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Microencapsulation of *Bacillus* and Yeast Aquaculture Probiotics: Viability, Digestive Enzyme Activity, and Protective Efficiency

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Abstract

The delivery technique of probiotics in aquaculture is a crucial role in maintaining their viability and functionality during the processes of preparation, storage, and ingestion to ensure reaching the site of action in the host's intestines. Microencapsulation aids in protecting probiotics against unfavorable environments within the capsular matrix. This study was designed to improve the viability and beneficial activities of probiotics during storage through microencapsulation. Four shrimp probiotic strains: *Bacillus megaterium* BUU002, *B. polymyxa* BUU003 and *B. licheniformis* BUU004 and a yeast, *Debaryomyces hansenii* BUU01, were individually entrapped in an alginate microcapsule. Viable cell counts of the *Bacillus* and yeast probiotics in microcapsules ranged from 9.25±0.08 to 9.73±0.05 log CFU/g. The survivals of encapsulated *Bacillus* and yeast probiotics were significantly (P<0.05) higher than those of free cells under strongly acidic and basic conditions (pH 2.0, 4.0, and 10.0). Viabilities of encapsulated *Bacillus* and yeast probiotics remained high in the ranges of 8.60±0.03 - 8.87±0.02 log CFU/g, which were significantly (P<0.05) higher than those of free cells (4.52±0.13 - 6.82±0.05 log CFU/g) at 60 days of storage. Stability of amylase, protease, and lipase activities of the four encapsulated probiotics was also improved significantly (P≤0.05) during 60-day storage, compared to free-cell counterparts. Microencapsulated *Bacillus* probiotics also showed significantly higher cell viability than free cells following heat treatment at 70°C for 60 min (91.6–93.6% vs 82.4–85.6%) and 90°C for 5 min (84.6–89.4% vs 79.8–83.5%). Microencapsulation also increased significantly (P≤0.05) viable cells of yeast probiotic: *D. hansenii* BUU01 exposed to heat treatment at 70°C for 60 min (22.3±2.4% vs 0%). Our study suggests that alginate encapsulation is a viable choice for extending the shelf-life of probiotics without compromising their beneficial activity.

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Introduction

Marine shrimp farming is a vital economic pillar of Thailand, with an estimated production of 392,600 tons valued at nearly 58,000 million Baht (Fisheries Statistics of Thailand, 2024). In recent years, the prevalence of shrimp diseases caused by bacterial and viral infections has increased due to factors such as intensive farming practices, water pollution, and environmental degradation. These issues have led to significant declines in shrimp production, resulting in substantial losses in income, employment, consumer confidence, and, ultimately, the closure of many businesses. The common treatment methods in a classical way have relied on chemotherapeutic agents, including antibiotics and chemicals. However, these approaches are neither effective nor environmental-friendly as well as pose risks to human and animal health. A promising alternative is the use of probiotics as a bio-therapeutic strategy to prevent infectious diseases. Probiotics offer several advantages. For example, they leave no harmful residues in food products and do not contribute to the development of antibiotic-resistant pathogens.

Probiotics are diverse microbiological supplements consisting of single or multiple strains of bacteria and yeast that exist within the ecological systems of host animals (De et al., 2018; Nimrat et al., 2020, 2021a; Mathan Muthu et al., 2024). They provide metabolic, trophic, and protective functions essential for enhancing overall health, e.g. improved growth performance, enhanced disease resistance, increased digestive enzyme activity, stimulation of non-specific immune responses, and the optimization of bacterial community structure in the gastrointestinal tracts. Additionally, probiotics contribute to the stabilization of aquaculture systems by maintaining optimal water quality, which is critical for the successful cultivation of penaeid shrimp (Nimrat et al., 2020, 2021a; Ringø, 2020; Mathan Muthu et al., 2024). In aquaculture, spore-forming *Bacillus* species, e.g. *B. subtilis, B. licheniformis,* and *B. amyloliquefaciens* are the most commonly used probiotics due to their numerous advantages. These include their non-pathogenic and non-toxic nature, good immunomodulator, extended shelf life, antagonistic activity against pathogens, resistance to hostile environments, simple nutritional requirements, rapid metabolic rate, and ease of preparation (Ringø, 2020; Mathan Muthu et al., 2024). In addition, *Bacillus* probiotics can produce a wide range of extracellular digestive enzymes, including amylase, protease, lipase, and cellulase (De et al., 2018). Numerous studies have conclusively demonstrated significant improvement of growth, survival, digestive enzyme activity, immunomodulatory response, and disease resistance of whiteleg shrimp (*Litopenaeus vannamei*), and black tiger shrimp (*Penaeus monodon*) as a consequence of *Bacillus* probiotic administration (De et al., 2018; Nimrat et al., 2020, 2021a). Aside from *Bacillus* species, yeasts have also received much attention as a complementary tool for disease prevention in aquaculture. This is due to their rich compositions including digestive enzymes, essential organic compounds, vitamins, coenzymes, fatty acids, amino acids, and growth-promoting factors (Ceseña et al., 2021). For instance, dietary supplementation with mixed cultures of *Debaryomyces hansenii* and *Rhodotorula* sp. has shown to significantly enhance growth, survival rate, and feed efficiency. This supplementation also modulates the microflora community structure within the gastrointestinal ecosystems of postlarval and juvenile whiteleg shrimp (Nimrat et al., 2021a).

To deliver health benefits to the host, probiotics must remain metabolically stable and active not only at the time of production, but also throughout their shelf life, and maintain viability until the expiry date. Additionally, they must survive in significant numbers while passing through the gastrointestinal tract of hosts. However, the viability of probiotic strains in commercial products is often questionable when used by farmers because of their low resistance to factors, such as pH fluctuations, post-acidification during storage, hydrogen peroxide production, oxygen toxicity (resulting from oxygen permeation through packaging), and suboptimal storage temperatures (Morales & Ruiz, 2016). A study conducted by Nimrat et al. (2021b) underscored this critical issue, emphasizing the need for greater scrutiny in evaluating the quality of commercial probiotics. They revealed discrepancies between the viable probiotic counts reported on product labels and those actually present in the products. Additionally, they observed that most probiotic products failed to meet the standard imposed by the Ministry of Agriculture and Cooperatives of Thailand. In this context, microencapsulation presents a promising alternative to produce probiotic products. It provides a high protective level for live probiotics against hostile environments, ensuring an adequate number of viable cells throughout the product's shelf life. This approach enhances both the application and efficacy of probiotics in the aquaculture industry.

Microencapsulation is an effective technique for entrapping probiotics within a protective coating material, shielding them from unfavorable environments and regulating the release of viable cells and their bioactive substances (Masoomi Dezfooli et al., 2019; Morales and Ruiz, 2016). Alginate microencapsulation has shown to obviously improve the viability of probiotic cells in simulated digestive tracts of shrimp, including the stomach, hepatopancreas and intestine (Vega-Carranza et al., 2021). In addition, dietary supplementation of *Bacillus* probiotic encapsulated in alginate resulted in a significant increase in growth performance, immune response, and disease resistance against *Vibrio* infection of whiteleg shrimp, *Litopenaeus vannamei* (Adilah et al., 2022)

A study focused on encapsulating bacterial and yeast probiotics for shrimp cultivation remains limited. In our previous study, alginate microcapsules containing a mixture of *Bacillus* probiotics demonstrated *in vitro* antagonism against pathogenic *Vibrio* species and significantly improved growth, survival, and disease resistance against *Vibrio harveyi* in whiteleg shrimp (Nimrat et al., 2020). Therefore, this study was designed to characterize the microcapsules containing *Bacillus* strains, namely *Bacillus megaterium* BUU002, *B. polymyxa* BUU003, *B. licheniformis* BUU004, and the yeast *Debaryomyces hansenii* BUU01 during storage.

Materials and methods

1. Probiotics and growth conditions

Probiotics used in this study were composed of three *Bacillus* strains and a non-pathogenic yeast species. Three *Bacillus* strains isolated from the intestinal tract of black tiger shrimp and pond sediment, namely *Bacillus megaterium* BUU002, *B. polymyxa* BUU003 and *B. licheniformis* BUU004, have shown to possess the ability to utilize nutrients (protein, starch, and lipids), exhibit *in vitro* antibacterial activity against pathogenic *Vibrio harveyi,* and contribute to improved growth performance, modulation of intestinal bacterial structure, and enhancement of culture water quality in black tiger shrimp and whiteleg shrimp (Boonthai et al., 2011; Nimrat et al., 2020, 2021a). Each *Bacillus* probiotic was grown in a 500-mL flask containing 200 mL of Trypticase Soy Broth (TSB; BD Biosciences, Sparks, Maryland, USA) in an incubator shaker at 200 rpm and 30±1°C for 24 h (Nimrat et al., 2021a). Cell suspension was centrifuged at 8,228 g and 4°C for 5 min, then washed thrice with sterile 1 g/L peptone water (pH 7.0). Cell pellet was re-suspended and adjusted to 1.5 A.U. at 580 nm using a spectrophotometer, which was approximately equivalent to 10^{10} CFU/mL. The resulting suspensions were prepared for subsequent microencapsulation.

A yeast probiotic strain, *Debaryomyces hansenii* BUU01, was isolated from shrimp gastrointestinal tract and pond sediment in Chon Buri Province, Thailand. It was selected due to its ability to degrade protein, lipid, and starch substrates, enhance growth and survival, reduce *Vibrio* abundance in the gastrointestinal tracts, and promote beneficial intestinal microbiota in larval and post-larval whiteleg shrimp. The stock culture was frozen at -80°C in Yeast extract Peptone Dextrose (YPD) broth (Becton BD) supplemented with 15% (v/v) glycerol. The active culture was propagated in YPD broth at 25°C for 24 h with shaking at 200 rpm (Nimrat et al., 2021a). Cell pellet was harvested by centrifugation at 3,214 g and 4°C for 5 min, and then adjusted to 10^{10} CFU/mL in sterile 1 g/L peptone water before use.

2. Microencapsulation of probiotics

All glassware and solutions used in this study were sterilized at 121°C for 15 min. Alginate microcapsules were prepared under aseptic conditions following an emulsion method described by Nimrat et al. (2020). The obtained microcapsules were washed threetimes with sterile 1 g/L peptone water, and then stored in sterile amber glass bottles at 10°C for further investigation (see below).

3. Microscopic examination of microcapsules

The dimension of the microcapsules was estimated using a light microscope (Olympus CH30RF200, Kyoto, Japan) connected to a digital camera (Nikon Digital Sight DS-5Mc). Diameters of 120 randomly selected microcapsules were measured in micrometer using an ASMA plug-in for open-source ImageJ software (available at http://rsb-web.nih.gov/ij/).

4. Evaluation of encapsulation efficiency

The encapsulation efficiency was calculated as follows:

$$\text{Encapsulation efficiency (\%)} = \frac{\text{Total probiotic numbers in microcapsule x 100}}{\text{Probiotic numbers added in the preparation process}}$$

5. Viability of free and encapsulated probiotics in acidic and alkaline solutions

Viability of free and encapsulated probiotics under various pH conditions was assessed based on a method described by Sultana et al. (2000) with some modifications. Free and encapsulated probiotics were

placed in a tube containing 10-mL peptone juice solution (0.1% (w/v) peptone, 0.08 M HCl and 0.2% (w/v) NaCl that was adjusted to the following pH values: pH 2.0, 4.0, 6.0, 8.0, and 10.0 using 5 M HCl or 1 M NaOH. During incubation at 30°C, samples were taken at 2, 30, 60, and 120 min-post exposure to evaluate viable cells using a 10-fold dilution plating method as detailed below.

6. Characterization of microencapsulated probiotics during storage

The viability and digestive enzyme activity of free and microencapsulated probiotics were evaluated during storage at 10°C. The un-encapsulated probiotic served as the control was prepared by re-suspending each probiotic strain in sterile 1 g/L peptone water and adjusting to 10^{10} CFU/mL. Free-cell suspension was subjected to the same storage conditions. During storage, the microencapsulated and un-encapsulated samples were withdrawn at 2 h, 7, 15, 30, 45 and 60 days post-storage for the analyses as follows.

6.1 Viability of free and encapsulated probiotics

Viable cells of free and microencapsulated probiotics were counted at each defined sampling interval using a spread plate technique (Truelstrup Hansen et al., 2002). The probiotic-loaded beads were liquefied in phosphate buffer saline (PBS), pH 7.2 with shaking at room temperature for 30 min to release the probiotics from microcapsules. A 10-fold dilution of microencapsulated and un-encapsulated probiotics was prepared in PBS prior to seeding onto Plate Count Agar (PCA; BD Biosciences) at 30°C, 24 h for *Bacillus* probiotics and YPD agar at 25°C, 48 h for yeast probiotic. All colonies grown on the media were counted and calculated as log colony forming unit (CFU) per gram of sample. The experiment was conducted in triplicate.

6.2 Evaluation of digestive enzyme activities

Three broth media were used to produce digestive enzymes of free and encapsulated probiotics. Soluble starch medium contained (g/L): soluble starch 40.0, yeast extract 5.0, KH_2PO_4 7.0, $CaCl_4 \cdot 6H_2O$ 0.3 and $MgSO_4 \cdot 7H_2O$ 0.3, pH 7.0. Composition of tyrosine medium was (g/L): tyrosine 10.0, peptone 5.0, yeast extract 5.0, $CaCl_2 \cdot 2H_2O$ 0.2, $MgSO_4 \cdot 7H_2O$ 0.25 and K_2HPO_4 5.0, pH 7.0. Olive oil medium was composed of (g/L): olive oil 10.0, yeast extract 0.2, $MgSO_4 \cdot 7H_2O$ 0.2, K_2HPO_4 0.9 and $(NH_4)_2SO_4$ 1.3, pH 7.0.

The free and microencapsulated probiotics were cultured in the three-broth media (100 mL) in a shaking incubator at 150 rpm, 30°C for 24 h to evaluate amylase, protease, and lipase activities. Cell suspension was centrifuged at 10,285 g, 4°C for 15 min. Cell-free supernatant was filtered using a 0.45 µm membrane filter (Sartorius, Bedford, Massachusetts, USA) and stored at -20°C until use. All measurements of enzyme activity were performed in triplicate.

Alpha-amylase activity was assayed using the dinitrosalicylic acid method (Bernfeld, 1955). One amylase unit is defined as the amount of enzyme per milliliter culture liberating one microgram glucose per minute. Protease activity was estimated based on a casein method using L-tyrosine as the standard (Walter, 1984). One enzyme unit is defined as the amount of enzyme that release 1 µmole of tyrosine from the substrate (casein) per minute. Lipase activity was assayed following a titrimetric assay using olive oil as the substrate and oleic acid as the standard (Gilbert et al., 1991). One unit of enzyme activity represents the amount of enzyme required to liberate 1 µmole oleic acid per minute.

7. Survival of heat-exposed microencapsulated probiotics

The free and microencapsulated probiotics were heated at 70°C for 60 min and 90°C for 5 min to simulate a condition in the shrimp feeding machine. Then, cell viability was evaluated using a spread-plating technique as previously described. Survival rate of the free and microencapsulated probiotics was calculated as follows:

$$\text{Survival rate (\%)} = \frac{\text{Viable cell number of probiotics after heat treatment x 100}}{\text{Initial number of probiotics}}$$

8. Data analysis

All data are expressed as mean±standard deviation. Normality of the data was tested, and transformation was applied when necessary. The student's *t* test or a One-way ANOVA was used to examine differences of the tested parameters between free and encapsulated probiotics at a significant level of $P<0.05$. All statistical analyses were conducted using the Statistical Package for the Social Sciences software (SPSS version 19.0, Chicago, Illinois, USA).

Results and discussion

1. Microcapsule sizes and encapsulation efficiency

Freshly microencapsulated beads, which contained *Bacillus* probiotics (e.g., *B. megaterium* BUU002, *B. polymyxa* BUU003, and *B. licheniformis* BUU004) and the yeast probiotic *D. hansenii* BUU01, exhibited a

spherical to elliptical shape with a smooth surface (Fig.1). Their diameters were 38.81±11.10, 40.60±11.24, 38.62±11.36, and 41.70±13.26 μm, respectively. Viable cell counts of *B. megaterium* BUU002, *B. polymyxa* BUU003, and *B. licheniformis* BUU004) and *D. hansenii* BUU01 in microcapsules were 9.25±0.08, 9.73±0.05, 9.37±0.12, and 9.28±0.09 log CFU/g, respectively. Encapsulation efficiencies of *B. megaterium* BUU002, *B. polymyxa* BUU003, *B. licheniformis* BUU004 and *D. hansenii* BUU01 were nearly the same, which were 91.49±1.41%, 91.25±1.27%, 90.42±1.64%, and 88.47±1.74%, respectively.

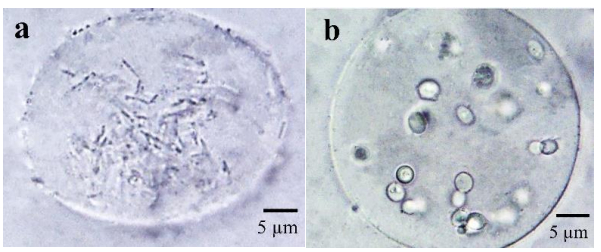

Fig. 1 Micrographs of alginate microcapsules containing (a) a *Bacillus licheniformis* BUU004 strain and (b) a yeast *Debaryomyces hansenii* BUU01. Bar = 5 μm

2. Viability of microencapsulated cells in acidic and basic conditions

Initial viable cell counts of entrapped *B. megaterium* BUU002, *B. polymyxa* BUU003, *B. licheniformis* BUU004, and *D. hansenii* BUU01 at pH between 2.0 and 10.0 were 9.24±0.04 to 9.55±0.03, 9.35 ± 0.04 to 9.58±0.07, 9.35±0.05 to 9.54 ± 0.04, and 9.30±0.05 to 9.55±0.03 log CFU/g, respectively. During incubation at 30°C for 2 h, the viable counts of free and encapsulated probiotics were similar when exposed to pH 6.0 and 8.0 solutions. At pH 2.0, 4.0, and 10.0, the viable cell counts of free and encapsulated probiotics significantly decreased ($P \leq 0.05$) with increasing exposure time. Notably, the survival rates of *Bacillus* and yeast probiotics entrapped in the alginate matrix were significantly higher ($P \leq 0.05$) than those of free cells under the same pH conditions (Fig. 2).

Despite pH values in the intestines of penaeid shrimp between 5.0 and 7.0 (Dall et al., 1990), probiotics are generally faced with pH variations, e.g. post-acidification of the products during storage, and alkaline environment of rearing water of shrimp (Morales & Ruiz, 2016). As such, protective technologies capable of withstanding a wide range of pH variations are necessary to improve the survival of probiotics throughout the processes of preparation, storage, and consumption until they are released to the site of action in the intestines of hosts. In the present study, survival of probiotics in free and microencapsulated forms remained stable under mild conditions at pH 6.0 and 8.0. However, a significantly higher viability of the entrapped probiotics was observed, compared to the unencapsulated cells at more hostile environments (pH 2.0, 4.0, and 10.0). This suggested that alginate encapsulation provided protective action through safeguarding the *Bacillus* and yeast probiotics from exposure to strong acidic and basic solutions. Praepanitchai et al. (2019) reported that survival of the *Lactobacillus plantarum* under hostile environments was significantly enhanced through alginate microencapsulation whereas the free cells did not survive at pH 2.0 after 3 h of incubation. Similarly, Pinpimai et al. (2015) observed that *Saccharomyces cerevisiae*, a baker's yeast, encapsulated in calcium alginate spheres, survived significantly better than free cells when exposed to simulated gastric juice (pH 1.5). Such a phenomenon is likely due to the physical and chemical protective action of alginate encapsulation, which forms a porous hydrogel structure that enhances probiotic survivability (Masoomi Dezfooli et al., 2019; Morales & Ruiz, 2016).

3. Stability of probiotics entrapped in alginate beads during storage

Similar viable counts of free and encapsulated *Bacillus* probiotics were observed, ranging from 8.82±0.01 to 9.92±0.08 log CFU/g during the first 15 days of storage. At 60 days of storage, the viabilities of *B. megaterium* BUU002, *B. polymyxa* BUU003, and *B. licheniformis* BUU004 entrapped in alginate beads remained high, ranging from 8.63±0.03, 8.60±0.03, and 8.68±0.06 log CFU/g, respectively. These values were significantly higher ($P \leq 0.05$) than those of the free cells, which were 4.57±0.10, 4.52±0.13, and 4.56±0.10 log CFU/g, respectively (Fig. 3a–c). Similarly, no significant difference ($P > 0.05$) in viable counts was observed between free and encapsulated *D. hansenii* BUU01 until 45 days of storage. However, at 60-day storage, the viability of encapsulated *D. hansenii* BUU01 was significantly ($P \leq 0.05$) better (8.87±0.02 log CFU/g) than that of free cells (6.82±0.05 log CFU/g; Fig. 3d).

In a previous study, Vega-Carranza et al. (2021) evaluated the protection efficiency of alginate microcapsules during storage at two different temperatures (4 and 25°C). They reported that alginate microcapsules containing *B. licheniformis* stored at

room temperature (25°C) provided minimal protection with cell viability declining over 90% after just 15 days. In contrast, storage at 4°C was optimal to preserve cell viability and probiotic functionality for at least 30 days. The present study exhibited the successful microencapsulation of *Bacillus* and yeast probiotics within the core matrix of calcium alginate spheres, as the viability of probiotic cells remained over 85% after 60 days of storage at 10°C. One of the hypotheses proposed to explain this phenomenon is that alginate can be covalently cross-linked with calcium ion to form a porous capsular structure, which helps protect the cells from stresses, thereby prolonging the viability of probiotics (Masoomi Dezfooli et al., 2019). In the present study, a gradual decrease in survival of *Bacillus* and yeast probiotics encapsulated in alginate spheres was observed as the storage period increased. To enhance the stability of alginate microcapsules, additives such as skim milk and chitosan should be incorporated as coating material to form a strong complex structure with alginate, thereby extending the viability of probiotics. Adilah et al. (2022) reported that incorporation of chitosan into alginate encapsulation significantly improved the viability of *Bacillus subtilis* E20 cells under simulated gastric and intestinal fluid conditions, as well as during storage at various temperatures (4, 25, and 23-37°C). Although the viable counts of microencapsulated probiotics in this study decreased over the storage period, the remaining populations exceeded 8 log CFU/g after 60-day storage, surpassing the threshold required for their application as bio-nutraceuticals in shrimp farming. These findings demonstrate the potential application of alginate-based microencapsulation in maintaining stable product viability during storage.

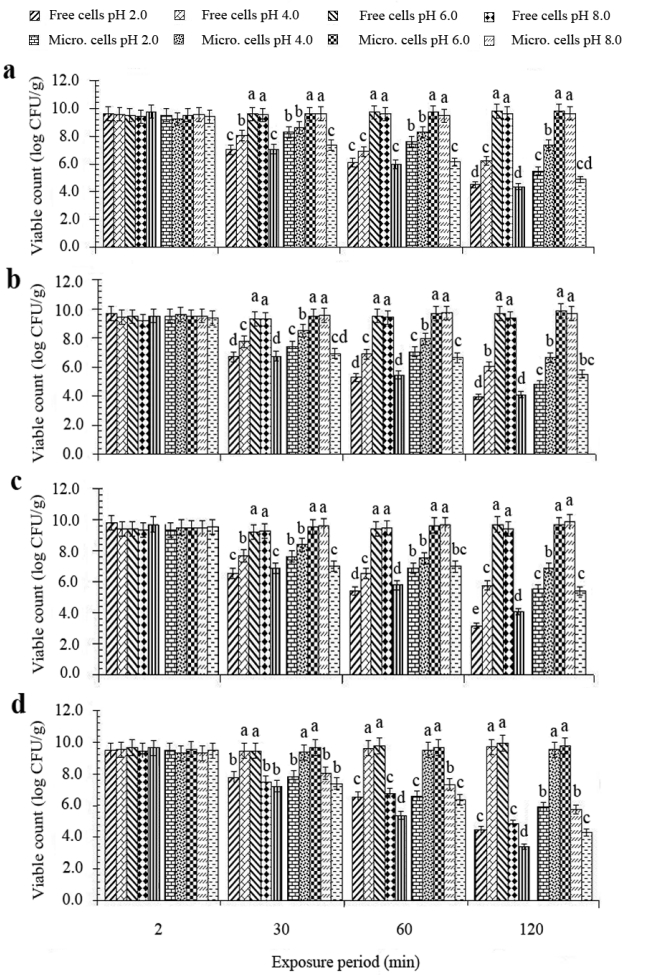

Fig. 2 Viable counts of free and encapsulated cells of (a) *B. megaterium* BUU002, (b) *B. polymyxa* BUU003, (c) *B. licheniformis* BUU004, and (d) *D. hansenii* BUU01 during 120-min incubation in simulated gastric solution at pH between 2.0 and 10.0. Superscript letters indicate significant difference ($P \leq 0.05$)

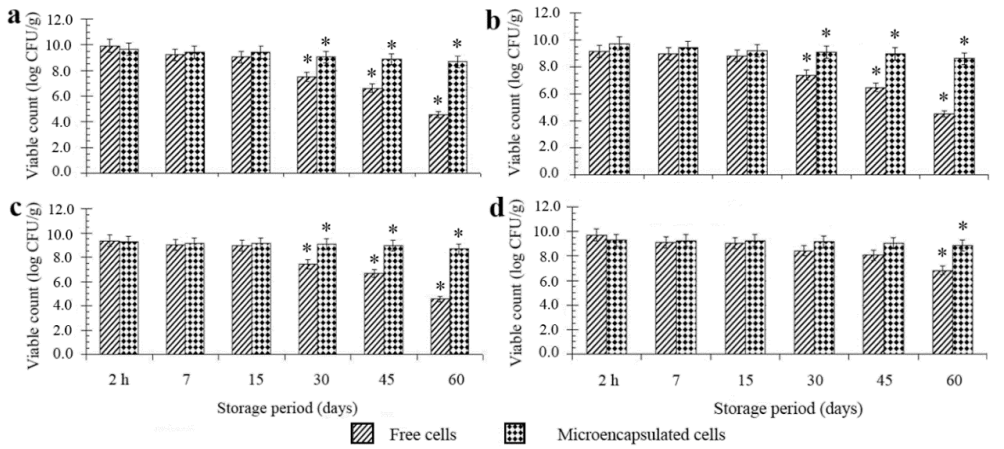

Fig. 3 Viability of free and encapsulated of (a) *B. megaterium* BUU002, (b) *B. polymyxa* BUU003, (c) *B. licheniformis* BUU004, and (d) *D. hansenii* BUU01 during 60-day storage at 10°C. Means with asterisk indicate significant difference ($P \leq 0.05$)

4. Digestive enzyme activities of encapsulated probiotics during storage

Similar alpha-amylase activity was observed in free and encapsulated *B. megaterium* BUU002, *B. polymyxa* BUU003, and *D. hansenii* BUU01 cells during the first 45 days of storage. At 60-day storage, amylase activities of microencapsulated *B. megaterium* BUU002, *B. polymyxa* BUU003, and *D. hansenii* BUU01 were 11.07±0.01, 9.17±0.02, and 2.41±0.01 U/mL, respectively, which were significantly higher ($P \leq 0.05$) than those of free counterparts (9.00±0.03, 7.30±0.04, and 2.17±0.01 U/mL, respectively shown in Fig. 4). For *B. licheniformis* BUU004, a significant decrease ($P \leq 0.05$) in amylase activity was observed in the encapsulated cells after 45 days of storage (Fig. 4).

Protease activity of free *B. megaterium* BUU002 and *D. hansenii* BUU01 significantly declined from 305.29±0.01 and 21.21±0.01 to 300.69±0.14 and 15.60±0.18 U/mL, respectively at 60-day storage (Fig. 5). In contrast, a significant reduction in protease activity of free *B. polymyxa* BUU003 and *B. licheniformis* BUU004 was observed by 45 days of storage (197.44±0.10 and 85.52±0.08 U/mL, respectively), compared to those of entrapped cells (199.79±0.05 and 87.75±0.06 U/mL, respectively).

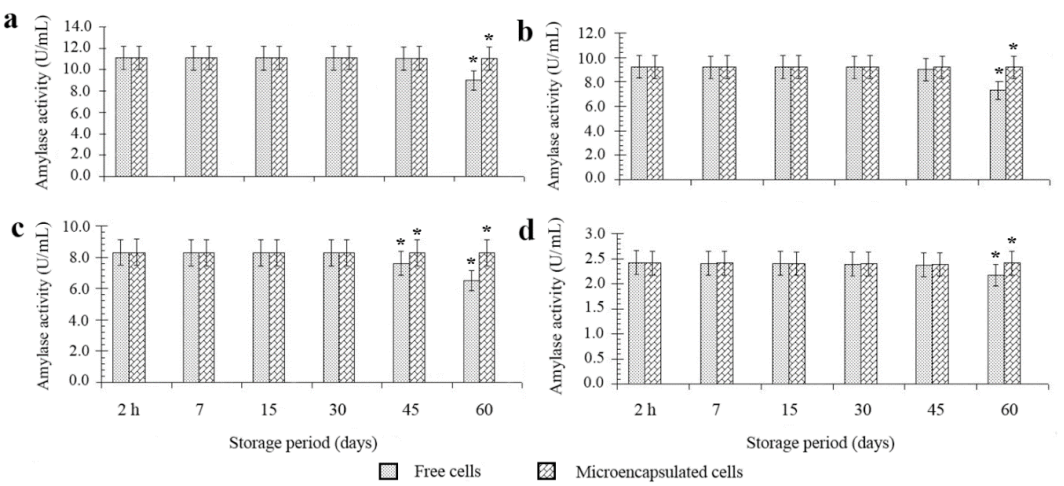

Fig. 4 Amylase activity of free and encapsulated (a) *B. megaterium* BUU002, (b) *B. polymyxa* BUU003, (c) *B. licheniformis* BUU004, and (d) *D. hansenii* BUU01 during 60-day storage. Means with asterisk indicate significant difference ($P \leq 0.05$)

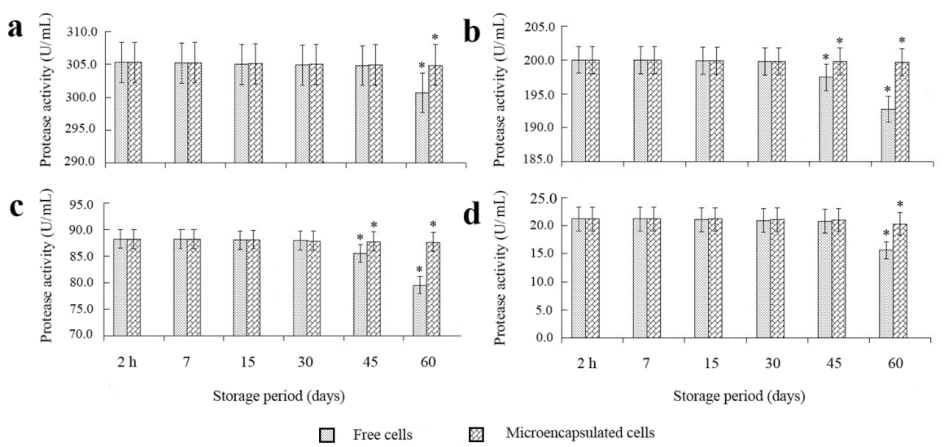

Fig. 5 Protease activity of free and encapsulated (a) *B. megaterium* BUU002, (b) *B. polymyxa* BUU003, (c) *B.licheniformis* BUU004, and (d) *D. hansenii* BUU01 during 60-day storage. Means with asterisk indicate significant difference ($P \leq 0.05$)

Similar to alpha-amylase and protease activities, lipase activity of all free and microencapsulated probiotics remained unchanged during the first 30 days of storage (Fig. 6). At 45-day storage, lipase activities of encapsulated *B. megaterium* BUU002, *B. polymyxa* BUU003, *B. licheniformis* BUU004, and *D. hansenii* BUU01 were 0.36±0.02, 0.30±0.03, 1.03±0.03 and 106.59±0.20 U/mL, respectively. These values were significantly ($P \leq 0.05$) higher than those of the corresponding free cells (0.29±0.02, 0.21±0.01, 0.83±0.03, and 101.28±0.16 U/mL, respectively).

An improvement in digestive enzyme activity reflects the development of the digestive tract, as well as the digestibility and assimilation of nutrients in aquatic animals. Amylase, lipase, and protease activities are well-known to enhance nutrient digestion and absorption, promote the growth of penaeid shrimp, support the molt cycle, and improve environmental adaptability (Duan et al., 2017). Stability of digestive enzymes of probiotics within commercial products is important to ensure beneficial health effects upon delivery to the shrimp's intestines. In this study, digestive enzyme activities-such as amylase, protease, and lipase - of the four probiotics encapsulated in alginate beads showed significant improvement compared to the free cells over a 60-day storage period. Alginate-based encapsulation is widely recognized as a promising method for stabilizing enzyme activity. Encapsulation of enzymes within the Ca-alginate hydrogels protects them from degradation, improves thermal and storage stability, and enables targeted delivery (Weng et al., 2023). Likewise, the entrapment of probiotics in an alginate gel matrix helps shield probiotic cells and their enzymes from harsh environments, such as extreme pH, temperature fluctuations, and toxins (Masoomi Dezfooli et al., 2019). Additionally, a significant decline in viable counts of free probiotic cells may account for such a decrease in digestive enzyme activities in the present study, which is supported by a positive correlation between enzyme activity and probiotic numbers (data not shown). Encapsulated probiotics with stable digestive enzyme activity can be used as functional supplements for shrimp culture. Beneficial effects on nutrient digestibility, feed utilization, and growth performance in shrimp following probiotic administration have been reported by several investigators (Nimrat et al., 2021a; Tsai et al., 2019). However, the *in vitro* results obtained in this study may not fully reflect *in vivo* outcomes. Further investigation, including *in vivo* supplementation of *Bacillus* and yeast probiotics, along with studies on enzyme activity and nutrient digestibility in penaeid shrimp, is needed.

5. Survival of heat-exposed microencapsulated probiotics

All free and encapsulated *Bacillus* probiotics were resistant to heat treatments at 70°C for 60 min and 90°C for 5 min. However, the survival rates of microencapsulated *Bacillus* species exceeded 79% and were significantly ($P \leq 0.05$) higher than those of free cells

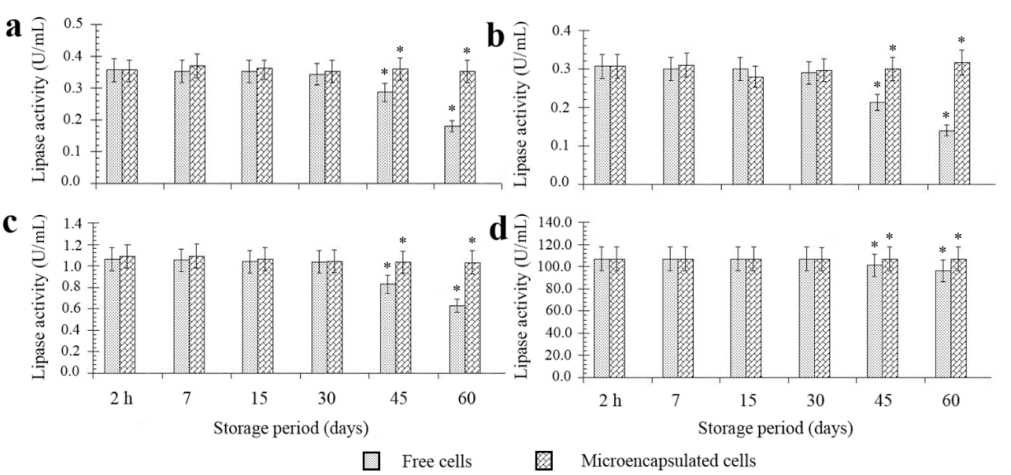

Fig. 6 Lipase activity of free and encapsulated (a) *B. megaterium* BUU002, (b) *B. polymyxa* BUU003, (c) *B. licheniformis* BUU004, and (d) *D. hansenii* BUU01 during 60-day storage. Means with asterisk indicate significant difference ($P \leq 0.05$)

(Table 1). On the other hand, the yeast probiotic, *D. hansenii* BUU01, was highly sensitive to heat treatment. Survival rate of the microencapsulated yeast was significantly improved (22.3±2.4%), while no viable cell of the free yeast was observed after exposure to 70°C for 60 min. However, *D. hansenii* BUU01 cells were completely eliminated after exposure to 90°C for 5 min, even in the microencapsulated form. In accordance with Qi et al. (2019), survival of the yeast *Saccharomyces boulardii* was markedly enhanced through alginate microencapsulation supported by approximately a 25% increase in the cell viability following heat treatment at 75°C and 85°C, compared to non-encapsulated cells. Alginate encapsulation generally protects cells from external stressors including oxygen, chemicals, acids, and interactions with other materials, thereby enhancing stability of their physical and chemical properties during storage.

This study demonstrated that alginate microencapsulation obviously enhanced thermal resistance of *Bacillus* probiotics. However, it provided minimal protection for the yeast probiotic, *D. hansenii* BUU01 during heat treatments at 70°C for 60 min. Therefore, further study is needed to improve the viability of microencapsulated *D. hansenii* BUU01 following heat exposure and to enhance its protection against thermal stresses encountered during feed preparation and storage. Santos and Machado (2021) previously reported that encapsulating *S. boulardii* with alginate and an additional chitosan coating resulted in the highest survival rates, compared to free cells and those encapsulated with sodium alginate alone.

Table 1 Survival rates (%) of free and microencapsulated probiotics after heat treatment

Probiotic species	70°C, 60 min		90°C, 5 min	
	Free	Microcapsule	Free	Microcapsule
B. megaterium BUU002	85.6±3.2^{b}	93.2±3.1^{a}	80.2±3.1^{b}	85.4±2.8^{a}
B. polymyxa BUU003	84.1±2.5^{b}	91.6±2.7^{a}	79.8±2.8^{b}	84.6±2.2^{a}
B. licheniformis BUU004	82.4±2.8^{b}	93.6±2.2^{a}	83.5±2.4^{b}	89.4±3.0^{a}
D. hansenii BUU01	0^{b}	22.3±2.4^{a}	0^{a}	0^{a}

Remark: Superscript letters indicate significant difference ($P \leq 0.05$) at each heat treatment.

Conclusion

In conclusion, this study aimed to improve the viability and beneficial activities of the four aquaculture probiotic strains, including *B. megaterium* BUU002, *B. polymyxa* BUU003, *B. licheniformis* BUU004, and yeast *D. hansenii* BUU01 through alginate microencapsulation. The results suggest that alginate microencapsulation has promising potential for maintaining the viability of *Bacillus* and yeast probiotics under acidic and basic conditions, enhancing digestive enzyme activity (amylase, protease and lipase), improving probiotic stability during 60-day storage, and increasing the survival of heat-exposed probiotics. Consequently, alginate microencapsulation is advisable as a delivery vehicle to ensure probiotics reaching their sites of action in the shrimp gastrointestinal tract. Further investigation is required to evaluate its effects on shrimp health, farm productivity, management practices, and the long-term cost benefits for sustainable shrimp aquaculture.

We declare no conflict of interest pertaining to authorship or research publication of this article.

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