



# The Use of Raw Poultry Waste as Soil Amendment Under Field Conditions Caused a Loss of Bacterial Genetic Diversity Together with an Increment of Eutrophic Risk and Phytotoxic Effects

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## Abstract

Poultry waste has been used as fertilizer to avoid soil degradation caused by the long-term application of chemical fertilizer. However, few studies have evaluated field conditions where livestock wastes have been used for extended periods of time. In this study, physicochemical parameters, metabarcoding of the 16S rRNA gene, and ecotoxicity indexes were used for the characterization of chicken manure and poultry litter to examine the effect of their application to agricultural soils for 10 years. Poultry wastes showed high concentrations of nutrients and increased electrical conductivity leading to phytotoxic effects on seeds. The bacterial communities were dominated by typical members of the gastrointestinal tract, noting the presence of pathogenic bacteria. Soils subjected to poultry manure applications showed statistically higher values of total and extractable phosphorous, increasing the risk of eutrophication. Moreover, while the soil bacterial community remained dominated by the ones related to the biogeochemical cycles of nutrients and plant growth promotion, losses of alpha diversity were observed on treated soils. Altogether, our work would contribute to understand the effects of common local agricultural practices and support the adoption of the waste treatment process in compliance with environmental sustainability guidelines.

**Keywords** Soil bacterial diversity · Poultry manure · Chicken litter · Metabarcoding · Organic amendment

## Introduction

Soil microbial community plays key roles in soil functions, such as nutrient cycling, bioremediation, and plant growth and health promotion [1]. For these reasons, high levels of microbial diversity in soil are of crucial importance for sustainable agriculture [2].

In particular, the structure of the soil bacterial community is highly sensitive to environmental changes caused by natural or human activities, and for this reason, could be used as a biomarker of land management [3, 4]. It has been proven that long-term chemical fertilization regimens decreased soil bacterial diversity [5], highlighting the suitability of organic amendments as an alternative to prevent the excessive use of synthetic fertilizers [6]. Traditionally, livestock manures have been used as fertilizers and soil amendments, increasing microbial activity and biomass, improving porosity, aeration, water holding capacity, structural stability, and nutrient availability [7, 8]. Specifically, the poultry industry produces two kinds of nutrient-rich waste requiring costly and proper management: chicken manure (M) from egg

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production and poultry litter (L) from the meat one. Though the use of M and L as amendment appeared as a poultry industry by-product, it could turn out to be a risky practice because of manure-borne pathogenic microorganisms and potentially hazardous chemical constituents, such as metals, hormones, antibiotics, antiprotozoals, probiotics, and among others [9, 10].

On the other hand, environmental factors such as soil nutritional status and pH, which are especially prone to amendment applications, are acknowledged as critical for bacterial community assembly [11]. Moreover, the over-application of animal waste on farmlands could lead to leakage into the surface water or leach into the groundwater [12, 13] as long as a high load of nutrients could not be efficiently absorbed. That is, the excess of phosphorous can provoke algal blooms and cyanobacterial growth together with fish mortality due to hypoxia/anoxia conditions in the water column [13]. Furthermore, to account for the acute toxicity of raw livestock waste on terrestrial organisms, seed germination and root elongation toxicity tests have been developed to determine the ecotoxicological effect of complex mixtures over vascular plants, in the context of integrated monitoring strategies of waste management [14].

The goal of the present study was to examine the effect of the application of poultry waste over agricultural soils for extended periods of time, using amplicon metagenomics for the integration of bacterial population profiling with physicochemical and toxicological parameters, after field applications by local producers. To this purpose, we evaluated poultry waste (chicken manure and poultry litter) and amended soils, in order to (i) characterize poultry waste and (ii) evaluate the impact of its application on soils.

## Methods

### Research Site

The study was carried out in Crespo, Entre Ríos, Argentina (32°01'52.1"S, 60°18'15.5"W), acknowledged as one of the main poultry production areas, as well as crop production. The region has a subhumid (annual rainfall ~ 1000 mm) and a temperate climate (annual temperature approximately 18.3 °C). The soil of the area was classified as thermic Vertic Argiudoll [15] of the Crespo Series. The texture of the A horizon was silty clay loam with 354 and 614 g kg<sup>-1</sup> of clay and silt, respectively [16].

### Poultry Waste Sampling

Poultry manure (M) and chicken litter (L) were collected from an egg production farm with 20,000 laying hens and a poultry farm of 30,000 broiler chickens, respectively. The

chicken litter was rice husk with wood chips used at least along 3 breeding cycles.

Poultry manure samples (triplicates) were obtained after pooling subsamples selected haphazardly from separated sites of the house (under the cages) and taken at different depths of the manure pyramid. Similarly, litter fractions were collected from the periphery and center of a pile located on the side of the shed.

All samples were homogenized according to standardized specifications [16] and were kept at 4 °C until physicochemical analysis or stored at -80 °C for DNA extraction.

### Amended Soil Sampling

Field sampling was conducted in farms with equivalent edaphic characteristics and subjected to similar agricultural cycles, representative of the productive practice in the area (wheat/soybean-corn rotation).

Each site was carefully selected after corroborating, and they had been subjected to different organic amendments application management over a period of 10 years:

- SC, control soil (no amendments added);
- SM, soil + poultry manure (5 applications of 10 tons ha<sup>-1</sup> y<sup>-1</sup> in the last 10 years);
- SL, soil + chicken litter (3 applications of 6 tons ha<sup>-1</sup> y<sup>-1</sup> in the last 10 years).

SC was subject to inorganic fertilization (average 150 kg ha<sup>-1</sup> urea), as well as amended soils in those years in which no organic amendment was applied.

Three samples per treatment, each one formed by pooling 10 random subsamples, were grabbed by soil boring (the diameter was 5 cm) at a depth of 0–10 cm. In order to avoid contamination, the auger was cleaned, then wiped with 70% alcohol, and thoroughly rinsed with sterile water after each sampling. Soil samples were collected in the third corn leaf stage (V3) in October 2018, whereas the last poultry waste application (SM and SL) was accomplished one week before the sowing of corn.

### Physicochemical Analysis and Stability of Waste and Soil Samples

Poultry manure, chicken litter, and soils were stored at 4 °C for physicochemical analysis. The characterization of the poultry waste (M and L) was performed following standard methods [17]. The following physicochemical parameters were evaluated: dry matter (DM), organic matter (OM), total Kjeldahl nitrogen (TN), pH, electrical conductivity (EC), nitrate-N (NO<sub>3</sub><sup>-</sup>-N), ammonium-N (NH<sub>4</sub><sup>+</sup>-N), and total phosphorous (TP). Biological activity was measured using the static respiration index (SRI) [17, 18].

For the soil samples (SC, SM, and SL), we determined the following parameters, according to standard methods [19]: DM, OM, TN, pH, EC,  $\text{NO}_3^-$ -N,  $\text{NH}_4^+$ -N, TP, and extractable P (eP).

### Seed Germination and Root Elongation Toxicity Test

Acute toxicity tests of the poultry waste on seeds were carried out according to Young et al. [14]. Briefly, aqueous extracts were prepared by mixing a sample of poultry waste (M or L) with deionized water (1:10 w/v) to simulate water-extractable substances present in leachate or runoff. Untreated seeds of five species were used as test organisms: lettuce (*Lactuca sativa* variety “Gallega”), radish (*Raphanus sativus* variety “Puntas blancas”), globe squash zucchini (*Cucurbita maxima* variety “Veronés”), arugula (*Eruca sativa*), and chicory (*Cichorium intybus*).

A completely randomized experimental design was carried out with three treatments for each plant species ( $n = 15$ ). Treatments consisted of aqueous extracts of poultry manure, chicken litter, and deionized water (negative control) by triplicate. Ten seeds of *C. maxima* or fifteen of *L. sativa*, *R. sativus*, *E. sativa*, and *C. intybus* were exposed to 4 mL of extract in Petri dishes with filter paper (Munktell AB Box 300, SE-790 20 GRYCKSBO, Sweden) for 120 h under controlled conditions ( $22 \pm 1$  °C in darkness).

After exposure, the number of germinated seeds and the root length were recorded to determine the percentages of seed germination inhibition and root elongation inhibition. Also, two phytotoxicity indices were calculated: relative growth index (RGI; Eq. 1) and germination index (GI; Eq. 2), according to Alvarenga et al. [20] and Zucconi et al. [21], respectively. RGI values between 0 and 0.8 are categorized as inhibition of root elongation (I), values  $> 0.8$  and  $< 1.2$  as no-significant-effect (NSE), and values  $> 1.2$  as stimulation of root elongation (S) [22]. GI values lower than 80% were considered inhibition [23].

$$\text{RGI} = \text{RLPS}/\text{RLC} \quad (1)$$

$$\text{GI}(\%) = \text{RGI} \times \text{GSPS}/\text{GSC} \times 100 \quad (2)$$

where, RLPS is the root length in the poultry waste (manure or litter), RLC is the root length in the negative control, GSPS is the number of germinated seeds in the poultry waste (manure or litter), and GSC is the number of germinated seeds in the negative control.

### Bacterial Community Analysis

#### DNA Extraction

Poultry manure, chicken litter, and soils were stored at  $-80$  °C for molecular analysis. DNA extractions from

samples were performed with the QIAmp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the protocol provided by the manufacturer. DNA was stored at  $-20$  °C until sequencing analysis. A total of 15 DNA samples (6 poultry waste -M and L- and 9 soil samples—SM, SL, SC-), 3 replicates per treatment, were used for 16S rRNA gene-based microbial community analysis.

#### 16S rRNA Gene Metabarcoding

PCR amplification was performed using a Fluidigm Access Array (Fluidigm Corporation, South San Francisco, USA) in combination with the Roche High Fidelity Fast Start Kit (Roche, Basel, Switzerland) following Lange et al. [24], using target-specific primers mix for the V3-V4 region of the 16S rRNA gene (with a final concentration of 250 nM each, 341F: 5'-CCTACGGGNGGCWGCAG-3' and 805R: 5'-GAC TACHVGGGTATCTAATCC-3'). The thermal profile was 95 °C for 10 min, followed by 10 cycles at 95 °C for 15 s, 55 °C for 30 s and 72 °C for 60 s, 2 cycles at 95 °C for 15 s, 80 °C for 30 s, 55 °C for 30 s and 72 °C for 60 s, 8 cycles at 95 °C for 15 s, 55 °C for 30 s and 72 °C for 60 s, 2 cycles at 95 °C for 15 s, 80 °C for 30 s, 55 °C for 30 s and 72 °C for 60 s, 8 cycles at 95 °C for 15 s, 55 °C for 30 s and 72 °C for 60 s, 5 cycles at 95 °C for 15 s, 80 °C for 30 s, 55 °C for 30 s, and 72 °C for 60 s, and a finishing step at 72 °C for 5 min.

The amplicons were sequenced using Illumina MiSeq with a MiSeq Sequencing Reagent Kit v2 to obtain 250 bp paired-end reads at the Unidad de Genómica (UGB) of the Instituto Nacional de Tecnología Agropecuaria (INTA, Hurlingham, Buenos Aires, Argentina).

#### Data Analysis of 16S rRNA Gene Amplicons

Poultry waste and soil samples were analyzed separately. Raw reads were processed by the QIIME2 software package (version 2018.2). Quality control and denoising were performed using the Divisive Amplicon Denoising Algorithm 2 (DADA2) [25]. DADA2 uses a parametric model to infer true biological sequences from reads, removes chimeras, and low-quality sequences. Reads were dereplicated, and amplicon sequence variants (ASVs) were inferred. Representative sequences were classified using a pre-trained naïve Bayes classifier for the V3-V4 region of the 99% SILVA v.128 database.

The ASVs were aligned with Mafft [26] and placed into a phylogenetic tree with FastTree [27]. Metrics of alpha diversity (observed ASVs, Faith-pd, Shannon diversity index, and Pielou's evenness) and beta diversity (Bray Curtis, Jaccard, weighted UniFrac, and unweighted UniFrac) distance indices [28] were estimated after samples were rarefied to the minimum depth of sequences observed in any given sample.

## Statistical Analysis

Physicochemical parameters of poultry waste and soils were analyzed using one-way analysis of variance (ANOVA) and Tukey's test when differences between means were observed ( $p < 0.05$ ). Data were processed using InfoStat software (version 2016) [29]. Similarly, for the ecotoxicological parameters, ANOVA was used to test differences between poultry manure and chicken litter for each evaluated species of seed.

Microbial analysis was complemented with an analysis of composition of microbiomes (ANCOM) test to determine if there were significant differences in the relative abundance of any taxa between treatments [30].

Statistical analyses for alpha and beta-diversity distance matrices were completed using QIIME2. Boxplot figures for alpha diversity were created using the Kruskal–Wallis test to estimate the median difference among all groups or pairwise groups. Correlations between the alpha diversity index and physicochemical and phytotoxic parameters were calculated using the Spearman correlation. The beta diversity distance matrix as inputs for assessing group significance and plotting Principal Coordinate Analysis (PCoA) charts were completed using QIIME2. Permutational multivariate analysis of variance (PERMANOVA, with 999 permutations) was used to test the significance among poultry wastes (M and L) and amended soils (SC, SM, and SL).

## Results

### Characterization of Poultry Waste

#### Physicochemical Analysis and Stability

Differences were observed in the physicochemical properties of the poultry waste (Table 1). Litter samples showed higher

values of DM, OM, pH, and  $\text{NO}_3^-$ -N, while the manure samples have a higher value for EC and  $\text{NH}_4^+$ -N. Poultry manure and chicken litter showed high SRI values, indicating that both samples were not biologically stable ( $> 0.5 \text{ mg O}_2 \text{ g MO}^{-1} \text{ h}^{-1}$ ).

#### Seed Germination and Root Elongation Toxicity Test

The phytotoxicity indices and toxicological endpoints allowed us to observe a difference in sensitivity between the tested plant species. Lettuce was the most sensitive species to poultry waste, showing the lowest germination percentage and root length which indicated a strong inhibition (Table 2). In contrast, zucchini (*C. maxima*) was the most tolerant species, with the highest GI and RGI in litter samples, showing a slight inhibition on germination but stimulation on root elongation. In addition, when the results of the 2 poultry waste (L and M) were compared, it was observed that M was more toxic than L for the 5 studied species. However, this effect was only significantly different on the seed germination of lettuce and zucchini, root elongation and RGI of lettuce and chicory, and GI of lettuce, arugula, and chicory.

#### Bacterial Community Analysis of Waste

Differential abundance testing with ANCOM revealed two phyla, four classes, one order, four families, and two genera were differentially abundant among poultry waste (Fig. S1 and Table S1).

The main phyla present in both types of poultry waste (L and M) were Bacteroidetes and Proteobacterias. Particularly for M, we observed higher levels of Firmicutes, with more than 23% of the bacterial belonging to this phylum (Fig. 1a and Table S2). The main families founded in L and M samples were Sphingobacteriaceae, Flavobacteriaceae,

**Table 1** Poultry waste properties

| Parameter          | Units  | Differences | Chicken litter       | Poultry manure         |
|--------------------|--|-------------|----------------------|------------------------|
| SRI                | ( $\text{mg O}_2 \text{ g}^{-1} \text{ OM h}^{-1}$ ) | ns          | $2.31 \pm 0.33$      | $2.87 \pm 0.91$        |
| DM                 | (%)  | **          | $76.49 \pm 6.72$     | $38.52 \pm 4.8$        |
| OM                 | (%)  | ***         | $74.80 \pm 2.27$     | $55.17 \pm 0.57$       |
| TN                 | (%)  | ns          | $1.72 \pm 0.43$      | $4.05 \pm 1.68$        |
| pH                 |  | **          | $8.43 \pm 0.23$      | $7.60 \pm 0.2$         |
| EC                 | ( $\text{mS cm}^{-1}$ )                              | **          | $5.65 \pm 0.55$      | $9.24 \pm 0.79$        |
| $\text{NH}_4^+$ -N | ( $\text{mg Kg}^{-1}$ )                              | *           | $14.37 \pm 8.19$     | $127.83 \pm 61.17$     |
| $\text{NO}_3^-$ -N | ( $\text{mg Kg}^{-1}$ )                              | *           | $22.18 \pm 22.86$    | $3.80 \pm 0$           |
| TP                 | ( $\text{mg Kg}^{-1}$ )                              | ns          | $7456.91 \pm 564.15$ | $10378.73 \pm 2077.67$ |

Statistical significant differences among samples based on one-way ANOVA: ns, not significant; \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ . Mean values ( $n = 3$ )  $\pm$  SD

SRI, static respiration index; DM, dry matter; OM, organic matter; TN, total Kjeldahl nitrogen; EC, electrical conductivity;  $\text{NH}_4^+$ -N, ammonium-N;  $\text{NO}_3^-$ -N, nitrate-N; TP, total phosphorous. All results are expressed on a dry weight basis

**Table 2** Seed germination and root elongation toxicity test

|                                 | Sample | <i>Lactuca sativa</i>        | <i>Eruca sativa</i>          | <i>Raphanus sativus</i>      | <i>Cichorium intybus</i>     | <i>Cucurbita maxima</i>       |
|---------------------------------|--------|------------------------------|------------------------------|------------------------------|------------------------------|-------------------------------|
| Seed germination inhibition (%) | Litter | 53.5 ± 10.7 <sup>a</sup>     | 14.6 ± 11.2 <sup>a</sup>     | 2.2 ± 3.8 <sup>a</sup>       | 6.1 ± 5.2 <sup>a</sup>       | 6.9 ± 10.3 <sup>a</sup>       |
|                                 | Manure | 86.0 ± 12.1 <sup>b</sup>     | 19.5 ± 26.4 <sup>a</sup>     | 6.7 ± 6.7 <sup>a</sup>       | 24.2 ± 13.9 <sup>a</sup>     | 49.4 ± 8.0 <sup>b</sup>       |
| Root elongation inhibition (%)  | Litter | 71.2 ± 2.4 <sup>a</sup>      | 53.7 ± 22.8 <sup>a</sup>     | 46.7 ± 10.7 <sup>a</sup>     | 33.4 ± 13.2 <sup>a</sup>     | -37.5 ± 42.2 <sup>a</sup>     |
|                                 | Manure | 95.3 ± 1.6 <sup>b</sup>      | 90.3 ± 6.0 <sup>a</sup>      | 59.8 ± 20.3 <sup>a</sup>     | 84.5 ± 16.8 <sup>b</sup>     | -23.8 ± 15.3 <sup>a</sup>     |
| GI                              | Litter | 13.6 ± 4.1 <sup>a</sup> (I)  | 37.9 ± 13.2 <sup>a</sup> (I) | 51.8 ± 8.3 <sup>a</sup> (I)  | 62.8 ± 14.0 <sup>a</sup> (I) | 130.4 ± 49.6 <sup>a</sup> (S) |
|                                 | Manure | 0.7 ± 0.8 <sup>b</sup> (I)   | 8.8 ± 7.4 <sup>b</sup> (I)   | 38.4 ± 21.8 <sup>a</sup> (I) | 13.1 ± 16.0 <sup>b</sup> (I) | 62.7 ± 13.5 <sup>a</sup> (I)  |
| RGI                             | Litter | 0.29 ± 0.02 <sup>a</sup> (I) | 0.46 ± 0.23 <sup>a</sup> (I) | 0.53 ± 0.11 <sup>a</sup> (I) | 0.67 ± 0.13 <sup>a</sup> (I) | 1.37 ± 0.42 <sup>a</sup> (S)  |
|                                 | Manure | 0.05 ± 0.02 <sup>b</sup> (I) | 0.10 ± 0.06 <sup>a</sup> (I) | 0.40 ± 0.20 <sup>a</sup> (I) | 0.15 ± 0.17 <sup>b</sup> (I) | 1.24 ± 0.15 <sup>a</sup> (S)  |

Different letters indicate significant differences ( $p < 0.05$ ) between samples at each toxicity endpoint or phytotoxicity index evaluated at each plant species. RGI, relative growth index; GI, germination index; I, inhibition; NS, no significant effect; S, stimulation

Porphyromonadaceae, Xanthomonadaceae, Halomonadaceae, Alcaligenaceae, Clostridiales-Family XI, and Bacillaceae (Fig. 1b and Table S2).

The diversity community analysis of the bacterial presents on samples showed no difference in the alpha diversity parameters evaluated between both types of poultry waste using Kruskal–Wallis (observed ASVs:  $p = 0.83$ ; Faith-pd:  $p = 0.83$ ; Shannon index,  $p = 0.28$ ; Pielou’s evenness:  $p = 0.28$ ) (Fig. S2).

Beta diversity showed no statistically differences using weighted UniFrac distance ( $pseudo-F = 4.21$ ;  $p = 0.101$ ), unweighted UniFrac distance ( $pseudo-F = 2.522$ ;  $p = 0.125$ ) (Fig. S3), Jaccard distance ( $pseudo-F = 2.611$ ;  $p = 0.095$ ) or Bray–Curtis distance ( $pseudo-F = 5.262$ ;  $p = 0.102$ ).

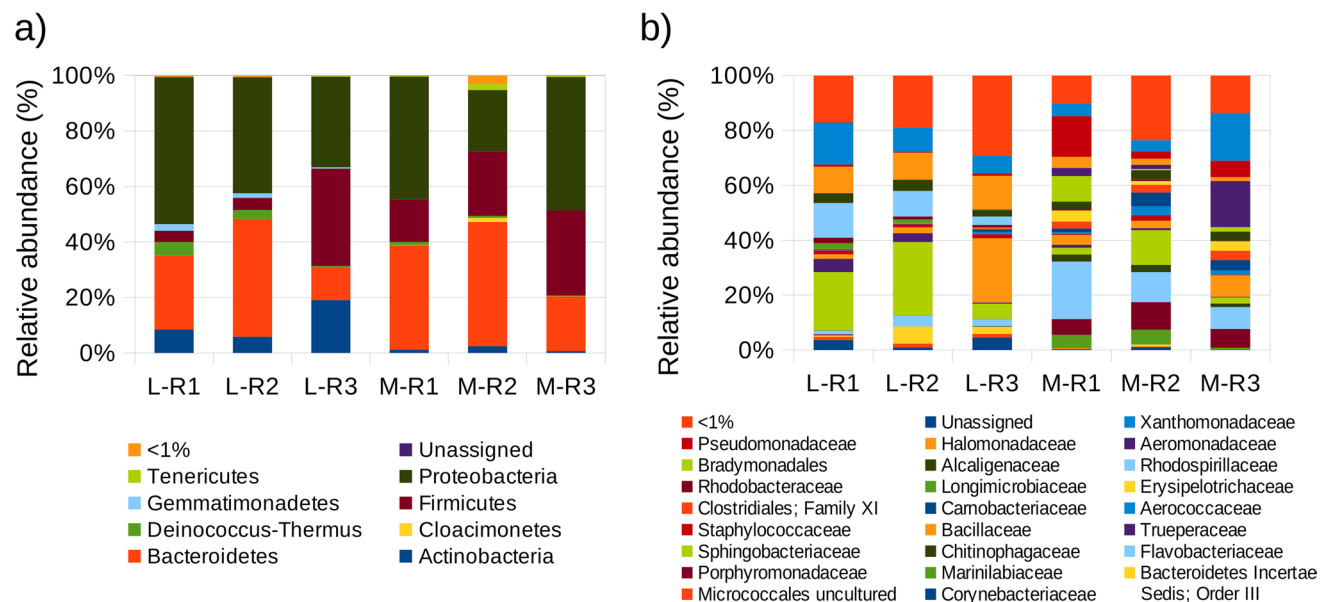
## Effects of Poultry Waste on Agricultural Soils

### Physicochemical Analysis

The soil samples did not show differences in OM, TN,  $NH_4^+$ -N, and  $NO_3^-$ -N (using one-way ANOVA,  $p > 0.05$ ). We only found differences in the manure treatment (SM) where modifications in the physicochemical patterns of soil were observed with an increase in pH, EC, eP, and TP (Table 3).

### Bacterial Community Analysis of Soils

Differential abundance through ANCOM revealed no statistically significant differences between soil samples.



**Fig. 1** Phylum **a** and family **b** barplots of the community structure of poultry waste. M, poultry manure; L, chicken litter. R1, R2, and R3 represent three different replicates by treatment



The main phyla present in soils were Acidobacteria, Actinobacteria, Proteobacteria, and Gemmatimonadetes (Fig. 2a and Table S3). The most abundant families present in soil samples were uncultured Acidimicrobiales, Gaiellaceae, Blastocatellaceae (subgroup 4), Acidobacteria (uncultured subgroup 6), Solibacteraceae (subgroup 3), Sphingomonadaceae, Nitrosomonadaceae, and Gemmatimonadaceae (Fig. 2b and Table S3).

Alpha diversity parameters showed differences between SC and SL samples using Kruskal–Wallis (observed ASVs,  $p=0.049$ ; Faith-pd,  $p=0.049$ ; and Shannon index,  $p=0.049$ ) (Fig. 3a). No statistically significant differences were found when evaluating Pielou's evenness ( $p=0.51$ ).

Spearman's linear correlation coefficient was calculated between the alpha diversity parameters of soils and the physicochemical factors. The complete results are shown in the supplementary documents (Table S4). We could only found a statistically significant Spearman

negative correlation between the observed ASVs and the TP ( $\rho = -0.6833$ ,  $p=0.0424$ ) (Fig. 3b).

The analysis of beta diversity showed statistically significant dissimilarities between soil samples (Jaccard distance,  $pseudo-F=1.301$ ;  $p=0.008$ , Bray–Curtis distance,  $pseudo-F=1.895$ ;  $p=0.002$ , and unweighted UniFrac distance,  $pseudo-F=1.417$ ;  $p=0.004$ ) (Fig. 3d). No statistically differences were observed using weighted UniFrac distance ( $pseudo-F=1.516$ ;  $p=0.147$ ) (Fig. 3c).

## Discussion

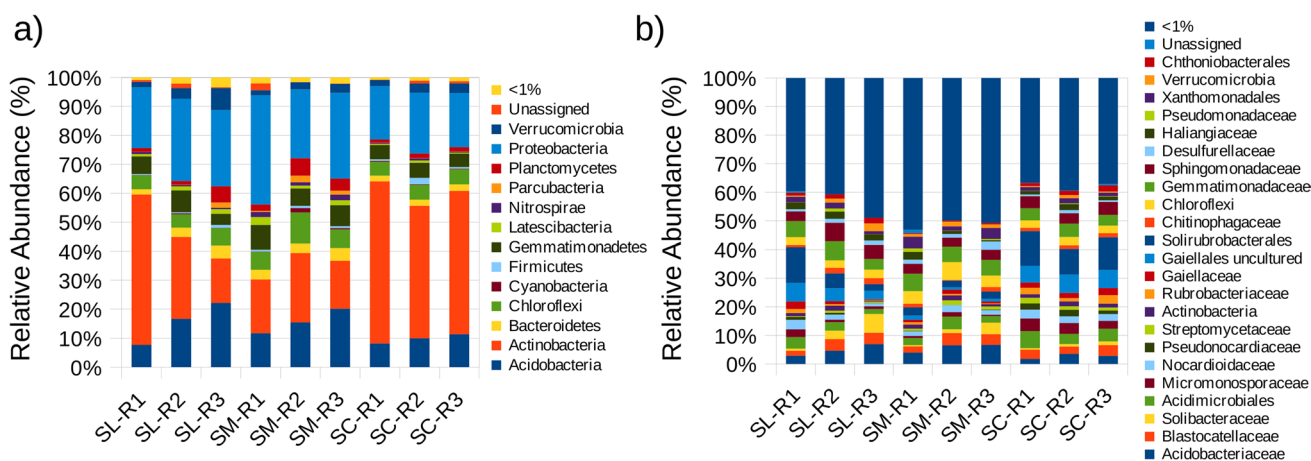
In this work, we approach the use of wastes from poultry farming as organic amendments, portraying the two scales implicated in the process as a whole. We carry out the characterization of the poultry waste at physicochemical, ecotoxicological, and microbiological levels. Furthermore, we

**Table 3** Soil physicochemical properties

| Parameter                       | Units                  | Differences | Soil control               | Soil + Litter               | Soil + Manure               |
|---------------------------------|------------------------|-------------|----------------------------|-----------------------------|-----------------------------|
| DM                              | (%)                    | **          | 87.73 ± 0.93 <sup>c</sup>  | 84.29 ± 0.54 <sup>b</sup>   | 82.55 ± 0.25 <sup>a</sup>   |
| OM                              | (%)                    | ns          | 7.38 ± 0.37                | 7.37 ± 0.79                 | 7.72 ± 0.13                 |
| TN                              | (%)                    | ns          | 0.37 ± 0.05                | 0.26 ± 0.23                 | 0.18 ± 0.17                 |
| pH                              |                        | **          | 6.22 ± 0.29 <sup>a</sup>   | 5.98 ± 0.06 <sup>a</sup>    | 7.22 ± 0.13 <sup>b</sup>    |
| EC                              | (mS cm <sup>-1</sup> ) | **          | 0.09 ± 0.04 <sup>a</sup>   | 0.14 ± 0.04 <sup>a</sup>    | 0.27 ± 0.03 <sup>b</sup>    |
| NH <sub>4</sub> <sup>+</sup> -N | (mg Kg <sup>-1</sup> ) | ns          | 0 ± 0                      | 0 ± 0                       | 0.67 ± 1.16                 |
| NO <sub>3</sub> <sup>-</sup> -N | (mg Kg <sup>-1</sup> ) | ns          | 0 ± 0                      | 1.66 ± 2.88                 | 0 ± 0                       |
| TP                              | (mg Kg <sup>-1</sup> ) | ***         | 239.10 ± 8.49 <sup>a</sup> | 304.07 ± 19.07 <sup>a</sup> | 801.99 ± 79.39 <sup>b</sup> |
| eP                              | (mg Kg <sup>-1</sup> ) | **          | 28.90 ± 13.99 <sup>a</sup> | 39.48 ± 29.33 <sup>a</sup>  | 128.60 ± 19.95 <sup>b</sup> |

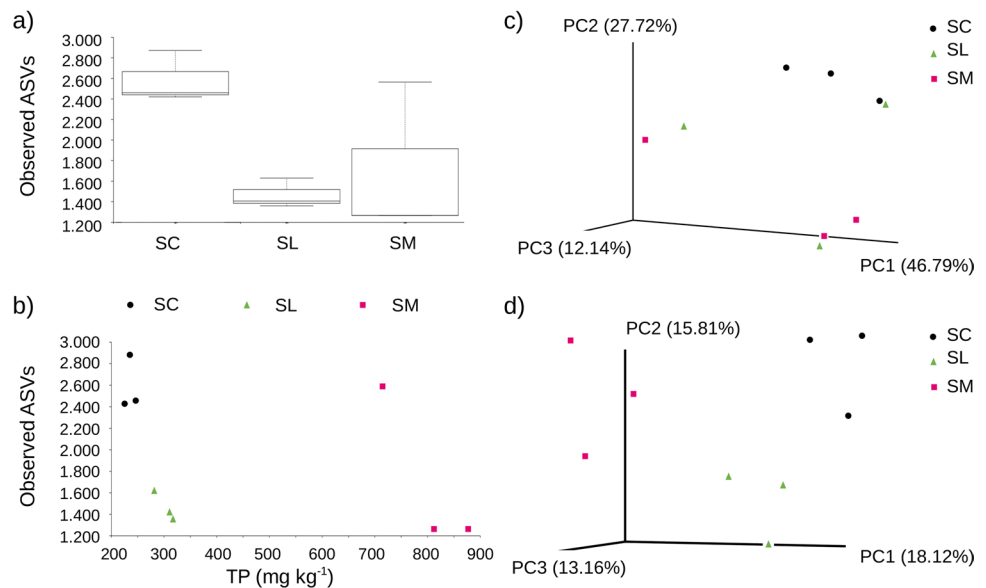
Different letter indicate significant differences among samples based on one-way ANOVA: ns, not significant; \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ . Mean values ( $n=3$ ) ± SD

DM, dry matter; OM, organic matter; TN, total Kjeldahl nitrogen; EC, electrical conductivity; NH<sub>4</sub><sup>+</sup>-N, ammonium-N; NO<sub>3</sub><sup>-</sup>-N, nitrate-N; TP, total phosphorous; eP, extractable phosphorous. All results are expressed on a dry weight basis



**Fig. 2** Phylum **a** and family **b** barplots of the community structure of soils. SL, soil + litter; SM, soil + manure; SC, soil control. R1, R2, and R3 represent three different replicates by treatment

**Fig. 3** Diversity analysis of the soil bacterial community. Observed ASVs Boxplots **a**, Spearman correlation of soils richness and total phosphorous **b**, and PCoA 3D plots from weighted **c**, and unweighted **d** UniFrac metrics. SL, soil + litter; SM, soil + manure; SC: control soil



retrospectively evaluate the impact of using each kind of waste over the currently producing agricultural soils.

### Characterization of Poultry Waste

Considering the poultry waste as the first scale of the analysis, statistical differences were detected between some physicochemical parameters.

The DM contents reflect the composition of the different waste, with higher values for the chicken litter which is made up of wood chips and seed shells, in addition to the remains of food, feathers, and poultry manure.

The SRI parameter reflects the stability of the poultry waste and the biodegradability of OM. In the case of our samples, the values found demonstrate their ongoing transformation since they have not undergone any type of treatment prior to their application, such as being composted or anaerobic digested [31]. In that sense, SRI values of less than 0.5 are required to guarantee the stability of treated amendments to be commercialised [32, 33].

Another parameter that showed differences between poultry waste was electrical conductivity (EC). We observe high values of EC for both types of waste, with higher values for M samples. High EC values indicate high salt concentrations (principally Na<sup>+</sup>, K<sup>+</sup>, Ca<sup>2+</sup>, Mg<sup>2+</sup>, Cl<sup>-</sup>, and SO<sub>4</sub><sup>2-</sup> ions or potentially toxic elements such as Cr, Co, Ni, Cu, Zn, As, Se, Mo, Cd, and Pb) that could generate changes in the physical properties of soils, osmotic stress, and ionic toxicity, which could affect crop germination and many physiological processes in plants [34]. The high EC values observed in this study could result in crop yield losses depending on the frequency and quantity with which they are applied to agricultural soil. In this sense, losses of up to 20% in spinach

and carrot growth have been reported in three different types of soils when applying poultry manure [35].

Finally, the correlation of pH with the composition of the microbial community and with the availability of nutrients in the soil is very well acknowledged [36]. In our study, L samples were always more alkaline than M samples. Usually, the pH is a good indicator of the quality of the amendments, being the neutral range (from 5.8 to 8.5) the better condition for the availability of the nutrients for crops [34]. Our results are in accordance to the expected value for suitable soil amendments.

Additionally, the pH is related to other evaluated metrics, such as TN, NO<sub>3</sub><sup>-</sup>-N, and NH<sub>4</sub><sup>+</sup>-N. Values of pH higher than 7 could cause nitrogen losses via the volatilization of NH<sub>3</sub>. Most N in poultry waste comes from uric acid, which is easily transformed to ammonium and therefore susceptible to N losses due to highly volatility [37]. In our study, L showed the lowest values of NH<sub>4</sub><sup>+</sup>-N and the highest of NO<sub>3</sub><sup>-</sup>-N, while the other way around in M. However, this inverse trend did not involve any statistically significant differences between the TN content in both types of waste.

The high values of many of the described parameters, especially higher amounts of nutrients and EC, alert us to possible phytotoxic effects that could generate yield losses in crops and a source of contamination. Plant biological endpoints such as seed germination and root elongation are commonly used to measure soil contaminant bioavailability and toxicity [14]. We used five different plants to evaluate the toxicity of the poultry waste, knowing that each species can show a different level of sensitivity to contaminants. Phytotoxicity indices found by seed exposure to poultry waste are consistent with those reported by other authors [14, 34].

The direct application of manure or litter without previous treatment could generate leachates with phytotoxic effects. Moreover, several authors have shown difficulties in reducing the toxicity caused by poultry waste treated by composting [14, 38] and anaerobic digestion [39]. In our study, manure was shown to be more toxic than litter, which could be related to higher values of EC and ammonium according to Young et al. [22] and Bitsánszky et al. [40].

At the microbiological level, the bacterial community analysis for both types of samples showed the predominance of Bacteroidetes and Proteobacteria, and particularly, M samples showed higher values of Firmicutes. Several authors have described the bacterial composition of M and L samples, finding similar results, with the presence of the same main phyla [41–45].

Although some *Bacteroides* spp. can be opportunistic pathogens, many Bacteroidetes are mutualistic species highly adjusted to the gastrointestinal tract. They perform metabolic conversions that are essential for the host, such as the degradation of proteins or complex sugar polymers [46]. Meanwhile, Proteobacteria include a wide variety of pathogenic genera for humans, animals, and plants. Others are free-living (nonparasitic) and include many of the bacteria responsible for nitrogen fixation [47]. Members of the Firmicutes phylum are typically present in the gastrointestinal tract of animals. This phylum has been reported as copiotrophs, with higher rates of growth in C- and nutrient-rich environments [6].

The main families found in the poultry waste were shown to be typical members of the gastrointestinal tract of the birds. They are usually involved in the degradation of food substances, and some of them could be potential sources of contamination and show pathogenic effects (Table 4).

The presence of these microorganisms with the ability to affect the animal, plant, and human health is one of the main risks of the application of poultry waste without prior treatment. This risk is higher in man-made environments, because they are less diverse than pristine ones [48]. Some potential reasons for pathogen and disease suppression in microbiologically diverse ecosystems are the greater chance to find antagonists, resource competitors, and predators of pathogens in soil, rhizosphere, plants, or animals [48].

However, to circumvent the release of pathogenic microorganisms and parasites into the environment, the application of raw wastes in the soil should be avoided. In this sense, composting and anaerobic digestion are the two most frequently studied technologies for the treatment of poultry waste and may contribute to reducing the transfer of this type of pollutant to the environment [49].

### Effects of Poultry Waste on Agricultural Soils

Considering the analysis of amended soils with poultry waste, we detected statistical differences in some physico-chemical parameters between treatments.

Although the chicken litter shows higher  $\text{NO}_3^-$ -N values, no differences in TN,  $\text{NH}_4^+$ -N, or  $\text{NO}_3^-$ -N soil

**Table 4** Principal families found in the community structure of poultry waste

| Family                  | Description   |
|-------------------------|---|
| <b>Bacteroidetes</b>    |   |
| Sphingobacteriaceae     | Composed by eight species of genus <i>Sphingobacterium</i> . Some species have been associated with bacteremia, peritonitis, and chronic respiratory infection in patients with severe underlying condition and showed multidrug resistance [44]                    |
| Flavobacteriaceae       | <i>Flavobacteria</i> and <i>Pseudomonas</i> are traditionally known to cause spoilage in food and food products [66]  |
| Porphyromonadaceae      | This family was suggested to play a role in degradation of accumulated volatile fatty acids [67]  |
| <b>Proteobacterias</b>  |   |
| Xanthomonadaceae        | A widespread family of bacteria with 22 genera, including plant-pathogenic genera <i>Xanthomonas</i> , <i>Xylella</i> , and <i>Stenotrophomonas</i> , increasingly recognized as an important cause of severe disease of crops [56]                                 |
| Halomonadaceae          | Contains two genera, <i>Halomonas</i> and <i>Deleya</i> . <i>Halomonas</i> were isolated from saline environments and exhibit extreme tolerance to NaCl [68]  |
| Rhodospirillaceae       | These bacteria comprise different morphological types of phototrophic bacteria that can photoassimilate simple organic compounds under anaerobic conditions. They are widely distributed in nature and man-made environments. They are stimulated by pollution [69] |
| Alcaligenaceae          | They have been reported to harbor tetracycline resistant and class 1 integron genes [70]  |
| <b>Firmicutes</b>       |   |
| Clostridiales-Family XI | Typical members of the gastrointestinal tract of chicken [71]   |
| Staphylococcaceae       | One of the most common microbiota members present in chicken's fecal [72]   |
| Bacillaceae             | Widely distributed in nature. Exhibit resistance to changes in pH, salinity, and temperature, as well as having resistance to many chemicals. They are common contaminants of man-made habitats [73]  |



content were found after waste application. We speculate that waste could have undergone nitrogen loss during the storage period because of leaching. In the case of poultry manure, though it is high in  $\text{NH}_4^+$ -N the contribution to TN might be negligible due to possible volatilization losses as a consequence of alkaline pH.

Soils amended with chicken manure (SM) show the highest values of EC, pH, TP, and eP (Table 3). In spite of using the waste of high EC content (Table 1), the increment in SM is below the recommended threshold for EC to avoid any risk of plant growth inhibition in amended soils ( $\text{EC} > 4 \text{ mS cm}^{-1}$  affect the productivity of most crops) [35], while SL does not show statistically significant differences in comparison to the untreated soil (Table 3). Similarly, SM and SL exhibit statistically significant modified soil pH after waste application. Still, the pH shift fits into the optimal range for healthy soils.

On the contrary, the amended soils show TP and eP significantly different even when the TP content in both poultry waste samples is equivalent. Most notably, SM exhibits the highest values of TP ( $SM = 801.99 \pm 79.39$ ), far exceeding the SC and SL ones ( $SC = 239.10 \pm 8.49$ ,  $SL = 304.07 \pm 19.07$ ). This result is well supported by previous reports, suggesting that poultry manure provides a source for replenishment of soil solution P [50, 51]. In that sense, Waldrip et al. [50] reported the incorporation of poultry manure into soil promoted transformation and mineralization of less-labile inorganic and organic P into labile-Pi in the rhizosphere, which result in higher root P concentrations.

On the other hand, the accumulation of P in agricultural soils resulting from fertilizer, organic manure, and sewage sludge applications, as observed in this study, might enhance the potential for P losses, even despite the high P fixation capacity of soils. Vertical movement of P through the soil profile is generally considered of little importance, unless soils become P saturated, especially following heavy manure applications [52]. Heckrath et al. [52] described the Olsen-P environmental critical value of  $60 \text{ mg kg}^{-1}$  as the tipping point above which P leaching has been shown to be significant. Our results of eP show values exceeding this threshold for SM ( $eP = 128.60 \pm 19.95 \text{ mg kg}^{-1}$ ). This high value of P could increase the risk of contamination, leading to enhanced phosphate loss, and subsequent degradation of freshwater resources where eutrophication can be triggered by additional P inputs [53]. Additionally, the selected fields are gently undulating plains with edaphic characteristics prone to runoff and soil loss, increasing the risk of eutrophication [54, 55].

Even when SM treatment shows the greatest physicochemical differences with respect to SC, the bacterial community analysis depicted no differences between the amended and control soil. The community profiles of soils (SC, SL, and SM) do not change after treatment. The

composition of treated and control soil samples shows higher proportions of Actinobacteria (with values ranging between 15 and 55%), Acidobacteria (7–22%), and Gemmatimonadetes (3–8%) compared to the poultry waste samples.

Actinobacteria was one of the dominant phyla in soil, helping to decompose the organic matter of dead organisms and solubilize phosphate and calcium carbonate [56]. Some Actinobacteria (such as *Frankia* sp.) live associate with a broad spectrum of plants, fixing nitrogen to the host plant in exchange for reduced carbon [57]. Other species, such as many members of the genus *Mycobacterium*, are well known as human and livestock pathogens [58]. The genus, *Streptomyces* is a major contributor to the biological buffering of soils, been also the source of many antibiotics [59].

Acidobacteria is a physiologically diverse and ubiquitous phylum, especially in soils. Acidobacteria dynamics are highly connected to environmental factors such as pH and nutrients [60].

The Gemmatimonadetes phylum makes up about 2% of the soil bacterial communities and has been identified as one of the top nine phyla found in soils [61].

The prominent families found in soils comprised bacteria involved in soil fertility (mainly participating in the cycles of nutrients such as C and N), as well as in plant growth promotion (inhibiting pathogens through antibiotic secretion, competing for space, or stimulating the production of phytohormones or producing siderophores) (Table 5).

In our study, the application of poultry waste along time shows a decrease in the alpha diversity values from SL. In the SM samples, the loss of diversity is observed though not statistically significant because of one out of the three replicate should be discarded as a real outlier.

We also detect a negative correlation between the richness (ASVs observed) and the TP values present in the soil samples. Maintaining stable and high values of diversity in the soils after the application of amendments is of great importance so as not to negatively impact the functions that these communities carry out in the soils for stimulating the growth of crops [62]. There are a limited number of functions to be performed within an ecosystem, and soil communities exhibit a high degree of functional redundancy [62]. This characteristic is crucial for maintaining ecosystem functioning in response to perturbation, as long as it increases the probability that some species capable of performing a certain function remain present [63].

In this regard, Celestina et al. [64] found that the application of fertilizers or manure in the field has no significant lasting impacts on soil microbial communities, concluding that the differences found are due to their location within the soil profile, and not due to the type of amendment applied or its method of spreading. Additionally, Sun et al. [5] and Semenov et al. [65] found similar results, with negligible effects of introduced bacteria from livestock manures on

**Table 5** Predominant families found in soil samples

| Family                                | Description   |
|---------------------------------------|---|
| <b>Actinobacterias</b>                |   |
| Uncultured Acidimicrobiales           | Improve soil fertility and plant growth efficiency [74]   |
| Micromonosporaceae                    | Improve soil fertility and plant growth efficiency [74]   |
| Nocardioideae                         | Degrade Sulfamethoxazole (one of the most frequent antibiotics in wastewater, surface water, and soils) [75]. They have been associated with plant development and specific root morphological traits [76]  |
| Solirubrobacteraceae                  | They are aerobes that utilize many sugars and a few other compounds as sole carbon sources [77]   |
| Solirubrobacterales Elev-16S-1332     | Degrade pollutants and inhibit pathogens [78]   |
| Pseudonocardiaceae                    | Contain a range of metal resistance and tolerance mechanisms [76]   |
| Gaiellaceae                           | Degrade pollutants and inhibit pathogens [78]   |
| Rubrobacteriaceae                     | Specific to semi-arid environments [79]. They may be important in inorganic carbon acquisition in desert soils, due to high plasticity in chemoautotrophic metabolism [80]  |
| Streptomycetaceae                     | Secrete antimicrobial by inhibiting pathogens or other taxa. Contain a range of metal resistance and tolerance mechanisms. Produce many metabolic products affecting host (phytohormones, siderophores) [76]  |
| <b>Acidobacterias</b>                 |   |
| Blastocatellaceae (subgroup 4)        | Beneficial to soil recovery because their soil carbon substrate decomposition abilities. They are oligotrophic bacteria, with higher efficiency in the utilization of recalcitrant organic pool and enhance nutrients cycling. They are acidophilic bacteria, and negatively correlated with soil pH [81] |
| Acidobacteria (uncultured Subgroup 6) | Positively correlated to nutrient availability and negatively correlated to soil acidity [60]   |
| Solibacteraceae (subgroup 3)          | They have been shown to associate with the resistance of some fungal pathogens ( <i>Fusarium oxysporum</i> ) and its relative abundance increases with water content. It has been found to involve in the carbon cycle of the soil [82]   |
| Acidobacteriaceae (subgroup 1)        | Common copiotrophs and potentially contribute a consortium of taxa consuming cellulose, hemicellulose, and chitin within burnt soils. Moderate acidophilic heterotrophs capable of reducing Fe (III) [83]   |
| <b>Gemmatimonadetes</b>               |   |
| Gemmatimonadaceae                     | Improve soil fertility and plant growth efficiency [74]   |
| <b>Proteobacterias</b>                |   |
| Sphingomonadaceae                     | Participate in the nitrogen cycle [74], degrade pollutants and inhibit pathogens [78]   |
| Nitrosomonadaceae                     | Participate in the nitrogen cycle [74]  |
| Haliangiaceae                         | Grow on insoluble organic substrates. Some of them are producers of important medical antibiotics   |
| Desulfurellaceae                      | Sulfur respiration  |
| <b>Bacteroidetes</b>                  |   |
| Chitinophagaceae                      | Degrade polysaccharides such as cellulose and chitin [84]   |
| <b>Verrucomicrobia</b>                |   |
| DA101 soil group (Chthoniobacterales) | Abundant and ubiquitous in soils, being characterized as an aerobic heterotroph with many putative amino acid and vitamin auxotrophies [85]   |
| <b>Planctomycetes</b>                 |   |
| Planctomycetaceae                     | Part of the nitrogen cycle, to carry out the anaerobic oxidation of ammonium, and metabolize C1 compounds [86]  |
| <b>Nitrospirae</b>                    |   |
| Nitrospiraceae                        | N transformation, essential for soil nitrification [84]   |

bacterial soil community. Semenov et al. [65] observed a rapidly increased in microbial biomass, gene abundance, taxonomic diversity, and respiration activity after manure application, but most of these properties tended to decrease after 2 weeks. The majority of manure-associated bacteria died almost immediately, and only a few genera survived in the soil after several months.

Our data reveal that though differences in beta diversity are observed, the bacteria introduced through the application of the poultry waste do not modify the structure of the resident soil bacterial community, revealing its resilience capacity to perturbations.

Although the analysis of soil bacterial communities is a powerful tool to understand the impact of agricultural

practices over the soil, the resilience capacity of these communities could conceal the potential risk of the use of poultry waste application as a soil amendment. Thus, multidisciplinary studies are needed, combining physicochemical, ecotoxicological, and microbiological approaches to unveil the current and potential impact of waste use in agricultural systems.

To our knowledge, the current results are a contribution to the portrait of current local agricultural practices, and on top of that, regarding the advantage of using microbiota as a biomarker for tracking the environmental impact of the animal production waste. This kind of study should pave the way for understanding the complexity of the waste reutilization process as well as stimulating the adoption of sustainable and safe practices among stakeholders.

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**Data availability** The raw sequences of the 16S rRNA gene reported in this article have been deposited in the NCBI Short Read Archive and are accessible there under accession number PRJNA689563.

## Declarations

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**Consent to participate** Not applicable.

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