Dietary supplementation with Acanthopanax senticosus extract enhances gut health in weanling piglets

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The present study was conducted to investigate the effects of Acanthopanax senticosus extract (ASE) as a dietary additive on gut health in weanling piglets by examining diarrhea frequency, intestinal microbiota and morphology. A total of 96 Duroc× (Landrace×Yorkshire) piglets weaned at 21 days of age with an average initial body weight (BW) of 5.6±0.4 kg were randomly assigned to 3 treatment groups with 4 duplicates of 8 piglets each. The piglets were fed basal diet to which had been added 0 or 1 g/kg of ASE, or 0.7 g/kg antibiotics, respectively. Fecal consistence was monitored twice daily and the frequency of diarrhea was calculated. On day 21 after the initiation of supplementation, 8 piglets were randomly selected from each treatment group (2 piglets per pen) and slaughtered. The jejunum, ileum, colon and cecum were then excised and fixed in 10% neutral formalin solution to determine villus height and crypt depth, after their contents were collected to determine microbiota. The results showed that dietary supplementation with ASE increased (\( P<0.05 \)) the density of bacterial populations that co-migrated with Lactobacillus amylovorus, Lactobacillus salivarius, Bacillus subtilis, and Clostridium lituseburens, but decreased (\( P<0.05 \)) those co-migrating with Staphylococcus aureus, Salmonella typhimurium, Ruminococcus forques, and E. coli O157:H7 in the PCR-DGGE profiling analysis when compared with the control group. The villus height of the duodenum, jejunum and ileum increased (\( P<0.05 \)) by 14.8, 13.7 and 10.0%, while the crypt depth decreased (\( P<0.05 \)) by 17.9, 9.1 and 12.1%, respectively, in response to dietary ASE supplementation. Additionally dietary supplementation with ASE or an antibiotic decreased (\( P<0.05 \)) the frequency of diarrhea by 55.6 and 52.2%, respectively, compared with the control group. In conclusion, these findings suggest that dietary supplementation with ASE could regulate the microbiota composition and maintain a normal morphology of gut mucosa in weanling piglets, thereby decreasing diarrhea that resulted from weaning stress.

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Keywords:
Acanthopanax senticosus extract
Diarrhea
Gut health
Weanling piglets

1. Introduction

In modern intensive swine-production systems, piglets are weaned between 14 to 28 days of age to maximize whole-herd production. Early weaning is commercially advantageous but is generally considered to be a stressful event and is often associated with a period of depressed feed intake and growth
2. Materials and methods

2.1. Preparation of ASE

ASE was prepared by decocting the dried herb in boiling distilled water (200 g/L) for 2 h. The ASE decoction was filtered, lyophilized and kept at 4 °C. The yield of extraction was about 25% (w/w). The water-extracted powder was dissolved in sterile saline (1 g/ml). The contents (g/kg) of total polysaccharides, flavone and organic acids in the ASE were 29.4, 1.9 and 10.4, as determined by the vitriol-anthracene ketone, rutin (Kong et al., 2004) and alkaliometric titration (Cai et al., 2000) methods, respectively. The contents (g/kg) of AA in the extract, as analyzed by high-pressure liquid chromatography (HPLC, Hitachi L-8800 Auto-Analyzer, Tokyo, Japan) method were: Phe 4.11; Leu 2.32; Ile 0.67; Val 0.77; Ala 2.14; Gly 1.87; Asp 2.86; Glu 4.71; Cys 2.45; His 0.41; Lys 0.95; Arg 3.78; Thr 1.30; Ser 2.47 and Met 0.28.

2.2. Animal housing and treatment

A total of 96 Duroc × (Landrace × Yorkshire) piglets weaned at 21 days of age with an average initial body weight (BW) of 5.6 ± 0.4 kg were randomly assigned into one of 3 groups based on litter, BW and sex. There were 4 pens per treatment group, with 8 piglets (4 barrows and 4 gilts) per pen. The 3 treatment groups received a maize-soybean-based diet [based on National Research Council (NRC, 1998) requirements] that had been supplemented with 0 or 1 kg/kg of ASE, or 0.7 g/kg antibiotics (10% bacitracin zinc 400 mg + 15% carboxad 300 mg) (Table 1). The basal diet did not contain antibiotics or growth-promoting levels of Cu and Zn. The piglets were housed in an environmentally-controlled nursery facility (temperature, 20–27 °C; relative humidity, 60–70%; lighting cycle, 12 h/d) with a hard-plastic completely slatted flooring, and had free access to food and drinking water. The study was carried out in accordance with the Chinese guidelines for animal welfare and experimental protocols.

### Table 1

Dietary ingredients and main nutrient levels (g/kg, as-fed basis).

<table>
<thead>
<tr>
<th>Dietary ingredients</th>
<th>Control</th>
<th>Acanthopanax senticosus extract</th>
<th>Antibiotics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn (CP 78.4%)</td>
<td>572.0</td>
<td>571.1</td>
<td>571.3</td>
</tr>
<tr>
<td>Soybean meal (CP 43%)</td>
<td>180.0</td>
<td>180.0</td>
<td>180.0</td>
</tr>
<tr>
<td>Ferrum soybean meal (CP 51.5%)</td>
<td>68.0</td>
<td>68.0</td>
<td>68.0</td>
</tr>
<tr>
<td>Whey powder</td>
<td>50.0</td>
<td>50.0</td>
<td>50.0</td>
</tr>
<tr>
<td>Soybean meal oil</td>
<td>30.0</td>
<td>30.0</td>
<td>30.0</td>
</tr>
<tr>
<td>Fish meal (CP 63.5%)</td>
<td>60.0</td>
<td>60.0</td>
<td>60.0</td>
</tr>
<tr>
<td>Zeolit meal</td>
<td>9.0</td>
<td>9.0</td>
<td>9.0</td>
</tr>
<tr>
<td>Acanthopanax senticosus extract</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Antibiotics</td>
<td>0</td>
<td>0.7</td>
<td>0.7</td>
</tr>
<tr>
<td>Calcium phosphate dibasic</td>
<td>12.0</td>
<td>12.0</td>
<td>12.0</td>
</tr>
<tr>
<td>CaCO₃</td>
<td>6.0</td>
<td>6.0</td>
<td>6.0</td>
</tr>
<tr>
<td>Premix a</td>
<td>12.5</td>
<td>12.5</td>
<td>12.5</td>
</tr>
</tbody>
</table>

**Main nutrient levels**

| Digestible energy (MJ/kg) | 14.30 | 14.30 | 14.30 |
| Crude protein            | 199.6 | 199.4 | 198.6 |
| Calcium                  | 7.8   | 7.8   | 7.8   |
| Available phosphorus     | 5.8   | 5.8   | 5.8   |
| Lysine                   | 13.6  | 13.6  | 13.6  |
| Methionine               | 4.3   | 4.3   | 4.3   |
| Threonine                | 9.0   | 9.0   | 9.0   |

* a The premix provides the following for kilogram of diets: VD₃ 386 IU; VA 3086 IU; VE 15.4 IU; VK₃ 2.3 mg; VB₆ 3.9 mg; D-calcium pantothenate 15.4 mg; nicotinic acid 23 mg; choline 500 mg; VB₁₂ 0.016 mg; Cu (Cu-Cu, 21%) 17 mg; Fe (Fe-Cu 14%) 133 mg; Zn (Met-Zn 17.5%) 133 mg; Mn (Mn-Mn 22%) 33.3 mg; I (I₂I₃·I₅) 0.83 mg; choline chloride (50%) 1000 mg; antimied/acidifying agent (propanic acid) 2.5 g; antioxidant (ethylene vitamin) 200 mg; edulcorant (crystallose) 400 mg; flavor 600 mg; salt 1.3 g; lysine·HCl 2.7 g; methionine 660 mg; threonine 440 mg. All of the feedstuffs were provide by Hunan Guang'an Biology Technology Co. Ltd., Changsha, Hunan, China.
2.3. Sampling and preparations

At the beginning of experiment, another 8 piglets at 21 days of age with an average initial BW of 5.6 ± 0.4 kg from the same pig barn were slaughtered under general anaesthesia and then immediately eviscerated. General anaesthesia was administered via intravenous injection of 4% sodium pentobarbital solution (40 mg/kg BW) and killed by jugular puncture (Kong et al., 2007b). At the end of the experiment, 8 randomly selected pigs from each treatment (one barrow and one gilt per pen) were slaughtered as described above. Samples of the digesta from the same parts of the jejunum, ileum, colon, and cecum were collected and placed in separate sterile tubes on ice until they were transported to the lab for an analysis of intestinal microbiota. Digesta collection and bacterial sample preparation were performed as described previously. The specimens from the same intestine (duodenum, jejunum, and ileum) were excised, flushed with physiological saline and fixed in 10% neutral formalin solution to determine gut morphology.

2.4. Diarrhea frequency

To evaluate the frequency of diarrhea, the number of pigs per pen that suffered diarrhea was recorded daily throughout the study. Fecal consistence was monitored twice daily and quantified using a scale ranging from 0 to 3, with 0 = normally shaped feces, 1 = shapeless (loose) feces, 2 = thick, liquid (soft) feces, and 3 = thin, liquid feces (watery diarrhea). When the score was higher than 1, the piglet was considered to have diarrhea. The frequency of diarrhea per pen was determined as described by Vente-Spreeuwenberga et al. (2004).

Diarrhea frequency = pigs per pen suffering diarrhea / (8 experimental pigs per pen × 21 days) × 100%.

The frequency of diarrhea per treatment group is shown as the mean ± SEM calculated using the data from 4 pens per treatment group.

2.5. Intestinal microbiota

2.5.1. Extraction and purification of DNA from gut digesta

A global assessment of gut microbiota and their development was conducted by a molecular analysis of 16S rRNA genes using the PCR-DGGE technique. All of the digesta from the 8 pigs per group were pooled and mixed as one sample, kept on ice, and processed further within 2 h. After being washed 3 times in saline containing 0.1% Tween 80 with vigorous shaking by hand for 5 min per wash, bacterial cells were released from the digesta, and then pelleted by centrifugation (27,000 × g for 20 min) at 4 °C. The total DNA from ~200 mg of digesta was isolated and purified by a QIAamp DNA Stool Kit (stool protocol; Qiagen, Hilden, Germany) (Gong et al., 2002). Briefly, digesta in TE buffer was thawed on ice, centrifuged and resuspended in 60 µl of PBS. The samples were treated with buffer ALT, proteinase K, RNase A and buffer AL according to the manufacturer's instructions. The DNA was precipitated with ethanol, purified on a QIAamp spin column and eluted in 50 µl of AE buffer (10 mM Tris–HCl, 0.5 mM EDTA, pH 9.0).

2.5.2. PCR amplification

For PCR-DGGE analysis of total bacteria, each DNA sample was standardized to 20 µg/ml and then amplified using primers specific for conserved sequences flanking the variable V3 region of 16S rDNA (Li et al., 2003a,b). Some of the 16S rRNA genes were amplified by PCR using a pair of universal PCR primers to bacteria, followed by DGGE analysis. The DGGE profiles of microbiota from the piglets with different treatments were compared and analyzed. 16S rRNA genes were amplified by PCR from genomic DNA of content bacteria using universal bacterial primers HAD1–GC (5′–CCG CGG CGC GCC CGC CGG GGG GCG GCG GGG GCG GGG GCC GCA CGT CAC CTT TAC GGG AGG CAG CAG T-3′) and HAD2 (5′–GTA TTA CCG CCG CTG CTG GCA C-3′). PCR mixtures were the same as described previously (Gong et al., 2002). The amplification program was 93 °C for 2 min and 30 cycles of 93 °C for 30 s, 57 °C for 30 s, and 72 °C for 30 s, followed by 2 min at 72 °C.

2.5.3. DGGE analysis

Parallel DGGE was performed essentially as described previously (Simpson et al., 2000) using a Bio-Rad D-Code System™ (Bio-Rad, CA, USA). PCR fragments were separated using 8% polyacrylamide gels with 1.0×TAE buffer (20 mM Tris–acetate, pH 7.4, 10 mmol/L sodium acetate, 0.5 mmol/L Na2EDTA) with 35–60% linear gradients of denaturant (100% denaturant corresponds to 7 mol/l urea and 40% deionized formamide). The polyacrylamide was diluted from a nondeionized 40% acrylamide/bis stock solution 37:5: 1 (Bio-Rad, CA, USA). Gradients were formed using a Bio-Rad Gradient Former™ Model 385 and gels were polymerized onto a gel support using 8% polyacrylamide gels with 1.0×TAE buffer (20 mM Tris–acetate, pH 7.4, 10 mmol/L sodium acetate, 0.5 mmol/L Na2EDTA) with 35–60% linear gradients of denaturant (100% denaturant corresponds to 7 mol/l urea and 40% deionized formamide). The polyacrylamide was diluted from a nondeionized 40% acrylamide/bis stock solution 37:5: 1 (Bio-Rad, CA, USA). Gradients were formed using a Bio-Rad Gradient Former™ Model 385 and gels were polymerized onto a gel support film (FMC, ME, USA). PCR samples were applied to gels in aliquots of 5 µl per lane. Electrophoresis was performed at 60 °C for 16 h at 100 V. Additionally, bacterial reference ladders representing known bacterial strains were loaded to allow standardization of band migration and gel curvature among different gels. The reference ladder included the following species, listed in order from the top of the gel to the bottom: Staphylococcus aureus, Lactobacillus amylovorus, Lactobacillus salivarius, Ruminococcus japonica, Bacillus subtilis, E. coli O157:H7, Clostridium perfringens, Salmonella typhimurium, and Clostridium lituseburens. After electrophoresis, the gels were silver-stained and scanned using a GS-710 calibrated imaging densitometer (Bio-Rad). Each individual amplicon was then visualized as a distinct band that represented at least one bacterial species on the gel.

2.5.4. DGGE image analysis

DGGE images were analyzed using Quantity One v. 4.5.2 software (Bio-Rad, CA, USA). The software was configured to automatically detect bands on gels. Automatic band detection criteria were identical on all lanes for each gel. When gel imperfections and features were automatically detected as bands by the software, these false bands were manually removed and not included in subsequent numerical analyses. Anomalous staining residues (spotting and peppering) were removed from digital images of gels as necessary. DGGE profiles were also compared using Sorenson’s index, a pairwise similarity coefficient Cj, which was determined by: Cj = |2m + 2| / (ac + b) × 100, where a is the number of DGGE bands in lane 1, b is the number of DGGE bands in lane 2, and j is the number of common DGGE bands (Gillan et al., 1998; Simpson et al., 2000).
2.6. Histomorphometry

Three cross-sections were prepared for each intestinal sample after staining with hematoxylin and eosin using standard paraffin embedding procedures (Xu et al., 2003). A total of 10 intact, well-oriented crypt-villus units were selected in triplicate for each intestinal cross-section (30 measurements for each sample, total of 240 measurements per dietary treatment). Villus height and crypt depth were determined using an image-processing and analysis system (version 1, Leica Imaging Systems Ltd., Cambridge, England).

2.7. Statistical analysis

The experimental unit in the present study was the pen and there were 4 replicates per treatment group. Data were expressed as mean ± SEM and statistically analyzed using GLM procedure of the Statistical Analysis System (2000, SAS Institute, Cary, NC). Duncan’s multiple range test was used to compare differences among the 3 treatment groups. The alpha level for determining significance was 0.05.

3. Results

3.1. Effects of ASE on diarrhea frequency

The effects of ASE and antibiotic on the frequency of diarrhea in the weaned pigs are presented in Fig. 1. Dietary supplementation with ASE or antibiotic decreased ($P < 0.05$) the frequency of diarrhea by 55.6 and 52.2%, respectively, compared with the control group. There was no significant difference between the antibiotic- and ASE-supplemented groups.

3.2. Effects of ASE on intestinal microbiota

Fig. 2A shows PCR-DGGE profiles of V3 amplicons of 16S rRNA genes obtained from microbiota in the digesta of the jejunum (5–8) and cecum (1–4) of piglets in the antibiotic (2, 5), ASE (3, 7) and control (1, 6) groups on day 21, as well as day 0 (4, 8). Fig. 3A shows PCR-DGGE profiles of V3 amplicons obtained from microbiota in the digesta of the ileum (2–5) and colon (6–9) of piglets in the antibiotic (2, 9), ASE (3, 8) and control (4, 7) groups on day 21, as well as day 0 (5, 6). The changes in the number and intensity of bands in the DGGE profiles suggested the changes in the richness of bacterial populations in the different treatment groups, as also indicated in Figs. 2B and 3B. The effects of ASE and antibiotic on the richness of intestinal bacterial populations in weaned piglets are summarized in Tables 2 and 3.

As shown in Table 2, the number of bands of intestine microbiota gradually increased from the foregut to hindgut in...
both pre-weaned piglets and weaned piglets, and the colon had the richest bacterial populations. The jejunum (11 vs 6), ileum (13 vs 9), cecum (13 vs 10) and colon (16 vs 10) all had richer bacterial populations on day 21 after weaning than on day 0 (pre-weaning). Dietary supplementation with ASE or antibiotic for 21 days had poorer bacterial populations in the jejunum (10 or 8), ileum (11 or 9), cecum (8 or 10) and colon (13 vs 12) compared to those in the control group (11, 13, 13, and 16).

As summarized in Table 3, the change in the intensity of bands in DGGE images (Figs. 2A and 3B) suggested that dietary supplementation with ASE increased the abundance of bacterial species with a similar distance of migration to the DNA references, including *L. amylovorus*, *L. salivarius*, *B. subtilis* and *C. lituseburens* in the jejunum, *B. subtilis* in the ileum, *L. amylovorus* and *L. salivarius* in the cecum, and *L. amylovorus* in the colon. The change also suggested a decrease of the abundance of bacterial species with a similar distance of migration to the DNA references, including *S. aureus*, *R. forques*, *C. perfringens* and *S. typhimurium* in the jejunum, *E. coli* O157:H7 and *C. perfringens* in the ileum, and *S. typhimurium* in the colon, compared with the control group. The bacterial species co-migrating with the DGGE band of *E. coli* O157:H7 in the cecum and with *C. perfringens* in the cecum and colon decreased in response to antibiotic supplementation, as did those with *R. forques* and *E. coli* O157:H7 in the jejunum, while the bacterial species co-migrating with *L. salivarius* in the jejunum, cecum and colon, with *L. amylovorus* in the jejunum decreased compared to the levels in pre-weaned piglets.

3.3. Effects of ASE on morphology in the small intestine

Data from histometric analysis (Table 4) indicated that both villus height and crypt depth in the ASE-supplemented piglets were greater (*P*<0.05) than those in pre-weaned piglets. Dietary supplementation with ASE increased (*P*<0.05) the villus height in the duodenum, jejunum and ileum by 14.8, 13.7, and 10.0%, and decreased (*P*<0.05) crypt depth in the duodenum, jejunum and ileum by 17.9, 9.1, and 12.1% respectively, in comparison with the control group. The piglets that were fed the ASE-supplemented diet had a greater (*P*<0.05) villus height in the duodenum and jejunum, and a

<table>
<thead>
<tr>
<th>Item</th>
<th>Day 0</th>
<th>Day 21 after supplementation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>ASE</td>
</tr>
<tr>
<td>Jejunum</td>
<td>6</td>
<td>11</td>
</tr>
<tr>
<td>Ileum</td>
<td>9</td>
<td>13</td>
</tr>
<tr>
<td>Cecum</td>
<td>10</td>
<td>13</td>
</tr>
<tr>
<td>Colon</td>
<td>10</td>
<td>16</td>
</tr>
</tbody>
</table>

The data are numbers of bands in DGGE lanes which showed that the complexity of bacterial population in the different treatment groups, as also indicated in Figs. 28 and 38, respectively.
low (P<0.05) crypt depth in the jejunum and ileum compared with the antibiotic-supplemented group.

4. Discussion

It is well known that the abrupt changes in feed composition and feeding conditions at weaning cause a dramatic change in digestive function, which often results in the intestinal malabsorption of nutrients (Wu, 1998). Weaning piglets exhibit an increased susceptibility to gram-negative bacterial (such as E. coli) infections (Naburu, 1995). Therefore, weaning is associated with growth retardation as well as an increase in both morbidity and mortality in piglets (Wilson et al., 1989). Weaning-induced damage to the gastrointestinal tract and the inadequate development of digestive enzymes affect nutrient absorption, and result in diarrhea. Madec et al. (1998) reported that more than 80% of the mortality in piglets during the first month post-weaning seems to be caused by digestive disorders alone.

A novel and important finding in the present study is that dietary supplementation with ASE or antibiotic decreases the frequency of diarrhea by 55.6 and 52.2%, respectively, compared with the control group (Table 4). Diarrhea results from an increase in water secretion from the intestinal epithelial cells and/or a decrease in the absorption of water and nutrients from the intestinal lumen. Thus, it is likely that ASE regulates these two physiological processes by improving the metabolism of nutrients (especially AA) and enhancing the anti-oxidant activity in the small-intestinal mucosa (Yang et al., 2004). Alternatively, dietary supplementation with Chinese herbal extracts may modulate immune function (Kong et al., 2007b), thereby reducing the inflammation in the small-intestinal mucosa that often occurs in weanling piglets (Yamazaki et al., 2007). Although the precise mechanisms are not clear, our results demonstrate the feasibility of using Chinese herbal extracts as natural dietary additives for early-weaned piglets, as a replacement for feed antibiotics.

The gut plays an important role in the digestion, absorption, and metabolism of nutrients. Findings from the present study demonstrate that piglets showed a decrease in villus height and an increase in crypt depth (P<0.05) during the weaning process (Table 4), which are consistent with the results reported by McCracken et al. (1999). Villus height and crypt depth are indirect indications of the maturity and functional capacity of enterocytes, and larger villi and crypts are associated with a greater number of enterocytes (Hampson et al., 1985). The above findings suggest that ASE could effectively improve the
recovery of stress-induced damage to the gut morphology, which might be a potential mechanism by which ASE increases gut health and prevents diarrhea in weaned piglets.

Many studies have focused on the changes in microbial communities in weaned piglets and found that nutritional manipulation during the weaning period could alter the diversity or composition of microbiota in the gastrointestinal tract (Hampson et al., 1985; Dritz et al., 1996; Gong et al., 2008). In this study, the PCR-DGGE technique was used to determine the microbial diversity and banding patterns in the intestinal tract of piglets. The PCR-DGGE approach is not used to quantify bacterial species, but can provide information on shifts in the bacterial community by showing changes in the predominant bacterial species (Muyzer and Smalla, 1998). In the present study, the results of PCR-DGGE indicated that the weaned piglets have a semi-developed and unstable gastrointestinal microbial ecology with richer microbiota (Table 2). There are several possible explanations for these changes: 1) functional disturbances in digestion and absorption may cause a nutrient surplus in the posterior segment of the small intestine, which leads to bacterial overgrowth; 2) a change in the diet from milk to feed may decrease the pH of the gastrointestinal tract; and 3) this may result in a net decrease in bacterial population coexisting with L. amylovorus and an increase with E. coli as revealed by DGGE profiling.

Xu et al. (2003) reported that Chinese herbs could reduce the density of enterotoxigenic E. coli and increase the density of Bacillus acidilactici or Bacillus bifidus. The results from the present experiments also suggest the potential of ASE as a dietary additive could suppress pathogenic bacteria and enriching beneficial bacteria, such as lactobacilli. Our previous study demonstrated that ASE as a dietary additive modified the immune responses of weaned piglets by modulating the production of immunocytes, cytokines and antibodies, which had inhibitory effects against a wide range of bacteria in vitro (Kong et al., 2007b).

In summary, dietary supplementation with ASE effectively increased villus height in the duodenum, jejunum and ileum, and decreased crypt depth in the gut, which resulted in a decreased incidence of diarrhea, compared with the control group. These findings suggest that ASE as a dietary additive could enhance gut health by regulating the microbiota composition and maintaining a normal morphology in weaning piglets, thereby decreasing the incidence of diarrhea resulting from weaning stress.

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