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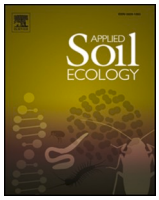

New insight into the soil bacterial and fungal microbiome after phosphorus biofertilizer application as an important driver of regenerative agriculture including biodiversity loss reversal and soil health restoration

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ABSTRACT

At present, the exploitation of biofertilizers is revealing the vast potential for the advancement of sustainable and organic agriculture and the improvement of arable soil quality. Therefore, we investigated the capacity of an innovative phosphorus biofertilizer to enhance the activity and diversity of the soil microbiome inhabiting chemically degraded soil (type Abruptic Luvisol). The two-year field experiment comprised a control treatment (FC) devoid of microbial enrichment, an optimal dose of fertilizer containing beneficial microorganisms (FA100) and a dose of fertilizer that was 40 % below this optimal level and contained microorganisms (FA60). Phosphorus biofertilizer increased soil enzymatic activity immediately after application and between the corresponding treatments in subsequent sampling times, it alleviated the effects of metabolic stress, improved phytoavailable phosphorus content and increased maize yield. Identification based on the terminal restriction fragments size revealed the presence of microorganisms important for soil health such as phosphorus solubilizers, nitrogen fixers, biological control agents, entomopathogens, mycorrhizal fungi, bioremediators and plant growth promoters. Next Generation Sequencing (NGS) showed that the application of phosphorus biofertilizer changed the relative abundance of different microbial phyla, classes and orders, increased the diversity of soil microorganisms and indicated that the composition of the soil microbiome was also dependent on the sampling time. The prediction of bacterial community function using PICRUSt demonstrated that the application of biofertilizer raised the number of functional sequences associated with metabolism and cell processes, including phosphorus compound pathways. FUNGuild showed that saprotrophic and symbiotrophic fungi were more abundant in microbiologically enriched treatments. Our results proved that the phosphorus biofertilizer used offers a sustainable and promising solution to the problem of reducing traditional mineral fertilizer inputs while ensuring soil microorganism welfare and enhancing land productivity, and, what is more, it can be effectively exploited in regenerative agriculture and as a factor used to enhance resilience to climate change.

1. Introduction

It is beyond doubt that modern agriculture has become strongly dependent on chemical inputs to intensify crop production and to meet the food requirements of an ever-growing world population which is predicted to hit >9 billion people by 2050 (Fasusi et al., 2021; Pirttilä et al., 2021). Mineral fertilizers assist in restoring the chemical balance in nutrient-deficient soils and increase crop biomass, however, their unreasonable and excessive application may lead to environmental concerns associated with soil quality and productivity and also with climate change. Moreover, plants use only a limited amount (30–40 %) of the nutrients that fertilizers provide and the rest remains unavailable and contributes to environmental pollution (Kumar et al., 2022). The deterioration in farmland soil quality has become a global concern which poses a threat to the further progress of agricultural economics (Kopittke et al., 2019).

Adverse environmental impacts (e.g. the accumulation of heavy metals, water eutrophication, nutrient leaching) arising from the overuse of mineral fertilizers and synthetic pesticides within a policy environment of regulating the quantities of applied agrochemicals has led to the development of non-hazardous alternative approaches (Mącik et al., 2020a). The application of beneficial microorganism strains, including

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bacteria and fungi, in the form of biofertilizer is currently one of the "hot topics" in the field of sustainable, organic and regenerative agriculture and is increasingly gaining attention (Canfora et al., 2021). The main ideas of the regenerative agriculture (RA), in addition to enhancing soil ecosystem services such as carbon sequestration, include soil health restoration and soil biodiversity loss reversal and these targets may be achieved through the adoption of practices such as intercropping, leaving crop residues on soil surface, application of organic amendments, avoidance of insecticides, fungicides and herbicides and limitation the use of chemical, mineral fertilizers (EASAC, 2022; Newton et al., 2020). Furthermore, the soil health restoration is inextricably linked to the enhancement of the soil microbiome quality as the complex communities of soil bacteria, archaea and fungi shape the soil environment and regulate ecosystem sustainability by directing the processes encompassing the decomposition of organic matter, chemical element cycling and plant nutrition (Feng et al., 2021; Frąc et al., 2018; Frąc et al., 2022; Jurys and Feizienė, 2021). Soil microorganisms are also well known as plant growth promoters (PGP) due to certain beneficial traits including the synthesis of phytohormones and antibiotics, the alleviation of biotic/abiotic stresses and providing protection against phytopathogens (Fan and Smith, 2021). Therefore, the welfare of soil microorganisms is an important factor determining the success of regenerative agriculture practices and the RA objectives may be achieved by increasing soil microbial biodiversity through the use of preparations containing beneficial microorganisms (Hermans et al., 2023). In our work we proposed the application of phosphorus mineral fertilizer enriched with beneficial microorganisms and, as the high doses of mineral fertilizers are recognized as a driver of biodiversity decline in ecosystems (Jin et al., 2022), we included a treatment with 40 % reduced dose of mineral component which complies not only with the rules of the regenerative agriculture, but also with the targets of the Biodiversity and Farm to Fork strategies (European Commission, 2020).

The exploitation of microbial strains is also justified in the regulation of phosphorus bioavailability, a macronutrient which determines proper cell functioning and is in fact indispensable for all plant life (Meng et al., 2021). Regrettably, most soil phosphorus remains unassimilable for plants; this challenge can be overcome by using phosphorus solubilizing microorganisms (PSM) (Alori et al., 2017; Hallama et al., 2021). The representatives of *Bacillus* spp., *Pseudomonas* spp., *Micrococcus* spp., *Xanthomonas* spp., *Serratia* spp., *Rhizobium* spp., *Burkholderia* spp., *Penicillium* spp., *Aspergillus* spp., *Mortierella* spp., *Saccharomyces* spp., *Paecilomyces* spp. and *Cephalosporium* spp. were found to process phosphorus compounds to form highly assimilable orthophosphates (PO_4^{3-}, HPO_4^{2-}, $H_2PO_4^-$) due to traits including the synthesis of organic acids, the excretion of phytases and phosphatases and the reduction of soil pH (Kumar et al., 2022; Mitra et al., 2020; Mitter et al., 2021; Tian et al., 2021). The utilization of PSM in place of chemical fertilizers is considered to be an attractive option and compatible with the principles of a sustainable approach to agriculture. Also, this applies in view of the fact that some portion of the phosphorus provided with mineral fertilizers becomes unavailable through binding with metal ions occurring in the soil environment (Nacoon et al., 2020).

Soil microorganisms are considered to be a reliable bioindicator of ecosystem functioning due to their considerable vulnerability to external factors and environmental perturbations (Schloter et al., 2018; Tahat et al., 2020). The high degree of biodiversity within soil microbial communities is undeniably related to the preservation of stability and the continuity of soil exploitation (Bastida et al., 2021). Current soil management practices are more frequently integrating microbiome status with the overall soil condition and involve the adoption of microbial-based formulations to enhance biodiversity, and consequently, soil health (Ray et al., 2020). Beneficial features innate to microorganisms have given them the potential to be used as a powerful and effective tool to counteract the harmful outcomes of human agricultural activity (Mohanty et al., 2021). Biofertilizers improve the heavy metals tolerance level of plants (Ahemad, 2019) and increase the soil organic matter (SOM) content (Dębska et al., 2016). The adoption of biofertilizers is also gaining momentum in the face of incessant climate change and the threats associated with agroecosystems including droughts, salinity, high temperature stress and the spread of phytopathogens. It goes without saying that such phenomena impair the productive potential of the soil and diminishes the quality and quantity of crop yields (Fadiji et al., 2022; Fiodor et al., 2021; Velásquez et al., 2018). Climate change also affects the structure, functionality and the stability of soil microbial communities, although it is difficult to clearly define the direction of these alterations due to the enormous heterogenity of terrestial ecosystems with respect to the plant diversity and the psychico-chemical soil properties (Tiedje et al., 2022). Despite the fact that soil microorganisms quickly adapt to the environmental factors, phenomena such as an increased temperature, drought, increased precipitation, flooding and salinity may decrease the biodiversity of the microbial communities which results in the decline in the long-term soil potential to perform ecosystem functions (Jansson and Hofmockel, 2020). Therefore, it is essential to deploy practices, such as the application of biofertilizers, which will contribute to the preservation of the high biodiversity against risk linked to the climate change (Shah et al., 2021). Taking also into account that soil phosphorus has a very limited availability and mobility, the aplication of a P mineral fertilizer enriched with benefical *Bacillus* sp. and *Paenibacillus* sp. strains offers a response to the problems related to climate change induced biodiversity loss.

Agriculture remains a sector which is highly sensitive to climate change, but at the same time the intensification of agricultural practices contributes significantly to global warming, taking into consideration the fact that the world food system generates ~21–37 % of annual greenhouse gas (GHG) emissions (Lynch et al., 2021; Nelson et al., 2014). Abiotic stresses that are triggered by climate change can be mitigated with the help of beneficial microbial strains (Sarker et al., 2021). Microorganisms work through several mechanisms to safeguard plants against stressful situations including exopolysaccharide synthesis (enhancing water holding capacity and protection from dessication), the emission of volatile organic compounds (protection from thermal stress and phytopathogens), ACC deaminase activity (alleviation of ethylene synthesis), the excretion of phytohormones, the synthesis of osmoprotectants, induced systemic resistance and the production of enzymes (superoxidase dismutase, catalase, ascorbate peroxidase and guaiacol peroxidase) that remove reactive oxygen species (Fadiji et al., 2022; Fiodor et al., 2021). An improvement in the microbiological parameters of the soil is provided by the application of biofertilizers, this would also seem to be an appealing solution given that a greater diversity in the soil microbiome may strengthen the general resilience of the soil to environmental stresses (Bertola et al., 2021). Furthermore, it was documented that the application of microbial inoulants decreased CH_4 and N_2O emissions in field studies (Akiyama et al., 2016; Kantachote et al., 2016). The GHG emissions may also be reduced by exploiting the phenomenon of biological nitrogen fixation by representatives of *Rhizobium* spp., *Azotobacter* spp. and *Azospirillum* spp., which would simultaneously limit the use of nitrogen mineral fertilizers (Aasfar et al., 2021; Wen et al., 2021). Biofertilization can also stimulate soil carbon sequestration, thereby potentially not only reducing the amount of CO_2 in the atmosphere, but also increasing the soil organic carbon content in arable lands (Gayathri et al., 2021). The members of *Pseudomonas* spp., *Bacillus* spp., *Paraburkholderia* spp., *Paenibacillus* spp., *Streptomyces* spp. and *Penicillium* spp. were found to demonstrate properties which enhance plant tolerance to environmental stresses arising from the climate change (Fadiji et al., 2022).

Fluctuations in temperature, precipitation and aridity induced by a changing climate may influence phosphorus availability in soils as extreme precipitation can lead to P leaching whereas high temperature stimulates the conversion of available P into forms which cannot be assimilated by plants. Climate change may also affect soil properties (such as pH and organic matter content) and the activity of soil microbial communities, and the acidification, low organic matter content and

diminished biodiversity and activity of soil microorganisms are limiting factors in phosphorus availability and assimilation. What is more, reduced plant cover in arid lands increases the likelihood of wind erosion and the consequent loss of fine soil particles, with which, unlike coarse particles, more phosphorus is associated. Therefore, it is essential to increase the P content in soils, e.g. by using phosphorus biofertilizers, also in the view of the fact that excessive amounts of conventional fertilizers generate greater GHG emissions (Bhat et al., 2017; Hou et al., 2018; Ockenden et al., 2017).

Soil organic fertilization is a longstanding and clearly advantageous agronomic procedure (Geng et al., 2019). Numerous reports have described the influence of organic amendments such as compost, chicken manure and spent mushroom substrate on the status of the soil microbiome (Frąc et al., 2021; Gryta et al., 2020; Tan et al., 2022). In addition, studies which describe the shifts in soil microbial communities that are triggered by the introduction of beneficial bacterial and fungal strains are also appearing with increasing frequency (Schütz et al., 2018). On the other hand, research concerning the refinement of microbial-based practices and the enhancement of their efficiency are still underway in order to fill the remaining knowledge gaps and to explore the entire spectrum of possibilities provided by microorganisms (Sudheer et al., 2020). In this work we propose the application of phosphorus mineral fertilizer enriched with strains of beneficial bacteria which represents a cutting edge approach in the field of biofertilization and broadens our existing and somewhat fragmentary understanding on this topic. We chose the phosphorus fertilizer since the degraded Abruptic Luvisol soil showed a very low P content, not meeting the nutritional requirements of maize, and a low pH. In acidic soils phosphorus remains unavailable for plants due to the fixation with aluminium and iron ions (Bouray et al., 2021). Replenishment of phosphorus is also essential due to the low availability of this macroelement in the soil (only 5 % of the total soil P is available for plant uptake) and the vital importance of P in processes such as cell division, photosynthesis and energy storage and as an integral component of ATP, NADPH, nucleic acids and proteins (Carstensen et al., 2018; Hallama et al., 2021; Siedliska et al., 2021). Phosphorus fertilizer used in this study was enriched with three bacterial strains (*Paenibacillus polymyxa* CHT114AB, *Bacillus amyloliquefaciens* AF75BB and *Bacillus* sp. CZP4/4) that were chosen on the basis of their beneficial properties (Mącik et al., 2020b; Mącik et al., 2022), encompassing the synthesis of plant hormones (auxin, cytokinin), increasing nutrient acquisition (P, N, Fe), the excretion of antifungal/antimicrobial compounds, the formation of biofilms and the alleviation of biotic and abiotic stresses (drought, soil salinity, phytopathogens) (Langendries and Goormachtig, 2021; Radhakrishnan et al., 2017; Sheteiwy et al., 2021).

In this study, we investigated whether the application of phosphorus biofertilizer containing beneficial bacterial strains could improve the microbiological parameters of degraded soil, including the soil enzymatic activity and the genetic and functional diversity of the soil microbiome. In accordance with the regulations concerning the limitation of mineral fertilizer inputs in agroecosystems, we also suggested a 40 % reduced dose of fertilizer containing microbial enrichment in the expectation of its effectiveness in soil health restoration and in the reversal of microbial biodiversity loss. We hypothesize that phosphorus biofertilizer can improve the quality of degraded soil and that the results obtained will provide scientific guidelines for the development of eco-friendly agricultural practices promoting the welfare of soil microorganisms.

2. Materials and methods

2.1. Soil characteristics and the field study site

The field experiment was carried out in Poland in 2018–2019 on Abruptic Luvisol (AL) degraded soil located in Basznia, South-East Poland (50°39′ N, 22°65′ E), under maize cultivation (variety P9241, FAO: K280, Z270, PIONEER). Soil chemical degradation (characterized by acidification (pH_{KCl} of 4.9) and a decrease in the nutrient content) occurred as a result of the past activity of a former sulphur mine situated nearby. The AL presented a low content of Mg (3.6 mg 100 g^{-1} of soil) and a very low content of P (4.8 mg 100 g^{-1} of soil) and K (5.3 mg 100 g^{-1} of soil). The study site was located at an altitude of 230 m above sea level.

The AL soil was fertilized with the following mineral fertilizers: phosphorus mineral fertilizer SUPER FOS DAR 40 (Grupa Azoty, Puławy, Poland), nitrogen fertilizer PULREA PUŁAWSKI MOCZNIK 46 N (Grupa Azoty, Puławy Poland) and potassium salt (BIALCHEM, Poland). The microbial consortium for the phosphorus fertilizer contained beneficial bacterial strains including *Paenibacillus polymyxa* (CHT114AB), *Bacillus amyloliquefaciens* (AF75BB) and *Bacillus* sp. (CZP4/4), they were mixed in the proportion of 1:1:1 and coated on fertilizer granules (Borowik et al., 2019). The bacterial strains were selected from the SYMBIOBANK Collection in the Research Institute of Horticulture (Skierniewice, Poland). The biofertilizers were provided by the Łukasiewicz Research Network - New Chemical Syntheses Institute (Puławy, Poland). The doses of applied (bio)fertilizers were calculated according to the nutritional requirements of maize and the soil mineral content and were described in detail by Mącik et al. (2020b).

The field experiment comprised the following treatments: FC (optimal dose of phosphorus mineral fertilizer without microbial enrichment), FA100 (optimal dose of fertilizer enriched with microorganisms) and FA60 (dose reduced by 40 % of the fertilizer enriched with microorganisms). Reducing the fertilizer dose in the FA60 treatment follows the principles of sustainable and organic agriculture. Each treatment included three experimental subplots with 15 m × 10 m dimensions and with a 6-m interval between the application of each particular fertilization method. Soil samples (0-25 cm) were collected from five randomly selected sites at each subplot and then averaged by intensive mixing. Samples were taken on the following terms: before bio (fertilizers) application in April 2018 and April 2019 (A18/A19), one week after (bio)fertilization in June 2018 and June 2019 (J18/J19) and after the maize harvest in October 2018 and October 2019 (O18/O19). The results obtained from J18 were thoroughly analyzed and discussed in a previous study concerning the immediate effect of the application of phosphorus biofertilizer on the status of the soil microbiome (Mącik et al., 2020b). However, for this study, an analysis of Next Generation Sequencing results was conducted comprehensively for the whole experimental period, thereby excluding the sampling times before (bio) fertilizer application.

2.2. Soil enzyme assays

β-glucosidase activity was determined using the method of Eivazi and Tabatabai (1988), as modified by Alef and Nannipieri (1995), with the measurement of the concentration of p-nitrophenol (PNP) released by the soil after 1 h of incubation at 37 °C with p-nitrophenol glucoside (PNG). The PNP concentration was measured at 400 nm. The acid phosphomonoesterase activity was determined according to Tabatabai and Bremner (1969) with *p*-nitrophenyl phosphate as a substrate. This activity was determined by the PNP released after 1 h of incubation at 37 °C. The concentration of PNP was measured at 400 nm. For the protease analysis, Tris-HCl (pH 8.1) sodium caseinate was used and after 1 h of incubation at 50 °C, the concentration of tyrosine was measured at 578 nm (Ladd and Butler, 1972 with modifications by Alef and Nannipieri, 1995). The urease activity was assessed using urea as a substrate and the release of ammonia was determined after incubation for 18 h at 37 °C. The concentration of ammonia was measured colorimetrically at a wavelength of 410 nm (Zantua and Bremner, 1977). The enzyme activities were calculated based on the dry (105 °C) weight of the soil.

2.3. Phytoavailable phosphorus content and maize yield

The content of available phosphorus (P_2O_5) was determined using the Egner-Riehm method (according to Polish Standard PN-R-04023:, 1996) using a Sherwood flame photometer and Genesys 6 spectrophotometer in the District Agricultural Chemical Station (Lublin, Poland). At full maturity, the maize was harvested and its yield was measured in terms of the weight of the grains and straw from each subplot with a particular fertilization method being applied.

2.4. Catabolic fingerprinting of microbial communities

The metabolic abilities of the soil microbial communities were investigated using Biolog™ ECO plates for the bacterial community and FF plates in the case of the fungal community (Biolog Inc., Hayward, CA, USA). A one gram portion of fresh soil was shaken in 99 ml of saline peptone water for 20 min at 20 °C and this was followed by incubation for 30 min at 4 °C (Gryta et al., 2014). Then the soil suspension was transferred into ECO and FF plate wells (120 µl and 100 µl, respectively). After inoculation, the plates were incubated at 23 °C for 216 h. The plates were read after every 24 h of the incubation period at 590/750 nm (ECO) and 490/750 nm (FF) with a Biolog MicroStation™.

2.5. Total genomic DNA extraction from soil samples

A total genomic DNA extraction of 0.5 g of soil was performed using a FastDNA SPIN Kit for Feces (MP Biomedicals, Solon, OH, USA) according to the protocol provided by the manufacturer. The DNA was quantified spectrophotometrically at 260 nm (NanoDrop 2000/2000c Thermo Scientific, West Palm Beach, FL, USA). The extracted DNA was then stored at −20 °C for further analyses including multiplex terminal restriction fragment length polymorphism and Next Generation Sequencing.

2.6. Analysis of the soil microbial communities using a multiplex terminal restriction fragment length polymorphism (M-tRFLP) approach

M-tRFLP DNA profiling consisted of the following steps: multiplex PCR, the purification of the PCR products, restriction enzyme digestion and the detection of the terminal restriction fragments (T-RFs). Multiplex PCR was conducted using literature primer sets for bacteria (63f/1087r HEX) (Hauben et al., 1997; Marchesi et al., 1998), archaea (Ar3f/Ar9r ROX) (Jurgens and Saano, 1999; Jurgens et al., 1997) and fungi (ITS1F 6-FAM/ITS4R) (Gardes and Bruns, 1993; White et al., 1990) on a Veriti Fast Thermalcycler (Applied Biosystems, Foster City, CA, USA) using the following program: 95 °C for 5 min, 30× (95 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min), 72 °C for 10 min. The obtained PCR products were purified using an Exo-BAP-Mix (EURx, Gdańsk, Poland) and then Performa® DTR (Dye Terminator Removal) Gel Filtration Cartridges (EdgeBio, San Jose, CA, USA) were used according to the protocol supplied by the manufacturer. The purified DNA was quantified using a spectrophotometer at 260 nm (NanoDrop 2000/2000c Thermo Scientific, West Palm Beach, FL, USA). The purified PCR products were digested using restriction enzyme *Hae*III (EURx, Gdańsk, Poland). After digestion, 1 µl of the digest was mixed with 9 µl of Hi-Di™ formamide and 0.5 µl of DNA size standard GS-600LIZ (Applied Biosystems, Foster City, CA, USA). Prior to fragment analysis, the samples were denatured and chilled on ice. The sizes of the fluorescently labelled terminal restriction fragments (T-RFs) were determined on an automated ABI 3130 DNA genetic analyzer through capillary electrophoresis. A detailed specification of M-tRFLP was provided by Gryta and Frąc (2020) and Mącik et al. (2020b).

2.7. Analysis of the soil microbial communities using next generation sequencing (NGS)

Next Generation Sequencing was conducted at Genomed S.A. (Warsaw, Poland) using the MiSeq instrument (Illumina Inc., San Diego, CA, USA). Amplification of bacterial V3-V4 16S rDNA and fungal ITS1 rDNA hypervariable regions was performed using primer sets 341F/785R (Klindworth et al., 2013) and ITS1FI2/5.8S (Schmidt et al., 2013; Vilgalys Mycology Lab, 1992), respectively, and also Q5 Hot Start High-Fidelity 2× Master Mix, according to the manufacturer (NEB Inc., Ipswich, MA, United States). Sequencing was performed in paired-end mode 2 × 250 bp using the v2 Illumina kit.

2.8. Statistical and bioinformatics analyses

The metabolic efficiency of the soil bacterial and fungal communities was calculated as the ratio between the mean values of OD 590 nm/OD 750 nm and OD 490 nm/OD 750 nm (Pinzari et al., 2016) from the 0–216 h incubation period, respectively, and visualized in terms of heat maps.

Matrix2png web interface (Pavlidis and Noble, 2003) was used to generate heat maps representing the relative abundance of T-RFs. The identification of the microorganisms based on the size of the T-RFs was conducted using the TRiFLe tool (Junier et al., 2008). The Jaccard and Sorensen coefficients were calculated based on the number of T-RFs between particular treatments. The Jaccard coefficient (J) was calculated according to the formula: $J = N_{AB} / (N_A + N_B - N_{AB})$, where N_{AB} - is the number of common T-RFs in both profile A and B, N_A- is the number of T-RFs in profile A, N_B - is the number of T-RFs in profile B (Gryta and Frąc, 2020). The Sorensen (S) coefficient was calculated using the following formula: $S = 2C / (A + B)$, where C- is the number of common T-RFs in both profile 1 and 2, A- is the number of T-RFs in profile 1, B- is the number of T-RFs in profile 2 (Johnston-Monje et al., 2014). Venn diagrams were constructed using the online tool http://bioinformatics.psb.ugent.be/webtools/Venn/. Cluster analysis dendrograms were prepared based on the relative abundance of T-RFs with a scaled similarity (%) on the axis using Ward's algorithm and Euclidean distance with Sneath's criteria (33 % and 66 %) being highlighted.

The automatic preliminary step of NGS data analysis was conducted with the MiSeq platform using the MiSeQ Reporter (MSR) v. 2.6 software (Illumina Inc., San Diego, CA) and included the demultiplexing and generation of the fastq files. The taxonomical classification of the OTUs was performed with Quantitative Insights into Microbial Ecology (QIIME) (Caporaso et al., 2010) software using the Basic Local Alignment Search (BLAST) tool (Altschul et al., 1990) and UNITE v. 8 database (ITS1 region) (Kõljalg et al., 2013; Nilsson et al., 2019) and uCLUST algorithm (Edgar, 2010) and also the GreenGenes v. 13.8 database (16S V3-V4 region) (DeSantis et al., 2006).

Variations in beta-diversity within the soil microbial communities were visualized using an unweighted pair group mean (UPGMA) clustering algorithm and principle coordinate analysis (PCoA) on Bray-Curtis distance matrices of bacterial and fungal operational taxonomic units (OTUs). The bacterial functional profile was predicted from 16S rDNA data using the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) algorithm (Douglas et al., 2020) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Kanehisa and Goto, 2000). The FUNGuild tool was used to parse fungal OTUs with the trophic mode and ecological guild (Nguyen et al., 2016).

Statistical tests were used to determine the differences between the various soil enzyme activities, the number of functional sequences, the maize yield and the available phosphorus content between treatments. The first step referred to the verification of analysis of variances (ANOVA) assumptions including dataset normality and the homoscedasticity of the variance with Shapiro–Wilk and Levene tests, respectively. Afterwards, the data were analyzed with both ANOVA and post

hoc Tukey tests when ANOVA assumptions were met (maize yield) and with F-Welch and RIR Tukey tests for unequal numbers when dataset normality was maintained, however, the homogeneity of the variance was violated (enzymatic activities, PICRUSt/KEGG pathways, phosphorus content). A statistical analysis was performed using Statistica Software (version 13, StatSoft Inc., Tulsa, OK, USA, 2011) and R software (version 1.0.5.999, R Core Team, 2018, Vienna, Austria).

3. Results

3.1. Soil enzymatic activity

The application of biofertilizers in J19 increased the activity of protease, urease and acid phosphomonoesterase in both FA100 and FA60, whereas the enhancement of β-glucosidase activity was only reported in FA100, as compared to the control treatment. A similar trend, namely increased soil enzymatic activity in FA100 and FA60 was observed in A19 for every enzyme studied, except for urease, an improvement in activity for this enzyme was only noted in FA60 (Table 1).

Protease activity throughout the two-year experiment remained higher in FA100 and FA60 for every sampling time apart from FA60 (O19). The highest protease activity was reported in FA100(J19), directly after biofertilizer application and, what is more, an upward trend between the equivalent FA100 and FA60 treatments was observed from the O18 to the J19 sampling times. Relatively elevated protease activities were also observed in the A18 treatments.

As for urease, the samples collected in A19 demonstrated the highest activity. In considering the variations within the individual sampling times, the urease activity remained at similar levels for the FC and FA100 treatments at A18, A19 and O19.

Through analyzing variations in β-glucosidase levels across a two year experimental period it was found that the samples collected in O19 exhibited the highest level of activity, with no statistically significant changes occurring between particular treatments. Interestingly, FA60 experienced a stepwise growth (by 112.03 %) in aforementioned enzyme activity, from A18 to O19. A comparable tendency was also observed for FA100, in which the β-glucosidase activity increased by 158.95 % between O18 and O19. Concerning interannual shifts, 2019 witnessed an increase in β-glucosidase activity between the corresponding fertilization treatments with successive soil sampling times.

The highest acid phosphomonoesterase activities were recorded within the J19 (FA100 and FA60) and O19 (FC and FA100) sampling times. An identical trend was observed between the consecutive O18, A19 and J19 sampling times, namely, acid phosphomonoesterase activity increased along with a reduction in the mineral fertilizer dose within a particular sampling time period.

3.2. Phytoavailable phosphorus content and maize yield

The phytoavailable phosphorus (P_2O_5) content remained at a higher level in all FA100 treatments throughout the experimental period with statistically significant differences occurring in comparison with FC and FA60. The highest P_2O_5 content was recorded in FA100(J19), directly after biofertilizer application (an increase of 49.89 % occurred with respect to FC(J19)). Furthermore, at the end of the experiment in O19, the soil phosphorus level increased in FA100 and FA60 as compared to the corresponding treatments from A18. With regard to temporal fluctuations in soil P content, FA100 and FA60 experienced upward trends from A18 to J19 and from O18 to O19, respectively. Compared with the control, the maize yield under FA60 treatment in 2018 and under FA100 treatment in 2019 increased with no statistically significant differences occurring between particular treatments (Table 1).

3.3. Metabolic potential of soil microbial communities

3.3.1. Metabolic efficiency of the soil bacterial community

The ratio between substrate utilization (OD 590 nm) and bacterial growth (OD 750 nm) was calculated for the individual substrates located on BIOLOG™ ECO plates and revealed the diversification in the metabolic efficiency of the soil bacterial communities and furthermore indicated that C-compounds caused metabolic stress. The application of biofertilizers in J19 lowered the OD590/OD750 ratio for D-xylose, D,L-α-glycerol phosphate, γ-hydroxybutyric acid, L-phenylalanine, tween 40, α-cyclodextrin and phenylethylamine in both FA100 and FA60 as compared to the control soil. A decline in the OD590/OD750 ratio was also reported for α-ketobutyric acid, D-malic acid, i-erythritol and D-glucose-1-phosphate in FA100(J19) and for itaconic acid, D-glucosaminic acid, tween 80, L-asparagine, D-mannitol and L-threonine in FA60 (J19). At the beginning of the field experiment in April 2018 2-hydroxy benzoic acid, 4-hydroxy benzoic acid, D-glucosaminic acid, glycyl-L-glutamic acid, L-serine and γ-hydroxybutyric acid were found to be stressful to the soil bacterial community, however, the OD590/OD750 ratio for the aforementioned substrates decreased at the end of the experiment in October 2019. No stressful situation occurred for β-methyl-D-glucoside, D-mannitol, tween 40, D-xylose, i-erythritol and phenylethylamine across all of the analyzed samples (Fig. 1).

Table 1
Variations in soil enzymatic activity, maize yield and phytoavailable phosphorus content. Different letters indicate statistically significant differences ($p < 0.05$) calculated for each sampling time separately. ± means standard deviation. Explanation: FC-optimal dose of fertilizer, FA100- optimal dose of fertilizer enriched with microorganisms, FA60-fertilizer enriched with microorganisms (dose reduced by 40 %), A18-April 2018, O18-October 2018, A19-April 2019, J19-June 2019, O19-October 2019, PNP-p-nitrophenol.

Treatment	β-glucosidase (mg PNP kg^{-1} h^{-1})	Urease (μg N-NH_4 kg^{-1} h^{-1})	Protease (mg tyrosine kg^{-1} h^{-1})	Acid phosphomonoesterase (mmol PNP kg^{-1} h^{-1})	Maize yield (t ha^{-1})	P_2O_5 content (mg 100 g^{-1})
FC(A18)	1.95 ± 0.05 a	52.47 ± 11.02 a	13.44 ± 1.81 b	31.14 ± 1.08 a	–	3.93 ± 0.25 b
FA100(A18)	1.88 ± 0.19 a	50.31 ± 0.93 a	19.39 ± 2.43 a	28.75 ± 2.22 a	–	5.30 ± 0.20 a
FA60(A18)	1.33 ± 0.43 a	50.94 ± 6.06 a	17.14 ± 0.37 ab	32.29 ± 6.14 a	–	3.47 ± 0.21 b
FC(O18)	1.18 ± 0.15 b	61.34 ± 1.73 a	7.67 ± 0.22 a	23.82 ± 6.16 a	13.53 ± 2.91 a	4.37 ± 0.25 b
FA100(O18)	0.95 ± 0.06 b	54.56 ± 6.27 a	8.55 ± 1.30 a	24.54 ± 2.97 a	12.42 ± 2.15 a	5.47 ± 0.21 a
FA60(O18)	1.61 ± 0.17 a	62.53 ± 0.79 a	8.93 ± 0.74 a	27.39 ± 4.93 a	13.60 ± 0.87 a	2.83 ± 0.15 c
FC(A19)	1.36 ± 0.64 a	86.37 ± 16.51 a	6.23 ± 1.22 b	8.73 ± 1.28 b	–	3.83 ± 0.25 b
FA100(A19)	1.85 ± 0.63 a	84.28 ± 4.83 a	11.67 ± 2.39 a	16.03 ± 2.66 a	–	5.90 ± 0.17 a
FA60(A19)	1.84 ± 0.54 a	95.84 ± 9.77 a	10.97 ± 1.21 a	17.90 ± 0.96 a	–	3.13 ± 0.12 c
FC(J19)	1.94 ± 0.33 a	48.71 ± 2.13 a	10.15 ± 0.69 b	28.96 ± 3.74 a	–	4.67 ± 1.27 b
FA100(J19)	2.09 ± 0.22 a	59.26 ± 13.51 a	20.42 ± 2.97 a	34.02 ± 4.83 a	–	7.00 ± 0.10 a
FA60(J19)	1.89 ± 0.29 a	55.57 ± 5.04 a	15.12 ± 4.90 ab	39.81 ± 7.25 a	–	3.53 ± 0.06 b
FC(O19)	2.86 ± 0.50 a	71.89 ± 7.77 a	8.81 ± 1.11 a	39.86 ± 2.96 a	14.18 ± 2.29 a	3.97 ± 0.21 b
FA100(O19)	2.46 ± 0.90 a	72.31 ± 7.38 a	9.86 ± 1.08 a	36.78 ± 1.22 a	14.78 ± 1.90 a	5.33 ± 0.23 a
FA60(O19)	2.82 ± 0.27 a	54.53 ± 4.59 b	8.10 ± 0.21 a	14.80 ± 4.06 b	13.89 ± 0.88 a	4.03 ± 0.23 b

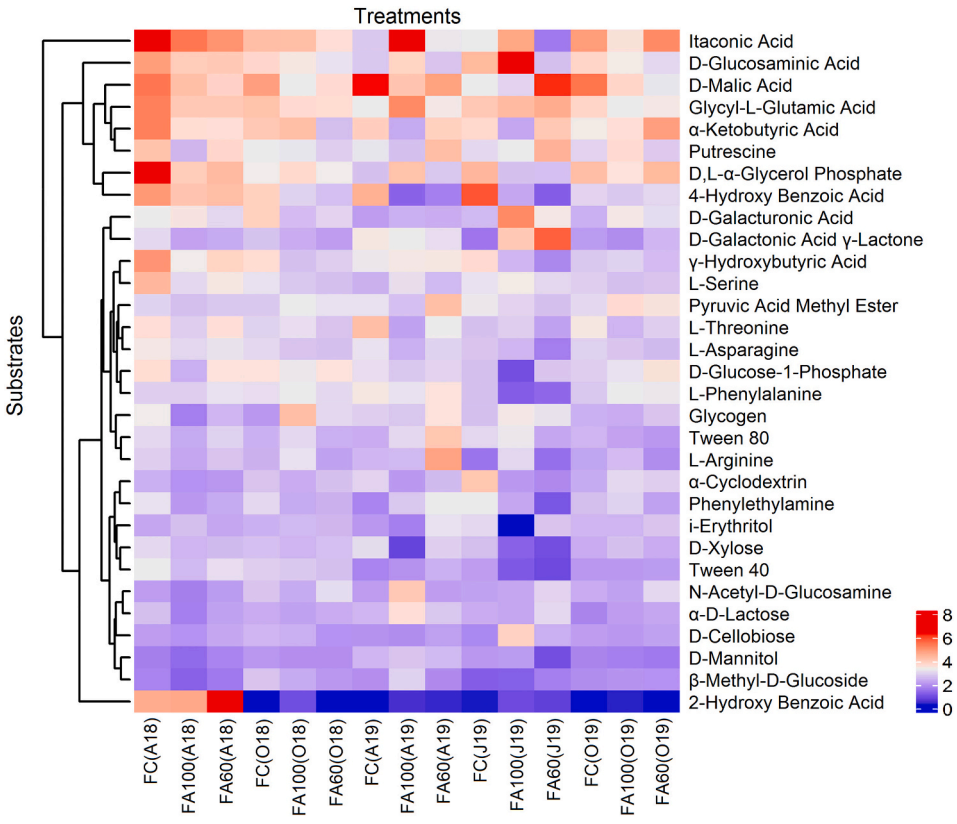

Fig. 1. The ratio between the values of substrate utilization (OD 590) and biomass production (OD 750) used to determine the theoretical metabolic efficiency of the soil bacterial community on different carbon sources located on Biolog™ ECO plates. A ratio of >4 indicates a stressful metabolic situation for soil bacterial community functioning. A ratio of <4 points to a balance between substrate utilization and biomass production. Explanation: FC-optimal dose of fertilizer, FA100-optimal dose of fertilizer enriched with microorganisms, FA60-fertilizer enriched with microorganisms (dose reduced by 40 %), A18-April 2018, O18-October 2018, A19-April 2019, J19-June 2019, O19-October 2019.

3.3.2. *Metabolic efficiency of the soil fungal community*

The metabolic efficiency of the soil fungal community was expressed as the ratio between the values of substrate utilization (OD 490 nm) and biomass production (OD 750 nm) for each substrate placed on BIOLOG™ FF plates. Directly after biofertilizer application in June 2019, the OD490/OD750 ratio values decreased for α-D-Lactose, maltose, D-mannitol, α-keto-glutaric acid, L-fucose, D-fructose and D-galactose in both FA100 and FA60. Metabolic stress was also reduced for acetamido-acetic acid and for arbutin, D-glucaric acid and D-sorbitol in FA100(J19) and FA60(J19), respectively. No stressful metabolic situation across the experimental period was observed for e.g. L-arabinose, D-ribose, α-D-glucose, D-arabitol, *N*-acetyl-D-galactosamine, 2-amino ethanol, D-mannose, xylitol, D-melezitose, stachyose, maltitol, D-raffinose, *N*-acetyl-D-glucosamine, D-cellobiose, turanose, L-proline, *N*-acetyl-L-glutamic acid, D-mannose, adonitol and adenosine. Relatively low values for OD490/OD750 ratio values, as compared to other sampling times, were noted in the case of dextrin, D-gluconic acid, L-asparagine, D-melibiose, putrescine, L-threonine, D-trehalose and sucrose in J19 (Fig. 2).

3.4. *Multiplex terminal restriction fragment length polymorphism*

With regard to the relative abundance of terminal restriction fragments, PCA analysis was used to arrange the fertilization treatments in response to the soil sampling time. PCA clustered treatments from particular soil sampling times occurred separately for all three of the investigated microorganism groups. Samples collected in A18 and O18 were grouped together, whereas A19, J19 and O19 formed three distinctly separated groups. PC1 explained 23.82 %, 35.05 % and 22.90 % of the variance, while PC2 accounted for 13.70 %, 16.28 % and 15.07 % of the variance for bacteria, archaea and fungi, respectively (Fig. S1). A cluster analysis also pointed to a clearly defined grouping of the fertilization treatments according to the soil sampling time. At both 33 % and 66 % of Sneath's criterion, the samples collected in June 2019 were clearly separated from the remaining sampling times in the bacterial and fungal communities (Figs. S2, S4).

The distribution of terminal restriction fragments during the two-year field experiment was expressed through Venn diagrams which showed the number of T-RFs common to the three fertilization treatments, which constituted the "core microbiome" and T-RFs which only appeared in selected treatments, called the "satellite microbiome". Archaea was the most abundant group in terms of "core" T-RFs and, simultaneously, the least diversified among the specific T-RFs (Fig. 3B). The bacterial community was characterized by the lowest number of T-RFs (Fig. 3A), while fungi exhibited the highest number of fragments characteristic of a particular fertilization method (Fig. 3C). On the other hand, only archaea experienced an increase in the total number of T-RFs in FA100 and FA60 as compared to the control soil (FC-51, FA100-57, FA60-52). The number of bacterial and fungal T-RFs remained at a higher level in FA100 than in FA60 (bacteria: FC-29, FA100-24, FA60-20; fungi: FC-54, FA100-47, FA60-41). The identification of microorganisms based on the size of selected T-RFs revealed the presence of various microbial genera. The fungal community was characterized by the highest number of identified microorganisms. In the restriction profile of each microbial group some genera were affiliated to T-RFs with different sizes, e.g. *Paracoccus* (66 bp, 70 bp, 71 bp) (Table S1), *Cenarchaeum* (150 bp, 160 bp, 170 bp) (Fig. S3) and *Lobulomyces* (170 bp, 240 bp) (Fig. S4). Furthermore, the TRiFLe tool assigned several different microbial genera to a fragment of a defined size (e.g. 117 bp and 214 bp among bacteria, 273 bp, 345 bp and 373 bp within archaea and 54 bp, 88 bp, 157 bp and 452 bp inside fungi) (Tables S1–S3). Within the bacterial and archaeal restriction profiles there were some T-RFs common to all treatments and sampling times (e.g. archaea: 60 bp, 100 bp, 120 bp, 200 bp, 320 bp and 360 bp; bacteria: 168 bp and 174 bp), whereas for fungi no fragment appeared simultaneously in all treatments throughout the experimental period. Only in June 2019 was the occurrence of fungal T-RFs 100 bp and 114 bp not observed, however, the aforementioned fragments were the most abundant in October 2019. The microorganisms identified among the fragments were

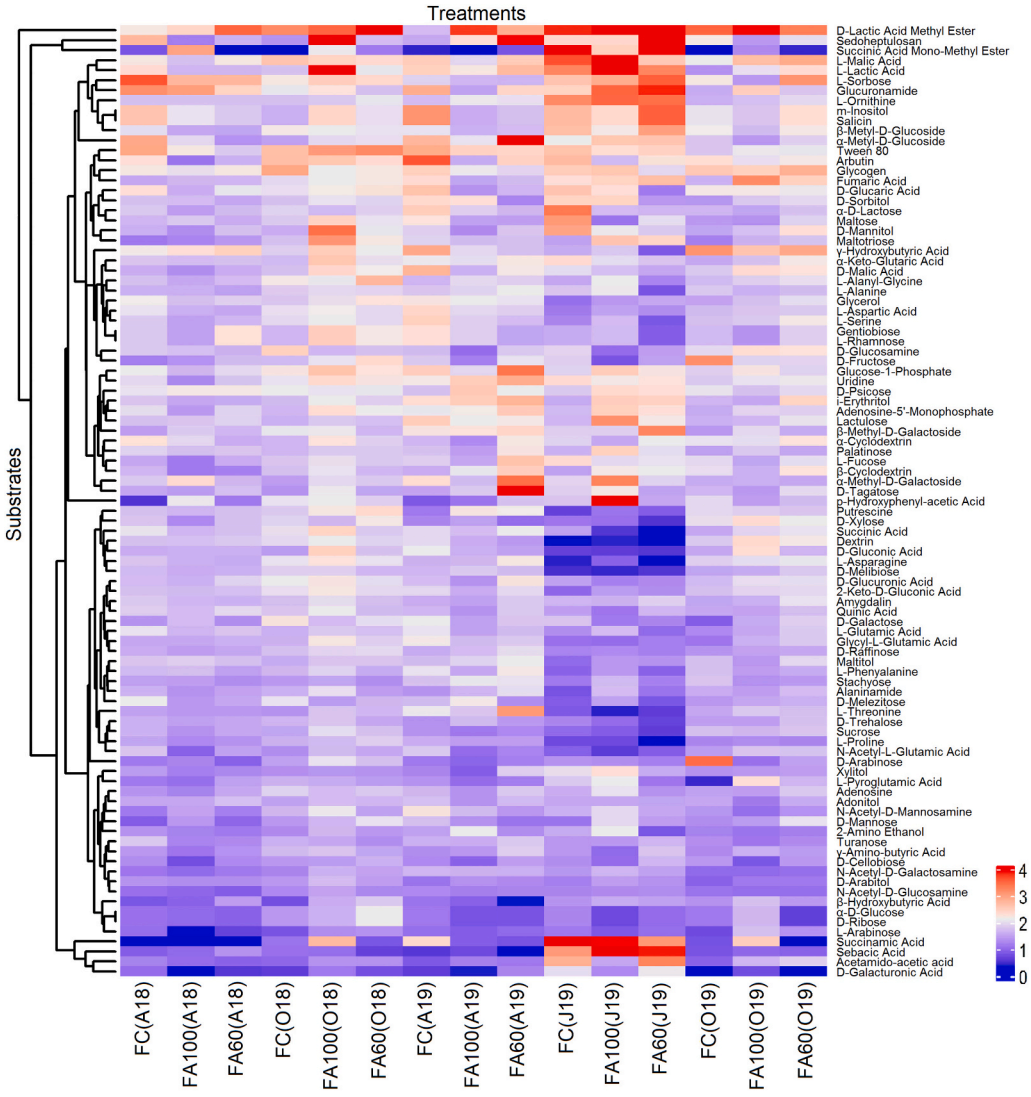

Fig. 2. The ratio between the values of substrate utilization (OD 490) and biomass production (OD 750) used to determine the theoretical metabolic efficiency of the soil fungal community on different carbon sources located on Biolog™ FF plates. A ratio of >2 indicates a stressful metabolic situation for soil fungal community functioning. A ratio of <2 points to a balance between substrate utilization and biomass production. Explanation: FC-optimal dose of fertilizer, FA100-optimal dose of fertilizer enriched with microorganisms, FA60-fertilizer enriched with microorganisms (dose reduced by 40 %), A18-April 2018, O18-October 2018, A19-April 2019, J19-June 2019, O19-October 2019.

classified as a "core microbiome" and were also shown in heatmaps (Figs. S2–S4), they represented variations in the relative abundance and number of individual T-RFs. After biofertilizer application in J19, the number of T-RFs decreased within the bacterial and fungal communities, and then increased in O19. Based on the number of T-RFs, the Sorensen and Jaccard coefficients were calculated to indicate the similarities between the fertilization treatments. The highest values of the aforementioned indices were reached between FA100 and FA60 within all of the investigated microbial groups (Fig. 3).

3.5. Next generation sequencing

3.5.1. The composition of bacterial and archaeal communities

The structure of the bacterial and archaeal communities in AL soil was analyzed at the phylum, class and order level. NGS revealed the presence of a total of 46 phyla with 41 being common to all fertilization treatments ("core microbiome"), 4 were shared between FA100 and FA60 (Deferrisomatota, Nanoarchaeota, TX1A-33 and WS4) and 1 was unique to FA60 (Dadabacteria) (Fig. S5A). At the phylum level the soil was dominated by Actinobacteriota (25.43 %–36.21 %), Proteobacteria (20.56 %–26.59 %), Acidobacteriota (12.38 %–16.55 %) and Chloroflexi (5.81 %–8.37 %). Acidobacteriota were more abundant in FA100 and FA60 throughout the whole experiment, while Chloroflexi decreased only in FA100-FA60(O18), as compared to the control treatments (Fig. 4A).

123 classes were shared between FC, FA100 and FA60. The classes common to FA100 and FA60 included Coriobacteriia, D8A-2, Defferrisomatia, Nanoarchaeia, Syntrophobacteria, Thermodesulfovibrionia, TX1A-33 and WS4, whereas Dadabacteriia and Sulfobacillia were unique to FA60 (Fig. S5B). The dominant soil bacterial classes were Alphaproteobacteria (12.74 %–15.52 %), Actinobacteria (10.58 %–16.33 %), Gammaproteobacteria (6.92 %–11.83 %) and Thermoleophilia (9.37 %–12.38 %). Gammaproteobacteria and Vicinamibacteria were present in a higher abundance in FA100 and FA60 across all sampling times in comparison with the controls. The relative abundance of Blastocatellia was enhanced (>3 %) in FA100(O18, J18, O19) and FA60(J18, J19) (Fig. 4B).

300 OTUs at the order level were common to all fertilization treatments. FA100 and FA60 included 5 (B55-F-B-G02 (Firmicutes), Candidatus_Buchananbacteria, Ga0077536 (Proteobacteria), Leptolyngbya les, Piscirickettsiales) and 2 (Dadabacteriales, Sulfobacillales) unique orders, respectively, while FC was characterized by the presence of 1 individual order (JTB23 (Proteobacteria)). 20 orders that were shared between FA100 and FA60 included Arenicellales, Defferrisomatales, Dehalococcoidales, Euzebyales, Puniceispirillales, Syntrophobacterales and Woesearchaeales while Ammonifexales and Gloeobacterales were reported in both FC and FA60 (Fig. S5C). At the order level, Rhizobiales predominated in the bacterial community (7.64 %–9.58 %), followed by

Gaiellales (5.85 %–7.94 %), Burkholderiales (5.09 %–7.54 %), Vicinamibacteriales (3.16 %–6.31 %), Gemmatimonadales (3.25 %–4.48 %) and Solirubrobacterales (3.00 %–4.16 %). Burkholderiales and Vicinamibacterales were more abundant in FA100 and FA60 across all of the sampling times as compared to FC. The relative abundance of Rhizobiales was only higher in FA60(J18) and FA100(O18) in comparison with the corresponding controls (Fig. 4C).

A principle coordinate analysis, which was based on Bray-Curtis distances, pointed to a well-defined clustering of samples with respect to the fertilization treatment and soil sampling time. The samples collected directly after biofertilizer application (J18, J19) were clearly separated from the autumn sampling times (O18, O19), and furthermore, PCoA highlighted the distinction of the controls from FA100 and FA60 not only in J18 and J19, but also in O19. Regarding the sample arrangement in the PCoA space, it was found that FC(O18) and FA60 (O18) formed one cluster, while the second one comprised FA100(O18) and FA100-FA60(O19) (Fig. 4D). On the other hand, the distribution of samples in the circular UPGMA dendrogram revealed that the main clustering factor was the fertilization regime. FA100 and FA60 from all of the sampling times (except FA60(O18)) formed a single group within which FA100 was separated from FA60 (Fig. 4F). An increased Shannon diversity index (H) was reported in FA60(J18), FA100-FA60(O18), FA100-FA60(J19) and FA100(O19) in comparison with the corresponding FC. The H index reached its highest values in FA60(J19), while FC(O18) and FA60(O19) were characterized by the greatest dispersion of H values (Fig. 4E).

3.5.2. *Predictive functions of the bacterial community*

The predictive functional profile of the bacterial community was dominated by sequences connected to "Metabolism" (~81.09 %), "Genetic Information Processing" (~12.08 %), "Cellular Processes" (~4.12 %) and "Environmental Information Processing" (~2.04 %). "Organismal Systems" and "Human Diseases" pathways represented the least abundant main KEGG classes, and accounted for ~0.38 % and ~0.30 % of the total, respectively (Fig. 5A). The application of biofertilizers increased the number of sequences in FA100(J18), while FA100(J19) and FA60(J19) experienced a decline in the number of functional sequences in comparison with the control treatments. However, directly after biofertilizer application in J19, the number of sequences increased as compared with J18 and O18. FA100 and FA60 in O18 and O19 recorded an upward trend in the number of sequences associated with the main KEGG classes as compared to the control treatments. Furthermore, FA100(O18) and FA100(O19) displayed an approximately 150 % and 250 % higher number of sequences, respectively, than the corresponding FC(O18) and FC(O19). The number of functional OTUs remained at a similar level between FA100(J18)-FA100 (O18) and between FA100(J19)-FA100(O19). In general, the total number of functional sequences was higher in 2019 than in 2018 (Fig. 5B). "Metabolism" was the most diverse main KEGG class and included 11 sub-classes with the highest number of functional sequences being attributed to amino acids and carbohydrate metabolism, this was followed by the "Metabolism of cofactors and vitamins" and the "Metabolism of terpenoids and polyketides". A relatively high number of sequences was also assigned to "Xenobiotics biodegradation and metabolism". By contrast, the pathways associated with nucleotide and glycan biotransformations were characterized by the lowest number of sequences (Fig. 5C). In considering the remaining main KEGG classes, "Cellular Processes" were dominated by "Cell growth and death" and "Cell motility", "Environmental Information Processing" was mainly composed of sequences related to "Membrane transport", while the sequences linked to processes including "Folding, sorting and degradation", "Replication and repair" and "Translation" were the most abundant within "Genetic Information Processing" (Fig. S6). For each sub-class, the number of functional sequences increased in FA100 and

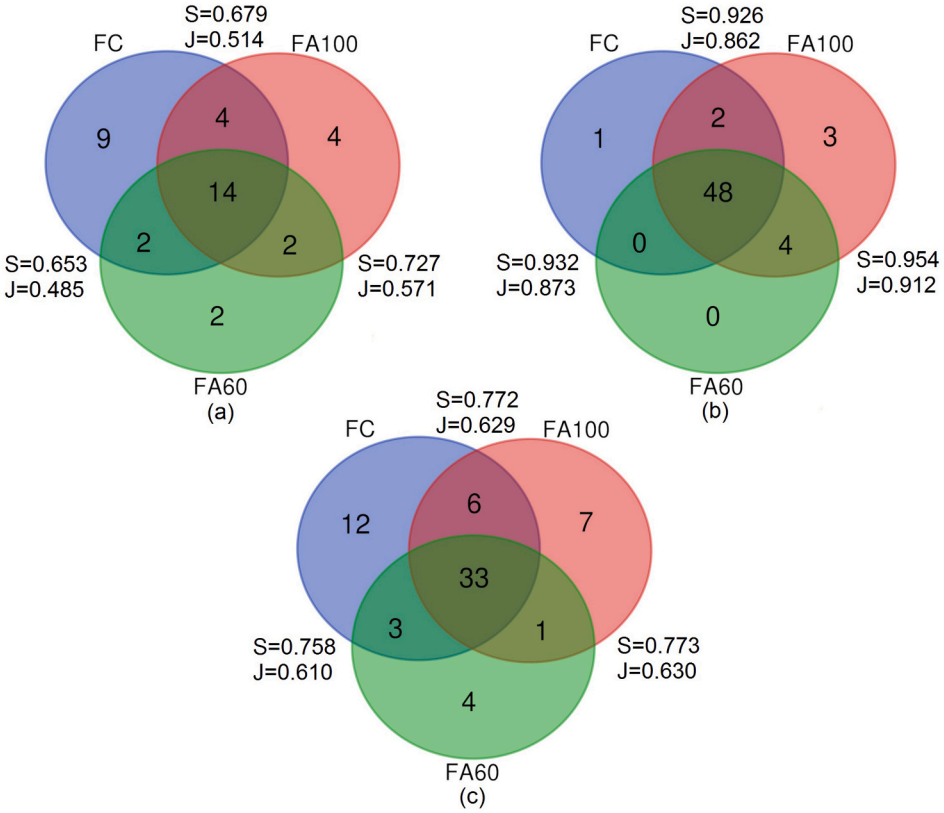

Fig. 3. Venn diagrams showing the distribution and number of common and unique terminal restriction fragments (T-RFs) within bacterial (A), archaeal (B) and fungal (C) communities. Explanation: FC-optimal dose of fertilizer, FA100-optimal dose of fertilizer enriched with microorganisms, FA60-fertilizer enriched with microorganisms (dose reduced by 40 %), S-Sorensen coefficient, J-Jaccard coefficient.

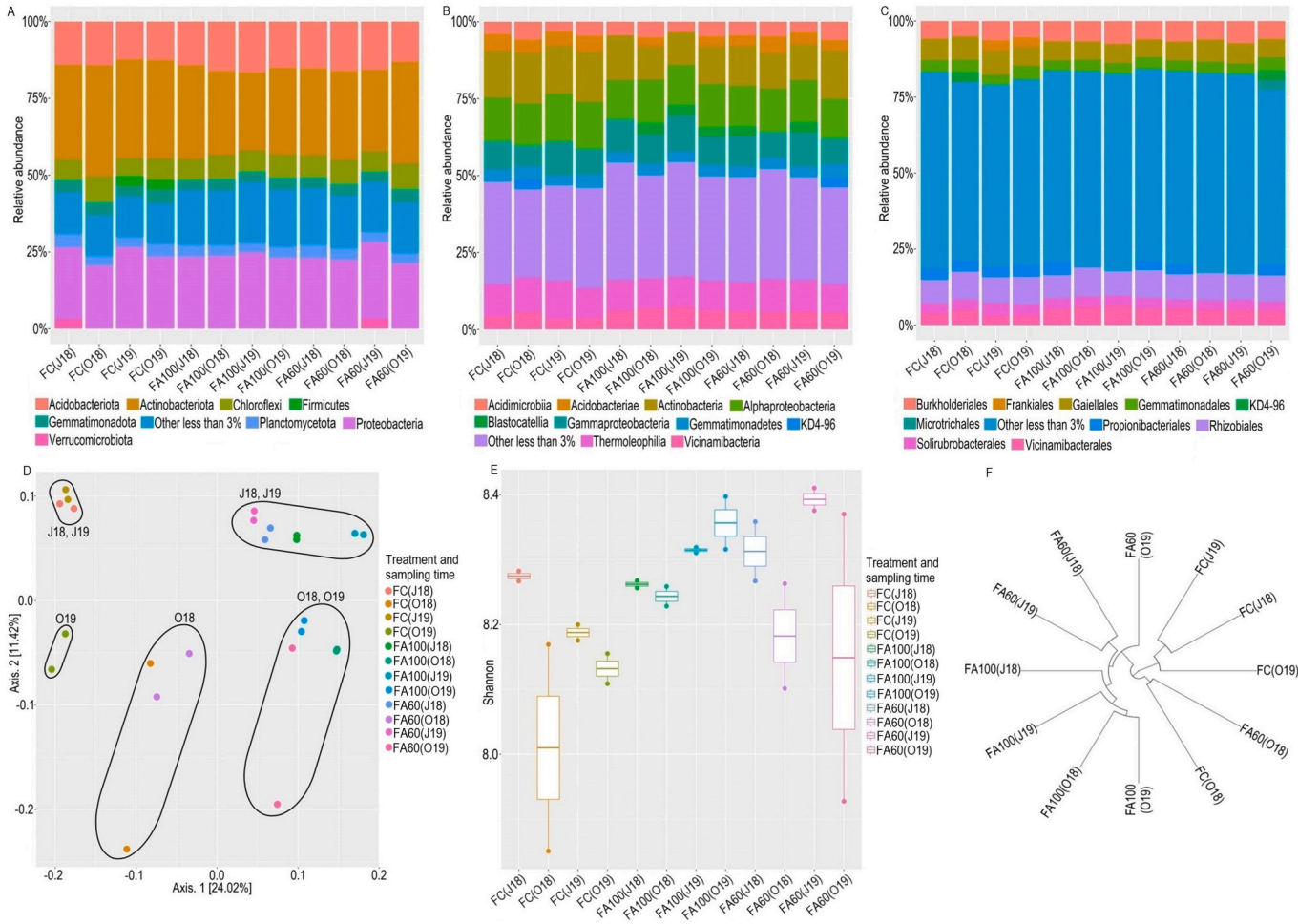

Fig. 4. Effects of the phosphorus biofertilizer on the composition of bacterial and archaeal communities. (A) - the relative abundance of the dominant bacterial phyla, (B)- the relative abundance of the dominant bacterial classes, (C) - the relative abundance of the dominant bacterial orders, (D) - principle coordinate analysis (PCoA) of bacterial and archaeal OTUs based on Bray-Curtis distances, (E) - Shannon diversity index, (F) - UPGMA dendrogram constructed from Bray-Curtis distances of the bacterial and archaeal OTUs. Explanation: FC-optimal dose of fertilizer, FA100-optimal dose of fertilizer enriched with microorganisms, FA60-fertilizer enriched with microorganisms (dose reduced by 40 %), J18-June 2018, O18-October 2018, J19-June 2019, O19-October 2019.

FA60 at O18 and O19 as compared to the corresponding controls. A depth analysis of the bacterial functional profile revealed that biofertilization influenced phosphorus compound biotransformations. Six different P-related biochemical pathways were distinguished: "Phosphotransferase system (PTS)", "Pentose phosphate pathway", "Inositol phosphate metabolism", "Oxidative phosphorylation", "Glycerophospholipid metabolism" and "Phosphonate and phosphinate metabolism". Similarly to the main classes and sub-classes, the number of functional sequences associated with P-compound processes remained at a higher level in FA100 and FA60 for the autumn dates (O18 and O19) (Fig. 5D).

3.5.3. *Fungal community composition*

The composition of the fungal community was analyzed in a similar way to bacteria and archaea, at the phylum, class and order level. The "core mycobiome" consisted of 13 phyla, with 1 phylum unique to FC (Calcarisporiellomycota) and 1 phylum shared between FA100 and FA60 (Blastocladiomycota) (Fig. S7A). The fungal assemblages were dominated by Ascomycota (46.63 %–66.86 %), Mortierellomycota (8.16 %–21.95 %) and Basidiomycota (>3.00 %–22.92 %). The relative abundance of Ascomycota increased in selected treatments where biofertilizers were applied (FA100(J18), FA100-FA60(O18), FA60(J19) and FA100-FA60(O19)), likewise for Basidiomycota (FA100-FA60(J18), FA100-FA60(O18) and FA100(J19)) (Fig. 6A).

36 OTUs at a class level were common among FC, FA100 and FA60. Both FC and FA60 included 3 characteristic classes encompassing Calcarisporiellomycetes, GS37 (Ascomycota), Wallemiomycetes, Chytridiomycetes, Endogonomycetes and Tritirachiomycetes respectively. Simultaneously, Laboulbeniomycetes and Olpidiomycetes were common to FA100 and FA60 (Fig. S7B). Sordariomycetes were found to be the most abundant class (20.48 %–39.26 %), followed by Mortierellomycetes (8.16 %–21.95 %), Dothideomycetes (3.63 %–16.91 %), Agaricomycetes (>3.00 %–21.45 %) and Eurotiomycetes (3.32 %–11.43 %). The application of biofertilizers stimulated the occurrence of Agaricomycetes (FA100-FA60(J18), FA100(O18, J19)), Eurotiomycetes (FA100-FA60(O19)), Leotiomycetes (FA100-FA60(J18, O18)), Pezizomycetes (FA100(J19), FA60(O19)) and Sordariomycetes (FA100-FA60 (J18, J19, O19)). Conversely, a decline in the relative abundance of Archaeorhizomycetes and Mortierellomycetes was observed in FA100 and FA60 (Fig. 6B).

88 orders out of a total of 108 constituted the "core mycobiome", 4 orders were specific to both FC (Calcarisporiellales, GS37, Microstromatales, Wallemiales) and FA60 (Chytridiales, Corticiales, GS20 (Mucoromycota), Tritirachiales); at the same time Botryosphaeriales, Gigasporales and Phacidiales were unique to FA100 (Fig. S7C). The dominant orders included Hypocreales (11.31 %–23.75 %), Mortierellales (8.16 %–21.95 %), Sordariales (3.64 %–14.30 %) and Agaricales (>3.00 %–20.91 %). Selected FA100 and FA60 had a greater abundance

of Agaricales (FA100-FA60(J18), FA100(J19)), Coniochaetales (FA100 (O18), FA60(O19)), Helotiales (FA100(O18)), Hypocreales (FA100-FA60(J18), FA60(O18), FA100-FA60(J19, O19)), Pezizales (FA100 (J19), FA60(O19)), Pleosporales (FA100(O19)) and Sordariales (FA100-FA60(J18), FA60(J19)) (Fig. 6C).

A PCoA analysis showed that the samples were grouped according to the soil sampling time, with J18 being vividly distinct from O18, J19 and O19. The samples collected in J19 and O19 were in relatively close proximity to each other in the PCoA space (Fig. 6D). A grouping of the treatments according to the sampling time was also evidenced by the UPGMA circular dendrogram; the samples collected in J18, O18 and J19 formed three clearly separated clusters. Furthermore, most of the samples collected in O18, J19 and O19 formed one cluster (Fig. 6F). Within the fungal community, the Shannon diversity index increased in FA100-FA60(J18), FA100(O18), FA60(J19) and FA100-FA60(O19) as compared to the controls. The highest values in the H index were reported in FA100(J18) and FA60(J18) (Fig. 6E).

3.5.4. Fungal functional profile

FUNGuild revealed that the AL soil was dominated by the representatives of the following trophic modes: "Pathotroph-Saprotroph-Symbiotroph" (15.79 %–44.58 %), "Saprotroph" (22.25 %–38.91 %) and "Saprotroph-Symbiotroph" (18.72 %–33.52 %). As compared to the corresponding control treatments, the relative abundance of "Saprotroph" increased in FA100(J18), FA60(O18) and FA100(O19), while "Saprotroph-Symbiotroph" was more abundant in FA100-FA60(O19) and "Symbiotroph" recorded an upward trend in FA100-FA60(J18), FA100-FA60(O18), FA60(J19) and FA60(O19). Meanwhile, "Pathotroph" noted a decline in relative abundance in FA100(J19) and FA60 (O19) (Fig. 7A). A deeper investigation into the fungal functional profile showed that the application of biofertilizer increased the relative abundance of "dung saprotroph" (FA60(J18), FA60(O18), FA100-FA60 (J19), FA100(O19)), "dung saprotroph-plant saprotroph" (FA100(J19), FA100-FA60(O19)), "plant saprotroph-wood saprotroph" (FA60(J18), FA60(O18), FA100(O19)), "undefined saprotroph" (FA100(J18), FA60 (O18), FA100(O19)), "arbuscular mycorrhizal" (FA60(O18), FA60

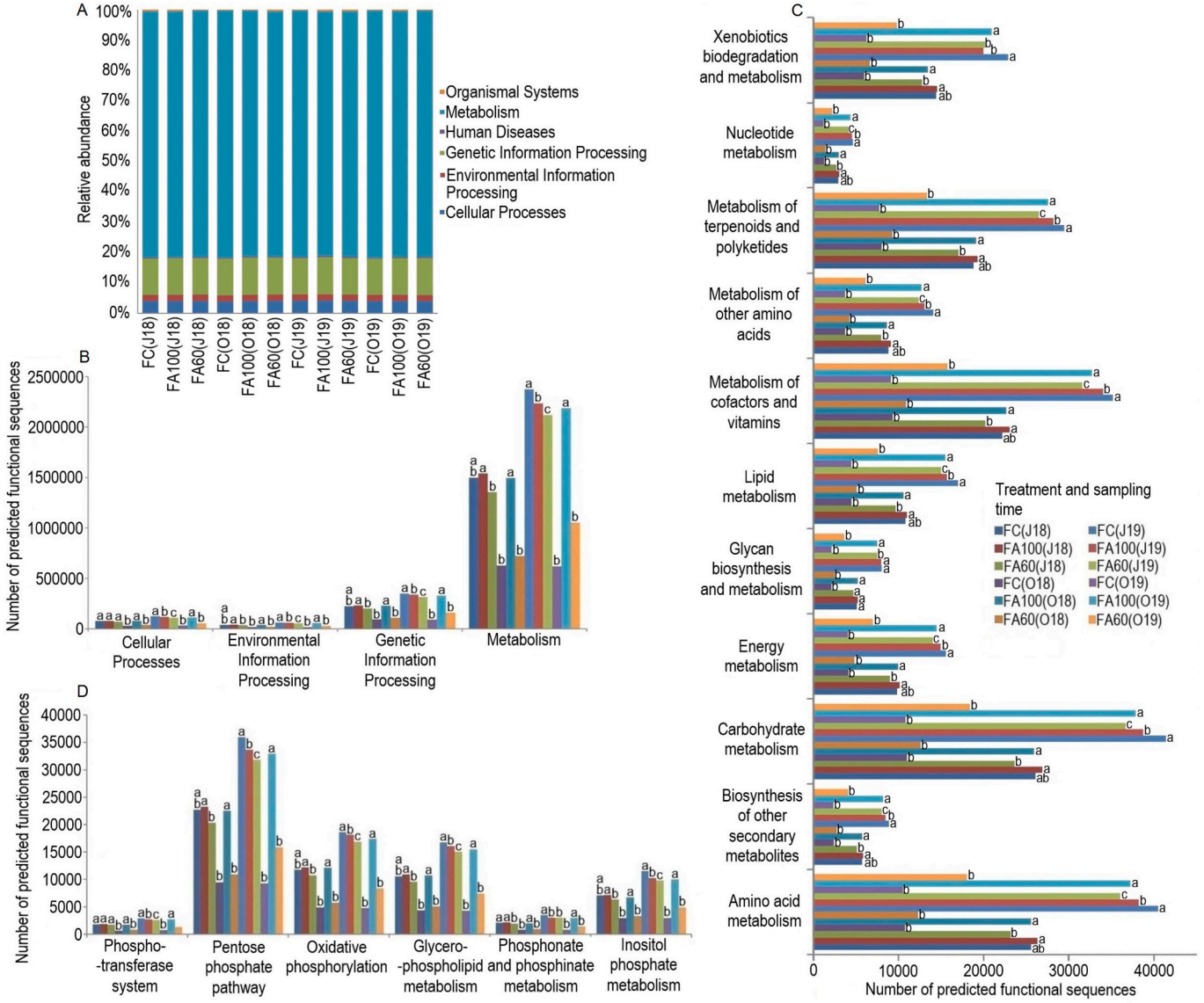

Fig. 5. PICRUSt prediction of the bacterial functional profile. (A) - the relative abundance of the main KEGG classes, (B) – the number of functional sequences assigned to the main KEGG classes, (C) – the number of functional sequences associated with the metabolism of various compounds, (D) – the number of functional sequences associated with phosphorus processes. Explanation: FC-optimal dose of fertilizer, FA100-optimal dose of fertilizer enriched with microorganisms, FA60-fertilizer enriched with microorganisms (dose reduced by 40 %), J18 - June 2018, O18- October 2018, J19-June 2019, O19-October 2019. Different letters indicate statistically significant differences ($p < 0.05$) calculated for each sampling time separately.

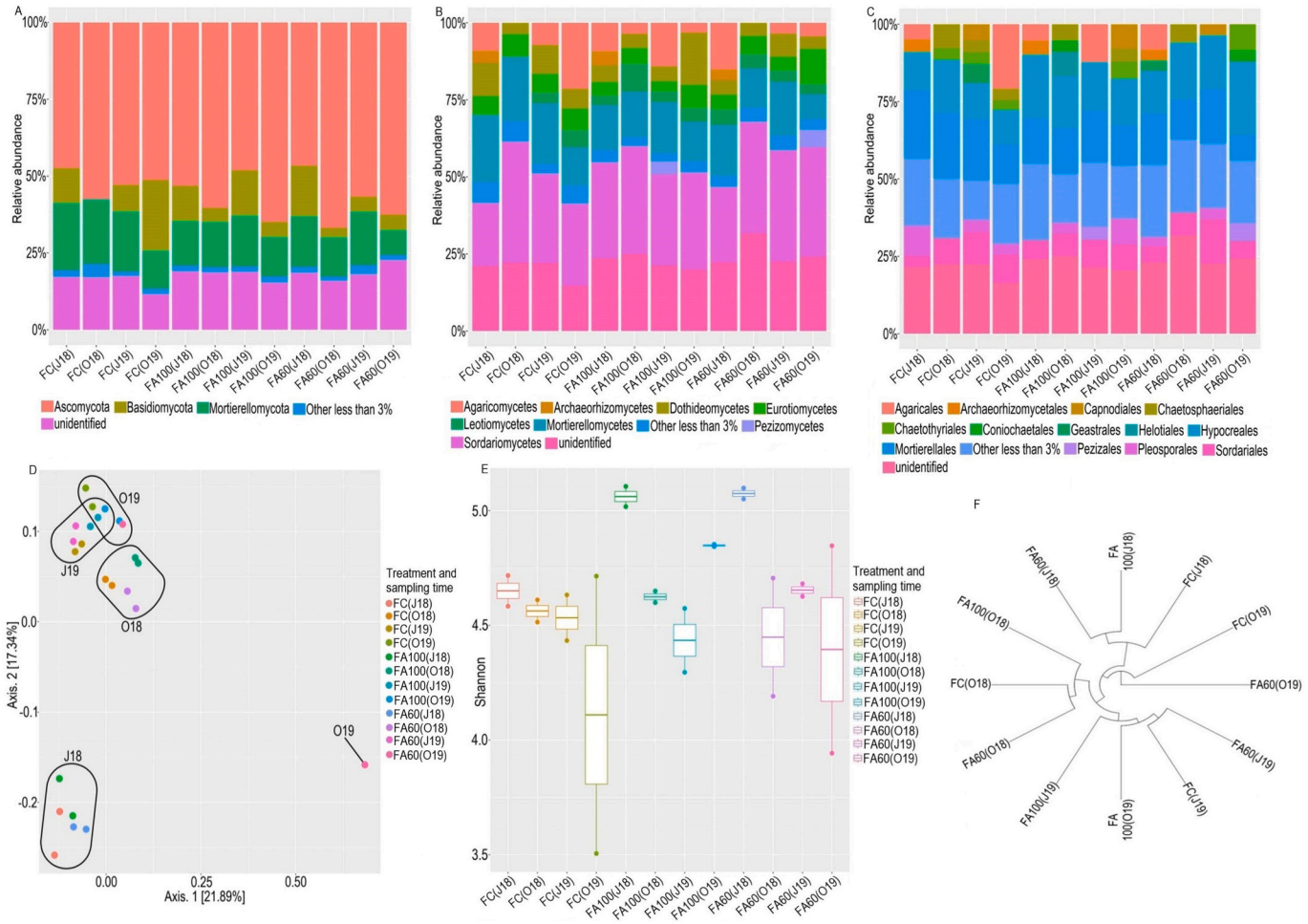

Fig. 6. Effects of the phosphorus biofertilizer on the composition of the fungal community. (A) - the relative abundance of the dominant fungal phyla, (B)- the relative abundance of the dominant fungal classes, (C) - the relative abundance of the dominant fungal orders, (D) – the principle coordinate analysis (PCoA) of fungal OTUs based on Bray-Curtis distances, (E) - Shannon diversity index, (F) - UPGMA dendrogram constructed from Bray-Curtis distances of the fungal OTUs. Explanation: FC-optimal dose of fertilizer, FA100- optimal dose of fertilizer enriched with microorganisms, FA60-fertilizer enriched with microorganisms (dose reduced by 40 %), J18- June 2018, O18-October 2018, J19-June 2019, O19-October 2019.

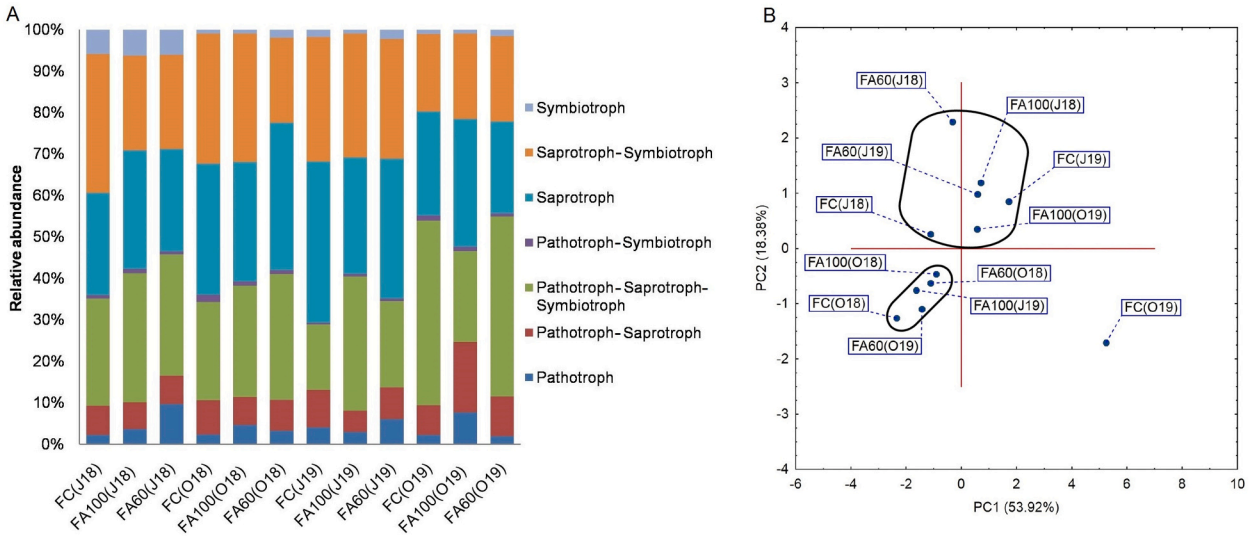

Fig. 7. Fungal functional profile inferred by FUNGuild. (A) - the relative abundance of the fungal trophic modes, (B) - principle component analysis (PCA) of the number of operational taxonomic units (OTUs) associated with fungal trophic modes. Explanation: FC-optimal dose of fertilizer, FA100- optimal dose of fertilizer enriched with microorganisms, FA60-fertilizer enriched with microorganisms (dose reduced by 40 %), J18- June 2018, O18-October 2018, J19-June 2019, O19-October 2019.

(J19), FA60(O19)), "ectomycorrhizal" (FA100(J18), FA60(J18), FA60 (J19)), "endophyte" (FA100(J18)), "ectomyccorhizal-undefined saprotroph" (FA100(O19)), "endophyte-litter, saprotroph-soil, saprotroph-undefined saprotroph" (FA100(O19), FA60(O19)) and "animal pathogen" (FA100(O18)). Plant pathogens were more abundant in almost all FA100 and FA60 treatments except for FA100(J19) and FA60(O19), however, the abundance of phytopathogens decreased between the corresponding FA100-FA60 treatments in 2018 and the FA60 treatments in 2019 (Fig. S8). A PCA analysis of the fungal functional OTUs showed a clear clustering of treatments with respect to the soil sampling time (Fig. 7B).

4. Discussion

The enzymatic response of the soil microbial communities, due to their sensitivity to management practices, is a commonly used bioindicator of soil quality, and reflects variations in the soil environment under the influence of external factors (Lee et al., 2020). During the field experiment variations were observed in the activity of acid phosphomonoesterase, urease, protease and β-glucosidase and they were triggered by the introduction of phosphorus biofertilizer and, as Dick and Kandeler (2005) has stated, enzymatic activity may change rapidly within a two year period after the implementation of a new fertilization regime. The improvement in enzymatic activity in FA100(J19) and FA60(J19) remains in agreement with other studies concerning the influence of biofertilizers on soil microbial properties. Acid phosphomonoesterase activity increased in soil inoculated with *Azotobacter chroococcum, Acetobacter diazotrophicus and Aspergillus awamori* (Srivastava and Singh, 2022), enhanced urease activity was observed in soil amended with a microbial inoculant containing *Frankia casuarinae CcI3* (Qi et al., 2022), the application of *Bradyrhizobium* sp. significantly improved protease activity during the growing seasons in 2018 and 2019 (El-Sawah et al., 2021), while an upward trend in β-glucosidase activity was reported in the case of seed-applied biofertilizers TripleN®, Rhizosum N® and Rhizosum PK® in common wheat cultivation (Dal Cortivo et al., 2020). A higher level of soil enzymatic activity may be associated with an elevated activity level of soil microorganisms and, concomitantly, with the remobilization and increased availability of nutrients (Chaudhary et al., 2022). In addition, enhanced acid phosphomonoesterase activity may facilitate P uptake from organic sources (El-Sawah et al., 2021). A study conducted by Ramesh et al. (2011) revealed the improvement of acid and alkaline phosphatase activity in the rhizosphere of soybean treated with *Bacillus* sp. strains, *Bacillus amyloliquefaciens* and *Bacillus cereus*. Improved soil enzymatic activity and a greater availability of essential nutrients is of particular importance in the light of climate change, as environmental stresses and incremental increases in greenhouse gases concentration in the atmosphere may contribute to a reduced mineral content in plants (Elbasiouny et al., 2022; Sangiorgio et al., 2020). The relatively high enzymatic activity in A18 and A19, however, may refer to the acceleration of chemical transformations occurring in the soil at the beginning of the growing season. The increase in soil enzymatic activity within the corresponding treatments between particular sampling times may suggest that the application of phosphorus biofertilizer not only improves this parameter but also maintains such an outcome over time.

Phosphorus biofertilizer improved the level of available P in the FA100 treatment. An increased P content in the soil was also observed after inoculation with the P-solubilizing bacterium *Paenibacillus* sp. (Li et al., 2020), *B. licheniformis* and *B. amyloliquefaciens* (Wang et al., 2021a) and also the *Bacillus* sp. strain SD01N-014 (Liu et al., 2018). An enhanced level of phytoavailable phosphorus content may result from the P-solubilizing activity of the microorganisms provided with the biofertilizer. Beneficial microorganisms may also stimulate the activity of the native microbiome in favour of biotransformations of the P compounds. Furthermore, the gradual increase in P content in FA100 and FA60 across the field experiment may suggest that the biofertilizer favours the accumulation of this element in the soil. Climate change may limit P availability in the soil which, in turn, impairs the drought tolerance of plants. Phosphorus increases root hydraulic conductivity and enhances development of root system which facilitates water uptake from deeper soil layers. What is more, the higher availability of phosphorus is thought to stimulate the synthesis of osmotically active compounds (e.g. carbohydrates) which are involved in maintaining leaf water potential in the face of drought stress. The application of inorganic phosphorus is also known to regulate the synthesis of abscisic acid and indoleacetic acid and to reduce the concentration of the reactive oxygen species in plant tissues, which also alleviates the drought stress effects. An increase in the mineral component content of the soil through biofertilization may not only increase its uptake but also promote resistance to environmental disturbances (Bechtaoui et al., 2021; Elbasiouny et al., 2022; Jin et al., 2015; Tariq et al., 2018). An improved maize yield may be associated with a higher soil enzymatic activity and, concomitantly, enhanced nutrient availability. According to Schmidt and Gaudin (2018) maize yield increased by an average of 15.3 % after biofertilization under field experiment conditions. Higher yields are thought to be reflected in the increased number of functional sequences associated with amino acid and carbohydrate metabolism in the soil, compounds that can be assimilated and utilized by plants (Moe, 2013). It is worth mentioning that, although the maize yield increase after phosphorus biofertilizer addition was not statistically significant, the 40 % reduction in mineral fertilizer application is more beneficial from an environmental protection point of view and without any loss in yield. This observation is very valuable for farmers and also within the context of assumptions concerning modern, sustainable and regenerative agriculture in view of the deteriorating state of crop yields as a result of climate change.

The metabolic potential of soil bacterial and fungal communities were evaluated in terms of the ratio between substrate utilization and growth pattern. According to Pinzari et al. (2016) lower values of the abovementioned ratio indicate that more dynamic microbial growth was accompanied by a minor amount of substrate consumption. By contrast, a higher ratio points to a stressful metabolic situation wherein a small amount of microbial biomass is developed with a relatively high utilization of carbon compounds. Our results showed that phosphorus biofertilizer has the potential to alleviate the metabolic stress of some C-substrates belonging to different compound groups (carbohydrates, carboxylic acids, polymers, amino acids) thereby enhancing the metabolic efficiency of soil microorganisms. Such a change may be a result of the different metabolic activity of beneficial microorganisms provided with a biofertilizer as compared with indigenous microbiota. The application of biofertilizer not only exerts an immediate effect on metabolic stress, but it also has a prolonged impact, especially on the carboxylic acids in ECO plates. The absence of a stressful situation across the experimental period for some substrates (e.g. carbohydrates) indicates that certain compounds can be effectively metabolized regardless of the soil sampling time and constitute a preferred carbon source. However, variations in the metabolic efficiency of soil microbial communities throughout the field experiment may be due to seasonal temperature fluctuations, maize photosynthetic activity and root exudates. The broadening of the catabolic potential of soil microorganisms may also entail a greater resistance to various environmental stresses, including those induced by climate change.

Along with enzymatic activity and metabolic potential, an analysis of the genetic diversity of bacterial, archaeal and fungal communities using the M-tRFLP approach was also carried out. Consequently, restriction profiles, known as "genetic fingerprinting", unique to each of the microbial groups were obtained. Throughout the experimental period variations in both the number of T-RFs and their relative abundance were observed, which may reflect the susceptibility of microorganisms to changes induced by the introduction of beneficial microorganisms to the soil. A reduction in the number of T-RFs in the bacterial community in J19 remains in agreement with the results of Zhang et al. (2010) who

presented a decline in the richness within bacteria inhabiting soil that was inoculated with the *Rhizobium leguminosarum* strain. In the case of bacteria and fungi, an increased number of T-RFs in O19 was observed in comparison with J19, which may indicate that the favourable impact of biofertilizer becomes apparent after a certain time. Such a trend was also reported by Mącik et al. (2022) where a different soil type (Brunic Arenosol) was treated with the same phosphorus biofertilizer. The archaeal "core microbiome" consisted of a relatively high number of T-RFs accompanied by a low degree of diversity among specific fragments, possibly indicating that archaea are the most stable microbial group when confronted by a novel fertilization technique. Furthermore, an increase in the total number of archaeal T-RFs in FA100 and FA60 may indicate that biofertilizer stimulates a selected microbial group. Higher number of T-RFs in FA100 than in FA60 among bacteria and fungi may suggest, instead, that a preferable solution is the application of phosphorus fertilizer at an optimal dose, but microbiologically enriched. The higher values of the Sorensen and Jaccard indices between the FA100 and FA60 treatments indicate a greater similarity of the restriction profiles with respect to the number of T-RFs (Gryta and Frąc, 2020; Johnston-Monje et al., 2014). A PCA analysis showed a clear clustering of treatments with respect to the soil sampling time. The arrangement of the samples in the PCA space indicated that changes in the t-RFLP patterns were more pronounced in the second year of the field experiment. The grouping of different sampling times within a particular year mainly resulted from dissimilarities in relative abundance and the number of individual T-RFs in the restriction profile. The common clustering of the A18 and O18 sampling times may suggest that the microbial communities rapidly achieved their pre-biofertilization status. Nevertheless, the subsequent application of biofertilizer in 2019 resulted in a greater degree of diversity within the restriction profiles as evidenced by the vivid distinction of samples according to their sampling time.

The TRiFLe tool, which is based on the size of the selected T-RFs, allowed for the identification of different microorganisms providing essential services for the preservation of soil productive potential and soil health. Ecologically important archaea may be represented by members of *Nitrososphaera* spp., ammonia oxidizing archaea, with wide metabolic abilities are involved in the nitrification process (Miranda et al., 2019; Mukhtar et al., 2019). Bacterial genera demonstrating beneficial traits included *Rhizobium* and *Bradyrhizobium* (biological nitrogen fixation) (Hara et al., 2019; Wang et al., 2018c), *Lysobacter* (biological control agent against fungal phytopathogens) (Nian et al., 2021), *Pseudomonas* (phytohormones synthesis) (Hassen et al., 2018), *Bacillus* (mitigation of abiotic and biotic stress factors) (Saxena et al., 2020), *Anabaenopsis* (phytohormones synthesis) (Kollmen and Strieth, 2022), *Aeromonas* (siderophore synthesis) (Seenivasagan and Babalola, 2021), *Sodalis* (decomposition of deadwood and plant biopolymers) (Tláskal et al., 2021) and *Burkholderia* (bioremediation) (Min et al., 2017). Furthermore, some representatives of the *Citrobacter* spp., *Lysobacter* spp., *Pseudomonas* spp., *Bacillus* spp., *Burkholderia* spp. and *Klebsiella* spp. were found to exhibit phosphate solubilizing properties (Castagno et al., 2021; Chen et al., 2019; Liu et al., 2020; Mažylytė et al., 2022; Nacoon et al., 2020; Suleman et al., 2018). Among the fungal community, selected T-RFs were associated with saprotrophic fungi (*Psathyrella*, *Conocybe*) (Padamsee et al., 2008; Ogura-Tsujita et al., 2009), entomopathogens (*Cordyceps*, *Metarhizium*) (Hussain et al., 2021; Brunner-Mendoza et al., 2019), nematophagous fungi (*Dactylellina*) (Degenkolb and Vilcinskas, 2016), lichenized fungi (*Caloplaca*, *Biatora*, *Cladonia*) (Vargas Castillo and Beck, 2012; Printzen, 2014; Tuovinen et al., 2015), mycorrhizal fungi (*Suillus*, *Sarcodon*, *Tomentella*, *Paraglomus*) (Li et al., 2021; Grupe et al., 2015; Suvi et al., 2010; Błaszkowski et al., 2012), plant growth promoting microorganisms (*Penicillium*, *Trichoderma*) (Zhang et al., 2018; Radhakrishnan et al., 2014) and fungi exerting bioremediation properties (*Solicoccozyma*) (Du et al., 2022). What is more, TRiFLe revealed the presence of fungi which may be potentially involved in phosphate solubilization and mobilization within the soil (*Oidiodendron*, *Penicillium*, *Trichoderma*, *Pichia*, *Aspergillus* and *Saccharomyces*) (Alori et al., 2017). Some microorganisms identified with TRiFLe may also participate in the mitigation of climate change induced stresses in crops including cold and freezing temperatures (*Pantoea* spp., *Pseudomonas* spp.), high temperatures (*Bacillus* spp., *Pseudomonas* spp.), salinity (*Pseudomonas* spp., *Burkholderia* spp.) and drought (*Klebsiella* spp., *Citrobacter* spp., *Serratia* spp., *Penicillium* spp.) (Fadiji et al., 2022).

Next Generation Sequencing was employed to gain a deeper understanding of the microbial community structure and functionality in soil amended with phosphorus biofertilizer. The composition of the bacterial and archaeal communities was analyzed at phylum, class and order level and each taxonomic level showed a higher number of OTUs in the FA100 and FA60 treatments, indicating the beneficial impact of the biofertilizer on the diversity of soil microorganisms. It is probable that the phosphorus biofertilizer stimulated the growth of specific microorganisms and boosted their proliferation, thereby broadening the range of services provided by the microbiome. The higher number of OTUs at the phylum, class and order level within the bacterial community was also observed by Wang et al. (2021b) in soil inoculated with biocontrol strains *Paenibacillus jamilae* HS-26 and *Bacillus amyloliquefaciens* subsp. *plantarum* XH-9. Specific orders in FA100 and FA60 included Dehalococcoidales, representatives of which are able to remediate halogenated ethenes and chloroorganic aromatic compounds (Löffler et al., 2013) and also Syntrophobacterales, a sulfate-reducing bacteria (Plugge et al., 2011). The higher degree of biodiversity within the bacterial community may also be supported by increased Shannon index values. Higher values of the Shannon index within the soil bacterial community were also observed after inoculation with *B. subtilis* (Wang et al., 2021a) and *Trichoderma* biofertilizer (Zhang et al., 2022). According to Pang et al. (2017) a higher degree of diversity within the microbial community ensures a well-balanced microbiome with a greater degree of resistance to environmental stresses and pathogen activity which is essential for fostering adaptation to climate change.

The application of biofertilizer modified the relative abundance of bacterial groups from the phylum to the order level. It is not easy to pinpoint the driver of such a change, however, shifts in the community structure may result from competition for an ecological niche, a certain imbalance arising from the introduction of active, beneficial microorganisms and interactions between the indigenous microbiome and the bacteria provided with the biofertilizer. Proteobacteria and Actinobacteriota are ubiquitous in soils, and also, Actinobacteriota were found to synthesize hydrolytic enzymes degrading plant polymers (Gao et al., 2019; Zhang et al., 2019). Acidobacteriota members colonize both acidic and nutrient deficient environments (Wang et al., 2018c), and, as Kalam et al. (2020) described, Acidobacteriota are involved in soil organic matter decomposition, biogeochemical cycles and also affect plant growth. An increased relative abundance in Chloroflexi and Acidobacteriota was also observed by Chen et al. (2021) after soil inoculation with *B. subtilis* and *P. polymyxa*. In selected FA100 and FA60 treatments the biofertilizer stimulated the occurrence of Blastocatellia, members of which can break down complex proteins (Ivanova et al., 2020). A similar trend was reported by Pongsilp and Nimnoi (2020) wherein the distribution of Blastocatellia increased in soil amended with a liquid medium containing *Ensifer fredii*. Members of Rhizobiales participate in N_2 fixation and are commonly used as ecofriendly biofertilizers, however, certain species are plant pathogens (Wang et al., 2018b), therefore a decline in their relative abundance may be considered to have a favourable impact.

Shifts in the fungal community, similarly to bacteria and archaea, were analyzed at the phylum, class and order level. Fungi belonging to Ascomycota are widely distributed soil organic matter decomposers (Muneer et al., 2021), Basidiomycota can break down lignocellulose and form symbiotic relationships with plants (Wang et al., 2022) while Mortierellomycota exerts plant growth promoting properties including phosphorus solubilization (Ozimek and Hanaka, 2021; Wolińska et al., 2022). The application of biofertilizer promoted the abundance of

Ascomycota, especially in O18 and O19, however, such a trend may derive from the accumulation of organic matter in the soil and in the enhanced activity of microorganisms involved in its breakdown. Cao et al. (2022) also observed that Ascomycota was more abundant in soil treated with a microbial inoculant containing *Bacillus* spp. strains. The biofertilizer used in this study stimulated the occurrence of some fungal orders including microorganisms important for soil microbial health such as Helotiales (saprotrophs and ericoid mycorrhizal fungi) (Wang et al., 2006), Sordariales (saprotrophs growing on dung or decaying plants and exerting an antifungal activity) (Luo et al., 2022) and also Agaricales (saprotrophs synthesizing hydrolytic and oxidative enzymes involved in lignocellulose breakdown) (Ruiz-Dueñas et al., 2020). Variations in the composition of the soil mycobiome between particular treatments may be attributed to the fact that the expansion of certain fungal groups inhibits the development of other fungi due to nutrient consumption (Yin et al., 2022). The increased Shannon diversity index within the fungal community remains in line with results obtained by Zhao et al. (2022) where soybean seeds were inoculated with *Bradyrhizobium japonicum* 5038 and a combination of *Bradyrhizobium japonicum* 5038 and *Bacillus aryabhattai* MB35-5.

PICRUSt-based functional inference revealed variations in bacterial biological functions after fertilization with phosphorus biofertilizer. In general terms, an increased number of functional sequences was associated with the main KEGG classes and sub-classes in both FA100 and FA60 in O18 and O19, this may suggest that the positive effect of the biofertilizer emerges gradually. Furthermore, a similar number of sequences between the FA100 treatments within a particular year may indicate that the activity of an optimal dose of fertilizer in combination with beneficial bacteria is maintained over time. It was speculated that an upward trend in the number of functional sequences in J19 in comparison with J18 and O18 resulted from soil supplementation with nutrients contained in fertilizers and with the acceleration of the processes involved in their breakdown. In other studies, it was observed that biofertilizers increased the abundance of genes involved in energy production and conversion, lipid transport and metabolism, cell motility, secondary metabolites biosynthesis, transport, and catabolism (Qi et al., 2022), xenobiotics biodegradation and metabolism (Wang et al., 2021a), signal transduction mechanisms and nucleotide and coenzyme transport and metabolism (Tian et al., 2022). A higher number of functional sequences in FA100 and FA60 in the autumn sampling times may indicate that microorganisms gradually adapted their metabolic pathways to altered environmental conditions.

The majority of the obtained functional sequences were assigned to metabolic-related pathways, which indicated that the metabolism of various compounds may be a determinant of the proper functioning of the soil microbiome and an indicator of soil health (Qi et al., 2022). A relatively high number of sequences was also assigned to “Genetic Information Processing” and this suggests that processes associated with DNA replication and gene expression are also fundamental to soil microorganism prosperity. Different sub-classes within “Metabolism” the main KEGG class indicated the broad spectrum of the metabolic capabilities of bacteria inhabiting AL soil. The highest number of functional sequences was connected to “Amino acids metabolism” and “Carbohydrates metabolism” which suggested that the biotransformation of these compounds occurs with the greatest intensity. For the improvement of degraded soil quality, important metabolic pathways included the degradation of xenobiotics, which are compounds responsible for soil contamination, and the synthesis of secondary metabolites such as antibiotics (Mishra et al., 2021; Sharrar et al., 2020). As Singh et al. (2014) and Crits-Christoph et al. (2018) stated, the enzymes involved in xenobiotics degradation and the metabolism of terpenoids and polyketides are harboured by a small group of soil microorganisms and, according to Xun et al. (2019), their specialized functions play a crucial role in the mitigation of environmental stresses and thereby ensure a high-quality microbiome. On the other hand, the synthesis of antibiotics may broaden the antagonistic capacity of microorganisms towards phytopathogens. A more profound insight into the predictive functional profile revealed that biofertilizer also affected the pathways associated with P compounds. In a long-term study conducted by Wang et al. (2021c) inoculation with *Burkholderia cepacia* ISOP5 and *Rhodopseudomonas palustris* ISP-1 decreased the abundance of genes associated with P uptake and transport, but increased the number of genes responsible for organic P mineralization and inorganic P solubilization such as *phoN* (acid phosphatase), *phnA* (phosphonoacetate hydrolase) and *phnFGHIJKLMNOP* (C—P lyase subunit). In our study, phosphorus biofertilizer increased the number of functional sequences associated with processes that are connected with the formation of cell wall membranes (“Glycerophospholipid metabolism”) (Kondakova et al., 2015), the decomposition of compounds constituting the P source (“Phosphonate and phosphinate metabolism”) (Tapia-Torres et al., 2016), the synthesis of NADPH and ATP (“Pentose phosphate pathway” and “Oxidative phosphorylation”) (Borisov et al., 2011; Spaans et al., 2015) and the transport of carbohydrates and sugar derivatives (“Phosphotransferase system”) (Somavanshi et al., 2016). The increased number of functional sequences associated with “Oxidative phosphorylation” in soil amended with biofertilizers was also observed by Cao et al. (2022). In general, the results obtained from PICRUSt indicated that phosphorus biofertilizer may be beneficial to the soil bacterial community by promoting the abundance of functional sequences associated with basic and specialized biochemical processes that determine the resilience and adaptability of soil bacteria. The enhanced metabolism of P compounds may strengthen the bacterial cells and support the establishment of a high quality soil microbiome.

The assignment of trophic modes and ecological functional groups (guilds) using the FUNGuild database revealed that phosphorus biofertilizer may stimulate the occurrence of fungi that are important for the protection and preservation of soil health, which is one of the most relevant challenges for regenerative agriculture. Saprotrophic fungal communities are involved in the decomposition of organic matter of various origins while mycorrhizal species improve the nutrient uptake in plants and display an antagonism towards phytopathogens (Fang et al., 2020; Frąc et al., 2018). Mycorrhizal fungi can also increase drought tolerance in plants by improving water retention, root biomass and the synthesis of certain substances (e.g. glomalin) and thereby exerting aggregating properties on the soil structure (Fadiji et al., 2022). Moreover, mutualistic interactions between arbuscular mycorrhizal fungi and plant roots can increase drought resilience by regulating glucose exudation and rhizosphere expansion (Hoang et al., 2022). An increase in the relative abundance of saprotrophs and mycorrhizal fungi was also observed by Mącik et al. (2022) in soil amended with the same phosphorus biofertilizer as the one used in this study. Biofertilization also promoted an increase in plant pathogen abundance, however, we assume that such an outcome can be controlled due to a higher genetic and improved functional diversity as well as the presence of microbial biocontrol agents.

Both PCoA and UPGMA clustering revealed that not only fertilization treatment, but also soil sampling time affected the composition of the soil microbiome. The distinction between the controls from microbiologically amended treatments within the bacterial community in the PCoA space was also observed by Wang et al. (2018a, 2021b). Yang et al. (2020) observed that the soil samples inoculated with microbial fertilizer were grouped according to the wheat growing periods. The arrangement of the samples within the bacterial community in both the PCoA and UPGMA tree may indicate that bacteria are more sensitive to seasonal environmental changes and new fertilization regimes as compared to fungi. The distribution of samples in the PCoA space may also indicate that the fungal community underwent shifts after the first application of biofertilizer and afterwards remained more resilient to subsequent fertilizer applications showing a simultaneously greater stability under fluctuating environmental conditions. The close proximity of fungal samples collected in O18, J19 and O19 and their common grouping may indicate that variations after biofertilizers

application were maintained over time.

5. Conclusions

The development of sustainable soil management practices through the application of microbial-based bioformulations is highly recommended at a time of agricultural intensification, climate change and in order to meet certain targets for regenerative agriculture including soil health restoration and the reversal of biodiversity loss. Due to the invaluable contribution of soil microorganisms to the maintenance of the ecological balance of the ecosystem, we suggested the application of an innovative phosphorus biofertilizer as a tool to improve the biodiversity of the microbiome inhabiting degraded soil. The obtained results confirmed the favourable influence of the biofertilizer on the microbiological properties of the soil, these included the enzymatic activity, the presence of microorganisms exhibiting plant growth promoting properties, and the composition of functional profiles and metabolic efficiency. What is more, we showed that phosphorus biofertilizer has the potential to increase the phytoavailable phosphorus content and enhance maize yield. These results revealed the comprehensive effect of biofertilizer on the soil environment. In summary, this work showed that a microbiologically enriched phosphorus mineral fertilizer may be adapted in sustainable and regenerative agriculture. This would potentially combine the status of the soil microbial communities with general soil health and the beneficial properties provided by microorganisms which could reveal novel pathways for the improvement of arable soil quality and productivity. Finally, enhanced soil ecosystem biodiversity and the replacement of agrochemicals with microbial inoculants could support the formation of a healthier and stronger microbiome that is more resistant to the damaging consequences of climate change.

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CRediT authorship contribution statement

Mateusz Mącik: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. **Agata Gryta:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – review & editing. **Lidia Sas-Paszt:** Conceptualization, Funding acquisition, Project administration. **Magdalena Frąc:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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