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Effect of early antibiotic administration on cecal bacterial communities and their metabolic profiles in pigs fed diets with different protein levels

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ABSTRACT

This study investigated the effects of early antibiotic administration (EAA) on cecal bacterial communities and their metabolic profiles in pigs fed diets with different protein levels. Eighteen litters (total 180) of piglets on day (d) 7 were fed either a commercial creep feed or commercial creep feed + antibiotic (Olaquindox, Oxytetracycline Calcium and Kitasamycin) until d 42. On d 42, pigs within each group were further randomly fed a normal crude protein (CP) diet (20% and 18% CP from d 42 to d 77 and d 77 to d 120, respectively) or a low-CP diet (16% and 14% CP from d 42 to d 77 and d 77 to d 120, respectively), generating 4 groups, control-low CP (Con-LP), control-normal CP (Con-NP), antibiotic-low CP (Ant-LP) and antibiotic-normal CP (Ant-NP), respectively. On d 77 and d 120, 5 pigs per group were slaughtered and cecal materials were collected for bacterial analysis. With cecal bacteria, principle component analysis (PCA) of the denaturing gradient gel electrophoresis (DGGE) profile showed two distinct groups of samples from low-CP diet and samples from normal-CP diet. Real-time PCR showed that EAA did not have significant effect on major bacterial groups, only showed significant interactions ($P < 0.05$) with CP level for *Lactobacillus* counts on d 77 and *Clostridium* cluster XIVa counts on d 120 with higher values in the Con-NP group compared to the Ant-NP groups. Low-CP diet increased ($P < 0.05$) short-chain fatty acids (SCFA) producing bacteria counts (Bacteroidetes on d 77 and d 120; *Clostridium* cluster IV and *Clostridium* cluster XIVa on d 77), but decreased ($P < 0.05$) *Escherichia coli* counts on d 77 and d 120. For metabolites, EAA increased ($P < 0.05$) protein fermentation products (*p*-cresol, indole and skatole on d 77; ammonia, putrescine and spermidine on d 120), and showed significant interactions ($P < 0.05$) with CP level for *p*-cresol and skatole concentrations on d 77 and putrescine and spermidine concentrations on d 120 with higher values in the Ant-LP group compared to the Con-LP groups. Low-CP diet increased ($P < 0.05$) SCFA concentration (propionate and butyrate) on d 77, but reduced ($P < 0.05$) the protein fermentation products (ammonia, phenol and indole on d 77; branched chain fatty acid (BCFA), ammonia, tyramine, cadaverine and indole on d 120). These results indicate that EAA had less effect on bacterial communities, but increased bacterial fermentation of protein in the cecum under low-CP diet. Low-CP diet altered bacterial communities with an increase in the counts of SCFA-producing bacteria and a decrease in the counts of *Escherichia coli*, and markedly reduced the protein fermentation products.

1. Introduction

Low doses of antibiotics are widely used in feed in livestock to prevent disease and improve feed efficiency, particularly in weaning and starter piglets. This is because around weaning, pigs usually exhibit impaired growth, increased incidence of diarrhea and other diseases [1,2], as well as undergo severe changes in the histomorphology of the intestine [3]. The effect of in-feed antibiotics on the host is believed to change ecologic and metabolic functions of the intestinal microbiota [4,5]. In-feed antibiotics are commonly used in creep feed for suckling and weaning piglets and most studies have been focused on the short-term effects of antibiotic administration on gut bacteria and bacterial metabolites in several-weeks-old pigs [6–8]. However, it is essential to understand the

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short and long-term effects of early antibiotics administration (EAA), which is usually used in creep feed for piglets, on the development of gut bacteria and their metabolism in the pigs.

Diet composition could affect the composition and metabolic activities of the microbiota that adapts to the intestinal luminal environment and substrate availability [9]. Recently, dietary protein level has been reported to affect gut bacteria [10,11]. Undigested protein in the large intestine can be fermented by indigenous bacteria to form amino acid-derived metabolites [12], such as ammonia, hydrogen sulphide, branched chain fatty acid (BCFA), phenolic and indolic compounds which have been associated with toxic impacts on the intestinal epithelium [13,14]. In pigs, researches have shown that protein levels in the diet affected the bacterial composition and their metabolism in the large intestine. Previous studies indicated that a low crude protein (CP) diet with crystalline amino acid (AA) supplement may reduce the formation of protein fermentation products and shift bacterial communities in the large intestine [15,16]. It would be interesting to understand whether in-feed antibiotics have differential effects on the bacterial fermentation under different protein level diets.

Therefore, the present study aimed at determining the subsequent effect of in-feed antibiotics administrated during sucking and around weaning period on bacterial communities and their metabolic profiles in the cecal digesta of later growing pigs fed diets with different protein levels.

2. Materials and methods

The experimental proposals and procedures were approved by the Animal Care and Use Committee of Nanjing Agricultural University in compliance with Chinese guidelines for animal welfare.

2.1. Animals, diets and sampling

18 litters of crossbred (Duroc × Landrace × Large White) newborn piglets (180 piglets in total) were used in this study. The piglets on day (d) 7 were randomly allocated to two groups (N = 9 litters) and offered creep feed either without antibiotics (control group) or with antibiotics (50 mg/kg Olaquindox, 50 mg/kg Oxytetracycline Calcium, 50 mg/kg Kitasamycin) (antibiotic treatment group). Olaquindox is one of the quinoxaline-*N*,*N*-dioxides with activity against gram-positive and gram-negative bacteria. Oxytetracycline Calcium is one of the tetracycline antibiotics, which can act against gram-positive and gram-negative bacteria. Kitasamycin, as a macrolide antibiotic, exhibits activity mainly against gram-positive bacteria. Olaquindox, Oxytetracycline Calcium and Kitasamycin are used to promote growth and improve feed efficiency for piglets in China. Piglets were weaned on d 23 and transported to an environmental-controlled nursery, with 1.8 × 2.5 m pens that had a hard plastic fully slotted floor, and fed the same diets until d 42. On d 42, the pigs (11.3 ± 0.3 kg) of each group were mixed and then further randomly assigned to either a normal or low CP level diet on the basis of equal average BW respectively, which generated 4 groups, control-low crude protein (Con-LP), control-normal crude protein (Con-NP), antibiotic-low crude protein (Ant-LP) and antibiotic-normal crude protein (Ant-NP), respectively. There were 5 pens (replicates) per treatment group and 9 pigs per pen. The two diets were formulated to provide a normal-CP diet (20% and 18% CP from d 42 to d 77 and d 77 to d 120, respectively) or a low-CP diet (16% and 14% CP from d 42 to d 77 and d 77 to d 120, respectively). Corn, soybean meal, wheat bran (from d 77 to d 120), wheypower (from d 42 to d 77) and fishmeal (from d 42 to d 77) were major nutrient ingredient of experimental diets. Crystalline AA (lysine, threonine, methionine and tryptophan) were added to the diets to meet amino acids standards, according to the NRC (2012) [17]. After d 77, pigs were moved to a total confinement house with 2.5 × 3.0 m pens that had partial concrete slatted floors. Water and feed were consumed *ad libitum*. On d 77 and d 120, one pig from each pen (5 pigs from each group) was slaughtered, the digesta from the cecum was sampled and immediately frozen at −20 °C for later bacterial DNA isolation and metabolite analysis.

2.2. DNA extraction and PCR amplification

The total DNA was extracted from each sample (0.3 g) using the bead-beating method with a mini-bead beater (Biospec Products, Bartlesville, OK, USA), followed by phenol-chloroform extraction [18]. The DNA was then precipitated with ethanol and the pellets were dissolved in 80 μL of Tris EDTA (TE). Bacterial primers U968-GC and L1401 (Table 1), were used to amplify the V6–V8 regions of the bacterial 16S ribosomal RNA (rRNA) gene. PCR was performed with the Premix EX Taq version 2.0 (Takara Biotechnology, Dalian, China). The PCR mixtures (20 μL) contained 10 μL of Premix EX Taq, 0.5 μL of the primers U968-GC and L1401, 1 μL of DNA template and 8 μL sterile water. The reactions were performed in a Biometra TProfessional Thermocycler (Göttingen, Germany) using the following programme: 94 °C for 7 min and 35 cycles of 94 °C for 30 s, 56 °C for 20 s, 68 °C for 40 s and 68 °C for 7 min (last extension). Aliquots of 5 μL were analyzed by electrophoresis on 1.2% agarose gel (weight/volume) containing GoldView (Solarbio, Shanghai, China) to check the size of the amplicons.

2.3. Denaturing gradient gel electrophoresis

The amplicons obtained from the cecum-extracted DNA were separated by denaturing gradient gel electrophoresis (DGGE) using a DcodeTM system (Bio-Rad Laboratories, Hercules, CA, USA) [19]. Similarity analysis, based on the unweighted pair group mean average (UPGMA), and principle component analysis (PCA) were performed using GelCompar II (Applied Maths, Gent, Belgium) and Canoco software (Microcomputer Power, Ithaca, NY, USA). The structural diversity of the microbial community (Shannon diversity index) H was calculated using the following function: $H = -\Sigma Pi \cdot \ln (Pi)$, where Pi is the importance probability of the bands in a lane. H was calculated on the basis of the bands on the gel tracks that were applied for the generation of the dendrograms by using the intensities of the bands as judged by the peak height in the densitometric curves. The importance probability Pi was calculated as: $Pi = ni/H$, where ni is the height of a peak and H is the sum of all peak heights in the densitometric curve.

2.4. Quantitative real-time PCR

Numbers of total bacteria, Firmicutes, Bacteroidetes, *Bifidobacterium*, *Clostridium* cluster IV, *Clostridium* cluster XIVa, *Escherichia coli* and *Lactobacillus* were quantified by real-time quantitative PCR (qPCR) using specific primers (Table 1). qPCR assay was performed on StepOnePlus™ Real-Time PCR System (Life Technologies, California, USA) by using SYBR Premix Ex Taq dye (Takara). Quantification of 16S ribosomal RNA (rRNA) gene copies in each sample was performed in triplicate, and the mean value was calculated. Standard curves of each bacterial group were generated from serial dilutions of a known copy number of the target gene cloned into a plasmid vector. For each reference strain, the 16S rRNA gene was cloned into a pMD-19T Vector System (Takara Biotechnology, Dalian, China). An *Escherichia coli* strain was transformed with the recombinant plasmid, and plasmid DNA was extracted from *Escherichia coli* by the miniprep method [20].