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Wood- and Manure-Derived Biochars Reduce Antibiotic Residues and Shift Antibiotic Resistance Genes and Microbial Communities in Manure Applied Forage–Soil Systems

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Abstract: The increasing use of antibiotics in livestock poses environmental risks, leading to contamination of agricultural soils and propagation of microbial antibiotic-resistant genes (ARGs). This study examined the impacts of wood- and manure-derived biochar (BC) on antibiotic residues, ARGs, and microbial communities in sandy loam and clay loam soils amended with manure in *Cynodon dactylon* pastures. We hypothesized that BC amendments would influence the degradation of antibiotics and the structure of microbial communities based on their physicochemical properties and soil types. Our results demonstrated that wood BC reduced the concentrations of tetracycline and sulfonamides, particularly in sandy loam soil, due to its larger surface area and hydrophobic properties. In contrast, manure BC provided additional nutrients and supported atmospheric nitrogen-fixing microbial groups, especially in clay loam soil, while exhibiting variable efficiency in reducing antibiotic residues due to its lower surface area and higher ash content. These findings underscore the differential impacts of each BC type, emphasizing the need for tailored BC applications based on soil type to effectively mitigate antibiotic contamination and promote sustainable agricultural practices. In conclusion, wood BC was more effective in enhancing soil health by reducing antibiotic residues and improving microbial diversity, particularly in sandy loam soils, while manure BC was beneficial for nutrient cycling in clay loam soils.

Keywords: antibiotics; antibiotic-resistant genes; biochar; microbial community structure; soil remediation; sustainable agriculture

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1. Introduction

Since the discovery of penicillin, over 250 different antibiotics have been registered for the treatment of human and veterinary diseases [1]. Notably, the use of antibiotics in food-producing animals raised and used for food production, such as livestock, pork, and poultry, was estimated at 99,502 Mg/year of active ingredients and is projected to increase by 8% to 107,472 Mg by 2030 globally [2]. The use of antibiotics in livestock poses environmental risks as animals are unable to effectively metabolize these substances, leading to the excretion of incompletely metabolized antibiotics via urine or feces [3,4]. Consequently, the application of animal manure as fertilizer, a common agricultural practice, can be a major source of antibiotic contamination in agricultural soils. Antibiotics adversely affect microbial communities and activities, such as respiration, nitrification, and denitrification,

by inhibiting specific enzyme functions [5,6]. Additionally, antibiotics can be transported into surface or groundwater, plants, and animals through runoff, leaching, and uptake, respectively, from not only agricultural field but also manure storage and livestock facilities, leading to their accumulation and distribution across various matrices [7].

The accumulation of antibiotics in agricultural soils further induces resistance in pathogens and bacteria through long-term exposure, genetic variation, and the transfer of antibiotic resistance genes (ARGs) between non-pathogenic and pathogenic bacteria [8]. Soils account for 30% of ARGs, due to the inherent presence of antibiotics [9]. Furthermore, ARGs can spread from agricultural environments to human pathogens through multiple pathways, such as direct contact or indirect transfer via the food chain, water systems, and/or aerosols, posing significant public health risks [10]. This increases the prevalence of antibiotic-resistant infections in humans, making bacterial infection treatments more challenging, thereby contributing to the global ARG health crisis. In the United States of America alone, more than 2.8 million antibiotic-resistant infections occur annually in humans, resulting in over 35,000 deaths [11]. A critical issue with ARGs is their dispersion through horizontal gene transfer mediated by mobile genetic elements such as plasmids, integrons, and transposons [12]. This transfer allows ARGs to spread not only among bacteria within the soil but also to human and animal pathogens, further exacerbating the issue.

Biochar (BC), a carbonaceous material produced by pyrolyzing organic feedstock, particularly byproduct biomass, at 300 to 700 °C under oxygen-deficient conditions, has been widely used in agriculture to enhance soil quality and crop yield due to its economic feasibility and physicochemical properties, including high specific surface area, pore structure, and abundant functional groups [13–16]. Furthermore, BC is a potential solution to mitigate the spread of ARGs. By immobilizing antibiotics and other contaminants in the soil, BC can reduce the mobility of these compounds, thereby decreasing the likelihood of ARGs being transferred to pathogenic bacteria. Biochar can enhance soil microbiome diversity, potentially suppressing ARG-carrying bacteria and reducing the overall abundance of ARGs in agricultural soils. [17–20]. However, the effectiveness of BC amendments is highly dependent on the physicochemical characteristics of the BC and soil type [21–23]. Therefore, it is important and necessary to compare different types of biochar to provide practical references for real applications.

In a previous study, we investigated the effects of wood- and manure-derived BC (wood BC and manure BC, respectively) on *Cynodon dactylon*, a widely distributed forage plant in tropical and subtropical regions, and on soil properties in manure-amended sandy loam and clay loam soils [15]. Our findings indicated that only manure BC increased the concentrations of essential nutrients for plant growth, such as NO_3-N and P, in sandy loam soil, which typically has low nutrient content, by providing nutrients derived from the manure itself. We hypothesized that the effects of each BC on antibiotics, ARGs, and microbial communities in each soil type would also differ. Our study focused on the microbial community structure and ARGs using PICRUSt to understand the changes in two representative antibiotics, tetracycline (TC) and sulfonamides including sulfamethazine (SMZ) and sulfamethoxazole (SMX), i.e., SMZ/SMX, in *C. dactylon* pastures on sandy loam and clay loam soils amended with dairy manure. Our goal was to evaluate the impact of wood versus manure BC on antibiotics and ARGs in different soil types and provide a reference for BC application in *C. dactylon* and manure-amended soils.

2. Materials and Methods

2.1. Soil and Manure Preparation

Soil samples were collected from the top 20 cm of a Windthorst fine sandy loam and a clay loam at Stephenville (32.2454° N, −98.1970° W) and Temple (31.0982° N, −97.3327° W), TX, USA. After homogenizing and air-drying under ambient conditions, 3 kg of each soil type was distributed into 4 L plastic nursery pots. Dairy manure, obtained from Tarleton State University Dairy, was screened from dairy stall flush spillways for collection. After

removing the sand, the manure was dried in the sun for 2 weeks before application. A manure application rate of 10 Mg/ha (dry matter weight basis, approximately 2% of soil mass) was used in this study because it met forage phosphorus requirements and equated to 45 Mg/ha before drying. The amount of manure was determined by grass phosphorus (P) requirements. According to [24], the recommended maximum annual compost application rate for manure as fertilizer should not exceed 44.8 Mg/ha in the southern USA when applied to *C. dactylon*. Both soils and manure were analyzed for pH, electrical conductivity, nitrate (NO_3-N), P, K, Ca, Mg, S, Na, Fe, Zn, Mn, and Cu values by Texas A&M AgriLife Extension Service Soil, Water, and Forage Testing Laboratory at the College Station using the standard methods practiced in this lab [25]. Phosphorus, K, Ca, Mg, Na, and S were extracted using Mehlich III extractant and were determined by ICP [26,27], and total C and total N were determined using a CN828 elemental analysis by combustion (LECO Corporation, St. Joseph, MI, USA) at the Texas A&M AgriLife Center at Stephenville. The physicochemical characteristics of soil and manure are presented in Table 1.

Table 1. Physicochemical characteristics of soils and manure.

Parameter	Unit	Sandy Loam	Clay Loam	Dairy Manure
pH		7.9	7.3	5.8
Conductivity	umhos/cm	166	2643	6530
Oxidizable C	ppm	198.7	1172.2	N/A [a]
NO_3-N	ppm	10.9	329.5	>400.00
P	ppm	34.8	320.4	1015.0
K	ppm	198.2	1965.3	1070.0
Ca	ppm	1820.5	15,967.1	2668.0
Na	ppm	47.0	262.2	380.0
Mg	ppm	176.0	160.0	2052.0
S	ppm	26.4	7.8	130.0
Fe	ppm	3.4	13.0	N/A
Zn	ppm	0.9	4.6	N/A
Mn	ppm	4.3	11.1	N/A
Cu	ppm	0.4	0.7	N/A
Organic matter	%	0.8	3.2	71.4
Total C	%	3.2	8.7	41.4
Total N	%	0.3	0.7	2.83
TC	ppb	14.9	9.7	23,020.4
SMZ/SMX [b]	ppb	934.4	725.3	11,446.2

[a] Not applicable. [b] SMZ/SMX: sulfamethazine and sulfamethoxazole.

2.2. BC Preparation

BC derived from wood (Waste to Energy, Inc., South Slocomb, AL, USA) and manure (Ecochar, Evansville, IN, USA), referred to as wood BC and manure BC, respectively, were ground using a Thomas Wiley Mill (Swedesboro, NJ, USA) fitted with a 2 mm screen before being added into the soil. Each BC was incorporated into the pots, replacing 2% of the soil on a dry matter weight basis.

Proximate analysis was conducted to measure fixed carbon, volatile carbon, and ash contents in wood and manure BC, following ASTM D7582-15 standards [28]. Elemental compositions and contents of both BCs were analyzed using an element analyzer (Robert Microlit Lab, NJ, USA) and inductively coupled plasma optical emission spectroscopy (Soil, Forage and Water Testing Lab, Texas A&M University, USA). The surface areas of both BCs were determined using the Brunauer–Emmett–Teller (BET) method [29] by measuring N_2 adsorption–desorption isotherms (Article Technology Lab, Downers Grove, IL, USA). The physicochemical characteristics of each BC are presented in Table 2.

Table 2. Physicochemical characteristics of wood BC and manure BC.

Parameter	Unit	Wood BC	Manure BC
Carbon	%	85.7	55.8
Hydrogen	%	1.4	0.9
Oxygen	%	6.6	11.8
Nitrogen	%	0.2	0.7
Phosphorus	%	0.0	1.1
Potassium	%	0.2	4.4
Calcium	%	0.2	6.4
Magnesium	%	0.0	2.6
Sodium	%	0.1	0.7
Ash	%	5.8	40.1
Fixed Carbon	%	60.7	23.8
Volatile Matter	%	27.8	32.6
Zinc	ppm	36.6	285.9
Iron	ppm	775.4	7708.7
Copper	ppm	12.6	153.7
Manganese	ppm	139.1	432.5
Sulfur	ppm	13.7	3167.2
Boron	ppm	2.3	29.7
S_{BET} [a]	m^2/g	419.0	7.1
pH		8.8	10.2

[a] S_{BET}: specific surface area measured by using the Brunauer–Emmett–Teller method.

2.3. Experimental Design

The greenhouse pot test was conducted at the Texas A&M AgriLife Center in Stephenville, TX, USA over a 90 d period. The experiment was arranged in a randomized complete block design with multiple factors including manure application (0 and 2% of soil), soils (clay loam, CL; sandy loam, SL) and biochar (wood biochar, WB; manure biochar, MB). Six treatment combinations were tested: SL-NB, CL-NB, SL-WB, CL-WB, SL-MB, and CL-MB. The SL-NB treatment involved the application of 2% manure to SL soil, while the CL-NB treatment applied 2% manure to CL soil based on dry weight. The SL-WB and CL-WB treatments included the addition of 2% manure and 2% wood BC to SL and CL soils, respectively, based on a dry weight basis. The SL-MB and CL-MB treatments included the addition of 2% manure and 2% manure BC to SL and CL soils, respectively. Biochar and manure were homogeneously mixed with the whole soil to ensure distribution throughout the soil in pots. A 15 cm sprig of pre-cultured *C. dactylon* was transplanted into each pot of these six treatments. During the experiment, the pots were watered as needed (~5–7 days) to maintain near-field capacity, and leachate was recycled back into the soil. All treatments were conducted in triplicate (replications). To investigate the effects of dairy manure and *C. dactylon* on soil microbial community structure, we established and analyzed two groups of SL and CL soils. Blank groups (i.e., bare soil) were named SL-Blank and CL-Blank, and control groups with manure applied were named SL-Control and CL-Control for sandy loam and clay loam, respectively. These groups were analyzed to assess their microbial community structures.

2.4. Antibiotics Measurement

At the initiation and termination of the greenhouse pot experiment, soil sub-samples representing 0.5% of the total pot soil were taken from each experimental unit (pot) using a small soil probe to minimize root loss and account for a complete cross-section of soil. The samples were air-dried under ambient conditions until the weight stabilized. To extract antibiotics from soil samples, a modified extraction buffer referring to [30], which is a mixture of 50% McIlvaine-ethylenediaminetetraacetic acid (EDTA) buffer (11.88 g of citric acid anhydrous, 11.96 g of Na_2HPO_4 anhydrous, and 37.224 g of Na_2EDTA in 1 L of reverse-osmosis water), 37.5% acetonitrile, and 12.5% methanol, was prepared. A mass of 1 g of the soil samples was incubated in 10 mL of extraction buffer and shaken at ambient temperature

for 1 day. After centrifugation at 5000 rpm for 10 min, each supernatant was taken and dried in a vacuum oven and then re-suspended in the same volume of redissolving solution provided by the manufacturer of ELISA kits. The re-suspended samples were measured for TC and SMZ/SMX using the Tetracycline ELISA Kit (Abcam, Cambridge, UK) and the Sulfamethoxazole (SMZ/SMX) ELISA BioAssay™ Kit (US Biological, Swampscott, MA, USA), respectively, following the manufacturer's instructions.

2.5. Microbial Community Structure

The microbial community structure of soil samples was analyzed as described previously [31]. Undried bulk soil samples were collected from each pot, and genomic deoxyribonucleic acid (DNA) was extracted in triplicate using DNeasy PowerSoil Kits (Qiagen, Hilden, Germany) from 0.5 g of soil, following the manufacturer's instructions. The 16S ribosomal ribonucleic acid (rRNA) V4 hypervariable region and the internal transcribed spacer (ITS) region were polymerase chain reaction (PCR)-amplified using Illumina adaptor-ligated universal primers. The thermocycler conditions were 94 °C for 3 min, followed by 25 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, and a final extension at 72 °C for 3 min. PCR products were size-selected (400–600 bp) on a Pippin prep instrument (Sage Science, Beverly, MA). Size-selected sequencing libraries were sent to the Genomics and Bioinformatics Service, College Station, TX, USA, for sequencing on an Illumina MiSeq (Illumina, San Diego, CA, USA) using a v3 600 cycle sequencing kit to produce 300 bp paired-end reads.

Taxonomy assignment and function annotation analyses were performed using the snakemake qiime2 pipeline available at https://github.com/olabiyi/snakemake-workflow-qiime2 (Access date 20 January 2024) with advanced computing resources provided by Texas A&M High Performance Research Computing. Briefly, raw sequences were processed using QIIME 2 (q2) version 2021.2 [32]. Sequences were imported and demultiplexed using the PairedEndFastqManifestPhred33 format of qiime2. Primers and adaptors were trimmed using cutadapt [33] through the q2-cutadapt plugin. Quality control, merging, chimera removal, denoising, and amplicon sequence variant (ASV) feature table generation were performed using the q2-dada2 plugin [34]. Paired-end sequences were used for prokaryotes (16S rDNA), while only the forward reads were used for fungi (ITS) to maximize the number of sequences after running dada2. Hence, the dada2 denoise-paired command was run for 16S, while the dada2 denoise-single command was run for ITS with default settings except for the max_ee parameter set to 4. For prokaryotes, the p-trunc-len-f and p-trunc-len-r dada2 parameters were set to 260 and 200, respectively. For fungi, the p-trunc-len and p-trim-left parameters were set to 200 and 36, respectively. These parameters were set only after observing the sequence quality distribution by length plot. For prokaryotes, ASV taxonomic classifiers were generated using the Silva 138-nb 99% reference database [35], while for fungi, the UNITE database [36] for eukaryotes v8.3 was used. The q2-feature-classifier plugin was used to assign taxonomy to the representative ASV sequences using scikit-learn [37] with the generated classifiers. Singletons, rare ASVs, i.e., ASVs with sequences < 0.005% of the total number of sequences [38], were excluded from the analyses, and non-target ASVs were filtered out of the ASV table. The sequence data generated in this study have been deposited in the NCBI Sequence Read Archive (SRA) under the BioProject accession number PRJNA1140274.

2.6. Statistical Analyses

Duncan's multiple range test was performed to examine the statistical differences among data of each treatment using the R software package *agricolae* ver. 1.3-5 [39]. A non-metric multidimensional scaling (NMDS) ordination was performed based on the Bray–Curtis distance using the R software package *vegan* ver. 0.6-2 to investigate the relatedness of microbial community structure [40]. The *Shannon* and *Simpson* indices were analyzed using the R software package *vegan* ver. 0.6-2. The *Shannon* index represents both species richness and community evenness, with a higher *Shannon* index indicating greater

species diversity. In contrast, the *Simpson* index is often used to describe biodiversity in ecological environments, where a higher *Simpson* index suggests lower diversity or greater dominance by a few species. Both indices were calculated using previously established methods [41]. The formulas are as follows (Equations (1) and (2)):

$$H_{shannon} = -\sum_{i=1}^{S_{obs}} \left(\frac{n_i}{N} \cdot \ln \frac{n_i}{N} \right) \quad (1)$$

$$D_{simpson} = \frac{\sum_{i=1}^{S_{obs}} n_i (n_i - 1)}{N(N-1)} \quad (2)$$

where n_i is the abundance of species i, N is the total abundance of all species, and S_{obs} represented the number of species observed in the sample.

The *Chao1* index was analyzed using the R software package *iNEXT* ver. 3.0.1 [42]. The *Chao1* index reflects the richness of the microbial community [41]. A larger *Chao1* index means a higher community richness. The formula is as follows (Equation (3)):

$$S_{chao} = S_{obs} + \frac{n_1(n_1 - 1)}{2(n_2 + 1)} \quad (3)$$

where S_{obs} represented the number of species observed in the sample, n_1 is the number of species observed once, and n_2 is the number of species observed twice.

The functional potential of the prokaryotic community based on the 16S rRNA gene sequences was predicted using PICRUSt2 v2.3.0-b [43] by running the picrust2_pipeline.py pipeline script on the unrarefied ASV abundance table and representative sequences using default settings [31]. Pearson's correlation analysis was performed on the relative abundance of microbial communities, antibiotics, and ARGs using PAST software ver. 3.06.

3. Results and Discussion

3.1. Changes in Residual Antibiotics in Sandy Loam and Clay Loam Soils

The residual antibiotic concentration in soil after 90 d is presented in Figure 1. The residual TC concentration in SL-NB and CL-NB was 37.1 ± 0.5 ppb and 40.3 ± 0.5 ppb, achieving 92.2% and 91.4% removal compared to the initial concentration at Day 0 (475.0 ± 1.0 ppb and 469.9 ± 0.7 ppb, respectively), which was a high removal efficiency. This might be due to several removal pathways including plant sorption, photodegradation, and biodegradation. The TC groups are well known to be more susceptible to photodegradation than the sulfonamide group [44], resulting in high removal in TC in control groups.

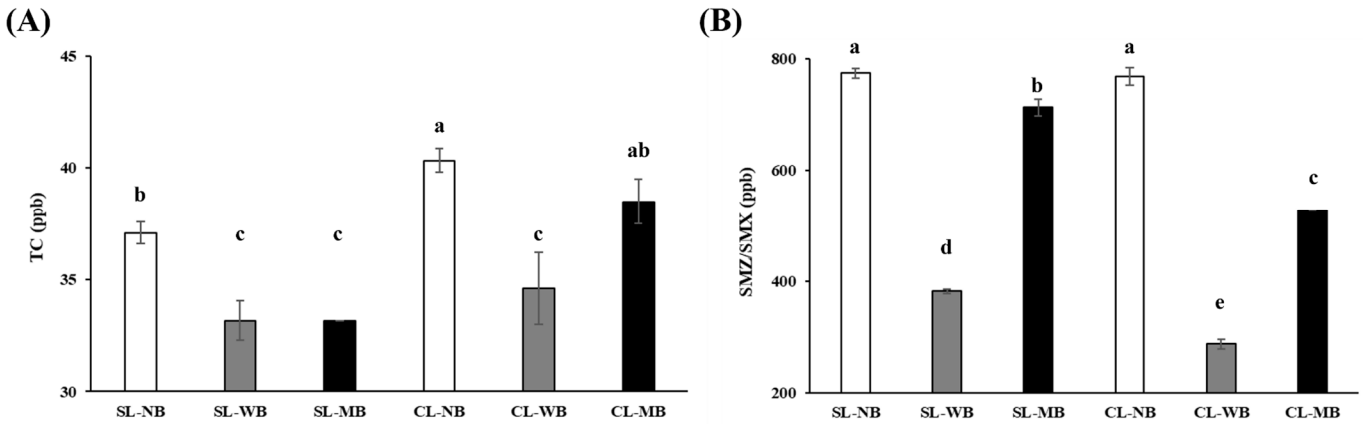

Figure 1. Residual tetracycline (**A**) and SMZ/SMX (**B**) concentration in each soil after 90 days. Different letters above bars indicate significant differences between conditions ($p < 0.05$, Duncan's test). Sandy loam, SL; clay loam, CL; no biochar, NB (white); wood biochar, WB (gray); manure biochar, MB (black).

Both SL-WB and SL-MB demonstrated similar residual TC concentrations (33.2 ppb in average; 93.0% removal), which was a slightly lower residual concentration compared to SL-NB. This result indicated that both wood and manure BC contributed to TC removal in the soil. However, while CL-WB showed a reduction in residual TC concentration (34.6 ± 1.6 ppb; 92.6% in removal), CL-MB still contained a relatively higher residual TC concentration (38.5 ± 1.0 ppb; 91.8% in removal) compared to CL-NB. One possible reason for the different trends between the soils might be the difference in physicochemical characteristics between the two soils. Sandy loam has a coarser texture, resulting in lower water-holding capacity [45,46] compared to CL. Therefore, BC could efficiently interact with water due to its high surface area, further increasing the water-holding capacity of sandy loam soil. This might improve water-mediated adsorption of TC on the surface of BC and biological removal by the soil microbiome.

Notably, wood BC has a high specific surface area and porous structure with less ash content (Table 2), which provides a wide area for physical adsorption [47]. In the case of manure BC, due to its relatively lower specific surface area and higher ash content containing functional groups, metals, and nutrients, manure BC is less hydrophilic than wood BC and might encourage chemical adsorption through mechanisms such as surface complexation, hydrogen bonding, and electrostatic interactions between the functional groups of manure BC and TC [14,48,49]. Meanwhile, CL loam soil has a relatively high water-holding capacity due to its small particle size and high surface area, which makes BC less effective in enhancing water-holding capacity compared to coarse-textured soils like SL [46]. Therefore, in clay soil, the large surface area of wood BC might be advantageous for interaction with TC due to the high competency for water retention of the clay soil. TC is a less hydrophobic compound with multiple polar functional groups such as hydroxyl, amine, and keto groups [38].

Even though the polar nature of TC induces chemical adsorption as a dominant mechanism [14,50], manure BC, which primarily adsorbs TC through chemical adsorption, might face limitations in water contact, reducing its adsorption effectiveness due to its low surface area. In summary, the effect of BC on TC removal in soil might be influenced by a combination of surface area and functional groups of BC. The large surface area of wood BC may play a critical role in environments with limited water movement, while the functional groups of manure BC may be more effective in conditions with adequate water contact.

BC-amended soils also showed lower residual SMZ/SMX concentration compared to soils without any BC in both SL (774.3 ± 8.2 ppb, 32.4 ± 0.2% in removal) and CL (768.6 ± 16.3, 22.3 ± 0.6% in removal) (Figure 1B). Unlike TC profiles, wood-BC-amended soils, i.e., SL-WB and CL-WB, showed better removal of SMZ/SMX, achieving 66.0 ± 0.2% (382.3 ± 4.1 ppb) and 70.5 ± 0.3% (287.4 ± 9.2), compared to manure-BC-amended soil, i.e., SL-MB and CL-MB (36.7 ± 0.4%, 713.0 ± 15.1 ppb and 45.8 ± 0.1%, 528.0 ± 0.0, respectively). Both SMZ and SMX are more hydrophobic compared to TC due to containing aromatic rings and other hydrophobic moieties, making them less soluble in water [44]. Due to their hydrophobicity, SMZ and SMX might be more effectively adsorbed on the surface of wood BC through physical adsorption, facilitated by its larger surface area and hydrophobic characteristics, compared to manure BC.

Furthermore, water contact with wood BC might facilitate physical adsorption. In summary, the effect of BC on SMZ/SMX removal in soil might be influenced by surface area and hydrophobicity due to its favorable water-mediated physical adsorption properties. In conclusion, wood BC efficiently increased the removal of both hydrophilic TC and hydrophobic SMZ/SMX across a range of soil textures, from coarse-textured sandy loam to fine-textured clay loam, due to its high surface area and hydrophobicity.

3.2. Dynamics in Microbial Community Structure

A total of 490,337 reads for prokaryotes and 806,747 reads for fungi were obtained from the sequencing analysis. In the prokaryotic community (Figure 2A), 11 phyla were predominantly distributed among the 31 detected phyla. The prokaryotic community structure differed between SL-Blank and CL. *Proteobacteria* (44.0%), followed by *Cyanobacteria* (31.6%), *Bacteroidota* (7.9%), and *Acidobacteriota* (6.5%), were predominant in SL (i.e., SL-Blank) (relative abundance > 1%). Meanwhile, the prokaryotic community structure in clay loam (i.e., CL-Blank) was more diverse, consisting of *Proteobacteria* (41.0%), followed by *Cyanobacteria* (13.7%), *Planctomycetota* (12.1%), *Myxococcota* (10.7%), *Actinobacteriota* (9.9%), *Chloroflexi* (4.9%), *Acidobacteriota* (5.4%), *Bacteroidota* (3.4%), and *Gemmatimonadota* (2.7%). Additionally, at the genus level, CL-Blank exhibited higher alpha diversity indices, including *Shannon*, *Simpson*, and *Chao1*, compared to SL-Blank (Table 3).

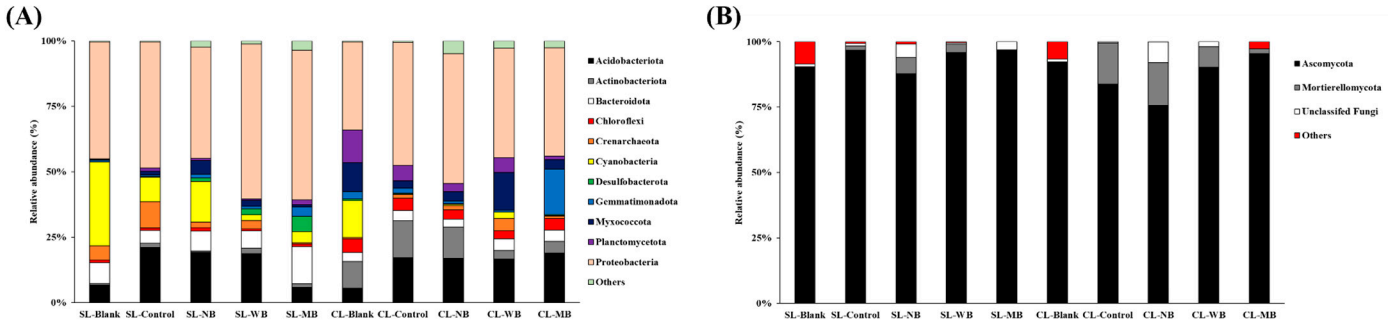

Figure 2. Taxonomic distribution of prokaryotic (**A**) and fungal (**B**) sequences at the phylum level for each condition. Sequences with a relative abundance of <1% in all samples were classified as "Others". SL = sandy loam soil; CL = clay loam soil; MB = manure biochar; WB = wood biochar; NB = no biochar applied.

Table 3. Alpha diversity of microbial community structure in each condition.

Condition	Prokaryote			Fungi		
	Shannon	***Simpson***	***Chao1***	***Shannon***	***Simpson***	***Chao1***
SL-Blank	3.14	0.89	168	1.94	0.76	24
SL-Control	3.81	0.96	215	2.55	0.90	27
SL-NB	4.00	0.96	215	2.37	0.86	30
SL-WB	3.89	0.96	157	2.35	0.88	22
SL-MB	4.09	0.97	145	1.81	0.71	14
CL-Blank	3.83	0.96	178	1.84	0.75	27
CL-Control	3.54	0.93	193	1.96	0.82	29
CL-NB	4.13	0.97	223	2.07	0.84	26
CL-WB	3.23	0.93	80	1.52	0.68	11
CL-MB	3.16	0.91	144	1.76	0.69	19

Sandy loam, SL; clay loam, CL; no biochar, NB; wood biochar, WB; manure biochar, MB.

These results indicate that CL soil had a more diverse and evenly distributed microbial community with higher richness compared to SL. The high nutrient and water retention capacity of CL likely created favorable conditions for fostering a more diverse microbial community structure. In both soils, manure amendment without *C. dactylon* led to increased relative abundances of *Acidobacteriota*, *Actinobacteriota*, *Crenarchaeota*, and *Proteobacteria* after 90 d of incubation, suggesting that these phyla originated from the manure. Furthermore, residual antibiotic residues in manure might partially contribute to the development of microbial community structure. Antibiotics can inhibit the growth of microorganisms by inhibiting synthesis of structure of microorganisms, such as cell wall/membrane, proteins, and/or nucleic acid [51,52]. Conversely, antibiotics induce predominance or stimulation of some species belonging to *Acidobacteriota*, *Actinoacteriota*,

Crenarchaeota, and *Proteobacteria* [53–55]. Therefore, shifts in microbial community structure might be affected by combined effects of manure as the original microbial community source and residual antibiotics in manure.

However, when *C. dactylon* was seeded and grown, the prokaryotic community changed (Figure 2). In SL-NB, *Bacteroidota*, *Chloroflexi*, *Cyanobacteria*, *Desulfobacterota*, *Gemmatimonadota*, and *Myxococcota* showed higher relative abundances compared to SL-Control. Similarly, CL-NB showed higher relative abundances of *Crenarchaeota*, *Cyanobacteria*, *Myxococcota*, and *Proteobacteria* compared to CL-Control. Generally, plants, including *C. dactylon*, add organic matter to soil through root exudates and decomposed plant materials, promoting nitrogen fixation and nutrient cycling, which enhance microbial nutrition and activity [56,57]. Furthermore, the root system improves the soil structure and increases pore space, promoting oxygen and water penetration [58]. These impacts likely created favorable conditions for some prokaryotic groups. Additionally, higher Shannon indices in both SL-NB and CL-NB suggest that *C. dactylon* positively influenced microbial community diversity in both SL and CL soils.

Both wood BC and manure BC also affected the prokaryotic community structure. For example, both SL-WB and SL-MB showed higher abundances of *Proteobacteria*, achieving 56−58%, compared to SL-NB (42.1%). *Proteobacteria* is a key bacterial phylum for organic matter mineralization and carbon/nitrogen cycling [59,60], suggesting that both BC types provided favorable conditions for *Proteobacteria*, facilitating nutrient utilization from manure. Furthermore, the decrease in richness (i.e., *Chao1* index) in SL-WB and SL-MB compared to SL-Control and SL-NB indicated that both BC types created a more competitive environment where only certain species thrived, leading to a decline in the number of different species.

However, while SL-WB showed negligible differences in prokaryotic community structure at the phylum level, SL-MB showed clear differences in the abundances of specific phyla, such as *Acidobacteriota* and *Bacteroidota*, compared to SL-Control and SL-NB. The NMDS plot supported the distinct differences in the prokaryotic community structure in SL-MB from other SL groups (Figure 3). This might be because the additional nutrients provided by manure BC positively affected the prokaryotic community in sandy loam soil (Table 2). Both BC types clearly and distinctly affected the prokaryotic community structure in different ways depending on soil type (Figures 2 and 3). While CL-WB showed a significant increase in the abundance of *Myxococcota* (12.7%) compared to CL-Control (2.5%) and CL-NB (3.5%), CL-MB showed an increase in the abundance of *Gemmatimonadota* (16.3%) compared to CL-Control (1.7%) and CL-NB (1.0%).

Myxococcota is known as a predator of other bacteria, helping to regulate bacterial populations and nutrient cycling [61]. By preying on pathogenic bacteria and producing bioactive compounds, *Myxococcota* can help suppress soil-borne diseases, contributing to the overall health of the soil ecosystem [62,63]. Our results suggest that wood BC and nutrient-rich CL soil provided favorable conditions for enriching various microorganisms, while, simultaneously, wood BC helped control soil health by selectively enriching *Myxococcota*.

Meanwhile, *Gemmatimonadota* may play a crucial role in nitrogen fixation and decomposition, increasing nitrogen availability in the soil and supporting plant growth [50,51]. Specifically, in relation to nitrogen, a previous study observed that the CL/SL-MB condition showed a significant increase in NO_3-N, which matches the high abundance of *Gemmatimonadota* [13]. Therefore, manure BC may enhance soil nitrogen availability by selectively enriching *Gemmatimonadota* in CL soil. We hypothesize that BC adsorbs N in sandy soils, which can be deficient in nutrients. By holding N in the rhizosphere, BC provides increased opportunity for plant N uptake, either directly or through rhizophagy of soil microbes, increasing plant growth [15].

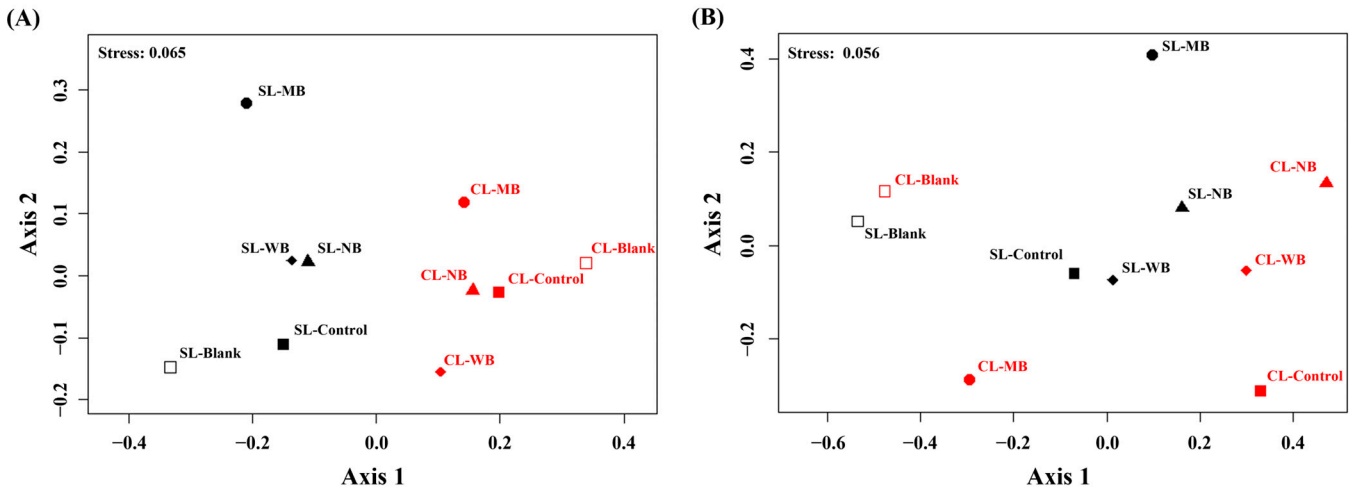

Figure 3. NMDS plot for prokaryotic community structure (**A**) and fungal community structure (**B**) based on phylum-level distribution. Samples are labeled with the corresponding condition. SL = sandy loam soil; CL = clay loam soil; MB = manure biochar; WB = wood biochar; NB = no biochar applied.

Diversity, evenness, and richness were remarkably lower in CL-WB and CL-MB compared to CL-NB, indicating that both BC types created conditions favoring specific microbial groups over a diverse community in clay loam (Table 3). The lower *Shannon*, *Simpson*, and *Chao1* values in the WB and MB groups compared to the NB group in the CL group, relative to the SL group, can be explained by the inherent differences in soil properties between clay loam and sandy loam. High water retention, nutrient retention, and potential for compaction in clay loam might create a more challenging environment for diverse microbial communities, which could be exacerbated by BC amendment.

In the fungal community structure (Figure 2B), Ascomycota, unclassified fungi, and Mortierellomycota were the three main phyla among the seven detected. Among them, Ascomycota was predominant in all groups (75.7−96.7%). Ascomycota play diverse roles in soil ecosystems, including organic matter decomposition, suppression of pathogenic bacteria and fungi, and formation of symbiotic relationships with plant roots, aiding in nutrient absorption by plants [64,65]. Meanwhile, another fungal phylum, Mortierellomycota, was present in all manure-amended soil groups. Mortierellomycota also play crucial roles in soil ecosystems, related to the decomposition of organic matter, contribution to nutrient cycling, and formation of symbiotic relationships with plants [66,67].

Therefore, the increase in the proportion of Mortierellomycota in manure-amended soil might be primarily due to the abundant organic matter and nutrients provided by manure [68,69]. Both WB and MB groups showed higher abundances of Ascomycota compared to the NB group, suggesting that both BC types could enhance interactions between Ascomycota and organic matter such as cellulose, improving organic matter conversion [70]. Similar to the prokaryotic community structure, the richness of the fungal community structure decreased when BC was amended, while manure and plants increased the richness (Table 3). Specifically, manure BC negatively affected the diversity, evenness, and richness of the fungal community structure in sandy loam, making the fungal community remarkably different from SL-Control, SL-NB, and SL-WB (Figure 3B).

Diversity, evenness, and richness were notably lower in CL-WB and CL-MB compared to CL-NB, indicating that both BC types might create conditions favoring specific fungal groups. In summary, manure and *C. dactylon* enhanced microbial diversity and richness in both SL and CL soils. Conversely, both wood BC and manure BC amendments tended to create competitive environments favoring specific microbial groups, leading to decreased overall diversity and richness while promoting soil health in both sandy loam and clay loam soils. Biochar can mitigate the negative effects of antibiotics on microbial community structure by adsorbing and immobilizing antibiotics as previously discussed in Section 3.1,

thereby reducing their bioavailability [71,72]. This might reduce the direct exposure of soil microbes to antibiotics. In short, biochar affected microbial community structure through a balance between direct benefits (i.e., increased surface area and nutrient) and indirect benefits (i.e., reduced bioavailability of antibiotics) to the soils.

3.3. Antibiotic Resistance Gene Distribution

To evaluate the functional changes in microbial community structure in terms of ARGs, functional metagenomic prediction using PICRUSt was conducted based on 16S rRNA gene HTS data (Table S1). A TC efflux pump gene (*tetA*) and a dihydropteroate synthase (DHPS, encoded by *folP*) were identified as the main resistance genes for TC and SMZ/SMX, respectively (Figure 4). Typically, bacterial resistance to TC is mediated by efflux pump genes (*tetA*, *tetB*, *tetC*, *tetD*, *tetE*, *tetG*, and *tetK*), ribosomal protection proteins (*tetM*, *tetO*, *tetQ*, and *tetS*), and enzymatic modification (*tetX*) [73]. The presence of only *tetA* across all groups is consistent with other studies that detected or quantified a single efflux pump gene or observed the highest abundance of an efflux pump gene among all *tet* genes in biological systems [73–76].

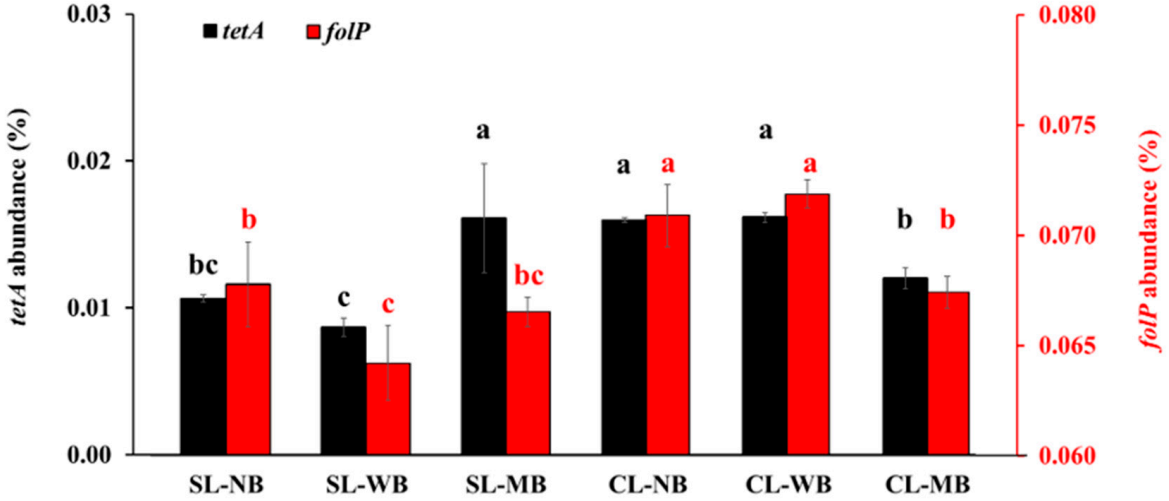

Figure 4. Relative abundance of *tetA* and *folP* based on the PICRUSt prediction model. Different letters above bars indicate significant differences between conditions ($p < 0.05$, Duncan's test). SL = sandy loam soil; CL = clay loam soil; MB = manure biochar; WB = wood biochar; NB = no biochar applied.

Sulfonamide resistance genes, such as sul1 and sul2, are commonly detected with the highest frequency in biological systems [77,78]. In this study, the main detected sulfonamide resistance gene was *folP*. Although *folP* itself does not mitigate the effects of sulfonamides, point mutations in the *folP* gene induced by frequent exposure to sulfonamides lead to structural changes that confer resistance [79–81]. Several studies have reported that some microbial groups can resist sulfonamide antibiotics through mutated *folP* genes [82–84], indicating that *folP* was the main contributor to SMZ/SMX resistance in this study.

In SL-WB, *tetA* and *folP* showed lower abundance compared to other groups, indicating that wood BC efficiently reduced the bioavailability of TC and SMZ/SMX. In contrast, SL-MB showed a higher abundance of *tetA* than SL-NB, despite achieving a significantly lower residual TC concentration. In the short duration of this study, it is possible that there was insufficient time for attenuation of *tet* resistance gene abundance, or other factors maintained their numbers. One possible mechanism is that the high concentrations of heavy metals (e.g., Zn, Fe, Co, and Mn) in manure BC might enhance the growth of the potential hosts of *tetA* [85]. Furthermore, Zn has a positive relationship with TC resistance genes, especially *tetA* [86], supporting the hypothesis that high Zn content in manure BC induced an increase in the abundance of *tetA* regardless of its efficient adsorption of TC

in SL soil. Unlike *tetA*, the abundance of *folP* in SL-MB followed the trends of residual SMZ/SMX in SL soil, indicating that the host of *folP* might be directly affected by the residual SMZ/SMX.

In CL, the abundance of *tetA* and *folP* did not correlate with the residual antibiotics in the soil. While there was no difference in ARG abundance between CL-NB and CL-WB despite the lower residual antibiotics in CL-WB, CL-MB showed lower abundance in both ARGs. Clay loam soils tend to support higher microbial concentrations compared to SL soils due to their superior nutrient and moisture retention capabilities [87,88]. This difference in microbial density means that when both soil types are exposed to the same amount of antibiotics, SL soils often exhibit a greater impact on their microbial communities. Therefore, in CL, changes in microbial community structure induced by BC might more strongly affect ARG abundance rather than residual ARGs in the soil.

3.4. Correlation between Antibiotics, ARGs, and Microbial Communities

A correlation analysis between antibiotics, ARGs, and microbial communities (Table 4) indicated that residual TC was positively correlated with *Micromonosporaceae* and *Vicinamibacteraceae*, which, as families, positively correlated with TC and resistance to various antibiotics, respectively [89,90]. Conversely, residual TC was negatively correlated with *Sphingomonadaceae* and *Lecanoromycetes*. The relative abundance of *Sphingomonadaceae* increases in BC-amended soil [91]. Although there is no reported observation that BC affects the abundance of *Lecanoromycetes* specifically, BC increases the relative abundance of the fungal phylum *Ascomycota*, which includes *Lecanoromycetes*, in soil [92]. Furthermore, *Ascomycota* ($p < 0.1$ and r = −0.73) was negatively correlated with residual TC. Considering the lower residual TC in BC-amended soils, both *Sphingomonadaceae* and *Lecanoromycetes* were likely positively affected by BC. Residual SMZ/SMX was positively correlated with *Nectriaceae* and unclassified fungi, while being negatively correlated with *Myxococcaceae* and *Nitrososphaeraceae*. *Myxococcales* ($p < 0.1$ and r = −0.73), which includes *Myxococcaceae*, and *Nitrososphaeraceae*, inhibited behavior or negative correlation with SMX [93,94]. In summary, the microbial community was affected by each ARG type and BC amendment, regardless of soil and BC type.

Table 4. Pearson correlation coefficients r and the level of the *p* values (** < 0.05; * < 0.1) for antibiotics and ARGs against microbial groups.

Parameter	Microbial Group [a]	Coefficient *r*
Antibiotic		
TC	*Micromonosporaceae*	0.75 *
	Vicinamibacteraceae	0.74 *
	Sphingomonadaceae	−0.81 *
	Lecanoromycetes	−0.82 **
SMZ/SMX	Unclassified Fungi	0.74 *
	Nectriaceae	0.77 *
	Myxococcaceae	−0.75 *
	Nitrososphaeraceae	−0.73 *
ARG		
tetA	*Planctomycetota*	0.78 *
	Chitinophagaceae	−0.89 **
	Microscillaceae	−0.73 *
	Steroidobacteraceae	−0.70 *
	Thermoanaerobaculaceae	−0.90 **
	Chaetomiaceae	−0.81 **
	Ostropales	−0.91 **
folP	*BIrii41*	0.73 *
	Xanthomonadaceae	0.88 **
	Planctomycetota	0.88 **

[a] Only the results for the lowest taxonomic rank are presented if similar correlation results were observed across family, order, and phylum. TC = tetracycline; ARG = antibiotic-resistant genes.

Specific microbial groups were also correlated with ARGs depending on the type of ARG. For example, *tetA* was negatively correlated with *Chitinophagaceae*, *Microscillaceae*, *Steroidobacteraceae*, *Thermoanaerobaculaceae*, *Chaetomiaceae*, and *Ostropales*, implying that these families might not be hosts of *tetA* in this study. Meanwhile, *tetA* showed a positive correlation with only *Planctomycetota*, which was also significantly and positively correlated with *folP*. *Planctomycetota* has been reported as a universal host for various ARGs, including TC and sulfonamide resistance genes, with positive correlations [78,95–97]. Therefore, it can be suggested that *Planctomycetota* was one of the key microbial indicators for comprehensive ARGs in this study. Additionally, *BIrii41* (belonging to the order *Burkholderiales*) and *Xanthomonadaceae* were also positively correlated with the abundance of *folP*, indicating that these microbial groups might also be potential hosts for the SMZ/SMX resistance gene *folP* in this study [98–100]. In conclusion, BC amendment did not seem to directly affect the proliferation and dissemination of ARGs, as it was strongly influenced by microbial community structure. To evaluate the effects of BC on ARGs quantitatively, further investigation through quantitative analysis of ARGs is necessary.

4. Conclusions

Our study demonstrated that BCs, derived from both wood and manure, can mitigate the environmental risks associated with antibiotic residues in agricultural soils, aligning with prior studies. However, our results show that the effectiveness of BC can vary depending on its physicochemical properties and the type of soil, a factor that is less emphasized in some previous studies. Wood BC, with its larger surface area and hydrophobic properties, was particularly effective in reducing tetracycline and sulfonamide concentrations, enhancing microbial diversity and nutrient cycling, especially in the sandy loam soil. Manure BC, while providing additional nutrients and supporting beneficial microbial groups for nitrogen fixation, exhibited variable efficiency in reducing antibiotic residues due to its lower surface area and higher ash content, performing better in clay loam soil. The differentiated impacts of wood and manure BC underscore the importance of selecting appropriate BC types based on soil characteristics to optimize antibiotic degradation and improve soil health. These findings contribute to the development of sustainable agricultural practices by offering a viable solution for reducing antibiotic contamination and supporting microbial community health in manure-amended soils. Additional field research is needed to further explore the long-term effects of biochar on antibiotic resistance gene dynamics and microbial community structure in forage fields across different soil types and environmental conditions. Comparative studies on various biochar types and soil properties will be essential in refining biochar application strategies in pastures for effective soil remediation.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy14092100/s1, Table S1: E-supplementary data for this study can be found in the online version of the paper.

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