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Characterization of potential probiotic strain, *L. reuteri* B2, and its microencapsulation using alginate-based biopolymers

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Abstract

In this study, *Lactobacillus reuteri* B2 was isolated from the feces of C57BL/6 mice and assessed on probiotic activity. *L. reuteri* B2 was identified by 16S rDNA sequencing, which the cell viability in acidic conditions at pH 2.0 was 64% after 2 hours, and in the presents of 0.30% of the bile salts, after 6h, was 37%. Antimicrobial assay with *L. reuteri* B2 showed maximum diameters against *Klebsiela oxytoca* J7 (12.5 ± 0.71mm). We further hypothesized if *L. reuteri* B2 strain in the free form can survive all conditions in the gastrointestinal tract (GIT) then the utilization of the appropriate biomaterials would ameliorate its stability and viability in GIT. *L. reuteri* B2 was microencapsulated into sodium alginate-(Na-alg) and different content of Na-alg and sodium maleate (SM) beads. Characterization materials enveloped their thermal characteristics (TGA/DTA analysis) and structure using: scanning electron microscopy (SEM), FTIR, and particle size distribution. The high survival rate of *L. reuteri* B2 at low pH from 2.0 to 4.0 and in the presence of the bile salts, at concentrations up to 0.30%, was obtained. *L. reuteri B2* showed

strong antimicrobial activity and the best protection microencapsulated with Na-alg+SM in simulated gastric juices (SGJ).

Keywords: Probiotics, *Lactobacills reuteri*, alginate, starch maleate, microencapsulation

1. Introduction

The major part of the human gastrointestinal tract (GIT) is inhabited with beneficial bacteria that belong to lactic acid bacteria (LAB) which are usually used as probiotics. The Food and Agricultural Association of the United Nations (FAO) as well as, the World Health Organization (WHO) defined probiotics as: "live microorganisms which when administered in adequate amounts confer a health benefit on the host" [1]. Generally, probiotics from the intestinal origin should be used for human or animal consumption performing a beneficial effect on host health and prevention of diverse disorders [2]. Different metabolites that are produced by these beneficial microbes can inhibit pathogens by antimicrobial components and in that way they modulating the immune system reaching equilibrium of the host [3,4].

Lactobacillus genus is the most researched bacterial genus with beneficial effects on human health in recent 40 years. Thanks to their beneficial effects these species are classified as probiotics where *Lactobacillus* species play the main role in the vital physiological processes including digestion, development of the immunity of the gut, and colonization of the gut against incoming pathogens. The functional requirements of *Lactobacillus* strains with probiotic characteristics include tolerance to human gastric juice and bile, exert immune stimulation, as well as microbial and antibiotic assays, that also make a selection of these strains that influence on population in the GIT [5] and have an important rule on microbial diversity. The genus *Lactobacillus* consists of a large heterogeneous group of Gram-positive, nonsporulating, facultative anaerobic bacteria that include *Lactobacillus reuteri, Lactobacillus acidophilus*, *Lactobacillus fermentum, Lactobacillus plantarum Lactobacillus salivarius*, and *Lactobacillus paracasei*. The most important role of this genus is in food fermentation, and it should be found in the different parts of the GI system of humans and animals in variable amounts depending on the age of the host , species, or position and location within the gut [6].

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According to these beneficial effects of the genus *Lactobacillus*, in this study, *L. reuteri* was chosen for assays of probiotic activity because it has multiple beneficial effects on the host health [7]. *L. reuteri* has been isolated and characterized in 1962. as heterofermentative species which grows in anaerobic conditions and colonizing GIT of the humans and animals [8]. This strain is mostly found in the digestive tract of the host [9] and becomes one of the most assessed organisms as a potential probiotic in humans. Numerous studies of *L. reuteri* showed that this strain obtains benefits and the dose~ 10^9 colony-forming units (cfu)/day. Those benefits include promoting health, reduction of susceptibility to infections, modulating host immune response, ameliorate feed tolerance, increasing the absorption of vitamins, minerals, and nutrients [7,10,11]. Survival in a wide different range of pH and the ability to synthesize antimicrobial intermediaries, such as reuterin, allows it to inhibit pathogenic microorganisms [12]. This strain produces compounds with health-promoting effects in the GIT such as reuterin [13,14], histamine [15], vitamins [16–18], and exopolysaccharides (EPS) [19].

To maintain all of these benefits, like the viability of the cells during the passage through the cruel environment of the GIT (gastric acid, digestive enzymes, and bile salts), it is important to make biocompatible material that can be used for the encapsulation of probiotics. Biopolymers are the most used compounds for the encapsulation process, because of their physic-chemical properties and the possibility to maintain beneficial effects of the probiotic cells through the GIT. Using biopolymers, encapsulation provides a lot of advantages for the cells that are encapsulated, including the maintenance of activity, stability, viability, function at the site, and high volumetric productivity, as well as reduced susceptibility to contamination and protection against mechanical damage. The most widely used biomaterial for encapsulation is alginate [20]. This biopolymer possesses all requirements properties to maintain beneficial effects of the cells such as: simplicity, nontoxicity, acceptance as a supplement for food, biocompatibility [21,22], wide availability and cheap [23]. Microencapsulated beads with Ca-alginate ameliorate the survival of the LAB cells under cruel conditions [23–25], as they are in the environment of the GIT. In the last years, increasing interest in researches in this camp, especially for biocomposite materials that are used for encapsulation besides alginate, such as: starch, xanthan, chitosan, gelatin, and cellulose-based materials [26,27]. In the many studies, the increased resistance of viable cells of *L. reuteri* species in alginate and alginate-starch microcapsules have been studied in the presence of the acid, where microencapsulated cells obtain protection from the polymer networks

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generated during capsule formation. Likewise, the use of the microcapsule formation of alginate or alginate plus starch or the materials such as, xanthan or κ-carageenan -containing polymers provide better protection of *L. reuteri* cells from acid challenge [24,28,29]. Likewise, the use of the Ca-alginate beads, improves the survival *L. reuteri* species, in harsh conditions (at low pH and bile salts treated), reducing an injury to the cell membrane and preserves the probiotic properties of the cells.

Consequently, in this study, the strain, *L. reuteri* B2, was isolated from fecal samples of C57BL/6 mice and systematically assessed for probiotic activity. As a potential probiotic, this strain has been tested and encapsulated into two different carrier biocomposite materials, sodium alginate (Na-alg) and sodium alginate-starch maleate (Na-alg+SM). They have been tested for bacterial cell viability in simulated gastrointestinal conditions (SGC).

2. Materials and methods

2.1 Materials

In the supplementary material (subsection 2.1), there are full data of materials used in this study.

2.2 Laboratory isolation of ricinoleic acid

The method is presented in Supplementary material (subsection 2.2)

2.3 Laboratory preparation of starch maleate (SM) monoester

The starch modification with maleic anhydride (MA) was performed applying two procedures: with and without the use of plasticizer ricinoleic acid (RA). In a typical experiment, wheat starch (adjusted at ~50% of moisture) and plasticizer RA, were vigorously grinded/homogenized at 90 ^{o}C for 30 min in a hot mixer (Papenmeier, Germany) at 3000 o/min. This process was performed to achieve two benefits: appropriate gelatinization degree in the first period followed by drying which results in plasticized, i.e. easier processable, starch powder. When the fluffy material was obtained, in the second step the powdered MA was added in five portions with vigorous mixing at room temperature (RT) for 30 minutes (3000 o/min), after

continued at 80 °C for 30 minutes. In case of the process without the use of the plasticizer, wheat starch (~14% moisture) was mixed with MA (five portions) at RT for the next 30 minutes (3000 o/min), and then continued at 80 °C for 120 minutes. After synthesis, the obtained material was transferred to Soxhlet extractor and subjected to continual extraction for 24 h using acetone. The amount of used reactants and experiment conditions in Table S1. The Exps. 1-4 (Table S1) were performed to investigate the influence of the MA amount on the degree of starch modification. The Exps. 5-8 (Table S1) were performed to investigate the influence of the plasticizer at different amounts of MA on the efficiency of starch modification. Plasticizer was introduced before MA addition to improving reagent distribution into starch grain interior. The chemical modification of starch with MA is shown in Fig. S2. Before of production of alginate-based carrier for *L. reuteri* B2 encapsulation, SM was neutralized using 0.5 mol dm^{-3} NaOH at the volume calculated according to the determination of the quantity of introduced carboxylic group (Table 6). After filtration obtained material was dried at 80 °C for 8 h and 60 °C for 6 h at reduced pressure (2000 Pa).

2.4 Characterization methods

A description of the methods used for the characterization of the tested materials is given in Supplementary material (subsection 2.4).

2.5 Probiotic assays

2.5.1 Isolation of fecal bacteria. To isolate pure bacterial colonies from the fecal samples of C57BL/6 mice, each fecal sample was aseptically measured up to 100 mg and transferred to a sterile tube, tenfold diluted with sterile phosphate buffered saline (PBS), and homogenized using vortex. Serial dilutions in PBS were prepared and inoculated on de Man Rogosa Sharpe (MRS) agar plates (Torlak, Serbia). These plates were incubated anaerobically at 37°C for 48 h. Anaerobic conditions were maintained by AnaeroGen anaerobic atmosphere generation bags (Fluka, St. Louis, MO, USA) in AnaeroJar jars (Fisher, Hampton, NH, USA). The level of oxygen (<1%) was monitored using anaerobic indicator strips (Fisher, Hampton, NH, USA). Colonies with particular morphological differences, such as size, color, and shape were selected

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and purified by streaking two passages onto MRS plates. The resulting pure strains were grown in MRS broth with 50% glycerol (Sigma - Aldrich) and stored at −80°C.

2.5.2 API 50CHL. Using API 50CHL kit (bioMérieux, France) carbohydrate fermentation profiles were discovered according to the manufacturer's recommendations.

2.5.3 Strain identification. Representative isolates were identified using the 16S rDNA sequencing, using universal primers (UNI16Sfw and UNI16Srev) [30]. Amplification was carried out in a thermal cycler (Applied biosystems, ThermoFisher Scientific) and DNA fragments were amplified as follows: initial denaturation at 94 °C for 5 min, followed by 30 cycles consisting of: denaturation at 94 °C for 1 min, annealing at 55°C for 1 min, and polymerization at 72°C for 1 min, and a final extension at 72°C for 7 min. The expected length was 1549 bp. Aliquots (5 µl) of the amplified products were subjected to electrophoresis in 1% agarose gel (ThermoFisher Scientific) in TAE buffer (40 mM Tris acetate, 1 mM EDTA, pH 8.2). Gels were stained with ethidium bromide (500 ng/mL) and visualized under UV light (BioDoc Analyze). All amplicons were eluted and purified using GeneJet PCR Purification Kit (ThermoScientific) by following the manufacturer`s protocol. The PCR products that we obtained were sequenced by the Macrogen Sequencing Service (Macrogen, Amsterdam, The Netherlands) and analyzed by using BLAST algorithm (http://www.ncbi.nlm.nih.gov/index.html). Selected isolates were identified as follows: isolate B2 - *Lacotbacillus reuteri* (GenBank accession no. **CP015408.2** and **CP029615.1**); isolate H10 - *Lactobacillus murinus* (GenBank accession no. **EU006153.1** and **EU006157.1**); isolate J7 - *Klebsiella oxytoca* (GenBank accession no. **CP029128.1** and **CP033844.1**). The most numerous colonies belong to isolate B2, hence it was chosen for further characterization as a potential probiotic strain.

2.6 Acid resistance and Bile Salt Tolerance.

The isolate of *L. reuteri* B2 was grown overnight, in sterile MRS broth at 37°C for 24 h, and was harvested by centrifugation at 4000 x g for 10 min at 4°C. The cell pellet was washed in (PBS, pH = 7.2) twice, and then was suspended at the concentration of 10^8 CFU/mL. 200 µl of cell suspension were mixed with 1.8 mL of sterile MRS broth which pH was adjusted to 3.0 and 2.5 for acid challenge studies. Then these suspensions were incubated anaerobically at 37°C for the next 2h. Bacterial viability was evaluated on two time points, t=0h and at the end of the treatment

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(t=2h), onto MRS agar plates, at 37°C for 48h anaerobically. Incubation at pH 6.4 was used as a control. To evaluate a bile tolerance, overnight MRS cultures of *L. reuteri* B2 were utilized to inoculate (10% v/v) bile salts supplemented MRS media, containing 1.5 g/L, and 3.0 g/L bile salts (Difco™ Oxgall, BD). These cultures and control MRS cultures (without bile salts) were maintained for 6h at 37°C, then bacterial viability was evaluated onto MRS agar plates, at 37°C for 48h anaerobically.

2.7 Antimicrobial activity of *L. reuteri* B2

All pathogen organisms belong to the collection of Torlak, Serbia. *Escherichia coli* ATCC25922, *Klebsiella oxytoca* J7, *Klebsiella pneumonie* sub. *pneumonie* ATCC 13883, *Schigella flexneri* ATCC 12022, *Pseudomonas aeruginosa* ATCC 27853, *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 6538, *Streptococcus pneumoniae* ATCC 6301, *Enterococcus faecalis* ATCC 29212 were selected pathogens that we used for antimicrobial activities of *L. reuteri* B2. The agar disk diffusion method [31], according to Raimondi et al., was used to determinate the antibacterial activity of *L. reuteri* B2, with some modifications. Briefly, cell-free supernatants from overnight cultures of *L. reuteri* (~10^8 CFU/mL) in MRS broth were collected by centrifugation at 2800 g at 4°C for the next 10 minutes, and adjusted to pH 6.0 with 1M NaOH, that was filter sterilized (0.22-μm pore-size filter). Fifty μL of cell-free supernatant was spotted on 6-mm-diameter filter paper disks, and placed over the surface of Mueller Hinton agar (MHA) plates (Torlak, Serbia), seeded with 100 μL of each pathogen species containing 10^5 CFU/mL. The incubation of the plates was performed at 37°C for 24 h. The radius of the inhibition zone was measured at the net of the radius of the disk. The value obtained from this experiment was reported as the mean of three different experiments.

2.8 Antibiotic susceptibility

The disk-diffusion method was used to determine the antibiotic susceptibility of *L. reuteri* B2. Overnight culture of *L. reuteri* B2 was inoculated into MRS broth at 37°C. 100 μL of diluted culture (10^8 CFU/mL) was spread onto MRS agar plate, and the antibiotic discs were applied onto the surface of the plate. The concentrations of indicator antibiotics are shown in Table 3. Plates were incubated at 37°C and evaluated after 24 h. To determinate, the inhibition zones around the discs a ruler was used and results were recorded in millimeters.

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2.9 Optimization of probiotic encapsulation

In order to achieve the highest encapsulation, yield the optimization procedure, according to RSM methodology, was applied. The experimental design of the *L. reuteri* B2 encapsulation is performed using Commercial software Design-Expert, Software Version 9 (Stat-Ease, Inc. 2021 E. Hennepin Ave. Suite 480 Minneapolis, USA). RSM methodology was applied as an empirical statistical technique for analyzing the simultaneous relationship between a set of experimental factors (the content of Na-alg and SM) and measuring the effects of such factors on responses (encapsulation yield) through the minimum number of runs. In this study, to investigate the effects of content Na-alg+SM on encapsulation yield, the D-Optimal design surface response methodology was used. Other parameters affecting the process are fixed. The proposed D-Optimal design requires 16 runs for modeling a response surface (**Table 1**). The predicted values of the using model matched well with the output parameters obtained in the experiments. To check the adequacy of the model for the responses in the experiment the analysis of variance (ANOVA) was used [32,33].

Thus, encapsulation of *L. reuteri* B2 has been done according to *Yeung et al.* (2016), with some modifications [20]. Briefly, *L. reuteri* B2 was routinely taken from glycerol stock where those were stored at −80 °C. The bacteria were grown in MRS broth (Torlak, Serbia) at 37 °C for 16 h, and cultivated on MRS agar (Torlak, Serbia). Anaerobic conditions were maintained using AnaeroGen anaerobic atmosphere generation bags (Fluka, St. Louis, MO, USA) in AnaeroJar jars (Fisher, Hampton, NH, USA). Overnight cell cultures were harvested by centrifugation (centrifuge, insert company) at 4000 g for 15 min followed by separation from the supernatant, washed with sterile PBS, and concentrated to ~10^8 CFU/mL.

In the subsequent step, a volume of 96 mL of each, sterile Na-alg solution (Sigma - Aldrich) and neutralized SM solution were mixed (Table 1) and autoclaved. A suspension of *L. reuteri* B2 strain was prepared and agitated with each mixture using a magnetic stirrer. The mixtures were agitated for 5 min to distribute uniformly cells throughout the sterile suspensions. Each alginate solution were drawn into a 5 mL syringe with 18G needle and dropped manually in 300 mL of 0.1 M $CaCl_2$ solution (Sigma - Aldrich) to form the beads. Under continuous agitation after 1 hour it came to cross-linking, beads were vacuum filtered, and rinsed twice with deionized water (200 mL), and then filtered again. At Petri dishes at 24-26 °C the resulting samples were stored aerobically. In the same conditions, unfilled alginate beads were prepared in parallel

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exception of not adding *L. reuteri* B2 to both alginate solutions [20]. The encapsulation yield (EY), was calculated according to Chávarri *et al*. (2010):

$$EY = (N / No) \times 100 \qquad (1)$$

where **N** is the number of viable entrapped cells released from the microcapsules, and **No** is the number of free cells added to the biopolymer mixtures during the production of the microcapsules [34].

Table 1.

2.10 Enumeration of viable cells

In the following way, the viable counts of encapsulated bacteria were determined. Alginate beads of 0.1 g were re-suspended in 9.9 mL of 10% sodium citrate dehydrate solution (Sigma - Aldrich) followed by vortexing for 10 minutes at RT. The plate count technique on MRS agar was used to determine the number of released cells. Dissolved beads were serially diluted (10^{-2} - 10^{-8}) and plated on Petri dishes in duplicate, and incubated at 37 °C for the next 48h anaerobically. Before the bead formation, the initial cell count was determined by plating serial dilutions (10^{-5}–10^{-8}) in duplicate of the beginning alginate-based carrier-bacteria mixtures before gelation on MRS agar. The incubation of these cells at 37° C for 48 h under anaerobic conditions, was followed by cell enumeration. The spread plate count technique on MRS agar was used for the determination of survival of free cells. In 50 mL sterile distilled water cell pellets were re-suspended and vortexed. Finally, the enumeration of the serial dilutions (10^{0} - 10^{-8}) of the cell suspension was sampled in triplicate and incubated at 37 °C for 48 h anaerobically [20].

2.11 Survival of free cells of *L. reuteri* B2 in simulated gastric juices (SGJ).

Determination of viable cells in SGJ was studied in a simulation model with some modifications, Huang *et. al*., 2015. SGJ was prepared with 3 g/L of pepsin (Merck) in saline solution (0.15M NaCl) and was adjusted to pH 2.5 with 1 M HCl [24]. Overnight samples were harvested by centrifugation (7,500 × g, 5 min, 4°C) and washed with PBS twice, then a 1.0 mL of suspension was centrifuged (7,500 × g, 5 min, 4°C), supernatants were discarded. Samples (400

mL) of *L. reuteri* B2 (~10^8) that were microencapsulated with Na-alg and Na-alg+SM, were mixed with 20 mL of SGJ and incubated for 5, 60 and 120 min at 37 °C with constant agitation at 50 rpm in an orbital shaker. For the free *L. reutei* B2, for all of the time points one flask was used; 10 µL of the homogenized sample was taken at each time point. The colonies were counted after each time point and expressed as CFU/mL (or log10 of CFU) [35].

2.12 Statistical analysis

Microsoft Excel® was used to enter and collect data on measured test powder sizes as well as antimicrobial inhibition zones. Data were then exported to non-commercial Computer software JASP version 0.13.1 was employed for statistical analysis [JASP Team (2020). JASP (Version 0.13.1)] [36], for further analysis. Various graphs and tables were extracted from this data and presented as mean ± SD of three replicates. Additional explanation on applied statistics is given in Supplementary material (Section 2.12).

3. Results

3.1 Isolation and identification of *L. reuteri* B2

After isolation of the pure colonies from the feces of C57 BL/6 mice, the size, color, shape, and number of colonies of assessed strain were criteria on which observed strain was chosen to be tested on probiotic activity. The strain morphology was follow: rod-shaped, facultative anaerobic and Gram-positive organism. API 50 CHL was used as biochemical tests, where this strain utilized 97% of the carbohydrates known to be used as a profile that is distinctive for *Lactobacillus* species. This strain was sequenced and identified as *L. reuteri* B2 using 16SrDNA sequencing with universal primers (UNI16Sfw and UNI16Srev) [30].

Survival to acidic conditions and bile salts tolerance of *L. reuteri* B2

The resistance under extreme conditions (low pH, high concentration of the bile salts) was performed and tested further for its sensitivity to acid and bile salts. In Table 2, is shown acid resistance of *L. reuteri* B2 strain was evaluated by measuring the survival rate after 2 h treatment

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at different pH values, in the range of 2.0- 3.5 with 0.5 holds, and at 6.4. Incubation at pH 6.4 was used as a control since at this pH value the strain did not present any significant loss in viability, 8.05 log CFU/mL, or 100% percentage viability. The viability of *L. reuteri* B2 was assessed at pH 3.5 and 3.0 after 2 hours of incubation, where the bacterial count did not change significantly, while at pH 2.5 the viability of *L. reuteri* B2 decreased slowly, 6.22 log CFU/mL (78%), to final pH during the same incubation time. At pH 2 the *L. reuteri* B2 showed the lowest viability 5.16 log CFU/mL (64%).

Table 2.

Therefore, in the presence of the bile salts the evaluation of the viability of *L. reuteri* B2, and as target concentrations, we used 0.15% and 0.30% of bile salts after 6 hours of incubation. After exposure on 0.15% MRS supplemented with bile salts, the viability of *L. reuteri* B2 cells decrease to 3.97 (48%) while a reduction trend cell counts on increasing bile salt concentration obtained the lowest viability of *L. reuteri* B2 and was, 3.04 (37%) on 0.30% MRS supplemented with bile salts. Incubation at MRS, pH 6.4 without bile salts was used as control.

3.3 Antimicrobial activity of *L. reuteri* B2

The antagonistic effect against pathogens was the main probiotic trait for the evaluation of the potential probiotic organism. There have been already established that LAB are probiotic strains that generally have antibacterial effects because these strains have the ability to produce several metabolic compounds (short-chain fatty acids, H_2O_2, and bacteriocins) that were found in the supernatants of these cultures [37]. The antibacterial activity, in the present study, was measured as the diameter zone of the transparent inhibition zone against different pathogens. Our results indicated that *L. reuteri* B2 can inhibit 6 of 9 pathogens, including *Escherichia coli* ATCC25922 (9.5 ± 0.71mm), *Klebsiella oxytoca* J7 (12.5 ± 0.71mm), *Klebsiella pneumonie* sub. *pneumonie* ATCC 13883 (5.5 ± 0.71mm), *Schigella flexneri* ATCC 12022 (10.5 ± 0.71mm), *Pseudomonas aeruginosa* ATCC 27853 (8 ± 2.83mm) and *Staphylococcus aureus* ATCC 6538 (11 ± 1.41mm). While, *Bacillus subtilis* ATCC 6633 (0.3 ± 0.43mm), *Streptococcus pneumoniae* ATCC 6301 (0.15 ± 0.21mm), *Enterococcus faecalis* ATCC 29212 (0.1 ± 0.14mm) were not

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significantly inhibited by the cell-free supernatant of *L. reuteri* B2. Table 3. Likewise, it is important to mention that *L. reuteri* B2 obtained the highest inhibition activity against *K. oxytoca* J7 (12.5 ± 0.71mm), the pathogen, isolated from the same source as itself. The results obtained from this assay revealed that cell-free supernatant of *L. reuteri* B2 can inhibit the tested pathogens significantly with exception of *Bacillus subtilis* ATCC 6633, *Streptococcus pneumoniae* ATCC 6301, and *Enterococcus faecalis* ATCC 29212.

Inhibition zone measurements by three assessors for each microorganism were analyzed using one-way ANOVA. As significant, P-value < 0.05 was considered. The results are shown in **Fig. S**3 and **Table 3.** The mean values of the inhibition zones depending on the evaluator are given in **Fig. S3.**

Table 3.

Subjectivity in measuring the size of the inhibition zone by the person performing the measurement was examined by comparing the two evaluators using the Paired Samples T-Test. The setting of the null hypothesis X_0 is that two evaluators give equal measurement results. The values (Table 4) at the 0.05 level of the population mean that they are significantly different from the test mean that the null hypothesis is rejected, ie there are differences in the results during the measurement.

Table 4.

Other statistical values are given in the Supplement - subsection 3.3 antimicrobial activity (Table S3).

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3.4 Antibiotic susceptibility of *L. reuteri* B2

Susceptibility of *L. reuteri* B2 strain to 5 generally used antibiotics was determined by disc diffusion method and presented in Table 5. After this assay, *L. reuteri* B2 was displayed intermediate resistance to tetracycline (30 µg) and amoxicillin and clavulanic acid (20 µg), while it was highly susceptible to ampicillin (10 µg) and cephalothin (30 µg). Vancomycin [38] was used as a negative control, as a general antibiotic of which lactobacilli have high natural resistance.

Tabel 5.

3.5 Results of SM characterization

In order to produce alginate-based bio-carrier with appropriate addition of SM it was necessary to characterize synthesized materials and further to perform a series of experiments to obtain beads with the best encapsulation efficiency. The results of degree of substitution (DS; Equation S1), viscosity, reaction efficiency (RE; Equation S3), swelling capacity (SC; Equation S4), and moisture adsorption (MAd; Equation S6) of pure starch and synthesized starch maleate are given in Table 6.

Table 6.

The calculated DS values (Table 6) showed that the esterification of starch by MA (Figure S2) is highly limited because of the presence of the intermolecular hydrogen bonding between starches macromolecule. To increase, the extent of starch macromolecules modification reactive RA plasticizer was used. Effectiveness of the starch granule gelatinization/structure development and thus plasticization, i.e. insertion and distribution between the polymer chains, is highly affected by plasticizer structure/properties and thus overall intensity change of inter-molecular interactions which influence matrix softening [39]. The process applied helped in the distribution of low size RA and MA molecules into starch granules interior by forming ester linkage. In that manner, both modifiers occupy intermolecular spaces between polymer chains contributing to the increase of starch macromolecules flexibility. Thus, the introduction of both structural segments: RA and MA cause an increase in the free volume due to molecular mobility and appropriate compatibility, and thus increase of segmental motion is a consequence [40]. Due to this, significant improvement in modification efficiency was obtained (RE for exps. 5-8; Table 6). According to ester and acid values determination it was estimated that MA unit/~5.6 AGU and RA unit/~228 AGU (m/l = 41; Fig. S2) are introduced into SM obtained according to exp. 7 (Table 6). Plasticizers and water break out the hydrogen bonding between starch macromolecules helping in improvement modification efficiency, and due to RA and MA structure/properties

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slightly increase the hydrophobicity of the product obtained. The result obtained indicates that modification degree increases cause lowering SC and MA of the produced SM [41]. Also, SEM micrographs of SM, given at **Fig. S4,** show irregularly shaped SM granule in comparison to regular one obtained for native starch [42].

3.6 Optimization of microencapsulation of *L. reuteri* B2

Obtained SM materials, results of characterization given in subsection 3.5, were used in preliminary encapsulation experiments. The significantly better encapsulation capability showed SM materials were obtained by experiments 5-9 (Tables 6 and S1), and among them, we selected SM material from exp. 7 to be used in an optimization study. Graphical presentation of the results of the optimization of the encapsulation with the output variable, i.e. encapsulation yield, concerning the amount of Na-alg and SM solution (in %) is given in **Fig. 1**. Regions that meet critical properties are colored intensely red, while the regions without optimization criteria are cut off from the graphical area. Using point prediction through the software based on the factors or components included in the model are obtained more precise optimization conditions. Based on the prediction equation displayed at the ANOVA output **(Table S4)** are calculated the expected responses and associated trust intervals. Additional results from the optimization of the encapsulation of *L. reuteri* B2 into alginate-based beads are given in the Supplementary material (subsection 3.6).

Fig. 1.

According to the results the initial concentration of 2% Na-alg and 3% SM was selected to provide an optimal condition for the production of alginate-based beads 1% Na-alg – 1.5% SM (Na-alg+SM) with the highest encapsulation yield of *L. reuteri* B2 (Fig. 1 and S5).

In order to estimate the effect of SM contribution in Na-alg+SM, concerning Na-alg, the effect on the viability of the probiotic bacteria microencapsulated with these biobased carriers is presented in Table 4. In the case of Na-alg microencapsulated beads with *L. reuteri* B2, the average of the initial viable count of 7.96 log CFU/mL was reduced to 7.80 log CFU/mL after encapsulation. While the average of the initial viable count of 7.94 log CFU/mL was reduced to

7.88 log CFU/mL with Na-alg+SM beads with *L. reuteri* B2 cells. The encapsulated yield of microencapsulated bacteria, Table 7., in these biomaterials were 70.2% and 88.4%, respectively. Therefore, these results confirm that the SM as a natural component improves higher viability of *L. reuteri* B2 cells as well as the yield of this biomaterial than biomaterial with Na-alg. The stability of beads with starch maleate indicates that the viability of the probiotic culture remains stable after microencapsulation.

Table 7.

Additionally, the comparative experiment was performed according to the literature method [43], at a 1:3:1 molar ratio of anhydroglucose unit:MA:sodium hydroxide. The obtained material was used in the production of alginate-based *L. reuteri* B2 carrier with somewhat lower success (encapsulation yield of 81.8%). Following that presented method of SM production, i.e. dry blending method, offer a better alternative for encapsulation of *L. reuteri* B2.

3.7 Size distribution of alginate beads in acidic conditions

The size determination of alginate beads of two different materials has been prepared in acidic conditions, at pH 2.5 for 2 hours. Herein, we obtained results where the Na-alg beads had the same diameter reduction as the diameter of Na-alg+SM beads after incubation of 2 hours at pH 2.5, while the initial size of Na-alg beads was higher than in the Na-alg+SM beads. Similarly, the weight loss of these two materials was the same.

Descriptive statistics of the results obtained by measuring the grain sizes of the material before and after exposure to acidic conditions obtained using JASP software are given in Table 8. Statistical examination of the frequencies of normality was performed using the Shapiro-Wilk test. Many consider this test to be the best test of normalcy. Normal population distribution is the null hypothesis of our test. Thus, the null hypothesis is rejected if the p-value is less than the chosen alpha level, and there is evidence tested data are not normally distributed. On the other hand, the null hypothesis cannot be rejected if the p-value is greater than the chosen alpha level, (the data came from a normally distributed population). According to the results in Table 8, the null hypothesis can be rejected, i.e. we do not have a normal distribution. If the sample size is large enough, this test can detect even a trivial deviation from the null hypothesis, like most tests

of the statistical significance, then the additional study of the effect is typically recommended, e.g. Q–Q plot (**Figs. S6** and **S7**) [44], and plot of density versus diameter (**Figs. S8 and S9**).

Table 8

The Q-Q plot (quantile-quantile plot) is one of the best ways to compare the distribution of sample x with some theoretical distribution. In this way, we can sense the distribution of the sample. On the diagram (Fig. S6 and S7) we notice that at all points -values of our data the distribution follows the line x=y which indicates that the distribution is normal. Other graphics, the density distribution of grains size, the density distribution of grains mass, scatter plots of grain size ratio before and after exposure to acidic conditions and scatter plots are the ratio of grain size to grain mass, are given in Fig. S8 and S9, and Table 8.

3.8 FTIR analysis

FTIR spectra of four different materials, Na-alg, starch, SM, and Na-alg+SM, used for microencapsulation of *L. reuteri* B2 are presented in **Fig. 2**. The broad peak at 3250 cm^{-1} in the spectra of all analyzed materials represents the stretching vibration of the intra-molecularly bonded primary and secondary hydroxyl groups. As a result of starch modification with MA it would be expected appearance the bands related to =C−H and C=C stretching vibrations at >3000 and ~1620 cm^{-1}, respectively. Instead, high overlapping was noticed. The peaks, observed at ~2928 cm^{-1} and ~2856 cm^{-1}, relate to asymmetric and symmetric –CH and –CH_2 stretching vibrations in all studied materials, respectively.

The new peaks observed at 1715 cm^{-1}, present in SM spectrum, originate from the C=O stretching vibration of the carboxyl and ester C=O group. These two peaks confirm successful starch hydroxyl group esterification with MA and ricinoleic acid. A small shift of this peak to 1711 cm^{-1} was observed in the spectrum of Na-alg+SM beads.

The two peaks, observed at 1593 and 1417 cm^{-1}, are assigned to the asymmetric and symmetric stretching vibration of carboxylate anion, respectively, in the spectrum of Na-alg. Analogous assignment of the peaks at 1599 cm^{-1} and 1417 cm^{-1}, in the spectrum of Na-alg+SM beads, were done. The band at 1610 cm^{-1}, assigned to the -OH bending vibration of hydroxyl

groups in cellulose and residual water, was observed in the spectra of starch and SM. This band, considering spectrum of Na-alg+SM beads, is strongly overlapped with asymmetric carboxylate vibration from Na-alg. In order to resolve contribution of of –OH bending, C=C stretching and COONa asymmetric stretching vibration deconvolution of Na-Alg+SM spectrum in the region from 1540 to 1800 cm^{-1} was applied **(Fig. S10).** The deconvoluted region indicates the presence of carbonyl from the carboxylic and ester group at 1705 and 1734 cm^{-1}, respectively. Also, the C=C stretching vibration from maleic anhydride and ricinoleic acid residues at 1650 cm^{-1} and asymmetric carboxylate vibration at 1598 cm^{-1} was observed. Additionally, the band at 1147 cm^{-1} represents the asymmetric C–O–C stretching vibration which indicated that the glycosidic linkages among molecules were unchanged after modification [45,46]. Following peaks at 1011 cm^{-1}, 929 cm^{-1}, and 860 cm^{-1} are associated with the C-H group in all materials, that has been already shown in previous researches [47,48].

Fig. 2.

3.9 Optical and SEM microscopy

The images that we obtained from optical microscopy of unfilled and field beads with two different materials were presented in Figure 3 for comparison. In both materials, unfilled beads did not have any visible microorganisms, while the pictures of the filled beads obtained the cells of the probiotic that can be clearly seen. Probiotics trapped in the alginate beads obtained similar cell morphology. SEM also showed the presence of probiotic cells that were in the range from 2 to 5 μm in length in filled alginate beads (**Fig. 4.**). Dimensions of the probiotic bacteria trapped in the Na-alg beads were between 1 to 2 μm in length that was also confirmed by optical and electron microscopy images. These results are consistent with a previous microstructural study on encapsulated bacteria [49]. For a comparison SEM micrograph of Na-alg + SM beads with *L. reuteri* B2 cells at 10kx magnification is given on **Fig. S11**.

Fig. 3

Fig. 4.

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3.10 Thermogravimetry analysis (TGA) of alginate-based beads

Further material characterization was carried out by thermogravimetry (TGA). Investigation of the terminal stability of the polymeric systems is performed by a suitable TGA method. The range of processing temperature applied should be based on the knowledge of the decomposition pattern of the material subjected to heating [50]. The TGA curves for the alginate-based beads, given in **Fig. 5**, show a dehydration process in the initial stage, and subsequent decomposition consisted of two overlapping steps under nitrogen. This result is in agreement with DTG data. Soares et al. [51] were also observed a similar behavior of the alginic acid, and the product obtained at ~400 °C was defined as a carbonaceous product [52]. The similar behavior indicates similar structural characteristics, i.e. thermal properties, of both materials used.

Fig. 5.

3.11 Survival of microencapsulated and free *L. reuteri* B2 cells in SGJ

Encapsulated probiotic bacteria and free form of *L. reuteri* B2 were exposed to *in vitro* SGJ conditions. The data of survivals of bacterial cells in microcapsules and in the free form obtained are presented in Fig. 2. The survival of probiotics in the free form was lower in gastric juice and decreased beyond as the incubation period increased than in the microcapsules. After 60 min, free *L. reuteri* B2, microencapsulated *L. reuteri* B2 with Na-alg, and microencapsulated *L. reuteri* B2 in Na-alg+SM, showed that their viability decreased slowly, while in the second hour their viability decreased abruptly. Therefore, microcapsules of *L. reuteri* B2 with Na-alg+SM decreased from 9.36 log CFU/mL to 8.88 log CFU/mL, while the microcapsules of *L. reuteri* B2 with Na-alg decreased from 9.36 log CFU/mL to 8.76. Free cells of *L. reuteri* B2 after 120 min, decreased to 7.72 log CFU/mL. However, the cell number microencapsulated and non-microencapsulated cells of *L. reuteri* B2 decreased to 8.88, 8.76, and 7.72 log CFU/mL respectively after 120 min (Figure 6). In the case of *L. reuteri* B2, irrespective of the material used for microencapsulation the survival of microencapsulated cells of *L. reuteri* B2 was better

than the cells in the free form. Comparison of the viability of microencapsulated *L. reuteri* B2 revealed better protection by Na-alg+SM calcium cross-linked beads.

Fig. 6.

4.0 Discussions

In the last few decades, the increasing interest in probiotics is significant because of the increasing health problems, as well as, in both developed and developing countries. The most investigated microorganisms that obtained probiotic characteristics are LAB and Bifidobacteria where the genus *Lactobacillus*, in particular, is commercially sold as a probiotic with health-promoting properties [53]. Specifically, probiotics may beneficially affect inhibiting the growth of pathogens, augmenting the intestinal microbial population of the host, thereby surviving the environment in GIT [54]. and inhibiting the growth of pathogens [54]. Hence, the isolation of novel bacterial strains that obtained probiotic activity and producing antibacterial substances become a very important step for potential industrial application.

The suitability of the strain *L. reuteri* B2 as potential probiotic were assessed including all probiotic characteristics, as well as resistance to artificial gastric juice and bile acid, antibacterial activity, and antibiotic susceptibility. Small amounts of microorganisms can survive the strongly acidic conditions of the stomach [55]. In this current study, we investigated the probiotic activity of the wild strain *L. reuteri* B2, and its stability and viability in the GIT. *L. reuteri* B2 exhibited obtained resistance to artificial acidic conditions with the viability of 64%, while the viability in the presence of bile salts was 37%. Hong et al., 2018., demonstrated that *Lactobacillus curvatus* PA40 [56] strain has the high survival rates (97.8%) at pH 2.5 while the viability was (99.1%) after assay at 24h in the presence of the bile salts. Similar results have been presented previously for *Lactobacillus casei, Lactobacillus acidophilus*, *Lactobacillus reuteri, Lactobacillus salivarius Lactobacillus rhamnosus*, *Lactobacillus bulgaricus*, *Lactococcus lactis*, and *Streptococcus thermophilus*, all of which grow well in the presence artificial gastric juice and bile acid [57]. According to these results, it should be concluded that differences between *Lactobacillus* species of viability in the acidic conditions and the presence of the bile salts are strain dependent.

Likewise, to provide beneficial effects for human or animal health, LABs as probiotics, must be resistant to various antibiotics. [58]. *L. reuteri* B2 displayed intermediate resistance to

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antibiotics, including amoxicillin and clavulanic acid, and tetracycline, in contrast, it proved highly susceptible to antibiotics, including ampicillin and cephalothin, while it was resistant to vancomycin [38]. Results that we obtained are in accordance with Hyacinta et al. [59], where they detected susceptibility in lactobacilli to antibiotics. Furthermore, there has been investigated the antimicrobial activity of *L. reuteri* B2 strain with nine pathogens. It is shown that *L. reuteri* B2 obtained high antimicrobial activity against *K. oxytoca* J7, *S. flexneri* ATCC 12022, and *S. aureus* ATCC 6538 strains. *L. reuteri* B2 obtained the highest antimicrobial activity versus *K. oxytoca* J7, the strain that was isolated from the same source as *L. reuteri* B2. Beneficial effects of *L. reuteri* strain have been noted previously, also by Mu et al. 2018., and showed that *L. reuteri* producing different antimicrobial molecules (ethanol, reuterin, and organic acids) increases its antimicrobial activity [7] inhibiting pathogens.

Furthermore, the encapsulation process improves the survival of probiotic microorganisms during passage through the specific environment of the GIT [24,60]. Hence, in the presence of simulated gastric acid and bile salts, the important protective effects of the encapsulation of probiotics with alginate have been reported previously [61]. The viability of bacterial cells varies depending on the encapsulation method, biomaterials that have been used for microencapsulation bacterial cells, and probiotic strains that were used. We investigated the probiotic activity of the wild strain *L. reuteri* B2, and its stability and viability in the SGJ, in the presence of two different biomaterials: Na-alg and Na-alg+SM after microencapsulation. Our hypothesis was if free cells of *L. reuteri* B2, can survive all conditions in the GIT (acidic conditions, presence of bile salts, SGJ) and in that way to improve the gut function, then the use of appropriate biomaterials for microencapsulation would improve its stability and viability in SGJ. Consequently, microencapsulation using different polymers such as alginate, gums, carrageenan, and starch is an attractive area of researches to make the most suitable biomaterial that can maintain the stability and viability of probiotics cells. Results obtained from this study showed that the encapsulation process with Na-alg+SM is the most effective for obtaining viable *L. reuteri* B2 cells.

Alginate alone as a biomaterial for encapsulation of the *Lactobacillus* species is competent to encapsulate different *Lactobacillus* species by cross-linking *via* calcium ions, although the beads formed typically have a relatively low mechanical stability. Then, these beads are sensitive to chelating compounds such as citrate, lactate, EDTA, and phosphate, or anti-

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gelling cations such as Na^+ and Mg^{2+} [62]. Modifying alginate with different polymers is an important way to ameliorate the encapsulation process and survival in the gastric environment. Hence, material characterization has been done by analyzing: beads size, optical microscopy, SEM, FTIR, and TGA. Dimensions of probiotic bacteria cells of about 1 to 2 μm in length that were trapped in Na-alg beads were confirmed using optical and electron microscopy images. The results obtained in our study are in agreement with previous studies where the importance of bacterial cell encapsulation has already been showed [63]. The size of the beads did not present a difference between the loaded beads with probiotic and unloaded beads by Na-alg and Na-alg+SM. FTIR and SEM analysis confirmed that the stability of Na-alg+SM beads as carbonaceous material higher than Na-alg beads. The viability of *L. reuteri* B2 in Na-alg+SM after the microencapsulation process was 7.88 log CFU/mL, or encapsulation yield was 88.4%, while in the Na-alg was 7.80 log CFU/mL, or encapsulation yield was 70.2%. Likewise, the highest viability obtained after exposure in gastric juices environment for 120 min, was in Na-alg+SM microcapsules with *L. reuteri* B2 and was confirmed. Require of the results, the viability of *L. reuteri* B2, microencapsulated with Na-alg+SM was higher in SGJ than in Na-alg beads. The finding of Lee *et al*. in 2004, where the strain *Lactobacillus bulgaricus* KFRI 673 ensured greater survival in the gastric environment microencapsulated by chitosan-coated calcium alginate is in agreement with our study [64]. Likewise, Mokarram *et al*. 2009, showed that *L. acidophilus* and *L. rhamnosus* had higher viability exposed to SGJ without pepsin when they are encapsulated in calcium alginate with double coating Na-alg [65]. They showed that the distribution of gastric juice in the membrane with double-layer and the reduction of pore size leads to limitation of interaction between cells with the gastric juice. According to our study microcapsules coated with Na-alg+SM provide the best protection in SGJ.

Conclusion

The present study showed that microencapsulation of *L. reuteri* B2 in Na-alg+SM resulted in better survival in SGJ of cells in adverse conditions as compared to free cells. Likewise, the survival of the bacterial cells in the gastrointestinal environment is significantly improved and allows viable cells are enabled to achieve beneficial effects as a probiotic. Therefore, for the delivery of probiotic cultures in simulated human gastrointestinal juices, the approach from this

study could be used in the future. In this research, the best protection of the cells in the presence of the gastric juice provided Na-alg+SM, from the two types of microcapsules that were assessed. The development of new equipment that could be used in large commercial processes, both in the food industry and in the pharmaceutical processing industry, presents the challenge for researchers in this field, as well as related researches into assessing the efficiency of microencapsulation of GIT using an animal model.

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Conflict of interest statement

The authors declare no conflict of interest.

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Tables

Table 1. D-optimal design for the experiment for the dependence of Encapsulation yield from Na- alg and SM concentration.

Run	*Factor 1* *A: Na-alginate* solution (%)	*Factor 2* *B: Starch maleate solution* (%)	*Response* *Encapsulation yield* (%)	*Predicted* *Encapsulation yield* (%)
1	1.125	4.2	57.2	55.53
2	2.5	2.0	55.1	54.66
3	1.125	6.0	52.3	52.48
4	0.5	4.8	49	48.60
5	0.5	2.0	54	52.64
6	1.625	3.0	70.2	69.47
7	1.625	3.0	70.2	69.47
8	1.875	5.1	65.2	67.76
9	0.625	3.0	50.1	53.13
10	1.125	6.0	52.3	52.48
11	2.5	3.6	60.2	61.34
12	2.5	6.0	56	54.98
13	1.375	2.0	58.8	69.60
14	1.375	2.0	58.8	59.60
15	0.5	4.8	49	48.60
16	1.625	3.0	70.2	69.47

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Table 2. Acid resistance and bile salts tolerance of *L. reuteri* B2 cells

	pH	Initial cells log CFU/mL, t = 0h	Log CFU/mL* t = 2h	[a]Viable percentages, %
Acid resistance	6.4	8.05 ± 0.04	8.05 ± 0.04	100
	3.5	8.05 ± 0.01	7.30 ± 0.03	91
	3.0	8.04 ± 0.01	7.28 ± 0.01	90
	2.5	8.02 ± 0.01	6.22 ± 0.06	78
	2.0	8.03 ± 0.01	5.16 ± 0.06	64
	MRS + Oxagall (MRSO)	**Initial cells log CFU/mL, t = 0h**	**Log CFU/mL* t = 6h**	**[a]Viable percentages, %**
Bile salts	MRS	8.29 ± 0.01	8.29 ± 0.01	100
	MRS 0.15%	8.29 ± 0.01	3.97 ± 0.01	48
	MRS 0.30%	8.23 ± 0.01	3.04 ± 0.03	37

*Values are the means of triplicate measurements

[a]Data are expressed as % survival

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Table 3. ANOVA one way descriptive statistics

	Assessor 1			Assessor 2			Assessor 3		
	Mean	SD	SE of Mean	Mean	SD	SE of Mean	Mean	SD	SE of Mean
E. Coli ATCC25922	9.6667	0.5774	0.3333	9.6667	0.5774	0.3333	10.333	0.5774	0.3333
K. oxytoca J7	12.333	0.5774	0.3333	12.333	0.5774	0.3333	12.667	0.5774	0.3333
K. pneumonie sub. pneumonie ATCC 13883	5.3333	0.5774	0.3333	5.3333	0.5774	0.3333	6	1	0.5774
S. flexneri ATCC 12022	11	1	0.5774	11	1	0.5774	10.667	0.5774	0.3333
P. aeruginosa ATCC 27853	8	2	1.1547	8	2	1.1547	8.6667	1.1547	0.6667
B. subtilis ATCC 6633	0.43333	0.37859	0.21858	0.43333	0.37859	0.21858	0.33333	0.28868	0.16667
S. aureus ATCC 6538	11	1	0.5774	11	1	0.5774	10.667	0.5774	0.3333
S. pneumoniae ATCC 6301	0.2667	0.2516	0.1453	0.2667	0.2516	0.1453	0.1667	0.2887	0.1667

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E. faecalis ATCC 29212	0.2333	0.2516	0.1453	0.2333	0.2516	0.1453	0.1333	0.2309	0.1333
Negative control	0.0667	0.1155	0.0667	0.0667	0.1155	0.0667	0.2	0.2	0.1155

Table 4. Paired Samples T-Test

Measure 1	Measure 2	*p* value
Assessor 1	Assessor 2	0.153
Assessor 1	Assessor 3	0.27
Assessor 2	Assessor 3	0.92

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Tabel 5. Antibiotic susceptibility of *L. reuteri* B2 strain

Antibiotics	Concentration, μg/mL	Diameter, mm	Sensitivity*
Ampicillin	10	22.3 ± 0.58	S
Amoxicillin and clavulanic acid	20	16.3 ± 0.63	I
Cephalotin	30	23.5 ± 0.81	S
Vancomycin	30	0.51 ± 0.58	R
Tetracycline	30	15.7 ± 0.64	I

Diameters of inhibition zones are expressed in millimeters; the diameter of the disc was 6 mm. Data are arithmetical means of three measurements. *S – sensitive, I – intermediate, R – resistant

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Table 6. DS, viscosity, RE, SC, and MA of pure starch and starch maleate

Experiment	DS	Viscosity, Pa · s	RE (%)	SC (g/g)[***]	MAd, %
0[*]	-	1.063± 0.084[**]		11.80± 0.45[*]	71.42[**]
1	0.078	1.138± 0.072	[illegible].9	4.82± 0.12	34.26
2	0.062	1.122± 0.062	6.3	5.12± 0.15	38.19
3	0.054	1.106± 0.060	8.2	5.66± 0.20	43.22
4	0.041	1.092± 0.091	8.3	6.02± 0.22	46.56
5	0.228	1.386± 0.068	17.2	3.88± 0.14	32.29
6	0.202	1.371± 0.06[illegible]	20.4	4.96± 0.17	34.29
7[****]	0.194	1.342± 0.054	29.3	4.78± 0.19	37.44
8	0.172	1.328± 0.051	26.3	4.36± 0.17	36.21
9	0.190	1.3[illegible]± 0.038	28.6	3.51± 0.12	25.41

[*] unmodified starch; [**] [37]; [***] SC and S was difficult to determine for a mostly soluble fraction of SM neutralized with sodium hydroxide; [****] SM acid value: 62 mg KOH/g

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Table 7. Encapsulation yield and viability of *L. reuteri* B2 in Na-alg and Na-alg+SM beads**.**

Microsphere type	Encapsulation yield (%)	Viability (log CFU/mL)
Na – alg with *L. reuteri* B2	70.2 ± 2.36	7.80 ± 0.93
Na – alg+SM with *L. reuteri* B2	88.4 ± 1.54	7.88 ± 0.96

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Table 8. Results of descriptive statistics of bead size (diameter - d and mass - m) obtained by the statistical program JASP. Size and mass of the Na-alg and Na-alg+SM beads at pH 2.5 after 2 hours

	Na-alg - d (mm)	Na-alg - d for 2 h pH 2.5 (mm)	Na-alg+SM - d (mm)	Na-alg+SM d for 2 h pH 2.5 (mm)	Na-alg - m (mg)	Na-alg - m for 2 h pH 2.5 (mg)	Na-alg+SM m (mg)	Na-alg+SM m for 2 h pH 2.5 (mg)
Minimum	3	2.55	2.1	1.7	11.7	10.58	7.35	7
Maximum	3.5	3	2.45	2.05	13.65	12.45	8.57	8
Mean	3.246	2.791	2.271	1.878	12.671	11.587	7.95	7.546
Std. Error of Mean	0.028	0.024	0.02	0.018	0.108	0.098	0.07	0.062
Median	3.25	2.8	2.25	1.9	12.65	11.6	7.9	7.5
Mode	3.25	2.7	2.3	1.9	11.7	11.2	8.05	7.65
Std. Deviation	0.146	0.123	0.104	0.091	0.559	0.51	0.365	0.324
Variance	0.021	0.015	0.011	0.008	0.312	0.26	0.133	0.105
Shapiro-Wilk α	0.96	0.958	0.956	0.963	0.968	0.96	0.954	0.938
p-value of Shapiro-Wilk	0.362	0.339	0.291	0.433	0.561	0.369	0.275	0.109

Figures

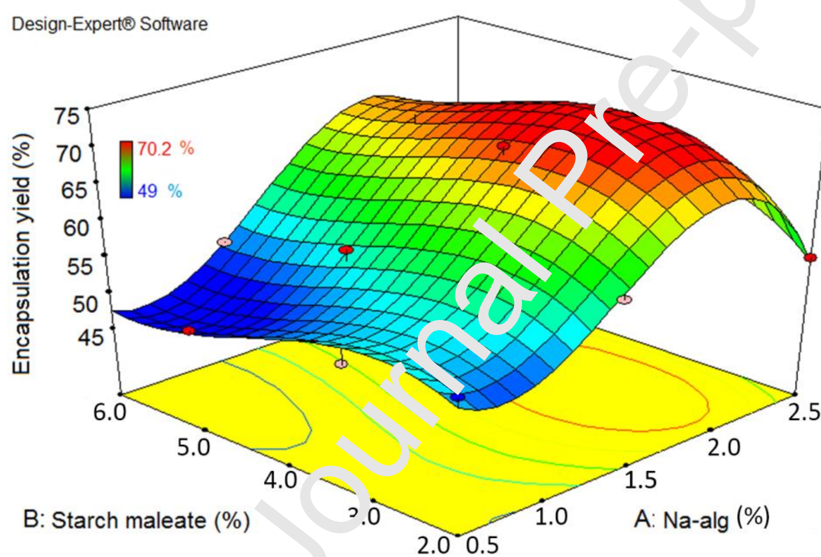

Figure 1. Contour diagram representing Encapsulation yield vs. concentration of Na-alg and starch maleate (SM) solution (%)

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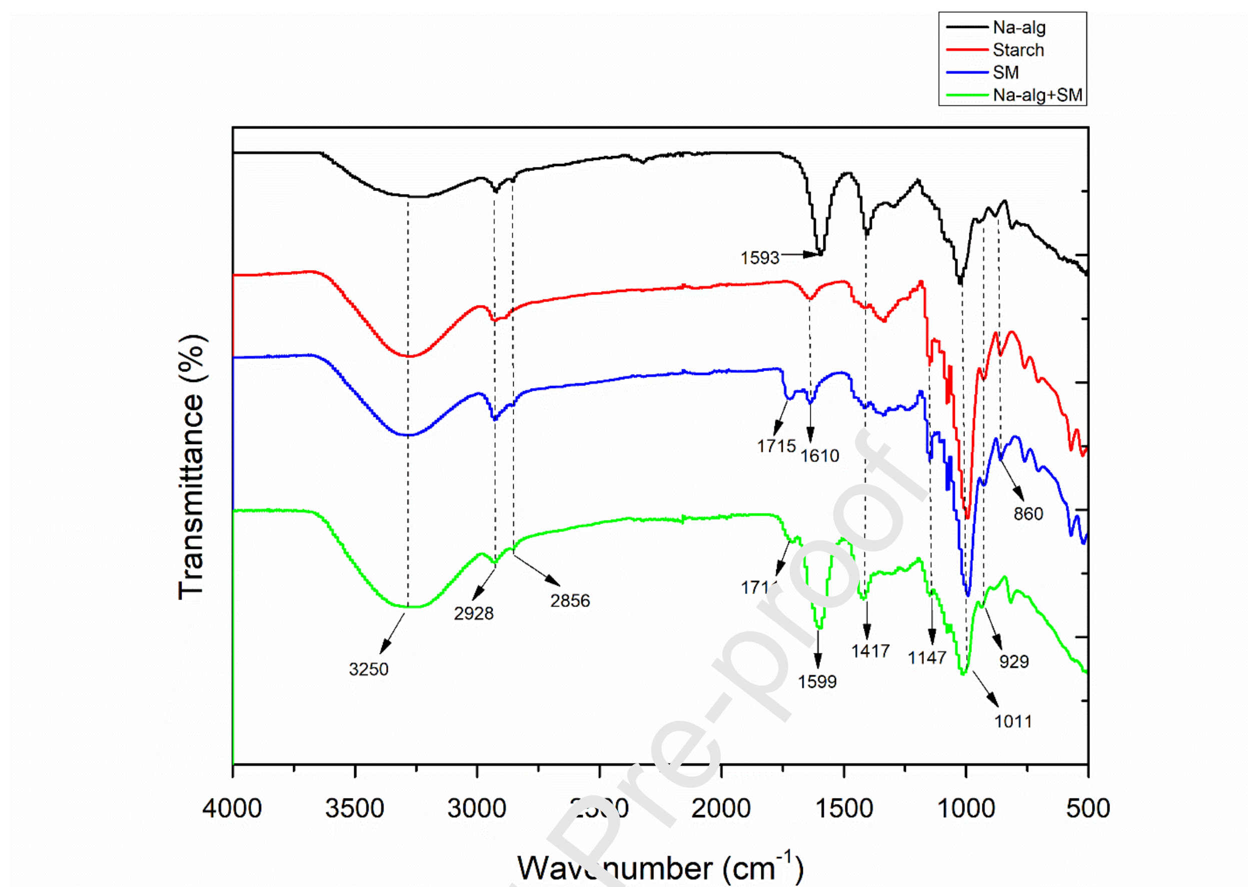

Figure 2. FTIR spectra of the Na-alg, starch, SM and Na-alg+SM materials

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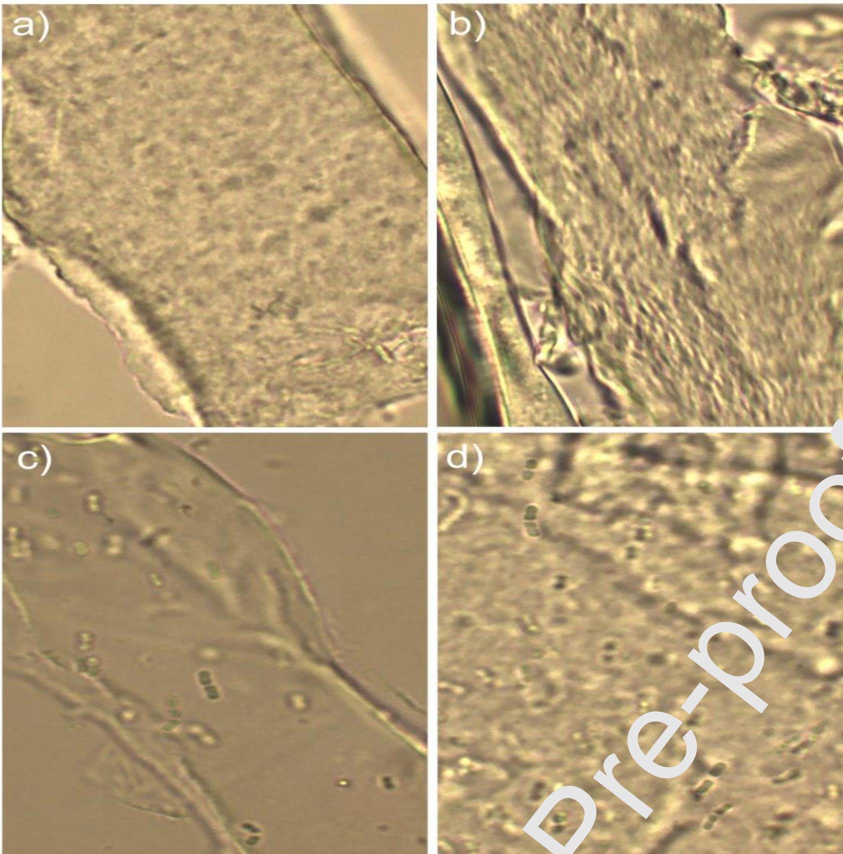

Figure 3. Optical micrograph of: a) Na-alg, b) Na-alg + SM, c) Na-alg + *L. reuteri* B2, d) Na-alg + SM + *L. reuteri* B2 (100x magnification).

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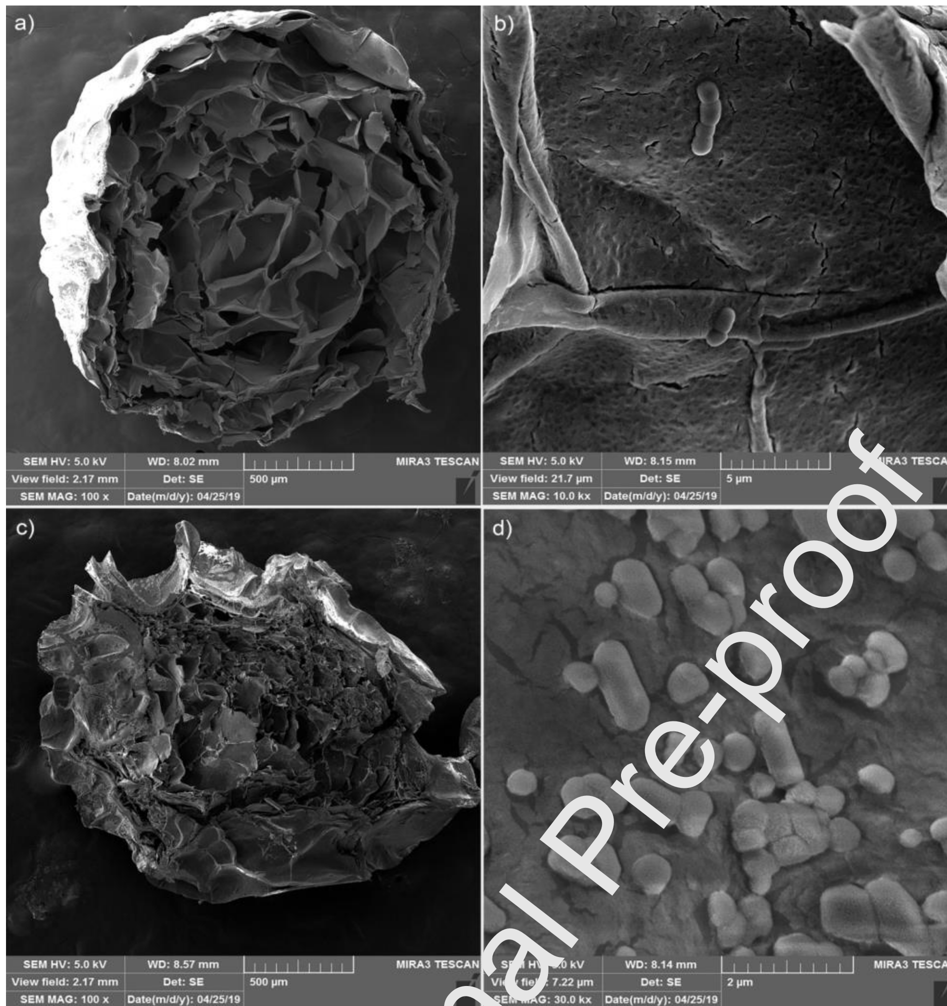

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Figure 4. SEM micrograph of: a) unfilled Na-alg beads, b) filled Na-alg beads with *L. reuteri* B2 cells, c) unfilled Na-alg + SM beads, d) filled Na-alg + SM beads with *L. reuteri* B2 cells

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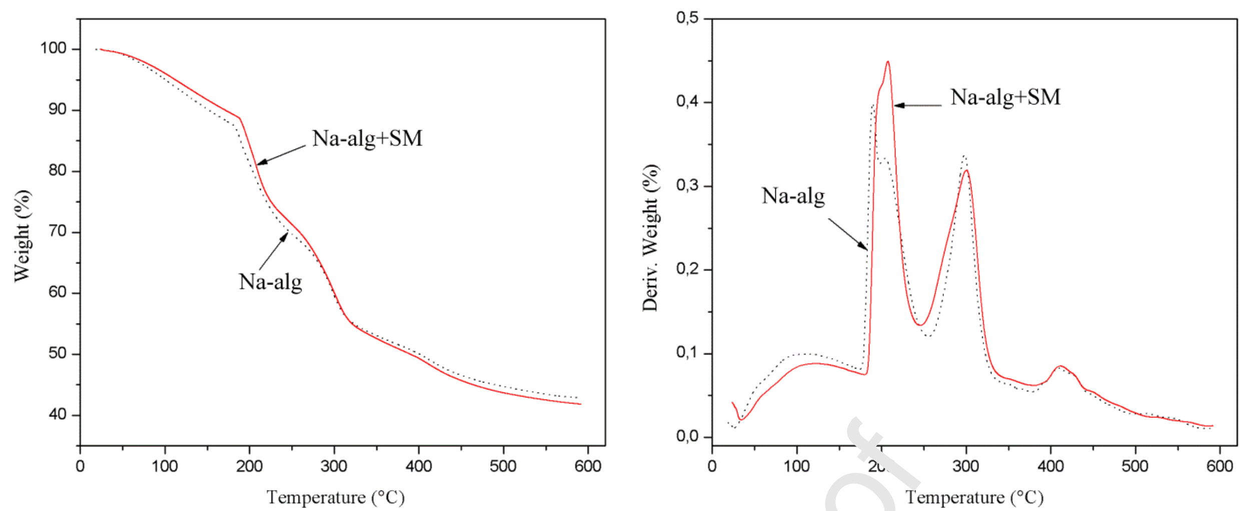

Figure 5. a) TGA and b) DTG curves of Na-alg and Na-alg+SM materials

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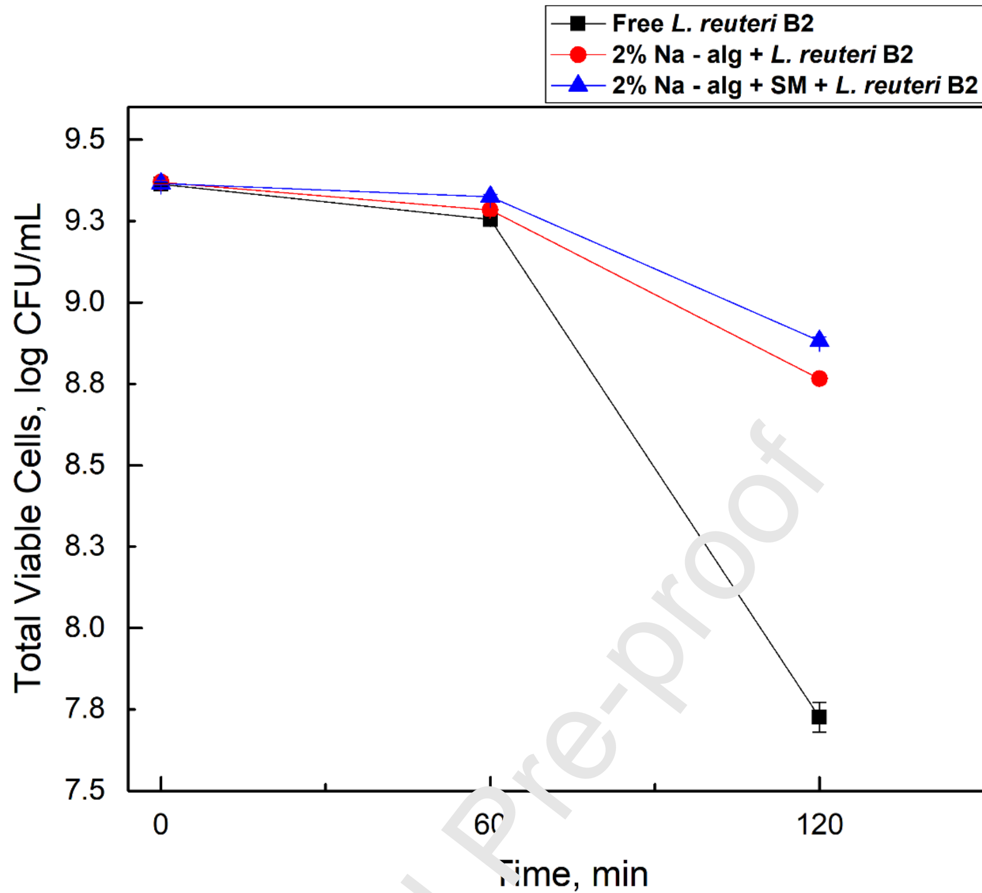

Figure 6. Survival of *L. reuteri* B2 cells in the free and encapsulated form in simulated gastric juices over time. Counts are based on samples plated on MRS and incubated at 37 °C anaerobically. Error bars represent the mean ± standard deviation of replicate samples.

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Conflict of interest statement

The authors declare no conflict of interest.

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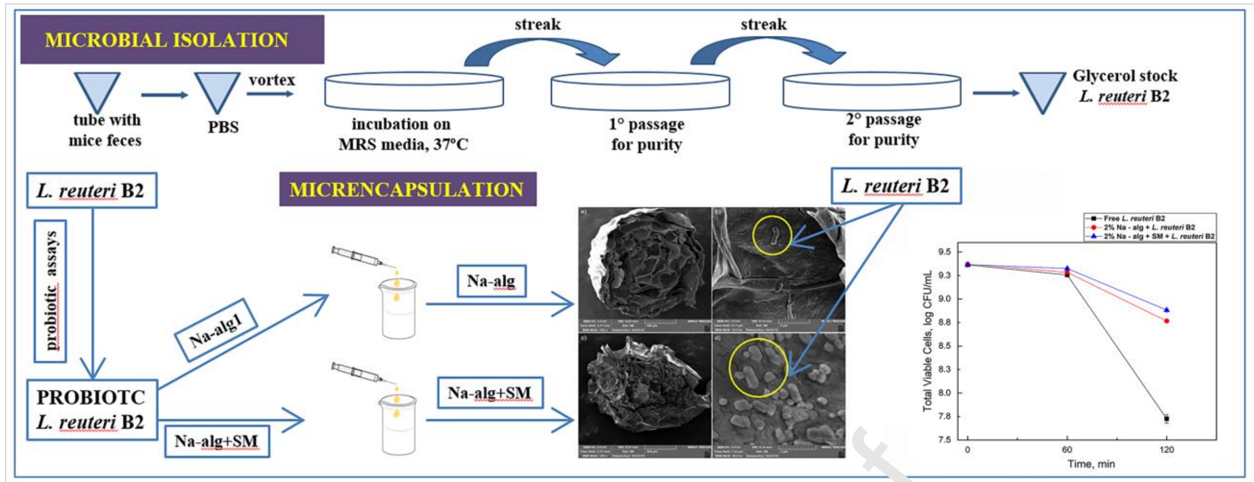

Graphical abstract

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Highlights:

- Probiotic activity and characterization of *L. reuteri* B2 strain
- Characterization and microencapsulation of probiotic cells of *L. reuteri* B2 with Na-alg and Na-alg+SM
- Viability of *L. reuteri* B2 in microencapsulated and free form in SGJ

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