Gel formation and rheological properties of fermented milk with in situ exopolysaccharide production by lactic acid bacteria

Gentès, St-Gelais, Sylvie Turgeon

Dairy Sci. & Technol. (2011) 91:645–661
DOI 10.1007/s13594-011-0039-0

ORIGINAL PAPER

Gel formation and rheological properties of fermented milk with in situ exopolysaccharide production by lactic acid bacteria

Marie-Claude Gentès · Daniel St-Gelais · Sylvie L. Turgeon

Received: 1 February 2011 / Revised: 28 June 2011 / Accepted: 1 July 2011 /
Published online: 11 August 2011

Abstract Exopolysaccharides (EPS) produced by lactic acid bacteria can be used as natural stabilizers in fermented milk. The ability of EPS to modulate viscosity has not been correlated to their concentration but rather to their structure and their interactions with other milk compounds. The development of a product with desirable characteristics requires knowledge of the EPS structure–function relationship. The aim of this study was to compare the influence of various EPS structures on the gel formation and rheological/physical properties (firmness, apparent viscosity, elastic modulus, syneresis) of fermented milk. Three *Streptococcus thermophilus*—HC15 (control); 0131 (neutral, flexible, branched EPS); 2104 (anionic, stiff, linear EPS)—and four *Lactobacillus delbrueckii* subsp. *bulgaricus*—210R (control); 11842 (neutral, flexible, branched EPS); 702074 (neutral, flexible, branched EPS); 291 (neutral, stiff, branched EPS)—were compared. Strains were grown at 42 °C in skim milk until pH reached 4.6. Gel formation and elastic modulus were modified by the anionic and linear EPS from strain 2104 as compared with the other strains. Higher values for apparent viscosity, firmness and whey retention were obtained with strains 0131, 2104 and 291 producing EPS with linear or few branching and high molecular weight. This work showed that the gel formation and rheological/physical properties of fermented milk are modified by the structural characteristics of

M.-C. Gentès · D. St-Gelais · S. L. Turgeon (✉)
Dairy Science and Technology Research Centre (STELA), Institute of Nutraceuticals and Functional Foods (INAF), 2425 De l'Agriculture Street, Pavillon Paul-Comtois, Room 1316, Université Laval, Quebec City, Quebec, Canada G1V 0A6
e-mail: sylvie.turgeon@fsaa.ulaval.ca

M.-C. Gentès · D. St-Gelais
Food Research and Development Centre, Agriculture and Agri-Food Canada, 3600 Casavant Boulevard West, Saint-Hyacinthe, Quebec, Canada J2S 8E3

EPS, especially negative charge, flexibility, degree of branching and molecular weight. This study contributes to a better understanding of the EPS structure–function relationship in view to provide tools to develop fermented products with desirable characteristics.

乳酸菌胞外多糖对发酵乳凝胶形成和流变特性的影响

摘要 乳酸菌产生的胞外多糖(EPS)是发酵乳中天然的稳定剂。EPS调解黏度的能力与EPS的浓度无关,而与EPS的结构和EPS与乳成分相互作用的程度有关。因此将EPS用于乳制品中首先要了解EPS结构与功能之间的关系。本文对比了不同EPS结构对发酵乳凝胶的形成和流变/物理特性(硬度、表观粘度、弹性模量、收缩作用)的影响。选择了不同来源的EPS进行了比较,其中3株*Streptococcus thermophilus*中HC15 (对照组)、0131(产中性、柔软、分枝EPS)、2104(产阴离子、硬的、线性EPS),4株*Lactobacillus delbrueckii* subsp. *Bulgaricus*中210R (对照组)、11842 (产中性、柔软、分枝EPS)、702074 (产中性、柔软、分枝EPS), 291 (产阴离子、硬的、线性EPS)。所有菌株在脱脂乳中培养(42 °C)到pH4.6。用菌株2104所产的阴离子和线性的EPS来修正凝胶形成和弹性模量,并与其他菌株进行对比,能够产生线性或者少量分枝及高分子量EPS的菌株0131、2104和291所产生的EPS具有较高的表观粘度、硬度和乳清保持力。本研究结果表明通过调整EPS的结构特性,如负电荷、弹性、分枝度和分子量,可以改变发酵乳的凝胶形成和流变/物理特性。因此,基于EPS结构与功能之间的关系,将有助于发酵乳制品的质量改善。

Keywords Exopolysaccharide · Gel formation · Rheological/physical properties · Fermented milk

关键词 胞外多糖 · 凝胶形成 · 流变/物理特性 · 发酵乳

1 Introduction

Lactic acid bacteria (LAB) can produce a wide range of exopolysaccharides (EPS) during milk fermentation. Because of their ability to bind water and modulate viscosity, EPS have been intensely studied for their potential as natural stabilizers in fermented milk. No clear correlation between EPS concentration and the positive effect of these polymers on the rheological properties of fermented milk has been found (Petry et al. 2003; van Marle and Zoon 1995; Cerning et al. 1986). It has been postulated that the EPS structure (monosaccharide composition, charge, molecular weight, degree of branching and rigidity of backbone) and the interactions of EPS with milk components, especially milk proteins, could be responsible for the various properties that have been reported (Hassan 2008; Folkenberg et al. 2006; Ruas-Madiedo et al. 2002). The production of fermented milk with desirable rheological properties requires comprehension of the EPS structure–function relationship. Some research has been done regarding the EPS produced in situ by LAB in fermented milk. For instance, a comparative study of two EPS from *Streptococcus thermophilus* strains with an identical structure (monosaccharide composition, degree of branching, charge and rigidity of backbone) but different molecular weight has shown a positive correlation between high molecular weight and viscosity (Faber et al. 1998). Petry et al. (2003) partially characterized the EPS produced by four *Lactobacillus delbrueckii* subsp. *bulgaricus* strains. In that study, a

strong correlation was also observed between high molecular weight and viscosity. Girard and Schaffer-Lequart (2007) compared the influence of three EPS from mesophilic and thermophilic LAB on the gelation process and the resistance to shearing of fermented milk. Negative charge seemed to be the most important structural characteristic in terms of increasing the resistance to shearing of fermented milk.

Almost all of the studies in the literature were limited to the effect of EPS structure on the viscosity of fermented milk. For a better understanding of the principles underlying the EPS structure–function relationship, it is important to measure the rheological properties (viscosity, firmness and elastic modulus) and syneresis of fermented milk in addition to taking traditional viscosity measurements. This information should make it possible to determine which structural characteristics are important in terms of modulating a given rheological property. Furthermore, a comparison of several EPS with well-known structures produced in situ by LAB in the same study under the same fermentation conditions has never been carried out. The aim of this work was to study the effect of EPS structures from five thermophilic EPS-producing LAB on the gel formation, rheological properties (apparent viscosity, firmness and elastic modulus) and physical properties (syneresis) of fermented milk. Strains were selected based on their known EPS structural characteristics (charge, stiffness, degree of branching and more). EPS production was also determined during the fermentation process.

2 Materials and methods

2.1 Bacterial strains, media and growth conditions

Three *S. thermophilus* strains—namely, HC15 (purchased from Chr. Hansen, Mississauga, ON, Canada), NIZO0131 (**0131**) and 2104 (**2104**, obtained from NIZO Food Research, Ede, the Netherlands)—and four *L. delbrueckii* subsp. *bulgaricus* strains—namely, 210R (purchased from Waterford, Gist Brocades, Millville, UT, USA), ATCC11842 (**11842**, purchased from ATCC, Manassas, VA, USA), NCIMB702074 (**702074**, obtained from NCIMB, Aberdeen, Scotland, UK) and DGCC291 (**291**, graciously lent by Danisco, Buxière, Orsay, France)—were used in this study. The structural characteristics of the EPS have been determined previously in other studies (Robitaille et al. 2009; Van Calsteren et al. 2008; Harding et al. 2005; Lamboley et al. 2003; Faber et al. 2001a, b, 2002) and are presented in Table 1. Strains HC15 and 210R were chosen for their potential as non-EPS-producing strains and were designated as the controls. Genotype was confirmed (>97% identification) by biomolecular techniques (PCR; Tabasco et al. 2007). Reconstituted skim milk (RSM) from low-heat skim milk powder (34.4% total protein and 98% dry matter) was used for the culture media (Crino, Agropur, Granby, QC, Canada). The strains were stored at −80 °C in 20% (*w*/*w*) RSM from skim milk powder rehydrated in distilled water supplemented with 5% (*w*/*w*) sucrose (Fisher Scientific, Nepean, ON, Canada) and 0.35% (*w*/*w*) ascorbic acid (Sigma-Aldrich, Toronto, ON, Canada) and sterilized at 110 °C for 10 min.

A 12% (*w*/*w*) RSM was prepared by dissolving skim milk powder in distilled water and stirring for 2 h at room temperature. The RSM preparation was sterilized at 110 °C for 10 min in an autoclave and then stored at 4 °C until use. Active strains were

Table 1 Structural characteristics of EPS produced by *S. thermophilus* (HC15, 0131, 2104) or *L. delbrueckii* subsp. *bulgaricus* (210R, 11842, 702074, 291)

Strain	Structural characteristics						
	Sugar composition	Sugar ratio	Charge	Molecular weight ($g.mol^{-1}$)	Branching[a]	Flexibility	References
HC15	Control strain						Lamboley et al. (2003)
0131	Galactose/rhamnose	2:1	Neutral	5.9×10^6	+	Flexible	Faber et al. (2001b)
2104	Galactose/ribose/ glucose/*N*-acetyl[b]	2:1:1:1	Negative	NA[c]	−	Stiff	Faber et al. (2002)
210R	Control strain						Robitaille et al. (2009)
11842	Galactose/glucose	3:1	Neutral	1.7×10^6	+	+/− Flexible	Van Calsteren et al. (2008)
702074	Galactose/glucose	4:3	Neutral	1.8×10^6	++	Flexible	Harding et al. (2005)
291	Galactose/glucose	2:3	Neutral	1.4×10^6	+	Stiff	Faber et al. (2001a)

NA information not available

[a] Branching: linear (−), one branching (+), more than two branching (++)

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

prepared by inoculating the culture stock at 10% (*v*/*v*) in the RSM. Incubation was carried out at 37 °C for 16 h to obtain a population of 1×10^8 CFU.mL^{-1}. For strains 0131 and 2104, a 3% (*v*/*v*) subcultivation (42 °C for 6 h) was also necessary to achieve a population of 1×10^8 CFU.mL^{-1}. All active strains were stored overnight at 4 °C before use. Lactobacilli and streptococci were enumerated on acidified MRS and M17 agar medium, respectively (Difco Laboratories, Detroit, MI, USA) and incubated under anaerobic condition for 48 h at 37 °C. Milk acidified with 1.4% (*w*/*w*) glucono-δ-lactone (GDL; Sigma-Aldrich) was used as an EPS-free control.

2.2 Ropy character of exopolysaccharides in milk during fermentation

The ropy character of EPS according to pH (pH 5.7, 5.5 and 5.3±0.05) was monitored during fermentation consisting in the measurement of the time required for 30 mL of fermented milk (in duplicate) to pass through a plastic funnel (33-cc reservoir, no. WAT011390, Waters, Mississauga, ON, Canada) at room temperature. The ropy character of EPS was also confirmed by the formation of filaments between the fermented milk (pH 5.7, 5.5 and 5.3) and a sterile inoculating loop, as described by Hess et al. (1997). A volume of 50 mL of pre-warmed (42 °C) RSM was inoculated at 3% (*v*/*v*) with the active strains. RSM acidified at pH 5.7, 5.5 and 5.3 with 0.4%, 0.5% and 0.8% (*v*/*v*) GDL were used as controls. During the fermentation, samples at the desired pH were rapidly immersed in ice (for 30 min) to stop fermentation and then left at room temperature (for 30 min) before the start of the experiments. For EPS quantification, 10 mL (in duplicate) of each fermented milk was frozen at −20 °C until use.

2.3 Exopolysaccharide production

EPS production was quantified at different pH during the fermentation as described by Robitaille et al. (2009) with slight modifications. Milk chemically acidified at pH 5.7, 5.5, or 5.3 with 10% (*v*/*v*) lactic acid (Fisher Scientific) were used as controls for EPS quantification. For protein removal, equal volumes of 40% (*w*/*v*) trichloroacetic acid (Laboratoire Mat, Quebec City, QC, Canada) were added to the milk samples at room temperature and the mixtures boiled for 5 min. The samples were centrifuged for 10 min at 3,700×*g* (21 °C). The supernatant was removed and the pellet washed with 10 mL 20% (*w*/*v*) trichloroacetic acid and then centrifuged as described above. The supernatants were pooled and 10-g amounts were dialyzed in 1 L distilled water for 72 h at 4 °C (6,000- to 8,000-g.mol^{-1} MWCO membrane, Spectra/Por). The water was changed twice a day. The samples were weighed after dialysis to evaluate dilution. Total sugar content (milligrams glucose per litre fermented milk) was quantified by the phenol–sulphuric acid method using glucose as the standard (Dubois et al. 1956). Total sugar in the chemically acidified milk and the EPS-free control milk was 10 mg.L^{-1}. This value was subtracted from all data. The protein content of each dialyzed sample was quantified as described by Bradford (1976).

2.4 Gel formation of fermented milk

The gel formation of each strain was recorded by monitoring the elastic modulus (*G*′) over time with an AR1000 dynamic rheometer (TA Instruments, New Castle, DE,

USA). Rugged plate geometry (4 cm) was used to minimize the effect of slippage due to syneresis. To avoid contamination, the plates were disinfected with ethanol (70%) prior to each experiment. Pre-warmed volumes of RSM (100 mL) at 42 °C were inoculated at 3% (*v*/*v*) with every strain. For EPS-free control, milk was acidified with 1.4% (*w*/*w*) GDL. A 3-mL volume of the inoculated or acidified RSM was used to monitor the changes in G' over 8–10 h as described by Girard and Schaffer-Lequart (2007). To limit dehydration of the samples, a solvent trap was used. The remaining volume of the inoculated RSM was used to monitor pH changes over time by means of a glass electrode and a standard pH meter (model 140, Corning, Fisher Scientific). Gel point was defined as the time (T_{gel}) and the pH (pH_{gel}) when G' was >1 Pa (van Marle and Zoon 1995).

2.5 Rheological and physical measurements of fermented milk

New pre-warmed (42 °C) volumes of RSM (1.2 L) were inoculated at 3% (*v*/*v*) with every strain. For EPS-free control, milk was acidified with 1.4% (*w*/*w*) GDL. Inoculated or acidified RSM (90 mL) was transferred into 100-mL plastic cups (no. 14-375-148, Fisher Scientific), and fermentation was carried out at 42 °C. Fermented milk at pH 4.6±0.05 (to simulate the pH value of yoghurt) was stored at 4 °C overnight before the rheological measurements. The hardness was measured with a TA-XT2 texture analyzer (Texture Technology Corp., Scarsdale, NY, USA), as described by Hess et al. (1997). Analyses were conducted at 4 °C on five different plastic cups using a cylindrical geometry made in Plexiglass with a flat base (25 mm in diameter). Maximum peak force (Newton) at a displacement of 20 mm was recorded as firmness (Newton·per square metre). To evaluate viscosity, a steady stress sweep test with a shear stress of 1–100 Pa was carried out with a dynamic stress rheometer using a bob and cup (Rheometric, model SR-2000, TA Instruments). Apparent viscosity at 10 and 100 s^{-1} was calculated according to the power law model (Everett and McLeod 2005). A dynamic stress sweep test was used to determine the linear viscoelastic region of yoghurt at an oscillation of 1 Hz and under an increasing strain from 0.02 to 30 Pa (Everett and McLeod 2005). The viscoelastic properties were measured at 1 Pa (linear viscoelastic region) between 0.1 and 10 Hz with the dynamic stress rheometer, also using a bob and cup. A new sample was used for each rheological test and taken with the stainless cylinder. Measurements were performed at 4 °C in duplicate. Susceptibility to syneresis was quantified by a centrifugation technique (Everett and McLeod 2005). Samples (25 g), in duplicate, were taken directly from the plastic cups with a homemade stainless cylinder to minimize disruption of the gel and were centrifuged at 210×*g* (20 min, 4 °C). The clear supernatant was poured off, weighed and recorded as syneresis (in percent).

2.6 Statistical methods

A completely randomized design was applied to compare the ropy character and the EPS production for every strain and to determine the effect of EPS structures on the gel formation and rheological/physical properties of fermented milk. Significant differences were tested at $P \leq 0.05$. Statistical analysis was carried out with ANOVA

using the general linear models procedure of SAS (version 9.1.3, Cary, NC, USA). All experiments were performed in triplicate.

3 Results

3.1 Acidification profile during fermentation

The acidification profile of the milk differed between the strains and GDL (Fig. 1). Initial pH (6.38±0.03) did not differ significantly between the strains and GDL. After storage overnight at 4 °C, the pH of all the fermented milk did not vary significantly (pH 4.63±0.03) as compared with the pH obtained directly at the end of fermentation (pH 4.64±0.01). Acidification was faster in the GDL milk (255 min). The streptococci had significantly longer fermentation times compared with the lactobacilli. The milk fermented with HC15 control strain (630 min) had significantly faster fermentation times than those fermented with strains 0131 (740 min) and 2104 (805 min). The milk fermented with lactobacilli strain 702074 (205 min) had a significantly shorter fermentation time than the milk fermented with control strain 210R (340 min), strain 11842 (350 min) and strain 291 (340 min).

3.2 Ropy character and exopolysaccharide production during fermentation

Evaluation of the ropy character of the EPS in milk is presented in Table 2. A significant interaction between strains and pH was observed for flow times ($P<0.05$). The milk fermented with the control strains (HC15 and 210R) and strains 11842 and 702074 took 12–15 s to pass through the funnel, no matter the pH. In addition, no filaments were observed (Table 2). These strains were considered to be non-ropy. For the milk fermented with strains 0131 and 291, the time needed to pass through the funnel increased significantly as the pH decreased. The formation of filaments was observed with these strains for each pH. The time needed to pass

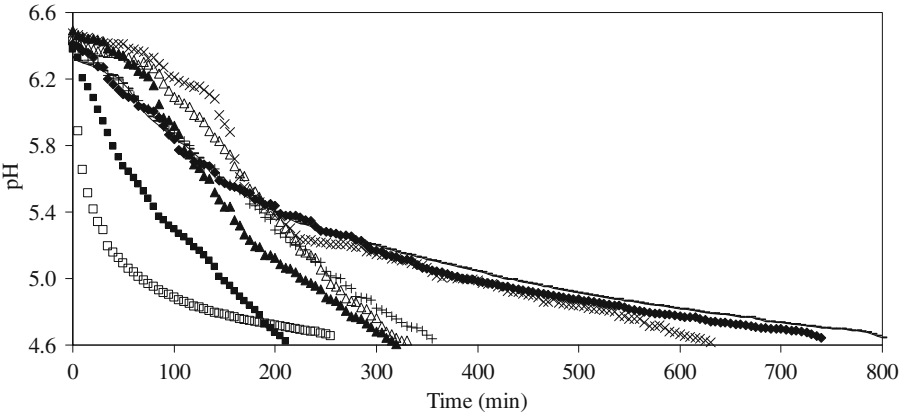

Fig. 1 Change of pH upon time of milk fermented with *S. thermophilus* HC15 (*ex mark*), 0131 (*diamond*) and 2104 (*dashed line*) and *L. delbrueckii* subsp. *bulgaricus* 210R (*filled triangle*), 11842 (*plus sign*), 702074 (*filled square*), and 291 (*empty triangle*) or acidified with GDL (*empty square*) during fermentation at 42 °C. Each data point is the mean of three experiments

Table 2 Ropy character of EPS in milk fermented with *S. thermophilus* (HC15, 0131, 2104) and *L. delbrueckii* subsp. *bulgaricus* (210R, 11842, 702074, 291) or acidified with GDL by measuring the time of 30 mL of milk to pass through a funnel according to pH during fermentation process

Strain	pH	Flow time (s)	Ropy character* in milk	EPS production (mg L^{-1})
HC15	5.7	13^{e}	−	n.d.
	5.5	13^{e}	−	46^{bc}
	5.3	12^{e}	−	52^{b}
0131	5.7	52^{d}	+	53^{b}
	5.5	58^{d}	+	71^{a}
	5.3	97^{b}	+	78^{a}
2104	5.7	13^{e}	−	29^{c}
	5.5	13^{e}	+	33^{c}
	5.3	17^{e}	+	45^{bc}
210R	5.7	13^{e}	−	n.d.
	5.5	12^{e}	−	n.d.
	5.3	11^{e}	−	53^{b}
11842	5.7	13^{e}	−	n.d.
	5.5	13^{e}	−	26^{d}
	5.3	13^{e}	−	60^{ab}
702074	5.7	12^{e}	−	n.d.
	5.5	14^{e}	−	n.d.
	5.3	14^{e}	−	36^{c}
291	5.7	53^{d}	+	20^{d}
	5.5	82^{c}	+	33^{c}
	5.3	153^{a}	+	38^{c}
GDL	5.7	12^{e}	−	n.d.
	5.5	13^{e}	−	0^{e}
	5.3	Gel**	−	0^{e}

Means within a line followed by different letters differ ($P<0.05$)

n.d. not determined

* +=visible filament formation by touching the milk with a sterile inoculating loop (ropy); −=no visible filament (non-ropy)

** Gel: visible gel formed at this pH

though the funnel was significantly longer for strain 291, especially at pH values below 5.5. Strain 2104 had a different profile from the other ropy strains. At pH 5.7, no filaments were visible, unlike at pH 5.5 and 5.3 where visible filaments were obtained. However, the time required to pass through the funnel was not affected by the presence of these filaments. At pH 4.6, the ropy character of EPS was no longer observable in the milk fermented with strain 2104, whereas the ropy character was still observable with strains 0131 and 291 (data not shown). No significant difference between the control strains (HC15 and 210R) or GDL was observed at pH 5.7 and 5.5. At pH 5.3, however, a gel formed in the GDL milk.

For all strains, no EPS were quantified immediately after inoculation (data not shown). At pH 5.3, EPS production ranged between 36 and 78 mg.L^{-1} (Table 2). No sugar was quantified in the GDL milk (Table 2) or in the milk chemically acidified with lactic acid at pH 5.7, 5.5 and 5.3 (data not shown). Significantly higher quantity of EPS was measured in fermented milk with 0131 and 11842 strains. For all the strains and all the milk samples taken at different times during fermentation, the protein content in the dialyzed fermented milk samples was very low (<0.9 mg.L^{-1}). Extraction with trichloroacetic acid successfully removed most of the initial protein content. Consequently, the protein was not considered to interfere in the phenol–sulphuric acid method. No further corrections were applied to the data.

3.3 Gel formation and rheological/physical properties of fermented milk

The gel formation during acidification of milk with all strains and GDL is presented in Fig. 2. The onset of gel formation (pH_{gel}) occurred between pH 5.54 and 5.50, except for 2104 strain that formed gel at pH 5.0. The formation of gel in milk acidified with GDL occurred at pH 5.28.

Apparent viscosity was significantly affected by strains and GDL (Table 3). The milk fermented with strains 0131, 291 and, especially, 2104 had the highest apparent viscosity values at 10 s^{-1}. The milk fermented with GDL had an intermediate value. For the milk fermented with the control strains (HC15 and 210R), the apparent viscosity was not significantly different (2.88 Pa s), whereas the milk fermented with strains 11842 and 702074 had the lowest values (2.41 and 2.67 Pa s, respectively). Apparent viscosity at 100 s^{-1} decreased for all strains and GDL as compared with 10 s^{-1}. The milk fermented with strains 0131 and 2104 still had the highest apparent viscosities. In contrast, the apparent viscosity of strain 291 at a high shear rate decreased significantly. The apparent viscosity values for the milk fermented with strains HC15 and 210R and with GDL were not significantly different (2.20–

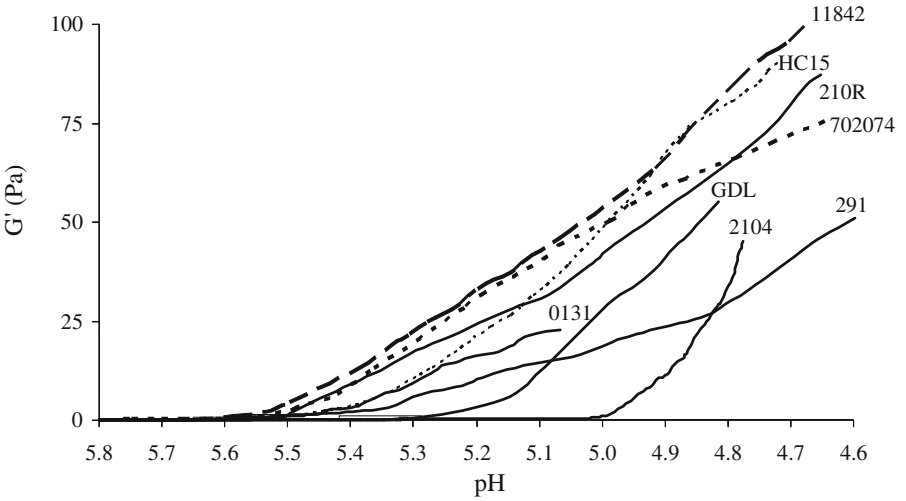

Fig. 2 Change of elastic modulus (*G'*) upon acidification of milk with *S. thermophilus* HC15, 0131 and 2104; *L. delbrueckii* subsp. *bulgaricus* 210R, 11842, 702074, and 291; or with glucono-δ-lactone (*GDL*) during fermentation at 42 °C. Each data point is the mean of three experiments

Table 3 Firmness, apparent viscosity and syneresis of milk fermented with *S. thermophilus* (HC15, 0131, 2104) and *L. delbrueckii* subsp. *bulgaricus* (210R, 11842, 702074, 291) or acidified with GDL at pH 4.6

Parameter	Strain							GDL	SEM
	HC15	0131	2104	210R	11842	702074	291		
Firmness ($N.m^{-2}$)	1185^{bc}	1314^{ab}	1253^{b}	1023^{cd}	932^{d}	892^{d}	1435^{a}	1189^{c}	60.1
Apparent viscosity at 10 s^{-1} (Pa.s)	2.88^{d}	4.03^{b}	4.65^{a}	2.88^{d}	2.41^{e}	2.67^{de}	3.45^{c}	3.16^{c}	0.11
Apparent viscosity at 100 s^{-1} (Pa.s)	2.22^{cd}	2.36^{b}	2.60^{a}	2.20^{cd}	2.01^{ef}	2.15^{ed}	1.93^{f}	2.33^{bc}	0.05
Syneresis (%) at 210×*g*	23.67^{a}	7.13^{d}	11.26^{cd}	14.72^{bc}	17.96^{b}	13.26^{bc}	1.43^{e}	16.25^{bc}	1.93

Means within a row followed by different letters differ ($P<0.05$)

SEM standard error of the mean

2.33 Pa s). The milk fermented with strains 11842 and 291 had the lowest apparent viscosities. The firmness of the fermented milk was significantly affected by strains and GDL (Table 3). The assessed firmness values were significantly higher for strains 0131, 2104 and 291 than for the other strains and for GDL. Firmness values were not significantly different among milk fermented with the control strains (HC15 and 210R) and GDL. The milk fermented with strains 11842 and 702074 had the lowest firmness values.

The ability of strains and GDL to limit syneresis in fermented milk is presented in Table 3. The milk fermented with strain 0131 and, especially, strain 291 had a greater ability to decrease syneresis under milder centrifugation conditions (210×*g*). The whey expelled from the milk fermented with strains 0131 and 291 was ropy (data not shown).

The elastic modulus (*G*′) of the fermented milk is presented in Fig. 3. *G*′ values significantly increased at higher frequencies. The viscous modulus (*G*″) was not presented because the *G*″ values had a similar profile to the *G*′, with the exception that the values were lower for all strains and GDL. A *G*′ value that is higher than the *G*″ indicates the elastic or solid-like character of a gel. Between 0.1 and 10 Hz, the *G*′ values did not differ significantly between the milk fermented with GDL and strains HC15, 2104 and 210R. The *G*′ values for the milk fermented with strains 0131, 11842, 702074 and 291 were similar but lower than the values obtained for the other strains and GDL.

4 Discussion

4.1 Exopolysaccharide production and functionality

This study aimed to investigate the structure–function relationship of EPS produced in situ by LAB in fermented milk. HC15 and 210R strains were chosen as the

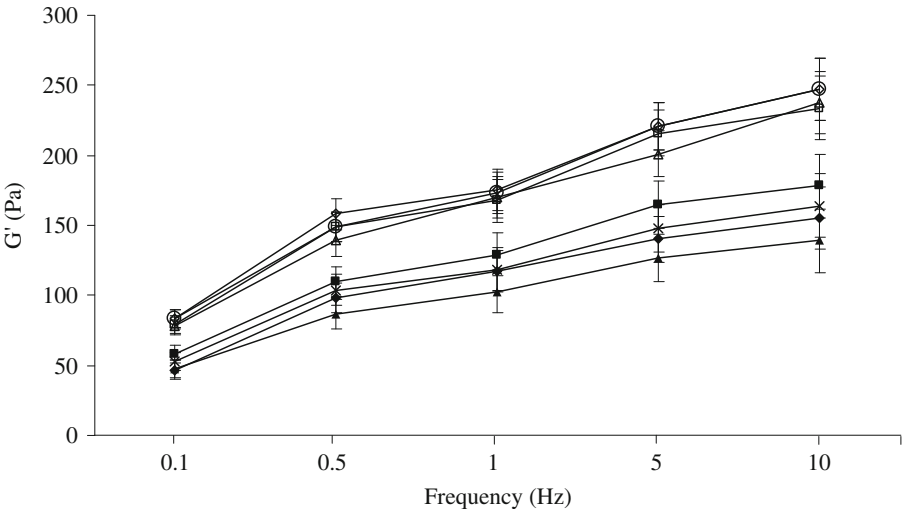

Fig. 3 Elastic modulus (*G*′) as a function of frequency of milk fermented with *S. thermophilus* HC15 (*vertical line*), 0131 (*ex mark*) and 2104 (*open triangle*); *L. delbrueckii* subsp. *bulgaricus* 210R (*empty square*), 11842 (*filled square*), 702074 (*triangle*) and 291 (*filled diamond*); or acidified with GDL (*empty diamond*) at pH 4.6. Each data point is the mean of three experiments

control strains and as non-EPS-producing strains. However, sugar quantification showed that these strains did in fact produce EPS. Some researchers have reported that non-EPS-producing or non-ropy strains from LAB can produce EPS in quantities ranging from as low as 10 $mg.L^{-1}$ to higher concentrations (Petry et al. 2003; van Marle and Zoon 1995; Cerning et al. 1986), as observed in this study. To ensure HC15 and 210R strains as adequate controls, bacterial gels were compared with GDL gel (EPS-free control) despite the well-known difference among these types of gel (Lucey et al. 1998). Even with this EPS production, the rheological/physical properties of the milk fermented with the control strains (HC15 and 210R) were similar to those of the GDL milk. Similar pH_{gel} and G' values for milk fermented with a non-ropy LAB strain (90 $mg.L^{-1}$) and acidified with GDL were also observed by van Marle and Zoon (1995). In addition, Hassan et al. (2003) found that some non-EPS-producing strains yielded higher G' values than EPS-producing strains. In this study, the EPS produced by the control strains (HC15 and 210R) did not influence the gel formation and the rheological/physical properties of fermented milk and may act as non-active fillers, as proposed by van Marle and Zoon (1995).

Our results did not show any evidence of the relationship between the amount of EPS produced and the resulting effect on rheological/physical properties as reported in the literature (Petry et al. 2003; van Marle and Zoon 1995; Cerning et al. 1986). For instance, strain 702074 that produced the higher amount of EPS did not show any improvement on the rheological or physical properties of fermented milk.

4.2 Ropy character of exopolysaccharides

The funnel test can be useful to give a quick idea of EPS production by ropy strains during fermentation until pH 5.2 is reached, e.g. before casein aggregation occurs. For strains 0131 and 291, the ropy character was expressed as high as at pH 5.7. Results from EPS quantification confirmed the presence of EPS at this pH for these strains.

Measurement of flow time by the funnel test combined with filament formation with a sterile inoculating loop can also be useful to classify strains based on the degree of ropy character. Ropy character was high for strain 291, moderate for strain 0131, low for strain 2104 and non-existent for the other strains. To our knowledge, this is the first study to develop a method for classifying ropy character among different EPS. Using the funnel test to compare several strains producing different EPS structures revealed different behaviours among the ropy strains (0131, 2104 and 291). Ropy character was visually observed in the milk fermented with strain 2104 at pH 5.5–5.3, but no notable change in flow time was noted for that strain as compared with the other ropy strains. In addition, the ropy character of strain 2104 was no longer observable at pH 4.6. These funnel test results showed that the ropy character does not seem to be the only factor affecting the rheological properties of fermented milk. For instance, the different structure of the EPS from strain 2104 (anionic, stiff and linear) as compared with the EPS from strains 0131 (neutral, flexible and slightly branched) and 291 (neutral, stiff and slightly branched) might explain this difference instead. Faber et al. (1998) also found a positive correlation between ropy character, molecular weight and viscosity. Between two EPS with identical repeating units, a higher viscosity was obtained with the EPS with the higher molecular weight. Furthermore, those authors observed the ropy character

only in the fermented milk containing the EPS with the highest molecular weight. Finally, the comparison of many EPS with known structures has revealed the importance of the structural characteristics of EPS in the modulation of the rheological properties of fermented milk.

4.3 Effect of exopolysaccharide structure–function relationship on gel formation

The pH of gelation (pH_{gel}) of the milk fermented with all strains was found to be from 5.54 to 5.50, in agreement with the results reported in the literature (Lucey et al. 1998; van Marle and Zoon 1995; Heertje et al. 1985). However, the milk fermented with strain 2104 formed gel at pH 5.0. The pH_{gel} is related to the attractive interactions between milk proteins and thus depends on the heat treatment of milk, which controls whey protein denaturation, incubation temperature and acidification rate (Haque et al. 2001; Lucey et al. 1998; Heertje et al. 1985). Among these parameters, the acidification profile was the sole uncontrolled one in this experiment. Despite strains 0131 and 2104 having similar acidification profiles, the pH_{gel} values for those strains were different. Girard and Schaffer-Lequart (2007) also observed that the acidification profile did not influence the pH_{gel} of milk fermented with EPS-producing strains. Consequently, the pH_{gel} of fermented milk measured in this study seemed to be more influenced by the structural characteristics of EPS than by the acidification profile.

During acidification, several physicochemical changes occur in milk. Acidification progressively destabilizes the initial structured organization of casein micelles, leading to aggregation (Heertje et al. 1985). At neutral pH and up to proteins' isoelectric point, milk proteins are negatively charged (Heertje et al. 1985). The quantification of EPS during fermentation showed that strain 2104 produced EPS at a pH as high as 5.7. Repulsive electrostatic interactions between anionic EPS from strain 2104 and the negative charge of milk proteins may have affected the gel formation. The linearity and stiffness of this EPS may also have contributed to delay the gel formation. Linear and stiff polysaccharides occupy a larger relative volume than branched and flexible molecules (Whistler and BeMiller 1997). The EPS produced by the strain 2104 contain a nonionic acid on its backbone. The possibility of lactone formation in the EPS repeating units in response to pH might alter the functional properties, as reported in the literature (Lifely et al. 1981, 1984). Girard and Schaffer-Lequart (2007) found higher pH_{gel} values in milk fermented with EPS-producing strains, although one of the three EPS studied was anionic. Those authors compared EPS from mesophilic and thermophilic strains, and milk fermentation was consequently carried out at different incubation temperatures (25 or 40 °C) depending on the strains used, a difference that is known to play an important role in the gel formation (Haque et al. 2001; Lucey et al. 1998). Stronger hydrophobic interactions due to higher temperatures may have caused earlier gelation. Furthermore, Girard and Schaffer-Lequart (2007) calculated the pH_{gel} with a G' value of 0.1 Pa instead of 1 Pa (as in the present study), a difference that makes it difficult to compare those authors' results with those obtained in the present study. Hassan et al. (2003) also observed higher pH_{gel} values in fermented milk with EPS (unknown structures) as compared with non-EPS-producing strains, possibly because of the incompatibility between EPS and milk proteins due to depletion

interactions. The resulting phase separation can lead to earlier gel formation, as reported in the literature (Tuinier et al. 2000). Finally, the pH_{gel} values for the fermented milk measured in this study were influenced by the structural characteristics of the EPS produced by LAB, especially those with a negative charge and produced at the early stage of fermentation, e.g. before the gel formation.

According to dynamic oscillatory rheology, the gel formation of all the fermented milk occurred at pH 5.5–5.3, a finding that may seem to contradict the funnel test results where no gels could be visually observed. Furthermore, no changes in flow time or pH were observed for the milk fermented with the non-ropy strains (Table 2). These differences may be due to the disruption of weak gels during sample handling in the funnel test as the samples in sterile tubes were carefully transferred to the funnel before testing. Furthermore, dynamic oscillatory rheology is more sensitive than the funnel test.

4.4 EPS structure–function relationship on rheological/physical properties

The elastic modulus (G') is related to protein–protein interactions and is an indication of the gel stiffness (Lucey 2001). Irrespective of their structural characteristics, the presence of neutral EPS (strains 0131, 11842, 702074 and 291) caused a decrease of G' as compared with the fermented milk with control strains (HC15 and 210R) and strain 2104. These results suggest that neutral EPS molecules modify casein gel network possibly through a phase separation between the proteins and the non-absorbing polysaccharides (neutral EPS), as reported in the literature (Girard and Schaffer-Lequart 2007; Hassan et al. 2003; Tuinier et al. 2000). The anionic EPS from strain 2104 seemed to modify the casein network too, but in a way different from neutral EPS. Interactions between positive charges of milk proteins and anionic EPS from strain 2104 in addition to protein–protein interactions may contribute to reinforce the casein network. Strengthened fermented milk has been observed by the addition of pectin (anionic and linear polysaccharide) due to the electrostatic interactions between caseins and pectin (Everett and McLeod 2005).

The viscosifying effect of a polysaccharide in solution depends on its shape (degree of branching and flexibility of the backbone) and its molecular weight (Whistler and BeMiller 1997). For instance, comparing two polysaccharides with similar molecular weight, a stiff and linear polysaccharide will lead to higher viscosity than a highly branched and flexible polymer (Whistler and BeMiller 1997). The higher apparent viscosity values were obtained with strain 2104. The linearity and the stiffness of the backbone of this EPS may have contributed to the apparent viscosity improvement. Electrostatic interactions between the anionic EPS from strain 2104 and milk proteins may also contribute to the enhancement of apparent viscosity. The molecular weight of EPS from strain 2104 has not been determined. Intermediate value of apparent viscosity was obtained in milk fermented with the EPS from strain 0131 known to have a higher molecular weight, which is related to viscosity enhancement (Whistler and BeMiller 1997). The neutrality, the flexibility and the branching of EPS from strain 0131 as compared with strain 2104 may explain the lower viscosity value. Milk fermented with strain 291 led to an increase of viscosity as compared with strains 11842 and 702074, but the values were lower than with strain 0131. This effect may be explained by the difference in the molecular weight. The EPS from strain 291 had a similar molecular weight than

strains 11842 and 702074, but lower than strain 0131. The flexibility (Ruas-Madiedo et al. 2002; Whistler and BeMiller 1997) of the EPS from strains 11842 and 702074 might explain the lowest apparent viscosities as compared with the EPS from strain 291. In addition, the EPS from strain 702074 is flexible and highly branched, which is not related to viscosity enhancement (Whistler and BeMiller 1997).

Although the firmness and syneresis results were slightly different from apparent viscosity values, the same trends concerning the impact of structural characteristics can be made. An exception was observed for syneresis values for the EPS from strain 2104, e.g. syneresis values were comparable to those obtained for strains 210R control, 11842 and 702074. Lower whey retention was also observed with an anionic EPS added as a bio-ingredient in powder form (at a high concentration) in milk acidified with GDL (Turgeon and Plesca 2009; Girard and Schaffer-Lequart 2007). The reason is likely that the electrostatic interactions between the anionic EPS and milk proteins may be favoured instead of hydrogen bonds, thus leading to less whey retention, or electrostatic interactions may have hindered casein–casein interactions, leading to a casein network with a lower ability to retain serum.

5 Conclusions

This work has shown that gel formation and rheological/physical properties are influenced by the structural characteristics of the EPS produced by LAB rather than the concentration of EPS produced. However, the EPS concentration measured must be taken carefully due to the limitation of the EPS quantification method used in this study as well as for the other methods available in the literature related to the possible extraction of bacterial cell, causing some sugar background. This effect needs to be taken in consideration in future studies.

Measurement of various rheological/physical properties of fermented milk allowed highlighting some important structural characteristics of EPS to modulate a given rheological or physical property. The presence of a negative charge on EPS structures modified the gel formation process and contributed to the elasticity of the casein network. The apparent viscosity, firmness and whey retention of fermented milk were improved by the EPS with a high molecular weight, a stiff chain and few branching. The lowest values for apparent viscosity and firmness were obtained with flexible and highly branched EPS with a low molecular weight. More investigation is needed regarding the structure–function relationship of EPS produced in situ by LAB in a dairy model system that allows the adjustment of milk protein composition in order to elucidate the role of EPS structure in terms of protein–polysaccharide interaction. In addition, it would be worthwhile to combine each EPS-producing strain (291, 0131, 2104, 11842, 702074) with its complementary control strain HC15 or 210R (e.g. a streptococci–lactobacilli combination) to simulate fermentation time in yoghurt manufacturing.

Acknowledgements This research was jointly funded by the research programmes of the Fonds québécois de la recherche sur la nature et les technologies, Novalait Inc., the Ministère de l'Agriculture, des Pêcheries et de l'Alimentation du Québec, and Agriculture and Agri-Food Canada. The principal author would like to thank the Canadian Dairy Commission, Novalait Inc. and the Fonds québécois de la recherche sur la nature et les technologies for her graduate scholarship. The authors would also like to thank Pierre Ward, of the Food Research and Development Centre, for his expertise in and help with biomolecular techniques.

References

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal Biochem 72:248–254

Cerning J, Bouillanne C, Desmazeaud MJ, Landon M (1986) Isolation and characterization of exocellular polysaccharide produced by *Lactobacillus bulgaricus*. Biotechnol Let 8:625–628

Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F (1956) Colorimetric method for determination of sugars and related substances. Anal Chem 28:350–356

Everett DW, McLeod RE (2005) Interactions of polysaccharide stabilisers with casein aggregates in stirred skim-milk yoghurt. Int Dairy J 15:1175–1183

Faber EJ, van Haaster DJ, Kamerling JP, Vliegenthart JFG (2002) Characterization of the exopolysaccharide produced by *Streptococcus thermophilus* 8S containing an open chain nononic acid. Eur J Biochem 269:5590–5598

Faber EJ, Kamerling JP, Vliegenthart JFG (2001a) Structure of the extracellular polysaccharide produced by *Lactobacillus delbrueckii* subsp. *bulgaricus* 291. Carbohydr Res 331:183–194

Faber EJ, van den Haak MJ, Kamerling JP, Vliegenthart JFG (2001b) Structure of the exopolysaccharide produced by *Streptococcus thermophilus* S3. Carbohydr Res 331:173–182

Faber EJ, Zoon P, Kamerling P, Vliegenthart JFG (1998) The exopolysaccharides produced by *Streptococcus thermophilus* Rs and Sts have the same repeating unit but differ in viscosity of their milk cultures. Carbohydr Res 310:269–276

Folkenberg DM, Dejmek P, Skriver A, Skov Guldager H, Ipsen R (2006) Sensory and rheological screening of exopolysaccharide producing strains of bacterial yoghurt cultures. Int Dairy J 16:111–118

Girard M, Schaffer-Lequart C (2007) Gelation and resistance to shearing of fermented milk: role of exopolysaccharides. Int Dairy J 17:666–673

Harding LP, Marshall VM, Hernandez Y, Gu Y, Maqsood M, McLay N, Laws AP (2005) Structural characterisation of a highly branched exopolysaccharide produced by *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB2074. Carbohydr Res 340:1107–1111

Hassan AN (2008) ADSA Foundation scholar award: possibilities and challenges of exopolysaccharide-producing lactic cultures in dairy foods. J Dairy Sci 91:1282–1298

Hassan AN, Ipsen R, Janzen T, Qvist KB (2003) Microstructure and rheology of yogurt made with cultures differing only in their ability to produce exopolysaccharides. J Dairy Sci 86:1632–1638

Haque A, Richardson RK, Morris ER (2001) Effect of fermentation temperature on the rheology of set and stirred yogurt. Food Hydrocoll 15:593–602

Heertje I, Visser J, Smits P (1985) Structure formation in acid milk gels. Food Microstruct 4:267–277

Hess JS, Roberts RF, Ziegler GR (1997) Rheological properties of nonfat yoghurt stabilized using *Lactobacillus delbrueckii* ssp. *bulgaricus* producing exopolysaccharides or using commercial stabilizer systems. J Dairy Sci 80:252–263

Lamboley L, St-Gelais D, Champagne CP, Lamoureux M (2003) Growth and morphology of thermophilic dairy starters in alginate beads. J Gen Appl Microbiol 49:205–214

Lifely MR, Gilbert AS, Moreno C (1984) Rate, mechanism, and immunochemical studies of lactonisation in serogroup B and C polysaccharides of *Neisseria meningitides*. Carbohydr Res 134:229–243

Lifely MR, Gilbert AS, Moreno C (1981) Sialic acid polysaccharide antigens of *Neisseria meningitidis* and *Escherichia coli*: esterification between adjacent residues. Carbohydr Res 94:193–203

Lucey JA (2001) The relationship between rheological parameters and whey separation in milk gels. Food Hydrocoll 15:603–608

Lucey JA, Tamehana M, Singh H, Munro PA (1998) A comparison of the formation, rheological properties and microstructure of acid skim milk gels made with a bacterial culture or glucono-delta-lactone. Food Res Int 31:47–155

Petry S, Furlana S, Waghornec E, Saulnierd L, Cerning J, Maguin E (2003) Comparison of the thickening properties of four *Lactobacillus delbrueckii* subsp. *bulgaricus* strains and physicochemical characterization of their exopolysaccharides. FEMS Microbiol Lett 221:285–291

Robitaille G, Tremblay A, Moineau S, St-Gelais D, Vadeboncoeur C, Britten M (2009) Fat-free yogurt made using a galactose-positive exopolysaccharide-producing recombinant strain of *Streptococcus thermophilus*. J Dairy Sci 92:477–482

Ruas-Madiedo P, Hugenholtz J, Zoon P (2002) An overview of the functionality of exopolysaccharides produced by lactic acid bacteria. Int Dairy J 12:163–171

Tabasco R, Paarup T, Janer C, Peláez C, Requena T (2007) Selective enumeration and identification of mixed cultures of *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *L.*

acidophilus, *L. paracasei* subsp. *paracasei* and *Bifidobacterium lactis* in fermented milk. Int Dairy J 17:1107–1114

Tuinier R, Dhont JKG, De Kruif CG (2000) Depletion-induced phase separation of aggregated whey protein colloids by an exocellular polysaccharide. Langmuir 16:1497–1507

Turgeon SL, Plesca V (2009) Study of interactions between exopolysaccharides produced by strain *Lactobacillus rhamnosus* RW-9595M and milk proteins. Proceedings of the 5th International Symposium Food Rheology Structure, Zurich, Switzerland, pp 416–419

Van Calsteren M-R, Gagnon F, Guertin N, Broadbent JR (2008) High-resolutions ^{1}H and ^{13}C NMR structure determination of a polysaccharide produced by *Lactobacillus delbrueckii* subsp. *bulgaricus* strain ATCC 11842. Proceedings of the 9th International Conference on the Applications of Magnetic Resonance in Food Science (poster presentation), Iceland

van Marle ME, Zoon P (1995) Permeability and rheological properties of microbially and chemically acidified skim-milk gels. Neth Milk Dairy J 49:47–65

Whistler RL, BeMiller JN (1997) In: Chemist AACC (ed) Polysaccharides. Eagan Press, Minnesota