Response of antibiotic and heavy metal resistance genes to two different temperature sequences in anaerobic digestion of waste activated sludge

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Graphical abstract

Sewage sludge

Temperature phased anaerobic digestion (TPAD)

- ❖ Scenario 1: Mesophilic-thermophilic
- ❖ Scenario 2: Thermophilic-Mesophilic

Responses of antibiotic and heavy metal resistance genes

Fate of human pathogenic bacteria

Abstract

Response of antibiotic resistance genes (ARGs) and heavy metal resistance genes (HMRGs) to two different temperature sequences (i.e., mesophilic-thermophilic and thermophilic-mesophilic) were investigated. Higher removal of total ARGs (twenty-one targeted subtypes) and HMRGs (three targeted subtypes) was achieved by the mesophilic-thermophilic sequence than by the thermophilic-mesophilic sequence. The sequence of mesophilic-thermophilic showed the highest removal of total ARGs, but the sequence of thermophilic-mesophilic proved more suitable for removal of class 1 integrons (*intI1*). Correlation analysis indicated that *intI1* correlated significantly with *tetG*, *tetQ*, *tetX*, *sul2*, *aac(6')-lb-cr*, *bla_{TEM}*, *ermB* and *floR*. High-throughput sequencing revealed that the mesophilic-thermophilic sequence TPAD removed more human bacterial pathogens (HBPs) than did the thermophilic-mesophilic sequence. Also, significantly positive correlation was observed between ARGs and HBPs. For instance, *Mycoplasma pneumonia* showed significantly positive correlation with several ARGs including *tetE*, *tetQ*, *tetX*, *tetZ*, *sul1*, *sul2*, *aac(6')-lb-cr* and *floR*.

Keywords: antibiotic resistance genes (ARGs); heavy metal resistance genes (HMRGs); temperature phased anaerobic digestion (TPAD); high-throughput sequencing; human bacterial pathogens (HBPs)

1. Introduction

One of the major drawbacks of any activated sludge process is that huge amounts of waste activated sludge (WAS) are produced as a by-product ([Appels et al., 2011](#)). According to [Zhang et al. \(2017b\)](#), approximately 45 million dry tons of global sewage sludge are produced annually. Prior to 2013, the cost for sludge disposal accounted for nearly 50 % of total wastewater treatment plant (WWTP) operating costs ([Appels et al., 2011](#)), representing

up to 10 billion USD annually ([Zhang et al., 2017b](#)). To reduce this cost, various physical, chemical and biological processes can be applied. Among these, anaerobic digestion (AD) is considered as one of the most cost-effective alternatives for WAS treatment because of its ability to convert organic matter to biogas, especially methane ([Appels et al., 2011](#)). About 90 % and 66 % of sewage sludge are treated via AD in Germany and the UK, respectively ([Tao et al., 2017](#)).

According to recent studies ([Auerbach et al., 2007](#); [LaPara et al., 2011](#); [Munir et al., 2011](#)), proliferation of antibiotic resistance genes (ARGs) has become a serious threat to both animals and humans and a growing body of evidence has suggested that WWTPs are considered to be potential reservoirs of diverse ARGs because most ARGs-contaminated wastewater originate from livestock, agriculture, industry and hospitals. Moreover, environmental factors (e.g., high microbial diversity and nutrition) in WWTPs may promote the proliferation of ARGs through vertical or horizontal gene transfer (HGT) with mobile genetic elements such as plasmids, transposable elements or integrin-integrase gene cassettes ([Schlüter et al., 2007](#)). Results of the high-throughput sequencing approach has recently suggested that a large proportion of ARGs in WWTPs are concentrated in WAS ([Yang et al., 2014](#)). For these reasons, considerable attention has been paid to the efficient treatment of WAS via AD, especially, because it is considered essential to reduce environmental burdens as well as operating costs.

In a positive sense, large amounts of ARGs can be reduced by AD. Recently, significant efforts have been made to evaluate the effect of operating parameters on the removal of ARGs in AD. [Diehl and LaPara \(2010\)](#) reported that digestate from thermophilic AD (TAD) showed a lower quantity of genes encoding tetracycline resistance (*tetA*, *tetL*, *tetO*, *tetW*, *tetX*) and class 1 integrons (*intII*) than that in mesophilic AD (MAD). Also, [Ma et al. \(2011\)](#)

achieved more effective reduction of two erythromycin (*ermB*, *ermF*) and tetracycline (*tetO*, *tetW*) resistance genes in TAD compared to MAD. However, MAD showed higher removal of other resistance genes (*sul1*, *sul2*, *tetC*, *tetG*, *tetX*) and *int11* than that obtained in TAD. These results suggest that temperature is critical to determining the distribution of ARGs in AD, but their response to temperature in AD is not universal (Zhang et al., 2015). By extension, various effects by pretreatment (Ma et al., 2011; Zhang et al., 2016b) of AD or two-stage (acidogenic-methanogenic phase or different temperature phased) AD (Ghosh et al., 2009; Wu et al., 2016) on ARGs have been reported. However, understanding of the fate of different types of ARGs in temperature-phased anaerobic digestion (TPAD) is still limited, although the presence of three tetracycline resistance genes (*tetA*, *tetO*, *tetX*) and *int11* in TPAD has been reported by Ghosh et al. (2009).

Interestingly, experimental evidence indicates that heavy metals can exert selection pressure not only on heavy metal resistance genes (HMRGs) but also ARGs (Berg et al., 2010; Di Cesare et al., 2016; Seiler and Berendonk, 2012). There are two potential mechanisms involved in the co-selection of antibiotic and heavy metals resistance (Baker-Austin et al., 2006): (1) cross-resistance (genes share a common route of access to their respective targets) and (2) co-resistance (genes specifying resistant phenotypes are located together on the same genetic element such as a plasmid, transposon or integron). Considering the characteristics of domestic wastewater (i.e., generally higher concentration and lower degradability of heavy metals than for antibiotics) (Di Cesare et al., 2016), co-selection of resistance genes might be enhanced.

In this study, TPAD of WAS using two different sequences (i.e., MAD-TAD, TAD-MAD) was operated to investigate variations in ARGs, HMRGs and *int11*. Using quantitative PCR (qPCR), twenty-one ARGs encoding tetracycline resistance (*tetA*, *tetB*, *tetD*, *tetE*, *tetG*, *tetH*,

tetM, *tetQ*, *tetX*, *tetZ*, *tetBP*), sulfonamide resistance (*sul1*, *sul2*), quinolone resistance (*qnrD*, *aac(6')-Ib-cr*), β -lactam antibiotics resistance (*bla*_{SHV}, *bla*_{TEM}, *bla*_{CTX}), macrolide resistance (*ermB*), florfenicol resistance (*floR*) and multidrug resistance (*oqxA*) and *intI1* were quantified. In addition, three HMRGs encoding copper resistance (*copA*, *pcoA*, *tcrB*) were quantified by qPCR. To gain insight into the potential mechanisms impacting the fate of ARGs, HMRGs and *intI1* in TPAD, the bacterial community structure was characterized using high-throughput sequencing of 16S rRNA genes. To our knowledge, limited studies to date have reported the fates and co-occurrence of ARGs, HMRGs and *intI1* during TPAD. Thus, current research is essential to obtain insight into the potential mechanisms of these genes in TPADs.

2. Materials and methods

2.1. Preparation of WAS and inoculum

The highly concentrated WAS and inoculum used in this study were collected from the secondary clarifier and mesophilic anaerobic digester in a municipal wastewater treatment plant in Daegu, Korea. The physico-chemical characteristics of WAS and inoculum were as follows: pH 5.59 ± 0.11 and 7.24 ± 0.11 ; total solids (TS) 45.60 ± 0.28 and 24.33 ± 0.89 g/L; volatile solids (VS) 33.67 ± 0.09 and 16.21 ± 0.57 g/L; total chemical oxygen demand (TCOD) 48.34 ± 0.44 and 26.65 ± 0.13 g/L; soluble COD (SCOD) 1.52 ± 0.04 and 0.38 ± 0.08 g/L; ammonium (NH_4^+ -N) 197.94 ± 0.45 and 702.51 ± 0.07 mg/L; nitrate (NO_3^- -N) 9.15 ± 0.83 and 9.86 ± 0.05 mg/L; phosphate (PO_4^{3-} -P) 483.13 ± 18.06 and 130.28 ± 0.82 mg/L; sulfate (SO_4^{2-}) 54.20 ± 2.19 and 60.05 ± 0.05 mg/L.

2.2. Reactor set up and operation

To evaluate the effect of temperature sequences on the fate of ARGs, HMRGs and *intI1* in TPADs, two different sequential ADs (MAD-TAD and TAD-MAD) of WAS were carried out in batch reactors with 200 mL working volume. Briefly, the procedure for operation of both TPAD experiments was as follows: (1) both single stages of MAD (M^{1st}) and TAD (T^{1st}) of WAS were conducted under batch mode for 30 days and then (2) digested sludge from M^{1st} and T^{1st} was sequentially digested in different temperature stages with 200 mL working volume. Each second stage of TAD (T^{2nd}) and MAD (M^{2nd}) was conducted under batch mode for 25 days. All AD experiments were conducted until daily biogas production was less than 1 % of total biogas production. The initial pH of all experimental sets was adjusted to 7. In this study, an inoculum to substrate ratio (ISR) of 2 (VS basis) was used to eliminate any potential inhibition during AD ([Raposo et al., 2012](#)). All bottles were capped with rubber stoppers with aluminum crimp seals to maintain anaerobic conditions. Afterwards, the bottles were purged with pure nitrogen gas and incubated under mesophilic (35 °C) and thermophilic (55 °C) temperatures. All experiments were conducted in triplicate.

2.3. DNA extraction and qPCR

Samples from both the initial and end points of each digestion stage were taken for qPCR and bacterial community analyses. The sample from the mixed inoculum and substrate for the M^{1st} and T^{1st} experiments was designated as “initial”. DNA from sludge samples was extracted and purified using a Nucleo Spin® Soil kit (MACHEREY-NAGEL, Germany) according to instructions and stored at -25 °C until further analysis.

To select the sub-type of ARGs and HMRGs for qPCR analysis, conventional PCR screening was conducted with twenty-one ARGs and eleven HMRGs primers. After the PCR screening step, twenty-one ARGs (*tetA*, *tetB*, *tetD*, *tetE*, *tetG*, *tetH*, *tetM*, *tetQ*, *tetX*, *tetZ*, *tetBP*, *sul1*, *sul2*, *qnrD*, *aac(6')-Ib-cr*, *bla_{CTX}*, *bla_{SHV}*, *bla_{TEM}*, *ermB*, *floR*, *oqxA*) and three HMRGs (*copA*,

pcoA, *tcrB*) were detected and quantified by qPCR (CFX96 Touch™, Bio-Rad, USA). Also, *intI1* and 16S rRNA genes were quantified by qPCR. Tenfold serial diluted plasmid DNA from cloned *Escherichia coli* (TOPO® TA Cloning® Kit) was used for qPCR standard curves for each target genes. All amplifications were performed in triplicate and included a non-template control (NTC). Detailed information about the PCR and qPCR is described in previous research ([Jang et al., 2018](#); [Jang et al., 2017](#)).

2.4. High-throughput sequencing analysis based on 16S rRNA genes

High-throughput sequencing of bacterial 16S rRNA genes was conducted as previously described ([Jang et al., 2017](#)). Briefly, equal amounts of amplicons derived from PCR of the V3-V4 region of 16S rRNA genes were pooled and then high-throughput sequencing was conducted using a Illumina Miseq platform as described by the manufacturer (Macrogen, Seoul). After merging of raw paired-end reads from Illumina Miseq through FLASH (fast length adjustment of short reads) software ([Magoč and Salzberg, 2011](#)), both clustering and denoising steps were conducted using CD-HIT-OUT. Finally, taxonomic composition and community diversity indices were determined by QIIME (quantitative insights into microbial ecology) ([Caporaso et al., 2010](#)) and UCLUST ([Edgar, 2010](#)) based on the 16S rRNA data base in RDP (ribosomal database project).

Classification of human bacterial pathogens (HBPs) in this study was conducted based on a constructed HBPs 16S database ([Cai and Zhang, 2013](#)). The database contained a total of 259 16S rRNA gene sequences corresponding to 32 HBPs species ([Cai and Zhang, 2013](#)).

Qualified 16S rRNA sequences from each sample were annotated with criteria as follows: 1) over 90% sequence match identity and 2) alignment length > 400 bp. Relative abundance of classified HBPs was calculated at the species level.

2.5. Analytical methods

Using standard methods ([APHA-AWWA-WEF, 1998](#)), TS, VS, TCOD and SCOD were determined and cation and anion concentrations including NH_4^+ , NO_3^- , PO_4^{3-} and SO_4^{2-} were quantified using ion chromatography (930 Compact IC Flex, Metrohm, Switzerland). The pH was measured using a pH meter (Orion star A221, Thermo Fisher Scientific). Using a microsyringe, biogas production from each serum bottle was quantified. Pearson correlation coefficients were calculated using R software with a Hmisc package. It should be noted that two ARGs (i.e., *tetH* and *tetBP*) and five HBPs (i.e., *Neisseria meningitides*, *Pseudomonas aeruginosa*, *Streptococcus pneumonia*, *Streptococcus pyogenes* and *Clostridium botulinum*) were not included in the correlation analysis due to insufficient samples.

3. Results and discussion

3.1. Responses of ARGs, HMRGs and *intI1* to TPAD

The profile of the residual gene for tetracycline resistance is shown in Fig. 1a. The abundance of *tetH* and *tetBP* decreased below the limit of quantification (LOQ) (4.35×10^2 copies/ μL for *tetH* and 5.96×10^4 copies/ μL for *tetBP*) after all types of AD in this study. In addition, abundance of *tetE* decreased below LOQ (4.67×10^3 copies/ μL) in $\text{T}^{1\text{st}}$ and $\text{T}^{2\text{nd}}$, but the residual gene fractions of *tetE* in $\text{M}^{1\text{st}}$ and $\text{M}^{2\text{nd}}$ were 0.59 and 0.50, respectively. Interestingly, proliferation and rebound (i.e. residual gene fraction > 1) of *tetA* (in $\text{M}^{1\text{st}}$), *tetB* (in $\text{M}^{1\text{st}}$, $\text{T}^{1\text{st}}$ and $\text{M}^{2\text{nd}}$), *tetD* (in $\text{M}^{1\text{st}}$, $\text{T}^{1\text{st}}$ and $\text{M}^{2\text{nd}}$) and *tetM* (in $\text{M}^{2\text{nd}}$) were observed. Rebound of some tetracycline resistance genes (e.g., *tetA*, *tetD*, *tetO* and *tetW*) after MAD has also been reported ([Ghosh et al., 2009](#); [Jang et al., 2017](#); [Ma et al., 2011](#)). However, to our best knowledge, this is the first study reporting enrichment of *tetB* and *tetD* after TAD. This is inconsistent with our previous research ([Jang et al., 2017](#)) which reported significant

removal of these genes during TAD of different types of sewage sludge (i.e. chemically enhanced primary sludge). Considering that the fate of ARGs is closely connected to the microbial community, the reason for this inconsistency might be different sources of substrate and inoculum for AD ([Luo et al., 2017](#)). Although proliferation and rebound of some tetracycline resistance genes occurred in M^{1st}, significant removal of these genes was achieved in T^{2nd}. This result shows the advantages of TPAD in removal of tetracycline resistance genes which are frequently detected in WWTPs ([LaPara et al., 2011](#)).

The abundance of *sul1* and *sul2* in the initial samples was 6.13×10^{12} and 2.30×10^{11} copies/g VS, respectively (Table 1). It should be noted that *sul1* showed the highest abundance among targeted ARGs. This is consistent with data from [Zhang et al. \(2015\)](#) and [Lee et al. \(2017\)](#) who reported high abundance of sulfonamide resistance genes in WWTP. Significant reduction of *sul1* and *sul2* was observed in all experimental sets (Fig. 1a). The residual gene fractions of *sul1* and *sul2* were 0.46 and 0.38 in M^{1st}, 0.16 and 0.17 in T^{1st}, 0.18 and 0.09 in T^{2nd} and 0.30 and 0.15 in M^{2nd}, respectively. This is consistent with previous research ([Ma et al., 2011](#); [Xu et al., 2018](#); [Yang et al., 2014](#)) reporting the significant removal of these genes via both MAD and TAD. One may conclude that maintain constant temperature was more effective than sequencing the temperature in controlling the abundance of *sul1* and *sul2*, unlike for tetracycline genes.

According to [Zhang et al. \(2016b\)](#), increased amounts of β -lactam antibiotics resistance genes (*bla*_{TEM}) and macrolide resistance (*ermB*) were observed in MAD of sewage sludge. There was no enrichment of these genes; however, other β -lactam antibiotics resistance genes (*bla*_{SHV} and *bla*_{CTX}) were enriched in M^{1st} (Fig. 1b). After T^{2nd}, a considerable amount of ARGs encoding *qnrD*, *aac(6')-Ib-cr*, *bla*_{TEM}, *bla*_{CTX}, *ermB* and *floR* was removed, but the residual gene fractions of *bla*_{SHV} and *oqxA* exceeded 1. Increased amounts of *bla*_{SHV} were

also observed in M^{2nd}, but there was no enrichment of β -lactam antibiotics and quinolone resistance genes in T^{1st}.

Recent research ([Di Cesare et al., 2016](#); [Luo et al., 2017](#); [Zhang et al., 2016a](#)) has shown that changes in the abundance of HMRGs can provide us new insights into the fate of ARGs. [Luo et al. \(2017\)](#) revealed significant correlation between ARGs and HMRGs in full-scale AD using a high-throughput sequencing approach. In addition, [Zhang et al. \(2016a\)](#) and [Di Cesare et al. \(2016\)](#) reported co-occurrence of ARGs and HMRGs in sludge composting and in WWTPs, respectively. Based on this information, we tried to quantify eleven HMRGs encoding copper (*copA*, *pcoA*, *tcrB*, *cueO*, *cusA*), cobalt/zinc/cadmium (*czcA*, *czcC*, *czcD*), nickel/cobalt (*nccA*), arsenic (*arsB*) and lead (*pbrT*) resistance genes, but only three HMRGs (*copA*, *pcoA*, *tcrB*) were detected in our samples. As shown in Fig. 1b, a considerable amount of *copA*, *pcoA* and *tcrB* was removed in T^{1st}. However, subsequent MAD caused a rebound of these genes (i.e., the residual gene fractions of *pcoA* and *tcrB* in M^{2nd} were higher than T^{1st}). This finding is inconsistent with data from [Zhang et al. \(2016b\)](#) who reported reduction of *copA* and *pcoA* in MAD. Because research about the fate of HMRGs in AD is limited, further study is required to understand their fates in TPAD.

Integrations are genetic elements and a common component of bacterial genomes that allow efficient capture and expression of exogenous genes ([Gillings, 2014](#)). As an essential part of integrations, *intI* is a gene encoding an integrase, and especially *intII* (encoding Class I integrations) is most widely used as a biomarker for HGT ([Burch et al., 2016](#); [Ghosh et al., 2009](#); [Mazel, 2006](#); [Zhang et al., 2016b](#)). In this study, *intII* was detected in the initial sample with an abundance of 9.91×10^{10} copies/ g VS. High abundance of *intII* in the initial sample was expected because this gene is prevalent in WWTPs ([Di Cesare et al., 2016](#); [LaPara et al., 2011](#); [Lee et al., 2017](#)). The residual gene fractions of *intII* in T^{2nd} (0.20) and M^{2nd} (0.17)

were lower than those of M^{1st} (0.32) and T^{1st} (0.46) (Fig. 1). The abundance of total ARGs (sum of targeted ARGs) in T^{2nd} (2.33 x 10¹² copies/ g VS) was lower compared to all other samples (ranged from 2.98 x 10¹² to 5.43 x 10¹²copies/ g VS). Fig. 1b shows that the residual gene fraction of total ARGs in M^{1st} (0.51) and M^{2nd} (0.40) is higher compared to that in T^{1st} (0.28) and T^{2nd} (0.22). Our findings are consistent with data from [Diehl and LaPara \(2010\)](#) and [Luo et al. \(2017\)](#) who observed positive correlation between the removal efficiency of ARGs and temperature in lab- and full-scale AD reactors treating sewage sludge and manure, respectively. Lower levels of total ARGs found in thermophilic conditions, especially in T^{2nd}, indicate that one efficient way to decrease the abundance of total ARGs might be the application of TAD during the final stage of AD. Collectively, the sequence of M^{1st} – T^{2nd} showed the highest removal of total ARGs, but the sequence of T^{1st} – M^{2nd} proved more suitable for removal of *intI1*. This suggests adding another stream (e.g. internal or external recycle) would achieve efficient removal of ARGs and *intI1* simultaneously.

3.2. Correlation of ARGs, HMRGs and *intI1*

As shown in Table 1, *intI1* showed significantly positive correlation with *tetG* ($r=0.940$), *tetQ* ($r=0.922$), *tetX* ($r=0.924$), *sul2* ($r=0.933$), *aac(6')-lb-cr* ($r=0.980$), *bla_{TEM}* ($r=0.965$), *ermB* ($r=0.920$) and *floR* ($r=0.911$). Positive correlation between *intI1* and the above genes during AD has also been reported ([Wu et al., 2016](#); [Zhang et al., 2016b](#)). This suggests that HGT may be associated with these genes' evolution during TPAD. In contrast, there was no significantly positive correlation between *intI1* and rebounded ARGs and HMRGs (i.e., *tetA*, *tetB*, *tetD*, *tetM*, *qnrD*, *bla_{SHV}*, *bla_{CTX}*, *oqxA*, *pcoA* and *tcrB*). This is inconsistent with previous research ([Jang et al., 2017](#)) which reported positive correlation between *intI1* and *tetA*, *tetM*, *bla_{SHV}* and *bla_{CTX}* in single stages of MAD and TAD. This inconsistency has often been observed during AD of sewage sludge due to different microbial structures (i.e., host

bacteria) in AD and should be studied further to reveal possible mechanisms for the genes' evolution in AD.

Although there was no significant correlation between HMRGs and *intI1*, HMRGs showed significant correlation with some ARGs (Table 1). Both [Amachawadi et al. \(2013\)](#) and [Zhang et al. \(2016a\)](#) have reported significant correlation of *tcpB* with *ermB* and five ARGs (*sul2*, *bla_{TEM}*, *bla_{CTX}*, *ereA* and *ermB*), respectively. In our study, however, the *tcpB* encoding copper resistance gene showed significant positive correlation with *tetM* ($r=0.891$) and *qnrD* ($r=0.951$). Unlike *tcpB*, the *pcoA* encoding copper resistance gene showed significant positive correlation with *tetE* ($r=0.899$), *tetG* ($r=0.889$), *tetZ* ($r=0.922$), *sul1* ($r=0.904$), *sul2* ($r=0.911$), *aac(6')-lb-cr* ($r=0.898$) and *bla_{TEM}* ($r=0.936$). This is partially consistent with data from [Zhang et al. \(2016a\)](#) who reported significant correlation of *pcoA* with *sul2*, *bla_{TEM}*, *bla_{CTX}*, *ereA* and *tetG*. Additionally, [Pal et al. \(2015\)](#) and [Luo et al. \(2017\)](#) reported the co-occurrence of *sul1* and *sul2* with several HMRGs including *pcoA* via a bioinformatics approach based on NCBI database and metagenomic analysis of full-scale AD, respectively. Interestingly, *pcoA* and *copA* showed significant positive correlation ($r=0.928$). This might result from the *pco* system (encoded a periplasmic multi-copper oxidase) being linked to activity of *copA*-, thereby conferring resistance. Recently, [Zhang et al. \(2016b\)](#) reported that proteins encoded in *pco* genes are highly linked with copper delivered by *copA* from the cytoplasm. As a result, significant correlation between some ARGs and HMRGs could provide mechanisms for the evolution of several ARGs in AD ([Luo et al., 2017](#); [Pal et al., 2015](#)).

3.3. High-throughput sequencing data

Using high-throughput sequencing analysis, a total 872 OTUs (based on 3 % dissimilarity) was obtained based on a total of 192,522 clean sequences from all samples ranging from

35,180 to 41,180. The relative abundance of bacterial phyla and genera in all samples is shown in Fig. 2. In the initial sample, the phyla *Proteobacteria* (23.57 %), *Bacteroidetes* (21.26 %), *Cloacimonetes* (11.11 %), *Chloroflexi* (6.64 %), *Verrucomicrobia* (6.64 %), *Spirochaetes* (4.43 %), *Aminicenantes* (2.43 %), and *Firmicutes* (2.59 %) were predominant, which is often the case in AD ([Jang et al., 2017](#)). Compared to the initial sample, the relative abundance of *Bacteroidetes* and *Proteobacteria* in T^{1st} and T^{2nd}, unlike in M^{1st} and M^{2nd}, decreased to 9.89 and 15.97 % and to 9.58 and 9.95 % respectively. In contrast, increments of *Cloacimonetes* (24.37 % for T^{1st} and 13.54 % for T^{2nd}), *Spirochaetes* (12.33 % for T^{1st} and 14.84 % for T^{2nd}) and *Aminicenantes* (13.39 % for T^{1st} and 11.09 % for T^{2nd}) were observed compared to the initial sample. Interestingly, the abundance of *Firmicutes* in T^{2nd} significantly increased to 10.04 %, while being less than 3 % in other samples. Considering that changes in bacterial communities trigger variations in ARGs during AD ([Sun et al., 2016](#)), the different responses of ARGs among the experimental sets can be attributed to the existence of different bacterial communities under different temperature conditions.

As shown in the Venn diagram for shared OTUs analysis (Fig. 3), 340 OTUs (about 39 % of the total detected numbers of OTUs) were shared by all the samples, accounting for 44.68 %, 58.02 %, 64.15 %, 64.39% and 60.28 % of the total detected numbers of OTUs in the initial, M^{1st}, T^{1st}, T^{2nd} and M^{2nd} samples, respectively. These OTUs were classified into 22 phyla and 92 genera. The phylogenetic relationships and relative abundance of the top 100 shared OTUs are shown in Fig. 4. Among these OTUs, OTU1 (Accession No. HQ133054) assigned to the genus *Candidatus Cloacamonas* showed the highest abundance of all samples (Fig. 2b and Fig 4).

3.4. Correlation among bacterial community, ARGs, HMRGs and *intI1*

In previous studies ([Jang et al., 2017](#); [Sun et al., 2016](#)), *Bacteroidetes* has exhibited significantly positive correlations with tetracycline (*tetA*, *tetB*, *tetC*, *tetG*, *tetH*, *tetM*, *tetQ*, *tetX*, *tetZ*), sulfonamide (*sul1* and *sul2*), quinolones (*qnrD* and *gyrA*), β -lactams (*bla_{SHV}*), macrolides (*ermB*) and florfenicol (*floR*). However, in this study, *Bacteroidetes* showed significantly positive correlations with *qnrD* ($r=0.904$), *copA* ($r=0.884$) and *tcrB* ($r=0.925$) (Table 2). This is highly consistent with the previous study ([Zhang et al., 2015](#)) which reported that ARGs are not universally responsive to biological treatment. Interestingly, both *Proteobacteria* and *Planctomycetes* showed similar correlation patterns with several ARGs (i.e., *tetE*, *tetG*, *tetH*, *tetQ*, *tetX*, *tetZ*, *tetBP*, *sul1*, *sul2*, *aac(6')-lb-cr*, *floR* and *pcoA*) excluding *bla_{TEM}* (correlated only with *Planctomycetes*). In addition, *oqxA* showed significantly positive correlations *Firmicutes* ($r=0.976$). Interestingly, this correlation is consistent with significant increases in both *Firmicutes* and *oqxA* (residual gene fraction of *oqxA* in T^{2nd} was 1.77) in T^{2nd} (Fig. 2a). Among the 17 phyla, only two phyla *Candidatus Saccharibacteria* and *Ignavibacteriae* had a significantly positive correlation with *intI1* ($r=0.962$ for *Candidatus Saccharibacteria* and $r=0.939$ for *Ignavibacteriae*). This suggests a possible contribution of those two phyla in HGT of ARGs although their relative abundance in all samples was less than 5 %. Considering the *intI1* showed significantly positive correlation with some ARGs including *tetG*, *tetQ*, *tetX*, *sul2*, *aac(6')-lb-cr*, *bla_{TEM}*, *ermB* and *floR* (as described in section 3.2.), it is reasonable to assume that the two phyla *Candidatus Saccharibacteria* and *Ignavibacteriae* served as potential hosts for these ARGs in our study.

As shown in Table 2, five phyla including *Bacteroidetes*, *Proteobacteria*, *Verrucomicrobia*, *Planctomycetes* and *Parcubacteria* showed significantly positive correlation with HMRGs. Among them, *Parcubacteria*, which has been identified in a broad range of anoxic environments ([Nelson and Stegen, 2015](#)), had significantly positive correlation with both

copA ($r=0.910$) and *pcoA* ($r=0.886$). Interestingly, the relative abundance of these five phyla significantly decreased in T^{1st} and T^{2nd}. Thus, significant decreases in these five phyla may partially explain the higher removal of HMRGs in T^{1st} and T^{2nd} than in M^{1st} and M^{2nd}.

3.5. Correlation of HBPs, ARGs, HMRGs and *intI1*

Among the thirty-two HBPs, a total of sixteen was detected in this study (Fig. 5). Some commonly detected genera including HBPs such as *Legionella*, *Mycobacterium*, *Clostridium*, *Corynebacterium* and *Enterococcus* were found after AD treatment, but genus *Streptococcus* was found only in the initial sample. In addition, the intestinal HBPs such as *Escherichia*, *Salmonella*, *Shigella* and *Yersinia* and the well-known genera *Vibrio* were not detected in our study. Compared to the initial sample (13 species), lower numbers of species of HBPs were detected in M^{1st} (10 species), T^{1st} (7 species), T^{2nd} (10 species) and M^{2nd} (12 species). The relative abundance of total HBPs in the initial sample was 1.36%, while lower levels of relative abundance of total HBPs were found in T^{1st} (0.15 %) and T^{2nd} (0.23 %) than those of M^{1st} (0.66 %) and M^{2nd} (0.44 %). This decrease in HBPs was consistent with previous research (Wu et al., 2016; Zhang et al., 2017a), indicating the potential role of AD for the removal of HBPs.

Recently, increased dissemination of multidrug-resistant *Enterococcus faecium* has become a serious threat to human health (Arias et al., 2010). According to previous metagenomic analysis (Fang et al., 2014), *Enterococcus faecium* carry ARGs impacting aminoglycoside antibiotics (i.e., carrying aminoglycoside phosphotransferase). It would be of interest to conduct future studies in which target ARGs could be expanded including aminoglycoside antibiotics resistance genes in AD. In this study, correlation analysis revealed that *Enterococcus faecium* had potential correlation not only with several ARGs and HMRGs (i.e., *tetG*, *tetQ*, *tetX*, *tetZ*, *sul1*, *sul2*, *aac(6')-lb-cr*, *bla*_{TEM}, *floR* and *pcoA*) but also *intI1*. The

relative abundance of *Mycobacterium tuberculosis*, the causative agent of tuberculosis (Gagneux et al., 2006), increased from 0.088 % (initial sample) to 0.15 % (M^{1st}) and to 0.11 % (M^{2nd}), but a decrease in this species was observed in T^{1st} (0.036 %) and T^{2nd} (0.047 %).

Fang et al. (2014) reported that *Mycobacterium tuberculosis* can carry macrolide-lincosamide-streptogramin (MLS) resistance protein in soil. However, in this study, there was no positive correlation between *ermB* which also encodes MLS resistance (Saribas et al., 2006). This study did show positive correlation with *tetB*, *qnrD* and *bla_{SHV}*, suggesting the potential of carrying other ARGs. Interestingly, *Mycoplasma pneumonia*, a causative agent of atypical pneumonia (Chopra and Roberts, 2001), showed significantly positive correlation with several ARGs including *tetE*, *tetQ*, *tetX*, *tetZ*, *sul1*, *sul2*, *aac(6')-lb-cr* and *floR*. Especially, positive correlation with tetracycline resistance genes has the potential to complicate antibiotic treatment of some human diseases because tetracycline is currently being used as an antibiotic to treat atypical pneumonia caused by *Mycoplasma pneumonia* (Pereyre et al., 2016). Considering the growing body of evidence for the potential of re-emergence of HBPs in various environments (Aw and Rose, 2012; Cleaveland et al., 2001), additional studies regarding their evolution in treatments such as air-drying and landfills are urgently needed. Furthermore, some types of HBPs may be overlooked due to the potential of PCR bias. Thus, other approaches (e.g., direct shotgun sequencing) should be considered in further studies.

4. Conclusion

Considering the abundance of total ARGs as well as the rebound of some ARGs after a mesophilic stage, the application of TAD as the final digestion stage of TPAD could be reasonable. However, in terms of *iniII*, MAD as the final digestion stage of TPAD might be appropriate. An internal or external recycle could solve this dilemma. Given that TAD is

usually used in the first stage of TPAD to achieve higher methane production, recycling digested sludge from M^{2nd} to T^{1st} might result in the efficient removal of both ARGs and *intII* as well as the efficient production of methane.

Acknowledgements

This work was supported by GIST Research Institute(GRI) grant funded by the GIST in 2018.

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Figure and Table captions:

Figure 1. Residual gene fractions of (a) tetracycline and sulfonamide resistance genes and (b) others including quinolones, β -lactams, macrolides, florfenicol, multidrug resistance genes,

intI1 and metal resistance genes. (C= Abundance of gene copies (copies/g VS) in treated samples, C₀= Abundance of gene copies (copies/g VS) in initial samples).

Figure 2. Taxonomic compositions of (a) bacterial communities at the phylum level (sequences showing percentages of reads < 1 % in all samples were grouped into ‘Others’) and (b) at the genus level (top 25 genera).

Figure 3. Venn diagram showing the number of shared bacterial OTUs in all samples.

Figure 4. Phylogenetic relationships and relative abundance of the top 100 shared OTUs.

Figure 5. Detection frequency and relative abundance of the human bacterial pathogens found in this study.

Table 1. Correlation between ARGs, HMRGs and *intI1* (* Significant at P < 0.05, * * Significant at P < 0.01).

Table 2. Correlation between ARGs, HMRGs and *intI1* and bacterial phyla (relative abundance > 1.0 %; * Significant at P < 0.05, * * Significant at P < 0.01).

Table 3. Correlation between ARGs, HMRGs and *intI1* and human bacterial pathogens (* Significant at P < 0.05, * * Significant at P < 0.01).

Table 1. Correlation between ARGs, HMRGs and *intI1* (* Significant at $P < 0.05$, * * Significant at $P < 0.01$).

	<i>tetA</i>	<i>tetB</i>	<i>tetD</i>	<i>tetE</i>	<i>tetG</i>	<i>tetM</i>	<i>tetQ</i>	<i>tetX</i>	<i>tetZ</i>	<i>sul1</i>	<i>sul2</i>	<i>qnrD</i>	<i>aac(6') -lb-cr</i>
<i>intI1</i>	0.541	-0.079	-0.096	0.645	0.94*	0.212	0.922*	0.924*	0.733	0.863	0.933*	0.314	0.98**
<i>copA</i>	0.532	0.485	0.298	0.96**	0.726	0.794	0.733	0.722	0.888*	0.841	0.737	0.947*	0.63
<i>pcoA</i>	0.812	0.4	0.316	0.899*	0.889*	0.571	0.862	0.848	0.922*	0.904*	0.911*	0.72	0.898*
<i>tcrB</i>	0.557	0.683	0.492	0.847	0.441	0.891*	0.444	0.421	0.724	0.58	0.459	0.951*	0.354
	<i>bla_{SHV}</i>	<i>bla_{TEM}</i>	<i>bla_{CTX}</i>	<i>oqxA</i>	<i>ermB</i>	<i>floR</i>	<i>intI1</i>	<i>copA</i>	<i>pcoA</i>	<i>tcrB</i>	16S rRNA		
<i>intI1</i>	-0.12	0.965**	0.321	-0.142	0.92*	0.911*		0.463	0.837	0.179	0.737		
<i>copA</i>	0.585	0.528	0.788	-0.376	0.274	0.724	0.463		0.928*	0.786	0.667		
<i>pcoA</i>	0.242	0.936*	0.329	-0.6	0.833	0.814	0.837	0.928*		0.649	0.824		
<i>tcrB</i>	0.606	0.345	0.484	-0.694	0.139	0.393	0.179	0.786	0.649		0.459		

Table 2. Correlation between ARGs, HMRGs and *intI1* and bacterial phyla (relative abundance > 1.0 %; * Significant at $P < 0.05$, ** Significant at $P < 0.01$).

	<i>tetA</i>	<i>tetB</i>	<i>tetD</i>	<i>tetE</i>	<i>tetG</i>	<i>tetM</i>	<i>tetQ</i>	<i>tetX</i>	<i>tetZ</i>	<i>sul1</i>	<i>sul2</i>	<i>qnrD</i>	<i>aac(6')-lb-cr</i>
<i>Bacteroidetes</i>	0.317	0.584	0.376	0.749	0.326	0.821	0.345	0.330	0.612	0.491	0.336	0.904*	0.198
<i>Cloacimonetes</i>	0.137	0.101	0.301	-0.580	-0.517	-0.612	-0.574	-0.579	-0.464	-0.573	-0.456	-0.531	-0.380
<i>Proteobacteria</i>	0.624	0.219	0.114	0.916*	0.980**	0.515	0.969**	0.967**	0.946*	1.000**	0.987**	0.708	0.949*
<i>Spirochaetes</i>	-0.706	-0.468	-0.315	-0.961**	-0.806	-0.810	-0.801	-0.782	-0.905*	-0.862	-0.811	-0.863	-0.769
<i>Chloroflexi</i>	-0.518	-0.541	-0.411	-0.835	-0.613	-0.488	-0.599	-0.600	-0.835	-0.760	-0.664	-0.872	-0.516
<i>Aminicenantes</i>	-0.654	-0.582	-0.424	-0.971**	-0.738	-0.699	-0.727	-0.718	-0.939*	-0.862	-0.773	-0.963**	-0.657
<i>Firmicutes</i>	-0.817	-0.716	-0.653	-0.637	-0.362	-0.578	-0.323	-0.295	-0.607	-0.428	-0.412	-0.667	-0.389
<i>Verrucomicrobia</i>	0.643	0.692	0.513	0.916*	0.555	0.848	0.550	0.530	0.825	0.691	0.583	0.984**	0.475
<i>Caldiiserica</i>	0.016	0.305	0.308	-0.285	-0.577	0.172	-0.580	-0.606	-0.388	-0.557	-0.573	-0.085	-0.539
<i>Thermotogae</i>	-0.597	-0.027	-0.048	-0.431	-0.647	-0.211	-0.620	-0.607	-0.491	-0.545	-0.642	-0.158	-0.741
<i>Planctomycetes</i>	0.699	0.277	0.166	0.915*	0.935*	0.640	0.924*	0.912*	0.911*	0.938*	0.933*	0.709	0.927*
<i>Synergistetes</i>	-0.923*	-0.630	-0.583	-0.884*	-0.789	-0.381	-0.737	-0.729	-0.964**	-0.869	-0.866	-0.797	-0.791
<i>Actinobacteria</i>	0.346	-0.233	-0.093	-0.049	0.337	-0.331	0.297	0.297	0.093	0.183	0.343	-0.333	0.479
<i>Parcubacteria</i>	0.799	0.584	0.475	0.970**	0.826	0.563	0.795	0.788	0.995**	0.923*	0.878	0.902*	0.784
<i>Atribacteria</i>	-0.833	-0.761	-0.635	-0.941*	-0.645	-0.689	-0.614	-0.597	-0.919*	-0.772	-0.703	-0.966**	-0.601
<i>Candidatus Saccharibacteria</i>	0.417	-0.136	-0.209	0.723	0.981**	0.371	0.985**	0.987**	0.762	0.919*	0.950*	0.405	0.979**
<i>Ignavibacteriae</i>	0.520	0.024	-0.067	0.827	0.999**	0.452	0.997**	0.997**	0.860	0.973**	0.984**	0.552	0.985**

Table 2. (Continued)

	<i>bla_{SHV}</i>	<i>bla_{TEM}</i>	<i>bla_{CTX}</i>	<i>oqx_A</i>	<i>erm_B</i>	<i>flo_R</i>	<i>int_{II}</i>	<i>cop_A</i>	<i>pco_A</i>	<i>tc_RB</i>
<i>Bacteroidetes</i>	0.690	0.101	0.693	-0.394	-0.157	0.332	-0.001	0.884*	0.414	0.925*
<i>Cloacimonetes</i>	-0.240	-0.147	- 0.962**	-0.241	0.118	-0.610	-0.239	-0.744	-0.258	-0.495
<i>Proteobacteria</i>	0.286	0.871	0.664	-0.233	0.699	0.968**	0.870	0.832	0.902*	0.567
<i>Spirochaetes</i>	-0.340	-0.776	-0.464	0.663	-0.613	-0.744	-0.654	-0.882*	-0.939*	-0.855
<i>Chloroflexi</i>	-0.784	-0.376	-0.839	0.119	-0.120	-0.639	-0.357	-0.905*	-0.564	-0.708
<i>Aminicenantes</i>	-0.670	-0.575	-0.737	0.403	-0.336	-0.724	-0.499	- 0.984**	-0.781	-0.863
<i>Firmicutes</i>	-0.339	-0.557	0.146	0.976**	-0.533	-0.230	-0.322	-0.494	-0.747	-0.730
<i>Verrucomicrobia</i>	0.641	0.456	0.541	-0.659	0.243	0.508	0.305	0.934*	0.735	0.987**
<i>Caldiserica</i>	-0.103	-0.331	-0.709	-0.655	-0.203	-0.675	-0.531	-0.313	-0.187	0.158
<i>Thermotogae</i>	0.311	-0.882*	0.223	0.538	- 0.960**	-0.546	-0.804	-0.176	-0.777	-0.156
<i>Planctomycetes</i>	0.168	0.928*	0.439	-0.514	0.800	0.880*	0.858	0.784	0.984**	0.652
<i>Synergistetes</i>	-0.575	-0.809	-0.391	0.502	-0.684	-0.726	-0.712	-0.758	-0.907*	-0.633
<i>Actinobacteria</i>	-0.522	0.633	-0.515	-0.177	0.824	0.257	0.630	-0.319	0.386	-0.379
<i>Parcubacteria</i>	0.614	0.741	0.613	-0.438	0.549	0.790	0.663	0.910*	0.886*	0.761
<i>Atribacteria</i>	-0.675	-0.616	-0.450	0.685	-0.438	-0.579	-0.460	-0.891	-0.846	-0.905*
<i>Candidatus Saccharibacteria</i>	-0.086	0.903*	0.541	-0.060	0.794	0.981**	0.962**	0.604	0.813	0.286
<i>Ignavibacteriae</i>	0.076	0.908*	0.602	-0.150	0.769	0.993**	0.939*	0.720	0.873	0.425

Table 3. Correlation between ARGs, HMRGs and *intI1* and human bacterial pathogens (* Significant at $P < 0.05$, ** Significant at $P < 0.01$).

	<i>tetA</i>	<i>tetB</i>	<i>tetD</i>	<i>tetE</i>	<i>tetG</i>	<i>tetM</i>	<i>tetQ</i>	<i>tetX</i>	<i>tetZ</i>	<i>sul1</i>	<i>sul2</i>	<i>qnrD</i>	<i>aac(6')-lb-cr</i>
<i>Legionella pneumophila</i>	0.031	-0.292	-0.142	-0.445	-0.170	-0.389	-0.193	-0.200	-0.372	-0.326	-0.183	-0.598	-0.026
<i>Mycoplasma pneumoniae</i>	0.690	0.292	0.200	0.920*	0.965	0.475	0.945*	0.943*	0.965*	0.996*	0.987**	0.726	0.941*
<i>Chlamydia trachomatis</i>	-0.166	0.220	0.001	0.332	-0.061	0.836	-0.001	-0.021	0.096	0.035	-0.121	0.508	-0.184
<i>Corynebacterium diphtheriae</i>	0.804	0.840	0.774	0.786	0.481	0.359	0.429	0.422	0.836	0.649	0.584	0.884*	0.434
<i>Mycobacterium tuberculosis</i>	0.642	0.929*	0.822	0.657	0.169	0.519	0.134	0.119	0.619	0.370	0.258	0.888*	0.097
<i>Enterococcus faecalis</i>	0.592	0.131	0.047	0.860	0.991**	0.424	0.979**	0.980**	0.909*	0.990*	0.996**	0.616	0.973**
<i>Bacillus anthracis</i>	-0.891	-0.474	-0.566	-0.429	-0.495	0.065	-0.416	-0.408	-0.584	-0.488	-0.584	-0.298	-0.598
<i>Staphylococcus aureus</i>	-0.318	0.237	0.064	0.055	-0.384	0.595	-0.330	-0.346	-0.163	-0.267	-0.424	0.330	-0.504
<i>Clostridium difficile</i>	-0.365	-0.571	-0.379	-0.795	-0.427	-0.734	-0.438	-0.429	-0.698	-0.593	-0.449	-0.919*	-0.300
<i>Clostridium perfringens</i>	-0.582	-0.029	0.027	-0.670	-0.844	-0.466	-0.835	-0.822	-0.684	-0.766	-0.818	-0.384	-0.888*
	<i>bla_{SHV}</i>	<i>bla_{TEM}</i>	<i>bla_{CTX}</i>	<i>oqxA</i>	<i>ermB</i>	<i>floR</i>	<i>intI1</i>	<i>copA</i>	<i>pcoA</i>	<i>tcrB</i>			
<i>Legionella pneumophila</i>	-0.683	0.186	-0.863	-0.229	0.451	-0.258	0.143	-0.662	-0.020	-0.507			
<i>Mycoplasma pneumoniae</i>	0.347	0.876	0.630	-0.265	0.710	0.945*	0.863	0.825	0.914	0.570			
<i>Chlamydia trachomatis</i>	0.217	-0.244	0.376	-0.359	-0.417	-0.050	-0.341	0.507	0.040	0.710			
<i>Corynebacterium diphtheriae</i>	0.906*	0.410	0.490	-0.415	0.231	0.445	0.299	0.777	0.622	0.718			
<i>Mycobacterium tuberculosis</i>	0.924*	0.102	0.364	-0.572	-0.077	0.124	-0.070	0.715	0.418	0.842			
<i>Enterococcus faecalis</i>	0.207	0.893*	0.633	-0.157	0.741	0.984**	0.915*	0.760	0.880*	0.460			
<i>Bacillus anthracis</i>	-0.195	-0.761	0.267	0.556	-0.835	-0.380	-0.642	-0.177	-0.717	-0.171			
<i>Staphylococcus aureus</i>	0.278	-0.559	0.234	-0.215	-0.697	-0.360	-0.646	0.274	-0.274	0.526			
<i>Clostridium difficile</i>	-0.749	-0.174	-0.794	0.279	0.098	-0.447	-0.106	-0.924	-0.456	-0.875			
<i>Clostridium perfringens</i>	0.203	-0.952	-0.119	0.501	-0.931*	-0.769	-0.894	-0.470	-0.900*	-0.376

Figure 1. Residual gene fractions of (a) tetracycline and sulfonamide resistance genes and (b) others including quinolones, β -lactams, macrolides, florfenicol, multidrug resistance genes, *intI1* and metal resistance genes. (C= Abundance of gene copies (copies/g VS) in treated samples, C₀= Abundance of gene copies (copies/g VS) in initial samples).

Figure 2. Taxonomic compositions of (a) bacterial communities at the phylum level (sequences showing percentages of reads < 1 % in all samples were grouped into ‘Others’) and (b) at the genus level (top 25 genera).

Figure 3. Venn diagram showing the number of shared bacterial OTUs in all samples.

Fig. 4. Phylogenetic relationships and relative abundance of the top 100 shared OTUs.

Fig. 5. Detection frequency and relative abundance of the human bacterial pathogens found in this study