Effect of oat β-glucan of different molecular weights on fecal bile acids, urine metabolites and pressure in the digestive tract – a human cross over trial

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ABSTRACT

While the development of oat products often requires altered molecular weight (MW) of β-glucan, the resulting health implications are currently unclear. This 3-leg crossover trial (n=14) investigated the effects of the consumption of oat bran with High, Medium and Low MW β-glucan (average >1000, 524 and 82 kDa respectively) with 3 consequent meals on oat-derived phenolic compounds in urine (LC-MS/MS), bile acids in feces (LC-QTOF), gastrointestinal conditions (ingestible capsule), and perceived gut well-being. Urine excretion of ferulic acid was higher ($p<0.001$, $p<0.001$), and the fecal excretion of deoxycholic ($p<0.03$, $p<0.02$) and chenodeoxycholic ($p<0.06$, $p<0.02$) acids lower after consumption of Low MW β-glucan compared with both Medium and High MW β-glucan. Duodenal pressure was higher after consumption of High MW β-glucan compared to Medium ($p<0.041$) and Low ($p<0.022$) MW β-glucan. The MW of β-glucan did not affect gut well-being, but the perceptions between females and males differed.

Keywords: oat; beta-glucan; molecular weight; enzyme treatment; avenanthramides; phenolic acids; bile acids; gastrointestinal pressure

1. Introduction

Oats (*Avena sativa*) are a nutritionally valuable food and food ingredient, and currently human consumption is rapidly rising, especially in the Nordic countries. The nutritional advantages of oats compared to many other grains include the gluten-free nature, the high content of polyunsaturated fatty acids, the protein composition which complements that of pulses, and the substantiated health effects of fibers, specifically oat β-glucan. EU has approved official health claims for β-glucan (EFSA Panel on Dietetic Products, 2011), which state that β-glucan reduces both blood cholesterol and postprandial glycemic response. Further, oat fiber in general increases fecal bulk. However, not all health benefits of oats are related to fibers. Phenolic compounds in oats are known to have bioactive functions as previously reviewed (Sang & Chu, 2017).

In addition to the substantiated health effects, the increase in the human consumption of oats is likely linked with the development of versatile novel oat products, which are often spoonable i.e. semi-solid or liquid. For such purposes, alterations of the physicochemical properties of oats are generally needed. These alterations can be achieved *via* bioprocessing by enzymatic means which often results in a reduction of the molecular weight (MW) of β-glucan. Although there are indications that β-glucan MW is related to the health effects (Q. Wang & Ellis, 2014), the current health claims do not name requirements for the state of β-glucan in the final product.

The cholesterol-lowering effect of β-glucan is linked with the re-absorption of bile acids (Ellegard & Andersson, 2007; Gunness & Gidley, 2010). In the intestine, β-glucan forms a viscous gel structure, which binds bile acids and prevents their re-absorption (Gunness & Gidley, 2010). Lack of reabsorption leads to increased bile acid synthesis in liver from the blood's cholesterol pool. Previously, an increase in the excretion of bile acids into human

66 feces has been detected after 5 weeks consumption of high MW barley beta-glucan in mildly
67 hypercholesterolemic individuals (Thandapilly, Ndou, Wang, Nyachoti, & Ames, 2018).
68 Some *in vitro* studies have suggested that reduced MW β-glucan binds bile acids equally well
69 or even better compared with high MW oat β-glucan (Kim & White, 2010; Sayar, Jannink, &
70 White, 2011). Nevertheless, the centrifugation methods used in these studies overlook the
71 viscosity effect, which is of importance in bile acid binding of soluble dietary fibers, as noted
72 in a recent *in vitro* study (Marasca, Boulos, & Nystrom, 2020). The few studies which have
73 followed subjects receiving products containing high or low MW oat β-glucan support either
74 the superiority of high MW β-glucan in decreasing blood cholesterol (Wolever et al., 2010) or
75 report no differences in cholesterol levels (Frank, Sundberg, Kamal-Eldin, Vessby, & Aman,
76 2004). However, the effect of β-glucan MW on bile acid excretion into feces has not
77 previously been studied *in vivo* in humans.

78 The positive health effects of phenolics in oats are caused by a pool of various compounds.
79 Avenanthramides, which are alkaloids found uniquely in oats, represent the largest group of
80 phenolics, followed by ferulic and *p*-coumaric acids (Antonini et al., 2016; Multari et al.,
81 2018). Human studies have shown that avenanthramides are bioavailable (Chen, Milbury,
82 Collins, & Blumberg, 2007) and have anti-inflammatory (Chen et al., 2007; Koenig et al.,
83 2014) and antioxidant (Chen et al., 2007) activity. It has even been suggested that they might
84 play a role in the cholesterol-lowering effect of oats (L. P. Liu, Zubik, Collins, Marko, &
85 Meydani, 2004). Since the majority of phenolic compounds in native oat bran are covalently
86 bound to macromolecules of the cell walls, they have compromised bioavailability (Antonini
87 et al., 2016; R. H. Liu, 2007; Multari et al., 2018). Previous studies have shown that
88 bioprocessing of wheat (Anson et al., 2011) and rye (Lappi et al., 2013) increases the
89 bioavailability of the phenolic compounds, especially ferulic acid. However, the effect of

bioprocessing on the bioavailability of oats´ phenolic compounds in humans has not been studied before.

The effect of dietary fiber on increasing the fecal bulk is considered to refer to improved bowel function and therefore improved “gut health” (EFSA Panel on Dietetic Products, 2011). However, apart from subjective experiences and the increase in the quantity of fecal bulk, gut health is challenging to evaluate. Previously, our research group (Nuora et al., 2018; Pirkola et al., 2018), in addition to others (Timm et al., 2011; Willis, Thomas, Willis, & Slavin, 2011) introduced an ingestible pH, pressure and temperature measuring capsule (SmartPill®) into nutritional intervention trials in order to link perceived gut wellbeing with the physiological, physical and chemical environment in the different parts of the digestive track. Thus far, no report exists that would link the oat β-glucan MW to intestinal pressure and subjective gut symptoms.

The hypothesis in this study was that bioprocessing of oat bran with enzyme treatment, causing depolymerisation of β-glucan, affects the nutritional properties of the bran and the functional properties of β-glucan in human gastrointestinal track. The hypothesis was tested by investigating 1) the extractability of phenolic compounds from the oats 2) the bioavailability of the phenolic compounds as measured from urine concentrations 3) the excretion of bile acids and cholesterol derivatives into the feces 4) the physiological, physical and chemical environment in the intestine as measured from the SmartPill® response 5) the self-reported perceived gut well-being and 6) the association between the pressure measured in the gut and the self-reported symptoms.

2. Materials and methods

2.1 Study subjects

Healthy participants (N=14), aged 26.8 ± 4.2, seven males and seven females, were recruited from the Turku area (Finland). The inclusion criteria were normal to overweight Body Mass Index (BMI) (18.5-30 kg/m2) and age between 18-64 years. The exclusion criteria were abdominal surgery, diseases affecting gastrointestinal tract and medication excluding oral contraceptives. All subjects gave written informed consent before enrolling in the study. The compatibility of study candidates was assured with an interview and questionnaires on health and diet. Candidates meeting the preliminary inclusion criteria received blood tests (blood count, thyroid function tests, transglutaminase antibodies and immunoglobulins for celiac disease), and were admitted if the tests were within the normal range. The study protocol was approved by the Ethics Committee of the Hospital District of Southwest Finland. The study was registered in ClinicalTrial.gov (identifier: NCT02764931).

2.2 Study design

A randomized, double-blind, postprandial cross-over study was applied. All participants attended the three study periods with a wash-out period of ≥2 weeks between each (Supplementary Figure 1). Each study period consisted of a run-in period of 48 hours (low-phenolic and low fiber diet, supplementary Table 1), a study day with three meals (breakfast, lunch and dinner with an oat bran concentrate) and a follow-up of 24h. Before the breakfast on study day, participants fasted for at least 10 hours. The order of the oat bran concentrates was randomized for each participant with a random number generator. A 24 h urine sample was collected before (control sample) and after (study sample) the study breakfast. In addition, subjects collected a fecal sample when the SmartPill® capsule exited. The subjects were asked to keep a gut symptom diary 5 days before and after the study day including the study day.

2.3. SmartPill® GI monitoring system

The SmartPill® GI monitoring system (Given Imaging LTD., Yoqneam, Israel) was used to measure gastrointestinal pH, temperature and pressure. The SmartPill® capsule (13 x 26 mm) was swallowed with the study breakfast and the subjects fasted for six hours after the meal in order for the capsule to proceed to the small intestine. The subjects were asked to avoid vigorous exercise as well as removal of the receiver from close proximity of the central body before the capsule exited. Of the 14 subjects who participated in the study, 4 chose not to ingest the capsule due to personal preference, and thus they participated in all legs of the study except the capsule ingestion. The study's fecal sample from these subjects was collected from the first feces defecated 24 hours after the study breakfast.

Measurement data was uploaded to a computer with the MotiliGI® software (Given Imaging LTD., Yoqneam, Israel). Mean pressure, contractions/min, median pH and transit times based on changes in pH and temperature for the different parts of the gastrointestinal tract were calculated. The temperature was used to follow the capsule ingestion and exiting. The pH was used to determine the timeframe of the parts of the gastrointestinal track and the pressure was used as an indicator of gut functions and gastrointestinal symptoms (Supplementary figure 2). The relationship between gastrointestinal symptoms and pressure was also studied. In detail, the pressure as area under curve value (AUC) of the time frame with perceived symptoms (1h) was compared to AUC pressure value of the nearest time frame (1h), when the participants did not perceive symptoms. Gastric, small bowel and colon phases were compared (total of 27 comparisons).

2.4. Study diets

The study meal consisted of oat bran concentrate (Fazer Mills, Finland) treated with a commercial food-grade cell wall degrading enzyme preparation (Depol 740L) at 1 or 50 nkat β-glucanase/g dm. A control sample was prepared in the same way without added enzymes.

The treatment was performed for 2 h at 50 °C and then heated to 90 °C to inactivate the enzymes. The concentrates were freeze-dried, ground, and packed in portions consisting of 9.33 g dietary fiber and stored at -20 °C until analyzed. The MW of β-glucan in the control oat bran concentrate was >1000 kDa (High MW), in 1 nkat/g dm treated 524 kDa (Medium MW) and in 50 nkat/g dm treated 82 kDa (Low MW). The oat bran concentrates were characterized as described earlier (Rosa-Sibakov, Mäkelä, Aura, Sontag-Strohm, & Nordlund, 2020) and the main characteristics are presented in Table 1.

On the morning of the test day, the study subjects ate a study breakfast, which included the oat bran concentrate mixed with 3 dl of lactose-free, fat-free yoghurt. Ten out of the 14 subjects ingested the SmartPill® capsule with the meal. Standardized lunch (macaroni casserole) and a portion of the study meal were consumed six hours later and dinner with an additional portion of the study meal 10-12 h after the breakfast. Thus, volunteers consumed a total amount of 28 g of oat fiber from the study meals during each study day.

The diet was standardized for three to four days. The standardized diet started for two days (-48h) before the study breakfast (0h), the study day and a further fourth day until the capsule exit. The standardized diet consisted of food with a low phenolic content (Supplementary Table 1). The participants were provided with food together with a list of additional permitted and non-permitted food choices. The low-phenolic study diet supplemented with the study meals was continued until the capsule exited and/or the fecal sample was collected.

2.5 Gut symptom diaries

Gut symptoms diaries were kept 5 days before and after the study day. The diary included the type of symptom (upper abdominal pain, lower abdominal pain, cramping, bloating, flatulence, diarrhea, constipation or other type of symptom), the severity of the symptom in a scale of 1 to 3 (one meaning mild, two being moderate and three being intense), and the

duration of the symptom. The diary was divided into time slots of three hours, except the night time, which was marked as a six-hour slot (from midnight until 6 am). The study day was marked in one hour slots except during the night time, which was one six-hour slot.

2.6 Chemicals and reagents

The commercial cell-wall degrading preparation (Depol 740L) was purchased from Biocatalysts Ltd, Cardiff, United Kingdom. Cholesterol, stigmasterol, cholic acid and lithocholic acid-2,2,3,4,4-d_5 were purchased from Sigma Aldrich, USA, desmosterol, deoxycholic acid, and chenodeoxy cholic acid from Steraloids Inc, USA. and cholesterol-d_7 from Avanti Polar Lipids. 7-hydroxycholesterol was purchased from Instruchemie, Netherlands. Anthranilic acid, 2,4-dihydroxybenzoic acid, 2,5-dihydrozybenzoic acid, vanillic acid, isovanillic acid, homovanillic acid, vanillin, *p*-coumaric acid, syringic acid, syringaldehyde, ferulic acid, 2-OH-hippuric acid, avenanthramide 2p, avenanthramide 2c and avenanthramide 2f were purchased from Sigma-Aldrich. The*Helix Pomatia* enzyme mix was purchased from Roche Diagnostics, Mannheim, Germany via Sigma-Aldrich and the SPE cartridges (OASIS® HLB 1 cc) used were purchased from Waters Corp., Milford, MA, USA. All the solvents and buffers were LC-MS grade.

2.7 Urine sample preparation

Urine samples were stored at -80 °C until analyzed. The urine samples (500 µL) were acidified and hydrolyzed by using a β-glucuronidase and arylsulfatase enzyme mix (*Helix Pomatia)* containing 15 µL of internal standard for 16 h at 37 °C (Vetrani et al., 2014). SPE extraction was conducted by following the instructions of OASIS® HLB 1 cc Cartridges. Briefly: The cartridge was conditioned with 1 mL methanol and equilibrated with 1 mL water. Sample solution was loaded and washed with 1 mL water and the analytes were eluted with 1 mL methanol.

2.8 Extraction of avenanthramides

Oat bran (2.5g) was extracted twice with 17.5 mL of 80 % methanol for 1 h using a Stuart roller mixer (Cole-Parmer, Staffordshire, UK). The samples were centrifuged for 10 min, 600 g at RT, and the supernatants were combined and dried under reduced pressure at a temperature not exceeding 40 °C. The extracts were redissolved in 1.5 mL of methanol and filtered through a 0.2 µm PTFE filter and analyzed by LC-MS/MS (Bryngelsson, Dimberg, & Kamal-Eldin, 2002).

2.9 Extraction of free phenolic acids

Oat bran (0.1 g) was dissolved in 3 mL of 0.2 M HCL and extracted twice with 6 mL of ethyl acetate for 1 h using a Stuart roller mixer. Samples were centrifuged at RT for 10 min at 600 g. Supernatants were combined and left to stand over anhydrous $NaSO_4$ followed by filtering. The samples were dried under reduced pressure at a temperature not exceeding 40 °C. The extracts were redissolved in 1 mL of methanol and analyzed by LC-MS/MS (Multari et al., 2018).

2.10 LC-MS/MS analysis of phenolic compounds

The LC-MS/MS method was modified from the method of Schar (2018). The UHPLC-ESI-MS/MS system consisted of Acquity UPLC (Waters) coupled with Xevo TQ-S electrospray ionization mass spectrometer (Waters) operated by Masslynx software (V. 4.1, Waters Inc, USA). Compound separation was performed using an Aquity UPLC HSS T3 1.8µm column (2.1 x 100mm) attached to a Van guard pre-column of the same material and pore size. The column oven temperature was set at 45°C and the flow rate was 0.65 mL min^{-1} and the sample injection volume was 2 µL. The mobile phase A was water and B was acetonitrile, both containing 0.1 % formic acid. The gradient of mobile phase B was following: 1% at 0

min, 1% at 1 min, 30 % at 10 min, 95 % at 12 min, 95% at 13 min, 1% at 13.10 min, 1% at 16 min.

A scheduled multiple reaction monitoring (sMRM) method was created based on a syringe infusion of 16 standards by using Masslynx Intellistart software (V. 4.1, Waters Inc, USA) (Table 2) to determine MRM transitions, collision energies and MRM modes (positive or negative ionization). The most intense MRM transition was chosen for the quantification of each analyte. Quantification was based on calibration curves of analytical standards. A blank quality control and a second blank were run after every 16 samples. Exact masses were determined in a UHPLC-QTOF system (Bruker Daltonik GmbH, Bremen, Germany) using the same LC conditions. The ESI source was operated in a positive mode with the following settings: ESI capillary voltage: 4500 V; ion source temperature: 300 °C, dry gas (nitrogen) 12 L min^{-1}.

The concentrations of phenolic compounds in the control samples were subtracted from the concentrations of phenolic compounds in the study samples in order to focus only on the oat-derived compounds. Concentrations were proportioned by the concentration of urine creatinine.

2.11 Fecal sample preparation

Fecal samples were freeze-dried and homogenized with a mortar and pestle. 5 mg of dry fecal powder was extracted and sonicated with 1000 µL of methanol containing internal standards. The fecal sample was centrifuged for 10 min, 24 400 g and the supernatant was dried under a nitrogen flow (John et al., 2014). Samples were redissolved in 200 µL of methanol for LC-QTOF-MS analysis.

2.12 LC-QTOF analysis for fecal bile acids and sterol derivatives

The LC-QTOF system consisted of Elute UHPLC paired with Impact II QTOF system (Bruker Daltonik GmbH, Bremen, Germany). Analytes were separated by an Accucore™ Polar Premium HPLC column (2.6 m, 150 mm × 2.1 mm i.d.) attached to an Accucore™ Polar Premium defender guard column (Thermo Fischer Scientific Inc., Waltham, MA, USA). The column oven temperature was set at 20 °C and a flow rate of 300 µL min^{-1} and the sample injection volume was 2 µL. The mobile phase A was water and B was methanol, both containing 0,2 % formic acid and 10 mM ammonium acetate. The gradient of the mobile phase B was following: 0 min 60 %, 2 min 60 %, 18 min 95 %, 22 min 100 %, 30 min 100 %, 33 min 60 % and 43 min 60 %. The ESI source was operated in positive mode with the following settings: ESI capillary voltage: 4500 V; ion source temperature: 250 °C, dry gas (nitrogen) 10 l min^{-1}. The system was controlled by Compass HyStar software (Bruker Daltonik GmbH, Bremen, Germany)

Quantification was based on the calibration curves of analytical standards. Standard solutions of cholesterol and bile acids were prepared in methanol, 7-hydroxycholesterol in chloroform and stigmasterol, desmosterol, and deuterated standards were dissolved in a Folch solution. Internal standards were used at each point of the calibration curve and for all the samples. Bile acids were corrected by lithocholic acid-2,2,3,4,4-d_5 while cholesterol-d_7 was used for correction of cholesterol derivatives. Blank and quality controls in three concentrations (0,8 ; 4 and 12 µg/mL) were run daily.

2.13 Data analysis

Statistical analyses of the gastrointestinal transit time, pH, contractions, mean pressure, AUC values of pressure-symptom comparisons, gut symptom diary, phenolic urine metabolites and fecal bile acids and cholesterol derivatives data were carried out using IBM SPSS Statistics

25 software, USA. Non-parametric tests of related samples (Wilcoxon, Friedman) were used to determine the statistical differences among the study groups.

3. Results and discussion

3.1 SmartPill® data

The median pH recorded by the SmartPill was similar between the study meals (High, Medium, Low MW) in all parts of the GI track (Figure 1A). There were no statistical differences between the meals in the transit times in the whole gut or the different parts of the gastrointestinal track (Figure 1B). Previously, in the study of Timm (Timm et al., 2011), the gut transit times of ten healthy subjects were compared after a 3-day consumption of high-fiber cereal or low-fiber cereal, and the colonial and whole gut transit times were significantly shorter after the consumption of high-fiber cereal. In the present study, unlike in the study of Timms et al, the fiber content of the study meals was identical. In addition, a shorter consumption period may be the reason for that no differences in transit times were detected in our study.

More contractions/min were measured in the duodenum after the High MW β-glucan meal compared to Medium MW β-glucan meal ($p<0.013$) and Low MW β-glucan meal ($p<0.022$) (Figure 1C). Moreover, the mean pressure in the duodenum was higher after the High MW β-glucan meal compared to Medium MW β-glucan meal ($p<0.041$) and Low MW β-glucan meal ($p <0.022$) (Figure 1D). Similar trends in the mean pressure and the frequency of contractions was observed in the small bowel, but the difference was not significant ($p =0.154$, Figure 1C and 1D). The mean pressures and the frequency of contractions in other parts of the GI tract were similar between the study meals. *In vitro,* the oat bran with High MW β-glucan was reported to show significantly higher viscosity of intestine digesta than measured in the Medium or Low MW β-glucan brans in the upper gut model (Rosa-Sibakov

et al., 2020, unpublished results). The higher pressure measured in the duodenum of the subjects was likely related to the increased viscosity that High MW β-glucan possibly caused in the small intestine.

The intestinal pressure could be an objective measurement of often subjective gastrointestinal sensations. However, the pressures calculated as areas under the curve did not differ during the time that the gut symptoms were perceived compared to the time without perceived symptoms (Figure 1E). We have also previously (Nuora et al., 2018) used the SmartPill® technology to correlate the gastrointestinal pressure with perceived gut symptoms. However, significant correlation between perceived gut symptoms and pressure has been scarcely substantiated. Earlier Cassilly et al (2008) and Nuora et al (2018) hypothesized that one explanation for varying results in pressures in relation to transit times could be delayed gastric emptying, which they recorded in 20-27 % of the cases. However, in the current study, only 3 out of 30 gastric emptying times were delayed, so the pressure recorded was the result from the study breakfast in 90 % of the cases. Moreover, since the subjects also had the study meal served with their lunch and dinner, even in the delayed cases the capsule moved to the small intestine with the β-glucan.

No difficulties were experienced with the ingestion of the capsule and no major data gaps were observed in this study. Previously our group detected significant patches of absent data in the response curve (Nuora et al., 2018), but in this study, only some minor few minute gaps were observed. This improvement may have resulted from the instructions to wear the receiver significantly closer to the body compared with the official instructions of the device or the different capsule batch.

3.2 Gut symptom diaries

In total 103 symptoms were reported in the study day after consumption of Low MW β-glucan meal, 118 after consumption of Medium MW β-glucan meal and 87 after consumption of High MW β-glucan meal and thus the perceived gut well-being was similar between the meals ($p=0.368$, Figure 1F). This does not support the hypothesis that bioprocessed oat bran induces more gut symptoms due to faster fermentation. On the contrary, the result encourages the future development of liquid and spoonable oat products with bioprocessed bran.

The background diet low in phenolic compounds and fiber caused gastrointestinal discomfort to the participants: less symptoms were reported 5 days before the study day (day -5) compared to the number of symptoms reported on the second day of the run-in period (day -1), on average in all the study periods ($p < 0.008$). The most commonly reported symptoms were flatulence and constipation, representing 23 % and 22 % of all the symptoms reported, respectively. Both of these symptoms could be associated with a diet low in fiber.

The types of perceived gut symptoms varied significantly by gender. The most commonly reported symptoms during the study day among female participants were flatulence, which was reported in total during the three study days 62 times, and bloating (43 times); while male participants reported flatulence in total 7 times and bloating once ($p < 0.001$; $p< 0.009$, respectively) during the three study days. In contrast, the male participants most frequently reported constipation (63 times during the three study days), which tended to differ from that of females (5 times, $p< 0.057$, Figure 1G and 1H).

Gender-related differences in self-reported gastrointestinal symptoms have been previously reported in irritable bowel syndrome studies (Mayer, Naliboff, Lee, Munakata, & Chang, 1999), but they have mainly focused on experienced pain. Moreover, the previous studies indicate that female subjects are more eager to report gastrointestinal symptoms (Mayer et al., 1999), while in our study the genders differed in the quality of symptoms, not in the quantity.

However, since the number of participants was rather small (n=14), further studies in gender-related differences in perceived symptoms are needed in the future.

3.3 Validation of LC-MS/MS and LC-QTOF methods

The validation of the methods was conducted by following the guidelines of The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH).

The chosen validation parameters for the LC-MS/MS method for the analysis of phenolic compounds in the oat bran concentrates and urine indicated good precision, repeatability and linearity. Intra-day and inter-day variabilities were analyzed by using spiked samples, being 2.6 % and 12.1 %, respectively. Linearity (linear correlation coefficient) ranged between 0.942 to 0.999 (Table 2). The standards were relatively stable, intra-day variation being 2.7 % and inter-day being 6.4 %. The limit of detection was determined from the calibration curves of spiked samples for each compound at the concentration of peak signal to noise being 3 (Table 2). The limit of the quantitation was at the low end of the linearity range. Recovery was established by adding an internal standard to the sample matrix before and after extraction (n=3) and the recovery rate was determined as recovery (%) = $\text{concentration}_{\text{before}}$/ $\text{concentration}_{\text{after}} \times 100$, being 113 %.

The validation parameters for the LC-QTOF method for the analysis of fecal bile acids were the following. Intra-day and inter-day variabilities were analyzed by using the analytical standards, being 1.8 % and 2.3 %, respectively. Linearity (linear correlation coefficient) ranged between 0.998 to 0.999 (Table 3). The limit of quantification was determined as the lowest standard in the linear calibration curve. Recovery rate was 80 %.

3.4 Identification of phenolic compounds in oat bran concentrates

372 The most abundant phenolic compounds extracted from oat bran concentrates were
373 avenanthramides 2p, 2c and 2f and ferulic acid (Table 4). Minor concentrations of *p*-
374 coumaric acid, syringic acid, syringaldehyde, 2,4-dihydroxybenzoic acid and vanillin were
375 detected from all concentrates. Overall, the phenolic composition of the oat bran concentrates
376 used in this study was in accordance with previous reports (Antonini et al., 2016; Bei, Liu,
377 Wang, Chen, & Wu, 2017; Multari et al., 2018). The total concentration of phenolic
378 compounds extracted was two-fold higher in Low MW β-glucan oat bran concentrate
379 compared to High and Medium MW β-glucan oat bran concentrates. Concentration of each
380 detected phenolic compound was higher in Low MW oat bran concentrate compared to
381 Medium and High MW β-glucan oat bran concentrates. The most remarkable difference
382 between the oat bran concentrates was the concentration of free ferulic acid, which was 2.08
383 ± 0.19; 0.38 ± 0.04; 0.08 ± 0.02 µg/g in Low, Medium and High MW β-glucan oat bran
384 concentrates, respectively. This was expected, since the enzyme used is known to have ferulic
385 acid esterase activity (Anson et al., 2009). (Table 4).

386 The higher extractable concentration of phenolic compounds in the Low MW β-glucan bran
387 concentrate compared to High and Medium MW β-glucan bran concentrates demonstrated
388 the release of bound phenolic compounds caused by the enzymatic treatment. However, a
389 moderate reduction of β-glucan MW from >1000 kDa to 524 kDa (Medium MW) did not
390 remarkably increase the concentration of free phenolic compounds. In oats, 75 % of phenolic
391 compounds are present in a bound form, primarily through ether linkages to lignin or through
392 ester bonds to the cell wall macromolecules, such as proteins and polysaccharides, including
393 β-glucan (Antonini et al., 2016; R. H. Liu, 2007). Previously, a release of bound phenolic
394 acids has been observed in bioprocessing of rye with cell wall degrading enzymes (Lappi et
395 al., 2013) and in the treatment of wheat with enzymes and yeast combined (Anson et al.,
396 2011). Comparable to bioprocessed rye and wheat, ferulic acid was found to be the dominant

free phenolic compound in the Low MW oat bran concentrate. The release of ferulic acid was 26-fold in oats, 12-fold in rye and 4-fold in wheat. However, since studies of rye and wheat used bread containing bioprocessed bran and the present study used plain bran, the results are not directly comparable. Ferulic acid is known to be the major phenolic acid in whole grains (Maillard & Berset, 1995; Sosulski, Krygier, & Hogge, 1982), which mainly exists in bound forms, for example covalently bound to arabinoxylan chains (Nara et al., 2008). Indeed, it was observed that 49 % of the arabinoxylan in the Low MW oat bran concentrate was hydrolyzed to oligosaccharide form, which correlates with the release of phenolic acids. (Table 1).

3.5 Identification of phenolic compounds in urine

The phenolic compounds resulting from the 3 oat brans were converted into an aglycon form due to enzymatic hydrolysis in the sample preparation and quantities of the phenolic compounds in the urine on the control day were subtracted from the quantities of the phenolic compounds on the study day. Ferulic acid was the most abundant oat-derived compound in urine after the ingestion of both Low and Medium MW β-glucan meals, while 2-hydroxyhippuric acid, syringic acid and vanillic acid were the most abundant oat derived compounds after the ingestion of High MW β-glucan meal (Table 4). Furthermore, an earlier study reported ferulic, hydroxyhippuric and vanillic acids as the dominant native oat-derived compounds in urine (Schar et al., 2018). A higher amount of ferulic acid was detected from urine after consumption of the Low MW β-glucan meal compared to the Medium and High MW β-glucan meals ($p < 0.001$; $p < 0.001$), (Table 4). The urine concentrations of other phenolic acids analyzed did not differ between the meals. However, the combined concentration of the 3 avenanthramides and 12 phenolic acids in urine was higher after consumption of Low MW β-glucan meal compared to Medium and High MW β-glucan meals ($p < 0.01$ and $p < 0.01$). Avenanthramide 2p and 2f were detected in very low concentrations

while avenanthramide 2c was not detected at all. The concentration of avenanthramide 2p was higher in the urine samples after the consumption of the Medium MW β-glucan meal compared to the Low MW β-glucan meal ($p < 0.042$). The reason for the very low concentrations of avenanthramides in urine was likely because they had been broken down to phenolic acids, as suggested previously (Schar et al., 2018; Y. X. Wang, Liu, Chen, & Zhao, 2013). In addition to breaking down into phenolic acids, a minor part of avenanthramides conjugates rapidly to sulfates, glucuronides or methylated forms mainly in the intestine, kidney and liver (Chen et al., 2007). The absorption of both avenanthramides and phenolic acids into plasma and tissues as well as further excretion into urine is known to be rapid. They have been detected in plasma even after 15 minutes of ingestion (Anson et al., 2011; Chen et al., 2007) and mainly excreted into urine between 0-2 and 4-8h after ingestion (Schar et al., 2018). This noted, the 24 h urine sample collected after the first study meal and including a total of three oat meals portrays well the excretion of oat metabolites. Our observation that a few major compounds were detected in the urine, namely vanillic, homovanillic and 2-hydroxyhippuric acids, and a few minor compounds, anthranilic and isovanillic acids, were present in the urine, but absent from the ingested oat bran concentrates supports an earlier finding that they are intestinal metabolites originating from avenanthramide (Schar et al., 2018). In addition, our results confirmed the earlier finding (Schar et al., 2018) of hippuric acid being the most common phenolic compound in urine, but its quantity was not increased in urine after ingestion of the oat-based study meals, indicating that it is not an oat metabolite (data not shown).

3.6 Identification of fecal bile acids and cholesterol derivatives

The most abundant bile acid and cholesterol derivative compound analyzed was cholesterol, followed by chenodeoxycholic acid (CDCA) and deoxycholic acid (DCA). Cholic acid (CA) was detected in minor concentrations (Table 5). In healthy adults, 99 % of cholesterol is

447 eliminated by the fecal route, of which approximately two thirds is excreted as cholesterol
448 and one third as bile acids (Hofmann, 1999). Primary bile acids produced in the liver from
449 cholesterol are delivered to the lumen of the small intestine where they facilitate the
450 absorption of cholesterol, dietary lipids and fat-soluble vitamins. Part of them undergoes
451 transformation to secondary bile acids by colon anaerobic bacteria and 95 % of all the bile
452 acids are transported back to the liver to be re-excreted (Lu, Feskens, Boer, & Muller, 2010).
453 In humans, CDCA and CA are the main primary bile acids, while DCA is a secondary bile
454 acid derived from CA (Hofmann, 1999; Russell, 2003). DCA is known to circulate with
455 primary bile acids and to be the dominant biliary acid in some adults (Hofmann, 1999), which
456 may explain its higher concentration over CA in our samples. The excretion of cholesterol
457 and cholesterol derivatives did not differ between the meals, indicating that their excretion
458 may be more dependent on the consumption of animal products (Table 5). However, as we
459 had already excluded all whole grains and vegetables from the background diet, also
460 excluding all animal-based products was not plausible. It is also worth noting that the genetic
461 differences, e.g. ApoE polymorphism largely influence the proportion of ingested and
462 excreted cholesterol originating from the diet (Marais, 2019).

463 The concentration of DCA was lower after consumption of the Low MW β-glucan meal
464 compared to the High ($p<0.02$) and Medium MW β-glucan meals ($p < 0.03$). Moreover, the
465 concentration of CDCA was lower after consumption of the Low MW β-glucan β-glucan
466 meal compared to the High MW β-glucan meal ($p<0.02$) and tended to differ compared to the
467 Medium MW β-glucan meal ($p< 0.06$). These results indicate that the bile acid binding
468 capability of β-glucan is related to its MW. Previous studies have proposed that the
469 cholesterol-lowering effect of oat β-glucan is linked to the prevention of bile acid
470 reabsorption from the intestines (Ellegard & Andersson, 2007; Gunness et al., 2016). The
471 effect has been suggested to result from the formation of viscous layer between the bile acid

micelles and the absorptive cells of the intestine by the β-glucan fiber, as well as the capture of the bile acids between the β-glucan chains or molecular level association of bile acids and β-glucan fiber (Gunness & Gidley, 2010). The influence of the physicochemical properties of β-glucan on its bile acid binding capacity have also been postulated (Q. Wang & Ellis, 2014). We observed that the excretion of DCA and CDCA was significantly lower after consumption of the Low MW β-glucan meal compared to the High MW β-glucan meal, but a moderate reduction of the β-glucan MW to 524 kDa (Medium MW) did not statistically affect the excretion of bile acids. Our results suggest that to maintain the cholesterol lowering health effect via bile acid binding, β-glucan could be moderately processed. This result is in accordance with an earlier clinical study measuring LDL cholesterol after consumption of High (2210 kDa), Medium (530 kDa) or Low (210 kDa) MW β-glucans: the LDL cholesterol of the participants was lowered more after consumption of High and Medium MW β-glucans compared to Low MW β-glucan (Wolever et al., 2010). A significant positive correlation between the MW of β-glucan in oat bran and the bile acid binding capacity was also detected *in vitro* (Rosa-Sibakov et al., 2020, unpublished results). Another recent *in vitro* study suggested that the essential factor for maintaining the bile acid binding capacity of β-glucan is viscosity, which is typically conserved after moderate processing (Marasca et al., 2020). However, since the measurement of the bile acid concentration in the earlier *in vitro* studies may have overlooked the viscosity effect, i.e. the enclosure of bile acids in the β-glucan gel (Kim & White, 2010; Sayar et al., 2011), and the present study is the first clinical trial studying the relationship of oat β-glucan MW to fecal bile acid excretion, studies are still needed in the future to obtain further evidence for the hypothesis.

4. Conclusion

The results of this study supported the hypothesis that alteration of the oat β-glucan MW with enzymatic treatment affects the nutritional properties of the oat bran and the functional

properties of the β-glucan in the human gastrointestinal tract. The consumption of a High MW β-glucan meal resulted in the highest excretion of fecal bile acids, the highest pressure in the duodenum, and the lowest excretion of phenolic compounds in the urine. Consumption of a Low MW β-glucan meal resulted in the lowest excretion of fecal bile acids and the lowest pressure in the duodenum, but the highest excretion of phenolic compounds, especially ferulic acid, in the urine. The behavior of the Medium MW β-glucan in the GI track was similar to the High MW β-glucan in that it resulted in high excretion of fecal bile acids and low excretion of phenolic compounds to urine, but the mean pressure in the duodenum was closer to the Low MW than to the High MW meal. These results are directly applicable in the evaluation of the health effects of moist products like yoghurts or drinks which require bioprocessing and depolymerisation of β-glucan. The perceived gut well-being after consumption of each meal did not differ between the meals, but varied between the genders, which should be further investigated.

Ethical statement

The study protocol was approved by the Ethics Committee of the Hospital District of Southwest Finland. The study was registered in ClinicalTrial.gov (identifier: NCT02764931).

Conflict of interest

The authors declare that they have no conflicting interests.

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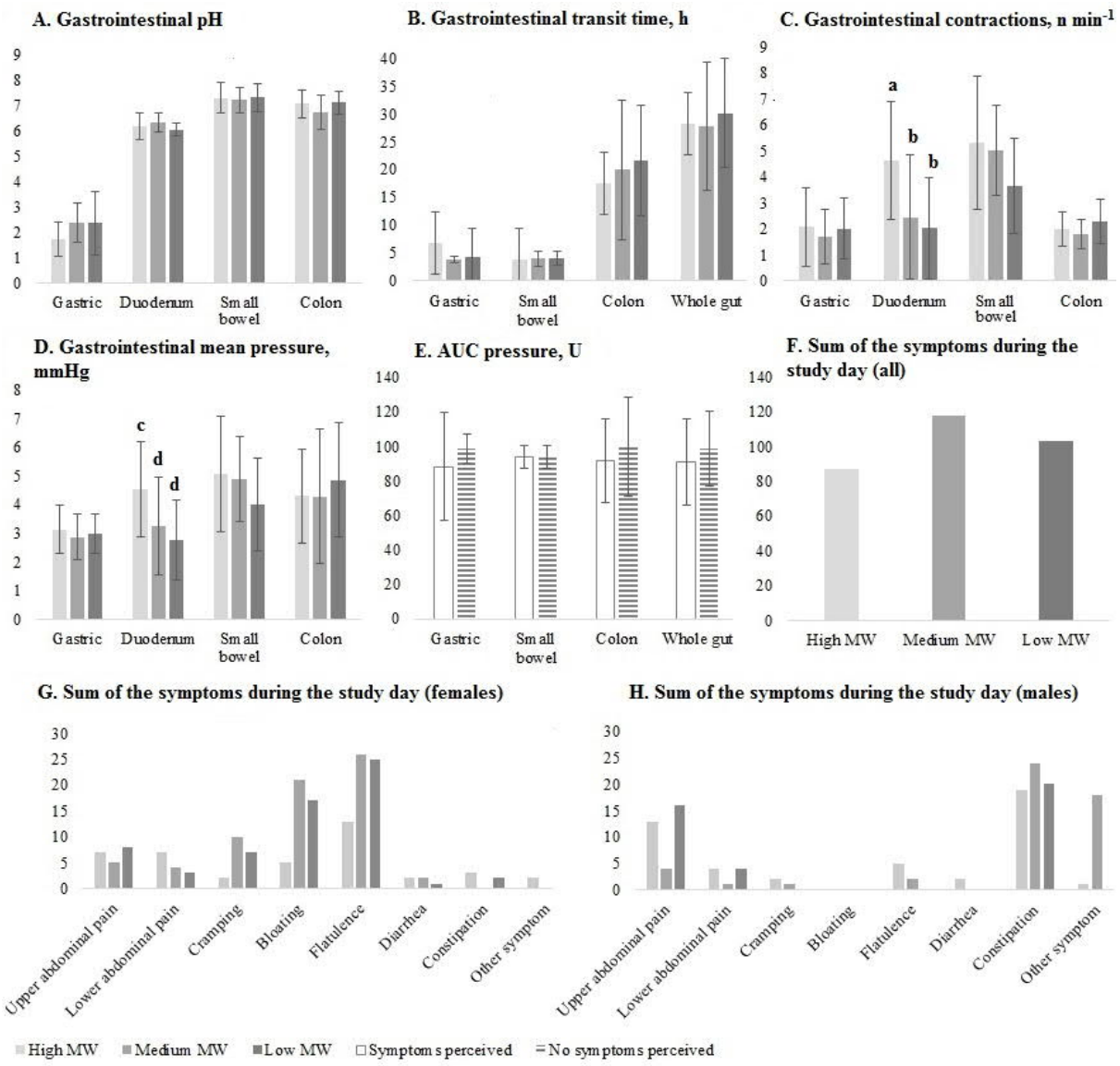

Figure Legend 1. A. pH B. Transit times/h C. Contractions/min D. Mean pressures/mmHg E. The averages of the AUC pressures of the time symptoms perceived compared to time of no symptoms perceived. F. Sum of the study day symptoms. G. Sum of the study day symptoms reported by females. H. Sum of the study day symptoms reported by males. [a] differs significantly from [b]; [c] differs significantly from [d].. AUC = area under curve.

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684

Table 1. β-glucan (MW and % dry mass), dietary fiber and arabinoxylan content of study meals.

	β-glucan MW (kDa)	β-glucan content (% dm)	Total DF (%)	Insoluble DF (%)	Soluble DF (%)	Total AX *g 100 g^{-1} dm^{-1}*	AX oligosaccharide forms *g 100g^{-1} dm^{-1}*
High MW	> 1000	19.9 ± 0.1	41.9	20.4	21.5	12.4	0.0
Medium MW	524	19.6 ± 0.1	36.6	14.4	22.2	12.0	0.0
Low MW	82	19.1 ± 0.2	33.9	7.1	26.8	11.5	5.6

DF = dietary fiber, MW = molecular weight, dm = dry mass, AX= arabinoxylans

Table 2. MRM transitions and method parameters of oat-derived metabolites.

Metabolite	**MW**	***m/z***	**Mass error (ppm)**	**sMRM transition *m/z***	**Cone Voltage (V)**	**Collision Energy (eV)**	**RT**	**LOD µg mL^{-1}**	**LOQ µg mL^{-1}**	**R**
Anthranilic acid	137.1	138.0548	1.30	138 > 65	30	22	4.83	0.001	0.003	0.996
2,4-dihydroxybenzoic acid	154.1	155.0336	2.00	155 > 53	18	26	4.35	0.001	0.003	0.998
2,5-dihydrozybenzoic acid	154.1	155.0338	0.71	155 > 81	20	20	3.71	0.002	0.004	0.996
Vanillic acid	168.1	169.0493	1.54	169 > 65	16	20	4.64	0.001	0.003	0.989
Isovanillic acid	168.1	169.0493	1.54	169 > 65	20	20	4.98	0.001	0.003	0.996
Homovanillic acid	182.2	183.0649	1.69	-181 > -122	24	14	5.08	0.003	0.04	0.990
Vanillin	152.2	153.0545	0.95	153 > 93	18	12	5.62	0.001	0.003	0.998
p-coumaric acid	164.2	165.0546	0.27	165 > 65	18	30	5.83	0.001	0.01	0.998
Syringic acid	198.2	199.0599	1.13	199 > 140	10	12	5.15	0.001	0.003	0.996
Syringaldehyde	182.2	183.0652	0.05	183 > 123	2	12	6.19	0.001	0.004	0.999
Ferulic acid	194.2	195.0651	0.56	195 > 89	22	26	6.52	0.001	0.001	0.997
2-OH-hippuric acid	195.2	196.0605	0.21	196 > 121	14	12	5.71	0.001	0.001	0.942
Avenanthramide 2p	299.3	n.a.	n.a.	300 > 147	4	16	9.03	0.001	0.003	0.996
Avenanthramide 2c	315.3	316.0828	3.52	316 > 163	4	10	8.10	0.001	0.001	0.995
Avenanthramide 2f	329.3	330.099	5.33	330 > 177	14	12	9.49	0.001	0.004	0.996

MW = molecular weight; sMRM = scheduled multiple reaction monitoring; RT = retention time; LOD = limit of detection; LOQ = limit of quantification; R = correlation coefficient; n.a. not analyzed

Table 3. Quantified bile acids and cholesterol derivatives.

Compound	**MW**	**M±X$^+$**	**M±X$^+$ (*m/z*)**	**Mass error (ppm)**	**RT**	**LOQ ug mL^{-1}**	**R**
Bile acids							
Deoxycholic acid (DCA)	392.2916	$[M + NH_4]^+$	410.3278	3.14	12.7	0.5	0.998
Chenodeoxy cholic acid (CDCA)	392.2916	$[M – 2\ H2O + H]^+$	357.2777	3.16	12.8	0.5	0.999
Cholic acid (CA)	408.2865	$[M + NH_4]^+$	426.3217	0.64	9.7	0.2	0.999
Cholesterol derivatives							
Cholesterol	386.3537	$[M – H2O + H]^+$	369.3522	1.62	25.0	0.5	0.998
7-hydroxycholesterol	402.3486	$[M – H2O + H]^+$	385.3469	1.00	19.7	0.1	0.998
Desmosterol	384.3381	$[M – H2O + H]^+$	367.3350	2.59	23.9	0.2	0.999
Stigmasterol	412.3693	$[M – H2O + H]^+$	395.3677	1.14	25.4	0.4	0.999

MW = molecular weight; RT = retention time; LOQ = limit of quantification; R = correlation coefficient

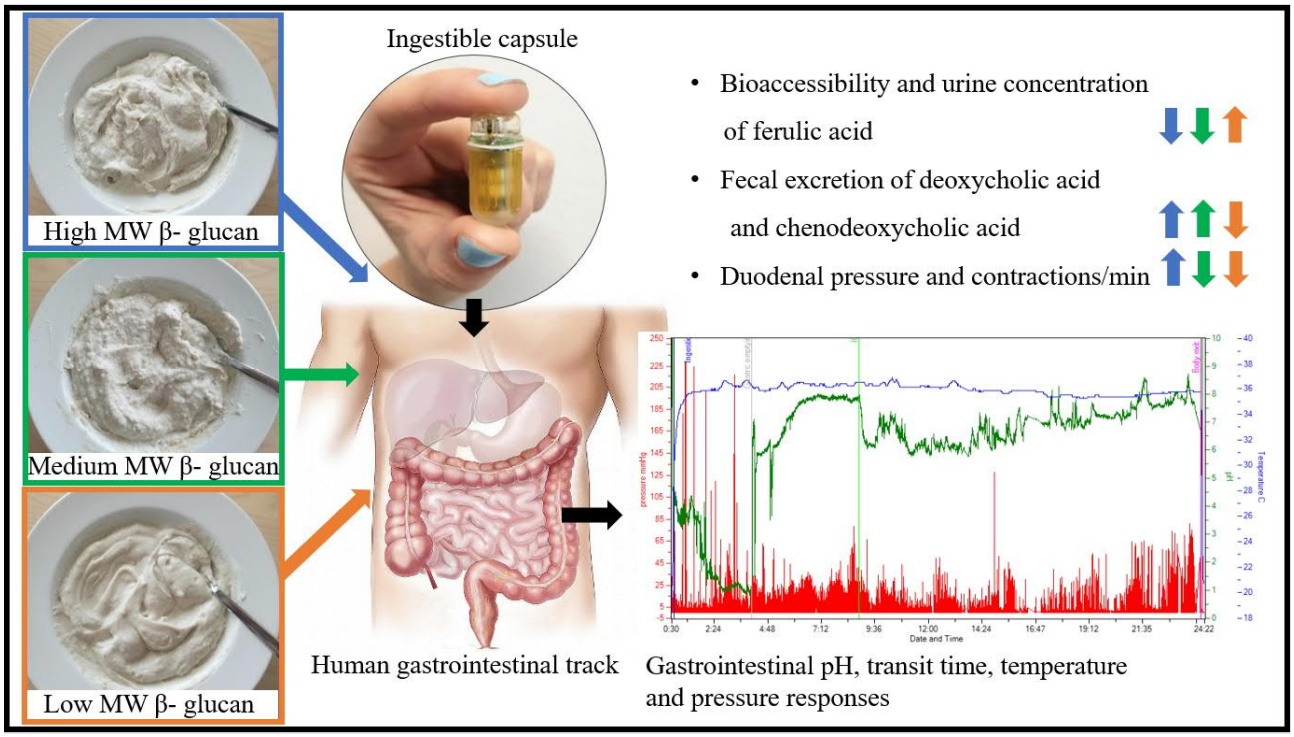
Table 4. Free phenolic compounds in oat bran concentrates (averages of 3 parallel samples) and their urine excretion (n=14). [a] differs significantly from [b]; [c] differs significantly from [d].

Compound	Oat bran concentrates μg g^{-1}			Urine excretion ng mL^{-1} creatinine^{-1}		
	Low MW	**Medium MW**	**High MW**	**Low MW**	**Medium MW**	**High MW**
Avenanthramide 2f	0.71 ± 0	0.48 ± 0	0.48 ± 0.03	0.06 ± 0.03	0.10 ± 0.05	0.07 ± 0.04
Avenanthramide 2c	0.75 ± 0.001	0.48 ± 0	0.48 ± 0.03	0	0	0
Avenanthramide 2p	0.78 ± 0	0.54 ± 0	0.54 ± 0.03	0.20 ± 0.11[a]	0.33 ± 0.11[b]	0.23 ± 0.20[ab]
Ferulic acid	2.08 ± 0.19	0.38 ± 0.04	0.08 ± 0.02	35.47 ± 15.64[c]	7.38 ± 4.11[d]	2.37 ± 2.08[d]
p-Coumaric acid	0.15 ± 0.05	0.05 ± 0.01	0.03 ± 0	0.03 ± 0.04	0.06 ± 0.15	0.02 ± 0.04
Syringic acid	0.08 ± 0.03	0.04 ± 0.01	0.04 ± 0.006	3.87 ± 3.96	4.34 ± 2.78	3.20 ± 2.80
Syringaldehyde	0.02 ± 0.006	0.02 ± 0	0.02 ± 0	0.006 ± 0.01	0.02 ± 0.03	0.03 ± 0.04
2,5-dihydroxybenzoic acid	0.02 ± 0	0	0	0.36 ± 0.67	0.19 ± 0.26	0.92 ± 2.80
2,4-dihydroxybenzoic acid	0.05 ± 0.007	0.04 ± 0	0.04 ± 0	1.04 ± 2.61	1.35 ± 2.58	1.82 ± 3.21
Anthranilic acid	0	0	0	0.05 ± 0.12	0.14 ± 0.23	0.19 ± 0.32
2-hydroxyhippuric acid	0	0	0	6.12 ± 5.80	6.65 ± 5.18	5.44 ± 6.93
Vanillin	0.15 ± 0.1	0.03 ± 0.007	0.04 ± 0	1.20 ± 6.01	0.03 ± 3.84	0.11 ± 11.1
Homovanillic acid	0	0	0	1.62 ± 3.57	2.63 ± 4.58	1.87 ± 4.68
Isovanillic acid	0	0	0	0.14 ± 0.15	0.10 ± 0.11	0.07 ± 0.10
Vanillic acid	0	0	0	5.55 ± 6.01	2.75 ± 3.84	3.86 ± 11.01
Total concentration	4.78 ± 0.39	2.05 ± 0.07	1.75 ± 0.11	55.7[e] ± 28.9	26.1[f] ± 16.4	20.2[f] ± 16.4

Table 5. Fecal excretion of bile acids and cholesterol derivatives µg mg^{-1} of dry weight (n=14). [a] differs significantly from [b]; [c] differs significantly from [d].

	Low MW		Medium MW		High MW	
Compound	**Average**	**Range**	**Average**	**Range**	**Average**	**Range**
Chenodeoxycholic acid (CDCA)	0.5[a]	0.04-1.27	0.69[ab]	0.24-1.20	0.78[b]	0.33-2.24
Deoxycholic acid (DCA)	1.49[c]	0.22-3.28	1.95[d]	0.88-3.58	2.3[d]	1.03-6.01
Cholic acid (CA)	0.17	0.0-1.69	0.17	0.0-2.02	0.08	0.0-0.35
Cholesterol	5.96	1.22-19.06	7.54	1.45-18.41	6.33	1.31-17.17
7-hydroxycholesterol	0.44	0.07-0.66	0.51	0.12-1.04	0.48	0.08-1.31
Stigmasterol	0.17	0.02-0.45	0.25	0.03-1.01	0.2	0.04-0.91
Desmosterol	0.014	0.0-0.08	0.025	0.0-0.09	0.016	0.0-0.09

Graphical abstract



Effect of oat β-glucan of different molecular weights on fecal bile acids, urine metabolites and pressure in the digestive tract – a human cross over trial Hakkola, Salla; Nylund, Lotta; Rosa-Sibakov, Natalia; Yang, Baoru, Nordlund, Emilia; Pahikkala, Tapio, Kalliomäki, Marko; Aura, Anna-Marja; and Linderborg, Kaisa M.

Supplementary Table 1. List of allowed and not-allowed food items in the background diet low in phenolics and fiber

	ALLOWABLE	AVOIDABLE
Drinks	Water and unflavored sparkling water	Coffee and tea Soft drinks and energy drinks Berry and fruit juices All alcohol beverages
Milk and other dairy products	Milk, sour milk, unflavored yoghurt and vanilla flavored yoghurt, unflavored quark and cream, cheeses	Vanilla (not vanilla aroma)
Fruits and berries	One banana per day	All
Vegetables	-	All
Desserts and sweets	Bun (no cinnamon or cardamom added), vanilla-flavored ice cream	All, also chocolate
Cereal products	White bread, white rice and white pasta	All whole grains, e.g. porridge Anything containing seeds All cereals and muesli Noodles Other baked products, such as bread, muffins, cake and cookies
Meat, poultry, fish and eggs	All unmarinated products	Breaded products, sausages, frankfurters, and ready-made meat products
Seeds, nuts, lentils, beans and legumes	-	All
Sweeteners and spices	Sugar Salt, pepper	Artificial sweeteners, honey All other spices and spice mixes
Fats	Butter	Oil and margarine

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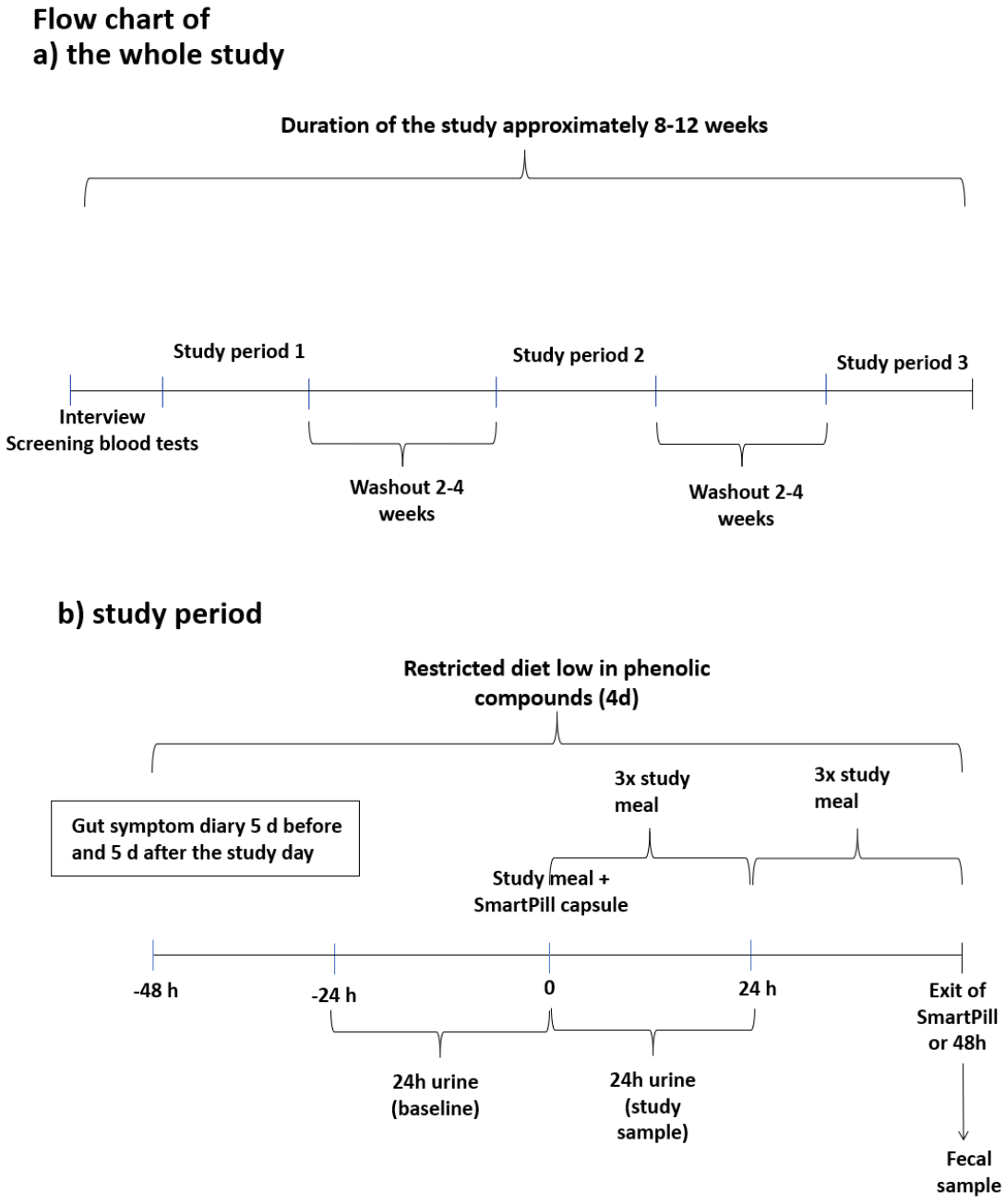
Supplementary Figure 1. The flow chart of the study.



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Supplementary Figure 2. A typical SmartPill data graph. Time of the activation of the capsule is shown on the X axis and the temperature, pH and pressure are shown on the Y axis. The blue largely horizontal line describes the temperature, the light green line the pH and the red bars the pressure. The blue vertical line represents the ingestion, the yellow lines the transit from the stomach to the small intestine, the green line the transit from the small intestine to the colon and the purple line the exit from the digestive tract.