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ENVIRONMENTAL MICROBIOLOGY - RESEARCH PAPER

Bacterial diversity changes in agricultural soils influenced by poultry litter fertilization

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Abstract

Poultry litter is widely applied as agricultural fertilizer and can affect the soil microbiome through nutrient overload and antibiotic contamination. In this study, we assessed changes in soil bacterial diversity using high-throughput sequencing approaches. Four samples in triplicate were studied: soils with short- and long-term fertilization by poultry litter (S1 = 10 months and S2 = 30 years, respectively), a soil inside a poultry shed (S3), and a forest soil used as control (S0). Samples S0, S1, and S2 revealed a relatively high richness, with confirmed operational taxonomic units (OTUs) in the three replicates of each sample ranging from 1243 to 1279, while richness in S3 was about three times lower (466). The most abundant phyla were Proteobacteria, Bacteroidetes, and Actinobacteria. Acidobacteria, Planctomycetes, and Verrucomicrobia were also abundant but highly diminished in S3, while Firmicutes was less abundant in S0. Changes in bacterial communities were very evident at the genera level. The genera *Gaiella*, *Rhodoplanes, Solirubacter*, and *Sphingomonas* were predominant in S0 but strongly decreased in the other soils. *Pedobacter* and *Devosia* were the most abundant in S1 and were diminished in S2, while *Herbiconiux*, *Brevundimonas*, *Proteiniphilum*, and *Petrimonas* were abundant in S2. The most abundant genera in S3 were *Deinococcus*, *Truepera*, *Rhodanobacter*, and *Castellaniella*. A predictive analysis of the metabolic functions with Tax4Fun2 software suggested the potential presence of enzymes associated with antibiotic resistance as well as with denitrification pathways, indicating that the S3 soil is a potential source of nitrous oxide, a powerful greenhouse gas.

Keywords Agricultural soil · Bacterial diversity · MiSeq · Poultry manure · Soil microbial communities

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Introduction

The soil microbiome constitutes a diversified ecological system, acting directly in the maintenance of important soil functions, such as the nitrogen (N) and other macro- and micronutrient cycles, improving soil fertility and crop production [1, 2]. Nevertheless, the impact of different types of agricultural management on the structure and metabolic function of this microbiome is still poorly understood [3]. A major challenge to soil health protection in agricultural systems is the high exposure of soils to agrochemicals such as antibiotics, pesticides, and fertilizers [4, 5]. In this context, the United Nations Sustainable Development Goals advocate sustainable practices in food production with the aim of improving food security and the resilience of agricultural systems. Among the sustainable options, the use of organic fertilizers, such as poultry litter and manure, is widespread to recycle the nutrients needed for good crop productivity and to improve soil properties (e.g., cation exchange capacity, organic matter content, and water-holding capacity) [6, 7].

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A usual way of quantifying the efficiency of applying poultry litter to crops is the measurement of its N content, since it is an important macronutrient present in high concentrations in this matrix [8, 9]. The N biogeochemical cycle is paramount in nature since organisms need N for cellular synthesis of enzymes, proteins, chlorophyll, DNA, and RNA. However, most of our planet's N is unavailable to organisms. Considering the Earth's surface, most of the N is in the atmosphere (66.4%), followed by the crust (33.2%), and only a small fraction is dissolved in water (0.4%) [10]. The problem is that the atmospheric N is in the molecular form N_2, and only a small group of prokaryotes has the ability to convert N_2 to an available N form (e.g., nitrate NO_3^-, nitrite NO_2^-, or ammonia NH_3). Notwithstanding, these specific prokaryotes are spread over several environmental compartments (air, freshwaters, marine waters, and terrestrial ecosystems) [11]. Therefore, any impact on the soil microbial diversity may disturb the homeostasis of this cycle. The microbial conversion of N is carried out by four reduction pathways (assimilatory nitrate reduction, dissimilatory nitrate reduction to ammonia, denitrification, and "N fixation") and two oxidation pathways (nitrification and anammox) [12].

On the other hand, poultry farming wastes are potential sources of sanitary products, such as veterinary antibiotics and feed additives, as well as pathogenic bacteria- and antibiotic-resistant strains [6, 13, 14]. A continuous input of poultry litter in soils can alter the soil physicochemical characteristics, such as pH, organic carbon content, and N availability, which have a great influence on soil microbiota diversity, abundance, and function [1, 3, 15].

Ashworth et al. [15] observed great changes in soil microbiome structure and an increased abundance of Bacteroidetes phylum after 2 years of treatment of legume cover crops with poultry litter. The Bacteroidetes phylum is one of the most representative, along with the Firmicutes, Proteobacteria, and Actinobacteria phyla, of the intestinal microbiome of chickens [16, 17]. In another study, Liu et al. [18] reported that application of pig manure during 5 months significantly reduced Actinobacteria phylum abundance (mainly *Gaiella* genus, which showed a 5.2% decrease) compared to an unfertilized soil. According to these authors, the physical-chemical changes from soil fertilization explained more than 40% of the variation in the structure of the bacterial community. The input of poultry litter in soils can also contribute to increased soil bacterial diversity. Jangid et al. [19] observed an increase in diversity in crop areas and pastures fertilized with poultry litter (Shannon index: 5.44 and 5.57, respectively) compared to a pine forest (*Pinus taeda* L.) soil (4.56). That is, previous studies have pointed out that the use of poultry litter or manure alters the bacterial community through changes in soil attributes, causing changes in diversity compared to other original plant coverings and increased antibiotic resistance genes due to long-term fertilization [15, 18]. However, these studies do not address different periods of land use, apart from being carried out in regions with a subtropical or temperate climate. Given the above, we hypothesized that the application of poultry litter in soils over the years can influence, in a cumulative way, bacterial diversity and richness, affecting important edaphic functions under tropical conditions.

Therefore, starting from microbial community diversity based on the 16S rRNA encoding gene, our main objective was to evaluate the influence of poultry litter application on soil microbiota over the short- and long-term (< 1 and 30 years), also including samples of a nearby non-impacted control area and a soil inside a poultry shed. As a sub-product, we used the metagenomic sequencing data to carry out a predictive analysis, by using the Tax4Fun2 algorithm, for the metabolic functions and routes, with the aim of identifying and studying the expected genes related to the N cycle and antibiotic resistance. It is noteworthy that the studied area, situated in the upland region of *Rio de Janeiro* state (RJ), in southeastern Brazil, is the most important poultry center of RJ, and we recently estimated that around 27,400 tons of poultry litter are produced and applied annually to its agricultural soils and surrounding areas [8].

Material and methods

Sampling area and experimental design

Soil samples were collected in *São José do Vale do Rio Preto* (SJVRP), RJ, Brazil (Fig. 1).

SJVRP has about 100 farms that produced 33,750 tons of chicken meat (*Gallus gallus domesticus*) in 2018 [8], and also has many agricultural areas with relevance for supplying fresh products to the *Rio de Janeiro* city metropolitan region. In addition, the sampling area has fragments of the Brazilian Atlantic Forest (BAF), a tropical biome under high pressure from anthropic actions and with great relevance for biodiversity conservation. Four soil samples (A horizon, 0–20 cm), classified as *Latossolo Vermelho* according to the Brazilian soil classification system [20] and equivalent to a Typic Hapludox in the US system [21], were collected in triplicate. Short-term-fertilized soil samples (S1.1, S1.2, S1.3) had been fertilized with poultry litter for only 10 months; long-term-fertilized soil samples (S2.1, S2.2, S2.3) had been regularly fertilized with poultry litter for 30 years. Crop (e.g., zucchini (*Cucurbita pepo*), cucumber (*Cucumis sativus*), tomato (*Solanum lycopersicum*), and bell pepper (*Capsicum annuum*)) rotation is common in the study area. The control soil was sampled (S0.1, S0.2, S0.3) as a non-impacted nearby soil microbiome inside a BAF fragment. Additionally, a soil inside the poultry shed was sampled (S3.1, S3.2, S3.3) and used as a reference for a highly impacted soil.

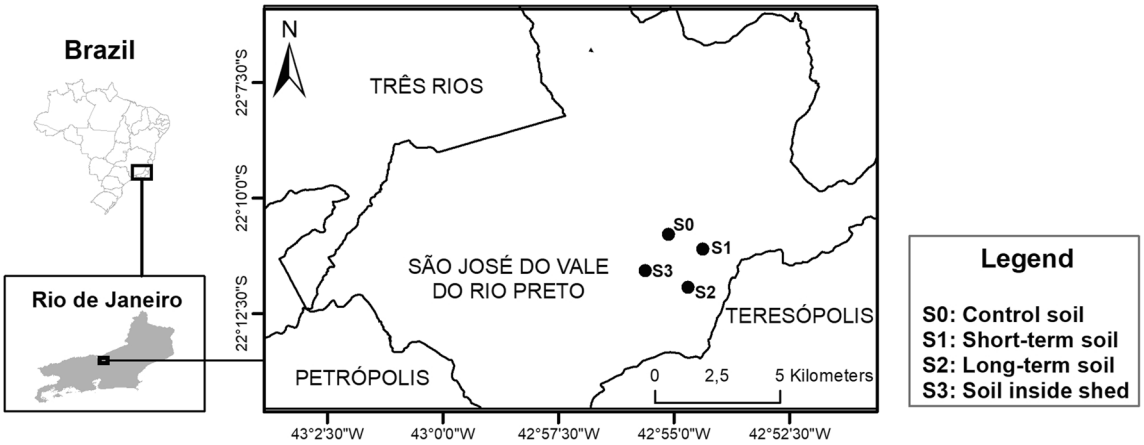

Fig. 1 Study area and sampling points

DNA extraction of soil samples

The total microbial community DNA was extracted directly from the 12 soil sub-samples (0.5 g of each) using DNeasy PowerSoil Kit (QIAGEN, USA). DNA preparations were visualized by electrophoresis in a 0.8% agarose gel in × 1 TBE buffer [22] to access integrity. The amplification of the V3–V4 hypervariable region of the 16S rRNA gene was accomplished by a PCR test with the bar-coded primers Bakt_341F (CC TAC GGG NGG CWG CAG) and Bakt_805R (GAC TAC HVG GGT ATC TAA TCC). High-throughput sequencing was performed using MiSeq (Illumina) in a commercial facility (Macrogen, Seoul, Republic of Korea) according to the manufacturer's protocols.

Analysis of soil microbial community based on 16S rRNA encoding gene

The extracted DNA was used to generate amplicons targeting the V3–V4 hypervariable regions of the 16S rRNA encoding gene. Amplicons were sequenced using the Illumina MiSeq platform at Macrogen Inc. (Seoul, Republic of Korea) according to the manufacturer's instructions. The quality of the reads was evaluated with FastQC v.0.11.5 software [23], while these reads were processed using the USEARCH 9.2 package [24]. The data were first filtered to eliminate small sequences and for trimming (at 425 bp) to guarantee an acceptable quality level. Reads with less than five occurrences were removed. The remaining sequences were clustered by similarity (on a 97% identity basis) in order to consider errors due to PCR, sequencing, and paralogs [25]. The obtained operational taxonomic units (OTUs) were then associated with the complete pool of sequences and checked for a preliminary taxonomy. The RDP 16S rRNA (training set v16) was used as a reference database. Richness and diversity indices, like the Shannon index and related parameters [26] and Simpson diversity [27], were estimated based on standard equations for sample community characterization [28].

Metabolism prediction

There are currently different algorithms available for the prediction of metabolic functional profiles for the bacterial communities from environmental or other samples, the most frequently used being PICRUSt and Tax4Fun. PICRUSt predictions use an ancestral-state reconstruction algorithm to infer functional composition [29], while in Tax4Fun the linking is performed with a nearest neighbor identification based on a minimum 16S rRNA sequence similarity [30]. Both methods use the Kyoto Encyclopedia of Genes and Genomes (KEGG) to predict the functional profiles of microbial communities. Since PICRUSt uses exclusively Greengenes database for the taxonomy classification, we chose Tax4Fun because it is more plastic in this sense—it accepts the taxonomy that we have done with the RDP database, among others.

The metabolic prediction based on the 16S rRNA gene sequences was done using the Tax4Fun2 package [31]. Tax4Fun2 software uses NCBI BLAST+ (US National Library of Medicine) for OTU labeling. A pre-computed association matrix based on KEGG organism functional profiles was used for association, followed by the abundance calculation. The identified KEGG ortholog functional genes (KOs) were then automatically associated with empirical metabolic pathways, paying special attention to the genes related to the N cycle due to the N input through poultry litter fertilization and the relevance of nitrification for soil fertility. In addition, as we verified the occurrence of antibiotics and antibiotic-resistant genes in the same set of samples in a previous study [6], we also included predictions related to antibiotic and multidrug resistance.

Many studies currently use functional predictions as tools for optimizing the use of high-throughput sequencing data [3, 32, 33]. However, some limitations must be considered. Among them, functional predictions are based only on taxonomic genes and do not take environmental limitations into account. Therefore, if they provide relevant information, the next step should be their confirmation by experimental analysis.

Results

Bacterial diversity

A total of 2,553,275 reads were obtained: 685,447 for S0, 622,641 for S1, 617,323 for S2, and 627,864 for S3, adding up the results of the three replicates of each sample. After removing 1896 chimeras, the remaining clustered reads resulted in 2699 OTUs, distributed into 1243, 1274, 1279, and 466 OTUs, respectively, for S0, S1, S2, and S3. These last numbers include what we call the confirmed OTUs, that is, those that were detected in the three replicates of each sample. The raw sequences of the present project were deposited in the Sequence Read Archive (SRA) database of the National Center for Biotechnology Information (NCBI) and assigned accession numbers SAMN13023011–SAMN13023014, respectively, for samples S0–S3, associated with the project PRJNA577278.

Several diversity indices were estimated for the OTUs found in the four sets of samples (Table 1). The values in the first line (for each sample) are the means for the triplicates and those in the second line the respective standard deviations (SD).

According to the Shannon H' index (also called Shannon entropy), there was a larger diversity of OTUs in the control soil and in the poultry litter fertilized soils (S0: 5.72, S1: 5.74, and S2: 5.62, respectively) as compared to the one in the poultry shed soil (S3: 4.74), for more than 6 SD. The Simpson index (a measurement of the probability of two individuals taken at random from the community being from different OTUs) reinforces the results of the Shannon index, with both revealing the relatively high bacterial diversity in the four samples, which was slightly smaller for S3. The distribution of dominating taxa (Shannon evenness) was relatively regular, ranging from 0.76 (S3) to 0.79 (S0), since the maximum value of 1.0 denotes complete regularity. Another important result of our analysis is that the uncertainty in these statistics (SD in Table 1), estimated from the use of triplicates, is at most 15%, with a typical value of 4%. This confirms the robustness of the techniques (both the sequencing and the statistical analysis) for detecting and evaluating the samples' diversity.

Concerning the taxonomy, more than 85% of bacterial communities are dominated by seven phyla: Proteobacteria, Bacteroidetes, Actinobacteria, Acidobacteria, Planctomycetes, Firmicutes, and Verrucomicrobia (Fig. 2). In addition, the pattern of abundance showed some distinct effects of the application of poultry litter: (i) Acidobacteria and Planctomycetes, which were common in the natural environment (S0), had their communities decreased in fertilized soils (S1 and S2), and were greatly diminished in the poultry shed (S3); (ii) Bacteroidetes and Firmicutes were more abundant in S3, were intermediately abundant in fertilized soils, and presented the smallest abundance in the natural environment; (iii) the dominant phylum of Proteobacteria was slightly less abundant in the control soil compared to the other soils.

The 2699 OTUs were associated with 607 distinct genera, distributed in the four samples as shown in the Venn diagram presented in Fig. 3. The most remarkable characteristic of this diagram is the absence of genera that are common to only S0 and S3. There are also genera exclusively present in each one of the samples: 35, 46, 56, and 51, respectively, in S0, S1, S2, and S3.

The distribution of the 15 most abundant genera is shown in Fig. 4. In terms of the phyla, they are distributed as follows: Proteobacteria (*Brevundimonas*, *Castellaniella*, *Devosia*, *Rhodanobacter*, *Rhodoplanes*, and *Sphingomonas*), Bacteroidetes (*Pedobacter*, *Petrimonas*, and *Proteiniphilum*), Actinobacteria (*Gaiella*, *Herbiconiux*, and *Solirubrobacter*), Deinococcus-Thermus (*Deinococcus* and *Truepera*), and Firmicutes (*Bacillus*).

Similar to the profiles based on phyla, there are genera that seem typical of the natural environment: control soil (S0) showed a predominance of only three genera (*Gaiella*, *Rhodoplanes*, and *Solirubrobacter*), which were not or almost

Table 1 Statistics of total number of sequence reads, estimated richness, and diversity indices for soil bacterial communities

Samplesa		Reads	Richness (OTUs)	Shannon H' index	Shannon $e^{H'}$ richness	Shannon evenness	Simpson's index
S0	Mean	**104,524**	**1418**	**5.72**	**307**	**0.79**	**0.99**
	SD	8104	37	0.16	46	0.02	0.003
S1	Mean	**104,080**	**1504**	**5.74**	**310**	**0.78**	**0.99**
	SD	1008	27	0.04	13	< 0.01	< 0.001
S2	Mean	**106,287**	**1490**	**5.62**	**278**	**0.77**	**0.99**
	SD	4498	35	0.13	35	0.02	0.002
S3	Mean	**114,937**	**521**	**4.74**	**115**	**0.76**	**0.98**
	SD	17,437	2	0.06	7	0.01	0.002

[a] *S0*, control soil; S*1*, short-term fertilized soil; *S2*, long-term fertilized soil; *S3*, soil inside poultry shed; *SD*, standard deviation

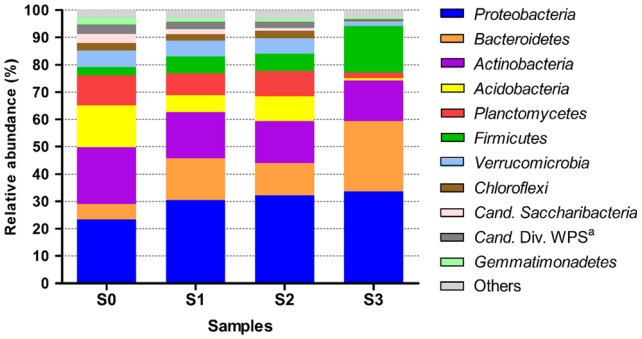

Fig. 2 Relative abundances of the most abundant bacterial phyla from control soil (S0), short-term fertilized soil (S1), long-term fertilized soil (S2), and soil inside poultry shed (S3). a: candidate division Wittenberg polluted soil

not present in the other samples. *Sphingomonas* can also be classified in this group; their abundance decreased with soil usage. On the other hand, some genera seemed to be typical of the poultry shed (S3): *Truepera*, *Castellaniella*, *Rhodanobacter*, and *Deinococcus*, but were almost inexistent in the other samples. Fertilized soils also had their particular profiles, with more similarity between them than with the others; while *Pedobacter* and *Devosia* decreased between S1 and S2, *Herbiconiux*, *Brevundimonas, Proteiniphilum*, and *Petrimonas* increased between S1 and S2.

Nitrogen metabolism

The predictions with Tax4Fun2 showed 27 functional genes associated with the N metabolism; their abundances are represented in Fig. 5.

Antibiotics resistance

Functional predictions related to antibiotic resistance were found for soil samples for specific classes, such as

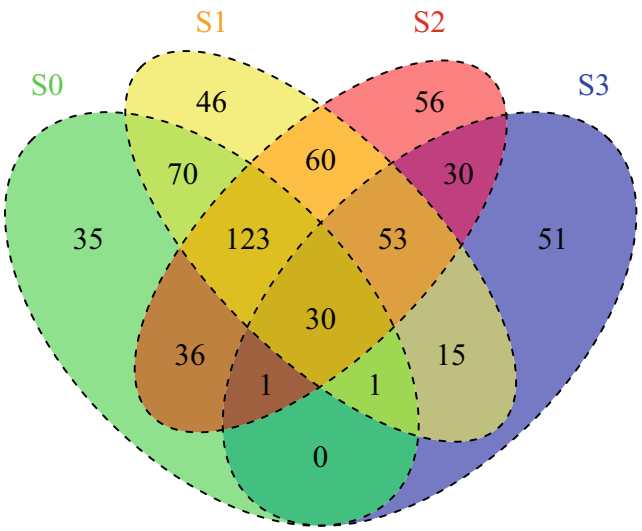

Fig. 3 Venn diagram of the 607 genera distributed over the four samples

fluoroquinolones, macrolides (erythromycin), tetracyclines, aminoglycosides (streptomycin), glycopeptide (vancomycin), polypeptide (bacitracin), streptogramins (virginiamycin), and *beta*-lactams (cephalosporin and penicillin) (Fig. 6). A large diversity of functional profiles related to resistance to other specific antibiotic classes was predicted to occur, such as erythromycin esterase, macrolide efflux protein, tetracycline resistance efflux pump, and tetracycline resistance. Functional profiles related to the tetracycline resistance protein *tetA* were found with higher abundance in outdoor soil samples (S1 and S2), including in control soil (S0). Also, virginiamycin enzymes (A acetyltransferase and B lyase) were predicted to occur on S0 and penicillin-binding proteins were predicted with equal abundance among soil samples.

Multidrug resistance functional profiles as multidrug and toxin extrusion (MATE) family and specific major facilitator superfamily (MFS) transporters related to multidrug resistance were predicted (Fig. 6). Moreover, mechanisms involved in xenobiotic bacterial resistance, such as multidrug efflux pump, were also predicted.

Discussion

Bacterial diversity

The indices in Table 1 indicate that soil samples from open environments (S0, S1, and S2) present similar abundances of OTUs and diversities, while the soil inside the poultry shed (S3) shows values indicating only about one-third of the richness and smaller diversity indices in relation to the previous ones. This suggests a certain resilience of the native bacteriome to the changes imposed by the input of the litter. Our results agree with those from Shange et al. [34], who reported lower richness in the bacterial community under poultry sheds compared to pasture soils. Both results suggest that an artificially maintained environment, such as the soil inside the shed, can be a selective environment for the soil bacterial community, affecting its abundance and diversity.

Concerning the Shannon H' index calculated for our samples, the results are consistent with other values presented in the literature, although not similar in their face values. Zhen et al. [35] reported lower Shannon diversity indices in control soil (2.53) and in soil fertilized with cattle manure (3.44) in samples from China. Vollú et al. [7] reported an increase of the Shannon H' index from 6.99 to 7.24 between 60 and 90 days of maize fertilization with mineral phosphate + poultry litter. Comparing these studies, the difference between results can be related to environmental characteristics and agricultural management, such as the background soil microbiome, source of fertilizer (mineral and/or organic), cropping system, type of soil and its physicochemical properties, and other environmental conditions (temperature, humidity, lighting) [15].

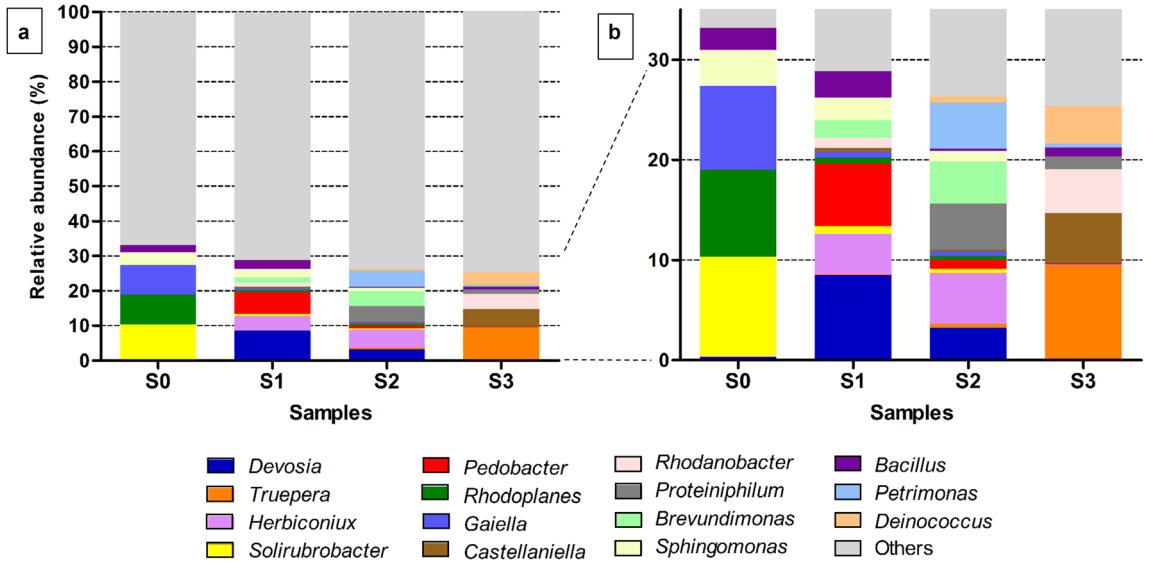

Fig. 4 **A** Relative abundances of the most abundant bacterial genera from control soil (S0), short-term fertilized soil (S1), long-term fertilized soil (S2), and soil inside oultry shed (S3); **B** zoom of the percentage distribution of the fifteen most abundant genera

Comparative sample profiles of both phyla (Fig. 2) and genera (Fig. 4) show the same three patterns: (i) Populations of Acidobacteria and Planctomycetes and some genera of Actinobacteria (*Gaiella* and *Solirubrobacter*) and Proteobacteria (*Rhodoplanes* and *Sphingomonas*) phyla, dominant in the pristine environment, were highly impacted by poultry litter fertilization: the ones dominating in S0 decreased or were extinguished towards fertilized soils (S1 and S2) and S3. Vollú et al. [7] also observed a reduction in abundance of *Rhodoplanes* and the family *Gaiellaceae* after

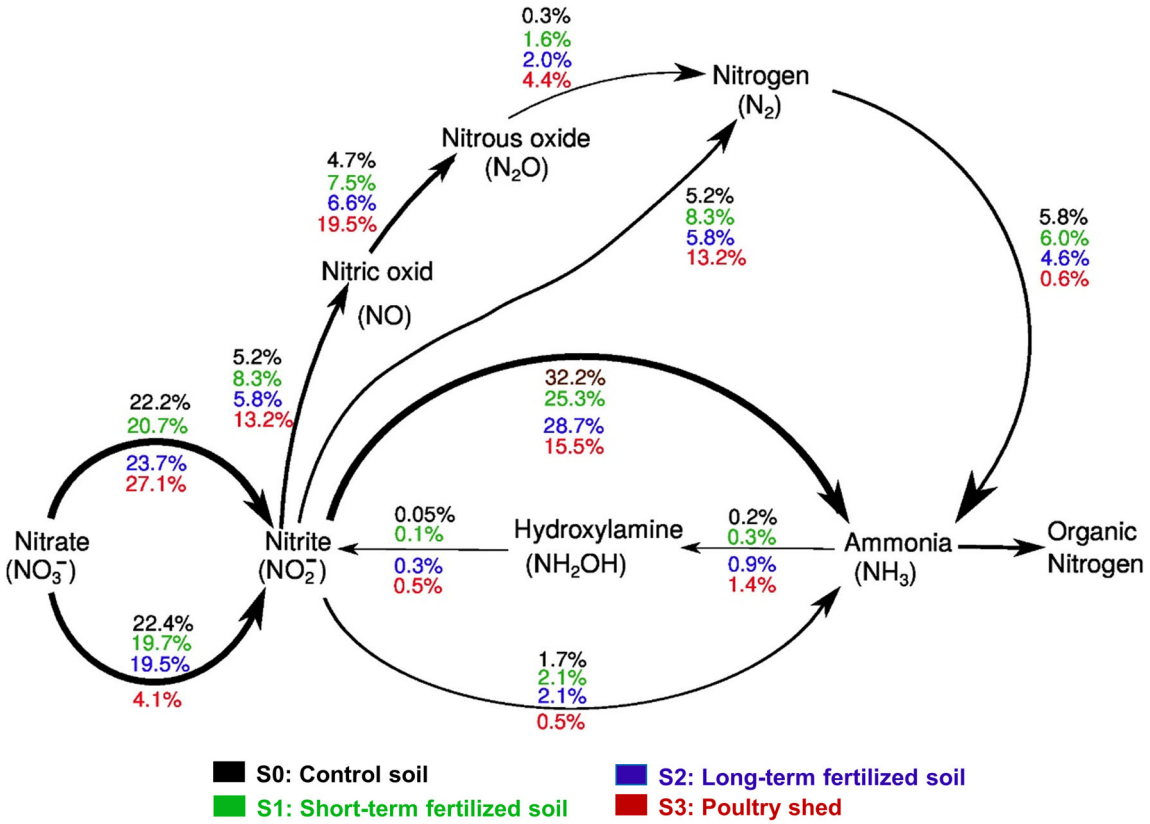

Fig. 5 The N cycle scheme based on KEGG metabolism pathways in soil samples. Thicker lines had greater relative abundance than thinner lines and relative percentages were calculated considering the total number of genes involved in the N cycle. Only the routes whose genes were detected on the prediction are pointed in this figure

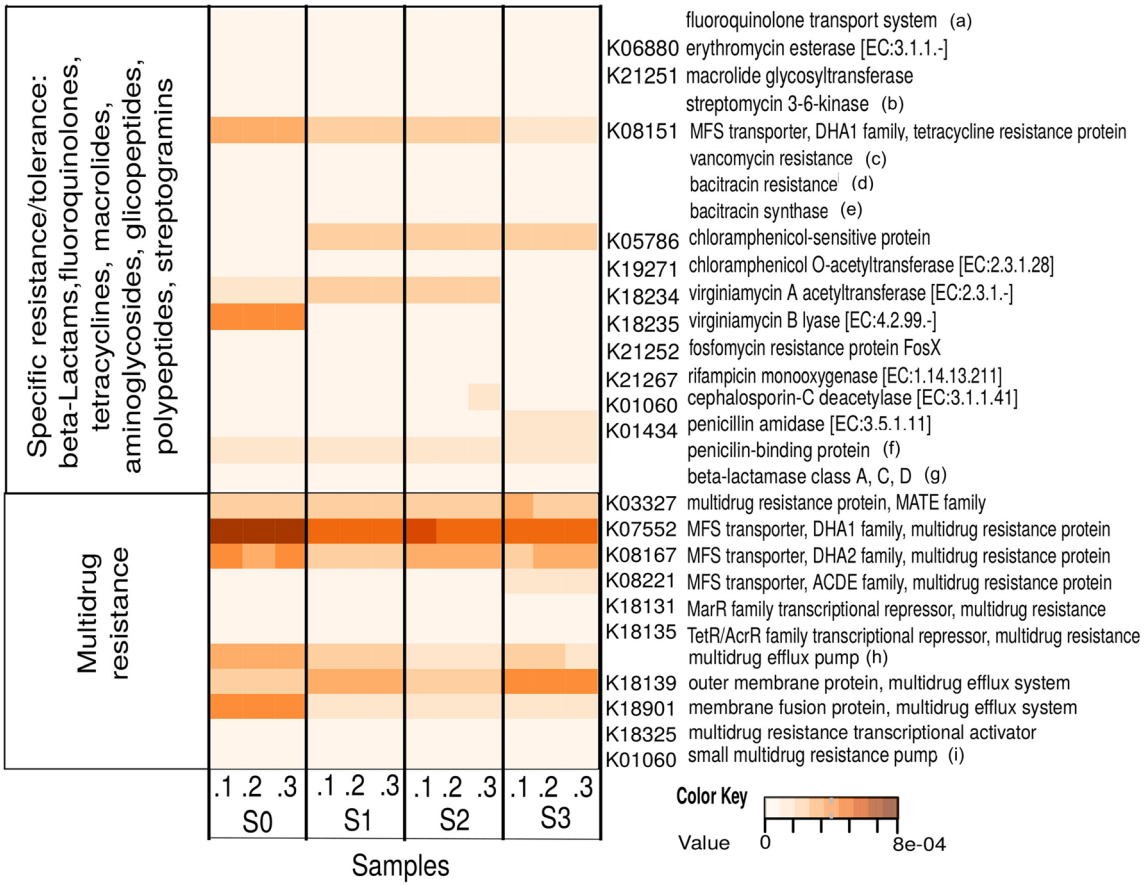

Fig. 6 Functional predictions based on bacterial metabolism from KEGG database from Control soil (S0.1–3), short-term fertilized soil (S1.1–3), long-term fertilized soil (S2.1–3), and soil inside poultry shed (S3.1–3) related to specific antibiotic resistance and multidrug resistance. Individual function predictions are presented with specific KEGG codes. Functions without KEGG codes are presented as means of the same (or similar) functional predictions found in the samples. Superscript letters represent KEGG codes: (a) K16905; K16906; K16907; (b) K10673 [EC:2.7.1.87]; K12570 [EC:2.7.1.72]; (c) K16444 [EC:2.4.1.310]; K18346; K18353; (d) K11631; K11632; (e) K16093; K16094; K16095; (f) K02545 [EC:3.4.16.4]; K05515 [EC:3.4.16.4]; K07337; K08724; K12552 [EC:3.4.-.-]; K12553 [EC:3.4.-.-]; K12555 [EC:2.4.1.129 3.4.16.4]; K12556; K18770 [EC:2.4.1.129 3.4.16.4]; K21464 [EC:2.4.1.129 3.4.16.4]; K21465; K21466; K21467; K21468; (g) K18698 [EC:3.5.2.6]; K18699 [EC:3.5.2.6]; K18766 [EC:3.5.2.6]; K18767 [EC:3.5.2.6]; K18768 [EC:3.5.2.6]; K18795 [EC:3.5.2.6]; K18797 [EC:3.5.2.6]; K18970 [EC:3.5.2.6]; K19097 [EC:3.5.2.6]; K01467 [EC:3.5.2.6]; K19095 [EC:3.5.2.6]; K19096 [EC:3.5.2.6]; K19100 [EC:3.5.2.6]; K19101 [EC:3.5.2.6]; K19215 [EC:3.5.2.6]; K20320 [EC:3.5.2.6]; K18790 [EC:3.5.2.6]; K18792 [EC:3.5.2.6]; K18793 [EC:3.5.2.6]; K18794 [EC:3.5.2.6]; K18971 [EC:3.5.2.6]; K18973 [EC:3.5.2.6]; K18976 [EC:3.5.2.6]; K19209 [EC:3.5.2.6]; K19211 [EC:3.5.2.6]; K19213 [EC:3.5.2.6]; K21266 [EC:3.5.2.6]; K21276 [EC:3.5.2.6]; K21277 [EC:3.5.2.6]; (h) K18139; K18147; K18308; K18323; K18903; K18904; K18141; K18145; K18298; K18302; K18306; K18321; K18898; K18901; K18990; K21135; K21136; K21137; K07788; K07789; K18138; K18142; K18146; K18296; K18299; K18303; K18307; K18322; K18324; K18899; K18902; K18908; K18989; K19585; K21133; K21134; (i) K18924; K18925; K18975

treatment for 60 days with poultry litter and fertilizers. (ii) In the poultry shed (S3), Proteobacteria, Bacteroidetes, and Firmicutes were the dominant phyla, being more abundant in this sample than in the fertilized soils and much more abundant than in S0. Shange et al. [34] also found a greater abundance of Bacteroidetes phylum in soils inside a poultry shed compared to a pasture soil. These three phyla match those usually found in the poultry gut microbiome [36, 37]. The *Bacteroides* genus, for instance, found exclusively in S3, is typical of this microbiome [38]. Wei et al. [16] reported that Proteobacteria was one of the most predominant phyla, accounting for 9.3% of the bacterial sequences in the intestinal tract of chickens. According to the authors, the genus *Desulfohalobium* was the most representative (0.7% of the sequences). This pattern is also presented by the genera *Castellaniella* and *Rhodanobacter* (Proteobacteria) as well as *Truepera* and *Deinococcus* (Deinococcus-Thermus), which were not present in the natural soil (S0) and not effectively transferred to fertilized soils (S1 and S2). Deinococcus-Thermus was described as one of the most extremophilic bacteria phyla, and *Truepera* is a halophilic genus [39]. Salt (NaCl) addition to the poultry feed can increase the Na^+ and Cl^- ions in the soil inside the shed and provides a suitable environment for halophilic bacteria. Indeed, higher concentrations of Na were found in S2 and S3 (1.5 and 1.2 $Cmol_c\ dm^{-3}$, respectively), both soils with great contact with poultry litter

(Online Resource – Table S2). (iii) Populations that grew in the fertilized soils but were abundant in neither S0 nor S3 (*Herbiconiux* genus from Actinobacteria phylum; *Devosia* and *Brevundimonas* genera from Proteobacteria phylum; and *Pedobacter*, *Petrimonas*, and *Proteiniphilum* genera from Bacteroidetes phylum). The genera *Pedobacter* and *Devosia* had high abundances in the short-term poultry litter fertilized soil (S1), while their abundances diminished in the long-term fertilized soil (S2). Ashworth et al. [15] observed a significant enrichment of *Pedobacter* genus in an agricultural soil with application of poultry litter. *Devosia* was found in different habitats, ranging from tropical to polar regions, and was reported as an abundant genus associated with composts of organic waste [40, 41].

The opposite occurred with *Herbiconiux*, with a slight increase in S2, and *Proteiniphilum*, *Brevundimonas*, and *Petrimonas*, with notable increases in S2. Previous studies reported the endophytic occurrence of *Herbiconiux* genus associated with roots, stems, and leaves [42, 43]. It is possible that the increased abundance of this genus is associated with the long-term agricultural use of S2 soil. *Brevundimonas* were isolated in a wide range of matrices, including activated sludge, aquatic environments, sediments, and soils [44]. Ryan and Pembroke [44] pointed out that this genus has opportunistic pathogenic species that can cause severe infections and that its clinical importance is still undervalued. It is worth mentioning that 30 years of poultry litter application in S2 soil contributed to changes in its physical-chemical attributes, such as increases in the trace elements concentration, macronutrients, pH, cation exchange capacity, and organic carbon content (Online Resource – Tables S1 and S2; Fig. S1). These changes are expected to have influenced the structure of the bacterial community in soil S2.

The phylum Actinobacteria showed the most stable abundance in all samples. It is a phylum of Gram-positive bacteria found mainly in soil that plays a significant role in organic matter decomposition [45]. This phylum is also known to have antibiotic- and pesticide-resistant species [46–48].

The strong reduction in Acidobacteria phylum in S3 soil is probably due to the influence of the alkaline pH generally found in poultry litter [6, 49]. According to Sait et al. [50], the pH strongly influenced the cultivation of Acidobacteria colonies from soil samples, with growth favored by slight to moderate acidic conditions.

Proteobacteria was the most abundant phylum in all soil samples, with slightly higher abundance in fertilized soils (S1 and S2) and in the poultry shed (S3). This phylum was the most abundant in agricultural soils treated with poultry litter, enriched with mineral fertilizers [7] and by crop rotation management [15]. Considering that agricultural soils are under constant management by farmers, it is important to mention that this phylum encompasses a very complex set of phenotypic and physiological attributes, including a large number of known human, animal, and plant pathogens [51].

Nitrogen metabolism

The functional prediction (Fig. 5) suggests a continuous change in the importance of the distinct N metabolic routes from the pristine soil (S0) to the hardly impacted one (S3). The metabolic pathways of N assimilation, either by assimilatory or dissimilatory nitrate reduction, which are the most important routes for soil fertility, are clearly impacted by the lower bacterial diversity of microorganisms involved in these routes in the poultry shed (S3). The assimilatory nitrate reduction decreased from above 20% to less than 5% in S3. The same pattern was also observed in the N fixation pathway. On the other hand, other detected pathways were enhanced in this direction: the denitrification, for example, which included only 10% of the genes involved in the N cycle in S0, increased in S2 and S1, presumably due to the poultry litter input since in S3 the same genes correspond to about 37%. By this pathway, the N passes to a gaseous phase of the N cycle: the nitrite is transformed into nitric oxide (NO), then to nitrous oxide (N_2O), and finally to N_2. This process can pose a problem for soil productivity due to soil fertility decrease, as well as being an important source of NO and N_2O to the atmosphere, contributing to the emission of greenhouse gases. Our results, based on functional predictions, coincide with previous experimental results which demonstrate that poultry farming wastes (poultry litter and manure) are important sources of N_2O in different soil types [52, 53]. Also, Davis et al. [54] reported an exponential increase of annual N_2O emissions in soils due to poultry litter application. Therefore, to validate the results predicted in the environmental conditions of the present study, techniques such as qPCR are required to study the activity of genes related to denitrification (*nirS* and *nirK*), nitrification (*amoA*), and nitrate reduction (*narG*) [55, 56].

Antibiotic resistance

A high variability in functional predictions related to resistance to veterinary antibiotics was found in soil samples (Fig. 6). The samples included in the present study received high loads of fluoroquinolone (FQ) antibiotics through poultry litter application [6]. FQ transport system permease proteins (e.g., K16905, K16906) were predicted in samples with previous contact with poultry litter contaminated with FQs (S1, S2, and S3). On the other hand, antibiotic and multidrug resistance was also predicted in the control soil (S0). Although some anthropogenic activities can contribute to increased antibiotic resistance in soils (e.g., application of manure and sewage sludge) [48, 57], studies have shown that pristine soils harbor an antibiotic resistance reservoir (the soil resistome) characterized by great diversity and abundance of resistant bacteria [58, 59]. Metabolic functions related to resistance to tetracyclines and macrolides were also predicted in our soil samples. These classes of antibiotics are applied mainly for

prophylactic and therapeutic purposes in animal production and can reach agricultural soils through manure and poultry litter [60, 61]. Functional predictions related to tetracycline resistance herein (e.g., K08151, K18221—tetracycline resistance proteins) are probably related to administration of this class of antibiotic on the poultry farms. However, the occurrence of *tet* genes (e.g., K08151, K18220, K18221, K18218) on control soil (S0) shows the importance of the soil resistome for the possible contribution to the emergence of antibiotic-resistant bacterial strains [58, 62]. Most of the 45 genes associated with tetracycline resistance are *tet* genes; among them, *tetA* encodes an efflux protein and is also related to resistance to other classes of antibiotics [63, 64]. In addition, functional predictions related to resistance to virginiamycin and penicillin were found in S0. Virginiamycin is administered as a growth promoter in poultry farming and, although there are still few studies on resistance genes in the environment [65], virginiamycin A acetyltransferase (K18234) was identified, occurring in different matrices, such as human feces and samples from the soil and oceans [65].

Penicillin-binding proteins (transpeptidases or carboxypeptidases) provide *beta*-lactams resistance by expressing low affinity to the target sites in critical human pathogens, such as *Staphylococcus aureus*, *Enterococci*, and *Streptococcus pneumoniae* [66]. Udikovic-Kolic et al. [67] observed increased *beta*-lactam resistance genes in a manure-treated soil compared to an untreated soil. According to the authors, the higher abundance of resistance genes in manured soil was probably related to the enrichment of *beta*-lactamases from resident soil bacteria.

MFS transporter proteins (DHA1 and DHA2 family), functional profiles related to multidrug efflux pump, and a multidrug resistance based on efflux pump *BpeEF-OprC* (K18901) had greater abundance in S0 compared to other soils. Podnecky et al. [68] reported that trimethoprim resistance in 60 isolates from clinical and environmental samples was related to *BpeEF-OprC* efflux pump expression. Trimethoprim is widely used in poultry farming associated with sulfamethoxazole.

The resistance mechanism identified as the multidrug efflux pump, with predicted greater abundance in S0, can be related to plasmid-mediated resistance genes (e.g., *OqxAB*), which confer resistance to FQs [69]. In addition to mechanisms related to antibiotic resistance, multidrug efflux pumps can act by extruding endogenous metabolites, heavy metals, and organic pollutants [70]. Given the above, it is important to emphasize that the predictions derived from the 16S rRNA analysis must be considered as an exploratory approach to the metabolic functions present in a microenvironment. Many antibiotic resistance genes are found in transposons, integrons, or plasmids, which can be mobilized and transferred to the environment regardless of bacterial populations. In our previous study [6], we verified the occurrence of plasmid-mediated quinolone-resistant genes (*qnrS*) in the long-term fertilized soil area (S2), but it is possible that poultry litter is influencing the occurrence of a greater diversity of genes associated with antibiotic resistance.

Conclusions

Our results strongly suggest that the application of poultry litter as organic fertilizer modifies the soil community profile, confirming our hypothesis that this agricultural practice can influence bacterial diversity and richness. A drastic reduction in the bacterial diversity was observed in the poultry shed soil sample (S3), possibly resulting from the management (e.g., periodic removal of topsoil, addition of new sawdust and treatment with sanitary products). At phyla taxonomic level, greater changes were observed between the control soil (S0) and S3. At the genus level, the changes were very evident in all soils, showing that some populations from natural soil (S0) are decreased or completely depleted by the poultry litter fertilization, others grow only in the fertilized soils—probably being existent in natural soils but as rare populations—and others develop only under the relatively extreme conditions of S3 soils. In summary, our study points to a real impact of large-scale poultry farming and the widespread use of its waste as agricultural fertilizer. This practice may increase the yield of N_2O and NO from indoor soil (S3) to the atmosphere. In view of the diversity of predicted functions associated with antibiotic resistance, confirmation by experimental techniques and evaluation of the transfer of these genes through the consumption of agricultural products are required.

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Authors' contributions All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Cláudio Ernesto Taveira Parente, Elcia Margareth Souza Brito, César Augusto Caretta, Renata Estebanez Vollú, and Lucy Seldin. The first draft of the manuscript was written by Cláudio Ernesto Taveira Parente, Elcia Margareth Souza Brito, and César Augusto Caretta. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability The raw sequences were deposited in the Sequence Read Archive (SRA) database of National Center for Biotechnology Information (NCBI) - project PRJNA577278.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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