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In vitro Survival of Microencapsulated Canine-Specific Probiotics Under Simulated Gastrointestinal Tract Conditions and During Storage

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Abstract

The survival of probiotics in the gastrointestinal system of dogs is crucial for them to provide health benefits. However, probiotics must also endure various physical conditions during commercial production and storage. Therefore, this study employed the microencapsulation technique to ensure the survival of probiotics using alginate as the encapsulation material, both alone and in combination with goat milk (alginate-goat milk). The study assessed the survival rates of two probiotic LAB strains, *Enterococcus hirae* Pom 4 and *Ligilactobacillus animalis* FB2, in both types of matrices under simulated dog gastrointestinal conditions, during food production, and 28 days of refrigeration at 4°C. The findings revealed that alginate-goat milk microcapsules had the highest encapsulation yield, and the viability of microencapsulated LAB cells in the alginate-goat milk matrix was the best protection for both probiotic strains under all conditions, including pasteurization temperature. Even after pasteurization, viable counts exceeding 6 log cfu/g were observed, indicating the promising application of alginate-goat milk microcapsules for optimal protection, enabling probiotics to survive until they reach the intended site and provide health benefits to dogs.

Keywords: *Enterococcus hirae*; *Ligilactobacillus animalis*; *probiotics*; *dogs*; *microencapsulation*; *goat milk*

1. Introduction

Probiotics are living microorganisms that, when consumed in sufficient quantities, can help promote the host's body health (FAO/WHO, 2002). At present, probiotics are commonly used as a dietary supplement for both humans and animals. Using probiotic bacteria in dogs or other pets not only helps maintain the balance of gut microbiota but also increases beneficial bacteria for dogs, resulting in good health and a longer lifespan (Lee et al., 2022). However, in order to efficiently promote the health of hosts, it is crucial that a significant quantity of them reaches their target area, such as the host's intestines. Moreover, probiotic cells must be able to survive in various unsuitable conditions during the production process, including the harsh conditions of the host's gastrointestinal system (Bhat et al., 2013; Moumita et al., 2017). Therefore, there is a need to explore methods that can help protect and increase the

survival rate of probiotic cells when passing through such unsuitable conditions. Micro-encapsulation is one such option that is used to protect probiotic cells from unsuitable conditions in the gastrointestinal system, during the production process, and during storage of products (Vivek et al., 2023). The microencapsulation process involves coating probiotic cells with a matrix or substance that can shield them from unfavorable environmental conditions. This process results in the formation of microcapsules that can control the release of probiotic cells in the required conditions.

The microencapsulation method used to encapsulate probiotic cells can be classified into four main types: extrusion, emulsion, spray drying, and freeze drying (Rajam &Subramanian, 2022). The extrusion method is a simple, low-cost, and mild encapsulation technique that does not significantly impact the viability of probiotic cells. Therefore, it is widely used for encapsulating probiotic cells, although the microcapsules produced by this method are relatively large, ranging from 0.5-3 millimeters in size. Hydrocolloids such as polysaccharides and proteins can be used as biopolymers for microcapsule preparation, which are stable, non-toxic, and biodegradable, without the use of organic solvents. Suitable polymers for encapsulation include alginate, chitosan, gellan gum, xanthan gum, casein, and whey protein (Zabot et al., 2022).

Alginate, a commonly used polymer for encapsulating bioactive cells, is typically calcium alginate with a concentration of 0.5-4% (w/v), as it is non-toxic, Generally Recognized as Safe (GRAS), compatible with bacterial cells, cost-effective, forms a mild gelation state, and can release encapsulated cells in the gastrointestinal tract. However, alginate microcapsules are sensitive to highly acidic environments, such as those found in the host's stomach, and the surface of the alginate microcapsules often contains pores or cracks, compromising the protection of probiotic cells from unsuitable environments (Heidebach, Först & Kulozik, 2012; Burgain et al., 2011). Consequently, researchers have studied other polymers in combination with alginate to reduce these drawbacks, such as alginate in conjunction with chitosan, zein, gum arabic, cellulose, starch, whey protein, gelatin, and pectin (Razavi et al., 2021). Additionally, researchers have investigated alginate-milk-based matrices, which can provide better protection for probiotic cells due to the milk proteins' ability to form a stronger barrier against harsh environments (Prasanna & Charalampopoulos, 2018).

Milk and milk proteins have good biological compatibility with bioactive cells, high buffering capacity, and a structure that can protect cells well during the gastrointestinal process and in the gastrointestinal tract. Milk protein is one of the interesting options for use as an encapsulating agent for probiotic cells together with alginate to improve the structural characteristics of alginate (Prasanna & Charalampopoulos, 2018; Mahmoud et al., 2020). The utilization of milk proteins as encapsulating agents in microcapsules can enhance the viability of probiotic cells in the gastrointestinal tract, as demonstrated by Burgain et al. (2014). When comparing different types of milk, it is advisable for dogs and pets to consume goat milk due to its lower lactose content and improved digestibility compared to cow's milk. Goat milk typically contains around 4.2-4.8% lactose, whereas cow's milk contains 4.7-5.0% lactose (Silanikove et al., 2015). Additionally, goat milk offers the benefits of prebiotics and probiotics, which can effectively supplement the health of the dogs. Considering these factors, this study aimed to assess the survival of both free cells and cells encapsulated in sodium alginate and sodium alginate-goat milk microcapsules. The evaluation was conducted in a simulated dog gastrointestinal tract, as well as during storage at 4°C for 28 days and under pasteurization temperature.

2. Objectives

The objectives of the study were to investigate the efficiency of encapsulating bacterial cells with probiotic properties using the extrusion method in the form of an alginate matrix and a matrix of alginate combined with goat milk. Additionally, the study aimed to investigate the survival of both free cells and cells encapsulated in microcapsules in a simulated dog gastrointestinal tract, during storage at 4°C for 28 days, and under pasteurization temperature.

3. Materials and methods

3.1 Identification of LAB strains

The LAB isolates Pom4 and FB2 were obtained from dog feces and characterized as probiotics (unpublished data). Identification of the isolates was performed by amplification of 16S rDNA using a standard PCR protocol with universal primers, following the method described by

Pringsulaka et al. (2011). DNA extraction was carried out using the method described by Sambrook et al. (1989), and the extracted DNA was used as a template for PCR amplification of the 16S rDNA region with the primers 27F (5' AGAGTTT GATC(A/C)TGGCTCAG 3') and 1492R (5'TACGG (C/T)TACCTTGTTACGAC TT 3') (Lane, 1991). PCR amplification was conducted using a Thermal Cycler Gradient TC1000-S (Scilogex, USA; Wongyoo et al., 2023). The amplified products were analyzed by 0.6% (w/v) agarose gel electrophoresis and visualized under UV light via ethidium bromide staining (1 mg/mL). The 16S rDNA fragments were purified using a gel extraction kit (MinElute Gel Extraction Kit; Qiagen, USA) and sequenced. BLAST analysis was performed on GenBank to determine the similarity of the sequences. The purified cultures were maintained in MRS broth and stored in glycerol at -20°C. For bacterial cultivation, the bacteria were cultured in MRS broth at 37°C for 48 hours in a candle jar to create microaerophilic conditions.

3.2 Preparation of probiotic bacterial cells for encapsulation

LAB cells were cultured in MRS broth at 37°C for 24 h. The cells were then centrifuged at 10,000 × *g* for 5 min and washed with 0.85% NaCl solution three times. The washed cells were resuspended in 0.85% NaCl solution and the absorbance was measured at OD600 = 1 for further testing in the next step.

3.3 Encapsulation of probiotic cells with alginate

The method of encapsulating probiotic cells with alginate using the extrusion method followed the protocol described by Prasanna & Charalampopoulos (2018). A 2% (w/v) solution of sodium alginate was prepared in distilled water, sterilized by autoclaving at 121°C for 20 minutes, and then mixed with concentrated probiotic bacterial cells at a ratio of 4:1 (sodium alginate: concentrated probiotic bacterial cells, v/v). The probiotic cells were prepared according to Section 3.2. To prepare the microcapsules, the cell suspension was dripped through a 21G syringe needle into a 0.5 M calcium chloride solution while gently stirring continuously. The dripping height was 10 cm, and the process continued for 30 min to allow the microcapsules to solidify. Afterward, the microcapsules were washed with a 0.85% (w/v) NaCl solution before being stored in sterilized tubes for further experiments.

3.4 Encapsulation of probiotic cells with alginate and goat milk

The method of Prasanna & Charalampopoulos (2018) was used to encapsulate probiotic bacteria with alginate and goat milk. To prepare the sodium alginate solution, 2% (w/v) sodium alginate was dissolved in filtered water and then sterilized by autoclaving at 121°C for 20 min. Sterile goat milk was then added to the sodium alginate solution in a ratio of 2:1 (sodium alginate: goat milk, v/v). The concentrated probiotic bacterial cells were then added at a ratio of 4:1 (sodium alginate in goat milk: concentrated probiotic bacterial cells, v/v). The microcapsules containing the probiotic cells were prepared according to the previously described method.

3.5 Determination of viable free and encapsulated probiotic cells

To determine the number of viable probiotic cells, both free and encapsulated cells were enumerated. For free cells, a 10-fold dilution was prepared in 0.85% (w/v) NaCl until an appropriate concentration was obtained, and the cells were counted using the spread plate method on MRS agar supplemented with 0.3% (w/v) calcium carbonate. The plates were incubated under microaerophilic conditions at 37°C for 48 h, and the viable cells were reported as cfu/mL. For encapsulated cells, the microcapsules were first weighed and then diluted in a 50 mM sodium citrate solution at pH 7.5. The microcapsules, which were in the form of calcium alginate, were converted to the form of sodium alginate that can be dissolved. The diluted microcapsules were then serially diluted and spread plated onto MRS agar supplemented with 0.3% (w/v) calcium carbonate. The plates were incubated under microaerophilic conditions at 37°C for 48 h, and the viable cells were reported as cfu/g.

3.6 Determination of encapsulation yield and size of prepared microcapsules

The encapsulating of the probiotics using each type of coating material can be determined by dissolving the microcapsules and counting them as previously described. Afterwards, the encapsulation yield can be calculated using the following equation:

Encapsulation yield (%) =

$$\frac{\text{Number of cells released from microcapsules}}{\text{Number of free cells before encapsulation}} \times 100$$

The size of the prepared microcapsules was determined by randomly selecting 30 microcapsules of each type and measuring their size using a Vernier caliper. The average size was then calculated.

3.7 Determination of the survival of free cells and microencapsulated cells in a simulated gastrointestinal system of dogs

To determine the survival of free cells and microencapsulated cells in a simulated oral condition, samples of sodium alginate and sodium alginate- goat milk microcapsules were weighed, or a free cell sample was placed in a simulated saliva juice (SSJ) with a pH value of 7.4, containing 0.77 g of 100 U α-amylase in 0.85% NaCl (w/v) (Bao et al., 2010). The samples were incubated in a water bath at 37°C for 0 and 5 min and collected. The surviving probiotic cells were counted as described in section 3.5.

To determine the survival of free cells and microencapsulated cells in simulated gastric conditions, microcapsules or free cells were placed in a simulated gastric juice (SGJ) with a pH value of 2.0, containing 3 g of pepsin in 0.2% NaCl (w/v) (Sun & Griffiths, 2000). The samples were incubated in a water bath at 37°C and collected after 0, 30, 60, 90, 120, 150, and 180 min. The surviving probiotic cells were counted as described in section 3.5.

To determine the survival of free cells and microencapsulated cells in simulated intestinal conditions, microcapsules or free cells were placed in a simulated intestinal juice (SIJ) containing 3 g of bile salt (Himedia, India) in 1,000 mL of intestinal solution containing 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). The samples were incubated in a water bath at 37°C and collected after 0, 30, 60, 90, 120, 150, and 180 min. The surviving probiotic cells were counted as described in section 3.5.

3.8 Determination of the survival of free cells and microencapsulated cells during refrigeration

Microcapsules containing 1 g of both alginate and alginate- goat milk, or 1 mL of free cells, were placed in sterile tubes and stored at 4°C for 28 days. For goat milk, 10 mL of sterilized goat milk was added to 1 g of microencapsulated cells or 1 mL of free cells in tubes, and then stored at 4°C for 28 days. Samples were taken on days 0, 7, 14, 21, and 28, and the surviving probiotic cells were enumerated using the method described in section 3.5.

3.9 Determination of the survival of free cells and microencapsulated cells during the heating process at pasteurization temperature

Microcapsules containing 1 g of both alginate and alginate- goat milk, or 1 mL of free cells, were placed in sterile goat milk and incubated at 63°C for 30 min and 72°C for 15 s in a water bath. Samples were rapidly cooled to approximately 20°C, and the surviving probiotic cells were enumerated using the method described in section 3.5.

3.10 Statistical analysis

All tests were performed in triplicate. The results of microcapsule size and encapsulation yield were analyzed using Student's t-test. Viable cell counts obtained from studies on simulated gastrointestinal conditions, storage, and pasteurization temperature were analyzed using one-way analysis of variance (ANOVA) with Tukey's multiple comparison tests in Alginate (version 9.2, Alginate S Institute Inc., Cary, NC, USA).

4. Results

4.1 Identification of LAB strains

Based on the identification of 16S rRNA genes, Pom4 belongs to *E. hirae* with a similarity of 99% (1,422/1,427 bp), and FB2 belongs to *Lig. animalis* with a similarity of 99% (1,382/1,383 bp). Additionally, the construction of a phylogenetic tree using the Maximum Likelihood (ML) method confirmed these assignments, with Pom4 belonging to the *E. hirae* cluster and FB2 belonging to the *Lig. animalis* cluster. The bootstrap values obtained for these assignments were 85% and 100% respectively (Figure 1).

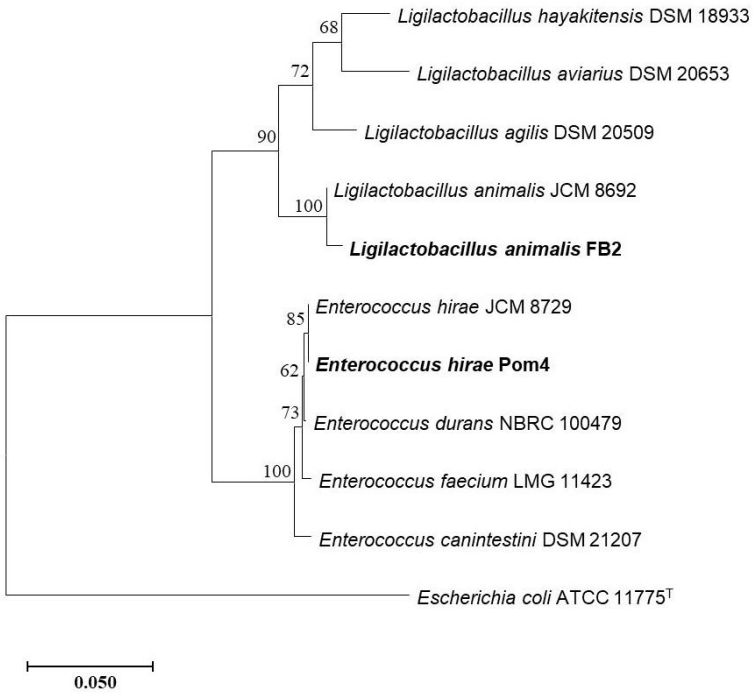

Figure 1 The phylogenetic tree 2 isolates, Pom 4 and FB 2, based on their 16S rRNA genes using the Maximum Likelihood (ML) method. The nodes display the bootstrap values (%) from 1,000 replicates, and the scale bar represents the substitution rate per nucleotide position.

Table 1 Encapsulation yield and size of microcapsules

Probiotic strains	Size (mm)		Encapsulation yield (%)	
	Alginate	Alginate- goat milk	Alginate	Alginate- goat milk
E. hirae Pom4	2.20 ± 0.04^{B}	2.26 ± 0.01^{A}	93.35 ± 0.98^{A}	93.64 ± 1.14^{A}
Lig. animalis FB2	2.23 ± 0.02^{B}	2.27 ± 0.01^{A}	95.13 ± 0.84^{B}	96.01 ± 0.16^{A}

Note: The results of the experiment were reported as the mean ± SD from three repetitions. Capital letters were used to indicate significant differences in size and encapsulation yield between the different types of encapsulating materials (rows), with statistical significance determined by Student's t-test at a significance level of $p \leq 0.05$.

4.2 Determination of encapsulation efficiency and size of microcapsules

Alginate and alginate-goat milk matrices were used to encapsulate two probiotic strains, *E. hirae* Pom 4 and *Lig. animalis* FB2, with an encapsulation yield ranging from 93.35% to 96.01%. The microcapsules had a size ranging between 2.20 mm and 2.27 mm, as measured using a vernier caliper (Table 1). Alginate-goat milk microcapsules encapsulating *Lig. animalis* FB2 had the highest encapsulation yield, suggesting that the extrusion method used mild conditions during the encapsulation process that did not significantly affect the cell viability of the probiotic cells. This method is commonly used in combination with a hydrocolloid solution (Krasaekoopt, Bhandari & Deeth, 2003). Significant differences in size were observed between alginate and alginate- goat milk microcapsules ($p \leq 0.05$). Furthermore, significant differences in encapsulation yield were observed between *Lig. animalis* FB2 microcapsules encapsulated in alginate and alginate-goat milk matrices, which is consistent with a study by Prasanna & Charalampopoulos (2018) on *Bifidobacterium longum* encapsulation using alginate alone, alginate with casein hydrolysate, alginate with cow's milk, and alginate-goat milk. The use of alginate with cow's milk and goat milk resulted in larger microcapsule sizes than using alginate with casein hydrolysate and alginate alone,

possibly due to the higher protein content in cow's milk and goat milk that contributes to the larger size of microcapsules.

4.3 Determination of the survival of free cells and microencapsulated cells in a simulated gastrointestinal system of dogs

A study conducted a comparison of the survival rate of two probiotic strains, one encapsulated using alginate and alginate- goat milk and the other being free cells, in a simulated SSJ. The results indicated that there was no statistically significant difference between the survival rates of the two strains. Both the encapsulated and free probiotic strains had a survival rate of more than 95% after being exposed to the SSJ for 5 min (Table 2).

After exposing both encapsulated probiotic strains and free cells to a simulated SGJ, it was observed that free cells had the lowest survival rate, which was less than 6 log cfu/mL - the minimum number of probiotics required in the product to provide health benefits to the host (Kechagia et al., 2013). In comparison, encapsulated probiotic cells with alginate and alginate- goat milk had a higher survival rate, especially those coated with alginate-goat milk. The study found that *E. hirae* Pom4 in alginate-goat milk had a higher survival rate of 89.02% than *Lig. animalis* FB2, which was 84.97% (Table 3). The simulated gastric fluid condition had a low pH and contained pepsin enzymes, which impacted the survival of probiotic cells, whether encapsulated or not, after 180 min of exposure. This is consistent with the findings of Dikit & Maneerat (2015), which reported that the low pH of the simulated gastric fluid condition affected the survival of *L. plantarum* D6SM3 cells, whether encapsulated or not. Additionally, there are reports that encapsulating *L. plantarum* D6SM3 cells with alginate resulted in higher survival rates compared to non-encapsulated cells. Furthermore, encapsulating probiotic cells with alginate combined with goat milk provided the highest protection, which may be due to the high buffering capacity of goat milk that helps protect probiotic cells (Prasanna & Charalampopoulos, 2018; Krasaekoopt et al., 2003; Guérin, Vuillemard & Subirade, 2003).

Similarly, in a simulated SIJ, the study compared the survival of encapsulated cells with alginate and alginate- goat milk to free cells for 180 min. It was found that the free cells of both strains had the lowest survival rate compared to encapsulated cells. Encapsulating probiotic cells with alginate goat milk had a higher survival rate than alginate alone (Table 4). The study concluded that probiotic cells encapsulated with alginate and alginate- goat milk had a higher survival rate than free cells in simulated SGJ and SIJ.

Table 2 Survival of free and microencapsulated probiotic cells in a simulated saliva fluid after exposure to 37°C for 5 min

LAB strains	Encapsulation material	Incubation period (min)[1]		
		0	5	Survival rate (%)[2]
E. hirae Pom4	Alginate	8.96±0.08^{A}	8.84±0.08^{A}	97.07^{A}
	Alginate-goat milk	9.10±0.28^{A}	9.05±0.08^{A}	97.97^{A}
	Free cells	9.09±0.04^{A}	8.95±0.03^{A}	97.48^{A}
Lig. animalis FB2	Alginate	8.96±0.13^{A}	8.88±0.27^{A}	97.98^{A}
	Alginate-goat milk	9.00±0.08^{A}	8.79±0.24^{A}	95.83^{A}
	Free cells	9.01±0.23^{A}	8.68±0.02^{A}	95.57^{A}

Note: The results of the experiment are presented as the mean ± SD of three replicates. Capital letters indicate significant differences within each time point (column) among the different matrices, as determined by Tukey's test with a significance level of $p \leq 0.05$.

[1]Log cfu/g for microencapsulated cells or log cfu/mL for free cells.

[2]Determined by dividing the final viable cells (cfu/g or cfu/mL) by the original viable cells (cfu/g or cfu/mL)

Table 3 Survival of free and microencapsulated probiotic cells in a simulated gastric juice (pH 2) at 37°C for 180 min

LAB strains	Encapsulation material	Incubation period (min)[1]							
		0	30	60	90	120	150	180	Survival rate (%)[2]
E. hirae Pom4	Alginate	8.31±0.08^{B}	8.13±0.10^{A}	7.63±0.17^{B}	7.24±0.08^{B}	7.17±0.05^{B}	7.11±0.05^{B}	7.01±0.07^{B}	84.33^{B}
	Alginate-goat milk	8.22±0.08^{B}	8.16±0.10^{A}	8.13±0.10^{A}	8.10±0.07^{A}	8.02±0.05^{A}	7.82±0.05^{A}	7.32±0.07^{A}	89.02^{A}
	Free cells	8.81±0.06^{A}	7.91±0.01^{B}	7.07±0.08^{C}	6.84±0.10^{C}	6.54±0.05^{C}	6.02±0.10^{C}	5.43±0.04^{C}	61.70^{C}
Lig. animalis FB2	Alginate	8.21±0.04^{C}	8.02±0.06^{B}	7.88±0.07^{A}	7.45±0.04^{A}	7.18±0.06^{B}	7.22±0.07^{B}	7.08±0.05^{B}	86.22^{A}
	Alginate-goat milk	8.77±0.06^{B}	8.49±0.05^{A}	8.15±0.04^{A}	8.02±0.07^{A}	8.03±0.05^{A}	7.53±0.07^{A}	7.45±0.04^{A}	84.97^{A}
	Free cells	8.79±0.06^{A}	7.47±0.02^{C}	6.73±0.08^{B}	6.06±0.08^{B}	5.46±0.04^{C}	5.44±0.17^{C}	5.15±0.04^{C}	58.57^{B}

Note: The results of the experiment are presented as the mean ± SD of three replicates. Capital letters indicate significant differences within each time point (column) among the different matrices, as determined by Tukey's test with a significance level of $p \leq 0.05$.
[1]Log cfu/g for microencapsulated cells or log cfu/mL for free cells.
[2]Determined by dividing the final viable cells (cfu/g or cfu/mL) by the original viable cells (cfu/g or cfu/mL).

Table 4 Survival of free and microencapsulated probiotic cells in a simulated intestinal juice (pH 7.4) at 37°C for 180 min

LAB strains	Encapsulation material	Incubation time (min)[1]							
		0	30	60	90	120	150	180	Survival rate (%)[2]
E. hirae Pom4	Alginate	8.46±0.05^{B}	8.29±0.04^{C}	7.86±0.06^{B}	7.63±0.08^{B}	7.61±0.03^{C}	7.24±0.10^{C}	7.01±0.08^{B}	82.91^{B}
	Alginate-goat milk	8.53±0.03^{B}	8.49±0.05^{B}	8.37±0.05^{A}	8.22±0.06^{A}	8.28±0.03^{A}	7.96±0.05^{A}	7.93±0.06^{A}	92.96^{A}
	Free cells	8.89±0.02^{A}	8.63±0.04^{A}	8.40±0.04^{A}	8.09±0.04^{A}	7.88±0.16^{B}	7.47±0.10^{B}	6.4±0.14^{C}	78.06^{C}
Lig. animalis FB2	Alginate	8.10±0.04^{A}	8.21±0.06^{B}	8.00±0.02^{C}	7.97±0.03^{B}	7.92±0.02^{B}	7.68±0.15^{B}	7.68±0.02^{B}	84.26^{A}
	Alginate-goat milk	8.68±0.05^{A}	8.65±0.08^{A}	8.55±0.04^{A}	8.22±0.10^{A}	8.26±0.05^{A}	8.14±0.07^{A}	7.99±0.13^{A}	94.81^{A}
	Free cells	8.56±0.4^{A}	8.60±0.02^{A}	8.36±0.04^{B}	7.99±0.11^{B}	7.82±0.06^{B}	7.33±0.10^{C}	7.21±0.02^{C}	79.86^{B}

Note: The results of the experiment are presented as the mean ± SD of three replicates. Capital letters indicate significant differences within each time point (column) among the different matrices, as determined by Tukey's test with a significance level of $p \leq 0.05$.
[1]Log cfu/g for microencapsulated cells or log cfu/mL for free cells.
[2]Determined by dividing the final viable cells (cfu/g or cfu/mL) by the original viable cells (cfu/g or cfu/mL).

Table 5 Survival of free and microencapsulated probiotic cells during refrigerated storage (4°C) for 28 days.

LAB strains	Encapsulation material	Days of storage[1]					
		0	7	14	21	28	Survival rate (%)[2]
E. hirae Pom4	Alginate	8.99±0.15^{A}	8.35±0.50^{A}	7.75±0.13^{B}	7.44±0.20^{B}	7.57±0.23^{B}	79.84^{B}
	Alginate-goat milk	9.17±0.26^{A}	8.70±0.14^{A}	8.57±0.12^{A}	8.23±0.18^{A}	7.89±0.26^{A}	86.06^{A}
	Free cells	9.30±0.17^{A}	8.70±0.12^{A}	8.02±0.30^{B}	8.29±0.27^{A}	6.90±0.23^{B}	74.24^{B}
Lig. animalis FB2	Alginate	9.06±0.20^{A}	8.92±0.03^{A}	7.98±0.02^{A}	7.57±0.07^{B}	6.25±0.04^{B}	69.05^{B}
	Alginate-goat milk	9.08±0.07^{A}	9.03±0.05^{A}	7.84±0.04^{A}	7.92±0.10^{A}	7.92±0.04^{A}	87.21^{A}
	Free cells	9.07±0.32^{A}	8.12±0.09^{B}	6.96±0.30^{B}	6.83±0.23^{C}	6.58±0.20^{B}	72.61^{B}

Note: The results of the experiment are presented as the mean ± SD of three replicates. Capital letters indicate significant differences within each time point (column) among the different matrices, as determined by Tukey's test with a significance level of $p \leq 0.05$.
[1]Log cfu/g for microencapsulated cells or log cfu/mL for free cells.
[2]Determined by dividing the final viable cells (cfu/g or cfu/mL) by the original viable cells (cfu/g or cfu/mL).

Table 6 Determination of the survival of free cells and microencapsulated cells in goat milk at 4°C for 28 days.

LAB strains	Encapsulation material	Days of storage[1]					
		0	7	14	21	28	Survival rate (%)[2]
***E. hirae* Pom4**	Alginate	8.87±0.20^{A}	8.32±0.53^{A}	8.15±0.62^{A}	8.07±0.19^{A}	7.80±0.54^{A}	89.15^{A}
	Alginate-goat milk	8.78±0.15^{A}	8.34±0.51^{A}	8.24±0.60^{A}	8.12±0.33^{A}	7.92±0.42^{A}	90.53^{A}
	Free cells	8.75±0.23^{A}	8.48±0.20^{A}	8.30±0.28^{A}	7.70±0.28^{A}	7.48±0.24^{A}	85.45^{A}
***Lig. animalis* FB2**	Alginate	9.03±0.38^{A}	9.21±0.49^{A}	8.80±0.28^{A}	8.77±0.40^{A}	8.56±0.26^{A}	95.10^{A}
	Alginate-goat milk	9.21±0.38^{A}	8.90±0.15^{A}	8.89±0.08^{A}	8.95±0.51^{A}	8.59±0.36^{A}	93.24^{AB}
	Free cells	9.73±0.23^{A}	9.43±0.13^{A}	8.34±0.11^{A}	8.62±0.29^{A}	8.45±0.04^{A}	86.84^{B}

Note: The results of the experiment are presented as the mean ± SD of three replicates. Capital letters indicate significant differences within each time point (column) among the different matrices, as determined by Tukey's test with a significance level of $p \leq 0.05$.
[1]Log cfu/g for microencapsulated cells or log cfu/mL for free cells.
[2]Determined by dividing the final viable cells (cfu/g or cfu/mL) by the original viable cells (cfu/g or cfu/mL).

4.4 Determination of the survival of free cells and microencapsulated cells during refrigeration

Table 5 presents the survival rates of both free cells and microencapsulated cells during storage at 4°C for 28 days. The results indicate that cells encapsulated with alginate alone and free cells of *E. hirae* Pom4 and *Lig. animalis* FB2 had survival rates of 74.24% and 79.84%, and 72.61% and 69.05%, respectively. In contrast, encapsulated probiotic cells combined with goat milk had the highest survival rates of 86.06% and 87.21% for the two strains, respectively. The results indicate that both free and microencapsulated forms of the strains can survive when stored in a refrigerator. However, it is worth noting that the free cells exhibited relatively lower survival rates compared to the encapsulated cells combined with goat milk. Despite this, the ability of the free cells to survive under refrigeration conditions suggests that they may possess inherent characteristics that enable them to tolerate the adverse environment of low temperatures and maintain viability. Further investigation is warranted to determine the specific mechanisms and factors contributing to the survival of free cells during refrigeration storage. This finding is consistent with a study by Shi et al. (2013), which investigated the survival of *L. bulgaricus* encapsulated and non-encapsulated cells during storage at 4°C for one month. They observed that encapsulated cells could maintain their cell count without a significant decrease, while non-encapsulated cells experienced a substantial decline in survival, dropping from 10 log cfu/mL to only 2.3 log cfu/mL after one month of storage.

4.5 Determination of the survival of free cells and microencapsulated cells in goat milk during refrigerated storage

Table 6 illustrates the survival rates of free cells and microencapsulated cells in alginate and alginate- goat milk during storage at 4°C for 28 days. The results showed no statistically significant differences in survival rates among the different strains of free cells and encapsulated cells of each strain. The survival rate of free cells in goat milk ranged from 80.36% to 84.44%, with a decrease in cell concentration of 1.44 to 1.84 log cfu/mL after 28 days of storage. Although the difference was not statistically significant, alginate- goat milk microcapsules provided greater cell protection compared to alginate and free cells. Moreover, all treatments maintained a viability level exceeding 6 log cfu in goat milk throughout the 28-day storage period. Additionally, the survival rate of both free and microencapsulated cells in goat milk was higher compared to the absence of goat milk (Table 5). The improved probiotic survival and growth in the digestive system can be attributed to the presence of prebiotic oligosaccharides in goat milk, as suggested by van Leeuwen et al. (2020). These prebiotic oligosaccharides create a favorable environment that supports the viability and proliferation of probiotics, enhancing their survival in the digestive system. These findings suggest that both free and microencapsulated cells with alginate

and alginate- goat milk in goat milk could be utilized in feed products, providing sufficient supply for the host and maintaining survivability during longer storage at 4°C for 28 days.

4.6 Microencapsulated cell survivability under pasteurization temperature

Table 7 shows the viability of *E. hirae* Pom4 and *Lig. animalis* FB2 microencapsulated cells at different pasteurization temperatures, as compared to free cells. The results show that the viability of free *E. hirae* Pom4 and *Lig. animalis* FB2 cells was significantly reduced after pasteurization at 63°C for 15 min and 72°C for 15 sec, whereas micro-encapsulated cells exhibited greater survivability. Specifically, encapsulation with alginate- goat milk was able to maintain the survivability of *E. hirae* Pom4 and *Lig. animalis* FB2 at around 7.10-8.25 log cfu/g and 7.37-7.91 log cfu/g, respectively. The pasteurization process is an important heat treatment in destroying pathogenic bacteria in food and beverage products. However, probiotic bacteria are not heat-resistant, resulting in a decrease in their survival rate. This poses a problem when using probiotics in the industrial setting. These findings suggest that the use of alginate- goat milk can effectively improve the thermal stability of *E. hirae* Pom4 and *Lig. animalis* FB2. Teoh et al. (2011) reported that *L. acidophilus* LA-5 and *B. pseudocatenulatum* G4, which were encapsulated with alginate and coated with chitosan and starch, had higher survival rates than free cells when exposed to temperatures of 55, 60, and 65°C. Similarly, Mahmoud et al. (2020) found that *L. plantarum* encapsulated with alginate and skim milk had higher cell survival rates than non-encapsulated cells when exposed to a temperature of 65°C for 30 min. Wang et al. (2015), which reported that encapsulating *L. kefiranofaciens* M1 with gellan gum and skim milk resulted in a stronger microcapsule structure, which helped protect *L. kefiranofaciens* M1 cells when exposed to a temperature of 75°C for 1 min. Ilha et al. (2015) also found that encapsulating *L. paracasei* FNU with skim milk and cheese whey could protect the cells when exposed to a temperature of 65°C for 30 min, compared to non-encapsulated cells.

5. Conclusion

Encapsulated probiotics *E. hirae* Pom4 and *Lig. animalis* FB2 using alginate and alginate-goat milk matrices offer a promising solution for commercial probiotic production in dogs. These microencapsulated probiotics exhibit exceptional survivability not only in the dog's gastrointestinal tract but also during commercial production, and they can maintain high viability rates during refrigerated storage (4 °C) for up to 28 days in goat milk. Furthermore, the use of alginate-goat milk encapsulation provides substantial protection for the probiotic bacteria, enabling them to maintain a viable count of over 10^6 cfu/g even under harsh pasteurization conditions. These findings indicate that these encapsulated probiotics can provide optimal protection, ensuring their survival until they reach the intended site, and thus deliver health benefits to dogs.

Table 7 Survival of free and microencapsulated probiotic cells in pasteurization temperature.

LAB strains	Encapsulation material	Before pasteurization	After pasteurization temperature (ºC)			
			63ºC, 15 min	Survival rate (%)[2]	72ºC, 15 s	Survival rate (%)[2]
E. hirae Pom4	Alginate	9.84±0.02^{A}	3.57±0.14^{B}	36.24±1.34^{B}	5.53±0.35^{B}	56.24^{A}
	Alginate-goat milk	9.46±0.01^{A}	7.10±0.02^{A}	75.10±0.16^{A}	8.25±0.15^{A}	87.23^{B}
	Free cells	9.64±0.05^{A}	3.53±0.03^{B}	36.58±0.43^{B}	4.79±0.16^{C}	49.73^{C}
Lig. animalis FB2	Alginate	9.02±0.05^{A}	4.81±0.07^{B}	51.10±0.67^{B}	5.52±0.10^{B}	61.11^{B}
	Alginate-goat milk	9.00±0.12^{A}	7.37±0.05^{A}	78.75±1.44^{A}	7.91±0.16^{A}	70.39^{A}
	Free cells	9.03±0.03^{A}	3.24±0.30^{C}	34.47±2.12^{C}	4.75±0.11^{C}	40.61^{C}

Note: The results of the experiment are presented as the mean ± SD of three replicates. Capital letters indicate significant differences within each time point (column) among the different matrices, as determined by Tukey's test with a significance level of $p \leq 0.05$.
[1]Log cfu/g for microencapsulated cells or log cfu/mL for free cells.
[2]Determined by dividing the final viable cells (cfu/g or cfu/mL) by the original viable cells (cfu/g or cfu/mL).

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