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The fate of insoluble arabinoxylan and lignin in broilers: Influence of cereal type and dietary enzymes

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ABSTRACT

Insoluble fiber degradation by supplemented enzymes was previously shown to improve fermentation in poultry, and has been further postulated to disrupt the cereal cell wall matrix, thus improving nutrient digestion. Here, we characterized insoluble feed-derived polysaccharides and lignin in digesta from broilers fed wheat-soybean and maize-soybean diets without or with xylanase/glucanase supplementation. Enzyme supplementation in wheat-soybean diet increased the yield of water-extractable arabinoxylan (AX) in the ileum. Still, most AX (> 73 %) remained insoluble across wheat-soybean and maize-soybean diets. Analysis of so-far largely ignored lignin demonstrated that a lignin-rich fiber fraction accumulated in the gizzard, while both insoluble AX and lignin reaching the ileum appeared to be excreted unfermented. More than 20 % of water-insoluble AX was extracted by 1 M NaOH and 11–20 % was sequentially extracted by 4 M NaOH, alongside other hemicelluloses, from ileal digesta and excreta across all diets. These findings showed that enzyme-supplementation did not impact AX extractability by alkali, under the current experimental conditions. It is, therefore, suggested that the degradation of insoluble AX by dietary xylanase *in vivo* mainly results in arabinoxylo-oligosaccharide release, which is not accompanied by a more loose cell wall architecture.

1. Introduction

Cereal grains are integral feedstocks for poultry, as they provide birds with energy in the form of starch [1]. Additionally, wheat and maize grains contain 13 and 10 % (w/w) fiber, respectively [1]. Fiber is a broad term that collectively describes feed-derived, indigestible and chemically distinct biomolecules, such as non-starch polysaccharides (NSP) and the alkyl-aromatic polymer lignin [1]. Studying the influence of dietary fiber on animal growth is of particular interest for improving cereal utilization, as well as for using alternative, fiber-rich feedstocks and for promoting intestinal health [2,3].

Arabinoxylan (AX) is the major NSP in wheat and maize grains (5–7 % w/w DM), followed by β-glucan (0.1–1.0 % w/w DM) and cellulose (2 % w/w DM) [1,4]. AX is composed of a β-(1→4)-linked D-xylosyl (Xyl) backbone and is mainly substituted by L-arabinofuranosyl (Ara) units at the *O*-2- and/or *O*-3-positions of the Xyl units. 4-*O*-D-methyl-glucuronoyl and acetyl moieties comprise additional substituents, while Ara units might be further *O*-5-esterified with feruloyl moieties [4,5]. Maize AX is more heavily substituted than wheat AX [1,6–8]. AX is the main component of endosperm and aleurone cell walls (CW), while pericarp cell walls additionally are rich in cellulose and lignin [9]. Lignin present in wheat and maize bran is mainly composed of syringyl (S) and guaiacyl

Abbreviations: NSP, Non-starch polysaccharides; CW, Cell wall; AX, Arabinoxylan; AXOS, Arabinoxylo-oligosaccharides; GIT, Gastrointestinal tract; WC, Wheat-soybean diet without enzyme addition; WE, Wheat-soybean diet with xylanase/glucanase addition; MC, Maize-soybean diet without enzyme addition; ME, Maize-soybean diet with xylanase/glucanase addition; WES, Water extractable solids (WES) corresponding to feed (WES_{Feed}), gizzard (WES_{Giz}), ileum (WES_{Ile}), ceca (WES_{Cec}) and excreta (WES_{Exc}) samples; WUS, Water unextractable solids (WUS) corresponding to feed (WUS_{Feed}), gizzard (WUS_{Giz}), ileum (WUS_{Ile}), ceca (WUS_{Cec}) and excreta (WUS_{Exc}) samples; AX/L, AX to Lignin weight ratio measured in WUS; 1M-ASS_F, The fraction of 1 M NaOH extractable solids from WUS_{Ile} (1M-ASS_{F_Ile}) or WUS_{Exc} (1M-ASS_{F_Exc}) that was recovered in the filtrate after ultrafiltration and was subsequently desalted by solid-phase extraction (SPE); 1M-ASS_R, The fraction of 1 M NaOH extractable solids from WUS_{Ile} (1M-ASS_{R_Ile}) or WUS_{Exc} (1M-ASS_{R_Exc}) that was recovered in the retentate after ultrafiltration; 4M-ASS, The fraction of 4 M NaOH extractable solids from WUS_{Ile} (4M-ASS_{Ile}) or WUS_{Exc} (4M-ASS_{Exc}) that was recovered after dialysis; RES, The fraction of WUS_{Ile} (RES_{Ile}) or WUS_{Exc} (RES_{Exc}) that remained unextractable during sequential alkali extraction.

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(G) units, and the Klason lignin contents amount to 5–7 and 1–3 % (w/w) of the wheat and the maize bran DM, respectively [1,6,10]. AX molecules may associate by hydrogen bonding with cellulose fibrils, and may also form covalent cross-links with other AX and lignin molecules *via* (esterified) ferulate and dehydrodiferulate cross-links [11–13]. AX plays an important role in tethering the CW matrix, providing the cells of cereal grains with structural integrity [9,11]. Typically, the characterization of CW polysaccharides requires their extraction from the matrix. In particular, alkali solutions (*e.g.*, 0.5–6 M NaOH) have been applied to extract pectin, heteroxylans and xyloglucan from plant material, while cellulose remains insoluble [8,14–19]. In the presence of alkali, the hydrogen bonds between hemicellulose and cellulose are known to be disrupted. If present, alkali-labile ester cross-links between cell wall polymers are also cleaved, which might lead to increased solubilization of water-unextractable AX [8,13].

Soluble and insoluble NSP can yield both adverse and beneficial effects during feed digestion in poultry. Soluble polysaccharides can increase digesta viscosity and compromise nutrient digestion in the small intestine [20]. At the same time, fermentation of soluble NSP in the ceca can contribute to gut health [21]. More than 70 % (w/w) of total NSP ingested are imbedded in the CW matrix [1,9,11]. These insoluble structures have been postulated to form a physical barrier that encapsulates nutrients (*e.g.*, starch and proteins), consequently limiting their digestion [1,22]. Moreover, the insoluble NSP fraction is largely excreted unfermented [23,24]. Nevertheless, inclusion of insoluble fibers in poultry diets has been linked with pronounced feed grinding in the gizzard and increased nutrient digestibility [25,26].

Dietary inclusion of NSP-degrading enzymes, predominantly xylanases, is widely performed to improve broiler performance [22,24,27,28]. In specific, xylanases belonging to the glycosyl hydrolase family 11 (GH11; CAzy.org [29]) have been shown to degrade both soluble and insoluble AX in the gastrointestinal tract (GIT) of broilers and pigs [30–33]. AX depolymerization has been associated with decreased intestinal viscosity, improved nutrient digestion and NSP fermentation in the ceca [27,31,33]. Our recent work further demonstrated that dietary xylanase released *in vivo* arabinoxylo-oligosaccharides (AXOS) with prebiotic potential, thus promoting cecal fermentation in broilers [33,34]. In addition, xylanase and β-glucanase have been associated with rupturing of the cell wall and subsequent nutrient release from cereals grains, *in vitro* [35,36]. Based on the outcomes of *in vitro* studies, CW degradation by enzymes has been hypothesized to alleviate the encapsulating effect of insoluble NSP, and to positively contribute to nutrient (*e.g.*, starch, protein) digestion [22,37]. Nevertheless, limited studies have focused on CW degradation *in vivo* by different dietary enzymes, as well as on the fate of insoluble CW components during feed digestion [23,38]. Investigating in detail the structure of insoluble cell wall components in the broiler GIT may be crucial in understanding and improving fiber utilization in poultry.

The present study aimed to investigate the amount and composition of insoluble, feed-derived cell wall components along the broiler GIT. Furthermore, insoluble NSP structure from wheat-based or maize-based diets, as well as the potential impact of enzyme supplementation on NSP structure and extractability, were investigated in detail. We hypothesized that the ratio between arabinoxylan and lignin content would provide further insight on the composition of insoluble fiber through the broiler GIT. It was also investigated whether enzyme supplementation could influence the interaction of insoluble CW polymers, and consequently alter AX extractability by alkali, compared to the control treatment.

2. Materials and methods

All reagents used were of analytical grade and supplied by Sigma Aldrich (St. Louis, MO, USA), Merck KGaA (Darmstadt, Germany) or VWR International B.V. (Amsterdam, The Netherlands), unless stated otherwise. The water used throughout laboratory experiments was purified with a Milli-Q Integral 5 (Millipore Corp., Billerica, MN, USA) purification system.

2.1. Samples from broiler GIT

Broiler digesta were obtained from our recent study [24], which was conducted at the facilities of the Laboratory for Animal Nutrition and Animal Product Quality (LANUPRO), Department of Animal Sciences and Aquatic Ecology, Ghent University (Belgium), in accordance with the ethical standards and recommendations for accommodation and care of laboratory animals covered by the European Directive 2010/63/EU on the protection of animals used for scientific purposes and the Belgian royal decree KB29.05.13 on the use of animals for experimental studies. In brief, 96 one-day old male broilers were fed either wheat-soybean (W) or maize-soybean (M) starter and grower diets (Table S1). On d 20, the birds were allocated to pens and were assigned to control or enzyme treatments following a randomized block design. Each dietary treatment consisted of 6 replicate pens, with 4 birds per pen. The finisher diets were provided as such (control: WC, MC) or supplemented with a commercially available enzyme preparation (enzyme: WE, ME) of GH11 endo-1,4-β-xylanase (EC 3.2.1.8) and endo-1,4-β-glucanase (EC 3.2.1.4) from *Trichoderma spp.* (Huvepharma NV, Berchem, Belgium) (Table S1). The excreta were collected per pen, daily (twice) between d 24 and 28 and immediately stored at −20 °C. On d 28, birds were euthanized, and the gizzard, ileum and ceca contents were collected, pooled per pen, and frozen at −20 °C. Frozen material was dried by lyophilization and homogenized with a MM 400 Mixer Mill (Retsch GmbH, Haan, Germany) prior to analysis. Three out of six replicate pens were randomly selected, and digesta samples originating from these three pens were used for the current study, as previously described [33].

2.2. Aqueous extraction of digesta

Aqueous extraction of digesta was performed as described in our recent publication [33]. In brief, 1 g digesta and finisher feed was dispersed in 35 mL water heated at 99 °C and was incubated at 99 °C for 20 min under head-over-tail rotation. Next, the supernatant was separated by centrifugation (30,000 ×*g*, 20 min, 20 °C), followed by filtration over 595 Whatman® filter paper. The residue was further washed with 30 mL water and the obtained supernatant was separated as described before. The washing step was repeated once and all resulting supernatants were combined. Supernatants (water-extractable solids; WES) and residues (water-unextractable solids; WUS) from the feed (Feed), gizzard (Giz), ileum (Ile), ceca (Cec) and excreta (Exc) were freeze-dried, and their dry weight was recorded.

2.3. Sequential alkali extraction and fractionation of digesta water-unextractable solids

WUS from ileal digesta (WUS_{Ile}) and excreta (WUS_{Exc}) were sequentially extracted with alkali solution (1 M NaOH and 4 M NaOH), as described by Murciano-Martínez and coworkers (2016) [18], with several modifications. A schematic overview of the extraction, including all fractions obtained (see Abbreviations), is presented in Fig. 1. Approximately 100 mg WUS was extracted with 12 mL 1 M NaOH solution containing 260 mM $NaBH_4$ at ambient temperature, for 2 h. The supernatant was separated by centrifugation (4000 ×*g*, 15 min, 20 °C). The procedure was repeated once. Per sample extracts were combined, placed in an ice bath and their pH value was adjusted to pH 5.0 by the addition of glacial acetic acid. The acidified pooled extract (1 M alkali soluble solids; 1M-ASS) was stored at 4 °C. The remaining residue was further extracted with 12 mL 4 M NaOH solution (+260 mM $NaBH_4$) at room temperature for 16 h. The 4 M NaOH extract was collected after centrifugation (4000 ×*g*, 15 min, 20 °C). Next, 12 mL water was added to the residue, the mixture was thoroughly vortexed and the supernatant

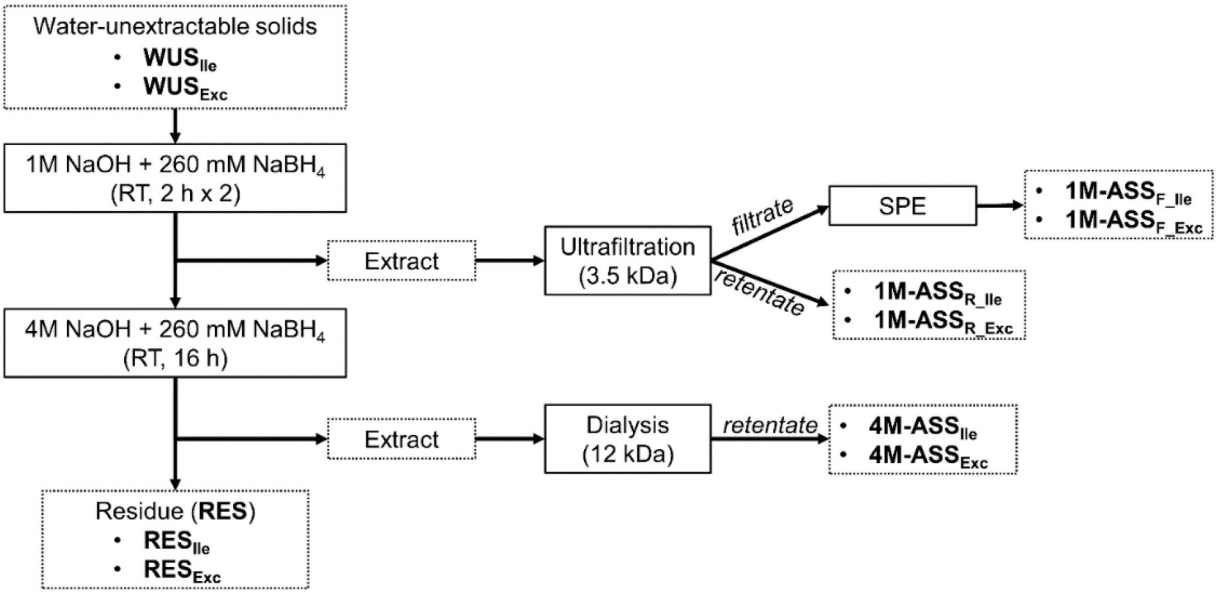

Fig. 1. Schematic overview of sequential alkali extraction of water-unextractable solids from the ileum (WUS_{Ile}) and excreta (WUS_{Exc}). The retentate (> 3.5 kDa) from ultrafiltration of the 1 M NaOH extract was labelled 1M-ASS_R and the filtrate after further desalting by SPE was labelled 1M-ASS_F. The retentate obtained from dialysis of the 4 M NaOH extract was labelled 4M-ASS. The alkali-unextractable residue was labelled RES.

(water wash) was separated from the residue by centrifugation (4000 ×*g*, 15 min, 20 °C). The 4 M NaOH and water wash fractions were combined (4 M alkali soluble solids; 4M-ASS), acidified at pH 5.0 and stored at 4 °C. The final residue (RES) was suspended in water, acidified to pH 5.0 with glacial acetic acid, washed twice with 10 mL water, stored at −20 °C, and freeze-dried.

The 1M-ASS fractions were extensively desalted with regenerated cellulose centrifugal filter units (molecular weight cut-off (MWCO): 3.5 kDa) (Amicon® Ultra-15: MilliporeSigma, Burlington, MA, USA), until the conductivity value of the retentate was at least tenfold lower than that of 50 mM sodium acetate (NaOAc) buffer solution (pH 5.0). The retentates (labelled: 1M-ASS_R) were collected in pre-weighed containers and were freeze dried. The filtrates were further subjected to solid-phase extraction (SPE) to separate oligosaccharides below 3.5 kDa from salts. To achieve that, the filtrates were quantitatively loaded on 500 mg Supelclean™ ENVI-Carb™ solid-phase extraction (SPE) cartridges (Supelco Inc., Bellefonte, PA, USA), pre-conditioned with 10 mL 80 % (v/v) acetonitrile (ACN) containing 0.1 % (v/v) trifluoroacetic acid (TFA) and washed with 10 mL water. Next, the retained analytes were eluted twice with 5 mL 40 % (v/v) ACN solution containing 0.1 % (v/v) TFA. Analytes eluting with 40 % (v/v) ACN (+0.1 % (v/v) TFA) solution (labelled: 1M-ASS_F) were collected in pre-weighed glass tubes, and were dried with a sample concentrator. The 4M-ASS fractions were desalted by dialysis against distilled water, using Visking dialysis membranes (12 kDa MWCO for proteins; Medicell Membranes Ltd., London, UK). The dialyzed material was transferred in pre-weighed containers, and was freeze-dried (labelled: 4M-ASS). Freeze dried materials were used for subsequent analyses.

2.4. Dry and organic matter yield determination during sequential alkali extraction

The total solids obtained in 1M-ASS_R, 1M-ASS_F, 4M-ASS and RES during alkali extraction were determined gravimetrically, after drying. Next, the organic matter (OM) was determined to account for residual salts present. Approximately 1–2 mg sample was weighed using an XP6 Excellence Plus Micro Balance (5 decimals) (Mettler-Toledo International Inc., Columbus, OH, USA) in Eco-Cup LF pyrolysis cups (Frontier Laboratories Ltd., Fukushima, Japan), followed by incineration at 550 °C, overnight. The ash content was determined gravimetrically, and the organic matter (OM) content was estimated as the difference between total solids and ash content. OM determination was not feasible for 1M-ASS_F due to the low sample amounts recovered. In this case, the dry matter content of 1M-ASS_F was assumed to equal OM content, given the complete salt removal by SPE.

2.5. Lignin content analysis

Lignin content and structural composition in WUS was determined by quantitative pyrolysis-GC–MS according to van Erven et al. (2019) [39]. ^{13}C-Labelled lignin isolated from uniformly ^{13}C labelled wheat straw (97.7 %, IsoLife, Wageningen, the Netherlands) was used as internal standard (^{13}C-IS) [40]. In brief, 100–200 μg sample was weighed in Eco-Cup LF pyrolysis cups with an XP6 Excellence Plus Micro Balance in duplicate, followed by the addition of 10 μL 1 μg/μL ^{13}C-IS solution (50/50 % v/v chloroform/ethanol). Pyrolysis, GC and MS settings were identical as previously described [39]. Processing excluded low-abundance lignin-derived pyrolysis products and *p*-hydroxyphenyl products from the samples (^{12}C only, Table S2), as the latter also substantially derives from aromatic amino acids, as deduced from the semi-quantification of the protein marker indole. Lignin content calculations also excluded the pyrolysis product 4-vinylguaicol, largely derived from ferulic acid and not necessarily incorporated into the lignin macromolecule [39].

2.6. Monosaccharide composition and content analysis

For WUS and RES samples (Fig. 1), neutral constituent monosaccharide analysis was performed according to Englyst and Cummings [41]. Samples were pre-hydrolyzed in 72 % (w/w) H_2SO_4 (30 °C, 1 h) and subsequently hydrolyzed in 1 M H_2SO_4 (100 °C, 3 h). The neutral monosaccharides released were derivatized to alditol acetates and analyzed by gas chromatography on a Trace 1300 GC system (Thermo Fisher Scientific Inc.) equipped with a DB-225 column (Agilent Technologies Inc., Santa Clara, CA, USA) and a flame/ionization detector (FID), using inositol as internal standard. Uronic acid content was determined by the colorimetric *m*-hydroxyphenyl assay with an automated analyzer (Skalar Analytical B.V., Breda, The Netherlands), according to Blumenkrantz and Asboe-Hansen (1973) and Thibault and Robin (1975) [42,43].

For 1M-ASS$_R$ and 4M-ASS samples (Fig. 1), neutral constituent monosaccharide analysis was performed according to Broxterman & Schols (2018) [15]. Following similar hydrolysis conditions as described above, samples were diluted 50 times and the neutral monosaccharides were determined by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD). The analysis was performed using an ICS5000 ED ion chromatography system (Dionex Corp., Sunnyvale, CA, USA), equipped with a CarboPac™ PA1 IC column (250 mm × 2 mm i.d.) and a CarboPac™ PA guard column (50 mm × 2 mm i.d.). Three mobile phases were used: A) 0.1 M NaOH, B) 1 M NaOAc in 0.1 M NaOH and C) Water. The injection volume was 10 μL, and neutral monosaccharides were eluted at 0.4 mL/min with 100 % C (isocratic) for 0–27 min, with post column addition of 0.5 M NaOH (0.1 mL/min) to enable PAD detection. The subsequent elution profile (v/v) was: 27.1–38 min linear gradient 10–17.3 % B (90–82.7 % A); 38.1–43 min isocratic 100 % B, 43.1–51 min isocratic 100 % A; 51.1–66 min isocratic 100 % C with post column addition.

For 1M-ASS$_F$ samples (Fig. 1), the material obtained after SPE was dissolved in water at 5 mg/mL. Aliquots (50 μL) were transferred in glass tubes, and dried with a sample concentrator. Neutral constituent monosaccharide analysis was determined by TFA hydrolysis, according to de Ruiter et al. (1992) [44]. In brief, 1 mL 2 M TFA solution was added, and the samples were hydrolyzed at 121 °C for 1 h. Next, TFA was removed by evaporation. The hydrolyzed samples were dissolved in 500 μL water and the neutral monosaccharides were determined by HPAEC-PAD as described above.

2.7. Molecular weight distribution analysis by HPSEC-RI

The molecular weight distribution of 1M-ASS$_F$, 1M-ASS$_R$ and 4M-ASS (see Fig. 1) was determined by high performance Size-Exclusion Chromatography using an Ultimate 3000 system (Dionex, Sunnyvale, CA, USA) coupled to an ERC Refractomax 520 detector (Biotech AB, Onsala, Sweden). A set of TSK-Gel super AW columns 4000, 3000, 2000 (6 mm × 150 mm) equipped with a TSK-Gel super AW guard column (6 mm ID × 40 mm) (Tosoh Bioscience, Tokyo, Japan) was used in series. The column temperature was set to 55 °C. Samples were injected (10 μL) and eluted with 0.2 M $NaNO_3$ at a flow rate of 0.6 mL/min. Prior to analysis, aliquots 1M-ASS$_R$ and 4M-ASS were dispersed at 5 mg/mL in 50 mM NaOAc (pH 5.0), heated at 80 °C for 30 min and centrifuged (15,000 ×*g*, 10 min, 20 °C). The obtained supernatants were analyzed by HPSEC (injection volume 10 μL). For analysis of 1M-ASS$_F$ samples, aliquots of solutions (5 mg/mL) used for sugar composition analysis (see Section 2.6) were used.

2.8. Oligosaccharide profiling by MALDI-TOF-MS

The presence of oligosaccharides released by alkali was determined in 1M-ASS$_F$ by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) using an UltrafleXtreme™ MALDI-TOF/TOF mass spectrometer (Bruker Daltonics Inc., Billerica, MA, USA). The equipment was controlled with FlexControl 3.3 software and operated in positive mode. The mass spectrometer was calibrated with maltodextrins (Avebe, Veendam, The Netherlands) in a mass range of *m/z* 700–2500. 1M-ASS$_F$ solutions (5 mg/mL; see Section 2.6) were diluted ten times with water. WUS samples were also examined to account for the presence of residual water-soluble oligosaccharides. For that, 5 mg WUS was suspended in 1 mL water for 20 min, and the supernatant was separated by centrifugation (20,000 x*g*, 10 min, 20 °C). Aliquots (100 μL) were desalted with Dowex 50 W-X8 resin (Sigma-Aldrich, St. Louis, MO, USA). After desalting, 10 μL was removed, followed by the addition of 1 μM NaCl to direct the formation of sodium adducts during ionization. Afterwards, 1 μL sample was co-crystallized with 1 μL matrix solution (25 mg/mL dihydroxy-benzoic acid in 50 % (v/v) acetonitrile) on a target plate under a stream of dry air.

2.9. Calculations

The proportion of AX (sum of Ara and Xyl) in whole digesta from the gizzard, ileum, ceca and excreta that was recovered in WUS and WES was determined according to Eq. (1):

$$AX\ in\ WUS\ (\%w/w) = \frac{AX_{WU}{*}WUS\%}{(AX_{WE}{*}WES\%) + AX_{WU}{*}WUS\%){*}100} \quad (1)$$

Where AX$_{WE,WU}$ is the measured AX content (% *w*/w dry matter) in WES or WUS of digesta from the gizzard, ileum, ceca and excreta. WES% and WUS% are the amount (% w/w dry matter) of WES and WUS out of total digesta. AX recovery in WES and glucan (Glc units) recovery in WUS and WES were determined in a similar manner.

2.10. Statistical analysis

The obtained data was subjected to analysis of variance (ANOVA) using R version 4.1.0, with pen being the experimental unit. The observations for WC, WE, MC and ME were used to model the effect of diet on arabinoxylan (AX) and glucan recovery and lignin content is WUS. The effect of diet and GIT location (gizzard, ileum, ceca, excreta) as well as their two-way interaction on AX to lignin weight ratio (AX/L) was also modelled. Finally, AX recovery during alkali extraction was modelled using diet as the sole factor. Normality of data residuals and homogeneity of variance were additionally checked. To test the significance of the differences between treatments, Tukey's post-hoc test was performed, with a significance threshold set at $P < 0.05$.

3. Results and discussion

3.1. Yield, content and composition of water-unextractable cell wall material

3.1.1. Insoluble carbohydrate content and recovery along the GIT

To understand the fate of insoluble fibers throughout the GIT of broilers, digesta from the gizzard, ileum, ceca and excreta were subjected to aqueous extraction (Fig. S1). More than 60 % (w/w) of the total solids was water-unextractable (WUS), while WES represented approximately 35–39 % (w/w) of the total solids in the gizzard, ileum and excreta, demonstrating >90 % dry matter recovery, for both wheat and maize diets. On the contrary, WES and WUS from the ceca showed comparable yields (~50 % w/w of total dry matter), indicating the presence of equal amounts of soluble and insoluble components.

The monosaccharide composition and content in WUS were determined to further distinct the types of insoluble NSP present (Table 1). Glc was the main constituent monosaccharide in WUS from the gizzard (WUS$_{Giz}$: 39–57 mol%) and the ceca (WUS$_{Cec}$: 54–47 mol%) for all four diets. Insoluble Glc may be of diverse origin, representing undigested starch, particularly in the gizzard, as well as cellulose and β-glucan [1,17]. Next to Glc, Ara (19–22 mol%) and Xyl (20–28 mol%) were highly abundant in both WUS$_{Ile}$ and WUS$_{Exc}$. This highlighted that AX was the second major constituent of unfermented insoluble carbohydrates, for both wheat and maize diets. Although AX is the main NSP in both wheat and maize grains [1,4], a proportion of the Ara, Gal and UA analyzed in WUS might still have derived from soybean meal in the diet [45]. Finally, Man (<3 mol%) and Fuc (traces; data not shown) were present at relatively low levels in all cases. Rha was present at ~1 mol% in WUS$_{Giz}$, WUS$_{Ile}$ and WUS$_{Exc}$ but was more abundant in WUS$_{Cec}$ (16–19 mol%), across all diets, probably due to the presence of bacterial glycans [46].

The recovery of AX and glucans in WUS during aqueous extraction was determined according to Eq. (1), as they were the main NSP present in all WUS fractions (Table 1). Water-unextractable AX recovered in WUS accounted for 90 %, 82 %, 44 % and 90 % (w/w) on average of total AX for the gizzard, ileum, ceca and excreta, respectively (Fig. 2).

Table 1
Constituent monosaccharide composition (mol%), Ara/Xyl ratio and total carbohydrate (Tot), arabinoxylan (AX: Ara + Xyl) and glucan (Glc) content (% w/w) of WUS obtained from the finisher diet (WUS_{Feed}), gizzard (WUS_{Giz}), ileum (WUS_{Ile}), ceca (WUS_{Cec}) and excreta (WUS_{Exc}) corresponding to wheat control (WC), wheat enzyme (WE), maize control (MC) and maize enzyme (ME) diets. Standard deviation ($n = 3$) is given within brackets.

Diet	Constituent monosaccharide composition (mol%)							Ara/Xyl	Carbohydrate content (% w/w)		
	Ara	Xyl	Glc	Gal	Man	Rha	UA		Tot	AX	Glc
WUS_{Feed}											
WC	5	6	82	2	1	1	3	0.8	60.7	5.4	51.6
	(<1)	*(<1)*	*(1)*	*(<1)*	*(<1)*	*(<1)*	*(<1)*	*(0.0)*	*(1.3)*	*(0.2)*	*(1.2)*
WE	5	6	83	2	1	1	3	0.8	60.4	5.6	50.6
	(<1)	*(<1)*	*(1)*	*(<1)*	*(<1)*	*(<1)*	*(<1)*	*(0.0)*	*(1.0)*	*(0.1)*	*(1.0)*
MC	5	4	84	3	1	0	3	1.2	67.4	5.1	57.4
	(<1)	*(<1)*	*(1)*	*(<1)*	*(<1)*	*(<1)*	*(<1)*	*(0.1)*	*(1.3)*	*(0.3)*	*(1.3)*
ME	5	4	84	3	1	0	3	1.1	71.1	5.2	61.1
	(<1)	*(1)*	*(1)*	*(<1)*	*(<1)*	*(<1)*	*(<1)*	*(0.0)*	*(3.5)*	*(0.2)*	*(0.5)*
WUS_{Giz}											
WC	14	22	49	6	3	1	6	0.6	55.3	17.0	29.2
	(2)	*(7)*	*(10)*	*(<1)*	*(1)*	*(<1)*	*(<1)*	*(0.1)*	*(3.2)*	*(3.6)*	*(7.3)*
WE	16	27	39	7	3	1	7	0.6	48.7	18.4	20.6
	(1)	*(2)*	*(4)*	*(1)*	*(<1)*	*(<1)*	*(1)*	*(0.1)*	*(1.9)*	*(1.0)*	*(2.6)*
MC	11	15	57	6	2	1	7	0.7	61.9	13.4	37.3
	(2)	*(3)*	*(9)*	*(2)*	*(1)*	*(<1)*	*(1)*	*(0.1)*	*(3.8)*	*(2.5)*	*(8.0)*
ME	11	16	56	6	2	1	7	0.7	62.7	14.4	36.9
	(2)	*(4)*	*(9)*	*(1)*	*(1)*	*(<1)*	*(2)*	*(0.1)*	*(6.2)*	*(1.8)*	*(8.9)*
WUS_{Ile}											
WC	20	28	28	11	2	1	8	0.7	50.4	21.7	15.3
	(1)	*(1)*	*(3)*	*(1)*	*(<1)*	*(<1)*	*(1)*	*(0.0)*	*(1.6)*	*(0.9)*	*(1.7)*
WE	21	26	24	13	3	1	10	0.8	48.0	20.3	12.8
	(<1)	*(2)*	*(<1)*	*(1)*	*(<1)*	*(<1)*	*(<1)*	*(0.1)*	*(1.0)*	*(0.3)*	*(0.4)*
MC	22	20	23	18	2	1	14	1.1	52.3	18.9	12.9
	(<1)	*(1)*	*(1)*	*(1)*	*(<1)*	*(1)*	*(<1)*	*(0.1)*	*(1.2)*	*(0.1)*	*(0.8)*
ME	21	21	22	17	2	1	14	1.0	52.9	19.5	12.8
	(1)	*(2)*	*(3)*	*(<1)*	*(<1)*	*(<1)*	*(<1)*	*(0.1)*	*(1.8)*	*(0.1)*	*(2.1)*
WUS_{Cec}											
WC	3	9	47	15	2	16	8	0.4	12.3	1.2	6.0
	(<1)	*(4)*	*(6)*	*(1)*	*(<1)*	*(1)*	*(<1)*	*(0.1)*	*(0.9)*	*(0.4)*	*(0.6)*
WE	2	2	53	16	2	18	7	0.9	12.3	0.4	6.8
	(<1)	*(1)*	*(6)*	*(3)*	*(1)*	*(1)*	*(2)*	*(0.2)*	*(2.9)*	*(0.1)*	*(2.3)*
MC	1	2	54	14	2	18	8	0.7	9.4	0.3	5.2
	(1)	*(1)*	*(3)*	*(1)*	*(<1)*	*(2)*	*(1)*	*(0.2)*	*(1.2)*	*(0.2)*	*(0.7)*
ME	1	2	51	16	2	19	9	0.8	9.1	0.2	4.7
	(<1)	*(<1)*	*(7)*	*(3)*	*(<1)*	*(3)*	*(2)*	*(0.1)*	*(1.4)*	*(0.1)*	*(1.2)*
WUS_{Exc}											
WC	19	25	33	11	2	1	8	0.8	51.8	20.0	18.5
	(1)	*(1)*	*(3)*	*(1)*	*(<1)*	*(<1)*	*(1)*	*(0.0)*	*(3.9)*	*(1.3)*	*(3.2)*
WE	20	24	29	13	2	1	9	0.8	44.5	17.3	14.4
	(1)	*(1)*	*(4)*	*(1)*	*(<1)*	*(<1)*	*(<1)*	*(0.0)*	*(1.9)*	*(0.3)*	*(2.0)*
MC	21	22	23	16	2	1	13	0.9	49.9	17.0	13.0
	(<1)	*(2)*	*(2)*	*(<1)*	*(<1)*	*(<1)*	*(1)*	*(0.1)*	*(1.0)*	*(3.6)*	*(1.4)*
ME	21	23	23	15	2	1	14	0.9	49.4	18.4	13.2
	(<1)	*(1)*	*(<1)*	*(1)*	*(<1)*	*(<1)*	*(1)*	*(0.0)*	*(0.8)*	*(1.0)*	*(0.9)*

The higher Ara/Xyl values observed in maize diets compared to wheat diets (Table 1) were expected given the more substituted structure of maize AX [1,7,8]. Insoluble AX present in WUS_{Giz} presented lower Ara/Xyl values (0.6–0.7) than in WUS_{Ile} (0.7–1.1) and WUS_{Exc} (0.8–0.9) across all diets. This could be a first indication that certain low-substituted AX populations were more retained in the gizzard compared to other AXs present in the diet.

Water-unextractable glucans represented 53 %, 60 %, 41 % and 82 % (w/w) on average of total Glc units for the gizzard, ileum, ceca and excreta, respectively. It was observed that enzyme supplementation did not impact AX solubilization in ME and glucan solubilization in both WE and ME. The lower recovery of AX with higher Ara/Xyl ratio for WE compared to WC observed in the ileum (WUS_{Ile}) was attributed to the xylanase-mediated release of low-substituted AXOS occurring in the upper GIT, as covered in our recent publication [33]. In contrast, similar recovery of Glc units in WUS_{Ile} for WC and WE implied that the supplemented glucanase did not release oligomers from insoluble glucans. The maize diets presented significantly higher AX recovery in WUS_{Giz}, WUS_{Ile} and WUS_{Exc} than the wheat diets ($P < 0.05$).

3.1.2. Lignin content in WUS along the GIT

Having established the prevalence of insoluble AX throughout the GIT, we further aimed at investigating the occurrence of lignin that is known to associate with AX [12,47]. Therefore, lignin was selectively quantified by using a recently developed pyrolysis-GC–MS technique relying on the use of uniformly ^{13}C labelled lignin as internal standard [40]. Pyrolysis products deriving from both lignin and aromatic amino acids or from both lignin and ferulic acid were excluded [39]. Although this method has been shown to accurately quantify lignin in lignified grass tissues [39], it should still be further validated for cereal grains and

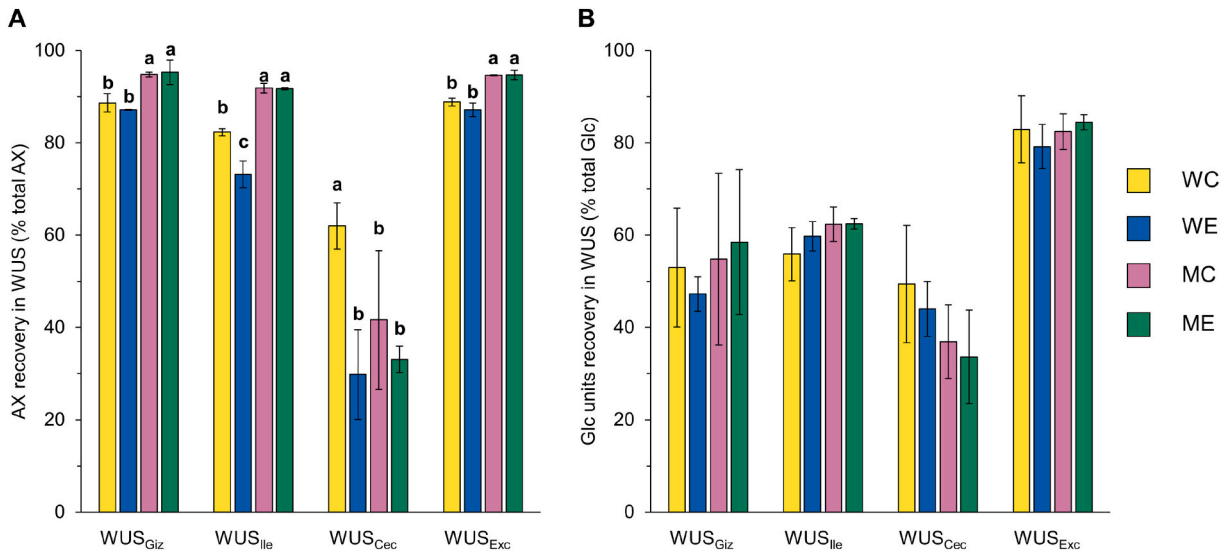

Fig. 2. Recovery of arabinoxylan (AX) (A) and Glc units (B) in WUS obtained from the gizzard (WUS_{Giz}), ileum (WUS_{Ile}), ceca (WUS_{Cec}) and excreta (WUS_{Exc}) corresponding to wheat control (WC), wheat enzyme (WE), maize control (MC) and maize enzyme (ME) diets, expressed as percentage of the total AX and Glc in whole digesta, respectively. Bars within GIT location not sharing common notation differ significantly ($P < 0.05$). The error bars indicate standard deviation ($n = 3$).

WUS from digesta samples before absolute lignin contents can be considered fully reliable. Nevertheless, the method can certainly be used to accurately and specifically monitor lignin presence throughout the GIT in a relative sense. The lignin contents for WUS_{Feed}, WUS_{Giz}, WUS_{Ile}, WUS_{Cec} and WUS_{Exc} are presented in Table 2. The values obtained for WUS_{Feed} after exclusion of the aforementioned pyrolysis products ranged between 0.08 and 0.12 % (w/w). For comparison, the values obtained including 4-vinylguaiacol were approximately two to nine times higher across WUS samples (Table S3). Interestingly, the values reported in Table 2 for WUS_{Feed} were tenfold lower than the Klason lignin values reported in broiler diets of similar composition [38] as well as those reported for wheat and maize whole grains and soybean meal [1]. As explained above, the current approach determined lignin in a more relative sense and revealed the specific lignin structures present in broiler digesta, while gravimetric methods provide an overall lignin content value, which can easily be influenced by the presence of other non-lignin acid-insoluble components [48].

Comparison between diets demonstrated that the lignin content in WUS_{Feed} tended to be higher for WC and WE, compared to MC and ME ($P < 0.1$) (Table 2). Next, the highest lignin content was found in WUS_{Giz}, ranging between 1.65 % and 2.10 % (w/w) (Table 2). The higher lignin content of WUS_{Giz} compared to WUS_{Feed} demonstrated that coarse, insoluble feed particles that are known to accumulate in the gizzard [49] contained relatively high lignin contents. The lignin contents in WUS_{Ile} were two to six times lower than in WUS_{Giz}. In particular, WC and WE presented significantly higher lignin content than both MC and ME in WUS_{Ile} and ME in WUS_{Exc} ($P < 0.05$), possibly corresponding to the higher lignin content of wheat diets (WUS_{Feed}). WUS_{ceca} presented relatively low lignin content compared to WUS from other GIT locations, specifically indicating the presence of insoluble feed-derived fiber in the ceca, that may have entered alongside soluble feed components [21,49].

Table 2
Lignin content (% w/w) in WUS obtained from the diet (WUS_{Feed}), gizzard (WUS_{Giz}), ileum (WUS_{Ile}), ceca (WUS_{Cec}) and excreta (WUS_{Exc}) ($n = 3$) corresponding to wheat control (WC), wheat enzyme (WE), maize control (MC) and maize enzyme (ME) diets, and determined based on lignin-specific pyrolysis products (Table S2) by ^{13}C-IS pyrolysis-GC–MS and excluding H-units and 4-vinylguaiacol.

Lignin content (% w/w)					
Diet	WUS_{Feed}[1]	WUS_{Giz}	WUS_{Ile}	WUS_{Cec}	WUS_{Exc}
WC	0.12	1.87	0.71^{a}	0.11^{a}	0.62^{a}
WE	0.17	1.66	0.71^{a}	0.11^{a}	0.60^{a}
MC	0.09	1.65	0.43^{b}	0.06^{b}	0.45^{ab}
ME	0.08	2.10	0.37^{b}	0.06^{b}	0.28^{b}
SEM[2]	0.03	0.42	0.06	0.01	0.08
P value	0.063	0.683	<0.001	<0.001	0.008

Values within GIT location not sharing common superscript letter differ significantly ($P < 0.05$).
[1] Mean values of analytical triplicates.
[2] Standard error of the mean, $n = 3$.

3.1.3. Insoluble AX and lignin contents along the GIT revealed fiber transit patterns

To the best of our knowledge, this is the first study to selectively determine the lignin contents of broiler digesta. The ^{13}C-IS pyrolysis-GC–MS analysis permitted us to use lignin as an indicator of the presence of insoluble cell wall material along the broiler GIT. For that, the weight ratio of AX and lignin (AX/L) in WUS was determined to obtain more insight into the chemical composition of insoluble cell wall components along the GIT (Fig. 3A). In addition, the ratio between syringyl (S) to guaiacyl (G) units (S/G), comprising the main lignin subunits in cereal grains [50], was evaluated to investigate lignin structure along the GIT (Fig. 3B).

Our analyses showed that AX/L values significantly depended on both diet and GIT location ($P < 0.001$). The low AX/L values obtained for WUS_{Giz} indicated that relatively more lignin than insoluble AX was present in the gizzard compared to WUS_{Feed}. WUS_{Giz} also presented lower S/G ratios (0.4–0.6) than WUS_{Feed} (0.6–1.1), pointing at the accumulation of a specific lignin fraction in this GIT segment. Interestingly, both content-wise and structure-wise, lignin in WUS_{Giz} is expected to increase fiber recalcitrance, as high S/G ratios have been associated with higher enzymatic lignocellulose degradation [10,51]. Both AX/L and S/G values increased in WUS_{Ile} compared to WUS_{Giz}. Still, wheat diets presented lower AX/L values in WUS_{Ile} than in WUS_{Feed}, while both maize diets presented similar AX/L values between WUS_{Ile} and WUS_{Feed}. This difference between diets was most likely due to the higher proportion of insoluble AX and lower lignin content determined for MC and ME compared to WC and WE (Fig. 2; Table 2). Overall, WUS_{Ile} appeared to contain the main insoluble AX and lignin fractions present in the diet. In contrast, WUS_{Giz} seemed to represent a specific fiber fraction originating from the diet that was characterized by relatively low AX/L, S/G as well as Ara/Xyl values (Table 1; Fig. 3). WUS_{Exc} presented similar AX/

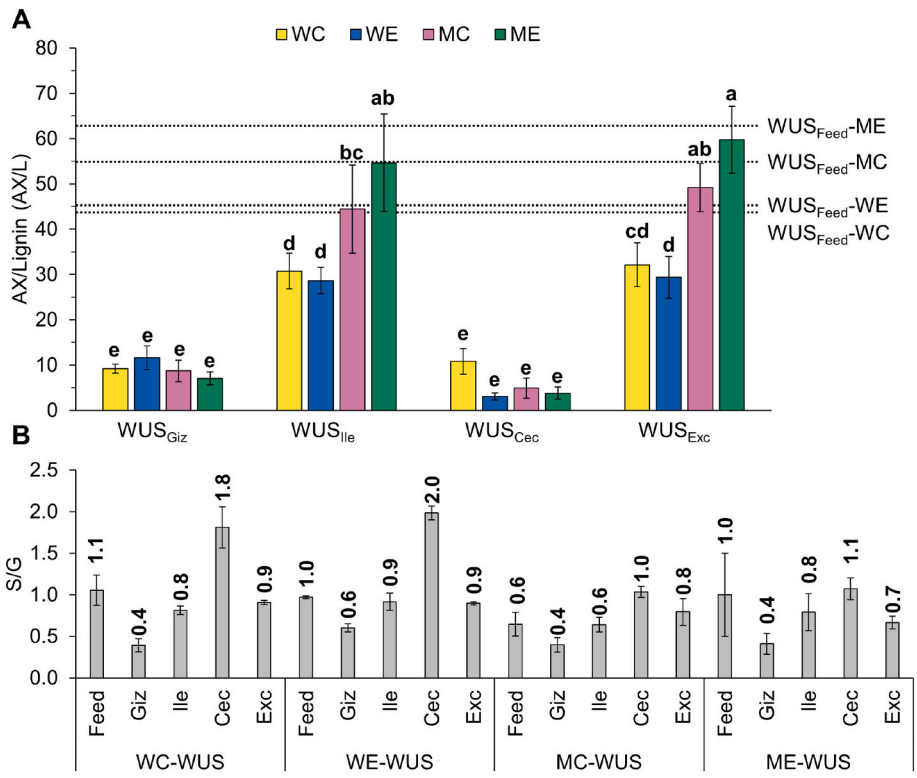

Fig. 3. Arabinoxylan (AX) to lignin (L) weight ratio (AX/L) (A) in WUS at different GIT locations. Bars corresponding to wheat control (WC), wheat enzyme (WE), maize control (MC) and maize enzyme (ME) diets across GIT locations (interaction effect Diet x GIT location, $P < 0.001$) not sharing common notation differ significantly ($P < 0.05$). Syringyl to guaiacyl units molar ratio (S/G) (B) in WUS obtained from the gizzard (WUS_{Giz}), ileum (WUS_{Ile}), ceca (WUS_{Cec}) and excreta (WUS_{Exc}). Error bars indicate standard deviations ($n = 3$). AX/L values in WUS_{Feed} (WC: 44.5 ± 8.1, WE: 44.1 ± 16.1, MC: 55.2 ± 5.7, ME: 63.2 ± 2.9) are shown as dashed lines (A), for comparison.

L and S/G values compared to WUS_{Ile} (Fig. 3), indicating that insoluble fiber complexes reaching the ileum were excreted unfermented. Previous research reported >98 % recovery of dietary lignin in broiler and ostrich excreta, as determined by non-specific gravimetric methods [52,53]. Although a lignin fraction presenting high S/G values was present in the ceca, our observations for WUS_{Ile} and WUS_{Exc} further confirmed the low fermentability of insoluble NSP in the broiler hindgut [21,23,49]. Finally, AX/L values were not found to be influenced by enzyme supplementation. Overall, by measuring both NSP and lignin in digesta, this work highlights the importance of interactions between cell wall components for NSP fermentation in boilers.

3.2. Influence of enzyme supplementation on the extractability by alkali of AX from WUS

Having established that WUS_{Ile} and WUS_{Exc} were representing insoluble fibers from the feed, these two WUS fractions were subjected to alkali extraction to further investigate the impact of enzyme supplementation on insoluble NSP structure. In a previous study, alkali extraction (6 M NaOH) of broiler excreta, followed by dialysis with 14 kDa membranes, resulted in significant loss of alkali-extractable oligosaccharides and polysaccharides [23]. To prevent such losses in our study, the 1 M NaOH extract was not subjected to dialysis, but instead was desalted with 3.5 kDa centrifugal filter units, resulting in a salt-free retentate (1M-ASS_R) and a salt-rich filtrate (Fig. 1). The salt from the filtrate that may also potentially contain 'alkali-extractable' oligomers was removed by SPE to yield the salt-free fraction 1M-ASS_F. Overall, 70–78 % (w/w) of OM in WUS was recovered during alkali extraction, regardless of diet (Fig. S2). The considerable losses of OM (25–30 % w/w) observed were partly attributed to the low amounts of starting material influencing handling accuracy. Most of alkali-extractable OM from WUS was extracted with 1 M NaOH, with 17–24 % (w/w) of OM being recovered in 1M-ASS_R, and 4–7 % (w/w) of OM being recovered in 1M-ASS_F. Subsequent extraction of WUS with 4 M NaOH further solubilized 12–17 % (w/w) of OM. The alkali-unextractable residue (RES) accounted for 29–37 % (w/w) of OM.

Continuing our efforts to disentangle the fate of insoluble AX in broilers, we focused on the impact of enzyme addition on AX extractability by alkali (Table 3). AX in WUS_{Ile} and WUS_{Exc} was mainly extractable by 1 M NaOH and recovered in 1M-ASS_R (19.1–27.9 % w/w), while AX below 3.5 kDa in 1M-ASS_F accounted for <0.4 % (w/w) of AX in WUS, irrespective of diet. Sequential extraction with 4 M NaOH solubilized additionally 17.5%–19.9 % and 11.0–12.3 % (w/w) AX from WUS_{Ile} and WUS_{Exc}, respectively, while unextractable AX recovered in RES accounted for 30.5–40.8 % (w/w) of total AX. Previous research has shown that 90 % (w/w) wheat endosperm AX and 40–60 % (w/w) wheat

Table 3
Arabinoxylan (AX) recovery in alkali extracts (1M-ASS_F, 1M-ASS_R, 4M-ASS) and residue (RES) expressed as percentage (%) of AX in WUS_{Ile} and WUS_{Exc} corresponding to wheat control (WC), wheat enzyme (WE), maize control (MC) and maize enzyme (ME) diets.

Diet	Arabinoxylan (AX) recovery (% AX in WUS)				
WUS_{Ile}	1M-ASS_{F_Ile}	1M-ASS_{R_Ile}	4M-ASS_{Ile}	RES_{Ile}	Losses[1]
WC	0.3	24.7	17.6	31.8	25.7
WE	0.4	26.0	18.6	38.3	16.7
MC	0.3	25.4	20.0	32.6	21.7
ME	0.3	20.5	19.2	34.0	26.0
SEM[2]	0.0	2.4	0.7	1.5	3.2
P value	0.325	0.403	0.186	0.059	0.225
WUS_{Exc}	1M-ASS_{F_Exc}	1M-ASS_{R_Exc}	4M-ASS_{Exc}	RES_{Exc}	Losses[1]
WC	0.3^{ab}	24.4	11.0	30.8^{b}	33.4
WE	0.4^{a}	27.9	12.3	40.5^{a}	18.8
MC	0.1^{c}	19.1	11.2	34.5^{ab}	35.0
ME	0.2^{bc}	21.1	11.1	31.3^{b}	36.3
SEM	0.0	2.5	1.5	1.5	3.9
P value	0.001	0.154	0.154	0.008	0.044

Values within column not sharing common superscript letter differ significantly ($P < 0.05$).
[1] Estimated by the difference between total AX in WUS and the sum of AX recovered in the different fractions.
[2] Standard error of the mean, $n = 3$.

bran and maize grain glucuronoarabinoxylan was alkali-extractable [7,8,17,19,54]. Overall, the alkali treatment of digesta containing various cereal fractions was expected to have solubilized structurally different AX populations [5,19].

In Table 3 it is shown that WC exhibited lower AX recovery in RES compared to WE in the ileum (RES_{Ile}, $P < 0.1$) and excreta (RES_{Exc}, $P < 0.05$). This meant that a relatively larger AX proportion was extracted for WC compared to WE. It is, therefore, indicated that AXOS release from insoluble AX by xylanase, previously documented for WE [33], currently resulted in the decrease of the relative amount of alkali-extractable AX in WUS. Nevertheless, this observation was not accompanied by significantly different AX recoveries in 1M-ASS_F, 1M-ASS_R and 4M-ASS between WC and WE ($P > 0.05$). On the contrary, WE presented elevated values for both 1M-ASS_{F_Ile} and 1M-ASS_{R_Ile} compared to WC, as well as for 1M-ASS_{F_Exc} and 1M-ASS_{R_Exc}. It should be noted that a considerable AX proportion (16.7–36.3 % w/w) was not recovered during extraction (Table 3; Losses), in line with the observed losses in OM (Fig. S2). WE presented numerically lower AX losses compared to WC for both WUS_{Ile} and WUS_{Exc}, but these differences did not reach statistical significance. Maize diets presented comparable AX recovery values to the wheat diets, while no differences were observed between MC and ME ($P > 0.05$).

The current findings were in agreement with previous work reporting that xylanase and cellulase pre-treatment of hardwood pulp decreased the relative amount of alkali-extractable xylan [55]. Furthermore, the decrease in the relative amount of alkali-extractable NSP from broiler excreta was previously observed when pectolytic enzymes were included in maize-rapeseed meal diets [23]. Overall, besides decreasing the relative amount of alkali-extractable AX, enzyme supplementation did not affect its extractability from WUS, as observed by the similar yields obtained for various alkali extracts, across diets (Table 3). Nevertheless, enzyme supplementation in WE did seem to decrease the estimated proportion of unrecovered AX (Losses) compared to WC in both WUS_{Ile} and WUS_{Exc}. However, the incomplete AX recovery did not permit further interpretation of those differences.

3.3. Characterization of alkali-extractable NSP

3.3.1. Molecular weight distribution in alkali extracts

The impact of enzyme addition on the structural characteristics of insoluble NSP recovered during alkali extraction was further investigated by determining the molecular weight (Mw) distribution of alkali extractable solids by HPSEC (Fig. 4). According to expectations, only compounds below 3.5 kDa were present in all 1M-ASS_F fractions. In contrast, HPSEC of 1M-ASS_R presented broad Mw distribution between 10 and 500 kDa. Despite such broad distribution, polymers with Mw distribution between 100 and 500 kDa were predominantly present in 1M-ASS_{R_Ile} and 1M-ASS_{R_Exc} for both WC and WE. Finally, polymers with Mw > 500 kDa were abundant in all 4M-ASS fractions. The signal observed between 12 and 3.5 kDa was attributed to borate salts. MC and ME presented similar chromatograms for all WUS_{Ile} and WUS_{Exc} fractions, while a more intense signal of Mw > 100 kDa compounds was noted for 1M-ASS_R and 4M-ASS compared to WC and WE. In conclusion, no effect of enzyme treatment on the Mw distribution of alkali extractable solids was observed, reflecting the similar AX recovery values obtained in the respective fractions (Table 3).

3.3.2. Oligosaccharide profiles in 1M-ASS_{F_Ile}

The 1M-ASS_{F_Ile} fractions obtained by SPE were further analyzed for oligosaccharide profiles by MALDI-TOF-MS (Fig. 5). This analysis was performed to assess the presence of enzymatically-formed oligosaccharides that remained embedded or associated in the cell wall matrix and could be released from WUS by alkali extraction, as previously suggested [23]. 1M-ASS_{F_Ile} fraction across all diets presented a homologous series

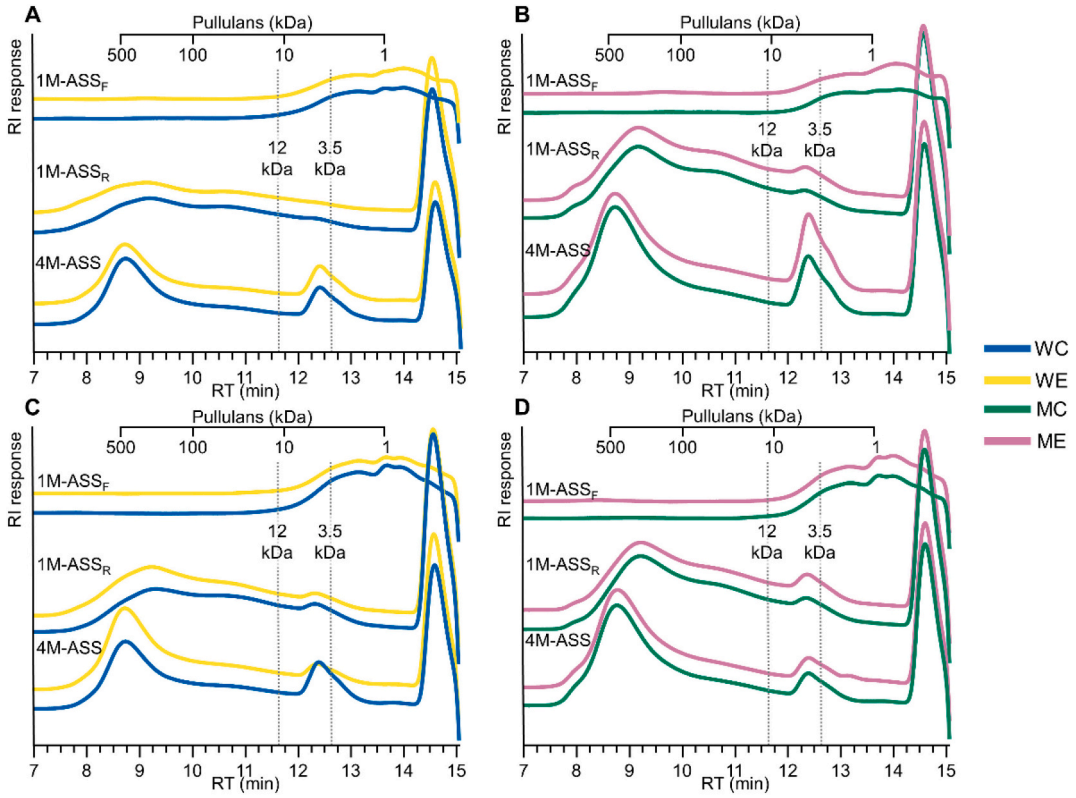

Fig. 4. HPSEC-RI chromatograms of alkali extracts obtained from WUS_{Ile} (A: wheat control (WC), wheat enzyme (WE) diets, B: maize control (MC), maize enzyme (ME) diets) and WUS_{Exc} (C: WC, WE, D: MC, ME). 1M-ASS_F were dissolved in water at 5 mg/mL whereas 1M-ASS_R and 4M-ASS were dissolved in 50 mM NaOAc (pH 5.0) at 5 mg/mL. Pullulan standards were used as calibrants.

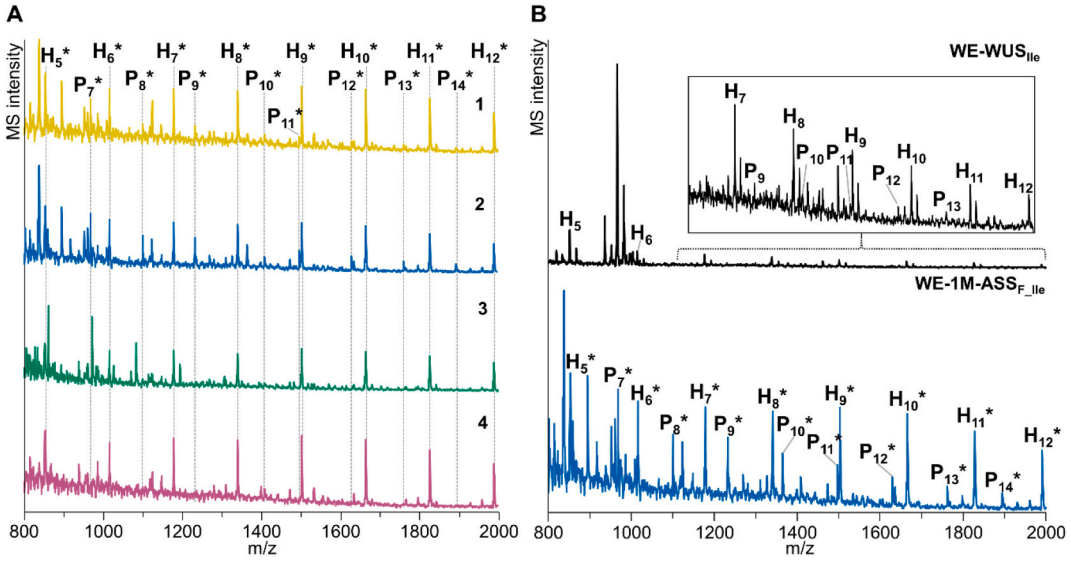

Fig. 5. MALDI-TOF mass spectra of 1M-ASS$_{F_IIe}$ solutions (A) corresponding to wheat control (WC: 1), wheat enzyme (WE: 2), maize control (MC: 3) and maize enzyme (ME: 4) diets. MALDI-TOF mass spectra (B) of WUS$_{IIe}$ dispersion (1) and 1M-ASS$_{F_IIe}$ extract (2) belonging to WE diet. The number of pentose (P_n) or hexose (H_n) units comprising the detected oligosaccharides are shown next to the *m/z* values; **m/z* values of reduced oligosaccharides formed due to the presence of $NaBH_4$ in the extractant present +2 mass increment compared to their neutral counterparts.

of hexose oligosaccharides (HexOS) with degree of polymerization (DP) 5–12. 1M-ASS$_{F_IIe}$ for WE also contained a series of pentose oligosaccharides (DP 6–14), representing AXOS [24,33]. Pentose oligomers were not detected in MC and ME 1M-ASS$_{F_IIe}$ fractions, as already indicated in our previous work [24], and presented only weak signal in WC. It therefore appeared that oligomeric AXOS accounted for part of the low Mw AX (< 3.5 kDa) recovered in 1M-ASS$_{F_IIe}$, which accounted for <0.4 % of AX in WUS$_{IIe}$ (Table 3). For WE, the presence of enzymatically-released AXOS in the MALDI-TOF mass spectrum of water dispersed WUS$_{IIe}$ (Fig. 5B), albeit at relatively low intensity, suggested that their signal became more intense after extraction and SPE purification and not that they were released from the cell wall matrix by alkali.

3.3.3. *Diverse insoluble NSP populations were present in digesta WUS*

Given the high Glc abundance in WUS (Table 1), we reported the recovery of Glc units during alkali extraction next to that of AX. As mentioned earlier, Glc in WUS represents a diverse population of glucans, such as insoluble starch, β-glucan and cellulose. Consequently, differences in Glc recovery most likely indicate differences in the relative amounts of the aforementioned glucans in WUS (Table S4). For example, the recovery above 90 % observed in RES$_{IIe}$ and RES$_{Exc}$ for MC and ME was indicative of cellulose abundance in WUS for maize diets. In contrast, WC and WE presented lower Glc recovery values in RES$_{IIe}$ (WC: 68.2 %, WE: 87.8 %, $P > 0.05$) and RES$_{Exc}$ (WC: 55.2 %, WE: 72.6 %, $P < 0.05$), highlighting the more abundant presence of glucans other than cellulose. The higher values obtained in RES for WE compared to WC suggested that lower amount of glucans remained insoluble upon enzyme addition in WE. This could be mainly explained by the higher starch digestibility observed for WE [24], as glucanase action did not seem to impact non-starch Glc solubility (Fig. 2). Swelling of RES by alkali could have caused more complete cellulose hydrolysis during sugar composition analysis compared to WUS, thus resulting in >100 % glucan recovery [56]. Further research is warranted to determine the impact of enzyme supplementation on the extractability of specific glucans.

Except for AX and glucan, other carbohydrates were extracted as well (Table S5, S6). Overall, 1M-ASS$_F$ extracts from WUS$_{IIe}$ and WUS$_{Exc}$ presented low total carbohydrate contents (7.7–11.6 % w/w), and were mainly composed of Glc (26–41 mol%), Gal (21–28 mol%), Man (18–26 mol%) and to a lower extent of Ara (7–13 mol%) and Xyl (2–10 mol%). Co-extraction of soybean NSP is expected to have confounded the final AX values, as evidenced by the unrealistically high Ara/Xyl values for AX in the 1M-ASS$_F$ fractions (Ara/Xyl = 1.3–4.1), especially for the maize diets. Higher carbohydrate contents were obtained in 1M-ASS$_R$ corresponding to WUS$_{IIe}$ (63.5–71.3 % w/w OM) and WUS$_{Exc}$ (39.3–61.4 % w/w OM) across all diets and GIT locations. For both WUS$_{IIe}$ and WUS$_{Exc}$, Ara and Xyl represented 20–28 mol% and 29–41 mol% of extracted carbohydrates, respectively. This indicated the prevalence in 1M-ASS$_R$ of AX with Ara/Xyl value of 0.6 and 0.9 for the wheat and maize diets, respectively. The lower Ara/Xyl values observed for 1M-ASS$_R$ compared to the corresponding WUS (Table 1) implied the extraction with 1 M NaOH of AX populations with relative low degree of Ara-substitution. Such AX populations could have partly been released from the aleurone layer of cereal grains upon cleavage of alkali-labile bonds (*e.g.* ferulate/diferulate cross-links) [5]. However, it should be noted that AX with Ara/Xyl ~0.6 has previously been extracted with 1 M NaOH from wheat endosperm WUS [19]. No additional differences in monosaccharide composition were observed between WC and WE and MC and ME. Finally, co-extraction of other NSP such as soybean arabinogalactans and β-glucan from wheat [16,17] from both WUS$_{IIe}$ and WUS$_{Exc}$ was evidenced by the abundant presence of Gal (12–22 mol%) and Glc (17–30 mol%).

The carbohydrates extracted in 4M-ASS from both WUS$_{IIe}$ and WUS$_{Exc}$ presented different amounts for wheat (41.8–54.8 % w/w) and maize (78.3–88.5 % w/w) diets, but similar carbohydrate composition (Table S5, S6). The main constituent monosaccharides were Ara (31–33 mol%), Xyl (27–30 mol%) and Gal (18–24 mol%), while Glc was less abundant (11–16 mol%) and Man and Rha were minorly present (< 4 mol%). Previously, the use of 4 M NaOH as final extractant in sequential extraction procedures solubilized a minor AX fraction (<10 %) from wheat flour, while it resulted in a hemicellulose-rich extract from soybean [16,17]. Overall, the similarities between 4M-ASS fractions from wheat and maize diets in terms of monosaccharide composition, Mw distribution and Ara/Xyl ratio (1.1–1.2) suggested the low prevalence of AX in this fraction. Finally, Glc prevalence in RES (49–55 mol%) was indicative of cellulose, while Ara (13–19 mol%) and Xyl (15–18 mol%) represented alkali-unextractable AX [8,16,17].

3.4. Implications of enzyme supplementation for insoluble cell wall polysaccharides in broiler digesta

The importance of cereal cell wall architecture for NSP utilization in broilers was currently reflected by the high proportion of insoluble AX present in digesta WUS (Fig. 2). Our work indicated that most AX present in the feed was not fermented in the broiler GIT, in line with recent findings [38]. Apart from limiting NSP fermentation, the cell wall matrix has been postulated to hinder nutrient digestibility as well [22]. AX extraction from WUS by alkali indicated that AX insolubility in digesta is mainly attributed to covalent AX-AX and AX-lignin associations *via* ferulate and di-ferulate cross-links, as well as to non-covalent interactions with cellulose [11–13]. This conclusion is supported by the occurrence of such covalent cross-links in cereal grains [12,13] and by the presence of bound hydroxycinnamic acids (*p*-coumarate, ferulate and diferulates) in digesta WUS, as confirmed for WC (Fig. S3). The abovementioned interactions were also expected to affect the action of dietary enzymes, as previously shown for xylanases [57]. Being that the case, the degradation of insoluble AX degradation by dietary xylanase was mainly reflected by AXOS release *in vivo*, as covered in our recent work [33]. However, AX depolymerization did not further impact insoluble AX extractability by alkali, under the present experimental conditions (Table 3). These findings infer that dietary enzymes play a more important role in promoting feed utilization by improving the fermentability of *insoluble* NSP, rather than by offsetting the encapsulating effect of the cell wall matrix, in line with previous studies [22,58]. Overall, further *in vitro* and *in vivo* research focusing on both NSP degradation and nutrient (*e.g.* starch, protein) digestion kinetics is warranted, in order to better understand the function of dietary enzymes. From this perspective, consideration of the interactions between cell wall components can further progress the application of NSP-degrading enzymes as means to improve NSP fermentability and promote intestinal health in broilers. Finally, optimization of NSP extraction and lignin analysis is necessary to further study the role of insoluble fibers in animal nutrition.

4. Conclusions

This study provided novel insights into insoluble arabinoxylan and lignin content and structure along the GIT of broilers fed wheat and maize diets. Investigating the chemical composition of cell wall material present in digesta demonstrated that lignin-rich fibers accumulated in the gizzard. Insoluble fibers reaching the ileum were unfermented in the broiler hindgut. Despite improving AX fermentability in the wheat diet, enzyme supplementation did not improve NSP extraction by alkali, in the current study. Consequently, associations between cell wall components that render the majority of AX insoluble and could potentially hinder nutrient digestibility, appeared unaffected by enzyme supplementation. Our work highlights that further research is needed to improve the utilization of insoluble NSP in broilers.

CRediT authorship contribution statement

Dimitrios Kouzounis: Conceptualization, Methodology, Formal analysis, Visualization, Writing – original draft. **Gijs van Erven:** Methodology, Formal analysis, Writing – review & editing. **Natalia Soares:** Resources, Writing – review & editing. **Mirjam A. Kabel:** Conceptualization, Supervision, Writing – review & editing. **Henk A. Schols:** Conceptualization, Supervision, Writing – review & editing.

Declaration of competing interest

Natalia Soares is employed by the funder. All other authors declare that they have no competing interest.

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Appendix A. Supplementary information

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