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Understanding the Direct and Indirect Mechanisms of Xylanase Action on Starch Digestion in Broilers

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ABSTRACT

The objective of the current study was to investigate the mechanisms of xylanase action in a maize-soya diet and its effect on starch digestion. A total of 60 broilers were divided into 6 treatment groups; a control group without xylanase, and five other groups supplemented with xylanase (Econase XT 25; 100 g/t) from 1, 2, 3, 4 or 5 weeks before slaughter. At the end of the experiment, digesta was collected from the gizzard, upper and lower small intestine, and both caeca. Digesta pH ranged from pH 2.2-4.4, 5.9-6.6, 6.7-7.8 and 5.7-7.3 in the gizzard, upper small intestine, lower small intestine, and both caeca, respectively, with no effect of xylanase ($P > 0.05$). Scanning Electron Microscope (SEM) images along with total starch measurements showed the progression of starch digestion through the tract. The SEM did not show any greater disruption to cell wall material with xylanase supplementation. This suggests that xylanase was not working directly on the cell wall and provides evidence for the hypothesis that xylanase works through an indirect mechanism. Peptide YY (PYY) concentration in the blood was higher during the first few weeks of supplementation, with longer periods of supplementation nulling this effect, implying that xylanase may be acting through a prebiotic mechanism. The RT-q PCR results revealed a numerical increase in glucose transporter (GLUT2 and SGLT1) expression at 2 and 3 weeks of xylanase supplementation, respectively, which might suggest a greater absorption capacity of birds. From these results, a potential mechanism of xylanase action in maize-based diets has been proposed.

Key words: Broiler, Maize, Starch, Xylanase

INTRODUCTION

With an increasing demand from the biofuels industry for cereals such as maize to be used for ethanol production, the cost of such raw feed materials has risen in recent years. Consequently, considerable attention has been given to improve nutrient utilisation of these diets through the use of exogenous enzymes, including xylanases, to release more energy from the diet. Xylanase enzymes hydrolyse arabinoxylans, a major component of cereal grain cell walls (Collins et al., 2005). Traditionally, exogenous xylanases have been added to viscous diets, such as those based on wheat, barley, and rye, whereby significant levels of soluble arabinoxylans have been demonstrated to have anti-nutritive effects on nutrient digestibility and absorption in poultry (Annison and Choct, 1991; Bedford and Schulze, 1998; Choct, 2006). These effects are often attributed to the ability of soluble non-starch polysaccharides (NSP) to increase digesta viscosity, thereby reducing digesta transit rate, digestibility, and increasing fermentation in the small intestine (Choct et al., 1996). Maize contains a relatively low concentration of soluble NSP (Choct, 1997) compared to other cereals, such as wheat, suggesting a possible

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explanation for the lower magnitude of broiler response to xylanase supplementation in maize-based diets. However, there is evidence to suggest a beneficial effect of xylanase inclusion in maize-based diets through improved broiler performance (Zanella et al., 1999; Cowieson, 2010), indicating that additional mechanisms other than reduction in viscosity are important. It is important to note however, that these studies focus on the use of multi-enzyme applications and therefore the mechanisms of xylanase action exclusively must also be considered.

It is still unclear as to how such enzymes improve digestibility in maize-based diets. One well-discussed explanation is cell wall dissolution, whereby xylanases hydrolyse the insoluble NSP fraction in maize (Meng and Slominski, 2005). This presumably leads to the release of components, including starch, protein and lipids, from within the cell thereby positively aiding digestibility (Bedford, 2002). However, the site of enzyme action in the gut combined with digesta transit time suggests that there is insufficient time for exogenous xylanases to significantly degrade cell wall material directly by the small intestine. Therefore, it has been suggested that xylanases may be working via an indirect mechanism (Singh et al., 2012).

Endo-xylanase hydrolysis of arabinoxylans leads to the production of beneficial arabinoxylo-oligosaccharides (AXOS) (Broekaert et al., 2011). Courtin et al. (2008) reported a comparable improvement in Feed Conversion Ratio (FCR) of birds that were fed a maize diet supplemented with either xylanase or wheat bran oligosaccharides derived from xylanase-treated wheat bran. This suggested that oligosaccharides produced from xylanase degradation of fibrous material are influential to broiler performance. An increase in movement of xylo-oligomers to the caeca has been linked to an increase in volatile fatty acids (VFA) concentration through fermentation in this section (Choct et al., 1996, 1999). The presence of VFA in the intestinal lumen is suggested to promote peptide YY (PYY) release from endocrine L-cells, located predominantly in the distal ileum and colon, into the blood (Cuche et al., 2000). PYY is a neuropeptide that acts on the hypothalamus in the brain to delay gastric emptying, a process also known as ileal brake or gastroparesia (Pironi et al., 1993; Lin et al., 1996). Feed is therefore retained in the proventriculus and gizzard for longer, resulting in a finer 'grind' and improved protein digestion. As the protein coating the starch granules is digested, it allows for a greater access of the amylase to the granules, thereby improving starch digestibility.

It has previously been reported that PYY administration is capable of increasing the active uptake of glucose in the small intestine (Bird et al., 1996; Croom et al., 1998). Glucose is transported across the enterocyte membrane via a sodium-glucose cotransporter (SGLT1). SGLT1 is a high affinity, low capacity transporter situated on the apical side of enterocytes (Braun and Sweazea, 2008). It actively transports glucose and galactose, along with two sodium ions down a concentration gradient from the intestinal lumen into the enterocyte (Kimmich and Randles, 1984). On the basolateral membrane of enterocytes, glucose transporter 2 (GLUT2) moves glucose out of the cell via facilitated diffusion. The bird's enhanced capacity for glucose absorption may be another possible explanation for the reported improvements in performance with xylanase supplementation, and therefore will be considered herein.

The current study was designed to investigate potential mechanisms by which xylanase may act in a maize-soya diet in order to elicit a beneficial response in broilers. The effects of xylanase on starch digestion were determined using both quantitative and qualitative methods to gain a more comprehensive understanding of the starch digestion process, and the factors influencing it.

MATERIALS AND METHODS

Ethical approval

The protocol for the experiment was reviewed and approved by Ethical Review Committee, University of Nottingham, and conducted according to the UK Home Office Animal (Scientific Procedures) Act of 2010.

Animals, housing and diets

A total of 60 day-old male Ross 308 broiler chicks were supplied from a commercial hatchery and raised on a maize-soya diet (Target Feeds, UK; Table 1) with or without xylanase (Econase XT 25 at 100 g/t). Birds were group-housed in separate pens for a specific treatment group, with 10 birds per treatment. The limitation of replication in this study should be remembered when considering the data present herein. Treatment groups (6) included a control group fed a maize-soya diet without xylanase, and groups fed maize-soya diets supplemented with xylanase from 1, 2, 3, 4 or 5 weeks before slaughter. This xylanase preparation (Econase XT) contained 160,000 units of endo-1,4-β-xylanase activity (EC 3.2.1.8) per gram. One xylanase unit (XU) is defined as the necessary amount of enzyme that liberates 1 nmol, reducing sugars from birch woodxylan, measured as xylose equivalents, under the conditions of the assay (AB Enzymes, Germany). The recovered xylanase activity in the diet was 18,100 BXU/kg, as measured using a

standardised ELISA courtesy of ESC (Enzyme Services and Consultancy). Chicks were kept in an appropriate warm environment with a progressive decrease in temperature from 35 to 21°C. Diets and water were provided *ad libitum* throughout the trial.

Five birds from each treatment group were euthanised on days 35 and 36 of age by an intravenous injection of pentobarbital, with cervical dislocation to confirm death. Digesta was collected by flushing contents from the gizzard, upper (to) and lower (from Meckel's Diverticulum) small intestine, and both caeca. Digesta pH was measured using an Inolab pH level 1 meter. The brachial vein under the wing was cut using a scalpel blade and blood glucose measured using an Accu-chek mobile blood glucose monitor (Roche, UK). Digesta samples were stored at -80 °C before being frozen in liquid nitrogen and freeze-dried. The empty weight of the gizzard and lengths of the upper and lower small intestine as well as both caeca were recorded. Approximately two sections of around 2 cm in length were removed from the proximal upper small intestine, snap frozen in liquid nitrogen, and stored at -80 °C, for later RNA extraction.

Table 1. Composition of raw materials and nutrient content of maize-soya diet

Ingredient	g/kg	Component	g/kg
Maize	624.5	AME MJ/kg	12.9
Soya extract hipro	260.0	Crude protein	204.3
Full fat soybean extruded	50.0	Calcium	9.4
L-lysine HCl	4.0	Available phosphorous	4.8
DL-methionine	4.0		
L-threonine	1.5		
Soya oil	20		
Limestone	12.5		
Monocalcium phosphate	15.0		
Sodium chloride	2.5		
Sodium bicarbonate	1.5		
Vitamin and Mineral premix	4.0		
Elancoban	0.5		

Peptide YY analysis

Following cervical dislocation, blood was drawn from the aorta using a needle and syringe, and collected in a centrifuge tube at room temperature, to allow coagulation. Samples were then centrifuged at 1623 xg for 10 min to allow separation of serum. Serum was then stored at -20 °C, prior to using a chick specific PYY ELISA (Cusabio, China). Samples were analysed in duplicate using 25 µl serum, and repeated at 50 µl per well where samples were below detection limits. Sample PYY concentrations were calculated using a specific plate reader program (Multiskan Ascent) calibrated with the kit standards, which were run for each plate assayed. Two quality control samples were also measured with each plate to monitor inter assay coefficient of variation. Samples with coefficients of variation greater than 15 % were repeated.

Sample morphology

The morphology of starch granules from the digesta was examined using a Scanning Electron Microscope (SEM; Jeol JSM-6490LV) with the energy capacity of 15 kV. Samples were mounted onto aluminium stubs and gold coated using the sputter coater technique, before being viewed and photographed in the SEM unit.

Starch fraction determination

A total starch assay (Megazyme International Ireland Ltd) was performed for the determination of starch, glucose and maltodextrins in digesta samples. Digesta samples were washed twice in 80 % ethanol and the precipitate digested with thermostable α-amylase (300 U) and amyloglucosidase (20 U). Glucose release was measured using a glucose analyser (Analox GM9 Analyser). Both ethanol washes, assumed to contain soluble dextrin and glucose, were collected and the ethanol evaporated. Glucose concentration in this fraction was measured using the glucose analyser. Next, this soluble ethanol fraction was digested with α-amylase (*A.oryzae;* 24 FAU; Sigma-Aldrich, UK; A8220) and amyloglucosidase (20 U), and glucose concentration measured again. The difference in glucose concentration before and after digestion gave the dextrin content.

Glucose transporter gene expression

Total RNA was isolated from small intestine tissue samples using an RNeasy Fibrous Tissue Mini Kit (Qiagen Ltd, UK; 74704). Isolated RNA concentration and quality was then checked using a DS-11 spectrophotometer (DeNovix, USA). Purity of the RNA was verified by measurement of absorbance ratios at 260/280 and 260/230 nm, with ratios of approximately 2.0 indicating purity. Once RNA concentration for all samples was determined, 10 µl of sample was diluted with RNase-free water to givea final concentration of 100 ng/µl. Confirmation of this final concentration was achieved using the DS-11 spectrophotometer.

Synthesis of single-stranded complementary DNA (cDNA) from total RNA was performed using a Revert Aid First Strand cDNA Synthesis kit (Thermo Scientific, UK; K1622). Each reaction was performed in duplicate:

one containing the reverse transcriptase enzyme (+RT) and one where the enzyme was substituted with nuclease-free water (-RT). Another negative control was used in which total RNA was replaced with 3 µl nuclease-free water. This was intended to control for any genomic contamination in the RNA samples as well as other reaction components. Each reaction consisted of 3 µl total RNA, 1 µl random primer, 8 µl nuclease-free water, 4 µl 5X reaction buffer, 1 µl RiboLock RNase Inhibitor, 2 µl deoxynucleotide triphosphate (dNTP) mix and 1 µl RevertAid Reverse Transcriptase, for a total reaction volume of 20 µl. Reactions were run in a 96 well plate that was sealed with a film and placed into an Eppendorf Mastercycler Gradient thermal cycler. Conditions for reverse transcription were set for random hexamer primed synthesis, whereby samples were incubated at 25 °C for 5 min, followed by 42 °C for 60 min. The reaction was terminated by heating the samples to 70 °C for 5 min. The resulting cDNA products were stored at -20 °C.

Quantitative PCR of cDNA samples was performed using an Applied Biosystems StepOne Real-time PCR system with the primer sets listed in table 2. PCR plates (Applied Biosystems MicroAmp® Fast Optical 48-Well Reaction Plate, 4375816) were designed to assay 4 +RT samples, including a sample from treatment group 1 (control sample). Each reaction was run in triplicate for each gene. PCR was also run on the RT samples and a no template control (NTC) in which cDNA was replaced with nuclease-free water, to check for any contamination in the PCR components. The cDNA was diluted 1:3 with nuclease-free water to lower template concentration and to ensure there was enough sample for any repeats. A mastermix for each gene was made consisting of 200 µl GoTaq qPCR Mastermix (Promega UK Ltd; A6001), 4 µl gene specific forward primer, 4 µl gene specific reverse primer and 141.2 µl nuclease-free water. This mix was then pulse centrifuged for 10 sec using a Thermo Scientific Espresso centrifuge. Into the appropriate wells, 18 µl of this mastermix and 2 µl cDNA were added. PCR was performed under the following conditions: 95 °C for 10 min and 40 cycles of 95 °C for 15 sec and 60 °C for 1 min. A melt curve was produced at the end of the run to determine single product amplification. The comparative Ct method, also known as the 'delta-delta Ct' method, was used to quantify qPCR results. This method involved comparing the Ct values of the xylanase treated samples with the non-supplemented (control) samples. Both treated and control sample Ct values were normalised to the house-keeping gene.

ΔCt=Ct value gene of interest−Ct value control gene (β−actin)

$\Delta\Delta$Ct=ΔCt value gene of interest−Average ΔCt value control sample

Fold change in gene expression was then calculated and averaged for the three replicates:

$$Foldchange = 2 \,\hat{}\, {-\Delta\Delta Ct}$$

Agarose gel electrophoresis was used to confirm that qPCR amplified solely the gene of interest. PCR products were resolved on a 3 % agarose gel containing 5 % (v/v) ethidium bromide. One sample was selected from two different PCR plates that produced good melt curves. One replicate for each gene was selected and 4 µl 6X purple loading dye (New England Biolabs Inc, UK; B7024S) was added. Each dyed product (10 µl) was placed in a separate well on the gel. The products were separated, alongside a 20 bp low ladder (Sigma-Aldrich, UK; P1598), for approximately 1 h using 80 V. The gel was visualised and imaged under ultraviolet (UV) light using a transluminator. The product band size was then determined by comparing positions relative to the ladder.

Table 2. Primer sequence used for qPCR analysis of glucose transporters

Gene	Forward Primer (5′-3′)	Reverse Primer (5′-3′)	Product Size, bp
GLUT2	GTCCACCGCAAATGCTTCTAA	TGTCCCTGGAGGTGTTGGT	88
SGLT1	TTTGTATACAGATGTGGTGGC	GCATAAGCTCCACTACCATT	113
β-actin*	GTCCACCGCAAATGCTTCTAA	TGCGCATTTATGGGTTTTGTT	78

*Gilbert et al. (2008)

Statistical analysis

Statistical significance was tested using the uni- and multi-variate platforms of IBM SPSS statistics v21 software. For statistically significant results ($P < 0.05$), Tukey's HSD Post-hoc test was performed to determine which groups in the population differ. For comparisons made between two sample groups, an independent sample t-test was performed.

RESULTS

Blood parameters

The effect of xylanase supplementation on blood glucose concentration of birds is shown in Figure 1. When comparing between treatment groups, no significant effect of supplementing diets with xylanase for any length of time was observed (P > 0.05). However, when data was split between before and after 3 weeks of supplementation, a significant increase in glucose concentration was determined for birds fed diets supplemented with xylanase for 3 weeks or more (P < 0.05).

Changes in the circulating concentration of the gastrointestinal PYY hormone was also measured (Figure 2). When just the mean values are considered (Figure 2a), there appears to be an effect of supplementing diets with xylanase for a shorter period of time. Birds supplemented for 3 weeks showed a significantly higher PYY concentration in the blood compared to the non-supplemented control birds (P < 0.05). Feeding xylanase over a longer time period appears to reverse this effect. However, when considering the raw data (Figure 2b), no significant effect was seen due to the large data variance (P > 0.05, R= 0.0931).

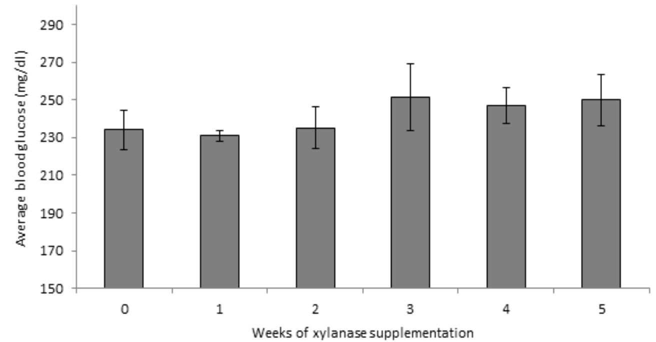

Figure 1. Average blood glucose concentration of broilers supplemented with xylanase for 0-5 weeks before slaughter. Error bars denote ± standard error of the mean (from 10 bird replicates).

Changes in gastrointestinal tract

For all treatment groups, digesta pH ranged from pH 2.2-4.4 in the gizzard, 5.9-6.6 in the upper small intestine, 6.7-7.8 in the lower small intestine and 5.7-7.3 in both caeca. For each traction section, digesta pH did not significantly differ with xylanase treatment for any length of time (P > 0.05).

Gizzard weight as well as intestine and caeca length were measured (Table 3) to assess the potential effect of xylanase supplementation on the physiology of digestive tract organs. The length of the upper small intestine and both caeca were not significantly affected by treatment (P > 0.05). However, the length of the lower small intestine significantly increased in birds fed a xylanase supplemented diet for 3 weeks before slaughter compared to the non-supplemented control birds (P > 0.05). Although birds supplemented with xylanase for 3 weeks had the highest mean gizzard weight, overall gizzard weight was not significantly affected by treatment (P > 0.05).

An increase in digesta weight collected from the caeca of birds supplemented with xylanase for longer periods of time was shown (Table 4). When data was split between before and after 3 weeks of xylanase supplementation there was a significant increase in caecal digesta weight in birds fed xylanase diets for 3 or more weeks (P < 0.05).

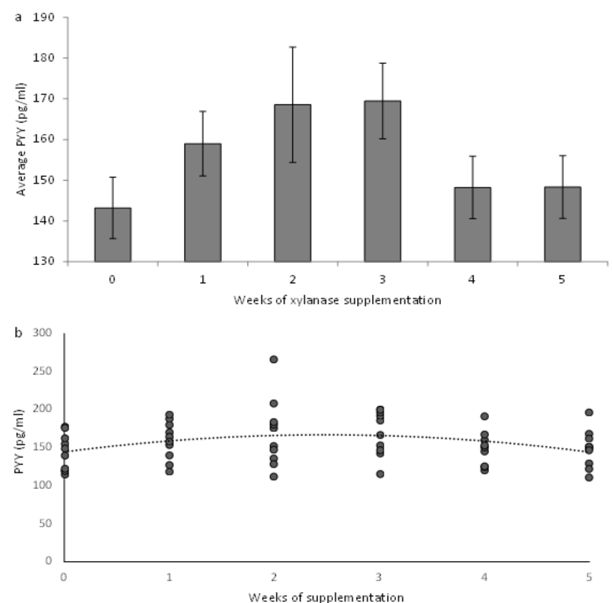

Figure 2. Peptide YY hormone concentration of broilers supplemented with xylanase for 0-5 weeks before slaughter. Graphs show averaged data (a) and raw data (b) sets. Error bars denote ± standard error of the mean (from 10 bird replicates, except week 4 of supplementation which has 9 replicates).

Table 3. Effect of length of xylanase supplementation on gizzard weight, and intestinal and caecal length of broilers at 5 weeks of age

Weeks of supplementation	Weight (g)				Length (mm)			
	Gizzard	SEM	USI	SEM	LSI	SEM	Caeca	SEM
0	42	1.49	632	20.78	669[b]	14.02	154	3.91
1	43	0.89	711	21.66	727[b]	30.34	159	4.40
2	41	1.68	663	15.00	686[b]	18.03	160	4.90
3	45	1.12	691	15.25	754[a]	16.12	158	3.77
4	42	1.69	637	18.81	713[b]	23.09	155	5.32
5	43	2.00	669	21.02	709[b]	13.99	158	5.58

SEM = Standard Error of the Mean (from 10 bird replicates); USI- upper small intestine; LSI- lower small intestine; [ab] Mean values not sharing a common superscript letter are significantly different at $P < 0.05$

Table 4. Effect of length of xylanase supplementation on the weight of digesta collected from each tract section of broilers at 5 weeks of age

Weeks of supplementation	Digesta weight (g DM)							
	Gizzard	SEM	USI	SEM	LSI	SEM	Caeca	SEM
0	6.35	0.68	3.08	0.28	3.21	0.22	0.31	0.03
1	6.28	0.45	3.12	0.39	2.74	0.34	0.30	0.03
2	6.78	0.27	3.18	0.22	2.66	0.36	0.27	0.03
3	6.56	0.30	3.50	0.33	3.17	0.22	0.34	0.05
4	5.74	0.50	2.63	0.34	2.48	0.21	0.34	0.04
5	6.57	0.55	3.14	0.33	3.16	0.19	0.41	0.07

SEM = Standard Error of the Mean (from 10 bird replicates); DM- dry matter; USI- upper small intestine; LSI- lower small intestine

Starch digestion

Total starch results demonstrated the progression of starch granule digestion as digesta passes through the digestive tract of birds (Figure 3). The most noticeable starch content was found in the gizzard (Figure 3a), with decreased levels within the upper small intestine and then less again in the digesta of the lower small intestine ($P < 0.05$) as starch becomes hydrolysed by endogenous amylases. Dextrin content varied with tract section and treatment with no consistent trend (Figure 3b). As expected, the glucose content (Figure 3c) of gizzard digesta was minimal, while a higher ($P < 0.05$) glucose content was found in the upper small intestine, as this is the major site of starch digestion. By the lower small intestine, glucose levels fall again as monomers are absorbed by enterocytes. The content of starch, dextrin and glucose in any tract section was not significantly affected by treatment ($P > 0.05$).

Total starch results were also supported by SEM imaging (Figure 4) where starch granules appear to become more hydrolysed and are fewer in number as digesta enters more distal tract sections. Interestingly, a small number of starch granules escaped digestion and entered the caeca (Figure 4d) for all treatment groups. All gizzard samples showed cell wall fractionation (Figure 5), however greater apparent cell wall destruction with xylanase treatment was not evident.

Glucose transporter expression in the small intestine

Fold change in expression of the glucose transporter genes, GLUT2 and SGLT1, are shown in Figure 6. Gel electrophoresis confirmed the GLUT2, SGLT1 and β-actin product sizes of 88 bp, 113 bp and 78 bp, respectively (Figure 7). Due to the large variation in qPCR results, GLUT2 and SGLT1 gene expression was not shown to vary significantly with length of xylanase supplementation ($P > 0.05$). However, results do show a numerical increase in GLUT2 expression at 2 weeks of xylanase supplementation. GLUT2 expression reduced and plateaued as the length of xylanase supplementation increased. A similar trend was observed for SGLT1 expression. However the initial increase in expression started slightly later, at 3 weeks of xylanase supplementation.

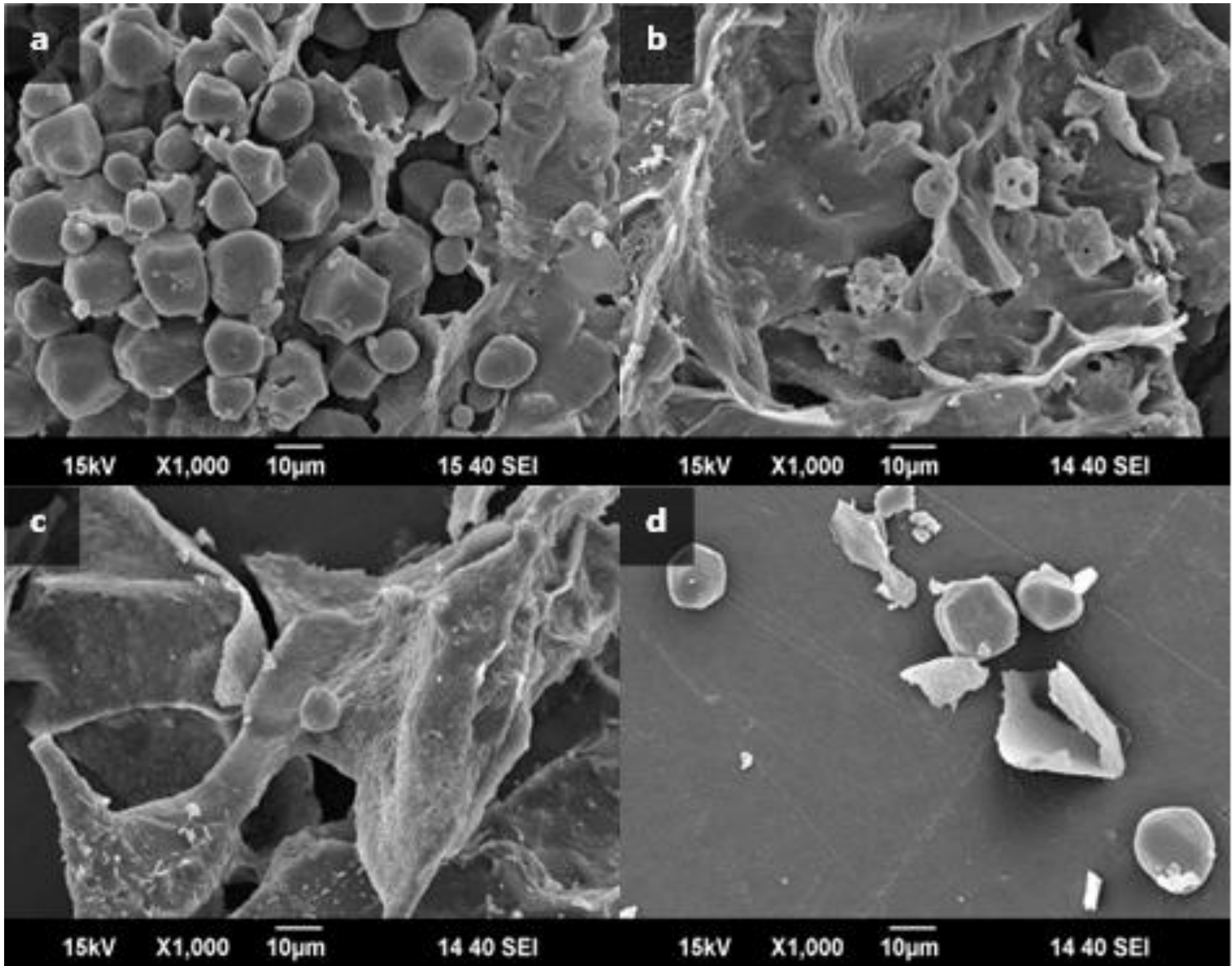

Figure 4. SEM images showing the progression of starch granule digestion through the tract of broilers at 5 weeks of age. Starch granules in digesta taken from the gizzard (a), upper small intestine (b), lower small intestine (c) and caeca (d).

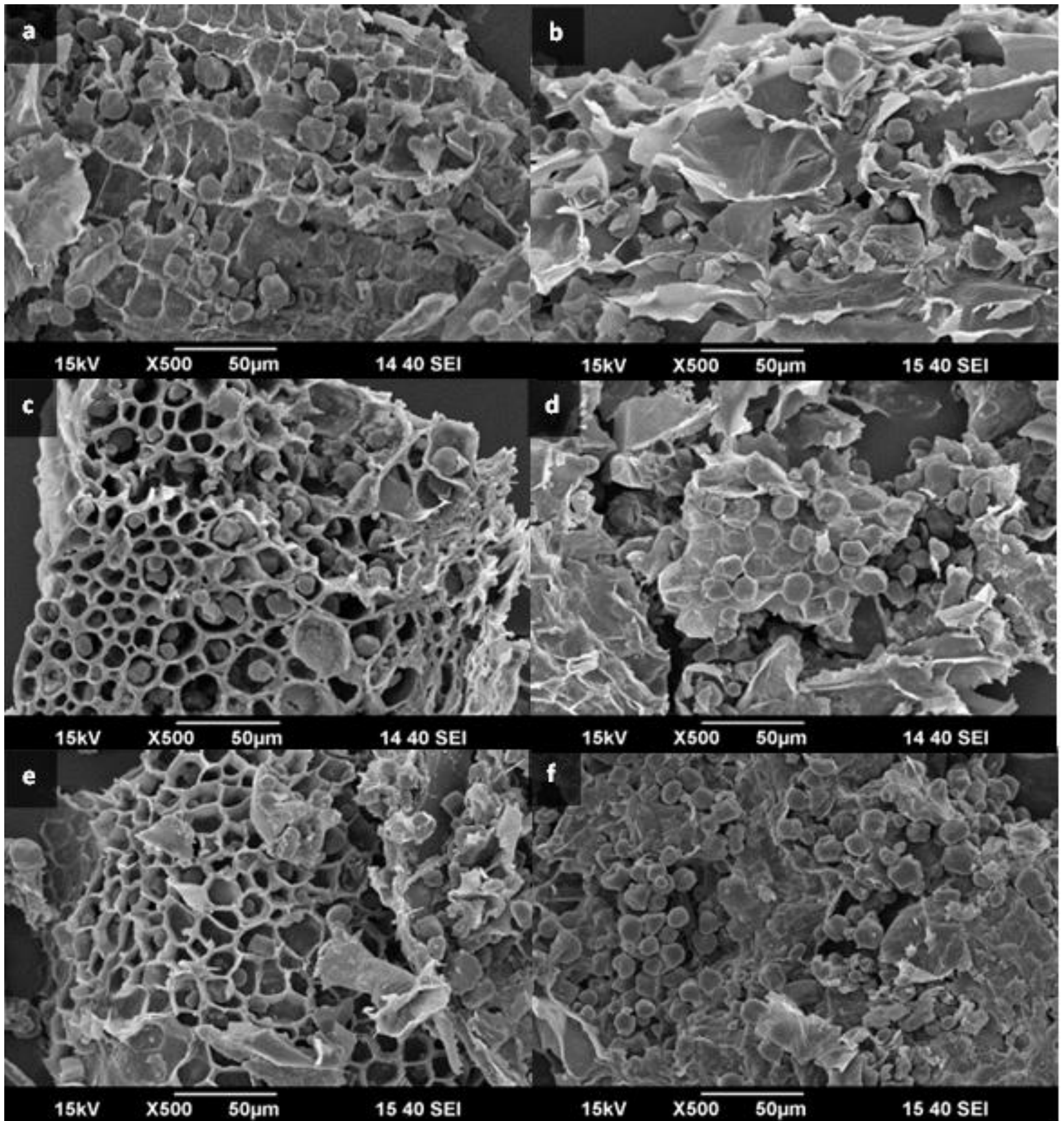

Figure 5. Starch granules in gizzard digesta taken from broilers supplemented with xylanase for 0-5 weeks before slaughter. SEM images show control broilers (a) and broilers supplemented with xylanase 1 (b), 2 (c), 3 (d), 4 (e) and 5 (f) weeks before slaughter.

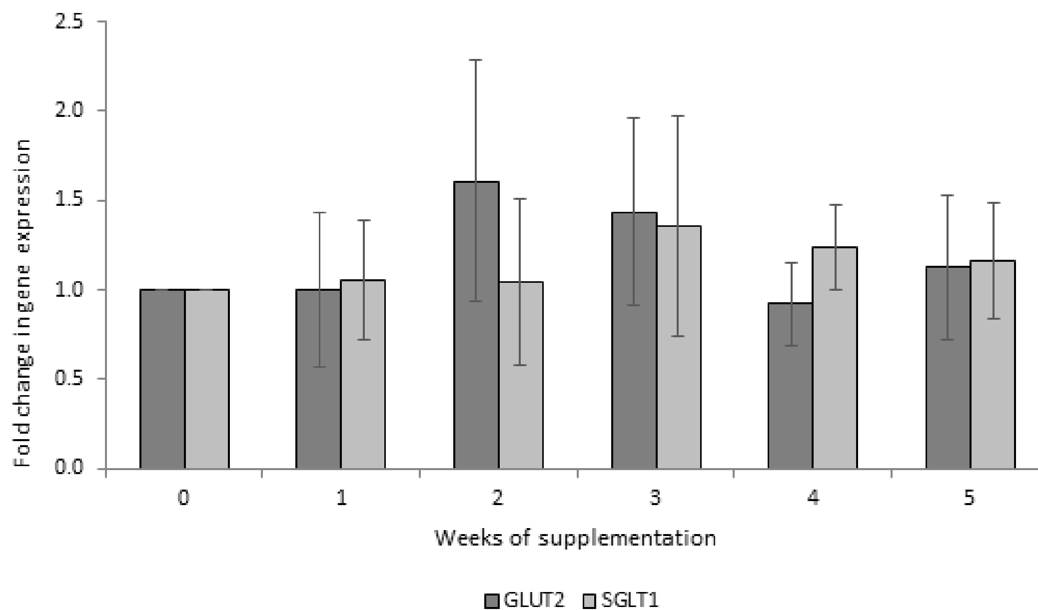

Figure 6. Expression of GLUT2 and SGLT1 gene in the small intestine of broilers supplemented with xylanase for 1-5 weeks before slaughter. Data are presented as a fold change in gene expression relative to control (non-supplemented) bird gene expression. Error bars denote ± standard error of the mean (from 10 bird replicates, except weeks 2 and 3 of supplementation which have 9 replicates).

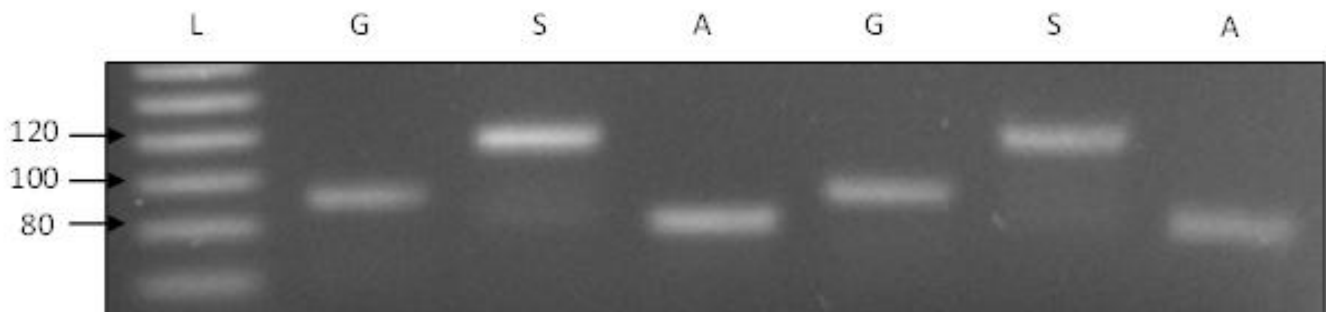

Figure 7. Agarose gel separation of qPCR reaction products. Products for the GLUT2 (G), SGLT1 (S) and β-actin (A) genes were run against a 20 bp ladder (L) for determination of product length.

DISCUSSION

NSP may play an important role in the physical entrapment of starches and proteins in cereal, thereby limiting their digestibility (Aftab, 2012). Disruption of the cell wall matrix through xylanase action is expected to release these entrapped components making them more available to endogenous enzymes (Bedford, 1996). This concept is supported by reports of improved starch, protein and fat digestibility in maize-soya diets when supplemented with exogenous xylanase containing enzyme cocktails (Zanella et al., 1999; Meng and Slominski, 2005; Cowieson et al., 2010). However, SEM images revealed no apparent difference in the matrices surrounding the starch granules for diets with or without xylanase supplementation, although the subjective nature of the SEM should be taken into account. Moreover, since birds were fed *ad libitum* prior to slaughter, feed particles at various stages of digestion would be expected to be present along the tract. Xylanase activity of 18,100 BXU/kg in feed was confirmed in the current study and with reports of fungal xylanase activity in digesta (Vahjen and Simon, 1999), although this was not measured in the current work, it can be assumed that the xylanase was also active in the tract. In contrast to the reported release of starch granules from their cell wall matrices with addition

of xylanase to maize *in vitro* (Masey O'Neill et al., 2012), the mechanism of xylanase action *in vivo* may not be as apparent due to the highly complex nature of the digestive tract. Moreover, the reasoning may not be solely dependent on plant cell wall material, but may also contain protein matrices that can form a continuous network that encapsulates starch granules, thereby preventing their release. Recently, however, Gonzalex-Ortiz et al. (2017) reported changes to the structural integrity of wheat cell walls in response to xylanase, using an auto-fluorescent technique. This method allows arabinoxylan structure to be specifically visualised (Jääskeläinen et al., 2013), unlike standard microscopy, and thus might explain the lack of findings when using the latter technique.

Total starch measurements also revealed no significant effect of xylanase on starch digestion through the tract. However, it was evident that starch still remained in the lower small intestine with some granules entering the caeca. Weurding et al. (2001) reported the same total tract starch digestion values as ileal starch digestion, indicating that the undigested starch fraction was not fermented in the caeca. Carre (2004) suggested that while soluble molecules or free granules can enter the caeca, as shown in the present study, the majority are expected to bypass the caeca. Therefore, caecal fermentation of starches may not be significant for the completion of starch digestion.

In addition to a direct action on cell wall material, xylanase may also play an indirect role on diet digestibility. Singh et al. (2012) reported increased secretion of PYY with xylanase supplementation to a maize-soya diet. In addition, Neyrinck et al. (2012) also described an increase in blood plasma PYY concentration with AXOS inclusion into the diet of mice, suggesting that it is the products of NSP hydrolysis that are affecting gastrointestinal hormone secretion. Caecal fermentation of AXOS produced from arabinoxylan breakdown by xylanase may promote PYY secretion leading to increased feed retention in the proventriculous and gizzard. This results in a ‘finer grind’ of feed material, with potential for greater gizzard development and improved protein digestion in the gastric phase. Since starch granules in the endosperm of the maize kernel are held within cellular and proteinaceous matrices, disruption of these materials can lead to granule release and enhancement of starch digestibility. In the current study, PYY secretion increased significantly when birds were fed maize-soya diets supplemented with xylanase for 1-3 weeks. This might suggest that the response is greater when birds are supplemented with the enzyme for a shorter period of time compared to birds supplemented over the whole trial period, indicating an adaptive effect due to the age of birds when supplementation begins. When birds were exposed to the enzyme at a young age they are able to adapt to these changes, most likely through modification of the microflora population. Enhanced fermentation in the small intestine and caeca, due to changes in the microbial population, leads to greater production of VFA and thus lactic acid in these sections, resulting in a reduced pH (Gao et al., 2008). Xylanase supplementation has been shown to reduce fermentation in the small intestine, while increasing caecal fermentation and VFA production (Choct et al., 1999). As no change in intestinal or caecal pH was observed in the current study, this might suggest that there had not been any change in gut microflora due to xylanase supplementation. However, laboratory conditions may have not allowed for a considerable change in microflora. Therefore, this could be the reason for limited enzyme response, particularly during short supplementation periods, although further microbial analysis would be needed to confirm these results.

Greater feed retention in the gizzard due to the ileal brake mechanism has been linked to increased gizzard weight (Masey O'Neill et al., 2014). However, in the current work, empty gizzard weight was unaffected by xylanase supplementation. This may also be due to the physical form of the diet, as mash diets, such as the feed in this study, have already been shown to increase gizzard weight when compared to pelleted diets (Niret al., 1995). Therefore, the change in gizzard weight may not be as noticeable when feeding a mash diet, when the gizzard is already well developed. Moreover, intestinal length was not shown to be significantly affected by xylanase addition. Xylanase supplementation at 1000 XU/kg diet has been shown to reduce the relative weight and length of the small intestine (Wuet al., 2004) in broilers fed a wheat-based diet for 21 days. As viscosity is less of a concern in a maize-based diet, this may be the reason why there is not a consistent change in intestinal length with enzyme addition.

Previously, PYY administration has also been shown to increase active uptake of glucose in the small intestine of mice (Bird et al., 1996). This was achieved without a significant change in energy expenditure, suggesting that PYY may also enhance the efficiency of glucose absorption in the small intestine. Interestingly, other reports have shown that PYY administration *in ovo* can improve growth and FCR of chicks during the first week post-hatch (Coles et al., 1999; 2001), an effect attributed to an enhanced absorptive capacity. Croom et al. (1999) postulated that *in ovo* PYY administration may increase glucose transporter maturation thereby enhancing glucose

absorption during post-hatch growth when digestive and absorptive processes are not yet fully developed. Glucose transporter response to PYY in older birds has not yet received much attention. As xylanase supplementation has been shown to increase PYY secretion in birds, there is consequently a potential for an enhanced absorptive capacity in these birds.

Glucose is absorbed from the intestinal lumen into epithelial cells by SGLT1, and then leaves at the basolateral membrane by GLUT2, before entering blood circulation. Guo et al. (2014) reported a decrease in SGLT1 expression in broilers fed a wheat-based diet when supplemented with xylanase. In contrast, in the current study, increased expression of the glucose transporters, GLUT2 and SGLT, although non-significant, may suggest a potential response to xylanase inclusion in maize-based diets. Miyamoto et al. (1993) reported an increase in SGLT1 and GLUT2 expression in the jejunum of rats fed a high-glucose diet compared to those fed a low-carbohydrate diet. This would suggest that a higher concentration of glucose in the intestine causes a response that elevates glucose transporter expression. As diet digestibility has been found to improve with xylanase supplementation of wheat and maize-based diets (Choct et al., 1999; Cowieson, 2005; Choct, 2006), this could increase glucose content of intestinal digesta. However in the current work, the glucose content of digesta taken from the upper small intestine was not significantly affected when birds were fed xylanase. However, if the absorptive capacity of birds increased with increasing glucose concentration then differences in luminal glucose content may be difficult to detect. When considering the concentration of absorbed glucose, studies have reported no effect of xylanase on blood glucose concentration in broilers (Gao et al., 2007; Luo et al., 2009). However, in the current study, blood glucose concentration increased in birds fed xylanase for 3 or more weeks. This is in agreement with Singh et al. (2012) who reported an increase in serum glucose concentration of broilers fed a xylanase supplemented maize-soya diet for 42 d. Xylanase supplementation is assumed to improve nutrient digestion and absorption in the small intestine of broilers (Choct et al., 1999). Therefore, the rise in blood glucose level after 3 weeks of feeding xylanase could be due to greater starch digestion and glucose absorption. Although it should also be noted that the number of bird replicates per treatment was relatively small (n = 10), from the aforementioned trends in treatment response a potential mechanism of xylanase action can be proposed. Xylanase induced secretion of PYY may enhance starch digestion, due to the aforementioned 'ileal-brake mechanism', thereby increasing glucose concentration in the intestinal lumen. Subsequent promotion of glucose transporter expression could increase glucose absorption and thus provide more energy to the bird for growth. With further investigation, this may give a potential mechanism for the improvement in performance seen in birds fed maize-based diets supplemented with xylanase.

CONCLUSION

In the current study, the progression of starch granule digestion was followed through the digestive tract, giving an indication as to where and how starches are being digested. Xylanase supplementation did not appear to have any significant effect on starch digestion in broilers nor on cell wall degradation. However, a potential indirect mechanism of xylanase action has been proposed that may explain the reported improvements in performance when birds are fed maize-based diets supplemented with xylanase.

Consent to publish

Not applicable

Competing interests

The authors declare that they have no competing interests

Authors' contributions

The present study was funded by AB Vista, Marlborough, Wiltshire, UK. Sophie Lee, Julian Wiseman, Helen Masey O'Neill and Sandra Hill contributed to the conception, design and interpretation of data. Sophie Lee was also involved in the collection of data, statistical analysis and drafting of the manuscript. Dawn Scholey and Emily Burton executed PYY analysis by ELISA at their facilities at Nottingham Trent University. All authors read and approved the final manuscript.

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