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The exopolysaccharides produced by *Streptococcus thermophilus* Rs and Sts have the same repeating unit but differ in viscosity of their milk cultures

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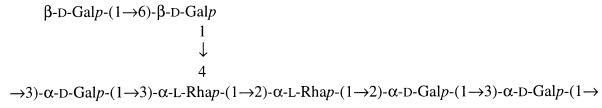
Abstract

The polysaccharides produced by *Streptococcus thermophilus* Rs and Sts in skimmed milk consist of D-Gal and L-Rha in a molar ratio of 5:2. Linkage analysis and 1D/2D NMR (^{1}H and ^{13}C) studies revealed that both polysaccharides have the same branched heptasaccharide repeating unit:



Remarkably, the two strains differ in their effects on the viscosity of stirred milk cultures. The milk culture of *S. thermophilus* Rs is non-ropy and affords 135 mg/L polysaccharide with an average molecular mass of 2.6×10^{3} kDa. In contrast, the milk culture of *S. thermophilus* Sts is ropy and produces 127 mg/L polysaccharide with an average molecular mass of 3.7×10^{3} kDa. Permeability measurements of non-stirred milk cultures of both strains suggest that both strains have a similar effect on the protein–polysaccharide network. Therefore, the only clear difference between both strains, which may cause the difference in ropiness of the milk cultures, is the difference in molecular mass of the polysaccharide.

Keywords: *Streptococcus thermophilus*; Exopolysaccharide; Ropiness; Permeability; Viscosity

1. Introduction

Microbial exopolysaccharides (EPSs) are widely applied as gelling and stabilizing agents in the food industry [1]. The EPSs produced by lactic acid bacteria which carry the GRAS (Generally Recognized as Safe) status, are promising as a new generation of food thickeners. For that reason, detailed structural studies have been performed on exopolysaccharides produced by several lactic acid bacteria strains including *Lactococcus lactis* subsp.

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cremoris [2,3], *Lactobacillus delbrückii* subsp. *bulgaricus* rr [4], *Lactobacillus helveticus* [5–9], *Lactobacillus acidophilus* [10], *Lactobacillus paracasei* [11], *Lactobacillus sake* [12], and *Streptococcus thermophilus* [13–15].

S. thermophilus strains are used in combination with *L. delbrückii* subsp. *bulgaricus* strains as commercial yoghurt starters. Since it is assumed that the ropiness of yoghurt is caused by the EPSs produced by these bacteria many investigations have been directed towards those EPSs. Polysaccharides produced by various *S. thermophilus* strains contain mainly rhamnose, glucose, galactose, and 2-acetamido-2-deoxygalactose [13–16]. Although the similarities in these structures are striking, little is known about the relationship between the structures and the consistency of the EPS-containing milk cultures. An exploratory study to establish the main factors determining the consistency of EPS-containing yoghurt showed that its apparent viscosity, which increases with increasing ropiness, was not simply related to the EPS concentration [17]. Furthermore, it is stated that the permeability of milk gels before stirring, which reveals information about the structure of the protein–EPS network, was affected by the type of yoghurt starter.

In this study, *S. thermophilus* Rs and Sts strains were selected on the basis of huge differences in viscosifying properties of their milk cultures. Both strains produce approximately 100 mg/L EPS which contain galactose and rhamnose in a molar ratio of 5:2. Because of these similarities the strains may give a better insight into the relationship between structures of exopolysaccharides and viscosity of milk cultures. Therefore, we have characterized the EPSs produced by both strains. Furthermore, we have studied the permeability and viscosity of milk cultures produced by both strains.

2. Results and discussion

Isolation, purification, and composition of the polysaccharides.—The exopolysaccharides produced by *S. thermophilus* Rs and Sts in skimmed milk were isolated via ethanol precipitation of the supernatant of the TCA-treated medium, followed by acetone precipitation and lyophilization of the precipitated polysaccharides. The purity of the polysaccharides was confirmed by 1D ^{1}H NMR spectroscopy (vide infra). From the *S. thermophilus* Rs culture 135 mg/L polysaccharide with an average molecular mass of 2.6×10^3 kDa was isolated, whereas the *S. thermophilus* Sts culture yielded 127 mg/L polysaccharide with an average molecular mass of 3.7×10^3 kDa (Table 1). The results show a difference of 1.1×10^3 kDa in molecular mass between both EPSs.

Table 1
Physical properties of milk cultures and concentration and molecular mass of EPS, produced by *S. thermophilus* Rs and Sts

Strain	B[a] (10^{-14} m^2)	Posthumus[b] viscosity (s)	EPS yield (mg/L)	Molecular mass (10^3 kDa)
Rs	11.2 (±0.2)	39 (±3)	135 (±8)	2.6 (±0.4)
Sts	13.0 (±0.6)	126 (±15)	127 (±13)	3.7 (±0.7)

[a] Permeability coefficient B measured at 20 °C.
[b] Posthumus viscosity measured at 5 °C.

Quantitative monosaccharide analysis using procedures I and II, together with the determination of absolute configurations, revealed the presence of D-Gal and L-Rha in a molar ratio of 5:2 for both EPSs. Methylation analyses (Table 2) indicated that the EPSs are composed of branched heptasaccharide repeating units, whereby, according to NMR experiments (vide infra), all monosaccharides are in the pyranose ring form.

The 1D ^{1}H NMR spectra (Fig. 1) of *S. thermophilus* Rs and Sts EPS are indistinguishable and contain six signals in the anomeric region (δ 5.4–4.4). Because the proton signal at δ 5.18 stems from two anomeric protons, the repeating units are heptasaccharides. The sugar residues are designated **A–G** according to increasing chemical shift of the anomeric protons. The chemical shifts and coupling constants of the anomeric signals at δ 4.480 (residue **A**, $^3J_{1,2}$ 7.8) and δ 4.671 (residue **B**, $^3J_{1,2}$ 7.8) indicate two β-Gal*p* residues, and the anomeric signal at δ 5.278 (residue **G**, $^3J_{1,2}$ 3.9 Hz) indicates an α-Gal*p* residue. Coupling constants of the

Table 2
Methylation analysis data of *S. thermophilus* Rs and Sts EPS

Derivative	Molar amounts[a]	
	Rs	Sts
3,4-Rha[b]	0.9	0.9
2-Rha	1.0	1.1
2,3,4,6-Gal	1.0	0.9
2,3,4-Gal	0.9	0.9
3,4,6-Gal	1.0	1.0
2,4,6-Gal	2.0	2.2

[a] 3,4,6-Gal is taken as 1.0.
[b] 3,4-Rha = 1,2,5-tri-*O*-acetyl-3,4-di-*O*-methyl-rhamnitol-1-*d*, etc.

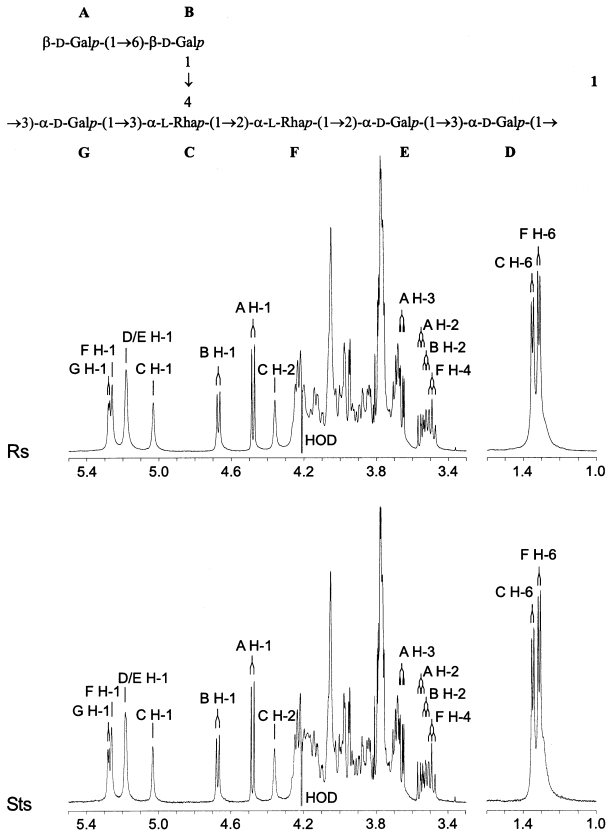


Fig. 1. 500-MHz ^{1}H NMR spectra of EPS from *Streptococcus thermophilus* Rs and Sts, recorded in D_2O at 80 °C.

remaining anomeric signals could not be measured. The spectra show two high-field doublets at δ 1.312 (residue **F**, $^3J_{5,6}$ 5.9 Hz) and δ 1.347 (residue **C**, $^3J_{5,6}$ 5.9 Hz), arising from the methyl groups of two Rha residues. The specific residue assignments follow from 2D NMR experiments (vida infra).

2D NMR spectroscopy.—Since 2D COSY, 2D TOCSY, 2D NOESY and ^{13}C–^{1}H HSQC spectra of both EPSs are essentially identical, assignments of ^{1}H and ^{13}C chemical shifts and coupling constants of the EPSs were performed using 2D NMR spectra of Sts EPS.

Assignments of the ^{1}H chemical shifts and coupling constants of Sts EPS (Table 3) were performed by means of 2D COSY, 2D TOCSY, 2D NOESY and ^{13}C–^{1}H HSQC-TOCSY experiments. The TOCSY spectrum with a mixing time of 250 ms and the ^{13}C–^{1}H HSQC-TOCSY spectrum are depicted in Figs. 2 and 3, respectively. Starting points for the interpretation of the spectra were the H-1 signals of residues **A–G** and the methyl signals of rhamnose residues **C** and **F**.

The TOCSY **A** H-1 track (δ 4.480) shows cross-peaks with **A** H-2,3,4,5. The chemical shifts of the **A** H-6 resonances were assigned through cross-peaks on the TOCSY **A** H-5 track. On the TOCSY **B** H-1 track (δ 4.671), cross-peaks were observed with **B** H-2,3,4. An intraresidual H-1,H-5 contact in the NOESY spectrum (Fig. 5) allowed the assignment of **B** H-5. The resonances for **B** H-6a,6b were found via connectivities with **B** H-5 in both the COSY and the TOCSY spectra. The chemical shifts of the H-6 protons of the Gal residue **B** could also be interpreted from cross-peaks on the **B** C-5 track in the HSQC-TOCSY spectrum. From the anomeric carbon signal of residue **D** (δ 98.4) a single cross-peak could be observed in the HSQC-TOCSY spectrum. The spectrum provides evidence that this signal derives from both the **D** H-2 and **D** H-3 atoms, since **D** C-2 and **D** C-3 have different chemical shifts. A connectivity with **D** H-4 was observed on the TOCSY **D** H-1 track, whereas the **D** H-5 signal could be deduced from the intraresidual H-1,H-5 contact in the NOESY spectrum. Finally the **D** H-6 signals were determined on the **D** C-5 track in the HSQC-TOCSY spectrum. For residue **E**, well resolved H-2 and H-3 connectivities were found on the HSQC-TOCSY **E** C-1 track (δ 97.8), whereas H-4 and H-5,6 were detected on the HSQC-TOCSY **E** C-3 and **E** C-4 track, respectively. On the TOCSY H-6 tracks of the Rha residues **C** (δ 1.347) and **F** (δ 1.312), the complete series

Table 3
^{1}H NMR chemical shifts[a] of Sts EPS, recorded in D_2O at 80 °C. Coupling constants (Hz) are included in parentheses

Residue	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b	CH_3
A	4.480 (7.8)	3.551 (9.8)	3.660 (3.4)	3.95	3.68	3.81	3.78	—
B	4.671 (7.8)	3.523 (9.3)	3.70	3.98	3.82	4.05	3.93	—
C	5.032	4.360	4.14	4.00	3.86	—	—	1.347 (5.9)
D	5.180	4.05	4.05	4.22	4.25	3.80–3.74		—
E	5.185	3.99	4.11	4.05	4.23	3.80–3.74		—
F	5.261	4.07	3.89	3.491 (9.3)	3.71	—	—	1.312 (5.9)
G	5.278 (3.9)	4.04	4.15	4.20	n.d[b]	3.80–3.74		—

[a] In ppm relative to the signal of internal acetone at δ 2.225.
[b] n.d., not determined.

of cross-peaks with H-1,2,3,4,5 are observed. The H-2,3,4 atoms of residue **G** were identified on the basis of cross-peaks on the TOCSY **G** H-1 track (δ 5.278). Assignment of the H-6 proton resonances of residue **G** was deduced from the HSQC-TOCSY spectrum, whereas the **G** H-5 signal could not be detected.

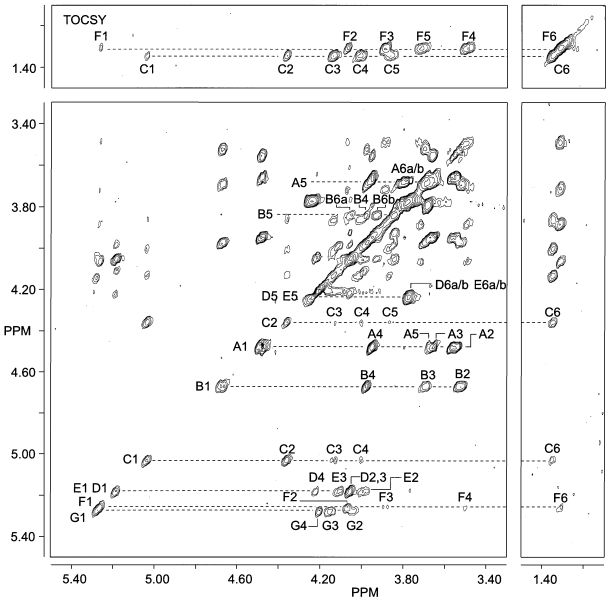


Fig. 2. 500-MHz 2D TOCSY spectrum (mixing time 250 ms) of Sts EPS, recorded in D_2O at 80 °C. Diagonal peaks of the anomeric protons, of H-2 of residue **C**, of H-5 of residue **A, B, D** and **E**, and of H-6 of residues **C** and **F** are indicated. Cross-peaks belonging to the same scalar-coupling network are indicated near a dotted line starting from the corresponding diagonal peak.

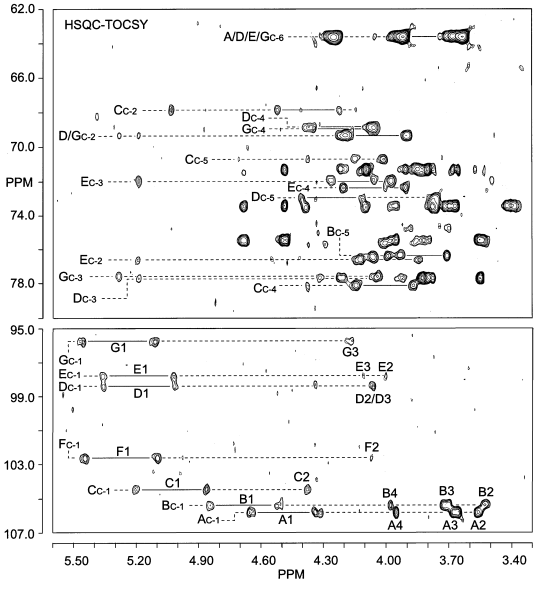


Fig. 3. 500-MHz 2D ^{13}C–^{1}H undecoupled HSQC-TOCSY spectrum (mixing time 80 ms) of Sts EPS, recorded in D_2O at 67 °C. The code Ac-1 denotes the chemical shift of C-1 of residue **A**, etc. Cross-peaks between carbons and protons belonging to the same scalar-coupling network are indicated near a dotted line.

The 2D ^{13}C–^{1}H HSQC spectrum of Sts EPS (Fig. 4) allowed the assignment of the ^{13}C resonances (Table 4). The one bond coupling constants of the anomeric carbon atoms of residues **A** and **B** ($^{1}J_{C\text{-}1,H\text{-}1}$162 Hz) prove that their anomeric configurations are β, and those of residues **C, D, E** and **G** ($^{1}J_{C\text{-}1,H\text{-}1}$ 170–171 Hz) are α [18]. The one bond coupling constant for residue **F** ($^{1}J_{C\text{-}1,H\text{-}1}$ 174 Hz) is indicative of an α configuration. The relatively high coupling constant of this residue is most likely caused by the 2-substitution.

In agreement with published chemical shift data of methyl aldosides [19], and methylation analysis, residue **A** was identified as the terminal β-Gal*p* residue, whereas the downfield chemical shift of **B** C-6 (δ 71.3) demonstrated residue **B** to represent the 6-substituted β-Gal*p* residue. The downfield chemical shifts for C-3 of residue **D** and **G** indicate that these α-Gal*p* units are 3-substituted, whereas the downfield chemical shifts of **E** C-2 (δ 76.7) and **F** C-2 (δ 81.4) show that both residues are 2-substituted. The remaining **C** residue could be assigned

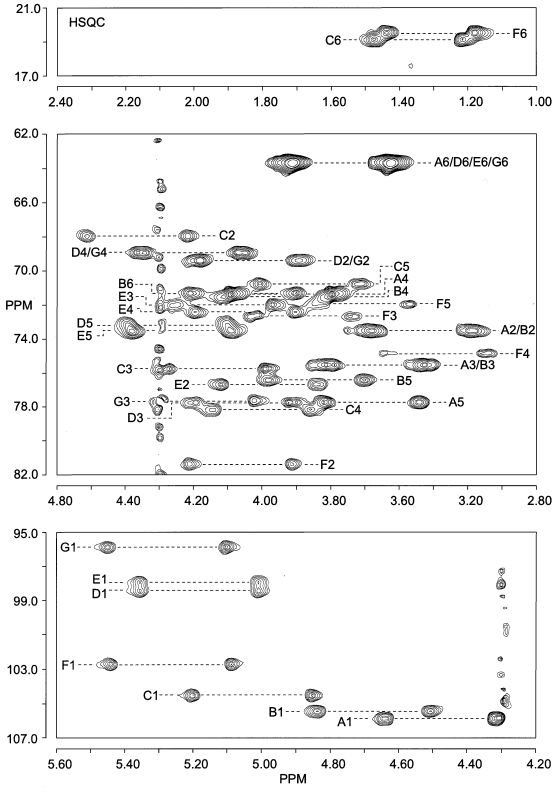


Fig. 4. 500-MHz 2D ^{13}C–^{1}H undecoupled HSQC spectrum of Sts EPS, recorded in D_2O at 67 °C. A1 stands for the set of cross-peaks between H-1 and C-1 of residue **A**, etc.

Table 4
^{13}C NMR chemical shifts[a] of Sts EPS, as determined from 2D ^{13}C–^{1}H HSQC experiments, recorded in D_2O at 67 °C. $^{1}J_{C\text{-}1,H\text{-}1}$. Values (Hz) are included in parentheses

Residue	C-1	C-2	C-3	C-4	C-5	C-6
A	105.8 (162)	73.5	75.5	71.4	77.7	63.7
B	105.4 (162)	73.5	75.5	71.5	76.1	71.3
C	104.5 (171)	68.0	75.8	78.2	70.8	19.8
D	98.4 (171)	69.6	77.7	68.9	73.0	63.7
E	97.8 (170)	76.7	72.0	72.4	73.5	63.7
F	102.6 (174)	81.4	72.7	74.9	71.9	19.3
G	95.8 (171)	69.6	77.6	68.9	n.d.[b]	63.7

[a] In ppm relative to the α-anomeric signal of external [1-^{13}C]glucose at δ 92.9.
[b] n.d., not determined.

as the 3,4-disubstituted α-Rha*p* unit, since **C** C-3 (δ 75.8) and **C** C-4 (δ 78.2) are downfield shifted.

By means of NOESY and HSQC-NOE experiments (Figs. 5 and 6) the sequence of the monosaccharide residues in the EPS was established. On the NOESY **C** H-1 track a cross-peak with **F** H-2 was observed, suggesting a **C**(1→2)**F** sequence, whereas a well-resolved interresidual connectivity **F** H-1,**E** H-2 in the HSQC-NOE spectrum demonstrates a **F**(1→2)**E** linkage. Likewise, the NOE between **E** H-1 and **D** H-2,3, observed in the HSQC-NOE spectrum, indicates a **E**(1→3)**D** linkage, since residue **D** has proven to be a 3-substituted galactose. The NOE between **D** H-1 and **G** H-3 in the HSQC-NOE spectrum reveals a **D**(1→3)**G** linkage. Furthermore, a strong NOE between **G** H-1 and **C** H-2 and a weak NOE between **G** H-1 and **C** H-3 were observed. The NOE cross-peaks indicate a **G**(1→3)**C** linkage, since methylation analysis in combination with carbon chemical shifts demonstrate that residue **C** is 3,4-disubstituted. The NOE cross-peak between **G** H-1 and **C** H-2 does not reflect the glycosidic linkage. The observation of such non-glycosidic NOE cross-peaks have been reported for several (1→3)-linked disaccharide methyl glycosides [20]. On the NOESY **B** H-1 track a NOE cross-peak with **C** H-3 was observed, suggesting a **B**(1→3)**C** sequence. The **A**(1→6)**B** linkage could not be confirmed by NOESY and HSQC-NOE analysis.

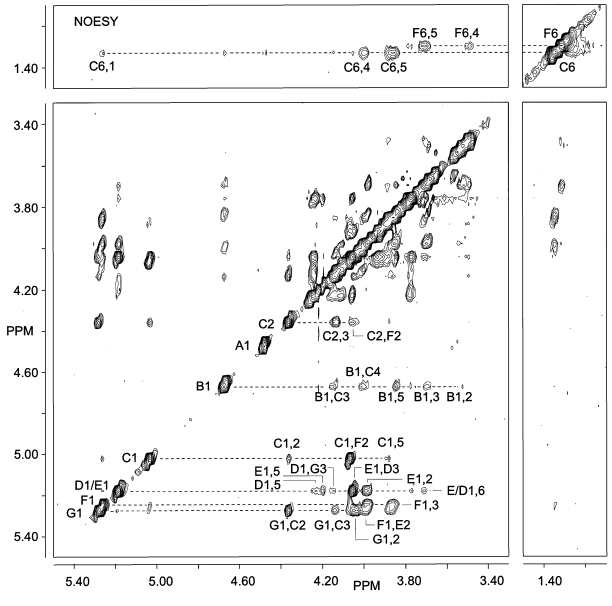
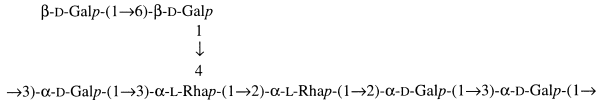


Fig. 5. 500-MHz 2D NOESY spectrum (mixing time 150 ms) of Sts EPS, recorded in D_2O at 80 °C. G1 corresponds to the diagonal peak belonging to residue **G** H-1; G1,2 refers to an intraresidual cross-peak between **G** H-1 and **G** H-2, and G1,C2 means an interresidual connectivity between **G** H-1 and **C** H-2, etc.

The intraresidual connectivities in the NOESY spectra of Sts EPS confirm the anomeric configurations of the various monosaccharide residues in the polysaccharide. Intraresidual connectivities between H-1 and H-5 of residue **C** and of H-1 and H-6 of residues **C, D** and **E** are brought about by spin diffusion.

The combined results, from chemical and NMR studies, have proven the EPSs of both *S. thermophilus* Rs and *S. thermophilus* Sts to be composed of a heptasaccharide repeating unit with the following structure:



This structure is identical to the EPS produced by *S. thermophilus* OR901 [14]. Since *S. thermophilus* Rs and Sts are distinct strains with different

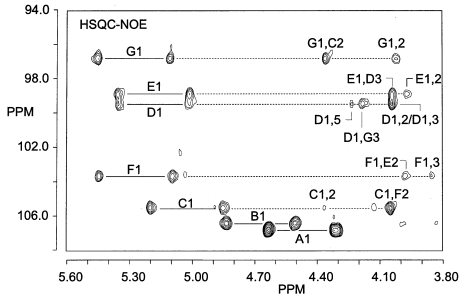


Fig. 6. 500-MHz 2D ^{13}C–^{1}H undecoupled HSQC-NOE spectrum (mixing time 80 ms) of the anomeric region of Sts EPS, recorded in D_2O at 67 °C. G1 denotes for the set of cross-peaks between C-1 and H-1 of residue **G**, G1,2 refers to an intraresidual cross-peak between **G** C-1 and **G** H-2, and G1,C2 means an interresidual connectivity between **G** C-1 and **C** H-2, etc.

viscosifying properties but the same repeating unit it is presumed that OR901 is a third strain with an identical repeating unit but other viscosifying properties.

Physical properties of the milk cultures.—Permeability and viscosity data from milk cultures of *S. thermophilus* Rs and Sts are shown in Table 1. The stirred milk culture of *S. thermophilus* Rs looked thin and lumpy, whereas that of *S. thermophilus* Sts looked very thick. The Posthumus viscosity (Table 1) of the stirred yoghurt increased with increasing ropiness of the milk cultures, which is in agreement with earlier findings [17]. The permeability coefficient B of the milk cultures is fairly equal, which suggests that the strains have a similar effect on the protein–EPS network in the milk cultures. Finally, the rate of protein digestion in the Rs and Sts milk cultures was evaluated by SDS-PAGE, showing a high similarity between both protein profiles.

Concluding remarks.—Polysaccharides produced by *S. thermophilus* Rs and Sts have the same branched heptasaccharide repeating unit. Permeability measurements of non-stirred milk cultures suggest that the cultures have a similar effect on the protein–EPS network. Consequently, the only detected difference which might explain the differences in ropiness of the milk cultures lies in the molecular mass of the polysaccharides.

3. Experimental

Organisms.—*S. thermophilus* Rs and Sts were obtained from the Netherlands Institute of Dairy Research (NIZO, Ede, The Netherlands).

Physical properties of milk cultures.—Milk and milk cultures were made as described [17]. *S. thermophilus* Rs or Sts were inoculated in such a concentration that the pH of the milk reached a value of 4.3 after 16 h at 32 °C. Permeability measurements, calculations of the permeability coefficient B and viscosity measurements were performed as described [17].

Exopolysaccharide concentration.—Trichloroacetic acid was added to milk cultures (final concentration 12% w/w). After stirring for 1 h, cells and precipitated proteins were removed by centrifugation (20 min, 16,300 *g*, 4 °C). Supernatants were collected, extensively dialyzed against tap water and subsequently lyophilized. Samples were re-dissolved in water and analyzed by HP-GPC with RI detection. Dextran was used as a standard to determine the EPS concentrations.

Culture conditions of microorganisms and isolation of exopolysaccharides.—Cultures were grown for 16 h at 32 °C in pasteurized reconstituted skim milk [17] containing 0.2% (w/w) casitone. After the addition of trichloroacetic acid to a final concentration of 4% (w/w), cultures were stirred for 1 h. Cells and precipitated proteins were removed by centrifugation (2×30 min, 16,300 *g*, 4 °C), and EPSs from the supernatants were precipitated with 2 vols of cold EtOH. Aqueous solutions of the precipitated materials were extensively dialyzed against tap water and after removal of insoluble material by centrifugation again 2 vols of EtOH were added. The precipitates formed were re-dissolved in water, and purified further by precipitation at 40% (v/v) acetone.

Molecular mass determination.—The average molecular masses of the polysaccharides were determined, using a modified method combining gel permeation chromatography, static light scattering, and differential refraction analysis, as described [21].

High performance gel permeation chromatography, gas–liquid chromatography, and mass spectrometry.—HP-GPC analyses were achieved on a TSK G6000 PW column (300×7.5 mm, Progel) using a RI detector (Erna ERC-7510). Elution was performed with 0.1 M $NaNO_3$ at a flow rate of 0.6 mL/min. GLC measurements were performed on a Chrompack CP9002 gas chromatograph, equipped with a CP-Sil 5CB fused silica capillary column (25 m×0.32 mm, Chrompack), using a temperature program of 120–240 °C at 4 °C/min, or with a CP-Sil 43CB fused silica capillary column (25 m×0.32 mm, Chrompack) using a temperature program of 140–200 °C at 3 °C/min. GLC–MS analyses were carried out on a MD800/8060 system (Fisons instruments; electron energy, 70 eV), using a DB-1 fused silica capillary column (30 m×0.32 mm, J&W Scientific) with a temperature program of 140–240 °C at 4 °C/min.

Monosaccharide analysis.—For monosaccharide analysis two procedures were followed: (I) Polysaccharides were hydrolyzed with 2 M CF_3CO_2H (2 h, 120 °C). After reduction of the monosaccharide mixtures with $NaBD_4$ in 0.5 M NH_4OH (1 h, room temperature), solutions were neutralized with HOAc and boric acid was removed by co-evaporation with MeOH. Alditol acetates, obtained by acetylation with acetic anhydride (3 h,

120 °C) were analyzed by GLC on CP-Sil 43CB. (II) Dry polysaccharides were subjected to methanolysis, followed by trimethylsilylation of the methyl glycoside mixtures and GLC analysis on CP-Sil 5CB as described [7,22,23]. Absolute configurations of monosaccharides were determined according to [24,25].

Methylation analysis.—Polysaccharides were permethylated as described [26]. After hydrolysis with 2 M CF_3CO_2H (2 h, 120 °C), the partially methylated monosaccharide mixtures were reduced and acetylated as in procedure I of the monosaccharide analysis. The partially methylated alditol acetates obtained were identified by GLC on CP-Sil 43CB and by GLC–MS on DB-1 [22,27].

NMR spectroscopy.—Prior to NMR-spectroscopic analysis, samples were exchanged twice in 99.9 atom% D_2O (Isotec) with intermediate lyophilization and finally dissolved in 99.96 atom% D_2O (Isotec). 1D ^1H and 2D NMR spectra were recorded on a Bruker AMX-500 spectrometer (Bijvoet Center, Department of NMR Spectroscopy) at a probe temperature of 80 °C for ^1H NMR experiments and 67 °C for heteronuclear NMR experiments. The HOD signal was suppressed either by applying a WEFT pulse sequence [28] in 1D ^1H NMR experiments, or by presaturation for 0.8–1 s in 2D experiments. Chemical shifts are expressed in ppm by reference to internal acetone (δ 2.225) for ^1H or to the α-anomeric signal of external [1-^{13}C]glucose ($\delta_{C\text{-}1}$ 92.9) for ^{13}C. Spectra were recorded with a spectral width of 4032 and 16,350 Hz for proton and carbon, respectively. Resolution enhancement of the spectra was performed by a Lorentzian-to-Gaussian transformation and when necessary, a fourth order polynomal baseline correction was performed.

The 2D DQF-COSY spectra were recorded according to ref. [29]. The 2D TOCSY spectra were recorded using a "clean" MLEV-17 mixing sequence with an effective spin-lock time of 15–250 ms. The 2D NOESY experiments were performed with a mixing time of 150 ms. The natural abundance ^{13}C–^1H 2D HSQC experiment, the 2D gradient-enhanced ^{13}C–^1H HSQC-TOCSY experiment [30] with a mixing time of 80 ms, and the 2D gradient-enhanced ^{13}C–^1H HSQC-NOE experiment consisting of a gradient-enhanced ^{13}C–^1H HSQC building block continued by a ^1H NOE step of 80 ms, were recorded without decoupling during acquisition of the ^1H FID.

All NMR data were processed using TRITON (Bijvoet Center, Department of NMR Spectroscopy) or Bruker UXNMR software.

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