Università Politecnica delle Marche

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DOCTORAL THESIS

Mitigating release of contaminants with manure land application

Author:

Biyensa Gurmessa Dubiwak

Supervisors:

Prof. Giuseppe Corti

Prof. Ester Foppa Pedretti

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Executive Summary

This study aimed to address three major objectives. Firstly, it was aimed at characterizing two major sources of manure: cattle manure and poultry litter in terms of nutrient content, microbial composition, and selected antibiotic resistance genes abundance (Chapter 1). These manure sources were widely used as fertilizers in silvopastural systems in Arkansas, USA, to improve productivity. While cattle manure is land applied in fresh, poultry litter, which was composed of poultry manure and bedding, is aerated in-house windrows to increase the temperature and kill pathogens before land application. The results indicated poultry litter had lower microbial diversity and antimicrobial resistance genes (ARGs) abundance compared to cattle manure, possibly was due to the handling prior to its land application. Secondly, since poultry litter was safer to use than cattle manure, the next study was aimed at understanding the effect of poultry litter application on soil microbial diversity (Chapter 2). The result showed that poultry litter applied to soils under a silvopasture system did not affect soil microbiota despite it improved soil fertility. The study by these two objectives suggested poultry litter is environmentally safer and richer in nutrients supply than cattle manure in Arkansas region setting. However, in global setting, studies show that poultry litter is believed to contain greater ARGs abundance and other pollutants, possibly due to the absence of the kind of handling in Arkansas.

Thus, the next step of the study was to conduct a thorough literature review, and unfortunately, the results showed that poultry litter contained greater pollutants (ARGs, antibiotics, etc.). Aerobic treatment is the most widely studied for its effect against such pollutants. However, it is inefficient, and sometimes it can be a hub for enrichment of ARGs. This was evidenced by the results of previous studies that found poor degradation of ARGs by aerobic digestion, where the abundance of ARGs in digestate (a byproduct of biogas production) is greater than in the feedstock (input for the biogas production). Consequently, the next chapter of the study was to look at a post-digestate treatment (Chapters 3 and 4). Composting was aimed to remove ARGs as well as mitigate release of trace elements from digestate. The results showed that composting reduced greater than 80% ARGs in digestate and immobilized trace elements up to 90%. In conclusion, with this study, it was revealed that cattle manure direct land application may pose greater environmental risk compared to poultry litter, and this can be mitigated by employing pre-treatments such as the

bedding and aerating in-house windrows, the technique that is used for handling poultry litter in Arkansas region, the USA.

However, this is not the case in Europe. For instance, in Italy, where part of this thesis was conducted, poultry litter comprises feedstock of biogas production, and it is used in fresh. It contained ARGs copies ranging from log(10) 6.46 to 11.46, which was the greatest in most of the other feedstocks. After 90 days of anaerobic digestion of mix of poultry litter, food processing byproduct and maize silage, log(10) 5.21 to 9.22 ARG copies were found, with the greatest copies of *tet*(M) in both scenarios. It implied anaerobic digestion effect against ARGs was not satisfactory. Thus, the next aim of the study was to further degrade ARGs in digestate before land application. Thus, composting and co-composting it with the other biogas feedstock elements was conducted, and up to 80% removal of ARGs was possible.

In conclusion, the study suggests manure handling approaches may affect ARGs, and aerating could be a good practice to suppress the abundance. In the biogas production pathway, where fresh poultry litter may be used, the anerobic digestion may not be effective against ARGs removal, as it was reported in many other previous reports, and the subsequent post-digestate treatment, such as composting and drying, may be sought.

General background

Following the increase in the need for food, livestock population is growing (Sims and Maguire, 2018). Consequently, manure production is increasing. Characterized as having high N, phosphorus (P), and potassium (K) contents (Zhou and Yao, 2020), manure is generally rich in nutrients despite significant variations can be observed among the different sources. It also improves soil organic matter essential for plant roots growth, buffers nutrients, and enhances functionality of soil biota-playing significant role in maintaining soil health. Manure use approaches lie in two broad categories: raw and after processing (Castellanos-Navarrete et al., 2015; Foged et al., 2011). The raw manure use approach is direct application on farmlands or pasturelands without treating. The major processing approaches are anaerobic digestion (AD) which targets both energy production and removal of toxic substances and aerobic composting. Acknowledging the significance of manure for its use as fertilizer and soil organic amendment, there are several environmental risks associated especially when it is directly applied to soils. Also, AD may not be effective against all kinds of the hazardous materials and antimicrobial resistance genes (ARGs) even could have more risk in terms the release of ammonium (NH_4^+).

Environmental challenges related to manure use include release of heavy metals, zoonotic bacteria, antimicrobial resistance genes (Li et al., 2017; Wohde et al., 2016). The different manure use approaches effect on reducing these environmental risks have got attentions by researchers from different fields including agronomists, ecologists, and environmentalists. Anaerobic digestion and composting are the main manure treatment approaches getting great attentions for nutrient release, trace elements immobilization, zoonotic bacterial composition reduction and removal of antimicrobial resistance genes. However, there are still knowledge gaps in the field, and the major research objectives addressed with this thesis are: 1) understanding variations of microbial composition and antimicrobial resistance genes abundance in cattle manure and poultry litter and whether the physicochemical compositions explain variations in microbial abundance (Chapter 1), evaluating the effect of poultry litter land application on soil microbial composition in a silvopatoral system (Chapter 2), understanding the effect of anaerobic digestion on antibiotics and resistance genes (Gurmessa et al., 2020), understanding the effect of anaerobic digestion and the subsequent aerobic composting on nutrient release, trace metals immobilization and enzyme

activities (Chapter 3), and evaluaitng digestate composting effect on microbial composition and antimicrobial resistance genes abundance (Chapter 4).

Chapter 1

Physicochemical compositions of cattle manure and poultry litter determine bacterial composition and ARGs abundance [1]

Abstract

Cattle manure and poultry litter are widely used as fertilizers because they are excellent sources of nutrients; however, potential adverse environmental effects exist during land applications, due to the release of zoonotic bacteria and antimicrobial resistance genes (ARGs). This study was conducted to understand linkages between physiochemical composition, bacterial diversity, and ARGs presence of cattle manure and poultry litter using quantitative polymerase chain reaction to enumerate four ARGs [erm(B), sul(I), intl(I), and bla$_{(ctx\text{-}m\text{-}32)}$], Illumina sequencing of the 16S region, and analysis of physical and chemical properties. Principal coordinate analysis of Bray–Curtis distance revealed distinct bacterial community structures between the two manure sources. Greater alpha diversity occurred in cattle manure compared to poultry litter ($p<0.05$). Redundancy analysis showed a strong relationship between manure physiochemical and composition and bacterial abundance, with positive relationships occurring among electrical conductivity and carbon/nitrogen, and negative associations for total solids and soluble fractions of heavy metals. Cattle manure exhibited greater abundance of macrolide erm(B) and sulfonamide (sulI) resistant genes. Consequently, fresh cattle manure applications may result in greater potential spread of ARGs to the soil-water environment (relative to poultry litter) and novel best management strategies (such as composting) may reduce the release of AMR genes to the soil-water environment.

Keywords: animal manure; bacterial diversity; total solid content; heavy metals; broiler litter.

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1.1. Introduction

Manure land applications may pose environmental health risks, despite its role in improving soil fertility and organic matter, by serving as a pathway for the release of zoonotic bacteria and antibiotic resistance genes (ARGs) to the environment (Biyensa Gurmessa et al., 2020b; Xie et al., 2018; Ziemer et al., 2010). However, there is lack of knowledge on the link between manure properties and microbial composition and the abundance of ARGs. Disparities in manure physiochemical properties, such as moisture content, pH, carbon to nitrogen (C/N) ratio, and nutrient composition (Zhou and Yao, 2020) may affect microbial composition and abundance (Xu et al., 2020). Trace element levels are also reportedly linked to bacterial community structure and ARGs, although specific relationships are largely unknown (Ding et al., 2017).

Animal manure is generally characterized as having high nitrogen (N), phosphorus (P), and potassium (K) contents (Zhou and Yao, 2020). Specifically, 310 mg kg^{-1} P (on dry matter basis) was reported for cattle manure (Giles and Cade-Menun, 2014), with approximately 1,500 mg kg^{-1} P being reported for poultry litter (consists of a combination of bedding material, feces, and litter) (Ashworth et al., 2020). Manure pH also varies depending on the manure type. A neutral to sub-alkaline pH range (6.8 to 7.9) has been reported for cattle manure (Huang et al., 2017; Whalen et al., 2000), whereas an average pH of 8.12 is typical for poultry litter (Ashworth et al., 2020). However, it is unknown how physiochemical properties are related with microbial abundance.

Previous studies have reported a wide range of antimicrobial resistant genes (ARGs) in manure and the most widely studied were genes resistant to tetracyclines (*tet*), sulfonamides (*sul*), macrolides-streptogramin B (*erm*), mobile genetic elements (MGEs), and integrons (*int*) (Biyensa Gurmessa et al., 2020b). However, these resistance genes may not be found in all manure sources. A detailed investigation of AMR in three manure sources (bovine, poultry, and swine) by Qian et al. (2018) revealed that about one-third of the 109 commonly found ARGs were identified in cattle manure and poultry litter. In this experiment, this set of targets was chosen to cover clinically, environmentally, and agriculturally relevant antibiotic resistance determinants. The specific targets were chosen by a panel of scientists working on antibiotic resistance in agriculture, and they aligned with an environmental antibiotic resistance gene surveillance effort in Europe. The bla(CTX-M) gene codes for third-generation cephalosporin resistance, one type of *β-lactamase*

resistant drug. The *erm*(B) codes for resistance to macrolide drugs, such as erythromycin. The *sul*(I) gene codes for sulfonamide-resistance and is one of the most commonly studied resistance genes in environmental samples. Finally, the *intl*(1) gene codes for an integron-integrase gene that helps ARGs spread from cell to cell. These drugs are classified as "Critically Important" (the top category) by the World Health Organization and used in large and small animals, and is approved for use in cattle, swine, and poultry, and is administered to food animals via food and water (Durso et al., 2012; Durso and Cook, 2014).

Reduction of ARGs in manure is essential to minimize the transfer and movement to the environment following land application. Although, it is largely unknown how microbial diversity and abundance of ARGs vary based on manure source (fresh cattle manure and poultry litter) that are typically applied to pasture soils to improve fertility (Yang et al., 2019). In the study region, typical poultry litter handling procedures include aerating in-house windrows to increase the temperature and kill pathogens before land application, whereas cattle manure is deposited directly to pastures during grazing. Therefore, understanding the bacterial diversity and abundance of ARGs based on local practices and their possible linkage with physiochemical composition may assist in developing manure best management strategies.

It is important to evaluate microbial composition, diversity, and abundance along with ARGs to employ a subsequent management approach that can be effective against reducing the transmission of ARGs to the environment and improving soil health. However, data are lacking on the linkages between physiochemical properties, manure source, and bacterial diversity (Wang et al., 2016). Hence, the current study aimed at 1) investigating the abundance of bacterial community composition in cattle manure and poultry litter prior to land application via Illumina sequencing, 2) quantifying four ARGs associated genes in cattle manure and poultry litter via quantitative polymerase chain reaction (qPCR), and 3) identifying linkages between bacterial alpha diversity and physiochemical properties for two manure sources.

1.2. Materials and Methods

1.2.1. Cattle manure and poultry litter sampling

In the spring of 2018 and 2019, 1 to 2 kg of poultry litter samples were collected from in-house piles gathered from a local typical broiler production system in Booneville, AR (n=6 per year,

n=12 total). Samples were collected from the center of in-house piles following 5-6 flocks (stored for up to four months), with bedding material, ventilation, and growth out days being representative of typical regional grower conditions (Ashworth et al., 2020). Following, typical cattle manure land application methods, fresh cattle (Angus crosses) manure was sampled at the United States Department of Agriculture, Agricultural Research Service, Dale Bumpers Small Farms Research Center (Pilon et al., 2019) during a grazing experiment (Yang et al., 2020) by collecting 1 to 2 kg of cattle manure following fresh deposition (within 24 hrs; n=7 per year, n=14 total) in the spring of 2018 and 2019 and represents regional pasture management procedures. Care was taken not to contaminate samples by sterilizing sampling equipment with 70% ethanol, placed in a cooler for transport, and stored at -80°C until DNA extraction.

1.2.2. Manure chemical analysis

Samples were analyzed for moisture content, pH, EC, soluble metals, ammonium-N (NH_4-N), nitric-N (NO_3-N), and total C and N (TC and TN). Moisture content was determined by oven drying a subsample at 65°C for 1 week (Nelson and Sommers, 1996), and total solids (TS) content was determined as the percentage fraction of dry mass to fresh sample. Water soluble metals were extracted on fresh aliquots using a 1:10 solid:liquid extraction ratio (Self-Davis and Moore, 2000). A subsample of the water extract was used to measure pH and EC. NH_4-N and NO_3-N were extracted on fresh aliquots by KCl 1M solution using a 1:10 solid:liquid extraction ratio (Self-Davis and Moore, 2000). Soluble metals in the water extracts were analyzed by inductively coupled plasma optical emission spectroscopy (ICP-OES) on an Agilent 5110 ICP-OES (Agilent Technologies, Santa Clara, CA, USA). Both NH_4-N and NO_3-N were obtained colorimetrically on a Skalar auto-analyzer (Skalar, Buford, GA, USA), using the salicylate-nitroprusside USEPA Method 351.2 (1983) for NH_4-N and the cadmium-reduction method according to APHA (1995) for NO_3-N. Total metals were determined by digesting oven-dried, ground litter samples with 70% nitric acid and 30% hydrogen peroxide according to the method by Zarcinas et al. (1987) followed by ICP-OES analysis. Total C and N were determined by dry combustion Elementar Vario Max Analyzer (Elementar Americas, Ronkonkoma, NY, USA).

1.2.3. DNA extraction, PCR amplification, and sequencing

DNA was extracted from manure and litter using the extraction kit MpBio FastDNA Spin Kit for Soil (MpBio Laboratories, SKU 116560200-CF) according to the manufacturer's directions. Extracted DNA was quantified using Quant-ItTM PicoGreen® (Invitrogen) dsDNA quantitation assay and stored at 20ºC. Samples were dried at 70°C for 48 h to determine gravimetric moisture content, which was used to present data per gram dry weight.

Bacterial community composition was determined using Illumina Miseq sequencing of 16S rRNA gene amplicons. Extracted DNA was sent to the University of Tennessee Genomic Services Laboratory, where the V4 region of the 16S rRNA gene was amplified with barcoded primers 515F and 806R (Caporaso et al., 2011). Amplicon libraries were pooled, and 291 base-paired end sequences were obtained on the Illumina MiSeq Platform, resulting in a total of 2,136,829 sequence reads. Reads were processed using the open source bioinformatics software Mothur V 1.40.0 following the Miseq protocol (Kozich et al., 2013). Sequences that did not match the primers were eliminated from demultiplexed sequence reads. These ambiguous base sequences with a length less than 100 bp were deleted and chimeric sequences were removed using the UCHIME algorithm implemented in Mothur. After the quality control pipeline, 1,677,430 sequence reads remained using a 97% similarity threshold to define ribotypes in Mothur (21.49% were deleted). Taxonomic assignment was performed using the Greengenes database. Microbial alpha diversity including Chao1, observed operational taxonomic unit (OTU), Shannon index, and Simpson index were calculated using Mothur. Beta-diversity was measured by using Bray-Curtis index, weighted and unweighted UniFrac distance metrics. Principal coordinate analysis (PCoA) plots were generated based on weighted and unweighted UniFrac distance metrics. Bacterial community structure was quantified in a matrix of Bray-Curtis similarities, which was then analyzed in a permutational analysis of variance (PERMANOVA) to compare bacterial communities at the phylum level in PRIMER-E.

Quantitative -PCR was performed to quantify these four ARGs in the DNA extracts from cattle manure and poultry litter samples. Amplifications were performed in a QuantStudio™ 3 Real-Time PCR system (ThermoFisher Scientific, Cat. A28137). Each 20 μL Q-PCR reaction included 5 μL of extracted DNA (approximately 100 ng) or standard, 10 μL of SYBR Green PCR

Master Mix, 1 μL of 100 mM of each primer and 3 μL of distilled water. The positive control (named gBlock2 4G with 16S *ermB* Florez 1-18-17) is an 808bp double stranded synthetic gBlocks® gene fragment synthesized by Integrated DNA technologies, lnc. (Blazejewski et al., 2019). It contains four genes of *erm*(B), *sul*(I), *intl*(I), and *bla*$_{(ctx\text{-}m\text{-}32)}$. The standard curves consisted of a serial dilution of known copy numbers of the gene fragment, ranging from 1.15 x 10^5 to 1.15 x 10^{11} copies per 5 µl. The quantities of gene copy numbers were calculated based on the standard curve using Quant Studio 3 real-time PCR system. (ThermoFisher Scientific). As a negative control, all sets of primers were tested with sterile water as the template and all of them were below the threshold. Each reaction was technically replicated three times per extracted sample DNA and standard DNA, resulting in an average cycle threshold (Ct) value used to estimate the initial quantity. The following cycling conditions were used: an initial denaturation step of 15 min at 95ºC, followed by 40 cycles of 15 s at 95ºC, 30 s at annealing temperature specific for each gene, and 10 s at 72ºC followed by 60-95ºC of melting curve.

The amplification efficiency was between 92% and 105%, and the R^2 value was above 0.98. Baseline and threshold calculations were performed using QuantStudio® Design & Analysis software. Gene copy abundances were normalized per volume of water. The quantities of gene copy numbers were then determined using standard curves. Gene copy abundances were normalized per gram dry weight of cattle manure or poultry litter after measuring the moisture content of each sample. Finally, the gene copy numbers per gram dry weight were transformed into log10 values for statistical analysis as they were not normally distributed.

1.2.4. Statistical analysis

To detect significant differences for fixed effects (manure source and year) on microbial diversity, richness, and evenness, an analysis of variance (ANOVA) was conducted on log transformed data using JMP software [JMP®12 (SAS Institute Inc., 2015)] with sample replicate as a random effect. Probability values less than 0.05 were considered significant. For the contents of nutrients, trace elements, and chemical properties [pH and electrical conductivity (EC)], Fisher's exact test followed by Wilcoxon rank-sum test were performed using gtsummary package (Sjoberg et al., 2020) in R for Windows, v. 4.0.1 (R Core Team, 2020). Redundancy analysis (RDA) was also conducted to understand the relationship between bacterial diversity indices

(Chao1, Simpson, and Shannon) and chemical properties (pH, EC, C, N, and major nutrients and trace elements) using the vegan package (Oksanen et al., 2019) in R for Windows, v. 4.0.1 (R Core Team, 2020). Except pH, all the other property variables were log transformed; whereas response variables (Chao1, Simpson, and Shannon indices) were Hellinger transformed, and RDA analysis was run and plotted. To further understand the relationship between Shannon index and those property variables that contributed to variability in the RDA analysis, Pearson correlations analysis was conducted and plotted using the ggpubr package (Kassambara, 2020) in R for Windows v. 4.0.1 (R Core Team, 2020).

1.3. Results and Discussions

1.3.1. Physiochemical properties of cattle manure and poultry litter as a fertility source

Cattle manure samples had lower total solids (TS), trace elements concentration, and pH, but greater EC and C/N ratio compared to poultry litter (Table 1.1). In a comparative study, Zhang et al. (2012) also found lower concentrations of trace elements in cattle manure relative to poultry litter. In contrast to the current findings, high amounts (about 1 g kg^{-1}) of Zn and Cu were reported for cattle manure (Xu et al., 2019). The feed source is predominantly the major determinant for the concentration of nutrients and trace elements in poultry litter and cattle manure (Pagliari et al., 2020; Sager, 2007; Zhang et al., 2012; S1), although the use of pharmaceutical products can also be a source (Sheppard and Sanipelli, 2012). Nevertheless, poultry litter had trace heavy metal levels, even in the total basis (Gurmessa et al., 2021b), which was far below the concentration limit for the USA (Mortvedt, 1995). However, to reduce possible environmental risks, alternative feed sources could be formulated to minimize the level of heavy metals in manure.

1.3.2. Bacterial community composition in cattle manure and poultry litter

Bacterial community composition varied at both phyla and genera levels in cattle manure and poultry litter (Fig. 1.1A and B). The three most abundant phyla constituted approximately 91 and 95% of the overall bacterial community composition in cattle manure and poultry litter, respectively. In particular, Firmicutes dominated microbial composition in both manure sources.

Table 1.1.Physiochemical properties of cattle manure and poultry litter. Numbers in parentheses represent the standard deviations.

Variable	CM[ǂ], N[1] = 12	PL[≠], N[1] = 14	p-value[2]
pH	7.95 (0.25)	8.33 (0.12)	<0.001
Total N (%)	3.14 (0.70)	4.23 (0.44)	<0.001
Total C (%)	47.0 (4.5)	36.7 (3.5)	<0.001
C/N	15.4 (2.5)	8.7 (0.4)	<0.001
TS (%)[¥]	14 (2)	76 (7)	<0.001
EC (dS m^{-1})	940 (114)	11 (2)	<0.001
Mo (mg kg^{-1})	<LOQ	2.00 (1.39)	<0.001
Mn (mg kg^{-1})	9 (5)	21 (4)	<0.001
Se (mg kg^{-1})	<LOQ	0.33 (0.15)	<0.001
Ti (mg kg^{-1})	<LOQ)	0.38 (0.25)	<0.001
Zn (mg kg^{-1})	10 (4)	70 (19)	<0.001
Ni (mg kg^{-1})	1.52 (0.41)	5.00 (1.19)	<0.001
Pb (mg kg^{-1})	<LOQ	0.035 (0.025)	<0.001
As (mg kg^{-1})	<LOQ	0.49 (0.25)	<0.001
Cd (mg kg^{-1})	<LOQ	0.047 (0.020)	<0.001
Co (mg kg^{-1})	<LOQ	0.59 (0.15)	<0.001
Cr (mg kg^{-1})	0.50 (0.67)	0.28 (0.10)	0.5
Cu (mg kg^{-1})	3 (1)	64 (41)	<0.001
Fe (mg kg^{-1})	20 (9)	83(42)	<0.001
K (g kg^{-1})	6 (3)	33 (8)	<0.001
Ca (g kg^{-1})	1.91 (0.97)	2.06 (1.7)	0.7
Mg (g kg^{-1})	2.38 (0.93)	0.39 (0.23)	<0.001
P (g kg^{-1})	1.77 (0.91)	1.40 (0.73)	0.2
S (g kg^{-1})	0.67 (35)	16 (7.6)	<0.001
NH_4-N (g kg^{-1})	0.76 (0.4)	5 (0)	<0.001
NO_3-N (mg kg^{-1})	<LOQ	449 (256)	<0.001

[ǂ]CM = Cattle manure, PL[≠] = poultry litter, TS[¥] = total solids. [1]N = number of replicates. <LOQ = below quantification limit. [2]Statistical tests performed: Fisher's exact test; Wilcoxon rank-sum test. Nutrients and trace elements concentrations are soluble fractions expressed on a dry matter basis.

Awasthi et al. (2019), Pandey et al. (2018), and Wang et al. (2020) reported similar findings, suggesting Firmicutes are the dominant microorganisms in manure, regardless of the source. According to Zhang et al. (2018), Firmicutes such as *Bacillus* and *Lentibacillus* were dominant in poultry litter and include known pathogens and may reach the soil ecosystems following direct application of cattle manure or poultry litter. Several zoonotic bacteria species including *Campylobacter jejuni, Escherichia coli,* and *Listeria monocytogenes* have also been detected in fresh cattle manure (Klein et al., 2010). Bacteroidetes inhabit the environment and gastrointestinal tract (Thomas et al., 2011). They are also found in fresh water and soils and are known to have a functional role in degrading soluble polysaccharides and organic substances of high molecular weight (Naas et al., 2014; Thomas et al., 2011). Because of their importance in depolymerizing or

degrading organic substances, their abundance in ecosystems might enhance the rate of organic matter turnover that could lead to enhanced CO_2 emissions. Moreover, some species of the phyla *Myroides*, *ordoratimimus*, and *Sphingobacterium* spp. are clinically important human pathogens that may also be resistant to antimicrobials (Yang et al., 2014). Given that fresh cattle manure had a higher proportion of Bacteroidetes, its land applications could pose greater environmental risks than poultry litter.

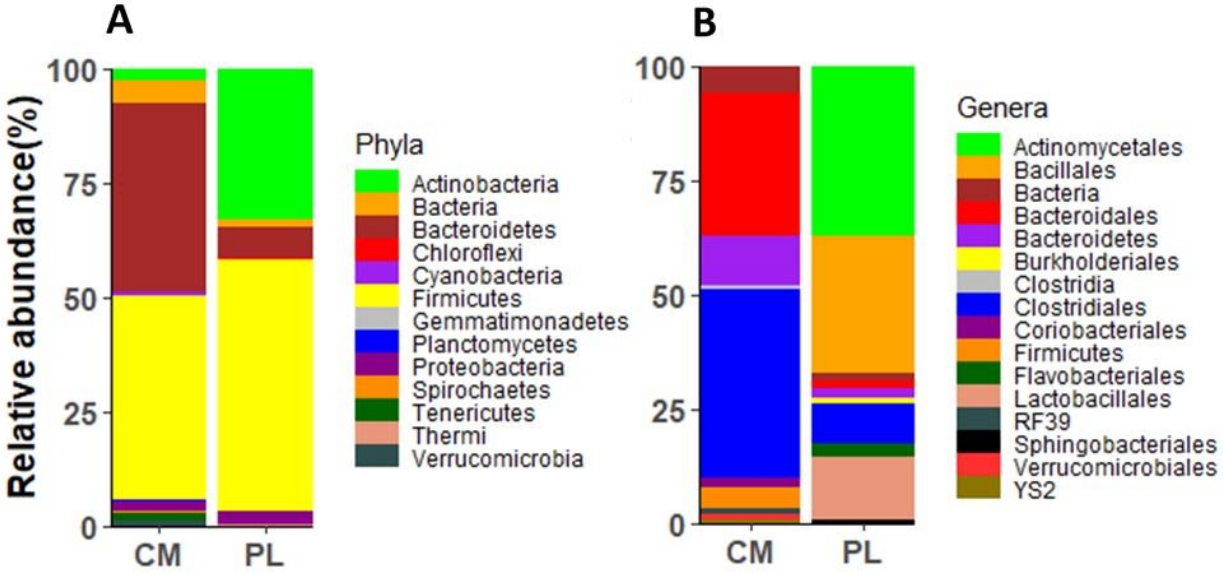

Fig. 1.1. Relative abundance of bacteria in cattle manure (CM) and poultry litter (PL) at phylum (A) and genus (B) levels

1.3.3. Differences in bacterial community structure and its implication on soil microbial structure

Source of manure affected bacterial structure as evidenced by Chao1, Simpson, and Shannon diversity indices (Table 2.1). Cattle manure exhibited greater bacterial diversity than poultry litter (Fig. 2.1).

Table 2.1.PERMANOVA for bacterial community structure of cattle manure and poultry litter.

Factor	Pseudo-F	*P*-value
Animal source	31.539	<0.001**
Year	8.26	0.019*
Animal source x Year	0.54	0.437

* Statistically significant (α = 0.05).
** Statistically significant (α = 0.01).

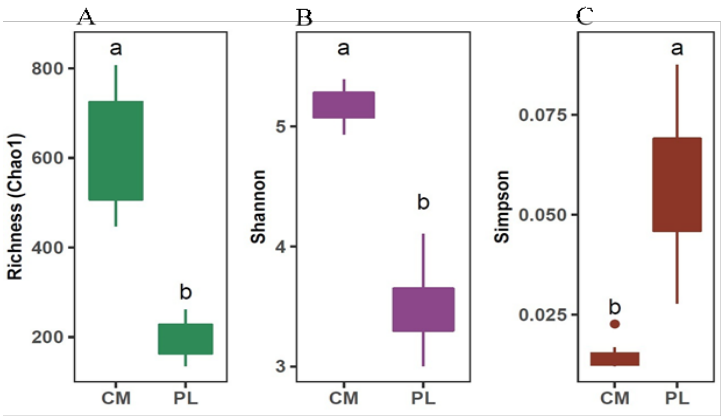

Fig. 2.1. Comparison of bacterial diversity indices of cattle manure (CM) and poultry litter (PL). A) Chao1, B) Simpson, and C) Shannon. Different letters reported on the bars indicate

Analysis of beta diversity also demonstrated distinct clustering differences between cattle manure and poultry litter (Fig. 3.1). The fact that poultry litter is a mixture of poultry manure and bedding (Ashworth et al., 2020), likely influences the physiochemical characteristics of poultry litter that, in turn, affects the bacterial community structure.

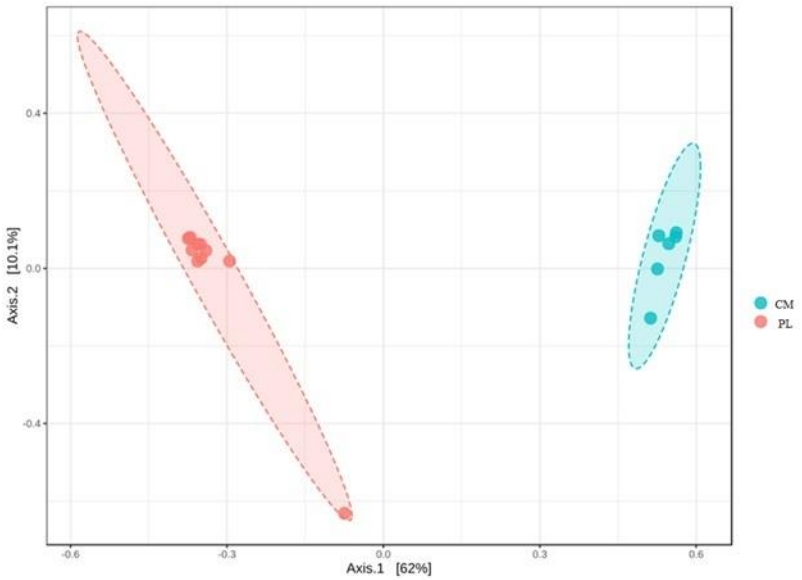

Fig. 3.1. Principal Coordinate Analysis (PCoA) of Bray–Curtis distances of bacterial community structure in cattle manure (CM) and poultry litter (PL).

Studies comparing microbial structure of manure sources are limited. The current study found a lower alpha bacterial diversity in poultry litter relative to cattle manure. Possible reasons for the

lower alpha diversity might be linked to the higher TS content of poultry litter (76%), (Schimel et al., 1999; Zealand et al., 2018), lower organic C content (Van Horn et al., 2014), and higher soluble trace elements (Tipayno et al., 2018).

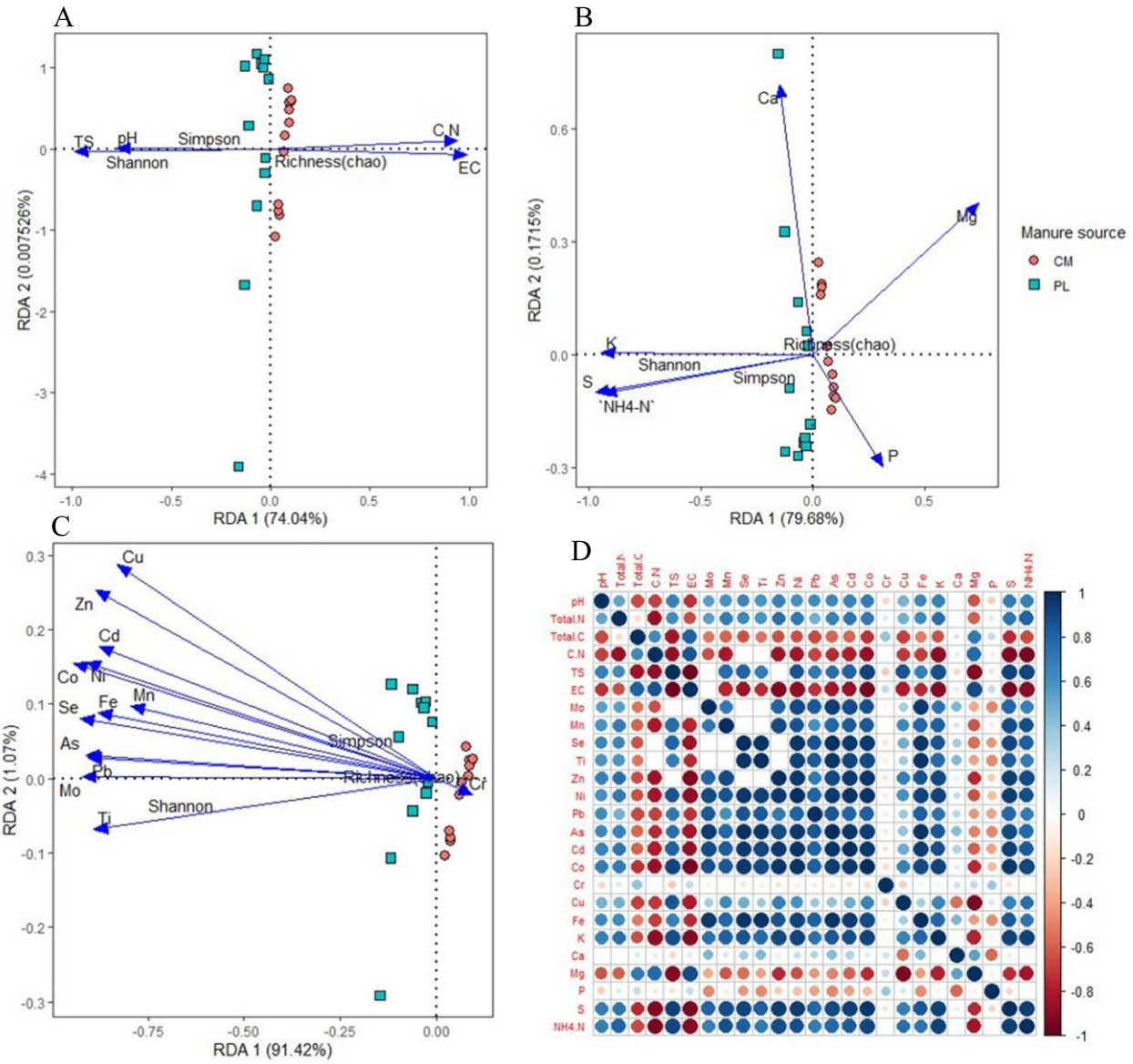

Fig. 4.1. Redundancy analysis (RDA) of bacterial diversity indices (Chao1, Shannon and Simpson) with physical and chemical properties of cattle manure (CM) and poultry litter (PL) [A: physical and chemical properties (pH, EC, C/N ratio, and TS); B: soluble fractions of major nutrients; C: soluble fraction of trace elements; D: co-occurrence of soluble heavy metals and their correlation with pH, C/N ratio, EC, and soluble fraction of nutrients]. Except the blank boxes in the correlation plot, all correlations are significant ($p < 0.05$).

1.3.4. RDA of bacterial diversity constrained by trace elements and physiochemical properties

A comprehensive assessment of physiochemical composition of manure, which included TS, pH, EC, soluble trace elements, and macronutrients, revealed differences between cattle manure and poultry litter. The RDA analysis illustrated these variables predicted bacterial alpha diversity (p<0.001) with pH, TS, EC, and C/N ratio explaining 74% of the total variation (Fig. 4.1A). Soluble fractions of available nutrients such as K, P, NH_4-N, and S, accounted for 83% of the total variation (Fig. 4B). Moreover, trace elements, mainly As, Ti, and Pb, accounted for 90% of the total variation (Fig. 4.1C). In addition to the RDA results, the correlation analysis showed a strong positive relationship between alpha diversity, C/N ratio, and EC (Fig. 5.1). However, TS, pH, trace elements and NH_4-N were negatively correlated with diversity indices.

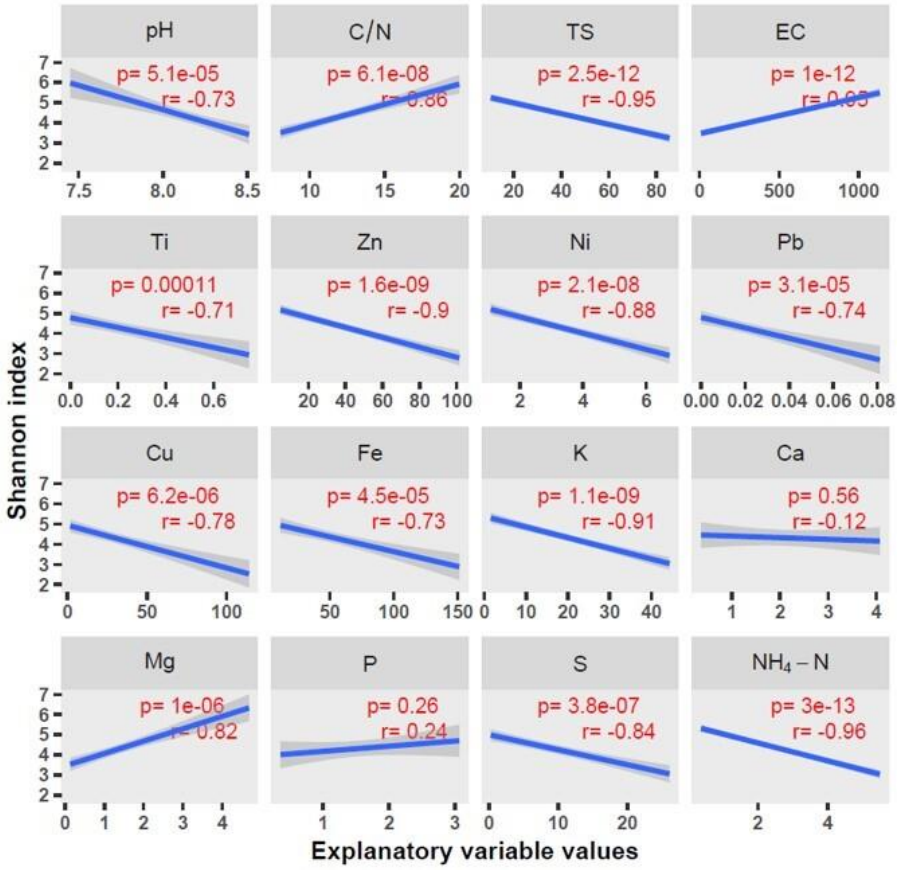

Fig.5.1. Shannon index of bacterial abundance correlated with pH, electric conductivity (EC), total solid content (TS%), and soluble nutrient and trace elements contents in cattle manure and poultry litter. Quantities of the chemical contents are on dry matter basis, and the unit for Ca, Mg, K, P, S and NH4-N is g kg-1; whereas the rest of trace elements is in mg kg-1.

This study provides insights into the relationships between physiochemical properties and bacterial community structure of manure sources. The cattle manure bacterial community may have benefited from a higher C/N ratio, lower TS (14%), higher EC (940 dS m^{-1}) and a slightly alkaline pH. A similar positive correlation between C/N ratio and bacteria diversity was also reported by Ge et al. (2010). The C/N ratio has been widely used as an index of stability or maturity of organic residue during composting, as C substrate having a C/N ratio of 16 or larger is not stable for supporting the growth of microorganisms (Macias-Corral et al., 2019). The EC and pH have been widely reported to affect microbial diversity (e.g., Geyer et al., 2013; Ye et al., 2016), with greater EC values in manure indicating nutrient mobility or availability to microbes. Thus, reducing EC could be a strategy to suppress pathogens in cattle manure (Kong et al., 2012). Trace elements can be stimulatory or inhibitory for microorganisms depending on the type and concentration level. The strong negative correlations between soluble trace element concentrations and bacterial diversity indices observed in this study may reveal an inhibitory functional role in poultry litter. However, this may be confounded by other factors since the total concentration of heavy metal was too low to be inhibitory or stimulatory (Ding et al., 2017; Mortvedt, 1995) to microorganisms. Moisture is also crucial for microorganisms and inadequate moisture content limits microbial diversity (Maestre et al., 2015). This could also be the case in this study as poultry litter had lower moisture content (higher TS content), which may have resulted in lower bacterial diversity compared to cattle manure.

1.3.5. Abundance of AMR genes in cattle manure and poultry litter

The two-year sampling resulted in ARGs abundance differences in both cattle manure and poultry litter, with variations in sampling year and source of manure (p<0.05, Table 3.1). Abundance of the two ARGs [*erm*(B) and *sul*(I)] and the integron *intl*(I) were greater in cattle manure in both 2018 and 2019, while they were rarely found in poultry litter samples during 2018 (Fig. 6.1). The relatively greater abundance of *erm*(B) and *intl*(I) in cattle manure compared to poultry litter corresponded to higher alpha diversity of the bacterial community.

The greater abundance of ARGs in cattle manure compared to poultry litter was not expected. In a study by Xu et al. (2020), more than two fold greater ARGs were found in poultry manure

compared to cattle manure. However, it must be noted that poultry manure rather than poultry litter (chicken manure and the bedding) was evaluated in the study of Xu et al. (2020).

Table 3.1.ANOVA of results testing for differences in quantities of three AMR associated genes by animal source (cattle manure and poultry litter), sampling year (2018 and 2019), and interaction between these two factors.

Parameter	Factor	Pseudo-F	*P*-value
erm(B)	Animal source	32.58854	3.23E-05**
	Year	0.280069	0.603921
	Animal source x Year	8.065261	0.010121*
Sul(I)	Animal source	48.14529	3.34E-06**
	Year	1.115031	0.306675
	Animal source x Year	3.590204	0.072671
Intl(I)	Animal source	25.34924	0.000122**
	Year	0.499597	0.489844
	Animal source x Year	0.001061	0.974335

* Statistically significant ($\alpha = 0.05$)
** Statistically significant ($\alpha = 0.01$)

Moreover, the very low TS content of cattle manure could underestimate the quantities of ARGs when estimated on a wet basis compared to solid poultry litter, which has three times less moisture (Table 1.1). Consistent with these results and knowing that the soil microbial community may shift in response to manure inputs, Yang et al. (2020) found that soils receiving long-term cattle manure deposition yielded greater *erm*(B), *sul*(I), and *intl*(I) gene abundances relative to soils receiving poultry litter. Therefore, using cattle manure for pastureland fertilization may pose a greater risk for ARGs dissemination to the environment than poultry litter.

Antimicrobial resistance has existed prior to the advent of antibiotics (Aminov, 2009; Brinkac et al., 2017; Wright, 2007). However, the fecal resistome in cattle manure and poultry litter depends largely on the feed (Liu et al., 2019) and the rate and type of antimicrobials provided to the animals for both therapeutic and non-therapeutic purposes (Barton, 2014; Hughes and Heritage, 2004; Wegener, 2003). Moreover, the quantity of ARGs released to the environment during application of feces is affected by post-handling and/or management activities.

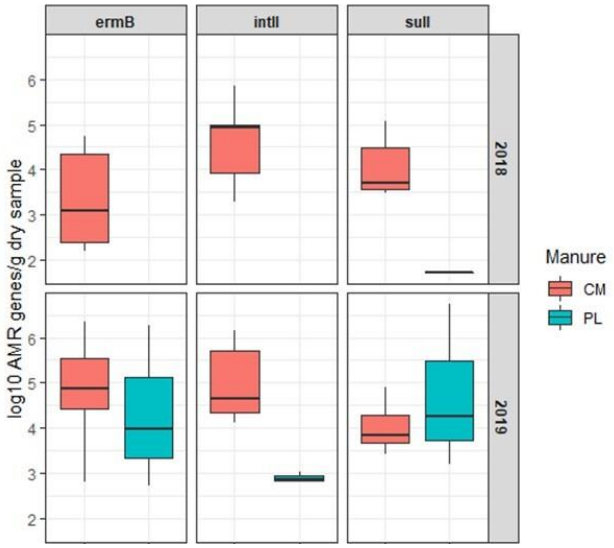

Fig. 6.1. Box plot of number of AMR genes in cattle manure (CM) and poultry litter (PL) collected in year 2018 and 2019.

In this study, the bedding that constituted the poultry litter may have increased TS content and suppressed resistant bacteria and genes. This is evidenced by the negative and significant correlation between TS and abundance of bacterial community (Fig. 5.1). In the USA, air drying is practiced to reduce pathogens in municipal wastewater and Burch et al. (2013) demonstrated reductions of ARGs in wastewater with this air-drying approach, although *sul*(1) and *intl*(I) were not affected. In the current study, the greater gene copies in poultry litter in 2019 samples also depicts that the in-house piling may not be useful at reducing sulfonamide resistant genes, implying not all ARGs respond to such treatment. The overall results indicate that increasing TS or aerating manure may be useful to reduce abundance of bacterial community and the chance of release of zoonotic bacteria associated with fresh manure use.

1.4. Conclusions

Fresh cattle manure deposition during grazing may increase agronomic productivity, as well as alter soil microbial structure and may be a source of ARGs entering soils. In contrast, poultry litter applications have greater advantages not only in terms of nutrient supply, but also environmental risks associated with disseminating ARGs to the environment based on the operations evaluated. The strong link between bacterial diversity and physiochemical properties of both cattle manure and poultry litter adds novel insights into the field of animal manure

management. A reduced C/N ratio and an increased TS may be drivers for low bacterial alpha diversity and ARGs abundance. Further work is needed to formally annotate antibiotic use and subsequent ARGs presence in various manure sources, as well as the potential for alternatives to antibiotics and manure composting to minimize the spread and dissemination of ARGs to soil and water bodies.

Chapter 2

Poultry litter application does not affect soil microbiota in short term field experiment[2]

Abstract

Soil microorganisms play crucial roles in nutrient cycling and provisioning ecosystem services. However, little is known about how soil microbial communities are affected by short term poultry litter land application in silvopastoral system. The current study was aimed to understand effects of short-term poultry litter land application and its interactions with forage species and moisture content on bacterial community in a silvopastural system. The experiment was arranged in factorial design, consisting poultry litter (applied and not applied), forage species [non-native, cool season orchardgrass (Dactylis glomerata L.) and a warm-season native grass mix (Andropogon gerardii L. and Schizachyrium scoparium L.) planted in strips between hedgerows], and soil moisture regime (aquic and udic). Illumina sequencing results identified little effect of poultry litter on soil microbial diversity. However, greater microbial diversity was observed under native grass and wet(aquic) soil. These results suggest an enhanced soil microbial diversity under native grasses with greater available soil water. Moreover, there was a strong negative correlation between microbial diversity and elevation, suggesting niche differentiation and microbial preference for lower elevations.

Key words: soil microbial diversity; forage systems; poultry litter; terrain attributes; microbial abundance.

[2] This work is published. Citation:

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2.1. Introduction

The importance of soil microorganisms are gaining attention, particularly in agricultural systems, as they interact with the mineral surface to govern availability of plant nutrients (Zhu et al., 2016) and overall ecosystem balance (Fierer, 2017). Although they have other unknown ecological attributes, they can be identified with state-of-the-art genomic approaches and manipulated or managed to increase ecosystem services (Sathya et al., 2017) and plant productivity (Fierer, 2017). In agroecosystems, different management practices could change soil microbial structure. For instance, increased aboveground diversity may bequest belowground ecosystems with greater species diversity that mitigates risk (Zak et al., 2003) and enhances climatic resiliency (Naeem, 1998). Unlike sole pastoral systems, silvopastoral systems are endowed with all these attributes.

Silvopasture, an agroforestry management practice that integrates trees and animal production under one system, is widely practiced in North America (Orefice and Carroll, 2017). It is generally considered a sustainable livestock production system (Jose and Dollinger, 2019) as it can address the three pillars of sustainability: planet, people, and profit (Tedeschi et al., 2015). It is also often more profitable (Broom et al., 2013) and preferable for improving forage quality (Ford et al., 2019; Jose and Dollinger, 2019; Neel et al., 2016) and minimizing cattle heat stress during hot summer months (Kay et al., 2018) than sole pastures. Apart from the system itself, management activities within, including selection of appropriate tree (Broom et al., 2013) and grass species (Jose, 2009), can improve grazing system (Xu et al., 2017), while the use of manure and/or fertilizer (Blazier et al., 2008; Lindgren and Sullivan, 2014) plays a crucial role in improving the net productivity of silvopasture. Such management practices improve productivity by enhancing CO_2 assimilation and improving nutrient availability (Lindgren and Sullivan, 2014). The latter is regulated by soil biophysical processes where soil microbes play an essential role. On the other hand, microbial composition and community structure, in turn, are influenced by soil nutrient status and ecological associations with plant roots (Q. Zhang et al., 2019).

The effect of silvopasture system on soil microbial activity, diversity, and abundance was previously studied and found to have advantages over sole pasture systems (Barros et al., 2018; Cubillos et al., 2016; Vallejo et al., 2012, 2010). The role of pasture manure applications on soil

bacterial diversity was also previously evaluated and indicated that an increased animal manure distribution results in enhanced microbial diversity (Yang et al., 2019). A study by Zhao et al. (2015) revealed that fertilizer applications and forage species altered microbial structure. Moreover, soil pH, C/N ratio, and available P were reported to be drivers of abundance of some specific groups of bacteria in soils (Hermans et al., 2017; Kaiser et al., 2016). Soil bacterial community structure also responds to changes to local topography that can be represented by terrain attributes, the elevation reportedly being the most strongly correlated with soil microbial richness and diversity (Peng et al., 2020; Singh et al., 2014; Yin and Yan, 2020). Further, recent studies revealed that microbial community structure showed strong correlation with soil moisture regime, temperature, pH, and P content along the elevation (Peng et al., 2020; Shen et al., 2019; Singh et al., 2014; Yin and Yan, 2020; Q. Zhang et al., 2019).

Despite knowledge on the influence of management on soil microbial community, evidence on the effects of poultry litter application, soil moisture regime, grass species, and their interaction with terrain attributes on diversity and abundance of soil bacterial community are lacking. Specifically, Adhikari et al. (2018) identified topographic influences on soil nutrient distribution, however, studies evaluating microbial abundance linkages with terrain attributes are few. Therefore, the current study is aimed to i) evaluate bacterial diversity and phylogenetic abundance in response to forage species, poultry litter applications, and soil moisture regime, and to ii) understand the interaction of these practices with terrain attributes in a silvopastoral system characterized by soils with a fine texture and neutral pH.

2.2. Materials and Methods

2.2.1. Site description

This study was conducted in a 4.25-ha paddock located at the University of Arkansas Agricultural Research and Extension Center in Fayetteville, AR (36.09°N, 94.19°W). The site is located in the Ozark Highlands, Major Land Resource Area 116A (Soil Survey Staff, 2019a). Information on previous site history is described by Sauer et al. (2015). Briefly, soil in most of the experimental area is mapped as Captina silt loam (fine-silty, siliceous, active, mesic Typic Fragiudult) with some Pickwick silt loam (fine-silty, mixed, semiactive, thermic Typic Paleudult)

and small areas of Johnsburg silt loam (fine-silty, mixed, active, mesic Aquic Fragiudult), and Nixa cherty silt loam (loamy-skeletal, siliceous, active, mesic Glossic Fragiudults) soils (Soil Survey Staff, 2019b).

Adhikari et al. (2018) provides details about the derivation of terrain attributes, and topographic functional units (TFUs) in the study area. Briefly, TFUs are derived using terrain attributes where the individual units are more homogeneous in terms of terrain properties and behave as a single functional unit within a landscape and can be used to describe functional behavior or soils. Based on terrain attributes, the study site could be divided into four TFUs, namely A, B, C, and D; TFU A had the highest nutrients present, whereas TFU B had the lowest P, K, Zn, Cu, Fe, and Ca but highest Na content. However, Mn, Mg, and B did not vary among TFUs (Adhikari et al., 2018). Topographic functional units are the landscape units that show more homogenous terrain properties within and less homogeneous properties between the units in terms of soil-terrain relationship (Adhikari et al., 2018). The wetter location within the study site has a fine, mixed, active, thermic Typic Endoaqualf and *aquic* soil moisture regime (virtually free of dissolved oxygen because it is saturated by ground water or by water of the capillary fringe), whereas the better drained soils have an *udic* soil moisture regime (classified as no evidence of saturation or reduction within 50 cm of the surface; Soil Survey Staff, 1999c). The site has a mean (30-yr mean) annual precipitation of 1,232 mm and a mean annual air temperature of 14.5°C (NCDC, 2019a 2019b). The mean and normal temperature and daily precipitation of the study area from January 2018 to July 2019 is reported in Gurmessa et al. (2021a).

2.2.2. Tree hedgerow and treatment placement

The hedgerows layout and the soil types of the experimental site were mapped and well described by Sauer et al. (2015) and (Adhikari et al., 2018). In the year 2000, hedgerows of a total of fifteen rows of three species, namely, northern red oak (*Quercus rubra* L.), eastern black walnut (*Juglans nigra* L.), and pecan (*Carya illinoinensis* Wangenh K. Koch) were established. Each species had five rows, and the rows were oriented east-west at 15-m spacing (Sauer et al., 2015). In 2014, the eastern black walnut trees rows were replaced with rows containing three species: American sycamore (*Platanus occidentalis* L.), cottonwood (*Populus deltoides* W. Bartram ex Marshall), and pitch/loblolly pine (*Pinus rigida* x *Pinus taeda*).

2.2.3. Experimental design

Treatments included poultry litter (applied and not applied), forage species (orcahrd and native), and moisture regime (*udic* and aquic), set up in a factorial design. Locally sourced poultry litter was applied at a rate of 84 kg N ha^{-1} on March 21, 2018 and April 12, 2019 (4.94 Mg ha^{-1}, fresh weight basis). Poultry litter of 2018 had a pH 6.2 and contained 1.98% N, 0.58% P, and 1.02% K on dry basis, while that of 2019 had pH 5.2 and contained 2.48% N, 0.69% P, and 0.94% K.

Two forage types were seeded in between the tree rows, and the experimental unit (the land between the hedgerows) was randomly allocated to grass species and poultry litter treatment. The grass treatments were: 1) a cool-season orchardgrass (*Dactylis glomerata* L., var. Tekapo), and 2) a mix of native warm-season grasses (*Andropogon gerardii* Vitman and *Sorghastrum nutans* L.) in 8:1 ratio. The orchardgrass was planted fall 2015 at 17 kg pure live seed (PLS) ha^{-1}, whereas the mix species were seeded spring in 2016 at 10 kg PLS ha^{-1}. Grasses were planted with a Haybuster 107C no-till drill (DuraTech, Jamestown, ND). Prior to establishment, Cornerstone® Plus (N-[phosphonomethyl] glycine) was used to kill existing vegetation at a 2.2 kg ha^{-1} rate (41% a.i.). Heifers (*Bos taurus* L.) grazed the site at a rate of 2.20 animal units (AU) ha^{-1} from May 24 to July 6 in 2018, and at rate of 2.42 AU ha^{-1} grazed the site from May 29 to July 11, 2019.

Soil moisture regime was determined by volumetric water content (VWC) TEROS 11 sensors (METER Group, Pullman, WA) at two soil depths (15 and 60-75 cm). Water content measurements were recorded every 4 h and logged on a Decagon EM50 data logger (METER Group, Pullman, WA) throughout the experimental period from May to July in 2018-2019. Soil moisture data were averaged each day and expressed as daily mean volumetric water content for further analysis (Gurmessa et al., 2021a). Weather variables were measured by a micro-meteorological weather station approximately 500 m far from the experimental site.

2.2.4. Soil sample collection and analysis

Per replicate, four soil samples (per species, poultry litter, and soil moisture regime) were collected by auger in triplicate on March 6, 2018 and May 17, 2019 from the Ap horizon (0 to 15 cm) in the center of grass alleys between two hedge rows (experimental unit). The sample points

were georeferenced. To prevent contamination, each soil sample was taken using auger sterilized with 70% ethanol between experimental units. Samples from each treatment combination (species, poultry litter, and soil moisture regime) and topographic position (depression and top slope) were collected and stored in a cooler for transport to the laboratory, where they were stored at -20ºC until DNA extraction.

After removing plant materials manually, soil samples were dried in a forced-air oven at 70°C for 48 hours. Dried samples were then ground and sieved to pass a 2-mm mesh. The pH was determined potentiometrically in deionised water (1:2.5 solid:liquid ratio). Weight-loss-on-ignition was used to determine soil organic matter (SOM) concentration after 2 hrs at 360°C (Schulte and Hopkins, 2015). Total C and N were determined via combustion using a VarioMax CN analyzer (Elementar Americas, Mt. Laurel, NJ). Mehlich-3 extractable soil nutrients were determined using a 1:10 solid: liquid ratio (w:v) (Tucker, 1992) and analyzed by inductively coupled argon-plasma spectrometry (ICP, Agilent Technologies, Santa Clara, CA).

2.2.5. DNA extraction and Illumina sequencing

DNA was extracted from each soil sample using the extraction kit of MpBio FastDNA Spin Kit for Soil (MpBio Laboratories, SKU 116560200-CF) according to the manufacturer's directions. Extracted DNA was quantified using Quant-It^{TM} PicoGreen® (Invitrogen) dsDNA quantitation assay and stored at -20ºC.

Bacterial community composition was determined using Illumina Miseq sequencing of 16S rRNA gene amplicons. Extracted DNA was sent to the University of Tennessee Genomic Services Laboratory, where the V4 region of the 16S rRNA gene was amplified with barcoded primers 515F and 806R (Caporaso et al., 2011). Amplicon libraries were pooled, and 291 base-paired end sequences were obtained on the Illumina MiSeq Platform, resulting in a total of 4,196,620 sequence reads. Reads were processed using the open source bioinformatics software Mothur V 1.40.0 following the Miseq SOP protocol (Kozich et al., 2013). Sequences that did not match the primers were eliminated from demultiplexed sequence reads. These ambiguous base sequences with a length less than 100 bp were deleted and chimeric sequences were removed using the UCHIME algorithm implemented in Mothur. After the quality control pipeline, 3,321,000

sequence reads remained using a 97% similarity threshold to define ribotypes in Mothur (7.91% were deleted).

2.2.6. Estimation of diversity and evenness indices

The greengenes database was used to classify the operational taxonomic unit (OTU) at the genus level using the Bayesian method (Cole et al., 2014); thereafter, relative abundance of all OTUs were summed within phylum and analyzed for relative abundance at both phylum and OTU levels. Except for the analysis of bacterial community structure, all other analyses were conducted based on OTU level. Based on this subsampled dataset, richness was calculated using the Chao index (Hughes et al., 2001) and diversity was calculated using the indexes of Simpson and Shannon from Mothur output files (Schloss et al., 2009). Beta-diversity was measured by using Bray-Curtis index, weighted and unweighted UniFrac distance metrics (Schroeder and Jenkins, 2018).

2.2.7. Topographic evaluation on microbial properties

The relationship between topography and soil microbial properties was explored using correlation analysis. Topographic information was provided with terrain attributes derived from a digital elevation model compiled from light detection and ranging (LiDAR). A 1-m grid digital elevation model was downloaded from the USDA-Geospatial Data Gateway site (https://datagateway.nrcs.usda.gov) and down sampled to 10 m resolution for developing the 13 terrain attributes: altitude above channel network, aspect, elevation, flow accumulation, mid-slope position, multi-resolution ridge top flatness index, multi-resolution valley bottom flatness index, normalized height, wetness index, slope percent, slope height, slope-length factor, and valley depth in SAGA GIS environment (Conrad et al., 2015). These terrain attributes represent topographic landscape variability and are related to water flow and distribution. Altitude above channel network measures the vertical distance of a point to the nearest channel. Elevation represents land surface elevation above mean sea level and its normalized value is the normalized height. Flow accumulation gives the number of upland pixels draining to a given raster, whereas wetness index determines a potential of a pixel to retain moisture. Similarly, valley depth determines the relative height difference to the immediate adjacent channel network, whereas the difference to the crest

lines is the slope height. Slope percent and slope-length factor show a maximum rate of change between pixels and neighbors, and the length of the slope is calculated per the universal soil loss equation. Aspect shows the direction of the steepest angle from the north direction. Multi-resolution ridge top and valley bottom flatness index identifies high and depositional areas in the landscape, respectively (Guo et al., 2019). Once the terrain attributes were derived, the raster values at soil sample locations were extracted and used to calculate Pearson's correlation coefficient of microbial properties (richness and diversity) at an alpha level of 0.1.

To characterize functional relationships between topography and microbial properties, the study area was divided into functional zones, topographic functional units, which in a previous study (Adhikari et al., 2018) showed similar terrain functional properties in terms of moisture and energy flow and distribution across the landscape. First, the terrain attributes were converted into principal components or factors, and the factors with eigen value >1 were used as inputs to k-means clustering technique to derive TFUs. that divided the study area into 4 TFUs (MacQueen, 1967). Principle component analysis of 13 terrain attributes provided 7 principle components or factors without losing much information in the data, and they were clustered into 2 to 10 potential clusters using JMP software (SAS Institute, 2016). To identify the optimum number of clusters, a cubic clustering criterion was calculated for each cluster and the one with the highest value was identified as an optimum cluster representing the TFUs in the study area. Detail statistical procedures followed in the derivation of TFUs in the study area including principle component analysis and k-means clustering techniques and are provided in Adhikari et al., 2018. The TFUs were named A, B, C, and D in which A TFU represents an accumulation zone where soils potentially tend to remain moist due to the higher values of wetness index, valley depth, and flow accumulation (aquic). Similarly, B TFU is an area with higher elevation and slope, but with lower values of wetness index, valley depth, and flow accumulation, indicating the dryer part of the study area (udic). While the TFU C and D are intermediate in terms of moisture retention compared to TFU A and TFU B, TFU D tends to remain slightly drier compared to TFU C. Once the TFUs were identified, the distribution of richness and diversity among TFUs were compared using student's t-test for their significant difference. The TFUs were grouped into *aquic* and *udic* soil moisture regimes for purposes of statistical analyses assuming the differences in water dynamics would affect the microbial responses.

2.2.8. Statistical analysis

Data on soil physiochemical properties were analyzed with a Mixed Model (V9.4; SAS Institute, Cary, NC) consisting of the random effects of replication and year, with fixed effects being forage species, poultry litter, and soil moisture regime. When main effect differences were found, pair-wise post hoc comparisons were performed by the SAS macro 'pdmix800' (Saxton, 1998); with Fisher's least significant difference (LSD) at a Type I error rate of 5% (SAS Institute Inc, 2009).

To evaluate differences in microbial diversity and evenness, ANOVA was carried using the statistical software R 3.5.1 (R Core Team, 2012) and JMP R 12 (SAS Institute Inc, 2015). Principal coordinate analysis (PCoA) plots were generated based on weighted and unweighted UniFrac distance metrics using MicrobiomeAnalyst (Dhariwal et al., 2017). Bacterial community structure quantified in a matrix of Bray-Curtis similarities was analyzed in a permutational analysis of variance (PERMANOVA) to compare bacterial communities at the OTU level in PRIMER-E (Clarke and Gorley, 2006).

2.3. Results and Discussion

2.3.1. Soil physiochemical variation based on treatments

Soil P was greater ($p<0.05$) under the fertilized native warm-season grass mix compared to fertilized or unfertilized orchardgrass but did not differ from the unfertilized native grass mix. Similarly, the greatest soil P and K occurred under the dry (*udic*) native grass mix (Table 1.2), likely owing to less loss due to overland flow and plant P and K uptake in drier areas. Concentrations of K and Mg were also greater under native grass species regardless of poultry litter application. In contrast, there were no three-way (forage species × poultry litter × soil moisture regime) interactions for any soil physiochemical properties ($p>0.05$; Table 1.2).

However, there was a two-way interaction for soil P and K (species × poultry litter; species × moisture regime). In addition to the absence of grass species effects, neither the soil moisture regime nor poultry litter impacted soil C and N concentrations. Similar findings were also reported previously by Bloor (2015) who evaluated the effects of manure amendments under different grass species and found little impact on soil N and C. This could be associated with cattle manure deposition across the site masking any treatment-induced changes in silvopastoral systems.

Table 1. 2. Soil properties measured at a silvopasture site in Fayetteville, AR in 2018 and 2019 (analyzed across years as there were minimal year effects; P≥0.05). Samples were collected at 0-15 cm at four factor levels: forage species (NG = native grass mix, and OG = non-native orchardgrass), moisture regime (W = wet/mesic, and D = dry/xeric), and poultry litter (F = fertilized with poultry litter, and NF = no poultry litter fertilization, control). [Spp = forage species, Fert = Fertility, OM = soil organic matter].

Forage	Fert/Moist	pH	OM	C	N	P	K	Ca	Mg	S
			——%——			——mg kg^{-1}——				
NG	F	6.75a	3.48a	1.94a	0.19a	77a*	125a	1657a	73a	13.2a
NG	NF	6.73a	3.27a	1.68a	0.16a	59ab	89ab	1646a	57ab	15.0a
OG	F	6.75a	3.17a	1.88a	0.18a	40b	76b	1850a	49b	13.8a
OG	NF	6.69a	3.56a	1.89a	0.19a	52b	68b	1538a	50b	12.6a
NG	D	6.75ab	3.38a	1.97a	0.19a	83a	137a	1741ab	75a	14.3a
NG	W	6.74ab	3.27a	1.65a	0.17a	52b	77b	1562ab	55ab	13.8a
OG	D	6.57b	3.38a	1.79a	0.17a	49b	77b	1470b	57ab	12.6a
OG	W	6.87a	3.45a	1.98a	0.19a	44b	67b	1918a	42b	13.8a

Comparison was within poultry litter application or grass types, not both, i.e., * P was the highest ($p < 0.05$) compared to the F and NF treatments).

2.3.2. Bacterial community composition based on treatments

The PERMANOVA test on the Bray-Curtis dissimilarity showed differences in soil bacterial community structure at the phylum level based on the interaction of moisture regime and forage species ($p<0.05$); however, there were no differences in bacterial communities based on the single factors of manure amendment, soil moisture regime, and forage species [($p>0.05$); (Table 2.2)].

Table 2.2. PERMANOVA in bacterial community structure by fertilization, moisture regime, and grass. PERMANOVA results illustrate differences in bacterial community structure by single factor of poultry litter (fertilized by poultry litter or non-fertilized), soil moisture regime (dry or wet), and forage species (orchardgrass or native grass), as well as two factors (poultry litter x moisture regime, poultry litter x forage and moisture regime × forage) and three factors (poultry litter x moisture regime x forage). *Significant at p = 0.05.

Factor	Pseudo-*F*	*P*-value
Poultry litter	1.074	0.322
Moisture regime	1.9608	0.148
Forage species	0.73523	0.496
Poultry litter x Moisture regime	0.96214	0.351
Poultry litter x Forage species	2.0381	0.123
Moisture regime x Forage species	3.805	0.042*
Poultry litter x Moisture regime x Forage species	1.8078	0.166

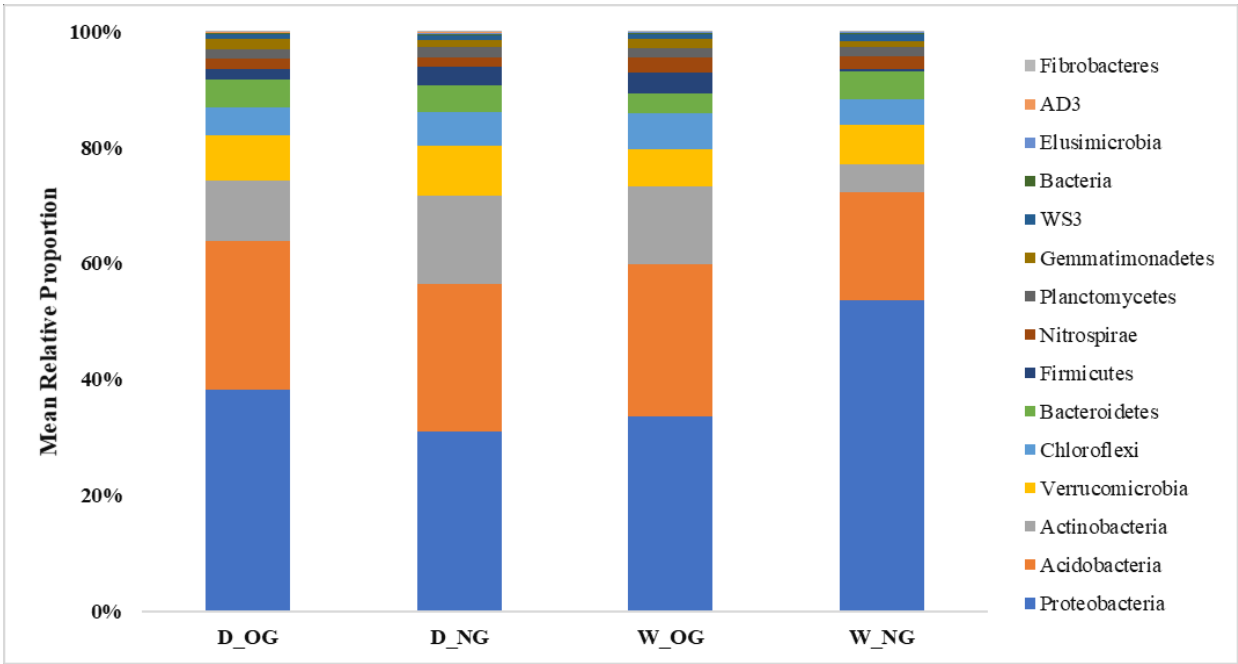

Fig. 1.2. Mean relative proportion of bacteria population in phylum. Factors include soil moisture regimes [udic (D) and aquic (w)], and forage species orchardgrass (OG) and native grass (NG).

The following top ten phyla dominated soil bacterial communities: Proteobacteria (mean relative abundance of all libraries was Protobacteria (37%), Acidobacteria (25.53%), Actinobacteria (12.07%), Verrucomicrobia (7.65%), Chloroflexi (5.50%), Bacteriodetes (4.37%), Firmicutes (2.58%), Nitrospirae (2.09%), Planctomycetes (1.64%), and Gemmatimonadetes (1.56%, Fig. 1.2). Even though the differences in terms of the relative abundance, for instance Proteobacteria, can be comparable across the treatments, the order in the level of composition is similar.

The findings by Estendorfer et al. (2017) on bacterial relative abundance under orchardgrass were consistent with the current findings. Protobacteria generally dominate the soil bacterial community under grasslands (Arenz et al., 2014; Singh et al., 2007; Spain et al., 2009), but the level of relative abundance of a bacterial phylum can be affected by soil management practices, which was also evident in this study (Fig.1.2).

In the current study, the interaction of *aquic* soil moisture regime and native grasses mix increased the relative abundance of protobacteria compared to other treatments. However, whole community evenness and diversity, expressed with Simpson's and Shannon indices, respectively, were reduced (Table 3.2). On the other hand, it is interesting that these diversity indices were

greater in *aquic* than *udic* soils under orchardgrass pastures, implying greater microbial preference to wet soil moisture regimes for these systems.

2.3.3. Bacterial community alpha diversity based on treatments

Alpha diversity was not influenced by poultry litter application, moisture regime (*udic* vs. *aquic*), and forage species (native vs. non-native) as estimated by three different algorithms (Chao1, Shannon, and Simpson's index) (Table 3.2). It was hypothesized that poultry litter applications would drive soil microbial community abundance, although this was rejected, given poultry litter application did not impact community structure ($p>0.05$). The Chao index was used to calculate the richness of soil bacterial community in this study. There was no influence from individual main effects, as well as for two and three-way interactions of these effects. The Simpson's index was used to calculate the evenness of soil bacterial community in this study. This result indicates, the moisture regime x forage species interaction affected bacterial community evenness ($p<0.05$). Fig.2.2A illustrates the influence of moisture regime and forage species on soil bacterial richness using Chao1; Fig. 2B shows bacterial evenness (calculated using Simpson index), and Fig. 2C presents the bacterial diversity (via Shannon index). The Shannon index was used to measure community diversity, including both richness and evenness. Overall, moisture regime x forage species interacted to affect bacterial community diversity ($p<0.05$).

Richness, evenness, and diversity indices illustrate the alpha diversity of bacterial community in response to changes in edaphic or anthropogenic factors. Unlike orchardgrass, the native grass mix was less effective in maintaining evenness and diversity of alpha diversity in *aquic* soils. Moreover, the variability of these diversity indices within a treatment (Fig.1.2) shows some bacterial phyla may be more sensitive to changes in soil conditions among the sampling points across the landscape. This may be reflected mainly in variabilities of soil nutrients (Peralta et al., 2010) or extremophile presence. This is demonstrated in the boxplot (Fig. 2.2). The highest proportion of Protobacteria may have reduced the evenness of bacterial community distribution under the same treatment. The absence of difference in richness due to the different management practices or their interactions might be attributed to niche differentiation of the bacterial species (Lennon et al., 2012).

Table 3.2. ANOVA of richness and diversity in bacterial community structure. ANOVA results illustrating richness and diversity in bacterial community structure by single factor of poultry litter (fertilized by poultry litter or non-fertilized), soil moisture regime (dry or wet), and forage species (orchardgrass or native grass), as well as two factors (poultry litter x moisture regime, poultry litterx forage and moisture regime × forage) and three factors (poultry litter x moisture regime x forage).

Parameter	Factor	*F*-value	*P*-value
Chao	Poultry litter	1.8245	0.1905
	Moisture regime	1.9448	0.1771
	Forage species	1.3148	0.2638
	Poultry litter x Moisture regime	1.2319	0.2790
	Poultry litter x Forage species	2.1094	0.1605
	Moisture regime x Forage species	4.1263	0.0545
	Poultry litter x Moisture regime x Forage species	2.9114	0.1020
Shannon	Poultry litter	3.5267	0.0737
	Moisture regime	2.1233	0.1592
	Forage species	1.2300	0.2794
	Poultry litter x Moisture regime	3.1843	0.0881
	Poultry litter x Forage species	3.5564	0.0726
	Moisture regime x Forage species	6.9957	0.0148*
	Poultry litter x Moisture regime x Forage species	3.9033	0.0609
Simpson	Poultry litter	1.9514	0.1764
	Moisture regime	2.4438	0.1323
	Forage species	1.1360	0.2980
	Poultry litter x Moisture regime	1.9529	0.1762
	Poultry litter x Forage species	2.9865	0.0980
	Moisture regime x Forage species	7.2339	0.0134*
	Poultry litter x Moisture regime x Forage species	2.8896	0.1033

*Significant at p = 0.05.

The positive interaction observed under the native prairie grass mix with *udic* moisture regime may be linked to the increased root biomass and the exudates from native grasses (Eisenhauer et al., 2017). Plant root exudates and biomass turnover are sources of amino acids, organic acids, and sugars that are essential food sources for soil microbes (Haichar et al., 2014). Since different plant species have different root-structures, the mixed native grass species roots assumedly cover a wider surface area, thus providing a more conducive environment for microbes compared to the sole non-native orchardgrass species.

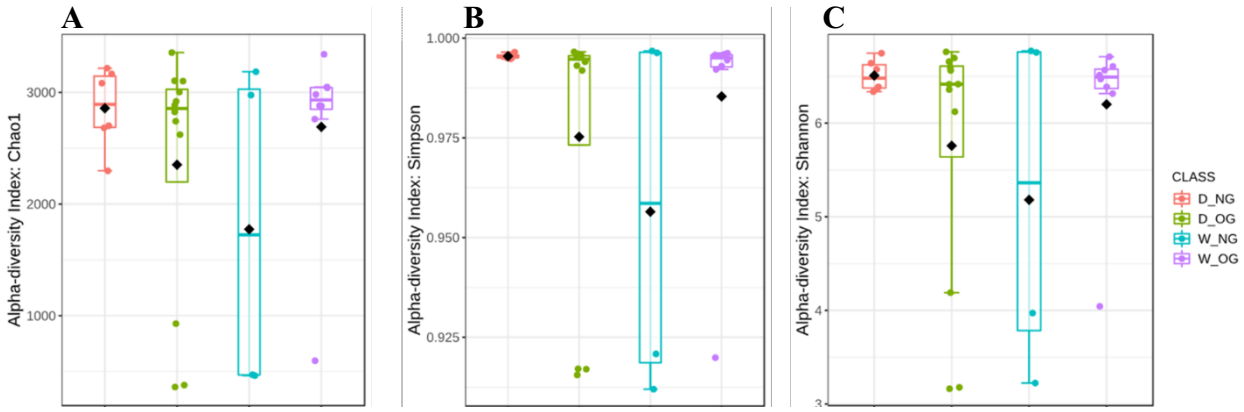

Fig. 2.2. Mean soil bacterial richness, evenness, and diversity [Chao1 (A), Simpson index (B), and Shannon index (C)] in different soil moisture regime (dry or wet) under different forage species (native and non-native) system. Factors include soil moisture regime [dry (D) and wet (w)], and forage species orchardgrass (OG) and native grass (NG).

2.3.4. Bacterial community composition based on treatments

Community structure was compared across moisture regimes, poultry litter treatments, and forage species to determine if moisture regime (*udic* vs. *aquic*) and forage species (native vs. non-native) impacted the bacterial community structure. Pairwise distances were calculated from all samples using the Bray-Curtis distance metric. These distances were visualized by principal coordinate analysis (PCoA), which showed overlaps between bacterial communities based on moisture regime and forage species. No significant shifts in microbial composition were observed in PCoA plots based on Bray-Curtis (PERMANOVA p>0.05) (Fig. 3.2). This result illustrates short term management practices are less likely to alter soil microbial composition, as previous studies reported shifts in soil microbial composition following 10+ years of continuous poultry litter applications (Ashworth et al., 2017; Yang et al., 2019).

2.2.1. Topographic linkages to soil microbiota

Among 13 terrain attributes used in the study, only 7 attributes were found to be significantly correlated with microbial properties (Table 4.2). Richness was positively correlated with elevation (r = 0.47), and mid-slope position (r = 0.52) in 2019 and the relationship was significantly, negatively correlated with flow accumulation (r = -0.22) and slope percent (r = -0.16). Moreover, richness was weakly correlated with multi-resolution ridge top flatness index, valley depth, and

altitude above channel network in both 2018 and 2019. In general, richness in 2018 had a weak correlation with terrain attributes, compared to 2019 except for aspect, multi-resolution valley bottom flatness index, and wetness index. These results indicate terrain attributes play a large role in soil microbial community abundances spatially and temporally.

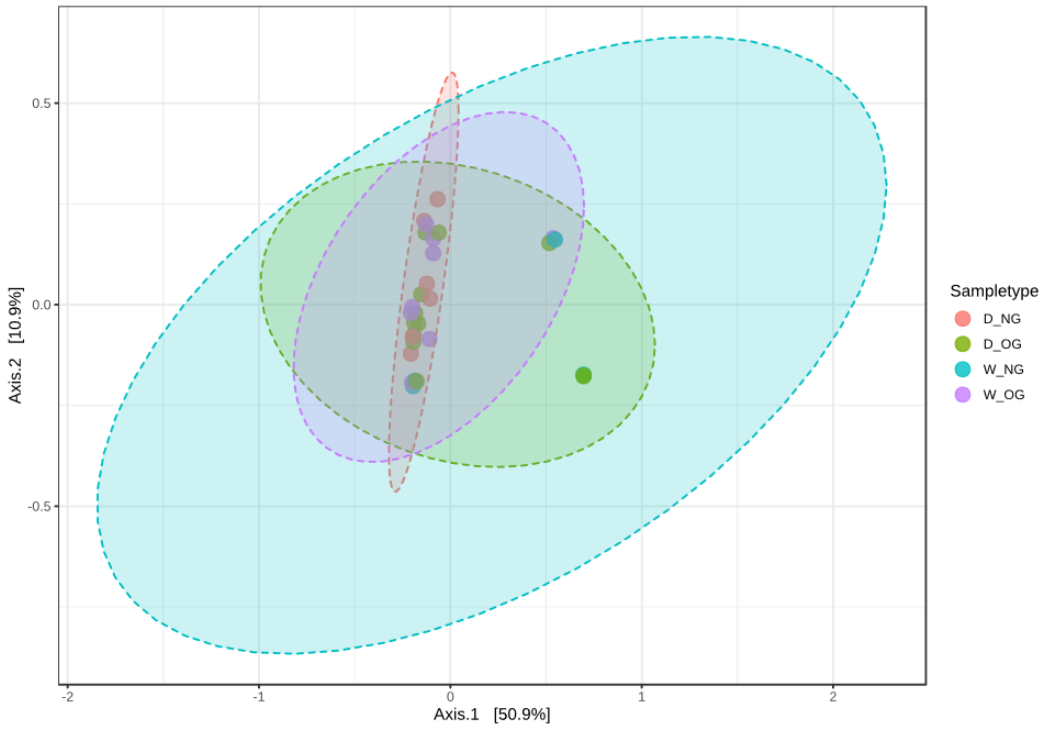

Fig. 3.2.Principal Coordinate Analysis (PCoA) of Bray–Curtis distances of bacterial community structures in different soil system. Factors include soil moisture regime [dry (D) and wet (w)], and forage species orchardgrass (OG) and native grass (NG).

Diversity had a significantly positive correlation with flow accumulation (r = 0.51), slope-length factor (r = 0.56), and altitude above channel network (r = 0.42) in 2018. In 2019, diversity was significantly negatively correlated with elevation (r = -0.59) and mid-slope position (r = -0.47); while slope height, multi-resolution ridge top flatness index, and normalized height showed a weak negative correlation with diversity (Table 4.2). The strong correlation between the Shannon index and elevation agrees with a recent finding (Merino-Martín et al., 2020). Elevation is among the most important terrain attributes and has been widely studied for its effect on soil microbial composition and structure (Merino-Martín et al., 2020; Q. Zhang et al., 2019), although interacting effects from management and inherent soil properties and elevation may occur (Z. Xu et al., 2020;

Q. Zhang et al., 2019). Terrain attributes alone illustrated little correlation with diversity; therefore, the TFU's which were developed based on combinations of multiple terrain attributes, were evaluated.

Table 4.2. Pearson's correlation coefficients among soil microbial properties and terrain attributes.

Terrain attribute	2018		2019	
	Richness (Chao)	Diversity (Simpson)	Richness (Chao)	Diversity (Simpson)
Aspect	0.22	0.29	0.07	-0.09
Elevation	-0.32	0.34	0.47*	-0.59*
Flow accumulation	-0.1	0.51*	-0.22	0.29
Slope-length factor	-0.09	0.56*	-0.15	0.32
Mid-slope position	0.18	0.14	0.52*	-0.47*
Multi-resolution ridge top flatness index	0.25	-0.27	0.35	-0.32
Multi-resolution valley bottom flatness index	-0.20	0.12	-0.08	0.03
Normalized height	-0.15	0.08	0.03	-0.21
Slope percent	0.14	-0.13	-0.16	0.21
Slope height	-0.12	0.19	0.16	-0.30
Wetness index	0.31	0.00	0.15	-0.11
Valley depth	0.11	0.22	0.25	0.03
Altitude above channel network	-0.18	0.42*	0.28	-0.41

*:Significant at $\lambda = 0.1$.

The distribution of richness and diversity in different TFUs are shown in Fig.4.2. Average richness in the study area was 3,803 (±1764), where it was above average in TFU A, B, and C. TFU A had the highest (5,804), and D had the lowest (2,626) richness. On the other hand, TFU B, C, and D had diversity lower than its overall mean (231) in this silvopasture study. TFU A had the greatest (373), and D (188) had the smallest diversity. Results showed that the richness was influenced by TFUs. For example, richness in TFU A was different than from TFU B and D, and that from D was different from TFU A, and C. Richness in TFU B and C were not different. However, diversity among the TFU B, C, and D were not different, except for TFU A which was different from the rest of the TFUs. Unlike management practices, richness was influenced by TFU, suggesting topographic effects may define soil microbial niches. The greatest soil bacterial richness was found in TFU A (Fig. 4.2) and may be attributed to this topographic position remaining moist for long periods (Adhikari et al., 2018). Moisture gradients along the landscape was also previously reported to be a determinant for variations in soil bacterial composition and

abundance (Lennon et al., 2012). The effect of topography on soil microbial composition and diversity of bacterial communities have also been reported by Liu et al. (2007, 2020).

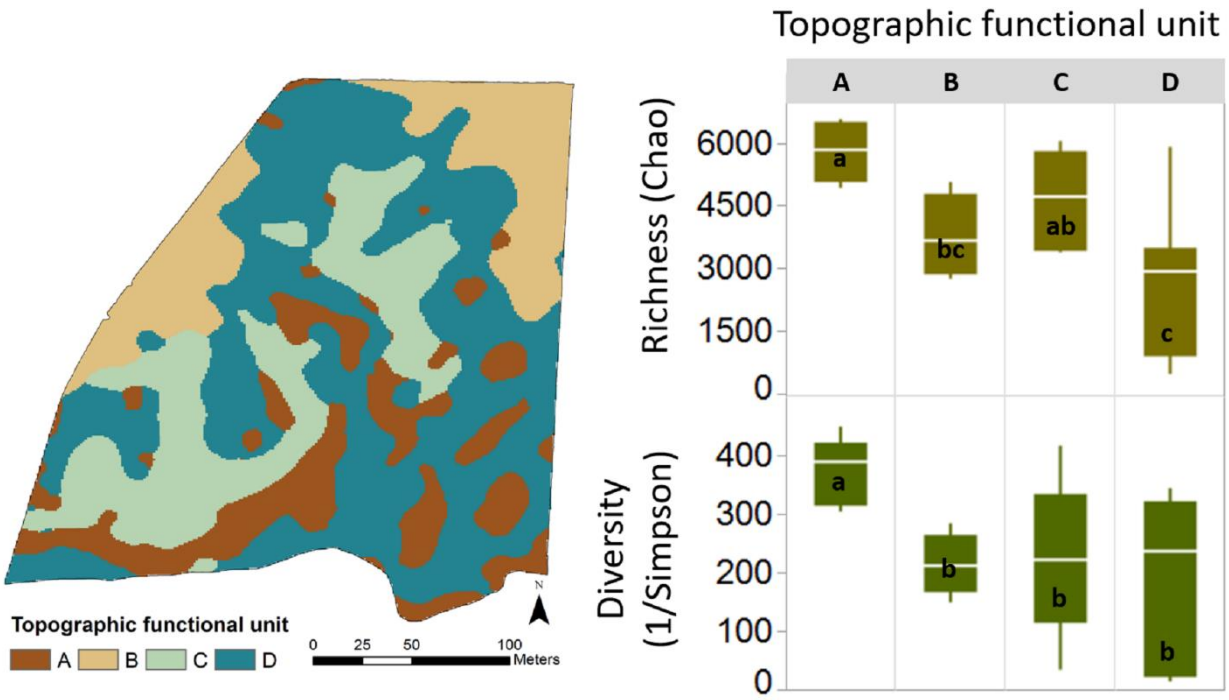

Fig.4.2. Richness and diversity as influenced by 4 topographic functional units (TFUs) (A, B, C and D). Letters a, b and c are notations for significance ($p < 0.05$).

2.3. Conclusions

This study evaluated the soil phylogenetic response to poultry litter under different scenarios: forage grass species types and soil moisture regimes, as well as soil microbiota interactions with topography using next generation sequencing to understand the interaction of management with terrain attributes in a silvopasture system. Two years poultry litter land application did not affect soil bacterial diversity. However, soil moisture regime interacted with forage species to effect bacterial community diversity, with native grass species under dry (*udic*) soil moisture regime resulting in the most diverse microbial assemblage relative to *udic* and wet (*aquic*) soil moisture regimes for the introduced forage (orchardgrass). In addition, a strong correlation was observed between soil bacterial diversity and terrain attributes (elevation and flow accumulation), thus suggesting the significance of considering TFUs to understand soil microbial diversity dynamics.

Therefore, TFUs controlled water distribution, with the unequal distribution of water controlling biologic responses. Hence, studies evaluating richness and diversity of soil microbes should consider the sample location within the context of landscape position.

Chapter 3

Post-digestate composting benefits and the role of enzyme activity to predict trace element immobilization and compost maturity[3]

Abstract

The current study evaluated the quality of agricultural waste digestate by composting or co-composting with biogas feedstock (maize silage, food processing waste, or poultry litter). Temperature, phytotoxicity, C/N ratio, water extractable trace elements and 14 enzyme activities were monitored for 90 days. Temperature dropped earlier in digestate and maize silage co-composting pile, reducing time to maturity by 20 days. Composting and co-composting reduced phytotoxicity and C/N ratio, but increased immobilization of Al, Ba, Fe, Zn, and Mn at least by 40% in all piles. All the enzyme activities, except arylsulfatase and α-glucosidase, were increased at the maturity phase and negatively correlated with organic matter content and most of trace elements. Post-digestate composting or co-composting with biogas feedstock is a promising strategy to improve digestate quality for fertilizer use, and selected enzyme activities can be indicators of compost maturity and immobilization of trace elements.

Keywords: C/N ratio; compost maturity, food processing waste, maize silage, poultry litter

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3.1. Introduction

In Europe, solid digestate, a byproduct of biogas production, is often directly used as fertilizer although legal status differs among member States. Some have policies that encourage direct use as fertilizer (Beggio et al., 2019; Tambone et al., 2015), while others do not consider it a fertilizer because of possible associated environmental risks such as ammonia emissions, odor, and high content of volatile fatty acids (Nkoa, 2014; PiotrZeng et al., 2016). Low nutrient supply and load of trace elements are other disadvantages associated with direct usage of digestate as fertilizer (Kupper et al., 2014; Torres-Climent et al., 2015). Digestates, especially those originating from manure or a mixture of manure and food processing wastes, can also be source of weeds, pathogens, pharmaceutical residues, and antibiotic resistant genes (Gurmessa et al., 2020).

Trace element loading with digestate at the farm-level could be high owing to continuous land applications as fertilizer (Kupper et al., 2014), thereby posing environmental risks. To avoid these and improve the quality of digestate for its use as an amendment, post-digestate treatment has been proposed (Bustamante et al., 2012; Rehl and Müller, 2011). It can be one possible treatment that has advantages in terms of increasing nutrient content, attenuating trace elements release, reducing the volume of biomass, and mitigating overall environmental risks. For instance, Karwal and Kaushik (2020) found significant reduction of Cd, Cu, Pb, and Zn contents with composting.

Thus, effective post-digestate composting may be sought for industrial level composting, which could involve the use of an appropriate co-composting material that is inexpensive and locally available. Moreover, it is essential to understand the benefits of composting digestate in terms of nutrient release, immobilization of trace elements, and phytotoxicity reduction. In addition to the commonly known compost monitoring indicators such as temperature, C/N ratio, pH, total solids content, total heavy metals content, and germination index (Tang et al., 2020), there is lack of knowledge on the dynamics of enzyme activities during post-digestate composting, which could be indicators of compost functioning, fate of trace elements, and maturity (a ready to use stable compost). Enzyme activities during composting were reported to be one of the best indicators of compost quality and maturity when the activities tend to increase (Wan et al., 2020) or become stable (Mondini et al., 2004) during the maturity phase. However, findings were not consistent,

and variations can be due to the differences in the sources of composting materials, the composting period, and enzyme activities.

Thus, the current study is aimed at understanding the role of post-digestate composting and co-composting with biogas feedstock on the final compost quality in terms of nutrient release, immobilization of trace elements, and phytotoxicity. The study is also aimed at investigating: *i*) the role of co-composting materials on post-digestate compost quality and maturity, *ii*) the trends of enzyme activities during composting and their use as proxy to define maturity of post-digestate compost, and *iii*) the relationship between enzyme activities and soluble nutrients and trace elements content during composting.

3.2. Materials and Methods

3.1.1. Composting, monitoring, and sampling

The digestate and co-composting materials were obtained from an industrial biogas plant in the Marche region, Italy. Digestate used is a byproduct of anaerobic digestion from a biogas plant that uses 10% poultry litter (85:15 chicken manure: wheat straw ratio; moisture ≈58%) and 90% of other biomass sources such as maize silage and food processing wastes (byproducts of cereal mill and fruit).

Five composting piles, 300 kg each (on wet basis) were prepared as follows: 1) solid digestate only (D00); 2) solid digestate + food processing waste (DCB); 3) solid digestate + maize silage (DMS); 4. solid digestate + poultry litter (DPL); 5) solid digestate + maize silage + poultry litter (DMP). Solid digestate was the target composting material and constituted 80% of the total biomass (w/w) of the piles; the other 20% was made by the co-composting material. The mix-ratio is described in Table 1.3.

Table 1. 3. Composition of the compost piles.

Pile	Composition	Mix ratio (w/w)
D00	Solid digestate only	-
DCB	Solid digestate + food processing waste	4:1
DMS	Solid digestate + Maize silage	4:1
DPL	Solid digestate + Poultry litter[¥]	4:1
DMP	Solid digestate + Maize silage + Poultry litter[¥]	8:1:1

[¥] The poultry litter was 85:15 chicken manure: wheat straw ratio; moisture ≈58%.

The composting materials were mixed thoroughly and stacked inside high-density polyethylene boxes, each having a volume of 1 m^3. To allow air movement, four holes of 3 cm diameter were drilled at 5 cm and 50 cm from the base on the corners of the box: two from one side and two on the opposite side. Two hard plastic tubes of 2 m length, each with holes every 20 cm, were inserted diagonally from the bottom hole of one corner to the upper hole of the other corner of the box. Piles were turned weekly during the thermophilic phase, every two weeks during the mesophilic period, and every three weeks during the maturity period, following standard industry procedures. Composting lasted for 90 days, and temperature was monitored daily from 4 to 5 o'clock p.m. using a temperature probe (A.M. Leonard Backyard Compost Thermometer) 30 cm depths in each pile; temperature monitoring stopped after 50 days of composting, when similar readings were observed across piles.

Three 1 kg samples were collected from every pile at ≈20 cm depths at 0, 7, 14, 21, 35, 49, 70, and 90 days of composting. The samples were dried at 40 °C and ground until it passed all through a 2 mm sieve. For each fragmented sample, an aliquot of about 100 g was stored at 4 °C for < 10 days to be analyzed for enzyme activities, while the rest of the sample was stored at room temperature for the physicochemical analyses.

3.1.2. Main physicochemical characteristics

The pH was determined potentiometrically in H_2O (1:8 w/v) after one night of contact time. Total solid (TS) content was estimated as the fraction of dry mass remaining after samples were dried at 105 °C for 24 h. The organic matter (OM) content was determined as the loss on ignition at 550 °C until a constant weight was reached (Heiri et al., 2001). Total C and N were determined by the dry combustion method using a CHNS analyzer (EA-1110, Carlo Erba Instruments, Milan, Italy).

3.1.3. Enzyme activities

The activities of 14 enzymes were determined according to the method described in Cardelli et al. (2019). Briefly, 150 mg of specimen was placed in a 2-ml Eppendorf tube with glass beads containing 1.2 ml of 50 mM tris–HCl solution at pH 7 containing 2% lysozyme as a desorbing protein. The tube was then subjected to bead-beating (3 min, 30 strokes s^{-1}) using a Retsch MM400 mill (Haan, Germany) and centrifuged for 5 min at 20,000 *g*. Enzyme activities were analyzed

fluorometrically in microplates using 4-methyl-umbelliferyl and 7-amino-4-methyl coumarine conjugated surrogate substrates (Sigma, St. Louis, MO, USA) at three pH ranges in three different solutions. Activities of acid phosphomonoesterase, α-glucosidase, arylsulfatase, β-glucosidase, β-galactosidase, cellulase, chitinase, glucuronidase, and xylosidase were determined in 200 mM MES (morpholineptansulfonic acid) solution at pH 5.8; whereas, the activities of leucine aminopeptidase, lipase nonanoate-esterase, pyrophosphatase-phosphodiesterase, and phosphodiesterase were determined in 100 mM tris–HCl solution at pH 7.5. The alkaline phosphomonoesterase activity was determined in 100 mM tris–HCl solution at pH 9.0.

3.1.4. Water-extractable elements

Ten g of each sample was added to 100 ml of distilled water (1:10 w/v) and shaken for about 1 h. Then, the suspension was centrifuged for five minutes at 300 *g* and the solution was filtered using a Whatman 42 filter. The amounts of macronutrients (Ca, K, Mg, P, and S) and trace elements (Al, Ba, Cd, Cu, Fe, Mn, Ni, Pb, and Zn) in the extracted solution were determined using Inductively Coupled Plasma Spectroscopy (Optima 8300, PerkinElmer, USA).

3.1.5. Phytotoxicity test

Germination index (GI) was used to evaluate the phytotoxicity of the original solid digestate (experimental control) and of the composting digestate at 7, 21, 51, and 90 days of composting. The experiment was conducted in a completely randomized design with three replications. One g of digestate or composting material was added to 5 ml deionized water in a 50 ml tube and shaken for 2 hours; the suspension was then centrifuged for 5 minutes at 300 *g*, and the solution filtered through a Whatman 42 filter. The germination test was conducted on petri dishes of 8.5 cm diameter using cress (*Lepidium sativum* L.) seeds, which are highly recommended because of their high sensitive to even low toxicity (Luo et al., 2018). Two Whatman™ 1442-125 grade 42 filter papers were moistened by applying 1 ml of the extract solution, whereas deionized water was used as a control. One filter paper was placed on the petri dish and 10 seeds were placed per dish. Then, the second filter paper was used to cover seeds to protect them from moisture loss. All Petri dishes were closed, sealed to avoid moisture loss, and incubated in the dark at temperature of 25 °C. Germinated seeds were counted after 48 hours of incubation, and root length was measured using

a digital caliper. GI was estimated as the percentage of seeds germinated multiplied by the average root length in the treated Petri dishes in relation to the number of seeds germinated multiplied by the average root length in the control Petri dishes.

3.1.6. Statistical analysis

Statistical analysis and visualizations were conducted using R packages. Pearson's correlation analysis was conducted and plotted between enzyme activities and other chemical compositions using the CorLevel package in R. Redundancy analysis (RDA) was conducted using Vegan package (Oksanen et al., 2019) in R for Windows, v. 4.0.1. and plotted using ggpot2 to understand the relationship between enzyme activities (response variables) and pH, OM, total C, and water extractable macronutrients and trace elements (environmental variables). The environmental variables were log transformed, whereas response variables were Hellinger transformed. PERMANOVA test was run to evaluate the statistical significance of the interaction effects of the environmental variables on response variables. The significance of composting on release and immobilization of elements were computed as the additional amount released or the amount reduced in the final composts as percentage of the initial concentration.

3.3. Results and discussion

3.3.1. Physicochemical characteristics of the digestate and co-composting materials

In Table 2.3, characteristics of digestate and co-composting materials are reported. The solid digestate had sub-alkaline pH (8.1), which was similar to previously studied digestate originating from feedstock mixtures containing pig-slurry, but higher than that of cattle slurry (Alburquerque et al., 2012a). In contrast, the pH of poultry litter was neutral, while it was slightly acidic for maize silage and food processing waste. Digestate and maize silage had the lowest TS content (38-41%), while food processing waste was the greatest (94%). OM content was the highest in food processing waste and maize silage, but the organic C content was similar in the four materials. Digestate mean C/N ratio was ≈26 (similar to that of maize silage), and exceeded the limit of <25 in use in several countries including Italy (Tambone et al., 2015) for direct use as fertilizer. This result was greater than that of by Alburquerque et al. (2012b), indicating possible variations among the different sources of digestate. Digestates with C/N values greater than 20, meaning with excess

of degradable organic C, could lead to immobilization of N (Teglia et al., 2011a), and may undermine the agronomic benefits of direct use of digestate. The lowest C/N ratio of the poultry litter was due to greater total N content. Water extractable macro-nutrients and trace elements mainly abounded in the poultry litter. Instead, digestate showed the highest content of Fe and the lowest of Mg, P (together with maize silage), and Ba.

Table 2.3. Physicochemical properties of digestate and the co-composting materials (Mean ± SD) (n = 3). For each parameter, mean values with different letters significantly differ (p< 0.05).

Variable	Digestate	Food processing waste	Maize silage	Poultry litter	*P*-value
pH	8.1 ± 0.0a	5.8 ± 0.1d	6.2 ± 0.0c	7.2 ± 0.1b	<0.001
TS (g kg^{-1})	384 ± 42c	941 ± 45a	413 ± 17c	565 ± 44b	<0.001
OM (g kg^{-1})	837 ± 2b	956 ± 1a	958 ± 0a	828 ± 0c	<0.001
Total C (g kg^{-1})	390 ± 35	416 ± 3	415 ± 6	387 ± 2	0.15
Total N (g kg^{-1})	15.1 ± 1.0b	18.2 ± 1.1b	16.1 ± 1.6b	35.3 ± 3.4a	<0.001
C/N ratio	25.8 ± 2.7a	22.9 ± 1.3a	25.8 ± 2.2a	11.0 ± 1.1b	<0.001
Ca (g kg^{-1})	3.0 ± 0.3c	0.8 ± 0.0d	6.0 ± 0.3b	12.6 ± 0.2a	<0.001
K (g kg^{-1})	141.0 ± 6.4b	36.2 ± 0.9d	83.5 ± 4.3c	259.6 ± 5.3a	<0.001
Mg (g kg^{-1})	2.9 ± 0.1d	4.2 ± 0.1c	7.7 ± 0.2b	15.3 ± 0.1a	<0.001
P (g kg^{-1})	10.2 ± 0.2c	17.9 ± 0.5a	10.2 ± 0.1c	14.4 ± 0.2b	<0.001
S (g kg^{-1})	11.7 ± 0.3b	2.0 ± 0.1d	4.4 ± 0.1c	57.5 ± 0.3a	<0.001
Al (mg kg^{-1})	108.2 ± 4.0b	145.9 ± 10.5a	113.1 ± 6.0b	143.1 ± 4.3a	<0.001
Ba (mg kg^{-1})	0.1 ± 0.0c	1.7 ± 0.5b	1.3 ± 0.3b	11.5 ± 0.3a	<0.001
Cd (mg kg^{-1})	0.8 ± 0.1b	1.2 ± 0.1ab	0.7 ± 0.1b	1.6 ± 0.3a	0.001
Cu (mg kg^{-1})	39.2 ± 1.5b	10.2 ± 0.3c	9.1 ± 0.2c	130.1 ± 0.4a	<0.001
Fe (mg kg^{-1})	1525.0 ± 65.9a	93.0 ± 4.9c	60.6 ± 3.1c	552.2 ± 1.5b	<0.001
Mn (mg kg^{-1})	28.8 ± 1.0b	20.7 ± 0.4b	25.9 ± 2.5b	141.9 ± 5.8a	<0.001
Ni (mg kg^{-1})	11.6 ± 0.1b	1.5 ± 0.2c	0.3 ± 0.3d	44.1 ± 0.4a	<0.001
Pb (mg kg^{-1})	36.7 ± 3.4	34.4 ± 2.4	35.4 ± 1.8	36.6 ± 2.6	0.672
Zn (mg kg^{-1})	95.3 ± 14.6b	41.1 ± 0.3c	4.3 ± 0.1d	258.0 ± 20.6a	<0.001

TS = total solid; OM = organic matter on a dry mass basis.

3.3.2. Changes of Temperature, pH, OM, total N, C/N, and GI during composting

In Fig. 1, temperature, pH, OM, total C, total N, C/N ratio, and GI changes are presented. These variables govern compost dynamics over the composting period and are also indicators of maturity. Except for D00, temperatures of all other piles increased to above 60 °C in the first two weeks of composting, but dropped quickly for DMS, thus reducing time to maturity by about 20 days compared to the control. The thermophilic phase lasted for about 49 days for D00, DCB, DPL, and DMP, and maturity was delayed. The pH decreased from alkaline to neutral in the control pile,

while it increased from slightly acidic to neutral in DCB. The pH changes in other piles were minimal. Both total C and OM showed a decreasing trend in all the piles, but the rate was lowest in D00 compared to the rest of the piles, suggesting the significance of the co-composting materials. Total N increased throughout the composting period with higher rates in all the piles with co-composting materials compared to the D00 pile.

The C/N ratio decreased over the experimental period in all piles, reaching the value of 17 or lower in the final composts, indicating compost maturity.

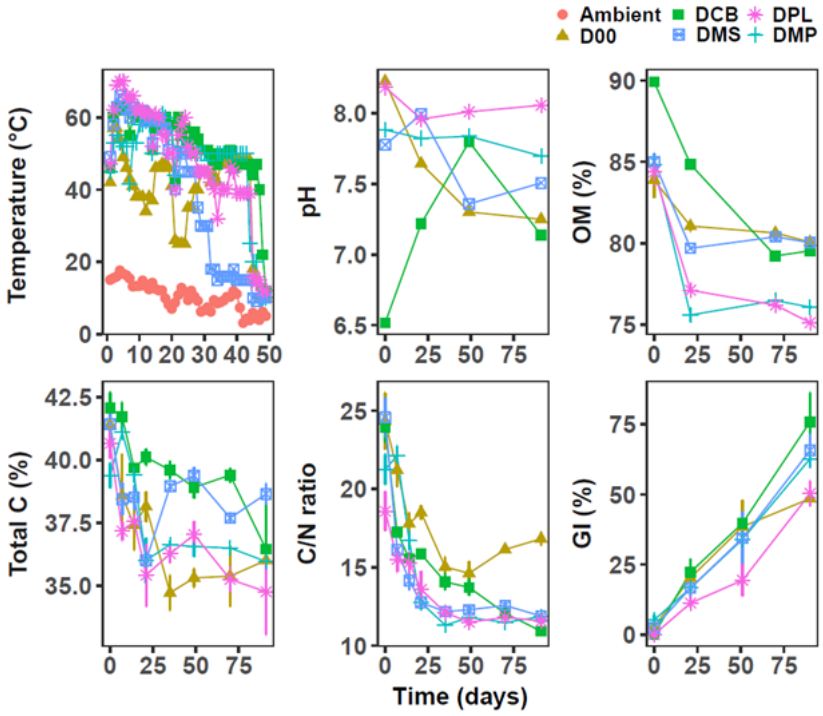

Fig.1.3. Trend of temperature, pH, total C, C/N ratio, organic matter (OM), and germination index (GI) during the composting period. D00 = solid digestate only, DCB = solid digestate + food processing waste, DMS = solid digestate + maize silage, DPL = solid digestate + poultry litter, DMP = solid digestate + maize silage + poultry litter.

GI increased in all piles over the composting period, but reached more than 60% only in DCB, DMS, and DMP, suggesting the significance of co-composting for reducing phytotoxicity of digestate. However, even after 90 days of composting, compared to the other piles, GI of D00 did not reach a satisfactory level, indicating the possible occurrence of phytotoxicity, especially when sensitive crops or vegetables are grown using digestate as fertilizer. A previous study also reported

possible ecotoxicity of digestate (Tigini et al., 2016), and our results suggest post-composting digestate, and co-composting materials may help mitigate phytotoxicity. A mature compost should have GI of 50% or more (Bernal et al., 2009), but it is commonly recommended to be 60% or more, which is generally believed to be indicator for a low toxicity level (Tambone et al., 2015), although there are differences among the seed types used for the test. The relatively low GI found in this study was similar to previous findings can be linked to the sensitivity to toxicity of the cress seeds used for the test, which is high even at low toxicity levels (Luo et al., 2018).

3.3.3. Composting effect on macronutrients release

The trends of water extractable values showed disparities among macronutrients during the composting period (Fig. 2.3). K and P showed an increasing trend in all the piles, while Mg showed an increasing trend only in D00. Instead, Ca displayed an inverse relationship in DCB and DPL, with few changes observed in D00. S concentration was reduced in DPL, while little changes were observed in the other piles.

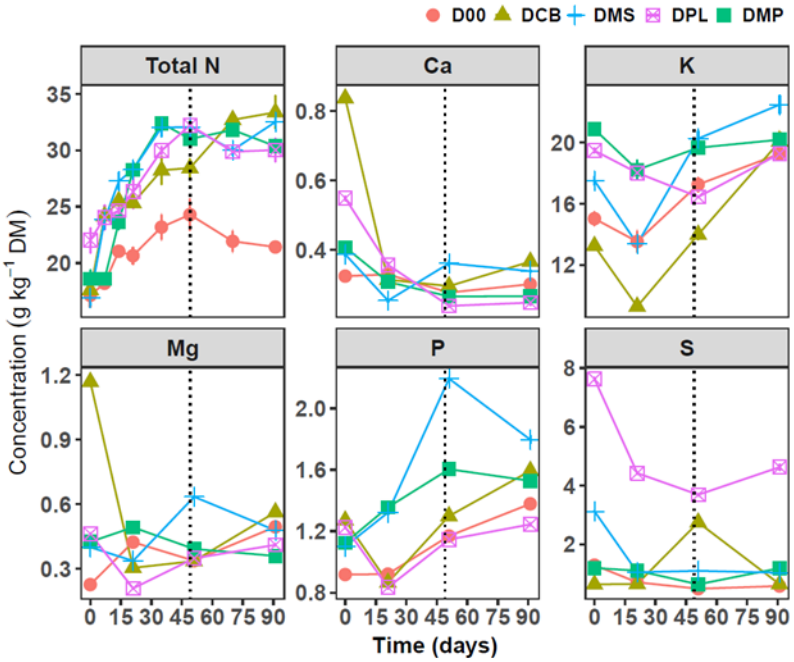

Fig.2.3. Dynamics of macro-nutrients during the composting period. D00 = solid digestate only, DCB = solid digestate + food processing waste, DMS = solid digestate + maize silage, DPL = solid digestate + poultry litter, DMP = solid digestate + maize silage + poultry litter.

Total N had increasing trends in all the piles and increased by about 30% in DPL and more than 50% in the other piles, suggesting a benefit from co-composting materials in reducing loss or

increasing mineralization of N during composting compared to the control. Previous research reported the recovery of N and other macro nutrients from wastes following composting (Rai and Suthar, 2020). Water extractable (available) was P enriched by 25, 30, 51, and 62% in the final composts for DCB, DMP, D00, and DMS, respectively (Table 3.3). On the other hand, DPL had little effect on P release, despite it having the highest amount of available P. Previous studies reported increases in available P release with composting, although the rate varies depending on the composted materials and the composting time (Sharma et al., 2018).

Table 3.3.Immobilization and release of trace elements and nutrients expressed as a percentage of the amount immobilized or released over the composting period from the concentration of the mix (mean ± SD) (n = 3). Negative values indicate immobilization, positive values indicate release.

Pile	D00	DCB	DMS	DPL	DMP
Ca	-9.1 ± 4.5	-53.5 ± 3.0	-22.3 ± 0.6	-50.9 ± 2.2	-33.0 ± 6.2
K	28.3 ± 3.9	51.3 ± 4.8	28.4 ± 2.8	-1.0 ± 1.5	-3.3 ± 1.2
Mg	124.2 ± 5.7	-38.5 ± 0.2	7.2 ± 1.5	-19.0 ± 2.4	-22.9 ± 3.6
P	50.7 ± 2.7	25.4 ± 3.0	61.8 ± 3.1	-1.5 ± 0.4	29.8 ± 6.7
S	-52.9 ± 1.6	2.6 ± 2.3	-47.7 ± 1.1	-18.1 ± 0.5	-32.4 ± 2.3
Total N	26.1 ± 16.2	89.8 ± 16.4	92.9 ± 14.2	36.7 ± 6.8	64.0 ± 15.6
Al	-93.4 ± 0.3	-94.6 ± 0.3	-93.6 ± 0.4	-16.2 ± 6.6	-93.2 ± 1.5
Ba	-74.8 ± 1.0	-95.3 ± 0.8	-91.3 ± 1.4	-97.7 ± 0.0	-93.8 ± 0.1
Cd	586.0 ± 76.1	475.9 ± 23.7	593.2 ± 83.9	-34.3 ± 8.6	528.9± 77.9
Cu	-55.7 ± 2.2	-47.9 ± 4.1	-43.2 ± 2.4	-25.7 ± 1.7	-49.4 ± 0.2
Fe	-89.3 ± 0.5	-82.3 ± 0.6	-86.5 ± 0.4	-75.3 ± 0.5	-85.9 ± 0.5
Mn	-74.6 ± 0.9	-60.6 ± 1.9	-68.6 ± 0.5	-72.6 ± 1.2	-72.3 ± 0.4
Ni	-42.5 ± 4.9	-42.6 ± 2.3	-40.1 ± 5.2	-70.4 ± 0.9	-71.0 ± 5.7
Pb	3.8 ± 16.8	-0.6 ± 4.7	-1.7 ± 8.4	-3.2 ± 10.2	-10.8 ± 1.8
Zn	-85.5 ± 2.2	-70.9 ± 3.9	-66.3 ± 4.1	-93.9 ± 1.0	-73.8 ± 2.5

D00 = solid digestate only; DCB = solid digestate + food processing waste; DMS = solid digestate + maize silage; DPL = solid digestate + poultry litter; DMP = solid digestate + maize silage + poultry litter.

In the current study, S content in the DPL pile was the greatest, although it gradually decreased over the composting period, suggesting the main source of S was poultry litter. The decreased in S content in all the piles over the composting period may be linked to the loss in the form of H_2S (Blazy et al., 2014). S breakdown during composting (and subsequent odor release) is sought to be mitigated to reduce S loss and pollution risk.

3.3.4. Composting effect on trace elements release

Water extractable contents of Ba, Cu, Fe, Mn, Ni, and Zn showed a decreasing trend in all the piles (Fig. 3.3), indicating immobilization with increased OM stability during composting. More than 60% of Al, Ba, Fe, Mn, and Zn was immobilized with composting across the piles (except Al

for DPL), while it was >40% for Ni; Cu showed the maximum immobilization (57%) in D00. In contrast, Pb showed little change, and Cd was enriched for all treatments except DPL (Table 3.3). Concentrations of water extractable Al, Ba, Cu, Fe, Mn, Ni, and Zn were reduced by 16-93, 74-95, 25-55, 75-89, 60-74, 42-70, and 66-85%, respectively. DPL was the least effective in reducing Al, Cu, and Fe release compared to the other treatments. This may be ascribed to the lower C/N ratio compared to the other piles (Wu et al., 2017). Cd level increased after the first week of composting in all the piles, except DPL, and remained constant until the maturity period. Pb mineralization or immobilization was little in all piles, suggesting composting digestate did not influence the release or immobilization of Pb. This could also be because of the relatively little concentration found in the co-composting and digestate materials.

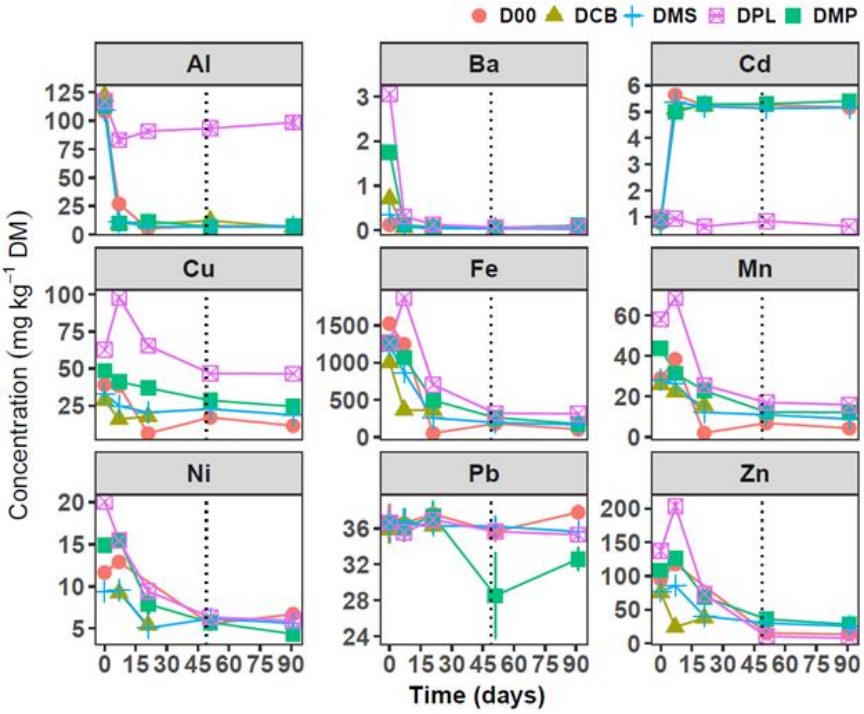

Fig.3.3. Changes in the content of trace elements during the composting period. D00 = solid digestate only, DCB = solid digestate + food processing waste, DMS = solid digestate + maize silage, DPL = solid digestate + poultry litter, DMP = solid digestate + maize silage + poultry litter.

Immobilization of trace elements is one of the benefits of composting. However, like the case of Cd in the current study, mineralization of trace metals could also be possible with post-digestate composting (Miaomiao et al., 2009). A previous study by Awasthi et al. (2020) reported that composting was effective in immobilizing Cu and Zn with biochar as a bulking agent, indicating

the importance of co-composting materials. Our study also gives an insight that stabilizing digestate with post-composting potentially stabilizes the material and reduces the release of trace elements, but also suggested careful selection of co-composting materials for optimization of nutrient digestate nutrient levels.

3.3.5. Effect of composting on enzyme activities

Among the 14 enzyme activities evaluated, arylsulfatase and xylosidase activities were either absent or very low in all piles over the composting period (Fig. 4.3).

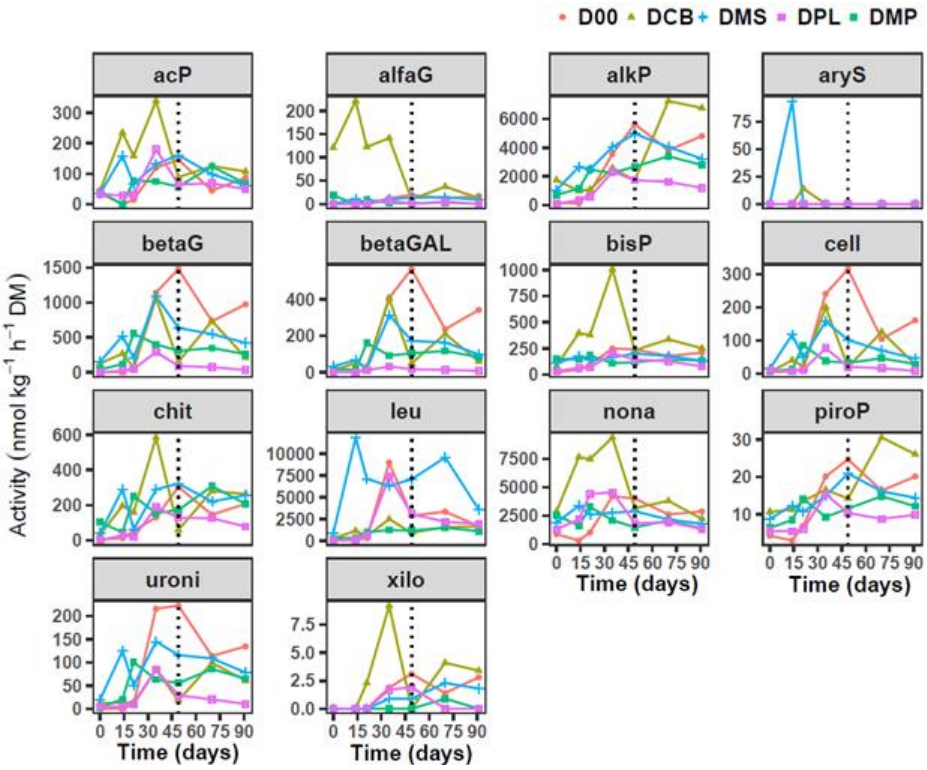

Fig. 4.3. Enzyme activity dynamics over the composting period. acP: acid phosphomonoesterase; alkP: alkaline phosphomonoesterase; alfaG: α-glucosidase; aryS: arylsulfatase; betaG: β-glucosidase; betaGAL: β-galactosidase; bisP: phosphodiesterase; cell: cellulase, chit: chitinase; leu: leucine aminopeptidase; nona: lipase nonanoate-esterase; piroP: pyrophosphate/phosphodiesterase; uroni: glucuronidase; xilo: xylosidase. D00 = solid digestate only, DCB = solid digestate + food processing waste, DMS = solid digestate + maize silage, DPL = solid digestate + poultry litter, DMP = solid digestate + maize silage + poultry litter.

In contrast, acid phosphomonoesterase, alkaline phosphomonoesterase, chitinase, and pyrophosphate/phosphodiesterase showed clear increasing trends in all piles during the 90 days of composting, with the greatest values achieved during the maturity phase, thus implying their role

in depolymerizing the most complex polysaccharides of the composting materials. The greatest activity was found for leucine aminopeptidase (11886 nmol kg^{-1} h^{-1}) in DMS during the second week of composting, followed by lipase nonanoate-esterase (9379 nmol kg^{-1} h^{-1}) and alkaline phosphomonoesterase (7250 nmol kg^{-1} h^{-1}) in DCB during the maturity phase. Acid phosphomonoesterase, α-glucosidase, β-galactosidase, β-glucosidase, glucuronidase, cellulase, and chitinase reached their peak values during the thermophilic phase, whereas pirophosphate and alkaline phosphomonoesterase, reached their peak during the maturity phase. Increased levels of activities of these latter two enzymes, indicates depletion of major unstable organic matter components (Herrmann and Shann, 1993), and may be indicators for stability of composts.

Some of our results agreed with the findings of Karwal and Kaushik (2020), Tiquia (2002), and Herrmann and Shann (1993), who conducted composting experiments with mixtures of buffalo dung and fly ash, manure, and municipal solid waste for 90, 154, and 90 days, respectively. In contrast, Castaldi et al. (2008) found a decreasing trend for dehydrogenase, urease, protease, and cellulase at maturity phase during municipal solid waste composting that lasted for 40 days. Ge et al. (2020) reported reduced activities of both alkaline and acid phosphatase during 60 days of cattle manure composting, but the trend of cellulase activity was consistent with our findings. This shows that enzymatic activity may serve as an indicator of compost maturity and stability, but with extended length of composting period (≥ 90 days) and when the most degradable organic materials are exhausted.

There were variations among the different co-composting materials. Interestingly, enzymatic activities in DPL changed little over the composting period, indicating the possible presence of inhibitory substances in the poultry litter. Increases in the activities of different enzymes like acid phosphomonoesterase, alkaline phosphomonoesterase, β-glucosidase, β-galactosidase, chitinase, and pyrophosphate/phosphodiesterase activities indicated maturity of composts, but their functional roles are different and, thus, they can be taken indicators of different chemical processes taking place during the composting process. For instance, cellulase are important indicators for cellulose degradation, which is expected to rise during the maturity stage of composting as easily degradable organic substances have already been degraded by microbes at the early stage of composting (Li et al., 2020). Cellulase had the highest activity in D00 and maintained levels until the end of composting, thus implying low availability for microbes.

3.3.6. *Correlations and RDA analysis*

Alkaline phosphomonoesterase, Chitinase, Glucuronidase, Leucine aminopeptidase, and Pyrophosphate/phosphodiesterase, and activities had strong positive correlation (p<0.05) with available P. Most of the enzyme activities had a negative correlation with C/N ratio, S, and most of the trace elements except Cd (Gurmessa et al., 2021c).

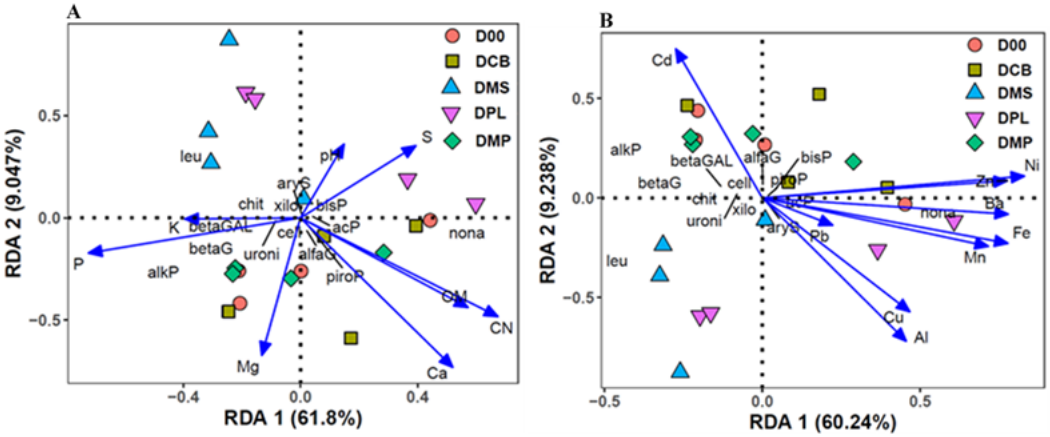

Fig. 5.3. Redundancy analysis of enzyme activities in relation to environmental factors (A: pH, C/N ratio, OM, and macro-nutrients; B: trace elements). acP: acid phosphomonoesterase; alkP: alkaline phosphomonoesterase; alfaG: α-glucosidase; aryS: arylsulfatase; betaG: β-glucosidase; betaGAL: β-galactosidase; bisP: phosphodiesterase; cell: cellulase, chit: chitinase; leu: leucine aminopeptidase; nona: lipase nonanoateesterase; piroP: pyrophosphate/phosphodiesterase; uroni: glucuronidase; xilo: xylosidase. D00 = solid digestate only, DCB = solid digestate + food processing waste, DMS = solid digestate + maize silage, DPL = solid digestate + poultry litter, DMP = solid digestate + maize silage + poultry litter.

The correlation was moderately strong with Al, Ba, Cu, Fe, Ni, Mn, and Zn, indicating that an increase of trace elements release during the early period of composting might inhibit enzyme activities (Aponte et al., 2020), which further suggests increased enzyme activities at later composting stages could be indicators of stability and immobilization of trace elements. A similar strong negative correlation of trace elements with enzyme activities was reported to occur during a cow manure vermicomposting by Malley et al. (2006) . However, in this study, positive correlations were found between Cd and all enzyme activities, implying that it could be rather stimulatory. Unlike all the other trace elements, Pb showed weak and positive correlations, possibly because Pb release was not affected during composting. RDA analysis of the enzyme activities as response variables and pH, OM, C/N ratio, and macro-nutrients as environmental factors showed a variance of 70.8 explained by RDA1 and RDA2 (Fig. 5.3B). The PERMANOVA

test conducted showed the contribution of variance explained by RDA1 was significant (p<0.01), but RDA2 was not. In Fig. 5.3C, trace elements explained 69.5% of total enzyme activity variation with RDA1 and RDA2. The overall results showed the significance of organic matter dynamics and the release or immobilization of elements in predicting the enzyme activities.

3.4. Conclusions

Post-digestate composting and co-composting reduced C/N ratio to 11-17. Phytotoxicity was reduced and greatest GI (76%) was obtained by co-composting with food processing waste. Up to 90% trace elements immobilization was found, and better results were obtained by co-composting with maize silage and food processing waste. Many of the 14 enzyme activities were low or absent in digestate but increased in the final composts. Alkaline phosphomonoesterase, and Pyrophosphate/phosphodiesterase had strong negative correlations (p<0.05) with Ba, Cu, Fe, Mn, Ni, and Zn. The increase in the activities of these enzymes also reveals P-cycling investments. The overall findings suggest the significance of post-digestate co-composting in improving quality and enzyme activities as compost maturity index.

Chapter 4

Post-digestate composting shifts microbial composition and degrades antimicrobial resistance genes[4]

Abstract

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

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

[4] This chapter was published:

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

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

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

4.2. Materials and Methods

4.1.1. Experimental setup and sampling

Digestate (fresh) was obtained from a biogas plant located in Marche Region, Italy. It is a byproduct of anaerobic digestion consisting of about 10% poultry litter (composed of chicken manure and wheat straw in a rough ratio of 85 and 15%, respectively) and 90% of mix of other biomass such as maize silage, food processing waste, and fruit processing byproducts.

Table 1.4. 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

Pilot level composting and co-composting piles were set up as described in Table 1.4, each amounting to 300 kg (on wet basis) and comprising of digestate and locally available materials in a ratio of 4:1 (w/w). The experiment was set up in completely randomized design with sub-sampling. The composting materials were stacked inside high-density polyethylene boxes, each

having volume of 1 m^3. The boxes were modified to allow air movement, and the piles were turned weekly during the thermophilic phase (≈49 days), every two weeks during the mesophilic phase (≈40 days), and every three weeks during the maturity phase. Composting lasted for 90 days, and samples were collected from each box at 0, 7, 35, 70, and 90 days of composting. Three samples were collected at about 5, 15, and 20 cm depth, after turning and thorough mixing. Then, 200 g of each sample was immediately stored at -20°C in a plastic bottle until analyzed for ARGs and microbial composition, whereas about 1 kg was dried at 40°C for chemical analysis.

4.1.2. Chemical analysis

Samples dried at 40 ^{0}C (about 1kg each) were ground, passed through a 2 mm sieve, and used to measure pH and total C and N content. The pH was determined potentiometrically in H_2O (1:8 w/v). Total C and N were determined by dry combustion method using a CHNS analyzer (EA-1110, Carlo Erba Instruments, Milan, Italy). Total solid content (TS) was estimated as the fraction of the dry mass after samples were dried at 105 ^{0}C for 24 h. Organic matter (OM) was determined as the loss on ignition (Heiri et al., 2001) at 550 ^{0}C until constant weight was obtained. Water extractable Al, Ca, Ba, Cd, Cu, Fe, Mg, Mn, Na, Ni, P, Pb, S, and Zn were determined by using 10 g of ground compost samples that were added to 100 ml of distilled water (1:10 w/v) and shaken for about 1 h at 10 rpm. The suspension was then centrifuged for 10 minutes at 300 *g* and the solution filtered using Whatman 42 filter. The extract was used to determine the concentration of the elements on an ICP-MS (Pröfrock and Prange, 2012).

4.1.3. DNA extraction and qPCR quantification of ARGs

DNA was extracted from 0.25 g of each compost sample using E.Z.N.A. ®Soil DNA Kit (OMEGA Bio-Tek, Inc., Norcross, GA, USA) according to the manufacturer's guidelines. Prior to qPCR analysis, the extracted DNA was checked for quantity and purity using a Nanodrop ND 1000 (Thermo Fisher Scientific, Wilmington, DE, USA). Moreover, the effective extraction of the bacterial DNA was checked by end-point PCR using the universal procaryotic primer pair 27f-1495r (Weisburg et al., 1991) targeting the bacterial 16S rRNA gene. DNA extracted from five reference bacteria strains, each carrying one of the ARGs under study were used as positive controls in the qPCR reactions as well as for the construction of qPCR standard curves as previously described by Vandeweyer et al. (2019). Each qPCR mixture was composed of 4 µL of

extract, 5 µL of Type-it 2X HRM PCR Master Mix (Qiagen, Hilden, Germany), 900 nm of forward and reverse primers for each ARG (Table 2.4), and nuclease-free water to reach the final reaction volume of 10 µL. The qPCR reactions were performed in a Mastercycler® ep realplex machine (Eppendorf, Hamburg, Germany) with an initial denaturation step of 5 min at 95 °C followed by 40 cycles of 95 °C for 15 s. The qPCR correlation coefficients (R^2) and amplification efficiencies were calculated automatically by Mastercycler® ep realplex software from the slopes of the standard curves. The qPCR detection limit for each AR gene in study was estimated from standard curves created in the range from $\sim 10^0$ to 10^7 gene copies per reaction.

Table 2.4. Primers used in the qPCR reactions targeting the five ARGs of interest (Flórez et al., 2014).

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

For absolute quantification of the five ARGs in study, each extract from the compost samples was run in triplicate along with tenfold serial dilutions of the standards. Each gene copy number detected in the analyzed samples was determined from the slope of the corresponding standard curve. The blank (nuclease-free water instead of DNA extract) and the negative control [DNA extracted from *Enterococcus faecalis* JH2-2 strain (Jacob and Hobbs, 1974)] were run together with the samples. The melt curve analysis with the temperature gradually increasing from 60 to 95°C by 0.4°C/s was performed to check amplification specificity. The results are presented as: *i*) the log of average gene copy number per gram of dry matter of each sample ± standard deviation and *ii*) the fold change, which was estimated to evaluate the reduction of ARGs abundance during the composting period. The latter was estimated as log of the quotient of ARGs copy number in composted sample divided by that in initial digestate sample as previously described by Qian et al. (2018).

4.1.4. 16S rRNA gene amplicon target sequencing and bioinformatics analysis

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

4.1.5. Statistical analysis

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

4.3. Results and discussions

4.3.1. Physicochemical characteristics of composts

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

4.3.2. ARGs abundance in anaerobic feedstocks, digestate and co-composting materials

qPCR standard curves created for each of the five ARGs were characterized by good amplification efficiencies, comprised between 0.91 and 0.99 for *tet*(K) and *tet*(S) genes, respectively, whereas R^2 values were 0.99 for all reactions. The lowest gene copy number per reaction in which the linearity was maintained (detection limit) was < 10^1 for the genes *erm*(B) and *tet*(O), and 10^2 for *tet*(K), *tet*(M), and *tet*(S).

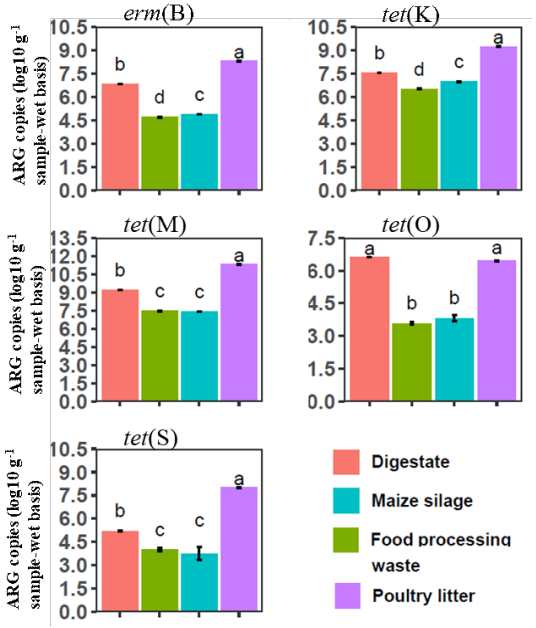

Fig. 1. 4. Abundance of ARGs in fresh digestate and co-composting materials. Bars indicated with different letters are statistically significant ($p<0.05$, $n = 3$).

All the five ARGs were detected in the biogas production feedstock, digestate, and co-composting materials (Fig. 1.4 and Table 3.4), but with great variations in number of copies among the types and co-composting materials, ranging from 3.6 log *tet*(O) in food processing waste to 11.3 log *tet*(M) in poultry litter. Poultry litter contained the greatest copies (p<0.05) of all ARGs followed by digestate, whereas maize silage and food processing waste had the lowest copies of ARGs. Generally, *tet*(M) was the most abundant gene (p<0.001) in digestate and co-composting materials followed by *tet*(K) and *erm*(B), whereas *tet*(O) and *tet*(S) were the least abundant gene in all the materials. The abundance of *erm*(B), *tet*(O), and *tet*(S) in poultry litter was about two-fold log higher than that of maize silage and food processing waste. Similarly, Agga et al. (2020) reported *tet*(M) as the most abundant ARG in digestates of three manure types suggesting possible differences in ARGs persistence under anaerobic digestion process.

Table 3.4. ARGs abundance (log10 per g sample, wet basis) in biogas feedstock. For ARG type, mean values with different letters significantly differ (p< 0.05) across the feedstock.

ARG	Food processing waste	Poultry litter	Maize silage
erm(B)	8.7± 0.00b	9.04± 0.04a	4.61± 0.12c
tet(K)	9.64 ± 0.03b	10.13 ± 0.10a	6.23 ± 0.01c
tet(M)	11.71 ± 0.02b	11.49 ± 0.06a	7.17 ± 0.06c
tet(O)	6.82 ± 0.01b	7.12 ± 0.08a	3.23 ± 0.04c
tet(S)	8.13 ± 0.06a	6.94 ± 0.17b	3.67 ± 0.35c

4.3.3. Dynamics of ARGs during post-digestate composting and co-composting

In Fig. 2.4, the fold change illustrates dynamics of ARGs abundance during the 90 days of composting. ARGs abundance was affected both by the composition of the piles and composting time. However, the change in the abundance of most of the ARGs was not consistent during the thermophilic and mesophilic phases. During the thermophilic phase, the abundance of all genes but *erm*(B) decreased. The abundance of *tet*(K) gene increased during the mesophilic phase although it was reduced at maturity. In contrast, *tet*(S) copies were potentially reduced after a week of composting and, unlike other ARGs, enrichment was rarely observed. This implies temperature dynamics over the composting period does not influence ARGs similarly. Compared to the digestate only pile (D00), ARGs abundance in the other piles was greater, and it was even the greatest in the DPL at the initial period, implying that the co-composting materials could be potential source of ARGs, particularly poultry litter (Fig.1.4). ARGs abundance in poultry litter,

however, largely relies on how it has been handled or managed before use (Gurmessa et al., 2021b).

Relative to the fresh digestate, *tet*(O) was reduced by the greatest fold compared to the other genes investigated (Fig. 2.4), which was -6.8, -7.6, -8.7, and -10.9 log in the final composts of DPL, DMS, DCB, and D00, respectively. In contrast, the least reduction was found for *erm*(B), indicating the relatively higher persistence of this gene compared to the tetracycline resistant genes, despite its relative abundance was lower than the other ARGs in the starting materials. Interestingly, ARGs abundance in D00 was reduced by greater fold than in the other piles. In the final composts, the lowest abundance of ARGs was found in D00 whereas the greatest was found in DPL(Gurmessa et al., 2021d). These results suggest that the materials, especially poultry litter, used for co-composting might have promoted or be the source of the ARGs. This can be further explained by the positive log change in the DPL (Fig. 2. 4), showing enrichment in ARGs with the addition of poultry litter to digestate.

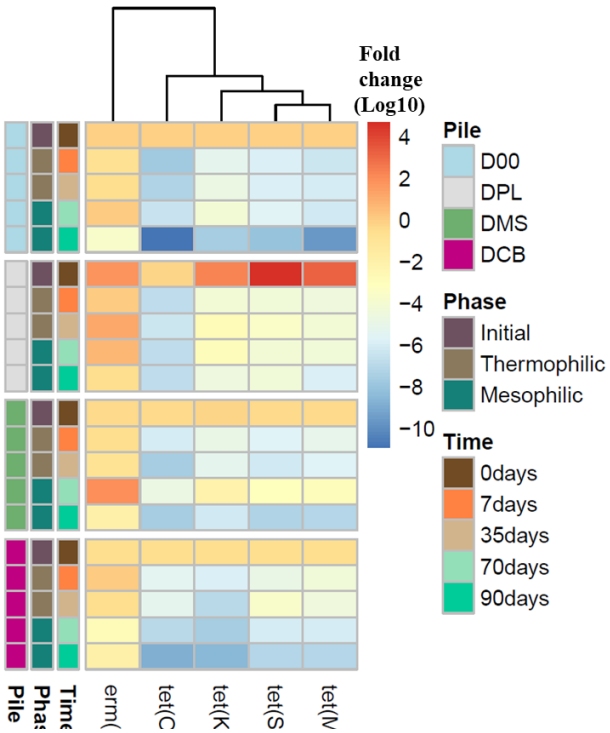

Fig.2. 4. Dynamics of ARGs in compost piles, expressed as log10 of fold change, during the composting period. Negative and positive results of the log fold change indicate reduction and enrichment, respectively.

It must be noted that fold change results discussed here were only in reference to the initial content in the piles. Compared to the copies number in the digestate, the benefit of using co-composting materials was not satisfactory, as *tet*(K) and *tet*(M) copies were enriched in DCB and DMS piles.

4.3.4. Bacteria community dynamics based on the 16S rRNA gene amplicon target sequencing

Three phyla dominated the bacteria community of fresh digestate: Firmicutes were the most abundant (73%), followed by Proteobacteria (18%), and Bacteroidetes (9%) (Fig. 3.4A). During composting, succession of Actinobacteria, Planctomycetes, and Verrucomicrobia formed a distinct bacteria community structure compared to that of fresh digestate. The emergence and presence of Actinobacteria in high proportion over the composting period indicates the well-functioning of the composts (Sundberg et al., 2013). At genus level, *Clostridium* (38%), *Sporosarcina* (33%), *Bacillus* (7%), and *Pseudomonas* (7%) were the top four dominant OTUs (Fig.3.4B).

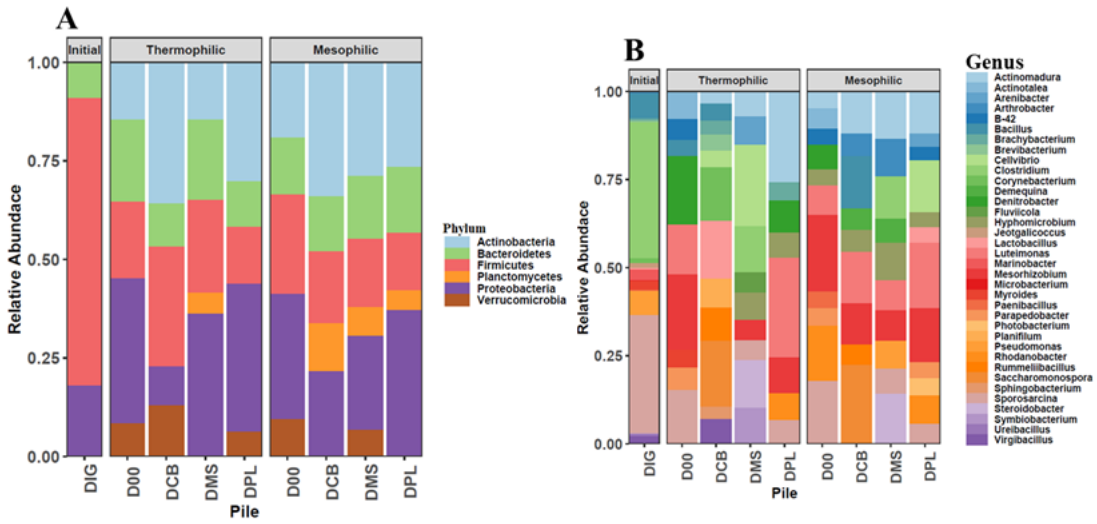

Fig.3. 4. Relative abundance of bacteria in the different piles (D00 = Solid digestate compost, DCB = Digestate + Food processing waste, DMS = Digestate + Maize silage, DPL = Digestate + Poultry litter) and fresh solid digestate (DIG) at phylum (A) and genus (B) level. DIG = Fresh solid digestate.

Non-metric multidimensional scaling (NDMS) of the Bray-Curtis dissimilarity analysis was conducted to understand the effect of co-composting or temperature regime on bacterial community structure. The influence of piles on bacterial community was significant (p<0.01) (Fig. 4. 4A), with significant effect on abundance of Firmicutes (p<0.001) and Bacteroidetes (p <0.05)

as MANOVA test revealed. The change in bacteria community structure was further elaborated with the non-metric multidimensional scaling (NDMS) of the Bray-Curtis dissimilarity analysis, which showed a significant (p<0.05) shift in the bacterial community structure between the digestate and the two phases of composting (Fig. 4. 4B). MANOVA test showed Firmicutes were significantly (p<0.001) affected, and their relative abundance was reduced during the shift from initial phase to the thermophilic and mesophilic phases. The shift in the abundance of these phylum and the succession of new phyla or genera over the composting period may be indictor of the compost status.

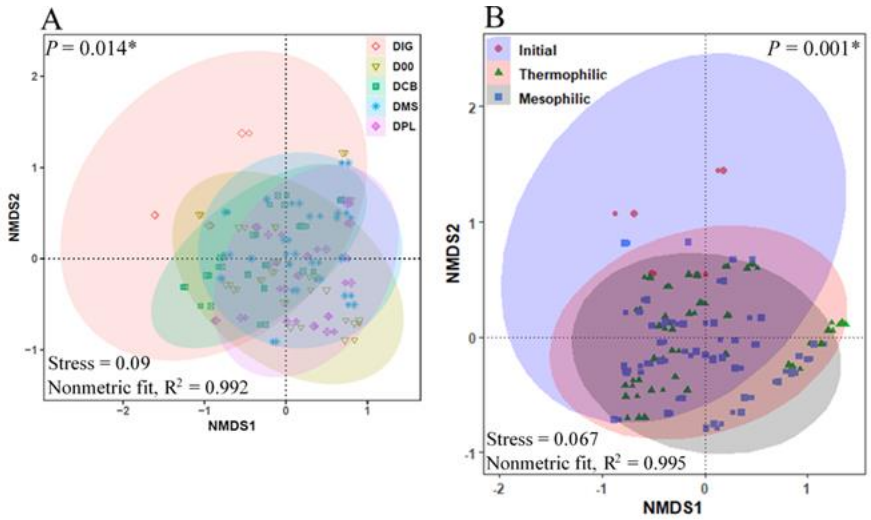

Fig. 4. 4. Differences in the composition of bacterial community among the piles (D00 = Solid digestate compost, DCB = Digestate + Food processing waste, DMS = Digestate + Maize silage, DPL = Digestate + Poultry litter) and fresh solid digestate (DIG) (A) and at the different compost phases (B), defined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

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

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

4.3.5. Co-occurrence of ARGs and bacteria

The co-occurrence of ARGs and bacteria (OTUs at genus level) was studied by Network analysis conducted on Pearson correlation coefficients (p<0.05) (Fig. 5.4). Despite more than 100 significant connections observed among the different genera, the results showed the exclusive co-occurrence of ARGs and the dominant bacterial phyla from the digestate such as Bacteroidetes (*Arenibacter* and *Fluvicola*), Proteobacteria (*Steroidobacter* and *Cellvibrio*) and Firmicutes (*Symbiobacterium*) (Fig. 3. 4A). *Arenibacter* showed a significantly strong correlation with all the ARGs, thus implying this genus could be their potential host. In addition, *Cellvibrio* displayed co-occurrence with all ARGs except for *tet*(O). Furthermore, *Fluviicola*, *Steroidobacter*, and *Symbiobacterium* co-occurred with *tet*(M) gene, which implies that *tet*(M) might have a broader host range compared to the other ARGs. Unlike what was previously reported during the composting of poultry litter by Cui et al. (2016), Actinobacteria, Verrucomicrobia, or Planctomycetes, which emerged during the composting period, did not show co-occurrence with any of the ARGs showing other bacteria in the digestate were the potential hosts, and the shift in the composition during the composting was responsible for the degradation of ARGs.

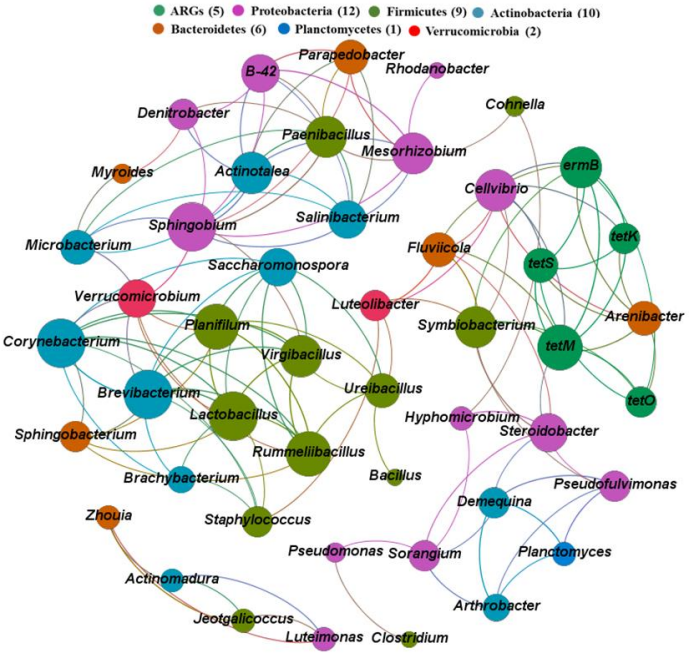

Fig. 5.4. Co-occurrence of ARGs and bacteria groups based on significant (p<0.05) Pearson's correlation.

Arenibacter is ubiquitous in various ecosystems and known for its capability of degrading high molecular weight substances including organic matter and pollutants (Roy et al., 2020; Stiborova et al., 2020). However, its potential as a host of ARGs has rarely been reported. In contrast, *Cellvibrio* was reported to be host of multiple ARGs in animal waste (Gou et al., 2021; Han et al., 2018), despite its significant functional role in lignocellulose degradation during composting (Raut et al., 2021). It also constitutes significant proportion of the bacteria communities in DPL and DMS, possibly due to the presence of lignocellulosic substrate (wheat straw and maize silage components, respectively). Within the Proteobacteria, *Steroidobacter* was the other potential host of *tet*(M). Previously, Wan et al. (2017) reported *Steroidobacter* as a potential host for selected ARGs in pig waste water. In the current study, it was found that the maize silage component might promote the abundance of *Steroidobacter*, as it was only found in the DMS pile without being affected by both the thermophilic and mesophilic temperature regimes. *Fluviicola* and *Symbiobacterium* belonging to Bacteroidetes and Firmicutes, respectively, co-existed and were found to be potential co-host for *tet*(M).

4.3.6. Relationship between ARGs abundance and chemical compositions

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

The CCA results were visualized in Fig. 6. 4. DCB formed distinct cluster, while there were potential overlaps among the other piles. In Fig. 6. 4A, dynamics of OM, pH, and extractable Ca, Mg, and K explained about 64% of the total variations in the ARGs abundance. However, both *tet*(M) and *erm*(B) had little relationship with these environmental variables. In contrast, *tet*(O) positively related to C/N ratio, OM, Ca, and Mg contents, and negatively related with pH. Both *tet*(S) and *tet*(K) had positive relationship with pH, K, and S. OM potentially contributed to the total variance explained, showing ARGs degradation could rely on the OM stability of the composts over the composting period.

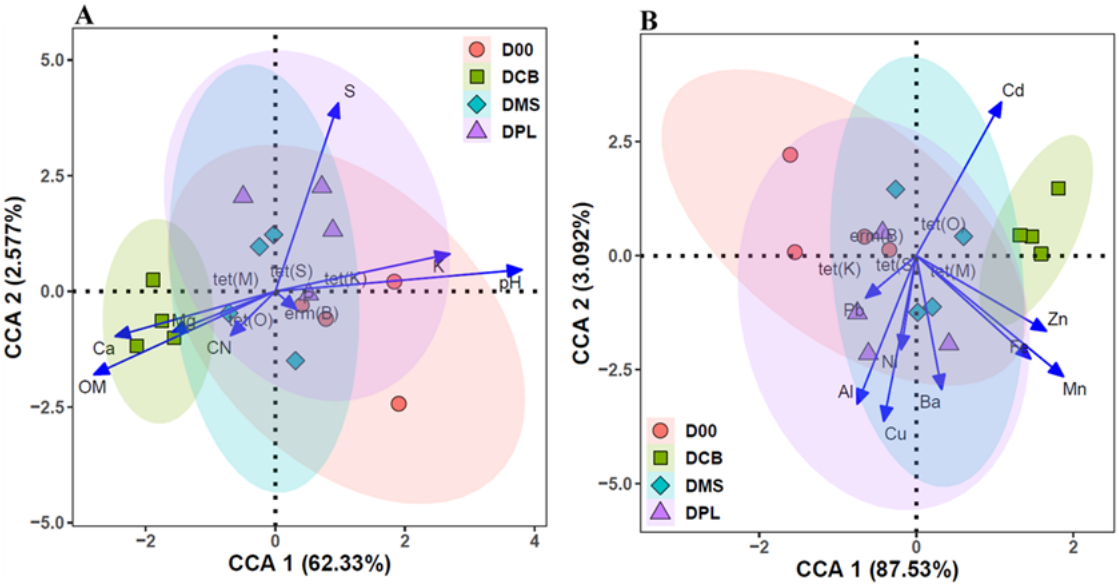

Fig. 4.4. Canonical coordinate analysis (CCA) of ARGs (response variables) and chemical composition (environmental variables) of composts. Two environments: A(C/N ratio, OM, pH, and macronutrients) and B(trace elements). D00 = Digestate, DCB = Digestate + Food processing waste, DMS = Digestate + Maize silage, DPL = Digestate + Poultry litter.

As reported in Fig. 6. 4B, trace elements (Al, Ba, Cd, Cu, Fe, Mn, Ni, Pb, and Zn) explained 90% of the total variations in ARGs abundance. Particularly, Al, Cd, and Cu had positive relationships (co-occurrence) with *tet*(K) and *tet*(S), whereas *tet*(M) had positive relationships with Fe, Mn, and Zn. Interestingly, *erm*(B) had no relationship with any of the trace elements, whereas *tet*(O) showed positive relationship with Cd. The greater contents of trace elements (Gurmessa et al., 2021c) and abundance of ARGs during the first phase and the significant reductions during the maturity period suggests co-occurrences. The weak relationship between trace elements and *erm*(B) could be linked to its weak response to composting as evidenced in Fig. 2.4. Similar to the these findings, Cui et al. (2016) reported a strong co-occurrence of bioavailable trace elements and ARGs during composting of poultry litter. These findings may further give an insight into the significance of composting on stabilizing organic substance that could lead to both immobilization of trace elements and reduction of ARGs.

4.4. Conclusions

Post-digestate composting removed more than 80% of ARGs from digestate, and it was at least as effective as co-composting with maize silage and food processing waste. Despite the strong co-

occurrence among the several bacteria groups, only a few genera were potential hosts of ARGs, and none of these belonged to those phyla (Actinobacteria, Planctomycetes, and Verrucomicrobia) that succeeded following composting. Results suggest the shift in microbial structure due to composting had little link with degradation of ARGs but could be an indicator of a well-functioning compost, rather, the physicochemical dynamics during composting or co-composting may be responsible for ARGs removal.

Major conclusions and recommendations

Manure has been widely used as organic fertilizer as it is rich in plant nutrients and organic matter content. In some cases, it is directly applied to the field, but in most cases it is either stored for some time or undergoes some processing before land application. In either of the scenarios, studies have widely reported possible environmental risks associated to manure land application as raw or processed. Thus, the current study was aimed to identify different manure usage scenarios and compared the relative benefits and risks. The core findings of the study are:

1) Direct manure land application could pose serious environmental risk especially in terms of release of ARGs, trace elements loading, and phytotoxicity.
2) While anaerobic digestion has been one of the common manure treatments, which results in energy production and digestate (byproduct that is widely used as a potential replacement for inorganic fertilizer), to mitigate environmental effects, it has been found to be not sufficient as some pollutants are persistent to such treatments.
3) Digestate was found to be not safe environmentally, and requires post-digestate treatments.
4) Post-digestate composting was found to potentially degrade ARGs and improve agronomic values of digestate.

As a recommendation, policies on use of digestate need to be revised, and trade-offs related to post-treatments need to be evaluated.

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Annex: First page of published papers that constitute the thesis.

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Variations in bacterial community structure and antimicrobial resistance gene abundance in cattle manure and poultry litter

Biyensa Gurmessa [a], Amanda J. Ashworth [b,*], Yichao Yang [c], Mary Savin [c], Philip A. Moore Jr. [b], Steven C. Ricke [d], Giuseppe Corti [a], Ester Foppa Pedretti [a], Stefania Cocco [a]

[a] Department of Agriculture, Food and Environmental Sciences, Università Politecnica Delle Marche, Via Brecce Bianche 10, 60131, Ancona, Italy
[b] USDA-ARS, Poultry Production and Product Safety Research Unit, 1260 W. Maple St, Fayetteville, AR, 72701, USA
[c] Department of Crop, Soil, and Environmental Sciences, University of Arkansas, 115 Plant Science Building, University of Arkansas, Fayetteville, AR, 72704, USA
[d] Meat Science & Animal Biologics Discovery Program (MSABD), Department of Animal and Dairy Sciences, University of Wisconsin-Madison, 1933 Observatory Drive, Madison, WI, 53706, USA

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ABSTRACT

Cattle manure and poultry litter are widely used as fertilizers as they are excellent sources of nutrients; however, potential adverse environmental effects exist during land applications, due to the release of zoonotic bacteria and antimicrobial resistance (AMR) genes. This study was conducted to understand linkages between physiochemical composition, bacterial diversity, and AMR gene presence of cattle manure and poultry litter using quantitative polymerase chain reaction to enumerate four AMR genes (*ermB*, *sulI*, *intlI*, and $bla_{ctx\text{-}m\text{-}32}$), Illumina sequencing of the 16 S region, and analysis of physical and chemical properties. Principal coordinate analysis of Bray–Curtis distance revealed distinct bacterial community structures between the two manure sources. Greater alpha diversity occurred in cattle manure compared to poultry litter ($P < 0.05$). Redundancy analysis showed a strong relationship between manure physiochemical and composition and bacterial abundance, with positive relationships occurring among electrical conductivity and carbon/nitrogen, and negative associations for total solids and soluble fractions of heavy metals. Cattle manure exhibited greater abundance of macrolide *(ermB)* and sulfonamide (*sulI*) resistant genes. Consequently, fresh cattle manure applications may result in greater potential spread of AMR genes to the soil-water environment (relative to poultry litter) and novel best management strategies (such as composting) may reduce the release of AMR genes to the soil-water environment.

1. Introduction

Manure land applications may pose environmental health risks, despite its role in improving soil fertility and organic matter, by serving as a pathway for the release of zoonotic bacteria and antibiotic resistance genes (ARGs) to the environment (Gurmessa et al., 2020; Xie et al., 2018; Ziemer et al., 2010). However, there is lack of knowledge on the link between manure properties and microbial composition and the abundance of ARGs. Disparities in manure physiochemical properties, such as moisture content, pH, carbon to nitrogen (C/N) ratio, and nutrient composition (Zhou and Yao, 2020) may affect microbial composition and abundance (Xu et al., 2020). Trace element levels are also reportedly linked to bacterial community structure and ARGs, although specific relationships are largely unknown (Ding et al., 2017).

Animal manure is generally characterized as having high nitrogen (N), phosphorus (P), and potassium (K) contents (Zhou and Yao, 2020). Specifically, 310 mg kg^{-1} P (on dry matter basis) was reported for cattle manure (Giles and Cade-Menun, 2014), with approximately 1500 mg kg^{-1} P being reported for poultry litter (consists of a combination of bedding material, feces, and litter) (Ashworth et al., 2020). Manure pH also varies depending on the manure type. A neutral to sub-alkaline pH range (6.8–7.9) has been reported for cattle manure (Huang et al., 2017; Whalen et al., 2000), whereas an average pH of 8.12 is typical for poultry litter (Ashworth et al., 2020). However, it is unknown how physiochemical properties are related with microbial abundance.

Previous studies have reported a wide range of antimicrobial resistant (AMR) genes in manure and the most widely studied were genes resistant to tetracyclines (*tet*), sulfonamides (*sul*), macrolides-streptogramin B (*erm*), mobile genetic elements (MGEs), and integrons (*int*) (Gurmessa et al., 2020). However, these resistance genes may not be found in all manure sources. A detailed investigation of AMR in three

* Corresponding author.
E-mail address: amanda.ashworth@usda.gov (A.J. Ashworth).

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Soil bacterial diversity based on management and topography in a silvopastoral system

Biyensa Gurmessa [a,b,f], Amanda J. Ashworth [a,*], Yichao Yang [b], Kabindra Adhikari [c], Mary Savin [b], Phillip Owens [d], Tom Sauer [e], Ester Foppa Pedretti [f], Stefania Cocco [f], Giuseppe Corti [f]

[a] USDA-ARS, Poultry Production and Product Safety Research Unit, 1260 W. Maple St., Fayetteville, AR 72701, USA
[b] Department of Crop, Soil, and Environmental Sciences, University of Arkansas, 115 Plant Science Building, Fayetteville, AR 72704, USA
[c] USDA-ARS, Grassland Soil and Water Research Laboratory, Temple, TX 76502, USA
[d] USDA-ARS, Dale Bumpers Small Farms Research Center, 6883 S. Hwy 23, Booneville, AR 72927, USA
[e] USDA-ARS, National Laboratory for Agriculture and the Environment, 1015 N University Blvd, Ames, IA 5001,1, USA
[f] Department of Agriculture, Food and Environmental Sciences, Università Politecnica delle Marche, Via Brecce Bianche 10, 60131 Ancona, Italy

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Poultry litter
Terrain attributes
Metagenomics
Soil moisture

ABSTRACT

Soil microorganisms play crucial roles in nutrient cycling and provisioning ecosystem services. However, little is known about how soil microbial communities are affected by soil management and landscape position in silvopastures. The current study aimed to understand effects of forage species [non-native, cool season orchardgrass (*Dactylis glomerata* L.) and a warm-season native grass mix (*Andropogon gerardii* L. and *Schizachyrium scoparium* L.) planted in strips between hedgerows], soil fertility (poultry litter and a control), and soil moisture regimes (*aquic* and *udic*) on soil bacterial communities to evaluate linkages between terrain attributes and soil bacterial assemblages. Thirteen terrain attributes representing topographic variability were clustered into four topographic functional units (TFUs) using the k-means method, and their impact on soil microbial diversity was evaluated. Illumina sequencing results identified a soil moisture regime × forage species interaction, with native grass species under wet (*aquic*) conditions resulting in the most diverse microbial assemblages relative to dry (*udic*) and wet soil conditions for the non-native forage (orchardgrass). These results suggest an enhanced soil microbial diversity under native grasses with greater available soil water. Overall, microbial diversity was negatively correlated with elevation, suggesting niche differentiation and microbial preference for lower elevations. Overall, TFUs and selected terrain attributes may be useful for predicting microbiota dynamics in integrated tree-livestock systems.

1. Introduction

The importance of soil microorganisms are gaining attention, particularly in agricultural systems, as they interact with the mineral surface to govern availability of plant nutrients (Zhu et al., 2016) and overall ecosystem balance (Fierer, 2017). Although they have other unknown ecological attributes, they can be identified with state-of-the-art genomic approaches and manipulated or managed to increase ecosystem services (Sathya et al., 2017) and plant productivity (Fierer, 2017). In agroecosystems, different management practices could change soil microbial structure. For instance, increased aboveground diversity may bequest belowground ecosystems with greater species diversity that mitigates risk (Zak et al., 2003) and enhances climatic resiliency (Naeem, 1998). Unlike sole pastoral systems, silvopastoral systems are endowed with all these attributes.

Silvopasture, an agroforestry management practice that integrates trees and animal production under one system, is widely practiced in North America (Orefice and Carroll, 2017). It is generally considered a

Abbreviations: PLS, pure live seed; AU, animal units; ANOVA, analysis of variance; LSD, least significant difference; SOM, soil organic matter; OTU, operational taxonomic unit; PERMANOVA, permutational analysis of variance; PCoA, principal coordinate analysis; PCA, principal component analysis; VWC, volumetric water content; TFUs, topographic functional units; LiDAR, light detection and ranging.

* Corresponding author.
E-mail address: Amanda.Ashworth@usda.gov (A.J. Ashworth).

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Review

Manure anaerobic digestion effects and the role of pre- and post-treatments on veterinary antibiotics and antibiotic resistance genes removal efficiency

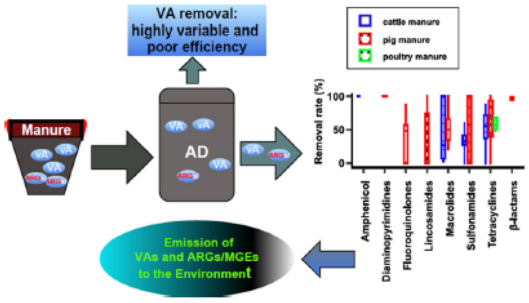
Biyensa Gurmessa *, Ester Foppa Pedretti, Stefania Cocco, Valeria Cardelli, Giuseppe Corti

Department of Agricultural, Food and Environmental Sciences, Università Politecnica delle Marche, 60131 Ancona, Italy

HIGHLIGHTS

- AD effect on VAs and ARGs removal was reviewed.
- AD does not guarantee complete removal of all types of VAs in manure.
- VAs and ARGs are entering soils with digestate compromising environmental quality.
- AAD followed by digestate composting can improve removal efficiency of VAs and ARGs.

GRAPHICAL ABSTRACT



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Solid digestate

ABSTRACT

This review was aimed to summarize and critically evaluate studies on removal of veterinary antibiotics (VAs), antibiotic resistance genes (ARGs) and mobile genetic elements (MGEs) with anaerobic digestion (AD) of manure and demonstrate areas of focus for improved removal efficiency. The environmental risks associated to the release of the same were also critically evaluated. The potential of AD and advanced AD of manure on removal rate of VAs, ARGs and MGEs was thoroughly assessed. In addition, the role of post and pre-AD treatments and their potential to support VAs and ARGs removal efficiency were evaluated. The overall review results show disparity among the different groups of VAs in terms of removal rate with relatively higher efficiency for β-lactams and tetracyclines compared to the other groups. Some of sulfonamides, fluoroquinolones and macrolides were reported to be highly persistent with removal rates as low as zero. Within group differences were also reported in many literatures. Moreover, removal of ARGs and MGEs by AD was widely reported although complete removal was hardly possible. Even in rare scenarios, some AD conditions were reported to increase copies of specific groups of the genes. Temperature pretreatments and temperature phased advanced AD were also reported to improve removal efficiency of VAs while contributing to increased biogas production. Moreover, a few studies also showed the possibility of further removal by post-AD treatments such as liquid-solid separation, drying and composting. In conclusion, the various studies revealed that AD in its current technological level is not a guarantee for complete removal of VAs, ARGs and MGEs from manure. Consequently, their possible release to the soils with digestate could threaten the healthcare and disturb soil microbial ecology. Thus, intensive management strategies need to be designed to increase removal efficiency at the different manure management points along the anaerobic digestion process.

* Corresponding author.
E-mail address: b.g.dubiwak@pm.univpm.it (B. Gurmessa).

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Post-digestate composting benefits and the role of enzyme activity to predict trace element immobilization and compost maturity

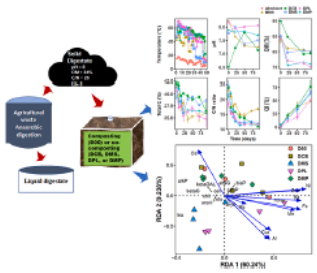
Biyensa Gurmessa [a,*], Stefania Cocco [a], Amanda J. Ashworth [b], Ester Foppa Pedretti [a], Alessio Ilari [a], Valeria Cardelli [a], Flavio Fornasier [c], Maria Letizia Ruello [d], Giuseppe Corti [a]

[a] Department of Agricultural, Food and Environmental Sciences, Università Politecnica delle Marche, 60131 Ancona, Italy
[b] USDA-ARS, Poultry Production and Product Safety Research Unit, 1260 W. Maple St. Fayetteville, AR 72701, USA
[c] Consiglio per la Ricerca e la Sperimentazione in Agricoltura, Centro di Ricerca per lo Studio delle Relazioni tra Pianta e Suolo (C.R.E.A.-R.P.S.), 34170 Gorizia, Italy
[d] Department of Materials, Environmental Sciences and Urban Planning, Università Politecnica delle Marche, 60131 Ancona, Italy

HIGHLIGHTS

- Pilot level aerobic co-composting of digestate and biogas feedstocks was conducted.
- Composting reduced C/N ratio, release of trace elements, and phytotoxicity.
- Enzyme activities were influenced by OM content and release of trace elements.
- Enzyme activities could be compost maturity indicators.

GRAPHICAL ABSTRACT



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Keywords:
C/N ratio
Compost maturity
Food processing waste
Maize silage
Poultry litter

ABSTRACT

The current study evaluated the quality of agricultural waste digestate by composting or co-composting with biogas feedstock (maize silage, food processing waste, or poultry litter). Temperature, phytotoxicity, C/N ratio, water extractable trace elements, and 14 enzyme activities were monitored. Temperature dropped earlier in digestate and maize silage co-composting pile, reducing time to maturity by 20 days. Composting and co-composting reduced phytotoxicity and C/N ratio, but increased immobilization of Al, Ba, Fe, Zn, and Mn at least by 40% in all piles. All the enzyme activities, except arylsulfatase and α-glucosidase, increased at the maturity phase and negatively correlated with organic matter content and most of trace elements. Post-digestate composting or co-composting with biogas feedstock is a promising strategy to improve digestate quality for fertilizer use, and selected enzyme activities can be indicators of compost maturity and immobilization of trace elements.

* Corresponding author.
E-mail addresses: biyensa@gmail.com, b.g.dubiwak@pm.univpm.it (B. Gurmessa).

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

Biyensa Gurmessa [a,*], Vesna Milanovic [a], Ester Foppa Pedretti [a], Giuseppe Corti [a], Amanda J. Ashworth [b], Lucia Aquilanti [a], Ilario Ferrocino [c], Maria Rita Corvaglia [c], Stefania Cocco [a]

[a] Department of Agriculture, Food and Environmental Sciences, Università Politecnica delle Marche, Via Brecce Bianche 10, Ancona 60131, Italy
[b] Agricultural Research Service, U.S. DEPARTMENT OF AGRICULTURE (USDA-ARS), Poultry Production and Product Safety Research Unit, 1260 W. Maple St., Fayetteville, AR 72701, USA
[c] Department of Agricultural, Forest, and Food Science, University of Turin, Largo Paolo Braccini 2, Grugliasco, Torino, Italy

HIGHLIGHTS

- Post-digestate composting effect on removal of ARGs was investigated.
- *tet*(M) was the most abundant ARG in fresh digestate compared to other ARGs.
- After 90 days of composting, ARGs abundance was reduced by more than 80%.
- Actinobacteria, Planctomycetes, and Verrucomicrobia succeeded with composting.
- *Arenibacter* and *Cellvibrio* were potential hosts for ARGs.

ARTICLE INFO

Keywords:
ARGs removal
Biogas feedstock
Co-composting
Poultry litter
Trace elements

ABSTRACT

Post-digestate treatments may reduce the risk linked to Antibiotic Resistant Genes (ARGs) release with digestate direct land application. Thus, this study aimed to evaluate post-digestate composting and co-composting with biogas production feedstock (maize silage, food processing waste, and poultry litter) effect on abundance of selected ARGs: *erm*(B), *tet*(K), *tet*(M), *tet*(O), and *tet*(S) genes. More than 80% of all ARGs were removed after 90 days of composting but removals from co-composting were lower. Bacteroidetes, Firmicutes, and Proteobacteria dominated fresh digestate, and a network analysis indicated that these were potential hosts of ARGs. The emergence of Actinobacteria (dominant), Planctomycetes, and Verrucomicrobia phyla during composting shifted the microbial composition. Moreover, canonical correspondence analysis showed trace elements explaining 90% variations in ARGs abundance. The study illustrates significance of post-digestate composting in mitigating ARGs release, and effectiveness could be linked to shift in microbial composition and trace elements release.

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

* Corresponding author.
E-mail addresses: biyensa@gmail.com, b.g.dubiwak@pm.univpm.it (B. Gurmessa).

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