



Probiotic encapsulation in CaCO_3 microparticles: Enhancement of viability under heat treatments and simulated gastrointestinal conditions

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ABSTRACT

The aim of this study was to evaluate the effect of microencapsulation on the viability and stability of *Lactobacillus plantarum* TISTR 543 using calcium carbonate microparticles (CaCO_3 -MPs) produced by co-precipitation. Free bacterial and encapsulated cells were freeze-dried with or without lactose and skim milk as lyoprotectants. The survivability of encapsulated probiotics was evaluated under different heat treatments, during storage, and under simulated gastrointestinal conditions, along with their intestinal adhesion to Caco-2 cells and their inhibition of *Escherichia coli* adhesion. The results showed that *L. plantarum*-encapsulated CaCO_3 -MPs were efficient, enabling the recovery of a high number of entrapped cells. Moreover, the presence of lyoprotectants significantly enhanced the survival rate of encapsulated *L. plantarum* during lyophilization ($P < 0.05$). The viability of the encapsulated probiotics was maintained at over 10^6 CFU/g under thermal conditions and simulated gastrointestinal conditions. In the adhesion experiment, encapsulated *L. plantarum* exhibited a concentration-dependent ability to adhere to Caco-2 cells. Encapsulated probiotics also effectively reduced *E. coli* in exclusion and competitive adherence tests. CaCO_3 -MPs serve as a robust delivery platform for protecting probiotics under various hostile conditions while maintaining probiotic viability in the gastrointestinal tract. These CaCO_3 -MPs can be included in suitable functional food products.

1. Introduction

Probiotics are live microorganisms widely regarded as having beneficial health effects when consumed appropriately (FAO/WHO, 2002). In the last two decades, interest in probiotics has consistently increased due to their benefits in improving intestinal health, enhancing immunological functions, reducing blood cholesterol levels, preventing and treating cancer, and modulating the human microbiome (Das et al., 2022; Ibrahim et al., 2023).

Live probiotics are naturally present in many fermented foods, such as traditional rice-based products, including appam, dairy-based items, such as yogurt and cheese, vegetable-based products, such as sauerkraut and kimchi, and fish-based foods including ngari and Som-fug (Dey, 2018; Ibrahim et al., 2023; Sivamaruthi et al., 2022). The fortification of processed foods and nutraceutical formulations offers an alternative method to deliver sufficient amounts of living probiotic cells orally. These fortified products have been applied to various food ingredients to

produce functional foods. The recommended minimum dose of probiotic bacteria in food products for conferring beneficial health effects at consumption is 10^6 CFU/g (Shori, 2016). However, the viability and survival rate of probiotics in functional foods (during processing and storage) and the gastrointestinal passage remain a significant challenge (Terpou et al., 2019).

Microencapsulation of probiotic bacteria is an efficient and novel technique for protecting probiotics from environmental degradation and enabling controlled release under specific conditions through entrapment within the matrix of carrier materials (Yao et al., 2020). Its goal is to shield probiotics from the harsh conditions encountered in food products during food processing and passage through the intestinal tract, such as low pH, bile salts, and other digestive products (Arenas-Jal et al., 2020; De Prisco & Mauriello, 2016). Nowadays, there are several microencapsulation methods, such as spray drying and alginate gelation, which show promise in maintaining the survival of probiotics through the gastrointestinal conditions and their use in food products

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(He et al., 2021; Obradović et al., 2022; Pupa et al., 2021). Spray drying is one of the most used techniques, since it is scalable, effective, and yields low moisture content, making it suitable for food storage. However, its high temperatures and energy costs are significant limitations, resulting in lower viability (Ngamekaue et al., 2024). Although alginate gelation is biocompatible and effective in preserving probiotics, the presence of bacteria in the gel can cause porosity formation in the matrix. This porosity may hinder the gels' capacity to maintain probiotics by facilitating the diffusion of H⁺ ions into the gels (Zhu et al., 2021). The effectiveness of these methods under different thermal conditions and their ability to protect probiotics through the entire gastrointestinal tract, especially in the harsh gastric environment, remain insufficiently explored. An innovative technique in encapsulation is needed to address these challenges, yielding a high survival rate of probiotics in the presence of different stress conditions.

Calcium carbonate microparticles (CaCO₃-MPs) represent a promising alternative for probiotic encapsulation. Biocompatible and biodegradable CaCO₃-MPs serve as versatile matrices for polymer-based carriers and drug encapsulation, as well as function as a food additive and pharmaceutical ingredient due to their abundance and cost-effectiveness (Hickman et al., 2019; Tan et al., 2022). CaCO₃ is stable under normal pH but dissociates in acid environments, so it is commonly used in antacids (Jung et al., 2021). Previous studies have revealed this pH sensitivity. *Bifidobacterium pseudocatenulatum* G7 and *L. acidophilus* have been encapsulated in microgels with the addition of CaCO₃ as an antacid to neutralize the gastric acids. This enhanced the probiotic survival under the simulated environments Afzaal et al., 2023; Gu et al., 2019). Moreover, the functional properties of CaCO₃ particles, such as their ability to fortify calcium and neutralize acids, can be further enhanced by converting these particles into absorbable calcium ions upon exposure to gastric acid (Guo et al., 2021).

Motivated by the excellent encapsulation of CaCO₃ as protective and delivery systems for probiotics, as well as food-grade encapsulating agents, to function as calcium fortification, *Lactobacillus plantarum* was selected as a model strain. The use of CaCO₃-MPs for encapsulating *L. plantarum* is based on their ability to neutralize stomach acid, creating a more favorable pH environment for the encapsulated probiotics. This neutralization protects the probiotics from harsh acidic conditions, thus improving their viability (Geng et al., 2023). However, while CaCO₃ protects probiotics during the gastric phase, it also dissolves in the stomach. This suggests that the release of probiotics occurs primarily in the stomach via decomposition, which may prevent them from reaching the intestines.

The present study highlights the challenges in maintaining the viability of probiotics during various stages of food processing, storage, and gastrointestinal conditions. Despite the fact that various encapsulation methods for probiotics have been explored, the study of CaCO₃-single-coated probiotics has recently gained attention, as previously reported by (Lee et al., 2024). The work reported has focused on morphology characteristics, survivability, and stability of probiotics under simulated gastrointestinal conditions; however, there still limited studies on the effectiveness of CaCO₃-MPs under thermal and storage. Therefore, this study aims to address these gaps and explore the morphology of probiotics encapsulated in CaCO₃-MPs, the impact of lyoprotectants on their viability during freeze-drying, and the resistance and survival of these encapsulated bacteria under various food processing conditions. The objective of this study is to assess the effectiveness of CaCO₃-MPs in improving the survivability of *L. plantarum* under various stress factors encountered during food processing, storage, and ingestion. In addition, the adhesion of the encapsulated bacteria to intestinal cell lines was assessed.

2. Materials and methods

2.1. Materials

Calcium carbonate (CaCl₂), sodium bicarbonate (NaHCO₃), and anhydrous sodium carbonate (Na₂CO₃) were obtained from Merck KGaA (Darmstadt, Germany). Triton X and phosphate buffered saline (PBS) were obtained from KemAus™ Chemicals Elago Enterprises Pty Ltd. (New South Wales, Australia). Sodium hydroxide (NaOH), sodium chloride (NaCl), De Man–Rogosa–Sharpes (MRS) agar, MRS broth, McConkey agar, nutrient broth (NB), and skim milk were obtained from HiMedia Laboratories Pvt. Ltd. (Mumbai, India). Lactose monohydrate was supplied by Sisco Research Laboratories (SRL) (Maharashtra, India). Dulbecco's Modified Eagle Medium (DMEM), Fetal bovine serum (FBS), streptomycin, penicillin, non-essential amino acids (NEAA), and sodium pyruvate were obtained from Gibco (Thermo Fisher Scientific, Waltham, MA, USA). Other chemicals used were of analytical grade and were directly used as received without further purification.

2.2. Microorganisms and cultivation

L. plantarum strain TISTR 543 was provided by the Thailand Institute of Scientific and Technological Research (TISTR) and cultivated in MRS broth at 37 °C for 24 h under anaerobic conditions.

2.3. Encapsulation of probiotics in CaCO₃

The production of CaCO₃ microparticles described by Vikulina (Vikulina et al., 2021) was adopted to encapsulate bacteria. *L. plantarum* cells were incubated in MRS broth at 37 °C for 18 h and then centrifuged at 5000×g for 10 min to isolate the cell pellet. The cells were washed twice and resuspended in sterile NaCl (9 g/L). The suspension was diluted to obtain 12 log CFU/mL with an optical density at 600 nm (OD₆₀₀) = 0.175–0.18. The cells were harvested by centrifugation (5000×g for 10 min) and suspended in Na₂CO₃ solution. After that, CaCl₂ was added to the mixture at an equal molar ratio and allowed to stand for 30 min at room temperature (RT) to produce *L. plantarum*-CaCO₃-MPs, with the concentrations of the Na₂CO₃ and CaCl₂ solutions being either 0.33 mol/L or 0.66 mol/L. The microparticle suspension was washed three times with sterile saline solution (9 g/L) by centrifugation at 3000×g for 5 min, followed by resuspension in a lyoprotectant solution.

The control CaCO₃-MPs were prepared following the same procedures without the addition of *L. plantarum*.

2.4. Enumeration of the viable probiotic cells

The viability of the *L. plantarum* entrapped in CaCO₃-MPs was evaluated. The lyophilized microparticles were resuspended in a 10-fold dilution in sterile acetate buffer (0.1 mol/L, pH 5.0) and vortexed until they were completely dispersed; after that, 30-µL aliquots of the dilute solution were plated in triplicate on the surface of MRS agar. All plates were incubated at 37 °C for 48 h in an anaerobic chamber before the encapsulated bacteria were counted as colony forming units per gram of microparticles (CFU/g). All samples were analyzed in three replicates (n = 3). The encapsulation efficiency (EE) was calculated according to (Maciel et al., 2014), and is presented in Eq. (1):

$$\text{EE (\%)} = (N/N_0) \times 100\%, \quad (1)$$

where N₀ is the initial number of free viable probiotic cells added to the mixture solution during co-precipitation, and N is the number of probiotic cells loaded in the microparticles.

2.5. Lyophilization of encapsulated cells and cell suspension of bacteria

Optimizing the probiotic production process is crucial for obtaining a product with desirable properties and an increased number of probiotic microorganisms. The suspension of free cells and microparticles were mixed with or without the combination of lyoprotectants (lactose (120 g/L) and reconstituted skim milk (60 g/L)) and frozen at -40 °C for at least 4 h (Juárez Tomás et al., 2015). Last, the samples were freeze-dried using a vacuum freezing dryer (Christ Alpha 2–4 LSCbasic, Osterode am Harz, Germany).

2.6. Morphological characterization of the microcapsules

2.6.1. Zeta potential measurements

The particle size and zeta potential of both free bacteria and the microencapsulated cells were assessed using a Zetasizer Nano ZSP (Malvern Instruments Ltd., Worcestershire, U.K.). The size and polydispersity index (PDI) analyses were conducted at 25 °C using a disposable sizing cuvette, while the zeta potential was determined using a reusable folded capillary zeta cell. Each value obtained represented the average of three parallel measurements from three batches.

2.6.2. Confocal microscopy

Probiotics were labelled with fluorescent dye by incubation with fluorescein isothiocyanate (FITC) at a final concentration of 1 mg/mL in PBS at pH 7.4 under agitation at RT with light protection for 1 h. Then, the bacterial suspension was centrifuged (5000×g for 10 min). After rinsing three times, aliquots of the samples were encapsulated in CaCO₃-MPs as described above. Both FITC-labelled free cells and FITC-labelled probiotics-encapsulated CaCO₃-MPs were observed under a Zeiss LSM 900 Airyscan confocal microscope (Zeiss, Oberkochen, Germany) under 488-nm laser excitation.

2.6.3. Field emission scanning electron microscope (FE SEM) with energy dispersive spectrometer (EDS)

Morphology and composition of samples were collected using a JMS-6335F electron microscope (JEOL, Tokyo, Japan) at a 15 kV accelerating voltage. The samples were prepared by depositing the particle suspension on a glass slide, followed by drying for 1 h at 45 °C. Subsequently, a thin layer of Au/Pd (5 nm thickness) was applied to the electrically conductive surface for optimal imaging. Micrographs were obtained using SEM at magnifications ranging from × 250 to × 6000.

2.7. Survivability of *L. plantarum* under simulated food processing conditions

2.7.1. Different heat treatments

Lyophilized free cells and *L. plantarum*-encapsulated CaCO₃-MPs were resuspended in sterilized NaCl solution (9 g/L) and then subjected to three different heat treatments, as described by Mahmoud et al. (Mahmoud et al., 2020): 1) exposure to a high incubation temperature (40 °C for 24 h); 2) application of heat typical in cheese production processes (45 °C for 30 min); and 3) subjecting the solution to pasteurization conditions (65 °C for 30 min). Finally, the viable cell count was determined using the plate count technique.

2.7.2. Different storage conditions

The lyophilized free cells and *L. plantarum*-encapsulated CaCO₃-MPs were packaged in airtight containers and stored at temperatures of -18 ± 2, 4 ± 2, and 25 ± 2 °C, as described by (Minj & Anand, 2022). During storage, the samples were examined for cell viability after 15, 30, and 60 days. The probiotic cell viability during storage was analyzed using the plate count technique.

2.8. Survival in gastrointestinal tract conditions and simulated intestinal fluid

Simulated gastric and intestinal fluids were prepared according to the method described by (Minekus et al., 2014), with some modifications. Briefly, the probiotic *L. plantarum* was added in both free and encapsulated forms (concentration approx. 10⁹–10¹⁰ CFU/g) to simulated gastric fluid (pepsin (3 g/L) and NaCl (5 g/L), dissolved in distilled water). The pH of simulated gastric fluid (SGF) was adjusted to value of 3.0 with 2 M HCl. The simulated intestinal fluid (SIF), consisting of NaHCO₃ (6.4 g/L), NaCl (1.28 g/L), KCl (0.239 g/L), bile salts (3.0 g/L), and pancreatin (1.0 g/L) dissolved in distilled water, was prepared with pH 7.0 (adjusted with NaOH). The samples were then incubated in SGF for 2 h at 37 °C. After the incubation, the supernatant was removed by centrifuged (7000×g for 5 min), and subsequently, SIF was added to resuspend the sediment. The suspensions were further incubated for 3 h at 37 °C. After incubation period, the initial *L. plantarum* count (0 h) and the counts after incubation in SGF (1 and 2 h) and SIF (3, 4, and 5 h) were determined using a drop plate technique to assess the bacterial survival in the gastric environment. All tests were conducted in triplicate.

2.9. In vitro acid neutralization capacity

To investigate the potential of the CaCO₃-MPs to neutralize gastric acid over time, the *in vitro* acid neutralization capacity of *L. plantarum*-encapsulated CaCO₃-MPs was evaluated by adding 10 µL of a suspension containing 15 mg/mL of *L. plantarum*-encapsulated CaCO₃-MPs into 10 mL SGF every minute for 1 h according to the protocol described by (Geng et al., 2023). Change in pH value of samples was recorded using a pH meter (Mettler-Toledo LLC, Columbus, OH, USA).

2.10. Adhesion of microencapsulated cells to epithelial cells

The adhesion of microencapsulated *L. plantarum* to the human colon adenocarcinoma, Caco-2 cells, was determined as previously reported by (Piatek et al., 2012) with slight modifications. The Caco-2 cell line was grown in DMEM medium supplemented with 10% heat-inactivated FBS and 1% each of NEAA, sodium pyruvate, and streptomycin and penicillin. When 90% confluence was reached, they were seeded to differentiate in 96-well plates at 37 °C in a humidified atmosphere of 95% air with 5% CO₂ for 15 days. The culture medium was changed every other day (Piatek et al., 2012).

The encapsulated cells were grown in 10 mL of MRS broth for 18 h under anaerobic conditions at 37 °C, followed by centrifugation (5000×g for 10 min). The bacterial cells were washed and suspended in DMEM medium (without FBS and antibiotics). The media was removed, and the Caco-2 cells were rinsed twice with PBS before bacterial cells were added (1 × 10⁷, 1 × 10⁸, and 1 × 10⁹ CFU/g). Following an incubation period of 1 h at 37 °C in 5% CO₂ in air, the plates were washed three times with PBS to eliminate the non-adhering bacteria. The monolayers were treated with 0.5% Triton X-100 in PBS to separate the Caco-2 monolayer and the adhered bacteria from the well. Lysis was performed on ice for 3 min. The microencapsulated *L. plantarum* cells were diluted and plated on MRS agar to quantify the number of adhered bacteria (Zhao et al., 2012).

2.11. Inhibition of *Escherichia coli* adhesion by probiotic bacteria

The *Escherichia coli* strain TISTR 073 used was grown overnight in NB medium at 37 °C with shaking. The overnight culture was then centrifuged at 5000×g for 10 min, and the bacterial pellet was washed twice with NaCl (9 g/L). The *E. coli* cells were resuspended in PBS to achieve an OD₆₀₀ of 0.5, which corresponds to approximately 10⁸ CFU/mL. The bacterial cells were centrifuged at 5000×g for 10 min, and the bacterial pellet was suspended in DMEM medium (without FBS and

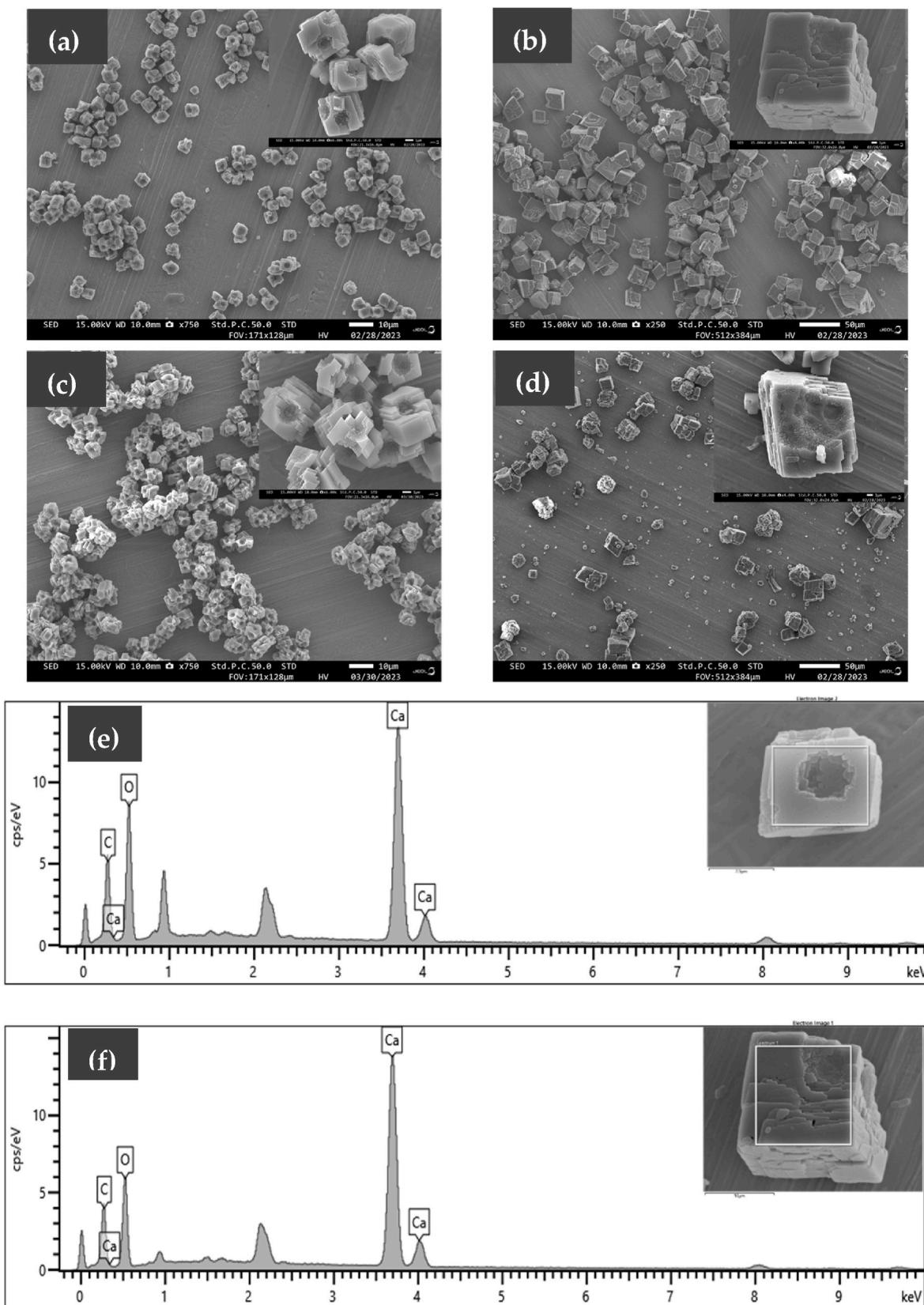


Fig. 1. Characterization of CaCO_3 -MPs in the absence and presence of $L. plantarum$ obtained with different $\text{Na}_2\text{CO}_3/\text{CaCl}_2$ concentrations. Representative SEM images of (a) control CaCO_3 -MPs at 0.33 mol/L $\text{Na}_2\text{CO}_3/\text{CaCl}_2$ concentration; (b) $L. plantarum$ -encapsulated CaCO_3 -MPs at 0.33 mol/L $\text{Na}_2\text{CO}_3/\text{CaCl}_2$ concentration; (c) control CaCO_3 -MPs at 0.66 mol/L $\text{Na}_2\text{CO}_3/\text{CaCl}_2$ concentration; (b) $L. plantarum$ -encapsulated CaCO_3 -MPs at 0.66 mol/L $\text{Na}_2\text{CO}_3/\text{CaCl}_2$ concentration. SEM micrographs and EDX spectra of (e) control CaCO_3 -MPs using 0.33 mol/L $\text{Na}_2\text{CO}_3/\text{CaCl}_2$ concentration; (f) $L. plantarum$ -encapsulated CaCO_3 -MPs using 0.33 mol/L $\text{Na}_2\text{CO}_3/\text{CaCl}_2$ concentration. (Ca, calcium; C, carbon; O, oxygen).

Table 1

Effect of $\text{Na}_2\text{CO}_3/\text{CaCl}_2$ concentration on particle size, zeta potential of CaCO_3 -MPs by DLS, and encapsulation efficiency (EE) of *L. plantarum*-encapsulated CaCO_3 -MPs.

$\text{Na}_2\text{CO}_3/\text{CaCl}_2$ concentration	Sample name	Z-Average Diameter (μm)	Zeta Potential (mV)	EE (%)
–	Free <i>L. plantarum</i>	1.7 ± 0.75	−7.19 ± 1.37	–
0.33 mol/L	Control CaCO_3 -MPs	3.5 ± 0.8	−18.92 ± 5.30	–
	<i>L. plantarum</i> -encapsulated CaCO_3 -MPs	5.15 ± 2.15	−14.54 ± 7.47	70.46
0.66 mol/L	Control CaCO_3 -MPs	10.83 ± 3.98	−23.26 ± 2.06	–
	<i>L. plantarum</i> -encapsulated CaCO_3 -MPs	21.27 ± 10.06	−12.96 ± 3.13	ND

Each value represents the average from three independent experiments ± standard deviation. (ND, not detected.)

antibiotics). This prepared suspension of *E. coli* was then used in the adhesion assay to evaluate the inhibitory effect of encapsulated *L. plantarum*.

The microencapsulated *L. plantarum* cells were tested on Caco-2 cell monolayers in 96-well plates for comparison purposes. Three different types of experiments were carried out as described by (Zhao et al., 2012), with minor modifications. To test the competition effect of *L. plantarum*, the probiotic bacterial cells (10^8 CFU/mL) and *E. coli* (10^8 CFU/mL) were co-incubated simultaneously with Caco-2 monolayers for 2 h at 37 °C. In the displacement and exclusion assay, Caco-2 cells were pre-incubated with *E. coli* (10^8 CFU/mL) and probiotic cells (10^8 CFU/mL), respectively, for 1 h. Thereafter, the non-adherent cells were removed by washing and the Caco-2 cells further incubated for 1 h with probiotics (10^8 CFU/mL) and *E. coli* cells (10^8 CFU/mL) for the displacement and exclusion assay. The Caco-2 and adhered cells were trypsinized and plated to enumerate viable colonies on MacConkey agar. The viability of *E. coli* cells was compared with that of the control group. Wells under identical conditions without the presence of probiotic cells were used as the control.

2.12. Statistics

The data analysis was performed using GraphPad Prism 8 software (GraphPad Software, La Jolla, CA, USA). The statistical analysis for cell adhesion and cell inhibition was performed using the one-way analysis of variance (ANOVA) test and Tukey's test. The effects of freeze-drying and thermal conditions were assessed using Student's t-test. All experiments were carried out in triplicate independently. The data are expressed as the mean values with standard deviation ($\pm \text{SD}$) of three independent experiments. The level of significance for experimental results was set at $P < 0.05$.

3. Results and discussion

3.1. Size and morphology of microencapsulation

The bacteria were encapsulated into CaCO_3 -MPs, and their properties were examined. Co-precipitation has previously been demonstrated with an optimized concentration of MnCl_2 and Na_2CO_3 for submicrometer-sized CaCO_3 crystals (5–20 μm) (Vikulina et al., 2021).

The average size of the microparticles produced by the co-precipitation method with/without *L. plantarum* under these preparation conditions was 4–27 μm as observed by SEM (Fig. 1). DLS measurements indicated that for the 0.33 mol/L $\text{Na}_2\text{CO}_3/\text{CaCl}_2$ concentration, the average diameter was $3.5 \pm 0.8 \mu\text{m}$, while for the

0.66 mol/L concentration, it measured $10.83 \pm 3.98 \mu\text{m}$ (Table 1). The PDI was found to be greater than 0.3, indicating a certain degree of variability in particle size (data not shown). Thus, these microparticles successfully entrapped *L. plantarum*, resulting in a diameter larger than the $1.7 \pm 0.8 \mu\text{m}$ size of *L. plantarum* itself, as determined by DLS. The larger size of the *L. plantarum*-encapsulated CaCO_3 -MPs compared to the control can be attributed to the presence of probiotics inside the calcium crystal matrix, providing a robust barrier against environmental stresses.

Positive Ca^{2+} accumulates on the slightly negative cell walls of *L. plantarum*, indicated by its negative zeta potential (De Wouters et al., 2015), shown in Table 1. Upon introducing CO_3^{2-} , CaCO_3 microshells nucleate and grow on these preadsorbed sites, completely coating the cells with a thick inorganic layer (Fakhrullin & Minullina, 2009).

The encapsulation yield for the 0.33 mol/L $\text{Na}_2\text{CO}_3/\text{CaCl}_2$ template was 70.46%, while for the 0.66 mol/L $\text{Na}_2\text{CO}_3/\text{CaCl}_2$ template, it was 0%. This difference in yield may be attributed to the high salt concentration in the 0.66 mol/L $\text{Na}_2\text{CO}_3/\text{CaCl}_2$ template, which could create lethal conditions for bacteria during dissolution in Na_2CO_3 before co-precipitation (Dong et al., 2017; Jarvis et al., 2001).

To confirm bacterial entrapment, the bacteria were stained with FITC and observed using confocal microscopy (Fig. 2a). The fluorescence of the FITC-labelled *L. plantarum* encapsulated CaCO_3 -MPs indicated successful encapsulation as shown in Fig. 2b. Additionally, the 3D spatially superimposed FITC fluorescence intensities of FITC-labelled *L. plantarum*-encapsulated CaCO_3 -MPs are shown in Fig. 2c. The images show that the *L. plantarum* cells were properly entrapped in the microcapsules. This was confirmed by the encapsulation yield of 70.46% for *L. plantarum*. The application of CaCO_3 -MPs to encapsulate *L. bulgaricus* was also explored to emphasize its broad potential and highlight opportunities for this technique to be applied to diverse strains, as depicted in Fig. 2d.

The SEM images showed that rhombohedral calcite crystals formed after CaCO_3 mineralization. Layered structures at the edges of the calcite crystals and an obvious cavity in the middle were observed in the control particles. The size and shape of the control particles differed from those of the encapsulated bacteria, with variations in shape observed; however, both were uniformly shaped (Fig. 1). The observed spherical holes at surfaces of the calcite particles may be attributed to the dissolution of vaterite particles (Kontrec et al., 2013). The microparticles obtained by the co-precipitation method with *L. plantarum* were rhombohedral calcite crystals ranging from 5 to 20 μm in size. They transformed from hollow to solid microparticles as the central cavities disappeared and solid calcium carbonate microspheres formed. The larger particle size and the disappearance of multilayered crystals suggested the presence of bacterial cells within the microparticles.

A comparison between templates with different concentrations showed that the 0.33 mol/L $\text{Na}_2\text{CO}_3/\text{CaCl}_2$ template produced more uniform particles, while the 0.66 mol/L $\text{Na}_2\text{CO}_3/\text{CaCl}_2$ template resulted in the appearance of debris. The particle size is influenced by the preparation temperature, time and speed of stirring, crystallization time, concentration of salts, and the presence of additives, along with the encapsulating procedures (Trushina et al., 2016; Vikulina et al., 2021; Volodkin et al., 2012).

The CaCO_3 -MPs were further characterized using EDX to detect the elements present in the pure calcite crystals, as shown in Fig. 1e. Additionally, to confirm the presence of CaCO_3 , EDX analysis was conducted on the precipitates produced in the *L. plantarum*-encapsulated CaCO_3 -MPs (Fig. 1f). The EDX spectra revealed the similarity between pure the CaCO_3 and the encapsulated CaCO_3 -MPs with carbon, calcium, and oxygen were the main elements (Seifan et al., 2016).

3.2. Cryoprotectant effect

The survival of the free cells and encapsulated *L. plantarum* during lyophilization was compared. The viable cell counts before and after the

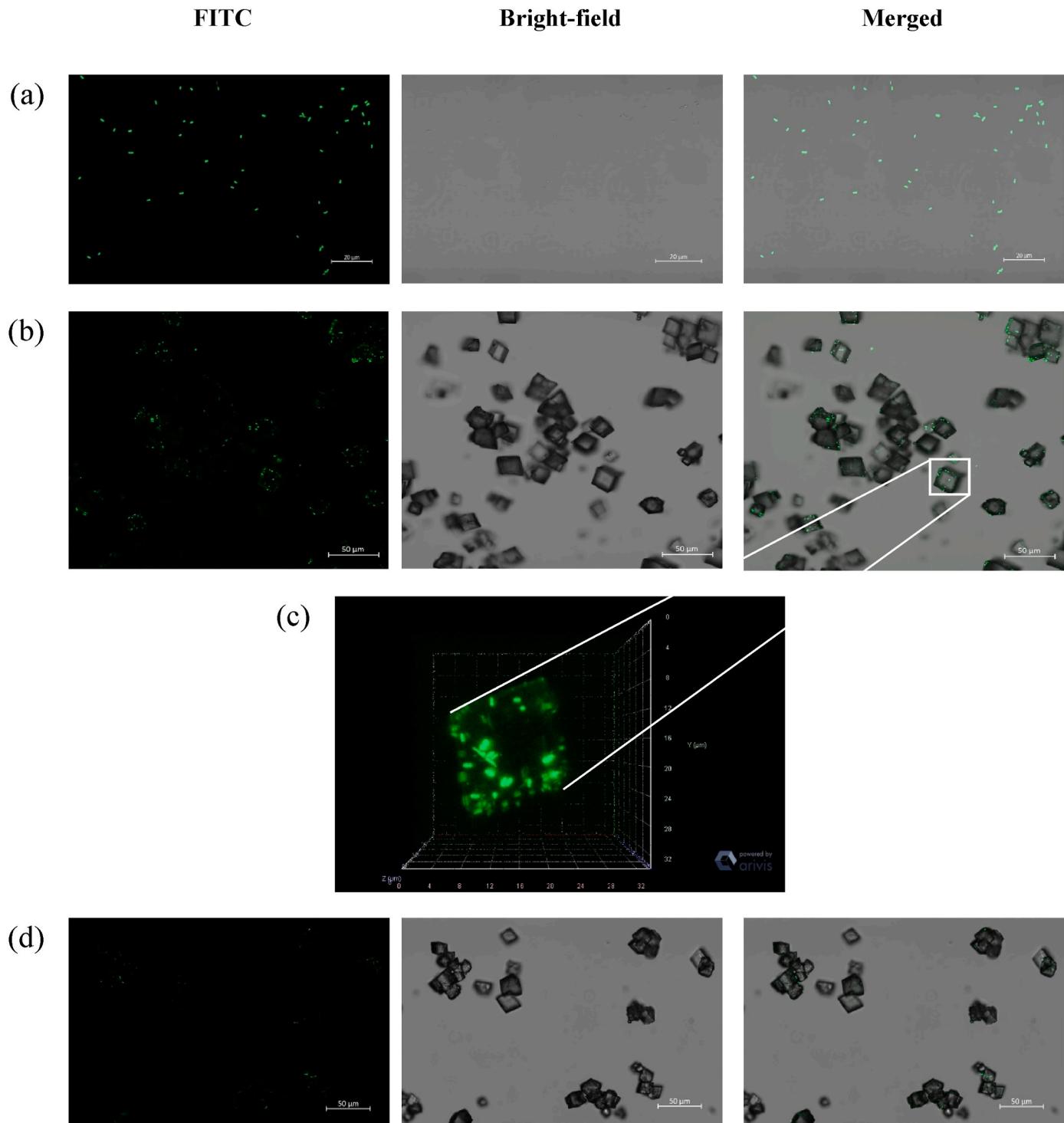


Fig. 2. Confocal micrographs of (a) FITC-labelled *L. plantarum*, (b) FITC-labelled *L. plantarum*-encapsulated CaCO_3 -MPs, (c) 3D confocal microscopy images of FITC-labelled *L. plantarum*-encapsulated CaCO_3 -MPs, and (d) FITC-labelled *L. bulgaricus*-encapsulated CaCO_3 -MPs. The CaCO_3 -MPs samples prepared at the condition of 0.33 mol/L $\text{Na}_2\text{CO}_3/\text{CaCl}_2$ concentration.

lyophilization process showed significant differences, as well as those with and without lyoprotectant (Fig. 3). During the freeze-drying process, encapsulated *L. plantarum* exhibited significantly higher survival rates ($P < 0.01$) when the lyoprotectants lactose and skim milk were added, in contrast to samples without it. However, the viability of the free cells after lyophilization with lyoprotectant was notably lower, dropping from 10.07 to 9.14 log CFU/g than that of microencapsulated cells lyophilized with lactose and skim milk, where the cell number

dropped from 9.7 to 9.63 log CFU/g. Thus, the survival rate of micro-encapsulated cells during lyophilization was higher than that of free cells, regardless of the presence of lyoprotectant.

Freeze-drying, used to produce probiotic powders, consists of three main steps: freezing the cell culture, primary drying to remove frozen water by vacuum sublimation, and secondary drying to eliminate the unfrozen water through desorption. However, crystal formation and osmotic stress during freeze-drying can lead to undesirable side effects

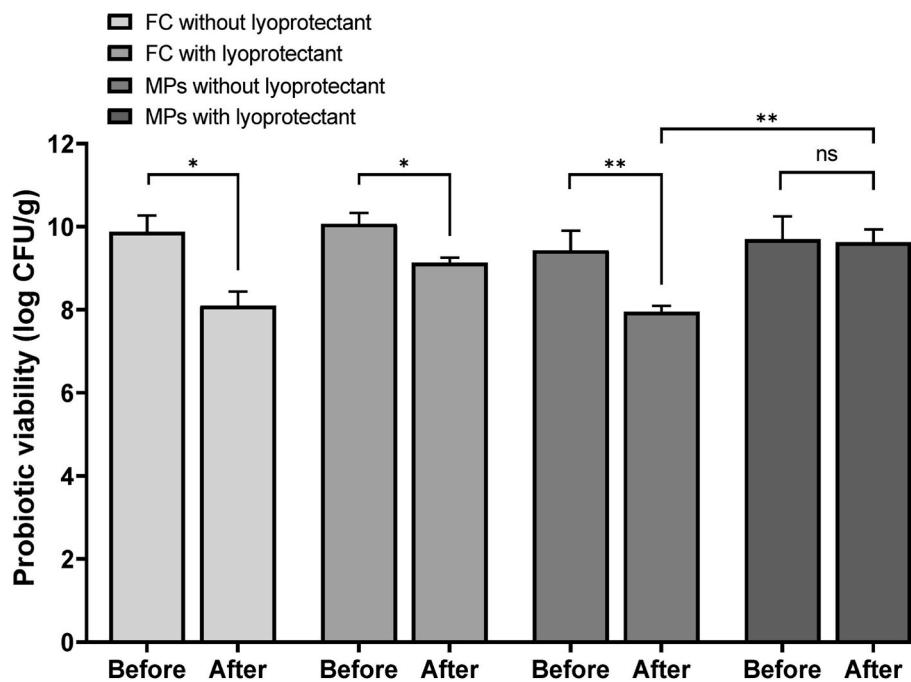


Fig. 3. Cell viability of *L. plantarum* before and after lyophilization with or without the lyoprotectant. * $P < 0.05$, ** $P < 0.01$, and ns $P > 0.05$ compared to sample before lyophilization. FC, free cells; MPs, Microparticles; Before, before lyophilization; After, after lyophilization.

such as membrane injury, protein denaturation, and DNA damage, affecting probiotic viability (Broeckx et al., 2016). Lyoprotectants from various substances have been widely reported for their lyoprotective effects against freeze-drying stress to prevent cell loss (Santivarangkna et al., 2008). Based on previous research studies (Juárez Tomás et al., 2015), the combination of lactose and skim milk was employed as a protective mixture for freeze-drying free and encapsulated *L. plantarum* cells.

During the freeze-drying process, the viability increased when the microparticles were suspended in lactose and skim milk before freeze-drying. For microencapsulated *L. plantarum*, the action of lyoprotectants during drying may be attributed to the formation of a protective layer over the CaCO_3 -MPs consisting of milk proteins, offering an additional physical barrier for the encapsulated cells. The encapsulated *L. plantarum* cells showed significantly higher survival during freeze-drying than the free cells with lyoprotectants, suggesting effective encapsulation by CaCO_3 for sufficient protection against freeze-drying stress. During lyophilization, lyoprotectants had a stronger effect when the cells were encapsulated, likely due to the enhanced interaction between the lyoprotectants and the bacterial cells within the microparticles (Juárez Tomás et al., 2015).

Skim milk has been extensively investigated as a lyoprotectant or rehydration media for preserving bacterial viability during long-term storage of probiotics (Carvalho et al., 2004). Comprising 50% (w/w) lactose and 30% (w/w) milk proteins, such as whey and casein, skim milk offers dual benefits. Lactose stabilizes cell membranes and proteins by preventing intracellular ice formation through hydrogen bonding with water and cellular structures and interacting with the polar head groups of the phospholipids and proteins in bacterial cell membranes. Additionally, the milk proteins form a protective layer over the cells, reducing cell membrane leakage and maintaining probiotic cell integrity, thus minimizing damage and cell inactivation during freezing-drying (Santivarangkna et al., 2008; Smetanková et al., 2018).

The highest recovery of viable *L. plantarum* cells with lyoprotectants during lyophilization was observed in this system; thus, free cells and encapsulated cells with lyoprotectants were used in most of the following assays.

3.3. Temperature effect

High temperatures can denature the proteins in probiotic cells, inhibiting enzymatic activity and leading to cell death (Corcoran et al., 2008). The heat resistance of probiotic cells is a crucial quality parameter, especially for various food manufacturing processes that involve the application of heat.

After exposure to different temperatures, the viability of both free cells and encapsulated cells increased compared to the initial count, which was approximately 9.4 log CFU/g for both samples after being exposed to 40 °C for 24 h, as shown in Fig. 4. This finding can be explained by the reported optimal growth temperature of *L. plantarum*, 45 °C (Smetanková et al., 2018).

In contrast, the viability of *L. plantarum* in both free and encapsulated form decreased slightly after exposure to 45 °C for 30 min. Furthermore, the survival rate of free cells significantly decreased after exposure to 65 °C for 30 min, corresponding to the pasteurization temperature. However, the viability of the encapsulated *L. plantarum* was maintained at over 10^6 CFU/g even after the 65 °C, 30 min heat treatment. This result is consistent with other studies indicating that incorporating skim milk powder in capsules formed a strong structure, enhancing heat tolerance and protecting *L. plantarum* from exposure to temperatures of 65 °C for 30 min (Mahmoud et al., 2020). Therefore, CaCO_3 -MP microencapsulation was an effective encapsulating matrix for protecting *L. plantarum* from the detrimental condition of 65 °C for 30 min.

3.4. Storage effect

Storage at RT is preferable to minimize handling and transportation costs. However, certain products containing probiotics require storage at freezing or low temperatures. Therefore, the viability of free cells and microencapsulated *L. plantarum* after lyophilization with lyoprotectants packed in hermetically sealed glass containers was evaluated at -18 ± 2 , 6 ± 2 , and 25 ± 2 °C.

The survival of *L. plantarum* in both free and encapsulated forms was not affected under all storage conditions. After 60 days of storage, the free cells and encapsulated *L. plantarum* showed a loss of viability of less than 1 log CFU/g (Fig. 5).

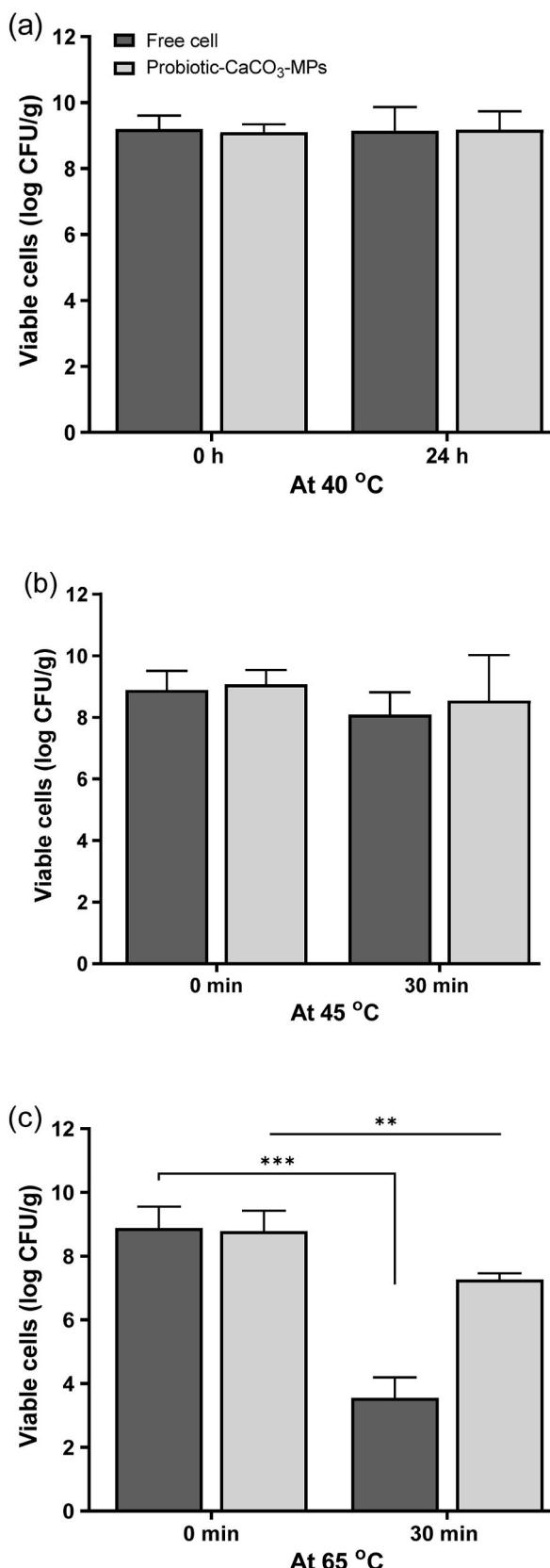


Fig. 4. The viability of *L. plantarum* under different conditions, including (a) incubation at 40 °C for 24 h, (b) 45 °C for 30 min, and (c) 65 °C for 30 min. **P < 0.01, ***P < 0.001 compared to data reported at 0 min.

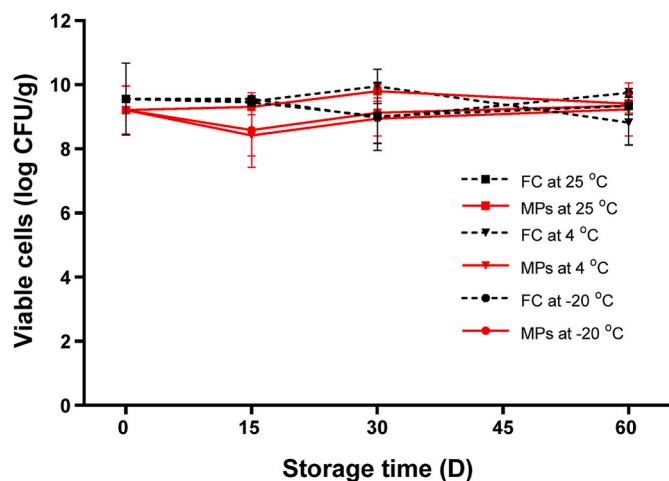


Fig. 5. Survival rate of encapsulated *L. plantarum* under different conditions, including refrigerated storage at RT, refrigerated storage at 4 ± 2 °C, and freezing storage at -18 ± 2 °C over 2 months. FC, free cells; MPs, Microparticles.

The survival rate of the free and encapsulated cells remained stable during the two-month storage period. This could be attributed to the presence of skim milk, which may serve as a nutritional component to promote cell growth (Liao et al., 2017). have also reported that using skim milk as an encapsulating agent resulted in little loss of *L. casei* LK-1 survival after 10 weeks of storage at -20, 4, and 25 °C (Maciel et al., 2014). also showed that skimmed milk beneficially maintains higher probiotic viability, with counts exceeding 10⁶ CFU/g after storage at 4 °C and 25 °C for 3 months.

Other studies have highlighted the advantages of using skimmed milk for microencapsulation: it possesses polysaccharide-based wall materials and amphiphilic properties, which fulfill the functional and physicochemical requirements for core encapsulation materials (Gul & Atalar, 2019). Skim milk proteins are highly effective in safeguarding *L. plantarum*. This is attributed to their ability to absorb onto the cell surface, forming a viscous layer that potentially triggers a partial efflux of water from the cell. Consequently, this process inhibits the growth of ice crystals and maintains the ice near the cell in an amorphous form, reducing the risk of cell injury and loss (Hubálek, 2003).

3.5. Survival of microencapsulated cells in simulated gastric and simulated intestine fluids

To assess the potential survival of free and encapsulated cells upon oral ingestion and passage through the stomach, their stability in SGF was evaluated (Fig. 6a). Both free and microencapsulated cells were resistant to SGF. More than 8 log CFU/g of the *L. plantarum* survived after 2 h. However, there was a rapid loss of free probiotic bacteria in SIF after 3 h of exposure; the free *L. plantarum* population rapidly decreased to less than 3.9 log CFU/g viable cells. The above observations indicate that free cells may not reach the intestine in sufficient quantities to confer probiotic benefits. In the case of microencapsulated *L. plantarum*, viability remained above 9 log CFU/g after 2 h and decreased to approximately 6.8 log CFU/g after 3 h. However, the cells encapsulated in CaCO₃-MPs maintained a viability of more than 10⁶ CFU/g for probiotic bacteria, which is the recommended probiotic dose level in carrier foods for effective product performance (Gu et al., 2022; Shori, 2016). Encapsulation in CaCO₃-MPs improved the survival of *L. plantarum*. This result is consistent with other research (Afzaal et al., 2023; Gu et al., 2019; Kim et al., 2017). This work demonstrated that co-encapsulation of probiotic cells with CaCO₃ enhances their viability. This indicates that CaCO₃ encapsulation effectively shields probiotics from the lethal conditions of the gastrointestinal tract.

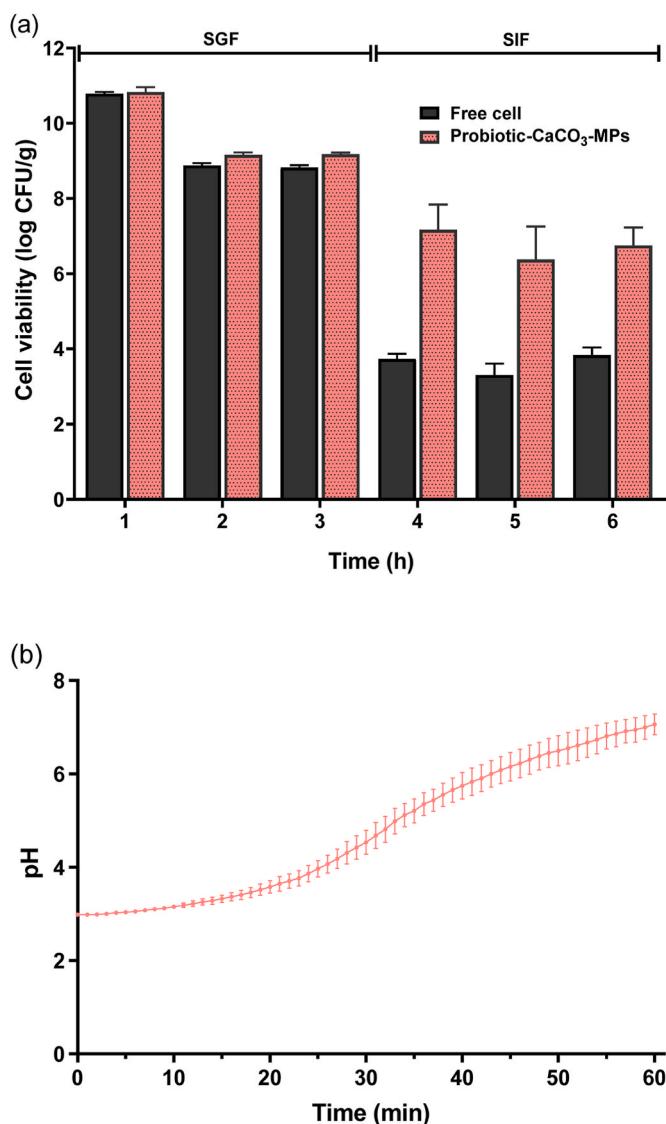


Fig. 6. Survival of microencapsulated *L. plantarum* in simulated gastric juice and simulated intestinal fluid (a); Average gastric pH profile after adding microencapsulated *L. plantarum* into 10 mL of SGF.

The mechanism for the protective effects of CaCO₃ remains unclear, with several possible contributing factors. First, CaCO₃ acts as an antacid, buffering the gastric acid in the stomach by dissociating into Ca²⁺ and CO₃²⁻ ions. These ions bind to free protons (H⁺) in the stomach, slightly increasing the pH and hindering the penetration of hydrogen ions into the microparticle core, protecting the bacteria from gastric acid degradation (Afzaal et al., 2023; Gu et al., 2019; Ma et al., 2012). This finding is in good agreement with the previous studies employing CaCO₃ as an antacid agent (Chehreara et al., 2022), demonstrated that multi-layer microcapsules containing CaCO₃ improved bacterial survival under gastrointestinal conditions. In both studies by (Gu et al., 2019; Zhang et al., 2021) shown that the viability and targeted release of Bifidobacterium *in vitro* gastric or small intestine environment can be improved by encapsulating with antacids in alginate microgels. Changes in pH value were observed as recorded in Fig. 6b. The pH of the gastric environment increased with addition of microencapsulated *L. plantarum* into SGF. The pH increased to >5.0 within 33 min, and reached pH 7 within 60 min. This result supported the hypothesis that *L. plantarum*-encapsulated CaCO₃-MPs can neutralize gastric acid. Our current results suggest that employing CaCO₃-MPs not only protects probiotics effectively but also neutralizes gastric acids, thus overcoming

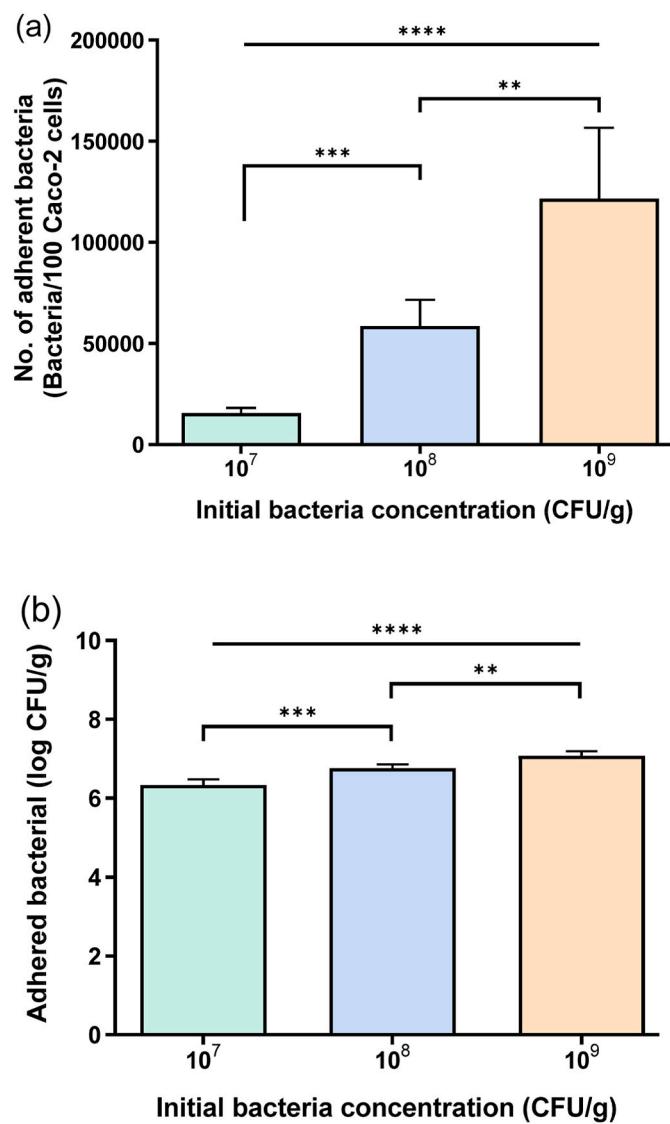


Fig. 7. Adhesion of encapsulated *L. plantarum* to Caco-2 cells as indicated by (a) number of adherent *L. plantarum* to 100 Caco-2 cells and (b) count of adhered viable *L. plantarum* cells. **P < 0.01, ***P < 0.001, ****P < 0.0001 compared to each concentration.

the limitations of the conventional encapsulation methods.

Second, gradually released calcium ions interact with bile and digestive salts, potentially reducing cell injury from interactions with bile salts or intestinal enzymes (Gu et al., 2019). (Lee et al., 2024; Shin et al., 2024) also stated that CaCO₃ reacts with phosphate in bile and converts to hydroxyapatite, providing additional protection to the probiotics. This may further protect the encapsulated cells from SIF. Third, the rate of matrix disintegration depends on the size and form of the CaCO₃ used: micro-sized CaCO₃ crystals likely reduce dissolution rates due to their size, potentially hindering hydrogen ion diffusion and slowing down diffusion processes overall (Meiron et al., 2011). The calcite form of CaCO₃ had a lower dissolution rate than vaterite, suggesting the potential for preparing composites with tailored drug release based on the CaCO₃ polymorph used (Ambrogi, 2023). Thus, CaCO₃ microstructures could protect encapsulated probiotics depending on size and geometry. Further research is needed to understand the protective properties of CaCO₃ relating to erodibility.

Additionally, according to (Borges et al., 2012), the effect of encapsulation on the survival of probiotics is highly dependent on the strain used and stress condition. Therefore, it would be worthwhile to

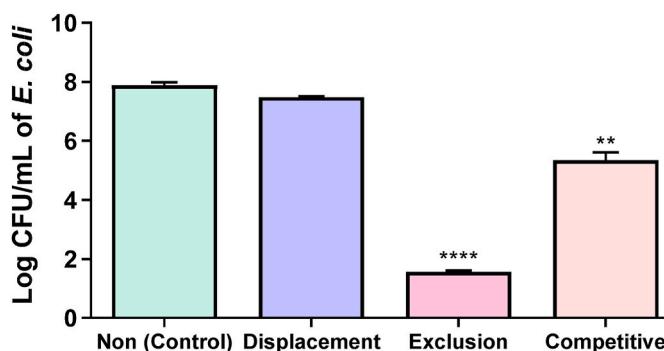


Fig. 8. The ability of the encapsulated *L. plantarum* cells to inhibit the adhesion of *E. coli* to Caco-2 cells. Values are shown as mean \pm SD determined from four independent experiments. ** P < 0.01, *** P < 0.0001 compared to each control.

investigate other probiotic strains to improve the viability of encapsulated probiotics during food processing and provide a broader understanding of the encapsulation efficacy.

3.6. Adhesion to Caco-2 epithelial cells

Fig. 7 presents the adhesion ability of encapsulated *L. plantarum* to the human intestinal Caco-2 cells. The average number of *L. plantarum* cells adhering to intestinal cells depended on bacterial concentration (Fig. 7a). The adhesion capacity also depended on the bacterial concentration, with adhered *L. plantarum* increasing from 6.67 log CFU/g at 10^7 inoculated bacteria to 7.00 and 7.24 log CFU/g at 10^8 and 10^9 inoculated bacteria, respectively (Fig. 7b). Similar concentration-dependent kinetics of adhesion have been reported by (Abedi et al., 2013).

The adhesion of probiotics to the intestinal mucosa is considered one of the most essential factors for probiotic selection (FAO/WHO, 2002). This research demonstrated that *L. plantarum* can effectively adhere to Caco-2 cells, in a similar to previous studies using different strains of *L. plantarum* on Caco-2 cells (Jang et al., 2019; Potočnjak et al., 2017).

3.7. Inhibition of *E. coli* adhesion to Caco-2 cells

The ability of the encapsulated *L. plantarum* to inhibit the adhesion of *E. coli* TISTR 073 to the human intestinal Caco-2 cells was measured (Fig. 8). The *E. coli* exhibited an adhesion rate of 7.88 log CFU/mL to Caco-2 cells. However, the adhesion capacity of *E. coli* decreased when *E. coli* and probiotics were added simultaneously and incubated with Caco-2 cell monolayers. A significant reduction was observed in the exclusion and competitive assay against *E. coli*. In the exclusion assay, probiotics performed the best, significantly decreasing the number of adhering *E. coli* (1.55 log CFU/mL) to the intestinal cells. In the competitive adhesion assay, *L. plantarum* also significantly reduced the number of adhering *E. coli* (5.33 log CFU/mL), while the displacement assay showed slightly lower inhibition effects at 7.48 log CFU/mL.

L. plantarum showed the ability to reduce *E. coli* adhesion to the intestinal cells in all tests. This inhibition was more effective in reducing the adhesion of *E. coli* when *L. plantarum* was pre-incubated before *E. coli* cells were added. However, simultaneously co-incubating *E. coli* with probiotic cells also showed promising outcomes. These findings are consistent with previous studies indicating that adding probiotics to epithelial cells before pathogens is more effective in inhibiting colonization than disrupting established colonization (Wang et al., 2018).

The inhibition of adherence of *E. coli* by *Lactobacilli* occurs through various mechanisms, including steric hindrance or binding-site competition, bacterial co-aggregation, substances present in the supernatant of *Lactobacilli*, and the secretion of bacteriocin and other antimicrobial

substances (Lebeer et al., 2010; Lee et al., 2000; Wong et al., 2013). However, the precise details of the proposed mechanisms remain unknown.

4. Conclusions

The delivery of potential probiotics has been consistently associated with a wide range of functional food products, including dairy and non-dairy products. However, incorporating probiotics into various food matrixes poses several technological challenges, particularly in preserving microbial viability. This study demonstrated a proof-of-concept strategy using a highly convenient and cost-effective co-precipitation method for synthesizing CaCO_3 microparticles. The structure evolution of *L. plantarum*-encapsulated CaCO_3 MPs, as confirmed by CSLM, demonstrates the encapsulation of *L. plantarum* within the CaCO_3 structure. The optimum 0.33 M $\text{Na}_2\text{CO}_3/\text{CaCl}_2$ concentration achieved a high encapsulation efficiency of 70.46%, providing high efficiency in protecting probiotics under thermal conditions and simulated gastrointestinal conditions (above the minimum value of 10^6 CFU/g). These microparticles have practical application potential because they demonstrate superior protection during freeze-drying process and enhance *L. plantarum* viability under heat treatments and *in vitro* gastrointestinal modeling. Nevertheless, further research is required to optimize the formulation and evaluate the efficacy of the delivery platform for protecting probiotics under *in vivo* conditions. In conclusion, microencapsulation is a promising approach to delivering probiotics and protecting probiotics, with potential applications in the health food industry.

CRediT authorship contribution statement

Krissana Khoothiam: Writing – review & editing, Supervision, Resources, Methodology, Investigation, Formal analysis, Conceptualization. **Napatsorn Supan:** Methodology, Formal analysis. **Chonthicha Uaongcharoen:** Methodology, Formal analysis. **Kittapas Yodthar:** Formal analysis. **Ausanai Prapan:** Formal analysis. **Chonthida Thephinlap:** Validation, Formal analysis. **Chutamas Thepmalee:** Validation, Investigation, Formal analysis. **Nittiya Suwannasom:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Methodology, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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