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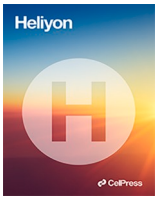

Research article

Application of biomacromolecules encapsulation systems for the long-term storage of *Lactobacillus plantarum* F1 and *Lactobacillus reuteri* 182

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ABSTRACT

The aim of this study was to improve the viability of lactic acid bacteria (LAB) during extended storage of 1 year and mechanical characteristics of the calcium alginate beads with co-encapsulation of prebiotics and chitosan coating and subsequent freeze drying. The results revealed that the addition of trehalose to alginate matrix effectively protects the LAB cells during freeze drying, i.e., the survival rate has increased up to more than 92.5 %. Chitosan coating reinforced Ca-alginate beads, therefore the sphericity and mechanical strength of the beads improved. The findings also showed that bacteria encapsulation with the prebiotics resulted in more cells stability during the prolonged storage of 1 year and were 4.82 ± 0.06 log CFU g^{-1} in the lyophilized alginate-trehalose beads for *Lactobacillus plantarum* and 5.64 ± 0.08 log CFU g^{-1} in the lyophilized alginate-trehalose-inulin beads for *Lactobacillus reuteri*. No survival, however, was noted for the LAB cells in wet capsules after the same period. This study demonstrated that prebiotics had a significant impact on the viability of cells during freeze drying and storage. What is more, physical properties of the alginate beads were enhanced by coating beads with the chitosan.

1. Introduction

Over the last few years, the significance of prebiotics has become clear in protecting human health and preventing diseases. The rapidly growing interest extended beyond simply having the gut microbiome to the skin microbiome with its positive effect for managing some skin disorders [1]. Using prebiotic strategies for skin microflora composition and/or function manipulation has an apparent interest in the dermatology since, in contrast to antibiotics, it may enable selective inhibition of detrimental while simultaneously protecting and/or stimulating the beneficial bacteria [2]. What is more, probiotic microorganisms have also been used as prophylactic agents for burn wound infection prevention. In this respect, several investigations that used *Lactobacillus plantarum* reported its great potential as a beneficial adjunct in the management of complicated burn injuries [3]. Cosmetic industry, understanding the great value, as well as promising opportunities of this concept, has ventured into this branch by focusing its efforts towards skincare

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[4]. This, in fact, is a rapidly growing proof that these kinds of probiotics, either viable or in extract forms, can improve skin barrier function, as well as modulate skin immune system, helping to maintain skin homeostasis [5].

The definition of the term probiotic has been changed and broadened over the years; the majority of the suggested descriptions underline the significance of the sufficient number of active cells during consumption. It is assumed that this viable cell quantity must be higher than or equal to 10^6 - 10^7 Colonia forming units (CFU) per milliliter or gram of product until the expiration date of the product [6]. There are multiple studies pointing out that probiotic bacteria added to the products as planktonic cells have very low survival rate for they cannot survive severe conditions of temperature and pH, and viability drops even more during the processing of product and further storage [7]. Therefore, probiotics are encapsulated with the appropriate protective methods and matrix materials to achieve the required cell viability. However, despite the large number of materials and methods used for the encapsulation, the maintenance of adequate amount of viable microorganism, while retaining the desired quality properties of the final product, especially during the long-term storage, is still unresolved.

Many polymers have been used to produce encapsulation systems. One of the most used polymers is sodium alginate. Ease of its handling, availability, biocompatibility, non-toxicity and low-cost are major advantages leading to such frequent use. However, alginate has disadvantages such as high porosity, sensitivity to extreme pH values and moisture permeability, which result in insufficient encapsulation efficiency and low quality [8]. Based on literature available, there are several ways to overcome these shortcomings and improve the stability of the microorganisms [9]. The combination of polysaccharide as encapsulation matrix and addition of prebiotics can contribute to the stability of the encapsulated probiotics [10]. Encapsulation with both alginate and prebiotics is referred to as co-encapsulation [8]. Inulin and trehalose were selected as prebiotics to assess the co-encapsulation protective effectiveness to probiotic bacteria. The soluble fiber inulin is one of the most widely known probiotics and can be found in many economically important plants such as wheat, rye, etc. [11]. Trehalose was chosen not only for its prebiotic properties, but also for its known protective effect on cells during freeze drying. Another available method to increase encapsulation efficiency is to coat the alginate capsules through complex coacervation. For this purpose, chitosan, a semi-synthetic polymer, is one material that has been used. Chitosan is deacetylated chitin, obtained from a natural source, non-toxic, and low-cost linear positive charge polysaccharide. and can interact electrostatically with calcium alginate, adhering to the particle's surface thus increasing particle stability [12–14].

As the production and maintenance of a sufficient number of encapsulated probiotics is still in a research stage, the present study addressed the investigating a few different compositions of the encapsulation matrix: pure alginate without additives and coating; with prebiotics as a component part of the encapsulation matrix and coating the capsules with chitosan. Two indigenous bacterial cultures (*Lactobacillus plantarum* F1 and *Lactobacillus reuteri* 182) isolated from natural bread sourdough, to which no commercial leaven has been added, were selected as the probiotic models. The morphological and physical characteristics, encapsulation efficiency, probiotic cell viability during the extended storage time were assessed.

Bearing in mind that the presence of a sufficient number of viable microorganisms in the probiotic formulation is considered to be an essential criterion for health claims, the purpose of this study is testing different compositions of calcium alginate macrocapsules as a potential encapsulation system suitable for use in products with long shelf-life. Findings of this study, which was carried out for 12 months, could provide significant information for the use of probiotics in shelf-stable pharmaceutical, food, and cosmetic products. What is more, as the use of probiotics has increased, scientific literature is extremely rich in publications describing different effective encapsulation methods and materials. However, both the technology and the encapsulation material must be carefully selected and compatible with the biological material to be encapsulated. In this context, another aim of this study is to compare different encapsulation systems, to determine their advantages and disadvantages and compatibility with specific probiotic cultures.

2. Materials and methods

2.1. Materials

Sodium citrate and calcium lactate (Ca-lactate) with a purity of 99 %, were received from REACHEM (Bratislava, Slovakia). Alginic acid sodium salt (with 15–25 cP viscosity and derived from the brown algae) that was used for the encapsulation of cells, was obtained from SIGMA-ALDRICH CHEMIE GmbH (Steinheim, Germany). As well as medium molecular weight (270 kDa) chitosan (extracted and purified from crab shell) and inulin (derived from chicory roots). Inulin and trehalose (from UAB Labochema LT, Lithuania) were used as prebiotics.

2.2. Microorganisms and growth conditions

Pure cultures of lactic acid bacteria (LAB) *Lactobacillus plantarum* F1 and *Lactobacillus reuteri* 182 were isolated from food products and were supplied by the Food Institute of Kaunas University of Technology (Lithuania). In order to ensure long-term viability of LAB cells, they were diluted with MRS broth (De Man-Rogosa-Sharpe, Biolife, Italia), placed into sterile 2 ml screw-top vials and stored at −80 °C. *L. plantarum* and *L. reuteri* were kept under the following conditions: at +1 °C temperature on the MRS agar and in the aerobic environment. For the culture activation, *L. plantarum* was pre-cultivated in the MRS broth at +30 °C temperature and *L. reuteri* at +37 °C temperature for 24 h. For the second subculture, an inoculum of 1 % pre-activated cells was made and re-incubated for another 24 h in the same medium at the same temperatures.

Table 1
Compositions, encapsulation efficiency (%) and cells survival after lyophilization (%) of *L.plantarum* and *L. reuteri* with various capsules compositions. Values (in the same column) followed by the different letters are significantly different ($p < 0.05$).

Formulation	Encapsulation efficiency (%)		Survival rate (%)	
	L. plantarum	L. reuteri	L. plantarum	L. reuteri
2 % Alginate (A)	100 ± 0.00^{a}	100 ± 0.00^{a}	89.19 ± 1.13^{a}	90.72 ± 0.75^{a}
2 % Alginate + 4 % Trehalose (A-T)	96.57 ± 1.84^{b}	100 ± 0.00^{a}	92.54 ± 0.45^{b}	92.88 ± 0.23^{b}
2 % Alginate + 4 % Trehalose + 2 % inulin (A-T-P)	97.95 ± 0.41^{b}	97.98 ± 0.55^{b}	93.13 ± 0.22^{b}	92.47 ± 0.59^{b}
2 % Alginate + 0.4 % Chitosan (A-Ch)	92.89 ± 0.77^{c}	94.57 ± 1.83^{c}	89.52 ± 0.87^{a}	90.66 ± 0.54^{a}

2.3. Encapsulation procedure

2.3.1. Preparation of LAB cells

After the second re-incubation, the LAB cells were collected in the late-log phase using centrifugation (MPW 260 RH centrifuge, MPW Med. Instruments, Poland). The collection was performed at +4 °C temperature at 6000 rpm (4427*g*) for 10 min. The supernatant of the culture broth was discarded, and the cells were suspended in the peptone water (0.1 % wt/vol) (Oxoid, UK). Afterwards, the cells suspension was centrifugated under the same conditions. In order to wash the cells, this procedure was repeated. The harvested biomass was diluted with the peptone water until the final LAB cells concentration of 1-2×10^{9} CFU ml^{-1}.

2.3.2. Preparation of the alginate capsules

The prepared LAB cell suspension was mixed with aqueous sodium alginate solution (A) (2 % wt/vol) in order to obtain a cell to polymer solution at a ratio of 1:10. Similarly two mixtures with cultures and prebiotics were prepared (Table 1). The resulting mixtures were used in the subsequent following encapsulation procedure.

In order to obtain beads by the extrusion technique [15], the calcium lactate was selected as crosslinking ions source [16]. The mixture of sodium alginate solution and cells, prepared as described above, was extruded into a solution of the divalent cation salt Ca-lactate (5 % wt/vol) using 22G needle. After dripping, the beads were stored in the hardening solution for 30 min under agitation to reach the complete gelation. The resulting capsules were washed with sterile distilled water, filtered and stored at the refrigerated conditions (at +4 °C temperature) for the further investigation.

Part of the prepared beads were frozen at −20 °C and freeze dried (Christ ALPHA 2–4 LSC freeze dryer, Germany) under vacuum at −110 °C temperature. The dried beads were kept in the sealed containers in the dark at +4 °C temperature.

2.3.3. Coating of the capsules

The aqueous solution of 0.4 % (wt/vol) chitosan was made as follows: chitosan was first dissolved in acidified distilled water with moderate heating under stirring. The solution was filtered through Whatman #4 filter paper to eliminate any undissolved precipitates. Then 10 g of the washed capsules were submerged in a 100 ml of chitosan solution for 30 min with gentle agitation to achieve even coating of the surface of macro- and microcapsules. The resulted chitosan-coated (A-Ch) capsules were parted by filtration, rinsed two times and kept in the sterile distilled water at the refrigerated conditions (at +4 °C temperature) for the further use. Part of the resulting A-Ch capsules were freeze dried.

2.4. Enumeration of the encapsulated bacteria and survival assay

2.4.1. Determination of the encapsulation efficiency and the survival after freeze drying

With the aim of determination of the encapsulation efficiency of the probiotic cells, the number of viable cells was calculated using pour plate method. To distort the three-dimensional alginate network and release the cells, 1 g of capsules was added to 9 mL of sterile 2 % (wt/vol) sodium citrate solution. The culture was plated on MRS. The plates were incubated for 48 h at +30 °C for *L. plantarum* and at +37 °C for *L. reuteri* and the encapsulated bacteria calculated as CFU g^{-1}.

The encapsulation efficiency (E), defined as a combined measurement of the efficacy of encapsulation and survival of the cells during the immobilization process, was calculated as follows (Equation (1)):

$$E = \left(\frac{N}{N_0}\right) * 100\ \% \tag{1}$$

where N represents the number of viable encapsulated cells, released from the alginate capsules, and N_0 is the number of free viable cells added to the polymer.

The survival rate of the LAB cells after freeze drying was calculated by using Equation (2):

$$Survival = \frac{N_L}{N} * 100\ \% \tag{2}$$

where N_L (CFU g^{-1}) is the number of viable encapsulated cells in 1 g of lyophilized capsules and N (CFU g^{-1}) is the number of viable encapsulated cells in 1 g of wet capsules.

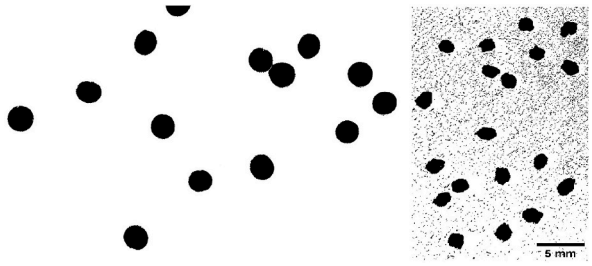

Fig. 1. The image analysis of calcium alginate (A) macrocapsules using ImageJ.

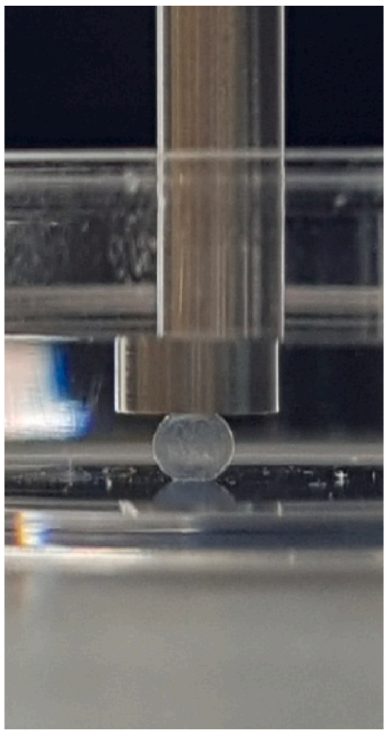

Fig. 2. The compression of a submerged calcium alginate (A) capsule.

2.4.2. Viability of LAB under refrigerated conditions during the prolonged storage

The viability of free and encapsulated LAB was monitored by counting CFU g^{-1} using the method described above. The survivals of bacteria were enumerated at 1 and 7 days, 1, 3 and 6 months and after 1 year of the storage at +4 °C temperature.

2.5. Characterization of the capsules

2.5.1. Morphology of the capsules and size determination

The beads microstructure was analysed using a scanning electron microscope (SEM) (JSM-7600 F, JEOL, Japan). An optical microscope (Primo Star, Zeiss, Germany) equipped with a digital camera (Axiocam ERc 5s, Zeiss, Germany) was used to observe the morphology of the capsules after the preparation and during disruption.

The size of the LAB-loaded calcium alginate macrocapsules was measured by taking the images of the macrocapsules and using an image analyser (ImageJ, USA) (Fig. 1). As a means to analyse the shape and morphology of the particles, the circularity (4 × pi × area/perimeter^2) was also obtained. The average beads diameters were defined by measuring thirty randomly chosen calcium alginate beads.

Below provided Equation (3) was used to calculate the shrinkage in beads size after freeze drying [17]:

$$k_{SF} = \frac{d_w - d_l}{d_w} \tag{3}$$

Table 2
Size and shape factors of various capsules compositions. Values shown are means ± standard deviation. Values (in the same column) followed by the different small letters and circularity factor values in the same row followed by the different capital letters are significantly different ($p < 0.05$).

Composition	Size, mm		Shrinkage factor k_{sf}	Circularity factor	
	Wet	Lyophilized		Wet	Lyophilized
A	2.75 ± 0.09^{a}	2.23 ± 0.18^{a}	0.19 ± 0.07^{a}	0.84 ± 0.07^{aA}	0.68 ± 0.08^{aB}
A-T	2.69 ± 0.14^{a}	2.21 ± 0.29^{a}	0.15 ± 0.07^{a}	0.87 ± 0.01^{abA}	0.76 ± 0.04^{aB}
A-T-P	3.08 ± 0.22^{b}	2.33 ± 0.13^{a}	0.24 ± 0.07^{a}	0.90 ± 0.01^{cA}	0.82 ± 0.06^{abB}
A-Ch	3.14 ± 0.10^{b}	2.45 ± 0.16^{a}	0.23 ± 0.05^{a}	0.86 ± 0.07^{bcA}	0.84 ± 0.04^{bA}

where k_{SF} represents the reduction factor related to the lyophilisation process; d_w (mm) is the average diameter of the wet calcium alginate beads before the freeze drying and d_l (mm) is the average diameter of beads after the freeze drying process.

2.5.2. Mechanical properties

Mechanical characteristics of the calcium alginate beads were established by the measurements of uniaxial compression of the beads with a 6 mm diameter cylindrical shape stainless-steel probe (TA-XT Plus Texture Analyzer, Stable Microsystems Ltd., Surrey, England). The capsules were compressed in the water to 30 % of their original height at +20 °C to prevent water loss during the compression (Fig. 2).

The compression rate was 1 mm/s. For the compression test six capsules were randomly picked from every group of samples. The Young's modulus (E) was calculated on the basis of the Hertz theory [18] (Equation (4)):

$$F = \frac{4R^{\frac{1}{2}}}{3}\frac{E}{1-\nu^2}\left(\frac{H}{2}\right)^{\frac{3}{2}} = \frac{4R^{\frac{1}{2}}}{3}E^{*}\left(\frac{H}{2}\right)^{\frac{3}{2}} \quad (4)$$

where F is the applied external force, R is the radius of the bead, E represents the Young's modulus, ν is Poisson ratio of the material and H is the displacement. $E^{*}=E/(1-\nu^2)$ is described as the reduced modulus of the sphere [19]. A linear graph of F against $H^{3/2}$ was plotted, and the reduced modulus was calculated as follows [20] (**Equation (5)**):

$$E^{*} = slope\left(\frac{3}{4}\right)2^{3/2} \Big/ R^{1/2} \quad (5)$$

Since the capsules are assumed to be incompressible under the high-speed compression, the Poisson ratio was considered to be 0.5 [21].

2.6. Statistical analysis

Data are expressed as means ± standard deviation. The tests were carried out in triplicates and data were subjected to one-way analysis of variance (ANOVA) followed by the Fisher LSD test using Statistica 8.0 software. A p-value <0.05 was considered statistically significant.

3. Results and discussion

3.1. Effect of composition of the capsules on the encapsulation efficiency and survival rate after the freeze drying

In this study, the encapsulation efficiency of probiotics was 92.89 ± 0.77–100 ± 0.00 % indicating low variation values of different compositions of the capsules (Table 1). The highest encapsulation efficiency of both LAB strains was achieved when using blank calcium alginate (A), while the survival rate of the encapsulated cells after lyophilization was the lowest. The significantly ($p < 0.05$) higher survival rate was achieved by adding trehalose to the polymer matrix, although the encapsulation efficiency in the A-T beads was lower for *L. plantarum*. In A-T-P compositions, the encapsulation efficiency via extrusion technique decreased for approximately 2 % (97.95 ± 0.41 % for *L. plantarum* and 97.98 ± 0.55 % for *L. reuteri*). This is in agreement with Gandomi et al. Kim et al., Ng et al. and Chan and Pui, who obtained lower encapsulation yield with the addition of prebiotics [22–25]. It might be explained by the capsules inner space being taken up by the prebiotics, thus resulting in lower number of the entrapped cells in the capsules as suggested by Ng et al. [24]. The survival rates of the LAB cells in the A-T-P capsules were similar to the A-T capsules. The results suggest that presence of trehalose during the microencapsulation of probiotics could improve the probiotics' survivability by acting as a cryoprotectant. Whereas most of the studies displayed no significant effect or higher encapsulation efficiency with the chitosan coating [26–28], this study demonstrated the significantly ($p < 0.05$) lower encapsulation efficiency (reduction of 7.11 % for *L. plantarum* and 5.25 % for *L. reuteri* compared to A beads) and no protective effect during freeze drying.

3.2. Size, shape, and morphology of the capsules

As shown in Table 2, the mean diameter of the fresh formed control A beads was 2.75 ± 0.09 mm. While the addition of trehalose

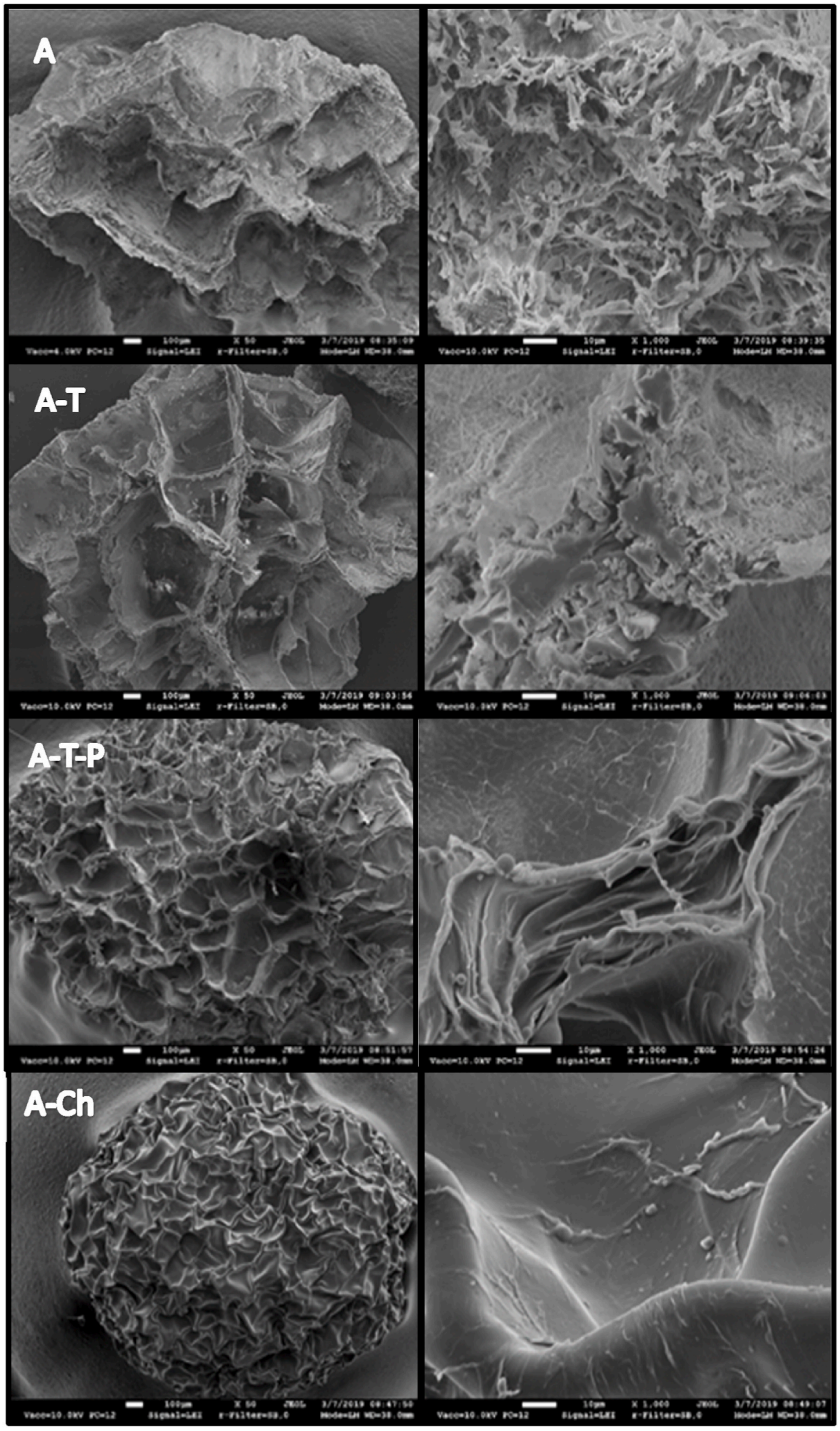

Fig. 3. Scanning electron microscopy images of the various compositions of the calcium alginate beads at 50x and 1000x magnification, respectively.

did not affect the size of the beads, the size of the capsules increased significantly ($p < 0.05$) with the co-encapsulation of the inulin. This could be due to the increase in viscosity of the polymer-inulin solution and in the surface tension of the drop [29]. Coating the capsules with chitosan yielded a significant increase in the particle diameter when compared to A beads and reached 3.14 ± 0.10 mm. This result indicated that the formation of thick layer of the oppositely charged polyelectrolytes was successful. The similar diameter of extruded calcium alginate beads was also obtained by Obradović et al. [14]. The study also confirmed an increase in the capsule size

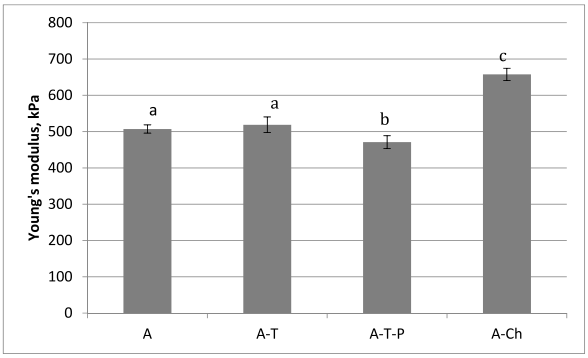

Fig. 4. The Young's modulus of the various compositions of calcium alginate beads. Error bars represent the standard deviation. Different letters on the columns indicate statistical difference ($p < 0.05$).

when coated with the chitosan layer. Regardless of the significant effect of the encapsulation solution composition on the size of wet beads, no differences were observed in the size of the beads after freeze drying. Our outcomes confirmed that lyophilization process caused reduction in the calcium alginate beads size. The size of the lyophilized macrocapsules varied from 2.21 ± 0.29 to 2.45 ± 0.16 mm with the highest shrinkage factor of the A-T-P and the lowest of the A-T composition. The k_{sf} values obtained in this study were similar to those reported by Chan et al. where starch was used as the bead filler [17]. It should be noted that in that Chan et al. study, the shrinkage factor was up to 0.6 [17], as well as in Balanč et al. [30]. Consequently, it can be considered that the shrinkage ratio could also be affected by the ratio of mannuronic and guluronic acids in the alginate composition and the cross-linker cation type.

The shape of the alginate capsules was described by the circularity factor (Table 2). The circularity value of 1.0 indicates a perfect sphere. All capsules had spherical shape before freeze drying irrespective of the capsule composition and the circularity values were above 0.8 in all cases. After lyophilization, the shape of the A and A-T beads were irregular. Yet, addition of the inulin and capsule coating with the chitosan helped to maintain the sphericity of the beads during lyophilization. Similar findings were reported in the previous studies [17,31].

As expected, microphotographs (Fig. 3) confirmed the reduced circularity of the A capsules.

During freeze drying process the water was eliminated from the hydrogel matrix, therefore the structure of the calcium alginate beads collapsed and the surface folded. Consequently, the surface was covered with the abundant protuberances and furrows. This rough texture in alginate microcapsules had been observed by Young et al. Song et al., and Yeug et al. [32–34]. Similar results were detected in the capsules with trehalose. With added inulin, the height ant thickness of the protuberances appears to lessen, which correspond to the literature data involved inulin's influence on the freeze dried capsules [30]. The A-Ch beads appeared spherical, with smoother wrinkles, without sharp jagged edges. This observation suggests that the chitosan coating was contributing to the hydrogel matrix resistance against the collapse.

3.3. Mechanical properties

The present results of the Young's modulus are in the range of 1–1000 kPa obtained from compression experiments [20]. According to the results presented in Fig. 4, the Young's modulus of the A-T beads remained similar to the control plain calcium alginate (A) beads, regardless of the trehalose addition to sodium alginate solution. Since the trehalose additive was found to have no influence on the Young's modulus of calcium alginate beads, the modulus had decreased significantly ($p < 0.05$) from 507 ± 11 kPa to 471 ± 18 kPa in the presence of inulin. Based on this observation, it could be stated that addition of the fiber inulin into the encapsulation solution led to the decreased stiffness, which contributed to the overall hardness. This result may be due to the reduced crosslinking density, because the hardness of the alginate beads depends on the degree of the crosslinking [35]. The Young's modulus value of the calcium–alginate beads with additive of the inulin obtained in this study is in a good agreement with that obtained by Balanč et al. where addition of the inulin to alginate hydrogel microbeads led to a lower stiffness [30]. As expected, due to the synergistic interactions between the polymers, the A-Ch beads presented increased Young's modulus compared to the control alginate beads (507 ± 11 kPa and 658 ± 17 kPa, respectively). This resulted in the ionic crosslinking between the functional groups of the chitosan and alginate, which created a rigid surface coating and strengthened the copolymer. This is in accordance to the observations of the several studies that determined the positive coating effect of the chitosan on the increase of Young's modulus [14,36–38]. It is known that the mechanical properties of the alginate capsules depend on the characteristics of the polymer, its molecular mass and the concentration, the cross-linker, the gelling environment, and the storage environment [35,39]. This study has shown that additives and/or modifications of the capsules also have a relevant influence on the mechanical properties of the capsules. These properties of the capsules are significant as they specify maximal forces that the capsules can withstand during processing and handling. Therefore, it is necessary to check the impact to the mechanical properties before making any changes on the composition of the capsules.

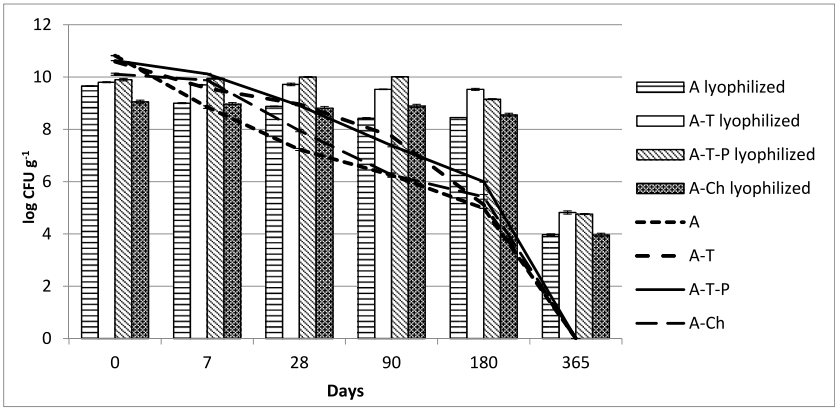

Fig. 5. The viability of the encapsulated *L. plantarum* in various capsules compositions during the extended storage at +4 °C. Error bars represent the standard deviation.

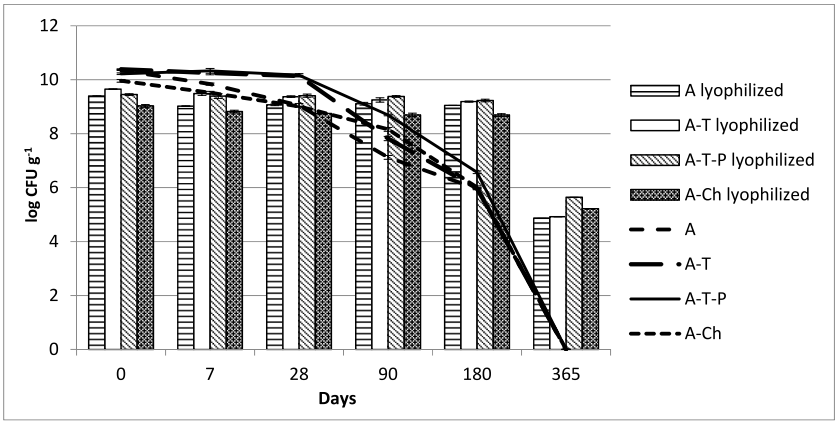

Fig. 6. The viability of the encapsulated *L. reuteri* in various capsules compositions during the extended storage at +4 °C. Error bars represent the standard deviation.

3.4. Influence of the beads composition on storage stability of the encapsulated LAB cells

Stability of the encapsulated LAB cells during 1 year of storage at +4 °C is shown in Fig. 5 (*L. plantarum*) and Fig. 6 (L. *reuteri*). *L. plantarum* seem to be more sensitive regarding storage at refrigerated conditions compared to *L. reuteri.* After 365 days of storage, the highest survival of encapsulated *L. plantarum* was detected in the freeze dried A-T and A-T-P capsules and was 4.82 ± 0.06 and 4.76 ± 0.02 lg CFU g^{-1} accordingly. The highest viability of the encapsulated *L. reuteri* cells after 1 year of storage was 5.64 ± 0.08 lg CFU g^{-1} in the freeze dried A-T-P capsules. No survival was, however, noted for the LAB cells in wet capsules after the same period.

The results also demonstrated that coating the beads with the chitosan had no additional protective effect on the encapsulated cells. After 1 year of storage viability in the A-Ch capsules was similar to the control A capsules. Taken together, the results revealed that the lyophilization and addition of the prebiotics enhanced the viability of the LAB cells under refrigerated conditions. These findings are in agreement with the results published earlier by Nami et al. [40] and Ng et al. [24]. The reason for this might be associated with prebiotics ability to act as cryoprotectant during the lyophilization. What is more, prebiotics being nutrients for the bacteria, might have had beneficial impact on the LAB cell recovery. However, a reasonable comparison is not possible since there are no studies on prolonged storage.

4. Conclusions

This study compared the influence of four different compositions of the encapsulation matrices on the mechanical properties of the calcium alginate beads and the effect on the survivability of two different lactic acid bacteria cells during the prolonged period of storage. To summarize, the results of this experiment showed that coating calcium alginate beads with chitosan was effective in improving beads mechanical and physical properties, such as Young's modulus, sphericity and surface smoothness. Although no major effect on the improvement of the bacterial viability has been observed when compared to the blank calcium alginate beads, viability remained moderately high after 1 year of storage. In contrast to the chitosan coating, addition of the trehalose to the encapsulation

matrix did not improve the properties of the capsules but increased survivability of encapsulated bacteria during the lyophilization process. Furthermore, the further addition of inulin excipients had a significant influence on the viability of the LAB cells during the prolonged storage. Thus, it can be employed industrially to produce probiotic products with improved storage stability. Also, further research should focus on a combination of addition of the prebiotics and coating capsules with chitosan to achieve the maximum quality. To our knowledge there are very few studies on the viability of encapsulated probiotics for 6–12 months or longer storage. Therefore, long term storage studies are particularly important in order to use probiotics in food, pharmaceutics and cosmetics that have a long shelf life. However, only two indigenous lactic acid bacterial cultures were tested. Knowing that even in biological classification closely related species have different characteristics, for example, different resistance to mechanical stress, oxygen, temperature, etc., the results of the study cannot be applied to the absolute majority of probiotic cultures.

Data availability statement

The data that support the findings of this study are available from the corresponding author, [S.J.], upon reasonable request.

CRediT authorship contribution statement

Sigita Jeznienė: Conceptualization, Methodology, Software, Formal analysis, Investigation, Data Curation, Writing – Original Draft, Visualization. **Ingrida Bružaitė:** Methodology, Investigation. **Aušra Šipailienė:** Conceptualization, Methodology, Validation, Writing – Review & Editing, Supervision, Project administration.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:Ausra Sipailiene reports financial support was provided by Research Council of Lithuania. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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