Combination of Xylan Depolymerizing and Debranching Enzymes Improves Digestion, Growth Performance and Intestinal Volatile Fatty Acid Profile of Piglets

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Simple Summary: Many plant feedstuffs (e.g. cereal brans) are abundant in arabinoxylan (Abx) that serves as a typical anti-nutritional factor for monogastric animals. Abx comprises a backbone of β-D-(1-4)-xylpyranosyl units onto which L-arabinose and ferulic acid groups are linked as the side chains. Due to the complex structure, the efficient degradation of Abx may demand an enzyme consortium consisting of depolymerizing enzymes such as endo-xylanase (Xyn) and debranching enzymes such as arabinofuranosidase (Afd) and feruloyl esterase (FE). To date, information about the potential synergy among Xyn, Afd and FE on degradation of Abx in plant ingredients along with the application of combination of these enzymes in pig diets is scarce. In this study, we found that combination of Xyn, Afd and FE displayed a superiority over Xyn alone and its combination with Afd or FE in promoting degradation of Abx in multiple brans. Treatment with combination of Xyn, Afd and FE had advantages over Xyn alone in improving nutrient digestion, growth performance and intestinal volatile fatty acid profile of postweaning piglets fed bran-added diet. These indicated that combination of Xyn, Afd and FE could be used as a prominent enzyme consortium for improving growth and gut health of piglets.

Abstract: This study was aimed to investigate the effect of xylan depolymerizing enzyme namely endo-xylanase (Xyn) combined with debranching enzymes namely arabinofuranosidase (Afd) and feruloyl esterase (FE) on digestion, growth performance and intestinal volatile fatty acid profile of piglets. The in vitro experiments were firstly conducted to examine the enzymological properties of Xyn, Afd and FE, the synergy among these enzymes, together with the effect of combination of these enzymes on digestion of piglet diet. The in vivo experiment was then implemented by allocating 270 35-d-old postweaning piglets into 3 treatment groups: control group, Xyn group and (Xyn+Afd+FE) group. Each group had 6 replicates (15 piglets/replicate). The results revealed a satisfying thermostability and pH stability of Xyn, Afd and FE. Combination of Xyn, Afd and FE had a superiority (P < 0.05) over Xyn alone and its combination with Afd or FE in promoting degradation of different bran fibers rich in arabinoxylan (Abx). Treatment with combination of Xyn, Afd and FE had advantages over Xyn alone to induce increasing trends (P < 0.10) of in vitro digestibility of dietary nutrients (dry matter, crude protein, crude ash and gross energy) and piglet growth performance (average daily gain, final body weight and feed efficiency), concurrent with a reduction (P < 0.05) of diarrhea rate and increases (P < 0.05) in cecal acetic acid, butyric acid and total volatile fatty acids concentrations as well as pH value of piglets. Collectively, combination of Xyn, Afd and FE was efficient in benefiting degradation of Abx in brans, as well as improving digestion, growth performance and intestinal volatile fatty acid profile of piglets.

Keywords: depolymerizing enzyme; debranching enzyme; xylanase; arabinofuranosidase; feruloyl esterase; arabinoxylan; digestion; growth performance; volatile fatty acid; piglet
1. Introduction

Arabinoxylan (Abx) exemplifies the typical non-starch polysaccharide in plentiful plant-derived feedstuffs such as cereal grains and brans [1], comprising a linear backbone of β-D-(1-4)-xylopyranosyl units onto which L-arabinose units are linked as the major side chains [2]. Generally, arabinose residues that attach to the C-2 and/or C-3 position of Abx backbone can be further substituted in the O-5 position by ferulic acid ester groups [2,3]. Abx is resistant to digestion of endogenous enzymes of animals, which causes viscous digesta with subsequent intestinal disorders such as reduced digestibility, increased pathogen load, gut leakage and inflammation, thus being characterized as a critical anti-nutritional factor for monogastric animals especially for those at young age [4,5]. These raise a necessity to explore strategies to degrade dietary Abx that may benefit intestinal digestion and growth performance of animals.

Enzymatic treatment has been evidenced as a promising approach to decompose polysaccharides in crop products [6,7]. Because of the complex molecular structure, the efficient degradation of Abx in diet may demand an enzyme consortium consisting of depolymerizing enzymes such as endo-xylanase (Xyn) and debranching enzymes such as arabinofuranosidase (Afd) and feruloyl esterase (FE) [3,8]. Thereinto, Xyn stochastically cleaves the β-1,4-glycosidic bonds within Abx backbone and produces smaller polysaccharides or oligosaccharides [3], which can be used as prebiotics to improve intestinal health by targeting host gut microbiota [4,9]. However, the presence of side chains fortifies the degradation resistance of Abx backbone through impeding recognition of cleavage sites in the backbone by Xyn [8,10]. This effect may be overwhelmed by the action of debranching enzymes, among which Afd removes the arabinose residues to expose more cleavage sites and provide a convenience for Xyn action [3,10], while FE breaks ferulic acid ester bonds cross-linked to arabinose residues to liberate ferulic acid [11,12]. These actions aid in simplifying the molecular structure of Abx and enhancing Xyn accessibility to produce reducing sugars [13]. Accordingly, there can be an efficient degradation of Abx in plant-sourced ingredients under the synergy among Xyn, Afd and FE, which may in turn diminish the viscosity of intestinal content, favoring a sufficient contact between digesta and digestive enzymes with a resultant benefit on digestion of dietary nutrients [4,5]. More importantly, the efficient degradation of Abx and the cleavage of ferulic acid ester bonds linking feruloyl residues of Abx with other cell wall components under the synergy among Xyn, Afd and FE probably prompt cell wall fragmentation of plant ingredients, which in turn profits the access of digestive enzymes to cell wall or intracellular components [14-16], presumptively triggering an enhanced digestion of plant ingredients with a subsequent promotion of growth and health of animals fed plant feedstuff-based diet. Although there were numerous studies confirming the positive effects of Xyn in combination with Afd on chicken growth performance and health condition [17-19], information about the synergistic action among Xyn, Afd and FE on the improvements of growth and gut health of piglets is scarce.

Comprehensively, we raised the assumption that the synergy among Xyn, Afd and FE was more favorable to result in an enhanced degradation of Abx with a destruction of cell wall of certain plant feedstuffs, possibly conducing to ameliorate the feeding effect of them. To test this hypothesis, the current study was conducted to investigate the effects of combination of Xyn, Afd and FE on degradation of cereal brans rich in Abx as well as growth and health of postweaning piglets received bran-added diet.

2. Materials and Methods

2.1. Materials

Xylose and arabinose were purchased from Aladdin (Shanghai, China). Beechwood xylan, p-nitrophenyl-α-L-arabinofuranoside, α-amylase, protease, glucosidase, pepsin and cellulase were purchased from Sigma-Aldrich (Shanghai, China). Methyl ferulate was purchased from Alfa Aesar (Beijing, China). Xyn (EC 3.2.1.8), Afd (EC 3.2.1.55) and FE (EC 3.1.1.73) were all provided by AsiaPac Co., Ltd (Dongguan, China). Trypsin and chymotrypsin were purchased from Amresco (Shanghai, China). Dialysis bag and pan-
creatin were derived from Viskase (Beijing, China) and Lanxu Biotech. Co. (Hefei, China), respectively. Wheat bran and oat bran were provided by Xingye Biotech. Co. (Dongguan, China). Destarched wheat bran (DSWB) was prepared according to the method of Mukherjee et al. [20]. All other chemicals and solvents used in this study were of analytical grade.

2.2. Enzymatic characteristics assay

2.2.1. Determination of enzyme activities

The activity of Xyn was determined using beechwood xylan as the substrate [21]. The reaction mixture consisted of 200 μL properly diluted enzyme and 1.8 mL of 10 mg/mL xylan in 0.05 M citric-Na₂HPO₄ (pH 5.0). After incubation at 40°C for 10 min, the enzymatic activity was terminated by adding 2 mL 3,5-dinitrosalicylic acid reagent (DNS) followed by boiling for 10 min. This mixture was then cooled down for quantifying reducing sugar using DNS method with xylose as a standard [21].

Afd activity was determined by using p-nitrophenyl α-L-arabinofuranoside as the substrate [22]. The reaction mixture consisted of 25 μL properly diluted enzyme and 175 μL of 2 mM substrate in 0.05 M citric-Na₂HPO₄ (pH 5.5). After incubation at 40°C for 15 min, the enzymatic reaction was terminated by adding 200 μL of 2 M Na₂CO₃. The concentration of p-nitrophenol was measured at optical density of 410 nm.

For FE activity assay, methyl ferulate was used as the substrate [21]. The reaction mixture consisted of 10 μL properly diluted enzyme and 190 μL of 0.1 M substrate in 0.05 M citric-Na₂HPO₄ (pH 5.5). After incubation at 40°C for 15 min, 100 μL acetonitrile was added into this mixture to terminate the enzymatic reaction. Ferulic acid was quantified by high performance liquid chromatography (Shimadzu SIL-20A, Japan) [21].

One unit of Xyn, Afd and FE was defined as the amount of enzyme required to generate 1 μmol of product equivalent per min from the corresponding substrates under the standard assay conditions. Each reaction was performed in triplicate.

2.2.2. Influence of pH and temperature on enzymes activities

The influence of pH on enzyme activities was detected by measuring the relative activities of Xyn, Afd and FE in 0.05 M citric-Na₂HPO₄ buffer (pH 3.0~8.0) and glycine-NaOH (pH 9.0~10.0) according to the method described above. Enzyme stability against pH was detected by determining the residual activities of these enzymes after incubation at pH 3.0 for 30 min.

The effect of temperature on enzyme activities was determined by measuring the relative activities of Xyn, Afd and FE at different temperatures (30°C~90°C) under the standard assay conditions described above. Thermostability stability of these enzymes was evaluated by determining the residual activities of these enzymes after incubation at 85°C for 3 min.

2.3. Effect of Xyn combined with Afd on degradation of Abx in DSWB

Several tubes with 0.5 g DSWB each were divided into 6 groups (5 replicates/group): control, X, (X+A1), (X+A2), (X+A3) and (X+A4), which received the following treatments. Control: no enzymes; X: 3 U/g Xyn; (X+A1): 3 U/g Xyn + 0.001 U/g Afd; (X+A2): 3 U/g Xyn + 0.003 U/g Afd; (X+A3): 3 U/g Xyn + 0.006 U/g Afd; (X+A4): 3 U/g Xyn + 0.03 U/g Afd. Each treatment group was then added with phosphate buffer solution (PBS) to obtain 10 mL reaction system, followed by incubation in a constant temperature shaker (40°C, 120 rpm/min) for 4 h. After centrifugation at 3000 rpm/min for 3 min, the supernatant was collected for quantifying the releasing of reducing sugar (RRS) using DNS method that represents the degradation of Abx [21].

2.4. Effect of Xyn combined with FE on degradation of Abx in DSWB

Several tubes with 0.5 g DSWB each were divided into 6 groups (5 replicates/group): control, X, (X+F1), (X+F2), (X+F3) and (X+F4), which received the following treatments. Control: no enzymes; X: 3 U/g Xyn; (X+F1): 3 U/g Xyn + 0.001 U/g FE; (X+F2): 3 U/g Xyn +
2.5. Evaluation of synergy among Xyn, Afd and FE on degradation of Abx in different brans

Wheat bran (WB) and oat bran (OB) were enzymolyzed as described previously [23]. The resulting zymolytes were used to extract the soluble fiber (SF) and insoluble fiber (IF) according to the report of Fahey et al. [24]. For evaluation of the synergy among Xyn, Afd and FE on the RRS from different Abx sources (WB-SF, WB-IF, OB-SF and OB-IF), several tubes with 0.5 g of each fiber were randomly allocated into 4 groups (5 replicates/group): X, (X+A), (X+F) and (X+A+F), which received the following treatments: X: 3 U/g Xyn; (X+A): 3 U/g Xyn + 0.001 U/g Afd; (X+F): 3 U/g Xyn + 0.006 U/g FE; (X+A+F): 3 U/g Xyn + 0.001 U/g Afd + 0.006 U/g FE (Note: the dosages of Xyn, Afd and FE were selected according to the results in Figures 3 and 4). Each treatment group was then added with PBS to obtain 10 mL reaction system, the supernatant was separated for quantifying the RRS using DNS method [21].

The value of synergy degree between multiple enzymes was calculated as the ratio of the sum of the activities (generation amount of hydrolysate) of all enzymes to the activity of one of these enzymes [25]. If the ratio is less than 1, it reveals a negative synergy between enzymes, while the ratio exceeding 1.0 suggests a positive synergy between enzymes.

2.6. Effect of combination of Xyn, Afd and FE on in vitro digestion of bran-added diet

2.6.1. Preparation of in vitro digestion juice

Referring to previous studies in pigs [26,27], the gastric digestion juice was simulated by preparation of pepsin (737.5 U/mL) with HCL solution (pH 2.0). The small intestinal digestion juice was simulated by preparing a mixture of amylase (221.4 U/mL), trypsin (69.1 U/mL) and chymotrypsin (8.7 U/mL) with deionized water. The large intestinal digestion juice was simulated by preparation of cellulase (0.4 U/mL) with deionized water.

2.6.2. In vitro digestion of bran-added diet

The composition and nutrient levels of bran-added diet based on the NRC requirement of swine [28] are shown in Table 1. The SDS-II simulated digestion system (Shenhua Biotech. Co. Ltd., China) was employed in simulating digestion of the above diet of piglets [26,27]. In brief, diet (2 g) was put into digestive tube sleeved with the MD34-14 dialysis bag, followed by digestion with 20 mL of simulated gastric juice together with one of three treatments (control, no enzymes; X, 3 U/g Xyn; (X+A+F), 3 U/g Xyn + 0.001 U/g Afd + 0.006 U/g FE) at 39°C for 4 h in the digestion system. Immediately after simulated gastric digestion, the diet was treated by 2.2 mL of simulated small intestinal juice at 39°C for 16 h and simulated large intestinal juice at 39°C for 3.5 h. Following these processes, the undegraded chyme was washed repeatedly with deionized water. Thereafter, the chyme residue was successively dried in an oven at 65°C and 105°C to constant weight, which was then cooled to measure the digestibility of nutrients (dry matter, crude protein, crude ash and gross energy) based on the method of Zhu et al. [27].

Table 1. The composition and nutrient levels of basal diet (as-fed basis)

| IngredientsContent | Content (%) | Corn60 Soybean meal | Bran1.5 Soybean oil | 0.45 Puffed soybean | 6.9 Fish meal | 4.5 Whey powder | 7.75 Dicalcium phosphate | 0.85 Limestone | 1.3 Salt | 0.3 Lysine | 0.35 Threonine | 0.15 Methionine | 0.1 Choline | 0.2 Silicon dioxide | 0.3 Premix | 0.55 Total | 100 | Nutrient lev-
|------------------|-------------|---------------------|--------------------|---------------------|--------------|----------------|------------------------|----------------|---------|---------|-----------|-----------|-----------|----------------|----------------|-------------|--------|-----------------|
Digestable energy (MJ/kg) 14.43
Metabolizable energy (MJ/kg) 13.90
Crude protein (%) 18.76
Digestible lysine (%) 1.14
Digestible methionine (%) 0.36
Available phosphorus (%) 0.33

Supplied per kilogram of diet: Cu, 9.9 mg; Zn, 142.5 mg; Fe, 242.5 mg; Mn, 68.7 mg; Se, 0.36 mg; I, 0.64 mg; vitamin A, 11500 IU; vitamin D3, 3250 IU; vitamin E, 91 mg; vitamin K3, 3 mg; thiamin, 8.6 mg; riboflavin, 10.9 mg; pyridoxine, 9.3 mg; cobalamin, 0.2 mg; niacin, 62.6 mg; pantothenic acid, 26.8 mg; folic acid, 1.9 mg.
| Ingredient                  | Amount   |
|----------------------------|----------|
| Corn                       | 60       |
| Soybean meal               | 15       |
| Bran                       | 1.5      |
| Soybean oil               | 0.45     |
| Puffed soybean            | 6.9      |
| Fish meal                  | 4.5      |
| Whey powder               | 7.75     |
| Dicalcium phosphate       | 0.85     |
| Limestone                 | 1.1      |
| Salt                       | 0.3      |
| Lysine                     | 0.35     |
| Threonine                 | 0.15     |
| Methionine                | 0.1     |
| Choline                    | 0.2     |
| Silicon dioxide           | 0.3     |
| Premix                     | 100.55   |
| Total                      | 100      |

**Nutrient levels**

- **Digestible energy (MJ/kg):** 14.43
- **Metabolizable energy (MJ/kg):** 13.90
- **Crude protein (%):** 18.76
- **Digestible lysine (%):** 1.14
- **Digestible methionine (%):** 0.36
- **Calcium (%):** 0.67
- **Available phosphorus (%):** 0.33

Supplied per kilogram of diet:
- Cu: 9.9 mg
- Zn: 142.5 mg
- Fe: 242.5 mg
- Mn: 68.7 mg
- Se: 0.36 mg
- I: 0.64 mg
- Vitamin A: 11500 IU
- Vitamin D3: 3250 IU
- Vitamin E: 91 mg
- Vitamin K3: 3 mg
- Thiamin: 8.6 mg
- Riboflavin: 10.9 mg
- Pyridoxine: 9.3 mg
- Cobalamin: 0.2 mg
- Niacin: 62.6 mg
- Pantothenic acid: 26.8 mg
- Folic acid: 1.9 mg

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Supplied per kilogram of diet:
- Cu: 9.9 mg
- Zn: 142.5 mg
- Fe: 242.5 mg
- Mn: 68.7 mg
- Se: 0.36 mg
- I: 0.64 mg
- Vitamin A: 11500 IU
Soybean meal, 15% Bran, 1.5% Soybean oil, 0.45% Puffed soybean, 6.9% Fish meal, 4.5% Whey powder, 7.75% Dicalcium phosphate, 0.85% Limestone, 1.1% Salt, 0.3% Lysine, 0.35% Threonine, 0.15% Methionine, 0.2% Choline, 0.2% Silicon dioxide, 0.3% Premix, 0.55% Total 100% Nutrient levels

| Digestible energy (MJ/kg) | 14.43 |
|----------------------------|-------|
| Metabolizable energy (MJ/kg) | 13.90 |
| Crude protein (%) | 18.76 |
| Digestible lysine (%) | 1.14 |
| Digestible methionine (%) | 0.36 |
| Calcium (%) | 0.67 |
| Available phosphorus (%) | 0.33 |

Supplied per kilogram of diet:
- Cu, 9.9 mg;
- Zn, 142.5 mg;
- Fe, 242.5 mg;
- Mn, 68.7 mg;
- Digestible lysine, 1.14%
mg; Se, 0.36 mg; I, 0.64 mg; gestible methionine (%)
0.36 Calcium
vitamin A, 11500 IU; vitamin
D3, 3250 IU; vitamin
E, 91 mg; vitamin K3, 3 mg;
thiamin, 8.6 mg; riboflavin,
10.9 mg; pyridoxine, 9.3
mg; cobalamin, 0.2 mg;
niacin, 62.6 mg; panto-
thenic acid, 26.8 mg; folic
acid, 1.9 mg.

Supplied per kilogram of
diet: Cu, 9.9 mg; Zn,
142.5 mg; Fe,
242.5 mg; Mn,
68.7 mg;
Se,
0.36 mg; I,
0.64 mg;
vitamin
A,
11500 IU;
vitamin
D3,
3250 IU;
vitamin
E, 91
mg;
vitamin
K3, 3
mg;
thiamin,
8.6
mg; riboflavin,
10.9
mg; pyridoxine,
9.3
mg; cobalamin,
Bran 1.5 Soybean oil 0.45 Puffed soybean 6.9 Fish meal 4.5 Whey powder 7.75 Dicalcium phosphate 0.85 Limestone 1.1 Salt 0.3 Lysine 0.35 Threonine 0.15 Methionine 0.1 Choline 0.2 Silicon dioxide 0.3 Premix 0.55 Total 100

Nutrient levels
Digestible energy (MJ/kg) 14.43
Metabolizable energy (MJ/kg) 13.90
Crude protein (%) 18.76
Digestible lysine (%) 1.14
Digestible methionine (%) 0.36
Calcium (%) 0.67
Available phosphorus (%) 0.33

Supplied per kilogram of diet:
Cu, 9.9 mg;
Zn, 142.5 mg;
Fe, 242.5 mg;
Mn, 68.7 mg;
Se, 0.36 mg;
I, 0.64 mg;
vitamin A, 11500 IU;
vitamin D3, 3250 IU;
vitamin E, 91 mg;
K3, 3 mg;
thiamin, 8.6 mg;
riboflavin, 10.9 mg;
pyridoxine, 9.3 mg;
cobalamin, 0.2 mg;
niacin, 62.6 mg;
pantothenic acid, 26.8 mg;
folic acid, 1.9 mg.
| Component               | Amount       |
|-------------------------|--------------|
| Soybean oil             | 0.45 P       |
| Puffed soybean          | 6.9          |
| Fish meal               | 4.5          |
| Whey powder             | 7.75         |
| Dicalcium phosphate     | 0.85         |
| Limestone               | 1.1          |
| Salt                    | 0.3          |
| Lysine                  | 0.3          |
| Threonine               | 0.15         |
| Methionine              | 0.1          |
| Choline                 | 0.2          |
| Silicon dioxide         | 0.3          |
| Premix                  | 0.55         |
| Total                   | 100.55       |

Nutrient levels:

| Digestible energy (MJ/kg) | 14.43 |
| Metabolizable energy (MJ/kg) | 13.90 |
| Crude protein (%)          | 18.76 |
| Digestible lysine (%)      | 1.14  |
| Digestible methionine (%)  | 0.36  |
| Available phosphorus (%)   | 0.67  |

Supplied per kilogram of diet:
- Cu, 9.9 mg
- Zn, 142.5 mg
- Fe, 242.5 mg
- Mn, 68.7 mg
- Se, 0.36 mg
- I, 0.64 mg
- vitamin A, 11500 IU
- vitamin D3, 3250 IU
- vitamin E, 91 mg
- vitamin K3, 3 mg
- thiamin, 8.6 mg
- riboflavin, 10.9 mg
- pyridoxine, 9.3 mg
- cobalamin, 0.2 mg
- niacin, 62.6 mg
- pantothentic acid, 26.8 mg
- vitamin A, 11500 IU
- vitamin D3, 3250 IU
- vitamin E, 91 mg
- vitamin K3, 3 mg
- thiamin, 8.6 mg
- riboflavin, 10.9 mg
- pyridoxine, 9.3 mg
- cobalamin, 0.2 mg
- niacin, 62.6 mg
| Nutrient        | Content     |
|-----------------|-------------|
| folic acid      | 1.9 mg      |
| Cu              | 9.9 mg      |
| Zn              | 142.5 mg    |
| Fe              | 242.5 mg    |
| Mn              | 68.7 mg     |
| Se              | 0.36 mg     |
| I               | 0.64 mg     |
| vitamin A       | 11500 IU    |
| vitamin D3      | 3250 IU     |
| vitamin E       | 91 mg       |
| vitamin K3      | 3 mg        |
| thiamin         | 8.6 mg      |
| riboflavin      | 10.9 mg     |
| pyridoxine      | 9.3 mg      |
| cobalamin       | 0.2 mg      |
| niacin          | 62.6 mg     |
| pantothenic acid|             |
Puffed soybean

Fish meal

Whey powder

Dicalcium phosphate

Limestone

Salt

Lysine

Threonine

Methionine

Choline

Silicon dioxide

Premix

Total

Nutrient levels

Digestible energy (MJ/kg)

Metabolizable energy (MJ/kg)

Crude protein (%)

Digestible lysine (%)

Digestible methionine (%)

Calcium (%)

Available phosphorus (%)

Supplied per kilogram of diet:

Cu, 9.9 mg;

Zn, 142.5 mg;

Fe, 242.5 mg;

Mn, 68.7 mg;

Se, 0.36 mg;

I, 0.64 mg;

vitamin A, 11500 IU;

vitamin D3, 3250 IU;

vitamin E, 91 mg;

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thiamin, 8.6 mg;

riboflavin, 10.9 mg;

pyridoxine, 9.3 mg;

cobalamin, 0.2 mg;

niacin, 62.6 mg;

pantothenic acid, 26.8 mg;

folic acid, 1.9 mg.
thiamin, 8.6 mg; riboflavin, 10.9 mg; pyridoxine, 9.3 mg; cobalamin, 0.2 mg; niacin, 62.6 mg; pantothenic acid, 26.8 mg; folic acid, 1.9 mg.

Fish meal 4.5, Whey powder 7.75, Dicalcium phosphate 0.85, Limestone 1.1, Salt 0.3, Lysine 0.35, Threonine 0.15, Methionine 0.2, Choline 0.2, Silicon dioxide 0.1, Premix 0.55, Total 100

Nutrient levels
Digestible energy (MJ/kg) 14.43, Metabolizable energy (MJ/kg) 13.90, Crude protein (%) 18.76, Digestible lysine (%) 1.14, Digestible methionine (%) 0.36, Available phosphorus (%) 0.33

Supplied per kilogram of diet: Cu, 9.9 mg; Zn, 142.5 mg; Fe, 242.5 mg; Mn, 68.7 mg; Se, 0.36 mg; I, 0.64 mg; vitamin A, 11500 IU; vitamin D3, 3250 IU; vitamin E, 91 mg; vitamin K3, 3 mg; thiamin, 8.6 mg; riboflavin, 10.9 mg; pyridoxine, 9.3 mg; cobalamin, 0.2 mg; niacin, 62.6 mg; pantothenic acid, 26.8 mg; folic acid, 1.9 mg.
Mn, 68.7 mg; Se, 0.36 mg; I, 0.64 mg; vitamin A, 11500 IU; vitamin D3, 3250 IU; vitamin E, 91 mg; vitamin K3, 3 mg; thiamin, 8.6 mg; riboflavin, 10.9 mg; pyridoxine, 9.3 mg; cobalamin, 0.2 mg; niacin, 62.6 mg; pantothenic acid, 26.8 mg; folic acid, 1.9 mg.
Nutrient levels

| Digestible energy (MJ/kg) | Metabolizable energy (MJ/kg) | Crude protein (%) | Digestible lysine (%) | Digestible methionine (%) | Calcium (%) | Available phosphorus (%) |
|---------------------------|-----------------------------|------------------|-----------------------|---------------------------|-------------|--------------------------|
| 14.43                     | 13.90                       | 18.76            | 1.14                  | 0.36                       | 0.67        | 0.33                     |

Supplied per kilogram of diet:
- Cu, 9.9 mg;
- Zn, 142.5 mg;
- Fe, 242.5 mg;
- Mn, 68.7 mg;
- Se, 0.36 mg;
- I, 0.64 mg;
- Vitamin A, 11500 IU;
- Vitamin D3, 3250 IU;
- Vitamin E, 91 mg;
- Thiamin, 8.6 mg;
- Riboflavin, 10.9 mg;
- Pyridoxine, 9.3 mg;
- Cobalamin, 0.2 mg;
- Niacin, 62.6 mg;
- Pantothenic acid, 26.8 mg;
- Folic acid, 1.9 mg;
- Phosphate, 0.85 g;
- Limestone, 1.1 g;
- Salt, 0.3 g;
- Lysine, 0.35 g;
- Threonine, 0.15 g;
- Choline, 0.2 g;
- Silicon dioxide, 0.3 g;
- Premix, 10.55 g;
- Total, 100 g.
Dicalcium phosphate0.85 Lime-stone1.1Salt0.3Lysine0.35 Threonine0.15 Methionine0.1 Choline0.2 Silicon dioxide0.3 Premix0.55 Total100 Nutrient levels Digestible energy (MJ/kg) 14.43 Metabolizable energy (MJ/kg) 13.90 Crude protein (%) 18.76 Digestible lysine (%) 1.14 Digestible methionine (%) 0.36 Calcium (%0.67 Available phosphorus (%) 0.33

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| Component        | Amount     |
|------------------|------------|
| Limestone        | 1.1 S     |
| Salt             | 0.3 L     |
| Lysine           | 0.35 T    |
| Methionine       | 0.15 C    |
| Choline          | 0.2 P     |
| Silicon dioxide  | 0.3 P     |
| Premix           | 0.55 T    |
| Total            | 100 N     |

**Nutrient levels**

| Digestible energy (MJ/kg) | 14.43 |
|---------------------------|-------|
| Metabolizable energy (MJ/kg) | 13.90 |
| Crude protein (%)         | 18.76 |
| Digestible lysine (%)     | 1.14  |
| Digestible methionine (%) | 0.36  |
| Calcium (%)               | 0.67  |
| Available phosphorus (%)  | 0.33  |

Supplied per kilogram of diet:

| Component        | Amount     |
|------------------|------------|
| Cu               | 9.9 mg     |
| Zn               | 142.5 mg   |
| Fe               | 242.5 mg   |
| Mn               | 68.7 mg    |
| Se               | 0.36 mg    |
| I                | 0.64 mg    |
| vitamin A        | 11500 IU   |
| vitamin D3       | 3250 IU    |
| vitamin E        | 91 mg      |
| vitamin K3       | 3 mg       |
| thiamin          | 8.6 mg     |
| riboflavin       | 10.9 mg    |
| thiamin          | 8.6 mg     |
| vitamin E        | 91 mg      |
| vitamin K3       | 3 mg       |
| thiamin          | 8.6 mg     |
| riboflavin       | 10.9 mg    |
| thiamin          | 8.6 mg     |
| vitamin E        | 91 mg      |
| vitamin K3       | 3 mg       |
| thiamin          | 8.6 mg     |
| riboflavin       | 10.9 mg    |
| thiamin          | 8.6 mg     |
| vitamin E        | 91 mg      |
| vitamin K3       | 3 mg       |
| thiamin          | 8.6 mg     |
| riboflavin       | 10.9 mg    |
| thiamin          | 8.6 mg     |
| vitamin E        | 91 mg      |
| vitamin K3       | 3 mg       |
| thiamin          | 8.6 mg     |
| riboflavin       | 10.9 mg    |
| thiamin          | 8.6 mg     |
| vitamin E        | 91 mg      |
| vitamin K3       | 3 mg       |
| thiamin          | 8.6 mg     |
| riboflavin       | 10.9 mg    |
| thiamin          | 8.6 mg     |
| vitamin E        | 91 mg      |
| vitamin K3       | 3 mg       |
| thiamin          | 8.6 mg     |
| riboflavin       | 10.9 mg    |
| thiamin          | 8.6 mg     |
| vitamin E        | 91 mg      |
| vitamin K3       | 3 mg       |
| thiamin          | 8.6 mg     |
| riboflavin       | 10.9 mg    |
| thiamin          | 8.6 mg     |
| vitamin E        | 91 mg      |
| vitamin K3       | 3 mg       |
| thiamin          | 8.6 mg     |
| riboflavin       | 10.9 mg    |
| thiamin          | 8.6 mg     |
| vitamin E        | 91 mg      |
| vitamin K3       | 3 mg       |
| thiamin          | 8.6 mg     |
| riboflavin       | 10.9 mg    |
| thiamin          | 8.6 mg     |
| vitamin E        | 91 mg      |
| vitamin K3       | 3 mg       |
| thiamin          | 8.6 mg     |
| riboflavin       | 10.9 mg    |
| thiamin          | 8.6 mg     |
| vitamin E        | 91 mg      |
| vitamin K3       | 3 mg       |
| thiamin          | 8.6 mg     |
| riboflavin       | 10.9 mg    |
| thiamin          | 8.6 mg     |
| vitamin E        | 91 mg      |
| vitamin K3       | 3 mg       |
| thiamin          | 8.6 mg     |
| riboflavin       | 10.9 mg    |
| thiamin          | 8.6 mg     |
| vitamin E        | 91 mg      |
| vitamin K3       | 3 mg       |
| thiamin          | 8.6 mg     |
| riboflavin       | 10.9 mg    |
| thiamin          | 8.6 mg     |
| vitamin E        | 91 mg      |
| vitamin K3       | 3 mg       |
| thiamin          | 8.6 mg     |
| riboflavin       | 10.9 mg    |
| thiamin          | 8.6 mg     |
| vitamin E        | 91 mg      |
| vitamin K3       | 3 mg       |
| thiamin          | 8.6 mg     |
| riboflavin       | 10.9 mg    |
| thiamin          | 8.6 mg     |
| vitamin E        | 91 mg      |
| vitamin K3       | 3 mg       |
| thiamin          | 8.6 mg     |
| riboflavin       | 10.9 mg    |
| thiamin          | 8.6 mg     |
| vitamin E        | 91 mg      |
| vitamin K3       | 3 mg       |
| thiamin          | 8.6 mg     |
| riboflavin       | 10.9 mg    |
| thiamin          | 8.6 mg     |
| vitamin E        | 91 mg      |
| vitamin K3       | 3 mg       |
| thiamin          | 8.6 mg     |
| riboflavin       | 10.9 mg    |
10.9 mg; pyridoxine, 9.3 mg; cobalamin, 0.2 mg; niacin, 62.6 mg; pantothenic acid, 26.8 mg; folic acid, 1.9 mg.

gram of diet: Cu, 9.9 mg; Zn, 142.5 mg; Fe, 242.5 mg; Mn, 68.7 mg; Se, 0.36 mg; I, 0.64 mg; vitamin A, 11500 IU; vitamin D3, 3250 IU; vitamin E, 91 mg; vitamin K3, 3 mg; thiamin, 8.6 mg; riboflavin, 10.9 mg; pyridoxine, 9.3 mg; cobalamin, 0.2 mg; niacin, 62.6 mg.
0.3 Lysine
0.35 Threonine
0.15 Methionine
0.1 Choline
0.2 Silicon dioxide
0.3 Premix
0.55 Total

Nutrient levels

Digestible energy (MJ/kg) 14.43
Metabolizable energy (MJ/kg) 13.90
Crude protein (%) 18.76
Digestible lysine (%) 1.14
Digestible methionine (%) 0.36
Calcium (%) 0.67
Available phosphorus (%) 0.33

Supplied per kilogram of diet:
Cu, 9.9 mg;
Zn, 142.5 mg;
Fe, 242.5 mg;
Mn, 68.7 mg;
Se, 0.36 mg;
I, 0.64 mg;
vitamin A, 11500 IU;
vitamin D3, 3250 IU;
vitamin E, 91 mg;
vitamin K3, 3 mg;
riboflavin, 10.9 mg;
pyridoxine, 9.3 mg;
cobalamin, 0.2 mg;
niacin, 62.6 mg;
pantothenic acid, 26.8 mg;
folic acid, 1.9 mg;
| Nutrient                   | Level       |
|---------------------------|-------------|
| Lysine                    | 0.35        |
| Threonine                 | 0.15        |
| Methionine                | 0.1         |
| Choline                   | 0.2         |
| Silicon dioxide           | 0.3         |
| Premix                    | 1.0         |
| Total                     | 1.0         |
| Digestible energy (MJ/kg) | 14.43       |
| Metabolizable energy (MJ/kg) | 13.90    |
| Crude protein (%)         | 18.76       |
| Digestible lysine (%)     | 1.14        |
| Digestible methionine (%) | 0.36        |
| Calcium                   | 0.67        |
| Available phosphorus (%)  | 0.67        |

Supplied per kilogram of diet: Cu, 9.9 mg; Zn, 142.5 mg; Fe, 242.5 mg; Mn, 68.7 mg; Se, 0.36 mg; I, 0.64 mg; vitamin A, 11500 IU; vitamin D3, 3250 IU; vitamin E, 91 mg; vitamin K3, 3 mg; thiamin, 8.6 mg; riboflavin, 10.9 mg; pyridoxine, 9.3 mg; cobalamin, 0.2 mg; niacin, 62.6 mg; pantothenic acid, 26.8 mg; folic acid, 1.9 mg.
| Nutrient          | Amount   |
|-------------------|----------|
| Se                | 0.36 mg  |
| I                 | 0.64 mg  |
| Vitamin A         | 11500 IU |
| Vitamin D3        | 3250 IU  |
| Vitamin E         | 91 mg    |
| Vitamin K3        | 3 mg     |
| Thiamin           | 8.6 mg   |
| Riboflavin        | 10.9 mg  |
| Pyridoxine        | 9.3 mg   |
| Cobalamin         | 0.2 mg   |
| Niacin            | 62.6 mg  |
| Silicon dioxide   | 0.3 mg   |
| Premix            | 10.55 T  |

**Nutrient levels**

| Nutrient          | Amount   |
|-------------------|----------|
| Digestible energy (MJ/kg) | 14.43 |
| Metabolizable energy (MJ/kg) | 13.90 |
| Crude protein (%) | 18.76 |
| Digestible lysine (%) | 1.14 |
| Digestible methionine (%) | 0.36 |
| Calcium           | 0.15    |
Available phosphorus (%)

Supplied per kilogram of diet: Cu, 9.9 mg; Zn, 142.5 mg; Fe, 242.5 mg; Mn, 68.7 mg; Se, 0.36 mg; I, 0.64 mg; vitamin A, 11500 IU; vitamin D3, 3250 IU; vitamin E, 91 mg; vitamin K3, 3 mg; thiamin, 8.6 mg; riboflavin, 10.9 mg; pyridoxine, 9.3 mg; cobalamin, 0.2 mg; niacin, 62.6 mg; pantothenic acid, 26.8 mg; folic acid, 1.9 mg.

Protein (%)

Digestible lysine (%)

Digestible methionine (%)

Calcium (%)

Available phosphorus (%)

Supplied per kilogram of diet: Cu, 9.9 mg; Zn, 142.5 mg; Fe, 242.5 mg; Mn, 68.7 mg; Se, 0.36 mg; I, 0.64 mg; vitamin A, 11500 IU; vitamin D3, 3250 IU; vitamin E, 91 mg; vitamin K3, 3 mg; thiamin, 8.6 mg; riboflavin, 10.9 mg; pyridoxine, 9.3 mg; cobalamin, 0.2 mg; niacin, 62.6 mg; pantothenic acid, 26.8 mg; folic acid, 1.9 mg.
Methionine, 0.1 Choline, 0.2 Silicon dioxide, 0.3 Premix, 0.55 Total, 100 Nutrient levels
Digestible energy (MJ/kg) 14.43
Metabolizable energy (MJ/kg) 13.90
Crude protein (%) 18.76
Digestible lysine (%) 1.14
Digestible methionine (%), 0.36
Calcium (%), 0.67
Available phosphorus (%), 0.33

Supplied per kilogram of diet:
Cu, 9.9 mg;
Zn, 142.5 mg;
Fe, 242.5 mg;
Mn, 68.7 mg;
Se, 0.36 mg;
I, 0.64 mg;
vitamin A, 11500 IU;
vitamin D3, 3250 IU;
vitamin E, 91 mg;
vitamin K3, 3 mg;
thiamin, 8.6 mg;
riboflavin, 10.9 mg;
pyridoxine, 9.3 mg;
cobalamin, 0.2 mg;
niacin, 62.6 mg;
pantothenic acid, 26.8 mg;
folic acid, 1.9 mg.
Choline 0.2 mg; silicon dioxide 0.3 mg; premix 0.55 kg of total 1 kg of nutrient levels of digestible energy (MJ/kg) 14.43, metabolizable energy (MJ/kg) 13.90, crude protein (%) 18.76, digestible lysine (%) 1.14, digestible methionine (%) 0.36, calcium (%), 0.67, available phosphorus (%), 0.33.

Supplied per kilogram of diet: Cu, 9.9 mg; Zn, 142.5 mg; Fe, 242.5 mg; Mn, 68.7 mg; Se, 0.36 mg; I, 0.64 mg; vitamin A, 11500 IU; vitamin D3, 3250 IU; vitamin E, 91 mg; vitamin K3, 3 mg; thiamin, 8.6 mg; riboflavin, 10.9 mg; pyridoxine, 9.3 mg; cobalamin, 0.2 mg; niacin, 62.6 mg; pantothenic acid, 26.8 mg; folic acid, 1.9 mg.
Silicon dioxide 0.3
Premix 0.55
Total 100
Nutrient levels

- Digestible energy (MJ/kg): 14.43
- Metabolizable energy (MJ/kg): 13.90
- Crude protein (%): 18.76
- Digestible lysine (%): 1.14
- Digestible methionine (%): 0.36
- Calcium (%): 0.67
- Available phosphorus (%): 0.33

Supplied per kilogram of diet: Cu, 9.9 mg; gestible methionine (%): 0.36 Calcium.
Zn, 142.5 mg; Fe, 242.5 mg; (%)
Mn, 68.7 mg; Se, 0.36 mg; I, 0.64 mg; vitamin A, 11500 IU; vitamin D3, 3250 IU; vitamin E, 91 mg; vitamin K3, 3 mg; thiamin, 8.6 mg; riboflavin, 10.9 mg; pyridoxine, 9.3 mg; cobalamin, 0.2 mg; niacin, 62.6 mg; pantothenic acid, 26.8 mg; folic acid, 1.9 mg.
Premix 0.55

| Nutrient levels | Digestible energy (MJ/kg) | Metabolizable energy (MJ/kg) | Crude protein (%) | Digestible lysine (%) | Digestible methionine (%) | Calcium (%) | Available phosphorus (%) |
|-----------------|---------------------------|------------------------------|------------------|-----------------------|---------------------------|-------------|-------------------------|
|                 | 14.43                      | 13.90                        | 18.76            | 1.14                  | 0.36                       | 0.67        | 0.33                    |

Supplied per kilogram of diet:
- Cu, 9.9 mg
- Zn, 142.5 mg
- Fe, 242.5 mg
- Mn, 68.7 mg
- Se, 0.36 mg
- I, 0.64 mg
- Vitamin A, 11500 IU
- Vitamin D3, 3250 IU
- Vitamin E, 91 mg
- Vitamin K3, 3 mg
- Thiamin, 8.6 mg
- Riboflavin, 10.9 mg
- Pyridoxine, 9.3 mg
- Cobalamin, 0.2 mg
- Niacin, 62.6 mg
- Pantothenic acid, 26.8 mg
- Folic acid, 1.9 mg
| Nutrient                  | Level       |
|--------------------------|-------------|
| Digestible energy (MJ/kg) | 14.43       |
| Metabolizable energy (MJ/kg) | 13.90     |
| Crude protein (%)         | 18.76       |
| Digestible lysine (%)     | 1.14        |
| Digestible methionine (%) | 0.36        |
| Available phosphorus (%)  | 0.67        |
| Calcium (%)               | 0.33        |
| Available per kilogram of diet: |            |
| Cu                        | 9.9 mg      |
| Zn                        | 142.5 mg    |
| Fe                        | 242.5 mg    |
| Mn                        | 68.7 mg     |
| Se                        | 0.36 mg     |
| I                         | 0.64 mg     |
| Vitamin A                 | 11500 IU    |
| Vitamin D3                | 3250 IU     |
| Vitamin E                 | 91 mg       |
| Vitamin K3                | 3 mg        |
| Thiamin                   | 8.6 mg      |
| Riboflavin                | 10.9 mg     |
| Pyridoxine                | 9.3 mg      |
| Cobalamin                 | 0.2 mg      |
| Niacin                    | 62.6 mg     |
| Pantotenic acid           | 26.8 mg     |
| Folic acid                | 1.9 mg      |
Nutrient levels

| Digestible energy (MJ/kg) | Metabolizable energy (MJ/kg) |
|--------------------------|-----------------------------|
| 14.43                     | 13.90                       |
| Crude protein (%)         | 18.76                       |
| Digestible lysine (%)     | 1.14                        |
| Digestible methionine (%) | 0.36                        |

Available phosphorus (%) = 0.67

Supplied per kilogram of gestible methionine (%) = 0.36

Calcium mg; Fe, 242.5 mg; Mn, 68.7 mg; Se, 0.36 mg; I, 0.64 mg;

Available vitamin A, 11500 IU; vitamin D3, 3250 IU; vitamin E, 91 mg;

Available vitamin K3, 3 mg; thiamin, 8.6 mg; riboflavin, 10.9 mg;

Available pyridoxine, 9.3 mg; cobalamin, 0.2 mg; niacin, 62.6 mg;

Available pantotheinic acid, 26.8 mg; folic acid, 1.9 mg.
E, 91 mg; vitamin K3, 3 mg; thiamin, 8.6 mg; riboflavin, 10.9 mg; pyridoxine, 9.3 mg; cobalamin, 0.2 mg; niacin, 62.6 mg; pantothenic acid, 26.8 mg; folic acid, 1.9 mg.
| Nutrient                        | Value  |
|--------------------------------|--------|
| Panthenic acid, mg             | 26.8   |
| Folic acid, mg                 | 1.9    |
| Digestible energy (MJ/kg)      | 14.43  |
| Metabolizable energy (MJ/kg)   | 13.90  |
| Crude protein (%)              | 18.76  |
| Digestible lysine (%)          | 1.14   |
| Digestible methionine (%)      | 0.36   |
| Calcium (%)                    | 0.67   |
| Available phosphorus (%)       | 0.33   |

Supplied per kilogram of diet:
- Cu, 9.9 mg
- Zn, 142.5 mg
- Fe, 242.5 mg
- Mn, 68.7 mg
- Se, 0.36 mg
- I, 0.64 mg
- Vitamin A, 11500 IU
- Vitamin D₃, 3250 IU
- Vitamin E, 91 mg
- Vitamin K₃, 3 mg
- Thiamin, 8.6 mg
- Riboflavin, 10.9 mg
- Pyridoxine, 9.3 mg
- Cobalamin, 0.2 mg
- Niacin, 62.6 mg
- Pantothenic acid, 26.8 mg
- Folic acid, 1.9 mg
Metabolizable energy (MJ/kg) 13.90
Crude protein (%) 18.76
Digestible lysine (%) 1.14
Digestible methionine (%) 0.36
Calcium (%) 0.67
Available phosphorus (%) 0.33

Supplied per kilogram of diet: Cu, 9.9 mg; Zn, 142.5 mg; Fe, 242.5 mg; Mn, 68.7 mg; Se, 0.36 mg; I, 0.64 mg; vitamin A, 11500 IU; vitamin D3, 3250 IU; vitamin E, 91 mg; vitamin K3, 3 mg; thiamin, 8.6 mg; riboflavin, 10.9 mg; pyridoxine, 9.3 mg; cobalamin, 0.2 mg; niacin, 62.6 mg; pantothenic acid, 26.8 mg; folic acid, 1.9 mg.
IU; vitamin D3, 3250 IU; vitamin E, 91 mg; vitamin K3, 3 mg; thiamin, 8.6 mg; riboflavin, 10.9 mg; pyridoxine, 9.3 mg; cobalamin, 0.2 mg; niacin, 62.6 mg; pantothenic acid, 26.8 mg; folic acid, 1.9 mg.

Crude protein (%) 18.76
Digestible lysine (%)
1.14
Digestible methionine (%)
0.36
Calcium (%)
0.67
Available phosphorus (%)
0.33

Supplied per kilogram of diet: Cu, 9.9 mg; Zn, 142.5 mg; Fe, 242.5 mg; Mn, 68.7 mg; Se, 0.36 mg; I, 0.64 mg; vitamin A, 11500 IU; vitamin D3, 3250 IU; vitamin E, 91 mg; vitamin K3, 3 mg; thiamin, 8.6 mg; riboflavin, 10.9 mg; pyridoxine, 9.3 mg; cobalamin, 0.2 mg; niacin, 62.6 mg;
pantothenic acid, 26.8 mg; folic acid, 1.9 mg.

| Component   | Amount  |
|-------------|---------|
| Fe          | 242.5 mg |
| Mn          | 68.7 mg  |
| Se          | 0.36 mg  |
| I           | 0.64 mg  |
| Vit A       | 11500 IU |
| Vit D3      | 3250 IU  |
| Vit E       | 91 mg    |
| Vit K3      | 3 mg     |
| Thiamin     | 8.6 mg   |
| Riboflavin  | 10.9 mg  |
| Pyridoxine  | 9.3 mg   |
| Cobalamin   | 0.2 mg   |
| Niacin      | 62.6 mg  |
| Pantothenic acid | 26.8 mg |
| Folic acid  | 1.9 mg   |
Digestible lysine (%) 1.14
Digestible methionine (%) 0.36
Calcium (%) 0.67
Available phosphorus (%) 0.33

Supplied per kilogram of diet: Cu, 9.9 mg; Zn, 142.5 mg; Fe, 242.5 mg; Mn, 68.7 mg; Se, 0.36 mg; I, 0.64 mg; vitamin A, 11500 IU; vitamin D3, 3250 IU; vitamin E, 91 mg; vitamin K3, 3 mg; thiamin, 8.6 mg; riboflavin, 10.9 mg; pyridoxine, 9.3 mg; cobalamin, 0.2 mg; niacin, 62.6 mg; pantothenic acid, 26.8 mg; folic acid, 1.9 mg.
Supplied per kilogram of diet: Cu, 9.9 mg; Zn, 142.5 mg; Fe, 242.5 mg; Mn, 68.7 mg; Se, 0.36 mg; I, 0.64 mg; vitamin A, 11500 IU; vitamin D3, 3250 IU; vitamin E, 91 mg; vitamin K3, 3 mg; thiamin, 8.6 mg; riboflavin, 10.9 mg; pyridoxine, 9.3 mg; cobalamin, 0.2 mg; niacin, 62.6 mg; pantothenic acid, 26.8 mg; folic acid, 1.9 mg.
Calcium (%)

Available phosphorus (%)

Supplied per kilogram of diet:
- Cu, 9.9 mg
- Zn, 142.5 mg
- Fe, 242.5 mg
- Mn, 68.7 mg
- Se, 0.36 mg
- I, 0.64 mg
- vitamin A, 11500 IU
- vitamin D3, 3250 IU
- vitamin E, 91 mg
- vitamin K3, 3 mg
- thiamin, 8.6 mg
- riboflavin, 10.9 mg
- pyridoxine, 9.3 mg
- cobalamin, 0.2 mg
- niacin, 62.6 mg
- pantothenic acid, 26.8 mg
- folic acid, 1.9 mg.
| Nutrient          | Amount   |
|------------------|----------|
| Vitamin A        | 11500 IU |
| Vitamin D3       | 3250 IU  |
| Vitamin E        | 91 mg    |
| Vitamin K3       | 3 mg     |
| Thiamin          | 8.6 mg   |
| Riboflavin       | 10.9 mg  |
| Pyridoxine       | 9.3 mg   |
| Cobalamin        | 0.2 mg   |
| Niacin           | 62.6 mg  |
| Pantothenic acid | 26.8 mg  |
| Folic acid       | 1.9 mg   |

Available phosphorus (%)

0.33\(^1\) Supplied per kilogram of diet: Cu, 9.9 mg; Zn, 142.5 mg; Fe, 242.5 mg; Mn, 68.7 mg; Se, 0.36 mg; I, 0.64 mg; vitamin A, 11500 IU; vitamin D3, 3250 IU; vitamin E, 91 mg; vitamin K3, 3 mg; thiamin, 8.6 mg; riboflavin, 10.9 mg; pyridoxine, 9.3 mg; cobalamin, 0.2 mg; niacin, 62.6 mg; pantothenic acid, 26.8 mg; folic acid, 1.9 mg.
Supplied per kilogram of diet: Cu, 9.9 mg; Zn, 142.5 mg; Fe, 242.5 mg; Mn, 68.7 mg; Se, 0.36 mg; I, 0.64 mg; vitamin A, 11500 IU; vitamin D3, 3250 IU; vitamin E, 91 mg; vitamin K3, 3 mg; thiamin, 8.6 mg; riboflavin, 10.9 mg; pyridoxine, 9.3 mg; cobalamin, 0.2 mg; niacin, 62.6 mg; pantothenic acid, 26.8 mg; folic acid, 1.9 mg.

1 Supplied per kilogram of diet: Cu, 9.9 mg; Zn, 142.5 mg; Fe, 242.5 mg; Mn, 68.7 mg; Se, 0.36 mg; I, 0.64 mg; vitamin A, 11500 IU; vitamin D3, 3250 IU; vitamin E, 91 mg; vitamin K3, 3 mg; thiamin, 8.6 mg; riboflavin, 10.9 mg; pyridoxine, 9.3 mg; cobalamin, 0.2 mg; niacin, 62.6 mg; pantothenic acid, 26.8 mg; folic acid, 1.9 mg.
2.7. Effect of combination of Xyn, Afd and FE on growth and health of piglets fed bran-added diet

2.7.1. Animal experiment design

The experimental animal protocols for this study were approved by the Animal Care and Use Committee of South China Agricultural University. Duroc×(Landrace×Yorkshire) crossbred piglets at 35 d of age were raised individually in stainless steel cages and maintained in an environmentally controlled room (25°C). After acclimation to the environment and basal diet for 1 week, a total of 270 postweaning piglets were picked out and allocated into 3 treatment groups: control group (received bran-added diet), X group (received bran-added diet supplemented with 1600 U/kg Xyn), and (X+A+F) group (received bran-added diet supplemented with 1600 U/kg Xyn, 0.8 U/kg Afd and 4 U/kg FE). The enzyme preparations were provided by AsiaPac Co., Ltd (Dongguan, China) and these dosages were selected based on several preliminary experiments in pigs. Each group had 6 replicates (15 piglets/replicate). The composition and nutrient levels of bran-added diet are shown in Table 1. The experiment lasted for 21 d. The initial body weight (9.70±0.50 kg) of piglets was similar across all replicates. Piglets had free access to water and feed. Feed consumption and final body weight (FBW) were recorded at 21 d of the experiment for calculating average daily gain (ADG), average daily feed intake (ADFI) and feed conversion ratio (FCR). Meanwhile, individual pigs were examined for diarrhea two times per day during the experimental period to calculate the diarrhea rate, which was evaluated by fecal consistency scoring using a four-grade system [29]. The occurrence of diarrhea was defined as maintaining a score of 3 for two days or a score of 4 for one day. The diarrhea rate (%) was calculated as the sum of the number of diarrheal piglets divided by the total number of piglets.

At 21 d of the experiment, one piglet per replicate was randomly selected and slaughtered by severing the jugular vein, followed by separation of the intestine and collection of the digesta of cecum and colon.

2.7.2. Assay of intestinal pH value and volatile fatty acid (VFA) concentrations of piglets

Immediately after cutting open the cecum and colon, the pH value of digesta in the mid-segments of cecum and colon was measured with a DELTA320 pH meter (Mettler Toledo, Switzerland). The VFA concentrations in cecal and colonic digesta were determined using gas chromatography GC-17A (Shimadzu, Japan) with a flame ionization detector fitted with a Nukol FFAP capillary column (30 m × 0.32 mm × 0.25 μm, Supelco, USA) according to the method reported elsewhere [30].

2.8. Statistical analysis

Data were presented as mean±standard deviation and analyzed by one-way ANOVA using SPSS 18.0 software. Differences among treatment groups were detected by using Duncan’s multiple range test. Significance was defined as $P < 0.05$ and $0.05 \leq P < 0.10$ was considered to be a tendency toward significance.

3. Results and Discussion

3.1. Enzymological characteristics of Xyn, Afd and FE

The activity of Xyn peaked at pH 5.0 and declined gently with the deviation of pH value from 5.0 (Figure 1A). Xyn maintained more than 50% relative activity in a wide temperature range (30°C–90°C), with the value peaking at 40°C (Figure 1B). In comparison, Afd had the highest activity at pH 6.0 and retained more than 60% relative activity when pH value ranged from 3.0 to 9.0 (Figure 1C). The relative activity of Afd was kept above 60% at the temperature range of 30°C–85°C and maximized at 40°C (Figure 1D). FE kept over 60% relative activity during pH 3.0 to 9.0 and the optimum pH for its activity was 5.0 (Figure 1E). FE retained more than 60% relative activity during 30°C to 80°C, with 50°C being the optimal temperature (Figure 1F).

Strikingly, both Xyn, Afd and FE retained more than 50% relative activity when incubated at pH 3.0 for 30 min (Figure 2). Xyn and Afd maintained more than 70% relative activity while FE retained approximately 40% relative activity when subjected to high
temperature (85°C) treatment for 3 min. The above results suggested that both Xyn, Afd and FE particularly the first two had a relatively high thermal endurance and acid resistance, which enabled a feasibility of these enzymes applied in diet to withstand the hostile conditions in diet processing and gastric digestion.
Figure 1. The relative activities of xylanase (A, B), arabinofuranosidase (C, D) and feruloyl esterase (E, F) at different pH values and temperatures.

Figure 2. The thermal endurance and acid resistance of xylanase (Xyn), arabinofuranosidase (Afd) and feruloyl esterase (FE).

3.2. Effect of Xyn combined with Afd on degradation of Abx in DSWB

Xyn treatment alone sharply increased \((P < 0.05)\) the RRS from DSWB (Figure 3), hinting that Xyn exerted a leading role among consortium of xylanolytic enzymes in degradation of Abx that subsequently released reducing sugars [3]. Despite the capacity of Xyn to stochastically break Abx backbone into smaller fragments, the existence of arabinose groups attached to the backbone could somewhat reduce the degradation efficiency of Abx through hampering the recognition of cleavage sites within the backbone by Xyn [9,10]. Nevertheless, this steric hindrance can be alleviated by the usage of Afd that removes arabinose groups from side chains, thus providing a convenience for further action of Xyn on Abx [3,10]. In support of this view, we noted that combination of Xyn and Afd at a ratio ranging from 3000:1 to 100:1 resulted in a more pronounced \((P < 0.05)\) RRS from DSWB relative to Xyn acting alone (Figure 3), validating a synergistic interaction between Xyn and Afd on degradation of Abx in DSWB [31,32]. Intriguingly, there was almost no elevation in the RRS with the increased ratio of Xyn dose to Afd dose. Thereby, the combination of Xyn treatment (3 U/g) and Afd treatment (0.001 U/g) at a ratio of 3000:1 was selected for further analysis.
3.3. Effect of Xyn combined with FE on degradation of Abx in DSWB

As exhibited in Figure 4, combination of Xyn and FE at varying ratios (from 3000:1 to 100:1) displayed an advantage (\(P < 0.05\)) over Xyn alone in promoting the RRS from DSWB, which emphasized a synergy between Xyn and FE on degradation of Abx in DSWB. It has been indicated that the arabinose residues linked in Abx backbone can further be substituted at the O-5 position by ferulic acid ester [3], an another essential side chain group of Abx to impede the depolymerization of Xyn, therefore retarding Abx degradation [33]. This steric hindrance effect can be attenuated by the action of FE capable of catalyzing hydrolysis of ferulic acid ester bonds attached to Abx side chains [34], which might account for the observed advantage of combination of Xyn and FE over Xyn alone. Similarly, it was reported that the release of xylo-oligosaccharide or total reducing sugars from Abx-riched crop products under the synergy between Xyn and FE was higher than that under Xyn acting alone [16,20]. Notably, due to generation of the highest RRS from DSWB, the combination of Xyn (3 U/g) and FE (0.006 U/g) at a ratio of 500:1 was used for further analysis.

3.4. Synergy among Xyn, Afd and FE on degradation of Abx in different brans

In the present study, WB-SF, WB-IF, OB-SF and OB-IF were employed as different Abx sources to validate the synergy among Xyn, Afd and FE at optimum doses (3, 0.001 and 0.006 U/g, respectively). The yield of WB-SF, WB-IF, OB-SF and OB-IF was 2.75%, 40.25%, 7.4% and 8.7%, respectively (Table 2), while the extraction ratio of these bran fibers was 94.82%, 95.83%, 98.67% and 95.18%, respectively.

As shown in Figure 5A, combining Xyn with Afd increased \((P < 0.05)\) the RRS from both WB-SF, WB-IF, OB-SF and OB-IF as compared with Xyn acting alone, while combination of Xyn and FE caused an increase in the RRS from OB-SF and OB-IF rather than WB-SF and WB-IF. The difference between the degrading actions on OB fiber and WB
fiber might be somewhat related to their different level of ferulic acid ester that can be targeted by FE [35]. Abx contains a linear backbone of xylopyranosyl residues that are substituted with various degrees of side chains including L-arabinose and ferulic acid groups [3,36]. For most cereals, arabinose monomer seems to be the main group linked as the side chain of Abx [36]. It was thus deduced that Afd might elicit a more momentous role than FE in removing the side chains of Abx, probably being more beneficial for depolymerization of Abx in cereal brans by Xyn. This might account for the current result that combining Xyn with Afd led to a higher RRS from Abx sources (especially WB fiber) in comparison with combination of Xyn and FE, as well as the result that the synergy degree between Xyn and Afd was obviously greater than that between Xyn and FE (Figure 5B). Strikingly, integration of Xyn, Afd and FE induced an increase ($P < 0.05$) in the RRS from all bran fibers relative to either combination of Xyn and Afd or of Xyn and FE, highlighting that combination of Xyn, Afd and FE had a pronounced superiority over combination of Xyn and Afd or of Xyn and FE in promoting Abx degradation of different brans, which corresponded to the higher synergy degree among these three enzymes than that between Xyn and Afd or between Xyn and FE (Figure 5B). The above results raised a necessity of combining Xyn with Afd and FE to promote full degradation of Abx in brans, most likely due to that cooperation of debranching enzymes (Afd and FE) was more efficient in eliminating the spatial obstacles (branch points) that limited the formation of enzyme-substrate complex in the backbone of Abx [37], thus favoring the access of Xyn to Abx and its depolymerization.

|                     | Wheat bran | Oat bran |
|---------------------|------------|----------|
|                     | Yield (%)  | Extraction ratio (%) | Yield (%) | Extraction ratio (%) |
| Soluble fiber       | 2.75       | 94.82     | 7.40       | 98.67     |
| Insoluble fiber     | 40.25      | 95.83     | 8.70       | 95.18     |
3.5. Effect of combination of Xyn, Afd and FE on in vitro digestion of bran-added diet

Xyn treatment was legitimately effective in promoting digestion of dietary fibers in animals [38-40], nevertheless, little study was available concerning the effect of Xyn combined with debranching enzymes on nutrient digestibility of piglets. In this study, we found that treatment with combination of Xyn, Afd and FE had a potential to improve nutrient digestibility, as manifested by the increasing trends ($P < 0.10$) of in vitro digestibility of dry matter, crude protein, crude ash and gross energy of bran-added diet (Figure 6). It was possible that the observed superiority of combination of Xyn, Afd and FE over Xyn alone in Abx degradation might translate into a benefit for cell wall destruction of brans or some other plant feedstuffs in diet, which potentially allowed an enhanced access of digestive enzymes to the cell wall and/or intracellular constituents of these ingredients [15,41], consequently favoring digestion of dietary nutrients. Alternatively, the mechanism underlying the increased nutrient digestibility might be associated with that the efficient degradation of Abx in diet under the synergy among Xyn, Afd and FE profited the lowering of digesta viscosity, which could facilitate a sufficient contact between digesta and digestive enzymes with a subsequent improvement of nutrient digestion [4,5].

Figure 5. Synergistic action among xylanase (Xyn), arabinofuranosidase (Afd) and feruloyl esterase (FE). (A) the release of reducing sugar from different arabinoxylan sources; (B) the value of synergy degree among Xyn, Afd and FE. *=Values with different superscripts differ significantly ($P < 0.05$). WB, wheat bran; WB-SF, soluble fiber of wheat bran; WB-IF, insoluble fiber of wheat bran; OB, oat bran; WB-SF, soluble fiber of oat bran; WB-IF, insoluble fiber of oat bran. X: 3 U/g Xyn; (X+A): 3 U/g Xyn + 0.001 U/g Afd; (X+F): 3 U/g Xyn + 0.006 U/g FE; (X+A+F): 3 U/g Xyn + 0.001 U/g Afd + 0.006 U/g FE.
3.6. Effect of combination of Xyn, Afd and FE on growth performance of piglets fed bran-added diet

Combination of Xyn, Afd and FE did not affect ADFI of piglets, but had an advantage over Xyn alone to induce increasing trends ($P < 0.10$) of FBW and ADG along with a decreasing trend ($P < 0.10$) of FCR of postweaning piglets fed bran-added diet (Figures 7A–D). Although numerous studies evidenced a variable efficacy of Xyn treatment alone in promotion of growth performance of pigs [38-40], there was little study regarding the effect of combination of Xyn, Afd and FE on pig growth performance. The results obtained herein revealed a potential of consortium of Xyn, Afd and FE to improve growth rate and feed efficiency of piglets, which was most likely responsible by the observed elevation in nutrient digestibility of diet treated with combination of Xyn, Afd and FE. With respect to the diarrhea rate of piglets, it was found to be reduced ($P < 0.05$) by the treatment with either Xyn alone or combination of Xyn, Afd and FE (Figure 7E), with a lower ($P < 0.05$) diarrhea rate in combined treatment group versus Xyn alone group. It was probable that the elevated nutrient digestibility of diet induced by these enzyme consortium led to reductions of nutrient residuals utilized by certain pathogens in hindgut, presumptively decreasing the prevalence of diarrhea of piglets [42,43]. Alternatively, the efficient degradation of Abx in diet under the synergy among Xyn, Afd and FE could lower the viscosity of chyme, which was hypothesized to avoid overgrowth of intestinal harmful bacteria and in turn reduce diarrhea rate of piglets [4, 44].
Figure 7. Effect of combination of xylanase (Xyn), arabinofuranosidase (Afd) and feruloyl esterase (FE) on growth performance of piglets received bran-added diet. (A) final body weight; (B) average daily gain; (C) average daily feed intake; (D) feed conversion ratio; (E) diarrhea rate. *Values with different superscripts differ significantly \((P < 0.05)\). Control: treatment without enzymes; X: treatment with 1600 U/kg Xyn; (X+A+F): treatment with combination of 1600 U/kg Xyn, 0.8 U/kg Afd and 4 U/kg FE.

3.7. Effects of combination of Xyn, Afd and FE on intestinal VFA profile and pH value of piglets fed bran-added diet

Treatment with combination of Xyn, Afd and FE or Xyn alone increased \((P < 0.05)\) the concentrations of acetic acid and total VFA in cecum and colon as well as butyric acid in cecum of piglets fed bran-added diet, with higher \((P < 0.05)\) concentrations of these parameters in combined treatment group versus Xyn treatment alone (Figures 8A–D). Moreover, there was an elevation \((P < 0.05)\) in cecal and colonic propionic acid concentration in response to the treatment with combination of Xyn, Afd and FE (Figure 8B). VFA produced by bacterial fermentation of dietary fibers have long been characterized as a typical improver of gut health of piglets [30]. It was reported that dietary Xyn treatment could modulate fermentation pattern of gut microbiota and increase the concentrations of certain VFA in gut of pigs fed Abx-rich diet [38,39]. However, the role of synergy among Xyn, Afd and FE on gut health of pigs remains unknown. Our results revealed a distinct advantage of combination of Xyn, Afd and FE over Xyn alone in improving intestinal VFA profile, which might be associated with that the degradation of dietary Abx into certain oligosaccharides under the synergy among Xyn, Afd and FE improved gut fermentation by several beneficial bacteria (namely the prebiotic effect) [4,39].

The elevated concentrations of certain VFA in gut induced by combination of Xyn, Afd and FE were postulated to translate into a lower pH value of gut. Although treatment with Xyn alone had little influence \((P > 0.05)\) on intestinal pH (Figure 8E), we indeed found a reduction \((P < 0.05)\) of cecal pH value together with a decreasing trend \((P < 0.10)\) of colonic pH value in piglets treated with combination of Xyn, Afd and FE when compared with either control or Xyn alone, which roughly coincided to the simultaneous increases in cecal and colonic concentrations of VFA as a result of treatment with combination of Xyn, Afd and FE. We speculated that the increased concentrations of VFA along with the decrease of pH value in gut due to treatment with combination of Xyn, Afd and FE were at least partially responsible for the observed reduction of diarrhea rate of piglets, because acid microenvironment in gut could resist pathogen invasion as well as boost gut health and growth performance of piglets [45,46].
Effects of combination of Figure 8. Effects of combination of xylanase (Xyn), arabinofuranosidase (Afd) and feruloyl esterase (FE) on volatile fatty acid concentrations and pH value in intestinal digesta of piglets received bran-added diet.

Values with different superscripts differ significantly (*P* < 0.05). Control: treatment
nation of Figure 8. Effects of combination of xylanase (Xyn), arabinofuranosidase (Afd) and feruloyl esterase (FE) on volatile fatty acid concentrations and pH value in intestinal digesta of piglets received bran-added diet. *Values with different superscripts differ significantly \((P < 0.05)\). Control: treatment without enzymes; X: treatment with 1600 U/kg Xyn; (X+A+F): treatment with combination of 1600 U/kg Xyn, 0.8 U/kg Afd and 4 U/kg FE.

4. Conclusions

The present study evidenced relatively high thermal endurance and acid resistance of Xyn, Afd and FE, which were suitable to be applied in animal diet. Combining these enzymes had a superiority over Xyn acting alone and its combination with Afd or FE in degradation of Abx in different bran fibers. Dietary treatment with combination of Xyn, Afd and FE had advantages over Xyn alone to improve nutrient digestion, growth performance and intestinal VFA profile of piglets received bran-added diet.

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