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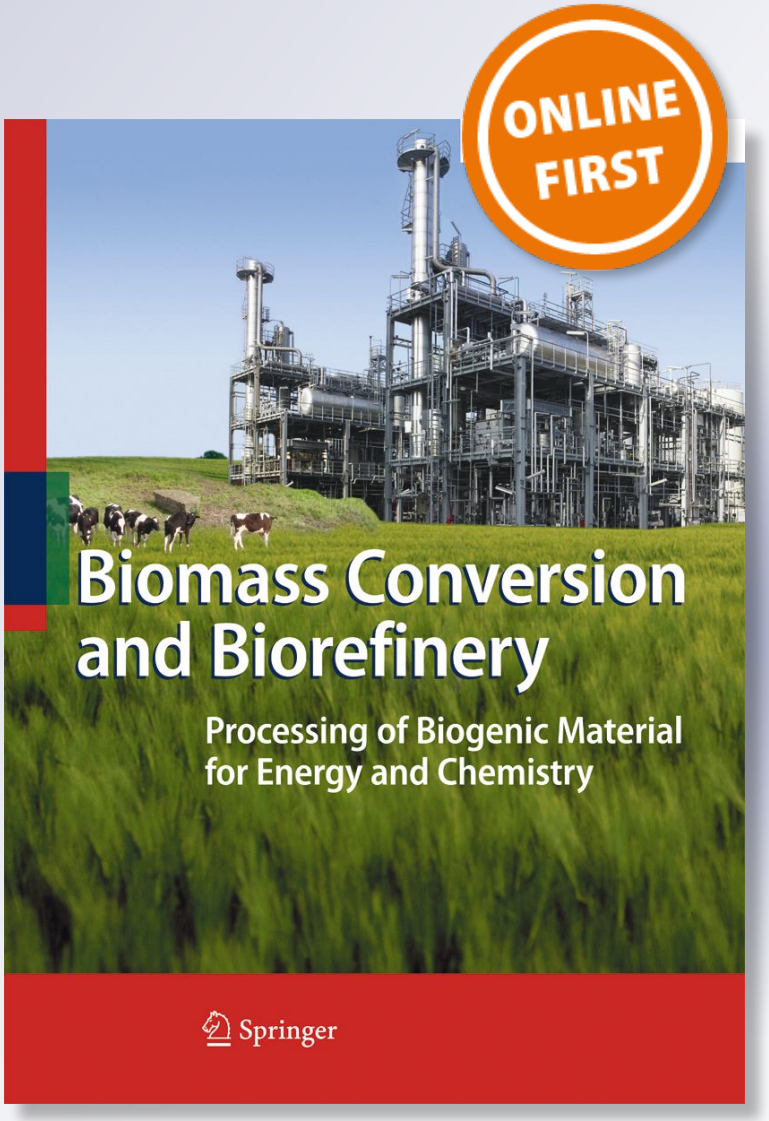

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ORIGINAL ARTICLE

Enzymatic production of wheat and ryegrass derived xylooligosaccharides and evaluation of their in vitro effect on pig gut microbiota

Gleb Dotsenko[1] · Anne S. Meyer[1] · Nuria Canibe[2] · Anders Thygesen[1] · Michael Krogsgaard Nielsen[1] · Lene Lange[1]

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Abstract
This study examines enzymatic production of linear xylooligosaccharides (XOS) and branched arabinoxylooligosaccharides (AXOS) from monocotyledonous biomass, wheat straw and ryegrass, and compares the in vitro effects of these XOS and AXOS on pig gut microbiota. XOS and AXOS were obtained from the biomass by treatment with different endo-1,4-β-xylanases. XOS of DP2-6 from wheat straw, obtained after treatment with *Aspergillus niger* endo GH11, suppressed growth of *Clostridium perfringens* and resulted in a high level of lactic acid production when fermented in vitro by pig fecal microbiota. Analogously, XOS ryegrass produced in the same way also suppressed *Cl. perfringens* growth, and more so than the corresponding ryegrass AXOS, but AXOS exhibited a more pronounced stimulation of lactic acid bacteria growth than XOS. The prebiotic potential, i.e., suppression of *Cl. perfringens* and stimulation of lactic acid bacteria, for the ryegrass oligosaccharides was as follows: XOS, produced by *A. niger* endo-1,4-β-xylanase (GH 11) ≥ AXOS, produced by *Thermotoga maritima* and *Cellvibrio mixtus* endo-1,4-β-xylanase s (GH10) > AXOS, produced by *Trichoderma viride* and *Aspergillus aculeatus* endo-1,4-β-xylanase s (GH11). These results indicate that wheat straw as well as green grass biomass such as ryegrass have potential as new sources of putative prebiotics for pig feed.

Keywords Xylooligosaccharides · Arabinoxylooligosaccharides · Prebiotics · Endo-1,4-β-xylanase · Ryegrass · Wheat straw

1 Introduction

In the twenty-first century, utilization of renewable raw materials and efficient exploitation of their chemical and biological potential have gained increasing importance and priority in scientific research and industry. Biomass biorefinery is a holistic approach in which biomass is considered to be a promising source of high-value products (food and feed ingredients, chemicals and pharmaceuticals, etc.) as well as energy for the sustainable development of human civilization [1, 2]. In Europe, green and yellow biomass, e.g., fresh plant leaves and cereal straw, respectively, is an abundant, sustainable and accessible raw material for multi-purpose biorefinery processing. According to the latest advances in green biomass biorefinery [3, 4], mechanical processing of green biomass enables its fractionation into two streams—green juice and pulp. Plant protein can be recovered from the green juice and used as a feed ingredient for monogastric animals. The pulp may be used either as a standard feed for ruminants or as a starting material for residual protein recovery [5] as well as for further upgrading of polysaccharides to high-value products. Upgrading of hemicellulose polysaccharides (mainly arabinoxylans) to feed and food ingredients (preferably with gut health stimulating effects) is a promising value chain for further valorization of the biomass in the green biorefinery.

Branched arabinoxylooligosaccharides (AXOS) and xylooligosaccharides (XOS) have recently been suggested as a promising alternative to fructooligosaccharide (FOS) prebiotics [6, 7]. AXOS and XOS are pentose oligomers derived from arabinoxylans by physico-chemical or/and enzymatic treatments [8, 9]. It is noteworthy that these compounds can

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be produced at an industrial scale from the majority of lignocellulosic materials containing arabinoxylans [9, 10]. Enzymatic production of AXOS and XOS can benefit from the exquisite selectivity of enzymes in relation to discrimination on substrate degradation, principally allowing specific desirable products to be produced. Moreover, such enzymatic processing seems to be more economically and environmentally feasible than physico-chemical processing approaches. The crucial enzyme for arabinoxylan main chain depolymerization and hence release of XOS and AXOS from xylan and arabinoxylan is endo-β-1,4-xylanase (EC 3.2.1.8), whereas α-L-arabinofuranosidase (EC 3.2.1.55) and various esterases attack the linkages of the polymer backbone substituents [11]. Two major glycoside hydrolase families of endo-1,4-β-xylanases (GH10 and GH11) have been identified based on the endo-1,4-β-xylanases structural and catalytic properties [11, 12]. GH10 endo-1,4-β-xylanases are generally considered better than GH11 endo-1,4-β-xylanases for catalyzing cleavage of glycosidic linkages near arabinose-substituents. Therefore, GH10 endo-1,4-β-xylanases are supposed to be able to produce shorter AXOS with higher yield than GH11 endo-1,4-β-xylanases.

Intake of XOS has several potentially beneficial effects, including selective growth stimulation of beneficial gut bacteria, reduction of blood glucose and cholesterol, reduction of pro-carcinogenic enzymes in the gastrointestinal tract, enhancement of mineral absorption from the large intestine, and immune-stimulation [10]. XOS can be used as an artificial sweetener in food applications and as a substitute for antibiotics in animal production [10].

Many studies have been published on general prebiotic properties of XOS [7, 13–15] but much less research has been devoted to the impact of AXOS on the pig gut microbiome [16]. As a consequence, this field still lacks clear understanding of the relationship between oligosaccharide structure and composition and the biological effect of these on the gut microbiome.

This study aimed to produce XOS and AXOS enzymatically from monocotyledonous biomass (ryegrass pulp (RG) and wheat straw (WS)) and compare the effect of XOS and AXOS on pig gut microbiota.

2 Materials and methods

2.1 Biomass materials and hydrothermal treatment

Mature WS was collected from an agricultural field (Aarhus, Denmark) in 2014 and dried at room temperature. Hydrothermally pretreated WS (190 °C, 10 min) was obtained from Novozymes A/S (Denmark). RG pulp, obtained as a solid material after RG screw pressing, was kindly provided by Aarhus University (Denmark). Prior to hydrothermal pretreatment and enzymatic production of oligosaccharides, RG was dried at 65 °C, milled (Retsch grindomix 200, 8000 rpm, 4 min). Hydrothermal pretreatment of RG for AXOS production was done at different degrees of severity by autoclaving at 100 °C for 60 min or at 140 °C for 60 min (Buch and Holm Sanoclav) or by 190 °C for 10 min in a loop autoclave set-up [17]. No chemicals or gases were added to the suspensions before or during these treatments. Afterward, the material was separated by filtration into insoluble and soluble fractions and the insoluble fractions were used for the enzymatic processing.

2.2 Compositional analysis

Two-stage sulfuric acid hydrolysis was utilized for determination of componential composition of WS and RG as described by Kaar et al. [18]. Acid hydrolysis was performed in glass tubes with screw caps (Pyrex, 60 ml). Next, high-performance liquid chromatography (HPLC) analysis was performed using a Dionex ICS3000 system consisting of an autosampler, a gradient pump (model DP-1), an electrochemical detector/chromatography module (model DC-1), and equipped with a Dionex CarboPac PA1 4 × 250-mm analytical column (for monosaccharide analysis) or a Dionex CarboPac PA20 4 × 250-mm analytical column (for oligosaccharide analysis). The eluent system for monosaccharide analysis employed MilliQ water (A), 0.5 M NaOH (B), and 0.5 M NaOAc with 0.02% (*w*/*v*) NaN_3 (C). Elution was performed in a linear gradient from 80:20:0 (% A:B:C) to 0:20:80 (% A:B:C) from 0 to 35 min, followed by isocratic elution at 0:20:80 (% A:B:C) for 5 min. The eluent system for oligosaccharide analysis was MilliQ water (A) and 0.5 M NaOH with 0.02% (*w*/*v*) NaN_3 (B). Elution was performed in a linear gradient from 97:3 (% A:B) to 99:1 (% A:B) from 0 to 1.5 min, isocratic elution at 99:1 (% A:B) for 1.5 min, linear gradient from 99:1 (% A:B) to 99.5:0.5 (% A:B) from 3 to 7 min, and isocratic elution at 99.5:0.5 (% A:B) for 10 min, followed by isocratic elution at 0:100 (% A:B) for 13 min. Arabinose, xylose, glucose, galactose, fructose, xylobiose (purchased from Sigma-Aldrich), and DP3-6 XOS (purchased from Megazyme) were used as standards; the presumed XOS of DP 7-15 were assessed by extrapolation from the retention times attained for the XOS standards of DP 3-6, and the peaks designated as AXOS peaks were peaks occurring between the XOS peaks.

2.3 Enzymatic XOS and AXOS production

Enzymatic hydrolysis of WS and RG was accomplished using the following buffers at a final concentration of 25 mM: sodium acetate buffer (pH 5.0), sodium phosphate buffer (pH 6.0–6.5), and sodium borate buffer (pH 9.0) [19]. Aliquots were taken periodically and analyzed by HPLC for oligosaccharides release. After 24 h the oligosaccharides concentration

remained constant. Prior to use, all enzymes were dialyzed using dialysis tubing "snake skin" with 3.5 kDa cut-off (Thermo Fisher Scientific) to remove low molecular weight compounds (such as sodium azide and ammonium sulfate in Megazyme enzymes).

An *Aspergillus niger* endo-1,4-β-xylanase (GH11, obtained from Megazyme, Bray, Ireland) was used for production of the XOS1 sample (65 U/g, 40 °C, pH 5.0, 24 h), the XOS2 sample (6 U/g, 40 °C, pH 5.0, 6 h), and the XOS3 sample (0.4 U/g, 40 °C, pH 5.0, 6 h) from hydrothermally pretreated WS (190 °C, 10 min), and for production of a XOS sample (70 U/g, 40 °C, pH 5.0, 24 h) from hydrothermally pretreated RG (190 °C, 10 min). The enzyme dosages employed were differentiated based on xylooligosaccharide yields and profiles obtained preliminary experiments (data not shown). The following four endo-1,4-β-xylanases were employed for enzymatic production of AXOS from hydrothermally pretreated RG (140 °C, 60 min): *Trichoderma viride* endo-1,4-β-xylanase (GH11, Megazyme (Bray, Ireland), 70 U/g, 40 °C, pH 5.0, 24 h), *Aspergillus aculeatus* endo-1,4-β-xylanase III (GH11, DTU, Center for Bioprocess Engineering, 70 U/g, 40 °C, pH 5.0, 24 h), *Thermotoga maritima* endo-1,4-β-xylanase (GH10, Megazyme (Bray, Ireland), 70 U/g, 40 °C, pH 5.0, 24 h), and *Cellvibrio mixtus* endo-1,4-β-xylanase (GH10, Megazyme (Bray, Ireland), 70 U/g, 40 °C, pH 6.5, 24 h).

Enzymatic hydrolysis of pretreated WS and RG was performed in quadruplicate in 2 l glass bottles (1 l per bottle in order to ensure proper mixing, substrate concentration 30 g dry weight substrate/l) under continuous shaking (200 rpm). The supernatants thus obtained were concentrated by evaporation at 75 °C, treated with 1% activated charcoal, and filtered through a 0.45-μm filter. Comparison of the xylooligosaccharide profiles by HPLC analysis of samples before and after charcoal treatment affirmed that no significant changes in the xylooligosaccharide profiles took place by the charcoal treatment. The final volume of each sample was approximately 40 ml. Prepared samples were stored at −20 °C for evaluation of their impact on pig gastrointestinal microbiota. The oligosaccharide yields (XOS and AXOS) were calculated as the ratio of total oligosaccharide weight (HPLC determination) to dry weight of spent substrate and expressed in percent.

2.4 Impact on gastrointestinal microbiota

For evaluation of the impact of the oligosaccharides on pig gut microbiota, feces from four grower pigs fed with a standard antibiotic-free diet were collected. Fifty percent feces slurries were prepared by adding 50 g of feces to 50 ml 0.1 M sodium phosphate buffer (pH 6.5) in a blender bag with an inner filter bag (VWR, no. 432-0003). The mixture was flushed with CO_2, homogenized in a Smasher paddle blender (bioMérieux Industry) for 4 min, and pressed through the filter to remove feed and other particulate material. Next, 3 ml pig fecal slurry and 7 ml oligosaccharide solution were mixed in Hungate tubes to give solutions containing 15% feces slurry and 1% weight/volume oligosaccharides. The tubes were flushed with CO_2 and incubated at 37 °C in anaerobic conditions and under stirring. Aliquots were taken after 0, 3, 6, and 24 h incubation for measuring pH and determination of organic acid concentrations. Furthermore, after 6 h incubation, an aliquot was taken for enumerating lactic acid bacteria, *Enterobacteriaceae*, *Cl. perfringens*, and fungal yeasts by plating. Sampling after 6 h of incubation was chosen to allow for identification of any possible differences among treatments (some of those may be difficult to assess after 24 h where bacterial levels may be more similar among tubes with different oligosaccharides). However, 24-h samplings were also included to ascertain that levels of short-chain fatty acids (SCFA) were measurable. A negative control was included by using a tube containing 7 ml sodium phosphate buffer and 3 ml fecal slurry, and a positive control was included by using a tube containing 7 ml chicory fructooligosaccharides (purchased from Sigma-Aldrich) and 3 ml fecal slurry. Each incubation set was performed using feces from each of the four pigs to give four replicates.

Concentrations of organic acids (formic, acetic, propionic, isobutyric, *n*-butyric, iso-valeric, *n*-valeric, DL-lactic, succinic, *n*-capronic, benzoic, iso-capronic, heptanoic, sorbic, hippuric acids) were quantified by GC as described by Canibe et al. [20]. Lactic acid bacteria were enumerated on de Man, Rogosa, and Sharp agar (Merck 10660) following anaerobic incubation for 2 days. *Enterobacteriaceae* were enumerated on McConkey agar (Merck 05465) following aerobic incubation for 1 day. Yeasts were enumerated on malt chloramphenicol/chlortetracycline agar (10 g glucose [Merck 08337]/L; 3 g malt extract [Merck 05397]/L; 3 g yeast extract [Merck 03753]/L; 5 g Bacto peptone [Merck 07224]/L; 50 mg chlortetracycline + 50 mg chloramphenicol [SR0177E, Oxoid LTD]/L; 15 g agar [Merck 01614]/L) following aerobic incubation for 2 days. Plates were incubated at 37 °C. *Cl. perfringens* was enumerated using the pour-plate technique on tryptose sulfit cycloserine agar (Merck 1.11972) supplemented with cycloserine (Oxoid SR088E) after anaerobic incubation for 1 day.

Bacteria count results were expressed in log cfu/g, and the deviated values were checked with Dixon's Q-test to reject outliers prior to calculating the mean and standard deviation. Confidence intervals were calculated for the 70% confidence level using the two-sided Student's *t* value for 3 degrees of freedom [21]. Results were represented as mean ± confidence interval.

2.5 Statistical analysis

For analyzing statistical difference of two data sets, Student's *t* test with unequal variances was performed using Microsoft Excel 2010 software. For analyzing statistical difference of

three and more data sets, single-factor ANOVA was performed using the same software.

3 Results and discussion

3.1 Composition of original and hydrothermally pretreated materials

The composition of the starting materials used in this study is shown in Table 1. As can be seen from the table, the pretreatment generally lowered the xylose and arabinose levels in both the WS and the RG, and RG had lower content of glucose, xylose, and lignin compared to WS, while arabinose and ash contents were similar in both materials. The arabinose/xylose ratio tended to be higher in the RG than in the WS (in the non-treated and the gently pretreated RG).

Hydrothermal pretreatment of RG at 100 °C (60 min) did not affect arabinoxylan branching, and arabinose content was found to be the same in non-treated and pretreated samples), but as assessed from the arabinose content, it was evident that increasing severity of the RG pretreatment resulted in gradual debranching of arabinoxylan accomplished by biomass enrichment in glucose and lignin (Table 1). Hence, at 140 °C (60 min), 34% of arabinofuranose substituents were removed, while at 190 °C (10 min), 87% of arabinofuranose substituents were removed. Hydrothermal pretreatment of WS at 190 °C (10 min) resulted in removal of 88% of total arabinofuranose substituents from original arabinoxylan (Table 1).

3.2 Enzymatic production of XOS and AXOS

In this study 190 °C (10 min) hydrothermally pretreated WS and RG were used for enzymatic production of linear XOS, while 140 °C (60 min) hydrothermally pretreated RG was used for enzymatic production of branched, arabinofuranose-substituted AXOS. The hydrothermal pretreatment of WS and RG at 190 °C deleted a major part of arabinofuranose branching (approximately 90%, Table 1), which enabled further enzymatic conversion of the resulting xylan into predominantly linear XOS [22]. Linear WS XOS of various lengths (XOS1–DP2-4, XOS2–DP2-5, and XOS3–DP2-6) and RG XOS (DP2-4) were thus prepared from hydrothermally pretreated WS and RG (190 °C, 10 min) using an *A. niger* endo-1,4-β-xylanase. XOS yields from WS and RG biomass were approximately 7 and 5% DM, respectively (data not shown).

For the AXOS in general, the GH10 xylanases catalyzed formation of shorter oligosaccharides with higher yield, while GH11 xylanases tended to catalyze formation of longer oligosaccharides with lower yield, which agrees with previous findings for cereal arabinoxylan depolymerization by GH10 and GH11 endo-1,4-β-xylanases [11].

Two GH10 and two GH11 endo-1,4-β-xylanases were chosen for large-scale preparation of four AXOS samples. After enzymatic treatment the following was observed: DP2-4 XOS and AXOS were the major oligosaccharide components after individual treatment of the biomass with endo-1,4-β-xylanases from *T. viride*, *Th. maritima*, and *C. mixtus*, respectively, and oligosaccharides produced by *T. viride* endo-1,4-β-xylanase comprised the full spectrum of DP2-12 oligosaccharides. *A. aculeatus* endo-1,4-β-xylanase III was not able to produce DP2-3 AXOS, and only linear DP2-3 XOS were present in the mixture. Furthermore, this same enzyme did not produce DP5-7 oligosaccharides but generated DP8-11 and DP15 oligosaccharides.

Th. maritima endo-1,4-β-xylanase action resulted in production of DP2-3 and DP10-15 oligosaccharides. In contrast, *C. mixtus* endo-1,4-β-xylanase generated DP2-4, DP7-8, and DP10-15 oligosaccharides. Total oligosaccharide yield varied in the range 4.1–5.5% DM, depending on the endo-1,4-β-xylanase employed.

Optimal choice of feed stock for production of XOS and estimate of (theoretical) yield can be guided by the algorithm-based method, described in Dotsenko et al. [22].

Table 1 Composition of non-treated (NT) and hydrothermally pretreated wheat straw (WS) and ryegrass pulp (RG) (%DM) presented as means ± standard deviation ($N = 3$). Values significantly different from each other in a row (ANOVA, $\alpha = 0.05$) are indicated by different superscript letters

Component	Feedstock (pretreatment conditions)					
	WS (NT)	WS (190 °C, 10 min)	RG (NT)	RG (100 °C, 60 min)	RG (140 °C, 60 min)	RG (190 °C, 10 min)
Glucose	38.4 ± 2.4^{c}	44.3 ± 2.7^{b}	24.3 ± 1.5^{d}	36.1 ± 1.3^{c}	44.3 ± 1.8^{b}	50.4 ± 2.3^{a}
Xylose	21.2 ± 1.7^{a}	10.1 ± 0.6^{c}	$12.1 \pm 1.1^{b,c}$	13.4 ± 0.9^{b}	13.1 ± 0.7^{b}	6.5 ± 0.6^{d}
Arabinose	2.9 ± 0.2^{a}	0.35 ± 0.03^{d}	3.2 ± 0.2^{a}	3.3 ± 0.2^{a}	2.1 ± 0.2^{b}	0.43 ± 0.03^{c}
Galactose	0.69 ± 0.03^{c}	0.13 ± 0.02^{d}	1.6 ± 0.1^{a}	1.6 ± 0.1^{a}	1.2 ± 0.1^{b}	0.69 ± 0.04^{c}
Lignin	22.1 ± 1.3^{a}	23.1 ± 1.4^{a}	14.7 ± 0.9^{c}	14.4 ± 1.0^{c}	18.5 ± 1.3^{b}	24.8 ± 1.7^{a}
Ash	5.9 ± 0.5^{a}	4.5 ± 0.3^{b}	$4.1 \pm 0.3^{b,c}$	$4.2 \pm 0.3^{b,c}$	3.9 ± 0.2^{c}	2.4 ± 0.2^{d}
Arabinose/xylose ratio	0.14	0.03	0.27	0.25	0.16	0.07

Table 2 Effect of length of enzymatically released wheat straw xylooligosaccharides (XOS) on in vitro growth of various microbial groups from pig faeces[1]. The XOS3 sample exerted a significantly positive effect for pig gut health through suppressing potentially pathogenic *Cl. perfringens* bacteria (indicated in bold). Values which are significantly different from each other in a column (ANOVA, $\alpha = 0.3$) are indicated by different superscript letters

Sample	Microbial group, log cfu/g		
	Enterobacteriaceae	*Cl. perfringens*	Lactic acid bacteria
Negative control[2]	6.3 ± 0.5^{a}	4.5 ± 0.1^{a}	8.8 ± 0.1^{a}
XOS1 (DP2-4)	5.9 ± 0.5^{a}	4.2 ± 0.5^{a}	8.8 ± 0.1^{a}
XOS2 (DP2-5)	6.1 ± 0.5^{a}	4.7 ± 0.2^{a}	8.9 ± 0.1^{a}
XOS3 (DP2-6)	5.8 ± 0.6^{a}	$\mathbf{3.1 \pm 0.9^{b}}$	8.7 ± 0.1^{a}

[1] The feces slurry contained 6.3 ± 0.5 log cfu/g *Enterobacteriaceae,* 3.0 ± 0.9 log cfu/g *Cl. perfringens*, 8.8 ± 0.1 log cfu/g lactic acid bacteria

[2] Negative control was a tube containing buffer instead of oligosaccharide

3.3 Oligosaccharide effect on pig fecal microbiota

A prebiotic concept was first introduced by Gibson and Roberfroid [23], where the term "prebiotic" was defined as "a nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health". However, several revisions of the original definition have been made to satisfy the scientific community, regulatory authorities, the food industry, and the final consumers [24]. In particular, the optimal definition should be valid not only for food but also for feed. Oligosaccharides

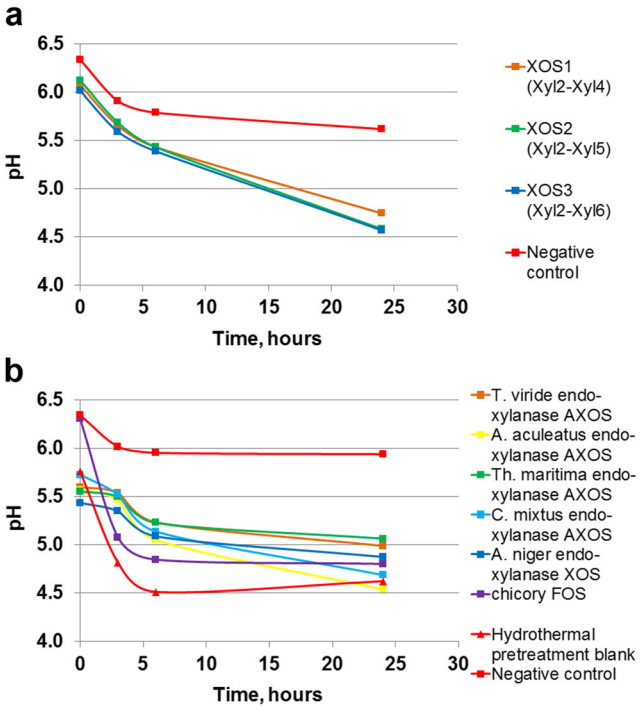

Fig. 1 pH progress in a batch fermentation of arabinoxylan-derived oligosaccharides with pig fecal microbiota: **a** wheat straw xylooligosaccharides (XOS1-3), **b** ryegrass xylooligosaccharides (XOS) and arabinoxylooligosaccharides (AXOS). All oligosaccharide samples demonstrated pH decrease due to bacterial production of organic acids

Table 3 Effect of arabinoxylooligosaccharides (AXOS) and xylooligosaccharides (XOS) prepared from hydrothermally pretreated ryegrass pulp using endo-1,4-β-xylanases from different sources on in vitro growth of various microbial groups from pig faeces[1]. Positive effects of oligosaccharides for pig gut health are indicated in italics. Values which are significantly different from each other in a column (ANOVA, $\alpha = 0.3$) are indicated by different superscript letters

Sample	Endo-1,4-β-xylanase source (GH family)	Microbial group, log cfu/g		
		Enterobacteriaceae	*Cl. perfringens*	Lactic acid bacteria
Negative control[2]	–	4.7 ± 0.3^{a}	2.4 ± 0.4^{a}	8.2 ± 0.3^{c}
Positive control[3]	–	4.4 ± 0.2^{b}	2.8 ± 0.6^{a}	8.2 ± 0.3^{c}
Hydrothermal pretreatment supernatant[4]	–	$3.9 \pm 0.4^{c,d}$	2.1 ± 0.4^{a}	9.2 ± 0.3^{a}
AXOS	*T. viride* (GH 11)	$3.5 \pm 0.3^{d,e}$	2.0 ± 0.6^{a}	$8.5 \pm 0.4^{b,c}$
AXOS	*A. aculeatus* (GH 11)	3.7 ± 0.2^{d}	2.1 ± 0.6^{a}	$8.6 \pm 0.4^{b,c}$
AXOS	*Th. maritima* (GH 10)	$\mathbf{3.4 \pm 0.2^{e}}$	2.1 ± 0.6^{a}	8.6 ± 0.1^{b}
AXOS	*C. mixtus* (GH 10)	$4.2 \pm 0.2^{b,c}$	*~1.0*	$8.7 \pm 0.4^{a,b}$
XOS	*A. niger* (GH 11)	$\mathbf{3.4 \pm 0.3^{d,e}}$	*< 1.0*	$8.4 \pm 0.3^{b,c}$

[1] The feces slurry contained 5.4 ± 0.2 log cfu/g *Enterobacteriaceae*, 2.6 ± 0.5 log cfu/g *Cl. perfringens*, 8.6 ± 0.5 log cfu/g lactic acid bacteria

[2] Negative control contained buffer instead of oligosaccharide

[3] Positive control contained fructooligosaccharides (FOS) from chicory instead of the experimental oligosaccharides

[4] Hydrothermal pretreatment supernatant was obtained after autoclaving milled RG at 140 °C for 60 min

derived from many natural polysaccharides have been shown to have prebiotic effects (e.g., FOS, galactooligosaccharides, maltooligosaccharides, and gentiooligosaccharides) [25]; and FOS are now accepted as the golden standard of prebiotics [26].

In order to evaluate the oligosaccharide effect on pig gut health, oligosaccharide samples prepared in this study were incubated with pig fecal microbiota under anaerobic conditions. pH and organic acids concentration were monitored after 0, 3, 6, and 24 h incubation, and four microbial groups (*Enterobacteriaceae*, *Cl. perfringens*, lactic acid bacteria, and yeasts) were enumerated after 6 h of incubation. Two of these groups, *Enterobacteriaceae* and *Cl. perfringens*, can be pathogenic for pigs [27, 28], and a reduction of their numbers is considered beneficial for maintaining a healthy gastrointestinal tract. In contrast, lactic acid bacteria are considered a beneficial microbial group with regard to gastrointestinal health [29, 30]. Besides bacterial species, fungal yeasts are also members of the commensal pig gut microbiota, and some yeast species are considered to have probiotic effects, such as some *Saccharomyces* species [29, 31]. When interpreting the data, it should be noted that in testing of both WS and RG oligosaccharides, pig feces were collected from different animals, and therefore the initial sample microbiota composition varied in these experiments (the microbiota composition for each case is detailed in footnote 1 of Tables 2 and 3).

3.4 Enzymatically produced XOS from wheat straw

After 24 h batch fermentation with pig fecal microbiota, all three WS XOS samples demonstrated a stronger decrease in pH than the negative control (Fig. 1a).

The observed pH decrease is mainly due to production of SCFA, and lactic acid and low pH exert an inhibitory effect on pathogenic microflora [7, 23]. SCFA (especially butyric acid) are moreover important energy sources for colonocytes (cells lining the mammalian colon) and thus contribute positively to the whole gut health and immune response [32]. Fermentation of the WS XOS1 sample by the pig fecal microbiota resulted

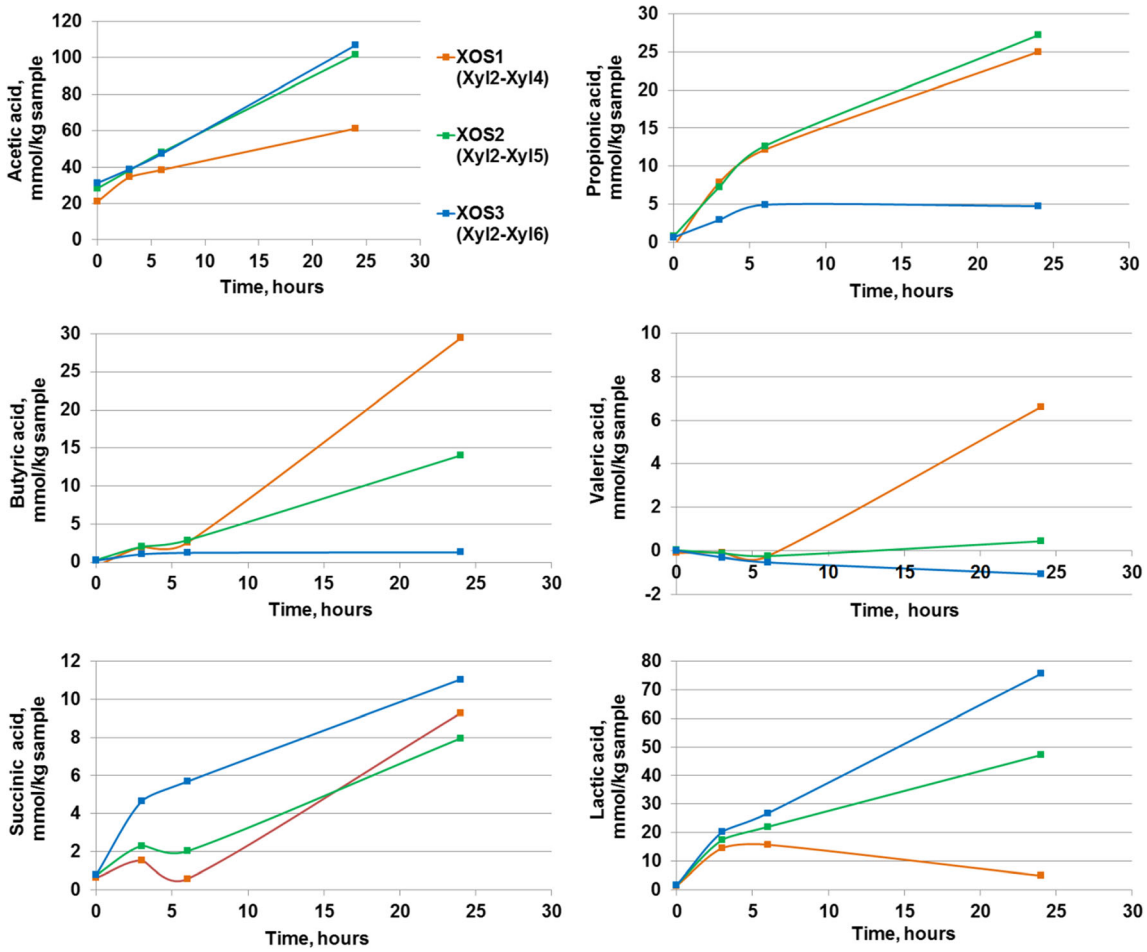

Fig. 2 Organic acids production in a batch fermentation of wheat straw xylooligosaccharides (XOS1-3) with pig fecal microbiota. All data are corrected for the negative control which contained buffer instead of oligosaccharides. Short-chain fatty acids (especially butyric acid) contribute to overall gut health and immune response. The highest production of butyric and valeric acids was observed in fermentation of XOS1 sample

in higher production of butyric and valeric acids than the XOS2 and XOS3 samples (Fig. 2).

Fermentation of WS XOS2 and XOS1 samples resulted in similar rates of propionic acid production which, however, were higher than those for the XOS3 sample. Acetic acid production rates were similar for WS XOS2 and XOS3 samples but higher than those for the XOS1 sample. Maximal lactic acid production was observed for WS XOS3 sample fermentation, the XOS2 sample gave a lower lactic acid production, while XOS1 sample fermentation resulted in the lowest level of lactic acid production. Some lactic acid bacteria strains have been reported to preferentially utilize DP2-4 XOS, while other stains are known to prefer xylose [7]. The opposite correlation of lactic acid production with XOS DP, as observed in the present study, can be explained by the longer XOS suppression effect on some bacterial species that probably compete with lactic acid bacteria. All three XOS samples contained xylose and DP2-4 oligosaccharides which are required for lactic acid bacteria growth, but it was the XOS3 sample that suppressed *Cl. perfringens* growth (data described below) and resulted in maximal lactic acid production.

As can be seen from the microbiological data (Table 2), WS XOS did not exert a significant effect on the growth of *Enterobacteriaceae* or lactic acid bacteria (no statistically significant difference was found between the WS XOS1, XOS2, XOS3 samples and the negative control). In contrast, the WS XOS3 sample suppressed *Cl. perfringens* growth, while no statistically significant effect was observed for the WS XOS1 and XOS2 samples. No statistically significant effect of the WS XOS1-3 samples was observed on the growth of yeasts (data not shown).

3.5 Enzymatically produced XOS and AXOS from ryegrass

RG XOS and AXOS samples demonstrated a stronger pH decrease, similar to that of Chicory FOS fermentation, after 24 h batch fermentation with pig fecal microbiota than was observed for the negative control (Fig. 1b). In contrast to FOS

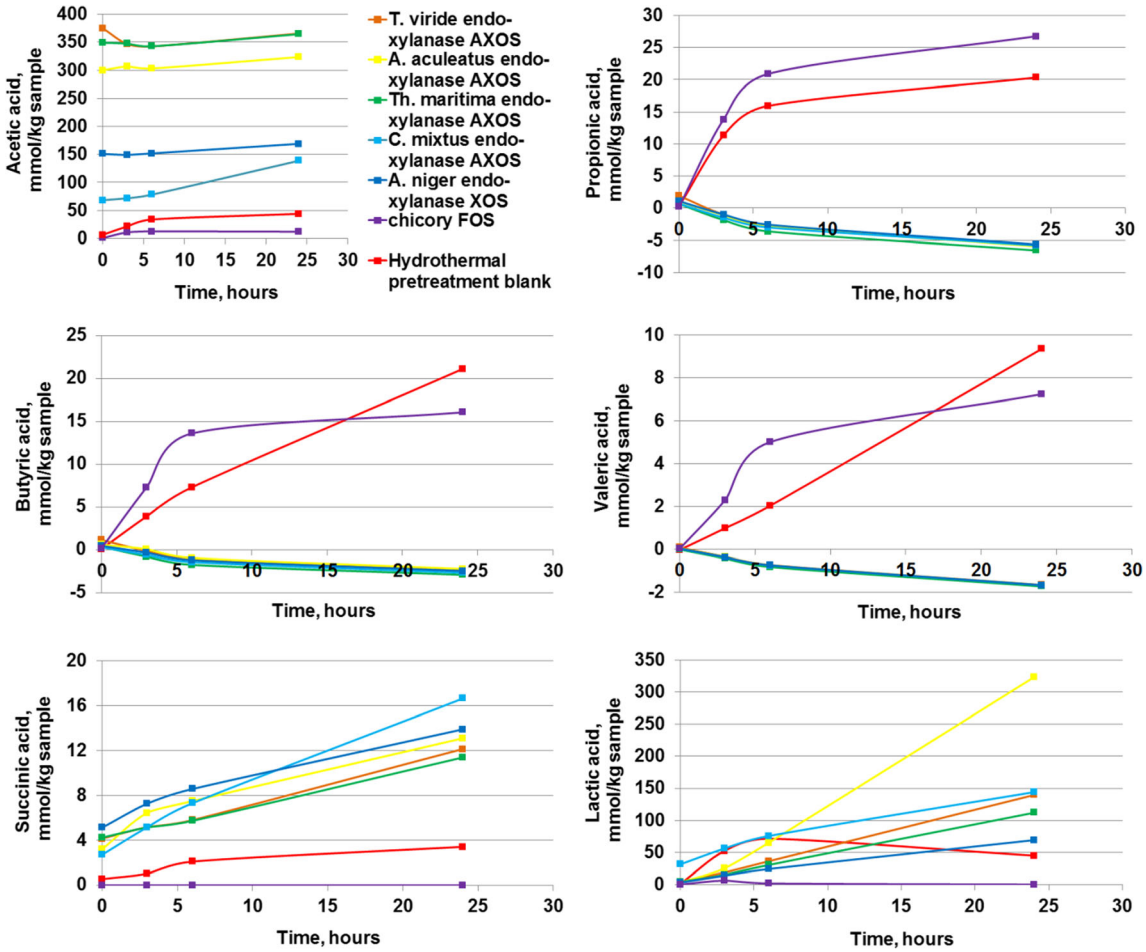

Fig. 3 Organic acids production in a batch fermentation of ryegrass xylooligosaccharides (XOS) and arabinoxylooligosaccharides (AXOS) with pig fecal microbiota. All data are corrected for the negative control which contained buffer instead of oligosaccharides. Observed differences in organic acids production profile reflect different microbial species, which are stimulated by oligosaccharides of various structures, as well as metabolic differences of microbial digestion of various oligosaccharides

fermentation, no production of propionic, butyric, and valeric acid was detected during RG XOS and AXOS fermentation (Fig. 3).

However, pig microbiota fermentation in the presence of RG XOS and AXOS resulted in high production levels of lactic acid and succinic acid, which was not observed for FOS fermentation. It should be mentioned that production of propionic, butyric, and valeric acids was observed during fermentation of a supernatant derived from ryegrass hydrothermal pretreatment. This supernatant consisted of five monosaccharides (xylose, arabinose, glucose, galactose, and fructose) and did not include any significant concentration of oligosaccharides.

Based on the microbiological growth data (Table 3), the AXOS produced with the endo-1,4-β-xylanase from *Th. maritima* and the XOS released by the *A. niger* endo-1,4-β-xylanase treatment demonstrated a substantial suppressing effect on *Enterobacteriaceae* growth. This effect differed significantly from that of the negative control and the result obtained with the hydrothermal pretreatment supernatant. Furthermore, the suppressing effect of *Th. maritima* AXOS on *Enterobacteriaceae* growth was stronger than the corresponding effect of FOS used in this work as the positive control. The suppressing effect of the other RG AXOS samples on *Enterobacteriaceae* growth also differed significantly from the negative control but not from the hydrothermal pretreatment supernatant effect. Thus, xylooligosaccharides present in the hydrothermal pretreatment supernatant and perhaps other components in the supernatant may also suppress *Enterobacteriaceae* growth.

The greatest suppressing effect on *Cl. perfringens* growth was registered for RG XOS produced by *A. niger* endo-1,4-β-xylanase and the RG AXOS produced by the *C. mixtus* endo-1,4-β-xylanase—both products essentially prevented the growth of *Cl. perfringens* in the pig microbiota (Table 3).

The data obtained do not allow any firm conclusions to be drawn with respect to explaining the reasons for the observed differences in the suppressing effects of the xylooligosaccharide fractions on *Cl. perfringens* growth in the pig microbiota, but our current interpretation is that differences in the backbone substitutions of the AXOS and XOS or variations in the oligosaccharide profiles of the samples may be the cause (oligosaccharide compositions of xylooligosaccharides and arabinoxylooligosaccharides are shown in Tables 4 and 5). Interestingly, linear RG XOS are the cheapest and simplest to produce. Surprisingly, the highest stimulatory effect on lactic acid bacteria observed in this part of the investigation resulted from fermentation with hydrothermal pretreatment supernatant, while RG AXOS samples also demonstrated a stimulatory but lower effect. These data did not correlate with lactic acid production (Fig. 3) where the minimal final lactic acid concentration was associated with hydrothermal pretreatment supernatant

Table 4 Componential composition of xylooligosaccharides (XOS), prepared from hydrothermally pretreated (190 °C, 10 min) wheat straw (WS), and ryegrass pulp (RG) using *A. niger* endo-xylanase. Linear XOS are abbreviated as Xyl*n*, where *n* is the degree of polymerization

Component	WS						RG	
	XOS1 (major DP2-4)		XOS2 (major DP2-5)		XOS3 (major DP2-6)		XOS (major DP2-4)	
	Concentration g/l	Ratio, %	Concentration, g/l	Ratio, %	Concentration g/l	Ratio, %	Concentration, g/l	Ratio, %
Xylose	1.17	8.1	0.93	6.4	1.1	7.9	6.85	19.3
Arabinose	0.42	2.9	0.52	3.6	0.74	5.3	4.41	12.4
Glucose	0.11	0.8	0.08	0.6	0.09	0.6	2.27	6.4
Galactose	0.12	0.8	0.13	0.9	0.13	0.9	1.03	2.9
Fructose	–	–	–	–	–	–	1.93	5.4
Xyl2	3.18	22.1	2.39	16.5	1.26	9.1	8.94	25.2
Xyl3	5.95	41.3	5.32	36.7	3.64	26.2	4.22	11.9
Xyl4	2.28	15.8	3.23	22.3	3.16	22.7	2.34	6.6
Xyl5	0.8	5.5	1.13	7.8	2.08	15.0	0.22	0.6
Xyl6	0.27	1.9	0.47	3.2	0.94	6.8	0.54	1.5
Xyl7	0.12	0.8	0.22	1.5	0.48	3.5	0.73	2.1
Xyl8	–	–	0.07	0.5	0.21	1.5	1.55	4.4
Xyl9	–	–	–	–	0.07	0.5	0.2	0.6
Xyl10	–	–	–	–	–	–	0.28	0.8
Oligosaccharide Ara/Xyl ratio	0.018 ± 0.004		0.015 ± 0.003		0.014 ± 0.003		0.032 ± 0.007	
Total XOS yield, %	7.4		7.2		7.3		5.3	

Table 5 Componential composition of arabinoxylooligosaccharides (AXOS), prepared from hydrothermally pretreated (140 °C, 60 min) ryegrass pulp (RG) using endo-xylanases from various sources. Xylooligosaccharides (XOS) and AXOS are abbreviated as Xyln and Xylns, respectively, where n is the degree of polymerization

Component	Endo-xylanase source (GH family)							
	T. viride (GH11)		*A. aculeatus* (GH11)		*Th. maritima* (GH10)		*C. mixtus* (GH10)	
	Concentration, g/l	Ratio, %	Concentration, g/l	Ratio, %	Concentration, g/l	Ratio, %	Concentration, g/l	Ratio, %
Xylose	1.48	4.8	1.77	5.5	1.62	5.3	1.53	5.0
Arabinose	3.45	11.1	3.32	10.4	3.13	10.3	3.24	10.7
Glucose	5.24	16.9	5.67	17.8	5.37	17.7	5.48	18.1
Galactose	0.12	0.4	0.08	0.3	0.15	0.5	0.13	0.4
Fructose	6.57	21.2	6.39	20.0	6.28	20.7	6.12	20.2
Xyl2	3.08	9.9	5.07	15.9	3.74	12.3	5.33	17.6
Xyl2s	1.59	5.1	–	–	2.55	8.4	1.67	5.5
Xyl3	2.44	7.9	4.08	12.8	2.09	6.9	2.17	7.2
Xyl3s	0.66	2.1	–	–	1.66	5.5	0.61	2.0
Xyl4 + Xyl4s	2.6	8.4	2.7	8.5	–	–	0.67	2.2
Xyl5 + Xyl5s	1.15	3.7	–	–	–	–	–	–
Xyl6 + Xyl6s	0.68	2.2	–	–	–	–	–	–
Xyl7 + Xyl7s	0.33	1.1	–	–	–	–	0.25	0.8
Xyl8 + Xyl8s	0.42	1.4	0.61	1.9	–	–	0.32	1.1
Xyl9 + Xyl9s	0.18	0.6	0.65	2.0	–	–	–	–
Xyl10 + Xyl10s	0.71	2.3	0.8	2.5	1.29	4.3	1.08	3.6
Xyl11 + Xyl11s	0.09	0.3	0.13	0.4	0.65	2.1	0.43	1.4
Xyl12 + Xyl12s	0.17	0.5	–	–	0.58	1.9	0.3	1.0
Xyl15+ Xyl15s	–	–	0.63	2.0	1.21	4.0	0.99	3.3
Oligosaccharide Ara/Xyl ratio	0.11 ± 0.02		0.06 ± 0.01		0.14 ± 0.03		0.09 ± 0.02	
Total XOS and AXOS yield, %	4.5		4.1		4.8		5.5	

while the maximal concentration was obtained with AXOS produced by *A. aculeatus* endo-1,4-β-xylanase. This part of the experiment is considered to be non-conclusive. Additional experiments must be done before valid interpretations can be made. It is noteworthy that the stimulatory effect on lactic acid bacteria growth of RG AXOS samples exceeded that of RG XOS, which in turn was higher than the FOS effect. Thus, in contrast to the suppressing effect on growth of *Cl. perfringens*, the higher stimulatory effect for lactic acid bacteria was associated with RG branched AXOS rather than RG linear XOS. No statistically significant effect of RG XOS and AXOS was observed for yeast growth (data not shown).

Interestingly, aqueous supernatant from RG hydrothermal pretreatment also exhibited a suppressing effect on *Enterobacteriaceae* and a stimulatory effect on lactic acid bacteria. As already mentioned, this supernatant consisted of five monosaccharides (xylose, arabinose, glucose, galactose, and fructose) and did not include any significant concentration of oligosaccharides. However, such monosaccharide mixtures cannot be considered as a prebiotic because of the well-accepted definition of prebiotic as a "host non-digestible food ingredient." Obviously, hydrothermal pretreatment supernatant may include biomass-derived compounds that could affect bacteria (e.g., cause the slight inhibition of *Enterobacteriaceae*). However, the effects of this supernatant also resonate with the many recent studies (albeit still not fully supported by scientific investigations) showing observed improved robustness in gut health in pigs fed with fermented feed ingredients (e.g., fermented soya and rapeseed used as feed component [33–35]). Full elucidation of the active ingredients in the hydrothermal pretreatment supernatant is required to gain full insight into the mechanisms underlying the observed effects.

4 Conclusions

Enzymatic production of arabinoxylan- and xylan-derived oligosaccharides from pretreated WS and RG fibers was

investigated, and their effect on pig gut microbiota was evaluated through in vitro fermentation of pig feces samples. Hydrothermal pretreatment was shown to enable various degrees of arabinoxylan debranching depending on process temperature and duration.

Endo-1,4-β-xylanase activity was found to be entirely adequate for oligosaccharide production from hydrothermally pretreated WS and RG. The WS XOS3 sample obtained after *A. niger* endo-1,4-β-xylanase GH11 treatment of pretreated WS (with DP2-6 as the major components) exhibited a suppressing effect on *Cl. perfringens*, a potential pathogen, in the pig gut microbiota fermentation; the WS XOS3 sample also resulted in the highest lactic acid production when fermented by pig fecal microbiota. Linear XOS as well as AXOS from RG (major components DP2-4) also showed a suppressing effect on *Cl. perfringens* bacterial growth in the pig microbiota fermentation, but the AXOS from RG exhibited a better stimulatory effect than the XOS on beneficial lactic acid bacteria. Apart from the lactic acid bacteria stimulating effect, both the XOS produced from RG by treatment with a GH11 *A. niger* endo-1,4-β-xylanase and the RG AXOS produced by *Th. maritima* endo-1,4-β-xylanase also demonstrated a suppressing effect on *Enterobacteriaceae*, which may be pathogenic gastrointestinal microbiota.

A further use of the results here reported could be to combine RG oligo, both the linear XOS and the branched AXOS DP2-4 produced by endo-1,4-β-xylanase s GH11 (*A. niger*), and GH 10 (*Th. maritima* and/or *C. mixtus*), for use as a gut health stimulating feed additive blend.

Seen from a broader perspective, the enzymatic reaping of parts of lignocellulosic biomass prior to use of the bulk biomass for bioenergy or bulk animal feed could provide added value for biorefining of both green and yellow lignocellulosic biomass.

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