Date: 2022-08-08

Final report

Validation of the prebiotic and antioxidant effects of wheat bran hemicelluloses in baking products

Project number: O-17-20-962

Budget: 2 993 000 kr

Project period: 20180101 - 20211231

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Part 1.1: Summary/Abstract

Wheat bran is the largest by-product of wheat flour production and is mainly used in animal feed. However, wheat bran contains valuable dietary fibres with great potential to produce food ingredients with prebiotic properties. In this project we have demonstrated the upscaled production of 4 different dietary fibre fractions from wheat bran in terms of molar mass and ferulic acid content. The process shows high reproducibility in yields and composition. The fibre fractions had distinct effects on microbiota functionality without affecting their composition. However, the metabolites from faecal fermentation did not show any protective effect on intestinal permeability. The addition of wheat bran fibres affected baking properties without significantly altering consumer acceptance of fresh or stored breads. The project contributes to the industrial valorization of wheat bran dietary fibres as bread improvers, with large implications for the sustainability and health of cereal production.

Vetekli är den största biprodukten från vetemjölsproduktionen och används främst som djurfoder. Vetekli innehåller värdefulla kostfibrer med stor potential för att tillverka livsmedelsingredienser med prebiotiska egenskaper. I projektet har vi demonstrerat uppskalningen av produktionen av 4 kostfiberfraktioner från vetekli med olika molekylvikten och ferulsyrainnehållet. Processen visade hög reproducerbarhet i utbyten och sammansättning. Veteklifiberfraktionerna hade en distinkt effekt på funktionalitet hos mikrobiota utan att påverka sammansättningen. Metaboliterna från fekal fermentering visade dock ingen skyddande effekt på tarmpermeabiliteten. Tillsatsen av veteklifibrer påverkade bakningsegenskaperna utan att förändra konsumenternas acceptans av färskt eller lagrat bröd. Detta projekt bidrar till den industriella tillverkningen av kostfibrerna från vetekli som brödförbättrare, med betydande konsekvenser för hållbarheten och hälsofördelarna hos spannmålsproduktion.

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Part 1.2: Main report (max. 10 pages)

Introduction

Wheat bran, the outer layer that surrounds the grain endosperm, is the largest by-product of wheat flour production with a total volume of approximately 300,000 tonnes/year in Sweden. The bran is primarily used in animal feed, as the human gastrointestinal system is not fully able to digest and absorb the bran components. Wheat bran contains valuable biomolecules, such as hemicellulose dietary fibre (arabinoxylans) and phenolic substances (ferulic acid), which could be used for high-quality applications in multifunctional food products. KTH has developed a bioprocess (1 - 5 g) to isolate dietary fibres - high molecular weight arabinoxylans (AX) and oligosaccharides -, using only water at high pressure and temperature (subcritical water) and enzymes. The process preserves the ferulic acid moieties that are covalently bound to the AX core, which confers antioxidant properties. Further processing of wheat bran using this technology therefore presents a large potential to develop bioactive food ingredients with beneficial effects for improved health and well-being.

The aim of the project was to evaluate and validate the prebiotic and antioxidative effects of the feruloylated arabinoxylan (FAX) dietary fibre fractions extracted from wheat bran in baking products. In order to do this, we have addressed three main scientific questions.

(1) Can we scale up to the kg scale the develop bioprocess to isolate bioactive FAX in polymeric and oligomeric form from wheat bran?
(2) What are the prebiotic effects of the isolated feruloylated polymeric and oligomeric fractions from wheat bran on the gut microbiota, the production of beneficial metabolites (short-chain fatty acids) and on improving gut barrier properties?
(3) Can the FAX fractions be used in bread products with prolonged shelf life compared to bread without baking improvers?

The project responds to the need from primary producers, here Lantmännen, to valorize a widely available side stream (wheat bran) from wheat milling into food ingredients with high value. The project demonstrates that wheat bran fibres can be incorporated in baking with health-promoting effects, with potential to replace bread improvers (enzymes and emulsifiers).

Materials and methods (full experimental details shown in Appendix 1)

Extraction and characterization of arabinoxylan fractions from wheat bran at pilot scale

Four arabinoxylan fractions from wheat bran were targeted and produced from laboratory to pilot scale: (i) high molecular weight AX with ferulic acid (FAX); (ii) high molecular weight AX without ferulic acid (AX); (iii) feruloylated xylo-oligosaccharides (FAXOs), and (iv) xylo-oligosaccharides without ferulic acid (AXOs). The process involved a pretreatment (destarching and β-glucan removal), subcritical water extraction (SWE) of FAX, alkaline treatment to obtain the AX fraction, precipitation and drying, and enzymatic hydrolysis of F-AX and AX into FAXOs and AXOs (**Figure 1a**).

Laboratory optimization of the pretreatment and extraction. Wheat bran was subjected to an enzymatic pretreatment (Celluclast, Novozymes) and the pretreated wheat bran was subjected to 6 different temperature programs during subcritical water extraction (**Figure 1b**).

Pilot scale pretreatment and extraction. Pilot scale pretreatments and extractions were performed in a 50 L reactor. 4 kg of wheat bran were enzymatically treated with Celluclast (37°C, 4 h), washed with cold tap water, and filtered. Six batches of subcritical water extraction were performed at 150°C (**Figure 1c**), the extract was filtered and cooled to 4 °C overnight prior to subsequent precipitation.

Precipitation. The laboratory and the pilot extracts were precipitated with 99.7% ethanol in a 4:1 ratio. The filter cake was then vacuum dried at 40 °C yielding the F-AX (and AX) fractions.

Alkaline treatment. The AX fraction was obtained by alkaline treatment (0.5 M NaOH, 4 hr, room temperature) of the dried F-AX, followed by precipitation and drying.

Enzymatic treatment. FAXOs and AXOs were produced from the FAX and AX fractions by incubation with a commercial endo-1,4-β-xylanase (Ronozyme, Novozymes) at 37°C for 4 hr.

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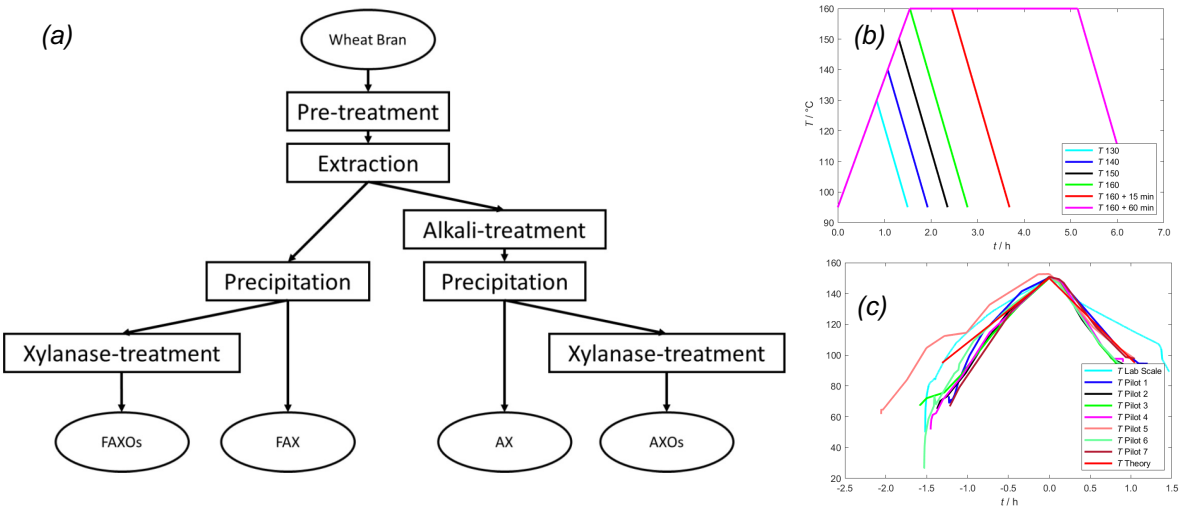

Figure 1. Production of dietary fibre fractions from wheat bran. (a) Process design; (b) Temperature profiles for laboratory scale; (c) Temperature profiles pilot batches.

Analysis. All the extracted batches were analyzed in order to determine the molecular composition and the molecular weight. Monosaccharide analysis by methanolysis and HPAEC-PAD analysis was performed to obtain the AX and total glucan content.[1] Starch quantification was performed enzymatically.[2] The β-glucan content was determined by subtracting the starch content from the total glucose content determined from monosaccharide analysis.[1] The soluble protein content was determined by the Bradford method[3] using bovine serum albumin as reference protein. The presence of ferulic acid (FA) was analyzed after overnight saponification in 2M NaOH, followed by HPLC-UV analysis of the neutralized supernatants at 325 nm against a reference of commercial FA.[4] The oligosaccharide profile of the FAXOs and AXOs was evaluated by HPAEC-PAD analysis.[5] The molar mass distributions of the AX fractions were evaluated by SEC-MALLS with DMSO/LiBr as mobile phase.[2]

Prebiotic and gut barrier properties of arabinoxylans and oligosaccharides from wheat bran

Study participants, questionnaires, and ethics. A total of 12 healthy subjects, between 18 and 45 years old, were enrolled in the study and all participants signed the informed consent. The study was approved by the Ethics Review Authority of Sweden (Dnr 2020-03943). The gastrointestinal symptoms rating scale (GSRS) confirmed absence of gastrointestinal problems. Participants completed a food frequency questionary (Meal-Q) prior to study start. Fecal samples were collected at home and delivered to Örebro University within 2 hrs of collection.

Collection of colonic biopsies. Study participants (n=12) underwent a sigmoidoscopy in the morning after 10 hours fasting to provide colonic biopsies for the intestinal barrier experiments.

Simulated in vitro gastrointestinal digestion. Fibre fractions (AX, FAX, FAXOs, AXOs and wheat bran) were homogenized in a stomacher (Seward, UK), subjected to simulated in vitro digestion for 3hr at 37°C under agitation, and further dialyzed for 26 hr.

In vitro fecal fermentation. The fermentation medium was as outlined by Fooks and Gibson,[6] replacing glucose by the freeze dried fiber fractions (5% w/v) as carbon source. After inoculation with 20% fecal slurry, fermentations were conducted at 37°C under anaerobic conditions with sample collection at 0, 4, 8 and 24 h. The samples were centrifuged, and pellet and supernatant were stored at -80°C for analyses.

Microbiota analysis. Microbiota composition analysis (Clinical Microbiomics, Denmark) were performed on the faecal fermentation samples. Alpha diversity was calculated as the number of entities detected (richness), or as the Shannon index based on natural logarithm.

SCFA analysis and polar metabolites (untargeted analysis). Samples were analyzed in an UHPLC-qToF-MS on negative mode (Agilent 1290) after methanol extraction (for the targeted SCFA and bile acid analysis) and acetonitrile extraction (for the polar metabolites by untargeted analysis). SCFA identification and quantification was performed using analytical standards. The polar metabolites (untargeted analysis) were putatively identified using an in-house library.

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Experimental setup and Ussing chamber experiments. A total of 24 biopsies from each participant were used per experiment. Treatments were run in duplicates in Ussing chambers (Harvard Apparatus, USA) at 37°C and oxygenized, and the biopsies were mounted[7] and treated with 100 uL of fecal fermentation supernatant of each fibre fraction, with and without sodium deoxycholate (DC; Sigma-Aldrich) as a stressor to induce intestinal hyperpermeability.[8] Tissue viability was monitored throughout the experiment. Permeability markers FITC-dextran 3000-5000 (2.5 nM, Sigma-Aldrich) and horseradish peroxidase (HRP, 5.34 µM, Sigma-Aldrich) were added for quantification as previously described.[9]

Validation of wheat bran AX fractions in bread products

Three different arabinoxylan fractions; non-purified arabinoxylan extract in suspension (AXE), freeze-dried feruloylated arabinoxylan (FAX) and unferuloylated arabinoxylan (AX), were used to validate the effect in bread products. Wheat pan breads were prepared with a farinograph using the straight-dough procedure. Part of the flour was replaced by arabinoxylan fractions to obtain an actual arabinoxylan content of 0.3 %, 1.0 % and 1.7 % (of flour), respectively. Each fraction and level combination were baked in duplicates. Bread with no addition of arabinoxylan was used as control. From each dough, four bread loaves were baked and tested for various parameters and after different storage times. A sensory evaluation was conducted day 1 and day 7, using an untrained in-house panel of 21 participants.

Results

Upscaling of the production of arabinoxylan fractions from wheat bran

We performed laboratory extractions with 6 different temperature profiles (**Figure 1b**), to assess the mass balances and molecular structure of the isolated AX fractions. Harsher conditions in terms of temperature (160°C) and longer hold time led to higher extraction yields (**Figure 2a**) at the expense of degradation, as observed by the resulting dark suspensions. The molecular weight of the extracts showed that the targeted high molecular fractions degraded at temperatures over 150 °C (**Figure 2b**). From the laboratory tests, we decided to continue the pilot scale runs at 150 °C, exhibiting similar monosaccharide composition and AX content between the laboratory and the pilot extractions (**Figure 2c**). Alkali treatment of the FAX successfully cleaved the ferulic acid attached to the AX, as expected (**Figure 2d**).

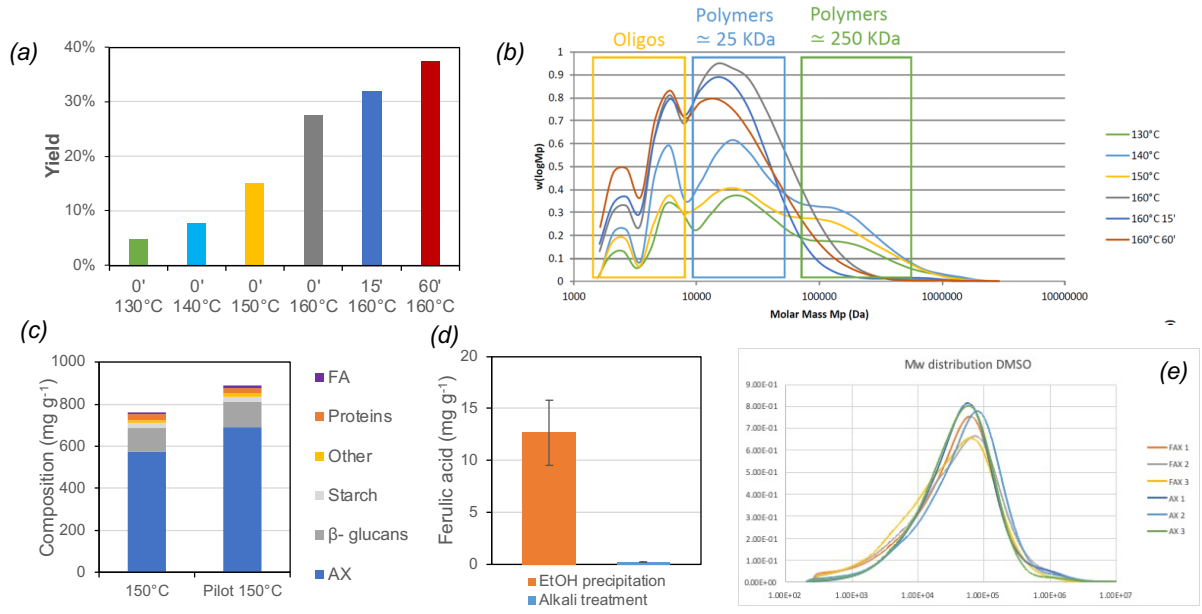

Figure 2. Mass balances and molecular structure of the AX from wheat bran. (a) Yields from the different temperature profiles at laboratory scale; (b) Molar mass distributions of the extracts at lab scale; (c) Monosaccharide composition at lab and pilot scale; (d) Ferulic acid content of the AX fractions after alkaline treatment; (e) Comparison of the molar mass distributions of the 6 pilot extraction batches.

After implementing the SWE and alkaline treatments at pilot scale, we repeated the processes in different batches, to obtain enough fibre fractions. The process showed excellent reproducibility at large scale, as the composition of the FAX and AX fractions was maintained in all batches (**Table 1**). The

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presence of ferulic acid was also very stable in the FAX batches, and the removal of ferulic acid in the AX batches was almost complete. The AX purity in all batches was around 80%, as previously obtained in process development at KTH.[1,2] The A/X ratio, indicating the substitution degree of the AX chains, was kept between 0.2 and 0.3 for all batches. The molar mass distributions of the batches were also very similar with averages between 70-80 kDa, in the range of the laboratory scale at KTH (**Figure 2e**).[1,2]

Table 1. Composition (mg/g) of the extracts from the different pilot scale batches

	FAX1	FAX2	FAX3	AX1	AX2	AX3
AX	619.29	638.97	604.71	575.87	579.24	574.10
Other sugars	11.98	11.95	12.10	10.29	11.47	2.55
Starch	83.88	99.69	64.63	88.76	76.58	57.26
β- glucans	65.97	40.16	44.52	52.10	71.10	79.01
Proteins	29.61	28.48	26.06	17.35	25.08	22.33
Ferulic acid	12.62	11.89	12.95	0.04	0.10	0.16

Finally, the enzymatic hydrolysis of the polymeric FAX and AX into FAXOs and AXOs, respectively, was successful, as evidenced in the reduction of the molar mass distributions (**Figure 3a**). The enzymatic deconstruction results in the production of oligomers of different sizes, from small oligosaccharides with degrees of polymerization (DP) between 3 - 4 and more complex oligosaccharides with DPs between 7 to 14 (**Figure 3b**), but also larger populations.

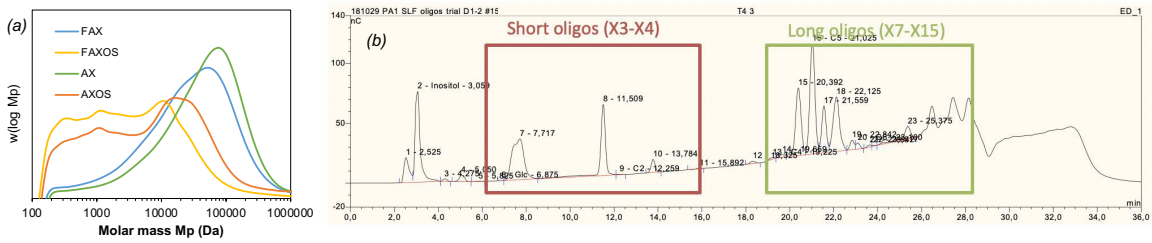

Figure 3: (a) Molar mass distributions of the dietary fibre fractions; (b) Oligomeric profile of FAXOs.

Prebiotic properties of wheat bran AX fractions using *in vitro fecal* fermentations

To investigate the effects of the wheat bran fibre fractions (WB, AX, AXOs, FAX and FAXOs) on microbiota composition, fecal fermentation samples obtained at baseline (T0) and after 24 hours (T24) were used for microbiota analysis. The differences in mcrobiota diversity between control and fibre-treated samples were more pronounced after 24 hours. However, there was no significant difference in alpha-diversity (number of taxa/richness) at T24 between any of the fibre fractions (**Figure 4a**). Significant differences in bacterial abundance were observed between the fermentation samples obtained at T24 (Figure 4b and 4c). Bacilli was significantly different at class level (Figure 4b) and *Enterococcus*, *Lysinbacillus* and *Parassuterella* were significant different at genus level (**Figure 4c**).

To explore the effects of the AX fibre fractions on microbiota functionality, metabolomic analyses were performed. Polar untargeted metabolomics detected 853 metabolites, of which 110 metabolites were identified using standards. A targeted approach was used to identify short chain fatty acids (SCFAs) and tricarboxylic acid (TCA) cycle metabolites. Using this approach, we were able to identify 19 SCFA and TCA cycle metabolites. From the 129 identified metabolites (110 polar metabolites and 19 SCFA/TCA metabolites), 35 metabolites were significant (ANOVA, $p<0.001$), and a clear segregation was observed on a Principal Component Analysis (PCA) and heatmap (**Figure 4d and 4e**). In the PCA results (**Figure 4e**), each dot represents a T24 fermentation sample obtained from one participant and the fibre substrates used for faecal fermentation. The fibre substrates are differentiated by the colours, where the wheat bran (WB) group (pink) is the most distant from the other groups. The FAXOs (light blue) is allocated on the right upper part whereas FAX, AXOs and AX are located at the lower part. The metabolite concentration levels responsible for distancing the samples in the PCA graph are shown in the heatmap (**Figure 4d**). WB fermentation resulted in higher production of phosphadylglycerols and taurine-conjugated bile acids, including taurocholic acid, taurochenodeoxycholic acid, and its glycine-conjugated forms (glycocholic acid and glycochenodeoxycholic acid). FAXO similarly to WB showed higher production of bile acid (BA) metabolites, but also taurine, coumaric acid, ferulic acid, salicylic acid and isovalerate. The other fibre fractions showed lower production of BA compounds and phosphadylglycerols.

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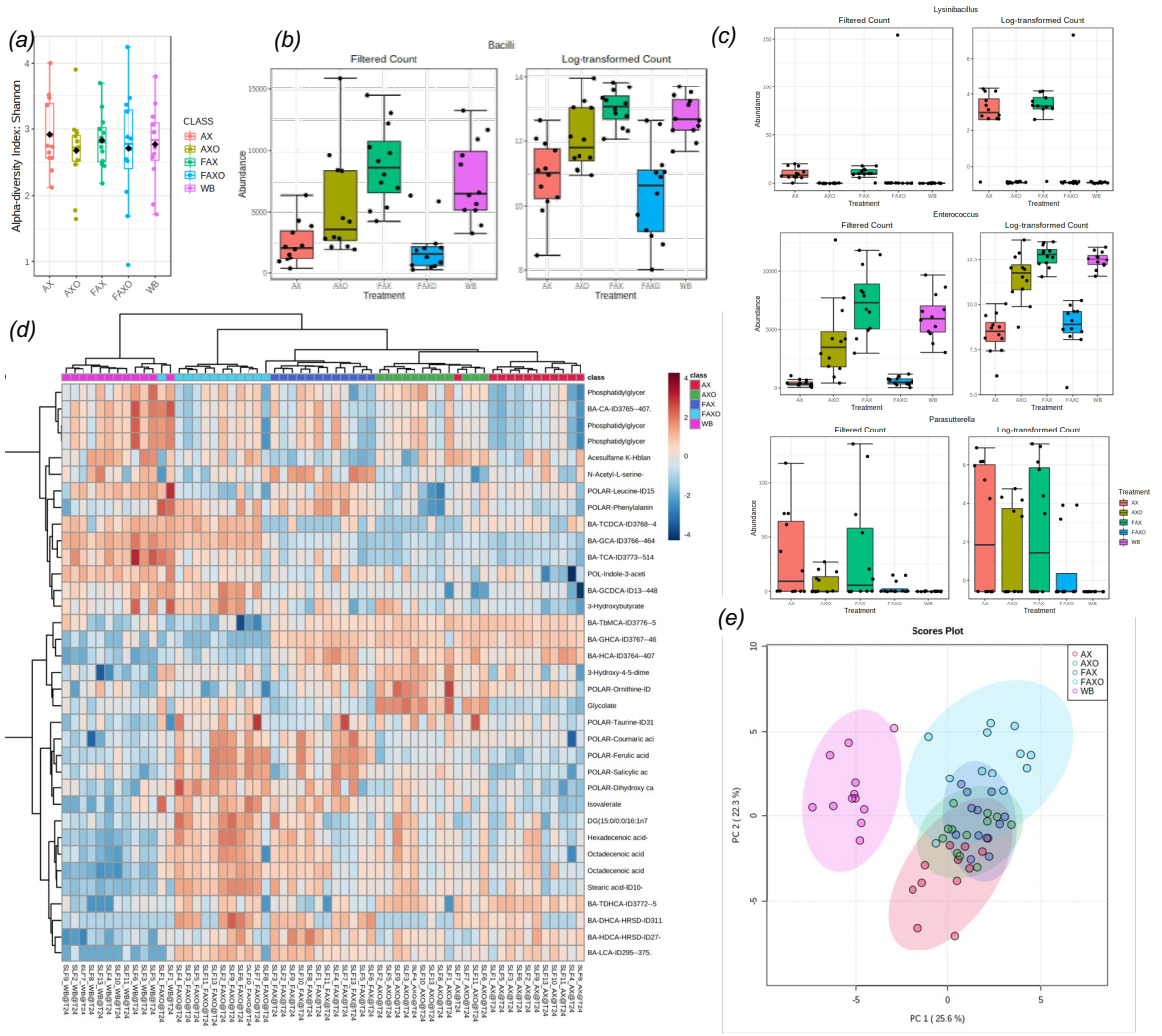

Figure 4. Metagenomic and metabolomic results from the faecal fermentations of wheat bran fibres: (a) Alpha-diversity Index: Shannon; DESeq2 significant differences $p<0.05$ for (b) class and (c) genus; Heatmap (d) and Principal Component Analysis (e) of significant identified metabolites ($p<0.001$).

Regulatory capacity of the wheat bran fibre fractions on colonic barrier function

We used an *ex vivo* Ussing chamber to assess if the supernatant from the fermented fibres protected human colonic biopsies against stress-induced hyperpermeability. To mimic an *in vivo* situation, each participant had their faecal material fermented with the different AX fibre fractions, which subsequently was added to mucosal biopsies from the same participant. The different dots in **Figure 5** represents the results obtained from each participant. Increased delta-passage of FITC and HRP indicates a higher permeability. No significant differences in paracellular or transcellular permeability were observed for any of the wheat bran fibre fractions when compared to stressor-stimulated biopsies (control) alone.

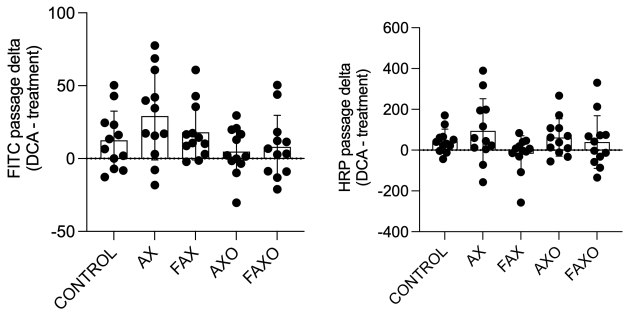

Figure 5. Effects of fecal fermented wheat bran fractions on intestinal permeability in colonic biopsies mounted in Ussing chambers. A: Paracellular permeability, B: Transcellular permeability.

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Incorporation of AX fractions in bread products and evaluation of their shelf life

The effect of the different AX fractions in the quality of bread products was evaluated in terms of colour, specific volume, crumb structure and moisture, and mechanical and morphological properties. The colour of the bread interior was influenced by arabinoxylan addition (**Figure 6**). Breads with addition of arabinoxylan had a darker crumb than control, particularly at high addition levels. AXE produced the darkest crumbs, while FAX and AX had a smaller effect on colour. AX addition had minor effects on the exterior of the breads. Breads with higher addition levels tended to have a slightly darker crust and a more uneven crust surface than control and low addition levels. The unevenness of the surface was likely due to the dough being stickier and thus more difficult to shape into even buns.

Figure 6. Example of bread slices from top to bottom: control, 1.7 % AXE, 1.7 % F-AX, 1.7 % AX. Illustrating the colour difference and difference in crumb structure.

Arabinoxylan addition did not affect significantly the specific volume of breads. The hardness of the breads increased over time for all samples, but there were no significant differences in hardness between the samples on any day depending on the addition of arabinoxylan. Addition of arabinoxylan had minor effects on the crumb structure of breads (**Figure 6**). The only significant effect was observed for 1.7 % AX, which gave a 22 % increase in total cell count compared to control. The crumb moisture content of the bread samples decreased from day 1 to day 14 with 10-14 %. Addition of 1.7 % F-AX and AX gave a significantly moister crumb than control and AXE breads on day 1, 7 and 14. Addition of 1.0 % F-AX increased the crumb moisture content on day 7 compared to control, and 1.0 % AX increased the crumb moisture content on day 1 and day 7. In general, the breads with high AX addition levels had slightly better moisture retention (i.e., a smaller moisture loss) than breads with medium or low addition level.

Analyzing the results by arabinoxylan fraction reveals possible structural differences between fractions, suggesting which fraction(s) may have potential as bread improvers. Significant differences between fraction averages were found for baking absorption, crumb moisture content (day 1, 7 and 14), crust moisture content (day 14), hardness (day 14), cohesiveness (day 7) and cell count. F-AX and AX gave a significantly higher baking absorption than AXE. AX had a higher moister crumb than the control and AXE on day 1. On day 7 and 14, the Tukey pairwise comparison test failed to group the arabinoxylan fraction means into significantly different groups, even though the p-value for the ANOVA (0.036 and 0.034, respectively) indicate that significant differences exist for crumb moisture content. F-AX gave significantly moister crust on day 14 than control. AXE breads were significantly softer than control breads on day 14. AX breads were significantly more cohesive than the other breads on day 7. Finally, AX had a significantly higher cell count than control, indicating an even crumb structure with many air cells. The sensory analysis was conducted on bread with no addition of arabinoxylan and bread with 1,0% AX without ferulic acid. No significant differences were found.

Discussion

Upscaling of the production of arabinoxylan fractions from wheat bran

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In this project we have successfully validated the upscale production of dietary fibres (FAX, AX, FAXOs and AXOs) from wheat bran using subcritical water extraction, alkaline treatment and enzymatic hydrolysis (**Figure 1a**). The process has high reproducibility in term of yields, composition, and molecular structure of the fibre fractions. The production of polymeric AX by a hydrothermal process with 50L reactor pilot scale has been demonstrated, being a step forward in their commercialization. This fulfills the first aim of the project - *Optimize the upscaling of the developed bioprocess to kg scale, to isolate bioactive F-AX in polymeric and oligomeric form from wheat bran* -.

However, several considerations and learned lessons must be taken from the upscaling process. The upscaling trials showed that the combined temperature and time in the subcritical water extraction affect the results greatly, which indicates that the product (here, polymeric FAX) is very sensitive towards heat fluctuations (no stirring, hot walls). This means that altering the configuration of the reactor will have a huge impact on the process mass balances. The volume efficiency of the FAX extraction method is limited, due to the low concentration in the extract and the high ratio between ethanol and water (4:1) during precipitation. This means that a reactor volume of 300 L will produce only 1 kg of dietary fibre product. Further work is required to increase the concentration of the extracts, either by increasing the solid ratio in the wheat bran slurry or by concentrating the liquid filtrate after extraction. Finally, the presence of glucose-rich fractions (residual starch and β-glucans) reduced the purity of AX extracts. If required for product development, the pretreatment steps should be improved to increase the AX yields and purity, i.e., with additional washing steps or by α-amylase addition before ethanol precipitation.

Prebiotic and colonic barrier effects of the dietary fibre fractions from wheat bran

To investigate the effects of the wheat bran dietary fibre fractions on microbiota composition and functionality, fecal fermentation samples obtained at baseline and after 24 hours fermentation were used for microbiota composition and metabolomics analysis. Although some differences were observed in bacterial abundance at class level and at genus level between the different fermented wheat bran fibre fractions, no difference in alpha-diversity, a marker of richness, was observed when comparing treatments. The results from the metabolomic analyses show that the metabolites produced are treatment-dependent and that the different wheat bran fibre fractions separates into distinct clusters in the PCA plot. Wheat bran (WB) and FAXOs had a more pronounced effect on the metabolites production compared with the other fibre fractions and resulted in higher production of bile acid metabolites such as taurocholic acid, taurochenodeoxycholic acid and its glycine-conjugated forms (glycocholic acid and glycochenodeoxycholic acid). Overall, the microbiota composition and metabolomics results demonstrate that although the wheat bran fibre fractions did not have a clear effect on the composition of the microbiota, they had a distinct effect on the functionality of the microbiota and that this effect was dependent on the specific wheat bran fibre fraction. These results addressed the second aim of the project proposal - *Prove, quantify, and understand the potential prebiotic and antioxidative effects of the isolated F-AX fractions by in vitro and ex vivo methods*-. Further studies using targeted metagenomic analysis are needed to understand how molecular size and the presence of phenolic compounds can modulate the microbiota function.

Intestinal permeability was assessed in human colonic specimens using paracellular and transcellular permeability markers. Mounted in Ussing chambers, the colonic specimens were stimulated with sodium deoxycholate (DC) to induce hyperpermeability. By adding the supernatant of the fermented AX fiber fractions before the stressor, a preventative effect of the fibers was evaluated. However, there were no differences in paracellular or transcellular permeability between the fibers and stressor compared with the stressor alone. This suggests that the supernatant from the fermented fibers did not have any protective effect on intestinal permeability under the conditions used in the present study.

Validation of the incorporation of wheat bran AX fractions in bread products

The conducted baking trials were not as large as planned, due to the limited amount of each arabinoxylan fraction. Therefore, it was decided to also bake with the arabinoxylan crude extract (AXE). This fraction contained a combination of high and low molecular weight arabinoxylan and since it was not purified it could be a more economic fraction to use. Statistical analyses of the data demonstrated the difficulties to achieve significant differences. Possibly, the number of replicates and the dosage used in this study were too low to reveal any effect of arabinoxylan addition on the parameters evaluated, as previous

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studies have seen significant effects of arabinoxylan addition on different quality parameters. Indeed, higher levels of low and high molecular weight AX (2-3 %) resulted in larger loaf volume increases.[10]

The results of this study indicate that wheat bran AX influences baking absorption, visual appearance, crumb moisture content and crumb texture. In the consumer acceptance test, arabinoxylan addition did not significantly influence acceptance of fresh or stored breads. This is very promising comparing our results with previous baking tests with 20% enzymatic treated wheat bran,[11] which resulted in an unacceptable colour and taste although giving a prolonged shelf-life. Here, we have demonstrated that all three arabinoxylan fractions have potential as bread improvers to retard staling while maintaining quality. This clearly fulfills the third aim of the project proposal - *Validate the use of F-AX fractions in bread products with prolonged shelf life compared to bread without baking improvers* -. However, the overall lack of significant results in this study calls for further research to elucidate the future role of wheat bran arabinoxylans as bread improvers as well as a source of fibre in industrial breadmaking.

Conclusions

We have successfully demonstrated the upscale production of four dietary fibres from wheat bran, with high reproducibility in term of yields, composition and molecular structure. Practical lessons have been learned regarding the sensitivity of the process towards heat fluctuations and the needs for further increase the volume efficiency and downstream processing step. This represents a step forward towards commercialization of AX dietary fibres from wheat bran.

The *in vitro* faecal fermentation tests demonstrated that the different wheat bran fibre fractions had a distinct effect on the functionality of the microbiota (in terms of the metabolites generated), without clearly affecting the microbiota composition. However, the ex vivo colonic permeability tests showed that the supernatant obtained from the fermented fibres did not have any protective effect on intestinal permeability under the conditions used in the present study.

The baking tests revealed that the addition of wheat bran arabinoxylan fibres affected the baking absorption, visual appearance, crumb moisture content and crumb texture, without significantly altering consumer acceptance of fresh or stored breads. The low number of replicates and AX dosage prevented us from obtaining significant results from the baking trials. However, this project has demonstrated the potential of wheat bran AX fractions as bread improvers to retard staling while maintaining quality.

Relevance and recommendations

This project has demonstrated the potential of arabinoxylan fibre fractions from wheat bran as bread improvers in baking applications, with beneficial effects on the functionality of the gut microbiota. This provides, on one hand, an extremely valuable route to valorize wheat bran, a significant side stream from wheat milling processes, into highly refined dietary fibre ingredients increasing the profitability for this stream. On the other side, this project offers the possibility to Lantmännen to produce new bread products with longer shelf life and higher gut functionality, which could improve overall sustainability by reducing bread and improving human health by its demonstrated prebiotic effects.

Important lessons have been learned towards the industrial implementation of the process. The stability of the high molecular weight fractions during extraction needs to be further understood. Moreover, the implementation of larger batch processes (beyond 50 L) will require longer heating and cooling profiles, which will influence the properties of the products. To circumvent these issues, the implementation of continuous extraction processes from the wheat bran should be explored. Additionally, ethanol precipitation only offered a small effect on the quality of the product. Therefore, it needs to be investigated whether the step might be skipped by spray drying the extraction liquor directly.

The evaluation of the *in vitro* prebiotic properties has revealed a distinct effect of each AX fibre on microbiome functionality and production of different metabolites. Further studies using targeted metagenomic analysis are required to understand how AX structure (molecular size and ferulic acid content) can modulate microbiota function and the biochemical mechanisms behind these effects. Finally, large scale clinical interventions should be planned, in order to claim the health effects of the AX fibres at relevant regulatory authorities, such as the European Food Safety Authority (EFSA).

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The overall lack of significant results in the baking trials calls for further research to elucidate the role of wheat bran AX as bread improvers as well as a source of fiber in industrial breadmaking. There is an interest to further study the effect the molecular weight of the added arabinoxylan. Lantmännen has therefore during the SLF-project period employed an industrial PhD on the subject "Fractionation of wheat bran to create functional ingredients", with the aim to dive deeper into this research.

References

1 Rudjito, R. C., Ruthes, A. C., Jiménez-Quero, A. & Vilaplana, F. Feruloylated Arabinoxylans from Wheat Bran: Optimization of Extraction Process and Validation at Pilot Scale. *ACS Sustainable Chemistry & Engineering* **7**, 13167-13177, doi:10.1021/acssuschemeng.9b02329 (2019).

2 Ruthes, A. C., Martinez-Abad, A., Tan, H.-T., Bulone, V. & Vilaplana, F. Sequential fractionation of feruloylated hemicelluloses and oligosaccharides from wheat bran using subcritical water and xylanolytic enzymes. *Green Chemistry* **19**, 1919-1931, doi:10.1039/C6GC03473J (2017).

3 Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**, 248-254 (1976).

4 Requena, R. *et al.* Integral Fractionation of Rice Husks into Bioactive Arabinoxylans, Cellulose Nanocrystals, and Silica Particles. *ACS Sustainable Chemistry & Engineering* **7**, 6275-6286, doi:10.1021/acssuschemeng.8b06692 (2019).

5 McKee, L. S. *et al.* A GH115 α-glucuronidase from Schizophyllum commune contributes to the synergistic enzymatic deconstruction of softwood glucuronoarabinoxylan. *Biotechnology for Biofuels* **9**, 1-13, doi:10.1186/s13068-015-0417-6 (2016).

6 Fooks, L. J. & Gibson, G. R. Mixed culture fermentation studies on the effects of synbiotics on the human intestinal pathogens Campylobacter jejuni and Escherichia coli. *Anaerobe* **9**, 231-242, doi:https://doi.org/10.1016/S1075-9964(03)00043-X (2003).

7 Wallon, C., Braaf, Y., Wolving, M., Olaison, G. & Söderholm, J. D. Endoscopic biopsies in Ussing chambers evaluated for studies of macromolecular permeability in the human colon. *Scandinavian Journal of Gastroenterology* **40**, 586-595, doi:10.1080/00365520510012235 (2005).

8 Münch, A., Ström, M. & Söderholm, J. D. Dihydroxy bile acids increase mucosal permeability and bacterial uptake in human colon biopsies. *Scandinavian Journal of Gastroenterology* **42**, 1167-1174, doi:10.1080/00365520701320463 (2007).

9 Tabat, M. W. *et al.* Acute Effects of Butyrate on Induced Hyperpermeability and Tight Junction Protein Expression in Human Colonic Tissues. *Biomolecules* **10**, 766 (2020).

10 Courtin, C. M. & Delcour, J. A. Physicochemical and Bread-Making Properties of Low Molecular Weight Wheat-Derived Arabinoxylans. *Journal of Agricultural and Food Chemistry* **46**, 4066-4073, doi:10.1021/jf980339t (1998).

11 Bach Knudsen, K. E. MERITS- Metabolic changes by carbohydrate and protein quality in the development and mitigation of metabolic syndrome. (Department of animal science (AU-ANIS), Aarhus University, 2015-2020).

Result dissemination:

Scientific publications, *published*	Solja Pietiäinen, Annelie Moldin, Anna Ström, Christian Malmberg, Maud Langton. Effect of physicochemical properties, pre-processing, and extraction on the functionality of wheat bran arabinoxylans in breadmaking – A review. Food Chemistry 383 (2022) 132584.
Scientific publications, *submitted*	Dongming Zhang, Reskandi C. Rudjito, Solja Pietiäinens, Francisco Vilaplana, Amparo Jiménez-Quero. *Fibre supplemented bread: from extraction of fibers to effect of baking, digestion, and fermentation.* Submitted to *Food Chemistry.*
Scientific publications, *manuscript*	Amparo Jiménez-Quero, Samira Prado, Rebecca Wall, Tatiana Marques, Francisco Vilaplana. *Wheat-derived Arabinoxylans: effect of their molecular structure in the human gut health.*

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	Samira Prado, Victor Castro-Alves, Amparo Jiménez-Quero, Tuulia Hyötyläinen, Francisco Vilaplana, Rebecca Wall, Tatiana M Marques. *Wheat-derived arabinoxylans differently affects human colonic fermentation metabolites.*
	Samira Prado, Mathias W Tabat, Selma Smrkovic, Patricia R Iglesias, Amparo Jiménez-Quero, Francisco Vilaplana, Robert J Brummer, Rebecca Wall, Tatiana M Marques. *Comparison of the effects of wheat-derived arabinoxylans and xylo-oligosaccharides on human colonic permeability.*
Conference publications/ presentations	F. Vilaplana. Hemicelluloses: Molecular Structure, Assembly in Plant Cell Walls and Food Applications. Nordic Lights on Food Conference 2021. 9-11 June 2021. Lund (Sweden) – virtual
	F. Vilaplana. Extraction of building blocks from lignocellulosic biomass. InnFOOD Conference 2021, 1 – 2 February 2021 (virtual), Aarhus University (Denmark)
	AC Ruthes, A Martínez-Abad, A Jiménez-Quero, RC Rudjito, S Yilmaz, F. Vilaplana Bioactive polysaccharides and oligosaccharides from cereal byproducts. ECO-BIO 2018. Dublin, 6th March 2018
Other publications, *media etc.*	Här blir livsmedelsavfall nästa generations mat och material. Dagens Nyheter, 2021-11-27 (https://www.dn.se/sthlm/har-blir-livsmedelsavfall-nasta-generations-mat-och-material/)
	Vetekli i nytt ljus. Grodden – En medlemstidning för Lantmännen. Issue 1. February 2022. Pages 18-19.
	Sidoström blir nya produkter. Grodden – En medlemstidning för Lantmännen. Issue 1. February 2022. Pages 20-21.
Oral communication, *to sector, students etc.*	TEDxKTH Salon "Food for thought" (2020-09-15). The audience consisted of KTH students and general public interested in sustainable food solutions.
	Dialogue. "Att äta gott och göra gott – ett livesänt samtal om maten på din tallrik och hållbar utveckling". Tranströmer Bibliotek, Stockholm (2021-06-02). The audience consisted of general public interested in sustainable food solutions.
	Lantmännens Stiftelsedagen (2022-03-31) Sidoströmmar – livsmedelsingrediens eller material?
Student theses	Zhang, Dongming; supervisors: Amparo Jiménez Quero and Reskandi Rudjito. 2021. Study of arabinoxylans dietary fiber in bread and their further effect on microbial fermentation after in vitro digestion. DiVA, id: diva2:1571931
	Wheat bran arabinoxylans in breadmaking – Their effect on staling and other quality aspects, Ylva Henriksson, Master-thesis, SLU/Department of Molecular Sciences, 2020:07, Uppsala 2020.

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Appendix 1. Full experimental details

Extraction and characterization of arabinoxylan fractions from wheat bran at pilot scale

Laboratory optimization of the pretreatment and extraction. 100 g wheat bran (Lantmännen) was suspended in 915 mL tap water at 37 °C and enzymatically treated with 3 mL Celluclast (Novozymes, 1 g/mL) for 4 h under agitation. The suspension was then filtered, and the filter cake was pressed out and suspended in fresh tap water to wash out the starch. The filter cake (30% dry content) was frozen at -20 °C until further SWE. For SWE, 60 g of the pre-treated bran was added into 130 mL hot water in a high-pressure steel reactor to give a 10% suspension and subjected to 6 heating and cooling ramp programs (Figure 1b). After extraction, the samples were filtered, and the extracts were frozen, dried and sent to KTH for analysis.

Pilot scale pretreatment and extraction. Based on the laboratory scale treatments, the pilot scale pretreatment and subcritical water extractions were performed with a 50 L reactor with a working capacity of 40 L equipped with an off-centred anchor-propeller stirrer at 150 rpm (Figure 1c). For the pre-treatment, 4 kg of wheat bran were added to preheated tap water at 37°C (approx. 37 kg of water were added to reach 10 % wheat bran) and were enzymatically treated with 120 mL Celluclast (Novoyzmes, 1 $g \cdot ml^{-1}$) for 4 h. After the pre-treatment, the reactor was emptied while being stirred into two filter socks (pore size 100 µm) and the socks were pressed out in a filter-press. The filter cake was washed in a 40 L barrel with 20 kg of cold tap water using a cement stirrer followed by a second filtration, resulting in a pre-treated filter cake with a DW of 35-40 %. For the subcritical water extraction, an estimated amount of tap water (typically 16 kg, adjusted to DW of the bran) were preheated to 95 °C and the wet pre-treated wheat bran was added, resulting in a DW of 10 %. After the reactor had been sealed, the subcritical water extraction program was performed in the reactor heated with 210 °C warm oil over a regulated valve. The temperature program included a controlled heating step to approach 150 °C in a 0.7 $°C \cdot min^{-1}$ slope, then the valve was closed until the oil temperature was below 150°C (approx. 7 min) and the cooling step was performed at approximately 1.1 $°C \cdot min^{-1}$ (Figure 1d). The mixture was filtered in one filter bag and squeezed out using a filter press. The filtrate was cooled to 4 °C overnight in a plastic 20 L barrel prior to subsequent precipitation. Six extraction batches were run, in order to yield enough F-AX for the project.

Precipitation. Precipitation of the laboratory and the pilot scale extracts was performed with 99.7% ethanol in a 4:1 liquid ratio. In the pilot scale, 15 – 18 L of the F-AX (or AX) fractions were precipitated with 65 – 75 L ethanol in a 100 L glass-lined reactor. After ethanol addition, the slurry was cooled down to 4°C and left overnight before decantation and filtration. The clear upper phase (approx. 60 L) was filtered first in a 10 L pressure filter (pore size 40 µm), followed by the precipitated slurry (approx. 30 L). The slurry was washed with 99.7% ethanol 3 times (using the same volume as the starting extract solution) and filtered. The precipitated filter cake was dried in a vacuum drying cabinet at 40 °C yielding the F-AX (and AX) fractions.

Alkaline treatment of the F-AX extract to produce the AX fraction. Ferulic acid (FA) removal requires a saponification step of F-AX extracted by SWE. The dried F-AX was mixed with 0.5 M NaOH in a 1:10 solid to liquid ratio, incubated for 4 hours at room temperature under constant stirring, and precipitated with ethanol following the same procedure as for F-AX.

Enzymatic treatment to produce FAXOs and AXOs. In order to generate oligosaccharides from the polymeric FAX and AX extracted in the different batches, a treatment with a commercial endo-1,4-β-xylanase (Ronozyme, Novozymes) was performed. After optimization of the conditions at small scale, overnight incubations in large batches of 60 g of FAX and AX, respectively, were performed at 37°C under stirring with a solid to liquid ratio of 1:6.

Prebiotic and gut barrier properties of arabinoxylans and oligosaccharides from wheat bran

Study participants, questionnaires, and ethics. A total of 12 healthy subjects, between 18 and 45 years old, were enrolled in the study. The study was approved by the Ethics Review Authority of Sweden (Dnr 2020-03943). Throughout the study, the principles of the Helsinki declaration were followed, and all participants signed the informed consent. The participants' gastrointestinal symptoms were assessed using a gastrointestinal symptoms rating scale (GSRS) to confirm absence of gastrointestinal problems.

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Participants completed a food frequency questionary (Meal-Q) prior to study start. Fecal samples for the *in vitro* fermentations were collected at home and delivered to Örebro University within 2 hrs of collection. On the evening before colonoscopies, the participants consumed a standardized low-fibre dinner.

Collection of colonic biopsies. Study participants (n=12) underwent a sigmoidoscopy in the morning after 10 hours fasting. No bowel cleansing procedure was performed to avoid affecting the mucosal integrity. Colonic biopsies were obtained from the unprepared sigmoid colon, at the crossing with the common ileac artery, using a non-spiked Captura biopsy forceps (DBF-2.4-230, Cook Medical, Bloomington, IN, USA) and were immediately transferred to ice-cold oxygenated modified Krebs-Ringer bicarbonate buffer (from now on called KRB). Biopsies were transported in KRB buffer to the laboratory within 10 minutes.

Simulated in vitro gastrointestinal digestion. Fibre fractions (AX, FAX, FAXOs, AXOs and wheat bran) were mixed with deionized water and homogenized in a stomacher (Seward, UK) at high speed for 5 min. Throughout the *in vitro* digestion process, the mixture was kept at 37ºC under constant stirring. After homogenization, 1.0 mM $CaCl_2$ (6,25 mL, pH 7) and 2000 U porcine pancreas α-amylase (Sigma-Aldrich, USA) were added to the mixture, then incubated for 5 min. After the incubation, pH was adjusted to 2, 2.7g pepsin from porcine gastric mucosa (Sigma-Aldrich) in 0.1M HCl (25 mL) was added, and the mixture was incubated for 2 h. Finally, pH was adjusted to 7, 0.5 M $NaHCO_3$ (125 mL), 3.5g of ox-bile (Sigma-Aldrich) and 560 mg pancreatin (Sigma-Aldrich) were added, and the mixture was incubated for a further 3 h. After simulated *in vitro* digestion, the mixture was divided into membrane bags (Spectra/por 100–200 Da MWCO dialysis membrane, Spectrum Europe B.V., Breda, The Netherlands) and dialyzed at 37°C with deionized water under constant stirring for 26 h. After dialysis, the content of the bags was combined, and the product was freeze-dried (Advantage ES-53). Dried fibre fractions were then pooled and stored at -20°C.

In vitro fecal fermentation. The fermentation medium used was as outlined by Fooks and Gibson;[6] however, freeze dried fiber fractions (5% w/v) were added as a replacement of glucose as carbon source. Fermentation vessels were inoculated with a 20% fecal slurry and fermentation were conducted at 37°C, under anaerobic conditions (10% H_2, 80% N_2 and 10% CO_2, Ruskinn, UK). Samples were collected at 0, 4, 8 and 24 h, centrifuged, and pellet and supernatant were stored at -80°C for posterior analyses. On the day of the Ussing experiment, supernatant samples collected after 24 h fermentation were thawed and centrifuged at 1800 g for 10 min. 100 uL of fecal fermentation supernatant obtained from the different wheat bran fibre fractions was used in the Ussing chambers to evaluate their effects on intestinal permeability in mucosal colonic biopsies.

Microbiota analysis. Samples from the fecal fermentation experiments with the different fibre fractions were sent to Clinical Microbiomics (Denmark) for microbiota composition analysis. DNA was extracted using the NucleoSpin® stool (Macherey-Nagel) kit. Bead beating was done on a Vortex-Genie 2 horizontally at 2700 rpm for 2x5 min. A minimum of one positive control (ZymoBIOMICS™ Microbial Community Standard, Zymo Research) and one negative control was included with each batch of samples. Sequencing was done on an Illumina MiSeq desktop sequencer using the MiSeq Reagent Kit V3 (Illumina) for 2 x 300 bp paired-end sequencing. A customized pipeline based on dada2 was used for bioinformatics processing of the sequence data into an ASV (amplicon sequence variant) abundance table (Callahan et al., 2016, PMID: 27214047). The default taxonomic assignment of the detected ASVs was done using a naïve Bayesian classifier algorithm comparing the ASV sequences to the SILVA reference database (v138.1). Alpha and beta diversity estimates were calculated from rarefied abundance matrices, created by random sampling of reads without replacement. Alpha diversity was calculated as the number of entities detected (richness), or as the Shannon index based on natural logarithm.

SCFA analysis and polar metabolites (untargeted analysis). Samples were extracted in methanol containing internal standard (IS, acetic acid-d4, butyric acid-d8, propionic acid-d2, succinic acid-d4) followed by derivatization using 3-NPH for the SCFA analysis and bile acid (BA)-Polar metabolites. For the polar metabolites (untargeted analysis), samples were extracted in acetonitrile extraction containing IS (glutamine-d5, succinic acid-d4, tryptophan-d5, cholic acid-d4, heptadecanoic acid).

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Samples were analyzed in an UHPLC-qToF-MS on negative mode (Agilent 1290) and the acquity was done using UPLC BEH C18 column (2.1 x 100 mm, 1.7 µm) (Waters). For the SCFAs, the data was analyzed using the Mass Hunter software, and metabolites were confirmed using analytical standards. For the polar metabolites, data were analyzed using MZmine 2.53 software and in-house library for identification and comparison with HMDB for putative identification.

Experimental setup and Ussing chamber experiments. A total of 24 biopsies from each participant were used per experiment. Treatments were run in duplicates and the biopsies were treated with 100 uL of fecal fermentation supernatant of each fibre fraction. Within the first set of specimens collected (12 biopsies), two were left untreated (control) and two biopsies were treated with fecal fermentation supernatant of each fibre fraction, respectively. Within the second set of biopsy specimens collected (12 biopsies), all specimens were stimulated with 1mmol/L sodium deoxycholate (DC; Sigma-Aldrich), a potent stressor to induce intestinal hyperpermeability.[8] Within these twelve biopsies, two were left stimulated with DC only (DC-control) and two biopsies were treated with fecal fermentation supernatant of each fiber fraction and were stimulated with DC. The mucosal specimens were mounted in a randomized order in 1.5 mL Ussing chambers (Harvard Apparatus, USA) and were held in between two polyester films that exposed a round area of 1.77 mm^2 of the mounted specimen. as previously described.[7] The serosal side of the biopsy was filled with ice-cold Krebs Buffer containing glucose whereas the serosal side contained mannitol. During the experiment, chambers were held at 37°C and oxygenized with 95% O_2 and 5% CO_2. To monitor tissue viability, the electrophysiological parameters transepithelial electrical resistance (TER), potential difference (PD) and short circuit current (Isc) were measured every 30 seconds throughout the experiment. After 10 min, the ice-cold buffers were replaced with fresh buffers with a temperature of 37°C. After an additional 10 min, buffers were replaced again with 37°C buffers. 1250µl mannitol Krebs was added to the mucosal side of the chambers without DC stressor and 950µl mannitol Krebs was added to the chambers with DC stressor. A sample was collected from the serosal side and regarded as the T0 sample. 100 uL fecal fermentation supernatant of the different fibre fractions was added into designated chambers and after 20 min, 1 mM DC was added to the mucosal side to increase the intestinal permeability. Permeability markers FITC-dextran 3000-5000 (2.5 nM, Sigma-Aldrich) and horseradish peroxidase (HRP, 5.34 µM, Sigma-Aldrich) were added directly after the DC to the mucosal side. 150µl of glucose buffer was added to the serosal side. 90 min after the permeability markers were added, samples were collected at the serosal side. Quantification of HRP and FITC-dextran was performed as previously described.[9]

Validation of wheat bran AX fractions in bread products

Three different arabinoxylan fractions; non-purified arabinoxylan extract in suspension (AXE), freeze-dried feruloylated arabinoxylan (FAX) and unferuloylated arabinoxylan (AX), were used to validate the effect in bread products. Wheat pan breads were prepared with a farinograph using the straight-dough procedure. The ingredients of the bread were as follows: wheat flour (or flour plus arabinoxylan fractions), 100; oil, 2.5; sugar, 5; salt, 1.5; yeast, 4,6; microencapsulated sorbic acid, 0.15, water, variable. The amount of flour in each dough was equivalent to 300 g on a 14 % moisture content basis. Part of the flour was replaced by arabinoxylan fractions to obtain an actual arabinoxylan content of 0.3 %, 1.0 % and 1.7 % (of flour), respectively. Each fraction and level combination were baked in duplicates. Bread with no addition of arabinoxylan was used as control. From each dough, four bread loaves were baked and tested for various parameters and after different storage times. The breads were packaged in plastic bags and stored in room temperature.

Dough properties were evaluated by baking absorption and dough developing time. Bread parameters were measured on the day of baking and during storage. The visual appearance was evaluated subjectively. Specific volume was determined on the day of baking. On day 1, 7 and 14 after baking, one loaf was taken out for determination of crumb structure, crumb texture and moisture content. A sensory evaluation was conducted day 1 and day 7, using an untrained in-house panel of 21 participants.

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