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Original Article

Gelation of different phases of probiotic-loaded water-in-oil-in-water emulsion to enhance probiotic survival stability

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Abstract

This study aimed to develop water-in-oil-in-water ($W_1/O/W_2$) double emulsion gels (DEGs) with *Lactiplantibacillus plantarum* subsp. *plantarum* and *Limosilactobacillus reuteri* encapsulated within the inner aqueous phase (W_1), with a focus on improving probiotic viability under adverse environmental conditions. The system incorporated gelling agents to enhance emulsion stability, including whey protein crosslinked with calcium chloride to gel the W_2 phase and carnauba wax as an oleogelator to solidify the O phase. Three formulations were investigated: W_2-gelled, O-gelled, and dual-phase gelled systems. Our study demonstrated that designed DEGs loaded with probiotics effectively maintained cell count in a sufficient amount (more than 6 log CFU/g) during 56-day storage, heat treatment (at 60 °C and at 72 °C for 1 hr), and four freezing–thawing cycles compared to free cells. During simulated digestion, free probiotic cells exhibited substantial cell reduction, particularly after intestinal digestion, with cell loss ranging from 3.00 to 3.50 lg colony forming unit (CFU)/g. However, encapsulation within DEGs effectively enhanced probiotic survival, minimising cell reduction throughout digestion with cell loss around 1 lg CFU/g. These findings highlight the practical application of W/O/W phase-specific gelling agents to enhance structural integrity and probiotic survival. The DEG matrix outperforms traditional encapsulation, providing superior probiotic stabilisation under stressors.

Keywords: digestion, double emulsion gels, freeze–thaw, microstructure, probiotic, stability, thermal treatment

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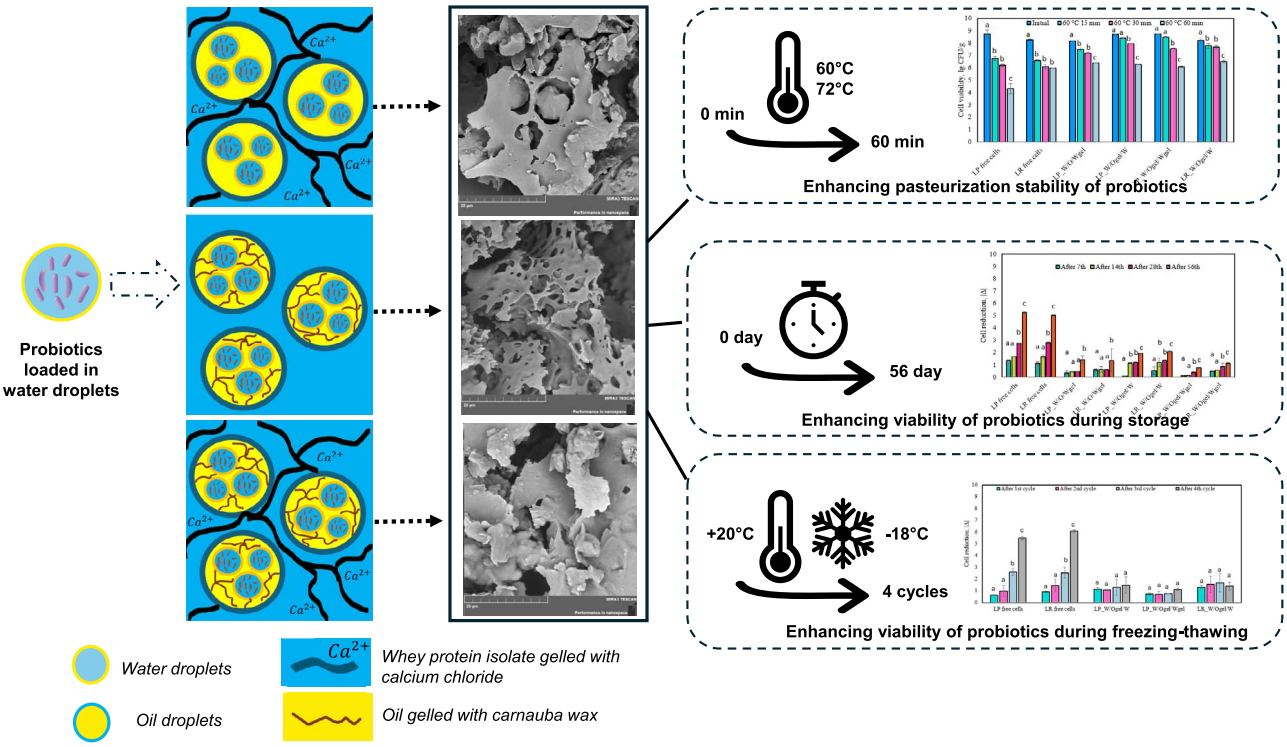
Graphical abstract



Introduction

Probiotics are essential in the human diet for promoting good health and normal organism functioning. Probiotics which provide health benefits to the host when administered in adequate amounts, have attracted significant interest for their potential to improve gastrointestinal (GI) health, enhance immune function, and prevent certain diseases (Milner et al., 2021). It is known that probiotic cells must survive in an acidic environment (pH 1–2) and reach the intestines in adequate amounts (more than 6 log CFU/g) to ensure colonisation (Araya et al., 2002). An acidic environment can be stressful for microorganisms; it can damage cell membranes, cellular proteins, and DNA, ultimately resulting in reduced bacterial viability (Melchior et al., 2021). Moreover, food storage and production, oxygen, the presence of bile salts and enzymes are anticipated to significantly hinder their transit through the GI tract (Lo Curto et al., 2011).

When searching for a suitable matrix for encapsulation of probiotics, it is necessary to pay attention to the following properties: non-toxic, inexpensive, compatible with probiotics, suitable for food, not changing, or perhaps even improving the texture and nutritional value of the food, if large quantities of probiotics are to be added. In addition, the structure of the encapsulation matrix must be designed to ensure maximum viability of the probiotics during production, storage, incorporation into the product, and suitability for probiotics intestine-targeted delivery. In this regard, double emulsions serve as an effective tool offering controlled release of active compounds, high entrapment efficiency, ability to encapsulate both hydrophobic and hydrophilic substances simultaneously, and an extra protective layer to enhance the viability of microorganisms within the inner water phase. Marefati et al. (2021) investigated the survival of *Limosilactobacillus reuteri* encapsulated within the internal water phase of a water-in-oil-in-water (W/O/W) emulsion over a storage period of 30 days at 6 °C. Their results showed a significant decline in viability during storage from 7.23 ± 0.07 to 2.82 ± 0.10 log CFU/g. Similarly, Yin et al. (2024) investigated the spray dried microcapsules of W/O/W loaded with *Lacticaseibacillus rhamnosus* GG. Their findings demonstrated that over an 8 week storage period at 25 °C, the viability of the encapsulated bacteria decreased significantly, dropping from approximately 9.91 to 5.32 log CFU/g. Regarding the heat treatment of double emulsions, Hua et al. (2024) observed that after 16 s at 75 °C, the survival rate of *Lactiplantibacillus plantarum* subsp. *plantarum* encapsulated in W/O/W emulsions was between 51.4% and 66.93%, significantly lower than the 100% survival rate observed before heat treatment. Additionally, this author subjected these emulsions to freezing–thawing, and the findings showed that the viability of probiotics decreased significantly from an initial 7.2–7.3 log CFU/g 2.3–2.9 log CFU/g after the final freeze–thaw cycle. As highlighted in the literature review, a key challenge in utilising W/O/W emulsions is their relatively low stability over prolonged storage periods, heat processing, and freezing, which makes them less favourable for practical use in food production. As a result, researchers have developed various strategies to improve the stability of double emulsions.

A potential solution to stabilise W/O/W emulsions during the storage, heating and freezing–thawing is the gelation of oil and water phases. During the gelation process the transition from emulsion liquid state to gel-like structure occurs. This transformation results in the formation of a three-dimensional network that traps dispersed droplets, providing structural integrity and reducing the mobility of emulsion phases. Additionally, it stabilises droplets and minimizes the coalescence, Ostwald ripening, and phase separation of the emulsion. Wang et al. (2025) demonstrated the successful use of W/O/W gelation for probiotic incorporation, developing a double emulsion gelled with cocoa butter. The emulsion, containing 20% cocoa butter, improved

bacterial survival to 92% over 20 days at 4 °C by preventing bacterial migration between phases, highlighting cocoa butter's efficacy as a gelator and protective agent. Another study by Taghrir et al. (2024) demonstrated that double emulsions gelled with basil seed gum significantly ($p < .05$) enhanced probiotic survivability during heat treatments at 72, 85, and 90 °C for 30 s, compared to free cells. Similarly, Abbasi et al. (2023) found that water-in-oil-in-water gels with different gelling materials (e.g., alginate, gelatin, carrageenan, and tragacanth) significantly ($p < .05$) improved *Lactiplantibacillus plantarum* subsp. *plantarum* viability during heat treatments at 30, 50, 63, and 72 °C for 2 min, compared to free cells. Additionally, Taghrir et al. (2024) investigated probiotic viability during *in vitro* digestion. It was observed that the emulsion gels enhanced probiotic survival, with the total cell count decreasing from approximately 9.3 to 5.5 log CFU/g, indicating substantial protection and retention of viability. The study by Zhu et al. (2024) investigated W/O/W emulsion nanogels and demonstrated that they successfully protect *Lactiplantibacillus plantarum* subsp. *plantarum* cells during *in vitro* gastric digestion and effectively release them in the intestinal phase in adequate amounts (more than 7 log CFU/g).

In our previous work, differently gelled phases in double emulsions enhanced physicochemical properties and improved stability during storage by preventing probiotic migration between phases (Varnaitė-Kapočė & Leskauskaitė, 2024). However, the effect of various processing parameters, such as heat treatment, freezing–thawing, and gastrointestinal environment on probiotic stability remains unclear. In our study, we explored the effect of gelation on enhancing the survival of probiotics in W/O/W emulsions, using whey protein and carnauba wax as gelling agents. Whey protein was selected for the gelation of the external water phase (W_2) due to its ability to form strong, heat-stable, and biocompatible gel networks, which can protect encapsulated probiotics during thermal processing and digestive transit. It is a food-grade material rich in essential amino acids, commonly used in various food applications, and known to enhance the nutritional profile of food products (Zhao et al., 2022a). Calcium chloride was used to induce gelation by cross-linking whey protein and enhance phase separation resistance. On the other hand, carnauba wax, a natural, non-toxic wax with excellent hydrophobic properties, was chosen to gel the oil phase. Its high melting point (Fei & Wang, 2017) and firm structure help to form a solid matrix that enhances the emulsion's thermal stability, reduces phase coalescence, and minimises probiotic migration across the emulsion phases. By combining these two gelling agents, we aimed to develop a stable double emulsion gel system that improves probiotic viability during storage, heating, freezing–thawing cycles, and gastrointestinal conditions.

In this study we assessed three $W_1/O/W_2$ gels (DEGs) loaded with *Lactiplantibacillus plantarum* subsp. *plantarum* and *Limosilactobacillus reuteri*. The emulsion types included (a) a W_2 phase gelled with whey protein and calcium chloride, (b) an oil phase gelled with carnauba wax, and (c) both phases gelled simultaneously. We evaluated the ability of these DEGs to maintain probiotic viability under various processing conditions with the aim of providing insights into the possibility of using gelled emulsions in functional foods.

Materials and methods

Materials

Lactiplantibacillus plantarum subsp. *plantarum* DSM 24624 and *Limosilactobacillus reuteri* 182 were generously supplied by the Food Institute of Kaunas University of Technology (Kaunas, Lithuania). Whey protein isolate (WPI) Lacprodan SP-9213 (Arla Foods Ingredients, Denmark); rapeseed oil (local market, Kaunas, Lithuania); polyglycerol polyricinoleate (PGPR) (Danisco, Copenhagen, Denmark); calcium chloride (AppliChem GmbH, Darmstadt, Germany); sodium chloride (EUROCHEMICALS, Vilnius, Lithuania); carnauba wax (Thermo Fisher GmbH, Kandel, Germany); MRS agar (De Man-Rogosa-Sharpe, Biolife, Italy); MRS broth medium (Biolife, Italia); hydrochloric acid (UAB Labochema, Vilnius, Lithuania); sodium hydroxide (EUROCHEMICALS, Vilnius, Lithuania); Nile red and fluorescein-5-isothiocyanate (FITC) were purchased from Fluorochem (Derbyshire, UK).

For simulated digestion, chemicals (pepsin A (600–1,200 U/mg, 77160); lipase from porcine pancreas (Type II, 100–500 U/mg, L3126); bile extract porcine (B8631), pancreatin (P1625-100G)), KCl, KH_2PO_4, NaCl, $MgCl_2(H_2O)_6$, $(NH_4)_2(CO_3)$ were supplied by Sigma-Aldrich Chemie GmbH, Steinheim, UK. All reagents used in this study were of analytical grade.

Methods

Preparation of microbial cultures

The lactic acid bacteria (LAB) microbial cultures of *Lactiplantibacillus plantarum* subsp. *plantarum* DSM 24624 (LP) and *Limosilactobacillus reuteri* 182 (LR) were maintained on De Man-Ragosa-Sharpe (MRS) agar at +1 ± 0.5 °C under aerobic conditions. Prior to experimentation, LP and LR cells (1%) were pre-cultivated in the MRS broth at +37 ± 0.5 °C for 24 hr. For the second subculture, an inoculum of 1% pre-activated cells was made and re-incubated under identical conditions for an additional 24 hr. LAB cells were subsequently harvested and centrifuged (MPW Med. Instruments MPW-260R, Warszawa, Poland) at 6,000 rpm for 10 min at +4 ± 0.5 °C. After removing the supernatant, the cell pellet was resuspended in 10 ml sterile water. The resuspended cells were vortexed and washed twice with sterile water. To achieve a final concentration of 1×10^{10} colony-forming units (CFU) per ml of LAB, the cells were diluted with sterile physiological saline (0.85% wt/vol NaCl). To achieve a 1:100 cell-to-emulsion ratio, the cell suspension was combined with sterile physiological saline (0.85% wt/vol NaCl), and the prepared mixture was utilised for the subsequent encapsulation process as W_1.

Formulation of double emulsion gels

$W_1/O/W_2$ emulsion gels were formulated according to our previous study by Varnaitė-Kapočė & Leskauskaitė (2024) with slight modifications. All samples were prepared using an Ultra-turrax rotor-stator system (IKA T-18 basic, Staufen, Germany) at room temperature (+20 ± 2 °C). W_1 served as bacterial suspension prepared as outlined in section Preparation of microbial cultures. W_2 was formed by mixing 12% wt/wt whey protein isolate with distilled water, magnetically stirred for 1 hr (at +20 ± 2 °C, 1,000 rpm). The WPI solution was then heated to +100 ± 0.5 °C at a temperature-controlled water bath (Wisd WiseBath, WITEG Labortechnik, Wertheim, Germany) for 1 hr until it thickened and was subsequently cooled to +20 ± 2 °C.

Preparation of W/O/Wgel

W_1/O (**1**): solubilised 4 wt% PGPR in rapeseed oil was served as an oil (O) phase. W_1/O was formulated by slowly adding W_1 to the O at a 1:4 ratio, followed by homogenisation (7,000 rpm, 10 min).

W/O/Wgel (**2**): W_1/O was homogenised with W_2 at a 2:3 ratio (10,000 rpm, 5 min). During homogenisation, 100 mM of $CaCl_2$ (salt and WPI solution ratio was 1:9) was gradually added to gel the W_2 phase.

Preparation of W/Ogel/W

W_1/O (**3**): O phase was prepared by dissolving 4 wt% PGPR and 10 wt% carnauba wax in rapeseed oil at +90 ± 0.5 °C until fully solubilised, followed by cooling to +20 ± 2 °C. W_1/O was then formulated as in **1**.

W/Ogel/W (**4**): W_1/O was homogenised with W_2 at a 2:3 ratio (10,000 rpm, 5 min).

Preparation of W/Ogel/Wgel

W_1/O (**5**) was formulated as in **3**.

W/Ogel/Wgel (**6**) was formulated as in **2**.

Creaming index assessment

A 20-g sample of DEG was transferred to a 50 ml centrifuge tube and subjected to centrifugation (MPW Med. Instruments MPW-260R, Warsaw, Poland) (6,000 rpm, 20 min, at +20 ± 0.5 °C). The leaked liquid (oil and water) was carefully removed and weighed using a laboratory analytical balance. Creaming index (CI) was calculated using the equation:

$$\text{CI}\ (\%) = 100 - \left(\frac{\text{Supernantant weight}}{\text{Sample weight}} \cdot 100\right) \quad (1)$$

Optical microscopy and confocal laser scanning microscopy analysis

For the optical microscopy (Motic B1 Advanced Series, China) samples were prepared according to Varnaitė-Kapočė and Leskauskaitė (2024). The microstructure of the emulsion gels was characterised using a confocal laser scanning microscope (OlympusFLUOVIEW FV1000) equipped with a 20× magnification oil immersion objective lens. In this case, Nile red (0.75 mg/ml) was added to the oil phase before preparation of the W_1/O emulsion. After emulsion preparation sample was mounted on glass slide. Proteins were physically labelled with fluorescein-5-isothiocyanate (FITC) (10 μl) dissolved in acetone (1 mg/ml). Then the sample was covered with cover glass and visualised using a confocal laser scanning microscopy (CLSM). The excitation wavelength of the FITC and Nile Red dyes was 488 and 543 nm, respectively. Image software ImageJ was used to analyse the CLSM micrographs.

Scanning electron microscopy analysis

For sample lyophilisation an Alpha 1–4 LSC model freeze dryer (Martin Christ, Germany) was used. Freeze-drying was performed for 18 hr at a pressure of 1 mbar, with the condenser temperature set to −55 °C. Subsequently, the oil phase was removed following the method described by Li et al. (2021). The lyophilised samples were immersed in petroleum ether for 24 hr to extract any remaining rapeseed oil. Subsequently, the deoiled gels were placed in an oven at 50 °C for 4 hr to completely evaporate the petroleum ether.

The microstructure of the samples was examined using a Tescan Mira3 XMU Scanning Electron Microscope (Tescan Inc., Czech Republic). The samples were mounted on scanning electron microsopy (SEM) aluminium stubs and coated with a 5 nm layer of gold using a Quorum Q150 model Au sputter coater (Quorum Tech, GB). The samples were imaged at a working distance of 10 mm with a Tescan Mira3 XMU (Czech Republic) operating at 10 kV field emission, using a back-scattered electron (BSE) detector. Representative images were captured at various magnifications ranging from 250× to 20,000×.

Viable lactic acid bacteria count

The viable cell count of LAB was assessed using the plate counting method. A 1 g sample was combined with 9 ml of sterile physiological saline (0.85% wt/vol NaCl), followed by serial dilutions, which were then plated on MRS agar (Biolife, Italia) for LAB enumeration. The plates were incubated aerobically at 37 ± 0.5 °C for 48 hr in a temperature-controlled incubator (Termaks, Norway). The number of LAB colonies was then counted and reported as the logarithmic CFU per gram.

Cell reduction and survival was calculated using the following equations:

$$|\Delta| = \frac{\text{CFU}}{\text{ml}}\ \text{initial} - \frac{\text{CFU}}{\text{ml}}\ \text{at a time point} \quad (2)$$

$$\text{Survival}\ (\%) = \frac{\frac{\text{CFU}}{\text{ml}}\ \text{at a time point}}{\frac{\text{CFU}}{\text{ml}}\ \text{initial}} \cdot 100 \quad (3)$$

Storage and thermal stability, thermal treatment

For storage stability, double emulsion gels (DEGs) with probiotics were stored at +4 ± 0.5 °C for 56 days and at +25 ± 0.5 °C temperature for 7 days. In the first case, samples were collected after 0 (initial), 7th, 14th, 28th, and 56th storage day. In the second case, samples were collected after 0 (initial), 3rd, 5th, and 7th storage day to assess the viable count of LAB.

For the thermal stability, samples were stored at +60 ± 0.5 and +72 ± 0.5 °C in the temperature-controlled water bath. Samples were collected after 0 (initial), 15, 30, and 60 min to assess the viable count of LAB. Suspensions of free *Lactiplantibacillus plantarum* subsp. *plantarum* and *Limosilactobacillus reuteri* were used as controls.

Freezing–thawing treatment

A total of 40 g of sample was placed in plastic container with airtight lid and stored in a freezer at −18 ± 0.5 °C for 7 days. Afterward, the sample was thawed at +20 ± 2 °C for 3 hr. The freeze–thaw (F-T) process was repeated up to four cycles. After each F-T cycle, the samples were collected to assess the viable count of LAB. Suspensions of free *Lactiplantibacillus plantarum* subsp. *plantarum* and *Limosilactobacillus reuteri* were used as controls.

In vitro digestion

In vitro digestion of the DEGs was performed according to Minekus et al. (2014). Suspensions of free *Lactiplantibacillus plantarum* subsp. *plantarum* and *Limosilactobacillus reuteri* were used as controls. This model is designed to mimic the gastrointestinal environment: the mouth, stomach, and intestine. For the simulated oral digestion phase, 5 g of the DEG sample and 2 g of glass beads were incubated with 5 ml of simulated saliva fluids (containing 10 ml KCl (0.6 M), 20 ml KH_2PO_4 (0.5 M), 1 ml NaCl (2 M), 1 ml $MgCl_2(H_2O)_6$ (0.15 M), 0.06 ml $(NH_4)_2(CO_3)$ (4.8 M)) for 2 min. Afterwards, the digesta was mixed for 120 min with 10 ml of simulated gastric juice (containing 28 ml KCl (0.6 M), 0.9 ml KH_2PO_4 (0.5 M), 10 ml NaCl (2 M), 2 ml $MgCl_2(H_2O)_6$ (0.15 M), 0.5 ml $(NH_4)_2(CO_3)$ (4.8 M) containing pepsin (2,000 U/ml) referred to as the post-gastric sample. Subsequently, sample was incubated for 240 min with 20 ml of intestinal juice (containing 5.4 ml KCl (0.6 M), 0.8 ml KH_2PO_4 (0.5 M), 8 ml NaCl (2 M), 1.1 ml $MgCl_2(H_2O)_6$ (0.15 M)) containing enzymes (lipase (2,000 U/ml), pancreatin (100 U/ml), and bile salts (10 mM), referred as the post intestinal sample. The stomach phase pH was adjusted to 3 using 6 M HCl, mouth and intestinal phases pH was

brought to 7 using 6 M NaOH. Throughout the process, samples were incubated in a shaking water bath (140 rpm) (Wisd WiseBath, WITEG Labortechnik, Wertheim, Germany) at 37 °C. The digestion process was halted by cooling the samples to 0–4 °C in ice water bath, after which they were collected for determining the total viable count of LAB.

Statistical analysis

Bacterial viability and creaming index experiments were performed twice with three repetitions. The microscopy process was repeated multiple times to achieve the optimal view. The *M* and *SD* were calculated using Microsoft Excel. Statistical significance was assessed using a *t*-test in GraphPad Prism software, with a *p*-value of less than .05 considered indicative of a significant difference.

Results and discussion

Microstructural characterisation of DEGs

The microstructure of DEGs is presented in Figure 1. Optical microscopy images (Figure 1A) illustrate the successful encapsulation of probiotics within the inner water phase across all samples. CLSM images (Figure 1B) of DEGs revealed the presence of oils and proteins, visualised in red and green colours, respectively. In W/O/W gel emulsion, the protein network formed by adding calcium chloride appears well-structured with uniformly dispersed oil droplets throughout the matrix. When the oil phase is gelled with carnauba wax (W/Ogel/W), there are larger and more irregular oil regions, indicating a less noticeable protein network and the solidification of the oil phase. W/Ogel/Wgel where both phases are gelled, a more heterogeneous structure is noticed, where areas of solidified oil coexist with a strong protein network. The differences in the microstructure vary because calcium chloride cross-links and strengthens the protein network while carnauba wax creates a crystalline form in the oil phase. These characteristics together shape gel's texture, its porosity, and its stability.

Observing the morphology of DEGs in SEM images (Figure 1C) confirms that all samples exhibit disordered, porous structures. W/O/Wgel displays a highly aggregated protein network with some large, rounded cavities. The existence of a porous microstructure indicates that the stability of the sample is low due to minimal lipid dispersion as well as weak protein-oil associations. This was also supported in our previous study (Varnaitė-Kapočė & Leskauskaitė, 2024) where it was found that during the 56 day storage period DEG destabilisation occurred, resulted in the lower value of the consistency index (490.01 ± 0.40 vs. 4.17 ± 0.46), the hardness (0.58 ± 0.06 N vs. 0.08 ± 0.01 N) and the ability to endure freeze–thaw cycles was lost. Holes in the protein network represent poor lipid dispersion that might appear due to a weaker oil binding capacity. During W/O/Wgel preparation, the addition of calcium chloride in to the external water phase causes the gel matrix to shrink due to strong interactions between protein molecules and Ca^{2+} ions (Zhao et al., 2022b). Apart from this, the electrostatic interactions between oil–water interface and protein molecules can be weakened or lost, resulting in oil phase destabilisation, induced Brownian movements, lipid coalescence and the formation of holes in SEM micrograph. In contrast, the W/Ogel/W, structured with carnauba wax, demonstrates a more fragmented, crystalline microstructure. Comparatively to the W/O/Wgel system, the SEM picture shows a dense, compact matrix with fewer holes. Apart from the micrograph, carnauba wax in the presence of water, creates three dimensional network that retains water droplets (Penagos et al., 2023). Gu et al. (2023) confirmed that emulsion gel with carnauba wax forms a crystalline network. Additionally, carnauba wax contributes to the stabilisation of the oil phase, giving the gel its characteristic stiffness and rigidity. In the presence of both structural ingredients (calcium chloride and carnauba wax), the W/Ogel/Wgel samples exhibited a more complex and tightly packed morphology and had a much smoother, denser wave-like structure with small pores. While carnauba wax increases the structural rigidity of the oil phase and prevents lipids from destabilising, hence producing compact and stable emulsion, calcium chloride promotes the formation of a protein gel network. This structural combination enhances stability under challenging conditions such as storage, thermal treatment, and freeze–thaw cycles (Varnaitė-Kapočė & Leskauskaitė, 2024). The rigidity imparted by the gelled oil phase reduces collapse during freezing or thawing, while the protein network with calcium chloride minimizes structural breakdown during thermal stress. This dual structuring results in enhanced mechanical properties, which could prove beneficial for controlled-release systems.

In summary, SEM analysis of DEGs shows significant microstructural variations based on the choice of structuring agents. These findings offer insights for optimising emulsion gels in food, where the viability of probiotics under different conditions is key. The microstructural differences among DEGs, such as porosity, protein network organisation, and crystalline density, correlate with stability during storage and processing. These features impact how the gels retain their structure under thermal cycles or mechanical stress, influencing the release and protection of probiotics.

Viability of LP and LR during storage at different temperatures

The extent of using probiotics in food production is closely related to ensuring their high activity during storage. Probiotic viability is significantly influenced by multiple factors during storage, including oxygen exposure, moisture content, pH, and temperature fluctuations (Wang & Zhong, 2024). The encapsulating matrix must have mechanical and chemical protection to withstand these stress factors. An increase in temperature can accelerate lipid oxidation in emulsions, destabilize the matrix, and reduce the viability of encapsulated bacteria. Therefore, the viability of probiotics loaded into different DEGs was tested during storage for 56 days at 4 °C. Figure 2 shows free probiotics' cell reduction ($|\Delta|$) and those loaded into different DEGs. As observed from Figure 2, the highest cell reduction occurred during the storage of free *Lactiplantibacillus plantarum* subsp. *plantarum* cells starting from the first days of storage. The viable counts of free LP decreased by 1.36, 2.73, and 5.24 lg CFU/g after 7, 28, and 56 days of storage, respectively. The reduction in the vitality of LP loaded into all DEGs was significantly ($p < .05$) lower during storage. W/O/Wgel and W/Ogel/W loaded with LP cells had about 2 log cycle reduction by 56 days of storage. Meanwhile, W/Ogel/Wgel was more stable during the entire storage registering only 0.76 lg CFU/g reduction of loaded LP cells. Similar trends in cell reduction during storage were found in *Limosilactobacillus reuteri* -loaded DEGs. The reduction of free LR was 5.02 lg CFU/g after 56 days of storage. The decrease of viable cell counts in 1.35, 2.07, and 1.12 lg CFU/g was registered for W/O/Wgel, W/Ogel/W, and W/Ogel/Wgel, respectively. Temperature and time have synergistic impact on the viability of probiotics since prolonged exposure to even moderate temperature increases can lead to greater stress on bacterial membranes, denaturation of protective proteins, and oxidative

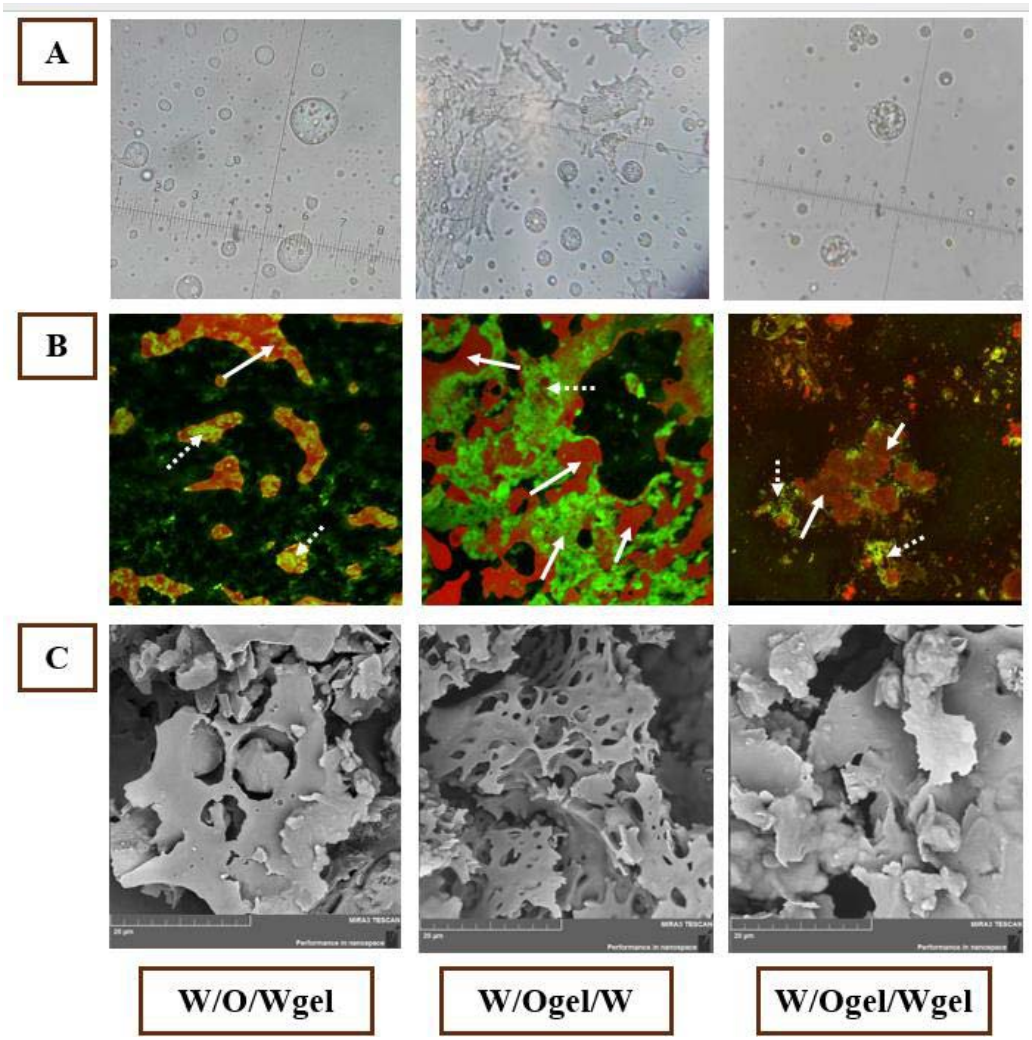


Figure 1. Optical (A), confocal laser scanning microscopy (B) and scanning electron microscopy (C) micrographs of double emulsion with gelled external water phase (W/O/Wgel), with gelled oil phase (W/Ogel/W), with gelled external water and oil phases (W/Ogel/Wgel). Optical images were captured using magnification of 40×. CLSM images were captured using magnification of 20×; dashed arrows represents proteins, straight arrows represents oils. SEM images were captured using magnification of 5,000×. W/O/W = water-in-oil-in-water.

degradation of lipid and gel phases, and therefore accelerate cell death (Kiepś et al., 2023; Wendel, 2022). In DEGs, probiotic bacteria are relatively isolated from the unwanted external environment because the gelled outer aqueous phase and/or oil phase form a safe physical barrier for the probiotic bacteria loaded in the inner aqueous phase (Liang et al., 2023). This means that encapsulated bacteria's survival depends on the stability of the DEG structure.

The creaming index of empty and probiotic-loaded DEGs during storage is presented in Figure 3. In most cases, the creaming index increases during storage of DEGs. After 28 days of storage, the creaming index of probiotic-loaded W/Ogel/Wgel was significantly higher than those of other DEGs ($p < .05$). Stability of emulsion is affected by factors like phase separation, droplet coalescence, and creaming. The creaming index shows how much an emulsion loses its homogeneity through the migration of droplets. This instability can impact probiotic protection if bacteria move between phases, resulting in uneven environmental exposure and loss of survival. Good stability of W/Ogel/Wgel guaranteed that probiotics loaded in the inner water phase of these DEGs showed the lowest cell reduction during storage. The higher viability of probiotics loaded in W/Ogel/Wgel may be attributed to the fact that there was a dense oleogel network surrounding them covered by the whey protein hydrogel. Such double protection of bacteria from environmental factors led to their higher viability during storage. Similarly, probiotics loaded W/O/W, in which the oil phase was composed of oil with different amounts of cocoa butter, showed a survival rate of strain to 92% after 15 days of storage (Wang et al., 2025). According to this study, the fat network formed by cocoa butter effectively prevented strains from migrating from the inner water phase to the outer water phase, thus protecting them from environmental factors.

In our study, it is obvious that loaded bacteria affect the creaming index of DEGs. As observed from Figure 3 significant differences among the creaming index of empty and loaded DEGs occurred at the end of the storage period, which was greater for the loaded DEGs. It could be due to the action of exopolysaccharides produced by LP and LR (Lynch et al., 2018) bacteria. Many experimental studies deliver observations that exopolysaccharides enhance the rheological properties and continuous network structure of different food gels (Mende et al., 2016). In this study, the increase of inner water phase viscosity in the probiotic loaded DEGs was the reason for the higher DEGs stability found.

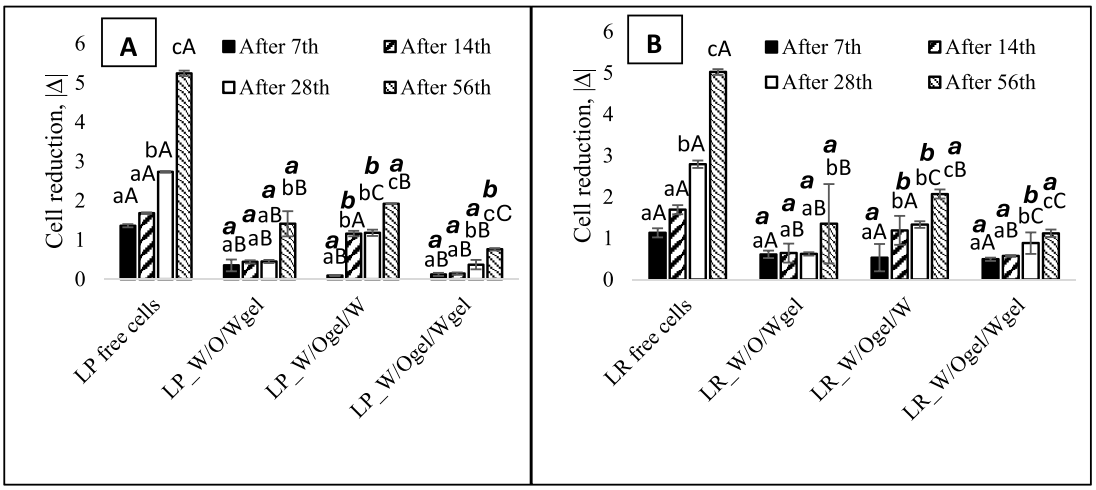


Figure 2. Cell reduction (|Δ|) of DEGs with (A) *Lactiplantibacillus plantarum* subsp. *plantarum*, (B) *Limosilactobacillus reuteri* during the storage time at 4 °C for 56 days. Values are reported as $M \pm SD$, $n = 6$; lower case letters indicate significant difference ($p < .05$) between storage days, upper case letters indicate significant difference ($p < .05$) between free and encapsulated cells; cursed bolded lower case letters indicate significant difference ($p < .05$) between differently gelled DEGs. LP = *Lactiplantibacillus plantarum* subsp. *plantarum* DSM 24624; LR = *Limosilactobacillus reuteri* 182; W/O/W = water-in-oil-in-water.

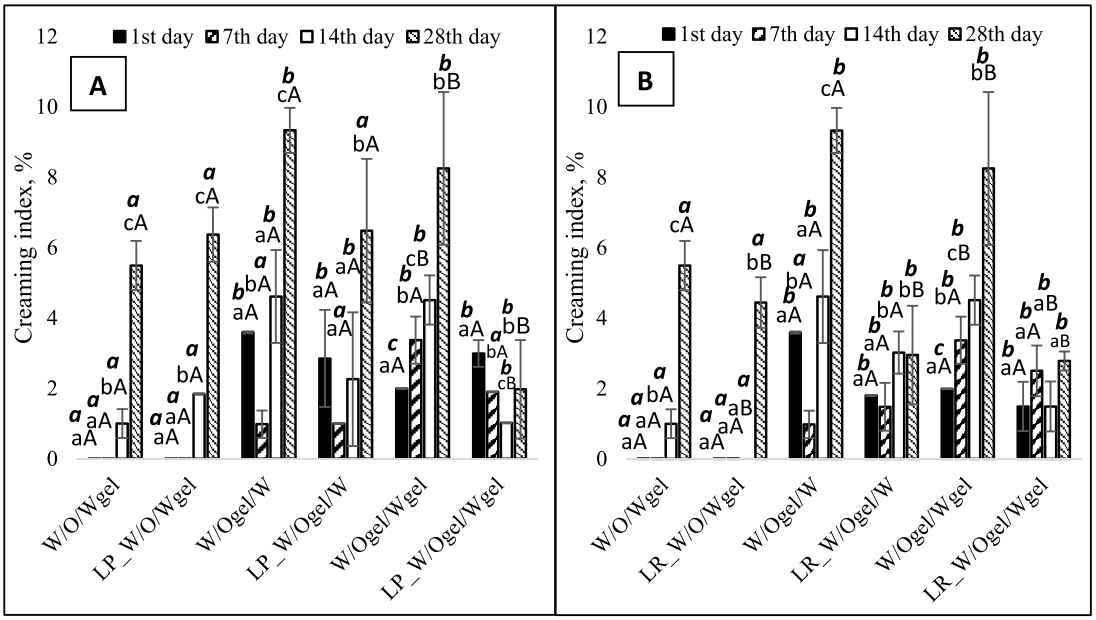


Figure 3. Creaming index (%) of double emulsion gels with (A) *Lactiplantibacillus plantarum* subsp. *plantarum*, (B) *Limosilactobacillus reuteri* during the storage time at 4 °C for 28 days. Values are reported as $M \pm SD$, $n = 6$; lower case letters indicate significant difference ($p < .05$) between storage days; upper case letters indicate significant difference ($p < .05$) between empty and probiotic loaded DEGs; cursed bold lower case letters indicate significant difference ($p < .05$) between differently gelled DEGs. LP = *Lactiplantibacillus plantarum* subsp. *plantarum* DSM 24624; LR = *Limosilactobacillus reuteri* 182; W/O/W = water-in-oil-in-water.

The survival of probiotics was reported to be negatively related to storage temperature (Mortazavian et al., 2011). Therefore, changes in viable counts of free LP and LR cells and those loaded into different DEGs during incubation at 25 °C for 7 days were investigated. Results are presented in Figure 4. The viability of LP and LR decreased from the initial number of 8.70 and 8.35 lg CFU/g to 5.79 and 4.06 lg CFU/g respectively, after 7 days of storage. During the same time period, LP loaded into W/O/Wgel, W/Ogel/W, and W/Ogel/Wgel fully retained their viability, showing no significant ($p < .05$) decrease in the cell count. LR loaded into W/Ogel/Wgel demonstrated a continual decreasing trend until the end of the storage period. After 7 days of storage the viable count of loaded cells was 5.88 lg CFU/g and remained about 2 lg cycles higher than free cells. Over the storage period, all DEGs hold their structure water in oil in water since their creaming index was 0% after 7 days of storage (results not presented). Such results indicated that the double action of the external whey protein hydrogel and structured oil phase around the internal water phase containing probiotics provided a good protective barrier for probiotics even when their storage temperature was 25 °C.

Viability of LP and LR during heat treatment

Since probiotics-loaded DEGs are developed for use in food products, it is important to evaluate the influence of various technological factors on the viability of probiotics. The most commonly

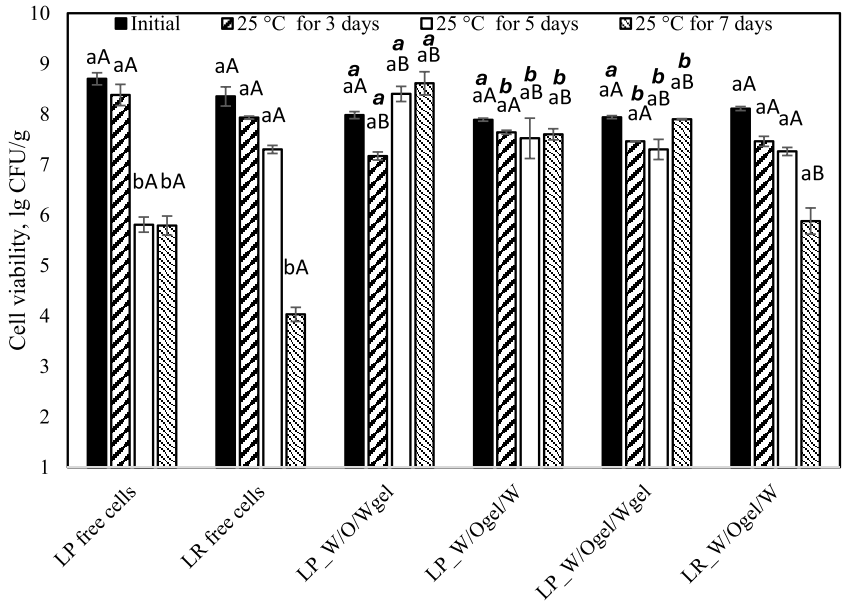


Figure 4. Cell viability (lg colony forming units, CFU/g) of DEGs with probiotic bacteria during incubation at 25 °C for 7 days. Values are reported as $M \pm SD$, $n = 6$; lower case letters indicate significant difference ($p < .05$) between storage days; upper case letters indicate significant difference ($p < .05$) between free and encapsulated cells; cursed bolded lower case letters indicate significant difference ($p < .05$) between differently gelled DEGs. LP = *Lactiplantibacillus plantarum* subsp. *plantarum* DSM 24624; LR = *Limosilactobacillus reuteri* 182; W/O/W = water-in-oil-in-water.

used process in food technology is heat treatment, the modes of which are very diverse and depend on the goals of this treatment, the place in the technological process, and the composition of the food product. Meanwhile, the optimal growth temperature for most probiotics is 37–43 °C, beyond which the viability of probiotics decreases (Śliżewska & Chlebicz-Wójcik, 2020). To simulate the effects of commonly used food heat treatment conditions on probiotic viability, free probiotic and probiotic-loaded DEGs were heated at 60 °C or 72 °C for 60 min. For this experiment, we selected the probiotic-loaded DEGs with the highest storage stability. Results are presented in Figure 5. The initial cell count in all samples was above 8.5 lg CFU/g without significant difference. After heat treatment at 60 °C for 60 min, 4.3 lg CFU/g viable *Lactiplantibacillus plantarum* subsp. *plantarum* cells count and 5.98 lg CFU/g viable *Limosilactobacillus reuteri* cells count was detected in the free probiotics samples. The viability of probiotics significantly ($p < .05$) increased when they were loaded into the inner water phase of DEGs. In W/O/Wgel, W/Ogel/W, and W/Ogel/Wgel *L. plantarum* showed high survival after heating at 60 °C for 60 min, and only decreased by 1.76, 2.41, and 2.67 lg CFU/g, respectively. LR loaded into W/Ogel/Wgel also demonstrated high heat resistance and decreased by 1.69 lg CFU/g after heating at 60 °C for 60 min.

The thermal damage of probiotics heated at 72 °C for 60 min is presented in Figure 6. The LP and LR concentrations in the free cell samples were 8.89 and 8.0 lg CFU/g, respectively. After heat treatment at 72 °C for 15 min, no viable cells were detected in these samples, which indicated poor heat tolerance of these probiotics. Some previous studies have achieved similar results (Pitigraisorn et al., 2017).

The thermal stability of encapsulated probiotics heated at 72 °C for 15 min was significantly ($p < .05$) improved. Specifically, after heating at 72 °C for 15 min, the decrease of LP encapsulated in W/O/Wgel, W/Ogel/W, and W/Ogel/Wgel was 0.65, 0.98 and 1.32 lg CFU/g, respectively. The decrease of LR loaded into W/Ogel/Wgel was only 0.6 lg CFU/g. Moreover, encapsulated probiotics showed good thermal stability after heating at 72 °C for 30 min. The count of viable *L. plantarum* cells was 7.47, 6.59 and 6.02 lg CFU/g for W/O/Wgel, W/Ogel/W, and W/Ogel/Wgel, and the viability of LR in W/Ogel/Wgel was 6.66 lg CFU/g after heating at 72 °C for 30 min, which was greater than recommended minimum level (6 lg CFU/g) for functional probiotic loaded foods. To further test the effect of encapsulation on the thermal stability of probiotics, heating at 72 °C for 60 min was applied. Viable probiotics were found to be significantly reduced in all DEG samples compared to samples heated at 72 °C for 15 min. Nevertheless, encapsulated probiotics still showed high viability, which was in the range 4.8–5.62 lg CFU/g. With rare exceptions, the highest survival after heating at 60 or 72 °C had probiotics encapsulated in DEGs characterised by the lowest creaming index (Table 1). These results prove once again that the gelation of double emulsion phases effectively protects bacteria from thermal degradation. The gelled outer water phase forms stable gel network as the surface hydrophobicity of whey protein increases during heating (Liang et al., 2017). This network effectively creates a protective barrier, restricting water mobility and limiting heat transfer. Although heat-induced whey protein aggregates lose more of their intermolecular β-sheet structure at the oil/water interface, they are still able to coat the oil droplet surface effectively (Liang et al., 2017). Similarly, oil phase solidification with carnauba wax, reduce heat diffusion and limits structural collapse. Due to that reason, gelation of the external water phase and/or oil phase can effectively slow down or confine the effect of heating on probiotics. The protective effect of solid fats on heat-sensitive probiotic bacteria to reduce heat damage during spray drying was revealed in a previous study (Liu et al., 2015; Yin et al., 2024). This was probably because the melting solid fat is a good heat absorber, which can decrease the internal temperature of a particle, thus minimising the heat shock to the probiotics.

The capability of probiotic cells loaded in the DEGs internal aqueous phase to survive at temperatures above 60 °C is a very important indicator when searching for suitable systems for their encapsulation and application in food systems. The minimum viable cell count of probiotic bacteria in food must be at least 6 log CFU/g to have a positive effect on consumer health

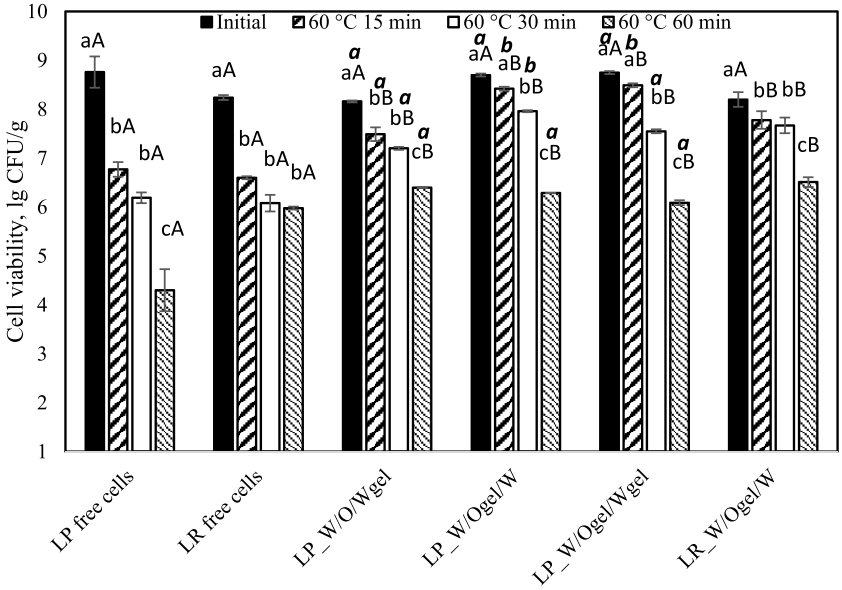


Figure 5. Cell viability (lg colony forming units, CFU/g) of DEGs with probiotic bacteria during heat treatment at 60 °C for 60 min. Values are reported as $M \pm SD$, $n = 6$; lower case letters indicate significant difference ($p < .05$) between storage time; upper case letters indicate significant difference ($p < .05$) between free and encapsulated cells; cursed bolded lower case letters indicate significant difference ($p < .05$) between differently gelled DEGs. LP = *Lactiplantibacillus plantarum* subsp. *plantarum* DSM 24624; LR = *Limosilactobacillus reuteri* 182; W/O/W = water-in-oil-in-water.

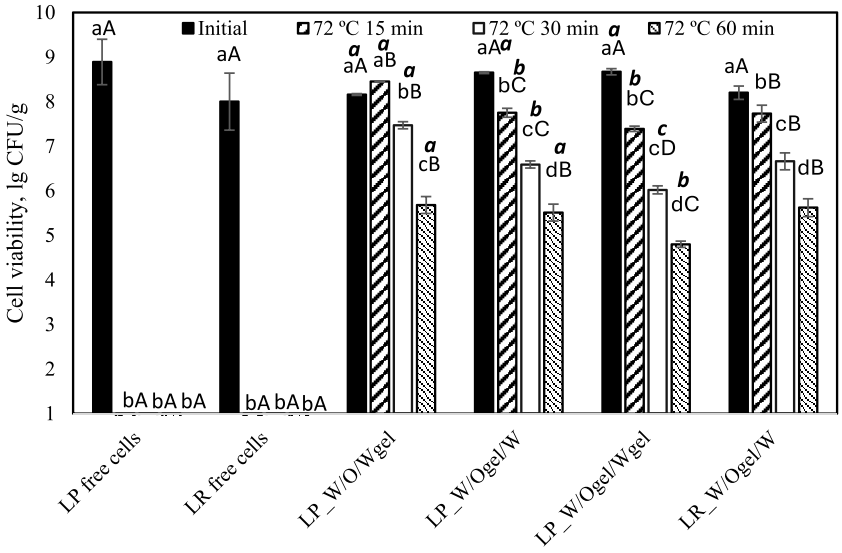


Figure 6. Cell viability (lg colony forming unit/g) of DEGs with probiotic bacteria during heat treatment at 72 °C for 60 min. Values are reported as $M \pm SD$, $n = 6$; lower case letters indicate significant difference ($p < .05$) between storage time; upper case letters indicate significant difference ($p < .05$) between free and encapsulated cells; cursed bolded lower case letters indicate significant difference ($p < .05$) between differently gelled DEGs. LP = *Lactiplantibacillus plantarum* subsp. *plantarum* DSM 24624; LR = *Limosilactobacillus reuteri* 182; W/O/W = water-in-oil-in-water.

(Araya et al., 2002). In this study, viable cell counts were greater than 8.0 and greater than 7.0 log CFU/g for probiotics-loaded DEGs after heating at 63 and 72 °C for 30 min, respectively.

Viability of LP and LR during freezing–thawing

Freezing–thawing cycle testing is a part of the stability test to determine whether DEG ensures probiotic viability under various conditions. Freezing can cause structural damage to DEGs due to the formation and growth of ice crystals in the water phases of DEGs. Ice crystals are capable of causing rupture, structural damage, and cell lysis of probiotics loaded in the inner water phase (Dalvi-Isfahan et al., 2019). In this study, the rates of microbial reduction between probiotics loaded into W/Ogel/W and W/Ogel/Wgel under four freezing–thawing cycles were compared. When subjected to freezing–thawing conditions, the free *Lactiplantibacillus plantarum* subsp. *plantarum* and *Limosilactobacillus reuteri* cells showed a significant decrease in number after three cycles—2.6 and 2.54 lg CFU/g, respectively (Figure 7). After fourth cycle, the reduction of free LP cells and LR was 5.49 and 6.08 lg CFU/g, respectively. The survival improvement was measured for probiotics loaded in the W/Ogel/W and W/Ogel/Wgel after four freezing–thawing cycles. As shown in Figure 7, the decrease in the number of living cells was in the range of 1.12–1.48 lg CFU/g, pointing out the protective effect of DEGs. The encapsulation matrix creates a physical barrier that protects probiotics against direct contact with ice crystals and reduces mechanical stress from freezing-induced dehydration. In this study, the oil phase

Table 1. Double emulsion gels with probiotics creaming index (%) during heat treatment at 60 and 72 °C for 60 min[a].

Storage time, min	0	15	30	60
	60 °C			
LP_W/O/Wgel	0.00 ± 0.00aA**a**	4.02 ± 0.03bA**a**	2.38 ± 0.51bA**a**	4.69 ± 0.09bA**a**
LP_W/Ogel/W	0.00 ± 0.00aA**a**	4.42 ± 0.62bA**a**	18.69 ± 1.18cA**b**	27.66 ± 3.06dA**b**
LP_W/Ogel/Wgel	0.00 ± 0.00aA**a**	3.71 ± 0.21bA**a**	24.03 ± 0.71cA**b**	29.24 ± 0.80cA**b**
LR_W/Ogel/W	0.00 ± 0.00aA	3.87 ± 0.09bA	26.39 ± 0.05cA	41.11 ± 2.24dA
	72 °C			
LP_W/O/Wgel	0.00 ± 0.00aA**a**	4.55 ± 0.78bA**a**	13.21 ± 1.57cA**a**	22.75 ± 0.03dB**a**
LP_W/Ogel/W	0.00 ± 0.00aA**a**	33.76 ± 1.88bB**b**	38.83 ± 1.85bB**b**	50.01 ± 1.30cB**b**
LP_W/Ogel/Wgel	0.00 ± 0.00aA**a**	33.09 ± 0.17bB**b**	42.67 ± 0.47cB**b**	49.31 ± 1.12cB**b**
LR_W/Ogel/W	0.00 ± 0.00aA	17.07 ± 0.10bB	33.88 ± 1.58cB	48.91 ± 0.18 dB

Note. LP = *Lactiplantibacillus plantarum* subsp. *plantarum* DSM 24624; LR = *Limosilactobacillus reuteri* 182; W/O/W = water-in-oil-in-water. [a]Values are reported as $M \pm SD$, $n = 6$; lower case letters indicate significant difference ($p < .05$) between storage time; upper case letters indicate significant difference ($p < .05$) between samples treated at different temperature; bolded lower case letters indicate significant difference between differently gelled DEGs.

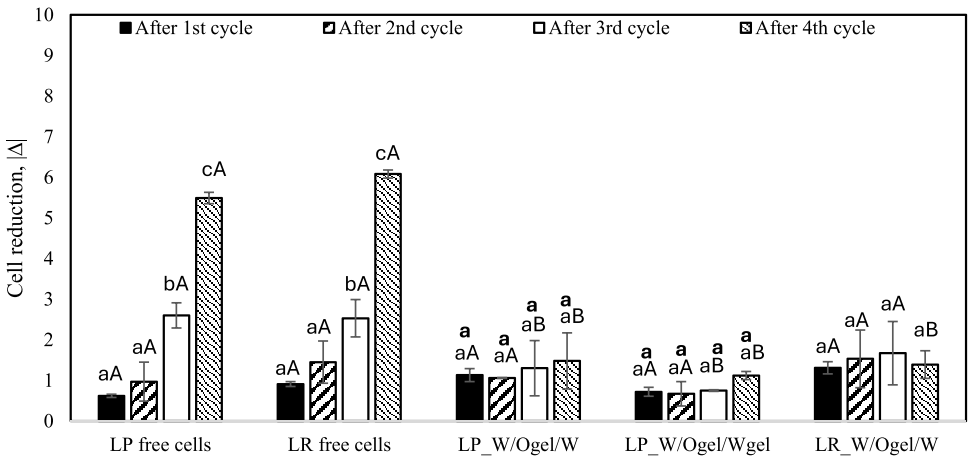


Figure 7. Cell reduction (|Δ|) of DEGs with probiotic bacteria during freezing–thawing (F-T) treatment. Values are reported as $M \pm SD$, $n = 6$; lower case letters indicate significant difference ($p < .05$) between F-T treatment cycles; upper case letters indicate significant difference ($p < .05$) between free and encapsulated cells; cursed bolded lower case letters indicate significant difference ($p < .05$) between differently gelled DEGs. LP = *Lactiplantibacillus plantarum* subsp. *plantarum* DSM 24624; LR = *Limosilactobacillus reuteri* 182; W/O/W = water-in-oil-in-water.

was solidified with carnauba wax, which provided structural integrity and prevented oil coalescence during freezing. Similarly, study conducted by Cho et al. (2023) demonstrated that biphasic gels made with rice bran wax and gelatin, significantly ($p < .05$) reduced water loss during F-T treatment compared to gels without wax. During freezing, not only the growth of ice crystals but also factors such as exposure to oxygen can have a significant impact on the probiotics cell (Tyutkov et al., 2022). The selection of an appropriate encapsulation matrix can provide effective protection of probiotics from the oxidative effects of atmospheric air. Encapsulation of probiotics in protein and/or polysaccharide matrixes has been successfully used to preserve cultures during freezing–thawing (Afzaal et al., 2019; Choudhury et al., 2021). In this study, the inner aqueous phase of DEGs acted as a reservoir for probiotics coated by the solidified oil phase and gelled outer water phase, leading to increased survival of microorganisms during freezing–thawing treatment.

Viability of LP and LR during *in vitro* digestion

Probiotics' ability to survive under gastrointestinal tract conditions, where they are exposed to lytic enzymes and improper pH conditions is crucial for their application in food. The gastric environment is acidic, with a pH ranging from 1.5 to 3.5 and containing digestive enzymes, such as pepsin, that hydrolyses proteins, including the cell wall of probiotics. The bile salts and pancreatic enzymes of the small intestine, with a pH ranging from 6.5 to 7.5, further affect probiotic survival by disrupting cell membrane components. The survival of free *Lactiplantibacillus plantarum* subsp. *plantarum* and *Limosilactobacillus reuteri* cells and those loaded in the inner water phase of different DEGs during simulated gastrointestinal digestion is shown in Figure 8. After incubating in the simulated gastric environment (pH 3.0, 120 min) the reduction of viable cells in free probiotics samples was 1.93 lg CFU/g of LP and 1.03 lg CFU/g of LR cells. In contrast, the decrease of viable LP cells encapsulated in DEGs was significantly ($p < .05$) lower: 0.6, 0.63, and 0.57 lg CFU/g for the W/O/Wgel, W/Ogel/W, and W/Ogel/Wgel, respectively. A very similar reduction of viable cells was determined for the encapsulated LR under gastric conditions. Such results suggest that DEGs were effective matrixes for increasing the gastric stability of probiotics. W/O/Wgel was able to protect probiotics from destruction due to the formation of whey protein gel that slowed down the diffusion of acids and proteolytic enzymes through the outer water phase of DEG and prevented cells from interacting with gastric juice. The effectiveness of W/Ogel/W in securing probiotics could be due to the solidified oil phase, which acted as a barrier to the spreading of gastric juices and reaching the inner water phase with probiotics located there. In the case of W/Ogel/Wgel, both above-mentioned approaches can be predicted in the protection of probiotics.

After intestinal digestion (pH 7, 240 min), probiotics viability significantly ($p < .05$) diminishes. The reduction of living cells in free probiotics samples was 3.00 lg CFU/g and 3.48 lg CFU/g of *Lactiplantibacillus plantarum* subsp. *plantarum* and *Limosilactobacillus reuteri*, respectively. However, the probiotics in DEGs were more

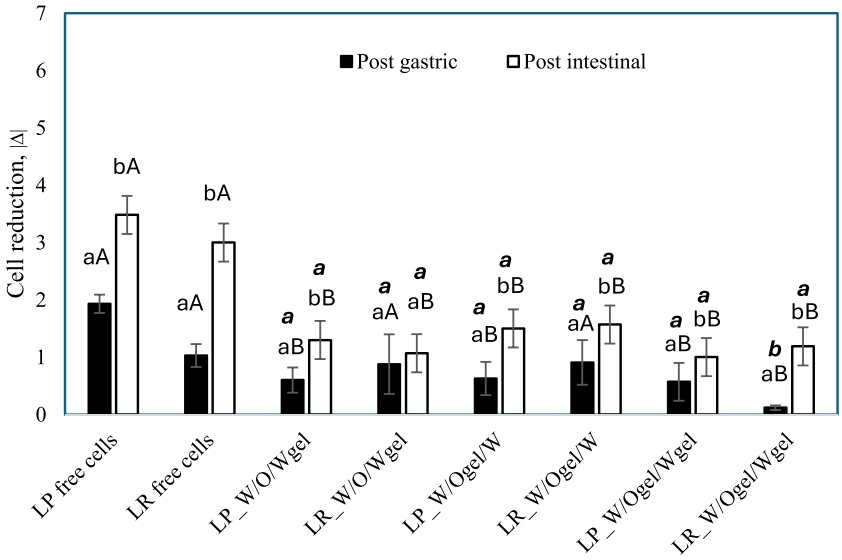


Figure 8. Cell reduction (|Δ|) of *Lactiplantibacillus plantarum* subsp. *plantarum* and *Limosilactobacillus reuteri* evaluation under *in vitro* gastrointestinal conditions. Values are reported as M ± SD, $n = 6$; lower case letters indicate significant difference ($p < .05$) between digestion stages; upper case letters indicate significant difference ($p < .05$) between free and encapsulated cells; cursed bolded lower case letters indicate significant difference ($p < .05$) between differently gelled DEGs. LP = *Lactiplantibacillus plantarum* subsp. *plantarum* DSM 24624; LR = *Limosilactobacillus reuteri* 182; W/O/W = water-in-oil-in-water.

alive and their reduction was around 1.0 lg CFU/g. The gelation of whey protein and oil in DEGs acted as a protective barrier, shielding the probiotics from direct exposure to harmful environmental conditions. It is clear that during the intestinal digestion phase, the whey protein gels and oleogels fully or partially retained their structure, thus protecting probiotics from contact with intestinal enzymes and bile salts and, therefore, keeping them alive. These results are similar to the conclusions of other researchers that encapsulation of probiotics into the inner water phase of W/O/W can improve their vitality during digestion (Liang et al., 2023; Zhou et al., 2024). However, the protection of probiotics was particularly efficient in these cases where uploaded emulsions were combined with hydrogel shell (Li et al., 2024; Zhu et al., 2024) or various prebiotics acted in W/O/W as wall materials (Yin et al., 2024).

Conclusion

This study shows that differently gelled double emulsions serve as an efficient tool for probiotics *Lactiplantibacillus plantarum* subsp. *plantarum* and *Limosilactobacillus reuteri*, offering protection against diverse adverse conditions, including storage, heating, freezing, and exposure to simulated digestive conditions. All evaluated DEGs showed improved probiotic viability during storage at 4 and 25 °C compared to free cells. Moreover, DEGs significantly ($p < .05$) improved probiotic stability during thermal treatment at 72 and 60 °C for 1 hr compared to non-encapsulated cells. Notably, DEGs with solidified oil phase and gelled outer water phase, increased microorganism survival during four freeze–thaw cycles. These properties indicate that encapsulating probiotics within the inner water phase of DEGs, effectively shields them and enhances their survival. Additionally, encapsulated probiotics successfully can stand *in vitro* digestion exhibiting a lower cell reduction than free cells.

Unlike conventional encapsulation methods, DEGs offer a tailored approach by combining multiple protective layers, significantly enhancing probiotic resilience. Given DEGs ability to maintain high probiotic viability and withstand heat and freezing conditions makes them suitable for use in probiotic-fortified baked goods, frozen desserts, or fat-replacing product. However, some limitations should be acknowledged. The long-term stability of encapsulated probiotics beyond the tested storage period must be studied, particularly in various food matrices and real food processing and distribution. Further study is required to understand how probiotics released from DEGs behave in the colon and whether they impact the gut microbiota. In conclusion, probiotic-loaded DEGs prove to be effective for food processing, ensuring the delivery of an adequate probiotic count (more than 6 log CFU/g). Moreover, their ability to withstand freezing and heat treatments extends shelf life. This contributes to improved gut health and supporting the development of novel functional products.

Data availability

The data that support the findings of this study are available from the corresponding author upon reason-able request.

Author contributions

Laurita Varnaitė-Kapočė (Formal analysis; Investigation; Methodology; Writing—original draft), Brigita Kabalinaitė (Formal analysis; Investigation), Vilma Petrikaitė (Investigation; Methodology), Evren Gölge (Investigation; Methodology), Aušra Šipailienė: (Conceptualization, Data curation), and Daiva Leskauskaitė (Conceptualization; Supervision; Writing—review & editing).

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Conflicts of 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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