Effect of different levels of copper on growth performance and cecal ecosystem of newly weaned piglets

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Abstract

The current study aimed to investigate the effects of different levels of copper sulfate on the growth performance and cecal ecosystem in newly weaned piglets. One hundred piglets weaned at 28±2 d were randomly allocated to four treatments with 5 replicates of 5 piglets each. Piglets received for 28 d the base diet with i) no addition (control) or with copper addition (from copper sulfate) at ii) 100, iii) 175 and iv) 250 mg/kg−1. On day 21, twenty piglets were randomly selected (one from each replicate) to slaughter and investigate the population and diversity of cecal microorganisms. The results showed that the diets containing 175 and 250 mg/kg−1 copper improved the average daily gain (ADG) by 51% and 60% and decreased the feed to gain ratio (F/G) by 21% and 16%, respectively. Adding 175 or 250 mg/kg−1 copper improved crude protein, ether extract, calcium and phosphorus digestibility. Viable counts of Enterobacteriaceae and Lactobacilli in cecum tended to be reduced, while the concentrations of cecal volatile fatty acids (VFA) were increased in pigs fed diet supplemented as copper level increased. Polymerase chain reaction (PCR) results showed that adding 175 or 250 mg/kg−1 copper reduced the lactobacilli in cecum. Denaturing gradient gel electrophoresis (DGGE) maps showed that band numbers and intensity of cecal bacterial 16S rDNA decreased as the copper levels increased. The results suggested that the effects of high dietary copper on microflora and their activities and metabolic products might contribute to the intestinal health and result in improved growth performance.

Material and methods

Animal care and experimental design

All experimental procedures were conducted with the approval of the Institutional Animal Care and Use Committee of Sichuan agricultural University. One hundred crossbred (Duroc × Large White × Yorkshire) piglets weaned at an average of 28±2 d of age and 7.5 kg body weight (BW), were fed a basal diet for a two days adaptation period. The composition of the basal diet is presented in Table 1. The experiment lasted for 28 days. Basal diet of the first 14-day period was produced according to phase I formula, and of the second 14-day period was produced according to phase II formula. Following the adaptation period, animals were randomly assigned to four groups, each comprising five replicates, based on body weight. Three replicates were barrows, and the other two were gilts. Groups were then randomly assigned to treatments. Piglets received the base diet (containing 10 mg/kg−1 copper) with i) no addition (control diet) or with the addition of copper (from copper sulfate) at ii) 100, iii) 175, and iv) 250 mg/kg−1. Piglets were penned in groups of five and feed intake and weight gain were measured on a per-pen basis. All procedures, care and handling of animals were performed according to routine management practices. Specifically, all pigs were housed in pens measuring approximately 2.8 m². Pens were daily cleaned. The barn temperature and relative humidity were maintained at 24 to 26°C and 50% to 65% during the experimental period, respectively. Piglets were weighed on day 14 and 28, and feed consumption was determined daily to calculate average daily gain (ADG), average daily feed intake (ADFI), and feed to gain ratio (F/G).

Sample collection

Clean faecal samples based on replicates were collected from the pen floor on day 17, 18, 19 and 20. Faecal samples collected on different days of the same replicate were pooled and stored at −4°C for determination of dry matter, crude protein, ether extract, calcium and phosphorus. On day 21, 20 randomly selected piglets (1 pig from each replicate) were slaughtered. The abdominal cavity was opened, and the entire gastrointestinal tract was removed. For determination of messenger ribonucleic acid (mRNA) abundance of insulin like growth factor 1 (IGF-1) and insulin like growth factor 1 receptor (IGF-1R), approximately 2 cm of middle jejunum were collected and quickly frozen in liquid nitrogen. Samples of the cecal contents were taken and kept in sterile tubes and stored at −4°C for culturing of bacteria or −70°C for polymerase chain reaction (PCR) quantification of bacteria, determination of cecal ecosystem diversity and cecal volatile fatty acids (VFA) concentration.

Analytical procedures

Nutrient digestibility

Dry matter, crude protein, ether extract, calcium and phosphorus of dietary and faecal samples were determined according to AOAC (1995). Acid-insoluble ash (AIA) was involved as an indigestible internal marker which was used of the ash from the feed components only (Yen et al., 1983). Nutrient digestibility was calculated as...
(percentage), 100-100*(A1/ A2*F1/F2). And A1 represented AIA content of feed, A2 represented AIA content of faeces, F1 represented nutrient content of feed, F2 represented nutrient content of faeces (Yang, 2002).

**IGF-1 and IGF-1R mRNA**

IGF-1 and IGF-1R gene expression was quantified by using real time PCR techniques. Sample of jejunum was ground in liquid nitrogen and a fraction of about 50 mg was used to extract total RNA with RNeasy plus kit (TaKaRa Biotechnology, Otsu, Japan), according to the manufacturer’s instruction. Two μg of total RNA was reverse transcribed with reverse transcribe kit (TaKaRa Biotechnology, Otsu, Japan) by incubation at 37°C for 15 minutes in a Master Cycler Gradient (Eppendorf, Hamburg, Germany). All real-time PCRs were carried out in triplicate on a DNA Engine thermal cycler (PTC-0200, Chromo4 Real-Time Detector, Bio-Rad Lab., Hercules, CA, USA). Two μL of 5-fold dilution of RT product was used for PCR in a final volume of 25 μL containing 12.5 μL SYBR Premix Ex Taq (Perfect Real Time) (TaKaRa Biotechnology, Otsu, Japan) and 0.2 to 0.8 μmol/L of each forward and reverse primer for IGF-1 and IGF-1R. Porcine β-actin mRNA was used as a reference gene for normalization purposes. The following PCR protocols were initial denaturation (20 s at 95°C), then a two-step amplification program (20 s at 95°C, 20 to 30 s at 60 to 64°C) repeated 45 times.

**Bacteria quantification by traditional methods**

Digesta samples from the cecum were serially diluted in sterile PBS and plated onto selective media. Enterobacteriaceae were enumerated on MacConkey agar plates incubated at 37°C for 24 h whereas lactobacilli were enumerated on Rogosa agar plates incubated at 37°C for 48 h in a 5% carbon dioxide atmosphere. Enterobacteriaceae were quantified using real-time PCR (PTC-0200, Chromo4 Real-Time Detector, Bio-Rad Lab., Hercules, CA, USA). Two (PTC-0200, Chromo4 Real-Time Detector, Bio-Rad Lab., Hercules, CA, USA). Two

**Statistical analysis**

Data were analyzed using the GLM procedure. In the experiment, pen means were treated as the experimental unit for PCR-DGGE analysis. The gel contained all samples from the four treatments. PCR-DGGE banding patterns were analyzed by measuring numbers and intensity of the bands within each lane of a gel.

**Results**

**Growth performance**

Feeding piglets with diets containing 175 and 250 mg/kg−1 copper improved ADG by 51% and 60% and decreased F/G by 21% and 16%, respectively (P<0.05; Table 2).

**Nutrient digestibility**

The supplementation of copper has positive effect (P<0.05) on apparent digestibility of protein, fat, calcium and phosphorus (Table 3).

**Quantification of mRNA expression by real-time PCR**

The abundance of IGF-1R mRNA in jejunum was higher in 250 mg/kg−1 copper supplemented group (P<0.05), whereas the abundance of IGF-1 mRNA was not affected (Table 4).

**Gastrointestinal microbiota**

Results for lactobacilli and Enterobacteriaceae in cecal samples using traditional and PCR methods are shown in Table 5 and Figure 1, respectively. The viable counts of lactobacilli and Enterobacteriaceae obtained by traditional methods tended to be reduced as the level of copper supplementation rose, however, no statistical significance was detected (Table 5). This was confirmed by the results obtained by PCR method that 175 and 250 mg/kg−1 copper supplementation reduces the amounts of lactobacilli (P<0.05) (Figure 1).

**Table 1. Composition of basal diet fed to weaning pigs at phase I and phase II.**

| Ingredients* | Phase I | Phase II |
|--------------|---------|---------|
| Corn, 7.8% CP | 49.37   | 50.70   |
| Soybean meal, 46% CP | 9.61   | 14.21   |
| Extruded soybean | 8.91   | 14.61   |
| Whey | 8.00   | 5.00    |
| Soy protein concentrate | 5.00   | 5.00    |
| Glucose | 5.00   | 3.00    |
| Homemade fish meal | 4.50   | –       |
| Whole egg powder | 3.00   | –       |
| Wheat middings | 2.00   | 2.00    |
| Soybean oil | 2.00   | 2.00    |
| Dicalcium phosphate | 0.75   | 1.46    |
| Limestone, powder | 0.66   | 0.73    |
| L-Iysine | 0.31   | 0.35    |
| Salt | 0.20   | 0.20    |
| Trace mineral mix, 0.2% | 0.20   | 0.20    |
| L-Threonine | 0.12   | 0.14    |
| Choline chloride, 50% | 0.10   | 0.10    |
| Mould inhibitor A, 0.5 kg/t | 0.06   | 0.06    |
| Antioxidant, 30% | 0.06   | 0.06    |
| Vitamin mix* | 0.06   | 0.06    |
| Aroma agent | 0.05   | 0.06    |
| Coating VC, 93% | 0.02   | –       |
| DL-methionine | 0.01   | 0.06    |
| L-tryptophan | –      | 0.04    |
| Calculated composition | –      | –       |
| Digestible energy, MJ/kg | 14.96  | 14.96   |
| Crude protein, % | 20     | 20      |
| Fat, % | 7.05   | 7.05    |
| Calcium, % | 0.8    | 0.8     |
| Total phosphorus, % | 0.02   | 0.02    |

*Percentage as-fed basis; “provided per kilogram of diet, 50 mg of Mn; 0.3 mg of Zn, 0.3 mg of Se, 100 mg of Fe; 100 mg of Cu; trace mineral mix (0.2%) supplemented Cu in the form of CuSO4/5H2O at levels of 10, 100, 175, 250 mg kg−1; respectively, in diet, provided per kilogram of diet, 4000 U of vitamin A, 400 U of vitamin D3, 24 μg of vitamin E, 3 μg of vitamin K (menadione dimethylpyrimidinol bisulfate), 6 mg of riboflavin, 15 mg of d-pantothenic acid, 24 μg of vitamin B6, 5 mg of d-biotin, and 0.51 mg of folic acid.

[Ital J Anim Sci vol.9:e71, 2010]
Comparison of cecal bacterial diversity via 16S rDNA PCR-DGGE

Band numbers decreased in response to copper supplementation. Besides, the band intensity also decreased in 100, 175, 250 mg/kg⁻¹ copper supplemented groups except three bands (Figure 2), the intensity of which increased by copper supplementation.

Volatile fatty acids concentrations

The concentrations of acetate, propionate and butyrate were higher in 250 mg/kg⁻¹ copper supplemented group (P<0.05), while no difference was found for 100 and 175 mg/kg⁻¹ copper supplemented groups (Table 6).

Discussion

It has been observed that supplementation of pharmacological levels of copper into the feed of piglets improved growth (Cromwell et al., 1989; Dove, 1993; Coffey et al., 1994). The increased feed intake observed may play an important role in this growth stimulating process. However, such appetite stimulating effect of high dietary copper is attenuated as the pig grows. Smith et al. (1997) reported that supplementation of 250 mg/kg⁻¹ copper in the diet of pigs weighing 19 kg had no effect on feed intake. In the current study, ADG, ADFI and F/G were improved during the first two weeks and over the entire experimental period. The supplementation of copper improved both piglet growth performance and apparent digestibility of protein, fat, calcium, and phosphorus and these findings are in good agreement with previous studies in which high dietary copper levels improved animal growth and digestibility of nutrients (Dove, 1995). Piglets fed diet with 250 mg/kg⁻¹ copper may improve the activities of digestive enzymes (Luo and Dove, 1996). It is well established from in vitro systems that the IGF system, which acts through the IGF-1R, has acute anabolic effects on metabolism as well as long-term effects on animal growth and skeletal development (Smith et al., 1998).

Table 2. Effects of supplemental copper on growth performance in weanling pigs.

| Cu, mg/kg⁻¹ | P | SEM |
|-------------|---|-----|
| Basal diet  | 100 | 175 | 250 |
| ADG, g/d 0 to 2 week | 116a | 153ab | 191b | 214b | 0.032 | 14 |
| 2 to 4 week | 249a | 309b | 360b | 370b | 0.036 | 23 |
| 0 to 4 week | 183a | 231b | 276b | 292b | 0.022 | 15 |
| ADFI, g/d 0 to 2 week | 262a | 345ab | 360ab | 405b | 0.033 | 25 |
| 2 to 4 week | 494a | 569b | 619b | 670b | 0.043 | 32 |
| 0 to 4 week | 406a | 483ab | 523ab | 587b | 0.028 | 26 |
| F/G 0 to 2 week | 2.33a | 2.27a | 1.85b | 1.87b | 0.035 | 0.65 |
| 2 to 4 week | 2.00 | 1.82 | 1.69 | 1.82 | 0.198 | 0.12 |
| 0 to 4 week | 2.27a | 2.08ab | 1.89b | 2.00b | 0.040 | 0.06 |

Table 3. Effects of supplemental copper on nutrient digestibility of weanling pigs.

| Cu, mg/kg⁻¹ | P | SEM |
|-------------|---|-----|
| Basal diet  | 100 | 175 | 250 |
| CP, % 0 to 2 week | 69.32a | 73.00b | 76.88b | 77.00b | 0.042 | 0.23 |
| 2 to 4 week | 68.44a | 72.25a | 75.74b | 77.25b | 0.034 | 1.02 |
| 0 to 4 week | 43.51a | 46.91a | 61.20b | 55.53b | 0.043 | 0.26 |
| EE, % 0 to 2 week | 40.71a | 49.09b | 53.20bc | 54.91c | 0.049 | 0.05 |
| 2 to 4 week | 2.00 | 1.82 | 1.69 | 1.82 | 0.198 | 0.12 |
| 0 to 4 week | 2.27a | 2.08ab | 1.89b | 2.00b | 0.040 | 0.06 |

Table 4. Effects of supplemental copper on IGF-1 and IGF-1R mRNA expression.

| Cu, mg/kg⁻¹ | P | SEM |
|-------------|---|-----|
| Basal diet  | 100 | 175 | 250 |
| IGF-1 0.6875 | 0.6443 | 0.6733 | 0.6825 | 0.276 | 0.0941 |
| IGF-1R 0.5934a | 0.5571a | 0.6199ab | 0.6794b | 0.048 | 0.0136 |

Table 5. Effects of supplemental copper on cecal microbial populations (log CFU/g⁻¹ FM).

| Cu, mg/kg⁻¹ | P | SEM |
|-------------|---|-----|
| Basal diet  | 100 | 175 | 250 |
| Enterobacteriaceae | 7.73 | 6.75 | 6.39 | 6.29 | 0.098 | 0.23 |
| Lactobacilli | 7.57 | 7.17 | 7.02 | 6.96 | 0.100 | 0.26 |

Table 6. Effect of dietary copper on volatile fatty acid concentrations.

| Cu, mg/kg⁻¹ | P | SEM |
|-------------|---|-----|
| Basal diet  | 100 | 175 | 250 |
| Acetate, mμmol/g⁻¹ | 267.1a | 234.0a | 266.2a | 340.4b | 0.043 | 9.97 |
| Propionate, mμmol/g⁻¹ | 155.2b | 149.5a | 177.7ab | 189.4b | 0.032 | 4.87 |
| Butyrate, mμmol/g⁻¹ | 53.5a | 48.4a | 57.8ab | 60.7b | 0.040 | 1.47 |

a,b Values with different superscript letters in the same row indicate significant difference (P<0.05).
cell replication and differentiation (Le Roith et al., 2001). IGF-1 exerts its biological effect through interaction with specific membrane-bound receptors. In the pig small intestine, IGF-1 is bound principally to IGF-1R. Thus, the increased IGF-1R expression in the small intestinal mucosa of weaned piglets indicates that the IGF-1R has a positive response to IGF-1 and supports the view that the IGF system plays a major role in mediating an anabolic effect of copper in the small intestine of weanling piglets. The increased expression of the IGF-1R genes that was observed in the small intestine of piglets receiving high dietary levels of copper may explain the beneficial effects on copper to intestinal integrity, function, and whole-body growth in weanling piglets.

The improvement in pig performance that accompanies pharmacological additions of copper in swine diets is often attributed to its enteric antimicrobial action. Højberg et al. (2005) reported that 175 mg/kg–1 copper supplementation reduced the amounts of cecal Escherichia coli and stomach lactobacilli, while the amount of cecal lactobacilli was not affected. In this study, copper supplementation has tendency to reduce the amounts of Enterobacteriaceae (P=0.09) and Lactobacilli (P=0.10), form the results of CFUs. From the PCR results, we found that adding 175 and 250 mg/kg–1 copper could decrease the amounts of Lactobacilli same with the traditional culture result. The results from the DGGE analysis suggest that copper supplementation reduced cecum microbiota diversity. Taken together, the result suggests that high dietary copper (100, 175 or 250 mg/kg–1) reduced cecal colonization which results in the alteration of the intestinal microflora, and this may be one mechanism by which copper improves growth performance in piglets. Large intestinal VFA production in the pig has been estimated to contribute between 5% and 28% of the total maintenance energy requirement (Pierce et al., 2005). The concentrations of VFAs were higher in 250 mg/kg–1 copper supplemented group, which may have significant effects on the energy available to the intestinal mass and further resulted in improved growth performance. However, the increased VFA concentrations that were found are likely to be the consequence of increased microbial fermentation and are therefore in contradiction with the reduced presence of bacteria in the cecum. Thus, we hypothesis that high dietary copper reduced cecal colonization by bacteria resulted in changes in fermentation pattern by making high fermentation activity strains predominant to yield more metabolic products as VFAs. This hypothesis is supported by the DGGE results that showed the presence of three new predominant strains in high dietary copper supplemented groups even if the microbiota diversity was decreased. Further study is needed to identify the strains that might have contributed to the increased yield of VFA.

Conclusions

Supplementing diets of weanling pigs with 175 or 250 mg/kg–1 copper resulted in improved animal growth performance and increased nutrient digestibility and IGF-1R mRNA expression. High dietary copper reduced microbiota diversity and cecal colonization but increased cecal VFA concentrations. Further research is needed to achieve a better understandin of the high dietary copper on growth performance and gastrointestinal health of weanling pigs.

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