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Survivability of Microencapsulation Probiotic Bacteria in Sodium Alginate-Goat Milk-β-Glucan Matrix under Freeze-drying Conditions for Dog Supplement

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Abstract

The successful oral delivery of probiotics to the canine gastrointestinal tract necessitates protection against harsh environmental conditions. This study investigated the efficacy of a novel alginate-goat milk-beta-glucan matrix for the microencapsulation of three canine-associated lactic acid bacteria (LAB) strains: *Agrilactobacillus fermenti* Pom1, *Limosilactobacillus fermentum* Pom5, and *Pediococcus pentosaceus* Chi8. High encapsulation efficiencies (88.25% to 99.33%) were achieved across all strains and cocktails, indicating successful cell entrapment. Following freeze-drying and two months of storage, microencapsulation significantly enhanced the survival of all probiotic strains and cocktails compared to their unencapsulated counterparts. Furthermore, microencapsulated probiotics demonstrated superior resilience under simulated gastrointestinal conditions. While all cells initially maintained high viability in simulated oral conditions, encapsulation provided robust protection in simulated gastric fluid, where only encapsulated cells remained viable after 60 minutes, whereas free cells were completely inactivated. *P. pentosaceus* Chi8 exhibited the highest survival in gastric conditions (76.23% after 120 minutes at 2 months), and both encapsulated cocktails survived up to 180 minutes. Similarly, under simulated intestinal conditions, encapsulated cells consistently maintained significantly higher viability than free cells, with *A. fermenti* Pom1 showing 83.38% viability after 180 minutes at 2 months, compared to 0% for its unencapsulated form. This comprehensive evaluation of the alginate-goat milk-beta-glucan matrix for these specific canine LAB strains under freeze-drying, storage, and simulated gastrointestinal conditions represents a novel contribution. The findings underscore the potential of this matrix as an effective delivery system for canine probiotics, paving the way for the development of stable formulations aimed at improving canine gut health.

Keywords: *microencapsulation; probiotics; canine; alginate; goat milk; beta-glucan; Agrilactobacillus fermenti; Limosilactobacillus fermentum; Pediococcus pentosaceus; Gastrointestinal conditions; Freeze-drying*

1. Introduction

The application of probiotics in canine health has significantly increased (Gopalakrishnan et al., 2025). Probiotics, defined as live microorganisms that confer health benefits when administered in adequate amounts, primarily modulate the canine gastrointestinal microbiota. This modulation leads to enhanced intestinal health, improved mucosal barrier integrity, and stimulated immune responses (Hardy et al., 2013). Furthermore, they improve the microenvironment for intestinal epithelial cells, strengthening resistance against pathogenic bacterial invasion (Xia et al., 2024). However, the efficacy of probiotics in dogs depends on delivering a sufficient number of viable colony-forming units (CFUs) to the intestinal tract.

Achieving high colony-forming unit (CFU) counts presents a considerable challenge, as probiotics are susceptible to degradation during processing and storage. Although freeze-drying is a preferred method for the long-term stabilization of starter cultures (Lodato et al., 1999), it exposes microbial cells to stressors such as freezing, high vacuum, and reduced water activity, potentially causing significant cellular damage. The survival of probiotics during freeze-drying is influenced by several factors, including strain resilience, initial cell concentration, cultivation conditions, and the choice of cryoprotectants (Agriopoulou et al., 2024; Arellano-Ayala et al., 2021).

Alginate encapsulation offers a promising strategy to protect probiotic bacteria, owing to alginate's biocompatibility, non-toxicity, and cost-effectiveness (Martin et al., 2015). Calcium-alginate hydrogels, in particular, provide a simple and safe encapsulation matrix (Colin et al., 2024). However, freeze-drying of encapsulated probiotics can still significantly reduce their viability (Nag & Das, 2013). Recent studies suggest that alginate-dairy-based matrices are especially effective for probiotic encapsulation. These matrices, composed of carbohydrates, fats, and proteins, contribute to enhanced viability and survival of microencapsulated probiotics (Maciel et al., 2014). When considering different milk options for dogs and pets, goat milk is recommended due to its lower lactose content and improved digestibility compared to cow's milk (ALKaisy et al., 2023). Moreover, goat milk is a promising prebiotic component, rich in oligosaccharides that selectively promote the growth of beneficial bacteria like *Lactobacillus* and *Bifidobacterium* (Leong et al., 2019). Goat milk's oligosaccharide profile, closely resembling that of human milk, suggests a potentially superior prebiotic effect compared to other dairy sources (ALKaisy et al., 2023).

Recent studies have isolated three lactic acid bacteria (LAB) strains-*Agrilactobacillus fermenti* Pom1, *Limosilactobacillus fermentum* Pom5, and *Pediococcus pentosaceus* Chi8-that exhibit promising probiotic characteristics. Safety evaluations confirmed that none of the strains displayed hemolytic activity, produced biogenic amines, or carried pathogenic genes. Moreover, all three strains-*A. fermenti* Pom1, *L. fermentum* Pom5, and *P. pentosaceus* Chi8-were susceptible to all tested antibiotics, consistent with the absence of antibiotic resistance genes. Genome analyses revealed that *A. fermenti* Pom1, *L. fermentum* Pom5, and *P. pentosaceus* Chi8 possessed key features of safe probiotic strains, including genome stability and the absence of transferable antibiotic resistance and virulence factor genes. Additionally, genes encoding proteins involved in stress response, acid and bile tolerance, and adhesion to host cells were identified, supporting their ability to survive gastric conditions and further enhancing their probiotic potential. Based on phenotypic and genomic analyses, *A. fermenti* Pom1, *L. fermentum* Pom5, and *P. pentosaceus* Chi8 are considered promising probiotic candidates for use in canines (Foongsawat et al., 2023a; Chonghan, 2024).

To address these challenges, a comprehensive strategy combining effective cryoprotection, prebiotics, and optimized freeze-drying protocols with robust probiotic lactic acid bacteria (LAB) strains is essential to enhance cell survival. This study employed extrusion encapsulation to improve microbial survival rates. Previous research indicated that alginate-goat milk microcapsules exhibited the highest encapsulation yield, and the viability of microencapsulated LAB cells within the alginate-goat milk matrix provided the best protection for the probiotic strains under all tested conditions: simulated gastrointestinal environments, refrigeration, and heating at pasteurization temperatures. Therefore, this study aimed to develop a powdered probiotic formulation using three strains and their combinations- *A. fermenti* Pom1, *L. fermentum* Pom5, and *P. pentosaceus* Chi8. This was achieved by freeze-drying and encapsulating them in microcapsules composed of sodium alginate, goat milk, and the prebiotic beta-glucan to enhance cell survival. Goat milk was employed as a cryoprotectant, as supported by Shu et al. (2018). The encapsulated probiotics were then subjected to freeze-drying. The resulting freeze-dried samples were evaluated for their ability to

withstand simulated gastrointestinal conditions and long-term storage.

2. Objectives

The objective of this study was to compare the viability of three lactic acid bacteria (LAB) strains, with and without encapsulation, after undergoing freeze-drying, exposure to simulated gastrointestinal conditions, and 2 months of storage.

3. Materials and Methods

3.1 Bacterial Strains and Culture Media

The lactic acid bacteria (LAB) strains, *Agrilactobacillus fermenti* Pom1, *Limosilactobacillus fermentum* Pom5, and *Pediococcus pentosaceus* Chi8**,** were sourced from a previous study in which their probiotic potential was characterized (Foongsawat et al., 2023a). These strains were cultured on de Man, Rogosa, and Sharpe (MRS) agar plates or in MRS broth (Himedia, India) and incubated at 37°C under anaerobic conditions.

3.2 Probiotic Bacterial Encapsulation via Extrusion

3.2.1 Probiotic Cell Preparation

All LAB strains were cultured in MRS broth at 37°C for 24 hours. Bacterial cells were then harvested by centrifugation at 10,000 × *g* for 5 minutes. The supernatant was discarded, and the resulting pellets were washed three times with 0.85% (w/v) NaCl. The washed cells were resuspended in peptone water, and their density was adjusted to an optical density (OD) of 1 at 600 nm. Due to its distinct colony morphology, *L. fermentum* Pom5 could be readily distinguished from *A. fermenti* Pom1 and *P. pentosaceus* Chi8. This characteristic allowed the preparation of specific two-strain cocktails: Cocktail 1 (*A. fermenti* Pom1 and *L. fermentum* Pom5) and Cocktail 2 (*L. fermentum* Pom5 and *P. pentosaceus* Chi8). A cocktail combining only Pom1 and Chi8 was not prepared, as the enumeration of these two strains individually within a mixed culture would have been challenging without a distinct morphological difference. For enumeration purposes, most viable cell counts obtained from the cocktails were attributed to *L. fermentum* Pom5. Individual LAB strain suspensions and the two-strain cocktails were prepared by mixing equal volumes of the standardized single-strain suspensions, then transferred to sterile tubes and stored at 4°C until use.

3.2.2 Encapsulation of LAB Strains

LAB strains were encapsulated in a sodium alginate-goat milk matrix following a modified method based on Prasanna and Charalampopoulos (2018) and Foongsawat et al. (2023b). A sterile alginate-based mixture was prepared by combining 2% (w/v) sodium alginate (Himedia), goat milk, beta-glucan (Betapet^{R}, Betapet Co., Ltd.), and a concentrated cell suspension in a volume ratio of 2:1:1:1 (v/v/v/v). This mixture was then extruded dropwise into a sterile 0.5 M calcium chloride ($CaCl_2$) solution (Thermo Fisher Scientific, USA) using a 21G needle (Nippo, Thailand) under gentle stirring to form microbeads. The formed microbeads were rinsed with sterile 0.85% NaCl solution and stored in sterilized bottles at 4°C. For the preparation of non-encapsulated cells, a concentrated cell suspension was prepared using sterile 0.85% NaCl, goat milk, and beta-glucan in the same 2:1:1:1 volume ratio (v/v/v/v). The initial bacterial concentration in both the microbeads and non-encapsulated suspensions was approximately 9 log CFU/g.

3.2.3 LAB Cell Enumeration and Encapsulation Efficiency

To determine cell counts, serial dilutions were prepared in peptone water and spread plated on MRS agar supplemented with 0.3% (w/v) $CaCO_3$. Plates were incubated anaerobically at 37°C for 48 hours, and results were expressed as log CFU/mL or log CFU/g as appropriate. For free (unencapsulated) cells in suspension, viability was expressed as percentage viability calculated using equation (1):

$$\text{Viability (\%)(Free cells)} = \frac{\text{Cell count after freeze-drying}}{\text{Initial cell count}} \text{ x } 100 \ldots\ldots\ldots\ldots \quad (1)$$

For encapsulated cells (prior to freeze-drying), 1 gram of microcapsules was dissolved in 9 mL of 50 mM sodium citrate solution (pH 7.5). The resulting suspension was serially diluted and spread plated on MRS agar with 0.3% (w/v) $CaCO_3$, followed by anaerobic incubation at 37°C for 48 hours. Results were expressed as log CFU/g of microcapsules. Encapsulation efficiency (EE%) was calculated using the equation (2):

$$\text{Encapsulation efficiency (\%)} = \frac{\text{Number of viable cells released from microcapsules (CFU/g)}}{\text{Number of viable cells added initially to encapsulation mixture}} \times 100 \quad (2)$$

3.3 Probiotic Freeze-drying and Storage Viability

Individual and cocktail LAB cells, both microencapsulated and non-encapsulated, were prepared for freeze-drying. Samples were frozen at −80°C and subsequently freeze-dried using a Delta 2-24 LSCplus model, CHRIST (Germany). The resulting freeze-dried products (encapsulated and/or non-encapsulated) were then stored at 4°C in sterile bottles for a period of two months. This duration was selected to represent a critical initial period for evaluating the stability of new probiotic formulations, as significant viability loss often occurs within the first few months of storage, particularly for freeze-dried products, with the 1-month mark providing an intermediate data point to assess the rate of decline and identify any rapid initial losses.

For cell enumeration of freeze-dried samples, both immediately after freeze-drying (0 months) and at subsequent storage time points (1 and 2 months), 1 gram of encapsulated freeze-dried microcapsules was dissolved in 9 mL of 50 mM sodium citrate solution (pH 7.5), while 1 gram of non-encapsulated freeze-dried samples was dissolved in 9 mL of sterile 0.85% NaCl. Both types of samples were then serially diluted and spread plated on MRS agar supplemented with 0.3% (w/v) $CaCO_3$. Plates were incubated anaerobically at 37°C for 48 hours, and results were expressed as log CFU/g of sample. Viability after freeze-drying and during storage was calculated by comparing the viable cell counts at 1 and 2 months of storage to the initial count immediately after freeze-drying (0 months), using equation (3):

$$\text{Viability during storage (\%)} = \frac{\text{Cell count post-freeze-drying or at specified storage time (1 or 2 months)}}{\text{Cell count immediately after freeze-drying (0 months)}} \text{ x } 100 \quad (3)$$

3.4 Determination of The Survival of Free and Microencapsulated Cells in A Simulated Gastrointestinal System for Dogs

3.4.1 Simulated Oral Conditions

One gram of freeze-dried microcapsules or non-encapsulated freeze-dried cells was incubated in 9 mL of simulated saliva juice (SSJ, pH 7.4) containing 0.77 g/L of α-amylase (100 U/mg) in 0.85% NaCl (w/v) (Foongsawat et al., 2023b) at 37°C. Samples were taken at 0 and 5 minutes, and viable cell counts were determined as described in section 3.2.3.

3.4.2 Simulated gastric conditions

One gram of freeze-dried microcapsules or non-encapsulated freeze-dried cells was incubated in 9 mL of simulated gastric juice (SGJ, pH 2.0) containing 3 g/L of pepsin (1,000 U/mg) in 0.2% NaCl (w/v) (Sun & Griffiths, 2000; Foongsawat et al., 2023b) at 37°C. Samples were taken at 0, 30, 60, 90, 120, 150, and 180 minutes, and viable cell counts were determined as described in section 3.2.3.

3.4.3 Simulated Intestinal Conditions

One gram of freeze-dried microcapsules or non-encapsulated freeze-dried cells was incubated in 9 mL of simulated intestinal juice (SIJ, pH 7.4) containing 3 g/L of bile salt (Himedia, India) in an intestinal solution composed of 6.5 g/L NaCl, 0.835 g/L KCl, 0.22 g/L $CaCl_2$, and 1.386 g/L $NaHCO_3$ (Chávarri et al., 2010; Foongsawat et al., 2023b) at 37°C. Samples were taken at 0, 30, 60, 90, 120, 150, and 180 minutes, and viable cell counts were determined as described in section 3.2.3.

3.5 Statistical Analysis

Cell viability data were analyzed using one-way Analysis of Variance (ANOVA) to determine if there were significant differences in viability between groups. Following a significant ANOVA result, Tukey's Honestly Significant Difference (HSD) post-hoc test was performed to identify specific group differences. A significance level of $p < 0.05$ was used for all analyses. All statistical computations were conducted using IBM SPSS Statistics, version 28.0.1.0, IBM Corp., Armonk, NY, USA.

4. Results

4.1 Encapsulation Efficiency

Encapsulation efficiency (EE) for the three probiotic strains encapsulated with alginate, goat milk, and prebiotics ranged from 88.25% to 99.33%, as shown in Table 1. This indicates a high level of successful probiotic cell entrapment across all treatments.

Table 1 Encapsulation yield of microcapsules

Probiotic strains	Encapsulation yield (%
A. fermenti Pom1	98.90 ± 1.01
L. fermentum Pom5	88.25 ± 1.02
P. pentosaceus Chi8	99.33 ± 0.65
Cocktail 1 (Pom1 and Pom5)	94.97 ± 0.77
Cocktail 2 (Pom5 and Chi8)	99.02 ± 0.75

Note: Values are expressed as mean ± standard deviation (n = 3)

4.2 Survival of Microencapsulated LAB in Alginate–Goat Milk–β-Glucan Matrix During Storage

The encapsulation matrix significantly enhanced the survival of all three tested LAB strains, both individually and in cocktails, during freeze-drying and subsequent two-month storage (Figure 1). Encapsulated *A. fermenti* Pom1 (94.11%, 88.46%, 86.24% at 0, 1, 2 months) and *L. fermentum* Pom5 (89.74%, 83.97%, 77.60%) consistently showed significantly higher survival rates compared to their unencapsulated counterparts (*A. fermenti* Pom1: 62.82%, 59.47%, 50.14%; *L. fermentum* Pom5: 52.34%, 51.59%, 45.76%). *P. pentosaceus* Chi8, the most resilient strain, exhibited encapsulated viabilities of 98.80%, 95.56%, and 90.05%; while its unencapsulated form initially performed comparably, it saw a significant drop to 74.64% by 2 months. Similarly, encapsulated Cocktail 1 (93.84%, 84.10%, 81.58%) and Cocktail 2 (97.91%, 95.21%, 89.55%) maintained significantly higher survival rates than their free-cell counterparts.

4.3 Survival of Freeze-dried Microencapsulated Cells in Simulated Gastrointestinal Tracts

4.3.1 Viability of Freeze-dried Microencapsulated Cells under Simulated Oral Conditions

Figure 2 illustrates the viability of freeze-dried microencapsulated cells under simulated oral conditions. Initially, both encapsulated and free cells exhibited 100% viability. After a 5-minute exposure to simulated saliva, we observed a slight viability reduction in the encapsulated groups. However, this decrease was significantly more pronounced in the unencapsulated control cells throughout the 2-month storage period. Despite these reductions, all treatments maintained viabilities above 90%, indicating superior survival of the encapsulated cells.

4.3.2 Viability of Freeze-dried Microencapsulated Cells under Simulated Gastric Conditions

Figure 3 illustrates the viability of freeze-dried microencapsulated cells compared to free cells under simulated gastric conditions. Encapsulation provided robust protection, as only encapsulated cells remained viable after 60 minutes, whereas free cells were completely inactivated. After 120 minutes of exposure to these harsh conditions, *P. pentosaceus* Chi8 demonstrated the highest survival rates, maintaining 79.83%, 77.48%, and 76.23% viability at 0, 1, and 2 months, respectively. In contrast, *A. fermenti* Pom1 showed lower survival rates of 56.09%, 54.74%, and 51.67%, and *L. fermentum* Pom5 exhibited similar viabilities at 57.56%, 56.51%, and 50.58% across the same time points. However, neither *A. fermenti* Pom1 nor *L. fermentum* Pom5 survived after 150 minutes of incubation. For the cocktails, both encapsulated Cocktail 1 and Cocktail 2 survived up to 180 minutes. Cocktail 1 displayed significantly higher viability percentages (78.31%, 72.00%, and 67.63% at 0, 1, and 2 months, respectively) compared to Cocktail 2 (67.65%, 61.57%, and 55.79% at 0, 1, and 2 months). Crucially, no free cell survival was observed beyond 60 minutes, clearly demonstrating that encapsulation significantly enhanced cell viability.

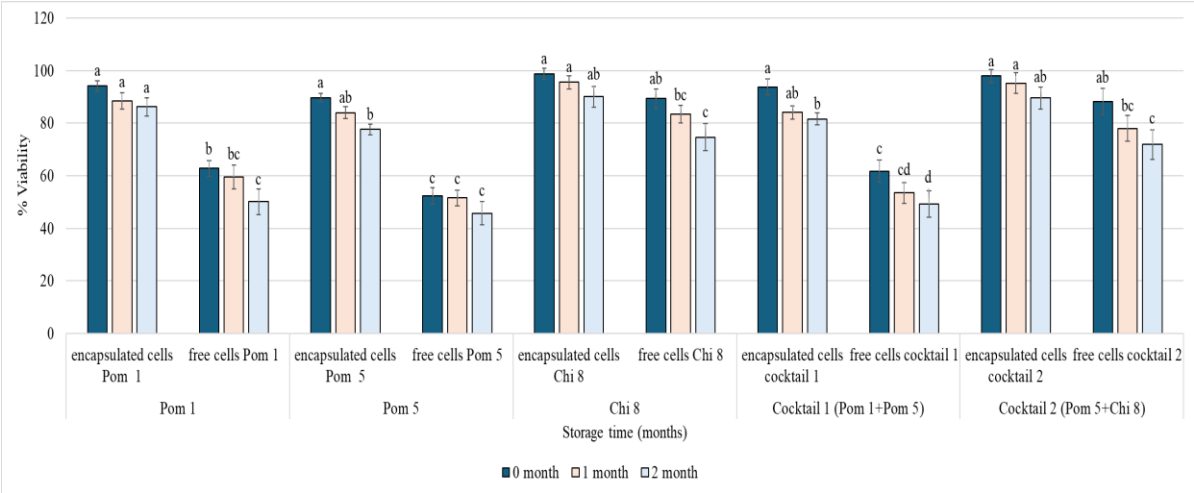

Figure 1 Viability of microencapsulated LAB cells compared with free cells after freeze-drying and during storage for 0, 1, and 2 months. Different letters above the bars indicate a significant difference (p <0.05) between storage time points within the same treatment group, as determined by Tukey's HSD analysis

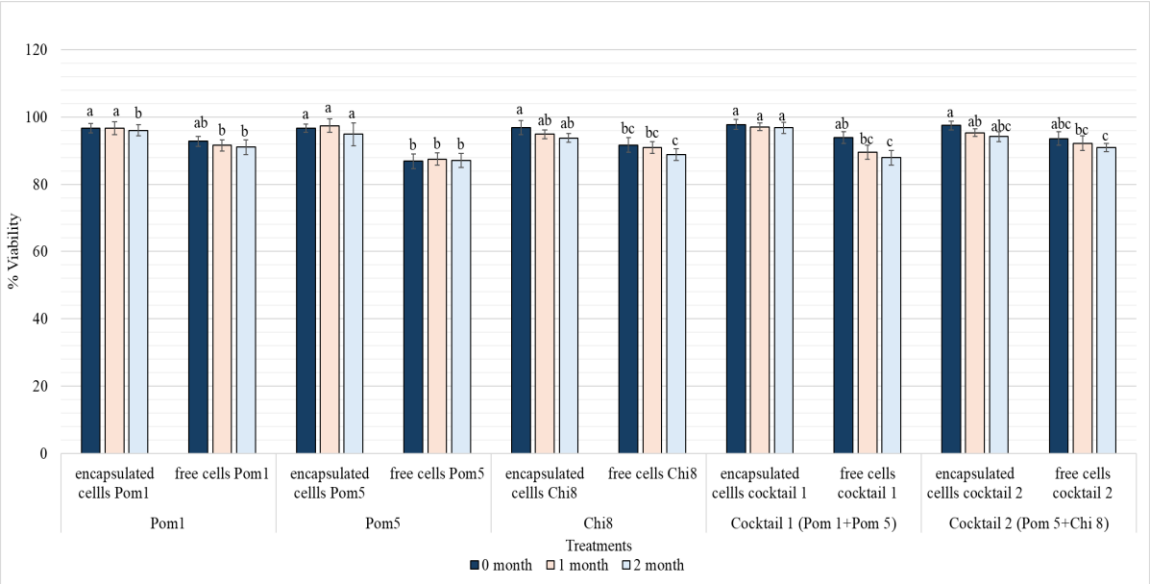

Figure 2 Viability of free and microencapsulated cells in simulated oral conditions at 5 minutes.
Different letters above the bars indicate a significant difference (p <0.05) between storage time points within the same treatment group, as determined by Tukey's HSD analysis

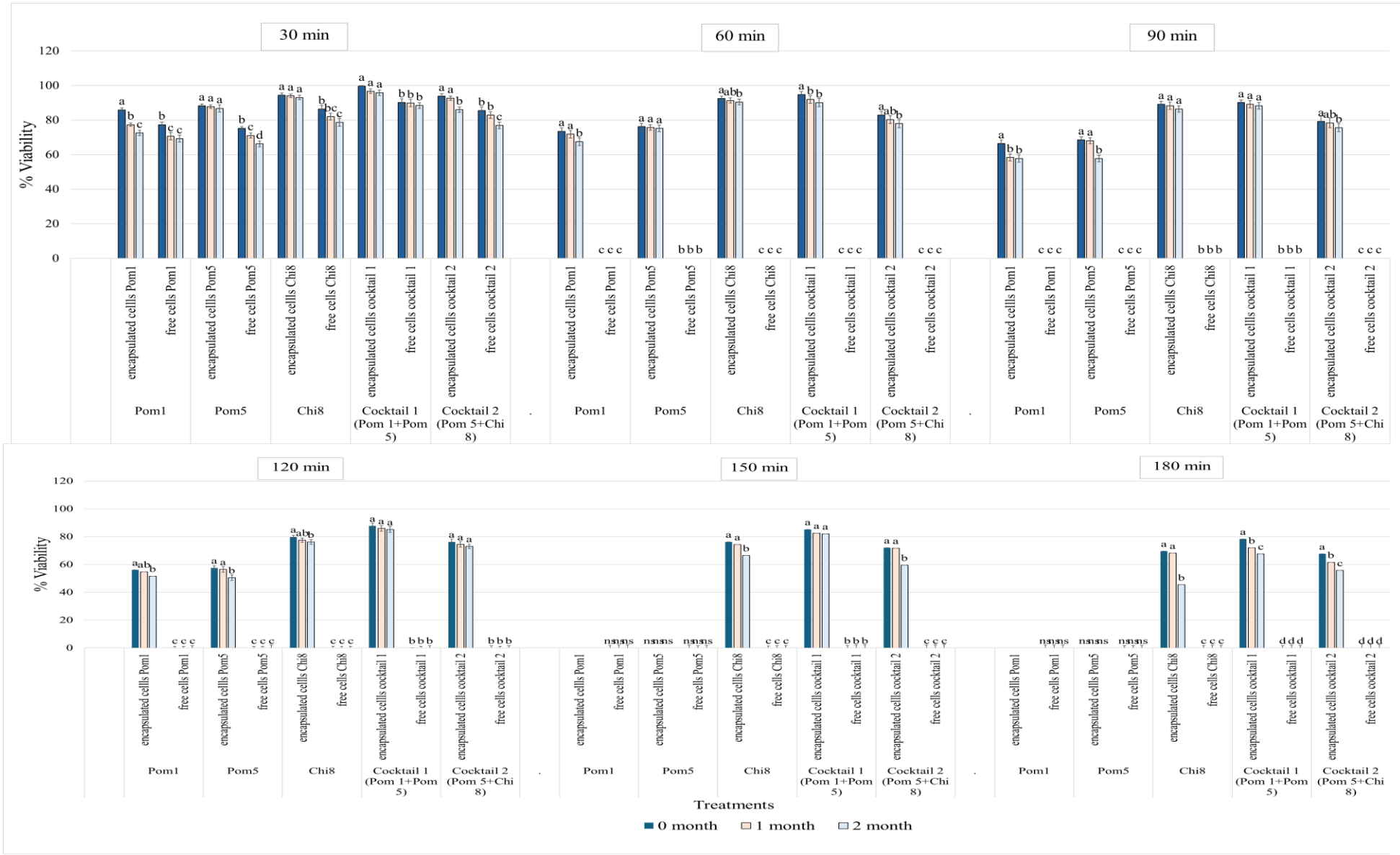

Figure 3 Viability of free and microencapsulated cells in simulated gastric conditions at 30, 60, 90, 120, 150, and 180 minutes.
Different letters above the bars indicate a significant difference (p <0.05) between storage time points within the same treatment group, as determined by Tukey's HSD analysis

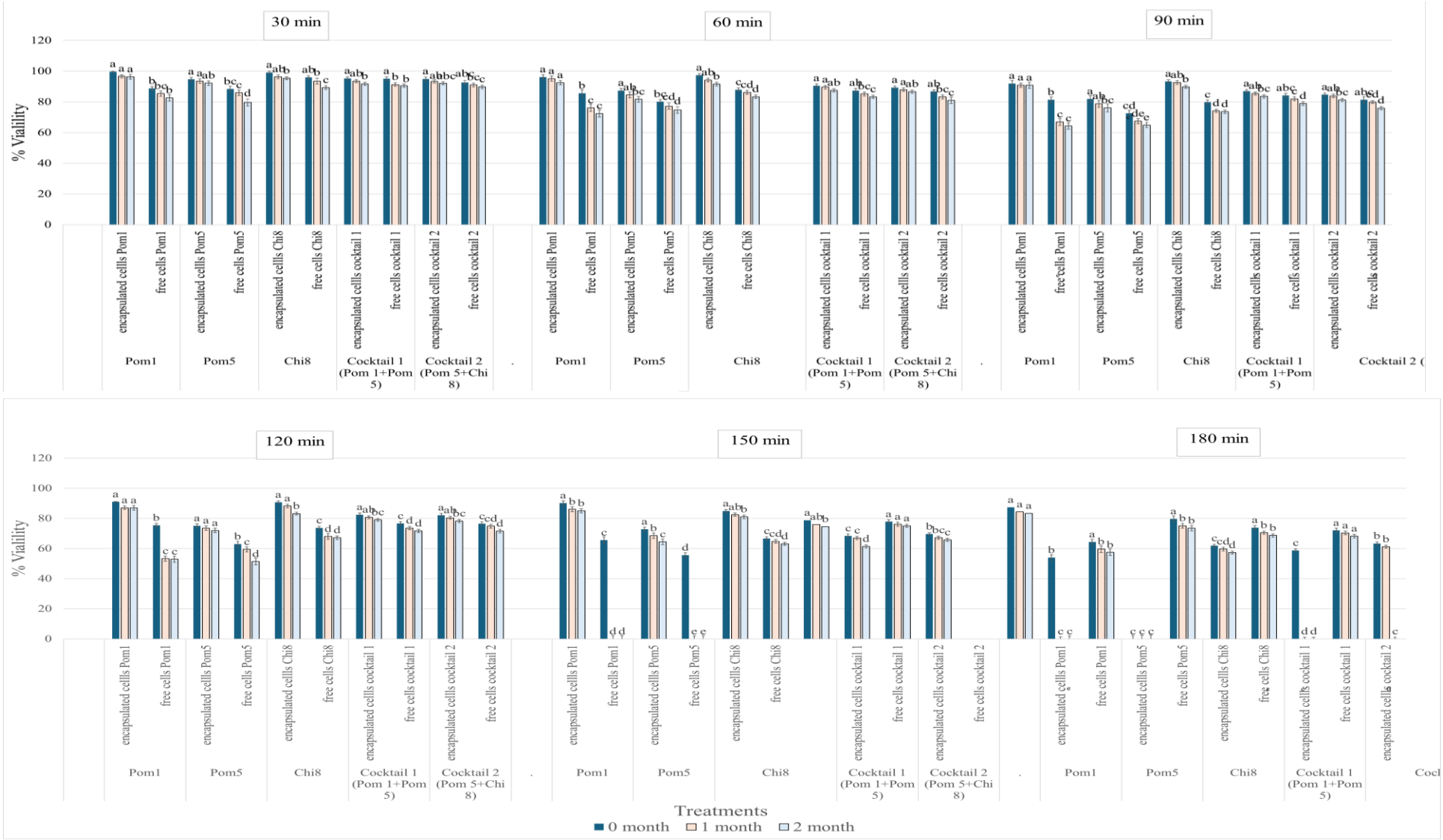

Figure 4 Viability of free and microencapsulated cells in simulated intestinal conditions at 30, 60, 90, 120, 150, and 180 minutes.
Different letters above the bars indicate a significant difference (p <0.05) between storage time points within the same treatment group, as determined by Tukey's HSD analysis

4.3.3 Viability of Freeze-dried Microencapsulated Cells under Simulated Intestinal Conditions

Figure 4 illustrates the viability of microencapsulated and free cells under simulated intestinal conditions. After 180 minutes of exposure, free cells of *A. fermenti* Pom1 and *L. fermentum* Pom5 had 0% viability, whereas *P. pentosaceus* Chi8 persisted. Encapsulated *A. fermenti* Pom1 maintained high survival rates of 87.33%, 84.34%, and 83.38% after 180 minutes at 0, 1, and 2 months of storage, respectively. This significantly outperformed its unencapsulated counterpart, which showed survival rates of 53.96%, 0.00%, and 0.00% over the same period. Following this, *P. pentosaceus* Chi8 exhibited survival rates of 79.73%, 75.10%, and 73.52% after 180 minutes at 0, 1, and 2 months of storage, respectively. *L. fermentum* Pom5 showed survival rates of 64.40%, 59.59%, and 57.47% under the same simulated intestinal conditions across the 0, 1, and 2-month storage periods. For the cocktails, both encapsulated Cocktail 1 and Cocktail 2 survived up to 180 minutes. Both cocktails displayed high viability percentages: Cocktail 1 at 73.93%, 70.50%, and 68.75% at 0, 1, and 2 months, respectively, compared to Cocktail 2 at 72.02%, 70.33%, and 68.23% at 0, 1, and 2 months. These results confirm that encapsulation effectively protects cell viability as the strains pass through the simulated gastrointestinal tract.

5. Discussion

The consistently high encapsulation efficiency (88.25% to 99.33%) across all treatments indicates that the alginate-goat milk-beta-glucan matrix is highly suitable for entrapping probiotic cells via extrusion. This aligns with previous research on similar alginate-based matrices (Foongsawat et al., 2023b), which also reported high EE values. The minor reduction in EE for *L. fermentum* Pom5 when beta-glucan was included might be due to its addition or subtle process variations, but the overall high EE confirms the efficacy of the modified matrix and the mild conditions of the extrusion method, which do not negatively impact probiotic viability (Mahmoud et al., 2020). The high EE values are also consistent with other studies using alginate-milk matrices (Prasanna & Charalampopoulos, 2019) or other polymers (Oberoi et al., 2019; Zubair et al., 2023), further validating our encapsulation approach.

The remarkable survival rates of the encapsulated LAB cells after freeze-drying and throughout two months of storage clearly demonstrate the protective effect of the alginate-goat milk-beta-glucan matrix. Immediately after freeze-drying, encapsulated cells showed significantly higher survival compared to unencapsulated controls, highlighting the matrix's instant protection against dehydration and ice crystal formation. This protective effect stems from the individual components of the matrix. Alginate effectively forms a physical barrier, shielding the cells from environmental stresses (Eckert et al., 2018). The inclusion of goat milk further enhances this protection; its milk proteins (casein, whey proteins) act as cryoprotectants, stabilizing cell membranes and enzymes during dehydration and rehydration, and providing a rich nutrient source (Prasanna & Charalampopoulos, 2019). Goat milk may also offer unique compositional benefits. Furthermore, beta-glucans, while known for their immunomodulatory properties, also serve as protective agents in the encapsulation matrix. They help stabilize the matrix structure, offering additional defense against environmental stressors and potentially acting as prebiotics (Guedes et al., 2019). The slower decline in viability observed for encapsulated bacteria during storage suggests the matrix continues to act as a barrier, likely by limiting oxygen exposure, maintaining cell integrity, and potentially offering nutritional support or buffering capacity. Variations in survival rates among the different LAB strains, with *P. pentosaceus* Chi8 consistently showing the highest viability, point to inherent differences in each strain's ability to withstand freeze-drying and storage stresses. The substantial difference in survival between encapsulated and control *A. fermenti* Pom1 particularly underscores the significant protective effect of the matrix for this specific strain. Even with this enhanced protection, the gradual decrease in viability over time within encapsulated groups suggests areas for further optimization. Many factors, such as residual moisture, storage temperature, and matrix-cell interactions, could help further improve long-term stability (Sun et al., 2023).

The simulated gastrointestinal tract experiments unequivocally confirm the enhanced survival of microencapsulated LAB cells. Even brief exposure to simulated saliva demonstrated the initial protective barrier provided by the encapsulation matrix. More critically, the alginate-goat milk-beta-glucan matrix offered robust protection against the harsh acidic conditions of the simulated stomach, preventing the complete inactivation observed in free cells. This aligns with established findings on polymer encapsulation protecting probiotics under acidic conditions (Atia

et al., 2017; Shi et al., 2013). The differential survival among individual strains in gastric conditions (*P. pentosaceus* Chi8 > *L. fermentum* Pom5 ≈ *A. fermenti* Pom1) emphasizes the importance of strain selection. A particularly compelling finding supporting the utility of multi-strain probiotic formulations is the enhanced resilience of Cocktail 1 (*A. fermenti* Pom1 and *L. fermentum* Pom5) under simulated gastric conditions. Despite the individual encapsulated strains of *A. fermenti* Pom1 and *L. fermentum* Pom5 demonstrating limited survival beyond 150 minutes of gastric exposure, their combination in Cocktail 1 extended survival to 180 minutes. This synergistic protective effect, where the combined strains exhibit greater survival than their individual components, is a strong justification for employing probiotic mixtures. Such phenomena in multi-strain probiotic formulations are often attributed to cross-feeding, where one strain produces metabolites beneficial or protective for another, or to collectively enhanced neutralization of gastric stressors. This observed synergy provides compelling evidence that the 'cocktail approach' can offer superior protective benefits, critical for efficacy during gastrointestinal transit.

Similarly, in simulated intestinal conditions, encapsulation significantly improved survival, with *P. pentosaceus* Chi8 again demonstrating superior resilience. The generally good survival of all encapsulated strains in the intestinal environment, even after two months of storage, indicates the stability of the formulations. The physical barrier created by alginate's gel-forming properties is crucial for protecting cells from enzymatic degradation, bile salt toxicity, and osmotic stress in the small intestine (Chaudhari et al., 2015). The slight decrease in viability over storage observed in both gastric and intestinal conditions further supports the need to optimize storage parameters to maximize long-term stability.

While this study primarily focuses on enhancing probiotic viability through encapsulation, it is important to acknowledge another crucial characteristic for probiotic efficacy: their ability to adhere to the intestinal mucosa. The immobilization of cells within a gel matrix, as employed in this encapsulation technique, raises pertinent questions regarding the subsequent release of viable cells and their capacity for effective adhesion post-release. However, it has been suggested that the encapsulated structure itself, or the materials used in encapsulation, may even enhance the adhesion properties of probiotics to the intestinal epithelium compared to free cells, by providing a larger surface area or by interacting favorably with mucin (Cook et al., 2011). Furthermore, encapsulation provides an additional protective layer for the probiotics, shielding them from various digestive enzymes present in the gut lumen, which could otherwise degrade cell surface components critical for adhesion (Ding & Shah, 2009). Although direct adhesion assays were beyond the scope of the current investigation, the sustained high viability of encapsulated cells after passage through simulated gastrointestinal conditions implies that a significant population of live cells would be available for interaction with the host intestinal environment. Upon reaching the intestine, the integrity of the alginate matrix can be affected by the physiological conditions, such as the presence of calcium-chelating agents or higher pH, potentially leading to a controlled release of encapsulated cells.

Overall, this study confirms that the alginate-goat milk-beta-glucan matrix effectively enhances the encapsulation efficiency and the survival of *A. fermenti* Pom1, *L. fermentum* Pom5, and *P. pentosaceus* Chi8 during freeze-drying, storage, and passage through simulated gastrointestinal conditions. This encapsulation technique holds significant potential for developing stable and effective probiotic formulations.

6. Conclusion

This study successfully demonstrated the significant advantages of microencapsulating probiotic strains (*A. fermenti* Pom1, *L. fermentum* Pom5, and *P. pentosaceus* Chi8) and their combinations within an alginate, goat milk, and prebiotic matrix. High encapsulation efficiencies, ranging from 88.25% to 99.33%, were consistently achieved, indicating excellent cell entrapment. Crucially, encapsulation significantly enhanced the survival of all probiotic strains and cocktails during freeze-drying and subsequent two-month storage, maintaining considerably higher viability compared to unencapsulated cells. Furthermore, this protective effect extended to simulated gastrointestinal conditions. While all cells initially showed high viability in oral conditions, microencapsulated probiotics exhibited superior resilience to harsh gastric environments, with free cells being completely inactivated after 60 minutes, whereas encapsulated cells remained viable for extended periods. Similarly, encapsulated cells maintained high viability under simulated intestinal conditions, far surpassing that of free cells. In summary, microencapsulation using the developed alginate, goat milk, and prebiotic matrix is a highly effective strategy for improving the stability

and viability of these probiotic strains during processing, storage, and transit through the canine gastrointestinal tract. This technology holds great promise for developing more robust and effective probiotic products.

7. Acknowledgements

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8. CRediT Statement

Onanong Pringsulaka: Conceptualization, methodology, writing original draft, writing review & editing, supervision, project administration, funding acquisition.
Kanokwan Thawornwiriyanan: Methodology, validation, formal analysis, investigation, data curation.
Chanoknan Tulathon: Investigation, data curation.
Sirinthorn Sunthornthummas: Methodology, visualization.
Siriruk Sarawaneeyaruk: Formal analysis.
Achariya Rangsiruji: Conceptualization, resources.
Natthida Sudyoung: Formal analysis, investigation.
Chatrudee Suwannachart: Investigation, resources.

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