Lactic-acid bacteria fermentation-induced effects on microstructure and interfacial properties of oil-in-water emulsions stabilised by goat-milk proteins

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ABSTRACT

An oil-in-water emulsion stabilized with goat milk proteins was subjected to processing from lactic acid bacteria fermentation and the effects on emulsion microstructure and droplet characteristics were investigated. Optical microscopy and Turbiscan analyses were used to monitor the microstructure and droplet size during the fermentation. pH-driven effects led to milk protein conformational changes as indicated by the determination of total thiol groups and affected the protein interfacial composition. This phenomenon coincided (pH<5.5, T>37.9 °C) with the increase in average droplet size, which was attributed to flocculation phenomena due to reduced electrostatic repulsion. Casein solubility was enhanced during the initial stages of fermentation (1 hr - 3 hr) and was precipitated at pH<4.8. LC-MS/MS analysis confirmed that caseins (α_{s1}-, α_{s2}-, β- and κ-casein) were the dominant protein species at oil-water interface at the end of the fermentation process and contributed to the stability of the emulsion by reducing the droplet diameter (1.24 μm).

Keywords: Emulsion; Fermentation; Lactic-acid bacteria; Whey; Caseins

1. Introduction

Fermentation is an ancient method of food production and preservation in which

controlled microbial growth is used to convert major and minor food components (Marco et al., 2017). The process of fermentation is known to exert several beneficial effects on foods which among others include the increase in shelf-life and enhancement of organoleptic properties, and therefore is a desirable method of food production from a food manufacturer perspective (Şanlier, Gökcen & Sezgin, 2017).

Recently, fermented foods have attracted additional attention due to their documented potential to reduce cholesterol levels, regulate blood glucose and alleviate the symptoms of lactose intolerance (Tamang & Kailasapathy, 2010). In addition, fermentation can result in the reduction of potentially harmful compounds in foods such as mycotoxins (Shetty & Jespersen, 2006). The health-promoting potential of fermented foods are attributed to various mechanisms linked to the delivery of commensal microbes to the gastrointestinal (GI) tract and the subsequent release of bioactive compounds such as vitamins, lactic acid and peptides (Derrien & van Hylckama Vlieg, 2015; Plé, Breton, Daniel & Foligné, 2015). Thus, fermented foods may provide health benefits to consumers which go well beyond those of basic nutrition.

Colloidal systems including food emulsions are currently exploited for their potential to serve as “vehicles” for the encapsulation, protection and delivery of bioactive compounds such as oil-soluble vitamins, nutraceuticals and lipids at different locations within the GI tract (McClements, 2018). The formulation of the distinct phases including the interfacial composition and the effects of processing are key factors which need to be carefully controlled when designing complex food matrices for the delivery of bioactive compounds (Raikos & Ranawana, 2017). Recently, the incorporation of

emulsions in fermented dairy foods has been investigated as a method of delivery of encapsulated lipophilic bioactives or bacteria and their metabolic by-products (Lalou, Kadri, & Gkatzionis, 2017; Lobato-Calleros et al. 2008; Tidona et al., 2015). The effects of bacterial metabolic activity, surface charge and hydrophobicity on emulsion stability have been previously documented and indicate that oil droplet aggregation and flocculation phenomena are predominantly driven by charge effects which can be modulated by carefully choosing the bacterial strain (Ly et al., 2006).

In this study, oil-in-water emulsions were formed and stabilized by goat milk proteins. The emulsions were inoculated with lactic acid bacteria and the fermentation process was carefully monitored. The purpose of this study was to investigate the combined effect of pH and temperature on the physicochemical properties of the emulsion. Emphasis is given on the changes on protein structure and composition at the interface and continuous phase because of the fermentation process. To the best of our knowledge, this is the first study to monitor and elucidate the processing effects on the microstructure and droplet characteristics of a fermented dairy-based emulsion.

2. Materials and methods

2.1. Materials

Spray dried goats milk (skimmed) and yogurt culture (Yo-Mix ABY 2-C) were purchased from Goat Nutrition Ltd (Ashford, England). Corn oil was purchased from the local supermarket (Tesco, UK). *N*-(1-Pyrenyl)maleimide, N-acetyl-L-cysteine, MRS agar and α-Lactalbumin, β-Lactoglobulin, alpha-S Casein and β-Casein standards (from

bovine milk) were purchased from Sigma Aldrich (St. Louis, MO, USA). Precast gels and all reagents used for protein electrophoresis were purchased from Bio-Rad Laboratories Ltd. (Hertfordshire, UK). All reagents used were of analytical grade.

2.2. Preparation of fermented beverage emulsion

Emulsion beverages were prepared using the following weight recipe (78.75% water, 16% goat milk powder, 5% corn oil, 0.25% freeze dried culture). A coarse emulsion (500 gr) was formed by adding corn oil at a steady rate and mixing the remaining of the ingredients by using an Ultra-Compact digital mixer system (Cole-Palmer, Cambridgeshire, UK) for 5 min at 1000 rpm. Emulsions were further homogenized by passing the samples through a high-pressure homogenizer (APV-1000, SPX Flow Technology, West Sussex, UK) at 100 MPa for 5 times. 1.25 gr (~ 9 Direct Culture Unit) of the freeze dried mixed culture were added to the beverage and mixed thoroughly. Samples were then poured in a sterile glass container and were placed in a yogurt fermenter (Lakeland, Aberdeen, UK) set at 50 °C for 6 hr. The samples were then stored at -20 °C before further analysis unless otherwise specified. Four batches of fermented beverages were prepared and used for subsequent analysis.

2.3. Monitoring of coagulum formation and particle size determination

The fermentation process of freshly prepared beverages was monitored using a Turbiscan MA2000 (Formulaction, Fullbrook Systems Ltd, Herts, UK). Samples were poured into a cylindrical borosilicate glass tube (25 mm inner diameter and 60 mm high)

and were scanned from top to bottom with a near-infrared light source (880 nm) at 5 min intervals for 10 hr to generate transmission and backscattering data every 40 μm. The temperature of the apparatus was set at 42 °C to simulate the fermentation process. The coagulation process was evaluated using the Turbiscan stability index (TSI) parameter calculated according to backscattering changes that indicate the particles aggregation and dynamic migration by Turbisoft Lab 2.2 software (Fullbrook Systems Ltd). TSI is a statistical factor and its value is calculated as the sum of all the destabilization processes in the sample by using the following formulae:

$$BS=\frac{1}{\sqrt{\lambda*}} \quad (1)$$

$$\lambda^*(\varphi, d) = \frac{2d}{3\varphi(1-g)Qs} \quad (2)$$

$$TSI=\sqrt{\frac{\sum_{i=1}^{n}(\chi i-\chi BS)^2}{n-1}} \quad (3)$$

where λ* is the photon transport mean free path in the analyzed dispersion, φ is the volume fraction of particles, d is the mean diameter of particles, g and Qs are the optical parameters given by the Mie theory. χi is the average backscattering for each minute of measurement, χBS is the average χi, and n is the number of scans. A series of scans (7) was repeated for the beverages at 5 min intervals from top to bottom and the intensity of light backscattered during a 30 min period at 25 °C was recorded. Backscattering profiles (%ΔBS) from the middle of the tube (10 mm - 30 mm) were used to monitor size variation (coalescence/flocculation) phenomena for particle size determination. The refractive indices of the dispersed and continuous phase which were used to compute the mean spherical equivalent diameter were 1.47 and 1.33 respectively.

132

133 *2.4. pH, temperature and microbiological analysis of fermented emulsion*

134 The pH and temperature of the samples was monitored every hr using a portable
135 food and dairy pH meter (Hanna Instruments Ltd, Leighton Buzzard, UK) and sampling
136 for lactic acid bacteria was performed during the fermentation process at 0, 1, 2, 4 and 6
137 hr. Total lactic acid bacteria (LAB) were incubated aerobically on MRS agar (OXOID)
138 and enumeration was performed after incubation at 37 °C for 3 days. The total viable
139 microbial populations capable of growing on agar plates were analyzed in duplicate and
140 the average values of colony forming units (CFUs) are depicted here.

141

142 *2.5. Determination of free sulfhydryl content*

143 The fermented emulsions were ultra-centrifuged at 25000 g for 1 hr using a Sorvall
144 Lynx 4000 apparatus (Thermo Scientific Ltd., Loughborough, UK) and the aqueous
145 phases were diluted 5 times with PBS (pH 7.4). Free sulfhydryl (SH) content was
146 measured by a fluorescent assay as described by Lacy, Baker and Brigham-Burke (2008)
147 with slight modifications. N-acetyl-L-cysteine was used as standard for free SH
148 determination. 50 µL of the appropriate solution was mixed with 250 µL of PBS,
149 followed by incubation for 30 min at 25 °C prior to addition of *N*-(1-pyrenyl)maleimide
150 (NPM). 3 µL of 10 mmol/L NPM were added into this mixture, and the samples were
151 incubated for 2 hr at 25 °C. 200 µL of samples were pipetted onto a black 96-well
152 microplate, and the fluorescence emission spectrum of the samples was then obtained
153 using the fluorescence (λ_{ex}=330 nm/ λ_{em}=380 nm) on a plate fluorimeter (SpectraMax,

154 GEMINI XS, Molecular Devices Ltd., Wokingham, UK).

155

156 *2.6. Recovery of adsorbed and non-adsorbed proteins*

157 Aliquots of 2 mL of the emulsions were diluted with 2 mL of 50% (w/v) sucrose
158 solution. After vortexing, 2 mL of the mixture were pipetted and carefully deposited at
159 the bottom of a tube containing 7 mL of 5% (w/v) sucrose solution. The samples were
160 centrifuged at 3500 g for 1 hr. Following centrifugation, three phases were formed: the
161 creamed oil droplets at the top, an intermediate separating phase corresponding to 5%
162 sucrose solution at the middle, and the aqueous phase of the emulsion at the bottom of
163 the tube. The cream phase was washed twice to remove non-adsorbed proteins, filtered
164 to remove lipid droplets and suspended in 10 mM phosphate buffer. The samples were
165 frozen at -20 °C for 24 hr and the creamed (adsorbed) and the aqueous (non-adsorbed)
166 phases were analysed by SDS-PAGE.

167

168 *2.7. Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE)*

169 SDS-PAGE was performed according to a method described previously (Laemmli,
170 1970) using a Mini-Protean® 3 electrophoresis cell unit (Bio-Rad Laboratories Ltd.).
171 Proteins were reduced with 2-mercaptoethanol, heat-denatured at 100 °C for 2 min and
172 were analyzed on a 4-20% Mini-Protean® TGX™ precast gel. Electrophoretic migration
173 was performed at 150 V (constant) for 40 min. The gel was stained with Coomassie
174 Brilliant Blue R-250 staining solution for 1 hr with gentle agitation and destained for 2
175 hr in 30% methanol, 10% acetic acid solution. The gel was scanned with a GS-

800™calibrated densitometer (Bio-Rad Laboratories Ltd). Precision Plus Protein™ unstained standards were used from protein band identification as molecular weight markers (MWM).

2.8. Determination of whey proteins and caseins using RP-HPLC

The quantitative determination of whey protein and caseins was performed by HPLC (Waters 2695 Separations module equipped with a diode array detector) according to the method described by Kiokias, Dimakou and Oreopoulou (2007). Samples were centrifuged at 25,000 g for 1 hr at 4 °C and filtered through a 5 mm filter. 20 ml of sample were injected into a polymeric reversed phase column (PRP-1, 250 mm x 4.1 mm; particle size 10 mm, Hamilton Bonaduz A.G., Switzerland) and eluted at constant flow rate (1 ml/min) using a gradient system (solvent A: 0.1% trifluoroacetic acid in water and solvent B: 95% acetonitrile - 5% water acidified with 0.1% trifluoroacetic acid) as previously described (Ferreira, Mendes, & Ferreira, 2001). Whey proteins and caseins were quantified by comparison with calibrated standards.

2.9. Proteomics

The protein samples were digested in-gel (SDS-PAGE) with trypsin according to the method of Shevchenko et al. (2007), adapted for use with a JANUS automated liquid handling workstation (Perkin Elmer, Beaconsfield, UK). The extracted peptide digestion products were dried in a vacuum centrifuge (SpeedVac) and dissolved in 10 µL LC-MS loading solvent. One-fifth (2 µL) was sampled for LC-MS analysis as described

198 previously by Ni et al. (2018) using a Q Exactive Plus LC-MS system (Thermo Scientific,
199 Hemel Hempstead, UK) and a 'Top 10' data-dependent MS2 method. All parameters
200 were as previously described by Ni et al. (2018). Database searches were carried out
201 against the *Capra hircus* (Goat) protein sequences, downloaded from
202 www.uniprot.org/uniprot in fasta format. A Mascot significance value of 0.05 was used
203 to filter unreliable peptide matches.
204
205 *2.10. Optical microscopy*
206 Light micrographs of the emulsion samples were obtained using a Leica DM IL LED
207 inverted laboratory microscope equipped with a Leica DFC295 digital color camera
208 (Leica microsystems Ltd, Milton Keynes, UK). The samples were observed through a
209 40× dry objective lens. Pictures were taken using the in-built 3 MP digital camera and
210 picture analysis was performed using Leica application suite software (V.3.6.0).
211
212 *2.11. Statistical analysis*
213 Results are expressed as mean±(SD) of at least three replicates (each replicate
214 corresponds to a different batch). Statistical analysis of the data was performed using the
215 statistical software SPSS Statistics 22 (IBM Corp, Armonk, NY, USA). Data were
216 analyzed by analysis of variance (ANOVA) followed by the Scheffè's post hoc test ($P < 0.05$).
217
218
219 **3. Results and discussion**

220 *3.1. Fermentation effects on emulsion microstructural evolution and droplet size*

221 During fermentation of dairy products, lactic acid is typically produced thanks to
222 the metabolic activity of bacterial cultures and pH is gradually decreased to
223 approximately 4.5. This acidification step is essential for the conversion of the liquid
224 dispersion into a soft gel network (Horne, 1999). Thus, fermentation of dairy products
225 may be considered as a food manufacturing process which can be monitored by the
226 evolution of pH and viscosity with time (Sfakianakis & Tzia, 2014). The primary
227 particles involved in gel formation are casein micelles, which aggregate through
228 isoelectric precipitation (Karam, Gaiani, Hosri, Burgain, & Scher, 2013; Oliveira et al.,
229 2015). In this study, an oil-in-water emulsion was subjected to fermentation which is
230 likely to contribute to the formation of a network with modified structure. The duration
231 of the fermentation process was 6 hr and the pH dropped gradually from 6.1 to 4.1 (Table
232 1). During the processing steps, there was an incremental change in temperature from
233 25.4 ºC to 46.0 ºC. Non-surprisingly, viable counts of total lactic acid bacteria also
234 increased from 7.4 Log CFU/Ml (0 hr) to 8.1 Log CFU/mL (6 hr), and a coagulum was
235 formed by the end of the fermentation process, as indicated by the flattening of the TSI
236 curve after 5 hr (Figure 1). The evolution of the emulsion microstructure was monitored
237 during the fermentation using optical microscopy (Figure 2). Following high pressure
238 homogenization, fine droplets were formed which were dispersed to the aqueous
239 medium. Changes in droplet size because of the fermentation were not detectable by
240 means of microscopy and intact droplets were still observed after processing for 6 hr.
241 The coagulum formation was visually detected after 4 hr of fermentation (Figure 1E)

and this effect was even more clearly observed at the end of the process (Figure 1F).

The droplet size of the dispersed phase constantly evolved during the 6 hr period of the fermentation process (Figure 3). The average droplet diameter showed a non-significant ($p>0.05$) decrease from 1.41 μm to 1.35 μm after the first 2 hr of the fermentation. During homogenisation, milk proteins adsorb at the interface to lower the interface tension and form a layer of interacting polymer molecules which confers protection against coalescence and provides physical stability for a defined period (Dickinson, 1997). The interfacial composition is determined by numerous factors including surface hydrophobicity, molecular flexibility and the quantities present in the aqueous phase (Dickinson, 1991; Hunt & Dalgleish, 1995). Protein unfolding and effective rearrangement at the interface is likely to occur following absorption at the interface (Dickinson 1992; Dalgleish 1996). This structural rearrangement also indicated by the increase in total -SH groups suggests protein denaturation and rearrangement at the interface which may explain the initial but non-significant decrease in droplet size at this stage (Dickinson, Rolfe & Dalgleish, 1988). The decrease in droplet size was followed by a non-significant ($p>0.05$) increase to 1.39 μm. This effect was consistent and was observed in all replicate measurements from different batches. During this stage of the fermentation process, the pH drops from 5.5 to 4.8 (Table 1), which is near the isoelectric point of the main goat milk proteins including α_{s2}-casein (4.7), β-casein (~5.2), κ-casein (~5.4), β-lactoglobulin (~5.6) and α-lactalbumin (~5.0) (da Costa et al., 2014). As a result, the net negative charge is reduced, and inter-droplet electrostatic repulsion is no longer sufficiently strong to overcome attractive forces leading to loss of

electrostatic stabilisation and droplet flocculation (Dickinson 2001; McClements 2004). Following this step, the droplet size continues to decrease until the end of the fermentation period to reach an average droplet diameter of 1.24 μm. This result agrees with the microstructural analysis of the fermented emulsion and suggests that the structural integrity of the droplets remains intact after the formation of the coagulum resulting to a gelled emulsion type of product. The effect of the fermentation process on the particle size of the emulsion is largely driven by the pH transition and the resulting changes in protein charge, solubility and interfacial composition.

3.2. Fermentation effects on protein structure and interfacial composition

Milk proteins are considered effective emulsifiers which are capable to adsorb at the oil-water interface and effectively lower the interfacial tension (Raikos, 2010). During the process of homogenization, individual milk proteins compete for adsorption and an interfacial layer is dynamically formed (Millqvist-Fureby, Eloffson, & Bergenstål, 2001). Caseins tend to predominate at the interface thanks to the higher proportion of hydrophobic residues and the more flexible structure compared to whey proteins which contain more rigid α-helix and β-pleated structures (Dalgleish, Goff, Brun & Luan, 2002; Singh & Sarkar, 2011). The preferential absorption of caseins over whey proteins at the interface is also concentration dependent and occurs at high protein concentrations (Ye, 2008). The protein composition of the interfacial film was determined by means of electrophoretic analysis and is presented in Figure 4. The protein profile obtained agrees with previously published data and confirms the abundant presence of α-lactalbumin, β-

286 lactoglobulin, κ-casein, β-casein, αs2-casein and bovine serum albumin in increasing
287 molecular weight order (da Costa et al., 2014; Yi et al., 2019). The main proteins
288 identified at the interface (Figure 4, lanes 3, 5, and 7) are identical with the ones detected
289 in the aqueous phase and an additional band of approximately 20 kDa, is detectable
290 which could correspond to γ-casein (da Costa et al., 2004). This confirms the presence
291 of both whey proteins and caseins at the interface. Non-surprisingly, the protein profile
292 in the aqueous phase changes dramatically at the end of the fermentation process (Figure
293 4, lanes 8 and 9) and indicates that a gel network is formed by casein clusters, chains
294 and strands. At this point, the casein micelles of the continuous phase entangle and form
295 insoluble aggregates due to attractive forces, which is supported by the disappearance of
296 the bands in the range between 25 kDa and 37 kDa (Figure 4, lane 8). On the other hand,
297 the solubility of whey proteins remains unaffected by the fermentation process.
298 Interestingly, the interfacial composition is affected by the pH transition and indicates
299 that by the end of the fermentation process caseins predominate the coating layer in
300 comparison to whey proteins. Although whey proteins can still be detected at the
301 interface after the fermentation process, it is likely that desorption occurs as indicated
302 by the disappearance of the bands corresponding to α-lactalbumin and β-lactoglobulin
303 monomers (Figure 4, lane 9). LC-MS/MS analysis of the main bands confirmed the
304 presence of α_{s2}- (top band), β-, α_{s1}- (middle band) and κ-casein (lower band) at the
305 interface at the end of the fermentation process (Figure 4, lane 9). The molecular weight
306 distribution of the identified bands agrees with previously published research (Greppi,
307 Roncada & Fortin, 2008). The analysis also indicates the presence of β-lactoglobulin in

308 the middle band, which suggests that dimers are formed and contribute to the interfacial
309 composition at the end of the process. This finding may account for the continuous
310 reduction of the droplet size during the last stages of the fermentation process (Fig. 3).
311 Caseins due to their disordered structure are known to form aggregates and thus provide
312 a high surface coverage around the droplet area, which can have a positive impact on
313 emulsion stability (Euston & Hirst, 2000).

314 Solubility is an important determinant of protein functionality including the ability
315 to form and stabilize emulsions (Pelegrine & Gasparetto, 2005). Protein charge and as a
316 result solubility can be dramatically affected by modifying the pH environment as it
317 occurs during fermentation. RP-HPLC has been successfully employed in the past to
318 monitor whey protein solubility, since the method is based on hydrophobic interactions
319 and therefore any denaturing effects induced by the method of analysis should be
320 negligible (Anandharamakrishnan, Rielly & Stapley, 2008). The effects of fermentation
321 on the solubility of the main casein and whey proteins is presented in Figure 5. The
322 solubility of whey proteins (α-lactalbumin, β-lactoglobulin) remains unaffected by the
323 decrease in pH. This result is consistent with the protein profile obtained from SDS-
324 PAGE analysis (Figure 4). Conversely, caseins (α and β) show a significant increase in
325 solubility between the 1st and 3rd of fermentation, followed by a sharp decrease which is
326 consistent until the end of processing period. The initial increase in casein solubility (pH
327 range: 5.9-4.8) may be attributed to the solubilization of colloidal calcium phosphate
328 which is known to increase with decreasing pH (from neutral) and reaches maximum
329 values at approximately pH 5 (Raynal & Remeuf, 2000; Post, Arnold, Weiss & Hinrichs,

330 2012). Further reduction of pH below 4.8, reduced the net negative charge of caseins and
331 decreased the solubility by inducing isoelectric precipitation. The effect of fermentation
332 on milk protein structure was investigated by determining the total SH-groups for the
333 non-adsorbed proteins (Figure 4). The determination of total SH-groups is a reliable
334 indicator of protein unfolding under conditions which can effectively modify the
335 structure (Siddique, Maresca, Pataro & Ferrari, 2016). Different methods have been
336 applied for analyzing sulfhydryl groups in milk related to processing effects and are
337 mainly associated with β-lactoglobulin (Owusu-Apenten, 2005). Thiol groups are
338 typically located in the hydrophobic core of the native protein structure, which during
339 processing can become exposed and reactive. The number of total SH-groups showed an
340 insignificant increase ($p>0.05$) during the first 2 hr of the fermentation, which may be
341 attributed to mild protein conformational changes induced by pH and temperature and/or
342 to the increasing solubility of casein proteins at this stage. A significant reduction in total
343 thiol groups followed from this point onwards until the end of processing (3-6 hr). This
344 phenomenon was effective prior to casein isoelectric precipitation (hr 4) and indicates
345 the formation of complex protein networks through intra- or intermolecular
346 thiol/disulfide interchange or thiol/thiol oxidation reactions. Denatured whey proteins
347 (and particularly β-lactoglobulin) can be involved in thiol-disulfide reactions with κ-
348 caseins at the micelle interface depending on the pH and temperature conditions (Anema
349 & Li, 2003; Mahomud, Katsuno & Nioshizu, 2017). Our results indicate that these type
350 of intermolecular covalent interactions between whey proteins and caseins occur below
351 pH 5.5 and above 37.9 °C and can take place before the formation of the gel network

induced by casein-casein interactions. Potential interactions between adsorbed proteins (whey-whey, casein-casein or whey-casein) may also be associated with the observed increase in droplet size which occurs simultaneously (Figure 3).

4. Conclusions

Light scattering analysis of the fermented emulsion revealed that the decrease of pH led to an increase of the droplet size near the isoelectric point of the milk proteins. At this stage of the fermentation process, reduced electrostatic repulsion may induce droplet flocculation and protein crosslinking via thiol/disulfide exchange reactions. The structural integrity of the oil droplets was retained until completion of the fermentation process. Caseins were the primary protein components involved in the formation of the gel network and their soluble fraction was greatly affected by the pH during the fermentation stages. The interfacial composition of the oil droplets was also pH-dependent. Whey and caseins were identified at the interface during the initial stages of the fermentation. Interestingly, caseins remained adsorbed and dominant at the interface following the formation of the gelled network and until the end of the fermentation and contributed to the stability of the oil droplets. Further studies are required to better understand the evolution of the colloidal structure and the mechanisms involved during the stages of lactic acid fermentation.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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Figure captions

Figure 1. Evolution of the gelation process during the fermentation process. Viable counts of total lactic acid bacteria are recorded at certain time intervals (0, 1, 2, 4 and 6 hr).

Figure 2. Optical microscopy images of oil-in-water emulsions at 0 hr (A), 2 hr (B), 3 hr (C), 4 hr (D), 5 hr (E) and 6 hr (F) of the fermentation process. Scale bar represents 50 μm.

Figure 3. Free -SH group content ()and oil droplet size () at different point intervals of the fermentation process. Different low case letters indicate significant differences between mean values±SD for each treatment ($p<0.05$).

Figure 4. Electrophoretic analyses of unadsorbed (Lanes: 1, 4. 6 and 8), adsorbed (Lanes: 3, 5, 7 and 9) at different time points of the fermentation process using SDS-PAGE under reducing conditions. Interfacial proteins at 6 hr of fermentation (Lane 9) were identified by LC-MS. Molecular weight markers are presented in Lanes 1 and 10.

Figure 5. RP-HPLC analyses of the main whey (α-lactalbumin, β-lactoglobulin) and casein (β-casein, α-casein) protein fractions in goat milk stabilized emulsions

396 during the fermentation process. Each point is the mean of two replicate measurements
397 from different batches.
398
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