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The effect of long-term application of nitrogen-rich fertilizers on soil resistome: A study of conventional and organic cropping systems

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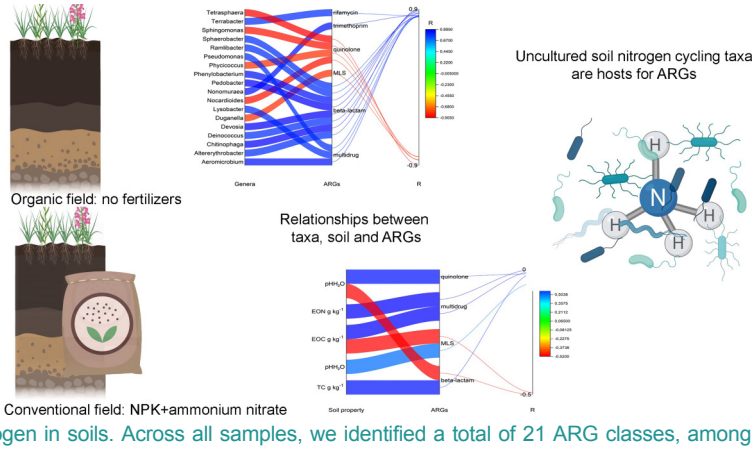
ABSTRACT

• Soil resistomes of conventional and organic systems were similar in terms of ARG biodiversity.
• Soil resistomes of conventional and organic systems were different regarding individual ARGs.
• Uncultivated bacteria and archaea can contribute significantly to soil resistome.



Metagenomic studies of various soil environments have previously revealed the widespread distribution of antibiotic resistance genes (ARGs) around the globe. In this study, we applied shotgun metagenomics to investigate differences in microbial communities and resistomes in Chernozem soils that have been under long-term organic and conventional cropping practices. The organic cropping system was seeded with *Triticum spelta* without any fertilizer. The conventional cropping system was seeded with *Triticum durum* Desf and used mineral fertilizer (NPK), that resulted in an increased amount of total and available carbon and nitrogen in soils. Across all samples, we identified a total of 21 ARG classes, among which the dominant were vancomycin, tetracycline and multidrug. Profiling of soil microbial communities revealed differences between the studied fields in the relative abundances of 14 and 53 genera in topsoil and subsoil, respectively. Correlation analysis showed significant correlations (positive and negative) among 18 genera and 6 ARGs, as well as between these ARGs and some chemical properties of soils. The analysis of metagenome-assembled genomes revealed that *Nitrospirota*, *Thermoproteota*, *Actinobacteriota* and *Binatota* phyla of archaea and bacteria serve as hosts for glycopeptide and fluoroquinolone/tetracycline ARGs. Collectively, the data obtained enrich knowledge about the consequences of human agricultural activities in terms of soil microbiome modification and highlight the role of nitrogen cycling taxa, including uncultivated genera, in the formation of soil resistome.

Keywords soil microbiome, inorganic fertilizer, nitrogen cycle, uncultured bacteria, chemolithotrophs, *Binatia*

1 Introduction

The concept of the resistome as the collection of all genes responsible for resistance to antibiotics and their precursors was introduced in 2006 in a study that demonstrated the extent of the spread of antibiotic resistance genes (ARGs) in soil ecosystems (D'Costa et al., 2006). The widespread use of metagenomic approaches in studying natural habitats has shown that resistance genes in natural microbial communities are diverse and ubiquitous, with presence in regions of Alaska and the Arctic untouched by human activity (Allen

et al., 2009; Van Goethem et al., 2018).

Active research is still being conducted to understand the natural and anthropogenic drivers that determine the resistome. The question of the contribution of anthropogenic activity to the resistome phenomenon is of particular interest, as the answer obtained can serve as the basis for any legislative decisions (Kaviani et al., 2022). The general questions can be formulated as follows: How exactly does the composition of the soil microbial community correlate with the composition of ARGs? What is the contribution of external factors (fertilizers, pesticides, etc.) to the richness and shape of the resistome?

Some studies have shown that anthropogenically modified lands such as croplands did not show significantly different levels of ARG richness or diversity compared with most other biomes (Qian et al., 2021; Delgado-Baquerizo et al., 2022). Other studies have provided evidence to the contrary. For example, the analysis of metatranscriptomic data sets from microbiomes revealed that soil from agricultural origins had significantly more diversity of ARGs per sample than was the case for forest soil (Lawther et al., 2022).

A study of soils collected from fields in the organic farming system compared to conventional farms could provide some answers to the aforementioned questions; that is, the organic soil microbiome can serve as a baseline for investigating the ecology of antibiotic resistance on farms and in fields (Cadena et al., 2018). Conventional farming systems use pesticides, which are one of the significant factors in the selection of antibiotic-resistant bacteria; these substances can come from different chemical (or functional) classes, but their use has led to an increase in the diversity and richness of ARGs (Qiu et al., 2022). On the other hand, in organic farming, biopesticides are used, which are reservoirs of clinically relevant antimicrobial resistance genes (Kaze et al., 2021). However, the most significant factor determining soil resistome is application of fertilizers, without which modern agriculture would be impossible. A number of studies have shown that the impact of organic fertilizers on soil resistome is more significant than that of mineral fertilizers. Those studies also confirmed that chemical fertilizer significantly modulates soil bacterial composition (Liu et al., 2017; Xie et al., 2018; Wang et al., 2020; Liu et al., 2022). Fertilizer application changed the physicochemical parameters of soils, leading to changes in the taxonomic profile of the microbiome (Dincă et al., 2022). Urea and ammonium bicarbonate significantly increased both soil pH and nitrification rates, while ammonium sulfate did not affect soil pH (Wang et al., 2020B). Zishu et al. (2023) convincingly demonstrated that soil pH plays a pivotal role in shaping the resistome.

Another important factor determining the richness and diversity of the soil microbiome is the vertical distribution of nutrients in the soil profile. For example, soils from fields fertilized with manure have been found to be enriched in phosphorus in the topsoil and nitrate in the subsoil (Edmeades, 2003). While the vertical distribution of nutrients in the soil and the related microbiome changes have been studied in many researches (Pathak and Reddy, 2021), much less is known about the vertical distribution of ARGs (Li et al., 2023).

In this study, we conducted a comparative investigation of the diversity and abundance of soil resistomes of the conventional and organic fields that received different amounts of nitrogen. Our main research tool was shotgun sequencing, which allowed coverage of a wide range of ARGs and direct identification of ARG hosts in the soil microbiome. We investigated how the microbiome and soil resistome are connected to each other and how the resistome is distributed when going deeper into the soil.

2 Materials and methods

2.1 Soil sampling

Soil samples were collected from two agricultural lands with Chernozems soils, located in the temperate continental climate zone in the Russian Federation, at the end of April 2022. The collected soils represented organic and conventional cropping systems. The chosen fields were seeded by wheat *Tríticum durum* Desf (conventional) and *Triticum spelta* (organic). The studied plots were located on arable soils used in the zonal crop rotation regime.

Conventional cropping system (CCS): Crop rotation was winter wheat−winter wheat−peas−winter wheat. Crop rotation had been carried out since 2003 (five full cycles of rotation). The field was treated with mineral fertilizers: diamofoska NPK(S) 10:26:26(2): N-10%, P_2O_5-26%, K_2O-26%, MgO-0.3%−1%, S-2% (PhosAgro, Russia)−100 kg ha^{-1}; ammonium nitrate−150 kg ha^{-1}. Seeds were treated with fungicides “Strike forte” (flutriafol + tebuconazole, 75 + 225 g L^{-1}). Coordinates of the field: 47°20′09.0" N, 38°18′21.3" E.

Organic cropping system (OCS): Crop rotation was peas−rye−lentils−spelt. Crop rotation had been carried out since 2011 (three full cycles of rotation). Seeds were treated with biological preparations “BSKA-3” (*Trichoderma viride, Pseudomonas koreensis, Bacillus subtilis, Bradyrhizobium japonicum*) and “Geostim” (*Trichoderma viride, Azomonas agilis, Azotobacter chrooccocum*) (Biotechagro, Russia). Fertilizers (organic or mineral) were not applied. Coordinates of the field: 47°20′55.7″ N, 38°18′46.3″ E.

Soil sampling took place at the end of the crop rotation during the growth of winter wheat (CCS) and spelta (OCS) Soil sampling was carried out during the middle stage of plant growth. At each study location (conventional and organic fields), four spatially remote sites were chosen. At each chosen site (1 m^2), the upper soil layer (0–5 cm) and

subsoil layer (5–15 cm) were collected using the "checkerboard" sampling method (four points in the corners and one in the center, then pooled in a plastic bag). Samples then were placed into plastic bags and delivered to the laboratory within 48 h. The samples were stored at −80°C prior to DNA extraction. Soil DNA was extracted from 250 mg of soil using a Quick-DNA Fecal/Soil Microbe Kit (Zymo Research, US) according to the manufacturer's protocol. DNA concentration was determined using a Qubit 4.0 Fluorometer (Thermo Fisher Scientific, US). DNA quality (A_{260}/A_{280}) was checked by NanoPhotometer N120 (Implen, US).

2.2 Physico-chemical soil analysis

The particle size distribution (soil texture) was determined by the Integral Suspension Pressure method (ISP) (Durner et al., 2017). The soil was dried to an air-dry state and treated with a 30% H_2O_2 solution and a 4% $Na_4P_2O_7$ solution. Physical dispersion was performed using a Scientz-IID ultrasonic probe homogenizer (Ningbo Scientz Biotechnology Co, China). The soil texture was determined using a Pario instrument (Meter Group, Inc, USA; instrument error 3%). The sand fraction was isolated from the suspension by wet sieving using an Analysette 3 vibrating screen (Fritsch, Germany) with 0.05, 0.25, and 0.5 mm sieves. The results were processed using the Pario Control program.

The soil texture was consisted of clay (28%), silt (26%), and sand (clay loam) (46%). Soil pH was measured according to the international standard ISO 10390. The pHH_2O value was determined in an aqueous solution at a soil:solution ratio of 1:5 using a pH-meter Orion Star A 111 (Thermo Scientific,Waltham, MA, USA) (Pansu and Gautheyrou, 2007). The total carbon (TC) and total nitrogen (TN) contents were measured using a Vario EL III elemental analyzer (Elementar, Langenselbold, Germany). Soil organic carbon (SOC) was determined by pretreatment of the samples with 10% HCl solution to destroy carbonates and bicarbonates (Pansu and Gautheyrou, 2007). Extractable organic carbon (EOC) and nitrogen (EON) in the soil were measured according to Vance et al. (1987) and Chen et al. (2021). A solution of 0.5 M K_2SO_4 (20 mL) was added to the soil samples (5 g), then shaken for 1 h on a rotator. The resulting extracts were filtered, frozen, and dried under vacuum, followed by determination of the extracted carbon on a Vario EL III C:N analyzer (Elementar, Germany). Determination of soluble phosphorus was performed as follows: 5 g sample of soil was transferred to a conical flask and 25 mL of 0.2N hydrochloric acid was added. 3 mL of the filtrate is transferred to a flask and added to 40 mL with distilled water. 2 mL of a 2.5% solution of ammonium molybdate in sulfuric acid were added, followed by 3 drops of stannous chloride solution. The contents of the flask were shaken and after 10 min the optical density of the colored solution was determined at 650 nm.

2.3 Metagenomic sequencing

Metagenomic sequencing was performed using HiSeq X Ten Illumina platform and a 2×150 bp paired-end sequencing reagent kit. Raw reads were deposited in the NCBI Sequence Read Archive (SRA) under the BioProject PRJNA974203. Preparation of reads for metagenome assembly was as follows. The quality of received reads using FastQC v.0.11.9 (available at bioinformatics.babraham) was checked. Reads from artificial sequences and nucleotides with poor read quality using Trimmomatic v.0.39 (Bolger, Lohse and Usadel, 2014) were cleared. The quality of cleaned reads using FastQC was checked. Thereafter, sequencing of 16 DNA samples yielded a total of 700 829 024 paired reads. After purification, 407 386 189 paired reads were used for subsequent analysis (Table S1). Reads filtered by quality were assembled *de novo* using metaSPAdes v. 3.15.0 using default options (Nurk et al., 2017). The quality of the resulting metagenomic assemblies was assessed using QUAST v. 5.0 (Gurevich et al., 2013). These assemblies had lengths from 144.3 to 202.9 Mb and their GC (guanine-cytosine) compositions varied from 62.7% to 65.05%.

Taxonomic affiliation was assigned to qualitatively filtered reads using Kaiju v. 1.5 with NCBI BLAST non-redundant protein database NR v.2017-05-16. The significance of difference among the structure of microbial communities at the phylum level was analyzed by a principal coordinate analysis (PCoA) using the Bray-Curtis distance with the Vegan and ggplot2 packages in R (v. 4.2.1). We implemented two-way PERMANOVA based on the Bray−Curtis similarity and 9 999 permutations using Past v.4.06b (Hammer et al., 2001).

The assembled metagenomes were separated into bins (MAGs) using MaxBin 2.0 (Wu, Simmons and Singer, 2016), MetaBAT 2 (Kang et al., 2019), and VAMB (Sieber et al., 2018) before dereplication and refinement with the DAS Tool (Okonechnikov et al., 2016). Genome coverage was assessed using QualiMap 2 v. 2.2.2 and Bowtie 2 v. 2.3.5.1. Completeness and contamination were assessed using CheckM v. 1.1.3 (Parks et al., 2015). Medium- and high-quality MAGs, determined by a completeness of $\geqslant$ 50% and a redundancy of < 10% according to the MIMAG standard (Bowers et al., 2017) were kept for downstream analyses. Open reading frames and genes were predicted using Prokka v.1.14.5 (Seemann, T. 2014). The taxonomic position of the assembled genomes was determined using GTDB-Tk v. 2.0.0 (Chaumeil et al., 2019). The metagenome-assembled genomes were annotated using online servers antiSMASH v.7.0 (Blin et al., 2021) and RGI (Resistance Gene Identifier)

6.0.1, Comprehensive Antibiotic Resistance Database (CARD) v.3.2.6 (Alcock et al., 2023). The protein functional annotation was performed using the domain-based annotation tool reCOGnizer (version 1.9.2) (available at anaconda.org). The results derived from the clusters of orthologous groups of proteins (COGs) database (Galperin et al., 2014) and Pfam domens (Finn et al., 2016) were used for functional categorization.

2.4 Metagenome analysis: analysis of ARGs

Clipped paired reads were analyzed for the presence of ARGs using the ARGs-OAP 2.0 pipeline as described (Yang et al., 2016; Yin et al., 2018) using the CARD, the Structured Antibiotic Resistance Genes (SARG) database. Greengenes database, comprising 16S rRNA genes, was used for the ARGs abundance normalization. The parameters used for ARG identification were alignment length cut-offs of 75 nucleotides, alignment *e* value cut-off of 10^{-7}, and alignment identity of 80%. The abundances of ARGs were normalized by 16S rRNA gene expressed using the formula (Li et al., 2015):

$$\text{Abundance} = \sum_{1}^{n} \frac{{}^{N}\text{ARG-like sequence} * {}^{L}\text{reads}/{}^{L}\text{ARG reference sequence}}{{}^{N}16\text{S sequence} * {}^{L}\text{reads}/{}^{L}16\text{S sequence}}$$

where ^{N}ARG-like sequence is the number of ARG-like sequences (reads) annotated as one specific reference ARG sequence; ^{L}ARG reference sequence is the length of the sequence corresponding to a specific ARG reference sequence; ^{N}16S sequence is the number of 16S rRNA sequences identified in the metagenomic data by mapping reads against the Greengenes database using minimap2 (Li et al., 2018); ^{L}16S sequence is the average length of the 16S rRNA sequence in the Greenenes database; and ^{L}reads is the length of the Illumina read sequence.

ARG results from ARGs-OAP 2.0 were analyzed for alpha and beta diversity using the Vegan package in R. The heat map was visualized using the pHeatmap package in R. Principal coordinate analysis using the Bray−Curtis distance was performed in the Vegan and ggplot2 packages in R.

2.5 Statistical processing

Averages, standard deviations, fold change values and Shannon indices of all data were calculated using Microsoft Excel. The correlation between microbial communities and ARGs was examined by Spearman's correlation coefficient R_s. A correlation matrix was constructed by calculating all possible pair-wise Spearman's rank correlations between the ARG classes and microbial genera to visualize the correlations in the network interface. A correlation between two items was considered statistically robust when R_s was greater than 0.8 and the *p*-value was less than 0.01. A network graph was visualized in OriginPro 2021 (OriginLab Corporation). Pearson's correlation coefficients (R_p) between ARGs and soil chemical properties were calculated and visualized using Past v.4.06b. Redundancy Analysis (RDA) was performed in Past v.4.06b

3 Results

3.1 The chemical properties of soils and functional potential of soil microbiome

According to pHH_2O, the studied soils were weakly alkaline and did not differ from each other (Table 1). A significant increase in the content of total carbon and total nitrogen (by 1.6 times, $p < 0.05$ and 3.5 times, respectively) was revealed for the subsoil of the conventional field (Table 1). There were no significant differences in the content of SOC

Table 1 Chemical properties of the soils.

Soil chemical properties	Cropping systems			
	Organic		Conventional	
	0−5 cm	5−15 cm	0−5 cm	5−15 cm
pHH_2O	7.82 ± 0.47	7.91 ± 0.15	7.65 ± 0.23	7.76 ± 0.28
TC g kg^{-1}	32.06 ± 2.39	29.52 ± 3.36	29.63 ± 4.13	47.37 ± 13.75 *
TN g kg^{-1}	4.24 ± 0.80	4.17 ± 1.00	4.14 ± 0.63	14.44 ± 8.27 #
TC:TN	7.96 ± 1.06	7.53 ± 1.15	7.56 ± 1.45	4.24 ± 2.06 #
EOC g kg^{-1}	2.38 ± 0.14	1.21 ± 0.28	2.80 ± 0.80	1.43 ± 0.12
EON g kg^{-1}	0.56 ± 0.09	0.37 ± 0.14	2.77 ± 2.25 *	1.50 ± 1.09 *
SOC g kg^{-1}	31.39 ± 11.63	27.1 ± 0.00	27.05 ± 3.64	27.83 ± 1.95
AP g kg^{-1}	74.63 ± 1.85	67.06 ± 3.36	15.97 ± 0.70*	13.82 ± 0.83*

The statistical difference between pair "organic-conventional". # $p < 0.05$ (two-sample *t*-test); * $p < 0.05$ (Wilcoxon signed ranks test).TC, total soil carbon; TN, total soil nitrogen; EOC, extractable organic carbon; EON, extractable organic nitrogen; SOC, soil organic carbon; AP, available phosphorus.

and EOC between the two cropping systems, but the content of EON was higher in the subsoil of the conventional system ($p < 0.01$) (Table 1). Interestingly, the content of extractable phosphorus in soils of the organic system was five times higher ($p < 0.05$) than that in soils of the conventional system (Table 1).

To understand the functional potential of soil microbiomes in nitrogen cycling, we analyzed the relative abundance of relevant functional genes. In the metagenomic pools, 10 genes were identified whose products are involved in the decomposition of organic nitrogen, and 13 genes involved in denitrification (Fig. S1). Among them, 3 genes had significantly different abundances in the two studied fields. The *nrtA* gene (ABC transporter nitrate binding protein) in the topsoil layer ($p < 0.05$) and the *narK* gene (nitrate/nitrite transporter) in the subsoil layer ($p < 0.05$) were more abundant in the microbiome of the conventional cropping system than in the organic microbiome. The *nifD* gene (nitrogenase Mo-Fe protein) was more abundant in both topsoil ($p < 0.01$) and subsoil ($p < 0.01$) of the conventional cropping system than in the organic one. No N-cycling gene was identified that was more prevalent in the organic cropping system than in the conventional cropping system.

3.2 Composition of prokaryotes in organic and conventional cropping systems

It was found that the soil microbial profile on phyla level did not depend on the cropping system, but differed in relation to the depth of soil sampling (Fig. 1A). Two-way PERMANOVA test indicated that the sampling depth variable significantly affected the abundance of soil microbial

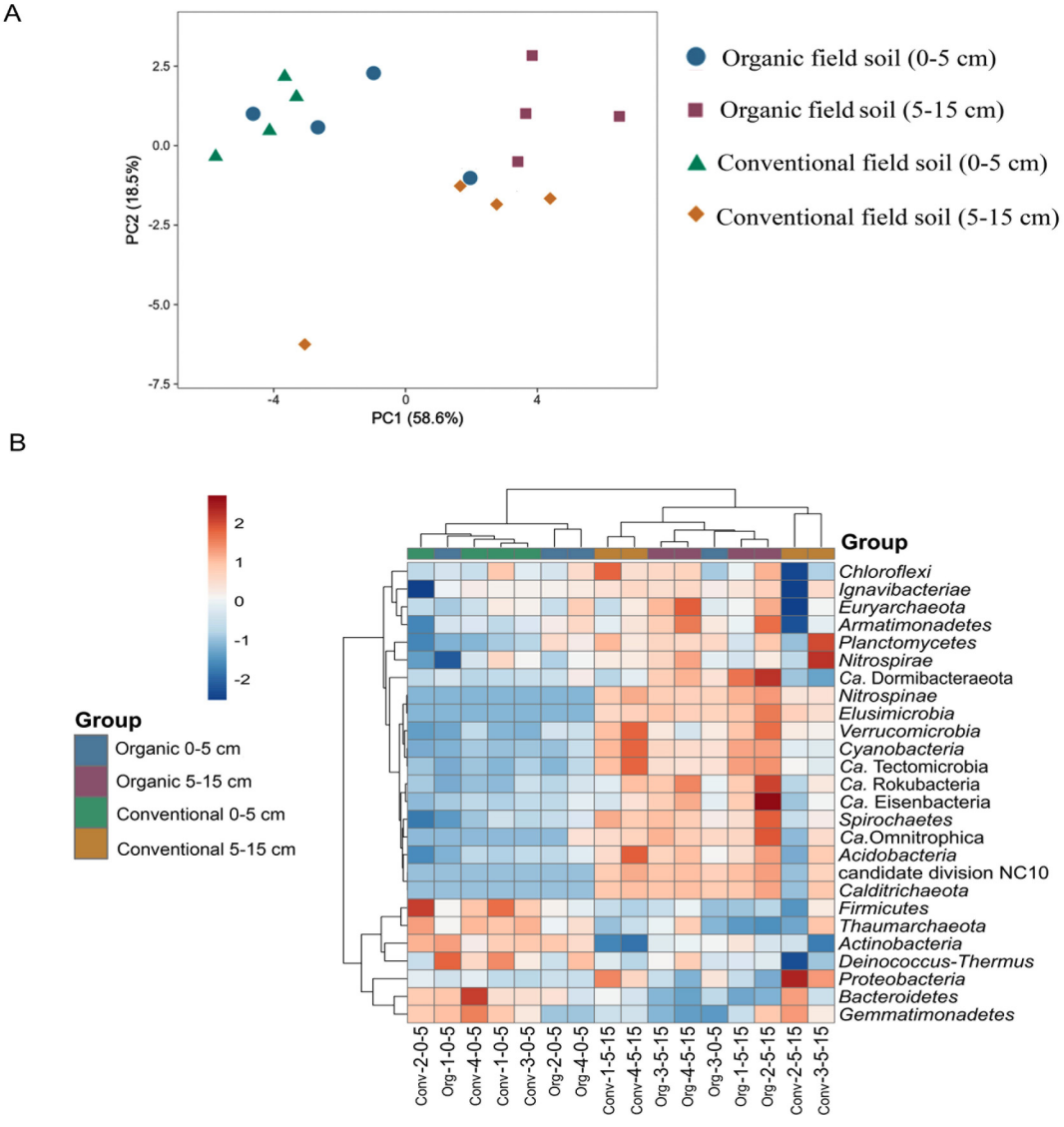


Fig. 1 Beta-diversity of soil microbial community of organic and conventional cropping systems. PCoA analysis of variations in the relative abundance of prokaryotic phyla (A). Heat map of the most common prokaryotic phyla, built on the basis of the average abundance for all samples (B).

community at phyla level (F = 9.78, p < 0.01) (Table 2).

Metagenomic shotgun sequencing revealed 28 prokaryotic phyla. Among these, *Actinobacteria* (*Actinomycetota*), *Proteobacteria* (*Pseudomonadota*), *Acidobacteria* (*Acidobacteriota*), *Chloroflexi* (*Chloroflexota*), *Bacteroidetes* (*Bacteroidota*), *Planctomycetes* (*Planctomycetota*), *Thaumarchaeota*, *Firmicutes* (*Bacillota*), and *Gemmatimonadetes* (*Gemmatimonadota*) (Fig. 1B) were dominant phyla and together accounted for 50%−55% of the total reads in the studied soil groups. *Thaumarchaeota*, *Firmicutes* and *Gemmatimonadetes* were found to be more abundant in the topsoil (0−5 cm) of the conventional field soils than in the organic field soils (p < 0.05) (Fig. 1B). The relative abundance of other dominant phyla did not significantly differ between the compared groups. In the subsoil (5−15 cm), *Actinobacteria* was more abundant in the organic soils, while *Proteobacteria* and *Bacteroidetes* prevailed in the conventional soils (p < 0.05) (Fig. 1B).

Eight phyla were identified as having a moderate level of presence, since they accounted for 2.86%−4.29% of the total reads in the studied soil groups. Only one phylum, *Candidatus* Rokubacteria, was significantly more abundant in the organic soils (p < 0.05). Among the minor presented phyla (0.23%−0.46%), candidate phyla *Eisenbacteria* and *Dormibacteraeota* were the taxa that were more abundant in the organic soils (5−15 cm) than in the soils of conventional field (Fig. 1B).

Analyzing the soil taxonomic compositions at the level of genera, we found that the Shannon indices of soil microbial diversity in the organic and conventional fields did not differ between the two cropping systems (p > 0.05) (Fig. 2A). However, differences were found in the relative abundances of 14 genera in the topsoils (Fig. 2B) and 53 genera in the subsoils (Fig. 2C, Table S2). In the topsoil, *Streptomyces* (−5.54 ± 3.86 $\log_2$fold, p < 0.05) was less abundant, while *Streptosporangium* (6.17 ± 2.68 $\log_2$fold, p < 0.01) were more abundant, in the conventional field soils than in the organic field soil (Fig. 2B). In the subsoil all 53 genera predominated in the conventional field soil (Fig. 2C). Among them, the difference in the relative abundance of *Massilia, Skermanlla, Pseudomonas, Gemmata*, and *Bradyrhizobium* was calculated as having a $\log_2$-fold change value greater than 1.0 (Fig. 2C).

Two-way PERMANOVA indicated that sampling depth (F = 2.84, p < 0.05) and cropping system (F = 2.50, p < 0.05) variables contributed significantly to the difference in the relative abundance of microbial genera (Table 2).

RDA was performed to analyze the relationships between the relative abundance of microorganisms at phylum level and environmental factors (soil sampling depth and cropping

Table 2 Two-way PERMANOVA based on Bray−Curtis similarity output of the effects of cropping systems, sampling depth and their interactions on soil microbial communities and ARGs.

Microbial phyla					
Source	Sum of sqrs	df	Mean square	*F*	*p*
Sampling depth	0.019 557	1	0.019 557	9.782 2	0.000 3
Cropping systems	0.005 148	1	0.005 148	2.574 7	0.071 1
Interaction	0.005 608	1	0.005 609	2.805 3	0.058 6
Residual	0.023 991	12	0.001 999		
Total	0.054 304	15			
Microbial genera					
Source	Sum of sqrs	df	Mean square	*F*	*p*
Sampling depth	0.091 005	1	0.091 01	2.837	0.016 5
Cropping systems	0.080 175	1	0.080 18	2.499	0.026 7
Interaction	0.128 887	1	0.128 89	4.018	0.000 7
Residual	0.384 935	12	0.032 08		
Total	0.685	15			
ARGs					
Source	Sum of sqrs	df	Mean square	*F*	*p*
Sampling depth	0.004 198	1	0.004 198	1.918	0.128 1
Cropping systems	0.007 532	1	0.007 533	3.441	0.031 1
Interaction	0.002 268	1	0.002 268	1.036	0.366 6
Residual	0.026 265	12	0.002 189		
Total	0.040 264	15			

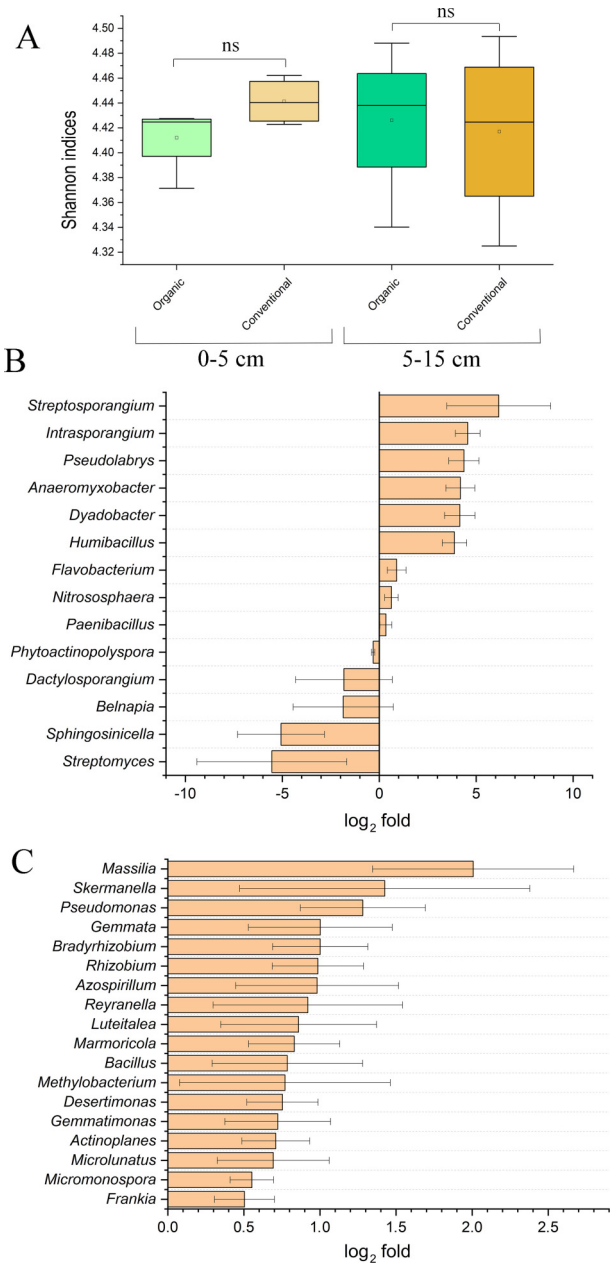


Fig. 2 The differences in soil bacterial community compositions of organic and conventional cropping systems. The Shannon biodiversity indices of soil microbial communities of the various cropping systems and sampling depths (A). The relative abundance of microbial genera expressed as the $\log_2$fold change values between conventional and organic cropping systems. Only genera with significance difference ($p < 0.05$, two-sample *t*-test) between two cropping systems are presented. Soils were sampled at the topsoil (0−5 cm) (B) and the subsoil (5−15 cm) (C).

system) showed dependence of *Actinobacteria* on the topsoil, while *Acidobacteria* showed stronger dependence on the subsoil; the abundance of *Proteobacteria* was strongly determined by the conventional cropping system (Fig. S2A). ($R = 0.64$, $F = 11.42$, $p < 0.01$).

3.3 Analysis of metagenome-assembled genomes

The raw reads were filtered and then *de novo* assembled using metaSPAdes. The resulting metagenomes were further divided into bins, resulting in the reconstruction of 46 metagenome-assembled genomes belonging to the bacterial and archaeal domains (Table S3). Analysis using Resistance Gene Identifier to predict AGR presence, revealed that genomes of JAFAQB01 (*Nitrososphaeraceae*) ($n = 7$), *Entotheonellia* bacterium ($n = 1$), did not contain any ARGs (Table 3).

The genomes ($n = 9$) of genus WHTF01, which belong to *Binatia* class in *Binatota* phylum (Chuvochina et al., 2019), were reconstructed with an average completeness of 72.06 ± 6.4%. Analysis using the CARD database revealed the presence of resistance genes *vanT* and *adeF*, which are associated with lipopeptide and tetracycline-fluoroquinone resistance, respectively (Table 3). Additionally, annotation of the genomes for the presence of biosynthetic gene clusters (BGCs) revealed the presence of the sequences that are associated with BGCs involved in biosynthesis of carotenoid, zeaxanthins and others (Table 3). Genomes ($n = 3$) of JACDAN01 bacterium belonging to *Gaiellaceae* family were assembled with average completeness 60.04 ± 7.25% and contained sequences assigned to tetracycline and vancomycin resistance genes (Table 3). *Nitrososphaera* sp. genomes ($n = 16$) were assembled with average completeness 91.27 ± 3.04%, and only one annotated genome contained a sequence identified as *catI* gene, which determines resistance to phenicol antibiotic (Table 3). Genome of *Nitrospira_C* sp. (*Nitrospiraceae*) was reconstructed from conventional (5−15 cm) field soil with completeness 60.03% and found to contain sequence assigned to the glycopeptide resistance gene cluster (Table 3). *Propionibacteriaceae* genomes ($n = 8$) were reconstructed with average completeness 76.53 ± 10.5% and contained the glycopeptide resistance gene cluster *vanW* (Table 3). It was found that many of the annotated taxa are potential producers of antimicrobial metabolites (Table 3). Genome annotation showed that only *Nitrososphaera* sp. and *Nitrospira_C* contained both ARGs and clusters for ribosomally synthesized and post-translationally modified peptide classes (RiPPs).

3.4 Resistome composition and abundance in soils of organic and conventional cropping systems

ARGs were identified across all sample types, with the ability to confer resistance to 21 different antibiotic classes. The abundance of these genes ranged from 10^{-4} to 10^{-1} copies of ARG per copy of the 16S rRNA gene. Vancomycin, aminoglycosides, and multidrug resistance were the most abundant ARG classes across all samples (Fig. 3A). While most of the ARGs were present in all samples, trimethoprim,

Table 3 Annotation of assembled genomes for ARGs and biosynthetic gene clusters.

Taxon	ARG family	Drug class	Resistance mechanism	BGCs
WHTF01 (class *Binatia*)	*vanT,* glycopeptide resistance gene cluster	Glycopeptide antibiotic	Antibiotic target alteration	Terpene (carotenoid) APE Vf, aryl polyenes
	adeF, Resistance-nodulation-cell division (RND) antibiotic efflux pump	Fluoroquinolone antibiotic, tetracycline antibiotic	Antibiotic efflux	
Not identified genus (class *Entotheonellia*)				Redox-cofactors (lankacidin C), phosphonate, hglE-KS, terpene, aryl polyenes
JACDAN01 (family *Gaiellaceae*)	*vanW*, glycopeptide resistance gene cluster	Glycopeptide antibiotic	Antibiotic target protection	
	otr(A)S.rim	Tetracycline-resistant ribosomal protection protein	Antibiotic target protection	
Nitrososphaera sp.	*catI,* chloramphenicol acetyltransferase (CAT)	Chloramphenicol antibiotic	Antibiotic inactivation	Thioamitides RRE-containing
JAFAQB01 (family *Nitrososphaeraceae*)				RRE-containing, RiPP-like
Nitrospira_C (family *Nitrospiraceae*)	*vanT* glycopeptide resistance gene cluster	Glycopeptide antibiotic	Antibiotic target alteration	RRE-containing, RiPP-like, phosphonate, terpene, aryl polyenes,
Not identified genus (family *Propionibacteriaceae*)	*vanW* glycopeptide resistance gene cluster	Glycopeptide antibiotic	Antibiotic target alteration	
Not identified genus (order *Solirubrobacterales*)				Beta-lactone

bleomycin, carbomycin, and pyromycin were only sporadically detected. Principal coordinate analysis revealed that ARG abundance was distributed differently across study groups (Fig. 3B). Two-way PERMANOVA showed that the soil cropping system is the significant variable (F = 3.44, p < 0.05), while the sampling depth is not significant (F = 1.92, p > 0.05) (Table 2). Calculated Shannon diversity indices suggested that all study groups had similar ARG alpha-diversity (Fig. 3C). RDA showed interrelationships of aminoglycoside, bacitracin, rifamycin with the topsoil, and of MLS and vancomycin with the subsoil. At the same time, the abundance of tetracyclin and multidrug ARGs was determined by the organic and conventional cropping systems, respectively (Fig. S2 B) (R = 0.34. F = 3.34, p < 0.05).

Comparing the relative abundance of ARGs between two cropping systems, we found that the studied samples had differences in the copy number of 6 ARGs (Fig. 3D). In the organic field topsoil, there were significantly more copies of genes resistant to tetracycline and macrolide-lincosamide-streptogramin (MLS) than in the corresponding layer of conventional field soil. In the organic field subsoil only quinolone ARG was found to be prevalent. Beta-lactams, fosmidomycin, and multidrug ARGs were significantly more abundant in the conventional field soil than in the organic soil (Fig. 3D).

The investigation into the resistome composition uncovered 23 distinct families of ARGs (Fig. 4A). The genes resistant to the glycopeptide antibiotic vancomycin were the most abundant, including *vanY, vanR, vanH, vanT*, *vanW* and *vanX*, among which *vanW* and *vanY* genes were present in the largest number of copies (Fig. 4A). In addition, genes encoding three types of different efflux pumps were found, among which the major facilitator superfamily (MFS) antibiotic efflux pump was dominant (Fig. 4A). Other common ARGs included the aminoglycoside O-phosphotransferase family gene APH(3′), which determines resistance to aminoglycoside antibiotics (Fig. 4A). Genes conferring resistance to tetracycline (tetracycline-resistant ribosomal protection protein) and chloramphenicol (chloramphenicol acetyltransferase (CAT)) also constituted a pool of abundant ARGs (Fig. 4A).

Analyzing the distribution of ARGs by resistance mechanism, four mechanisms were identified, with antibiotic target alteration being the predominant mechanism and antibiotic inactivation being a minor mechanism. When comparing the samples to each other, no predominance of any cell detoxification mechanism was found (Fig. 4B).

3.5 Correlation analysis

Spearman's correlation coefficient showed significant correlations (positive and negative) among 18 genera and 6 ARG abundances grouped by drug class. Beta-lactam drug class positively correlated with 8 genera (Fig. 5A). Interestingly, all these genera prevailed in their relative abundance in the subsoil layer of the conventional field. Multidrug class ARG showed positive Spearman's correlation with 3 genera, all of them were more significantly abundant in the subsoil layer of

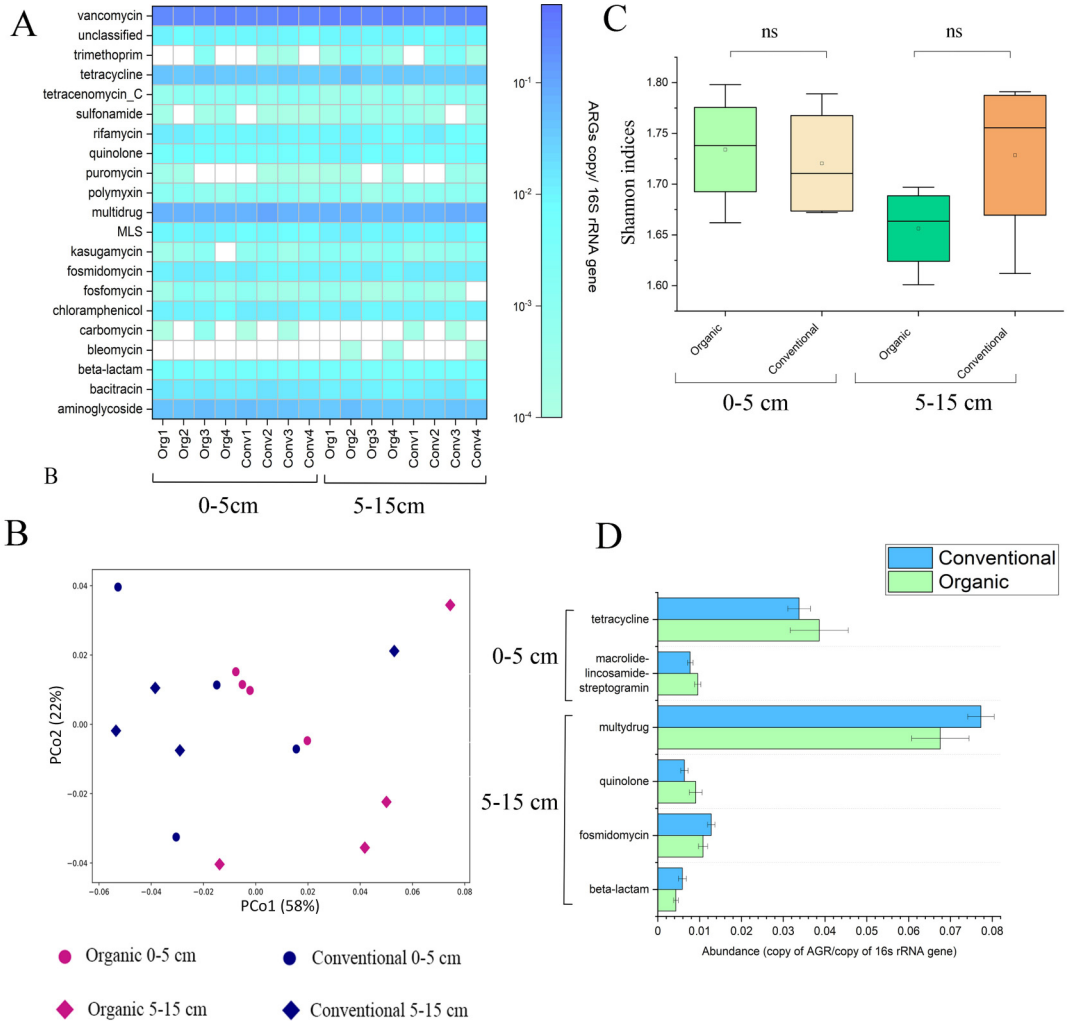


Fig. 3 Heatmap showing the abundance of ARG classes across various cropping systems and soil sampling depth (A). PCoA analysis of ARGs distribution across the samples (B). Shannon diversity indices of soil resistomes (C). The histogram of the distribution of ARGs among the soil samples studied, selected from the topsoil (0−5 cm) and subsoil (5−15 cm) of conventional and organic cropping systems. Only antibiotics for which differences between samples were significant ($p < 0.05$, two-sample *t*-test) are shown (D).

the conventional fieldthat that in the organic field (Fig. 5A). Rifamycin and trimethoprim positively correlated with *Terrabacter* and *Momomurae*, respectively (Fig. 5A), however the relative abundance of these genera did not differ between organic and conventional cropping systems. MLS and quinolone drug classes showed negative correlation with 1 and 4 genera, respectively (Fig. 5A).

Choosing an ARG and assuming the genera, which are highly correlated to the ARG, to be the ARG predictors, we may apply multivariate regression technique to establish the "weight" of each predictor in the corresponding approximate expression for the ARG quantitative characteristic (Alexopoulos 2010; Rubinfeld 2011). We considered multidrug and its expected predictors (according to Fig. 5A): *Aeromicrobium*, *Lysobacter*, and *Pseudomonas*. The normalized "weights" of *Aeromicrobium*, *Lysobacter*, and *Pseudomonas* were 1, 2.32, and 1.01, respectively, and the quantitative characteristic of multidrug ARG was approximated as follows:

multidrug = 6.27 × *Aeromicrobium* + 14.55 × *Lysobacter* + 6.33 × *Pseudomonas* + 11.97 (Fig. 5B).

The coefficient of determination R^2 in this approximation model is 0.83. According to the correlation analysis, beta-lactam may have eight predictors: *Altererythrobacter*, *Chitinophaga*, *Deinococcus*, *Devosia*, *Pedobacter*, *Phenylobacterium*, *Ramlibacter*, and *Sphaerobacter*. Similar analysis yields the results depicted in Fig. 5B, and an approximate model of the quantitative characteristic of beta-lactam ARG could have $R^2 = 0.9$. However, analysis of covariance of the quantitative characteristics of these predictors reveals that they are rather highly correlated (in the case of multidrug, there is no significant correlation between the predictors) (Fig. S3).

Pearson correlation analysis between ARGs and soil

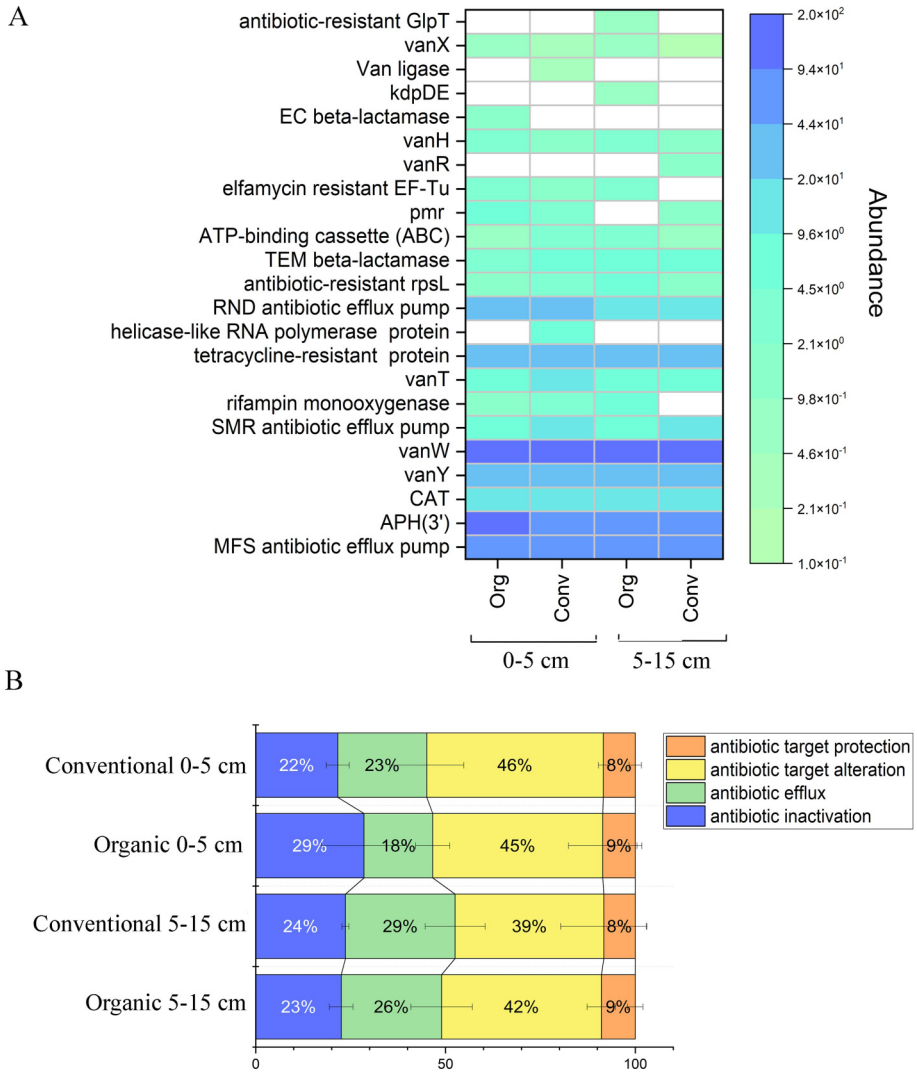


Fig. 4 Heatmap of distribution of ARG gene families (A) and resistance mechanisms (B) across the study soil groups. Data are presented as the mean gene copy numbers (A) and percent (B) in soils across various cropping systems and soil sampling depth.

chemical properties showed strong positive relationships among beta-lactam and TC (R_p = 0.61, p < 0.05), MLS and pHH_2O (R_p = 0.52, p < 0.05); multidrug and EOC (R_p = 0.63, p < 0.05), EON (R_p = 0.65, p < 0.05); quinolone with pHH_2O (R_p = 0.61, p < 0.05) and AP (R_p = 0.55, p < 0.05) tetracycline and AP (R_p = 0.54, p < 0.05) (Fig. 5C). Negative correlations were established for beta-lactam and pHH_20 (R_p = −0.52, p < 0.05); MLS and EOC (R_p = −0.50, p < 0.05) (Fig. 5 C). Thus, pH of soils and content of carbon and nitrogen were the most significant soil properties that determined ARG abundance.

4 Discussion

There are many factors that determine the shape of a soil resistome, among which the anthropogenic one is the most important because it can be legally controlled. In this work, we conducted a comparative study of soils of organic and conventional cropping systems in order to determine how differences in land use determine the differences in their resistome and which microorganisms are interconnected with these differences. The selected fields were characterized by similar parameters; the soils were represented by one type (Chernozems); the fields were sown with similar crops; the sites were located at a distance of 500 m from each other; crop rotation was used in both systems. The essential difference between them is the use of mineral nitrogen-rich fertilizers in the conventional cropping system.

Available studies indicate that the ARG classes that are widespread in soils include multidrug resistance, beta-lactam resistance, tetracycline resistance, and vancomycin resistance (Nesme and Simonet, 2015; Lawther et al., 2022; Delgado-Baquerizo et al., 2022; Liu et al., 2023). In our

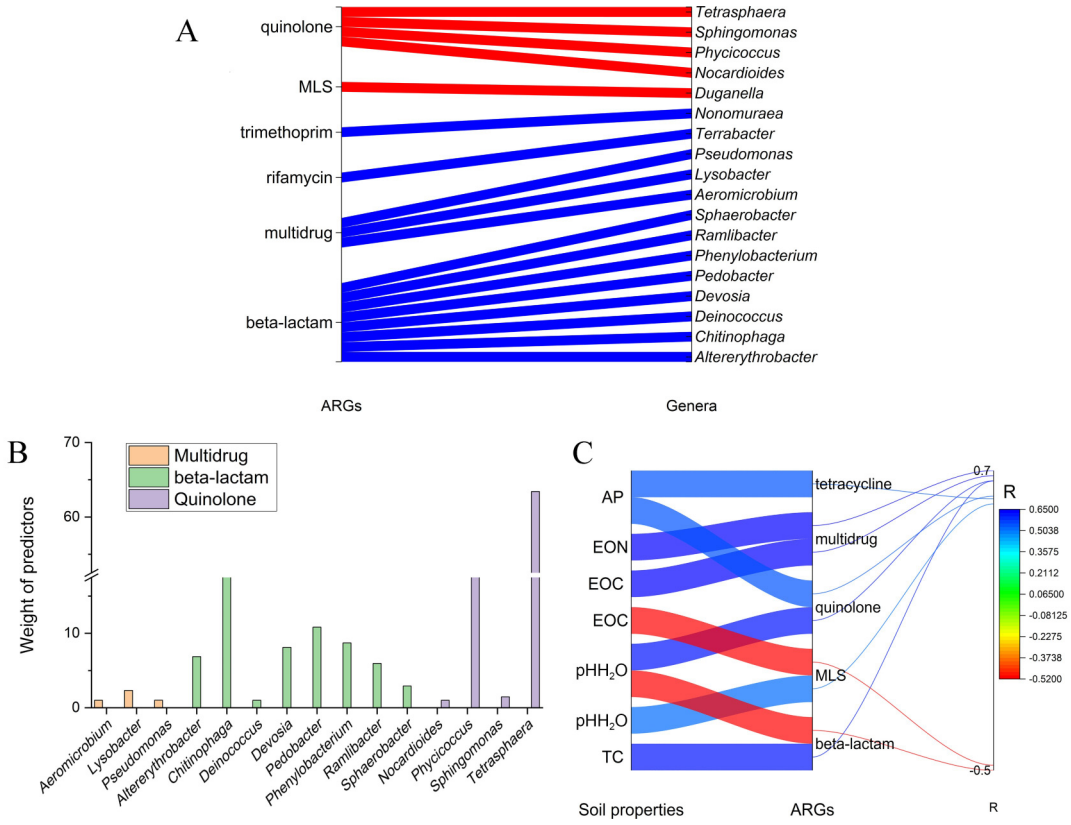


Fig. 5 Correlation analysis between genera, ARGs and soil chemical properties across all soil samples. Only genera with significative correlation (Spearman R_s > 0.8 and p < 0.01) with at least one ARGs are represented (A). Relative "weights" of predictors for multidrug and beta-lactam ARGs based on their abundance in soils (B). Heatmap of Pearson's correlation coefficients of the relative abundance of ARGs and the chemical properties of the soils (p < 0.05).

study the dominant classes of ARGs were vancomycin, tetracycline and multidrug resistance. The two studied cropping systems did not differ in biodiversity of ARGs, but did differ in the abundance of particular ARGs. It was found that the multidrug ARG was more abundant in the subsoil of the conventional cropping system; ARG for tetracycline was more abundant in the topsoil of organic system; the abundance of ARG for vancomycin did not differ between the compared groups. Two-way PERMANOVA calculations suggest that land use is the driving factor for resistome of the studied soils. Interestingly, soil depth also turned out to be a significant factor determining the differences between the compared groups.

There are limited number of works on the study of soil resistomes that take into account the soil depth. The depth-based data collected in the work of Cadena et al. (2018) revealed that *tet*(L) gene was detected more frequently in the surface soils (0–7.1 cm) than in subsoils (7.1–15.2 cm); the amounts of organic nitrogen, and of nitrate, were higher in surface soil than in subsoil (Cadena et al., 2018). Recently, a further study assessed the biodiversity and distribution of ARGs at different soil depths and it was found that the composition of ARGs in unfertilized soils and soils fertilized with inorganic fertilizers was similar at 0–40 cm depth (Li et al., 2023).

It is well known that the diversity and abundance of the resistome are closely dependent on the microbial community, which in turn is modulated by the physicochemical properties of soils (Fosberg et al., 2016; Hao et al., 2021; Rchiad et al., 2022). Availability of organic carbon, nitrogen, and other nutrients in soil varies with depth. For example, the work by Zhang et al. (2016) showed that the concentration of organic nitrogen, and of carbon, was greater in the surface layer (0−10 cm) than in the subsoil (10−30 cm), the same dependence on depth was shown by the analysis of the microbial biomass of these layers. Shotgun metagenomics have previously revealed that functional genes related to phosphorus metabolism, were particularly abundant at 30–60 cm, while functional genes related to metabolism of nitrogen, sulfur and carbohydrates, were more abundant in the topsoil depth (0−15 cm) (Rchiad et al., 2022).

Our study shows a similar relationship between the depth of the soil layers and the content of carbon and nitrogen. The conventional system was characterized by a large difference between the topsoil and the subsoil in terms of TC and TN content. We assume that the applied mineral fertilizers moved downward along the soil profile under the

influence of rainfall. Also, the applied N-rich mineral fertilizers determined a higher concentration of EON in the soils of the conventional system than in the organic systems.

The use of mineral fertilizers was reflected in a predominance in number of some microbial genera. In this work, a two-way PERMANOVA study showed that land use determined differences in ARG abundance and microbial genera abundance. Existing research shows that the use of fertilizers directly stimulates the growth of specific microbial populations that increase the organomineral content and fertility of the soil (Epelde et al., 2018; Dinca et al., 2022). It is necessary to understand how soil microbiomes specifically change under application of fertilizers, considering that organic fertilizers have a notable impact on altering resistomes, in contrast to mineral fertilizers, which do not exhibit significant effects (Liu et al., 2017; Xie et al., 2018; Wang et al., 2020[A]; Liu et al., 2022). In soils fertilized by organic amendments specific microorganisms that prefer nutrient-rich environments are prevalent (Francioli et al., 2016). NPK-fertilizers increased abundance of bacteria involved in N-cycling (Sun et al., 2015; Bill et al., 2021; Dinca et al., 2022). The potential of microbial communities for N-cycling has been identified in our work through metagenomic profiling of functional genes. It was found that the functional potential for decomposition of organic N and denitrification of the microbial community of the conventional system exceeds that of the organic one. Can typical soil microorganisms be a source of ARG, perhaps involving those taxa that are involved in the assimilation of inorganic fertilizers?

Spearman correlation analysis revealed a significant correlation between the ARGs profile and bacterial communities at the genera level. Three taxa of bacteria have a strong positive correlation with multidrug ARG: *Pseudomonas, Lysobacter* and *Aeromicrobium.* Interestingly, according to calculation, the contribution of the *Lysobacter* was significantly greater than that of the *Pseudomonas*. Bacteria of the genus *Lysobacter* are less studied in terms of beneficial properties than *Pseudomonas*, but there is a growing body of work reporting on the production of antibiotics and ability to fix nitrogen (Jochum et al., 2006; Ji et al., 2008; Iwata et al., 2010; Puopolo et al., 2010; Xu et al., 2016; Brescia et al., 2021). Thus, it is possible that rhizosphere and soil bacteria of the genus *Lysobacter* are even more important as carriers of ARGs than *Pseudomonas*. Overall, strong positive correlations were established for bacterial genera, among which there were taxa that produce antibiotics and are resistant to them, as well as typical soil microorganisms.

Shotgun sequencing provides opportunity to study a microorganism′s genome without isolation of microbial cells in pure culture (Rodríguez-Ramos et al., 2022; Zishu et al., 2023). One of the main taxa present in all the investigated soil samples was the genus WHTF01 belonging to the class *Binatia* (phylum *Candidatus* Binatota (UBP10)). The genome of this non-cultivable bacterium encodes the capacity for aerobic methylotrophy using methanol, methylamine, sulfomethanes, and chloromethanes as substrates (Murphy et al., 2021; Rodríguez-Ramos et al., 2022). In addition, this bacterium has genes involved in the oxidation of ammonia (Venturini et al., 2022). Previously, the presence of ARGs for *Binatia* was not reported. In our study, the assembled *Binatia* genomes were also characterized by the presence of biosynthetic gene clusters encoding terpenes. It is significant that in the assembled genomes of WHTF01, the sequence similar to vancomycin resistance gene (*vanT*) and gene coding multidrug efflux pump (*adeF*) were found. In addition to *Binatia*, annotation of the genomes of *Nitrospira_C* and *Nitrososphaera* sp., which are bacteria and archaea involved in N-cycling, revealed ARGs for chloramphenicol (*catI*) and glycopeptide (*vanT*) resistance. Thus, the hypothesis of the presence of ARG in soil microbial community represented by chemolithotrophs was confirmed.

5 Conclusion

In summary, this study showed that the shape of the microbial community and resistome of the organic cropping system without the use of mineral fertilizers can be quite similar to that of the conventional cropping system. However, the long-term use of nitrogen-rich mineral fertilizers in the conventional farming system is accompanied by the accumulation of microorganisms involved in the utilization of inorganic nitrogen, which leads to a difference in the ratios of microbial taxa between conventional and organic systems and determines the revealed differences in their resistomes. As has been shown, even typical nitrogen-assimilating chemolithotrophs are still an underestimated group of microorganisms in terms of dissemination of ARGs.

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Conflicts of interest

The authors declare no conflict of interest.

Author contributions

Conceptualization, A.S.V.; methodology, A.S.V., E.O.B., A.V.V.; formal analysis, A.S.V., E.O.B., A.V.V.; investigation, D.S.G., D.

V.P., S.V.K., A.V.I.; resources, A.S.V.; data curation, A.S.V., A. V.V.; writing—original draft preparation, A.S.V.; writing—review and editing, N.L.; funding acquisition, A.S.V. All authors have read and agreed to the published version of the manuscript.

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