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Distribution of antibiotic resistance genes in soil-plant ecosystem under manure fertilization

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Master in Applied Ecohydrology

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Declaration of authorship of work

I declare I am the author of this work, which is original and unpublished. The sources consulted have been duly cited in the text and included in the list of references.

Date: Signature:

Abstract

The global demand for food production has enhanced the application of animal manure as an organic fertilizer for sustainable soil management practices. However, recent studies have highlighted the problem of the occurrence of veterinary antibiotics in animal manure and the risk of the development of antibiotic resistance in terrestrial and aquatic ecosystems.

This study investigates the spread of antibiotic resistance genes (ARGs) in the microbiome of manure, soil, and plant-associated bacteria of cherry radish following the application of three kinds of animal manure (turkey, cow, and pig). A 45-day pot experiment was conducted, involving the sampling of manure-amended soil, rhizospheric bacteria, and endophytic bacteria inhabiting cherry radish roots and leaves. The samples were used to analyze the presence of *16S rRNA* genes and 21 ARGs belonging to 7 Antibiotic Resistance Phenotypes (ARPs). Soil samples from the end of the experiment (45 day) were additionally used to check the influence of manure on the functional diversity of the soil microbiome (Biolog Ecoplates) and determine soil dehydrogenase activity.

The most frequently detected ARPs in cow and pig manure were tetracyclines, beta-lactams, sulphonamides, macrolides, and fluoroquinolones. None of these ARPs were found in turkey manure. Pig manure-amended soil had the highest number of detected ARGs, while cow manure-amended soil had the fewest. Similarly among rhizospheric bacteria, pig manure-amended soil variant showed the highest number of detected ARGs, while cow-manure amended variant the lowest. No ARGs were detected in the endophytic bacteria in roots of turkey manure-amended soil, however 8 ARGs: *blaTEM, blaOXA, tet(A), tet(B), tet(C), tet(D), tet(D),* and *aac(6')-Ib-cr* were found in the endophytic bacteria in cherry radish leaves. The microbial functional diversity measured using the Biolog Ecoplates was higher in turkey-manured soil compared to the control, but it was lower in cow and pig-manured soil. This pattern was consistent with the Shannon-Weaver diversity index (H'). The soil dehydrogenase activity was higher in the control variant than in the manure-amended variants. This study indicates that ARGs present in animal manure have the potential to be transferred to vegetables and pose a threat to human health.

Keywords: antibiotic resistance phenotypes, antibiotic resistance genes, animal manure, Biolog Ecoplates, human health

ABSTRACT

A procura global pela produção de alimentos aumentou a aplicação de estrume animal como fertilizante orgânico. No entanto, estudos recentes realçaram o problema da ocorrência de antibióticos de uso veterinários no estrume animal, e o risco de desenvolvimento de resistência aos antibióticos em solos e plantas fertilizados.

Este estudo investiga a disseminação de genes de resistência a antibióticos (ARGs) no microbioma do estrume no solo e a presença de bactérias associadas a plantas de rabanete cereja após a aplicação de três tipos de esterco animal (peru, vaca e porco). Uma experiência em vaso de 45 dias foi realizada, envolvendo a amostragem de solo corrigido com esterco, bactérias rizosféricas e bactérias endofíticas que habitam raízes e folhas de rabanete cereja. As amostras foram utilizadas para analisar a presença de genes 16S rRNA e 21 ARGs pertencentes a 7 Fenótipos de Resistência a Antibióticos (ARPs). Amostras de solo do final da experiência (45 dias) foram adicionalmente utilizadas para verificar a influência do estrume na diversidade funcional do microbioma do solo (técnica Biolog Ecoplates) e para a determinação da atividade da desidrogenase do solo.

Os ARPs mais frequentemente detectados em estercos de vacas e suínos foram tetraciclinas, beta-lactâmicos, sulfonamidas, macrolídeos e fluoroquinolonas. Nenhum desses ARPs foi encontrado em esterco de peru. O solo corrigido com esterco de porco teve o maior número de ARGs detectados, enquanto o solo corrigido com esterco de vaca teve o menor número. Da mesma forma, entre as bactérias rizosféricas, a variante de solo corrigida com esterco de porco apresentou o maior número de ARGs detectados, enquanto a variante alterada com esterco de vaca apresentou o menor. Nenhum ARGs foi detectado nas bactérias endofíticas em raízes de solo alterado com esterco de peru, no entanto, 8 ARGs: blaTEM, blaOXA, tet(A), tet(B), tet(C), tet(D), tet(D), e aac(6')-Ib-cr foram encontrados em bactérias endofíticas em folhas de rabanete cereja. A diversidade funcional microbiana medida usando as Biolog Ecoplates foi maior em solo com esterco de peru em comparação com o controle, mas foi menor em solo com estrume de vaca e porco. Este padrão foi consistente no índice de diversidade de Shannon-Weaver (H’). A atividade da desidrogenase do solo foi maior na variante controle do que nas variantes alteradas com estrume. Este estudo indica que os ARGs presentes no estrume animal têm potencial para serem transferidos para vegetais e representam uma ameaça à saúde humana.

Palavras-chave: fenótipos de resistência a antibióticos, genes de resistência a antibióticos, esterco animal, Biolog Ecoplates, saúde human

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Abbreviations and Symbols

ARB	Antibiotic-Resistant Bacteria
ARG	Antibiotic-Resistance Gene
ARP	Antibiotic-Resistance Phenotype
g	grams
mL	milli liters
µL	micro liters
Mm	micro meter
h/H	hour
min	minutes
TET	tetracyclines
β	β-lactams
MLS	macrolides, lincosamides, streptogramins
SUL	Sulfonamides
FQ	Fluoroquinolones
I	Integrases resistance determinants
Van	Vancomycin
PCR	Polymerase Chain Reactions
A	amines
AA	amino acids
CA	carboxylic acids
CCS	complex carbon sources
CH	carbohydrates
PC	phosphate carbon
AWCD	Average well-color development
MGE	Mobile Genetic Elements
HGT	Horizontal Gene Transfer

1 Introduction

The world's growing population has heightened the demand for food production, leading to increased reliance on both natural and synthetic fertilizers to enhance crop yields. Seeking for enhanced crop productivity and agricultural intensification, the farmers have sustained indiscriminate inputs of synthetic agrochemicals, an overuse of water, and the alteration of the soil ecosystem, at great expense to the environment.

Protecting soil and monitoring its biological activity are pivotal in assessing soil productivity and fertility. Soil conditions significantly influence ecohydrological processes, affecting landscapes, river valleys, and aquatic ecosystems (United Nation, 2022). Ecohydrology principles highlight the potential of ecological biotechnologies for soil purification, enhancing soil productivity and preventing pollutant spread in aquatic ecosystems (Zalewski, 2002).

Replacing soil internal processes with external inputs has resulted in the progressive deterioration of the fundamental properties of agricultural soils, including the potential for self-regulation. Furthermore, the exponential growth of the human population has led to the extensive use of non-renewable resources, exerting greater pressure on the Earth’s finite reserves (Zou et al., 2022). Soil degradation, water pollution, and greenhouse gas emissions are some of the critical environmental concerns in the circular economy. Thus, adopting organic fertilizers, such as animal manure, to enrich the soil nutrient and organic matter content has gained a prominent sustainable alternative (FAO, 2022). Implementing environmentally sustainable agricultural practices is crucial for ensuring food security.

In this context, using animal manure or natural compost presents a promising solution with multifaceted benefits for enhancing soil fertility, reducing reliance on synthetic fertilizers, and fostering a thriving soil ecosystem (FAO, 2022). The use of animal manure outweighs other organic/green manures due to the presence of high proportions of nitrogen and potassium, medium proportions of calcium and phosphorus, and low proportions of magnesium and sulfur (Carvajal-Muñoz & Carmona-Garcia, 2012). The addition of manure to soils gives positive effects on soil physicochemical stability, promoting plants' growth and development of beneficial microbial populations. Furthermore, manure enhances soil organic carbon levels and biodiversity, which are closely linked to increasing food availability. Additionally, increasing market prices and strict policies regarding the use of mineral fertilizers have led farmers to use animal manure as a readily available nutrient resource, which is especially visible in lowincome countries (Figure *1*) (FAO & ITPS, 2015; Jiang et al., 2015). All these factors influence the widespread use of manure as an easily available and cheap fertilizer (Figure *1*).

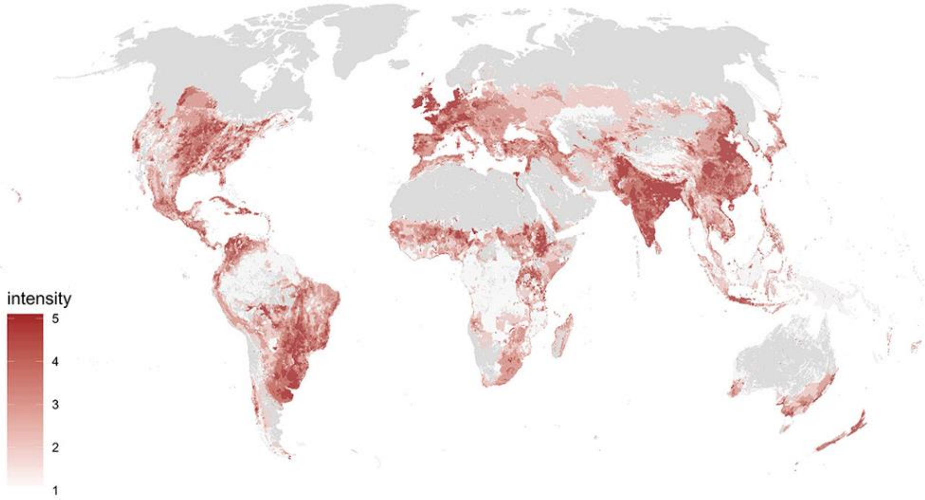


Figure 1*: The global map of manure*

(source: [This global map of manure could help save farming as we know it | Science | AAAS)](This global map of manure could help save farming as we know it | Science | AAAS)

Although the application of animal manure has been recognized as global best practice for restoring soil nutrients, the possible negative extremities such as foodborne and waterborne diseases due to soil-water contamination from animal manure are poorly recognized (Carvajal-Muñoz & Carmona-Garcia, 2012; Jiang et al., 2015). Studies have shown that animal manure can also pose a threat to human health due to the presence of contaminants such as heavy metals and organic pollutants (Carvajal-Muñoz & Carmona-Garcia, 2012; Jiang et al., 2015). Consequences of the unmanaged application of animal manure may also lead to the problem of contamination of soil and water ecosystems with compounds of emerging concern, which include antibiotics and antibiotic resistance determinants (Antibiotic-Resistant Bacteria (ARB), Antibiotic Resistance Genes ARGs).

1.1 Antibiotics as an emerging pollutant

In the year 1928, with the discovery of penicillin, the world advanced in the field of medicine by significantly reducing the mortality of both humans and animals due to bacterial infections (Ezzariai et al., 2018). Most antibiotics are natural products of bacteria, fungi, or semisynthetic modifications of natural compounds, and a few are entirely synthetic. Microorganisms that produce antibiotics have evolved antibiotic resistance to them (Xie et al., 2018).

Today between 100,000 to 200,000 tons of antibiotics are used in human and veterinary practices worldwide (Walia et al., 2019). A substantial proportion of administered antibiotics to humans and animals are excreted as unmetabolized or active antibiotic residues (Jia et al., 2023). According to the Centers for Disease Control and Prevention (CDC US), the widespread and indiscriminate use of antibiotics has led to the emergence of antibiotic resistance (CDC, 2019); Tasho & Cho 2016a).

The wide, and in many cases, unnecessary administration of antibiotics in both humans and animals has led to their spread in the environment. Residues of antibiotics and their metabolites are ubiquitously present in municipal, agricultural, and industrial wastewater, sewage sludge, treated wastewater, animal manure, and in water bodies receiving treated wastewater (Carvalho & Santos, 2016; Ezzariai et al., 2018; Godoy & Sánchez, 2020; Zhang et al., 2019). Antibiotics, due to their water solubility spread in the soil-water environment, and in this way may reach surface waters, groundwater, and subsequently sources of drinking water (Carvalho & Santos, 2016; Koniuszewska et al., 2020). As a consequence, antibiotics are considered as emerging pollutants associated with human-impacted environments (Zalewska et al., 2021; Zhang et al., 2019).

1.2 The use and fate of antibiotics in veterinary practices

Since the 1960s, antibiotics have been commonly used as feed additives in small doses to promote the growth of food-producing animals. This practice has led to a substantial increase in the global use of veterinary antibiotics (Xie et al., 2018). For example, in the US, approximately 80% of the total annual antimicrobial treatment is for food-producing animals (Zalewska et al., 2021), and in Europe, this amount is estimated to be 33% (Ezzariai et al., 2018). The US Food and Drug Administration (US FDA) has given permission to produce more than 2000 veterinary pharmaceutical products, within 18 classes of antimicrobials, to treat various species of animals as therapeutic, metaphylaxis, prophylaxis, and antibiotic growth promoting compounds (AGPs) (FAO, 2022; Mullard, 2013; Walia et al., 2019). In 2010, China was ranked the top country consuming antibiotics in livestock followed by the USA, Brazil, Germany, and India. In countries with growing economies, the high demand for protein-rich food is leading to the administration of antibiotics as growth promoters rather than for treatment purposes (Walia et al., 2019). The five mostly administered antibiotics in the animal industry are: tetracyclines, (β)beta-lactams, sulphonamides, macrolides, and fluoroquinolones (Table 1) (Xie et al., 2018).

Table 1: Some of the most used antibiotics in veterinary practice

Class of antibiotic	Common use in veterinary medicine	Amount [%]
Tetracycline	Respiratory infections, urinary tract infections, bacterial enteritis, tick-borne diseases. Growth promoter in cattle	33.4
β-lactams	Respiratory infections, skin, and soft tissue infections, urinary tract infections, and mastitis. Growth enhancement	Not specified
Sulphonamides	Respiratory infections, gastrointestinal infections, urinary tract infections, coccidiosis	11
Macrolides	Respiratory infections, skin infections, mastitis, and some gastrointestinal infections. Growth enhancement in some cases	7.5
Fluoroquinolones	Urinary tract infections, respiratory infections, skin infections, enteric infections	1.9

* The content of antibiotics in the bodies of animals that are intended for food purposes, data from 29 European Union countries. Data for the table has been adopted from (Dróżdż et al., 2020; Mullard, 2013; Tasho & Cho, 2016; The European Medicines Agency, 2020; Xie et al., 2018).

The fate of antibiotics used in veterinary practice is considerably important because they are bioactive organic compounds with a short retention and partial uptake into the animal intestines (Carvalho & Santos, 2016; Walia et al., 2019). Studies have shown that animals' average rate of antibiotic excretion is 58%, and some compounds can be excreted as much as 90% as their parent compound. For example, Tasho & Cho, (2016a) showed that the rate of excretion of tetracycline and sulphonamides varies from 40% to 90%, respectively.

In consequence, such a widespread and indiscriminate administration of veterinary antibiotics causes the problem of spreading not only antibiotics but also antimicrobial resistance in the environment. When antibiotics are used to treat animals, they may lead to develop ARB and

ARGs in animal intestines, and their further transmission through animal feces (manure) to the arable and edible crops, posing a threat to human health (CDC, 2019; Tasho & Cho, 2016; Xie et al., 2018; Zalewska et al., 2021). What is more, some of the antibiotics used for growth promotion are classified by WHO as critically useful in human medicine. Consequently, their widespread usage in veterinary practice may lead to the development of resistance to the so-called "last-change antibiotics", which pose a real threat to human health.

1.3 Antibiotics and antibiotic resistance determinants in manure and manure-fertilized soil

The fate of antibiotics' residues, ARB and ARGs released into the environment depends on the type of environment (soil/water/air) they are released and the interactions between bacteria. Soil is the primary reservoir of microbial communities and plays a crucial role in both the emergence and dissemination of antibiotic resistance. Consequently, antibiotics and their residues present in manure and disseminated to the fertilized soil in subinhibitory concentrations pose a risk of acquiring antibiotic resistance by native soil bacteria. In fact, according to several studies, one of the principal sources of ARB and ARGs in the soil is antibiotic-contaminated animal manure (Jia et al., 2023; Szczepanowski et al., 2009; Tasho & Cho, 2016a; Zhang et al., 2020; Urra et al., 2019). Xie et al. (2018) showed that fresh manure or manure compost is a source of antibiotics and ARB, and its land application causes the spread of antibiotic resistance in the soil microbial community (Xie et al., 2018). Subirats et al. (2021) showed that chicken litter and other manures carrying ARB and ARGs have the potential to contaminate soil due to antibiotics' residues. According to Marti et al., (2014) the application of manure and manure compost increased the abundance of viable antibiotic-resistant coliform bacteria and the frequency of detection of ARGs. Another study indicated also that when soil is repeatedly exposed to amendments carrying ARGs, it can lead to the emergence and propagation of these genes in the native soil bacteria (Urra et al., 2019). In this case, transfer of ARGs between manure and soil bacterial community occurs through horizontal gene transfer depending on mobile genetic elements (MGEs) such as plasmids, integrons, transposons, and gene cassettes (Xie et al., 2018; Zalewska et al., 2021).

Also, antibiotics' residues can affect the soil microflora in a range of ways altering soil microbial functions (Tasho & Cho, 2016). The detrimental impacts include the elimination of vital microbes responsible for e.g., providing nutrients to plants or degradation of harmful compounds, disruption of microbial populations through the selection of resistant species, altering the structure and function of soil microorganisms, and an increase in the presence and abundance of ARGs in different soil bacteria (Tasho & Cho, 2016; Zhang et al., 2020). Numerous literature data have indicated the problem of long-term persistence of antibiotic residues and thus the risk of development of antibiotic resistance in soil fertilized with manure – in this case, studies have shown that even after composting and stopping the use of antibiotics in veterinary practice, they residues have been still detected in the manure-amended soil (Ezugworie et al., 2021; Ezzariai et al., 2018; Liu et al., 2021; Urra et al., 2019).

1.4 Antibiotics and antibiotic resistance determinants in manure fertilized plants

Plants are subjected to various changes as an effect of the application of animal manure including accumulation of antibiotics' residues in plant tissues and transmission of ARGs to the plant-associated bacteria (rhizobacteria and endophytic bacteria).

In the case of the accumulation of antibiotics in plant tissues, numerous studies have shown the bioaccumulation and biomagnification of a range of veterinary antibiotics in plants after prolonged use of animal manure (Carvajal-Muñoz & Carmona-Garcia, 2012; Hu et al., 2010; Jiang et al., 2015; Kuppusamy et al., 2018; Tasho & Cho, 2016). The studied plants have included the following species *Cucumis sativus, Zea mays, Oryza sativa, Capsicum annum, Lactuca sativa,* and also tomato, and lettuce varieties (Tasho & Cho, 2016a; Zalewska et al., 2021; Zhang et al., 2020).

Several studies have also revealed transmission of ARGs to the plant microbiome as an effect of contaminated organic fertilizers application, eg. Chen et al., (2018) showed that ARGs in soil can be transmitted to the roots and leaves of plants. Wang et al. (2015) detected abundant ARGs in endophytes and phyllosphere microorganisms of livestock manure-amended vegetables at harvest. Plethora of data have demonstrated the presence of ARGs in bacteria inhabiting many plants including vegetables (cilantro, endive, lettuce, pak choi, tomato, cucumber, pepper, carrot, and radish) and crops (rice and wheat). A great concern is required especially for plants eaten raw and those in direct contact with the manure-fertilized soil such as carrots or radish, as they can be more dangerous to human health than others in terms of antimicrobial resistance food security (Marti et al., 2013a; Tasho & Cho, 2016; Zhang et al., 2020).

2 Aims of the Research

Nowadays, there is a great concern over the monitoring of animals in larger farms and the composition of manure produced in commercial farms; however, there is little to no monitoring of medium-scale and small-scale farms. Therefore, research should also be focused on the spread of ARGs from small-scale and family farms where the monitoring of antibiotic use is rare. Since the application of manure from small-scale farms in agricultural practice is a common practice worldwide, it may create a risk of transmission of antibiotic resistance from manure to soil and consequently to edible plants. Particular attention should be paid to plants whose edible parts are in direct contact with soil fertilized with manure - such plants (eg. radish) are most at risk of acquiring antibiotic resistance from manure.

In view of the above, the **goal of the present study is to monitor the prevalence of selected ARGs** (21 ARGs in total) from 7 antibiotic resistance phenotypes-ARP (tetracyclines-TET, β-lactams-β, macrolides, lincosamides, streptogramins-MLS, sulfonamides-SUL, fluoroquinolones-FQ, integrases-I, and glycopeptides) **in soil and radish microbiome under animal manure fertilization**. To achieve the above-mentioned study goal the following study aims have been proposed:

1. To analyze the occurrence of selected ARGs in the three types of animal manure (turkey, cow, pig).
2. To analyze the prevalence of selected ARGs found in animal manure in manure-fertilized soil.
3. To analyze the occurrence of ARGs in radish-associated bacteria (rhizosphere bacteria, and endophytic bacteria in roots and leaves) grown in manure-fertilized soil.
4. To analyze the changes in functional diversity in soil and radish-associated microbiome under animal manure fertilization.
5. To examine the enzymatic activity in soil under animal manure fertilization.

3 Materials and Methods

3.1 Manure-amended soil preparation

Manure for the current study was collected from three farms: a pig farm with 150 pigs, a cow farm with 30 cows, and a turkey farm with 82,000 turkeys. Collected manure samples of 5 L were well-mixed and then diluted (with distilled water) in a 1:10 ratio before being applied to the soil (Dróżdż et al., 2020; EU, 2001, 2009). For 300 g of dry soil, 120 mL of water (for control variant) and an equal amount of diluted manure (for manure-amended variants) were added.

3.2 Experimental setup

Each pot containing soil with either distilled water or diluted manure was planted with 5-6 seeds of cherry radish. Seeds were purchased from a certified supplier of garden seeds W. Legutko). Each variant (Table *2*) was prepared in 5 replicates and the pots were placed inside a grow box under ambient conditions. After two weeks extra plants from pots were removed by leaving out 1-2 plants at the same growth stadium. The experiment was conducted for 45 days.

Table 2: The variants used in the experiment and the number of replicates (The abbreviations stand for control soil CS, Turkey manure amended soil TM, Cow manure amended soil CwM, and Pig manure amended soil PM)

Control soil	Turkey manure amended soil	Cow manure amended soil	Pig manure amended soil
CS1	TS1	CwS1	PS1
CS2	TS2	CwS2	PS2
CS3	TS3	CwS3	PS3
CS4	TS4	CwS4	PS4
CS5	TS5	CwS5	PS4

3.3 Sampling

a) ***Soil sampling:*** Control and manure-amended soil samples were collected from each replicate on days: 0, 5, 15, 30, and 45. Samples were stored until further biochemical, molecular, and physicochemical analysis (Table *8*A).

Table 3: The order of sample collection and analysis done in the study

Sample type	Day	Molecular analysis	Biochemical analysis	Physico-chemical parameters analysis
Manure-amended and control soil samples (CS, TS, CwS, PS)	0	+	-	+
	5	+	-	-
	15	+	-	-
	30	+	-	-
	45	+	+	+
Plant-associated material (rhizospheric soil, roots, and leaves)	0	-	-	-
	5	-	-	-
	15	-	-	-
	30	-	-	-
	45	+	-	-

The abbreviations in the table are control soil (CS), turkey manure amended soil (TS), cow manure amended soil (CwS), and pig manure amended soil (PS).

b) Rhizospheric bacteria sampling: Plants were carefully removed after the separation of bulk soil and the above-ground plant parts (stems and leaves). Stems and leaves were separated from roots using sterile blades.

To extract rhizospheric bacteria, roots were placed in 50 mL sterile falcon tubes with 10 mL of sterile PBS-S buffer amended with Tween 20. The rhizospheric soil was then separated by shaking the falcon tubes on a shaker platform for 20 min at 180 rpm. The roots were then carefully transferred to new sterile 50 mL falcon tubes containing 25 mL of PBS-S buffer. The roots were then rinsed with tap water to remove any residuals and soil attached to the main root. Root samples were then used for surface sterilization. The rhizospheric soil was centrifuged for 20 min at 3500 rpm and then the supernatant was discarded, and the resulting pellet was used as the rhizospheric bacteria for further analysis (Bulgarelli et al., 2012; Mierzejewska et al., 2022).

b) Leaves endophytes sampling: The second, third, and fourth leaves (from the bottom) of each plant was taken for plant endophyte bacteria analysis.(Mierzejewska et al., 2023). Surface sterilization of leaves was done under the laminar flow in sterile conditions. Leaves sterilization was done first with 150 mL of 1x 70% ethanol (1min) followed by 150 mL of 1x 2.5% NaOCl + 0.1 % Tween 20 (2min), 150 mL of 1x 70 % ethanol (30 sec), and finally three separate sterilizations in 150 mL of distilled water (30sec). Then the sterilized leaves were stored in 50 mL sterile falcon tubes (Plaszkó et al., 2022). The sterility of the last wash was confirmed by inoculating 100µL of the third wash water on a petri dish containing an undiluted 869 medium (Mergeay et al., 1985). The surface-sterilized leaves tissues weighing approximately 50 mg were frozen (-80°C) in 2 mL eppendorf tubes for DNA extraction (Mierzejewska et al., 2023).

c)Roots endophytes sampling : Surface sterilization of roots was done under the laminar flow in sterile conditions. Only the main root was selected for further analysis. Roots were sterilized subsequently in 1x96% 100 mL ethanol (30 sec), 150 mL of 1x 2.5 % NaOCl + 0.1% Tween 20 10 min), and finally three separate sterilizations in 150mL distilled water (3 0sec each sterilization). Then the sterilized plant roots were stored in 50mL sterile falcon tubes (Plaszkó et al., 2022). The sterility of the last wash was confirmed by inoculating 100 µL of the third wash water on a petri dish containing an undiluted 869 medium (Mergeay et al., 1985). The surface-sterilized roots tissues weighing approximately 50 mg were frozen (-80°C) in 2 mL eppendorf tubes for DNA extraction (Mierzejewska et al., 2023).

Table 4:Sampling order of rhizospheric soil and endophytes (leaves and roots) at the end of experiment

Rhizospheric soil				Endospheric Leaves				Endospheric Leaves			
CS	TS	CwS	PS	CS	TS	CwS	PS	CS	TS	CwS	PS

The letters indicate the variant from which the samples were collected; CS-Control, TS-turkey manure amended soil, CwS-cow manure amended soil, PS-Pig manure amended soil.

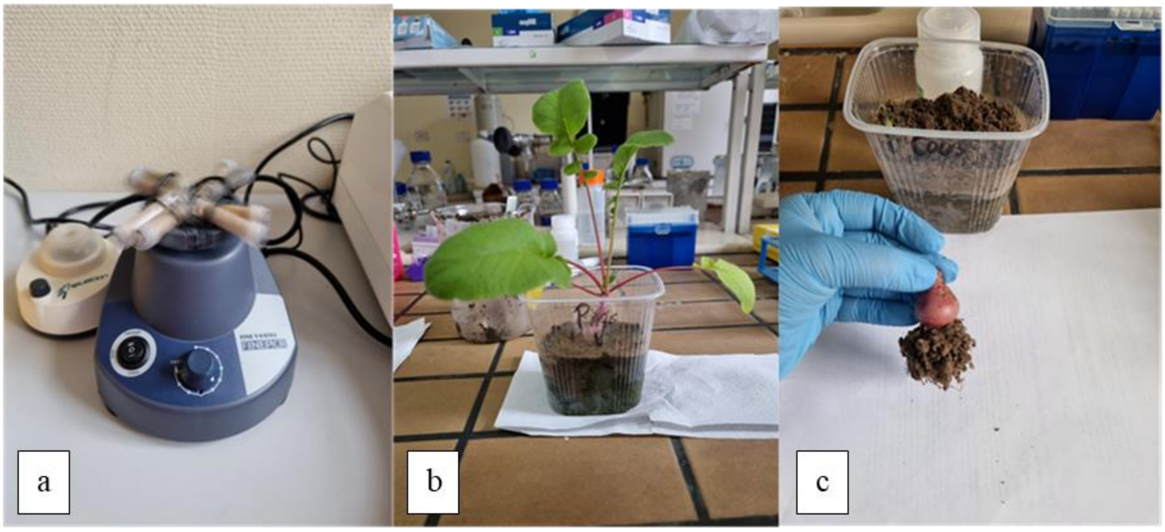


Figure 2:Sample preparation (a)Vortexing of soil samples for DNA isolation; (b)Healthy cherry radish plant grown in pig manure amended soil; (c)cherry radish at the end of the experiment.

3.4 Molecular analysis

3.4.1. DNA extraction

Total DNA from undiluted manure, control soil and manure-amended soil, as well as rhizosphere was isolated using the GeneMATRIX soil DNA purification Kit (Qiagen, Venlo, Netherlands). Endophytic DNA was isolated using the PowerPlant R Pro DNA Isolation Kit. DNA samples were quality-checked using Nanodrop 2000 (Thermo Fisher Scientific) and stored at -20°C.

3.4.2. Detection of ARGs: Polymerase Chain Reactions (PCR) and Gel Electrophoresis

Standard PCRs were first carried out for the 16S rRNA gene to confirm the presence of bacteria in the studied material. The occurrence of genes encoding resistance to selected antibiotics was determined in manure, manure-amended soil, rhizosphere bacteria, and in endophytes (from roots and leaves). The PCR reaction profiles are shown in Table *5*.

The analyzed ARGs are as follow: tetracyclines (tet(A), *tet*(B), *tet*(C), *tet*(D), *tet*€,*tet*(M), beta-lactams (*bla*$_{\text{OXA}}$, *bla*$_{\text{SHV}}$, *bla*$_{\text{TEM}}$, *bla*$_{\text{CTX-M}}$), macrolides, lincosamides, streptogramins (MLS) antibiotics (*erm*F, *erm*A, and *lin*A), fluoroquinolones *(aac(6 _)-Ib-cr, qnr*B1), sulfonamides (*sul*1), vancomycin (*vanA*, *van*B, *van*C-1, *van*C-2), and integrase (*intl*1).

Table 5: Primer sequence and PCR parameters used for analysis

	Target gene	Primer sequence (5’-3’)	Amplicon size (bp)	Annealing temperature in standard PCR (°C)	References
	16S rRNA	CCTACGGGNGGCWGCAG GACTACHVGGGTATCTAATCC	444	55	(Klindworth et al., 2013)
β	*bla*$_{\mathrm{TEM}}$	AGTGCTGCCATAACCATGAGTG CTGACTCCCCGTCGTGTAGATA	431	53	(Kim et al., 2009)
	bla$_{\mathrm{OXA}}$	ATTATCTACAGCAGCGCCAGTG TGCATCCACGTCTTTGGTG	296	61	
	bla$_{\mathrm{SHV}}$	GATGAACGCTTTCCCATGATG CGCTGTTATCGCTCATGGTAA	214		
	bla$_{\mathrm{CTX\text{-}M}}$	TCCAGAATAAGGAATCCCATGG TGCTTTACCCAGCGTCAGAT	501		
TET	*tet*(A)	GCTACATCCTGCTTGCCTTC GCATAGATCGCCGTGAAGAG	211	54	(Nawaz et al., 2006)
	tet(B)	TCATTGCCGATACCACCTCAG CCAACCATCATGCTATTCCATCC	391	50	
	tet€	CTGCTCGCTTCGCTACTTG GCCTACAATCCATGCCAACC	897	50	
	tet(D)	TGTGCTGTGGATGTTGTATCTC CAGTGCCGTGCCAATCAG	844	50	
	tet€	ATGAACCGCATCGTGATGATG ACCGACCATTACGCCATCC	744	50	
	tet(M)	AGTGCACTGTTGCAAGAAAAG GAAACCGAGCTCTCATACTGC	197	58	(Szczepanowski et al., 2009)
MLS	*erm*A	ATGTCTGCATACGGACACGG ACTTCAACTGCCGTTATCGC	185	58	(Szczepanowski et al., 2009)
	*erm*F	TAGATATTGGGGCAGGCAAG GGAAATTGCGGAACTGCAAA	126		(Eitel et al., 2013)
	*lin*A	CTGGGGAGTGGATGTCTTGT AGTTGGCTTGTTTGGAAGTG	230	60	
SUL	*sul*1	CGCACCGGAAACATCGCTGCAC TGAAGTTCCGCCGCAAGGCTCG	163	55.9	(Pei et al., 2006)

FQ	*aac* (6’)-Ib-cr	TTGCGATGCTCTATGAGTGGCTA CTCGAATGCCTGGCGTGTT	482	55	(Park et al., 2006)
	*qnr*B1	AAATATGGCTCTGGCACTCG CTTTCAGCATCGCACGACTA	191		(Szczepanowski et al., 2009)
Van	*van*(A)	GGGAAAACGACAATTGC GTACAATGCGGCCGTTA	732	54	Dutka-Malen et al., 1995)
	van(B)	ATGGGAAGCCGATAGTC GATTTCGTTCCTCGACC	635		
	van(C_1)	GGTATCAAGGAAACCTC CTTCCGCCATCATAGCT	822		
	van(C_2)	CTCCTACGATTCTCTTG CGAGCAAGACCTTTAAG	439		
I	*int*l1	CCTCCCGCACGATGATC TCCACGCATCGTCAGGC	280	54	(Goldstein et al., 2001)

PCR gradients were used to optimize conditions for the selected ARGs (*tetA*, *aac(6 _)-Ib-cr*, *ermA*, *ermF*) to adjust the optimal annealing temperature and DNA concentrations. The temperature gradient used for PCR optimization is given in (Table 6).

Table 6: Temperature gradients for selected genes

Target gene	Amplicon size (bp)	Annealing temperature in standard PCR (°C) *	Temperature Gradient PCR (°C)
*tet(*A)	211	54	52.1/ 54.6/ 56.1
aac(6 _)-Ib-cr	482	55	52.1/ 54.6/ 56.1/ 57.2/ 59.1
*erm*A	185	58	54.1/ 56.3/ 59/ 60.1
*erm*F	126	58	56.1/ 57.2/ 59.1

*Annealing temperatures in standard PCR are given in Table 5.

The PCR products were analyzed by transferring 5-10 µL of the product (depending on the comb size) with 1-2 µL of loading buffer to 1.5 % agarose gel stained with ethidium bromide (0.5 µg/mL concentration). Electrophoresis was conducted for 30-60 minutes at 100 V in 1x TBE buffer solution, and the products were visualized under UV light.

3.5 Biochemical analysis

3.5.1. Community-level physiological profiling (CLPP) using Biolog Ecoplates

In order to assess the microbial activity in control soil and manure-amended soil after 45 days, Biolog Ecoplates were used. Firstly, 1g of homogenized soil from each sample was dissolved in 99 mL of 1 % NaCl in Pyrex bottles (250 mL). The samples were then shaken for 20 min and allowed to settle for 30 min at 4°C. Samples were then filtered through 100 µm mesh cell filters in 50 mL falcon tubes. An adequate amount of suspension was taken to sterile petri dishes for the inoculation of microarray plates. Aliquots of 120 µL were dispensed into the microarray plates using a multichannel pipette and the plates were incubated for 7 days at 27°C. The optical density (absorbance at 590nm wavelength) was measured at 24h intervals starting from time '0", using a Thermo Scientific™ Multiskan™ Skyhigh Microplate Spectrophotometer (Mierzejewska et al., 2022). The obtained results for the microbial activity on use of 31 different carbon sources from 6 carbon groups (A- amines, AA- amino acids, CA- carboxylic acids, CCS- complex carbon sources, CH- carbohydrates, PC- phosphate carbon) were then analyzed using Microsoft excel 365.

3.5.2. Dehydrogenase activity in the soil

Dehydrogenase activity in the soil was measured following the method of (CASIDA, KLEIN and SANTORO, 1964). A soil sample of 1.5 g was weighed into 50mL falcon tubes and nearly 15mg of $CaCO_3$ was added. Then 750 µL of distilled water and 2.5 cm^3 of 3% TTC were added. Falcon tubes were then tightly closed and incubated for 24 h at 25°C. After 24 h incubation, 12.5 cm^3 of ethanol was added and mixed vigorously. Falcon tubes were then closed tightly and further incubated for 1h in the dark. Samples were then centrifuged, and the absorbance of the supernatant was measured at 485nm on a spectrophotometer plate reader Multiskan SkyHigh (Thermo Fisher Scientific) (CASIDA et al., 1964).

To measure dry soil weight, 5g of soil was added to each crucible and dried overnight at 100°C. The final weight of the crucibles with the dried soil was recorded and the dry weight of the soil was calculated. Dehydrogenase activity of soil (DHA) was calculated based on the standard curve and the obtained results from the spectrophotometer using the below formula.

$$DHA = \frac{(X - XO) * 12.5}{g.d.m}$$

X (µg/mL)-   final TPF concentration, calculated based on the standard curve

X0 (µg/mL)- final TPF concentration in blank sample

g.d.m. – grams of soil dry mass

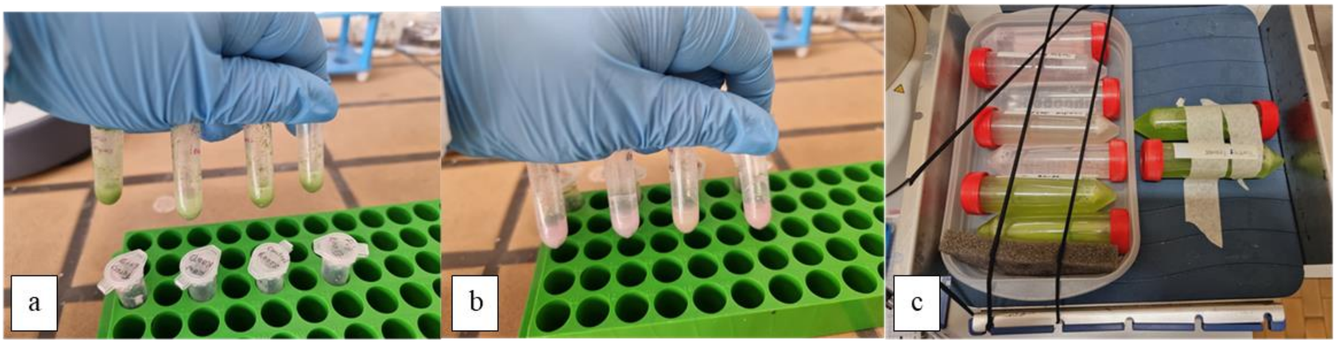


Figure 3: Preparation for DNA extraction from Leaves tissue extract (a); soil tissue extract (b); Falcon tubes with leaves and roots extract on shaking platform preparing for Biolog Ecoplates (c).

3.6 Data analysis

A comprehensive analysis of the data from PCR reactions, Biolog Ecoplates, and DHA was done using Microsoft Excel 365. In addition to basic data processing, Microsoft Excel 365's built-in statistical functions were used to perform statistical analysis on the data.

4 Results

4.1 Presence of bacterial DNA confirmed by *16SrRNA* gene.

The samples were initially analyzed for the confirmation of bacterial gene 16SrRNA. **Error! Reference source not found.** shows the results of standard PCR confirming the presence of 16SrRNA (444 bp) in manure, manure-amended soil, rhizosphere, and root and leaves endophytes. All the samples showed positive bands.

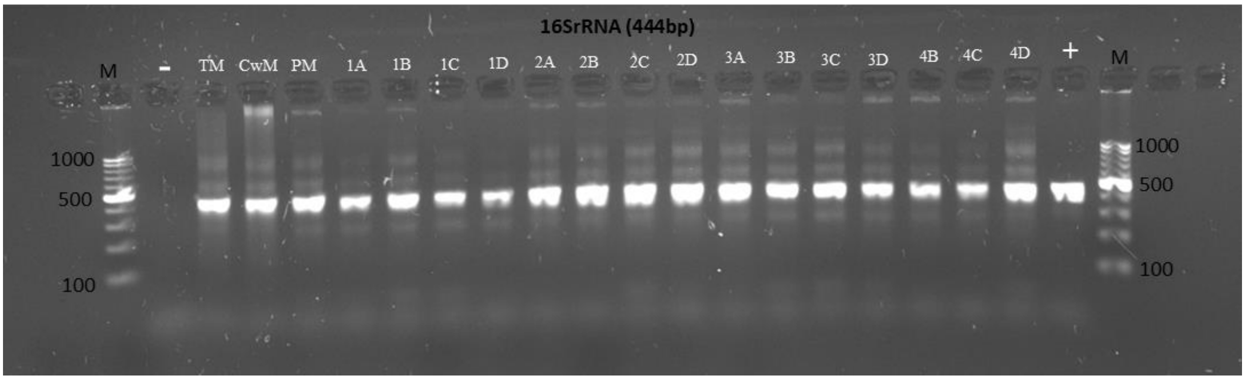


Figure 4: Electrophoresis results showing positive results confirming the presence of 16SrRNA in samples at 444bp. The abbreviations represent turkey manure TM, cow manure CwM, pig manure PM, and the letters represent control soil A, turkey manure amended soil B, cow manure amended soil C, and pig manure amended soil D. The numbers represent sampling day 0 as 1, day 5 as 2, day15 as 3, and day 30 as 4.

4.2 Occurrence of ARGs in manure

In order to find the best amplification conditions for the target genes, PCR conditions were optimized for each of the selected ARGs by performing gradient PCR for annealing temperature and changing the used DNA concentrations (Table *6*). As an example, Figure 15A shows electrophoresis results of temperature gradients used for optimizing the annealing temperature of *tet(*A) and *aac(6 _)-Ib-cr* genes. Optimized PCR conditions were used to detect a total of 21 ARGs (Table 9A), belonging to 7 ARPs (tetracyclines, β-lactams, MLS, sulfonamides, fluoroquinolones, integrases, and glycopeptides) in the turkey, cow, and pig manure samples.

The obtained results of the presence of ARGs are depicted in Figure 5 and Table 9A (see Annex). The results showed no ARGs in the turkey manure samples, while pig and cow manure displayed the presence of TET, SUL, Van, MLS, and FQ. Tetracycline resistance genes were detected at the highest frequency in cow and pig manure samples, constituting 34% and 40% of the total detected ARGs (Figure 5, Table 9A). The second most abundant genes were those from the MLS phenotype, constituting 25% in cow manure, and 20% in pig manure (Figure 5, Table 9A). The total percentage of β-lactams recorded in cow manure was higher compared to pig manure samples. The overall percentage of all the detected gene phenotypes was higher in cow manure than in pig manure. With the exception of vancomycin resistance genes found exclusively in pig manure samples. The percentage content of vancomycin resistance genes detected in pig manure samples was 13% of the total ARGs detected. *van*B and *van*C1 were the only detected vancomycin resistance genes in pig manure samples (Figure 5, Table 9A).

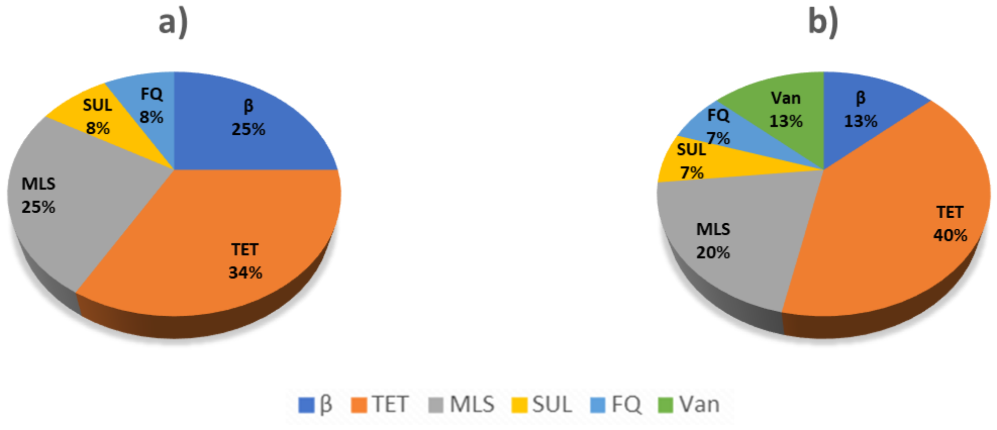


Figure 5: Total percentage of gene determinants detected in cow manure (a), and pig manure (b) used as manure amendments for the experiment. B-β lactam resistance determinants, TET- Tetracycline resistance determinants, MLS- Macrolides, lincosamides, streptogramins resistance determinants, SUL- sulphonamides resistance determinants, FQ- Fluoroquinolones resistance determinants, and Van- Vancomycin resistance determinants

4.3 Occurrence of ARGs in manure-fertilized soil

PCR results demonstrating the presence of selected ARGs in soil samples are depicted in **Error! Reference source not found.** and Table 9A. In the control soil, the only *ermF* gene b elonging to MLS phenotype was detected. Genes from MLS phenotype was also detected in turkey and pig manure-amended soil, but it was not present in cow manure-amended soil.

The most detected phenotype in the manure-amended soil variants was tetracycline which accounted for 75% in cow manure-amended soil and 50% in both turkey and pig manure-amended soil. Among the 6 tetracycline resistance genes tested, the most frequently detected were *tet(*M)> *tet(*A)> *tet(*C). *tet(*M) was detected in the pig variant throughout the entire 45-day experimental period. The pig variant prominently manifested the presence of the *tet*(B), *tet*(D), and *tet*€ genes, whereas these genes were not detected in the other two variants (Table 9A).

Fluoroquinolones were detected in all three manure-amended variants. Among the two ARGs belonging to fluoroquinolones phenotype (*aac(6 _)-Ib-cr* and *qnrB1*), *qnrB1* was not detected in any manure-amended variants (Table 9A).

β-lactams phenotype accounted for 16% in turkey manure-amended soil and 17% in pig manure-amended soil and was not detected in the cow manure-amended variant (**Error! Reference source not found.**).

In the pig variant, *bla*TEM following *bla*$_{\mathrm{OXA}}$ were detected (Table 9A). *bla*TEM was also the only ARG detected in turkey manure-amended soil.

The sulfonamide phenotype was detected only in the pig variant (accounting for 8% of the total percentage of genes detected) and was not detected in either turkey or cow manure-amended soil (**Error! Reference source not found.**).

Integrase determinants (*intl* 1) ARG was not detected in any of the variants (Table 9A).

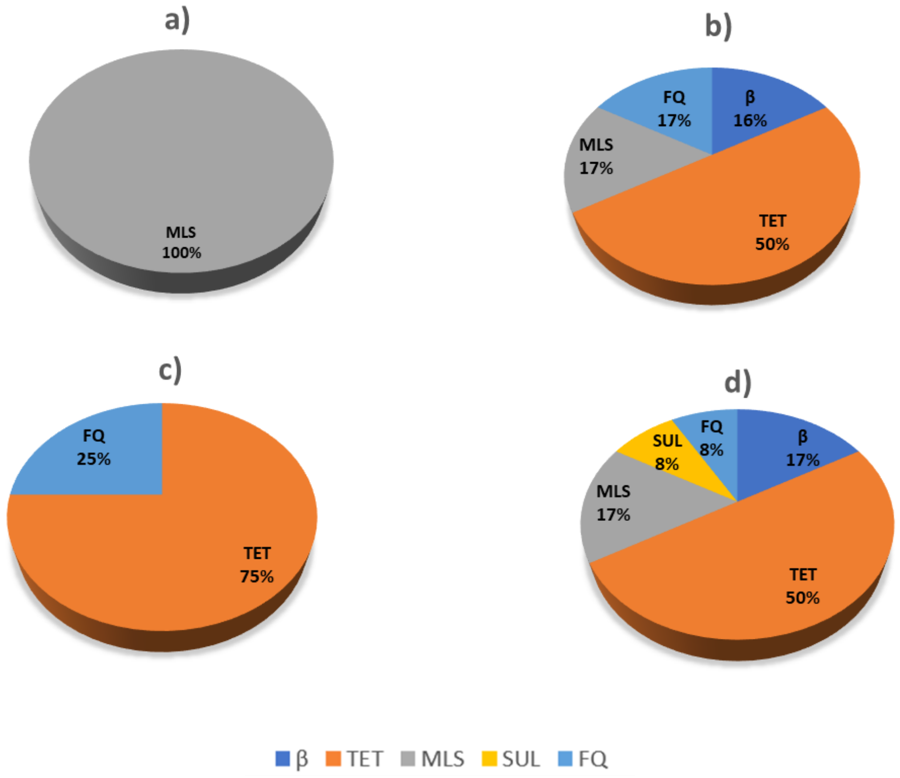


Figure 6: Total percentage of gene determinants detected in soil control (a), turkey manure amended soil (b), cow manure amended soil (c), and pig manure amended soil (d) throughout the experiment period of 45 days. β-β lactam resistance determinants, TET- Tetracycline resistance determinants, MLS- Macrolides, lincosamides, streptogramins resistance determinants, SUL- sulphonamides resistance determinants, and FQ- Fluoroquinolones resistance determinants

4.4 Occurrence of ARGs in plant-associated bacteria under manure fertilization

4.4.1. Rhizospheric bacteria

The occurrence of ARGs in rhizospheric bacteria collected at day 45 of the experiment is depicted in figure 7 and Table 10A. There were no ARGs detected in the rhizospheric bacteria in control soil. In the rhizospheric soil of cow manure-amended soil, only MLS phenotype was detected. Among the three genes form MLS (*erm*F, *erm*A, and *lin*A), only *erm*F was detected in turkey and cow manure-amended rhizospheric soil (Table *10*A). In the rhizospheric soil of turkey and pig variants, the tetracycline was the most prominent phenotype accounting for 50% of the total genes analyzed in both variants (Figure 7). From the 6 tet ARGs tested for the rhizospheric soil variants, *tet(*B), *tet(*D), and *tet(E)* were not detected in turkey and pig variants. *tet(*M) ARGs were present in turkey and pig variants but, *tet(*A) was detected only in pig variant (Table *10*A). Sulfonamides were detected 17% of the pig variant and were not detected in any other variant. Among the 7 antibiotic resistance phenotypes, integrases and glycopeptides were not detected in any of the variants (Table *10*A).

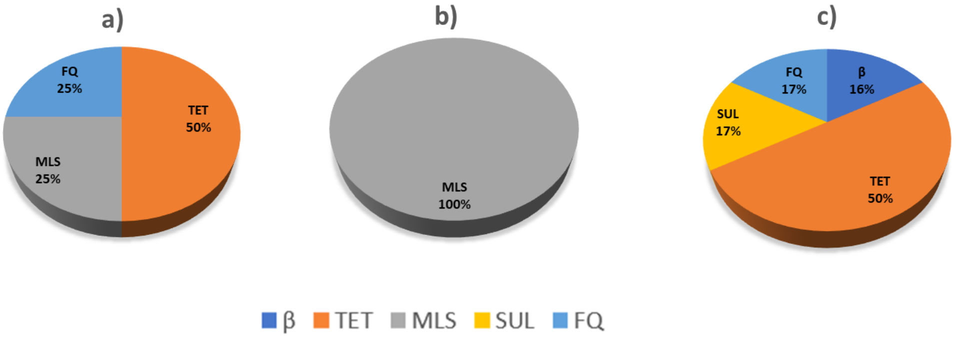


Figure 7:Total percentage of gene determinants detected in Rhizospheric soil 0f turkey manure amended soil (a), cow manure amended soil (b), and pig manure amended soil (c) at the end of the experiment period on day 45. β-β lactam resistance determinants, TET-Tetracycline resistance determinants, MLS-Macrolides, lincosamides, streptogramins resistance determinants, SUL- sulphonamides resistance determinants, and FQ- Fluoroquinolones resistance determinants

4.4.2. Root Endophytic bacteria

The presence of ARGs in root endophytes from all the variants (control soil, turkey, cow, and pig manure-amended soil) is presented in Figure 8. Finding show that there were no ARGs detected in the root endophytes of the control and turkey variant. Tetracycline phenotype was detected in 50% of samples in cow and pig manure-amended soil root endophytes (Figure 8). The other 50% of the genes detected in the cow variant were fluoroquinolones, and the detected ARG was *aac(6 _)-Ib-cr* (Table *10*A). In the pig variant, 25% of the detected phenotypes were fluoroquinolones and β-lactams. The ARPs: MLS, SUL, Van, and I were not detected in any of the variants (Table *10*A).

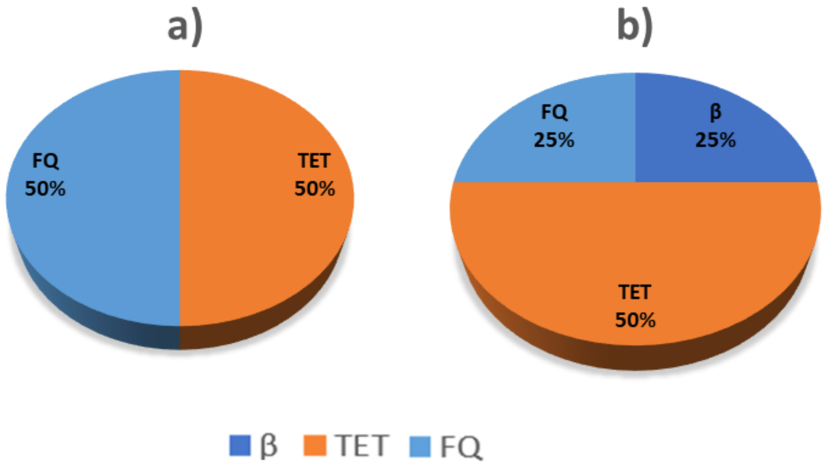


Figure 8: Total percentage of gene determinants detected in root endophytes of cow manure amended soil (a), and pig manure amended soil(b), at the end of the experiment period on day 45. β-β lactam resistance determinants, TET- Tetracycline resistance determinants, FQ- Fluoroquinolones resistance determinants

4.4.3. Leaf Endophytic bacteria

The percentage of ARP detected in control and turkey manure amended soil variant was similar: 20% of fluoroquinolones, 20% of β-lactams and 60% of tetracycline phenotype (Figure 9). The percentage of tetracycline ARP detected in leaves of cow and pig variants was 57%, and was lower than the percentage detected in the control variant. The tetracycline ARG: *tet(*B) and *tet(*D) were detected in leaves of all four variants (Table *10*A). *tet(*A) was detected in leaves of manure-amended soil variants but it was not detected in the control soil variant. *tet(*C) was not detected in the leaves of the turkey variant but was detected in the other three variants. *tet(*E) and *tet(*M) ARGs were not detected in any of the leaves' endophytic samples.

The ARG *aac(6 _)-Ib-cr* belonging to FQ phenotypes was found in all the leaf variants. *bla*TEM was the most frequently detected of the four β-lactams ARGs in all the variants, and *bla*SHV, *and bla*CTX-M were not detected in any of the leaf variants (Table *10*A). The phenotypes MLS, SUL, Van, and I resistance determinants were not detected in any of the leaf variants at the end of the experiment (Figure 9).

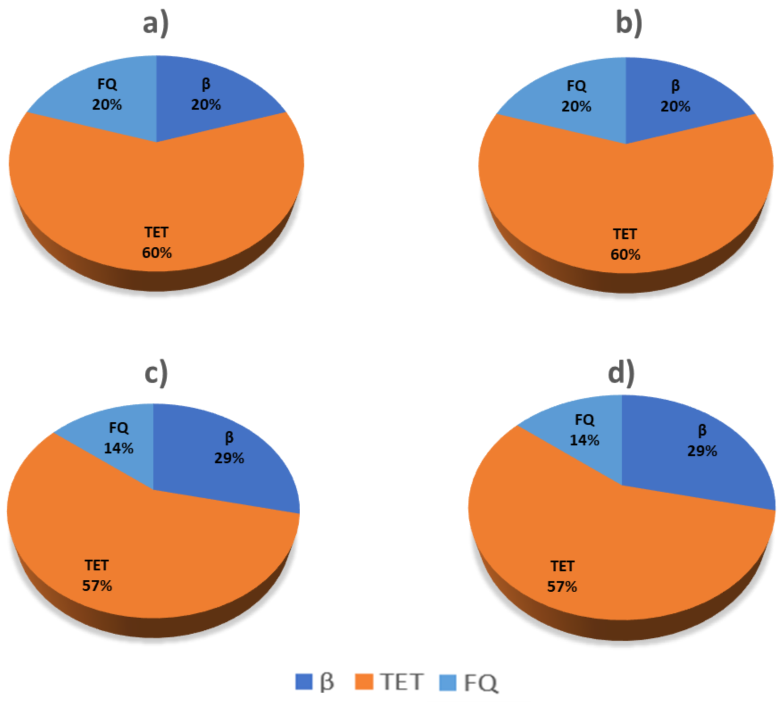


Figure 9: Total percentage of gene determinants detected in leaf endophytes soil control (a), turkey manure amended soil (b), cow manure amended soil (c), and pig manure amended soil (d) at the end of the experiment period on day 45. β-β lactam resistance determinants, TET- Tetracycline resistance determinants, , FQ- Fluoroquinolones resistance determinants

4.5 Changes in functional microbial diversity in soil

4.5.1. Community-level physiological profiling (CLPP)

The functional microbial diversity in soil was measured at 24 h intervals for 7 days (Figure *10*). The most profound change in the metabolism of carbon substrates was observed after 72 h of incubation (Figure 10). Among the four variants, turkey manure-amended soil shows higher AWCD at 72 h, and the lowest AWDC is observed in pig manure-amended soil. Over the incubation period of 168 h, the control and turkey variants showed higher activity compared to cow and pig variants.

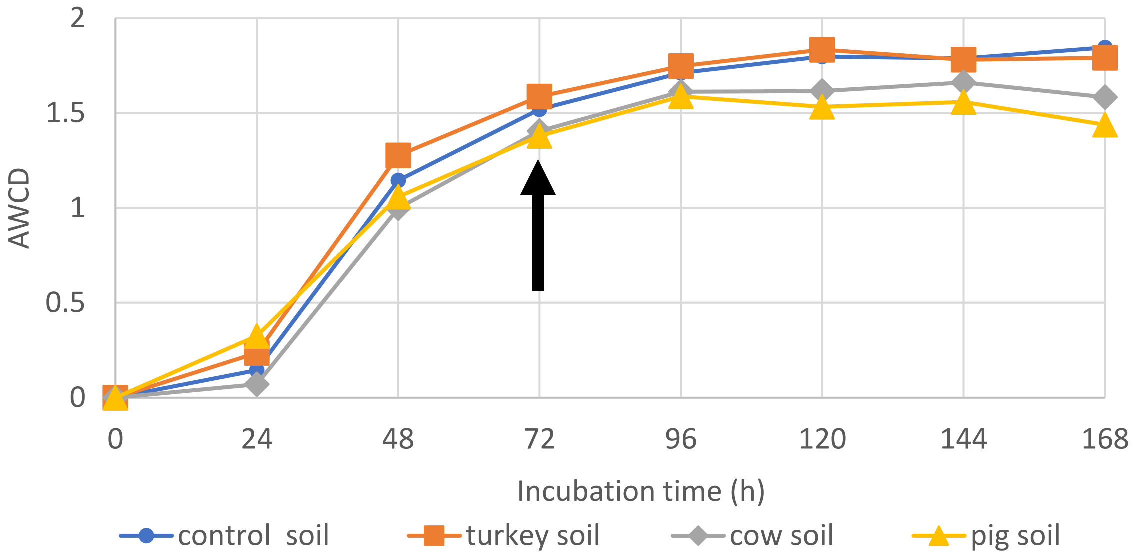


Figure 10: Functional microbial diversity in soil measured by the AWCD at 24 h intervals for 7 days of incubation. The black arrow represents the sampling time 72 h chosen for further analysis.

The functional metabolic diversity of samples was calculated at 72 h for each carbon source category (Figure 11 and Figure 12). The total AWCD of the 31 carbon substrates utilization by the variants after 72 h of incubation is given in Figure 11**Error! Reference source not found.**. T he AWCD measurements showed the highest total AWCD values in the following order: TS>CS>CwS>PS corresponding to 1.59, 1.52, 1.40, 1.38 for turkey manure-amended soil, control soil, cow manure-amended soil, and pig manure-amended soil variant, respectively. In comparison to the control variant, only turkey manure-amended soil had greater total AWCD, and the other two variants showed lower total AWCD than control soil (Figure 11**Error! R eference source not found.**).

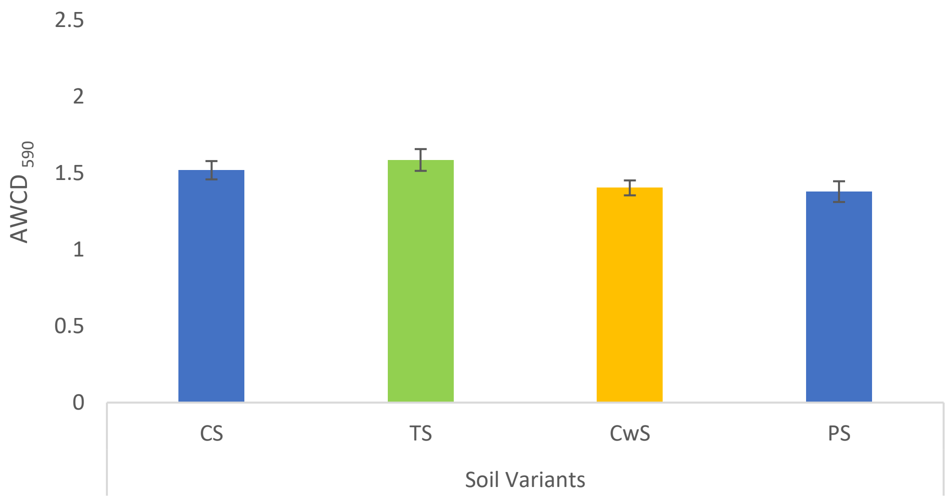


Figure 11: The total AWCD measure after 72h incubation in the Biolog® EcoPlate™ for the control soil CS; Turkey manure-amended soil TS; Cow manure-amended soil CwS; and Pig manure-amended soil PS.

The results obtained for the microbial activity on use of 6 carbon groups is depicted in Figure 12. Among these six carbon groups, the highest AWDC was observed for CH (1.83), while the lowest AWDC was noted for CCS group of substrates (1.22). The total average of carbon substrate utilization was in the order of CH>A>CA>PC>AA>CCS ranging from the highest to lowest utilization rate. For amines (A), the most substantial activity was recorded in the turkey manure-amended soil variant (TS= 2.16), whereas the lowest utilization was in pig manure-amended soil (PS= 1.26). In comparison to the control soil variant (CS=1.40), the utilization of A increased in the turkey variant (2.16) and decreased in both the cow (1.27) and pig variants (1.26) (Figure 12 a). The total average utilization of A across all the variants was 1.52, and was the second most utilized carbon substrate. In the case of AA, the highest utilization was observed in the TS (1.50), followed by the CwS (1.27), the PS (1.26), and finally the CS variant (1.07) (Figure 12 b). The CS variant had the lowest AA utilization among the four variants.

As for carboxylic acids (CA), these were the third most utilized carbon source within the four variants. Notably, the control variant (1.70) showed the highest metabolism of CA followed by the TS (1.35)> CwS (1.33) > PS (1.32) (Figure 12c).

The least metabolized carbon source was complex carbon source (CCS), with a total average of 1.22 for all the 4 variants. Within the four variants, the utilization of CCS was observed in the sequence as follows: PS<CwS<CS<TS with the highest utilization in TS variant (Figure 12 d).

Conversely, carbohydrates were the highest metabolized carbon source among the 6 carbon substrates, and the highest metabolism was observed ins TS variant (2.03) and the lowest in CS (1.70) variant (Figure 12 e).

In terms of phosphate carbon (PC) utilization, the CS variant showed the highest value(1.56), whereas PS variant showed the lowest value (1.21) (Figure 12 f). Furthermore, in the control soil variant, the most utilized carbon sources were CA and CH, and the least utilized was AA. In contrast, the turkey variant showed the highest metabolism of A and the lowest was recorded for the CSS source. Similarly, within the cow and pig variants, the highest metabolized carbon source was CH whereas, CCS was the lowest utilized carbon source (Figure 12).

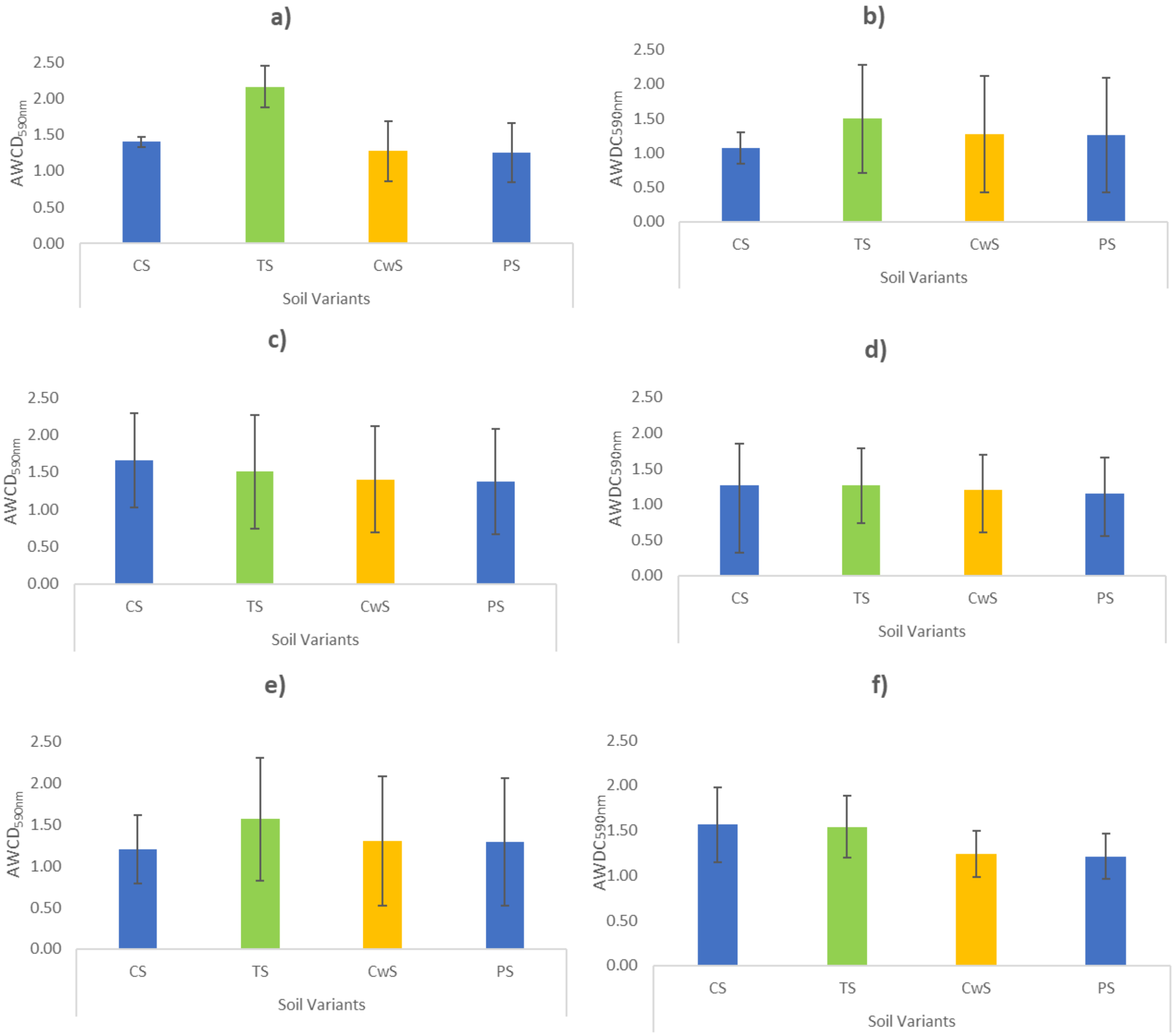


Figure 12:The AWCD for the six groups of carbon substrates after 72 h in the EcoPlate™ assay: amines (a); amino acids (b); carboxylic acids (c); complex carbon sources (d); carbohydrates (e); phosphate carbon (f).

The heatmap demonstrating the utilization of the 31 different carbon sources at 72h of incubation in the control soil, turkey manure amended soil, cow manure amended soil, and pig manure amended soil is given

Figure *13*. The turkey manure-amended soil variant showed notably higher utilization of phenylethyl amine from the A group of substrates compared to the control soil, cow manure-amended soil, and pig manure-amended soil (

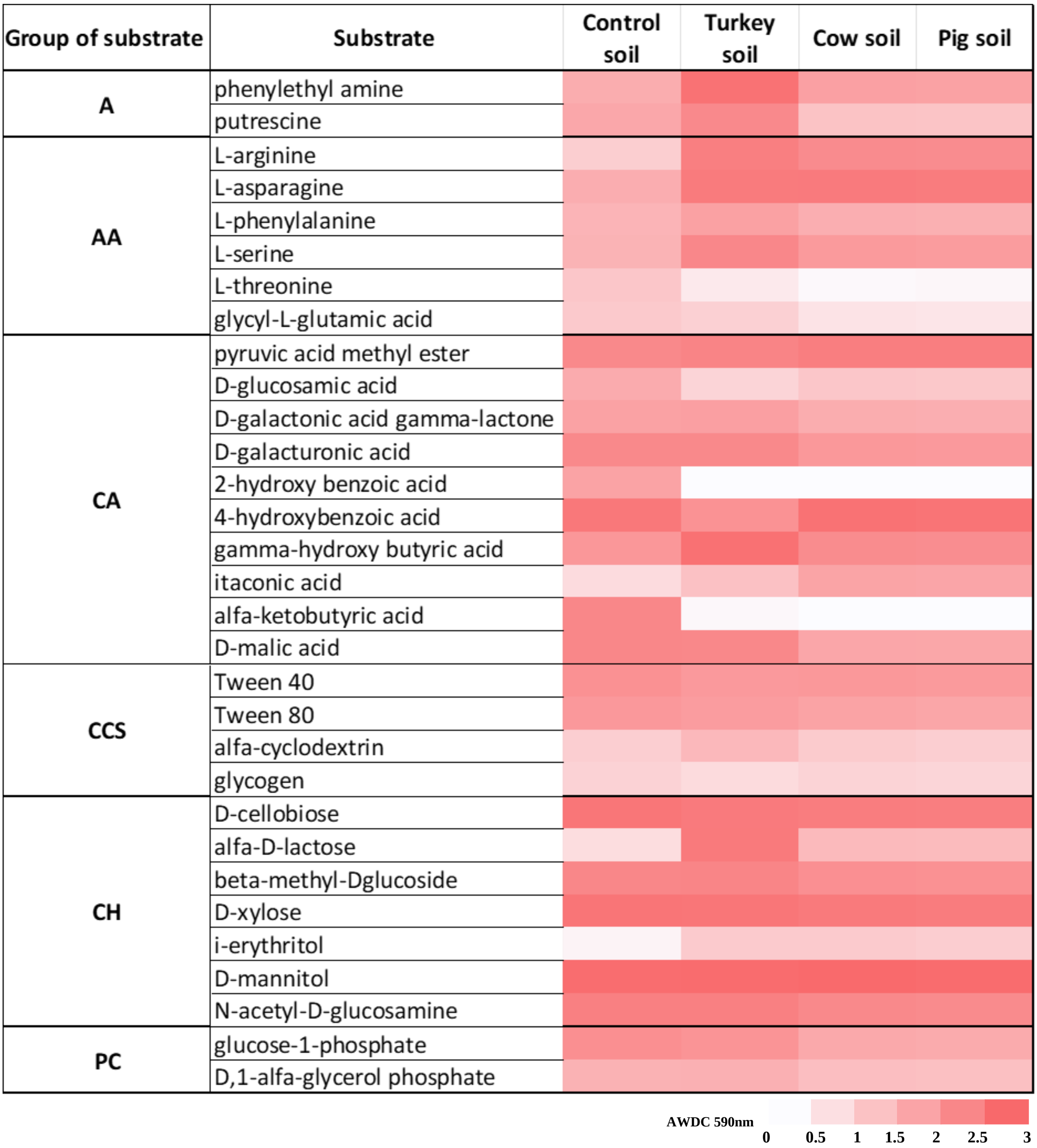
Figure *13*). Among the AA group of substrates, L-asparagine showed minimal utilization in the control soil (AWCD = 1.35). However, in turkey (AWCD = 2.20), cow (AWCD = 2.21), and pig (AWCD = 2.19) manure-amended soils, the microbiota showed relatively comparable higher rates of utilization of L-asparagine. Conversely, L-threonine and glycyl-L-glutamic acid displayed the lowest levels of carbon substrate use within the AA group (AWCD < 0.5) (Figure 13).

In the CA group of substrates, 2-hydroxy benzoic acid and alfa-ketobutyric acid were metabolized only in the control soil (AWCD =1.53; AWCD =2.00), while the microbiota in the three manure-amended variants did not utilize them (AWCD = 0). A higher microbial activity was detected in turkey manure-amended soil for gamma-hydroxybutyric acid than the other three soil groups. Conversely, the utilization of 4-hydroxybenzoic acid was lower in turkey manure-amended soil (AWCD =1.81) in comparison to the other three soil substrates.

The lower metabolism (AWCD < 1.5) of alfa-cyclodextrin and glycogen within the CCS substrate group contrasts with the higher metabolism observed for the other two substrates: Tween 40 and Tween 80. Meanwhile, among all six substrate groups, CH stands out as the most extensively utilized by the soil microbial community: notably, the carbohydrate substrate D-mannitol demonstrates the highest metabolic activity (AWCD > 2) across all four variants. In contrast, i-erythritol exhibits the least utilization among carbohydrate substrates (AWCD < 0.5).

The utilization of PC did not have specific variation across the variants. Both the control soil and turkey manure-amended soil (AWCD = 1.86 and AWCD = 1.78) demonstrated elevated utilization of glucose-1-phosphate when compared to the cow and pig manure-amended variants (AWCD = 1.42 and AWCD = 1.39) (

Figure *13*).



Group of substrate	Substrate	Control soil	Turkey soil	Cow soil	Pig soil
A	phenylethyl amine				
	putrescine				
AA	L-arginine				
	L-asparagine				
	L-phenylalanine				
	L-serine				
	L-threonine				
	glycyl-L-glutamic acid				
CA	pyruvic acid methyl ester				
	D-glucosamic acid				
	D-galactonic acid gamma-lactone				
	D-galacturonic acid				
	2-hydroxy benzoic acid				
	4-hydroxybenzoic acid				
	gamma-hydroxy butyric acid				
	itaconic acid				
	alfa-ketobutyric acid				
	D-malic acid				
CCS	Tween 40				
	Tween 80				
	alfa-cyclodextrin				
	glycogen				
CH	D-cellobiose				
	alfa-D-lactose				
	beta-methyl-Dglucoside				
	D-xylose				
	i-erythritol				
	D-mannitol				
	N-acetyl-D-glucosamine				
PC	glucose-1-phosphate				
	D,1-alfa-glycerol phosphate				

Figure 13: Biolog® EcoPlate™ substrate utilization after 72 h of incubation; substrate groups: A- amines, AA- amino acids, CA- carboxylic acids, CCS- complex carbon sources, CH- carbohydrates, PC- phosphate carbon.

4.5.2. Microbial catabolic diversity

The Shannon Weaver Index (H’) of the turkey variant (3.39) was observed to be the highest among the four variants. The H’ of pig manure-amended soil was the lowest (3.04). The H’ index of the control variant (3.23) was lower compared to the turkey variant but greater than for the cow and pig variants (Table 7).

The Shannon Evenness index (E) ranged from 0.91 to 1.00 for all the four variants. The E values were similar for cow and pig variant (0.91) and the highest E index was observed in the turkey variant (1.00). The control variant (0.96) had lower E value than turkey variant (Table 7).

The Substrate Richness index (S) values were higher for the manure-amended variants compared to the control variant (26.33). The highest value was observed in turkey manure-amended soil (28.33) and the S values for cow and pig manure-amended soil were similar (28.00) (Table 7).

Table 7:Microbial catabolic diversity indices calculated for the data obtained from the EcoPlate™. H′—Shannon-Weaver diversity index, E—Shannon evenness index, S—substrate richness index; AV-average value, SD-standard deviation

Variant	**H’ (AV ± SD)**	**E (AV ± SD)**	**S (AV ± SD)**
Control soil	3.23± 0.02	0.96±0.01	26.33±0.58
Turkey soil	3.39± 0.07	1.00±0.02	28.33±0.58
Cow soil	3.08±0.08	0.91±0.02	28.00±0
Pig soil	3.04±0.08	0.91±0.02	28.00±0

4.6 Changes in enzymatic activity in soil

4.6.2. Dehydrogenase activity of soil

The change in enzymatic activity of soil was measured by the dehydrogenase activity of soil. The results of dehydrogenase activity showed that the control variant had a higher DHA in the soil compared to the manure amended variants. The values of DHA varied from 0.96 in pig manure-amended soil to 6.10 in the control soil (Figure 14).

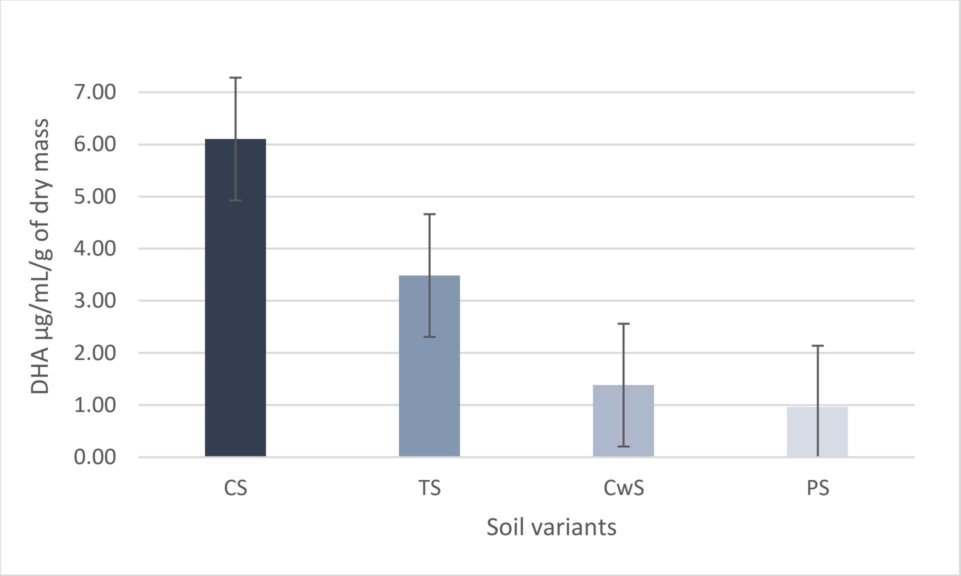


Figure 14: Change in enzymatic activity in soil measured using dehydrogenase activity (DHA) of soil

5. Discussion

Ecohydrology is an interdisciplinary field that explores the intricate relationship between ecosystems and water resources (Zalewski, 2002) . It seeks to understand how the availability and distribution of water affect ecosystems and, conversely, how ecosystems influence the movement and quality of water. One fundamental concept in ecohydrology is the need for sustainable soil management practices (United Nation, 2022; Zalewski, 2002). This concept along with SDG goals 2, 3, 6, and 13 which are interconnected to provide recognizes that healthy soils serve as a natural defense against water-related challenges(UNESCO & UN-Water, 2019; United Nation, 2022). Furthermore, preserving soil health and sustainability and feeding a growing population has become a global challenge in the agricultural field. Holistic approaches to agricultural practices offer the use of organic additives. However, the unintended consequences of organic fertilizers application, such as the spread of ARGs through animal fertilizers(Xie et al., 2018; Zalewska et al., 2021) should be taken into concern. The One Health Action Plan for global health proposed by the World Health Organization (WHO), Food and Agriculture Organization (FAO), World Organization for Animal Health (OIE), United Nations System Influenza Coordination, and the World Bank was adopted by the European Commission in 2017. The plan initiated to address the existing knowledge gap concerning the spread of antimicrobial resistance through ARB/ARGs. Studies of Walia et al., (2019) and Zalewska et al., (2021), highlight the urgent need for strategies to address this critical issue of spread of ARB and ARGs (Zalewska et al., 2021). The present study focuses on filling the research gap on the animal manure (turkeys, cows, and pigs manure) on occurrence and prevalence of ARGs in fertilized soil, rhizosphere and plant endophytic bacteria.

5.1. The presence of bacterial DNA confirmed by 16SrRNA gene.

Standard PCR analysis confirmed the presence of the bacterial gene *16S rRNA* (444 bp) across diverse samples: manure, manure-amended soil, rhizosphere, and root and leaf endophytes.. This broad distribution aligns with previous studies (Hardoim et al., (2015); Janda & Abbott, (2007)) emphasizing the significance of *16S rRNA* detection in understanding microbial communities in various ecosystems.

5.2. **Occurrence of ARGs in manure**

The optimization of PCR conditions is pivotal for reliable detection of targeted ARGs (Gumpert et al., 2017a; Zhu et al., 2013a). The present study involved annealing temperature gradients, varied DNA concentrations, and primer concentration to achieve optimal PCR amplification conditions. Bustin, (2004); and Holland et al., (1991) have shown the importance of PCR optimization, especially when using environmental soil samples, as they may contain different microorganisms, various PCR inhibitors and complex DNA compositions.

The worldwide studies have shown that the direct application of animal manure (as fresh manure or manure-based composts) can introduce substantial amounts of antibiotics and ARB to the environment, and in this way facilitate the spread of antibiotic resistance (Tasho & Cho, 2016; Xie et al., 2018; Zalewska et al., 2021; Zhu et al., 2013a). As mentioned in the Introduction section, the continuous introduction of antibiotics administered to animals for disease prevention and growth promotion, can exert greater pressure on ARGs dissemination in animal manure and soil (Tasho & Cho, 2016; Xie et al., 2018; Zhang et al., 2019). In the present study, the manure of turkeys, cows, and pigs was collected from large to small-scale family farms. The intriguing results of PCRs showed there were no ARGs detected in the turkey manure sample (from large farm), which could have resulted in long-term storage of manure (over a year) and the degradation and transformation of ARB/ARGs (Marti et al., 2013b). The presence of decrease number of ARGs in turkey manure can also be a reason why these determinants were not detected (Xie et al., 2018). Nonetheless, studies conducted in Portugal by Amador et al., (2019), in Greece by Tzavaras et al., (2012), in the US by Graham et al., (2009), and South Korea by Lee, (2006) have shown that ARGs belonging to following ARPs: FQ (*qnrB, qnrC, qnrD, qnrS*), TET(*tet(M), tet(A), tet(B), tet(C), tet (E), tet(K), tet(L), tet(O)*), SUL (*sul1, sul2, sul3*), Van (*vanA*), MLS (*ermB, ermA, mrsA/B*), and MRSA (methilicillin-resistant *S. aureus*) were detected in poultry manure (Zalewska et al., 2021).

Conversely to the results of turkey manure, cows and pigs manure samples showed the presence of ARGs belonging to the following ARPs: TET, SUL, β-lactams, MLS, and FQ. The investigations from the studies of Karami et al. (2007), Brichta-Harhay et al. (2011), and Wichmann et al. (2014) as cited in (Zalewska et al., (2021), show that antibiotics usage in dairy cattle can promote the spread of ARGs among various bacterial populations. This includes potentially harmful species that could pose risks to human health upon acquiring ARGs. Notably, even non-pathogenic bacteria from manure can facilitate the transfer of ARGs to pathogens in the environment and food chain (Zalewska et al., 2021). The ARGs: *tet(M), tet(B), ermF, ermA, linA, blaTEM, blaOXA, blaCTX_M, sul1,* and *aac(6)-Ib-cr* were detected in the present study. These ARGs were also detected in studies conducted in the US, Canada, China, and in France (Zalewska et al., 2021). However, the ARGs: *tet(A), tet(C),blaSHV, blaOXA, qnrB, and vanA, vanB,* and *vanC1* that were tested in the present study were not detected but these genes were recorded in the studies conducted in the above-mentioned countries (Zalewska et al., 2021). Vancomycin and integrase genes were not detected in the cow manure used for the present study. The utilization of antibiotics in cattle farming is primarily centered around tetracyclines, chosen to treat various health issues encompassing skin, respiratory, and gastrointestinal disorders (Zalewska et al., 2021). The high risk of resistance to tetracycline in cow farm was also confirmed in our study, with tetracyclines being the most detected ARP in cow manure. The emergence of tetracycline resistance could also be linked to their association with MGEs. The European cattle industry also frequently employs macrolides and lincosamides to combat common infections. This leads to prevalence of macrolides resistance genes in fecal microbial communities of treated animals. Additionally, β-lactams are extensively administered in dairy settings, particularly for managing mastitis cases (Zalewska et al., 2021). In our study β-lactams phenotype accounted to 25% of the total detected ARPs.

The ARGs detected in pig manure of the current study belonged to 6 ARPs and the obtained results comply with studies conducted in the US, China, and several European countries such as Germany, Belgium, Bulgaria, Denmark, Spain, France, Netherlands, Italy and Poland (Zalewska et al., 2021). Nevertheless, *blaCTX-M* which was detected in samples of pig manure in studies of above-mentioned European countries was not detected in the current study. On the other hand, in our study the presence of two glycopeptide resistance genes (*vanB*, *vanC1*) in the pig manure was detected, which was not mentioned in any of the studies considered in above mentioned European countries (Zalewska et al., 2021). However, in the study by Zhu et al., (2013a), the vancomycin resistance genes in pig manure was detected. The studies from European countries have shown that ARGs detection reflects the antimicrobial usage policy of each country: in countries with strict policies the tendency to locate ARGs was lower (Zalewska et al., 2021).

5.3. **Occurrence of ARGs in manure-fertilized soil**

Worldwide research highlighted that organic manure application impacts the soil resistome, either by introducing new ARGs or amplifying existing ones (Su et al. (2014), Udikovic et al. (2014;), Chen et al. (2016b),; and Yu et al. (2014); Xie et al., (2018); Zalewska et al., (2021). The current in-depth study, spanning both control and manure-amended soils, showed the dynamics of ARGs occurrence across a 45-day period in manure-fertilized soil. The studied ARGs demonstrate diverse trends among variants, highlighting the complex dissemination of antibiotic resistance within agricultural settings.

In the control soil, the exclusive detection of the MLS phenotype, represented by the *ermF* gene, suggests a subdued presence of resistance genes in this environment. However, manure-amended soils reveal a more complex scenario, displaying a diversified pattern of ARPs. Turkey manure-amended soil showed the presence of MLS, FQ, β, and TET ARPs even though they were not detected in the turkey manure. Possible reasons could be the complex soil bacterial matrix enhanced the thriving of any ARGs available in the manure (Marti et al., 2013b). According to Cycoń et al., (2019); Hu et al., (2010); and Martínez-Carballo et al., (2007) the microbial diversity of soil can enhance both the abundance and diversity of antibiotic resistance determinants. The highest prevalence of ARGs and ARPs were detected in pig manure-amended soil compared to the other two variants, which complies with the study conducted by Zhang et al., (2019). However, the percentages of the ARPs detected in pig manure vary in their study. Since the behavior of antibiotics and ARGs depends also on the soil properties, hence the comparison of the obtained data with different studies can be challenging. Possible reasons for detecting higher ARGs in one variant than the others could be hypothesized as the manure may have increased the soil organic matter content enhancing the growth of resistant bacteria, and the ARGs present in the manure bacteria were transferred to new hosts through horizontal Gene Transfer (HGT), or the active antibiotic compounds in manure enhanced new mutations in soil bacteria (Xie et al., 2018; Zalewska et al., 2021). Zhao et al., (2017) cited by Xie et al. (2018) showed *tet(M)* and *tet(A)* were detected in poultry manure; these genes were also detected in the turkey manure-amended soil in the present study.

A study from Finland involving cattle and swine manure showed the presence of *sul1* and *intl1* (Marti et al., 2014a); notably, while *sul1* and *intl1* were absent in the cow manure-amended soil in our study, *sul1* was detected in pig manure amended soil. Other frequently detected ARPs includes: β-lactam, aminoglycoside, macrolide-lincoside-streptogramine B (MLS), and tetracycline (Zhang et al., 2019). These ARPs were also detected in our study in pig manure-amended soil; however β-lactam and MLS genes were not detected in cow manure amended soil. The presence of *blaTEM* in turkey manure-amended soil and pig manure-amended soil among other tested β-lactam resistance genes also aligns with the results of Gumpert et al. (2017b) and Koniuszewska et al. (2020).

5.4. Occurrence of ARGs in plant-associated bacteria under manure fertilization

The findings of the current research on ARGs in the rhizospheric soil showed a similar pattern of the ARGs detected in the turkey manure-amended soil and pig manure-amended soil, and the contradictory results in the cow manure-amended soil. The results of this study show also that the rhizosphere is a hotspot for the spread of ARGs into plant microbiome due to the exudation of nutrients and root growth in the rhizosphere. This phenomenon is also demonstrated in the work by Zhang et al., (2019). The rhizospheric ARGs in the cow variant showed the presence of only MLS genes, while TET and FQ genes were detected in the other two variants. One possible explanation is the relatively brief transient period, which might have been insufficient for the gradual transfer of ARGs from manure to microbial communities in the cow-variant rhizosphere (Xie et al., 2018). Another plausible reason could be the competitive advantage of indigenous bacterial populations, which might have outcompeted the host bacteria carrying the ARGs from manure (Marti et al., 2014a). Notably, no ARGs were detected in the control variant however in the study by Zhang et al. (2020), the ARGs have been detected in the rhizospheric control soil.

The investigation of root endophytic bacteria among the variants showed that ARGs were not detected in the control and turkey variants. The importance of not detecting any ARGs in the turkey variant shows that though ARGs were found in the rhizospheric soil it has not been transferred to the root endophytes. The findings align with the research conducted by Zhang et al. (2020) on poultry manure. The authors observed an increase in the abundance of ARGs in the rhizosphere of plants after the application of poultry manure. However, they did not detect a significant increase in ARGs within the endophytes of plant roots (Zalewska et al., 2021). Nonetheless, the results (Zhang et al., 2020) do not comply with the results of our research wherein ARGs belonging to TET and FQ phenotypes were detected in the root endophytes of the cow variant.

Another study on ARGs in root endophytes of manure-amended soil by Wang et al. (2015) showed that they have detected TET, SUL, and int1 in lettuce and endive (Zalewska et al., 2021), and the study by Cerqueira et al. (2019b) showed presence of *blaTEM* in roots and leaves endophytes in tomato, lettuce, broad beans (Zalewska et al., 2021). A key finding of the present study shows that ARGs are present in the root endophytes of cherry radish plants, with TET and FQs phenotypes found prominently in cow and pig manure-amended soil. This finding can be related to the transfer of ARGs from the manure to the plant endophytes. A considerable amount of conducted research have detected several ARGs in leafy vegetables and the phyllosphere of lettuce, maize, and tomato (Zalewska et al., 2021). Furthermore, in vegetables that are eaten raw, such as tomatoes, cucumbers, peppers, carrots, and lettuce, the ARGs such as *tetB, ermF, ermA, qnrB, blaOXA* were detected on at least one vegetable sample grown in dairy and swine manure-amended soil (Marti et al., 2014a; Zalewska et al., 2021). An important finding in the current study is that ARGs were detected in the leaf endophytes of all the variants including the control variant. Tetracycline was the most ubiquitously found ARP in all the variants constituting more than 50% of the detected ARGs in all variants. *blaTEM* was detected in all leaf endophytes, *blaOXA* was detected in cow and pig variants, which complies with results found in (Marti et al., (2014a). In other studies, the prevalence of ARGs in leaf endophytes was higher compared to root endophytes (Marti et al., 2014a; Zhang et al., 2020). Likewise, the results of the present study showed that ARGs in leaf endophytes from all variants depicted higher detection frequency than for root endophytes. The possible explanation for detecting ARGs in the control variant and leaves of all manure-amended variants could be the potential ARGs transfer through the air (Bulgarelli et al., 2012; Zalewska et al., 2021; Zhang et al., 2020).

The findings of the present study clearly show that there is a possibility of ARGs transfer from contaminated manure to edible parts of cherry radish. Concerns should be given to this matter as there can be a potential threat to human beings exposed ARGs through consumption of raw vegetables (Hu et al., 2010; Liu et al., 2021; Marti et al., 2014b; Xie et al., 2018; Zalewska et al., 2021;Zhang et al., 2020; Zhu et al., 2013b).

5.5. Changes in functional microbial diversity and enzymatic in soil

The soil ecosystem is a complex network of microbial species that play a crucial role in nutrient cycling, organic matter degradation, and energy flow (Gomez et al., 2006). In the current study, the functional metabolic diversity showed that turkey manure-amended soil had the highest microbial activity whereas pig manure-amended soil exhibited the lowest value of AWCD. These results can also be supported by the microbial catabolic diversity (α diversity), in which the H’, E, and S of turkey manure-amended soil are higher than the other two manure-amended variants in the current study. (Sylwia Siebielecet al., (2023) showed in their research that cow manure-amended soil had a higher H’ than pig manure-amended soil but the E index was highest in poultry manure-amended soil. Furthermore, in study by (Sylwia Siebielec et al. , (2023) the highest AWDC has been observed in pig and cow manure amended soil which is contrary to the results of current study. It may be due to different factors such as the difference in manure content and observing the highest AWCD at a different time. The study conducted by (Kuppusamy et al., 2018) highlights the impact of manure amendments on agricultural soil. It emphasizes that while manure can enhance soil fertility through the introduction of beneficial microorganisms such as bacteria, it also has the potential to introduce ARGs into the soil microbial community changing the overall soil microbial community (Kuppusamy et al., 2018). The findings of the current research are further supported by the study (Zhang et al., (2019), in which the authors show that cattle and pig manure have a positive effect on the ARGs profile and a negative effect on the bacterial diversity. (Xie et al., (2018) highlighted that prolonged incubation of soil with manure with smaller concentrations of antibiotics can lead to enriching the antibiotic-resistant strains and exert selective pressure on bacteria under both laboratory and natural conditions. These may a reason for the lower AWCD and lower α diversity in pig and cow variants compared to the control variant in the current study.

In the current study, it was observed that the CH were the most metabolized in all four variants, with the highest activity observed in the turkey manure-amended soil. In contrast, CCS were the least metabolized carbon sources in all variants, with the highest utilization observed for the turkey variant. Furthermore, except for the utilization of CA and PC, all the other carbon source utilization was higher in turkey manure-amended soil compared to the control soil. On the other hand, except for AA and CH, the utilization of carbon sources in cow and pig manure-amended soil is lower compared to the control soil. The study conducted by Gamliel et al., (2000), with chicken manure showed that raw manure application could adopt a higher microbial community than in unmanured soil, which could be a possible explanation for the observations of the current study with turkey manure amended soil (Subirats et al., 2021).

The preference for simple carbon sources, such as AA and CH, over complex sugars in cow manure-amended soil is consistent with the findings of (Sradnick et al., (2013). Sradnick et al. (2013) suggest that higher levels of neutral sugars can lead to increased microbial activity, particularly among highly active microorganisms like r-strategy bacteria. Additionally, the presence of biotrophic arbuscular mycorrhizal fungi, observed in fields with cattle manure application, can influence how the soil microbial community responds metabolically (Sradnick et al., 2013). Another factor that could be deduced from their findings is that as soil pH increases, there's more microbial biomass and organic matter (Sradnick et al., 2013).

In the context of the study by P(Pengthamkeerati et al., (2011), they show that the carbon substrate utilization changed in pig manure-amended soil than in unamended soil. The DHA utilization in pig manure-amended soil from the current is lower than the other two manure-amended variants and the control variant. Additionally, the microbial catabolic diversity is lower compared to the other variants. It is worth considering that according to Degens et al., (2001), the humped-back model explanation for this scenario, suggests that soil microbes thrive the most when there's a moderate level of disturbance. However, if the disturbance becomes too extreme, it can reduce microbial growth and activity, making only a few microbes dominate, lowering overall diversity (Pengthamkeerati et al., 2011). Since, ARGs were most detected in the pig manure-amended soil in the current study we can hypothesize there is a relation between ARGs occurrence to the microbial activity of the soil.

Soil organic carbon content is a major determinant of soil microbial dynamics, thus variations in soil organic matter can cause variations in the soil microbial structure and functioning(Cycoń et al., 2019; Pengthamkeerati et al., 2011). In the aforementioned studies, the pig manure-amended soil increased the soil microbial activity and microbial growth, which contradicts to the results of the current study. One possible explanation could be, that there were other factors that caused stress to the microorganisms, and possibly the presence of ARGs could have an effect (Martínez-Carballo et al., 2007; Pengthamkeerati et al., 2011; Zalewska et al., 2021).The comparison of results from the present study with other studies is difficult due to the different soil/experiment conditions and scarce resource availability. However, the results obtained in this study comply with other related studies.

6. Conclusions

The obtained findings and the information gathered from numerous studies provide a valuable insight into a complex dynamic of soil and plant associated microbial communities, their responses to organic amendments like manure, and the impact of these changes on soil health, ARGs dissemination, and carbon substrate metabolism. Obtained results suggest an urgent need to manage the use of antibiotics, especially in unmonitored small family farms, and enforce strict legislation on the use of veterinary antibiotics. Understanding other environmental factors that could influence the dissemination of ARB and ARGs in manure amended soil and food products is crucial in understanding the paths of ARGs transmission to living organisms. It is crucial to recognize that the quality and quantity of water resources are intricately tied to the overall health of the soil. It is evident that soil microbiology is a multifaceted field with significant implications for agriculture, environmental sustainability, and human health. Further research in this area is necessary to broaden our understanding of the spread of ARGs between different matrices and its impact on human health.

7. References

Bulgarelli, D., Rott, M., Schlaeppi, K., Ver Loren van Themaat, E., Ahmadinejad, N., Assenza, F., Rauf, P., Huettel, B., Reinhardt, R., Schmelzer, E., Peplies, J., Gloeckner, F. O., Amann, R., Eickhorst, T., & Schulze-Lefert, P. (2012). Revealing structure and assembly cues for Arabidopsis root-inhabiting bacterial microbiota. In *Nature* (Vol. 488, Issue 7409, pp. 91–95). https://doi.org/10.1038/nature11336

Bustin, S. A. (2004). *A-Z of Quantitative PCR*. www.fivephoton.com

Carvajal-Muñoz, J. S., & Carmona-Garcia, C. E. (2012). Benefits and limitations of biofertilization in agricultural practices. *Livestock Research for Rural Development* , 1–8. http://www.lrrd.org/lrrd24/3/carv24043.htm

Carvalho, I. T., & Santos, L. (2016). Antibiotics in the aquatic environments: A review of the European scenario. In *Environment International* (Vol. 94, pp. 736–757). Elsevier Ltd. https://doi.org/10.1016/j.envint.2016.06.025

CASIDA, L. E. J. R., KLEIN, D. A., & SANTORO, T. (1964). SOIL DEHYDROGENASE ACTIVITY. *Soil Science*, *98*(6).

CDC. (2019). *Antibiotic resistance threats in the United States, 2019*. https://doi.org/10.15620/cdc:82532

Cycoń, M., Mrozik, A., & Piotrowska-Seget, Z. (2019). Antibiotics in the soil environment—degradation and their impact on microbial activity and diversity. In *Frontiers in Microbiology* (Vol. 10, Issue MAR). Frontiers Media S.A. https://doi.org/10.3389/fmicb.2019.00338

Dróżdż, D., Wystalska, K., Malińska, K., Grosser, A., Grobelak, A., & Kacprzak, M. (2020). Management of poultry manure in Poland – Current state and future perspectives. *Journal of Environmental Management*, *264*. https://doi.org/10.1016/j.jenvman.2020.110327

Dutka-Malen, S., Evers, S., & Courvalin, P. (1995). Detection of Glycopeptide Resistance Genotypes and Identification to the Species Level of Clinically Relevant Enterococci by PCR. In *JOURNAL OF CLINICAL MICROBIOLOGY* (Vol. 33, Issue 1). https://journals.asm.org/journal/jcm

Eitel, Z., Sóki, J., Urbán, E., & Nagy, E. (2013). The prevalence of antibiotic resistance genes in Bacteroides fragilis group strains isolated in different European countries. *Anaerobe*, *21*, 43–49. https://doi.org/10.1016/j.anaerobe.2013.03.001

EU. (2001). polish legislation on use of fertilizers. *Journal of Laws*. https://faolex.fao.org/docs/pdf/pol60152.pdf

EU. (2009). OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL laying down health rules as regards animal by-products and derived products not intended for human consumption and repealing Regulation (EC) No 1774/2002 (Animal by-products Regulation). *Official Journal of the European Union*.

Ezugworie, F. N., Igbokwe, V. C., & Onwosi, C. O. (2021). Proliferation of antibiotic-resistant microorganisms and associated genes during composting: An overview of the potential impacts on public health, management and future. *Science of The Total Environment*, *784*, 147191. https://doi.org/10.1016/J.SCITOTENV.2021.147191

Ezzariai, A., Hafidi, M., Khadra, A., Aemig, Q., El Fels, L., Barret, M., Merlina, G., Patureau, D., & Pinelli, E. (2018). Human and veterinary antibiotics during composting of sludge or manure: Global perspectives on persistence, degradation, and resistance genes. In *Journal of Hazardous Materials* (Vol. 359, pp. 465–481). Elsevier B.V. https://doi.org/10.1016/j.jhazmat.2018.07.092

FAO. (2022). The State of Food Security and Nutrition in the World 2022. In *The State of Food Security and Nutrition in the World 2022*. FAO. https://doi.org/10.4060/cc0639en

FAO, & ITPS. (2015). *INTERGOVERNMENTAL TECHNICAL PANEL ON SOILS INTERGOVERNMENTAL TECHNICAL PANEL ON SOILS Status of the World's Soil Resources*. https://www.fao.org/3/i5199e/I5199E.pdf

Godoy, M., & Sánchez, J. (2020). Antibiotics as Emerging Pollutants in Water and Its Treatment. In *Antibiotic Materials in Healthcare* (pp. 221–230). Elsevier. https://doi.org/10.1016/B978-0-12-820054-4.00012-4

Goldstein, C., Lee, M. D., Sanchez, S., Hudson, C., Phillips, B., Register, B., Grady, M., Liebert, C., Summers, A. O., White, D. G., & Maurer, J. J. (2001). Incidence of class 1 and 2 integrases in clinical and commensal bacteria from livestock, companion animals, and exotics. *Antimicrobial Agents and Chemotherapy*, *45*(3), 723–726. https://doi.org/10.1128/AAC.45.3.723-726.2001

Gomez, E., Ferreras, L., & Toresani, S. (2006). Soil bacterial functional diversity as influenced by organic amendment application. *Bioresource Technology*, *97*(13), 1484–1489. https://doi.org/10.1016/j.biortech.2005.06.021

Gumpert, H., Kubicek-Sutherland, J. Z., Porse, A., Karami, N., Munck, C., Linkevicius, M., Adlerberth, I., Wold, A. E., Andersson, D. I., & Sommer, M. O. A. (2017a). Transfer and persistence of a multi-drug resistance plasmid in situ of the infant gut microbiota in the absence of antibiotic treatment. *Frontiers in Microbiology*, *8*(SEP). https://doi.org/10.3389/fmicb.2017.01852

Gumpert, H., Kubicek-Sutherland, J. Z., Porse, A., Karami, N., Munck, C., Linkevicius, M., Adlerberth, I., Wold, A. E., Andersson, D. I., & Sommer, M. O. A. (2017b). Transfer and persistence of a multi-drug resistance plasmid in situ of the infant gut microbiota in the absence of antibiotic treatment. *Frontiers in Microbiology*, *8*(SEP). https://doi.org/10.3389/fmicb.2017.01852

Hardoim, P. R., van Overbeek, L. S., Berg, G., Pirttilä, A. M., Compant, S., Campisano, A., Döring, M., & Sessitsch, A. (2015). The Hidden World within Plants: Ecological and Evolutionary Considerations for Defining Functioning of Microbial Endophytes. *Microbiology and Molecular Biology Reviews*, *79*(3), 293–320. https://doi.org/10.1128/mmbr.00050-14

Holland, P. M., Abramson, R. D., Watson, R., & Gelfand, D. H. (1991). Detection of specific polymerase chain reaction product by utilizing the 5'-* 3' exonuclease activity of Thermus aquaticus DNA polymerase (ofigonucleotide probe/human immunodeficiency virus). In *Proc. Natl. Acad. Sci. USA* (Vol. 88). https://www.pnas.org

Hu, X., Zhou, Q., & Luo, Y. (2010). Occurrence and source analysis of typical veterinary antibiotics in manure, soil, vegetables and groundwater from organic vegetable bases, northern China. *Environmental Pollution*, *158*(9), 2992–2998. https://doi.org/10.1016/j.envpol.2010.05.023

Janda, J. M., & Abbott, S. L. (2007). 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: Pluses, perils, and pitfalls. In *Journal of Clinical Microbiology* (Vol. 45, Issue 9, pp. 2761–2764). https://doi.org/10.1128/JCM.01228-07

Jia, J., Dai, H., Wei, S., Xue, J., Skuza, L., Sun, Q., & Li, R. (2023). Toxicity of emerging contaminant antibiotics in soil to Capsicum annuum L. growth and their effects on it accumulating copper. *Plant Physiology and Biochemistry*, *196*, 661–667. https://doi.org/10.1016/j.plaphy.2023.02.019

Jiang, X., Chen, Z., & Dharmasena, M. (2015). The role of animal manure in the contamination of fresh food. In *Advances in Microbial Food Safety* (Vol. 2, pp. 312–350). Elsevier Inc. https://doi.org/10.1533/9781782421153.3.312

Kim, J., Jeon, S., Rhie, H., Lee, B., Park, M., Lee, H., Lee, J., & Kim, S. (2009). Rapid Detection of Extended Spectrum β-Lactamase (ESBL) for Enterobacteriaceae by use of a Multiplex PCR-based Method. *Infection and Chemotherapy*, *41*(3), 181. https://doi.org/10.3947/ic.2009.41.3.181

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., & Glöckner, F. O. (2013). Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Research*, *41*(1). https://doi.org/10.1093/nar/gks808

Koniuszewska, I., Korzeniewska, E., Harnisz, M., Kiedrzyńska, E., Kiedrzyński, M., Czatzkowska, M., Jarosiewicz, P., & Zalewski, M. (2020). The occurrence of antibiotic-resistance genes in the Pilica River, Poland. *Ecohydrology and Hydrobiology*, *20*(1), 1–11. https://doi.org/10.1016/j.ecohyd.2019.09.002

Kuppusamy, S., Kakarla, D., Venkateswarlu, K., Megharaj, M., Yoon, Y. E., & Lee, Y. B. (2018). Veterinary antibiotics (VAs) contamination as a global agro-ecological issue: A critical view. In *Agriculture, Ecosystems and Environment* (Vol. 257, pp. 47–59). Elsevier B.V. https://doi.org/10.1016/j.agee.2018.01.026

Liu, B., Yu, K., Ahmed, I., Gin, K., Xi, B., Wei, Z., He, Y., & Zhang, B. (2021). Key factors driving the fate of antibiotic resistance genes and controlling strategies during aerobic composting of animal manure: A review. In *Science of the Total Environment* (Vol. 791). Elsevier B.V. https://doi.org/10.1016/j.scitotenv.2021.148372

Marti, R., Scott, A., Tien, Y. C., Murray, R., Sabourin, L., Zhang, Y., & Topp, E. (2013a). Impact of manure fertilization on the abundance of antibiotic-resistant bacteria and frequency of detection of antibiotic resistance genes in soil and on vegetables at harvest.

Applied and Environmental Microbiology, *79*(18), 5701–5709. https://doi.org/10.1128/AEM.01682-13

Marti, R., Scott, A., Tien, Y. C., Murray, R., Sabourin, L., Zhang, Y., & Topp, E. (2013b). Impact of manure fertilization on the abundance of antibiotic-resistant bacteria and frequency of detection of antibiotic resistance genes in soil and on vegetables at harvest. *Applied and Environmental Microbiology*, *79*(18), 5701–5709. https://doi.org/10.1128/AEM.01682-13

Marti, R., Tien, Y. C., Murray, R., Scott, A., Sabourin, L., & Topp, E. (2014a). Safely coupling livestock and crop production systems: How rapidly do antibiotic resistance genes dissipate in soil following a commercial application of swine or dairy manure? *Applied and Environmental Microbiology*, *80*(10), 3258–3265. https://doi.org/10.1128/AEM.00231-14

Marti, R., Tien, Y. C., Murray, R., Scott, A., Sabourin, L., & Topp, E. (2014b). Safely coupling livestock and crop production systems: How rapidly do antibiotic resistance genes dissipate in soil following a commercial application of swine or dairy manure? *Applied and Environmental Microbiology*, *80*(10), 3258–3265. https://doi.org/10.1128/AEM.00231-14

Martínez-Carballo, E., González-Barreiro, C., Scharf, S., & Gans, O. (2007). Environmental monitoring study of selected veterinary antibiotics in animal manure and soils in Austria. *Environmental Pollution*, *148*(2), 570–579. https://doi.org/10.1016/j.envpol.2006.11.035

Mergeay, M., Nies, D., Schlegel, H. G., Gerits, J., Charles, ’ P, & Van Gijsegem3, A. F. (1985). Alcaligenes eutrophus CH34 Is a Facultative Chemolithotroph with Plasmid-Bound Resistance to Heavy Metals. In *JOURNAL OF BACTERIOLOGY*. https://journals.asm.org/journal/jb

Mierzejewska, E., Tołoczko, W., & Urbaniak, M. (2023). Behind the plant-bacteria system: The role of zucchini and its secondary metabolite in shaping functional microbial diversity in MCPA-contaminated soil. *Science of the Total Environment*, *867*. https://doi.org/10.1016/j.scitotenv.2022.161312

Mierzejewska, E., Urbaniak, M., Zagibajło, K., Vangronsveld, J., & Thijs, S. (2022). The Effect of Syringic Acid and Phenoxy Herbicide 4-chloro-2-methylphenoxyacetic acid

(MCPA) on Soil, Rhizosphere, and Plant Endosphere Microbiome. *Frontiers in Plant Science*, *13*. https://doi.org/10.3389/fpls.2022.882228

Mullard, A. (2013). 2012 FDA drug approvals. In *Nature Reviews Drug Discovery* (Vol. 12, Issue 2, pp. 87–90). https://doi.org/10.1038/nrd3946

Nawaz, M., Sung, K., Khan, S. A., Khan, A. A., & Steele, R. (2006). Biochemical and molecular characterization of tetracycline-resistant Aeromonas veronii isolates from catfish. *Applied and Environmental Microbiology*, *72*(10), 6461–6466. https://doi.org/10.1128/AEM.00271-06

Park, C. H., Robicsek, A., Jacoby, G. A., Sahm, D., & Hooper, D. C. (2006). Prevalence in the United States of aac(6′)-Ib-cr encoding a ciprofloxacin-modifying enzyme. *Antimicrobial Agents and Chemotherapy*, *50*(11), 3953–3955. https://doi.org/10.1128/AAC.00915-06

Pei, R., Kim, S. C., Carlson, K. H., & Pruden, A. (2006). Effect of River Landscape on the sediment concentrations of antibiotics and corresponding antibiotic resistance genes (ARG). *Water Research*, *40*(12), 2427–2435. https://doi.org/10.1016/j.watres.2006.04.017

Pengthamkeerati, P., Motavalli, P. P., & Kremer, R. J. (2011). Soil microbial activity and functional diversity changed by compaction, poultry litter and cropping in a claypan soil. *Applied Soil Ecology*, *48*(1), 71–80. https://doi.org/10.1016/j.apsoil.2011.01.005

Plaszkó, T., Szűcs, Z., Cziáky, Z., Ács-Szabó, L., Csoma, H., Géczi, L., Vasas, G., & Gonda, S. (2022). Correlations Between the Metabolome and the Endophytic Fungal Metagenome Suggests Importance of Various Metabolite Classes in Community Assembly in Horseradish (Armoracia rusticana, Brassicaceae) Roots. *Frontiers in Plant Science*, *13*. https://doi.org/10.3389/fpls.2022.921008

Sradnick, A., Murugan, R., Oltmanns, M., Raupp, J., & Joergensen, R. G. (2013). Changes in functional diversity of the soil microbial community in a heterogeneous sandy soil after long-term fertilization with cattle manure and mineral fertilizer. *Applied Soil Ecology*, *63*, 23–28. https://doi.org/10.1016/j.apsoil.2012.09.011

Subirats, J., Murray, R., Yin, X., Zhang, T., & Topp, E. (2021). Impact of chicken litter pre-application treatment on the abundance, field persistence, and transfer of antibiotic resistant bacteria and antibiotic resistance genes to vegetables. *Science of the Total Environment*, *801*. https://doi.org/10.1016/j.scitotenv.2021.149718

Sylwia Siebielec, Anna Marzec-Grządziel, Grzegorz Siebielec, Aleksandra Ukalska-Jaruga, Monika Kozieł, Anna Gałązka, Marcin Przybyś, Piotr Sugier, & Magdalena Urbaniak. (2023). Microbial community response to various types of exogenous organic matter applied to soil. *International Journal of Molecular Sciences*.

Szczepanowski, R., Linke, B., Krahn, I., Gartemann, K. H., Gützkow, T., Eichler, W., Pühler, A., & Schlüter, A. (2009). Detection of 140 clinically relevant antibiotic-resistance genes in the plasmid metagenome of wastewater treatment plant bacteria showing reduced susceptibility to selected antibiotics. *Microbiology*, *155*(7), 2306–2319. https://doi.org/10.1099/mic.0.028233-0

Tasho, R. P., & Cho, J. Y. (2016). Veterinary antibiotics in animal waste, its distribution in soil and uptake by plants: A review. In *Science of the Total Environment* (Vols. 563–564, pp. 366–376). Elsevier B.V. https://doi.org/10.1016/j.scitotenv.2016.04.140

The European Medicines Agency. (2020). *Categorisation of antibiotics for use in animals*. The Federation of Veterinarians of Europe. https://bit.ly/2s7LUF2

UNESCO, & UN-Water. (2019). *Leaving no one behind : the United Nations World Water Development Report 2019*. UNESCO.

United Nation. (2022). *The Sustainable Development Goals Report*.

Urra, J., Alkorta, I., & Garbisu, C. (2019). Potential benefits and risks for soil health derived from the use of organic amendments in agriculture. In *Agronomy* (Vol. 9, Issue 9). MDPI AG. https://doi.org/10.3390/agronomy9090542

Walia, K., Sharma, M., Vijay, S., & Shome, B. R. (2019). Understanding policy dilemmas around antibiotic use in food animals & offering potential solutions. In *Indian Journal of Medical Research* (Vol. 149, Issue 2, pp. 107–118). Wolters Kluwer Medknow Publications. https://doi.org/10.4103/ijmr.IJMR_2_18

Xie, W. Y., Shen, Q., & Zhao, F. J. (2018). Antibiotics and antibiotic resistance from animal manures to soil: a review. In *European Journal of Soil Science* (Vol. 69, Issue 1, pp. 181–195). Blackwell Publishing Ltd. https://doi.org/10.1111/ejss.12494

Zalewska, M., Błażejewska, A., Czapko, A., & Popowska, M. (2021). Antibiotics and Antibiotic Resistance Genes in Animal Manure – Consequences of Its Application in Agriculture. In *Frontiers in Microbiology* (Vol. 12). Frontiers Media S.A. https://doi.org/10.3389/fmicb.2021.610656

Zalewski, M. M. (2002). *ECOHYDROLOGY ECOHYDROLOGY--Integrative tool Integrative tool for for achieving achieving good ecological good ecological status status of freshwater ecosystems of freshwater ecosystems*.

Zhang, Y. J., Hu, H. W., Chen, Q. L., Singh, B. K., Yan, H., Chen, D., & He, J. Z. (2019). Transfer of antibiotic resistance from manure-amended soils to vegetable microbiomes. *Environment International*, *130*. https://doi.org/10.1016/j.envint.2019.104912

Zhang, Y.-J., Hu, H.-W., Chen, Q.-L., Yan, H., Wang, J.-T., Chen, D., & He, J.-Z. (2020). Manure Application Did Not Enrich Antibiotic Resistance Genes in Root Endophytic Bacterial Microbiota of Cherry Radish Plants. *Microbial Ecology* . https://doi.org/10.1128/AEM

Zhu, Y. G., Johnson, T. A., Su, J. Q., Qiao, M., Guo, G. X., Stedtfeld, R. D., Hashsham, S. A., & Tiedje, J. M. (2013a). Diverse and abundant antibiotic resistance genes in Chinese swine farms. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(9), 3435–3440. https://doi.org/10.1073/pnas.1222743110

Zhu, Y. G., Johnson, T. A., Su, J. Q., Qiao, M., Guo, G. X., Stedtfeld, R. D., Hashsham, S. A., & Tiedje, J. M. (2013b). Diverse and abundant antibiotic resistance genes in Chinese swine farms. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(9), 3435–3440. https://doi.org/10.1073/pnas.1222743110

Zou, T., Zhang, X., & Davidson, E. A. (2022). Global trends of cropland phosphorus use and sustainability challenges. *Nature*, *611*(7934), 81–87. https://doi.org/10.1038/s41586-022-05220-z

8. Annexes

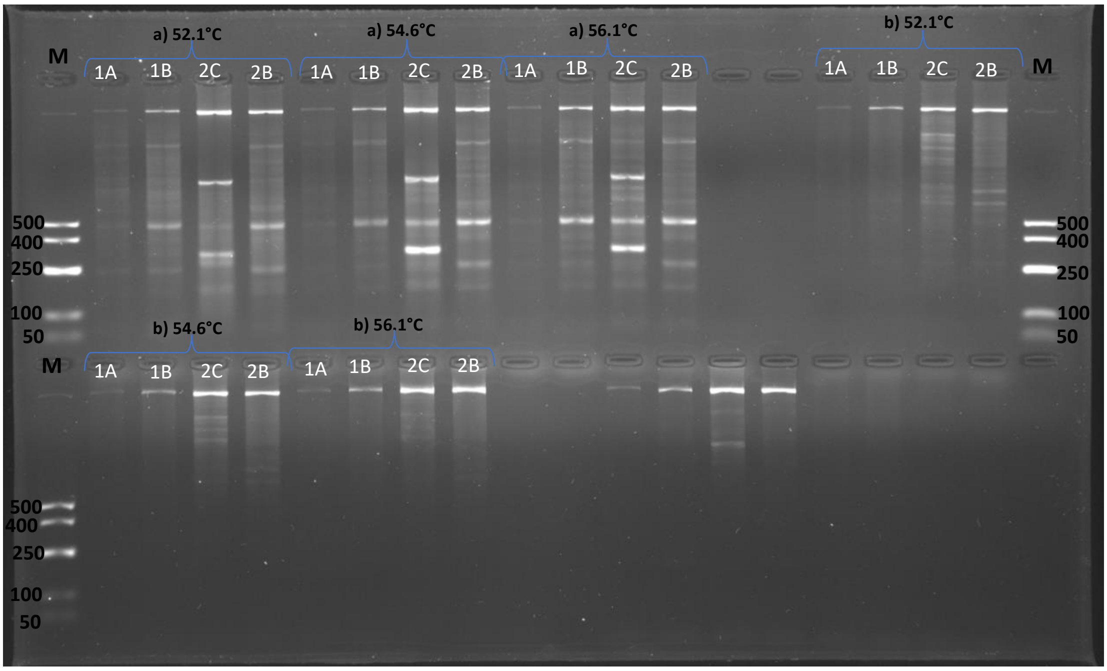


Figure 15A: Electrophoresis results showing PCR temperature gradients of selected samples for the genes tetA and aac. The superscripts represent the temperature gradient for tetA gene (a), and aac gene (b), and the numbers 1 and 2 represent homogenized soil samples.

Table 8A: Basic physical-chemical parameters of the soil and animal manure amended soil.

Sampling Time	**Sample name**	**C org [%]**	**Soil humus**	**pH 1M KCl**	**pH H2O**	**Soil salinity (as NaCl in 1L of soil)**	**Hh [cmol(+)/kg]**	**Cation exchange capacity**	
								Hh + basic cations	**Hh + exchangeable cations**
DAY 0	CS	1.09	1.88	6	6.91	0.2	2.2	7.6	7.06
	TS	0.88	1.51	6.3	7.04	0.27	1.9	7.5	6.76
	CwS	0.77	1.33	6.78	7.52	0.43	1.6	5.6	6.18
	PS	0.71	1.22	6.49	7.24	0.32	2.1	6.3	6.34
DAY 45	TS	0.94	1.63	6.45	7.18	0.285	2.3	7.5	7.71
	CwS	0.9	1.55	6.43	7.2	0.295	2	7.2	7.28
	PS	1.1	1.9	6.21	7.01	0.29	2.1	6.7	7.29

*results delivered by dr Wojcuech Tołoczko from the Department of Physical Geography, Faculty of Geographical Sciences, University of Lodz

Table 9A:The detected ARGs belonging to 7 ARPs from Day 0 to Day45 for soil, manure, and manure-amended soil variants; D- day

ARPs	ARGs	CS					TM	TS					CM	CwS					PM	PS				
		D 0	**D 5**	**D15**	**D 30**	**D 45**	**D 0**	**D 0**	**D 5**	**D 15**	**D 30**	**D 45**	**D 0**	**D 0**	**D 5**	**D 15**	**D 30**	**D 45**	**D 0**	**D 0**	**D 5**	**D 15**	**D 30**	**D 45**
β	***bla***$_{\mathrm{TEM}}$	-	-	-	-	-	-	+	-	-	+	-	+++	-	-	-	-	-	+++	++	+++	+++	+	++
	bla$_{\mathrm{OXA}}$	-	-	-	-	-	-	-	-	-	-	-	++	-	-	-	-	-	++	-	+++	+	-	-
	bla$_{\mathrm{SHV}}$	**n. a**	**n.a**	**n.a**	**n.a**	-	-	**n.a**	**n.a**	**n.a**	**n.a**	-	-	**n.a**	**n.a**	**n.a**	**n.a**	-	-	**n.a**	**n.a**	**n.a**	**n.a**	-
	bla$_{\mathrm{CTX\_M}}$	**n.a**	**n.a**	**n.a**	**n.a**	-	-	**n.a**	**n.a**	**n.a**	**n.a**	-	+	-	-	-	-	-	-	**n.a**	**n.a**	**n.a**	**n.a**	-
TET	***tet*(A)**	-	-	-	-	-	-	**n.a**	-	-	+	+	-	-	+	-	+	-	+	-	+	+	-	++
	***tet*(B)**	-	**n.a**	**n.a**	**n.a**	-	-	**n.a**	**n.a**	**n.a**	**n.a**	-	+	-	-	-	-	-	+	+++	+++	-	-	-
	***tet*(C)**	-	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	-	+	+	++	++	-	+
	***tet*(D)**	-	**n.a**	**n.a**	**n.a**	-	-	**n.a**	**n.a**	**n.a**	**n.a**	-	++	-	-	-	-	-	+	-	+	+	-	-
	***tet*(E)**	-	**n.a**	**n.a**	**n.a**	-	-	**n.a**	**n.a**	**n.a**	**n.a**	-	+	-	-	-	-	-	+	-	-	+	-	-
	***tet*(M)**	-	-	-	-	-	-	-	++	+	++	-	+++	+++	-	-	-	-	+++	+++	+++	+++	+	+
MLS	***erm*A**	-	-	-	-	**n.a**	-	-	-	-	-	**n.a**	+	-	-	-	-	**n.a**	+	-	-	-	-	**n.a**
	***erm*F**	-	+	+	-	-	-	-	-	-	-	-	++	-	-	-	-	-	++	-	-	-	-	+
	***lin*A**	-	-	-	-	-	-	+	+	-	-	-	++	-	-	-	-	-	+	-	+	-	-	-
SUL	***sul*1**	-	-	-	-	-	-	-	-	-	-	**n.a**	+	-	-	-	-	**n.a**	+	-	+	-	-	+
FQ	***aac* (6’)-Ib-cr**	-	-	-	-	-	-	-	+	-	-	-	+	-	-	+	-	-	+++	-	+	-	-	++
	qnrB1	-	-	-	-	**n.a**	-	-	-	-	-	**n.a**	-	-	-	-	-	**n.a**	-	-	-	-	-	**n.a**
Van	***van*A**	-	-	-	-	-	-	**n.a**	**n.a**	**n.a**	**n.a**	**n.a**	-	-	-	-	-	-	-	-	-	-	-	-
	***van*B**	-	-	-	-	-	-	**n.a**	**n.a**	**n.a**	**n.a**	**n.a**	-	-	-	-	-	-	++	-	-	-	-	-
	***van*C1**	-	-	-	-	-	-	**n.a**	**n.a**	**n.a**	**n.a**	**n.a**	-	-	-	-	-	-	+	-	-	-	-	-
	***van*C2**	-	-	-	-	-	-	**n.a**	**n.a**	**n.a**	**n.a**	**n.a**	-	-	-	-	-	-	-	-	-	-	-	-
I	***intl*1**	**n.a**	**n.a**	**n.a**	**n.a**	-	-	**n.a**	**n.a**	**n.a**	**n.a**	-	-	**n.a**	**n.a**	**n.a**	**n.a**	-	-	**n.a**	**n.a**	**n.a**	**n.a**	-

n.a. – not analyzed; -no signal, + weak signal; ++ strong signal; +++ very strong signal

Table 10A:The detected ARGs belonging to 7 ARP at Day45 for Rhizospheric soil and Endophytes (roots and leaves).

ARPs	ARGs	CS			TS			CwS			PS		
		RHIZOPSHERE	ROOTS	LEAVES	RHIZOPSHERE	ROOTS	LEAVES	RHIZOPSHERE	ROOTS	LEAVES	RHIZOPSHERE	ROOTS	LEAVES
β	*bla*$_{\mathrm{TEM}}$	-	-	+	-	-	+	-	-	+	++	+	+
	bla$_{\mathrm{OXA}}$	-	-	-	-	-	-	-	-	++	-	-	++
	bla$_{\mathrm{SHV}}$	-	-	-	-	-	-	-	-	-	-	-	-
	bla$_{\mathrm{CTX\_M}}$	-	-	-	-	-	-	-	-	-	-	-	-
TET	*tet*(A)	-	-	-	-	-	+	-	-	+	+	-	+
	tet(B)	-	-	++	-	-	++	-	-	++	-	-	++
	tet(C)	-	-	+	+++	-	-	-	+	+	+	+	+
	tet(D)	-	-	+	-	-	++	-	-	++	-	+	++
	tet(E)	-	-	-	-	-	-	-	-	-	-	-	-
	tet(M)	-	-	-	+	-	-	-	-	-	++	-	-
MLS	*erm*A	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a
	*erm*F	-	-	-	+	-	-	+	-	-	-	-	-
	*lin*A	-	-	-	-	-	-	-	-	-	-	-	-
SUL	*sul*1	n.a	-	-	-	n.a	n.a	n.a	n.a	n.a	+	-	-
FQ	*aac* (6')-Ib-cr	-	-	+	++	-	+	-	+	+	++	+	+
	qnrB1	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a
Van	*van*A	-	-	-	n.a	-	-	-	-	-	-	-	-
	*van*B	-	-	-	n.a	-	-	-	-	-	-	-	-
	*van*C1	-	-	-	n.a	-	-	-	-	-	-	-	-
	*van*C2	-	-	-	n.a	-	-	-	-	-	-	-	-
I	*intI*1	-	-	-	-	-	-	-	-	-	-	-	-

n.a. – not analyzed; -no signal, + weak signal; ++ strong signal; +++ very strong signal