Pak. J. Chem. 16(1-2): 65-73, 2026 Research article
ISSN (Print): 2220-2625
ISSN (Online): 2222-307X DOI: 10.15228/2026.v16.i1-2.p07

Impacts of Veterinary Antibiotics on Rhizosphere Soil Chemistry, Microbial Communities, and Enzyme Activities

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Abstract
Veterinary antibiotics (VAs) contamination has become a pressing concern for soil health and crop productivity. Therefore, this greenhouse study evaluated the effects of VAs on the rhizosphere soils of *Trifolium alexandrinum* and *Eruca vesicaria*. We compared different concentrations of enrofloxacin (ENR), penicillin (PCN) and sulfamethazine (SMZ) applied individually and as a mixture (MIX) at different concentrations: 0 mg kg^{-1}, 20 mg kg^{-1}, 50 mg kg^{-1}, 100 mg kg^{-1}, 250 mg kg^{-1}, 500 mg kg^{-1} and 1000 mg kg^{-1}. Results demonstrated clear concentration-dependent effects of VAs on soil physicochemical and biological properties, with the most pronounced effects observed under MIX treatment. High ENR concentrations (>250 mg kg^{-1}) significantly increased soil pH and bulk density, while reducing electrical conductivity and water-holding capacity. Increasing concentrations of ENR and MIX resulted in marked declines in calcium carbonate ($CaCO_3$), total organic carbon (TOC), total dissolved carbon (TDC), total nitrogen (TN), total phosphorus (TP), and exchangeable K, Ca, Na, and Ba. . Microbial analyses revealed a significant suppression of both bacterial and fungal populations with increasing antibiotic concentrations. Enzymatic activities showed differential sensitivity, with esterase being the most responsive indicator of antibiotic stress, phosphatase strongly affected by SMZ, and urease exhibiting a comparatively weaker response. Comparative analysis further indicated that *E. vesicaria* was more sensitive to VAs than *T. alexandrinum*, as evidenced by greater disruptions in soil physicochemical, biochemical, and microbial functions. Collectively, this study demonstrates the ecological risks of VAs and emphasize the need for regulated antibiotic use and improved manure management to protect soil health and sustain agroecosystem productivity.

Keywords: veterinary antibiotics; soil health; microbial abundance; enzyme activities; rhizosphere

1. Introduction
Veterinary antibiotics (VAs) use in livestock production has become a major environmental concern [1]. Application of VAs contaminated manure and biosolids alters soil physicochemical and biological processes [2,3]. Typically, VAs can alter nutrient cycling, and microbial diversity, thereby increasing antibiotic resistance [4-6]. Although some VA compounds degrade in soil naturally, repeated application of contaminated manure and biosolids results in prolonged soil contamination [7-10]. Furthermore, co-pollution of VAs and other natural pollutants represents a significant ecological threat to the soil–plant system [11].

Globally, annual VAs use is estimated at 63,000–105,000 tonnes annually, with approximately 60–90% excreted unmetabolized into the environment [2]. Thus, the VAs-related negative effects on agroecosystems may be exacerbated. Recent research has largely focused on the accumulation of VAs in plant tissues [3,12-13]. However, limited attention has been given to soil health. The effects of VAs on soil physicochemical parameters, enzymatic activities, and microbial community dynamics remain insufficiently explored. Furthermore, a substantial portion of recent research has been conducted under controlled laboratory conditions, thereby limiting the ecological relevance of the findings.

This study hypothesized that soil contamination of VAs exerts adverse effects on soil rhizosphere functioning. Such impacts may depend upon concentrations of VAs and plant species. This study aims to investigate the concentration-dependent and combined effects of veterinary antibiotics on rhizosphere soil functioning, with particular emphasis on plant-specific responses and integrated alterations in physicochemical properties, nutrient dynamics, microbial communities, and enzyme activities. This work provides critical insight into how VAs contamination threatens rhizosphere nutrient cycling, and the long-term sustainability of agroecosystems.

2. Materials and Methods
2.1. Experimental soil
Soil used in this study was collected from a greenhouse at the University of Science and Technology, Bannu, Pakistan. The soil was classified as loamy, containing a balanced mixture of sand, silt, and clay and had no history of antibiotic contamination. The soil was classified as alkaline, with pH of 8.4, and electrical conductivity (EC) of 0.67 dS m^{-1}.

2.2. Experimental design

This pot study comprised of three types of VAs: enrofloxacin (ENR), penicillin (PCN) and sulfamethazine (SMZ). Each VA was applied at following concentrations: 0 mg kg^{-1}, 20 mg kg^{-1}, 50 mg kg^{-1}, 100 mg kg^{-1}, 250 mg kg^{-1}, 500 mg kg^{-1} and 1000 mg kg^{-1}. Plastic cylindrical pots (10 cm diameter × 10 cm height; volume ≈ 785 cm^3) were used. Each pot was filled with 1 kg of air-dried experimental soil, corresponding to an approximate bulk density of 1.2 g cm^{-3}. All pots were perforated at the base to facilitate proper drainage and avoid waterlogging. To apply VAs in the pot soils, stock solutions of the VAs (1 mg mL^{-1} in methanol) were prepared, with the mixture containing 0.33 mg mL^{-1} of each antibiotic. The control treatment received only methanol. The antibiotics were thoroughly incorporated into the soil to ensure uniform distribution. Each treatment was conducted in triplicate and arranged in a completely randomized design to minimize positional effects. Following application, the soils were incubated at room temperature (25 ± 2 °C) for 25 days to allow equilibration and microbial adaptation. Soil moisture content was maintained at approximately 60% of field capacity throughout the incubation period.

Seeds of locally grown cultivars of *T. alexandrinum* and *E. vesicaria* were obtained from the District Agriculture Office, Bannu, Pakistan. Seeds were sown at a density of 15 seeds per pot and allowed to grow under greenhouse conditions for 30 days.

2.3. Soil physicochemical analyses

Soil samples were collected from each pot and analyzed for physicochemical properties. Soil pH was measured in a suspension of 20 g of soil and 50 mL of deionized water using a calibrated pH meter according to [14]. Electrical conductivity (EC) was determined from the same soil–water extract using a conductivity meter [14].

Bulk density (BD) was calculated according to standard protocol (FAO Soil Doctor Guide) using the following formula:

$$BD\left(\ g\ cm^{-3}\right) = \frac{W2 - W1}{V}$$

Where W_2 is the weight of oven-dried soil, W_1 is the weight of the empty container, and V is container volume.

Water holding capacity (WHC) was determined using the following formula:

$$WHC\ (\%) = \frac{W2 - W1}{W1}\ \times\ 100$$

Where W_2 is weight of moist saturated soil, and W_1 is weight of dry soil.

2.4. Nutrient and metal analysis

Total soil nitrogen was determined using the Kjeldahl method [15]. Olsen method was used to determine total phosphorus [16]. The back-titration method was used to determine $CaCO_3$ content [17]. For total dissolved carbon, soil samples mixed with deionized water were filtered (0.45 µm membrane) and analyzed using a TOC analyzer [18]. Total organic carbon was determined by the Walkley–Black dichromate oxidation method [19].

The concentrations of sodium (Na), calcium (Ca), potassium (K), and barium (Ba) were determined by acid digestion followed by analysis using a flame photometer [20].

2.5. Plant growth promoting bacteria and fungi

Plant growth promoting bacteria and fungi were cultured using plate culture techniques. For bacterial and fungal isolation, rhizosphere soil treated with a mixture of three antibiotics was selected for each plant. One gram of rhizosphere soil was suspended in 9 mL sterile distilled water and serially diluted up to 10^{-6}. Aliquots (0.1 mL) of each dilution were spread onto nutrient agar (NA) plates. Plates were incubated at 28 ± 2 °C for 48–72 hours. Distinct bacterial colonies were selected based on morphological differences (color, size, margin, elevation). Isolates were tested for plant growth–promoting trait, i.e., phosphate solubilization using Pikovskaya's agar: halo zone formation method [21]. For fungal isolation, the same serial dilutions were spread onto potato dextrose agar (PDA) plates supplemented with streptomycin (50 µg mL^{-1}) to inhibit bacterial growth. Plates were incubated at 28 ± 2 °C for 5–7 days after which dominant fungal colonies were purified by sub-culturing and identified microscopically based on colony morphology.

2.6. Soil enzyme activities

Acid phosphatase activity was assessed using p-nitrophenyl phosphate as substrate, recording absorbance at 405 nm [22]. Esterase activity was measured using p-nitrophenyl acetate as substrate, with absorbance recorded at 415 nm [23]. Urease activity was determined by the indophenol method, with absorbance measured at 630 nm [24].

2.7. Statistical Analysis

All experiments in this study were conducted in triplicate, and the data were represented as mean ± standard deviation (SD). One-way ANOVA was performed using Origin software (OriginLab, USA), with significance determined at $p < 0.05$. The different letters above the bars indicate statistically significant differences ($p < 0.05$).

3. Results and Discussion

The physicochemical properties like pH, EC, BD, and WHC of the soil rhizosphere under VAs application was studied.

3.1. Effects on soil physicochemical properties

VAs caused concentration-dependent alterations in soil rhizosphere pH, EC, BD and WHC of *T. alexandrinum* and *E. vesicaria* (Table 1). These changes demonstrate strong sensitivity of soil physicochemical properties to antibiotic

contamination. In both rhizospheres, soil pH increased progressively with increasing VAs concentrations. Among individual antibiotics, ENR induced the greatest increase in soil pH, particularly at higher concentrations, while PCN caused comparatively smaller changes and SMZ showed intermediate effects. The MIX treatment produced the highest pH values, reaching 11.1 in *T. alexandrinum* and 12.8 in *E. vesicaria*. This suggests a synergistic effect of combined antibiotics. The greater increase in soil pH caused by ENR might be attributed to its formulation and effect on soil N cycle. The greater pH elevation in *E. vesicaria* rhizosphere suggests higher sensitivity of this crop to antibiotics. This increase in soil pH may be attributed to suppression of microbial respiration and organic acid production, which normally contribute to soil acidification [25]. Furthermore, results indicated that EC declined consistently with increasing VA concentration in both plant rhizospheres (Table 1). Among individual antibiotics, ENR caused the greatest decrease in electrical conductivity (EC), particularly at higher concentrations, while SMZ had intermediate effects and PCN induced comparatively smaller reductions. The combined antibiotic treatment (MIX) produced the strongest overall decline, with EC in *T. alexandrinum* decreasing to 0.23 dS m^{-1}, and in *E. vesicaria* decreasing to 0.12 dS m^{-1} as compared to control (0.67 dS m^{-1}). Overall, EC reductions were consistently more pronounced in *E. vesicaria* than in *T. alexandrinum*, indicating greater disruption of soil ionic balance in the former. The decline in EC suggests a reduction in soluble salts and ionic strength of the soil solution. This effect is likely linked to inhibited microbial-mediated mineralization and nutrient release processes under antibiotic stress [25]. Bulk density is a key indicator of soil compaction that governs root penetration, water movement, aeration, and overall plant productivity [26]. In the present study, bulk density increased consistently with rising VA concentrations in the rhizospheres of both *T. alexandrinum* and *E. vesicaria* (Table 1), reflecting progressive soil compaction and degradation of soil structure. The strongest effects were observed under ENR and MIX treatments, with bulk density increasing by ~45% and ~40% in *T. alexandrinum*, and slightly higher increases of ~46% and ~42% in *E. vesicaria*, respectively. SMZ caused moderate increases (~20–25%), whereas PCN had minor effects. Overall, *E. vesicaria* soils were more sensitive to antibiotic-induced structural stress than *T. alexandrinum*. The increase in bulk density is likely associated with impaired soil aggregation and reduced organic binding agents due to antibiotic-mediated suppression of microbial activity and extracellular polymer production [4,9]. WHC is the ability of soil to retain water against gravity, and it is crucial for maintaining plant-available moisture, supporting microbial activity, and sustaining healthy plant growth [26]. The results of the present study showed that WHC was highest in control soils, reflecting intact soil structure, higher porosity, and adequate organic matter content (Fig. 1A and 1B).

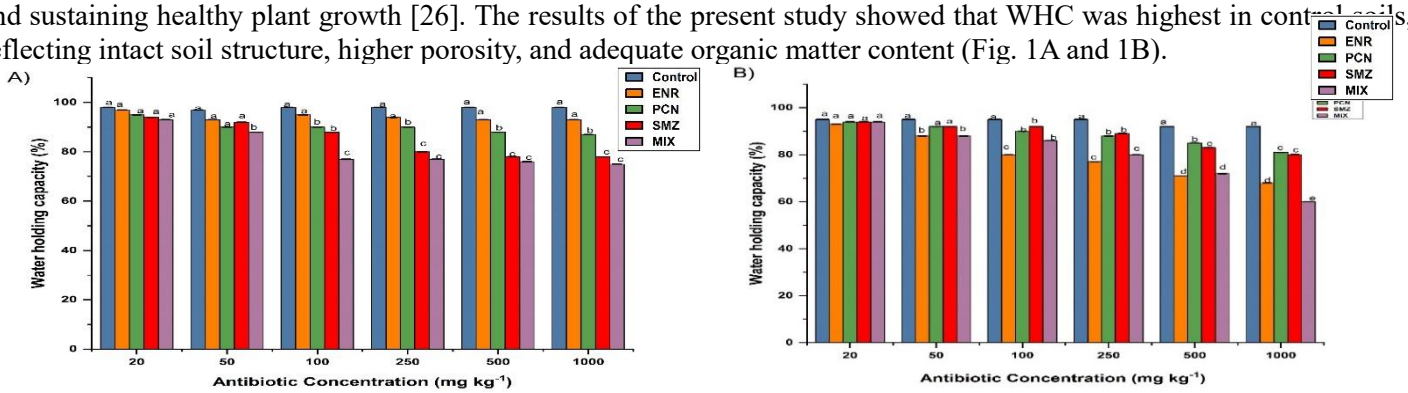

Figure 1: Effect of veterinary antibiotics on rhizosphere soil water holding capacity (WHC) in *Trifolium alexandrinum* (A) and *Eruca vesicaria* (B). Different letters on bars indicate significant differences among the treatments. n = 3, p < 0.05.

Application of VAs resulted in a progressive decline in WHC with increasing concentration in both rhizospheres. ENR, PCN, and SMZ caused moderate reductions (5–13%), while MIX had the strongest impact, reducing WHC by up to 23% in *T. alexandrinum* and 35% in *E. vesicaria* at the highest concentration.. The observed decline in WHC may be linked to structural changes in soil. These include reduced aggregate stability, lower pore connectivity, and loss of organic binding agents [4, 26]. Overall, these findings demonstrate that VAs particularly when applied in combination can significantly deteriorate soil physicochemical quality, highlighting the ecological risks posed by pharmaceutical residues in agricultural soils.

Table 1: Effect of veterinary antibiotics (VA) contamination on rhizosphere soil chemical properties of *Trifolium alexandrinum* (*T.A.*)and *Eruca vesicaria* (*E.V.*) at different concentrations

Treatments/Crops	*T.A.*	*E.V.*	*T.A.*	*E.V.*	*T.A.*	*E.V.*
	pH	pH	EC (dS m^{-1})	EC (dS m^{-1})	Bulk density (g cm^{-3})	Bulk density (g cm^{-3})
Control	8.5	8.5	0.67	0.67	1.22	1.22
ENR 20 mg kg^{-1}	8.4±0.43	8.7 ±0.53	0.55±0.07	0.54±0.005	1.53 ±0.34	1.57±0.01
ENR 50 mg kg^{-1}	9.6±0.22	9.3±0.33	0.55±0.08	0.47±0.08	1.61±0.23	1.64 ±0.15
ENR 100 mg kg^{-1}	9.7±0.11	9.7±0.77	0.49±0.04	0.42±0.08	1.64 ±0.22	1.65 ±0.1
ENR 250 mg kg^{-1}	9.9±0.08	10.5±0.53	0.43± 0.02	0.38±0.02	1.67±0.23	1.66 ±0.23
ENR 500 mg kg^{-1}	10.2±0.24	12.2±0.77	0.39±0.02	0.33±0.03	1.70±0.03	1.72 ±0.02
ENR 1000 mg kg^{-1}	10.5±0.22	12.6±0.05	0.30±0.03	0.28±0.07	1.77 ±0.03	1.79 ±0.08
PCN 20 mg kg^{-1}	9.3±0.66	8.9±0.11	0.58±0.01	0.61±0.03	1.57±0.15	1.58±0.31
PCN 50 mg kg^{-1}	9.6±0.97	9.1±0.76	0.53±0.06	0.60±0.03	1.55±0.43	1.61±0.05
PCN 100 mg kg^{-1}	9.8±0.07	9.5±0.13	0.46±0.01	0.59±0.01	1.60±0.91	1.65±0.32

PCN 250 mg kg^{-1}	9.9±0.13	9.2±0.34	0.39±0.06	0.54±0.09	1.64±0.09	1.68±0.55
PCN 500 mg kg^{-1}	10.1±0.11	10.3±0.19	0.36±0.02	0.49±0.08	1.67 ±0.43	1.70±0.15
PCN 1000 mg kg^{-1}	10.4±0.35	10.8±0.11	0.35±0.06	0.43±0.01	1.72±0.06	1.74 ±0.21
SMZ 20 mg kg^{-1}	9.3±0.63	9.3±0.33	0.55±0.05	0.53±0.05	1.55±0.23	1.53±0.03
SMZ 50 mg kg^{-1}	9.4±0.15	9.6±0.27	0.53±0.02	0.47±0.02	1.59 ±0.11	1.60±0.33
SMZ 100 mg kg^{-1}	9.6±0.55	9.8±0.43	0.49±0.08	0.41±0.08	1.64 ±0.05	1.63±0.05
SMZ 250 mg kg^{-1}	9.9±0.16	10.2±0.43	0.46±0.05	0.36±0.05	1.68±0.08	1.64 ±0.11
SMZ 500 mg kg^{-1}	10.3±0.55	10.3±0.67	0.37±0.08	0.27±0.08	1.73 ±0.55	1.68±0.23
SMZ 1000 mg kg^{-1}	10.8±0.13	10.7±0.15	0.33±0.04	0.21±0.04	1.74 ±0.56	1.74±0.24
MIX 20 mg kg^{-1}	9.8±0.43	9.7±0.15	0.46±0.02	0.41±0.08	1.28 ±0.22	1.77 ±0.56
MIX 50 mg kg^{-1}	10.0±0.69	10.2±0.88	0.44±0.02	0.35±0.04	1.46±0.02	1.59±0.13
MIX 100 mg kg^{-1}	10.4±0.85	10.6±0.33	0.42±0.05	0.31±0.03	1.55 ±0.08	1.64±0.03
MIX 250 mg kg^{-1}	10.5±0.99	11.9±0.22	0.37±0.03	0.26±0.07	1.61±0.13	1.68±0.03
MIX 500 mg kg^{-1}	10.7±0.03	12.2±0.33	0.32±0.12	0.20±0.07	1.69±0.22	1.73 ±0.03
MIX 1000 mg kg^{-1}	11.1±0.42	12.8±0.32	0.23±0.06	0.12±0.01	1.70 ±0.11	1.73±0.02

ENR- Enrofloxacin; PCN- Penicillin; SMZ-Sulfamethazine; MIX- mixture of three antibiotics. EC- Electrical conductivity. All values are expressed as mean ± standard deviation (SD) of three independent replicates (n = 3).

3.2. Effects on soil nutrient contents

Application of VAs caused pronounced alterations in soil chemical properties, carbon fractions, nutrient pools, and metal contents in the rhizosphere soils of *T. alexandrinum* and *E. vesicaria* (Table 2). In both rhizospheres, most parameters exhibited clear concentration-dependent responses, demonstrating the high sensitivity of soil biogeochemical processes to antibiotic contamination. Across all treatments, $CaCO_3$ content showed only minor fluctuations, suggesting that carbonate minerals are relatively stable and less responsive to antibiotic stress. Compared with the control, $CaCO_3$ decreased modestly at lower concentrations (20–100 mg kg^{-1}) but declined substantially at ≥500 mg kg^{-1}, with the largest reductions observed under MIX and SMZ treatments. Across all treatments, soils planted with *T. alexandrinum* maintained slightly higher $CaCO_3$ levels than those under *E. vesicaria*. At 1000 mg kg^{-1} MIX treatment, $CaCO_3$ declined to 9.3% in *T. alexandrinum* soil compared to 8.5% in *E. vesicaria*, indicating greater sensitivity of *E. vesicaria* to antibiotic stress. Total organic carbon (TOC) and total dissolved carbon (TDC) declined sharply with increasing antibiotic concentration. While minor variations were observed at lower concentrations (20–50 mg kg^{-1}), substantial reductions occurred at ≥250 mg kg^{-1}, particularly under SMZ and MIX treatments. The decline was more pronounced in *E. vesicaria* than in *T. alexandrinum*, indicating greater sensitivity of carbon dynamics and organic matter stability in the former under antibiotic stress. Concomitantly, TDC dropped drastically with increasing concentration. The sharpest declines were recorded under SMZ and MIX treatments, where TDC values at 1000 mg kg^{-1} dropped to nearly 10–15% of control levels (Table 2). Across all concentrations, *T. alexandrinum* soils maintained higher TDC compared with *E. vesicaria*. SMZ caused stronger reductions in $CaCO_3$, TOC, and TDC than ENR and PCN. The stronger reductions caused by SMZ is likely due to its higher mobility and persistence, which prolong microbial inhibition [27]. This also enhances carbonate dissolution and soil organic matter loss, whereas ENR and PCN have weaker effects due to stronger sorption or rapid degradation [4,9]. Nitrogen is a fundamental nutrient in ecosystems, and its biogeochemical cycle maintains the stability of ecosystems [28]. In this study, TN content of soil decreased progressively with increasing antibiotic concentration in both rhizospheres. The decline was moderate under PCN treatments but became pronounced under ENR, SMZ, and especially MIX treatments. At the highest concentration (1000 mg kg^{-1}), TN decreased by approximately 25–30% in *T. alexandrinum* and 35–40% in *E. vesicaria* relative to the control (Table 2).

Table 2: Effect of veterinary antibiotics (VA) contamination on rhizosphere soil nutrient contents of *Trifolium alexandrinum* (*T.A.*) and *Eruca vesicaria* (*E.V.*)at different concentrations

Treatments /crops	*T.A.* $CaCO_3$ (%)	*E.V.* $CaCO_3$ (%)	*T.A.* TOC (%)	*E.V.* TOC (%)	*T.A.* TDC (mg kg^{-1})	*E.V.* TDC (mg kg^{-1})	*T.A.* TN (g kg^{-1})	*E.V.* TN (g kg^{-1})	*T.A.* TP (mg kg^{-1})	*E.V.* TP (mg kg^{-1})
Control	12	12	2.7	2.7	850.3±0.02	850.3±0.02	1.85±0.02	1.85±0.02	615±0.02	615±0.02
ENR 20 mg kg^{-1}	11.9	11.8	2.6	2.7	830.6±0.03	818±0.05	1.76±0.04	1.78±0.03	610±0.2	603±0.02
ENR 50 mg kg^{-1}	11.3	11.2	2.7	2.6	807.6±0.02	801±0.02	1.72±0.05	1.70± 0.03	598±0.03	571±0.02
ENR 100 mg kg^{-1}	11.4	11.4	2.6	2.3	757± 0.02	771±0.03	1.68±0.05	1.62±0.04	585±0.02	568±0.02
ENR 250 mg kg^{-1}	11.21	11.1	2.5	2.1	652.1±0.03	654±0.02	1.60±0.06	1.50±0.04	565± 0.04	563±0.03
ENR 500 mg kg^{-1}	10.9	9.7	2.2	1.7	485.2±0.03	451±0.02	1.52±0.07	1.38±0.05	540± 1.1	558±0.03
ENR 1000 mg kg^{-1}	10.5	9.5	1.9	0.6	100±0.02	82.3±0.05	1.44±0.08	1.24±0.05	515±0.08	578±0.01
PCN 20 mg kg^{-1}	12.3	11.6	2.7	2.8	860.6±0.02	836±0.03	1.77±0.03	1.80±0.02	612±0.1	574±0.02
PCN 50 mg kg^{-1}	11.9	10.7	2.6	2.6	839.4±0.05	821±0.02	1.74±0.04	1.74±0.03	602±0.12	572±0.02
PCN 100 mg kg^{-1}	11.8	11	2.6	2.6	793±0.02	787±0.03	1.70±0.04	1.68±0.03	590±0.03	569±0.02
PCN 250 mg kg^{-1}	11.3	10.8	2.3	2.3	706.9±0.02	684±0.03	1.64±0.05	1.56±0.03	570±0.06	565±0.02
PCN 500 mg kg^{-1}	10.9	10.0	2.2	1.9	694.4±0.02	560±0.02	1.57±0.06	1.45±0.04	548±1	560±0.03
PCN 1000 mg kg^{-1}	10.3	9.7	2.2	1.0	454±0.03	259±0.05	1.49±0.07	1.32±0.05	520±0.6	498±0.03
SMZ 20 mg kg^{-1}	12.0	11.5	2.6	2.5	823.2±0.05	800±0.02	1.75±0.04	1.76±0.03	608±0.12	473±0.02
SMZ 50 mg kg^{-1}	11.8	11.2	2.6	2.4	787.7±0.02	773±0.03	1.71±0.05	1.68±0.02	595±0.03	470±0.02
SMZ 100 mg kg^{-1}	11.2	10.5	2.5	1.9	776±0.02	753±0.03	1.67±0.05	1.60±0.03	582±0.4	466±0.02
SMZ 250 mg kg^{-1}	10.8	10.6	2.2	0.9	659.9±0.02	567±0.03	1.59±0.06	1.48±0.04	560 ± 0.02	461±0.03

SMZ 500 mg kg^{-1}	10.0	9.5	1.89	0.6	261.5±0.03	162±0.02	1.51±0.07	1.36±0.04	535±0.01	456±0.03
SMZ 1000 mg kg^{-1}	9.8	9.6	1.1	0.13	100±0.02	96.8±0.03	1.43±0.08	1.22±0.05	510±0.7	450±0.03
MIX 20 mg kg^{-1}	12.1	11.7	2.7	2.7	829.5±0.05	829±0.02	1.74±0.05	1.72±0.03	605±0.4	451±0.02
MIX 50 mg kg^{-1}	11.7	10.9	2.6	2.5	781.9±0.02	785±0.02	1.69±0.05	1.62±0.03	590±0.03	455±0.02
MIX 100 mg kg^{-1}	11.2	9.5	2.3	2.3	713.4±0.03	708±0.02	1.64±0.06	1.52±0.04	575±0.1	449±0.03
MIX 250 mg kg^{-1}	10.6	9.1	1.9	1.7	506.6±0.02	512±0.05	1.55±0.07	1.38±0.04	550±0.7	450±0.03
MIX 500 mg kg^{-1}	9.5	8.8	1.28	0.6	373.7±0.02	185±0.02	1.46±0.08	1.24±0.05	520±0.01	452±0.03
MIX 1000 mg kg^{-1}	9.3	8.5	1.0	0.1	212±0.02	104±0.02	1.36±0.09	1.1±0.06	495±0.23	461±0.04

ENR- Enrofloxacin; PCN- Penicillin; SMZ-Sulfamethazine; MIX- mixture of three antibiotics. $CaCO_3$- Calcium carbonate; TOC- Total organic carbon; TDC- Total dissolved carbon; TN- Total Nitrogen; TP- Total Phosphorus. All values are expressed as mean ± standard deviation (SD) of three independent replicates (n = 3).

These results indicate inhibition of nitrogen-fixing bacteria and suppression of mineralization processes under antibiotic stress [29-30]. In addition, the total phosphorus (TP) also exhibited a concentration-dependent decline in both soils, though the magnitude of change was smaller than that observed for other parameters. Significant TP depletion occurred under SMZ and MIX treatment, particularly at ≥250 mg kg^{-1}. In *T. alexandrinum*, TP declined by approximately 16–20% at the highest concentration, whereas a stronger reduction of about 6–27% was observed in *E. vesicaria*, with the greatest decreases under SMZ and MIX treatments. Strong reduction in TP under SMZ treatment may be attributed to its higher mobility, persistence, and broad-spectrum microbial inhibition as compared to ENR and PCN [4,9]. Moreover, reduced TP availability may be linked to decreased abundance of phosphate-solubilizing microorganisms and lower phosphatase activity under antibiotic exposure [31]. VA treatments caused substantial reductions in exchangeable potassium (K), calcium (Ca), sodium (Na), and barium (Ba) in both rhizospheres (Table 3).

Table 3: Effect of veterinary antibiotics (VA) contamination on rhizosphere soil metal contents of *Trifolium alexandrinum* (*T.A.*) and *Eruca vesicaria* (*E.V.*) at different concentrations

Treatments /crops	*T.A.* K (mg kg^{-1})	*E.V.* K (mg kg^{-1})	*T.A.* Ca (mg kg^{-1})	*E.V.* Ca (mg kg^{-1})	*T.A.* Na (mg kg^{-1})	*E.V.* Na (mg kg^{-1})	*T.A.* Ba (mg kg^{-1})	*E.V.* Ba (mg kg^{-1})
Control	176.4± 0.21	176.4± 0.02	216.7±0.43	216.7±0.1	265.0± 0.02	265.0±0.43	240.9± 0.02	240.9±0.02
ENR 20 mg kg^{-1}	156.1±0.91	134.9± 0.2	196.5±0.93	186.3±0.1	247.7± 0.02	234.2±0.1	226.1± 0.02	203.8±0.02
ENR 50 mg kg^{-1}	138.7±0.23	118.7± 0.21	172.5±0.43	163.4± 0.55	221.3± 0.02	208±0.1	207.1±0.33	188± 0.21
ENR 100 mg kg^{-1}	121.5± 0.21	105.7± 0.02	155.4±0.22	137.2 ±0.55	185.0±0.23	110.0±0.1	178.7±0.33	94.0± 0.21
ENR 250 mg kg^{-1}	117.9±0.1	105.6±0.3	137.0±0.1	117.6±0.1	159.3±0.23	95.5± 0.21	147.7±0.1	73.2±0.02
ENR 500 mg kg^{-1}	83.2±0.1	85.9±0.1	104.8±0.02	88.2± 0.21	123.8±0.33	88.7±0.08	136.8±0.33	59.0±0.43
ENR 1000 mg kg^{-1}	61.7±0.05	42.5±0.43	94.0±0.02	64 ±0.55	145.0±0.33	78.5±0.91	132.3±0.1	33.4±0.02
PCN 20 mg kg^{-1}	158.0±0.22	132.6± 0.02	208.4±0.43	162.7± 0.27	253.1±0.22	217.2±0.23	192.1±0.33	224.2± 0.21
PCN 50 mg kg^{-1}	134.9± 0.21	117.9±0.23	186.3±0.22	138.4± 0.24	237.7± 0.21	179.3±0.33	168.1±0.1	216.1±0.04
PCN 100 mg kg^{-1}	118.7±0.22	83.2± 0.02	163.4±0.02	114.2±0.1	208.3±0.08	155.3±0.33	141.5±0.1	188.1±0.91
PCN 250 mg kg^{-1}	95.7±0.91	61.7±0.23	137.2±0.22	93.3± 0.22	185.0±0.93	117± 0.02	106.3±0.23	178.7±0.02
PCN 500 mg kg^{-1}	84.6±0.73	56.2± 0.02	117.6±0.1	200.7± 0.33	159.3±0.1	102± 0.02	229.8±0.23	147.7± 0.21
PCN 1000 mg kg^{-1}	76.0±0.23	23.3±0.93	88.2±0.02	193.0± 0.21	123.8±0.02	96±0.1	207.0± 0.02	116.8±0.22
SMZ 20 mg kg^{-1}	167.2±0.22	107.3±0.23	209.4±0.02	141.1±0.1	191.8± 0.02	245.0±0.06	232.3±0.22	172.5± 0.02
SMZ 50 mg kg^{-1}	143.7± 0.21	85.9± 0.02	187.1±0.1	123.1± 0.21	176.2±0.33	223.6± 0.21	212.5± 0.21	146.3±0.33
SMZ 100 mg kg^{-1}	132.6±0.43	54.5± 0.02	162.7±0.02	117.2± 0.22	138.6±0.33	217.2± 0.21	192.1± 0.21	123.1±0.23
SMZ 250 mg kg^{-1}	106.1±0.1	156.1± 0.02	138.4±0.93	106.5± 0.25	243.2±0.33	179.3±0.08	168.1±0.33	113.8± 0.02
SMZ 500 mg kg^{-1}	92.3±0.23	138.7± 0.02	114.2±0.06	102.5± 0.21	221.8±0.1	155.3±0.91	141.5±0.1	107.3±0.33
SMZ 1000 mg kg^{-1}	67.2± 0.02	121.5± 0.02	93.3±0.05	95.4± 0.24	110.0±0.1	100.7± 0.21	106.3±0.1	94.0±0.23
MIX 20 mg kg^{-1}	164.2±0.01	85.9± 0.02	200.7±0.27	104.8±0.1	250.2± 0.21	61.7±0.1	229.8±0.05	200.0±0.33
MIX 50 mg kg^{-1}	146.0± 0.21	42.5± 0.02	103.0±0.1	94.0± 0.21	235.5±0.1	38.5±0.33	207.0±0.91	193.4±0.23
MIX 100 mg kg^{-1}	127.3±0.93	41.5± 0.02	102.8±0.08	92 ±0.55	215.0±0.03	38±0.33	192.4±0.06	174±0.23
MIX 250 mg kg^{-1}	107.3±0.1	40.8± 0.02	95±0.1	92.4± 0.02	191.8±0.91	36±0.1	172.5± 0.21	162± 0.02
MIX 500 mg kg^{-1}	85.9±0.03	40± 0.02	101±0.05	91±0.1	176.2±0.25	30±0.1	146.3±0.01	129± 0.02
MIX 1000 mg kg^{-1}	54.5±0.1	36± 0.02	97.2±0.91	90± 0.22	138.6±0.07	28±0.1	123.1± 0.21	110±0.23

ENR- Enrofloxacin; PCN- Penicillin; SMZ-Sulfamethazine; MIX- mixture of three antibiotics. Ca- Calcium; Na- Sodium; Ba- Barium. All values are expressed as mean ± standard deviation (SD) of three independent replicates (n = 3).

These alterations in cation availability may be linked to chelation effects of antibiotics, affecting nutrient mobility and uptake [29]. ENR showed strong responses. PCN and SMZ showed similar but slightly weaker trends, whereas MIX caused pronounced declines across all metals. Across all concentrations, *T. alexandrinum* consistently maintained higher K levels than *E. vesicaria*. Calcium concentrations decreased progressively in *T. alexandrinum* soils but showed irregular responses in *E. vesicaria* soils, particularly under PCN and SMZ treatments. The variability in *E. vesicaria* may reflect disrupted microbial-mediated Ca mobilization and rhizosphere interactions including localized increases under high PCN and SMZ treatments, suggesting disturbed Ca mobilization and potential ionic imbalance. Barium (Ba) decreased across all treatments and concentrations. *T. alexandrinum* soils generally retained higher Ba compared to *E. vesicaria*, indicating stronger rhizosphere-mediated Ba buffering. Crop-specific responses emphasize the importance of plant-mediated soil buffering capacity in mitigating antibiotic stress. Nitrogen appeared slightly more sensitive than phosphorus, and carbon fractions were more strongly affected than mineral elements, indicating differential vulnerability of soil functional pools. The greater retention of soil cationic nutrients (K, Ca, Na, Ba) in *T. alexandrinum* soils highlights the buffering role of

leguminous crops. Enhanced rhizodeposition, higher microbial biomass, and improved cation exchange capacity associated with legumes likely mitigated antibiotic toxicity and stabilized nutrient pools [29]. Overall, these findings demonstrate that VAs contamination can simultaneously impair soil carbon storage, nutrient cycling, and elemental availability, leading to broad degradation of soil functional quality. The consistent responses observed in both rhizospheres suggest that antibiotic-driven disturbances are robust and not strongly plant-specific. The strong impact of antibiotic mixtures further highlights the ecological risks associated with combined pharmaceutical residues commonly present in manure-amended and wastewater-irrigated agricultural soils.

3.3. Effects on plant growth promoting bacteria and fungi

13 different bacterial isolates were isolated from the rhizosphere soil of *T. alexandrinum,* while 8 isolates from the rhizosphere soil of *E. vesicaria*. Control soils exhibited the highest bacterial counts (15), out of which 2 showed phosphate solubilizing activity (Fig. 2A and B).

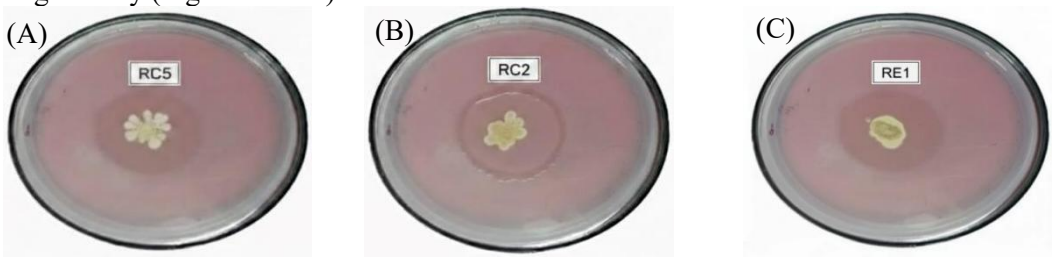

Figure 2. Isolation of phosphate-solubilizing bacteria from rhizosphere soils. Clear halo zones on Pikovskaya's agar indicate phosphate solubilization activity in control (A,B)and . and *E. vesicaria* (C).

Only one bacterial isolate, RE1, isolated from the rhizosphere of *E. vesicaria,* exhibited phosphate solubilizing activity (Figure 2C), while no bacteria from the rhizosphere soil of *T. alexandrinum* showed phosphate solubilizing activity. The results indicated that the total colony-forming units (CFUs) of bacteria decreased significantly with increasing antibiotic concentrations (Table 4). Fungal colony counts also decreased with increasing antibiotic concentrations (Table 4).

Table 4: Effect of veterinary antibiotics (VA) contamination on rhizosphere bacterial and fungal counts (log_{10} CFU g^{-1}) of *Trifolium alexandrinum* and *Eruca vesicaria* at different concentrations

Treatments/crops	*T. alexandrinum* Bacterial CFU counts (log_{10} CFU g-1)	*E. vesicaria* Bacterial CFU counts (log_{10} CFU g-1)	*T. alexandrinum* Fungal CFU counts (log_{10} CFU g-1)	*E. vesicaria* Fungal CFU counts (log_{10} CFU g-1)
Control	6.8 ± 0.2	6.4 ± 0.1	5.2 ± 0.2	5.5 ± 0.1
ENR 20 mg kg^{-1}	6.5 ± 0.1	6.2 ± 0.1	5.0 ± 0.2	4.4 ± 0.1
ENR 50 mg kg^{-1}	6.3 ± 0.2	6.0 ± 0.1	4.8 ± 0.2	3.0 ± 0.1
ENR 100 mg kg^{-1}	6.1 ± 0.2	6.2±0.1	4.6 ± 0.1	3.0 ± 0.1
ENR 250 mg kg^{-1}	5.8 ± 0.1	5.8±0.1	4.3 ± 0.2	3.2±0.1
ENR 500 mg kg^{-1}	5.4 ± 0.2	5.2±0.4	4.0 ± 0.1	2.8±0.1
ENR 1000 mg kg^{-1}	5.0 ± 0.2	5.5 ± 0.2	3.8 ± 0.2	3.2±0.4
PCN 20 mg kg^{-1}	6.6 ± 0.2	5.5 ± 0.2	4.7 ± 0.2	3.5 ± 0.2
PCN 50 mg kg^{-1}	6.3 ± 0.2	5.1 ± 0.2	4.3 ± 0.1	3.1 ± 0.2
PCN 100 mg kg^{-1}	5.9 ± 0.2	4.4 ± 0.2	4.0 ± 0.2	3.1 ± 0.2
PCN 250 mg kg^{-1}	5.5 ± 0.2	4.2±0.5	3.7 ± 0.2	2±0.5
PCN 500 mg kg^{-1}	5.0 ± 0.2	4.8±0.5	3.4 ± 0.1	2.8±0.5
PCN 1000 mg kg^{-1}	4.6 ± 0.3	4.2±0.1	3.0 ± 0.1	3.2±0.1
SMZ 20 mg kg^{-1}	6.4 ± 0.1	5.2± 0.2	4.9 ± 0.1	3.2± 0.1
SMZ 50 mg kg^{-1}	6.4 ± 0.1	4.9± 0.2	4.5 ± 0.2	3.2± 0.1
SMZ 100 mg kg^{-1}	6.0 ± 0.2	4.2± 0.1	4.1 ± 0.2	2.8± 0.1
SMZ 250 mg kg^{-1}	5.6 ± 0.1	3.8± 0.1	3.8 ± 0.2	2.2±0.1
SMZ 500 mg kg^{-1}	5.2 ± 0.2	3.2±0.1	3.5 ± 0.2	2.5± 0.1
SMZ 1000 mg kg^{-1}	4.8 ± 0.2	2.9± 0.1	3.1 ± 0.2	2±0.5
MIX 20 mg kg^{-1}	6.0 ± 0.1	4.2± 0.1	4.0 ± 0.1	3.2± 0.1
MIX 50 mg kg^{-1}	5.8 ± 0.2	4.0± 0.1	3.8 ± 0.2	3.0± 0.1
MIX 100 mg kg^{-1}	5.2 ± 0.2	3.9± 0.2	3.8 ± 0.2	2.5± 0.2
MIX 250 mg kg^{-1}	4.9±0.04	3.8± 0.1	3.2±0.04	2.1± 0.1
MIX 500 mg kg^{-1}	3.8±0.4	3.2± 0.1	3.2±0.4	2.2± 0.1
MIX 1000 mg kg^{-1}	3.3± 0.2	3.3± 0.1	3.3± 0.2	1.8± 0.1

ENR- Enrofloxacin; PCN- Penicillin; SMZ-Sulfamethazine; MIX- mixture of three antibiotics. All values are expressed as mean ± standard deviation (SD) of three independent replicates (n = 3).

These findings are consistent with previous studies reporting that fluoroquinolones like ENR have broad-spectrum antimicrobial activity and are highly persistent in soil, leading to strong suppression of microbial growth. Sulfonamides such as SMZ, though less toxic, can still alter community composition and select for resistant strains [27, 32]. Traits such as phosphate solubilization are essential for nutrient cycling and plant growth promotion [33, 34]. Its decline suggests that antibiotic residues in soils could indirectly reduce plant nutrient availability and productivity. Similar reductions in PGP activities under antibiotic exposure have been reported by others, highlighting the ecological risks of chronic antibiotic

inputs [4,27,30]. Since PGPB and fungi are integral to nutrient cycling, carbon turnover, and plant health, their suppression could have long-term consequences for agricultural productivity [34].

3.4 Effects on soil enzymatic activities

Soil enzymatic activities were differentially affected by VAs, reflecting disruption of microbial nutrient cycling. Phosphatases play a key role in soil P cycling. Produced by microorganisms and plant roots, phosphatase activity serves as a sensitive indicator of soil fertility and microbial health [35]. In the present study, phosphatase activity was most sensitive to SMZ, declining by 35–50% in *T. alexandrinum* and 40–55% in *E. vesicaria* soils at 500–1000 mg kg^{-1}, indicating strong inhibition of phosphate-solubilizing microbes and impaired P mobilization (Figure 3). These results are consistent with reductions in TP content of soil as described previously. Reductions in phosphatase activity reflect microbial stress and impaired nutrient turnover, making it a valuable biomarker for assessing the impact of environmental contaminants, including VAs. Furthermore, esterase activity was severely suppressed by all antibiotics, with reductions of 45–60% at 500 mg kg^{-1} and near-total inhibition (>90%) under ENR and MIX at 1000 mg kg^{-1} (Figure 4).

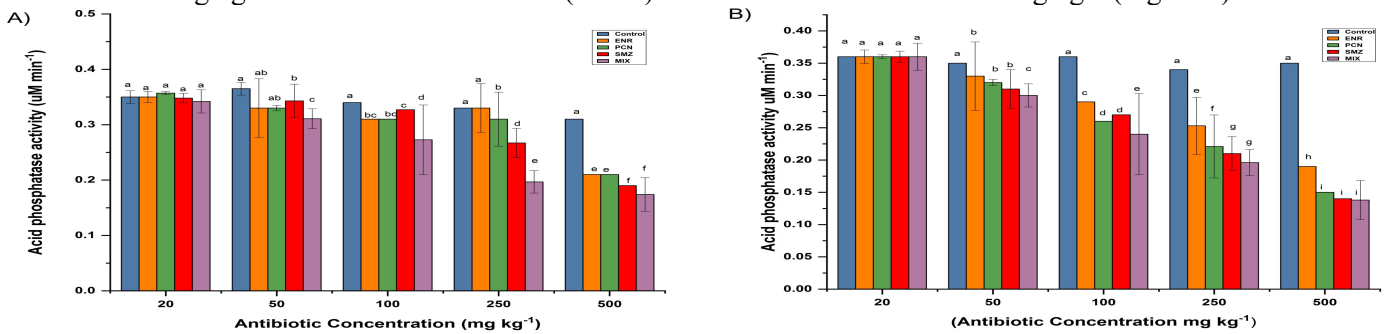

Figure 3: Effect of veterinary antibiotics on rhizosphere soil acid phosphatase activity in *Trifolium alexandrinum* (A) and *Eruca vesicaria* (B). Different letters on bars indicate significant differences among the treatments. n = 3, p < 0.05.

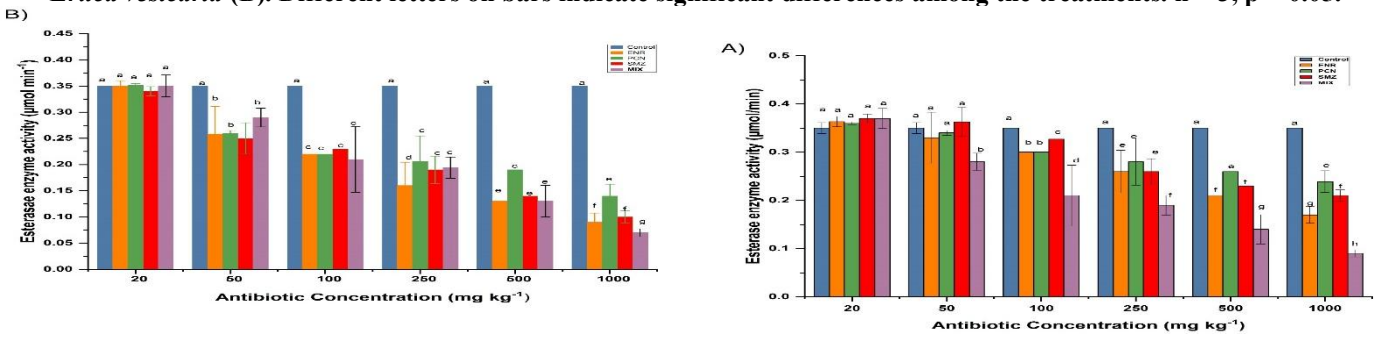

Figure 4: Effect of veterinary antibiotics on rhizosphere soil esterase activity in *Trifolium alexandrinum* (A) and *Eruca vesicaria* (B). Different letters on bars indicate significant differences among the treatments. n = 3, p < 0.05.

These results suggest that esterase enzymes can be used as an early biomarker of antibiotic contamination. Urease activity also declined, but less sharply than phosphatase or esterase, decreasing by 25–40% at the highest concentrations, suggesting partial suppression of urea-hydrolyzing microbes while N cycling remained relatively buffered (Figure 5).

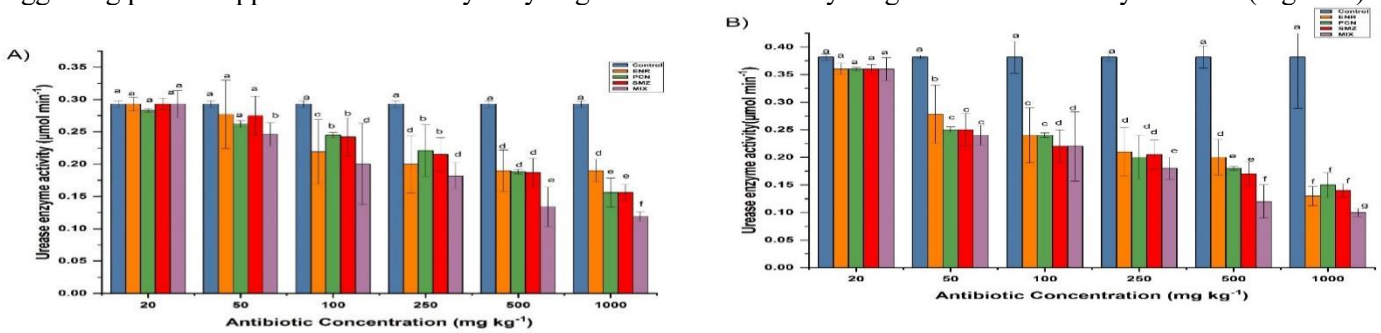

Figure 5. Effect of veterinary antibiotics on rhizosphere soil urease activity in *Trifolium alexandrinum* (A) and *Eruca vesicaria* (B). Different letters on bars indicate significant differences among the treatments. n = 3, p < 0.05.

Enzyme inhibition was consistently stronger in *E. vesicaria* rhizospheres than in *T. alexandrinum*, likely due to host-specific differences in root exudates and microbial recruitment, which modulate microbial resilience under antibiotic stress. Soil enzymes such as phosphatase, esterase, and urease play critical roles in nutrient cycling and microbial functioning (Hu et al. 2020). Monitoring these enzymes in the rhizospheres of *T. alexandrinum* and *E. vesicaria* provides essential insight into the impact of VAs exposure on microbial viability, nutrient turnover, and soil biochemical integrity, thereby offering a comprehensive assessment of soil functional health under anthropogenic stress [4,36].

4.0 Conclusion

This study concludes that veterinary antibiotics significantly impaired rhizosphere soil health by altering physicochemical properties, reducing nutrient availability, and suppressing microbial communities and enzyme activities. The pronounced effects of ENR and MIX highlights the roles of persistence and synergistic toxicity in driving soil degradation. The greater sensitivity of *E. vesicaria* compared to *T. alexandrinum* further highlights species-specific differences in rhizosphere resilience. Overall, these results demonstrate that VAs act as silent disruptors of soil ecosystems, affecting nutrient cycling, microbial stability, and sustainable agriculture. Hence, future research should focus on long-term field studies, antibiotic resistance dynamics, and advanced molecular approaches to better understand soil functional responses. Additionally, the development of sustainable remediation strategies and regulated use of antibiotics in agriculture are essential to mitigate environmental risks and ensure sustainable crop production.

Acknowledgment

The authors acknowledge the University of Science and Technology, Bannu, Pakistan, for providing research facilities.

Author's Contribution: Sadia Nadir, Sehroon Khan: Conceptualization, writing, supervision, Bakhtawar Hameed, Shahid Iqbal: Experimentation, data collection, Shahnaz Khan, Hidayatullah Khan and Laiq Zaman: Data analysis, review. All authors approved the final manuscript.

Conflict of Interest: Authors has no conflict of interest.

Funding Resource: This research received no external funding.

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Received: March 10th 2026 **Accepted: April 15th 2026**