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Design of alginate microsphere formulation as a probiotics carrier

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Abstract
The process of obtaining alginate microspheres (AMs) by emulsification method was optimized by applying statistical analysis software. Ten batches of microspheres were prepared using the fractional plan 3^{K-P}. AMs were obtained with two different methods: an ultrasonic homogenization (UH) process and a rotor-stator mechanical homogenization (MH). The amount of a cross-linking agent ($CaCl_2$), calcium chloride rate addition, and the sonication amplitude (UH) or the speed of rotor rotation (MH) were selected as formulation variables. All the batches were evaluated in terms of stability and size of the alginate microspheres. Approximation profiles were developed. As a result of the conducted research, stable alginate microspheres with sizes ranging from 10 to 30 micrometres were obtained. The obtained results showed that the quality of AMs was mainly affected by the concentration and the rate of calcium chloride addition into the system. Therefore, the role of calcium ions in the mechanisms of shell structuring was discussed. *Lactobacillus casei* bacteria were encapsulated into the batches found to be optimum. The high encapsulation efficiency (*EE*) of the bacteria (72–94%) depending on the form) and their viability over time were obtained. The model developed in the study can be effectively utilized to achieve the AMs formulations.

Keywords
alginate microspheres, optimization, *Lactobacillus casei*, encapsulation, emulsification

1. INTRODUCTION

Alginates are natural polysaccharides obtained from marine algae (Gomathi et al., 2017; Tŕnnesen and Karlsen, 2002). The polymers are unbranched copolymers of D-mannuronic acid (M) and L-guluronic acid (G) linked by β (1–4) glycosidic bonds (Asgari et al., 2020; Kim et al., 2019; Paques, 2014; Van Vlierberghe et al., 2014; Walczak et al., 2015; Wyrębska et al., 2014). The individual monomers in the chain are arranged in an irregular block pattern with different GG, MM and MG block proportions (Iravani et al., 2015; Paques, 2014; Tŕnnesen and Karlsen, 2002; Van Vlierberghe et al., 2014; Wyrębska et al., 2014).

The monovalent metal ions with the alginates form soluble salts, while the divalent and multivalent cations form gels or deposits. Alginates containing a large number of guluronic acid blocks form gels with much greater strength compared to alginates rich in manuronates, because G blocks have a stronger affinity for divalent ions than M blocks (Tŕnnesen and Karlsen, 2002). Biodegradable microspheres are obtained by cross-linking the alginate structure with divalent cations, in particular Ca^{2+}. The alginate microparticles (AMs) are biocompatible, biodegradable and nontoxic delivery systems used to encapsulate natural active substances, including probiotics (Choukaife et al., 2020; Iravani et al., 2015; Puscaselu et al., 2020).

Most of the scientific articles focus on application of alginate microparticles in the food and pharmaceutical industries (Dhamecha et al., 2019; Martău et al., 2019; Puscaselu et al., 2020; Yang et al., 2020). Recently, environmental application of alginate carriers as microspheres and microcapsules is more frequent (Wang et al., 2019). However, the AMs application in cosmetic industry has not been very popular. There are only a few reports regarding the use of alginate capsules as carriers of turmeric oil or vitamin E (Lebeer et al., 2018; Lin et al., 2016; Shalaka et al., 2009).

In the case of cosmetic products an application of different vehicles helps to combine various active substances in one formula, solves problems related to the incompatibility of the substances (Sikora et al., 2020) and protect the actives against oxidation (Martins et al., 2017). Additionally, microparticles are used to enclose unpleasant-smelling substances and, as a result, to obtain products with a low odour or completely without it (Łętocha et al., 2022). When probiotics are used in cosmetic formulations, the microencapsulation process allows survival of the microorganisms despite the presence of preservatives. Compared to other effective carriers of active compounds, such as nanoparticles, the advantage of microcarriers is that they do not penetrate into the interstitial tissue and thus act locally (Lengyel et al., 2019; Łętocha et al., 2022; Yang and Forrest, 2016). The choice of microparticles instead of nanoparticles for cosmetic

products may be an ideal solution for epidermal effect, where the encapsulated ingredient is to act on the top layer of the epidermis and thus positively affect the skin microbiome. In addition, nanocarriers are not suitable, due to their small size, for encapsulating active ingredients with sizes larger than nano, e.g. bacteria.

The most commonly used methods of encapsulating compounds in alginate capsules include techniques such as extrusion, emulsification and spray drying (Tyagi et al., 2011). Each method has its advantages and disadvantages. However, due to the different size of the particles obtained by these methods, emulsification was selected for testing, because this technique allows to obtain smaller capsules (μm) than other techniques (mm), which is the most desirable in the case of the cosmetics industry, where large particles negatively affect the texture of the cosmetic and the consumer feeling.

The purpose of this study was to obtain the high-quality polymer microspheres by emulsification process, especially in the potential use of the alginate microparticles in the cosmetics industry. The effect of the emulsification parameters on the size of the bacteria-loaded alginate microspheres was evaluated. In order to optimize the process parameters, for subsequent encapsulation in optimal forms of probiotic bacteria, a statistical method of experimental design (DOE) was applied.

2. MATERIALS AND METHODS

2.1. Materials

Alginic acid sodium salt from brown algae, sodium citrate, MRS broth and MRS agar (de Man Rogosa Sharpe) were purchased from Sigma Aldrich (Poland). Calcium chloride was bought in Avantor Performance Materials Poland S.A. The capric-caprylic triglycerides and ECO-Tween 80 were kindly supplied by Croda (Poland). *Lactobacillus casei strain* ATCC 393 was purchased from American Type Culture Collection, (USA).

2.2. Method for obtaining blank microdispersions

Microdispersions with alginate microspheres were prepared using a modified encapsulation method described by Sheu and Marshall (1993) and Sultana et al. (2000). All details of the methodology are described and claimed in patent application P. 443812 (Łętocha et al., 2023). Briefly, the water in oil (W/O) pre-emulsion was prepared by mixing the encapsulating material (2% sodium alginate solution) with capric-caprylic triglycerides and 1% ECO-Tween 80. To reduce the water phase particle size, the emulsion was mixed thoroughly using a homogenizer (Unidrive X 1000, CAT) or sonicator (UP200 Ht, Hielscher). Next, the obtained system was stirred with a magnetic stirrer (IKA C-MAG HS 7) and water solution of 0.1–0.5 M calcium chloride was slowly added dropwise to form a dispersion containing microspheres. After the addition of $CaCl_2$ solution, the stirring of alginate microsphere dispersion was continued for an additional period of time (5–30 minutes). The microdispersions were then stored in the refrigerator and their stability was observed over time. After a defined period of time (24 h, 10 days) the stability of the dispersions was macroscopically observed. Figure 1 shows the scheme of the production of alginate microspheres.

2.3. Optimization process

The process of obtaining alginate microspheres were optimized with the use of mathematical methods of experiment planning (Statistica® ver. 13, StatSoft, Poland). The aim of the optimization was to determine the process parameters allowing to obtain stable microcapsules of relatively small size ($d < 100$ μm). In order to develop the best parameters of AMs production the statistical method of experimental design (DOE) with the fractional plan 3^{K-p} was used. Where K is the number of variables and p always takes the value 1. First, the process structure and the variables influencing the final alginate microcapsules properties were determined (Figure 2).

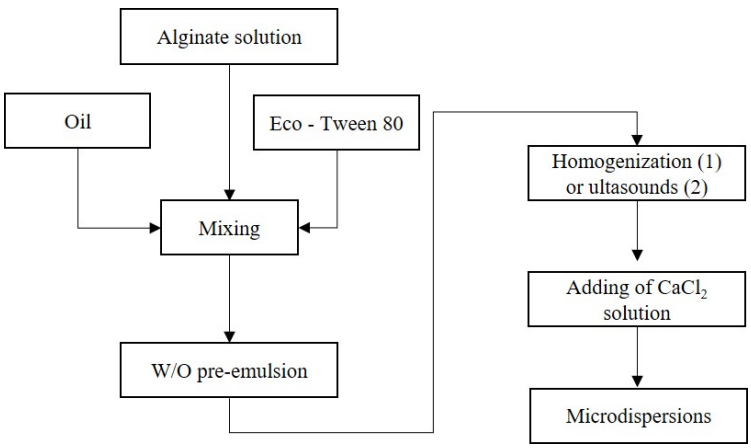

Figure 1. Block diagram of the microdispersion formulation process.

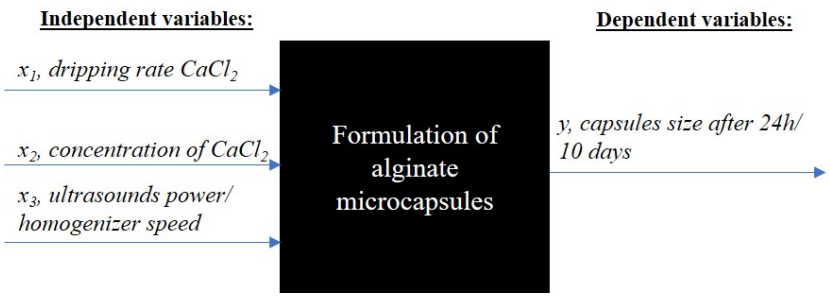

Figure 2. Schematic description of the optimization process.

2.4. Statistical analysis

Statistical analysis was performed based on one-way analysis of variance (ANOVA). The significance of differences was assessed using the F test. In all cases, a p value <0.05 was considered statistically significant. Approximation profiles have been developed taking into account independent parameters (dripping rate and concentration of calcium chloride, and homogenization method). By means of these profiles, it was possible to determine changes in dependent parameters (microcapsules size after 24 h and 10 days) depending on changes in the value of independent parameters. Approximation profiles were obtained, that allowed to determine which values of the input variables provide the most useful values of the output variables. The approximate values were then converted to a utility scale ranging from 0 to 1. The least desirable effect is 0 (the largest microcapsule size) and 1 (the smallest microcapsule size) is the most desirable effect. On the basis of the obtained approximation profiles, it was possible to optimize the usability of the composition.

2.5. Determination of microdispersion droplet size

Optical microscope measurements were performed to determine the droplet size of the microdispersion. A small amount of the dispersion to be tested was placed on a glass slide and covered with a coverslip. The observations were made using the Motic B1 Advanced Series Microscope equipped with a digital camera. The droplet diameter for each sample was determined from the mean of 200 measurements ±SD.

2.6. pH measurements

The pH of W/O microdispersions were measured using a Mettler Toledo S47 SevenMulti pH meter. First, the electrode was washed with a large amount of distilled water, and then it was placed in the test sample and the measurement was made. For each sample, the result was given as the mean of three measurements ±SD.

2.7. SEM analysis

The morphology of the obtained microspheres was observed with a scanning electron microscope (Mira3-FEG-SEM, Tescan, Brno—Kohoutovice, Czech Republic) with a pole emission (Schottky emitter), equipped with an X-ray energy dispersive spectrometer EDX (Oxford Instruments) and a cooling table (Peltier), operating in a temperature range from as low as −30 °C. The samples, for SEM investigations, were prepared by rapid freezing in liquid nitrogen followed by freeze-drying for 24 h (Al-Abboodi et al., 2019; Mao et al., 2019; Miastkowska et al., 2020).

2.8. TEM Analysis

The morphology of the microdispersions was investigated using a transmission electron microscope JEOL JEM 2100 HT (Jeol Ltd., Tokyo, Japan). Samples were collected on 300 mesh grids made from copper, additionally covered with formvar film. On each grid was applied 5 µL of samples. The excess was removed using the filter paper and was left to dry at ambient temperature. The transmission electron microscope was used for observation with accelerating voltage of 80 kV. Images were taken using a 4 k×4 k camera (TVIPS) equipped with EMMENU software ver. 4.0.9.87 (TVIPS GmbH, Gauting, Germany) (Ryś et al., 2022).

2.9. Bacterial strain and culture condition

Pure freeze dried culture of *Lactobacillus casei* strain ATCC 393 (LAB) was inoculated into MRS broth and incubated at 30 °C for 48 h in aerobic conditions. Harvesting of cells in the logarithmic phase was carried out by centrifugation at 3000× g for 10 min at 4 °C. The cells were washed twice using sterile saline solution (0.9%) (w/v). The cell pellets were resuspended in normal saline and prepared at a final concentration of 10,6 log CFU/g. The numbers of bacteria in the cell suspension was determined by counting on plates in MRS agar medium (37 °C, 72 h), using the pour plate inoculation technique (Arenales-Sierra et al., 2019). The obtained bacterial suspensions were used in the microencapsulation process.

2.10. Encapsulation of probiotics

The encapsulation of probiotics (*Lactobacillus casei* bacterias) was done in the similar way as the preparation of the blank microdispersions. The only difference was the composition of the water phase. In this case, the aqueous phase consisted of the aqueous solution of sodium alginate (2%)

and bacteria suspension. The mass ratio of the sodium alginate solution to the bacterial cell suspension was 4:1. In order to separate microspheres, the samples were centrifuged in a laboratory centrifuge (EBA 20 by Hettich Zentrifugen). Samples of the microspheres without centrifugation (1), microspheres spun at 500 rpm (2) and 30000 rpm (3) and stable microdispersion (4), were selected for further tests.

2.11. Assessment of microencapsulation efficiency and survival of probiotic bacteria during storage

The obtained microspheres (1, 2, 3) and microdispersion (4) were stored at room temperature for 1 month. The assessment of the viability of microdispersion and microcapsules with LAB was carried out immediately after encapsulation, and after 7 and 30 days after the process in line with the methodology described by Guimarães et al. (2013). Suspension of the bacteria cells not subjected to encapsulation was used as the control.

A one-gram portion of microcapsules was dissolved in 9 mL of 0.2 mol L^{-1} sterile sodium citrate solution (pH 6.0) and then vortexed.

Serial dilutions were performed and then the obtained solutions were inoculated by the pour plate method (1 mL) on plates containing MRS-agar medium. Viable cells were enumerated as the number of colonies after incubation for 72 h, at 37 °C,in aerobic condition. Results were shown as log colony forming units per gram (log CFU g^{-1}). All tests were conducted in triplicates.

The microencapsulation efficiency (*EE* [%]) of *L. casei* was calculated using Eq. (1) and expressed as the percent of the log CFU g^{-1} (Darjani et al., 2016).

$$EE = (N/N_o) \times 100\% \quad (1)$$

In this equation, N stand for the number of bacterial cells caught inside the microspheres or microdispersion, and N_o is the number of free *L. casei* cells added during encapsulation process (Darjani et al., 2016; Shafizadeh et al., 2020). The reduction in viability was characterized by the formula; $N_o - N$ and expressed in log CFU/g.

3. RESULTS

3.1. The influence of input parameters on the size of obtained microspheres

The process of obtaining alginate microparticles was optimized with the use of mathematical methods of experiment planning. Two fractional plans (ultrasound (UH) and high shear homogenization (MH)) were prepared, with one point at the centre of the plan. It was checked whether the input parameters (Table 1) significantly affect the output parameters.

The size of the capsules after 24 h and 10 days [μm] were classified as a group of output parameters for both plans. Tables 2 and 3 present the results of the optimization process

Table 1. The variability ranges of independent parameters.

Concentration of calcium chloride [mol/dm^3]		0.1; 0.3; 0.5
Dropping rate of calcium chloride [drop/min]		5; 10; 15
Homogenisation method	UH [%]	69; 79; 89
	MH [rpm]	8 000; 10 000; 12 000

Table 2. Matrix of the experimental design and experimental data obtained for the dependent variables with the use of ultrasound method.

No of the system	Input parameters			Physicochemical properties of microdispersions from experimental design				
				Output parameters				
	C_{CaCl2} [mol/l]	$V_{drop\ CaCl2}$ [drop/min]	Ultrasounds [%]	Capsule size after 24 h [μm] ±SD	Capsule size after 10 days [μm] ±SD	Stability after 24 h	Stability after 10 days	pH
1	0.1	5	69	10.76±4.25	10.77±5.65	+	+	6.05±0.02
2	0.1	10	89	14.16±8.47	15.31±8.69	+	–	6.05±0.02
3	0.1	15	79	28.87±14.62	30.78±17.17	+	–	6.05±0.02
4	0.3	5	89	16.91±6.99	17.23±7.69	+	–	5.90±0.02
5	0.3	10	79	15.65±8.01	16.14±10.64	–	–	5.90±0.02
6	0.3	15	69	23.88±13.65	32.56±22.66	–	–	5.90±0.02
7	0.5	5	79	10.74±4.39	13.71±11.79	–	–	5.84 ±0.01
8	0.5	10	69	16.31±9.95	29.75±18.41	–	–	5.84 ±0.01
9	0.5	15	89	30.30±15.89	30.30±18.95	–	–	5.84 ±0.01
10	0.3	10	79	15.65±8.01	16.14±10.64	–	–	5.90±0.02

Table 3. Matrix of the experimental design and experimental data obtained for the dependent variables with the use of high shear homogenisation method.

No of the system	Input parameters			Physicochemical properties of microdispersions from experimental design				
				Output parameters		Stability after 24 h	Stability after 10 days	pH
	C_{CaCl2} [mol/l]	$V_{drop\ CaCl2}$ [drop/min]	Homogenisation [rpm]	Capsule size after 24 h [μm] ±SD	Capsule size after 10 days [μm] ±SD			
1	0.1	5	8000	15.54±8.85	15.55±8.26	+	+	6.05±0.02
2	0.1	10	12000	16.29±7.99	16.58±9.26	+	–	6.05±0.02
3	0.1	15	10000	22.83±13.84	23.11±16.87	+	–	6.05±0.02
4	0.3	5	12000	18.14±6.62	18.45±13.79	+	–	5.90±0.02
5	0.3	10	10000	15.73±8.32	18.61±13.79	–	–	5.90±0.02
6	0.3	15	8000	24.07±24.98	30.39±20.22	–	–	5.90±0.02
7	0.5	5	10000	7.16±3.45	18.21±18.48	–	–	5.84 ±0.01
8	0.5	10	8000	13.12±10.26	19.34±15.94	–	–	5.84 ±0.01
9	0.5	15	12000	17.71±12.71	27.77±17.99	–	–	5.84 ±0.01
10	0.3	10	10000	15.73±8.32	18.61±13.79	–	–	5.90±0.02

obtained by ultrasound and rotor-stator mechanical homogenization methods, respectively.

On the basis of the obtained results it was found that the amount of a cross-linking agent ($CaCl_2$) and the calcium chloride rate addition are parameters influencing the microcapsules properties.

The most stable microdispersions with the smallest capsules size were obtained (independently of homogenization method used) for the samples at the concentration of calcium chloride solution amount 0.1 mol/dm^3 and for the lowest rate of adding the cross-linking agent (5 drop/min). The remaining samples delaminated spontaneously, thanks to which it was easy to isolate the microspheres from the obtained microdispersion that deposited at the bottom of the test tubes. In order to accelerate the isolation of the microcapsules, the dispersions were centrifuged (500 rpm to 30 000 rpm) and stored in a refrigerator (at 5 °C) in the next steps.

3.2. Statistical analysis

The obtained results were analysed statistically. Figure 3 presents Pareto charts for the input parameters mentioned. Statistically significant parameters for a fixed p value ($p < 0.05$) are marked with a red line.

Based on the graphs, it is stated that the parameters that have a statistically significant influence on the particle size after 24 hours and 10 days in the case of ultrasound are the dripping rate of calcium chloride as a linear function. However, when using homogenization to the group of parameters that have a statistically significant impact on the particle size after 24 hours, there are the rate of cross-linking agent drops addition as a linear function and the concentration of calcium chloride as a linear and quadratic function, which proves a strong influence of this parameter on the size of microparticles. On the other hand, after 10 days, the drop rate of calcium chloride, as a dimension of the square function, is an additional parameter significantly influencing the particle size. This proves the enhancement of the influence of those parameters on the size of the microspheres.

The full general regression equation is shown below. The Eq. (2) takes into account all independent parameters. The coefficients of the Eq. (2) are summarized in Table 4.

$$y = a + bx_1 + cx_1^2 + dx_2 + ex_2^2 + fx_{(3u/h)}b + gx_{(3u/h)^2} \quad (2)$$

where: x_1 – C_{CaCl2}, x_2 – V_{CaCl2}, x_{3u} – UH, x_{3h} – MH.

The next point of statistical analysis includes approximation profiles. The approximation profiles are presented in Figures 4 and 5. On the basis of the approximation profiles, the influ-

Table 4. The coefficients of regression equations for ultrasonification and homogenization method.

Coefficients	Ultrasonification		Homogenisation	
	after 24 h	after 10 days	after 24 h	after 10 days
a	20.0565	206.0285	72.4396	55.2031
b	8.0534	9.3917	43.0485	43.5368
c	–8.4832	7.8273	–94.8997	–58.5507
d	–2.3632	–0.4108	–1.2897	–2.3227
e	0.1926	0.1071	0.104	0.1645
f	–0.220	–4.8537	–0.0116	0.0073
g	0.0025	0.0296	$5.8 \cdot 10^{-7}$	$3.5 \cdot 10^{-7}$

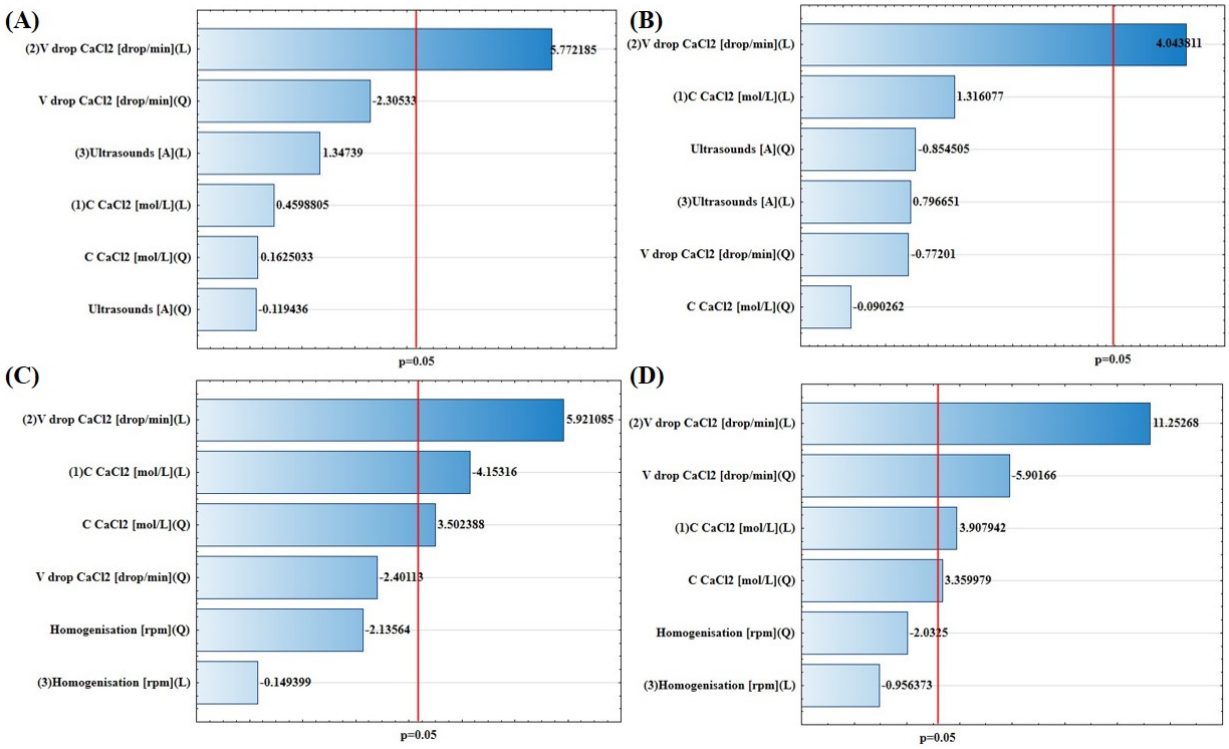

Figure 3. Pareto charts for prepared compositions: (A) capsule size after 24 h [µm] by ultrasonification, (B) capsule size after 10 days [µm] by ultrasonification, (C) capsule size after 24 h [µm] by homogenisation, (D) capsule size after 10 days [µm] by homogenisation.

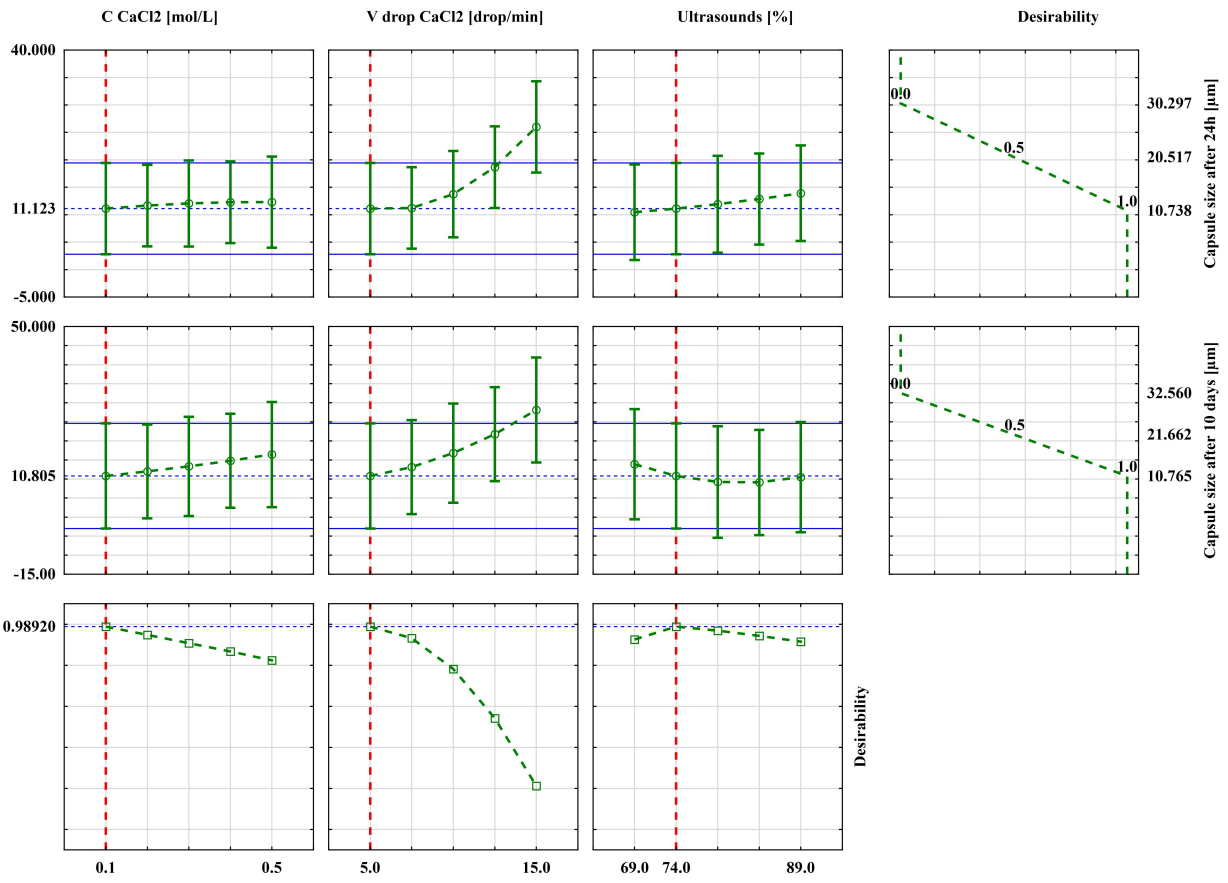

Figure 4. Approximation profiles for prepared compositions with ultrasonication method.

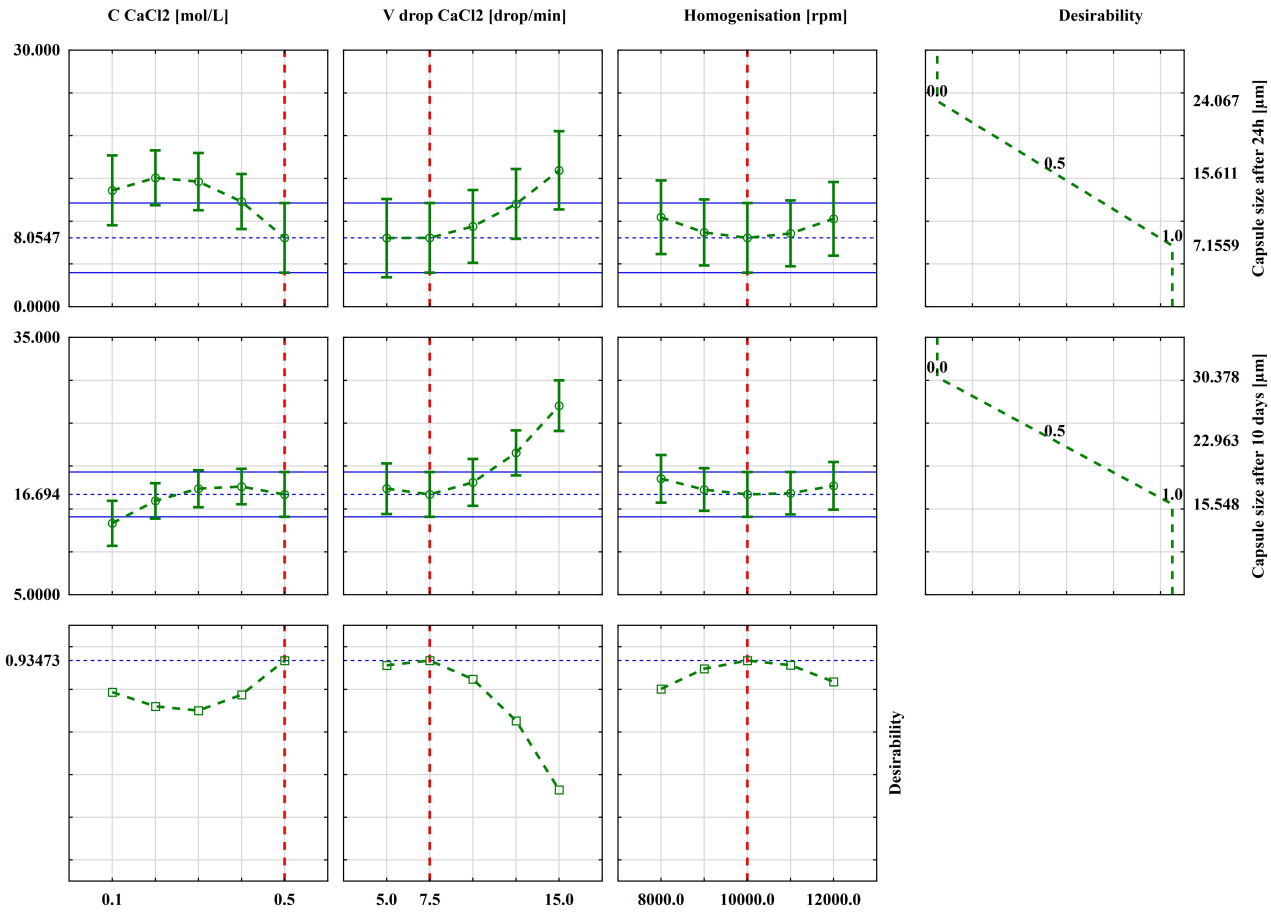

Figure 5. Approximation profiles for prepared compositions by homogenization.

ence of the input parameters on the output variables as well as the determination of specific values of the independent parameters ensure the possibility of achieving the desired values of the output variables. The most desirable values are the smallest particle size, both after 24 h and 10 days, because the smaller the size, the better the consistency of the cosmetic. However, on the other hand, the sizes cannot be nanometric, because if these carriers are to be used to encapsulate bacteria, the sizes must be micrometric.

The analysis of the approximation profiles for prepared compositions by ultrasonication method (Fig. 4) shows that microparticles with the smallest size of approx. 11 µm are obtained for the lowest concentration of calcium chloride used (0.1 M), the minimum rate of cross-linking addition at the level of 5 drop/min and amplitude values of 74%, after 10 days of storage. The charts show that the microsphere size are stable over time (similar dimensions after 24 h and 10 days).The graphs also show that the average microparticle size changes slightly with increasing concentration of cross-linking agent. In the case of rate of calcium chloride addition, the size of the microspheres increases as the rate of addition of the cross-linking agent increases. In turn, analysing the influence of the amplitude on the particle size, after 24 hours, an increase in their size is observed along with the increase in amplitude. However, after 10 days, the particle size decreases to an amplitude of 74% and then begins to increase.

The analysis of the approximation profiles for prepared compositions with homogenization (Fig. 5) shows that microparticles with the smallest size of approx. 8 µm (24 h) and approx. 17 µm (10 days) are obtained for the highest concentration of calcium chloride used, crosslinking at the level of 7.5 drop/min and an intermediate homogenization value of 10 000 rpm. The graphs also show that the average size of microparticles increases with increasing concentration of cross-linking agent to the value of 0.4 mol/dm^3, and then begins to decrease. When analysing the rate of dropping, an increase in particle size is observed after the value of 7.5 drop/min is exceeded. In the case of amplitude, the particle size decreases to an intermediate value and then begins to increase.

Figure 6 shows the surface response charts for the desirability in terms of dripping rate and concentration of calcium chloride, as a result of the ultrasonication method and the homogenization method (and this range was extended by model calculations).

The data presented in Figure 6 shows that in order to obtain microparticles with the smallest size with the use of ultra-

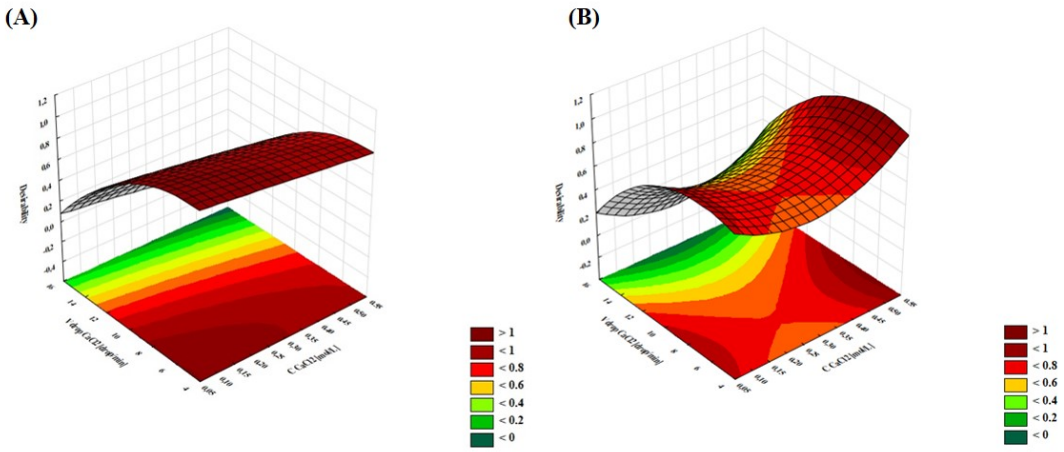

Figure 6. Response surface plots for the desirability with respect of dripping rate and concentration of $CaCl_2$: (A) as a result of the ultrasonification method, (B) as a result of the homogenization method.

sound, dripping rate and concentration of $CaCl_2$ should be in the range from 4 to 8 drop/min and 0.05 to 0.3 mol/dm^3, respectively. In the case of homogenization, dripping rate and concentration of cross-linking agent should be in the range from 4 to 10 drop/min and 0.5 to 0.55 mol/dm^3, respectively.

Based on the design of experiments (DOE), the ultrasonification method was chosen for further research (due to the shorter preparation time of the samples) and stable microdispersion (denoted as number 4 in the further part), and microspheres samples (marked as number 1, 2 or 3) (Table 5). The efficiency of encapsulation was checked when using different centrifugation speeds (no centrifugation (microspheres 1), 500 rpm (microspheres 2) and 30 000 rpm (microspheres 3)).

Although the amplitude did not have a statistically significant effect on the preparation of microspheres. the use of the smallest amplitude (69%) was chosen due to the lower energy consumption and thus the cost of the process.

3.3. AMs Morphology analysis

Morphology and size of obtained microparticles were analysed by transmission electron microscopy (TEM) (Fig. 7)

Table 5. Input and output parameters for optimal formulations.

	Concentration of calcium chloride [mol/dm^3]	Dropping rate of calcium chloride [drop/min]	Homogenisation method UH [%]	Capsule size after 24 h [μm]±SD	Capsule size after 10 days [μm]±SD
Microdispersion (ref. 4)	0.1	5	69	10.76±4.25	10.77±5.65
Mikrospheres (ref. 1/2/3)	0.3	10	69	15.87±7.03	16.78±9.13

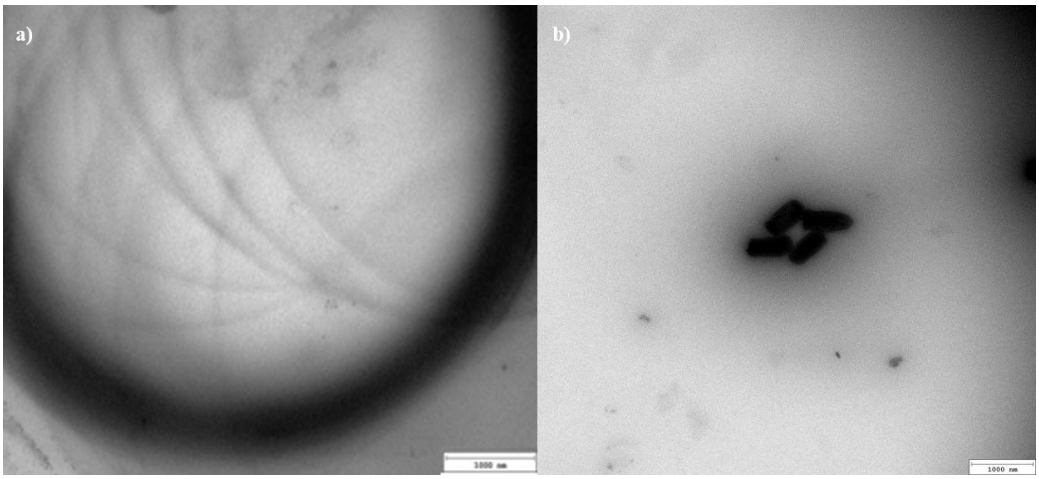

Figure 7. Transmission electron micrograph of a) structure of microdispersion, b) probiotic bacteria closed in microdispersion.

and scanning electron microscopy (SEM) (Fig. 8). The TEM image of the microdispersion confirmed that droplets were spherical with the micro-size range.

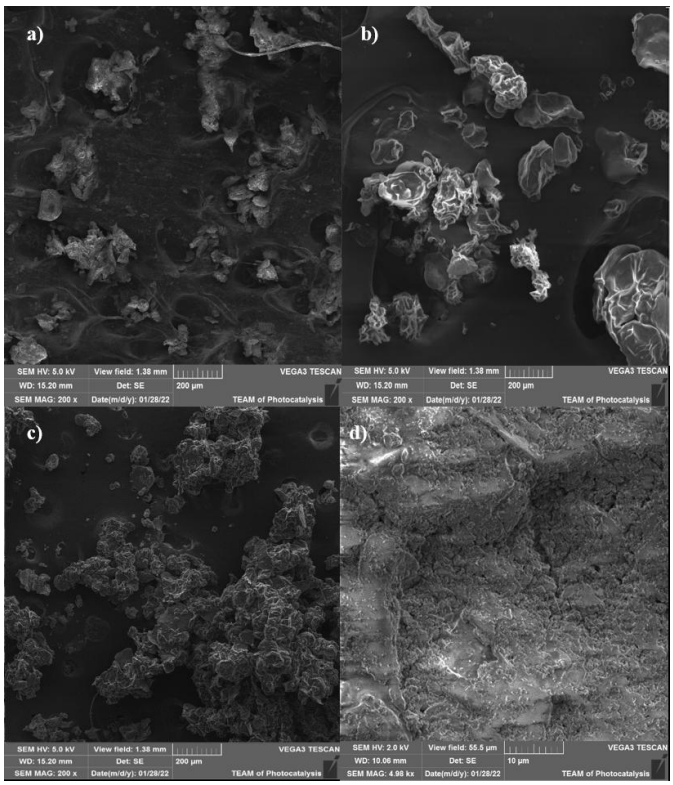

Figure 8. Scanning electron micrograph of a) structure of microsphere obtained without the centrifugation, b) structure of microsphere obtained with the 500 rpm, c) structure of microsphere obtained with the 30 000 rpm, d) cross-section through microsphere.

The scanning electron microscopy technique has been widely used to investigate a microparticle surface topography and its network structure. The SEM images (Fig. 8) of microspheres obtained by different centrifugation speed showed that the use of a centrifugation speed of 30 000 rpm caused the destruction of the microsphere structure as a result of strong centrifugal force. On the other hand. in the case of using the speed of 500 rpm, microspheres of spherical shape were obtained. In addition. the micrographs show the surface of the obtained structures. As can be see (Fig. 8). AMs microspheres have a porous structure, which is consistent with the studies of other research groups.

3.4. Viability of bacteria and encapsulation efficiency

Table 6 presents the results of viability assessment and encapsulation efficiency of *L. casei* species bacteria (LAB) enclosed in microspheres (1, 2, 3) and microdispersion (4). Microencapsulation efficiency was determined immediately after the process. The viability of probiotic cells was assessed after encapsulation. and after 7 and 30 days of storage at room temperature.

Suspension of LAB cells not subjected to encapsulation with the initial density of 10.6 log CFU g^{-1} was used as the control (N_o). Decapsulation was carried out with 0.2 mol/L sodium citrate solution with pH = 6. The assessment of viability of free and encapsulated probiotic cells was carried out by counting of bacterial colonies grown on plates with MRS agar medium (incubation conditions: 37 °C, 72 hours) using pour plate inoculation method. The results obtained were expressed in log CFU g^{-1}.

The studies showed that microencapsulation efficiency (*EE*) of live bacterial cells (LAB) enclosed in microspheres and microdispersion depends on the method of their preparation and varies from 72% to 94%. The highest microencapsulation efficiency (*EE*) was obtained in microspheres 2 (microspheres centrifuged at 500 rpm) and microspheres 3 (microspheres centrifuged at 30 000 rpm), which amounted to 94% and 87%, respectively. Slightly lower *EE* values were obtained in microspheres 1 (microspheres without centrifugation) and microdispersion 4, which amounted to 84% and 72%, respectively.

The reduction in numbers of live bacterial cells in formulations 1, 2, 3, 4 amounted to: directly after encapsulation process; from 0.4–2.97 log CFU g^{-1}. 7 days after encapsulation

Table 6. Assessment of viability of encapsulated probiotic cells (LAB) over time. Determination of the microencapsulation process efficiency (*EE*).

	Immediately after encapsulation			7 days after encapsulation		30 days after encapsulation	
Sample no.	Cell viability [log CFU g^{-1}]	Reduction [log CFU g^{-1}]	*EE* [%]	Cell viability [log CFU g^{-1}]	Reduction [log CFU g^{-1}]	Cell viability [log CFU g^{-1}]	Reduction [log CFU g^{-1}]
1	8.9±0.2	1.7	84±1.8	8.52±0.9	2.08	6.34±0.9	4.26
2	10.0±0.4	0.6	94±0.5	9.13±0.6	1.47	6.77±0.5	3.83
3	9.22±0.2	1.38	87±1.2	8.8±0.4	1.8	6.39±1.2	4.21
4	7.63±0.8	2.97	72±0.4	7.5±1.1	3.1	6.36±0.5	4.24

1 – microspheres without centrifugation, 2 – microspheres centrifuged at 500 rpm,
3 – microspheres centrifuged at 30 000 rpm, 4 – microdispersion. All values are mean ± standard deviation of three

process; 1.47–3.1 log CFU g^{-1}, 30 days after encapsulation; 3.83–4.26 log CFU g^{-1}.

The highest survival rate of *L. casei* strain up to day 30 of the study was found in microspheres 2. For these microspheres, the decrease in the number of live LAB cells was from 0.6 log CFU g^{-1} directly after encapsulation to 3.83 log CFU g^{-1} after 30 days of storage. The lowest values of probiotic bacteria survival were obtained for microdispersion 4, for which the reduction in numbers of LAB cells directly after encapsulation amounted to 2.97 log CFU g^{-1}. and 4.24 log CFU g^{-1} after one month of storage at room temperature.

4. DISCUSSION

In our study, probiotic-loaded alginate microspheres were obtained with the emulsification technique, The obtained microspheres were characterized by smaller sizes than previously reported (Ji et al., 2019; Pupa et al., 2021; Seyedain-Ardabili et al., 2016). The use of microscale delivery systems may be the ideal solution for epidermal action, where an encapsulated ingredient is intended to act on the *stratum corneum*. In addition, using the emulsification technique, smaller capsules are obtained than in the case of extrusion, where the particles obtained are a few millimeters in size (Lin et al., 2016), and a better texture of the cosmetic is associated with it. On the other hand, too small size (nanoscale) would be unsuitable for micrometer-sized bacteria. Considering the above, when planning this experiment, it was assumed that the size of microspheres is the most desirable parameter of the experimental plan.

Based on the results of the design of the experiment (DOE), the input parameters that significantly affect the size of the microparticles are found in the rate of cross-linking agent solution drops addition and its concentration (in case of ultrasonication method). Alginate gels cross-link in the presence of divalent cations. Previous studies have shown that several divalent cations can bind to alginates with different affinities, which are on the order of $Mg^{2+} < Mn^{2+} < Zn^{2+}$, Ni^{2+}, $Co^{2+} < Fe^{2+} < Ca^{2+} < Sr^{2+} < Ba^{2+} < Cd^{2+} < Cu^{2+} < Pb^{2+}$ (Li et al., 2007; Zhang et al., 2021). Moreover, in a study by Chan et al. (2011) gel strength was assessed. Gel strength expressed in Young's modulus showed that copper formed the strongest gels. followed by barium, strontium and calcium (Chan et al., 2011). Although cations such as barium and copper formed stronger gels with alginate in studies, calcium is the main cation used to cross-link alginates as it is considered clinically safe, readily available and economical (Łętocha et al., 2022; Reis et al., 2006). Therefore, in this study, calcium chloride was chosen as the source of divalent ions. In a study by Lotfipour et al. (2012) on the effect of calcium chloride concentration on bacterial microencapsulation in extrusion method, it was concluded that an increase in calcium chloride concentration (1–4%) had no significant effect on the size of the prepared balls. However, in our experimental plan based on microemulsification method, in the case of ultrasonication, one of the statistically significant parameters turned out to be the concentration of the cross-linking agent. In addition, during the experiment, it was noticed that with the increasing concentration of calcium chloride, the number of formed microcapsules decreased. This is consistent with the research of Walczak et al. (2015), who found that the amount of hydrogel obtained decreased with increasing concentration of calcium chloride (0.05–0.7 mol/dm^3), while the hardness of the microspheres increased. The decrease in cross-linking efficiency is probably related to the rapid exchange of ions on the surface of the droplet, which prevents the penetration of calcium cations into its interior (Walczak et al., 2015).

Literature sources indicated that increasing the mixing speed had a positive effect on reducing the size of microparticles. The high agitation speed produces finer microspheres due to the higher energy that ensures dispersion between the oil phase and the water phase in the emulsion (Łętocha et al., 2022; Zhai et al., 2013). On the contrary, our experimental method of emulsification (of high shear homogenization and ultrasonic homogenization) did not have an effect on the size of the obtained microspheres, in the tested area of the experiment. Most importantly, statistically significant input parameter appeared to be the dripping rate. This is in line with the research of Bennacef et al. (2022), who evaluated the properties of alginate capsules in the co-extrusion method. It was shown that the increase in the dripping rate significantly increased the diameters of the capsules, which can be explained by the fact that more material added to the capsule results in a larger diameter (Bennacef et al., 2022).

For further research, the ultrasonic homogenization process was used, due to the shorter time of preparation method. The data presented on the approximation profiles and saddle diagrams suggested that in order to obtain the smallest particles in the process of ultrasonic homogenization dripping rate and concentration of $CaCl_2$ should be in the range from 4 to 8 drop/min and 0.05 to 0.3 mol/dm^3, respectively. Taking all this information into account, the final samples of microspheres (1, 2, 3) and stable microdispersion (4) were obtained using the dripping rate and crosslinker concentration of 10 drop/min and 0.3 mol/dm3, respectively. The ultrasonication amplitude was 69%. The efficiency of encapsulation was checked when using different centrifugation speeds (no centrifugation (1), 500 rpm (2) and 30 000 rpm (3)).

In order to obtain alginate microspheres and microdispersions with a fairly high LAB cell density, *L.casei* cell suspension with the density of 10.6 log CFU g^{-1} was added to the alginate solution in the encapsulation process. The strategy was used to avoid a possible reduction in cell viability during sodium alginate cross-linking in calcium chloride. This approach resulted in a decrease in probiotic cells viability in the created structures (1, 2, 3, 4) in the range of 0.6–2.97 log CFU g^{-1} compared to the original *L. casei* cell suspension used to obtain them. The lowest reduction in the number of LAB was found in microspheres 2 and 3, which amounted to

0.6 log CFU g^{-1} and 1.38 log CFU g^{-1}, respectively. Slightly higher reduction values were obtained in microspheres 1 and microdispersion 4, which amounted to 1.38 log CFU g^{-1} and 2.97 log CFU g^{-1}.

The reduction in *L. casei* cells may be associated with debonding and non-entrapment of LAB during the emulsification process applied. The decrease in LAB viability might also have been associated with the use of calcium chloride for the cross-linking of sodium alginate during the immobilization process. Its high concentration could have caused osmotic stress and contributed to the damage of bacterial cells (Lasta et al., 2021). The amplitude of ultrasounds used to obtain microspheres and microdispersion was not without significance for the survival of LAB. According to some authors, the level of reduction of the number probiotic bacteria cells depends on the intensity of ultrasonic waves and the duration of their use (Yeo and Liong, 2011). The mechanism of ultrasound-induced stress is mainly associated with the phenomenon of cavitation. ultimately leading to membrane permeabilization. cell lysis and lipid peroxidation of the cell membrane in probiotic bacteria (Hayer, 2010; Tang et al., 2008). The differences in LAB reduction can also result from the way of centrifugation.

Although some cells died during encapsulation, a large number of viable bacteria survived because they were enclosed in alginate structures, which corresponded to the microencapsulation efficiency *EE* within the range of 72–94%. Similar *EE* values (73.64%–94.1%) were obtained by Pupa et al. (2021), whose objective was to develop a double-encapsulated alginate (1.5%) and chitosan (0.5%) shell by extrusion, emulsion and spray drying methods using *Lactobacillus plantarum* strains 31F, 25F, 22F. *Pediococcus pentosaceus* 77F and *P. acidilactici* 72N. Slightly lower *EE* values within the range of 62.54–72.48% were obtained during encapsulation of *Lactobacillus plantarum* NCDC201 and *L. casei* NCDC297 in the extrusion process by Rather et al. (2017). Even lower *EE* values within the range of 43–50% were obtained by encapsulation of (Zou et al., 2011).

It should be emphasize that different encapsulation methods can affect the values of LAB encapsulation and the viability of probiotic cells in different ways. According to some authors, the genus of probiotic strain used, the microcapsule size, the type of wall material used, the microbial cell load, as well as the time of curing in the calcium chloride solution may be essential for the above-mentioned parameters (Chávarri et al., 2010; Farias et al., 2019).

The assessment of *L. casei* survival in microspheres (1, 2, 3) and microdispersion (4) showed a reduction at the level of 1.47–3.1 log CFU g^{-1} after 7 days of storage and 3.83–4.26 log CFU g^{-1} after 30 days of storage. It should be highlighted, however, that the lowest LAB reduction was found in the case of microspheres 2 and microspheres 3, which amounted to 1.47 log CFU g^{-1} and 1.8 log CFU g^{-1} after 7 days, and 3.83 log CFU g^{-1} and 4.21 log CFU g^{-1}, respectively. A higher LAB reduction was found in microspheres 1 and microdispersion 4, which amounted to 2.08 log CFU g^{-1} and 3.1 log CFU g^{-1} after 7 days. and 4.26 log CFU g^{-1} and 4.24 log CFU g^{-1} after 30 days, respectively. The observed differences in *L. casei* survival mainly associated with the water content in the formulations and it is related to the method of the AMs systems obtaining. This is in accordance to Kim et al. (2017) and Sohail et al. (2013), whose observed that the reduction of water content in probiotic-loaded systems is the simplest way to increase the viability of LAB in microcapsules.

On the other hand, some authors suggest that the parameters such as: temperature, relative humidity, storage environment, wall material composition, and light exposure affect viability of the bacteria during storage of probiotic-loaded formulations (Meng et al., 2008; Morgan et al., 2006).

5. CONCLUSIONS

The subject of the work was the optimization of probiotic-loaded alginate microsphere production. The method of experimental design (DOE) was applied to select the most important process parameters affecting alginate microcapsule quality and probiotic bacteria viability. The statistical analysis provided an excellent tool to describe the parameters that significantly influenced the formation of alginate microparticles. The obtained results showed that the quality of AMs was mainly affected by the concentration and the rate of calcium chloride addition in to the system. Based on the DOE, stable microdispersion or microsphere samples were prepared by ultrasonification with the following parameters: calcium chloride concentration equal to 0.3 mol/dm^3, the rate of $CaCl_2$ addition of 10 drop/min and the ultrasonication amplitude of 69%. *Lactobacillus casei* bacteria were encapsulated with high efficiency (72–94%) in the elaborated systems.

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