 °C for 15 min) before spray drying can enhance the cell survival during drying and subsequent storage (Paéz et al., 2012). Pispan, Hewitt, and Stapley (2013) found lower survival rates of *E. coli* and *L. acidophilus* as the outlet air temperature was increased above 80 °C. However, the cells which survived spray drying at higher temperature were found to be more likely to survive during storage.

The tolerance to different stresses during spray drying varies from species to species, and hence it is important to select the appropriate strain for development of dried probiotic products. Gardiner et al. (2000) found *L. paracasei* NFBC 338 survived considerably better than *L. salivarius* UCC 118 under similar conditions of spray drying, which may be attributed to the greater thermal tolerance of the former strain. Pispan et al. (2013) attributed the thicker cell wall of the Gram positive cells like Lactobacillus, which resulted in better survival of the species during spray drying compared to *E. coli*. Secondly, cells in the early-stationery phase survived better during spray drying and subsequent storage than cells in the mid-log phase. In case of Bifidobacterium species, it was found that closely related species exhibited superior heat and oxygen tolerance and performed better during spray drying. *Bifidobacterium animalis* subsp. lactis showed more than 70% survival after spray drying in RSM (20%, w/v) at an outlet temperature of 85–90 °C (Simpson, Stanton, Fitzgerald, & Ross, 2005).

Based on the studies mentioned earlier, it may be stated that the survival rate of probiotic cultures during spray drying depends on factors such as the species and strain of probiotics used, the drying parameters (outlet air temperature, type of atomization), and the drying and growth medium.

3.1.3.1. *Growth media and probiotic culture survival during drying.* The survival rate of probiotic cultures during drying also depends upon the composition of the growth media, and the presence of carbohydrates plays an importance role in this respect. Panoff, Thammavongs, and Gueguen (2000) showed that cells of *L. delbrueckii* sub sp. *bulgaricus* can be adapted to freezing and thawing by an osmotic stress, when they are grown in the presence of sugars such as lactose, sucrose and trehalose. Similarly, the survival of spray dried *L. sakei* was enhanced when the cells were grown in the presence of sucrose (Ferreira et al., 2005).

The protective effect also depends upon the type of sugar. Carvalho et al. (2004a, 2004b) found *L. bulgaricus* to survive better after freeze drying when grown in the presence of mannose, compared to other sugars such as fructose, lactose or glucose. They also stated that glucose, which is used as the standard growth medium, is the least effective carbohydrate when compared to fructose and sorbitol. The difference in the effectiveness of lactose, sucrose and trehalose in the recovery of *L. delbrueckii* subsp. bulgaricus after drying has been reported by Tymczyszyn, Gomez-Zavaglia, and Disalvo (2007). Sucrose was found to be as effective as trehalose in preserving the dehydrated bacteria, after growing them in a low water activity medium. Anekella and Orsat (2013) demonstrated MRS as a better heating medium than raspberry juice during the sub-lethal heat shock pre-treatment. Microorganisms were able to withstand up to 50 °C (for *L. acidophilus*) and 52.5 °C (for

L. rhamnosus) in MRS as heating medium, whereas both the microorganisms were killed at 45 °C in raspberry juice as the heating medium.

The effect of medium concentration on the survival of probiotic cultures during and after dehydration has been demonstrated by Linders, Wolkers, Hoekstra, and van't Riet (1997). The growth and activity of *L. plantarum* after drying were found to be higher for cells grown in diluted MRS than for cells grown in enriched MRS. The presence of sodium chloride during growth of *L. plantarum* also resulted in a decreased residual activity after drying.

Carvalho et al. (2004b) stated that growth of bacteria in the presence of various sugar substrates produces cells with distinct morphological and physiological traits that may reflect the distinct resistances to various stresses. It has been reported that metabolites such as mannitol, sorbitol and glutamate found inside the bacterial cells are responsible for their distinct survival behavior during dehydration, and the carbon sources in the growth medium affects the formation of these metabolites (Wisselink, Weusthuis, Eggink, Hugenholtz, & Grobben, 2002).

3.1.3.2. *Addition of cell protectants*. Protectants are substances which when added to the drying medium before drying help in protecting the viability of probiotic cells. Some of these substances include skim milk powder, whey protein, glycerol, betaine, adonitol, lactose and polymers such as dextran and polyethylene glycol (Hubalek, 2003). Compatible cryoprotectants such as glycerol are added to the medium prior to freeze drying to assist in the adaptation of probiotics to the environment by reducing the osmotic difference between the internal and external environments (Capela, Hay, & Shah, 2006).

Desmond, Ross, O'Callaghan, Fitzgerald, and Stanton (2002) used gum acacia in the spray-drying medium that resulted in enhanced probiotic survival. *L. paracasei* NFBC 338 displayed 10-fold greater survival than the control cells when grown in a mixture of RSM (10%, w/v) and gum acacia (10%, w/v) prior to spray drying at the outlet temperature of 100–105 °C. Skim milk protein in RSM can prevent cellular injury by stabilizing cell membrane, and hence found to be a suitable medium for efficacious spray drying of probiotic cultures (Ananta, Volkert, & Knorr, 2005). RSM has also the ability to form a protective coating on the cell wall proteins, and milk calcium increases survival after dehydration (King & Su, 1993). On the other hand, the inclusion of polydextrose and inulin in the spray-drying RSM medium did not enhance viability during spray drying or powder storage (Corcoran, Stanton, Fitzgerald, & Ross, 2005). The protective impact of excipients on the cell damage during spray drying and storage was evaluated by Salar-Behzadi et al. (2013). Gum arabic, gelatin and pectin showed the best protective impact. Cells pre-treated with these excipients showed reduced membrane damage, enhanced stability and improved culturability during 1 month of storage time.

It is stated earlier that carbohydrates have protective effects for probiotic bacteria during freeze drying. They help in raising the glass-phase transition temperature, and thereby helping the viable cells to reach the glassy phase without nucleating intracellular ice (Fowler & Toner, 2005). The stability of probiotics in glassy protein–carbohydrate matrices depends on the composition of the matrix (Hoobin et al., 2013). The authors found a positive correlation between the inactivation rate constant for probiotics in freeze-dried matrices with moisture uptake and molecular mobility. The partial substitution of maltodextrin with glucose (D- or L-) improved microbial survival at 33% RH because of reduced molecular mobility and lower water uptake of the matrix (Hoobin et al., 2013). It has also been demonstrated that trehalose is an effective cryoprotectant during freezing and freeze drying due to its remarkably high glass transition temperature, and the strong ion–dipole interactions and hydrogen bonding between trehalose and the biomolecules, enabling better survival of *L. acidophilus* (Conrad, Miller, Cielenski, & de Pablo, 2000). Compatible solutes have also proven beneficial in probiotic viability protection in acidic environments. Corcoran et al. (2005) found that the presence of 19.4 mM glucose resulted in up to 6-log enhanced survival following 90 min of exposure to simulated gastric juice at pH 2.0 as compared to the control. Santivarangkna et al. (2006) reported that survival of *L. helveticus* during vacuum drying was improved by the addition of 1% sorbitol.

3.1.4. *Rehydration of dried probiotic products*

The dehydrated probiotic products are rehydrated for the revival of cells before consumption. According to Freudig, Hogekamp, and Schubert (1999), the reconstitution process takes place in four steps of wetting, submersion, dispersion and dissolving. Among these steps, wetting of the particles is often the controlling step (Vega & Roos, 2006). The rate of recovery of the probiotics to the viable state is significantly influenced by the rehydration conditions (temperature, volume of rehydrating media and rehydration time), physical properties of the material to be rehydrated, as well as properties like osmolarity, pH and nutritional energy of the rehydration solution (Carvalho et al., 2004b). Teixeira, Castro, and Kirby (1995a) recommended to dry the cells at their stationary phase of growth and to use slow rehydration procedures for optimum results. Increased cell recovery of *Saccharomyces cerevisiae* was achieved when the dried cells were rehydrated slowly (7–16 days) under controlled conditions rather than immediate rehydration (Poirier, Marechal, Richard, & Gervais, 1999).

The rehydration temperature is a critical factor influencing cell recovery of freeze dried and spray-dried probiotics. Various studies have indicated that there is not an 'ideal' single point rehydration temperature for optimum growth of cultures. For thermophilic cultures, temperature between 30 and 37 °C was found best for post hydration viabilities, while the optimum range is 22–30 °C for mesophilic bacteria (Mille, Obert, Beney, & Gervais, 2004; Sinha, Shukla, Lal, & Ranganathan, 1982). The rehydration temperature should not cross 40 °C in any case. Ray, Jezeski, and Busta (1971) found the highest number of recovered *Salmonella anatum* cells when rehydration was carried out at 15–25 °C, and cell recovery was lower at 35 and 45 °C.

The ratio of dried powder to liquid medium as well as the composition of the rehydration medium can significantly influence post hydration culture recovery. Viable count of different probiotic cultures was found to be higher 4–10 times when the powder was added to a small amount of water in the ratio of 1:3 as compared to 1:50 ratio of powder to liquid (De Valdez, De Giori, De Ruiz Holgado, & Oliver, 1985). Costa, Usall, Teixido, Garcia, and Vinas (2000) found a complex medium containing RSM, peptone/tryptone, and meat extract to produce

significantly higher bacterial cell recovery than a medium such as phosphate buffer, sodium glutamate and water. The same cryopreservation solution when used as rehydration medium resulted in increased viability (Abadias, Teixido, Usall, Benabarre, & Vinas, 2001; Ray et al., 1971). The reason may be the high osmotic pressure environment provided by these solutions could control the rate of hydration, and thus avoid osmotic shock. The behavior during rehydration also depends upon different species and strains of the probiotic bacteria. Hence it is necessary to standardize the rehydration procedure for each and every product.

3.1.5. *Microencapsulation*

Microencapsulation is the process of enclosing the cells by coating them with a proper substance in a way that results in appropriate cell release in the intestinal medium (Mortazavian et al., 2008). Microencapsulation helps in segregating the cells from surrounding environment. Materials used to encapsulate probiotic cells include different polysaccharides such as alginate, plant/microbial gums, chitosan, starch, K-carrageenan, cellulose acetate phthalate, gelatin, milk proteins, and fats (Burgain, Gaiani, Linder, & Scher, 2011; Ying et al., 2010). Recently, water-insoluble hydrogels based on proteins are successfully applied as a promising alternative to polysaccharide hydrogels for microencapsulation of probiotic cells (Annan, Borza, & Hansen, 2008; Heidebach, Först, & Kulozik, 2009). Many reviews have shown the potential of microencapsulation to improve probiotic survival during processing and storage in food products or in gastrointestinal transit (Anal & Singh, 2007; Burgain et al., 2006; Champagne & Fustier, 2007; Heidebach, Leeb, Först, & Kulozik, 2010b; Mohammadi et al., 2011; Wenrong & Griffiths, 2000).

Probiotic cells have successfully been microencapsulated to preserve them from detrimental factors during processing and storage such as low pH and high acidity (Wenrong & Griffiths, 2000), bile salts (Lee & Heo, 2000), heat shocks caused by spray drying and cold shocks induced by freezing (Shah & Ravula, 2004), molecular oxygen in case of anaerobic microorganisms (Sunohara, Ohno, Shibata, & Seki, 1995), bacteriophages (Steenson, Klaenhammer, & Swaisgood, 1987), and chemical antimicrobial agents (Sultana et al., 2000). In addition, microencapsulation may help in improvement and stabilization of sensory properties (Gomes & Malcata, 1999) and immobilization of the cells for their homogeneous distribution throughout the product (Krasaekoopt, Bhandari, & Deeth, 2003).

Ding and Shah (2007) tested eight strains of microencapsulated probiotic bacteria for their acid, bile, and heat tolerance. Microencapsulation (in alginate matrix) resulted in better survival of probiotic bacteria as compared to free cells (control) in MRS containing hydrochloric acid. Viability was reduced by 6.51 log CFU ml^{-1} when free probiotic bacteria were exposed to oxgall, whereas only 3.36 log CFU ml^{-1} was lost in microencapsulated strains. At 30 min of heat treatment at 65 °C, microencapsulated probiotic bacteria survived with an average loss of only 4.17 log CFU ml^{-1} as compared to 6.74 log CFU ml^{-1} loss with free probiotic bacteria. However, viability did not improve after 1 h of heating. It could be concluded that microencapsulation improved the survival of probiotic bacteria when exposed to acidic conditions, bile salts, and mild heat treatment (Desmond, Fitzgerald, Stanton, & Ross, 2004). Koo, Cho, Huh, Baek, and Park (2001) observed that both non-encapsulated and encapsulated cells stored at 4 °C had comparable stability, while encapsulation provided a greater degree of protection against increased storage temperature. Microencapsulation of free probiotic cells can increase their viability by more than 2 log cycles in fermented milks during refrigerated storage (Mortazavian et al., 2010). In fermented milk drinks with pH values of less than 4.2, free cells of *L. acidophilus* LA-5 lost their viability to less than 10^6 CFU ml^{-1} after 1 week; and in the case of *Bifidobacterium lactis* BB-12, a similar loss occurred after 2 weeks of storage. For encapsulated cells, viable population of *L. acidophilus* and Bifidobacteria remained higher than 10^6 CFU ml^{-1} after 42 days of refrigerated storage, whereas counts of free probiotic free cells were limited to 10^2 CFU ml^{-1} (Mortazavian et al., 2008).

The influence of casein-based microencapsulation on the viability of probiotic strains during freeze drying and subsequent storage was investigated by Heidebach, Först, and Kulozik (2010a). They took two different strains, Lactobacillus F19 and Bifidobacterium Bb12, which differ in their sensitivity against dehydration. No difference in water activities was found after drying between free and encapsulated samples, but both the strains survived in significantly higher numbers in the encapsulated state, compared to free cells (protein–cell mixture). After 90 days of storage at 4 °C and 11% RH, reduction in viability was only 1 and 2 log cycles for encapsulated Bifidobacterium Bb12 and Lactobacillus F19, respectively. The similar protective effect of encapsulation was not observed when a resistant corn starch was used as the encapsulating medium.

Stummer et al. (2010) used modified shellac as a microencapsulating agent to develop enteric coating formulations for probiotic microorganisms, including Bifidobacterium, Lactobacilli and Enterococci. Shellac plasticized with 5% glycerol or 5% sodium alginate showed the best result as encapsulating material that protected the microorganisms against acidic pH and provided the best release profile in simulated intestinal fluid. *B. bifidum* and *E. faecium* showed more resistant to manufacturing process than *L. reuteri*, indicating the effect of coating is strain specific. Ying et al. (2010) encapsulated commercial Lactobacillus rhamnosus GG (LGG) with an emulsion-based formulation stabilized by whey protein and resistant starch, and reported no difference in loss of probiotics viability after spray drying or freeze drying. Chen and Mustapha (2012) used a combination of κ-carrageenan and inulin at a proportion of 1.9:0.1 (w/w) as capsule wall materials that significantly retained the viability of *Lactobacillus acidophilus* LA-2 through freeze drying. Upon incorporation into soy protein bars, the freeze-dried microcapsules of *L. acidophilus* LA-2 remained in high numbers throughout 14 weeks of storage at 4 °C. However, Weinbreck, Bodnár, and Marco (2010) stated that encapsulation of *L. rhamnous* GG with whey protein and palm oil did not improve its survival in a high level of water activity (0.7) environment.

Prebiotics such as inulin, oligofructose, and oligofructose-enriched inulin (at a ratio of 1:1, 200 g L^{-1} total concentrations) have been tried as alternative encapsulating agent to RSM with partial replacement of RSM (Fritzen-Freire et al., 2012). It was observed that microcapsules produced with oligofructose were more hygroscopic, whereas inulin-based microcapsules took more time to dissolute in water. The partial replacement of RSM with prebiotics also decreased the water activ-

ity of the microcapsules. Results of their study indicated oligofructose-enriched inulin as the most appropriate prebiotic for partial replacement of RSM for encapsulation of Bifidobacterium BB-12. The use of inulin could prove beneficial in the encapsulation of probiotic strains since this carbohydrate is not hydrolyzed by human digestive enzymes and may act as prebiotic (Avila-Reyesa, Garcia-Suareza, Jiménez, Martín-Gonzalez, & Bello-Perez, 2014). However, inulin could not increase storage stability of *L. casei* CRL 431, when added as a fortifying agent (Nag & Das, 2013).

3.2. *Factors affecting survival of probiotics during storage*

The composition of the food, types of packaging material and storage environment (storage temperature, moisture content of powders, relative humidity, oxygen content, and exposure to light, among others) have significant influences on the survival of probiotics (Mattila-Sandholm et al., 2002).

3.2.1. *Food ingredients and additives*

Ingredients in food can be protective, neutral, or detrimental to probiotic stability (Mattila-Sandholm et al., 2002), hence the compatibility of probiotics with different food ingredients plays a major role in their survival. The additives generally used in the food industry include different types of sugars, sweeteners, salts, aroma compounds (diacetyl, acetaldehyde and acetoin), natural or artificial flavoring and coloring agents, nisin (a polypeptide-type antibiotic), natamycin, lysozyme and nitrite. These additives could drastically affect the growth and viability of probiotic bacteria used for fermented and non-fermented products (Vinderola, Costa, Regenhardt, & Reinheimer, 2002). Higher levels of certain ingredients can inhibit the growth of probiotics during storage (Boylston et al., 2004; Lee & Salminen, 2009). Curing agents such as sodium nitrite, usually added to the meat batter for preservation, poses a challenge to probiotic bacteria in meat fermentation (Kołozyn-Krajewskaa & Dolatowski, 2012).

Different growth promoters such as glucose, vitamins, minerals, casein, whey protein hydrolysates, yeast extract, and antioxidant are fortified in dairy products to increase the growth rate of probiotic species (Lactobacilli and Bifidobacteria), as these species are reported to grow poorly in milk (Korbekandi et al., 2011). These supplements have significant positive effects on the survival of probiotic microorganisms during storage (Mohammadi et al., 2011). Certain protein derivatives (whey protein concentrate, acid casein hydrolysate and tryptone) were also found to promote growth of the probiotic by providing nutrition for the cells, by reducing redox potential of the medium as well as increasing buffering capacity of the medium that results in a smaller decrease in pH (Dave & Shah, 1998; Mortazavian et al., 2010). The viability of *L. acidophilus* and Bifidobacteria was improved by the addition of L-cysteine, whey protein concentrate, acid casein hydrolysate and tryptone. These additives provided the required growth factors to the probiotic bacteria (Dave & Shah, 1998). Casein and whey protein hydrolysate reduced the growth rate of probiotic *L. acidophilus* La-5 and *L. rhamnosus* Lr-35 in fermented milks during the manufacturing stages, but the survival of these bacteria was improved after storage (Lucas, Sodini, Monnet, Jolivet, & Corrieu, 2004).

Studies have also shown that the presence of disaccharides can stabilize the cell membrane during storage (Carvalho et al., 2002; Önneby et al., 2013). For example, sorbitol prevents membrane damage by interaction with it, and stabilizes protein functionality and structure (Yoo & Lee, 1993). Linders et al. (1997) also found sorbitol as the most effective protectant for *L. plantarum* and *L. rhamnosus* during storage, and trehalose was not an effective protectant. Certain non-digestible or minimally digestible food ingredients (known as prebiotics) are metabolized selectively by beneficial intestinal bacteria that enhance their growth and/or activity. Some of these compounds like fructo-oligosaccharides and galacto-oligosaccharides have a positive effect on the retention of probiotics viability (especially Bifidobacteria) in food products during storage (Nobakhti, Ehsani, Mousavi, & Mortazavian, 2009; Rycroft, Jones, Gibson, & Rastall, 2001).

The matrices in solid food products, such as the gel structure in cheese, support probiotic cells by reducing their exposure to detrimental factors (Karimi et al., 2011). The high fat content, anaerobic environment and buffering capacity of the matrix in cheese help to protect the probiotic cells, both in the product and during intestinal transit (Lee & Salminen, 2009). Increasing the buffering capacity of milk leads to higher viability of probiotics in dairy fermented products during storage due to the maintenance of higher pH values. Moreover, the dry matter of the product matrix absorbs hydrogen ions, leading to an increase in the amounts of undissociated organic acids. This results in the reduction of bactericidal effect of these compounds on probiotics (Heydari, Mortazavian, Ehsani, Mohammadifar, & Ezzatpanah, 2011; Korbekandi et al., 2011). It has been found that for delivery of viable probiotic *Lactobacilli* and *Enterococci* to the gastrointestinal tract, Cheddar cheese showed a more protective effect as a food carrier as compared to the yogurt (Gardiner, Ross, Collins, Fitzgerald, & Stanton, 1998; Stanton et al., 1998).

3.2.2. *Oxygen content and redox potential*

Oxygen content and redox potential are among the important factors affecting the viability of probiotics especially during the storage period (Lee & Salminen, 2009). Molecular oxygen is harmful to probiotic survival and growth, as most of the species are strictly anaerobic and saccharoclastic (De Vuyst, 2000; Holzapfel, Haberer, Geisen, Bjo rkroth, & Schillinger, 2001). Oxygen affects probiotics in three ways i.e. (i) it is directly toxic to some cells, (ii) certain cultures produce toxic peroxides in the presence of oxygen, and (iii) free radicals produced from the oxidation of components (e.g., fats) are toxic to probiotic cells (Korbekandi et al., 2011). The level of oxygen within the package during storage of probiotic products should be as low as possible in order to avoid toxicity and death of the microorganism and the consequent loss of functionality of the product.

The degree of oxygen sensitivity varies considerably among different species and strains of probiotics (Kawasaki, Mimura, Satoh, Takeda, & Niimura, 2006; Talwalkar & Kailasapathy, 2003, 2004). Bifidobacteria are more vulnerable to oxygen damage than *L. acidophilus* due to their anaerobic nature. *B. lactis* is a moderately oxygen tolerant species of among *Bifidobacterium* that

was isolated from fermented milk by Meile et al. (1997), confirming the strain dependent phenomenon of oxygen sensitivity. In general, Lactobacilli are more tolerant to oxygen than Bifidobacteria, to the point where oxygen levels are rarely an important consideration in maintaining the survival of Lactobacilli. High levels of enzymes NAD-oxidase and NADH-peroxidase have been reported in aero-tolerant species, and these enzymes are responsible for removing oxygen from the intercellular medium (Roy, 2005). The modified relative bacterial growth ratio (RBGR) methodology developed by Talwalkar, Kailasapathy, Peiris, and Arumugaswamy (2001) can successfully be utilized to enumerate the oxygen tolerance of several probiotic bacteria, and can assist in differentiating the oxygen sensitive strains from oxygen tolerant strains.

In addition to the oxygen content in the product, it also permeates through the package and comes in contact with the product. This considerably reduced the viability of *L. acidophilus* and Bifidobacteria in fermented milk products (Klaver, Kingma, & Weerkamp, 1993). Dave and Shah (1997b) found that Bifidobacteria survived well over a 35 days period in yogurt, regardless of the oxygen content and redox potential of the yogurt. Even the dissolved oxygen of the yogurt was seen to rise steadily, counts of Bifidobacteria remained above the recommended level of 10^6 CFU g^{-1} throughout the shelf life of the yogurt, while *L. acidophilus* counts were found to decrease below 10^3 CFU g^{-1} by the third week of storage. The impairment of viability during storage is related to oxidation of membrane lipids (Teixeira, Castro, & Kirby, 1996). Products of lipid peroxidation have been shown to induce DNA damage in a model system (Akasaka, 1986) and in bacteria (Marnett et al., 1985). Therefore, to minimize oxidation and to maximize probiotic viability during storage, the presence of antioxidants in combination with storage under vacuum with controlled water activity should be effective (Teixeira, Castro, Malcata, & Kirby, 1995b).

Different methods have been attempted to reduce the oxygen content during packaging and storage of probiotic foods. These include vacuum packaging, using packaging materials with low oxygen permeability, adding antioxidants and oxygen scavengers to the product, and controlling the production process in such a way that minimum dissolved oxygen entered into product (Dave & Shah, 1997b; Korbekandi et al., 2011; Talwalkar, Miller, Kailasapathy, & Nguyen, 2004). Antioxidant compounds, such as catechins, could be used to limit negative effects of oxygen exposure on bacteria during their growth and storage in food products (Gaudreau et al., 2013). The authors measured the effects of different concentrations of (+)-catechin, green tea epigallocatechin gallate and green tea extracts (GTE) on the growth of probiotic strains with different oxygen sensitivities. Results obtained showed that medium enrichment with catechins did not stimulate the growth of the two Bifidobacteria. However, the growth of *L. helveticus* was greatly enhanced, under aerobic conditions, by supplementation of the medium with GTE. Similar results were obtained by fortification of vitamin-E in the stabilization matrix as an antioxidant that improved the stability of *L. casei* CRL 431 during 20 week storage period at 25 °C (Nag & Das, 2013).

3.2.3. *Moisture content/water activity*

The moisture content of probiotic products is another factor influencing shelf-life stability of live bacteria. Storage in the presence of both oxygen and moisture was detrimental for bacterial survival (Önneby et al., 2013). The amount of water remaining after drying affects not only the viability of bacteria as determined immediately after the process, but also the rate of loss of viability during subsequent storage. The optimum moisture content for storage of freeze-dried *L. salivarius* subsp. *salivarius* was reported to range between 2.8% and 5.6% (Zayed & Roos, 2004). Increasing the relative humidity of the environment at which the samples were stored caused an increase in water mobility and the rate of loss in viability (Ying et al., 2010). Weinbreck et al. (2010) reported that a water activity of 0.7 resulted in 10 log cycle reduction in viable conuts of *L. rhamnosus* GG within 2 weeks of storage. Hoobin et al. (2013) suggested that moisture uptake properties and molecular mobility of the matrix composition, as opposed to the relative humidity of the environment, are better determinants of probiotic viability during storage.

3.2.4. *Storage temperature*

Viability of probiotic bacteria during storage is inversely related to storage temperature (Gardiner et al., 2000). Probiotic food products should preferably be stored at a temperature of 4–5 °C (Mortazavian, Ehsani, Mousavi, Sohrabvandi, & Reinheimer, 2007a). Highest viability of *L. acidophilus* LA-5 in yogurt was observed for up to 20 days when stored at 2 °C, whereas for *Bifidobacterium lactis* BB-12, the optimum storage temperature was 8 °C (Mortazavian et al., 2007a; Mortazavian, Razavi, Ehsani, & Sohrabvandi, 2007b). This is attributed to the low resistance of Bifidobacteria cells to low refrigeration temperatures (Korbekandi et al., 2011). However, for long-term storage of freeze-dried probiotics, Bruno and Shah (2003) recommended a much lower temperature of –18 °C that maximized viability of Bifidobacteria. Storage temperature of 20 °C resulted in significant reductions in viable counts of this species in the dried products. Similar results were obtained by Simpson et al. (2005) during storage of spray-dried Bifidobacteria species at 15 and 25 °C.

The decrease in viability of probiotics in sugar-containing products during storage at high temperatures and/or relative humidity is related to their glass transition temperature (Passot, Cenard, Douania, Trelea, & Fonseca, 2012; Vega & Roos, 2006). A possible reason is that sugars form high viscous glasses at room temperature when they are dehydrated, and the presence of a glassy state improves storage life of anhydrobiotes.

3.2.5. *pH and titratable acidity*

Survival of probiotics during storage is considerably affected by pH and titratable acidity of the products (Mortazavian et al., 2010). A very low pH value increases the concentration of undissociated organic acids in fermented products, thereby enhancing the bactericidal effect of these acids. Beverages such as fruit juices with low pH values possess a significant challenge to probiotics.

Hood and Zottola (1988) were unable to recover cells of a *Lactobacillus acidophilus* culture after exposure to a pH of 2.0 for 45 min, while no significant reduction in the number of cells was observed even after 2 h exposure at a pH of 4.0. Goldin et al. (1992) found similar trends for survival of *Lactobacillus rhamnosus* GG in human gastric juice at pH values between 1.0 and 7.0. The optimum range of pH for growth of *Lactobacillus*

acidophilus and Bifidobacteria is in the range of 5.5–6.0 and 6.0–7.0, respectively (De Vuyst, 2000). Lactobacilli are capable of growing and surviving in fermented products with pH values between 3.7 and 4.3 (Boylston et al., 2004). Bifidobacteria species are reported to be less acid tolerant, and a pH level below 4.6 is detrimental to their survival (Dunne et al., 2001; Lee & Salminen, 2009).

The acid tolerance of Bifidobacterium spp. depends upon the strain of the species and characteristics of the substrate. For example, *B. longum* survived best in the presence of acids and bile salts, and *B. lactis* in fermented milks (Korbekandi et al., 2011). Sheehan, Ross, and Fitzgerald (2007) observed extensive differences in acid resistance of Lactobacillus and Bifidobacterium when added to orange, pineapple and cranberry juices. All of the strains survived better in orange and pineapple juices compared to cranberry juice. Among the different strains, *L. casei*, *L. rhamnosus* and *L. paracasei* survived for at least12 weeks in orange and pineapple juices at levels above 6.0 log CFU ml^{-1} (Rivera-Espinoza & Gallardo-Navarro, 2010). Reduction in pH of fermented meat products also poses a challenge for the survival of probiotics. A reduction in pH from 5.6 to 4.9 after fermentation affected the survival of probiotics (*L. rhamnosus* GG and E-97800) in the fermented sausage (Erkkila, Suihko, Eerola, Petaja, & Mattila-Sandholm, 2001). Several studies have indicated that cell viability in a fermented meat environment is strain dependent (Kołozyn-Krajewskaa & Dolatowski, 2012).

The earlier results indicate that strain selection is very much essential in the development of probiotic foods, and those strains which can remain viable for an acceptable shelf life should only be used to ensure actual benefits to the consumer. Tolerance to bile and acid stresses is a useful indictor of technological performance of the strain in probiotic foods (Park, So, & Heo, 1995).

3.2.6. *Packaging aspects*

Different aspects of packaging, such as the type and thickness of packaging materials, gas (O_2, CO_2 and water vapor) and light permeability through the material, and packaging technique (vacuum, modified, active/intelligent packaging systems) could influence survival of probiotics (Korbekandi et al., 2011). The temperature and relative humidity of the atmosphere may affect gas permeability of the packaging material, and thereby affecting the viability (Cruz et al., 2007). Most of the dairy probiotic and other products are stored and sold in the market in plastic packages with high oxygen permeability. This poses a serious problem to the growth and survival of the probiotic. Use of plastic films with high oxygen barrier properties and active packages with oxygen absorbers have been evaluated in many studies (Cruz et al., 2007).

The viability of *L. acidophilus* in yogurts packed in glass and high-density polyethylene (HDPE) containers was studied by Dave and Shah (1997a). The level of dissolved oxygen increased significantly in the HDPE packages, whereas the glass containers maintained the viability as the oxygen levels remained low in them during 35 days of storage. The superiority of glass bottles in maintaining viability of probiotics was also reported by Jayamanne and Adams (2004). The authors used clay pots, plastic cups and glass bottles to ferment and store buffalo milk, and found that Bifidobacteria survived best in the glass bottles, followed by the plastic packages and the clay pots, when stored at 29 °C. The difference in viability was attributed to the permeability of the packages which allowed diffusion of oxygen into the containers.

The permeability of polymeric materials is reduced with increase in crystallinity of the material. However, contrary to the expectations, the bacterial counts did not vary proportionally with the degree of crystallinity of the packaging material (Janson et al., 2002). Miller, Nguyen, Rooney, and Kailasapthy (2002, 2003) used different laminated polymeric materials with high oxygen and gas barrier properties along with oxygen scavenging film for storage of probiotic yogurt. Significant differences in the value of dissolved oxygen were found during storage period between the materials investigated. The oxygen level in polystyrene containers increased from 20 to 40 ppm, whereas the oxygen levels in the laminated film decreased to the level of 10 ppm after 42 days of refrigerated storage. The best conditions for creating a favorable anaerobic environment (less than 1 ppm oxygen) for the growth of viable probiotic cultures was obtained when yogurt was packaged in a container made of an oxygen barrier material integrated with an oxygen-scavenging agent (Miller et al., 2003; Talwalkar et al., 2004). The results clearly illustrate the importance and potential of using oxygen absorbers for packaging probiotic foods.

Hisiao, Lian, and Chou (2004) and Wang et al. (2004) studied the effect of packaging material with oxygen absorbent as well as storage temperature on the viability of microencapsulated Bifidobacteria. The authors evaluated samples filled in polyester bottles with and without oxygen absorbent, glass bottles, and in laminated bags during storage at 4 and 25 °C. The viable cell counts improved with the inclusion of an oxygen absorber when stored at 25 °C. However, the best results were obtained with the product in glass bottles stored at 4 °C, with a reduction of only 0.15–0.20 log CFU g^{-1} after 42 days storage. Kudelka (2005) analyzed the effect of package types on the acidity of probiotic yogurts during 21 days of refrigerated storage. The yogurt samples were pasteurized and subsequently filled in plastic (polypropylene, polystyrene and polyethylene) packages as well as in glass containers. Yogurt contained in polystyrene packages showed the lowest acidity values as compared to other packages evaluated throughout the storage period.

Cruz et al. (2013) evaluated the stability of probiotic yogurts added with glucose oxidase and packaged in different plastic packaging systems with different oxygen permeability transfer rates ranging from 0.09 to 0.75 ml O_2 day^{-1}. Plastic containers with lower oxygen permeability rates showed a lower content of dissolved oxygen and a higher count of the probiotic bacteria in yogurts during refrigerated storage. Additionally, these samples also presented a higher extent of post-acidification and organic acid production.

Extremely low oxygen permeability of glass packages favors the survival of probiotic cultures. However, due to the high cost of glass along with the hazards inherent to its handling, the manufacturers prefer to market probiotic fermented products in plastic packages. In this context, alternative approaches such as vacuum packaging, addition of oxygen absorbing compounds, active packages with incorporated oxygen barrier materials should be looked at for their potential applications in the packaging of probiotic food products.

4. Conclusions

Probiotics are being included in different food systems apart from the traditional fermented dairy products, and numbers of such probiotic foods are available in the market nowadays. However, the foremost challenge is maintaining the appropriate numbers of these probiotics in food during processing and storage, as insufficient doses at the time of consumption will not provide the intended health benefit. In this context, the use of microencapsulation, cell protective agents, growth promoting food ingredients, oxygen barrier packaging materials, antioxidants and modification of storage environments has enabled these microorganisms to survive better in several processes and formulations.

Identification of the proper encapsulating or cell protecting material for different probiotics is a key issue that determines the efficacy of the process. There is increasing interest in the use of synbiotic (probiotic/prebiotic combination) due to that when probiotics reach to colon, they could use the prebiotics for survival and implantation that beneficially affect the host. Further studies should aim at developing protein or starch based microcapsules with incorporation of additional protective substances into the matrix, and examining the interaction between the microencapsulating material and the protein–carbohydrate–probiotics. Another challenge is the scale-up of microencapsulation process for commercial production. Development of process/equipment for large scale microencapsulation will help industries in further improving the commercialization of their products.

As microencapsulation alone results in limited extensions of probiotic viability, a comprehensive approach is required incorporating emerging food processing technologies those may improve and maintain survival of probiotics during processing and storage, along with the recent knowledge on genotypes and expressed traits of probiotics. Novel processing and packaging technologies such as high-pressure processing (HPP), pulse electric field (PEF), active and smart packaging may prove beneficial for survival of the probiotics in food, after appropriate optimization of the involved processing/storage parameters. Gene technology will play a major role in future for developing new strains with increased stress resistant.

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