Effects of activated carbon on anaerobic digestion – methanogenic metabolism, mechanisms of antibiotics and antibiotic resistance genes removal

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Abstract:

Activated carbon (AC) is commonly used to enhance anaerobic digestion performance for methane production. However, AC has a beneficial effect that is commonly overlooked - its ability to remove antibiotics and influence the fate of antibiotic resistant genes (ARGs) through a combination of physisorption and biodegradation mechanisms. In this work, the use of AC in an anaerobic co-digestion system of food waste and chicken manure was explored. It was found that AC played an important role in antibiotics removal, effectively removing classes of LIN, CIPX, ERY and CLAR in the digester effluent. Specific to ofloxacin, a CIPX-class antibiotic, antibiotics removal was determined to be 33-60% without AC whereas it was close to 100% with AC. Furthermore, digesters with AC contained effluents with significantly lower ARG concentrations of *tetQ* and *tetW*. Changes in microbial community and biometabolic pathways were tracked using bioinformatics analysis, taxonomic trees and the KEGG pathway database. Results revealed that AC promoted the proliferation of essential anaerobic bacteria such as *Clostridrium perfringen, Prevotella sp* and *Firmicutes*. AC also increased the relative abundance of archaea; the dominant bacterial species was changed from *Methanolinea*, *Methanospirillum*, *Methanocelleus*, and *Methanosaeta* to *Methanosarcina*. Hence, methanogenesis could occur through both acetoclastic and hydrogenotropic pathways instead of through only a single pathway. Additionally, pathways involving ABC transporters and phosphotransferase systems became more prominent through supplementation with AC.

Keywords: Anaerobic digestion; Activated carbon; Antibiotics; Chicken manure; Food waste; Microbial community.

1. Introduction

Antibiotics are antimicrobial drugs which serve to treat and inhibit infections caused by bacteria [1, 2]. The extensive use of antibiotics over the years has resulted in large quantities being discharged into the environment. One major source of antibiotics being discharged into the environment is via animal manure as industrial farms are adding antibiotics into livestock feeds [3]. The feeding of antibiotics to animals promote faster growth rate among livestock, hence increasing profits among meat producing farms.

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The concerning consequence with increasing quantity of antibiotics being released into the environment is the formation of antibiotics resistance genes (ARGs) and antibiotic resistant bacteria. Antibiotics resistance refers to the ability of microbes to resist the effects of medication such as antibiotics, whereby the antibiotics are no longer an effective means to destroy or kill the intended bacteria. As of today, 271 subtypes of ARGs, originating from the 18 basic types, have been identified [4]. The occurrence of antibiotics resistance is a serious problem as it can result in various adverse impacts on human health and ecology.

Anaerobic digestion (AD) is a biological process which involves the breaking down of organic matter in the absence of oxygen by anaerobic microorganisms to produce biogas – methane and carbon dioxide. AD plays an important role in wastewater treatment processes, leading to the reduction of organic solids matter during effluent treatment. It has been suggested that AD can remove antibiotics and ARGs during stable operation, depending on the concentration and class of the antibiotics and ARGs, inoculum sources and other operating factors of the bioreactor [5]. In activated sludge, the removal routes of antibiotics are considered to be biodegradation, adsorption, volatilisation and hydrolysis. Arising from the various nature of antibiotic resistance, the degradation of antibiotics by bacteria during AD process is limited. In this research study, activated sludge was used to examine the adsorption and degradation effect of various classes of antibiotics with solid waste treatment.

Powdered activated carbon (PAC) is a form of amorphous carbonaceous materials with high porosity and large surface area. Due to its high adsorptive capacities, AC is extensively used in wastewater treatment to eliminate color, odor, taste and antibiotics via physical adsorption [6, 7]. According to Adams et al. (2002), the percentage removal of the tested antibiotics was more than 90% for a PAC dosage of 50mg/L in deionised water [8]. It was reported that the removal efficiency of ciprofloxacin was 87% at an initial concentration of 20mg/L with 250g/L of PAC [9]. Based on these results, it is highly likely that PAC is largely effective in removing antibiotics from wastewater. Moreover, adding AC facilitates microorganism enrichment [10] and alleviates organic shock loading impact, enhancing AD stability [11]. As a result, the use of AC in AD processes has been gaining momentum over the years. With increasing demand for renewable energy, a large majority of articles in the literature emphasizes the utilization of AC in anaerobic enhancement strategies in converting waste to biogas. Notwithstanding, little has been reported on the mechanisms that adding PAC into AD sludge have on both methane production and antibiotics removal. Hitherto, it is still not clear what effects PAC addition has on the fate of antibiotics and ARGs during the AD process.

The overall objective of this research was to evaluate the mechanisms that PAC has on the methane production and removal of antibiotics during AD process using food waste (FW) and chicken manure [12] as co-substrates. Furthermore, specific antibiotics were used to investigate the removal mechanisms of antibiotics in AD reactors by adding PAC; the fate of ARGs and biometabolic pathways were also explored.

2. Materials and Methods

2.1. Inocula and substrates

Seed sludge was collected from a large-scale anaerobic digester from the Ulu Pandan Water Reclamation Plant (UPWRP) in Singapore. The anaerobic digester at UPWRP currently treats waste activated sludge from the domestic sewage treatment. The ratio of volatile suspended sludge (VS) to total suspended sludge was 0.64 with initial TS of 18.9g/L.

FW was obtained from a canteen at the National University of Singapore. This comprised mainly rice, noodles,

meat, vegetables, and condiments. After removing any bones and non-biodegradable waste like plastic bags and utensils, the FW was homogenized by a blender and then stored at -20 °C freezer. The chicken manure (CM) used in this study was collected from Chew's Group Limited, Singapore. After collection, CM was homogenized by a blender. The detailed characteristics of FW and CM are listed in Table. 1.

Table 1 Characteristics of food waste and chicken manure

Components	Unit	Food Waste	Chicken Manure
Total Solids	wt %	31.70 ± 1.20	87.07 ± 6.20
Volatile Solids	wt %	29.59 ± 2.37	43.93 ± 3.12
VS/TS	wt %	93.34 ± 1.54	50.46 ± 1.30
Elementals			
Carbon	wt %	47.08 ± 2.01	29.82 ± 2.31
Hydrogen	wt %	7.04 ± 1.11	4.70 ± 0.43
Nitrogen	wt %	3.02 ± 0.32	3.70 ± 0.29
Sulphur	wt %	<0.5	0.6 ± 0.11
C/N ratio	-	15.58 ± 1.87	8.06 ± 0.27

2.2. Reactor specification and operation during anaerobic co-digestion of FW and CM

A glass anaerobic digester (Φ150 mm × 390 mm) was fabricated and operated with the addition of 15 g PAC/L (hereafter referred to as R1). The working volume of R1 is 5 L. The control digester was identical to R1 but without the addition of AC (hereafter referred to as R2). The pore volume and surface area of AC were 0.30 cc/g and 385 m^2/g, respectively. The size of the PAC was 100-400 mesh. The PAC was only added once in digester R1 at the beginning of the AD process. After being seeded with seed sludge, the two digesters were operated for anaerobic co-digestion of FW and CM in a semi-continuous mode (feeding every day) with a gradual increase in the organic loading rate (OLR): 1.8, 3.7, 5.5, 7.3, and 11.0 g VS_{FW+CM}/L/d. The VS ratio of FW to CM was 0.65 : 1. The two reactors were operated at 35 °C in parallel. The retention time was 30 d. All the experiments were conducted in triplicates.

2.3. Experimental procedure for the removal of specific antibiotics

Powered amoxicillin ($C_{16}H_{19}N_3O_5$) and ofloxacin ($C_{18}H_{20}FN_3O_4$) were weighed and diluted in deionized water to form the antibiotics solution. Depending on the concentration required for the experiment, specific volume of antibiotics solutions were added into the batch reactors. For batch experiments, 500 ml glass reactors were prepared with the addition of 15 g PAC/L (hereafter referred to as A1 and A2). The working volume of R1 was 400 ml. The control digester was identical to A1 but without the addition of PAC (hereafter referred to as B1 and B2). In addition, the inocula in both A2 and B2 were autoclaved at a temperature of 120°C to eliminate the presence of microorganisms, hence preventing the removal of antibiotics via biodegradation. After being seeded with seed sludge, PAC was added into reactors A1 and A2, separately. Antibiotic solution was added at the start of the experiment to achieve 0.1 ppm of antibiotics solution in each glass reactor. All reactors were then subsequently placed on a magnetic stirrer in the incubator at a pre-set temperature of 35 °C. All the experiments were conducted in triplicates.

2.4. Analytical methods

COD was determined using HACH color meter (DR900, USA) according to the manufacturer's instructions. The pH was recorded using a pH analyzer (Agilent 3200M, USA). TS and VS were determined based on the weighing method after being dried at 103-105 ^{o}C and burnt to ash at 550^{o}C. The CH_4 production was determined using a gas chromatograph (Clarus 580 Arnel, PerkinElmer, USA) equipped with a thermal conductivity detector. The concentration of the antibiotics was analysed directly via Acquity Ultra-High Performance Liquid Chromatography – tandem mass spectrometry (UHPLC, Agilent 1290 Infinity, USA). The UHPLC was subjected to the following mobile phase compositions: 0.1% formic acid in Milli-Q water (mobile phase A) and 0.1% formic acid in a 50:50 (v/v) mixture of methanol and acetonitrile (mobile phase B). Solid phase extraction and test of antibiotics were conducted according to the method described by Tran et al. (2016) [13]. C, N, S and H elemental analyses in FW and CM were determined using the vario MICRO cube (Elementar, HANAU, Germany). BET surface area and pore volume of activated carbons were measured by N_2 adsorption measurement using a Quantachrome Autosorb-6B. The one way analysis of variance (ANOVA) was used to analyze the data.

2.5. Quantification of ARGs by Real-time PCR analysis

A total of 11 ARGs - seven tetracycline resistance genes (*tetA*, *tetW*, *tetO*, *tetX, tetB*, *tetM*, *tetQ*), two sulfonamide resistance genes (*sul1*, *sul2*), one chloramphenicol resistance gene *cmlA*, one florfenicol resistance gene *floR*, and the integrase gene *intI1* of class I integrons were detected and quantified using a PCR Thermal Cycler Dice Real Time System by Sangon Biotech Shanghai Co., Ltd. Primers, the annealing temperature and amplification size are selected according to the report of Zhang et al. (2018) [14]. Each sample was analyzed in triplicate, which means that there were three separate AD runs for all reactors.

2.6. Metagenomic shotgun sequencing and metabolic pathways analysis.

Sequencing of metagenomic DNA was conducted by IIIumina Miseq^{TM} sequencer (IIIumina Inc., USA). The analytical methods refer to the reference [15] that included DNA extraction, DNA library construction and sequencing, screening of effective reads, assembling of high-quality reads of DNA samples, Gene taxonomic assignment, Gene functional classification, and other related analyses.

Briefly, the metagenomic DNA of the sample was extracted using an extraction kit (MO BIO Laboratories, Inc. Carlsbad, USA) according to the manufacturer's instructions. The quality of the extracted DNA was checked by determining its absorbance at 260 nm and 280 nm. To obtain the effective and clean sequencing data, raw sequencing results were processed by Trimmomatic as follows: 1) trimed paired-end sequences of reads; 2) removed sequences containing ambiguities ("Ns"); 3) removed reads shorter than 35nt. 4) removed low - quality sequences i.e. a sequencing quality value lower than 20; 5) removed sequences with the quality of the tail less than 20 bases by sliding window protocol. Effective reads were assembled by IDBA_UD software according to the relationships between reads and overlap to obtain contigs that were further translated into protein sequences. Subsequently, taxonomy was assigned by MetaPhlAn2 software through blasting marker genes with effective reads. Analysis of metabolic pathways and gene functional classification were conducted by DIAMOND [16] and HUMAnN [17] through comparing reads with gene database of Kyoto Encyclopedia of Genes and Genomes (KEGG) that provides sequences information of genes and proteins [18].

3. Results and Discussion

3.1. Enhancement of anaerobic co-digestion of FW and CM by adding activated carbon

From Fig. 1, the superior performance of the reactor with the addition of AC (R1) in comparison with that of the reactor without the addition of AC (R2) is evident through (i) larger accumulated methane yield at various OLRs and greater specific methane production (SMP).

From Fig. 1A, as the OLRs of FW and CM was increased from 1.8 to 11.0 g VS_{FW+CM}/L/d, the accumulated methane yield gradually increased for both reactors. However, the accumulative methane yield of the reactor R1 was 218 L at the end of the experimental period, while that of the reactor R2 was only 150 L. The large difference in the yield could be explained by the higher SMP of R1. At the lowest OLRs of 1.8 g VS_{FW+CM}/L/d, the average SMP in R1 and R2 was approximately 0.04 L CH_4/VS_{FW+CM}/d, presenting no statistically significant difference between R1 and R2 ($p>0.05$). Also, the pH was kept between 7 and 7.6, and COD concentration in the effluent was below 4100 mg/L. When the OLRs was increased to 11.0 g VS_{FW+CM}/L/d, the maximum SMP was 0.2 L CH_4/VS_{FW+CM}/d for R1, while reactor R2 was only able to achieve a SMP of 0.14 L CH_4/VS_{FW+CM}/d, showing statistically significant differences ($p<0.05$). Meanwhile, pH in R2 decreased to 6.5, while pH in R1 still maintained at a neutral range of 7.6. The significant difference in the cumulative methane yield and SMP could be attributed to the presence of AC. These results are in agreement with literature values, which demonstrated the ability of AC to enhance the electron exchange between syntrophs and methanogens to accelerate substrate consumption and methane production [19]. In addition, a study conducted by Aktas et al (2001) concluded that the large surface area and high adsorption capacity of activated carbon helped offset the shock in the AD system due to an increase in OLRs [20]. This report concurs with the results obtained in this study as Fig. 1B shows an increase in the SMP with an increase in OLRs for reactors with AC.

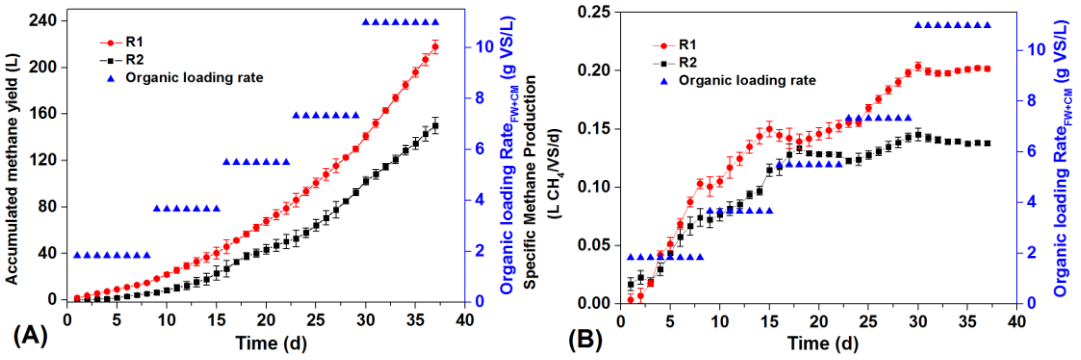

Fig. 1 (A) Accumulated Methane Yield, (B) Specific Methane Yield in R1 and R2.

3.2. Removal of antibiotics during anaerobic digestion process by adding activated carbon

As indicated in Table 2, although the quantity of antibiotics was negligible in FW, trace amounts were present in CM and a large amount of CIPX and TCS were present in the inoculum. This is not surprising as the inoculum was used to treat domestic wastewater which might have been contaminated with antibiotics. To investigate the effectiveness of antibiotics removal with and without the addition of PAC, the concentration of antibiotics in the effluents of R1 and R2 on day 37 were analyzed and the results are summarized in Fig. 2. CTC and TCS were found to be non-existent in R1 and R2; these classes of antibiotics were likely removed via biodegradation rather than physical adsorption with PAC. Meanwhile, the concentrations of LIN, CIPX, ERY and CLAR were found to be lower in the effluent of R1 compared to R2. Of the 4 classes of antibiotics removed, ERY displayed the greatest decrease. This suggests that ERY might have a stronger physical adsorption affinity with PAC as compared to the other 3

classes. While these results seem promising, it should be noted that Fig. 2 was based on one sample alone; it might not be conclusive to state that PAC assisted in the removal of the antibiotics.

To gain more insight into the role of PAC in effecting the removal of antibiotics in the anaerobic digestion system, batch experiments involving ofloxacin (a CIPX-class antibiotic) and amoxicillin (a β-lactam class antibiotic) were conducted. In this experiment, the initial concentrations of the two antibiotics were 0.1 mg/L. In addition, the inocula in both A2 and B2 were autoclaved to eliminate biodegradation. Consequently, any decrease in antibiotic concentration must be attributed to physical sorption to PAC and to inactivated bacteria. The results are shown in Fig. 3. From Fig. 3A, it can be seen that the concentrations of ofloxacin in reactors without PAC (i.e., B1 and B2) were consistently higher than those dosed with PAC (i.e., A1 and A2) throughout the experimental period, indicating the role of PAC in removing ofloxacin. In PAC dosed reactors, ofloxacin was not detected in the effluent after 2 hours, whereas, about half of the ofloxacin was removed in the digester without PAC. In addition, no significant difference was observed between autoclaved and live activated sludge in terms of effluent ofloxacin concentration. These results show that ofloxacin was primarily removed by sorption to sludge within the experimental period. When the reactors were dosed with PAC, the sorption was enhanced, leading to an ofloxacin free effluent. Hence, it can be concluded that biodegradation was insignificant in this experiment. This agrees with a previous study which reported that ofloxacin was only sparingly biodegradable [21]. One possible explanation for the limited biodegradation is that the tendency of digested biosolids to be adsorbed onto PAC reduces the bioavailability of antibiotics, resulting in low degradation rates [22]. It has been reported that sorption to activated sludge can remove 82-92% of fluoroquinolones, another antibiotics, in a sewage treatment plant in Zurich [23].

Results for amoxicillin were similar to that for ofloxacin (Fig. 3). Again, physical sorption played the dominant role in removing amoxicillin and biodegradation was negligible. A previous study reported that 50% percentage of amoxicillin was sorbed by activated sludge in 10 h [24]. Regarding the biodegradability of amoxicillin, the conclusion was still ambiguous. Gartiser et al. (2007) demonstrated that amoxicillin could be biodegraded provided a long incubation time [25]. Another study reported that only 9.5% amoxicillin could be degraded by a biofilm airlift suspension reactor during a 13 h operation [26]. However, Alexy et al. (2004) reported that amoxicillin would probably not be biodegraded efficiently in treatment plants and in surface water using closed bottle tests [27]. The contrasting results on biodegradation of amoxicillin suggest that more studies are needed.

Table 2. Amount of antibiotics in food waste, chicken manure and inoculum (ng/g dry weight)

Substrate	[1]LIN (n=3)	[2]CIPX (n=3)	[3]CTC (n=3)	[4]ERY (n=3)	[5]CLAR (n=3)	[6]TCS (n=3)
FW	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
CM	0.02 ± 0.004	< LOD	0.090 ± 0.005	0.03 ± 0.02	0.002 ± 0.001	< LOD
Inoculum	0.02 ± 0.001	1.0 ± 0.2	< LOD	< LOD	0.005 ± 0.002	0.160 ± 0.004

[1]LIN: Lincosamides – Lincomycin; [2]CIPX: Fluoroquinolone – Ciprofloxacin;
[3]CTC: Tetracycline Family – Chlortetracycline; [4]ERY: Macrolides – Erythromycin;
[5]CLAR: Macrolides – Clarithromycin; [6]TCS: Antiseptics – Triclosan.

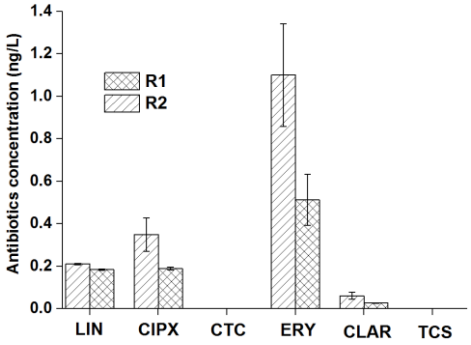

Fig. 2 Effluent concentration of several classes of antibiotics in R1 and R2.

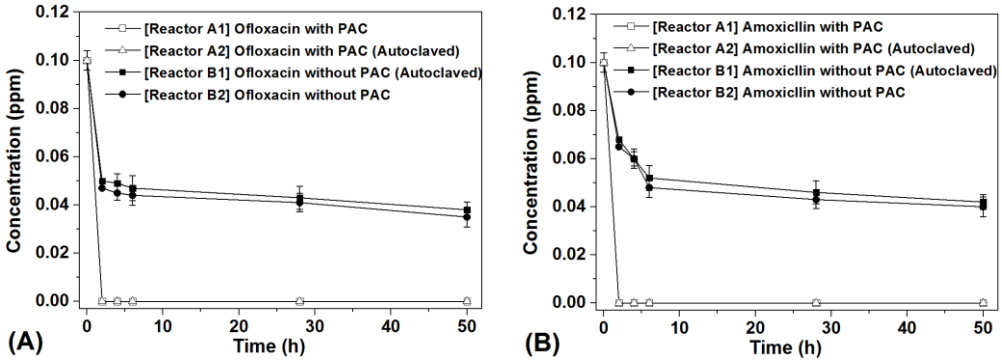

Fig. 3 Effluent Concentration of (A) Ofloxacin with and without AC and (B) Ofloxacin with and without Autoclave

3.3. Fate of ARGs during anaerobic digestion process by adding activated carbon

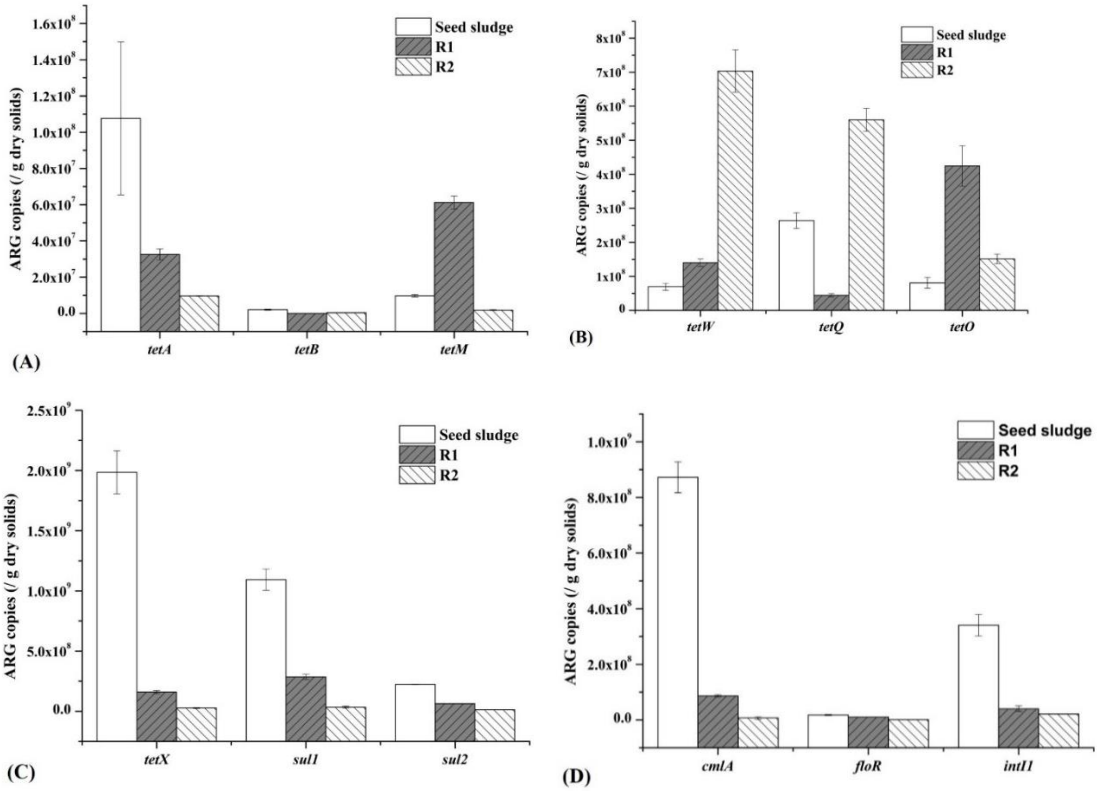

Fig. 4 Abundance and concentration changes of ARGs in seed sludge and reactors R1 and R2

As shown in Fig. 4, there were eleven ARGs and *intI*1 detected in all the samples of seed sludge, R1, and R2. ARGs were classified to four types - tetracycline resistance genes (*tetA*, *tetW*, *tetO*, *tetX, tetB*, *tetM*, *tetQ*),

sulfonamide resistance genes (*sul1*, *sul2*), chloramphenicol resistance gene *cmlA*, and florfenicol resistance gene *floR*. Based on the number of types of ARGs, the *tet* genes dominated among all ARGs detected in R1, which was consistent with previous studies [28].

The fate of each ARG was quite different in these two digesters. Anaerobic co-digestion in R1 and R2 could reduce some ARGs abundance in comparison with seed sludge in which the initial abundance of ARGs (*tetA*, *tetX, sul1*, *sul2* and *cmlA*) was very high, and the addition of AC in R1 showed a better reduction for the ARG of *tetQ*. In particular, *tetA*, with the function of encoding transporters with efflux pump function [5], was significantly decreased by co-digestion. The *tetX* exhibited similar tendency to *tetA*, that is, on the basis of decreasing effect of co-digestion, the addition of AC further decreased the abundance of *tetX*. The remaining ARG (*floR*) displayed no significant change during the anaerobic co-digestion process with or without AC addition, which indicated that AC could not affect this kind of ARGs. However, the abundance of most ARGs (*tetA*, *tetM*, *tetO*, *tetX, sul1*, *sul2, cmlA and floR*) in R1 was much higher than that of R2. For *tetO*, the ribosomal protection protein genes, anaerobic co-digestion improved their abundance to some extent while AC addition further enhanced increase of *tetO* to some extent, compared with corresponding *tet* genes in R2.

In anaerobic co-digestion, several ARGs and *intl*1 could be efficiently removed from seed sludge, suggesting the importance of anaerobic co-digestion process in controlling the release of ARGs. A slower growth of antibiotic resistant bacteria (ARB) might be one possible explanation for the decrease of ARG levels because methanogens tended to grow better in anaerobic digesters. This limited the horizontal gene transfer in the reactor. The decreased *intl*1 level further ascertained the reduced horizontal gene transfer potential (Fig. 4). After adding PAC, ARG removal efficiencies were complicated as reduction and enrichment of ARGs both happened. It was hypothesized that PAC could reduce the level of ARGs in wastewater solids because of more diverse microbial communities and higher microbial activity [29], as PAC could provide sufficient retention sites for various kinds of bacteria. Another advantage of adding PAC was to reduce bacterial mobility which might limit the exchange of genetic materials (i.e., DNA) [30]. Another possible reason for the enrichment of ARGs lie in the selective pressure posed by antibiotics presented in the substrates [4]. Previous studies have indicated the ubiquitous presence of various antibiotics in WAS [31] and CM [32]. In addition, the use of manure as fertilisers to agricultural fields could possibly increase the ARGs level and ARB [33]. In fact, Heuer and Smalla [34] reported that higher ARGs levels in the soil were observed after adding sulfadiazine (an antibiotic) into the manure. Hence, it was speculated that the antibiotics in the waste activated sludge and CM were responsible for the elevated ARGs levels in the digesters.

3.4. Evaluation of Microbial Communities through Bioinformatics Analysis

The addition of AC might have initiated changes on a micro-scale, giving rise to macro-scale observations in the previous sections. Bioinformatics analysis was conducted to better understand the differences in biometabolic pathways and microbial community. The taxonomic trees in Fig. 5 show that *Clostridium*, *Prevotella*, *Unclassified Firmicutes* and *Fibrobacter* exhibited a change in proportion when AC was added; AC increased the proportion of the former 3 bacterial species and reduced the proportion of *Fibrobacter*.

The functions of these bacterial classes are as follows: *Clostridrium perfringen* contain anaerobic fermentation enzymes that expedite biogas production [35]. *Prevotella sp.*, which thrive in anaerobic environments, facilitates the breakdown of proteins and carbohydrates into volatile fatty acids [36]. *Firmicutes* increase the uptake of fatty acids

and improve energy harvesting efficiency, thus exhibiting a positive correlation with biogas production [37]. These findings provide a deeper understanding of Fig. 1A on a micro-level; AC promoted the growth of bacterial classes that aid in converting carbon-containing compounds in FW and CM to methane, hence increasing methane yield. The impact on methane yield due to an increase in *Clostridium*, *Prevotella* and *Firmicutes* is also in good agreement with the literature – Zhu et al. discovered that raw glycerol pre-treated with AC experienced less inhibition to fermentation by *Clostridrium butyricum* [38]. In another experiment involving the mesophilic digestion of landfill leachate in a submerged anaerobic membrane bioreactor, Trzcinski and Stuckey pinpointed the dominant bacterial species to be *Prevotella* and *Thauera* [39]. According to Chen et al., *Firmicutes* dominated the bacterial community at low OLRs and directly affected digestion performance [40].

There are two types of methanogenesis: acetoclastic methanogenesis, where bacteria convert acetate to methane; and hydrogenotrophic methanogenesis, where bacteria utilize hydrogen and carbon dioxide in methane production. An example of an acetoclastic specie is *Methanosaeta*, whereas hydrogenotrophic species include *Methanospirillum*, *Methanothermus*, *Methanoculleus* and *Methanolinea* . Methanogens belong to the archaea family and are commonly termed methanogenic archaea. There was a noticeable difference between the relative proportion of archaea between R1 and R2, with R1 having relatively more archaea than R2. The translated to higher levels of methanogenesis and increased biogas production. Digging deeper, taxonomic trees in Fig. 5 depict a shift in dominant bacterial species from *Methanolinea*, *Methanospirillum*, *Methanocelleus*, and *Methanosaeta* to *Methanosarcina* when AC was added to the reactor. *Methanosarcina* has a higher maximum growth rate, half-saturation coefficient and yield coefficient than *methanosaeta*, making them prevalent when acetate concentrations are high [12]. *Methanosarcina* uses acetate, hydrogen and carbon dioxide as methanogenic substrates, producing methane through both acetoclastic and hydrogenotrophic methanogenesis [41]. Conversely, *Methanosaeta* only converts acetate to methane through the acetoclastic pathway [42]. Naturally, *Methanosarcina* has a lower affinity and a higher threshold for acetate compared to *Methanosaeta* [43]. As such, research has shown that *Methanosarcina*-dominated digesters perform better than *Methanosaeta-dominated* ones [44]. This, coupled with a larger relative proportion of archaea, led to the better digestion performance in R1 as observed in Fig. 1 previously.

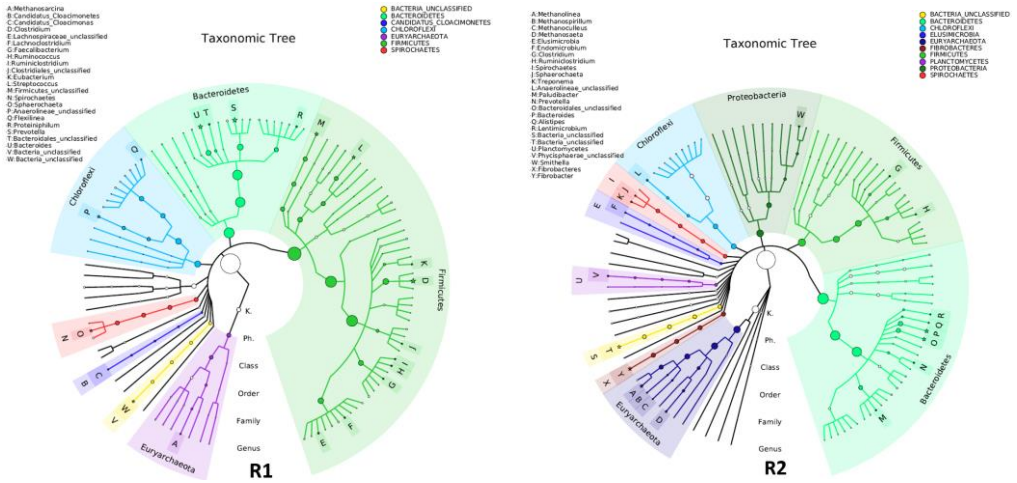

Fig. 5 Schematic diagram of phylogenetic and taxonomy trees of dominant AD microbial communities in reactor R1 and R2 according to GraPhlAn analysis. The top one hundred genus were selected to construct phylogenetic trees, and the corresponding phyla of top twenty genus (marked with asterisk) was marked in different color. The size of circles and asterisks represent the different relative abundance of microbial populations.

3.5. Evaluation of Biometabolic Pathways through Bioinformatics Analysis

The annotation for phylogenetically defined microorganisms with functional profiles were taken from the KEGG Orthologs (ko) database. Analysis and normalization of ko pathways and functional profiles are depicted in Fig. 6. It was found that the addition of AC modified only 1 out of 20 dominant pathways – from bacterial secretion system (ko03070) to a two-component system (ko02020), causing the AD system to accelerate cellular response to external influences by inducing changes in transcription [45]. Moreover, the addition of AC increased the relative abundance of environmental information processing pathways by 2% (data not shown). Within this category, there was a 6% increase in ATP-Binding Cassette (ABC) transporters (ko02010), 6% decrease in bacterial secretion system (ko03070) and 4% increase in phosphotransferase system (ko02060), a mechanism which aided the assimilation of carbohydrates where the energy source was derived from phosphoenolpyruvate. According to the literature, ABC transporters thrive in environments that are less nutrient-rich, implying that R1 had less nutrients than R2 [46].

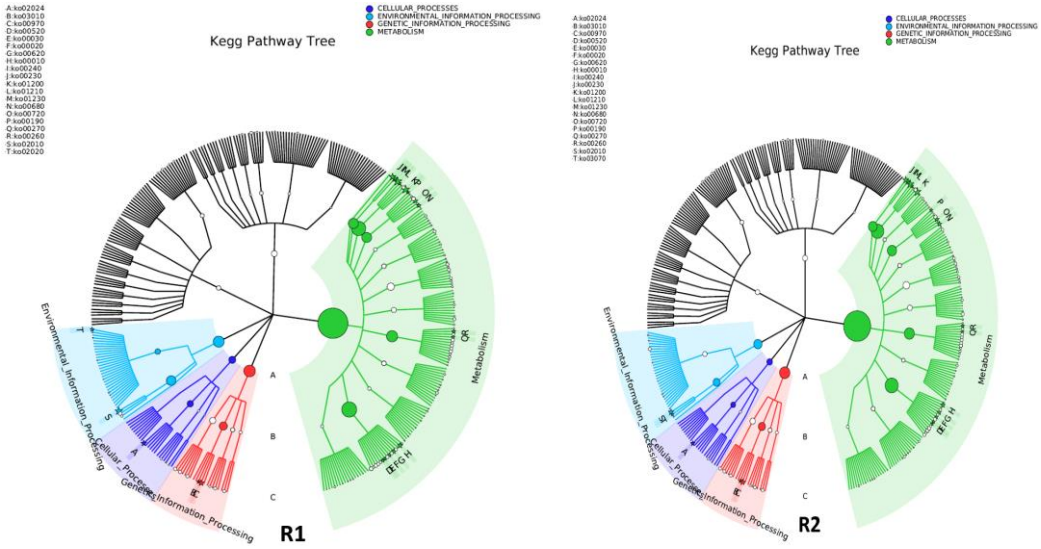

Fig. 6 KEGG pathway trees represent the dominant metabolic pathways during AD process in reactor R1 and R2. Asterisks in the outer ring indicate the dominant metabolic pathways of reactor R1, R2.

4. Conclusions

The addition of AC to an anaerobic co-digestion process simultaneously enhanced methane production while removing various classes of antibiotics. The concentration of certain ARGs such as *tetQ* and *tetW* were found to decrease with AC addition whereas other ARGs showed an increase. AC increased the relative abundance of archaea and altered the dominant species of methanogens, allowing methanogenesis through acetoclastic and hydrogenotropic pathways using multiple archaeal species. Membrane transport pathways also became more prominent with AC. This proves that AD systems do not only function as a waste-to-energy process, but they also possess the ability to reduce the negative impact of antibiotics.

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