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ORIGINAL PAPER

Exopolysaccharide–milk protein interactions in a dairy model system simulating yoghurt conditions

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Abstract Although exopolysaccharides (EPS) produced by lactic acid bacteria can be used to modulate the rheological and physical properties of fermented milk, the interactions between EPS and milk proteins in such complex system remain poorly understood. This work aimed to study the interaction between EPS with different structural characteristics and caseins in the absence or presence of whey proteins in a dairy model system simulating yoghurt conditions. The study was expected to highlight the contribution of whey proteins to the casein network and identify possible interactions of EPS with the casein network. Four starters were used: HC15/210R (control), HC15/291 (neutral, stiff, branched EPS), HC15/702074 (neutral, flexible, highly branched EPS), and 2104/210R (anionic, stiff, linear EPS). Fermentation was performed at 42 °C until the pH reached 4.6. Microstructure and rheological and physical properties (syneresis, elastic modulus, and apparent viscosity) were measured. The diversity of EPS functionalities depended on the specific structures of the EPS: stronger gels were formed with the anionic EPS from strain 2104 probably because of electrostatic interactions, although limitation of syneresis was more influenced by the neutrality and stiffness of the EPS backbone of strain 291. The sequential addition of casein and whey proteins to the dairy model system revealed their individual contribution to the microstructure of the protein network. This study showed that the rheological and physical

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properties of fermented milk can be modulated by the casein and whey protein concentrations and the use of different EPS with specific structural characteristics.

Keywords Exopolysaccharide · Yoghurt · Dairy model system · Rheological property

1 Introduction

Exopolysaccharides (EPS) are microbial polysaccharides that are naturally produced by some lactic acid bacteria (LAB) during the fermentation process. Because of consumer demand for natural foods, EPS are being studied more extensively as natural stabilizers in fermented dairy products such as yoghurts. The abilities of EPS to modulate viscosity and bind water are correlated not to the concentration of EPS but rather to their structure and interactions with milk components, especially proteins (Doleyres et al. 2005; Ruas-Madiedo et al. 2002). The molecular weight (Faber et al. 1998), backbone conformation (flexibility and degree of branching) (Tuinier et al. 2001), and charge (Girard and Schaffer-Lequart 2007; Turgeon and Plesca 2009) of EPS influence the rheological properties and microstructure of fermented milk.

Protein–polysaccharide interactions are well-known (Doublier et al. 2000; Tolstoguzov 1991; Turgeon et al. 2003). However, the interactions between milk proteins and EPS in a complex system such as fermented milk are poorly understood, likely because EPS are progressively produced during the fermentation process as compared to conventional stabilizer agents (modified starches and pectin), which are directly added to the mixture at the natural pH of milk. To overcome this problem, some authors have studied protein–polysaccharide interactions in model systems where EPS were used as bioingredients; that is, added in purified and freeze-dried form to milk at the beginning of the process (Girard and Schaffer-Lequart 2008; Tuinier et al. 1999, 2000). However, a comparative study has demonstrated different rheological properties of fermented milks with in situ or bioingredient EPS (Doleyres et al. 2005). Purification and freeze-drying steps cause the loss of some functional properties such as ropy character after rehydration (Girard and Schaffer-Lequart 2008).

Skim milk media are often used to study EPS production. However, the complexity of milk, which is made up of proteins (caseins and whey proteins), fat, sugars, and salts, adds to the difficulty of studying the interactions between milk proteins and EPS. Only one group of researchers has studied the interactions between one EPS with a well-known structure produced in situ by *Lactococcus lactis* and whey proteins in a simplified milk system, namely, milk permeate (Ayala-Hernández et al. 2008, 2009). Results from scanning electron microscopy have shown interactions between the anionic EPS and whey proteins (2–8%) at pH 4.5 after 12 h of fermentation at 30 °C. However, possible interactions with caseins were not studied.

To study the protein–EPS interactions in yoghurt, a dairy model system allowing the control of protein concentration and bacterial growth with a similar acidification rate and in situ EPS production should be used. The dairy model system should also have the same environmental conditions as milk in terms of sugars, minerals, and vitamins, making milk permeate a good system. Furthermore, a comparative study of several EPS with well-known structures produced in situ by LAB in the presence of different types and concentrations of milk proteins under the same fermentation

conditions has never been carried out. The aim of this work was to study the interactions between milk proteins (caseins and whey proteins) and three EPS having different structural characteristics (charge, stiffness, and degree of branching) produced in situ in a dairy model system.

2 Materials and methods

2.1 Materials

Skim milk powder was made up of 34.4% total protein and 98% dry matter (low-heat spray-drying process; René Rivet Inc., Terrebonne, QC, Canada). Whey protein isolate (89% whey proteins and 98% dry matter) was purchased from Davisco Foods International (Le Sueur, MN, USA). The lactose that was used contained 98% sugar (Saputo Dairy Products Canada, Saint-Léonard, QC, Canada). Milk permeate, containing 0.27% total protein, 0.75% ash, and 5.6% dry matter, was prepared by the ultrafiltration of fresh skim milk at 30 °C with a PM10 polysulfone membrane (5–10 kDa). Native phosphocaseinate (NPC) was prepared according to the method of Pierre et al. (1992). The NPC, previously frozen at −20 °C, was lyophilized under vacuum at 20 °C for 48 h (Model Y6-CAB; Lyo-San Inc., Lachute, QC, Canada). The NPC consisted of 85% total protein, 78% caseins, 7.2% ash, and 93% dry matter. Bacto casamino acids (Difco), sucrose, sulfuric acid, trichloroacetic acid, and magnesium sulfate were purchased from Fisher Scientific (Nepean, ON, Canada). Bacto casamino acid powder (69% total nitrogen, 18% ash, and 95% dry matter) was composed of highly hydrolyzed caseins (amino nitrogen-to-total nitrogen ratio of 0.87). Glycerol 2-phosphate disodium, isoamyl alcohol, sodium nitrate, and phenol (>98%) were obtained from Sigma-Aldrich (Toronto, ON, Canada). Acetone and chloroform were obtained from Tekniscience (Terrebonne, QC, Canada).

2.2 Preparation of bacterial strains and starters

Two *Streptococcus thermophilus* strains were used: HC15, from Chr. Hansen (Mississauga, ON, Canada), and NIZO2104 (referred to in this study as 2104), from NIZO Food Research B.V. (Ede, Netherlands). Three *Lactobacillus delbrueckii* subsp. *bulgaricus* strains were used: 210R, from Waterford (Gist Brocades, Millville, UT, USA), NCIMB702074 (referred to in this study as 702074), from NCIMB (Aberdeen, Scotland, UK), and DGCC291 (referred to in this study as 291), from Danisco (Buxière, Orsay, France). Strains HC15 and 210R were chosen for their ability to not influence the rheological properties of fermented milks although they produce EPS (Gentès et al. 2011). By mixing these single strains, four starters were prepared: HC15/210R (control), HC15/291 (neutral, stiff, branched EPS), HC15/702074 (neutral, flexible, branched EPS), and 2104/210R (anionic, stiff, linear EPS). Complete structural information on the EPS is presented in Table 1. The complete structural composition of EPS is presented in a previous publication (Gentès et al. 2011). Reconstituted skim milk (RSM) from a low-heat skim milk powder was used for strain preparation. Strains were stored at −80 °C in 20% (*w*/*w*) RSM supplemented with 5% (*w*/*w*) sucrose and 0.35% (*w*/*w*) ascorbic acid and sterilized at 110 °C for 10 min.

Table 1 Structural characteristics of EPS from LAB

	Charge	Molecular weight[a] ($g \cdot mol^{-1}$)	Branching[b]	Flexibility	References
HC15	Control				Lamboley et al. (2003)
2104	Negative[c]	0.9×10^6	−	Stiff	Faber et al. (2002)
210R	Control				Robitaille et al. (2009)
702074	Neutral	1.8×10^6	++	Flexible	Harding et al. (2005)
291	Neutral	1.4×10^6	+	Stiff	Faber et al. (2001)

[a] The molecular weight of strain 2104, the only unknown structural characteristic, was determined in this study

[b] Branching=linear (−), one branching (+), two or more branchings (++)

[c] *N*-acetyl=*N*-acetylgalactosamine plus another monomer: 6-*O*-(3′,9′-dideoxy-D-*threo*-D-*altro*-nononic acid-2′-yl)-α-D-glucopyranose

Stock cultures were inoculated at 10% (*v*/*v*) in sterilized 12% RSM (at 110 °C for 10 min) and incubated at 37 °C for 16 h. Active strains were prepared by the inoculation of culture stock at 3% (*v*/*v*) in sterilized 12% RSM until the pH reached 5.2 for streptococci (4.5–6 h) and 4.8 for lactobacilli (3–4 h). The populations of strains HC15, 291, and 702074 were 5×10^8 CFU·mL^{-1}. For strain 210R, the population was 4×10^8 CFU·mL^{-1}. For strain 2104, an additional 3% (*v*/*v*) subcultivation (at 42 °C for 6 h followed by storage overnight at 4 °C) was necessary to reach a population of 2×10^8 CFU·mL^{-1}. Active strains were stored overnight at 4 °C.

2.3 Determination of molecular weight of EPS from strain 2104

Molecular weight was determined for the EPS from strain 2104, as it was the only unpublished structural characteristic. Bacterial growth and EPS production were achieved as described previously in Section 2.2. Protein was precipitated and purification of the EPS in the supernatant was performed as described by Van Calsteren et al. (2008). For protein removal, equal volumes of 40% (*w*/*v*) trichloroacetic acid were added to the milk samples at room temperature, and the mixtures were agitated for 20 min under magnetic agitation. The samples were centrifuged for 30 min at 12,000×*g* at 4 °C (Avanti J-20 XPI, Beckman Coulter Inc., Fullerton, CA, USA). After filtration of the supernatant (Whatman paper no. 41), EPS were precipitated in chilled acetone (4 °C) to a final concentration of 50% (*v*/*v*) at 4 °C for 16 h and then centrifuged with the parameters described previously. The pellet was dissolved in 400 mL of deionized water at room temperature during 4 h of magnetic agitation and stored at 4 °C for 48 h. Residual protein was removed by a phenol/chloroform/isoamyl alcohol (25:24:1) extraction solution (400 mL). The phenol was removed by a chloroform/isoamyl alcohol (24:1) extraction solution (400 mL). EPS were precipitated with acetone as described previously. The pellet, dissolved in 100 mL of pure water, was dialyzed at 4 °C for 72 h with 5 L of pure water with four water changes per day (12,000–14,000 g·mol^{-1}, MWCO membrane, Spectra/Por). After filtration on a 0.22-μm filter under vacuum, EPS were lyophilized at −45 °C for 48 h (Freezone 2.5,

Labconco, Kansas City, MO, USA). The purity of EPS from strain 2104 was determined by size exclusion chromatography coupled with a multi-angle laser light scattering (MALLS) detector. Chromatographic separation was done with two 8×300 mm Shodex OHpak columns connected in series, as follows: SB-806 and SB-804 gel filtration columns, preceded by an SB-807G guard column (Showa Denko, Tokyo, Japan), eluted with 0.1 M $NaNO_3$ mobile phase at a flow rate of 0.5 mL·min^{-1}. Molecular weight was determined with a Dawn EOS MALLS (Wyatt, Santa Barbara, CA, USA) and with a differential refractometer RI 410 detector (Waters, Milford, MA, USA) using xanthan standards (Brandrup et al. 2005). The structure of EPS from strain 2104 was confirmed by nuclear magnetic resonance (data not shown).

2.4 Preparation of dairy model systems

The NPC powder was first sieved with a stainless sieve (mesh, 1×1 mm) to remove large dry particles formed during the freeze-drying process. An NPC solution at 7% caseins (*w*/*w*) was made by dissolving the NPC powder in milk permeate at 50 °C for 45 min under constant agitation of 200 rpm with the propeller stirrer 3 fl (R1381) of an IKA RW 20 digital mixer (Wilmington, NC, USA). The NPC solution was kept under constant magnetic agitation at 4 °C for 16 h. Because of the poor solubility of NPC powder, as mentioned by Pierre et al. (1992), a centrifugation step (690×*g*, 21 °C, 10 min) was carried out to remove insoluble particles from the NPC solution at 7% caseins. Casein level in the supernatant (3.5%) was measured with an infrared analyzer using Fourier transform infrared (FT-120, Foss North America, MN, USA). Neither the distribution of the casein profile (α, β, κ, and γ) evaluated by high-performance liquid chromatography (Jaubert and Martin 1992) nor the particle size (Nano-Zs, Malvern Instruments, Worcestershire, UK) had changed in the supernatant after centrifugation (data not shown). The supernatant (3.5% caseins) was diluted (*w*/*w*) in milk permeate to obtain 1%, 2%, and 3% caseins. In the dairy model at 0% caseins, no caseins were added but casamino acid powder (2% [*w*/*w*]) was added as nitrogen source to the milk permeate to promote bacterial growth. The buffering capacity of milk permeate was lower than that of milk owing to the absence of milk proteins (data not shown). To allow bacterial growth and obtain similar acidification profile, a buffering agent (glycerol 2-phosphate disodium) was added to the dairy model systems at 0% and 1% caseins to a concentration of 0.5% (*w*/*w*) and 1% (*w*/*w*), respectively. The dairy model systems at 0% and 1% caseins also required $MgSO_4$ (30 mM) to promote cellular division for lactobacilli, as reported in the literature (Lamboley et al. 2003). At 2% and 3% caseins, the addition of glycerol 2-phosphate disodium and $MgSO_4$ was not necessary. The final weight of the solutions was 100 g.

To study the effect of the addition of 0.5% (*w*/*w*) or 0.75% (*w*/*w*) as equivalent whey proteins, a casein concentration of 2% was used. Whey proteins came from whey protein isolate. Total solids content was adjusted to 10% (*w*/*w*) with lactose. The final weight of the solutions was 100 g. All solutions were left under magnetic agitation at 21 °C for 30 min to ensure complete dissolution. The solutions were homogenized (EmulsiFlex C5, Avestin Co., Ottawa, ON, Canada) by a single pass at 2,000 psi at 55 °C and heat-treated at 90 °C for 2 min in an automatic steam-controlled water bath usually used for dairy starter preparation (Laboratorium

Wiesby GmbH & Co., Niebüll, Germany). The mixtures were rapidly cooled to the fermentation temperature (42 °C) in the water bath (about 15 min) or stored at 4 °C until use (maximum of 24 h).

2.5 Fermentation of dairy model systems

The dairy model systems were inoculated with starters to obtain an initial population of 2×10^7 CFU·mL^{-1} with a streptococci-to-lactobacilli ratio of 50:50 for the HC15/210R (control), HC15/291, and HC15/702074 starters. For the 2104/210R starter, the streptococci-to-lactobacilli ratio was 40:60 to achieve an acidification profile similar to that of the other starters. After inoculation, 90 mL of the solution was divided equally among three sterile 50-mL tubes (Falcon, VWR, Montreal, QC, Canada). The first tube was for pH measurement and EPS quantification, the second tube was for titratable acidity and microbiological enumeration, and the third tube was for the funnel test (evaluation of ropy character). The remaining 10 mL of the inoculated solution was used for microscopy. Fermentation was done in a water bath at 42 °C until the pH reached 4.6±0.05. Titratable acidity (expressed as percent lactic acid), pH, microbiological enumeration, and EPS content were determined before and after fermentation.

2.6 Ropy character of EPS in dairy model systems

At a pH value of 4.6, tubes (30 mL) of the fermented dairy model systems were put on ice until the temperature reached 21 °C. The ropy character of EPS was evaluated by measuring the time (in seconds) required for 30 mL of the samples to pass through a funnel, as described by Gentès et al. (2011).

2.7 Analytical methods

Official standard methods were used to determine lactic acid production, pH, dry matter content, and ash content (AOAC 2000). The macro-Kjeldahl method was used to quantify total protein, noncasein nitrogen, and nonprotein nitrogen (St-Gelais et al. 1998). The noncasein nitrogen content in the unheated milk preparation was obtained by casein precipitation at pH 4.6 with H_2SO_4 (0.02 N). The acid solution was filtered (Whatman paper no. 40), and the filtrate was analyzed. The nonprotein content was obtained by protein precipitation with 12% trichloroacetic acid (*w*/*w*). The sample was filtered (Whatman paper no. 40) and analyzed (St-Gelais et al. 1998). The casein and whey protein contents were calculated by difference. A nitrogen conversion factor of 6.38 was used. The EPS concentration was measured as described by Gentès et al. (2011).

2.8 Rheological and physical properties of dairy model systems

To measure the rheological properties of the dairy model systems at 2% and 3% caseins with or without whey protein addition, a dynamic rheometer (AR1000, TA Instruments, New Castle, DE, USA) was used to monitor the gelation profile at 42 °C as described by Girard and Schaffer-Lequart (2007). To minimize the possible effect

of slipperiness due to syneresis, rugged plate geometry (4 cm) was used. The gap was 46 μm. To limit dehydration of the sample, a solvent trap was used. Prior to each experiment, plates were disinfected with ethanol (70%) to avoid contamination. After inoculation, 1.5 mL of the inoculated solution was transferred to the rheometer. The remaining volume of the inoculated solution was used to monitor the pH over time with a glass electrode and a standard pH meter (model 140, Corning, Fisher Scientific, Nepean, ON, Canada). The gelation point was defined as the time (T_{gel}) and the pH (pH_{gel}) when the elastic modulus (G') was >1 Pa (van Marle and Zoon 1995). Raw data for obtaining T_{gel} and pH_{gel} were analyzed by modeling curves (pH or G' as functions of time) with the following four-parameter logistic equation:

$$y = \frac{(A - D)}{1 + \left(\frac{x}{C}\right)^{B}} + D \quad (1)$$

where y is G' or pH, x is time, A is initial pH or initial G', B is the coefficient of slope, C is the coefficient of acidification rate or coefficient of G' rate, and D is final pH or final G'. The T_{gel} (x) was calculated from Eq. 1 at a G' of 1 Pa (y). The pH_{gel} was calculated using Eq. 1 with y as pH_{gel} and x as T_{gel}.

When the pH reached 4.60±0.05, measurement of the elastic modulus as a function of time was stopped, and a temperature ramp test (elastic modulus as a function of temperature) was applied (40 min at 1 °C·min^{-1}) at 0.1 Hz and 0.02% strain to simulate the cooling step to the storage temperature (4 °C) in yoghurt manufacturing. A conditioning step of 5 min at 4 °C was applied. After cooling to 4 °C, a continuous ramp test was applied to measure the viscosity as a function of shear rate ranges changing linearly from 0 to 100 s^{-1} in 2 min. Apparent viscosities at 10 and 100 s^{-1} were calculated according to the power law model as described by Gentès et al. (2011). Susceptibility to syneresis was quantified by a centrifugation technique as described by Gentès et al. (2011).

2.9 Microscopy

Confocal scanning laser microscopy operating in fluorescence mode was used to evaluate the microstructure of the dairy model systems (Nikon TE-2000E Eclipse, Nikon Canada, Mississauga, ON, Canada). Inoculated samples (10 mL) were transferred to 50-mL sterile tubes and stained with 30 μL of acridine orange (protein dye) at 0.2% (*w*/*w*) (Sigma-Aldrich, Toronto, ON, Canada) according to the method of Lee and Lucey (2004). The samples were gently mixed by inversion five times. The samples (20 μL) were transferred to slides with a cavity (single-depression microslides, no. CA48324-001, VWR, Montreal, QC, Canada), and a cover slip was fixed to each with Cytoseal 60 (Richard-Allan Scientific, Kalamazoo, MI, USA). The samples were put in Petri dishes covered with parafilm to prevent dehydration and were incubated in an incubator at 42 °C. When the pH reached 4.6, the samples were stored at 4 °C for 48 h before visualization. The samples were observed at an excitation wavelength of 488 nm with an He/Ne laser and a water-immersion ×60 objective lens (numerical aperture, 1.4). Three representative images of the samples were taken (depth of 10–20 μm).

2.10 Statistical methods

A split-plot design was applied to evaluate the effect of casein or whey protein concentrations on the composition, microbiological population, pH, titratable acidity, and rheological properties of dairy model systems fermented with starters that produce various EPS structures. Starter was the subplot factor. Significant differences were tested at $P \leq 0.05$. Statistical analysis was carried out with the General Linear Models procedure of SAS (Version 9.1.3, 2003, Cary, NC, USA). All experiments were made in triplicate.

3 Results

3.1 Effect of casein concentration

3.1.1 Composition

The composition, initial pH, and lactic acid production of the dairy model systems at different casein concentrations are presented in Table 2. Dry matter was significantly affected by casein concentration. Ash level was significantly higher for the dairy model system at 0% caseins owing to the higher ash content of the casamino acid powder added to the system. The initial pH of the dairy model systems at 0% and 1% caseins was significantly higher, likely because of the addition of glycerol 2-phosphate disodium. Lactic acid production was significantly affected by the casein level, although differences between values were small.

3.1.2 Bacterial growth, exopolysaccharide production, and ropy character

The initial populations in the dairy model systems were controlled at $2.3 \pm 0.09 \times 10^7$ CFU·mL^{-1} and a streptococci-to-lactobacilli ratio of 51±2 for the HC15/210R (control), HC15/291, and HC15/702074 starters. The initial population in the dairy model system fermented with the 2104/210R starter was $1.9 \pm 0.09 \times 10^7$ CFU·mL^{-1} with a streptococci-to-lactobacilli ratio of 40±2. The final pH values of all the dairy model systems were not significantly different (4.58±0.02). The fermentation time

Table 2 Composition (in percent), initial pH, and lactic acid production after fermentation of the dairy model systems at different casein concentrations

Item	Casein concentration (%)				SEM
	0	1	2	3	
Dry matter	7.35^{c}	7.55^{c}	8.59^{b}	9.79^{a}	0.17
Casein	0.02^{d}	1.03^{c}	2.03^{b}	3.04^{a}	0.02
Nonprotein nitrogen	1.46^{a}	0.15^{b}	0.17^{b}	0.18^{b}	0.01
Ash	1.61^{a}	0.75^{b}	0.68^{c}	0.79^{b}	0.02
Initial pH	6.54^{a}	6.51^{a}	6.18^{b}	6.22^{b}	0.01
Percent lactic acid	0.48^{a}	0.48^{a}	0.43^{b}	0.45^{b}	0.004

Values in the same row followed by the same letter are not significantly different ($P<0.05$). Data are the mean of three experiments. *SEM* standard error of the mean

and ropy character of EPS are presented in Table 3. As there were no significant differences in the streptococci population (log 8.48±0.04 CFU·mL^{-1}), the results are not shown. The lactobacilli population was significantly affected by starter and casein concentration. However, the difference was very small. The amplitude of variation was of 0.5 log between the highest and the lowest lactobacilli population (average of log 8.02±0.06 CFU·mL^{-1}). Fermentation time was significantly affected by starter and casein concentration. A significantly longer fermentation time was measured in the dairy model system at 0% caseins for all starters as compared to the other concentrations. EPS production was significantly affected by casein concentration but not by the type of starter used. The means of EPS concentration was significantly higher in the dairy model systems at 1% (54±3 mg·L^{-1}) and 3% (55±3 mg·L^{-1}) caseins as compared to those at 0% (37±3 mg·L^{-1}) and 2% (43±3 mg·L^{-1}) caseins.

A significant interaction between starter and casein concentration was observed for the ropy character of EPS (Table 3). At 0% and 1% caseins, no gels were formed at pH 4.6. For the dairy model systems at 0% and 1% caseins, the time required to pass through the funnel was significantly longer for the HC15/291 and 2104/210R starters, especially at 1% caseins. For the HC15/210R (control) and HC15/702074 starters, the

Table 3 Fermentation time and the evaluation of ropy character of EPS (by the measurement of the time required to pass through a funnel) for fermented dairy model systems at pH 4.6 with various casein concentrations

Casein (%)	Starter	Fermentation time (min)	Flow time (s)
0	Control	322^{a}	11^{d}
	HC15/291	307^{ab}	24^{c}
	HC15/702074	325^{a}	11^{d}
	2104/210R	287^{b}	37^{bc}
1	Control	235^{cde}	14^{d}
	HC15/291	218de	64^{a}
	HC15/702074	242^{c}	13^{d}
	2104/210R	205de	50^{ab}
2	Control	241^{c}	Gel*
	HC15/291	203de	Gel
	HC15/702074	240^{c}	Gel
	2104/210R	197^{e}	Gel
3	Control	240^{c}	Gel
	HC15/291	195^{e}	Gel
	HC15/702074	236^{cd}	Gel
	2104/210R	200^{e}	Gel
SEM		13.3	3.4

The control was the HC15/201R starter. Values in the same column followed by the same letter are not significantly different ($P<0.05$). Data are the mean of three experiments

SEM standard error of the mean

* The evaluation of the flow time (in seconds) by means of the funnel test was not possible for the dairy model systems at 2% and 3% caseins because of gel formation at pH 4.6

time required to pass through the funnel was statistically similar at 0% and 1% caseins. For the dairy model systems at 2% and 3% caseins, ropy character could not be measured by the funnel test because a gel was formed at pH 4.6 (Table 3).

3.1.3 Rheological and physical properties

The gel properties of the dairy model system at 3% caseins with all starters were not significantly different, as follows: T_{gel} (177±9 min), pH_{gel} (4.68±0.08), G' at 42 °C (3±0.9 Pa), and G' at 4 °C (16±8 Pa). At 2% caseins, a weak gel was formed (G' at 42 °C<1 Pa) and, consequently, the pH_{gel} and T_{gel} were not detectable for this casein concentration.

The ability of the starters to limit syneresis in the dairy model systems at 2% and 3% caseins was significantly influenced by an interaction between starter and casein concentration (Fig. 1a). At both casein concentrations, the dairy model systems fermented with the HC15/291 starter had the highest (P>0.05) ability to retain water as compared to the other starters. The HC15/210R (control) and HC15/702074 starters in the dairy model systems at both casein concentrations had the lowest

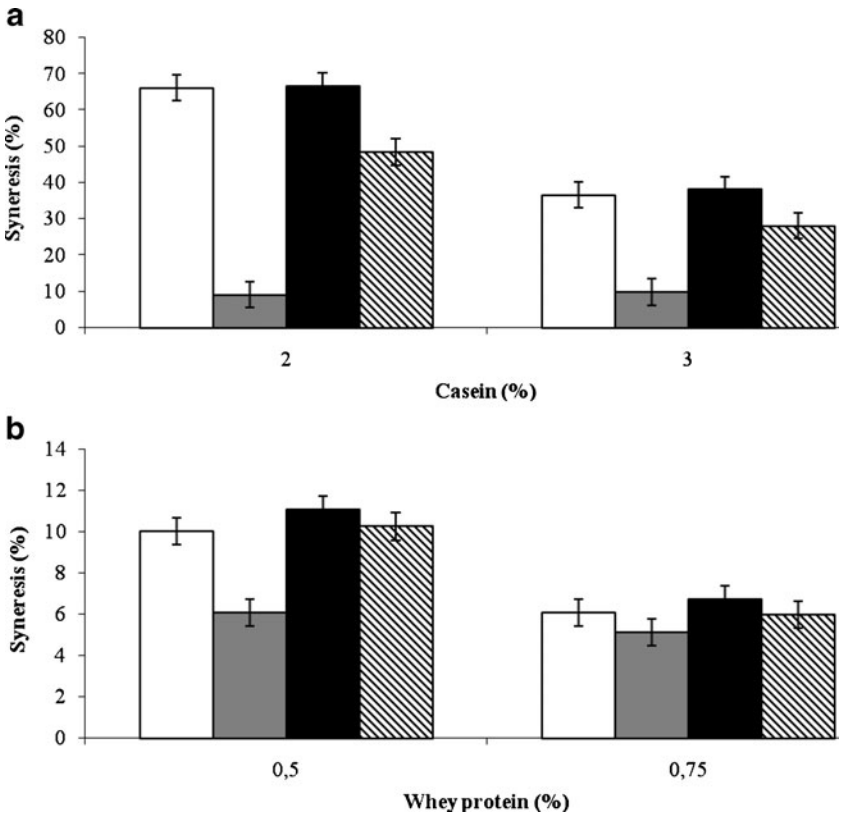

Fig. 1 Syneresis (210×*g*, 4 °C, 20 min) of the dairy model systems at 2% or 3% caseins (**a**) or with the addition of 0.5% or 0.75% whey proteins (**b**) and fermented with HC15/210R (control; *white bar*), HC15/291 (*gray bar*), HC15/702074 (*black bar*), or 2104/210R (*hatched bar*) starter. Data are the mean of three experiments

($P>0.05$) ability to retain water. The 2104/210R starter in the dairy model system at 2% casein concentration had a significantly lower syneresis value than HC15/210R (control) and HC15/702074 starters. At the highest casein concentration (3%), the ability of the starters to limit syneresis in the dairy model systems was significantly higher for all starters except HC15/291.

3.2 Effect of whey protein concentration

3.2.1 Composition

To study the impact of whey protein addition in the dairy model systems, a casein concentration of 2% was chosen as it was the lowest casein level that allowed gel formation. At 3% caseins, casein gels were stronger and, consequently, the effect of EPS was masked. The composition of the dairy model systems at 2% caseins with 0.5% and 0.75% whey protein was not significantly different for total solids content (9.76±0.01), casein content (2.01±0.03), or ash content (0.71±0.01). The whey protein levels were 0.5% and 0.75%, as targeted.

3.2.2 Bacterial growth and exopolysaccharide production

The initial population and streptococci-to-lactobacilli ratio for all starters were controlled at $2.2\pm0.06\times10^7$ CFU·mL^{-1} and 48±1, respectively, with the exception of the 2104/210R starter, for which the ratio was slightly lower (45±1). The initial pH value of all blends was 6.27±0.018. Fermentation was stopped when the pH reached 4.60±0.05 and the production of lactic acid was 0.38±0.01% for all dairy model systems. At the end of fermentation, streptococci and lactobacilli populations were log 8.48±0.04 CFU·mL^{-1} and log 7.93±0.04 CFU·mL^{-1}, respectively. At all whey protein concentrations, the fermentation time was significantly longer only for the dairy model systems inoculated with the HC15/702074 starter (Table 4). EPS production was similar (51±6 mg·L^{-1}) for all the systems.

3.2.3 Rheological and physical properties

The addition of whey proteins yielded stronger gels, and the rheological and physical properties can be compared (Table 4 and Fig. 1b). The pH_{gel} and T_{gel} were significantly affected by the starter used: 2104/210R had the lowest T_{gel}, while HC15/702074 had the longest T_{gel} and the lowest pH_{gel} (Table 4). The T_{gel} and pH_{gel} were significantly affected by whey protein concentration (Table 4). Adding 0.75% whey proteins as compared to 0.5% led to significantly faster T_{gel} and higher pH_{gel}. Starter was the main factor that significantly influenced G' values at both temperatures, that is, after gel formation and after the cooling step (Table 4). The dairy model system fermented with the 2104/210R starter had a higher G' at both temperatures. Apparent viscosities were significantly affected by starter irrespective of whey protein concentration (Table 4). The dairy model systems fermented with the HC15/291 and 2104/210R starters had higher viscosity than those fermented with the HC15/210R (control) and HC15/702074 starters at both whey protein concentrations.

Table 4 Fermentation time, gelation time (T_{gel}), pH of gelation (pH_{gel}), elastic modulus (G') before (42 °C) and after (4 °C) the cooling step, and apparent viscosity (10 and 100 s^{-1}) for dairy model systems at 2% caseins with the addition of 0.5% or 0.75% whey proteins and fermented with different starters

Item	Whey protein addition								SEM
	0.5%				0.75%				
	Control	HC15/291	HC15/702074	2104/210R	Control	HC15/291	HC15/702074	2104/210R	
Time (min)	190^{bcd}	197^{bc}	233^{a}	160^{d}	197^{bc}	183^{cd}	213^{ab}	177^{d}	9.29
T_{gel} (min)	157^{b}	140^{b}	187^{a}	114^{c}	132^{c}	124^{c}	165^{a}	120^{c}	8.20
pH_{gel}	4.91^{bc}	5.08^{abc}	4.79^{c}	5.11^{ab}	5.09^{ab}	5.24^{a}	5.23^{a}	5.18^{a}	0.08
G' (Pa) at 42 °C	6^{c}	18^{abc}	5^{c}	26^{a}	13^{bc}	19^{abc}	12^{bc}	28^{a}	5.51
G' (Pa) at 4 °C	31^{c}	109^{abc}	39^{c}	151^{a}	67^{bc}	100^{abc}	80^{bc}	178^{a}	30.5
Viscosity at 10 s^{-1} (Pa)	0.154^{c}	1.89^{ab}	0.163^{c}	1.05^{b}	0.262^{c}	2.88^{a}	0.184^{c}	0.714^{b}	0.39
Viscosity at 100 s^{-1} (Pa)	0.024^{c}	0.190^{ab}	0.027^{c}	0.113^{b}	0.055^{c}	0.241^{a}	0.031^{c}	0.083^{b}	0.03

The control was the HC15/201R starter. Values in the same row followed by the same letter are not significantly different ($P<0.05$). Data are the mean of three experiments

SEM standard error of the mean

The ability of the starters to limit syneresis in the dairy model systems with different whey protein concentrations was significantly influenced by the interaction between starter and whey protein concentration (Fig. 1b). Adding whey proteins to the dairy model system led to a significant decrease (by approximately fivefold) of syneresis for all starters except HC15/291, for which the values remained unchanged (Fig. 1a, b). At 0.5% and 0.75% whey proteins, syneresis values in the dairy model systems were not significantly different among the HC15/210R (control), 2104/210R, and HC15/702074 starters (Fig. 1b). The HC15/291 starter had the highest ($P<0.05$) ability to retain water in the dairy model system at 0.5% whey protein. At the highest whey protein concentration (0.75%), the ability of the starters to limit syneresis in the dairy model systems was significantly higher for all starters except HC15/291. For that starter, the syneresis value was similar at 0.5% and 0.75% whey proteins.

3.3 Microstructure

The fermented dairy model systems at 2% caseins showed different casein aggregates depending on the starter (Fig. 2a–d). The dairy model system fermented with the HC15/210R (control) starter led to large and interconnected casein aggregates. The aggregates were more loosely connected than in usual yoghurt (Tamine and Robinson 1999) because of the low casein content. Smaller dense casein aggregates were observed with the HC15/291, HC15/702074, and 2104/210R starters. Furthermore, the casein aggregates of the dairy model system fermented with the HC15/702074 starter were very small compared to those obtained with the HC15/291 and 2104/210R starters. Adding 0.75% whey

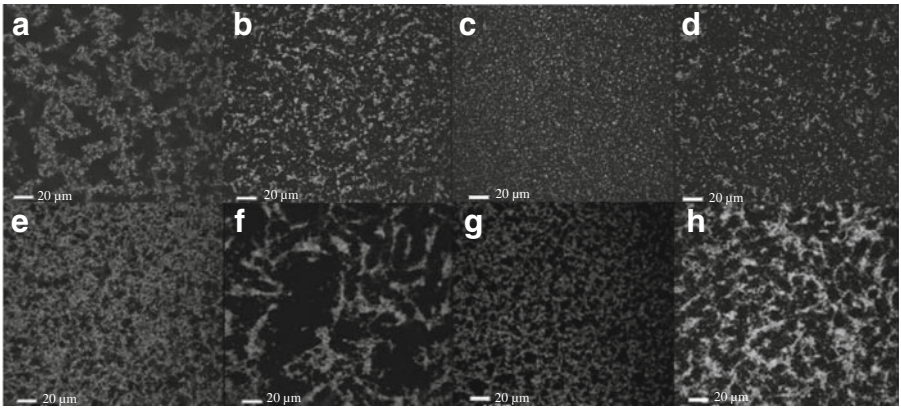

Fig. 2 Confocal scanning laser microscopy observations of the dairy model systems at 2% caseins without (**a**–**d**) or with (**e**–**h**) the addition of 0.75% whey proteins and fermented at pH 4.6 with HC15/210R (control; **a**, **e**), HC15/291 (**b**, **f**), HC15/702074 (**c**, **g**), or 2104/210R (**d**, **h**) starter

proteins to the dairy model system at 2% caseins led to the formation of a different casein network as compared to those without whey protein (Fig. 2). A highly interconnected casein network was observed with the HC15/210R (control) and HC15/702074 starters. When the HC15/291 and 2104/210R starters were used, the casein strands were denser but with a more open structure, and the darker, protein-depleted zones were wider.

4 Discussion

The lowest casein concentration that allowed gel formation and the measurement of rheological and physical properties in the dairy model system was 2% caseins when whey proteins were added. Higher viscosity was measured with EPS produced by the HC15/291 and 2104/210R starters, as previously observed in fermented milk with the single strains 291 and 2104 (Gentès et al. 2011). The ability of a polysaccharide to influence viscosity is a function of its total hydrated volume in solution (Whistler and BeMiller 1997). Molecular weight, degree of branching, and backbone flexibility influence the conformation of polysaccharides in solution (Whistler and BeMiller 1997). EPS produced by the 2104 and 291 strains had similar reported molecular weights. Strain 2104 produces linear, stiff, and anionic EPS, while strain 291 produces neutral, stiff, and branched EPS. It is known that, for the same molecular weight, linear polysaccharides occupy a larger volume in solution and thus produce higher viscosities (Whistler and BeMiller 1997). The stiffness of polysaccharides also contributes to viscosity, as polysaccharides able to adopt an extended conformation can occupy a larger volume in solution (Whistler and BeMiller 1997). The stiffness of the EPS backbone of strain 291 may have contributed to reach higher viscosity values.

The presence of EPS can cause phase separation in the casein network owing to depletion flocculation (Girard and Shaffer-Lequart 2007; Hassan et al. 2003; Tuinier

et al. 1999, 2000). In the present study, denser casein aggregates with large protein-depleted zones were linked to higher viscosity values. The structural characteristics and the larger radius of volume of these EPS in association with their higher viscosity and segregative conditions may have increased the compactness of the casein aggregates. Similar microstructures were also observed when EPS-producing strains were used to ferment milk (Girard and Schaffer-Lequart 2007; Hassan et al. 2003). However, molecular weight is not the only factor involved, given that no effect on viscosity was observed for the EPS produced by strain 702074 even though these EPS have the highest reported molecular weight value. The microstructure also showed a more compact structure. This could be explained by the high degree of branching and the flexibility of the backbone allowing compactness of the polysaccharide, with the result that the EPS occupy a smaller volume in solution. The microstructure of the system with strain 702074 was more compact and similar to the one fermented with the HC15/210R (control) starter. This type of microstructure was a typical casein network formed by chemical acidification of skim milk at 10% dry matter (Turgeon and Plesca 2009).

The whey protein concentrations under study did not significantly influence the viscoelastic properties (G') and viscosity values, probably because of the small difference between the two concentrations. Among the starters, higher G' was measured with the anionic EPS from the 2104/210R starter. Turgeon and Plesca (2009) have also observed higher G' values with the addition of the anionic EPS from *Lactobacillus rhamnosus* in skim milk. This effect was attributed to the associative phase separation favored by electrostatic interactions between EPS and caseins that reinforce the casein network. For the 2104/210R starter, the casein network seemed to be more interconnected as compared to the network achieved with the HC15/291 starter. An absorbing polysaccharide such as an anionic EPS may interact with positive charges on caseins through electrostatic interactions (Doublier et al. 2000; Tolstoguzov 1991; Turgeon et al. 2003) and thus strengthen the casein network, as observed in this study. Girard and Schaffer-Lequart (2007) have also observed a denser casein network with larger pores irrespective of the flexibility, degree of branching, and charge of EPS. However, direct comparison is not possible because the incubation temperatures (25 and 40 °C) and final pH (5.2–4.6) were different among strains in their study. These factors are well-known to influence the casein network (Tamine and Robinson 1999).

The ability to retain water, estimated by syneresis, was also influenced by the type of EPS used. The addition of caseins or whey proteins increased the water-retention ability but EPS functionalities remained the main factor. With respect to rheological properties, the flexible and highly branched EPS from the HC15/702074 starter did not contribute to the limitation of syneresis when compared to the HC15/210R control starter. Syneresis was lower when the HC15/291 starter was used as compared to the 2104/210R starter, which also had a positive impact on the limitation of syneresis. Consequently, neutral EPS contributes more to viscosity but has no effect on gel stiffness (G'), unlike anionic EPS, with which electrostatic interactions with caseins may occur as observed in this study. These findings underlining the importance of the charge of the EPS to modulate the rheological and physical properties are in accordance to general concepts of protein–polysaccharide interactions that such opposite charge proteins and polysaccharides form complexes leading to associative behavior and that

neutral polysaccharide do not absorb onto proteins leading to thermodynamic incompatibility (Doublier et al. 2000; Tolstoguzov 1991; Turgeon et al. 2003).

According to the literature, very few reports have studied the interactions between EPS and caseins in the absence of whey proteins in conditions simulating yoghurt manufacturing. In the system with casein only, the resulting network showed casein aggregates instead of a highly interlinked network as observed in the stronger gels obtained with whey proteins. This type of interconnected microstructure was also observed when whey proteins were added to a solution of 3.5% caseins (Famelart et al. 2004). The main effect of the lack of whey proteins in the systems at 3% caseins was a lower pH_{gel} at values close to pH 4.6 (isoelectric point of casein), as reported in the literature for unheated milk or for media with 3.5% caseins (Famelart et al. 2004; Lucey et al. 1998). The shift in the pH_{gel} to a higher pH when whey proteins were added is due to the higher isoelectric point of whey protein (pH 5.2) and the interactions between whey proteins and κ-casein (Famelart et al. 2004).

The behavior of EPS to modulate syneresis in the system with casein alone was similar to the system with whey proteins. The level of whey proteins added contributed to reduce syneresis but did not affect the contribution of EPS to water retention. This difference in EPS functionalities seem to be more related to the intrinsic ability of EPS to contribute to water retention due to specific structural characteristics. The results correspond to previous results obtained with individual strains (Gentès et al. 2011) and could be specific to structural EPS characteristics. However, in rich protein system (3% caseins), no difference can be observed among starters, although significant amounts of EPS were quantified for all starters. Consequently, a more dilute system (2% caseins) was more efficient to differentiate specific EPS functionalities.

In systems with 0% and 1% caseins even with all starters, no gel and no casein structural organization could be detected at pH 4.6. However, such systems did not influence the EPS functionalities of HC15/291 and 2104/210R starters. These starters had the ability to enhance viscosity values even without the presence of casein (conditions at 0% caseins). The water-retention ability of the EPS produced by strain 291 was also unique as compared to the other EPS because the presence of milk proteins (caseins and/or whey proteins) in the media is not required for a significant effect on the limitation of syneresis. This characteristic is interesting for application to a weak gel system (such as a matrix with low protein content) that is rich in water, in which controlling the limitation of syneresis is important.

5 Conclusions

In this study, a simplified dairy model system was used to study the effect of several thermophilic EPS starters in conditions mimicking yoghurt acidification rate and with various milk protein levels. This dairy model has allowed showing the sequential impact of the addition of casein and whey proteins and the use of bacterial EPS having different structural characteristics on the microstructure and rheological and physical properties. The addition of caseins and whey proteins into the dairy model system increased the rheological and physical property values, but the EPS functionalities remained. The EPS structural characteristics of charge, linearity, and stiffness

impacted the rheological and physical properties. The presence of linear, stiff, and anionic EPS contributed to gel stiffness (elastic modulus), possibly through associative behavior due to electrostatic interactions with caseins, as well as to viscosity. Neutral and stiff EPS contributed to viscosity because of their ability to retain water and increase the bulk volume but not to the gel stiffness probably due to the thermodynamic incompatibility. Lastly, this study made it possible to gain a deeper understanding of the EPS functionalities in a rich protein system. It might also be interesting to study other EPS-producing strains with other structural characteristics in the dairy model system to confirm these findings.

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