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In vivo formation of arabinoxylo-oligosaccharides by dietary endo-xylanase alters arabinoxylan utilization in broilers

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ABSTRACT

Previously, arabinoxylan (AX) depolymerization by dietary endo-xylanase was observed in the broiler ileum, but released arabinoxylo-oligosaccharides (AXOS) were not characterized in detail. This study aimed at extracting and identifying AXOS released *in vivo* in broilers, in order to delineate the influence of endo-xylanase on AX utilization. Hereto, digesta from the gizzard, ileum, ceca and excreta of broilers fed a wheat-soybean diet without (Con) or with endo-xylanase supplementation (Enz) were assessed. Soluble AX content in the ileum was higher for Enz diet (26.9%) than for Con diet (18.8%), indicating a different type and amount of AX entering the ceca. Removal of maltodextrins and fructans enabled monitoring of AX depolymerization to AXOS (Enz diet) using HPSEC-RI and HPAEC-PAD. A recently developed HILIC-MS^n methodology allowed AXOS (DP 4–10) identification in ileal digesta and excreta. Xylanase-induced AXOS formation coincided with decreased total tract AX recovery, which indicated improved AX hindgut utilization.

1. Introduction

Wheat grains are a major starch source in poultry nutrition. Next to starch, wheat grains contain about 9% non-starch polysaccharides (NSP) present in the cell walls (Bach Knudsen, 2014; Black, 2016). Arabinoxylan (AX) is the most abundant NSP, accounting for 5–7% of the grain dry matter (Saulnier et al., 2007). AX is a well characterized polymer composed of a β-(1 → 4) linked D-xylosyl (Xyl) backbone, mainly substituted with L-arabinosyl (Ara) units. For endosperm AX, approximately 66% of all Xyl residues are unsubstituted, 21% are mono-substituted mainly by *O*-3-linked Ara, while doubly substituted Xyl by Ara at both *O*-2 and *O*-3 account for 13% of backbone Xyl residues. *O*-2-linked Ara may also be present in minor amounts (Gruppen et al., 1992; Izydorczyk & Biliaderis, 1995; Saulnier et al., 2007). AX from wheat aleurone and pericarp tissues may present different Ara-substitution pattern compared to endosperm AX, and can be further substituted with 4-*O*-methyl-D-glucuronyl, acetyl and/or feruloyl moieties (Izydorczyk & Biliaderis, 1995; Saulnier et al., 2007).

Polymeric AX is reported to increase intestinal viscosity and hinder nutrient digestibility, thus negatively influencing broiler growth (Bedford, 2018; Choct & Annison, 1992). Nonetheless, certain soluble AX fractions can be readily fermented to short chain fatty acids (SCFAs) by gut microbiota in the ceca, thus benefiting the host (Pan & Yu, 2013; Sergeant et al., 2014; Svihus et al., 2013). Endo-xylanase supplementation has been a successful strategy to offset the aforementioned anti-nutritive effects of AX, predominantly in wheat-based diets. Xylanase-mediated improvements in nutrient digestibility and broiler performance have been associated with reduced digesta viscosity, decreased nutrient encapsulation by the cereal cell wall matrix and increased AX fermentation to SCFAs (Bedford, 2018; Bedford & Classen, 1992; Masey-O'Neill et al., 2014).

Endo-xylanases hydrolyze the β-(1 → 4) linkage between two Xyl

Abbreviations: AX, arabinoxylan; AXOS, arabinoxylo-oligosaccharides; XOS, xylo-oligosaccharides; DP, degree of polymerization; WES, water-extractable solids; WUS, water-unextractable solids; GH, glycosyl hydrolase; AMG, amyloglucosidase; FRM, fructanase; TFA, trifluoroacetic acid; SPE, solid phase extraction; W, SPE fraction containing analytes eluting with water; M, SPE fraction containing analytes eluting with 30% methanol; HPAEC-PAD, high-performance anion exchange chromatography with pulsed amperometric detection; HPSEC-RI, high-performance size-exclusion chromatography with refractive index detection; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; HILIC-ESI-CID-MS^n, hydrophilic interaction liquid chromatography - electrospray ionization - collision-induced dissociation - tandem mass spectrometry.

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residues of the AX backbone, depolymerizing AX and releasing (arabino) xylo-oligosaccharides ((A)XOS) (Biely et al., 1997; Kormelink et al., 1993). The (A)XOS formed are known for their prebiotic properties for humans (Broekaert et al., 2011). In particular, it has been demonstrated that the beneficial impact of (A)XOS on hindgut fermentation depends on their chemical structure (*i.e.* degree of polymerization (DP), degree/type of substitution) (Broekaert et al., 2011; Mendis et al., 2018). Similar to human studies, (A)XOS supplementation resulted in bifidobacterial growth as well as pronounced SCFAs formation in the broiler ceca (Courtin et al., 2008; Morgan et al., 2019). Likewise, endo-xylanase supplementation has been shown to influence microbiota ecology and to promote cecal SCFAs formation (Masey-O'Neill et al., 2014; Morgan et al., 2019; Singh et al., 2021). Such observations outline that improvement of hindgut fermentation can be expected as a result of the xylanase-driven degradation of AX to (A)XOS *in vivo*. Indeed, several studies have reported AX solubilization and size reduction upon endo-xylanase supplementation in broilers (Bautil et al., 2021; Choct et al., 2004; Lee et al., 2017). However, a detailed characterization of *in vivo* released AXOS has yet to be performed, mainly due to the challenge of analyzing such structures in complex digesta matrices.

Recently, we demonstrated that endo-xylanase supplementation in a wheat-based diet led to *in vivo* formation of pentose oligomers with degree of polymerization (DP) of 5–26 in the proximal GIT of broilers (Kouzounis et al., 2021). The current research aims at defining the detailed structure of AXOS released by dietary endo-xylanase in the broiler GIT. It is hypothesized that the detailed characterization of AXOS formed *in vivo* by dietary endo-xylanase will improve our understanding on how such dietary enzymes may promote AX fermentation in broilers. This work is considered to contribute to the more comprehensive study of carbohydrate fermentation in animals, and is of interest for academia, policy makers and the industry.

2. Materials and methods

Wheat flour arabinoxylan (medium viscosity) (WAX), linear XOS standards (DP 2–6; X_2-X_6), amyloglucosidase from *Aspergillus niger* (AMG) and fructanase preparation (FRM) were obtained from Megazyme (Bray, Ireland). Hostazym X (HX) containing a GH11 endo-1,4-β-xylanase (EC 3.2.1.8) from *Trichoderma sp.* was from Huvepharma NV (Berchem, Belgium). All reagents used were of analytical grade and were obtained by either Sigma Aldrich (St. Louis, MO, USA) or Merck (Darmstadt, Germany), 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 and excreta samples were obtained from our recent work (Kouzounis et al., 2021). The animal study 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, 48 one-day old male broilers were reared in a floor pen and were fed wheat-soybean starter (day 0–10) and grower (day 10–20) diets. On day 20, the birds were allocated to pens and were assigned to control (Con) or enzyme (Enz) diets following a randomized block design. Each diet consisted of 6 replicate pens, with 4 birds per pen. The wheat-soybean finisher diet (Table S1) was provided as such (Con) or supplemented with a commercially available enzyme preparation (Enz) 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 S2). The excreta were collected per pen, daily (twice) between day 24 and 28 and immediately stored at −20 °C. On day 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.

2.2. Aqueous extraction of broiler digesta

Approximately 1000 mg digesta was mixed with 35 mL water heated at 99 °C and was incubated at 99 °C for 20 min, with continuous mixing. Next, the mixture was centrifuged at 30,000 ×*g*, 20 min and the supernatant was filtered over 595 Whatman® filter paper. The residue was added to 30 mL water, mixed thoroughly and centrifuged at 30,000 ×*g*, 20 min. The washing step was repeated once and the resulting supernatants were combined with the filtrate. The extracts and residue were freeze-dried, and the weight of the water extractable solids (WES) and water unextractable solids (WUS) was recorded. All fractions were homogenized with a MM 400 Mixer Mill.

2.3. Amyloglucosidase and fructanase treatment of WES from digesta

A combination of AMG/FRM was applied to remove maltodextrins and inulin from WES from all digesta samples (*i.e.* gizzard, ileum, ceca and excreta). The individual activity of AMG and FRM was predetermined on WES from the ileum (see supplement 1.1). Potential side-activity of AMG and FRM on raffinose series oligosaccharides from soybean was also evaluated (see supplement 1.2). Hereto, WES was dissolved in MilliQ water (10 mg/mL), helped by heating at 80 °C for 30 min. The resulting solutions were cooled at ambient temperature, centrifuged at 20,000 ×*g* for 10 min, and 0.25 mL supernatant was diluted with 4.5 mL 50 mM NaOAc buffer solution (pH 5.0). Subsequently, pre-diluted AMG and FRM solutions were added (0.125 mL each). AMG and FRM were added in excess to ensure total or 'end-point' degradation. The mixture was incubated at 50 °C, overnight under continuous mixing. In parallel, 0.05 mL WES-in-buffer-solution was mixed with 0.95 mL NaOAc and incubated under otherwise similar conditions, but without AMG/FRM addition. The enzymatic reactions were stopped by heating at 99 °C for 15 min, and the samples were cooled at ambient temperature. The resulting solutions were then subjected to solid phase extraction (SPE).

2.4. Reversed-phase (C18) SPE of AMG/FRM-treated WES

SPE with C18 stationary phase was used to remove enzymatically released hexoses (see 2.3) from AMG/FRM-treated WES from the gizzard (G_{WES}), ileum (I_{WES}), ceca (C_{WES}) and excreta (E_{WES}), while retaining oligomers and polymers, and was performed as described elsewhere (Jonathan et al., 2017). Sep-Pak® C18 cartridges (6 cc, 1 g, Waters Corp., Milford, MA, USA) were activated with 80% (v/v) methanol (MeOH) and subsequently washed with water (5 mL; 3 times, respectively). The samples were quantitatively loaded onto the cartridges and washed with water (5 mL; 3 times). The effluents during the loading and washing steps were pooled. Next, C18-bound analytes were eluted with 30% (v/v) MeOH (5 mL; 2 times). Per sample combined water fractions (coded: G_{WES}-W, I_{WES}-W, C_{WES}-W and E_{WES}-W) were freeze dried. Per sample combined 30% MeOH fractions (coded: G_{WES}-M, I_{WES}-M, C_{WES}-M and E_{WES}-M) were dried under a constant air stream with a sample concentrator. The dried W and M fractions were dissolved in 1 mL water, centrifuged at 20,000 ×*g* for 10 min, and were analyzed for molecular weight distribution by HPSEC (as is), for their oligosaccharide profile by HPAEC (5 times dilution) and for sugar composition after methanolysis by HPAEC (see 2.6).

2.5. Preparative C18-SPE of AMG/FRM-treated pooled ileum and excreta WES

In a separate experiment, 150 mg ileum or excreta WES from the three biological replicates were pooled together (coded: IP_{WES}, EP_{WES}, respectively) and dissolved in 450 mL 50 mM NaOAc (pH 5.0) by heating at 80 °C for 30 min. Upon cooling at ambient temperature, AMG and FRM were added in excess and the solution was incubated overnight at 50 °C. The enzymatic reactions were stopped by heating at 99 °C for 15 min, and the samples were cooled at ambient temperature. Next, the samples were subjected to SPE, as follows; The Sep-Pak® C18 cartridges (35 cc, 10 g, Waters Corp.) were activated with 100 mL 80% (v/v) MeOH and subsequently washed with 100 mL water. The samples were quantitatively loaded onto the cartridges and washed with 100 mL water. The effluents during the loading and washing steps were pooled. Next, C18-bound analytes were eluted with 30% (v/v) MeOH (35 mL; 2 times). Eluates were dried under a constant air stream overnight followed by lyophilization, and the obtained solids were labelled as IP_{WES}-M and EP_{WES}-M. IP_{WES}-M and EP_{WES}-M were dissolved in water at 10 mg/mL, and the derived solutions were subjected to sodium borohydride ($NaBH_4$) reduction (see 2.10), followed by HILIC-MS^n analysis.

2.6. Neutral carbohydrate content and composition analysis

The neutral sugar composition of whole digesta and WUS fractions thereof, was determined after pre-hydrolysis with 72% (w/w) H_2SO_4 (1 h, 30 °C) and hydrolysis with 1 M H_2SO_4 (3 h, 100 °C), followed by derivatization of the released sugars to alditol acetates and subsequent analysis by gas-chromatography (Broxterman & Schols, 2018; Englyst & Cummings, 1984).

The neutral sugar composition of WES, and the SPE fractions thereof, was determined by methanolysis followed by TFA hydrolysis (Broxterman & Schols, 2018). Due to fructose (Fru) instability during methanolysis and TFA hydrolysis, the Fru content in WES was determined enzymatically according to Stöber et al. (2004), with certain modifications. 100 μL aliquots of 10 mg/mL WES solutions were mixed with 892 μL of 55 mM NaOAc buffer (pH 4.5), followed by the addition of 8 μL FRM. The mixtures were incubated at 60 °C for 1 h, and the reaction was stopped by heating at 99 °C for 15 min. The free monosaccharide content of untreated samples, and the monosaccharide composition of methanolized and FRM-treated WES, as well as that of methanolized SPE fractions was determined by HPAEC-PAD. Samples were dissolved/diluted in water prior to injection to achieve individual monosaccharide concentrations of 1–50 μg/mL. For quantification, a standard solution containing arabinose, xylose, glucose, fructose, galactose, mannose, fucose and rhamnose was prepared, treated similarly to unknown samples and diluted in the range of 1–50 μg/mL.

2.7. Monosaccharide analysis and oligosaccharide profiling by HPAEC-PAD

Monosaccharide composition and oligosaccharide profiles were determined by High-Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) using a ICS7000 HPLC system (Dionex, Sunnyvale, CA) with and ICS7000 ED PAD detector (Dionex), and 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.). The column temperature was set at 20 °C and the injection volume was 10 μL.

For monosaccharide analysis, three mobile phases were used: A) 0.1 M NaOH, B) 1 M NaOAc in 0.1 M NaOH and C) Water. Neutral monosaccharides were eluted at 0.4 mL/min with 100% C (isocratic) for 0–35 min, with post column addition of 0.5 M NaOH (0.1 mL/min) to enable PAD detection. The subsequent elution profile was: 35.1–50 min linear gradient 0–40% B (100–60% A); 50.1–55 min isocratic 100% B, 55.1–63 min isocratic 100% A; 63.1–78 min isocratic 100% C with post column addition.

For oligosaccharide characterization, 0.1 M sodium hydroxide (NaOH) (A) and 1 M sodium acetate in 0.1 M NaOH (B) were used as mobile phases. The flow rate was 0.3 mL/min. The separation was performed by using the following elution profile: 0–32 min from 0% to 38% B (linear gradient), 32–37 min from 32% to 100% B, 37–42 min at 100% B (isocratic), 42–42.1 min to 100% A (linear gradient) and 42.1–55 min 100% A (isocratic). Linear XOS were identified based on elution of analytical standards (Megazyme).

2.8. Molecular weight distribution analysis by HPSEC-RI

The molecular weight distribution was determined by high performance size-exclusion chromatography with refractive index detection (HPSEC-RI) as described elsewhere (van Gool et al., 2012), with an Ultimate 3000 HPLC System (Dionex Corp., Sunnyvale, CA, USA) equipped with a set of three TSK-Gel Super columns 4000AW, 3000AW, and 2500AW (6 mm ID × 150 mm per column, 6 μm), and a TSK Super AW-L guard column (4.6 mm ID × 35 mm, 7 μm) (Tosoh Bioscience Tokyo, Japan). The HPLC system was coupled to a Shodex RI-101 refractive index detector (Showa Denko KK, Kawasaki, Japan). The system was calibrated using a pullulan series of known Mw.

2.9. Oligosaccharide profiling by MALDI-TOF-MS

Oligomeric characterization by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was carried out as described elsewhere (Kouzounis et al., 2021), with an ultrafleXtreme™ MALDI-TOF/TOF mass spectrometer (Bruker Daltonics Inc., Billerica, MA, USA) operated at mass range *m/z* 900–2700. Mass calibration was performed with maltodextrins (Avebe B.V., the Netherlands).

2.10. Identification of (reduced) (A)XOS by HILIC-ESI-CID-MS^n

Structural analysis of AXOS and XOS isomers present in IP_{WES}-M and EP_{WES}-M solutions (10 mg/mL) (Section 2.5) was conducted by hydrophilic interaction chromatography - electrospray ionization - collision induced dissociation - tandem mass spectrometry (HILIC-ESI-CID-MS^n), according to Kouzounis et al. (2022). In brief, oligosaccharide reduction was performed by mixing 500 μL aliquots with 500 μL 0.5 M $NaBH_4$ in 1 M NH_4OH (4 h, ambient temperature), and was followed by acidification and sample clean-up (Supelclean™ ENVI-Carb™ SPE). Recovered analytes were dissolved in 100 μL 50% ACN and reduced oligosaccharides were analyzed with a Vanquish UHPLC system (Thermo Scientific, Waltham, MA, USA), equipped with an Acquity UPLC BEH Amide column (Waters, Milford, MA, USA; 1.7 μm, 2.1 mm ID × 150 mm) and a VanGuard pre-column (Waters; 1.7 μm, 2.1 mm ID × 50 mm), coupled with an LTQ Velos Pro mass spectrometer (Thermo Scientific). The mass spectrometer was equipped with a heated ESI probe, was operated in negative ion mode, and was run at three modes: Full MS (*m/z* 150–2000), MS^2 on selected MS ions, and MS^3 on selected MS^2 ions (Kouzounis et al., 2022). Mass spectrometric data were processed by using Xcalibur 2.2 software (Thermo Scientific).

2.11. AX distribution in WES and WUS and marker-based AX recovery

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

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

where $AX_{WE,WU}$ is the measured AX content (% dry matter) in WES or WUS of digesta from the gizzard, ileum, ceca and excreta. WES % and

WUS % represent the fraction of WES and WUS, out of whole digesta (% dry matter). AX recovery in WUS (WU--AX) was determined in a similar manner.

The proportion of AX (sum of Ara and Xyl) ingested by broilers, that was recovered in WES (WE--AX) in the ileum and excreta, was determined, according to Eq. (2):

$$marker\text{-}based\ WE-AX\ (\%) = AX_{Rec} * \frac{WE-AX}{100} \quad (2)$$

where AX_{Rec} (%) is the recovery of total AX in the ileum or excreta previously determined using acid insoluble ash (AIA) as a digestibility marker, as described in our recent publication (Kouzounis et al., 2021). Mean values of AX_{Rec} for Con and Enz are given in Table S3. WE-AX (%) was determined by Eq. (1). Marker-based AX recovery in WUS (WU--AX) was determined in a similar manner.

2.12. Statistical analysis

The obtained data was subjected to analysis of variance (ANOVA) using R version 4.0.2 (R Core Team), with pen being the experimental unit. Statistical analysis was carried out for each sampling site (gizzard, ileum, ceca, excreta) separately. Arabinoxylan solubilization and arabinose-to-xylose (Ara/Xyl) ratio were modelled using diet (D; Con or Enz) and fraction (F_{aq}; WES, WUS) as main effects including their two-way interaction term. Monosaccharide recovery during SPE of WES samples was modelled using D and SPE fraction (F_{SPE}; water (W), 30% (v/v) methanol (M)) as main effects including their two-way interaction term. Normality of data residuals and homogeneity of variance were additionally checked. The significance of differences between treatments was determined by Fisher's least significant difference procedure, with a significance threshold set at $p < 0.05$.

3. Results and discussion

3.1. Soluble carbohydrate level and composition varied along the GIT

The first step to characterize AX and xylanase-released AXOS was to subject digesta samples from the gizzard, ileum, ceca and excreta from broilers fed with Con and Enz diets, to aqueous extraction and subsequent analysis (Table S4). Water-extractable solids (WES) represented 32.3–39.0% of total solids in gizzard, ileum or excreta, while more than 59% of total solids was recovered in the corresponding water-unextractable solids (WUS) for both diets. In contrast, in the ceca, the WES and WUS presented similar yields, approximately 50% of the total dry matter. Next, the constituent monosaccharide composition and total carbohydrate content of WES was determined (Table 1). The monosaccharide composition of the corresponding WUS is provided in Table S5, in order to facilitate an overview of NSP distribution between WES and WUS.

WES fractions of the gizzard, ileum, ceca and excreta from both diets were composed of ~53%, ~44%, ~11% and ~17% carbohydrates (% w/w), respectively (Table 1). Soluble carbohydrates originated predominantly from wheat whole grains and soybean meal, that composed 66% and 17% of the diets (% as fed), respectively (Table S1). Glc, mainly representing starch, was the main water-extractable carbohydrate in the gizzard (79–81 mol% of neutral sugars), while non-starch carbohydrates Gal, Xyl, Ara and Fru were considerably less abundant (4.0–5.4 mol%). Starch digestion in the small intestine decreased the relative amount of Glc and revealed the building blocks of soluble NSP more clearly. Part of the Glc (17–18 mol%) was present in the ileum as unabsorbed monomer, while the majority of Glc was still present as building block of undigested carbohydrates. Soy-derived NSP accounted for the high Gal presence in the ileum (~19 mol%) and may have had a limited contribution to Ara content as well (Huisman et al., 1998). Next, Ara and Xyl mainly represented arabinoxylan (AX), which is the most abundant NSP in wheat (Saulnier et al., 2007), and together accounted for ~20% and ~30% (mol%) of the water-extractable carbohydrates present in the ileum in Con and Enz. This observation was already suggestive of the influence of enzyme addition on AX solubilization. Ceca WES presented high free monosaccharide content, while Rha abundance might be attributed to bacterial polysaccharides (Mäki & Renkonen, 2004). Finally, soluble NSP escaping fermentation were still present in the excreta, and were mainly composed of Glc (~37 mol%), Ara + Xyl (~32 mol%) and Gal (~14 mol%).

3.2. Xylanase-driven solubilization of AX species along the GIT

Next, we studied the impact of dietary endo-xylanase on the marker-

Table 1
Constituent neutral monosaccharide composition and total carbohydrate content (% w/w) of water-extractable solids (WES) in the gizzard, ileum, ceca and excreta, after acid and enzymatic (FRM) hydrolysis. Standard deviations ($n = 3$) are shown in Table S6. The values in parentheses represent the proportion (%) of each constituent monosaccharide present as free sugar.

	Constituent monosaccharide composition (mol%)								Carbohydrate content (% w/w)
	Ara	Xyl	Glc	Fru	Gal	Man	Fuc	Rha	
Gizzard									
Con[a]	4.0	4.5	81.1	4.4	5.0	1.0	0.1	0.0	52.9
	(6%)	n.d.[b]	(1%)	(23%)	(2%)	n.d.	n.d.	n.d.	
Enz[a]	5.4	6.0	78.7	3.6	5.2	0.9	0.1	0.0	52.5
	(3%)	n.d.	(1%)	(21%)	(3%)	n.d.	n.d.	n.d.	
Ileum									
Con	9.3	10.5	41.9	16.2	18.7	2.5	0.9	0.0	43.7
	(1%)	n.d.	(17%)	(10%)	(14%)	n.d.	n.d.	n.d.	
Enz	12.9	16.4	27.7	19.6	19.6	2.7	1.1	0.0	44.2
	(1%)	n.d.	(18%)	(18%)	(23%)	n.d.	n.d.	n.d.	
Ceca									
Con	2.8	6.7	60.9	8.8	9.9	3.5	1.7	5.6	10.1
	(95%)	(13%)	(48%)	(116%)	(14%)	(112%)	(1%)	n.d.	
Enz	2.7	5.5	64.7	8.3	8.4	4.4	1.3	4.7	13.0
	(95%)	(13%)	(48%)	(116%)	(14%)	(112%)	(1%)	n.d.	
Excreta									
Con	14.0	17.8	37.5	10.2	14.1	4.5	2.0	0.0	17.4
	(7%)	n.d.	(5%)	(28%)	(11%)	n.d.	n.d.	n.d.	
Enz	13.9	18.2	37.4	9.3	14.3	4.7	2.2	0.0	17.1
	(7%)	n.d.	(9%)	(51%)	(16%)	n.d.	n.d.	n.d.	

[a] Con: control diet; Enz: endo-xylanase supplemented diet.
[b] Not detected.

based recovery of AX (Eq. (2)) in WES (WE-AX) and WUS (WU-AX) for ileum and excreta (Fig. 1). Insoluble digestibility markers are differently retained in the gizzard than soluble feed components, and cannot follow soluble feed components in the ceca (De Vries et al., 2014; Vergara et al., 1989). Consequently, the relative WE-AX and WU--AX proportion in the gizzard, ceca and finisher diets was determined instead (Eq. (1), Table 2, Table S7). HPSEC-RI and MALDI-TOF-MS analysis of WES from both Con and Enz finisher diets only showed the presence polymeric material, while AX-deriving oligomers were not detected (data not shown). The Ara/Xyl ratio at the different GIT locations was determined as well (Table 3).

WE-AX in the ileum represented 18.9% and 26.8% of AX consumed by broilers in Con and Enz, respectively (Fig. 1). The observed increase of soluble AX by endo-xylanase in the ileum ($p < 0.05$) was accompanied by a concomitant decrease in WU-AX from 82.3% to 73.2% ($p < 0.05$). The Ara/Xyl values above 0.8 obtained for WES (Table 3) were higher than the ones reported for soluble wheat AX (Ara/Xyl 0.5–0.7), suggesting *co*-extraction of Ara-containing soybean polysaccharides (Bach Knudsen, 2014; Huisman et al., 1998). Still, comparing the Ara/Xyl values between Con and Enz may reveal structural aspects of enzymatically-released AX species. For example, WE--AX in the ileum was found to be less substituted for Enz than for Con (Ara/Xyl: 0.78 *vs* 0.88; $p < 0.05$). Our findings are in agreement with previous studies reporting the increased AX solubilization in the ileum of broilers and pigs upon GH11 endo-xylanase supplementation (Bautil et al., 2021; Lærke et al., 2015; Lee et al., 2017). In addition, endo-xylanase action resulted in significantly lower WU-AX recovery in excreta, compared to Con (Fig. 1; 65.8% *vs* 74.9%; $p < 0.05$). At the same time, Enz and Con presented similar WE--AX recovery in excreta, with similar Ara/Xyl ratio ($p > 0.05$). Overall, the decreased AX recovery from ileum to excreta (Fig. 1) indicated that xylanase-mediated WU-AX solubilization coincided with increased WE-AX fermentation. The latter has been confirmed by a more pronounced acetate and butyrate formation for Enz compared to Con, as was previously found for the same ceca samples (Kouzounis et al., 2021).

Finally, a similar WE--AX recovery in Con and Enz in the gizzard ($p > 0.05$) suggested limited enzymatic activity under gastric conditions (Gonzalez-Ortiz et al., 2017). Overall, the present findings indicated that endo-xylanase increased the proportion of AX becoming available for fermentation in the ceca (Fig. 1, Table 2), which could be beneficial for SCFAs formation and ultimately, for broiler health (Broekaert et al., 2011; Courtin et al., 2008).

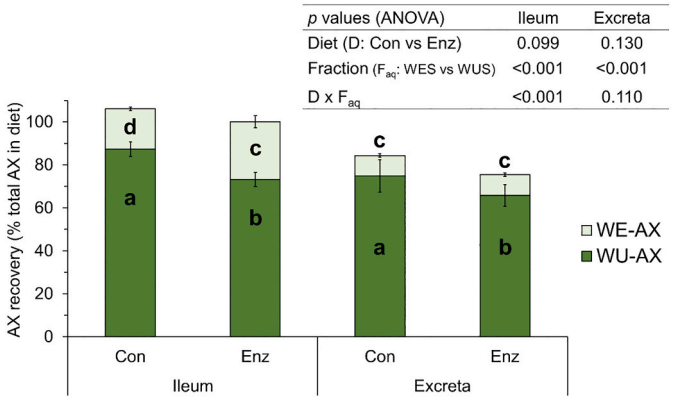

Fig. 1. Marker-based recovery of water-extractable (WE--AX) and water-unextractable (WU--AX) arabinoxylan in the ileum and excreta, expressed as percentage (%) of the total AX present in the control diet (Con) and endo-xylanase supplemented diet (Enz). Bars corresponding to ileum or excreta not sharing common notation differ significantly ($p < 0.05$). The error bars indicate standard deviation ($n = 3$); ANOVA results are presented in the table inserted.

Table 2
Total arabinoxylan (AX: sum of Ara and Xyl) recovery during aqueous extraction (% AX in digesta), and WE--AX and WU-AX recovery, expressed as percentage (%) of AX present in the gizzard and ceca. Values within GIT location not sharing common superscript letter differ significantly ($p < 0.05$). The ANOVA results are presented in Table S8.

	Arabinoxylan (AX) recovery (% AX in GIT location)		
	Total	WE-AX	WU-AX
Gizzard			
Con	91.1	11.7^{b}	88.3^{a}
Enz	88.2	13.2^{b}	86.8^{a}
Ceca			
Con	86.6	38.0^{b}	62.0^{a}
Enz	117.1	70.2^{a}	29.8^{b}

3.3. Maltodextrins and fructans were dominant oligosaccharide species in the ileum

To further delineate the influence of endo-xylanase on AX fermentability, we investigated by MALDI-TOF-MS the types of oligosaccharides released in the ileum, and recovered in WES. Representative mass spectra of *m/z* 900–2700 are shown in Fig. 2. AX hydrolysis by endo-xylanase was substantiated in Enz by the detection of soluble pentose oligosaccharides of DP 7–20 (Fig. 2; *m/z* series with 132 Da difference), as previously published (Kouzounis et al., 2021). In addition, hexose oligosaccharides (HexOS) of DP 6–15 were dominant in ileal WES for both Con and Enz (Fig. 2; *m/z* series with 162 Da difference). MS analysis at *m/z* 500–900 showed smaller pentose oligosaccharides (DP 4–6) and HexOS (DP 3–5) as well, but the resulting spectra were rather noisy (data not shown). HexOS enzymatic fingerprinting and removal was conducted to resolve AXOS, and was achieved by treatment with amyloglucosidase (AMG), fructanase (FRM), and their combination (AMG/FRM) (Figs. 2; S1). AMG/FRM combination resulted in complete Fru release, followed by the release of 65.5–67.5% of Glc and 26.8–28.5% of Gal present in ileal WES, regardless of diet. At the same time, HexOS disappeared, while (A)XOS, present only in Enz, gained in relevance. Consequently, the abundance of Glc and Fru in ileal WES (Table 1) was now explained as being part of HexOS maltodextrins and fructan oligosaccharides. In addition, Gal and part of Glc and Fru formed by AMG/FRM could be attributed to a series of raffinose oligosaccharides (Fig. S2), whose degradation may account for the apparent release of more than 100% Fru (Fig. 2). The present findings are in agreement with the detection of maltodextrins in the small intestine of pigs, as well as with the occurrence of fructans in wheat grains at approximately 1.5% of the dry matter (Bach Knudsen, 1997; Fincher, 2016; Martens et al., 2019). Overall, the enzymatic degradation of HexOS mixtures present in digesta now enables the further characterization of enzymatically released (A)XOS.

3.4. AX/AXOS-enriched fractions were obtained from digesta by solid phase extraction

AMG/FRM-treated G_{WES}, I_{WES}, C_{WES} and E_{WES} were subjected to reversed phase (C18) SPE, in order to separate monosaccharides (hexoses) from AX/AXOS (Figs. 3 and S4). The SPE procedure was evaluated beforehand for reference polymeric WAX and WAX digested with GH11 endo-xylanase (HX). It was found that WAX could only be partially recovered during SPE as polymer (~52%; Fig. S3), while it was fully recovered after endo-xylanase treatment. In particular, xylose and DP 2–4 linear XOS were eluted with water, while retained AXOS were eluted with MeOH up to 30% (v/v). Previously published C18-SPE behavior of soluble carbohydrates from corn stover demonstrated that Xyl-rich DP 3–4 pentose oligomers eluted with water and Ara-substituted DP 4–7 pentose oligomers eluted with 30% MeOH (Jonathan et al., 2017).

Approximately 47.5–64.5% of total Ara and 49.2–71.0% of total Xyl

Table 3
Arabinosyl to xylosyl ratio (Ara/Xyl) at the different GIT locations (gizzard, ileum, ceca, excreta). The ratio is corrected for free Ara and Xyl present. Values within GIT location not sharing common superscript letter differ significantly ($p < 0.05$). The ANOVA results are presented in Table S9.

	Ara/Xyl ratio							
	Gizzard		Ileum		Ceca		Excreta	
	Con	Enz	Con	Enz	Con	Enz	Con	Enz
WE-AX	0.85[a]	0.88[a]	0.88[a]	0.78[bc]	0.13[c]	0.29[bc]	0.73[b]	0.72[b]
WU-AX	0.65[b]	0.57[b]	0.72[c]	0.81[ab]	0.35[b]	0.87[a]	0.76[ab]	0.81[a]

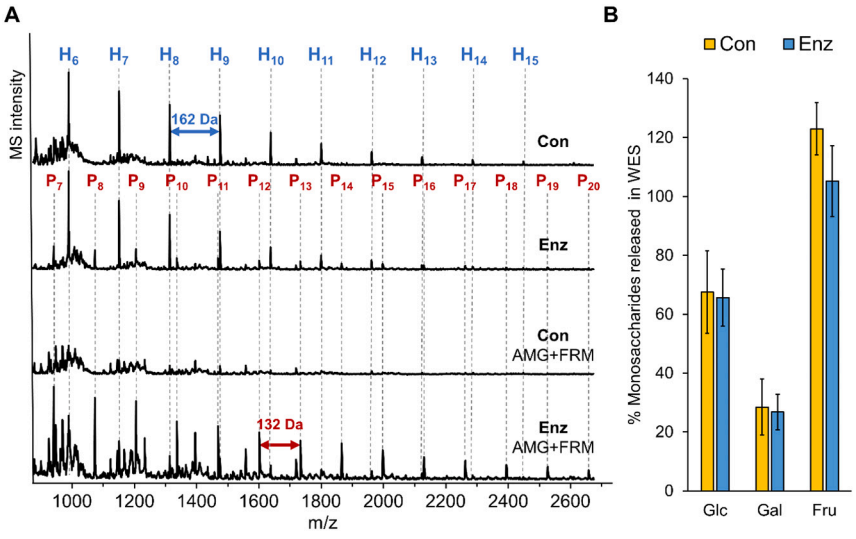

Fig. 2. MALDI-TOF-mass spectra (*m/z* 900–2700) (A) of ileal WES, from Con and Enz diets before (top two spectra) and after (bottom two spectra) incubation with AMG/FRM; Glc, Gal and Fru released by AMG/FRM (B), expressed as proportion of the corresponding total constituent monosaccharides present in WES. The number of hexose (H_n) or pentose (P_n) residues per oligomer is indicated.

was recovered in G_{WES}-M, I_{WES}-M and E_{WES}-M fractions, while less than 12.8% of total Ara and 12.1% of total Xyl was recovered in the corresponding G_{WES}-W, I_{WES}-W and E_{WES}-W fractions. Most of the Fru, Glc and Gal was recovered in the W fractions, according to expectations (Fig. S4). Consequently, the resulting M fractions were enriched in AX/AXOS, as indicated by their carbohydrate composition (Fig. 3; inserts). Interestingly, significantly higher Ara and Xyl recovery in both G_{WES}-M and I_{WES}-M was observed for Enz compared to Con ($p < 0.05$) (Fig. 3). Apparently, *in vivo* depolymerization of both WE--AX and WU-AX to AXOS resulted in higher AX recovery after SPE for Enz compared to Con, as already described in Fig. S3. Therefore, a correspondingly higher amount of polymeric AX is expected to be retained on the SPE column for Con than for Enz. The higher Xyl recovery observed in I_{WES}-W for Enz compared to Con ($p < 0.05$) was more likely due to low DP XOS removal by water. The ceca presented a distinct case, as low Ara and Xyl recovery and amounts were recorded in C_{WES}-M. Instead, C_{WES}-W presented elevated Ara and Xyl recovery compared to other GIT locations for both Con and Enz. C_{WES}-M also exhibited elevated Gal recovery compared to I_{WES}-M for both Con and Enz (Fig. S4). These observations highlight a rather complete utilization of (A)XOS entering the ceca. No differences in Ara and Xyl recovery in E_{WES}-M between Con and Enz were observed ($p > 0.05$). On the contrary, Enz presented significantly higher Xyl recovery in E_{WES}-W compared to Con ($p < 0.05$).

3.5. Characterization of AX and AXOS in SPE digesta fractions

3.5.1. Xylanase-mediated AX degradation began in the gizzard

To further detail the isolated (A)XOS structures in the M fractions (see 3.4), these M fractions were subjected to molecular weight distribution analysis by HPSEC-RI and the oligomers present were profiled by HPAEC-PAD (Fig. 4). G_{WES}-M presented different HPSEC profile for Enz compared to Con (Fig. 4A). Additionally, compounds eluting at similar retention times as AXOS were detected by HPAEC in G_{WES}-M for Enz only, although the signal was rather low (Fig. 4B). Still, only a marginal increase in WE-AX was observed in the gizzard for Enz compared to Con (Table 2). It can, therefore, be concluded that endo-xylanase mainly degraded soluble, polymeric AX in the gizzard to smaller fragments (Mw between 10 and 100 kDa). Soluble, polymeric WE-AX for wheat can exhibit molecular weight (Mw) up to 300 kDa, although showing broad polydispersity (70–655 kDa; Saulnier et al., 2007). Currently, high Mw AX populations (*i.e.* >100 kDa) potentially present in digesta, appeared to be retained on the C18-SPE column (Fig. S3). Therefore, such polymers may be underrepresented in the HPSEC profiles of M fractions, especially for Con (Fig. 4A). Furthermore, AX depolymerization by endo-xylanase may further explain the increased Ara and Xyl recoveries observed in G_{WES}-M for Enz, compared to Con (Fig. 3A). These findings demonstrate that AX hydrolysis by endo-xylanase began in the gizzard, in line with recent reports of AX degradation in the crop and gizzard (González-Ortiz et al., 2022; Matthiesen et al., 2021). Nevertheless, the acidic pH (1.0–4.5) and short feed retention time (30–60 min) in the gizzard were expected to result in limited activity of supplemented fungal xylanases (Gonzalez-Ortiz et al., 2017; Polizeli et al., 2005; Svihus, 2014).

3.5.2. Enzymatic fingerprint and chemical structure of in vivo formed AXOS

I_{WES}-M for Con mainly presented Mw populations between 10 and 100 kDa, while 10–1 kDa material was more abundant for Enz (Fig. 4A). This shift in size distribution was accompanied by AXOS presence in I_{WES}-M for Enz, as clearly demonstrated by HPAEC (Fig. 4B). In particular, the *in vivo* AXOS profile matched the one obtained during *in vitro* hydrolysis of soluble WAX by the same enzyme preparation (Fig. 4B;

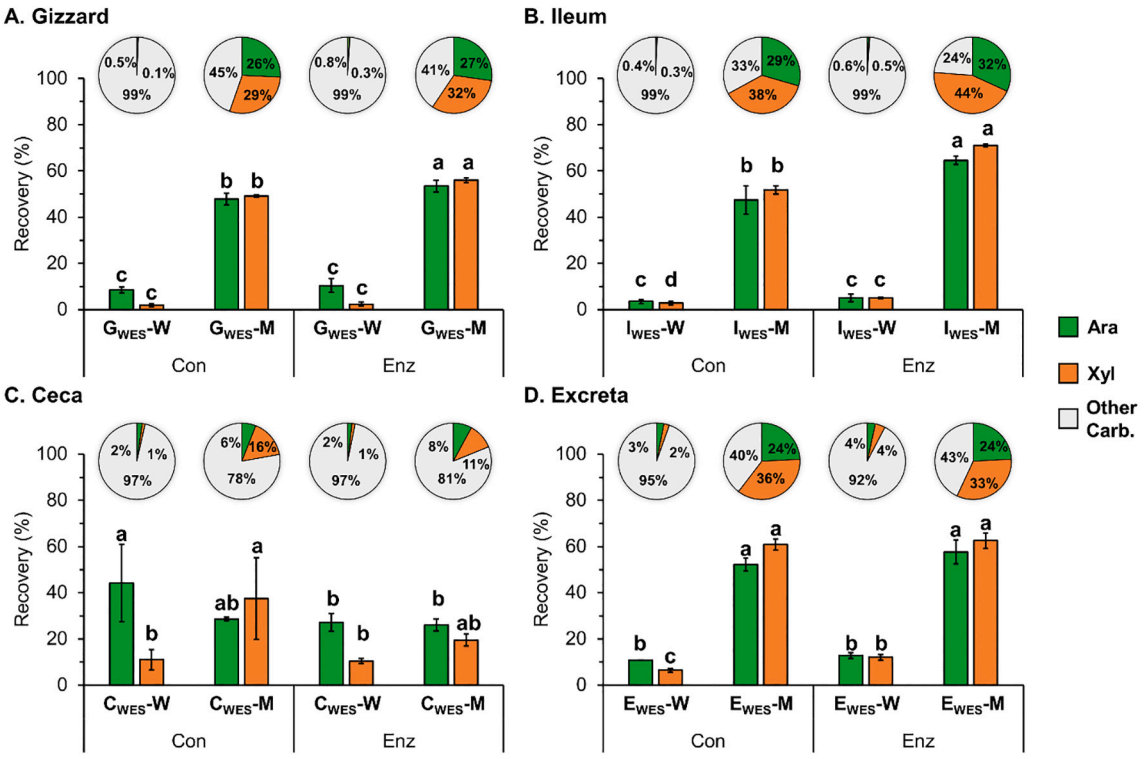

Fig. 3. Constituent arabinosyl (Ara: ■) and xylosyl (Xyl: ■) recovery (%) in the water (W) and 30% (v/v) MeOH (M) fractions, after SPE of AMG/FRM-treated WES from the gizzard (A: G_{WES}), ileum (B: I_{WES}), ceca (C: C_{WES}) and excreta (D: E_{WES}); Pie charts (inserts) show the carbohydrate composition (mol%) of Ara, Xyl and other carbohydrates (Other Carb.: ■). Bars representing either Ara or Xyl not sharing common notation differ significantly ($p < 0.05$). The error bars indicate standard deviation ($n = 3$). The ANOVA results are given in Table S10.

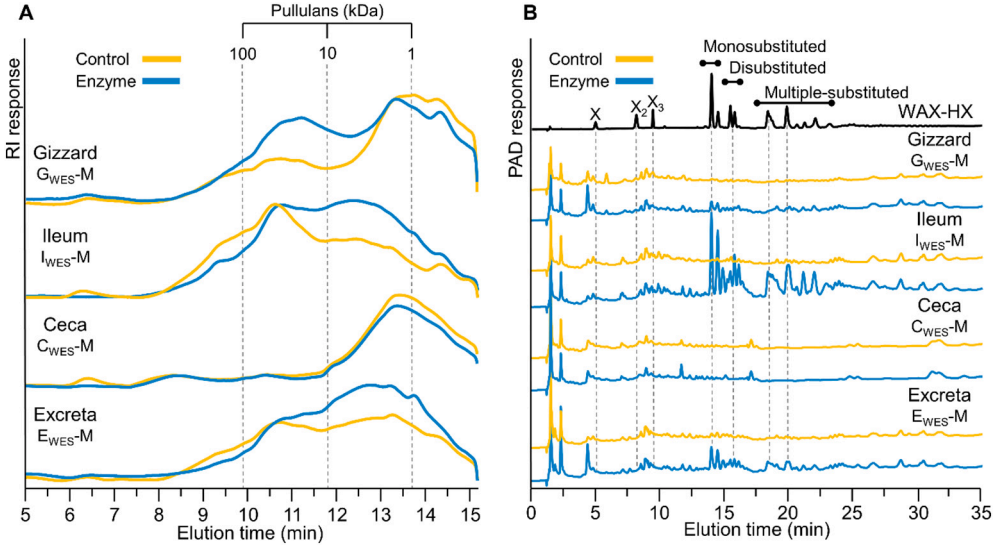

Fig. 4. HPSEC-RI (A) and HPAEC-PAD (B) elution patterns of SPE-M fractions of the WES from the gizzard, ileum, ceca and excreta of broilers fed with control and enzyme diets. Pullulans were used as calibrants for HPSEC. X: xylose, X_2: xylobiose, X_3: xylotriose were labelled according to analytical standards; AXOS were eluted in groups of monosubstituted, disubstituted and multi-substituted oligosaccharides (van Gool et al., 2013).

WAX-HX), and by a different GH11 endo-xylanase (not shown; van Gool et al., 2013). This corroborated that AXOS detected in I_{WES}-M for Enz were formed by the supplemented GH11 endo-xylanase. AXOS were also detected in excreta (E_{WES}-M) for Enz, which presented similar profiles as the ileum (Fig. 4). AXOS *in vivo* formation currently coincided with WE-AX depolymerization as well as AX solubilization from the water-unextractable cell wall matrix (Figs. 1, 4). These findings confirm previous studies reporting the ability of GH11 endo-xylanases to hydrolyze both soluble and insoluble AX *in vitro*, under optimal conditions (Bonnin et al., 2006; Courtin & Delcour, 2001; Moers et al., 2005). So far, our findings demonstrate that dietary GH11 endo-xylanase depolymerized both soluble and insoluble AX from wheat grains, and released AXOS *in vivo*. Depolymerization of WE-AX is consistent with the previously reported xylanase-mediated decrease in digesta viscosity (Bedford & Classen, 1992; Choct & Annison, 1992; Matthiesen et al., 2021). Besides, the enzymatic WU-AX degradation *in vivo* is postulated to offset nutrient

encapsulation by the cell wall matrix, thus promoting nutrient digestion (Bedford, 2018; Matthiesen et al., 2021).

The chemical structure of *in vivo* formed AXOS was further investigated for pooled ileal (IP_{WES}) and excreta (IP_{WES}) samples from Enz diet after SPE (Section 2.5). Specifically, the obtained M fractions (IP_{WES}-M, EP_{WES}-M) were analyzed by HILIC-MS^n (Fig. 5), and the individual AXOS present were identified based on a database built up with retention times and MS^2 and MS^3 mass spectra of known (A)XOS, as described by Kouzounis et al. (2022). Ileum and excreta samples presented similar HILIC-MS profiles, with various DP 4–10 (A)XOS isomers being resolved (Fig. 5; Figs. S5–7). Based on the present identification by HILIC-MS^n, the main identified AXOS contained no more than two Ara substituents, mainly present as single *O*-3-linked and double *O*-2,3-linked Ara, and were further composed of two or more contiguous unsubstituted xylosyl residues at the reducing end. The presence of at least one unsubstituted xylosyl adjacent to the cleavage site was in accordance with the mode of action of GH11 endo-xylanases targeting low-substituted AX populations (Beaugrand, Reis, et al., 2004; Biely et al., 1997; Kormelink et al., 1993). GH11 endo-xylanases were previously found to release low-substituted DP 5–7 AXOS from wheat bran and wheat flour WUS (Beaugrand, Chambat, et al., 2004; Courtin & Delcour, 2001; Gruppen et al., 1993). The low substitution degree of AXOS currently observed is in agreement with the lower Ara/Xyl ratio observed in ileal digesta for WE-AX for Enz diet compared to Con diet (Table 3). Moreover, the DP 8–10 AXOS currently detected were expected to be more complex, possibly containing multiple single and/or double Ara substitutions.

3.5.3. *The fate of in vivo formed AXOS in the broiler hindgut*

AXOS formation by dietary GH11 endo-xylanase in the upper GIT currently coincided with pronounced hindgut fermentation of AX for Enz compared to Con (Fig. 1, Table 2). Studies on AX and (A)XOS fermentation demonstrated that low Mw XOS and low-substituted AXOS are preferentially fermented by *Bifidobacterium* and *Bacteroides spp.* in human fecal inocula (Broekaert et al., 2011; Mendis et al., 2018; Rumpagaporn et al., 2015). On this basis, the release of relatively simple (A)XOS structures may further explain the pronounced cecal fermentation previously documented upon endo-xylanase supplementation in broilers (Kouzounis et al., 2021; Masey-O'Neill et al., 2014; Morgan et al., 2019; Singh et al., 2021). Soluble compounds with Mw < 10 kDa were present in the ceca (C_{WES}-M), for both Con and Enz (Fig. 4A). However, AXOS were not detected by HPAEC (Fig. 4B), confirming the low Ara, Xyl recoveries in C_{WES}-M (Fig. 3C) for both Con and Enz. In this view, it is demonstrated that extensive fermentation of both polymeric and oligomeric AX species had occurred in the ceca. Still, low Mw AX species (<DP 5) may still be present in the broiler ceca, as recently reported (Bautil et al., 2021). Finally, soluble unfermented structures mainly between 1 and 10 kDa were observed in excreta (Fig. 4A), partly corresponding to a similar WE-AX recovery for both Con and Enz (Fig. 1). Soluble feed components may first reach the cloaca and then enter the ceca *via* antiperistalsis, while insoluble and indigestible feed fractions will be excreted directly (Duke, 1989; Svihus et al., 2013). It, therefore, appeared that a WE-AX fraction, including AXOS, passed from the ileum to the hindgut and was excreted unutilized, alongside WU-AX, instead of entering the ceca. This observation became more evident by the similar HILIC profile of (A)XOS in excreta and the ileum (Fig. 5).

4. Conclusions

In this study, endo-xylanase supplementation in broilers resulted in AXOS formation *in vivo*. The detection and characterization of released oligosaccharides further delineated the xylanase-mediated improvement of AX fermentation in the broiler ceca. In particular, it is proposed that low-substituted AXOS and XOS, released *in vivo* by the dietary GH11 endo-xylanase, were more extensively fermented than polymeric AX by cecal microbiota. Still, further research in terms of AXOS quantification is warranted to better understand and optimize AX fermentation in livestock. Our work highlights the contribution of dietary endo-xylanase to broiler health and provides valuable insight on the utilization of AX and AXOS along the GIT. Overall, the detailed oligosaccharide analysis in complex digesta currently performed is expected to further progress the study of carbohydrate fermentation *in vivo*, in the GIT of animals. Such approach is of interest for academia, policy makers and the industry regarding the efficacy of feed additives.

CRediT authorship contribution statement

Dimitrios Kouzounis: Conceptualization, Methodology, Formal analysis, Visualization, Writing – original draft. **Melliana C. Jonathan:**

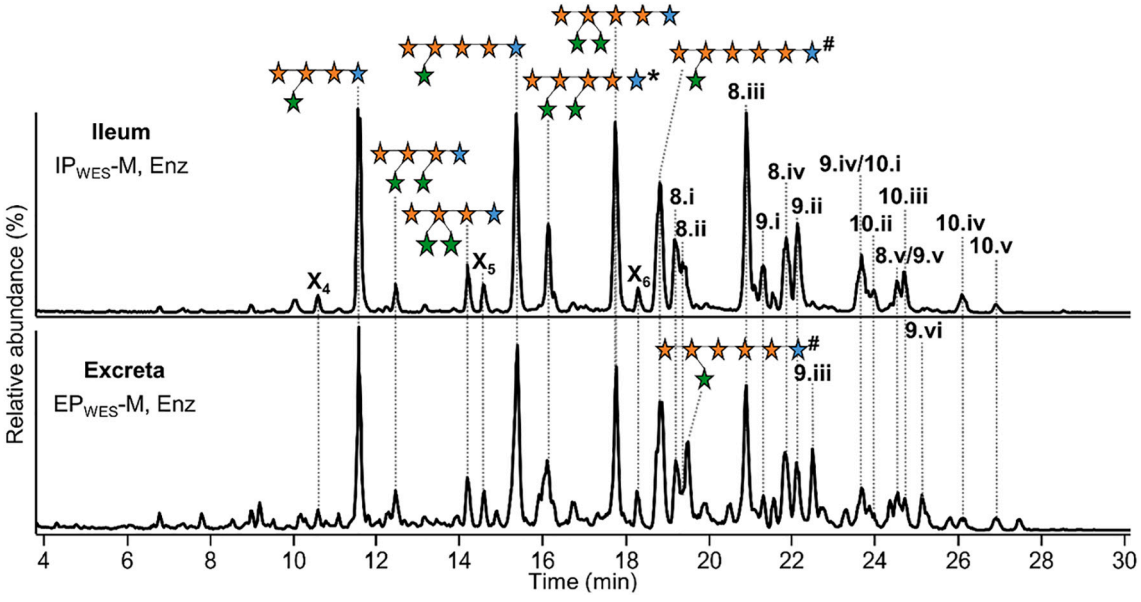

Fig. 5. HILIC-MS ion-extracted base peak chromatograms of $NaBH_4$-reduced DP 4–10 (A)XOS present in M fractions obtained by SPE of pooled ileum (IP_{WES}-M) and excreta (EP_{WES}-M) samples for Enz diet. Peak annotation was performed according to Kouzounis et al. (2022). *putative structure; #identification by MS^n is presented in Figs. S5–7; Unidentified DP 8–10 (A)XOS are labelled (i-v) according to their elution order; Arabinosyl (Ara: ★), xylosyl (Xyl: ★, xylitol: ★).

Methodology, 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

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Natalia Soares is employed by the funder. All other authors declare that they have no competing interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.carbpol.2022.119527.

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