Xylanase impact beyond performance: effects on gut structure, faecal volatile fatty acid content and ammonia emissions in weaned piglets

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Abstract

The addition of xylanase to piglet diets is known to improve performance and nutrient digestibility. The present study aimed to assess the impact of a new xylanase on the growth performance, nutrient digestibility, and gut function of weaned piglets. 144 piglets, weaned at 28 days (7.48 kg IBW), were randomly assigned to four treatments: a basal control diet based on corn, wheat, rice and soy, and the basal diet supplemented with 45,000, 90,000 and 135,000 U/kg xylanase from *Thermopolyspora flexuosa* and expressed in *Pichia* yeast. Performance was measured at day 0, 14 and 35. At day 35, samples were collected for assessment of intestinal histology, and volatile fatty acid and ammonia concentrations. In a further study, 12 piglets (11.34 kg IBW) were placed in metabolic crates for assessment of total tract nutrient digestibility using Cr_2O_3 and Fe_2O_3 as an indigestible marker. The addition of xylanase at 90,000 and 135,000 U/kg significantly improved average daily gain (333.6 g/day control, 364.86 g/day 90,000 U/kg, 405.89 g/day 135,000 U/kg, $P<0.05$), the Gain to Feed ratio (0.557 control, 0.612 90,000 U/kg, 0.692 135,000 U/kg, $P<0.05$), and reduced the incidence of diarrhoea. This was driven by significant improvements in nutrient digestibility at all levels of xylanase supplementation and increased villus height in the jejunum (372.87 μm control, 432.53 μm 45,000 U/kg, 465.80 μm 90,000 U/kg, 491.28 μm 135,000 U/kg, $P<0.05$). Xylanase supplementation also linearly increased the levels of butyrate in the faeces and had a quadratic relationship with propionate concentrations. Supplementation with xylanase also reduced faecal ammonia emissions compared to the control, with a significant difference found when supplementing 135,000 U/kg. In conclusion, dietary supplementation with xylanase improved growth performance and feed efficiency in weaning piglets, likely driven by improvements to gut structure and function.

Keywords: digestibility; histology; performance; piglets; volatile fatty acids; xylanase

1. Introduction

Increasing levels of non-starch polysaccharides (NSPs) in piglet diets, negatively affect nutrient digestibility and growth performance, as pigs lack endogenous enzymes capable of hydrolyse fibrous raw materials (Tsai et al., 2017; Yu et al., 2016; Berrocoso et al., 2015; Bartelt et al., 2002). This problem is particularly evidenced in weaning diets, when the combined stressors of removal from the sow, social mixing, and new feed sources and method of acquisitions cause the production of endogenous enzymes and stomach acid to drop (Inoue et al., 2015; Lindemann et al., 1986). High fibre diets can also increase greenhouse gas emissions from pig manure (Prenafeta-Boldú et al., 2017; Seradj et al., 2018). By contrast, low fibre diets are linked to numerous detrimental effects on gut health including thinning of the mucus layer, increasing susceptibility to infection, and induction of dysbiosis and probiotic extinction in the gut (Wang et al., 2021a; Riva et al., 2019; Desai et al., 2016).

To improve nutrient digestibility and performance, commercial nutritionists rely on the addition of exogenous enzymes, including xylanase. Xylanase degrades arabinoxylan, the main NSP found in the major cereals used in animal feed (O'Neill et al., 2014). Addition of xylanase to piglet diets has shown to reduce intestinal viscosity (He et al., 2020; Passos et al., 2015), increase nutrient digestibility (Mejicanos et al., 2020; Tsai et al., 2017) and improve performance (He et al., 2020; Tsai et al., 2017). Meta-analysis of xylanase impact on pig growth performance suggests that responses can be inconsistent between studies (Torres-Pitarch et al., 2017, 2019). The size of effect is influenced by several factors, including the specific xylanase molecule tested (Zhang et al., 2018) and the composition of the basal diet (Nørgaard et al., 2019; Lærke et al., 2015), among others.

It is thought that this is due to the interaction of xylanase and the gut environment and intestinal microbiota. By breaking down long arabinoxylan chains into shorter arabinoxylan-oligosaccharides (AXOS), xylanase addition changes the availability of substrates for microbiota growth (Lærke et al., 2015). This favours beneficial *Lactobacillus, Ruminococcus, Prevotella* and *Bifidobacteria* populations and reduces potentially pathogenic *Clostridia* and *Pasteurella* counts (Van

Hoeck et al., 2021a,b; Gonzalez-Ortiz et al., 2020; Luise et al., 2020; Wang et al., 2020; Zhao et al., 2018). These bacteria ferment NSP into short chain fatty acids, which are used in "cross-talk" feedback loops that encourage the proliferation of other, associated beneficial bacteria (). These shifting populations subsequently augment the barrier function of the host intestine (Kelly et al., 2015), allowing better nutrient absorption and disease resilience.

These changes in bacterial populations, combined with the traditional reductions in viscosity, drive a healthier, better functioning gut environment with longer villi and larger absorptive surfaces (He et al., 2020; Duarte et al., 2019), increased digestibility and absorption, and finally improved growth performance. Effects of xylanase supplementation on gaseous emissions are less clear: Kpogo et al (2021) saw no effect of a multi-enzyme blend on faecal gas emissions, and similarly Chen et al. (2020) found that a multi-enzyme blend had no significant effect on NH_3 levels, though significant reductions were seen in CO_2 production.

The link between xylanase supplementation, improved gut health and function and improved post-weaning performance is well established. However, as effects of xylanase differ between specific enzyme molecules, the current experiment was designed to study the effects of increasing levels of a new xylanase supplementation in weaned piglets fed complex diets on the production performance, nutrient digestibility, intestinal tract morphology and volatile fatty acid concentration in faeces.

2. Materials and methods

Two experiments were conducted to assess the effect of xylanase supplementation: a pen trial to assess growth performance and a metabolic trial to assess nutrient. Both studies took place in a commercial pig farm in Nakhon Pathom province, Thailand. All experimental procedures followed the guidelines of the National Research Council of Thailand and were approved by the Animal Care and Use Committee of Khon Kaen University (permission No. IACUC-KKU79/63).

105

106 2.1 Experimental diets

107 The same diets were used for both studies. A two-phase feeding program was used: a
108 pre-starter feed from day 1 to day 14 and a starter feed from day 15 to day 35. Basal diets were
109 formulated using corn, wheat and soybean meal. The composition of the basal experimental diets
110 was formulated to meet or exceed requirements (NRC, 1998) is presented in Table 1. All diets
111 were manufactured before the onset of the experiment. The experimental pellet feeds (4 mm in
112 both phases) were prepared at 75 ºC and were manufactured at the feed mill of Bangkok Animal
113 Research Center Co., Ltd (Samut Prakan, Thailand; #AF20/28A). Feed samples from each
114 treatment were provided to Kemin Europa N.V. for recovery of xylanase as per Van Hoeck et al.
115 (2021a).

116 For each phase, one basal diet was made, which was then split equally into different
117 experimental products: 1) a control diet without supplemental xylanase, 2) the control diet
118 supplemented with 45,000 U/kg xylanase, 3) the control diet supplemented with 90,000 U/kg
119 xylanase, and 4) the control diet supplemented with 135,000 U/kg xylanase. The xylanase used in
120 the study was XygestTM HT (Kemin Animal Nutrition and Health, Herentals, Belgium). Xygest
121 HT is an intrinsically thermostable, monocomponent xylanase produced by Thermopolyspora
122 flexuosa expressed in Pichia pastoris. It is an endo-beta-1,4-xylanase, belonging to the GH11
123 family, particularly designed to further improve the degradation of dietary fibre to maximize the
124 energy utilization of the diet.

125

126 2.2 Experiment 1 – performance study

127 A total of 144 weaned pigs ([Large white x Landrace] x Duroc; 7.48±0.24 kg initial BW)
128 weaned at day 28 were used in a 5-week growth trial. Upon arrival, piglets were blocked by body
129 weight and gender and were randomly allotted to one of four dietary treatments in a randomized

130 complete block design. Each treatment consisted of nine replicates, with four piglets (two gilts
131 and two barrows) per pen.

132 During the experiment, all pigs were housed in an environmental controlled building
133 with half-slatted concrete floors (0.96 m x 2.16 m) with a stocking density of 0.52 m^2 per pig as
134 per Li et al. (2020). The pen was equipped with heating using an electric bulb, feeder and nipple
135 drinker to provide water and feed with *ad libitum* access throughout the study. Unused rice straw
136 was provided for two weeks postweaning to minimize temperature stress, which conformed to
137 the European regulations (EU Directive 2010/63/EC for animal experiments. During the first
138 week the housing temperature was maintained at 30 °C, after which it was gradually reduced by 1
139 °C every week. Microclimate conditions (temperature and relative humidity) were recorded daily.

140 Performance parameters were recorded throughout the experimental period. Pig body
141 weight and feed intake were recorded on weeks 0, 2, and 5 post weaning, and used for evaluating
142 average daily gain (ADG) and average daily feed intake (ADFI) observations. Per pen, the ADG
143 and ADFI were calculated by dividing the total weight gain and total feed intake, respectively, by
144 the total number of experimental days. The gain-to-feed ratio (G:F) was calculated for each pig
145 by dividing the ADG by the ADFI. Mortality was registered when occurred (date and weight of
146 dead pigs was recorded). The number of diarrheic piglets per pen was recorded daily at 0700 to
147 calculate the diarrhoea rate. Diarrhoea occurrence was defined by the faeces being soft with a
148 moisture content over 75 %. Diarrhoeal rate (%) was calculated by [total number of diarrheic
149 piglets/(total number of piglets × days of experiment)] × 100.

150

151 2.3 Intestinal morphology

152 To determine the histomorphological changes in the small intestine, segments of
153 duodenum and jejunum were collected from 28 pigs (seven pigs per treatment) at 35 days of
154 experiment. Tissue samples were washed with a 0.85% saline solution and fixed in 10%
155 formaldehyde solution for 18 hours and then transferred to 70% (v/v) ethanol until performing.

156 Fixed intestinal samples were embedded in paraffin. Each fixed sample was sectioned into 5-μm
157 slices by microtome and stained with Harris' Alum hematoxylin and counterstained with eosin
158 following standard procedures. Villus height (VH) and crypt depth (CD) were assessed using
159 Scion image software (Scion Corporation, Frederick, MD). The VH was defined from the villus
160 tip to the villus-crypt junction between each villus, while the CD was measured as the depth of
161 the invagination between adjacent villi and the villus width. The villus height/crypt depth ratio
162 (VH: CD ratio) was calculated.
163
164 2.4 Volatile fatty acid and ammonia concentration
165 For assessment of volatile fatty acid (VFA) contents, faecal samples were taken from 35
166 individual pigs (seven samples per treatment) at the end of experiment. VFA concentrations
167 were determined after metaphosphoric acid derivation as per the modified methods of Zhuang
168 et al. (2014). Briefly, 0.2 g of thawed sample was mixed with 0.2 mL of H_2O in a capped plastic
169 centrifugal tube for 10 min and then centrifuged at 1,872× g for 30 min. A volume of 0.2 mL of
170 supernatant was vortexed with 40 μL of a 25 % metaphosphoric acid solution (containing 7.5
171 mM crotonic acid solution as the internal standard) to adjust the pH to approximately 2.0. This
172 solution was kept at − 20 °C for 12 h and centrifuged at 12,000x g for 10 min before analysis.
173 The deproteinized supernatant of 0.1 mL of 4-methyvaleric acid (catalog # SHBL3457) was
174 added as an internal standard prior to quantify by an Agilent 7890A gas chromatograph (Agilent
175 Technologies, Santa Clara, CA, USA) with a column packed SP-24107 column (30 m × 0.25 mm
176 × 0.25 μm, Supelco Inc., Bellefonte, PA, USA). The injector-port and flame ionization detector
177 were maintained at 230 and 250 ºC (run time 25 min), respectively. The initial temperature was
178 held at 120 ºC for 4 min after injection, and increased at 4 ºC/min to 160 ºC. Helium was used
179 as the carrier gas.
180 For assessment of ammonia emissions, fresh faecal samples were collected at the end of
181 experiment by direct rectal massage of 32 pigs (eight samples per treatment). A total of 50 g of

182 fresh faecal samples were collected, kept in a 2.6-L sealed plastic box, and then delivered directly
183 to a commercial laboratory (Betagro Science Centre, Pathum Thani, Thailand) for subsequent
184 analysis. The samples were incubated at room temperature for seven days to allow for
185 fermentation using the method described by Cho et al. (2008). A total of 100 mL of headspace
186 air was sampled for ammonia quantification using a gas detector (4 LK Detector tube; Gastec
187 Corp).
188
189 2.5 Experiment 2 – nutrient digestibility
190 For evaluating nutrient total tract digestibility, a total of 12 male piglets (11.34±0.67 kg
191 initial BW) reared alongside those of Experiment 1 were randomly selected and assigned to four
192 treatments following a completely randomized design for a 6-day adaption period and five days
193 of collection. Prior to entry into the metabolism crates, pigs had been fed a commercial diet. All
194 pigs were housed in individual metabolic crates (0.85 m x 1.15 m x 0.68 m) at a controlled
195 temperature of 27 ºC. The experimental diets, identical to the starter diets of Experiment 1, were
196 provided twice daily at 0700 and 1900 according to the rate of 2.0 times the maintenance
197 requirement for ME (NRC, 1998) based on initial BW, with *ad libitum* access to water. Chromic
198 oxide and ferric oxide (5 g/100 g of feed) were included in all experimental diets as indigestible
199 markers. Collected excreta were kept at -20 ºC during the collecting period and dried at 60 ºC
200 for 72 hours. Then, the representative samples were ground (2 mm screen, Wiley mill) using a
201 centrifugal mill for later chemical analysis.
202 Homogeneous samples of feed and faeces were analysed in duplicate to determine
203 nutrient digestibility of organic matter (OM, method # 930.15), crude protein (CP, method #
204 984.13; N x 6.25), ether extract (EE, method 920.39), crude fiber (CF), acid detergent fibre
205 (ADF) and starch following the protocol of AOAC (2000) and Van Soest et al. (1991). Gross
206 energy was determined using an adiabatic oxygen calorimeter. Neutral detergent fibre (NDF) was
207 defined using Ankom fibre analyser (Ankom Technology, Macedon, NY). Total amino acid was

208 analyzed by high-performance liquid chromatography (as per Boontiam et al., 2019). Apparent
209 total tract digestibility was calculated as the following equation:

210 $$X_{apparent\ digestibility} = \frac{X_{ingested} - X_{excreted}}{X_{ingested}} \times 100$$

211 where X represents OM, DM, CP, EE, CF, ADF and total amino acids (AA). Digestibility of 17
212 amino acids (Asp, Thr, Ser, Glu, Gly, Ala, Val, Ile, Leu, Tyr, Phe, Lys, His, Arg, Pro, Met, and
213 Cys) was reported as the total AA digestibility.
214
215 *2.6 Statistical analysis*
216 Data were analysed in the Fit Model platform of JMP 15, with treatment as the fixed
217 effects. The experimental unit was the sample replicate or individual pen (as per He et al., 2020).
218 Means separation was conducted using Tukey's HSD. To assess the linear and quadratic effects
219 of increasing xylanase dose, a second model was run using U/kg and U/kg squared as model
220 effects. Significance was determined at $P<0.05$; $P<0.1$ was taken to indicate a near-significant
221 trend.
222
223 **3. Results**
224 Analysed enzyme activities in feed samples were all close to expected values (Table 2).
225
226 *3.1 Growth performance and diarrhoea incidence*
227 The effects of xylanase supplementation on the growth performance of weaned piglets
228 are shown in Table 3. In the prestarter diets, ADG and G:F were significantly improved by
229 135,000 U/kg xylanase: ADG was increased by 24.5 % (285.69 g/day XYL vs 229.52 g/day
230 CON, $P<0.01$) and G:F was increased by 29.8 % (0.810 g gain/g feed XYL vs 0.624 g gain/g
231 feed CON, $P<0.001$). Supplementation of 45,000 and 90,000 U/kg xylanase, resulted in
232 numerical improvements of 7.0 and 17.4 % for ADG and 2.6 and 18.3 % for G:F, respectively.

Significant increases in ADG did not lead to significant improvements in BW at d14. In the starter period, ADG and G:F were again significantly improved by 135,000 U/kg xylanase; ADG was increased by 20.6 % (486.02 g/day XYL vs 402.98 g/day CON, $P<0.0001$) and G:F was increased by 22.7 % (0.653 g gain/g feed XYL vs 0.532 g gain/g feed CON, $P<0.001$). Supplementation of 45,000 and 90,000 U/kg led to numerical improvements of 6.3 and 7.7 % for ADG and 6.8 and 7.5 % for G:F, respectively. At day 35, BW was significantly increased by supplementation with 135,000 U/kg xylanase (21.67 kg XYL vs 19.14 kg CON, +13.2 %, $P<0.001$).

When considering the whole study period, all levels of xylanase inclusion increased the ADG compared to the control. At 45,000 U/kg, the improvement was near significant, when supplementing the xylanase at 90,000 and 135,000 U/kg the difference became even significant (respectively +9.4% and +21.7%). The ADG achieved at the highest dose was also significantly higher compared to the lower doses of xylanase applied. G:F significantly increased compared to the control at inclusion of 90,000 (+9.9 %) and 135,000 (+24. 2%) U/kg of xylanase, with a near significant increase following supplementation with 45,000 U/kg. Xylanase addition also reduced the number of days of diarrhoea recorded per pen, with the control suffering 15.22 pig days of diarrhoea on average, compared to respectively 9.78, 7.89 and 5.89 pig days in the 45,000, 90,000 and 135,000 U/kg groups.

Looking at the linear and quadratic response of growth performance to increasing xylanase levels, most measured performance parameters demonstrated significant linear responses (data not shown). Significant quadratic relationships were only seen for ADFI in the starter phase ($P=0.0182$) and across the whole study ($P=0.0156$). Pigs supplemented with 45,000 U/kg ate marginally more (+0.78 % in the starter phase and +1.6 % across the whole study) than pigs fed the control diet, while 90,000 and 135,000 U/kg reduced ADFI below the level of the control. The same pattern was also seen in the starter diet, but the quadratic relationship was not significant.

259

260 *3.2 Nutrient digestibility and nitrogen balance*

261 Nutrient digestibility response to xylanase supplementation is summarized in Table 4.
262 Xylanase supplementation significantly increased the digestibility of all measured nutrients except
263 crude fibre. Significant increases in the digestibility of dry matter, crude protein, ether extract,
264 gross energy and starch were seen at the lowest level of xylanase supplementation. Energy
265 digestibility showed the largest response to 45,000 U/kg, increasing from 54.96 % in the control
266 to 68.40 % with 45,000 U/kg. At 135,000 U/kg gross energy digestibility was 78.76 %, though
267 this was not significantly different from 45,000 U/kg. At the highest level of xylanase
268 supplementation, 135,000 U/kg, the digestibility of both dry matter and crude protein was
269 significantly increased compared to both the control diet and the lower levels of xylanase
270 supplementation. At 135,000 U/kg, crude protein digestibility was 6.9 % higher than that of pigs
271 fed the control diet (93.33 XYL vs 87.29 CON, P=0.0002) and 2.9 % higher than that of pigs
272 supplemented with 90,000 U/kg.

273 Interestingly, although pigs fed diets supplemented with 135,000 U/kg xylanase showed
274 an excellent growth performance, overall, they had the lowest levels of crude protein intake. This
275 can be explained by a lower overall feed intake, as the dosage of xylanase supplementation
276 increased. Crude protein intake was highest in pigs supplemented with 45,000 U/kg xylanase.
277 However, N content in the faeces did not reflect overall CP and N intake, as the highest levels
278 were seen in the control, non-xylanase fed pigs and decreased linearly with increasing xylanase
279 supplementation (P<0.0001). Urine N was not affected by treatment, meaning that N
280 digestibility and retention were also linearly as the levels of xylanase supplementation went up
281 (P=0.0224 digestibility, P=0.0314 retention). Quadratic responses were not seen for N balance
282 parameters.

283

284 *3.3 Intestinal morphology*

285 The effects of xylanase supplementation on duodenal and jejunal morphology are
286 summarised in Table 5. There were no significant differences between treatments on duodenal
287 morphology, due to large variation between individual pigs. However, there was a significant
288 linear relationship between increasing xylanase dose and villus height in the duodenum
289 (P=0.0480). Crypt depth was not affected by xylanase supplementation.

290 In the jejunum, there was a significant treatment effect on villus height. Supplementation
291 with 90,000 and 135,000 U/kg xylanase, significantly increased villus height versus the control,
292 with a near significant numerical increase in the villus height of pigs supplemented with 45,000
293 U/kg xylanase. There was a significant linear relationship between jejunal villus height and
294 xylanase supplementation (P<0.0001), and a near significant quadratic relationship (P=0.0828).
295 There was also a near significant linear relationship between jejunal crypt depth and xylanase
296 supplementation (P=0.0870), with crypt depth tending to increase with increasing levels of
297 xylanase in the diet.

298

299 *3.4 Faecal volatile fatty acid and ammonia concentrations*

300 The effects of xylanase on VFA concentrations in the faeces of weaning pigs at 35 days
301 of age are summarized in Table 6. While there were limited significant between treatments, there
302 were strong linear and quadratic relationships between increasing xylanase supplementation
303 levels and VFA concentrations. Propionate concentrations in the faeces had a significantly
304 positive linear relationship (P=0.0364) and a significant, negative quadratic relationship
305 (P=0.0284) with increasing xylanase dose; this suggested that the highest levels of propionate
306 would be recovered in the faeces of pigs fed approximately 55,000 U/kg, as shown in Figure 1.
307 The relationship between xylanase and butyrate levels was less significant than that for
308 propionate, and only the linear relationship was significant (P=0.0161). No significant
309 relationships were seen between xylanase dose and acetate, isobutyrate and isovalerate

concentrations. There was a near significant, positive linear relationship between xylanase dose and total VFA concentration (P=0.1187).

As shown in Figure 2, there was a significant effect of treatment on ammonia emissions from the faeces of weaned piglets (P=0.0178). Faeces from piglets fed the control diets had the highest ammonia emissions, with 45,000 and 90,000 U/kg xylanase diets tending to reduce emissions. Faeces from pigs fed diets supplemented with 135,000 U/kg xylanase had significantly lower ammonia emissions compared the control diet (0.490 mg/g CON vs 0.361 mg/g XYL). There was a significant, negative linear relationship between faecal ammonia emissions and increasing xylanase dose (P=0.017).

4. Discussion

Although the complete mechanism by which xylanase increases nutrient digestibility is not fully understood, the use of exogenous enzymes, including xylanase, to improve nutrient digestibility and retention is an established commercial practice. Torres-Pitarch et al. (2017) found that, on average, the addition of xylanase to the diets of weaned piglets improved ADG by 28.1 g and G:F by 0.016 g/g. In the current study, the lowest level of supplementation with a new xylanase from *Thermopolyspora flexuosa* and expressed in *Pichia pastoris* tended to improve ADG by 24.97 g and G:F by 0.031 g/g. Higher levels of supplementation were able to further improve growth performance, with significant improvements seen versus control at 90,000 U/kg. At 135,000 U/kg ADG and G:F were significantly improved compared to both the control and lower doses of xylanase, with increases compared to the control diet of 72.29 g ADG and 0.135 g/g G:F across the whole study. These improvements in performance followed a linear relationship with increasing xylanase dose. Many studies report classic non-linear responses to increasing levels of enzyme supplementation, with higher doses reporting limited further advantages (Dong et al., 2018; Jang et al., 2017; Wealleans et al., 2016; Kiarie et al., 2012). The lack of quadratic response in the present study is in line with the findings of Kiarie and

Petracek (2015) and He et al. (2020), who saw significant linear responses to xylanase submission but non-significant quadratic responses.

Most xylanases are able to improve the performance of wheat-based diets to a greater extent than of diets based on corn (Abelilla and Stein, 2019); although corn accounts for nearly half of the dietary arabinoxylans found in a diet (Jaworski et al., 2015). Corn-derived arabinoxylans are often poorly degraded due to their structure, the degree of interaction with other components, abundant phenolic cross linkages, arabinose substitutions, and lignification (Bach Knudsen, 2014). However, the comparative efficacy on different dietary substrates is enzyme specific (Ndou et al., 2015). The xylanase used in the present study was able to improve the performance of both broilers and layers fed corn-based diets (Van Hoeck et al., 2021a,b). This can be possibly explained by two factors: an increase in feed efficiency through the release of encapsulated nutrients in the plant cell wall, and microbiome modulation via the prebiotic effect of the released xylo-oligosaccarides (XOS) from arabinoxylan hydrolysis. Improvements in digestibility of starch and crude protein observed in both the present study, and a previous study in broilers using the same xylanase, could be related to the reduction in cage-effect with the release of encapsulated nutrients such as starch and protein (Van Hoeck et al 2021a).

Traditionally, these performance improvements following xylanase supplementation were assumed to be driven by changes in gut viscosity (He et al., 2020) and nutrient digestibility. In the present study, supplementation with xylanase significantly improved the apparent total tract digestibility (ATTD) of dry matter, crude protein, ether extract, NDF, ADF, starch and gross energy. Gross energy was substantially increased by the lowest level of xylanase supplementation, jumping from 54.96 % in the control diet to 68.4 % with 45,000 U/kg xylanase ($P<0.05$). Higher levels of xylanase supplementation resulted in further numerical increases, to a maximum of 78.76 % with 135,000 U/kg xylanase. When looking at the efficiency of energy conversion, the increase in apparent energy digestibility is clear: pigs fed the control diet consumed 6019.16 kcal digestible energy (DE) per kilogram of bodyweight gain, with stepwise improvements at each

362 level of xylanase supplementation (5,689.61 kcal DE/kg BWG at 45,000 U/kg, 5,438.46 kcal
363 DE/kg BWG at 90,000 U/kg, 4,825.36 kcal DE/kg BWG at 135,000 U/kg).

364 However, recent research has exposed a more complicated mode of action for
365 supplemental xylanase than simple viscosity reduction and digestibility improvement. By
366 breaking down the long arabinoxylan chains into shorter oligosaccharides, the substrate available
367 for bacterial growth changes. This drives shifts in the microbial community in the gut, increasing
368 VFA concentrations, reducing intestinal pH, and reducing inflammation. Combined with
369 reductions in viscosity and reductions in nutrient caging by long fibre chains, this results in large
370 and more resilient villi which promotes better nutrient uptake across the gut barrier. In the
371 present study, supplementation with xylanase significantly increased the levels of propionate in
372 the faeces and tended to increase butyrate. Other VFAs were not significantly affected by
373 xylanase supplementation, in line with the results of Tsai et al. (2017), who reported limited
374 effect of xylanase supplementation on faecal VFA concentrations in weaning pigs. Tsai et al.
375 suggested that the limited observed response may be due to the rapid absorption of VFAs in the
376 intestine (Bergmann, 1990), with only 10 % of VFA excreted in faeces (Wolin and Miller, 1983).
377 It is possible that larger shifts in the VFA profile between treatments would be apparent in the
378 analysis of ileal or caecal contents, compared to faeces, and this poses a future avenue of
379 investigation.

380 Increasing levels of VFA may suggest a shift in the microbiome: Zhao et al. (2018)
381 reported piglet diets that promoted higher faecal concentrations of butyrate were linked to
382 greater abundances of Actinobacteria and Firmicutes. Zhang et al. (2018) reported decreased
383 abundance of Bacteroidetes and increased abundance of Firmicutes following xylanase
384 supplementation, while Sutton et al. (2021), Luise et al. (2020) and Gonzalez-Ortiz et al. (2020)
385 reported increased levels of *Lactobacilli*; similar responses have been seen following xylanase
386 supplementation in poultry (Singh et al., 2021; Van Hoeck et al., 2021a,b; Wang et al., 2021b).
387 The genera involved in these shifts are associated with fibre-degrading mechanisms, breaking

388 down complex polysaccharides and shorter chain arabinoxylo-oligosaccharides, into lactic acid,
389 hydrogen and SCFAs (Pryde et al., 2002). These products of fibre degradation are then
390 consumed by *Clostridium* cluster XIV and other butyrate producing bacteria in a mechanism
391 known as cross-feeding (Onrust et al., 2015). The prebiotic mode of action of xylanase could
392 also explain the improved diversity and altered microbiota ecology in the large intestine of pigs
393 fed corn-based feed ingredients supplemented with xylanase (Zhang et al., 2017; 2018).
394 Furthermore, there are increasing reports of positive impact of xylanase supplementation on the
395 markers of improved gastrointestinal health in swine with reduction in finishing pig mortality
396 (Petry and Patience, 2020), probably resulting from modulation of microbial populations in the
397 gut.

398 These changes in bacterial populations and metabolic pathways may also explain the
399 linear decrease in faecal ammonia emissions seen with increasing xylanase supplementation.
400 Supplementation with 45,000 and 90,000 U/kg xylanase, led to large, near-significant reductions
401 of respectively 13.5 and 15.9 % in ammonia excretion, while supplementation with 135,000 U/kg
402 xylanase significantly reduced faecal ammonia emissions (0.490 mg/g control vs 0.361 mg/g
403 135,000 U/kg xylanase, -26.3 %, $P<0.05$). By contrast, McAlpine et al. (2012) saw no significant
404 effect when supplementing xylanase only however, when xylanase and protease were fed
405 together, a significant reduction in ammonia emissions was observed.

406 In conclusion, supplementation with the xylanase molecule assessed in this study was
407 able to improve growth and nutrient digestibility at the lowest dose (45,000 U/kg). The
408 beneficial effects of xylanase addition increased with increasing dose, including improvements in
409 FCR, nutrient utilization and faecal ammonia emissions. These improvements are likely driven
410 both by classic mechanisms of viscosity reduction and the reduction of the caging effects of
411 fibre, but also through driving changes in the intestinal microbial population of the piglets, as
412 evidenced by changes in VFA profile between treatments. Further work should focus on
413 elucidating the complementarity between these different modes of action.

Acknowledgements

This study was supported by Kemin Industries (Asia), Asia Pacific, Singapore (grant no. KIA1121-19). The funder, Kemin Industries (Asia) provided support in the form of salaries for authors ALW, VVH, DW, BLV, IS but did not have any additional role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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623 **Table 1.** Ingredient and nutrient composition (% as fed) of the basal diets given in the
624 prestarter (d0-14) and starter (d15-35) phases

	Prestarter	Starter
Raw materials, %		
Corn, 8% CP	34.45	34.29
Wheat	20.00	30.00
Broken rice	10.00	5.50
Full-fat soybean meal	8.00	3.50
Sweet whey	8.00	2.70
Soybean meal, 48% CP	5.00	13.10
Soy protein concentrate	5.00	1.00
Wheat bran	5.00	5.00
Rice bran oil	1.00	1.50
Limestone	0.61	0.92
Acid Lac[1]	0.50	-
Monocalcium phosphate	0.30	0.45
Sodium chloride	0.40	0.35
L-lysine HCl	0.50	0.52
DL-methionine	0.24	0.21
L-threonine	0.20	0.20
L-valine	0.15	0.12
L-tryptophan	0.08	0.08
L-isoleucine	0.06	0.05
Phytase[2]	0.01	0.01
Vitamin-mineral premix[3]	0.50	0.50
Total	100.00	100.00
Nutrient composition, %		
Metabolisable energy, kcal/kg	3,296.21	3,244.49
Digestible energy, kcal/kg	3,446.08	3,467.15
Net energy, kcal/kg	2,496.60	2,482.61
Crude protein	17.35	17.03
Ca	0.61	0.71
Available P	0.40	0.39
Digestible Lys	1.11	1.09
Digestible Met + Cys	0.67	0.64
Digestible Thr	0.66	0.64
Digestible Ile	0.61	0.58
Ash	4.75	4.77
Crude fibre	2.59	2.67

625 [1] Acidifier (Anhui Cofco Biochemical & Galactic Lactic Acid Co., Ltd, China)

626 [2] Provided 500 FTU/kg product (Quantum Blue, AB Vista, Marlborough, UK)

627 [3] Contains vitamin A, 4,000,000 (4,000,000) IU; vitamin D 600,000 (360,000) IU; vitamin E, 8 (2.5) g; vitamin K3,
628 0.40 (0.40) g; vitamin B1, 0.30 (0.25) g; vitamin B2, 1 (0.70) g; vitamin B6, 0.50 (0.40) g; vitamin B12, 4 (4) mg;
629 niacin, 4 (3.6) g; choline chloride, 30 (19.77) g; calcium pantothenate, 3 (1.8) g; biotin, 10 (14) mg; folic acid, 0.10

(0.10) g; cobalt, 0.20 (0.09) g; copper, 40 (36) g; ferrous 36 (23) g; manganese, 16 (9.6) g; zinc, 20 (20) g; iodine, 0.20 (0.10) g; selenium, 0.02 (0.02) g; ethoxyquin, 10 (0.267) g; and silicon dioxide, 2 (10) g for prestarter (starter diet).

635 **Table 2.** Xylanase recovery in pelleted weaned piglet feeds compared to expected levels

	Pre Starter, U/kg		Starter, U/kg	
Expected U/kg	Cold Mash	Pellet	Cold Mash	Pellet
0	n.d.[1]	n.d.	n.d.	n.d.
45000	36376	26369	41343	67068
90000	88844	63204	86437	100459
135000	136607	104139	173287	189339

636 [1] not detected

637

638 **Table 3.** Effect of increasing levels of xylanase addition on piglet growth performance

		Xylanase, U/kg					P-value		
		0	45,000	90,000	135,000	SEM	Trt	Lin	Quad
d0-14	BW d0, kg	7.46	7.48	7.49	7.46	0.237	0.9997	0.9890	0.9227
	BW d14, kg	10.68	10.92	11.27	11.46	0.314	0.3114	0.0601	0.9389
	ADG, g	229.52^{b}	245.69^{ab}	269.52^{ab}	285.69^{a}	10.979	0.0051	0.0004	1.000
	ADFI, g	369.22^{ab}	384.11^{a}	366.22^{ab}	353.11^{b}	5.525	0.0044	0.0129	0.0182
	G:F	0.624^{b}	0.640^{b}	0.738^{ab}	0.810^{a}	0.032	0.0007	<0.0001	0.3833
d15-35	BW d35, kg	19.14^{b}	20.03^{b}	20.26^{ab}	21.67^{a}	0.381	0.0006	<0.0001	0.5053
	ADG, g	402.98^{b}	428.43^{b}	433.82^{b}	486.02^{a}	10.908	<.0001	<0.0001	0.2498
	ADFI, g	756.78^{ab}	762.67^{a}	746.56^{bc}	743.00^{c}	3.357	0.0007	0.0010	0.1949
	G:F	0.532^{b}	0.568^{b}	0.572^{b}	0.653^{a}	0.014	<.0001	<0.0001	0.1300
d0-35	ADG, g	333.60^{c}	358.57^{bc}	364.86^{b}	405.89^{a}	7.525	<.0001	<0.0001	0.3047
	ADFI, g	601.67^{ab}	611.22^{a}	594.44^{bc}	587.00^{c}	3.079	<.0001	0.0003	0.0156
	G:F	0.557^{c}	0.588^{bc}	0.612^{b}	0.692^{a}	0.013	<.0001	<0.0001	0.0679
	Days diarrhoea per pen	15.22^{a}	9.78^{ab}	7.89^{b}	5.89^{b}	1.628	0.0021	--	--

639 $^{a-c}$ Means in a row without a common superscript are significantly different (P<0.05)

640

641

642 **Table 4.** Effect of increasing levels of xylanase addition on apparent total tract digestibility of

643 nutrients, energy, and nitrogen retention in weaned piglets[1]

		Xylanase, U/kg				SEM	P-value		
		0	45,000	90,000	135,000		Trt	Lin	Quad
Nutrient Digestibility, %	Dry Matter	91.49[c]	93.61[b]	93.37[b]	95.19[a]	0.240	<.0001	0.0002	0.7098
	Crude Protein	87.29[c]	90.61[b]	90.68[b]	93.33[a]	0.505	0.0002	0.0002	0.6291
	Ether Extract	82.60[b]	88.90[a]	87.24[ab]	89.45[a]	1.034	0.0060	0.0106	0.1534
	Crude Fibre	67.11	70.94	72.22	74.26	1.625	0.0721	0.0095	0.5781
	NDF	66.15[b]	66.85[ab]	67.21[a]	67.49[a]	0.220	0.0128	0.0011	0.3436
	ADF	65.90[c]	67.49[bc]	70.36[ab]	70.81[a]	0.672	0.0024	0.0003	0.4306
	Gross Energy	54.96[b]	68.40[a]	74.11[a]	78.76[a]	2.869	0.0020	0.0001	0.1448
	Starch	51.34[b]	62.51[a]	64.82[a]	70.74[a]	2.301	0.0022	0.0003	0.2952
N Balance	Crude Protein Intake	75.97[b]	76.57[a]	75.57[c]	74.5[d]	8.60E-09	<.0001	0.1256	0.6005
	N Intake	12.15	12.25	12.09	11.92	--	--	--	--
	N-faeces	1.54[a]	1.14[b]	1.12[b]	0.81[c]	0.061	0.0002	0.0001	0.5668
	N-urine	5.63	5.33	5.57	4.63	0.346	0.2329	0.1107	0.3851
	N-retention	4.98[b]	5.78[ab]	5.23[ab]	6.66[a]	0.363	0.0470	0.0314	0.4786
	N-digestibility, %	40.96[b]	47.15[ab]	43.90[ab]	55.04[a]	3.007	0.0497	0.0224	0.4784

644 [a-c] Means in a row without a common superscript are significantly different (P<0.05)

645 [1]A total of 12 crossbred pigs with an average BW of 10.22 kg were used in the digestibility trial

646

647 **Table 5.** Effect of increasing levels of xylanase addition on duodenal and jejunal morphology of

648 weaned piglets[1]

	Xylanase, U/kg					P-value		
	0	45,000	90,000	135,000	SEM	Trt	Lin	Quad
Duodenum								
Villus height, μm	386.36	422.44	430.42	452.39	23.201	0.2669	0.0480	0.7598
Crypt depth, μm	313.23	322.93	327.90	327.48	18.849	0.9408	0.5685	0.7864
VH:CD	1.24	1.32	1.36	1.43	0.113	0.6878	0.2268	0.9745
Jejunum								
Villus height, μm	372.87[b]	432.53[ab]	465.80[a]	491.28[a]	15.547	0.0001	<0.0001	0.0828
Crypt depth, μm	297.79	332.46	334.63	332.40	15.921	0.3177	0.0870	0.2496
VH:CD	1.26	1.34	1.42	1.49	0.090	0.3224	0.0618	0.9745

649 [a-c] Means in a row without a common superscript are significantly different (P<0.05)

650 [1]Values are expressed as means of seven weaned pigs represented from each treatment (N = 28)

651 **Table 6.** Effect of increasing levels of xylanase addition on volatile fatty acid concentrations in

652 the faeces of weaned piglets[1]

	Xylanase, U/kg					P-value		
	0	45,000	90,000	135,000	SEM	Trt	Lin	Quad
Volatile Fatty Acids, mmol/L								
Acetate	17.79	16.89	19.55	18.56	2.629	0.9055	0.6715	0.9855
Propionate	8.55[b]	9.16[b]	14.40[a]	10.59[b]	0.933	0.0011	0.0364	0.0284
Butyrate	3.98	5.49	5.29	6.14	0.583	0.0971	0.0161	0.5785
Isobutyrate	1.79	2.29	1.00	2.13	0.328	0.0511	0.8753	0.4118
Isovalerate	2.64	2.03	2.01	1.97	0.456	0.6919	0.3167	0.5316
Total VFA	34.75	35.86	42.25	39.39	2.664	0.2102	0.1187	0.4689

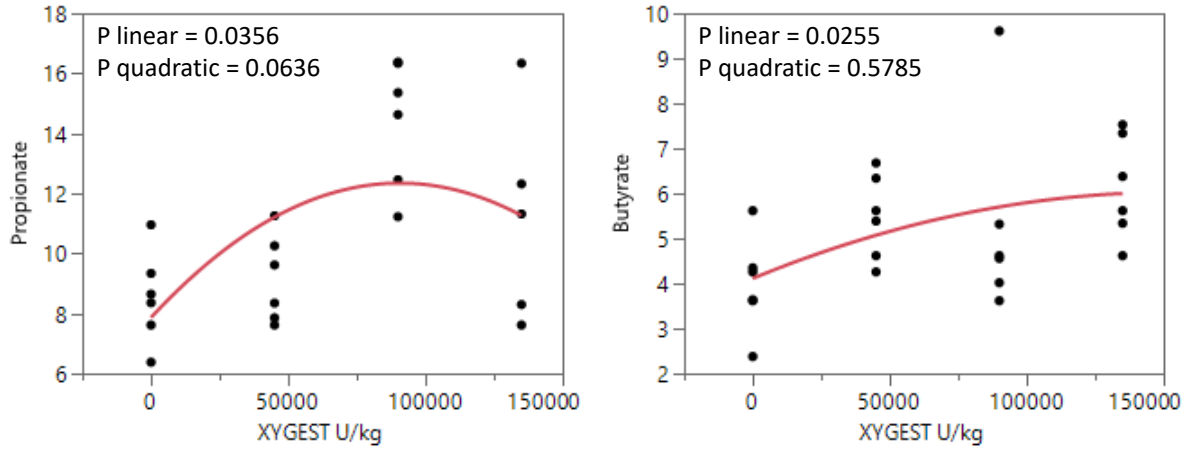
653 [a,b] Means in a row without a common superscript are significantly different ($P<0.05$)

654 [1]Values are expressed as means of seven weaned pigs represented from each treatment ($N = 28$)

655

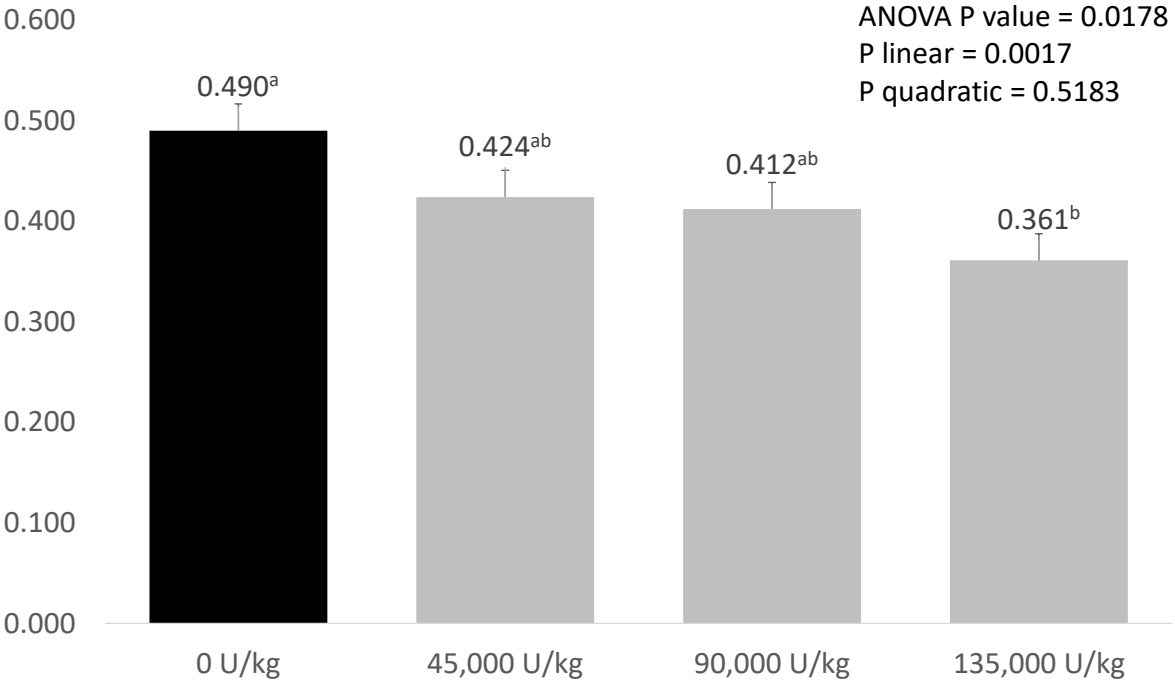
656

Figure 1. Relationships between increasing xylanase dose and propionate (A) and butyrate (B) concentrations (mmol/L) in the faeces of weaned piglets[1]



[1]Values are expressed as means of eight weaned pigs represented from each treatment (N = 32)

Figure 2. Effect of increasing levels of xylanase addition on ammonia emissions (mg/g) from the faeces of weaned piglets[1]



[1]Values are expressed as means of eight weaned pigs represented from each treatment (N = 32)