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Improving stabilising effect of *Enterococcus faecalis* HZNU S1 microbeads with rice milk and alginate as wall materials

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Abstract

The utilisation of *E. faecalis* HZNU S1 as a probiotic is hindered by its sensitivity to gastrointestinal and storage environments. Therefore, the aim of the present work was to encapsulate *E. faecalis* HZNU S1 using alginate (ALG) and rice milk (RM) as encapsulating materials through the extrusion method. The resulting microbeads were characterised in terms of their size, encapsulation efficiency, and probiotic resistance under simulated gastrointestinal conditions, and also with regard to various storage conditions. The microbeads had a mean particle size of 1.60 ± 0.20 mm, and an encapsulation efficiency of 97.40%. The viability of free-state probiotics exposed to simulated gastric juice (SGJ) was found to be poor. After the treatment with SGJ under pH 2.0 and 2.5 for 1.0 h, the viability of encapsulated cells was found to be greater than 6.0 log CFU/g. Furthermore, encapsulation led to an enhancement in the viable rate of cells when incubated in bile salt solution. After exposure to bile salt solution (0.30%), the viable count entrapped cells significantly decreased from 10.01 to 6.92 log CFU/g after 1 h of exposure. The full release of entrapped cells was observed when exposed to simulated intestinal juice (SIJ) within 120 min. Furthermore, encapsulation also was able to improve the storage stability of probiotics. After 12 days of storage, viable counts of encapsulated cells were 9.1 and 6.3 log CFU/g at 4 and 25°C, respectively. Therefore, the present work suggested that encapsulating *E. faecalis* HZNU S1 within ALG/RM microbeads could enhance its survival during both gastric-intestinal tract and storage conditions.

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Introduction

Probiotics are living microorganisms that, when consumed in appropriate quantities, can offer health benefits to the host (Eratte *et al*., 2018; Zhang *et al*., 2022). Probiotics have been shown to offer a number of significant health benefits, including enhanced immune response, prevention of ulcerative colitis recurrence, alleviation of allergic and inflammatory diseases, reduction in serum cholesterol levels, promotion of vitamin synthesis, and the ability to exhibit anti-carcinogenic and anti-bacterial properties (Amiri *et al*., 2021; De Mauri *et al*., 2022; Houttu *et al*., 2022; Kumar *et al*., 2022; Zhang *et al*., 2022). Due to these benefits, there has been a significant increase in the consumption of probiotic supplements. According to the market estimates, the global probiotics market is expected to reach USD 91.1 billion by 2026, exhibiting a forecasted CAGR of 8.3% over this period. The inclusion of probiotics in functional foods is of paramount importance, with a minimum requirement of 6.0 log colony-forming units (CFU) of probiotics per gram or millilitre of each product. This ensures that the product contains sufficient probiotics to exert the intended health benefits (Lillo-Pérez *et al.*, 2021; Tianwitawat and Klaiprasitti, 2023). However, the food industry faces numerous challenges, particularly in relation to the viability of the probiotics during food processing and storage, as well as their low rate of viability in the gastrointestinal environment (Yoha *et al*., 2020; Zhen *et al*., 2020; Misra *et al*., 2021; Tianwitawat and Klaiprasitti, 2023). At present, various strategies have been attempted to overcome

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these limitations, including the selection of appropriate strains, adaptation to adverse conditions, and encapsulation (Ramos *et al*., 2016; Sun *et al*., 2023; Tang *et al*., 2023).

Encapsulation represents a promising technology with regard to maintaining the viability of probiotics, since it is able to protect them from stomach acid and bile juices found in the small intestine (Kent and Doherty, 2014; Sun *et al*., 2023). Moreover, the release of probiotics can be regulated in designated and appropriate locations (Tang *et al*., 2023). The material utilised for encapsulation should exhibit strong emulsification characteristics, and function as an effective protective agent during storage. Often, meeting these requirements necessitates the use of multiple encapsulating materials. Therefore, it is common practice to combine polysaccharides and proteins. One of the most commonly used polysaccharides in encapsulation process is alginate (ALG) (Dong *et al*., 2013). ALG is a naturally occurring, non-toxic, water-soluble, and edible biopolymer (Sun *et al*., 2023; Tang *et al*., 2023). Microbeads composed primarily of ALG possess several advantageous properties, including the ability to entrap a significant number of probiotics, and importantly, to provide probiotic protection (Pan *et al*., 2013; Chen *et al*., 2014). According to Dong *et al*. (2013), ALG can enhance the viability of probiotic cells in tough gastric environments. However, some studies have shown that ALG alone is not sufficient to withstand the low pH levels in the stomach. To address this issue, one effective solution is to combine ALG with some other biopolymers that possess resistance to low pH levels in the gastric environment (Pan *et al*., 2013; Sun *et al*., 2023). Recently, milk-based proteins have been widely employed as wall materials for encapsulating bioactives due to their good physicochemical characteristics, which include good buffering and excellent emulsification abilities, as well as other properties (Shi *et al*., 2013a; Prasanna and Charalampopoulos, 2018; 2019). The probiotics are encapsulated by blending ALG and milk proteins, which provide effective protection to probiotics during the digestive process (Prasanna and Charalampopoulos, 2018; 2019; Sun *et al*., 2023). In our group, the microbeads such as milk/ALG, soy milk/ALG, and flaxseed milk/ALG, were constructed to provide probiotic protection (Shi *et al*., 2013b; 2015; Sun *et al*., 2023). These milk-based carriers demonstrated the capability to enhance the number of viable probiotic cells, even under the adverse gastrointestinal and storage conditions. Rice is a staple diet for more than 50% of the global population, especially in Asian countries, making it a significant source of daily protein for many people. Rice, a widely cultivated cereal grain, is a cost-effective source of numerous nutritious compounds, including proteins, carbohydrates, minerals, fats, fibres, and vitamins (Padma *et al*., 2018; El Sayed and Mabrouk, 2023). During the 21-day storage period, probiotic cells (*e.g*. *Bifidobacterium animalis* subsp. *lactis* BB-12) exhibited enhanced viability when rice was incorporated into yogurt, compared to plain yogurt (Kumari *et al*., 2015). Additionally, Zanjani *et al.* (2018) found that the viability of *Lactobacillus casei* ATCC 39392 was enhanced by wheat and rice starches. Rice protein, which provides essential functional characteristics including emulsifying, foaming, and gelation abilities, is present in rice milk (RM) (Han *et al*., 2015). Therefore, RM shows great potential for application in probiotics encapsulation. To the best of our knowledge, the use of ALG/RM as wall materials for probiotics encapsulation has never been reported.

Enterococcus spp. are among the most prevalent bacterial strains found in numerous fermented dairy products (Zheng *et al*., 2015; Kouhi *et al*., 2022). The most prevalent species identified within foods are *E. faecium* and *E. faecalis* (Gaglio *et al*., 2016; Oruc *et al*., 2021). These bacteria have been associated with a number of benefits, including the contribution to the sensory properties of cheeses, the possession of probiotic characteristics, and the production of antimicrobial compounds (Carlos *et al.*, 2010; Oruc *et al*., 2021). *Enterococcus faecalis* is a type of lactic acid bacterium that is frequently employed as a potential probiotic (Panthee *et al*., 2021; Lou *et al*., 2024). *Enterococcus faecalis* HZNU S1 (*E. faecalis* HZNU S1) is a strain that exhibits high resistance to intestinal conditions and adhesion to gut cells. However, its survival is poor when it is exposed to simulated gastric juice (SGJ) and bile juice (Sun *et al*., 2023; Tang *et al.*, 2023). Efforts have been made to enhance the added value of rice grain in many countries due to declining rice consumption. Since RM has the potential to be utilised as a wall material, the effective utilisation of RM for probiotic encapsulation should be considered for enhancing its value. Therefore, the purpose of the present work was to encapsulate *E. faecalis* HZNU S1 in ALG/RM using the extrusion method, to evaluate the properties

of probiotics, and to enhance their viability in the simulated gastrointestinal environments. Additionally, the viability of probiotic cells when stored under various conditions was also evaluated.

Materials and methods

Activation and preparation of *E. faecalis HZNU S1 suspension*

The culture, obtained from Hangzhou Normal University, was stored at -80°C in 20% glycerol. The culture was activated and adapted by cultivating it twice in MRS broth at 37°C for 24 h. The activated culture was obtained *via* centrifugation at 3,000 *g* for 10 min at 4 ± 1°C. It was then rinsed with sterile water, and added to a 0.85% sodium chloride solution at 4 ± 1°C for encapsulation.

Encapsulation of E. faecalis HZNU S1 by extrusion method

The *E. faecalis* HZNU S1 cells were thoroughly mixed with a 2.0% ALG (Sigma Aldrich, Shanghai, China)-RM (Shanghai Chuangshi Co. Ltd, Shanghai, China) matrix (2:1). The mixture was then introduced into a 0.10 M $CaCl_2$ solution using 26-gauge nozzle syringe with low stirring speed (100 rpm). The resulting microbeads were left undisturbed for 30 min at room temperature, then collected by filtration, washed with sterile water, and stored at 4°C for further study.

Morphological properties of microbeads

The morphology of the produced microbeads was observed using an optical light microscope. The microbeads were selected at random to determine their size.

Enumeration of viable cells and encapsulation efficiency (EE)

The microbeads' encapsulation efficiency (EE) was evaluated following the method described by Sun *et al*. (2023). Briefly, 4.5 mL of sterile trisodium citrate solution (50 mmol/L) was mixed with 0.50 g of microbeads. The microbeads were dissolved for 30 min. After that, the viable cells (colony forming units) were enumerated by plating 10-fold serial dilutions of dissolved microbeads and free *E. faecalis* HZNU S1 cell suspensions onto MRS agar plates. Afterwards, the cells were cultivated at 37°C for 24 h. The EE for probiotics was calculated using Eq. 1:

$$EE = (\text{Log } N / \text{Log } N_0) \times 100 \quad \text{(Eq. 1)}$$

where, N = count of entrapped probiotics; and N_0 = count of free probiotics added into ALG and RM mixture.

Survival of non- or entrapped E. faecalis HZNU S1 in SGJ

The viable rate of non- or entrapped cells in SGJ (0.20% NaCl), with or without pepsin (0.30 g/L), was tested by exposing them to SGJ (pH 2.0 and 2.5) heated to 37°C for 30 min. Briefly, 4.5 mL of SGJ was mixed with the produced microbeads (0.50 g). At predetermined times (0, 30, 60, 90, and 120 min), the samples were filtered and washed twice by sterile water, and then broken *via* the addition of the microbeads into a trisodium citrate solution (50 mmol/L). Meanwhile, probiotic cells were collected *via* centrifugation at 3,000 *g* for 10 min. They were then rinsed twice using sterile water, and dissolved in a sodium chloride solution (0.85%). The cells were serially diluted, and then the suspension (100 μL) was spread plated on MRS agar. After being cultured at 37°C for 24 h, the viable cells were enumerated.

Survival of non- or encapsulated E. faecalis HZNU S1 in bile salt solution

In order to examine the impact of bile juice (Sigma-Aldrich, Shanghai, China) on the survival of non- or entrapped *E. faecalis* HZNU S1, 0.10 and 0.30 g of bile salt were dissolved in 100 mL of sterile water, and the pH was adjusted into 6.8 (Sun *et al*., 2023). After sterilisation by filtration, either non-entrapped *E. faecalis* HZNU S1 or the microbeads (0.50 g) was incubated in 4.5 mL of bile salt solution. The suspension was then cultured at 37°C with low speed (100 rpm) for 60 and 120 min. The viable cells were enumerated at predetermined time intervals, as described earlier. The viable rate of either non- or encapsulated cells was calculated using Eq. 2:

$$\text{Survival rate} = (\text{Log } N / \text{Log } N_0) \times 100 \quad \text{(Eq. 2)}$$

where, N = amount of viable cells after being incubated in bile salt solution; and N_0 = amount of viable cells before being exposed to bile salt solution.

Release of encapsulated E. faecalis HZNU S1 in SIJ

The release of entrapped *E. faecalis* HZNU S1 cells from ALG/RM microbeads in SIJ (pH 6.8,

50 mmol/L KH_2PO_4), with or without pancreatin (1.0 g/L; Sigma-Aldrich, Shanghai, China), was determined by the modified method of Sun *et al*. (2023). Entrapped probiotics in the microbeads were added to SIJ at pH 6.8 with stirring using a mechanical stirrer, with low agitation (100 rpm) at 37°C for 3 h. Sampling was performed at 0, 10, 30, 60, 120, and 180 min, and the constant volume was maintained through adding the same volume of fresh SIJ. The quantity of released viable probiotic cells was determined by plate count.

Viability of non- or encapsulated probiotics in storage conditions

To assess the survival rate of the cells during storage, both free cells and microbeads were stored at 4 and 25°C for 4 w. At predetermined intervals, 1.0 g of wet microbeads was added into 9.0 mL of trisodium citrate solution (50 mmol/L) to break the microbeads. Viable cell numbers were determined following the method described earlier.

Statistical analysis

The studies were replicated three times, and the data were reported as mean and standard deviation. Statistical differences were determined using analysis of variance (ANOVA), and the data with significant differences at a confidence level of 95% ($p < 0.05$) were differentiated by Student's *t*-test.

Results and discussion

EE and size of microbeads

The EE of *E. faecalis* HZNU S1 was 97.40% for ALG/RM microbeads, indicating that ALG/RM microbeads were an effective agent for encapsulating *E. faecalis* HZNU S1. The high EE achieved may be attributed to the mild conditions used during the extrusion process, including mild temperature and the absence of organic solvents. This was also attributed to strong gelling characteristics of the wall materials. The EE depends on various factors, such as encapsulation methods, wall materials, and the specific probiotics used. Gebara *et al*. (2013) found that encapsulation of *L. acidophilus* using pectin coated with whey protein as a wall material had 84.35% EE. Similarly, Trabelsi *et al*. (2013) reported that EE of ALG/chitosan microbeads containing *L. plantarum* was 81%. Premjit and Mitra (2021) used the electrospraying technique to prepare microparticles for encapsulating *Leuconostoc lactis*. The EE of probiotics was found to range from 89.7 to 92.9%. Herein, the EE levels were higher than those reported in previous studies. This indicated that ALG and RM were effective wall materials for generating microbeads to encapsulate *E. faecalis* HZNU S1. Additionally, the encapsulation procedure used in the present work has demonstrated efficacy in maintaining probiotic viability.

The distribution of ALG/RM microbeads was observed to range from 1.60 to 2.00 mm, with an average mean diameter of 1.80 ± 0.20 mm (Figure 1). The size of the microbeads was influenced by a number of factors, including syringe diameter, the content of encapsulating materials, $CaCl_2$ content, the viscosity of encapsulating materials, and the distance between Ca^{2+} solution and the syringe (Voo *et al*., 2011; Valero-Cases and Frutos, 2015). The large size of the microbeads in the present work was primarily due to the large diameter of the syringe (Klemmer *et al*., 2011; Valero-Cases and Frutos, 2015; Huang *et al*., 2017; Pauluk *et al*., 2019). Shi *et al*. (2013b) used the encapsulator with varying nozzle sizes to prepare ALG/milk microbeads. The investigation revealed that ALG/milk microbeads generated using nozzle sizes of 0.45 and 0.20 mm, had mean sizes of 830 ± 10 and 310 ± 8 μm, respectively. Klemmer *et al*. (2011) used 20 and 27 G needles to prepare pea protein-based microcapsules. The size of pea protein microcapsules ranged from 1.75 to 2.17 mm, which was comparable to the results reported in the present work. According to Lawless and Heymann (2010), the microbeads employed in encapsulated foods should be within the range of 100 to 200 μm. This size range facilitates the direct incorporation of encapsulated probiotics into various food products. Therefore, it would be advisable to attempt to reduce the size of the microbeads in order to ensure optimal usage in food products.

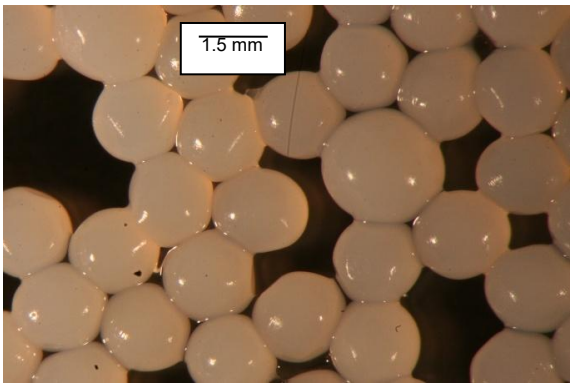

Figure 1. Optical photograph of alginate-rice milk microbeads.

Survivability of encapsulated microbeads under SGJ

The environment within the gastric tract varies depending on the contents and location. The stomach is particularly importance for pH-sensitive substances, such as probiotics. In healthy individuals, the pH level of the stomach typically ranges from 1.3 to 2.5 during the fasting state. However, the pH level can increase to a range of 4.5 to 5.8 after the ingestion of food (Chotiko and Sathivel, 2016).

The survivability of entrapped *E. faecalis* HZNU S1 in SGJ, with and without pepsin, was determined as presented in Figure 2. The results indicated that encapsulation with ALG and RM enhanced the viable rate of cells under SGF in the absence of pepsin at pH 2.0. Viable numbers reached 6.89 log CFU/g after 1 h of exposure. After 2 h of exposure at the same SGF pH, the viable count of entrapped cells was reduced to 5.23 log CFU/g. The results indicated that encapsulated cells with viable numbers of more than 6.0 log CFU/g could survive exposure to SGF for 1 h. Additionally, a higher survival rate of cells was observed when exposed to SGF at pH 2.5. The viable amount of cells encapsulated in ALG/RM microbeads decreased from 10.0 log CFU/g to 8.82 and 6.03 log CFU/g, when exposed to SGF with pepsin for 1 and 2 h, respectively. The enhanced probiotic protection capacity of ALG/RM was attributable to the buffering capacity of RM (Tan *et al*., 2022). Additionally, the interaction of RM and ALG resulted in the formation

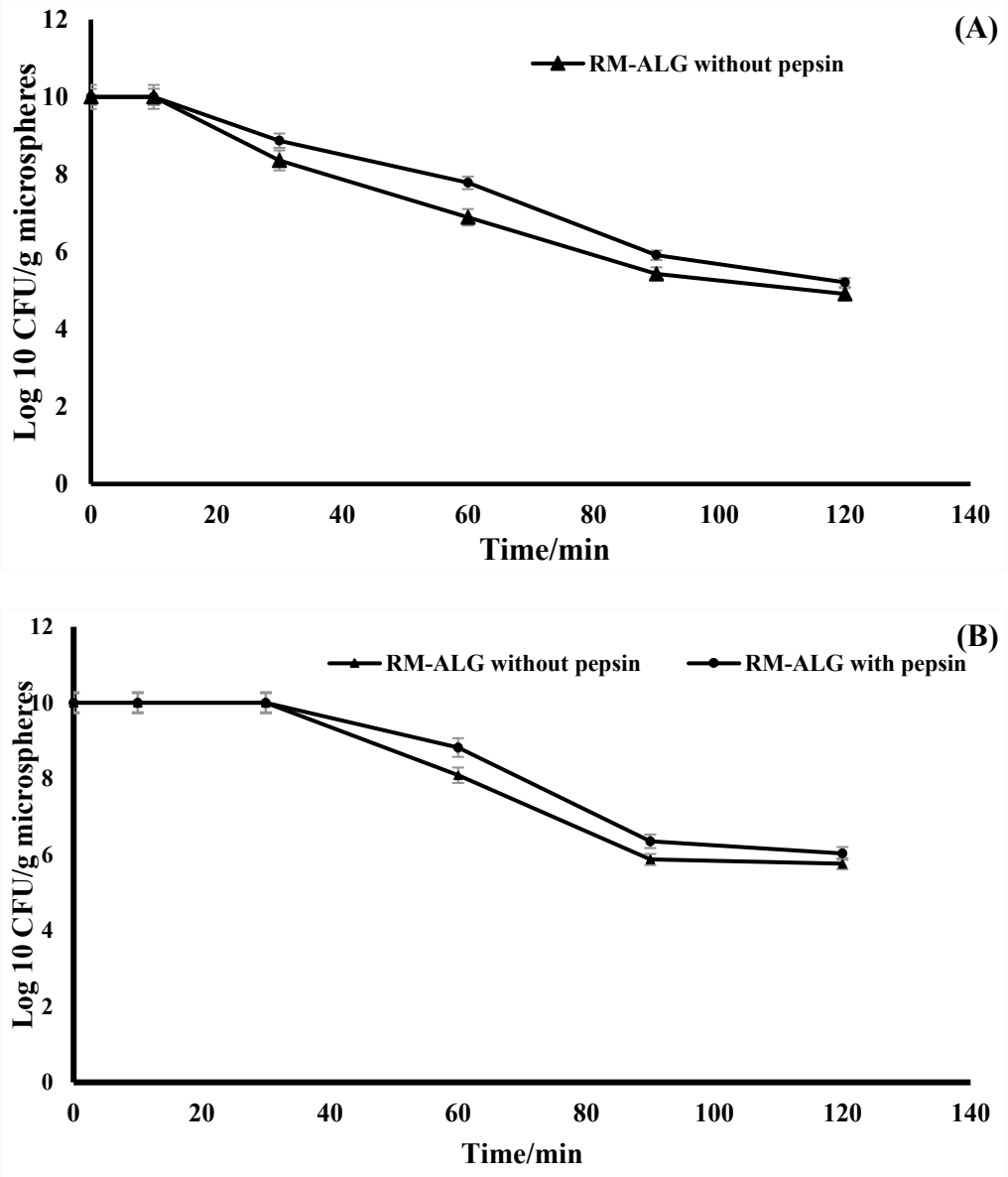

Figure 2. Survivability of entrapped *E. faecalis* HZNU S1 exposed to simulated gastric juice (SGJ), with/without pepsin, at pH 2.0 **(A)** and 2.5 **(B)**.

of filling materials, which could potentially reduce the porous structure of ALG/RM microbeads. These results agreed with previous research, which has shown that encapsulating probiotics using ALG-based microbeads increased their stability within SGJ (Chen *et al*., 2014; Malmo *et al*., 2021; Misra *et al*., 2022). According to Shi *et al*. (2013a), the viable counts of *Lactobacillus bulgaricus* entrapped in carrageenan-locust gum/milk microbeads were still greater than 8.0 log CFU/g after being exposed to SGJ (pH 2.0) for 2 h. In the same group, Shi *et al*. (2013b) investigated the stability of *Lactobacillus bulgaricus* that was encapsulated in ALG/milk microspheres when being incubated in SGJ (pH 2.0). The amounts of viable encapsulated probiotics did not change within 30 min. Afterwards, the survival counts of cells gradually decreased to 8.3 log CFU/g after 2 h of exposure. The authors indicated that the high buffering capacity of milk provided adequate protection for *Lactobacillus bulgaricus*. In our previous work, *E. faecalis* HZNU S1 was loaded in ALG/flaxseed milk microbeads. The study found that the encapsulated cells survived fully even after being incubated in pH 2.5 SGJ for 2 h. Only a 1.0 log CFU/g reduction was achieved after entrapped cells were incubated in SGJ (pH 2.0) for 2 h (Sun *et al.*, 2023). In the present work, wall materials of ALG and RM could provide good protection to *E. faecalis* HZNU S1 when compared to non-encapsulated cells in the same condition (El-Shafei *et al*., 2015; Misra *et al*., 2021).

Survivability of E. faecalis HZNU S1 in bile salt solution

It is widely accepted that the primary challenges to viability of probiotics are the gastric acid and bile juice in the small intestine, with bile juices being particularly problematic due to their ability to disrupt probiotic cells (Yao *et al*., 2018; Sun *et al*., 2023). The survivability of *E. faecalis* HZNU S1 was assessed under bile salt condition, and the results are showed in Table 1. It was found that encapsulation had the potential to enhance cell viability during exposure to bile salt solution (60 and 120 min). When ALG/RM microbeads were incubated under bile salt conditions, there was a slight decrease in the number of probiotic cells. The most significant decrease was observed in free cells. The number of viable non-entrapped cells was significantly reduced, dropping to a non-detectable level within the incubation period (Table 1). The survival numbers of encapsulated cells were found to decrease from 10.01 to 6.92 log CFU/g under bile salt solution (0.10%) for 120 min. When entrapped cells were exposed to 0.30% bile salt solution for 120 min, the survival rate of entrapped probiotics was reduced from 9.98 to 5.52 log CFU/g. The data regarding the survivability of encapsulated cells under bile salt solution were consistent with other reported findings (Shi *et al*., 2013a; 2013b; Sun *et al*., 2023). Various studies demonstrated that encapsulation could enhance the survivability of the entrapped probiotic cells under bile salt conditions. The viability of encapsulated *Lactobacillus acidophilus* LA1 within ALG/starch microbeads only decreased by 2.16 log CFU/mL after being exposed to bile salt solution (2.0%) (Sabatini, 2010). Our group found that probiotics were able to withstand unfavourable conditions, including high levels of bile salts (1.0 and 2.0%) after being entrapped in ALG/milk microbeads (Shi *et al*., 2015). Similarly, Sun *et al*. (2023) demonstrated that the initial viable amounts of encapsulated cells in ALG/flaxseed milk decreased from 10.01 to 9.81 log CFU/g when exposed for 1 h, and then further to 9.54 log CFU/g when exposed for 2 h in a bile salt solution (1.0%). Likewise, in the present work, the decrease in the viability of entrapped probiotic cells after being incubated in bile salt solution (0.10 and 0.30%) for 1 and 2 h was found. Probiotics were immobilised in the

Table 1. Survivability of non- or entrapped *E. faecalis* HZNU S1 after being incubated in 0.10 and 0.30% bile salt solutions for 1 and 2 h (log CFU/mL or g microbeads).

Condition/time	Bile salt content (%)		
	0	0.10	0.30
Non-entrapped cells (1 h)	10.01 ± 0.11	0	0
Entrapped cells (1 h)	10.03 ± 0.09	8.86 ± 0.09	6.92 ± 0.08
Non-entrapped cells (2 h)	10.02 ± 0.08	0	0
Entrapped cells (2 h)	9.98 ± 0.13	7.27 ± 0.15	5.52 ± 0.08

microbeads through the encapsulation method, thereby reducing the interaction of bile salt with probiotics, and ultimately achieving the aim of probiotic protection.

Release of entrapped E. faecalis HZNU S1 cells in SIJ

The release of cells from ALG/RM microbeads in SIJ, with and without pancreatin, was investigated at 37°C over a 3 h period. It was observed that ALG/RM microbeads exhibited a rapid release property when exposed to SIJ. It was found that approximately 60% of encapsulated cells were released within 30 min incubation. Almost all entrapped cells within the microbeads were released in 120 min (Figure 3). The survival of free cells suggested that once released, *E. faecalis* HZNU S1 would be able to survive in SIJ conditions. Various investigations have reported the release property of entrapped probiotics in SIJ (Shi *et al*., 2013a; 2013b; 2015; 2016). The release of encapsulated probiotics in SIJ might be induced by destroying ALG matrix due to the presence of Na^+ (Klemmer *et al*., 2011). Klemmer *et al*. (2011) obtained pea protein/ALG microcapsules by extrusion method and also observed that the viable numbers of 5.5 to 7.0 log CFU/mL from encapsulated *B. adolescentis* were released gradually in SIJ over a 1.5 h period. Shi *et al*. (2016) found that *E. faecalis* HZNU P2 loaded in ALG/milk microbeads was fully released in SIJ over a 1 h period. Our previous work indicated that ALG/flaxseed milk and ALG/soy milk microbeads also exhibited a rapid release profile within SIJ (Shi *et al*., 2015; Sun *et al*., 2023). Therefore, our results indicated that ALG/RM microbeads could have the potential to deliver probiotics to the intestinal tract in a targeted manner, allowing them to colonise and provide health benefits to the host.

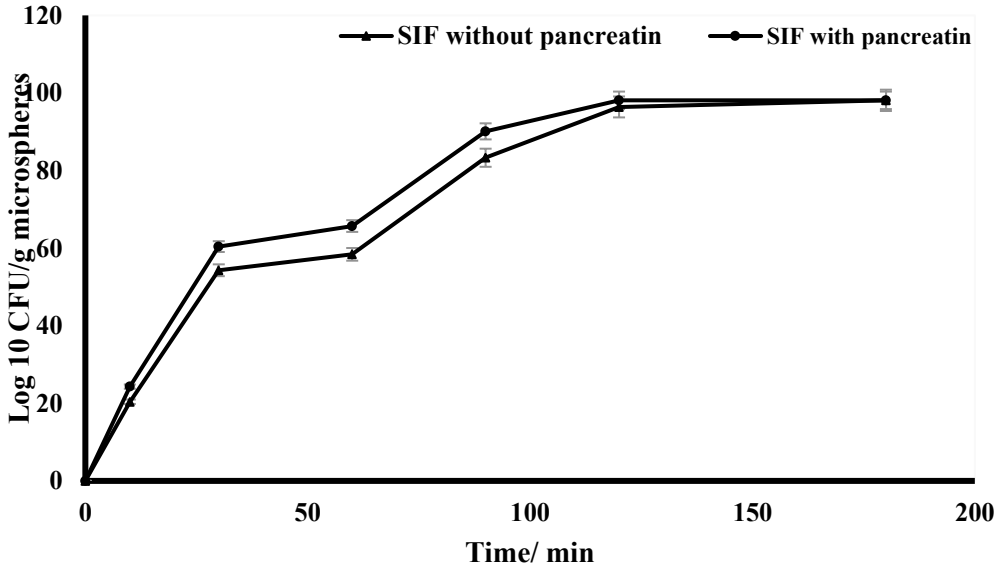

Figure 3. Release curve of entrapped *E. faecalis* HZNU S1 in simulated intestinal juice (SIJ), with/without pancreatin, at 37°C.

Stability of non- or entrapped E. faecalis HZNU S1 during storage conditions

Figure 4 presents the storage stability of non- or entrapped cells at 4 and 25°C for 30 days. It was found that the survival rates of probiotics decreased under the investigated storage conditions. Additionally, an increase in storage temperature led to a further decrease in survival rates. For instance, even following 18 days of storage under refrigeration conditions (4°C), the survivability of non-entrapped cells was still higher than 6.0 log CFU/mL (Figure 4A). But, at 25°C, the survivability of non-entrapped cells was 6.30 and 2.61 log CFU/g after storage for six and ten days, respectively (Figure 4B). Over one month of preservation at 4°C, the number of cells entrapped in ALG/RM microbeads remained at 8.8 log CFU/g after being stored for 14 days, and further dropped to 8.3 log CFU/g after 30 days storage period. During the storage at 25°C, the viable cells in ALG/RM microbeads slightly deceased from 10.01 to 8.50 log CFU/g after eight days of storage, and then further decrease to 2.6 log CFU/g after storage for 30 days. Therefore, it was indicated that encapsulating *E. faecalis* HZNU S1 within ALG/RM microbeads was

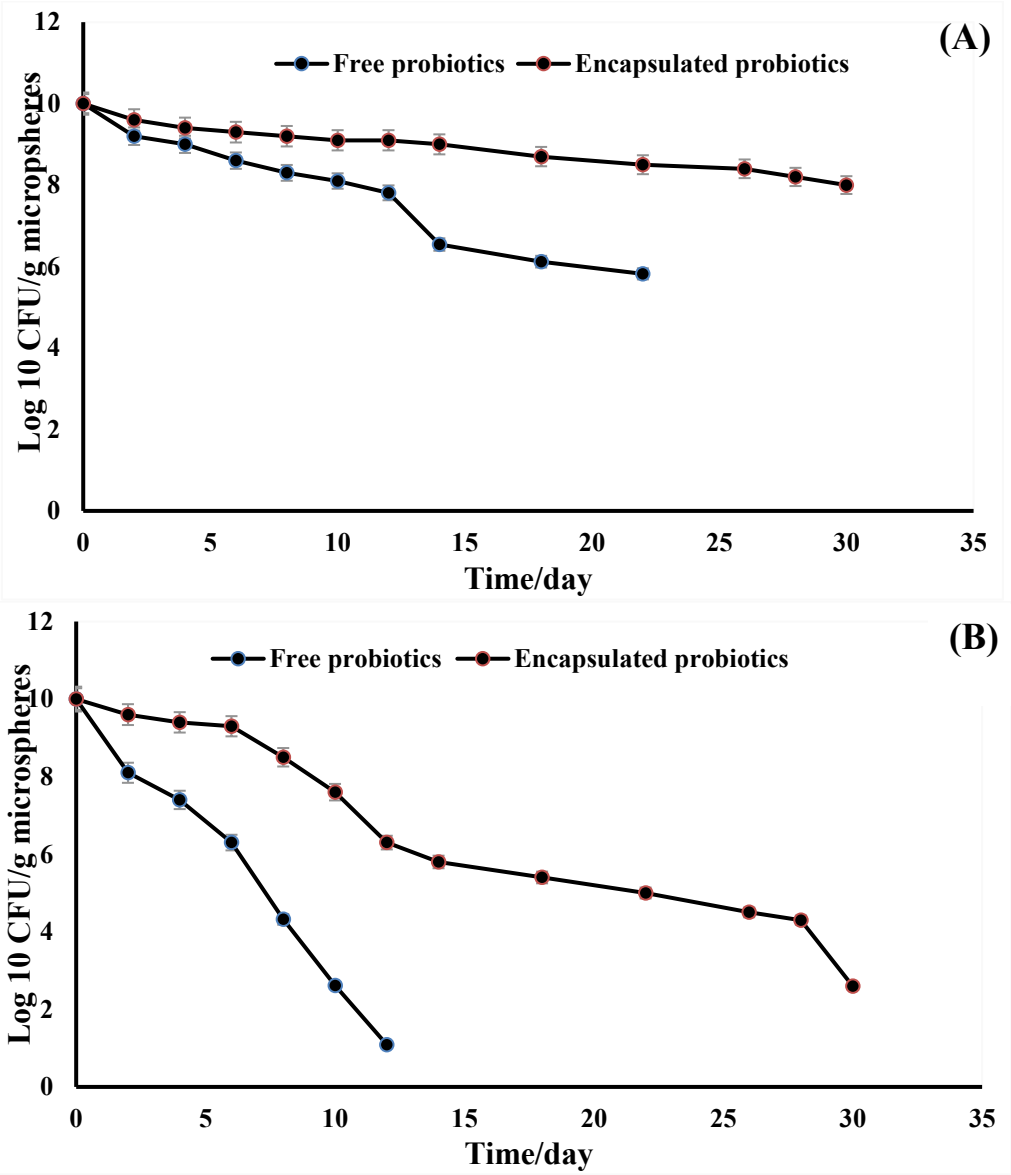

Figure 4. Survivability of non- or entrapped *E. faecalis* HZNU S1 in alginate-rice milk microbeads at 4°C **(A)** and 25°C **(B)**.

able to effectively enhance the viability of probiotic cells under the investigated conditions. RM was able to transform a porous structure of ALG microbeads into a more interconnected network, thereby resulting in a reinforced structure of ALG/RM microbeads. This structure likely offered protection to the entrapped probiotic cells *via* creating an effective barrier against the mobility of solutes, moisture, and oxygen, thereby enhancing the storage stability of *E. faecalis* HZNU S1 (Yao *et al*., 2018). Similarly, a number of studies have demonstrated that encapsulating probiotics in milk-based wall materials could enhance their survival under the storage conditions (Shi *et al*., 2013a; 2013b; 2015; 2016). Prasanna and Charalampopoulos (2019) reported that viable cell amounts in ALG-goat milk-inulin microcapsules did not decrease below the recommended level at 4°C over a period of 28 days, whereas, the viable numbers of non-encapsulated bifidobacteria decreased by around 3.67 log CFU/g. In the same group, they also found that the viable numbers of more than 6.0 log CFU/g in ALG/cow milk (-goat milk) microbeads were maintained after being preserved for 28 days at 4°C. It was indicated that ALG/dairy microcapsules could enhance the storage stability of probiotics (Prasanna and Charalampopoulos, 2018). Shi *et al*. (2016) showed that ALG/milk microbeads offered enhanced protection for entrapped *E. faecalis* HZNU P2 compared to non-entrapped cells, even after being stored for two weeks at 4°C. In our previous work, we found that after being stored eight days at 25°C, the

survival rate of entrapped *E. faecalis* HZNU S1 only decreased by 0.81 log CFU/mL (from 10.01 to 9.2 Log CFU/g). Entrapped *E. faecalis* HZNU S1 exhibited good storage stability, which could be attributed to the dense layer of the microbeads resulting from the interaction ALG and flaxseed milk (Sun *et al*., 2023). In the present work, the encapsulation significantly improved the viability of probiotics at 25°C compared to non-encapsulated cells. This enhancement in viability would lead to an improvement in the profitability of probiotic products by extending their shelf life.

Conclusion

In the present work, ALG and RM were employed to successfully encapsulate *E. faecalis* HZNU S1 *via* the extrusion technique. Entrapped cells exhibited higher survival rates when exposed to SGJ at pH 2.0 and 2.5, as well as bile salt solution at 0.10 and 0.30%. Entrapped *E. faecalis* HZNU S1 experienced a rapid release when exposed to SIJ. The present work also determined the survival of non- or entrapped *E. faecalis* HZNU S1 cells during storage. Survival rates were found to vary depending on the storage temperature. The use of ALG/RM microbeads to encapsulate *E. faecalis* HZNU S1 cells was found to be an effective method for improving the viability of probiotics under the tested storage conditions. This encapsulation technique, using the ALG/RM system, has the potential to deliver probiotics in foods with the aim of optimising health benefits.

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