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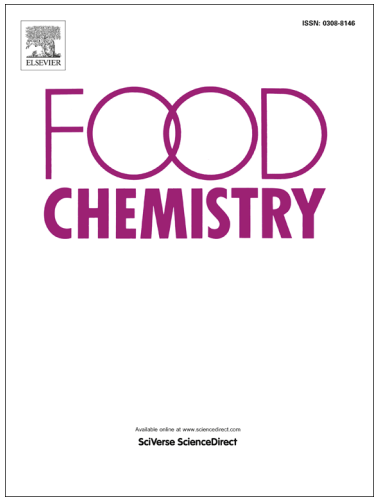

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Performances of different protocols for exocellular polysaccharides extraction from milk acid gels: application to yogurt

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Abstract

Dextran or xanthan were used as model exocellular polysaccharides (EPS) to compare the extraction efficiency of EPS from skim milk acid gels using three different protocols. Extraction yields, residual protein concentrations and the macromolecular properties of extracted EPS were determined. For both model EPS, the highest extraction yield (~ 80 %) was obtained when samples were heated in acidic conditions at the first step of extraction (Protocol 1). Protocols that contained steps of acid/ethanol precipitation without heating (Protocols 2 and 3) show lower extraction yields (~ 55 %) but allow a better preservation of the EPS macromolecular properties. Changing the pH of acid gels up to 7 before extraction (Protocol 3) improves the extraction yield of anionic EPS without effect on the macromolecular properties of EPS. Protocol 1 was then applied for the quantification of EPS produced during the yogurt fermentation, while Protocol 3 was dedicated to their macromolecular characterization.

Highlights:

- Dextran or xanthan were used to compare 3 extraction protocols of EPS from acid gel
- Extraction yields and molecular properties of EPS after extraction were determined
- Heat treatment in acid condition allows a high extraction yield but degrades EPS
- Soft extraction is needed to avoid EPS degradation
- Protocols were used to quantify or characterize EPS during milk lactic fermentation

Keyword: Exocellular polysaccharides (EPS), extraction protocols, EPS production, yogurt, milk acid gelation, HPSEC-MALS

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1. Introduction

Yogurt is one of the most popular milk fermented product in the world. The yogurt texture depends on various factors, especially milk compositions, manufacturing processes and starter cultures (Lucey & Singh, 1997; Tamime, 2006). The lactic acid bacteria (LAB) influence the gel formation and the final texture by their acidification rate and/or the production of exocellular polysaccharides (EPS) (Lucey & Singh, 1997). EPS are either associated with the cell surface in the form of capsules or excreted into the extracellular environment in the form of slime (De Vuyst & Degeest, 1999; Sutherland, 1972). The quantities and the structure of EPS produced in milk depend on (i) the specific strains, (ii) the fermentation conditions, i.e. temperature, incubation time, initial and final pH, and (iii) the medium composition, i.e. carbon source and nitrogen source (Ayala-Hernández, Hassan, Goff, Mira de Orduña, & Corredig, 2008; Cerning, 1990; De Vuyst & Degeest, 1999; Vaningelgem et al., 2004). Due to their ability to bind water and modulate viscosity, EPS have been considered as a potential natural thickening agent in fermented milk (De Vuyst & Degeest, 1999). EPS from LAB increase the viscosity and firmness, improve the texture, reduce susceptibility to syneresis and contribute to the "mouth-feel" of low-fat products (Cerning, 1995; Doco et al., 1990; Folkenberg, Dejmek, Skriver, & Ipsen, 2005; Leroy & De Vuyst, 2004; Ruas-Madiedo, Tuinier, Kanning, & Zoon, 2002). The intrinsic factors which determine the effects of EPS on fermented milks include both their concentration and the EPS structural and physico-chemical properties, i.e. their molar mass, the stiffness of their backbone, the number of side chains and their charge (De Vuyst & Degeest, 1999; Mende, Rohm, & Jaros, 2016). In addition, the evolution of the physico-chemical environment during the yogurt processing, i.e. the decrease of pH and milk protein negative charges, the increase of EPS concentration, affects the EPS functionality in

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yogurt and changes the protein-protein and protein-EPS interaction (Mende et al., 2016). Only few studies have been focused on the role of EPS in the gel formation and gel structure (Ayala-Hernández et al., 2008; Girard & Schaffer-Lequart, 2007; Mende, Mentner, Thomas, Rohm, & Jaros, 2012b). It is therefore of utmost importance to conduct researches on the EPS production and characterization "*in situ*" in order to determine more accurately the role of EPS in yogurts. For this purpose, their extraction and quantification were major issues in EPS researches. The various steps required to obtain EPS must be carefully adjusted for each strain, type of EPS, fermentation medium and the purpose of isolation (Mende et al., 2016). Many EPS isolation procedures were proposed and reviewed in various papers, e.g. Mende et al. (2016) and Ruas-Madiedo & de los Reyes-Gavilán (2005). Trichloracetic acid (TCA) and/or proteases are usually used for protein removal, organic solvents for EPS precipitation and dialysis, filtration or size exclusions chromatography (SEC) for removing minerals and monomeric/dimeric sugars (Ayala-Hernández et al., 2008; De Vuyst, F. Vanderveken, S. Van de Ven, & B. Degeest, 1998; Enikeev, 2012; Goh, Haisman, Archer, & Singh, 2005). Challenges during the EPS extraction and quantification are the low extraction yield due to the co-precipitation with milk proteins or residual protein (Cerning, 1990; Mende et al., 2016). To obtain highly purified EPS, the extraction is tedious and time-consuming and requires specific equipments for sugar analyses that are not always available in microbiology laboratories. Furthermore, EPS production by LAB starter culture is low in fermented milk, which ranges from 10 to 170 mg L^{-1} (De Vuyst et al., 1998; Mende et al., 2012b; Mende et al., 2016). This makes the analytical approach and the EPS isolation from complex fermentation medium such as fermented milk particularly difficult. Several studies were carried out on the optimization of EPS extraction protocols from milk-based products (Enikeev, 2012; Goh et al., 2005).

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Recently, Miao (2015) compared different protocols of EPS extraction from fermented milk with TCA/ethanol precipitations and/or proteolysis steps. The author suggested that proteolysis treatment before TCA/ethanol precipitation leads to a higher recovery of EPS but with a poor purity (high content in proteins) whereas protocols with TCA/ethanol at the first step allow to obtain less EPS with a higher degree of purity. However, when the author worked on EPS produced in fermented milk, the real extraction yields of each protocol were not known. The aim of this research is to compare three different protocols of EPS extraction from skim milk acid gel in terms of i) extraction yields, ii) residual protein concentration, and iii) preservation of macromolecular properties of extracted EPS. For this purpose, two model EPS were used: the dextran – a low molar mass, neutral homopolysaccharide and the xanthan gum – a high molar mass, anionic heteropolysaccharide (García-Ochoa, Santos, Casas, & Gómez, 2000; Naessens, Cerdobbel, Soetaert, & Vandamme, 2005). The model EPS milk acidification was carried out chemically with glucono-δ-lactone (GDL). The influence of milk composition i.e. the enrichment in whey protein on EPS extraction was also investigated. The most effective extraction protocol was then applied on yogurt produced by a commercial starter culture to monitor the production of EPS and their macromolecular properties.

2. Materials and methods

2.1. Solvents and reagents

Low-heat skim milk powder (Spray 0) and whey protein concentrate powder (from the permeate of microfiltred milk - Promilk 802 FB) were purchased from Ingredia (France). A high EPS-producing starter culture (YF-L901) was provided by Chr. Hansen A/S, Arpajon (France). Lactose monohydrate and TCA were from GPR

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Rectapur, VWR chemicals (Belgium). GDL, D(+)glucose monohydrate, sodium hydroxide (NaOH) 1N, n-hexane, phosphorus pentoxyde (P_2O_5), trifluoroacetic acid (13.05 N), sodium borohydride, ethyl acetate, perchloric acid, acetic anhydride, ammonia solution (13.35 N), acetone, glacial acetic acid, chloroform and 1-methylimidazole ($C_4H_6N_2$) were from Merck (Germany). Dextran (D-1537, average M_w ≈ 76 kDa, from *Leuconostoc mesenteroides*), xanthan gum (G-1253, from *Xanthomonas campestris*), phenol, lithium nitrate ($LiNO_3$), sodium azide (NaN_3), β-D-allose, myo-inositol and acetyl chloride were from Sigma (USA). Bicinchoninic acid (BCA) protein assay was from G-Biosciences (USA). Ethanol (95% v/v) was from TechniSolv, VWR chemicals (France). Concentrated sulphuric acid (95% w/w) was from Normapur, VWR chemicals (France).

2.2. Milk base preparation

Two different milk formulas were used: skim milk (SM) or skim milk supplemented with whey proteins (SMWP). SM is reconstituted from low-heat skim milk powder of 4% (w/w) of total protein. SMWP is the reconstituted skim milk supplemented in whey proteins and lactose. Each milk formulation presents the same content of dry matter (11.8% w/w), total proteins (4% w/w), lactose (7.65% w/w) but different ratio of caseins/whey proteins: 80/20 and 54/46, for SM and SMWP, respectively.

The model EPS were dextran (from *Leuconostoc mesenteroides*) and xanthan gum (from *Xanthomonas campestris*). After the dissolution of the milk powders by stirring during 1h at room temperature, the model EPS were incorporated at a final concentration of 100 mg kg^{-1} of milk. Reconstituted milks with or without model EPS were kept at 4 °C for 12h, then pasteurised at 95°C during 6 min. After the heating step, the milk was held at 43 °C before the chemical acidification or fermentation.

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2.3. Milk acid gel manufacture

GDL was used as the chemical acidifying agent to determine the efficiency of the EPS extraction at acid pH. 15 g of GDL was added in 1 kg of milk with or without model EPS and the acidification was carried out at 43 °C. The pH was monitored during the acidification using a Consort C833 pH meter (Consort, Turnhout, Belgium). When the pH reached 4.65, the milk acid gels were cooled down and stored at -20 °C before the EPS extraction.

2.4. Set-type yogurt fermentation

The fermentation was performed at 43 °C by inoculating SM or SMWP with 0.02% (w/w) of high EPS-producing starter culture (YF-L901) until reaching a final pH equal to 4.65 (Buldo et al., 2016). The gel formation was monitored using an Anton-Paar Physica MCR 300 rheometer (Anton Paar, St Albans, UK) equipped with a concentric cylinder geometry (CC27). The rheometer was operated in continuous oscillation mode at the frequency of 1 Hz and 1% strain. The gel point was defined as the moment when the storage modulus (G') exceeds the loss modulus (G'') (Winter, 1987). In parallel, the pH was recorded during the acidification kinetic to determine the pH at the gel point. The kinetic of acidification was measured in triplicate.

During the fermentation, the samples were collected at different pH values (i.e. 6.2; 5.5; 5.0; 4.65), cooled and kept at -20 °C before analysis. The milk at the start point was used as negative control and subtracted from all data.

2.5. EPS extraction protocols

EPS extractions were carried out using three different protocols described in Fig. 1.

Protocol 1: This is the protocol described by Gentès et al. (2011) with some modifications. This protocol is selected for its simplicity, its rapidity and its low

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requested quantity of sample. For protein removal, 10 g of thawed milk sample were mixed with TCA (at a final concentration of 20% w/v) and stirred for 30 min at room temperature with magnetic stirring at a high speed (700 rpm). Then the mixture was heated in boiling water for 10 min and cooled down to the room temperature. The samples were then centrifuged for 30 min at 10000 ×*g*. The supernatants were collected and the pellets were washed with TCA at 20% (w/v) by stirring at 700 rpm for 30 min and was then centrifuged as described above. The supernatants were pooled and 10 g of them were placed in a 6000 - 8000 g mol^{-1} MWCO membrane (Spectra/Por) and dialyzed against 1 L of distilled water for 72h at 4 °C with 3 water changes per day. For molar mass analysis, extracted model EPS were purified and concentrated by TCA/ethanol precipitations in order to decrease the protein content.

Protocol 2: This is the protocol described by Ayala-Hernández et al. (2008) with some modifications. This protocol is also easy to be implemented, practical but requires more time, more sample and chemicals. It consists in two steps with a protein precipitation by TCA followed by EPS precipitation obtained by ethanol addition. More precisely, TCA was added to 100 g of thawed milk samples at the final concentration of 10% (w/v). The mixture was stirred for 30 min at room temperature with magnetic stirring at a high speed (700 rpm). Samples were then centrifuged for 30 min at 10000 ×*g*. The supernatant was collected and mixed with 2 volumes of chilled ethanol 96% (v/v) in order to precipitate the EPS. After 12h at 4 °C, the mixture was centrifuged for 1h at 10000 ×*g* and the pellet was dissolved in TCA of 20% (w/v) by stirring at 700 rpm for 30 min to remove residual proteins. The sample was then centrifuged for 30 min at 10000 ×*g* and the EPS precipitation was repeated as described above. After the centrifugation, the pellet was dissolved in 10 mL of ultrapure water, placed in a 6000 -

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8000 g mol^{-1} MWCO membrane (Spectra/Por) and dialyzed against distilled water (1L) for 48h at 4 °C, with 3 water changes per day.

Protocol 3: This protocol was developed from Protocol 2 by adding a pH change step. The pH of the 100 g of thawed milk acid gel was adjusted to 7 with NaOH (1 N). EPS extraction was then carried out as described in Protocol 2.

Dialysis allows the elimination of a maximum of lactose and is necessary to improve the purity of the extracted EPS. In preliminary experiments (not presented herein), the total duration and the number of water changes in the dialysis step were determined to reduce as much as possible the amount of residual lactose. Thereby, for all protocols, the residual lactose was inferior to 10 mg L^{-1} which allowed the quantification of small EPS concentration.

Each extraction was performed in triplicate.

2.6. Quantification of EPS and total proteins

The total sugar content was determined by the phenol-sulfuric acid method (PSA method) as described by Dubois et al. (1956) using glucose as the standard (range of concentration from 0 to 200 mg L^{-1}). In hot acidic medium, the carbohydrates are dehydrated to furfural derivatives which form a colored product with phenol. 200 µL of EPS extracted solution was mixed with 200 µL of 5% w/v aqueous solution of phenol. Subsequently, 1 mL of concentrated sulphuric acid was added rapidly to the mixture and then vortexed. The sample was incubated at room temperature in 30 min before the measurement of absorbance at 490 nm. The PSA method detects virtually all classes of carbohydrates, including mono-, di-, oligo- and polysaccharides. For this reason, the residual milk sugars (Q_R) were evaluated on extracted samples from milks without model EPS and subtracted from all data. To evaluate the extraction yield, the quantity of

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added model EPS (Q_T) (dextran or xanthan gum solutions at 100 mg kg^{-1}) were also determined and expressed in mg glucose per kg milk acid gel. The quantity of extracted EPS was expressed in equivalent of mg of glucose in order to facilitate the comparison to others studies on this topic.

The EPS extraction yield (EY) was determined by Eq. (1):

$$EY(\%) = (Q_E - Q_R)/Q_T \times 100 \quad (1)$$

in which Q_E was the total amount of EPS extracted, Q_R, the residual milk sugars and Q_T was the added amount of model EPS. Each measurement was performed in triplicate.

The protein content of dialyzed samples was quantified by the BCA protein assay with bovine serum albumin as standard (range of concentration of 0 to 2000 mg L^{-1}). The principle of the reaction is the formation of a Cu^{2+}-protein complex under alkaline conditions, followed by reduction of the Cu^{2+} to Cu^{+}. BCA forms a purple-blue complex with Cu^{+} in alkaline environments, thus providing a basis to monitor the reduction of alkaline Cu^{2+} by proteins at absorbance maximum 562 nm. 50 µL of extracted EPS solution was mixed with 1 mL of working solution then incubated at room temperature for 2 hours, unexposed to the light, before the measurement of absorbance at 562 nm. The protein content was expressed in mg L^{-1} of dialysed extract. Each measurement was performed in triplicate.

2.7. EPS macromolecular properties

The macromolecular properties of EPS were investigated using a high performance size exclusion chromatography coupled with multi-angle laser light scattering detector (HPSEC-MALS) to determine the molar mass distribution and the intrinsic viscosity. HPSEC-MALS experiments were performed using a Shimadzu HPLC system (Shimadzu, Kyoto, Japan) coupled on line to a Dawn Heleos II multi-angle laser light

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scattering (Wyatt Technology Corp., Santa Barbara, Ca, USA), a VISCOSTAR II viscosimeter (Wyatt Technology Corp., Santa Barbara, Ca, USA) and an Optilab T-rEX refractometer (Wyatt Technology Corp., Santa Barbara, Ca, USA). The EPS were separated using a Shodex OHPAK SB-G pre-column followed by two or four columns in series. For dextran samples, four columns (Shodex OHPAK SB 806 HQ, OHPAK SB 805 HQ, OHPAK SB 804 HQ and OHPAK SB 803 HQ) were used, but only the first two columns were used for xanthan and bacterial EPS samples. Dextran or xanthan gum solutions were prepared at 1 mg mL^{-1} in ultra-pure water as control samples. The concentration of extracted EPS (including xanthan and dextran) from skim milk acid gels ranged from 100 to 250 mg L^{-1}. The extracted EPS and the control samples were centrifuged at 15300 ×*g* for 15 min to remove insoluble materials, and then 100 µL of the supernatant were injected. The macromolecules were eluted with 0.1 M $LiNO_3$ solution containing 0.02% (w/w) NaN_3 at a constant flow rate of 1 mL min^{-1} and 30 °C. The weight-average molar mass (M_w), the intrinsic viscosity ([η]), the radius of gyration (R_g) and the hydrodynamic radius (R_h) were calculated using the software ASTRA 6.1.2 (Wyatt Technologies, Santa Barbara, CA). The M_w and R_g were calculated using the Zimm's model (1st order) for dextran and the Berry's model (2nd order) for xanthan and bacterial EPS. The data were analyzed using a refractive index increment (dn/dc) of 0.145 ml g^{-1} according to the literature (Lambo-Fodje et al., 2007). Each sample was analysed in duplicate.

2.8. Neutral sugar monomer analysis of EPS

Neutral sugar monomers of bacterial extracted EPS were determined by the alditol acetates method (Harris, Henry, Blakeney, & Stone, 1984). Briefly, dialyzed extracted EPS was lyophilized and then dried at 50°C for 12h in vacuum condition with P_2O_5. After different steps of hydrolysis, reduction and acetylation, neutral sugar monomers

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were released and transformed to their alditol acetate derivatives. Then, they were separated and quantified by gas chromatography (GC) analysis using a fused silica DB-225 capillary column (30 m × 0.25 mm i.d.; film thickness of 0.25 µm; Reference: 122-2232; J&W Scientific) with hydrogen as carrier gas (Shimadzu GC-2010 Plus Gas Chromatograph, Kyoto, Japan). The alditol acetates were identified from their retention times by comparison with standard monosaccharides. Inositol was used as internal standard. Each sample was analyzed in duplicated.

3. Results and discussions

3.1. Comparison of protocols of EPS extraction

Two model EPS were used to compare the extraction efficiency of the three protocols selected: i) the dextran, an uncharged homopolysaccharide and ii) the xanthan, a charged heteropolysaccharide. They were introduced in milk before chemical acidification by GDL, at a concentration (100 mg kg^{-1}) similar to that usually reported for EPS produced in yogurt by LAB. Two formulations of milk were also used, i.e. skim milk (SM) or skim milk enriched in whey proteins (SMWP), to determine the influence of the milk protein composition on the efficiency of the three extraction protocols.

3.1.1. Extraction yields and purity of model EPS

EPS extraction yields and residual protein contents obtained using the three different protocols are presented on Fig. 2 for dextran and Fig. 3 for xanthan, respectively. Protocol 1 leads to the highest extraction yield (~ 80 %) for both model EPS, regardless of the milk protein composition (Fig. 2). Protocols 2 and 3, which do not include a heat treatment step, exhibit extraction yields for dextran or xanthan that are significantly lower (< 60%) than those observed for Protocol 1 (Fig. 2 and 3). These findings are in great agreement with the results reported by Rimada et Abraham (2003) who compared

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different protocols of extraction of LAB EPS from kefir grains. The authors concluded that heat treatment of the samples (without TCA) as the first step of isolation procedure was critical for complete recovery of the EPS. They suggested that heat treatment could promote the separation and the dissolution of EPS attached to cell wall or to milk proteins and/or could inactivate EPS-degrading enzymes. The enrichment of milk in whey proteins has little or no influence on the extraction yield of model EPS for a given protocol (Fig. 2). Furthermore, Protocol 2 results in a significantly lower extraction yields for xanthan than for dextran. The difference may be due to the electrostatic attraction between xanthan (negatively charged) and milk proteins (partially positively charged) at pH of acid milk gel (4.65) (Sanchez, Zuniga-Lopez, Schmitt, Despond, & Hardy, 2000). This hypothesis is confirmed by the results obtained with Protocol 3. The extraction yields of dextran using Protocols 2 and 3 were similar whereas that of xanthan was improved with Protocol 3. Indeed, this protocol was developed from Protocol 2 with the addition at the first step of an increase of the milk gel pH from 4.65 to 7. At this pH, milk proteins and xanthan are negatively charged and can no longer interact with each other. Thus, the extraction yields of xanthan obtained with Protocol 3 increase to 55 % and reach the same level as for dextran (Fig. 2A and 3A). The lower extraction yields observed for Protocol 3 as compared to Protocol 1 could also be due to the co-precipitation of EPS with proteins during the TCA treatment. Furthermore, it is worth noting that xanthan and dextran have different structural and physico-chemical properties but their extraction yields with Protocol 3 was similar. This result suggests that the extracted EPS by this protocol would be representative for the total produced EPS.

Regarding the purity of the extracts, the residual protein contents measured in the different extracts were 61 ± 3, 15 ± 4 or 3 ± 6 mg L^{-1} of extract for Protocol 1, 2 or 3

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respectively. The higher purity observed for Protocol 2 or 3 could be due to the additional protein removal and EPS precipitation steps as compared to Protocol 1. Moreover, it can be suggested that the pH change at the first step of Protocol 3 could improve the separation of EPS from the protein network, resulting in a very low concentration in residual proteins. No significant difference was observed between dextran or xanthan extracts from SM or SMWP (data not shown).

3.1.2. Macromolecular properties of extracted EPS

The molar mass distributions of extracted EPS are presented and compared to native EPS on Fig. 2B for dextran and Fig. 3B for xanthan, respectively. During the elution of xanthan, a rise of the molar mass was observed between 15 and 17 minutes. This might be due to an abnormal elution of xanthan which was delayed in the column. The molar mass distributions of dextran or xanthan extracted by Protocol 1 are strongly modified with a shift towards small molar masses, as compared to native EPS, which indicates a strong degradation of EPS during the extraction procedure. The mean molar mass of dextran or xanthan decreased from 7.0×10^4 g mol^{-1} or 3.6×10^6 g mol^{-1} for the native molecules to 2.5×10^4 g mol^{-1} or 3.2×10^5 g mol^{-1} for the extracted EPS by Protocol 1, respectively. The intrinsic viscosity of dextran and xanthan was also strongly affected by Protocol 1, since its decrease from ~25 to ~14 mL g^{-1} and from ~1800 to ~350 mL g^{-1}, respectively, supporting an important molecular degradation. This EPS degradation is probably due to the acid hydrolysis in the first isolation step of Protocol 1 that consists in heating the sample up to 100°C in drastic acid conditions. On the contrary, Protocol 2 or 3 seem to better preserve the macromolecular properties of the extracted EPS. Indeed, extracted dextran presents the same M_w distribution as native molecule (Fig. 2B), while the M_w distributions of xanthan show only few modifications after extraction by Protocol 2 or 3 (Fig. 3B). In the same way, the intrinsic viscosity of both model EPS

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was not changed after extraction by Protocol 2 or 3, indicating a good preservation of molecular properties. The composition of milk (SM versus SMWP) did not have a significant influence on the M_w distribution model EPS extracted by a given protocol (data not shown).

All these results indicate that the extraction procedure has a great influence on the purity and on the macromolecular integrity of extracted EPS. Therefore, the choice of the EPS extraction protocol should consider the final goal of the analysis (quantification, molecular characterisation, etc.). Only few studies have investigated the influence of extraction protocols from dairy products on the macromolecular integrity of extracted EPS (Miao, 2015; Pop et al., 2016). Recently, Miao (2015) compared four purification protocols of EPS (which are different from those presented in the present study) from milk fermented by LAB and concluded that the sugar composition and their ratio as well as the molar mass distribution of extracted EPS may be influenced by EPS isolation procedures. However, as the native structure of extracted EPS was not known, Miao (2015) could only compare the relative influence of each protocol against the others. Pop et al. (2016) observed different molar mass of kefiran polymers under different conditions. The kefirans were extracted from kefir grains in hot water at four temperatures (70°C, 80°C, 90°C, 100°C) using different extraction time. Subsequently, the mixture was cooled and centrifuged to remove microbial cells and proteins. The high purity of the kefiran solutions was obtained by twice purification steps involving freezing, slow thawing and centrifugation. With low temperature extraction (70°C, 80°C and 90°C), the molar mass ranged from 1.4 - 1.5 × 10^7 Da. The high extraction temperature (100°C) demonstrated a degradation of the polymer structure with molar mass ranging from 0.2 – 0.7 × 10^7 Da. This result shows that heat treatment during EPS

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extraction could increase the extraction yield but also modify EPS macromolecular properties.

Among the three extraction protocols investigated in the present study, Protocol 1 provides high extraction yields of both model EPS but induces a high degradation of these polysaccharides and then should be only adequate for EPS quantification. On the contrary, Protocol 2 leads to lower extraction yields of both model EPS (especially xanthan) but does not induce hydrolysis of these polysaccharides. Protocol 3 presents also a good preservation of EPS structure and a higher extraction yield of xanthan than Protocol 2. Moreover, the same extraction yield for xanthan and dextran was obtained with Protocol 3 suggesting that this protocol provide the same efficiency for the extraction of EPS featuring different macromolecular and physico-chemical properties. Hence, Protocol 3 appeared to be convenient for charged polysaccharides.

It can be considered that the results obtained hereby could be applied to EPS produced by LAB in acidified milks and that makes the Protocol 3 appropriate for macromolecular EPS characterisation. Finally, it should be noted that Protocol 1 requires little milk gel sample (10 g) whereas Protocols 2 and 3 use more milk gel samples (100 g). However, the EPS concentration during the ethanol precipitation steps in Protocol 2 and 3 is convenient for their macromolecular characterization.

3.2. Application to EPS produced by LAB during set-yogurt fermentation

Protocols 1 and 3, which were studied in the previous section, were then used to monitor and characterize the production of EPS during set-type yogurt fermentation. Fermentations were performed at 43 °C using a commercial starter culture YF-L901 which provides ropy yogurts (Buldo et al., 2016). The starter culture contains several *Streptococcus thermophilus* (*S. thermophilus*) and *Lactobacillus delbrueckii* subsp. *bulgaricus* (*L. bulgaricus*) strains. The yogurt texture from this starter culture was

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known to be medium ropy and smooth in skim milk gel (based on the manufacturer information).

3.2.1. Monitoring of EPS production during acidification

The kinetic of acidification and the evolution of rheological parameters of milk during fermentation were monitored simultaneously to determine the characteristics of the gel point. To monitor the production of EPS during the lactic fermentation, milk samples were taken at pH 6.2, 5.5, 5.0 or 4.65 and processed with the Protocol 1 for EPS quantification. Samples from final yogurt were also processed with Protocol 3 to characterize the produced EPS by HPSEC-MALS analysis. The kinetics of acidification and the EPS production during LAB fermentation in SM or SMWP are presented in Fig. 4. The characteristic parameters of the fermentation are shown and compared in Table 1A. When the milk is enriched in whey proteins (SMWP), the fermentation is significantly faster than for classic skim milk (SM) (Fig. 4, Table 1A). As the total amount of proteins is the same in both milks, these findings are due to the reduction of milk buffering capacity when the ratio casein/WP decreased (Puvanenthiran, Williams, & Augustin, 2002). Indeed, the casein micelles contain phosphoserine and histidine residues and their highest buffering capacity is obtained at pH 5-5.5 (Clark, Thompson, & and Rokahr, 1983). In contrast, the whey proteins contain amino acids that give a highest buffering capacity of about pH 3 - 4 (Clark et al., 1983). Furthermore, the gel point occurs at slightly higher pH in SMWP as compared to SM (Table 1A). This phenomenon is well described in the literature and it could be explained by the binding of denatured whey proteins on the casein micelles during heat treatment of milk (pasteurization) and/or by the gelation of heat-induced whey proteins aggregates around their isoelectric point (at 5.2 – 5.3) which is higher than casein ones (pHi = 4.6)

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(Puvanenthiran et al., 2002). These trends being amplified when the milk is enriched in whey proteins.

EPS production started before pH 6.2 and continued until pH 4.65 without slowing down (Fig. 4). It is worth noting that at pH 5.5, a half of EPS was produced in the milk before the gelation point. The presence of EPS could increase the pH of gelation by the depletion interaction between EPS and milk proteins (Mende et al., 2016). After 5 - 6 hours of acidification, the EPS production was up to 68.8 ± 2.4 mg glucose kg^{-1} for SM gel and 67.8 ± 1.6 mg glucose kg^{-1} for SMWP gel. The similar final EPS amount in both milk formulations showed the independence of EPS production to milk composition and time of acidification. Our results were in agreement with those of Mende et al. (2012b) who quantified the EPS content in 7 commercial yogurts and found final EPS concentrations ranged between 48 ± 19 to 168 ± 22 mg glucose kg^{-1}. The EPS production kinetic was strain-dependent and the maximum EPS production were observed at either the beginning or the end of the stationary growth phase (Mende et al., 2016). However, the majority of the studies on the kinetics of EPS production by LAB are carried out in model media and/or in milk but at constant pH which makes difficult the comparison of our results with the literature (Degeest, Vaningelgem, & De Vuyst, 2001; Mende, Krzyzanowski, Weber, Jaros, & Rohm, 2012a; Mende et al., 2012b; Urshev, Dimitrov, Fatchikova, Petrova, & Ishlimova, 2008; Welman & Maddox, 2003). Mende et al. (2012a, b) studied individual EPS production from 3 *S. thermophilus* and 1 *L. bulgaricus* in semi-defined medium and in whey permeate medium at a controlled pH of 6.0. They observed that the majority of EPS from *S. thermophilus* was produced in the stationary phase rather than during the exponential growth like *L. bulgaricus*. In our case, the starter culture contains both *S. thermophilus* and *L. bulgaricus* which probably have different kinetics of growth and EPS production.

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After inoculation, *S. thermophilus* develops rapidly and is responsible for the initial drop in pH (Tamime, 2006). Over the next 2 hours, *L. bulgarius* starts to grow exponentially by the synergistic influence with the streptococci. From pH 5.2, *S. thermophilus* stops growing while *L. bulgarius* continues to grow until pH reaches 4.4 (Beal & Corrieu, 1991), taking over on the acidification and probably also the EPS production. This could explain the continuous EPS production observed in the present study (Fig. 4).

In the present study, the EPS quantification was carried out with the PSA method. However, HPSEC-MALS analysis could also be used for the EPS quantification (Mende et al., 2016; Ruas-Madiedo & de los Reyes-Gavilán, 2005). The PSA method is simple, practical and requires inexpensive materials. However, it cannot provide the absolute polysaccharide content because the measured absorbance depends on the sugar type (Dubois et al., 1956). When glucose was used as standard, galactose and rhamnose would be underestimated and mannose would be overestimated (Dubois et al., 1956). In contrast, HPSEC-MALS requires expensive materials and a high sample purity but it is capable to estimate the absolute EPS content (Ruas-Madiedo & de los Reyes-Gavilán, 2005). In the present study, the amount of extracted dextran determined by HPSEC-MALS was equal to 97.6 ± 3.7% of that determined by PSA method, while for xanthan, this value was about 85.0 ± 6.4%. This difference could be explained by the distinct monomer composition of these 2 model EPS. Dextran contains mainly glucose so the PSA method (with glucose as standard) presents a good estimation for this macromolecule. Xanthan is a heteropolysaccharide with two glucose units, two mannose units and one glucuronic acid, so the PSA method will overestimate the xanthan content as compare to HPSEC-MALS analysis.

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Moreover, we found that the amounts of extracted LAB EPS determined by HPSEC-MALS were equal to 126.7 ± 12.0% or 131.5 ± 14.6% of those determined by the PSA method, for SM gel or SMWP gel, respectively. These results show that, in our case, the PSA method underestimated the EPS content. These findings could be explained by the high content of galactose and rhamnose in the monomer composition of extracted EPS (section 3.2.3). However, the PSA method expressed in mg of glucose per kg of milk was preferred to HPSEC-MALS analysis for the quantification of EPS in the present study, to normalize the results and facilitate the comparison with the other studies.

3.2.2. Macromolecular properties of EPS

The macromolecular properties of EPS produced in both gel types and extracted with the Protocol 3 were characterized using HPSEC-MALS. The M_w of EPS extracted from SM and SMWP gels ranged from 2.5×10^5 to 3.8×10^6 g mol^{-1} and 1.4×10^5 to 3.8×10^6 g mol^{-1}, respectively. This range of M_w is in a good agreement with the M_w determined for EPS produced by *S. thermophilus* and *L. bulgaricus* strains and extracted using different protocols (Mende et al., 2012b; Urshev et al., 2008; Vaningelgem et al., 2004). Vaningelgem et al. (2004) found that EPS extracted from milk medium using a quite similar protocol of our study (two protein precipitation step by TCA and two EPS precipitation step by acetone) have mean M_w ranging from 10×10^4 to up 2×10^6 g mol^{-1}. More recently, Mende et al. (2012b) characterized the EPS produced by LAB in semi-defined and whey permeate medium using a more complex protocol combining several steps of pH variation, proteolysis, heating in acid conditions (TCA) and ethanol precipitation. They found that the mean M_w was 1.8×10^4 and 4.3×10^6 Da depending on the strain (Mende et al., 2012b). However, it is necessary to confirm that the heating step in acidic medium of the extraction protocol does not affect the macromolecular

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properties of the EPS as observed in our study and others (Pop et al., 2016; Rimada & Abraham, 2003).

According to Vaningelgem et al. (2004), the EPS can be classified into two main populations based on their M_w: the high M_w EPS showing a $M_w > 10^6$ g mol^{-1} and the low M_w EPS showing a $M_w < 10^6$ g mol^{-1}. These two EPS populations could be produced by different strains present in the starter culture YF-L901 or by the same strain as already observed by others studies (Cerning, 1995; Vaningelgem et al., 2004). The structural parameters (M_w, [η], R_g, R_h) of these two EPS populations extracted from SM and SMWP gels were determined from HPSEC-MALS chromatograms (Table 1). The high M_w EPS populations extracted from both milk formula (SM or SMWP) present similar structural parameters (M_w, [η], R_g, R_h) with a mean $M_w \sim 2.5 \times 10^6$ g mol^{-1} and a mean [η] ~ 1150 ml g^{-1} (Table 1B and Fig. 5). The conformation of the high M_w EPS was estimated by calculating the structural factor ρ ($\rho = R_g/R_h$). Theoretical ρ values obtained in good solvents are 0.778 for homogeneous hard spheres up to 2 for extended coils and linear chains (Adolphi & Kulicke, 1997; Burchard, 1999). For the high M_w EPS, the ρ value is around 1.47-1.48 that is consistent with a random coil and linear chain conformation in θ solvent (Burchard, 1999). In contrast to the high M_w EPS, the low M_w EPS populations extracted from SM and SMWP gels differ in their structural parameters. The low M_w EPS population from SMWP gel has lower mean M_w (3.0×10^5 vs. 4.4×10^5 g mol^{-1}) and lower mean [η] (137 vs. 225 ml g^{-1}) than low M_w EPS population from SM gel (Table 1B). The proportion of low M_w EPS is also different in SM and SMWP gel. In mass, it is equal to 7.6 ± 1.0 % and 13.3 ± 4.3 % of total EPS extracted from SM and SMWP gels, respectively (Table 1B). If we consider the distribution of EPS in number of macromolecules, the proportion of low M_w EPS represents a noticeable part of the total number of macromolecules: 25.5 ± 1.2 % and up

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to 50.0 ± 8.0 % of total EPS extracted from SM and SMWP gels, respectively (Table 1B). These results suggest that the enrichment in whey protein induced the production of a higher proportion of low M_w EPS, while the total amount of EPS was similar in both gels. This trend could be linked to the lower time of fermentation and the higher rate of EPS production observed in SMWP gels as compared to SM gels.

3.2.3. Monomer composition of extracted EPS

The EPS monomer composition was investigated by GC analysis. The extracted EPS produced by starter culture in SM milk or SMWP milk consisted only of neutral sugars: glucose/galactose/rhamnose at ratio of 1/6.6/2.6 or 1/5.3/1.7, respectively. No presence of acid sugars (galacturonic acid and glucuronic acid) and of glucosamine is observed by GC analysis of their per-O-trimethylsilylated methyl derivatives (data not shown). The neutral charge of produced EPS was confirmed by the measurement of zeta potential (data not shown). This EPS monomer composition is in a good agreement with the data reported for *S. thermophilus* and *L. bulgaricus* strains (De Vuyst et al., 1998; Mende et al., 2016; Urshev et al., 2008; Vaningelgem et al., 2004). According to the literature, the majority of LAB used in fermented milk produces uncharged hetero-polysaccharides which content mainly glucose, galactose with or without others monomers such as rhamnose, N-acetylglucosamine and N-acetylgalactosamine (Mende et al., 2016; Vaningelgem et al., 2004). In the present study, the extracted EPS is a mixture of EPS produced by the different strains of the starter culture. The monomer ratio changed when the acidification occurred in the milk supplemented in whey proteins. This finding could be related to the different EPS M_w distribution observed in SM and SMWP gels (see section 3.2.2).

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4. Conclusion and perspective

The exocellular polysaccharides (EPS) extraction and quantification from fermented milk represent a challenging issue because of the low extraction yield due to the co-precipitation with milk proteins, the low purity (high residual proteins) and the degradation of EPS that modify their structural properties. We compared different protocols of EPS extraction from skim milk acid gel for the EPS quantification and characterization, using 2 model EPS: dextran and xanthan. The present results obtained on model EPS underline the importance of the choice of the protocol regarding to the purpose of isolation. Indeed, Protocol 1 which has a high extraction yield (80 %) induces a strong hydrolysis of EPS and is then only suitable for EPS quantification. Meanwhile, Protocol 3, which provides a lower extraction yield (55 %), preserves the structural properties of EPS and is then more suitable for the EPS characterization. Despite differences in their structural and physico-chemical properties (molar mass, charge), the extraction yield of added dextran and xanthan is the same using Protocol 3, which suggests that LAB EPS extracted by this protocol might be representative of total EPS produced. The composition of milk, and more precisely, the ratio of whey proteins, has no significant influence on the extraction efficiency of the tested protocols. Protocols 1 and 3 were applied on yogurt produced by a ropy starter culture. EPS production was detected at very low concentration (< 10 mg glucose kg^{-1}) at pH 6.2 and was monitored until final pH. HPSEC-MALS analysis shows 2 groups in each extracted EPS from 2 milk formulas: low ($<10^6$ g mol^{-1}) and high ($>10^6$ g mol^{-1}) M_w EPS. The enrichment of whey proteins increases the proportion of low M_w EPS. Protocols of extraction suitable for quantification or characterization of EPS could be then applied to study *in situ* the role of EPS on gel formation and gel texture in future studies.

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Figure captions

Figure 1: The 3 protocols used for EPS extraction from skim milk (SM) or acid milk gel (*TCA: Trichloroacetic acid)

Figure 2: Comparison of 3 different extracted dextran protocols from GDL-acid milk gels enriched with 100 mg kg^{-1} of dextran (A): Dextran extraction yield (% w/w) by Protocol 1 (□), Protocol 2 (■) and Protocol 3 (■) from two milk formulations: skim milk (SM) or skim milk supplemented in whey proteins (SMWP). Mean values ± standard deviation (n = 3). Values affected with different letters were significantly

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different for p = 0.05. (B) Molar mass distribution (M_w, thick lines) and differential refractive index (thin lines) obtained by HPSEC-MALS analysis of native dextran (──) and extracted dextran from SM GDL-acid milk gels using Protocol 1 (──), Protocol 2 (- - -) or Protocol 3 (─ ▪ ─).

Figure 3: Comparison of 3 different extracted xanthan protocols from of GDL-acid milk gels enriched with 100 mg kg^{-1} of xanthan (A): Xanthan extraction yield (% w/w) by Protocol 1 (□), Protocol 2 (■) and Protocol 3 (■) from two milk formulations: skim milk (SM) or skim milk supplemented in whey proteins (SMWP). Mean values ± standard deviation (n = 3). Values affected with different letters were significantly different for p = 0.05. (B) Molar mass distribution (M_w, thick lines) and differential refractive index (thin lines) obtained by HPSEC-MALS analysis of native xanthan (──) and extracted xanthan from SM GDL-acid milk gels using Protocol 1 (──), Protocol 2 (- - -) or Protocol 3 (─ ▪ ─).

Figure 4: Kinetic of acidification (continuous line) and EPS production (dotted line) monitored during skim milk (SM, black) or skim milk supplemented with whey proteins (SMWP, grey) fermentation obtained by LAB YF-L901 starter culture. EPS extraction was carried out with Protocol 1, mean values ± standard deviation (n = 3).

Figure 5: Molar mass distribution (M_w, thick lines) and differential refractive index (thin lines) obtained by HPSEC-MALS analysis of EPS produced by YF-L901 starter culture during fermentation of skim milk (SM, ──) and skim milk supplemented in whey proteins (SMWP, ──). EPS were extracted from acid milk gels using Protocol 3.

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Figure 1

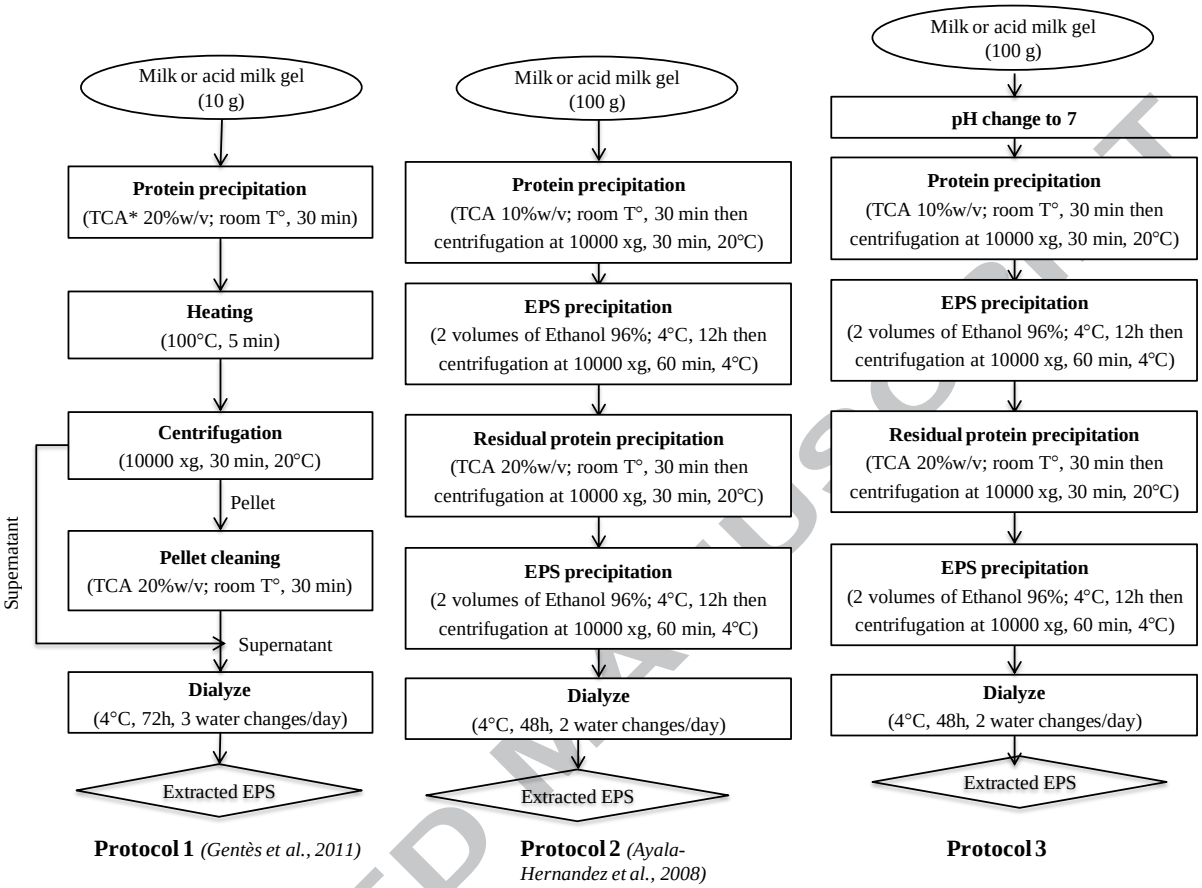

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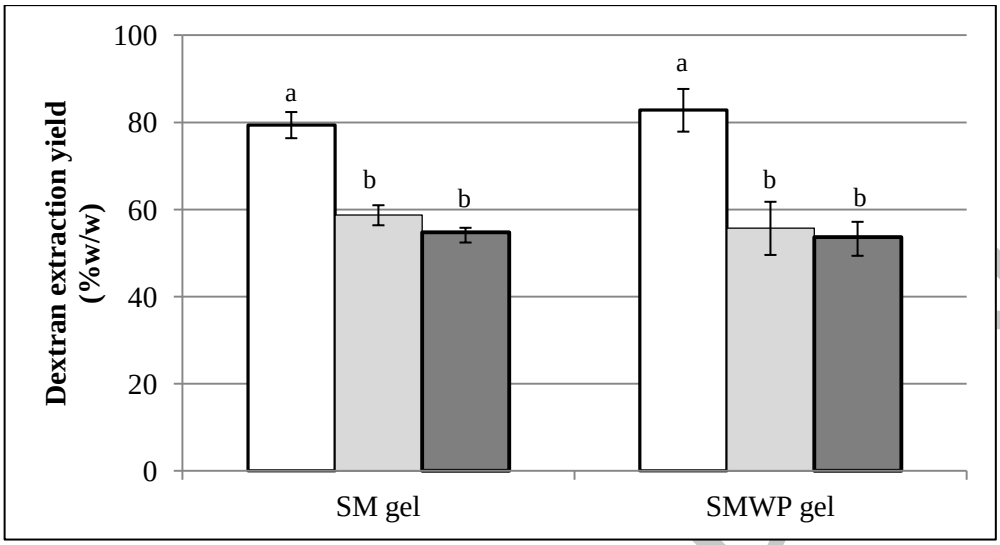
Figure 2



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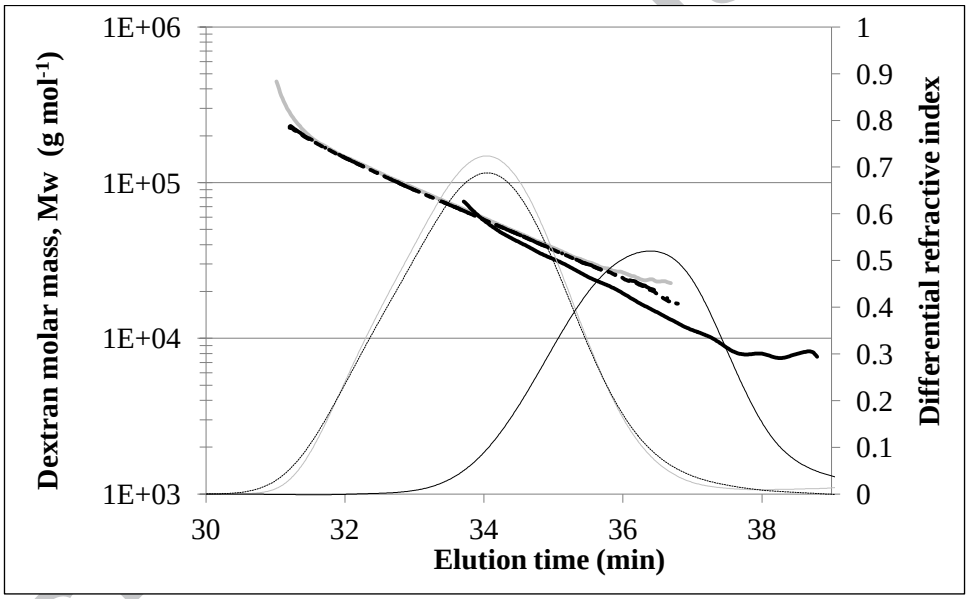

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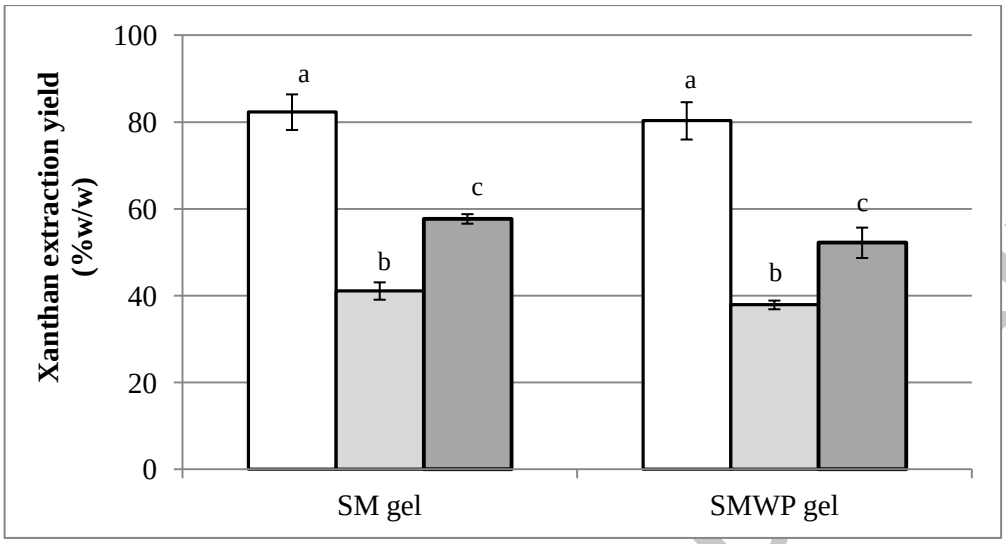
Figure 3



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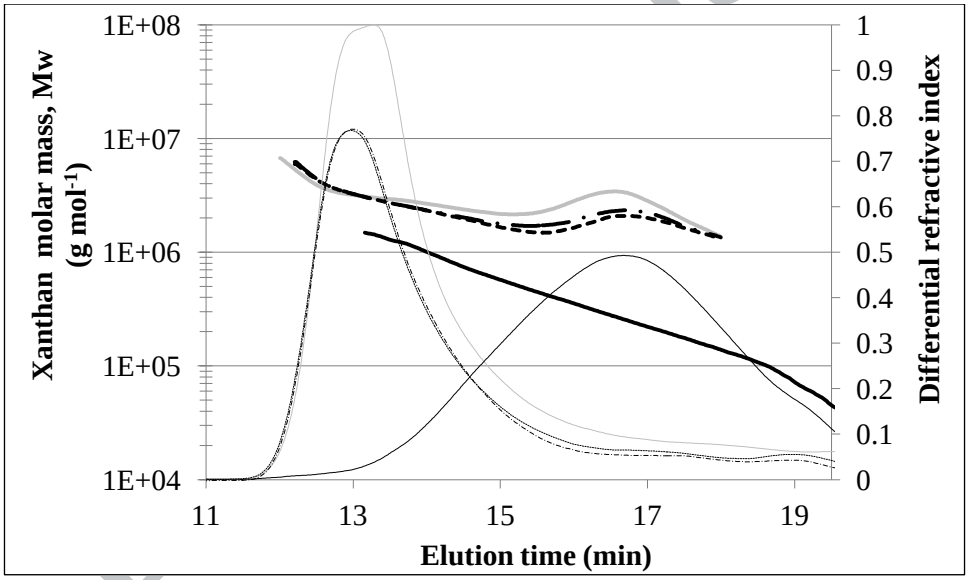

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Figure 4

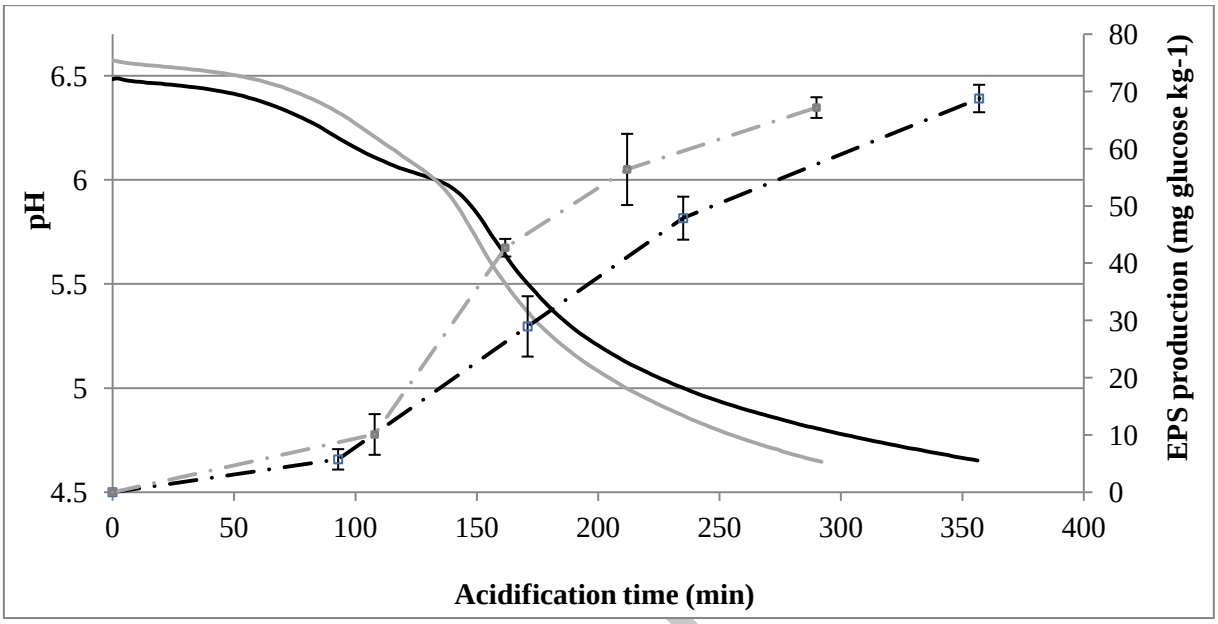

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Figure 5

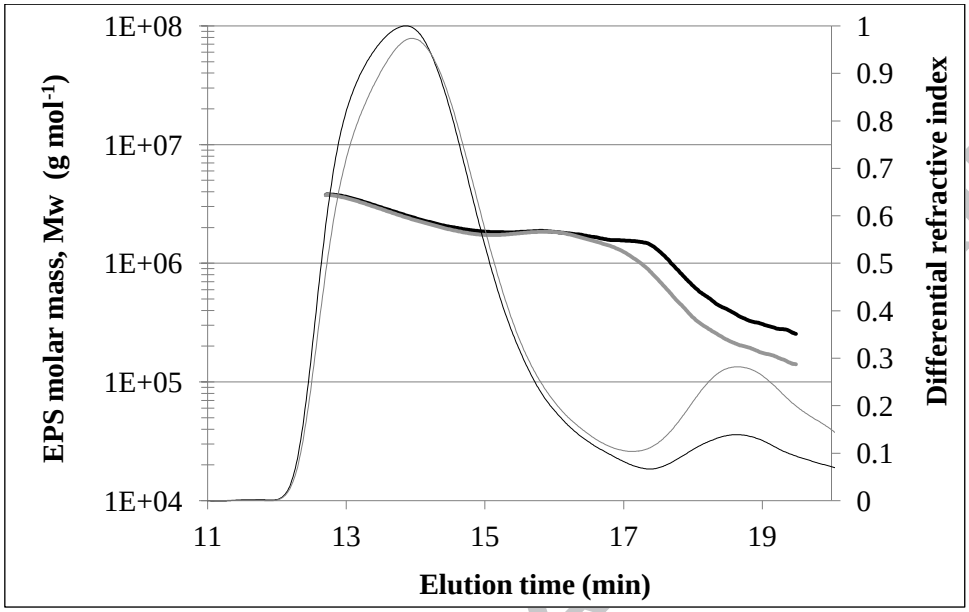

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Table 1: Characteristics parameters of yogurt fermentation (A) and macromolecular properties of extracted EPS (B). Values of each characteristic affected with different letters were significantly different for p = 0.05.

	SM gel[1]		SMWP gel[2]	
(A) Characteristic parameters of yogurt fermentation[3]				
Acidification time (min)	351.9 ± 4.4[a]		298.6 ± 9.0[b]	
Time of gelation point (min)	188.7 ± 7.5[a]		164.7 ± 5.7[b]	
pH of gelation point	5.26 ± 0.10[a]		5.40 ± 0.05[a]	
Quantity of EPS at pH 4.65 (mg glucose kg^{-1})	68.8 ± 2.4[a]		67.8 ± 1.6[a]	
(B) Macromolecular properties of extracted EPS[4]				
	Low M_w EPS	High M_w EPS	Low M_w EPS	High M_w EPS
Average M_w (g mol^{-1})	$(6.0 \pm 0.7) \times 10^{5}$ [a]	$(2.9 \pm 0.3) \times 10^{6}$ [b]	$(4.0 \pm 0.6) \times 10^{5}$ [c]	$(2.6 \pm 0.3) \times 10^{6}$ [b]
Percentage in mass (%)	7.6 ± 1.0[a]	92.4 ± 1.0[b]	13.3 ± 4.3[a]	86.7 ± 4.3[b]
Percentage in number of particle (%)	25.5 ± 1.2[a]	74.5 ± 1.2[b]	50.0 ± 8.0[c]	50.0 ± 8.0[c]
Intrinsic viscosity [η] (mL g^{-1})	253 ± 32[a]	1253 ± 88[b]	163 ± 13[c]	1189 ± 58[b]
Radius of gyration (R_g) (nm)	NA[5]	127.2 ± 10.3[a]	NA[5]	120.3 ± 7.7[a]
Hydrodynamic radius (R_h)(nm)	30.0 ± 2.1[a]	85.9 ± 6.1[b]	23.8 ± 1.7[c]	81.7 ± 4.7[b]
$\rho = R_g/R_h$	ND[6]	1.48	ND[6]	1.47

[1] SM = skim milk.

[2] SMWP = skim milk supplemented with whey proteins.

[3] (A) = data obtained by monitoring rheological parameters (G', G"), and acidification kinetics during fermentation at 43°C, culture YF-L901.

[4] (B) = Data obtained by HPSEC-MALS analysis of EPS extracted by using protocol 3.

[5] NA: No applicable.

[6] ND: No determined.

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Highlights:

- Dextran or xanthan were used to compare 3 extraction protocols of EPS from acid gel
- Extraction yields and molecular properties of EPS after extraction were determined
- Heat treatment in acid condition allows a high extraction yield but degrades EPS
- Soft extraction is needed to avoid EPS degradation
- Protocols were used to quantify or characterize EPS during milk lactic fermentation

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