Chapter 10
Encapsulation of Probiotics for use in Food Products

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10.1 Probiotics From Research to Consumer

The history of the role of probiotics for human health is one century old and several definitions have been derived hitherto. One of them, launched by Huis in't Veld and Havenaar (1991) defines probiotics as being "mono or mixed cultures of live microorganisms which, when applied to a man or an animal (e.g., as dried cells or as a fermented product), beneficially affect the host by improving the properties of the indigenous microflora". Probiotics are living microorganisms which survive gastric, bile, and pancreatic secretions, attach to epithelial cells and colonize the human intestine (Del Piano et al. 2006). It is estimated that an adult human intestine contains more than 400 different bacterial species (Finegold et al. 1977) and approximately 10^{14} bacterial cells (which is approximately ten times the total number of eukaryotic cells in the human body). The bacterial cells can be classified into three categories, namely, beneficial, neutral or harmful, with respect to human health. Among the beneficial bacteria are *Bifidobacterium* and *Lactobacilli*. The proportion of bifidobacteria represents the third most common genus in the gastro-

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N.J. Zuidam and V.A. Nedović (eds.), *Encapsulation Technologies for Active Food Ingredients and Food Processing*,
DOI 10.1007/978-1-4419-1008-0_10,

intestinal tract, while *Bacteroides* predominates at 86% of the total flora in the adult gut, followed by *Eubacterium*. Infant-type bifidobacteria *B. bifidum* are replaced with adult-type bifidobacteria, *B. longum* and *B. adolescentis*. With weaning and aging, the intestinal flora profile changes. Bifidobacteria decrease, while certain kinds of harmful bacteria increase. Changes in the intestinal flora are affected not only by aging but also by extrinsic factors, for example, stress, diet, drugs, bacterial contamination and constipation. Therefore, daily consumption of probiotic products is recommended for good health and longevity. There are numerous claimed beneficial effects and therapeutic applications of probiotic bacteria in humans, such as maintenance of normal intestinal microflora, improvement of constipation, treatment of diarrhea, enhancement of the immune system, reduction of lactose-intolerance, reduction of serum cholesterol levels, anticarcinogenic activity, and improved nutritional value of foods (Kailasapathy and Chin 2000; Lourens-Hattingh and Viljoen 2001; Mattila-Sandholm et al. 2002). The mechanisms by which probiotics exert their effects are largely unknown, but may involve modifying gut pH, antagonizing pathogens through production of antimicrobial and antibacterial compounds, competing for pathogen binding, and receptor cites, as well as for available nutrients and growth factors, stimulating immunomodulatory cells, and producing lactase (Kopp-Hoolihan 2001).

Probiotics can be delivered commercially either as nutritional supplements, pharmaceuticals or foods. A large number of probiotic products are available in the market in the form of milk, drinking and frozen yoghurts, probiotic cheeses, ice-creams, dairy spreads and fermented soya products. Also, special freeze-dried pharmaceutical dietary preparations are available in the form of tablets, but the marketing as a pharmaceutical product requires long, complex and costly research, and a demonstration of a well-defined therapeutic target. Together with prebiotics, probiotics are often consumed as functional foods, demonstrated to be effective for the treatment or control of several diseases. Prebiotic substances, such as lactulose, lactitol, xylitol, inulin and certain non-digestive oligosaccharides, selectively stimulate the growth and activity of, for example, bifidobacteria in the colon (Zubillaga et al. 2001). Most widely and commercially used probiotic species are *Lactobacillus* (*L. acidophillus, L. casei, L. fermentum, L. gasseri, L. johnsnli, L. lactis, L. paracasei, L. plantarum, L. reuteri, L. rhamnosus, L. salivarius*), *Bifidobacterium* (*B. bifidum, B. breve, B. lactis, B. longum*), *Streptoccocus* (*S. thermophilus*) species, yeasts and molds (*Saccharomyces boulardii*). The presence of a specific enzyme, the fructose-6-phosphate-phospoketolase (F6PPK) in bifodobacteria, is the main criteria to distinguish them from Lactobacillus.

International standards (e.g., from the International Dairy Federation) require that products claimed to be ‘probiotic products’ contain a minimum of 10^7 viable probiotic bacteria per gram of product or 10^9 cells per serving size when sold, in order to provide $10^{6–8}$ cells/g feces. However, many products failed to meet these standards when they are consumed. This is due to death of probiotics cells in food products during storage, even at refrigerating temperatures. Consequently, industrial demand for technologies ensuring stability of bifidobacteria in foods remains strong, which leads to the development of immobilized cell technology to produce probiotics with increased cell resistance to environmental stress factors (Doleyres and Lacroix 2005).

This chapter will briefly review the isolation and selection of probiotic strains, and then focus on the use of several microencapsulation techniques to protect probiotics. The use of these microencapsulates in several food applications and their future developments are then discussed.

10.2 Isolation and Selection of Probiotic Strains

The isolation of potential probiotic strains proceeds from animal or human planktonic flora, or adhesive bacteria (adhered on the surface of the epithelial cells and interacting with the intestinal mucosa and surfactants). This can be achieved by preendoscopic biopsies or brushing. The procedure of brushing is more physiological, less invasive, requires optimal intestinal preparation and permits the withdrawn of almost complete planktonic flora. After a strain has been isolated, and purified to obtain a pure culture, it must be taxonomically classified. The identification of a strain is performed by comparison of rDNA gene sequences with those available in the GeneBank database. The minimum DNA genomic similarity of 70% and a 16 S rRNA sequence similarity of 97% are required to recognize a probiotic strain. After taxonomic classification, growth curves are developed and duplication parameters are determined for the specific strain. The presence of plasmid DNA is also assessed during the preliminary stage in order to obtain information on the genomic stability of the strain. As a general rule, the presence of plasmids is not a reason to discard the strain as a potential probiotic, but the role of this extra-chromosomal DNA in establishing phenotypes relevant for the technological and probiotic properties must be assessed (Del Piano et al. 2006).

A probiotic strain must be resistant to stomach and upper intestine microenvironment, to be able to reach the colon and be effective by conferring health benefits to the host. Therefore, in vitro studies are conducted to test the survival of a potential probiotic strain to gastric, bile or pancreatic juices. The survival of a strain depends on both strain characteristics and intestinal juice type (simulated gastric, bovine or pig bile and various types of animal pancreatic extracts).

Except the stability, the safety of a novel and existing starter, and probiotic cultures must be evaluated. There was a constant requirement for antibiotic resistant probiotics in the past few decades. This, on the other hand, led to the prevalence of multi-drug resistant strains that caused diseases in humans. The establishing of a safety profile implies determination of strain resistance to a wide variety of common classes of antibiotics and subsequent confirmation of non-transmission of drug resistance genes or virulence plasmids. Ideally, probiotic bacteria should exhibit tolerance to antimicrobial substances, but should not be able to transmit such resistance to other bacteria (Charteris et al. 2000; Kheadr et al. 2004; Moubareck et al. 2005). Although studies on safety of probiotics are necessary, in general most of *Lactobacillus* and *Bifidobacterium* strains are recognized as safe and have long history of safe use in foods or present in normal human intestinal microflora. Cases of infection pathologies or allergic reactions caused by probiotics or food substances employed for their processing are very rare.

Since probiotic bacteria are very sensitive to the environmental factors, stability tests are a prerequisite to define conditions under which they should be produced and stored. Stability of probiotics depends on many factors, including the genus, species, strain biotype and the formulation, as well as parameters such as temperature, water, pH, osmotic pressure, mechanical stresses and oxygen. Especially, the viability of lactic acid bacteria is jeopardized after freezing. Therefore, a strain must be tested on growth conditions during fermentation (alkali used to neutralize pH), harvesting conditions (cell washing, medium in which cells are re-suspended after concentration) and freezing conditions (cryoprotectants, freezing temperature, rate, duration).

Last but not least, the health benefits of potential probiotics strains should be assessed. Some potential health benefits, ranging from maintenance of normal intestinal flora to anti-cancer effects, have already been mentioned in the previous section. However, such benefits might be very strain-specific, are relatively small (compared to drugs) and may be affected by the food matrix. Long-term clinical studies with many people are therefore required to get fully proven health effects, especially when people are generally healthy.

10.3 Microencapsulation Technology for Probiotics

10.3.1 Protection Needs of Probiotics

During the time from processing to consumption of a food product, probiotics in that food product need to be protected against the following:

- Processing conditions, like high temperature and shear.
- Desiccation if applied to a dry food product.
- Storage conditions in the food product on shelf and in-home, like food matrix, packaging and environment (temperature, moisture, oxygen).
- Degradation in the gastrointestinal tract, especially the low pH in stomach (ranging from 2.5 to 3.5) and bile salts in the small intestine.

Microencapsulation technologies have been developed and successfully applied to protect the probiotic bacterial cells from damage caused by the external environment at the conditions mentioned above.

Encapsulation technology is widely used for various food applications such as stabilizing food compounds, controlling the oxidative reactions, sustained or controlled release of active ingredients (probiotics, minerals, vitamins, phytosterols, enzymes fatty acids and antioxidants), masking unpleasant flavors and odors, or to provide barriers between the sensitive bioactive materials and the environment (see other chapters of this book). Encapsulation technology is based on packing solid, fluid or gas compounds in milli-, micro- or nano-scaled particles which release their contents upon applying specific treatments or conditions (e.g., heating, salvation, diffusion and pressure). Sealed capsules are coated with semipermeable, spherical, thin, strong membrane around the solid or liquid core. A coating can be

designed to open in specific areas of the human body and microcapsules can gradually release active ingredients. For engineering probiotic containing capsules, a coating is usually employed which can withstand acidic conditions in the stomach and bile salts from the pancreas after consumption. In this way, the protection of the biological integrity of probiotic products is achieved during gastro-duodenal transit, which is a prerequisite for delivery of a high concentration of viable cells to the jejunum and the ileum. Probiotics should ideally be released in segments of the gastrointestinal tract where Peyer's patches and other mucosa-associated lymphatic tissues are found that are said to play a critical role in immunostimulation (Rescigno et al. 2001). Since encapsulates should provide protection of sensitive microorganism against harsh conditions in the gut environment, the produced particles should be tested on swelling, erosion, disintegration in simulated gastric/intestinal fluids prior to industrial and real-life applications. Another purpose of microencapsulation of probiotic bacteria is to stabilize them, that is, to ensure prolonged viability during storage. The so-called stabilization of microorganisms means providing metabolic activity after storage and intake by a new host (Viernstein et al. 2005). An average rate loss found for sophisticated formulations under excellent storage conditions is one log unit of cell number reduction per year, which still means a loss of 90% per year.

There are two main problematic issues when considering microencapsulation of probiotics: (1) the size of probiotics (between 1 and 5 μm diameter) which immediately excludes nanotechnologies, and (2) difficulties to keep them alive. The most common techniques currently used for microencapsulation of probiotics will be presented in this section (Sect. 10.3) and their application in food products in Sect. 10.4.

10.3.2 Spray-Drying

Microencapsulation by spray-drying is a well-established technique suitable for large-scale, industrial applications: a liquid mixture is atomized in a vessel with a nozzle or spinning wheel and the solvent is then evaporated by contacting with hot air or gas. The resulting particles are collected after their fall to the bottom. Spray-drying is probably the most economic and effective drying method in industry. It can be used for dehydration of materials and/or encapsulation. However, to our best knowledge spray-drying has not been developed commercially for probiotics for food use yet, because of low survival rate during drying of the bacteria and low stability upon storage. The conventional procedure requires exposing of cells to severe temperature and osmotic stresses due to dehydration, which results in relatively high viability and activity losses immediately after spray-drying and most likely also affects storage stability. Main parameters that affect these include the following:

Type of strain: One strain survives spray-drying much better than the other. Preferably stationary phase cultures should be used (Corcoran et al. 2004).

Drying temperature: The logarithmic number of probiotics decreases linearly with outlet air temperature (in the range of 50–90° C) of the spray-dryer (Brian and Etzel 1997; Chavez and Ledeboer 2007), and to a lesser extent with the inlet air

temperature (typically in the range of 150–170° C). An optimal outlet air temperature might be as low as possible (using a low feed rate, also allowing low inlet air temperatures like 80° C); however, one should take care that the powder obtained has been dried sufficiently at such low temperature conditions. Alternatively, a second drying step in, for example, a fluid bed (Meister et al. 1999) or vacuum oven (Diguet 2000; Chavez and Ledeboer 2007) might be applied.

Drying time: The shorter the heating time, the better the viability of probiotics. Optimal drying time, however, is affected by the droplet size of the atomized liquid, which is influenced by viscosity and flow rate of the feed solution.

Type of atomization: High shear must be avoided during atomization, and the air pressure applied might also influence the droplet size and thus the optimal drying time.

Carrier material: Typically a mixture of about 20% (w/v) (dairy) proteins and/or carbohydrates are used, which may be in the glassy state at storage temperatures to minimize molecular mobility and thus degradation. Examples include skim milk powder (SMP), non-fat dry milk solids (NFDM), soy protein isolates, gum arabic, pectin, (modified) starch, maltodextrin and sugars.

Osmotic, oxidative and mechanical stresses should be minimized, during both spray-drying and rehydration. Antioxidants and osmoprotectants might be included in the carrier material. Furthermore, the use of 'pre-stressed' bacteria may improve survival. Desmond et al. (2001) found that heat-adapted (52°C for 15 min) or salt-adapted (0.3 M NaCl for 30 min) *Lactobacillus paracase*i had a, respectively, 18-fold or 16-fold greater viability upon spray-drying than controls.

Storage conditions: Survival of probiotics is optimal at low water activity (<0.25) and low temperatures. Oxygen and light might be detrimental, so a nitrogen or vacuum-sealed package with a proper barrier function should be selected.

Unfortunately, the conditions need to be optimized for each different type of probiotic strain, and a good survival upon spray-drying may not indicate a good survival upon storage in a spray-dried form.

Picot and Lacroix (2004) spray-dried fresh and freeze-dried bifidobacteria in the presence of an o/w emulsion composed of anhydrous milk fat and an aqueous solution of 10% heat-denaturated whey protein isolate. This resulted in the production of water-insoluble microcapsules (<100 μm). However, the viability of the probiotics was low and slightly better results were obtained in the absence of the milk fat (26 or 1.4% survival for fresh *Bifidobacterium breve* R070 and *Bifidobacterium longum* R023, respectively, in the absence of fat; the experiments with freeze-dried ones resulted in survival rates <1%). The authors used a relatively high outlet temperature of 80° C, which might be the cause of the low survival found upon spray-drying. Another reason for the low survival might be the sensitivity of their probiotics towards the spray-drying process. The authors claim higher storage stability in yoghurt (+2.6 log cycles after 28 days at 4° C) and survival in gastrointestinal (GI) tract of the encapsulated probiotics (+2.7 log cycles) compared to free ones.

Crittenden et al. (2006) also spray-dried probiotics in the presence of an o/w emulsion, but combined this with Maillard reaction products between protein and carbohydrates to improve film-forming and oxygen-scavenging properties of the shell. First, emulsions were prepared of canola vegetable oil, caseinate, fructo-

oligosaccharides (a prebiotic), dried glucose syrup or resistant starch, and heated to 98° C for 30 min to promote Maillard reactions. The emulsion was then cooled to 10° C, probiotics were added and finally this mixture was spray-dried. Final formulations of the dried powders were 8% (w/w) probiotics, 32% (w/w) oil, 20% (w/w) caseinate, 20% (w/w) fructo-oligosaccharides and either 20% (w/w) dried glucose syrup or 20% (w/w) resistant starch. The encapsulated probiotics were more stable upon storage at 25° C and 50% relative humidity than non-encapsulated ones. The encapsulated probiotics were also more stable in in vitro gastrointestinal tract conditions.

10.3.3 Freeze- or Vacuum-Drying

Freeze-drying is performed by freezing probiotics in the presence of carrier material at low temperatures, followed by sublimation of the water under vacuum. In this way, water phase transition and oxidation are avoided. The addition of cryoprotectants helps to retain probiotic activity upon freeze-drying and stabilize them during storage. Many investigators have used SMP as the major drying medium, but other compounds like fructose, lactose, mannose, monosodium glutamate, sorbitol (Champagne et al. 1991; Carvalho et al. 2002, 2003, 2004a, b), trehalose (Garcia De Castro et al. 2000), 30% maltodextrin (Brian and Etzel 1997) and a mixture of 20% soy protein isolate and 20% maltodextrin (Chavez and Ledeboer 2007) have also been used as protective additives in recent investigations. The obtained dried mixture can be grounded (Picot and Lacroix 2003) and the final particles are of a wide size distribution and with a low surface area.

Freeze-dried probiotics are well stable upon storage, especially at low temperatures and in an inert atmosphere (nitrogen or vacuum). In general, the choice of optimal water content (in the order of 3–8%) is a compromise between high survival rates immediately after drying (more survival at higher water contents) and low inactivation upon storage (more survival at low water contents although not necessarily at 0%). The decrease in survival of freeze-dried bacteria under vacuum may follow first-order kinetics and the rate constants can be described by an Arrhenius equation (King et al. 1998). Extrapolation from results obtained at higher temperatures allows one to predict the degradation at any selected temperature. Based on the study of King et al. (1998) one can calculate that at 70, 60, 50, 20 and 4° C, a 50% reduction in cell viability of freeze-dried *Lactobacillus acidophilus* in originally 4.15% glycerol, 10% NFDM and 0.53% $CaCO_3$ and with a final moisture content of 3% is obtained after 0.2 h, 50 h, 9.6 days, 5.2×10^6 days and 1.3×10^{10} days, respectively. The Arrhenius relationship might be affected by phase transition (if any) and atmosphere (oxidation by oxygen may not follow first-order kinetics). Maybe water content will play a role as well.

Not much is known about the rehydration medium. When probiotics suspended in water are freeze-dried, the rehydration medium has a considerable effect on viability (Champagne et al. 1991). The situation is more complex when a better drying medium has been used. The rehydration medium must be free of RNase and

probably near a neutral pH. Different temperature effects have been reported, depending on the type of strain. It has been recommended to rehydrate the culture back to the volume it had prior freeze-drying. Long rehydration periods might be detrimental as the bacteria themselves might form inhibitory compounds.

Unfortunately, freeze-drying is a very expensive technology [about 4–7 more expensive than spray-drying (Chavez and Ledeboer 2007)]. However, freeze-drying is one of the least harmful drying methods of probiotics and is therefore probably most often used to dry probiotics, also as a standard to compare with other drying techniques. Most freeze-dried probiotics only provide stability upon storage and not or limited in the gastrointestinal tract. An exception might be freeze-dried probiotics from Cell Biotech in Korea, which are called Duolac™ (http://www.cellbiotech.com/sub06/img/duolac.pdf). Bacteria with a soy protein coating (most likely made by a precipitation process prior freeze-drying) get a further polysaccharide coating (= dual coating) and are then freeze-dried in the presence of cryoprotectant. After grinding, the particle sizes are around 125–250 μm. Cell Biotech claims that the coating material shrinks and coagulates together at stomach pH to protect lactic acid bacteria. The coating material dissolves in the small intestine due to its neutral pH conditions, but it should still protect the bacteria for bile salts.

Some years ago, a new starch-based technology for probiotic microencapsulation was developed by VTT Biotechnology (Myllarinen et al. 2000, using freeze-drying; O'Riordan et al. 2001, using spray-drying). Both steps, the bacterial production and their encapsulation were preformed in one batch process (Myllarinen et al. 2000). Starch is a dietary component, having an important role in the colonic physiology. Starch consists of two types of molecules, amylose and amylopection (see Chap. 3). There are different forms: starch entrapped within food matrix, granular starch structure and retrograded starch formed after food processing. In the VTT technology large potato starch granules (50–100 μm), enzymatically treated to obtain a porous structure, were used as a carrier. The enzyme attacked the inside of the granules, making them porous. Subsequently, amylose, the linear polymer of starch was solubilized, cooled and precipitated over the bacteria-filled starch granules. The strength of adhesion of bifidobacteria to starch granules varied for different starches (Crittenden et al. 2001). Finally, the whole product together with the growth media was freeze-dried to a powder form. Different amylases (bacterial, malt, fungal, pancreatic) have been tested using a range of conditions to establish the optimal method to produce internal hollows for the encapsulating bacteria inside starch granules. Several probiotic strains have been used in starch encapsulation studies. In addition, the viability of encapsulated bacteria stored at room temperature was at least 6 months and when frozen, at least 18 months. The capsule material appeared to be resistant to intestine milieu in in vitro and in vivo studies. A new, interesting approach is to use starch granules that naturally form aggregates, such as small barley starch granules (Mattila-Sandholm et al. 2002).

Vacuum drying is a similar process as freeze-drying, but it takes place at 0–40° C for 30 min to a few hours. The advantages are that the products are not frozen, which prevents freezing damage and energy consumption, and that the drying is fast. King and Su (1993, 1995), and King et al. (1998) used controlled low-temperature vacuum dehydration (CLTVD) to dry *Lactobacillus acidophilus* at about 0° C. This temperature was

maintained by a controlled combination of shelf heating and vacuum adjustment in a freeze-dryer. Vacuum drying at about 40°C resulted in poor survival numbers. At about 0°C the bacterial survival upon CLTVD was just slightly lower than the one upon freeze-drying (King and Su 1995). A lower decrease in viability of *Lactobacillus acidophilus* with time was found when 0.57% $CaCO_3$ and 4.1% glycerol was added to the growth medium and 10% NFDM prior CLTVD (King and Su 1995; King et al. 1998). The freeze-dried or CLTVD-dried bacteria could be stored at 5 or –20°C for more than 120 days without much degradation, in contrast to storage at room temperature. Based on the study of King et al. (1998) one can calculate that at 70, 60, 50, 20 and 4°C, a 50% reduction in cell viability of vacuum-dried *Lactobacillus acidophilus* in originally 4.1% glycerol, 10% NFDM and 0.57% CaCO3 and a final moisture content of 3% is obtained after 0.5 h, 4 h, 22 h, 999 days and 7.5×10^4 days, respectively.

10.3.4 Fluid Bed Coating

In spray-coating techniques, the core material needs to be in a solid form and is kept in motion in a specially designed chamber, either by injection of air at the bottom (fluid bed coating, see also Sect. 2.2.2 of this book) or by rotary action (e.g., pan coating). Solid forms of probiotics can be obtained by spray-drying or freeze-drying (see previous sections). A liquid coating material is sprayed through a nozzle over the core material in a hot environment. The film formation then begins, followed by successive wetting and drying or solidification stages which result in a solid, homogenous layer on the surface of a core. The small droplets of the sprayed liquid contact the particle surface, spread on the surface and coalesce. The spray liquid, also referred as shell, wall or coat material can be a solution, a suspension, an emulsion or a melt. Any edible material with a stable molten phase can be sprayed at high deposition rates, allowing coatings with a thickness of 100 μm up to 10 mm. The coating material can be injected from many angles and this influences the properties of the coating. In Fig. 10.1 three fluid bed coating technologies are presented, principally differing in the type of air fluidization employed and the site in the vessel where the coating material is sprayed: the top spray-, the bottom spray- and the tangential spray-coating. The probiotic bacteria are presented in fine powder particles prepared by traditional methods (fermentation, concentration, freeze-drying and granulation). The coating material is introduced into the vessel under compressed air. In food applications the coating of probiotics is mostly lipid based (e.g., waxes, fatty acids and specialty oils), but proteins (e.g., gluten and casein) or carbohydrates (e.g., cellulose derivatives, carrageenan and alginate) can also be used (Champagne and Fustier 2007).

Spray-coating technique is suitable for particles with a diameter from 50 μm to 5 mm. Product quality characteristics depend on numerous variables, which affect different steps of the process. The film characteristics, through the evaporation or congealing rate, are function of fluidization air velocity, temperature and humidity (Jacquot and Pernetti 2004; Sect. 2.2.2 of this book). The coating homogeneity and success are influenced by the stickiness of the coating material, the wettability of particles by the coating liquid and the operating conditions. The thickness of the

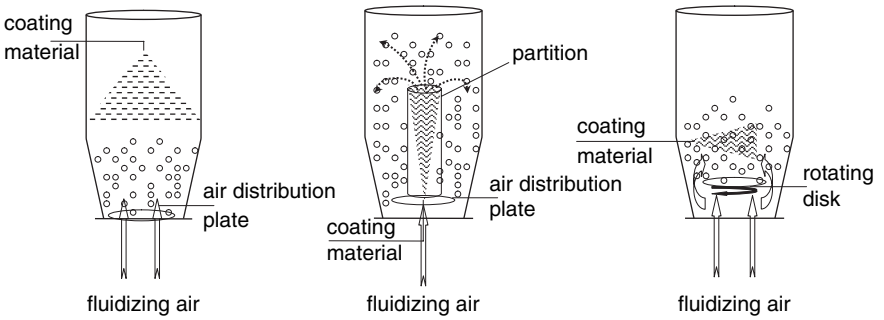

Fig. 10.1 Fluid bed coating methods for the microencapsulation of probiotics. (**a**) Fluid bed top spray-coating; (**b**) Fluid bed bottom spray-coating with the Würster device; (**c**) Fluid bed tangential spray-coating. Adopted from publications of Champagne and Fustier (2007) and Jacquot and Pernetti (2004)

final film coat is determined by the number of coating cycles (passages of the particles in the coating zone). An adequate choice of the coating material (with respect to viscosity and hygroscopicity), and control of the operating conditions, such as the particle velocity and bed moisture content, prevent collision between particles and agglomeration. During the spraying process bubbles might form due to shear and be trapped in the coating film, which affects porosity, permeability and mechanical properties of the shell layer.

In the top fluid bed coating mode, the spray liquid and the air flow are counter current (Fig. 10.1a) and the distance between powder particles and liquid droplets are relatively large. Therefore, there is a risk of spray liquid drying or solidifying before they coat the particles. Particles should travel fast to prevent agglomeration and the liquid droplets should be small enough to sediment on the core and create dense coating film. In practical applications, the motions of fluidized core particles are random, resulting in a nonuniform coating. In the bottom fluid bed coating, the spray liquid is introduced in the vessel through spray nozzles placed at the bottom, thus in the concurrent direction with the air flow (Fig. 10.1b). Würster (1950) improved the device by adding a cylindrical partition centrally placed and an air distribution plate. This improved device brings the powder particles in circulation and enables dense and homogenous coating. Collisions between spray liquid droplets and powder particles are increased, resulting in higher coating efficiency, lower droplets drying or solidifying before they coat the particles and minimal risk of agglomeration. In addition, the production capacity of the Würster coating device is increased compared to a conventional top spraying coating system. The tangential fluid bed coating device is also called rotary spray-coating system (Fig. 10.1c). A rotary disk, placed at the bottom of the chamber, maintains a complex fluidization pattern and the particles movement is influenced by centrifugal force, air stream and gravity (Jacquot and Pernetti 2004). The coating liquid is brought in tangentially, while air streams pass through the gap between the rotor disk and inside chamber wall, maintaining fluidization of the core particles. As with bottom spray device, the achieved coating is homogeneous. The main disadvantage of the

technique is the high shear stress applied to the particles, thus it is limited to sturdy and resistant materials.

Fluid bed coating is among all, probably the most applicable technique for the coating of probiotics in industrial productions, since it is possible to achieve large batch volumes and high throughputs. As written above, most coatings used are lipid based. Commercially available encapsulates include Probiocap™ of Lallemand (Institut Rosell, see http://www.lallemand.com/HNAH/eng/probiotics.shtm; Makhal and Kanawjia 2003; Durand and Panes 2001). These particles are made by fluid bed coating of freeze-dried probiotics with low melting lipids, around 250 μm in size, and developed by Lallemand in collaboration with Balchem (see http://www.balchem.com/encapsulates and Wu et al. 2000).

10.3.5 Spray-Cooling

In spray-cooling, a molten matrix with low melting point containing the bioactive compound is atomized through a pneumatic nozzle into a vessel (see also Sect. 2.3.3 of this book). This process is similar to spray-drying with respect to the production of fine droplets. However, it is based on the injection of cold air into the vessel to enable solidification of the gel particle rather than on hot air which dries the droplet into a fine powder particle. The liquid droplet solidifies and entraps the bioactive product. Spray-cooling is considered as the least expensive encapsulation technology and offer few advantages over other encapsulation techniques. It may expand the range of matrices used. Further, it is possible to produce very small particles. However, so far it has been used rarely for probiotics (rather more suitable for encapsulation of other food ingredients, like water-soluble vitamins, fatty acids, antioxidants, fatty acids, yeasts, enzymes), since other technologies are easier to establish in laboratories. One example is the spray-cooling of a slurry of freeze-dried probiotics and molten lipids (e.g., 60–75% stearic acids at 60° C), which was atomized by a rotary disk in a cooling chamber to give 75–300 μm encapsulates (Rutherford et al. 1993). The contact time of the freeze-dried probiotics should remain very short, but no details about the survival rate of freeze-dried probiotics at 60° C were given by the inventors. Section 10.3.3 may indicate more about the storage stability of freeze-dried probiotics at this high temperature, assuming that molten lipids have no further detrimental effect on probiotics.

10.3.6 Encapsulation of Probiotics in Microspheres

10.3.6.1 Gel-Particle Techniques

Probiotics can be encapsulated in microspheres (gel beads or polymeric matrix beads), often coated with an outer layer which may be designed to dissolve under specific conditions allowing release of the encapsulated bacteria (Anal and Singh 2007).

Polymeric matrices are utilized mainly to protect probiotics against low pH and high bile concentrations, but they also ease handling and allow propagation of the probiotics in application.

Extrusion or emulsification techniques may be applied to produce spherical polymer beads ranging from 0.3 to 3 mm in diameter (Krasaekoopt et al. 2003). The first step in both techniques is mixing of bacterial culture with a polymer solution to create bacteria-polymer suspension (Fig. 10.2), which is then extruded through a needle to produce droplets collected in a bath where gelation occurs (ionotropic or thermal), or dispersed in a continuous phase applying mixing to create stable w/o emulsion.

Extrusion is the oldest and the most common approach to making capsules with hydrocolloids, and might be achieved by simply dropping an aqueous solution of probiotics into a gelling bath. Extrusion bead production techniques (like electrostatic, coaxial-air flow, vibration, atomization or jet-cutter) are based on applying the additional force to generate smaller spheres compare to those produce by simple dropping; the size of the particles can be adjusted by choosing needle diameter and manipulating the distance between the outlet and the coagulation solution and electric or piezzo parameters. Extrusion technology is more popular than emulsion technology due to its simplicity, easy to handle with the equipment, low cost at small scale and gentle formulation conditions ensuring high retention of cell viability. The main problem with respect to their applications on probiotics is the relatively large particle size, although it is possible to generate microspheres of very narrow size distribution.

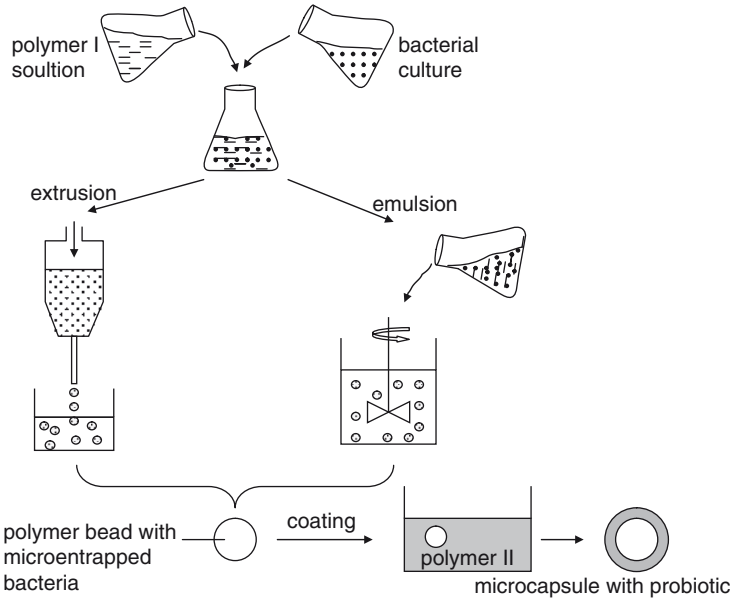

Fig. 10.2 Gel-particle technologies for the microencapsulation of probiotics

In the emulsion technique, a small volume of cell-polymer suspension (discontinuous phase) is added to a large volume of a vegetable oil (continuous phase) such as soybean oil, sunflower oil, canola oil or corn oil. In some studies, white light paraffin oil (Rao et al. 1989) and mineral oil (Groboillot et al. 1993) have been used. The mixture is homogenized to form a water-in-oil (w/o) emulsion. In some cases emulsifiers are added to form more stable emulsions, since these agents lower the surface tension of droplets leading to smaller spheres. The most common emulsifier used is Tween 80 at low concentrations. Once the emulsion is formed, solidification occurs after the addition of an adequate solidifying agent to the emulsion. In the emulsion procedure, adjustment of agitation speed and phase ratio enables production of the targeted bead size. The size of the beads can vary between 25 μm and 2 mm. The double emulsion technique (water-in-oil-in water, w/o/w), a modification of the basic technique in which an emulsion is made of an aqueous solution in a hydrophobic wall polymer can also be appropriate for incorporation of probiotics (Shima et al. 2006). The relative viability of the encapsulated microbial depends on operating parameters, such as inner phase volume ratio and the median diameter of the oil droplets.

The obtained polymer beads with entrapped microbials can be further introduced into a second polymer solution to create a coating layer which provides an extra protection to the cells and/or gives sensorial properties to the product. Another way to perform coating is to use co-extrusion devices, where beads formation and wrapping occur simultaneously (see Sect. 10.3.7). Coating can be performed with cationic polymers, such as polyethylenimine, polypropyleneimine, chitosan or combination of these. However, these polymers have no or limited food grade status. Formation of the membrane around the beads results in stronger microcapsules and minimized cell release.

Storage stability of obtained microspheres can also be enhanced by fluidization drying or by freeze-drying using cryoprotective additives like skimmed milk with or without 5% saccharose and/or 0.35% ascorbic acid (Goderska and Czarnecki 2008).

10.3.6.2 Encapsulation of Probiotics in Alginate

Alginate is the most widely used encapsulation matrix for various food-grade and non-food compounds. Alginate is used in the form of a salt of alginic acid. Alginates are naturally derived linear copolymers of 1,4-linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) residues (Martinsen et al. 1989; Gombotz and Wee 1998; Sect. 3.2.1.4 of this book). The ratio and sequential distribution of uronic acid residues, along the length of the alginate chain, vary in alginates of different origins (brown seaweeds, certain bacteria) (Martinsen et al. 1989; Gombotz and Wee 1998). There is no regular repeat unit in alginate polymers, and the chains can be described as a varying sequence of regions which usually denotes as M blocks, G blocks and MG blocks. Aqueous solutions of polysaccharides form hydrogels in the presence of divalent Ca^{2+} ions via ionic interactions between the acid groups on G blocks and the gelating ions. As a result, calcium alginate gels are physically cross-linked polymers

with mechanical and hosting properties dependant on the alginate composition. The mechanism of calcium-induced alginate gel formation occurs due to orderly alignment of the alginate polymers which interact with divalent cations such as calcium, where calcium ions occupy the space between two alginate polymers like an egg placed inside an egg box and is known as "egg box" gelling mechanism (Smidsrod and Skjak-Braek 1990; Skjak-Braek et al. 1986).

Both techniques (extrusion and emulsion) can be applied to generate calcium alginate microspheres. In the emulsion technique, the addition of an oil-soluble acid, such as acetic acid, reduces the alginate pH from 7.5 to approximately 6.5, enabling initiation of gelation with Ca^{2+} (Poncelet et al. 1993).

The survivability of probiotic cultures in calcium alginate beads in general depends on many factors, such as concentration of alginate and gelation solution ($CaCl_2$), the duration of gelation and cell concentration (Chandramouli et al. 2004; Lee et al. 2004; Lee and Heo 2000; Sheu et al. 1993). For example, the survival of *Lactobacillus casei* increased proportionately with increasing alginate concentrations from 2% to 4% (Mandal et al. 2006). The probiotic strain *L. acidophilus* encapsulated in Ca–alginate beads showed higher survival level under different conditions compared to the non-encapsulated cultures. The viability of encapsulated bacteria in simulated gastric fluid appeared to increase with the increase in bead size. Lee and Heo (2000) proposed a model to express the influences of gel concentration, bead size and initial cell numbers on the survival of bifidobacteria in calcium alginate beads in *in vitro* gastrointestinal conditions. While large alginate beads (>1 mm) get rough textural structure in the real microbial feed solution, small capsules (<100 μm) allow fast and easy diffusion of water and other molecules in and out of the matrix (Truelstrup Hansen et al. 2002). Out of nine different strains of *Bifidobacterium spp* encapsulated in calcium alginate spheres, only the strain *B. lactis* Bb-12 was found to be resistant to low pH and bile salts (Truelstrup Hansen et al. 2002). The loaded, 20–70 μm in diameter calcium alginate microspheres were produced by emulsification procedure using 2% alginate and showed good stability of Bb. 12 after storage up to 16 days in various surrounding media ($CaCl_2$, milk, yoghurt, sour cream) and for 1 h in simulating gastric fluid (37° C, pH 2.0). However, the small alginate spheres could not provide good protection to the other, more acid-sensitive bifidobacteria strains against low pH or upon storage in milk.

Selmer-Olsen et al. (1999) found that addition of protective solutes was very important when drying *Lactobacillus helveticus* CNRZ 303 in calcium alginate beads by a fluid bed. The bacteria fall in viability when the water content decreased below 100% (w/w). The best survival upon drying was found in the presence of adonitol (a sugar alcohol derived from ribose) and non-fat milk solids (respectively, 71% and 57% survival after drying to 20–30% water content). These were also the best for survival upon storage. Rehydration conditions also affected the survival rate; best results were obtained by Selmer-Olsen et al. (1999) when the cells were rehydrated in cheese whey permeate between 20 and 30° C and pH 6–7.

In vitro laboratory studies have shown that with alginate hydrogel microcapsules, the release of the probiotic bacteria can be accomplished by shaking the gel beads in 0.1 M phosphate or citrate buffer solutions in a laboratory stomacher blender.

The calcium ions holding the alginate polymers are pulled out of the beads due to their affinity for hydroxyl ions and hence the orderly gel structure disintegrates and releases the probiotic bacteria. This method of release of the bacteria is used to determine the encapsulation efficiency (Chandramouli et al. 2004).

Coated alginate microcapsules approve to have better protective characteristics compared to uncoated ones. Krasaekoopt et al. (2004) encapsulated three different probiotic strains in alginate particles coated with three types of materials (chitosan, sodium alginate and poly-L-lysine) and determined that chitosan-coated alginate beads provided the best protection for the strains *L. acidophilus* 547 and *L. casei* 01, while sensitive *B. bifidum* ATCC 1994 did not survive the acidic conditions of gastric juice. Chitosan, a positively charged polyamine, forms a semipermeable membrane around a negatively charged polymer such as alginate. This membrane does not dissolve in the presence of Ca^{2+} chelators or antigeling agents and thus enhances the stability of the gel (Smidsrod and Skjak-Braek 1990). As a consequence, the cell release is lowered down to 40% (Zhou et al. 1998). Low-molecular-weight chitosan diffuses more readily into the calcium alginate gel matrix resulting in a denser membrane than with high-molecular-weight chitosan (McKnight et al. 1988). A whey protein and pectin conjugation has also been used as a protective membrane around calcium alginate beads (Guérin et al. 2003). Protein–alginate composite beads were covalently bound by a transacylation reaction (Levy and Edwards-Levy 1996). The reaction involved the formation of amide bonds between protein and alginate, producing a membrane on the bead surface, which resisted gastric pH and pepsin activity. The bifidobacteria immobilized in the mixed gel were more resistant to simulated gastrointestinal tract conditions (Guérin et al. 2003).

Except conventional polymers, polysaccharides (fructo-oligosaccharides, isomalto-oligosaccharides) and peptides may also be used as an outer coating layer (Chen et al. 2005). Introducing an additional enteric coating (made from methacrylic acid copolymer, which is not food-grade) together with the outer coating layer (mixture of sodium alginate and hydroxypropyl cellulose in the weight ratio 9:1) enabled 10^4- to 10^5-fold increase in cell survival in simulated gastrointestinal tract fluids. In addition, the use of the non-food grade toluene diisocyanate as a cross-linking agent provided membranes which were more resistance to breakage (Hyndman et al. 1993).

The release of encapsulated probiotic bacteria from calcium alginate and chitosan-coated-alginate–starch encapsulates (CCAS) under ex vivo and in vivo conditions have been reported (Iyer et al. 2004, 2005). In these studies, the release profiles of different bacteria, *L. casei* strain Shirota (LCS) and green fluorescent protein (GFP)-tagged *Escherichia coli* K12 (*E. coli* GFP^+ K-12), from encapsulates were investigated in porcine gastrointestinal contents by an ex vivo method. In another study by the same authors, calcium alginate and CCAS encapsulates were fed to mice and bacterial release at different sites in the gastrointestinal tract was monitored for up to 24 h. In the latter experiment, LCS was used as a model probiotic strain because of the specific selective media used that allowed differentiation of the inoculated bacteria from food and from the gastrointestinal tract microbiota. The results showed that there was no detectable release of encapsulated bacteria from the capsules in the acidic gastric contents. In contrast, there was a

complete release of *E. coli* within 1 h of incubation in the small intestinal contents (pH 6.5–6.8) at 37° C, while it took nearly 8 h to completely release the *E. coli* in the colon contents (pH 6.9) under similar conditions. In the case of LCS, there was no significant release of LCS in gastric porcine contents (pH 2.5) even after 24 h of incubation. There was a complete release of LCS in the ileal contents (pH 6.8) after 8 h of incubation. As in the ileum, there was a complete release of LCS from capsules in colon contents, but it took approximately 12 h. The results reported indicate that while there was a complete release of *E. coli* GFP^+ from calcium alginate encapsulates within 1 h in porcine ileal contents ex vivo, it took approximately 8 h to completely release LCS from CCAS capsules. The difference between the release of *E. coli* and LCS was reported to be due to the chitosan coating of the capsules. *E. coli* GFP^+ was encapsulated with alginates while alginate capsules containing LCS were coated with chitosan polymer. It can be said that microencapsulation in alginate gel beads with or without coating effectively minimizes the bactericidal effects of the gastric pH and maximize the number of encapsulated bacterial cells reaching the ileum and subsequently to the colon.

10.3.6.3 Encapsulation of Probiotics in κ-Carrageenan

Carrageenan is a natural polysaccharides isolated from marine macroalgae, commonly used as food additives (see Sect. 3.2.1.4). Carrageenan dissolves at high temperatures (60–80° C) in concentrations of 2–5% (Klein and Vorlop 1985). Dispersion of the carrageenan gel into small droplets has to be carried out at elevated temperatures (40–45° C) and gelation occurs during cooling procedure down to room temperatures. After the beads are formed, K ions in the form of KCl are used to stabilize the gel, prevent swelling or to induce gelation (Krasaekoopt et al. 2003). Audet et al. (1988) reported inhibitory effect of KCl on some bacteria such as *Streptococcus thermophilus* and *L. bulgaricus*. The presence of monovalent ions such as Rb^+, Cs^+, and NH_4^+ makes stronger gels (Tosa et al. 1979). *Lactobacillus acidophilus* survived freezing, freeze-drying and storage in a freeze-dried form much better in 3 mm 4% (w/v) κ-carrageenan gel beads made in 0.3 M KCl than free cells (Tsen et al. 2002). Locus bean gum in ratio to carrageenan of 1:2 significantly increases the strength of the gel through specific interaction of its galactomannan chains with carrageenan. Carrageenan/locus bean gum mixture has been frequently tested for microbial encapsulation (Audet et al. 1990, 1991; Ouellette et al. 1994; Doleyres et al. 2002a, b, 2004). Encapsulated cells proliferate in high biomass concentration in dairy products and exhibited increased tolerance to stresses, such as freeze-drying, hydrogen peroxide and simulated gastrointestinal conditions.

10.3.6.4 Encapsulation of Probiotics in Chitosan

Chitosan is a positively charged, linear polysaccharide formed by deaceylation of chitin (see Sect. 3.2.1.5). It is water soluble below pH 6 and forms a gel by ionotropic

gelation. The terms chitin and chitosan refer not to specific compounds, but to two types of copolymers containing the two monomer residues anhydro-*N*-acetyl-D-glucosamine and amino-D-glucosamine, respectively. Chitin is a polymer of β-(1,4)-2-acetamido-2-deoxy-D-glucopyranose and one of the most abundant organic materials. Chitosan, a polycation with amine groups, can be cross-linked by anions and polyanions, such as polyphosphates (Anal and Stevens 2005), $[Fe(CN)_6]^{4-}$ and $[Fe(CN_6)]^{3-}$ (Anal and Singh 2007), polyaldehydrocarbonic acid (Klein and Vorlop 1985), and sodium alginate (Anal et al. 2003). It is an important biomaterial in food and pharmaceutical applications due to its favorable properties, such as good biocompatibility, biodegradability and non-toxicity. However, chitosan's food-grade status is not clear in many countries and does not taste well in a free form. Furthermore, it exhibits inhibitory effects on different types of lactic acid bacteria (Groboillot et al. 1993). Thus, chitosan is mainly used as coating for conventional alginate gel beads (Krasaekoopt et al. 2003, 2004; Lee et al. 2004; Zhou et al. 1998; see also Sect. 10.3.6.2). Various chitosans (different molecular weights) in combination with alginate can be used to achieve high cell loadings (up to 10^{10} cfu g^{-1}, Zhou et al. 1998). Nevertheless, the viability of the encapsulated microorganisms depends on the way by which chitosan cross-links with alginate (whether they interact and form matrix together, i.e., chitosan is the inner polymer or chitosan creates an outer layer around alginate sphere, i.e., chitosan is the outer polymer). Calcium alginate–chitosan microcapsules can be made by one- or two-step processes, based on the presence or absence of Ca^{2+} in the receiving chitosan solution (Lacík 2004). The beads can be prepared in a way to differ in a level of homogeneity of the alginate concentration gradient through the cross-section of the bead by addition of sodium chloride to the calcium chloride solution. Capsules' mechanical strength and permeability strongly depend on the process of capsule preparation (Gaserod et al. 1998, 1999). In the one-step process (in the absence of Ca^{2+} in chitosan solution), chitosan is located only at the interface, as a thin-alginate–chitosan membrane with a weak mechanical resistance. The capsules were much stronger when the two-step protocol was used. This difference between two protocols of capsule formation is due to the ability of chitosan to penetrate through the membrane (Lacík 2004). The kinetics of membrane formation and the capsule parameters (like thickness, permeability and mechanical strength) depend on the concentration of components, molar masses of both, alginate and chitosan, reaction time, pH and ionic strength. Sprayed particles coated with chitosan are recommended as impressively effective vehicles in delivering viable bacterial cells to the colon and stable shells during refrigerated storage.

10.3.7 Submerged Co-extrusion

Seamless capsules containing probiotics are available from Morishita Jintan Co. Ltd in Japan. These capsules are composed of three layers: a core of freeze-dried probiotics in solid fat (m.p of 35°C), with an intermediate hard fat layer (m.p. of

40° C) and a gelatin–pectin outer layer (Asada et al. 2003, and http://www.jintan-world.com). They are made with a concentric, multi-nozzle via a submerged co-extrusion technique (see also Sect. 2.3.9 of this book). The size of the capsules is quite large (1.8–6.5 mm) and the technique is relatively expensive, which may be a barrier for use in many food products. Capsules with different bifidobacteria and lactobacillus strains are available, and these reach the intestine alive without being too much affected by stomach acid or oxygen. Other actives, such as fish oil, vitamin C and iron sulfate might be encapsulated as well in these kind of capsules.

10.3.8 Twin Screw Extrusion

Some publications have also shown that probiotics can be processed in a twin screw extruder at moderate pressures and low temperatures. Example, Van Lengerich (1999) disclosed that pellets with *Lactobacillus acidophilus* can be prepared by twin screw extrusion. First, cookies were ground and this flour was fed into the extruder at 4 kg/h, followed by mixing with water and citrus juice (7/1 w/w) at 0.8 kg/h, and feeding in the next barrel of the extruder a preblend of 0.118 kg of probiotics, 0.375 kg of vegetable fat and 0.188 kg of vegetable oil at 0.75 kg/h. The extruder was operating at 150 rpm, 45 bar and 20° C, and equipped with a 20 × 1 mm die. The product temperature reached 31° C, and the pellets were dried afterwards in a convection batch dryer for 1 h at 30° C to 5.9% moisture. Optionally, pellets can be coated with a 25% shellac solution in alcohol to give 5–10% shellac coating. The patent claims that the starch should have been preprocessed (i.e., mixed and heat treated) to avoid gelatinization of the starch and provide a pleasantly taste and texture. Van Lengerich (2000) also entrapped *Lactobacillus acidophilus* in 0.5–1 mm pellets by feeding into a twin screw extruder semolina/wheat gluten 70/25 (w/w) at 2.5 kg/h, vegetable oil at 0.29 kg/h, water at 0.06 kg/h and 20% (w/w) *Lactobacillus acidophilus* at 0.82 kg/h. All barrels of the extruder were kept at 21° C, and a screw speed of 67 rpm and a die with 40 circular openings of 0.5 mm each were used. The temperature of the product remained in this way below 40° C. After extrusion, the pellets were dried for about 30 min under vacuum or carbon dioxide to prevent access of oxygen. Jongboom-Yilmaz (2002) disclosed in her patent that probiotics can be extruded in destructurized potato starch and/or sugar at 100 rpm, 13–17% torque, 8–17 bars and 33–38° C (die temperature). Ten percent of glycerol might be added as a plasticizer, which improved the survival of probiotics during the process and afterwards during storage.

10.3.9 Compression Coating

Recently, compressing coating has been developed as a promising technique which permits the stabilization of lyophilized cells during storage (Chan and Zhang 2002, 2004; Ubbink et al. 2003). This technique involves compressing dried cell powder

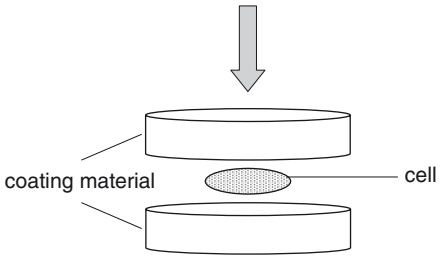

Fig. 10.3 Compression coating of cells (Chan and Zhang 2004)

into a core tablet or pellet with a 10 mm die, and then compressing coating material around the core to form the final compact. The compressed cell pellet should be positioned on the center of the die before the rest of the coating material is poured on the top of it and the punch applied, as shown in Fig. 10.3. In this way, two coatings may be formed: one enteric and the other an outer coating layer. Investigations of the bacteria immobilized by this procedure showed very good results with regard to cell protection against gastrointestinal tract in in vitro studies. Sodium alginate or pectin can be used as coating material with the addition of a binder compound (such as hydroxypropyl cellulose) to make a more rigid compact (Chan and Zhang 2002). Alternatively, 50% flour, 25% maltodextrin and 25% semi-humid pet food, or direct compressible starch, or 50% lactose and 50% maltodextrin can be used (Ubbink et al. 2003). An additional outer coating can be applied by dipping the pellets in a barrier solution or by fluid bed coating [e.g., using dipping in a melt of fat or fluid bed coating using an aqueous solution of 15% Sepifilm LP010 (= hydroxypropyl methylcellulose + 10% stearic acid)] (Ubbink et al. 2003). Above 90 MPa, the viability of microbial culture after the compression to form a pellet, gradually decreased with the pressure applied during compression procedure (upper punch pressure) (Chan and Zhang 2002). Since the compression pressure could have harmful effects on the cells during compaction, careful selection of a pressure which will be employed is needed. Pellets with probiotics might be useful as pharmaceuticals, food supplements or feed.

10.4 Food Applications

10.4.1 Challenges for Probiotics in Food Products

A number of technological challenges exists to successfully incorporate probiotics into foods and to maintain their viability:

1. *Stability of probiotics during processing and storage*. Processing of probiotic foods may involve mild heat treatment (e.g., low temperature and long-term pasteurization), pumping, homogenizing and stirring (incorporating air), freezing (frozen

products), addition of ingredients that can be antimicrobial (e.g., salt in cheese manufacture), drying (osmotic dehydration, e.g., powdered foods), packaging (oxygen ingress through packaging during storage), unfavorable storage conditions (e.g., post-acidification in yoghurt or presence of oxygen), large ice crystals formations (e.g., thawing and freezing of stored ice-cream) and the possible development of anti-microbial compounds secreted by the starter cultures during fermentation. In the past, culture companies select probiotic strains to withstand these conditions; however, the recent trend has been for these companies to focus the selection of strains on the basis of health-enhancing and therapeutic effects. Therefore the latest probiotic strains may have lost their ability to withstand unfavorable processing and storage conditions. Hence the viability of probiotic bacteria is of paramount importance in the marketability of probiotic incorporated products. In the development of functional foods, microencapsulation is especially used for incorporation and protecting viable cells into the products.

2. *Protection in the gastrointestinal tract and controlled release of probiotics in the intestines*. Most of the probiotics cannot stand the acid in the stomach (ranging from pH 2.5 to 3.5), and also the bile salts in the small intestine might be harmful. Microencapsulation and also components of the food matrix (like fat) may provide protection of probiotics against these harsh conditions. In addition, it is important that bacterial cells end up in large numbers in areas of the gastrointestinal tract where they are beneficial. Controlled release of bacterial cells from micro, encapsulates at the target site is therefore critical. It is beneficial for encapsulated bacteria to be released in the small intestine, where Peyer's patches exist, to activate the immune system. Therefore the polymers used as shell material for microencapsulation should be able to protect the bacteria in the acidic stomach and release the bacteria under the alkaline conditions in the small intestine. Many reports show that microencapsulation in, for example, alginate or pectin based beads can be used for controlled release of bioactive substances (Champagne and Kailasapathy 2008). Other examples are fat coated ones (see below).
3. *Clinical proof of health effects of the food product containing the probiotics*. The food matrix may affect the health benefit(s) of probiotics and ideally clinical studies using the final food product application should be performed to demonstrate them.

Co-encapsulation of probiotic cultures with certain food ingredients may be beneficial in two ways. First, it enables introducing multiple bioactive compounds. In addition, with the right selection of compounds, probiotic beneficial activity can be enhanced, prolonged or complemented by interactions between cells and co-encapsulated ingredients. Co-encapsulation can be performed by adding the second bioactive ingredient to the polymer solution, polymerizing solution or coating solution. Co-encapsulation with prebiotics, antioxidants, peptides or immune-enhancing polymers is becoming especially attractive in future perspectives. It has been determined that at least 3 g of prebiotics in a sample is needed to cause detectible activity improvement of the probiotic culture in the gastrointestinal tract (Krasaekoopt et al. 2003). This high amount is hardly possibly to achieve in real-life microencapsulation systems. On the other hand, some other compounds are active in much lower concentrations.

A combination with antioxidants is especially beneficial, due to the extending of probiotic stability in the gut and during storage caused by the effect of an antioxidant. Bioactive peptides, like bacteriocins, could enhance or complement the antimicrobial activities of probiotic bacteria.

Not only co-encapsulation, but also the presence of (ingredients in) a food product may improve the viability of probiotics (Ross et al. 2005), for example, by feeding the probiotics during storage, by the presence of probiotics which can be consumed during storage or upon consumption, by neutralizing partly the low pH in the stomach or by 'hiding' of probiotics in the food matrix during passage through gastrointestinal tract.

There are two classical ways of bacterial culture distribution in supply chains. One way is the storage and delivery of fresh, concentrated, chilled or frozen probiotic cultures for direct use. This has the advantage of very limited loss of viability, but the limit of a short storage time, similar to milk products. For fresh products containing probiotics, storage time is usually limited to 4 up to 6 weeks under refrigerated conditions. In fresh dairy products, the probiotics may multiply upon storage, even at low temperatures (except if the products get frozen), and may compensate for some probiotic deaths. The use of semipermeable encapsulates might be then a good option; feed is able to penetrate slowly into the encapsulates and the shell is still able to protect the probiotics against some harsh (sub-lethal) conditions in the gastrointestinal tract.

Another way of bacterial culture distribution in supply chains is the storage and delivery of dried probiotics, optionally in combination with microencapsulation techniques, which give microorganisms more stability and flexibility. The demands for probiotic stability are quite large when they do not multiply in the food product upon storage, as is often the case in non-dairy products, or when longer storage times are required. Probiotic viability in a food product depends on, for example, pH, storage temperature, oxygen levels, presence of competing microorganisms, presence of inhibitors (Mattila-Sandholm et al. 2002), and these factors are even more important when probiotics do not multiply. Bringing probiotics in a dormant state, by drying in the presence of additives and optionally coating them with an impermeable barrier during storage, might be a way to meet the demands for probiotics stability. For the food and pharmaceutical industries, a period of 1 year is often a minimum requirement to supply a marketable dry probiotic product. Capsule fillings, sachets and tablets with dried probiotics are very popular among consumers and inexpensive to produce, thus manufactured in the pharmaceutical or food supplement area. Application of dormant probiotics in both dry and liquid food products is possible, as discussed below in the following subsections which exemplify the potential of using microencapsulates containing probiotics in food products.

10.4.2 Yoghurt

It has been reported that microencapsulation using calcium-induced alginate–starch polymers (Godward and Kailasapathy 2003; Sultana et al. 2000), potassium-induced κ-carrageenan polymers (Adhikari et al. 2000, 2003) and whey protein polymers

(Picot and Lacroix 2004) have increased the survival and viability of probiotic bacteria in set and stirred yoghurts during storage. Kailasapathy (2006) reported that incorporation of calcium-induced alginate–starch microencapsulates containing probiotic bacteria (*L. acidophilus* and *B. lactis*) did not substantially alter the overall sensory characteristics of yoghurts. Microencapsulation also appears to provide anoxic regions inside the encapsulates thus reducing oxygen trapped inside the encapsulates which prevented the viability losses of oxygen-sensitive strains (Talwalkar and Kailasapathy 2003, 2004) in addition to protecting the cells against the detrimental effects of the acid environment in the yoghurt. McMaster et al. (2005) also showed increased oxygen tolerance by bifidobacteria in gel beads. The efficiency of microencapsulation in protecting the probiotic bacteria, however, depends on the oxygen sensitivity of the bacteria and the dissolved oxygen levels in the product. The addition of starch as a filler material in the alginate capsule matrix (Sultana et al. 2000), co-encapsulation with prebiotic substances such as inulin (Iyer and Kailasapthy 2005), or coating the microbeads with chitosan (Krasaekoopt et al. 2006) appear to improve the viability of probiotic cultures. A filler material used in preparing microencapsulated probiotic cultures is, for example, Hi Maize™ starch. Because of its cross-linked structure it will swell and absorb water but it will not gelatinize fully during pasteurization of yoghurt mix. This swollen starch therefore will contribute to increased viscosity and firmness. The formation of exopolysaccharides by the yoghurt starter cultures and probiotic cultures may contribute to prevention of syneresis and an increase in viscosity, combined with a better mouthfeel. The exopolysaccharides produced during fermentation may themselves form natural encapsulant for the yoghurt and probiotic bacteria.

The encapsulates above are semipermeable, and protect the still active probiotics against harsh conditions (oxygen, low pH of around 4). Another approach has been disclosed by Tessier (2005), who used granules composed of dormant, dehydrated lactic acid bacteria and coated with a solid fat in fermented milk (e.g., yoghurt, but also other liquid foods were claimed). The granules were coated on a fluidized bed in a 50/50 (w/w) mixture of stearic acid and palmitic acid, and had an average particle size between 150 and 200 μm. The encapsulated probiotics had no effect on the fermentation by other, non-encapsulated bacteria. Larger granules (1–3 mm) can also be used (Shin et al. 2002), but then the granules must have a density very close to that of the yoghurt. Furthermore, one may need to place the granules first at the bottom of the container prior to the filling of it with the yoghurt, which is a considerable manufacturing constraint.

10.4.3 Cheese

Among the traditional dairy foods, cheddar cheese has a markedly higher pH (4.8–5.6) than fermented milks and yoghurt (pH 3.7–4.3) and thus help in providing a more stable medium to support the long-term survival of acid-sensitive probiotic bacteria (Stanton et al. 1998). The metabolism of various lactic acid bacteria in

cheddar cheese results in anaerobic environment within a few weeks of ripening, favoring the survival of probiotic bacteria (Van den Tempel et al. 2002). Furthermore, the matrix of cheddar cheese and its relatively high fat content offers protection to probiotic bacteria during passage through gastrointestinal tract (Vinderola et al. 2002). Thus it appears that microencapsulation may be only marginally beneficial in protecting probiotic bacteria in cheddar cheese. However, compared to yoghurt, cheddar cheese has a longer ripening, storage and shelf life during which the pH decrease, making the cheese acidic in nature during ripening. The combination of long maturation periods and acidic conditions could make it difficult for probiotic bacteria to survive during the 6–12 month ripening period. Additionally, compared to yoghurts, cheddar cheese also contains starter and non-starter lactic acid bacteria which may affect the survival of probiotic bacteria.

Dinakar and Mistry (1994) reported improved survival of *B. bifidum* in cheddar cheese over a 6 month ripening period. Gardiner et al. (2002) reported improved and increased survival as well as an increased growth rate of *L. paracasei* in cheddar cheese after 3 months of ripening. Similar results have been reported by McBrearty et al. (2001), Godward and Kailasapathy (2003) and Darukaradhya (2005). Cheese containing encapsulated *Bifidobacterium* was shown to possess similar flavor, texture and appearance compared to the control (Dinakar and Mistry 1994; Desmond et al. 2002). Kailasapathy and Masondole (2005) have reported that production of feta cheese incorporating encapsulated probiotic bacteria (*L. acidophilus* and *B. lactis*) is technologically feasible; however, selection of probiotic strains that are acid and salt tolerant and produces exo-polysaccharides as well as using food polysaccharides as shell materials for encapsulation will allow the production of a better quality feta cheese with greater survival rate of probiotic bacteria and an improved texture.

10.4.4 Frozen Desserts

Several studies have reported that probiotics entrapped in alginate or carrageenan beads have greater viability following freezing in dairy desserts (Kebary et al. 1998; Sheu et al. 1993; Godward and Kailasapathy 2003; Shah and Ravula 2000). In the manufacturing of frozen ice milk, probiotics microencapsulated with 3% calcium alginate are blended with milk and the mix is frozen continually in a freezer. The incorporation of microencapsulated probiotics has no measurable effect on the overrun and the sensory characteristics of the products with 90% probiotic survival (Sheu et al. 1993). Addition of encapsulated cultures (*L. acidophilus* and *B. infantis*) did not show any effect on the amount of air incorporated into the ice-cream (Godward and Kailasapathy 2003). The high fat content of ice-cream and the neutral pH of dairy desserts may be the main factors responsible for the additional protection provided to probiotic bacteria. However, the addition of cryoprotectants such as glycerol (Sheu et al. 1993; Sultana et al. 2000) seems to improve the viability of probiotic bacteria during freezing of the dairy desserts. The milk fat in ice-cream

formulations may also act as an encapsulant material for probiotic bacteria during the homogenization of the ice-cream mix. The high total solids in ice-cream mix, including the fat (emulsion), may provide protection for the bacteria (Kailasapathy and Sultana 2003). However, full-fat ice-cream offered no extra protection for probiotic bacterial cultures (*L. acidophilus* LAFTI™ L10, *B. lactis* BLC-1 and *L. casei* subsp *paracasei* LCS-1) over the low-fat product during storage, with the low-fat formulation showing improved survival of all three cultures during the freezing process (Haynes and Playne 2002).

10.4.5 Powdered Formulations

In powdered milk products, the challenge is to protect the probiotics from the excessive heat and osmotic degradation during spray-drying. Improved viability upon conjointly spray-drying of milk and probiotics might be achieved by the use of a second drying step in two fluidized bed compartments operating at 60–90° C and a last, third compartment to cool to about 30° C (Meister et al. 1999).The addition of a thermoprotectant such as trehalose (Conrad et al. 2000) may help to improve the viability during drying and storage. Some studies have examined the stability of encapsulated probiotics in dried milk. Incorporation of the soluble fiber gum acacia into a milk-based medium prior to spray-drying the probiotic *L. paracasei* enhanced its viability during storage, compared with milk powder alone (Desmond et al. 2002). However, not all soluble fibers enhanced the probiotic viability during spray-drying of milk or milk powder storage, for example, inulin and polydextrose did not influence the viability (Corcoran et al. 2005). Freeze-drying of probiotics in micro-encapsulated hydrogel beads seems to be more stable than non-encapsulated ones during yoghurt incubation at room temperature (Kailasapathy and Sureeta 2004; Capela et al. 2006). Spray-coating of a freeze-dried culture seems to be more effective for additional protection (Siuta-Cruce and Goulet 2001). When a lipid coating is used, it may form a barrier to moisture and oxygen entry into the microcapsules. The nature of the packaging materials (e.g., yoghurt packaging) including their oxygen scavenging capacity, together with addition of antioxidants, desiccants, etc., may need to be considered for effective protection of probiotic cells during storage (Hsiao et al. 2004).

10.4.6 Meat Products

While dairy products are the most commonly used food vehicles for delivery of probiotics, their use in meat is not reported widely (Incze 1998; Chap. 13). Meat emulsion for the manufacture of small goods such as dry fermented sausages with their low water activity, pH, curing salts and competing starter culture organisms presents a challenging environment for the survival of introduced probiotics during processing. When *Lactobacillus plantarum* and *Pediococcus pentosaeccus* were

immobilized in alginate micro capsules, the fermentation rate was much rapid with the encapsulated cells (Kearney et al. 1990). The rapid fermentation performance of the immobilized cells was caused by available nutrients (i.e., skim milk) and more protective re-hydration environment within the alginate capsules. Similar results can be obtained when microencapsulated probiotics are incorporated into a meat fermenting product mix. Muthukumarasamy and Holley (2006) showed that microencapsulated *Lactobacillus reuteri* can be used in dry fermented sausages to ensure that a desirable level of probiotic organisms is maintained in the final product at consumption without altering the sensory quality of these traditional small goods. In this study, alginate microcapsules prepared by either emulsion or extrusion were added to the salami batter (meat ingredients, starter cultures, cure mix, spice mix and salt) at 1% (w/w). The batter was stuffed in casings, transferred to a smoke house and allowed to ferment at 26° C and 88% relative humidity for 24 h, to reach pH less than 5.3. Fermentation was followed by drying at 13° C and 75% relative humidity for 25 days. It has been shown that *L. casei* cells when microencapsulated in alginate beads were more resistant to heat processing at 55–65° C (Mandal et al. 2006). This was also demonstrated when microencapsulated alginate beads containing cultures were heat treated to 55° C for 15 min; the encapsulated cells showed more stability than free cells in MRS broth acidified to pH 5.0 (Lemay et al. 2002). These data suggest that probiotic cells microencapsulated in alginate gel beads could be used in meat processing which require moderate heat treatments. For meat small goods where a meat emulsion is initially prepared (e.g., salami, sausages) the high fat in the system may also envelop the alginate gel particles containing the bacterial cells to provide additional protection to heat during processing.

10.4.7 Fermented Plant-Based (Vegetarian) Probiotic Products

With regard to plant-fermented products, probiotics are most frequently incorporated into soy products (Wang et al. 2002), although interest is increasing in the use of probiotics in fermented cereals (Charalampopoulos et al. 2003; Laine et al. 2003) and vegetable pickles (Savard et al. 2003). For stabilization of bifidobacteria during a traditional African-fermented corn product, the bacterial cells were encapsulated in mixed polymer (gellan/xanthan) beads (McMaster et al. 2005). Microencapsulation improved the survival of *L. rhamnosus* subjected to freezing in a cranberry juice concentrate and during storage of the frozen product (Reid et al. 2006). Microencapsulation can be of benefit to the stability of probiotic cultures; however, the way the bacteria are grown, harvested and dried for subsequent industrial use can be as important in promoting the viability of the cultures in food systems as the microencapsulation itself. Although the probiotic bacteria show good stability in products having a low water activity such as peanut butter ($a_w = 0.24$), spray-coating of *L. rhamnosus* using hard fat and incorporating into peanut butter formulations (incubated at 21° C), showed decreased cell viability (Belvis et al. 2006). In bakery applications, stabilizing viability of probiotics is a challenge, due to the high temperature treatment during

processing. Microencapsulation by spray-coating in hard fat did not improve the survival and stability of added lactobacilli during bread-making (Belvis et al. 2006). However, microencapsulation in a whey protein particle was reported to be effective at enhancing the survival of probiotic lactobacilli during the heat treatment applied during biscuit manufacture (Reid et al. 2006).

10.4.8 Mayonnaise

Bifidobacterium bifidum and *Bifidobacterium infantis* survived only for 2 weeks in mayonnaise at pH 4.4 and 5° C (Khalil and Mansour 1998). However, within calcium alginate beads they survived for 12 and 8 weeks, respectively. This also resulted in lower total bacterial, yeast and mold counts. The mayonnaise containing encapsulated bifidobacteria also had a higher titratable acidity (due to acid production of the surviving bifidobacteria) and lower thiobarbituric acid (TBA, a measure for oxidation) values. These lower TBA values might be due to lower lypolytic activity as a result of lower bacterial, yeast and mold growth in the presence of the encapsulated bifidobacteria. Finally, the sensory properties were improved by the use of encapsulated bifidobacteria.

10.5 Future Perspectives

Despite the lack of industry standardization, and potential safety issues, there is obviously considerably potential for the benefits of probiotics. Ongoing basic research will continue to identify and characterize existing strains of probiotics, identify strain-specific properties, determine optimal doses needed for the aspired results, and assess their stability through processing and digestion. Parallel with the basic research, gene and industry-centered research are essential. Gene technology plays a role in developing new strains, with gene sequencing allowing an increased understanding of mechanisms and functionality of probiotics. The assessment of the industrial feasibility of a microencapsulation technology is mandatory for providing cost-effective, large-scale quantities of a probiotic product for specific clinical and/or commercial use. There are sequential steps, which from the identification of a possible probiotic strain, through laboratory tests, investigations in animal models and finally in humans, leads to its production and marketing.

The therapeutic effect of probiotic bacteria and their use in preventive medicine is increasingly being reported. As clinical evidence of the beneficial effects of probiotics accumulate, the food, nutraceutical and pharmaceutical industries will formulate new and innovative probiotic-based therapeutic products. New innovative ways of administering, delivering and controlled-releasing of probiotics will be developed in the near future. In addition to food and nutraceutical products, personal products, sports and health products, and cosmetics containing specific strains of

probiotics are currently being either developed or planned and more innovative products will be developed in the future. Designer probiotic products delivering specific therapeutic strains will be the next phase of development. This will include food, pharmaceutical and nutraceutical products. These products may take the form of tablets, pills, re-constitutable single-serve sachet products, or convenient packs with instructions on how to prepare and administer them. Some food companies have already developed formulations to prepare probiotic yoghurts in the kitchen at home using a yoghurt maker.

An important issue in the development of functional foods is the stability and functionality of bioactive cultures. The viability of probiotic bacteria is important for their efficacy and a large number of reports have shown that many probiotic-based food products do not have the cell numbers in recommended number of viable cells (Iwana et al. 1993; Rybka and fleet 1997; Shah et al. 1995; Vinderola et al. 2000; Shah et al. 2000). Microencapsulation is an effective way of protecting and improving the viability of probiotic bacteria. It has been shown that non-protected cells consumed in a dried form have lower recovery levels in stools than those consumed in milk or cheese (Saxelin et al. 2003). The high viability losses that occur when free cells in a powder enter the stomach explains why microencapsulation is beneficial for the functionality of probiotics in nutraceuticals (Champagne and Fustier 2007). Microencapsulation or enteric-coated probiotic nutraceuticals may deliver the recommended number of viable cells. Microencapsulation offers the potential to reduce the adverse effects on probiotic viability of the food and gastrointestinal tract environment as well as during food or nutraceutical processing, storage and consumption. A number of efficient shell materials and controlled release trigger mechanisms have been developed in microencapsulation and this trend will continue, particularly with reference to food grade materials and the controlled and targeted release of probiotic bacteria in the gastrointestinal tract. For example, spray-drying of probiotics is not commercially used for probiotics yet (as far as we know), but this may change in the future and it might be combined with shell materials that do not only protect probiotics in a dry state but also in the gastrointestinal tract. Co-encapsulation with prebiotics, antioxidants, peptides, or immune-enhancing polymers might also be further explored. Furthermore, more research is needed of the stability and release of microencapsulated probiotics in food products.

The biological activity of probiotic bacteria is due in part to their ability to attach to enterocytes and thereby prevent binding of pathogens. The attachment of probiotic bacteria to receptors on the cell surface of intestinal epithelial cells can activate signaling processes leading to the synthesis of cytokines that affect the function of mucosal lymphocytes. Many of these receptors, such as, glycosphingolipids, mannosylated glycoproteins and TOLL, are already utilized by pathogens. This could be used to develop designer probiotic bacteria by coating with the selected receptor compound and targeting and directing the probiotic bacteria to areas in the gastrointestinal tract, such as the Payer's patches (small intestine) for maximum activation of the immune system. Further selection of suitable receptor polymers and microencapsulation can also help to direct the probiotic bacteria to access areas of medical interest such as tumors in the colon. More research is needed to study the adhesion

properties of probiotic bacteria and the selection of polymers that can trigger successful adhesion to targeted intestinal cells and to design these polymers as capsular wall materials or coatings. This could achieve targeted delivery of probiotic bacteria to various sites within the gastrointestinal tract.

In addition to efficacious capsular wall materials or coatings, cell loading of the capsules is an important challenge. Capsules larger than 20–50 μm may influence the texture of the food products and hence the overall sensory characteristics. However, the microbial cells are already 1–5 μm in size and therefore could limit the cell loading within the capsules. Another challenge is to improve the heat resistance of these probiotic cells. There appears to be no commercial probiotic product available that is stable at high temperatures. Discovering or manipulating strains that are heat stable and developing new heat-insulating-encapsulating systems are two of the major challenges in this area of functional food development.

The sensory aspects of foods are critical in the acceptance of new products. Food scientists have generally tried to prevent sensory changes related to the addition of probiotics (Champagne et al. 2005), but in many instances there are no major changes in texture or organoleptic quality that significantly affect the sensorial properties of food (Kailasapathy 2006). An emerging marketing strategy is to develop food products that clearly show the microcapsules (possibly colored) distributed within the product. Then microencapsulation could also become a future marketing tool for the food and nutraceutical industry.

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