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## Development of functional hemp seed milk enriched with encapsulated probiotics

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**Abstract** Hemp seed milk has recently gained significant attention due to its high protein content and low allergenicity. Proximate analysis of hemp seed milk revealed a composition of 91.51% moisture, 3.33% protein, 0.57% fat, 0.78% ash, and 4.84% carbohydrate, derived from dehulled hemp seeds. The physicochemical properties of the hemp seed milk included a pH of 5.9, total soluble solids of 3°Brix, and a viscosity of 0.047 Pa.s, with no detectable levels of cannabidiol (CBD) and tetrahydrocannabinol (THC). Under *in vitro* simulated gastrointestinal conditions, a combination of alginate and chitosan effectively protected *Lactobacillus rhamnosus* TISTR 372 from simulated gastric juice, and enhanced its release in simulated intestinal conditions. After 120 minutes of exposure, the alginate-chitosan encapsulated probiotics-maintained viability at 10.21 log CFU/g in simulated gastric juice and 6.59 log CFU/g in simulated intestinal juice, whereas encapsulation with alginate alone resulted in complete loss of viability. When encapsulated probiotics were incorporated into hemp seed milk, *L. rhamnosus* TISTR 372 could survive in hemp seed milk for 16 days during refrigerated storage. These findings suggest that the functional hemp milk developed in this study delivers an adequate number of probiotics capable of exerting beneficial effects on gut health.

**Keywords:** Hemp seed milk, Prebiotic, Probiotic, Synbiotic

### Introduction

Consumers are increasingly aware of the environmental and health impacts of food production and consumption. The consumption of animal-based products, particularly dairy, significantly contributes to greenhouse gas emissions, especially carbon dioxide. Consequently, consumers are seeking sustainable alternatives, driving global demand for plant-based proteins, especially non-dairy milk products. This trend is influenced by health-conscious consumers seeking to avoid animal products, those with lactose intolerance or cow's milk allergy, and younger generations looking for new, plant-based options tailored to their lifestyle and nutritional preferences.

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Hemp (*Cannabis sativa* L.) seeds have emerged as a promising plant-based protein source. They contain 25-30% high-quality protein, and equivalent proportion of oil rich in omega-3, along with omega-6 and omega-9 fatty acids. Furthermore, hemp seeds are naturally free of cholesterol and lactose, with a low glycemic index (Hartsel *et al.*, 2016). Importantly, hemp seeds only contain trace levels of cannabidiol (CBD) and tetrahydrocannabinol (THC) (0-0.2%), making them safe for consumption. Interestingly, hemp seed milk is a good choice for people who are allergic to cow's milk, soy, or nut-based milks.

In Thailand, the recent approval of hemp seed use in food products has opened new avenues for the development of high-protein, plant-based alternatives. Given these reasons, it is of interest to develop a synbiotic hemp seed milk-a product combining probiotics and prebiotics for enhanced health benefits. Synbiotics are defined as a combination of synergistically acting live microorganisms (probiotics) and substrates (prebiotics) that are selectively utilized by host microorganisms conferring a health benefit (Gibson *et al.*, 2017; Gibson and Roberfroid, 1995). Prebiotics help sustain probiotic viability during product storage and gastrointestinal transit.

Among probiotic candidates, *Lactobacillus* spp. are widely recognized for their health-promoting effects. However, many probiotic strains, including *Lactobacillus rhamnosus*, are sensitive to lower pH, bile salt, dissolved oxygen, and digestive enzymes. These factors can significantly reduce their viability during food processing, storage, and gastrointestinal passage (Kailasapathy and Chin, 2000).

To address this challenge, the encapsulation technique has been developed to enhance the survival of probiotic bacteria from undesirable environmental conditions. Various coating materials have been used including alginate, chitosan, gelatin, and whey protein (Krasaekoopt *et al.*, 2004; Krasaekoopt *et al.*, 2006). Alginate is the most common coating material used as a biopolymer because of its non-toxicity, ability to form a gel with calcium ions to trap sensitive materials, low cost, acceptance as a food additive, and applicability in dairy preparations (Krasaekoopt *et al.*, 2006). However, using alginate alone is limited by its low stability under highly acidic conditions, causing the release of the core material. Therefore, the addition of other polymers including chitosan as a secondary coating has been shown to improve their stability and enhance encapsulation efficiency (El-Abd *et al.*, 2018).

The objective was to develop a functional hemp seed milk product enriched with encapsulated probiotic *Lactobacillus rhamnosus* TISTR 372. The hemp seed milk product was evaluated for the viability of encapsulated probiotics during 16 days of refrigerated storage.

## **Materials and methods**

### ***Materials***

Whole hemp seeds and dehulled hemp seeds (RPF-1 cultivar) were obtained from the Royal Project Foundation (Thailand). Sodium alginate and calcium chloride were obtained from NerdyGummy (Thailand). De Man Rogosa Sharpe (MRS) media and peptone were obtained from Himedia (India). Phosphate-buffered saline (PBS) solution was obtained from SRL (India). Pepsin, sodium chloride, and bile salts were obtained from Sigma-Aldrich (USA). Chitosan was obtained from Sinudom Agriculture Products Ltd. (Thailand).

### ***Hemp seed milk production***

Dehulled hemp seeds were mixed with water at a 1:5 (w:v) ratio (Wang *et al.*, 2018). The seeds were mixed with blender (Panasonic, Japan) for 5 min and filtered through cheesecloth 4 times. The hemp seed milk was pasteurized at 80 °C for 10 min. The hemp seed milk was kept at 4 °C until further analyzed.

### ***Physicochemical properties of hemp seed and hemp seed milk***

Hemp seeds and hemp seed milk were analyzed for their nutrition values according to the AOAC official method (2000). pH was determined using a pH meter (Sartorius, Germany). Total soluble solids (TSS) was analyzed using a hand refractometer (Atago, Japan). Viscosity was determined using a Brookfield viscometer DV-III. L\*a\*b\* values were determined using a colorimeter (Konica Minolta, Japan). Cannabidiol (CBD) and tetrahydrocannabinol (THC) were determined using high-performance liquid chromatography (HPLC) according to the AOAC official method 2018.11 (2018).

### ***Probiotic culture***

The experiment used *Lactobacillus rhamnosus* TISTR 372 probiotic bacteria obtained from Thailand Institute of Scientific and Technological Research (TISTR). *L. rhamnosus* TISTR 372 was cultured in MRS medium and incubated at 37 °C for 24 h until the initial probiotic bacteria count reached 10<sup>14</sup> CFU/mL.

### ***Microencapsulation preparation***

To encapsulate probiotic *L. rhamnosus* TISTR 372, 40 mL of 2% (w/v) sodium alginate solution was prepared, and then 1% of a prebiotic (inulin) was added to the solution. A homogenizer was used to mix the solution for 15 minutes at a speed of 10,000 rpm. Then, 10 ml of the probiotic culture (with an initial count of  $10^{14}$  CFU/mL) was added, and the mixture was mixed thoroughly for 15 minutes at a speed of 150 rpm. The encapsulation process was performed via extrusion. The mixture was drawn into a syringe with an 18G needle and dropped into a 2% (w/v) of calcium chloride bath to form synbiotic microcapsules. Then, encapsulated probiotics were washed twice with PBS.

Chitosan was dissolved in 100 mL of distilled water acidified with glacial acetic acid to get a final chitosan concentration of 1 % (w/v) and the pH was adjusted to 5.7 (Khimmakthong *et al.*, 2020). The encapsulated probiotics were incubated in chitosan solution for 40 min. After that, the encapsulated probiotics was washed with PBS twice and stored at 4 °C until further use.

### ***In vitro digestion of encapsulated probiotic (L. rhamnosus TISTR 372)***

The survival rate of the microbeads was determined after exposure to simulated gastrointestinal conditions. The solution was prepared by using 9 g/L of NaCl and 3 g/L of pepsin (Chávarri *et al.*, 2010). Then, the mixture was adjusted to pH 2. One gram of microbeads was added to 9 ml of PBS solution and incubated at 37 °C for 0, 30, 60, 90, and 120 min. The plate count technique was then performed to determine viable bacterial counts and expressed as log CFU/g.

Simulated intestinal juice was prepared in PBS (pH 8.0) containing 3 mg/mL pancreatin and 1 % bile salts (Krasaekoopt *et al.*, 2004). The samples were incubated at 37 °C for 0, 30, 60, 90, and 120 min. Cell counting was performed and reported as CFU/g. All simulated solutions were prepared using analytical grade reagents under aseptic conditions.

### ***Encapsulated L. rhamnosus TISTR 372 survival in hemp seed milk***

*L. rhamnosus* TISTR 372 microcapsules at a concentration of 5% were added to pasteurized hemp seed milk and kept at 4 °C for a 16-day test duration. Microcapsules were collected on days 0, 4, 8, 12, and 16. One gram of microcapsule beads was washed with sterile distilled water twice. Subsequently, 0.01% peptone was added and the beads were ground with a glass rod to release the probiotics from the capsules. Released bacteria were counted using the pour

plate method in MRS agar at 37 °C for 48 h. Cell counting was performed and reported as CFU/g.

### ***Statistical analysis***

All experiments and analyses were performed in triplicate. Data were expressed as mean  $\pm$  standard deviation (SD) and were subjected to one-way analysis of variance (ANOVA). Multiple comparisons were performed using Duncan's multiple range test. A  $p$ -value of  $< 0.05$  was considered statistically significant. The analysis was conducted using SPSS version 17.

## **Results**

### ***Proximate analysis of dehulled hemp seeds and hemp seed milk***

The proximate composition analysis revealed that the dehulled hemp seeds used in this study contained on average 8.07% moisture, 0.15% ash, 23.15% fat, and 39.87% protein. In comparison, the prepared hemp seed milk contained 3.33% protein, 0.57% fat, and 0.78% ash, as shown in Table 1.

**Table 1.** Proximate analysis of dehulled hemp seeds and hemp seed milk

| Parameters       | Dehulled hemp seeds (%) | Hemp seed milk (%) |
|------------------|-------------------------|--------------------|
| Moisture content | 8.07 $\pm$ 0.00         | 91.51              |
| Protein          | 39.87 $\pm$ 1.82        | 3.33               |
| Fat              | 23.15 $\pm$ 0.03        | 0.57               |
| Ash              | 0.15 $\pm$ 0.00         | 0.78               |
| Fiber            | 18.70 $\pm$ 0.65        | -                  |
| Carbohydrate     | 10.07 $\pm$ 2.18        | 4.84               |



**Figure 1.** Dehulled hemp seeds

### ***Physicochemical properties of hemp seed milk***

The prepared hemp seed milk had a pH of 5.9, a total soluble solids (TSS) content of 3°Brix, and a viscosity of 0.047 Pa.s, no CBD or THC compounds were detected in the hemp seed milk.

### ***Viability of the encapsulated *Lactobacillus rhamnosus* TISTR 372 after *in vitro* digestion***

The viable cell counts during incubation at 37 °C in simulated gastric juice (pH 2) and intestinal juice (pH 8) are shown in Table 2. When encapsulated with a combination of alginate and chitosan, *L. rhamnosus* TISTR 372 successfully survived after 120 min of exposure to *in vitro* gastric conditions. Encapsulated *L. rhamnosus* with alginate and chitosan exhibited higher survival rates in simulated gastrointestinal conditions compared to those encapsulated with alginate alone. Exposure to simulated intestinal juice containing 1% bile salts significantly reduced the viability of the encapsulated probiotics, with notable decline occurring within the first 30 minutes of exposure. Complete loss of cell viability in simulated intestinal conditions was observed in probiotics encapsulated with alginate alone.

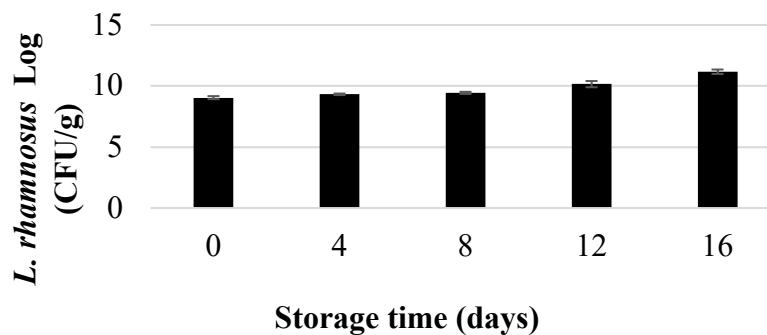
**Table 2.** Number of surviving cells (log CFU/g) of encapsulated *L. rhamnosus* during incubation at 37 °C in simulated gastric juice (pH 2) and intestinal juice (pH 8)

| Treatment           | Simulated gastric juice    |              |              |              |              |
|---------------------|----------------------------|--------------|--------------|--------------|--------------|
|                     | 0 min                      | 30 min       | 60 min       | 90 min       | 120 min      |
| alginate            | 7.97 ± 0.22                | 7.92 ± 0.27  | 7.57 ± 0.14  | 6.70 ± 0.15  | 0.00 ± 0.00  |
| alginate + chitosan | 10.01 ± 0.10               | 10.12 ± 0.17 | 10.15 ± 0.26 | 10.25 ± 0.20 | 10.21 ± 0.10 |
| Treatment           | Simulated intestinal juice |              |              |              |              |
|                     | 0 min                      | 30 min       | 60 min       | 90 min       | 120 min      |
| alginate            | 0.00 ± 0.00                | 0.00 ± 0.00  | 0.00 ± 0.00  | 0.00 ± 0.00  | 0.00 ± 0.00  |
| alginate + chitosan | 10.21 ± 0.10               | 7.52 ± 0.28  | 6.70 ± 0.31  | 6.66 ± 0.12  | 6.59 ± 0.50  |

### ***The viability of encapsulated *Lactobacillus rhamnosus* TISTR 372 in hemp seed milk during storage at 4 °C for 16 days***

The viable cell counts determined using standard culture methods are presented in Figure 2. In the hemp seed milk beverage, the initial concentration of encapsulated *L. rhamnosus* TISTR 372 was 9.04 log CFU/g.

The viability of the *L. rhamnosus* TISTR 372 strain was well-maintained, remaining above the therapeutic threshold of 6 log (CFU/g) until day 16 of storage. Additionally, no coliforms or *E. coli* were detected in the hemp seed milk containing the encapsulated probiotics. These results indicate that the encapsulated probiotics exhibited strong survivability in the hemp seed milk throughout the refrigerated storage period.



**Figure 2.** Number of surviving cells (log CFU/g) for encapsulated *L. rhamnosus* in hemp seed milk during 16 days of refrigerated storage

## Discussion

The values of the proximate analysis were similar to other studies (Callaway, 2004; Gram and Mortas, 2023). Typical hemp seed milk produced by mixing ground hemp seeds with water at a ratio of 1:5 (w/v) has been reported to contain 4% protein and 5% fat (Wang *et al.*, 2018). The protein content of the hemp seed milk in this study was within the range of previous reports. However, the fat content of hemp seed milk was noticeably lower than in previous reports. The physicochemical properties of hemp seed milk were similar to a previous study (Thakur *et al.*, 2024). Variations in the proximate components of hemp seed milk may depend on the seed variety, geographical origin, and climatic conditions (Karabulut and Yemiş, 2022).

For probiotics to exert their beneficial effects in the intestine, they have to survive the harsh conditions of the host's hostile digestive tract (Maragkoudakis *et al.*, 2006). In the stomach, the primary obstacles to probiotic viability are the low pH and the antimicrobial activity of pepsin. In addition, probiotic bacteria need to survive the environment in the small intestine, where they are exposed to pancreatin and bile salts at a pH of approximately 8.0. Probiotics have a lower tolerance to bile than to gut acid, which agrees with in previous study (Aboulfazli and Baba, 2015). The viability data indicated that the alginate-chitosan encapsulated beads could enhance the protection, likely due to reduced acid

penetration and stronger interactions between alginate and chitosan (Jumazhanova *et al.*, 2023). Although alginate is widely regarded as a suitable material for probiotic encapsulation, it presents some limitations. One of the primary restrictions is its high porosity, which results from its open lattice structure and broad pore size distribution, making cell retention challenging (Jumazhanova *et al.*, 2023). This structural characteristic can result in the premature release of cells into the medium, leading to reduced initial cell concentrations (Lee and Mooney, 2012). However, these limitations can be effectively overcome by combining alginate with other materials or applying a secondary coating, such as chitosan (Khimmakthong *et al.*, 2020). The use of alginate-chitosan capsules provides a protective barrier, enhancing the targeted delivery of probiotics to the intestinal tract.

An important parameter for assessing the quality of health-promoting beverages is determining the viability of lactic acid bacteria throughout the entire shelf life (Skryplonek and Jasinska, 2016). It has been suggested that a daily intake of 6-7 log (CFU/g) of viable probiotic cells is necessary to confer health benefits (World Health Organization, 2003). In support of these findings, a previous study determined the increased survival rates of non-encapsulated *Lactobacillus casei* subsp. *rhamnosus* in hemp seed milk. After the fermentation process, the total count of viable lactic acid bacteria (LAB) cells increased from 8.41 log (cfu/mL) to 10.92 log (cfu/mL) in hemp seed milk; however, after storing non-fermented hemp seed milk at 4 °C, it was found that LAB cells decreased from 8.41 log (CFU/mL) to 3.54 log (CFU/mL) (Szparaga *et al.*, 2019). These findings suggest that the encapsulation process in this study played a protective role in maintaining probiotic viability.

This study has successfully developed a functional hemp seed milk enriched with encapsulated probiotics (*L. rhamnosus* TISTR 372). The extrusion technique can be effectively used for probiotic encapsulation, with alginate-chitosan serving as coating materials that offer enhanced protection to the probiotics during gastrointestinal transit. Moreover, the synbiotic alginate-chitosan encapsulated beads significantly improved the stability of *L. rhamnosus* TISTR 372 in hemp seed milk during 16 days of refrigerated storage. Additionally, the final product maintained a viable probiotic number exceeding 10<sup>6</sup> CFU/g, achieving the recommended level for potential health benefits.

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## Conflicts of interest

The authors declare no conflict of interest.

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