Structure-function relationships of bacterial and enzymatically produced reuterans and dextran in sourdough bread baking application

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ABSTRACT

Exopolysaccharides from lactic acid bacteria may improve texture and shelf life of bread. The effect of exopolysaccharides on bread quality, however, depends on properties of the EPS and the EPS producing strain. This study investigated structure-function relationships of EPS in baking application. The dextransucrase DsrM and the reuteransucrase GtfA were cloned from *Weissella cibaria* 10M and *Lactobacillus reuteri* TMW1.656, respectively, and heterologously expressed in *Escherichia coli*. Site-directed mutagenesis of GtfA was generates reuterans with different glycosidic bonds. NMR spectrum indicated reuteranPI, reuteranNS and ReuteranPINS produced by GtfA-V1024P:V1027I, GtfA-S1135N:A1137S and GtfA-V1024P:V1027I:S1135N:A1137S, respectively, had a higher proportion of α-(1→4) linkages when compared to reuteran. ReuteranNS has the lowest molecular weight as measured by asymmetric flow-field-flow fractionation. The reuteransucrase negative mutant *L. reuteri* TMW1.656Δ*gtfA* was generated as EPS-negative derivative of *L. reuteri* TMW1.656. Cell counts, pH, and organic acid levels of sourdough fermented with *L. reuteri* TMW1.656 and TMW1.656Δ*gtfA* were comparable. Reuteran produced by *L. reuteri* TMW1.656 during growth in sourdough and reuteran produced *ex situ* by GtfA-ΔN had comparable effects on bread volume and crumb hardness. Enzymatically produced dextran improved volume and texture of wheat bread, and of bread containing 20% rye flour. ReuteranNS but not reuteranPI or reuteran was as efficient as dextran in enhancing wheat bread volume and texture. Overall, reuteran linkage type and molecular weight are determinants of EPS effects on bread quality. This study established a valuable method to elucidate structure-function relationships of glucans in baking applications.

KEYWORDS: Sourdough; reuteransucrase, bread quality; reuteran, dextran, exopolysaccharides

Highlights

- *Lactobacillus reuteri* TMW1.656 and TMW1.656Δ*gtfA* produce same amount of organic acids.
- EPS from mutant reuteransucrases have different linkage type and molar mass.
- The effect of *in situ* produced reuteran on bread quality is not different from added reuteran
- ReuteranNS, reuteranPI, and reuteran have different effects on wheat bread quality
- EPS effects on bread quality depend on polymer size and structure.

1. INTRODUCTION

Sourdough improves texture, flavour, shelf life, and nutritional properties of baked and steamed bread (Gänzle et al., 2007; Gänzle, 2014; Liu et al., 2016). These beneficial effects are related to the metabolism of lactic acid bacteria (LAB) during sourdough fermentation. Exopolysaccharides (EPS) produced by LAB increase bread volume, decrease bread firmness, and function as prebiotics (Galle et al., 2010; Kaditzky et al., 2008; Katina et al., 2009; Rühmkorf et al., 2012; Tieking and Gänzle, 2005). Because of their beneficial effects on bread quality, EPS from LAB may replace or reduce the use of hydrocolloids as bread improvers (Galle and Arendt, 2014).

Most EPS produced in sourdough fermentation are high molecular weight polymers composed of glucose (glucan) or fructose (fructan). Among these EPS, α-(1→6) linked dextrans have been regarded as the most promising bread improvers. Dextran from *Weissella cibaria* 10M decreased firmness and improved freshness of sorghum bread (Schwab et al., 2008); however, levan produced by *Lactobacillus reuteri* in the same bread formula showed no effect on bread quality. Reuteran, an α-(1→4) and α-(1→6) linked glucan produced by *L. reuteri*, and dextran from *W. cibaria* MG1 also exhibited differential effect on wheat bread quality (Galle et al., 2012a). It remains unclear, however, whether differences relate to functional differences of the EPS, or to other differences between the EPS producing strains. For example, *L. reuteri* but not *W. cibaria* produce high levels of acetate in presence of sucrose (Galle et al., 2010; Schwab et al., 2008). Thus, different acidification levels may confound the beneficial impact of *in situ* produced EPS. To assess the effect of EPS that differ in linkage type and molecular

weight on sourdough bread quality, it is necessary to eliminate the differential impact of EPS producing strains on bread quality as confounding factor.

Reuteran and dextran are synthesized by the glycoside hydrolase family 70 (GH70) enzymes reuteransucrase and dextransucrase, respectively. Glucansucrases catalyze the alternative reactions sucrose hydrolysis, EPS synthesis, and oligosaccharide synthesis when suitable acceptor sugars are present (Korakli and Vogel, 2006, Van Hijum et al., 2006). Glucansucrases harbor of four distinct domains, a signal peptide, an N variable region, a catalytic domain and a C-terminal domain (Monchois et al., 1999). Sequence alignments, site directed mutagenesis and three-dimensional structure analysis identified the catalytic sites of glucansucrases, namely the catalytic nucleophile, the acid/base catalyst, and the transition-state stabilizer (van Hijum et al., 2006). Linkage specificity is determined by several catalytic residues. The amino acids V1024, V1027 in nucleophile region, and S1135, N1136, A1137 in the transition-state stabilizer region (GtfA of *L. reuteri* TMW1.656 numbering) determined the linkage specificity of glucansucrases (Kralj et al., 2005; van Leeuwen et al., 2008). Knowledge on the amino acids that determine glucan structure thus allows the generation of mutant glucansucrases producing glucans differing in linkage type and / or molecular weight.

This study aimed to investigate structure-function relationships of reuteran produced from *L. reuteri* TMW1.656 and dextran produced from *W. cibaria* 10M in baking application. The primary structure of reuteransucrase was modified to produce reuterans with different linkage types and molar masses, and the effect of these EPS on bread quality was determined. Moreover, the effect of *in situ* produced reuteran on bread quality was compared to the effect of reuteran used as additive.

2. MATERIAL AND METHODS

2.1. Bacteria strains, plasmids, media and growth condition

L. reuteri TMW1.656 was cultivated anaerobically at 37 ˚C in modified DeMan-Rogosa-Sharpe (mMRS) medium (Stolz et al., 1995) containing either 10 g/L maltose, 5 g/L glucose and 5 g/L fructose, or 100 g/L sucrose. *W. cibaria* 10M was cultivated anaerobically at 30 ˚C in the same medium. *E. coli* strains TOP10 (Invitrogen, Toronto, ON, Canada), *E. coli* XL1 Blue (Agilent Technologies, Santa Clara, CA, USA) containing plasmids pUC18, pUC19 (Thermo scientific, Burlington, ON, Canada) were cultivated aerobically at 37 ˚C in LB (BD, Mississauga, ON, Canada) medium with 50 mg/L ampicillin. *E. coli* strain BL21 Star (DE3) (Invitrogen) with plasmid pET28a^{+} (Novagen, Etobicoke, ON, Canada) was cultivated aerobically at 37 ˚C in LB broth with 50 mg/L kanamycin (Invitrogen) for the purpose of expression *dsrM*, *gtfA* and derived mutant genes.

2.2. General molecular techniques

Bacterial DNA was isolated with DNeasy Blood & Tissue Kit (Qiagen, Toronto, ON, Canada). Bacterial plasmid DNA was isolated with QIAprep Spin Miniprep Kit (Qiagen). DNA was amplified by PCR using PfuUltra High-Fidelity DNA polymerase (Agilent Technologies). DNA fragments were purified from agarose gel using MinElute Gel Extraction Kit (Qiagen). Cloning, *E. coli* transformation, DNA manipulations and agarose gel electrophoreses were performed as described (Green and Sambrook, 2012). Restriction endonuclease (Thermo Scientific, Ottawa, Canada) digestion and DNA ligation with T4 ligase (Thermo Scientific) were performed following procedures provided by the suppliers. Chromosomal and plasmid DNA were sequenced by service of

126 Macrogen (Macrogen, Rockville, MD, USA). Nucleotide and amino acid sequences
127 analysis was performed using DNAMAN software (Lynnon Biosoft, San Ramon, CA,
128 USA).

129 2.3. Glucansucrase gene cloning and plasmid construction

130 The reuteransucrase *gtfA* in *L. reuteri* TMW1.656 was identified in the genome of that
131 strain by nucleotide blast with *gtfA* from *L. reuteri* TMW1.106 (EF189716) as query
132 sequence. GtfA from *L. reuteri* TMW1.656 is identical to GtfA from *L. reuteri*
133 TMW1.106. For construction of the expression plasmid, *gtfA* without the N-terminal
134 variable region was cloned into two fragments with a site silent mutation to inactive NcoI
135 restriction site and ligated into pUC19 plasmid. pUC19-GtfA-ΔN-2571 and GtfA-ΔN-
136 616 were ligated after the digestion at BamHI and PaeI sites to allow the generation of
137 pUC19-GtfA-ΔN. pE-GtfA-ΔN was constructed by ligating GtfA-ΔN with pET28a$^+$
138 plasmid at NcoI and NotI sites after digestion (Table 1). To simplify further mutagenesis,
139 pUC18-gtfA1500 was constructed by ligating pUC18 plasmid and 1500 bp catalytic
140 domain of *gtfA* at PstI and KpnI sites (Table 1).

141 The dextransucrase gene *dsrM* from *W. cibaria* 10M was amplified by using primer pair
142 (DSRf 5'-TTACCAAGTGAACAACGTGCAA -3' and DSRr 5'
143 TTAAAWCGTCACCAACGTACC-3'); these primers were designed based on the
144 alignment the dextransucrase genes from *W. cibaria* LBAE-K39 (GU237484), *W. cibaria*
145 CMU (EU885339), *W. cibaria* CH2 (CP012873), *W. cibaria* TN610 (HE8118409) and *L.*
146 *fermentum* strain Kg3 (AY697433). The amplified fragment was sequenced (KU363982).
147 The signal peptide truncated *dsrM* was cloned into pUC18 plasmid (Table 1). DsrM-ΔSP
148 was ligated with pET28a$^+$ plasmid at BamHI and NotI sites.

149 2.4. Amino acid sequence alignment and site-directed mutagenesis of GtfA

150 The amino acid sequence of GtfA was aligned together with 8 glucansucrases and 3 GtfA
151 mutants with known catalytic preferences by using DNAMAN software (Lynnon Biosoft)
152 (Table S1 of the online supplementary material). Catalytic residues with putative
153 glucosidic bond preference were identified. QuikChange II Site Directed Mutagenesis Kit
154 (Agilent Technologies) was used to construct site-directed mutants in pUC18-gtfA1500
155 (Table 1). Successful mutants resulted in the generation or elimination of restriction sites,
156 followed by sequence confirmation. The desired mutant pUC18-gtfA1500 was ligated
157 into the corresponding site of pUC19-GtfA-ΔN after the digestion by PstI and FspI
158 restriction endonucleases. Whole sequence of the mutated GtfA-ΔN was ligated into
159 pET28a^{+} plasmid to generate GtfA-ΔN-V1024P:V1027I, GtfA-ΔN-S1135N:A1137S and
160 GtfA-ΔN-V1024P:V1027I:S1135N:A1137S.

161 2.5. Heterologous expression and purification of glucansucrases

162 DsrM-ΔSG, GtfA-ΔN and its mutant derivatives were expressed and purified as
163 described previously (Kralj et al., 2011). The purity of glucansucrases was determined by
164 SDS-PAGE (Bio-Rad, Mississauga, ON, Canada).

165 2.6. Recombinant enzyme characterization

166 Protein concentration was determined by using protein assay reagent (Bio-Rad) with
167 bovine serum albumin as standard. Enzyme activities of reuteransucrase GtfA-ΔN and
168 mutant enzymes were evaluated essentially as described (Kralj et al., 2004). In brief,
169 enzyme activities were determined in 25 mM sodium acetate buffer (pH = 4.7) containing
170 1 mM $CaCl_2$, 100 mM sucrose, and 1 μM enzyme. Samples were taken in 5 min intervals

and the reaction was stopped by heating to 95 ˚C for 10 mins. The concentration of glucose and fructose was determined enzymatically (Glucose and Fructose Assay Kit, Sigma-Aldrich, Oakville, ON, Canada). The amount of free glucose represents hydrolysis activity and the amount of fructose represents the total enzyme activity. Transferase activity was calculated as difference between total and hydrolysis activity. One unit of enzyme activity was defined as the release of 1 µmol of monosaccharides per min.

2.7. Analysis of acceptor reaction products

Oligosaccharides were synthesized from acceptor reaction of 1 µM of GtfA-ΔN or mutant derivatives incubated with 500 mM sucrose and 500mM maltose in reaction buffer for 24 h. Samples were analyzed by HPAEC-PAD with a Carbopac PA20 column coupled to an ED40 chemical detector (Dionex, Oakville, Canada) (Galle et al., 2010). Fructose, glucose, sucrose, maltose, maltotriose and panose were identified by using external standards (Sigma Aldrich).

2.8. Glucan production and purification

To purify enzymatically produced glucans, 50 nM purified DsrM-ΔSP, GtfA-ΔN and derivative mutant enzymes were incubated with 500 mM sucrose in 25 mM sodium acetate buffer (pH = 4.7) containing 1 mM $CaCl_2$ for 2 days. Reuteran and dextran were harvested via 2 volume ethanol precipitation and purified by dialysis. The retentate was freeze dried and stored at -20 ˚C. To purify glucans produced by *L. reuteri* TMW1.656, the strain was cultured in sucrose mMRS broth for 16 h and subcultured overnight in 1 L sucrose-mMRS broth. Reuteran was harvested via 2 volume ethanol precipitation and purified by dialysis as described (Chen et al., 2014). The retentate was freeze dried and stored at -20˚C.

2.9. Characterization of EPS

One-dimensional ^{1}H-NMR spectra were recorded on an Agilent/Varian Inova three-channel 400 MHz spectrometer at the University of Alberta NMR facility. All spectra were recorded at 353K with Z-gradient probe. EPS samples were dissolved in 99.97% D_2O. Chemical shifts were expressed in parts per million (ppm) by reference to internal standard 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS). The proportion of α-(1→4) and α-(1→6) glucosidic linkages was determined at 5.3 and 5.0 ppm, respectively (Kralj et al., 2005).

Molar mass determination of glucan was performed with asymmetrical flow field-flow-fractionation (AF4) coupled to multi-angle light scattering (MALS) and RI detectors (Postnova, Salt Lake City, UT, USA). The regenerated cellulose membrane (Postnova) of the accumulation wall had a molecular weight cut off of 10 kDa. Glucan samples were dissolved to 10 g/L in 10mM NaCl and injected onto the channel at a flow rate of 0.2 mL/min and a cross flow of 1 mL/min for 6 mins. After injection, the cross flow rate remained constant for 2 min, decreased exponentially to 0.1 mL min over 10 mins, and was then maintained at 0.1 mL/min for 10 mins. The molar mass was calculated MALS signals and RI signal by AF 2000 software (Postnova). A value of 0.146 ml/g was employed as refractive index increment (dn/dc) (Vilaplana and Gilbert, 2010). Poly-styrolsulphonate standards and BSA were used for calibration of detectors.

2.10. In frame deletion of *gtfA* from *L. reuteri* TMW1.656

The conserved catalytic domain of *gtfA* was truncated according to a deletion strategy described previously (Su et al., 2011). In brief, an upstream 1494 bp fragment and a downstream 1132 bp fragment were amplified from genomic DNA of *L. reuteri*

TMW1.656 (Table 1) and ligated together into pJRS233 at PstI and XbaI sites. The first and second crossover were selected as described (Su et al., 2011). The genotype of *L. reuteri* TMW1.656Δ*gtfA* was confirmed by PCR and DNA sequencing (KU363983).

2.11. Sourdough fermentation

L. reuteri TMW1.656 or *L. reuteri* TMW1.656Δ*gtfA* were incubated at 37 ˚C overnight in mMRS broth and subcultured with 1% inoculum in 10% sweet wort (Zhao et al., 2015). Sourdoughs with a dough yield of 200 [100 x (wheat flour + malt solution) / wheat flour] were fermented for 24 h (Galle et al., 2012b). Cell counts and pH were determined at 0 h and 24 h of fermentation. After 24 h fermentation, organic acids and EPS were quantified as described (Galle et al., 2010). EPS formation was analyzed and quantified by size exclusion chromatography using a Superdex 200 column (GE Healthcare Life Sciences, Mississauga, ON, Canada). Fermented sourdough was directly used for baking at a dosage level of 10%.

2.12. Bread baking

White wheat flour with 13% protein content and whole rye flour with 10% protein content were purchased from local supermarket. Bread dough was prepared with 55% (w/w flour base) tap water; 10% (w/w flour base) sourdough; 2% (w/w flour base) dry yeast; 1.5% (w/w flour base) table salt, and 0%, 0.09% or 0.19 % (w/w flour base) glucan produced from GtfA-ΔN and its mutant derivatives. To keep a constant water/flour ratio, flour and water present in sourdough replaced equal amount of flour and water for control dough (Galle et al., 2012b). Control bread was prepared by replacing sourdough with equal amount of commercial flour and tap water.

239 Dough ingredients were blended with a C-dough hook kneader for 2 min at low speed
240 (level 2) and 3 min at high speed (level 5) in a KitchenAid mixer (Mississauga, ON,
241 Canada). After a dough rest at 30°C for 75 min, dough was scaled to 230 g, shaped, and
242 placed in baking pans greased with sunflower oil. Dough was proofed at 30°C for 20 min
243 before baking at 177°C for 25 min in a preheated convection oven (Bakers pride, Allen,
244 Texas, USA). Loaves were removed from pans after baking and cooled for at least 2 h at
245 room temperature before further analysis. Breads were stored in sealed plastic boxes at
246 room temperature. Two to six independent repetitions on different baking days were
247 performed for each type of bread.

248 2.13. Evaluation of bread characteristics

249 Bread volume was measured according to the AACC method 10-05 (AACC International,
250 2001) with four loaves for each bread type. Bread hardness was measured by texture
251 profile analysis (TPA) according to the AACC method 74-09 (AACC International,
252 1995). TPA was performed using a TA.XT Express Enhanced texture analyser (Stable
253 Micro System, Godalming, Surrey, UK) equipped with a 2 kg load cell and an aluminum
254 plunger with a diameter of 12.7 mm. Measurements were performed with a test speed of
255 2.0 mm/s and a trigger force of 20 g. Slices were compressed to 50% of their original
256 height. For TPA, loaves were cut into 20 mm thick slices. Two slices were cut from the
257 centre of the bread and each slice was measured 3 times at different sites. Hardness was
258 measured after 0, 2, 5, and 8 d of storage, or after 0, 5, and 7 d of storage.

259 2.14. Statistical analysis

260 Differences of glucans effect on bread volume and hardness (n = 2, 4 or 6) were analyzed
261 using PROC MIXED of SAS Version 9.4 (SAS Institute Inc, USA). Model included the

fixed effect of glucan treatment and random effect of baking day. Results were expressed as least square mean ± standard error of the means. Significant differences are reported with a 5% probability of error ($P < 0.05$).

3. RESULTS

3.1. Biochemical characterization of wild-type glucansucrase and derived mutants

To generate different reuterans and dextran, reuteransucrase from *L. reuteri* TMW1.656 was cloned as N terminal truncated enzyme GtfA-ΔN. Dextransucrase DsrM-ΔSP was cloned from *W. cibaria* 10M as signal peptide truncated enzyme (Figure S1 of the online supplementary material). DsrM-ΔSP, GtfA-ΔN and derived mutants were expressed in *E. coli*. The purity and molecular weight of recombinant enzymes were verified by SDS-PAGE (data not shown). No background activity was detected from *E. coli* with the empty pET28a$^+$ plasmid itself (data not shown). GtfA-ΔN and mutants enzymes had a molecular weight of 118 KDa. The molecular weight of DsrM-ΔSP was 160 kDa. Total enzyme activity and hydrolysis activity of GtfA-ΔN and derived mutants were determined with 500 mM sucrose. GtfA-ΔN-V1024P:V1027I and GtfA-ΔN-S1135N:A1137S yielded the same total activity but a lower ratio of transferase activity to hydrolysis activity compared to the wild-type GtfA-ΔN. GtfA-ΔN-V1024P:V1027I:S1135N:A1137S showed a significant decreased total activity and relative transferase activity compared to GtfA-ΔN (Figure S2 of the online supplementary material). The difference of enzyme activity among wild-type and mutant reuteransucrase indicated that site-directed mutations of the catalytic domain decreased total activity and transferase activity of the reuteransucrase GtfA.

3.2. Glucan linkage type and molar mass determination

285 Glucan structure and size may relate to their effects in bread quality; therefore, the
286 linkage type and the molar mass of enzymatically produced dextran, reuteran and derived
287 mutant reuterans were evaluated (Table 2). ^{1}H NMR analysis of EPS produced by DsrM-
288 ΔSP, GtfA-ΔN and mutant derivatives indicated that DsrM-ΔSP produced dextran with
289 mainly α-(1→6) linkages. GtfA produced reuteran with 14% α-(1→4) and 86% α-(1→6)
290 linkages, in keeping with the reuteran produced from GtfA from *L. reuteri* TMW1.106
291 (Kaditzky et al., 2008). Reuteran synthesized from GtfA-ΔN-V1024P:V1027I (reuteranPI)
292 showed an elevated level of α-(1→4) linkages compared to wild type reuteran. Reuteran
293 from GtfA-ΔN-S1135N:A1137S (reuteranNS) and GtfA-ΔN-
294 V1024P:V1027I:S1135N:A1137S (reuteranPINS) also had an increased content of α-
295 (1→4) linkages (Table 2).

296 The molar mass distribution of bacterial and enzymatic produced glucan was determined
297 by AF4 (Table 2 and Figure 2). Dextran produced with DsrM-ΔSP had the highest molar
298 mass. The molar mass of the reuteran produced enzymatically with GtfA-ΔN, except for
299 reuteranNS, was higher than the molar mass of reuteran produced by fermentation with *L.*
300 *reuteri* TMW1.656. ReuteranPI and ReuteranPINS had a similar molecular weight as
301 enzymatically produced reuteran.

302 3.3. Oligosaccharides synthesis with 500 mM maltose as acceptor sugar

303 Analysis of enzyme properties was complemented by determination of oligosaccharide
304 synthesis. All reuteransucrases synthesized panose. GtfA-ΔN and GtfA-ΔN-
305 V1024P:V1027I also yielded the α-(1→6) linked panose series of oligosaccharides with
306 DP4 to DP8. GtfA-ΔN-S1135N:A1137S and GtfA-ΔN-

V1024P:V1027I:S1135N:A1137S, however, produced several new oligosaccharides that could not be identified, indicating a glucosidic bond preference other than α-(1→6), in accordance with NMR result (Figure 2). GtfA-ΔN-V1024P:V1027I:S1135N:A1137S produced maltotriose, in keeping with the preference for synthesis of α-(1→4) linkages (Figure 1).

3.4. In frame deletion of *gtfA* in *L. reuteri* TMW1.656

To obtain an EPS-negative derivative of *L. reuteri* TMW1.656, *gftA* was disrupted by deletion of the section corresponding to the GtfA catalytic domain by double crossover mutagenesis. The disruption of *gtfA* in *L. reuteri* TMW1.656Δ*gtfA* was verified by sequencing (Figure S3 of the online supplementary material). A comparison of polysaccharides in culture supernatants of *L. reuteri* TMW1.656 and *L. reuteri* TMW1.656Δ*gtfA* was performed by AF4. *L. reuteri* TMW1.656 but not *L. reuteri* TMW1.656Δ*gtfA* produced a high molecular weight polysaccharide (Figure 3), confirming that the phenotype *L. reuteri* TMW1.656Δ*gtfA* matches the genotype.

3.5. Sourdough fermentation and EPS quantification

After 24 h fermentation of *L. reuteri* TMW1.656 and TMW1.656Δ*gtfA* with 15% sucrose, a similar pH of 3.4 ± 0.1 and 3.3 ± 0.1 was observed. A comparable organic acid profile was observed in sourdough fermented with *L. reuteri* TMW1.656 and *L. reuteri* TMW1.656ΔgtfA; the concentration of lactate (116.4 ± 3.5 mM and 107.0 ± 20.5 mM), acetate (45.8 ± 1.2 mM and 34.0 ± 5.5 mM) and ethanol (7.6 ± 0.2 and 16.5 ± 3.8 mM) were similar. The results indicate *L. reuteri* TMW1.656 and *L. reuteri* TMW1.656ΔgtfA have same fermentation behavior.

Quantification of bacterial glucan production in sourdough was performed to guide the addition of enzymatically produced glucan to bread. Purification of water soluble polysaccharides from sourdough fermented with *L. reuteri* TMW1.656 and TMW1.656Δ*gtfA*, followed by acid hydrolysis, confirmed that the wild type strain but not the mutant strain produced EPS composed of glucose. Quantification of glucan by size exclusion chromatography, and by quantification of glucose after hydrolysis revealed that *L. reuteri* TMW1.656 produced 15.8 ± 4.2 g reuteran/kg sourdough dry weight. For the subsequent studies, enzymatically produced glucans were added at two levels corresponding to 9.0 or 19 g/kg sourdough dry weight.

3.6. Impact of bacterial and enzymatically produced glucans on sourdough bread volume and texture

To compare the influence of *in situ* produced reuteran and enzymatically produced reuteran used as additive, bread quality was evaluated by the determination of loaf volume and crumb hardness. Enzymatically produced glucans were added at 0.09 or 0.19% to the bread dough. EPS was added to bread dough produced with 10% sourdough fermented with the EPS-negative *L. reuteri* TMW1.656Δ*gtfA* to account for metabolic properties of the strain. Sourdough bread was baked with two types of flours, 100% white wheat flour, or with addition of 20% whole rye flour to weaken the gluten structure. Bread volume and crumb hardness of wheat breads produced with sourdough and EPS addition are shown in Table 3; bread characteristics of 80% wheat and 20% rye bread produced with sourdough and EPS addition are shown in Table 4.

Wheat sourdough bread had a higher bread volume when compared to control bread (Table 3), except for the bread with 0.09% reuteran addition. The loaf volume of bread with 0.19% reuteran addition and *L. reuteri* TMW1.656Δ*gtfA* sourdough was not different from the bread baked with *L. reuteri* TMW1.656 sourdough. However, volume enhancement by 0.19% reuteran was accompanied by uneven pore formation in bread (Figure 4). The hardness of the crumb of bread produced with *L. reuteri* TMW1.656 or with *L. reuteri* TMW1.656Δ*gtfA* and addition of 0.19% reuteran remained comparable throughout 5 days of storage (Table 3); bread produced with *L. reuteri* TMW1.656Δ*gtfA* and addition of 0.09% reuteran exhibited a higher crumb hardness. *In situ* produced and *ex situ* added reuteran thus had comparable effects on the volume and crumb hardness of wheat sourdough bread.

The loaf volume of 100% wheat sourdough bread was not increased by the addition of dextran when compared to *L. reuteri* TMW1.656Δ*gtfA* sourdough bread (Table 3). The volume of bread produced with 80% wheat flour and 20% rye flour, however, increased after addition of 0.09% dextran (Table 4). Remarkably, 0.09% addition of reuteran decreased the volume of sourdough bread when compared to sourdough bread produced with *L. reuteri* TMW1.656Δ*gtfA* (Table 4). Thus, weakening of gluten structure promoted EPS impact on sourdough bread volume. Hardness measurement on both 100% wheat and 20% rye sourdough bread revealed dextran addition decreased crumb hardness and reduced bread staling (Table 3 and 4).

Reuterans produced from reuteransucrase mutants differed in their linkage type and molar mass (Table 2). To compare the effect of EPS structure on bread volume and hardness, 0.19% reuteran, reuteranPI, reuteranNS, and reuteranPINS were added to

373 sourdough bread produced with *L. reuteri* TMW1.656Δ*gtfA*. Remarkably, sourdough
374 bread produced with reuteranNS and reuteranPINS were higher in volume when
375 compared to bread produced with reuteran addition (Table 3), demonstrating that reuteran
376 structure impacts bread volume. The effect of reuteranNS and reuteranPINS on bread
377 volume was comparable to the effect of dextran (Table 3). Addition of reuteranNS and
378 reuteranPINS, however, resulted in uneven pore formation, particularly the formation of
379 large pores under crust, an effect that was also observed with reuteran but not with
380 dextran (Figure 4). The crumb hardness of sourdough bread produced with reuteranNS
381 was comparable to dextran and lower than the crumb hardness of bread produced with
382 reuteranPINS or reuteran (Table 3). Thus, reuteran linkage type and molar mass both
383 contributed to bread quality improvement.

384 **4. DISCUSSION**

385 EPS production during sourdough fermentation may beneficially affect bread quality.
386 Dextran production by *W. cibaria* in sourdough increased bread volume and reduced
387 staling (Galle et al., 2012b; Katina et al., 2009). A beneficial effect of reuteran, however,
388 was not demonstrated, possibly because acetate formation by *L. reuteri* in presence of
389 sucrose compensated any beneficial effects of reuteran (Galle et al., 2012b). Likewise,
390 beneficial effects of levan from *L. sanfranciscensis* were obscured by increased acetate
391 formation, which reduced bread volume (Kaditzky et al., 2007). Most heterofermentative
392 lactobacilli preferentially metabolize fructose as electron acceptor, which results in the
393 formation of mannitol and acetate (Korakli et al., 2001; Zheng et al., 2015). However, the
394 absence of mannitol dehydrogenase in most strains of *Weissella* spp prevents excessive
395 acidification even when sucrose is present (Galle et al., 2010). The differential effect of

sucrose on metabolism of heterofermentative lactobacilli and *Weissella* confounds the comparison of EPS with different composition and structure. The present study added enzymatically produced reuterans and dextran to sourdough fermented with the EPS-negative *L. reuteri* TMW1.656Δ*gtfA*, thus eliminating confounding factors related to bacterial metabolism.

The application of glucan in bread can be achieved either by *in situ* production with starter culture or *ex situ* addition of the same compound. It was suggested that *in situ* produced EPS is superior to addition of *ex situ* produced EPS with respect to its effect on bread quality (Brandt et al., 2003 as cited in Tieking and Gänzle, 2005). The use of the levansucrase mutant strain *L. sanfranciscensis* LTH2590, however, indicated that *in situ* produced levan was not as effective as addition of *ex situ* produced levan (Kaditzky et al., 2007). In *L. sanfranciscensis*, the deletion of levansucrase activity also abolishes sucrose metabolism and thus reduces acetate formation (Tieking and Gänzle, 2005); any effects related to levan formation are thus confounded by the different acetate concentrations. *L. reuteri* TMW1.656Δ*gtfA* retains sucrose phosphorylase as second sucrose metabolic enzyme (Teixeira et al., 2012) and deletion of glucansucrases in *L. reuteri* has thus only a minor effect on sucrose metabolism and acetate formation (Schwab et al., 2007; Walter et al., 2008). Accordingly, lactate production by *L. reuteri* TMW1.656Δ*gtfA* was comparable to *L. reuteri* TMW1.656. *L. reuteri* TMW1.656Δ*gtfA* formed slightly less acetate and more ethanol, which may relate to the observation that metabolic turnover by extracellular glycansucrases is faster than metabolism by intracellular enzymes (Teixeira et al., 2012). Nevertheless, the cell counts, the pH, and organic acid levels in bread dough produced with *L. reuteri* TMW1.656 were comparable to bread dough produced with *L.*

419 *reuteri* TMW1.656Δ*gtfA*, eliminating the confounding effect of acetate formation. This
420 improved experimental setup demonstrated that the effect of *in situ* produced reuteran on
421 bread volume and crumb hardness was similar to the effect of addition of reuteran.

422 This study compared bread produced with 100% wheat flour to bread produced with 20%
423 rye flour. EPS effects on bread quality were more pronounced in the bread with rye
424 addition. This appears to contrast previous studies reporting beneficial effects of EPS in
425 wheat bread (Galle et al., 2012a). Galle et al. (2012), however, used wheat flour with a
426 protein content of 8-10% while the protein content of the wheat flour used in the present
427 study was 13%. Both studies thus consistently indicate that EPS effects are more
428 pronounced when a strong gluten network is absent. Remarkably, rye bran was recently
429 shown to be an excellent substrate to boost dextran formation by *W. cibaria* (Kajala et al.,
430 2016). Therefore, dextran-enriched rye or rye bran sourdoughs may serve as functional
431 ingredient in bread production to improve sensory and nutritional properties without
432 compromising texture and volume.

433 Only few previous studies compared the effect of linkage type, molecular weight, and
434 branching on the technological functionality of glucans in baking. It was suggested that
435 dextran with a linear chain structure may be more effective in increasing bread volume
436 when compared to dextran with a similar molar mass but more branching (Lacaze et al.,
437 2007). Moreover, reuteran produced from *L. reuteri* TMW1.106 with 19 % α-(1→4)
438 linkage was more efficient in increasing bread volume than dextran produced from
439 *L. curvatus* TMW 1.624 with 8-9 % α-(1→4) linkage (Rühmkorf et al., 2012). Different
440 from previous studies, the use of *L. reuteri* TMW1.656Δ*gtfA* in combination with
441 addition of EPS from mutant reuteransucrases provided an unprecedented opportunity to

442 study structure-function relationships of EPS. Reuterans from mutant enzymes with
443 increased α-(1→4) linkage (25% to 51%) were more effective in enhancing wheat bread
444 volume than wild type reuteran with 14% α-(1→4) linkages. Moreover, the effect of
445 reuteranNS with 39% α-(1→4) linkage and the lowest molecular weight of 12.2 MDa
446 was comparable to the effect of dextran with a molecular weight of 82.9 MDa. The
447 present study thus demonstrates that size as well as structure of glucans relate to their
448 effects on bread quality. Remarkably, specific effects of EPS structure extended to the
449 crumb porosity. Addition of reuteran, reuteranNS and reuteranPINS resulted in an uneven
450 pore distribution while reuteranPI and dextran improved bread texture without leading to
451 formation of large pores (Table 3 and Figure 4). The effect of hydrocolloids on crumb
452 porosity is poorly documented in the literature. It was previously reported that high
453 concentrations of pectin increased crumb porosity when compared to addition of low
454 levels of pectin because pectin was not able to stabilize gas cells in the dough and leaded
455 to the coalescence of the cells (Lazaridou et al., 2007).

456 In conclusion, this study investigated the effect of glucans on sourdough bread quality.
457 EPS was added to bread produced with sucrose metabolizing *L. reuteri* TMW1656Δ*gtfA*,
458 thus eliminating confounding effects of (sucrose) metabolism during sourdough
459 fermentation on bread quality. In wheat bread, *in situ* produced reuteran and addition of
460 enzymatically produced reuteran exerted comparable effects on bread quality. Effects of
461 dextran on bread volume and texture were superior to reuteran. Site-directed mutagenesis
462 of reuteransucrase generated reuterans which differed in the linkage type and the molar
463 mass. Noticeably, reuteranNS was as efficient as dextran in bread quality improvement.
464 Therefore, a structure function relation is suggested high percentage of α-(1→4) linkage

and low MW of reuteran promoted the improvement of bread volume and softness. Our experiment allowed a fast screening of suitable EPS on bread quality enhancement in baking application.

ACKNOWLEDGEMENTS

The Alberta Livestock and Meat Agency are acknowledged for funding. Xiao Yan Chen acknowledges support from the China Scholarship Council.

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586

Figure legends

Figure 1. HPAED-PAD analysis of oligosaccharides products produced from acceptor reaction of GtfA-ΔN and derived mutants. Oligosaccharides were formed by incubation of 2µM GtfA-ΔN enzyme with 500 mM sucrose and 500 mM maltose for 24 h. Chromatographic traces are offset by 600nC. GtfA = oligosaccharides produced from GtfA-ΔN; PI = oligosaccharides produced from GtfA-ΔN-V1024P:V1027I; PINS = oligosaccharides produced from GtfA-ΔN-V1024P:V1027I:S1135N:A1137S; NS = oligosaccharides produced from GtfA-ΔN-S1135N:A1137S. Oligosaccharides identified by external standards are indicated; "?" indicates unknown oligosaccharides.

Figure 2. Analysis of polysaccharides purified from culture supernatant of *L. reuteri* TMW1.656 and *L. reuteri* TMW1.656Δ*gtfA* by AF4 coupled with multi angle laser scattering detector. Shown are signals from the light scattering at 90° angle (black line) and RI (grey line)

Figure 3. Cumulative molar mass distribution of glucans as determined by AF4 coupled with multi angle laser scattering detector. Shown is the cumulative molar mass distribution of reuteran produced from *L. reuteri* TMW1.656 (black dotted line) or enzymatically produced reuterans (black solid lines) and dextran (grey solid line). 1.656 = reuteran produced from *L. reuteri* TMW1.656; NS = reuteran produced from GtfA-ΔN-S1135N:A1137S; PINS = reuteran produced from GtfA-ΔN-V1024P:V1027I:S1135N:A1137S; PI = reuteran produced from GtfA-ΔN-V1024P:V1027I; GtfA = reuteran produced from GtfA-ΔN; DsrM = dextran produced from DsrM-ΔSP

Figure 4. Crumb structure of wheat sourdough bread produced with 10% sourdough fermented with *L. reuteri* TMW1.656Δ*gtfA* and addition of 0.19% dextran, reuteran, reuteranPI, reuteranNS, or reuteranPINS. Pictures are representatives of 50% of all breads baked with 0.19% EPS addition.

Table 1. Primers and plasmids used in this study.

Wild type / Mutation	plasmid	Primer pair (5'-3')	Restriction site
GtfA-ΔN-2571	pUC19	For ATTCGAGCTCCCATGGGCACTATTAACG	SacI NcoI
		Rev: TGAAGGATCCATAGCAACCCCAGT	Inactivation of NcoI
GtfA-ΔN-616	pUC19	For TCAACTGGGGTTGCTATGGATCCTT	Inactivation of NcoI
		Rev ACATGCATGCGGCCGCTAGTTTTTTCTGA	PaeI NotI
GtfA-ΔN	pET-28a$^+$		NcoI NotI
GtfA-1500	pUC18		PstI KpnI
DsrM-ΔSP	pUC19 / pET-28a$^+$	For GCCGGGATCCGATACTGTATTACCAAGTGAA	BamHI
		Rev ATTCTAGAGCGGCCGCAATCGTCACCAACGTA	XbaI NotI
GtfA-ΔN V1024P:V1027I	pUC18-GtfA-1500 pUC19/ pET-28a$^+$	For CGAGTAGATGCGCCGGATAACATCGATGCAGATC	Inactivation of AclI
		Rev GATCTGCATCGATGTTATCCGGCGCATCTACTCG	
GtfA-ΔN S1135N:A1137S	pUC18-GtfA-1500 pUC19/ pET-28a$^+$	For CCGTGCGCATGATAATAATTCACAAGATC	FspI
		Rev GATCTTGTGAATTATTATCATGCGCACGG	
GtfA-ΔN V1024P:V1027I: S1135N:A1137S	pUC18-GtfA-1500 (V1024P:V1027I) pUC19/ pET-28a$^+$	For CCGTGCGCATGATAATAATTCACAAGATC	FspI
		Rev GATCTTGTGAATTATTATCATGCGCACGG	
L. reuteri TMW1.656Δ*gtfA*	pUC18/ pJRS233	For-up CGCAAGCTTAATGATTTACAACCGGGTC	HindIII
		Rev-up ATTCGAGCTCCCTTGGAATTGAATACC GCTA	PstI
		For-down AACTGCAGTACTTATCTGGTCATAGCCTAG	PstI
		Rev-down CTAGTCTAGACTCCACGGAATGATACACC	XbaI

Plasmids and oligonucleotides used for *gtfA* and *dsrM* cloning, site-directed mutagenesis of *gtfA* and *L. reuteri* TMW1.656Δ*gtfA* knock out.

Underlined nucleotides stand for silent mutation or site-directed mutation.

Table 2. Chemical properties of enzymatically produced reuterans and dextran.

Enzyme used for synthesis	Chemical shift %			Mw	Mn	Mz
	α-(1→3)	α-(1→4)	α-(1→6)	(10^6 g·mol^{-1})		
GtfA-ΔN	/	14	86	48.6	33.9	99.8
V1024P:V1027I	/	25	75	43.5	36.9	50.6
S1135N:A1137S	/	39	61	12.2	9.6	13.8
V1024P:V1027I:S1135N:A1137S	/	51	49	21.4	20.2	22.7
DsrM-ΔSP	≤2*	≤2*	98	82.9	47.3	192

400 MHz ^{1}H NMR recorded at 300 K in D_2O and AF4 molar masses analysis of the glucans produced by purified wild-type GtfA-ΔN and DsrM-ΔSP enzymes and derived mutants. Displayed are the anomeric signals at ~4.96 ppm [α-(1→6) linkages], ~5.33 ppm [α-(1→4) linkages] and ~5.29 ppm [α-(1→3) linkages]

* Dextran produced from DsrM-ΔSP with trace amount (no more than 2%) of α-(1→3) and α-(1→4) linkages

Table 3. Effect of added EPS on the impact of wheat sourdough bread volume and crumb hardness. Bread was produced with 10% wheat sourdough containing 15% sucrose and fermented with *L. reuteri* TMW1.656, or with 10% sourdough containing 15% sucrose and fermented with *L. reuteri* TMW1. 656Δ*gtfA*.

L. reuteri strain used for fermentation	Glucan added to bread dough	Volume [mL/g]	Hardness day 0 [gf]	Hardness day 5 [gf]	Hardness day 7 [gf]	Hardness day 8 [gf]
TMW1.656	no addition*	3.02 ± 0.08^{b}	$251 \pm 25^{c,d}$	$775 \pm 30^{c,d,e}$	ND	921 ± 37^{c}
TMW1.656Δ*gtfA*	0.09% reuteran*	2.86 ± 0.07^{c}	$297 \pm 18^{b,c}$	896 ± 25^{b}	ND	1110 ± 32^{b}
TMW1.656Δ*gtfA*	0.19% reuteran	3.03 ± 0.08^{b}	$329 \pm 25^{a,b}$	827 ± 31^{c}	1170 ± 20^{a}	ND
TMW1.656Δ*gtfA*	0.09% dextran	$3.04 \pm 0.08^{a,b}$	224 ± 25^{d}	$659 \pm 30^{g,h}$	ND	765 ± 37^{d}
TMW1.656Δ*gtfA*	0.19% dextran	$3.08 \pm 0.08^{a,b}$	$273 \pm 25^{b,c,d}$	$698 \pm 31^{e,f,g}$	806 ± 20^{c}	ND
TMW1.656Δ*gtfA*	0.19% reuteran-PI	$3.06 \pm 0.08^{a,b}$	$272 \pm 25^{b,c,d}$	$736 \pm 31^{d,e,f}$	907 ± 20^{b}	ND
TMW1.656Δ*gtfA*	0.19% reuteran-NS	3.18 ± 0.08^{a}	231 ± 25^{d}	$684 \pm 31^{f,g}$	754 ± 20^{c}	ND
TMW1.656Δ*gtfA*	0.19% reuteran-PINS	3.19 ± 0.08^{a}	$277 \pm 25^{b,c,d}$	600 ± 31^{h}	867 ± 20^{b}	ND
TMW1.656Δ*gtfA*	no addition	2.99 ± 0.07^{b}	$289 \pm 15^{b,c}$	$784 \pm 23^{c,d}$	897 ± 20^{b}	908 ± 32^{c}
No sourdough	no addition	2.80 ± 0.08^{c}	375 ± 25^{a}	1050 ± 30^{a}	ND	1250 ± 37^{a}

Data are shown as least square means ± standard error, n = 2, 4 or 6. Mean values in the same column with unlike letters are significantly different, $P < 0.05$.

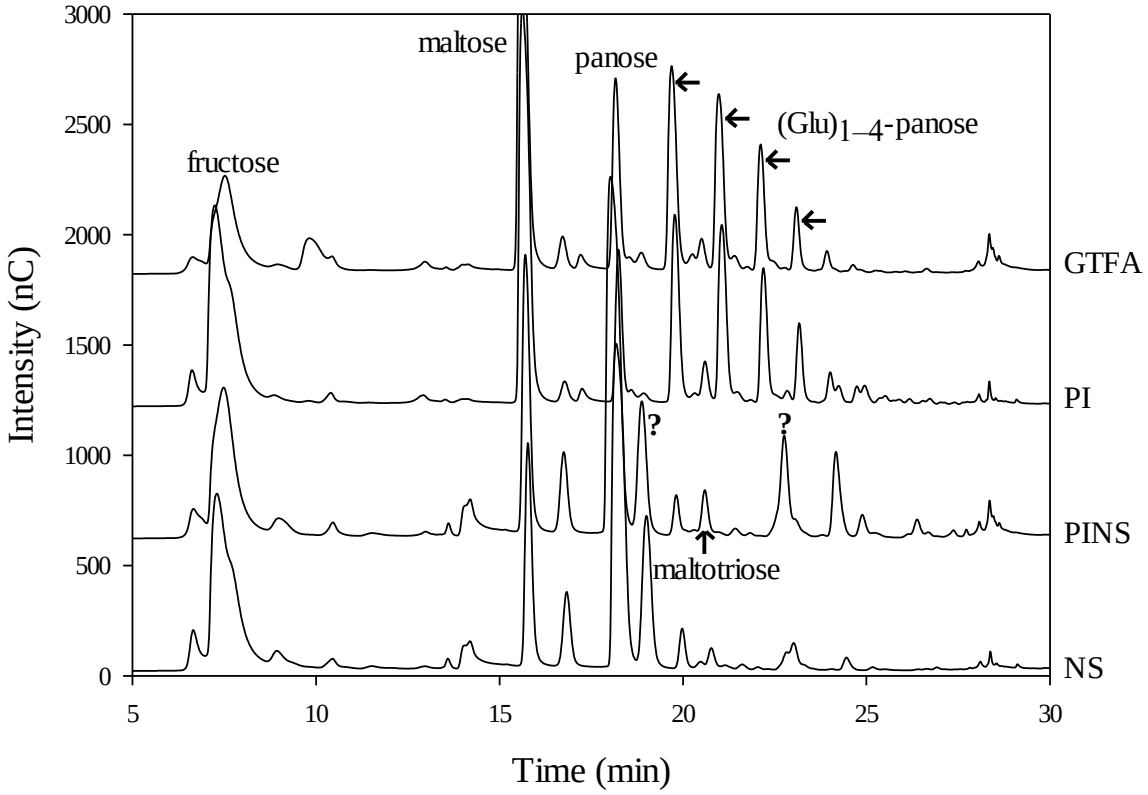
*Sourdough fermented with *L. reuteri* TMW1.656 contained 15.8 ± 4.2 g reuteran/kg sourdough dry weight. Sourdough fermented with *L. reuteri* TMW1.656Δ*gtfA* contained no EPS

Table 4 Effect of added EPS on the impact of 20% rye sourdough bread volume and crumb hardness. Bread was produced with 70% wheat flour, 20% rye flour, and 10% wheat sourdough containing 15% sucrose and fermented with *L. reuteri* TMW1. 656Δ*gtfA*, and addition of 0.09% enzymatically produced EPS.

Glucan added to bread dough	Volume [mL/g]	Hardness day 0 [gf]	Hardness day 2 [gf]	Hardness day 5 [gf]	Hardness day 8 [gf]
Reuteran	2.47 ± 0.03^{c}	457 ± 8.46^{a}	888 ± 13^{a}	1150 ± 31^{a}	1410 ± 12^{a}
Dextran	2.59 ± 0.03^{a}	382 ± 8.46^{c}	717 ± 13^{c}	853 ± 31^{b}	1140 ± 12^{c}
no EPS	2.53 ± 0.03^{b}	423 ± 8.46^{b}	757 ± 13^{b}	1056 ± 31^{a}	1250 ± 12^{b}

Data are shown as least square means ± standard error, n = 2. Mean values with unlike letters in the same column were significantly different, $P < 0.05$.

637 Figure 1



638

639 **Figure 1.** HPAED-PAD analysis of oligosaccharides products produced from acceptor reaction of
640 GtfA-ΔN and derived mutants. Oligosaccharides were formed by incubation of 2μM GtfA-ΔN
641 enzyme with 500 mM sucrose and 500 mM maltose for 24 h. Chromatographic traces are offset
642 by 600nC. GtfA = oligosaccharides produced from GtfA-ΔN; PI = oligosaccharides produced
643 from GtfA-ΔN-V1024P:V1027I; PINS = oligosaccharides produced from GtfA-ΔN-
644 V1024P:V1027I:S1135N:A1137S; NS = oligosaccharides produced from GtfA-ΔN-
645 S1135N:A1137S. Oligosaccharides identified by external standards are indicated; "?" indicates
646 unknown oligosaccharides.

647

Figure 2

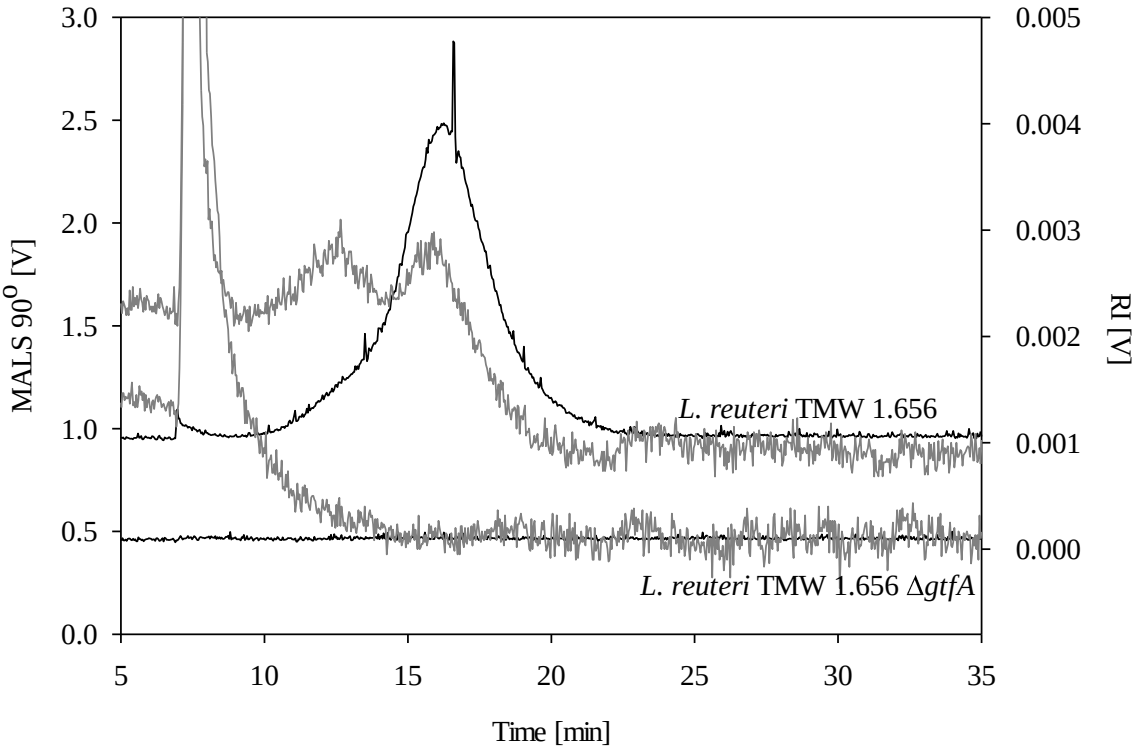
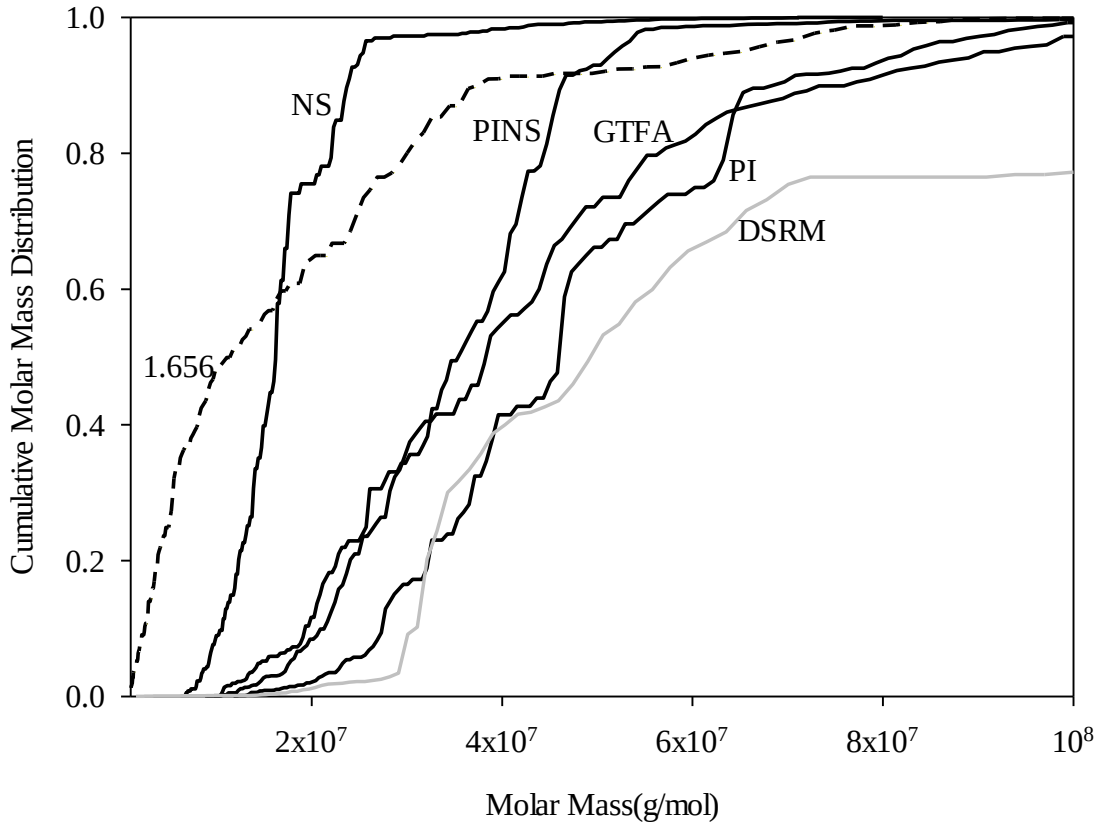


Figure 2. Analysis of polysaccharides purified from culture supernatant of *L. reuteri* TMW1.656 and *L. reuteri* TMW1.656Δ*gtfA* by AF4 coupled with multi angle laser scattering detector. Shown are signals from the light scattering at 90° angle (black line) and RI (grey line)

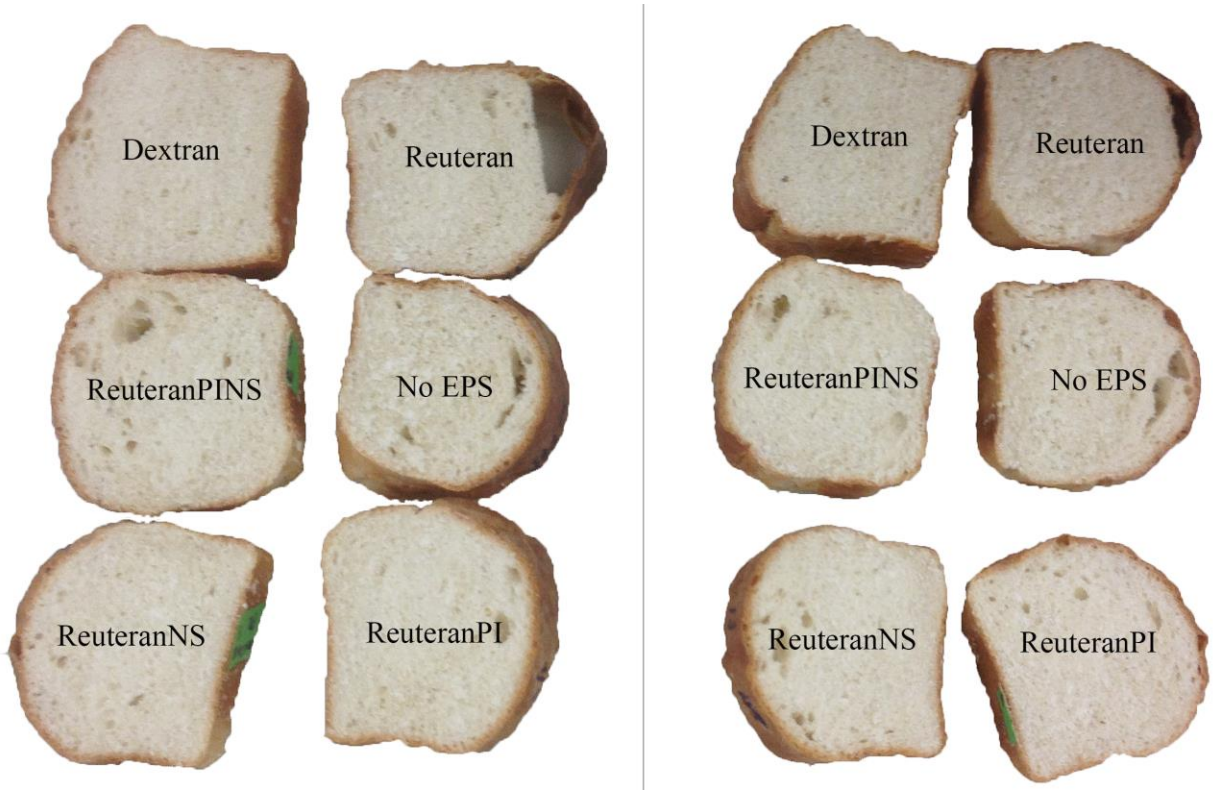
654 Figure 3



655

656 **Figure 3.** Cumulative molar mass distribution of glutans as determined by AF4 coupled with
657 multi angle laser scattering detector. Shown is the molar mass distribution from 10^6 to 10^8 g/mol
658 of reuteran produced by *L. reuteri* TMW1.656 (black dotted line) or enzymatically produced
659 reuterans (black solid lines) and dextran (grey solid line). 1.656 = reuteran produced from *L.*
660 *reuteri* TMW1.656; NS = reuteran produced from GtfA-ΔN-S1135N:A1137S; PINS = reuteran
661 produced from GtfA-ΔN-V1024P:V1027I:S1135N:A1137S; PI = reuteran produced from GtfA-
662 ΔN-V1024P:V1027I; GtfA = reuteran produced from GtfA-ΔN; DSRM = dextran produced from
663 DsrM-ΔSP

664 Figure 4



665

666 **Figure 4.** Crumb structure of wheat sourdough bread produced with 10% sourdough fermented with *L. reuteri* TMW1.656Δ*gtfA* and addition of
667 0.19% dextran, reuteran, reuteranPI, reuteranNS, or reuteranPINS. Pictures are representatives of 50% of all breads baked with 0.19% EPS
668 addition.