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Utilisation of water-in-oil-water ($W_1/O/W_2$) double emulsion in a set-type yogurt model for the delivery of probiotic *Lactobacillus paracasei*

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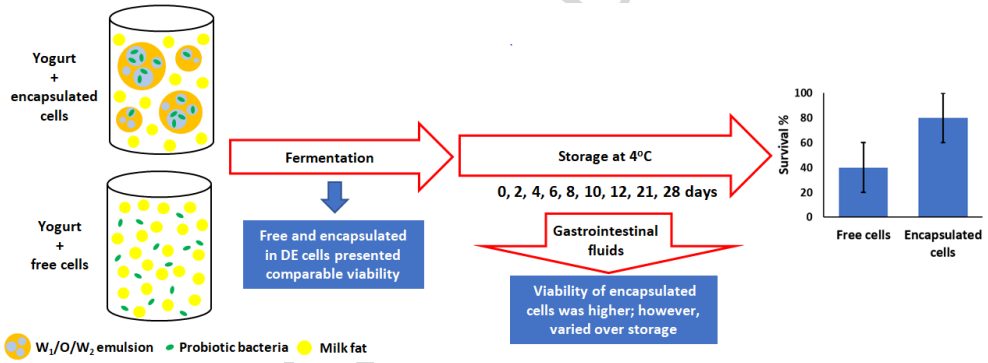
Abstract

$W_1/O/W_2$ emulsion in set-type yogurt has the potential to segregate probiotics in order to avoid interference with the starter culture as well as protection against harsh processing and digestion conditions. *Lactobacillus paracasei* subsp. *paracasei* DC 412 probiotic cells in milk-based $W_1/O/W_2$ emulsions were incorporated in yogurt, in addition to starter cultures *Lactobacillus bulgaricus* and *Streptococcus thermophilus*, and the effect on the fermentation, bacterial growth kinetics, physicochemical properties, and structural characteristics was investigated. Stability of $W_1/O/W_2$ was monitored with optical microscopy and cryo-SEM and localisation of encapsulated *L. paracasei* in yogurt was monitored using fluorescent microscopy. During fermentation, starter culture was not affected by introduction of *L. paracasei* and/or $W_1/O/W_2$ emulsion. The viability of *L. paracasei* encapsulated in $W_1/O/W_2$ emulsion was enhanced during storage and after exposure to simulated gastrointestinal conditions. *L. paracasei* remained within the inner W_1 phase till the end of the storage period (28 days at 4^oC). Moreover, $W_1/O/W_2$ emulsion altered physicochemical and textural properties; however, these were within acceptable

range. These results demonstrate the capability of $W_1/O/W_2$ emulsion to be utilised for probiotic fortification of yogurt to increase functionality without interfering with starter culture and fermentation.

Keywords: yogurt, $W_1/O/W_2$double emulsion, stability, *Lactobacillus paracasei*, viability

Graphical abstract



1. Introduction

Yogurt is one of the most widely consumed fermented milk products associated with the intake of probiotics which are added either as part of the starter culture or by fortification prior the fermentation process, simultaneously or sequentially with the yogurt culture or into the final product (Lourens-Hattingh and Vijoen, 2001). Set-type yogurt is produced by milk fermentation directly into containers without any further stirring aiming at increased firmness, high consistency and cohesiveness. Thus, the challenge is to incorporate probiotic bacteria without compromising texture development.

Furthermore, probiotic bacteria in yogurt need to remain viable and resist stresses i.e. resist manufacture processes (Rodríguez-Huezo*et al*., 2014), storage at refrigeration temperature (Xin et al., 2009), and digestion conditions (Shima et al., 2006), in order to reach the gut in functional concentrations (>10^8 $\log_{10}$colony forming units (CFU)/g) (Kechagia et al. 2013). However, probiotic bacteria can interfere with starter cultures and compete during fermentation (Vinderola et al., 2001) and/or alter process performance and quality of final product. Therefore, encapsulation of probiotic bacteria can be applied to increase their viability in foods and post-digestion as well as enabling the spatial separation of strains that negatively affect yogurt fermentation but present beneficial effects post-consumption.

Water-in-oil-in-water ($W_1/O/W_2$) emulsions could be a suitable alternative for encapsulation of probiotics compared to polymers (e.g. alginate) as they can be made from ingredients that are highly compatible with yogurt. Furthermore, encapsulation using $W_1/O/W_2$ emulsion can protect probiotics from cytotoxic gastric juice (Shimaet al., 2006; Pimentel-González et al., 2009) bile salts, (Shima et al.,

2009) and prolonged storage at low temperatures (Rodríguez-Huezo et al., 2014). However, studies on the ability of $W_1/O/W_2$ emulsion to protect probiotics during fermentation, storage and digestion of yogurt are lacking. Moreover, $W_1/O/W_2$ emulsion made with vegetable oils rich in mono- and polyunsaturated fatty acidscan be used to replace milk fat, thus reducing the risk of cardiovascular disease (Chen et al., 2016; Ryeo-Eun et al., 2015).

The presence of $W_1/O/W_2$ emulsion during fermentation may alter the physicochemical and textural properties of the yogurt. $W_1/O/W_2$ emulsion was incorporated in a stirred-type yogurt for the encapsulation of caffeine (Hernandez-Marín et al., 2016) and inlow-fat stirred yogurt made with canola oil and stabilized by adding edible polymers in combination with a food grade lipophilic surfactant (PGPR) in the inner W_1 phase (Lobato-Calleros et al., 2009). It was found that stirred yogurts containing $W_1/O/W_2$ emulsion showed higher stability compared to full milk-fat stirred yogurts depending on the type of polymer used to stabilize the $W_1/O/W_2$ emulsion. Also, the lacunarity values (a measure of size distribution of gaps) and viscosity were higher in stirred yogurts containing $W_1/O/W_2$ emulsions compared to full milk-fat stirred yogurts. Recently, the authors investigated the incorporation of $W_1/O/W_2$ emulsion in set-type yogurt (Lalou et al. 2017). The addition of $W_1/O/W_2$ emulsion altered the fermentation process and texture properties within an acceptable range. However, there is a lack in understanding how the set-type yogurt would behave in the presence of probiotics added in free form or encapsulated within the W_1 phase of the $W_1/O/W_2$ emulsion. Also, the interaction between probiotics and $W_1/O/W_2$emulsion affect the stability of the system and viability of the cells.

Bacteria can alter the stability of emulsions and this mainly depends on the

characteristics of the species such as metabolic activity, surface charge and hydrophobicity (Li et al., 2001; Dorobantu et al., 2004; Ly et al., 2006; Boitard et al., 2012; Firoozmand and Rousseau, 2014). On the other hand, emulsion structure can affect the bacteria by limiting the diffusion rate of nutrients and cause a reduction in the growth rates of bacteria (Brocklehurst et al., 1995; Charteris, 1996).

In this study *L. paracasei* a probiotic bacterial species, typically found in dairy products, such as yogurt, kefir, and infant formulas was encapsulated in set- type yogurt using $W_1/O/W_2$ emulsion and survival was assessed during fermentation, storage, and in gastrointestinal fluids. The interaction of encapsulated *L. paracasei* with starter cultures *Streptococcus salivarius* subsp. *thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* at ratio 1:1 was assessed. The stability of the system over storage was investigated by monitoring bacterial survival, physicochemical and textural properties. Finally, the stability of the $W_1/O/W_2$ emulsion was monitored using optical microscopy and cryo-SEM while the localization and spatial distribution of *L. paracasei* was observed using fluorescent microscopy. Milk as W_1 and W_2 phases and thus the O-W_2 interface was stabilised solely by the milk proteins without using synthetic hydrophilic surfactants.

2. Materials and Methods

2.1 Materials and microbial cultures

Fresh whole milk and food grade sunflower oil were purchased from a local retailer (United Kingdom). The oil soluble emulsifier polyglycerol polyricinoleate (PGPR) was provided by Danisco (Denmark). Skimmed milk powder, MRS broth, MRS agar and M17 were purchased from Fisher Scientific (United Kingdom). The two stains 2-(4-

amidinophenyl)-1H-indole-6-carboxamidine (DAPI) and 3,6-Acridinediamine(AO) were purchased from Sigma-Aldrich (United Kingdom).

A commercially available yogurt starter culture (*Streptococcus salivarius* subsp. *thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* at ratio 1:1*)* was purchased from Micromilk (Cremosano, Italy). Microbial inoculum was prepared by transferring aseptically 0.01 g of the freeze-dried yogurt starter culture in 150mLof sterile 10% w/v SMP solution. The cultures were grown anaerobically at 37 °C overnight to ~10^9 colony forming units (CFU)/mL. The cultures were grown anaerobically at 37 °C overnight to ~10^9 CFU/ml. The probiotic strain used in this study was *L. paracasei* subsp. *paracasei* DC412 (Xanthopoulos et al., 2000).

2.2 Preparation of set-type yogurt models with cells of *Lactobacillus paracasei* free and encapsulated in $W_1/O/W_2$ emulsions

Yogurt models were prepared with $W_1/O/W_2$ emulsion encapsulating *L. paracasei* in the inner water phase (W_1) and *L. paracasei* in free form without $W_1/O/W_2$ dispersed in the aqueous phase.

Fortified milk for yogurt production was prepared by adjusting the total soluble solid content to 16% w/v with the addition of skimmed milk powder. Aliquots of 150 mL were transferred in sterile Duran bottles (250mL) with screw caps and were pasteurized at 80 °C for 30 min. Fermentation was carried out by inoculating the substrates with 6% w/v of activated starter culture to a final concentration of ~8.4 $\log_{10}$CFU/mL, in a water bath at 42°C and pH was monitored every 15 min as described by Lazaridou et al. (2014).

$W_1/O/W_2$ emulsions were prepared using a high shear mixer homogeniser (Silverson

L5M) at 25°C. A two-step emulsification process was followed as described by El Kadri et al. (2015) with slight modification. Briefly, in the first step primary W_1/O (milk in oil) emulsions were made. An oil phase was prepared by dissolving 2 % w/w PGPR in sunflower. The inner aqueous phase (W_1) consisting of milk and *L. paracasei* cells (8,1±0,08log_{10}CFU/ml) was emulsified into the oil phase (W_1:O phase ratio of 40:60) at 4000 rpm for 120 sec. In the second step $W_1/O/W_2$ emulsion was made using milk as (W_2). The previously prepared primary W_1/O emulsion was emulsified into W_2 to form the $W_1/O/W_2$ emulsion (W_1/O:W_2 ratio of 20:80) at 2700 rpm for 60 sec. The $W_1/O/W_2$ emulsion composed 33% v/v of the final product. Specifically, 50 mL of double emulsion were introduced in 100 mL of fermenting milk at pH 5.7±0.1 at 180min after initiation of fermentation. At this pH value the viscosity of the milk is high enough to prevent creaming of the $W_1/O/W_2$ emulsion and thus the oil globules can be homogenously dispersed. The samples were mixed gently and left to stand until the pH reached 4.6±0.05. On completion of fermentation, the samples were cooled by immersing in ice water and were stored at 4°C for 24h (day 0 of storage).

L. paracasei cells were grown in MRS broth for 24h, washed twice with PBS and either inoculated in the fermenting milk or resuspended in the inner phase of the $W_1/O/W_2$ emulsion as to achieve the same initial population at the fermenting milk. $W_1/O/W_2$ emulsion with probiotic cells and free probiotic cells were introduced to the fermenting milk after 180 min (i.e. when the suitable pH value was reached).

2.3 Monitoring acidification, microbial growth kinetics and encapsulation efficiency during fermentation and storage

Maximum acidification rate (V_{max}) was calculated as the time variation of pH (dpH/dt).

At the end of each fermentation, kinetic parameters were calculated according to Mishra and Mishra (2013): (1) time to reached Vmax (h), t_{Vmax}; (2) pH at Vmax, pH_{Vmax}; (3) time to complete the fermentation (h)$t_{pH4.6}$.

During fermentation, samples were analysed every 1 h for cell growth and physicochemical characteristics. During post-fermentation storage, samples were analysed at 0, 2, 4, 6, 8, 10, 12, 14, 21 and 28 days.

For bacterial enumeration yogurt samples (1 g) were collected aseptically, serially diluted in phosphate buffered saline (PBS) buffer solution and were analysed by culture on media using the Miles and Misra technique (Miles & Misra, 1938). Enumeration of *L. bulgaricus* and *L. paracasei* was conducted on MRS agar media supplemented with bromophenol blue that detects pH values from 3 to 5 developed by starter culture by producing yellow colour at pH 3.0 and violet at pH 4.6 after incubation aerobically at 37 °C for 48 h. *L. bulgaricus* and *L. paracasei* were differentiated based on colony morphology (bluish elongated and white round colonies respectively) (Lee and Lee, 2008). *S. thermophilus* was incubated aerobically on M17 agar media at 45°C for 48 h, as elevated temperature prevented the growth of *L. paracasei* (Kristo et al., 2003; Tabasco et al., 2007).

2.4 Physicochemical determinations

Total acidity: Samples of yogurt (9 g) were diluted in 18mL of water and titrated using 0.1 N NaOH and phenolphthalein solution (1% w/v) as an indicator. Titratable acidity was measured according to official method (AOAC, 2005). Results were expressed as g of lactic acid /100 g of fermented sample.

Water retention capacity: 10g of sample were transferred in a plastic conical tube

(15mL) and centrifuged at 20000g for 10 min. The supernatant was discarded and the water retention capacity was calculated as % w/w of the sediment over the initial weight of the sample.

Syneresis: 5 g of samples were weighted on Whatman filter paper No1 (11 μm) and were drained under vacuum for 10min. Syneresis was expressed as % w/w of the drained liquid over the initial weight of the sample.

Viscosity measurements: Rheological characterisation of the yogurt samples with and without $W_1/O/W_2$ emulsions during and after fermentation was performed at 4°C using AR-G2 rheometer (TA instruments, New Castle, Delaware USA) equipped with a 14mm vane spindle. Viscosity of a representative yogurt sample (~30mL) was measured over a shear rate 0-100s^{-1}.

Texture Analysis: Samples of yogurt (30mL) were distributed to cylindrical plastic vessels (diameter 140mm) immediately after preparation (day 0) and left to set for another 24h at 4°C.Texture profile analysis (TPA) of the samples was conducted using a Texture Analyzer TAXT2i (Stable Micro Systems, Surrey, England) with accompanying computer software (Exponent). Samples were compressed under a cylindrical probe (P/40) at a test speed of 1 mm/s and a trigger force of 1 g, using the Texture Analyzer. Two compression cycles at 50% of the initial height were applied using a post-test speed of 4 mm/s. The data obtained from the force–time curves were used to calculate the hardness (g), cohesiveness, adhesiveness (g*s) and gumminess (g).

2.5 Viability of *L. paracasei* in yogurt exposed to simulated gastrointestinal conditions

Simulated gastric juice (SGJ) and simulated intestinal juice (SIJ) were prepared according to Mantzouridou et al. (2012). Briefly, SGJ was prepared by dissolving 0.3 g/L pepsin in a 0.2% NaCl w/v solution. The pH was adjusted to 2.5 with concentrated HCl. SIJ was prepared by suspending pancreatin and bile salts in phosphate buffer (PB, 0.05 mol/L Na_2PO_4) to achieve a final concentration of 1 g/L and 4.5 g/L, respectively. The pH of the solution was adjusted to 7.4 with 0.1 N NaOH. Both SGJ and SIJ were prepared fresh and were used on the same day after filter sterilization through a 0.45-μm pore size cellulose–ester membrane. Samples of yogurt (2 g) were withdrawn aseptically at 0, 7, 14, 21 and 28 days of storage and were subjected to simulated digestion conditions as described by Mantzouridou et al. (2012). Briefly, the yogurt sample was mixed with 18 mL of SGJ and incubated in a shaker incubator at 100 rpm and 37 °C for 2 h., followed by the addition of 20 mL of SIJ to the mixture and further incubation for 4 h under the same conditions. *L. paracasei* enumeration was conducted immediately after exposure as previously by culture on MRS agar media supplemented with bromophenol blue. In each case, 100% represents the living cell number (log_{10}CFU/g yogurt) before exposure to simulated gastrointestinal conditions.

2.6 Monitoring of $W_1/O/W_2$ emulsion structure and encapsulation efficiency in yogurt with optical and fluorescent microscopy

Yogurt samples with or without $W_1/O/W_2$ emulsions were observed on microscope slides and images were acquired under 10x magnification using optical microscopy (Zeiss Axioplan) at room temperature coupled with a digital colour camera system (10megapixelMoticMoticam CMOC camera) via Motic Images Plus video acquisition software.

For fluorescent microscopy the *L. paracasei* cells were stained in W_1 phase with AO (10 µl mL^{-1}) before encapsulation. At day 0, 7, 14, 21 and 28 a 10mL yogurt sample was stained with DAPI (4 µl mL^{-1}) and incubated in the dark for 30 minutes to stain the starter culture. For imaging, the sample was placed on a microscope slide and gently covered with a cover slip. The image was acquired under objective lens 100x magnification (oil immersion) with a digital camera system Axiocam ICm1 using a 1.4 megapixel monochrome CCD camera via AxioVision Software (Zeiss). The light source used to excite the DAPI and AO was a mercury arc lamp and the emission was observed at 461 nm (DAPI) and 590 nm (AO). Micrographs were overlaid using analysis software (ImageJ).

The microstructure of yogurts samples containing $W_1/O/W_2$ emulsions was visualised using cryogenic scanning electron microscopy (Cryo-SEM; Philips XL30 FEG ESSEM). One drop of each sample was frozen to -180 °C in liquid nitrogen slush. Fractuation and etching of the frozen sample was performed for 5 min at -195 °C in a preparation chamber. Subsequently, samples were sputter coated with gold and scanned at -160 °C.

2.7 Statistical Analysis

Two independent experiments were carried out in all cases and at least three replicate measurements were carried out for each sample. Statistical comparison of the mean values was performed by student's *t*-test ($p<0.05$ confidence level) using the SPSS 21.0 software (SPSS Inc., Chicago, IL, USA). Error bars represent the standard deviation (sd) of the mean value.

3. Results and Discussion

3.1 Encapsulation of *L. paracasei* and incorporation of $W_1/O/W_2$ emulsion during milk fermentation

In this study sunflower oil was used as dispersed phase since it can form a more stable $W_1/O/W_2$ emulsion during fermentation compared to milk fat (i.e. butterfat) (Lalou et al., 2017) and presents a healthier substitute of milk fat in set-type yogurts (Farmani et al., 2016).The average mean size distribution [D (4, 3)] of W_1 droplets and oil globules (10-15 μm and 50-70 μm, respectively) (Fig S1) was sufficient to encapsulate *L. paracasei* cells (Fig.S2). Immediately after the fermentation process the oil globules were dispersed homogeneously throughout the yogurt and no flocculation or coalescence was observed (Fig. S3).

3.2 Acidification kinetics during set-type yogurt formation with *L. paracasei* and $W_1/O/W_2$ emulsion

The kinetics of milk acidification were determined in this study by monitoring changes in pH during the fermentation process (Fig. 1). Some variation was observed in the values of the parameters $V_{max,}$ T_{Vmax}, pH_{Vmax}, and $t_{pH4.6}$ values between fermenting milk with free *L. paracasei* cells (control) and encapsulated in $W_1/O/W_2$ emulsion (Table 1). The introduction of $W_1/O/W_2$ emulsion encapsulating *L. paracasei* varied the rate of acidification. A gradual decrease in pH was observed in both samples. However, when $W_1/O/W_2$ emulsion with *L. paracasei* was introduced to the system (at pH 5.7 ± 0.1, 180 min) a slight increase in pH (~0.2 pH units) was observed which did not occur when free *L. paracasei* cells were added, However, the latter followed a faster decline in pH (Fig. 1).

The fermenting milk with $W_1/O/W_2$ emulsion had higher V_{max} and T_{Vmax} compared to

that with free probiotic ($14.63x10^{-3}$ *vs* $14.00x10^{-3}$ pH units/min and 4.5 *vs*3.5 h, respectively) while pH_{Vmax} was similar in both samples (5.33±0.07 *vs* 5.36±0.09, respectively) (Table 1). The V_{max} values recorded in both yogurt samples were lower compared to those reported for yogurts fermented with other starters and cow milk (19.89-23.44 $x10^{-3}$ pH units/min) (Medeiros et al., 2015). Lower rate of acidification was observed with probiotic cultures like *L. plantarum* and *L. paracasei* subsp. *tolerans* grown in association with yoghurt cultures (Managkoudakis et al., 2006). After adding $W_1/O/W_2$ emulsion encapsulating *L. paracasei* the Tv_{max} was delayed and $T_{pH4.6}$was slightly prolonged compared to yogurt with free *L. paracasei* (5 *vs* 5.25h). The addition of ingredients during fermentation can interfere with the buffering capacity of the milk leading to lower V_{max} values (Do Espírito et al., 2012). In the case of $W_1/O/W_2$ emulsion, the constituents of non-fermented milk (e.g. soluble phosphate, colloidal calcium phosphate, caseins, and whey proteins) in the outer W_2 phase can act as such ingredient (Salaün et al., 2005) and when introduced into the fermenting milk can alter its acidification kinetics (Lalou et al., 2017). Furthermore, physicochemical, and sensorial properties in yogurt can be affected negatively by prolonged fermentation of milk (Mishra and Mishra, 2013).

The differences in fermentation behaviour of set-type yogurt observed between the samples with and without $W_1/O/W_2$ emulsion is comparable to set-type yogurt in literature. For example, the addition of $W_1/O/W_2$ emulsion to the fermenting milk resulted in similar V_{max} (13±1.2), T_{Vmax} (4.5h), pH_{Vmax} (5.24±0.06) and $T_{pH4.6}$ (5.25h) values in comparison to those for fermenting milk with $W_1/O/W_2$ emulsion encapsulating *L. paracasei* in this study (Lalou et al., 2017). However, in literature (Lalou et al., 2017) fermenting milk without $W_1/O/W_2$ emulsion or *L. paracasei* showed higher V_{max} (20.9±3.5) and pH_{Vmax} (5.24±0.06) but similar T_{Vmax} (3.5h) and

$T_{pH4.6}$ (5h) to fermenting milk with free *L. paracasei* in this study. These results show that differences in acidification behaviour are mainly due to addition of $W_1/O/W_2$ emulsion regardless of *L. paracasei*.

3.3 Viability of probiotic *L. paracasei*

The viability of free *L. paracasei* cells co-present with the *S. thermophilus* and *L. bulgaricus* starter culture in the continuous phase and encapsulated *L. paracasei* in $W_1/O/W_2$ emulsion, was monitored during fermentation(Table S1) and 28 days of storage at 4^{o}C (Fig. 2) and after exposure to gastrointestinal conditions (Fig. 3 and Table S2). During fermentation, the initial viability of *L. paracasei* was similar (~8 $\log_{10}$CFU/g yogurt) and developed at a lower rate in $W_1/O/W_2$ emulsion compared to free cells until the end of the fermentation process (4 and 6h) (Table S1). Since the initial viability of *L. paracasei* in $W_1/O/W_2$ emulsion was unchanged compared to the initial viability before encapsulation this suggests that the the emulsification process as well as the surfactants used were not harmful to the bacteria. Similar observations were reported in studies on other bacterial species (El Kadri et al., 2015; Shima et al., 2006). Also, *L. paracasei* grows optimally at pH 4.5-5.7 (Mahboubi and Kazempour, 2016), therefore, in free form it is expected to grow during fermentation (pH <5.7) while the encapsulated cells are present in higher pH (pH 6-7) within the inner W_1 phase (milk) which would not encourage their growth.

In contrast to the trend observed during fermentation, the viability of encapsulated *L. paracasei* was significantly ($P<0.05$) higher compared to free cells, throughout the storage period reaching a population of~7.5 $\log_{10}$CFU/g by the end of the storage (28 days), ~1-log higher than free *L. paracasei* cells (Fig. 2). In a study by Ng et al. (2011), *Lactobacillus bulgaricus* had negative effects on the survival of the probiotic

L. acidophilus in yogurt by producing inhibitory metabolites such as H_2O_2 during storage for 28 days at 4 ^oC. Therefore, the encapsulation in $W_1/O/W_2$ emulsion could be protecting *L. paracasei* from antagonistic interactions as well as nutrient competition with the starter culture. The oil phase slows down mass transport and biological signalling between the cells and environment resulting in a molecular gradient that makes microorganisms go into a non-dividing resting state (Wang et al., 2008) which makes cells more resistant to environmental stress (e.g. limited nutrients) and prolong viability in starvation (Herman, 2002). Xin et al. (2009) found that encapsulation in $W_1/O/W_2$ emulsion can maintain high viability of *Lactobacillus* E1 for an extended shelf life of 37 days at refrigerated storage temperatures (4-10 ^oC).

The viability of *L. paracasei* encapsulated in $W_1/O/W_2$ emulsion was monitored after 2 hours exposure to SIJ and SGJ on day 0, 8, 14, 21 and 28.The viability of *L. paracasei* encapsulated in $W_1/O/W_2$ emulsion was monitored after 2 hours exposure to SIJ and SGJ on day 0, 8, 14, 21 and 28. Encapsulated *L. paracasei* cells showed significantly ($P<0.05$) higher survival rates (86-105% of the population before treatment) compared with free cells (72-92%) depending on the length of storage (Fig 3). Similar observations were recorded during storage. Namely, the viability of *L. paracasei* remained rather stable (~8 $\log_{10}$CFU/g) throughout storage compared to the steadily decreasing population of free probiotics. These results agree with previous studies that reported an enhancement in viability of probiotics after exposure to gastrointestinal conditions by encapsulation in $W_1/O/W_2$ emulsion (Shima et al. 2006; Shima et al., 2009; Pimentel-González et al., 2009; Rodríguez-Huezo et al., 2014).The results in this study indicate that encapsulated *L. paracasei*

cells in $W_1/O/W_2$ emulsion in set-type yogurt, can resist harsh gastrointestinal conditions, and potentially reach the colonization site in sufficient numbers.

3.4 Starter culture growth kinetics and viability during storage

The population of *S. thermophilus* and *L. bulgaricus* starter culture was monitored during fermentation and storage at 4^oC for 28 days (Table S2 and Fig. 4a and b). During fermentation ,*L. bulgaricus* population was significantly ($P<0.05$) lower in samples with $W_1/O/W_2$ emulsion throughout the fermentation (Table S1), however, it remained higher after day 12 and towards the end of storage (Fig. 4a). During fermentation, the pattern in *S. thermophilus* population did not differ dramatically between samples with encapsulated and free *L. paracasei* (Table S1). During storage, *S. thermophilus* population was similar between both samples with no significant differences (Fig. 4b).

The addition of $W_1/O/W_2$ emulsion to the fermenting milk affected the growth of *L. bulgaricus* and may have altered its proteolytic activity. This in turn could affect the growth of *S. thermophilus* which is stimulated by the bioavailability of free amino acids and peptides present within the milk, released due to the proteolytic activity of *L. bulgaricus* (Tamime et al., 2007). *S. thermophilus* growth is known to be stimulated by the bioavailability of free amino acids and peptides present within the milk, released due to the proteolytic activity of *L. bulgaricus* (Tamime et al., 2007). Also, *L. bulgaricus* is stimulated by S*. thermophilus* in symbiotic fermentation (Zourari et al., 1992). This might explain the significant reduction in *L. burgaricus* population after 3 hours which occurred due to the interruption of symbiosis during fermentation upon adding $W_1/O/W_2$ emulsion.

3.5 Physicochemical properties of set-type yogurt during formation and storage at 4^{o}C

The physicochemical properties of yogurt changed rapidly during the fermentation process. Evolution in pH and lactic acid concentration during fermentation and storage are key criteria of quality and acceptability of yogurts (Tamime et al., 2007). During milk fermentation, samples showed a decline in pH, associated with a gradual increase in acidity content (Fig. 5a) with values that were comparable for yogurt with and without $W_1/O/W_2$ emulsion, and to literature for yogurt fermentation made with cow milk (Kristo et al., 2003; do Espírito Santo et al., 2012; Mishra and Mishra 2013; Medeiros et al., 2015). The accumulation profile of lactic acid was not affected by the addition of free *L. paracasei* or $W_1/O/W_2$ encapsulating *L. paracasei* and reached a maximum of ~0.8 % w/w by the end of the fermentation process (5h).

During storage, the pH was lower in yogurts with free *L. paracasei* within the first 6 days and then followed a similar trend until day 28 (pH 4.2-4.3) (Fig. 6a). The acidity profiles followed a similar pattern in both samples and maximum (1.4% w/w) was achieved after 12 days of storage. During storage, the decrease in the acidity content was accompanied by pH increase from day 14 onwards suggesting that the presence of $W_1/O/W_2$ emulsion encapsulating *L. paracasei* or free form did not affect the pH values and the acidity content of the yogurt system during storage.

As expected water retention capacity was increased during the fermentation process, however, it was significantly lower in yogurt samples with $W_1/O/W_2$ emulsion encapsulating *L. paracasei* (Fig. 5b). When $W_1/O/W_2$ emulsion is added to the fermenting milk the oil globules may partially disrupt the gel formation, altering the ability of the structure to retain water (Lalou et al., 2017). During storage, the

water retention capacity followed a similar pattern in all samples within the first 14 days of storage (Fig. 6b). On day 21 onwards, yogurt with $W_1/O/W_2$ showed a higher water retention capacity. Set-type yogurts are known for loss in the water retention capacity during storage (Sahan et al., 2008; Supavititpatana et al., 2010; Tamjidi et al., 2012) which seems to be related with amino acid composition, protein conformation and surface polarity/hydrophobicity.

Syneresis (i.e. the whey separation) is one of the key quality parameters for yogurt and did not differ between samples throughout the fermentation process (Fig. 5c) and followed a similar trend during storage (Fig. 6c). Syneresis marks the deterioration of the protein network and the subsequent loss of the serum phase from the yogurt gel (Lucey, 2002). The stirring of the fermenting milk upon introducing the $W_1/O/W_2$ emulsion encapsulating *L. paracasei* was expected to stimulate syneresis (Ozturkoglu-Budak et al., 2016), however, this was not the case in the present study. The addition of fruit pulp caused a decrease of syneresis in yogurts due to their ability to absorb the water (Matter et al., 2016). However, oil globules are not capable of absorbing water from the system. In literature, syneresis values seem to be inversely related to fat content, i.e. increased fat content reduces the whey released due to the increased interactions between the fat globules and the protein network (Akgun et al., 2016). Lower syneresis values were recorded for yogurt with O/W emulsion over a 28-day storage (Izadi et al., 2015). The results in this study show that the addition of $W_1/O/W_2$ emulsion encapsulating *L. paracasei* do not alter syneresis which is advantageous since higher levels of syneresis is associated with low quality yogurt (Matter et al., 2016).

During the fermentation of milk casein micelles start to aggregate at a ~ pH 5.3, which also causes the solubilisation of colloidal calcium phosphate and change in viscosity (Mishra and Mishra, 2013). In this study, the viscosity of pasteurised milk (16%w/v total soluble solids) prior to fermentation was 9.98 mPa.s (data not shown). As expected the viscosity in fermenting milk gradually increased during the fermentation process (Fig. 5d), however, the introduction of the $W_1/O/W_2$ emulsion encapsulating *L. paracasei* to the fermenting system at 3h (pH~5.7) led to a decrease in the apparent viscosity after 4h. After 3h of fermentation, the viscosity values were tripled marking the onset of the formation of acid induced gel and remained unchanged with $W_1/O/W_2$ emulsion encapsulating *L. paracsei* while it quadrupled with free *L. paracasei* at the end of the fermentation process.

As the pH drops, a well-defined 3-D network is formed which is initiated by caseins that form aggregates at pH<5.2 (Tamime et al., 2007)and solubilisation of colloidal calcium phosphate leading to changes in viscosity (Mishra and Mishra, 2013).The viscosity of the yogurt is affected by the strength and number of bonds between the micelles as well as their structure and spatial distribution (Lucey, 2002). During fermentation, the change in viscosity of yogurt with free *L. paracasei* (Fig. 5d) verified the three-step structure formation process proposed by Parnell-Clunies et al. (1988), i.e. an initial lag period of low viscosity followed by a period of rapid increase and a final stage of high viscosity. A disturbance in formation of the yogurt structure was determined by a slight decrease in viscosity observed at 4 hours of fermentation after addition of $W_1/O/W_2$ emulsion to the fermenting milk (at ~3.5 hours). Similar observations were reported by Izadiet al. (2015) with the addition of O/W emulsion to yogurt. In this study, to achieve a homogenous dispersion of oil globules within the yogurt matrix, the $W_1/O/W_2$ emulsion was mixed with the fermenting milk at pH

values close to 5.7, whereby the fermented milk is semi-solid and viscous enough to prevent creaming of oil globules. At such pH values the structure that is forming is still weak and was probably partially disrupted by the presence of the oil globules resulting in a yogurt with a significantly ($P<0.05$) lower viscosity value compared to yogurt with free *L. paracasei*. During the first 2 days of storage there was a pronounced difference in viscosity values between both yogurts (Fig. 6d), which then equilibrated until the end of the storage period.

3.6 Textural properties of set-type yogurt during storage

The post-fermentation texture profile of yogurt during storage at 4^{o}C was monitored for 28 days (Table 2). Set-type yogurts should be firm but spoonable (Tamime et al., 2007), thus hardness, cohesiveness, adhesiveness, and gumminess are considered important for consumer acceptability (Domagala et al., 2006). Hardness, adhesiveness, cohesiveness, and gumminess were examined with texture analysis. Yogurt with $W_1/O/W_2$ emulsion encapsulating *L. paracasei* exhibited very different adhesiveness, cohesiveness and gumminess profiles compared to yogurts with free *L. paracasei*.

Hardness values fluctuated in both samples throughout the storage period, however, set-type yogurt with $W_1/O/W_2$ emulsion encapsulating *L. paracasei* seemed to be harder (i.e. firmer) than yogurts with free *L. paracasei*. Yogurt with $W_1/O/W_2$ emulsion encapsulating *L. paracasei* were more adhesive and less cohesive throughout the storage period. Yogurt with $W_1/O/W_2$ emulsion encapsulating *L. paracasei* were more adhesive throughout the storage period. Cohesiveness decreased gradually in yogurt with free *L. paracasei*, while it was stable in yogurt with $W_1/O/W_2$ emulsion encapsulating *L. paracasei*, however, it remained higher in

the former than in the latter in all cases. Yogurts containing the $W_1/O/W_2$ emulsion encapsulating *L. paracasei* exhibited higher values of gumminess (i.e. required more energy to disintegrate), however, this trend was reversed at the end of storage. Gumminess values remained comparable until 6 days of storage, thereafter, sample with $W_1/O/W_2$ emulsion presented higher values recorded for all the samples during the storage period.

Overall, set-type yogurt containing *L. paracasei* had comparable textural properties of set-type yogurts in literature (Lalou et al., 2017). Therefore, the textural changes observed in this study are due to the presence of the oil globules and not *L. paracasei*. Further work that includes sensory (Fonseca et al., 2016) and descriptive (Torres et al., 2017) analysis with consumers is required to better evaluate the quality and acceptability of the set-type yogurt containing $W_1/O/W_2$ emulsion and/or *L. paracasei*.

3.7 Microscopic observation of $W_1/O/W_2$ emulsion and probiotic *L. paracasei* during storage

To determine the stability of $W_1/O/W_2$ emulsion within yogurt the structure was monitored with light microscopy (Fig. S4) and cryo-SEM during storage (Fig. 7). The $W_1/O/W_2$ emulsion incorporated within the yogurt remained stable throughout the storage. The inner W_1 phase of the oil globules was maintained throughout the 28 days of storage (Fig. S4 and 7). Although inner W_1 phase was partially lost in some oil globules, the majority retained their inner W_1 phase until the end of the storage period. Furthermore, no flocculation or aggregation between the oil globules was observed over time (Fig. S4). The $W_1/O/W_2$ emulsion was incorporated successfully into the yogurt structure as the oil globules seemed to be part of the gel network (Fig.

7). Moreover, cryo-SEM analysis confirmed the observations made using light microscopy that the inner phase was still retained within the oil globules until the end of the storage period (Fig. 7). These results suggest that $W_1/O/W_2$ emulsion incorporated within the yogurt matrix exhibited prolonged stability under the storage conditions. This corroborates with previous studies showing that yogurt fermentation and storage conditions (Lalou et al., 2017) as well as the presence of bacteria in the W_1 phase does not affect the stability of $W_1/O/W_2$ emulsion (El Kadri et al., 2015; 2016).

Also, encapsulated *L. paracasei* cells in yogurt were monitored with fluorescence microscopy during storage (Fig. 8 and S5). All *L. paracasei* cells remained within the oil globules and no *L. paracsei* cells were observed in the outer phase throughout the storage period. The release of bacteria from W_1 to W_2 phase was shown to only occur as a result of oil globule bursting (El Kadri et al., 2016; 2017). Furthermore, since no *L. paracasei* were found within the continuous phase this indicates high stability of $W_1/O/W_2$ emulsion in the yogurt system as bursting of oil globules did not occur.

4. Conclusions

L. paracasei cells encapsulated in the inner W_1 phase of $W_1/O/W_2$ emulsion in yogurt enhanced their viability during storage at refrigeration temperature and simulated gastric juice with amounts above the generally accepted minimum concentration. The yogurt with $W_1/O/W_2$ emulsion encapsulating *L. paracasei* had comparable physicochemical characteristics to yogurt with *L. paracasei* in free form, stability in texture, and retrained high bacterial survival throughout the storage period. The incorporation of $W_1/O/W_2$ emulsion in yogurt structure caused no major alterations in

the values of key textural properties. However, consumer tests are necessary to assess any variation in perception. These results demonstrate the suitability of $W_1/O/W_2$ emulsion for developing functional foods by providing a compartmentalized environment allowing the fortification with non-starter cultures, i.e. probiotics, without interfering with starter culture and fermentation. Furthermore, it demonstrates the feasibility of enchanting yogurt with non-starter cultures other than probiotics, for example, anti-Listeria cultures, contributing to food safety, especially if combined with selective release.

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Tables

Table 1. The corresponding fermentation parameters: acidification rate (V_{max}), time to reached V_{max} (h), t_{Vmax}; pH at V_{max}, pH_{Vmax}; and the time to complete the fermentation (h), $t_{pH4.6}$.

Product	V_{max}(10^{-3}pHunits/min)	T_{vmax}(h)	pH_{vmax}	$t_{pH4.6}$(h)
Probiotic yogurt with no $W_1/O/W_2$ emulsion	14.00 ± 0.51[a]	3.5	5.36 ± 0.09[a]	5.00
Yogurt with encapsulated probiotic in $W_1/O/W_2$ emulsion	14.63 ± 0.83[a]	4.5	5.33 ± 0.07[a]	5.15

Mean value of three independent experiments ± sd; Mean values in the same column with the same superscript indicate no significant differences ($P < 0.05$).

Table 2. Effect of encapsulation of *L. paracasei* cells in $W_1/O/W_2$ emulsion on the texture profile of yogurts during storage at 4 ^{o}C.

Variables	Days	Yogurt samples	
		Probiotic Yogurt	Encapsulated Probiotic Yogurt
Hardness	0	65.13 ± 11.01 ^{a}	70.83 ± 5.51 ^{b}
	2	41.27 ± 4.97 ^{a}	57.38 ± 4.25 ^{b}
	4	48.60 ± 1.63 ^{a}	49.62 ± 0.71 ^{a}
	6	65.87 ± 11.05 ^{a}	79.18 ± 7.62 ^{b}
	8	48.95 ± 1.86 ^{a}	69.22 ± 4.92 ^{b}
	10	45.82 ± 1.80 ^{a}	62.17 ± 2.40 ^{b}
	12	43.37 ± 1.32 ^{a}	60.90 ± 3.10 ^{b}
	14	43.67 ± 2.30 ^{a}	61.08 ± 3.71 ^{b}
	21	50.02 ± 5.33 ^{a}	63.50 ± 3.10 ^{b}
	28	94.60 ± 10.63 ^{a}	95.68 ± 10.54 ^{a}
Adhesiveness	0	-91.68 ± 5.19 ^{b}	-97.48 ± 8.38 ^{a}
	2	-28.29 ± 4.22 ^{a}	-33.28 ± 5.15 ^{a}
	4	-19.51 ± 2.45 ^{b}	-22.78 ± 2.53 ^{a}
	6	-36.86 ± 3.81 ^{b}	-92.65 ± 9.32 ^{a}
	8	-18.63 ± 3.63 ^{b}	-68.47 ± 13.05 ^{a}
	10	-18.06 ± 0.91 ^{b}	-52.46 ± 7.53 ^{a}
	12	-4.52 ± 0.76 ^{b}	-42.95 ± 3.98 ^{a}
	14	-4.73 ± 0.76 ^{b}	-50.27 ± 7.11 ^{a}
	21	-4.61 ± 0.86 ^{b}	-47.25 ± 4.17 ^{a}
	28	-147.50 ± 17.16 ^{a}	-105.50 ± 10.60 ^{b}
Cohesiveness	0	1,24 ± 0,07 ^{b}	0,75 ± 0,06 ^{a}
	2	1,14 ± 0,06 ^{b}	0,82 ± 0,04 ^{a}
	4	1,14 ± 0,08 ^{b}	0,83 ± 0,03 ^{a}
	6	1,01 ± 0,13 ^{b}	0,82 ± 0,05 ^{a}
	8	0,92 ± 0,04 ^{b}	0,83 ± 0,05 ^{a}
	10	0,87 ± 0,05 ^{a}	0,84 ± 0,04 ^{a}
	12	0,92 ± 0,01 ^{b}	0,84 ± 0,03 ^{a}
	14	0,89 ± 0,04 ^{b}	0,82 ± 0,03 ^{a}
	21	0,87 ± 0,09 ^{a}	0,87 ± 0,03 ^{a}
	28	0,83 ± 0,04 ^{b}	0,75 ± 0,04 ^{a}
Gumminess	0	61,14 ± 6,92 ^{b}	53,25 ± 5,03 ^{a}
	2	46,82 ± 5,47 ^{a}	49,76 ± 4,27 ^{a}
	4	55,43 ± 2,83 ^{a}	51,14 ± 4,39 ^{a}
	6	67,87 ± 20,29 ^{b}	53,15 ± 5,26 ^{a}
	8	45,28 ± 3,50 ^{a}	57,42 ± 1,74 ^{b}
	10	39,89 ± 2,41 ^{a}	52,14 ± 3,59 ^{b}
	12	39,72 ± 1,51 ^{a}	50,87 ± 2,69 ^{b}

14	38,88 ± 2,24 [a]	50,08 ± 3,54 [b]
21	43,35 ± 6,66 [a]	54,97 ± 3,27 [b]
28	78,11 ± 7,32 [a]	71,61 ± 7,48 [a]

Mean value of six independent measurements ± standard deviation (sd); Mean values in the same row with the same superscript indicate that there are no significant differences between them ($P < 0.05$).

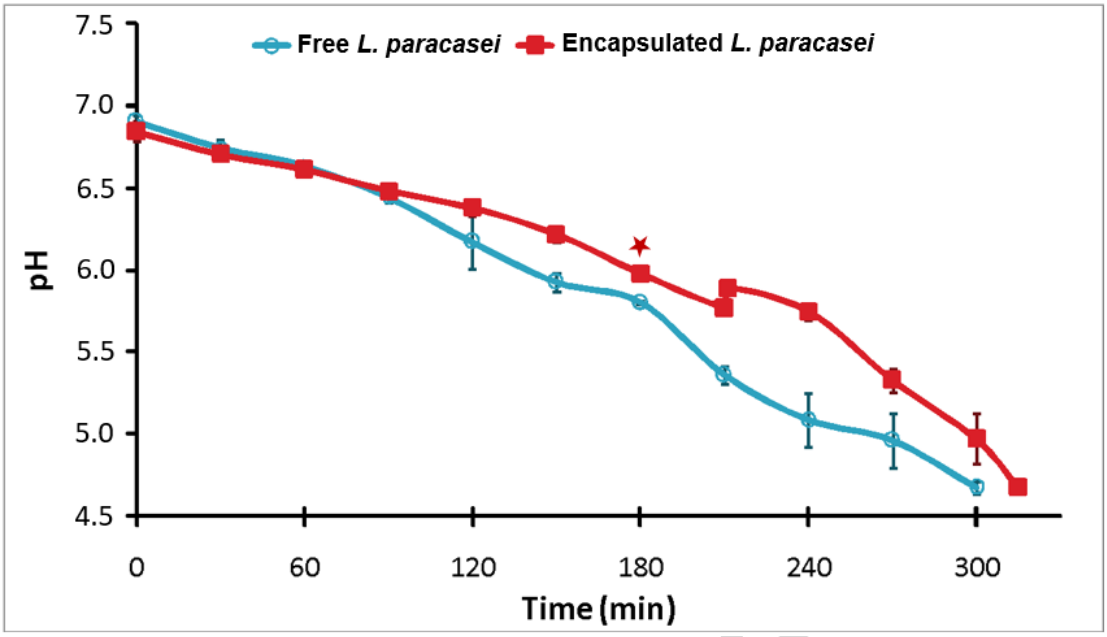


Fig. 1. Acidification profiles of milk fermented with bacterial *L. paracasei* encapsulated in $W_1/O/W_2$ emulsion (squares) and free in continuous phase (circles). Error bars represent the standard deviation (sd) of the mean value (n=3).
* Time point of incorporating the probiotic bacteria encapsulated in $W_1/O/W_2$ emulsion or in free form (180 min) is represented by two pH measurements before and after addition.

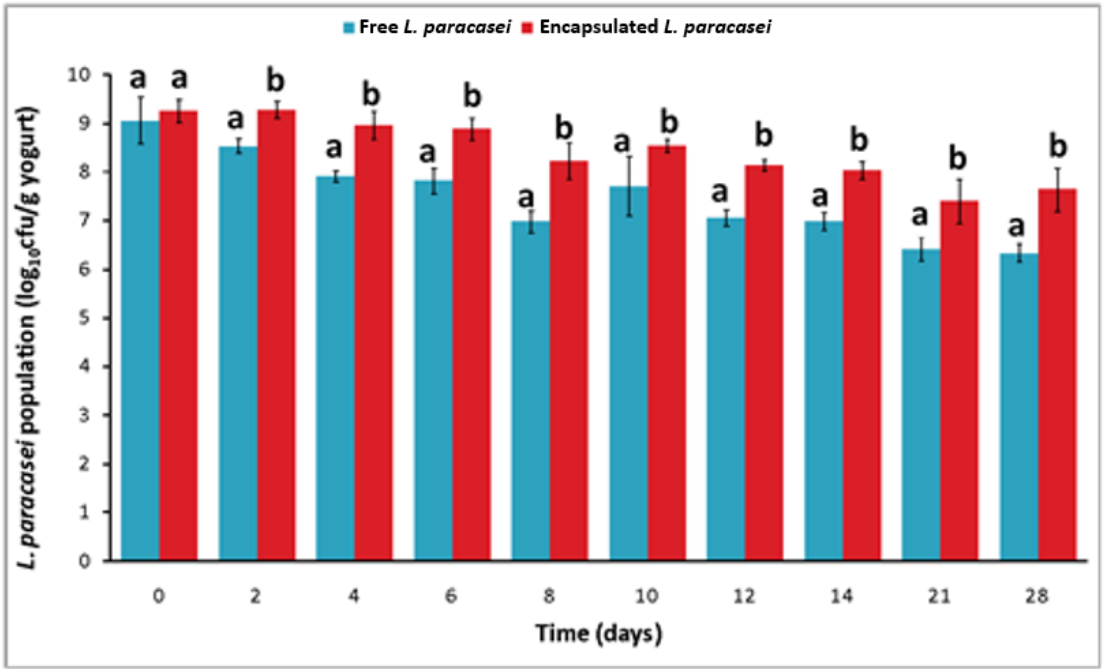


Fig. 2. Cell viability free (blue) and encapsulated *L. paracasei* cells in $W_1/O/W_2$ emulsion (red) during storage of yogurt at 4 ^{o}C. Error bars represent the standard deviation (sd) of the mean value (*n*=6). Mean values with different letters are significantly different (P < 0.05).

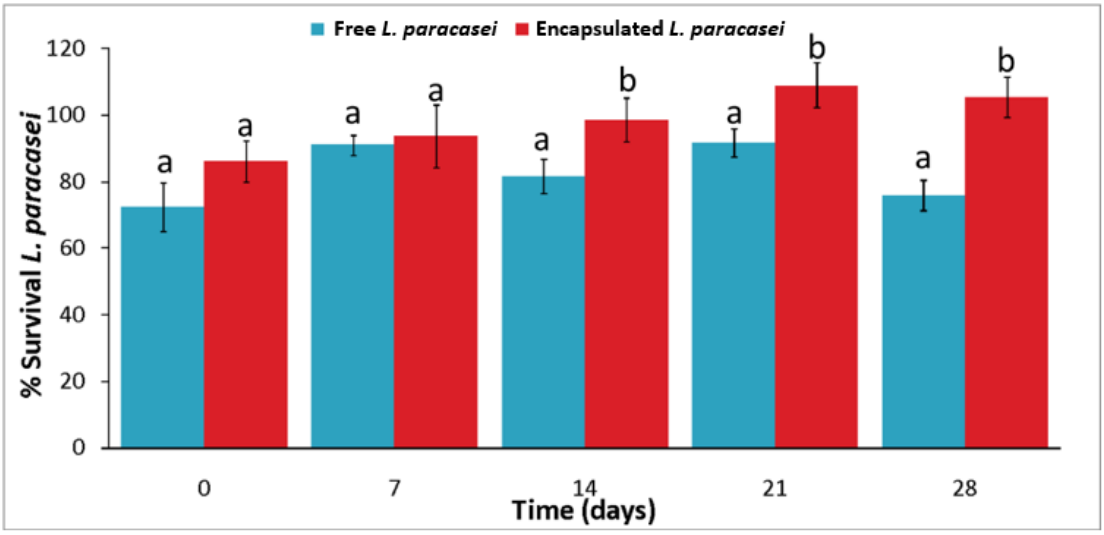


Fig. 3. Survival of free (blue) and encapsulated *L. paracasei* cells in $W_1/O/W_2$ emulsion (red) after exposure to SIJ and SGJ. Bars represent mean ± standard deviation (*n*=6). Mean values with different letters are significantly different (P < 0.05).

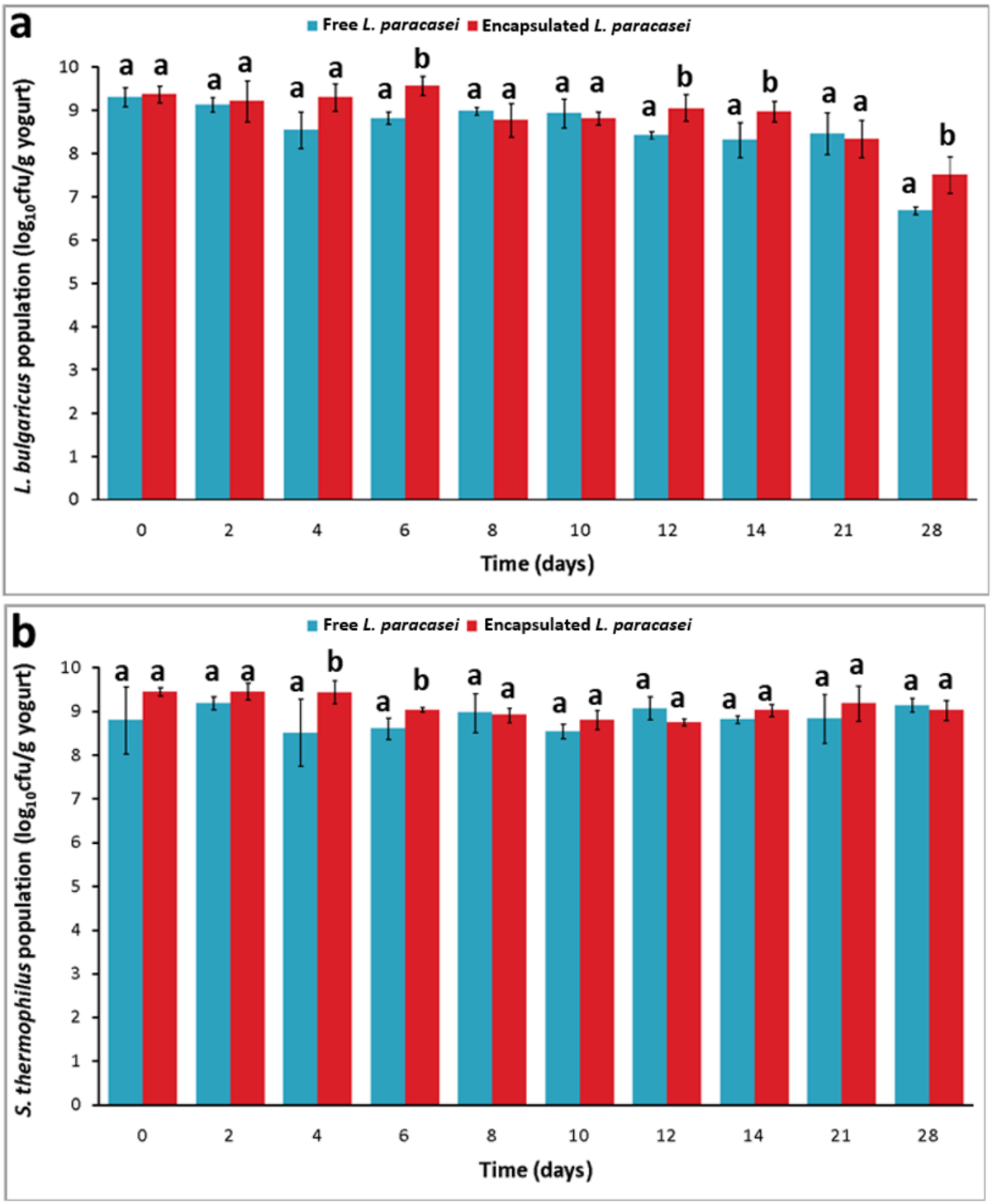
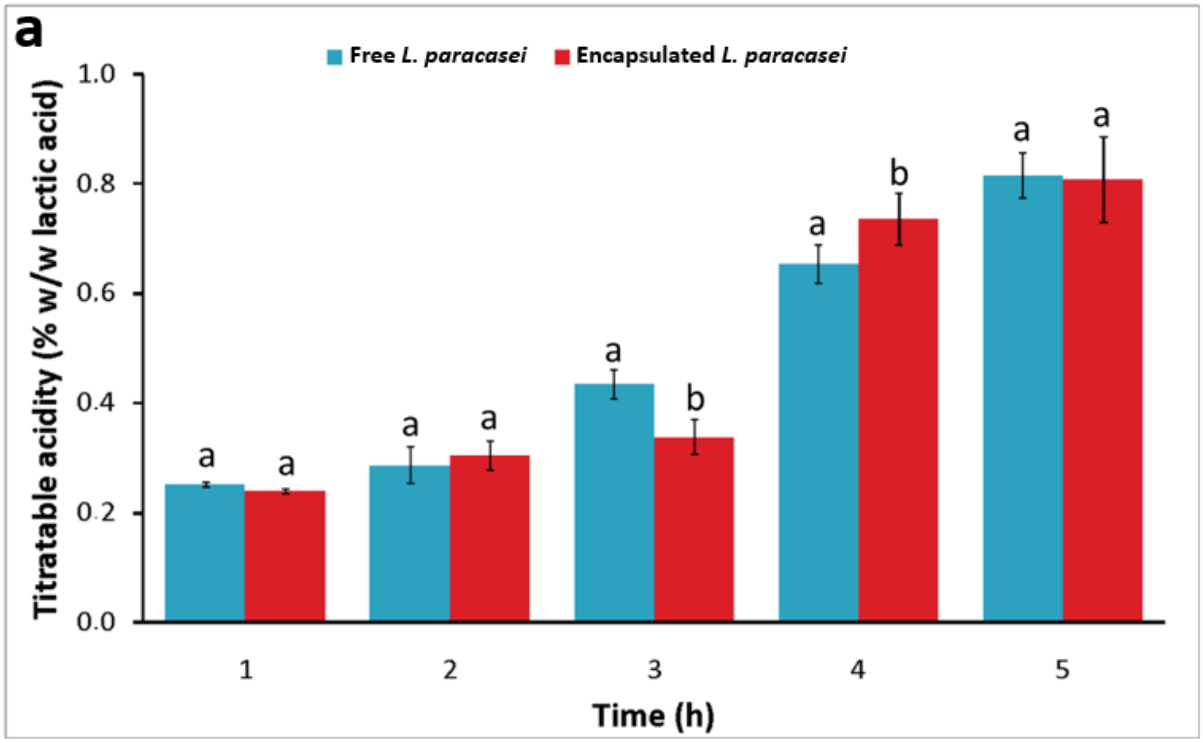
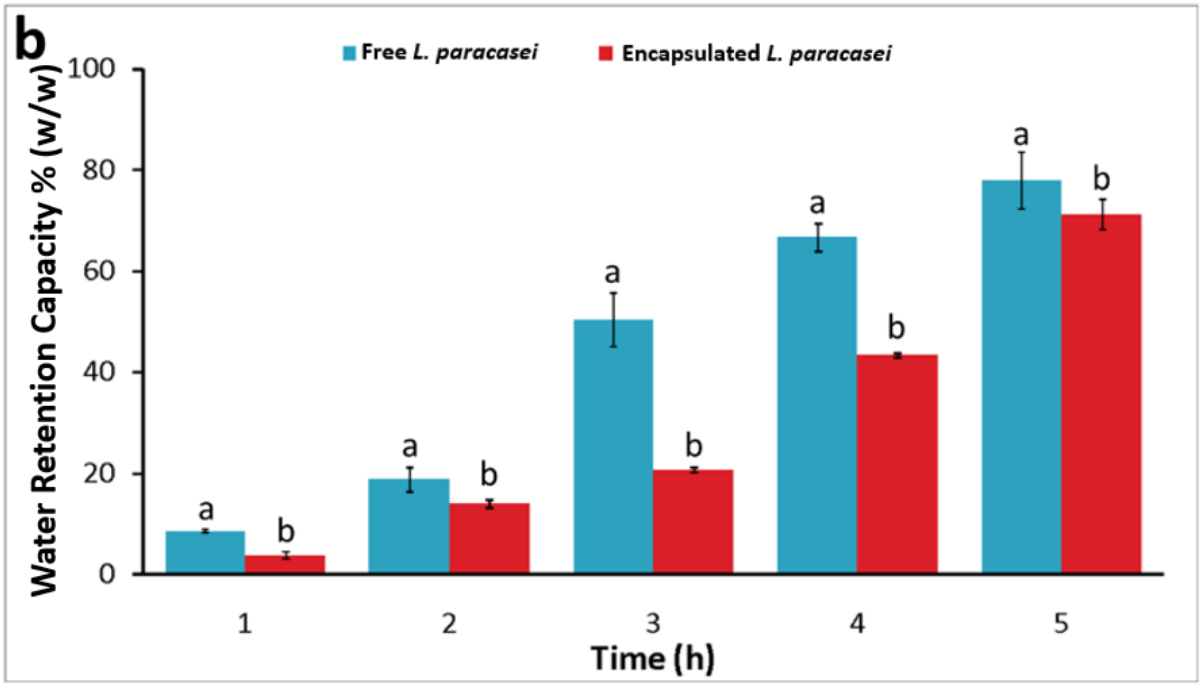


Fig. 4. Cell viability of (a*) L. bulgaricus* and (b) *S. thermophillus* during storage at 4 ^{o}C of yogurt with free (blue) or encapsulated *L. paracasei* cells (red). Error bars represent the standard deviation (sd) of the mean value (*n*=6). Mean values with different letters are significantly different ($P < 0.05$).





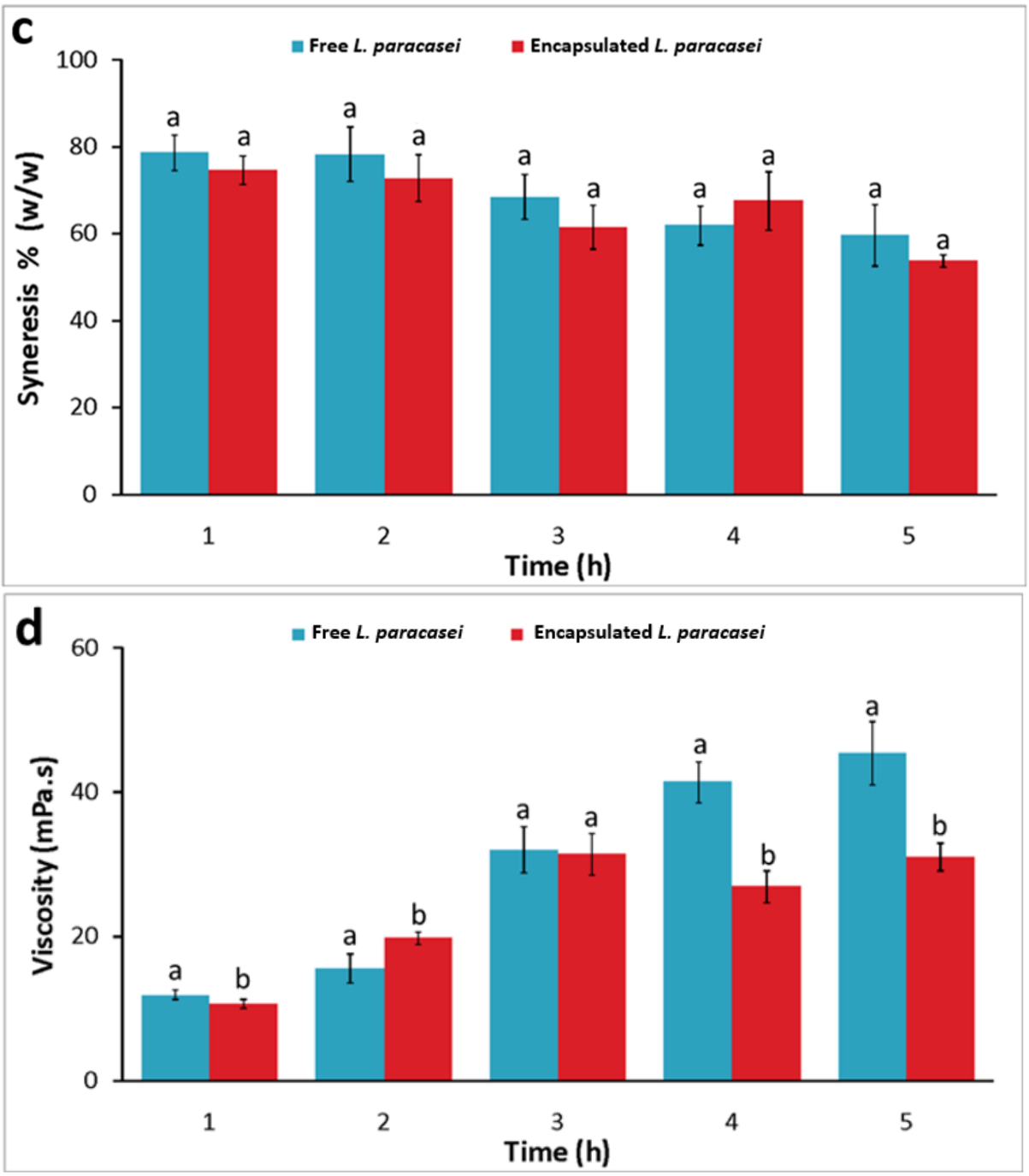
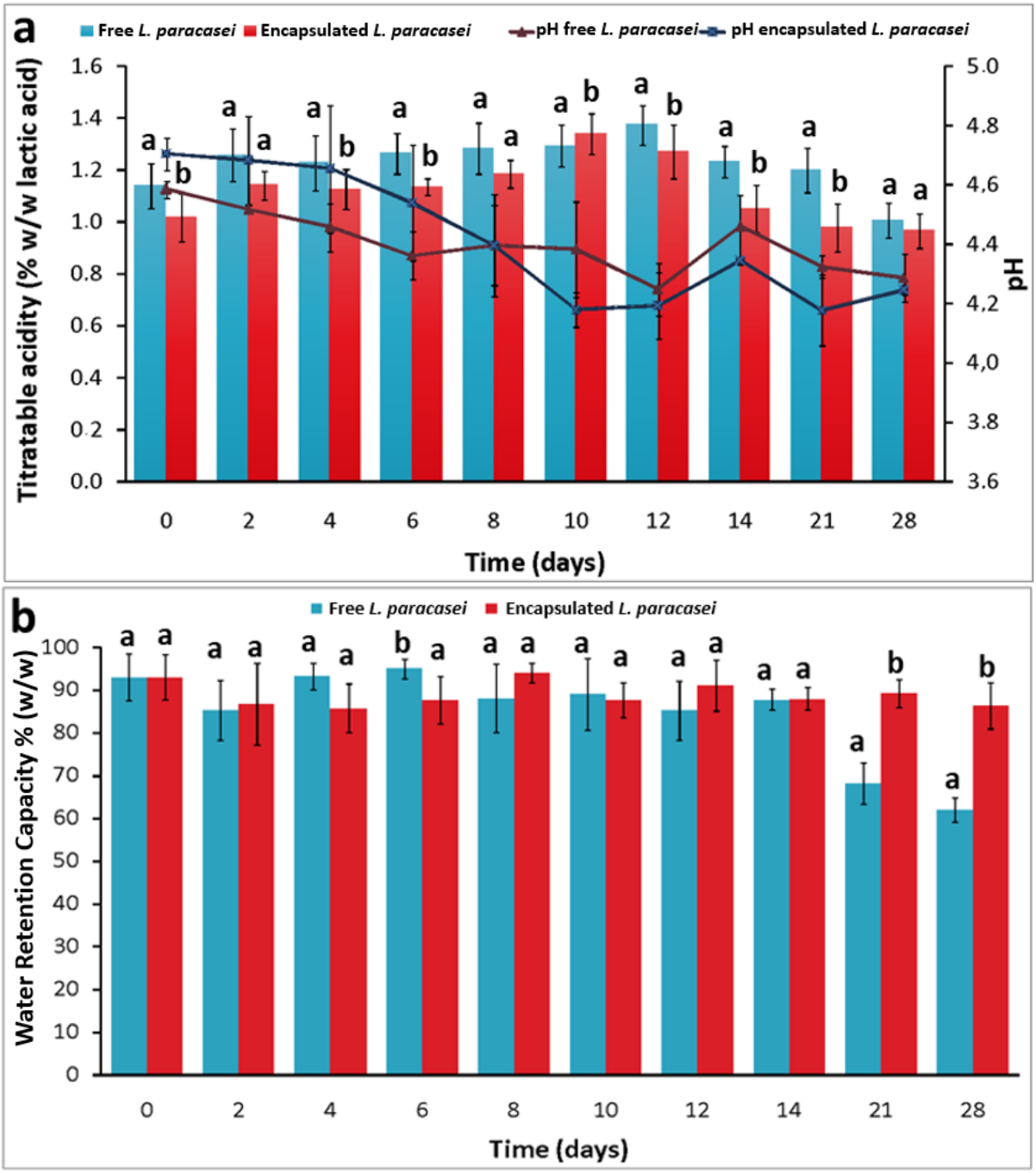


Fig. 5. Kinetics of (a) titratable acidity, (b) water retention capacity, (c) syneresis (d) viscosity, during the acidification process of milk with free (blue) and encapsulated *L. paracasei* in $W_1/O/W_2$ emulsion (red). Error bars represent the standard deviation (sd) of the mean value (n=3). Mean values with different letters are significantly different ($P < 0.05$).



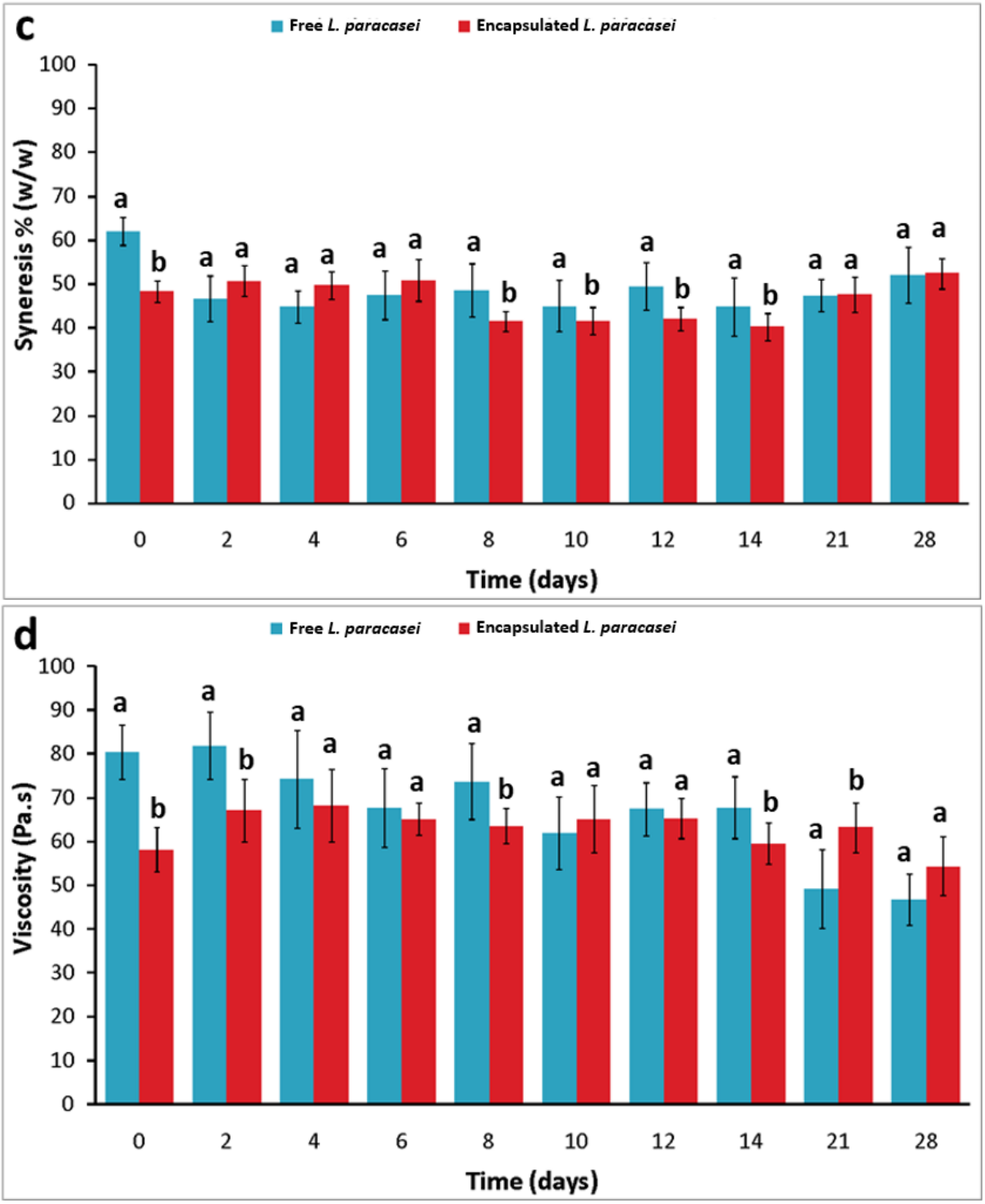


Fig. 6. Kinetics during storage of yogurt with free (blue) and encapsulated *L. paracasei* in $W_1/O/W_2$ emulsion (red) of (a) pH values and titratable acidity, (b) water retention capacity (c) syneresis and(d) viscosity. Error bars represent the standard deviation (sd) of the mean value (n=6). Mean values with different letters are significantly different ($P < 0.05$).

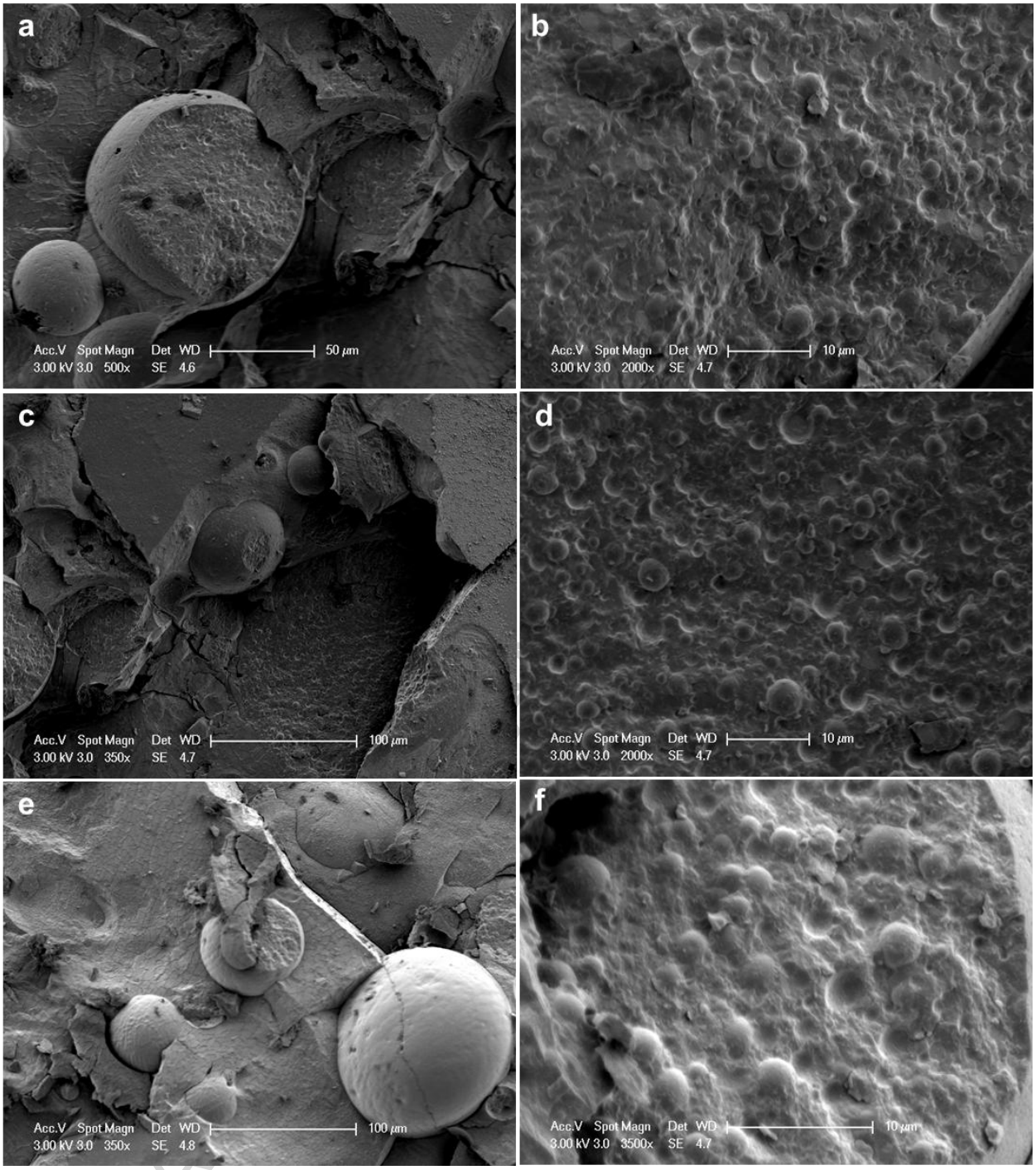


Fig. 7. Scanning electron microscope (SEM) image of the cryo-fractured yogurt samples containing $W_1/O/W_2$ emulsion encapsulating *L. paracasei* (a) at the beginning of the storage period (0 days), Scale bar, 50 µm. (b) Zoomed SEM image of a. Scale bar, 10 µm (c) after 2 weeks of storage at 4 ^{o}C, Scale bar, 100 µm (d) Zoomed SEM image of c. Scale bar, 20 µm and (e) at the end of the storage (after 28 days) at 4 ^{o}C. (f) Scale bar, 100 µm (f) Zoomed SEM image of e. Scale bar, 10 µm.

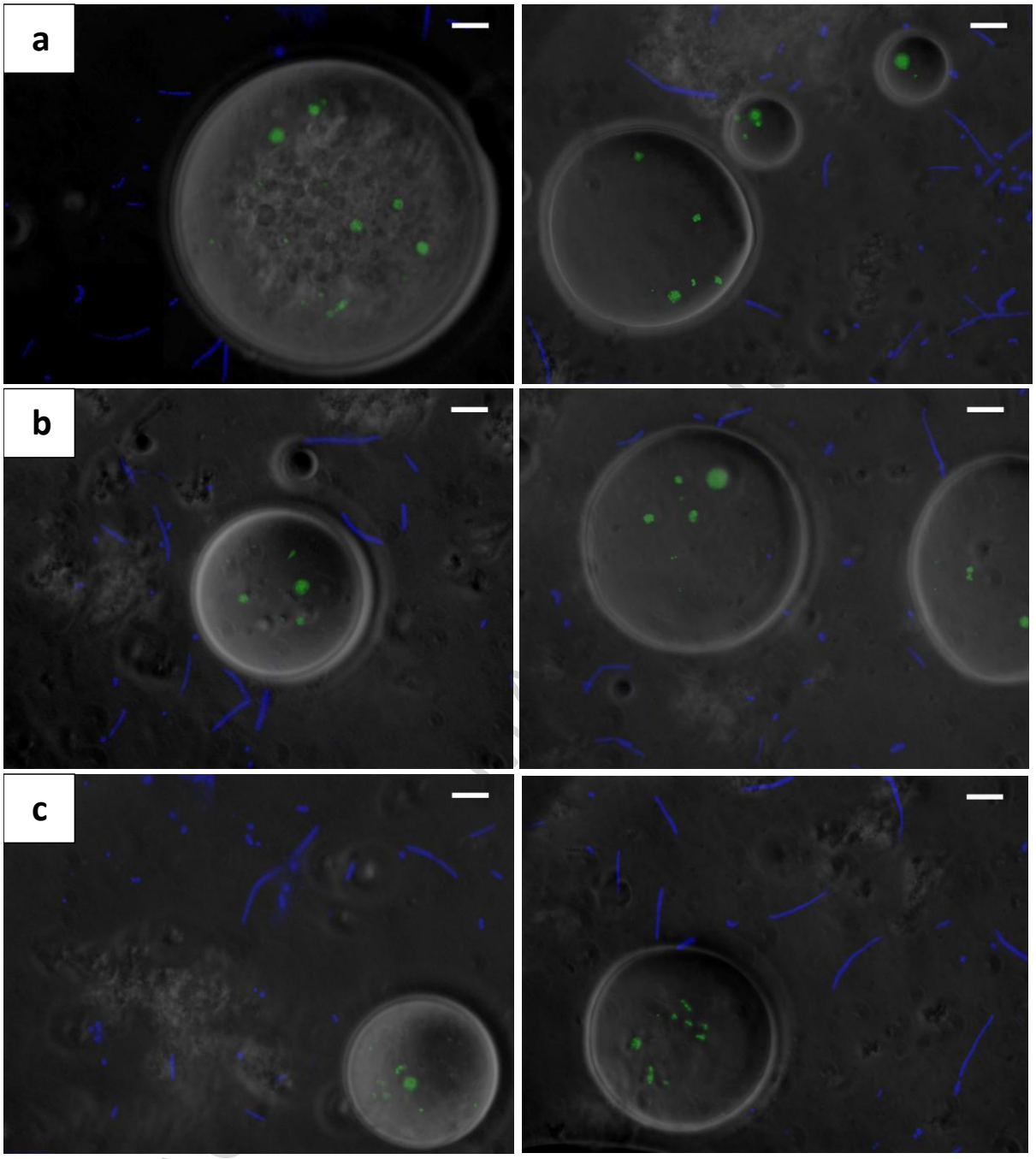


Fig. 8. Fluorescence microscopy images of yogurt with $W_1/O/W_2$ emulsion encapsulating *L. paracasei* (green) and starter culture (blue) during storage at day 0, 14, and 28 (a, b, and c, respectively).

Highlights

- First study to utilise $W_1/O/W_2$ emulsion for probiotic delivery in set-type yogurt.
- *Lactobacillus paracasei* viability increased during 28-day storage at $4^{O}C$.
- *L. paracasei* remained encapsulated throughout storage.
- *L. paracasei* viability in gastric juice increased throughout storage.
- $W_1/O/W_2$ emulsion affected set-type yogurt physicochemical and textural properties.