International Journal of Food Science +Technology

Original article

Digestive protection of probiotic *Lacticaseibacillus rhamnosus* in Ricotta cheese by monoglyceride structured emulsions

Sofia Melchior,[1] Sonia Calligaris,[1*] Marilena Marino,[1] Francesca D'Este,[2] Giorgio Honsell,[1] Maria Cristina Nicoli[1] & Nadia Innocente[1]

1 Dipartimento di Scienze Agroalimentari, Ambientali e Animali, Università di Udine, via Sondrio 2/A, Udine 33100, Italy
2 Dipartimento di Area Medica, Università di Udine, P.le Kolbe 4, Udine 33100, Italy

(Received 7 December 2021; Accepted in revised form 7 February 2022)

Summary This research aimed at studying the potential use of monoglyceride (MG) structured emulsions (MSEs) as delivery and protective systems for probiotic bacteria in Ricotta cheese. To this purpose, a low-fat commercial Ricotta cheese was added with MSEs formulated with milk, as water phase, and sunflower oil (MSE-SO) or anhydrous milk fat (MSE-AMF), as lipid phase. A commercial whole milk Ricotta cheese (W-RC) was considered as reference. A probiotic *Lacticaseibacillus rhamnosus* strain was inoculated as free cells in W-RC or embedded into the MSEs and added to the low-fat Ricotta at the same reference fat content. After physico-chemical characterisation, *L. rhamnosus* viability and sample destructuring behaviour upon *in vitro* digestion were evaluated. At the end of *in vitro* digestion, both W-RC and sample containing MSE-SO were unable to protect cells. By contrast, sample with AMF ensured a sufficient probiotic viability, even after 14 days of storage at 4 °C. This result was attributed to system composition and structure. During the gastric phase, the presence of caseins and MG-AMF mixed structures induced the formation of clots, entrapping and protecting cells against the acidic pH of the stomach, as confirmed by confocal micrographs and particle size. During the intestinal phase, cell viability was guaranteed by the formation of mixed micelles promoted by MG. It was demonstrated that microbial cells located near MG structures where they found protection.

Keywords *in vitro* digestion, monoglyceride structured emulsions, probiotic protection, Ricotta cheese.

Introduction

Products containing probiotics represent about 60–70% of the total functional food sale with an estimated annual growth rate of 7.9% for the period 2019–2025 (Grand View Research, 2020). This is due to the increasing number of evidence of their health benefits (Sarao & Arora, 2017; Roobab *et al.*, 2020). As widely accepted by the scientific community, to claim the health benefits of a probiotic product it is required a cell viability in the product to be consumed higher than 10^6–10^7 CFU g^{-1}, so that the intake of a portion of about 100 g allows the ingestion of 10^8–10^9 probiotic cells (Akhtar *et al.*, 2021; Marino *et al.*, 2021). At the same time, the health benefits of probiotic bacteria are guaranteed only if probiotics survive the gastrointestinal transit. It should be highlighted that the gastrointestinal tract (GIT) is stressful for probiotic cells due to the presence of enzymes and bile salts as well as the strong acidic pH of the stomach (Ranadheera *et al.*, 2010; Sumeri *et al.*, 2010; Melchior *et al.*, 2020). The ability of a probiotic strain to survive depends firstly on its tolerance to acidity and bile salts, which is an intrinsic feature of the strain (Hu *et al.*, 2018). However, the characteristics of the food matrix could deeply influence the resistance of the microbial cells to the stressful conditions suffered during digestion. The structure of the matrix in this context has been indicated as a pivotal factor impacting the microbial behaviour upon digestion due to the presence of supramolecular structures made of fats, proteins, carbohydrates or a mixture of them able to give protection to microbes (Ranadheera *et al.*, 2010; Klu & Chen, 2015; Marino *et al.*, 2021). It is likely that the destructuring behaviour of these structures in the gut could deeply affect the microbial survival against gastrointestinal stresses.

The most suitable candidates to become probiotic foods are dairy derivatives, such as yoghurt, fermented milk, ice cream, cheese and cream cheese (Saarela,

*Correspondent: E-mail: sonia.calligaris@uniud.it

doi:10.1111/ijfs.15641

2018). This is mainly due to the ability of these matrices to maintain a good viability of probiotic bacteria during processing and storage thanks to their intrinsic properties, such as pH, buffering capacity, nutrient availability and structure (Karimi *et al*., 2011; Kisan *et al*., 2021; Marino *et al*., 2021). The changes of these characteristics deeply impact the final viability of the microbial cells and the further susceptibility upon digestion (Coman *et al*., 2012; Terpou *et al*., 2019; Rolim *et al*., 2020).

In the case of cheese, the dense protein matrix and the fat clusters may offer protection to microbial cells during the transit through the stomach to the intestine (Pitino *et al*., 2012; Rolim *et al*., 2020). For instance, Ricotta cheese was shown to be an effective food matrix to maintain the viability of *L. acidophilus* La-05 and *B. lactis* BB-12 upon the gastrointestinal transit (Meira *et al*., 2015; Kisan *et al*., 2021; Lopes *et al*., 2021). Similar results were also observed in other cheeses. *L. acidophilus* La-05 viability in Mascarpone-type cheese was slightly reduced at the end of the intestinal phase, while a stretched-curd cheese containing *L. rhamnosus* exhibited a worst but still acceptable protective efficacy (Pitino *et al*., 2012; de Almeida *et al*., 2018). However, other authors reported a reduction below 10^6 UFC g^{-1} of *L. acidophilus* inoculated in whey cheese matrix during the gastric phase (Madureira *et al*., 2011). This inconsistency can be due to the combined effects of microbial strain sensitivity and food chemical, physical and structural properties.

To reduce the detrimental effects of these stressful conditions, a series of protection systems are under study (Burgain *et al*., 2011; Sarao & Arora, 2017; Terpou *et al*., 2019). All of them could found application in the food context since the most suitable delivery strategy should be designed and tailored on the basis of the target food properties. Recently, our research group demonstrated the ability of monoglyceride (MG)-based structured emulsions (MSEs) to protect a probiotic *L. rhamnosus* strain during processing, storage and digestion (Marino *et al*., 2017; Calligaris *et al*., 2018; Melchior *et al*., 2021). Probiotics were prevalently situated in the aqueous phase near MG lamellar structures, where they found at the same time physical protection against the environmental stresses and nutrients to remain metabolically active. The formulation of the system in terms of the composition of the aqueous phase and lipid phase greatly affected the protection capacity of the system during digestion (Melchior *et al*., 2021).

Based on these recent findings, MSEs seem to be promising candidates as probiotic delivery and protection systems in foods. In the present study, we studied their applicability to deliver a probiotic *L. rhamnosus* strain in Ricotta cheese. Ricotta is the most popular unripened variety of whey cheese characterised by high moisture, soft texture and mild flavour. In this study, a low-fat commercial Ricotta cheese was added with MSEs formulated with milk, as water phase, and sunflower oil or anhydrous milk fat (AMF), as lipid phase and enriched with *L. rhamnosus* strain. The latter was selected for its well-known probiotic properties (Pandey *et al*., 2015) and compatibility with chemical characteristics of Ricotta cheese (Boylston *et al*., 2004; Borba *et al*., 2014; Sattin *et al*., 2016). The final fat content of the reconstituted sample was comparable to that of a reference sample that was a commercial whole milk Ricotta cheese. Samples were stored at 4 °C for up to 14 days. After physico-chemical characterisation of Ricotta (pH, confocal laser scanning micrographs and rheological properties), the destructuring behaviour upon *in vitro* digestion was assessed in concomitance with *L. rhamnosus* viability during ricotta cheese storage and at the end of gastric and intestinal phases of the *in vitro* digestion.

Materials and methods

Materials

α-amylase from *Bacillus* sp. (EC 3.2.1.1), porcine pepsin (EC 3.4.23.1), porcine pancreatin (EC 232-468-9, 8xUSP), porcine bile extract, NaH PO (2H O), NaOH, $CaCl_2(H_2O)_2$, Na_2CO_3, $NaHCO_3$, NaCl, KCl, KH_2PO_4, $MgCl_2(H_2O)_6$, $(NH_4)_2CO_3$, phosphate buffered saline (PBS), Fast Green FCF, Nile Red, stearic acid and palmitic acid were purchased from Sigma Aldrich (Milano, Italy); HCl and NaOH were provided by J. T. Baker (Center Valley, USA); Hoechst 33342 was purchased from Thermo Fisher Scientific Inc. (Massachusetts, USA); saturated monoglycerides (fatty acid composition: 1.4% C14:0, 59.8% C16:0, 38.8% C18:0; melting point 68.05 ± 0.5 °C) were provided by Kerry Ingredients and Flavour (Bristol, United Kingdom). Maximum Recovery Diluent (MRD), MRS agar and MRS broth were purchased from Oxoid (Milan, Italy). *L. rhamnosus* (Lyofast LRB) was purchased from Sacco Srl (Cadorago, Como, Italy). Sunflower oil (SO), low-fat Ricotta cheese (2.9% fat content), whole milk Ricotta cheese (W-RC; 11% fat content), anhydrous milk fat (AMF; fat content 99.8% w/w) and UHT skim milk (pH = 6.70) were purchased in a local market. Deionised water (System advantage A10®, Millipore S.A.S, Molsheim, France) was used.

Culture preparation

Lacticaseibacillus rhamnosus was stored at −80 °C as 30% (v:v) glycerol stock cultures in MRS broth. Before each experiment, overnight cultures were prepared by sub-culturing 100 μL of stock cultures in 100 mL of MRS broth at 37 °C for 18 h in anaerobic conditions. Cells were then recovered by centrifugation

at 13 000*g* for 10 min at 4 °C, washed three times with PBS and resuspended in PBS to a final viability of about 10^{11} CFU mL^{-1}.

Monoglyceride structured emulsion preparation

Monoglyceride structured emulsions (MSEs) enriched with probiotic bacteria were prepared accordingly to the procedure reported by Marino *et al.* (2017). In particular, the lipid phase contained 7.2% (w:w) of a mixture of saturated monoglycerides added with palmitic and stearic acid (co-surfactants) in a ratio of 5:1:1 (w:w), 56.4% (w/w) of UHT skim milk as water phase, and 36.4% (w/w) of SO or AMF. The *L. rhamnosus* suspension was added to MSEs at a concentration of about 10^{10} CFU mL^{-1}.

Ricotta cheese preparation

18.5% w/w of MSEs containing *L. rhamnosus* were added to commercial low-fat Ricotta cheese allowing to obtain the same fat content of the W-RC. After manual well-mixing for 2 min, samples were moulded in a 100 g container and stored at 4 °C until further analysis. Based on the lipid phase composition, the samples were named RC-SO and RC-AMF for Ricotta cheese containing MSEs with oil and anhydrous milk fat respectively. W-RC was used as reference sample. In this case, *L. rhamnosus* suspension was added to W-RC at a final concentration of about 10^9 CFU g^{-1} and the sample was well-mixed as previously reported for the other samples. The composition of the systems is reported in Table 1. Samples were stored at 4 °C for up to 14 days.

Analytical determinations

Sample characterisation

Moisture content (AACC, 2010), fat (ISO/TS, 2008) and total nitrogen (ISO/IDF, 2004) of samples were analysed. Total nitrogen was converted to protein using a factor of 6.38.

pH measurement

Sample pH was measured by a standard pH meter (Hanna Instruments pH 301, Padua, Italy) at 25 °C. Buffer solutions (pH 4, 7 and 9) were used for calibration.

Table 1 Composition of Ricotta cheese samples

Sample	Composition
W-RC	Whole Ricotta cheese added with free *L. rhamnosus* cells
RC-SO	Low-fat Ricotta cheese added with MSE containing sunflower oil, skim milk and *L. rhamnosus*
RC-AMF	Low-fat Ricotta cheese added with MSE containing anhydrous milk fat, skim milk and *L. rhamnosus*

Rheological determinations

Rheological properties of Ricotta cheese were evaluated at 20 °C using an RS6000 Rheometer (Thermo Scientific RheoStress, Haake, Germany) equipped with a Peltier cell and a parallel plate geometry with the measuring gap of 2 mm. To determine the linear viscoelastic region for each sample, stress sweep tests were performed increasing stress from 0.1 to 100 Pa at 1 Hz frequency. Frequency sweep tests were conducted increasing frequency from 0.1 to 10 Hz using a fixed stress value included in the linear viscoelastic region. All measures were conducted in triplicate at the beginning and at the end of storage at 4 °C.

Confocal laser scanning microscopy

Samples before *in vitro* digestion and after the gastric phase were analysed with confocal laser scanning microscopy in accordance with Melchior *et al.* (2021). The reported micrographs are single optical sections (pinhole size 1 AU) displayed in pseudocolour.

Viability of probiotic bacteria upon storage

Ricotta cheese samples were stored at 4 °C in sterile 120 mL airtight containers for up to 14 days. At the beginning and after 14 days of storage at 4 °C, aliquots of 1 g of each sample were suspended in 9 mL of MRD and homogenised in a LabBlender 400 (PBI International, Milan, Italy) for 2 min. Decimal dilutions in MRD were then pour plated in MRS agar and incubated at 37 °C for 48 h under anaerobic conditions.

In vitro digestion of ricotta cheese samples

In vitro digestion was performed in accordance with the INFOGEST protocol (Brodkorb *et al.*, 2019) on samples at the beginning and at the end of storage (14 days) at 4 °C.

Briefly, 2.5 g of sample was weighted in 50-mL Falcon tubes and the oral phase was performed by adding 13 µL of 0.3 M $CaCl_2$ $(H_2O)_2$, 112 µL of water, 2 mL of simulated salivary fluid and 375 µL of a solution of α-amylase from *Bacillus* sp in water (final activity 75 U mL^{-1}). The entire mixture was maintained at 37 °C under stirring at 13 rpm for 2 min. Subsequently, 3 µL of 0.3 M $CaCl_2$ $(H_2O)_2$, 664 µL of water, 4 mL of simulated gastric fluid and 333 µL of porcine pepsin solution in water (2000 U mL^{-1} in the final mixture) were added. The pH was adjusted to 3 by adding 6 M HCl to start the gastric phase. The chime was maintained under stirring at 13 rpm at 37 °C for 2 h. Finally, the

gastric chime was mixed with 20 μL of 0.3 M $CaCl_2(H_2O)_2$, 1.98 mL of water, 4 mL of simulated intestinal fluid (SIF), 1.50 mL of 160 mM porcine bile extract in SIF and 2.5 mL of porcine pancreatin solution in SIF (100 U mL^{-1} in the final mixture). The pH was adjusted to 7 by adding 1 M NaOH, and the mixture was stirred at 13 rpm at 37 °C for 2 h. Samples were collected at the end of each digestion phase, and probiotic viability was evaluated as previously reported. *L. rhamnosus* viability was reported as survival rate calculated by the following equation:

$$\text{survival rate}\,(\%) = \frac{\log N}{\log N_0} \times 100$$

where N and N_0 are the probiotic viability (CFU g^{-1}) at the end of the digestion phase and before *in vitro* digestion respectively.

Particle size and zeta potential of digested samples

The particle size distribution and ζ-potential of the digested samples were measured in accordance with Melchior *et al.* (2021).

Statistical analysis

All determinations were expressed as mean ± standard deviation (SD) of at least two measurements from two experimental replications. Analysis of variance (ANOVA) and *t*-test were performed by using R v. 4.1.2 for Windows (The R foundation for statistical computing). A Tukey's post hoc test was used to assess differences between means ($P < 0.05$).

Results and discussion

Physico-chemical characterisation of the probiotic Ricotta cheese

The chemical composition, the pH and the rheological properties of the reconstituted Ricotta cheese containing MSEs with SO (RC-SO) or AMF (RC-AMF) as lipid phase are reported in Table 2. As previously described, samples were formulated to maintain the fat content at 11% (w/w) and thus comparable to that of a commercial full-fat sample (W-RC). As expected, the pH of all samples was approximately 6.5, which is typical for Ricotta cheese and reported as the optimal condition for *Lactobacillus* growth (Boylston *et al.*, 2004; Borba *et al.*, 2014; Sattin *et al.*, 2016). Considering rheological properties, the viscoelastic behaviour of all samples can be ascribed to that of weak gel being the storage modulus (G') higher than the loss modulus (G''). In the W-RC, this is due to the acid-thermal destabilisation of whey proteins during Ricotta cheese processing and the formation of a protein network (Fox *et al.*, 2017; Rubel *et al.*, 2019). In this system, the fat phase (green pseudocolour) was present as irregular fat globules embedded within the protein network (magenta pseudocolour), as evident in confocal laser scanning micrographs (Fig. 1a).

W-RC resulted in a stronger gel compared with samples containing MSEs with SO or AMF. This result can be probably attributed to the different structural organisation of the lipid phase (Buriti *et al.*, 2005). In the reformulated samples, the lipid phase appeared as spherical droplets uniformly distributed in the matrix. The protein network resulted less dense in comparison with the W-RC (Fig. 1b and c) probably due to the interactions between MGs and proteins competing at the lipid droplet surface, as previously demonstrated by different authors (Mao *et al.*, 2014; Valoppi *et al.*, 2015; Chen *et al.*, 2020). It should be highlighted that MG in this system is expected to behave as emulsifiers covering the surface of lipid droplets forming crystalline lamellar bilayers (Calligaris *et al.*, 2010). These lipid droplets contain lipids that were partially crystallised in the case of sample containing AMF or liquid lipids when sunflower oil was used, as previously reported by Marino *et al.* (2017). This is obviously due to the different fatty acid composition of AMF, rich in saturated fatty acids, and sunflower oil, and rich in unsaturated fatty acids. The presence of AMF crystals in conjunction with MG ones was probably responsible of the higher consistency of the RC-AMF than that of RC-SO (Marino *et al.*, 2017; Melchior *et al.*, 2021). Within this structural organisation, *L. rhamnosus* cells (grey signal) were prevalently entrapped within the protein network (Fig. 1a) in the W-RC. Differently and in accordance

Table 2 Chemical composition, pH, storage modulus (G'), loss modulus (G'') and probiotic viability (log CFU g^{-1}) of whole Ricotta cheese (W-RC) and Ricotta cheese containing MSEs made by oil (RC-SO) and anhydrous milk fat (RC-AMF)

Sample	Moisture content (g/100 g)	Fat content (g/100 g)	Protein content (g/100 g)	pH	G' (Pa)	G'' (Pa)	Viability (log CFU g^{-1})
W-RC	74.06 ± 0.0^{b}	11.70 ± 0.60^{a}	6.24 ± 1.10^{a}	6.5 ± 0.1^{a}	4156±454^{a}	730±73^{a}	8.97 ± 0.40^{a}
RC-SO	77.44 ± 0.04^{a}	11.23 ± 0.13^{a}	6.25 ± 0.14^{a}	6.4 ± 0.1^{a}	2054±185^{c}	426±29^{c}	9.14 ± 0.08^{a}
RC-AMF	77.61 ± 0.4 ^{a}	10.97 ± 0.25^{a}	6.27 ± 0.24^{a}	6.5 ± 0.1^{a}	3592±318^{b}	632±40^{b}	9.08 ± 0.25^{a}

$^{a\text{-}c}$ indicated a significant difference ($P < 0.05$) within means in the same column.

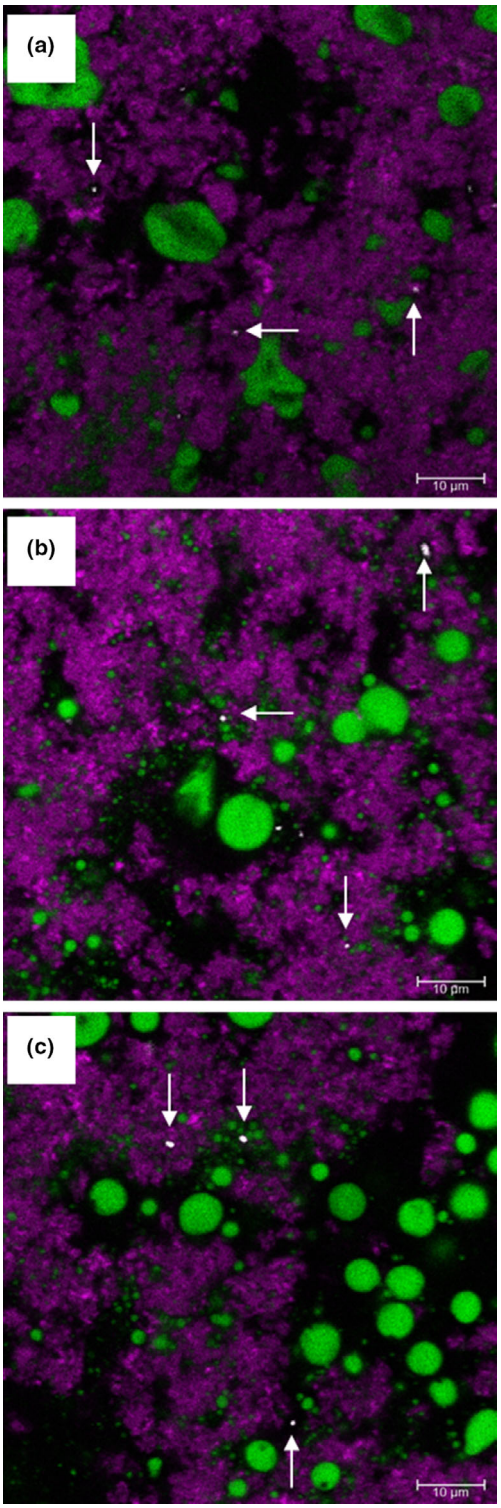

Figure 1 Confocal laser scanning micrographs of W-RC (a), RC-SO (b) and RC-AMF (c) stained with Nile Red (green), Fast Green (magenta) and Hoechst (grey) to visualise lipids, proteins and bacterial cells respectively. Arrows denote bacterial cells. Scale bar, 10 µm.

with previous results (Melchior *et al.*, 2021), *L. rhamnosus* cells were mainly detected near the MG structures in samples containing MSE (Fig. 1b and c). Considering probiotic viability, all samples presented a viable count of about 10^9 CFU g^{-1} (Table 2) indicating that the process operations did not impact *L. rhamnosus* survival.

After 14 days of storage at 4 °C, W-RC did not show significant ($P > 0.05$) modifications in pH and rheological properties, while in both RC-SO and RC-AMF a decrease in rheological moduli without syneresis was observed, accounting for 15% and 35% respectively. These results could be attributed to the MSEs structure that undergo to gel network rearrangement upon cold storage (Melchior *et al.*, 2021). Despite this, after 14 days of cold storage, no significant changes were detected in the viable counts (data not shown) indicating that the temperature, the pH of the medium, the storage duration and the sample structure did not affect probiotic viability.

L. rhamnosus viability upon *in vitro* digestion

Samples were exposed to *in vitro* digestion to evaluate the survival of *L. rhamnosus* during the gastrointestinal transit (Fig. 2a). As expected and in accordance with the literature, no viability reduction was recorded at the end of the oral phase due to the short exposure to enzyme activity and neutral pH (Melchior *et al.*, 2021). After the gastric phase, a survival rate reduction of 17.52% ± 1.09 and 17.15% ± 1.31 was observed in W-RC and RC-AMF, respectively, while in RC-SO it was significantly higher (39.59% ± 2.17). These results confirmed the deleterious role of enzymes and acid environment typical of the stomach that are responsible for the damage of cell DNA, membrane and cellular proteins leading to the cell survival decrease (Gbassi *et al.*, 2009; Sohail *et al.*, 2011; Gómez-Mascaraque *et al.*, 2016). Thus, probiotic cells resulted less protected upon gastric conditions in the softer gel structure of RC-SO.

At the end of the intestinal phase, no further modifications were observed in MSEs containing samples, while in W-RC an additional survival rate reduction of 17.64% ± 1.33 was detected. Summarising, at the end of the digestive simulation, only RC-AMF was able to ensure a viability higher than 10^6 CFU g^{-1} that has been established as the concentration limit to guarantee the health benefits of the probiotic cultures on the host (Akhtar *et al.*, 2021; Marino *et al.*, 2021).

L. rhamnosus viability upon *in vitro* digestion was also evaluated considering samples stored for 14 days at 4 °C (Fig. 2b). Obtained results mimic the behaviour previously observed (Fig. 2a) corroborating the ability of MSE containing AMF to protect probiotics against the stressful conditions of the GIT.

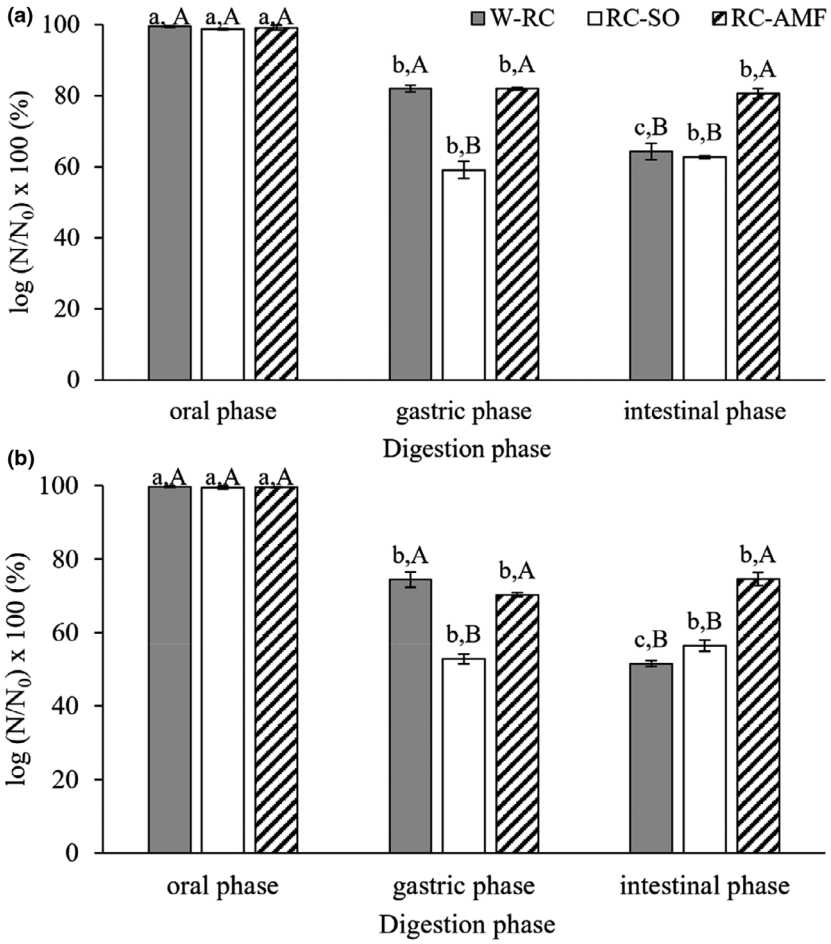

Figure 2 Survival rate (%) of *L. rhamnosus* incorporated in whole Ricotta cheese (W-RC) and Ricotta cheese with MSEs containing sunflower oil (RC-SO) and anhydrous milk fat (RC-AMF) after oral, gastric and intestinal phases of *in vitro* digestion. Data reported refer to the beginning (a) and the end (b) of storage at 4 °C. [a-c] indicated a significant difference ($P < 0.05$) among different digestion phases within the same sample. [A-B] indicated a significant difference ($P < 0.05$) between samples within the same digestion phase.

Probiotic viability as a function of destructuring behaviour upon *in vitro* digestion

To understand the role of food structure in protecting probiotic bacteria during digestion, the destructuring behaviour of samples upon digestion was studied firstly with the evaluation of the particle size distributions of digesta (Fig. 3). At the end of the gastric phase (Fig. 3a), a polydisperse distribution was observed in all samples. A main peak was detected between 220 and 260 nm in both W-RC and RC-AMF, while it was shifted at 524 nm ± 64 in the case of RC-SO digesta. Moreover, systems containing MSEs exhibited an additional particle family at approximately 5300 nm, while a peak around 60 nm was detected in W-RC and RC-AMF. These results could be attributed to different aggregation pathways resulting in protein–lipid aggregates with different sizes induced by the acidic pH and high ionic strength typical of the gastric phase. Considering ζ-potential (Fig. 4), which is correlated with the surface electrical charge (Salvia-Trujillo *et al.*, 2017), the systems containing MSEs exhibited a less positive value than W-RC probably due to the pepsin action that would reduce the droplet charge thus promoting aggregation (Sarkar *et al.*, 2009; Singh *et al.*, 2009). These hypotheses were confirmed also by confocal laser scanning micrographs of gastric digesta (Fig. 5). W-RC chime (Fig. 5a) was characterised by small aggregates and strand-like structures composed of proteins and small lipid droplets. By contrast, in both RC-SO (Fig. 5b) and RC-AMF digesta (Fig. 5c), large protein aggregates entrapping lipid droplets and monoglycerides were imaged. Such result could be attributed to the presence of proteins, deriving from milk, able to form clots in the stomach through the combined action of acidic pH and digestive enzymes. This coagulum is retained in the stomach for a longer period than whey proteins that remain soluble and rapidly pass in the intestine (Wang *et al.*, 2018; Dupont & Tomé, 2020). Interestingly, it should be noted that the RC-SO clots (Fig. 5b) were characterised by a jagged structure while RC-AMF (Fig. 5c) presented more dense and homogeneous aggregates probably due to the presence of AMF whose crystals strengthened the protein–lipid network.

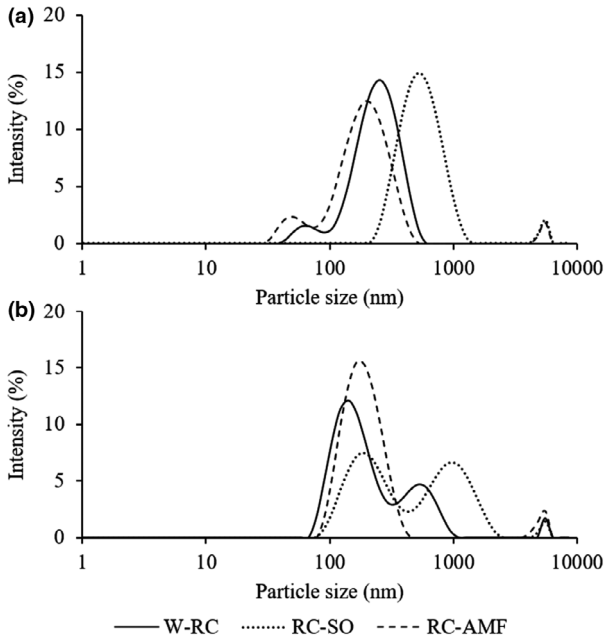

Figure 3 Particle size distribution of whole Ricotta cheese (W-RC) and reformulated Ricotta cheese with MSEs containing oil (RC-SO) and anhydrous milk fat (RC-AMF) after the gastric (a) and intestinal (b) phases of *in vitro* digestion.

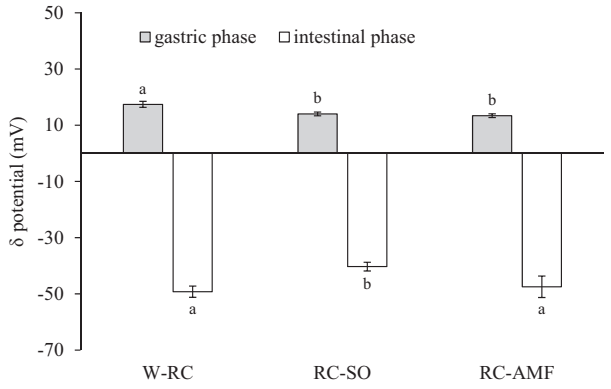

Figure 4 ζ potential of whole Ricotta cheese (W-RC) and reformulated Ricotta cheese with MSEs containing oil (RC-SO) and anhydrous milk fat (RC-AMF) after the gastric and intestinal phases of *in vitro* digestion. [a-b] indicated a significant difference ($P < 0.05$) between samples within the same digestion phase.

As for probiotics, in W-RC digesta, *L. rhamnosus* cells were mainly located near small aggregates and protein–lipid network (Fig. 5a) while in RC-AMF they were physically entrapped in the dense protein clots (Fig. 5c). The location of probiotics probably ensures physical protection against the severe conditions of the stomach, safeguarding cell viability. Moreover, the dense matrix and the solid lipid phase of the sample may offer additional protection to probiotic bacteria, as previously observed in the case of Mascarpone-type cheese (Bergamini *et al.*, 2005; Pitino *et al.*, 2012; de Almeida *et al.*, 2018). By contrast, in RC-SO sample, cells were detected outside the protein aggregates (Fig. 5b) and were thus more exposed to the acidic pH and enzyme action.

Also, after the intestinal phase, a multimodal distribution was detected for all samples with an average diameter between 100 and 6000 nm (Fig. 3b). The main peak, observed between 150 and 220 nm, was attributed to the presence of milk proteins and mixed micelles formed upon digestion (Salvia-Trujillo *et al.*, 2017; Calligaris *et al.*, 2020; Melchior *et al.*, 2021) while the particle families detected around 5000 nm and between 500 and 1000 nm indicated the presence of bile salts, aggregates of fatty acids, undigested lipids and digested milk proteins (Singh *et al.*, 2009; Salvia-Trujillo *et al.*, 2013; Alongi *et al.*, 2019; Melchior *et al.*, 2021). Comparing the particle distributions in Fig. 3, the three samples showed a slight diversity, especially for the R-SO samples. This sample demonstrated also a different ζ-potential in comparison with W-RC and RC-AMF after both gastric and intestinal phases. These samples exhibited a more negative parameter compared with that of RC-SO indicating a better absorption of the bile salt at the emulsion interface as well as the presence of surface-active molecules derived from triglyceride digestion (monoglycerides, diglycerides and free fatty acids) or interfacial materials, such as proteins (Wickham *et al.*, 1998; Singh *et al.*, 2009; Sarkar *et al.*, 2010; Ye *et al.*, 2010). The ability of digested components to bind bile salts probably provided additional protection to probiotics from bile salts toxicity (Begley *et al.*, 2005; de Almeida *et al.*, 2018). This intestinal behaviour seems to be mainly attributed to the presence of fat crystal aggregates than to the presence of monoglycerides. It is likely that the softer structure of RC-SO allowed an easier destructuring in comparison with other samples.

Conclusions

This study demonstrated the applicability of MSEs as delivery system of probiotic bacteria in Ricotta cheese, guaranteeing microbial survival during processing and further storage. Both composition and structure of MSEs determined their performances upon digestive simulation. During the gastric phase, the destructuring behaviour of the samples was mainly associated to the Ricotta gel strength. An easier destructuring was noted for the sample with sunflower oil resulting in a reduced protection of microbial cells against stressful gastric conditions. In any case, the presence of MG structures was crucial in favouring microbial survival thanks to their ability to guarantee microbial physical protection while the protein network underwent hydrolysis. Results here reported

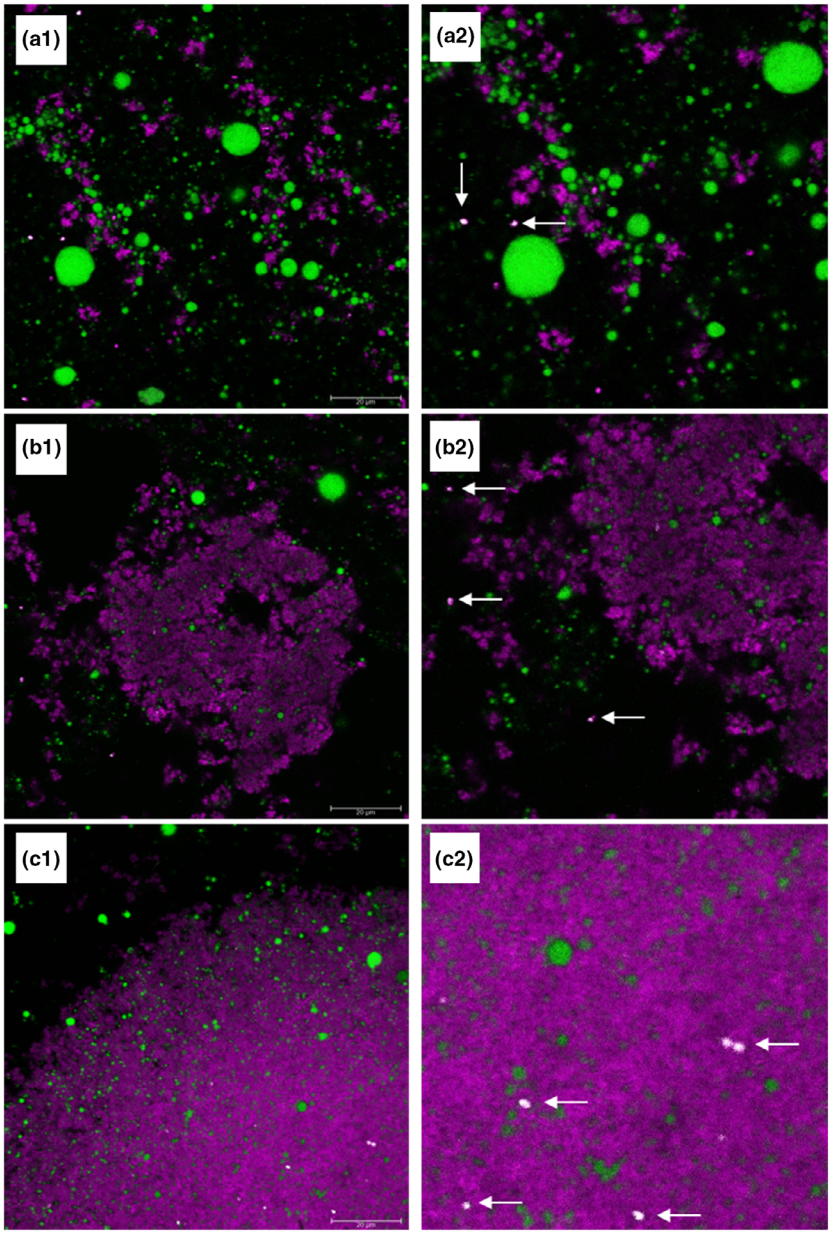

Figure 5 Confocal laser scanning micrographs of W-RC (a_1), RC-SO (b_1) and RC-AMF (c_1) digesta after the gastric phase. Samples were stained with Nile Red (green), Fast Green (magenta) and Hoechst (grey) to visualise lipids, proteins and bacterial cells respectively. Magnified views (a_2, b_2 and c_2) are also reported to allow clearer visualisation of bacterial cells (indicated with arrows). Scale bar, 20 μm.

demonstrated the critical role of food structure in determining microbial survival during both gastric and intestinal phases. Thus, the engineering of the best performing food structure could allow to improve probiotics' survival and food health functionality.

Acknowledgment

This work was partially supported by University of Udine - I, Research Quality Enhancement Plan 2018. Action 3b Competitive research funding. Open Access Funding provided by Universita degli Studi di Udine within the CRUI-CARE Agreement. [Correction added on 30 May 2022, after first online publication: CRUI funding statement has been added.]

Conflict of interest

The authors declare that they have no conflict of interest.

Author contribution

Sofia Melchior: Conceptualization (equal); Formal analysis (equal); Investigation (equal); Visualization

(equal); Writing – original draft (equal); Writing – review & editing (equal). **Sonia Calligaris:** Conceptualization (equal); Resources (equal); Supervision (equal); Writing – original draft (equal); Writing – review & editing (equal). **Marilena Marino:** Formal analysis (equal); Investigation (equal); Resources (equal); Supervision (equal); Visualization (equal); Writing – review & editing (equal). **Francesca D'Este:** Formal analysis (equal); Writing – review & editing (equal). **Giorgio Honsell:** Formal analysis (equal); Writing – review & editing (equal). **Maria Cristina Nicoli:** Conceptualization (equal); Writing – review & editing (equal). **Nadia Innocente:** Conceptualization (equal); Resources (equal); Supervision (equal); Writing – review & editing (equal).

Peer review

The peer review history for this article is available at https://publons.com/publon/10.1111/ijfs.15641.

Data availability statement

Research data are not shared.

References

AACC. (2010). *Approved methods of analysis, n° 44-15.02* pp. 11th edn., St. Paul, MN: AACC International, –.

Akhtar, G., Bhat, N.A., Masoodi, F.A. & Gani, A. (2021). Small- and large-scale production of probiotic foods, probiotic potential and nutritional benefits. In: *Advances in Probiotics Microorganisms in Food and Health* (edited by D. Dhanasekaran & A. Sankaranarayanan) Pp. 365–395. 1st edn., London: Academic Press.

Alongi, M., Calligaris, S. & Anese, M. (2019). Fat concentration and high-pressure homogenization affect chlorogenic acid bioaccessibility and α-glucosidase inhibitory capacity of milk-based coffee beverages. *Journal of Functional Foods*, **58**, 130–137.

Begley, M., Gahan, C.G.M. & Hill, C. (2005). The interaction between bacteria and bile. *FEMS Microbiology Reviews*, **29**, 625–651.

Bergamini, C.V., Hynes, E., Quiberoni, A., Suárez, V. & Zalazar, C.A. (2005). Probiotic bacteria as adjunct starters: Influence of the addition methodology on their survival in a semi-hard Argentinean cheese. *Food Research International*, **38**, 597–604.

Borba, K.K.S., Silva, F.A., Madruga, M.S., de Cássia Ramos do Egypto Queiroga, R., de Souza, E.L. & Magnani, M. (2014). The effect of storage on nutritional, textural and sensory characteristics of creamy ricotta made from whey as well as cow's milk and goat's milk. *International Journal of Food Science and Technology*, **49**, 1279–1286.

Boylston, T.D., Vinderola, C.G., Ghoddusi, H.B. & Reinheimer, J.A. (2004). Incorporation of bifidobacteria into cheeses: Challenges and rewards. *International Dairy Journal*, **14**, 375–387.

Brodkorb, A., Egger, L., Alminger, M. *et al.* (2019). INFOGEST static *in vitro* simulation of gastrointestinal food digestion. *Nature Protocols*, **14**, 991–1014.

Burgain, J., Gaiani, C., Linder, M. & Scher, J. (2011). Encapsulation of probiotic living cells: From laboratory scale to industrial applications. *Journal of Food Engineering*, **104**, 467–483.

Buriti, F.C.A., Da Rocha, J.S. & Saad, S.M.I. (2005). Incorporation of *Lactobacillus acidophilus* in Minas fresh cheese and its implications for textural and sensorial properties during storage. *International Dairy Journal*, **15**, 1279–1288.

Calligaris, S., Alongi, M., Lucci, P. & Anese, M. (2020). Effect of different oleogelators on lipolysis and curcuminoid bioaccessibility upon *in vitro* digestion of sunflower oil oleogels. *Food Chemistry*, **314**, 126146.

Calligaris, S., Marino, M., Maifreni, M. & Innocente, N. (2018). Potential application of monoglyceride structured emulsions as delivery systems of probiotic bacteria in reduced saturated fat ice cream. *LWT*, **96**, 329–334.

Calligaris, S., Da Pieve, S., Arrighetti, G. & Barba, L. (2010). Effect of the structure of monoglyceride-oil-water gels on aroma partition. *Food Research International*, **43**, 671–677.

Chen, H., Mao, L., Hou, Z., Yuan, F. & Gao, Y. (2020). Roles of additional emulsifiers in the structures of emulsion gels and stability of vitamin E. *Food Hydrocolloids*, **99**, 105372.

Coman, M.M., Cecchini, C., Verdenelli, M.C., Silvi, S., Orpianesi, C. & Cresci, A. (2012). Functional foods as carriers for SYNBIO®, a probiotic bacteria combination. *International Journal of Food Microbiology*, **157**, 346–352.

de Almeida, J.D.S.O., Dias, C.O., Pinto, S.S. *et al.* (2018). Probiotic Mascarpone-type cheese: Characterisation and cell viability during storage and simulated gastrointestinal conditions. *International Journal of Dairy Technology*, **71**, 195–203.

Dupont, D. & Tomé, D. (2020) Milk proteins: Digestion and absorption in the gastrointestinal tract. In: *Milk Proteins: From Expression to Food* (edited by Academic Press). 3rd ed. pp. 701–714. London: Academic Press.

Fox, P.F., Guinee, T.P., Cogan, T.M. & McSweeney, P.L.H. (2017) Overview of Cheese Manufacture. *Fundamentals of Cheese Science*. pp. 11–26. 2nd edn. Boston, MA: Springer.

Gbassi, G., Vandamme, T., Ennahar, S. & Marchioni, E. (2009). Microencapsulation of *Lactobacillus plantarum* spp in an alginate matrix coated with whey proteins. *International Journal of Food Microbiology*, **129**, 103–105.

Gomez-Mascaraque, L.G., Morfin, R.C., Pérez-Masiá, R., Sanchez, G. & Lopez-Rubio, A. (2016). Optimization of electrospraying conditions for the microencapsulation of probiotics and evaluation of their resistance during storage and *in-vitro* digestion. *LWT - Food Science and Technology*, **69**, 438–446.

Grand View Research. (2020). *Probiotic ingredients market size & share, global industry report, 2025*. Available at: https://www.grandviewresearch.com/industry-analysis/probiotic-ingredients-market Accessed: August 16, 2021.

Hu, P.-L., Yuan, Y.-H., Yue, T.-L. & Guo, C.-F. (2018). A new method for the in vitro determination of the bile tolerance of potentially probiotic lactobacilli. *Applied Microbiology and Biotechnology*, **102**, 1903–1910.

ISO/IDF (2004) Cheese and processed cheese products — Determination of fat content — Gravimetric method. ISO 1735:2004 [IDF 5:2004].

ISO/TS (2008) *17837:2008 - Processed cheese products — Determination of nitrogen content and crude protein calculation — Kjeldahl method.*

Karimi, R., Mortazavian, A.M. & Da Cruz, A.G. (2011). Viability of probiotic microorganisms in cheese during production and storage: A review. *Dairy Science and Technology*, **91**, 283–308.

Kisan, B.S., Ganguly, S. & Khetra, Y. *et al.* (2021). Effect of Ricotta matrix on viability of probiotic organism exposed to simulated gastro-intestinal (GI) digestion. *LWT*, **151**, 112099.

Klu, Y.A.K. & Chen, J. (2015). Effect of peanut butter matrices on the fate of probiotics during simulated gastrointestinal passage. *LWT - Food Science and Technology*, **62**, 983–988.

Lopes, L.A.A., Pimentel, T.C., Carvalho, R.D.S.F. *et al.* (2021). Spreadable goat Ricotta cheese added with *Lactobacillus*

acidophilus La-05: Can microencapsulation improve the probiotic survival and the quality parameters? *Food Chemistry*, **346**, 128769.

Madureira, A.R., Amorim, M., Gomes, A.M., Pintado, M.E. & Malcata, F.X. (2011). Protective effect of whey cheese matrix on probiotic strains exposed to simulated gastrointestinal conditions. *Food Research International*, **44**, 465–470.

Mao, L., Calligaris, S., Barba, L. & Miao, S. (2014). Monoglyceride self-assembled structure in O/W emulsion: Formation, characterization and its effect on emulsion properties. *Food Research International*, **58**, 81–88.

Marino, M., Innocente, N., Calligaris, S., Maifreni, M., Marangone, A. & Nicoli, M.C. (2017). Viability of probiotic *Lactobacillus rhamnosus* in structured emulsions containing saturated monoglycerides. *Journal of Functional Foods*, **35**, 51–59.

Marino, M., Innocente, N., Melchior, S., Calligaris, S. & Maifreni, M. (2021). Main technological challenges associated with the incorporation of probiotic cultures into foods. In: *Advances in Probiotics Microorganisms in Food and Health*. (edited by D. Dhanasekaran & A. Sankaranarayanan) Pp. 479–495. London: Academic Press.

Meira, Q.G.S., Magnani, M., de Medeiros Júnior, F.C. *et al*. (2015). Effects of added *Lactobacillus acidophilus* and *Bifidobacterium lactis* probiotics on the quality characteristics of goat ricotta and their survival under simulated gastrointestinal conditions. *Food Research International*, **76**, 828–838.

Melchior, S., Marino, M., Innocente, N. *et al*. (2020). Effect of different biopolymer-based structured systems on the survival of probiotic strains during storage and *in vitro* digestion. *Journal of the Science of Food and Agriculture*, **100**, 3902–3909.

Melchior, S., Marino, M., D'Este, F. *et al*. (2021). Effect of the formulation and structure of monoglyceride-based gels on the viability of probiotic *Lactobacillus rhamnosus* upon *in vitro* digestion. *Food & Function*, **12**, 351–361.

Pandey, K.R., Naik, S.R., & Vakil, B.V. (2015). Probiotics, prebiotics and synbiotics- a review. *Journal of Food Science and Technology*, **52**, 7577–7587.

Pitino, I., Randazzo, C.L., Cross, K.L. *et al*. (2012). Survival of *Lactobacillus rhamnosus* strains inoculated in cheese matrix during simulated human digestion. *Food Microbiology*, **31**, 57–63.

Ranadheera, R.D.C.S., Baines, S.K. & Adams, M.C. (2010). Importance of food in probiotic efficacy. *Food Research International*, **43**, 1–7.

Rolim, F.R.L., Freitas Neto, O.C., Oliveira, M.E.G., Oliveira, C.J.B. & Queiroga, R.C.R.E. (2020). Cheeses as food matrixes for probiotics: *In vitro* and *in vivo* tests. *Trends in Food Science and Technology*, **100**, 138–154.

Roobab, U., Batool, Z., Manzoor, M.F., Shabbir, M.A., Khan, M.R. & Aadil, R.M. (2020). Sources, formulations, advanced delivery and health benefits of probiotics. *Current Opinion in Food Science*, **32**, 17–28.

Rubel, I.A., Iraporda, C., Gallo, A., Manrique, G.D. & Genovese, D.B. (2019). Spreadable ricotta cheese with hydrocolloids: Effect on physicochemical and rheological properties. *International Dairy Journal*, **94**, 7–15.

Saarela, M. (2018). Future development of probiotic dairy products. In: *Probiotics in Dairy Products*. (edited by A.Y. Tamime & L.V. Thomas) Pp. 389–394. Wiley-Blackwell: Hoboken, NJ.

Salvia-Trujillo, L., Qian, C., Martín-Belloso, O. & McClements, D.J. (2013). Influence of particle size on lipid digestion and β-carotene bioaccessibility in emulsions and nanoemulsions. *Food Chemistry*, **141**, 1472–1480.

Salvia-Trujillo, L., Verkempinck, S., Sun, L., Van Loey, A.M., Grauwet, T. & Hendrickx, M.E. (2017). Lipid digestion, micelle formation and carotenoid bioaccessibility kinetics: Influence of emulsion droplet size. *Food Chemistry*, **229**, 653–662.

Sarao, L.K. & Arora, M. (2017). Probiotics, prebiotics, and microencapsulation: A review. *Critical Reviews in Food Science and Nutrition*, **57**, 344–371.

Sarkar, A., Goh, K.K.T., Singh, R.P. & Singh, H. (2009). Behaviour of an oil-in-water emulsion stabilized by β-lactoglobulin in an *in vitro* gastric model. *Food Hydrocolloids*, **23**, 1563–1569.

Sarkar, A., Horne, D.S. & Singh, H. (2010). Interactions of milk protein-stabilized oil-in-water emulsions with bile salts in a simulated upper intestinal model. *Food Hydrocolloids*, **24**, 142–151.

Sattin, E., Andreani, N.A., Carraro, L. *et al*. (2016). Microbial dynamics during shelf-life of industrial Ricotta cheese and identification of a Bacillus strain as a cause of a pink discolouration. *Food Microbiology*, **57**, 8–15.

Singh, H., Ye, A. & Horne, D. (2009). Structuring food emulsions in the gastrointestinal tract to modify lipid digestion. *Progress in Lipid Research*, **48**, 92–100.

Sohail, A., Turner, M.S., Coombes, A., Bostrom, T. & Bhandari, B. (2011). Survivability of probiotics encapsulated in alginate gel microbeads using a novel impinging aerosols method. *International Journal of Food Microbiology*, **145**, 162–168.

Sumeri, I., Arike, L., Stekolštšikova, J. *et al*. (2010). Effect of stress pretreatment on survival of probiotic bacteria in gastrointestinal tract simulator. *Applied Microbiology and Biotechnology*, **86**, 1925–1931.

Terpou, A., Papadaki, A., Lappa, I., Kachrimanidou, V., Bosnea, L. & Kopsahelis, N. (2019). Probiotics in food systems: significance and emerging strategies towards improved viability and delivery of enhanced beneficial value. *Nutrients*, **11**, 1591.

Valoppi, F., Calligaris, S., Barba, L. & Nicoli, M.C. (2015). Compositional phase diagram, rheological and structural properties of systems containing UHT skim milk, sunflower oil, saturated monoglycerides and co-surfactants. *Food Biophysics*, **10**, 94–102.

Wang, X., Ye, A., Lin, Q., Han, J. & Singh, H. (2018). Gastric digestion of milk protein ingredients: Study using an *in vitro* dynamic model. *Journal of Dairy Science*, **101**, 6842–6852.

Wickham, M., Garrood, M., Leney, J., Wilson, P.D.G. & Fillery-Travis, A. (1998). Modification of a phospholipid stabilized emulsion interface by bile salt: Effect on pancreatic lipase activity. *Journal of Lipid Research*, **39**, 623–632.

Ye, A., Cui, J. & Singh, H. (2010). Effect of the fat globule membrane on *in vitro* digestion of milk fat globules with pancreatic lipase. *International Dairy Journal*, **20**, 822–829.