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Effects of different swine manure to wheat straw ratios on antibiotic resistance genes and the microbial community structure during anaerobic digestion

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HIGHLIGHTS

- Antibiotic resistance genes (ARGs) analyzed in manure/straw anaerobic digestion.
- Abundances of ARGs were decreased with manure/wheat straw at a mass ratio of 7:3.
- Firmicutes may be the main potential hosts of ARGs.
- Bacterial composition/environmental factors mainly determined the fate of ARGs.

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ABSTRACT

This study explored the effects of different mass ratios of swine manure relative to wheat straw (3:7, 5:5, and 7:3, i.e., control reactors C1, C2, and C3, respectively) on variations in antibiotic resistance genes (ARGs) and the microbial community during anaerobic digestion (AD). The cumulative biogas production volumes were 1711, 3857, and 3226 mL in C1, C2, and C3, respectively. After AD, the total relative abundance of ARGs decreased by 4.23 logs in C3, whereas the reductions were only 1.03 and 1.37 logs in C1 and C2, respectively. Network analysis showed that the genera *Solibacillus*, *Enterococcus*, *Facklamia*, *Corynebacterium_1*, and *Acinetobacter* were potential hosts of *ermB*, *sul1*, and *dfrA7*. Redundancy analysis showed that the bacterial communities and environmental factors played important roles in the variation in ARGs. Thus, reductions in ARGs should be considered before reusing animal manure treated by AD.

1. Introduction

Antibiotics are used widely in intensive animal husbandry to prevent diseases and promote growth. A recent survey showed that in China, 52% of all antibiotics (162,000 tons) are consumed by animal production (Sui et al., 2016; Zhang et al., 2015a). However, 50–80% of these antibiotics are excreted in feces and urine as metabolic products (Sarmah et al., 2006); thus, animal manure is an important reservoir of antibiotics and antibiotic resistance genes (ARGs) (Zhao et al., 2010). Furthermore, ARGs will be transferred into the receiving environment after the application of manure onto land, which is a serious and growing issue that might affect contemporary medicine and pose risks for human health.

Anaerobic digestion (AD) is an effective and economic approach for manure management because it can convert organic solid waste into fuel gas and fertilizer (Ward et al., 2008). However, swine manure contains higher concentrations of ammonia than are suitable for microbial growth, which may reduce the efficiency of AD (Jiménez et al., 2015). Wheat straw is a possible substrate for mixing with manure before AD because it can yield a more suitable carbon:nitrogen ratio (Zhang et al., 2015b). Furthermore, studies have shown that AD is a potential method for reducing the abundance of ARGs before the application of the AD product to the soil as fertilizer. Ma et al. (2011) observed that thermophilic AD was effective in removing most of the ARGs that they investigated. Similarly, Diehl and Lapara (2010) found that the removal of *tetA*, *tetO*, *tetW*, and *tetX* by thermophilic AD fitted a first-order kinetic model. By contrast, other studies have indicated that AD reactor can increase the abundance of ARGs (Aydin et al., 2015; Cheng et al., 2016). A previous study have reported that the AD residues are a reservoir of ARGs (Sui et al., 2016), and their application to agricultural soils is assumed to increase ARGs and select for resistant bacterial populations in soils (Cheng et al., 2016; Sui et al., 2016),

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which may pose a potential risk to the ecological environment. Previous studies have reported the effects of various AD parameters (e.g. pH, temperature, mass ratio, and total solids) on biogas production (Zhang et al., 2015b; Chae et al., 2008; Liu et al., 2009), but little is known about the dynamics of ARGs during the AD process, particularly when using different mass ratios of swine manure relative to wheat straw.

Currently, the mechanisms that underlie the variations in ARGs during AD are not well understood, several studies have reported a role for vertical gene transfer during reproduction by bacterial hosts. Su et al. (2015) and Zhang et al. (2016) showed that the succession of the bacterial community had a great influence on the variations in ARGs in environmental samples. A previous study has reported that the presence of different ratios of materials can affect the evolution of bacterial community during AD (Zhang et al., 2015c). Thus, in the present study, we hypothesized that different mass ratios of manure relative to wheat straw may affect the abundances of ARGs. By contrast, other studies have suggested that horizontal gene transfer (HGT) may be involved in the transfer of ARGs between different bacterial cells via mobile elements. For instance, Makowska et al. (2016) suggested that the wastewater treatment process may select for resistant microorganisms and favor the spread of ARGs by HGT. Therefore, this study explored the main drivers of the vertical or horizontal transfer of mobile elements (mobilome or bacterial hosts) during the changes in ARGs in AD using different swine manure to wheat straw ratios.

The objectives of this study were to investigate the variations in ARGs during AD using three different proportions of swine manure relative to wheat straw, and to explore the underlying mechanisms responsible for the variation in ARGs. This study analyzed the fate of ARGs in different control reactors during AD as well as the changes in the microbial community by high throughput sequencing. The relationships between ARGs and microorganisms were determined by network analysis, which is a powerful tool for obtaining new insights into ARGs and their possible hosts in complex environmental examples (Li et al., 2015a). The results of this study provide insights into the dissemination of ARGs in AD using different mass ratios of swine manure relative to wheat straw.

2. Material and methods

2.1. Experimental setup

Dried wheat straw was chopped using a grinder (Hummer 900) to approximately 0.1–0.5 mm. The swine manure used in this study was collected from a medium-sized farm (sulfonamides, tetracyclines and macrolide were used in this farm) in Yangling, Shaanxi, China. The characteristics of the substrates are shown in Table S1. The digestion reactors comprised 36 identical 500-mL triangular flasks, each with a working volume of 400 mL. A schematic model of the AD system is shown in Fig. S1. Three mixed mass ratios of swine manure to wheat straw were established, i.e., control reactors C1 (swine manure:wheat straw = 3:7), C2 (5:5), and C3 (7:3). Each mixture was combined thoroughly to obtain a digestion system with uniform properties. In each control reactor, the mixture had the same total solids content of 8%. The bottles were incubated in a water bath to control the temperature at 37 ± 0.5 °C. To prevent the accumulation of materials that might affect AD, each control reactor was mixed manually once a day (Kafle and Sang, 2013). This study was a five day pre-digestion period. The system defined the sixth day as day 1, and the biogas production process continued for about 55 days. All of the experiments were finished when the biogas production rate was below 5% of the total cumulative production (Abudi et al., 2016). Each control reactor was repeated in triplicate.

2.2. Sample collection

Each control reactor comprised 12 identical 500-mL triangular flasks and three flasks were randomly sampled as triplicates on days 0, 3, 25, and 55. The digestion mixture samples were transferred to centrifuge tubes and centrifuged for 15 min at 5000 rpm. The supernatant was used to analyze the soluble chemical oxygen demand (SCOD), ammonium nitrogen (NH_4^+) content, pH, and volatile fatty acid (VFA) contents. The precipitate was freeze-dried using a vacuum freeze dryer (Songyuan, China), ground to 1 mm with an ultra-centrifugal mill (Retsch Z200, Germany), and stored at −80 °C until DNA extraction. Biogas was collected during the AD process using the water displacement method. The volume of the biogas produced was determined by measuring the volume of displaced water.

2.3. Determination of chemical properties

The NH_4^+ and SCOD contents were determined using a flow injection analyzer (Westco Scientific, USA) and AQ4001 COD analyzer (Thermo Orion, USA), respectively. The pH was determined with a pH meter (Mettler Toledo, Switzerland). The concentrations of VFAs, including acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, and valeric acid, were determined by gas chromatography (Shimazu GC2010, Japan) (Tian et al., 2015).

2.4. DNA extraction and quantitative PCR (qPCR)

DNA was extracted from 0.1 g of each sample using a Fast DNA SPIN Kit for Soil (MP Biomedicals, USA), according to the manufacturer's instructions.

Three classes of ARGs, i.e. sulfonamides, tetracyclines and macrolide resistance genes, are frequently detected in the swine manure (Zhu et al., 2013; Ji et al., 2012). Thus, standard PCR was employed to determine the presence of 11 tetracycline resistance genes (*tetA*, *tetB*, *tetC*, *tetE*, *tetG*, *tetM*, *tetO*, *tetQ*, *tetT*, *tetW*, and *tetX*), five sulfonamide resistance genes (*sul1*, *sul2*, *sulA*, *dfrA1*, and *dfrA7*), seven macrolide resistance genes (*ermA*, *ermB*, *ermC*, *ermF*, *ermQ*, *ermT*, and *ermX*), and the integrase gene of class 1 integrons (*intI1*). Five tetracycline resistance genes (*tetC*, *tetG*, *tetQ*, *tetW*, and *tetX*), three sulfonamide resistance genes (*sul1*, *sul2*, and *dfrA7*), four macrolide resistance genes (*ermB*, *ermF*, *ermQ*, and *ermX*), and the integrase gene of class 1 integrons (*intI1*) were detected by standard PCR, and then quantified by qPCR. The qPCR reaction mixture comprised 1 μL of DNA template, 0.25 μL of each 20 pM primer (ShengGong, China), 10 μL of SuperReal PreMix Plus (TianGen, China), and 8.5 μL of RNase–free water. The thermal cycling steps for qPCR amplification were as follows: (1) 95 °C for 15 min; (2) 95 °C for 10 s; (3) annealing temperature (Table S2) for 20 s; (4) 72 °C for 32 s; (5) plate read, where steps (2) to (4) were repeated 39 times. To eliminate the effects of inhibitory compounds, the DNA template comprised a tenfold dilution of the extracted DNA. qPCR was performed using a Bio-Rad IQ5 system (Bio-Rad, USA). The quantitative limit of qPCR was 10^4 copies per gram dry solid. Melting curve analysis was used to detect nonspecific amplification. The absolute abundance (AA) was expressed as gene copies per gram dry solid. The relative abundance (RA) of an ARG was calculated as: copy number of an ARG/copy number of 16S rRNA.

2.5. 16S rRNA gene sequencing

16S rRNA gene high-throughput sequencing was performed by Novogene Genomics Institute (Beijing, China) using the Illumina HiSeq platform. PCR primers 515F and 806R targeting the bacterial 16S rRNA V4 region were selected for bacterial community analysis using the Illumina high-throughput sequencing method