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Effect of gelled phases on the relevance of double emulsion gels for probiotics encapsulation, rheological properties, and stability

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ABSTRACT

This study aimed to develop a stable double emulsion gel (DEG) based on whey protein cold-set gelation of the external water phase and carnauba wax-based solidification of the oil phase as a potential delivery system for probiotics in high-protein food products. The effect of the gelation of different phases on the microstructure, gel properties, and stability of DEGs under storage and freezing–thawing conditions and the viability of *L. plantarum* loaded in the inner water phase of DEGs were evaluated. The oil phase crystallization allowed the formation of stable DEGs during storage, showing negligible changes in consistency index, G′, and particle size after 56 d of storage and under four freeze–thaw cycles. The gelation of both the oil and external water phases allowed a stronger gel network formation, with a six times lower creaming index than non-gelled double emulsions. All samples demonstrated that the encapsulation of *L. plantarum* into the inner water phase of the DEG is beneficial for the storage stability of probiotics.

1. Introduction

In the past few decades, double emulsions (DEs) have shown significant progress in both fundamental research and practical applications as vehicles for bioactive ingredients (Choi, Choi, Lee, & Jo, 2020; Díaz-Ruiz, Valdeón, Álvarez, Matos, & Gutiérrez, 2021) and probiotic bacteria (Jiang et al., 2023; Marefati, Pitsiladis, Oscarsson, Ilestam, & Bergenståhl, 2021). Additionally, DEs offer a unique composition of water-in-oil-in-water, possibly reducing the fat content of food products. Our previous study successfully prepared DE, achieving the co-encapsulation effect of the respective hydrophilic and hydrophobic vitamins and high milk protein content in the external water phase (Keršienė et al., 2020a,b). This DE has the potential to be used in the production of yogurt to fortify it with beneficial nutrients (vitamins and proteins) (Keršienė et al., 2020a,b). However, DEs are unsuitable for encapsulating bacteria due to the high temperature used in their production. Another disadvantage is that DEs break down and separate water or oil during food processing (e.g., freezing, heating, and mechanical behavior). It is hypothesized that converting DEs into DE gel will increase their stability and enable their use for probiotic bacteria delivery and in high-protein yogurt production.

Notably, the cold-gelation process is preferable for DE gels applied as carriers of bacteria. Polysaccharides (Ren et al., 2024) and proteins (Habibi, Dekiwadia, Kasapis, & Truong, 2023) with a cold gel-forming ability are often exploited to induce the gelation of the aqueous phase of DE. Cold-set whey protein gels can be established by the addition of divalent salts since whey protein at temperatures above 75 °C can unfold and aggregate in the presence of Ca^{2+} ions due to the electrostatic shielding, ion-specific hydrophobic interaction, and cross-linking of adjacent anionic groups via protein-Ca^{2+}- protein bridges (X. Zhang et al., 2023). To structure the oil (O) phase, a solid or semi-solid structure must be created without modification of the oil's chemical characteristics. Carnauba wax is widely applied as an oil-structuring agent due to its high melting point (Fei & Wang, 2017) and strong gel formation (Lim, Hwang, & Lee, 2017). Carnauba wax can be dissolved directly in oil above its melting temperature. Then, the mixture is cooled and begins to undergo a sol–gel transition due to the self-assembly of carnauba wax, forming a network (Hu, Jiang, Du, & Meng, 2023).

Since most research has been performed on the stability of double emulsion gel (DEG) (W/O/W) with external water phase gelled by different polysaccharides (Chen et al., 2022) or proteins cross-linked with polysaccharides (Li et al., 2024; L. Wei et al., 2024), the comprehension of their structural stability in the presence of high protein content gel is still low, which is a drawback for the rational use of such structures for high-protein products. DEs have been used to encapsulate probiotics within the internal water phase (Ding et al., 2022; Qin, Luo, &

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Li, 2021; Wang et al., 2020). There are no reports on employing DEs to encapsulate probiotics and then execute gelation in the oil phase and external water phase to provide dual bacteria protection.

This study aimed to develop a stable DE gel based on whey protein isolate (WPI) cold-set gelation of the external water phase and carnauba wax-based solidification of the oil phase, which could be a potential delivery system for probiotics in high-protein food products. The impact of the gelation of different phases on the formation and properties of the DEGs was assessed, emphasizing their structural, textural, and rheological properties. The stability of DEGs was evaluated during the storage time (56 d) and under freeze–thaw treatment to ensure a longer product shelf life. Finally, the viability of probiotic cells loaded in the inner water phase of DEGs was measured during storage.

2. Materials and methods

2.1. Materials

Distilled water was used to prepare the emulsion's aqueous phases. WPI was supplied by Arla Foods Ingredients (Lacprodan DI-9213, Denmark) and contained 85.0 ± 3.0 g/kg of protein, 60.0 ± 1.0 g/kg of moisture, 40.0 ± 1.0 g/kg of ash, and 1.0 g/kg of lactose. Rapeseed oil was soured from the local market in Kaunas, Lithuania; polyglycerol polyricinoleate (PGPR) from Danisco, Copenhagen, Denmark; sodium chloride from EUROCHEMICALS, Vilnius, Lithuania; calcium chloride from AppliChem GmbH, Darmstadt, Germany; carnauba wax from Sigma-Aldrich, Steinheim, Germany; MRS agar from De Man-Rogosa-Sharpe, Biolife, Italy; and MRS broth medium from Biolife, Italia. All chemicals and reagents used in this study were of analytical grade.

2.2. Methods

2.2.1. Preparation of double emulsion gels

All DE samples were prepared using a two-step emulsification process with an Ultra-turrax rotor-stator system (IKA® T-18 basic, Staufen, Germany) at room temperature (+20 ± 2 °C).

2.2.1.1. Double emulsion without gelation (control). The internal phase (W_1) consisted of 0.5 % NaCl solution. For the external phase (W_2), 12 % w/w of whey protein isolate (WPI) was mixed with distilled water on a magnetic stirrer (IKA C-MAG HS 7 Package, IKAMAG) for 1 h at +20 ± 2 °C and 1000 rpm and left for a night to completely hydrate in a fridge (+4 ± 2 °C). The following day, the whey protein isolate solution was heated in a water bath (Wisd WiseBath, WITEG Labortechnik, Wertheim, Germany) at 74 ± 0.5 °C for 10 min and cooled at room temperature. The O phase was prepared by dissolving 4 wt% PGPR in rapeseed oil at +50 ± 0.5 °C for 15 min in a water bath.

W_1/O (**1**): The primary emulsion W_1/O was prepared by a dropwise addition of W_1 to the O at a ratio of 1:4, followed by homogenization at 7000 rpm for 10 min.
W/O/W (**2**): The DE was prepared by homogenizing the primary emulsion (W_1/O) with W_2 at a ratio of 2:3 at 10000 rpm for 5 min.

2.2.1.2. Double emulsion gels. The internal phase (W_1) consisted of 0.5 % NaCl solution. The external phase (W_2) contained 12 % w/w WPI mixed with distilled water, stirred magnetically for 1 h at +20 ± 2 °C, at 1000 rpm speed, and left overnight at +4 ± 2 °C. The WPI solution was then heated at +82 ± 0.5 °C for 10 min until it became viscous and was subsequently cooled to room temperature.

2.2.1.3. Double emulsion with gelled external water phase (W/O/Wgel).

W_1/O (**3**): Prepared as described in step **1**.

W/O/Wgel (**4**): The DEG was prepared by homogenizing W_1/O with W_2 at a 2:3 ratio at 10000 rpm for 5 min. During homogenization, 100 mM of $CaCl_2$ (in a 1:9 salt-to-WPI solution ration) was added dropwise to structure the W_2 phase.

2.2.1.4. Double emulsion with gelled oil phase (W/Ogel/W).

W_1/O (**5**): The O was prepared by dissolving 4 wt% PGPR and 10 wt % carnauba wax in rapeseed oil at +90 ± 0.5 °C until complete solubilization, followed by cooling to +20 ± 2 °C. The primary emulsion was then prepared as in step **1**.

W/Ogel/W (**6**) was prepared as described in step **2**.

2.2.1.5. Double emulsion with gelled external water and oil phases (W/Ogel/Wgel).

W_1/O (**7**) was prepared as described in step **5**.

W/Ogel/Wgel (**8**) was prepared as described in step **4**.

2.2.2. Preparation of microbial cultures

Lactobacillus plantarum was kindly provided by the Food Institute of Kaunas University of Technology (Kaunas, Lithuania). Microbial cultures were stored on MRS agar at +1 ± 0.5 °C. *L. plantarum* was stored in aerobic conditions. Before experimental use, *L. plantarum* cells (1%) were pre-cultivated in the MRS broth medium at +37 ± 0.5 °C temperature for 24 h and subcultured in the same medium at the same conditions for another 24 h.

Afterwards, the harvested cells were centrifuged (MPW Med. Instruments MPW-260R, Warszawa, Poland) at 6000 rpm for 20 min at +4 ± 0.5 °C temperature. After centrifugation, the supernatant was discarded and the cell pellet was suspended in the centrifugal tube. The suspended cells were vortexed, and the washing procedure with sterile water was repeated twice. The collected cells were diluted with sterile physiological saline (0.85 % wt/vol NaCl) to reach the final 1×10^{10} colony-forming unit (CFU) ml^{-1} concentration of the viable lactic acid bacteria (LAB) cells. To obtain a cell-to-emulsion ratio of 1:10, the cell suspension was added to sterile physiological saline (0.85 % wt/vol NaCl), and the resulting mixture was used in the subsequent encapsulation procedure. Bacteria suspension was used for encapsulation as W_1 for W/Ogel/W, W/O/Wgel, and W/Ogel/Wgel emulsions.

2.2.3. Characterization of double emulsion gels

2.2.3.1. Microstructure. Each DEG sample underwent visual documentation through photography and was observed with an optical microscope (Optika IM-3 Series equipped with an IM-3 Series mechanical stage and a 6.3 MP CMOS P6 Pro Kamera) (Optika S.r.l., Ponteranica [BG]—Italy). The sample was positioned on a standard 75 × 25 mm microscope slide. A drop of distilled water was then added, followed by carefully placing the cover slip over the sample. To enhance the microscope view, the software PROVIEW (version 3.7) was utilized to adjust the exposure and color balance. The microscopy process was repeated multiple times to achieve the optimal view.

2.2.3.2. Rheological measurements. All rheological measurements were carried out with an MCR92 rheometer (Anton Paar, Graz, Austria) using a parallel-plate measuring system with a PP25 plate (Anton Paar, Graz, Austria) (diameter: 25 mm; length: 100 mm; torsional compliance: 0.00113 rad/Nm), and the measurement parameters were based on preliminary experiments.

Approximately 5 g of the sample was placed on the rheometer plate using a spatula and then preincubated at room temperature (20 ± 0.5 °C) for 10 min. Samples were subjected to a logarithmically

increasing shear rate with a continuous ramp from 1 to 200 s^{-1} to register the flow curves. Data were analyzed using the Ostwald regression model, and the coefficient of determination (R^2) was calculated automatically using RheoCompass software (Anton Paar, Graz, Austria). A frequency sweep test was conducted within the linear viscoelastic region at a constant shear strain (0.05 %) to determine the changes in the elastic (G′) and viscous (G″) moduli within the increasing frequency range of 0.1–100 rad/s (temperature +20 ± 0.5 °C).

Each measurement was conducted at least three times.

2.2.3.3. Texture analysis. Texture analysis was conducted with a TA-XT2 texture analyzer (Stable Micro Systems, TA.XT Plus, Godalming, UK), according to Baltuonytė et al. (2022), with slight modifications. Emulsion gels were prepared in small plastic containers loaded with 40 g of sample, with a diameter of 5.5 cm and a height of 2.5 cm. The spherical probe (SMS P/0.5S, Stable Micro Systems, Godalming, UK) was inserted into the emulsion sample up to 5 mm, with a speed of 1 mm/s.

The texture parameters (hardness [expressed in Newtons] and cohesiveness [dimensionless]) were calculated using the Exponent Stable Micro Systems software. Measurements were performed in triplicate at room temperature (+20 ± 2 °C).

2.2.3.4. Particle size and distribution. The droplet size distribution of the emulsions was determined by laser diffraction with a Mastersizer 2000 (Malvern Instrument Ltd., Worcestershire, UK), according to Pan, Ma, Sun, and Bai (2024), with slight modifications. A sample was added to circulating distilled water (1750 rpm) induced with ultrasound (5 %) to produce a 10–15 % obscuration rate. The average droplet size was expressed as the surface moment mean diameter ($D_{3,2}$) (dimensionless) and volume-based size distribution (S_{pan}) (dimensionless).

2.2.3.5. Creaming assessment. Creaming assessment was performed using the centrifugation method, which accelerates the destabilization of DEGs. Five grams of DE was centrifuged into a centrifugal tube (MPW Med. Instruments MPW-260R, Warszawa, Poland) at 5000 rpm for 20 min at room temperature (+20 ± 2 °C). After centrifugation, the supernatant was drained and weighed on a laboratory analytical scale. The creaming index was expressed according to the following equation:

$$\text{Creaming index } (\%) = \frac{\text{weight of supernatant}}{\text{weight of sample}} \bullet 100 \quad \text{Eq. 1}$$

2.2.3.6. Freezing–thawing (F–T) cycles. A freezing–thawing treatment was conducted according to the method described by Zang et al. (2019) with slight modifications. Forty grams of samples were placed in 120 mL plastic (PP) containers with closed lids, frozen at −20 ± 2 °C for 7 d, and thawed at +20 ± 2 °C for 3 h. This freeze–thaw treatment was repeated for up to four cycles. The samples were collected after thawing to evaluate rheology and particle size.

2.2.3.7. Viable lactic acid bacteria count. Viable LAB cell counts in emulsions were determined using the plate counting method. A sample of 1 g was mixed with 9 ml of sterile physiological saline (0.85 % wt/vol NaCl), and a series of dilutions were plated to count the LAB on MRS agar (Biolife, Italia). Incubation was performed under aerobic conditions using a temperature-controlled incubator (Termaks, Norway) at 37 ± 0.5 °C for 48 h. LAB colonies were counted and expressed as the logarithmic CFU per gram.

2.3. Statistical analysis

All experiments were conducted twice, with three repetitions. The average value and standard deviation were calculated using Microsoft Excel. Significant differences were evaluated by *t*-test with GraphPad Prism software, assuming a significant statistical difference when the *p-value* was less than 0.05.

3. Results and discussion

3.1. Basic characterization of double emulsion gels with different gelled phases

Preliminary tests were conducted to determine the DEG preparation conditions enabling the formation of a shape-holding gel with good stability. DEG samples with a liquid, lumpy, sour cream-like texture were rejected. Some preliminary research results are shown in Fig. 1. Preliminary experiments have shown that critical parameters for the texture and stability of DEG are the concentration of whey protein isolate in the external water phase and the temperature and duration of its pretreatment, as well as the amount and time of addition of gelling agents to the different phases of DE (carnauba wax, calcium chloride, and mono- and diglycerides of fatty acids). By systematically adjusting these parameters, conditions were found that enabled the production of the following DEG samples: 1) DEG in which the external water phase was cross-linked using cold-gelation of whey proteins by adding $CaCl_2$, 2) DEG in which the oil phase was cross-linked with carnauba wax, and 3) DEG in which both the external water and oil phases were cross-linked. Non-networked DE was used as a control in further studies.

First, DEGs' structural, mechanical, and rheological properties with different gel phases were determined. As illustrated in Fig. 2, all manufactured DEGs form a homogeneous, smooth, and shape-maintaining gel. Microscopic images of DEG and DE prove the existence of a three-phase structure typical of DEs in all cases.

The viscosity of DE gelled in different phases was determined. The results are presented in Fig. 3 and Table 1. Non-gelled DE rheological behavior was close to pseudoplastic liquids, where viscosity changed slightly with an increasing shear rate. All DEG samples showed pronounced shear-thinning behavior, with apparent viscosity gradually decreasing with an increased shear rate due to the disruption of the whey protein gel structure or oleogel morphology at high shear rates. The viscosity increased significantly after gelating one or two DE phases ($p < 0.05$). The Ostwald equation was adjusted to the rheological behavior of DEGs because this model fitted well to the shear stress and shear rate data ($R^2 \geq 0.84$). The highest consistency index was determined in W/O/Wgel (K = 490.01 ± 0.40), and the lowest in W/Ogel/W (K = 77.84 ± 11.67). The maximum viscosity of the external water phase gelled DEG was also reported by Chen et al. (2022). In this study, the higher viscosity of W/O/Wgel indicates that the addition of calcium chloride ions induced the networking of aggregated whey proteins that led to significantly ($p < 0.05$) increased viscosity compared with the control sample (K = 58.51 ± 2.25) (Table 1) (Ye & Singh, 2000). The lower viscosity of DEG produced with only a structured oil phase may be due to the relatively low oil phase volume in the W/Ogel/W, which had less influence on the viscosity of the formed structure.

As the frequency sweep test indicates, DEGs with different phases networked demonstrate gel-like behavior as G' > G'' throughout the entire frequency range, with moduli almost parallel to each other (Fig. 4). However, the viscoelastic properties of DEGs gelled in different phases varied. The W/Ogel/W and W/Ogel/Wgel had the largest G′ values in the frequency sweep at 2.51 rad/s—1.37E + 04 ± 2.70E + 03 Pa and 62E + 04 ± 1.93E + 03 Pa, respectively (Table 1). It can be seen in the micrographs of these DEGs that carnauba wax crystallizes at the oil–water interface and forms a thicker interface film, giving DEG a more vital anti-deformation ability (Chen et al., 2022). A continuous network of aggregated whey proteins formed by Ca cross-linking in the outer water phase contributes to a stronger W/Ogel/Wgel formation. However, DEG with a gelled external water phase only (W/O/Wgel) appeared as a soft gel; its G′ was significantly ($p < 0.05$) lower—3.79E+03 ± 2.06E+02 Pa. When heat-induced gelation of whey proteins occurs at higher Ca ion concentrations, denatured proteins

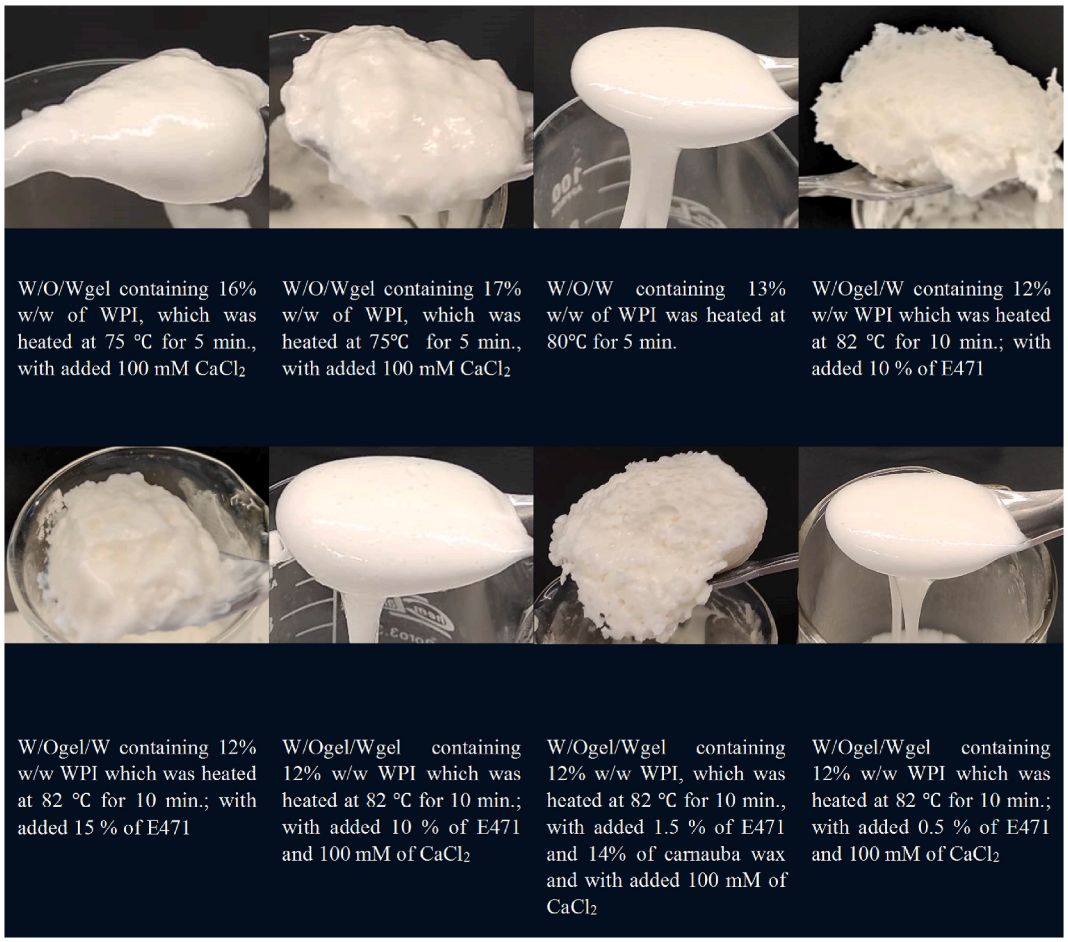

Fig. 1. Photographs of some DEGs were obtained during the preliminary experiments.

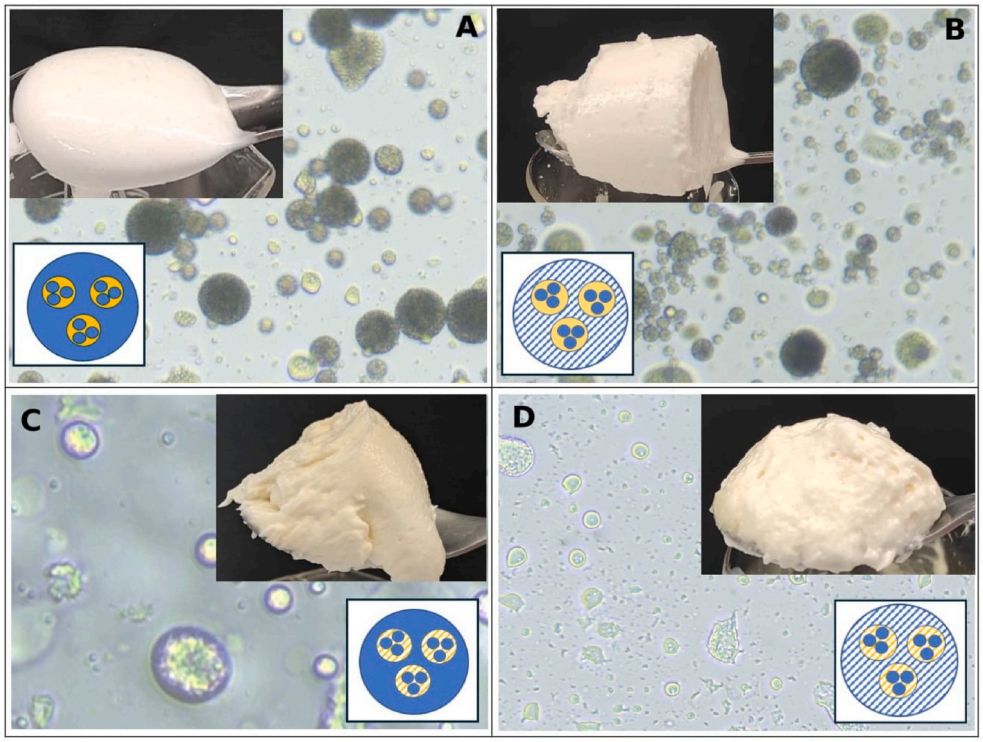

Fig. 2. Photographs and optical micrographs of DE and DEGs: (A) W/O/W, (B) W/O/Wgel, (C) W/Ogel/W, (D) W/Ogel/Wgel.

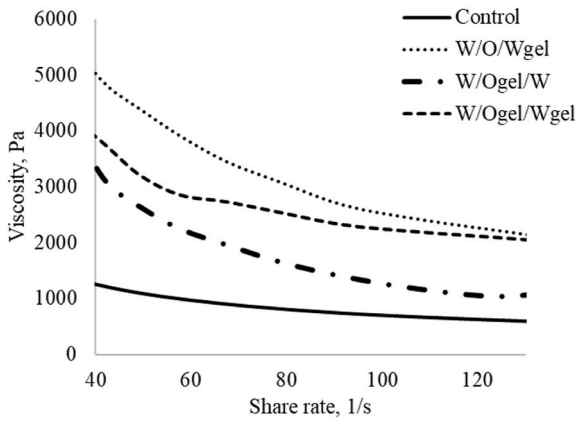

Fig. 3. Viscosity curves of DE and DEG with different phases gelled.

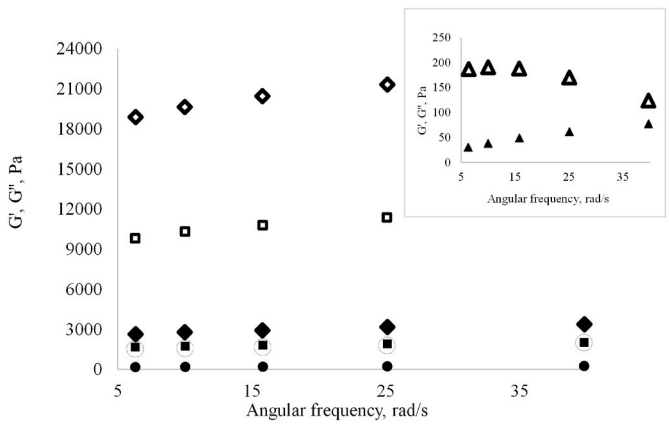

Fig. 4. Frequency sweep tests of DE and DEG with different phases gelled. ▲,Δ - Control; ●, ○ – W/O/Wgel; ■, □ – W/Ogel/W; ◆, ◊ - W/Ogel/Wgel. Empty symbols represent storage modulus (G′), filled symbols represent loss modulus (G′′).

aggregate randomly by physical interactions into particulate turbid soft gels (Alting, Hamer, de Kruif, & Visschers, 2003), which explains the lower viscoelastic modulus of the external water phase gelled DE than that of the oil phase gelled DE. Even when the DEs were not gelled, the G′ of DE was still slightly larger than G′′. However, G′ declined at high frequencies, suggesting weakened elasticity and damage to the gel network.

Texture is a relevant property of food that contributes to the sensory experience of eating, such as mouthfeel and taste. According to the data presented in Table 1, the non-gelled DE was soft (0.07 ± 0.00 N) and tended to flow. When the external water and/or oil phases were gelled, a significant ($p < 0.05$) hardness increase was determined. The hardest was W/Ogel/Wgel (0.82 ± 0.04 N), while the least hard was W/O/Wgel (0.28 ± 0.03 N). These results are comparable with those of G′ values determined for DEGs. W/Ogel/W and W/Ogel/Wgel show a significant ($p < 0.05$) decrease (−0.23 ± 0.03 and −0.22 ± 0.03, respectively) of cohesiveness compared with the control sample (−0.13 ± 0.01). This means that samples with carnauba wax, when chewed, hold together mass better and resist falling apart.

The droplet size is a substantial parameter that predetermines DEGs' stability and textural and rheological properties. Gelation of the external water, oil, and both phases led to a significantly ($p < 0.05$) smaller averaged droplet size with higher polydispersity than non-gelled DE. Presumably, adding carnauba wax into the oil phase caused crystalline associations to develop on the interphase oil–water, giving a thicker interface film, which causes the oil droplets to shrink. Smaller oil droplets in W/O/Wgel could result from entrapping oil droplets, inhibiting their movement in the three-dimensional whey protein network and preventing their enlargement. The Span parameter indicated that all DEG samples are polydisperse systems. W/Ogel/W and W/Ogel/Wgel showed higher numbers of Span (3.41 ± 0.37 and 3.98 ± 0.60, respectively), which offers a broader, more heterogeneous particle distribution than the control sample (1.50 ± 0.04), which is more uniform with a narrow particle distribution. Similarly, P. Zhang, Sun, Hou, Wang, and Tan (2024) observed a more uniform droplet size distribution in single-network DEGs than in double-network DEGs. The creaming index of DEGs correlates with the results of the viscoelastic properties and oil droplet size of gels. All DEG samples indicating smaller oil droplets and stronger gel formations showed a significantly ($p < 0.05$) lower creaming index (less than 4 %) than that of the control sample (13.28 ± 1.32 %) (Table 1).

Combined with the data in Figs. 3 and 4 and Table 1 and it can be concluded that the gelation of the external water phase had the most negligible impact on the viscoelastic properties of DE but resulted in the system's high viscosity. Gelling both the external water and oil phases had a more significant influence on DEG viscosity and elasticity.

3.2. *Effect of the gelling site on the storage stability of double emulsion gels*

When evaluating the stability of DEG during storage (56 d), it was found that the rheological indices decreased in all samples but differently (Table 2). As expected, the control non-gelled DE showed minor stability; its consistency index (K) decreased to 0.34 ± 0.08, and its storage modulus G′ decreased to 16 ± 1 Pa after 56 d of storage. For W/O/Wgel emulsion, the consistency index and storage modulus decreased significantly ($p < 0.05$) every 14 d. In this formulation, the whey protein isolate placed into the external water phase of DE was treated above 80 °C, which unfolds and promotes protein aggregation (Demetriades & McClements, 1998; Nicolai, 2019). Adding salts, such as calcium chloride, increases electrostatic repulsions between the droplets and allows them to get closer together (Demetriades & McClements, 1998). These bonds can undergo ion exchange reactions or reversibility during

Table 1
Physico-chemical characteristics of DEG samples measured at 1st day of storage time[a].

Sample	Hardness, N	Cohesiviness	D[3,2]	Span	Creaming index[b], %	K	G', Pa (at 2.51 rad/s)	tan δ (at 2.51 rad/s)
Control	0.08 ± 0.00a	−0.12 ± 0.01a	17.29 ± 0.24a	1.50 ± 0.04a	13.28 ± 1.32a	58.51 ± 2.25a (R = 0.98 ± 0.01)	3.94E+02 ± 1.80E+01a	0.15 ± 0.03a
W/O/Wgel	0.58 ± 0.06b	−0.13 ± 0.01a	12.35 ± 0.37b	2.37 ± 0.05b	3.39 ± 0.52b	490.01 ± 0.40b (R = 0.95 ± 0.01)	3.79E+03 ± 2.06E+02b	0.12 ± 0.00a
W/Ogel/W	0.45 ± 0.01c	−0.23 ± 0.03b	13.62 ± 0.70b	3.41 ± 0.37c	1.85 ± 0.18b	77.84 ± 11.67a (R = 0.87 ± 0.04)	1.37E+04 ± 2.70E+03c	0.14 ± 0.01a
W/Ogel/Wgel	0.70 ± 0.04d	−0.22 ± 0.03b	14.34 ± 0.90b	3.98 ± 0.60c	1.02 ± 0.13b	205.57 ± 8.04c (R = 0.88 ± 0.02)	1.62E+04 ± 1.93E+03c	0.15 ± 0.01a

[a] Values are reported as means ± standard deviation, n = 3 (but Span and D[3,2] n = 6). Letters indicate significant difference ($p < 0.05$) between samples.
[b] Creaming index was measured after 14th day of storage time.

Table 2
Rheological parameters and hardness of DE and DEG with different phases gelled during the storage time (56 days)[a].

Storage day	Emulsion			
	Control	W/O/Wgel	W/Ogel/W	W/Ogel/Wgel
	Consistency index (K)[b]			
1	58.51 ± 2.25aA	490.01 ± 0.40aB	77.84 ± 11.67aC	205.57 ± 8.04aD
14	12.75 ± 0.55bA	305.99 ± 6.61bB	104.74 ± 0.16bC	171.48 ± 4.33bD
28	1.98 ± 0.28cA	123.44 ± 16.14cB	100.70 ± 18.00bB	182.86 ± 22.93bC
42	2.26 ± 0.16cA	31.99 ± 4.16dB	100.07 ± 5.01bC	177.49 ± 13.10bD
56	0.34 ± 0.08dA	4.17 ± 0.46eB	108.05 ± 20.38bC	164.62 ± 15.93bD
	Storage modulus (G′, Pa) at 2.51 rad/s			
1	3.94E+02 ± 1.80E+01aA	3.79E+03 ± 2.06E+02aB	1.37E+04 ± 2.70E+03aC	1.62E+04 ± 1.93E+03aC
14	1.78E+02 ± 9.00E+00bA	1.96E+03 ± 9.20E+01bB	1.45E+04 ± 6.58E+02aC	1.75E+04 ± 9.26E+02aC
28	7.80E+01 ± 3.30E+01cA	1.22E+03 ± 1.75E+02cB	1.30E+04 ± 5.72E+02aC	2.10E+04 ± 1.54E+03aD
42	6.50E+01 ± 1.00E+01cA	4.69E+02 ± 8.40E+01dB	1.29E+04 ± 8.60E+0aC	2.38E+04 ± 3.65E+03aD
56	1.60E+01 ± 1.00E+00dA	1.80E+01 ± 1.00E+00eA	1.33E+04 ± 1.92E+02aC	1.99E+04 ± 4.95E+03aD
	Hardness, N			
1	0.08 ± 0.00aA	0.58 ± 0.06aB	0.45 ± 0.01aC	0.70 ± 0.04aD
14	0.07 ± 0.00aA	0.28 ± 0.03bB	0.67 ± 0.06bC	0.82 ± 0.04bD
28	0.07 ± 0.01aA	0.17 ± 0.01cB	0.60 ± 0.02bC	0.94 ± 0.04cD
42	0.07 ± 0.01aA	0.07 ± 0.01dA	0.91 ± 0.01cB	0.97 ± 0.04cB
56	0.05 ± 0.02aA	0.08 ± 0.01dA	0.49 ± 0.02aB	0.67 ± 0.28abB

[a] Values are reported as means ± standard deviation, n = 3; lower case letters indicate significant difference ($p < 0.05$) between storage days; upper case letters indicate significant differences ($p < 0.05$) between samples.
[b] The K values obtained after fitting the flow curves Ostwald equation ($R^2 \geq 0.84$).

storage, resulting in a weaker gel structure. Another interpretation was suggested by Sala, de Wijk, van de Velde, and van Aken (2008), who stated that whey protein gel weakens due to oil droplets that are not incorporated into the gel.

However, gelation of the oil phase resulted in more stable emulsions W/Ogel/W and W/Ogel/Wgel. Here, the apparent viscosity and G′ values of W/Ogel/W significantly increased ($p < 0.05$) after the 14th day of storage and remained almost unchanged during further storage. Oil gelling with plant-based wax creates a three-dimensional network that entraps liquid and greatly influences gel strength and rigidity (Penagos, Murillo Moreno, Dewettinck, & Van Bockstaele, 2023; Thakur, Singh, Prabhakar, Meghwal, & Upadhyay, 2022). During the early storage stage, the fat crystals in the structure of oleogels might grow or change form to decrease the free volume between crystals and make the network more compact (Doan, Tavernier, Okuro, & Dewettinck, 2018). After fat crystal crystallization was established, the structure of the oleogel did not change during further storage, so no significant changes in the consistency index and storage modulus values were observed. Similar behavior was found in the case of W/Ogel/Wgel. In this formulation, the gelation process occurs in the external water phase of the emulsion, with a smaller proportion of the oil phase where the internal water phase is entrapped in the compact fat crystal network. This study's results show that gelation of the oil and external water phases allowed a stronger gel network formation and consequently exhibited a higher G′ value throughout the storage period for 56 d.

The changes in the texture properties of DEG during storage confirmed the previously reported results of rheological parameters. During the storage, W/O/Wgel indicated a significant hardness ($p < 0.05$) decrease (from 0.58 ± 0.06 N to 0.08 ± 0.01 N) every 14 d. The opposite results were observed in emulsions with a gelled oil phase: the firmness decreased after 42 d of storage. Otherwise, it was pretty stable.

Monitoring the changes in D[3,2] over 56 d showed that the non-gelled DE droplet size remained stable, starting at 17.29 ± 0.24 and ending at 17.21 ± 0.37. According to the data presented in Table 3, gelation led to different changes in droplet size during storage depending on the gelling sites. In the W/O/Wgel, the particle size increased significantly (27.05 ± 0.75) on day 14, decreasing slightly during storage. However, no changes in the polydispersity of droplet size were indicated by the Span during storage. A more moderate increase in droplet size was observed in other DEGs, but at a later stage of storage: the 42nd day for W/Ogel/W and 28th day for W/Ogel/Wgel. Moreover, in DEGs containing oleogel in their structure, there was a more diverse droplet size distribution, indicating changes in fat crystal morphology and junction zone formation via the crystallization process as the emulsion ages. However, it did not affect the creaming stability of DEG, whereas an observed postponed droplet size aggregation led to the

Table 3
The oil droplet size of DE and DEGs with different phases gelled during the storage time (56 days)[a].

Storage day	Emulsion			
	Control	W/O/Wgel	W/Ogel/W	W/Ogel/Wgel
	D[3,2]			
1	17.29 ± 0.24aA	12.35 ± 0.37aB	13.62 ± 0.70aB	14.34 ± 0.90aB
14	18.51 ± 0.27bA	27.05 ± 0.75bB	11.84 ± 1.99aC	14.53 ± 0.54aC
28	16.47 ± 0.24cAB	23.10 ± 2.54bB	15.10 ± 0.47aA	17.04 ± 0.72bB
42	18.65 ± 0.14bA	24.08 ± 1.12bB	17.00 ± 0.99bC	17.10 ± 0.01bC
56	17.21 ± 0.37 aA	24.50 ± 1.34bB	17.20 ± 0.45bA	17.35 ± 0.11bA
	Span			
1	1.50 ± 0.04 aA	2.37 ± 0.05aB	3.41 ± 0.37aC	3.98 ± 0.60aC
14	2.19 ± 0.08bA	2.63 ± 0.15bB	4.85 ± 0.31bC	5.25 ± 0.34bC
28	1.98 ± 0.01bA	2.66 ± 0.32bB	5.28 ± 0.61bC	6.36 ± 0.57bC
42	3.01 ± 0.16cA	2.78 ± 0.26bA	5.62 ± 1.21bB	5.93 ± 1.25bB
56	2.68 ± 0.12dA	2.46 ± 0.21 aA	5.75 ± 0.58bB	5.49 ± 0.91bB

[a] Values are reported as means ± standard deviation, n = 6. Lower case letters indicate significant differences ($p < 0.05$) between storage days; upper case letters indicate significant differences ($p < 0.05$) between samples.

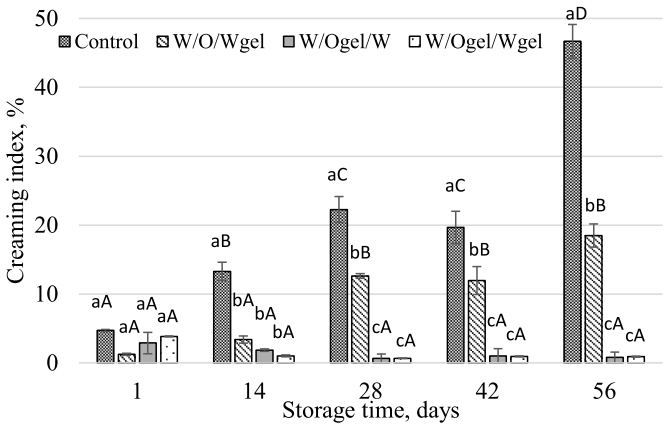

Fig. 5. The creaming index of DE and DEGs with different phases gelled during the storage time (56 days). Values are reported as means ± standard deviation, n = 3. Lower case letters indicate significant differences ($p < 0.05$) between samples; upper case letters indicate significant differences ($p < 0.05$) between storage days.

excellent creaming stability of DEG with the structured oil phase. After 56 d of storage, the W/Ogel/W and W/Ogel/Wgel creaming indexes were about six times lower than those of non-gelled DE and W/O/Wgel (Fig. 5). Different data are presented by P. Zhang et al. (2024), who found that the storage stability of DEG developed using gelatine and beeswax to gelatinize the internal aqueous and oil phases, respectively, was 50 times greater than that of DE prepared by simply adding rice bran wax.

The results suggest that gelling both the oil and external water phases is more efficient in improving the storage stability of DE than gelling one of these phases. The creaming index of DEGs highly correlates with the gelled emulsions' rheological characteristics, texture, and oil droplet size. While the emulsion gel shrinks and the liquid separates from the DEG, the consistency index, storage modulus, firmness, and droplet size decrease.

3.3. Effect of gelling site on double emulsion gel stability under freezing–thawing (F–T) conditions

Many food emulsions undergo freezing to ensure long shelf lives. However, emulsions frequently experience destabilization upon thawing, leading to the separation of the oil and aqueous phases. Therefore, it is essential to assess their stability during these processes. As for the results above, DEGs such as W/Ogel/W and W/Ogel/Wgel have been identified as exhibiting the most stable characteristics. Therefore, these particular formulations were selected to evaluate DEG stability under four cycles of F–T treatment. Stability was evaluated by measuring the rheological characteristics and DEG oil droplet size after each cycle of F–T treatment. The results are presented in Table 4.

The changes in storage modulus during F–T treatment demonstrated a significant ($p < 0.05$) decrease after the 4th F–T cycle of both tested DEGs. The consistency index of W/Ogel/Wgel was stable (from 281.66 ± 5.38 to 275.18 ± 10.26) during F–T treatment although the viscosity of the W/Ogel/W sample decreased significantly ($p < 0.05$) after the 4th F–T cycle (from 176.49 ± 2.94 to 122.80 ± 4.73). The oil droplet size for the W/Ogel/W emulsion decreased progressively across the four freeze–thaw cycles, starting at 25.37 ± 1.38 and ending at 16.92 ± 0.84 (Table 4). W/Ogel/Wgel also showed reduced oil droplet size after the first cycle but stabilized at 17.86 ± 0.80 from the second cycle onwards. Polydispersity (Span) showed an increasing trend for both types of DEGs; the W/Ogel/W sample reached a Span of 7.48 ± 0.83 after three cycles. However, the W/Ogel/Wgel exhibited a generally higher Span throughout the cycles, suggesting greater diversity in oil droplet size distribution, particularly notable with a Span of 7.42 ± 0.23 after four cycles. Oleogel structural stability is presumably responsible for the observed stability of DEGs during the F–T process. The carnauba wax oleogel crystalline microstructure remains unchanged during F–T cycles because the melting point of this wax is about 80 °C (Yi, Kim, Lee, & Lee, 2017). The freezing point of rapeseed oil is minus 20 to minus 18 °C (Miyagawa, Shintani, Katsuki, Nakagawa, & Adachi, 2017); therefore, the oil phase in the oleogel persists in liquid and is entrapped into the crystalline structure of wax. According to studies by Z. Wei and Huang (2019), such semi-solid interfacial crystalized oleogels can maintain

Table 4
Rheological characteristics and oil droplet size during freezing-thawing cycles of DEG samples[a].

Freeze-thaw cycles	1	2	3	4
Emulsion		Storage modulus (G′, Pa) at 2.51 rad/s		
W/Ogel/W	1.87E+04 ± 2.36E+03aA	1.69E+04 ± 9.02E+02abA	1.67E+04 ± 1.72E+03abA	1.40E+04 ± 8.79E+02bA
W/Ogel/Wgel	2.36E+04 ± 7.28E+02aB	2.96E+04 ± 1.67E+02bB	2.98E+04 ± 6.04E+02bB	1.21E+04 ± 5.40E+02cA
		Consistency index (K)[b]		
W/Ogel/W	176.49 ± 2.94aA	141.83 ± 30.17abA	129.10 ± 3.32bA	122.80 ± 4.73bA
W/Ogel/Wgel	281.66 ± 5.38aB	288.10 ± 41.12aB	308.08 ± 1.62aB	275.18 ± 10.26aB
		D[3,2]		
W/Ogel/W	25.37 ± 1.38aA	22.48 ± 0.92aA	17.86 ± 0.80bA	16.92 ± 0.84bA
W/Ogel/Wgel	31.73 ± 3.17aB	24.25 ± 0.86bA	17.86 ± 0.80 cA	17.78 ± 0.36cA
		Span		
W/Ogel/W	4.63 ± 0.45aA	4.95 ± 0.06aA	7.48 ± 0.83bA	6.36 ± 0.20bA
W/Ogel/Wgel	6.89 ± 1.35aA	6.35 ± 0.91aA	6.96 ± 0.82aA	7.42 ± 0.23aA

[a] Values are reported as means ± standard deviation, n = 3 (for D[3,2] and Span n = 6). Lower case letters indicate significant differences ($p < 0.05$) between F-T cycles; upper case letters indicate significant differences ($p < 0.05$) between samples.
[b] The K values obtained after fitting the flow curves Ostwald equation ($R^2 \geq 0.84$).

Table 5
Viable cell numbers (log CFU/g) in DEGs loaded with *Lactobacillus plantarum* during the storage time (56 days)[a].

Storage day	0	7	14	28	56
W/O/Wgel	7.78 ± 0.20a	7.57 ± 0.01a	7.27 ± 0.08b	7.59 ± 0.39ab	7.35 ± 0.02b
W/Ogel/W	7.27 ± 0.09ac	7.68 ± 0.13bc	7.44 ± 0.08c	7.43 ± 0.09c	6.69 ± 0.11d
W/Ogel/Wgel	7.13 ± 0.09a	7.90 ± 0.07b	7.10 ± 0.04a	7.10 ± 0.04a	7.42 ± 0.14a

[a] Values are reported as means ± standard deviation, n = 4; lower case letters indicate significant difference ($p < 0.05$) between storage days.

stability after several F–T cycles by providing a rigid physical shell around oil droplets. The behavior of the external water phase is no less critical for the stability of DEG during the freezing and thawing processes. However, in this study, this was not crucial. Whether or not whey proteins were connected to a network in this phase, their aggregates protected the system from disruption due to large and irregular ice crystal formation. The stability of frozen aqueous protein systems depends mainly on their constituents' physical states and the proportionality between them (Aider, de Halleux, & Akbache, 2007). It has been observed that the freezing–thawing stability of proteins in an aqueous solution increases with protein concentration (Meza, Verdini, & Rubiolo, 2010). Moreover, whey protein hydrogels (10 % w/w) demonstrated apparent changes in the gel network structure but moderate changes in viscoelastic and textural properties after F–T treatment (Zhao, Yan, Xue, Zhang, & Shen, 2022). This may be due to the gradual decrease in protein–water interactions, which are replaced by protein–protein interactions.

3.4. Viability of probiotic cells loaded in the inner water phase of DEGs during storage

L. plantarum viable cell numbers in DEGs loaded with cells during storage at +4 °C are presented in Table 5. The probiotics were immobilized in the inner water phase of DE. At the initial time point, the viable cell numbers of all tested samples varied between 7.13 and 7.78 log CFU/g. This indicates that *L. plantarum* retained resilience throughout the entire DEG preparation process. Throughout the storage period of up to 56 d, *L. plantarum* viable cell numbers changed slightly from 7.13 to 7.42 log CFU/g when loaded into W/Ogel/Wgel. A greater decrease in viable cell numbers was found when *L. plantarum* was loaded into W/O/Wgel, from 7.78 to 7.35 log CFU/g. Viable cell numbers loaded into W/Ogel/W were detected at the level of 6.69 log CFU/g, showing a 0.58 log cycle reduction after 56 d of storage. This demonstrates that the DEG structure worked as a physical barrier to protect probiotics from the environment. The migration of probiotics from the inner water phase was protected by the oil phase transforming into the platelet-like crystalline network due to the presence of carnauba wax (Gu, Du, & Meng, 2023). Additionally, the whey protein network formed in the external water phase helped prevent probiotic migration from phase to phase, as has already been reported for semi-solid and liquid emulsions (Zhuang, Gaudino, Clark, & Acevedo, 2021). The results demonstrate that the encapsulation of *L. plantarum* into the inner water phase of DEG is beneficial for the storage stability of probiotics. It is essential to note that probiotic viability remained above 6.0 log CFU/g throughout the entire storage period, which is in accordance with the recommended levels of viable probiotic cell counts in food at the time of consumption (Araya, Magdalena, et al., 2002; Razavi, Janfaza, Tasnim, Gibson, & Hoorfar, 2021).

4. Conclusion

In this study, DEGs—in which (i) the external aqueous phase was gelled with whey protein by adding $CaCl_2$ (W/O/Wgel), (ii) the oil phase was gelled with carnauba wax (W/Ogel/W), (iii) both external water and oil phases were gelled (W/Ogel/Wgel)—were prepared. All gels were viscous and demonstrated gel-like behavior with increased hardness, reduced creaminess, and broad, heterogeneous particle distribution compared with the control (non-gelled) DE sample. During the storage period (56 d), W/O/Wgel was not stable due to the weaking of the protein gel. Otherwise, W/Ogel/W and W/Ogel/Wgel emulsions were identified with the most stable characteristics and underwent four freezing–thawing cycles. Carnauba wax is a good structuring agent for DEs that enhances stability, creates a strong barrier, and maintains a semisolid food structure during storage. The encapsulation of *L. plantarum* into the inner water phase of DEG ensured the survival of cells for 56 d in all tested gels. This effect was attributed to the ability of the structured phases to prevent probiotic migration from phase to phase. Considering everything, this study demonstrates that it is possible to produce DEGs with good stability under storage and freezing–thawing conditions, using high protein content in the external water phase and crystallized oil phase with carnauba wax. By structuring different phases of DE, it is possible to tune their characteristics, expanding their application for the delivery of probiotic bacteria. Therefore, the developed DEGs have great potential as a delivery system for probiotics in high-protein food products.

CRediT authorship contribution statement

Laurita Varnaitė-Kapočė: Writing – original draft, Methodology, Investigation. **Daiva Leskauskaitė:** Writing – review & editing, Supervision, Funding acquisition, 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.

Data availability

Data will be made available on request.

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