

AperTO - Archivio Istituzionale Open Access dell'Università di Torino

Post-digestate composting shifts microbial composition and degrades antimicrobial resistance genes

This is a pre print version of the following article:

Original Citation:

Availability:

This version is available <http://hdl.handle.net/2318/1795358> since 2021-07-30T09:02:03Z

Published version:

DOI:10.1016/j.biortech.2021.125662

Terms of use:

Open Access

Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)

1 Post-digestate composting shifts microbial composition and degrades
2 antimicrobial resistance genes

3
4 Biyensa Gurmessa^{1*}, Vesna Milanovic¹, Ester Foppa Pedretti¹, Giuseppe Corti¹, Amanda J.
5 Ashworth², Lucia Aquilanti¹, Ilario Ferrocino³, Maria Rita Corvaglia³, Stefania Cocco¹

6 ¹ Department of Agriculture, Food and Environmental Sciences, Università Politecnica delle
7 Marche, Via Brecce Bianche 10, 60131 Ancona, Italy

8 ² USDA-ARS, Poultry Production and Product Safety Research Unit, 1260 W. Maple St.,
9 Fayetteville, AR 72701, USA

10 ³ Department of Agricultural, Forest, and Food Science, University of Turin, Largo Paolo
11 Braccini 2, Grugliasco, Torino, Italy

12 *Corresponding author: biyensa@gmail.com

13

14

15

16

17

18

19

20

1 **Abstract**

2 Post-digestate treatments **may** reduce the risk linked to **Antibiotic Resistant Genes (ARGs)**
3 **release with digestate direct land application. Thus, this study** aimed to evaluate post-digestate
4 composting and co-composting with biogas production feedstock (maize silage, food
5 processing waste, and poultry litter) effect on abundance **of selected ARGs: *erm* (B), *tet* (K),**
6 *tet* (M), *tet* (O), and *tet* (S) genes. More than 80% of all **ARGs** were removed after 90 days of
7 composting but removals from co-composting were **lower**. Bacteroidetes, Firmicutes, and
8 Proteobacteria dominated fresh digestate, but a network analysis indicated only a few genera
9 were potential hosts of ARGs. Canonical correspondence analysis showed more than 90%
10 variations in ARGs abundance were explained by water extractable trace elements, indicating
11 a strong relationship. The study **illustrates** the **potential** of post-digestate composting to
12 mitigate ARGs **in the environment.**

13

14 Keywords: ARGs removal; biogas feedstock; co-composting; poultry litter; trace elements

15

16

17

18

19

1. Introduction

Antibiotic resistance genes (ARGs) have been threatening the global health sector and some claim inappropriate use of antibiotics are the main drivers (He et al., 2020). Efforts made to reduce or mitigate the phenomenon of antibiotic resistance include banning growth promoters (Callens et al., 2018), avoiding inappropriate use of drugs (Holmes et al., 2016; Llor and Bjerrum, 2014), and developing novel drugs from natural products (Jackson et al., 2018). Moreover, mitigating the spread of ARGs in the environment is essential to reduce potential health risks. There are several pathways through which ARGs can be released to the environment such as manure land application (Tien et al., 2017), disposal of poorly treated pharmaceutical sludge (Tong et al., 2018), and digestate land application (Derongs et al., 2020).

Anaerobic digestion (AD) of organic wastes is widely reported to mitigate the spread of ARGs despite the rate of removal varying not only by the type of ARGs but also the type and scale of the reactor, retention time, temperature, feedstock type, etc (Gurmessa et al., 2020). The ultimate goal of AD is energy production, but it has been widely reported that changes in the biological and physicochemical processes could remove ARGs (Couch et al., 2019; Sun et al., 2018; Zhang et al., 2018). However, AD does not always help remove ARGs. It has been reported that AD plants can also be a hub for the emergence of new or enrichment of the existing ARGs, promoted by the favorable conditions in the reactors, such as the possible accumulation of antibiotic residues and microbial community dynamics (Ma et al., 2011; Wallace et al., 2018). There is therefore a concern related to the possible spread of ARGs with direct use of digestate as a fertilizer, although this is largely not been studied.

Where AD could suppress abundance of ARGs in organic wastes, inefficiency is possible, thus post-digestate treatments have been suggested to reduce the release of ARGs with digestate use.

1 Post-digestate composting is one of such strategies (Zhang et al., 2019), and it has been reported
2 to remove about 75% of selected tetracycline resistance genes *tet(G)*, *tet(C)* and *tet(Q)* from
3 cattle manure (Qian et al., 2018). ARGs removal with composting is affected by changes in
4 temperature (thermophilic and mesophilic), dynamics of chemical compositions (heavy metals,
5 organic matter content, etc.), and shifts in microbial composition (Oliver et al., 2020). Variation
6 in removal rate could also be due to waste material being composted and composting time
7 length.

8 Solid digestate has poor nutrient and low total solids (TS) content to effectively support air
9 movement and functionality of microbial activities during composting, which could negatively
10 influence final compost quality and time to maturity (Gurmessa et al., 2021b). On the other hand,
11 co-composting could have better advantages over composting a sole material, including
12 improving ARGs removal efficiency (Chen et al., 2021). Thus, co-composting digestate with
13 fresh and locally available inexpensive material having a better TS content and greater nutrient
14 supply for microbes could be sought for economically feasible effective industrial level
15 composting.

16 The current study aimed at evaluating post-digestate composting and co-composting with biogas
17 production feedstock effect on the removal of ARGs encoding the resistance to antibiotics
18 conventionally used in animal husbandry and clinical practice, such as macrolide-lincosamide-
19 streptogramin B (MLS_B) [*erm(B)*] and tetracyclines [*tet(K)*, *tet(M)*, *tet(O)*, *tet(S)*] using
20 quantitative PCR (qPCR) assays. The study hypothesized composting solid digestate or co-
21 composting of the same with inputs for biogas production further removes significant
22 proportions of ARGs and alters microbial composition.

1 2. Materials and Methods

2 2.1. Experimental setup and sampling

3 Digestate (fresh) was obtained from a biogas plant located in Marche Region, Italy. It is a
4 byproduct of anaerobic digestion consisting of about 10% poultry litter (composed of chicken
5 manure and wheat straw in a rough ratio of 85 and 15%, respectively) and 90% of mix of other
6 biomass such as maize silage, food processing waste, and fruit processing byproducts. Pilot level
7 composting and co-composting piles were set up as described in **Table 1, each amounting to 300**
8 **kg (on wet basis)** and comprising of digestate and locally available materials in a ratio of 4:1
9 (w/w). The experiment was set up in completely randomized design with sub-sampling. The
10 composting materials were stacked inside high-density polyethylene boxes, each having volume
11 of 1 m³. The boxes were modified to allow air movement, and the piles were turned weekly
12 during the thermophilic phase (**≈49 days**), every two weeks during the mesophilic phase (**≈40**
13 **days**), and every three weeks during the maturity phase. Composting lasted for 90 days, and
14 samples were collected from each box at 0, 7, 35, 70, and 90 days of composting. **Three** samples
15 were collected at about 5, 15, and 20 cm depth, after turning and thorough mixing. Then, 200 g
16 of each sample was immediately stored at -20°C in a plastic bottle until analyzed for ARGs and
17 microbial composition, whereas about 1 kg was dried at 40°C for chemical analysis.

18 2.2. Chemical analysis

19 Samples dried at 40 °C (**about 1kg each**) were ground, passed through a 2 mm sieve, and used to
20 measure pH and total C and N content. The pH was determined potentiometrically in H₂O (1:8
21 w/v). Total C and N were determined by dry combustion method using a CHNS analyzer (EA-
22 1110, Carlo Erba Instruments, Milan, Italy). Total solid content (TS) was estimated as the
23 fraction of the dry mass after samples were dried at 105 °C for 24 h. Organic matter (OM) was

1 determined as the loss on ignition (Heiri et al., 2001) at 550 °C until constant weight was
2 obtained. Water extractable Al, Ca, Ba, Cd, Cu, Fe, Mg, Mn, Na, Ni, P, Pb, S, and Zn were
3 determined by using 10 g of ground compost samples that were added to 100 ml of distilled
4 water (1:10 w/v) and shaken for about 1 h at 10 rpm. The suspension was then centrifuged for 10
5 minutes at 300 g and the solution filtered using Whatman 42 filter. The extract was used to
6 determine the concentration of the elements on an ICP-MS (Pröfrock and Prange, 2012).

7 2.3. DNA extraction and qPCR quantification of AR genes

8 DNA was extracted from 0.25 g of each compost sample using E.Z.N.A. ®Soil DNA Kit
9 (OMEGA Bio-Tek, Inc., Norcross, GA, USA) according to the manufacturer's guidelines. Prior
10 to qPCR analysis, the extracted DNA was checked for quantity and purity using a Nanodrop ND
11 1000 (Thermo Fisher Scientific, Wilmington, DE, USA). Moreover, the effective extraction of
12 the bacterial DNA was checked by end-point PCR using the universal procaryotic primer pair
13 27f-1495r (Weisburg et al., 1991) targeting the bacterial 16S rRNA gene. DNA extracted from
14 five reference bacteria strains, each carrying one of the ARGs under study were used as positive
15 controls in the qPCR reactions as well as for the construction of qPCR standard curves as
16 previously described by Vandeweyer et al. (2019). Each qPCR mixture was composed of 4 µL of
17 extract, 5 µL of Type-it 2X HRM PCR Master Mix (Qiagen, Hilden, Germany), 900 nm of
18 forward and reverse primers for each AR gene (Table 2), and nuclease-free water to reach the
19 final reaction volume of 10 µL. The qPCR reactions were performed in a Mastercycler® ep
20 realplex machine (Eppendorf, Hamburg, Germany) with an initial denaturation step of 5 min at
21 95 °C followed by 40 cycles of 95 °C for 15 s. The qPCR correlation coefficients (R^2) and
22 amplification efficiencies were calculated automatically by Mastercycler® ep realplex software

1 from the slopes of the standard curves. The qPCR detection limit for each ARG gene in study was
2 estimated from standard curves created in the range from $\sim 10^0$ to 10^7 gene copies per reaction.
3 For absolute quantification of the five ARGs in study, each extract from the compost samples
4 was run in triplicate along with tenfold serial dilutions of the standards. Each gene copy number
5 detected in the analyzed samples was determined from the slope of the corresponding standard
6 curve. The blank (nuclease-free water instead of DNA extract) and the negative control [DNA
7 extracted from *Enterococcus faecalis* JH2-2 strain (Jacob and Hobbs, 1974)] were run together
8 with the samples. The melt curve analysis with the temperature gradually increasing from 60 to
9 95°C by 0.4°C/s was performed to check amplification specificity. The results are presented as:
10 *i*) the log of average gene copy number per gram of dry matter of each sample \pm standard
11 deviation and *ii*) the fold change, which was estimated to evaluate the reduction of ARGs
12 abundance during the composting period. The latter was estimated as log of the quotient of
13 ARGs copy number in composted sample divided by that in initial digestate sample as previously
14 described by Qian et al. (2018).

15 2.4. 16S rRNA gene amplicon target sequencing and bioinformatics analysis

16 DNA was used as a template for the amplification of the V3-V4 region of the 16S rRNA gene
17 using primers and PCR condition described by Klindworth et al. (2013). PCR amplicons were
18 then purified, tagged, and pooled following the Illumina metagenomic flow. Sequencing (paired
19 end mode 2X250bp) was performed on a MiSeq Illumina platform according to the
20 manufacturing instructions. After sequencing, reads were assembled by using the FLASH
21 software. Joined reads were quality filtered with QIIME software and USEARCH was then used
22 for removing chimeric sequencing. Sequences were clustered into operational taxonomic units
23 (OTUs) at 97% of similarity and after the picking step of each centroids sequence taxonomy was

1 performed against the greengenes database by means the RDP classifier. OTUs table generated
2 by QIIME was rarefied at the lowest number of sequences for sample.

3 2.5. Statistical analysis

4 Analysis of variance (ANOVA) conducted on log₁₀ of ARGs abundance in digestate and final
5 composts using Agricolae package in R, and least significance difference (LSD) tests were
6 conducted to separate means when statistically significant results were obtained in the general
7 ANOVA model. To understand the relationship between ARGs abundance and the
8 environmental variables (chemical compositions and enzyme activities), Canonical
9 correspondence analysis (CCA) was conducted using the vegan package in R (Oksanen et al.,
10 2019). Non-metric multidimensional scaling (NMDS) was analyzed and plotted on the OTUs
11 using the Bray-Curtis dissimilarity distances using ecodist package in R to evaluate differences
12 in bacterial community structure among the different piles or shifts in the same from the fresh
13 digestate to thermophilic and mesophilic phases of the composts. Multivariate analysis of
14 variance (MANOVA) test was performed to evaluate the significance of the factors (piles or
15 phases) on the OTUs (response variable). Both the CCA were conducted using and plotted using
16 ggplot2 in R. Additionally, network analysis was conducted to understand co-occurrence of the
17 ARGs and bacteria at phylum and genus level using R (for generating the weight values) and
18 Gephi (for visualization). The weight values were positive and significant ($P < 0.05$) coefficient
19 values of Pearson's correlation.

1 3. Results and discussions

2 3.1. Physicochemical characteristics of composts

3 Temperature, pH, C/N ratio, organic matter (OM) content, water extractable macro nutrients, and
4 trace elements were analyzed for digestate and the co-composting materials, and these were
5 monitored over the composting period. Thermophilic temperature lasted for about 49 days.
6 Organic matter declined over the composting period. While major macro nutrients, except Ca
7 and S, increased, trace elements, except Cd and Pb, reduced over composting period. Details of
8 the dynamics of these parameters were reported in Gurmessa et al. (2021b).

9 3.2. ARGs abundance in digestate and co-composting materials

10 qPCR standard curves created for each of the five ARGs were characterized by good
11 amplification efficiencies, comprised between 0.91 and 0.99 for *tet(K)* and *tet(S)* genes,
12 respectively, whereas R² values were 0.99 for all reactions. The lowest gene copy number per
13 reaction in which the linearity was maintained (detection limit) was < 10¹ for the genes *erm(B)*
14 and *tet(O)*, and 10² for *tet(K)*, *tet(M)*, and *tet(S)*.

15 All the five ARGs were detected in the digestate and co-composting materials (Fig. 1), but with
16 great variations in number of copies among the types and co-composting materials, ranging from
17 3.6 log *tet(O)* in food processing waste to 11.3 log *tet(M)* in poultry litter. Poultry litter contained
18 the greatest copies ($p < 0.05$) of all ARGs followed by digestate, whereas maize silage and food
19 processing waste had the lowest copies of ARGs. Generally, *tet(M)* was the most abundant gene
20 ($p < 0.001$) in digestate and co-composting materials followed by *tet(K)* and *erm(B)*, whereas
21 *tet(O)* and *tet(S)* were the least abundant gene in all the materials. The abundance of *erm(B)*,

1 *tet(O)*, and *tet(S)* in poultry litter was about two-fold log higher than that of maize silage and
2 food processing waste.

3 Similarly, Agga et al. (2020) reported *tet(M)* as the most abundant ARG in digestates of three
4 manure types suggesting possible differences in ARGs persistence under anaerobic digestion
5 process.

6 3.3. Dynamics of ARGs during post-digestate composting and co-composting

7 In Fig. 2, the fold change illustrates dynamics of ARGs abundance during the 90 days of
8 composting. ARGs abundance was affected both by the composition of the piles and composting
9 time. However, the change in the abundance of most of the ARGs was not consistent during the
10 thermophilic and mesophilic phases. During the thermophilic phase, the abundance of all genes
11 but *erm(B)* decreased. The abundance of *tet(K)* gene increased during the mesophilic phase
12 although it was reduced at maturity. In contrast, *tet(S)* copies were potentially reduced after a
13 week of composting and, unlike other ARGs, enrichment was rarely observed. **E-supplementary**
14 **data of this work can be found in online version of the paper. This implies temperature dynamics**
15 **over the composting period does not influence ARGs similarly.** Compared to the digestate only
16 pile (D00), ARGs abundance in the other piles was greater, and it was even the greatest in the
17 DPL at the initial period, implying that the co-composting materials could be potential source of
18 ARGs, particularly poultry litter (Fig.1). **ARGs abundance in poultry litter, however, largely**
19 **relies on how it has been handled or managed before use (Gurmessa et al., 2021a).**

20 Relative to the fresh digestate, *tet(O)* was reduced by the greatest fold compared to the other
21 genes investigated (Fig. 2), which was -6.8, -7.6, -8.7, and -10.9 log in the final composts of
22 DPL, DMS, DCB, and D00, respectively. In contrast, the least reduction was found for *erm(B)*,
23 indicating the relatively higher persistence of this gene compared to the tetracycline resistant

1 genes, despite its relative abundance was lower than the other ARGs in the starting materials.
2 Interestingly, ARGs abundance in D00 was reduced by greater fold than in the other piles. In the
3 final composts, the lowest abundance of ARGs was found in D00 whereas the greatest was found
4 in DPL. E-supplementary data of this work can be found in online version of the paper. These
5 results suggest that the materials, especially poultry litter, used for co-composting might have
6 promoted or be the source of the ARGs. This can be further explained by the positive log change
7 in the DPL (Fig. 2), showing enrichment in ARGs with the addition of poultry litter to digestate.
8 It must be noted that fold change results discussed here were only in reference to the initial
9 content in the piles. Compared to the copies number in the digestate, the benefit of using co-
10 composting materials was not satisfactory, as *tet(K)* and *tet(M)* copies were enriched in DCB and
11 DMS piles.

12 3.4. Bacteria community dynamics based on the 16S rRNA gene amplicon target 13 sequencing

14 Three phyla dominated the bacteria community of fresh digestate: Firmicutes were the most
15 abundant (73%), followed by Proteobacteria (18%), and Bacteroidetes (9%) (Fig. 3A). During
16 composting, succession of Actinobacteria, Planctomycetes, and Verrucomicrobia formed a
17 distinct bacteria community structure compared to that of fresh digestate. The emergence and
18 presence of Actinobacteria in high proportion over the composting period indicates the well-
19 functioning of the composts (Sundberg et al., 2013). At genus level, *Clostridium* (38%),
20 *Sporosarcina* (33%), *Bacillus* (7%), and *Pseudomonas* (7%) were the top four dominant OTUs
21 (Fig.3B).

1 Non-metric multidimensional scaling (NDMS) of the Bray-Curtis dissimilarity analysis was
2 conducted to understand the effect of co-composting or temperature regime on bacterial
3 community structure. The influence of piles on bacterial community was significant ($p < 0.01$)
4 (Fig. 4A), with significant effect on abundance of Firmicutes ($p < 0.001$) and Bacteroidetes (p
5 < 0.05) as MANOVA test revealed. The change in bacteria community structure was further
6 elaborated with the non-metric multidimensional scaling (NDMS) of the Bray-Curtis
7 dissimilarity analysis, which showed a significant ($p < 0.05$) shift in the bacterial community
8 structure between the digestate and the two phases of composting (Fig. 4B). MANOVA test
9 showed Firmicutes were significantly ($p < 0.001$) affected, and their relative abundance was
10 reduced during the shift from initial phase to the thermophilic and mesophilic phases. The shift
11 in the abundance of these phylum and the succession of new phyla or genera over the
12 composting period may be indicator of the compost status.

13 Verrucomicrobia was not found in DCB during the thermophilic and mesophilic phases, nor in
14 DPL during the mesophilic phase. This phylum is ubiquitous in soils, but its habitat is influenced
15 by temperature, pH, and chemical composition (Freitas et al., 2012). Since it appeared during the
16 composting period, it could be an indicator of a well-functioning compost.

17 Planctomycetes were observed in DCB, DMS, and DPL only during the mesophilic phase. The
18 presence of this bacteria phyla could be an indicator of the transitioning from thermophilic to
19 mesophilic phase. In contrast, its absence in the D00 might be due to the lack of carbohydrates
20 in this pile, unlike the other piles which contained fresh materials (Buckley et al., 2006).

21 3.5. Co-occurrence of ARGs and bacteria

22 The co-occurrence of ARGs and bacteria (OTUs at genus level) was studied by Network analysis
23 conducted on Pearson correlation coefficients ($P < 0.05$) (Fig. 5). Despite more than 100

1 significant connections observed among the different genera, the results showed the exclusive
2 co-occurrence of ARGs and the dominant bacterial phyla from the digestate such as
3 Bacteroidetes (*Arenibacter* and *Fluviicola*), Proteobacteria (*Steroidobacter* and *Cellvibrio*) and
4 Firmicutes (*Symbiobacterium*) (Fig. 3A). *Arenibacter* showed a significantly strong correlation
5 with all the ARGs, thus implying this genus could be their potential host. In addition, *Cellvibrio*
6 displayed co-occurrence with all ARGs except for *tet(O)*. Furthermore, *Fluviicola*,
7 *Steroidobacter*, and *Symbiobacterium* co-occurred with *tet(M)* gene, which implies that *tet(M)*
8 might have a broader host range compared to the other ARGs. Unlike what was previously
9 reported during the composting of poultry litter by Cui et al. (2016), Actinobacteria,
10 Verrucomicrobia, or Planctomycetes, which emerged during the composting period, did not
11 show co-occurrence with any of the ARGs showing other bacteria in the digestate were the
12 potential hosts, and the shift in the composition during the composting was responsible for the
13 degradation of ARGs.

14 *Arenibacter* is ubiquitous in various ecosystems and known for its capability of degrading high
15 molecular weight substances including organic matter and pollutants (Roy et al., 2020; Stiborova
16 et al., 2020). However, its potential as a host of ARGs has rarely been reported. In contrast,
17 *Cellvibrio* was reported to be host of multiple ARGs in animal waste (Gou et al., 2021; Han et
18 al., 2018), despite its significant functional role in lignocellulose degradation during composting
19 (Raut et al., 2021). It also constitutes significant proportion of the bacteria communities in DPL
20 and DMS, possibly due to the presence of lignocellulosic substrate (wheat straw and maize silage
21 components, respectively). Within the Proteobacteria, *Steroidobacter* was the other potential host
22 of *tet(M)*. Previously, Wan et al. (2017) reported *Steroidobacter* as a potential host for selected
23 ARGs in pig waste water. In the current study, it was found that the maize silage component

1 might promote the abundance of *Steroidobacter*, as it was only found in the DMS pile without
2 being affected by both the thermophilic and mesophilic temperature regimes. *Fluviicola* and
3 *Symbiobacterium* belonging to Bacteroidetes and Firmicutes, respectively, co-existed and were
4 found to be potential co-host for *tet(M)*.

5 3.6. Relationship between ARGs abundance and chemical compositions

6 Canonical Correspondence Analysis (CCA) results showed variations in abundance of ARGs,
7 except *erm(B)*, could be well explained by the dynamics of chemical compositions over the
8 composting period. It was interesting that abundance *erm(B)* showed little relationships with the
9 chemical compositions, and this gene was also least affected by composting and had the lowest
10 removal rate.

11 The CCA results were visualized in Fig. 6. DCB formed distinct cluster, while there were
12 potential overlaps among the other piles. In Fig. 6A, dynamics of OM, pH, and extractable Ca,
13 Mg, and K explained about 64% of the total variations in the ARGs abundance. However, both
14 *tet(M)* and *erm(B)* had little relationship with these environmental variables. In contrast, *tet(O)*
15 positively related to C/N ratio, OM, Ca, and Mg contents, and negatively related with pH. Both
16 *tet(S)* and *tet(K)* had positive relationship with pH, K, and S. OM potentially contributed to the
17 total variance explained, showing ARGs degradation could rely on the OM stability of the
18 composts over the composting period.

19 As reported in Fig. 6B, trace elements (Al, Ba, Cd, Cu, Fe, Mn, Ni, Pb, and Zn) explained 90%
20 of the total variations in ARGs abundance. Particularly, Al, Cd, and Cu had positive
21 relationships (co-occurrence) with *tet(K)* and *tet(S)*, whereas *tet(M)* had positive relationships
22 with Fe, Mn, and Zn. Interestingly, *erm(B)* had no relationship with any of the trace elements,
23 whereas *tet(O)* showed positive relationship with Cd. The greater contents of trace elements

1 (Gurmessa et al., 2021b) and abundance of ARGs during the first phase and the significant
2 reductions during the maturity period suggests co-occurrences. The weak relationship between
3 trace elements and *erm(B)* could be linked to its weak response to composting as evidenced in
4 Fig.2. Similar to these findings, Cui et al. (2016) reported a strong co-occurrence of
5 bioavailable trace elements and ARGs during composting of poultry litter. These findings may
6 further give an insight into the significance of composting on stabilizing organic substance that
7 could lead to both immobilization of trace elements and reduction of ARGs.

8 3. Conclusions

9 Post-digestate composting removed more than 80% of ARGs from digestate, and it was at least
10 as effective as co-composting with maize silage and food processing waste. Despite the strong
11 co-occurrence among the several bacteria groups, only a few genera were potential hosts of
12 ARGs, and none of these belonged to those phyla (Actinobacteria, Planctomycetes, and
13 Verrucomicrobia) that succeeded following composting. Results suggest the shift in microbial
14 structure due to composting had little link with degradation of ARGs but could be an indicator of
15 a well-functioning compost, rather, the physicochemical dynamics during composting or co-
16 composting may be responsible for ARGs removal.

17

18

19

20

21

1 References

- 2
- 3 Agga, G.E., Kasumba, J., Loughrin, J.H., Conte, E.D., 2020. Anaerobic Digestion of
4 Tetracycline Spiked Livestock Manure and Poultry Litter Increased the Abundances of
5 Antibiotic and Heavy Metal Resistance Genes. *Front. Microbiol.* 11.
6 <https://doi.org/10.3389/fmicb.2020.614424>
- 7 Buckley, D.H., Huangyutitham, V., Nelson, T.A., Rumberger, A., Thies, J.E., 2006. Diversity of
8 Planctomycetes in soil in relation to soil history and environmental heterogeneity. *Appl.*
9 *Environ. Microbiol.* 72, 4522–4531. <https://doi.org/10.1128/AEM.00149-06>
- 10 Callens, B., Cargnel, M., Sarrazin, S., Dewulf, J., Hoet, B., Vermeersch, K., Wattiau, P., Welby,
11 S., 2018. Associations between a decreased veterinary antimicrobial use and resistance in
12 commensal *Escherichia coli* from Belgian livestock species (2011–2015). *Prev. Vet. Med.*
13 157, 50–58. <https://doi.org/10.1016/j.prevetmed.2017.10.013>
- 14 Chen, Z., Li, Y., Ye, C., He, X., Zhang, S., 2021. Fate of antibiotics and antibiotic resistance
15 genes during aerobic co-composting of food waste with sewage sludge. *Sci. Total Environ.*
16 784, 146950. <https://doi.org/10.1016/j.scitotenv.2021.146950>
- 17 Couch, M., Agga, G.E., Kasumba, J., Parekh, R.R., Loughrin, J.H., Conte, E.D., 2019.
18 Abundances of tetracycline resistance genes and tetracycline antibiotics during anaerobic
19 digestion of swine waste. *J. Environ. Qual.* 48, 171–178.
20 <https://doi.org/10.2134/jeq2018.09.0331>
- 21 Cui, E., Wu, Y., Zuo, Y., Chen, H., 2016. Effect of different biochars on antibiotic resistance
22 genes and bacterial community during chicken manure composting. *Bioresour. Technol.*
23 203, 11–17. <https://doi.org/10.1016/j.biortech.2015.12.030>
- 24 Derongs, L., Druilhe, C., Ziebal, C., Le Maréchal, C., Pourcher, A.M., 2020. Characterization of

1 clostridium perfringens isolates collected from three agricultural biogas plants over a one-
2 year period. *Int. J. Environ. Res. Public Health* 17, 1–12.
3 <https://doi.org/10.3390/ijerph17155450>

4 Flórez, A.B., Alegría, Á., Rossi, F., Delgado, S., Felis, G.E., Torriani, S., Mayo, B., 2014.
5 Molecular Identification and Quantification of Tetracycline and Erythromycin Resistance
6 Genes in Spanish and Italian Retail Cheeses. *Biomed Res. Int.* 2014.
7 <https://doi.org/10.1155/2014/746859>

8 Freitas, S., Hatosy, S., Fuhrman, J.A., Huse, S.M., Mark Welch, D.B., Sogin, M.L., Martiny,
9 A.C., 2012. Global distribution and diversity of marine Verrucomicrobia. *ISME J.* 6, 1499–
10 1505. <https://doi.org/10.1038/ismej.2012.3>

11 Gou, C., Wang, Y., Zhang, X., Zhong, R., Gao, Y., 2021. Effects of chlorotetracycline on
12 antibiotic resistance genes and the bacterial community during cattle manure composting.
13 *Bioresour. Technol.* 323, 124517. <https://doi.org/10.1016/j.biortech.2020.124517>

14 Gurmessa, B., Ashworth, A.J., Yang, Y., Savin, M., Moore, P.A., Ricke, S.C., Corti, G., Pedretti,
15 E.F., Cocco, S., 2021a. Variations in bacterial community structure and antimicrobial
16 resistance gene abundance in cattle manure and poultry litter. *Environ. Res.* 197, 111011.
17 <https://doi.org/10.1016/j.envres.2021.111011>

18 Gurmessa, B., Cocco, S., Ashworth, A.J., Foppa, E., Ilari, A., Cardelli, V., Fornasier, F., Letizia,
19 M., Corti, G., 2021b. Post-digestate composting benefits and the role of enzyme activity to
20 predict trace element immobilization and compost maturity. *Bioresour. Technol.* 338,
21 125550. <https://doi.org/10.1016/j.biortech.2021.125550>

22 Gurmessa, B., Pedretti, E.F., Cocco, S., Cardelli, V., Corti, G., 2020. Manure anaerobic digestion
23 effects and the role of pre- and post-treatments on veterinary antibiotics and antibiotic

1 resistance genes removal efficiency. *Sci. Total Environ.* 721.
2 <https://doi.org/10.1016/j.scitotenv.2020.137532>

3 Han, X.M., Hu, H.W., Chen, Q.L., Yang, L.Y., Li, H.L., Zhu, Y.G., Li, X.Z., Ma, Y.B., 2018.
4 Antibiotic resistance genes and associated bacterial communities in agricultural soils
5 amended with different sources of animal manures. *Soil Biol. Biochem.* 126, 91–102.
6 <https://doi.org/10.1016/j.soilbio.2018.08.018>

7 He, Y., Yuan, Q., Mathieu, J., Stadler, L., Senehi, N., Sun, R., Alvarez, P.J.J., 2020. Antibiotic
8 resistance genes from livestock waste: occurrence, dissemination, and treatment. *npj Clean*
9 *Water* 3, 1–11. <https://doi.org/10.1038/s41545-020-0051-0>

10 Heiri, O., Lotter, A., Lemcke, G., 2001. Loss on ignition as a method for estimating organic and
11 carbonate content in sediments. *J. Paleolimnol.* 25, 101–110.

12 Holmes, A.H., Moore, L.S.P., Sundsfjord, A., Steinbakk, M., Regmi, S., Karkey, A., Guerin,
13 P.J., Piddock, L.J.V., 2016. Understanding the mechanisms and drivers of antimicrobial
14 resistance. *Lancet* 387, 176–187. [https://doi.org/10.1016/S0140-6736\(15\)00473-0](https://doi.org/10.1016/S0140-6736(15)00473-0)

15 Jackson, N., Czaplewski, L., Piddock, L.J.V., 2018. Discovery and development of new
16 antibacterial drugs: Learning from experience? *J. Antimicrob. Chemother.* 73, 1452–1459.
17 <https://doi.org/10.1093/jac/dky019>

18 Jacob, A.E., Hobbs, S.J., 1974. Conjugal transfer of plasmid borne multiple antibiotic resistance
19 in *Streptococcus faecalis* var. *zymogenes*. *J. Bacteriol.* 117, 360–372.
20 <https://doi.org/10.1128/jb.117.2.360-372.1974>

21 Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., Glöckner, F.O., 2013.
22 Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-
23 generation sequencing-based diversity studies. *Nucleic Acids Res.* 41, 1–11.

1 <https://doi.org/10.1093/nar/gks808>

2 Llor, C., Bjerrum, L., 2014. Antimicrobial resistance : risk associated with antibiotic overuse and
3 initiatives to reduce the problem 5, 229–241. <https://doi.org/10.1177/2042098614554919>

4 Ma, Y., Wilson, C.A., Novak, J.T., Ri, R., Aynur, S., Murthy, S., Pruden, A., 2011. Effect of
5 Various Sludge Digestion Conditions on Sulfonamide , Macrolide , and Tetracycline
6 Resistance Genes and Class I Integrons 7855–7861. <https://doi.org/10.1021/es200827t>

7 Oksanen, A.J., Blanchet, F.G., Friendly, M., Kindt, R., Legendre, P., Mcglinn, D., Minchin, P.R.,
8 Hara, R.B.O., Simpson, G.L., Solymos, P., Stevens, M.H.H., Szoecs, E., 2019. vegan:
9 Community Ecology Package. R package version 2.5-6. [https://CRAN.R-](https://CRAN.R-project.org/package=vegan)
10 [project.org/package=vegan](https://CRAN.R-project.org/package=vegan)’.

11 Oliver, J.P., Gooch, C.A., Lansing, S., Schueler, J., Hurst, J.J., Sassoubre, L., Crossette, E.M.,
12 Aga, D.S., 2020. Invited review: Fate of antibiotic residues, antibiotic-resistant bacteria, and
13 antibiotic resistance genes in US dairy manure management systems. *J. Dairy Sci.* 103,
14 1051–1071. <https://doi.org/10.3168/jds.2019-16778>

15 Pröfrock, D., Prange, A., 2012. Inductively coupled plasma-mass spectrometry (ICP-MS) for
16 quantitative analysis in environmental and life sciences: A review of challenges, solutions,
17 and trends. *Appl. Spectrosc.* 66, 843–868. <https://doi.org/10.1366/12-06681>

18 Qian, X., Gu, J., Sun, W., Wang, X.J., Su, J.Q., Stedfeld, R., 2018. Diversity, abundance, and
19 persistence of antibiotic resistance genes in various types of animal manure following
20 industrial composting. *J. Hazard. Mater.* 344, 716–722.
21 <https://doi.org/10.1016/j.jhazmat.2017.11.020>

22 Raut, M.P., Pandhal, J., Wright, P.C., 2021. Effective pretreatment of lignocellulosic co-
23 substrates using barley straw-adapted microbial consortia to enhanced biomethanation by

1 anaerobic digestion. *Bioresour. Technol.* 321, 124437.
2 <https://doi.org/10.1016/j.biortech.2020.124437>

3 Roy, D., Lemay, J.F., Drogui, P., Tyagi, R.D., Landry, D., Rahni, M., 2020. Identifying the link
4 between MBRs' key operating parameters and bacterial community: A step towards
5 optimized leachate treatment. *Water Res.* 172, 115509.
6 <https://doi.org/10.1016/j.watres.2020.115509>

7 Stiborova, H., Strejcek, M., Musilova, L., Demnerova, K., Uhlik, O., 2020. Diversity and
8 phylogenetic composition of bacterial communities and their association with
9 anthropogenic pollutants in sewage sludge. *Chemosphere* 238, 124629.
10 <https://doi.org/10.1016/j.chemosphere.2019.124629>

11 Sun, W., Gu, J., Wang, X., Qian, X., Tuo, X., 2018. Impacts of biochar on the environmental risk
12 of antibiotic resistance genes and mobile genetic elements during anaerobic digestion of
13 cattle farm wastewater. *Bioresour. Technol.* 256, 342–349.
14 <https://doi.org/10.1016/j.biortech.2018.02.052>

15 Sundberg, C., Yu, D., Franke-Whittle, I., Kauppi, S., Smårs, S., Insam, H., Romantschuk, M.,
16 Jönsson, H., 2013. Effects of pH and microbial composition on odour in food waste
17 composting. *Waste Manag.* 33, 204–211. <https://doi.org/10.1016/j.wasman.2012.09.017>

18 Tien, Y.C., Li, B., Zhang, T., Scott, A., Murray, R., Sabourin, L., Marti, R., Topp, E., 2017.
19 Impact of dairy manure pre-application treatment on manure composition, soil dynamics of
20 antibiotic resistance genes, and abundance of antibiotic-resistance genes on vegetables at
21 harvest. *Sci. Total Environ.* 581–582, 32–39.
22 <https://doi.org/10.1016/j.scitotenv.2016.12.138>

23 Tong, J., Lu, X., Zhang, J., Angelidaki, I., Wei, Y., 2018. Factors influencing the fate of

1 antibiotic resistance genes during thermochemical pretreatment and anaerobic digestion of
2 pharmaceutical waste sludge *. *Environ. Pollut.* 243, 1403–1413.
3 <https://doi.org/10.1016/j.envpol.2018.09.096>

4 Vandeweyer, D., Milanović, V., Garofalo, C., Osimani, A., Clementi, F., Van Campenhout, L.,
5 Aquilanti, L., 2019. Real-time PCR detection and quantification of selected transferable
6 antibiotic resistance genes in fresh edible insects from Belgium and the Netherlands. *Int. J.*
7 *Food Microbiol.* 290, 288–295. <https://doi.org/10.1016/j.ijfoodmicro.2018.10.027>

8 Wallace, J.S., Garner, E., Pruden, A., Aga, D.S., 2018. Occurrence and transformation of
9 veterinary antibiotics and antibiotic resistance genes in dairy manure treated by advanced
10 anaerobic digestion and conventional treatment methods. *Environ. Pollut.* 236, 764–772.
11 <https://doi.org/10.1016/j.envpol.2018.02.024>

12 Wan, J., Ye, M., Jiang, X., Jiao, J., Tian, D., Wu, J., Li, H., Hu, F., 2017. Dynamic interplay
13 between microbial denitrification and antibiotic resistance under enhanced anoxic
14 denitrification condition in soil. *Environ. Pollut.* 222, 583–591.
15 <https://doi.org/10.1016/j.envpol.2016.10.015>

16 Weisburg, W.G., Barns, S.M., Pelletier, D.A., Lane, D.J., 1991. 16S ribosomal DNA
17 amplification for phylogenetic study. *J. Bacteriol.* 173, 697–703.
18 <https://doi.org/10.1128/jb.173.2.697-703.1991>

19 Zhang, J., Lin, H., Ma, J., Sun, W., Yang, Y., Zhang, X., 2019. Compost-bulking agents reduce
20 the reservoir of antibiotics and antibiotic resistance genes in manures by modifying bacterial
21 microbiota. *Sci. Total Environ.* 649, 396–404.
22 <https://doi.org/10.1016/j.scitotenv.2018.08.212>

23 Zhang, J., Mao, F., Loh, K.-C., Gin, K.Y.-H., Dai, Y., Tong, Y.W., 2018. Evaluating the effects

1 of activated carbon on methane generation and the fate of antibiotic resistant genes and
2 class I integrons during anaerobic digestion of solid organic wastes. *Bioresour. Technol.*
3 249, 729–736. <https://doi.org/10.1016/j.biortech.2017.10.082>

4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23

1 **Figure captions**

2 **Fig. 1.** Abundance of ARGs in fresh digestate and co-composting materials. Bars indicated with
3 different letters are statistically significant ($p < 0.05$, $n = 3$).

4 **Fig. 2.** Dynamics of ARGs in compost piles, expressed as log₁₀ of fold change, during the
5 **composting period.** Negative and positive results of the log fold change indicate reduction and
6 enrichment, respectively.

7 D00 = Digestate, DCB = Digestate + Food processing waste, DMS = Digestate + Maize silage,
8 DPL = Digestate + Poultry litter.

9 **Fig. 3.** Relative abundance of bacteria in the different piles (D00 = Solid digestate compost,
10 DCB = Digestate + Food processing waste, DMS = Digestate + Maize silage, DPL = Digestate +
11 Poultry litter) and fresh solid digestate (DIG) at phylum (A) and genus (B) level. DIG = Fresh
12 solid digestate.

13 **Fig. 4.** Differences in the composition of bacterial community among the piles (D00 = Solid
14 digestate compost, DCB = Digestate + Food processing waste, DMS = Digestate + Maize silage,
15 DPL = Digestate + Poultry litter) and fresh solid digestate (DIG) (A) and at the different compost
16 phases (B), defined by non-metric multidimensional scaling (NMDS) analysis.

17 **Fig. 5.** Co-occurrence of ARGs and bacteria groups based on significant ($P < 0.05$) Pearson's
18 correlation.

19 **Fig. 6.** Canonical coordinate analysis (CCA) of ARGs (response variables) and chemical
20 composition (environmental variables) of composts. **Two environments: A(C/N ratio, OM, pH,**
21 **and macronutrients) and B(trace elements).** D00 = Digestate, DCB = Digestate + Food
22 processing waste, DMS = Digestate + Maize silage, DPL = Digestate + Poultry litter.

23

24

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17

Table 1 Composition of the compost piles.

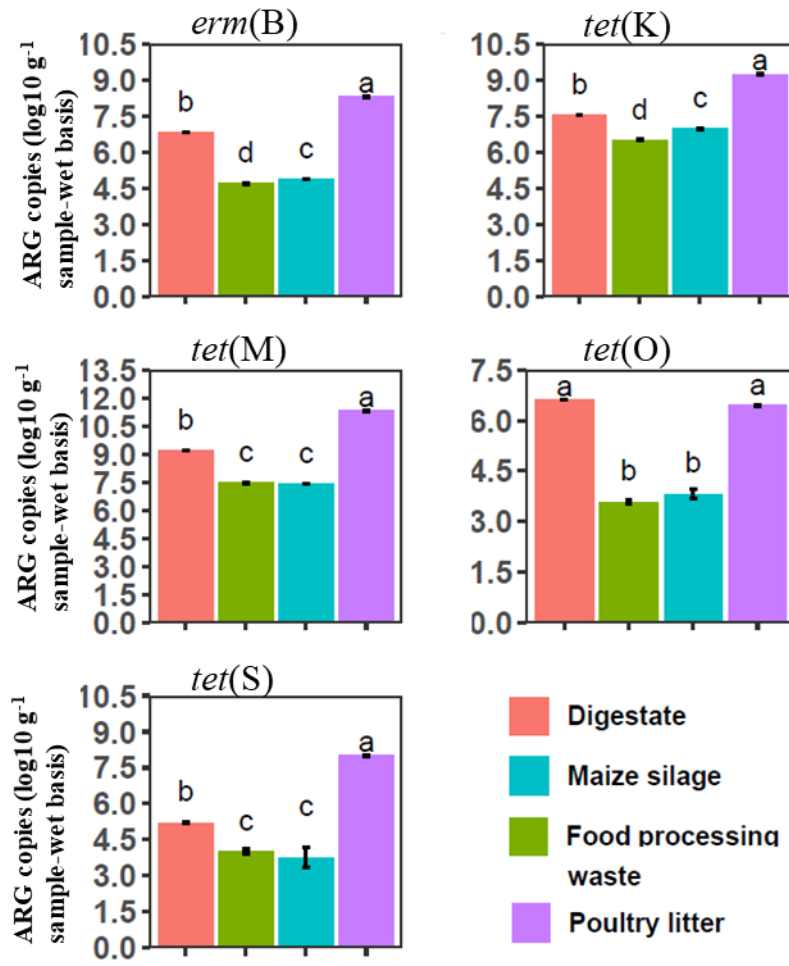
Pile	Composition	Mix ratio (w/w)
D00	Solid digestate	
DCB	Solid digestate + Food processing waste	4:1
DMS	Solid digestate + Maize silage	4:1
DPL	Solid digestate + Poultry litter	4:1

1
2
3
4
5
6
7
8
9
10
11
12

Table 2 Primers used in the qPCR reactions targeting the five ARGs of interest (Flórez et al., 2014).

ARG	Primer sequence (5'-3')	Product size (bp)
<i>erm</i> (B)	F- GGATTCTACAAGCGTACCTTGGA R- AATCGAGACTTGAGTGTGCAAGAG	69
<i>tet</i> (K)	F- TGCTGCATTCCCTTCACTGA R- GCTTTGCCTTGTTTTTTTTCTTGTA	69
<i>tet</i> (M)	F- CAGAATTAGGAAGCGTGGACAA R- CCTCTCTGACGTTCTAAAAGCGTAT	67
<i>tet</i> (O)	F- AATGTCAGAACTGGAACAGGAAGAA R- CGTGATAAACGGAAATAACGTT	59
<i>tet</i> (S)	F- CGAGGTCATTCTCATTGGTGAA R- CAGACACTGCGTCCATTTGTAAA	84

1



16

17

18

Fig. 1

19

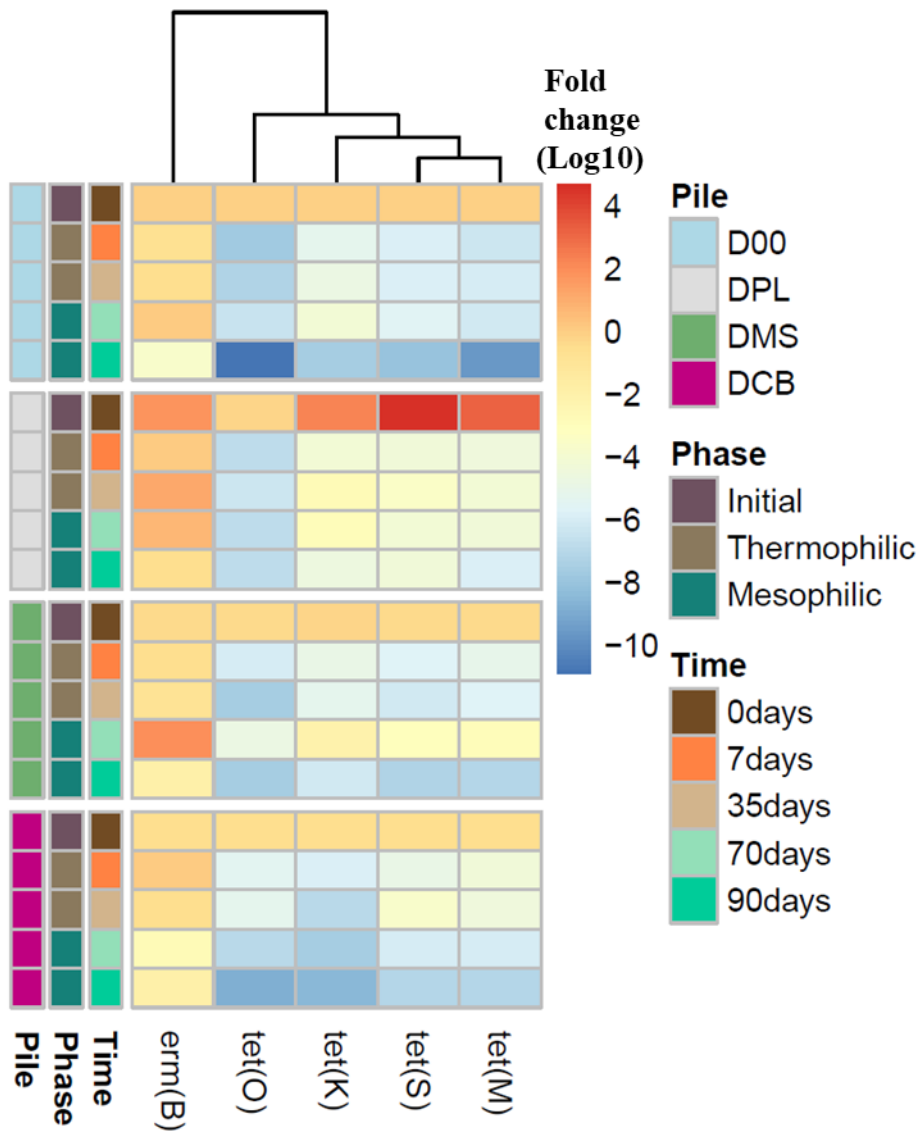


Fig. 2

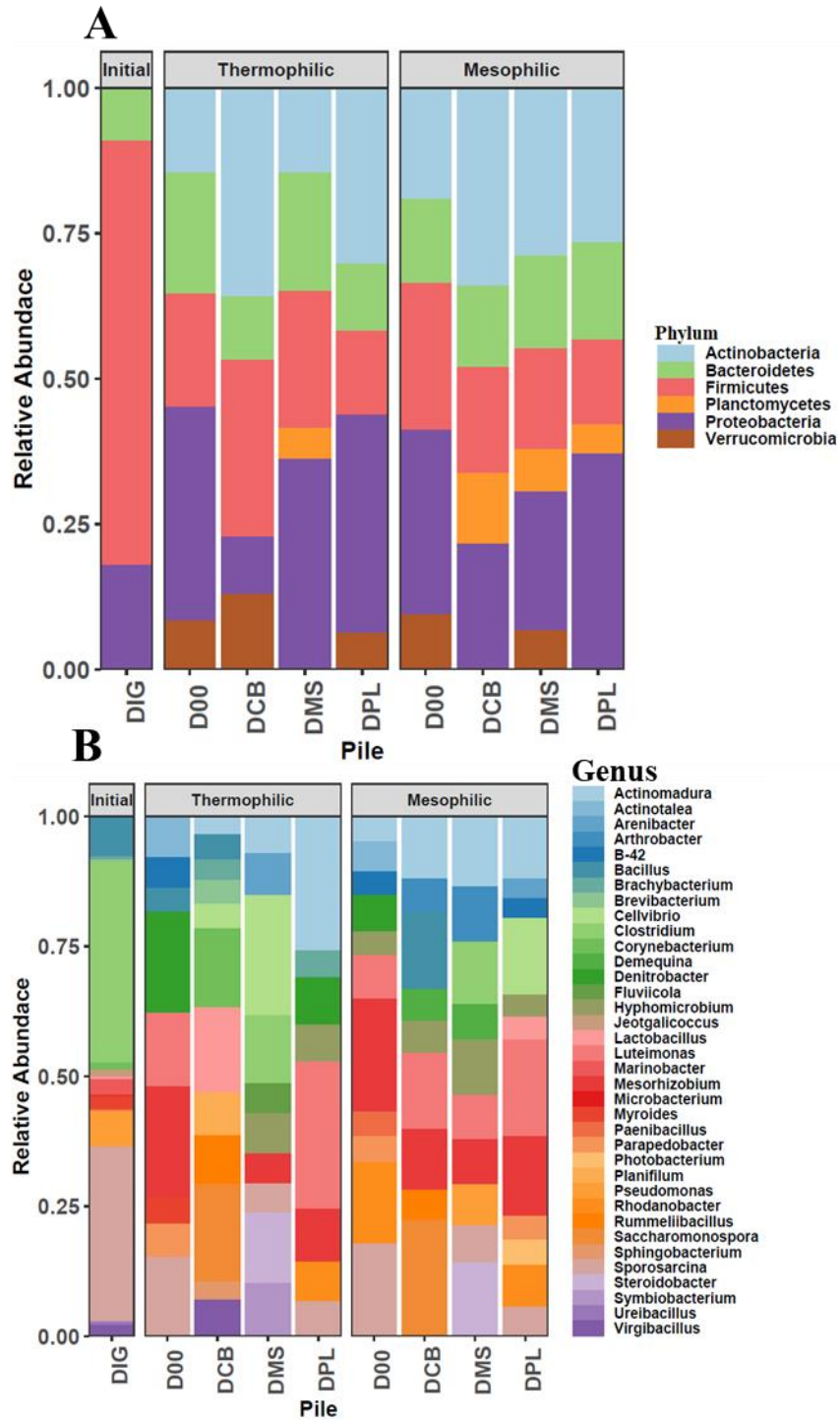


Fig. 3

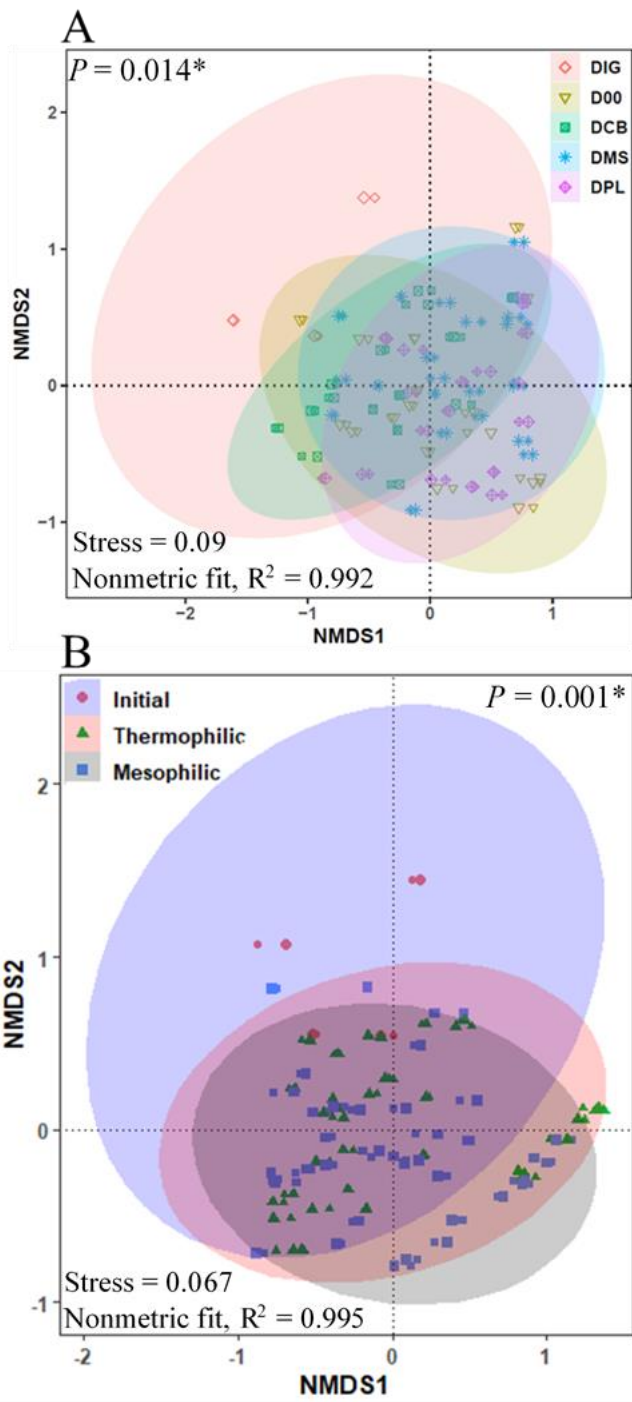


Fig. 4

- ARGs (5) ● Proteobacteria (12) ● Bacteroidetes (6) ● Planctomycetes (1)
- Firmicutes (9) ● Actinobacteria (10) ● Verrucomicrobia (2)

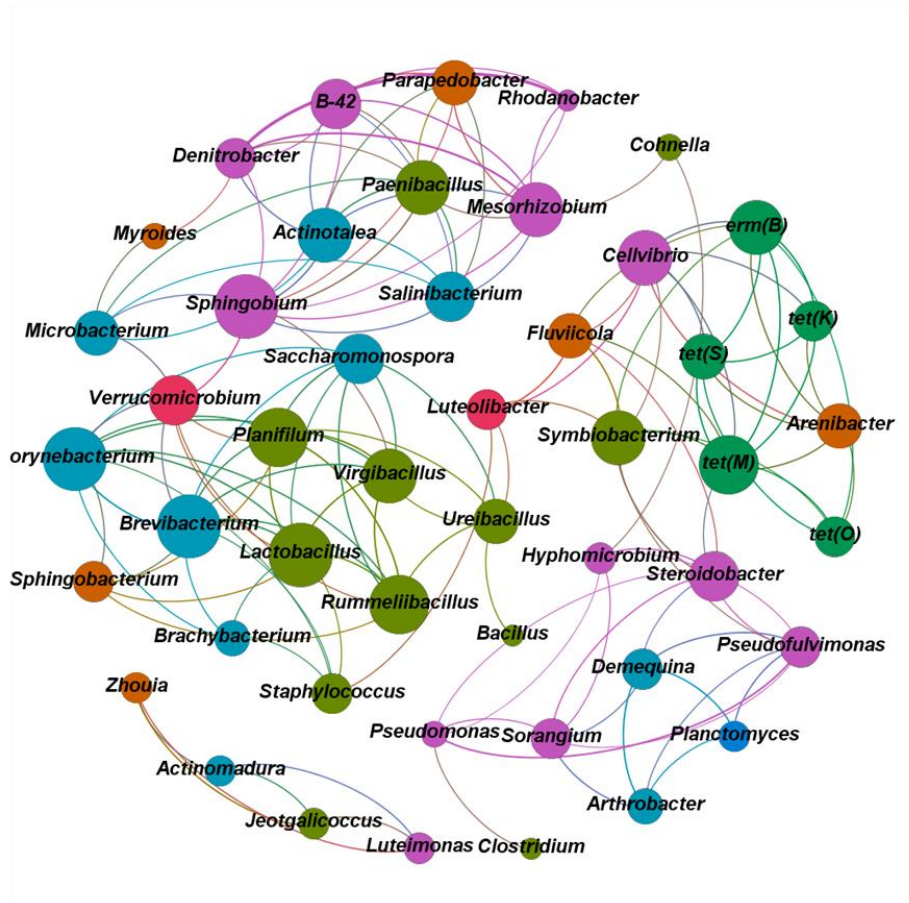


Fig. 5.

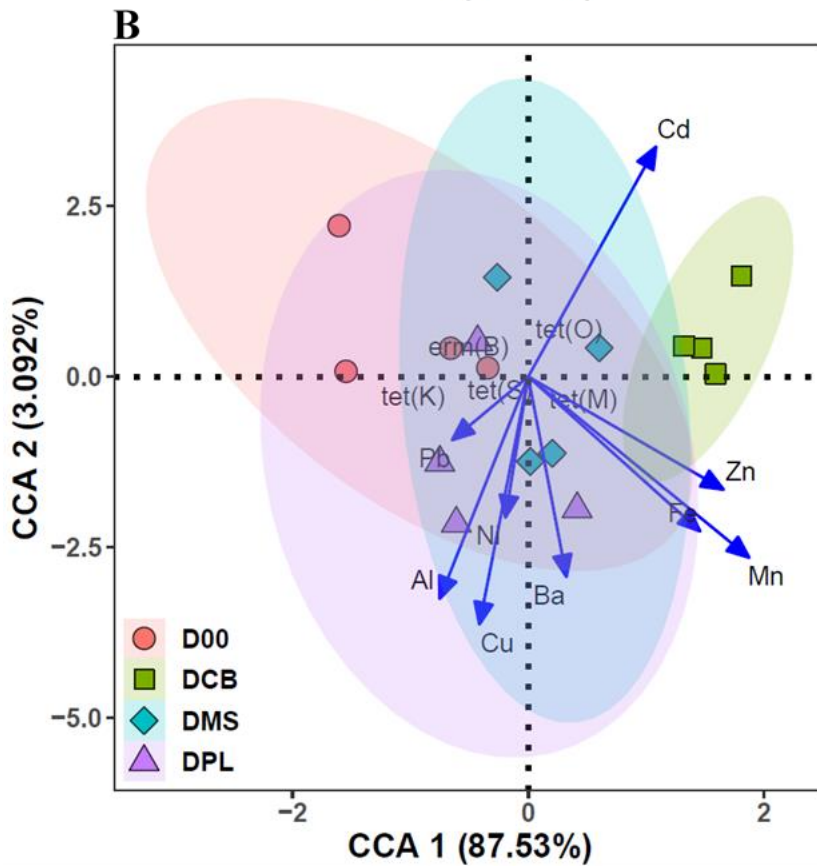
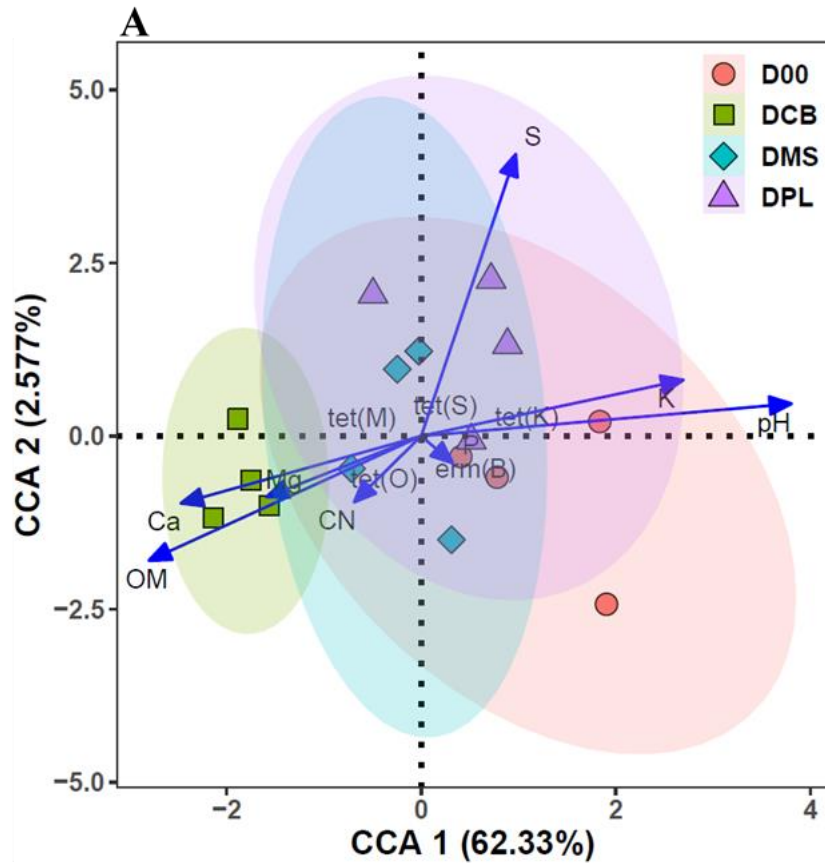


Fig. 6.