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# Methods for improving survival of probiotics against harsh environments

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**Abstract**

Probiotics are live microorganisms which pass through the gastrointestinal tract and survive the harsh environments of the GI tract tolerating low pH in stomach, bile salts, limited space preoccupied by the pathogens etc. Therapeutic benefits have led to an increase in the incorporation of probiotic bacteria such as lactobacilli and bifidobacteria in dairy products, especially fermented dairy products. Encapsulation techniques enhanced the survival of probiotic cultures compared to free cells in different dairy products stored for a longer periods. It also protects the bacteria from harsh conditions (low pH, antibiotics, bacteriocins, bile salt concentration etc) in the gut. Different coating materials for encapsulations for various bacterial cultures including probiotics has been a common practice for extending their storage life *in vitro* and also survivability in the GI tract *in vivo* to provide the health benefits to the consumers consuming different functional fermented dairy foods.

***Keywords*:** probiotics, encapsulation, yoghurt, coating, protection, lactic acid bacteria

Probiotics, live organisms that have multiple functions and effects on the human body, play an important role in maintaining the precise balance of microbiota controlling desirable and undesirable bacteria in the human digestive system. The potential health-promoting effects of dairy products, which incorporate probiotic organisms, mainly *Lactobacillus* spp. and *Bifidobacterium* spp. has stimulated a foremost research exertion in recent years. All of us carry in our intestinal tracts a complex ecosystem of microbes. These bacteria are highly important to our health, providing us with protection against intestinal infections, supplying us with additional nutritional value from the food we eat, and contributing to the development of our immune system whenever they are in live and active in the delivery site. Probiotic foods are the most important discipline of functional food category, which are defined as "foods containing live microorganisms, which actively enhance the health benefits of consumers by improving the balance of microflora in the gut when digested live in sufficient numbers" (Fuller, 1992 and IDF, 1997). There have been long-term interests in the use of cultured milks products with various strains of lactic acid and other probiotic bacteria to improve health of humans (Salminen *et al.* 1998a). Studies relate the possible health benefits of consuming cultured and culture containing milks. Understanding the relationship between microbial populations in the colon and health continues to increase. Clearly, it is important to comprehend the effects of colonic bacteria on host health in order to fully exploit potential applications of prebiotics and probiotics. The colon can be both an organ of health and of disease, especially with regard to the microbiota. Thus the interest in probiotics and prebiotics arises, in part, from the desire to manipulate or enhance the 'beneficial' gut microbiota in a manner that decreases the peril of developing bacterial associated colonic diseases. The selection criteria for a lactic acid bacteria to be used as 'probiotic' include the following ability to: (i) exert a beneficial effects on the host; (ii) survive into a food products at high viable cell counts, and remain viable throughout the shelf-life of the product; (iii) withstand transit through the GI tract; (iv) adhere to the intestinal epithelium cell lining and colonize the lumen of the tract; (v) produce antimicrobial substances towards pathogens; and (vi) stabilize the intestinal microflora and be associated with health benefits. Probiotics must have a good shelf-life in food or preparations, containing a large number of viable cells at the time of consumption, and be nonpathogenic and nontoxic in their preparation. The most extensively studied and widely used probiotics are the lactic acid bacteria, particularly the *Lactobacillus sp.* and *Bifidobacterium* sp. The probiotic strains should maintain their viability in dairy foods as well as in the human gut (Ishibashi and Shimamura, 1993). The development of spray dried skim milk powders harbouring probiotics has been proved useful as direct-vat inoculation, thereby providing more convenient way of incorporation of beneficial biocultures into cheese or other fermented dairy products (Berner and Dannell, 1998). One of the novel challenges in developing probiotic foods is to get survivability during storage period without hampering the normal cultures responsible for the development of proper body and texture and flavour as well as in time of preservation treatments given used to enhance the shelf life of the products. In addition, it is obvious that their ability to reproduce in the GI tract is an important necessity for their overall efficacy. "Probiocap technology" for the improved stabilization of probiotics is now being evolved, which may dramatically enhance the viability of probiotic bacteria. That is why technology to develop "Probiocap" may be used as promising itinerary for transporting high concentration of biomass ensuring maximum protection of the biological integrity of the probiotic products.

## Probiotics

Microorganisms reside the intestines and colon in numbers 10 times greater than the total number of cells in the body itself - over 10 billion per gram of stool. One half of the dry weight of stools is a microorganism. This cell counts are being increasingly found to have profound effects on health. There is a delicate balance, however, between those organisms which contribute to health in assisting digestion, synthesizing nutrients, and inhibiting cancer-causing biochemicals, for example - and those which can cause disease. Beneficial (probiotic) organisms in the diet can help rebalance the digestive tract. It is only recently, that the interrelationship between intestinal microorganisms and the health benefits deriving from it is beginning to be understood. At present, it is generally recognized that an “optimum” balance in microbial population in our digestive tract is associated with good nutrition and health (Rybka and Kailasapathy, 1995). The microorganisms primarily associated with this balance are Lactobacilli and Bifidobacteria. Increasing evidence indicates that consumption of probiotic microorganisms can help maintain such a favourable microbial profile and results in several therapeutic benefits (Lourens-Hattingh and Vijoen, 2001). In recent years, probiotic bacteria have increasingly been incorporated into foods as dietary adjuncts.

The human intestinal tract constitutes a complex bionetwork of microorganisms. More than 400 bacterial species have been identified in the faeces of a single subject (Finegold et al., 1977). The bacterial populations in the large and small intestine are very high and reach maximum counts of $10^{12}$ cfu/g and $10^4$ – $10^8$ cfu/g, respectively (Hoier, 1992). All these intestinal microflora exist in dynamic balance with one another. Some are useful, some detrimental and some neutral to the physiological functions of the body. Thus, intestinal microflora can influence health in a number of ways, both positive and negative (Sandine, 1979). These include impacts on nutrition and physiological functions. For instance, one of the short chain fatty acids produced by colonic bacteria (butyrate) is important in determining the rate of colonic cell growth and differentiation, drug efficacy, carcinogenesis, immunological responses, resistance to infection and resistance to endotoxins and other stresses (O’Sullivan, 1996 & Buttriss, 1997). The beneficial bacteria tend to predominate during periods of good health. If the ecological balance of the gut is disturbed due to prolonged disease, dispossession from foods and water, travel (especially by air), antibiotics, radiation etc. (Hanevaar and Huis in’t Veld, 1992), certain microorganisms with negative roles in the human system may dominate. In such cases, there is an increase in the products of putrefaction, toxins and carcinogens. Pathogenic bacteria, which are normally present at low levels, also boom, if the resistance of the body is lowered for any reason, manifesting their pathogenicity and causing diseases. Living probiotic cultures help uphold the critical balance and can stabilize a disturbed intestinal flora (Gibson and Roberfroid, 1995).

There is mounting evidence of health benefits of probiotic foods and the reverberation in market is to combine these health benefits with product appeal and versatility without any procrastination. Current clinical applications of probiotic bacteria in the well documented areas, such as treatment of acute rota virus diarrhoea, lactose maldigestion, constipation, colonic disorders and side-effects of pelvic radiotherapy and more recently, food allergy including milk hypersensitivity and changes associated with colon cancer development (Salminen *et al*. 1998). There are myriad evidences to support the view that oral administration of some *Lactobacillus* and *Bifidobacterium* species are able to restore the normal balance of probiotic populations in the intestine. In addition to their established role in GI therapy, the probiotic foods are claimed to serve several nutritional and therapeutic benefits, such as antimicrobial properties (Shah, 2000), antimutagenic properties (Lankaputra and Shah, 1998), anticarcinogenic properties (Mitsuoka, 1989), improvement in lactose metabolism (Vesa *et al*. 1996), reduction in serum cholesterol (Fukushima and Nakano, 1996) and immune system simulation (Schiffrin *et al.* 1994). Therefore, in the near future probiotic foods will be seen in many different markets beyond what is seen today.

## Market of Probiotics

Functional dairy products with probiotics are promising in the Indian market, as major players such as Yakult, Danone, Nestle, Amul, Mother Dairy has launched more health drinks and yoghurts in the country. The market for products containing probiotics is expected to grow as Indians become more aware of natural, healthy ingredients in foods. Probiotics drinks are available for purchase mainly in big cities. Rest of the areas including rural are now being penetrated by systematic marketing strategies to increase awareness of the country people and to educate them about the probiotics benefits. The size of the dairy market is expected to grow at almost 40 % annually or even more, reaching ₹ 5,20,780 crores ($ 122,825 million) by 2011 (Roy and Langerholc, 2010). The global market of probiotic ingredients, supplements and food was worth $14.9 billion in 2007 and it was expected to reach 15.9 billion in 2008, and 19.6 billion in 2013, representing a compound annual growth rate of 4.3 % (Agheyisi, 2008). The most commonly found commercial probiotic drinks for human consumption are found in the form of probiotic drinks, fluid yoghurt, cultured butter milk and special milk with probiotic supplement with or without fruit juices. Requirement of probiotic drinks is supported by importing probiotic cultures from other countries, especially Europe and United States where this segment has registered tremendous success. Probiotic products are gaining acceptance mostly in urban areas as they are better connected to information network and the society is more aware about health. The Indian market is big, but difficult to reach, since cold storage and cold distribution chain are underdeveloped, especially in the rural areas.

According to market research report, global probiotics market generated US $ 15.9 billion in 2008 and is expected to be worth US $ 32.6 billion by 2014 with a compound annual growth rate of 12.6 % from 2009 to 2014 (Table 1). The probiotic product industry in India is estimated worth ₹ 20.6 million with a projected annual growth rate of 22.6 % until 2015 (Frost and Sullivan, 2009; ICMR-DBT, 2011). Table 2 depicts value of fermented milks in USA.

**Table 1: Probiotic market evaluation in US**

| Product category | Microorganisms involved | Market worth (US Dollar) |
|---|---|---|
| Regular Yoghurt | *Lactobacillus delbrueckii* *Streptococcus thermophilus* | 11,348 |
| Probiotic Yoghurt | Bifidobacterium Acidophilus | 4,127 |
| Probiotics Drinks | Not specified | 1,662 |
| Kefirs | Not specified | 350 |

A report in Functional Food weekly states that around 30 % of the global population buys into the probiotic dairy sector on a regular basis, representing a major part of the US $ 85 billion global functional foods market, of which the US, Western Europe and Japan account for over 70 %. In 2008, the global probiotics market (including both foodstuffs and supplements) was worth over US $ 15.7 billion, or over 18 % of the global functional foods market. Since 2003, the global probiotics market has more than doubled in value terms, and is currently rising by almost 15 % per annum. Although probiotics remain best suited to dairy products such as eatable and drinking yoghurts, probiotic products have emerged in sectors such as breakfast cereals, infant formula and soft drinks. Probiotic bacteria offer new dietary alternatives for the management of bacterial disease conditions through stabilization of intestinal microflora, promotion of colonization resistance, regulation of the immune response and preservation of intestinal integrity (Salminen et al., 1998)

### *Hurdles with Probiotics*

Probiotic foods formulators, now are confronted with a myriad of obstacles in getting an effective product to market, of which most consumers are blissfully unaware. Stability, heat and cold sensitivity, rancidity, unpalatable flavors, unappealing colors, odors, and adulteration are just the beginning (Kailasapathy and Rybka, 1997). While manufacturers have long been fortifying products with probotics, they have faced significant processing challenges regarding the stability and survivability of probiotics during processing and preservation treatments as well as during their passage through the stomach. In fact, many active cultures die even before the consumer receives any of the health benefits. If these beneficial microorganisms stay alive during food processing conditions, they must also survive during their passage from the mouth to intestine (Davis *et al*. 1971). Specially, probiotics are extremely susceptible to environmental conditions viz. water, oxygen, processing and preservation treatments, acidity and salt concentration, which collectively affect the overall viability of probiotics. Probiotics can lose upto 95 per cent of their viability when unprotected. This is why the concentration of viable cells in the duodenum rarely exceeds $10^5$ cfu/g of product, while as high as $10^8$ and $10^{11}$ cfu/g have been found in the ileum and the colon, respectively (Siuta-Cruce and Goulet, 2001). These unprotected microflora are also typically destroyed during such processing treatments as sterilization, pasteurization, microwave treatments, thermization, retorting disinfections, and irradiation, washing and peeling. Hence, maintaining the probiotic viability is central to any probiotic food manufactures.

### *Materials for "protection"*

One of the most studied technology to improve viability of probiotics is microencapsulation of cells along with various wall materials. The wall protects the core against deterioration and limits evaporation of volatile core materials (Kadian *et al*. 1999). The encapsulating agents should have certain ideal characteristics, depending on the objectives and requirements, process of encapsulation, chemical characteristics of the core material, the intended use of the core material, the conditions under which the product will be stored, and the processing conditions to which it will be exposed (Kanawjia *et al.* 1992). Some general characteristics of the encapsulating agent are that it is insoluble in and non-reactive with the core material, have solubility in the end-product food system, and be able to withstand high temperature of the spray-drying process. Some typical encapsulation agents are dextrans, gums, starches or proteins (Table 2).

**Table 2: Coating Materials Used to Produce Microcapsules**

| Class of Coating Material | Specific Types of Coatings |
|---|---|
| Gums | Gum arabic, agar, sodium alginate, carageenan |
| Carbohydrates | Starch, dextran, sucrose, corn syrup |
| Celluloses | CMC, methycellulose, ethylcellulose, nitrocellulose, acetylcellulose, cellulose acetate-phthalate, cellulose acetate-butylate-phthalate |
| Lipids | Wax, paraffin, tristearin, stearic acid, monoglycerides, diglycerides, beeswax, oils, fats, hardened oils |
| Inorganic materials | Calcium sulfate, silicates, clays |
| Proteins | Gluten, casein, gelatin, albumin |

(Jackson and Lee, 1991).

**Entrapment of Microorganisms**

Several studies have reported the microencapsulation to develop "Probiocap" by using gelatin or vegetable gum to provide protection to acid sensitive bifidobacteria (Rao *et al*. 1989 & Ravula and Shah, 1999). Entrapment of living microbial cells in calcium alginate is simple and low cost. Furthermore, alginate is non-toxic so that it may be safely used in foods. Alginate gels can be solubilized by sequestering calcium ions thus releasing entrapped cells (Rao *et al*. 1989). Ravula and shah (1999) have developed a microencapsulation technique with sodium alginate. Encapsulated organisms were incorporated in fermented frozen dairy desserts and investigated the viability of probiotic bacteria (Hati et al., 2013). The counts of *L. acidophilus* and *Bifidobacteria* decreased to $<10^3$ cfu/g in the control batch, whereas the counts were $>10^5$ cfu/g in the products made with encapsulated organisms.

Rao et al. (1989) developed a technology for microencapsulation of *Bifidobacterium longum* with cellulose acetate phthalate (CAP) using phase searation coacervation method. Microbiological analysis indicated that microencapsulated cell survived the simulated gastric environment in large numbers than non-encapsulated. In another report, Kim *et al*. (1988) described a method for the preparation of stable microencapsulated lactic acid bacteria using polyvinyl acetate phthalate. Due to the presence of ionizable phthalate group, this polymer is insoluble in acid media at pH 5 or below but is soluble when the pH is increased to 6 or higher. In addition, this compound is physiologically inert when administered *in vivo* and is, therefore widely used as an enteric coating material for release of drugs and other pharmaceutical substances in the intestine, This is successfully used to prepare microcapsules of active viral antigens and other proteins for oral consumption (Moharaj *et al*. 1984). Microencapsulation of bifidobacteria in K-carrageenan appeared to increase the viability of bifidobacteria in yoghurt (Adhikari *et al*. 2000).

In another investigation, *L.delbruecki* spp. *bulgarius* cells were entrapped in beads of calcium alginate and found increased ability to survive freezing process (Sheu *et al*. 1993). Significantly, lower β-galactosidase activities were observed with Ca-alginate encapsulated cells (Sheu and Marshall, 1992). Forty per cent more lactobacilli survived freezing ice milk when they were entrapped in calcium alginate than when they are not entrapped (Sheu and Marshall, 1993). Hyndman *et al*. (1993) microencapsulated *Lactococcus lactis* spp. *cremoris* with in gelatin membranes cross-linked with toluenc-2, 4-di-isocyanate at an oil/water interface. Microencapsulation resulted in acidification of milk to pH 5.5 within 2.8 hr, similar to that achieved in free cell fermentations. A team of Chinese scientists studied microencapsulation of *L. delbruecki* spp. *bulgarius* and *Streptococcus thermophilus* by spray drying and observed improved survival rates of microorganisms significantly, with survival rates of up to 50 per cent (Hua *et al*. 1998).

Demos *et al*. (1998) attempted to microencapsulate different lactic acid bacteria by spray drying and observed that survival rate after spray drying was correlated with the outlet air temperature and storage temperature. Lee *et al*. (1998) encapsuled lactic acid bacteria with hydrogenated maize oil by injecting coat-core emulsions into a chilled dispersion fluid. They also studied their stability using different emulsifiers and observed that the microcapsules were not disrupted after heat treatment of 37$^{o}$C for 1 h. Microencapsulation of three different strains of bifidobacteria in alginate or K-carrageenan beads has been also proved effective in improving the survival throughout the storage for 10 weeks at-20$^{o}$C from 43-44% to about 50-60% with better survivability in alginate beads than in K-carrageenan beads (Kebary *et al*. 1998). Addition of glycerol and mannitol during preparing of alginate beads increased the survival of bifidobacteria from 58.8 to 88.5%.

With the aims to derive better retention and protection of the cells, recovery and reuse of the starter cultures, *Lactococcus lactis* spp. *lactis* was microencapsulated within cross-linked chitosan membranes formed by emulsification/interfacial polymerization (Groboillot et al., 1993). Champrgne *et al*. (1992) investigated the cell release during fermentation by *Lactococcus lactis* entrapped in calcium alginate beads with poly-L-lysine (PLL) and observed that it did not significantly reduce the release of cells during consecutive fermentations. In another work, Khalil and Mansour (1998) evaluated the survival of bifidobacteria microencapsulated in alginate and their effect on the quality of mayonnaise. They observed that the viability of the free cells disappeared after two weeks, however encapsulated *B. bifidum* survived well for 12 and *B. infantis* for 8 weeks. Mayonnaise containing encapsulated bifodobacteria had lower total bacterial counts compared to other treatments. Even, sensory properties of mayonnaise were improved by the addition of encapsulated bifidobacteria.

The entrapment of *Lactococcus lactis* spp. *cremoris* CRA-1 in alginate/poly-L-lysine (Alg/PLL) nylon or cross-linked polyethyleneimine (PEI) membranes has been investigated (Larisch *et al*. 1994). They reported that Alg/PLL encapsulation resulted in viable and active cell preparations, which acidified milk at a rate proportional to cell concentration, but at rates, less than that of free cell preparations. Hong (1997) studied the enhancing survival of lactic acid bacteria in ice cream by natural encapsulation and observed that *Streptococcus thermophilus* strains survived better than their non-encapsulated mutants did in reduced fat ice cream during freezing and frozen storage at –29 $^{0}$C for 16 days. He reported harvest of cells in late log phase at 37 $^{0}$C, keeping overrun at 50 % and storage at –17 $^{0}$C, gave maximum survival of *S. thermophilus*.

### *"Probiocap": A Novel Approach in Improving Probiotic Survivability*

The probiotic market shows great potential for manufactures and has continued to gain unprecedented momentum, despite the complex processing challenges of developing probiotic foods with those beneficial microorganisms. The bacteria often die during manufacturing process, storage treatments to be given or during passage to the intestine. Shelf life is also unpredictable, and the industry has had difficulty making up label claims. Manufacturers who want to inflate the use of probiotic must have to think over probiotic stability and survival rates during production, preservation and gastric transient to ensure actual benefits to the consumer. Even in India, very few companies have proper health claims with highest survivability in their fermented probiotic milk foods.

Traditional freeze-dried probiotic bacteria are sensitive to high moisture, extreme temperatures and other physical and chemical stresses. Patent-pending microencapsulation technology, "Probiocap", for the improved stabilization of probiotics has been reported from Canada. This new technology dramatically enhances the viability of probiotic bacteria. "Probiocap": increases heat resistance, improves resistance to compression, enhances acid resistance, extends shelf life specially designed equipment is used to coat and entrap probiotic bacteria in a matrix of food grade vegetable fatty acids. This allows for the use of probiotics in new applications: powders, cream type formulas, such as chocolate bars, and processed foods as it withstands high temperatures. ""Probiocap"" uses a food-grade fatty acid coating and therefore protects probiotic strains against the action of excessive stresses such as temperature, oxygen, stomach acidity, moisture, and pressure. In addition, "Probiocap" also expands probiotics into many new applications in the functional-food industry, including creamy formulas, powders and nutritional bars, allowing them to be mixed with other food ingredients that could have inhibited their viability. The technology is triggered to separate cells when the release of this functional ingredient is desired for maximum benefit viz in the intestines, directly after exposure to the low pH of the stomach.

Recently, a new microencapsulation technology, called "Probiocap" that applies a coating to the probiotic to protect it from moisture, high humidity and acidity. The coating allows the probiotic to pass through the gastrointestinal tract without being destroyed by gastric juices and is triggered for release in the intestine based on pH conditions. It has been applied successfully to several strains of probiotics including *L. acidophilus, L. rhamnosus* and *Bifidobacterium longum.* However, clinical trials has been revealed that tablets with the encapsulated probiotics had an unprecedented recovery rate. In standard industry testing of tablet compression, 50-75 percent of probiotics die. Initial research suggests that Balchem's encapsulation technology may triple shelf life for probiotics in certain applications and help manufacturers deliver a reliable quantity of probiotics to meet label claims. This process would allow probiotic foods formulators to guarantee stability and to recover all the active bugs. It also eliminates the need to overdose. It has been the practice of manufacturers to recommend a dose of one to 10 billion live cells to ensure the delivery of at least 100 million live cells in the GI tract. Using “Probiocap” technology they wouldn’t need to overdose to this extent. The technology is said to ensure a ten-fold increase in bacterial survival. Manufacturers using this technology can include lower concentrations, thereby reducing production costs with greater live cells.

## Conclusion

The bacteria often die during manufacturing process, storage treatments to be given or during passage to the intestine. Shelf life is also unpredictable, and the industry has had difficulty baking up label claims. Manufacturers who want to inflate the use of probiotic must have to think over probiotic stability and survival rates during production, preservation and gastric transient to ensure actual benefits to the consumer. Probiotic products have been marketed in recent years, primarily in the fermented food category or as live microbial dietary supplements whose biological activity needed to be preserved. Microencapsulation has been investigated to be the best accessible technology to preserve the potency of probiotics to be ultimately delivered into the GIT. The novel application of microencapsulated probiotics would allow the beneficial microorganisms to be incorporated readily in high dosage and allow the probiotic food designers to provide confidence on viability and quantity of probiotics upto the GIT, even after the processing and some preservation treatments. Keep in mind by encapsulating probiotics we can prevent unwanted ingredient interaction and increase shelf life and viability as well. This process is opening up avenues previously not possible to manufacturers. It may also allow the medical industry in the field of bacteriotherapy, wherein very high doses of viable probiotic bacteria are required for the treatment of patients with chronic paucities, diarrhea etc.

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