Bioresource Technology 225 (2017) 343–348

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Relationships between sulfachloropyridazine sodium, zinc, and sulfonamide resistance genes during the anaerobic digestion of swine manure

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HIGHLIGHTS

- Dissipation of sulfachloropyridazine-Na delayed by Zn during anaerobic digestion.
- Abundances of *intI1* and *intI2* decreased after anaerobic digestion for 52 days.
- Zn increased *sul1* and *sul2* abundances by 1.3–10.7 times after anaerobic digestion.
- Sulfachloropyridazine-Na and Zn changed the microbial community during digestion.

ARTICLE INFO

Article history:
Received 15 August 2016
Received in revised form 13 October 2016
Accepted 19 October 2016
Available online 21 October 2016

Keywords:
Antibiotic resistance gene
Mesophilic anaerobic digestion
Sulfachloropyridazine sodium
Zinc

ABSTRACT

In this study, swine manure containing sulfachloropyridazine sodium (SCPS) and zinc was subjected to mesophilic (37 °C) anaerobic digestion (AD). The absolute abundances (AAs) of antibiotic resistance genes (ARGs) were evaluated, as well as *intI1* and *intI2*, and the degradation of SCPS according to variation in the amount of bio-available zinc (bio-Zn). In digester that only contained SCPS, the concentrations of SCPS were lower than that digesters both contain SCPS and Zn. Compared with the control digester, the addition of SCPS increased the AAs of *sul1*, *sul3*, *drfA1*, and *drfA7* by 1.3–13.1 times. However, compared with the digester with SCPS but no added Zn, the AAs of *sul3*, *drfA1*, and *drfA7* were decreased by 21.4–70.3% in the presence of SCPS and Zn, whereas *sul1* and *sul2* increased 1.3–10.7 times. There were significant positive correlations ($P < 0.05$) between the concentrations of SCPS with several ARGs and bio-Zn.

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1. Introduction

For many years, antibiotics have been used extensively for preventing and/or treating infections, as well as being employed as food additives to promote growth during animal husbandry (Martinez, 2009). According to a survey performed in China during 2013, 52% of the total consumption of antibiotics (162,000 t) was due to animal production (Zhang et al., 2015). In addition, to obtain economic benefits, producers of domestic animals and poultry frequently add heavy metal additives to their food (Xiong et al., 2010). However, a significant fraction of the antibiotics and heavy metals fed to animals cannot be absorbed (Huang et al., 2011), so their dung and urine contain high amounts of antibiotics and heavy metals.

Wang et al. (2016) showed that due to the growing use of antibiotics, the incidence of antibiotic resistance has also increased because antibiotics are potent selectors of antibiotic-resistant bacteria (ARB). Thus, large amounts of antibiotics and their metabolites are released into various environments, where they are regarded as the main factors responsible for the selection and spread of antibiotic resistance genes (ARGs) (Allen et al., 2010). ARGs can be transferred widely among bacterial species and once they are present in the environment, they can increase the chances of human pathogens acquiring resistance, thereby posing a threat to human health (Martinez, 2009).

Heavy metals are considered to be co-selection factors for antibiotic resistance (Martinez, 2008) and they have been shown to select for antibiotic resistance among commensal and pathogenic bacteria from different environments (Becerra-Castro et al., 2015). If heavy metals can come in contact with large numbers

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http://dx.doi.org/10.1016/j.biortech.2016.10.057
0960-8524/© 2016 Published by Elsevier Ltd.

of ARGs and their host bacteria, then heavy metal-driven selection of ARB can occur (Becerra-Castro et al., 2015).

During the treatment of organic waste, anaerobic digestion (AD) requires less space and provides improved biogas recovery, which can be used as fuel for heating or the generation of electricity. AD is an efficient technique for the treatment of organic waste such as livestock manure (Aydin et al., 2015), which is a reservoir for ARGs (Sun et al., 2016). Previous studies have shown that AD is considered to be a promising method for controlling ARB (Beneragama et al., 2012; Ma et al., 2011), but the effects of the antibiotics and heavy metals in livestock manure on ARGs during AD are unclear. AD products are applied in agriculture and this may increase the risk of ARG transfer to pathogens via horizontal gene transfer, thereby making antibiotics ineffective. Therefore, it is necessary to understand how the antibiotics and heavy metals present in livestock manure might affect ARGs during AD.

Sulfachloropyridazine sodium (SCPS) is a sulfonamide antibiotic and it is used widely on farms. In addition, Wang et al. (2013) investigated the high concentrations of zinc found in manure. Therefore, in the present study, we investigated the combined effects of SCPS and zinc in swine manure during mesophilic (37 °C) AD, where we determined the degradation of SCPS as well as the changes in sulfonamide ARGs, especially *int11* and *intl2*, using real-time quantitative PCR (qPCR). The aim of our study was to clarify the relationships between SCPS, zinc, and sulfonamide resistance genes during the AD of swine manure. In addition, the results of this study provide insights into the potential effects on ARGs due to the presence of SCPS and zinc in swine manure when subjected to AD.

2. Materials and methods

2.1. Description of raw materials

The swine manure (produced by swine fed with leftovers containing no additives) and wheat straw (as a regulating substance) used in this experiment were obtained from a small local farm in Yangling, Shaanxi, China. The wheat straw was crushed and passed through a 2-mm sieve. The physical and chemical properties of the substrates are shown in Table S1. SCPS was obtained from Hubei Tuochukangyuan Pharm and Chem Co. Ltd, China. The SCPS was soluble in water and its purity was 98%. Zinc sulfate ($ZnSO_4$, 99.0% ACS grade) was purchased from Sigma Chemicals Co. (St Louis, MO, USA).

2.2. Experimental digester and design

The AD experiments were conducted at 37 °C using a constant temperature water bath pot (DK-600, Shanghai Jing Hong Laboratory Instrument Co. Ltd, China). The digestion reactions were conducted in 4-L digestion tanks with working volumes of 2.8 L (Fig. S1). Deionized water was added to the digesters to maintain the total solids content at 8%. The raw materials were stirred for fifteen minutes to ensure that they were mixed homogeneously. The anaerobic digesters were wrapped with black plastic bags to prevent antibiotic photolysis.

Four digestion trials were performed, i.e., CK: control digestion with no additives; S: SCPS at 630 mg kg^{-1} dry weight (DW); SL: SCPS at 630 mg kg^{-1} with $ZnSO_4$ solution to obtain a zinc content of 500 mg kg^{-1} DW; and SH: SCPS at 630 mg kg^{-1} with $ZnSO_4$ at 5000 mg kg^{-1} DW. Each treatment was repeated in triplicate. The concentration of SCPS was calculated based on the minimum residues in the manure (adult swine weighed about 50 kg during therapy). The concentration of zinc was determined according to that found on different swine farms, as shown in detail in Table S2.

2.3. Sample collection

The digester tank was shaken slightly before sampling and 100 mL samples were collected in brown bottles on days 0, 7, 16, 31, 46, and 52 for all treatments. All of the samples were divided into two parts, where one was stored in a refrigerator at 4 °C. This subsample was used for determining the physical and chemical properties. The other subsample was freeze-dried using a vacuum freeze dryer (Songyuan, China) and stored at −80 °C before determining the SCPS levels and DNA extraction.

2.4. Physicochemical analyses

The methods employed for determining the concentrations of ammonium nitrogen (NH_4^+-N), nitrate nitrogen (NO_3-N), soluble chemical oxygen demand (sCOD), and volatile fatty acids (VFAs), as well as the pH were the same as those used by Sun et al. (2016). Zinc was extracted using diethylenetriaminepentaacetic acid (DTPA) and analyzed with a flame atomic absorption spectrometer (Hitachi, Japan). DTPA-extractable Zn was defined as bio-available Zn (bio-Zn) (Roosa et al., 2014).

2.5. Extraction and analysis of antibiotics

Antibiotics were extracted from the samples using USEPA (2007) method 1694 with some modifications. Briefly, 1.0 g of sample was placed in a 50 mL centrifuge tube and extracted with 10 mL of buffer solution containing 0.1 mol L^{-1} Na_2EDTA-McIlvaine and acetone with 20% 0.01 mol L^{-1} oxalic acid/methanol. After vortexing for 1 min, 5 mL of acetonitrile was added to increase the extraction efficiency, before shaking intensively for 30 min, and then centrifugation at 5000 r min^{-1} for 10 min at 4 °C. The same extraction procedure was repeated twice and the supernatants were combined in a new centrifuge tube. The combined supernatant was concentrated to about 5 mL under nitrogen, diluted to 50 mL with ultra-pure water, and concentrated using an Oasis HLB cartridge (Waters, USA). The extract was concentrated by nitrogen evaporation and reconstituted in 1 mL of the mobile phase (acetonitrile: water (1:3), where the pH was adjusted to 4.0 after adding glacial acetic acid) for chromatographic analysis. Ultraviolet detection was conducted at 270 nm. SCPS was quantified using an external standard, where the correlation coefficient was 0.9998 and the recovery was 80–92%.

2.6. DNA extraction and qPCR

The total genomic DNA was extracted using a Fast DNA Kit for Soil (MP Biomedicals, USA) according to the manufacturer's instructions and then stored at 20 °C until use. Traditional qualitative PCR was used to determine the presence of six sulfonamide resistance genes (*sul1*, *sul2*, *sul3*, *sulA*, *drfA1*, and *drfA7*) and the integrase genes *intI1* and *intI2*. The primers and standard PCR conditions employed are described in the Supporting Information (S and Table S3). The PCR products were analyzed by 1.5% (w/v) agarose gel electrophoresis.

The five sulfonamide genes (*sul1*, *sul2*, *sul3*, *drfA1*, and *drfA7*) and two integrase genes (*intI1* and *intI2*) were quantified by real-time qPCR using a BioRad iQ5 Real-Time PCR Detection System (BioRad). 16S rRNA was also quantified to minimize the variance in the abundance of ARGs caused by differences in the background bacterial abundance and the DNA extraction efficiency. The qPCR conditions are described in the Supporting Information (S). The absolute abundances (AAs) of the genes were expressed as copies g^{-1} DW. The qPCR detection limit was 8×10^3 copies g^{-1} DW.

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