Curr. Issues Intest. Microbiol. (2002) 3: 39-48.

Microencapsulation of Probiotic Bacteria: Technology and Potential Applications

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Abstract

In the recent past, there has been an explosion of probiotic health-based products. Many reports indicated that there is poor survival of probiotic bacteria in these products. Further, the survival of these bacteria in the human gastro-intestinal system is questionable. Providing probiotic living cells with a physical barrier against adverse environmental conditions is therefore an approach currently receiving considerable interest. The technology of micro-encapsulation of probiotic bacterial cells evolved from the immobilised cell culture technology used in the biotechnological industry. Several methods of micro-encapsulation of probiotic bacteria have been reported and include spray drying, extrusion, emulsion and phase separation. None of these reported methods however, has resulted in the large numbers of shelf-stable, viable probiotic bacterial cells necessary for use in industry for development of new probiotic products. The most commonly reported micro-encapsulation procedure is based on the calcium-alginate gel capsule formation. Kappa-carrageenan, gellan gum, gelatin and starch are also used as excipients for the micro-encapsulation of probiotic bacteria. The currently available equipment for micro-encapsulation is not able to generate large quantities of uniform sized micro or nano capsules. There is a need to design and develop equipment that will be able to generate precise and uniform micro or nano capsules in large quantities for industrial applications. The reported food vehicles for delivery of encapsulated probiotic bacteria are yoghurt, cheese, ice cream and mayonnaise. Studies need to be done on the application of micro-encapsulation of probiotic bacteria in other food systems. The number of probiotic supplements will increase in the future. More studies, however, need to be conducted on the efficacy of micro-encapsulation to deliver probiotic bacteria and their controlled or targeted release in the gastro-intestinal tract.

Introduction

Probiotics are living microorganisms which when ingested have beneficial effects on the equilibrium and the physiological functions of the human intestinal microflora (Fuller, 1992). Probiotics have been recently defined as "live microbes which transit the gastro-intestinal tract and in doing so benefit the health of the consumer (Tannock *et al.*, 2000) differing from the earlier definitions which focused on probiotic interactions with indigenous intestinal microbes (Fuller, 1989). These definitions of probiotic bacteria generally agree that probiotic bacteria should be living organisms to confer health benefits. Probiotics have been reported to play a therapeutic role by modulating immunity, lowering cholesterol, improving lactose tolerance and preventing some cancers (Kailasapathy and Chin, 2000).

In the recent past, there has been an explosion of probiotic-based health products mostly in the form of fermented dairy products as well as dietary supplements. The markets for probiotic products and supplements are increasing worldwide (Playne, 1997). Today there are more than 70 "Bifidus"- and "Acidophilus"-containing products worldwide, including several fermented dairy products (Shah, 2001).

Viability of probiotic bacteria in a product at the point of consumption is an important consideration for their efficacy, as they have to survive during the processing and shelf life of food and supplements, transit through high acidic conditions of the stomach and enzymes and bile salts in the small intestine. The consumption of probiotics at a level of 10^8-10^9 cfu/g per day is a commonly quoted figure for adequate probiotic consumption, equating to 100 g of a food product with 10^6-10^7 cfu/g (Kebary, 1996; Lee and Salminen, 1996; Dave and Shah, 1997c). Analysis of probiotic products in many different countries has confirmed that probiotic strains exhibit poor survival in traditional fermented dairy products (Shah, 2000, Lourens-Hattingh and Viljoen, 2001). The probiotic preparations such as tablets, powders etc. may contain lower viable counts. Of the 15 feed supplements examined, viable probiotic counts varied greatly, with 3 products containing no lactobacilli at all (Gilliland, 1981), although the supplements were supposed to contain *L. acidophilus*.

Probiotic survival in products is affected by a range of factors including pH, post-acidification (during storage) in fermented products, hydrogen peroxide production, oxygen toxicity (oxygen permeation through packaging), storage temperatures, stability in dried or frozen form, poor growth in milk, lack of proteases to break down milk protein to simpler nitrogenous substances and compatibility with traditional starter culture during fermentation (Dave and Shah, 1997a, b, c; Kailasapathy and Rybka, 1997; Shah, 2000). Oxygen plays a major role in the poor survival of probiotic bacteria (Brunner *et al.*, 1993).

Providing probiotic living cells with a physical barrier against adverse external conditions is an approach currently receiving considerable interest. In the past, micro-organisms were immobilised or entrapped in polymer matrices for use in bio-technological applications. The

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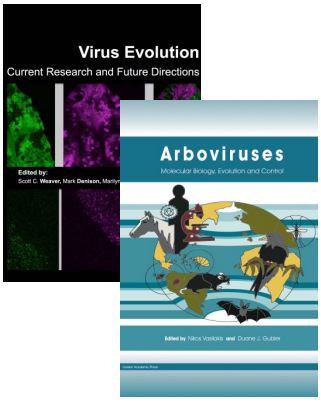

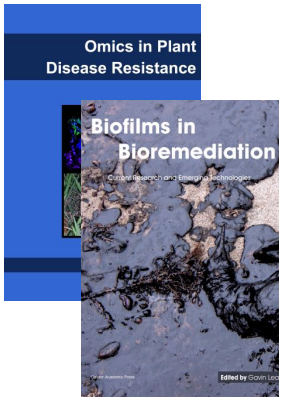

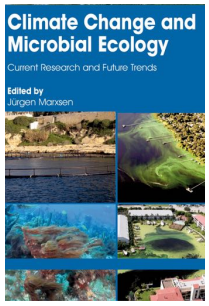

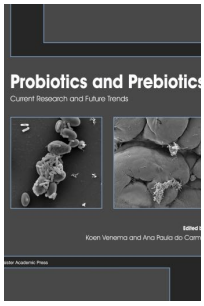

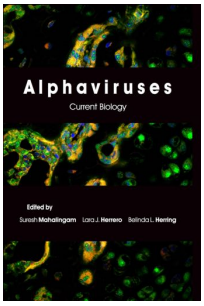

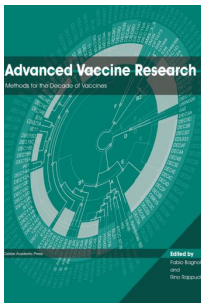

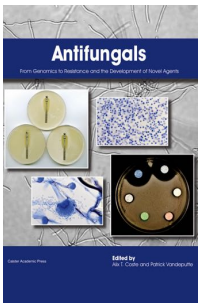

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physical retention of cells in the matrix facilitated the separation of the cells from their metabolites. As the technique of immobilisation or entrapment became refined, the immobilised cell technology has evolved into encapsulation of cells. Encapsulation tends to stabilise cells, potentially enhancing their viability and stability in the production, storage and handling of lactic cultures. An immobilised environment also conferred additional protection to lactobacilli and bifidobacterial cells during re-hydration and lyophilization (Kim *et al*., 1996).

Immobilisation and Encapsulation

Entrapment of cells in a gel matrix of alginates is the most popular system of immobilisation reported (Champagne *et al*., 1994). The terms immobilisation and encapsulation were used interchangeably in most reported literature. While encapsulation is the process of forming a continuous coating around an inner matrix that is wholly contained within the capsule wall as a core of encapsulated material, immobilisation refers to the trapping of material within or throughout a matrix. A small percentage of immobilised material may be exposed at the surface, while this is not the case for encapsulated material (King, 1995).

Encapsulation occurs naturally when bacterial cells grow and produce exo-polysaccharides. The microbial cells are entrapped within their own secretions that act as a protective structure or a capsule, reducing the permeability of material through the capsule and therefore less exposed to adverse environmental factors. Many lactic acid bacteria synthesise exo-polysaccharides, but they produce insufficient exo-polysaccharides to be able to encapsulate themselves fully (Shah, 2002).

Microencapsulation helps to separate a core material from its environment until it is released. It protects the unstable core from its environment, thereby improving its stability, extends the core's shelf life and provides a sustained and controlled release (Figure 1). The structure formed by the micro-encapsulation agent around the core substance is known as the wall. The properties of the wall system are designed to protect the core and to release it at controlled rates under specific conditions while allowing small molecules to pass in and out of the membrane (Franjione and Vasishtha, 1995; Gibbs *et al*., 1999). The capsules may range from submicron to several millimetres in size and can be of different shapes (Shahidi and Han, 1993; Franjione and Vasishtha, 1995).

Compared to immobilisation/entrapment techniques, micro-encapsulation has many advantages. The microcapsule is composed of a semipermeable, spherical, thin and strong membranous wall. Therefore the bacterial cells are retained within the microcapsules (Jankowski *et al*., 1997). More over, compared to an entrapment matrix, there is no solid or gelled core in the microcapsule and its small diameter helps to reduce mass transfer limitations. The nutrients and metabolites can diffuse through the semipermeable membrane easily. The membrane serves as a barrier to cell release and minimises contamination. The encapsulated core material is released by several mechanisms such as mechanical rupture of the cell wall, dissolution of the wall, melting of the wall and diffusion through the wall (Franjione and Vasishtha, 1995).

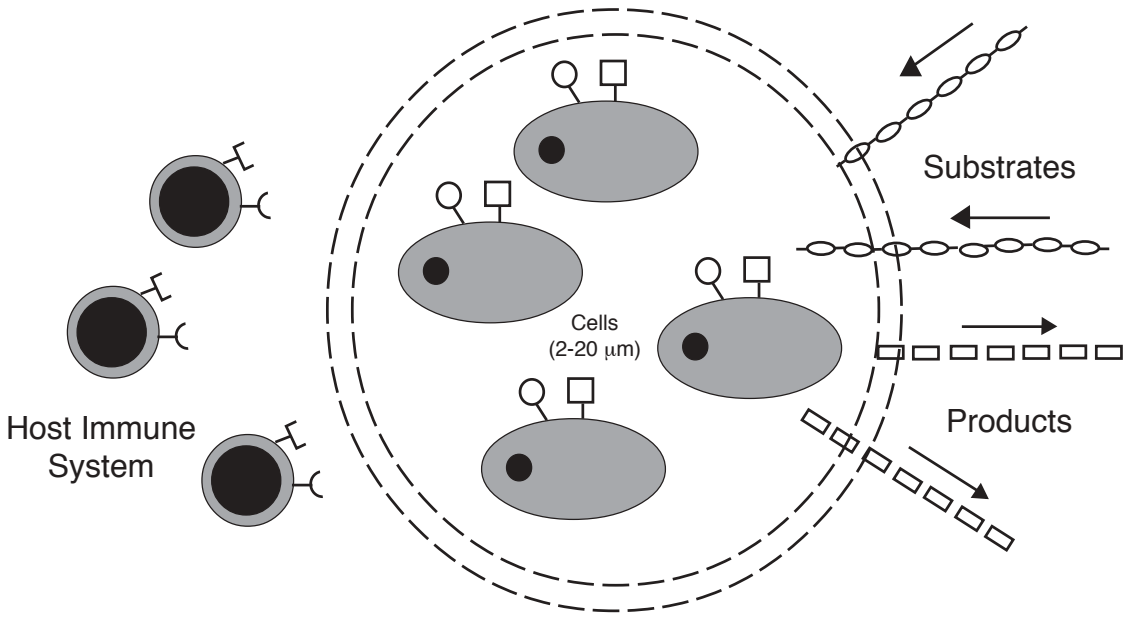

Figure 1. Principle of Encapsulation: Membrane barrier isolates cells from the host immune system while allowing transport of metabolites and extracellular nutrients. Membrane with size selective pores (30-70 kDa). Source: INOTECH Encapsulation.

Methods of Micro-Encapsulation

Spray Drying

Spray drying is the most commonly used micro-encapsulation method in the food industry, is economical and flexible, and produces a good quality product (Dziezak, 1988). The process involves the dispersion of the core material into a polymer solution, forming an emulsion or dispersion, followed by homogenisation of the liquid, then atomisation of the mixture into the drying chamber (Jackson and Lee, 1991). This leads to evaporation of the solvent (water) and hence the formation of matrix type micro capsules.

The advantage of the process is that it can be operated on a continuous basis. The disadvantage is that the high temperature used in the process may not be suitable for encapsulating probiotic bacterial cultures. However, proper adjustment and control of the processing conditions such as the inlet and the outlet temperatures can achieve viable encapsulated cultures of desired particle size distribution.

At an inlet temperature of 100°C and low outlet temperature of 45°C, *Bifidobacterium* cells were encapsulated satisfactorily to produce micro spheres with gelatinised modified starch as a coating material (O'Riordan *et al*., 2001). In this study, spray drying was found to be a valuable process for encapsulating bifidobacteria. The process of spray drying is economical, easily scaled up and uses equipment readily available in the food industry (Gibbs *et al*., 1999). A previous report indicated that survival of probiotic bacteria during spray drying decreased with increasing inlet temperatures (Mauriello *et al*., 1999). Inlet temperatures of above 60°C resulted in poor drying and the sticky product often accumulated in the cyclone and sometimes in the receiving flask. Higher inlet temperatures (>120°C) resulted in higher outlet temperatures (>60°C) and significantly reduced the viability of encapsulated bifidobacteria (O'Riordan *et al*., 2001). Other strains of probiotic bacteria have also been reported to lose viability at higher inlet temperatures (Gardiner *et al*., 2000). The spray dried encapsulated cells showed consistent coverage of cells and good average micro sphere size (>10 micro meters)(O'Riordan *et al*., 2001).

Extrusion, Emulsion and Phase Separation

Micro-encapsulation by extrusion involves projecting an emulsion core and coating material through a nozzle at high pressure. Extrusion of polymer solutions through nozzles to produce capsules is mainly reported on a laboratory scale, where simple devices such as syringes are applied. If the droplet formation occurs in a controlled manner (contrary to spraying) the technique is known as prilling (Heinzen, 2002). This is preferably done by pulsation of the jet or vibration of the nozzle. The use of coaxial flow or an electrostatic field is the other common technique to form droplets.

Mass production of beads can either be achieved by multi-nozzle systems, rotating disc atomizers or by the jet cutting technique. Centrifugal systems using either a multi-nozzle system or a rotating disc have also been developed for the mass production of microcapsules (Heinzen, 2002).

The centrifugal extrusion process is a liquid co-extrusion process utilising a nozzle consisting of concentric orifices located on the outer circumference of a rotating cylinder (Schlameus, 1995). A liquid or core material is pumped through the inner orifice and a liquid shell material through the outer orifice forming a co-extruded rod of core material surrounded by shell material. As the system rotates, the extruded rod breaks up into droplets that form capsules.

Most of the literature reported on the encapsulation of probiotic bacteria has used the emulsion technique to produce small amount of capsules. The capsules or beads are formed in a two-step procedure involving dispersion and hardening. The dispersion can be performed either by extrusion or by emulsification (Groboillot *et al*., 1994). In this method, the bacterial cells and polymer suspension is extruded through a needle or a nozzle, generating spherical droplets that fall into a hardening solution. The emulsion technique involves the dispersion of an aqueous phase containing the bacterial cells and polymer suspension into an organic phase, such as oil, resulting in a water in oil emulsion. The dispersed aqueous droplets are hardened by cooling or by addition of a gelling agent or a cross-linking agent in the case of polyacrylamide gels. Following gelation, the beads are partitioned into water and washed to remove oil. The emulsion technique results in smaller diameter beads, and is better suited to scale up applications. Residual oil in the capsules, however, may not be suitable for development of low-fat food product applications.

Materials Used as Excipients

For the encapsulation of viable bacterial cells, the materials used as excipients should be gentle and non-toxic. By far, the most commonly used bio gum for micro-encapsulation of bacterial cells is alginate. The advantages of using alginate as an encapsulating agent includes: non-toxicity, forms gentle matrices with calcium chloride to trap sensitive materials such as probiotic bacteria, the viability of bacteria is not affected during the encapsulated shelf life and the reversibility of immobilisation as gels can be solubilized by sequestering calcium ions thus releasing the entrapped cells (Sheu and Marshall, 1993; Shah and Ravula, 2000). Several gelling agents including pectate, kappa-carrageenan-locust bean gum, gellan gum and agar were tested for gel strength. The results showed that alginates formed a firmer gel with good mechanical stability and demonstrated easy release of the encapsulated bacteria when suspended in an alkaline buffer (Nicetic *et al*., 1999). When used in lactic acid fermentation, chelating agents such as lactate may cause weakening of calcium-alginate gel and may allow cell leakage (Smidsrod and Skjack-Braek, 1990).

Since alginates are a heterogenous group of polymers, with a wide range of functional properties, their success as immobilisation matrices will rely on an appropriate choice of materials and methodology for each application. Alginates with a high content of guluronic acid blocks (G blocks) are preferable for capsule formation because of their high mechanical stability, high porosity and tolerance to salts and chelating agents (Nicetic *et al*., 1999).

Micro-Encapsulation Laboratory Procedures

Most of the reported literature on micro-encapsulation of probiotic bacteria was based on small-scale laboratory procedures involving emulsion, extrusion and/or coacervation.

Among the entrapment procedures reported, cell entrapment in gelled biopolymer is commonly used with kappa-carrageenan or calcium-alginate as matrices. The main advantage of gel immobilisation is the biocompatibility; although scaling up is difficult, the beads are often permeable to cells (Lacroix et. al., 1990) and alginate containing lactic acid bacteria tends to be liquefied by lactic acid (Roy *et al*., 1987). To reduce mass transfer effects, poly-L-lysine (PLL) membrane coating of alginate beads followed by liquefaction of the alginate core was reported (Lim, 1983), however leakage of cultures from the matrix was still observed with PLL-alginate beads (Champagne *et al*., 1992).

An alternative micro-encapsulation technique that involves a single step process is interfacial polymerisation. The interfacial polymerisation technique involves the formation of an emulsion with an aqueous suspension of the cells as the discontinuous phase and an organic solvent as the continuous phase. The droplets contain the cell and the reaction is initiated when a biocompatible reagent, soluble in the continuous organic phase, is added to the emulsion.

Micro capsule formation by emulsification/interfacial polymerisation results in liquid droplets enveloped within a thin and strong membrane. Compared to the gels, the membrane should have the potential to retain bacterial cells and minimise mass transfer limitations (Groboillot *et al*., 1993). Micro-encapsulation should also reduce the volume occupied by the immobilised cells. Synthetic polymers such as nylon or cross-linked polyethyleneimine membranes are unsuitable for live cell immobilisation due to toxicity of the reagents and harsh conditions of encapsulation (Larisch *et al*., 1994).

Rao *et al*., 1989 reported a microencapsulating procedure for *Bifidobacterium pseudolongum* which involved mixing the bacterial cells with starch and suspending the powder in light paraffin oil. The mixture was stirred, a solution of cellulose acetate phthalate was added to the suspension and stirred to obtain the microspheres. The microspheres were then coated with beeswax.

Shue and Marshall (1993) reported an emulsion method to encapsulate lactobacilli in calcium alginate gels. In this method the bacterial cells were mixed with sodium alginate solution (one part of the culture concentrate with 4 parts of 3 % sodium alginate). One part of this mixture was added dropwise to 5 parts of vegetable oil containing an emulsifier such as Tween 80 (0.2%), and stirred. Calcium chloride solution (0.05M) was then added to this turbid emulsion down the sides of a beaker until the water-in-oil emulsion was broken. The formed calcium-alginate beads were collected by gentle centrifugation and washed with sterile water. Similar procedures were used to encapsulate *B. bifidum, B.infantis* and *B. longum* for incorporation in the production of Crescenza cheese (Gobbetti *et al*., 1997), encapsulating *B. bifidum* and *B. infantis* into mayonnaise and frozen milk (Khalil and Mansour, 1998; Kebary *et al*., 1998) and microencapsulating *L. acidophilus* LAMJLA1 and *Bifidobacterium* spp. BBBDBB2 for incorporation into frozen dairy desserts (Shah and Ravula, 2000).

A two-polymer micro-encapsulation reported by Khalida *et al*. (2000) used a mixture of 2% alginate solution and 2% HiMaize™ resistant starch and 1% probiotic culture cells (*L. acidophilus* and *Bifidobacterium* spp).

The encapsulation of *Lactobacillus casei* subsp. *rhamnosus* (ATCC 10863) was reported using alginate capsules that possess an interphasic membrane and a liquid core (Yoo *et al*., 1996). Compared to gels, micro capsules consist of a liquid core surrounded by a semi permeable membrane that retains the cells, reduces mass transfer limitations and minimise phage contamination (Hsu and Chu, 1992). Jankowski *et al*. (1997) developed biocompatible capsules consisting of a liquid core with calcium alginate membranes for encapsulating lactic acid bacteria. They concluded that the alginate-starch liquid core capsules offer the possibility to encapsulate lactic acid bacteria without loss of viability.

Prevost and Divies (1992) reported a double needle system to entrap lactococci in cream fermentation. In this procedure, a concentric flow of sterile sodium-alginate solution through 0.5 mm tubing was used to mix alginate cells off the nozzle into a well-mixed 0.2M calcium chloride solution. The resulting capsules contained a two-layered calcium alginate gel.

Chitosan, a water-soluble polymer (pH <6) has been used to microencapsulate *Lactococcus lactis* (Groboillot *et al*., 1993). In this study, a cross-linked chitosan membrane was formed by emulsification/interfacial polymerisation using biocompatible reagents with oil-soluble cross-linking agents at low concentrations to minimise cell contact. The anti-bacterial property of chitosan, however, limits its use as coating material in encapsulation (Sudarshan *et al*.,, 1992).

Lactococcus lactis cells were encapsulated within gelatin membrane cross-linked with toluene-2, 4 diisocyanate at an oil/water interface. Reagent toxicity was avoided by use of vegetable or silicone oil as a dispersant, and by minimising cell exposure to the water insoluble cross-linking agent during membrane formation (Hyndman *et al*., 1993). Larisch *et al*. (1994) however, observed toxic effects with solvents and reagents when *Lactococcus lactis* subspp. *cremoris* was encapsulated within poly-L-Lysine membranes formed on alginate micro spheres.

Another bio gum that has been used as an encapsulant was gellan gum. Camelin *et al*. (1993) observed that the gellan gum produced by dissolving in a citrate solution was a better entrapment matrix for temperature-sensitive cells such as mesophilic lactic acid bacteria. In this study, *B. longum* (ATCC 15707) cells were successfully immobilised using a sterilised 2.5% gellan gum solution supplemented with 0.2% citrate or metaphosphate to prepare gel beads with immobilised living cells.

In some other studies, equal volumes of bacterial suspension and kappa-carrageenan were mixed and the mixture was added drop wise to a gently stirred 0.1M

potassium chloride solution, the beads were collected by gentle centrifugation (Buyukgungor, 1992). A similar procedure for micro entrapment of *B. bifidum* and *B. infantis* was reported using kappa-carrageenan as the gelling polymer. In this procedure, one part of bacterial cells was mixed with 3% kappa-carrageenan and the mixture was then added drop-wise using a syringe into 10 parts of 3M potassium chloride solution to form the gelled beads (Kebary *et al*., 1998). Adhikari *et al*. (2000) reported an emulsion method involving kappa-carrageenan for encapsulating *B. longum* ATCC 14708 for incorporation into set yoghurt. The method used was similar to that reported earlier (Kebary *et al*., 1998), however, in this method the bacterial cell/kappa-carrageenan mix was added to vegetable oil to which Tween 80 was added as an emulsifier, and stirred. Potassium chloride solution (0.3M) was then added down the sides of a beaker to break the emulsion. The oil phase was removed from the top with an aspirator and the micro capsules were harvested from the potassium chloride solution by gentle centrifugation.

Development of Micro-Encapsulation Equipment

For immobilising living bacterial cells, the capsule size is crucial and should be carefully controlled. Depletion of nutrients like oxygen in the interior of the capsules may lead to cell death as a result of consumption of oxygen from the surrounding cells, however, it may be advantageous for anaerobic bacteria such as bifidobacteria. Small beads under controlled conditions are useful for research purposes, and also will not affect the texture of food products.

Pronova BioMedical, Norway, had developed a small bench-top capsule generator (Melvik *et al*., 1999). This electrostatic bead generator uses an electric potential to pull the droplets from a needle tip. The electrostatic potential is established between the needle feeding the mixture of alginate and bacterial cell solution and the gelling bath. Capsule size may be controlled by adjusting the magnitude of the voltage. The alginate (or other polymer solution) with cells may be fed into the instrument using a syringe pump. A magnetic stirrer placed underneath the gelling bath will keep the capsules separated during gelling. The capsule sizes range from 1mm to 400 micro meters. The capsule size depends on voltage and distance between the needle tip and the gelling bath, solution viscosity, flow rate of the solution as well as needle diameter.

A jet cutter technology was developed by Genia Lab BioTechnology, Germany, to allow production of small (<1mm) monodisperse beads with high production rates from high viscous polymer solutions (Wittlich *et al*., 1999). In this method, the fluid is pressed through a nozzle in the form of a solid liquid jet. This jet is cut into uniform cylindrical segments by a means of a rotating cutting tool. Due to the surface tension these segments form spherical beads while falling down. The diameter of the resulting bead is determined by the number of cutting wires, the number of rotations of the cutting tool and the mass flow through the nozzle, the mass flow in turn depending both on the nozzle diameter and the fluid velocity.

Laminar jet break-up technique (Seifert and Philips, 1997) is another method reported in the literature to overcome low productivity rates of capsules as well as to produce smaller particles of equal size. Based on the laminar jet break-up, a 13- nozzle system for encapsulation and immobilisation of micro-organisms is reported (Brandenberger and Widmer, 1998). This system is designed to produce mono disperse beads of calcium alginate under sterile and reproducible conditions in the range between 0.2 and 1mm. To ensure mono disperse droplets a stroboscopic light is used to optimise frequency and volume flow. An in situ cleaning of the nozzle is designed in order to guarantee several batch process cycles and productivity up to 5000 ml per hour.

A similar technology based on the principle that a laminar liquid jet is broken into equal-sized droplets by a superimposed vibration was reported (Heinzen, 2002). The polymer-cell mixture is forced into the pulsation chamber by a syringe pump. The liquid then passes through a precisely drilled sapphire nozzle and separates into equal size droplets on exiting the nozzle. These droplets then pass through an electrical field between the nozzle and the electrode resulting in a surface charge. Electrostatic repulsion forces disperse the beads as they drop into the hardening solution. The uniformity of the bead chain is seen in a stroboscope light allowing for the immediate manipulation and determination of optimal bead formation parameters. At the conclusion of the production run, the hardening solution is drained off (waste port), while capsules are retained on the filtration grid.

Most of the micro-encapsulation laboratory procedures reported involve water-in-oil emulsion technology. For example, an aqueous solution of alginate or carrageenan in vegetable oil containing Tween 80 (emulsifier) and sodium lauryl sulphate (surfactant) was used to encapsulate probiotic bacterial cells (Shue and Marshall, 1993; Adhikari *et al*., 2000; Shah and Ravula, 2000; Khalida *et al*., 2000). The bacterial cells were mixed in a solution of alginate and dropped into oil to accomplish encapsulation. The emulsifier and surfactant were added to promote capsule formation.

This technique may not be suitable for food product development application because, 1. The residual oil in the encapsulated material is detrimental to texture (Godward, 2000) and organoleptic characteristics, and may not be suitable for the development of low-fat dairy products. 2. The residual oil, emulsifier and surfactant in the encapsulated material can be toxic to live bacterial cells and may interact with sensitive food components (Godward, 2000). The resulting capsules (0.1-0.5mm in diameter) are considered to be too large and not uniform (Khalida *et al*., 2000). This can affect mouth feel and creates a coarse texture, and will therefore not be suitable for incorporation into food.

A new prototype micro-encapsulation system was developed at South West Research Institute, USA in collaboration with University of Western Sydney, Australia. This system is capable of generating micro capsules between 20 and 100 micrometers using only aqueous gelling without use of emulsifiers, surfactant or oil. This prototype consists of a feed vessel and an encapsulation

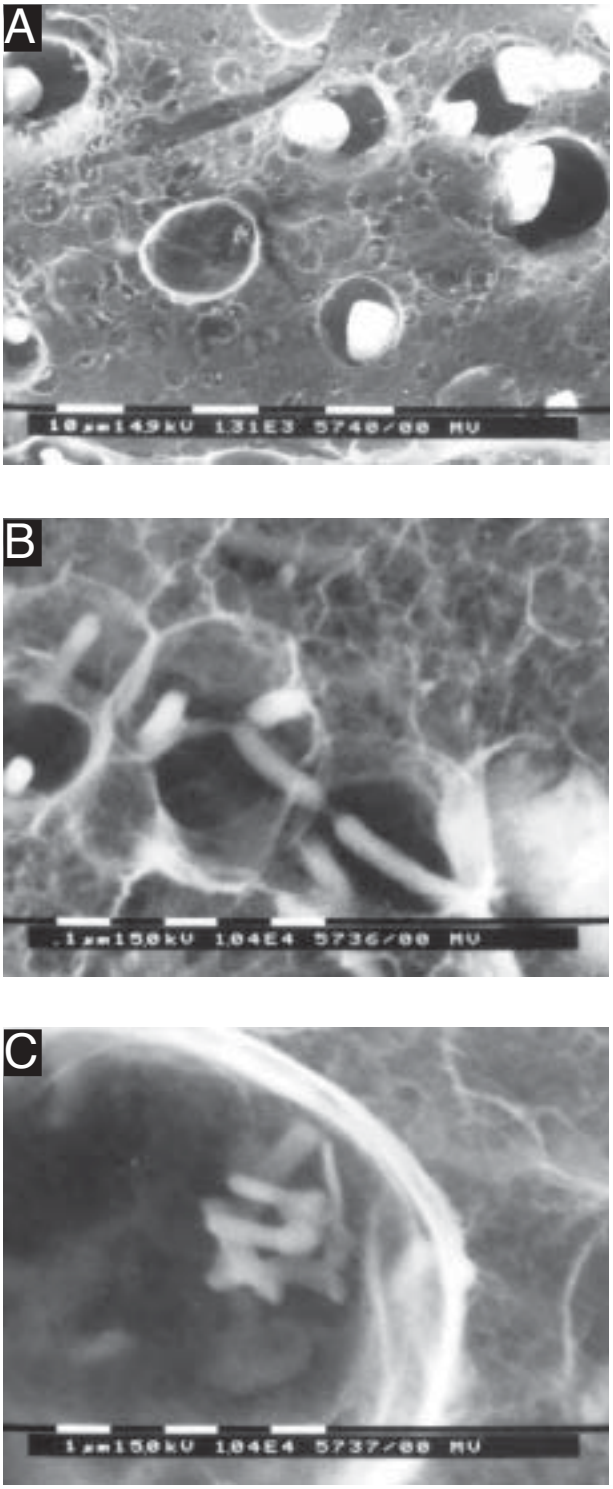

Figure 2. Section of alginate microcapsules showing: A). the starch grains in cavities, B). *L. acidophilus*, and C). *B. infantis* located in the alginate matrix.

vessel. The solution of aqueous alginate and the bacterial cells is mixed in the feed vessel and pumped on top of the encapsulation vessel which has smoother walls to allow the free flow of calcium chloride solution as a thin film, ensures uniform wetting and prevents channelling.

A rotating spray device located on top of the encapsulation vessel operated by a variable speed motor sprays the solution into fine droplets. As the alginate droplets are sprayed onto the calcium chloride falling film, they will gel with calcium and form capsules. The semi-gelled capsules will fall into calcium chloride solution at the bottom of the encapsulation vessel and will remain there for further hardening. The calcium chloride solution will be recycled and the capsules will be filtered using suitable sterile nylon filters. Nitrogen gas replaces air while the equipment is in operation. This helps oxygen sensitive probiotic bacteria such as bifidobacteria to survive during exposure to high levels of oxygen (unpublished data). Encapsulated probiotic bacteria (*L. acidophilus* and *B. infantis*) in a calcium-alginate-Hi Maize™ starch (a prebiotic) matrix produced by this encapsulation prototype are shown in Figure 2 (A, B, C).

Applications of Micro-Encapsulation of Probiotic Bacteria

The early literature on immobilised culture technology for biotechnological and food applications is summarised in Table 1. Early reviews on the immobilisation of bacterial cells for application in the food industry (Groboillot *et al*., 1994) and in dairy technology (Champagne *et al*., 1994) cover most of the immobilised culture technologies.

Shue and Marshall (1993) reported 40% more survival of lactobacilli during freezing of ice milk when they were entrapped in calcium alginate than free non-entrapped cells. Thus addition of entrapped probiotic bacteria to frozen dairy deserts could be advantageous in providing desirable marketing and health benefits. These findings encouraged studies on the incorporation of encapsulated probiotic bacteria in frozen desserts (Shue *et al*., 1993) and ice creams.

Kebary *et al*. (1998) also reported that entrapment of *Bidobacterium bifidum* and *B. infantis* in alginate or kappa-carrageenan beads significantly improved ($P \leq 0.05$) their survival in frozen ice milk throughout the storage period (-20°C for 10 weeks) from about 43-44% to about 50-60% and the bifidobacteria survived better in beads made from alginate than those made from kappa-carrageenan. The numbers of bifidobacteria that survived at the end of 10 weeks of frozen storage in ice milk made with added entrapped bifidobacteria were higher ($6.1 – 8.9 \times 10^7$ cfu/g) than that should be present to achieve the therapeutic effects of bifidobacteria ($>10^6$ cfu/g).

L. acidophilus and *Bifidobacterium* spp. were encapsulated in calcium alginate beads, freeze dried and incorporated into a mix for making frozen fermented dairy desserts prior to freezing (Shah and Ravula, 2000). The frozen desserts were stored at -20°C for 12 weeks. It was observed that counts of *L. acidophilus* and Bifidobacterium spp in the frozen desserts containing encapsulated bacteria were higher at the end of 12 weeks storage than in those

Table 1. Immobilisation/ Encapsulation of cells for food/biotechnological application

Culture	Technique/Mechanism	Product	Reference
B. bifidum, B. infantis	Calcium alginate	Mayonnaise	Khalil and Mansour, 1998
L. paracasei	Milk fat	Cheddar cheese	Stanton *et al.,* 1998
Enterococcus faecium	Milk fat	Cheddar cheese	Gardiner *etal., 1998*
B. bifidum, B. adolescentis	Cream	White brined cheese	Ghoddusi and Robinson, 1998
B. bifidum, B. infantis, and *B. longum*	Calcium alginate gels	Crescenza cheese	Gobbeti *et al.,* 1997
L. lactis subspp. *lactis*	k-Carrageenan and locust bean gum	Fresh cheese	Sodini *et al., 1997*
L. casei	Liquid core alginate capsule	Lactic acid	Yoo *et al*., *1996*
Lactobacilli	Calcium alginate	Frozen dessert	Sheu and Marshall*, 1993*
Lactococci	Calcium alginate	Cream	Prevost and Divies, 1992
L casei	k-Carrageenan and locust bean gum	Yoghurt	Lacroix *et al.,* 1990

containing non-encapsulated bacteria. This study, therefore, shows that encapsulation of probiotic bacterial cells in calcium alginate provided protection to these organisms in fermented frozen dairy desserts.

Godward (2000), however, studied the survival of probiotic bacteria including *L. acidophilus* and *Bifidobacterium* strains in ice creams stored for six months at -20°C and found that there is no significant difference between the survival of encapsulated probiotic bacteria and the free cells. The high survival of free cells in ice cream would be due to protection to the cells by the high total solids (>10% fat) in the ice cream mix, making ice cream a suitable food for delivering probiotics to consumers. This shows that the probiotic bacteria are comparatively more prone to cell death in acidic environment than during freezing. The pH of the frozen dairy desserts as reported by Shah and Ravula (2000) was ≤ 4.5, however, the pH of fermented ice cream as reported by Godward (2000) was 5.5-6.0. This highlights the need for encapsulation or enteric coatings for probiotic bacteria to survive the human gastric juice secretion in the stomach, where the pH can be as low as 2. Shah and Ravula (2000) also reported that the encapsulated probiotic bacteria also survived in acidic conditions (at pH 2.5) in calcium alginate beads, however, the organisms were released in the presence of bile. These studies suggest that the encapsulated probiotic bacteria incorporated into fermented, frozen dairy desserts could survive the low pH of the product and possibly in acidic conditions such as is encountered in the human stomach, and could be delivered in the intestine.

Probiotic bacteria must be able to colonise the gut and therefore survive gastric acidity, bile salts, enzymes, toxic metabolites, bacteriophages, antibiotics and anaerobic conditions. Post-production acidification, in yoghurt, due to the decrease in pH after fermentation and during storage at refrigerated temperatures causes major cell death of probiotic bacteria (Kneifel *et al*., 1993; Lourens-Hattingh and Viljoen, 2001). Micro-encapsulation and added prebiotic substances in probiotic products have been used satisfactorily to increase the survival of probiotic organisms in high acid fermented products such as yoghurts.

Khalida *et al* (2000) reported a modified method involving calcium-alginate-starch micro-encapsulation. In this study the encapsulated *L. acidophilus* and *Bifidobacterium* spp were incorporated and set yoghurt was made and stored for 8 weeks at 4°C. This study demonstrated that survival of encapsulated cultures of *L. acidophilus* and bifidobacteria showed a better survival over an 8 weeks storage period compared to the survival of free cells.

Adhikari *et al*. (2000) reported a significant difference between the viable counts of microencapsulated *B. longum* compared to free cells in set yoghurt at 4.4°C for 30 days. Godward (2000) observed that micro-encapsulation of *L. acidophilus* and *Bifidobacterium* spp enhanced the survival in yoghurt. These studies clearly show that acid labile probiotic bacteria such as bifidobacteria are protected by micro-encapsulation.

Khalil and Mansour (1998) added *B. bifidum* and *B. infantis* either as free cells or encapsulated to prepare a probiotic mayonnaise. The viability of free cells disappeared after 2 weeks, but encapsulated *B. bifidum* survived well for 12 weeks and *B. infantis* survived for 8 weeks. The mayonnaise in this study had a pH of 4.42 (due to the presence of acetic acid) throughout the storage period of 4 weeks. This study also confirms the protective capability of micro-encapsulation against harsh acid conditions. Therefore it may be concluded that the survival of encapsulated probiotic bacteria would not be decreased by low pH value of yoghurt or human gastric juice. These reports show that micro-encapsulation will be useful for ensuring high number of cells survive the transit through the gastro-intestinal tract, particularly the acidic stomach and delivery to the lower part of the intestine in a viable state to colonise and confer beneficial probiotic effects.

The pH of cheese, especially Cheddar cheese, is relatively high (pH=5.5) as a fermented product, hence it is an advantage over more acidic conditions present in the yoghurt. The matrix of the cheese, greater buffering capacity, and the high fat content may help protect the cells from enzymatic degradation and low pH during passage through the acidic stomach (Stanton *et al*., 1998; Playne, 2002). The Valio company of Finland markets probiotic cheese (Gefilus Swiss and Edam). It has been reported that the probiotic organism *Lactobacillus* GG (LGG) grew at the same rate as non-starter lactic acid bacteria in these cheeses, and in ripened cheeses, the level of LGG was > 10^7 cfu/g which was maintained during the shelf life of the cheese. Daily consumption of only 4-6 slices (30g) of these cheeses was adequate for faecal recovery of the strain in 7 human volunteers (Playne, 2002).

Godward (2000) observed that in Cheddar cheese, free probiotic cells (*L. acidophilus* and *B. infantis)* survived better than encapsulated cells, possibly due to the build up of metabolites such as organic acids within the calcium-alginate capsules hence causing cell death. It is possible

that the dense matrix of Cheddar cheese is not conducive for free exchange of metabolites and nutrients to and from the entrapped capsules. These studies demonstrate that micro-encapsulation may not be necessary for increasing the viability of probiotic bacteria in high pH, hard cheeses such as Cheddar. This may not be the case, however, in acidic, fresh, low pH type cheeses such as cottage cheese.

Legislation, especially in the USA, allows probiotic supplements under Dietary Supplement Health and Education Act of 1994. Probiotic supplements are available in different forms, the two most popular being capsules and freeze dried powders. Probiotic strains of *L. acidophilus* 50 ME are sold as micro-encapsulated by Institut Rosell/ Lallemand The Americas, Montreal, Canada (www.lallemand.com). Probiocap ™ (micro encapsulated *L. acidophilus* 50 ME in an hydrophobic matrix) marketed by this company claims to have increased tolerance to gastric juices, improved survival during tableting, enhanced temperature resistance during food processing and extended shelf life at room temperatures. Cerbios-Pharma (www.cerbios.ch) markets Cernivet® LBC ME 10, is a pelletable microbial feed additive for the stabilisation of the intestinal microflora. This product contains micro encapsulated probiotic strain of *E. faecium* SF 68. Nutraceutix (www.nutraceutix.com) offers a number of probiotic bacteria in the form of tablets (Live Bac®) as dietary supplements as well as ingredient for formulating nutraceuticals, sports nutrition products, and animal feed supplements. Chr Hansen (www.chbiosystems.com) markets Probio-Tec® capsules for innovative probiotic product solutions for dietary supplements and infant formulas. The Jintan capsule Technology (www.jintanworld.com) manufactures encapsulated probiotic bifidobacteria with enteric function (Bifina Tablet). Biogaia and Chr. Hansen A/S Denmark provides Life Top™ capsules that contain a minimum of 100 million active, health promoting *Lactobacillus reuteri.*

Geneflora™ manufactured and sold by America's Bio-Plus Corporation (www.yeastbuster.com) is a robust friendly intestinal microflora, classified by the USDA as a probiotic. It contains encapsulated "Lactobacillus sporogenes" as probiotic and fructo-oligosaccharides as prebiotic. The product is claimed to control candida and yeast infections. Bio-Three tablets are being marketed by Japanese TOA Pharmaceutical Co. Ltd (E-Mail: toabio3@blue.ocn.ne.jp). These tablets contain a mixture of three probiotic organisms: *Enterococcus* T-110, *Clostridium butyricum* TO-A and *Bacillus. mesentericus* TO-A. It is claimed that a symbiosis of these 3 bacteria strongly inhibits harmful bacteria in the intestine, facilitates proliferation of bifidobacteria and normalises intestinal microflora.

Conclusions and Future Trends

The technology of micro-encapsulation has developed from a simple immobilisation or entrapment to sophisticated and precise micro capsule formation. The advances in this field have been tremendous with nutraceuticals and food ingredients, however, as to the micro-encapsulation of live probiotic bacterial cells, the technology seems to be not well developed. Probiotic therapy (or microbial intervention) is based on the concept of healthy gut microflora. The delivery of viable micro encapsulated probiotic bacteria will become important in the near future.

Micro-encapsulation will assume importance in delivering viable strains of probiotic bacteria in large numbers to consumers. It will be used as a tool to co-encapsulate both prebiotic ingredients and probiotic bacteria within the same capsule to enhance growth and multiplication of these bacteria through symbiotic effects when they are released in the gastro-intestinal tract.

In the future multiple-delivery may be developed, such as co-encapsulating prebiotics and probiotics as well as nutraceuticals, thus a new area of more complex nutritional matrices will need to be investigated.

In the food processing industry, preservation and storage, and micro-encapsulation will increasingly play a role to protect the viability and enhance the survival of bacteria against adverse environmental conditions. New food regulations may specify labelling including the strain and the number of viable probiotic bacteria at the end of shelf life of a food or supplement claimed to be probiotic. Studies (clinical data) will need to be conducted on the effect of encapsulation on the safety of probiotic bacteria.

Fermented and non-fermented dairy, cereals, meat small goods, sous-vide products as well as prepared home meal solutions could become food vehicles using micro-encapsulation technology to protect probiotic bacteria as a means of delivering large quantities to consumers.

In the health food industry, capsules, tablets, suspensions, creams and powders will be increasingly using micro-encapsulation technology for direct consumption and for external application of probiotics. They will increasingly be used to treat patients with medical problems. The technology of micro-encapsulation, however, needs to be developed with more precise machinery, capsule and better delivery systems. Nano-encapsulation may assume importance in the near future to develop designer probiotic bacterial preparations that could be delivered to certain parts of the gastro-intestinal tract where they interact with specific receptors. These nano-encapsulated designer probiotic bacterial preparations may act ac *de novo* vaccines, with the capability of modulating immune responses. Improved techniques need to be developed to track these micro- or nano-encapsulated probiotic bacterial cells for their delivery, persistence, sustained release and immune enhancing effects in the gastro-intestinal system. More in vivo studies should be conducted using human subjects to confirm the efficacy of micro or nano encapsulation in delivering probiotic bacteria and their controlled release in the gastro-intestinal system.

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