The effects of exogenous xylanase supplementation on the *in vivo* generation of xylooligosaccharides and monosaccharides in broilers fed a wheat-based diet

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ABSTRACT

1. This study aimed to quantify xylanase-induced changes in soluble monosaccharides, xylooligosaccharides (XOS) and volatile fatty acid (VFA) contents of the different sections of the GIT and whether these relate to altered bird performance.
2. An *in vitro* digestion of the wheat-based diet was carried out with the xylanase (Econase XT at 16,000BXU/kg diet) to compare the *in vitro* and *in vivo* generation of these XOS and monosaccharides. For the *in vivo* study, 80 male Ross 508 broiler chicks were split into two groups fed a wheat-based diet with or without Econase XT (16,000BXU/kg diet) for 21 days.
3. There were no effects of Econase XT inclusion on growth performance characteristics, likely a result of the high-quality wheat diet and corresponding high performance of the control group (FCR average of 1.45 in controls), but also the relatively young age (from 4 to 26 days of age).
4. Econase XT supplementation increased the xylotetraose (X_4) content in the colon ($p=0.046$, enzyme x GIT section interaction) and the xylose contents in the colon and caeca ($p<0.001$, enzyme x GIT section interaction).
5. The trend for increased acetate proportion in the caeca of Econase XT treated birds ($p=0.062$) suggests that the XOS generated were subsequently fermented in the caeca, potentially impacting upon the types of microbiota present.
6. The present study suggests that wheat arabinoxylan degradation is enhanced by xylanase supplementation, which may increase the production of beneficial VFA in the caeca, and thereby potentially modulate the caecal microbiome, but without affecting bird performance (at this early stage).

INTRODUCTION

Arabinoxylan (AX) is the most abundant hemicellulose in the endospermic cell wall of wheat (JMares and Stone 1973). Non-starch polysaccharides (NSP) like AX are anti-nutritive in monogastrics, who lack the endogenous enzymes to break down plant cell walls (Choct and Annison 1990). The result is an increase in digesta viscosity, which has negative effects on performance (Bedford 2000). The supplementation of endo- β1, 4-xylanases effectively hydrolyse the xylan backbone of AX, generating arabinoxylan-oligosaccharides (AXOS) which encompasses both arabino-xylooligosaccharides and xylooligosaccharides (XOS) (Jommuengbout et al. 2009). Monosaccharides are generated if any contaminating xylosidase or arabinofuranosidase is present in an enzyme product or within the diet itself, cleaving β- xylosidic glycosidic linkages into the monomeric pentoses (e.g. xylose and arabinose) and hexoses (e.g. glucose and galactose). This results in a reduction in viscosity and improved growth rates and feed efficiency (Bedford and Classen 1992). It is also hypothesised that the resulting short-chain AXOS from enzyme hydrolysis are utilised for fuel by the microbiota occupying the distal gastro-intestinal tract (GIT), thereby having positive effects on the microbiome (Choct et al. 1999a).

NSP account for 10-12% of the dry matter (DM) in wheat (Knudsen 1997), of which 19-21% is soluble (Rodehutscord et al. 2016). The insoluble NSP in wheat mainly provides protection in the form of the cell wall (Simon et al. 2015). As per the cell wall degradation hypothesis, xylanase shows significant cell wall destruction *in vivo* (Bedford and Autio 1996), which enhances access to cell contents by the endogenous enzymes, increasing their efficacy, thereby increasing amino acid retention,

pancreatic amylase and mucin secretion to aid digestion (Cowieson and Bedford 2009).

There is also evidence that feeding xylanase influences the caecal microbiome, where beneficial bacteria ferment the end products of enzyme hydrolysis (McCracken et al. 2006; Masey-O'Neill et al. 2014). This is presumably from the generation of AXOS during hydrolysis, as feeding near-pure AXOS results in similar performance benefits to xylanase inclusion itself (Morgan et al. 2019). AXOS are thus displaying prebiotic effects, in that they are fermented into beneficial volatile fatty acids in the microbiome, particularly acetate and butyrate in the caeca and colon (Choct et al. 1999b). Walker et al (2005) suggested that AXOS substrate fermentation in the colon significantly lowered the pH balance, which gave a boost to butyrate producing bacteria, whilst hampering the proliferation of *Bacteroides* spp. which have the capacity to cause harm to their host. This subsequently improves the gut environment and could also lead to better mineral absorption and immune response along with increasing gizzard grinding, which all lead to more efficient nutrient absorption (Kim et al. 2011).

One theory is that there is increased recovery of energy from the diet as volatile fatty acids (VFAs). The issue with this theory is that the AXOS studies so far used doses ranging from 0.1g – 10g per kilogram of diet (Ribeiro et al. 2018; Suo et al. 2015; Eeckhaut et al. 2008). The lower levels of inclusion are unlikely to provide enough substrate to generate significant amounts of energy in the form of VFA. The other issue is that in chickens, it is necessary to feed xylanase over an extended period for a response to be observed. If the enzyme were generating significant quantities of AXOS instantaneously, which were then fermented, there would not be a delay in the response seen after enzyme inclusion. However, studies show performance

benefits in wheat-based diets are often not seen until the birds reach 21d of age (Mendes et al. 2013).

Hence a new hypothesis (Bedford 2018), suggests that the chicken microbiome is 'trained' or adapted over time as a result of xylanase supplementation. Rather than NSPases producing more fermentable sugars, it may be that NSPases produce AXOS that signal to the microbiome, thereby increasing the capacity to degrade fibre. This theory is supported by a recent study showing that chickens fed xylanase for 35d had greater fermentation of pentoses and AXOS in their caecum than controls (Bedford and Apajalahti 2018).

Whether it is more efficacious that AX are hydrolysed into AXOS *in situ* via endogenous enzyme supplementation or to supplement diets with AXOS that has been prepared *in vitro* remains to be established.

The aim of this study was to quantify the XOS and monosaccharides generated when a commercially available xylanase (Econase XT) was fed to broilers on a wheat-based diet between d4 and d25/26 of age, to establish where the nutrients are generated and/ or utilised along the GIT. In addition, the *in vitro* digestion of the wheat-based diet was compared to the contents of the different sections of the GIT *in vivo*. Caecal VFA contents were also determined as an indication of possible effects of Econase XT on the microbiome.

MATERIALS AND METHODS

Diet and Enzyme used

The diet was formulated by a commercial feed manufacturer in mash form (Target Feed Ltd, England) and the ingredients and nutritional values are shown in Table 1. Econase XT 25 is a commercially available xylanase with reported β-1,4 endo-xylanase activity (160,000 BXU/g) provided by AB Vista (Marlborough, UK) and used at the recommended dose of 100µg/g diet, which provides 16,000 BXU/kg, where BXU represents the amount of enzyme required to release 1 µmol of reducing sugar per min from xylan under defined test conditions.

Total hydrolysis of non-cellulosic polysaccharides in the diet

Total non-cellulosic sugar contents in the diet were determined by total hydrolysis of non-cellulosic polysaccharides using Trifluroacetic acid (TFA), as previously described (Fry 1988). Briefly, diet samples were ground to a fine powder (0.5mm) and suspended to 10mg/ml in 2M TFA in triplicate. Tubes were sealed and heated to 120°C for 1 hr in an autoclave, then allowed to cool to room temperature before being centrifuged at 2236×g for 10 min at room temperature. The supernatant was then diluted 1:100 with 10mM NaOH and transferred into 2ml clear vials for sugar analysis. The content of the 4 monosaccharides (arabinose, galactose, glucose and xylose) was quantified, but there were no measurable XOS.

In vitro digestion of the wheat-based diet with Econase XT

The diet samples were individually ground to a fine powder (0.5mm) and 0.2g was resuspended (in 4 replicates) in 40mls 50 mM sodium citrate buffer (pH 5.2) either without (Control – no enzyme) or with Econase XT 25 at 16,000 BXU/kg. The buffer was chosen to represent the average pH of the broiler digestive tract

(Mabelebele et al. 2014). Digestion reactions were then placed in a shaking incubator at 150RPM and a temperature of 41°C for up to 24 h. At each time point (0, 3, 6, 9, 12 or 24hrs), 1ml of each digest was removed and added to 9ml of 10 mM NaOH at room temperature, mixed, centrifuged at 2236×g for 10 min at room temperature and then frozen at -20°C prior to sugar analysis.

Chicken trial

The trial was conducted at the University of Nottingham Bio-Support Unit using 80 one-day-old male Ross 308 broiler chickens (average body weight 42g), obtained from P D Hook Hatcheries Limited (Cote, Bampton OX18 2EG). Birds were housed and cared for according to the UK Animals (Scientific Procedures) Act 1986 (ASPA) Code of Practice for the care and accommodation of animals (February 2013), approval reference number 197. Birds were wing tagged for identification, acclimatised in one group, and fed the control diet (without enzyme) between days 1-4. On day 4 they were allocated to 20 pens in groups of 4 birds per pen, matched with birds of similar weights. Hence there were 10 replicate pens per diet (control and enzyme treated) each with four birds.

All birds were given *ad libitum* access to diet and water throughout the study and were raised under controlled conditions of light, temperature and humidity as recommended by the breeder. Temperature was maintained at 32° on arrival and reduced by approximately 1°C per day until 21°C was reached as per the Ross 308 Management Guidelines.

Body weight for individual birds was monitored and recorded following the start of access to the experimental diets on 4, 11, 18 days of age and immediately following culling on days 25 or 26 (5 pens per treatment were culled on 2 consecutive days).

Diet consumption and body weight gain were measured between 4 and cull at 25 or 26 days of age to calculate feed conversion ratio (FCR). Birds from pens 1-10 were culled on 25 days of age and pens 11-20 on 26 days of age by Schedule 1 method (Animals (Scientific Procedures) Act 1986). Samples of intestinal digesta were collected from 5-cm segments of the mid-jejunum, proximal ileum and colon, as well as the caecal contents for each bird. Digesta samples were snap frozen using liquid nitrogen immediately after collection and stored at -80 °C prior to analysis. One representative bird closest to average pen weight from each pen was analysed for monosaccharide and XOS contents, whereas the first bird from each pen was selected for VFA analysis on the caecal contents and processed accordingly, before being sent to Alimetrics Ltd (Finland) for analysis.

Identification and quantification of sugars using HPAEC-PAD

The sample contents of arabinose, galactose, glucose and xylose, as well as the XOS (g/100g), were determined using High-Performance Anion-Exchange Chromatography coupled with Pulsed Amperometric Detection (HPAEC-PAD) following the method of Xu et al (Xu et al. 2013). Analysis was carried out using a Dionex ICS-3000 with a Dionex CarboPac PA20 Column (3mm x 150mm) and CarboPac PA20 Guard (3x30mm) for the monosaccharide analysis. A CarboPac PA200 column (3mmx250mm) and CarboPac PA200 guard (3mm x 50mm) were used for the oligosaccharide analysis. An injection volume of 10µl was used throughout for all standards and samples. Monosaccharide standards (arabinose, galactose, glucose and xylose) were purchased from Sigma-Aldrich, UK and XOS standards (xylo-biose, -triose and –tetraose) from Megazyme, Ireland. Serial dilutions for each standard (2.0, 1.0, 0.5 and 0.25g/L for monosaccharides and 2, 1, 0.5 and 0.25g/L for XOS) were made fresh for each batch of analyses, diluted 1:100.

For the monosaccharides, a single eluent, containing 10mM NaOH solution, was used as the mobile phase at 0.5ml/min for 14 min. For oligosaccharides, 2 eluents were used in a gradient for the mobile phase, 0.1M sodium hydroxide (Solution A) and 0.1M NaOH containing 0.5M sodium acetate (solution B) in standard quadruple waveform, as described by Xu et al (2013). The gradient program used for XOS determination was 100% solution A at 0 minutes, rising to 80% solution A and 20% Solution B at 25 minutes, before returning to 100% Solution A after 25 minutes elapsed. Both eluents were stored in plastic pressurised bottles with inert nitrogen gas at 6-9 psi. Data were collected with Dionex Chromeleon software (Version 6.7). Dry matter content of the digesta was determined by oven drying the digesta at 60°C for 36h. Monosaccharide and XOS contents from *in vivo* digesta samples were then adjusted to dry matter contents.

Measurement of Volatile Fatty Acids

The VFAs in the caeca of one randomly selected bird from each pen were analysed as free acids by Alimetrics Ltd (Espoo, Finland), using gas chromatography, as described previously (González-Ortiz et al. 2019; Holben et al. 2002). In brief, 1g caecal contents were vigorously mixed with 1ml H_2O for 5 minutes, before 1ml of 0.8M perchloric acid was added and the mix shaken to extract the VFAs. The acids measured were acetic, butyric, lactic, propionic, valeric, and total branched chain fatty acids.

Data and Statistical analysis

For the *in vitro* digests, one source was used for the diet and all analyses (digestions and Dionex analysis) were carried out in four batches of control and Econase XT

combinations. The data was then processed in excel (Microsoft, 2013) and expressed as means and standard error of the mean (SEM).

Standards for the four monosaccharides or XOS were run at the start and end of each batch, standard curves were generated from the areas under the curve and presented as g/100g of diet or digesta. Data was then analysed by one- (enzyme) or two-way (enzyme x time or enzyme x GIT section) ANOVA, as appropriate, using Genstat statistical software (19th Edition), with blocking for batch and tube. Bonferroni post-hoc tests were used to identify significant differences between groups following a significant ANOVA. $p<0.05$ was taken as statistically significant, and $p<0.10$ was described as a trend.

For average daily feed intake (AFDI), average daily gain (ADG) and feed conversion ratio (FCR), data was analysed by one-way ANOVA (Genstat statistical software, 19th Edition). For body weight gain, data were analysed by two-way (treatment x time) ANOVA with repeated measures (Genstat statistical software, 19th Edition). $p<0.05$ was taken as statistically significant in both instances.

For the correlations, data were analysed by Pearson correlation coefficients, assuming gaussian distribution, using GraphPad Prism (Version 8.1.2). A two-tailed test was undertaken to determine statistical significance with a 95% confidence interval. $p<0.05$ was taken as statistically significant, and $p<0.10$ was described as a trend.

RESULTS

Total hydrolysis of non-cellulosic polysaccharides in trial diet

The total sugar contents were determined by TFA hydrolysis of the control diet (Table 2). As expected, the highest monosaccharide in the diet was glucose, while the xylose and arabinose contents were similar (Table 2). It is assumed that the vast majority of these two monosaccharides are present as arabinoxylan (AX), and as such the total AX content and arabinose to xylose ratio were calculated. Galactose was present in fairly high amounts, presumably due to the use of soyabean meal in the diet, which is known to have high levels of galactose (Irish and Balnave 1993). These total hydrolysis values for each monosaccharide were subsequently used to calculate the proportion that was released during the *in vitro* digestions and in the *in vivo* experiment with and without Econase XT supplementation.

In vitro digestion of the wheat-based diet with or without Econase XT – release of xylooligosaccharides (XOS) and Monosaccharides

There were significant enzyme x time interactions seen for the release of xylotetraose (X_4), xylotriose (X_3) and xylobiose (X_2, all $p<0.001$). X_4 was released from the diet in the absence of Econase XT but *post hoc* Bonferroni tests revealed that the release was higher with Econase XT at 3h, 9h, 12h and 24h ($p<0.001$, figure 1A). Similarly, X_3 was also released from the diet in the absence of Econase XT, peaking at 3h (Figure 1B) and then either declining (Control) or remaining flat (Econase XT). *Post hoc* Bonferroni tests, indicated higher concentrations with Econase XT only at 12h and 24h, with no differences at earlier timepoints. In contrast, there was no release of X_2 from the diet in the absence of Econase XT (Figure 1C), with significant release of X_2 only observed after 24h incubation with Econase XT.

There were significant enzyme x time interactions for the release of xylose ($p=0.034$) and arabinose ($p<0.001$). As observed for X_3, xylose was released in the absence of Econase XT and both groups peaked at 3h, before declining (Figure 1D). *Post hoc* Bonferroni tests indicated higher levels with Econase XT only at 24h, with no differences at earlier timepoints. In contrast, arabinose was released linearly from 0-9 hours, both with and without Econase XT (Figure 1E). *Post-hoc* Bonferroni tests showed significantly more was released with Econase XT at 12h and 24h. Unlike the release of xylose, there were no significant enzyme x time interactions for the release of galactose or glucose (Figures 1F and G respectively), nor were there any effects of enzyme, but the release of both increased with time (both $p<0.001$).

The effect on Broiler performance of Econase XT in a wheat-based diet

There were no differences in average daily feed intake (ADFI), average daily gain or feed conversion ratio (FCR) of broiler chickens fed the diets for 3 weeks (Table 3). There were no mortalities.

The effect on release of xylooligosaccharides (XOS) and Monosaccharides at different sections of the broiler gastro-intestinal tract (GIT) of Econase XT in a wheat-based diet

There was a significant enzyme x GIT section interaction for X_4 release ($p=0.038$, Figure 3A), such that X_4 concentration was highest in the colon of the birds supplemented with Econase XT, but there was no X_4 observed in the caeca. There was no enzyme x GIT section interaction for X_3 release (Figure 3B) and no effect of Econase XT, but there was a highly significant effect of GIT section ($p<0.001$). The X_3 content was highest in the ileum followed by the jejunum and colon, with the lowest concentrations observed in the caeca. As for X_3, there was no enzyme x GIT section

interaction nor any effect of Econase XT for X_2 release (Figure 3C), but there was a highly significant effect of GIT section ($p<0.001$). The concentration of X_2 declined down the GIT, although the concentrations in the ileum and colon were not significantly different nor were those in the colon and caeca.

As observed for X_4, there was a significant enzyme x GIT section interaction for xylose ($p<0.01$, Figure 4A). Econase XT increased xylose content in the colon and caeca, with no detectable xylose in the jejunum or ileum of any of the birds irrespective of whether the enzyme was present. Unlike xylose release, there were no significant enzyme x GIT section interactions for arabinose, galactose or glucose (figure 4B, C and D respectively). Econase XT significantly increased the release of arabinose ($p=0.012$) and tended to increase Galactose ($p=0.071$) in all sections of the GIT. There were significant differences between the sections of the GIT for release of arabinose, galactose and glucose ($p<0.001$). Arabinose concentrations were higher in the colon than in the jejunum and ileum, with the caeca being intermediate (Figure 4B), whereas galactose was highest in the colon (Figure 4C). In contrast, glucose concentrations declined down the GIT but with no difference between colon and caeca (Figure 4D).

As xylanase increased X_4 in the colon (but not in the caeca) and xylanase increased xylose in the caeca, correlations were done between colonic X_4, X_3 or X_2 contents and caeca xylose or other monosaccharides. The correlations for X_3 or X_2 were not significant (Supplemental 1). There appeared to be positive relationships between X_4 and both xylose and arabinose contents, but negative relationships with galactose and glucose contents. However only colonic X_4 and caecal arabinose contents showed a trend for positive correlation ($p=0.089$, figure 5B), whereas none of the other correlations were significant (Figure 5).

Proportions of Volatile Fatty Acids in the Caeca

Acetic acid was the most abundant VFA in the caeca (Figure 5). Total concentration of Acetic acid was not statistically different between control birds (91.7mM) and Econase XT birds (97.8mM, p= 0.338). However, control birds had a slightly lower (p= 0.062) proportion of acetic acid (74%) than birds supplemented with Econase XT (78%). The other VFA were in the order Butyric acid > Lactic acid > Propionic acid > BCFAs > Valeric acid (Figure 6), but there were no significant differences in proportions in the caeca of birds fed the Control and Econase XT supplemented diets.

DISCUSSION

To our knowledge, this is the first *in vivo* study to quantify the generation of XOS and release of monosaccharides through the GIT of broilers and to compare that to an *in vitro* model of digestion for the same enzyme and diet combination. Hence, there is little published literature to compare the findings to. The main findings of this study are that Econase XT supplementation of a wheat-based diet increased the X_4 content in the colon, the arabinose content throughout the GIT and the xylose content in the colon and caeca of male broilers. Although other XOS and monosaccharides were detected in the GIT, they were unaffected by Econase XT supplementation.

There was a higher quantity of X_4 in the colon of broilers fed Econase XT, but this was lost in the caeca, where X_4 was undetectable in both treatment groups. Econase XT increased the generation of X_4 along the GIT from jejunum to ileum before peaking in the colon and vanishing in the caeca. There are currently no reports of X_4 transporters and AXOS are resistant to digestion via saliva, gastric juices, pancreatin

and the intestinal mucosa, limiting their absorption (Fujikawa et al. 1991). Therefore, the reduced X_4 content between colon and caeca was likely a result of factors acting on AXOS in the GIT, potentially the microbiome. These factors may also be responsible for the increased xylose contents seen in the colon and caeca. AXOS are known to be readily fermented by the microbial populations that inhabit the caeca (Kiriyama et al. 1992). Their fermentation, along with that of xylose, can lead to greater acetate production, so the increased proportion of acetate observed with Econase XT potentially indicates greater fermentation of AX products in the caeca (Johnson et al. 2006).

Interestingly there were increases in X_4 generation associated with Econase XT, but not X_2 or X_3. This might suggest that the endogenous xylanase enzymes present in both the wheat source used and the broilers GIT were able to hydrolyse bonds in the AX chain associated with X_2-X_3, but the addition of Econase XT was necessary to create AXOS products with more than 3 degrees of polymerisation. Since X_2, X_3 and X_4 are not thought to be absorbed due to a lack of transporter system, then concentrations of each XOS might be expected to increase down the GIT, before reaching the caeca where they may be metabolised by the microbiome. This was true for X_4, but X_2 declined and X_3 remained constant across the GIT, suggesting that X_2 may be more easily degraded to xylose in the lower small intestine or that there are differential specificities for oligosaccharide fermentation with different degrees of polymerisation. It is important to remember that the concentrations along the GIT reflect a single time point, that reflect a balance between generation of XOS and monosaccharides by enzymatic action and disappearance by absorption/utilisation *in vivo*, whereas there was no disappearance mechanism with the *in vitro* model.

Comparing the *in vitro* data to the *in vivo,* the X_4 generation *in vitro* was most like the *in vivo* findings. At the 9hr time point *in vitro* there was a clear increase in X_4 associated with Econase XT addition to the diet, which may reflect the digesta reaching the colon *in vivo,* where X4 contents were also increased by Econase XT. There were no effects of Econase XT on the *in vitro* levels of X_3, which was also reflected *in vivo.* In contrast there was an increase in X_2 *in vitro,* but only at the 24hr time point, which did not reflect *in vivo* digests. This could be because broiler GIT transit time is more rapid, with marker first appearing in the faeces 90 minutes after consumption and a peak at 220 minutes (Summers and Leeson 1986).

The monosaccharides arabinose and galactose, both increased linearly over 24 hours during *in vitro* digests, with arabinose release being greater with Econase XT during the final 12hrs of digestion than controls. This contrasts with the *in vivo* profiles, where arabinose and galactose were either very low or undetectable in the jejunum or ileum, yet markedly higher in the colon, before decreasing again in the caeca. This could be because enzyme hydrolysis of arabinose and galactose does not occur before reaching the colon, or because arabinose and galactose are being rapidly removed from the GIT by their corresponding transporter proteins in the jejunum and ileum. Arabinose and galactose appear to be well utilised in the early stages of GIT, based on data from other species (Schutte 1990; Csáaky and Ho 1965; Wagh and Waibel 1966).

Glucose release also increased linearly throughout the 24hr time course *in vitro*, contrasting with *in vivo,* where glucose levels decreased down the GIT, with no effect of Econase XT both *in vitro* and *in vivo*. The gradual decrease down the GIT is likely due to glucose being released from the starch component of the diet followed by rapid absorption in the jejunum and ileum (Klasing 1998), via both glucose

366 transporter (GLUT) and sodium-glucose cotransporter (SGLT) proteins (Braun and
367 Sweazea 2008), resulting in very little getting through to the colon and caeca.

368 There were increases in xylose release due to Econase XT supplementation only at
369 24hrs *in vitro*, whereas xylose contents were significantly higher in the colon and
370 caeca of Econase XT supplemented birds. The lack of xylose seen in the jejunum
371 and ileum was probably due to rapid absorption in the chicken small intestine as
372 previously described (Schutte et al. 1991; Longstaff et al. 1988). The increase in
373 xylose in the colon and caeca could result from the colonic and caecal bacteria
374 producing their own xylanases after being activated by Econase XT, which then
375 more aggressively attack the fibre component of the diet. This suggests that Econase
376 XT supplementation may be inducing adaptations to the microbiome in the colon
377 and caeca over time, rather than providing an acute effect of increasing AX
378 hydrolysis. This is supported by the ratio of free arabinose and xylose being closer to
379 one, much higher than would be susceptible to Econase XT attack, suggesting
380 activity other than the added Econase XT may be responsible for the release of these
381 monomers.

382 One question is why X_3 and X_2 concentrations along the GIT were not different
383 between controls and Econase XT treated birds? It has previously been shown that
384 wheat can contain endogenous xylanase activity (Dornez et al. 2008), suggesting that
385 the wheat-based diet used may also contain endogenous xylanase activity that
386 generated XOS without inclusion of exogenous Econase XT. The generation of XOS
387 without any exogenous enzyme may also be due to the birds being fed a mash rather
388 than a pelleted diet. Pelleting requires high temperature and high pressure, which
389 would be expected to denature the proteins present, thereby reducing any
390 endogenous xylanase activity. The results from the *in vitro* digests of the same diet

would appear to confirm this, with similar release of xylose, galactose and glucose observed with or without Econase XT addition. This suggests endogenous xylanase causes the release of certain monosaccharides *in vitro*, but this is not enhanced further by exogenous xylanase. We therefore suggest there might be greater effects of Econase XT in broilers fed a pelleted diet or a mash diet where the wheat contains either less endogenous xylanase activity or more xylanase inhibitors. Despite this, there were still significant effects of Econase XT on the generation of X_4, xylose and arabinose, particularly in the colon and caeca. We propose that the Econase XT produces more X_4 in the colon, thereby activating the gut microbiome through provision of substrate, leading to further enzyme attack to produce the higher levels of xylose and arabinose seen in the colonic digesta and caeca. This appeared to be supported by the correlation analysis, where there tended ($p=0.089$) to be more arabinose in the caeca of broilers with the highest X_4 contents in the colon, with a similar positive relationship seen for caecal xylose and colonic X_4 although this was not significant ($p=0.112$). The fact that similar relationships were not seen for X_3 and X_2 suggests that undigested X_4 produced in the colon is being utilised in the caeca, potentially resulting in the increased xylose and proportion of acetate seen in the caeca of Econase XT supplemented broilers. This increase in proportion of acetate in the caeca with Econase XT supplementation agrees with previous studies (Kabel et al. 2002; Ravn et al. 2018), showing that acetate and butyrate are produced by the fermentation of pure AXOS or via xylanase action. However, there was no effect of Econase XT on butyrate in the present study. Whether the observed increase in acetate represents a change in the microbiome is unclear but does appear to support the hypothesis that the chicken microbiome is ‘trained’ or adapted over time as a potential mechanism for the effects of xylanase supplementation.

The lack of effect on bird performance could be due to the limited time frame, and late exposure to Econase XT, as many studies have supplemented xylanase from day of hatch, when the microbiome is rapidly developing. Birds were given Econase XT supplementation from 4 days of age for a total of 21d. This relatively short exposure implemented from d4 may be too short or may be before the birds' microbiome had established or had completely adapted. A recent study (Figueiredo et al. 2012) showed that xylanase supplementation from d1 of age only had significant effects on FCR at 28 days of age.

The present study shows that there was degradation by Econase XT of AX in the wheat-based diet, as indicated by higher xylose, arabinose and X_4 levels present, particularly in the colon and caeca of male broilers. This may then be responsible for the observed increase in acetate production by the caecal microbiome and may be indicative of the microbiome adapting. Although these effects did not result in improved performance of the birds, this could be explained by a) the presence of endogenous xylanase activity in the diet, particularly as it was not pelleted, and b) the relatively short exposure time to Econase XT at such an early stage of development.

Table 1 Ingredient constituents and calculated nutritional values of the experimental diet**.**

% Ingredient unless otherwise specified			
Diet **ingredient constituents** [1]		**Calculated nutritive values** [1]	
Raw ground wheat	55	**Protein**	20.2
HiPro soya	23	**Energy ME, MJ**	12.96
Raw ground barley	9.37	**Oil**	6.36
Full fat soya	5	**Fibre**	3.07
Soya oil	4	**Ash**	5.35
Limestone flour	0.95	**Calcium**	0.80
Monocalcium phosphate	1.18	**Available P**	0.41
Vitamin and mineral premix[b]	0.4	**Sodium**	0.18
DL-Methionine	0.35	**Lysine**[a]	1.19
L-Lysine HCl	0.2	**Methionine**[a]	0.63
L-Threonine	0.15	**Methionine + Cysteine**[a]	0.94
Salt	0.25	**Threonine**[a]	0.85
Sodium bicarbonate	0.15	**Tryptophan**[a]	0.24

[1] The same diet was used for the 2 treatments: Control (diet as shown) and Econase XT (Control diet supplemented with 16,000 BXU/kg of Econase XT 25).

[a] Amino acid levels are expressed as standardised ileal digestible content.

[b] Supplying: retinoic acid 3mg/kg, cholecalciferol 125μg/kg, α-tocopherol 100mg/kg, thiamine 3 mg/kg, riboflavin 10 mg/kg, pyridoxine 3 mg/kg, cobalamin 30 μg/kg; nicotinic acid 60 mg/kg, pantothenic acid 15 mg/kg, folic acid 1.5 mg/kg, biotin 250 μg/kg, choline chloride 25 mg/kg, Fe 20 mg/kg, Cu 10 mg/kg, Mn 100 mg/kg, Co 1.0 mg/kg, Zn 80mg/kg, I 1 mg/kg, Se 0.25 mg/kg and Mo 0.5 mg/kg.

Table 2 Monosaccharide composition, total Arabinoxylan (AX) content and Arabinose: Xylose (A:X) ratio of the wheat-based diet after Trifluroacetic acid hydrolysis of non-cellulosic polysaccharides.

Monosaccharide[1]	g/100g diet
Arabinose	9.21 ± 1.48
Galactose	6.22 ± 1.04
Glucose	18.11 ± 0.17
Xylose	10.59 ± 0.28
Total	44.16 ± 0.67
Total AX[2]	19.80 ± 1.63
A:X[3]	0.87 ± 0.13

[1]Values of monosaccharides are expressed as mean grams per 100g of diet ± SD of four replicates. [2]Total AX values are calculated as mean grams of Arabinose + Xylose per 100g cereal ± SD of four replicates.

[3]A:X ratio calculated as Arabinose (g/100g cereal) divided by Xylose (g/100g cereal) ± SD of four replicates.

457 **Table 3 Effect of inclusion of Econase XT in diet on broiler performance.**

458

	Control[1]	Econase XT[1]	SED [2]	P-Value
ADFI [3] (d0-cull) (g/bird/d)	61	68	14.171	0.11
ADG [4] (d0-cull) (g/bird/d)	42	43	9.093	0.67
FCR [5] (d0-cull)	1.45	1.61	0.126	0.23

459

460 [1]Data are means for ten replicate pens per treatment, with four birds per pen.

461 [2]SED, Standard error of the difference of the means;

462 [3]ADFI, average daily feed intake;

463 [4]ADG, average daily gain;

464 [5]FCR, feed conversion ratio.

465

466

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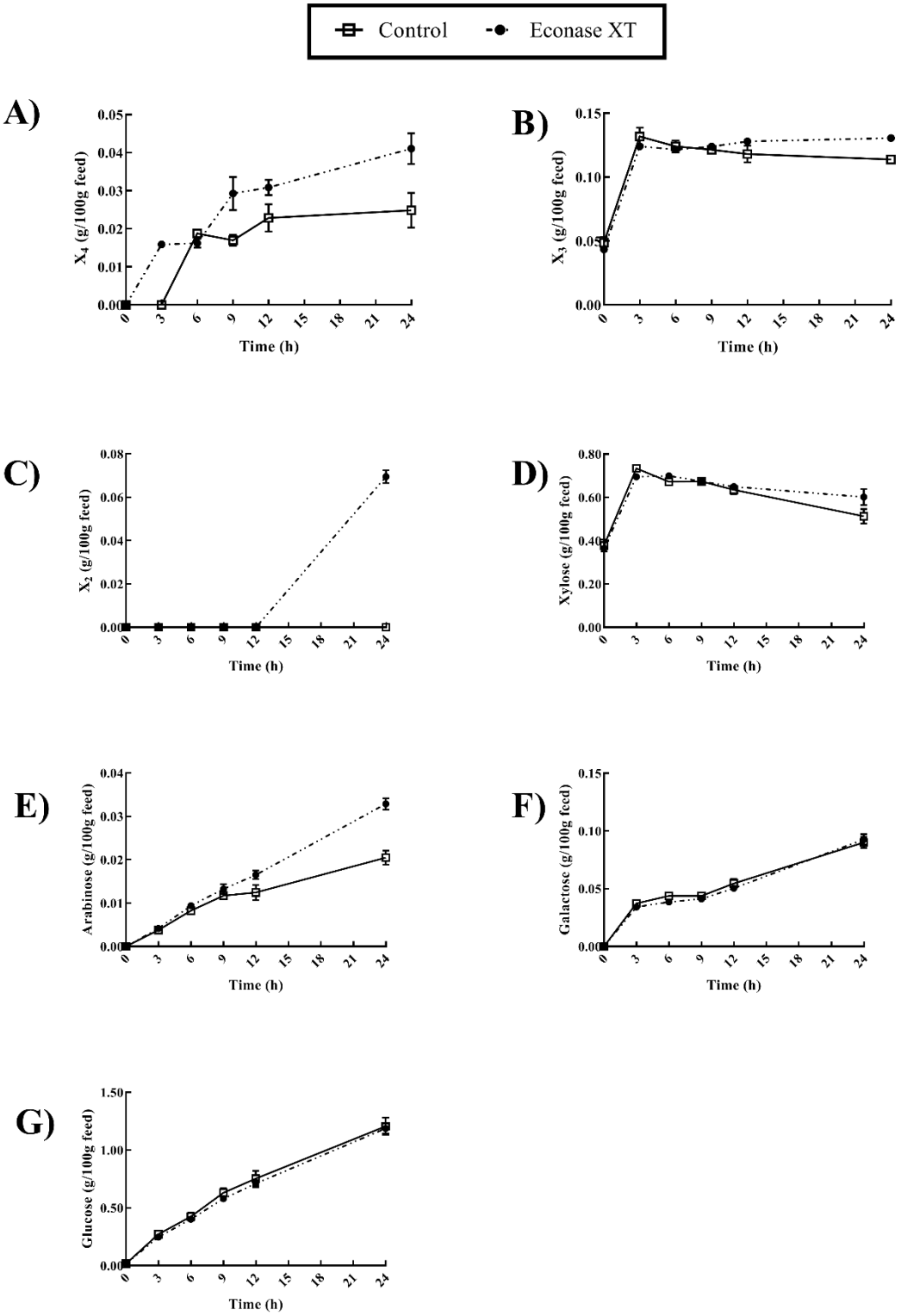
622 **Figures**



623

624 **Figure 1 Time-dependent release of Xylo-oligosaccharides and monosaccharides from**
625 **the wheat-based diet during an *in vitro* incubation at 41°C in the presence or absence of**
626 **Econase XT for 24 hours. A:** Xylotetraose (X_4) **B:** Xylotriose (X_3) **C:** Xylobiose (X_2) **D:**
627 Xylose, **E:** Arabinose, **F:** Galactose, **G:** Glucose. Data show mean values ± standard error of
628 the mean (SEM). Two-way ANOVA indicated significant enzyme x time interactions for X_4,

X_3, X_2 (all $p<0.001$), Xylose ($p=0.034$) and Arabinose ($p<0.001$). There were significant effects of time for Galactose ($p<0.001$) and Glucose ($p<0.001$), but no effects of enzyme.

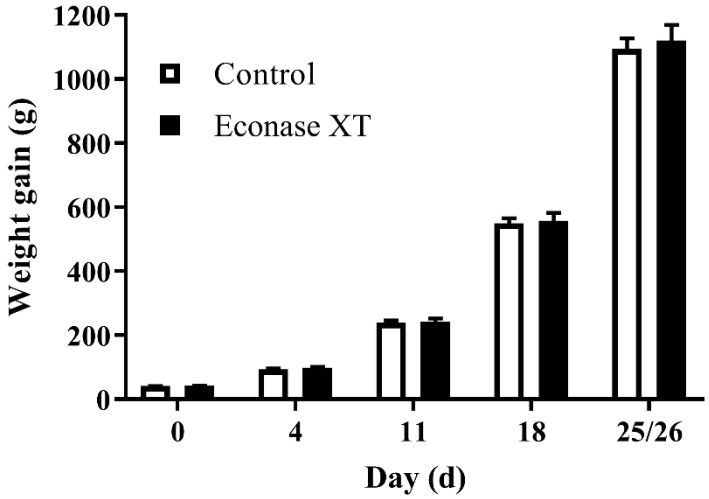


Figure 2 Effect of inclusion of Econase XT in diet on broiler growth. Data show mean values ± standard error of the mean (SEM). Data represent ten replicate pens per treatment, with four birds per pen. There was no enzyme x time interaction ($p=0.716$), nor any effect of enzyme ($P=0.686$), but there was a significant effect of time ($p<0.001$).

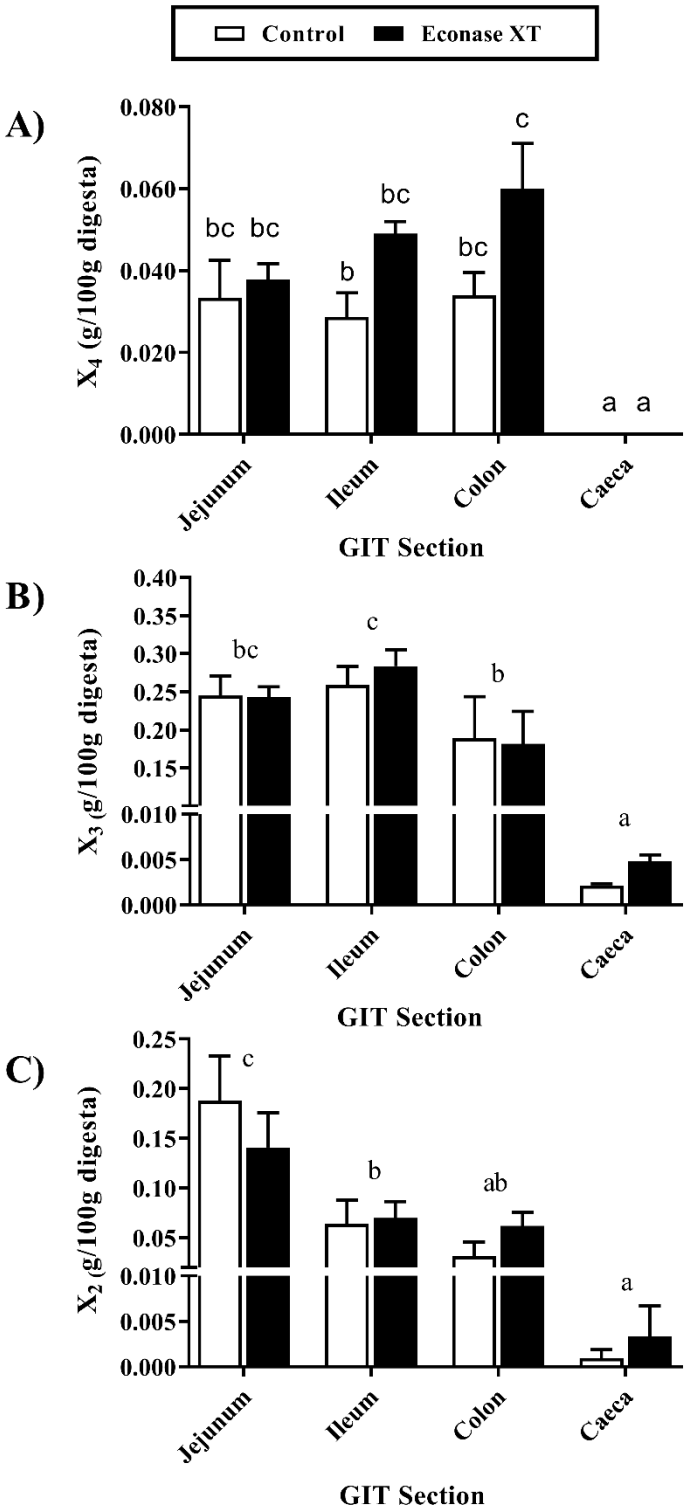


Figure 3 Effects of Econase XT inclusion in the diet on release of Xylooligosaccharides at different sections of the broiler gastro-intestinal tract (GIT). Data show mean g/100g of digesta dry matter ± standard error of the mean (SEM) for 10 replicates per treatment (1 bird per pen per treatment) for **A:** Xylotetraose (X_4) **B:** Xylotriose (X_3) **C:** Xylobiose (X_2). Two-way ANOVA indicated a significant enzyme x gut section interaction for X_4 ($p=0.038$) and significant effects of gut section for both X_3 and X_2 (both $p<0.001$). ^{a,b,c,d} Columns with

different superscript letters were significantly different ($p<0.05$, Bonferroni *post-hoc* test). In **B** and **C** *post-hoc* Bonferroni tests were for effects of gut section.

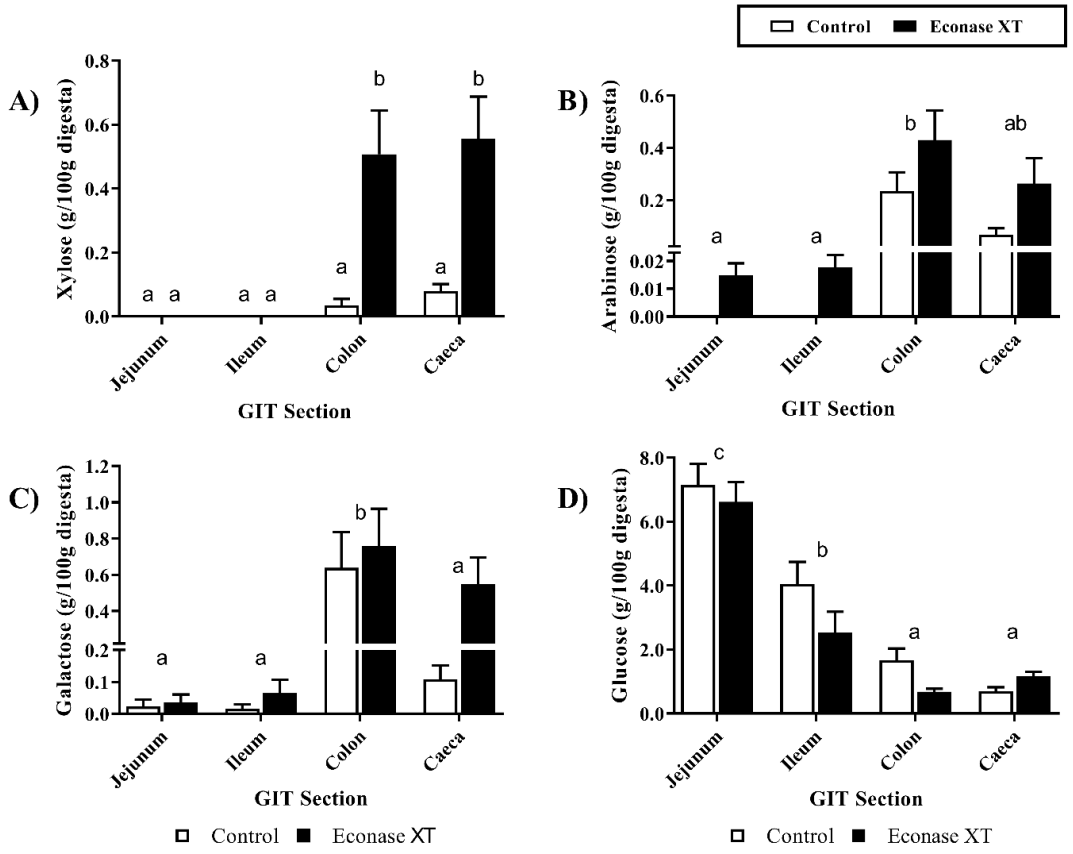
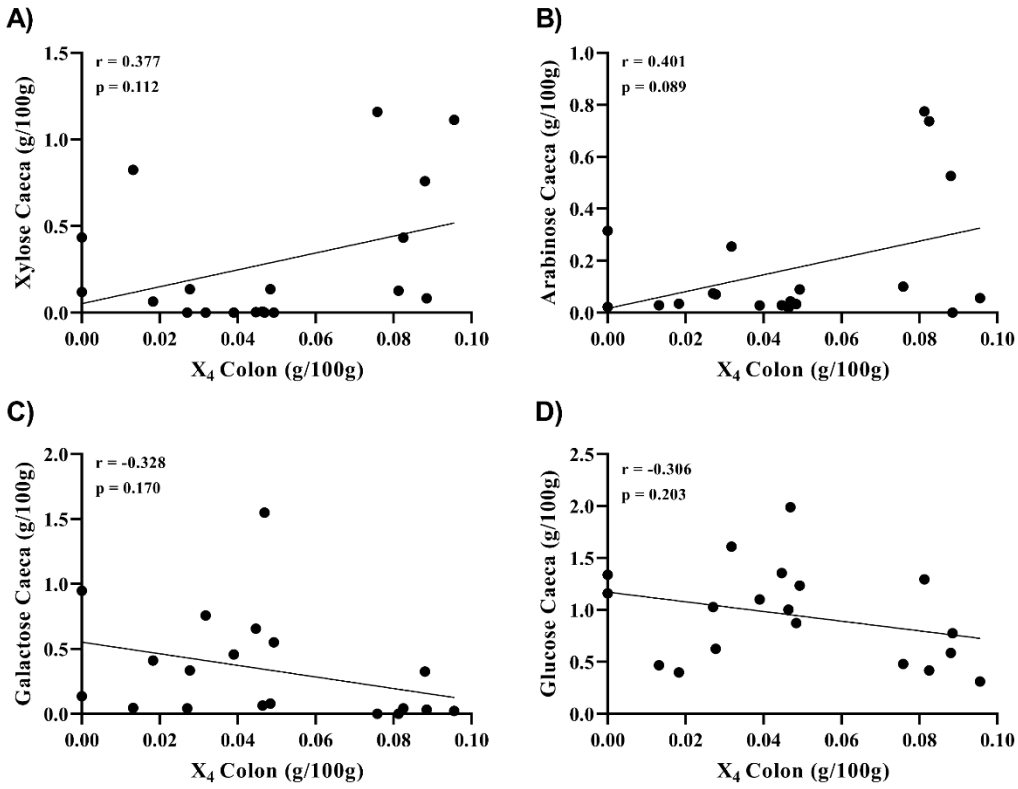


Figure 4 Effects of Econase XT inclusion in the diet on release of Monosaccharides at different sections of the broiler gastro-intestinal tract (GIT). Data show mean g/100g of digesta dry matter ± standard error of the mean (SEM) for 10 replicates per treatment (1 bird per pen per treatment) for **A**) Xylose, **B**) Arabinose, **C**) Galactose and **D**) Glucose. Two-way ANOVA indicated a significant enzyme x gut section interaction for Xylose ($p<0.001$) and significant effects of gut section for Arabinose, Galactose and Glucose (all $p<0.001$). There was also a significant effect of enzyme for Arabinose ($p=0.012$) and a trend for Galactose ($p=0.071$). ^{a,b,c,d} Columns with different superscript letters were significantly different ($p<0.05$, Bonferroni post-hoc test). In **B, C** and **D** *post-hoc* Bonferroni tests were for effects of gut section.



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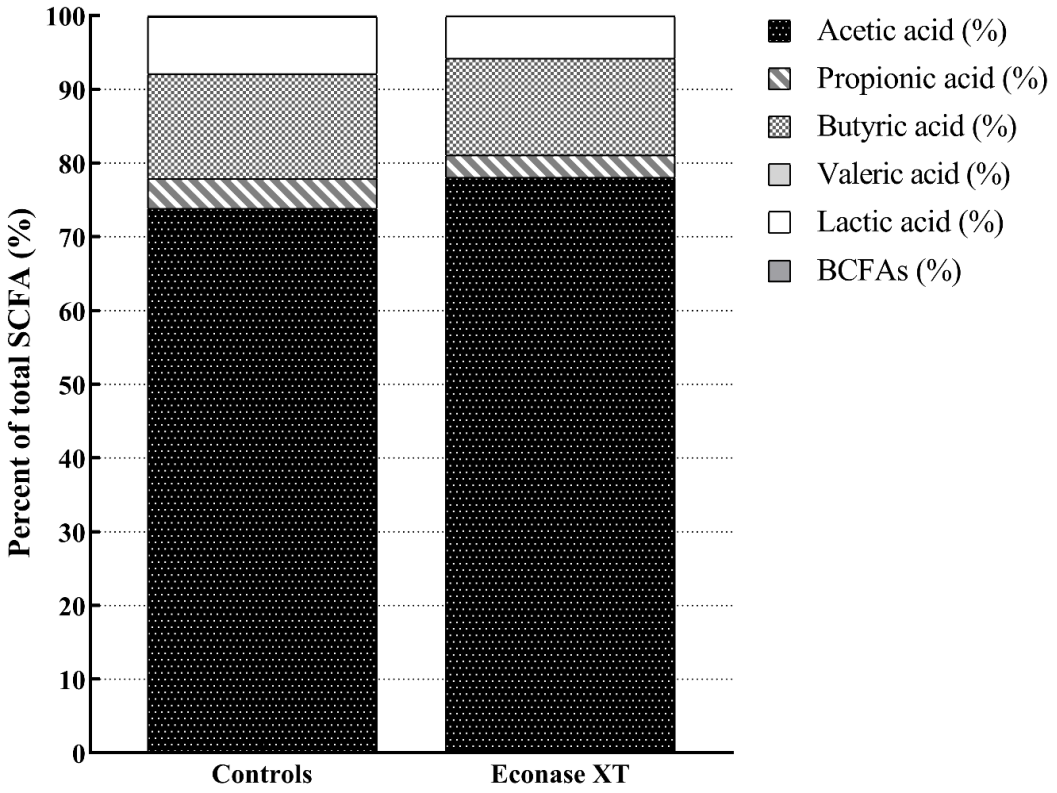
659 **Figure 5 Correlations between colonic xylotetraose (X_4) contents and caecal**
660 **monosaccharide contents.** Data show colonic X_4 contents (g/100g of digesta dry matter)
661 against caecal contents for **A**) Xylose, **B**) Arabinose, **C**) Galactose and **D**) Glucose (all
662 g/100g of digesta dry matter). Pearson correlation coefficients indicated a positive trend for
663 colonic X_4 against caecal arabinose (p=0.089), but not for xylose (p=0.112), galactose
664 (p=0.170) or glucose (p=0.203).



665

666 **Figure 6 Effects of Econase XT inclusion in the diet on the proportions of Volatile Fatty**
667 **Acids (VFA) in the caeca of male Broilers.** Data are means of 10 randomly selected birds
668 (one from each pen) expressed as % of the total VFA content. There was a trend for Econase
669 XT supplementation to increase the proportion of acetic acid in the caeca (p=0.062).

670