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Complete List of Authors:	Li, Huizhi; Nanjing Agricultural University, College of Animal Science and Techonolgy Liang, Tingting; Nanjing Agricultural University, College of Animal Science and Techonolgy Chu, Qingpo; Nanjing Agricultural University, College of Animal Science and Techonolgy Xu, Feilong; Nanjing Agricultural University, College of Animal Science and Techonolgy Li, Yuan; Nanjing Agricultural University, College of Animal Science and Techonolgy Fu, Lingling; Nanjing Agricultural University, College of Animal Science and Techonolgy Zhou, Bo; Nanjing Agricultural University, College of Animal Science and Technology
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**Effects of several in-feed antibiotic combinations on the abundance and diversity
of fecal microbes in weaned pigs**

Authors: Huizhi Li, Tingting Liang, Qingpo Chu, Feilong Xu, Yuan Li, Lingling Fu
and Bo Zhou *

College of Animal Science and Technology, Nanjing Agricultural University, Nanjing
210095, P. R. China

***Corresponding author:** College of Animal Science and Technology, Nanjing
Agricultural University, Nanjing 210095, P.R. China.

Tel: +86 25 84395362; Fax: +86 25 84395314; E-mail: zhoubo@njau.edu.cn

All authors disclose any conflict of interest

Abstract:

To characterize the effects of several subtherapeutic antibiotic combinations on the abundance and diversity of fecal microbes, 400 weaned pigs were selected and randomly assigned to 8 groups, where they were continuously fed different antibiotic combinations for 28 days. Then, a total of 48 pigs were randomly selected to individually collect the feces for DNA extraction and 16S rRNA high-throughput sequencing. Compared with the pigs without antibiotic administration, the diversities of fecal microbes were decreased in the pigs fed chlortetracycline (**CTC**), olaquinox (**OLA**) and enramycin (**ER**)/ virginiamycin (**VIR**), where the relative abundance of the phylum *Bacteroidetes* and the genus *Prevotella* were increased. Compared with the pigs without antibiotic administration, the relative abundance of the phylum *Proteobacteria* and the genus *Succinivibrio* were decreased in the pigs fed CTC, zinc bacitracin (**ZB**) and colistin sulphate (**CT**), a mixture of CTC, ZB and CT, CTC and OLA, or a mixture of CTC, OLA and ER. Alpha-diversity and β -diversity were decreased ($P < 0.05$) in the pigs fed ZB and CT, or a mixture of CTC, OLA and ER/VIR. This study provides experimental data to deepen our understanding on the effects of antibiotic combinations on intestinal microbes.

Keywords: 16S rRNA gene; antibiotic combination; fecal microbes; Illumina; pig

Introduction

The use of subtherapeutic antibiotic in-feed has been reported to increase growth rates and feed efficiency of pigs (Cromwell 2002; van der Fels-Klerx et al. 2011). However, in-feed antibiotics have worsen the already alarming scenario of antibiotic resistance (Ahmed et al. 2014). In pig production, antibiotic combinations are typically used in-feed as antibiotic growth promoters (AGPs) (Cromwell 2002) in some countries, although in parts of the world the procedure of using antimicrobials for growth promotion is banned. However, the impact of in-feed antibiotic combinations on the relative abundance and diversity of intestinal microbiota in pigs has not been well studied.

There are several antimicrobial agents permitted for use in pig production in China, such as chlortetracycline (**CTC**), zinc bacitracin (**ZB**), colistin sulphate (**CT**), olaquinox (**OLA**), enramycin (**ER**), and virginiamycin (**VIR**). CTC belongs to the tetracycline class, which are broad-spectrum agents, exhibiting antibacterial activity against bacteria, mycoplasmas, chlamydiae, and protozoan parasites (Chopra and Roberts 2001). ZB, a water-soluble and non-toxic antibiotic, is active chiefly against gram-positive bacteria, such as streptococcal and staphylococcal infections (Johnson et al. 1945). In chickens, ZB in the diet significantly reduced the number of coliform bacteria in the ileum (Engberg et al. 2000) and the abundance of lactobacilli in the caeca (Gong et al. 2008). COL is active chiefly against gram-negative organisms by alteration of permeability of membrane (Nakajima and Kawamata 1965). Until now OLA, a member of quinoxaline-1,4-dioxides derivatives with antibacterial activity

against bacteria and toxicity, is still used in China for promoting pig growth (Chen et al. 2009). ER has antibacterial activity against gram-positive bacteria (Kawakami et al. 1971). VIR may reduce the concentration of gram-positive bacteria and increase the apparent ileal digestibility of amino acid in pigs (Stewart et al. 2010). As an alternative to antibiotics, *Macleaya cordata* extract contains considerable amounts of sanguinarine, chelerythrine, dihydrochelerythrine, dihydrosanguinarine, allocryptopine, and protopine, which have broad antimicrobial activity as well as antiinflammatory properties (Kosina et al. 2010).

In the mammalian gastrointestinal tract, a large and diverse microbial population plays a major role in health and nutrient digestion (Kim et al. 2012; Yatsunenkov et al. 2012). Previous studies showed that tylosin and CTC altered the gut microbial community of pigs (Kim et al. 2012; Rettedal et al. 2009). Nevertheless, Castillo et al. (2006) reported that the types of feed additives had little impact on bacteria counts in gastrointestinal tracts but changed the community structure. A mixture of CTC, sulfamethazine, and penicillin (APS250) decreased the relative abundance of *Bacteroidetes* (Looft et al. 2012). However, administration of carbodox increases the relative abundance of genus *Roseburia*, *Prevotella*, and *Fecalibacterium* (Looft et al. 2014b).

We hypothesized that antibiotics can differentially affect the intestinal microflora of pigs because of their different antimicrobial mechanisms. The objective of this study was to investigate effects of in-feed antibiotic combinations on the abundance and diversity of fecal microbes in weaned pigs. In the present study, we sequenced the

V3-V4 regions of 16S rRNA genes in the *Bacteria/Archaea* to investigate the impacts of antibiotic combinations on pig fecal microbiota using Illumina HiSeq 2500 sequencer.

Materials and Methods

Pigs and fecal collection

The present study was reviewed and approved by animal use committee of Nanjing Agricultural University. The present study was carried out from October to November in 2014 at Yong-kang Agricultural and Animal Husbandry Co., Ltd. in Jiangsu Province, China (31.74°N).

Four hundred 35-day-old weaned pigs (Duroc × Landrace × Large White, body weight 12.1 ± 0.3 kg) were selected and randomly assigned to eight treatments: (Table 1). There were three replicates (pens) in each treatment and 16-17 pigs per pen. All the experimental pigs were raised in the same room. Each pen (1.7×3.5 m) was equipped with a feeder (1.2 m long) and two nipple drinkers to allow *ad libitum* access to feed and water. After 28 days of continuous antibiotics administration, a total of 48 pigs (two pigs per pen, 1 barrow and 1 female) were randomly selected to individually collect the fresh feces into 2 mL centrifugal tubes and stored at -20°C until DNA extraction.

DNA extraction and high-throughput sequencing

Microbial genomic DNA extraction was performed using a bead-beating and CTAB (cetyltrimethyl ammonium bromide) method referring to our previous study (Li et al. 2016). The concentration of extracted genomic DNA was measured using a

spectrophotometer (NanoDrop 2000, Thermo, Waltham, MA, USA).

Based on previous studies (Caporaso et al. 2011; Kim et al. 2012; Youssef et al. 2009), the V3-V4 hypervariable regions of the 16S rRNA gene were amplified and then sequenced, the broadly conserved primer 314F (CCT AYG GGR BGC ASC AG) and 806R (GGA CTA CNN GGG TAT CTA AT) targeting the *Bacteria* and *Archaea* 16S V3-V4 regions were selected for the microbial community structure analysis (Caporaso et al. 2011). The PCR amplifications of the 16S rRNA gene were performed in a total volume of 50 μ L containing 25 μ L rTaq DNA polymerase (Takara Biotech, Co., Ltd., Dalian, China), 1.0 μ M of each primer, 120 ng template DNA. The thermal cycling conditions were as follows: 95 $^{\circ}$ C for 5 min, 35 cycles of 95 $^{\circ}$ C for 30 s, temperatures of annealing for 30 s, and 72 $^{\circ}$ C for 45 s, followed by 72 $^{\circ}$ C for 7 min. All PCR reactions were carried out using Phusion High-Fidelity PCR Master Mix (New England Biolabs). Mix same volume of 1 $\times$ loading buffer (contained SYB green) with PCR products and operate electrophoresis on 2% agarose gel for detection. Samples with bright main strip between 400-450bp were chosen for further experiments. Only PCR products without primer dimers and contaminant bands were used for the high-throughput sequencing. Barcoded V3-V4 Amplicons were sequenced using the Illumina HiSeq platform (Illumina, USA) at Novogene Institute (Beijing, China) according to the manufacturer's instructions.

Based on their unique barcode, paired-end reads were assigned to samples, truncated by cutting off the barcode and primer sequence, and merged using FLASH software (V1.2.7). According to the QIIME (V1.7.0) quality controlled process,

quality filtering on the raw tags were performed to obtain the clean tags. The tags were compared with the reference database Gold using UCHIME algorithm to detect and remove chimera sequences. Then the Effective Tags finally obtained. Sequences analysis were completed using Uparse. Sequences with $\geq 97\%$ similarity were assigned to the same OTUs. For each OUT, representative sequence was screened for further annotation. The taxonomic information of each representative sequence was annotated using the GreenGene Database based on RDP classifier algorithm. In order to study phylogenetic relationship between OTUs and dominant species in different samples or groups, multiple sequence alignments were conducted using the MUSCLE software. Abundance information of OTU was normalized using a standard of sequence number corresponding to the sample with the least sequences. Subsequent analysis of alpha diversity and beta diversity were all performed basing on this output normalized data using QIIME and displayed with R software. Cluster analysis was preceded by principal component analysis (PCA), which was used to reduce the dimension of the original variables using the FactoMineR package and ggplot2 package in R software. Principal Coordinate Analysis (PCoA) was performed to get principal coordinates and visualize from complex, multidimensional data. A distance matrix of weighted or unweighted unifracs among samples obtained before was transformed to a new set of orthogonal axes, by which the maximum variation factor is demonstrated by the first principal coordinate, the second maximum one by the second principal coordinate, and so on. PCoA analysis was displayed by WGCNA package, stat packages and ggplot2 package in R software. Unweighted Pair-group

Method with Arithmetic Means (UPGMA) Clustering was performed as a type of hierarchical clustering method to interpret the distance matrix using average linkage and was conducted by QIIME software.

Results

Sequence analysis

A total of 3,861,058 raw reads were obtained from the Illumina HiSeq sequencing. Following quality control to remove chimera, 2,535,291 high quality sequences with an average length of 414 bp were remained (Table S1). The average number of sequences per sample was 52,819. Fig. S1 shows the rarefaction curves of treatments of antibiotic combinations that indicate a high level of microbial diversity. Based on 97% species similarity, the superior quality sequences were clustered into operational taxonomic units (OTUs) using the *de novo* method. The removal of low abundance OTUs (< 0.005%) yielded a total of 1166 OTUs for analysis. Good's coverage of all samples was $\geq 99.7\%$ (Table S2).

Microbial abundance of the pig fecal microbiota

At the phylum level, the 10 most relatively abundant bacterial phyla were *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Euryarchaeota*, *Spirochaetes*, *Tenericutes*, *Cyanobacteria*, *Actinobacteria*, *Elusimicrobia*, and *Fibrobacteres* (Table S3). *Firmicutes* and *Bacteroidetes* dominated, accounting for 54.2% and 40.4% of sequences, respectively. Only 1.2% of sequences could not be classified at the phylum level. Compared to the group without antibiotics, the relative abundances of *Firmicutes* were increased ($P < 0.05$) in the groups fed with CTC, or a mixture of ZB

and CT, and decreased ($P < 0.05$) in the group fed with a mixture of CTC, OLA and VIR. The relative abundances of *Bacteroidetes* were increased ($P < 0.01$) in the groups fed with a mixture of CTC, OLA and ER, and a mixture of CTC, OLA and VIR, and the relative abundances of *Proteobacteria* were decreased in the groups fed with CTC ($P < 0.05$), a mixture of ZB and COL ($P < 0.01$), a mixture of CTC, ZB and COL ($P < 0.05$), a mixture of CTC and OLA ($P < 0.05$), and a mixture of CTC, OLA and ER ($P < 0.01$) (Fig. 1a and Table 2).

Prevotella was the most relatively abundant genus accounting for 18.89 % of all sequences followed by *Roseburia* (8.45 %), *[Prevotella]* (5.39 %), *Faecalibacterium* (4.12 %), *Succinivibrio* (3.17 %), *Lachnospira* (3.12 %), *Ruminococcus* (2.97 %), *Oscillospira* (2.81 %), *Coproccoccus* (0.95 %), *CF231* (0.93 %), *Phascolarctobacterium* (0.90 %), *p-75-a5* (0.57 %), *Parabacteroides* (0.54 %), *Bacteroides* (0.52 %), and *Methanobrevibacter* (0.46 %).

The sequences were assigned into 118 different genera, with an average of 43.3% of sequences being unclassified at the genus level. Compared to the control group, the relative abundances of *Prevotella* increased ($P < 0.01$) in the groups fed with a mixture of CTC, OLA and ER, and a mixture of CTC, OLA and VIR. Compare with the control group, the relative abundances of *Succinivibrio* were decreased in the groups fed with CTC ($P < 0.05$), a mixture of ZB and COL ($P < 0.01$), a mixture of CTC, ZB and COL ($P < 0.05$), a mixture of CTC and OLA ($P < 0.05$), and a mixture of CTC, OLA and ER ($P < 0.05$). The relative abundance of *Faecalibacterium* was less ($P < 0.01$) in the group fed with a mixture of CTC, OLA and ER than in the

control group (Fig. 1b and Table 3).

The distribution of predominant bacterial OTUs in which the relative abundance was over 1% is shown in Fig. S2 and the OTU Taxonomy is shown in Table S4.

Among the 1,168 OTUs identified in the present study, 62 OTUs were significantly ($P < 0.01$) affected by antibiotic combinations (Fig. S3). Among them, 23 and 35 OTUs belong to *Bacteroidetes* and *Firmicutes*, respectively. Two OTUs belong to *Actinobacteria* and two belong to *Proteobacteria*. In the group fed with CTC, 8 OTUs (7 OTUs belong to *Firmicutes*) had the highest ($P < 0.01$) normalized relative abundance (NRA), but no OTUs had the lowest NRA among 8 treatments. In the group fed with a mixture of CTC, ZB and CT, 15 OTUs had the lowest ($P < 0.01$) NRA, only one OTUs had the highest ($P < 0.01$) NRA. In the groups fed with a mixture of CTC, OLA and ER, and a mixture of CTC, OLACOV, 7 and 9 OTUs that belong to *Bacteroidetes* had the highest ($P < 0.01$) NRA, 8 and 12 OTUs that belong to *Firmicutes* had the lowest ($P < 0.01$) NRA, respectively.

Microbial diversity of the pig fecal microbiota

Alpha-diversity was used in microbial diversity analysis within-community. The overall average Chao1 of all samples, a nonparametric estimator of species richness, was 641.85 ± 5.97 (SEM). The Chao1 was less ($P < 0.05$) in the groups fed with a mixture of ZB and CT, a mixture of CTC, OLA and ER, and a mixture of CTC, OLA and VIR than in the control group (Table 4). The average number of OTUs per sample was 597.15 ± 5.59 (SEM). The number of observed species became less ($P < 0.05$) in the groups fed with a mixture of ZB and CT, a mixture of CTC, OLA and ER, and a

mixture of CTC, OLA and VIR, than in the control group (Table 4). The average value for the Shannon and Simpson index, which measures both species richness and abundance, were 6.44 ± 0.05 and 0.966 ± 0.002 , respectively (SEM of the mean).

UPGMA (Unweighted pair-group method with arithmetic mean) UniFrac clustering of antibiotic combination groups was based on weighted unifrac distance matrix, in which pigs fed with a mixture of CTC, OLA and VIR, and a mixture of CTC, OLA and ER are clustered together and differ with other pigs. The same results are shown in the cluster distribution of fecal microbiota of pigs at the level of bacterial genus ($> 0.1\%$) by heatmap (Fig. S4).

Weighted UniFrac distances were used to as β -diversity and to compare the eight treatments. The weighted UniFrac distances were less in the groups fed with a mixture of ZB and COL ($P < 0.001$), a mixture of CTC, OLA and ER, and a mixture of CTC, OLA and VIR ($P < 0.01$) than in the control group (Fig. 2a). The principal coordinates analysis (PCoA) plot of the weighted UniFrac distances (PC1=43.33%, PC2=15.62) suggested there was a greater clustering in the groups fed with a mixture of ZB and CT, a mixture of CTC, OLA and ER, and a mixture of CTC, OLA and VIR (Fig. 2b).

Discussion

The pig gut microbiota is comprised of a large number of diverse microorganisms, such as *Bacteria*, fungi, *Protozoa*, viruses and *Archaea*, which contribute to the animal health (Lamendella et al. 2011; Sommer and Bäckhed 2013). The structure of the gut microbiota depends on some factors, such as diet, age, genetics, and in some cases antimicrobial exposure (Holman and Chénier 2015; Scott

et al. 2013). Antimicrobial growth promoters cannot be well absorbed in the gut and can affect directly on the gut microbiota (Erik and Knudsen 2001).

In the previous study, Allen et al. (2013) found that combination of CTC, sulfamethazine and penicillin altered the fecal bacterial microbiota and bacteriophage community in the feces of pigs. For example, the relative abundance of genera *Coprococcus*, *Succinivibrio*, *Streptococcus*, *Treponema*, and *Turicibacter* was decreased. After continuous subtherapeutic levels of in-feed CTC administration, the relative abundance of *Bacteria* at 3 weeks of age, the genus *Lactobacillus* at 6 weeks of age and the phylum *Firmicutes* at 19 weeks of age were decreased (Holman and Chenier 2014), while the relative abundance of phylum *Firmicutes* was increased and *Proteobacteria* was decreased at 9 weeks of age in the present study.

In addition, Holman and Chenier (2014) found that in-feed tylosin increased the relative abundance of genera *Coprococcus* (3 to 9 weeks of age) and *Streptococcus* (6 weeks of age), and decreased *Fibrobacteres* (3 weeks of age), *Bacteroidetes* (9 weeks of age), *Succinivibrio* (9 weeks of age). However, Kim et al. (2012) showed that tylosin (44 mg kg⁻¹ feed) administration decreased the relative abundance of the genera *Prevotella*, *Megasphaera*, *Blautia*, *Sarcina* but increased *Lactobacillus*, *Sporacetigenium*, *Acetanaerobacterium*, and *Eggerthella*. The results of these two studies are lacking good consistency, which suggests that continuous subtherapeutic antibiotic administration is not the determinant of intestinal microflora diversity.

In China, CTC, B, CT, OLA, ER, and VIR are widely used in pig production. Therefore, the effects of these antibiotic combinations on the abundance and diversity

intestinal microflora have important significance for the pig production. In the present study, the α -diversity indexes Chao1 and observed species of groups fed with a mixture of ZB and COL, a mixture of CTC, OLA and ER, and a mixture of CTC, OLA and VIR was lower than that of control group, which means that the mixture of ZB and CT, the mixture of CTC, OLA and ER, and the mixture of CTC, OLA and VIR reduced the diversity of fecal microbes. The Shannon index ranged from 6.18-6.53, similar to the values of previously studies (Holman and Chenier 2014; Kim et al. 2012).

Weighted UniFrac is a β -diversity measure that uses phylogenetic information to compare environmental samples. Weighted UniFrac, coupled with standard multivariate statistical techniques including PCoA, identifies factors explaining differences between microbial communities (Lozupone et al. 2011). A smaller weighted UniFrac value indicates lower the microbial diversity within the group. In the present study, weighted UniFrac distances were less in the groups fed with a mixture of ZB and CT, a mixture of CTC, OLA and ER, and a mixture of CTC, OLA and VIR than in the control group, which is consistent with the α -diversity indexes. The PCoA plot of the weighted UniFrac distances had greater clustering in the groups fed with a mixture of ZB and CT, a mixture of CTC, OLA and ER, and a mixture of CTC, OLA and VIR. The results indicate that the combination ZB and CT, CTC combination with OLA and ER or CTC combination with OLA and VIR reduced the diversity of fecal microbes within the group. Previous studies found that B, ER and VIR are microbicide with activity against Gram-positive bacteria (Abudabos 2012;

Kawakami et al. 1971); COL and OLA are microbicide with activity against Gram-negative bacteria (Guyonnet et al. 2010; Jeong et al. 2009). These suggest that combination of antimicrobial growth promoters with different antibacterial mechanisms should have a better antimicrobial effect.

In the present study, the large majority of sequences were classified as either *Firmicutes* or *Bacteroidetes*, which are in agreement with several other studies (Holman and Chenier 2014; Looft et al. 2014b; Looft et al. 2012). Similarly, previous studies showed that *Prevotella* was the most abundant genus (Holman and Chenier 2014; Looft et al. 2014a), which is in agreement with our study. In our present study, the relative abundance of the phylum *Bacteroidetes* was increased in the COE and COV groups, and the genus *Prevotella*, which belongs to the phylum *Bacteroidetes* (Leser et al. 2002), was increased in the COE and COV groups. Our results showed that CTC combination with OLA and ER, or CTC combination with OLA and VIR, increased the relative abundance of the genus *Prevotella*. Interestingly, Ley et al. (2006) found that the relative proportion of the phylum *Bacteroidetes* was decreased in the obese people, which suggest that *Bacteroidetes* is connected with growth performance. But in our present study, the increase of *Bacteroidetes* did not increase the growth performance of pigs (Results not presented).

In the present study, the microbial genomic DNA extraction was performed using a bead-beating and CTAB method, which was used by the previous studies (Li et al. 2016; Simpson et al. 1999; Wang et al. 2007). But the DNA-isolation protocol of samples influences the distribution of fecal microbes (Knudsen et al. 2016), which

means that some phyla may be underrepresented simply due to bias of the isolation protocol.

Conclusions

Understanding the mechanism of how AGPs regulate gut microbiota of animals will aid in the research and development of alternatives to antibiotics. In the present study, the effects of subtherapeutic antibiotic combinations on the relative abundance and diversity of fecal microorganisms were complicated. At the phylum level, the relative abundance of *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* has differences between antibiotic combination treatments. The mixture of CTC, OLA and ER, and the mixture of CTC, OLA and VIR reduced the microbial diversity in the feces of pigs, in which the relative abundance of the phylum *Bacteroidetes* and the genus *Prevotella* were increased. Compared with the pigs without antibiotics, the relative abundance of the phylum *Proteobacteria* and the genus *Succinivibrio* were decreased except in the pigs fed combination of CTC and *Macleaya cordata*, and a mixture of CTC, OLA and VIR. Since the effects of antibiotic combinations are complicated, we should not only consider additive effects but also the interaction effects between antibiotics.

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Figure Legends

Fig. 1. The relative abundances of top 10 phylum (a) and genus (b) in the eight groups (n=6)

CT: Control group; CTC: Chlortetracycline; BC: bacitracin zinc and colistin sulphate; CBC: chlortetracycline, bacitracin zinc and colistin sulphate; CS: chlortetracycline and *Macleaya cordata* extracts; CO: chlortetracycline and olaquinox; COE: chlortetracycline, olaquinox and enramycin; COV: chlortetracycline, olaquinox and virginiamycin.

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Fig. 2. Weighted Unifrac distances in the eight groups (a) and PCoA of the weighted UniFrac distances (b). Principal components (PCs) 1 and 2 explained 43.3% and 15.6% of the variance, respectively.

CT: Control group; CTC: Chlortetracycline; BC: bacitracin zinc and colistin sulphate; CBC: chlortetracycline, bacitracin zinc and colistin sulphate; CO: chlortetracycline and olaquinox; CS: chlortetracycline and *Macleaya cordata* extracts; COE: chlortetracycline, olaquinox and enramycin; COV: chlortetracycline, olaquinox and virginiamycin.

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Table 1. Treatments for in-feed antibiotic additives for weaned pigs.

Groups	Weaned pigs	Antibiotics additives
CT	50	Fed basal diet (without antibiotics)
CTC	50	75 mg/kg chlortetracycline (CTC) (feed grade premixed agent)
BC	50	40 mg/kg zinc bacitracin (ZB) and 20 mg/kg colistin sulphate (COL)
CBC	50	75 mg/kg CTC, 40 mg/kg ZB and 20mg/kg CT
CS	50	75 mg/kg CTC and 50 mg/kg <i>Macleaya cordata</i> extracts
CO	50	75 mg/kg CTC and 100mg/kg olaquinox (OLA)
COE	50	75 mg/kg CTC, 100 mg/kg OLA and 20mg/kg enramycin (ER)
COV	50	75 mg/kg CTC, 100 mg/kg OLA and 15mg/kg virginiamycin (VIR)

Table 2. Comparison of the proportions of dominant *phyla* in the eight groups of pigs fed different antibiotic combinations for 28 days.

Groups	<i>Firmicutes</i>	<i>Bacteroidetes</i>	<i>Proteobacteria</i>
CT	0.518±0.032	0.381±0.006	0.076±0.031
CTC	0.594±0.016*	0.352±0.013	0.027±0.007*
BC	0.604±0.011*	0.369±0.011	0.018±0.003**
CBC	0.571±0.025	0.389±0.022	0.026±0.009*
CS	0.560±0.019	0.385±0.017	0.039±0.014
CO	0.577±0.039	0.365±0.023	0.030±0.014*
COE	0.470±0.025	0.497±0.023**	0.019±0.003**
COV	0.437±0.010*	0.496±0.008**	0.051±0.015

Note: CT: Fed basal diet (without antibiotics); CTC: 75 mg/kg chlortetracycline (CTC) (feed grade premixed agent); BC: 40 mg/kg zinc bacitracin (ZB) and 20 mg/kg colistin sulphate (COL);CBC: 75 mg/kg CTC, 40 mg/kg ZB and 20mg/kg CT; CS: 75 mg/kg CTC and 50 mg/kg *Macleaya cordata* extracts; CO: 75 mg/kg CTC and 100mg/kg olaquinox (OLA);COE: 75 mg/kg CTC, 100 mg/kg OLA and 20mg/kg enramycin (ER);COV: 75 mg/kg CTC, 100 mg/kg OLA and 15mg/kg virginiamycin (VIR). The data are presented as mean ± SEM. Compared with the CT group: * $P < 0.05$; ** $P < 0.01$.

Table 3. Comparison of dominant genus in the eight groups.

Groups	<i>Prevotella</i>	<i>Roseburia</i>	<i>Succinivibrio</i>	<i>Faecalibacterium</i>
CT	0.158±0.014	0.061±0.010	0.071±0.032	0.047±0.012
CTC	0.123±0.017	0.116±0.031	0.023±0.007*	0.036±0.004
BC	0.151±0.015	0.107±0.009	0.013±0.003**	0.060±0.011
CBC	0.160±0.029	0.085±0.036	0.022±0.009*	0.038±0.004
CS	0.181±0.026	0.090±0.010	0.034±0.014	0.056±0.005
CO	0.145±0.011	0.068±0.022	0.027±0.013*	0.039±0.008
COE	0.307±0.021**	0.080±0.017	0.016±0.003*	0.019±0.003**
COV	0.286±0.031**	0.069±0.013	0.048±0.015	0.033±0.007

Note: CT: Fed basal diet (without antibiotics); CTC: 75 mg/kg chlortetracycline (CTC) (feed grade premixed agent); BC: 40 mg/kg zinc bacitracin (ZB) and 20 mg/kg colistin sulphate (COL); CBC: 75 mg/kg CTC, 40 mg/kg ZB and 20mg/kg CT; CS: 75 mg/kg CTC and 50 mg/kg *Macleaya cordata* extracts; CO: 75 mg/kg CTC and 100mg/kg olaquinox (OLA); COE: 75 mg/kg CTC, 100 mg/kg OLA and 20mg/kg enramycin (ER); COV: 75 mg/kg CTC, 100 mg/kg OLA and 15mg/kg virginiamycin (VIR). The data are presented as mean ± SEM. Compared with the CT group: * $P < 0.05$; ** $P < 0.01$.

Table 4. Comparison of Alpha diversity index in the eight groups.

Groups	observed_species	shannon	simpson	chao1	goods_coverage
CT	624.83±17.01	6.52±0.19	0.967±0.0078	673.16±16.2	0.9980±0.00
CTC	609.17±13.47	6.48±0.18	0.965±0.0083	650.70±13.7	0.9980±0.00
BC	583.17±12.61*	6.49±0.14	0.973±0.0045	625.75±12.7*	0.9980±0.00
CBC	613.00±22.71	6.18±0.16	0.952±0.0080	663.05±24.8	0.9978±0.00
CS	603.67±9.96	6.38±0.14	0.968±0.0056	649.68±9.8	0.9980±0.00
CO	606.00±11.12	6.49±0.16	0.967±0.0076	653.02±11.5	0.9980±0.00
COE	557.50±9.71**	6.41±0.13	0.969±0.0042	603.10±9.4**	0.9983±0.00
COV	579.83±14.62*	6.53±0.13	0.971±0.0040	616.37±17.7*	0.9983±0.00

Note: CT: Fed basal diet (without antibiotics); CTC: 75 mg/kg chlortetracycline (CTC) (feed grade premixed agent); BC: 40 mg/kg zinc bacitracin (ZB) and 20 mg/kg colistin sulphate (COL);CBC: 75 mg/kg CTC, 40 mg/kg ZB and 20mg/kg CT; CS: 75 mg/kg CTC and 50 mg/kg *Macleaya cordata* extracts; CO: 75 mg/kg CTC and 100mg/kg olaquinox (OLA);COE: 75 mg/kg CTC, 100 mg/kg OLA and 20mg/kg enramycin (ER);COV: 75 mg/kg CTC, 100 mg/kg OLA and 15mg/kg virginiamycin (VIR). The data are presented as mean ± SEM. Compared with the CT group: **P* < 0.05; ***P* < 0.01.

Supporting Information

Fig. S1. The rarefaction curves of treatments in antibiotic combinations.

Fig. S2. OTU-level composition (the relative abundance > 1%) of fecal samples in antibiotic combination groups.

Fig. S3. OTUs with significant difference at 1% level ($P < 0.01$) between antibiotic combination treatments.

Fig. S4. Cluster distribution of fecal microbiota of pigs at the level of bacterial genus (> 0.1%) by heatmap.

Table S1. Raw reads, Effective Tags and OTUs from each groups.

Table S2. OTUs with significant difference between different antibiotics combinations.

Table S3. Effect of each group on the relative proportion (percentage $\pm$ standard deviation of the mean) of the most abundant phyla and genera in the each treatment pigs (n=6).

Table S4. OTU Taxonomy.

Fig. 1. The relative abundances of top 10 phylum (a) and genus (b) in the eight groups (n=6)[†] CT: Control group; CTC: Chlortetracycline; BC: bacitracin zinc and colistin sulphate; CBC: chlortetracycline, bacitracin zinc and colistin sulphate; CS: chlortetracycline and *Macleaya cordata* extracts; CO: chlortetracycline and olaquinox; COE: chlortetracycline, olaquinox and enramycin; COV: chlortetracycline, olaquinox and virginiamycin.

Fig. 1
191x243mm (300 x 300 DPI)

Fig. 2. Weighted Unifrac distances in the eight groups (a) and PCoA of the weighted UniFrac distances (b). Principal components (PCs) 1 and 2 explained 43.3% and 15.6% of the variance, respectively.!! † CT: Control group; CTC: Chlortetracycline; BC: bacitracin zinc and colistin sulphate; CBC: chlortetracycline, bacitracin zinc and colistin sulphate; CO: chlortetracycline and olaquinox; CS: chlortetracycline and *Macleaya cordata* extracts; COE: chlortetracycline, olaquinox and enramycin; COV: chlortetracycline, olaquinox and virginiamycin.

Fig. 2

260x422mm (300 x 300 DPI)

Supporting Information

Figure S1 The rarefaction curves of treatments in antibiotic combinations.

CT: Control group (n=6); CTC: Chlortetracycline (n=6); BC: a mixture of bacitracin zinc and colistin sulphate (n=6); CBC: a mixture of chlortetracycline, bacitracin zinc and colistin sulphate (n=6); CS: a mixture of chlortetracycline and *Macleaya cordata* extracts (n=6); CO: a mixture of chlortetracycline and olaquinox (n=6); COE: a mixture of chlortetracycline, olaquinox and enramycin (n=6); COV: a mixture of chlortetracycline, olaquinox and virginiamycin (n=6).

Figure S2 OTU-level composition (the relative abundance > 1%) of fecal samples in antibiotic combination groups.

CT: Control group (n=6); CTC: Chlortetracycline (n=6); BC: a mixture of bacitracin zinc and colistin sulphate (n=6); CBC: a mixture of chlortetracycline, bacitracin zinc and colistin sulphate (n=6); CS: a mixture of chlortetracycline and *Macleaya cordata* extracts (n=6); CO: a mixture of chlortetracycline and olaquinox (n=6); COE: a mixture of chlortetracycline, olaquinox and enramycin (n=6); COV: a mixture of chlortetracycline, olaquinox and virginiamycin (n=6).

Figure S3 OTUs with significant difference at 1% level ($P < 0.01$) between antibiotic combination treatments.

CT: Control group (n=6); CTC: Chlortetracycline (n=6); BC: a mixture of bacitracin zinc and colistin sulphate (n=6); CBC: a mixture of chlortetracycline, bacitracin zinc and colistin sulphate (n=6); CS: a mixture of chlortetracycline and *Macleaya cordata* extracts (n=6); CO: a mixture of chlortetracycline and olaquinox (n=6); COE: a mixture of chlortetracycline, olaquinox and enramycin (n=6); COV: a mixture of chlortetracycline, olaquinox and virginiamycin (n=6).

Figure S4 Cluster distribution of fecal microbiota of pigs at the level of bacterial genus (> 0.1%) by heatmap.

CT: Control group (n=6); CTC: Chlortetracycline (n=6); BC: a mixture of bacitracin zinc and colistin sulphate (n=6); CBC: a mixture of chlortetracycline, bacitracin zinc and colistin sulphate (n=6); CS: a mixture of chlortetracycline and *Macleaya cordata* extracts (n=6); CO: a mixture of chlortetracycline and olaquinox (n=6); COE: a mixture of chlortetracycline, olaquinox and enramycin (n=6); COV: a mixture of chlortetracycline, olaquinox and virginiamycin (n=6).

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Table S2 OTUs with significant difference between different antibiotics combinations

OTU	P	Microbial group	CT	CTC	BC	CBC	CS	CO	COE	COV	P Value
OTU276	Actinobacteria	f__Coriobacteriaceae	0.005±0.008	0.014±0.017	0.023±0.008	0.002±0.005	0.007±0.009	0.012±0.011	0.009±0.007	0.002±0.005	0.006
OTU281		f__Coriobacteriaceae; g__Slackia	0.002±0.005	0.008±0.009	0.030±0.015	0.005±0.008	0.020±0.012	0.002±0.005	0.000±0.000	0.003±0.008	0.000
OTU82	Bacteroidetes	o__Bacteroidales	0.247±0.214	0.135±0.030	0.388±0.157	0.164±0.069	0.372±0.253	0.129±0.038	0.150±0.056	0.182±0.052	0.004
OTU73		f__[Paraprevotellaceae]	0.290±0.172	0.126±0.023	0.108±0.028	0.174±0.071	0.150±0.029	0.092±0.020	0.156±0.021	0.254±0.054	0.000
OTU222		f__Bacteroidaceae	0.060±0.033	0.051±0.017	0.027±0.013	0.044±0.014	0.036±0.015	0.042±0.015	0.067±0.023	0.067±0.018	0.008
OTU110		f__Bacteroidaceae;g__ Bacteroides	0.383±0.242	0.292±0.084	0.185±0.081	0.395±0.158	0.186±0.097	0.355±0.186	0.436±0.162	0.641±0.385	0.007
OTU11		f__Prevotellaceae;g__ Prevotella	1.539±0.626	1.670±1.444	1.311±0.684	1.759±1.014	1.439±0.896	2.236±0.544	3.819±1.587	3.346±1.413	0.001
OTU66		f__Prevotellaceae;g__ Prevotella	0.556±0.253	0.699±0.542	0.333±0.185	0.297±0.141	0.192±0.096	0.511±0.413	1.289±0.971	0.691±0.408	0.007
OTU102		f__Prevotellaceae;g__ Prevotella	0.247±0.126	0.178±0.083	0.198±0.068	0.139±0.048	0.184±0.070	0.124±0.060	0.368±0.183	0.469±0.303	0.001
OTU140		f__Prevotellaceae;g__ Prevotella	0.062±0.017	0.176±0.137	0.060±0.046	0.052±0.025	0.056±0.020	0.093±0.059	0.225±0.173	0.321±0.191	0.000
OTU146		f__Prevotellaceae;g__ Prevotella	0.331±0.266	0.201±0.082	0.091±0.042	0.086±0.039	0.110±0.051	0.109±0.023	0.200±0.044	0.244±0.061	0.002
OTU154		f__Prevotellaceae;g__ Prevotella	0.047±0.034	0.063±0.056	0.054±0.037	0.020±0.032	0.041±0.047	0.034±0.034	0.411±0.320	0.334±0.403	0.002
OTU224		f__Prevotellaceae;g__ Prevotella	0.064±0.036	0.033±0.020	0.017±0.014	0.022±0.016	0.026±0.014	0.040±0.037	0.071±0.030	0.074±0.059	0.009

OTU	P	Microbial group	CT	CTC	BC	CBC	CS	CO	COE	COV	P Value
OTU244	Bacteroidetes	f__Prevotellaceae;g__Prevotella	0.049±0.082	0.015±0.016	0.041±0.030	0.020±0.004	0.027±0.018	0.007±0.008	0.046±0.031	0.107±0.052	0.002
OTU254		f__Prevotellaceae;g__Prevotella	0.037±0.015	0.031±0.012	0.018±0.011	0.022±0.014	0.035±0.014	0.031±0.015	0.165±0.133	0.068±0.025	0.000
OTU382		f__Prevotellaceae;g__Prevotella	0.030±0.012	0.014±0.007	0.014±0.012	0.009±0.015	0.017±0.016	0.004±0.006	0.019±0.010	0.033±0.020	0.006
OTU384		f__Prevotellaceae;g__Prevotella	0.026±0.019	0.014±0.018	0.005±0.007	0.002±0.005	0.002±0.005	0.010±0.011	0.034±0.027	0.019±0.018	0.006
OTU395		f__Prevotellaceae;g__Prevotella	0.007±0.010	0.005±0.009	0.009±0.010	0.004±0.009	0.015±0.017	0.007±0.013	0.010±0.011	0.035±0.012	0.001
OTU442		f__Prevotellaceae;g__Prevotella	0.000±0.000	0.000±0.000	0.000±0.000	0.000±0.000	0.000±0.000	0.022±0.023	0.010±0.012	0.000±0.000	0.001
OTU1164		f__Prevotellaceae;g__Prevotella	0.000±0.000	0.000±0.000	0.000±0.000	0.000±0.000	0.000±0.000	0.000±0.000	0.007±0.007	0.000±0.000	0.000
OTU306		f__Prevotellaceae;g__Prevotella;s__copri	0.276±0.243	0.180±0.080	0.277±0.085	0.152±0.052	0.253±0.147	0.137±0.058	0.751±0.718	0.757±0.304	0.001
OTU30	Firmicutes	f__S24-7	1.241±1.033	1.541±1.130	2.919±1.128	0.854±0.258	1.961±1.589	1.456±0.962	0.545±0.180	0.765±0.288	0.003
OTU201		f__S24-7	0.055±0.080	0.052±0.043	0.183±0.135	0.013±0.011	0.104±0.074	0.021±0.013	0.037±0.020	0.034±0.035	0.001
OTU424		f__S24-7	0.000±0.000	0.019±0.021	0.002±0.005	0.002±0.006	0.000±0.000	0.007±0.011	0.000±0.000	0.000±0.000	0.006
OTU644		f__S24-7	0.018±0.011	0.024±0.018	0.029±0.033	0.023±0.014	0.029±0.052	0.162±0.180	0.030±0.018	0.010±0.012	0.010
OTU256		c__Clostridia;o__Clostridiales	0.015±0.003	0.023±0.008	0.030±0.011	0.008±0.014	0.022±0.015	0.015±0.010	0.020±0.004	0.005±0.009	0.003
OTU263		c__Clostridia;o__Clostridiales	0.025±0.008	0.036±0.019	0.029±0.011	0.015±0.013	0.038±0.011	0.016±0.006	0.020±0.008	0.023±0.007	0.004

OTU	P	Microbial group	CT	CTC	BC	CBC	CS	CO	COE	COV	P Value
OTU273	Firmicutes	c__Clostridia;o__Clostridiales	0.005±0.008	0.017±0.005	0.015±0.008	0.000±0.000	0.018±0.011	0.005±0.008	0.014±0.012	0.012±0.010	0.004
OTU117		c__Clostridia;o__Clostridiales	0.099±0.028	0.133±0.042	0.159±0.083	0.073±0.015	0.095±0.025	0.169±0.098	0.085±0.034	0.054±0.009	0.003
OTU212		c__Clostridia;o__Clostridiales	0.032±0.034	0.048±0.046	0.101±0.035	0.022±0.018	0.071±0.042	0.029±0.030	0.032±0.017	0.023±0.027	0.001
OTU70		f__[Mogibacteriaceae]	0.121±0.054	0.296±0.191	0.713±0.707	0.063±0.021	0.207±0.052	0.137±0.103	0.169±0.091	0.109±0.070	0.004
OTU1003		f__[Mogibacteriaceae]	0.002±0.005	0.011±0.012	0.015±0.014	0.000±0.000	0.012±0.007	0.011±0.006	0.009±0.007	0.022±0.005	0.003
OTU1125		f__[Mogibacteriaceae]	0.033±0.013	0.030±0.015	0.016±0.010	0.020±0.008	0.003±0.008	0.012±0.010	0.009±0.007	0.000±0.000	0.000
OTU28		f__Clostridiaceae	0.455±0.301	0.446±0.126	0.382±0.109	0.285±0.076	0.332±0.114	0.378±0.129	0.697±0.386	0.663±0.165	0.008
OTU58		f__Lachnospiraceae	0.081±0.080	0.135±0.086	0.085±0.033	0.326±0.217	0.107±0.070	0.477±0.334	0.164±0.110	0.101±0.059	0.000
OTU312		f__Lachnospiraceae	0.022±0.019	0.013±0.007	0.014±0.008	0.000±0.000	0.008±0.007	0.005±0.007	0.015±0.008	0.027±0.010	0.000
OTU367		f__Lachnospiraceae	0.013±0.021	0.030±0.027	0.006±0.009	0.002±0.006	0.000±0.000	0.004±0.006	0.002±0.005	0.000±0.000	0.003
OTU315		f__Lachnospiraceae;g__Coprococcus	0.008±0.010	0.012±0.011	0.007±0.007	0.011±0.009	0.007±0.008	0.008±0.009	0.021±0.013	0.037±0.029	0.004
OTU194		f__Lachnospiraceae;g__Coprococcus;s__cat us	0.032±0.004	0.067±0.041	0.023±0.005	0.019±0.017	0.020±0.009	0.012±0.010	0.027±0.014	0.036±0.013	0.000
OTU48		f__Ruminococcaceae	0.125±0.043	0.259±0.160	0.341±0.109	0.443±0.242	0.254±0.069	0.294±0.107	0.129±0.031	0.137±0.075	0.000
OTU120		f__Ruminococcaceae	0.140±0.080	0.121±0.043	0.067±0.010	0.076±0.042	0.082±0.025	0.064±0.018	0.061±0.013	0.061±0.019	0.004
OTU200		f__Ruminococcaceae	0.027±0.015	0.041±0.012	0.053±0.014	0.037±0.023	0.027±0.007	0.027±0.010	0.015±0.008	0.017±0.011	0.000
OTU326		f__Ruminococcaceae	0.015±0.014	0.014±0.015	0.003±0.009	0.003±0.007	0.002±0.005	0.000±0.000	0.000±0.000	0.000±0.000	0.007
OTU808		f__Ruminococcaceae	0.011±0.009	0.025±0.010	0.010±0.008	0.013±0.011	0.011±0.011	0.016±0.011	0.002±0.005	0.013±0.002	0.009
OTU13		f__Ruminococcaceae	0.395±0.153	0.516±0.380	1.223±0.835	0.955±0.475	1.507±0.840	0.939±0.643	0.516±0.292	0.440±0.161	0.005

OTU	P	Microbial group	CT	CTC	BC	CBC	CS	CO	COE	COV	P Value
OTU79	Firmicutes	f__Ruminococcaceae	0.047±0.037	0.126±0.057	0.203±0.057	0.058±0.014	0.096±0.057	0.155±0.104	0.090±0.034	0.051±0.019	0.000
OTU1066		f__Ruminococcaceae	0.225±0.092	0.164±0.072	0.072±0.037	0.077±0.030	0.073±0.029	0.143±0.085	0.098±0.051	0.063±0.020	0.000
OTU6		f__Ruminococcaceae; g__Faecalibacterium;s__prausnitzii	4.152±2.776	2.905±1.050	4.734±1.975	3.087±0.880	4.874±1.550	2.753±1.059	1.353±0.386	2.641±1.411	0.004
		f__Ruminococcaceae; g__Oscillospira	0.689±0.161	1.654±0.950	1.006±0.608	0.322±0.084	0.697±0.168	0.458±0.109	0.522±0.214	0.597±0.309	0.000
OTU45		f__Ruminococcaceae; g__Oscillospira	1.021±0.830	0.638±0.295	0.627±0.289	0.262±0.080	0.428±0.122	0.274±0.131	0.251±0.073	0.360±0.230	0.004
OTU128		f__Ruminococcaceae; g__Oscillospira	0.077±0.016	0.201±0.192	0.053±0.046	0.044±0.016	0.029±0.018	0.059±0.041	0.040±0.039	0.022±0.013	0.004
OTU218		f__Ruminococcaceae; g__Oscillospira	0.015±0.014	0.047±0.041	0.025±0.007	0.016±0.011	0.007±0.008	0.033±0.015	0.009±0.011	0.007±0.010	0.003
OTU220		f__Ruminococcaceae; g__Oscillospira	0.052±0.019	0.034±0.008	0.037±0.006	0.043±0.011	0.037±0.012	0.031±0.009	0.019±0.012	0.024±0.014	0.001
OTU405		f__Ruminococcaceae; g__Oscillospira	0.018±0.009	0.014±0.008	0.005±0.009	0.005±0.008	0.004±0.007	0.002±0.005	0.000±0.000	0.000±0.000	0.000
OTU908		f__Ruminococcaceae; g__Oscillospira	0.005±0.007	0.010±0.009	0.002±0.006	0.000±0.000	0.002±0.005	0.000±0.000	0.000±0.000	0.000±0.000	0.007
OTU15		f__Ruminococcaceae; g__Ruminococcus	0.252±0.132	0.305±0.157	1.410±0.816	0.595±0.510	0.651±0.202	0.438±0.165	0.190±0.098	0.210±0.110	0.000
OTU93		f__Ruminococcaceae; g__Ruminococcus	0.095±0.056	0.186±0.148	0.177±0.161	0.177±0.054	0.056±0.032	0.576±0.489	0.130±0.114	0.045±0.026	0.001

OTU	P	Microbial group	CT	CTC	BC	CBC	CS	CO	COE	COV	P Value
OTU153		f__Ruminococcaceae; g__Ruminococcus	0.046±0.028	0.105±0.063	0.066±0.034	0.113±0.042	0.025±0.020	0.276±0.176	0.069±0.034	0.022±0.016	0.000
OTU54		f__Ruminococcaceae; g__Ruminococcus;s__ bromii	0.224±0.079	0.389±0.105	0.318±0.056	0.352±0.183	0.178±0.063	0.827±0.514	0.454±0.080	0.259±0.147	0.000
OTU17		f__Veillonellaceae;g__ Phascolarctobacteriu m	1.346±0.505	1.319±0.554	0.647±0.168	0.749±0.352	1.197±0.508	0.587±0.362	0.656±0.361	0.660±0.481	0.004
OTU170	Proteobacteria	f__Alcaligenaceae;g__ Sutterella	0.027±0.024	0.021±0.012	0.073±0.049	0.033±0.014	0.069±0.038	0.025±0.010	0.028±0.022	0.037±0.016	0.005
OTU317		f__Xanthomonadaceae ;g__Stenotrophomona s	0.021±0.013	0.023±0.009	0.029±0.006	0.008±0.013	0.035±0.027	0.012±0.009	0.014±0.013	0.004±0.006	0.003

Table S1 Raw reads, Effective Tags and OTUs from each groups

Groups	Raw reads	Effective Tags	OTUs	Average Length
CT	85468	57237	689	413
CTC	80596	53948	667	413
BC	76418	50169	636	413
CBC	83335	55040	670	413
CS	79889	52676	663	414
CO	80874	52920	663	413
COE	79605	50792	608	416
COV	77326	49767	626	416

CT: Control group; CTC: Chlortetracycline; BC: bacitracin zinc and colistin sulphate; CBC: chlortetracycline, bacitracin zinc and colistin sulphate; CS: chlortetracycline and *Macleaya cordata* extracts; CO: chlortetracycline and olaquinox; COE: chlortetracycline, olaquinox and enramycin; COV: chlortetracycline, olaquinox and virginiamycin.

Table S3 Effect of each group on the relative proportion (percentage $\pm$ standard deviation of the mean) of the most abundant phyla and genera in the each treatment pigs (n=6).

Taxon	CT	CTC	BC	CBC	CS	CO	COE	COV
Phylum								
Firmicutes	51.8 $\pm$ 3.2	59.4 $\pm$ 1.6*	60.4 $\pm$ 1.1*	57.1 $\pm$ 2.5	56.0 $\pm$ 1.9	57.7 $\pm$ 3.9	47.0 $\pm$ 2.5	43.7 $\pm$ 1.0*
Bacteroidetes	38.1 $\pm$ 0.6	35.2 $\pm$ 1.3	36.9 $\pm$ 1.1	38.9 $\pm$ 2.2	38.5 $\pm$ 1.7	36.5 $\pm$ 2.3	49.7 $\pm$ 2.3**	49.6 $\pm$ 0.8**
Proteobacteria	7.6 $\pm$ 3.1	2.7 $\pm$ 0.7*	1.8 $\pm$ 0.3**	2.6 $\pm$ 0.9*	3.9 $\pm$ 1.4	3.0 $\pm$ 1.4*	1.9 $\pm$ 0.3**	5.1 $\pm$ 1.5
Euryarchaeota	0.72 $\pm$ 0.41	1.32 $\pm$ 1.20	0.03 $\pm$ 0.01	0.08 $\pm$ 0.06	0.32 $\pm$ 0.29	1.06 $\pm$ 0.94	0.10 $\pm$ 0.09	0.12 $\pm$ 0.09
Spirochaetes	0.65 $\pm$ 0.44	0.34 $\pm$ 0.13	0.11 $\pm$ 0.02*	0.19 $\pm$ 0.09	0.24 $\pm$ 0.06	0.35 $\pm$ 0.19	0.18 $\pm$ 0.04	0.24 $\pm$ 0.08
Tenericutes	0.21 $\pm$ 0.03	0.32 $\pm$ 0.12	0.19 $\pm$ 0.04	0.37 $\pm$ 0.11	0.35 $\pm$ 0.15	0.65 $\pm$ 0.30	0.54 $\pm$ 0.21	0.36 $\pm$ 0.12
Cyanobacteria	0.51 $\pm$ 0.29	0.24 $\pm$ 0.07	0.25 $\pm$ 0.13	0.40 $\pm$ 0.14	0.23 $\pm$ 0.07	0.32 $\pm$ 0.08	0.27 $\pm$ 0.08	0.47 $\pm$ 0.16
Actinobacteria	0.43 $\pm$ 0.03	0.43 $\pm$ 0.05	0.35 $\pm$ 0.03	0.26 $\pm$ 0.02**	0.40 $\pm$ 0.06	0.31 $\pm$ 0.04*	0.23 $\pm$ 0.02**	0.26 $\pm$ 0.03**
Elusimicrobia	0.054 $\pm$ 0.043	0.016 $\pm$ 0.006	0.005 $\pm$ 0.002	0.006 $\pm$ 0.002	0.012 $\pm$ 0.006	0.029 $\pm$ 0.022	0.011 $\pm$ 0.006	0.011 $\pm$ 0.003
Fibrobacteres	0.0000 $\pm$ 0.0000	0.0012 $\pm$ 0.0012	0.0012 $\pm$ 0.0012	0.0015 $\pm$ 0.0008	0.0004 $\pm$ 0.0004	0.0251 $\pm$ 0.0200	0.0031 $\pm$ 0.0023	0.0027 $\pm$ 0.0019
Others	0.027 $\pm$ 0.012	0.030 $\pm$ 0.006	0.026 $\pm$ 0.005	0.043 $\pm$ 0.021	0.054 $\pm$ 0.019	0.032 $\pm$ 0.007	0.042 $\pm$ 0.006	0.078 $\pm$ 0.038
Genus								
Prevotella	15.8 $\pm$ 1.4	12.3 $\pm$ 1.7	15.1 $\pm$ 1.5	16.0 $\pm$ 2.9	18.1 $\pm$ 2.6	14.5 $\pm$ 1.1	30.7 $\pm$ 2.1**	28.6 $\pm$ 3.1**
Roseburia	6.1 $\pm$ 1.0	11.6 $\pm$ 3.1	10.7 $\pm$ 0.9	8.5 $\pm$ 3.6	9.0 $\pm$ 1.0	6.8 $\pm$ 2.2	8.0 $\pm$ 1.7	6.9 $\pm$ 1.3
Succinivibrio	7.1 $\pm$ 3.2	2.3 $\pm$ 0.7*	1.3 $\pm$ 0.3**	2.2 $\pm$ 0.9*	3.4 $\pm$ 1.4	2.7 $\pm$ 1.3*	1.6 $\pm$ 0.3*	4.8 $\pm$ 1.5
[Prevotella]	6.7 $\pm$ 2.1	5.0 $\pm$ 1.5	5.8 $\pm$ 0.6	4.7 $\pm$ 0.7	4.8 $\pm$ 0.9	5.3 $\pm$ 0.6	6.0 $\pm$ 0.7	4.7 $\pm$ 0.4
Faecalibacterium	4.7 $\pm$ 1.2	3.6 $\pm$ 0.4	6.0 $\pm$ 1.1	3.8 $\pm$ 0.4	5.6 $\pm$ 0.5	3.9 $\pm$ 0.8	1.9 $\pm$ 0.3**	3.3 $\pm$ 0.7
Lachnospira	3.3 $\pm$ 0.6	2.6 $\pm$ 0.2	3.7 $\pm$ 0.7	2.5 $\pm$ 0.2	3.4 $\pm$ 0.4	3.3 $\pm$ 0.8	2.8 $\pm$ 0.3	3.3 $\pm$ 0.3
Methanobrevibacter	0.72 $\pm$ 0.41	1.30 $\pm$ 1.20	0.03 $\pm$ 0.01	0.07 $\pm$ 0.06	0.32 $\pm$ 0.29	1.06 $\pm$ 0.94	0.10 $\pm$ 0.09	0.12 $\pm$ 0.09
Ruminococcus	2.1 $\pm$ 0.2	2.3 $\pm$ 0.2	3.4 $\pm$ 0.4*	4.3 $\pm$ 0.8**	2.9 $\pm$ 0.1	4.0 $\pm$ 0.5**	2.5 $\pm$ 0.2	2.3 $\pm$ 0.2
CF231	1.74 $\pm$ 0.90	0.94 $\pm$ 0.08	0.89 $\pm$ 0.09	0.62 $\pm$ 0.11	0.75 $\pm$ 0.19	0.79 $\pm$ 0.13	0.86 $\pm$ 0.09	0.88 $\pm$ 0.12
Oscillospira	3.4 $\pm$ 0.5	4.3 $\pm$ 0.5	3.6 $\pm$ 0.5	2.0 $\pm$ 0.2*	2.6 $\pm$ 0.1*	2.3 $\pm$ 0.3	2.0 $\pm$ 0.3	2.2 $\pm$ 0.3
Others	48.2 $\pm$ 4.0	53.8 $\pm$ 3.8	49.4 $\pm$ 2.2	55.4 $\pm$ 6.2	49.0 $\pm$ 3.3	55.3 $\pm$ 4.3	43.5 $\pm$ 3.2	42.9 $\pm$ 3.5

CT: Control group; CTC: Chlortetracycline; BC: bacitracin zinc and colistin sulphate; CBC: chlortetracycline, bacitracin zinc and colistin sulphate; CS: chlortetracycline and *Macleaya cordata* extracts; CO: chlortetracycline and olaquinox; COE: chlortetracycline, olaquinox and enramycin; COV: chlortetracycline, olaquinox and virginiamycin. The relative abundances of phyla and genera are presented as mean $\pm$ SEM. Statistical significance was analyzed by the Student's t tests. Statistically significant comparison results are marked with * $P < 0.05$, and ** $P < 0.01$.

Table S4. OTU Taxonomy.

OTU_num	Taxonomy
OTU1	k_Bacteria;p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae
OTU10	k_Bacteria;p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_;s_
OTU1003	k_Bacteria;p_Firmicutes;c_Clostridia;o_Clostridiales;f_[Mogibacteriaceae];g_;s_
OTU102	k_Bacteria;p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Prevotellaceae;g_Prevotella;s_
OTU1066	k_Bacteria;p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_;s_
OTU1099	k_Bacteria;p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_Roseburia;s_faecis
OTU11	k_Bacteria;p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Prevotellaceae;g_Prevotella;s_
OTU110	k_Bacteria;p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Bacteroidaceae;g_Bacteroides;s_
OTU1125	k_Bacteria;p_Firmicutes;c_Clostridia;o_Clostridiales;f_[Mogibacteriaceae];g_;s_
OTU1164	k_Bacteria;p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Prevotellaceae;g_Prevotella;s_
OTU117	k_Bacteria;p_Firmicutes;c_Clostridia;o_Clostridiales;f_;g_;s_
OTU120	k_Bacteria;p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae
OTU128	k_Bacteria;p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_Oscillospira;s_
OTU13	k_Bacteria;p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_;s_
OTU14	k_Bacteria;p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_[Paraprevotellaceae];g_[Prevotella];s_
OTU140	k_Bacteria;p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Prevotellaceae;g_Prevotella;s_
OTU146	k_Bacteria;p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Prevotellaceae;g_Prevotella;s_
OTU15	k_Bacteria;p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_Ruminococcus;s_
OTU153	k_Bacteria;p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_Ruminococcus;s_
OTU154	k_Bacteria;p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Prevotellaceae;g_Prevotella;s_
OTU16	k_Bacteria;p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_Oscillospira;s_
OTU17	k_Bacteria;p_Firmicutes;c_Clostridia;o_Clostridiales;f_Veillonellaceae;g_Phascolarctobacterium;s_
OTU170	k_Bacteria;p_Proteobacteria;c_Betaproteobacteria;o_Burkholderiales;f_Alcaligenaceae;g_Sutterella;s_
OTU18	k_Bacteria;p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Prevotellaceae;g_Prevotella;s_
OTU19	k_Bacteria;p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Prevotellaceae;g_Prevotella;s_
OTU194	k_Bacteria;p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_Coprococcus;s_catus
OTU2	k_Bacteria;p_Proteobacteria;c_Gammaproteobacteria;o_Aeromonadales;f_Succinivibrionaceae;g_Succinivibrio;s_
OTU200	k_Bacteria;p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae
OTU201	k_Bacteria;p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_S24-7;g_;s_
OTU21	k_Bacteria;p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_S24-7;g_;s_

OTU212	k_Bacteria;p_Firmicutes;c_Clostridia;o_Clostridiales;f_ ;g_ ;s_
OTU214	k_Bacteria;p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Prevotellaceae;g_Prevotella;s_copri
OTU218	k_Bacteria;p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_Oscillospira;s_
OTU220	k_Bacteria;p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_Oscillospira;s_
OTU222	k_Bacteria;p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Bacteroidaceae
OTU224	k_Bacteria;p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Prevotellaceae;g_Prevotella;s_
OTU244	k_Bacteria;p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Prevotellaceae;g_Prevotella;s_
OTU254	k_Bacteria;p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Prevotellaceae;g_Prevotella;s_
OTU256	k_Bacteria;p_Firmicutes;c_Clostridia;o_Clostridiales
OTU263	k_Bacteria;p_Firmicutes;c_Clostridia;o_Clostridiales
OTU27	k_Bacteria;p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Prevotellaceae;g_Prevotella;s_copri
OTU273	k_Bacteria;p_Firmicutes;c_Clostridia;o_Clostridiales
OTU276	k_Bacteria;p_Actinobacteria;c_Coriobacteriia;o_Coriobacteriales;f_Coriobacteriaceae;g_ ;s_
OTU28	k_Bacteria;p_Firmicutes;c_Clostridia;o_Clostridiales;f_Clostridiaceae;g_ ;s_
OTU281	k_Bacteria;p_Actinobacteria;c_Coriobacteriia;o_Coriobacteriales;f_Coriobacteriaceae;g_Slackia;s_
OTU3	k_Bacteria;p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_Roseburia;s_
OTU30	k_Bacteria;p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_S24-7;g_ ;s_
OTU30	k_Bacteria;p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_S24-7;g_ ;s_
OTU306	k_Bacteria;p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Prevotellaceae;g_Prevotella;s_copri
OTU312	k_Bacteria;p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae
OTU315	k_Bacteria;p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_Coproccoccus;s_
OTU317	k_Bacteria;p_Proteobacteria;c_Gammaproteobacteria;o_Xanthomonadales;f_Xanthomonadaceae;g_Stenotrophomonas;s_
OTU32	k_Bacteria;p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_[Paraprevotellaceae];g_[Prevotella];s_
OTU326	k_Bacteria;p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae
OTU34	k_Bacteria;p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Prevotellaceae;g_Prevotella;s_
OTU367	k_Bacteria;p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae
OTU382	k_Bacteria;p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Prevotellaceae;g_Prevotella;s_
OTU384	k_Bacteria;p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Prevotellaceae;g_Prevotella;s_
OTU395	k_Bacteria;p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Prevotellaceae;g_Prevotella;s_
OTU4	k_Bacteria;p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae
OTU405	k_Bacteria;p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_Oscillospira;s_
OTU424	k_Bacteria;p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_S24-7;g_ ;s_
OTU442	k_Bacteria;p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Prevotellaceae;g_Prevotella;s_

OTU45	k_Bacteria;p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_Oscillospira;s_
OTU48	k_Bacteria;p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae
OTU5	k_Bacteria;p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_Lachnospira;s_
OTU51	k_Bacteria;p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_[Paraprevotellaceae];g_[Prevotella];s_
OTU54	k_Bacteria;p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_Ruminococcus;s_bromii
OTU58	k_Bacteria;p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae
OTU6	k_Bacteria;p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_Faecalibacterium;s_prausnitzii
OTU62	k_Bacteria;p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_;s_
OTU644	k_Bacteria;p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_S24-7;g_;s_
OTU66	k_Bacteria;p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Prevotellaceae;g_Prevotella;s_
OTU7	k_Bacteria;p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales
OTU70	k_Bacteria;p_Firmicutes;c_Clostridia;o_Clostridiales;f_[Mogibacteriaceae];g_;s_
OTU73	k_Bacteria;p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_[Paraprevotellaceae];g_;s_
OTU79	k_Bacteria;p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_;s_
OTU808	k_Bacteria;p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae
OTU82	k_Bacteria;p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_;g_;s_
OTU9	k_Bacteria;p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_S24-7;g_;s_
OTU908	k_Bacteria;p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_Oscillospira;s_
OTU93	k_Bacteria;p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_Ruminococcus;s_