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Soil enzyme activities and microbial community modulation after addition of poultry litter amendment enriched with *Bacillus* spp.

Modulação de atividades enzimáticas e da comunidade microbiana do solo após adição de condicionador de solo a base de cama de frango e enriquecido com *Bacillus* spp.

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ABSTRACT

The global consumption of mineral fertilizers has increased in the last 60 years. However, these fertilizers can negatively affect the soil and the atmosphere. The application of soil amendments focusing on sustainable farming practices can reduce the effects of mineral fertilization. In this study, we investigated the effects of increasing the dose of a commercial amendment using poultry litter enriched with *Bacillus* (SMARTGRAN) in different types of soils in Brazil under microcosm conditions. These different types of soils were classified as *Nitossolo*, *Argissolo arênico*, *Argissolo alumínico*, *Latossolo distroférrico*, and *Latossolo distrófico*. The biological activity in the soil was quantified by measuring the enzymes arylsulfatase, beta-glucosidase, and acid phosphatase. Additionally, bacterial diversity was evaluated by amplicon sequencing of the 16S rRNA gene and conducting phylogenetic analyses of three types of soils, which were selected because of their occurrence and fertility profiles. The results showed an increase in those enzyme activities under all conditions. The results of the analysis of the bacterial community in *Nitossolo*, *Argissolo arênico*, and *Latossolo distrófico* soil types showed a direct relationship between the bacterial composition in the soil and the increase in the amendment dosage. The alpha diversity indices decreased considerably because some plant-growth-promoting bacteria, such as *Bacillus*, *Massilia*, *Paenibacillus*, and *Rhizobium*, increased in relative abundance. The results indicated that an organic amendment enriched with *Bacillus* had a beneficial effect on different types of soil in Brazil.

Index terms: Ecology; soil bacterial community; organic matter; alpha diversity; sustainable agricultural practices.

RESUMO

Nos últimos 60 anos o consumo global de fertilizantes aumentou, porém estes produtos podem causar efeitos negativos ao solo e atmosfera. A aplicação de condicionadores de solos visando práticas sustentáveis pode reduzir os impactos da fertilização mineral. No presente estudo, os efeitos de doses crescentes de um condicionador comercial feito a partir de cama de frango enriquecida com *Bacillus* (SMARTGRAN) foram investigados em microcosmo para diferentes tipos de solos brasileiros. Os solos foram classificados como *Nitossolo*, *Argissolo arênico*, *Argissolo alumínico*, *Latossolo distroférrico* e *Latossolo distrófico*. A atividade biológica foi quantificada através da medição das enzimas arilsulfatase, beta-glicosidase e fosfatase ácida. Além disso, a diversidade de bactérias foi avaliada com sequenciamento de *amplicons* de gene para rRNA 16S e análises filogenéticas em três dos cinco solos, selecionados devido à ocorrência e perfis de fertilidade. Os resultados mostraram aumento das enzimas em todas as condições. A análise de comunidades de bactérias do *Nitossolo*, *Argissolo arênico* e *Latossolo distrófico* demonstrou relação direta entre a composição de bactérias do solo e o aumento da dose de condicionador. Os índices de alfa diversidade reduziram drasticamente devido ao aumento da abundância relativa de algumas bactérias promotoras de crescimento de plantas, como *Bacillus, Massilia, Paenibacillus e Rhizobium*. Os dados demonstraram efeitos benéficos de um condicionador de solos enriquecido com *Bacillus* em diferentes solos brasileiros.

Termos para indexação: Ecologia; comunidade bacteriana do solo; matéria orgânica; diversidade alfa; práticas sustentáveis na agricultura.

INTRODUCTION

Global consumption of mineral fertilizers composed of nitrogen, phosphorus, and potassium has increased significantly in the last 60 years (Ritchie; Roser; Rosado, 2022), reaching 192 million tons in 2019 (Food and Agriculture Organization of the United Nations - FAO,

2019). Although fertilizers play a key role in supplying nutrients to plants, the negative effects of overfertilization need to be further studied (Bisht; Chauhan, 2020; Dimkpa et al., 2020; UN Environment Programme, 2022). Biofertilizers and organic amendments, such as manure, composts, and crop residues, are eco-friendly and inexpensive alternatives to synthetic fertilizers (Kawalekar, 2013; Ling et al., 2016; Zheng et al., 2011). They can regulate the biological properties of the soil, provide plants with nutrients (Abdel-Raouf; Al-Homaidan; Ibrabeem, 2012; Mahanty et al., 2017; Mukhtar et al., 2017; Sevilla-Perea; Mingorance, 2015), and impart additional benefits to the soil, for example, increase soil carbon stock (Xie et al., 2014), improve soil structure, enhance water retention (Yu et al., 2012), increase soil enzyme activities, and modulate the microbial community (Bowles et al., 2014; Kotroczó et al., 2014).

Microorganisms living in the soil play key roles as plant protectors and growth promoters through phytohormone synthesis, antifungal activities, nitrogen fixation, and the solubilization of phosphorus and other nutrients (Bender et al., 2022; Biggs et al., 2021; Shi et al., 2018; Thind et al., 2022; Vuong; Zeng; Man, 2020; Zhang et al., 2017; Zhang et al., 2022). Adding beneficial microorganisms to the soil can maximize nutrient uptake by plants (Kirankumar et al., 2008), increase the growth of plants (Cummings, 2009; Guiñazú et al., 2009), enhance their resistance to abiotic stress (Selvakumar; Panneerselvam; Ganeshamurthy, 2012) and prevent various diseases (De Vleesschauwe; Höfte, 2009).

The decomposition of organic matter and nutrient cycling are directly related to the enzymatic activity of the soil (Tabatabai, 1994). Arylsulfatase, acid phosphatase, and beta-glucosidase catalyze key reactions involved in the biogeochemical cycle of sulfur, phosphorus, and carbon (Henry, 2012; Liu et al., 2021; Sinsabaugh et al., 2008; Turner et al., 2016). These enzymes have been used as bioindicators of soil health in Brazil (Chaer et al., 2023; Sobucki et al., 2021). The activities of these enzymes were reported to increase after the soil was fertilized with pig manure and sawdust, pig slurry, and farmyard manure (Balota; Machineski; Truber, 2011; Chang; Chung; Tsai, 2007; Siwik-Ziomek; Lemanowicz; Koper, 2016), and organic composts (Chang; Chung; Tsai, 2007; Song et al., 2019).

To understand the importance of the response of the soil microbiota to an amendment treatment, in this study, we tested whether soil enzymes and bacterial communities can increase in activity and be reshaped, respectively, after the application of organic amendments. We investigated the effects of an amendment made of poultry litter enriched with *Bacillus* spp. applied at doses of 150, 300, 600, and 1,200 kg/ha to the soil on the activities of arylsulfatase, beta-glucosidase, and acid phosphatase in five soil types. We selected three of these soil types (because of their occurrence in Brazil and fertility profiles) to investigate the modulation and diversity of the bacterial communities in response to the application of the amendments. Our findings provided valuable information for developing and refining soil amendment strategies, which can improve the condition of the soil and crop productivity.

MATERIAL AND METHODS

Soil sampling

Soil samples were collected from five agricultural areas in Piracicaba, São Paulo State, Brazil (22°43'S and 47°39'W; altitude: 547 m). All samples were stored in foam boxes and transported to the laboratory on the same day. The soil type was previously characterized according to the Brazilian System of Soil Classification (Santos et al., 2018) as follows: 1) *Nitossolo* (*Nitossolo vermelho eutroférrico típico*), 2) *Argissolo arênico* (*Argissolo vermelho-amarelo distrófico arênico abrúptico*), 3) *Argissolo alumínico* (*Argissolo vermelho-amarelo alumínico abrúptico*), 4) *Latossolo distroférrico* (*Latossolo vermelho distroférrico típico*), and 5) *Latossolo distrófico* (*Latossolo vermelho-amarelo distrófico típico*). *Argissolo arênico* and *Argissolo alumínico* are similar to Oxisols from Soil Taxonomy (ST) and Lixisols from the World Reference Base (WRB). *Latossolo distrófico* and *Latossolo distroférrico* are similar to Oxisols from ST and Ferrasols from WRB, and *Nitossolo* is similar to Oxisols and Alfisols from ST and Nitosols from WRB.

The chemical and physical characteristics of the different types of soils are shown in Table 1. The texture was measured using the Bouyoucos method (Dane; Topp, 2002). Phosphorus, potassium, calcium, and magnesium were extracted with an ion-exchange resin and measured using an atomic absorption spectrophotometer, following the method described by Raij et al. (2001).

Based on the amount of clay in *Nitossolo*, *Argissolo alumínico*, and *Latossolo distroférrico*, they were classified as clay soils, whereas *Argissolo arênico* and *Latossolo distrófico* were classified as average sandy soils. The fertility of the different soil types varied considerably; the pH ranged from 3.49 to 5.11, the organic matter ranged from 2.7 to 20.2 $g.dm^{-3}$, and calcium ranged from 6.6 to 35.6 $mg.dm^{-3}$. Overall, all soil types lacked phosphorus,

while the aluminic soil had higher levels of Al (83.7 mmolc.dm^{-3}). *Nitossolo* and *Latossolo distroférrico* were the most fertile, whereas, *Argissolo arênico* was the least fertile.

Microcosm setup and treatments

For microcosm incubation, 50 g of the five types of soils were placed individually in hermetically sealed 100-mL glass jars. A commercial soil amendment composed of poultry litter enriched with non-OGM *Bacillus* spp. (1×10^7 CFU/g and 2% each of total N, total P, and total K) was applied at five doses (kg/ha): control (0), 150, 300, 600, and 1,200, with six replicates per treatment (SMARTGRAN, Superbac Biotechnology Solutions, Brazil; registration ID: PR 000638–6.000053 - Ministry of Agriculture, Cattle and Supplying). The soil microcosms were incubated at 26 °C for seven days, and the soil was maintained at 60% of its maximum water-holding capacity.

Soil enzyme assays

The assays to determine the activity of arylsulfatase, beta-glucosidase, and acid phosphatase were performed for the five types of soils by the colorimetric determination of p-nitrophenol (Tabatabai, 1994).

DNA extraction, amplicon sequencing, and analysis

DNA extraction

DNA from *Argissolo arênico*, *Latossolo distrófico*, and *Nitossolo* was extracted using the DNEasy PowerSoil Pro kit (Qiagen) following the manufacturer's protocol and quantified by a Qubit platform (Invitrogen). These soil samples were selected for analyzing the bacterial community because of their opposite fertility profiles and occurrence rates in Brazil. *Latossolo* is the most abundant soil in the country (covering 39% of the area), followed by *Argissolo* (covering 24% of the area). In contrast, *Nitossolo* is found in only 1.5% of the area in Brazil (Santos et al., 2018).

Constructing 16S rRNA libraries and amplicon sequencing

The 16S rRNA sequences underwent two rounds of PCR, following the method described by Bender et al. (2022). In the first round of PCR, the V3-V4 region of the 16S rRNA was amplified with the primers 341F-CCTACGGGNGGCWGCAG-30 and 785R-GACTACHVGGGTATCTAATCC (Klindworth et

Table 1: Soil texture and fertility profiles.

		Soil texture profile				
		Nitossolo	*Argissolo arênico*	*Argissolo alumínico*	*Latossolo distrófico*	*Latossolo distroférrico*
Sand	g.kg^{-1}	329	825	384	774	346
Silt		182	24	175	50	194
Clay		489	151	441	175	460
Texture		Clay	Average sandy	Clay	Average sandy	Clay
		Soil fertility profile				
pH		4.92	4.10	3.49	4.37	5.11
Organic matter	g.dm^{-3}	13.9	2.7	8.0	6.8	20.2
Phosphorus	mg.dm^{-3}	7.8	<7.0	<7.0	<7.0	<7.0
Potassium	mmolc.dm^{-3}	2.98	0.59	1.96	1.16	1.95
Calcium		35.6	6.60	35.6	6.8	22.8
Magnesium		7.9	4.0	36.8	5.0	11.6
H + Aluminum		35.7	44.5	83.7	29.8	23.9
SEB*		46.5	11.2	74.4	13.0	36.4
CEC**		82.2	55.7	158.1	42.8	60.3
V***	%	57	20	47	30	60

*Soil exchangeable bases **Cation exchange capacity ***CEC saturation by bases.

al., 2013). The reaction included 2.5 µL of DNA, 12.5 µL of 2 × Kappa Hifi HotStart Mix, and 0.2 µM of each primer; the final volume was 25 µL. The amplification conditions were as follows: 3 min of initial denaturation at 95 °C, followed by 25 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 30 s, and a final extension at 72 °C for 5 min. In the second round of PCR, the indices were added to each sample. The reaction consisted of 25 µL of 2 × Kappa Hifi HotStart Mix, 5 µL of each index (Illumina TruSeq i5 and i7), and 5 µL of the previously amplified product; the final volume was 50 µL. The amplification conditions were as follows: 3 min of initial denaturation at 95 °C, followed by eight cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 30 s, and a final extension at 72 °C for 5 min.

After the two rounds of PCR were completed, the DNA samples were purified using magnetic beads (Agencourt AMPure XP) on a magnetic rack. The samples were then adjusted to equimolar concentrations and quantified via qPCR assays (Collibry Library Quantification Kit). Sequencing was performed on a MiSeq V2–300 platform

Bioinformatics and amplicon analyses

The raw sequencing reads are available in the NCBI Short Read Archive (SUB12914232). Read processing was performed following the method described by Bender et al. (2022). Multiplexed raw single-end reads were quality-checked, denoised, and filtered for chimeras with DADA2 (Callahan et al., 2016) with max_ee = 2. Finally, amplicon sequence variants (ASVs) and feature tables were obtained.

Taxonomy assignment was performed with Vsearch (Rognes et al., 2016) against the SILVA v.138 database (https://www.arb-silva.de/) for ASVs ≥ 270 bases. Only sequences with ≥ 99% identity with some sequence in the database were assigned. Sequences with identities lower than 99% were classified as unassigned.

Bacterial communities were analyzed and the alpha diversity was calculated in R 4.1.0 (R Core Team, 2021) with the following packages: qiime2R (Bisanz, 2018), Biostrings (Pagès et al., 2021), tidyverse (Wickham et al., 2019), phyloseq (McMurdie; Holmes, 2013), vegan (Oksanen et al., 2020), and picante (Kembel et al., 2010), and following the method described by Swenson (2015). Linear models were constructed to determine differences in the alpha diversity indices among different types of soils and treatments. The models were simplified to build the most parsimonious model. The estimated marginal means and contrasts between models were calculated using the emmeans package (Lenth, 2021), and letters were assigned using the rcompanion package (Mangiafico, 2021).

Principal coordinate analysis (PCoA) (with weighted UniFrac distances) was performed using the phyloseq package. PERMANOVA was performed using the vegan package (Oksanen et al., 2020) with 10,000 permutations.

Statistical analysis

All statistical analyses were performed using R 4.1.0. The package Hmisc was used for Pearson's correlation tests (Harrel, 2022), and the package vegan was used to perform PERMANOVA. Plots were generated using the package ggplot2 (Wickham, 2016). ANOVA was performed using the R base function *lm*, and the model constructed included an interaction effect between soil and dosage (variable ~ soil * dosage). Non-significant factors were discarded using the function *drop1*, and a new model was assessed. ANOVA tables were generated using the function *anova.*

RESULTS AND DISCUSSION

Soil enzyme activities increased in response to organic amendment

We assessed samples from different types of soils based on their occurrence in Brazil and their diverse fertility profiles (Table 1). Arylsulfatase, acid phosphatase, and beta-glucosidase were closely related to organic matter metabolism (Chaer et al., 2023; Chang; Chung; Tsai, 2010; Chen et al., 2019; Jian et al., 2016; Liu et al., 2021). In this study, we found that applying soil poultry litter amendment enriched with *Bacillus* spp. significantly increased these three enzymes in all evaluated soil samples ($p < 0.05$) (Figure 1). The doses from 600 kg/ha could induce enzyme activities in all tested soil samples. Specifically, lower doses could induce activities in *Argissolo alumínico* and *Latossolo distrófico*; arylsulfatase was induced at the minimum dose (150 kg/ha) compared to the control in both soil types. A dose of 300 kg/ha increased the activities of acid phosphatase in *Latossolo distrófico* and beta-glucosidase in *Argissolo alumínico*.

The greatest effects of soil amendment were observed in *Argissolo alumínico* and *Latossolo distrófico*, as most amendment doses increased enzyme activities in these soils (Figure 1). Higher enzyme activities were measured in these soils and in *Latossolo distroférrico* than in the control. *Nitossolo* and *Argissolo*

arênico had 10.21–17.60% and 9.40–19.38% higher mean enzyme activity (Supplementary Table 1). *Argissolo alumínico*, *Latossolo distrófico*, and *Latossolo distroférrico* had 9.76–28.39%, 11.16–22.60%, and 10.95–24.23% higher mean enzyme activity than the control soil, respectively .

The results of the regression analysis showed positive and significant correlations between an increase in amendment doses and enzyme activities in the five types of soils (Figure 1). Arylsulfatase activity was mostly correlated with an increase in the amendment dose in *Argissolo alumínico* (R^2 = 0.39, $p < 0.001$), but it was not correlated with the amendment dose in *Argissolo arênico*, although at a dose of 600 kg/ha, a significantly higher activity was recorded compared to the activity in the control (Figure 1). The dose curve was more strongly correlated with the activity of beta-glucosidase in *Latossolo distroférrico* (R^2 = 0.71, $p < 0.001$) and *Nitossolo* (R^2 = 0.58, $p < 0.001$). It was also correlated with acid phosphatase activity in *Argissolo alumínico* (R^2 = 0.71, $p < 0.001$), followed by *Argissolo arênico* (R^2 = 0.47, $p < 0.001$) and both *Latosolos* (R^2 = 0.49, $p < 0.001$).

Overall, these results showed a positive response of different types of soils to the application of amendments, indicating the capacity of the evaluated amendment to modulate the activity of bacterial communities in different types of soils. Our results were similar to those of other studies in which enzyme activities were found to increase after the application of mineral/organic fertilizers (Balota; Machineski; Truber, 2011; Chang; Chung; Tsai, 2007; Chen et al., 2019; Jian et al., 2016; Shi et al., 2018; Siwik-Ziomek; Lemanowicz; Koper, 2016; Song et al., 2019). A meta-analysis of 65 studies showed that beta-glucosidase and acid phosphatase overaccumulated at an average rate of 11.2% and 10.6%, respectively, after fertilization (Jian

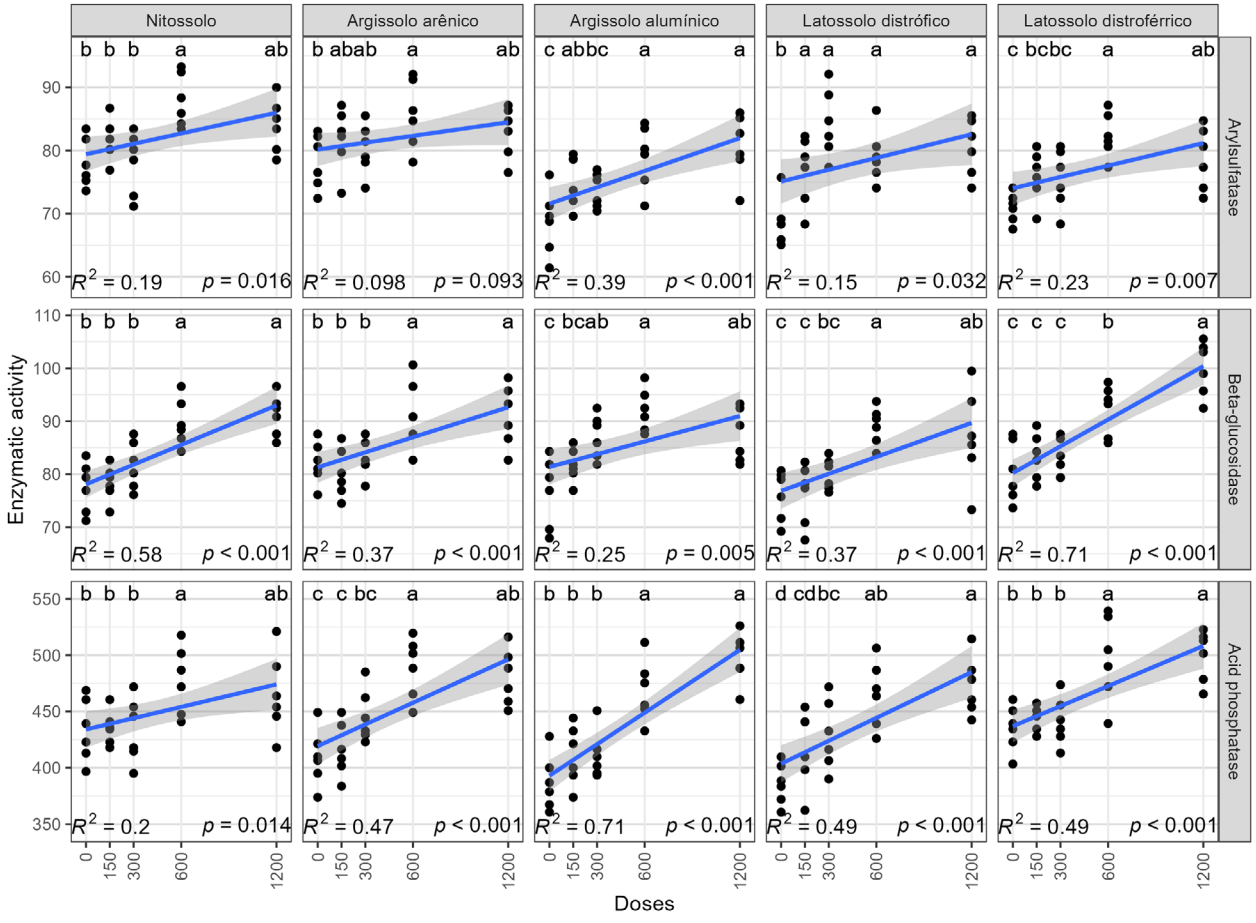

Figure 1: The results of linear regression analysis of arylsulfatase, beta-glucosidase, and acid phosphatase activities measured after treatment with different doses of soil amendment in each soil type.

et al., 2016). We found that an organic soil amendment could significantly increase the activity of arylsulfatase from 9.4% to 22.6%, acid phosphatase from 10.21% to 28.39%, and beta-glucosidase from 10.70% to 24.23% in the five evaluated soils, in some cases, even at a lower dose (150 kg/ha).

Organic compost treatment showed a positive correlation between beta-glucosidase activity and soil organic matter and total carbon (Chang; Chung; Tsai, 2007; Song et al., 2019). We found a very strong and significant correlation between organic matter and the beta-glucosidase activity at a dose of 1,200 kg/ha ($r = 0.98$, $p = 0.002$). *Latossolo distroférrico* contained more organic matter and showed the highest increase in beta-glucosidase activity at 1,200 kg/ha. No correlation was found between the 300 kg/ha dose and organic matter ($r = –0.03$, $p = 0.95$) independent of the soil type, while a positive relationship was found with magnesium ($r = 0.90$, $p = 0.03$).

Another study found that arylsulfatase activity was responsive to the soil pH level and texture; however, the relationship was a more complex quadratic correlation (Chen et al., 2019). A higher arylsulfatase activity was found in the presence of organic fertilizers made of pig manure and sawdust, pig slurry, and farmyard manure (Balota; Machineski; Truber, 2011; Chang; Chung; Tsai, 2007; Siwik-Ziomek; Lemanowicz; Koper, 2016) because those residues contain a high content of organic matter. In this study, arylsulfatase activity was positively and linearly correlated with the soil amendment dose ($R^2 = 0.15–0.39$) (Figure 1), which could be a response to the organic matter in the amendment. Similar to the results reported by Chen et al. (2019), we found no significant correlation between arylsulfatase activity and soil components, and only weak negative correlations were detected. These results indicated that arylsulfatase activity might be responding to similar complex relationships, such as quadratic correlation, when considering soil fertility.

Soil acid phosphatase plays a key role in converting soil organic phosphate into plant-absorbable P (Liu et al., 2021). Similar to the activities of beta-glucosidase and arylsulfatase, we found that the acid phosphatase activity was positively and significantly correlated with the soil organic amendment dosage, which indicated that increasing the organic matter content in the soil system increased enzyme activity. Acid phosphatase was the most highly induced enzyme in this study. Its activity increased by 10% in *Nitossolo* and by 19% in *Argissolo arênico* at a dose of 600 kg/ha and by over 20% in *Latossolo distrófico*, *Latossolo distroférrico*, and *Argissolo alumínico*. The latter soil type showed a 28% increase in acid phosphatase activity at an amendment dose of 1,200 kg/ha. Fertilization was found to increase acid phosphatase activity in several studies conducted by Jian et al. (2016), which probably occurred due to an increase in N supply, leading to an accumulation of more C-acquisition enzymes (Jian et al., 2016). Organic composts applied to the soil were found to more strongly induce acid phosphatase and double its activity compared to a mineral fertilizer (Chang; Chung; Tsai, 2007). Although acid phosphatase is not directly involved in soil carbon turnover, total carbon is positively correlated with its activity and is an important factor in inducing the enzyme (Liu et al., 2021).

Soil amendment changed the soil bacterial community

The most abundant phyla found were Proteobacteria, Actinobacteriota, Firmicutes, and Acidobacteriota. This finding was similar to those of other studies that investigated soil microbiomes with different planting systems, including wheat (Asad et al., 2021), tomato (Readyhough; Neher; Andrews, 2021), and runner bean (Stavridou et al., 2022). Firmicutes was not reported as one of the most abundant phylum in those studies. Its abundance was also not high after *Bacillus* inoculation in soils planted with rice (Ali et al., 2022) and lettuce (Krober et al., 2014). However, in this study, after an amendment with *Bacillus* was applied to the soil, the abundance of Firmicutes increased considerably, and it was one of the most abundant phyla in the soil after an amendment of 300 kg/ha was applied to *Argissolo arênico* and *Latossolo distrófico* and after an amendment dose of 600 kg/ha was applied to *Nitossolo*.

The genera identified from all treatments and replicates are shown in Figure 2. In total, 188, 276, and 289 genera were found in *Argissolo arênico*, *Latossolo distrófico*, and *Nitossolo*, respectively, which reflected the fertility and texture profiles of these soils. Among the various genera found in the soils and amended doses, we filtered the following most abundant and prevalent groups to discuss their presence. The most abundant bacterial genera in *Argissolo arênico* were *Massilia*, *Bacillus*, *Paenibacillus*, and *Burkholderia*. The most abundant bacterial groups in *Latossolo distrófico* were *Bacillus*, Unclassified Acidobacteriales, *Massilia*, and *Paenibacillus*. The most abundant bacterial groups in *Nitossolo* were Unclassified Acidobacteriales, *Bacillus*, *Pseudomonas*, and Unclassified Gaiellales.

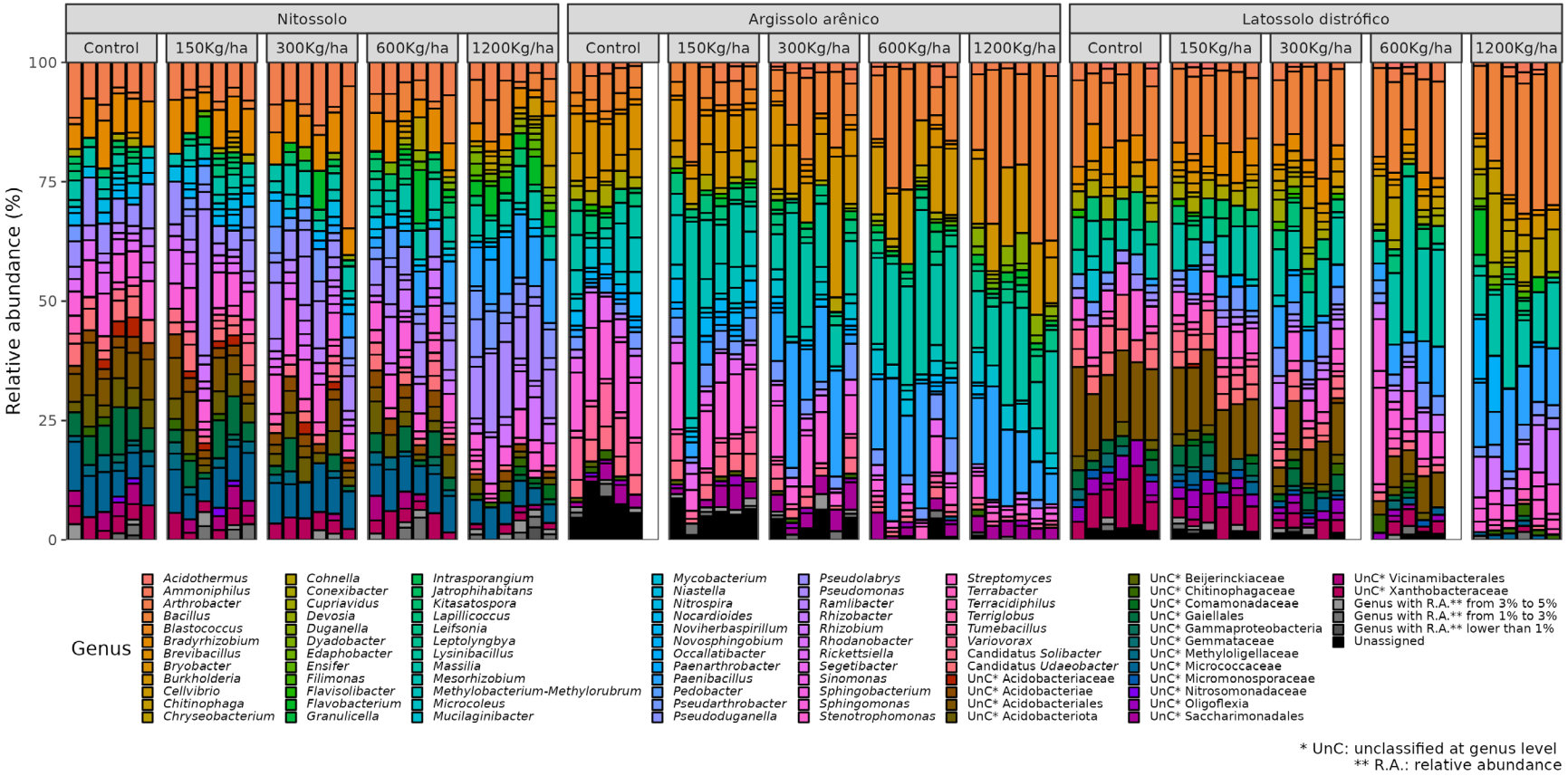

Figure 2: Relative abundance (%) of the genera detected in the bacterial communities of *Argissolo arênico*, *Latossolo distrófico*, and *Nitossolo*. Treatments included control and different soil amendment doses (150, 300, 600, and 1,200 kg/ha). Blank columns indicate sample loss.

Soil management can improve the positive functions performed by microorganisms. Long-term phosphate fertilization can increase soil pH, P levels, and the abundance of P-solubilizing bacteria (Ndabankulu et al., 2022), which can deliver P to plants. However, organic fertilization by applying manure is more likely to change microbial composition (Bhattacharyya et al., 2022). In the tomato rhizosphere, organic production systems could distinguish the microbiome composition from conventional management (Schmidt et al., 2019). Tillage with straw increased microbial activity, probably because of the addition of organic matter (Hao et al., 2019). We found that each type of soil contained an initial and individual particular bacterial composition and underwent reshaping in community assembly. Those communities became more similar as the amendment dose increased in all types of soils (Figure 2). These results were similar to those of other studies, which reported that a straw-derived biochar amendment favored the diversity and richness of bacteria (Bai et al., 2020), and manure and slurry improved microbial carbon biomass, ensuring higher stability of soil aggregates (Bhattacharyya et al., 2022).

Bacillus, *Massilia*, *Paenibacillus*, and *Rhizobium* are effective plant growth-promoting rhizobacteria (PGPR) (Ali et al., 2022; Bender et al., 2022; Biggs et al., 2021; Ndabankulu et al., 2022; Xiong; Lu, 2022; Wei et al., 2019). We found that their abundance increased in response to an increase in the amendment dose (Figure 3).

The results of ANOVA showed that the changes in the relative abundance were highly significant according to the amendment dosage and type of soil for the four genera (Figure 4). We also found significant interactions between both factors for *Massilia, Paenibacillus*, and *Rhizobium*, which indicated that those genera responded differently to the amendment depending on the soil type. The curves corresponding to the variation in *Bacillus* were similar in all types of soils analyzed because of the lack of interaction (Figure 4).

The abundance of *Bacillus* changed in response to the soil type (ANOVA, $p = 2.1 \times 10^{-23}$) and dosage (ANOVA, $p = 7.1 \times 10^{-12}$). The relative abundance of *Bacillus* at amendment doses starting at 300 kg/ha was significantly different from that found in the control treatment. The mean relative abundance of *Bacillus* ranged from 4.4 ±1.0% to 22.0 ±7.2% in *Argissolo arênico*, 6.5 ±0.7% to 15.8 ±2.7% in *Latossolo distrófico*, and 1.6 ±0.3% to 4.2 ±1.9% in *Nitossolo*. Similarly, Dong, L. et al. (2019) found an increase in the abundance of *Bacillus* spp. after fertilization with manure. *Bacillus* plays various roles as a PGPR, including plant defense against pathogens, phosphate solubilization, nitrogen fixation, biofilm production (Xiong; Lu, 2022), and the biosynthesis of indoleacetic acid and abscisic acid (Chobotarov et al., 2017). Several *Bacillus* spp. and *B. thuringiensis* gene clusters were found to be associated with sorghum

Figure 3: Relative abundance of *Bacillus, Massilia, Paenibacillus*, and *Rhizobium*, known as plant growth-promoting bacteria, in each soil type and treatment.

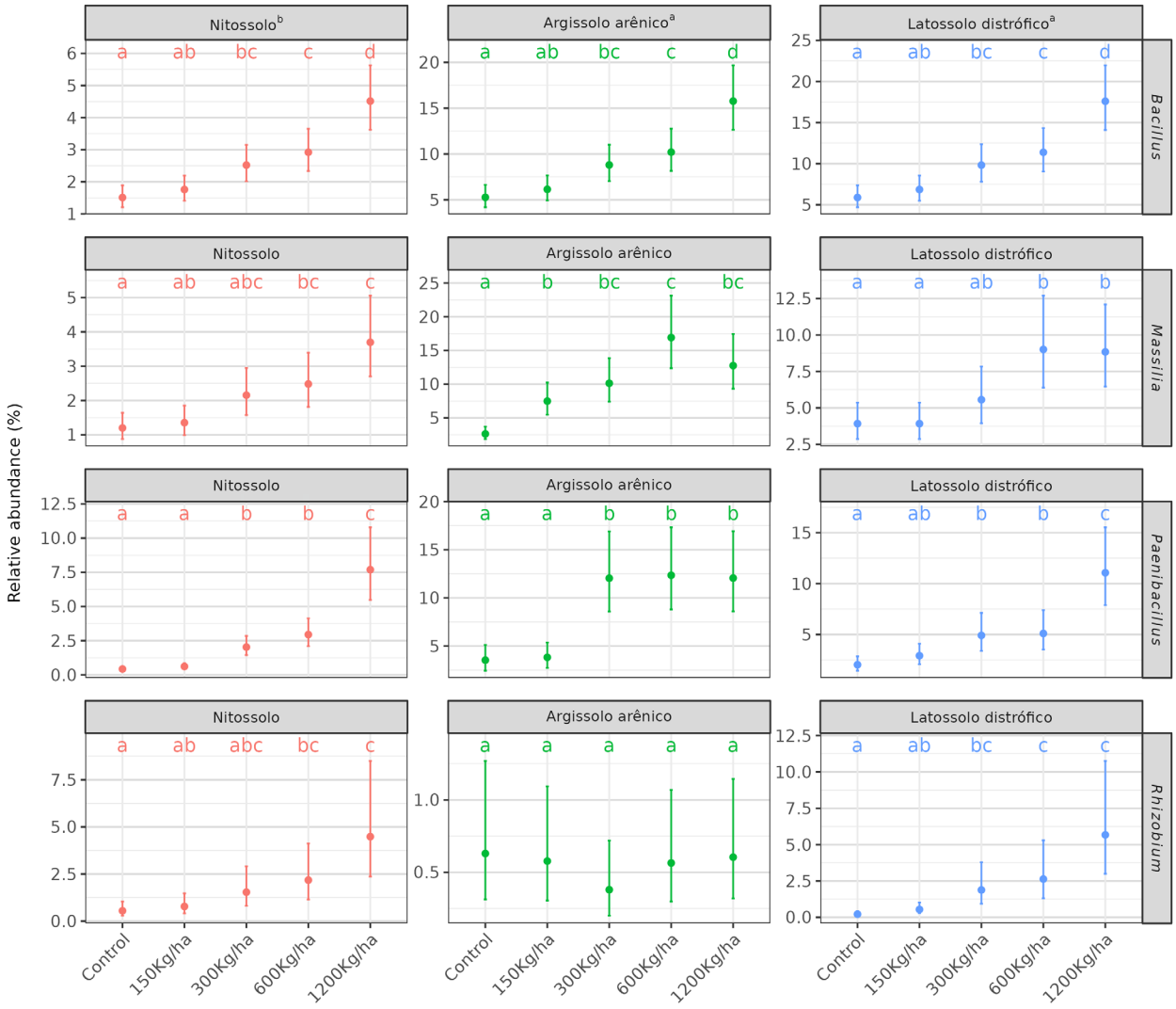

Figure 4: The results of ANOVA with estimated marginal means of the relative abundance of *Bacillus, Massilia, Paenibacillus*, and *Rhizobium* in *Argissolo arênico*, *Latossolo distrófico*, and *Nitossolo* after treatment with different doses of the amendment. Different superscript letters represent different soil types in the absence of interactions between soils and amendment doses after testing bacterial variation. Different letters associated with the data points represent significantly different means (ANOVA, Tukey *post hoc*; $p < 0.05$).

anthracnose control (Biggs et al., 2021). *Bacillus* spp., *B. velezensis, B. pumilus*, and *B. safensis* were found to produce biofilm under salt stress, for example, 16% salt concentration, as well as, rice growth promoters (Ali et al., 2022). These bacteria were also reported as one of the main endophytes and rhizoplane bacteria in soils planted with runner beans and were found to produce gamma-aminobutyric acid (GABA) (Stavridou et al., 2022).

The relative abundance of *Massilia* varied in response to soil type (ANOVA, $p = 3.0 \times 10^{-23}$), amendment dosage (ANOVA, $p = 3.8 \times 10^{-14}$), and the interaction between soil type and amendment dosage ($p = 0.01$). The mean relative abundances changed from 3.3 ±2.0% to 17.6 ±5.9% in *Argissolo arênico*, 3.9 ±0.74% to 10.1 ±5.2% in *Latossolo distrófico*, and 1.2 ±0.2% to 3.7 ±0.5% in *Nitossolo*. As *Massilia* is a keystone taxon related to the initial soil microbiome of healthy plants, they were suggested as able to suppress pathogens (Wei et al., 2019). Their functions may take place after direct plant-bacteria interactions, as

Massilia is a major root-colonizing bacteria (Stavridou et al., 2022). These bacteria were found to be highly abundant in bulk soil (1.6%) and their relative abundance increased to 7.4% in the rhizoplane and 7.4% in the root (Readyhough; Neher; Andrews, 2021). *Massilia* was also found to be highly abundant (1.2%) in the soil planted with runner beans, and this high abundance might be associated with the summer season (Stavridou et al., 2022). Our results showed the presence of *Massilia* in soils with no plants. Further, its abundance increased in treatments, for example 5.3 fold in *Argissolo arênico*, which suggested that the bacteria belonging to *Massilia* were soil-borne and their abundance was strongly shaped by the amendment.

Members of the genus *Paenibacillus* are important to the soil-plant system. Biggs et al. (2021) found isolates acting against black sigatoka and sorghum anthracnose. Ali et al. (2022) found that *Paenibacillus* was positively correlated with an increase in the growth of rice and enzymatic activity. We found that *Paenibacillus* was consistently present in the soil, and its abundance increased after the amendment. The relative abundance of *Paenibacillus* varied in response to the soil type (ANOVA, $p = 5.3 \times 10^{-22}$), amendment dose (ANOVA, $p = 1.9 \times 10^{-23}$), and the interaction between soil type and amendment dose (ANOVA, $p = 8.2 \times 10^{-5}$). Higher abundances were found in *Argissolo arênico*, ranging from 3.6 ±0.5% in the control to 14.5 ±8.0% at an amendment dose of 600 kg/ha. However, its relative abundance also increased in *Latossolo distrófico* from 2.0 ±0.2% to 11.2 ±1.7% and in *Nitossolo* from 0.3 ±0.3% to 7.8 ±1.2%, indicating a 22-fold change.

Rhizobium is a known nitrogen-fixing genus whose members live inside roots (Bender et al., 2022). They are also found in soil (Dong, C. J. et al, 2019; Dong, L. et al., 2019), considering that soil is a reservoir for microorganisms that further colonize plants (Furtak; Gajda, 2018; Kaminsky et al., 2021). The relative abundance of *Rhizobium* was higher when an organic biofertilizer was added to the soil (Dong, L. et al., 2019). Similar to the relative abundance of *Bacillus, Massilia*, and *Paenibacillus,* we also found in this study that the abundance of *Rhizobium* increased according to the amendment dose (ANOVA, $p = 1.4 \times 10^{-5}$), soil type (ANOVA, $p = 9.3 \times 10^{-9}$), and the interaction between soil type and amendment dose (ANOVA, $p = 2.1 \times 10^{-4}$). *Rhizobium* was less abundant than those genera and almost absent in the control treatment. Its relative abundance ranged from 0.02 ±0.04% in the control to 0.5 ±0.07% at an amendment dose of 1,200 kg/ha in *Argissolo arênico*; from 0.16 ±0.16% in the control to 5.9 ±1.7% in *Latossolo distrófico*, indicating a 36-fold change; from 0.6 ±0.1% to 4.8 ±2.0% in *Nitossolo*, indicating an eight-fold change.

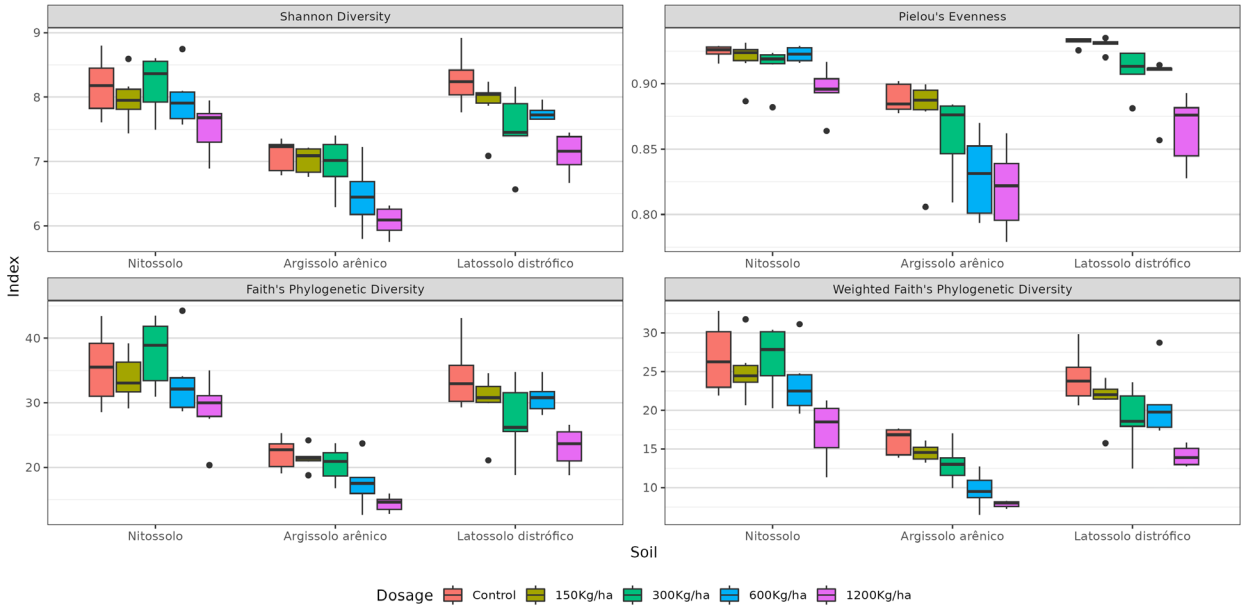

Figure 5: Bacterial alpha diversity in the soil from the control group (without amendment) and in amended soils at doses of 150 to 1,200 kg/ha.

Diversity of soil bacterial communities after amendment

To further investigate the changes in the community composition after the soil was treated (Figure 2), we estimated the Shannon, Pielou, Faith, and weighted Faith alpha-diversity indices and found different levels of those indices in each type of soil. We also found that the soil type was the main source of variation followed by the amendment dose, and all the diversity indices decreased considerably in response to the amendment dose (Figure 5).

Increasing the amendment dose decreased the diversity in the soil (ANOVA; Shannon, $p = 1.60 \times 10^{-8}$; Pielou, $p = 1.42 \times 10^{-9}$; Faith, $p = 1.76 \times 10^{-6}$; weighted Faith, $p = 2.21 \times 10^{-11}$). This reinforced the reassembly of the bacterial composition in response to treatment. The applied amendment contained *Bacillus*, and as its dose increased, the abundance of *Bacillus* also increased along with other PGP bacteria, as previously discussed. This might be a reason for the substantial decrease in diversity since the bacterial communities changed from a natural composition to an amended state. A similar reduction in Shannon diversity was reported in the strawberry amendment-treated soil and rhizosphere but not in the controls during the berry cycle in the field (Deng et al., 2019). These patterns suggested that applying amendments can alter microbial communities (Deng et al., 2019). Similar to the findings of the above-mentioned study, the inoculated rice rhizosphere in another study was found to have a lower Shannon index than the control group but higher yields (Ali et al., 2022). Overall, these findings suggested that the decrease in alfa diversity after the application of these classes of products might be a common response in the soil, which needs to be further investigated.

The results of PERMANOVA showed significant differences within treatments ($p = 1.00 \times 10^{-4}$) and soil type ($p = 1.00 \times 10^{-4}$) and a weak effect of the interaction between soil type and dose ($p = 1.00 \times 10^{-4}$). Further investigation of the bacterial composition associated with each treatment and soil type showed a common changing pattern for all bacterial groups, i.e., individual assemblies existed in the control for each type of soil, which became more similar as the amendment dose increased (Figure 6A). Soils clustered individually according to their bacterial composition and further clustered according to the amendment dose (Figure 6B). This finding confirmed the amending activity of the product made of poultry

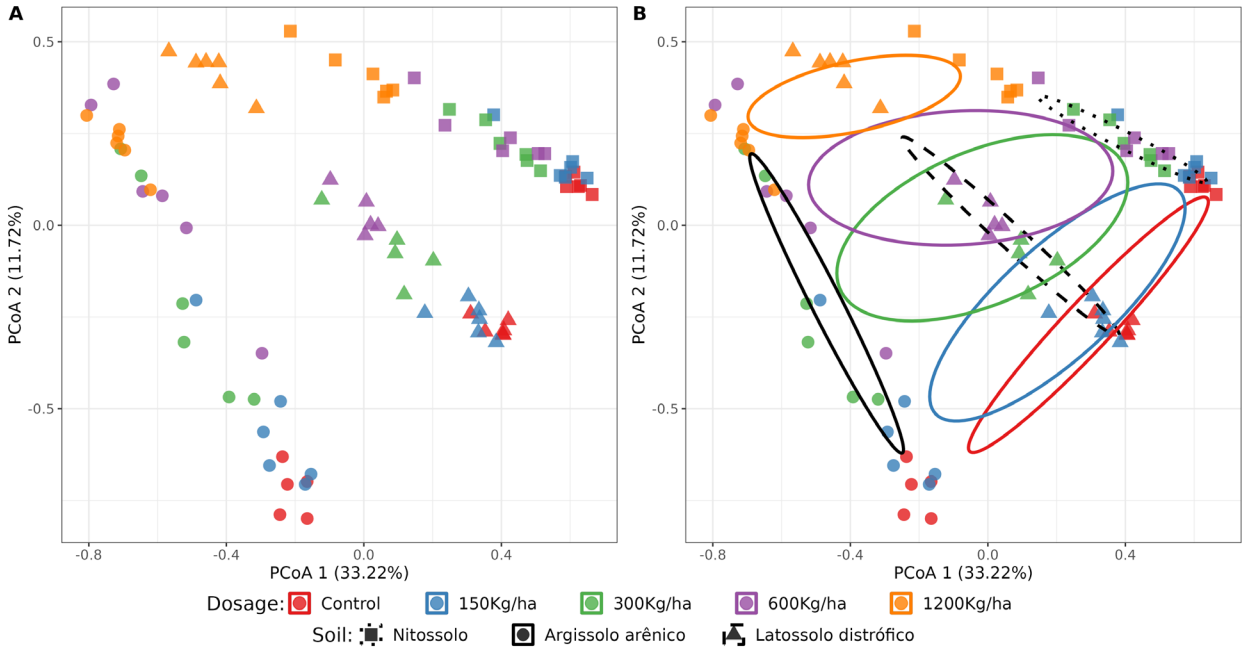

Figure 6: The weighted UniFrac beta diversity distances in the bacterial communities from soils in the control group (without amendment) and amended soils at doses of 150 to 1,200 kg/ha. A: The distribution of bacterial communities. B: The distribution of bacterial communities with clusters representing the grouping of similar assemblies based on the soil type and amendment dose.

litter and *Bacillus* evaluated in this study and matched the findings of other studies; in one study, the amendment was found to reshape the soil microbial community in strawberry fields (Deng et al., 2019), while in another study, the amendment was found to result in a similar microbiota composition after 113 days in sugarcane soil (Lourenço et al., 2018).

Amendments may influence microbial communities via several mechanisms. Microbes are involved in the cycling of soil metabolites, especially central carbon metabolism and ammonia assimilation (Krober et al., 2014), altering the carbon-nitrogen balance and the available phosphate (Liu et al., 2021), biofilm production in the rhizosphere under salt stress (Ali et al., 2022), and resistance to toxic compounds (Krober et al., 2014). The results of the network analysis showed that amendments can lead to the positive functional potential of soil microbiota at the cost of decentralized microbial functions assigned to small groups (Ling et al., 2016), which might be related to a decrease in diversity and an increase in the abundance of plant growth-promoting bacteria.

The effect of microorganism-derived soil treatments on microbiome assembly might be related to treatment composition more closely than expected. For example, inoculation of bacteria in the soil did not significantly change the microbiome in lettuce-planted soil (Krober et al., 2014) but changed the microbiome in rice-planted soil (Ali et al., 2021). Crop-rotated soils treated with sewage sludge amendment showed changes in the microbiome diversity and assembly (Li et al., 2021), and the amended soils in this study showed a significant change in the composition of the bacterial community.

CONCLUSIONS

An amendment enriched with *Bacillus* applied to different soils increased the activity of different enzymes. Our results also showed a particular bacterial community in each soil, and they changed to a more similar assembly as the amendment dose increased. We found that alpha diversity decreased considerably because some PGP bacteria increased in abundance, and thus, the communities changed to a more beneficial assembly. Our results indicated that organic amendments with *Bacillus* had beneficial effects on the soil.

AUTHOR CONTRIBUTIONS

Conceptual Idea: Alves, L.C.; Pauli, G.; Andreote, F.D.; Junior, E.B., Methodology design: Tornisiello, D.C.; Junior, E.B.; Andreote, F.D., Data collection: Andreote, F.D.; Alves, L.C., Data analysis and interpretation: Alves, L.C.; Silva, J.F.M. and Writing and editing: Alves, L.C.; Andreote, F.D.; Tornisiello, D.C.

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