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ABSTRACT

One acidifying (ST1) and two texturing strains (ST2 and ST3) of *Streptococcus thermophilus* were used as pure or co-cultures to identify and understand their effects on the structuring of acid milk gels and on the appearance of texture defects, i.e., syneresis and graininess. Symbiosis between specific texturing and acidifying strains reduced acidification time and increased exocellular polysaccharide (EPS) content. The texturing strain could simultaneously produce low and high molar mass EPS and their distribution in mass and/or in number were influenced by the proportion of acidifying to texturing strain used. The results of this study suggest that the high molar mass EPS contributes to acid gel firmness, but less so compared with the acidification rate. The ability of strain ST3 to prevent texture defects, specifically graininess, did not depend on the acidification kinetics or final EPS content, but rather on the structural properties of EPS and/or the bacterial chain morphology.

1. Introduction

Fermented milk such as yoghurt is one of the most popular dairy products in many countries. In the literature, set-type fermented milk is defined as a viscoelastic gel formed from the aggregation of casein micelles due to milk acidification (Tamime, Robinson, & Latrill, 2001). For manufacturers, a set-type acid milk gel must have a firm body, smooth texture, thick mouthfeel and structural stability. The most common texture defects in milk gels are spontaneous syneresis and graininess. Spontaneous syneresis, or wheying-off, indicates the separation of serum on the yoghurt surface, and graininess usually refers to lumpiness or granular texture due to the presence of large protein aggregates that range in size from 1 to 5 mm (Folkenberg, Dejmek, Skriver, & Ipsen, 2006; Lucey & Singh, 1997). The three main factors determining gel texture and the appearance of defects are the milk composition, the manufacturing process and the starter cultures (Lucey & Singh, 1997; Sodini, Remeuf, Haddad, & Corrieu, 2004). A positive correlation between graininess and high content of denatured whey proteins was reported in the literature (Lucey, 2004; Nguyen et al., 2018a; Remeuf, Mohammed, Sodini, & Tissier, 2003). However, the effect of the starter culture on the graininess has not been clarified.

Starter cultures can influence milk gel texture by their acidification rate and/or by their exocellular polysaccharide (EPS) production. EPS from “texturing” or “ropy” cultures are well known to improve the gel viscosity and reduce syneresis (Folkenberg et al., 2006; Marshall & Rawson, 1999; Mende, Mentner, Thomas, Rohm, & Jaros, 2012). Some authors have also observed a reduction in graininess when using a ropy strain or a high-level EPS-producing culture, but no further details on the mechanism of this phenomenon have been reported (Hassan, Ipsen, Janzen, & Qvist, 2003; Küçükçetin, Weidendorfer, & Hinrichs, 2009). In a previous study (Nguyen et al., 2018a), the effects of heat treatment, milk

63 composition and two EPS-producing starter cultures on the microstructure and the texture
64 defects of acid milk gel have suggested that the presence of denatured whey proteins is
65 critical for grain formation. Gel viscosity and texture defects appeared to be culture-
66 dependent and were correlated with the amount, molar mass and intrinsic viscosity of the
67 EPS produced. Furthermore, the gel microstructure obtained from the two cultures showed
68 major differences in the location of bacterial cells in the gels, suggesting that simultaneously
69 with the EPS effect, the length of bacterial cell chains could also interfere with the formation
70 of the protein network during acidification due to steric hindrance. However, the different
71 acidification kinetics of the two cultures used might influence gel structuring.

72 In industrial applications, commercial starter cultures for yoghurt fermentation usually
73 consist of several strains of *Streptococcus thermophilus*, which are chosen for their specific
74 properties such as fast initial acidification or texture promoting capacity. The interaction
75 between different *S. thermophilus* strains could affect the total EPS production, but different
76 trends were observed and no details on the acidification kinetics or EPS characterisation have
77 been reported (Folkenberg et al., 2006). In addition, the influence of co-cultures of *S.*
78 *thermophilus* strains on milk gelation has not yet been studied. The aim of this study was to
79 identify the effects of mono or co-culture of EPS-producing *S. thermophilus* on the formation
80 of acid milk gels and the appearance of texture defects. For this purpose, three *S.*
81 *thermophilus* strains were used alone or in combination: one for its fast acidification
82 characteristics and the other two for their texturing properties. Hence, seven starter cultures
83 were formed with different ratios between acidifying and texturing strains to study the
84 correlations between the gel texture characteristics (e.g., firmness, viscosity, syneresis,
85 graininess), the kinetics of acidification and gelation, but also the quantity and structural
86 features of EPS produced.

2. Materials and methods

2.1. Solvents and reagents

Low-heat skim milk powder (Spray 0) was purchased from Ingredia (France). The three *S. thermophilus* strains were provided by Chr. Hansen A/S, Arpajon (France). Lactose monohydrate and trichloroacetic acid (TCA) were from GPR Rectapur, VWR chemicals (Belgium). D(+)Glucose monohydrate and 1 N sodium hydroxide (NaOH) were from Merck (Germany). Phenol, $LiNO_3$ and NaN_3 were from Sigma (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. Starter cultures

Three different frozen lyophilised strains of *S. thermophilus* ST1, ST2, ST3 from the Chr Hansen culture collection (Chr Hansen A/S) were used alone (pure culture) or in combination (co-culture) in seven different starter cultures. From manufacturer information, strain ST1 possess a cell wall protease (PrtS+) and could play a role as acidifying strain. ST2 or ST3 were texturing strains without cell wall protease (PrtS-) and were chosen to provide different textural types of milk gels: firm or viscous, respectively.

Cultures ST1, ST2 and ST3 were composed of a single *S. thermophilus* strain corresponding to the name of the culture. The four co-cultures ST1+2 (1/1.7), ST1+2 (5/1), ST1+3 (1/1.7)-and ST1+3 (5/1) consisted of the combination of acidifying strain ST1 with a texturing-strain (ST2 or ST3) with the cfu ratios of acidifying strain/texturing strain

corresponding to the name of the culture. All strains were stored at –80 °C. Strains were thawed and mixed then 2 g of the final culture were added to 100 mL of UHT skim milk. Ten millilitres of this pre-inoculated milk were immediately added to 1 L of pasteurised milk to start the lactic fermentation. The final inoculation rate for the seven starter cultures was 10^6 cfu mL^{-1} pasteurised milk. Sampling, thawing and formulation were performed in the laboratory under sterile conditions.

2.3. Acid milk gel manufacture

The milk-based preparation was carried out as described by Nguyen et al. (2018b). The milk formulation contained 11.8% (w/w) dry matter, 4.0% (w/w) proteins and 6.7% (w/w) lactose. The skim milk powder was dissolved in ultra-pure water during 1h at room temperature and then kept for 12 h at 4 °C before the pasteurisation at 95 °C for 6 min. After the heating step, milk suspensions were cooled to 43 °C and immediately inoculated with the starter cultures. Fermentation procedures, pH monitoring and gel structuring have previously been reported by Nguyen et al. (2018b). In brief, the fermentation was carried out at 43 °C with pH control until a final pH of 4.65 was reached. In parallel, gel formation was monitored using a Physica MCR 300 rheometer (Anton Paar, St Albans, UK) equipped with concentric cylinder geometry (CC27) that operated in continuous oscillation mode at a frequency of 1 Hz and 1% strain. The gel point was defined as the moment when the storage modulus (G') first exceeds the loss modulus (G'') (Winter, 1987). After acidification, the gels were stored undisturbed in plastic cups at 4 °C for 7 days before determination of texture properties.

2.4. Gel texture properties

137

138 The textural parameters of set-type acid milk gel at day 7 including the firmness, the
139 viscosity, the spontaneous syneresis and the grain defect were characterised as described by
140 Nguyen et al. (2018a).

141 The firmness of acid milk gel was determined immediately after removing out of the
142 cold room (4 °C) using a texture analyser equipped with an acrylic cylinder compression
143 probe (diameter = 40 mm, thickness = 5 mm). The compression test was carried out with a
144 speed of 5 mm s^{-1}, the trigger force of 5 g and a penetration depth of 15 mm. The gel firmness
145 (N) corresponding to the maximum compression force, i.e., the rupture point of the gel, was
146 calculated with the Exponent software (Stable Micro Systems, Godalming, UK).

147 For the viscosity measurement, the set-type acid milk gels were stirred 20 times with a
148 spoon and 20 g of sample was placed in the rheometer cup. The viscosity of the stirred gel (η,
149 Pa s) at a shear rate of 300 s^{-1} (13 °C) was determined using Physica MCR 300 rheometer
150 equipped with concentric cylinder geometry and the shear rates γ (s^{-1}) increased with a linear
151 ramp from 0.271 s^{-1} to 300 s^{-1} in 210 s.

152 The spontaneous syneresis (% w/w) was the percentage of whey on the surface of the
153 undisturbed gel to the total gel weight and was measured according to the siphon method
154 (Amatayakul, Sherkat, & Shah, 2006).

155 The grain defect was determined by image analysis. After stirring 5 times with a
156 spoon, 1 g of acid gel was dispersed in 100 mL of distilled water by stirring at 400 rpm for
157 2 min. 10 mL of diluted sample was poured into a plastic Petri dish and visualised with a
158 digital camera in black and white mode. The image analysis was performed using ImageJ
159 1.49v software (National Institutes of Health, USA) with Fiji plug-in. Grains with a diameter
160 > 1 mm were enumerated (Remeuf et al., 2003). The grains were classified into different size

groups by their diameter: 1–1.5 mm, 1.5–2.0 mm, 2.0–2.5 mm, 2.5–3 mm or > 3 mm. The results were expressed as the number of grains of each size per gram of gel.

2.5. EPS quantification

EPS quantification was performed using the protocol 1 described by Nguyen et al. (2018b). The EPS were extracted in hot-acidic medium and dialysed to obtain a high extraction yield. The total sugar content was determined by the phenol-sulfuric acid method using glucose as the standard (range of concentration from 0 to 200 mg L^{-1}). The acidified milk with glucono-δ-lactone (GDL) was used as blank and the residual milk sugars after dialysis were subtracted from all data.

2.6. EPS characterisation

EPS were extracted without a heating step using the protocol 3 described by Nguyen et al. (2018b) to avoid EPS denaturation. The extraction process included a step of pH correction to 7, the protein precipitations in an acidic medium, followed by the precipitations of EPS by ethanol before dialysis and lyophilisation. The structural properties of extracted EPS were characterised using high-performance size exclusion chromatography coupled with multi-angle laser light scattering detector (HPSEC-MALS). The material and operating conditions were reported in Nguyen et al. (2018b). The weight-average molar mass (M_w) and the intrinsic viscosity ([η]) were determined using the software ASTRA 6.1.2 (Wyatt Technologies, Santa Barbara, CA). The M_w was calculated using the Berry's model (2^{nd} order). The data were analysed using a refractive index increment (dn/dc) of 0.145 mL g^{-1} according to the literature (Lambo-Fodje et al., 2007).

186

187 *2.7. Statistical analysis*

188

189 All analyses were conducted at least in triplicate and the values were expressed as the
190 mean ± standard deviation. The experimental data were analysed statistically using the
191 Fischer's and Student's tests with 95% confidence level and using Pearson's correlation
192 coefficient functions for the correlation between means.

193

194 **3. Results and discussion**

195

196 *3.1. Characterisation of starter cultures*

197

198 *3.1.1. Acidification kinetics*

199 The kinetics of milk acidification induced by lactic fermentation were monitored by
200 pH evolution between pH 6.5 and 4.6 (Fig. 1). The parameters evaluated were the total
201 acidification time and the maximum acidification rate (V_{max}) (Table 1). As an acidifying
202 strain, ST1 presented the highest V_{max} value of all pure cultures and the shortest acidification
203 time (~300 min) compared with ST2 and ST3. The acidification curve profiles of strains ST2
204 and ST3 (Fig. 1) were very different from that of strain ST1. ST2 and ST3 had significantly
205 slower acidification rates during the first 160 min and also a stronger slowdown in
206 acidification after the shoulder observed around pH 6.25. This specific profile corresponded
207 to the strains' urease activity, which produced ammonia from milk urea and induced a slight
208 alkalisation of the medium. Furthermore, the final count of bacterial cells at the end of the
209 fermentation was ~10^9, 10^7 or 10^6 cfu g^{-1} for ST1, ST2 or ST3, respectively, and was
210 consistent with the kinetics observed. These differences in acidification kinetics and cell

growth were related to the proteolytic activity of these 3 strains. Indeed, strain ST1 has a cell envelope-associated proteinase (PrtS+) that hydrolyses caseins into peptides and essential amino acids to ensure high cell density growth and thereby generating high acidification activity (Galia, Perrin, Genay, & Dary, 2009). When this enzymatic activity is lacking, such as for ST2 and ST3 (PrtS-), the growth of *S. thermophilus* strains in milk is less optimal depending on the specific intracellular peptidases and amino acids requirements of the strain (Galia et al., 2009).

The addition of ST1 to ST2 or ST3 in four co-cultures resulted in a considerable decrease in acidification time and a significant increase in V_{max} (Fig. 1, Table 1). For example, the total acidification time for cultures of ST2 was reduced from 1351 min (pure culture) to 528 min or 304 min when ST1 was added with a ST1/ST2 ratio of 1/1.7 or 5/1, respectively. Furthermore, while pure culture of ST3 had the slowest acidification kinetics of all cultures tested, the ST1+3 co-cultures had better acidification performance as compared with ST1+2 co-cultures for the same ST1 ratio. These results suggested that the proteinase activity of ST1 produced peptides and essential amino acids that stimulated growth of both texturing strains. However, the amino acid requirements seemed to be different between ST2 and ST3, as indicated by their differing rates of acidification. ST1 metabolites may be more adapted to the needs of ST3 than to those of ST2, which allowed for a better symbiotic relation between ST1 and ST3 than between ST1 and ST2.

3.1.2. EPS production and characterisation

The EPS produced by the seven starter cultures were extracted for quantification (Fig. 2) and for macromolecular characterisation (molar mass distribution (M_w) and intrinsic viscosity ($[\eta]$)) (Fig. 3). Although the strain ST1 was not considered as textural strain, it was able to produce a substantial amount of EPS of ~55 ± 4 mg glucose kg^{-1} of gel in a short time

of acidification (~300 min) (Fig. 2). However, the EPS produced have low M_w of ~9.2×10^4 g mol^{-1} and low *[η]* of ~42 mL g^{-1}, which could explain the poor texturing property of this strain. These results also confirmed that macromolecular properties are a more critical characteristic for the functional properties of EPS than the total amount of polymers (Mende, Rohm, & Jaros, 2016). Even after an acidification time that was 4–5 times longer than for culture ST1, cultures ST2 and ST3 did not produce a greater quantity of EPS (~30 mg glucose kg^{-1} and ~52 mg glucose kg^{-1}, respectively), particularly for ST2 (Fig. 2). However, the EPS produced by these two texturing strains had very different macromolecular properties from each other and from those of the ST1 culture. Based on elution profiles of HPSEC-MALS analysis, EPS produced by ST2 or ST3, could be divided into two groups of low and high M_w as also observed in the literature (Petry et al., 2003; Vaningelgem et al., 2004). The EPS produced by the strain ST3 has a M_w and intrinsic viscosity significantly higher than those produced by cultures ST2 or ST1 (Fig. 3). Furthermore, EPS from ST3 consisted of 80% high M_w polymers in number, in contrast to EPS from ST2, which contained only 35% high M_w molecules.

Regarding the strain ST2 and its co-cultures with ST1, the increase in the proportion of ST1 from 0 to 37% [culture ST1+2 (1/1.7)] and to 83 % [culture ST1+2 (5/1)] induced a significant increase in the amount of EPS produced from 30 to ~52 and to ~67 mg glucose kg^{-1}, respectively (Fig. 2), while the time of acidification was significantly decreased (Table 1). The same trend was also observed when ST1 was added to strain ST3. Furthermore, the macromolecular characteristics of the EPS produced by ST2 and its co-cultures were broadly similar (Fig. 3A,D). For the strain ST3 and its co-cultures ST1+3, a significant decrease of EPS M_w was observed when the acidification time decreased (Pearson's $r = 0.951$) due to the increase in ST1/ST3 ratio. These results suggested a chain length extension of EPS from ST3 during longer acidification time. Laws, Leivers, Chacon-Romero, and Chadha (2009)

reported a similar trend in which EPS M_w produced by *Lactobacillus acidophilus* increased 3.7 times from 6 h to 24 h of fermentation in skim milk supplemented with glucose at 42 °C and at a controlled pH of 5.8. In association with cell counting, the authors observed that EPS synthesis and chain length extension continued after the end of the exponential phase, using sugar nucleotides already produced during in the latter phase.

When looking in more details at the results of EPS M_w distribution (in mass or in number, Fig. 3B,C), different trends could be observed. Firstly, the presence of the acidifying strain ST1 at a level of 37% in culture ST1+2 (1/1.7) and ST1+3 (1/1.7) did not change the M_w distribution characteristics as compared with pure strains (Fig. 3B,C), suggesting that in these conditions ST1 produced a negligible quantity of EPS, but had an enhancing effect on the EPS production of texture strains. In contrast, a significant change in the M_w distribution of EPS was observed with 83% ST1 in cultures ST1+2 (5/1) and ST1+3 (5/1), in that the low M_w EPS then became predominant. These findings could be related to the short acidification time observed with these co-cultures and/or to the prevalence of the acidifying strain over the texturing strain. Furthermore, we observed that the high M_w EPS group from culture ST1+3 (5/1) presented a lower average M_w, but a higher $[\eta]$ than for ST1+2 (5/1). These findings suggested that the structure of EPS produced by ST2 could be more flexible and/or branched than those produced by ST3. It has been shown that for two EPS with identical structure (i.e. repeating units branching, stiffness of side chain), a higher M_w could lead to a higher $[\eta]$ and therefore to a higher gel viscosity (Faber, Zoon, Kamerling, & Vliegenthart, 1998; Looijesteijn, van Casteren, Tuinier, Doeswijk-Voragen, & Hugenholtz, 2000; Mende et al., 2016). However, other structural factors such as side chain stiffness and branching also made a considerable contribution to the $[\eta]$ of EPS. Generally, for a given M_w, a rigid linear polysaccharide will tend to extend under shearing, as opposed to a flexible branched

285 molecules which will tend to compact, and will then contribute to increase the viscosity of the
286 solution (Whistler & BeMiller, 1997).
287 Overall, the results presented in this section showed that the acidification kinetics, the
288 production and the macromolecular characteristics of EPS depended not only on the strains
289 but also on the interaction between textural and acidifying strains. The combination between
290 ST1 and ST3 seemed to be more efficient than the combination between ST1 and ST2.
291 Furthermore, all pure cultures could produce EPS, but these EPS differed in terms of
292 quantity, distribution in mass or in number and intrinsic viscosity. In the same way, the
293 overall distribution of low and high M_w EPS is strongly correlated with the interaction
294 between acidifying and texturing strains. It is quite difficult to compare these findings with
295 other studies because very few data are reported on the EPS production of mixtures of *S.*
296 *thermophilus*. Folkenberg et al. (2006) observed different trends of EPS production in
297 fermented milk by 5 *S. thermophilus* strains in pure or in mix cultures of two strains. Their
298 results suggested that the interaction between two *S. thermophilus* strains could be
299 compatible or not, depending on the strain used. However, they did not report acidification
300 time or molecular characteristics of EPS, which made difficult the comparison with our
301 results.
302
303 *3.2. Influence of starter cultures on milk gelation and textural properties of gels*
304
305 *3.2.1. Gelation kinetics*
306 The kinetics of gelation were monitored by the evolution of the storage modulus (G')
307 and the loss modulus (G''), characterised by time and pH of the gel point. As presented in
308 Table 1, the time and pH of gelation depended on the culture used. The pH of the gel point
309 was approximately 5.2 for the cultures ST1, ST2, ST1+2 (1/1.7) and ST1+2 (5/1), and was

not influenced by the rate of acidification. The same pH value was reported for the gel point of heated milk acidified chemically using glucono-delta-lactone (Lucey, Tamehana, Singh, & Munro, 1998; Tamime et al., 2001). In the case of ST3, the pH of the gel point was found to be significantly higher at 5.57 and decreased to 5.4 and 5.2 when strain ST1 was added at 37 and 83%, respectively (Table 1). The occurrence of the gel point at a higher pH could be related to the presence of EPS and their interactions with milk proteins (Mende et al., 2013b).

In the case of strain ST3 and its co-cultures, a positive correlation (Pearson's r = 0.992) was observed between the pH of gelation and EPS macromolecular properties (M_w, $[\eta]$), but not with the EPS quantity. The decrease in the pH of the gel point could be related to a decrease of the average M_w of EPS and/or of the proportion of high M_w EPS (Fig. 3). On the other hand, for all cultures, a predominant proportion in number of low M_w EPS corresponded to a gel point occurring at a lower pH of ~5.2 (Fig. 3C). Mende et al. (2013a,b) also reported a similar trend. The authors observed early gelation due to the depletion effect between casein micelles and added bacterial EPS or commercial dextran (Tuinier, Grotenhuis, Holt, Timmins, & de Kruif, 1999). These two neutral polysaccharides, bacterial EPS and dextran had different M_w of 2.6×10^6 Da and 5×10^5 Da, respectively. Compared with bacterial EPS, addition of 15 – 80 times more dextran was required to achieve a similar effect on gelation point.

3.2.2. Gel texture

Gel texture was characterised by four parameters: (i) firmness, (ii) viscosity, (iii) spontaneous syneresis (Table 2) and (iv) graininess (Fig. 4). Gels from the ST3 culture are not mentioned in this section because after 24 h of acidification, the gels obtained at a final pH of ~4.8 were heterogeneous with an upper gel-like part and a lower liquid-like part.

335 *Firmness.* Gel firmness is the main texture property of set-type acid milk gel and this
336 parameter is mainly governed by the protein network and not by bacterial EPS (Folkenberg et
337 al., 2006; van Marle & Zoon, 1995). For the monocultures used in the present study, ST1
338 provided the lowest gel firmness while ST2 produced the highest gel firmness, which could
339 be related to its long acidification time of nearly 24 h. Moreover, it was observed for strain
340 ST2 and its co-cultures ST1+2 that the higher the proportion of ST1, the faster the
341 acidification and the lower the gel firmness (Pearson's r = 0.998). According to the literature,
342 slow protein aggregation could result in continuous network formation with more connected
343 proteins, reducing the rearrangement of particles during gel formation and contributing to an
344 increase in gel rigidity during fermentation at low temperatures (Lucey & Singh, 1997;
345 Sodini et al., 2004).
346 In the present study, it is interesting to note that although the cultures ST1, ST1+2
347 (5/1) and ST1+3 (5/1) induced similar acidification times (Table 1), the resulting gel firmness
348 was significantly different in the order: ST1+2 (5/1) > ST1+3 (5/1) > ST1, consistent with the
349 order of the average M_w of EPS produced. In other words, the M_w of EPS from ST1+2 (5/1)
350 was higher than from those of ST1+3 (5/1) and ST1 (Pearson's r = 0.987). These findings
351 suggested that the presence of high M_w EPS was also an important determining parameter for
352 gel firmness, though not as much as the acidification rate.
353 When comparing the gels of co-cultures with the same ST1 ratio, the gels containing
354 ST2 had a higher firmness than those containing ST3 (Table 2). Other studies have also
355 reported a decreased gel firmness when using a ropy culture which is defined as a high EPS
356 producing culture with high [η] such as the culture ST3 (Hassan et al., 2003; Hess, Roberts,
357 & Ziegler, 1997; Mende et al., 2012). Using microscopic analyses, Hassan et al. (2003)
358 observed that the EPS of ropy cultures were mainly located and segregated in the pores of the
359 gel rather than attached to the protein network. Hence, the gel structure from a ropy culture

360 was less dense than that of non-ropy culture. Mende et al. (2012) and Rohm and Kovac
361 (1994) also suggested that the large amount of uncharged EPS produced by ropy culture
362 could have partially prevented the development of protein-protein bonds during gel formation
363 and thus reduced the rigidity of acid milk gel.
364
365 *Stirred gel viscosity*. Stirred gel viscosity is correlated with a thick mouthfeel and with
366 the smoothness of fermented milk products (Folkenberg et al., 2006). In this study, the stirred
367 gel viscosity varied depending on the culture and the macromolecular properties of the
368 produced EPS. ST1 produced only low M_w EPS with low [η] and formed gels presenting the
369 lowest viscosity. When ST2 was used, even if a low quantity of EPS was produced, the
370 viscosity of the stirred gels formed was higher than for strain ST1. This result could be
371 explained by the high percentage (80%) in mass of EPS with high M_w produced by culture
372 ST2.
373 Concerning the co-cultures of ST2 or ST3, increasing the ratio of the acidifying strain
374 decreased the stirred gel viscosity from 193 to 164 mPa s for ST1+2 (1/1.7) and (5/1),
375 respectively, or from 301 to 239 mPa s for ST1+3 (1/1.7) and (5/1), respectively (Table 2).
376 These results could be directly linked to the macromolecular properties of the EPS produced
377 (Fig. 3) and suggested a positive correlation between the quantity of high M_w EPS and the
378 stirred gel viscosity (Pearson's r = 0.890). Petry et al. (2003) also reported a striking
379 correlation between an elevated proportion of the high M_w EPS fraction and viscosity of acid
380 milk gel. In the present study, we also observed that even though the amount and molar mass
381 distribution of EPS were similar, gels from culture ST1+3 (1/1.7) were more viscous than
382 those from culture ST1+2 (1/1.7). This result could be partially explained by the higher [η] of
383 the EPS produced by ST3 and probably by a different molecular rigidity as already
384 mentioned in section 3.1.2. Also when comparing ST1+2 (5/1) and ST1+3 (5/1), despite both

385 co-cultures exhibiting a low proportion of high M_w EPS and similar [η] of EPS, the viscosity
386 of stirred gels was significantly different, suggesting that EPS were not the only factor
387 contributing to this textural property. In a previous study based on gel microstructure
388 observations, Nguyen et al. (2018a) suggested that in parallel to EPS production, the
389 morphology of bacterial cell chain length should also be taken into account to understand the
390 texture results.
391
392 *Spontaneous syneresis defect.* Spontaneous syneresis is the contraction of a gel
393 without exerting external forces and is related to an instability of the gel network that results
394 in the loss of ability to entrap the entire serum phase (Lucey & Singh, 1997). Gels obtained
395 with pure culture of ST1 presented a medium level of syneresis while those from ST2 had the
396 highest syneresis, possibly related to the long acidification time. For co-cultures of ST2, this
397 texture defect decreased from 2.7 to 1.4% when the ratio of ST1/ST2 changed from 1/1.7 to
398 5/1. In this case, a positive correlation was observed between syneresis, gel firmness and time
399 of acidification (Pearson's r = 0.999) suggesting that gel contraction could occur during the
400 slow fermentation, thereby hardening the gel and expelling the serum. In contrast, low
401 syneresis levels were observed for co-culture of ST1+3. Decreased gel syneresis has been
402 reported with the use of ropy strains that produce a high quantity of high M_w EPS
403 (Amatayakul et al., 2006; Kristo, Miao, & Corredig, 2011). It can be assumed that the ropy
404 EPS located in the gel pores could bind the serum and thus reduce the syneresis.
405
406 *Graininess defect.* Graininess or lumpiness is considered an undesirable defect
407 because consumers usually expect a smooth product (Lucey, 2004). Remeuf et al. (2003)
408 measured graininess by the number of grains with a diameter above 1 mm in 1 g of acid milk
409 gel. In the present study, the graininess defect was determined by the number of grains, but

410 also by their diameter from 1 mm to above 3 mm. Larger grains were more damaging for
411 texture perception even in low number (Fig. 4). The graininess was high when using pure
412 cultures ST1 and ST2. In the mixtures, regardless of the strain ratio, co-cultures of ST3
413 presented a low graininess while the co-cultures of ST2 showed a medium granular texture
414 (Fig. 4).

415 According to the literature, grains are large aggregates of proteins that appear under
416 certain conditions such as a high fermentation temperature, excessive whey protein to casein
417 ratio or with the use of certain types of starter culture (Lucey, 2004). However, only few
418 studies have focused on the impact of starter culture on the appearance of grain. In agreement
419 to our result, Küçükçetin et al. (2009) reported that the number of grains and their size were
420 significantly reduced when using a high-level EPS-producing starter culture, rather than a
421 medium- or low-level EPS-producing culture. Results from the present study provided
422 additional data on this phenomenon. Indeed, it is worth noting that cultures ST1+2 (5/1) and
423 ST1+3 (5/1) had a similar kinetics of acidification and gelation (Table 1) and similar
424 production of EPS (Fig. 2) that presented identical intrinsic viscosity and EPS molar mass
425 distribution (Fig. 3 B,C,D). However, despite these similarities, the texture of the resulting
426 gels from ST1+2 (5/1) presented more major defects (syneresis and graininess) as compared
427 with gels from culture ST1+3 (5/1). These new data support the inhibitory effect of strain
428 ST3 against appearance of texture defects, specifically graininess, which has been observed
429 in a previous study (Nguyen et al., 2018a). Therefore, we suggested that this interesting
430 technological property of culture ST3 could be related to the bacterial chains morphology
431 and/or the structural properties of the EPS produced and that it did not depend on the
432 acidification kinetics or final EPS content.

4. Conclusion

It is known that the symbiosis between texturing and acidifying strains of *S. thermophilus* reduces the acidification time and has an impact on the final acid milk gel structure. In this work, we selected one acidifying strain (ST1) and two texturing strains (ST2, ST3) strains, and used them singly or in co-cultures (ST1+2 or ST1+3) at two different ratios (1/1.7 or 5/1) in pasteurised skim milk to assess and understand the influence of their interaction on the acidification kinetics, EPS production, gel structure and appearance of texture defects.

In pure culture, due to the presence of proteinases in the cell wall, the acidifying strain provided rapid acidification and produced a medium amount of EPS. However, these EPS had a low M_w and intrinsic viscosity $[\eta]$ hence the gel obtained had the lowest firmness and viscosity, and the most graininess defect. Both texturing strains did not have the proteinase activity and showed a slow acidification. They both produced EPS with high molar mass but with different macromolecular properties.

In co-culture, the strains ST2 and ST3 had positive symbiotic relations with ST1, as showed by the reduction in acidification time and the increase in EPS production. Furthermore, the viscosity of gels increased significantly, probably due to the production of high M_w EPS by the texturing strains. Texture defects such as syneresis and graininess disappeared with use of the co-cultures of ST1+3. Moreover, co-cultures ST1+3 (5/1) and ST1+2 (5/1) had some similarities in terms of (i) acidification, (ii) gelation kinetics, (iii) EPS production and molar mass distribution and (iv) gel firmness. However, despite these similarities, gels from co-culture ST1+2 (5/1) showed worse texture defects as compared with ST1+3 (5/1).

These results highlight the fact that the inhibitory effect of a strain against the appearance of texture defects is not correlated to the acidification rate or EPS content, but that it could instead be explained by differences in the structural properties of EPS and/or in the morphology of bacterial chains.

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Figure legends

Fig. 1. Kinetics of acidification monitored during skim milk fermentation by cultures ST1 (— ▪ ▪ —), ST2 (——), ST1+2 (1/1.7) (▪▪▪▪▪), ST1+2 (5/1) (— —), ST3 (——), ST1+3 (1/1.7) (▪▪▪▪▪), ST1+3 (5/1) (— —). Skim milk suspensions were pasteurised at 95 °C for 6 min.

Fig. 2. Quantity of EPS (mg glucose kg^{-1}) produced by the 7 cultures/co-cultures (ST1, ST2, ST1+2 (1/1.7), ST1+2 (5/1), ST3, ST1+3 (1/1.7), ST1+3 (5/1)) during fermentation of skim milk pasteurised at 95 °C for 6 min. Mean values ± standard deviation (n = 3). Values affected with different letters were significantly different for $p = 0.05$.

Fig. 3. Macromolecular properties of extracted EPS from the 7 cultures/co-cultures (ST1, ST2, ST1+2 (1/1.7), ST1+2 (5/1), ST3, ST1+3 (1/1.7), ST1+3 (5/1): A, average molar mass; B, molar mass distribution in mass; C, molar mass distribution in number of particles; D, intrinsic viscosity. The EPS were classified in high molar mass (□) or low molar mass (□).

Fig. 4. Graininess defect of acid milk gels made by 6 different cultures [ST1, ST2, ST1+2 (1/1.7), ST1+2 (5/1), ST1+3 (1/1.7), ST1+3 (5/1)]. Grains were classified by their diameters: 1–1.5 mm (□), 1.5–2 mm (□), 2–2.5 mm (□), 2.5–3 mm (□) and > 3 mm (□). Mean values ± standard deviation (n = 9).

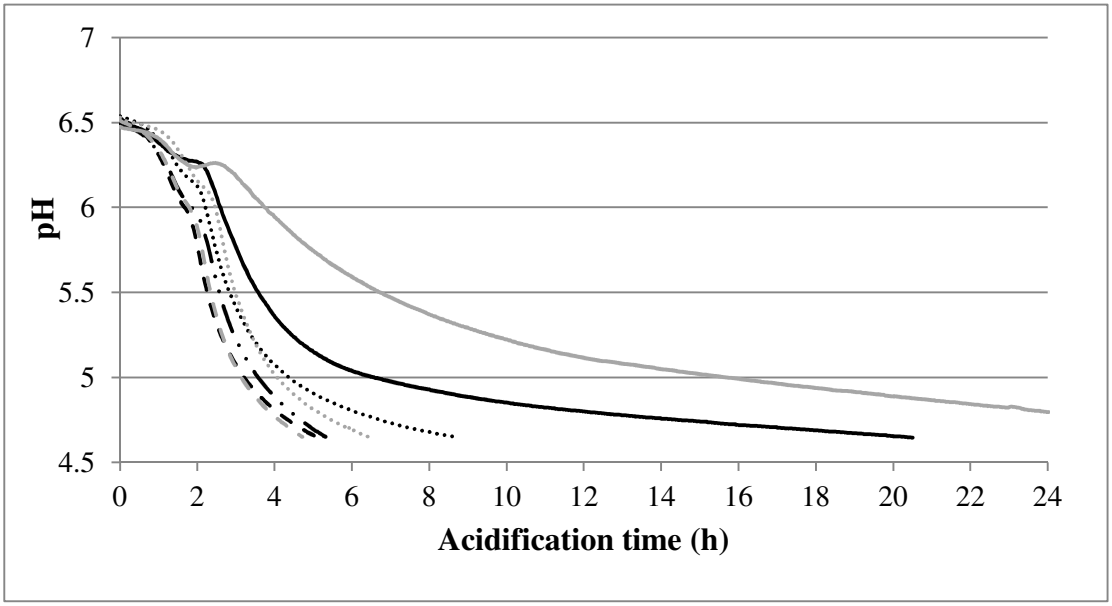

7
6.5
6
5.5
5
4.5
pH
0
2
4
6
8
10
12
14
16
18
20
22
24
Acidification time (h)

Figure 1

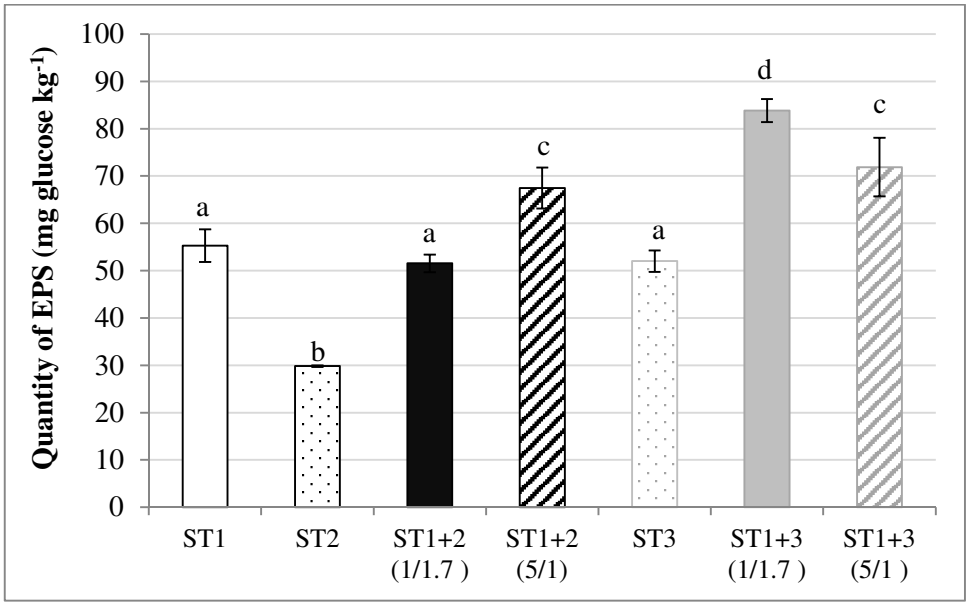
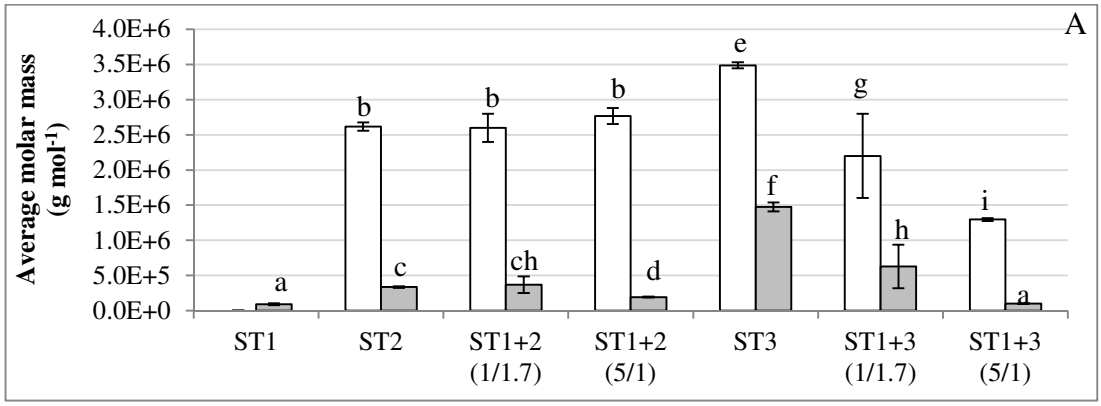
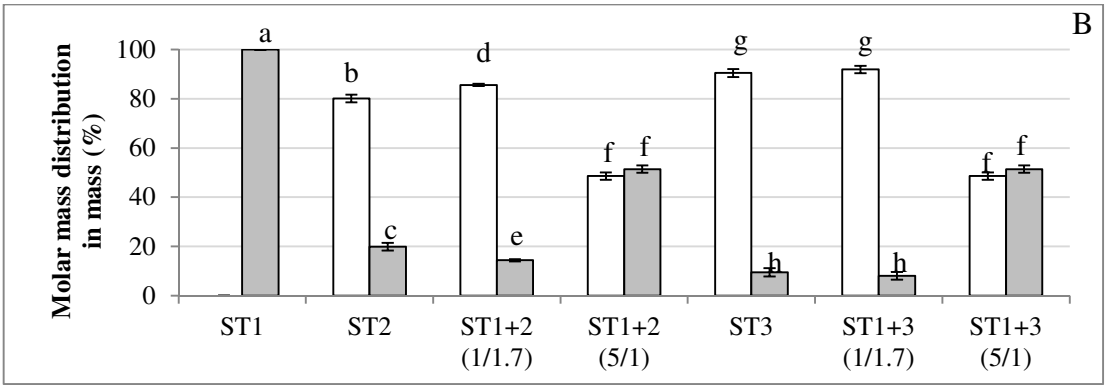
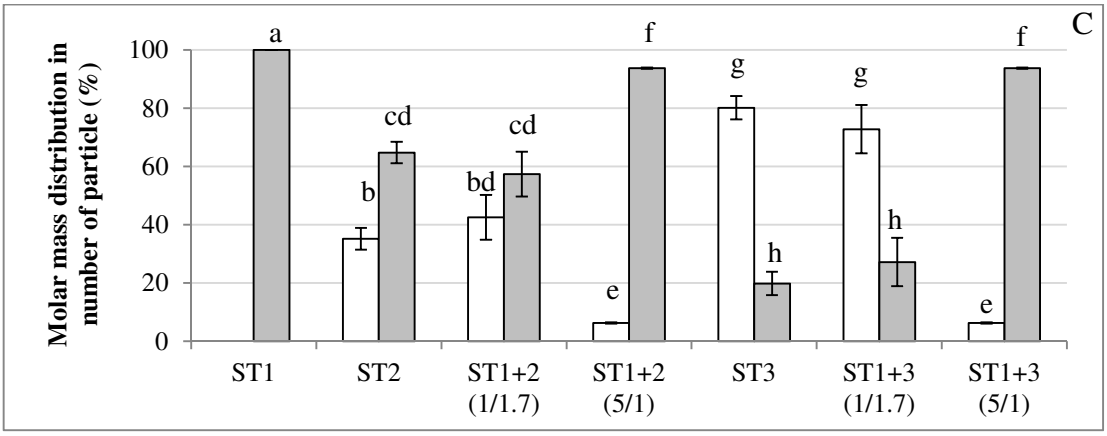

Quantity of EPS (mg glucose kg-1)
100
90
80
70
60
50
40
30
20
10
0
a
b
a
c
a
d
c
ST1
ST2
ST1+2 (1/1.7)
ST1+2 (5/1)
ST3
ST1+3 (1/1.7)
ST1+3 (5/1)

Figure 2


A
Average molar mass (g mol-1)
4.0E+6
3.5E+6
3.0E+6
2.5E+6
2.0E+6
1.5E+6
1.0E+6
5.0E+5
0.0E+0
a
b
c
b
ch
b
d
e
f
g
h
i
a
ST1
ST2
ST1+2 (1/1.7)
ST1+2 (5/1)
ST3
ST1+3 (1/1.7)
ST1+3 (5/1)


B
Molar mass distribution in mass (%)
100
80
60
40
20
0
a
b
c
d
e
f
f
g
h
g
h
f
f
ST1
ST2
ST1+2 (1/1.7)
ST1+2 (5/1)
ST3
ST1+3 (1/1.7)
ST1+3 (5/1)


C
Molar mass distribution in number of particle (%)
100
80
60
40
20
0
a
b
cd
bd
cd
e
f
g
h
g
h
e
f
ST1
ST2
ST1+2 (1/1.7)
ST1+2 (5/1)
ST3
ST1+3 (1/1.7)
ST1+3 (5/1)

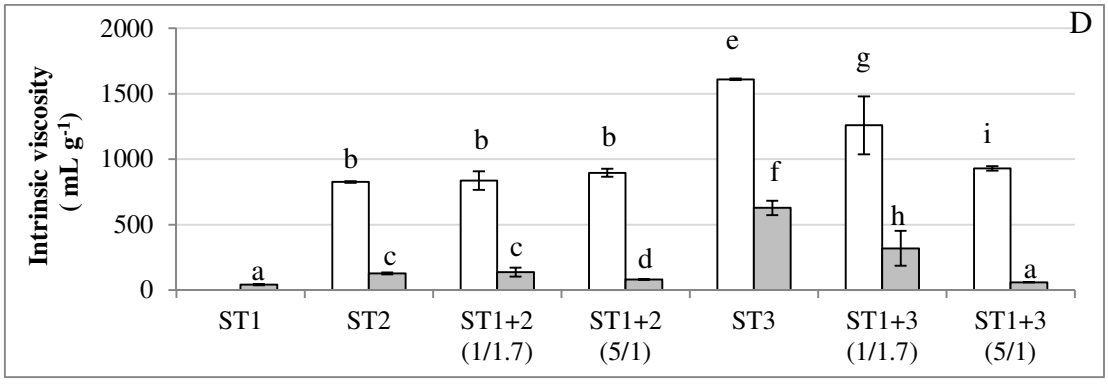

D
Intrinsic viscosity (mL g-1)
2000
1500
1000
500
0
a
b
c
b
c
b
d
e
f
g
h
i
a
ST1
ST2
ST1+2 (1/1.7)
ST1+2 (5/1)
ST3
ST1+3 (1/1.7)
ST1+3 (5/1)

Figure 3

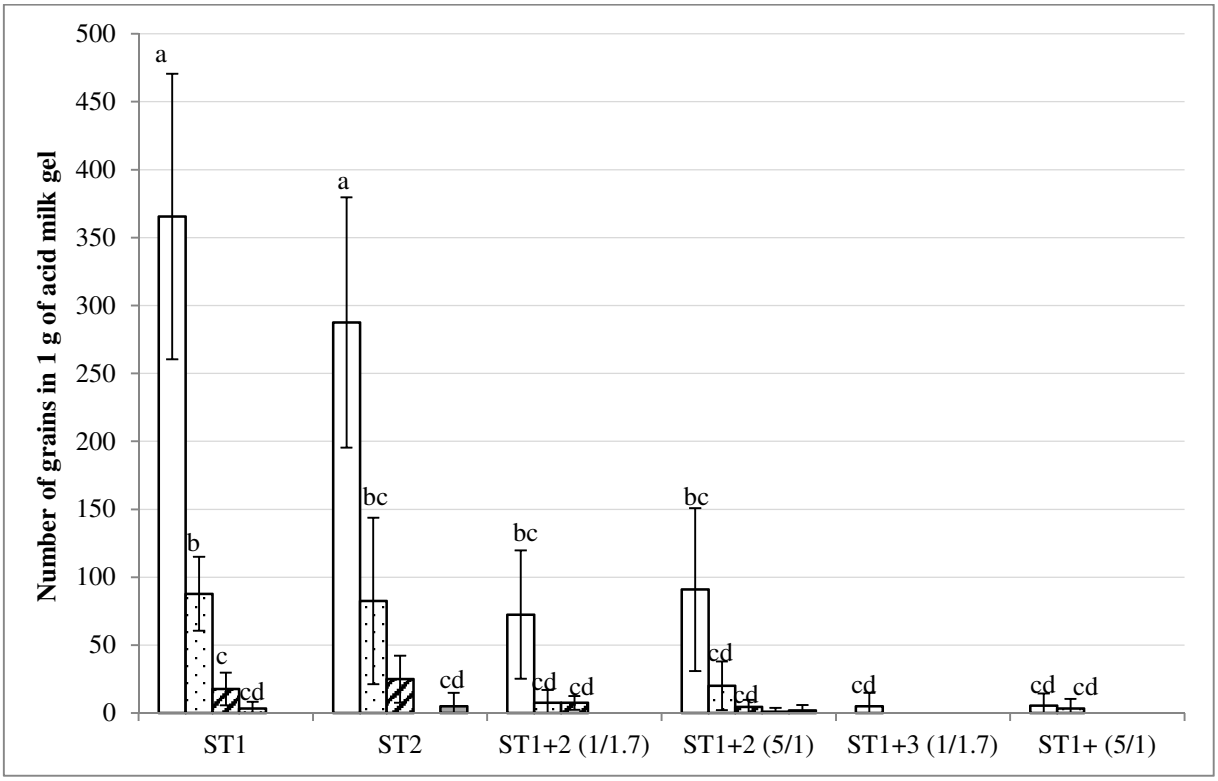

Number of grains in 1 g of acid milk gel
500
450
400
350
300
250
200
150
100
50
0
a
b
c
cd
a
bc
cd
bc
cd
cd
bc
cd
cd
cd
cd
cd
ST1
ST2
ST1+2 (1/1.7)
ST1+2 (5/1)
ST1+3 (1/1.7)
ST1+ (5/1)

Figure 4

Table 1

Acidification parameters of skim acid milk gel obtained using 3 pure strain cultures (ST1, ST2, ST3) and 4 co-cultures [ST1+2 (1/1.7), ST1+2 (5/1), ST1+3 (1/1.7), ST1+3 (5/1)]. [a]

Parameters	ST1	ST2	ST1+ST2		ST3	ST1+ST3	
			1/1.7	5/1		1/1.7	5/1
Acidification							
Acidification time (min)	348 ± 49^{ad}	1351 ± 109^{b}	528 ± 33^{c}	304 ± 24^{a}	ND	395 ± 27^{d}	287 ± 11^{a}
V_{max} (10^{-3} pH unit min^{-1})	17.1 ± 1.7^{a}	14.0 ± 0.3^{b}	16.9 ± 1.3^{a}	20.0 ± 0.3^{c}	6.5 ± 0.1^{d}	18.1 ± 1.7^{ac}	20.0 ± 0.1^{c}
Gelation							
Time of gel point (min)	206 ± 13^{a}	388 ±36^{c}	208 ± 10^{a}	157 ± 11^{b}	371 ±18^{c}	179 ± 11^{b}	164 ± 10^{b}
pH of gel point	5.16 ± 0.05^{a}	5.15 ±0.05^{a}	5.23 ± 0.01^{a}	5.21 ± 0.05^{a}	5.57 ± 0.01^{c}	5.39 ± 0.06^{b}	5.22 ± 0.01^{a}

[a] Reconstituted skim milk was pasteurised at 95 °C, 6 min. V_{max} is the maximum acidification rate. Data were obtained by monitoring the pH evolution (acidification) and rheological parameters (gelation) during fermentation at 43 °C; values of each characteristic affected with different letters were significantly different for $p = 0.05$ (ND, not determined).

Table 2

Textural parameters of pasteurised acid milk gel using 6 different cultures [a]

Cultures	ST1	ST2	ST1+ST2		ST1+ST3	
			1/1.7	5/1	1/1.7	5/1
Firmness (N)	3.36 ± 0.18 [a]	6.44 ± 0.15 [d]	4.78 ± 0.08 [c]	4.14 ± 0.13 [b]	4.43 ± 0.25 [b]	3.85 ± 0.11 [e]
Viscosity (mPa s) at $\gamma = 300\ s^{-1}$	138 ± 12 [a]	186 ± 4 [c]	193 ± 3 [c]	164 ± 2 [b]	301 ±10 [e]	239 ± 15 [d]
Spontaneous syneresis (%, w/w)	1.8 ± 0.3 [a]	6.3 ± 0.3 [c]	2.7 ± 0.4 [b]	1.4 ± 0.4 [a]	0.4 ± 0.2 [e]	0 [d]

[a] Reconstituted skim milk was pasteurised at 95 °C for6 min; values of each characteristic affected with different letters were significantly different at $p = 0.05$.