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Role of β-glucan content, molecular weight and phytate in the bile acid binding of oat β-glucan

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ABSTRACT

There is controversy about the role of viscosity and co-migrating molecules on the bile acid binding of beta-glucan. Thus, this study aimed to investigate the impact of β-glucan molecular weight and the content of both β-glucan and phytate on the mobility of bile acids by modelling intestinal conditions *in vitro*. Two approaches were used to evaluate factors underlying this binding effect. The first studied bile acid binding capacity of soluble β-glucan using purified compounds. Viscosity of the β-glucan solution governed mainly the mobility of bile acid since both a decrease in β-glucan concentration and degradation of β-glucan by enzyme hydrolysis resulted in decreased binding. The second approach investigated the trapping of bile acids in the oat bran matrix. Results suggested trapping of bile acids by the β-glucan gel network. Additionally, hydrolysis of phytate was shown to increase bile acid binding, probably due to better extractability of β-glucan in this sample.

1. Introduction

Cereal mixed linkage (1 → 3)(1 → 4)-β-D-glucan (hereafter β-glucan) is a major non-starch polysaccharide in oat and barley (Wood, 2010). Due to its water-solubility, β-glucan has the ability to form viscous solutions, a factor linked to its physiological functionality. The authorities have approved health claims for the cholesterol-lowering and blood glucose-attenuating effects of oat and barley β-glucan, and both of these functions have been mainly linked to the increased viscosity of digesta (EFSA, 2009; 2010; 2011; 2005;; FDA, 1997; Wood, 2010). The viscosity of β-glucan in solution is affected by the solubility, concentration and molecular weight of β-glucan (Lazaridou, Biliaderis & Izydorczyk, 2007; Wood, 2002, and thus degradation of β-glucan during food production and storage could lead to a reduction in physiological functionality. However, results are contradictory, as there are studies showing lowering of cholesterol even when consuming β-glucan with low molecular weight (Keenan, Goulson, Shamliyan, Knutson, Kolberg & Curry, 2007; Yokoyama, Knuckles, Stafford & Inglett, 1998). This suggests that there are several mechanisms for the cholesterol-lowering effect of β-glucan.

In their review, Grundy, Fardet, Tosh, Rich and Wilde (2018) reported that increased viscosity may lead to reduced uptake of dietary cholesterol but also hindered reabsorption of bile acids. However, there have been studies demonstrating that only the viscosity of the surrounding matrix does not govern the mobility of bile acids, but additionally this physical entrapment or hindrance of movement, some mechanisms including chemical interactions may occur. Zacherl, Eisner and Engel (2011) showed increased *in vitro* bile acid binding with increased oat fibre concentration even when the oat fibre was heat treated to produce a sample with negligible viscosity. Thus, Zacherl et al. (2011) suggested that, in addition to the viscosity, the bile acid binding is also affected by other binding forces. Kim and White (2010) showed increased binding of bile acids when the molecular weight of β-glucan was decreased. They also indicated that oat flour with rather low β-glucan concentration had relatively high binding, suggesting that there might be other compounds in oat flour affecting bile acid. One hypothesised mechanism for decreased reabsorption of bile acids, is the formation of a complex or other kind of association between bile acids and dietary fibre molecule (Gunness and Gidley, 2010). Nuclear magnetic resonance studies have shown dynamic interactions between the

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bile salt micelles and β-glucan, and these interactions have been suggested to result in stabilisation of the bile salt micelles (Gunness, Flanagan, Mata, Gilbert & Gidley, 2016; Mikkelsen, Cornali, Jensen, Nilsson, BeYuangeren & Meier, 2014). The noted contradiction in the reported results may have resulted from the differences in the methodology and design of the studies, and this suggests that further systematic studies are required to reveal the β-glucan-derived bile acid binding mechanism.

In addition to the direct influence of β-glucan, other factors in oats can also have an effect on cholesterol metabolism. Oat ingredients contain higher amounts of phytate compared with most of the other grain ingredients, firstly, because of the originally higher levels in oat (Bartnik and Szafrańska, 1987). Secondly, oat material is usually kilned (a process that includes both increased moisture content and heat treatment) to inactivate the endogenous enzymes, and as heating has been shown to decrease endogenous phytase activity (Slominski, Davie, Nyachoti & Jones, 2007), kilning-treated oat is likely to contain a relatively high phytate content. Phytate (or phytic acid, if not in the salt form) co-migrates with β-glucan, resulting in high phytate levels in β-glucan rich fractions (Wang, Yang & Sontag-Strohm, 2020). Generally, phytate is considered an antinutrient due to its mineral binding capacity, which hinders absorption of dietary minerals (Weaver & Kannan, 2001; Zhou & Erdman, 1995), but positive physiological functionalities have also been reported. A phytate-containing diet has been linked to increased faecal bile acid excretion and decreased cholesterol levels in liver and serum, but the mechanisms remain somewhat unclear (Katayama, 1995; Yuangklang, Wensing, Lemmens, Jittakhot & Beynen, 2005).

Thus, it is clear that there is some controversy about the role of viscosity and lack of knowledge about the role of phytate in the bile acid binding of β-glucan. To address these questions, this study aimed to investigate the mobility of bile acids by modelling the intestinal conditions *in vitro,* focusing on the effect of β-glucan and phytate contents and the degradation of β-glucan on bile acid mobility. The decreased concentration of β-glucan and phytate in the samples as well as degradation of β-glucan were hypothesised to decrease the ability of β-glucan matrix to bind or trap bile acids. Two approaches were used to evaluate the factors underlying the bile acid binding effect of β-glucan. The first approach used model system with purified compounds to investigate the role of viscosity for the bile acid binding of soluble β-glucan. The second approach studied the binding and entrapment ability of the oat bran matrix and the influence of enzymatic hydrolysis of β-glucan on this bile acid binding. In both approaches, the role of phytate in bile acid binding was also studied.

2. Material and methods

2.1. Bile acid binding of β-glucan modelled using a dialysis-based method

2.1.1. Extraction and purification of β-glucan

β-glucan was extracted from oat bran concentrate (β-glucan content 14%, Swedish Oat Fiber, Bua, Sweden) and the extract was further purified to produce purified oat β-glucan extract (OBG). Bran and MilliQ water were shaken for 30 min at 40 °C, at ratio of 6–7 g of bran per each 100 ml of water. The suspensions were centrifuged (15,200 g, 10 min), and the supernatants were collected and heated in a boiling water bath for 10 min to precipitate proteins. The solution was centrifuged (15,200 g, 10 min), and 1.5 ml of protease solution (18.75 mg pancreatin [Pancreatin from porcine pancreas; Sigma-Aldrich, St. Louis, MO, USA] per ml of 150 mM $NaHCO_3$ (Sodium hydrogen carbonate for analysis, Merck, Germany), prepared by incubating at 37 °C for 20 min and centrifuging at 3,200 g for 10 min) per each 100 ml of supernatant were added. The solutions were incubated at 37 °C for 10 min, after which the reaction was stopped by heating the solution in a boiling water bath for 10 min. The solutions were centrifuged (16,000 g, 10 min), and the supernatants were collected.

The extracted β-glucan was precipitated by slowly adding 96% ethanol (Altia, Rajamäki, Finland) in a ratio of 2 volumes of ethanol per 1 vol of β-glucan extract. The solution was mixed thoroughly while adding ethanol and then kept overnight at 4 °C to allow precipitation of β-glucan. The excess ethanol was then decanted, and the precipitate in a smaller amount of ethanol was transferred to centrifuge tubes. The suspensions were centrifuged (3,200 g, 10 min) and the ethanol was discarded. Next, 99% ethanol (Altia, Rajamäki, Finland) was added to the β-glucan precipitate in a ratio of 2 volumes of ethanol per 1 vol of β-glucan. The mixture was thoroughly mixed and centrifuged again (3,200 g, 10 min). The 99% ethanol washing was repeated three times prior to suspending the precipitate in 99% ethanol and transferring the suspension to a wide dish. The solution was left to dry overnight in an oven at 60 °C. The dried purified oat β-glucan was homogenised using a mortar.

2.1.2. Phytate removal treatment for the purified β-glucan

A 0.4% solution was prepared from the purified β-glucan by wetting the solution with 99% ethanol, adding MilliQ water and mixing the solution at 85 °C for 2 h prior to letting the solution cool, adjusting the volume with MilliQ water and mixing the solution for an hour at room temperature. The pH of this solution was adjusted to 4 with hydrochloric acid (HCl). Residual phytate was removed from this solution by the ion exchange method described by Wang, Maina, Ekholm, Lampi and Sontag-Strohm (2017) with some modifications. The activation of the ion exchange resin (Amberlite IRA-410, Chloride form 20–25 mesh; Sigma-Aldrich, St. Louis, MO, USA) was conducted by washing the resin with 1 N HCl, deionised water, 1 N sodium hydroxide (NaOH), deionised water, 1 N HCl, deionised water, and finally with 1 N HCl. The activated resin and the 0.4% β-glucan solution (pH 4) (0.05 g resin/ml β-glucan solution) were mixed at 4 °C for 2 h. The mixture was filtered through cotton cloth to remove the resin, and the filtrate was dialysed against MilliQ water overnight. The phytate-removed β-glucan was dried overnight in an oven at 60 °C. It should be noted that this method also removes lower inositol phosphates than phytate (inositol hexaphosphate), but for simplification, this sample is called phytate-removed OBG (PR-OBG).

2.1.3. β-glucan content of the purified β-glucans

The β-glucan content of the purified and dried OBG and PR-OBG extracts were measured using the β-glucan assay kit for mixed linkage β-glucan (β-glucan Assay Kit [Mixed Linkage], Megazyme, Bray, Ireland). For this analysis, 0.6% solutions were prepared from the dried β-glucan extracts using a similar dissolution protocol as for the preparation of 0.4% solution in subsection 2.1.2. The β-glucan content of these solutions was then measured using procedure C of the assay kit, and the results were used to calculate the β-glucan content of the dried OBG and PR-OBG extracts, which were 66% and 73%, respectively. These values were used to calculate the actual β-glucan content in the bile acid binding model samples instead of reporting the concentration based on the total amount extract, which also includes the impurities.

2.1.4. Bile acid binding model

For *in vitro* bile acid binding tests, taurocholic acid sodium salt hydrate (<95% purity; Sigma-Aldrich, Auckland, New Zealand) was purchased. Taurocholic acid was chosen to be used as a representative of bile acids, as Zacherl et al. (2011) tested the performance of different bile acids in an *in vitro* system and showed no or only minor differences in the viscosities and permeation and diffusion rates. A 20 mM bile acid stock solution was prepared by dissolving the taurocholic acid in MilliQ water using a volumetric flask. This stock solution was stored at –20 °C and used for the bile acid binding trials.

For bile acid binding tests, 1.2% β-glucan stock solutions from both the OBG and PR-OBG were prepared similarly as in the phytate removal process (described in 2.1.2). The sample amount was calculated taking into account the purity of the β-glucan extracts and adjusting the

weighed extract amount to gain the desired β-glucan content. Part of the 1.2% OBG solution was enzyme hydrolysed using Depol 740L (Biocatalysts Ltd, Cardiff, UK) enzyme preparate, which contains β-glucanase activity. The enzyme hydrolysis was conducted by adding 1.25 nkat of β-glucanase activity/g of BG (taking into account the purity of the extracted OBG) and incubating the solution at 40 °C for 1.5 h. The enzyme was inactivated by placing the sample in boiling water for 10 min. The amount of added enzyme was only 1:1,000 of the total volume, and thus this was not considered to affect the β-glucan concentration significantly.

The bile acid binding solutions were prepared in three replicates from the stock solutions of 20 mM taurocholic acid and 1.2% OBG, 1.2% PR-OBG and 1.2% OBGenz. For each sample solution, 6 ml of 1.2% β-glucan solution (OBG, PR-OBG or OBGenz) was mixed thoroughly with 4.8 ml of buffer (50 mM Na_2HPO_4 including 25 mM NaCl). Then, 0.6 ml of 20 mM taurocholic stock solution and 0.6 ml of MilliQ water were added to the samples, and the solutions were mixed. The final β-glucan concentration was thus 0.6% and the taurocholic acid concentration was 1 mM. Additionally, bile acid binding was tested with 0.4% OBG by adding only 4 ml of 1.2% OBG and increasing the amount of added MilliQ water to 0.8 ml. The solutions were mixed at room temperature for 20 h to enable proper binding.

Ten millilitres from each sample solution were transferred into dialysis tube using a dialysis membrane with a cut-off of 8 kDa (BioDesignDialysis Tubing TM, cellulose, D102, wet diameter 15.5 mm; BioDesign Inc., Carmel, NY, USA). The dialysis tubes were placed into 250 ml plastic centrifuge tubes into which 150 ml of MilliQ water had been weighed.

2.1.5. Bile acid analysis

The quantification of the taurocholic acid in the dialysates (containing the non-retained taurocholic acid) was done with the liquid chromatographic method according to a method reported by Roda, Minutello, Angellotti and Fini (1990) with some modifications. For the separation, Nova-Pak C18 reversed-phase column (4 μm, 3.9 mm × 150 mm; Waters Corp., Milford, MA, USA) was used. The mobile phase consisted of 35% 0.01 M potassium phosphate monobasic (KH_2PO_4; Sigma-Aldrich, St. Louis, MO, USA) and 65% methanol (Sigma-Aldrich, St. Louis, MO, USA) and an isocratic elution with 0.5 ml/min flow rate using a 15 min run time was conducted at 25 °C. Taurocholic acid was detected using UV detection at 200 nm and quantified by comparing the peak areas with the ones obtained from the standard curve (including taurocholic acid concentrations of 0.01 mM, 0.05 mM, 0.1 mM, 0.5 mM and 1 mM).

Bile acid binding of the samples (%) was calculated from the difference of the dialysed taurocholic acid amount in the sample and the dialysed taurocholic acid amount in the control sample, which contained only taurocholic acid, but no β-glucan. The amount of dialysed taurocholic acid in the control sample was considered to describe the situation where no binding at all occurred, but maximum transfer of taurocholic acid through the dialysis membrane was enabled.

2.2. Bile acid binding of β-glucan in a digestive tract simulation

2.2.1. Preparation of enzyme treated oat bran samples

Oat bran (OB) containing 20.1 g of β-glucan in 100 g dry matter (DM) was kindly provided by Fazer Mills (Lahti, Finland). The OB was treated with enzymes to produce samples with lower amounts of phytic acid and/or depolymerised β-glucan. β-Glucanase-treated OB was prepared by mixing OB with distilled water at 5% DM content. Enzyme Depol 740L (Biocatalysts Ltd., Cardiff, UK) was added at a 50 nkat/g dosage, and the sample was incubated for 2 h at 50 °C with constant mixing (blade mixing). Phytase-treated OB was prepared by mixing OB with distilled water (5% DM content) with phytase 100 U/g (Ultra Bio-Logics Inc., Châteauguay, GC, Canada) for 1 h at 50 °C with constant mixing. OB treated with both the β-glucanase and phytase was prepared by adding both of the enzyme dosages described above and incubating the sample for 2 h at 50 °C. All the enzyme treatments were performed at pH 4 (adjusted with 1 M HCl). At the end of the incubation time, the samples were heated to 100 °C (10 min) in order to inactivate the enzymes. After inactivation, the samples were freeze-dried, ground and stored in sealed plastic bags.

2.2.2. Bile acid retention using an in vitro model

The retention of bile acids by the oat bran samples was evaluated using an *in vitro* digestion model according to our previous paper (Rosa-Sibakov, Mäkelä, Aura, Sontag-Strohm & Nordlund, 2020). Briefly, the OB samples with and without enzyme treatments were digested in simulated conditions of the upper intestinal model (oral, gastric and intestinal phases) according to Minekus et al. (2014) with the following modification: porcine bile extract (B-8631; Sigma-Aldrich, St. Louis, MO, USA) was added in the duodenal phase to reach a 6 mM concentration of bile acids. At the end of the duodenal phase, samples were centrifuged (5,000 rpm, 15 min, 4 °C) and the volume of the whole supernatant was calculated by measuring its mass and density. Bile concentration in the supernatant (mM) was determined using a Total Bile Acid Assay kit (DZ042A; Diazyme Laboratories, Poway, CA, USA). The total amount of bile acids (μmol) was calculated by multiplying the bile acid concentration (mM) with the total volume of the supernatant (mL). The amount of bile acids (μmol) retained by the sample matrix was expressed by the difference between the total bile acid amount in the supernatant of the enzyme blank (i.e. digestive enzymes without any sample) and the total bile acid amount in the supernatant of the OB samples. This was further calculated in relation to the dose of the sample (μmol/g DM).

2.2.3. Molecular weight analysis

OB samples (with and without enzyme treatments) were dissolved in 0.1 M NaOH and 0.1% $NaBH_4$ under magnetic stirring at room temperature overnight. The samples were diluted, filtered (0.45 μm syringe filter) and analysed by high performance size exclusion chromatography (*SEC*). The system consisted of an Alliance 2690 separation module, using Calcofluor white (Fluorescent Brightener 28, Steinheim, Germany) and Scanning Fluorescence 474 detector (Waters Corp., Milford, MA, USA), as described by Suortti (1993). Three *SEC* columns, Ultrahydrogel 2000, 500 and 250 (Waters Corp., Milford, MA, USA), were connected in series and maintained at 60 °C. The eluent was aqueous 50 mM NaOH at a flow rate of 0.5 ml/min. The linear size exclusion calibration curve was made with β-glucan M_w standards from Megazyme (Bray, Ireland) as follows: 667000, 375000, 247000, 160000, 67,100 and 33600 g/mol as peak molecular weight.

2.3. Phytic acid content analysis

The phytic acid contents of the samples were calculated using the Phytic Acid Assay Kit (Megazyme, Bray, Ireland) with some modifications in the sample extraction phase. The samples were expected to have high phytic acid contents, and thus the samples were extracted at a lower concentration than that noted in the kit instructions. Also, due to the low amount of samples, the extractions were scaled down, and a lichenase treatment was included in the extraction protocol to prevent the viscosity caused by β-glucan hindering the extraction of phytic acid. The lichenase solution was prepared by diluting the lichenase enzyme (1,000 U/ml, Megazyme, Bray, Ireland) with MilliQ water to reach 50 U/ml.

For extraction of the phytic acid, 0.15 g of each sample was weighed in appropriate-sized disposable tubes. For the OBG sample with the highest expected phytic acid content, 8.4 ml of MilliQ water and 0.4 ml of lichenase (50 U/ml) were added to the sample; for all of the other samples, the pipetted amounts were 4 ml and 0.4 ml, respectively. The samples were incubated at 50 °C for 1 h, after which the reaction was stopped by adding 1.2 ml of 5.5 M HCl to the OBG and 0.6 ml to the other

samples. Thus, the HCl concentration in all the samples reached 0.66 M. The samples were mixed at room temperature overnight to extract phytic acid and other inositol phosphates.

After the extraction, the measurement was done according to the kit instructions. Next, 1 ml of each sample was transferred to Eppendorf tubes, and the transferred samples were centrifuged at 13,000 rpm for 10 min. After this, 0.5 ml of the supernatants were transferred to clean tubes and neutralised by adding 0.5 ml of 0.75 M NaOH. The extraction of phytic acid was done in duplicate, and from each of the neutralised samples, two replicate analyses were done according to scheme 1 to reach a total of four replicate results. The standards were prepared according to the kit instructions. For all the samples, both the free and total phosphorus contents were determined, and their difference was calculated for the phytic acid content (mg/g).

2.4. Statistical analyses

Most of the analyses were conducted in three replicates. However, in phytic acid analyses, two replicate phytate extractions were done, of which two replicate measurements were conducted, resulting in a total of four replicate results. The results are reported as an average ± standard error of mean (SEM). Statistical software (IBM SPSS Statistics, version 25, IBM, Armonk, NY, USA) was used to carry out an analysis of variation (ANOVA) with a post-hoc Tukey test. Differences were considered significant if $p < 0.05$.

3. Results

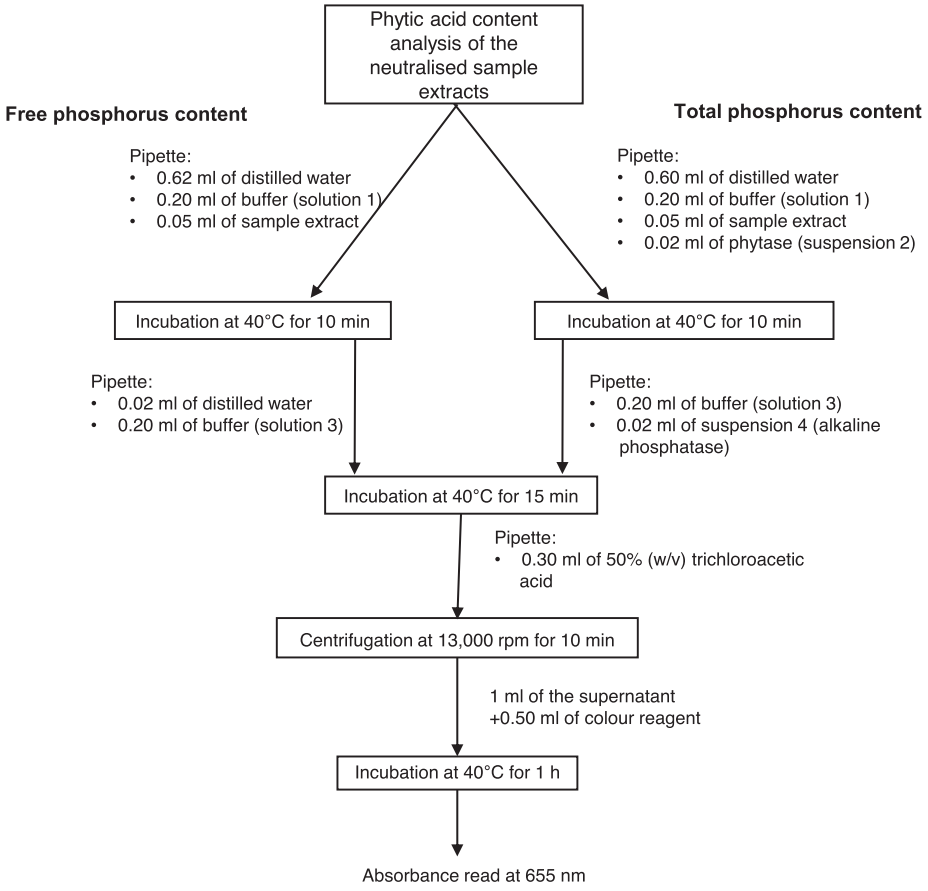
3.1. Phytic acid content of β-glucan samples

The OBG, which was used in the model system with purified compounds, contained a significantly higher amount of phytic acid than the OB sample of the digestive tract simulation (Fig. 1): 55.1 mg/g and 20.0 mg/g, respectively. The ion-exchange treatment removed 82% of the original phytic acid of the OBG sample resulting in a phytic acid content of 9.8 mg/g in the PR-OBG. A similar decrease in the phytic acid content was observed with phytase hydrolysis, since the phytase-treated OB contained 16% of the original content of the OB. However, the result of the phytase-treated sample may have been somewhat overestimated if total hydrolysis to inositol and free phosphate was not reached during the enzyme treatment. The OB hydrolysed with both β-glucanase and phytase had the lowest phytic acid content (2.1 mg/g), although the content did not differ significantly from the content of the phytase-treated OB (3.3 mg/g).

3.2. Bile acid binding studied with a dialysis-based method

In the dialysis-based method, a simplified model system with purified compounds was used to exclude the effect of other compounds present during the digestion of grain ingredients. In this model, taurocholic acid was used to represent bile acids. The theoretical maximum of taurocholic acid in the dialysate, if all the added taurocholic acid in the samples had been transferred through the dialysis membrane, would have been 66.7 μM. However, the concentration of taurocholic acid in the dialysate of the control sample was 34.2 μM after the 3 h dialysis and 60.4 μM after the 20 h dialysis. This indicates that complete dialysis was not obtained, but 49% and 10% of the taurocholic acid was retained in the dialysis bag or within the membrane, respectively. This showed that the increased length of the dialysis period significantly decreased the amount of retained taurocholic acid.

When calculating the bile acid binding results, the bound or retained taurocholic acid amount (%) was calculated by comparing the amount of



Scheme 1. Outline of the phytic acid content analysis for the neutralised sample extracts.

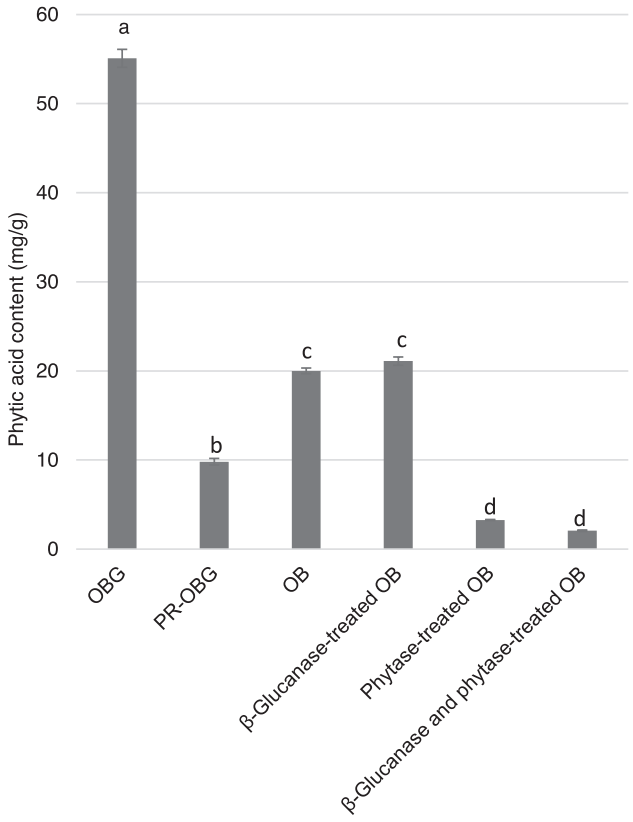


Fig. 1. Phytic acid content (mg/g) of the samples of both experiment.

the dialysed taurocholic acid of the samples with the corresponding amount in the control sample, where no binding occurred (Fig. 2). After the 3 h dialysis, the concentrations of taurocholic acid in the dialysate were 28.5 μM, 26.4 μM, 26.2 μM and 29.5 μM in 0.4% OBG, 0.6% OBG, 0.6% PR-OBG and 0.6% OBGenz, respectively. When comparing these values with the taurocholic acid content of the dialysate when no binding occurred (control sample), the respective bile acid binding was shown to be 16.7%, 22.9%, 23.4% and 13.9%. After the 20 h dialysis, the bile acid binding was significantly less than after the 3 h dialysis. After the 20 h dialysis the bile acid binding of enzyme-hydrolysed OBG was negligible and in 0.4% OBG, 0.6% OBG and 0.6% PR-OBG, the

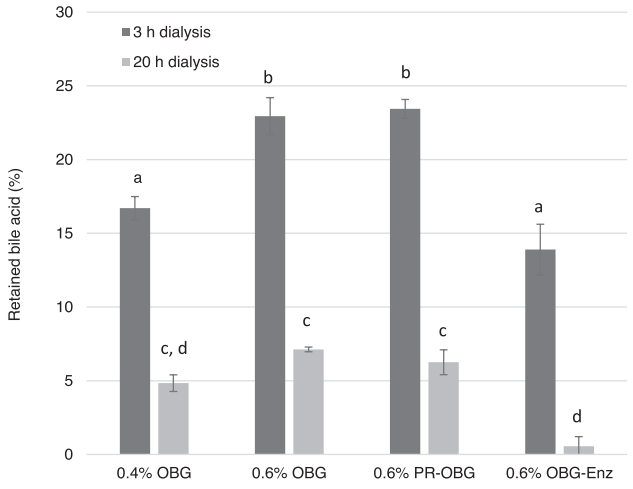


Fig. 2. The amount (%) of bile acid retained by 0.4% oat β-glucan (0.4% OBG), 0.6% oat β-glucan (0.6% OBG), 0.6% oat β-glucan where the intrinsic phytate had been removed (0.6% PR-OBG), and 0.6% enzyme-hydrolysed oat β-glucan (OBG-Enz).

binding was only 4.8–7.1%.

3.3. Bile acid entrapment in the grain matrix studied with an in vitro digestive tract model

The effects of the molecular weight of β-glucan and phytate content on bile acid binding were further studied by hydrolysing these components of the sample both one at a time and simultaneously using β-glucanase and phytase. The M_w of the β-glucan in OB was originally 1,840 $\times 10^3$ g/mol and a single peak in the Mw distribution curve (Fig. 3), but it decreased during all the tested enzyme treatments (Table 1; Fig. 3). As expected, the β-glucanase treatment led to a significant degradation, resulting in a 91% decrease in M_w of β-glucan leading to M_w of 173 $\times 10^3$ g/mol. The treatment of OB with phytase appeared to slightly degrade β-glucan resulting in M_w of 1,180 $\times 10^3$ g/mol. However, since the measurement of β-glucan by *SEC*-calcofluor was prepared with β-glucan standards ranging from 33.6 $\times 10^3$ to 667 $\times 10^3$ g/mol, the values above this threshold were based only on an estimation of the linear calibration curve. Polydispersity, calculated as a ratio of M_w to M_n, was originally 2.0 in OB, but it increased to about 4, 20 and 20 in samples treated with phytase, β-glucanase and β-glucanase + phytase, respectively.

The retained bile acids were measured from the supernatant, taking into account the different volumes of supernatant in different samples. The volumes of the supernatants in OB, β-glucanase-treated OB, phytase-treated OB and β-glucanase- and phytase-treated OB were 9.2 ml, 12.2 ml, 8.6 ml and 12.8 ml, respectively). The amount of retained bile acids was more pronounced in the samples without any enzyme treatments with 44 μmol of retained bile acids per g of DM (Fig. 4). When the OB was hydrolysed with the enzyme preparate containing β-glucanase, the amount of retained bile acids decreased by 31%. The reduction was also similar in the sample, which was treated with both the β-glucanase and phytase. When the OB was treated with phytase only, the reduction of bile acids increased slightly by 18% compared with the OB.

4. Discussion

The study used two *in vitro* approaches for investigating the mobility of bile acid in gut conditions. In the first approach, a simplified model system using purified compounds was utilised to exclude the effect of other compounds present during digestion. Solubilised β-glucan was used in the study, thus focusing on the effect of the viscosity-forming ability of β-glucan on the mobility of bile acid. In this method, the mobility of bile acid was studied by a dialysis-based method, and the bile acid that was transferred through the dialysis membrane was considered unbound and non-trapped. In the second approach, β-glucan was present in the form of an oat bran, which also included other components of the grain ingredient, such as protein and starch. Oat bran ingredient (OB) was enzymatically hydrolysed in order to reduce phytate and depolymerize β-glucan. These ingredients were digested in an *in vitro* model, where a mixture of bile acids was added. Subsequently, a mild centrifugation was used to separate the water-absorbing grain matrix from the free liquid phase, and the amount of bile acid retained by the grain matrix was calculated. Utilising these two methods, the potential mechanisms of the bile acid binding of β-glucan were investigated, while also considering the potential role of phytate in the matrix.

4.1. Role of viscosity in bile acid binding

The dialysis method models the digestion phase during which the β-glucan-containing digesta remains in the small intestine. During that phase, the bile acids are either absorbed or trapped in the non-absorbed matrix, which would lead to excretion of bile acids. The digesta remains in the small intestine for about 2–6 h (Lee, Erdogan & Rao, 2014), thus the 3-h dialysis time represents this digestion phase well, and gives an indication that the viscosity of the digesta in the small intestine might be able to hinder the absorption of bile acids in the gut. However, while bile

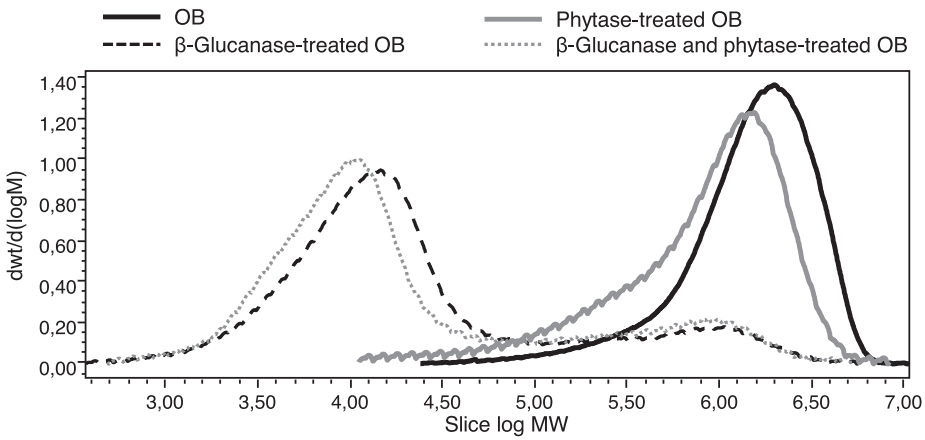


Fig. 3. Molecular weight distribution of the β-glucan in oat bran (OB) with and without enzyme hydrolysis treatments.

Table 1
Number-average (M_n) and weight-average (M_w) molecular weights and polydispersity (M_w/M_n) of the oat bran (OB) with and without enzyme treatments.

Sample	M_n ($\times 10^3$ g/mol)	M_w ($\times 10^3$ g/mol)	Polydispersity
OB	914.7 ± 0.6	1840 ± 20	2.01 ± 0.03
β-Glucanase-treated OB	8.9 ± 0.4	173 ± 11	19.5 ± 0.4
Phytase-treated OB	320 ± 40	1180 ± 10	3.8 ± 0.5
β-Glucanase and phytase-treated OB	7.77 ± 0.04	158 ± 2	20.3 ± 0.4

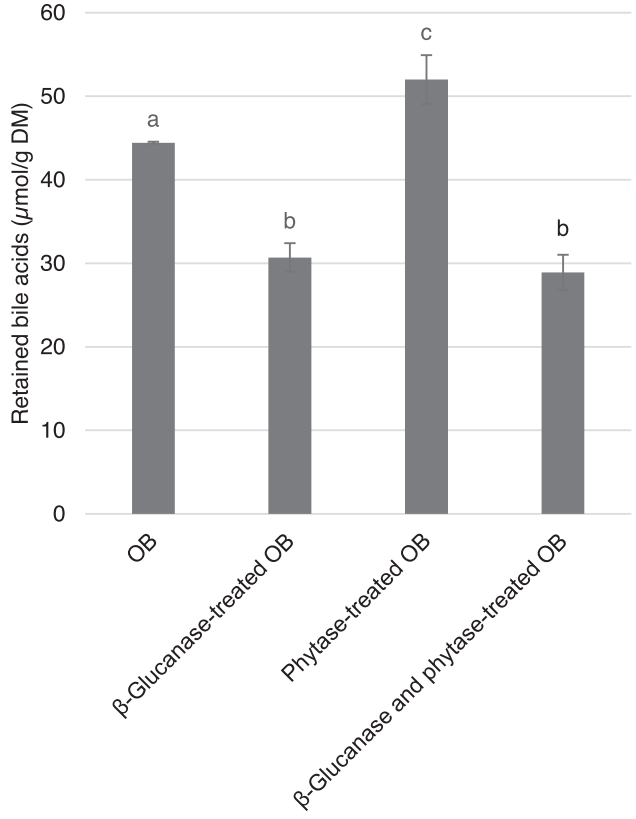


Fig. 4. Amount of bile acids retained by the oat bran (OB) with and without enzyme hydrolysis treatments.

acid absorption occurs partly through passive diffusion, there are also active transport mechanisms in the ileum part of the small intestine (Schiff, Small & Dietschy, 1972); thus, this model is only a simplified version showing the potential capability of β-glucan to hinder the absorption of bile acids.

The soluble β-glucans were shown to have a significant effect on bile acid mobility. All the tested β-glucans decreased the amount of bile acid that transferred through the dialysis membrane, and the effect was more pronounced in the samples with higher viscosity. Both the concentration and molecular weight of β-glucan are known to affect its viscosity (Wood, 2010), and these factors were found to have a significant effect on the mobility of bile acids. The decrease in the β-glucan content from 0.6% to 0.4% had a similar effect on the bile acid binding than the enzymatic degradation of β-glucan, indicating that viscosity was most likely the major reason for the hindered mobility of bile acids in the samples. These results correspond well with the studies by Zacherl et al. (2011) and Ellegård and Andersson (2007). Zacherl et al. (2011) used an *in vitro* method to study the bile acid binding of dietary fibres and reported that viscosity played a significant role in the bile acid binding capacity of oat β-glucan. Also, based on their results, Ellegård and Andersson (2007) suggested that bile acid excretion was mostly linked with the viscosity of oat β-glucan in the small intestine instead of the total amount of β-glucan.

The dialysis time significantly affected the binding, which also indicated that the bile acid binding was mostly influenced by the viscosity, causing a physical hindrance. No significant binding was observed in the degraded β-glucan sample after the 20-h dialysis time (bile acid binding of this sample was 0.6%), and thus the mechanism did not appear to rely on the covalent bonds between the β-glucan and bile acid molecules. This was similar to the findings of Bowles, Morgan, Furneaux and Coles (1996), who studied bile acid binding of barley β-glucan with NMR spectroscopy, which showed no direct binding between the two molecules. Zacherl et al. (2011) showed some binding when the oat fibre had lost its viscosity due to heat damage and suggested that direct binding by the fibre may have occurred. However, their study was done with oat fibre containing only 22% β-glucan; thus, other fibre components in the matrix may also have affected the binding. Our dialysis-based method studied only soluble β-glucan, which is most likely the reason for the observed differences. About 25% of the β-glucans are water-unextractable (Wood, 2010), and even for the water-extractable ones, the solubility may differ based on the dissolution conditions (Mäkelä, Maina, Vikgren & Sontag-Strohm, 2017). In the gastrointestinal conditions, complete dissolution is not likely; therefore, to further study the role of different factors for bile acid binding of oat β-glucan, an *in vitro* gastrointestinal simulation was conducted.

4.2. Bile acid binding of oat fibre concentrate in the gastrointestinal simulation

In this second approach, whole OB (with 20% β-glucan) was digested using an *in vitro* digestive tract simulation, and thus the binding capacity was studied in a more complex system. Instead of studying the viscosity of the whole matrix, here the emphasis was on the part of the sample containing the solid matrix (thus, also the non-soluble β-glucan), which was shown to trap large amounts of water.

In all samples, a significant proportion of the added bile acids was retained by the sample structure. The amount of bile acids retained by the matrix decreased significantly when the OB matrix was hydrolysed with β-glucanase-containing enzyme preparate, which was similar to the observations done in the dialysis method. However, in the dialysis method, the mechanism of binding was concluded to be through the formation of viscosity, but in this model, other kinds of mechanisms applied. The formation of a viscous solution would require the β-glucan to be extracted from the grain matrix and solubilised during the *in vitro* digestion. However, efficient dissolution is not likely to occur in these conditions. In our previous work (Mäkelä et al., 2017), we studied the effect of the dissolution temperature on the gelation of oat β-glucan at low concentrations (1.5%) and showed that oat β-glucan was able to form a gel structure when it was dissolved at physiological temperature (37 °C). If the dissolution was done at a high temperature (85 °C), β-glucan was completely solubilised and formed a viscous solution instead of a gel-structure. In the *in vitro* model of the current study, the temperature was not sufficient for efficiently solubilising the β-glucan. Thus, partial dissolution, which in our previous study was suggested to be the reason for the gelation at low concentration, was likely to occur.

The OB retained bile acids most, and a significant reduction in this retention capacity was observed when the β-glucan was degraded by β-glucanase. This was as expected, since the gel strength has been shown to decrease when β-glucan is degraded (Mäkelä et al., 2017), and the decreased gel strength here would cause weaker entrapment of the bile acids in the matrix. Also, the data about the volumes of the supernatants support this, since in both β-glucanase-containing samples, these volumes were larger than in OB and phytase-treated OBG, indicating less trapping of water when β-glucan was degraded. However, it should be noted that, even in the case of degraded β-glucan, significant trapping was maintained. This may be partly due to the degraded β-glucan molecules being able to form a gel structure, even though weaker, but the effect of the other components in the grain matrix should be considered. The enzyme preparation used in the present study contains dietary fibre-degrading activities (*endo*-xylanase, *endo*-glucanase; Sibakov, Myllymäki, Suortti, Kaukovirta-Norja, Lehtinen & Poutanen, 2013), and we have previously detected that solubilisation of the other dietary fibre components can also increase viscosity to some extent (Rosa-Sibakov et al., 2020). Sayar, Jannink and White (2005) reported that, while the β-glucan content of oat flour did not have a significant effect on bile acid binding, the bile acid binding corresponded with the insoluble fibre content of the flour. However, in their study, oat flours with a β-glucan content of 4.8–8.1% were used, and thus the β-glucan concentration in the *in vitro* samples may have been too low for either viscosity or gel structure formation. Based on the results of our study, the viscosity and gel structure formation are able to have a significant effect on the bile acid binding, but when the viscosity or gel formation is disabled due to insufficient β-glucan concentration or harsh degradation of β-glucan, other factors may dominate.

4.3. Effect of the phytate on the bile acid mobility

The phytic acid content of the samples from both experiments was analysed using a phytic acid assay kit. The method hydrolyses all the inositol phosphates (including phytate, inositol hexaphosphate) and measures the released phosphorus, which is then used to calculate the phytic acid content (McKie & McCleary, 2016). Thus, the method assumes that all phosphorus is cleaved from phytate, which results in overestimation of phytic acid in samples with a significant amount of lower inositol phosphate forms (inositol phosphates with one to five phosphate groups). In the dialysis-based method, phytate removal was conducted by ion exchange resin, which does not cause formation of lower inositol phosphates, and thus the inositol phosphates present in the samples should be mostly in inositol hexaphosphate (=phytate) form. In the digestive tract simulation, the materials were oat ingredients, which should not contain significant amounts of degradation of phytic acid due to the inactivation of enzymes during the kilning treatment of oat. In these samples, the phytic acid was phytase-hydrolysed to such an extent that no significant amount of inositol phosphates were assumed to remain, and thus, overestimation of phytic acid was not expected to occur.

The effect of the phytate content of the β-glucan-containing matrix was studied in both study approaches that were used to investigate bile acid binding. In the dialysis-based method, the extracted β-glucan contained a significantly higher amount of phytate than the bran concentrate used in the *in vitro* simulation. This agreed with the findings of Wang et al. (2020) who showed that phytate actually concentrated in the β-glucan-rich fractions when β-glucan was extracted from the bran concentrate. In both of the approaches used in our study, the phytate content was decreased to a similar extent, but due to the differences in the original phytate contents in OBG and OB, the PR-OBG contained a more significant amount of phytate after its removal.

When studying the viscosity effect of β-glucan on bile acid binding, the role of phytate was also studied to clarify whether phytic acid could be trapped or bound by the β-glucan, consequently resulting in bile acid binding of the β-glucan-rich matrix. However, the results of this first approach showed no effect of phytate on bile acid binding efficacy. The results did not suggest any actual covalent binding, but the bile acid binding was more affected by the viscosity hindering the movement and dialysis of the bile acid. However, these results do not exclude the possibility of the binding of bile acid by free phytate (which is not linked to β-glucan).

In the *in vitro* gastrointestinal simulation, the phytase-treated sample retained more bile acids than the OB sample. This was in contrast to the original hypothesis according to which the phytate was considered able to bind bile acids, thus partly explaining the binding effect linked to the β-glucan. A potential reason for the increased bile acid binding, is the increased gel formation in these samples, due to the potentially better extractability of β-glucan. Phytase treatment also led to a slight degradation of β-glucan, which implies that some fibre-degrading side activities were present. The side activities may have led to a breakage of the grain structure to such an extent that better extractability could be gained but without such a harsh degradation that would lead to a loss of ability to build a gel structure. In addition, the possibility of phytate disturbing digestive enzyme activity was raised, as it is known to be able to interact with proteins (Schlemmer, Frølich, Prieto & Grases, 2009). Thus, decreased phytate content may have led to increased activity of the digestive enzymes and, consequently, more efficient *in vitro* extraction of β-glucan, which would indeed be seen as an elevated bile acid binding in the phytase-treated OB.

5. Conclusions

The dialysis-based method enabled us to investigate the role of viscosity and phytate content of purified β-glucan and showed that factors affecting the viscosity – namely, the concentration and molecular weight of β-glucan – had a major effect on the bile acid binding. However, the phytate did not significantly affect the bile acid binding of solubilised β-glucan in this dialysis-based model. Thus, when the β-glucan is in the solubilised form, the mobility of bile acid is mainly governed by the physical hindrance caused by the viscosity. This indicates that, in food products where the β-glucan is in the solubilised form, its ability to form viscosity is essential for the health functionality related to the bile acid

binding. However, β-glucan may be present in the food matrix in a non-solubilised form or might still be trapped in the food matrix when consumed. In these kinds of food systems, the viscosity is not likely to be the main factor affecting the bile acid binding capacity, but the gelation of β-glucan may partly explain the increased retention of bile acids in the food matrix, as indicated by the results we found using the second approach of this study.

CRediT authorship contribution statement

Noora Mäkelä: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing - original draft, Writing - review & editing. **Natalia Rosa-Sibakov:** Conceptualization, Investigation, Methodology, Writing - review & editing. **Yu-Jie Wang:** Formal analysis, Investigation, Methodology, Writing - review & editing. **Outi Mattila:** Formal analysis, Investigation, Methodology, Writing - review & editing. **Emilia Nordlund:** Conceptualization, Funding acquisition, Resources, Supervision, Writing - review & editing. **Tuula Sontag-Strohm:** Conceptualization, Funding acquisition, Resources, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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