Development of Triple-Polymer Beads for the Delivery of Probiotics under Simulated Gastrointestinal Digestion

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Abstract. This study developed triple polymer beads composed of alginate, inulin and chitosan for encapsulating *Bifidobacterium bifidum* TISTR 2129 and *Lactobacillus sporogenes* BC 208. Four formulations were prepared by the extrusion method, representing alginate (A), alginate with chitosan coating (AC), alginate with inulin (AI), and alginate with both inulin and chitosan coating (AIC). The beads were evaluated for encapsulation efficiency, swelling behavior, and probiotic release under simulated gastrointestinal digestion using the INFOGEST protocol. AI and AIC beads exhibited higher encapsulation efficiency (up to 92%) compared to A and AC, suggesting that inulin improved the interaction between the matrix and probiotic cells. All formulations showed contraction in acidic conditions (pH 3), with AI and AIC showing the lowest swelling, indicating a more compact network. In intestinal conditions (pH 7), swelling increased, particularly in A bead. Notably, AIC beads demonstrated the highest probiotic release (7.32 ± 0.03 log CFU/mL) at 10.25 h, despite exhibiting the lowest swelling, indicating a sustained and controlled release. These results confirm that the combination of inulin and chitosan enhances bead stability, encapsulation efficiency, and targeted delivery, supporting the application of triple polymer systems in functional food development.

1 Introduction

Recently, probiotic-enriched foods have grown to be an important market in the functional food industry due to their potential to enhance human health beyond primary nutrition. Probiotics are living bacteria that provide health benefits when ingested in adequate amounts (at least 6 log CFU/g) and are crucial for maintaining gastrointestinal microbiota and improving host immunity [1-3]. Among the various probiotics, *Bifidobacterium bifidum* TISTR 2129 and *Lactobacillus sporogenes* BC 208 are important for their beneficial effects on gut microbiomes. However, the effective delivery of probiotics to the intestine was an important challenge because these bacteria must endure the harsh conditions of the gastrointestinal tract, especially the acidic pH of the stomach and bile salts, which can significantly reduce their survival. [4].

Encapsulation is known as a technique for enhancing the stability and viability of probiotics during storage and gastrointestinal transit [5]. Sodium alginate is a naturally generated anionic polysaccharide consisting of β-D-mannuronic acid (M) and its epimer, α-L-guluronic acid (G), with the 1, 4-glycosidic linkages commonly used in probiotic encapsulation due to its ability to form hydrogels that interact with divalent cations such as calcium ion. [6]. Nevertheless, alginate beads could be at risk for disintegration in acidic environments, especially in the stomach (pH ~2), since the protonation of carboxyl groups diminishes electrostatic connections and weakens the gel network [7]. To enhance protective efficiency, researchers have incorporated inulin, a fructooligosaccharide that acts as a prebiotic and a non-digestible dietary fiber for beneficial gut microbiota, into encapsulation systems. This addition improves matrix strength and supports selective probiotic growth in the colon [8]. Moreover, the application of chitosan, a naturally sourced cationic polysaccharide produced through the deacetylation of chitin, has attracted considerable attention for its ability to strengthen the structural integrity of encapsulated beads. Chitosan consists of repeated units of β-(1→4)-linked D-glucosamine and N-acetyl-D-glucosamine, containing free amino groups ($–NH_3^+$) in the glucosamine units providing a positive charge under acidic environment. [9-11]. The positive charge causes chitosan to form strong ionic links with the negatively charged alginate matrix, leading to a stable and cohesive coating layer [12]. This coating enhances the strength of the beads and protects them against harmful gastric conditions, particularly low pH and digestive enzymes, thus preventing the leakage of internal bacteria and maintaining bead stability at varying pH levels [13]. Alginate constructs the hydrogel structure, inulin acts as a prebiotic and matrix enhancer, and chitosan offers a protective cationic coating that increases stability in acidic conditions, thereby utilising the unique properties of each polymer to establish a strong encapsulating matrix. This combination offers controlled release in the colon and has improved probiotic viability

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during gastrointestinal transit. These encapsulating techniques have great potential to advance dietary supplements and functional foods where the preservation of bacterial viability is crucial. Moreover, studying the swelling behaviour of beads is essential since it influences their probiotic release and interaction with intestinal fluids.

Recent studies in probiotic delivery have investigated the integration of inulin into encapsulation systems with diverse materials. Guo et al. (2024) [14] examined the thermo-kinetic properties of co-encapsulated inulin and *Lactobacillus rhamnosus* GG within alginate/pectin composite beads during *in vitro* digestion and colonic fermentation. The results indicated that inulin improved thermal stability and controlled the release of encapsulated probiotics, although it also promoted colonic fermentation action, thereby maintaining probiotic viability and prebiotic efficacy throughout the gastrointestinal tract. Sakoui et al. (2022) [15] found that encapsulated *Enterococcus mundtii* SRBG1 within an inulin–maltodextrin–sodium alginate matrix increased probiotic viability and improved the rheological properties of fermented milk. Maleki et al. (2023) [16] developed synbiotic ice cream by microencapsulating *Lactobacillus rhamnosus* with a composite of whey protein, bacterial cellulose, and inulin. The research demonstrated that this encapsulation method successfully maintained probiotic viability during freezing and storage, while simultaneously improving the functional and sensory attributes of the ice cream.

This study aims to evaluate the production of triple-polymer beads based on alginate (A), alginate with chitosan coating (AC), alginate with inulin (AI), and alginate with both inulin and chitosan coating (AIC) for the encapsulation of two probiotic strains, *Bifidobacterium bifidum* TISTR 2129 and *Lactobacillus sporogenes* BC 208. The encapsulation efficiency, release under simulated gastrointestinal conditions, and swelling behaviour of the beads were assessed to evaluate their efficacy for protecting probiotics and promoting targeted delivery in the gastrointestinal tract.

2 Materials and methods

2.1 Chemicals, probiotics and pathogenic strains

Alginate, chitosan and inulin were purchased from CTi & Science Co., Ltd., Thailand. De -man, rogosa and sharpe (MRS broth and agar) were purchased from Sisco Research Laboratories Pvt. Ltd. (Mumbai, India). The probiotic strains used in this study were *Bifidobacterium bifidum* TISTR 2129 derived from Thailand Institute of Scientific and Technological Research (TISTR) (Pathum Thani, Thailand) and *Lactobacillus sporogenes* BC 208 purchased from New Bellus Enterprises Co., LTD. (Taiwan).

2.2 Probiotic culture preparation

The fermentation of *B. bifidum* TISTR 2129 and *L. sporogenes* BC 208 was carried out in a 5L stirred tank bioreactor (BioFlo 120, Eppendorf, USA) that contained MRS medium. *B. bifidum* TISTR 2129 was maintained at a pH of 6.0 with an agitation rate of 100 rpm, while *L. sporogenes* BC 208 was maintained at a pH of 6.5 with an agitation speed of 150 rpm. Both strains were inoculated with a 10% inoculum size, and the fermentation was maintained at a constant temperature of 37°C. After fermentation, the broth was centrifuged at 3000 rpm for 10 min at 4°C, followed by washing twice with 0.85% NaCl. The probiotic cells were prepared for the encapsulation process.

2.3 Encapsulation process

The beads were encapsulated using the extrusion method, as described by Thinkohkaew et al. (2024) [17], with slight modifications. Four bead formulations were developed to evaluate the individual and combined effects of inulin and chitosan. The formulations were designated as A, AC, AI, and AIC, indicating alginate only, alginate with chitosan coating, alginate with inulin, and alginate with both inulin and chitosan coating, respectively. To prepare the base solution, 2% (w/v) sodium alginate was solubilized in deionized water and heated to 80°C for 30 min. The solution was afterwards sterilized at 121 °C for 15 min. In formulations containing inulin (AI and AIC), 3% (w/v) inulin was added to the sterile alginate solution, and the mixture was then heated at 80°C for 30 min to achieve complete dissolution. After the alginate or alginate–inulin solution had cooled to room temperature, a probiotic mixture consisting of *L. sporogenes* BC 208 and *B. bifidum* TISTR 2129 was added at a final concentration of approximately 10 log CFU/mL. The mixture was then gently stirred for 2 h to ensure homogeneous distribution of the bacterial cells. Encapsulation was conducted using a Büchi B-390 encapsulator (Switzerland) equipped with a 450 µm nozzle. The operational settings were a vibration frequency of 1000 Hz, an electrode potential of 1000 V, and an air pressure of 600 bar. The extrusion procedure was carried out in a 0.1 M calcium chloride ($CaCl_2$) solution with stirring for 30 min. The formed beads were collected and rinsed with sterile distilled water. The produced beads were collected and washed with sterile distilled water. For formulations with chitosan coating (AC and AIC), the beads were immersed in a 0.4% (w/v) chitosan solution for 30 min to form a surface coating. The composition of the prepared bead formulations is summarized in Table 1.

Table 1. The composition of the prepared bead formulations.

Formulations	Alginate (% w/v)	Inulin (% w/v)	Chitosan coating (% w/v)
A	2	-	-
AC	2	-	0.4
AI	2	3	-
AIC	2	3	0.4

2.4 Encapsulation efficiency

Mahmoud et al. (2020) [18] explained the process of assessing the number of viable cells encapsulated in the beads. In brief, 1 g of beads was suspended in 9 mL of sterile 2% (w/v) trisodium citrate solution and vortexed until completely dissolved. Subsequently, 1 mL of the resulting solution was serially diluted using phosphatebuffered saline (PBS). The number of viable cells was determined by plating the diluted samples on MRS agar media, followed by incubation at 37°C for 48 h. Viable cell counts were expressed as colony-forming units per milliliter (CFU/mL). The encapsulation efficiency of beads was subsequently determined using Equation (1).

$$\%EE = Log_{10}N / Log_{10}N_0 \times 100 \qquad (1)$$

Where N_0 and N were the initial number of probiotic cells (CFU/mL) and the number of cells encapsulated in beads, respectively.

2.5 Release study of probiotics under *in vitro* gastrointestinal digestion

The *in vitro* gastrointestinal digestion was carried out following the standardized INFOGEST protocol established by Brodkorb et al. (2019) [19], with slight adaptations to suit the experimental conditions. The *in vitro* digestion phase involved three consecutive phases: the oral phase, the gastric phase, and the intestinal phase, using simulated salivary fluid (SSF), simulated gastric fluid (SGF), and simulated intestinal fluid (SIF), respectively. For the oral phase, the sample was mixed with SSF at a 1:1 weight ratio, supplemented with 0.3 M $CaCl_2$ and salivary amylase to achieve a final enzymatic activity of 75 U/mL. The pH of the fluid was adjusted to 7.0 by 1 M NaOH. The mixture was subsequently incubated at 37°C with continuous agitation at 100 rpm for 2 min to mimic the limited exposure duration to salivary amylase. The gastric phase started with a solution of SGF containing pepsin at an activity of 2000 U/mL and gastric lipase at an activity of 60 U/mL. The pH was adjusted to 3.0 with 1 M HCl, and the mixture was incubated at 37°C with shaking at 100 rpm for 2 h to simulate stomach conditions. The intestinal phase was carried out by providing SIF with bile extract at a final concentration of 10 mM and pancreatin at a final enzymatic activity of 100 U/mL. The pH was adjusted to 7.0 using 1 M NaOH, and the mixture was shaken continuously at 100 rpm at 37°C for 8 h to simulate intestinal digestion. Upon completion of each digestion phase, samples were taken for analysis. The total number of living cells released was counted and shown as colony-forming units per millilitre (CFU/mL).

2.6 Swelling studies

The swelling behaviour of beads under simulated gastrointestinal digestion was previously described by Gbassi et al. (2011) [20], with modifications. Initially, 1 g of beads was immersed in 50 mL of simulated gastric fluid (SGF) adjusted to a pH of 3.0 by 1M NaOH and incubated at 37°C with continuous shaking at 100 rpm for 2 h. After the gastric simulation, the beads were placed into simulated intestinal fluid (SIF) maintained at pH 7.0 and incubated for an additional 8 hours. At certain times, the swollen beads were extracted and carefully wiped with filter paper to remove excess surface moisture before weighing. The swelling index (SI) was determined by calculating the weight gain according to Equation (2). All experiments were conducted in triplicate.

$$S_i = [M_2 - M_1] / M_1 \qquad (2)$$

Where M_1 was the initial weight of the beads before immersion in the fluid. M_2 was the weight of the beads after immersion in the fluid.

2.7 Statistical analysis

All experiments were conducted in triplicate. Data were expressed as mean values ± standard deviation (SD). One-way analysis of variance (ANOVA) was employed to assess differences among treatments, followed by Tukey's test for multiple comparisons. Statistical significance was defined at $p < 0.05$. All analyses were performed using SPSS 21.0 software (Chicago, IL, USA).

3 Results and discussion

3.1 Encapsulation efficiency

As shown in Figure 1, encapsulation efficiency of A, AC, AI and AIC were 82.11 ± 0.07%, 83.41 ± 0.01%, 90.06 ± 0.10% and 92.56 ± 0.09%, respectively, with AI and AIC exhibiting higher encapsulation efficiency compared to A and AC. No significant difference was found between A and AC ($p = 0.544$), nor between AI and AIC ($p = 0.112$). The results indicated that the incorporation of inulin produced a greater impact on encapsulation efficiency than the chitosan coating under the studied conditions. Although chitosan was recognized for improving the barrier characteristics and mechanical strengths of alginate beads [21], the chitosan layer was more likely to improve long-term stability, protect against environmental stress, and help with controlled release in the digestive system rather than significantly increase encapsulation efficiency [22–24]. Importantly, previous studies have reported that the encapsulation efficiency of similar systems incorporating inulin ranged from 83% to 90% [17, 25, 26]. In comparison, the encapsulation efficiency achieved in our study was notably higher, indicating the superior performance of the developed encapsulation system. The enhanced efficiency might be attributed to the addition of inulin, which reinforces the alginate matrix and promotes stronger interactions between the encapsulating shell and probiotic cell surfaces, thereby improving cell retention. Figure 2 shows the freshly produced probiotic-encapsulated beads. All exhibited a consistently spherical form and a milky-white hue. These attributes are crucial for ensuring regularity in probiotic delivery and efficient protection. Pamela et al. (2024) [27] indicated that probiotic-encapsulated beads composed of 3% alginate and 10% inulin presented a spherical morphology, attaining an encapsulation efficiency of 99% and enhanced probiotic viability in low pH environments.

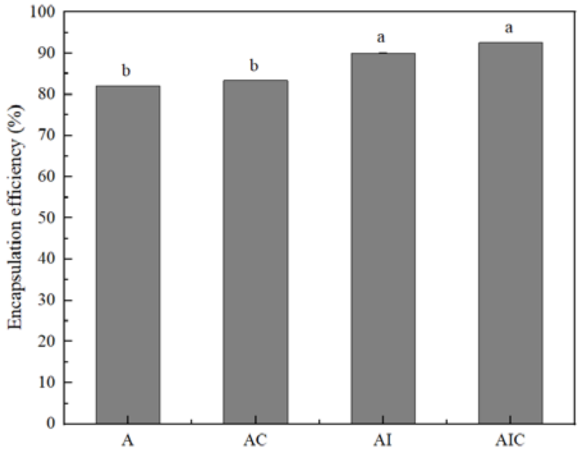

Fig. 1. Encapsulation efficiency for four formulations: alginate (A), alginate with chitosan coating (AC), alginate with inulin (AI), and alginate with both inulin and chitosan coating (AIC). Different letters represent statistically significant differences (p < 0.05).

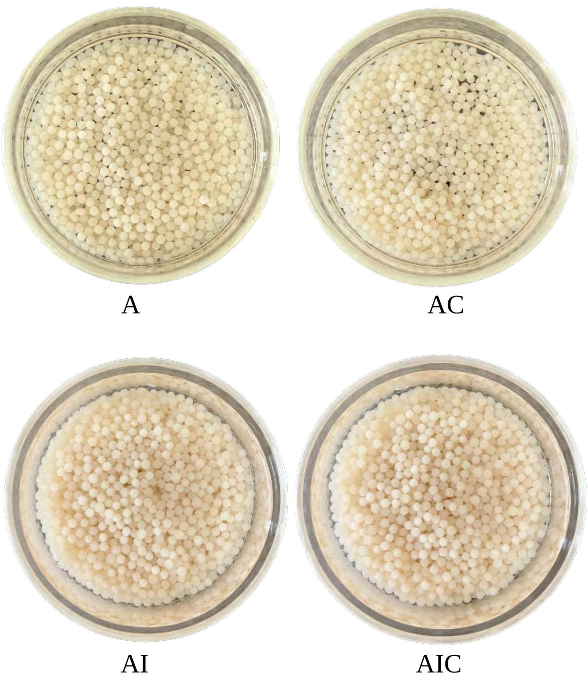

Fig. 2. Apparent images of freshly probiotic-encapsulated beads.

3.2 Swelling behavior

The swelling index of the beads was determined in simulated gastrointestinal digestion, initially with the stomach phase and then transitioning to the intestinal phase, as shown in Figure 3. For the gastric phase at pH 3, all beads demonstrated negative swelling behavior during a 2 h digestion period. A and AC beads presented slight differences in swelling, which were -0.10 ± 0.04 and -0.11 ± 0.04, respectively, with no significant difference (p = 0.97), indicating that the chitosan coating did not substantially alter their swelling behavior in an acidic environment. On the other hand, AI and AIC beads were -0.32 ± 0.06 and -0.27 ± 0.05, proving significantly more reduced than A and AC beads (p = 0.003, p = 0.011, p = 0.017 and p = 0.004), reflecting a more compact structure. However, there was no significant distinction between AI and AIC beads, indicating that the addition of a chitosan coating to the inulin-loaded beads did not significantly affect their swelling behavior under these conditions.

In the intestinal phase transition at pH 7, all beads indicated significantly enhanced swelling behavior. At 5.25 h, A beads exhibited the highest swelling index of 1.07 ± 0.05, followed by AC beads at 0.69 ± 0.09, AI beads at 0.64 ± 0.03, and AIC beads at 0.62 ± 0.02. Statistical analysis indicated substantial disparities between the alginate beads and all other beads (p < 0.0001). At the final time point of 9.25 h, the swelling indexes attained their greatest. A bead showed the highest swelling index of 1.58 ± 0.10, while AC, AI and AIC beads demonstrated similar swelling indices of 1.39 ± 0.03, 1.41 ± 0.04 and 1.38 ± 0.09, respectively.

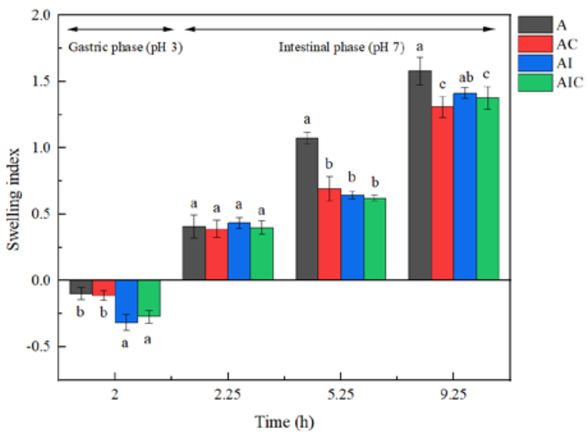

Fig. 3. Swelling behavior of beads under simulated gastrointestinal digestion. Different letters represent statistically significant differences (p < 0.05).

3.3 *In vitro* release under the gastrointestinal digestion

The *in vitro* release of probiotic-loaded beads was assessed according to the INFOGEST protocol to simulate gastrointestinal conditions, as shown in Figure 4. In the oral phase (pH 7, 2 min), free probiotic cells exhibited the maximum release of 9.62 ± 0.10 log CFU/mL. Conversely, all encapsulated beads exhibited no quantified release, signifying successful encapsulation and protection within the oral environment. In the gastric phase transition (pH 3, 2 h), a slight increase in release was observed in all beads, with A and AI exhibiting releases of 2.86 ± 0.03 log CFU/mL and 2.69 ± 0.04 log CFU/mL, respectively, while the other beads presented no probiotic release. In the intestinal phase (pH 7, 2.25–10.25 h), all beads exhibited a slow enhancement in probiotic release. At 10.25 h, AIC achieved the highest release among the encapsulated, at 7.32 ± 0.03 log CFU/mL. AC and AI showed moderate release at 6.98 ± 0.01 log CFU/mL and 6.79 ± 0.14 log CFU/mL, respectively. A showed the lowest release among the encapsulated beads, with release at 6.02 ± 0.03 log CFU/mL. The results indicate that the composition of the encapsulating matrix, specifically alginate and inulin coated with chitosan, significantly influences probiotic release during gastrointestinal digestion. The probiotic release observed in this study demonstrates that the encapsulation method effectively protected the probiotics, ensuring their viability throughout gastrointestinal delivery. The probiotics maintained a viability of a minimum of 6 log CFU/mL, which is the required concentration for providing health benefits [1]. The results of the present study corresponded with those of a previous study that confirmed the improved survival of *Lactobacillus casei* when encapsulated in alginate/inulin coated with chitosan by the extrusion method [17].

The swelling behavior of the beads, as observed under simulated gastrointestinal conditions, strongly correlates with their probiotic release profiles, as shown in Figure 3. All beads showed negative swelling induced in the stomach phase at pH 3, which indicated that the gel matrix contracted. This behavior was typical of alginate-based hydrogels, where network contraction and reduced electrostatic repulsion result from carboxyl groups becoming protonated in an acidic environment [28]. As a result, limited probiotic cell release was seen during this phase, especially in AC and AIC beads, where the presence of inulin or chitosan coating caused the matrix to become denser and further prevented diffusion. On the other hand, all beads indicated significant improvement during the intestinal phase at pH 7. According to Jin et al. (2009) [29], this was explained by the ion exchange mechanism between Na^+ in PBS and Ca^{2+} in the alginate matrix, particularly within the polymannuronate. Probiotic diffusions were assisted by chain relaxation and increased gel swelling caused by the consequent increase in ionized carboxyl groups and lower cross-linking hardness. Interestingly, AIC beads demonstrated the maximum overall release at 10.25 h even though they had the lowest swelling index of all beads. This suggests that additional inulin assisted continuous probiotic release along with controlling swelling, possibly because of its porous structure and prebiotic-enhancing properties. The importance of composite matrix design for reaching desired delivery performance was highlighted by the less controlled release of the pure alginate beads (A), which had the greatest swelling index.

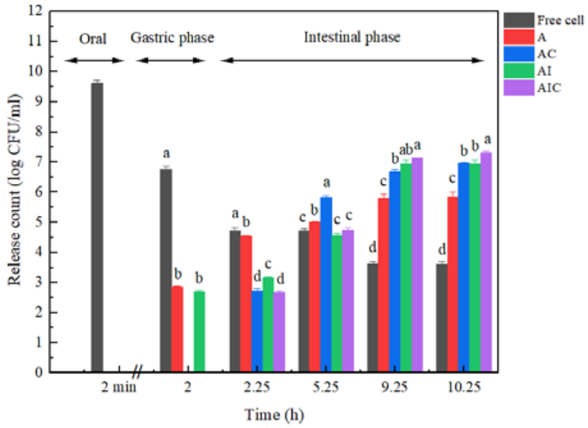

Fig. 4. *In vitro* release profile under simulated gastrointestinal digestion (INFOGEST protocol. Different letters represent statistically significant differences ($p < 0.05$).

4 Conclusion

The triple-polymer encapsulation system comprising alginate, inulin, and chitosan successfully enhanced the structural integrity and functionality of probiotic beads under simulated gastrointestinal digestion. The inclusion of inulin notably improved encapsulation efficiency and contributed to a more compact bead structure, while chitosan coating offered additional protection in acidic conditions. Swelling studies revealed that all beads contracted in gastric conditions and expanded in intestinal fluid, correlating closely with their probiotic release behavior. Among all formulations, AIC beads demonstrated the most desirable performance, with a controlled swelling profile and the highest probiotic release during intestinal digestion. These results emphasize the synergistic potential of combining biopolymers to optimize probiotic encapsulation and delivery, offering promising applications in the development of functional foods and synbiotic products.

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