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Research paper

Formulation and investigation of *Lactobacillus rhamnosus* cek-R1 filled alginate microspheres with different excipients

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Abstract

Microspheres are spherical particles containing the active substance, in our case, the bacterial probiotic strain *Lactobacillus rhamnosus* (*L. rhamnosus*), in an individually coated form. *L. rhamnosus* is a natural constituent of the human intestinal flora and is known to improve immune function, enhance healing of the intestinal mucosa, reduce inflammation, bloating, and diarrhea.

The aim of our experimental work was to formulate sodium alginate microspheres containing L. rhamnosus bacterial strain as active ingredient and prebiotics (galactooligosaccharide, pectin, inulin). The microspheres were formulated using Büchi Encapsulator equipment, and the entrapment efficiency was subsequently measured. The lyophilized product was filled into hydroxypropylmethylcellulose (HPMC) capsules and was subjected to a dissolution assay using Erweka equipment. The number of viable *L. rhamnosus* was determined from samples of the dissolution fluid. The microspheres are lyophilised to improve shelf-life and facilitate filling into traditional capsules. The number of viable bacteria in the lyophilizate was determined by inoculation on medium and a standard microdilution test. Microspheres containing different compositions of pro- and prebiotics were formulated, and their antioxidant capacity was detected by 2,2-diphenyl-l-1-picrylhydrazyl (DPPH). We tested the anti-inflammatory effect of the microspheres using a human IL-4 ELISA Kit on the colon adenocarcinoma (CaCo-2) cell line. Our results showed that the cek-R1 strain had a strong self-adhesion, Polyvinylpyrrolidone (PVP) enhanced encapsulation efficiency and pH-responsive release, inulin co-formulation increased antioxidant activity, and *L. rhamnosus* dose-dependently suppressed IL-4 production in Caco-2 cells, indicating immunomodulatory potential.

1. Introduction

Probiotics are live microorganisms that when administered in adequate quantities, confer health benefits to the host [1]. According to research, between 10^{10} and 10^{12} live microorganisms live in the human colon[2]. This group includes bacterial species from genera such as *Lactobacillus, Bifidobacterium, Bacillus*, and *Escherichia*, as well as yeast species like *Saccharomyces cerevisiae*[3]. They contribute to gut microbiome homeostasis, enhancing digestion and immune responses. *Lactobacillus* exhibits superior acid tolerance (pH 4.5–6.4) compared to *Bifidobacterium*, favoring its survival and viability during gastrointestinal transit[4].Prebiotics are non-digestible dietary fibers that selectively stimulate the growth and activity of beneficial gut microbiota, promoting host health[5]. Prebiotics include various natural and synthetic compounds such as oligosaccharides (e.g., FOS, GOS) and polysaccharides (e.g., inulin, resistant starch), which support beneficial gut bacteria. GOS are composed of galactose units linked to glucose by β-(1-4) or β-(1-6) bonds, while inulin consists of fructose units mainly linked by β-(2-1) bonds, often terminating in glucose[6,7]. Krasaekoopt et al. demonstrated that the addition of GOS and inulin prebiotics during microencapsulation not only provided better protection of various *Lactobacillus* strains but also enhanced the growth of these microorganisms in the simulated digestive system[8]. Oral administration of *Lactobacillus* probiotics is limited by poor mucosal adherence, acid sensitivity, and microbial competition. Advanced delivery methods including biofilm-based systems and microsphere encapsulation, enhance bacterial adhesion, viability, and therapeutic outcomes [9–11]. Alginate-based microspheres are an effective carrier system for *Lactobacillus* species, as they enhance probiotic survivability by protecting cells from harsh gastrointestinal conditions, improving encapsulation efficiency, and enabling controlled release in the intestines[12,13]. Alginate-based formulations protect bacteria by forming biocompatible, ionically crosslinked hydrogels that maintain viability and shield against environmental stress more effectively than harsher encapsulation methods[14]. Polyvinylpyrrolidone (PVP) stabilizes and solubilizes bioactive in microencapsulation, promoting uniform dispersion and sustained release. Its film-forming properties improve microcapsule stability and protection against degradation[15,16].

Lactobacillus species mitigate oxidative stress by enhancing antioxidant enzyme activity (e.g., SOD, catalase) and modulating Nrf2 signaling, while reducing inflammation through downregulation of pro-inflammatory cytokines like TNF-α and IL-

6 via NF-κB and MAPK pathways[17,18]. *Lactobacillus* strains downregulate NF-κB pathway genes (*TIRAP, IRAK4, NEMO, RIP*) and JAK/STAT components, suppressing cytokine production in inflamed intestinal cells[19,20].

2. Materials and methods

2.1 Materials

Low-viscosity-grade sodium alginate was obtained from BÜCHI Labortechnik AG (Flawil, Switzerland). The human adenocarcinoma cancer cell line (Caco-2) originated from the European Collection of Authenticated Cell Cultures (ECACC, Public Health England, Salisbury, UK). Dulbecco's Modified Eagle's Medium (DMEM), phosphate buffered saline (PBS), heat-inactivated fetal bovine serum (FBS), L-glutamine, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 96% ethanol and L-ascorbic acid (CAS No.: 50-81-7), human IL-4 ELISA Assay Kits were purchased from Sigma-Aldrich (Budapest, Hungary). Nonessential amino acid solution and penicillin–streptomycin mix, TrypLE™ Express Enzyme (no phenol red), 96-well plates, and cell culture flasks were obtained from Thermo-Fisher (Darmstadt, Germany, CAS number: 156499). All other products were purchased from Sigma-Aldrich (St. Louis, MI, USA).

2.2 Selection of Lactobacillus species

2.2.1 Microbial adhesion to hydrocarbons (MATH) test

The MATH assay involves mixing the bacterial suspension with hexane, followed by vigorous agitation to promote interaction between the two phases and evaluate cell surface hydrophobicity. The hydrophobic bacterial cells adhere to the hexane phase, and their hydrophobicity is quantified by measuring the change in optical density (OD_{600}) of the aqueous phase before and after mixing. The hydrophobic property was calculated using the following equation, where H_0 represents the result measured at point 0 and H_x represents the results of the aqueous phase tested after a specific time[21]:

$$H = \frac{H_0 - H_x}{H_0} \times 100 \quad (1)$$

2.2.2. Auto aggregation

Auto-aggregation measurement assesses the ability of probiotic cells to self-associate and form clumps, which is important for their adhesion to host tissues and pathogen exclusion. The method involves suspending the bacteria in a growth medium, followed by incubation to allow for aggregation over a specific period. After incubation, the

optical density (OD_{600}) of the supernatant is measured using a spectrophotometer at specific times (3h, 6h, and 24 h). The auto-aggregation percentage is calculated by comparing the (OD_{600}) of the supernatant ($A_{3h,6h,24h}$) to the initial (OD_{600}) of the bacterial suspension (A_0). This method provides valuable information about the surface properties of probiotics and their functional capabilities in adhesion and colonization[22].

$$A = [1 - \frac{A_{(3h,6h,24h)}}{A_0} \times 100] \quad (2)$$

2.3 Formulation of Lactobacillus-loaded microspheres

In this study, bacterial cultures were lyophilized and encapsulated in microspheres using various excipients. Lyophilization was performed at −110 °C for 24h with a Scanvac CoolSafe Touch 110-4 Freeze Dryer. Encapsulation was performed using a Büchi Encapsulator B-395 Pro to form calcium cross-linked sodium alginate beads, protecting *Lactobacillus* from environmental stress. A 1.5% sodium alginate solution (prepared overnight under light-free conditions) was mixed with a bacterial suspension (10^{15} CFU/mL), and solidified in 100 mM $CaCl_2$. Production parameters are listed in **(Table 1).** The formulations included GOS, pectin, and inulin at various concentrations **(Table 2)**. Microspheres were washed, filtered (0.20 µm), and lyophilized again under the same conditions [23].

Diameter of the Nozzle [µm]	Vibration Frequency [Hz]	Electrostatic Voltage [V]	Flow Rate (mL/min)
150	1600	1000-1500	5,06

Table 1.: The applied parameters of the encapsulator.

	SA	$CaCl_2$	IN	GOS	PVP	cek-R1
I	-	-	-	-	-	+
Ia	-	-	+	-	-	+
Ib	-	-	-	+	-	+
II	+	+	-	-	-	+
IIa	+	+	+	-	-	+
IIb	+	+	-	+	-	+
IIc	+	+	+	+	+	+

Table 2.: Formulation of microspheres containing the bacterial species cek-R1 with different excipients

2.4 Encapsulation Efficiency

To determine the encapsulation efficiency (EE%), 1 mL sampling of the hardening solution is collected and the amount of active ingredient inside the bead is calculated according to the amount of active ingredient in the solution. For the determination of the active ingredient concentration UV-VIS spectrophotometer was used[24]. The following equation was used to calculate the exact amount of probiotic strain entrapped, where m0 is the amount of initial probiotic strain (mg) and m1 is the amount of probiotic strain measured in the hardening solution (mg):

$$(EE\%) = \frac{m_0 - m_1}{m_0} \times 100 \qquad (3)$$

2.5 In vitro dissolution test

In vitro dissolution testing was conducted using simulated gastric fluid (SGF, pH 1.2) and simulated intestinal fluid (SIF, pH 6.8) according to the European Pharmacopoeia IX. Formulations encapsulated in size 0 HPMC capsules were evaluated using a USP dissolution apparatus (Erweka DT800) at 37 ± 0.5°C with agitation at 100 rpm. Capsules were exposed to SGF for 1 hour, followed by transfer to SIF for 23 hours. Samples were withdrawn at predetermined intervals (0, 2, 4, 6, 8, and 24 h) to assess bacterial viability[25].

2.6 DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay

The antioxidant activity of the formulations was evaluated using the DPPH assay. Microspheres with various *L. rhamnosus* strains were dissolved in PBS and centrifuged at 4000 rpm. From the supernatant, 50 µL was added to a 96-well plate, followed by 100 µL of 0.06 mM DPPH in 96% ethanol. Samples were incubated in the dark at room temperature. The color change from purple to yellow indicated antioxidant activity, and absorbance was measured at 517 nm using a Thermo Scientific Multiskan GO spectrophotometer[26]. Antioxidant capacity (% inhibition) was calculated as:

$$AA\% = 100 - \frac{(Abs_{sample} - Abs_{blank}) \times 100}{Abs_{control}} \qquad (4)$$

Where Abs_{sample} is the absorbance of the sample and the DPPH solution mixture, Abs_{blank} is the absorbance of the 96% alcohol, and $Abs_{control}$ is the absorbance of the

DPPH solution mixture. The DPPH solution itself was used as the negative control, and an absolute alcohol solution of ascorbic acid at a concentration of 0.25 mg/mL was used as the positive control.

2.7 *Anti-inflammatory effect of Lactobacillus rhamosus cek-R1*

The anti-inflammatory effect of *L. rhamnosus* isolates in different concentrations was tested on the CaCo-2 cell line. Cells were seeded in 96-well plates and treated with TNF-α inflammatory cytokine for 24 h after reaching full confluence. After in vitro inflammation was induced, cells were incubated with the sample solutions for 24 hours and then tested for anti-inflammatory activity using IL-4 ELISA assays, as recommended by the manufacturer.

2.8 Statistical analysis

The data were analyzed using GraphPad Prism (version 6, GraphPad Software, San Diego, CA, USA). For comparisons between two groups, an unpaired *t*-test was performed, while for comparisons involving three or more groups, analysis of variance (ANOVA) was used, followed by Tukey or Dunnett post-testing. Differences were considered significant at $p < 0.05$. Statistically significant differences are indicated as $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$(***), $P \leq 0.0001$ (****).

3. Results

3.1. Selection of Lactobacillus species

3.1.1. MATH

The assessment of bacterial cell surface hydrophobicity facilitates the selection of specific strains. It offers critical insights into their adhesion to epithelial surfaces, a key determinant of their colonization capacity and pathogenic potential. In this study, three *L. rhamnosus* species, DSM 20021, cek-R1, and UB-T1 were tested. The results of the test are shown in **(Table 3)**, where the species cek-R1 had the highest value at the base of the hydrophobicity.

***L. rhamnosus* bacterial strain**	**Hydrophobic properties (%)**
DSM 20021	45,21 ± 0,67
cek-R1	61,36 ± 0,46

ub-T1	34,68 ± 0,53

***Table 3**.:* Hydrophobicity properties of different *L. rhamnosus* bacterial species. Results are presented as mean ± SD.

3.1.2. Auto aggregation

Auto-aggregation measurement is crucial for evaluating the self-adhesion ability of probiotic strains, which is directly linked to their capacity to form stable biofilms and persist in the host environment. The percentage results of auto-aggregation are shown in **Figure 1.** According to the literature, bacterial strains below 10% are considered as non-aggregating and above 80% as well aggregating. The results showed 81% auto aggregation was measured for cek-R1[27].

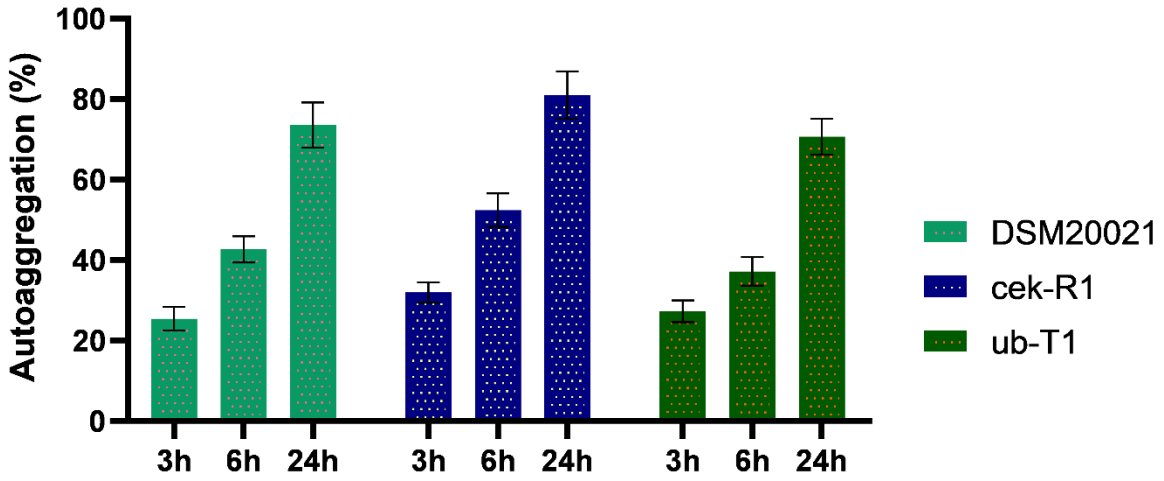

Figure 1.: Auto-aggregation of different *L. rhamnosus* species expressed as percentage. All results are represented of the mean ± SD.

3.2. Encapsulation efficiency

The active ingredient content of microspheres was calculated using the equation in section 2.3. The results showed that the encapsulation efficiency was between 58% and 69%, as shown in **(Table 4)**. The encapsulation efficiency was highest for formulation IIc, which also contained PVP as an excipient. PVP is able to form a coating on the surface of the microspheres, which can prevent drug leaching through the microbeads, thus improving the encapsulation efficiency.

Formulation	Encapsulation efficiency (EE%)
II	58,5 ± 3,64

IIa	60,1 ± 5,39
IIb	59,2 ± 2,21
IIc	68,7 ± 3,89

Table 4.: Encapsulation efficiency of different sodium alginate microbeads containing cek-R1 with different excipients. All results are represented of the mean ± SD.

3.3. In vitro dissolution study

In vitro dissolution experiments were performed in SGF and SIF. The drug release in the acid medium remained below 10% for all formulations at the beginning of the study. At higher pH levels, the release exceeded 50% for microcapsulated APIs based on 8 h results. Microspheres containing PVP showed the highest release after 24 hours. **Figure 2.** shows the percentage of *L. rhamnosus* cek-R1 released compared to the initial encapsulated bacterial suspension.

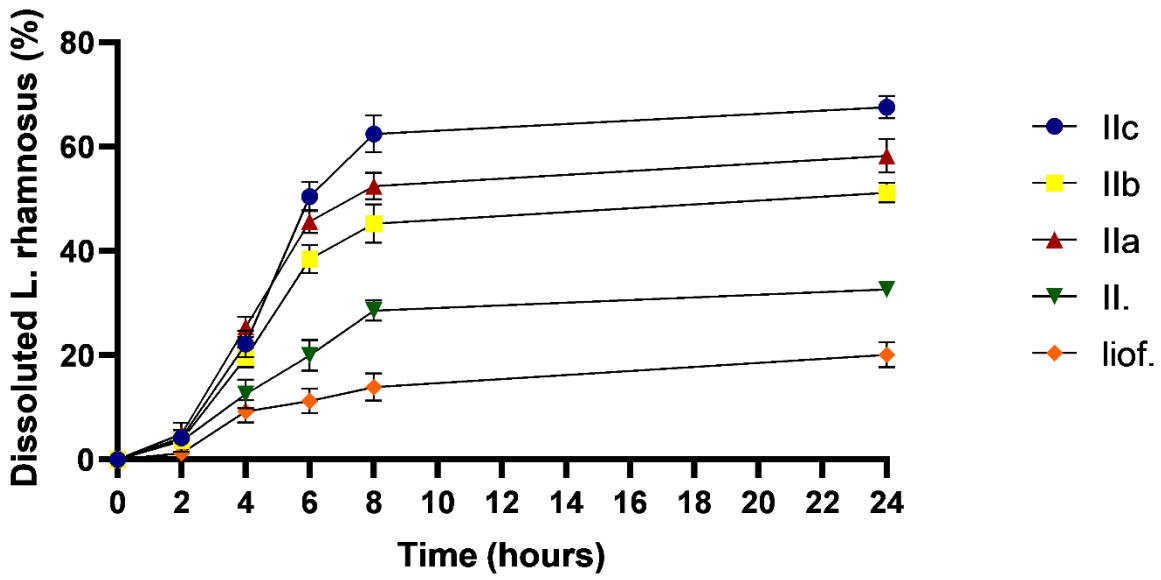

Figure 2.: In vitro dissolution profile of *L. rhamnosus*-loaded microspheres and lyophilizate in simulated gastric fluid (pH 1.2) and simulated intestinal fluid (pH 6.8). All results are expressed as the mean ± SD, (n = 5).

3.4. Radical scavenging activity of L. rhamnosus microspheres

The DPPH radical scavenging activity of the tested samples was evaluated relative to 0.25% ascorbic acid, which served as the positive control and exhibited the highest inhibition of reactive oxygen species (ROS), reaching nearly 100%. Among the test groups, sample **IIa** showed the strongest scavenging activity, inhibiting approximately

60% of ROS relative to the control. Other samples displayed moderate antioxidant capacities, ranging from roughly 40% to 55% inhibition. **Figure 3.,** shows that the samples containing inulin showed the highest radical scavenging property, and microencapsulated probiotic formulations showed higher radical scavenging property compared to lyophilizates

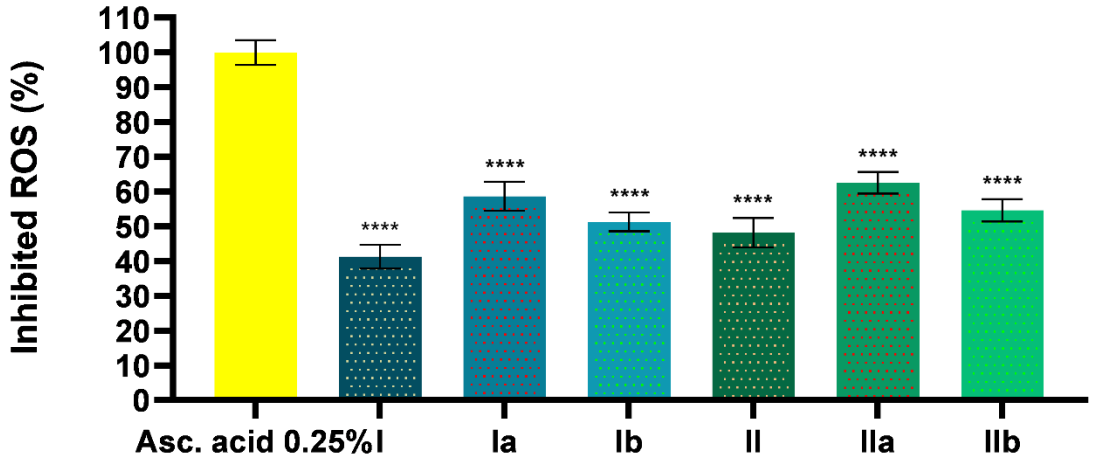

Figure 3.: DPPH-scavenging activity of the formulation. Data are mean ± SD (n = 6). The different formulations were represented in relation to ascorbic acid.

3.5. In vitro anti-inflammatory effect of L. rhamnosus cek-R1

L. rhamnosus significantly reduced IL-4 concentrations at all test concentrations compared to the PBS-treated group. The reduction in the inflammation level was concentration-dependent, as shown in **Figure 4,** and was found to be directly proportional to the concentration. The results of the anti-inflammatory effect study for IL-4 are shown in **Figure 4.**

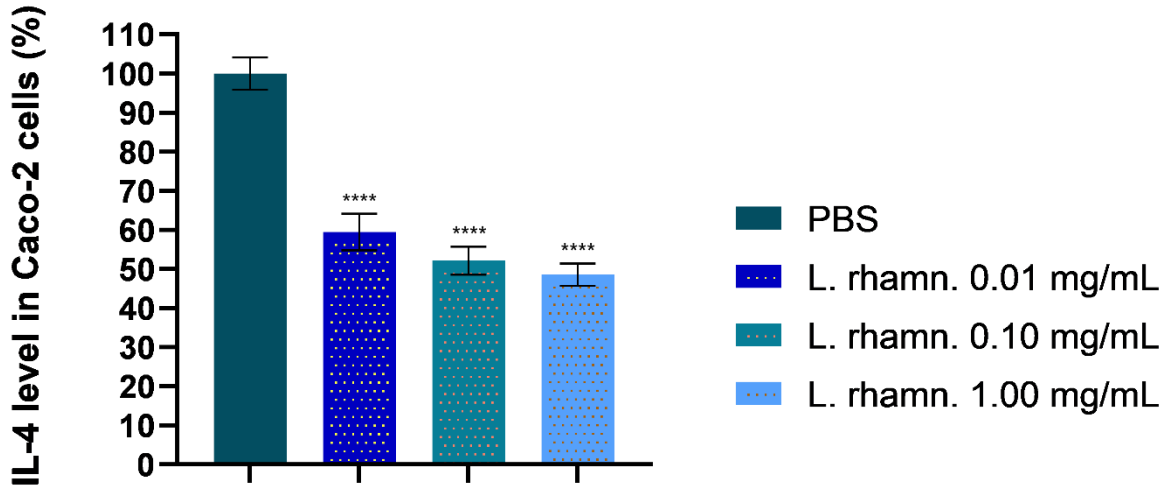

Figure 4.: Results of the IL-4 level in the Caco-2 cell line. Results are shown as mean ± SD (n = 6).

4. Discussion

The development of micro-formulations containing both pro- and prebiotics, and their physical and chemical properties, with the optimal formulation steps were determined experimentally. Dietary fiber is an essential component of the human system as it is responsible for maintaining a healthy intestinal flora, and several studies have confirmed its important role in the maintenance of the gastrointestinal barrier[28,29]. These substances contribute to the proper functioning of the immune system as nutrients for probiotic bacterial strains that form the normal flora[30]. In our formulations, we used inulin, a biopolymer of the fructan group, a galacto-oligosaccharide consisting of short chains of sugar molecules with proven prebiotic activity, as a key component[31]. Kaewarsar et al. demonstrated in their work that the studied prebiotics (inulin 1.33 (W/V), FOS 2.00 W/V, and GOS 2.67 W/V%) were able to stimulate the growth of *L. rhamnosus* in all cases[32].

The formulation was developed by studying the physical and chemical properties of the components and selecting the correct technological steps for the formulation.

Hydrophobicity is an important factor in predicting the ability of a probiotic's to adhere to intestinal epithelial cells, which is crucial for colonization and exerting beneficial effects[33]. The hydrophobicity measurements indicated that the **cek-R1** strain of *L. rhamnosus* exhibits the highest hydrophobic properties (61.36 ± 0.46%), suggesting a stronger potential for bacterial adhesion to epithelial cells and possibly a higher colonization ability compared to the **DSM 20021** and **UB-T1** strains.

Cell surface hydrophobicity influences the adhesion and colonization of the probiotics. The cek-R1 strain showed 81% auto-aggregation, indicating strong self-adhesion, stable biofilm formation, and high persistence, as values above 80% reflect strong aggregation[34,35].

Encapsulation efficiency ranged from 58% to 69%, with the highest observed in formulation IIc, likely due to the presence of PVP. PVP enhances efficiency by increasing the viscosity of the cross-linking solution, reducing pore size, and minimizing active compound leakage. In the dissolution studies, all microspheres exhibited controlled release under acidic conditions, while PVP-containing formulations demonstrated the highest release at elevated pH levels over 24 h, indicating the role of PVP in modulating pH-responsive drug release[36,37].

Several studies have shown that microcapsules containing *Lactobacillus* species exhibit greater antioxidant capacity when inulin is included, as it enhances probiotic survival, metabolic activity, and resistance to gastrointestinal stress while enabling sustained release. This prebiotic-probiotic synergy not only improves bacterial viability but also amplifies antioxidant effects through increased enzyme activity and overall bioavailability[38,39]. Our study has also confirmed that in cases the formulation contained both the bacterial strain and inulin, higher antioxidant properties were measured

Lui et al. demonstrated in their study that *L. rhamnosus* in combination with inulin can reduce dextran sodium sulphate-induced artificial colitis in male Balb/c mice, reduce pathological tissue damage, regulate the expression of inflammatory cytokines, and induce anti-inflammatory effects in mice[40]. *L.* rhamnosus suppresses IL-4 production in Caco-2 cells in a dose-dependent manner, with all tested concentrations showing a similar reduction, and the most pronounced effect at the highest dose, indicating potential anti-inflammatory or immunomodulatory properties.

5. Conclusion

The developed microformulations combining L. rhamnosus and prebiotic components such as inulin showed favorable physicochemical properties, efficient encapsulation, and controlled, pH-responsive release. These formulations also demonstrated enhanced probiotic viability, antioxidant capacity, and potential anti-inflammatory effects, supporting their use in gut health applications.

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Data Availability Statement:

All measurement data are available at the corresponding author upon request.

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