Effects of magnetite on anaerobic digestion of swine manure: Attention to methane production and fate of antibiotic resistance genes

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\textbf{GRAPHICAL ABSTRACT}

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\textbf{ABSTRACT}

Effects of magnetite on methane production and fate of antibiotic resistance genes (ARGs) during anaerobic digestion (AD) of swine manure were investigated. Results showed that methane production was increased by maximum 16.1%, and magnetite could enhance the acetoclastic methanogenesis not hydrogenotrophic methanogenesis reflected by the functional gene quantification and microbial community analysis. The propionate degradation rate was improved, and it was syntrophic oxidized into H\textsuperscript{+}/e\textsuperscript{-}/CO\textsubscript{2} for direct interspecies electron transfer (DIET) and acetate, where DIET was further enhanced by magnetite and the acetate was transformed into methane through syntrophic acetate oxidation (SAO) pathway. Magnetite mainly influenced the ARGs at the interim period of AD, where ARGs especially \textit{ermF} were significantly enriched. Magnetite did not influence the total ARGs abundance at the end, although the \textit{tetM} was enriched and \textit{mefA} was reduced finally. Statistical analysis indicated that magnetite influenced the ARGs fate mainly through the changes of microbial community.

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1. Introduction

The suitable disposal of swine manure has become an increasing challenge for the environment especially in China, where half of the pork in the world were consumed, and about 618 billion kilograms of swine manure were produced every year (Larson, 2015). Thus, China have launched the Technical Specifications for Pollution Treatment Projects of Livestock and Poultry Farms since 2009, and anaerobic digestion (AD) was the core for the basic treatment processes for swine wastewater and manure (Hu et al., 2017). However, up to data, AD is still limited by the long start-up times, low acidification efficiencies and slow syntrophic metabolism of fermentative intermediates (Bharathiraja et al., 2018). The improvement of AD efficiency on methane production is always an important research hotspot in the swine manure disposal either through the use of pretreatments or additives supplementation (Li et al., 2019).

Iron-based AD was supposed to be an effective way for the improvement of AD efficiency due to its properties as an electron donor and its low toxicity and low cost, but more efforts were devoted to focusing on the AD of sewage sludge and zero valent iron (Wei et al., 2013). AD was generally considered as a promising process for the control of ARGs on different stages of the AD (Zhang et al., 2017). Furthermore, there are already studies on the enhancement AD with magnetite, the range of doses has not yet been defined with consideration for the characteristics of swine manure, and the information on the role of magnetite on the changes of functional genes in AD is limited.

Besides, swine manure is an important reservoir of antibiotic resistance genes (ARGs) which threatens the public health increasingly, and the control of ARGs in the environment has become one of the most important themes for world health organization (WHO) (Pruden et al., 2013). AD was generally considered as a promising process for the control of ARGs spread, while concerning the enhancement of antibiotics degradation and heavy metal passivation by magnetite (Li et al., 2017; Suannon et al., 2016), whether magnetite addition could enhance the ARGs reduction in AD should be clarified. Heavy metal resistance genes (MRGs) could well represent the selective pressure from heavy metals, and the role of magnetite on the heavy metal passivation from the perspective of MRGs has not been determined. Meanwhile, as an important co-selection factor influencing the fate of ARGs, how the MRGs contributed to the occurrence of ARGs response to magnetite is not answered.

Thus, AD of swine manure under different dosage of magnetite were established to: 1) clarify the effects of magnetite on the AD performance of swine manure; 2) figure out the fate of ARGs response to magnetite; 3) decipher the potential mechanisms of the effects of magnetite on the performance and ARGs fate.

<table>
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<tr>
<th>Table 1</th>
<th>Changes of physical and chemical parameters response to the magnetic during anaerobic digestion of swine manure.</th>
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<tbody>
<tr>
<td>TS (%)</td>
<td>pH</td>
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<tr>
<td>29.28</td>
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- **Table 1**: Changes of physical and chemical parameters response to the magnetic during anaerobic digestion of swine manure.
2. Materials and methods

2.1. Experiment setup

Swine manure and the inoculum sludge were collected from a large-scale swine farm with the feedstock of 10,000 in Beijing, China, where an AD reactor was run treating the swine manure. The basic characteristics of the swine manure and inoculum sludge were shown in Table 1. 99% metals basis of magnetite was obtained in the form of powder from Aladdin Reagent Co., Ltd., China. Batch experiments were set up through the Automatic Methane Potential Test System II (Bioprocess Control, Sweden) as previously described (Zhang et al., 2018b).

Briefly, swine manure and inoculum sludge were mixed at the ratio of 3:1 (Total solids, TS), and then transferred to each bottle (working volume, 0.4L) with the final TS of about 8%. Appropriate magnetite was added to the bottles to attain the final concentrations of iron element at 0 mmol (Control), 5 mmol (M5), 75 mmol (M75), 150 mmol (M150) and 350 mmol (M350). Each treatment was set in triplicate and incubated in a water bath at the temperature of 37 ± 0.1°C. Methane added to the bottles to attain the final concentration of iron element at 0.4L (volume, 0.4L) with the final TS of about 8%. Appropriate magnetite was added to the bottles to attain the final concentrations of iron element at 0 mmol (Control), 5 mmol (M5), 75 mmol (M75), 150 mmol (M150) and 350 mmol (M350). Each treatment was set in triplicate and incubated in a water bath at the temperature of 37 ± 0.1°C. Methane production was automatically recorded, and CO2 was removed through 3 M NaOH solution before the record. The batch experiments lasted for 30 days, and 2 mL of samples were sucked out directly from the sampling hole of each bottle during sampling which was conducted on days 0, 5, 13 and 30 according to the status of methane production involving the initial stage, intermediate stage and end of AD for further analysis.

2.2. Physicochemical analysis

Samples were centrifuged at 4000 rpm for 10 min and filtered through 0.45-μm cellulose membrane. The filtrate was analyzed for pH, NH3-N, Total soluble chemical oxygen demand (TOC and SCOD), proteins, polysaccharides and VFAs (volatile fatty acids) as previously described (Zhang et al., 2016b). The concentration of iron ions in the filtrate was determined through ICP-OES to consider the possibility of iron ions release from magnetite, and the PO4-3 and SO4-2 were quantified through ion chromatography.

Before the three-dimensional excitation-emission matrix (EEM) analysis, the filtrate was diluted by the same amount (∗1000) for all samples to keep the dissolved organic carbon (DOC) below 20 mg L−1. Fluorescence EEM spectroscopy was performed as previously suggested (Zhang et al., 2015). Briefly, excitation and emission were simultaneously scanned at wave-lengths from 200 to 400 nm and from 220 to 550 nm, respectively, at 5-nm intervals. The slit widths were set to 5-nm for both the excitation and emission monochromators, and the scan speed was set to 12000 nm min−1.

2.3. Quantitative PCR (qPCR)

0.2 mL of each sample was used for DNA extraction using FastDNA Spin Kit for Soil (MP Biomedicals, USA), and quality and concentration of the extracted DNA were determined through 1% agarose gel electrophoresis and NanoDrop ND-1000 (NanoDrop, USA), respectively. The triplicate extracts of the same treatment were composited to get a representative DNA sample.

Twelve typical ARGs in swine manure (two sulfonamide resistance genes: sulI, sulII; four macrolide resistance genes: ermB, ermF, ereA, mefA; two β-lactam resistance genes: blaCTX-M, blaTEM; three tetracycline resistance genes: tetM, tetG, tetX and one colistin resistance gene: mcr-1) were selected to represent the changes of ARGs (Zhang et al., 2019a). The int1 gene was chosen to represent the mobile genetic elements (MGEs) and multi-resistance (Amos et al., 2018). The functional genes (FTHFS, ACAS, mcrA) were quantified to reflect the effects of magnetite on the acetogenesis, specifically acetoclastic methanogenesis and non-specific methanogenesis, respectively (Aydin et al., 2015). Four typical MRGs (mreA, pcoA, arcC and ccoA) in swine manure were also chosen to represent the selection pressure from heavy metals (Zhang et al., 2019a).

The qPCR process was described as previously (Zhang et al., 2018a), and the primers, annealing temperature and the corresponding amplification efficiencies were summarized in the supporting information.

2.4. Microbial community analysis

The primers 515F/806R were selected for bacterial community analysis (Caporaso et al., 2010), and archaeal community was analyzed using the nested PCR primers (Arch340F/Arch1000R and Arch349F/Arch806R) as previously described (Zhang et al., 2016b). The samples were sent to Sangon Co., Ltd., (Shanghai, China) for the small-fragement library construction and pair-end sequencing (Illumina Miseq, USA). Pair-end reads were merged (PEAR: -x, 0.1) and assigned to each sample according to the unique barcode; the merged reads were quality controlled (PRINSEQ) and chimers filtered (USEARCH) to get the clean sequences; the clean sequences were normalized and submitted to the NCBI Sequence Read Archive (SRA) under the project number of PRJNA510701. The taxonomic classification was carried out using the Ribosomal Database Project (RDP) Classifier with the taxon below the 0.01% being removed (Wang et al., 2007), and the diversity indexes were calculated by the RDP associated modules.

2.5. Statistical analysis

The maximum production potential and rate were calculated through the modified Gompertz model (Lizama et al., 2017). Parallel factor (PARAFAC) analysis was conducted using MATLAB R2016b (MathWorks, Natick, MA) with the DOMFluor toolbox as previously suggested (Song et al., 2019). Four components were identified based on the split-half validation, and the relative concentration of each PARAFAC component was estimated by the Fmax output from DOM-Fluor. Mantel test showing the correlation between ARGs and microbial community was determined through the PAST 3.0. Principal component analysis (PCA), redundancy analysis (RDA) and Procrustes analysis were performed using Canoco 5.0. The Kruskal-Wallis H-test with the Benjamini-Hochberg FDR correction was adopted to figure out the OTUs with significant difference between groups (p < 0.05) using STAMP 2.1.3, and the ternary plot was further drawn to show the significantly enriched OTUs through the R package of ggttern. Network analysis based on the spearman correlation was constructed through the Gephi platform (Bastian et al., 2009).

3. Results and discussion

3.1. The performance response to magnetite during AD of swine manure

The methane production was significantly impacted by the magnetite (p < 0.05), the maximum methane production potential (P) was increased by maximum 16.1% from 285.9 mL CH4 g−1 VSadded to 332.0 mL CH4 g−1 VSadded, and the maximum methane production rate (Rmax) was increased by 8.0% through the modified Gompertz model analysis (Fig. 1a and Table 2). The improvement of methane production happened at two peaks: days 5–10 and days 20–25. The maximum enhancement caused by magnetite happened at days 20–25 (the later stage of AD), while it was days 5–10 (early stage of AD) for nano-magnetite (Zhang et al., 2019b). It was speculated that magnetite might better improve the degradation of the refractory organics, whose consumption generally happened at the later period of AD, compared to nano-magnetite (Kafle and Chen, 2016). However, there was no big leap for the methane production even the magnetite addition was increasing significantly from 5 mmol to 350 mmol. This indicated that the enhancement efficiency was not completely dose dependent and other factors like the effective contact area and the dispersibility of magnetite in the AD system might cause the limited bioavailability of magnetite.

The VFAs accumulation was not relieved or VFAs transformation was not enhanced significantly by magnetite, which could also be
reflected by the limited improvement of the $R_{\text{max}}$ value (8.0%). However, nano-magnetite could significantly reduce the VFAs accumulation and enhance the VFAs transformation to CH$_4$, and the $R_{\text{max}}$ value was improved significantly by 47.8% (Zhangetal., 2019b). These further indicated the quite different mechanisms of the enhancement of methane production between magnetite and nano-magnetite.

The TS increased after AD, because magnetite could not be degraded and the dissolution was also limited as reflected by the concentration of soluble Fe (Table 1). While the VS reduction were significantly enhanced along with the magnetite addition, and the VS reduction efficiency correlated significantly with the magnetite addition ($p < 0.05$). The VS reduction was increased by maximum 45.6% at M350, and the reduction of the TCOD concentration was also enhanced, but the reduction of SCOD was limited. The pH and concentration of NH$_4^+$-N, TIC, proteins and polysaccharides were reduced slightly after the magnetite addition. The pH buffer system as indicated by the VFAs-TIC-NH$_4^+$-N could well reflect the changes of the AD stability (Yuetal., 2018), and it seemed that magnetite did not impact the changes of the pH buffer system significantly (Fig. 1d). The concentration of soluble Fe generally decreased along with magnetite addition, and it seemed that the precipitation of soluble Fe was enhanced by the magnetite. The behind mechanism of the phenomenon might be associated with the magnetism of the magnetite, which need further investigation. Besides, most Fe(II) would precipitate with an insoluble form in neutral pH, and the slight reduction of pH by the magnetite can facilitate the precipitation (Straub et al., 2000). The decrease of the concentration of NH$_4^+$-N could be ascribed to the enhanced microbial activity through the stronger N-assimilation and increased ammonia adsorption by the formed iron-precipitates with a high specific surface area like ferrhydrite, goethite, hematite, lepidocrocite, maghemite and strengite (Yang et al., 2018). These well explained the decrease of the soluble Fe concentration along with magnetite addition, and the role of nutriments in the AD of swine manure due to magnetite addition could be excluded. Limited release of soluble Fe also explained the limited effects of magnetite on the changes of the concentration of SO$_4^{2-}$ and PO$_4^{3-}$.

\begin{table}[ht]
\centering
\begin{tabular}{|c|c|c|c|c|}
\hline
Treatment & $R^2$ & $R_{\text{max}}$ & P & $\lambda$ \\
\hline
CK & 0.995 & 13.7 & 285.9 & < 0.01 \\
M5 & 0.994 & 14.1 (2.9%) & 302.8 (5.9%) & < 0.01 \\
M75 & 0.995 & 14.8 (8.0%) & 290.6 (1.6%) & < 0.01 \\
M150 & 0.994 & 14.5 (5.8%) & 318.0 (11.2%) & < 0.01 \\
M350 & 0.994 & 14.8 (8.0%) & 332.0 (16.1%) & < 0.01 \\
\hline
\end{tabular}
\caption{Kinetic parameters obtained from fitting biogas production of the modified Gompertz model.}
\end{table}

\textsuperscript{a} $R_{\text{max}}$ indicated the maximum methane production rate mL CH$_4$ g$^{-1}$ VS$_{\text{added}}$ per day; P indicated the maximum methane production potential (mL CH$_4$ g$^{-1}$ VS$_{\text{added}}$); $\lambda$ is the lag phase (days) defined as delayed period for the methane production.

Fig. 1. Dynamics of the accumulative methane production (a), daily methane production (b), concentration of volatile fatty acids (VFAs, c) and the ternary pH buffer system (d) response to magnetite during AD of swine manure.
because soluble Fe could have reduced the biogenic sulphide by pyrite (FeS) precipitation and form vivianite (Fe₃(PO₄)₂·8H₂O) to entrap the soluble phosphorus in AD (Puyol et al., 2017), but it did not in this study shown in Table 1.

3.2. PARAFAC analysis indicated the role of magnetite on the performance of AD

It had been reported that iron oxides could enhance the AD performance by improving the properties of extracellular polymeric substances (EPS) (Wang et al., 2018a,b). It was related that magnetite could facilitate the secretion of EPS which are natural macromolecule polymers accounting for 80% of the total mass of activated sludge and key mediators to conduct electron exchange between intra- and extracellular (Wang et al., 2019). Furthermore, humic substances may constitute a natural carrier system that transfers electrons from bacterial cells to barely soluble electron acceptors like magnetite, and it was demonstrated that fermenting bacteria are capable of transferring electrons from anaerobic oxidations, via humic substances, towards iron oxide reduction (Straub et al., 2000).

The EEM results indicated that the release of the fluorescent substances was increased along with the AD of swine manure, and it was the microbial byproducts and tryptoghan-like substance that were increased significantly (Fig. 2). At D5 and D13, magnetite improved the release of the proteins like substances, and the overall intensity of the fluorescent was reduced slightly compared to the CK as indicated in the supporting information. This was well corresponded to the changes of the concentration of the proteins and SCOD. Through the PARAFAC analysis, four components were collected, and Tyrosine-like and Tryptoghan-like are the two important components of the EPS, while soluble microbial by-product-like substances are the SMP parts (Fig. 2). It indicated that tryptoghan-like substances are the most important re-fractory proteins not fulvic acid-like substances during AD of swine manure. However, the tryptophan- and tyrosine-like materials are present as “free” molecules or else bound in proteins, peptides, or humic structures (He et al., 2014). Generally, the former was easily degraded by microbes, which indicated that the tryptoghan-like and tyrosine-like substances at the end of AD generally bound to the humic structures. Magnetite could enhance the release of EPS and SMP at D5 and D13 (Fig. 2), the most activated phase of the methane production. Besides, there existed the transformation from the tryptophan-like and tyrosine-like substances as free molecules or bound in proteins and peptides to humic structures bound, and magnetite could have enhanced the transformation, which could facilitate the electron transfer by humic acids as indicated previously (Straub et al., 2000) and well explained the enhancement of methane production at the later period of anaerobic digestion (Fig. 1a and b).

3.3. Fate of ARGs, intI1 and MRGs response to magnetite during AD of swine manure

The fate of total ARGs in AD of swine manure could be divided into three stages corresponding to D5, D13 and D30, and the effects of magnetite on the ARGs mainly happened at D13 but with limited impacts at D5 and D30 (Fig. 3a). The abundance of ARGs at D5 changed little compared to D0, but the abundance of ARGs increased significantly at D13. The magnetite further enriched the ARGs abundance at D13, and there existed significant correlation between ARGs abundance and magnetite addition (p < 0.05). Nonetheless, the abundance of ARGs at D30 was comparable between each other, and magnetite did not significantly influence the ARGs abundance at the end of AD. Interestingly, this is contrary with the effects of magnetite on the methane production, where the methane production was enhanced at D5 and D30 stages.

The types of ARGs showed different fate at different AD stages response to magnetite (Fig. 3a). The macrolide resistance genes (ermB, ermF, mefA) dominated the swine manure and subsequent AD, and AD significantly increased the relative abundance of ermF, but significantly reduced the ermB, which was in accordance with previous studies (Zhang et al., 2019b, 2017). The ereA was also reduced along with AD, and magnetite could reduce the abundance of mefA at the end of AD and enrich the ermF at D13 significantly. The relative abundance of the dominant tetracycline resistance genes (tetX and tetM) were also enriched in AD and magnetite further increased the abundance of them at D30, but tetG changed little along with AD. The changes of sulfonamides resistance genes (sulI and sulII) was limited along with AD response to magnetite, so did the β-lactam resistance genes (blaCTX-M and blaTEM) and mcr-1.

As for the dynamics of the abundance of intI1, no regular changes could be concluded response to the magnetite (Fig. 3b). Although the abundance of ARGs was significantly enriched at D13, the abundance of intI1 was the lowest compared to other stages. This indicated that the
increase of ARGs abundance at D13 was not associated with the horizontal gene transfer (HGT). The dominant MRGs in the swine manure and subsequent AD were \textit{arsC} (resistance to As) and \textit{pcoA} (resistance to Cu) (Fig. 3c), which could be attributed to the widely use of As and Cu in the swine farming (Zhang et al., 2019a). The reduction of the MRGs could be significantly enhanced by magnetite, especially for \textit{pcoA}. The MRGs could reflect the bioavailability of the corresponding heavy metals (Zhang et al., 2016a), and the enhancement of the MRGs reduction indicated that magnetite could have increased the passivation of the heavy metals. The passivation of the heavy metals by magnetite has been demonstrated previously (Liang et al., 2017; Suanon et al., 2016), this study further confirmed the hypothesis from the perspective of MRGs, which could better reflect the selective pressure emphasized by heavy metals.

### 3.4. Fate of the key functional genes response to magnetite during AD of swine manure

The \textit{FTHFS} (formyltetrahydrofolate synthetase) gene represents the dynamics of the acetogens, which function the production of the acetate during AD (Merlino et al., 2013). Magnetite improved the abundance of \textit{FTHFS} significantly at D5 and D30 ($p < 0.05$), while reduce the abundance significantly at D13 ($p < 0.05$) (Fig. 3d). This was in accordance with the two enhancement peaks of methane production caused by magnetite, where the enhancement by magnetite mainly happened at earlier and later period (Fig. 1b). It was hypothesized that before D5, the acetate was the limited factor for the methane production, thus, the acetate production was enhanced by magnetite. Along with the accumulation of VFAs, acetate production was not the limited factor but the methane production rate, but at the end of the AD, acetate was again become the limited factors, and the acetate production was further enhanced by magnetite.

The \textit{ACAS} encoding the acetyl-coA synthetase could represent the acetoclastic methanogenesis, which used the acetate for the methane production produced by acetogens (Aydin et al., 2015), and the \textit{ACAS} showed the similar pattern with \textit{FTHFS}, where the abundance was improved at D5 and D30 but reduced at D13. This demonstrated the hypothesis that magnetite enhanced the acetoclastic methanogenesis. The \textit{mcrA} encoding methylcoenzyme M reductase could represent the abundance of the methanogens (Zhang et al., 2017), and the ratio \textit{ACAS/mcrA} could reflect the ratio of the acetoclastic methanogenesis in the methanogens. The abundance of \textit{mcrA} also showed the similar pattern with \textit{FTHFS}, which indicated that the methanogenesis could also be enhanced, but the reduction at D13 indicated that the dominant methanogenesis could be different and the enhancement of methanogenesis could be specific. The results of the \textit{ACAS/mcrA} showed that the acetoclastic methanogenesis dominated at D0 (>90%), but reduced significantly during the BMP test, and only 6.0%–22.0% of methanogens is acetoclastic during the BMP tests, but the ratio increased significantly at D5, D13 and D30 due to the magnetite addition. This indicated that magnetite improved the acetoclastic methanogenesis not hydrogenotrophic methanogenesis. While the hydrogenotrophic methanogenesis dominated at D13, where the daily methane production varied little between treatments. However, before D5, the acetoclastic methanogenesis dominated due to the inoculum, at D30, the acetoclastic methanogenesis increased gradually reflected by the ratio of \textit{ACAS/mcrA}, and this is in accordance with the time phase of the improvement of methane production by magnetite.
3.5. The dynamics of microbial community response to magnetite during AD of swine manure

The alpha diversity of microbial community generally decreased along with AD, and there existed limited effects of magnetite on the diversity as shown in the supporting information. The phyla of Firmicutes and Bacteroidetes dominated the AD process, the abundance of Firmicutes ranged from 60.0% to 81.7%, and it was 10.4%–35.9% for Bacteroidetes shown in the supporting information. Magnetite could increase the abundance of Bacteroidetes and decrease the Firmicutes significantly at D13, and there existed positively significant correlation between Bacteroidetes and magnetite ($p < 0.05$). This was in accord with the fate of ARGs response to magnetite, where the abundance of ARGs was enriched by magnetite addition at D13.

The abundant genera under the phyla of Bacteroidetes consisted of unclassified_Bacteroidales and Petrimonas, while it was Clostridium sensu stricto and Terrisporobacter that were significantly reduced for the phyla of Firmicutes at D13 (Fig. 4A and B). The Clostridium sensu stricto was a kind of fermentative microbes that degraded the macro-substances, producing both acid and alcohol during fermentation (Peng et al., 2018). Amounts of CO2 and H2 are also generated during fermentation, which led to the accumulation of H2 and CO2 in the anaerobic systems. As for the Terrisporobacter, the main products of the fermentation are acetate and CO2, while the main products of the fermentation by Petrimonas are also acetate, H2 and CO2 (Deng et al., 2015). The Syntrophomonas belonging to the phyla of Firmicutes increased significantly due to the magnetite addition at D13. The Syntrophomonas belonging to Firmicutes degraded butyrate in co-culture with methanogens or hydrogen-utilizing sulfate-reducing bacteria, which indicated that butyrate was degraded through the interspecies electron transfer (Zhang et al., 2004). Clostridium is also important syntrophic bacteria for the acetate along with methanogens. Thus, the accumulation of acetate and butyrate is not severe, but the degradation of propionate degradation happened after D13, where the magnetite addition increased the methane production rate, and the methane production peak came to 5 days ahead of CK (Fig. 1b). Nonetheless, the Syntrophobacter, Pelotomaculum, Smithella which were important syntrophic bacteria along with methanogens for the use of propionate were not detected, and DIET maybe very important for the use of propionate, which deserve further investigation.

The PCA analysis indicated that the role of magnetite on the dynamics of microbial community was limited at D5, but high dosage of magnetite (150 mM and 350 mM) could influence the microbial community at D13 and D30 (Fig. 4C). Nonetheless, the difference of microbial community caused by the maximum magnetite addition could still not obscure the difference along with AD time. The unclassified_Syntrophomonadaceae and Saccharofermentans were enriched by the magnetite at D30. These microbes could enhance the syntrophic association with methanogens for the methanee production. Through the PIRUST analysis, no significant correlation was found between functions and magnetite at D5, and more than 14 COG function taxa correlated significantly with magnetite at D30 like amino acid transport and metabolism.

The dynamics of archaeal community showed similar pattern response to magnetite, and the dominant archaea changed from Methanosphaera to Methanorevibacter and Methanosarcina (Fig. 4D).
dominant archaea varied from stages. At D5, the dominant archaea are Methanosphaera and Methanosaicna, while it was Methanosarcina and Methanospirillum at D13, and Methanosarcina and Methanobrevibacter become dominant at D30. The Methanospirillum and Methanobrevibacter could only use H2 and CO2 for the methane production, and the Methanosphaera need the H2 and methanol for the methane production, while the Methanosarcina could use both H2/CO2 and acetate (Garcia, 1990).

As shown in the PCA analysis of archael community in the supporting information, the effects of magnetite mainly happened at D30 especially for the M150 and M350 where the Methanosarcina became dominant and Methanobrevibacter was enriched response to magnetite. It was reported that Methanobrevibacter are hydrophilic methanogens, but it was indicated that the methane production is mainly conducted via syntrophic acetate oxidation (SAO) by hydrophilic methanogens at high ammonia concentrations (De Vrieze et al., 2015), where the magnetite could enhance the SAO through the DIET.

3.6. Mechanism discussion of magnetite on the methane production

This study investigated the methane production process through the BMP test, it was quite different from the continuous feeding but one cycle of the methane production. It was hypothesized that one cycle of methane production could be divided into three stages no matter from the physico-chemical role or the microbial community during AD of swine manure. At the first stage (D0-D5), the easily degradable substances were quickly used, and degradation of acetate into methane dominated, thus, acetoclastic methanogenesis was the key for the methane production. Nonetheless, the H2/CO2 and propionate accumulated along with AD. In this study, the unclassified Ruminococcaceae, Clostridium sensu stricto, unclassified Bacteroidales were responsible for the hydrolysis, the proteins, polysaccharides and lipids were degraded into glucose and amino acids along with VFAs and amounts of H2 and CO2 (Li et al., 2018). Then, the Petrimonas use the glucose, amino acids and long-chain fatty acids to produce the VFAs along with amounts of H2 and CO2 (Grabowski et al., 2005). The Methanosarcina and Methanosphaera consumed the acetate and part of the H2 and CO2 for the methane production at this stage.

At the second stage (D5-D15), the refractory substances began the degradation, and the VFAs and H2/CO2 was consumed, thus, microbial community changed significantly to consume the accumulated H2/CO2 due to the first stage, but due to the higher partial H2 pressure, propionate further accumulated, because low H2 concentrations is essential for the propionate oxidation energetically favorable (Jing et al., 2017). The Syntrophomonas further degraded the VFAs along with methanogens to produce methane. The H2 accumulation reached the maximum at D13, and hydrophilic methanogens including Methanospirillum and Methanosarcina dominated the methane production. The first enhancement peak by magnetite happened at this stage, and magnetite could have promoted the production of hydrogen by enhancing the biological activity of hydrogen producers, while both direct interspecies electron transfer and interspecies H2 transfer were thermodynamically feasible with the addition of magnetite (Jing et al., 2017). Thus, the transformation of these easily used substrates for methane production was further enhanced and accelerated by magnetite.

At the third stage, the propionate was degraded due to the consumption of H2 at the second stage from the perspective of thermodynamic analysis. The propionate should be syntrophic oxidized into H2/CO2 (IHT), H+/e−/CO2 (DIET) and acetate first (Jing et al., 2017). But this study found no syntrophic propionate bacteria, and it was supposed that H+/e−/CO2 (DIET) and acetate were the dominant products for the propionate oxidase. The DIET could be enhanced by the magnetite addition, and acetate were further degraded through the SAO pathway due to the higher ammonia concentration (De Vrieze et al., 2015). The hydrogenotrophic methanogens of Methanospirillum and Methanobrevibacter are reported be involved in DIET in the propionate degradation and SAO pathway (Jing et al., 2017). This well explained of the second enhancement peak of the methane production caused by magnetite, but it should be noticed that more evidence and experiments are needed for the direct elucidation of DIET enhancement.

The role of iron-reducing bacteria (IRB), which could facilitate the degradation of the refractory organics (Xu et al., 2017), on the enhancement of VS reduction should also be emphasized, and the dominant genus of Clostridium species are well-known IRB and have the type IV pili for extracellular electron transfer to the insoluble iron oxides with the reduction of Fe3+ to Fe2+. But how the surface biochemistry happened on the magnetite and whether the Saccharofermentans and unclassified Syntrophomonadaceae enriched by the magnetite addition are IRB need further investigation. Besides, considering the limited concentration of soluble Fe after magnetite addition, the role of trace elements on AD should be limited.

3.7. Deciphering the fate of ARGs response to magnetite

The ARGs could existed in two forms: extracellular ARGs (eARGs) and intracellular ARGs (iARGs). The dissemination of eARGs was through the transformation into the bacteria and become the iARGs. Most of eARGs were degraded in the environment, and previous study indicated that ARGs existed mainly in the form of iARGs in AD (Zhang et al., 2013). The dissemination of iARGs was through the HGT between microbes and vertical gene transfer (VGT) through the changes of microbial community (Shao et al., 2018). While no significant correlation was found between ARGs and intI1 in this study, which indicated that the HGT might be restricted during AD. Other co-selection factor like the heavy metals were also reduced after AD, and magnetite could further enhance the reduction. Compared to the VGT, the influence on the ARGs fate from co-selection of heavy metals is limited.

Mantel test and Procrustes analysis indicated that there existed significantly positive correlation between ARGs and microbial community (R = 0.8468, p = 0.0001). The factor concerned in this study (intI1, MRGs, microbial community and environmental variables) could explain 87.9% of changes of the fate of ARGs through the RDA analysis (Fig. 5), and microbial community (54.7%) followed by intI1 (26.3%) and MRGs (24.5%) was the dominant factors through the pRDA analysis. Thus, the changes of the VGT caused by magnetite could have triggered the dynamics of ARGs, then the potential host of ARGs were further identified through the network analysis.

Fig. 5. Redundancy analysis (RDA) showing the relationship between ARGs and influencing factors. MC-1 and MC-2 indicated the two principal components of the bacterial community, and EV1 and EV2 indicated the two principal components of the environmental parameters concerned in this study.
From the perspective of the phyla level, the significantly negative correlation between ARGs and Firmicutes were identified \((p < 0.01)\), but positively significant correlation between ARGs and Bacteroidetes were confirmed \((p < 0.01)\) (Fig. 6A). This indicated that the reduction of ARGs could be enhanced through the enrichment of Firmicutes and reduction of Bacteroidetes during AD of swine manure. Although the average number of ARG homologs in the phyla of Firmicutes (28.10) was much higher than Bacteroidetes (16.92) (Bahram et al., 2018), the condition could be converse in the AD of swine manure.

Network analysis showed the potential hosts of ARGs and the relationship between ARGs and other factors (Fig. 6B). Meanwhile, the dominant factors influencing the specific ARGs could be varied, there existed significantly positive correlation between \(\text{mer}\text{A}, \text{arsC}, \text{pcoA}, \text{czcA}\) and \(\text{sulI, mefA, blu}_{\text{CTX-M}}\), which indicated that the co-selection from heavy metals could have contributed the fate of these ARGs, while \(\text{intI1}\) could influence the fate of \(\text{tetG}\) and \(\text{sulII}\), which were generally located together with \(\text{intI1}\). But the reduction of \(\text{ermB}\) during AD of swine manure could be attributed to the changes of microbial community. However, as for the enrichment of \(\text{ermF}\) and \(\text{tetX}\), the role of microbial community maybe limited. It was hypothesized that the eDNA could have contributed to the enrichment of \(\text{ermF}\) and \(\text{tetX}\), that is, they were more persistent in the environment of AD of swine manure. The magnetite could reduce the co-selection from heavy metals, and the microbial community were also changed response to magnetite which contributed the most to the ARGs fate during AD of swine manure.

4. Conclusions

This study indicated that the methane production was increased by maximum 16.1% through the magnetite addition during AD of swine manure. Improvement of the methane production could be attributed to the DIET enhancement of the propionate degradation and SAO pathway. The functional genes and microbial community analysis indicated that magnetite enhanced the acetoclastic methanogenesis not hydrogenotrophic methanogenesis. The abundance of ARGs was significantly enriched by magnetite at D13 especially \(\text{ermF}\). Although the \(\text{tetM}\) was enriched and \(\text{mefA}\) was reduced at the end, the total abundance of ARGs changed little. Microbial community changes caused by magnetite well explained the ARGs fate.

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Conflict of interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2019.121847.

References


Fig. 6. The linear fitting (A) showing the relationship between ARGs and Firmicutes along with Bacteroidetes; Network analysis (B) indicating the factors influencing the target ARGs.


