

Research Article

Immobilization of *Bifidobacterium infantis* Cells in Selected Hydrogels as a Method of Increasing Their Survival in Fermented Milkless Beverages

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The aim of the study was to examine whether immobilization of *Bifidobacterium infantis* inside hydrogels could prolong their survival in fermented milkless beverages. The starter culture *Streptococcus thermophilus* was used to obtain fermented nonmilk beverages: oat, oat-banana, and oat-peach. The biota of beverages were supplemented with *Bifidobacterium infantis* cells, free and immobilized, in three types of spherical hydrogel particles: microcapsules with a liquid and gelled core, microbeads of 0.5 mm diameter, and beads of 2.5 mm diameter. As a carrier material, low-methoxylated pectin and alginate were used. Microbeads and microcapsules were obtained using extrusion techniques: vibrating and electrostatic method, and beads were obtained using manual method with a syringe. A significantly lower decrease in the count of cells immobilized in hydrogels compared to free cells was observed during storage of fermented beverages at 4°C. Microcapsules were more effective compared to microbeads in terms of bacterial cells protection. The observed effect was better for higher biopolymer concentration. The highest survival of the strain was noted in cells immobilized in low-methoxylated pectin beads of 2.5 mm diameter. Supplementing the biota of fermented beverages with microencapsulated bacteria did not negatively affect the overall sensory quality of beverages during the entire storage period.

1. Introduction

Recently, a constant increase is being observed in the consumption of milk products, recognized as the best carriers of probiotic bacteria [1, 2]. At the same time, there is a growing population with allergies or intolerance to milk proteins, thus creating the need to introduce milkless beverages of plant origin, which contain bacterial strains beneficial for human body and can also be considered to be a functional food, onto the market. The most common probiotics introduced into food are *Bifidobacterium* and *Lactobacillus* spp. [3–5].

Keeping the probiotic bacterial cells, especially *Bifidobacterium*, alive in fermented products during storage is difficult and very challenging for the food industry, considering the unfavourable physicochemical characteristics of the environment, especially the low pH and the presence of oxygen [1, 6]. One of the possible solutions to this problem is the immobilization or encapsulation of bacterial cells [1, 7, 8] using appropriate carrier materials. However, the effectiveness of applying encapsulated bacteria depends on many factors, such as the type and concentration of the material used for immobilization, the encapsulation/immobilization method, the size of the capsules obtained, and the

physicochemical characteristics of the environment [9]. The material most commonly used to immobilize bacterial cells is naturally occurring polysaccharide sodium alginate, mainly because of its safety, its good gelling properties (temperature and pH), and biocompatibility [4, 10]. Low-methoxylated pectin is also a nontoxic polymer which, like alginate, forms gels with divalent metal ions, that is, calcium [11, 12]. Pectin is a cheap and easily available compound of plant origin. Some literature data suggest that pectin provides better protection for bacterial cells than alginate [13], which may result due to its lesser sensitivity to chemical interactions of the environment [14]. Moreover, also other research studies show that pectin from fruits may also be used as an effective prebiotic [15], thereby positively affecting human health.

Bacterial cells are immobilized in hydrogels using different methods and equipment, that is, the electrostatic and vibrating technique, which enables us to obtain, in a one-step procedure, uniform, spherical microcapsules or microbeads with a diameter of 0.15–2.0 mm (vibrating technique) or 0.2–3.0 mm (electrostatic technique), with a small size distribution [16–19]. Both methods belong to the extrusion techniques, which provide the most favourable conditions for the survival of encapsulated microorganisms, and they are relatively easy to apply [8, 20–22]. It should be noted that the size of the capsules or beads may affect not only the number of surviving bacteria but also the physicochemical and sensorial properties of food products [23].

There is a lack of reports on the supplementation of fermented milkless beverages with immobilized probiotic bacteria cell. Therefore, the aim of this work was to examine, if immobilization of *B. infantis* ATCC15697 KKP2029p cells in selected hydrogels can increase their survival in such environments during refrigerated storage. The impact of immobilized bacteria on sensory quality of beverages was also the scope of the work.

2. Materials and Methods

2.1. Biological Material. The biological material consisted of two strains from the IBPRS Culture Collection of Industrial Microorganisms: *Bifidobacterium infantis* ATCC15697 KKP2029p and *Streptococcus thermophilus* T_{KM}₃ KKP2030p. The strains were stored at -70°C using Viabank (Abtek Biologicals Ltd., Liverpool, GB), propagated on a suitable medium: *S. thermophilus* T_{KM}₃ KKP2030p on modified LAB (lactic acid bacteria) medium without agar [24] and *B. infantis* ATCC15697 KKP2029p on modified Garche medium [25], and incubated at 37°C for 24 h. The cells were harvested by centrifugation at 6000 rpm for 10 min at 4°C and then washed twice and suspended in a small volume of saline. The bacterial suspension was used for inoculation immediately after its preparation.

2.2. Preparation of Fermentation Matrices. The matrices for fermentation were prepared by mixing the dry components, introducing the mixture into the amount of water (Table 1) given in the recipe and gelatinization of the oat (*Avena sativa* L.) flour (Mlyny Stojsław, Poland) suspension at 85°C for 5 min while

TABLE 1: Composition of the fermentation matrices.

Ingredient (%)	Oat-banana	Oat	Oat-peach
Banana puree	10.0	—	—
Peach filling	—	—	30.0
Oat flour	4.0	4.0	4.0
Sucrose	4.5	4.5	4.5
Water		Up to 100	

stirring. Banana (*Musa paradisiaca* L.) puree (Maspex, Poland) was introduced into the cereal matrix. The matrices were sterilized for 10 min at 118°C, cooled to the fermentation temperature (37°C), and subsequently inoculated with *S. thermophilus* T_{KM}₃ KKP2030p at approximately 6 log cfu·g⁻¹.

The oat-banana and oat beverages were obtained as a result of the fermentation process. For further experiments, a portion of the oat beverage was combined with peach (*Prunus persica* (L.) Batsch) yoghurt filling with large fruit pieces (PRPH Kandy, Poland) in the amount of 30%. The biota of all three beverages was subsequently supplemented with free and immobilized cells of *B. infantis* ATCC15697 of approximately 9 log cfu·g⁻¹, and their survival during storage at 4°C was determined.

2.3. Immobilization of *B. infantis* ATCC15697 KKP2029p Cells in Biopolymers. The immobilization of *B. infantis* ATCC15697 KKP2029p cells was conducted in aseptic conditions, using sterile solutions and vibrating and electrostatic techniques in calcium alginate and low-methoxylated pectinate gel, using disposable syringes. The process conditions for each technique were chosen experimentally.

An encapsulator B-395 Pro (Büchi, Switzerland) was used in the vibrating technique following the Büchi [19] procedure to produce microbeads and microcapsules. Spherical microbeads were obtained with a freshly prepared suspension of bacterial cells in saline combined with a 1.5% sodium alginate solution (low viscosity; Büchi, Switzerland) with a viscosity of 98 mPa·s, in a 1:5 proportion. A 300 µm diameter single nozzle was used. The one-step procedure of microcapsule production with a liquid core was carried out using a set of concentric nozzles, comprising an inner nozzle of 150 µm diameter and an outer nozzle of 300 µm diameter. The liquid core of the microcapsules was a bacterial suspension in saline, and the shell was a 1.5% sodium alginate gel.

Cell encapsulation using the electrostatic technique [16, 17] was conducted with an electrostatic droplet generator—developed in the Nalecz Institute of Biocybernetics and Biomedical Engineering, PAS, Warsaw. This apparatus enables the formation of uniform microbeads as well as obtaining, in a one-step procedure, uniform microcapsules, in which the hydrogel core containing the bacterial cells is surrounded by an additional polysaccharide shell. A sterile 2% sodium alginate (medium viscosity; Sigma-Aldrich Co., USA) solution in saline was used with viscosity differing depending on the method of sterilization: viscosity of 170 mPa·s for sterilization of alginate solution at 121°C for 15 min or viscosity of 240 mPa·s for sterilization of alginate

powder at 121°C for 15 min. To produce microbeads, a freshly prepared bacterial suspension in saline was combined (1:10) with a sodium alginate solution (2%) with a viscosity of 240 mPa·s, and then the cell suspension in alginate was forced through a single nozzle with an outer/inner diameter of 330/600 µm. To produce microcapsules, a head consisting of two concentric nozzles with the following diameters: inner nozzle 330/600 µm and outer nozzle 680/1000 µm, was used. The core liquid (pressed through the inner nozzle) was sodium alginate solution (viscosity of 240 mPa·s) containing bacterial cells, and the shell was pure alginate solution with a viscosity of 170 mPa·s.

Alginate microbeads and microcapsules, obtained using the vibrating and electrostatic technique, were gently stirred in a bath with gellifying solution (0.1 M aqueous solution of CaCl₂; POCH, Polska) for 10 min, filtered through a stainless steel sieve (0.4 mm) and washed with sterile water and used immediately after preparation.

The immobilization of bacterial cells in a 5% aqueous solution of low-methoxylated pectin (Aglupectin LA-S20; Hortimex, Poland) and a viscosity of 180 mPa·s and in a 2% sodium alginate solution with a viscosity of 240 mPa·s was carried out by combining 1 g of freshly prepared bacterial suspension with 10 g of pectin solution or 10 g of alginate solution. The suspension thus prepared was manually pressed using a 20 mL syringe through a medical needle with a diameter of 0.2/0.4 mm (BD Microlance 27 G 3/6"; BD Dicardit I) into the gellifying solution, 0.1 M CaCl₂. The suspension was left for 1/2 h with gentle stirring until hardening of the pectin or alginate droplets, filtered through a stainless steel sieve (0.4 mm) and washed with water.

2.4. Examination of the Appearance and Size of Alginate Microbeads/Microcapsules and Pectin Beads. The appearance of the alginate microbeads and microcapsules obtained using the vibrating technique was assessed using an Olympus CX40 optical microscope, at 60x magnification. Photographs were taken with an Olympus C5060 digital camera. The size of the alginate microgels before storage in fermented beverages was determined by the laser diffraction method, using a Mastersizer 3000 (Malvern Instruments Ltd., GB) and was expressed as the average sphere diameter equal in terms of surface D (3,2) and in terms of volume D (4,3) [10, 23]. However, measurements of diameters of alginate microgels after 28 days of storage were made using the scale from the images.

The alginate microbeads and microcapsules produced using the electrostatic technique and pectin and alginate beads were characterized using an Olympus CKX 41 inverted optical microscope with CellSens software for making measurements and taking pictures. Magnifications of 20x, 40x, and 100x were used.

2.5. Determination of the Bacterial Count. Counts of *S. thermophilus* T_KM₃ KKP2030p in fermented beverages were determined using the plate count method according to ISO 7889:2003 [26], spread on selective M-17 agar (BTL, Łódź, Poland) medium. The count of free *B. infantis*

ATCC15697 KKP2029p cells was determined according to ISO 29981:2010 [27], spread on TOS-MUP agar (Merck, Darmstadt, Germany).

To determine the count of immobilized *B. infantis* ATCC15697 KKP2029p, the hydrogels with bacterial cells were drained off in a sieve and washed out with sterile deionised water. Next, 1 g of capsules or beads was placed, in order to gel liquefaction, in 29 mL of 0.1 M sodium citrate (Sigma-Aldrich Co., St. Louis, MO, USA) and was vortexed for 5 min. The bacteria released from the hydrogels were serially diluted with Ringers liquid and then spread on agar medium, similarly to the free cells population. The cell count was expressed as cfu·g⁻¹.

2.6. Determination of pH. The pH value measurement was carried out using the potentiometric method and a Mettler Toledo MP235 pH meter (Switzerland).

2.7. QDA Sensory Profile of the Beverages. The sensory profile and overall sensory quality of the beverages were evaluated in a sensory laboratory, designed in accordance with PN-ISO 8589:1988 [28], by a trained [29] 6-person panel in one-week intervals, over a one-month period. The evaluation was carried out using quantitative descriptive analysis (QDA) [30], that is, setting the descriptors of the sensory notes perceived as the most important for texture, odour, and taste attributes. The intensity of particular notes, as well as the overall sensory quality (SQ) of the assessed samples, was quantified using a 10-unit nonstructured linear scale with defined border restraints denominated as "very weak/none" to "very strong" [31].

2.8. Statistical Analysis. A statistical analysis of the results was carried out using a one-way ANOVA and Duncan test ($P \leq 0.05$) (Statistica 7.1 StatSoft).

3. Results and Discussion

3.1. The Appearance and Size of Alginate Microbeads/Microcapsules and Pectin Beads Containing *B. infantis* ATCC15697 KKP2029p Cells. Producing microcapsules and microbeads of narrow size distribution is the greatest challenge of all the microencapsulation technologies [17]. When the process parameters are incorrectly set, the undesirable satellite fraction of very small beads may be formed, and there may also be a nonuniform distribution of the cell suspension inside the capsules. However, despite the immobilization method used, the authors managed to achieve alginate microbeads and microcapsules uniform in terms of their size and shape, preserving their size and shape during the whole storage period. Examples of the microgels obtained using the vibrating technique are shown in Figure 1 and using the electrostatic technique in Figures 2 and 3.

Laser diffraction measurements confirmed that the initial average diameters of the spherical alginate microgels obtained using the vibrating technique were uniform (Table 2) and slightly lower for microbeads: 499 µm (D (3,2)) and

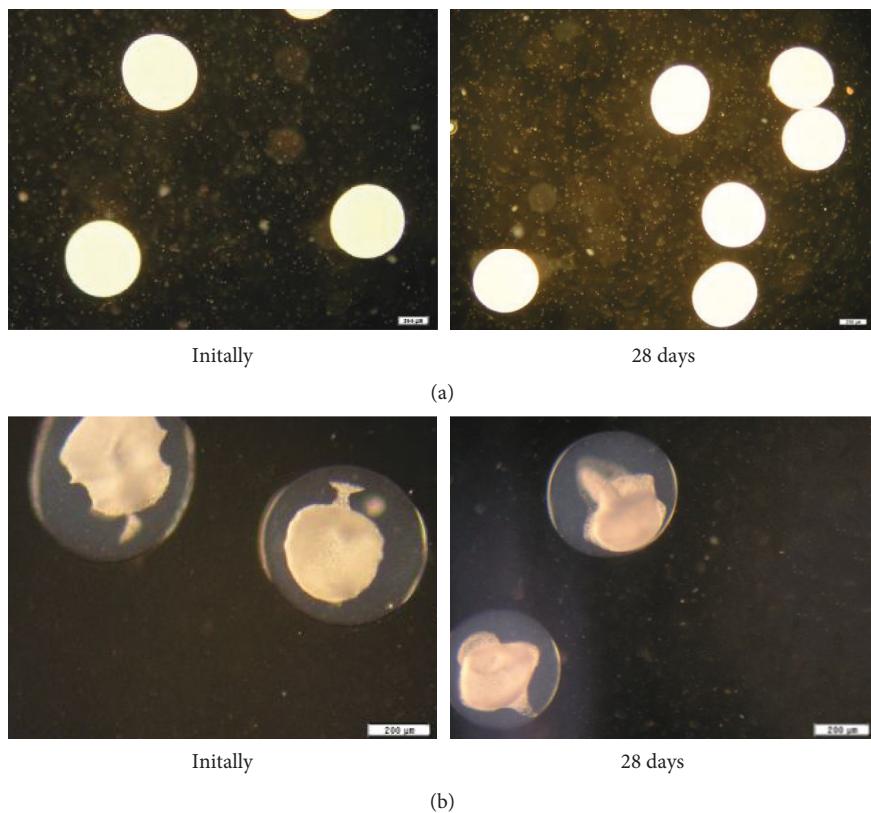


FIGURE 1: Optical microscopic images (reflected light) of the spherical microgels made with 2% calcium alginate and obtained using the vibrating technique (60x): microbeads (a) (initially and after 28 days) and microcapsules (b) with a liquid core (initially and after 28 days).

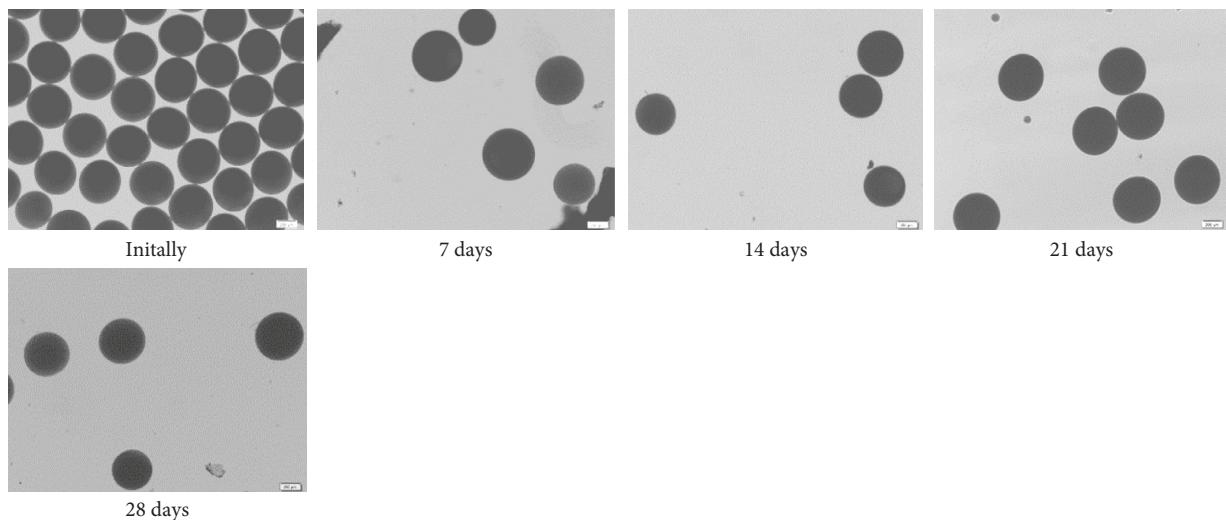


FIGURE 2: Optical microscopic images (40x): microbeads made with 2% calcium alginate containing immobilized bacterial cells directly after formation using the electrostatic technique and during 4-week storage at a temperature of 4°C in fermented beverage. The irregular fragments visible in the images are the beverage ingredients.

511 μm ($D(4,3)$) for microbeads and 528 and 568 μm for microcapsules with a liquid core. These diameters did not change significantly after 28 days of storage and amounted to 500 and 520 μm for microbeads and microcapsules, respectively.

The diameters of the alginate microgels obtained using the electrostatic technique were also uniform (Table 3).

Moreover, these diameters did not change significantly ($P \leq 0.05$) during storage. Initially, the average diameter of the microbeads was $527 \pm 12 \mu\text{m}$ and after 28 days of storage $517 \pm 31 \mu\text{m}$ (a 2% reduction). The initial outer diameter of the microcapsules was $551 \pm 38 \mu\text{m}$, and the inner (core) diameter was $424 \pm 34 \mu\text{m}$. These values decreased slightly during storage at 4°C and after 4 weeks amounted to $520 \pm$

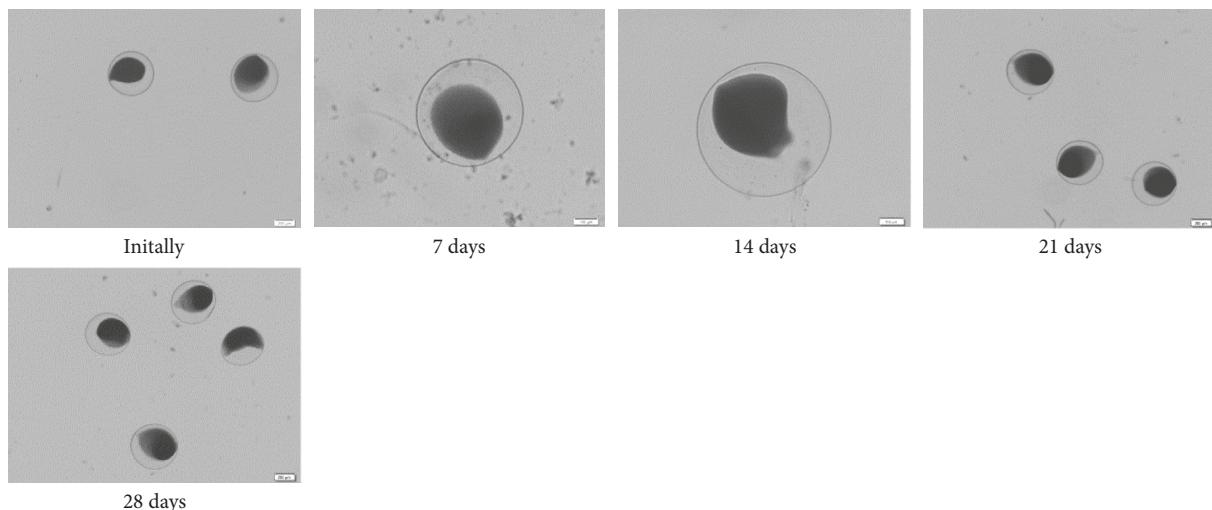


FIGURE 3: Optical microscopic images (40x: initially and after 21 and 28 days; 100x: after 7 and 14 days) of microcapsules made with 2% calcium alginate with bacteria in an alginate core directly after formation using the electrostatic technique and during 4-week storage at a temperature of 4°C in fermented beverage.

TABLE 2: Changes in the diameter of the microbeads and microcapsules made with 1.5% calcium alginate using the vibrating technique during refrigerated storage (4°C) of fermented beverage.

Alginate microgels	Diameter (μm)		
	Initially*	D (4,3)	28** days
Microbeads	499 \pm 17	511 \pm 23	500 \pm 42
Microcapsules with liquid core	528 \pm 14	568 \pm 13	520 \pm 44

*The size of the alginate microgels was determined by the laser diffraction method. The values are the mean of three replicates \pm standard deviation. **The size of the alginate microgels was measured using the scale from the images. The values are the mean of 20 replicates \pm standard deviation.

58 μm and 516 \pm 40 μm , respectively (a reduction of 6 and 2%, resp.).

As in the case of alginate microgels, the appearance of pectin and alginate beads obtained using a syringe (Figure 4) as well as their size (Table 4) did not change significantly during the whole storage period. The initial average diameter of pectin beads was 2.47 \pm 0.09 mm, and after 28 days, it was 2.56 \pm 0.11 mm (an increase of 3.6%); in the case of alginate beads, their initial average diameter was 2.36 \pm 0.07 mm, and after 28 days, it was 2.30 \pm 0.14 mm (a reduction of 2.5%).

3.2. Acidity and Count of the Population of *S. thermophilus* T_{KM₃} KKP2030p Starter in Beverages. The characteristics of oat and banana-oat fermented beverages, obtained as a result of the lactic acid fermentation of cereal and fruit-cereal matrices using *S. thermophilus* T_{KM₃} KKP2030p as a starter culture, are shown in Table 5. The acidity of the beverages was pH 4.22 and 4.26, and the count of the starter culture in the beverage biota was 7.60 and 7.65 log cfu·g⁻¹, respectively. Oat beverage with peach filling achieved a pH of 4.07 and a bacteria count of 7.47 log cfu·g⁻¹.

Acidity, expressed as a pH value, was stable during the whole period of beverage storage (Table 5), which can be attributed to the fact that the streptococci of *S. thermophilus* spp. do not exhibit any postacidification activity [32]. The

pH values did not change after supplementation with *B. infantis* ATCC15697 KKP2029p. Similar observations were made by Sohail et al. [33], who supplemented the biota of fermented peach dessert with immobilized and free cells of *L. rhamnosus* GG; these samples retained the same pH during storage (4°C) as the nonsupplemented sample. According to Quero et al. [34], the decrease in the pH of fermented milk beverages after completion of fermentation led to generally unfavourable sensory changes. As shown in Table 5, introducing free and immobilized *B. infantis* ATCC15697 KKP2029p cells into the beverage did not cause a substantial decrease in the count of *S. thermophilus* T_{KM₃} KKP2030p starter (only of approximately 0.5 log) during storage, similar to the results obtained in nonsupplemented beverages (a decrease of approximately 0.5 log).

3.3. Survival of Free and Immobilized *B. infantis* ATCC15697 KKP2029p Cells in Fermented Beverages during Refrigerated Storage. For studying the survival of free and immobilized cells of *B. infantis* ATCC15697 KKP2029p during storage, first the oat-banana beverage was used with 0.5 mm microbeads and microcapsules (with liquid and gelled core) obtained with 1.5% and 2% sodium alginate (Table 6). The reduction of living bacterial cells count was significantly lower in all alginate microgels, compared to free cells. Nonencapsulated cells did not survive 14 days of storage. However, bacterial

TABLE 3: Changes in the diameter of the microbeads and microcapsules made with 2% calcium alginate using the electrostatic technique during refrigerated storage (4°C) of fermented beverage.

Alginate microgels	Diameter (μm)				
	Initially	7 days	14 days	21 days	28 days
Microbeads	527 \pm 12	487 \pm 53	505 \pm 32	490 \pm 39	517 \pm 31
Microcapsules with alginate core	Outer diameter 551 \pm 38	502 \pm 39	507 \pm 30	510 \pm 31	520 \pm 58
	Inner (core) diameter 424 \pm 34	—	—	407 \pm 23	416 \pm 40

The values are the mean of 40 replicates \pm standard deviation.

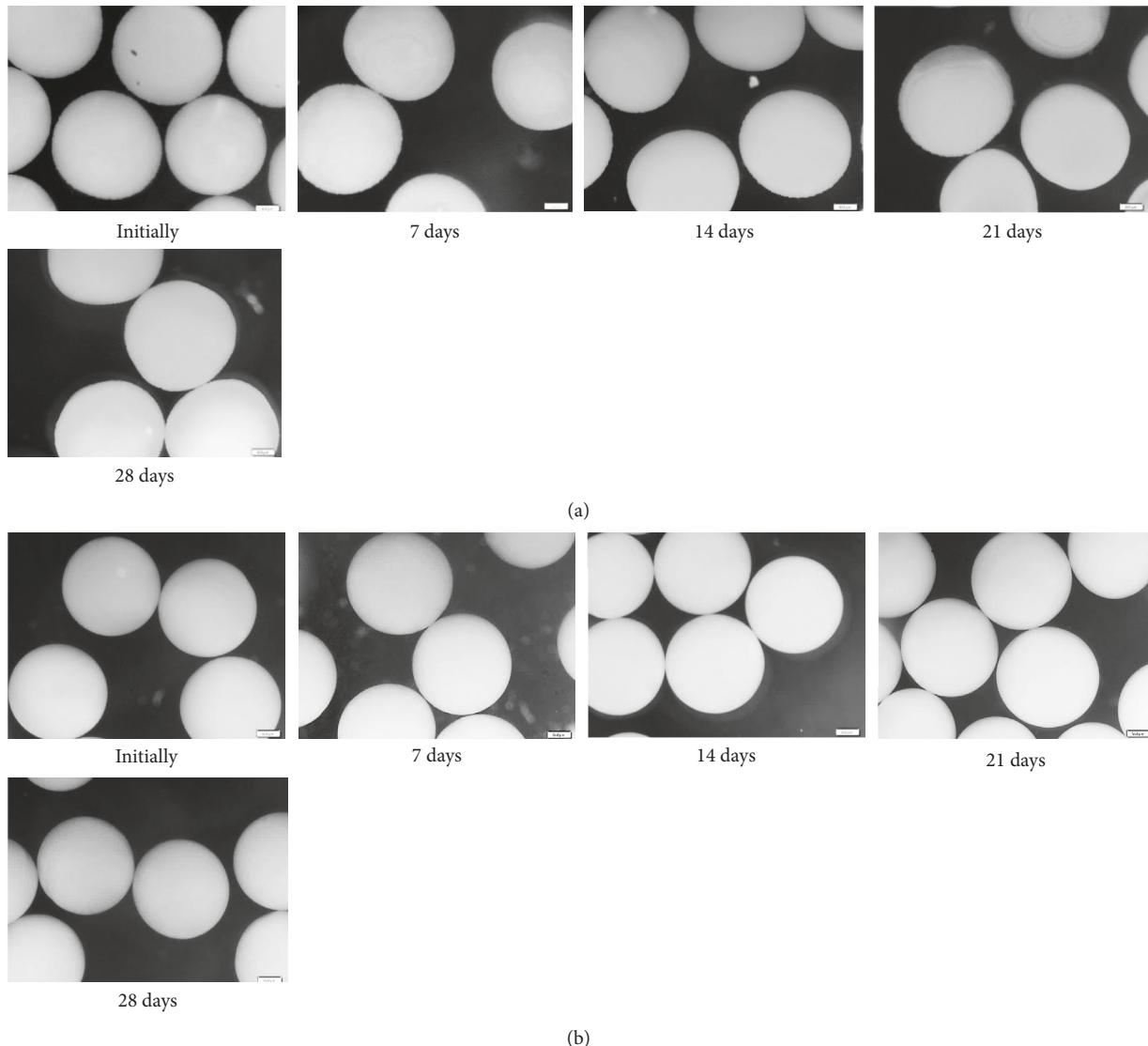


FIGURE 4: Optical microscopic images (20x reflected light) of beads made with (a) 5% low-methoxylated pectin and (b) 2% alginate-containing immobilized bacterial cells directly after formation with a syringe and after 4-week storage at temperature 4°C in fermented beverage.

cells immobilized in microbeads and microcapsules made with 1.5% calcium alginate completely disappeared after 21 days while the cells protected by microbeads and microcapsules made with 2% calcium alginate died after 28 days of storage. There was also a significant ($P \leq 0.05$) difference in the survival of bacterial cells immobilized in 1.5% and 2% calcium alginate between microcapsules and microbeads.

The count reduction of living cells in microcapsules was approximately 1 log lower compared to the reduction in microbeads up to 21 days of storage. This finding was confirmed by other researchers: Bakula et al. [35] reported that capsules better sustain a bacterial population count, more efficiently preventing their migration into the environment. Mokarram et al. [36] also found that bifidobacteria were better

TABLE 4: Changes in the size of beads made with 5% low-methoxylated pectin and 2% alginate during refrigerated storage (4°C) in fermented beverage.

Beads	Diameter (mm)				
	Initially	7 days	14 days	21 days	28 days
Beads with 5% pectin	2.47 ± 0.09	2.52 ± 0.08	2.51 ± 0.11	—	2.56 ± 0.11
Beads with 2% alginate	2.36 ± 0.07	2.34 ± 0.10	2.34 ± 0.09	2.36 ± 0.07	2.30 ± 0.14

The values are the mean of 20 replicates ± standard deviation.

TABLE 5: The acidity and *S. thermophilus* T_KM₃ KKP2030p count of fermented beverages without supplementation and of beverages supplemented with free and immobilized *B. infantis* ATCC15697 KKP2029p during storage at 4°C.

Beverage	Storage time (days)	Acidity (pH)			<i>S. thermophilus</i> T _K M ₃ KKP2030p (cfu·g ⁻¹)		
		A	B	C	A	B	C*
Oat	0	4.22	4.22	4.22	7.65 ± 0.08	7.65 ± 0.08	7.65 ± 0.08
	28	4.19	4.20	4.18	7.19 ± 0.17	7.16 ± 0.15	7.14 ± 0.16
Oat-banana	0	4.26	4.26	4.26	7.60 ± 0.06	7.60 ± 0.06	7.60 ± 0.06
	28	4.20	4.24	4.25	7.18 ± 0.24	7.06 ± 0.22	7.12 ± 0.12
Oat with peach filling	0	4.07	4.08	4.07	7.47 ± 0.10	7.47 ± 0.10	7.47 ± 0.10
	28	4.04	4.09	4.05	6.88 ± 0.19	6.86 ± 0.28	6.94 ± 0.06

A: beverage without supplementation with *B. infantis* ATCC15697 KKP2029p; B: beverage supplemented with free cells of *B. infantis* ATCC15697 KKP2029p; C*: -beverage supplemented with immobilized cells of *B. infantis* ATCC15697 KKP2029p; oat and oat with peach filling: supplementation with beads made with 5% pectin; oat-banana: supplementation with microbeads made with 1.5% alginate.

TABLE 6: Survival of free *B. infantis* ATCC15697 KKP2029p cells immobilized in calcium alginate and pectinate in fermented beverages during storage at 4°C.

<i>B. infantis</i> ATCC15697	Initial cell count (log cfu·g ⁻¹)	Cell count reduction (log)				
		4 days	7 days	14 days	21 days	28 days
<i>Oat-banana beverage</i>						
Free	9.28 ± 0.27	3.75 ± 0.24 ^d	5.12 ± 0.13 ^d	9.28 ± 0.27 ^h	—	—
Microcapsules ⁽¹⁾ with 1.5% alginate	9.21 ± 0.26	2.24 ± 0.25 ^c	3.67 ± 0.23 ^b	6.20 ± 0.24 ^a	9.21 ± 0.26	—
Microbeads with 1.5% alginate	9.71 ± 0.20	3.24 ± 0.29 ^a	4.82 ± 0.06 ^g	7.02 ± 0.11 ^g	9.71 ± 0.20	—
Microcapsules ⁽²⁾ with 2% alginate	8.75 ± 0.18	1.47 ± 0.20 ^e	2.53 ± 0.19 ^e	5.10 ± 0.23 ^f	>7	8.75 ± 0.18
Microbeads with 2% alginate	9.14 ± 0.35	2.05 ± 0.38 ^c	3.16 ± 0.24 ^f	5.91 ± 0.46 ^a	>8	9.14 ± 0.35
<i>Oat beverage</i>						
Free	9.41 ± 0.10	4.06 ± 0.17 ^d	5.40 ± 0.37 ^d	9.41 ± 0.10 ^h	—	—
Beads with 5% pectin	9.77 ± 0.15	0.93 ± 0.08 ^b	2.11 ± 0.07 ^a	3.32 ± 0.29 ^b	5.08 ± 0.24 ^a	>7
Beads with 2% alginate	9.62 ± 0.04	1.09 ± 0.07 ^b	2.19 ± 0.12 ^a	3.76 ± 0.10 ^c	5.60 ± 0.16 ^b	>8
<i>Oat-peach beverage</i>						
Free	9.41 ± 0.10	5.15 ± 0.32 ^f	6.88 ± 0.29 ^h	9.41 ± 0.10 ^h	—	-
Beads with 5% pectin	9.77 ± 0.15	2.88 ± 0.15 ^a	3.93 ± 0.24 ^{bc}	4.28 ± 0.26 ^d	5.96 ± 0.10 ^c	>8
Beads with 2% alginate	9.62 ± 0.04	2.92 ± 0.10 ^a	4.08 ± 0.19 ^c	4.70 ± 0.11 ^e	6.51 ± 0.16 ^d	9.62 ± 0.04

The values are the mean of two replicates. The values with the same superscript letter in columns do not differ significantly ($P \leq 0.05$). ⁽¹⁾Microcapsules with liquid core. ⁽²⁾Microcapsules with alginate core.

protected in microcapsules with a double alginate shell, which provides anaerobic conditions more favourable for micro-organisms than microbeads. Taking into account the results presented in this study, it can be concluded that *B. infantis* ATCC15697 cells immobilized in 2% calcium alginate microcapsules with an alginate core remain alive in fermented oat-banana beverages at a significantly higher level compared to cells encapsulated/immobilized in other microgels (a reduction of 2.52 log).

The important objective in this research was to ensure the good sensory acceptance of milkless beverages enriched with immobilized cells as well as high bacterial count during storage. Literature data show that the bigger the gel particles, the better the cell protection they provide [8, 23]; but, at the

same time, they are more sensed during consumption, what is not accepted by the consumers. The sensory scores of oat-banana beverage with 0.5 mm microcapsules were high (Figure 5), but preliminary supplementation of this beverage with microbeads of high diameter (ca. 2.5 mm) was not positively perceived by the panel. Therefore, at the next stage of experiments, the oat beverage with big particles of peach filling (of pH similar to oat-banana beverage, Table 5) was used to make microbeads not detectable in the mouth. For comparison, the survival of bacteria was also checked in oat beverage without fruit filling.

On this stage of research, we decided to check the protective action of pectin on cells of *B. infantis* ATCC15697 KKP2029p strain compared to 2.5 mm alginate beads.

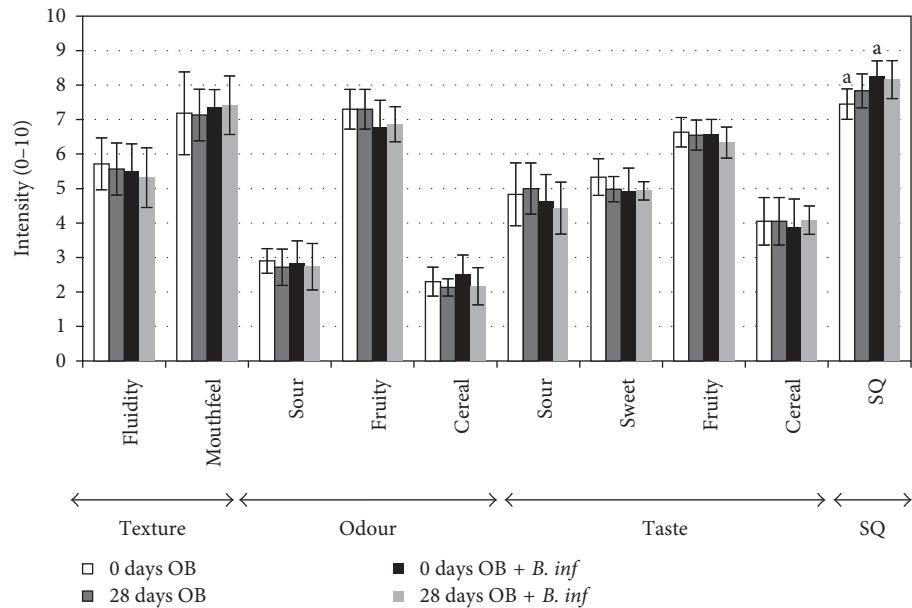


FIGURE 5: Sensory profile of oat-banana beverage with biotum supplemented with *B. infantis* ATCC15697 KKP28p cells immobilized in calcium alginate (OBan. + *B. inf.*) compared with beverage without supplementation (OBan.) after storage at 4°C (SQ is the overall sensory quality). (a) Differences of mean (of two independent experiments) values of descriptors significant at $P \leq 0.05$.

Studying the survival of *B. infantis* ATCC15697 KKP2029p in oat-peach beverage and oat beverage during refrigerated storage (Table 6), a significantly lower reduction of immobilized cells compared with free cells was observed in beverages. Nonencapsulated cells did not survive 14 days of storage, while the lowest reduction of immobilized bacteria in this period was observed in the pectin beads introduced into the oat beverage (a decrease of 3.32 log) and slightly higher in the pectin beads introduced into the oat-peach beverage (a decrease of 4.28 log). A similar trend was observed in alginate beads, although the reduction of cell count was significantly higher compared to pectin beads, both in oat (a decrease of 3.76 log) and in oat with peach-filling beverage (a decrease of 4.70 log). The pH of all the beverages was similar (4.22–4.07) (Table 5), so it can be stated that factors other than acidity influenced the viability of bacteria, such as the phenolic compounds present in fruit [2, 37].

Although after 4 and 7 days of beverage storage there was no significant difference in the decrease of bacterial count in pectin and alginate beads, the protective effect of alginate weakened in the following terms.

Nualkaekul et al. [13] studied the viability of *Bifidobacterium longum* in cranberry juice (pH 2.77) and in pomegranate juice (pH 3.16) at a temperature of 4°C. They observed the complete inactivation of free cells and cells immobilized in 4% low-methoxylated pectin and in 4% sodium alginate with a low viscosity already after 7 days of storage in cranberry juice, while in the pomegranate juice the count of free cells decreased by more than 4 log after 7 days, and after 14 days, they were completely inactivated, with only a slight decrease in the immobilized cells. After 14 days of storage in pomegranate juice, the count of cells immobilized in 4% pectin decreased by approximately 1 log, while the cells immobilized in 4% sodium alginate by approximately 1.5 log. Phoem et al. [38]

noted a decrease in the *B. longum* free cell count of more than 6 log after 15 days of storage in pineapple juice (pH 3.8), and 1 log in the case of cells encapsulated in 2% sodium alginate with *Eleutherine americana* extract.

Maintaining the population of probiotic bacteria in food at a desirably high level is not an easy task. Even in fermented milk products, which provide the best protection for bacteria because of their buffer properties, the survival of bacteria still remains a challenge for the industry, requiring new technological solutions [2, 39]. Hansen et al. [40] studied the viability of two bifidobacteria strains during storage in milk with a 2% fat content. They observed that the count of free *B. longum* Bb-46 cells decreased by 3 log after 14 days, and cells immobilized in calcium alginate (microbeads of 20 μm diameter) by 1.5 log, unlike in the case of the *B. lactis* Bb-12 strain, whose cell count, both free and immobilized, increased slightly during that period.

Taking into account all results presented in Table 6, it can be said that the survival of immobilized *B. infantis* ATCC15697 KKP2029p cells was significantly higher in all milkless fermented beverages compared to free cells. The best protective effect was observed in beads made with 5% low-methoxylated pectin; hence, it can be stated that pectin is a promising biopolymer for immobilization of bacterial strains in this kind of beverages. Further research is needed to optimize the biopolymer concentration and the size of microgel particles.

3.4. Assessment of the Sensory Profile of Fermented Beverages with Biota Supplemented with Immobilized *B. infantis* ATCC15697 KKP2029p Cells. A sensory assessment was carried out on two fermented beverages: oat-banana with microcapsules made with 1.5% calcium alginate of approximately 0.5 mm diameter containing *B. infantis* ATCC15697 KKP2029p cells

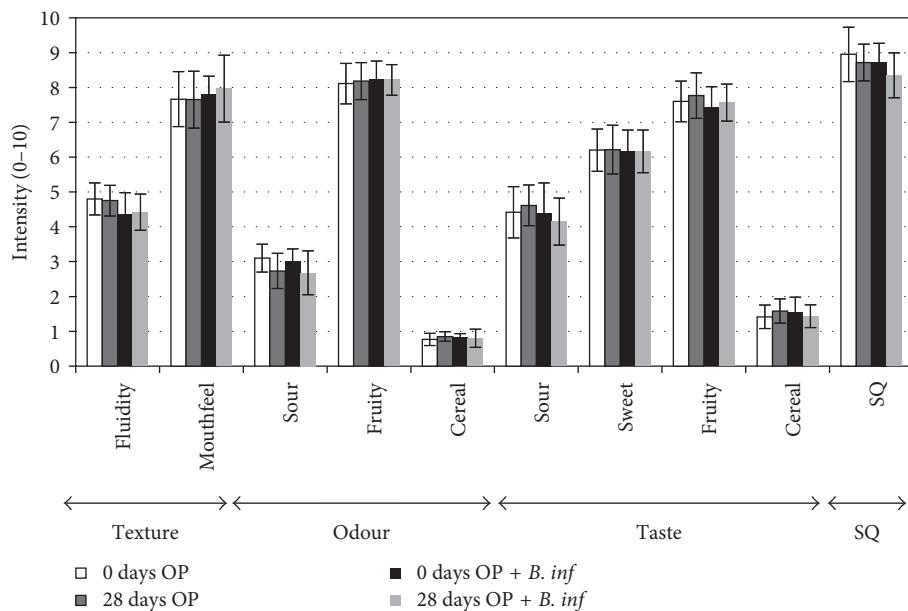


FIGURE 6: Sensory profile of fermented oat-peach beverage with biotum supplemented with *B. infantis* ATCC15697 cells immobilized in pectin beads (OP + *B. inf.*) compared with beverages without supplementation (OP) stored at 4°C. The values are the mean of two independent experiments.

and oat-peach beverage with the same strain immobilized in pectin beads of approximately 2.5 mm diameter. The aim of the evaluation was to check whether the introduction of gel microbeads or beads into the fermented cereal or fruit-cereal beverage would influence their sensory quality after 4 weeks of storage at 4°C.

The sensory profile assessment of the oat-banana beverage without biotum supplementation and with biotum supplemented with *B. infantis* ATCC15697 cells immobilized in microcapsules did not show any significant ($P \leq 0.05$) differences with regard to texture, odour, and taste during the entire storage period (Figure 5). However, the beverage with microcapsules obtained higher scores for overall sensory quality SQ (8.3 points) compared to the reference without supplementation (7.4 points), but the difference was significant ($P \leq 0.05$) only after the first storage period. Some authors suggest that the capsule size affects the product's sensory quality; that is, microcapsules (made with alginate and corn starch) of 0.3 mm diameter were considered the cause of better yoghurt smoothness compared to samples with free cells [41]. Generally, it can be stated that the addition of microcapsules of a size of approximately 0.5 mm to fermented cereal-fruit beverages did not affect the sensory assessment of the product, and microcapsules were not perceptible by the panelists.

The sensory profile of the oat-peach beverage without supplementation and with biotum supplemented with *B. infantis* ATCC15697 immobilized in 2.5 mm low-methoxylated pectin beads (Figure 6) did not show any significant ($P \leq 0.05$) differences between the samples for any storage period. Supplementing the biotum of the fermented beverage with bacterial cells immobilized in pectin beads of a diameter of approximately 2.5 mm did not affect the product's overall sensory quality (SQ). Fermented beverages

received high scores in terms of SQ, both directly after preparation, 8.95–8.73 points, and after 4 weeks of storage, 8.72–8.35 points, for beverages without supplementation and beverages supplemented with *B. infantis* ATCC15697, respectively.

It should be emphasized that the results presented indicate that there were no perceptible differences in the texture attributes (fluidity and mouthfeel) between a beverage with biotum supplemented with bifidobacteria encapsulated in relatively large pectin beads and a beverage without beads. This finding has not been confirmed by other researchers [6, 42], who observed that capsules of a diameter of over 1 mm gave a sandy texture mouthfeel. This divergence and lack of differences noted herein between the sensory assessment of a beverage with 2.5 mm pectin beads and a beverage without beads probably arise from the fact that the panelists associated the pectin beads with fruit particles present in the beverage, and they were not negatively perceived.

4. Conclusions

The immobilization of *B. infantis* ATCC15697 KKP2029p cells in alginate or low-methoxylated pectin hydrogel particles significantly increased the survival rate of these strains in fermented nonmilk beverages during storage compared with free cells. The highest survival of the strain was noted in cells immobilized inside low-methoxylated pectin beads of 2.5 mm diameter. It was proved that microcapsules provided better protection to bacterial cells compared to microbeads. It was also stated that the higher the alginate concentration, the better the protection effect was observed. Supplementation of fermented beverages with immobilized bacterial

cells did not affect the sensory quality of beverages during the whole storage period.

The results of this work create a basis for further research on the technologies for the production of probiotic supplements using pectin, stable in the environment of fermented nonmilk beverages.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

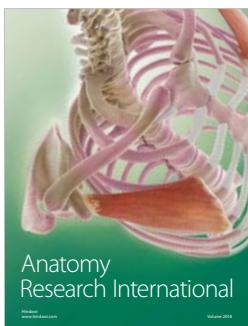
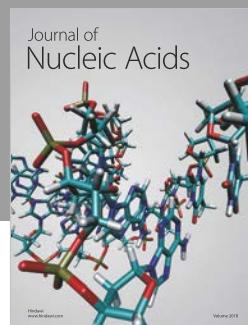
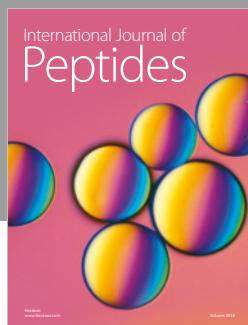
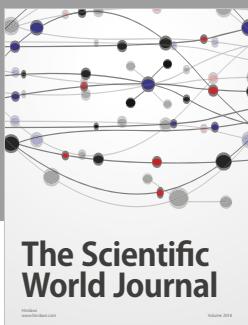
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