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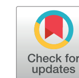
Effect of arabinoxylo-oligosaccharides and arabinoxylans on net energy and nutrient utilization in broilers



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ABSTRACT

Arabinoxylo-oligosaccharides (AXOS) are hydrolytic degradation products of arabinoxylans (AX) that can be fermented by the gut microbiota, thus potentially displaying prebiotic properties. This study examined the effects of AX and AXOS on net energy (NE) and nutrient utilization in broilers. Ross 308 broilers (n = 90, 30 birds per treatment) were fed wheat-soybean diets supplemented with pure AX, AXOS produced by exposing the AX to xylanase *in vitro* (AXOS), or AX with xylanase (AX + E) from d 10 to 21. Performance parameters were measured from d 10 to 21. On d 15, 10 birds per treatment were allocated to closed-circuit net energy chambers to assess the impact of AX and AXOS on dietary energy utilization, through assessment of both metabolisable energy (ME) and NE. Ileal and caecal digesta samples were collected on d 21 to determine the effect of AX and AXOS on ileal and total tract dry matter digestibility, ileal digestible energy, digesta pH, short chain fatty acids (SCFA) and microbiota concentration. Feed conversion ratio was numerically the lowest in birds fed the diet supplemented with AXOS, which is 1.26 compared to 1.37 and 1.30 for AX and AX + E, respectively. Ileal dry matter digestibility was higher in birds fed AXOS than those fed AX (P = 0.047). Ileal digestible energy and total tract dry matter digestibility were higher in birds fed AXOS than those fed AX or AX + E (P = 0.004 and P = 0.001, respectively). Birds fed AXOS had higher ME intake (P = 0.049) and nitrogen retention (P = 0.001) and a strong trend of higher NE (P = 0.056), NE intake (P = 0.057) and retained energy (P = 0.054) compared to those fed AX. Ileal total SCFA, lactic and formic acid concentrations were higher in birds fed AXOS than those fed AX (P = 0.011, P = 0.012 and P = 0.023, respectively). Birds fed AXOS or AX + E had higher caecal total SCFA, acetic, butyric and isovaleric acid concentrations compared to those fed AX (P = 0.001, P = 0.004, P = 0.016 and P = 0.008, respectively), and caecal propionic acid concentration was higher in birds fed AX + E than those fed AX (P = 0.050). Ileal and caecal microbiota concentrations were numerically higher and pH was lower in birds fed AXOS and AX + E than those fed AX. Results from this study indicate that feeding AXOS directly is more efficient than AXOS generation in the gastrointestinal tract, and suggest that AXOS has a potential to be an efficacious prebiotic in broiler diets.

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1. Introduction

Xylans, also known as arabinoxylans (AX) and pentosans, are the most abundant hemicelluloses in the cell walls of monocotyledonous plants, such as cereals. The presence of these polysaccharides has a direct negative impact on energy availability of monogastric diets (Choct and Annison, 1990), largely due to the direct effect of soluble AX on increasing digesta viscosity. These negative effects can be combatted by supplementing the diet with endo-β1, 4-xylanases, which hydrolyses the xylan backbone. These enzymes cleave the internal β-

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xylosidic glycosidic linkages uninterrupted by side chains to short-chain xylans or xylo-oligosaccharides (Jommuengbout et al., 2009), resulting in a mixture of low-molecular weight xylans, arabinose-substituted xylo-oligosaccharides (arabinoxylan-oligosaccharides, AXOS) and non-substituted xylo-oligosaccharides (XOS). Partial depolymerisation of AX by enzymes reduces molecular chains containing more than 5,000 sugars to just over 1,000 sugars. The resulting short-chain xylans and xylo-oligosaccharides can be utilized more efficiently by gut microbiota, which have a direct positive impact on the overall energy utilization of the cereals. Access of endogenous digestive enzymes to cell contents is also improved and there is reduced loss of endogenous amino acids, namely through modifications to pancreatic amylase and mucin secretion (Cowieson and Bedford, 2009; Meng et al., 2005).

Caecal and colonic bacteria generate energy by fermenting the end products of hydrolysis of polysaccharides. Selective fermentation of AXOS and XOS by intestinal bacteria positively influences the composition and activity of the gastrointestinal microbiota, improving health and performance of the host. Therefore, XOS fulfils the definition of a prebiotic. The potential prebiotic effects of XOS include optimizing colon function, increasing or changing composition of short chain fatty acids (SCFA), increasing mineral absorption, immune stimulation and increased ileal villus length (Kim et al., 2011). Fermentation of XOS results in production of SCFA, including butyrate and lactate, which stimulates butyrate production. Butyrate fuels epithelial cells and increases intestinal epithelial integrity, which results in improved growth performance and positive changes to intestinal microbiota composition and metabolic activity (De Maesschalck et al., 2015; Sanchez et al., 2008). Indeed, Mäkeläinen et al. (2010a,b) reported that XOS were fermented with high specificity by strains of *Bifidobacteria*. This suggests that feeding poultry AXOS could potentially result in improved gut heath, energy and nutrient utilization. This study examines whether it is more advantageous for AX to be hydrolyzed into AXOS *in situ* via supplemental enzymes or to supplement diets with AXOS that has been prepared *in vitro*.

The anti-nutritive effects of soluble non-starch polysaccharides (NSP) on energy utilization are evidenced by the negative relationship between soluble AX and apparent metabolizable energy (AME) and that *in situ* degradation of cell wall NSP by enzymes increases AME (Hughes and Choct, 1999). The accuracy of methods used to measure metabolizable energy (ME) may be questionable as they do not take into consideration the efficiency of nutrient utilization. Additionally, AME is often corrected for nitrogen, but this system is unable to fully take into account the energy value of high protein ingredients and it partitions energy use into meat production, waste and heat production (Swick et al., 2013). As a result, net energy (NE) was used in this study to determine the true energy value of the diets, as this method takes into account energy lost as heat and differences in metabolic utilization of ME of nutrients for maintenance and production requirements (Noblet et al., 2010). It was predicted by van der Klis et al. (2010) that when a NE system is used instead of a ME system cost savings as high as €4.00 to 4.50 per tonne could be achieved without any detrimental impact on production performance. This suggests that using a NE system to accurately assess the amount of energy provided from polysaccharides and oligosaccharides in feed ingredients could have significant economic value. The aim of this study was to examine the effects of AX and AXOS on NE and nutrient utilisation in broilers. This was assessed by feeding broilers diets containing pure AX, AXOS produced by exposing AX to xylanase *in vitro* or AX in combination with xylanase from d 10 to 21.

2. Materials and methods

2.1. Extraction of AX and AXOS

This study examined whether it was more efficacious to feed broilers with a dietary supplement of AXOS produced in the laboratory or to feed broilers with intact AX with xylanase. Arabinoxylan was isolated from a starch milling by-product that contained 191 g AX, 681 g starch and 43 g crude protein per kg dry matter. The AX were extracted by adjusting the milling by-product to 60% to 70% ethanol (according to the water content), leaving the mixture at room temperature for a minimum of 24 h, centrifuging it at 13,000 × *g* for 15 min and then freeze-drying the residue. The AXOS was prepared from the resulting AX by hydrolysing it with 16,000 BXU/g xylanase (Econase XT 25, AB Vista Feed Ingredients, Marlborough, UK); the AX was suspended in citrate buffer (50 mmol/L, pH 5.4) containing 0.02% azide, the pH was adjusted to pH 2.5 with HCl and then the xylanase was added. The solution was then shaken at 50 °C for 24 h, centrifuged at 13,000 × *g* for 15 min and the residue was freeze-dried.

2.2. Birds and husbandry

Ross 308 male broilers ($n = 90$) were obtained from a commercial hatchery at day of hatch. Chicks were randomized by weight and placed in 120 cm × 75 cm floor pens, 15 pens of 6 birds per pen, 30 birds per treatment, bedded on clean wood shavings. All birds received vaccination against Marek's disease, infectious bronchitis and Newcastle disease at the hatchery under Australian code of practice for the distribution of broiler chickens. On d 10, birds were individually weighed and allocated to a pen. Pen allocation was randomised across the room. Total pen weight and mean chick body weight (BW) were calculated and diet allocation was arranged to ensure there was no significant difference in BW by pen across diets. Birds were allowed *ad libitum* access to the treatment diets and water for the duration of the trial. The room was thermostatically controlled to produce an initial temperature of 34 to 35 °C (50% to 60% relative humidity) upon arrival and reduced in steps of approximately 0.5 °C per day, reaching 22 to 24 °C by d 21. The lighting regimen used was 24 h light on d 1, with darkness increasing by 1 h a day until 6 h of darkness was reached, which was maintained throughout the remainder of the study. All birds sampled were euthanised by cervical dislocation on d 21 post hatch. This occurred after at least 6 h of light, to ensure maximum gut fill. Total pen weight and feed intake (FI) were determined on d 21 post hatch and were used to calculate feed conversion ratio (FCR). Mortality was recorded daily and any birds culled or dead were weighed. Institutional and national guidelines for the care and use of animals were followed and all experimental procedures involving animals were approved by the university's animal ethics review committee.

2.3. Dietary treatments

All birds were fed a standard wheat-soybean starter crumble diet from arrival to d 10 (Table 1). From d 10 to 21, birds were allocated to 1 of 3 dietary treatments: a standard wheat-soybean meal based grower diet supplemented with an additional 2% AX, 2% AXOS or 2% AX with 16,000 BXU xylanase (Econase XT 25, AB Vista Feed Ingredients, Marlborough, UK) (AX + E). Diets were composed of wheat, sorghum and soybean meal and were formulated to be adequate in all nutrients, based on the Ross 308 nutrient specifications. The diets were mixed in house using a ribbon mixer and cold-pelleted (3 to 3.5 mm, 50 to 70 °C).

The analysed nutrient values of the diets are presented in Table 2. Nitrogen content of the diets was determined with a

Table 1
Composition (%) of basal diets (DM basis).

Ingredient	Starter	Grower
Wheat	56.8	63.1
Soybean meal 45.2%	26.1	19.9
Canola meal 37%	8.0	10.0
Meat and bone meal 53%	3.5	2.5
Tallow	3.5	2.4
Limestone	0.732	0.766
Dicalcium phosphate (18P:21Ca)	0.039	0.059
Salt	0.199	0.159
Sodium bicarbonate	0.150	0.150
Premix[1]	0.200	0.200
Choline Cl 60%	0.074	0.068
L-lysine HCl 78.4%	0.257	0.245
DL-methionine	0.292	0.232
L-threonine	0.174	0.138
Phytase 5,000 U/g[2]	0.010	0.010
TiO_2	0.50	0.50

[1] Vitamin-mineral concentrate supplied per kilogram of diet: retinol, 12,000 IU; cholecalciferol, 5,000 IU; tocopheryl acetate, 75 mg, menadione, 3 mg; thiamine, 3 mg; riboflavin, 8 mg; niacin, 55 mg; pantothenate, 13 mg; pyridoxine, 5 mg; folate, 2 mg; cyanocobalamine, 16 μg; biotin, 200 μg; cereal-based carrier, 149 mg; mineral oil, 2.5 mg; Cu (sulphate), 16 mg; Fe (sulphate), 40 mg; I (iodide), 1.25 mg; Se (selenate), 0.3 mg; Mn (sulphate and oxide), 120 mg; Zn (sulphate and oxide), 100 mg; cereal-based carrier, 128 mg; mineral oil, 3.75 mg.
[2] Phyzyme XP (Feedworks, Australia).

combustion analyser (Leco model FP-2000N analyser, Leco Corp., St. Joseph, MI, USA), using EDTA as a calibration standard, and was multiplied by 6.25 to determine the protein content of the diet. Calcium and phosphorus content were analysed using inductively coupled plasma optical emission spectrometer (ICP-OES, Model-725 radial viewed) and gross energy was determined using an adiabatic bomb calorimeter (IKA Werke, C7000, GMBH and Co., Staufen, Germany), with benzoic acid as a calibration standard. Extractable fat content was analysed by the Soxhlet method (AOAC official method 2003.05), and dry matter and ash were determined by the AOAC standard methods (930.15 and 942.05, respectively). Titanium dioxide was added at a rate of 0.5% to act as an inert marker for nutrient digestibility evaluation and the dietary content quantified by UV-spectroscopy, by the method of Short et al. (1996). The soluble and insoluble NSP contents were analysed in each diet. Briefly, the sample was fat extracted and the oligosaccharides were removed. The starch in the resulting residue was gelatinised and α-amylase and amyloglucosidase added. The prepared sample was then incubated and centrifuged at 2,000 × *g* for 10 min and the resulting supernatant and residue used for the analysis of soluble and insoluble NSP, respectively. For the soluble NSP analysis, the sugars released were removed using ethanol, the residue was dried and trifluoroacetic acid added. The supernatant from the soluble NSP analysis was used to analyse the total starch content of the diets by Megazyme Total Starch Assay (AA/AMG) kit (Megazyme, Wicklow, Ireland, UK). For the insoluble NSP analysis, the glucose released from starch digestion was removed and then acetone was added. The sample was centrifuged at 3,000 × *g* for 20 min, the resulting supernatant was removed and the residue was dried. Dilute H_2SO_4 was added and the sample was heated, cooled and then centrifuged at 3,000 × *g* for 15 min to sediment the insoluble materials. Ammonium (28%) was added to an aliquot of the resulting supernatant. For all the resulting samples, an internal standard was added (allose, 4 mg/mL) and the sample was evaporated to dryness and re-dissolved in water with slight alkalinity. $NaBH_4$ was then added, after incubation, excess decomposed with $C_2H_4O_2$. 1-methylimidazole and 5 mL of $C_4H_6O_3$ were added followed by water, after which, the pellet was dried and reconstituted before a final centrifugation step. The supernatant was then analysed by gas chromatography. The NSP content of the starch milling by-product was also analysed, and it was shown that 20.83%, and 91.84% of it was AX. The AXOS content of the diets was analysed by liquid chromatography-mass spectrometry (LCMS). Briefly, diet samples were suspended in citrate buffer (pH 5.4) and were shaken at 42 °C for 8 h. The pH was then adjusted to 7 with 1 mol/L NaOH, the sample was centrifuged at 3,000 × *g* for 15 min and 1 mL of the supernatant was collected. Xylose (X_1; Chemsupply, Gillman, South Australia), xylobiose (X_2; Prod Code. O-XBI), xylotriose (X_3; Prod Code. O-XTR) and xylotetraose (X_4; Prod Code. O-XTE) were obtained from Megazyme (Wicklow, Ireland, UK) and standards of 0, 1, 5, 10, 25 and 50 mg/L were prepared with QH20. The following steps were conducted on both the diet samples and standards. To 1 mL of the sample or standard, 50 μL 3 mol/L NaOH and 500 μL of 0.5 mol/L 1-phenyl-3-methyl-5-pyrazolone (PMP) (Chemsupply, Gillman, South Australia) in methanol were added. The sample was then incubated at 70 °C for 30 min, cooled and then 50 μL 3 mol/L HCl was added. Chloroform (500 μL) was then added to 1 mL of the resulting solution, and it was centrifuged at 3,000 × *g* for 10 min and the organic phase discarded. This step was repeated 3 times, and then the supernatant was filtered through 22 μm syringe filters. Analysis of the standards and samples was carried out on an Agilent 1260 Infinity HPLC with G1329B 1260 ALS, G1312B 1260 Binary Pump, G1379B 1260 Degasser, G1316A 1260 TCC and G7117C 1260 DAD HS coupled with Agilent 6120 Quadrupole LCMS. The HPLC column used was a Varian Polaris C18 A 3u column (150 × 2 mm), maintained at 40 °C. The mobile phases are 20 mmol/L ammonium acetate buffer (pH 5) (A) and LCMS grade acetonitrile (B). The flow rate was set to 0.3 mL/min and the chromatographic program was as follows: 0 to 14 min isocratic 20% B, 14 to 19 min linear gradient 20% to 100% B, 19 to 25 min isocratic 100% B, 25 to 30 min linear gradient 100% to 20% B and 30 to 35 min isocratic 20% B. The separation occurred in initial isocratic part of the program between 5 and 14 min. The gradient was added to wash the column reduce column fouling. To prevent non-volatile salts and other contaminates entering the MS, the mobile phase diverted to waste for the first 5 min and after 14 min. The UV was monitored at 245 nm and compared well to MS data but not used in calculation of final concentrations due to low sensitivity and baseline ambiguity. The MS analysis was performed on an Agilent 6120 LCMS using electrospray ionisation (ESI). The ESI was set to the following conditions: nebuliser gas (N_2) pressure 50 psi (1 psi = 6.895 kPa), capillary voltage 3,000 V, drying gas (N_2) temperature 350 °C with flow rate of 10 L/min and fragmentor

Table 2
Analysed proximate composition (g/kg DM) of the experimental diets containing 2% AX, AXOS or AX + E.

Item	Starter	AX	AXOS	AX + E
Dry matte, g/kg	919.13	924.27	921.13	923.41
Ash, g/kg	59.58	60.06	60.12	59.58
Protein	242.17	254.26	255.37	254.71
Total P	7.38	6.52	6.54	6.53
Total Ca	11.01	10.05	9.96	10.07
Gross energy, MJ/kg DM	20.24	19.97	20.01	19.80
Fat	67.66	65.25	65.73	65.87
Starch	340.40	367.09	375.27	381.88
Soluble NSP	16.13	16.37	17.52	20.30
Insoluble NSP	73.38	77.57	74.68	74.26
Total AXOS, mg/kg DM		140.02	514.69	152.7
X_1		6.44	10.12	5.48
X_2		32.85	119.16	26.37
X_3		42.86	227.42	48.31
X_4		57.87	157.99	72.54

AX = arabinoxylan; AXOS = arabinoxylo-oligosaccharides; AX + E = AX + xylanase; NSP = non-starch polysaccharides; X_1 = combined xylose; X_2 = xylobiose; X_3 = xylotriose; X_4 = xylotetraose.

voltage 70 V. The 1-phenyl-3-methyl-5-pyrazolone (PMP)-derivatives were detected using positive selected-ion monitoring (SIM) of the dominate $[M + H]+$ ions. These were determined prior to HPLC analysis by direct infusion of single standards. The following SIM values were used in the HPLC analysis: PMP- Xyl4, m/z 877.5; PMP- Xyl3, m/z 745.5; PMP- Xyl2, m/z 613.5 and PMP- Xyl, m/z 481.5.

2.4. Response variables

2.4.1. Net energy

On d 15, 2 birds per pen were allocated to 1 of 15 closed-circuit calorimeter chambers, 5 replicate chambers per dietary treatment. Birds were acclimatised to the calorimeter chambers for 4 days prior to collection of data and calculation of heat production (HP). All birds had *ad libitum* access to feed and water throughout the NE trial period. Feed intake and total excreta output during the 3-day period (d 19, 20 and 21) were measured. The total excreta collected was weighed and was thoroughly homogenized, and subsamples were taken for analysis of dry matter and gross energy, as described above for the diet analysis. As described by Swick et al. (2013), chambers were approximately 100 cm long, 76 cm high and 70 cm wide and were made of stainless steel. Each chamber housed a wire mesh cage that was approximately 89 cm long, 60 cm high and 61 cm wide. Water was used to seal the chambers, as highlighted by Farrell (1972). The pressure in the chamber was controlled using a barometric sensor connected to an electronic switch that activated a solenoid valve. Temperature and humidity in each chamber were monitored continuously using temperature and humidity sensors. Humidity was maintained at less than 70% for the run. Chamber air was circulated by a 28 L/min diaphragm pump through a bottle containing 2 L of 320 g/kg KOH solution and a bubbler assembly, to absorb the CO_2 produced by the birds. The air was then passed through a trap containing 3 kg of dried silica, to absorb the humidity, and was then returned to the chamber. CO_2 concentrations were maintained at less than 4 mL/L. Each chamber was equipped with a 490 L cylinder of medical grade O_2 fitted with a regulator and a reducing valve to replenish the O_2 as it was consumed. O_2 consumption was calculated by subtracting the weight of the O_2 cylinder at the end of each daily run from its weight at the beginning of the run. The conversion of weight to volume was based on the density of the O_2, which was approximately 1.331 g/L at 20 °C and 101.325 kPa. Subsamples of the KOH from each chamber were collected at the end of each daily run to analyse CO_2 production by the birds. Recovery of the CO_2 was determined based on a $BaCl_2$ precipitation technique, as described by Annison and White (1961) and Swick et al. (2013). Apparent metabolisable energy was determined by the total excreta collection method. Total heat production was measured during the whole 3-day trial period and was estimated from the O_2 consumed and CO_2 produced by the birds, using the equation: Total heat (kcal) $= 3.866 \times O_2$ consumed (L) $+ 1.200 \times CO_2$ produced (L). The respiratory quotient (RQ) of the trial period was calculated as the ratio of CO_2 produced to the volume of O_2 consumed. Heat increment (HI) was calculated by subtracting fasting heat production from the total heat production. To correct for zero activity, a value of 450 kJ/(kg $BW^{0.70}$·day) was used, which corresponds to the asymptotic heat production (at zero activity) during a 24-h fasting, as proposed by Noblet et al. (2015) with $BW^{0.70}$ being metabolic body weight of the birds. Net energy was calculated as ME intake minus HI divided by feed consumed on an as-is basis.

2.4.2. Ileal and caecal pH

On d 21, 2 birds per pen were randomly selected and euthanized by cervical dislocation to measure ileum and caeca pH and collect ileum and caeca digesta for analysis of SCFA and microbiota concentration and dry matter and energy digestibility. Immediately post-euthanasia the ileum and caeca were removed intact, and a digital pH meter (Ecoscan, Eutech Instruments, Singapore) with a spear tip piercing pH electrode (Sensorex S175CD) was directly inserted into the digesta in the lumen, whilst ensuring the pH electrode did not touch the intestinal wall, and pH was recorded. This was repeated 3 times, putting the probe in different areas of the section of tract each time. The probe was then rinsed with ultra-pure water.

2.4.3. Ileal and total tract digestibility

Following the pH measurements, the digesta was collected on an individual bird basis and was weighed. It was then freeze-dried and re-weighed to determine the dry matter content. The dried digesta was then ground into a fine powder, and the energy and TiO_2 content was measured, using the same methods as described earlier for the diet and excreta analysis. Ileal dry matter digestibility and total tract dry matter digestibility was determined using the equation: Digestibility (%) $= [1 - (TiO_2$ diet/TiO_2 ileal digesta or excreta$) \times ($DM ileal digesta or excreta/DM diet$)] \times 100$. Ileal digestible energy was calculated by multiplying the percent digestibility by the diet energy content.

2.4.4. Ileal and caecal SCFA concentration

To determine the SCFA concentration in the ileal and caecal digesta, briefly, 1 mL of internal standard (0.01 mol/L ethylbutyric acid) was added to approximately 2 g of fresh homogenized digesta sample and the solution was then mixed and centrifuged at $38{,}625 \times g$ at 5 °C for 20 min. Approximately 1 mL of the resulting supernatant, 0.5 mL of concentrated HCl and 2.5 mL of ether were then combined. An internal standard solution and a blank were also prepared using 1 mL of the standard acid mixture and 1 mL of water respectively in place of the supernatant. The mixture was then centrifuged at $2{,}000 \times g$ at 5 °C for 15 min and 400 µL of the resulting supernatant was combined with 40 µL of N-tert-butyldimethlsilyl-N-methyltrifuoroacetamide (MTBSTFA). The samples were then heated at 80 °C for 20 min, left at room temperature for 48 h and were then analysed on a Varian CP3400 CX gas chromatograph (Varian Analytical Instruments, Palo Alto, CA, USA). Total SCFA concentration was derived as the sum of all the SCFA measured in the sample, expressed as µmol/g digesta.

2.4.5. Ileal and caecal microbiota

Immediately post-collection, approximately 1 g of digesta was snap-frozen in liquid nitrogen and stored at −20 °C for DNA extraction. PCR amplification of 16S ribosomal DNA was used to quantify the chromosomal DNA counts of the total microflora, *Lactobacillus* spp. and *Enterobacteria* spp. Template DNA samples were prepared from the digesta using Bioline Isolate II Plant DNA Kit (Bioline, Alexandria, NSW, Australia). For DNA preparation, approximately 200 mg of ileal digesta was accurately weighed and vigorously shaken with 0.2 g of 0.1 mm glass beads prior to the extraction step. For the caecal samples, 60 mg of digesta was processed by a Qiaxtractor automated DNA extractor robot (Qiagen, Australia). A NanoDrop ND-8000 UV spectrophotometer was used to assess the DNA purity in all the samples (Thermo Fisher Scientific, Waltham, USA). Only DNA elutions that emitted ratios of between 1.6 and 1.8 in 260/280 nm wavelength were used for PCR analysis. The quantitative PCR analysis was performed on a Rotorgene-6500 real-time PCR machine (Corbett, Sydney, Australia). Duplicate samples of 10 µL were used in each PCR reaction. SensiMix SYBR No-ROX (Bioline, Meridian Life Science, Memphis, USA) was used to amplify the 16S ribosomal DNA for analysis. A SensiMix SYBR No-ROX Kit was used to quantify the total bacteria, Enterobacteriaceae, and *Lactobacilli*. Species-specific

16 rRNA annealing primers were used as follows. Enterobacteriaceae F: 5'-CATTGACGTTACCCGCAGAAGAAGC-3' and R: 5'-CTCTACGAGACTCAAGCTTGC-3', *Lactobacillus* spp. F: 5'-CACCGCTACACATGGAG-3' and R: 5'-AGCAGTAGGGAATCTTCCA-3' and total bacteria F: 5'-CGGYCCAGACTCCTACGGG-3' and R: 5'-TTACCGCGGCTGCTGGCAC-3'. Serial dilutions of linearised plasmid DNA (pCR 4-TOPO Vector, Life Technologies, Carlsbad, USA) inserted with respective amplicons were used to construct a standard curve. A threshold cycle average from the replicate samples was assigned for quantification analysis. The number of target DNA copies was calculated from the mass of the DNA, taking into account the size of the amplicon insert in the plasmid. Bacteria numbers were expressed as $\log_{10}$ (genomic DNA copy number)/g digesta.

2.5. Statistical analysis

All data were analysed using IBM SPSS statistics version 23. After Kolmogorov–Smirnov testing to confirm normality, one-way ANOVA was used to determine the equality of the means, with diet as the factor. Treatment means were separated using Tukey post-hoc test where appropriate. Statistical significance was declared at $P < 0.05$.

3. Results

3.1. Performance

The effect of diets containing AX, AXOS or AX + E on broiler performance from d 10 to 21 is illustrated in Table 3. Dietary treatment had no significant effect on bird performance.

3.2. Ileal and total tract digestibility

As illustrated in Table 4, dietary treatment had no significant effect on the dry matter content of the ileal digesta or excreta. Ileal dry matter digestibility was higher ($P = 0.047$) in birds fed AXOS than those fed AX. Ileal digestible energy and total tract dry matter digestibility was higher ($P = 0.004$ and $P = 0.001$, respectively) in birds fed AXOS than those fed AX or AX + E.

3.3. Net energy

The dietary treatment effects on energy balance and efficiency of energy utilisation are shown in Table 5. Metabolisable energy intake [kJ/(kg $BW^{0.70}\cdot$d)] was higher ($P = 0.049$) in birds fed AXOS than those fed AX. Retained nitrogen [g/(bird$\cdot$d)] was lower ($P = 0.001$) in birds fed AX than those fed AXOS or AX + E. Retained energy and NE intake [kJ/(kg $BW^{0.70}\cdot$d)] and net energy [kJ/(bird$\cdot$d)] presented a strong trend of being higher in birds fed AXOS or AX + E compared to those fed AX ($P = 0.054$, $P = 0.057$ and $P = 0.056$, respectively).

Table 3
Effect of diets containing 2% AX, AXOS or AX + E on individual bird performance from d 10 to 21.

Item	FI, g	BWG, g	FCR
AX	1,015.69	746.67	1.37
AXOS	967.00	766.43	1.26
AX + E	987.55	760.36	1.30
SEM	11.52	4.77	0.02
P-value	0.267	0.818	0.167

AX = arabinoxylan; AXOS = arabinoxylo-oligosaccharides; AX + E = AX + xylanase; FI = feed intake; BWG = body weight gain; FCR = feed conversion ratio.

3.4. Ileal and caecal SCFA and microflora concentration

Dietary treatment had no significant effect on the pH or microbiota content of the ileum or caeca. Table 6 shows that total SCFA concentration, along with lactic and formic acid concentration, was higher in the ileum of birds fed the diets with AXOS than those fed AX ($P = 0.011$, $P = 0.012$ and $P = 0.023$, respectively). Table 7 shows that total SCFA, acetic, butyric and isovaleric acid concentrations were lower in the caeca of birds fed AX than those fed AXOS or AX + E ($P = 0.001$, $P = 0.004$, $P = 0.016$ and $P = 0.008$, respectively). Propionic acid concentration in the caeca was higher in birds fed AX + E than those fed AX ($P = 0.050$), and lactic acid concentration was higher in birds fed AXOS than those fed AX or AX + E ($P = 0.005$). Generally, microbiota content was numerically higher in birds fed AXOS and AX + E than those fed AX.

4. Discussion

Results from this study suggest that AXOS has the capacity to be an efficacious prebiotic in broiler diets, as highlighted by its positive effects on broiler performance, intestinal SCFA production and energy utilisation. An interesting observation from this study was that feeding AXOS prepared *in vitro* was generally more advantageous than feeding AX + E, particularly when observing ileal and total tract digestibility. This was probably because depolymerisation of NSP *in situ* is not instantaneous, hence AXOS generation in the gut via the use of enzymes is not as efficient as feeding AXOS directly. The concept of using AXOS as a feed additive to reduce the reliance on in-feed antibiotics is noteworthy. However, in order to develop further in this research area, technologies will need to be developed that can amass large volumes of AXOS as it is highly costly to produce on a laboratory scale to conduct a feeding experiment as we have done here. Additionally, it may be even more profitable to produce specific sized AXOS *in situ*, resulting in customized prebiotic activities in broiler diets. In order to do this, a deeper understanding of the gastrointestinal microbiota is required to determine the substrate requirements and hence tailor the prebiotic capabilities to reflect the specific function and activity of the microbiota.

Performance and energy utilisation was lower in birds fed the diet containing AX, presumably because more digestive and metabolic effort was required for the birds to utilise this diet, meaning it was less efficient at providing energy for maintenance and production. This may be partly because the weight and relative proportion of energetically active organs, such as the gastrointestinal tract and pancreas, was greater in birds fed this diet (Wu et al., 2004), which increased the total cost of maintenance. This was illustrated by Gao et al. (2008) who showed that supplementing a wheat-based diet with xylanase resulted in reduced relative weights of the duodenum, jejunum, colon and pancreas in 21-day-old broilers. Future analysis is therefore warranted into quantifying the additive effects of AXOS. Arabinoxylans contributes towards heat production and this study showed that if AX is hydrolysed *in vitro* there was numerically reduced heat increment and total cost of maintenance. Apparent metabolizable energy systems are traditionally used to evaluate dietary energy utilisation in broilers, but this system does not take into consideration the efficiency of nutrient utilization and partitioning into meat, waste (namely depot fat), losses of chemical energy in the solid, liquid and gaseous excreta and energy and chemical losses due to heat production during digestion and absorption (Swick et al., 2013). The NE:ME ratio presented in this study suggests that the net energy system may provide a more sensitive measure of energy utilisation compared to the ME system, but the low number of replicates used in this study means that this cannot be confirmed and requires further investigation. In order to improve energy utilization from

Table 4
Effect of diets containing 2% AX, AXOS or AX + E on ileal and total tract digestibility in broilers from d 10 to 21.

Item	DM, %		Ileal DM digestibility, %	IDE, MJ/kg	Total tract DM digestibility, %
	Ileal digesta	Excreta			
AX	18.59	25.81	85.89[b]	17.16[b]	91.15[b]
AXOS	17.55	24.56	86.84[a]	17.38[a]	91.58[a]
AX + E	18.31	26.60	86.18[ab]	17.06[b]	91.32[b]
SEM	0.25	0.48	0.23	0.08	0.10
P-value	0.169	0.313	0.047	0.004	0.001

AX = arabinoxylan; AXOS = arabinoxylo-oligosaccharides; AX + E = AX + xylanase; IDE = ileal digestible energy.
[a,b]Means within the same column with no common superscript differ significantly ($P \leq 0.05$).

Table 5
Effect of diets containing 2% AX, AXOS or AX + E on energy balance and efficiency of energy utilization in broilers from d 10 to 21.

Item	AX	AXOS	AX + E	SEM	*P*-value
Energy value (DM basis)					
ME feed, kJ/g	13.51	13.48	13.47	0.15	0.995
MEn feed, kJ/g	12.73	12.64	12.49	0.15	0.825
NE feed, kJ/g	10.50	10.88	10.63	0.16	0.662
NE:ME	0.77	0.81	0.79	0.01	0.143
Energy partition, kJ/(bird·d)					
ME	1,547	1,631	1,631	32.02	0.532
NE	1,163	1,358	1,289	21.56	0.056
HI	431	423	449	7.59	0.410
Energy/nitrogen balance, kJ/(kg $BW^{0.70}$·d)					
ME intake	1,497[b]	1,682[a]	1,631[ab]	32.03	0.049
NE intake	1,090	1,240	1,186	25.92	0.057
HP	814	817	831	3.55	0.122
HI	388	402	413	4.59	0.081
RE	683	865	801	31.27	0.054
Retained N, g/(bird·d)	2.50[b]	3.06[a]	3.46[a]	0.12	0.001
RQ	1.03	1.02	1.02	0.00	0.363

AX = arabinoxylan; AXOS = arabinoxylo-oligosaccharides; AX + E = AX + xylanase; ME = metabolisable energy; NE = net energy; HI = heat increment; HP = heat production; RE = retained energy; RQ = respiratory quotient.
[a,b] Means within the same column with no common superscript differ significantly ($P \leq 0.05$).

carbohydrate fractions, further knowledge on the NSP substrates that are present in different diets needs to be improved, which is currently constrained by a lack of rapid methods available to detect substrates in real time.

Supplemental xylanase and degradation of AX *in vitro* not only enhanced nutrient digestibility but also increased the concentration of ileal and caecal SCFA, associated with increased flow of xylo-oligomers. The results presented in this study provide evidence that AXOS is readily fermented in the ileum and caeca, producing SCFA that can be absorbed and used as an energy source, namely acetic, propionic and butyric acid which are recognized for their role in enhancing energy metabolism (den Besten et al., 2013). Thus, heightened SCFA production may partly explain the observed improvements in energy utilization observed in birds fed AXOS and AX + E compared to those fed AX. Butyric acid has anti-inflammatory properties, fuels epithelial cells and increases intestinal epithelial integrity (Guilloteau et al., 2010), and hence may also have contributed to the observed numerical improvements in performance. Additionally, De Maesschalck et al. (2015) observed longer villi in the ileums of chickens fed XOS, which may be due to butyrate stimulating glucagon-like peptide 2 production. This warrants further investigation. Additionally, acetic acid in the form of acetyl-coenzyme A

Table 6
Effect of diets containing 2% AX, AXOS or AX + E on pH, SCFA concentration and $\log_{10}$ DNA enumeration of gut bacteria using 16S rDNA qPCR quantification in the ileum of broilers at d 21.

Item	pH	SCFA, µmol/g			Microbiota, $\log_{10}$ counts/g digesta		
		Total	Lactic acid	Formic acid	Total anaerobic	*Lactobacillus*	Enterobacteriaceae
AX	6.64	21.25[b]	18.61[b]	0.51[b]	9.61	8.15	6.01
AXOS	6.46	49.18[a]	43.58[a]	1.42[a]	9.98	8.47	6.14
AX + E	6.53	38.79[ab]	35.59[ab]	0.70[ab]	10.03	8.51	6.19
SEM	0.04	6.65	6.01	0.23	0.11	0.09	0.04
P-value	0.831	0.011	0.012	0.023	0.088	0.375	0.499

AX = arabinoxylan; AXOS = arabinoxylo-oligosaccharides; AX + E = AX + xylanase; SCFA = short chain fatty acids.
[a,b] Means within the same column with no common superscript differ significantly ($P \leq 0.05$).

Table 7
Effect of diets containing 2% AX, AXOS or AX + E on pH, SCFA concentration and $\log_{10}$ DNA enumeration of gut bacteria using 16S rDNA qPCR quantification in the caeca of broilers at d 21.

Item	pH	SCFA, µmol/g						Microbiota, $\log_{10}$ counts/g digesta		
		Total	Acetic acid	Propionic acid	Butyric acid	Isovaleric acid	Lactic acid	Total anaerobic	*Lactobacillus*	Enterobacteriaceae
AX	6.29	46.42[b]	30.88[b]	1.79[b]	11.47[b]	0.05[b]	0.21[b]	10.51	8.69	7.70
AXOS	6.21	100.75[a]	65.47[a]	5.05[ab]	22.48[a]	0.21[a]	0.66[a]	10.53	8.90	7.80
AX + E	6.27	105.28[a]	67.87[a]	7.51[a]	24.39[a]	0.19[a]	0.30[b]	10.61	8.83	7.92
SEM	0.02	15.44	9.76	1.35	3.29	0.04	0.11	0.02	0.05	0.05
P-value	0.869	0.001	0.004	0.050	0.016	0.008	0.005	0.411	0.290	0.881

AX = arabinoxylan; AXOS = arabinoxylo-oligosaccharides; AX + E = AX + xylanase; SCFA = short chain fatty acids.
[a,b] Means within the same column with no common superscript differ significantly ($P \leq 0.05$).

in cells is particularly integral in energy production and is vital for ATP production and biosynthesis of long chain fatty acid, as well as playing a role in improving growth performance and increasing intestinal epithelial cell division and villus width, height and area (Hudha et al., 2010). According to Cuche et al. (2000), fermentation of oligosaccharides into SCFA potentially triggers a neurohormonal response, through stimulating peptide YY, resulting in delayed gastric emptying and duodenal transit time rates and hence heightened diet digestion and nutrient absorption in the small intestine. Findings from this study suggest that microbial metabolites such SCFA have the potential to be indicators of generation and prevalence of fermentative oligosaccharides and could hence be used to measure the effects of xylanase on nutrient digestibility and retention.

Microbiota hydrolyse indigestible carbohydrates into oligosaccharides and then into monosaccharides, which they then ferment in the anaerobic environment of the gut. Arabinoxylo-oligosaccharides selectively stimulate beneficial bacteria, namely *Bifidobacteria*, and non-digestible carbohydrates act as the main source of energy during microbial proliferation in the hindgut (Mäkeläinen et al., 2010a, b). The impact of diet on microbiota was not significant in this study, likely due to the low number of replicates. Ileal and caecal lactic acid concentration was higher in birds fed AXOS and AX + E than those fed AX. *Lactobacilli* readily ferment AXOS into lactic acid which lowers pH, but in this study there was a lack of significant effect of dietary treatment on pH, which may be partly because the lactic acid was absorbed in the intestine or used as a substrate for lactate-utilizing bacteria. The SCFA produced by *Lactobacilli* can also directly stop harmful bacteria from reproducing, by acting as bacteriostatic agents, producing bacteriocins with microbicidal or microbiostatic properties and modifying the receptors used by pathogenic bacteria, which increases resistance against pathogenic microbes (Adil and Magray, 2012). Also, propionic and formic acid, which were stimulated by AXOS and AX + E, have high bacteriostatic properties due to their pH reduction activity both in feed and in the gastrointestinal tract, through pharmacological actions on microflora (Haque et al., 2009; Hernández et al., 2005). Reduced digesta viscosity in birds fed AXOS and AX + E likely increased the rate of digesta passage and hence stomach emptying decreased fermentation of the pathogenic microbial populations, enabling the beneficial bacteria to flourish. Bacteria compete with the host for nutrients within the tract, eliciting an immune response that can dictate appetite, muscle catabolism, disease prevalence and nutrient absorption (Bedford, 2000). It is important therefore to promote the growth of bacteria that can provide nutrients for the host and reduce the growth of bacteria that are detrimental to the host. Findings from this study suggest there is potential to use AXOS to aid and control this, but further investigation in this research area is required.

5. Conclusion

In conclusion, AXOS appear to be efficacious prebiotics that have positive effects on net utilization of dietary energy and bird performance. This appears to be largely due to the ability of AXOS to stimulate beneficial bacteria and SCFA production. In this study, it was found feeding broilers with AXOS that had been prepared *in vitro* was more effective than *in situ* AXOS production, particularly with regards to enhancing diet digestibility, highlighting the potential for using AXOS as a feed additive in the future. Further studies are required to examine the effect of the source and structure of AXOS on their effects in broilers, and to investigate the response and impact of AXOS in different environmental conditions, namely focusing on pH, transit time and microflora composition.

References

Adil S, Magray SN. Impact and manipulation of gut microflora in poultry: a review. J Anim Vet Adv 2012;11:873–7.

Annison E, White R. Glucose utilization in sheep. Biochem J 1961;80:162.

Bedford M. Removal of antibiotic growth promoters from poultry diets: implications and strategies to minimize subsequent problems. World's Poult Sci J 2000;56:347–65.

Choct M, Annison G. Anti-nutritive activity of wheat pentosans in broiler diets. Br Poultry Sci 1990;33(4):821–34.

Cowieson AJ, Bedford MR. The effect of phytase and carbohydrase on ileal amino acid digestibility in monogastricdiets: complementary mode of action? World's Poult Sci J 2009;65:609–24.

Cuche G, Cuber JC, Malbert H. Ileal short-chain fatty acids inhibit gastric motility by a humoral pathway. Am J Physiol Gastrointest Liver Physiol 2000;279:G925–30.

De Maesschalck CD, Eeckhaut V, Maertens L, De Lange L, Marchal L, Nezer C, et al. Effects of xylo-oligosaccharides on broiler chicken performance and microbiota. Appl Environ Micrbiol 2015;81:5880–8.

Den Besen G, van Eunen K, Groen AK, Venema K, Reijngoud D-J, Bakker BM. The role of short-chain fatty acids in the interplay between diet, gut microbiota and host energy metabolism. J Lipid Res 2013;54:2325–40.

Farrell D. An indirect closed circuit respiration chamber suitable for fowl. Poult Sci 1972;51:683–8.

Gao F, Jiang Y, Zhou GH, Han ZK. The effects of xylanase supplementation on performance, characteristics of the gastrointestinal tract, blood parameters and gut microflora in broilers fed on wheat-based diets. Anim Feed Sci Technol 2008;142:173–84.

Guilloteau P, Martin L, Eeckhaut V, Ducatelle R, Zabielski R, Van Immerseel FV. From the gut to the peripheral tissues: the multiple effects of butyrate. Nutr Res Rev 2010;23:366–84.

Hernández F, García V, Madrid J, Orengo J, Catalá-Megías MD. Effect of formic acid on performance, digestibility, intestinal histomorphology and plasma metabolite levels of broiler chickens. Br Poult Sci 2005;47:50–6.

Haque MN, Chowdhury R, Islam KMS, Akbar MA. Propionic acid is an alternative to antibiotics in poultry diet. Bangladesh J Anim Sci 2009;38:115–22.

Hudha MN, Ali MS, Azad MAA, Hossain MM, Tanjim M. Effect of acetic acid on growth and meat yield in broilers. Int J Biol Res 2010;1:31–5.

Hughes RJ, Choct M. Chemical and physical characterisitcs of grains related to variability in energy and amino acid availability on poultry. Aust J Agric Res 1999;50:689–701.

Jommuengbout P, Pinitglang S, Kyu KL, Ratanakhanokchai K. Substrate-binding site of Family 11 xylanase from *Bacillus firmus* K-1 by molecular docking. Biosci Biotechnol Biochem 2009;73:833–9.

Kim G-B, Seo YM, Kim CH, Paik IK. Effect of dietary prebiotic supplementation on the performance, intestinal microflora, and immune response of broilers. Poult Sci 2011;90:75–82.

Meng X, Slominski BA, Nyachoti CM, Campbell LD, Guenter W. Degradation of cell wall polysaccharides by combinations of carbohydrase enzymes and their effect on nutrient utilization and broiler chicken performance. Poult Sci 2005; 84:37–47.

Mäkeläinen H, Forssten S, Saarinen M, Stowell J, Rautonent N, Ouwehand AC. Xylo-oligosaccharides enhance the growth of bifidobacteria and Bifidobacterium lactis in a simulated colon model. Benef Microbes 2010a;1:81–91.

Mäkeläinen H, Saarinen M, Stowell J, Rautonen N, Ouwehand AC. Xylo-oligosaccharides and lactitol promote the growth of Bifidobacterium lactis and Lactobacillus species in pure cultures. Benef Microbes 2010b;1:139–48.

Noblet J, Dubois S, Labussiere E, Carré B, van Milgen J. Metabolic utilization of energy in monogastric animals and its implementation in net energy systems. In: Crovetto GM, editor. 'Proceedings of the 3rd international symposium on energy and protein metabolism and nutrition, Parma Italy'. European Federation of Animal Science. Wageningen, The Netherlands: Wageningen Academic Publishers; 2010. p. 573–82.

Noblet J, Dubois S, Lasnier J, Warpechowski M, Dimon P, Carré B, Van Milgen J, Labussière E. Fasting heat production and metabolic BW in group-housed broilers. Animal 2015;9:1138–44.

Sanchez JI, Marzorati M, Grootaert C, Baran M, Craeyveld VV, Courtin CM, et al. Arabinoxylan-oligosaccharides (AXOS) affect the protein/carbohydrate fermentation balance and microbial population dynamics of the simulator of human intestinal microbial ecosystem. Microb Biotechnol 2008;2:101–13.

Short FJ, Gorton P, Wiseman J, Boorman KN. Determination of titanium dioxide added as an inert marker in chicken digestibility studies. Anim Feed Sci Technol 1996;59:215–21.

Swick RA, Wu S-B, Zuo J, Rodgers N, Barekatain MR, Choct M. Implications and development of a net energy system for broilers. Anim Prod Sci 2013;53: 1231–7.

van der Klis JD, Kwakernaak C, Jansman A, Blok M. Energy in poultry diets: adjusted AME or net energy? In: Selle P, editor. Proceedings of the 21st annual Australian poultry science symposium. Sydney: Poultry Research Foundation; 2010. p. 44–9.

Wu YB, Ravindran V, Thomas DG, Birtles MJ, Hendriks WH. Influence of phytase and xylanase, individually or in combination, on performance, apparent metabolisable energy, digestive tract measurements and gut morphology in broilers fed wheat-based diets containing adequate level of phosphorus. Br Poult Sci 2004;45:76–84.