Problematic effects of antibiotics on anaerobic treatment of swine wastewater

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Abstract

Swine wastewaters with high levels of organic pollutants and antibiotics have become serious environmental concerns. Anaerobic technology is a feasible option for swine wastewater treatment due to its advantage in low costs and bioenergy production. However, antibiotics in swine wastewater have problematic effects on micro-organisms and the stability and performance of anaerobic processes. Thus, this paper critically reviews impacts of antibiotics on pH, COD removal efficiencies, biogas and methane productions as well as the accumulation of volatile fatty acids (VFAs) in the anaerobic processes. Meanwhile, impacts on the structure of bacteria and methanogens in anaerobic processes are also discussed comprehensively. Furthermore, to better understand the effect of antibiotics on anaerobic processes, detailed information about antimicrobial mechanisms of antibiotics and microbial functions in anaerobic processes is summarized in this review. Future research on deeper knowledge of the effect of antibiotics on anaerobic processes are suggested to reduce their adverse environmental impacts.

Keywords: Antibiotics, Anaerobic processes, Swine wastewater, Impacts, Micro-organisms

1. Introduction

In the last few decades, swine farming has changed from small family farms to large concentrated animal feeding operations (CAFOs) to increase pig production at lower cost (Moses and Tomaselli, 2017). According to the research conducted by the Worldwatch Institute, CAFOs now account for 55% of pork production worldwide. However, intensive pig production requires frequent use of antibiotics for disease control and growth promotion. Consequently, large amounts of wastewater with high concentrations of organic pollutants, antibiotics and other toxicants was produced by

CAFOs (Cheng et al., 2018). Anaerobic treatment technology has been widely used to treat swine wastewater (the concentrations of COD, NH_3-N, TN and TP are 3000-15,000, 400-1400, 600-2100 and 100-250 mg/L, respectively) (Cheng et al., 2018). As a cost-effective technology, anaerobic treatment consumes less energy to remove pollutants and generate methane rich biogas (Guo et al., 2015b; Sui et al., 2017). Besides, the smaller amounts of sludge production in anaerobic processes also minimize the risks associated with discharging antibiotics and antibiotic resistance genes in sludge to the environment (Xiong et al., 2017).

However, we cannot ignore the possibility that antibiotics in swine wastewater could pose serious problems to micro-organisms in anaerobic processes. They could reduce the microbial activities or change microbial populations, influencing the pollutants removal and biogas production (Ji et al., 2013). Currently, some studies have confirmed that the introduction of antibiotics influences the performance of anaerobic systems (Loftin et al., 2005; Poels et al., 1984). Others conclude that the presence of antibiotics in the anaerobic processes could result in a changed microbial structure by shifting microorganisms to less sensitive ones to specific antibiotics or by developing strains with antibiotic resistant genes (Angenent et al., 2008; Shimada et al., 2008).

The most frequently detected classes of antibiotics in swine wastewater are sulfonamides, tetracyclines and macrolides, with the concentration of 324.4, 388.7 and 72 μg/L, respectively (Cheng et al., 2018; Li et al., 2017; Tong et al., 2009; Wei et al., 2011). These antibiotics have different modes of action on microorganisms, like interfering with the functions of cell membranes, blocking protein synthesis and preventing nucleic acid synthesis (Walsh, 2003). In anaerobic processes, several microbial groups convert complex organic compounds to simple, chemically stabilized ones. The coaction of bacteria and methanogens is critical to high-rate and stable

anaerobic treatments (Aydin et al., 2015e; Town et al., 2014). The presence of antibiotics during the anaerobic treatment process could disrupt the balance. In this case, the accumulation of metabolic intermediates like VFAs would seriously inhibit the anaerobic treatment efficiency (Ji et al., 2013).

Considering the prevalence of anaerobic technologies for treating swine wastewater and the adverse effect of antibiotics on the environment, researchers started to investigate the performance of anaerobic treatment processes in removing antibiotics and their corresponding resistance genes (Cheng et al., 2018). In order to improve the treatment performance, the inhibition effect of antibiotics on the anaerobic treatment processes has aroused great concern. In this review paper, the impact of antibiotics on the treatment performance of anaerobic systems and the shift of microbial communities will be discussed respectively. It will help greatly to improve the stability and efficiency of the anaerobic treatment of swine wastewater with antibiotics, and reduce the emission of these antibiotics to the environment. Additionally, this review is helpful for understanding: firstly, the microbial relationships in anaerobic processes; and secondly, the impacts of antibiotics on the dynamics of anaerobic microbial communities.

1.1 Antibiotic classes and antimicrobial mechanisms

Based on the antimicrobial mechanisms, the classifications of antibiotics are summarized in Fig. 1 (Cheng et al., 2018; Kapoor et al., 2017). The classes of sulfonamides, tetracyclines and macrolides are bacteriostatic antibiotics. They limit the growth of microorganisms by interfering with their protein production, DNA replication, or other aspects of cellular metabolism, but do not necessarily kill them. Meanwhile, β-lactam antibiotics like amoxicillin and penicillin have the ability to kill microorganisms by inhibiting cell wall synthesis or inhibiting enzymes or protein translation (Kohanski et al., 2010). Nonetheless, there is not always a precise distinction

between bacteriostatic and bactericidal antibiotics. Some high concentrations of bacteriostatic antibiotics also may kill microorganisms (Ocampo et al., 2014).

Antibiotics with different modes of action are known to have various effects on micro-organisms in anaerobic treatment processes. In particular, sulfonamides inhibit the synthesis of folic acid required for synthesis of purines and nucleic acid by preventing the addition of para-aminobenzoic acid into the folate molecule through competing for the enzyme dihydropteroate synthetase. Tetracyclines and macrolides inhibit protein synthesis by reversibly binding to receptors on the 30S and 50S ribosomal subunit of microbes (Tenover, 2006). The ribosomes of archaea and bacteria are relatively dissimilar in terms of size and shape, and consist of between 50 and 70 proteins depending on the species. They incorporate three rRNA molecules: 16S, 23S and 5S rRNA. Some archaeal r-proteins are also closer in characteristics to the eukaryote ribosomal proteins (Ramirez et al., 1993). All cells require folic acid, but as it cannot enter bacterial cells, they have to manufacture folic acid themselves. Sulfamethoxazole (SMX) inhibits a critical enzyme, dihydropteroate synthase, and therefore restricts the growth of the bacteria (Hong et al., 1995). These antibiotics impact only on bacterial ribosomal proteins and bacterial cells and do not affect archaea ribosomal proteins and cells (Aydin et al., 2015c). Conversely, β-lactams are able to inhibit bacterial cell wall synthesis, and then lead to the death of bacterial cell due to osmotic instability or autolysis (Kohanski et al., 2010).

1.2 Microbial functions in anaerobic processes

Under the action of micro-organisms, anaerobic processes are divided into four phases: hydrolysis, acidogenesis, acetogenesis, and methanogenesis, as shown in Fig. 2. Micro-organisms in each phase co-operate with each other to convert organic materials to methane and CO_2 in a step-wise reaction (McInerney et al., 2009). Hydrolysis and

acidogenesis are the initial phases of anaerobic digestion. Hydrolytic bacteria degrade complex polymers like carbohydrates, proteins and fats into sugars, amino acids and long chain fatty acids, respectively. Subsequently, this phase is followed by the action of acidogens, which convert water-soluble chemical substances and end products of hydrolysis to short-chain organic acids (formic, acetic, propionic, butyric, and pentanoic), methanol, ethanol, CO_2, and H_2 (Ali Shah et al., 2014). Acetate, H_2, CO_2, and methyl compounds can be directly used by methanogens, while other intermediates formed via acidogenesis, such as propionate, butyrate, isobutyrate, valerate, isovalerate, and ethanol, have to be further bio-degraded through syntrophic acetogenesis to form acetate, H_2, and CO_2 before methanogens utilize them to produce methane (Venkiteshwaran et al., 2016).

As described in Fig.3 (Ariesyady et al., 2007; González-Fernández & García-Encina, 2009; Guo et al., 2015a; Lang et al., 2015; Sánchez-Andrea et al., 2014; Stone et al., 2009; Suhadolnik et al., 2017; van de Werken et al., 2008; Vanwonterghem et al., 2016; Wang et al., 2017a; Wang et al., 2016), bacteria species in *Firmicutes, Bacteroidetes, Thermotogae, Proteobacteria, Actinobacteria, Chloroflexi* and *Spirochaetes* phyla are the function bacteria in anaerobic processes (Ali Shah et al., 2014; Guo et al., 2015a; Venkiteshwaran et al., 2016; Yang et al., 2014). In particular, within the previously mentioned phyla, the genera of *Caldicellulosiruptor*, *Butyrivibrio*, *Acetivibrio*, *Anaerococcus*, *Clostridium*, *Spirochaeta*, *Halocella and Bacteroides* have the ability to degrade cellulose-, hemicellulose- and pectin-containing biomass to acetate, CO_2, and hydrogen or ethanol (Blumer-Schuette et al., 2008; van de Werken et al., 2008). The genera of *Bacteroides*, *Escherichia*, *Thermotoga* and *Halothermothrix* can convert particulate materials like carbohydrates, protein and animal fats into dissolved materials. The microbes in *Proteobacteria phylum* also play important roles in

anaerobic digestion process, because most of *them* are well known propionate, butyrate, and acetate-utilizing microbial communities (Ariesyady et al., 2007).

As is already known, the accumulation of VFAs can destroy methanogens activity and result in the failure of anaerobic processes. Thus, converting intermediary metabolites into acetate and other substrates used by methanogens is important to retain the balance of anaerobic processes. Some members belonging to the *Firmicutes*, *Proteobacteria* -and *Chloroflexi* phyla, like *Syntrophomonas*, *Streptococcus*, *Pelotomaculum*, *Syntrophobacter*, and *Smithella* are syntrophic bacteria. They can degrade various VFAs into acetates and hydrogen used by methanogenic bacteria (González-Fernández & García-Encina, 2009; Schink, 1997; Stone et al., 2009; Wang et al., 2017a). However, the release of hydrogen may be toxic to the microbial community at this stage, since the build-up of hydrogen partial pressure to more than 10^{-4} atm will inhibit the destruction of propionate and butyrate intermediates (Venkiteshwaran et al., 2016). Therefore, a symbiosis between acetogenic bacteria and autotrophic methane bacteria which consume hydrogen to produce methane is necessary (Ali Shah et al., 2014).

The last phase of anaerobic processes is occupied by methanogenic archaea, which degrade the products of previous phases, such as acetic acid, H_2, CO_2, formate, methanol, methylamine, or dimethyl sulphide to methane (Ali Shah et al., 2014). During this phase, species mainly belong to four phylogenetic orders of methanogens, namely, *Methanomicrobiales*, *Methanobacteriales*, *Methanococcales*, and *Methanosarcinales.* They are known to be responsible for the production of methane (Demirel & Scherer, 2008). There are mainly three recognized methanogenic pathways, these being acetoclastic, hydrogenotrophic and methylotrophic pathways (Vanwonterghem et al., 2016). The acetoclastic pathway is an extremely important one for methane production

185 (70%) in anaerobic digestion processes (Stams et al., 2012). It is executed by members
186 of the genera *Methanosaeta* and *Methanosarcina*, which are known to use acetate for
187 methanogenesis. *Methanosaeta* is a specialist that uses acetate exclusively,
188 while *Methanosarcina* is a relative generalist genus that can utilize methanol,
189 methylamine and acetate as well as hydrogen and carbon dioxide for methane
190 production (Guo et al., 2015a). For hydrogenotrophic methanogenesis, members of
191 *Methanospirillum, Methanoculleus* and *Methanoregula* genera can reduce CO_2
192 successively to methane with H_2 as the primary electron donor, and formate through a
193 series of intermediates, including formyl, methylene, and methyl levels. To maintain the
194 stability of anaerobic systems, these species play a crucial role in maintaining the very
195 low partial pressures of H_2 necessary for the syntrophic communities of bacteria and
196 archaea to function properly (Stams et al., 2012). Among the above three pathways,
197 methylotrophic methanogens have the smallest number of species belonging to
198 *Methanosphaera*, *Methanococcoides*, *Methanohalophilus* and *Methanolobus* genera
199 (Vanwonterghem et al., 2016).

200 **2. Impacts of antibiotics on the performance of anaerobic processes**

201 Anaerobic processes are widely applied to the treatment of swine wastewater
202 (Cheng et al., 2018). However, the inhibition effects of antibiotics on the performance
203 of anaerobic treatment processes have been recently documented (Álvarez et al., 2010;
204 Aydin et al., 2015c; Aydın et al., 2015). As reported earlier (Table 1), the antibiotics
205 reveal a wide range of differences regarding the performance of anaerobic processes.
206 This is due to the variations of antibiotics concentrations and types as well as the
207 combination of different antibiotics (Mitchell et al., 2013; Ozbayram et al., 2015).

208 **2.1 Impact on pH**

209 The level of pH is an important process parameter during anaerobic treatment
210 processes. It should be noted that both acidogenic and methanogenic micro-organisms
211 have their optimal pH value. Failing to maintain the optimal range of pH (6.7-7.4) in the
212 anaerobic reactor could break down the anaerobic systems (Chen et al., 2008; Lay et al.,
213 1997). The presence of antibiotics in the anaerobic reactors could result in the
214 accumulation of VFAs, which would cause a decrease in the pH value (Beneragama et
215 al., 2013; Ma et al., 2013). For example, Shi et al. (2011) and Aydin et al. (2015b)
216 indicated that the pH value in reactors with antibiotics decreased more than that in
217 control reactors. In the pig slurry anaerobic digestion process, the pH of the reactor with
218 chlortetracycline (CTC) was lower than that in the control reactor (Stone et al., 2009).

219 Additionally, it is notable that the pH value in the anaerobic reactor was sensitive
220 to high concentrations of antibiotics. As reported by Miller et al. (2013), the pH value
221 remained stable, which maintained between 7.4 and 7.6, with the addition of 1-5 mg/L
222 SMX to the reactor, however, the value of pH decreased to 6.3 immediately in response
223 to an increase in VFAs after 50 mg/L of SMX was added. Also demonstrated that the
224 pH value did not decrease from neutral (6.8–7.2) to 5.9 until the concentration of SMX
225 and tetracycline (TC) was up to 20 and 1.5 mg/L, respectively. Thus, the stability of
226 anaerobic reactors could be effected by adding high concentrations of antibiotics.
227 However, the sharp decrease of pH might only happen at the beginning of the anaerobic
228 process with a short contact time between antibiotics and the anaerobic sludge, which
229 would become stable as the reaction progressed. This has been confirmed by the report
230 of Beneragama et al. (2013) and Shi et al. (2011), who stated that the presence of
231 antibiotics (cefazolin and OTC) wielded no effect on the pH value during anaerobic
232 digestion processes after a sharp decrease of pH in the beginning. As well, the study

about effects of antibiotics on anaerobic digestion of swine slurry showed similar results, since the pH in bioreactors with antibiotics did not show any difference with the control reactors at the end of the treatment cycle (Masse et al., 2000). The reason might be that the short contact time between high concentrations of antibiotic and the anaerobic sludge can promote the organic acid production (acidogens) which results in the accumulation of organic acids due to the failure of methanogens to convert the organic acids to methane (Ma et al., 2013). Such results demonstrate that anaerobic treatment processes are sufficiently buffered to minimize pH fluctuations and have undergone digestion without a failure.

2.2 Impact on COD removal efficiencies

The COD removal efficiency could be affected by the presence of antibiotics in anaerobic treatment processes, which is significantly related to the concentrations of antibiotics (Aydin et al., 2015b). Previous research concluded that removing efficiencies of COD in anaerobic treatment processes can be significantly inhibited by high concentrations of antibiotics. For example, the COD removal efficiency only decreased from 97.8 ± 2.5% to 92.9 ± 1.3% as the addition of SMX from 1 to 40 mg/L. However, the ASBR system began to collapse when 45 mg/L of SMX was added with only 25.0 ± 1.1% of COD elimination (Cetecioglu et al., 2016). Sponza and Demirden (2007) also reported that the COD removal efficiencies in an upflow anaerobic sludge blanket reactor (UASB) reactor only varied from 89% to 82% when the sulfamerazine concentration rose from 10 to 65 mg/L. Once the concentration increased to 90 mg/L, only 68% of COD could be removed.

Meanwhile, tetracycline antibiotics reveal similar inhibition trends with sulfonamide antibiotics in the anaerobic processes. Cetecioglu et al. (2013) indicated that TC had no effect on the removal efficiency of COD in an ASBR reactor with the

concentration ≤5.7 mg/L, while adding higher dose of TC (8.5 mg/L) in the reactor resulted in a significant inhibition of the overall COD removal efficiencies (only 9%) compared with that in the control reactor (≥ 96%), and the metabolic activity of the biomass could not be reactivated at the end of the operation. Xiong et al. (2017) also reported that the COD concentration in the effluent of the anaerobic process was not affected in the presence of tetracycline with the concentration less than 150 μg/L, but increased in greater amounts when adding 20 mg/L of TC.

Frequently, however, antibiotics are present as mixtures in the environment. The effects of mixtures differ from the individual compounds, due to the antagonistic and/or synergistic effects among them (Ozbayram et al., 2015). For instance, Aydin et al. (2015c), Aydin et al. (2014) and Aydin et al. (2016) indicated that the combination of antibiotics (erythromycin-tetracycline-sulfamethoxazole (ETS), sulfamethoxazole-tetracycline (ST), erythromycin-sulfamethoxazole (ES) and erythromycin-tetracycline (ET)) had more serious inhibition than the individual antibiotics on the COD utilization and methane production. As well, the study also indicated that the joint inhibition of combined antibiotic on the performance of anaerobic treatment was more serious at higher concentrations (Aydin et al., 2015b; Aydin et al., 2015c). In particular, Aydin et al. (2015b) concluded that the COD removal efficiency was not significantly affected if the added concentration of SMX and TC mixtures was less than 10 mg/L, whereas, the performance of the reactor declined substantially after 30 mg/L of the SMX and TC mixture was added to the reactor. The authors also concluded that less than 17 mg/L of ETS did not exert a noticeable effect on the overall COD removal efficiency, while the effluent soluble COD concentration would increase to more than 2000 mg/L (82 ± 21.2 mg/L in control reactor), corresponding to an overall COD reduction of only 10% when 46 mg/L of ETS were added. What is worse, such inhibition cannot be balanced since

283 the metabolic activity of the biomass could not be reactivated to induce noticeable
284 substrate utilisation.

285 As discussed above, it is notable that the inhibitory influence of antibiotics on the
286 COD removal efficiencies of the anaerobic process resulted in the accumulation of
287 soluble COD in reactors. This is most likely due to the presence of antibiotic
288 stimulated activities of fermentative or acid-forming bacteria in the anaerobic reactors
289 which produce more soluble COD. In addition, the utilization of the soluble COD
290 might decrease due to the inhibition of antibiotics on the interrelated activities of
291 hydrogen producing acetogenic bacteria, methane producing aceticlastic methanogens,
292 and or a combination of all these processes (Stone et al., 2009).

293 **2.3 Impact on biogas and methane production**

294 Biogas and methane generation is the inherent outcome of COD removal under
295 anaerobic conditions, their production is parallel to the effluent COD concentration of
296 anaerobic treatment processes. As the terminal end-products produced from
297 biodegradable materials, biogas and methane production is good indicators of digestive
298 performance (Boe et al., 2010). However, the effect of antibiotics on methane and COD
299 might be different. As reported earlier, the addition of TC started to inhibit biogas
300 production with the dosing of 1.65 mg/L in an ASBR despite achieving complete COD
301 removal. Lu et al. (2016) also reported that the presence of TC (250 μg/L) in an
302 anaerobic baffled reactor (ABR) had less influence on the degradation of organic matter
303 but had a strong influence on biogas generation and the accumulation of VFAs. This
304 might be the reason that the methanogenesis process was sensitive to TC presence, but
305 the acidogenesis process was insensitive, so that only part of the substrate is utilized for
306 methane production.

Similarly, there is a wide range of inhibition from complete biogas inhibition to slight biogas enhancement based on the antibiotic types and concentrations. For example, Sanz et al. (1996) indicated that the presence of penicillin and TC in swine slurries reduced methane production by 35% and 25%, respectively. However, other antibiotics in slurries from pigs, including tylosin, lyncomycin, sulfamethazine, and carbadox, did not significantly affect methane production. In addition, higher concentrations of antibiotics showed more serious influence on methane production. This is explained by their bactericidal characteristics that kill bacteria instead of inhibiting bacterial growth at high concentrations, which is very different to bacteriostatic characteristics at lower concentrations (Bauer et al., 2014; Shi et al., 2011). According to the report by Cetecioglu et al. (2015) and Mohring et al. (2009), biogas production was not affected significantly by adding sulfonamide antibiotics (less than 18 mg/L) in the anaerobic system; however, the inhibition became noticeable with the concentration of SMX higher than 45 mg/L.

Compared to other types of antibiotics, CTC and OTC showed a relatively serious effect on the biogas and methane production in anaerobic digesters (Wang et al., 2016; Yin et al., 2016). As reported by Sun et al. (2012), CTC was the most significant inhibitor among the antibiotics of CTC, amoxicillin, florfenicol, and sulfamethazine. In contrast, doxycycline (DC) showed less activity and resulted in a 25%-45% reduction on methane production with the concentration of 10-100 mg/L. Stone et al. (2009) stated that the CTC (27mg/L) reactor inhibited the production rate of methane and resulted in 27.8% reduction of total production, although total methane production increased in all anaerobic reactors of swine slurry. Sanz et al. (1996) also revealed that CTC was a powerful inhibiter for acetoclastic methanogenic bacteria with 50% reduction of methane at 40 mg/L of CTC concentration. Similar to CTC,

oxytetracycline (OTC) indicates a significant effect on methane production, and inhibition increased with the growing concentration of OTC (Ince et al., 2013). For instance, Arikan et al. (2006) revealed that OTC could reduce methane production by 27% even at 3.1 mg/L in the anaerobic digestion system. Xin et al. (2014) also indicated that the generation rate of methane and total production of biogas during the anaerobic digestion process decreased with the concentrations of OTC stepwise increasing from 0, 20, 50 to 80 mg/L. As well, the methane production fell by 56%, 60% and 62% at OTC and CTC concentrations of 10, 50 and 100 mg/L during the anaerobic digestion of pig slurry, respectively (Álvarez et al., 2010). Similarly, a highly significant decrease in methane production in the presence of OTC (around 3 mg/L) was also observed in two-phase and single-phase digesters of cattle manure, with 43% and 52% lower than control digesters, respectively. Interestingly, higher methane yields and OTC reductions were achieved with two-phase configuration, due to much higher cell activity was observed than that in single-phase digester (Akyol et al., 2016). This could be explained that CTC and OTC are effective against both gram-negative and gram-positive organisms by inhibiting bacterial protein synthesis through binding to the 30S ribosomal subunit. Therefore, at high CTC and OTC concentrations, they would impose a significant inhibition effect on the activity of microorganisms in anaerobic treatment processes (Beneragama et al., 2013; Wang et al., 2016).

As for tylosin, its inhibition behavior was only obvious at higher concentrations. It may be attributed to its limited spectrum of activity toward gram-negative bacteria, although tylosin is active against many gram-positive bacteria (Sanz et al., 1996). In an anaerobic sequencing batch reactor (ASBR), Angenent et al. (2008) and Shimada et al. (2008) both indicated that tylosin at 1.6 and 1.67 mg/L had negligible effects on the total methane production and propionate degradation, though the rate of both decreased.

Nonetheless, a reduction of methane production occurred after its concentration increased to 167 mg/L, because its high inhibition on propionate- and butyrate-oxidizing syntrophic bacteria and fermenting bacteria resulted in unfavourable effects on methanogenesis. The inhibition of high concentrations of tylosin on the anaerobic treatment process was also confirmed by Mitchell et al. (2013), who wrote that tylosin at a concentration of less than 100 mg/L did not show any inhibition on the total production of biogas, while as the concentration increased stepwise to 130, 260, 520 and 913 mg/L, biogas production reduced by 10%, 20%, 30% and 38%, respectively. However, penicillin showed inhibitions on the methane production at all concentrations evaluated since this type of antibiotics can inhibit the cell wall synthesis (Rodríguez et al., 2017). Specifically, masse et al. (2000) found that bioreactors with penicillin or TC experienced a significant decrease of methane production (35% and 25%, respectively), with respect to the control ones. Gartiser et al. (2007) also discovered that amoxicillin, a comparable penicillin antibiotic, lowered biogas production by 10% and 20% with 12.3 and 95.9 mg/L amoxicillin added in the reactor.

Again, the combination of antibiotics demonstrated higher inhibition on the methane production than the individual antibiotic. For example, Álvarez et al. (2010) reported the significant inhibition of anaerobic digestion in the swine slurry containing a combination of CTC and OTC at concentrations of 10, 50 and 100 mg/L; methane production reduced by 56%, 60% and 62%, respectively. Aydin et al. (2015b) also reported that the biogas and methane production were almost completely inhibited by the combined antibiotics of ERY, TC and SMX. The research conducted by Aydin et al. (2015a), Aydin et al. (2015b), and Ozbayram et al. (2015) showed the synergistic and antagonistic effect between different antibiotics. In the mixture of ETS, SMX can have an antagonistic effect on ERY and TC, however, the synergistic effect was observed in

almost all antibiotic mixtures that included TC as a component (Aydin et al., 2015b; Aydın et al., 2015).

However, some reports concluded that antibiotics had no or only a limited effect on the production of biogas even at high concentrations (Chelliapan et al., 2011; Mitchell et al., 2013). As described previously, SMX did not inhibit biogas production at 6-100 mg/L (Gartiser et al., 2007). The author also stated that the reactor with sulfamethazine and ampicillin up to 280 mg/L and 350 mg/L exhibited no impact on total biogas production compared with the control reactor, although they did inhibit biogas production rates during early stages of anaerobic digestion. Similarly, Lallai et al. (2002) demonstrated that OTC (125 and 250mg/L) had no varied effect on methane production in the anaerobic digestion of swine slurry in comparison to the control value. They concluded that both the acid-forming and methane-forming microbes were not affected by the presence of OTC. Chelliapan et al. (2011) also found negligible biogas inhibition with 100–800 mg/L tylosin in an up-flow anaerobic stage reactor (UASB). The reason for the above varied results may be attributed to the differences in the histories of sludge used, the acclimatization period, the microbial structures and the operational conditions. Álvarez et al. (2010) reported that the less inhibitive behavior of sulfonamides was due to their reduced antibacterial activity resulting from the large size of fresh inoculum and the complexity of the sludge (inherently including endogenous protein-degradation products). These organic compounds prevented sulfonamides from causing bacteriostasis on susceptible bacteria. It is known that methanogens are responsible for producing methane in an anaerobic treatment process. As advances are made in the anaerobic digestion process, the methanogen populations become more established, which leads to more methane being produced.

406 Moreover, according to the review paper by Chen et al. (2008), higher
407 concentrations of antibiotics could be tolerated after a period of adaptation by microbes
408 in anaerobic processes. Nevertheless, certain concentrations of antibiotics would exert
409 some pressure on methanogens at the beginning, so methanogens had to undergo an
410 acclimatization phase (Beneragama et al., 2013). Acclimatization is a selection and
411 multiplication of specialized microorganisms capable of biodegrading or co-
412 metabolizing the compounds or only surviving in the presence of relevant compounds
413 after a certain adaptation time. Hence, the acclimatization phase was considered vital
414 for the biodegradation of antibiotics and their impact on the performance of anaerobic
415 treatment processes (Wang et al., 2017b). As a result, the antibiotics might inhibit the
416 initial methane production, but this inhibition could be recover following the
417 acclimatization period. This has been confirmed by Masse et al. (2000), the inhibition of
418 antibiotics on methane production decreased because the reactors exhibited patterns of
419 recovery or acclimation as time progressed.

420 The historical sludge and the acclimatization phase might therefore be potentially
421 influential factors in the impact of antibiotics on methane production, and less inhibition
422 on methane production was noted in sludge including a historical sludge with multiple-
423 antibiotic used before (Huang et al., 2014). For instance, the absence of inhibition of
424 antibiotics on the production of methane and treatment efficiency of anaerobic
425 processes, reported by Loftin et al. (2005) and Dreher et al. (2012), might be the
426 consequence of the acclimation experienced by their microbial consortia, since it has
427 adapted to the presence of antibiotics. García-Sánchez et al. (2016) also revealed that
428 for biomass that had no contact with an antibiotic, the presence of tylosin inhibited the
429 generation of methane even at concentrations as small as 0.01 mg/L. Whereas, in the
430 digesters acclimating the presence of tylosin at a concentration of 0.01 - 0.065 mg/L,

methanogenesis was not inhibited and the generation of methane improved. It may imply that the microorganisms have developed not only a resistance to the antibiotic but also the ability to metabolize it.

The increasing concentrations of antibiotics do not always cause the elevated inhibition of methane production; conversely, the use of an appropriate dose of antibiotics could aid in enhancing methane production due to their metabolism by microorganisms (Sponza & Demirden, 2007). The authors indicated a relatively high concentration of sulfamethazine could increase the total amount of methane production. In the UASB reactor, with the addition of 10 and 90 mg/L of sulfamerazine, the daily methane gas production was recorded as 1558 ml and 2000-2275 ml, respectively, although the maximum methane percentage decreased from 76% to 60%. Yin et al. (2016), Lu et al. (2014) and Yin et al. (2015) also demonstrated the beneficial role of CTC, OTC, cefalexin and colistin sulfate in methane production in anaerobic treatment. These results might be attributed to the utilization of antibiotics as co-substrates together with glucose-COD, which were used as primary carbon and energy sources for micro-organisms in the anaerobic processes (Sponza & Demirden, 2007).

2.4 Impact on the accumulation of VFAs

VFAs, as short chain fatty acids that include formate, acetate, propionate, butyrate etc., are intermediate and or end products of the anaerobic process. Their accumulation is important for the performance of anaerobic systems (Lins et al., 2015). One research group observed that adding SMX, ERY, and TC at 15 - 20, 1.5, and 1.5 mg/L concentrations, respectively, could accumulate 400 - 600 mg/L of VFAs (Aydin et al., 2015d). Similarly, Cetecioglu et al. (2015) and Miller et al. (2013) stated that increasing SMX concentration to 45 mg/L and 50 mg/L could result in the accumulation of VFAs and the decrease of pH, alkalinity in anaerobic processes.

Stone et al. (2009) indicated the VFAs accumulation in the CTC (27mg/L) treatment reactor was the greatest about 37.4% - 47.0% more than the tylosin and control reactors, and the acetate concentration in the CTC treatment (12,269 mg/L) was greater than either the tylosin (7687 mg/L) or the no-antimicrobial control (6498 mg/L) treatments. This might be the reason that the generation of acetate through soluble organic fermentation is efficient, however, the utilization of acetate by either homoacetogenic bacteria or aceticlastic methanogens was inhibited by CTC. As well, both propionate and butyrate concentrations in the CTC and tylosin (1.67 mg/L) treatment reactor were greater than those in control, implying that CTC and tylosin inhibited propionate and butyrate degraders, such as the genera of *Pelotomaculum*, *Psychrobacter* and *Streptococcus* (as shown in Table 2). Similarly, Sanz et al. (1996) also indicated the butyrate degrading bacteria were affected by CTC at low concentration, and died as the concentration of CTC increased above 100 mg/L. This resulted in a greater accumulation of short-chained VFAs during the anaerobic digestion compared to a no-antimicrobial control system. The anaerobic reactors with OTC (30, 60, 90 mg/L) also exhibited an increasing trend in the accumulation of total VFAs compared to the control reactor (Beneragama et al., 2013).

The accumulation of VFAs showed a positive correlation with antibiotic concentrations. This has been confirmed by Aydin et al. (2015a), who revealed that the VFAs concentration increased in a linear manner with the increase of antibiotics concentration. Xiong et al. (2017) also indicated that TC had no impact on the accumulation of VFAs with the concentrations less than 150 μg/L, while a significant accumulation of VFAs and increase in propionate were observed in the reactor subjected to the highest concentration of TC (20 mg/L). The accumulation of VFAs

may result from the stimulation of the acidogenesis and/or the subsequent inhibition of VFAs degradation as well as methanogenic activities (Beneragama et al., 2013).

As well, the effect of a combination of antibiotics in swine wastewater is higher than the effect of the individual antibiotic. For example, Cetecioglu et al. (2012) examined the individual inhibitory effects of antibiotics on the ASBR process. They indicated that the VFA accumulation started from 25 mg/L within the tetracycline dosage and 250 mg/L within the SMX and ERY dosage. However, Aydin et al. (2015a) found VFAs started to accumulate even in the presence of very low concentration of antibiotic mixtures (1 mg/L of ET–ST). Their results indicate that antibiotic combinations have an effect on acetate, propionate and butyrate degradation pathways, leading to the accumulation of VFAs and soluble microbial products, which results in a decrease in the total methane production.

In summary, different classes of antibiotics reflect different impacts on the performance of anaerobic treatment processes related to their mode of actions. Generally, lower dose of antibiotics shows less impact on the stability, the removal efficiencies and the production of biogas and methane in anaerobic processes. In contrast, relatively higher concentrations of antibiotics cause toxic effects on microorganisms in the anaerobic process, affect the substrate removal and the biogas production, and even result in a total collapse of the reactors. The various combinations of antibiotics increase inhibition effects over the individual antibiotics. Additionally, the synergistic and antagonistic effects are also identified in the reactor within the antibiotic mixtures.

3. Impact of antibiotics on the microbial communities in anaerobic processes

The anaerobic system contains a complex microbial culture, and the conversion of organic compounds to methane is carried out by various microbial communities in acidogenic and methnogenic processes (Town et al., 2014). There is a significant correlation between the microbial community (both bacterial and archaeal) and the performance of anaerobic reactors such as COD removal efficiency, biogas production, and VFAs accumulation. Failure to maintain the stability of these microorganisms would result in a decrease in the performance and stability of anaerobic reactors (Aydin et al., 2015e; Beneragama et al., 2013; Cetecioglu et al., 2016). Based on all of the above, we can conclude that the presence of antibiotics has a negative effect on biogas and methane production, yet appears to have a positive effect on the accumulation of VFAs. This indicates that the methanogenesis process is sensitive to the exposure to antibiotics, while the acidogenesis process is not. As reported, the decrease in methane and biogas production was closely related to the disappearance of the acetoclastic methanogens represented by the *Methanosarcinales* order in the reactor with antibiotics (Aydin et al., 2015e). Venkiteshwaran et al. (2016) and Aydın et al. (2015) did report the change in the composition and diversity of the microbial community was linked to the performance of anaerobic reactors. Hence, in anaerobic treatment processes, a relatively higher balance and diversity in their bacterial communities resulted in a higher biogas and methane production. Nonetheless, the existence of antibiotics in anaerobic systems may change the structure of the microbial community (Xin et al., 2014; Table 2). The reason is that antibiotics in general, even those broad-spectrum medications, have their selective effects on various groups of microbes. As a result, the selective antibiotic effects alter the relative abundance of microbial species, and

526 subsequently interfere with the interactions among different species (Wang et al.,
527 2017b).

528 **3.1 Impact on the bacteria in anaerobic processes**

529 During the initial phase of anaerobic treatment processes, hydrolytic and
530 fermentative bacteria, belonging to *Bacteroidetes*, *Firmicutes*, *Thermotogae*,
531 *Actinobacteria* and *Spirochaetes* phyla (in Fig. 3) showed positive correlation with the
532 presence of antibiotics. For example, Cetecioglu et al. (2016) demonstrated that
533 *Clostiridum* species in the *Firmicutes* phylum which were known to produce lactic acid,
534 ethanol and volatile fatty acids, became dominant in the ASBR with SMX. As well, the
535 number of *Acinetobacter* species increased along with the rising concentration of SMX
536 (Cetecioglu et al., 2016). This may explain why *Acinetobacter* species were reported as
537 the hosts of sulfonamide resistance genes. Xiong et al. (2017) also found the changes in
538 microbial communities in anaerobic treatment processes when exposed to different
539 concentrations of TC. In the anaerobic reactor with 20 mg/L of TC, the relative
540 abundance of bacteria belonging to *Bacteroidetes*, *Spirochaetes* and *Firmicutes* phyla
541 increased significantly in comparison with the reactor with 150 μg/L of TC (Xiong et
542 al., 2017). Specifically, the VFAs producing bacteria increased significantly in the
543 reactor with the high concentration of TC, including *Clostridium aurantibutyricum*,
544 *Microbacter margulisiae*, *Porphyromonas pogonae*, *Treponema zuelzerae* and
545 *Proteiniphilum acetatigenes* (propionate-producing bacteria). Similarly,
546 *Spirochaetaceae*, in the phylum *Spirochaetes*, could ferment glucose to acetate, ethanol,
547 and small amounts of lactate which present an obviously increasing trend during the
548 anaerobic digestion reactors with the addition of CTC (Wang et al., 2017a). The
549 positive effect of antibiotics on some bacteria might relate to the contact time between

antibiotics and biomass. This was demonstrated by faster growth kinetics and a better adaption rate of antibiotics and the bacteria (Aydin et al., 2015c; Ma et al., 2013).

Yet, a negative relationship between antibiotics and the VFAs degrading bacteria in anaerobic processes was found by previous researchers. Aydin et al. (2016) indicated that *Bacteroidetes*, *Acinetobacter* and *Proteobacteria* were negatively affected by different antibiotic combinations present in the anaerobic reactor compared to the control samples. This is despite the fact that the population of the *Firmicutes* in the reactor did not significantly change in comparison to the control reactor according to antibiotic concentration (3.0 mg/L). Xin et al. (2014) and Akyol et al. (2016) also reported that the total microbial diversity decreased with the addition of OTC in the anaerobic digestion reactor, and a higher concentration of OTC would decrease the relative abundance of *Gammaproteobacteria* in *Proteobacteria* phylum and result in the disappearance of *Sphingobacteriaceae* in *Bacteroidetes* phylum, although the genus *Flavobacterium* revealed great resistance to the increase in antibiotic loadings, and existed throughout the entire digestion process. Bacterial phyla, including *Proteobacteria*, *Cloacimonetes*, *Ignavibacteriae*, and *Chloroflexi*, showed the significantly less number in the reactor with high concentrations of TC than those in the control reactor (Xiong et al., 2017). According to one study about the acute effects of antibiotics on syntrophic butyrate and propionate-oxidizing bacteria in ASBR, antibiotics could cause inhibitory effects on butyrate and propionate degradation bacteria, including the species of *Syntrophomonas*, *Syntrophospora*, *Syntrophobacter* and *Pelotomaculum* (Aydin et al., 2015a). As a result, the utilization of VFAs by bacteria is affected by the addition of antibiotics, and the propionic acid utilization is much more affected than butyric acid utilization.

3.2 Impact on methanogens in anaerobic processes

During the methnogenic phase, the impact of antibiotics on the structures of acetogenotrophic, hydrogenotrophic and methylotrophic methanogens could dominate the performance of the whole anaerobic treatment processes. As reported, the presence of antibiotics in the anaerobic reactors had adverse effects on acetogenotrophic methanogens but positive effects on the abundance of hydrogenotrophic methanogens. For instance, Xiong et al. (2017), Cetecioglu et al. (2013) and Wang et al. (2017a) concluded that the long-term exposure of TC and CTC to the anaerobic reactor had negative effects on the relative abundances of acetogenotrophic methanogens like *Methanothrix, Methanoculleus*, and *Methanobacterium* genus. However, an increase in the relative abundance of *Methanomassiliicoccus* and *Methanoculleus* (hydrogenotrophic methanogens) was observed. Similarly, the abundance of hydrogenotrophic methanogens, especially *Methanobacterium species*, and *methanogenic archeons* in the ASBR with the high concentration of SMX (40 mg/L) also became dominant through the operation, while the acetoclastic methanogens disappeared in the last phase (Cetecioglu et al., 2016; Shin et al., 2010). The order *Methanomicrobiales*, which utilizes hydrogen or formate as electron acceptors to produce methane, is the most abundant methanogenic group in the anaerobic digestion processes with high concentrations of OTC (Wang et al., 2016).

In addition, Aydin et al. (2015e) and Cetecioglu et al. (2015) also reported that the total number of methanogenic population was not affected in the processes with long-term exposure to high concentrations and combined antibiotics, probably due to the shift of the major pathway to hydrogenotrophic methanogenesis. Hydrogenotrophic methanogens were higher in substrate utilization rate, growth rate and cell yield to exposed toxic substances compared to acetoclastic methanogens (Aydin et al., 2015c).

The reason is that the inhibition effect of antibiotics on the acetoclastic methanogens makes homoacetogenic bacteria more competitive to transfer acetate to H_2 and CO_2, and this procedure would provide a substrate for hydrogenotrophic methanogens to produce methane. Consequently, *homoacetogenesis* coupled with *hydrogenotrophic methanogenesis* enables the microbial community to maintain the system's stability (Aydin et al., 2015a; Cetecioglu et al., 2016). The research reported by Aydin et al. (2015c) also confirmed that antibiotic combination did have a dramatic effect on the acetoclastic methanogens present in the ST and ETS reactors. In particular, as the ancestral form of methane production, hydrogenotrophic methanogenesis was reported to be the most widespread in all methanogenic orders (Bapteste et al., 2005).

Methanogenic archaea show more tolerance than bacteria when exposed to antibiotics which inhibiting protein synthesis like tetracyclines and macrolides (Aydın et al., 2015). The reason is that archaeal ribosomes have a heterogeneous protein composition, enabling them to adapt to harsh environmental conditions in comparison with bacterial ribosomes (Hilpert et al., 1981). Furthermore, sulfonamides are bacteriostatic inhibitors designed to prevent bacterial infections and therefore reveal a more obvious effect on bacteria than archaea (Aydin et al., 2015e). Thus, both bacteria and methanogenic archaea can be affected by the presence of antibiotics according to the classes and concentrations of antibiotics. Previous research revealed that even though some species in the anaerobic process were negatively affected by higher antibiotic concentrations, the surviving species continued the degradation of substrate and the production of methane (Cetecioglu et al., 2016).

4. Future perspectives

Anaerobic processes are widely applied to treat wastes from swine farms. So far, studies mainly focused on the occurrence, fate, and removal of antibiotics from swine

624 wastewaters through anaerobic processes (Cheng et al., 2018). However, the toxic
625 effect of antibiotics on micro-organisms in anaerobic processes should be given more
626 attention considering the important role of micro-organisms in these processes. In this
627 review, we can see the complicacy of microbial communities responding to antibiotics.
628 Hence, it is important and necessary to study the impact of antibiotics on the treatment
629 process and microorganisms in anaerobic processes, to understand the fate of antibiotics
630 in anaerobic processes, to know the removal mechanism of antibiotics by the anaerobic
631 treatment, and to improve their removal efficiencies from wastewater.

632 To date, there are still large gaps in our knowledge on the impact of antibiotics on
633 anaerobic processes. Only a few types of antibiotics, such as SMX, TC, CTC, etc., were
634 studied previously, whilst studies on other antibiotics in swine wastewater are very
635 limited. Furthermore, these analyses mainly focused on the potential inhibiting
636 mechanisms of individual antibiotic. However, we should not ignore the fact that
637 antibiotics do not appear in swine wastes individually, but rather together with many
638 other types of antibiotics and toxic pollutants (hormones and/or heavy metals) (Zhang et
639 al., 2017b). The current studies on the impact of the coexistence of different types of
640 antibiotics and metals on the anaerobic treatment processes only touch the tip of the
641 iceberg; much more investigation should be done in the future due to their synergistic
642 and or antagonistic effects (Aydin et al., 2015c; Guo et al., 2012; Zhang et al., 2017a).
643 In addition, only a few studies focused on the impact of the metabolites of antibiotics on
644 micro-organisms in anaerobic processes, and noted their potential toxic effects
645 (Baumann et al., 2015). Thus, further studies are needed to obtain more comprehensive
646 data about the impact of the combination of many antibiotics as well as their
647 metabolites on microbial communities in anaerobic treatment processes.

Furthermore, the proliferation and dissemination of antibiotic resistance genes in the anaerobic swine wastewater treatment process are the most serious threats of antibiotics to the environment and people's health. Therefore, to reduce the threats of antibiotic resistance genes in anaerobic treatment processes, more studies are urgently required to explore the relationship between antibiotics, microorganisms and antibiotic resistance genes.

5. Conclusion

The key conclusions in this review article are as follows:

- Higher doses and combined antibiotics revealed more inhibition effects on anaerobic processes than that of lower concentration and individual antibiotics.
- The VFAs degrading bacteria and acetoclastic methanogens were more sensitive to antibiotics than other hydrolytic bacteria, VFAs producing bacteria, and hydrogenotrophic methanogens in the anaerobic processes.

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954 **Figure Captions**

955 Fig. 1 Classes and antimicrobial mechanisms of antibiotics in swine wastewater
956 Fig. 2 Phases of the anaerobic digestion process
957 Fig.3 Microorganisms ((a) bacteria; (b) methanogens) and their functions in anaerobic
958 process

Table Captions

Table 1 Impact of antibiotics on the performance of anaerobic treatment processes

Table 2 Impact of antibiotics on the microbial communities in anaerobic processes

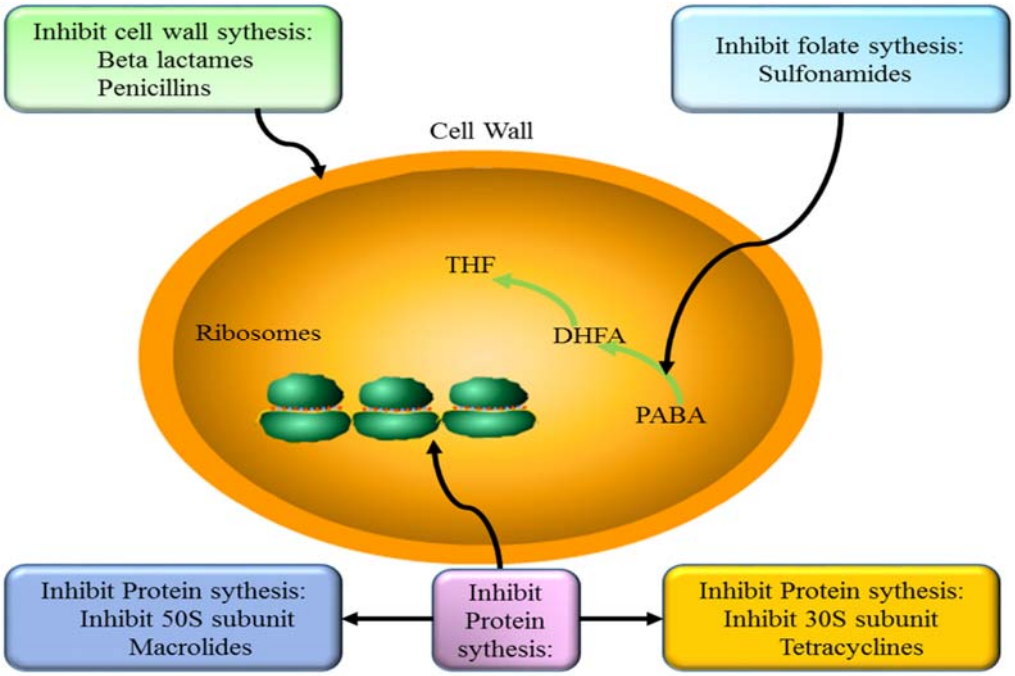

Fig. 1 Classes and antimicrobial mechanisms of antibiotics in swine wastewater

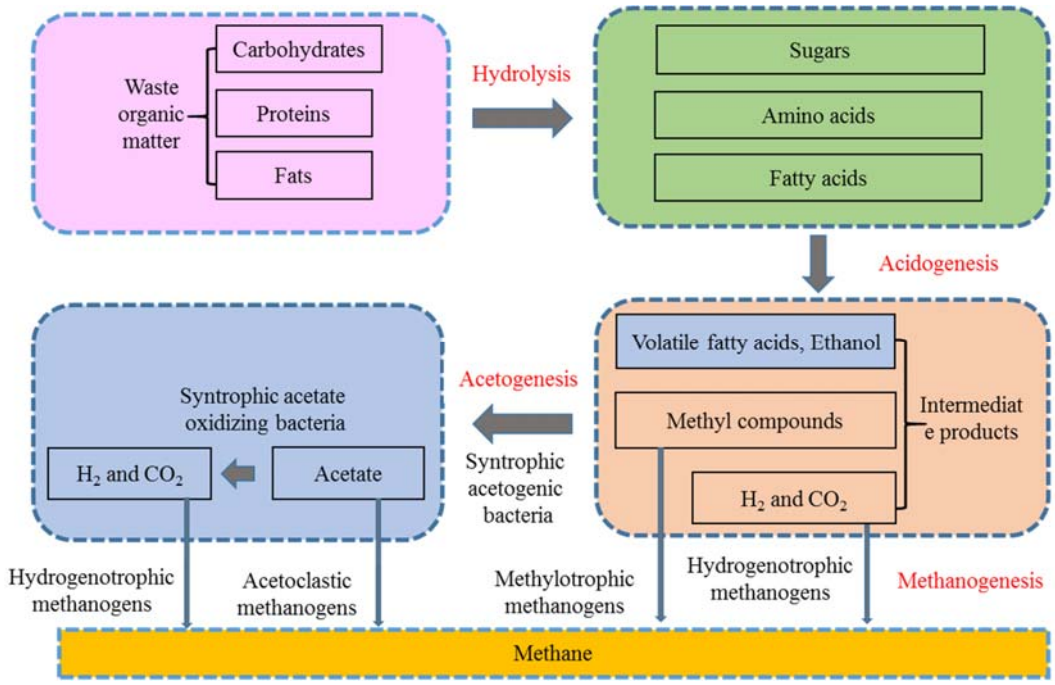

Fig. 2 Phases of the anaerobic digestion process

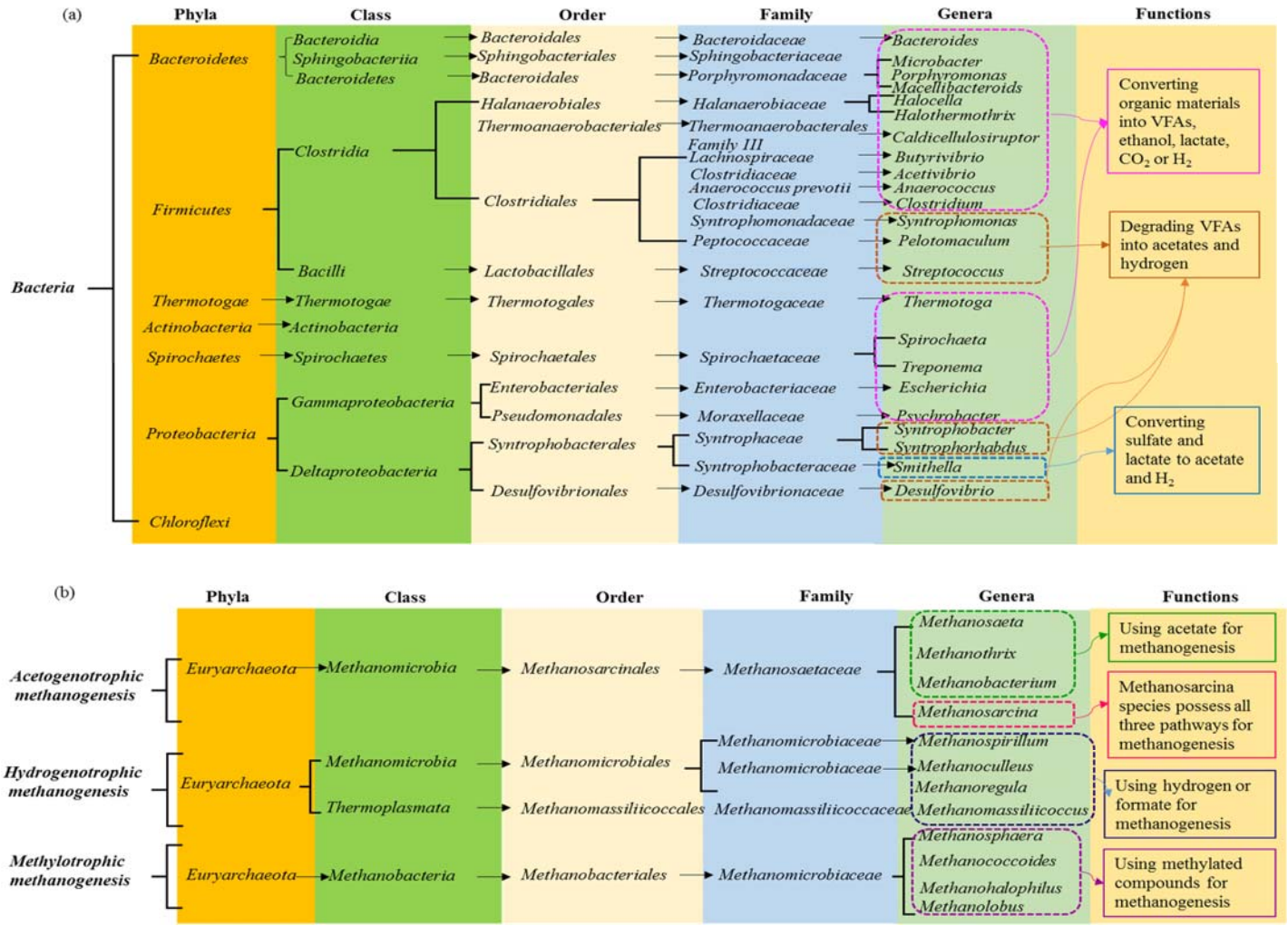

Fig.3 Microorganisms ((a) bacteria; (b) methanogens) and their functions in anaerobic processes

Table 1 Impact of antibiotics on the performance of anaerobic treatment processes

Antibiotic	Concentrations (mg/L)	Effects			References
		COD removal efficiencies	Biogas/methane production	VFAs accumulation (mg/L)	
Sulfamethoxazole	1–45	96.8 ± 2.6%–25.0 ± 1.1%	Biogas: 1004 ± 129 mL/d–96 mL/d	Propionic acid : not detected–438; Acetic acid: not detected–342	(Cetecioglu et al., 2016)
	6–100	–	No inhibition	–	(Gartiser et al., 2007)
Sulfamerazine	10–90	88%–68%	Methane content: 76%–60%	200–600	(Sponza & Demirden, 2007)
	⩽280	–	No impact on total biogas production	–	(Mitchell et al., 2013)
Sulfamethazine	5.0–160	–	Inhibited	Accumulated	(Sun et al., 2012)
Tetracycline	1.65–5.7–8.5	96%–9%–0%	Biogas: 951–853–71 mL/day	Acetic acid: not detected–457; Propionic	(Cetecioglu et al., 2013)

				acid: not detected–(750–385); Butyric acid: 4–20; Valeric acid: 14–70	
	0.15–20	Decreased	Methane: 160–110 mL	propionic acid: 0.7–44	(Xiong et al., 2017)
	550 mg/kg in pig diet	No statistically affected	25% reduction of methane	Not statistically affected	(Masse et al., 2000)
Chlortetracycline	500 mg/kg in pig slurry	Decreased	Increased	Accumulated	(Wang et al., 2017a)
	28	Decreased	27.8% reduction of methane	Accumulated	(Stone et al., 2009)
	40	–	50% reduction of methane	–	(Sanz et al., 1996)
Oxytetracycline	60, 100, 140 mg/kg in pig slurry	–	Reduce biogas production by 9.9, 10.4, and 14.1%	–	(Wang et al., 2016)
	30, 60, 90	–	79.1, 70.3, 68.6% of the control values	Increased	(Beneragama et al., 2013)

	125, 250	–	No inhibition	–	(Lallai et al., 2002)
	3.1	No significant effect	Reduce methane production by 27%	–	(Arikan et al., 2006)
Oxytetracycline – Chlortetracycline	10, 50 and 100	–	Reduced methane production by 56, 60 and 62%	–	(Álvarez et al., 2010)
Tylosin	130, 260, 520, 913	–	Reduced biogas production by 10, 20, 30, 38%	–	(Mitchell et al., 2013)
	1.67–167	Negligible effects to completely inhibited	Negligible effects to completely inhibited	Accumulated	(Shimada et al., 2008)
Amoxicillin	60, 120	–	75% and 68% of the control	–	(Lallai et al., 2002)
	12.3, 95.9	–	Reduced biogas production by 10% and 20%	–	(Gartiser et al., 2007)

Penicillin	16 mg/kg used in pig diets	No statistically affected	35% reductuin of methane production	not statistically affected	(Masse et al., 2000)
Erythromycin–Tetracycline (ET) combinations	(0.1 + 0.1)–(3+3)	90%–12%	Production rate decreased by more than 97%		(Aydin et al., 2016)
Sulfamethoxazole –Tetracycline	20 + 1.5	Decreased	–	–	(Aydin et al., 2015c)
	12–43	80%–10%	Biogas production: 1247 mL/day–0	–	(Aydin et al., 2015b)
Sulfamethoxazole – Erythromycin – Tetracycline	2.5 + 2.5 + 25	Decreased	–	–	(Aydin et al., 2015c)
	18– 46	75%–10%	Biogas production: 1247 mL/day–0; methane yield dropped from 0.32 L/g COD removed to 0	Acetic acid : 50–1000; Butyric acid: 140–710	(Aydin et al., 2015b)

Table 2 Impact of antibiotics on the microbial communities in anaerobic processes

Antibiotic	Concentrations (mg/L)	Microbies	Effects	References
Sulfamethoxazole	1–45	*Clostridium* *Acinetobacter* *Acetoclastic methanogenic* *Methanobacterium*, *Methanogenic archeons*	Decreased Increased Disappeared Higher than acetoclastic methanogens	(Cetecioglu et al., 2016)
Tetracycline	1.65–8.5	*Bacteroidetes* , *Spirochaetes*, *Syntrophomonas*, *Clostridium aurantibutyricum*, *Microbacter margulisiae*, *Porphyromonas pogonae, Treponema zuelzerae*, *Proteiniphilum acetatigenes*, *Proteobacteria*, *Cloacimonetes*, *Ignavibacteriae*,	Increased Decreased	(Xiong et al., 2017)

		Chloroflexi, *Syntrophomonas,* *unclassified* *Syntrophobacterace* *ae,* *Syntrophobacter* *wolinii,* *Methanomassiliicocc* *us, Methanoculleus* *acetate-utilizing* *Methanothrix,* *Methanoculleus,* *Methanobacterium*	Stable	
Chlortetracyc line		*Spirochaetaceae ,* *Acinetobacter,* *Pseudomonas,* *Comamonadaceae,*	Increased	
	0.5 g/kg (pig slurry)	*Methanomassiliicocc* *us* *Syntrophomonas* *spp., Syntrophospora* *spp.,* *Syntrophobacter spp*	Decreased	(Stone et al., 2009; Wang et al., 2017a)
	28	*Methanosaetaceae ,* *Methanosarcinaceae*	Decreased	

Oxytetracycline	20, 50, and 80 60, 100, 140mg/kg (pig manure)	*Sphingobacteriaceae* *Gammaproteobacteria* *Flavobacterium* *Methanomicrobiales*	Disappeared Decreased Increased Decreased	(Stone et al., 2009; Xin et al., 2014)
Tylosin	1.67	*Syntrophomonas spp., Syntrophospora spp.*	Decreased	(Shimada et al., 2008)
Erythromycin–tetracycline (ET) combinations	0.1–4	*Firmicutes* *Bacteroidetes,* *Acinetobacter,* *Proteobacteria* *Actinobacteria,* *Fusobacterium*	Stable Decreased Increased	(Aydin et al., 2016)
Sulfamethoxazole - Tetracycline	20 + 1.5	*Methanosarcinales*	Decreased	(Aydin et al., 2015c)
Sulfamethoxazole - Erythromycin - Tetracycline	2.5 + 2.5 + 25	*Methanosarcinales*	Decreased	