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ORIGINAL RESEARCH

Development of Chitosan-Polyacrylic Acid Complex Systems for Enhanced Oral Delivery of *Lactobacillus Gasseri* and *Bifidobacterium Bifidum* Probiotics

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Introduction: The beneficial effects of probiotics are encountered by their low viability in gastrointestinal conditions and their insufficient stability during manufacturing, throughut the gastrointestinal transit, and storage. Therefore, novel systems are highly required to improve probiotics delivery.

Methods: In this study, Lactobacillus gasseri (L), Bifidobacterium bifidum (B), and a combination of L+B were encapsulated in chitosan (CS)-polyacrylic acid (PAA) complex systems (CS-PAA). The CS-PAA systems were analysed on basis of morphology, size, and zeta potential. The loaded CS-PAA systems were evaluated for their morphology, particle size, zetapotential, vaiability in both simulated gastroic and intestinal fluids, and stability at 4°C storage temparature. Moreover, the antibiofilm activity of the probiotics-loaded systems were evaluated againt Campylobacter jejuni, Pseudomonas aeruginosa and Escherichia coli.

Results: Probiotic strains were successfully incorporated into the porous structures of the CS-PAA systems, either individually or in combination. The Loaded L, B, and L+B showed higher particle size than the unloaded particles and excellent viability in simulated gastric and intestinal fluids, where the free probiotic species were undetected. Additionally the loaded probiotic exhibited an anti-biofilm effect at 0.5 mg/mL concentration level.

Conclusion: The CS-PAA complexes demonstrate a promising mechanism for the effective delivery of incorporated probiotics. The probiotics exhibited high viability and maintain stability under physiological conditions, and showed a remarkable anti-biofilm activity. These characteristics suggest that CS-PAA could serve as an alternative system for probiotics, enhancing gut microbiota health and offering a robust protection against microbial pathogens.

Keywords: probiotics, *Lactobacillus gasseri*, *Bifidobacterium bifidum*, chitosan, encapsulation, polyacrylic acid

Introduction

Probiotics are viable bacteria that beneficially improve the host's health.[1,2] Probiotics can play an important role in immunological, respiratory, and digestive functions, as well as their significant impact on alleviating infectious disease.[3–7] Probiotics must, however, be metabolically stable and active in the food product, survive in significant numbers in the digestive system, and have a favorable impact on the host's gut since they are thought to be necessary for optimal functionality.[8–10]

The most commonly used probiotic strains, as oral probiotics, are *Lactobacillus* and *Bifidobacterium*.[11] Lactic acid bacteria species inhibit the growth of pathogens such as *Salmonella, Staphylococcus, Listeria, Clostridium, Enterococcus* and SARS-CoV-2 by producing bacteriocins, small cationic peptides cause pore formation, which results in the death of target cells.[12,13] *Bifidobacterium bifidum* is the common probiotic bacteria found in the digestive and urinary tracts in

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addition to *Lactobacillus gasseri*.[14] Both strains play a role in improving overall immunity, reducing and treating gastrointestinal infections, as well as boosting conditions such as diarrhea, constipation, and infection by *Helicobacter pylori*.[15,16] Further, *B. bifidum* reduces allergy symptoms by discouraging the production of histamine.[17] Other probiotic strains such *as Enterococcus, Streptococcus, Pediococcus, Leuconostoc, Bacillus, Escherichia coli*, or yeast are used to a lesser extent.[18–21]

Various studies have reported the reduction of viable probiotics below the recommended intake dose, 10^8–10^{11} colony-forming units (CFU), upon storage.[22] Several factors influence the survival and colonization of probiotics, including low pH, bile acids, and digestive enzymes.[23] Recently, pharmaceutical delivery systems have been exploited as new methods for targeting and delivery of probiotics with improved stability.[24,25] Encapsulation technologies are the most commonly applied method to protect the probiotics from harsh conditions and deliver probiotics in viable numbers into the colon to exert their activity.[26,27] It is a reliable, simple, and safe method for the stable encapsulation of live cells, resulting in a controlled and continued release of probiotics.[28] The most reported microencapsulation methods include extrusion, coacervation, emulsion and spray drying. Method selection should be able to produce microspheres or microcapsules with the required physical and/or chemical attributes while causing minimal damage to cell viability and integrity. Further, it should be easy to scale up with acceptable processing costs[29,30].

Natural and synthetic polymers are usually the biomaterials that used for probiotics encapsulation.[31] Chitosan, the N-deacetylated form of chitin, has gained advanced importance in the pharmaceutical field due to the unique properties of the cationic polymer, good biocompatibility, non-toxicity, and biodegradability.[32] The applications of chitosan as a coating material in probiotic microencapsulation as discussed thoroughly in various studies and reviews.[33] Despite the characteristic features of chitosan, there are difficulties in delivering the coated materials under physiological conditions. The pKa of the primary amine group of chitosan is approximately 6.5, resulting in a neutral charge at physiological pH, which renders it water-insoluble. This would limit the release of the coated agents. Therefore, chemical modifications are essential to enhance the solubility and functionality of chitosan. Hydrophilic polymers such as polyethylene glycol or sodium alginate have been introduced to retain the (NH_2) and (OH) functional groups of chitosan and protect it from the gastrointestinal environment.[34,35] The advantages of PAA over the other polymers are based on the structural density they attain via electrostatic interaction with chitosan. As interactions unfolded, a high degree of entanglement could result in a compact structure. The enhancement effects of PAA on the structural, mechanical, and thermal properties of polyelectrolytes have been studied and proven for drug delivery. Moreover, achieving extended drug release potentiates the usage of these systems via the different routes of administration.[36–38] PAA demonstrates superior stability across diverse environmental conditions. In contrast to alginate, which is vulnerable to the influence of divalent cations, PAA exhibits reduced susceptibility to degradation by enzymes and other biological factors. Additionally, PAA can be easily modified chemically to enhance the chitosan's properties and introduce specific functionalities for targeting. These factors make PAA a compelling choice for advanced carrier systems in delivery and biomedical applications. Despite the extensive reporting of CS-PAA systems in drug delivery, limited studies involving bacteria encapsulation have been done and rarely has any study discussed the usage of these systems in the delivery of probiotics.

In this study, probiotics were introduced within the matrix of CS-PAA nanoparticles. This work is focusing on introducing a new carrier system for probiotics with enhanced stability and viability. The novelty of this work lies on extending application of robust, versatile, and well-studied systems in drug delivery toward probiotics delivery. The stability of these systems and their ability to protect coated material motivated the research for their application to protect the probiotics from the harsh physiological conditions. Moreover, these systems were proven to have antibiofilm activity, which could boost the antibiofilm activity of the loaded probiotics.

Materials and Methods

Materials

Two strains of probiotics, *L. gasseri* ATCC 19992 and *B. bifidum* ATCC 29521 (German Collection of Microorganisms and Cell Cultures GmbH, Germany) were used. Man Rogosa and Sharpe (MRS) broth (Lab supply, Egypt) an MRS supplement (Lab supply, Egypt). Polyacrylic acid (PAA) of MW 72.06 g/mol was obtained from Sigma-Aldrich (USA),

Chitosan with deacetylation degree of 95% was purchased from Santa Cruz Biotechnology (USA), Trehalose was obtained from Combi Blocks (USA), Sodium triphosphate (TPP) was obtained from Thermo Fisher Scientific (China), carbodiimide was purchased from Thermo Fisher Scientific (China). Sodium hydroxide (NaOH) and acetic acid (CH_3COOH) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). All the reagents used were analytical grade. Crystal violet dye (Merck KGaA, Germany) and a microplate reader from Bio-Tek, USA were used to measure the optical density.

Methods

Preparation of the Bacterial Strain

Probiotic strains, *L. gasseri* (ATCC 19992) and *B. bifidum* (ATCC 29521), were grown in Man Rogosa and Sharpe (MRS) broth. Freeze-dried cells were rehydrated in 5 mL MRS broth and incubated for 24 h under aerobic conditions for *L. gasseri* at 37°C and under anaerobic conditions for 72 h using CO_2 incubator for *B. bifidum* at 39°C. Centrifugation to harvest cells at 1500 ×g for 5 min at 4°C was applied. The cell pellet was washed twice with sterile saline solution. The cell suspensions of probiotics were represented as the following: F1: used for encapsulation of *L. gasseri* (L). F2: Used for encapsulation of *B. bifidum* (B), F3: used for encapsulation of *L. gasseri* and *B. bifidum* together. The encapsulated probiotics strains were compared to relative free cells. All experiments were repeated six times and were reported as six separate independent experiments. Aliquots were prepared with 20% glycerol and stored at −80°C as a master seed.[39]

Preparation of CS-PAA

Chitosan was firstly cross-linked with sodium tripolyphosphate (TPP).[40] One mg of chitosan was dissolved in 100 mL acetic acid and stirred for 24 h. Then, the chitosan solution pH was adjusted to 6.0 using sodium hydroxide (NaOH) and cross-linked with 1% TPP. The mixture was ultracentrifuged at 15,000 rpm at 4°C for 1.5 h and resuspended with Poly-Acrylic acid (PAA) using an ultrasonic processor (Vibra-Cell™, SONICS & MATERIALS, INC, USA) for 5 s. Then, 10 mg/mL **Carbodiimide solution(CB) was added** to the previously prepared CS-PAA to cross-linked CS with the PAA and left overnight on stirring to fix the interaction of PAA with chitosan-TPP via the amide bond between the carboxyl group of PAA and the amino group on the chitosan surface. Finally, the pH was controlled between 4.2 and 6.5. The mixture was again ultracentrifuged for 1.5 hours at 4°C. The supernatant was discarded and the ultrasonic processor was used to resuspend the CS-PAA particles in sterile water.

Freeze Drying of CS-PAA

CS-PAA polyelectrolyte complexes were dried by freeze-drying utilizing a Telsar, Lyo Alfa 15–85 plus type equipment (Spain). Prior to deep freezing, 3% w/v trehalose was added to the reconstituted CS-PAA. The suspensions were placed in a round-bottom flask and frozen for 24 hours at −80°C in a deep freezer. To create the dry powder, the samples were then freeze-dried for 24 hours at 0.1 mbar.

Determination of Probiotic Viability and the Encapsulation Efficiency

To find the viable counts, the entrapped probiotic was taken out of the beads. After homogenizing for 15 minutes, one gram of beads will be re-suspended in nine milliliters of phosphate buffer (0.1 mol/L, pH 7.0). Colony forming units (CFU/g) were calculated by plating the cultures on appropriate agar plates and then incubating them for 24 to 48 h at 37°C.[41]

The encapsulation efficiency (EY) which is a combined measurement of the efficacy of entrapment and survival of viable cells during the encapsulation procedure was calculated as EY = (N /No) × 100. Where N is the number of viable entrapped cells released from the microspheres, and No is the number of free cells added during the production of the microspheres.

Scanning Electron Microscopy (SEM)

Quanta FEG 450 SEM was used to capture images of CS-PAA and CS-PAA probiotic-loaded samples at 10.000 Kv. Samples were mounted on aluminum stubs by double-sided sticky disks of conductive carbon, then they were coated with palladium by sputter coater (Quorum Q 150R, Sussex, UK) for a 5 nm thickness coat.

Mean Particle Size and Zeta Potential Analysis

The mean particle size and zeta potential of the unloaded and probiotics-loaded systems were measured by laser diffraction using a Nicomp® Nano ZLS System (Entegris, USA), using water as the dispersant and setting the refractive index to 1.33. Each system suspension was diluted by distilled water and measured at 25°C. Twelve parallel measurements were carried out.

Attenuated Total Reflectance -Fourier-Transform Infrared Spectroscopy (ATR-FTIR)

ATR-FTIR (Brucker Alpha, USA) spectrometer was applied to check the interactions between PAA-CS. The CS, PAA, and CS-PAA powdered samples were tested. CS-PAA samples were freeze-dried for this analysis without trehalose added.

Viability in Intestinal Fluid and Simulated Gastric Juice

Small intestinal juices and simulated gastric were freshly prepared. A simulated gastric juice was prepared by suspending 3 mg/mL pepsin (1:3000) in a sterile saline. The pH was adjusted to 3.0 with 1.0 mol/L HCl. Two separate rounds of sterile saline washings were performed on free cells. CS-PAA systems were used to ascertain the impact of storage in the intestinal environment. Viable colonies were incubated for two hours at 37°C before being counted and numerated. Following cell harvesting, the cells were suspended in simulated intestinal fluid containing 1 mg/mL pancreatin and 7 mL v/v fresh bile that had been pH-8.0-adjusted using a 0.1 M NaOH solution. After two hours of incubation at 37°C, the viable count of the suspension was calculated and reported as log CFU/mL.

Biofilm Formation

Three strains of bacteria were cultivated: *P. aeruginosa* ATCC 12453, *E. coli* ATCC 25922, and *C. Jejuni* ATCC 33560. Three concentrations (0.005, 0.05, and 0.5 mg/mL) of F1, F2, and F3 CS-PAA systems were applied to a mixed culture of bacterial strains in 96-well plates. 180 µL of MH broth and 20 µL of bacterial culture were placed in each well. At 37°C, the bacteria were grown aerobically for 72 hours. To get rid of the medium, the 96-well plates were gently washed three times with distilled water. After that, they were dried for about 30 minutes at 55°C. Each well was stained by adding 200 µL of 0.1% crystal violet dye and letting it sit at room temperature for ten to fifteen minutes. After washing the wells with sterile water to remove any leftover crystal violet, they were dried at 55°C. The optical density was measured at 630 nm using a microplate reader following the application of 300 µL of an eluent consisting of 80% ethanol and 20% acetone.[42]

Stability of Microencapsulated Probiotic Bacteria During Storage at 4°C

The stability of free and encapsulated probiotic bacteria during 4 weeks of storage in the refrigerator at 4°C was evaluated. One concentration for all trails was determined (10^5) to start with. Probiotic strains were grown in Man Rogosa and Sharpe (MRS) broth and the viable count was determined as log CFU/mL.

Statistical Analysis

All data were presented as the means ± standard errors. Statistical and graphical analyses were performed using GraphPad Prism 10 software (San Diego, CA, USA). Two-way ANOVA tests were conducted. The threshold for statistical significance was set at a p value < 0.05.

Results

ATR-FTIR

CS-PAA polyelectrolyte complexes were formed via intermolecular interaction between amino groups on CS and carboxyl groups on PAA. The ART-FTIR spectra (Figure 1) confirmed this complexation. CS spectra showed characteristic peaks at 3261 cm^{-1} related to O-H and at 1682 cm^{-1} related to C=O (amide), whereas PAA spectra showed peaks at 2928 and 1200 cm^{-1} related to C-H and C-O bond stretching, respectively.[43] The CS-PAA spectra showed a new absorption peak at 1628 cm^{-1} related to the $–NH_3^+$ absorption of CS. A broad peak also appeared at 2500 cm^{-1} that also confirmed the presence of NH_3^+ in CS-PAA. Moreover, the absorption peaks at 1532 cm^{-1} and 1414 cm^{-1} could be

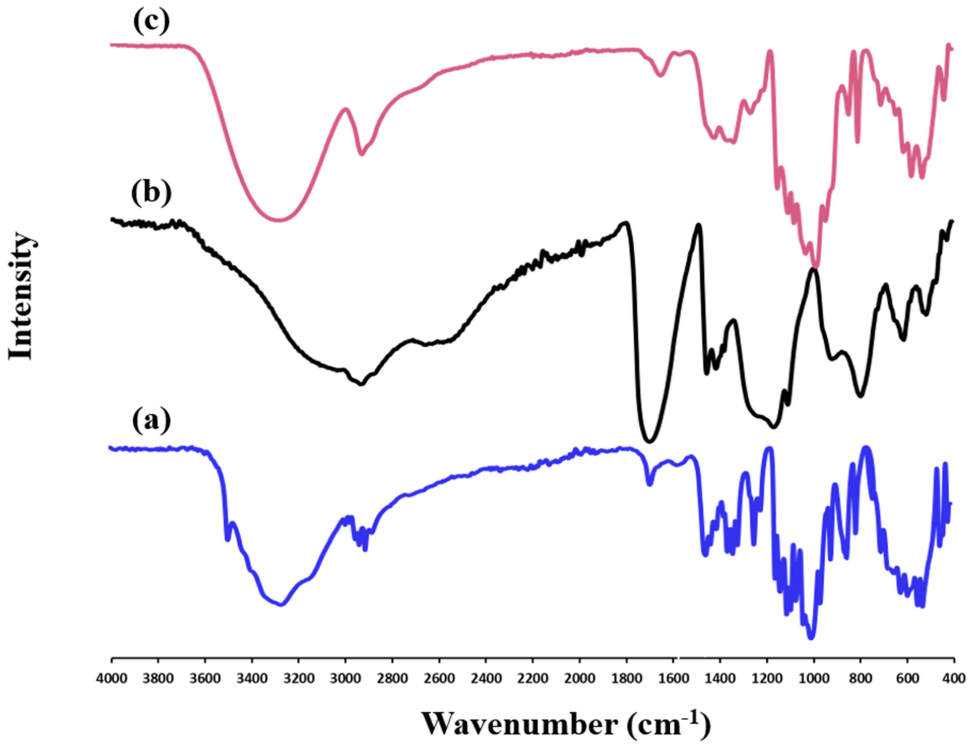

Figure 1 FTIR spectra of different compounds: (a) CS, (b) PAA, and (c) CS-PAA particles.[42]

related to asymmetric and symmetric stretching vibrations of COO^- groups. The superimposed peak at 3261 cm^{-1} represented stretching vibrations of –OH, –NH and intermolecular hydrogen bonding, confirming the interaction.

Scanning Electron Microscopy (SEM)

Characterization of the Probiotics Loaded CS-PAA

The morphology of the CS-PAA and the encapsulated probiotics shown in Figure 2. The CS-PAA nanoparticles exhibited a spherical shape that were uniformly distributed within the matrix established by the CS chains (Figure 2a). The encapsulated systems showed the probiotics residence within the interconnected pores of micrometer-scale that formed by the porous architecture of the CS-PAA structure (Figure 2b–d).

Particle Size and Zeta Potential

The CS-PAA showed a particle size of approximately 2.98 ± 0.57 µm using the particle size analyzer. The loaded system showed 10.68, 5.737, and 7.306 µm for L, B, and L-B loaded particles, respectively. The zeta potential of CS-PAA was +44±8 Mv. However, the loaded systems of L, B, and L+B were −36.41, −34.85, and −60.21 Mv, respectively.

The Encapsulation Efficiency (EY)

The encapsulation efficiency of *L. gasseri, bifidum* and *B. bifidum*+ *L. gasseri* with CS-PAA were 82.6%, 93.96% and 87.55%, respectively (Table 1). These high encapsulation efficiency values indicated the safety of encapsulation process and the efficiency of the CS-PAA to preserve the probiotics.[24]

Viability of Free and Encapsulated Cells During Storage at 4°C

In this study, the viability of encapsulated cells was different at the same storage conditions, indicating the discrepancy in the stability of these encapsulated cells. The survival of *L. gasseri* microcapsule decreased slowly from 10^5 to $9.8*10^4$ CFU/mL with approximately 2% loss within 14 days, shown in Figure 3. However, the survival rate after 28 days showed a significant cell loss reached 98%. Similarly, *B. bifidum* in microcapsule decreased from 10^5 to $8.1*10^4$ CFU/mL in the

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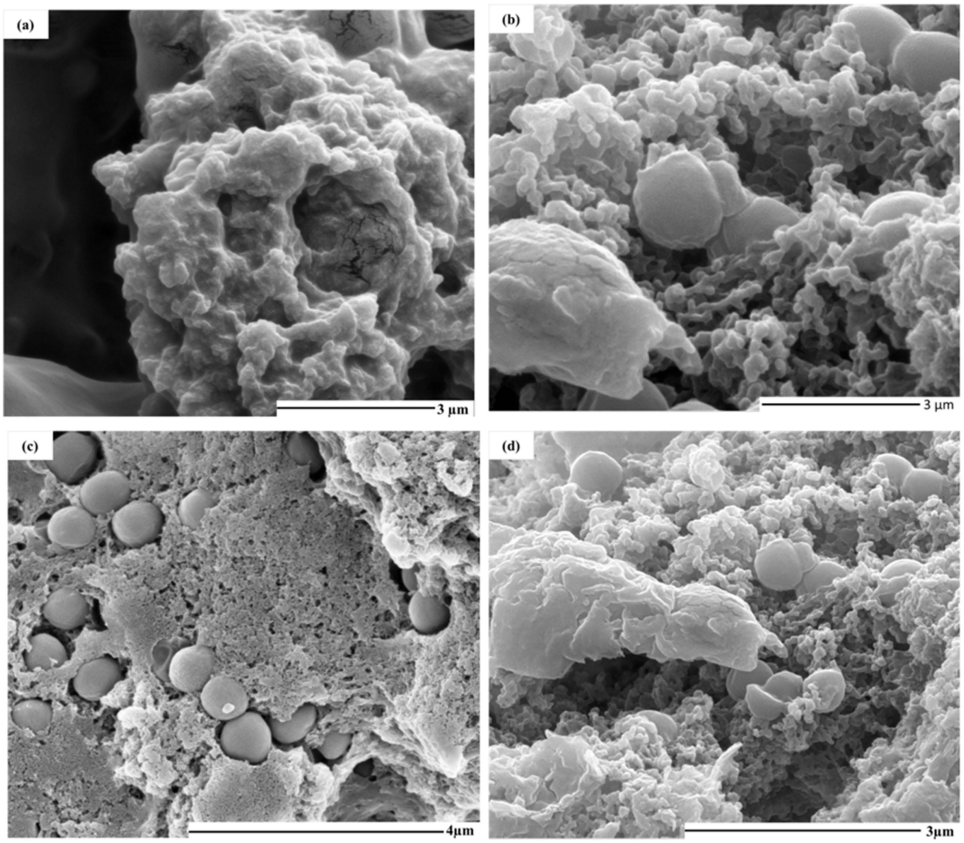

Figure 2 Representative SEM images of (**a**) CS-PAA, (**b**) CS-PAA loaded with L probiotics strain, (**c**) CS-PAA loaded with B probiotics strain, and (**d**) CS-PAA loaded with L+B probiotics strains.

first two weeks, and the loss was 95% after 28 days. The combination of the two stains L&B with CS-PAA had the highest loss of 91% and 98% after 14 and 28 days, respectively. Notably, there was a significant difference ($p < 0.0001$) in survival between the free and CS-PAA encapsulated strains. The CS-PAA systems showed superior viability when the two probiotics types were concomitantly encapsulated compared to chitosan-alginate systems, where these strains were not detected after 2 hr at 4°C. Moreover, CS-PAA showed higher encapsulation efficiency compared to chitosan-alginate capsules where 39% and 40% were reported for *L. gasseri* and *B. bifidum*, respectively[26].

Survival of Free and Encapsulated B. Bifidum and L. Gasseri in SGJ

The stability of both free and encapsulated probiotic bacteria in simulated gastric fluid was assessed to ascertain their chance of surviving oral administration and making it through the stomach (Figure 4). The survival of lactobacilli and bifidobacteria was significantly ($p < 0.0001$) increased by encapsulation in CA-PAA carrier systems. Both *B.ifidum* and *L. gasseri* that were encapsulated showed resistance to the stomach simulation conditions. After two hours, more over half of the *L. gasseri* and *B. bifidum* capsules remained viable. Nonetheless, free probiotic bacteria in SGJ at pH 2.0 were

Table 1 Encapsulation Efficiency of CS-PAA Systems

Sample	CFU Before Encapsulation	CFU After Encapsulation	Efficiency (%)
L. gasseri	$2.3(10^8)$	$1.9(10^8)$	82.60%
B. bifidum	$1.16(10^7)$	$1.09(10^7)$	93.96%
B. bifidum+ *L. gasseri*	$9(10^7)$	$7.88(10^7)$	87.55%

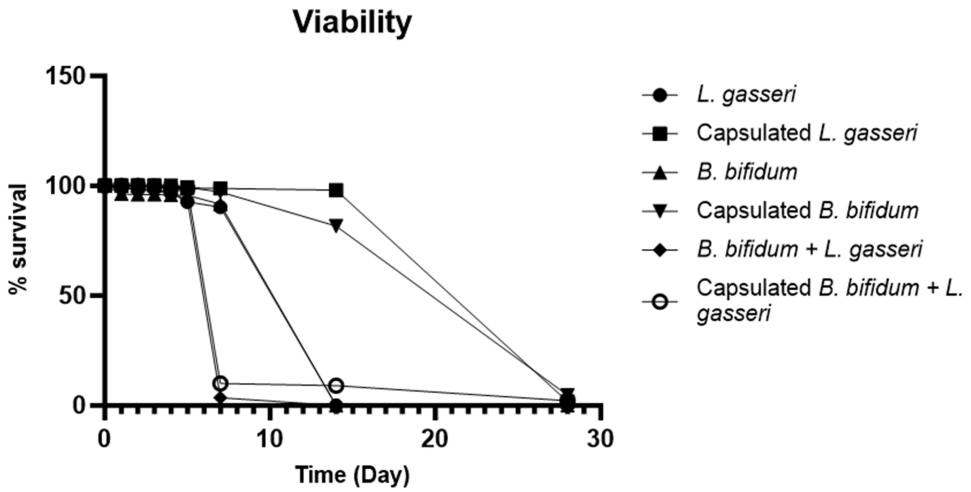

Figure 3 Stability of free and encapsulated *L. gasseri* and *B. bifidum* during 4 weeks of storage at 4 °C.

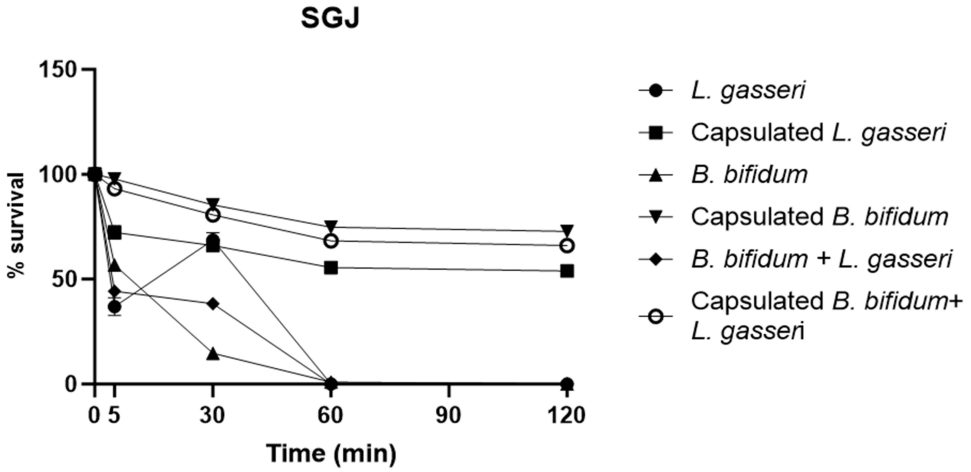

Figure 4 Probiotic survival of free and encapsulated with CS-PAA in SGJ conditions in time interval [0, 5, 30, 60, 90, and 120 minutes].

rapidly lost; after one hour of exposure, starting counts of 10^5 CFU/mL for free *L. gasseri* and *B. bifidum* rapidly dropped to fewer than 10 CFU/mL.

Survival of Microencapsulated Probiotic Bacteria in Simulated Intestinal Juices (pH 6.0)

Figure 5 shows the impact of the bile salt on both free and microencapsulated probiotic bacteria's viability. The initial concentration 10^5 viable CFU/mL. In the case of free *L. gasseri*, free *B. bifidum*, and free L&B, the concentrations of the cells were reduced to less than 7% after 120 min. Microencapsulated bacteria survival after 120 min exposure to SGI was 90%, 85%, and 75% of the initial population found in encapsulated *L. gasseri, B. bifidum*, and L+B, respectively. Probiotic bacteria were best shielded from bile salt by CA-PAA systems. Because the chitosan coating causes an ion-exchange reaction when the beads absorb bile salt, it offers the best protection in bile salt solution.[44] Consequently, there may be a limit to the bile salt's diffusion into the CS-PAAA systems. Our results concur with other studies that used similar concentrations of bile salts.

Effect of Formula on Pathogen Biofilm Formation

At various concentrations of capsulated probiotics (L, B, and L+B), pure and mixed cultures of pathogenic bacteria were assessed for biofilm inhibition under aerobic conditions compared to (Figure 6). Two-way ANOVA p-values revealed that

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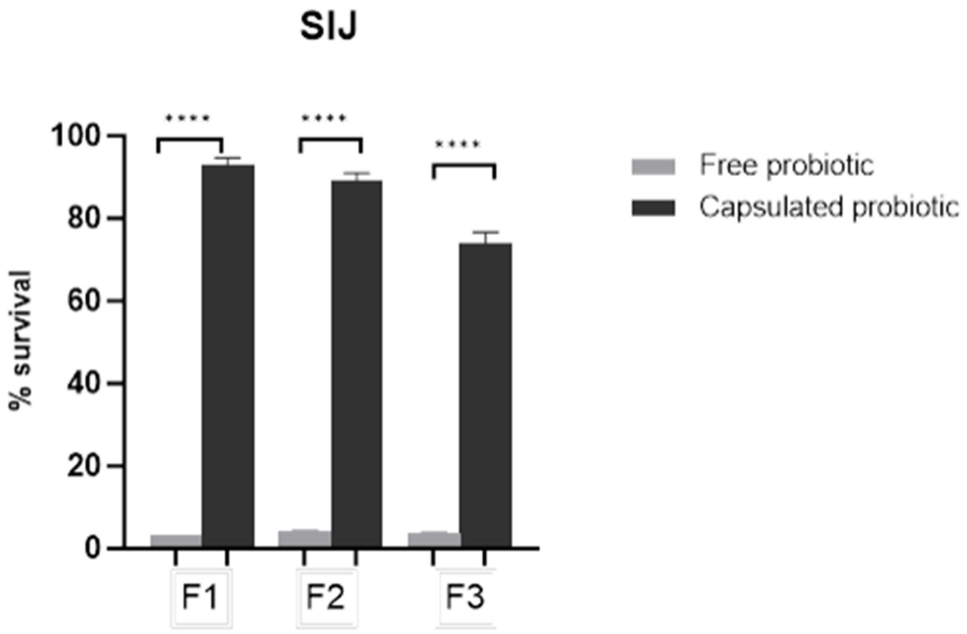

Figure 5 Probiotic survival of free and encapsulated with CS-PAA in SIJ conditions. ****P≤ 0.0001.

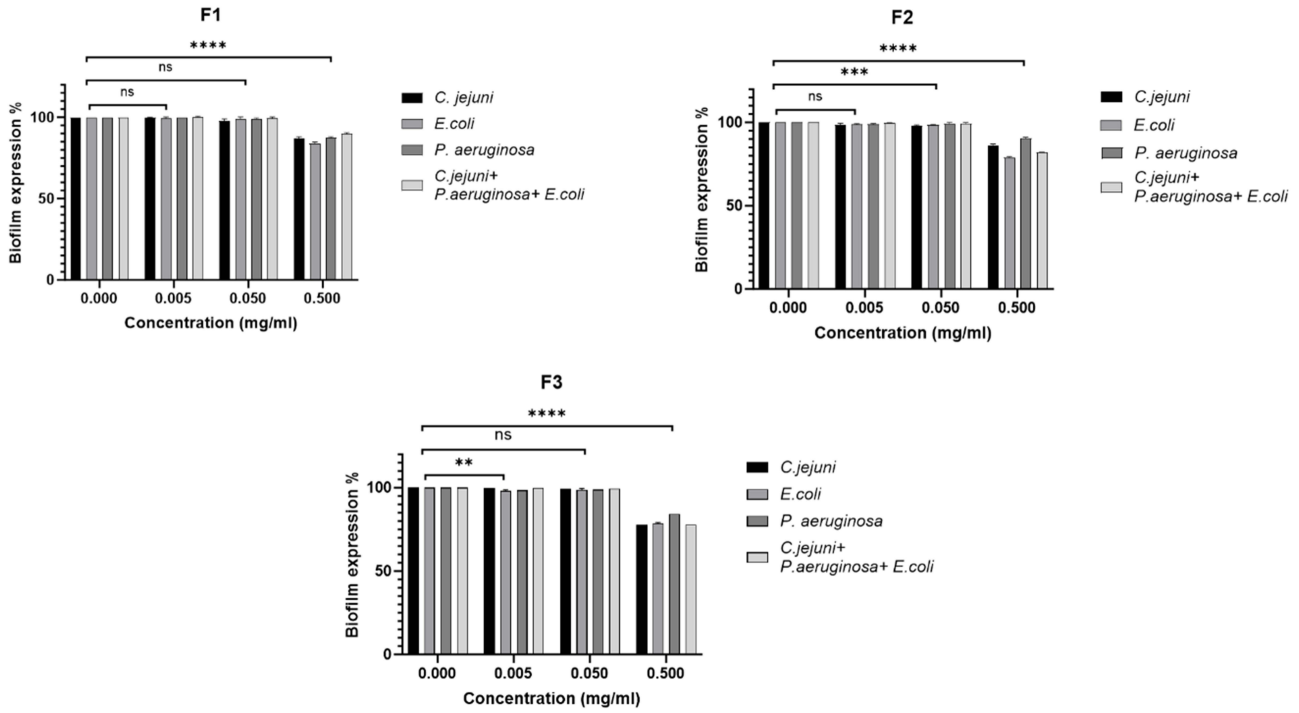

Figure 6 The effect of concentration (0.005, 0.05, 0.5 mg/mL) of the encapsulated probiotics on the biofilm formation of pure and mixed pathogen *C. Jejuni, (**P**) aeruginosa* and E. coli (0.000 concentration represent the control group. F1: represents the different oncentrations of capsulated *L. gasseri*. F2: represents the different concentrations of capsulated *B. bifidum*. F3: represents the different concentrations of capsulated *L. gasseri* +*B. bifidum*. **p ≤ 0.01,***p ≤ 0.001, ****P≤ 0.0001.
Abbreviation: ns, non- significant.

the inhibition in biofilm expression was not always significant with concentrations 0.005 and 0.05 mg/mL of capsulated probiotics in F1, F2, and F3 (Supplementary 1), however, the concentration 0.5 mg/mL showed significant inhibition of biofilm expression compared to control group in all formulas (F1, F2, and F3) with p-value <0.0001). Therefore, the concentration 0.5 mg/mL of capsulated probiotic was selected for further investigations.

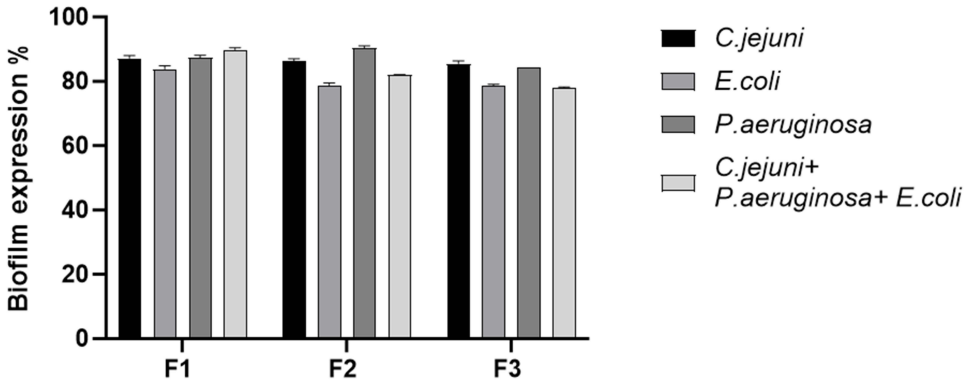

Figure 7 Anti-biofilm activity of 0.5 mg/mL of encapsulated probiotics with CA-PAA against the selected pathogenic bacteria in pure and mixed cultures. F1: represent different concentrations of capsulated *L. gasseri*. F2: represent different concentrations of capsulated *B. bifidum*. F3: represent different concentrations of capsulated *L. gasseri* +*B. bifidum*..

In comparison to evaluate the most effective encapsulated strain that inhibit the pathogenic bacteria at 0.5 mg/mL concentration, results revealed that encapsulated *L. gasseri and B. bifidum* has a significant inhibition rate on pure and mixed culture (Figure 7). In addition, results revealed that encapsulated *B. bifidum* with CS-PAA has a significant inhibition rate on pure culture of *E. coli* in comparison to encapsulated *L. gasseri*.

Discussion

To effectively reach the colon in substantial quantities, probiotic strains must survive and transit through the digestive system.[45] This study aims to investigate the efficacy of CA-PAA nanoparticles in protecting selected probiotic strains when administered orally under adverse conditions. In our research, chitosan (CS) was incorporated at a concentration of 1% (w/v), which exceeds the concentrations previously examined in studies on probiotic bacteria encapsulation.[46,47] The results indicate that the prepared CS-PAA nanoparticles were stable enough to provide superior defense against unfavorable conditions. This suggests that the chitosan tested herein advantageously incorporated in the PAA, thereby imparting increased rigidity to the CS-PAA systems.

The formation of the spherical CS-PAA morphology could be attributed to the strong affinity between these polymers, leading to the formation of polyelectrolyte complexes.[26] This process facilitated the creation of composite polyelectrolyte multilayers, where excess CS chains allowed the diffusion of the PAA. Such interactions could form an island-like morphology characterized by an increased porosity. Consequently, the probiotics effectively resided within the formed pores and attached to the surrounding nanoparticles[48–50] .

The FTIR analysis revealed significant interactions among the components of CS-PAA polyelectrolytes, as indicated by the results. These complexes formed primarily due to the intermolecular interactions between the amino groups of CS and the carboxyl groups of PAA. These results correspond with those previously obtained, whereby similar spectral features in the CS-PAA system have been matched, recording strong interactions induced by the hydrogen bond and electrostatic force between the two polymers' functional groups.[51,52]

The encapsulated system showed a substantial increase compared with unloaded CS-PAA composites due to the large size of bacteria. This observation highlighted the effectiveness of CS-PAA composite as a protective matrix for the probiotics bacteria.[53] Initially, CS-PAA composites exhibited a positive charge related to the electrostatic interactions. However, upon the encapsulation of the probiotics, the positive zeta potential of the CS-PAA unloaded composites shifted to the negative charge because these bacteria carry a negative charge. When the probiotics were encapsulated within the CS-PAA matrix, they affected the net surface charge of the nanoparticles by contributing an additional negative charge. This change could potentially lead to improved stability against environmental factors that might otherwise compromise probiotic viability.[54]

The encapsulation efficiency values of *L. gasseri, bifidum* and *B. bifidum*+ *L. gasseri* with CS-PAA was founded to be statistically significant ($P < 0.05$), indicating both the safety of the encapsulation process and the efficiency of the CS-PAA to preserve these probiotics.[24] Despite the well-documented antimicrobial properties of chitosan, the majority of L. gasseri and

B. bifidum were successfully encapsulated within CS-PAA capsules, indicating that chitosan did not inactivate the probiotic cells during the encapsulation process. Notably, these probiotic bacteria remained viable even in a 1% chitosan solution.[27]

Further investigation revealed that adding chitosan to the coencapsulated probiotic bacteria significantly enhanced their viability.[55] It is also essential to consider that encapsulation yield could significantly affect the stability under simulated digestive conditions.[56] Various variables, such as capsule size, concentration, probiotic cell weight, and hardening time in PAA, can affect chitosan encapsulation. The current study also explored the effect of different types of probiotics on diameter of the CS-PAA systems. Other studies have reported the improved survival of the encapsulated probiotic bacteria encapsulated with prebiotics like Hi-maize starch and subsequently coated with chitosan.[57] This evidence ensure the potential of chitosan-coated microcapsules to enhance probiotic stability and viability during gastrointestinal transit, thereby supporting their application in functional food products.

Results of storage stability at (4°C) indicates the stability of the strains after encapsulation. Encapsulation provides a protective effect against the viability loss. Previous reports have demonstrated that during the storage at 4°C, the microencapsulated bacteria showed a superior survival in CS microparticles compared with non-encapsulated cells. The increased viability is attributed to the low temperature that inhibits the enzymatic activity of probiotic cells, thereby restricting the absorption of nutrients. Consequently, low temperatures affect the viability of probiotics, whether they were in free or encapsulated forms.[58]

Recent studies have reported the significance of encapsulation in boosting the probiotic bacteria's stability at low temperatures.[55,59] Specifically, freeze-drying has been identified as a superior method for encapsulating probiotics, yielding better long-term stability and production outcomes compared to spray-drying.[60] The differences among probiotic strains regarding their survival has an impact on the long-term stability and production capabilities.[61–64]

Several types of polymers have been tested as microencapsulation materials to improve the storage period and yield quantity. For instance, the survival of microencapsulated bacteria was notably higher in alginate microparticles compared to the free bacteria throughout the proposed storage period.[65,66] Chávarri et al show that *L. gasseri* and *B. bifidum* loaded in chitosan- coated alginate microparticles exhibited a higher storage stability than their free cell culture.[26]

Exposure to simulated gastric juice resulted in a considerable decrease in the total number of free *B. bifidum* due to its low acid resistance. Within 30 min, the free *B. bifidum* cell numbers decreased to an undetectable level, corroborating previous studies that reported no free *B. bifidum* survival for 15 minutes in a simulated stomach pepsin-free environment at pH 1.55.[67,68] In contrast, encapsulation in chitosan-coated alginate microspheres considerably increased the survival of *L. gasseri* and *B. bifidum* in simulated gastric juice in addition to pepsin, according to study by Chávarri et al (2010).[26] Notably, Sultana et al[69] found that probiotics were not tolerably protected from high acidity by coating probiotics in alginate beads. However, several studies have reported that coating with alginate matrix improved the bacterial viability by reducing calcium ion transport outside of capsules.[63,70] A study conducted in 2009 indicated that when *L. acidophilus* and *L. rhamnosus*, encapsulated in calcium alginate and double-coated with sodium alginate, a greater vitality was achieved when exposed to simulated gastric juice without pepsin. The reduction of gastric juice distribution and pore size in the double layer membrane limited the ability of cells to interact with the gastric juice.[70]

Our findings suggest that coated microcapsules provide superior protection in simulated gastric juice and reduce probiotic leakage; this structure can show greater resistance in harsh conditions like the acidic pH of simulated gastric juice.

The results of this study indicate that CS-PAA significantly enhances the protection of the probiotic bacteria from simulated intestinal juice, with statistical significance established ($P < 0.05$). This aligns with previous studies that indicated that microencapsulated probiotics were able to maintain viability in gastro-intestinal conditions.[26,57,71] Khosravi et al[68] reported that probiotic bacteria encapsulated in alginate-chitosan or poly-L-lysine markedly improved the survival of probiotic bacteria in such environments. Furthermore, previous studies have confirmed the enhanced probiotic bacterial viability under gastric simulations for probiotics microencapsulated in alginate beads.

Notably, Bifidobacterium in chitosan-containing alginate beads had a greater survival rate than those in regular alginate beads.[72–74] Compared to uncoated microcapsules, the additional coating provided probiotic organisms with superior overall protection.

The advancement of novel biofilm-fighting strategies is critical in the clinical setting due to the inadequacy of the existing methods. According to recent studies, probiotics have created new avenues for combating pathogenic biofilms.

Probiotics can be considered as the perfect alternative for anti-virulence drugs as they exhibit lower cytotoxic than quorum sensing (QS)-suppressing agents and cannot exert the same level of selection pressure on resistant isolates as conventional antibiotics.

Probiotics can inhibit pathogenic bacteria's activity and surface attachment through multiple mechanisms. They disrupt biofilm integrity, impede QS, inhibit biofilm development and biofilm pathogen survival, and ultimately result in biofilm eradication.[75–77] Several studies have demonstrated that probiotics can prevent foodborne bacteria from forming biofilms.[78,79] Biofilms are linked to infection [78] due to their structural properties that confer increased resistance to environmental stresses and antimicrobial treatments.

Different compounds found in probiotics possess antibacterial activity that influence pathogens and components of the biofilm matrix. Examples include hydrogen peroxide, oxygen metabolites, exopolysaccharide, saturated fatty acids acting as bio-surfactants, and various lactic acid bacteria components and their metabolites .[80] Additionally, CS-PAA nanoparticles demonstrated a strong antibiofilm activity, which would increase the overall antibiofilm activity of probiotics when encapsulated .[42]

Extensive studies have demonstrated the antibiofilm efficacy of probiotics strains against a range of pathogens as well as multidrug-resistant pathogens. Probiotics deploy a variety of mechanisms to counteract harmful microorganisms.[81] In this regards, many exometabolites, including bacteriocins, oxygen reactive species (ROS), extracellular polymeric substances (EPSs) and biosurfactants with anti-biofilm action, are produced by *Lactobacillus* species. The polysaccharides generated by lactic acid bacteria exhibits immune- stimulatory effect, antioxidant capabilities, and anti-biofilm properties.[82] Both Gram-positive (such as *L. monocytogenes* and *S. aureus*) and Gram-negative (such as *P. aeruginosa, E. Coli* and *S. typhimurium*) bacteria show susceptibility to EPS produced by Lactobacillus spp., highlighting the potential of probiotics in combating biofilm-associated infection.[83,84]

Conclusions

The present study provides robust preliminary evidence supporting the formulation and efficacy of a synthesized delivery system utilizing 1% chitosan and polyacrylic acid for encapsulating probiotics *L. gasseri* and *B. bifidum* and their combination for oral administration. The unique structure of this system combined the nanoparticles of chitosan with tripolyphosphate to give nanoparticles, which then complexed with polyacrylic acid to form a matrix with pores suitable for residing the probiotics This configuration significantly enhances the delivery of viable probiotics, ensuring they maintain sufficient activity to positively impact human health.

The Chitosan coating provided a superior protection of the probiotic cells. Our data highlights the effectiveness of CS-PAA nanoparticles against SIJ and SGJ. Specifically, in bile salt solutions, the chitosan-coated formulations markedly increased the survival rates *of L. acidophilus, B. bifidum*, separately and combined, as compared to the in uncoated cells. Moreover, this system preserved the adequate viability of the probiotics for a long time, which can be assigned to their shelf life. The probiotic-loaded systems inhibited the biofilm formation by pathogenic bacteria. The presence of nanoparticles could boost the antibiofilm activity of these systems. Despite the advantages of the CS-PAA systems, we recognize that strain sequencing and colonization investigations in in vivo model systems are necessary. Further work to demonstrate the ability to regulate the transcription level of biofilm-associated gene and protein expression before moving on to clinical trials is recommended. This study lays a strong foundation for future developments in probiotic delivery systems, highlighting the potential for improved therapeutic applications in gut health management.

Data Sharing Statement

Data available from the corresponding author on reasonable request.

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Disclosure

The Authors declare that there is no conflict of interest.

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