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Original Research Article

Dietary supplementation of xylanase and protease on growth performance, digesta viscosity, nutrient digestibility, immune and oxidative stress status, and gut health of newly weaned pigs

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ABSTRACT

This study was to investigate the effect of dietary supplementation with xylanase and protease on growth performance, digesta viscosity, apparent ileal digestibility (AID) of nutrients, and gut health in nursery pigs. Forty-eight pigs (24 barrows and 24 gilts at 21 d of age with 7.2 ± 0.4 kg BW) were randomly allotted to 4 dietary treatments (2 × 2 factorial arrangement) in a randomized complete block design and fed in 2 phases (phase 1 for 10 d and phase 2 for 14 d). Factors were xylanase (0 or 45,000 XU/kg) and protease (0 or 300,000 U/kg). Feed intake and BW gain were measured on d 10 and 24. Titanium dioxide (0.25%) was added to all diets as an indigestible external marker from d 20 to 24. On d 24, all pigs were euthanized to obtain jejunal and ileal digesta to measure viscosity and apparent ileal digestibility. The jejunal mucosa was collected to measure immune and oxidative stress status. Jejunal tissues were used to measure morphology and crypt cells proliferation. In phase 2, xylanase increased ($P < 0.05$) the average daily gain (ADG) which was further increased ($P < 0.05$) when combined with protease. Overall, combinational use of xylanase and protease increased ($P < 0.05$) ADG compared with the use of xylanase or protease alone, whereas protease improved ($P < 0.05$) feed efficiency. In jejunum, xylanase reduced ($P < 0.05$) viscosity of digesta, mucosal malondialdehyde (MDA), crypt depth and crypt cells proliferation, and protease increased ($P < 0.05$) villus height, and decreased ($P < 0.05$) crypt depth and crypt cells proliferation. Collectively, xylanase improved growth performance, digesta viscosity, and oxidative stress, whereas protease improved feed efficiency and gut morphology. The combinational use of xylanase and protease enhanced growth performance of newly weaned pigs.

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1. Introduction

The early weaning is a delicate proceeding for pigs since pigs are suddenly exposed to nutritional, immunologic, and psychologic changes. Among these changes, the diet based on vegetables, dryer, less digestible and containing antinutritional factors, replaces the sow milk which is rich in protein, fat and lactose, nutrients highly digestible. This promotes physiologic alteration and consequently affects the performance of newly weaned pigs.

It is well known that corn and soybean meal is the most used feedstuffs in the diet of non-ruminants in the world. However, these ingredients have a significant quantity of antinutritional factors that can interfere in the growth performance of the animals (Berrocoso et al., 2015; Gutierrez et al., 2014; Kim et al., 2010; Taliercio and Kim, 2013). In young pigs, these effects are even more pronounced due to their low digestive capacity to vegetable-based diets (Lindberg, 2014).

The content of non-starch polysaccharides (NSP) in the plant cell wall limits the use of vegetable feedstuffs in the diet of pigs (Kerr and Shurson, 2013). The major NSP in the swine corn-soybean

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diets are arabinoxylan, present in corn (Jaworski et al., 2015), and xyloglucan and xylan in soybean (Karr-Lilienthal et al., 2005). Non-ruminant animals do not produce endogenous enzymes capable to digesting NSP. Once ingested, NSP can increase the digesta viscosity, alter the epithelial morphology of the intestine and reduce the digestibility of nutrients (Lindberg, 2014; Passos et al., 2015).

As well as, glycinin and β-conglycinin, the main storage globulins in soybean (Krishnan et al., 2007) have an allergenic effect that can also reduce the growth performance, affect the immune system and change the morphology of the intestinal epithelium (Krishnan et al., 2009; Wang et al., 2010; Zhao et al., 2010; Taliercio and Kim, 2013). Besides that, these proteins show resistance to hydrolysis by endogenous enzymes due the presence of disulfide bond, especially in young animals (Wang et al., 2011). Due to the immaturity of the digestive system of young animals, the immunoreactivity of these proteins and their partially digested segments are even more pronounced when compared to adult animals (Wang et al., 2010, 2009; Zhao et al., 2008).

Thus, the dietary supplementation with exogenous enzymes, such as xylanase, reduces the NSP content in the digesta and, consequently, enhances the use of nutrients (Kim et al., 2003; Lærke et al., 2015; Pedersen et al., 2012), whereas the protease complements the proteolytic activity, mainly hydrolyzing those proteins that are resistant to digestion by endogenous enzymes (Wang et al., 2011). These enhancements can also reduce the nutrient excretion in the environment (Ferket et al., 2002).

Therefore, it was hypothesized that by eliminating or reducing the effects of antinutritional factors, these enzymes enhance the growth performance of newly weaned pigs through the benefits in the gastrointestinal tract and the improvement in the nutrient digestibility. Thus, this study was conducted to evaluate the effect of the supplementation with xylanase and protease on growth performance, digesta viscosity, oxidative stress, immune status, digestibility of nutrients and gut health of pigs from 21 to 45 d of age.

2. Materials and methods

The protocol used in this study was approved by the Institutional Animal Care and Use Committee of North Carolina State University.

2.1. Animals, experimental design, and diets

A total of 48 newly weaned pigs (24 barrows and 24 gilts), with an initial body weight (BW) of 7.2 ± 0.4 kg, were allotted in a randomized complete block design with sex and body weight at weaning as blocking criteria. Four dietary treatments were arranged based on a 2 × 2 factors. First factor was supplementation of xylanase (0 or 45,000 XU/kg, Xylamax, BRI, Durham, NC) and the second factor was supplementation of protease (0 or 300,000 U/kg, Versazyme, BRI).

The experimental diets were formulated meeting the requirement for nursery pigs suggested by NRC (2012) in 2 phases: phase 1 (10 d) and phase 2 (14 d). The composition of basal diets is shown in Table 1.

Pigs had free access to the feed and water. Titanium dioxide (0.25%) was added to all diets from d 20 to 24 as an indigestible marker to determine the digestibility. The weight of the pigs and feed disappearance were recorded individually on d 10 and 24 to determine BW, average daily gain (ADG), average daily feed intake (ADFI), and gain to feed ratio (G:F).

2.2. Samples collection, and processing

Blood samples were collected from the jugular vein of all pigs at d 21 of the experiment. The blood samples (7 mL) were drawn into vacutainers without anticoagulant (BD, Franklin Lakes, NJ). Sera were obtained after centrifugation (3,000 × *g*, 15 min) at room temperature, and then stored at −80 °C in Eppendorf tubes until the analysis determining the concentration of tumor necrosis factor α (TNF-α), as an indicator of local inflammatory response, protein carbonyl and malondialdehyde (MDA), as an indicator of local oxidative stress status, and immunoglobulin G (IgG), as an indicator of local humoral immune status.

At the end of the d 24 of feeding, all the pigs were euthanized followed by removal of the gastrointestinal tract to samples collection. Digesta from ileum was collected in containers (50 mL), weighted and stored at −20 °C. Before analysis, all samples were lyophilized, finely ground and stored until analyses to measure nutrient content to determine the apparent ileal digestibility (AID). Digesta from proximal jejunum was collected into falcon tubes (50 mL), placed on ice and immediately carried to the lab to measure its viscosity. As well as, approximately 10 mL of digesta from mid-colon (second third of colon) was collected into falcon tubes (15 mL) and placed on ice to measure pH.

Segments mid-jejunum (3 m after the duodenojejunal junction) were collected, rinsed with a 0.9% saline solution and then fixed in 10% buffered formaldehyde to be used for Ki-67 staining, and histological evaluation measuring the ratio of Ki-67 positive cells to total cells in the crypt (%), as an indicator of crypt cells proliferation, villus height, crypt depth, villus width, and villus height to crypt depth ratio.

Table 1
Composition of basal diets.

Item	Phase 1	Phase 2
Ingredient, %		
Corn	32.04	43.72
Soybean meal	23.00	27.00
Blood plasma	3.30	0.00
Corn DDGS	20.00	20.00
Whey permeate[1]	12.00	2.00
Poultry meal	5.00	3.00
L-Lys·HCl	0.35	0.35
DL-Met	0.08	0.05
L-Thr	0.03	0.03
Limestone	1.30	1.10
Vitamin premix[2]	0.03	0.03
Mineral premix[3]	0.15	0.15
Salt	0.22	0.22
Zinc oxide	0.25	0.25
Dicalcium phosphate	0.15	0.50
Poultry fat	2.00	1.50
Supplement (corn or enzyme)	0.10	0.10
Calculated composition		
DM, %	90.80	89.70
ME, MJ/kg	14.27	14.10
SID Lys, %	1.35	1.24
SID Met + Cys, %	0.74	0.68
SID Trp, %	0.25	0.22
SID Thr, %	0.79	0.73
Ca, %	0.80	0.71
STTD P, %	0.41	0.34
Total P, %	0.63	0.59

DDGS = distillers dried grains with solubles; SID = standardized ileal digestible; STTD = standardized total tract digestible.

[1] DairyLac80 (International Ingredient Corporation) was used as a source of whey permeate containing (79.3 ± 0.8)% lactose.

[2] The vitamin premix provided the following per kilogram of complete diet: 6,613.8 IU of vitamin A as vitamin A acetate, 992.0 IU of vitamin D_3, 19.8 IU of vitamin E, 2.64 mg of vitamin K as menadione sodium bisulfate, 0.03 mg of vitamin B_{12}, 4.63 mg of riboflavin, 18.52 mg of D-pantothenic acid as calcium pantothenate, 24.96 mg of niacin, and 0.07 mg of biotin.

[3] The trace mineral premix provided the following per kilogram of complete diet: 4.0 mg of Mn as manganous oxide, 165 mg of Fe as ferrous sulfate, 165 mg of Zn as zinc sulfate, 16.5 mg of Cu as copper sulfate, 0.30 mg of I as ethylenediamine dihydroiodide, and 0.30 mg of Se as sodium selenite.

Mucosal samples from mid-jejunum were scraped, placed in Eppendorf tubes (2 mL) and stored at −80 °C (after snap-freezing in liquid nitrogen, immediately after collection). To measure the concentration of total protein, TNF-α, protein carbonyl, MDA, and immunoglobulin A and G (IgA and IgG), each sample was weighed (500 mg), suspended in 1 mL of phosphate-buffered saline (PBS) and homogenized on ice using a tissue homogenizer (Tissuemiser; Thermo Fisher Scientific Inc. Waltham, MA USA). After homogenization, samples were centrifuged (14,000 × g for 3 min) and the supernatant was divided in 5 aliquots and stored at −80 °C until analyses.

2.3. Viscosity and pH

Samples of jejunum digesta from 50 mL tubes were divided into 2 falcon tubes (15 mL) and centrifuged at 1,000 × g at 4 °C for 10 min to obtain the liquid phase. Then, the liquid phase was removed to an Eppendorf tube (2 mL) to centrifuge at 10,000 × g at 4 °C for 10 min. The supernatant obtained was transferred to another Eppendorf tube (1.5 mL) for further measurement. 0.5 mL of digesta supernatant was placed in the viscometer (Brookfield Digital Viscometer, Model DV-II Version 2.0, Brookfield Engineering Laboratories Inc., Stoughton, MA), set at 25 °C. The viscosity measurement was the average between 45.0/s and 22.5/s shear rates, and the viscosity values were recorded as apparent viscosity in millipascal-seconds (mPa s).

The pH was measured immediately after collecting using a pH meter (AB15 Basic, Thermo Fisher Scientific Inc., Waltham, MA), placed directly in the digesta.

2.4. Measurement of apparent ileal digestibility

The concentration of titanium dioxide of all samples (feed and digesta) was measured according to Myers et al. (2004), to determine the AID of nutrients. Gross energy (GE) content was measured in calorimeter bomb (Parr 6200 − Parr instrument company, Moline, Illinois). The nitrogen content was measured using a TruSpec N Nitrogen Determinator (LECO Corp., St. Joseph, MI) to calculate crude protein (CP, 6.25 × N). The AID of the nutrients was calculated using the equation:

$$AID = \{1 - [(TiO_{2diet}/TiO_{2digesta}) \times (Nutrient_{digesta}/Nutrient_{diet})]\} \times 100,$$

in which TiO_{2diet} and $TiO_{2digesta}$ were the concentrations of titanium dioxide in the diet and in the digesta; $Nutrient_{digesta}$ and $Nutrient_{diet}$ were the concentrations of nutrient in the digesta and in the diet.

2.5. Measurement of oxidative stress and immune status

The concentrations of MDA, protein carbonyl, IgG, IgA, and TNF-α were measured by ELISA methods using commercially available ELISA kits according to instructions of the manufacturers. The absorbance was read using an ELISA plate reader (Synergy HT, BioTek Instruments, Winooski, VT) and software (Gen5 Data Analysis Software, BioTek Instruments). The concentration was calculated based on the standard curve created from concentration and absorbance of the respective standard.

The concentration of mucosal total protein was determined using the kit Pierce BCA Protein Assay (23225#, Thermo Fisher Scientific Inc. Rockford, IL). The samples were diluted (1:80) in PBS to reach the working range for 20 to 2,000 μg/mL. The absorbance was measured at 562 nm.

Malondialdehyde concentrations in the mucosa and serum were measured using the Thiobarbituric Acid Reactive Substance Assay Kit (STA-330, Cell Biolabs, San Diego, CA). The MDA standard was used in a working range for 0 to 125 μmol/L. Aliquots of 100 μL of each standard and sample were pipetted into a microcentrifuge tubes, followed by addition of 100 μL of SDS lysis and incubation for 5 min at room temperature. Then, 250 μL of thiobarbituric acid reagent was added into each sample and standard, and incubated at 95 °C for 60 min in water bath. All tubes were cooled to room temperature in an ice bath for 5 min before being centrifuged (10,000 × g for 15 min). Aliquots of 300 μL of supernatant were transferred to another tube with 300 μL of butanol and vortex for 1 min and centrifuge for 5 min at 10,000 × g. Aliquots of 200 μL of samples and standards were transferred to a 96 well microplate. The absorbance was measured at 532 nm, and the MDA concentration in the serum and mucosa was expressed as μmol/mL and μmol/mg protein, respectively.

The concentration of protein carbonyl was measured using the ELISA kit (STA-310, Cell Biolabs, San Diego, CA). Before measurement, serum and mucosa samples were diluted in PBS to reach the protein concentration at 10 μg/mL. The standard was prepared by mixing the oxidized BSA and reduced BSA to reach the working range for 0.375 to 7.5 nmol/mg protein. Aliquots of 100 μL of standard or sample were added into the 96-well Protein Binding Plate. The content of protein carbonyl in the samples and standard were derivated to dinitrophenyl (DNP) hydrazine and probed with an anti-DNP antibody, followed by an incubation with a horseradish peroxidase (HRP) conjugated with the secondary antibody. The absorbance was measured at 450 nm, and the concentration of protein carbonyl expressed as nmol/mg protein for both serum and mucosa.

The concentrations of TNF-α in mucosa and serum were measured using the Porcine Immunoassay ELISA Kit (PTA00; R&D System Inc. Minneapolis, MN), as described by Chaytor et al. (2011). The standard was used in a working range for 0 to 1,500 pg/mL. Fifty microliter of sample, standard and control was added to microplate wells coated with capture antibody in conjunction with biotinylated antibody reagent. Detection occurred using HRP, TMB (3,3′, 5,5″-tetramethylbenzidine) substrate, and a stop solution of 2 mol/L H_2SO_4. Absorbance was read at 450 nm and 550 nm and the TNF-α concentration in serum and mucosa was expressed as pg/mL and pg/mg protein, respectively.

Analysis to detect the concentrations of IgG and IgA was previously described by Chaytor et al. (2011). The serum and mucosa samples were diluted with PBS to 1:1,600 and 1:1,000, respectively, to analyze IgG using the kit ELISA Pig IgG (E101-104, Bethyl Laboratories, Inc, Montgomery, TX). The standard was used in a working range for 0 to 500 ng/mL. The absorbance was measured at 450 nm and the concentration of IgG in the serum and mucosa was expressed as μg/mL and mg/μg of protein, respectively. The mucosal samples were diluted to 1:400 with PBS to analyze IgA using the kit ELISA Pig IgA (E101-102, Bethyl Laboratories, Inc). The standard was used in a working range for 0 to 1,000 ng/mL. The absorbance was measured at 450 nm and the concentration of IgA in the mucosa was expressed as μg/mg of protein.

2.6. Histological evaluation

Two sections of jejunum per pig fixed in 10% buffered formalin were transferred to a 70% ethanol solution and sent to North Carolina State University Histology Laboratory (College of Veterinary Medicine, Raleigh, NC) for dehydration, embedment, staining and Ki-67 assay according to their internal standard protocol, for morphological evaluation and Ki-67 immunohistochemistry staining. Villus height, villus width, and crypt depth were measured

using a camera Infinity 2-2 digital CCD attached to a microscope Olympus CX31 (Lumenera Corporation, Ottawa, Canada). Lengths of 15 well-oriented intact villi and their associated crypts were measured in each slide. The villi length was measured from the top of the villi to the villi-crypt junction, the villi width measured in the middle of the villi, and the crypt depth was measured from the villi-crypt junction to the bottom of the crypt. Then, the villus height to crypt depth (VH:CD) ratio was calculated. Images of 15 intact crypts from each slide were cropped and the Image JS software was used for calculating the ratio of Ki-67 positive cells to total cells in the crypt (%). All analyses of the intestinal morphology were executed by the same person.

2.7. Statistical analyses

A randomized complete block design was used. Main effects were 2 factors (xylanase, protease) and their interaction which were handled fixed effects. Sex and initial BW were blocks which were handled random effects. Data were analyzed using the MIXED procedure in SAS version 9.4 (SAS Inc., Cary, NC, USA). The experimental unit was the pig as pigs were individually housed and fed. The means were separated using the LSMEANS statement in SAS. When an interaction between 2 factors was significant or tended to be significant, a pairwise comparison was made using the PDIFF option in SAS. Statistical differences were considered significant with $P < 0.05$. Tendency was considered when $0.05 \leq P < 0.10$.

3. Results

3.1. Growth performance

The using of either xylanase or protease did not affect the BW of pigs at d 10 after weaning (Table 2). However, at d 24 after weaning, the BW was increased ($P < 0.05$) when xylanase and protease were used together, whereas it was not affected when each enzyme was used individually. In phase 1, the individual or combined use of xylanase and protease did not affect the ADG of pigs. Meanwhile, in phase 2, xylanase increased ($P < 0.05$) the ADG, however, the benefit of xylanase on the ADG was further enhanced ($P < 0.05$) when protease was used together. Overall, combinational use of xylanase and protease increased ($P < 0.05$) the ADG compared with the use of xylanase or protease alone.

The ADFI was not affected by the enzymatic supplementation during phase 1. However, during phase 2, the ADFI tended to be increased ($P = 0.063$) by the xylanase supplementation. Whereas, during the entire period, ADFI tended to be increased ($P = 0.099$) by the combinational use of xylanase and protease. There was no significant effect on G:F by dietary supplementation of either enzymes when each phase was analyzed separately, but in overall, the G:F was improved ($P < 0.05$) by protease supplementation.

3.2. Apparent ileal digestibility and digesta characteristics

The AID of dry matter (DM), GE, and CP was not affected using either enzymes (Table 3). The use of xylanase in the diet of pigs reduced the ileal digesta viscosity ($P < 0.05$), but the pH of colon digesta was not affect by the use of either enzymes.

3.3. Immune and oxidative status

Serum and mucosal concentrations of TNF-α, IgG and IgA were not affected by the use of either enzymes, individually or combined (Table 4). However, in the mucosa of jejunum, the use of xylanase reduced ($P < 0.05$) the concentration of MDA and tended to decrease ($P = 0.066$) the concentration of protein carbonyl. The using of protease in the diet of newly weaned pigs did not affect the parameters of immune and oxidative status measured in this study.

3.4. Histology and immunohistochemistry

The using of protease increased ($P < 0.05$) the villi length in jejunum, whereas the using of protease tended to increase ($P = 0.09$) it (Table 5). The individual using of xylanase and protease reduced ($P < 0.05$) the crypt depth, and crypt cells proliferation, and increased the VH:CD ratio ($P < 0.05$). The villi width was not affected by both factors. There was no interaction between 2 factors.

4. Discussion

The use of exogenous enzymes in the diet of pigs is an important tool to reduce antinutritional factors, improve digestion and nutrient absorption efficiency, and consequently enhance the growth performance (Adeola and Cowieson, 2011; Kiarie et al.,

Table 2
Performance of pigs fed diets with xylanase (Xyl) and protease (Pro) supplementation.

Item	Treatment				SEM	*P*-value		
	Xyl−		Xyl+					
	Pro−	Pro+	Pro−	Pro+		Xyl	Pro	Xyl + Pro
Body weight, kg								
Initial	7.2	7.2	7.2	7.2	0.4	0.742	0.951	0.789
d 10	10.7	10.6	10.5	11.0	0.5	0.726	0.442	0.155
d 24	18.1[ab]	17.7[b]	17.8[b]	19.3[a]	0.7	0.142	0.243	0.046
ADG, kg/d								
d 0 to 10	0.354	0.335	0.321	0.380	0.025	0.799	0.416	0.121
d 10 to 24	0.526[a]	0.510[a]	0.528[a]	0.591[b]	0.022	0.032	0.212	0.041
d 0 to 24	0.454[ab]	0.437[b]	0.442[b]	0.503[a]	0.020	0.149	0.222	0.036
ADFI, kg/d								
d 0 to 10	0.414	0.390	0.391	0.445	0.028	0.536	0.566	0.132
d 10 to 24	0.713	0.674	0.723	0.773	0.033	0.063	0.842	0.121
d 0 to 24	0.589[ab]	0.556[b]	0.585[ab]	0.636[a]	0.029	0.135	0.711	0.099
G:F								
d 0 to 10	0.850	0.855	0.814	0.869	0.031	0.727	0.343	0.424
d 10 to 24	0.740	0.760	0.733	0.771	0.018	0.897	0.118	0.615
d 0 to 24	0.773	0.787	0.756	0.799	0.014	0.830	0.035	0.279

[a, b] Within a row, means without a common superscript letter differ ($P < 0.05$).

Table 3
Apparent ileal digestibility of GE, CP and DM of diets and characteristics of digesta with xylanase (Xyl) and protease (Pro) supplementation on DM basis.

Item	Treatment				SEM	P-value		
	Xyl−		Xyl+					
	Pro−	Pro+	Pro−	Pro+		Xyl	Pro	Xyl + Pro
AID GE, %	67.2	66.1	66.7	68.8	2.9	0.705	0.855	0.586
AID CP, %	78.5	79.4	78.7	79.6	1.6	0.888	0.557	0.982
AID DM, %	67.1	61.1	64.4	60.1	3.2	0.635	0.138	0.720
pH digesta	6.27	6.35	6.30	6.45	0.10	0.529	0.258	0.725
Viscosity of digesta, mPa·s	2.67	2.71	2.48	2.24	0.14	0.016	0.452	0.307

GE = gross energy; CP = crude protein; DM = dry matter.

Table 4
Oxidative stress and immune parameters of pigs fed diets with xylanase (Xyl) and protease (Pro) supplementation.

Item	Treatment				SEM	P-value		
	Xyl−		Xyl+					
	Pro−	Pro+	Pro−	Pro+		Xyl	Pro	Xyl + Pro
Protein carbonyl								
Mucosa, nmol/mg of protein	1.15	1.07	0.96	0.79	0.12	0.066	0.322	0.724
Serum, nmol/mg of protein	0.62	0.58	0.55	0.51	0.09	0.443	0.647	0.991
MDA								
Mucosa, μmol/mg of protein	1.21	1.07	0.98	0.92	0.11	0.022	0.212	0.500
Serum, μmol/mL	23.12	20.85	23.42	18.46	3.91	0.763	0.301	0.699
IgG								
Mucosa, μg/mg of protein	3.55	3.48	3.72	3.44	0.42	0.846	0.597	0.755
Serum, μg/mL	5.34	5.17	4.76	4.82	0.49	0.288	0.895	0.791
IgA								
Mucosa, μg/mg of protein	1.12	0.82	1.16	1.10	0.13	0.187	0.143	0.315
TNF-α								
Mucosa, pg/mg of protein	1.17	0.93	0.94	1.00	0.19	0.664	0.625	0.432
Serum, pg/mL	126.70	112.64	121.74	115.40	7.22	0.881	0.171	0.600

MDA = malondialdehyde; Ig = immunoglobulin; TNF-α = tumor necrosis factor α.

Table 5
Jejunum morphology and the ratio of Ki-67 positive cells to total cells in the crypt of pigs fed diets with xylanase (Xyl) and protease (Pro) supplementation.

Item	Treatment				SEM	P-value		
	Xyl−		Xyl+					
	Pro−	Pro+	Pro−	Pro+		Xyl	Pro	Xyl + Pro
Villus height, μm	422.1	474.1	456.3	512.5	21.4	0.092	0.014	0.919
Villus width, μm	97.1	98.1	101.6	97.0	3.9	0.649	0.624	0.439
Crypt depth, μm	238	202	220	176	9	0.019	<0.001	0.695
VH:CD ratio[1]	1.77	2.38	2.12	2.94	0.12	<0.001	<0.001	0.346
Ki-67 positive, %[2]	23.3	18.3	19.7	15.5	1.4	0.011	<0.001	0.739

[1] Villus height to crypt depth ratio.
[2] Ratio of Ki-67 positive cells to total cells in the crypt.

2013). However, the data related to the use of xylanase and protease shown in the literature is not consistent yet. Apparently, this is due the interactions between the age of the animals, diet composition and the way in which enzymes are used, individually or combined (Adeola and Cowieson, 2011; Kerr and Shurson, 2013; Kiarie et al., 2013; Lindberg, 2014).

This study showed that the final BW enhanced only when xylanase and protease were used together. This effect was due the ADG has similar effect in second phase and in overall. The improvement in the ADG can be partially explained by the tendency of enhancement in the ADFI in the phase 2 using xylanase and in overall when both enzymes were used together, as well as by the higher feed efficiency promoted by the use of protease in overall.

One of the factors that influence the feed intake are the physicochemical properties of the NSP, as water solubility and fermentability and their interactions (Kerr and Shurson, 2013). In corn-soybean diets, the major fraction of NSP is insoluble (Gutierrez et al., 2014; Jaworski et al., 2015; Ngoc et al., 2012). The insoluble NSP acts preventing the digestive enzymatic activity on nutrients inside of the plant cell wall (Nortey et al., 2007; O'Neill et al., 2012; Owusu-Asiedu et al., 2010; Tervilä-Wilo et al., 1996) and improving the digesta passage rate and the chyme bulk (Jha and Berrocoso, 2015; Nortey et al., 2007). On the other hand, soluble NSP have high water holding capacity and viscosity which can improve the digesta bulk (Berrocoso et al., 2015; Jha and Berrocoso, 2015; McDonald et al., 2001). It is important to emphasize that there is a crescent availability and use of coproducts, such as dried distillers grains and soluble (DDGS) to feed, and the NSP content in this ingredients is higher than in the respectively unprocessed grain (Choct, 2015; Gutierrez et al., 2014; Jaworski et al., 2015). The distention in wall of the digestive tract due the improvement of the digesta bulk stimulates the production of cholecystokinin (CCK), an hormone that acts in the control of satiety, as well as stimulates the

pancreatic synthesis and secretion (McDonald et al., 2010). This could improve endogenous losses, and consequently affects the growth performance (Agyekum et al., 2015; Agyekum and Nyachoti, 2017). Thus, the ADG improvement in the phase 2 due the use of xylanase can be explained by the degradation of the arabinoxylan which was proven by the reduction of 13% of the ileal digesta viscosity. The capacity to improve the viscosity has been related to the deleterious effect of NSP in the intestinal lumen (Choct et al., 2004; McDonald et al., 2001; Wellock et al., 2008). Besides that, the partial hydrolysis of the NSP results in oligosaccharides that can act as prebiotics, as well as releasing encapsulated nutrients from the cell (Agyekum et al., 2015; Montagne et al., 2003; Pedersen et al., 2012), what can also improve the growth performance (Kiarie et al., 2016, 2013).

Another factor is the content of glycinin and β-conglycinin in the corn-soybean diet of pigs (Zhao et al., 2008). Nishi et al. (2003) demonstrated, in rats, that β-conglycinin binds some components of the gastrointestinal cell wall and stimulates the synthesis of CCK. Thus, the protease supplementation, capable of hydrolyzing proteins with disulfide bonds and reducing their effects on the intestinal mucosa, may increase feed intake (Zuo et al., 2015). However, the effect of protease used in the current study on the ADFI tended to be higher when combined with xylanase, due the reduction of viscosity, since, according to Adeola e Cowieson (2011), the higher viscosity reduces the access of enzymes to substrates.

The improvement of feed efficiency with the using of protease in this study indicates a reduction of the maintenance nutritional requirements due the degradation of glycinin and β-conglycinin. Beyond stimulating the CCK secretion, the presence of that globulins in the diet can activate the immune system and it causes damage on enterocytes, reduces villi length, improves crypts depth and the proliferation of enterocyte (Jun et al., 2009; Pluske et al., 1997; Zhang et al., 2013; Zhao et al., 2010). This induces the organism to mobilize nutrients to repair the intestinal epithelium, to immune function and to the cell proliferation (Xu, 2014).

Even with enhanced growth performance observed in this study, the dietary supplementation with xylanase and protease did not affect the AID of DM, CP or GE. Although many studies have reported that the use of xylanase (Cadogan and Choct, 2015; Passos et al., 2015; Yin et al., 2001) and protease (Chen et al., 2017; Wang et al., 2011; Yin et al., 2001; Zuo et al., 2015) in the swine diet improves the nutrient digestibility. This can be due the high ratio between insoluble and soluble NSP in corn-soybean meal diets, since, according to Urriola e Stein (2010), insoluble NSP interfere less in the digestibility than soluble NSP. Besides that, the short chain fat acid (SCFA) produced by the fermentation of carbohydrates are not totally counted in the analysis of AID, although they contribute to the growth performance (Owusu-Asiedu et al., 2010, 2006). The major part of the NSP fermentation occurs in the cecum-colon portion (Montagne et al., 2003; Serena et al., 2008), mainly in newly weaned pigs (Knudsen et al., 2012), because they do not have the digestive tract totally developed and have high digesta passage rate (Cadogan and Choct, 2015; Lindberg, 2014).

The dietary supplementation with xylanase and protease did not affect the immune status of the pigs, despite the reduction in the viscosity which is correlated to the decrease in the pathogenic microorganism proliferation in the small intestine (Agyekum et al., 2015; Agyekum and Nyachoti, 2017; Kim et al., 2012; McDonald et al., 2001; Montagne et al., 2003). Probably, this is due the high ratio between insoluble and soluble NSP, as well as the high digesta passage rate in pigs, that reduces the proliferation of microorganism in the small intestine (Choct, 1997; Molist et al., 2014; Montagne et al., 2003).

However, the physicochemical properties of the digesta, due the NSP and low digestibility proteins content (Adeola and Cowieson, 2011), can act directly on molecules in the cell wall of the intestine (Ruemmele et al., 2002). This can explain the reduction of the oxidative stress due the supplementation with xylanase. The MDA and the protein carbonyl are the final product of the oxidation of lipids and proteins, respectively (Kwiecien et al., 2014) that have been used as indicators of oxidative stress (Shacter, 2000).

Products of the oxidative stress can affect important compounds in the cell wall, such as lipids and proteins, causing the cell destruction and, consequently, reduction of villi length (Assimakopoulos et al., 2011; 2007; Sido et al., 2017). According to Pluske et al. (1997), the villi reduction caused by the increasing of cell losses is associated to the improvement of the crypt cells proliferation and, consequently, the crypt depth. Thus, the reduction of the oxidative stress found in this study due the xylanase supplementation can explain the enhancement in the morphology in jejunum, such as the tendency to improve villi length and the decrease of crypt depth and cell proliferation. Also, the enterocyte proliferation may have been reduced by the use of xylanase due the decrease in the viscosity, once the lower viscosity results in a lower production of SCFA (Serena et al., 2008). Some fatty acids in the lumen stimulate the enterocyte proliferation (Knudsen et al., 2012).

The protease was more efficient in the intestinal morphology maintenance than the xylanase in this study, once it increases the villi length and reduces the crypt depth and cell proliferation. According to Chen et al. (2011), the content of β-conglycinin on the intestinal cell stimulates the synthesis of mitogen-activated protein kinases 8 (MAPK8), which induces both apoptosis and enterocyte proliferation (Rao, 2001). This was confirmed by Zhao et al. (2010), who observed an increasing in the proliferation rate, apoptosis and enterocyte migration when adding glycinin and β-conglycinin in the diets of pigs.

5. Conclusion

Supplementation of xylanase (45,000 XU/kg) enhanced growth performance and gut morphology, reduced digesta viscosity, and reduced intestinal oxidative stress. Supplementation of protease (300,000 U/kg) enhanced feed efficiency and the gut morphology. The combined use of xylanase and protease enhanced the growth performance of newly weaned pigs.

Conflicts of interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

References

Adeola O, Cowieson AJ. Board-invited review: opportunities and challenges in using exogenous enzymes to improve nonruminant animal production. J Anim Sci 2011;89:3189–218. https://doi.org/10.2527/jas.2010-3715.

Agyekum AK, Nyachoti CM. Nutritional and metabolic consequences of feeding high-fiber diets to swine: a review. Engineering 2017;3:716–25. https://doi.org/10.1016/J.ENG.2017.03.010.

Agyekum AK, Sands JS, Regassa A, Kiarie E, Weihrauch D, Kim WK, Nyachoti CM. Effect of supplementing a fibrous diet with a xylanase and glucanase blend on growth performance, intestinal glucose uptake, and transport-associated gene expression in growing pigs. J Anim Sci 2015;93:3483–93. https://doi.org/10.2527/jas.2015-9027.

Assimakopoulos SF, Scopa CD, Vagianos CE. Pathophysiology of increased intestinal permeability in obstructive jaundice. World J Gastroenterol 2007;13:6458–64. https://doi.org/10.3748/wjg.v13.i48.6458.

Assimakopoulos SF, Tsamandas AC, Louvros E, Vagianos CE, Nikolopoulou VN, Thomopoulos KC, et al. Intestinal epithelial cell proliferation, apoptosis and expression of tight junction proteins in patients with obstructive jaundice. Eur J Clin Investig 2011;41:117–25. https://doi.org/10.1111/j.1365-2362.2010.02379.x.

Berrocoso JD, Menoyo D, Guzmán P, Saldaña B, Cámara L, Mateos GG. Effects of fiber inclusion on growth performance and nutrient digestibility of piglets reared under optimal or poor hygienic conditions. J Anim Sci 2015;93:3919–31. https://doi.org/10.2527/jas2015-9137.

Cadogan DJ, Choct M. Pattern of non-starch polysaccharide digestion along the gut of the pig: contribution to available energy. Anim Nutr 2015;1:160–5. https://doi.org/10.1016/j.aninu.2015.08.011.

Chaytor AC, See MT, Hansen JA, de Souza ALP, Middleton TF, Kim SW. Effects of chronic exposure of diets with reduced concentrations of aflatoxin and deoxynivalenol on growth and immune status of pigs. J Anim Sci 2011;89:124–35. https://doi.org/10.2527/jas.2010-3005.

Chen F, Hao Y, Piao XS, Ma X, Wu GY, Qiao SY, Li DF, Wang JJ. Soybean-derived β-conglycinin affects proteome expression in pig intestinal cells *in vivo* and *in vitro*. J Anim Sci 2011;89:743–53. https://doi.org/10.2527/jas.2010-3146.

Chen H, Zhang S, Park I, Kim SW. Impacts of energy feeds and supplemental protease on growth performance, nutrient digestibility, and gut health of pigs from 18 to 45 kg body weight. Anim Nutr 2017;3:359–65. https://doi.org/10.1016/j.aninu.2017.09.005.

Choct M. Feed non-starch polysaccharides for monogastric animals: classification and function. Anim Prod Sci 2015;55:1360–6. https://doi.org/10.1071/AN15276.

Choct M. Feed non-starch polysaccharides: chemical structures and nutritional significance. Feed Milling Int 1997;191:13–26.

Choct M, Kocher A, Waters DLE, Pettersson D, Ross G. A comparison of three xylanases on the nutritive value of two wheats for broiler chickens. Br J Nutr 2004;92:53–61. https://doi.org/10.1079/BJN20041166.

Ferket PR, Van Heugten E, Van Kempen TATG, Angel R. Nutritional strategies to reduce environmental emissions from nonruminants. J Anim Sci 2002;80:168–82. https://doi.org/10.2527/ANIMALSCI2002.80E-SUPPL_2E168X.

Gutierrez NA, Serão NVL, Kerr BJ, Zijlstra RT, Patience JF. Relationships among dietary fiber components and the digestibility of energy, dietary fiber, and amino acids and energy content of nine corn coproducts fed to growing pigs. J Anim Sci 2014;92:4505–17. https://doi.org/10.2527/jas2013-7265.

Jaworski NW, Lærke HN, Knudsen KEB, Stein HH. Carbohydrate composition and in vitro digestibility of dry matter and nonstarch polysaccharides in corn, sorghum, and wheat and coproducts from these grains. J Anim Sci 2015;93:1103–13. https://doi.org/10.2527/jas2014-8147.

Jha R, Berrocoso JD. Review: dietary fiber utilization and its effects on physiological functions and gut health of swine. Animal 2015;9:1441–52. https://doi.org/10.1017/S1751731115000919.

Jun X, Anguo Z, Zhisheng W, Dawei A. Influence of glycinin and Β-conglycinin of soybean on the proliferation and immune function of suckling piglets peripheral blood mononuclear cells in in vitro culture. J Anim Plant Sci 2009;19:115–8.

Karr-Lilienthal LK, Kadzere CT, Grieshop CM, Fahey GC. Chemical and nutritional properties of soybean carbohydrates as related to nonruminants: a review. Livest Prod Sci 2005;97:1–12. https://doi.org/10.1016/j.livprodsci.2005.01.015.

Kerr BJ, Shurson GC. Strategies to improve fiber utilization in swine. J Anim Sci Biotechnol 2013;4:1–12. https://doi.org/10.1186/2049-1891-4-11.

Kiarie E, Romero LF, Nyachoti CM. The role of added feed enzymes in promoting gut health in swine and poultry. Nutr Res Rev 2013;26:71–88. https://doi.org/10.1017/S0954422413000048.

Kiarie E, Walsh MC, Nyachoti CM. Performance, digestive function, and mucosal responses to selected feed additives for pigs. J Anim Sci 2016;94:169–80. https://doi.org/10.2527/jas2015-9835.

Kim JC, Hansen CF, Mullan BP, Pluske JR. Nutrition and pathology of weaner pigs: nutritional strategies to support barrier function in the gastrointestinal tract. Anim Feed Sci Technol 2012;173:3–16. https://doi.org/10.1016/j.anifeedsci.2011.12.022.

Kim SW, Knabe DA, Hong KJ, Easter RA. Use of carbohydrases in corn–soybean meal-based nursery diets. J Anim Sci 2003;81:2496–504. https://doi.org/10.2527/2003.81102496x.

Kim SW, van Heugten E, Ji F, Mateo RD. Fermented soybean meal as a vegetable protein source: I. Effects on growth performance of nursery pigs. J Anim Sci 2010;88:214–24. https://doi.org/10.2527/jas.2009-1993.

Knudsen KEB, Hedemann MS, Lærke HN. The role of carbohydrates in intestinal health of pigs. Anim Feed Sci Technol 2012;173:41–53. https://doi.org/10.1016/j.anifeedsci.2011.12.020.

Krishnan HB, Kim W, Jang S, Kerley MS. All three subunits of soybean β-conglycinin are potential food allergens. J Agric Food Chem 2009;57:938–43. https://doi.org/10.1021/jf802451g.

Krishnan HB, Natarajan SS, Mahmoud AA, Nelson RL. Identification of glycinin and β-conglycinin subunits that contribute to the increased protein content of high-protein soybean lines. J Agric Food Chem 2007;55:1839–45. https://doi.org/10.1021/jf062497n.

Kwiecien S, Jasnos K, Magierowski M, Sliwowski Z, Pajdo R, Brzozowski B, Mach T, Wojcik D, Brzozowski T. Lipid peroxidation, reactive oxygen species and antioxidative factors in the pathogenesis of gastric mucosal lesions and mechanism of protection against oxidative stress - induced gastric injury. J Physiol Pharmacol 2014;65:613–22.

Lærke HN, Arent S, Dalsgaard S, Bach Knudsen KE. Effect of xylanases on ileal viscosity, intestinal fiber modification, and apparent ileal fiber and nutrient digestibility of rye and wheat in growing pigs. J Anim Sci 2015;93:4323–35. https://doi.org/10.2527/jas2015-9096.

Lindberg JE. Fiber effects in nutrition and gut health in pigs. J Anim Sci Biotechnol 2014;5:1–7. https://doi.org/10.1186/2049-1891-5-15.

McDonald DE, Pethick DW, Mullan BP, Hampson DJ. Increasing viscosity of the intestinal contents alters small intestinal structure and intestinal growth, and stimulates proliferation of enterotoxigenic Escherichia coli in newly-weaned pigs. Br J Nutr 2001;86:487–98. https://doi.org/10.1079/BJN2001416.

McDonald P, Edwards RA, Greenhalgh JFD, Morgan CA, Sinclair LA, Wilkinson RG. Animal nutrition. 7th ed. Harlow, United Kingdom: Pearson Education Limited; 2010.

Molist F, van Oostrum M, Pérez JF, Mateos GG, Nyachoti CM, van der Aar PJ. Relevance of functional properties of dietary fibre in diets for weanling pigs. Anim Feed Sci Technol 2014;189:1–10. https://doi.org/10.1016/j.anifeedsci.2013.12.013.

Montagne L, Pluske JR, Hampson DJ. A review of interactions between dietary fibre and the intestinal mucosa, and their consequences on digestive health in young non-ruminant animals. Anim Feed Sci Technol 2003;108:95–117. https://doi.org/10.1016/S0377-8401(03)00163-9.

Myers WD, Ludden PA, Nayigihugu V, Hess BW. Technical Note: a procedure for the preparation and quantitative analysis of samples for titanium dioxide1. J Anim Sci 2004;82:179–83. https://doi.org/10.2527/2004.821179x.

Ngoc TTB, Len NT, Lindberg JE. Chemical characterization and water holding capacity of fibre-rich feedstuffs used for pigs in Vietnam. Asian Australas J Anim Sci 2012;25:861–8. https://doi.org/10.5713/ajas.2011.11294.

Nishi T, Hara H, Tomita F. Soybean β-conglycinin peptone suppresses food intake and gastric emptying by increasing plasma cholecystokinin levels in rats. J Nutr 2003;133:352–7. https://doi.org/10.1093/jn/133.2.352.

Nortey TN, Patience JF, Simmins PH, Trottier NL, Zijlstra RT. Effects of individual or combined xylanase and phytase supplementation on energy, amino acid, and phosphorus digestibility and growth performance of grower pigs fed wheat-based diets containing wheat millrun. J Anim Sci 2007;85:1432–43. https://doi.org/10.2527/jas.2006-613.

NRC. Nutrient requirements of swine. 11th revis. Washington, D.C: National Academies Press; 2012. https://doi.org/10.17226/13298.

O'Neill HVM, Liu N, Wang JP, Diallo A, Hill S. Effect of xylanase on performance and apparent metabolisable energy in starter broilers fed diets containing one maize variety harvested in different regions of China. Asian Australas J Anim Sci 2012;25:515–23. https://doi.org/10.5713/ajas.2011.11314.

Owusu-Asiedu A, Patience JF, Laarveld B, Van Kessel AG, Simmins PH, Zijlstra RT. Effects of guar gum and cellulose on digesta passage rate, ileal microbial populations, energy and protein digestibility, and performance of grower pigs 1, 2. J Anim Sci 2006;84:843–52. https://doi.org/10.2527/2006.844843x.

Owusu-Asiedu A, Simmins PH, Brufau J, Lizardo R, Péron A. Effect of xylanase and glucanase on growth performance and nutrient digestibility in piglets fed wheat-barley-based diets. Livest Sci 2010;134:76–8. https://doi.org/10.1016/j.livsci.2010.06.102.

Passos AA, Park I, Ferket P, von Heimendahl E, Kim SW. Effect of dietary supplementation of xylanase on apparent ileal digestibility of nutrients, viscosity of digesta, and intestinal morphology of growing pigs fed corn and soybean meal based diet. Anim Nutr 2015;1:19–23. https://doi.org/10.1016/j.aninu.2015.02.006.

Pedersen NR, Azem E, Broz J, Guggenbuhl P, Le DM, Fojan P, Pettersson D. The degradation of arabinoxylan-rich cell walls in digesta obtained from piglets fed wheat-based diets varies depending on digesta collection site, type of cereal, and source of exogenous xylanase. J Anim Sci 2012;90:149–51. https://doi.org/10.2527/jas.53832.

Pluske JR, Hampson DJ, Williams IH. Factors influencing the structure and function of the small intestine in the weaned pig: a review. Livest Prod Sci 1997;51:215–36. https://doi.org/10.1016/S0301-6226(97)00057-2.

Rao KMK. MAP kinase activation in macrophages. J Leukoc Biol 2001;69:3–10. https://doi.org/10.1189/jlb.69.1.3.

Ruemmele FM, Beaulieu JF, Dionne S, Levy E, Seidman EG, Cerf-Bensussan N, Lentze MJ. Lipopolysaccharide modulation of normal enterocyte turnover by toll-like receptors is mediated by endogenously produced tumour necrosis factor α. Gut 2002;51:842–8. https://doi.org/10.1136/gut.51.6.842.

Serena A, Hedemann MS, Bach Knudsen KE. Influence of dietary fiber on luminal environment and morphology in the small and large intestine of sows. J Anim Sci 2008;86:2217–27. https://doi.org/10.2527/jas.2006-062.

Shacter E. Quantification and significance of protein oxidation in biological samples. Drug Metab Rev 2000;32:307–26. https://doi.org/10.1081/DMR-100102336.

Sido A, Radhakrishnan S, Kim SW, Eriksson E, Shen F, Li Q, Bhat V, Reddivari L, Vanamala JKP. A food-based approach that targets interleukin-6, a key regulator of chronic intestinal inflammation and colon carcinogenesis. J Nutr Biochem 2017;43:11–7. https://doi.org/10.1016/j.jnutbio.2017.01.012.

Taliercio E, Kim SW. Epitopes from two soybean glycinin subunits are antigenic in pigs. J Sci Food Agric 2013;93:2927–32. https://doi.org/10.1002/jsfa.6113.

Tervilä-Wilo A, Parkkonen T, Morgan A, Poutanen K, Heikkinen P, Autio K. In vitro digestion of wheat microstructure with xylanase and cellulase from trichoderma reesei. J Cereal Sci 1996;24:215–25. https://doi.org/10.1006/jcrs.

Urriola PE, Stein HH. Effects of distillers dried grains with solubles on amino acid, energy, and fiber digestibility and on hindgut fermentation of dietary fiber in a corn-soybean meal diet fed to growing pigs. J Anim Sci 2010;88:1454–62. https://doi.org/10.2527/jas.2009-2162.

Wang D, Piao XS, Zeng ZK, Lu T, Zhang Q, Li PF, Xue LF, Kim SW. Effects of keratinase on growth performance, nutrient utilization, intestinal morphology, intestinal ecology and inflammatory response of weaned piglets fed diets with different levels of crude protein. Asian Australas J Anim Sci 2011;24:1718–28. https://doi.org/10.5713/ajas.2011.11132.

Wang T, Qin G, Sun Z, Zhao Y, Zhang B. Comparative study on the residual rate of immunoreactive soybean glycinin (11S) in the digestive tract of pigs of different ages. Food Agric Immunol 2010;21:201–8. https://doi.org/10.1080/09540100903563597.

Wang T, Qin GX, Zhao Y, Sun ZW. Comparative study on the stability of soybean (*Glycine max*) β-conglycinin in vivo. Food Agric Immunol 2009;20:295–304. https://doi.org/10.1080/09540100903203004.

Wellock IJ, Fortomaris PD, Houdijk JGM, Wiseman J, Kyriazakis I. The consequences of non-starch polysaccharide solubility and inclusion level on the health and performance of weaned pigs challenged with enterotoxigenic Escherichia coli. Br J Nutr 2008;99:520–30. https://doi.org/10.1017/S0007114507819167.

Xu J. Effect of glycinin and β-conglycinin on the absorbing capacity of mouse intestinal epithelial cells. Asian J Anim Sci 2014;8:73–8. https://doi.org/10.3923/ajas.2014.73.78.

Yin YL, Baidoo SK, Jin LZ, Liu YG, Schulze H, Simmins PH. The effect of different carbohydrase and protease supplementation on apparent (ileal and overall) digestibility of nutrients of five hulless barley varieties in young pigs. Livest Prod Sci 2001;71:109–20. https://doi.org/10.1016/S0301-6226(01)00215-9.

Zhang JX, Guo LY, Feng L, Jiang WD, Kuang SY, Liu Y, Hu K, Jiang J, Li SH, Tang L, Zhou XQ. Soybean β-conglycinin induces inflammation and oxidation and causes dysfunction of intestinal digestion and absorption in fish. PLoS One 2013;8:1–12. https://doi.org/10.1371/journal.pone.0058115.

Zhao Y, Qin G, Sun Z, Zhang X, Bao N, Wang T, Zhang B, Zhang B, Zhu D, Sun L. Disappearance of immunoreactive glycinin and beta-conglycinin in the digestive tract of piglets. Arch Anim Nutr 2008;62:322–30. https://doi.org/10.1080/17450390802190318.

Zhao Y, Qin GX, Sun ZW, Zhang B, Wang T. Effects of glycinin and β-conglycinin on enterocyte apoptosis, proliferation and migration of piglets. Food Agric Immunol 2010;21:209–18. https://doi.org/10.1080/09540101003596644.

Zuo J, Ling B, Long L, Li T, Lahaye L, Yang C. Effect of dietary supplementation with protease on growth performance , nutrient digestibility , intestinal morphology , digestive enzymes and gene expression of weaned piglets. Anim Nutr 2015;1:276–82. https://doi.org/10.1016/j.aninu.2015.10.003.