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Diversification of Probiotics through Encapsulation Technology

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Abstract

Probiotics are live bacteria and dietary concepts to improve the intestinal microbial balance. Microencapsulation technique significantly improves the stability of probiotics during food processing and gastrointestinal transit. However, the matrix has a positive impact on survival without affecting the release of entrapped cells in simulated colonic pH solution. Maximum survival of cells has been noticed in encapsulated bacteria compared to the normal cells during different processing treatments as well as acid and bile tolerance and resistance to the gastric juices.

Keywords: Microencapsulation technique, probiotics, cells, tolerance, resistance

Probiotics, "live microbial feed supplements that have beneficial effects on the host by improving its intestinal microbial balance", compete with and suppress the growth of undesirable microorganisms in the colon and small intestine and thus prevent of intestinal infections, expression of antitumour activities and lactose utilization in the human gut. For these beneficial effects, probiotics should survive in human gastrointestinal tract after ingestion, reach colon and should get established there.

During their passage through gastrointestinal tract, probiotic organisms are confronted with many harsh physicochemical conditions, i.e., low pH of stomach, bile salts, pancreatic enzymes, etc. Beside these, incorporation of probiotics in food products have also been limited due to industrial food processes where elevated temperatures, compression, and the presence of oxygen and moisture, can adversely affect their survival rates. Also for probiotic foods to be beneficial for human health, the probiotics should maintain their viability in the product until the time of consumption and during the passage through the gastrointestinal tract (GIT).

Thus, challenges in developing probiotic-foods is the survival of organisms during storage without hampering the normal body and texture, and flavor of the product as well as in time of preservation treatments used to enhance the shelf life of the products. One technology that makes it possible to "have high dose of your probiotics and eat it alive, too!" is microencapsulation. Microencapsulation protects probiotics against stress in processing, storage conditions and against gastrointestinal conditions, thereby increasing recovery rates. Encapsulation and preservation techniques make products capable of being directed to specific sites in the body. 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 assurance on viability and quantity of probiotics upto the GIT, even after the processing and some preservation treatments.

Probiotics

The concept of probiotics was evolved at the turn of the 20th Century from a hypothesis proposed by Russian Scientist, Elie Metchenikoff, that the long healthy life of Bulgarian peasants was resulted from the consumption of fermented milk products. According to Metchenikoff, the regular consumption of live beneficial bacteria such as lactic acid bacteria (LAB) through fermented dairy products are needed to maintain a good equilibrium of the intestinal microflora that minimize putrefactive microbial fermentations. Probiotic is a "live microbial (feed) supplement, which beneficially affects the host (animal) by improving its intestinal microbial balance" (Fuller, 1989).

Probiotics are basically mono or mixed culture of live microorganisms which when applied to animal or man decreases the number of intestinal infections and/or improves the general health by contributing to a better gastrointestinal environment (Fuller, 1992). Probiotics have been reported to have several health benefits such as balancing of intestinal microflora, stimulation of the immune system, prevention of diarrhoea, and anti-carcinogenic activity (Mandal *et al.,* 2012).

However, several studies have shown that non-viable probiotics can have beneficial effects such as immune modulation and carcinogen binding in the host (Ouwehand and Salminen, 1998).

Selection of probiotics strains

Different probiotic species and even different strains within a species exhibit distinctive properties that can markedly affect their survival in foods, fermentation characteristics and other probiotic properties. Strain selection becomes, therefore, a critical parameter to ensure the culture's fermentation or probiotic performance (Hati *et al.,* 2015, Mandal *et al.,* 2006).

Desirable selection criteria for probiotic strains

(A) Appropriateness:

- ◊ Taxonomic identification known by phylogenetic analysis and rRNA sequencing.
- ◊ Origin – normal inhabitant of the species targeted and isolated form a healthy individual.
- ◊ Safety – nontoxic, nonpathogenic, "generally recognized as safe" status.

(B) Technological suitability:

- ◊ Amenable to mass production and storage: adequate growth, recovery, concentration, freezing, dehydration, storage and distribution.
- ◊ Viability at high population (preferred at 10^7 to 10^9 cfu/ g).
- ◊ Stability of desired characteristics during culture preparation, storage and delivery.
- ◊ Provide desirable organoleptic qualities (or no undesirable qualities) when included in foods or fermentation processes.
- ◊ Genetically stable to maintain phenotypic properties.
- ◊ Genetically accessible for potential modification.

(C) Competitiveness:

- ◊ Capable of survival, proliferation and metabolic activity at the target site *in-vivo.*
- ◊ Resistant to bile.
- ◊ Resistant to acid.
- ◊ Able to compete with the normal microflora, including the same or closely related species; potentially resistant to bacteriocins, acids and other antimicrobial agents produced by residing microflora.
- ◊ Adherence, colonization and retention evaluated.

(D) Performance and functionality:

- ◊ Able to exert one or more clinically documented health benefits.
- ◊ Antagonistic toward pathogenic/cariogenic bacteria.
- ◊ Production of antimicrobial substances (bacteriocins, hydrogen peroxide, organic acids or other inhibitory compounds).
- ◊ Immunostimulatory
- ◊ Anti-inflammatory.
- ◊ Antimutagenic.
- ◊ Anticarcinogenic.
- ◊ Production of bioactive compounds (enzymes, vaccines, peptides).

(Klaenhammer and Kullen, 1999).

Currently use probiotics

These include Lactobacilli, such as *Lactobacillus acidophilus*, *L. casei*, *L. delbrueckii* ssp. *bulgaricus*, *L. reuteri*, *L. brevis*, *L. cellobiosus*, *L. carvatus*, *L. fermentum* and *L. plantarum*; Gram-positive cocci, such as *Lactococcus lactis* ssp. *cremoris*, *Streptococcus thermophilus*, *Enterococcus faecium*, *S. diacetylactis* and *S. intermedius* and Bifidobacteria, such as *B. fifidum*, *B. adolescentis*, *B. animalis*, *B. infantis*, *B. longum* and *B. thermophilum*. Nonpathogenic microorganisms that occupy important niches in the host gut or tissues, such as yeasts, enterococci and *Enterobacteriaceae,* are used as human and animal probiotics. Though, *Lactobacillus* and *Bifidobacterium* are the most commonly species of bacteria used as probiotics for the production of fermented milks and other dairy products (Hati *et al.,* 2014).

- **Human probiotic species and strains**
  - ◊ *Bifidobacterium breve* Yakult
  - ◊ *Bifidobacterium lactis* (BB12)
  - ◊ *Bifidobacterium longum* (SBT2928, BB536)
  - ◊ *Lactobacillus acidophilus* (NCFM, SBT2062)
  - ◊ *Lactobacillus casei* (Shirota, CRL431, DN014001, immunits)
  - ◊ *Lactobacillus delbrueckii* ssp. *bulgaricus* (2038)
  - ◊ *Lactobacillus fermentum*
  - ◊ *Lactobacillus johnsonii* (La1, Lj 1)
  - ◊ *Lactobacillus paracasei* (CRL431, F19)
  - ◊ *Lactobacillus plantarum* (299V)
  - ◊ *Lactobacillus reuteri* (SD 2112)
  - ◊ *Lactobacillus rhamnosus* (GG, 271, GR1)
  - ◊ *Lactobacillus salivarius* (UCC118)
  - ◊ *Streptococcus thermophilus* (1131)

Recommendation of effective probiotic foods

Currently, there are no legal recommendations for consumption of probiotics through foods. Adequate numbers of viable cells, namely the "therapeutic minimum" needs to be consumed regularly for transfer of the probiotic effect. The IDF (1997) proposed that in probiotic foods, "the specific microorganisms shall be viable, active and abundant at the level of at least 10^7 cfu/ g in the product to the date of minimum durability" (Ouwehand and Salminen, 1998). It has been suggested that approximately 10^9 cfu/ d of probiotics is necessary to elicit health effects. Based on daily consumption of 100 gram of a probiotic food, it has been suggested that a product should contain at least 10^7 cfu/ g, a level paralleling current Japanese recommendations (Ishibashi and Shimamura, 1993). The ingestion of 10^6 to 10^9 viable cells per day is necessary for humans in order to develop beneficial effects (Lee and Salminen, 1995). Fermented Milks and Lactic Acid Bacteria Beverage Association of Japan has developed a standard, which requires a minimum of 10^7 viable bifidobacteria cells/ mL to be present in fresh dairy products. The National Yoghurt Association (NYA) of the United States specifies 10^8 cfu/ g of lactic acid bacteria at the time of manufacture as a prerequisite to use the NYA "*Live and Active culture*" logo on the containers of products (Kailasapathy and Rybka, 1997).

Probiotic functional foods

The term functional food was first introduced in Japan in the mid-1980s and refers to "processed foods containing ingredients having specific health beneficial functions in addition to being nutritious". Functional foods have been variously termed as *neutraceuticals, designed foods, medicinal foods, therapeutic foods, superfoods, foodiceuticals, and medifoods* (Finley, 1996). The functional foods market is flourishing at a rate of 15-20 per cent per annum and the industry is claimed to be worth $ 33 billion (Hilliam, 2000). Probiotic foods, the important discipline of functional foods, are defined as "foods containing live microorganisms, which actively enhance the health of consumers by improving the balance of microflora in the gut when ingested live in sufficient numbers" (Fuller, 1992). Traditionally, probiotics have been added to yogurt and other fermented foods.

Prebiotics

Prebiotics are "non-digestible dietary components that pass through to the colon and selectively stimulate the proliferation and/or activity of populations of desirable bacteria *in-situ*" (Gibson and Roberfroid, 1995). Food ingredients classified as prebiotics must not be hydrolyzed or absorbed in the upper GIT, need to be a selective substrate for one or a limited number of beneficial colonic bacteria, must alter the microbiota in the colon to a healthier composition and should induce luminal or systematic effects that are beneficial to host health (Mishra *et al*., 2001). Crittenden and Playne (1997) described food-grade oligosaccharides in commercial production; these include lactulose, galactosaccharides, fructo-oligosaccharides, isomalto-oligosccharides, lacto-sucrose, gentio-oligosaccharides and xylooligosaccharides.

Targets for development of prebiotics

- ◊ Expand avenues for incorporation into appropriate food vehicles.
- ◊ Improved stimulation of beneficial floras.
- ◊ Exhibit anti-pathogenic properties; anti-adhesive and attenuation.
- ◊ Identify low-dosage forms.
- ◊ Derived from dietary polysaccharides.
- ◊ Non-cariogenic.
- ◊ Good preservative and drying characteristics.
- ◊ Low caloric value.
- ◊ Controllable viscosity.

(Klaenhammer, 2003).

Synbiotic foods

Synbiotic is where probiotics and prebiotics are used in combination, to manage microflora. Due to the potential synergy between probiotics and prebiotics, foods containing a combination of these ingredients are often referred to as synbiotics (Collins and Gibson, 1999). Gibson *et al*. (1995) reported a significant effect on the composition of fecal flora on feeding a group of volunteers a daily supplement of a synbiotic. Number of synbiotic products containing Bifidobacteria and lactulose are already available in Japanese markets. Some of these products are Hounyu Milk Powder for adults [lactuloe – 8.3 g/ 100 g and Bifidobacteria >3 × 10^7], Sawayaka sour milk [lactulose 4 g/ 100 g and Bifidobacteria > 10^8], etc. (Mizota *et al*., 1987). Some synbiotic dairy products e.g., Symbalance, mixture of *L. reuterii*, *L. acidophilus* and *L. casei* along with RAFTILINE, an inulin and John après Jour a UHT skimmed milk with ACTILIGHT, etc. have also been marketed in Europe (Young, 1998).

Hurdles affecting probiotics' viability

Careful screening of probiotic strains for their technological suitability can also allow selection of strains with the best manufacturing and food technology characteristics. However, even the most robust probiotic bacteria are currently in the range of food applications to which they can be applied. Additionally, bacteria with exceptional functional heath properties are ruled out due to technological limitations. New process and formulation

technologies will enable both expansion of the range of products in to which probiotics can be applied and the use of efficacious stains that currently cannot be manufactured or stored with existing technologies. Viability of probiotics has been both a marketing and technological concern for many industrial produces. Probiotics are difficult to work with, the bacteria often die during processing and shelf life is unpredictable. Probiotics are extremely susceptible environmental conditions such as oxygen, processing and preservation treatments, acidity and salt concentration, which collectively affect the overall viability of probiotics. Manufactures have long been fortifying products with probiotics; they have faced significant processing challenges regarding the stability and survivability of probiotics during processing and preservation treatments, storage as well during their passage through GIT.

Food processing, preservation and storage

Several factors have been claimed to affect the viability of probiotic bacteria including acid and hydrogen peroxide produced by yogurt bacteria, oxygen content in the product and oxygen permeation through package (Ishibashi and Shimamura, 1993; Lankaputhra and Shah, 1994; Lankaputhra *et al*., 1996). Oxygen content and redox potential have been shown to be important factors for the viability of bifidobacteria during storage of fermented milk (Brunner *et al*., 1993 a,b).

Probiotic microorganisms are anaerobic and some micro-aerophillic in nature and oxygen toxicity is an important problem. The viability also depends on the availability of nutrients, growth promoters and inhibitors, concentration of sugars (osmotic pressure), dissolved oxygen and oxygen penetration through package, inoculation level, incubation temperature and time and also storage temperature (Costello, 1993). The survival of bifidobacteria in fermented dairy products depends on the strain of bacteria used, fermentation conditions and storage temperature (Laroia and Martin, 1991; Blanchette *et al*., 1996; Lankaputhra *et al*., 1996; Shin *et al*., 1996).

Passage through "GIT"

Many strains of *L. acidophilus* and *Bifidobacterium* spp. intrinsically lack the ability to survive harsh conditions in the gut and may not be suitable for use as dietary adjuncts in fermented foods (Lankaputhra and shah, 1995). Rao *et al.* (1989) documented that one of the major barriers to the survival of ingested microorganisms is low pH of the stomach. Many reports indicated that there is poor survival of probiotic bacteria in the products and the survival of these bacteria in human GI system is questionable (Kailasapathy, 2002). Several investigations have studied the survival of *L. acidophilus* and *Bifidobacterium* spp. in presence of acid and bile salts (Ibrahim and Bezkorovainy, 1993).

Approaches in improving probiotics' viability

Probiotic suppliers have developed a variety of proprietary technique to preserve and protect the integrity of these tiny living organisms. Viability of probiotics can be improved by appropriate selection of acid and bile resistant strains, use of oxygen impermeable containers, two-step fermentation, stress adaptation, incorporation of micronutrients such as peptides and amino acids, sonication of yogurt bacteria and microencapsulation (Shah, 2000).

Microencapsulation – in improving probiotics' viability

Microencapsulation is a process where droplets of liquids, solids, or gases (core) are coated by thin films (coatings), which protect the core until it is needed (Sheu and Rosenberg, 1995). In the foods alone, a large number of substances have been microencapsulated, such as acidulants, amino acids, antimicrobials, bases, colorants, edible oils, flavor, enzymes, microorganisms, flavor enhancers, leavening agents, minerals, sugars, salts, and vitamins (Kanawjia *et al*., 1992). The core can be released at different times as and when occasion demands by any desired mechanisms, such as disruption, dissociation, dissolution or diffusion and with any desired rates, such as instantaneous, delayed, controlled or sustained release (Kanawjia *et al.,* 1992) depending on the properties of the coatings that are applied. The

coating on a core is semi-permeable and protects the core from severe conditions and controls substances flowing into the core and the release of metabolites from the core (Jackson and Lee, 1991).

During microencapsulation, specially designed equipment coats probiotic bacteria in a matrix. This will increase formulation possibilities, broadening the range of ingredients with which probiotics can be blended. Institut Rosell/Lallemand's microencapsulation provides protection at 50°C for several hours. The hydrophobic coating surrounding microencapsulated bacteria protects the fragile microbial cells, allowing them to pass into the intestine. In order to make this technology successful to entrap probiotics, the protective wall materials should be such that it would afford protection to the probiotics against the processing and storage conditions, GIT transient but release them in post-stomach in the human body. It has been investigated that some lipid coatings are not only an efficient protective barrier against chemical entities, such as moisture, oxygen, and acids but also a good protector against short exposure to high temperature and pressure (Suita-Cruce and Goulet, 2001).

Microencapsulation of various bacterial cultures including probiotics has been a common practice for extending their storage live and converting them into a powder form for ease of their use. There are several techniques such as spray drying, freeze-drying, and fluidized bed drying for encapsulating the cultures and converting them into a concentrated powdered form. However, the bacteria encapsulated by these techniques are completely released in the product. In this case, the cultures are not protected from the product environment or during the passage through the stomach or intestinal tract. Encapsulation in hydrocolloid beads entraps or immobilizes the cells within the bead matrix, which in turn provides protection in such an environment. The cells are retained within an encapsulating membrane to reduce cell injury or cell loss. The encapsulation techniques applied to probiotics for the use in fermented milk products or biomass production can be classified in to two groups: extrusion (droplet method) and emulsion or two-phase system. Both extrusion and emulsion techniques increase the survival of probiotics by up to 80-95 per cent (Audet *et al*., 1988; Rao *et al*., 1989; Sheu and Marshall, 1991; Sheu and Marshall, 1993; Sheu *et al*., 1993; Jankowski *et al*., 1997 and Kebary *et al*., 1998).

Consideration of materials for microencapsulation

The structure formed by microencapsulating agent around the core material is called the wall material which protects the core against deterioration, limits the 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).

Table 1: Coating materials used to produce microcapsules (Jackson and Lee, 1991)

Class of coating materials	Specific types of coatings
Gums	Gum arabic, agar, sodium alginate, carrageenan
Carbohydrates	Starch, dextran, sucrose, corn syrup
Celluloses	CMC, methylcellulose, 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

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 processing. Some typical encapsulation agents are dextrans, gums, starches or proteins. Many coating materials have been used for encapsulation

of microorganisms. These include a mixture of k-carrageenan and locust bean gum (Audet *et al*., 1988, 1989; Arnaud *et al*., 1992), cellulose acetate phthalate (Rao *et al*., 1989), alginate (Sheu and Marshall, 1993; Sheu *et al*., 1993; Larisch *et al*., 1994; Kebary *et al*., 1998), alginate-starch mixture (Sultana *et al*., 2000), k-carrageenan (Adhikari *et al*., 2000).

Additional treatments of microcapsules

Entrapment in hydrocolloid gels, such as alginate, k-carrageenan, etc. have some limitations due to less stability of microcapsules in the presence of chelating agents such as phosphate, lactate, citrate etc., which share the affinity for ions such as Ca^{+2}, K^{+}, etc. and destabilize the gel (Smidsrod and Skjak-Braek, 1990). The problems are encountered during lactic acid fermentation (Roy *et al*., 1987) and cause cell release from the beads. In other matrix material, such as chitosan, the entrapped cells can be released form the beads during fermentation and cause low initial loading for the next fermentation. Therefore, additional treatments, such as coating the beads, are applied to improve the properties of beads. Coated beads not only prevent cell release but also increase mechanical and chemical stability. Cross-linking with cationic polymers, coating with other polymers, mixing with starch and incorporating additives improve stability of beads.

Microencapsulation of microorganisms

The benefits offered by encapsulation, entrapped microorganisms can be used to advantage for producing dairy products. Several studies have reported on the microencapsulation to by using gelatin or vegetable gum to provide protection to acid sensitive bifidobacteria (Rao *et al*., 1989; Ravula and Shah, 1999). Ravula and shah (1999) have encapsulated organisms in sodium alginate and incorporated in fermented frozen dairy desserts. They reported that *L. acidophilus* and bifidobacteria decreased to $<10^3$ cfu/ g in the control, whereas the counts were $>10^5$ cfu/ g in the products made with encapsulated organisms. *L. delbruecki* spp. *bulgarius* cells were entrapped in calcium alginate beads and evaluated their survival during freezing (Sheu *et al*., 1993), and they observed the higher survival of entrapped cells. Lactobacilli survived higher (>40%) during freezing ice milk entrapped in calcium alginate (Sheu and Marshall, 1993). Khalil and Mansour (1998) observed that the viability of the free cells disappeared after two weeks, however alginate encapsulated *B. bifidum* survived well for 12 and *B. infantis* for 8 weeks in Mayonnaise. Sensory properties of mayonnaise were improved upon addition of encapsulated cells. The entrapment of *L. lactis* spp. *cremoris* CRA-1 in alginate/ poly-L-lysine (A/ PLL), nylon or cross-linked polyethyleneimine (PEI) membranes has been investigated (Larisch *et al*., 1994).

They reported that A/ 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) observed that *S. thermophilus* strains survived better than their non-encapsulated mutants did in reduced fat ice cream during freezing and frozen storage at –29°C for 16 d. Kushal (2001) encapsulated *B. bifidum* NCDC 255 and *L. acidophilus* NCDC 13 as single and co-culture in calcium alginate beads and reported higher effect by ingesting the encapsulated probiotics as encapsulation ensures higher number of probiotics delivery into colon. Encapsulated probiotics survived the LTLT pasteurization (63°C for 30 min.) and improveed the storage stability. Lee and Heo (2000) studied the survivability of calcium alginate *B. longum* in simulated gastric juices and bile salt. They reported that the death rate of the cells in the beads decreased proportionally with an increase in both the alginate concentration and bead size.

Microencapsulation of bifidobacteria in k-carrageenan increased the viability of bifidobacteria in yoghurt (Adhikari *et al*., 2000). 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°C from 43-44 per cent to about 50-60 per cent with better survivability in alginate beads than in k-carrageenan beads (Kebary *et al*., 1998).

Rao *et al*. (1989) developed a technology for microencapsulation of *B. longum* with cellulose-acetate-phthalate (CAP) using phase separation and coacervation method. They reported that microencapsulated *B. pseudolongum* survived the simulated gastric environment in large numbers. Kim *et al*. (1988) described a method for the preparation of stable microencapsulated lactic acid bacteria using polyvinyl-acetate-phthalate.

Encapsulated probiotics have been used into a number of applications with very promising results. These include yoghurt-covered resins, nutrient bars, chocolate bars and tablets. Stability testing of compressed tables with encapsulated probiotics has shown and unprecedented 100 per cent delivery rate, whereas in standard industry testing of tablets of compression, 50-75 per cent of the probiotics do not survive. Testing in chocolate bar has also showed a good recovery rate (Suita-Cruce and Goulet, 2001). Encapsulated *B. longum* ATCC 15696 was added to Cheddar cheese during milling of the curd, and the organism remained viable over a ripening period of 24 weeks.

Scanning electron microscopy showed that capsules containing the bacteria were relatively intact and well dispersed in the cheese matrix (Dinkar and Mistry, 1994). By monitoring the levels of acetic acid during the ripening period, they were also able to establish that the encapsulated organisms were metabolically inactive. The cells made not significant contribution to the flavor profile of the cheese, as determined by sensory evaluation.

Conclusion

Thus, problems associated with probiotics incorporation into foods are survival and stability during processing; preservation, storage and GI transient and probiotic foods should include probiotic strains at suitable level until the time of consumption. Microencapsulation has been found the most suitable and accessible technology to protect the tiny living organisms towards the fermented, non-fermented and heat-treated food product categories. Further clinical studies are required to establish the *in vivo* survivability of encapsulated probiotics for a specific health claims.

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