Effects of antibiotics on anaerobic digestion resistance genes and archaeal communities in cattle manure

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Abstract. In this paper, the effects of antibiotics on key enzymes genes of gas production pathway, diversity of microbial communities and ARGs changes during anaerobic digestion of cattle manure are investigated by adding antibiotics of different kinds and concentrations. TYL and SM2 increased the abundance of Methanosarcina in the acetic acid methanogens pathway by 29.04% and 52.79%, respectively, and CIP decreased the abundance of Methanobrevibacter and Methanosarcina. At the same time, CIP has a strong inhibitory effect on mcrA gene, and its abundance is 49% lower than that of the control group. SM2 increases ermF gene by 2 logs, and TYL and CIP by 1 log. Sul1 increased by 1-2 logs in the process of adding antibiotics to the anaerobic digestion process.

1. Introduction
Antibiotics are commonly used medicinal products on farms and have a wide range of uses in disease treatment and growth promotion [1]. Many of these compounds have poor retention in the intestine during animal digestion, and 17% to 76% of antibiotics are excreted in urine and feces in undegraded form or as metabolites of the parent compound [2-3]. Tylosin (TYL), sulfamethazine (SM2), and ciprofloxacin (CIP) are the most commonly used types of antibiotics in humans and animals, and their use in 2013 was 7,920 t, 15560 t, and 27300 t [4]. In recent years, some studies have found that the residual level of sulfonamide antibiotics in livestock and poultry manure is mg/kg, of which SM2 is the main sulfonamide antibiotic found in livestock and poultry manure. TYL has also been found in the manure of some large farms, and its content in pig manure can reach 89 mg/kg [5-6].

Methanogenic archaea are responsible for the biological production of methane, and they typically use H2/CO2 and acetate as substrates under anaerobic conditions [7]. As methanogens grow slowly and are susceptible to environmental changes and inhibitors, methanogens are important microbial communities in the stable operation of anaerobic digestive system. The pollution of antibiotics in
agricultural waste will affect the microbial community structure and reduce the efficiency of the biogas fermentation system. The methanogen community determined by the mcrA and 16S rRNA genes is basically the same. Both genes have been used to clarify the diversity and phylogeny of methanogens in anaerobic digestion [8-9]. Methane production is significantly positively correlated with the abundance of mcrA transcripts in biogas digesters used to treat sludge and wastewater samples, supporting the mcrA gene as a biomarker for methane production. In this study, three antibiotics, tylosin (TYL), ciprofloxacin (CIP), and sulfamethazine (SM$_2$), were selected to study resistance genes and archaeal microbial communities during anaerobic digestion.

2. Materials and Methods

2.1. Methods

The anaerobic digestion device is shown in Fig.1. The effective working volume of each fermentation tank is 4L. Use distilled water to adjust the total solids content of cow dung to 8%, and stir the raw materials for 15 minutes to ensure uniform stirring. Anaerobic digestion tanks are wrapped in black plastic bags to prevent photolysis of antibiotics. Add 800ml of seed liquid and ferment at 35 ℃ for 45 days. Set four treatments according to the type of antibiotic selected, namely CK: no antibiotics added; TYL: 50mg / kg dry weight of cow dung; CIP: 30 mg / kg dry weight of cow dung; SM$_2$: 20 mg / kg dry weight of cow dung. Each treatment was repeated three times. The concentration of antibiotics is calculated based on the average residual level in the stool.

![Figure 1. Schematic diagram of anaerobic digestion](image)

Note: 1 5L digestion flask, 2 cattle manure, 3 thief hatch

2.2. Sample collection and determination

Immediately after the anaerobic digestion substrate was stirred and mixed, three parallel samples were collected as the samples on day 0, and then 100 ml samples were collected on the fifth, 15, 30, and 60 days, and three parallel samples were processed. Transfer the fermentation sample to a centrifuge tube and centrifuge at 5000 rpm for 15 minutes. The precipitate was freeze-dried using a vacuum freeze dryer (Matsuhara, China) and pulverized to a particle size of less than 1 mm (ultracentrifuge, Retsch Z200, Germany), and stored in a -80℃ refrigerator for subsequent DNA extraction. CH$_4$ was measured using a gas chromatograph (7890A Gas Chromatograph, USA). High-throughput sequencing of 16S rRNA was performed by Meiji Biological Co., Ltd. (Shanghai, China). The sequencing platform was Illumina MiSeq PE250/PE300. The primers were 524F-10-ext (TGYCAGCCGCCGCGGTAA) and Arch958R-mod (YCCGGCGTTGAVTCCAATT).

3. Results and discussion

3.1. Resistance gene abundance analysis

It can be seen from Fig.2 that the ermQ genes of all treatments did not change significantly during fermentation. Except for CK, the ermF genes of anaerobic digestion treated with antibiotics were increased, indicating that the addition of antibiotics increased the size of the ermF abundance of cyclic
lactone resistance genes. Among them, SM2 increased the \textit{ermF} gene by 2 logs, and TYL and CIP increased by 1 log.

According to the analysis of the change in the relative amount of resistance genes from Fig.3, the relative amount of \textit{ermQ} during the fermentation process was reduced by 1 to 2 logs, and the relative content of \textit{ermF} did not change significantly. This indicates that the addition of antibiotics has no significant effect on the removal of \textit{ermQ} and inhibits the removal of \textit{ermF}.

![Figure 2. Absolute quantification of macrolide resistance genes](image)

![Figure 3. Macrolides resistance genes are relatively quantitative](image)

As can be seen from Fig.4, except for CK treatment, \textit{sul1} increased by 1-2 logs in the process of anaerobic digestion with antibiotic treatment, and the absolute abundance of \textit{sul2} increased with the addition of SM2. The absolute abundance of \textit{sul2} increased with TYL.

It can be seen from the analysis of Fig. 5 that the relative abundance of \textit{sul1} in the addition of TYL and CIP is reduced by 1 log. Combined with the change in the absolute abundance number, it shows that TYL and CIP can promote the removal of \textit{sul1}; the relative abundance of \textit{sul2} is in addition to the addition of TYL. Treatments were significantly reduced, and TYL inhibited the removal of \textit{sul2} during fermentation.
3.2. Quantitative analysis of methanogen genes

It can be known from Fig. 6 that the absolute copy number and change law of the methanogenic gene mcrA in the various processes during the fermentation are basically the same, and they all increase first and then decrease.

It can be seen from Fig. 7 that the relative abundances of mcrA genes treated by CK and TYL are basically the same, and they increase first and then decrease during the fermentation process, and the relative abundance is the highest on the 20th day, indicating that there is a higher potential for methanogenesis; The relative abundance of mcrA gene was lower when the antibiotics CIP and SM2 were added, and the inhibitory effect of CIP on methanogen microorganisms was the most significant. This is consistent with the gas production results.
Figure 6. Absolute quantification of the methanogen gene mcrA

Figure 7. Relative quantification of the methanogenic gene mcrA

3.3. Quantitative analysis of methanogen genes

As shown in the Fig.8, the archaeal communities were analyzed, and the treatments at 20d and 40d were grouped into one group. S treatment and T treatment were grouped into one group on the 5th and 10th days of fermentation, respectively. *Methanocorpusculum* persists in the fermentation process and is a dominant flora, which belongs to hydrogen-trophic methanogenic microorganisms. *Methanobrevibacter* mainly exists in the pre-fermentation stage and uses CO$_2$ as a substrate to generate CH$_4$. *Methanosarcina* exists in large quantities only at 45 days, and belongs to the acetic acid type methanogen.

The results showed that the hydrogen-trophic alkane-producing bacteria became the main alkane-producing bacteria group in the anaerobic treatment of cow manure. It is mainly time that affects the evolution of microbial communities in the later stages of fermentation.
Figure 8. Genmap-based heatmap

4. Conclusion
TYL and CIP promote the removal of sul1 during anaerobic digestion. Addition of antibiotics increases ermF abundance of macrolide resistance genes during anaerobic digestion. CIP decreased the abundance of Methanobrevibacter and Methanosarcina. At the same time, CIP has a strong inhibitory effect on mcrA gene, and its abundance is 49% lower than that of the control group. CIP affects fermentation gas production by inhibiting the abundance of mcrA gene in the fermentation system.

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