Contribution of antibiotics to the fate of antibiotic resistance genes in anaerobic treatment processes of swine wastewater: a review

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Abstract

Antibiotic resistance genes (ARGs) in water environment have become a global health concern. Swine wastewater is widely considered to be one of the major contributors for promoting the proliferation of ARGs in water environments. This paper comprehensively reviews and discusses the occurrence and removal of ARGs in anaerobic treatment of swine wastewater, and contributions of antibiotics to the fate of ARGs. The results reveal that ARGs' removal is unstable during anaerobic processes, which negatively associated with the presence of antibiotics. The abundance of bacteria carrying ARGs increases with the addition of antibiotics and results in the spread of ARGs. The positive relationship was found between antibiotics and the abundance and transfer of ARGs in this review. However, it is necessary to understand the correlation among antibiotics, ARGs and microbial communities, and obtain more knowledge about controlling the dissemination of ARGs in the environment.

Keywords: Antibiotics, antibiotic resistance genes, anaerobic treatment processes, swine wastewater

1. Introduction

Antibiotics are the most effective agents used in pig farms to prevent and treat disease, as well as improve the growth of pigs (Li, 2017; Sarmah et al., 2006). Landers et al. (2012) reported that approximately 88% of growing pigs in the United States receive antibiotics in their feed for disease prevention and growth promotion purposes. In Japan, 175 tons of antibiotics were used as growth promotion to animals in 2001 (Li, 2017). Nearly half of the antibiotics consumed (162,000 tons in 2013) were used in animal husbandry (Zhang et al., 2015b). However, most antibiotics are poorly absorbed by pigs and hence are excreted through faeces and urine of pigs in unchanged forms or as metabolites (Álvarez et al., 2016; Cheng et al., 2018b). Therefore, swine wastewater has been reported as an important reservoir of antibiotics due to the frequent use of antibiotics for controlling disease and in the growth of pigs (Apley et al., 2012; Chen et al., 2018; Sui et al., 2017).

The high frequency and detection of ARGs in swine wastewater reflected the positive relationship between ARGs and their corresponding antibiotic concentrations (Wang et al., 2016a). He et al. (2016) states that ARGs in swine wastewater samples were at least 31 times higher than those in well water and fishpond water. Ben et al. (2017) investigated the ARGs' encoding resistance to tetracycline antibiotics in nine swine feedlots located in Shandong Province of China. Results indicate that targeted ARGs were widely distributed in swine wastes, with the mean relative abundance (copies per 16S rRNA gene) ranging from 3.3×10^{-5} (tetC) to 5.2×10^{-1} (tetO) in swine manure and from 7.3×10^{-3} (tetC) to 1.7×10^{-1} (tetO) in swine wastewater. Moreover, high levels of ARGs have been detected in soil and water environments adjacent to swine farms (Cheng et al., 2016; He et al., 2016; Hsu et al., 2014; Wu et al., 2010). Wu et al. (2010) found that the absolute tetracycline resistance gene copies (sum of tetM, tetO, tetQ, tetW genes) in soils, which received pig wastes strongly correlated with the concentrations of tetracycline residues. Li et al. (2018b) explained that

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antibiotics together with ARGs (i.e., *qnr*A*, sul*1*, sul*2*, tet*G, tet*M*, and tet*O) discharged from swine feedlots through wastewater could disseminate into surrounding groundwater environments. The total relative abundance of ARGs also increased in the river water after receiving swine wastewater discharge (Jia et al., 2017). The presence of ARGs in the environment could pose a serious risk to aquatic and terrestrial life because of their spread in the environment through bacteria, and/or mobile genetic elements (Li et al., 2018a; Xie et al., 2016). As emerging environmental contaminants, ARGs have become one of the world's most serious clinical and public health issues as indicated by the first global report of the World Health Organization (WHO) on antimicrobial resistance (WHO, 2014). According to the latest report, a new superbug, *Staphylococcus epidermidis*, which is resistant to all known antibiotics has been discovered by Australian scientists (Lee et al., 2018). The increased resistance, including multi-drug resistance, supports urgent research that the factors and hot spots involved in its diffusion and development should be better understood (Lupo et al., 2012).

Anaerobic treatment technology is one of the main methods for treating swine wastewater with high organic pollutants, since biogas generated could be used as bioenergy for households and anaerobic digestion residue usually applied as organic fertilizers to facilitate the growth of crops (Jechalke et al., 2014; Skouteris et al., 2012; Sui et al., 2016). However, anaerobic treatment processes not only have limited capability to completely remove antibiotics and ARGs received from swine wastewater they can, in turn, create favorable conditions for ARGs development and transfer (Miller et al., 2016; Pu et al., 2018; Zhang, 2016). Hence, products from the anaerobic treatment process have been regarded as the source of antibiotics, and ARGs brought into the environment (Sui et al., 2016).

To date, previous studies have focused on factors contributing to the proliferation and spread of ARGs in anaerobic treatment processes, such as antibiotics, heavy metals, operational conditions, and microbial communities (Ma et al., 2011; Sun et al., 2016; Yin et al., 2017). Exposure to antibiotics has been considered as the most important factor influencing the emergence and spread of ARGs (Allen et al., 2010; Gao et al., 2012b; Levy, 2002). Therefore, it is necessary to focus on the contribution of antibiotics on ARGs in the anaerobic treatment of swine wastewater due to the significantly high concentrations of antibiotics in swine wastewater (Cheng et al., 2018b). Horizontal Gene Transfer (HGT) has been confirmed as a major contributor to the spread of ARGs, which have specific structures and ability to capture genes by a site-specific recombination system (Lopatkin et al., 2016a). Furthermore, ARGs are carried by microbes in anaerobic treatment processes, so changes in the structure of the bacterial community is the dominant mechanism responsible for the variation of ARGs and during anaerobic processes (Tong et al., 2016; Zhang et al., 2016). It should be noted that antibiotics in swine wastewater show significant effects on the microbial community in anaerobic processes (Akyol et al., 2016; Cheng et al., 2018a). It can be considered that the impacts of antibiotics on HGT and the microbial communities can also demonstrate diverse influences on ARGs. Therefore, the goal of this paper is to figure out the influence of antibiotics on ARCs through their impacts on HGT and the microbial communities related to ARGs. Although studies have focused on the removal of antibiotics and ARGs during the anaerobic process, the occurrence and spread of ARGs continues to be a problem (He et al., 2016; Sui et al., 2016; Sun et al., 2016). Understanding the contribution of antibiotics to the fate of ARGs during anaerobic treatment of swine wastewater is critical for reducing the spread of ARGs through the wastewater treatment process. Hence, this review is focused on the occurrence and removal of ARGs in swine

wastewater treatment by anaerobic processes, and the contributions of such antibiotics to the abundance and transfer of ARGs.

2. Occurrence and removal of ARGs in the anaerobic treatment of swine wastewater

Antibiotics in the class of tetracyclines, sulfonamides, and macrolides are known as the frequently used antibiotics in swine farms (Cheng et al., 2018b; Hsu et al., 2014). High concentrations of such antibiotics have been found in swine wastewater. As reviewed by Cheng et al. (2018b), antibiotics, such as sulfamethoxazole (SMX), sulfadiazine (SDZ), sulfamonomethoxine (SMN), tetracycline (TC), oxytetracycline (OTC), chlortetracycline (CTC) and tylosin, were detected at alarmingly high levels in swine wastewater with the average concentration of 316.5, 98.9, 45.4, 130.08, 16.25, 12.16 and 72 μg/L, respectively. In this case, ARGs, including tetracycline resistance genes, sulfonamide resistance genes, and macrolide resistance genes have been frequently detected in swine wastewater (Ben et al., 2017; Yuan et al., 2018). The class 1 integron-integrase gene (*Intl*1) is part of class 1 integrons that could capture ARGs as part of gene cassettes. The review paper by Gillings et al. (2015) suggested that *Intl*1 could be proposed as a good proxy for monitoring ARGs in the environment. Thus, *Intl*1 was linked to the dissemination of various ARGs (Aydin et al., 2015b; Tian et al., 2016; Zarei-Baygi et al., 2019). As reported, *Intl*1is also prevalent in swine wastewater with a proportion of up to 30% (Yuan et al., 2018).

All these ARGs were detected and highly abundant in swine wastewater. Their abundance in raw swine wastewater (10^{11}-10^{14} copies/L) is much higher than that in drinking water sources (10^4–10^9 copies/L) (Su et al., 2018; Sui et al., 2016). Based on the various amounts of antibiotics used and the management of pig farms, the mean relative abundance of the selected ARGs in swine wastewater from pig farms is shown in Fig. 1 (Ben et al., 2017; Cheng et al., 2013; Joy et al., 2014; Sui et al., 2016; Wang et al., 2016a). As can be seen from Fig. 1, the mean relative abundance of the selected ARGs in swine wastewater

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ranges between 10^{-3} and 10^{-1} copies/16S rRNA, which is higher than the abundance in global

lakes (10^{-5} to 10^{-2}) (Yang et al., 2018). In particular, tetracycline resistance genes *tet*C and

*tet*O were the most detected ARGs in swine wastewater with the mean relative abundance of

6.39×10^{-2} and 1.13×10^{-1} copies/16S rRNA, respectively (Cheng et al., 2016; Sui et al., 2016;

Tao et al., 2014). Except to *tet*C, the abundance of the ribosomal protection protein genes

(*tet*M, *tet*O, *tet*Q, *tet*W) were higher than the efflux protein genes (*tet*A, *tet*B, *tet*G) in swine

wastewater. Two plasmid-borne genes, *sul*1 and *sul*2, were also highly detected in swine

wastewater, with means of 5.48×10^{-2} and 3.23×10^{-2} copies/16S rRNA, respectively (Cheng

et al., 2016; McKinney et al., 2010; Sui et al., 2016; Tao et al., 2014). The *sul*1 and *sul*2 are

associated with sulfonamide resistance in gram-negative bacteria, which produce

dihydropteroate synthetase and induce resistance against sulphonamides (Arabi et al., 2014).

The macrolide resistance genes, such as *erm*B, *erm*F, and *mef*A, were also detected in swine

wastewater with high abundance (Sui et al., 2016). Meanwhile, the mean relative abundance

of *intl*1was 8.76×10^{-3} copies/16S rRNA in swine wastewater (Sui et al., 2016).

Figure 1

Considering high residuals of antibiotics and ARGs in swine wastewater, recent studies

about the treatment of swine wastewater by anaerobic treatment processes not only

considered the removal of common contaminants and biogas production, but also their

efficiencies for removing antibiotics and ARGs (Ma et al., 2018; Sui et al., 2014; Sui et al.,

2016). The ARGs removal in anaerobic processes needs more attention due to the usual land

application of products from the anaerobic process. As reports, the shift of ARGs in the

anaerobic process was related to their resistance mechanisms and different operating

conditions of anaerobic processes (Sui et al., 2016; Sui et al., 2017; Wang et al., 2017b).

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Accordingly, the intrinsic resistance mechanism of the most detectable ARGs in swine wastewater, the main hosts of these ARGs in anaerobic processes, and their shift are summarized in Table 1 (Aydin et al., 2015b; Sui et al., 2016).

Table 1

As shown in Table 1, *tet*X encoding tetracycline inactivation enzyme did consistently decrease in the anaerobic treatment of swine wastewater (Chen et al., 2015a; Cheng et al., 2013). The shift of macrolide antibiotic resistance genes (*erm*B and *erm*F), mainly documented as 23S rRNA methyltransferase genes, showed a similar trend with *tet*X after the treatment of anaerobic process (Yang et al., 2014). Whereas, the removal of the ribosomal protection protein genes (*tet*W and *tet*O) was variable in different operating conditions (Wang et al., 2017b; Wu et al., 2016). Temperature was essential for ARGs removal by anaerobic treatment, the removal rates of ARGs and *intl*1 increased with increasing the temperature. For instance, Wang et al. (2017b) stated that predominant ARGs (*tet*O, *tet*W, and *tet*X) almost reduced by one order of magnitude through mesophilic anaerobic digestion (37 °C). The removal rates of genes (*tet*O, *tet*X, *tet*W and *int*I1) could be enhanced when the temperature increased from 37 to 55 °C, but quantities of some ARGs (*tet*A, *tet*O, and *tet*X) increased in anaerobic effluent under the temperature of 22 °C (Diehl & LaPara, 2010). Similar results have been concluded by Wu et al. (2016), who indicated that ARGs could be much more decreased in thermophilic digestion than that in mesophilic one.

However, the relative abundances of tetracycline resistance genes (*tet*A, *tet*G, *tet*C and *tet*M), as well as the sulfonamide resistacne genes (*sul*1 and *sul*2) increased after anaerobic treatment of swine wastewater (Cheng et al., 2016; Sui et al., 2016; Tao et al., 2014). Ju et al. (2018) also observed the increase of the relative abundance of ARGs and

*intI*1 in the effluent of WWTPs. This might be due to proliferation of their host bacteria during the wastewater treatment process or horizontal transfer of the ARGs to other species (Hultman et al., 2018; Koike et al., 2007). For example, Hultman et al, (2018) demonstrated that the effluent of the wastewater treatment plants contained resistance genes in bacteria not carrying these genes in the influent. Anaerobic treatment processes with a high density of bacteria have been shown to be hotspots for ARGs transfer among different microorganisms through HGT (Lopatkin et al., 2016b; Miller et al., 2016; Sørensen et al., 2005). HGT is the process of genetic movement among species, which is another major mechanism responsible for the spread of antibiotic resistance (Lopatkin et al., 2016b). As reported, *tet*A, *tet*C and *tet*G genes can easily spread among bacteria species. This is mainly because they are carried by main mobilizable vectors, self-transmissible transposon and smaller plasmids (Jiang et al., 2013). *sul*1 is often associated with *IntI*1, it was located in the 3′-conserved segment (3′-CS) of class 1 integrons, while *sul*2 gene was usually related to small non-conjugative plasmids or large transmissible multi-resistant plasmids, which could facilitate their dissemination (Ma et al., 2018). Class 1 integrons were often used to represent the HGT as it can regulate the expression of exogenous genes. Although they cannot mobilize and transfer themselves between microbes, they are often associated with genetic elements which can, such as conjugative plasmids, transposons and insertion sequences (Berglund, 2015).

Rich nutrients in swine wastewater and relatively permissive conditions for the anaerobic bacteria also can explain the inability of the anaerobic process to substantially reduce such ARGs, because the spread of ARGs could be increased by the synergistic effect of the antibiotics and nutrients in wastewater. (Chen et al., 2010; Subirats et al.,

2018). For instance, the study by Subirats et al. (2018) found that the abundance of *sul*1 and *intI*1 genes increased when bacterial communities exposed to both emerging contaminants and a high nutrient concentration (1, 25 and 1 mg/L of phosphate, nitrate and ammonium, respectively). In addition, antibiotics in swine wastewater demonstrated statistically significant correlations with the ARGs, in which their fate in the anaerobic treatment process was linked to the level of residual antibiotics (Cheng et al., 2016). Thus, it is difficult to give a general conclusion about the removal vs. enrichment of ARGs in anaerobic treatment processes. Exploring the impact factors on the fate and transfer of ARGs in anaerobic processes is significant in future research.

3. Contribution of antibiotics to the fate of ARGs in anaerobic processes

High concentrations of antibiotics in swine wastewater have great effects on the fate of ARGs in the anaerobic treatment processes, although some studies have reported that besides antibiotics, other influential factors also affect the fate of ARGs (He et al., 2017; Li et al., 2010; Wang et al., 2016a). In most cases, antibiotics in swine wastewater were positively correlated with the prevalence of ARGs in the treatment process (Huang et al., 2017; Jia et al., 2017; Zhang et al., 2015a). For ARGs, these antibiotics pose strong selective pressures which facilitate their horizontal transfer among bacterial cells by plasmids or integrons during anaerobic treatment processes (Ghosh & LaPara, 2007; Looft et al., 2012). Previous literature indicated that antibiotics even in concentrations below the minimal inhibitory concentration can increase the abundance of ARGs (Aydin et al., 2015b; Gullberg et al., 2011). The appearance of antibiotics in anaerobic reactors could show a negative effect on the removal efficiencies of ARGs (Wang et al., 2017b). Furthermore, the microbial community carrying ARGs could be influenced by

antibiotics, which would result in the change of ARGs in anaerobic processes (Luo et al., 2017; Tong et al., 2016; Wang et al., 2014a; Zhang et al., 2013a).

3.1 Contribution of antibiotics to the abundance of ARGs

3.1.1 Contribution of sulfonamides to their corresponding ARGs

The presence of antibiotics in the anaerobic treatment process exerts direct pressure in the accumulation of ARGs. Some reports indicated that the concentration of tetracycline and sulfonamide antibiotics correlate with their corresponding ARGs (Zarei-Baygi et al., 2019). That is, the abundance of ARGs increased with the increasing dose of antibiotics (Aydin et al., 2016; Cheng et al., 2018a; Zarei-Baygi et al., 2019). For instance, Jiang et al. (2013) found more sulfonamide resistance genes and tetracycline resistance genes in the water samples containing higher concentrations of total sulfonamide and tetracycline antibiotics. A significant increase of total ARG abundance, *sul*1 and *erm*F in particular was observed by the addition of SMX and the increment of its concentration from 10 to 250 μg/L (Zarei-Baygi et al., 2019). Aydin et al. (2015b) predicted that ARGs develop rapidly as they become stable in bacterial populations in reactors of higher or lethal antibiotic concentrations in comparison to reactors with lower or non-lethal antibiotic concentrations. In particular, along with the swine wastewater treatment process, a strong positive correlation between the total relative abundance of sulfonamide resistance genes and the total sulfonamides concentration was observed (Ma et al., 2018; Wang et al., 2016a). In a constructed wetlands system for treating swine wastewater, the author also indicated that the increase of the relative abundance of *tet*W, *tet*O, *tet*M, *sul*1 and *sul*2 genes was mainly caused by SMZ and TC accumulation in the system (Cheng et al., 2018a).

These similar results were found in the conventional wastewater treatment plant and fish pond waters, as the increased quality of *sul*1 genes were observed in the samples with a higher concentration of sulfonamides (Gao et al., 2012b; He et al., 2017). It is reported that the relatively high water solubility and stability of sulfonamides could promote the spread of antibiotic resistance (Chee-Sanford et al., 2009). More explicitly, according to the transfer mechanism of sulfonamide resistance genes, the *sul*1 gene was usually accompanied by the presence of integrons as it contributes to the widespread of *sul*1 in the aquatic environment (Gao et al., 2012a).

3.1.2 Contribution of tetracyclines to their corresponding ARGs

Compared with sulfonamides, tetracyclines can be easily absorbed into the anaerobic sludge through adsorption (Cheng et al., 2018b). Thus, the abundance of tetracycline resistance genes was reported as being higher in the residual sludge rather than those in the effluent wastewater (Aydin et al., 2016; Wu et al., 2016). Huang et al. (2015a) reported that in the anaerobic reactor with TC, almost all target tetracycline resistance genes (*tet*A, *tet*B, *tet*C, *tet*E, *tet*M, *tet*O, *tet*S) proliferated in the sludge more than those in the reactor without TC. Smith et al. (2004) also concluded that tetracycline resistance genes were positively selected after exposure to high levels of TC, the total abundance of ARGs was significantly increased at high TC levels. Therefore, the adsorption of tetracyclines in biomass during the treatment process plays an important role in increasing tetracycline resistance genes in the anaerobic sludge (Aydin et al., 2016). The appearance of OTC in the anaerobic reactor also benefit the proliferation of ARGs. For example, there were more abundant tetracycline resistance genes presented in an OTC wastewater treatment system than those in sewage treatment systems (Liu et al., 2012). Interestingly, the abundance of efflux pump genes (*tet*A, *tet*B, and *tet*C) in

the reactor with OTC (2 mg/L) and TC were significantly higher than in the control reactor (He et al., 2017; Shi et al., 2017). This explains how most of the efflux pump genes could be acquired or transferred through mobile elements, which is the dominant mechanism for the dissemination of ARGs (Jiang et al., 2013; Wang et al., 2014b).

When antibiotics show different influences on various ARGs, the abundance of some ARGs could be improved while other ARGs were not affected. This explains the different responses of various ARGs to antibiotics (Wu et al., 2015). As reported earlier, although the abundance of efflux pump genes (*tet*A, *tet*B, and *tet*C) increased with the increase of OTC concentration, the enzymatic modification gene (*tet*X) did not fluctuate significantly (Shi et al., 2017). The reason might be that the resistance mechanism of efflux pump is only a self-protection mechanism of microbes, which cannot reduce antibiotic content, whereas the enzymatic modification gene is one of the main mechanisms of tetracycline degradation that can chemically modify tetracycline to inactivate it via synthetic oxidoreductase expression (Chopra & Roberts, 2001). In the anaerobic processes, *Bacteroides spp*. which carry *tet*X, modifies antibiotics and degrades it via the *tet*X protein (a flavin-dependent monooxygenase) (Yang et al., 2004). According to the report by Wang et al. (2016b), the abundance of *tet*Q and *tet*W genes increased when OTC concentration also increased, while *tet*M and *tet*C genes decreased due to the OTC inhibition of microbes.

3.1.3 Co-resistance and cross-resistance of antibiotics

Alternatively, some reports also concluded that the correlation between total ARGs and their corresponding antibiotics was not as significant as previously thought; other factors may also influence the abundance of ARGs in addition to the selection pressure of antibiotics (Ji et al., 2012; McKinney et al., 2010; Peak et al., 2007; Selvam et al.,

2012; Wang et al., 2016a). For instance, Selvam et al. (2012) found that regarding the treatment of swine manure, in most cases, the copy number of ARGs in the reactor with antibiotics was higher than the control reactor. However, in some aspects, more ARGs were detected in control reactors compared with antibiotic spiked ones. As well, in both swine treatment lagoons and the municipal wastewater treatment plant, although a positive correlation was observed between total sulfonamide resistance genes and total sulfonamides in wastewater, no significant correlation was found between total tetracycline resistance genes and total tetracyclines (Gao et al., 2012b; Zhang et al., 2013b). The reason might be that many other factors could also affect the spread of ARGs in wastewater at low concentrations of antibiotics, which would result in a weak correlation between antibiotics and their corresponding resistance genes throughout the wastewater treatment process (Gao et al., 2012b). Probably, other factors, for instance heavy metals and other types of antibiotics presenting in the wastewater, can result in co-selection and cross-selection of resistance, which may affect correlations between ARGs and their corresponding antibiotics (McKinney et al., 2010; Stepanauskas et al., 2006).

As reported by Ji et al. (2012), due to the coexistence of antibiotics and heavy metals, significant positive correlations were found between sulfonamide resistance gene and typical heavy metals (Cu, Zn and Hg), while the presence of ARGs was relatively independent of their respective antibiotic inducer. Li et al. (2015) also indicated that the correlation not only just occurred between ARGs and their corresponding antibiotics, but is still true for other types of antibiotics, for instance, the slufamerazine indicated a positive relationship with tetracycline resistance genes (*tet*B and *tet*C). In a landfill, OTC and TC showed positive correlations with *tet*O and *sul*1,

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while SMX was not found to be associated with the ARGs investigated (Song et al., 2016). Also, a high concentration of OTC could improve the relative abundance of *drfA*7, a sulfonamide resistance gene (Qian et al., 2016). All of the above may be due to the co-resistance and cross-resistance of the two classes of antibiotics.

During the anaerobic treatment process, the number of ARGs would reduce due to the effective degradation of antibiotics (Mohring et al., 2009). Aydin et al. (2015b) and Aydin et al. (2016) indicated that the concentration of tetracycline resistance genes was always higher than the erythromycin resistance genes in the reactor with tetracycline and erythromycin (ERY), even though the dosage of tetracycline was the same as that of ERY. This is mainly because the biodegradation of tetracycline could be lower than ERY in biological wastewater treatment. According to the report by Shi et al. (2017), OTC did benefit the proliferation and accumulation of ARGs in the anaerobic reactor, but the abundance of ARGs displayed a downward trend when the antibiotic selective pressure was removed. This also has been confirmed by the treatment of swine wastewater by constructed wetlands, which demonstrated that the higher influent TCs concentration selected more copy numbers of *Intl*1, *tet*A and *tet*W in the effluent. Thereafter, however, this selection effect was alleviated probably because of elevated antibiotics removal (Huang et al., 2015b).

Additionally, ARGs increased with an increased dosage of combined antibiotics (sulfamethoxazole-tetracycline-erythromycin (STE) and sulfamethoxazole-tetracycline (ST)) (Aydin et al., 2015a). This outcome has been confirmed by Aydin et al. (2015b), who compared the proliferation of ARGs in the reactors with individual and combined antibiotics, respectively. These findings indicated that the presence of antibiotic combinations resulted in higher rates of ARG acquisition than individual antibiotics.

The author also stated that the effluent from the STE reactor had a higher number of ARGs than in the ST reactor due to the synergistic effects of ERY. He et al. (2017) have also concluded that the total relative abundance of ARGs in combined stresses exposure tanks (tetracycline-sulfanilamide, tetracycline-sulfanilamide-cefotaxime, tetracycline-sulfanilamide-Cu, and tetracycline-sulfanilamide-Zn) was about 1.01–1.55 times more than the sum of their individual ones.

3.2 Contribution of antibiotics to the transfer of antibiotic resistant genes

One of important factors for the proliferation and transfer of ARGs is the HGT with MGEs, as they have specific structures and the ability to capture genes by a site-specific recombination system (Hershberg, 2017). Anaerobic treatment processes with a high density of bacteria have been shown to be hotspots for ARGs transfer among different bacteria through HGT (Lopatkin et al., 2016b; Miller et al., 2016; Sørensen et al., 2005). The transfer of ARGs among bacteria mainly include three pathways: 1. Bacteria transformation; 2. Bacteria transduction; 3. Bacteria conjugation (see Fig. 2). The figure explains how the process of transformation, naked DNA and ARG-harbouring mobile genetic elements (MGEs) released by the donor cell and taken up by bacteria and incorporated into the genome (Chen & Dubnau, 2004). While in transduction, phages can act as vectors for genetic exchange, whereby ARGs are carried by phage particles from a donor bacterial cell to a recipient cell (Balcazar, 2014).

Figure 2

Previous studies reported that antibiotics could contribute to antibiotic resistance by enhancing their spread among the bacterial community under non-lethal concentrations (Beaber et al., 2004; Jutkina et al., 2018; Úbeda et al., 2005). Thus, it

was believed that the selective pressure from antibiotics could accelerate the ARGs'

transmission in the sensitive bacteria (Li et al., 2009). For example, Aydin et al. (2015b)

concluded that high levels of antibiotics have shown abilities to increase and stimulate

HGT, and activate MGEs among the bacterial community. The study by Jutkina et al.

(2018) also concluded that SMX (1 mg/L) and gentamicin (0.1 mg/L) significantly

increased the transfer frequency of antibiotic resistance. Therefore, the transfer of ARGs

could be enhanced by the presence of antibiotics in anaerobic processes.

3.2.1 Contribution of antibiotics to transfer through conjugation

Conjugation plays an important role in the association of ARGs with MGEs, which

occurs either through plasmid transfer or transmission of chromosomally integrated

conjugation elements, including conjugative transposons (Aminov, 2011; Frost et al.,

2005; Wozniak & Waldor, 2010). As reported in one study, the abundance of

sulfonamide resistance genes significantly correlated with the abundance of plasmids

(Tao et al., 2016). In addition, most efflux protein tetracycline resistance genes, such

as *tet*A, *tet*B, *tet*C and *tet*G, can easily spread among bacteria species. This is mainly

because they are carried by main mobilizable vectors, these being self-transmissible

transposon and smaller plasmids (Jiang et al., 2013). In addition, *tet*W, *tet*O, *tet*S, *sul*2

and *sul*3 are commonly found on conjugative plasmids or chromosomes transferred

horizontally between microbial communities (Billington et al., 2002; Wu et al., 2016).

Due to the high prevalence and the broad host ranges of plasmids, as well as the ARGs

carried by many plasmids, conjugation is thought to be responsible for the majority of

antibiotic resistance spread (Lopatkin et al., 2016b)

Some studies supposed that antibiotics could promote HGT through conjugation.

They can the increase the conjugation efficiency through activating the excision of

transferrable genes from the host chromosome and/or by inducing the expression of conjugation machinery (Beaber et al., 2004; Stevens et al., 1993). The study by Scornec et al. (2017) indicated that some ribosome-targeting antibiotics could increase the conjugation frequency of a specific conjugative transposon more than a thousand-fold. Lopatkin et al. (2016b) showed that antibiotics determine conjugation dynamics primarily by serving as a selective driver in a broad range of conjugative systems. Tetracycline and SMX have been reported as potent inducers of conjugation by recent researches (Jutkina et al., 2018; Jutkina et al., 2016). However, the effect of antibiotics on HGT by conjugation is still controversial. For instance, antibiotics ciprofloxacin CIP and ERY have no inductive effects on conjugal transfer (Lopatkin et al., 2016a; López et al., 2007). In particular, antibiotics can promote and suppress HGT dynamics, depending on how antibiotics affect growth rates of populations undergoing conjugation (Lopatkin et al., 2016b). Lu et al. (2017) investigated the effect of antibiotics on conjugational transfer of a mobilizable gentamicin resistance plasmid, and concluded that the conjugation-promoting ability of antibiotics was controlled by the quorum sensing. The study by Lopatkin et al. (2016a) also indicated that the contribution of antibiotics to the promotion of conjugation might be over-estimated. The reason is that: firstly, the efficiency of conjugation antibiotics was not significantly increased by antibiotics, and secondly, antibiotics might reduce the frequency of conjugation by reducing the population sizes of either or both parental populations, which would negate the effect of positive selection for the transconjugant.

3.2.2 Contribution of antibiotics to transfer through integrons

An integron is a typical gene capture and dissemination system. Integrons are ubiquitous in chromosomes and MGEs, like plasmids and transposons (Chen et al.,

2015b; Guerin et al., 2009). They play a key role in the dissemination of ARGs by facilitating horizontal transfers of ARGs between microbes through combination with mobile gene platforms and/or incorporating ARGs into microbial chromosomes (Gillings et al., 2015; He et al., 2016). Class 1 integrons were often used to represent the HGT as it can regulate the expression of exogenous genes. Although they cannot mobilize and transfer themselves between microbes, they are often associated with genetic elements which can, such as conjugative plasmids, transposons and insertion sequences (Berglund, 2015). The most detectable antibiotics in swine wastewater and class 1 integrase genes have been considered to be key markers of HGT in the anaerobic process (Ghosh et al., 2009; Ma et al., 2011; Miller et al., 2013). *Intl*1 is part of class 1 integrons which could capture ARGs as part of gene cassettes, thus *Intl*1 was linked to the transfer of ARGs (Aydin et al., 2015b; Tian et al., 2016).

For example, for ARGs in the treatment of swine wastewater when utilizing the anaerobic process, *tet*A, *tet*C, *tet*G, *tet*Q, *tet*X, *sul*1, *erm*B, and *dfrA*1 were found to be positively correlated with *Intl*1 (Chen et al., 2015b; Cheng et al., 2013; Liu et al., 2012; Sui et al., 2017; Wu et al., 2016). Specifically, *sul*1 gene was located in the 3′ -conserved segment (3′ -CS) of class 1 integrons, while *sul*2 gene was usually related to small non-conjugative plasmids or large transmissible multi-resistant plasmids (Ma et al., 2018). Although tetracycline resistance genes have not been found as gene cassettes in class 1 integrons, they have been associated with class 1 integrons on self-transferable plasmids in both gram-positive and gram-negative bacteria (Agersø & Sandvang, 2005). Significant correlations between *Intl*1 and *tet*O, *tet*W and *sul*3 were also found in fish tanks water with exposure to antibiotics (He et al., 2017). Such correlation indicated that these ARGs could have been on conjugative plasmids and

could transfer among microbes using the class 1 integron as a carrier, which means that *Intl*1 plays an important role in the propagation of ARGs (Agersø & Sandvang, 2005; Bennett, 2008; Wang et al., 2017a).

The relationship between the abundance of *Intl*1 and the concentration of antibiotics has been studied previously (Chen et al., 2015b; Subirats et al., 2018). Chen et al. (2015b) demonstrated that the relative abundance of *Intl*1 was significantly correlated with the total concentration of antibiotics, and the higher concentration of antibiotics resulted in the greater abundance of *Intl*1 gene in both water and sediment samples. Subirats et al. (2018) also indicated that bacterial communities exposed to antibiotics resulted in higher copy numbers of *sul*1 and *Intl*1 genes than those not exposed. To some extent, the increase in the relative abundance of *sul*1 may be related to integron mobilization since this gene is usually linked to class 1 integrons (Gillings et al., 2015). This has been confirmed by He et al. (2017) and Rafraf et al. (2016), who found a significant correlation between sul1 gene and *Intl*1 genes when exposed to sulfanilamide. Furthermore, the combination of antibiotics and metals in swine wastewater may provide a stronger selection for realized horizontal gene transfer among the microbial community than either alone (Baker-Austin et al., 2006; Zhu et al., 2013).

3.3 Impacts of antibiotics on the host of ARGs in anaerobic processes

In anaerobic processes, the bacterial community is significantly correlated to the fate of ARGs (Song et al., 2018; Tong et al., 2016). The bacterial community drives the distribution of ARGs during anaerobic digestion, and the level of ARGs in anaerobic processes is attributed to the survival or death of antibiotic resistant bacteria (Miller et al., 2016; Tao et al., 2016). Previous reports have conducted that most ARGs are carried by the function bacteria in anaerobic processes, which belong to the phylum of

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Bacteroidetes, Firmicutes, Proteobacteria, Actinobacteria and *Spirochaetes* (Aydin et al., 2015b; Forsberg et al., 2014; Resende et al., 2014; Sun et al., 2016). Hence, understanding the effect of antibiotics on the above functional bacteria is important for exploring the contribution of antibiotics to the fate of ARGs during anaerobic processes.

Bacteroidetes and *Clostridium* were the dominant hosts of tetracycline and sulfonamide resistance genes (Cetecioglu et al., 2016; Huang et al., 2015a; Sun et al., 2016). Specifically, *Bacteroidetes* were reported as the important hosts of *tet*C, *tet*M, *tet*Q, *tet*X, *erm*B, *erm*F and *sul*1 (Tao et al., 2016). Aydin et al. (2015b) reported that almost 50% of the tetracycline resistance genes were located in the *Clostridium* genus. Their abundance were reported to be increased with the increase of TC and SMX concentrations in the anaerobic reactor (Cetecioglu et al., 2016; Xiong et al., 2017). Wang et al. (2017c) demonstrated that the presence of CTC in anaerobic reactors might promote the growth of species in family of *Moraxellaceae* and *Pseudomonadaceae* because of their strong resistance to CTC. It is known that *Moraxellaceae* and *Pseudomonadaceae* belong to the phylum of *Proteobacteria*, which had significant positive correlations with all ARGs and integrase genes, except for *tet*W and *tet*X (Sun et al. 2016, Wu et al. 2016). According to the report of Aydin et al. (2016), the number of *Actinobacteria* and *Fusobacterium* rose significantly with the increasing concentration of tetracycline and erythromycin, due to these phyla possibly acquiring antibiotic resistance and disseminating easily across the phylum. The authors also stated that the *Acinetobacter*-related populations and *Bacteroidetes*-related population in the anaerobic reactor played a functional role in the elimination of tetracycline and erythromycin antibiotics, and the acquisition of resistance genes.

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In the methanogenic phase, more diverse microbial community structure and more potential microbes could contain ARGs, because most of ARGs were observed to rebound significantly and increase in this stage (Wu et al., 2016). Therefore, an abundance of bacteria carrying ARGs might be increased with the addition of antibiotics into the anaerobic reactor. In other word, failure to maintain the stability of bacteria in anaerobic processes would result in the increasing occurrence of ARGs.

4. Future perspectives

It is clear that antibiotics in swine wastewater can affect the proliferation of ARGs in the anaerobic treatment process. Although most research studies have proved the positive correlation between antibiotics and their corresponding genes, other analyses still concluded differently due to the probable co-selection and/or cross-selection of other factors in swine wastewater (McKinney et al., 2010; Stepanauskas et al., 2006). In order to better characterize and understand their correlations to further explore the contribution of antibiotics to the fate of ARGs in the anaerobic treatment process, additional studies with respect to the selection pressure of present antibiotics in swine wastewater on the occurrence and accumulation of ARGs are needed. Similarly, HGT is an important mechanism for the transmission of ARGs among different microorganisms. Antibiotics have been believed to accelerate the horizontal transfer of ARGs (Aydin et al., 2015b; Li et al., 2009). However, it is still necessary to study the effect of antibiotics on mechanisms of HGT in the future.

In anaerobic treatment processes, microorganisms show an important responsibility for the fate of ARGs, but it is very difficult to clearly understand their relationship with ARGs. The microbial community in anaerobic treatment processes is very complex and most microorganisms are uncultivated. As well, the microbial structure could vary

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depending on different operation conditions of the anaerobic treatment process (Tao et al., 2016; Tian et al., 2016). Thus, to further clarify the correlation between ARGs and microbial communities and provide more information on the treatment of ARGs by anaerobic treatment processes, it is necessary to learn more about ARGs profiles as well as microbial community composition during the swine wastewater treatment process.

Currently, metagenomic sequencing or qPCR were usually used to detect ARGs in the environment (Guo et al., 2017; He et al., 2016; Tao et al., 2014). However, as reported by Tian et al. (2016), metagenomic sequencing or the qPCR method cannot distinguish ARGs from live or dead cells in anaerobic treatment processes, which could result in partially distorting the temporal and spatial patterns of the detected ARGs. In order to evaluate the removal efficiency of ARGs in a treatment process and their abundance in different environmental media, the more accurate method need to develop. In addition, it is difficult to evaluate whether the treatment technology is safe for the treatment of ARGs because there is no standard for their discharge, although they were proposed as emerging contaminants more than 10 years ago (Pruden et al., 2006). Therefore, more and suitable studies on the risk of ARGs are needed to draft a discharge standard for ARGs in swine wastewater treatment plants in future research. Finally, as reported by Lopatkin et al. (2017), ARGs could act to stabilize and persist for many years in the environment even in the absence of antibiotics. That is why treatment technologies are urgently requires to remove antibiotic and ARGs effectively from q much higher than that in drinking water sources and lakes. The removal or enrichment of ARGs in anaerobic treatment processes is difficult to give a general conclusion. Antibiotics in swine wastewater showed a positive effect on the abundance of ARGs, the transfer of ARGs, and the abundance of bacteria carrying ARGs and *Intl*1.

Understanding the contribution of antibiotics to the proliferation of ARGs is significant to control their dissemination in the environment. Further studies are necessary to explore the correlation among antibiotics, ARGs and microbial communities.

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Figure Captions

Fig. 1 Mean relative abundances of ARGs in swine wastewater

Fig. 2 Mechanisms of horizontal gene transfer among bacteria

Table Captions

Table 1 Intrinsic resistance mechanisms and hosts of ARGs and their shift in anaerobic
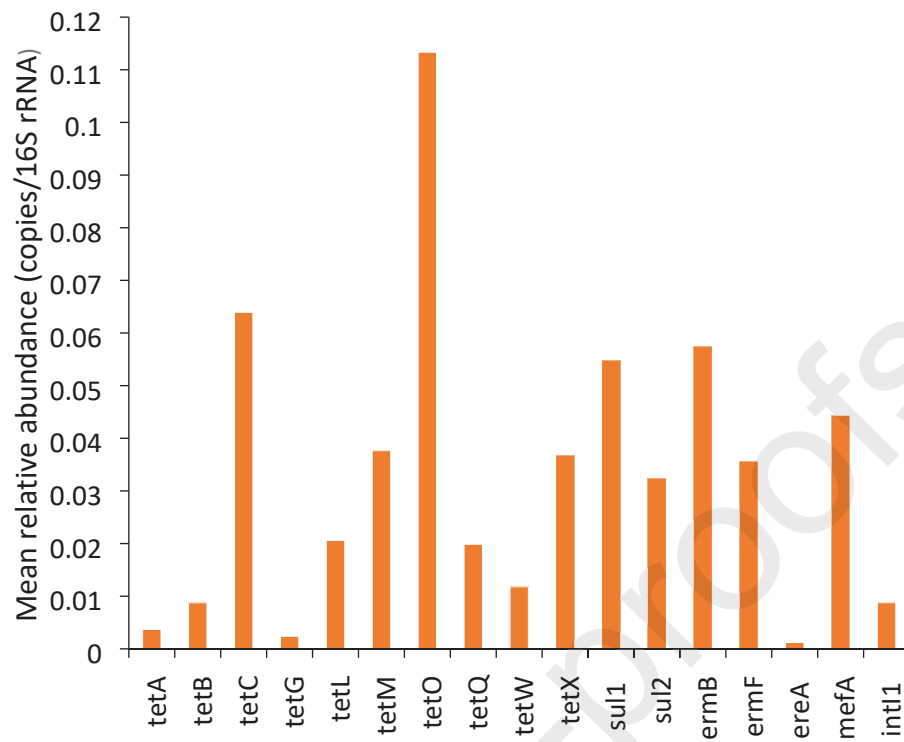
treatment processes

Fig. 1 Mean relative abundances of ARGs in swine wastewater

1. Bacterial transformation

2. Bacterial transduction
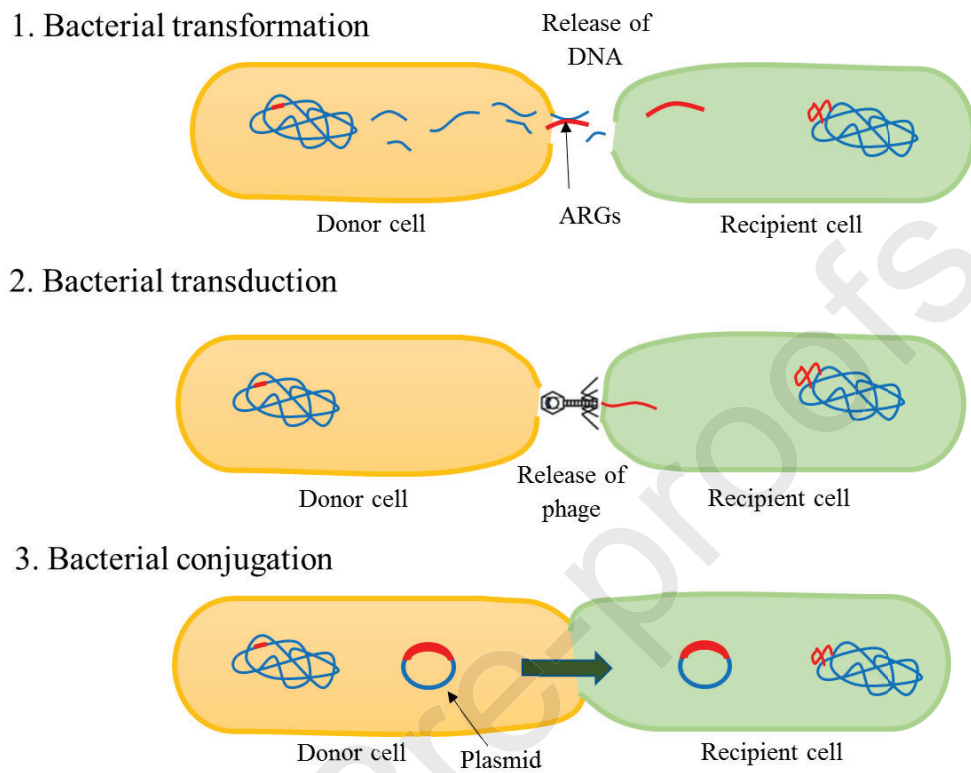
3. Bacterial conjugation

Fig. 2 Mechanisms of horizontal gene transfer among bacteria (modified from Furuya and Lowy (2006))

Table 1 Intrinsic resistance mechanisms and hosts of ARGs and their shift in anaerobic treatment processes

Classes of antibiotics	Antibiotic resistance mechanism							Hosts[a]	References
	Efflux pumping protection		Target site protection			Antibiotic inactivation			
	Increased	Decreased	Increased	Decreased	Unchanged	Increased	Decreased		
Tetracyclines	tetA		tetW						(Tao et al., 2014)
	tetA,			tetW, tetO, tetL,	tetC, tetM		tetX	Clostridium, Bacteroides, Flavobacteriacea,	(Wang et al., 2017b)
	tetA, tetG		tetW, tetO				tetX	Paenibacillus,	(Wu et al., 2016)
	tetG		tetM				tetX	Streptococcus,	(Sui et al., 2017)
	tetG		tetM				tetX	Bacillus,	(Sui et al., 2016)
	tetC, tetG		tetM	tetO, tetQ, tetW				Unclassified	(Cheng et al., 2016)
Sulfonamides	sul1, sul2							Bacteroides, Acinetobacter, Bacillus,	(Sui et al., 2016)
	sul1, sul2							Aeromonas, Unclassified	(Tao et al., 2014)
Macrolides		mefA		ermB, ermF		ereA		Bacteroides, Bacillus,	(Sui et al., 2016)
		mefA		ermB,		ereA		Aeromonas,	(Sui et al., 2017)
	ermF			ermB				Unclassified	(Wu et al., 2016)

[a]: (Aydin et al., 2015b; Forsberg et al., 2014; Sun et al., 2016)