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Research Article

Comparative Evaluation of Viability of Encapsulated *Lactobacillus casei* Using Two Different Methods of Microencapsulation

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Abstract

Microencapsulation using two different methods, spray-drying and emulsion technique were applied to preserve the viability of the probiotic *Lactobacillus casei* during manufacture and refrigerated storage. As coating materials to encapsulate the probiotic by spray-drying method, compatible biopolymers alginate and chitosan were utilized, while as a cross-linking agent, $CaCl_2$ was used. In addition to the probiotic, oligofructose enriched inulin (Synergy 1®) as prebiotic was added to the medium intended for spray-drying. For microencapsulation of the probiotic by emulsion method, alginate and whey proteins were applied. Further, protective effects of four potential cryoprotectants (oligofructose enriched inulin, sorbitol, sucrose, lactose) were investigated when added to the whey proteins-Ca-alginate microparticles before freeze-drying. Experiments showed that chitosan-Ca-alginate microparticles and whey protein-Ca-alginate microparticles with high viability of *L. casei* were obtained using spray-drying and emulsion method, respectively. *Lactobacillus casei* encapsulated by emulsion method survived better during 6-months storage at refrigerated conditions than *L. casei* encapsulated by spray-drying. Cryoprotectants have no additional effect on the cells survival during freeze-drying, while sorbitol has shown negative impact on the probiotic viability encapsulated in whey protein-alginate and subsequently freeze-dried. No significant differences in viability of *L. casei* during storage at refrigerated conditions were observed, both in whey proteins-Ca-alginate microparticles as control and formulations containing cryoprotective agent.

1. INTRODUCTION

Beneficial health properties of probiotics, prebiotics and other functional compounds strengthened the production, the offer and requirement of functional food products and/or pharmaceuticals worldwide. The efficacy of the administered functional products containing probiotic bacteria largely depends on the viability of the cells and their release in the lower intestine in sufficient number. Different techniques of microencapsulation have been used as a potential tool to enhance the viability of probiotics and to control release of these cells across the intestinal tract[1,8]. It is important the encapsulation method applied does not reduce viability of the cells and does not inhibit their activity which includes resistance of the cells to gastrointestinal environment and their ability to adhere to intestinal mucosa.

Spray-drying is a method of microencapsulation where an aqueous solution containing the sensitive active core material and solution of wall material are atomized into hot air. The production of stable microparticles with low diameters and homogenous size distribution can be considered as advantage of the spray-drying method in comparison to extrusion or emulsion methods[3,4,10-17]. The main disadvantage of spray-drying technique is viability loss resulting from simultaneous dehydration and thermal inactivation of probiotic cells during the process[5,18-25]. Emulsion technique does not involve severe conditions, both in terms of temperature and solvents used, while has the benefit of producing very small (< 100 µm) microparticles[6,7,26-31]. Examples of biomaterials often applied to encapsulate bacterial cells include alginate, gelatin, chitosan, carrageenan, whey proteins, cellulose acetate phthalate, acacia gum, gellan and xanthan gum and starches[1,2, 32-35]. Alginate beads are sensitive to the acidic environment, while cellulose acetate phthalate and mixture of gellan-xanthan are not soluble at acidic pH and enable high resistance towards acid conditions. Amphoteric nature of gelatin and whey protein allows favorable interaction with anionic polysaccharides when the pH is adjusted below the isoelectric point of the protein component thus the net charge of the protein becomes positive[8,36-40]. The behavior of the proteins below their isoelectric point encourages cooperation between proteins and polysaccharides in terms of higher survival rate of probiotics during processing and after consumption. Due to certain advantages and disadvantages of an applied method of encapsulation and coating agents, it is important to compare different biomaterials and encapsulation methods using the same probiotic strain.

Lactobacillus casei is an acid sensitive, rod-shaped, facultative heterofermentative lactic acid bacterium that can be isolated from a variety of environments including raw and fermented milk and meat or plant products, as well as the oral, intestinal, and reproductive tracts of humans and animals. It is a beneficial microorganism that helps to promote other beneficial bacteria and prevents the overgrowth of pathogenic bacteria in the human body. It has been reported that it can improve and intensify digestion, control diarrhea, reduce inflammations of the gut, reduce lactose intolerance and alleviate the symptoms of constipation, all leading to better function of the immune system[9,10,41-44]. Recently, there has been intensive usage of this probiotic in functional food products. However, many reports indicate poor survival of the probiotic in these products and also the survival in the human gastrointestinal system is questionable[11,45-57]. In our preliminary studies, 3-hour exposure to simulated gastric acid at 37°C using a horizontal shaking water bath with 75 strikes/min (0.08 M HCl; 2 g/L NaCl; 3 g/L pepsin; pH 1.5) resulted in poor survival of *L. casei,* 2.68±0.28 log cfu/g *vs.* 10.51±0.58 log cfu/g as initial cell count, while with 10- hour continuous exposition to bile salts in pH 6.8 (0.05 M KH_2PO_4;

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10 g/L bile salts), the viability of the probiotic decreased to 4.18±0.13 log cfu/g[12,58-62]. Considering that, providing *L. casei* with a physical barrier against adverse environmental conditions could significantly improve its stability thus ensuring its health effects.

The aim of this study was to evaluate the effects of coating materials and applied methods of encapsulation on the viability of probiotic strain *Lactobacillus casei* - 01 during preparation and storage of chitosan-Ca-alginate and whey protein-Ca-alginate microparticles. The probiotic whey protein-Ca-alginate microparticles were obtained using an emulsion method, while probiotic/synbiotic chitosan-Ca-alginate microparticles were prepared by spray-drying method. As the spray-drying method is associated with aggressive conditions of encapsulation, it is worthwhile to add prebiotic or other protective agent in the medium before drying. Thus, to improve the viability of probiotic *L. casei* during preparation and storage of the chitosan-Ca-alginate microparticles, the medium for spray-drying was supplemented with oligofructose enriched inulin (Synergy 1®) as prebiotic. Further, the viability of *L. casei* using different cryoprotective agents was determined and compared with *L. casei* loaded in whey protein-Ca-alginate microparticles as control.

2. MATERIALS AND METHODS

2.1 Microencapsulation by spray-drying

Probiotic/synbiotic chitosan-Ca-alginate microparticles were prepared by modified method of spray-drying[13] that was previously successfully applied for microencapsulation of sensitive drug 5-aminosalicylic acid[14,15, 63-65]. The feed suspension was prepared of carrier agent sodium alginate (4% *w/w*) (Protanal LF 10/60 LS, fG 35-45%, FMC BioPolymer, IMCD, Ayrshire, UK) and oligofructose enriched inulin (1.5% *w/w*) (Orafti® Synergy 1, Orafti-Rue L. Maréchal, Tienen, Belgium) which is the mixture of oligofructose (DP 2-8) and long-chain inulin fraction (DP 10-60). Then, overnight inoculated culture of *Lactobacillus casei* - 01 (Chr. Hansen, Hoersholm, Denmark) into MRS broth at 37 °C was added to alginate-prebiotic mixture. Alginate suspension enriched by prebiotic and probiotic *L. casei* with a cell load *ca*. 12 log cfu/g was infused into a spray-dryer (Büchi Mini Spray Dryer B-290, Flawil, Switzerland) at a constant air inlet temperature of 120 °C and outlet temperature at 58±3 °C. The flow rate of the spray-drying process was 6 ml/min, nozzle diameter 0.7 mm, aspirator pressure 90% and atomizer pressure 600 Nlh^{-1}. The dried powder samples were collected in sterile containers and then slowly added into previously prepared solution of 5% (*w/w*) $CaCl_2$ (Merck, KGaA, Darmstadt, Germany) and 0.5% (*w/w*) chitosan (France Chitine, Marseille, France) in 1% (*v/v*) acetic acid under continuous stirring using a magnetic stirrer for at least 3 h at room temperature. The hardened microparticles were separated by centrifugation at 1500 x *g* for 10 min, washed with sterile saline solution and frozen at -20 °C. Afterwards, the microparticles were freeze-dried at 0.070 mbar and -50 °C for 24 h (FreeZone Freeze Dry System, Labconco, Kansas City, USA).

2.2 Microencapsulation by emulsion method

For the preparation of probiotic whey protein-Ca-alginate microparticles, emulsion technique adapted from Mandal et al.[16] combined with subsequent coating according to the method proposed by Gbassi et al.[17] was used[12]. Namely, aqueous suspension of sodium alginate (2.5% *w/w*) and *L. casei* (cell load *ca*. 12 log cfu/g) was emulsified in olive oil (Sigma-Aldrich, St. Louis, USA) with 0.2% Tween 80 (Merck, KGaA, Darmstadt, Germany) while stirring magnetically. The emulsion droplets were hardened by quickly added $CaCl_2$ and the mixture was allowed to stir for additional 15 min. The particles were removed from the aqueous phase by centrifuging at 1000 x *g* for 5 min and subsequently rinsed with sterile water until complete removal of the oil phase. Additional coating of the alginate particles was performed into 3% *w/w* native whey protein solution (100% Hydrolyzed whey protein isolate – ISO 100, Dymatize Nutrition, Farmers Branch, USA) by stirring at initial speed of 900 rpm to disperse the particles. After 60 min, the coated particles were separated by centrifugation at 1000 x *g* for 5 min, rinsed twice with sterile water, frozen at -20 °C and freeze-dried at conditions given in the previous section. Further, the cryoprotective effects of oligofructose enriched inulin, sorbitol, sucrose and lactose during freeze-drying of *L. casei* loaded whey protein-Ca-alginate microparticles were investigated. Thus, 1 ml of 10% (*w/v*) solution of tested cryoprotectants in phosphate buffered saline was mixed with 5 g of prepared microparticles before freeze-drying and cell viability determined after freeze-drying and 8-week of cold storage.

2.3 Enumeration of encapsulated *L. casei*

For quantitative determination of cell viability, it was necessary to release the encapsulated *L. casei* from the particles. Thus, one gram of particles was suspended in 9 ml phosphate buffer solution (pH 6.9) at room temperature until complete release of the probiotic cells. Measurements of viable cells in the suspensions were determined using plate-count method on selective MRS agar with incubation at 37 °C under aerobic conditions for 72 h. The average of the results obtained in triplicates was expressed as colony-forming units per gram or ml of sample (cfu/g or cfu/ml) using following equation[18]:

$$a = \frac{a_n \times C_n}{V}$$

Where, a is the number of colonies expressed as cfu/ml, a_n is the number of colonies determined at dilution *n*, *V* is the volume of the sample in ml and C_n is reciprocic value of the dilution *n*. Afterwards, results calculated in cfu/g were transformed to log cfu/g to make the reading of the paper more friendly.

RESULTS AND DISCUSSION

3.1 Viability of *L. casei* encapsulated by spray-drying versus emulsification

Probiotic/synbiotic chitosan-Ca-alginate microparticles using a spray-drying method and probiotic whey protein-Ca-alginate microparticles using an emulsion method with high cell rate were produced. The encapsulation efficiency of the emulsion method applied was higher (95.8±0.7%) compared with the spray-drying method, 89.5±1.2. The increased efficiency of microencapsulation of the probiotic cells using an emulsion technique is probably due to the mild conditions of the procedure applied. Herewith, the reduction of the survival rate of encapsulated probiotic cells in whey protein-Ca-alginate microparticles was lower than it was in probiotic/synbiotic loaded chitosan-Ca-alginate microparticles before freeze-drying (Table 1), although, the medium intended for spray-drying was supplemented by prebiotics as inulin and oligofructose, which may be useful for enhancing probiotic viability during spray-drying and storage[19].

Table 1: Viability of *L. casei* during microencapsulation; (A) using spray-drying technique, biopolymers, alginate (4% *w/w*) and chitosan (0.5% *w/w*), $CaCl_2$ (5% *w/w*) as cross-linking agent and oligofructose enriched inulin as prebiotic (1.5% *w/w*), and (B) using emulsification, encapsulating materials, alginate (2.5% *w/w*) and whey protein (3% *w/w*), and $CaCl_2$ (3% *w/w*).

Sample	Viability of *L. casei* in initial alginate suspension (log cfu/g)	Viability of *L. casei* before coating by chitosan/whey proteins (log cfu/g)	Viability of *L. casei* after freeze-drying (log cfu/g)
A	12.52±0.15	11.84±0.11	11.28±0.26
B	11.26±0.27	10.91±0.19	10.55±0.21

Spray-drying process is advantageous in view of rapidity and relatively low cost[20] as well high reproducibility and suitability for industrial applications[1]. However, high temperature and rapid evaporation of water during atomization of a suspension of probiotics and encapsulating agents in a drying chamber may destroy the integrity of the cell membrane provoking significant inactivation of the cells despite short residence time in the hot air drying chamber. Thus, the initial viability of *L. casei* in alginate suspension prepared for spray-drying was significantly higher in respect to the alginate bacterial suspension prepared to emulsifying. The concentration of sodium alginate as encapsulating agent applied in both methods used to encapsulate the probiotic *L. casei* is also different in accordance with our previous investigations. Namely, the optimization procedures using a full-factorial experimental design have shown that the probiotic/synbiotic chitosan-Ca-alginate microparticles and probiotic whey protein-Ca-alginate microparticles with advanced physicochemical (particle size, zeta potential and Ca content) and enhanced functional properties (probiotic viability during

microencapsulation and in simulated gastrointestinal conditions) can be obtained when alginate at respective concentration of 4% *w/w*[13] and 2.5% *w/w*[12] was applied.

3.2 Effects of protective polymers on viability of encapsulated *L. casei*

Sodium alginate is often applied for encapsulation of probiotic cells due to its low cost, biocompatibility and suitability to form gels easily by selective binding of divalent ions, while properly resolve in the intestine and release entrapped cells. Alginate microparticles improved survival of *L. casei* NCDC-298 in simulated GI conditions and exposure to heat[16] and had no negative effect on adhesion of probiotic cells to HT-29 cell line of the human epithelium[21]. However, alginate particles are sensitive to acid medium and they can be easily disintegrated in the presence of monovalent ions or salts as phosphates, lactates and citrates which bond calcium ions. Additional disadvantage of alginate particles is their porous surface[22]. On the other hand, the polyelectrolyte nature of alginate enables improving its properties as encapsulating agent by creating of electrostatic interaction with other polymers or by using additives to cause structural modifications of alginate[23]. Physical and chemical stability of negatively charged alginate particles may be improved when they are coated with chitosan due to the structure obtained by cross-linking of opposite charged polymers where disorientation of the calcium ions is not possible, even the particles are subjected to monovalent ions or antigelling agents. Chitosan-alginate particles have more strength and possess increased resistance to environmental conditions[24]. There are studies indicating that high molecular chitosan creates particles with better mucoadhesive properties[25] and improves the survival of probiotic cells under gastrointestinal conditions[26,27].

The protective properties of alginate may be enhanced through combination of proteins due to various functional groups and significant number of potential interactions. Electrostatic interactions between polysaccharides and proteins produce complex structures that can be resolved or precipitated depending on the polymer properties (cationic, anionic), pH, ionic strength and ratio polysaccharide/protein[8]. Physicochemical properties of the obtained microparticles (elasticity, swelling, resistance) using a combination of polysaccharide and protein mainly depend of the conditions of manufacture, thus convenient encapsulating matrix may be designed by optimizing the conditions. Whey proteins as biocompatible and biodegradable components are potential candidates to improve protective effects of alginate, especially synergizing the surface properties of whey proteins with stabilization properties of polysaccharides in water-oil-water and oil-water-oil double emulsions[28]. Other studies have already shown whey protein as effective protectant of probiotic survival during exposure to simulated gastrointestinal fluids and characterize whey protein as suitable carrier of viable and active probiotic cells[17,29-31]. In our study, the loss of viable cell number of encapsulated *L. casei* was 0.36 log after freeze-drying of whey protein-alginate microparticles, while for chitosan-Ca-alginate microparticles it was 0.56 log. The increased survival of *L. casei* during freeze-drying in presence of whey protein is probably due to increased stability of proteins compared to sugars at temperature of transition of ice to liquid form[32].

3.3 Cryoprotective effects of sugars during freeze-drying of encapsulated *L. casei*

The study of Desmond et al.[33] has showed better survival of *L. paracasei* during drying when mixture of reconstituted skim milk (10%) as protein matrix and acacia gum (10%) as sugar compound was applied. Thus, to investigate whether adding of sugars or polyhydric alcohol to proteins may increase survival of encapsulated *L. casei*, probiotic whey protein-alginate microparticles were supplemented by oligofructose enriched inulin, lactose, sucrose and sorbitol before freeze-drying. The experimental results of the viability of encapsulated *L. casei* during freeze-drying and subsequent storage at 4°C using oligofructose enriched inulin (OEI), sorbitol (SORB), lactose (LACT) and sucrose (SUCR) as protective agents, are shown in Fig. 1, with respect to the whey protein-alginate microparticles (WP-ALG) as control and chitosan-Ca-alginate microparticles loaded with *L. casei* and oligofructose enriched inulin (CTS-ALG-OEI). Survival of encapsulated *L. casei* during the freeze-drying process was similar for whey protein-alginate microparticles and for the three of the tested cryoprotective agents (oligofructose enriched inulin, lactose, and sucrose). Sorbitol showed lowest protection of encapsulated cells from drying injuries, although its penetrating effect as polyalcohol should be more efficient than the effect of non-penetrating substances as oligosaccharides. During storage at 4°C, there was no significant difference observed either among the tested protective agents, or in comparison to the control. The addition of protective compounds as sucrose and trehalose and prebiotics as Raftilose® (fructooligosaccharides), Hi-maize®, and Raftiline® (inulin) maintains the viability of probiotics during dehydration and storage of freeze-dried yoghurt, although the viability was not significantly lower in the samples without added protectants[34]. These indicate that the tested prebiotics have no additional cryoprotective function, thus the use of prebiotics as cryoprotective agents should be further established.

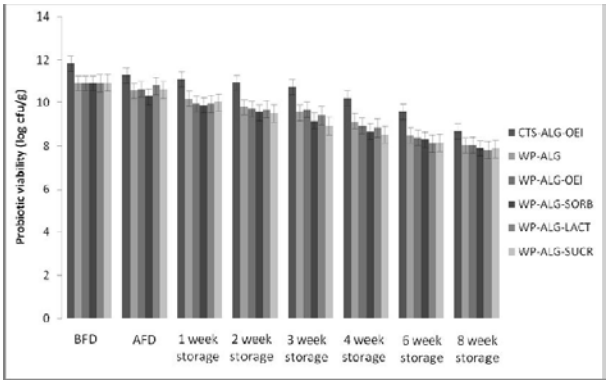

Figure 1: Viability of encapsulated *L. casei* during freeze-drying (BFD - before freeze-drying and AFD - after freeze-drying) and subsequent cold storage for 2 months of microparticles obtained by spray-drying method (CTS-ALG-OEI) and emulsification (WP-ALG) and whey protein-Ca-alginate particles using protective agents, oligofructose enriched inulin (WP-ALG-OEI), sorbitol (WP-ALG-SORB), lactose (WP-ALG-LACT), and sucrose (WP-ALG-SUCR). Values are presented as averages (n=3)±standard error.

3.4 Comparative viability of encapsulated *L. casei* during storage

In our study, to preserve the viability of *L. casei* using a spray-drying technique, polyelectrolyte complexation between alginate and chitosan was used, while using an emulsion technique, an electrostatic interaction between alginate and whey protein was applied. During 8-week of cold storage, the reduction of viable cell number encapsulated in synbiotic chitosan-Ca-alginate microparticles and probiotic whey protein-alginate microparticles was similar. After 3 months of storage at 4°C, the viability of *L. casei* encapsulated by emulsifying method was significantly higher (for approximately 0.8 log) with respect to the viability of *L. casei* encapsulated by spray-drying, although the second method includes prebiotic during drying. This may be due to the more stressful conditions of the spray-drying process, but also owing to different properties of the encapsulating materials applied. Conjugates obtained between amino groups in proteins and the reducing-end carbonyl residues in saccharides, generally have superior surface stabilization properties[28], compare to their components before conjugation. The high protective potential of whey proteins in a view of probiotic viability is probably due to their buffering capacity which provided good shielding for encapsulated *L. casei* against harsh conditions during drying and exposure to simulated gastrointestinal fluids.

The synbiotic microparticles manufactured using a spray-drying method and probiotic whey protein-Ca-alginate microparticles produced using an emulsion method were able to preserve the therapeutic level of viable cells within 4 months (6.9±0.24 log cfu/g) and 6 months (6.95±0.18 log cfu/g) of refrigerated storage, respectively. The polyelectrolyte complex between alginate and chitosan cross-linked with calcium ions is relatively porous, while whey proteins create strength anchorage for the cells that are embedded into the protein milieu[30], thus the cells may retain their stability longer. The comparative results of the applied methods and encapsulating materials with respect to tolerance of *L. casei* during

processing and storage conditions presented in this study are very valuable, considering that previously published work never considered comparison of methods, using a same probiotic strain and literature is relatively poor with these type of data.

In conclusion, synbiotic chitosan-Ca-alginate microparticles and *L. casei* loaded whey protein-alginate microparticles showed potential for further implementation as functional ingredients to manufacture probiotic and/or synbiotic products with prolonged expiry date considering the proposed level of viable cells in the commercial product at the end of the shelf-life to be at least 10^6 cfu/g or ml in order probiotics to achieve positive effects[35, 66, 68].

4. CONCLUSION

The effective preservation of the probiotic cells applying spray-drying and emulsion techniques as encapsulation methods and appropriate selection of coating materials provided the survival rate of microencapsulated *L. casei* in chitosan-Ca-alginate microparticles and whey protein-Ca-alginate microparticles to be above the therapeutic level within 4 and 6 months of cold storage, respectively. No further improvement in survival of encapsulated cells coated by whey protein was observed when cryoprotective agents were added to the formulation before freeze-drying.

Nevertheless, both microparticulate formulations, the synbiotic chitosan-Ca-alginate microparticles and whey protein-Ca-alginate microparticles, are potential candidates to be further used as functional ingredients in pharmaceutical and food industry.

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