Xylo-oligosaccharides display a prebiotic activity when used to supplement wheat or corn-based diets for broilers

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ABSTRACT It is now well established that exogenous β-1,4-xylanases improve the nutritive value of wheat-based diets for poultry. Among other factors, the mechanism of action of exogenous enzymes may involve a microbial route resulting from the generation of prebiotic xylo-oligosaccharides (XOS) in the birds' gastro-intestinal (GI) tract. In a series of three experiments, the effect of XOS on the performance of broilers fed wheat or corn-based diets was investigated. In experiment 1, birds receiving diets supplemented with XOS displayed an increased weight gain ($P = 0.08$). The capacity of XOS to improve the performance of animals during a longer trial (42 d) was investigated (Experiment 2). The data revealed that diet supplementation with XOS, tested at two incorporation rates (0.1 and 1 g/kg), or with an exogenous β-1,4-xylanase resulted in an increased nutritive value of the wheat-based diet. An improvement in animal performance was accompanied by a shift in the microbial populations colonizing the upper portions of the GI tract. XOS were also able to improve the performance of broilers fed a corn-based diet, although the effects were not apparent at incorporation rates of 10 g/kg. Together these studies suggest that in some cases the capacity of β-1,4-xylanases to improve the nutritive value of wheat-based diets is more related to their ability to produce prebiotic XOS than to their ability to degrade arabinoxylans. The extremely low quantities of XOS used in this study also challenge the depiction of a prebiotic being a quantitatively fermented substrate. These data also bring into question the validity of the "cell wall" mechanism, as XOS elicited an effect with clearly no action on endosperm cell wall integrity and yet the performance effects noted were equivalent or superior to the added enzymes.

Key words: xylo-oligosaccharides, prebiotics, feed enzymes, wheat and corn-based diets, β-1,4-xylanases, broilers

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INTRODUCTION

Cereal-based diets contain soluble non-starch polysaccharides (**NSP**) that, due to its intrinsic viscosity, display significant anti-nutritive properties for poultry (Bedford et al., 1991; Bedford and Classen, 1992; Chesson, 1993; Choct, 2006). It is now well established that the addition of β-1,4-xylanases or β-1,3-1,4-glucanases to wheat- or barley-based diets, respectively, leads to a significant depolymerization of the indigestible NSPs resulting in improved bird performance. There has been considerable debate about the mechanisms by which exogenous enzymes improve the nutritive value of cereal-based diets for poultry. In diets containing a high proportion of soluble NSPs, exogenous enzymes reduce the resulting high digesta viscosity, thus promoting feed intake and the efficacy of endogenous digestive enzymes and leading to an improvement in nutrient digestibility (Bedford and Morgan, 1996; Bedford, 2000). In low viscosity diets, the action of exogenous enzymes has been attributed to their ability to degrade cereal cell walls, thus enabling enhanced access to cell contents by digestive enzymes (Bedford, 2000). Another, more subtle, action mechanism could involve the gut microbiota route (Apajalahti and Bedford, 1999; Fontes et al., 2004; Figueiredo et al., 2012). In the case of wheat-based diets, which are rich in arabinoxylans

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(Fengler and Marquardt, 1988), the resulting effect of exogenous β-1,4-xylanases would be the generation of a range of xylo-oligosaccharides (**XOS**) (Biely et al., 1997). Such oligosaccharides would have a beneficial effect on the microflora colonizing the distal portion of the gastro-intestinal (**GI**) tract (Apajalahti and Bedford, 1999). Thus, the beneficial effects resulting from inclusion of β-1,4-xylanases in wheat-based diets could result from the production of XOS in addition to the direct activity on the soluble, viscous arabinoxylans.

XOS are xylose oligomers with a number of residues that can vary from 2 to 15, or even 20. It has been suggested that XOS may improve gut health and stimulate the animals´ immune response, thus functioning as typical prebiotics (De Maesschalck et al., 2015). In general, prebiotics stimulate selectively the growth and/or activity of those gut bacteria that contribute to health and well-being (Gibson and Roberfroid 1995; Grizard, and Barthomeuf, 1999; Gibson et al. 2004). Prebiotics are not degraded or absorbed in the small intestine and are thus available for fermentation in the lower gut by bacterial species adept at using such carbohydrate sources. This typically shifts the composition of the intestinal microbiome towards a relative increase in bifidobacterium and/or lactobacillus species and a decrease in pathogenic bacteria. In addition, prebiotics may stimulate the production of anti-bacterial metabolites such as lactic acid, volatile fatty acids, and bacteriocins (Ššković et al., 2001). The presence of short-chain fatty acids (SCFA) in the intestines contributes to a lower pH, a better bio-availability of calcium and magnesium, and the inhibition of potentially harmful bacteria (Teitelbaum and Walker, 2002; Wong et al., 2006). SCFA are further used by the host organism as an energy source. A variety of compounds have been proposed to act as prebiotic compounds. These include inulin, fructo-oligosaccharides, galacto-oligosaccharides, isomalto-oligosaccharides, amylase-resistant gluco-oligosaccharides, lactosucrose, mannan-oligosaccharides, and soybean oligosaccharides (Monsan and Paul, 1995; Orban et al., 1997; Patterson et al., 1997; Piva, 1998; Collins and Gibson, 1999; Macfarlane et al., 2006; Baurhoo et al., 2007a,b).

Several studies have demonstrated that different Bifidobacterium species, when grown in pure cultures, can efficiently utilize XOS (Okazaki et al., 1990; Yamada et al., 1993; Jaskari et al., 1998; Van Laere et al., 2000; Crittenden et al., 2002; Palframan et al., 2003; Moura et al., 2007). Some strains of Bifidobacterium species grow more efficiently on XOS than on xylose (Okazaki et al., 1990; Crittenden et al., 2002; Palframan et al., 2003). This suggests that Bifidobacterium species possess a specific oligosaccharide uptake mechanism for XOS (Palframan et al., 2003), which may provide a competitive advantage over strains that can only take up monosaccharides. Less efficiently, XOS can be utilized by some *Bacteroides* species and Lactobacillus species, but not by a range of other intestinal bacteria including *Escherichia coli* and *Clostridia* (Yamada et al., 1993; Van Laere et al., 2000; Moura et al., 2007). The increase in the levels of colonic or caeca *Bifidobacterium* spp has been reported in several studies using XOS on rats or on mice (Campbell et al., 1997; Hsu et al., 2004; Santos et al., 2006). In these studies, increases in Bifidobacterium spp levels were also accompanied by significant decreases in levels of Enterobacteriaceae and/or sulfite-reducing bacteria. Here, in a set of 3 experiments, we have investigated the prebiotic activity of XOS. The data suggest that, *per se*, XOS can contribute to improve the nutritive value of both wheat- and corn-based diets for poultry. The implication of the data in view of the putative role of β-1,4-xylanases used to supplement cereal-based diets for poultry is discussed.

MATERIALS AND METHODS

Enzymes and Oligosaccharides

A series of 3 experiments were performed to probe the capacity of β-1,4-xylanases and XOS to improve the nutritive value of wheat and corn based diets for broilers. The enzyme selected for these studies was DuPont/Danisco Xylanase 40000G® (Marlborough, United Kingdom), a dried *Trichoderma reesei* fermentation product displaying a minimum xylanase activity of 40,000 U/g. The XOS preparation (XOS95P®) and purified D-xylose used to supplement the poultry diets were acquired from Shandong Longlive Biotechnology Corporation, China.

Oligosaccharide and Monosaccharide Composition Analysis

Monosaccharides were released from XOS by hydrolysis with 1 M H_2SO_4 for 2.5 h at 100°C. Neutral sugars were analyzed as their alditol acetates by gas-chromatography-flame ionization detection (Coelho et al., 2016). The hydrolysis was performed in duplicate. For the determination of free sugars composition, the samples were not submitted to the acid hydrolysis step prior to the derivatization to alditol acetates. The XOS profile was determined by reduction and acetylation of the sample as performed for monosaccharides. The reduced and acetylated XOS were separated and analyzed by gas chromatography–mass spectrometry (GC–MS) on an Agilent Technologies 6890N Network. The GC was equipped with a 400-1HT, dimethylpolysiloxan capillary column (25 m length, 0.22 mm of internal diameter, and 0.05 μm of film thickness). The GC was connected to an Agilent 5973 mass quadrupole selective detector operating with an electron impact mode at 10 and 70 eV, scanning the m/z range 50 to 800 at 2 scans s^{-1}, in a full scan mode acquisition (Simões et al., 2011).

Determination of the Presence of Starch

In order to evaluate the presence of starch in XOS mixture, 19 mg of sample were treated with α-amylase from *Bacillus subtilis* (EC 3.2.1.1, 62 U/mg, Sigma-Aldrich; St. Louis) at 37°C overnight, at pH 7. Amylose and cellulose were used as positive and negative controls, respectively. The sugars released by enzymatic hydrolysis were analyzed after the derivatization to alditol acetates.

Oligosaccharide Ethanol Fractionation and Matrix-Assisted Laser Desorption/Ionization (MALDI) Mass Spectrometry Analysis

The XOS sample was suspended in absolute ethanol and the ethanol insoluble material was separated from the supernatant by centrifugation, giving origin to an ethanol soluble fraction and an ethanol insoluble fraction (Coelho et al., 2014). The ethanol insoluble fraction was dissolved in water and absolute ethanol was added until reaching 50% (v/v) of ethanol, allowing separation by centrifugation of the material insoluble in 50% ethanol (Et50). The material remaining dissolved in the 50% ethanol solution was then precipitated adding more ethanol, giving origin to fractions Et75 and Et80. The material remaining soluble in the ethanol 80% solution was named EtSn. The fractions Et50 and Et75 were pooled, suspended in water, and re-precipitated in 50% ethanol. The XOS sample and the precipitated ethanol fractions were dissolved in ultrapure water (1 mg/mL) and 10 μL of each sample was added to 10 μL of 2,5-dihydroxybenzoic acid (DHB) matrix (15 mg/mL in methanol). From this mixture, 0.5 μL were deposited on the top of a layer of crystals formed by the deposition of 0.5 μL of DHB solution on the MALDI plate and letting it dry at ambient conditions. MALDI-MS spectra were acquired using a MALDI-TOF/TOF Applied Biosystems 4800 Proteomics Analyzer (Applied Biosystems, Framingham, MA) instrument equipped with a nitrogen laser emitting at 337 nm and operating in a reflectron mode. Full-scan mass spectra ranging from m/z 500 to 4,000 were acquired in the positive mode.

Diets

The composition of the basal wheat (Experiments 1 and 2) and corn (Experiment 3) based diets used in this study, which were formulated to contain adequate nutrient levels as defined by the NRC (1994), is presented in Tables 1 and 5, respectively. The wheat used to prepare diets used in experiment 1 and 2 were from different sources. In experiment 1, the wheat basal diet was provided with no supplement (treatment C-), or with the xylanase mixture (0.0625 g/kg incorporation, treatment XYL), the XOS mixture (0.06 g/kg incorporation, treatment XOS) or xylose (0.06 g/kg incorporation, treatment OSE). In experiment 2, the wheat basal diet was also provided with no supplement (treatment C-), with the xylanase mixture (0.0625 g/kg incorporation, treatment XYL) or the XOS mixture at 2 levels of incorporation, 0.1 g/kg (treatment XOS1) or 1 g/kg (treatment XOS2). In experiment 3, the corn basal diet was provided with no supplement (treatment C-) or was supplemented with the XOS mixture at 3 levels of incorporation, 0.1 g/kg (treatment XOS1), 1 g/kg (treatment XOS2), or 10 g/kg (treatment XOS3). Diets were provided in the pelleted form after pelletizing at approximately 90°C. The mineral and vitamin premix was previously mixed with the enzyme/XOS preparations and used to supplement the feed just before administration to the animals.

Table 1. Ingredient composition and calculated analysis of the wheat-based feed of experiments 1 and 2.

Ingredients	%
Wheat	60.00
Soybean meal 47%	29.00
Corn	4.17
Soybean oil	2.75
Sodium chloride	0.30
Calcium carbonate	1.43
Dicalcium phosphate 18%	1.80
Lysine	0.15
DL-Methionine	0.20
Mineral and vitamin premix[1]	0.20
Calculated nutrient content	
Energy (Kcal/Kg)	3009
Crude Protein (%)	19.90
Ether extract (%)	4.70
Crude cellulose (%)	2.93
Ash (%)	6.55

[1]Mineral-vitamin premix provided the following per kilogram of diet: vitamin A, 9000 IU; vitamin D_3, 2100 IU; vitamin E, 20 mg; nicotinic acid, 30 mg; vitamin B_{12}, 0,12 mg; calcium pantothenate, 10 mg; vitamin K_3, 2 mg; thiamin, 1 mg; riboflavin, 4,2 mg; vitamin B_6, 1,7 mg; folic acid, 0,5 mg; biotin, 0,5 mg; Fe, 80 mg; Cu, 10 mg; Mn, 100 mg; Zn, 80 mg; Co, 0,2 mg; I, 1,0 mg; Se, 0,3 mg; monensin, 100 ppm.

Animals and Management

For each of the 3 experiments, 360 1-d-old Ross 308 male broiler birds were individually weighed at the beginning of the experiment and were randomly divided into 24 pens of 15 birds each. Chicks were wing-banded for individual identification. The 24 pens were randomly allocated to each of the 4 dietary treatments, leading to 6 pens per treatment. Throughout the experiment, chicks were given free access to water and feed, which were provided with drinking nipples and hanging feeders, respectively. Broilers were raised in floor pens that were located in an environmentally controlled room adjusted daily to the recommended temperatures and ventilation rates, according to standard brooding practices, and were exposed to constant light for the duration of the trial. Although experiment 1 lasted for only 28 d, in experiments 2 and 3 animals were

raised until 42 d. Weekly feed consumption and individual body weights were recorded. Feed conversion ratios were calculated by dividing the weight gain per pen, per week and at the end of the experiment, including the weight gain of any dead birds, by the total feed consumed during the respective period. Bird mortality was recorded daily. At the end of the experiments, when animals were 28 (Experiment 1) or 42 (Experiments 2 and 3) d of age, 2 birds per pen were slaughtered by cervical dislocation. The sizes of the various empty GI compartments were measured or weighed, and digesta samples were collected and stored at –20°C for subsequent analysis. Levels of β-glucanase activity in the GI tract were measured and microbial gDNA extracted from caecum samples as described below. Animal experiments were conducted in accordance with the Ethics Committee of CIISA, Faculdade de Medicina Veterinária, and approved by the Animal Care Committee of the National Veterinary Authority (Direcção Geral de Veterinária, Lisboa, Portugal), following the appropriate European Union guidelines (Council Directive 86/609/EEC).

Analytical Procedures

Digesta samples were centrifuged and the supernatant recovered and frozen at −80 °C for subsequent analysis of β-1,4-xylanase activity. Initially, qualitative analysis of β-1,4-xylanase activity in the digesta samples recovered from the various GI compartments was assessed in agar plates, using wheat arabinoxylan (Megazyme, Ireland) at 0.1% (w/v) final concentration, in 10 mM Tris-HCl pH 7.0. Catalytic activity was detected after 16 h of incubation at 37°C through the Congo Red assay plate, as described by Ponte et al. (2004) and Mourão et al. (2006). For measuring the viscosity of the small intestine contents, samples were centrifuged for 10 min at 7500 × g and the viscosity of the supernatant was measured using a Brookfield viscometer (Model LVDVCP-II, Brookfield Engineering Laboratories, Middleboro, MA) with a cup maintained at 24°C. Analyses for dry matter (DM; method 934.01), crude fat (920.39), crude protein (954.01), NDF (2002.04), and ADF/ADL (973.18) were performed according to the methods specified by Association of Official Analytical Chemists (1980).

Microbiome Profiling Through 16 s rRNA Sequencing

Caecum samples of 3 animals from each treatment (12 extractions in total) were subjected to genomic DNA extraction through Qiagen DNA extraction kit for stool samples, following the manufacturer instructions. DNA from caecum samples were then amplified for the region V3–V4 of the 16s rRNA as described previously (Vaz-Moreira et al., 2013), using the following primers: V3_titF ACTCCTACGGGAGGCAG and V4_titR TACNVRRGTHTCTAATYC. Resulting PCR products were sequenced at Biocant (Cantanhede, Portugal), in the 454 sequencing platform (Roche), according to manufacturers' instructions. Data analysis was carried out as previously described (Pinto et al., 2014) at Biocant (Cantanhede, Portugal). The raw pyrosequencing reads were assigned to the appropriate samples based on the respective barcode. Reads were quality filtered by elimination of sequence reads with <120 bp and that contained more than 2 undetermined nucleotides. Sequences in which the reverse primer was reached were additionally cut. Finally, sequences with more than 50% of low complexity regions, determined by DustMasker (Sogin et al., 2006), and chimera sequences, identified by UChime (Edgar et al., 2011), were discarded. The sequences were grouped by USearch (Edgar, 2010) according to a phylogenetic distance of 3%, creating the operational taxonomic units (OTU). Richness of population (rarefaction curves) and the diversity indices (Chao1) were calculated using the Mothur package (Schloss et al., 2009). The taxonomy of each OTU was identified through a BLAST search against the nt@ncbi/SILVA database. The best hits were selected and subjected to further quality control. All sequences with an alignment of less than 40% as well as those with an E-value greater than $1 \times e^{-50}$ were rejected. Additionally, a bootstrap test was applied to the OTUs to identify the least common taxonomy level. Only the sequences with a bootstrap greater than 70% after 100 replicates, as obtained by seqBoot from Phylip package (Felsenstein, 1989), were kept. The taxonomic assignment of the OTUs was completed with the attribution of the NCBI taxonomy identification number, which allowed the complete taxonomy construction of all identified organisms. Finally, for each taxon identified in the sample, the total number of sequences was summed up, providing the abundance of all identified organisms, for population statistics analysis.

Statistical Analysis

Statistical analysis of data concerning birds' performance was conducted by analysis of variance, using the General Linear Models procedure of SAS (SAS Inst. Inc., Cary, NC). Means with a significant F ratio were separated by the least significant difference test. The experimental unit was the pen of 15 birds (n = 6 per treatment). Unless otherwise stated, differences were considered significant when $P < 0.05$. A principal component analysis was established using the microbiota data following standard procedures (Edgar, 2010).

RESULTS AND DISCUSSION

Characterization of xylo-Oligosaccharides (XOS)

The XOS sample used to supplement the diets included 85% of xylose residues, determined as alditol

acetates after hydrolysis, of which 5% were monosaccharides. The analysis of XOS by GC-MS showed that xylobiose was the most abundant oligosaccharide (55 to 58%), followed by xylotetraose (26 to 33%) and xylotriose (12 to 16%). These results are in accordance with the high solubility of the sample in ethanol solutions, as the material that remained soluble in 80% ethanol accounted for 90% of the XOS. In order to characterize the 10% of XOS material with lower solubility in ethanol, which should present higher degree of polymerization, an MALDI-MS analysis was performed. The MALDI spectrum of the XOS mixture (Figure 1A) showed sodium adduct ions ($[M+Na]^+$) attributed to pentose oligosaccharides ($Pent_n$), from n = 4 to 12. These data, together with GC-MS data suggest the presence of XOS in the sample with the degree of polymerization from 2 to 12. Also, the MALDI spectrum of XOS mixture showed $[M+Na]^+$ ions assigned to hexose oligosaccharides (Hex_n), from n = 3 to 13. In order to evaluate if the glucose present came from starch, the XOS mixture was submitted to an extensive hydrolysis with α-amylase, releasing only 24% of the initial glucose that accounts for residual starch. MALDI-MS spectrum of fraction Et50 (Figure 1B) showed the presence of $Pent_3Hex$, $Pent_2Hex_2$, $Pent_4Hex$, and $Pent_6Hex_m$ from m = 1 to 8. In addition, XOS present in Et50 showed polymerized structures until 19 pentoses (Figure 1B), which allows us to conclude for the occurrence of hexoses linked to XOS. These glucose residues, not affected by α-amylase treatment, should not contribute to any glycogenic effect.

Inclusion of Exogenous xylanases or XOS in Wheat-Based Diets Promote Broiler Performance

In experiment 1, the capacity of a microbial β-1,4-xylanase, an XOS preparation and xylose to modulate the performance of broilers grown for 28 d was investigated. The data, presented in Table 2, revealed that birds fed on the wheat-based diet supplemented with the XOS mixture reached a higher final body weight ($P = 0.08$) and presented a higher 0 to 28 d weight gain ($P = 0.036$) when compared with non-supplemented animals. Under identical circumstances, both the microbial xylanase and xylose were unable to affect bird's final body weight and weight gain when compared with the negative control group. No differences were found in feed intake although the feed conversion ratio of animals receiving the oligosaccharide mixture was significantly lower when compared with the other groups. In addition, birds fed on the 4 different diets presented no differences in the weight and length of the various GI compartments (Table S1). The addition of xylose, XOS, or the microbial enzymes had no impact in the viscosity of the intestinal contents, which were low (Table S1). Taken together, the results suggest that XOS used to supplement a wheat-based diet have a positive effect on performance, possibly through a prebiotic effect. However, the short duration of the trial (28 d), the majority of which was conducted when the birds still have an under-developed gut microflora, compromises the ability of the prebiotic XOS to promote animal performance. Similar data were reported by Courtin et al. (2008) in a study using arabinoxylo-oligosaccharides (**AXOS**) to supplement wheat and corn-based diets for broilers. In animal trials that lasted 21 d, the authors reported an improvement in feed utilization, whereas body weights were unchanged (Courtin et al., 2008). The lack of response to enzyme supplementation observed in the present study was not due to an inactivation of the microbial xylanase through the GI tract, as samples collected in the different digestive compartments revealed capacity to degrade arabinoxylans (data not shown). It is possible that the lack of response to the addition of the exogenous enzyme results from the lower levels of NSPs present in the diet, which is supported by the lower viscosity of the digesta (see above). Lower NSPs would result in a limited release of oligosaccharides upon polysaccharide hydrolysis, thus reducing the prebiotic effect. Finally, the end product of xylan hydrolysis, xylose, had no impact on animal performance, suggesting that this sugar has no major effect in gut microbiota.

A similar experiment was performed using a diet with identical composition to experiment 1 (Table 3) but excluding xylose as a supplement. This allowed testing the effect of XOS at two different incorporation rates (0.1 and 1 g/kg). These incorporation rates were higher than those of Experiment 1 when XOS were incorporated at a 0.06 g/kg rate. A different batch of wheat was used to prepare the diets for Experiment 2; all other remaining components of the diet were of the same origin as in Experiment 1 (Table 1). The trial lasted for 42 d. Bird body weight, weight gain, feed intake, and feed conversion ratio of Experiment 2 are summarized in Table 3. The data revealed that final body weight of birds fed the basal diet supplemented with XOS, at the two levels of incorporation, or the commercial enzyme was significantly higher than those of birds fed on the non-supplemented diet. Differences in body weight were visible as soon as day 7 and remained significant for the duration of the experiment. However, although differences in weight gain were observed in the first 2 wk of the experiment, they were particularly acute in the last 2 wk of the trial, from days 28 to 42, when caeca fermentation was presumably far more extant than in younger birds. There was no difference in performance between the two levels of XOS incorporation. In addition, all of the supplemented diets resulted in markedly higher feed intake than the control diet in the last week of the trial, although when the entire period of the experiment was considered no differences between groups were observed. Finally, there were no differences in feed conversion ratios although in the initial 2 wk of the experiment birds supplemented with both the enzyme and lower dose of XOS converted

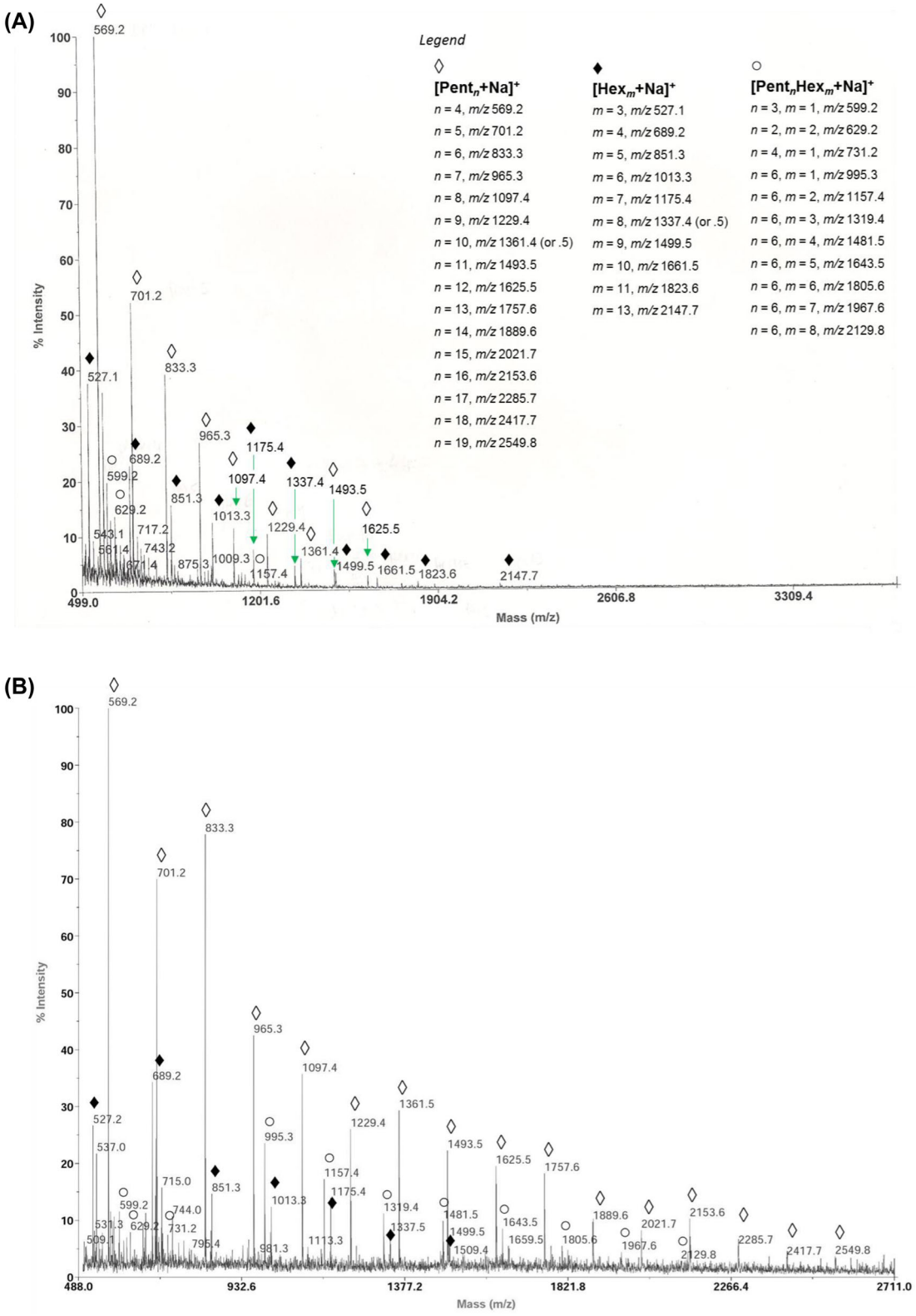

Figure 1. MALDI spectra of (A) XOS and (B) the XOS insoluble in solutions of 50% of ethanol.

feed more effectively. This may indicate that the direct result of XOS incorporation is through the improvement of feed intake rather than from an improvement in feed efficiency. Taken together the results revealed that, as it has been extensively described previously, the exogenous xylanase was able to improve the nutritive value of the wheat-based diet of experiment 2. The fact that animals consuming diets supplemented with XOS and the microbial enzyme achieved similar performance suggests that the exogenous biocatalysts may mediate their effects through mechanisms that do not involve a decrease in the concentration of the anti-nutritive

Table 2. Performance of broilers fed on the wheat-based diet supplemented with a β-1,4-xylanase mixture (XYL), a xylo-oligosaccharide preparation (XOS) and xylose (OSE) (**Experiment 1).** A fourth group of birds was fed on a basal non-supplemented diet (C-).

	C-	XYL	XOS	OSE	SEM	p(*F*)
Body Weight (g)						
0d	37.9	37.9	37.9	37.9	0179	0991
7d	152	146	149	148	4278	0823
14d	406	401	402	397	9208	0933
21d	876	881	880	875	15.10	0991
28d	1508	1503	1558	1483	20.5	0082
Weight Gain (g)						
0–7d	114	108	112	110	4296	0822
7–14d	248	250	243	244	8416	0901
14–21d	465	481	480	478	9958	0630
21–28d	616	619	631	605	13.82	0617
0–28d	1470^{b}	1465^{a,b}	1534^{a}	1445^{a,b}	21.75	0036
Feed Intake (g)						
0–7d	131	125	125	127	4598	0765
7–14d	348	348	350	338	8027	0935
14–21d	676	681	693	690	13.31	0779
21–28d	955	994	955	955	23.33	0566
0–28d	2081	2143	2123	2110	36.04	0664
Feed Conversion Ratio						
0–7d	1.15	1.16	1.13	1.16	0029	0842
7–14d	1.40	1.41	1.45	1.39	0033	0667
14–21d	1.46	1.42	1.45	1.44	0015	0284
21–28d	1.56	1.58	1.55	1.44	0041	0523
0–28d	1.42^{a}	1.46^{a}	1.39^{b}	1.46^{a}	0021	0052

Means in the same row with different superscripts (a,b) are statistically different ($P \leq 0.05$).

Table 3. Performance of broilers fed on the wheat-based diet supplemented with a β-1,4-xylanase mixture (XYL), and a XOS preparation provided at two different incorporation rates, 0.1 g/kg (XOS1) or 1 g/kg (XOS2) (**Experiment 2)**. A fourth group of birds was fed on a basal non-supplemented diet (C-).

	C-	XOS1	XOS2	XYL	SEM	p(*F*)
Body Weight (g)						
0d	45.5	46.3	46.5	45.5	0391	0203
7d	160^{b}	168^{a}	168^{a}	172^{a}	2163	0012
14d	419^{b}	450^{a}	441^{a}	451^{a}	6598	0004
21d	864^{b}	928^{a}	903^{a,b}	916^{a}	15.250	0029
28d	1526	1614	1566	1602	26.223	0081
35d	2266^{b}	2388^{a}	2348^{a,b}	2370^{a}	30.407	0032
42d	2854^{b}	3048^{a}	2994^{a}	3021^{a}	35.386	0003
Weight Gain (g)						
0–7d	115^{b}	121^{a,b}	122^{a}	126^{a}	2136	0013
7–14d	2589^{b}	283^{a}	273^{a,b}	279^{a}	5275	0019
14–21d	446	478	462	465	9579	0169
21–28d	661	686	663	686	14.663	0403
28–35d	741	774	782	768	11.476	0061
35–42d	588^{b}	660^{a}	647^{a}	651^{a}	20.165	0053
0–42d	2808^{b}	3001^{a}	2948^{a}	2975^{a}	35.291	0003
Feed Intake (g)						
0–7d	207	206	218	215	5367	0111
7–14d	469	439	467	443	17.484	0298
14–21d	832	797	779	870	29.939	0132
21–28d	1091	1062	1047	1079	42.602	0865
28–35d	1370	1328	1355	1341	25.239	0648
35–42d	1188^{b}	1373^{a}	1364^{a}	1333^{a}	28.213	0001
0–42d	5156	5204	5230	5280	93.969	0783
Feed Conversion Ratio						
0–7d	1.80^{a}	1.70^{b}	1.79^{a}	1.70^{b}	0036	0050
7–14d	1.82^{a}	1.56^{b}	1.72^{a}	1.59^{b}	0083	0033
14–21d	1.88	1.67	1.68	1.87	0087	0142
21–28d	1.66	1.55	1.58	1.58	0076	0719
28–35d	1.85^{a}	1.71^{b}	1.74^{b}	1.75^{b}	0031	0017
35–42d	2.03	2.09	2.11	2.05	0044	0461
0–42d	1.84	1.74	1.77	1.78	0035	0253

Means in the same row with different superscripts (a,b) are statistically different ($P \leq 0.05$).

arabinoxylans or the release on cell-wall trapped nutrients, but rather involves the generation of XOS that are used as prebiotics by gut microbiota.

It has been extensively observed that diets presenting high levels of soluble NSP induce a considerable enlargement of some portions of the GI tract (Brenes et al., 1993) and stimulate an increase in protein turnover rates (Dänicke et al., 2000). Generally, enzyme supplementation leads to a reduction in the relative weight of the digestive tract compartments, leading to an increase in carcass yield (Van der Klis et al., 1993). Reduction in the size of GI tract of animals receiving exogenous xylanases results from a decrease in digesta viscosity (Fuente et al., 1998). Thus, the effects of dietary treatments in the relative length and weight of GI tract compartments of broiler chickens of experiment 2 were evaluated. The data, presented in Table 4, suggest that none of supplements had an effect on crop, gizzard, and liver relative weights or in the length of the small and large intestines. The beneficial effects that result from the inclusion of XOS in poultry diets seem to be mediated through the microflora route (see below), and thus it is surprising to observe that the size of the caeca remains unchanged in animals receiving the oligosaccharide supplement. As observed in experiment 1, these data suggest that the wheat lot used in experiment 2 was not viscous enough to affect GI tract dimensions as has been noted in the past (Fontes et al., 2004).

Table 4. Relative weight and length of the GI tract of broilers fed on a wheat-based feed supplemented with a β-1,4-xylanase mixture (XYL), and an XOS preparation provided at 2 different incorporation rates, 0.1 g/kg (XOS1) or 1 g/kg (XOS2) (data from Experiment 2). C-, birds fed on a non-supplemented diet.

	C-	XOS1	XOS2	XYL	SEM	p(*F*)
Relative weight (g/100 g BW)						
Crop	2.08	2.34	2.48	2.22	0.138	0.228
Gizzard	9.56	10.00	11.46	10.14	0.904	0.488
Liver	22.86	22.02	21.74	21.56	0.931	0.750
Duodenum	4.32	4.47	4.24	4.34	0.147	0.748
Jejunum	9.30	9.37	9.64	9.46	0.349	0.914
Ileum	7.86	7.92	8.03	7.71	0.324	0.919
Cecum	1.72	1.79	1.77	1.73	0.081	0.934
Relative length (cm/kg BW)						
Duodenum	10.57	10.11	10.27	10.19	0.296	0.714
Jejunum	26.66	23.56	26.06	25.90	0.976	0.135
Ileum	27.64	25.35	25.95	25.51	0.725	0.114
Cecum	7.08	7.25	6.75	6.77	0.225	0.337

Data presented above suggest that in wheat-based diets exogenous xylanases may influence animal performance through the production of XOS rather than by reducing the concentration of soluble, viscous arabinoxylans or by reducing the integrity of cereal cell walls. XOS resulting from the activity of feed xylanases

on arabinoxylans leads to a mixture of unsubstituted and arabinose substituted XOS, usually termed AXOS. In addition to arabinose side chains, arabinoxylans may be branched with a combination of side groups such as α-D- glucopyranosyl uronic acid or its 4-*O*-methyl derivative and acetyl groups (Coelho et al., 2016). The presence of these side groups in arabinoxylan hydrolysis products may result in branched XOS with potentially diverse biological properties. The data presented here and by other groups (De Maesschalck et al., 2015) show that unbranched XOS displayed beneficial effects in bird performance. Here, we also reveal that this effect is similar when compared with the complex mixture of putative oligosaccharides that result from the hydrolysis of complex arabinoxylans, after hydrolysis by endo-β-1, 4-xylanases. These data suggest that beneficial effect of XOS and AXOS in broiler performance is identical. It is also possible that exogenous xylanases may attack not only the soluble arabinoxylans but also the more recalcitrant and insoluble forms of the polysaccharide thus contributing to the production of large concentrations of XOS despite the apparently reduced concentrations of soluble arabinoxylans in modern wheat varieties.

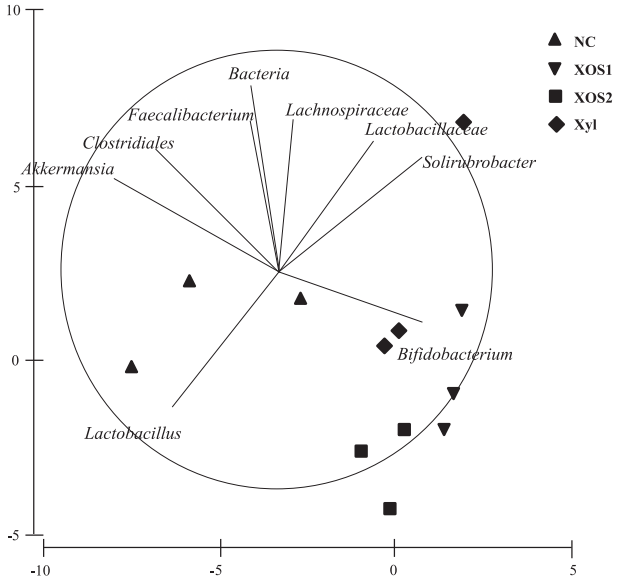

Figure 2. Principal Component Analysis (PCA) of bacterial microbiota in the caecum of birds fed on a wheat-based diet supplemented with a β-1,4-xylanase mixture (XYL), and xylo-oligosaccharides provided at two different incorporation rates, 0.1 g/kg (XOS1) or 1 g/kg (XOS2). The fourth group of birds was fed on a basal non-supplemented diet (NC). NC (▲), XOS1 (▼), XOS2 (■), XYL (◆).

XOS Modulate Gut Microbial Populations

Data presented above suggest that XOS and AXOS indirectly generated by β-1,4-xylanases used to supplement wheat-based diets, may modulate an improvement in broiler performance through a prebiotic mechanism. To test this hypothesis, we investigated how β-1,4-xylanases and XOS modulate gut microflora populations. Thus, bacterial genomic DNA was extracted from caecal samples of 12 animals from each of the 4 treatments of experiment 2 (3 animals per treatment) to allow profiling bacterial populations using second generation sequencing technology. Thus, 16S rRNA region V3–V6 was amplified through PCR using caecum nucleic acids as a template and the resulting amplification products were sequenced through a pyrosequencing method. A multivariate analysis using principal component analysis was implemented in order to evaluate differences in bacterial profile. The data, presented in Figure 2, revealed that non-supplemented animals were characterized primarily by microorganisms of *Anaerotrumcus*, *Akkemansia* and *Faecalibacterium* genera. In contrast, samples from the supplemented birds (XOS and xylanase) were characterized by microrganisms of the *Solirubrobacter* and *Bifidobacterium* genera. Regarding the abundance of the microflora, the data revealed that samples from the non-supplemented animals can be distinguished from samples of the supplemented animals. This further suggests an overlap between the XOS and xylanase treatments, suggesting the latter is functioning in large part by generation of XOS/AXOS. Control samples were characterized by *Lactobacillus*, *Akkermansia*, *Clostridiales*, *Faecalibacterium*, among other bacteria, whereas the supplemented samples were characterized by *Bifidobacterium*, *Solirubrobacter* and also members from the family *Lactobacillaceae* and *Lachnospiraceae*. Overall the data suggest that XOS are the preferential substrate for the beneficial bacteria, mainly bifidobacteria, contributing to their predominance in the GI ecosystem. Nonsubstituted XOS and AXOS are fermented more quickly than more complex structures (methylated glucuronic acid, glucuronic acid, and acetyl branching) releasing higher concentrations of lactate (Kabel et al, 2002). Also, commercial XOS resulted in greater production of lactate and increased bifidobacterium populations (Moniz et al. 2016). Taken together, results suggested that XOS and also AXOS resulting from exogenous xylanase activity on arabinoxylans can modulate the caecal microbiota profile of chickens. These results demonstrate a prebiotic effect of XOS/AXOS that leads to an improvement of animal performance as reflected by higher body weights and weight gains. It is possible that the improvement in the gut microbiota profile also contributes to an improvement in feed intake, thus promoting performance.

XOS Promote the Performance of Broilers Fed on Corn-Based Diets

In experiment 3, the capacity of XOS to improve the performance of broilers fed a corn-based diet was investigated. A typical corn-based diet was prepared (Table 5) and used to feed broiler chickens throughout a 42 d trial. Three levels of XOS incorporation were compared (0.1, 1, or 10 g/kg) with a non-supplemented version of the basal diet. Bird performance in

Table 5. Ingredient composition and calculated analysis of the corn-based feed of experiment 3.

Ingredients	%
Corn	56.96
Soybean meal 47%	35.90
Soybean oil	3.15
Sodium chloride	0.25
Calcium carbonate	1.48
Monocalcium phosphate 22.7%	1.70
DL-Methionine	0.25
Elancoban 200	0.05
Mineral and vitamin premix[1]	0.20
Calculated nutrient content	
Energy (kcal/kg)	3000
Crude Protein (%)	20.00
Ether extract (%)	5.60
Crude cellulose (%)	4.10
Ash (%)	5.88

[1]Mineral-vitamin premix provided the following per kilogram of diet: vitamin A, 9000 IU; vitamin D_3, 2100 IU; vitamin E, 20 mg; nicotinic acid, 30 mg; vitamin B_{12}, 0,12 mg; calcium pantothenate, 10 mg; vitamin K_3, 2 mg; thiamin, 1 mg; riboflavin, 4,2 mg; vitamin B_6, 1,7 mg; folic acid, 0,5 mg; biotin, 0,5 mg; Fe, 80 mg; Cu, 10 mg; Mn, 100 mg; Zn, 80 mg; Co, 0,2 mg; I, 1,0 mg; Se, 0,3 mg; monensin, 100 ppm.

Table 6. Performance of broilers fed on the corn-based diet supplemented with a XOS preparation provided at three different incorporation rates, 0.1 g/kg (XOS1), 1 g/kg (XOS2) or 10 g/kg (XOS3) (**Experiment 3)**. A fourth group of birds was fed on a basal non-supplemented diet (C-).

	C-	XOS1	XOS2	XOS3	SEM	p(F)
Body Weight (g)						
0d	46.5	46.6	46.1	46.3	0368	0777
7d	134^{a,b}	136^{a}	137^{a}	127^{b}	2571	0044
14d	292^{b}	319^{a}	313^{a}	291^{b}	6329	0009
21d	536^{b}	646^{a}	607^{a}	566^{b}	15.774	0.0001
28d	906^{c}	1128^{a}	1036^{b}	964^{c}	27.381	0001
35d	1480^{c}	1781^{a}	1646^{b}	1536^{c}	43.536	0001
42d	2084^{b}	2406^{a}	2262^{a}	2136^{b}	56.732	0003
Weight Gain (g)						
0–7d	87^{a,b}	90^{a}	91^{a}	81^{b}	2442	0033
7–14d	158^{b}	183^{a}	176^{a}	164^{b}	4251	0004
14–21d	245^{c}	327^{a}	294^{b}	274^{b}	10.388	0002
21–28d	370^{b}	482^{a}	429^{a}	398^{b}	12.640	0.0001
28–35d	574^{b}	653^{a}	615^{a}	573^{b}	18.579	0019
35–42d	604	625	616	604	20.058	0856
0–42d	2038^{b}	2359^{a}	2216^{a}	2090^{b}	56.696	0003
Feed Intake (g)						
0–7d	130	133	134	126	2747	0210
7–14d	312^{b}	339^{a}	334^{a}	311^{b}	6653	0009
14–21d	539^{b}	592^{a}	585^{a}	5385^{b}	11.916	0005
21–28d	952^{b,c}	1009^{a,b}	1039^{a,b}	888^{c}	38.188	0053
28–35d	1229	1234	1319	12.020	36.775	0161
35–42d	1294	1269	1370	1234	40.600	0144
0–42d	4455^{b}	4576^{a,b}	4781^{a}	4299^{b}	102.386	0023
Feed Conversion Ratio						
0–7d	1.49^{b}	1.49^{b}	1.47^{b}	1.57^{a}	0022	0026
7–14d	1.98	1.86	1.91	1.90	0048	0418
14–21d	2.21^{a}	1.83^{b}	1.99^{b}	1.99^{b}	0082	0036
21–28d	2.59^{a}	2.10^{b}	2.42^{a}	2.27^{b}	0115	0042
28–35d	2.15	1.91	2.15	2.10	0091	0206
35–42d	2.17	2.04	2.25	2.09	0111	0571
0–42d	2.19	1.95	2.16	2.07	0074	0119

Means in the same row with different superscripts (a,b and c) are statistically different ($P \leq 0.05$).

experiment 3, expressed as body weight, weight gain, feed intake and feed conversion ratios are summarized in Table 6. From day 14, birds receiving the corn-based feed supplemented with XOS at the lowest incorporation level (0.1 g/kg) presented a body weight higher than the non-supplemented animals. In contrast, animals that were fed on the diet containing the highest incorporation rate of XOS (10 g/kg) presented a body weight similar to birds that were not exposed to the XOS additive. From day 7, the body weight of birds supplemented with the lower levels of XOS was always higher than those of birds receiving XOS at 10 g/kg. Although birds fed on the diets containing the intermediate levels of XOS (1 g/kg) presented a final body weight similar to birds of the XOS1 treatment, at days 28 and 35 they presented a significantly lower weight. Thus, the data suggest that while XOS have a beneficial effect in broilers´ weight, the optimum is only observed at lower incorporation rates (0.1 g/kg); at higher incorporation levels XOS have a marginal or no effect on animal performance. Similar such "overdosing" effects have been demonstrated by Zhenping et al. (2013). A similar trend is observed for weight gain, with animals receiving XOS at the lowest incorporations levels presenting a higher final weight gain than non-supplemented birds or birds fed on the corn diet supplemented with 10 g/kg of XOS. The addition of exogenous XOS resulted in a higher feed intake for birds receiving the oligosaccharide preparation at an intermediate level when compared with non-supplemented animals although there are no differences in the supplemented groups. In contrast, no differences in FCRs were observed between the four treatments. Taken together, the data suggest that XOS are able to improve the performance of broiler chicks although only when incorporated at the lowest rates tested (0.1 to 1 g/kg). Evaluation of body weight and weight gain evolution throughout the trial suggest that the lowest incorporation rate (0.1 g/kg), or perhaps even lower than tested here, might lead to the best results. The effects of the different dietary treatments on the relative length or weight of different organs and GI tract compartments of broiler chickens of experiment 3 were evaluated. The data, presented in Table S2, revealed that feed supplementation did not affect GI tract dimensions, as animals receiving the XOS preparation displayed a similar GI size as non-supplemented animals.

Data from experiments 1, 2, and 3 suggest that the exogenous XOS used as feed supplements managed to pass through the most aggressive GI compartments, evading gastric denaturation and attack by the endogenous repertoire of digestive enzymes that is a property required for an effective prebiotic mixture (Gibson and Roberfroid 1995; Grizard, and Barthomeuf, 1999; Gibson et al. 2004). Overall, previous data describing the biochemical properties of XOS suggest that they indeed remain intact during passage through the GI tract (Okazaki et al., 1990; Jaskari et al., 1998; Kajihara

et al., 2000; Van Laere et al., 2000; Crittenden et al., 2002; Palframan et al., 2003; Chung et al., 2007; Moura et al., 2007; Na and Kim, 2007). Yamada et al. (1993) studied the resistance of XOS to low pHs and found that these molecules were only weakly hydrolyzed at pH 2.0 at 100 °C, whereas sucrose and inulin were completely hydrolyzed under those conditions. Courtin *et al.* (2009) mimicked gastric conditions (pH 2.0, 37°C) *in vitro* and found less than 10% hydrolysis of XOS after up to 14 d, whereas more than half of the fructo-oligosaccharides were hydrolyzed after 3 d. Furthermore, previous *in vitro* tests revealed that xylobiose is not hydrolyzed to xylose upon addition of either saliva, pepsin-containing artificial gastric juice, pancreatin, or intestinal mucosa homogenate (Okazaki et al., 1991), indicating that at least the β-1,4-linkage in arabinoxylans resists hydrolysis in the upper GI tract. Taken together, the data presented here corroborates previous observations suggesting that XOS remain largely unabsorbed in the small intestine. The production of SCFA in *in vitro* fermentation XOS studies has been corroborated through several *in vivo* studies. Increased SCFA levels in the caeca of rats or mice were observed after feeding the animals with diets enriched with XOS (Imaizumi et al., 1991; Campbell et al., 1997; Van Craeyveld et al., 2008). The data presented here corroborate these reports and suggest XOS function as prebiotics. The beneficial effects of XOS on broiler performance have already shown stimulation of butyrate-producing bacteria through cross-feeding of lactate and subsequent effects of butyrate on GI function (De Maesschalck et al., 2015).

CONCLUSIONS

Data presented here revealed that exogenous xylanases used to supplement wheat-based diets for poultry may mediate their effects by modulating the gut microflora colonizing the upper portions of the birds´ GI tract. Thus, XOS resulting from the hydrolysis of wheat arabinoxylans by exogenous xylanases or used directly as a feed supplement, display a prebiotic effect in broiler chicks. Significantly, the prebiotic effect of XOS was also observed when these oligosaccharides were used to supplement corn-based diets, resulting in a significant growth promoting activity. Overall these data suggest that XOS used as feed supplements resist the passage through the upper regions of the GI tract, a condition required to their use as effective prebiotics in poultry nutrition. In addition, the bioactivity of unbranched XOS seems to be similar to the oligosaccharides resulting from the hydrolysis of feed arabinoxylans that are mostly branched with arabinose side-chains (AXOS). Finally, data of experiment 3 suggest that the incorporation rates of XOS need to be carefully fine-tuned, since XOS used at a 10 g/kg level had no effect on performance. Thus, optimum incorporation rates may range between 0.1 and 1 g kg of XOS, although animals fed on corn-based diets supplemented with XOS at a 0.1 g/kg rate resulted in more than 350 grams of additional body weight gain when compared with the control group, suggesting even lower doses may be effective. This observation suggests that XOS modulate an improvement in animal performance by optimizing feed digestion, feed intake and by triggering the evolution of the microbiome to a more favorable construction. An important consideration of the mode of action of this prebiotic relates to the quantities used. At 0.1 g/kg, this is equivalent to less than 0.3 Kcal/kg that is clearly so little that the XOS alone cannot be responsible for the scale of response noted in all experiments. Even if all the added XOS were converted with 100% efficiency to SCFA's this would not result in a measurable effect. Thus, we suggest that XOS are effectively pump primers, sending a signal to encourage those bacteria that can digest feed xylan to become much more active, digest the xylan in the diet more effectively and interact with the digestive tract in such a way that efficiency of digestion as a whole is increased. Such an evolution of the microbiome would not be immediate and develops slowly which is in concert with the performance responses being greater later on in life. One proposal is that the SCFA produced elicit a peptide tyrosine-tyrosine release that delays gastric emptying and thus enhances gastric digestive efficiency. If XOS and other prebiotics are acting in such a "pump priming" manner, it suggests the definition of a prebiotic may also need revisiting, as their role may simply be to guide the microbiome to more effectively utilize a fermentable substrate (i.e., in this case feed derived xylan) that is already present in the intestine rather than by quantitatively acting as a substrate for fermentation.

SUPPLEMENTARY DATA

Supplementary data are available at *Poultry Science* online.

Table S1. Relative weight and length of the GI tract of broilers diet supplemented with a β-1,4-xylanase mixture (XYL), a xylo-oligosaccharide preparation (XOS) and xylose (OSE). A fourth group of birds was fed on a basal non-supplemented diet (C-). Data from **Experiment 1**.

Table S2. Relative weight and length of the GI tract of broilers diet supplemented with a XOS preparation provided at three different incorporation rates, 0.1 g/kg (XOS1), 1 g/kg (XOS2) or 10 g/kg (XOS3). A fourth group of birds was fed on a basal non-supplemented diet (C-). Data from **Experiment 3**.

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