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REVIEW

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Microencapsulation of Live Probiotic Bacteria

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Scientific research regarding the use of live bacterial cells for therapeutic purposes has been rapidly growing over the years and has generated considerable interest to scientists and health professionals. Probiotics are defined as essential live microorganisms that, when administered in adequate amounts, confer a health benefit on the host. Owing to their considerable beneficial health effects, these microorganisms are increasingly incorporated into dairy products; however, many reports have demonstrated their poor survival and stability. Their survival in the gastrointestinal tract is also questionable. To overcome these problems, microencapsulation techniques are currently receiving considerable attention. This review describes the importance of live probiotic bacterial microencapsulation using an alginate microparticulate system and presents the potentiality of various coating polymers such as chitosan and polylysine for improving the stability of this microencapsulation.

Keywords: Live probiotic bacteria, microencapsulation, alginate, chitosan, polylysine

LIVE PROBIOTIC BACTERIA

Live bacterial cells have been paid considerable attention for treating several diseases including kidney failure uremia, cancer, inflammatory bowel disease, cholesteremia, and others [10–11, 35, 58]. Probiotic live bacteria are recognized as good or friendly bacteria and thought to reduce potentially harmful bacteria from the intestine [27]. Therefore, these live bacterial microorganisms can improve microbial balances in intestine and exert positive health effects on the host [24]. *Lactobacillus* and *Bifidobacteria* are the two most common types of microbes used as probiotics and are extensively investigated for their beneficial importance [72].

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Lactobacillus or lactic acid bacteria, a heterogeneous group of Gram-positive, microaerobic, or anaerobic species, are found to be the most beneficial bacteria in the digestive tract. The name "lacto" represents that they can convert milk sugar to lactic acid. When they produce lactic acid, an unsuitable environment exists in the intestine for the harmful bacteria and forces them to leave the niches [73]. For the production of yogurt, cheese, and fermented milk, they also play an important role. Lactic acid bacteria can participate in the synthesis of thiamine, riboflavin, folic acid, niacin, vitamin B complex, and absorption of minerals [21]. During the digestion of food, they also partially break down protein, fats, and carbohydrates [28, 48]. *Lactobacillus* facilitates several essential beneficial effects such as immunomodulatory, anticarcinogenic, and antimicrobial actions. Certain *Lactobacillus* spp. have been shown to significantly suppress intestinal tumors [4, 50, 65]. Several other studies suggest that lactobacilli have a possible effect on lowering cholesterol when consumed by humans [4, 65]. In another study, freeze-dried live *Lactobacillus acidophilus* consumed by patients with advanced chronic kidney failure was shown to lower the elevated levels of uremic toxins [64]. Recently, it has been reported that *Lactococcus lactis* can be genetically engineered to produce interleukin-10 (IL-10) and used for the treatment of inflammatory bowel disease by oral delivery [67]. Bifidobacteria, another essential probiotic bacteria and normal inhabitants in the human gut, have been shown to play beneficial roles in human health [43]. They are Gram-positive, strictly anaerobic, and grow at pH 4.5 to 8.5 [72]. Found usually in the large intestine in humans, bifidobacteria have been reported to function against many intestinal pathogens including *E. coli* [80], *Salmonella typhimurium*, and rotaviruses [62, 63]. Lyophilized strains of *B. breve* or *B. longum*, while consumed by premature infants, can restore the imbalance in the gut microflora [3, 65]. There is evidence that ingesting *Bifidobacterium lactis* can enhance general immunity [5]. Table 1 shows the applications of different live probiotic bacteria for various therapeutic purposes.

Table 1. Applications of different live probiotic bacteria for various therapeutic purposes.

Live probiotic bacteria	Applications	Delivery system	References
Lactococcus lactis	Genetically engineered to produce interleukin-10 for the treatment of inflammatory bowel disease	Oral	[67]
Lactobacillus lactis	Activation of mucosal immune system	Oral (with sterile non-fat milk)	[55]
Lactobacillus casei Shirota	Activation of cellular immune system; inhibition of tumor incidence and IgE production	Oral	[80]
Lactobacillus acidophilus	Activation of mucosal immune system	Oral (with sterile non-fat milk)	[55]
Lactobacillus acidophilus (L1 and ATCC43211)	Reduction of serum cholesterol concentration and the risk for coronary heart disease	Oral (with fermented milk product)	[4]
Lactobacillus fermentum CECT5716	Immunostimulatory effect	Oral (with skimmed milk)	[22]
Lactobacillus salivarius CECT5713	Anti-inflammatory effect; inflammatory bowel disease treatment	Oral (with skimmed milk)	[22, 57]
Lactobacillus casei, *Lactobacillus rhamnosus*, *Lactobacillus delbrueckii*, *Lactobacillus plantarum*	Activation of mucosal immune system	Oral (with sterile non-fat milk)	[55]
Lactobacillus plantarum L137	Protection against influenza virus infection	Oral	[47]
Bifidobacterium longum	Colon cancer inhibition; strong antitumor activity	Oral (with fed diet)	[65]
Bifidobacterium breve YIT4064	Activation of humoral immune system; protection against rotavirus or influenza infections	Oral	[80]
Bifidobacterium lactis (HN 019)	Reduction of the severity of weanling diarrhea caused by rotavirus and *E. coli*; enhancement of the resistance to oral *Salmonella typhimurium* infection	Oral	[62, 63]
	Enhancement of natural immune function	Oral (with milk supplement)	[5]
Bifidobacterium breve	Stabilization of intestinal microflora	Oral	[39]
Pediococcus acidilactici UL5	Inhibition of listeriosis caused by *Listeria monocytogenes*	Oral	[20]
Streptococcus salivarius	Activation of mucosal immune system	Oral (with sterile non-fat milk)	[55]

Delivery System of Live Probiotic Bacteria

To achieve positive health effects by using probiotic bacteria, they must be delivered alive through oral administration to the intestine, which is the target site for their action. After reaching the intestine, they should establish themselves in certain numbers to exert positive health effects [15]. Thus, it appears that there are considerable evidences to support the importance of oral feeding of live probiotic bacteria for diverse therapeutic applications, although their oral delivery has several limitations. When probiotic bacteria are administered orally, they must be protected from the stomach acidic condition [66]. They can also be denatured by bile acid, antimicrobial compounds, and degradative enzymes before reaching the target site. These obstacles limit the survival and stability of the live bacterial cells before their arrival to the intestine alive. Thus, for an efficient oral delivery of live probiotic bacteria, an effective carrier system is mandatory to protect them from the unfavorable conditions. The microencapsulation technique facilitates a suitable carrier system for this purpose.

Microencapsulation Technique

Microencapsulation of live bacterial cells has received considerable research interest because of its growing and promising potential in therapeutic applications against many diseases [10, 50]. This technique, hypothesized as a means to protect encapsulated active contents from the external environment, has been successfully used to entrap live probiotic bacteria or other therapeutic live cells for oral delivery to protect from the harsh gastric conditions and to deliver them with improved survival rate [12].

However, the success of microencapsulation for oral delivery of live bacteria depends on the suitability of the membrane of the microparticulate systems. It is worth to note that the safety of this system is very important when live cells are intended for use for oral delivery to the intestine. This is because the live cells have to be protected during the encapsulation process, and the system should remain intact at the site of harsh environment of the gastrointestinal (GI) tract. Moreover, the survival of live cells must be ensured during their passage. Therefore, the membrane of the microparticulate system must have the properties to not only provide sufficient permeability for nutrients to pass through but also prevent the entry of hostile molecules that could destroy the encapsulated live bacterial cells [61, 70]. Considering all these factors, therefore, the oral delivery of probiotic bacteria is considered as a challenging and difficult field in the research arena.

Several microencapsulation systems have been proposed for the oral delivery of live probiotic bacteria [6, 52, 53, 71]. Among these, alginate microparticulate systems have been widely used to encapsulate these live microorganisms, because they are non-toxic, easy to handle, bioavailable, biocompatible, and cost effective [2, 56].

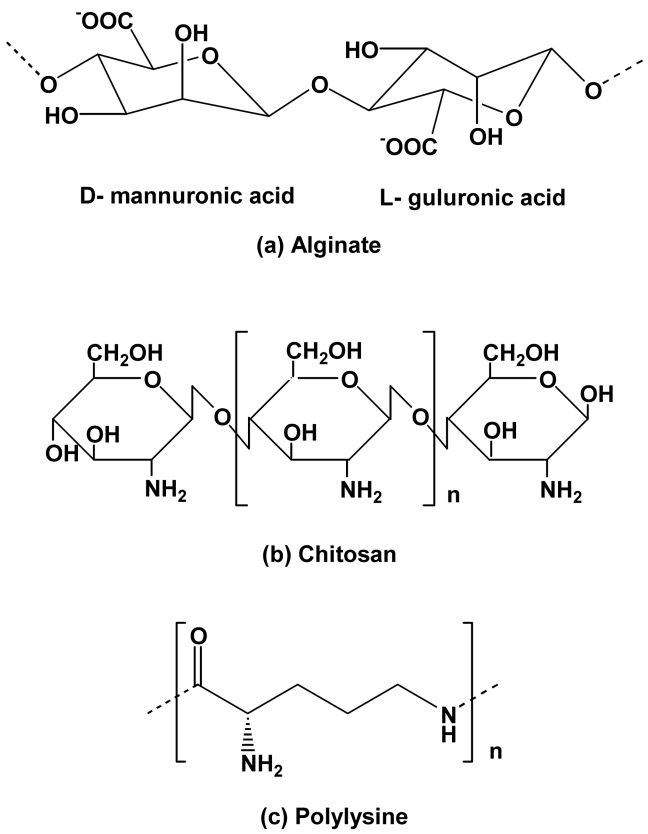

Fig. 1. Structures of alginate (**A**), chitosan (**B**), and polylysine (**C**).

GENERAL ASPECTS OF ALGINATE MICROENCAPSULATION OF LIVE PROBIOTIC BACTERIA

Alginate is commonly obtained from brown seaweed as a natural polysaccharide, which forms a physical hydrogel in the presence of divalent cations such as calcium or barium. Because of its biocompatibility, non-toxicity, mildness of gelation conditions, and low immunogenicity, purified alginate has been widely used in the pharmaceutical and food industries, as well as for biomedical and therapeutic purposes [9, 34, 74]. Alginate, an anionic polysaccharide composed of D-mannuronic and L-guluronic acids (Fig. 1A), forms simple gelation with calcium, where the polyguluronic acid blocks bind effectively with calcium ions better than the polymannuronic acid blocks. The strength of the binding depends on both the nature of the cations and the properties of the polymer. Because of the cooperative nature of crosslinking by the polyguluronic units and crosslinking ions, gelation of alginate can occur very rapidly. For example, aqueous alginate solution added dropwise into a calcium-containing aqueous bath will form gel beads *via* rapid diffusion of calcium into the alginate. Such "external gelation" method has been employed for entrapment of live cells or macromolecules into microbeads for the delivery of therapeutic agents [9, 34, 77]. Alginate forms a gel in contact with calcium in solution by crosslinking between the carboxylate anions of alginate guluronate units and the calcium ions [41]. This crosslinking mechanism can be defined in terms of an "egg box" model, which is a cooperative binding of calcium ions between the aligned polyguluronate ribbons of alginate (Fig. 2) [79]. In addition to the ionic bonding with the carboxyl groups, the calcium ion binding is strong because various rings and hydroxyl oxygen atoms are able to chelate the cations.

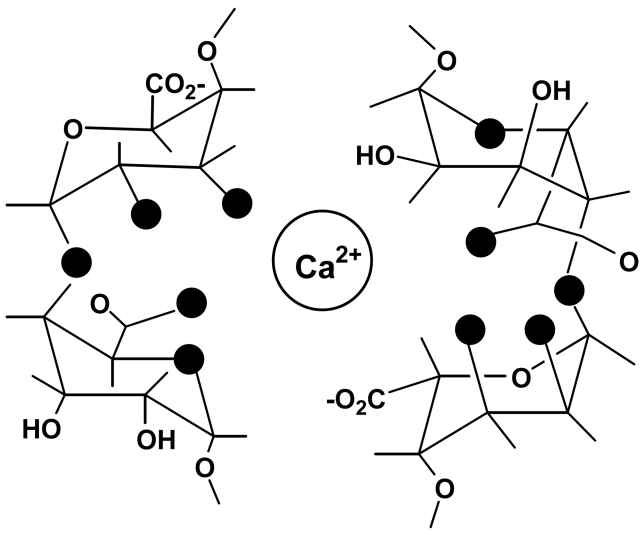

Fig. 2. Schematic representation of the association of polyguluronate within the alginate molecule with calcium [79].

Alginate is used as a salt form, mostly as sodium alginate, to crosslink with calcium ions in order to regulate a controlled reaction [79]. After gel formation between sodium alginate and calcium crosslinking, alginate microparticles can be formed, which are not chemically stable to protect the encapsulated contents because of the presence of non-gelling cations such as sodium or magnesium ions and chelators such as phosphate and citrate [25]. Sultana *et al.* [68] reported that encapsulation of probiotic bacteria in alginate beads was not able to effectively protect the organisms from high acidity. Therefore, to increase the stability of alginate microparticles and prevent the loss of encapsulated contents, various cationic polymers are usually used. Chitosan, a well-known and most widely used cationic polymer, has been successfully used as a coating agent [68]. Chitosan is a cationic polysaccharide derived from alkaline deacetylation of natural polymer chitin (Fig. 1B). It is also non-toxic, biocompatible, mucoadhesive, and bioavailable. Coating with cationic chitosan provides strength to alginate microparticles [30]. Chitosan not only can bind with any anionic polymers but also can adhere with a negatively charged mucosal surface, which are very important properties for oral delivery of drugs. Polylysine is another useful cationic polymer (Fig. 1C) that has been successfully used as a coating material for alginate microparticles in the microencapsulation of live bacterial cells.

In the present manuscript, we have focused on several microencapsulation systems for oral delivery of live probiotic bacteria. The importance of chitosan and polylysine are discussed, as coating polymers for the alginate microparticulate system to improve their stability for microencapsulation of probiotic microorganisms. Several other biopolymers including whey protein, xanthan gum, and carrageenan gum are also discussed.

Microencapsulation of Live Probiotic Bacteria Using Alginate Microparticulate Systems: *In Vitro* and *In Vivo* Studies

Chitosan-Coated Alginate Microparticulate System

Chitosan coating provides stability to alginate microparticles for effective microencapsulation of therapeutic live cells. The positively charged amino groups of chitosan and negatively charged carboxylic acid groups of alginate form a membrane on the microparticle surface, which reduces the leakage of entrapped materials from the particles [31]. Various research works were carried out to investigate the potentiality of a chitosan-coated alginate microparticulate system for increasing the survival and stability of entrapped live probiotic bacterial cells [14, 32, 42, 68, 75].

The survival and stability of probiotic bacteria loaded into chitosan-coated alginate microparticles are largely dependent on the molecular weight of chitosan. *Lactobacillus bulgaricus* KFRI 673-loaded alginate microparticles were coated with chitosans of three different molecular weights to investigate the survival and stability of *Lactobacillus bulgaricus* KFRI 673 in simulated gastric (pH 2.0) and intestinal fluids (pH 7.4) [42]. Before microencapsulation, the authors examined the survival of free *L. bulgaricus* KFRI 673 in simulated gastric fluid (SGF) of pH 2.0 and in simulated intestinal fluid (SIF) of pH 7.4. In SGF, none of the cells survived after 60 min. On the other hand, survival of the *Lactobacillus* strain was fully maintained in SIF over the time period until 120 min, suggesting that *L. bulgaricus* KFRI 673 is pH-sensitive and cannot survive in acidic pH conditions. Therefore, microencapsulation of the *Lactobacillus* is essential for its survival when given orally. After microencapsulation, the survival of *L. bulgaricus* KFRI 673 was investigated for all microparticle batches after sequential incubation in SGF and SIF. The incubation time in SGF was optimized at 0, 30, 90, and 180 min. After then, 180 min incubation was carried out in SIF as for sequential incubation. The microparticles prepared with high molecular weight chitosan provided a higher survival rate (46%) compared with the microparticles made with low molecular weight chitosan (36%). Chitosan-uncoated alginate microparticles showed lower survival (25%) of *L. bulgaricus* KFRI 673. The prepared microparticles stability was also investigated at 4°C and 22°C during a four weeks period. Both the free and the microencapsulated cells showed similar stabilities at 4°C, whereas high molecular weight chitosan-coated alginate microparticles appreciably improved the *Lactobacillus* stabilities at 22°C compared with free cells and the other respective batches. This was due to the thicker membrane of the microparticles made with high molecular weight chitosan, which protected the microencapsulated *Lactobacillus* better than the microparticles made with low and medium molecular weight chitosans and non-encapsulated cells [42].

In 2007, Urbanska *et al.* [75] reported the survival and stability of *Lactobacillus acidophilus* encapsulated into chitosan-coated alginate microcapsules (CCAMs) in different pH conditions. They investigated this formulation in yogurt for therapeutic delivery of *L. acidophilus*. Microcapsules loaded with *L. acidophilus* were observed as a homogeneous spherical shape after preparation. The fixed bacterial cells loaded in each subsequent microencapsulation were kept constant in a concentration of 10^{10} CFU/ml. *L. acidophilus*-loaded CCAMs were incorporated in yogurt and their survival was investigated in comparison with free cells in SGF for 2 h, which was the estimated retention time of capsules in acidic stomach. Encapsulated *L. acidophilus* suspended in yogurt showed better survival compared with free cells in SGF. After the gastric transit, the microcapsules were exposed to SIF for 6 h, and the results showed that *L. acidophilus*-loaded CCAMs and their incorporation in yogurt also retained their viability best compared with free

cells as well as the free cells suspended in yogurt. Thus, it is obvious that in both SGF and SIF, the encapsulated bacteria can survive better compared with non-encapsulated cells and yogurt-inherent cells because of the protective chitosan-coated alginate membrane. They also reported that CCAMs prepared with 0.5% chitosan coating provided the highest survival of bacteria after 4 weeks. Through this study, they claimed that CCAMs and their incorporation in yogurt provided a suitable oral delivery system for *Lactobacillus* [75]. They also investigated the mechanical stability of microcapsules loaded with *L. acidophilus* in MRS broth exposed to mechanical shaking at 150 rpm at 37°C for 72 h. After intense mechanical stress, it was observed that there were no physical damage of the microcapsules and changes in their shapes, suggesting that these microcapsules are potent enough to protect the encapsulated bacterial cells from the harsh GI conditions. To verify their stability in oral delivery, a human GI model was also used to investigate the microcapsules loaded with *L. acidophilus* by subsequently exposing to SGF of pH 1.98 and SIF of pH 6.5 after shaking at 150 rpm, 37°C. To investigate this, microcapsules were first incubated in SGF for 3 h and thereafter subsequently incubated in SIF for 3 h, 12 h, and 24 h. The results showed that the capsules successfully maintained their structure in SGF and decreased their integrity while exposed to SIF, indicating their potentiality in oral administration of live probiotic bacteria.

To investigate the potential of the chitosan-coated alginate microparticulate system in retaining the viability of probiotic bacteria, an *ex vivo* experiment was done by Iyer *et al.* [32] where they examined the release of *Lactobacillus casei* strain Shirota (LCS) from chitosan-coated alginate starch (CCAS) microcapsules in different sections of *ex vivo* porcine GI contents. LCS-loaded CCAS microcapsules (containing 10^8 CFU of LCS) were incubated in different sections of *ex vivo* porcine GI contents at 37°C under anaerobic condition up to 24 h. It was found that there was a complete release of LCS in the ileal content within 8 h, and about 12 h was needed for colon content under the similar condition. In contrast, a partial release occurred from duodenal and jejunal contents, while no significant release was found in gastric content even after 24 h of incubation. This study revealed that the capsules were able to release the live probiotic bacteria completely in *ex vivo* porcine ileal and colon contents, whereas release was insignificant in the gastric environment, indicating that probiotic bacteria can be protected from the adverse gastric conditions to reach the target site alive through the chitosan-coated alginate microparticulate carrier system.

Recently, Chen *et al.* [14] reported on genipin crosslinked chitosan-coated alginate (GCCA) microcapsules for live cell therapy application. They used bacterial *Lactobacillus plantarum* 80 $P^{(CBH1)}$ (LP80) and mammalian HepG2 cells, which were successfully encapsulated into GCCA microcapsules. The morphological stability and physical integrity of LP80-loaded GCCA microcapsules were attained after exposure to physiological medium and during long-term storage of more than 6 months post-encapsulation. The viability of encapsulated LP80 cells into the microcapsules was also counted after 6 months of storage at 4°C. A high LP80 cell viability was obtained (9.03 log CFU/ml beads) for GCCA microcapsules when stored in medium with 50% broth, whereas cell viability was reduced after being stored in physiological saline without any nutrient supply, albeit a considerable number of viable LP80 cells (5.38 log CFU/ml beads) were achieved. In contrast, however, free LP80 cells did not survive under the similar storage condition. The results demonstrated that LP80-loaded GCCA microcapsules effectively protected the encapsulated live microorganisms against death during long-term storage and also established a favorable microenvironment for bacterial growth and proliferation, which might be accelerated by cell–cell communication and cell–biopolymer interactions. On the other hand, HepG2 cells also remained viable to a certain extent even when encapsulated into GCCA microcapsules, although the metabolic activity of HepG2 cells apparently decreased within the microcapsules for a prolonged period of time. Although this microcapsule formulation introduced a novel microencapsulation system, further researches are required for live cell therapy application.

Polylysine-Coated Alginate Microparticulate System

It has been shown that microencapsulation of live probiotic bacteria into alginate-polylysine (AP) microparticulate systems provides effective protection against harsh environments and improves survival and stability of the encapsulated cells. Cui *et al.* [17–19] reported several works on alginate-polylysine microparticulate systems loaded with bifidobacteria to investigate their survival and stability *in vitro* and *in vivo* after oral administration. They used *Bifidobacterium bifidum* (BB) as live probiotic bacteria to encapsulate into AP microparticles [17]. The survival of *Bifidobacteria* was highly dependent on the pH of the exposing media, since they are very labile in low pH condition but quite stable at physiological pH. The number of BB increased gradually for 8 h (10^8 CFU/g) and then reached about 10^9 to 10^{10} CFU/g when incubated in SIF (pH 6.8) for over 12 h, suggesting that AP microparticles can be completely dissolved over 12 h in intestinal fluid without losing their activities. Encapsulation of BB into AP microparticles highly enhanced their survival in the low pH conditions ($>10^8$ CFU/g) compared with free BB ($<10^3$ CFU/g) after incubation in SGF (pH 1.5). The stability of BB-loaded into AP microparticles successfully maintained their survival at over 10^7 CFU/g during 16 weeks of storage at 4°C. This stability was significantly higher than free BB. They concluded that BB-loaded AP microparticles could be applied to various dairy products without significantly

losing the viability of the encapsulated probiotic bacteria at low pH condition [17].

The *in vivo* investigation with BB-loaded AP microparticles was studied in human volunteers [19]. BB-loaded AP microparticles significantly ($P<0.05$) increased the viability of bifidobacteria in human volunteers (approx 11.5 to 30 times) as compared with the control, unloaded bacterial cultures, suggesting that microencapsulation of bifidobacteria into AP microparticles highly improved the resistance to gastric acid. They claimed that oral delivery of AP microparticles loaded with bifidobacteria could be used without losing their viability in the acidic stomach condition in humans.

Alginate-Polylysine-Alginate Microcapsules

Although numerous microencapsulation systems have been investigated, one of the most promising formulations is the alginate-polylysine-alginate (APA) microcapsules, which have been successfully used for live cell therapy and other biomedical applications [40, 45, 69, 70]. The APA microcapsule membrane was first proposed in 1980 [44]. Since then, this microencapsulation system has proven to be an effective strategy for live cell immobilization. The APA microcapsules can be prepared by extruding the alginate mixture in a stirred $CaCl_2$ solution. The resulting alginate beads are then immersed in a polylysine solution and finally coated again by alginate to prepare APA microcapsules.

Chen *et al.* [13] investigated the potential use of APA microcapsules *in vitro* for oral delivery of live probiotic bacteria, using a dynamic simulated human GI model. This unique apparatus mimics the GI environments close to the actual human situation. The original APA microcapsules were spherical and uniform in shape with smooth surfaces and morphologically stable in the simulated stomach condition, although they did not retain their structural integrity after a 3-day exposure in simulated human GI medium. In the simulated stomach (pH<2), they remained intact during 2 h incubation but became weak when passing the simulated small intestine (pH>6.5) 4 h later. The microcapsule beads were still found intact at the phase representing the transverse colon, although forming a ghost-like structure. The integrity of microcapsules declined continuously as they passed through the colon, and after 72 h, only traces of the microcapsules were detected at the phase representing the descending colon, indicating that the APA microcapsules maintain their physical stability and integrity in acidic condition and gradually lose their structure as the pH increases. Furthermore, they investigated the survival of live *Lactobacillus plantarum* 80 (LP80)-loaded into APA microcapsules against the harsh gastric environment. After being exposed for 60 min in SGF (pH 2.0), no severe morphological damage of LP80-loaded APA microcapsules was found. About 80% of the encapsulated cells remained viable after a 5 min incubation of LP80-loaded APA microcapsules in SGF (pH 2.0), although viability was considerably decreased to 8.3, 2.6, and 0.2% after 15, 30, and 60 min, respectively, indicating that the APA microencapsulation system was effective but not properly sufficient to protect the entrapped live probiotic bacteria for oral delivery application.

Martoni *et al.* [49] also investigated the APA microcapsules loaded with bile salt hydrolase active LP80 cells, using a simulated human GI tract model. Microcapsules protected the entrapped cells in the simulated stomach and maintained cell viability above 10^9 CFU/ml at pH 2.5 and 3.0 after 2 h residence time, whereas viability decreased linearly over time at pH 2.0 although it was maintained above 10^6 CFU/ml under similar conditions. In simulated stomach condition at pH 1.5, microencapsulated cells were not viable after 30 min exposure time. These results suggest that microencapsulation of live bacterial cells into APA microcapsules has potential in oral delivery but is not fully effective to protect entire encapsulated cells against an acidic condition.

Recently, Urbanska *et al.* [76] reported the immunomodulatory and antitumorigenic effects of live probiotic bacterial cells microencapsulated into APA microcapsules in yogurt formulation, in mice carrying a germline *APC* mutation. *Lactobacillus acidophilus* was used as a therapeutic live probiotic bacterium to encapsulate into the APA microcapsules. Oral administration of *L. acidophilus*-loaded APA microcapsules in yogurt formulation in mice resulted in significant suppression of colon tumor incidence and tumor multiplicity, and reduced the tumor size as well. Furthermore, it was shown that treated animals exhibited fewer gastrointestinal intra-epithelial neoplasias with a lower grade of dysplasia in detected tumors. The results suggest that oral administration of probiotic *L. acidophilus* exerts antitumor activity even when encapsulated into APA microcapsules, which consequently leads to the reduction of tumor mass.

Although the APA microcapsules have been widely used for live cell immobilization and therapy, they have a limitation in oral administration because of their inadequate stability in the GI tract [36, 37, 46, 59]. To overcome this problem, Ouyang *et al.* [54] reported APPPA (alginate-polylysine-pectinate-polylysine-alginate) as multilayer microcapsules, which were designed, prepared, and characterized *in vitro* for oral delivery of *Lactobacillus reuteri* as a model probiotic bacterium [54]. The APPPA microcapsules' integrity, stability, and GI survival were investigated in SGF and SIF, and compared with the APA microcapsules. The result showed no damage of APPPA microcapsules for 12 h at 250 rpm mechanical shaking when exposed to SGF and SIF. The stability studies in different pH conditions revealed that 92.8±3.1% of APPPA microcapsules loaded with *L. reuteri* remained intact at pH 1, 3, 5, and 7, and no damages were observed

at pH 9 when challenged for 24 h. Furthermore, the microcapsules remained undamaged at pH 1, 3, 5, 7, and 9 after exposed to SGF and SIF, respectively, for 3 h with 250 rpm shaking at 37.2°C. The mechanical stability of APPPA microcapsules in SGF and SIF showed that 93.1±3.1% of APPPA microcapsules remained intact in SGF after shaking for 24 h at 150 rpm at 37.2°C, whereas 90.2±3.5% APA microcapsules remained intact under similar condition. Moreover, these microcapsules were exposed initially to SGF for 3 h and subsequently to SIF for 24 h at a mechanical shaking of 150 rpm at 37.2°C, where 95.4±3.6% APPPA microcapsules were found undamaged whereas 88.9±4.3% were calculated for APA microcapsules. The APPPA microcapsules showed better GI stability and permeability for cell encapsulation compared with the APA microcapsules, although *in vivo* investigation is needed for proper evaluation of these results.

Afkhami *et al.* [1] also investigated APPPA microcapsules *in vitro*, using a computer-controlled dynamic human GI model to show the impact of orally administered APPPA microcapsules on GI microbial flora. The results indicated that the biomaterials used to prepare APPPA microcapsule membranes do not significantly affect the bacterial flora of the GI tract. They summarized that although the APPPA microcapsule has shown encouraging results for oral delivery of live bacterial cells, further research is required for their therapeutic applications.

Alginate-Chitosan-Alginate Microcapsules

E. coli DH5, a genetically engineered bacterial strain containing the gene encoding urease was encapsulated into alginate-chitosan-alginate (ACA) microcapsules for oral therapy of uremia [35]. The stability of the ACA microcapsules was compared with APA microcapsules. The survival of entrapped live bacterial cells as well as the *in vitro* urea removal capacity for both the microcapsules was also investigated. The ACA microcapsules remained intact and with a spherical shape with smooth surface after shaking in SGF at 150 rpm for 24 h. On the contrary, APA microcapsules were observed as a wrinkled shape after being incubated in a similar condition. The survivals of live *E. coli* DH5 cells loaded in ACA and APA capsules were examined in SGF at 37°C for 2 h. Both ACA and APA microcapsules showed similar results, and a much higher survival rate (55%) was found for both microcapsules than for free cells (8.4%). It was found that alginate is more protective between both capsules, although no significant differences of urea removal capacity were found between both microcapsules loaded with live *E. coli* DH5 cells.

ACA and APA microcapsules were orally administered to rats to examine microcapsules stability *in vivo*. After 6 h of intestine residence time, the ACA microcapsules remained intact and spherical, whereas the APA capsules turned to a wrinkle shape and broke open, indicating that the stability of ACA microcapsules to protect the entrapped cells was higher than for APA. To support this *in vivo* result, they further investigated the *in vitro* stability of both capsules in SIF mixed with trypsinase enzyme. The result indicated that the APA microcapsule membranes were disrupted by the degradation of the peptide bond of lysine residues by trypsinase. On the other hand, no significant effects were found on ACA microcapsule membranes, suggesting that ACA microcapsules are a more effective and protectable carrier system for live bacterial cells against enzymatic degradation compared with APA microcapsules.

Modified Alginate for Microencapsulation of Live Probiotic Bacterial Cells

Modified alginates were also investigated for microencapsulation of live probiotic bacteria to improve their survival in acidic condition. In this regard, succinylated alginate and *N*-palmitoylaminoethyl alginate were prepared [8]. *Lactobacillus rhamnosus* was microencapsulated into unmodified and modified alginate beads to investigate their acid resistance and viability in acidic condition. To investigate the acid resistance of free cells and encapsulated cells, all the formulations loaded with *Lactobacillus rhamnosus* were incubated in SGF (pH 1.5) for 30 min. For free cells, the initial count was dropped from 1.0×10^8 CFU/ml to an uncountable level after 30 min. Moderate protection was achieved by the unmodified alginate beads loaded with *L. rhamnosus*. Succinylated alginate (SA) and succinylated chitosan (SC) beads loaded with the probiotic bacteria showed better protection in SGF, with a slight decrease of viability, although no significant ($P>0.05$) differences were achieved in protection of encapsulated cells between these two formulations. The best protection in SGF was obtained for *N*-palmitoylaminoethyl alginate with a slight decrease in bacterial cell viability from 2.5×10^7 to 2.2×10^7 CFU/ml. The minor loss of encapsulated cells from *N*-palmitoylaminoethyl alginate beads could have occurred from near or on the bead surface. *N*-Palmitoylaminoethyl alginate beads showed a promising formulation to protect the live bacteria from acidic environment and to improve their survival and stability.

Recently, Rao *et al.* [60] reported on a functionalized alginate to immobilize a *Lactobacillus* strain for lactic acid production. Lactic acid has a wide range of applications in pharmaceutical, agro-, food, and textile industries. Microbial fermentation has great advantages in producing an optically pure lactic acid compared with the chemical synthesis procedure of lactic acid production [29]. However, this method has limitations in large-scale commercial production because of the reduction of cell growth and increase in fermentation time [7]. To solve this problem, Rao *et al.* used an optically pure lactic-acid-producing *Lactobacillus delbrucekii* and immobilized it into succinylated alginate. The microencapsulation of *L. delbrucekii* cells into

succinylated alginate beads provided better stability and durability of the encapsulated live bacterial cells under an acidic environment compared with unmodified alginate. Moreover, the modified alginate beads loaded with *L. delbrucekii* showed increased cell mass entrapment and production of lactic acid with higher yields.

The investigations therefore suggest that modified alginates are able to provide a potentially promising microencapsulation system for therapeutic as well as industrial uses of live probiotic bacteria.

MISCELLANEOUS

Whey Protein-Coated Alginate Beads

Viable probiotic bacteria contained in dietary supplements are being well recognized for their health benefits and, therefore, are increasingly popular in the marketplace. These benefits are dependent on several factors such as bacterial strain selection including their repopulation in the gut, support of the intestinal health and function, offset of lactose intolerance, and support of the immune system [33, 51]. Although the beneficial effects of probiotic live bacteria are well reported, the problems of their survival and stability in various food products still remain a concern. By applying improved methods to increase the stability and survival of probiotic bacteria for their protection, using biopolymeric carriers, an increased delivery of the viable probiotic bacterial cells can be achieved in the human intestine. As a biopolymer used for a coating agent of probiotic live bacteria, whey protein also appears as a potential candidate because it is entirely biodegradable and frequently used in many types of food products. Kitabatake *et al.* [38] reported that whey protein derived from bovine milk was not digested in the gastric fluid when they were in the native state.

Recently, Gbassi *et al.* [26] demonstrated the microencapsulation of *L. plantarum* strains in an alginate matrix coated with whey protein. They used whey protein as a coating material to examine its ability to improve the survival of various *L. plantarum* strains such as *L. plantarum* 299v (LP 299v), *L. plantarum* CIP A159 (LP A159), and *L. plantarum* 800 (LP 800). SGF of pH 1.8 and SIF of pH 6.5 were used to test the viability of the encapsulated bacteria. From an initial count of 10.04±0.01 Log_{10} CFU/g for LP 299v, 10.12±0.04 Log_{10} CFU/g for LP A159, and 10.03±0.01 Log_{10} CFU/g for LP 800, the bacterial loads in coated beads after 60 min of exposure in SGF were 7.76±0.12, 6.67±0.08, and 5.81±0.25 Log_{10} CFU/g, respectively, whereas the loads of bacterial cells in uncoated beads under similar conditions were 2.19±0.09, 1.89±0.09, and 1.65±0.10 Log_{10} CFU/g for LP 299v, LP A159, and LP 800, respectively. In addition, no survival was noted for all the *L. plantarum* strains in uncoated beads after 90 min of exposure in SGF. Thus, a significant ($P<0.05$) difference was found in the survival of bacterial cells between coated and uncoated beads for all the strains. Their study further revealed that only bacteria in the coated beads survived in the SIF medium (37°C, 180 min) after SGF treatment, suggesting that whey coating significantly improved the bacterial survival in alginate beads.

Recently, Weinbreck *et al.* [78] studied the shelf-life of probiotic bacteria in dry products by microencapsulation. To investigate this, they encapsulated the probiotic *Lactobacillus rhamnosus* GG (LGG) strain with whey protein solution onto a core particle, and the viability of LGG was examined over time at 37°C by exposing the LGG preparation to different water activities (a_w=0.15 or 0.7). The results showed that the encapsulation did not improve the survival of encapsulated LGG in high water activity; however, their survival decreased less rapidly when the water activity was lower (<0.15), similar to the unencapsulated LGG. In contrast to these results, Crittenden *et al.* [16] provided contradictory evidence that the survival of probiotic bacteria can be improved at high water activities by microencapsulation. In their study, however, the effectiveness of the microencapsulation was not described at different water activities, temperatures, or with different bacterial strains. Therefore, questions to be raised are which methods, encapsulating materials, or water activities should be used to maintain the viability of various probiotic bacterial strains in dry products. Hence, more investigations should be carried out to increase the viability of probiotic bacteria in dry products by microencapsulation.

Other Encapsulating Materials for Live Probiotic Bacterial Microencapsulation

Recently, Ding and Shah [23] investigated various coating materials such as xanthan gum, carrageenan gum, guar gum, and locust gum to examine their effects on the stability of various microencapsulated live probiotic bacteria. Ten kinds of probiotic bacteria, namely *Lactobacillus rhamnosus*, *Bifidobacterium longum*, *L. salivarius*, *L. plantarum*, *L. acidophilus*, *L. paracasei*, *B. lactis* type Bl-04, *B. lactis* type Bi-07, HOWARU *L. rhamnosus*, and HOWARU *B. bifidum*, were microencapsulated using the above-mentioned coating materials. All encapsulated probiotic bacteria were studied for their acid and bile tolerance. Acid tolerance of the probiotic bacteria was investigated at pH 2.0 for a 2 h incubation period, and taurocholic acid was used for an 8 h incubation period to examine the bile tolerance of encapsulated probiotic organisms. The results indicated that probiotic bacteria encapsulated in alginate, xanthan gum, and carrageenan gum showed significantly better survival rate ($P<0.05$) compared with free bacteria under acidic condition. Moreover, when free probiotic bacteria were exposed to taurocholic acid, the viability of the free

bacterial strains was decreased by 6.63 Log CFU/ml, whereas only 3.63, 3.27, and 4.12 Log CFU/ml were reduced in probiotic organisms encapsulated in alginate, xanthan gum, and carrageenan gum, respectively. The results suggest that among the various encapsulating materials, xanthan gum and carrageenan gum appear to be as effective as alginate in protecting probiotic live bacterial cells from harsh acidic conditions.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Microencapsulation appears to be a promising technology to retain the potency of probiotic bacteria or other live bacterial cells to be delivered orally into the GI system in terms of therapeutic means. In this review, alginate microparticulate systems have been discussed to improve their stability and efficiency in live probiotic bacterial microencapsulation for oral delivery. It appears that the chitosan-coated alginate microparticulate system has effective applications for oral delivery of probiotic bacteria because chitosan coating showed good results in terms of the survival and stability of encapsulated live cells. *In vitro* studies demonstrated that they are physically stable to protect the encapsulated contents from enzymatic degradation and can provide better survival in the harsh stomach condition as well as in the intestine. *In vivo* studies further indicated that the stability and survival of entrapped probiotic bacterial cells can be improved with this encapsulating system. The potentiality of polylysine as a coating polymer for the alginate microparticulate system was also discussed. The modified alginate showed higher survival and protection of *Lactobacillus* in harsh acidic environment, and therefore it can be speculated that they are able to bring promising prospects in probiotic microencapsulation for various therapeutic purposes. The extensive researches in molecular biology and microbiology, and advances in biotechnology will continue to bring more valuable progresses in the field of probiotics. The novel probiotic strains isolation as well as their applications in metabolic induction and genetic engineering will lead to the generation of probiotic live bacteria with improved properties that can be utilized to enhance more health benefits. Therefore, it can be hoped that proper investigations into microencapsulation systems may provide efficient, effective, and frequent uses of live probiotic bacterial cells in advanced therapeutics and food product systems in the near future.

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