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MICROENCAPSULATION OF *LACTOBACILLUS CASEI* IN CHITOSAN-Ca-ALGINATE MICROPARTICLES USING SPRAY-DRYING METHOD

Tanja Petreska Ivanovska[1], Lidija Petruševska-Tozi[1], Margita Dabevska Kostoska[2], Nikola Geškovski[1], Anita Grozdanov[3], Chris Stain[4], Trajče Stafilov[5], Kristina Mladenovska[1]*

[1]*Ss. Cyril and Methodius University, Faculty of Pharmacy, Vodnjanska 17, 1000 Skopje, Republic of Macedonia*
[2]*Center for Public Health – Tetovo, 29 Noemvri bb, Tetovo, Republic of Macedonia*
[3]*Ss. Cyril and Methodius University, Faculty of Technology and Metallurgy, Rudjer Bošković 16, 1000 Skopje, Republic of Macedonia*
[4]*University of Reading, Centre for Advanced Microscopy, Reading RG6 6AH, Whiteknights, United Kingdom*
[5]*Ss. Cyril and Methodius University, Faculty of Natural Sciences and Mathematics, Arhimedova 5, 1000 Skopje, Republic of Macedonia*
e-mail: krml@ff.ukim.edu.mk

In this study, the probiotic *Lactobacillus casei* was microencapsulated using the method of spray-drying combined with polyelectrolyte complexation of alginate, fructooligosaccharide and chitosan, and cross-linking with calcium chloride, followed by freeze-drying. Survival rate and physicochemical properties of the prepared microparticles were evaluated. In addition, viability of *Lactobacillus casei* in simulated gastric and intestinal juices was investigated. Positively charged microparticles with average size of 11.08±1.1 μm and high cell viability of 10.98±0.11 log cfu/g were prepared. The synbiotic microparticles were stable during exposure to simulated gastric and intestinal juices, while release of viable cells above the therapeutic value (8.31±0.14 log cfu/g) in the simulated colonic pH was observed.

The presented method for microencapsulation of synbiotics shows potential for effective protection of viable probiotic cells during exposure to harsh environmental conditions.

Keywords: *Lactobacillus casei*; chitosan-Ca-alginate microparticles; spray-drying method

МИКРОИНКАПСУЛИРАЊЕ НА *LACTOBACILLUS CASEI* ВО ЦИТОЗАН-Ca-АЛГИНАТНИ МИКРОЧЕСТИЧКИ СО ПРИМЕНА НА МЕТОДОТ НА СУШЕЊЕ СО РАСПРСНУВАЊЕ

Во рамките на ова истражување се подготвени синбиотски микрочестички со *L. casei* со примена на методот на сушење со распрснување комбиниран со полиелектролитно комплексирање на алгинат, фруктоолигосахарид и цитозан, вкрстено поврзување со $CaCl_2$ и последователно лиофилизирање на честичките. Одредени се физичко-хемиските својства на микрочестичките, виталноста на *Lactobacillus casei* во честичките и во симулирани желудочни и интестинални услови. Подготвените микрочестички се карактеризираат со позитивен полнеж, среден волуменски дијаметар од 11,08±1,1 μm и високо ниво на преживување на *L. casei* (10,98±0,11 log cfu/g). Синбиотските микрочестички покажаа значителна стабилност во симулиран желудочен и интестинален сок, додека нивото на

ослободени витални клетки во симулирани услови на колон го надминува терапевтскиот минимум (8,31±0,14 log cfu/g).

Опишаниот метод за микроинкапсулирање на синбиотици покажува висок потенцијал за ефикасна заштита на пробиотските клетки од агресивните услови на средината.

Клучни зборови: *Lactobacillus casei;* цитозан-Са-алгинатни микрочестички; метод сушење со распрснување

1. INTRODUCTION

Probiotics are living microorganisms which upon ingestion with food or food supplements in adequate number confer a health benefit on the host [1–2]. Regarding the incorporation of probiotics into pharmaceutical and food products, attaining the satisfactory number of viable probiotic cells during the product's shelf life is the most important challenge from commercial and technological point of view [3]. Any probiotic product must contain at least 10^6 –10^7 cfu of viable probiotic bacteria per g of the product at the time of it consumption to exert beneficial effects on human health [4].

Development of new formulation that would provide better probiotic's viability during processing and especially during the passage through gastrointestinal tract is the essence [5]. Microencapsulation is a promising technique for enhancing the probiotic's viability and several studies have been carried out investigating the protective role of microencapsulation against adverse conditions to which probiotics can be exposed [6–12]. There are various techniques widely used for microencapsulation of probiotics such as spray-drying, emulsion, extrusion, adhesion to starch, matrix encapsulation and coating of microcapsules [13]. The protective biopolymers used as coating agents during microencapsulation reduce the injury and cell loss by decreasing the influence of the numerous factors of the environment, such as atmospheric moisture, oxygen, acid, high temperature, pressure, attack by bacteriophages and enable targeted delivery of probiotics to the colon [14]. Although different protective materials are applied during the microencapsulation process [5], natural biopolymers alginate and chitosan are of continuous interest due to their biocompatibility, potential for effective preservation of probiotics and targeted release of viable cells in the colon [15].

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 with extrusion or emulsion methods [16–18]. The main disadvantage of spray-drying technique is viability loss resulting from simultaneous dehydration and thermal inactivation of probiotic cells during the process [5, 16]. One approach to improve the survival of probiotics is to add prebiotics [19] or protectants [20] to the media prior to drying. The addition of prebiotics in the medium can decrease the negative effect of spray-drying process by providing carbon and nitrogen sources that are important factors for enhancing the growth of probiotics [21]. In this study microparticles were prepared by spray-drying of alginate matrix enriched with *Lactobacillus casei* as probiotic and fructooligosaccharide as prebiotic, followed by polyelectrolyte complexation of alginate with chitosan, cross-linking with calcium chloride and subsequent freeze-drying. The method and the protective polymers used were previously successfully applied for microencapsulation of sensitive drug 5-aminosalicylic acid [22, 23].

2. EXPERIMENTAL

The probiotic strain used in this study was pure freeze-dried *Lactobacillus paraca-*

sei subsp. *paracasei* with commercial name FD-DVS *L.casei*-01 (Chr. Hansen, Denmark). The prebiotic fructooligosaccharide (FOS) was supplied from Sigma-Aldrich. As encapsulation agent, alginate (Protanal LF 10/60 LS, fG 35-45%) was applied, while for additional coating of spray-dried microparticles, chitosan with deacetylation degree ≥ 85% and low viscosity 342, M_w 150 kDa (Chitine, France) and for cross-linking procedure, calcium chloride (Merck, Germany) were used. The Man Rogosa Sharpe (MRS) broth, MRS agar and peptone water were provided from Merck, KGaA, Germany. Bile salts used for preparation of simulated intestinal juice were purchased from Sigma-Aldrich (Ox gall, Sigma-Aldrich, Pool, UK). For production of spray-dried microparticles, a Büchi spray dryer (Büchi Mini Spray Dryer B-290, Switzerland) was used, while freeze-drying process was performed using Freeze-Dryer, Labconco, USA. Morphology of the microparticles was observed using two different techniques: high vacuum SEM (Leo 1450 VP, Germany) and cryo-SEM (Quanta 600 FEG, USA). Furthermore, following physicochemical properties were determined: particles size (Mastersizer Hydro-2000G, Malvern Instruments Ltd., UK), calcium-content (atomic emission spectroscopy-inductively coupled plasma (AES-ICP), Varian, USA) and zeta-potential (Zeta-sizer Nano ZS, Malvern Instruments Ltd., UK). The viability tests of *L.casei* in simulated gastrointestinal conditions were performed using a water bath with horizontal shaker (HAAKE, SWB 20, Denmark).

2.1. *Microencapsulation and physicochemical characterization of L. casei loaded microparticles*

A slant of the freeze-dried probiotic culture was inoculated into 5 ml MRS broth and incubated at 37 °C for 24 h under aerobic conditions. The cells were harvested by centrifugation at 3000 rpm for 10 min and washed with sterile 0.1 % peptone solution. The bacterial cell count in the initial suspension was adjusted to 12.2 log cfu/g. Washed bacteria suspension (0.005 % *L. casei*) was added to previously prepared solution of 2.5 % w/w sodium alginate and 1.5 % w/w FOS. The conditions of the spray-drying process were: inlet temperature of 120 °C and outlet temperature of 60 °C, aspiration of 90% and air flow velocity of 5–6 ml/min. Obtained microparticles were slowly added into solution of 0.3 % w/w chitosan and 2.75 % w/w calcium chloride during continuous stirring using a magnetic stirrer for 3 h. After polyelectrolyte complexation of the oppositely charged polymers, alginate and chitosan and cross-linking with calcium chloride, the particles were separated by centrifugation at 3000 rpm for 10 min. The washed microparticles were frozen at –20 °C and submitted to freeze-drying at constant temperature of –50 °C and pressure of 0.070 mbar for 24 h.

To measure the particle size of microparticles, an aqueous suspension of the particles was dispersed into ultrasound cell for 15 min. and then the samples were added to water cell of Mastersizer until laser obscuration exceeded 10 %. The mean particle size of the microparticles was expressed as d_{50}. Using high vacuum SEM technique, the microparticles previously coated with chromium were scanned at an accelerating voltage of 30 kV. For cryo-SEM observations, the samples were embedded in low temperature adhesive, frozen at –180 °C and coated with platinum. The zeta-potential of suspended microparticles in 0.1 mM phosphate buffer (pH 6.8) was measured by dynamic light scattering. Sodium ions originated from sodium alginate were substituted with calcium ions and the degree of substitution was calculated determining the content of Ca^{2+} and Na^{+} by atomic emission spectroscopy-inductively coupled plasma (AES-ICP), after degradation of microparticles with nitric acid (2.5 mg/ml).

2.2. *Enumeration of L. casei*

Viability of probiotic cells entrapped in microparticles was followed during the process of microencapsulation and in simulated gastric and intestinal juices in comparison with uncoated cells. For that purpose 0.1 g of microparticles were liquefied with 9.9 ml phosphate buffer solution (pH 6.9) and vortexed 30 s. The suspension was allowed to stand at room temperature for 15 min to dissolve. After serial dilutions in 0.1% sterile peptone solution, *L. casei* was enumerated on selective MRS agar in a triplicates and incubated at 37 °C under aerobic conditions for 72 h [24]. The average of the results was expressed as colony-forming units per gram of sample (cfu/g).

2.3. *Viability of L. casei in simulated gastrointestinal conditions*

Viability of microencapsulated cells of *L. casei* was determined in a simulated gastric juice (0.08 M HCl; 0.2 % NaCl; pH 1.5) by using a horizontal shaker with 75 rpm according to the method described by Mokarram *et al.* [25]. Freshly prepared microparticles were treated in simulated gastric solution in proportion 1:10. Viability of probiotic cells, encapsulated or alone was determined in different time intervals of 0.5, 1.5 and 3 h and enumerated as it is described above.

In order to compare the viability of encapsulated cells of *L. casei* with free ones in bile salts solution, the microparticles treated in simulated gastric juice firstly were washed with 0.1% sterile peptone solution and then transferred in 9 ml of sterile simulated intestinal juice (0.05 M KH_2PO_4; pH 6.8) with 1 % filter sterilized bile salts. The tubes were incubated at 37 °C for additional 0.5, 1.5 and 3 h and then enumerated on MRS agar.

Release test of viable cells of encapsulated and free *L.casei* in colon was carried out as described by Mandal *et al.* [26]. Washed microparticles, after being incubated in simulated intestinal juice, were placed in simulated conditions of colon (0.1 M KH_2PO_4; pH 7.4) and then incubated up to 24 h. The viability of *L.casei* was determined in different time intervals of 10, 12 and 24 h. Non-encapsulated cells of *L.casei* were treated in the same conditions and enumerated as it is described in a previous section.

3. RESULTS AND DISCUSSION

3.1. *Viability of L. casei during microencapsulation and physicochemical properties of the microparticles*

The average size of the microparticles obtained after freeze-drying was 11.08±1.1 μm. In general, the morphological analysis of the freeze-dried microparticles showed spherical shape. However, the surface of the microparticles was wrinkled due to the encapsulated cells within the particles and loss of water content during spray- and freeze-drying process (Figure 1). Characteristic complex microstructure of the fractured chitosan-Ca-alginate microparticles loaded with *L. casei* was obtained (Figure1b). The inner part of the microparticles was built of a mesh-like alginate network through which the bacteria groups were distributed and sequestered in voids, that is in agreement with the results of Allan-Wojtas *et al.* [27].

The influence of each phase of the microencapsulation process on viability of probiotic bacteria was observed separately. When comparing the viability of *L. casei* after spray-drying alone or with alginate and fructooligosaccharide, increased survival in the microparticles for 4 logs was observed. After the microencapsulation process, high cell viability of the probiotic in the particles was achieved (10.98±0.11 log cfu/g), which is within the established therapeutic value [4]. Although the most often used techniques for microencapsulation of *L. casei* are extrusion and emulsion [5, 14], the high survival rate of *L. casei* within the microparticles obtained in this study shows

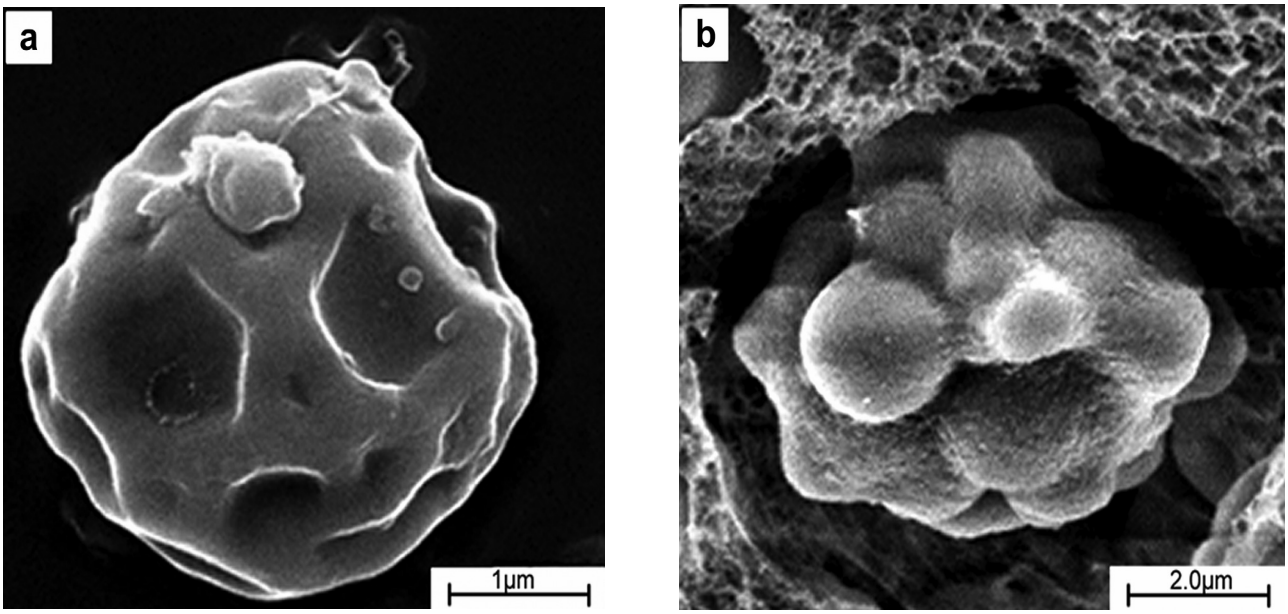

Fig.1. Micrographs of the whole (a) and fractured (b) chitosan-Ca-alginate microparticles loaded with *L. casei*; a) high vacuum SEM; b) cryo-SEM technique

the potential of the spray-drying method to be successfully applied for probiotic preservation. Due to the mild conditions of the extrusion method, high cell loading of *L. acidophilus*, *B. bifidum* and *L. casei* in chitosan-coated alginate beads was achieved with viability loss lower than 1 log [28]. Another study showed variable entrapment efficiency of *L. casei* in beads made with sodium alginate, low methoxyl pectin and alginate-low methoxyl pectin depended on the total biopolymers concentration of the beads, although *L. casei* was entrapped by the extrusion method [12]. An emulsion method applied to encapsulate *L. casei* resulted in slight decrease of the cell viability about 0.5 logs [26], while the same method used to encapsulate *L. acidophilus* and *L. rhamnosus* showed entrapment efficiency of 99.8% [25]. Further, there are literature data showing viability loss of *L. rhamnosus* encapsulated in double emulsions formulated with sweet whey as emulsifier for almost 1.5 logs [29] and survival rate of encapsulated *L.paracasei* higher than 60% after spray freeze drying [30]. In comparison, the viability of *L. casei* in this study was reduced for approximately 1 log which is acceptable cell loss in view to the high temperature associated with the spray-drying process. Additionally, increased content of Ca^{2+} in the microparticles increased the strength and stability of alginate coating that affects the survival rate of *L. casei* during exposure to high acidity and bile salts. The content of Ca^{2+} was 0.88±0.02 mg/10 mg of microparticles or 9.77 expressed as degree of substitution of Na^{+} ions with Ca^{2+} ions. The degree of substitution was calculated from stoichiometric relationship of sodium alginate and $CaCl_2$ according to eq. DS = [198 / (3*a* + 2000)] 100, where *a* is content of Ca^{2+} in 100 g of microparticles [22, 31]. The zeta potential of the freeze-dried microparticles was 14.5±0.7 mV indicating the ability of microparticles to adhere to the negatively charged intestinal mucosa.

3.2. *Viability of microencapsulated L. casei in simulated gastrointestinal conditions*

Viability assays in simulated gastrointestinal conditions were carried out on non-encapsulated cells of *L. casei* and freeze-dried particles loaded with *L. casei*. The survival rate

of non-encapsulated cells after 3 h exposure to gastric juice was 8.12±0.2 log cfu/g and after additional 3 h exposure in bile salts solution it was 6.66±0.13 log cfu/g. From an initial count of 10.98 log cfu/g, the probiotic load in microparticles after 3 h exposure in gastric pH was 8.64±0.11 log cfu/g and after additional 3 h exposure in pH 6.8 it was 8.04±0.03 log cfu/g. After incubation in simulated colonic pH significant difference in survival between the free and encapsulated cells was observed, 5.85±0.1 log cfu/g and 8.31±0.07 log cfu/g, respectively (Figure 2).

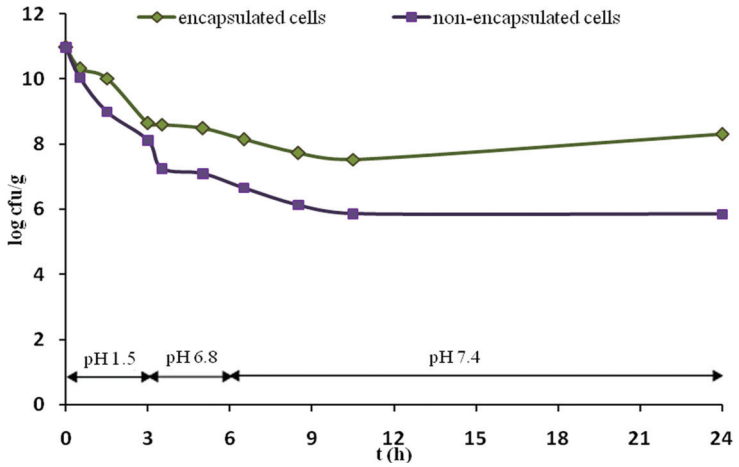

Fig. 2. Viability of non-encapsulated and microencapsulated *L. casei* in simulated gastric conditions (pH 1.5), bile salts solution (pH 6.8) and colonic pH (7.4)

The results indicated that microencapsulation of *L. casei* by spray-drying method using alginate as encapsulating material, chitosan as additional coating agent and calcium chloride for polyelectrolyte complexation, offered significant protection of probiotic cells from high acidity and antimicrobial effects of bile salts. This result is consistent with that of Krasaekoopt *et al.* [6], who found that bacterial cells of *L. acidophilus* and *L. casei* encapsulated in chitosan-alginate beads using extrusion technique survived better in low pH conditions. Another study showed that microencapsulation of *L. gasseri* and *B. bifidum* with alginate and chitosan coatings when using extrusion technique maintain their viability during simulated gastric and intestinal juices, which is important factor for effective delivery of viable cells to the colon [15]. Various studies showed protective potential of alginate coatings in reduction of viability loss of probiotics during exposure in acidic conditions [21, 26, 32]. Chitosan-alginate microparticles ensure higher protection of viable probiotic cells in intestinal conditions due to their ion-exchange reaction resulting in formation of insoluble complex between chitosan and bile salts which probably limits the diffusion of bile salts into the particles [33]. Increased viability of *L. casei* in bile salts solutions was observed when cells were encapsulated with alginate and chitosan in comparison with non-encapsulated cells or alginate-coated cells [34]. In addition, the effect of an advanced emulsion method of encapsulation on the survival rate of *L. casei* under harsh gastrointestinal conditions has been studied [35]. Namely, the viability of the encapsulated cells in artificial gastric and bile juices was higher when comparing with non-encapsulated ones and the *L. casei* preservation was better in comparison with the encapsulated *L. casei* in this study. However, the absence of micropores on the surface of the Ca-alginate microcapsules prepared with microporous glass membrane emulsification system does not ensure effective cell release in the colon.

The presented microencapsulation method shows potential for effective preservation and targeted release of viable probiotic cells in the colon. Beside stability in simulated gastrointestinal conditions, obtained chitosan-Ca-alginate particles loaded with *L. casei* showed optimal moisture content of 3.77 %, which is in accordance with approved level of moisture content of up to 5% [36]. Low content of moisture is necessary to keep live probiotic cells in long term period. Additionally, spherical microparticles with mean sizes smaller than 100 μm can be incorporated in different food products without negative influence on the texture of the food. Unlike, there is literature data reported that small particles might negatively influence the cell viability in simulated gastric fluids [37]. In order to achieve Ca-alginate microbeads with size below 40 μm able to provide effective protection in acid and bile environment, a novel technique based on dual aerosols of alginate solution and calcium chloride cross-linking solution was successfully applied [38]. The microparticles obtained in this study, although smaller than 100 μm, efficiently protected the encapsulated *L. casei* under investigated conditions thus indicate the potential for application in pharmaceutical and food products. This suggestion is much valuable due to the favorable safety profile of alginate and chitosan based on proven biocompatibility, biodegradability, bioactivity [39, 40] and their approval for human use [41]. However, further studies are needed for optimal formulation to be prepared and *in vivo* effects of the probiotic to be confirmed.

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