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SOIL DEGRADATION CONTROL, REMEDIATION AND RECLAMATION

Long-term organic amendments regulate *cbbL*-harboring bacterial community via soil physicochemical properties and enzyme activities in a paddy soil

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Abstract

Purpose Organic amendments improve soil physicochemical and microbial properties, but the effects vary by fertilizer type. These amendments also modulate the autotrophic CO_2-fixing microbial community, particularly those harboring the *cbbL* gene, which encodes the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) form I. Nevertheless, how *cbbL*-harboring autotrophs respond to different organic amendments and their associations with soil enzyme activities are still not well understood.

Materials and methods A long-term organic amendment experiment was established in a double-cropping rice paddy field in Southern China, including four treatments: without organic fertilizer input (control), green manure (GM), pig manure (PM), and rice straw returning (RS). Soil C-, N-, and P-acquisition enzyme activities were analyzed using a fluorometric method. The *cbbL*-harboring bacterial community was characterized by quantitative PCR (qPCR) and high-throughput sequencing. Partial least squares path modeling (PLS-PM) was used to determine the relationships among physicochemical properties, enzyme activities, and the *cbbL*-harboring community.

Results and discussion The organic amendments improved soil physicochemical properties, including pH and soil organic C (SOC). Soil C-, N-, and P- acquisition enzyme activities responded variably to the amendments. Although the *cbbL* gene number did not significantly change, all organic amendments reduced the diversity of *cbbL*-harboring bacterial community. Shifts in the *cbbL*-harboring community composition were also observed: GM enriched *Afipia*, PM favored *Pseudonocardia*, and RS exhibited increased abundances of *Methylotenera* and *Sulfuricaulis*. PLS-PM indicated that soil pH, SOC, and C- and N-acquisition enzyme activities negatively influenced the diversity and the composition of the *cbbL*-harboring community, whereas P-acquisition enzyme activity had a positive effect on the community diversity.

Conclusions Our study highlights the complex interactions among soil physicochemical properties, enzyme activities, and *cbbL*-harboring bacterial community under organic amendments. The results address the critical factors shaping the *cbbL*-harboring bacterial community, advancing our understanding of CO_2-fixing microorganisms in agricultural ecosystems.

Keywords *cbbL*-harboring microorganisms · Paddy soil · Organic fertilizer · Enzyme activity

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1 Introduction

Organic amendment is a widely used strategy to enhance plant growth, improve soil physicochemical properties, and increase C sequestration in agricultural systems. Soil microorganisms play pivotal roles in ecosystem functioning by regulating organic matter decomposition and mediating the effects of organic amendment (Zhou et al. 2023), and their taxonomic shifts have been widely associations with soil productivity (Cui et al. 2023). However, the impacts of organic amendments on soil microorganisms vary substantially depending on the organic fertilizer type. Generally, organic fertilizers with low C:N ratios (high quality) are more bioavailable to microorganisms than high C:N ratio fertilizers, inducing more profound changes in soil properties (Zhou et al. 2019a; Cui et al. 2023; Chang et al. 2014). Therefore, the type of organic fertilizer must be considered when evaluating amendment effects on soil microbial processes.

CO_2-fixing microorganisms are ubiquitous in terrestrial ecosystems, playing a crucial role in enhancing soil C sequestration and regulating atmospheric CO_2 concentrations, thereby mitigating the climate warming effect (Akinyede et al. 2022). Currently, six primary CO_2-fixing pathways have been identified in soil microorganisms, contributing an estimated 0.6–4.9 Pg C annually to microbial biomass, which constitutes approximately 0.5%–4.1% of the total terrestrial C fixation (Liu et al. 2022; Akinyede et al. 2022). Among these, the Calvin-Benson-Bassham (CBB) cycle is considered the ancestral CO_2 assimilation pathway, with the *cbbL* gene that encodes the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) serving as the key protein for CO_2 assimilation. Both obligate and facultative *cbbL*-harboring microorganisms contribute markedly to this process (Wang et al. 2021a; Zhao et al. 2018). Obligate autotrophs exclusively assimilate CO_2 for cellular synthesis, whereas facultative autotrophs exhibit metabolic flexibility by utilizing organic substrates as alternative C and energy sources (Zhou et al. 2019b). Organic substrates can profoundly influence the *cbbL*-harboring bacterial community by altering the distribution of obligate to facultative autotrophs, thereby changing the microbially driven CO_2-fixing processes (Badger and Bek 2008; Xiao et al. 2019; Long et al. 2024). While the temporal dynamics and dosage effects of organic amendments on *cbbL*-harboring microorganisms have been well characterized (Liu et al. 2022; Xu et al. 2023), the critical role of fertilizer quality in shaping these CO_2-fixing microorganisms remains to be elucidated.

The CO_2-fixing process mediated by *cbbL*-harboring microorganisms in soil is governed by multiple factors, including soil physicochemical properties such as pH, soil organic carbon (SOC), and nutrient availability. SOC promotes *cbbL* gene abundance and *cbbL*-harboring microbial diversity (Xiao et al. 2021), whereas N deposition exerts opposite effects on both (Qin et al. 2021). Soil pH and available P both negatively influence the *cbbL*-harboring community diversity (Xu et al. 2023). In an environment abundant in organic materials, extracellular enzymes serve as crucial regulators of the *cbbL*-harboring community by mediating organic matter decomposition and niche differentiation (Zhang et al. 2022). However, the current understanding of the relationship between soil enzyme activities and *cbbL*-harboring community remains inconsistent. While Liang et al. (2025) report a positive correlation between C-acquisition enzyme activities (β-glucosidase and cellobiose hydrolase) and CO_2-fixing microorganisms across several phyla, Zhou et al. (2024b) observe a negative relationship between such activities and *cbbL* gene abundance. As to the organic amendments, the substantial compositional variability of organic fertilizers makes their relationship more complex. Thus, a systematic investigation is needed to elucidate how organic amendments influence *cbbL*-harboring communities via soil enzyme activities.

Paddy soil has been shown to exhibit an approximately four-fold higher rate of CO_2 fixation in comparison to upland and forest soils (Liao et al. 2023), with the CBB cycle identified as the predominant CO_2-fixation pathway (Xiao et al. 2021). This makes it an ideal ecosystem for studying the characteristics of CO_2-fixing microbial community in response to environmental changes. In rice cultivation, organic fertilizers are routinely applied to boost crop productivity and improve soil quality. However, their application fundamentally alters the soil microenvironment and microbial nutrient acquisition strategies, thereby restructuring the niches and community composition of CO_2-fixing microorganisms. The complex interactions among these changes are not yet well understood. To this end, a representative paddy soil subjected to long-term organic amendments, i.e., straw return, green manure, and livestock manure, was selected in this study. We measured soil C-, N-, and P- acquisition enzyme activities and the composition of the *cbbL*-harboring bacterial community. We hypothesized that: i) soil enzyme activities for C-, N-, and P-acquisition would respond differently to different organic amendments due to their distinct chemical compositions; ii) organic amendments would decrease the diversity and alter the structure of the *cbbL*-harboring bacterial community, as the increased nutrient availability would favor heterotrophic microorganisms over these autotrophs; and iii) soil C-, N-, and P- acquisition enzyme activities, together with soil physicochemical properties, would exert varying impacts on the *cbbL*-harboring bacterial community, given that they

function through distinct mechanisms linked to specific soil microbial processes.

2 Materials and methods

2.1 Experimental site

A long-term field experiment has been conducted since 1990 at the Red Soil Ecological Research Station (28°15′30"N, 116°55′30"E) of the Chinese Academy of Sciences in Yingtan, Jiangxi (China). This experimental site is characterized by quaternary red clay parent material and represents a typical Ultisol under the USDA classification system. The region has a typical subtropical monsoon climate with an annual precipitation of 1,795 mm, an annual temperature of 17.6 °C, and an annual evaporation of 1,318 mm. Prior to experimental establishment, the paddy fields existed as uncultivated wastelands with naturally developed soil profiles.

2.2 Experimental design and soil sampling

Since 1990, the paddy field has been leveled and flooded on an annual basis for the purpose of cultivating double rice (*Oryza sativa L.*) crops. The rice cultivation period extends from early April to the end of October, with the field remaining fallow for the remainder of the year (unless stated otherwise). Early rice is sown in April, and both the grain and straw are harvested in late July. Late rice is then sown in July, and its grain and straw are harvested in early November, as reported in previous studies (Li et al. 2010).

The field experiment included four treatments: without organic fertilizer input (control), green manure amendment (GM), pig manure amendment (PM), and rice straw amendment (RS). To meet the nutrient requirements for plant growth, additional mineral fertilizers (N, P, and K) were applied to all treatment plots. The mineral fertilizers were applied in the form of urea, calcium magnesium phosphate, and potassium chloride, at annual application rates of 239 kg N ha^{-1}, 344 kg P_2O_5 ha^{-1}, and 360 kg K_2O ha^{-1}, respectively. In the GM amendment, a legume crop, Chinese milk vetch (*Astragalus sinicus* L.), was planted during winter and then returned to the field in situ. In the PM amendment, the composted pig manure was purchased from a local livestock farm. In the RS amendment, the rice straw was sourced from the *in-situ* plot after the rice harvest. All organic fertilizers were applied at a rate of 2,250 kg ha^{-1} (dry weight) on an annual basis before the cultivation of the early rice crop. The experimental design employed a randomized complete block configuration, with three replicates for each treatment. Each plot has an area of 30 m^2. Agricultural management, including subsurface drainage, irrigation, herbicide application, and pesticide utilization, remains constant across all treatment plots. The chemical properties of the organic fertilizers are detailed in Table 1.

In October 2017, the rice crop in each plot was harvested manually. Then, the air-dried grain from each plot was weighed for yield determination. Surface soil samples were collected from a depth of 0–20 cm. Within each plot, a homogeneous composite sample was obtained by thoroughly mixing five separate soil cores (6 cm in diameter). The soil samples were then placed in a portable fridge (4°C) and transported to the laboratory within 24 h. In the laboratory, each sample was divided: one portion was stored at −80°C for molecular analysis, while the other was sieved (2 mm) to remove coarse roots, debris, and stones. The sieved samples were then separated into two subsamples: one was stored at 4 °C for analyses of soil enzyme activities and available nutrients (completed within one month), and the other was air-dried, ground, and passed through a 0.15-mm sieve for determination of other physicochemical properties.

2.3 Soil physicochemical properties analyses

Soil physicochemical properties were analyzed following the methods described by Lu (1999). The soil pH was determined by using a pH meter with a 1:2.5 soil-to-water suspension (w/v). The SOC was measured by the sulfuric acid–potassium dichromate oxidation (H_2SO_4-$K_2Cr_2O_7$) method. The total nitrogen (TN) and available nitrogen (AvailN) were measured by the Kjeldahl method. Total phosphorus (TP) was assayed using the molybdenum blue method after the digestion of hydrofluoric-hydrochloric oxide four acid (HF-$HClO_4$), while available phosphorus (AvailP) was determined using the same procedure but following sodium bicarbonate ($NaHCO_3$) extraction. Total potassium (TK) and available potassium (AK) were measured by flame emission spectrometry. TK was analyzed

Table 1 The chemical properties of the organic fertilizers used in this study

Fertilizer	TC	TN	TP	TK	C:N	C:N:P
	(g/kg)					
Green manure	373.55	31.27	3.24	31.81	11.95	115.9:9.7:1
Pig manure	261.33	28.50	33.56	12.84	9.17	8.3:0.9:1
Rice straw	325.61	9.23	1.51	30.49	35.28	215.2:6.1:1

TC, Total C; *TN*, Total nitrogen; *TP*, Total phosphorus; *TK*, Total potassium

after an HF-$HClO_4$ digestion, while AvailK was determined following an ammonium acetate extraction.

2.4 Analyses of soil enzyme activities

The enzyme activities were determined by using the 96-well plate fluorescence spectrophotometry method as described by Sinsabaugh et al. (2008). The tested enzymes included C-acquisition enzymes (β−1,4-glucosidase, BG; β-xylosidase, BX; β-D-cellobiohydrolase, CBH), N-acquisition enzymes (leucine aminopeptidase, LAP; β-N-acetylglucosaminidase, NAG), and the P-acquisition enzyme (acid phosphatase, AP). BG catalyzes the hydrolysis of cellobiose and cellodextrins into glucose (Veres et al. 2015), the last step of cellulose degradation. CBH cleaves cellulose into cellobiose, while BX breaks down xylose-harboring oligosaccharides into xylose (Banerjee et al. 2010). For the N-acquisition enzymes, NAG degrades chitin—a major component of fungal cell walls and arthropod exoskeletons—yielding N-acetylglucosamine, whereas LAP hydrolyzes peptide bonds to release free amino acids (Daunoras et al. 2024). AP is responsible for mineralization of organic matter to release phosphate ions ($H_2PO_4^-$ and HPO_4^{2-}) in soil (Criquet and Braud 2008). The detailed methodology for determining enzyme activities is provided in Table S1. The final enzyme activities were expressed as nanomoles per gram of dry soil per hour (nmol/g/h).

2.5 Soil DNA extraction, quantification, and sequencing of *cbbL*-harboring bacteria

Soil DNA was extracted by using a Fast® DNA SPIN Kit (MP Biomedicals, CA, USA) with a subsequent purification step utilizing a PowerClean® DNA Clean-up Kit (MoBio, CA, USA), in accordance with the manufacturer's instructions. The concentration and quality of the extracted DNA were subsequently measured using a spectrophotometer (NanoDrop One, DE, USA). Soil *cbbL*-harboring bacterial community were characterized by the high-throughput sequencing technique. The amplification of the *cbbL* region was conducted using the primers K2f (5'-ACCAYCAAGCCSAAGCTSGG-3')/V2r (5'-GCCTTCSAGCTTGCCSACCRC-3'). Purified PCR products were quantified by Qubit 3.0 (Invitrogen, CA, USA), mixed equally, and sequenced on an Illumina MiSeq platform carried out by Shanghai Genesky Biotechnologies Inc. (Shanghai, China).

The *cbbL* gene was quantified via qPCR using the primer pair K2f/V2r (Wu et al. 2017). Each 10 μL reaction mixture consisted of 5 μL SYBR Premix ExTaq (TaKaRa, Dalian, China), 1 μL template DNA of approximately 5 ng, 0.15 μM respective primer, and nuclease-free water. The thermocycling conditions were: initial denaturation at 95 °C (2 min), followed by 40 cycles of denaturation at 95 °C (30 s), annealing at 62 °C (30 s), and extension at 72 °C (20 s). The standard curves were generated from ten-fold serial dilutions of recombinant plasmids carrying the *cbbL* gene. The copy numbers of the *cbbL* gene were calculated according to the standard curves, with qPCR efficiency at 97% ($R^2 = 0.998$).

2.6 Bioinformatics analysis

The raw sequencing data underwent initial processing with Trimmomatic (v0.36) for filtering and FLASTQ software for assembly. All chimaeras were removed by the USEARCH tool according to the UCHIME algorithm. Operational taxonomic units (OTUs) were clustered at a 97% identity level by UPARSE. The representative OTU sequences were annotated by the National Center for Biotechnology Information (NCBI) database. The raw sequencing reads were deposited into the NCBI Sequence Read Archive (SRA) database under the accession number PRJNA967816.

All subsequent bioinformatics analyses, data visualization, and statistical computations were conducted using R (v4.2.1). Significant differences among treatments were analyzed using a one-way analysis of variance (ANOVA) incorporating Duncan's Multiple Range test at $P < 0.05$ unless otherwise specified. Principal coordinates analysis (PCoA) was applied to evaluate the structural difference within the *cbbL*-harboring bacterial community based on the Bray–Curtis distances. Pairwise PERMANOVA was conducted to discern differences in the *cbbL*-harboring bacterial community structure between the treatments using the pairwiseAdonis package. The Bonferroni *P*-value correction was employed as the prevailing method for multiple comparisons. Linear discriminant analysis (LDA) effect size (LEfSe) in the microeco package (Liu et al. 2021) was used to identify the *cbbL*-harboring bacterial biomarkers classified from Phylum to Genus that were more dominant in each treatment than the others based on a $P < 0.05$ from the Kruskal–Wallis sum rank test and LDA score > 3.0. The cladogram illustrated the taxonomic relationship of the identified taxa. The soil physicochemical and enzymatic effects on the *cbbL*-harboring bacterial community variations (pairwise Bray–Curtis dissimilarity) were determined by variation partitioning analysis (VPA). Spearman's correlation was used to analyze the associations between the soil properties (physicochemical and enzymatic properties) and the specific *cbbL*-harboring biomarkers using the "rcorr" function in the Hmisc package. A random forest model, which included multiple decision tree classifiers, was employed to assess the relative importance of both soil physicochemical and enzymatic properties in changing the individual biomarker.

The random forest model was constructed using the randomForest package, with the number of trees set to 500 to ensure stability and reliability of the results. A partial least squares-path modeling analysis (PLS-PM) was used to explore the direct and indirect relationships among the physicochemical variables, the enzymatic properties, and the *cbbL*-harboring bacterial community traits using the plspm package. An initial hypothesized conceptual model was considered that included all reasonable pathways among environmental factors, *cbbL*-harboring community characteristics, and enzyme activities. Non-significant pathways were then sequentially eliminated until all remaining paths were significant. The variables with loadings < 0.7 were removed, and the goodness of fit index (GOF) and R^2 were used to estimate the performance of the model.

3 Results

3.1 Grain yields and soil physicochemical properties

The rice grain yields did not significantly differ among the organic amendments, but were significantly higher than those in the control ($P < 0.05$, Table 2). Soil pH, SOC, TN, AvailN, and AvailK were all significantly increased by the organic amendments compared to the control ($P < 0.05$), but the differences among the organic amendments were not significant ($P > 0.05$). On average, the organic amendments increased SOC by 44.81% and pH by 17.71% compared to the control ($P < 0.05$). TP and AvailP showed a consistent pattern across treatments, with the highest levels in PM, followed by plant residue-derived amendments (GM and RS), and the lowest in the control. Pairwise comparisons indicated significant differences between each adjacent treatment group (i.e., PM *vs*. plant residue-derived, and plant residue-derived *vs*. control). ($P < 0.05$).

3.2 Soil enzyme activities

Organic amendments altered the enzyme activities to varying degrees (Fig. 1). All organic amendments significantly increased AP compared to the control, with RS, PM, and GM showing 1.33-, 0.19-, and 0.18-fold increases, respectively. Among the C-acquisition enzymes, BG followed a similar pattern to AP, with RS, PM, and GM showing 2.58-, 0.78-, and 0.88-fold increases compared to the control. CBH showed the following trend: RS > GM > control > PM, with significant differences between each of the adjacent amendments ($P < 0.05$). BX showed no significant variation across treatments ($P > 0.05$). For the N-acquisition enzymes, NAG was significantly higher in

Table 2 Soil physicochemical properties and *cbbL* gene copies under different organic amendments

Treatment	Grain yields ($\times 10^3$ kg/ha)	pH	SOC (g/kg)	TN (g/kg)	TP (g/kg)	TK (g/kg)	AvailN (mg/kg)	AvailP (mg/kg)	AvailK (mg/kg)	*cbbL* gene copies ($\times 10^8$/g soil)
Control	3.31 b	5.59 b	7.69 b	0.93 b	0.86 c	15.21 a	73.85 b	44.39 c	102.50 b	2.59 a
GM	5.56 a	6.61 a	10.88 a	1.34 a	1.61 b	14.77 a	108.79 a	102.95 b	211.67 a	4.44 a
PM	5.68 a	6.55 a	10.24 a	1.24 a	1.85 a	14.71 a	95.55 a	134.61 a	198.33 a	2.43 a
RS	5.63 a	6.58 a	10.81 a	1.23 a	1.45 b	13.26 b	105.89 a	88.95 b	208.33 a	4.02 a

Different letters in the same row denote significant differences among treatments ($P < 0.05$). Control, without organic fertilizer input; GM, green manure amendment; PM, pig manure amendment; RS, rice straw amendment. SOC, soil organic carbon; TN, total nitrogen; TP, total phosphorus; TK, total potassium; AvailN, available nitrogen, AvailP, available phosphorus; AvailK, available potassium

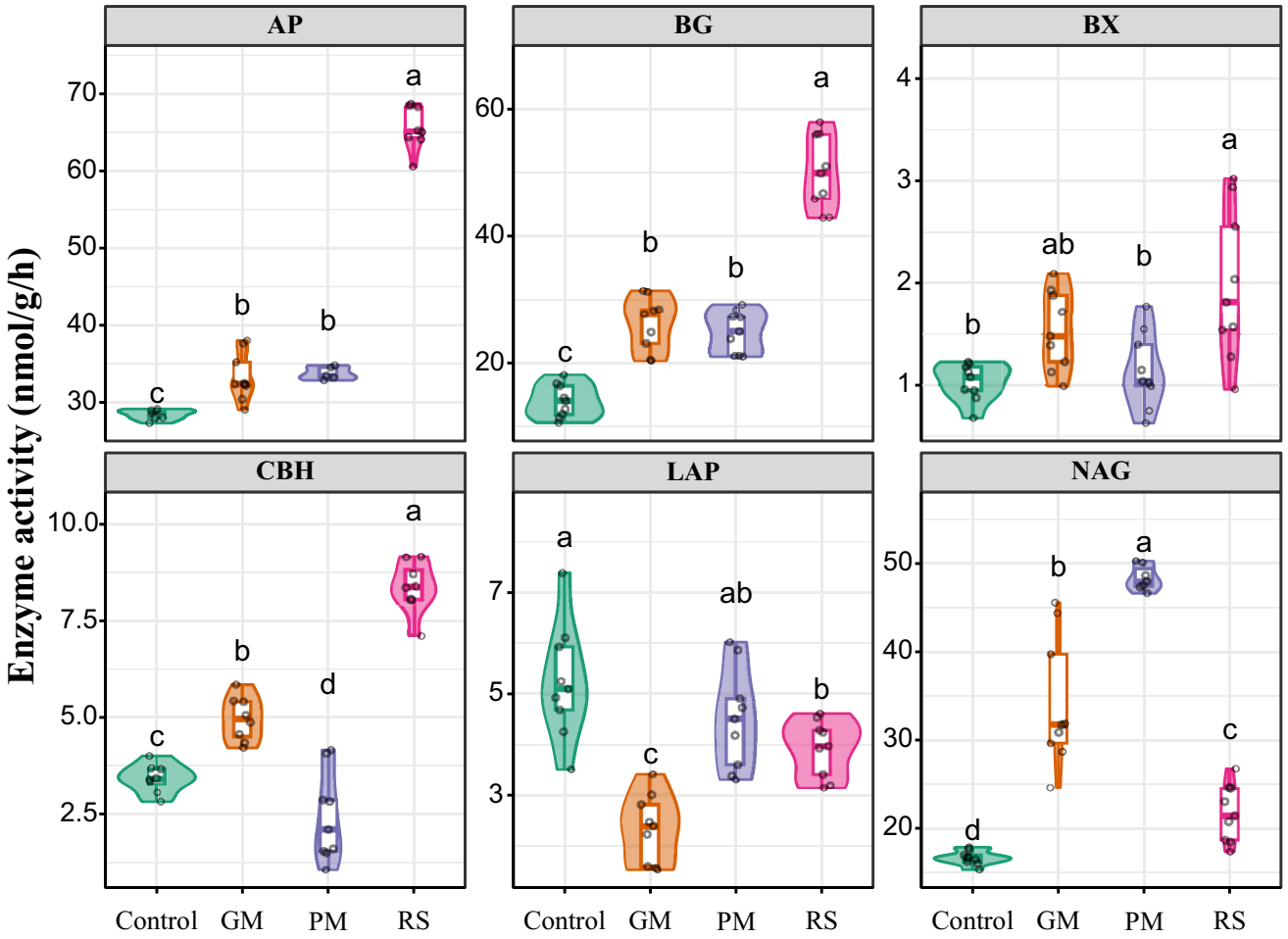


Fig. 1 Soil enzyme activities under different treatments. Different letters denote the significant differences among the treatments. Control, without organic fertilizer input; GM, green manure amendment; PM, pig manure amendment; RS, rice straw amendment. AP, acidic phosphatase; BG, β−1,4- glucosidase; BX, β-xylosidase; CBH, β-D-cellobiohydrolase; LAP, leucine aminopeptidase; NAG, β-N-acetylglucosaminidase

PM (1.9-fold increase) and GM (1.05-fold increase) compared to the control, while LAP was significantly reduced in GM (0.56-fold decrease) and RS (0.25-fold decrease) as compared to the control.

3.3 Gene abundance and community diversity of *cbbL*-harboring bacteria

The abundance of the *cbbL* gene ranged from 2.43×10^8 to 4.44×10^8 copies/g soil (Table 2). Although most organic amendments (except PM) showed an increasing trend in the *cbbL*-harboring abundance, the differences between treatments were not statistically significant ($P > 0.05$). The organic amendments did not significantly alter the Chao1 richness and the observed OTUs of the *cbbL*-harboring bacterial community (Fig. 2, $P > 0.05$). In contrast, the plant residue-derived amendments (GM and RS) significantly reduced Shannon diversity by 8.28–8.65% compared to the control ($P < 0.05$).

PCoA results showed that the first two axes explained 71.7% of the total variance in the *cbbL*-harboring bacterial community (Fig. 3). Plant residue-derived organic amendments clustered together and separated from the control along the first coordinate, while PM diverged from both the control and the plant-derived organic amendments along the second coordinate. PERMANOVA analysis (pairwiseAdonis) revealed that the plant residue-derived amendments significantly affected the soil *cbbL*-harboring community as compared to the control ($P = 0.047$), whereas PM had no significant effect ($P = 0.15$).

3.4 Composition of *cbbL*-harboring bacterial community

At the phylum level, Proteobacteria and Actinobacteria dominated the *cbbL*-harboring bacterial community, comprising average relative abundances of 81.2% and 17.6%, respectively (Fig. S1). Rare phyla included Chloroflexi

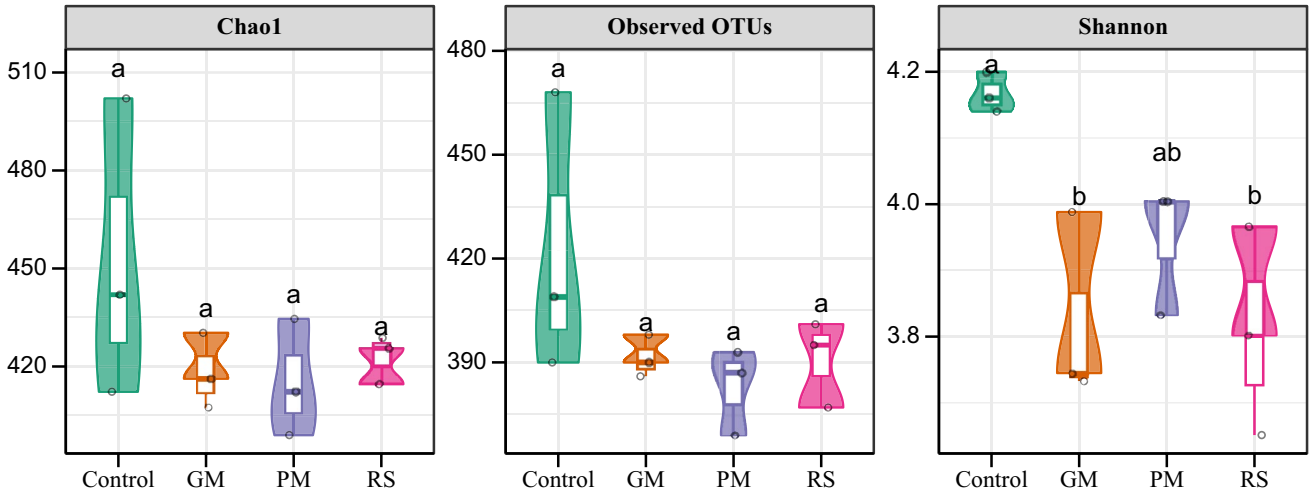
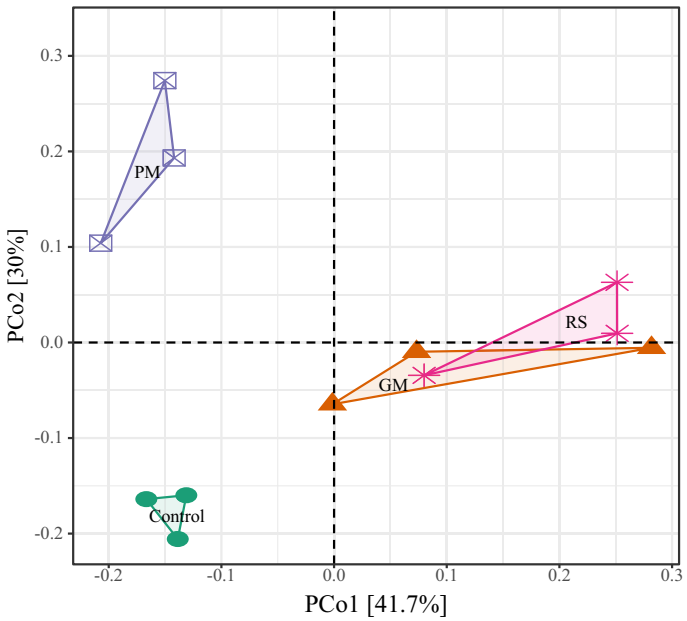


Fig. 2 Alpha-diversity indices (Chao1, Observed OTUs, and Shannon) of *cbbL*-harboring bacterial community under different treatments. Different letters above the boxes indicate significant differences among treatments ($P<0.05$). Control, without organic fertilizer input; GM, green manure amendment; PM, pig manure amendment; RS, rice straw amendment



Pairs	F Model	R^2	*P* value	*P* adjusted
control *vs.* Plant residue-derived	4.62	0.40	0.021	0.047
Control *vs.* PM	4.67	0.54	0.1	0.15
Plant residue-derived *vs.* PM	1.88	0.21	0.159	0.159

Fig. 3 Principal coordinates analysis (PCoA) based on the Bray–Curtis distance matrix illustrates the differences in the soil *cbbL*-harboring bacterial community across treatments. PairwiseAdonis is used to assess whether the differences between treatments are significantly larger than those within treatments. *P*-values are derived from a permutation test (with 999 permutations) to indicate the presence of a significant difference between treatments. Control, without organic fertilizer input; GM, green manure amendment; PM, pig manure amendment; RS, rice straw amendment. Plant residue-derived treatment includes GM and RS

(0.03%–0.05%), Cyanobacteria (0.21%–0.94%), Verrucomicrobia (0.06–0.07%), and Firmicutes (0.06–0.4%). Compared to the control, PM significantly increased the proportion of Proteobacteria by 7.60%, but decreased that of Actinobacteria by 6.84% ($P<0.05$). RS showed a trend similar to PM, but the impact was not statistically significant, with corresponding changes of 6.84% for Proteobacteria and 2.16% for Actinobacteria ($P>0.05$). The proportions of those phyla did not significantly differ between GM and the control ($P>0.05$).

LEfSe results showed the treatment-specific biomarkers in the *cbbL*-harboring bacterial community (Fig. 4). Specifically, *Bradyrhizobium* and *Acidithiobacillus* were enriched and served as the biomarkers in the control ($P<0.05$, LDA score > 3). By contrast, the biomarkers were *Afipia* in GM, *Methyloferula*, *Pseudonocardia*, and *Burkholderia* in PM,

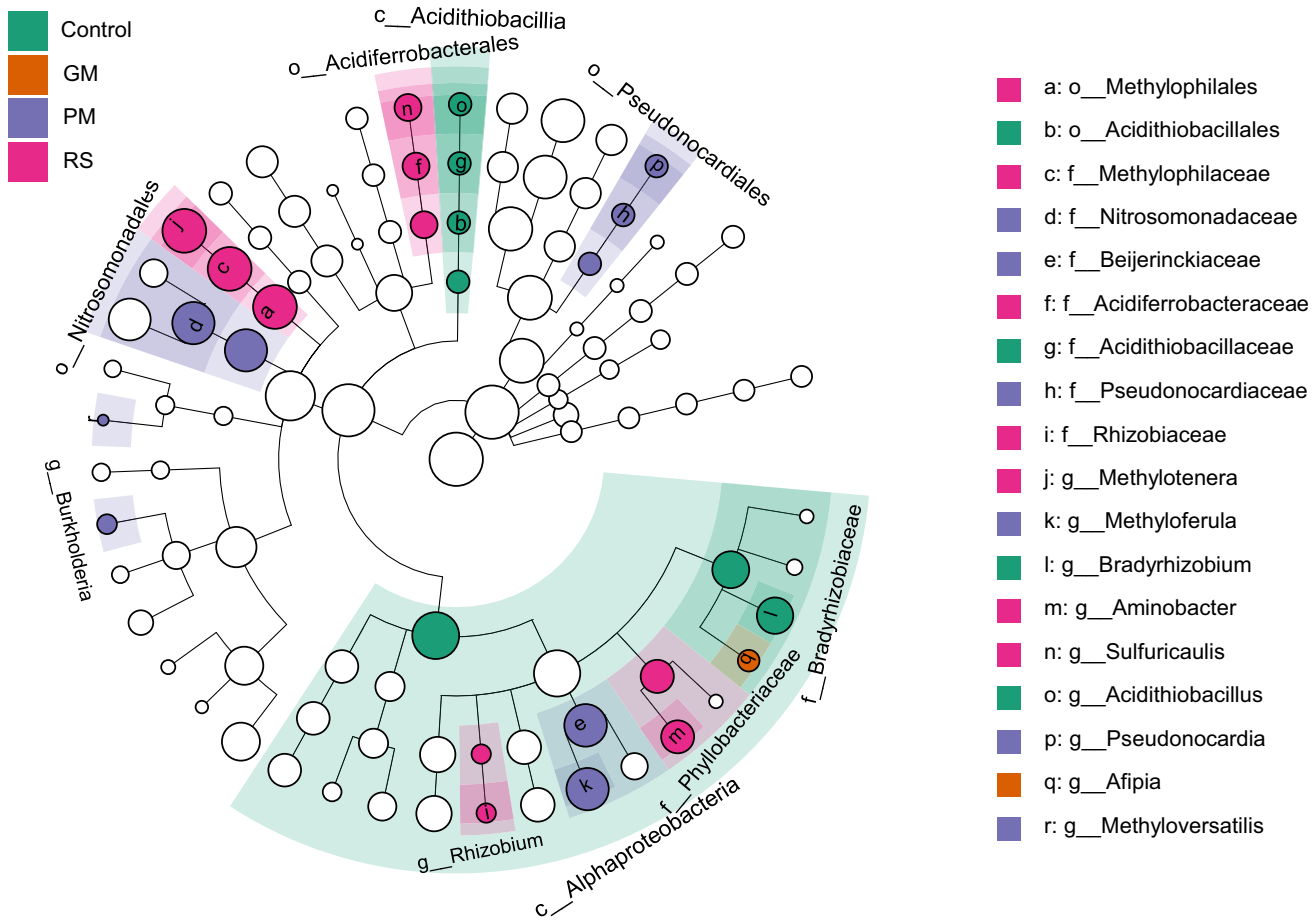


Fig. 4 The LEfSe hierarchical diagram of the *cbbL*-harboring bacterial community among the treatments. The circles are used to represent taxonomic levels ranging from phylum to genus, with each small circle at a given taxonomic level signifying a specific taxon within that level. The diameter of these small circles is proportional to the relative abundance of the taxa. The coloring principle is as follows: taxa without significant differences are uniformly colored white, while those with distinct biomarkers are colored according to their group. Specifically, green nodes denote microbial groups that are vital to the control, orange nodes highlight those that are crucial to the GM, purple nodes signify those that are important to the PM, and pink nodes indicate those that are essential to the RS. The nomenclature of the taxa, as indicated by the letters in the diagram, is presented on the right side. The prefixes c_, o_, f_, and g_ in taxonomic groups denote class, order, family, and genus, respectively

and Phyllobacteriaceae, *Rhizobium*, *Methylotenera*, and *Sulfuricaulis* in RS ($P<0.05$, LDA score > 3).

3.5 *CbbL*-harboring biomarkers associated with soil physicochemical and enzyme activities

The correlations between the *cbbL*-harboring biomarkers and the soil properties (physicochemical and enzymatic properties) were shown in Fig. 5. It was apparent that soil physicochemical properties (e.g., pH, SOC, TN, and TP) had significant negative correlations with *Acidithiobacillus* and *Bradyrhizobium* ($P<0.05$). For the soil enzyme activities, the C-acquisition enzymes (i.e., BG, CBH, and BX) were partly or entirely positively correlated with *Methylotenera* and *Sulfuricaulis* ($P<0.05$), but negatively correlated with *Burkholderia*, *Acidithiobacillus*, *Methyloferula*, and *Bradyrhizobium* ($P<0.05$). For the N-acquisition enzyme activities, NAG and LAP were found to be negatively correlated with *Acidithiobacillus* and *Afipia*, respectively ($P<0.05$). AP was negatively correlated with *Burkholderia*, but positively correlated with *Methylotenera* and *Sulfuricaulis* ($P<0.05$). Notably, these correlations exhibited minimal overlap between soil physicochemical properties and enzyme activities, implying their distinct influences on the *cbbL*-harboring biomarkers.

The random forest results revealed the importance of both soil physicochemical and enzymatic properties in shaping the *cbbL*-harboring biomarkers (Fig. 5). The physicochemical and enzymatic properties totally explained 54.27% variations in *Acidithiobacillus*, 45.73% variations in *Methyloferula*, 42.99% variations in *Burkholderia*, 35.73% variations in *Bradyrhizobium*, and 33.65% variations in *Methylotenera*. Other biomarkers, such as *Afipia* and *Sulfuricaulis*, were less influenced by the soil properties,

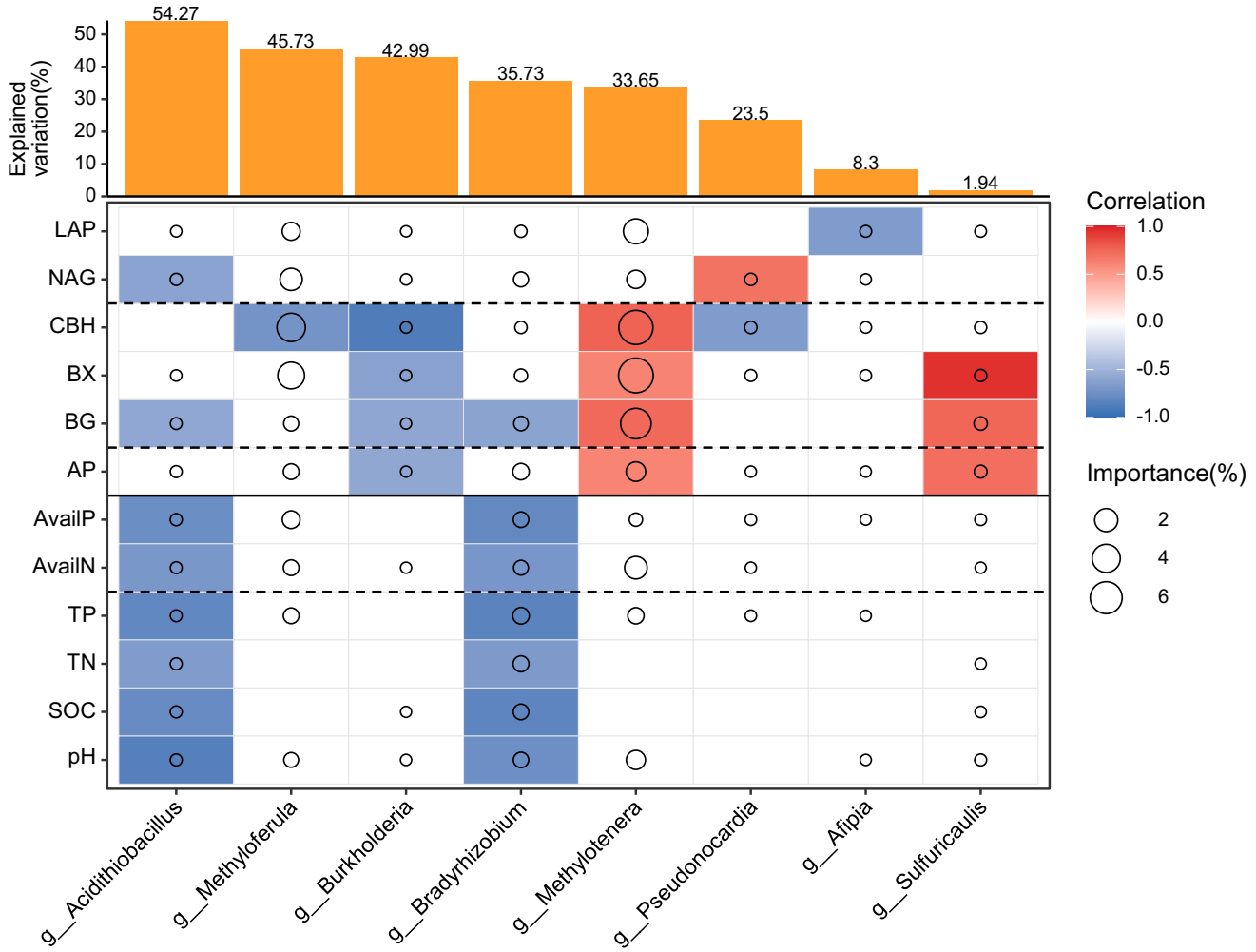


Fig. 5 The importance of soil physicochemical properties and enzyme activities to the *cbbL*-harboring lineages is highlighted. The heatmap plots displayed the Spearman correlations, with non-significant correlations left blank ($P>0.05$), and the circles over diamonds indicated the importance of soil physicochemical properties and enzyme activities on the respective *cbbL*-harboring biomarker. The total explained variations of those soil properties are summed and presented over the heatmap via the bar chart

suggesting the existence of more undetermined factors in shaping those *cbbL*-harboring biomarkers.

3.6 Ecological linkages between *cbbL*-harboring bacterial community, soil physicochemical properties, and soil enzyme activities

According to VPA results, soil physicochemical properties (i.e., SOC and pH) and enzyme activities explained 35.8% and 22.4% of the variations in the *cbbL*-harboring bacterial community, respectively, with 13.7% attributed to their combined effects (Fig. S2).

PLS-PM was subsequently employed to verify the impact of the filtered soil physicochemical properties and enzyme activities on the *cbbL*-harboring community (Fig. 6). The results demonstrated that the organic amendments positively influenced the soil SOC and pH ($P<0.05$), which then negatively influenced the Shannon index and the composition of the *cbbL*-harboring community ($P<0.05$). All tested soil enzyme activities significantly affected the *cbbL*-harboring bacterial community. In particular, P-acquisition enzyme activity positively impacted the Shannon index of the *cbbL*-harboring bacterial community ($P<0.05$), whereas C-acquisition enzyme activities negatively influenced the community diversity and N-acquisition enzyme activities negatively affected community composition ($P<0.05$).

4 Discussion

Consistent with previous findings (Chang et al. 2008), our results confirmed that the organic amendments effectively increased crop yields and improved soil physicochemical properties, although the magnitude of these effects

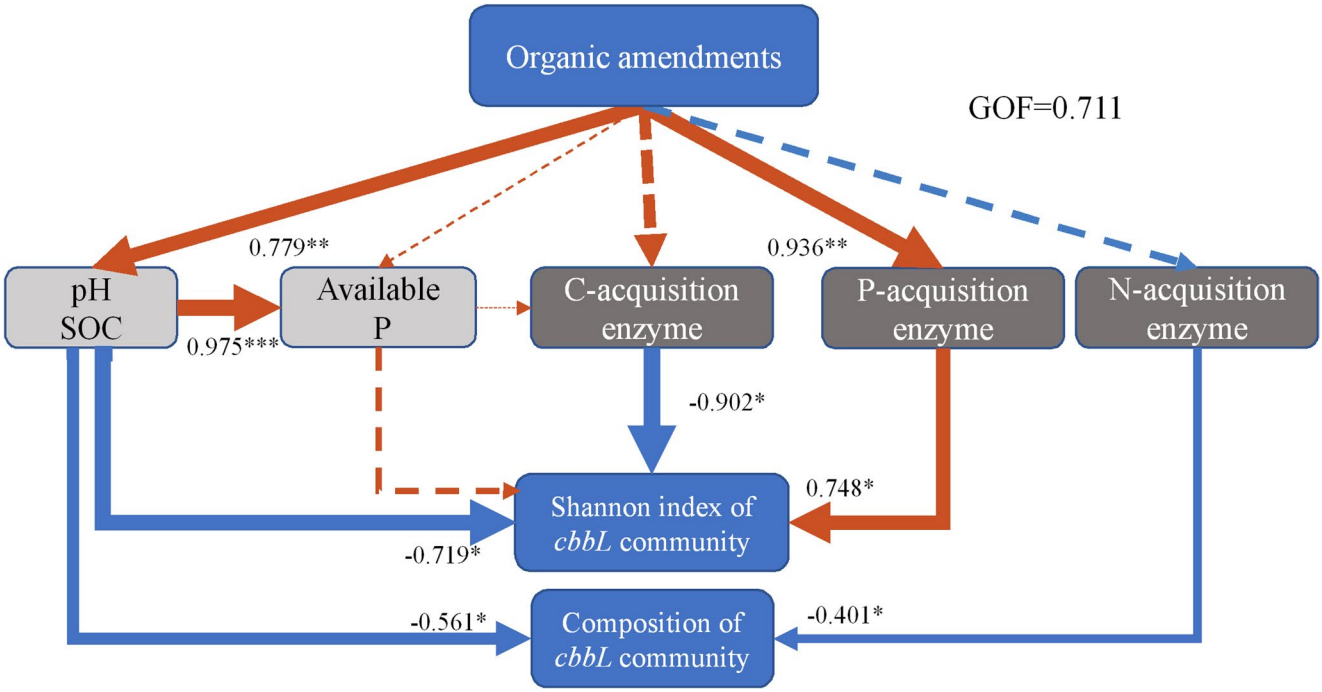


Fig. 6 PLS-PM shows the deterministic relationships among the filtered soil physicochemical properties, enzyme activities, and the *cbbL*-harboring community. The numbers adjacent to the arrows indicate standardized path coefficients, with red and blue arrows denoting positive and negative effects, respectively. Solid and dashed lines denote significant and non-significant effects, respectively. *, $P<0.05$, **, $P<0.01$, ***, $P<0.001$

varied by fertilizer types. Only AvailP and TP exhibited statistically significant differences among different organic amendments, while other parameters remained unaffected. Such features suggested that soil physicochemical properties had limited sensitivity to organic fertilizer types. We then shifted our focus to more sensitive indicators, i.e., soil enzyme activities.

4.1 Soil enzyme activities were sensitive to organic amendments

Soil enzymes, primarily secreted by soil microorganisms, are sensitive to environmental changes. In this study, we focused on the typical C-, N-, and P-acquisition enzymes as their activities are directly relevant to the decomposition of the organic matter supplied by the amendments. For the C-acquisition enzymes (BG, BX, and CBH), the most significant increase occurred in RS, followed by GM and PM, compared to the control. These enzymes are known to play active roles in depolymerizing crystalline cellulose (Sorouri and Allison 2022; Banerjee et al. 2010). The pronounced effect in the RS treatment was expected, given its high C: N ratio (Table 1) and its lignocellulosic composition, which necessitated greater C-acquisition enzymes for decomposition.

The N-acquisition enzymes (LAP and NAG) mediate protein and chitin depolymerization, respectively (Zheng et al. 2020). Both of them are involved in recalcitrant organic matter breakdown. In agricultural soil, chitin primarily originates from fungal cell walls and arthropod exoskeletons (Wieczorek et al. 2014). The observed increase in NAG following organic amendments likely reflected the enhanced fungal and arthropod growth, thereby providing more chitinous substrates for decomposition. This response was particularly pronounced in GM and PM treatments as compared to RS, likely due to their lower C: N ratios of raw materials (Table 1) that favored N mineralization and fungal growth (Organo et al. 2022). By contrast, all the organic amendments consistently reduced LAP compared to the control. This pattern might be ascribed to the abundant available N sources in the organic amendments, which decreased microbial reliance on organic matter decomposition. The opposite responses of NAG and LAP to organic amendments have also been documented previously in other agroecosystems (Zheng et al. 2020; Wang et al. 2022).

Phosphatase activity is expected to increase following the organic amendment, because soil microorganisms have to exude more phosphatase to mineralize organic P to maintain high C mineralization (Campdelacreu Rocabruna et al. 2024; Feyissa et al. 2022). In this study, the magnitude of elevated AP varied among the organic amendments, with the highest level observed in RS, followed by GM and PM. By contrast, Wang et al. (2022) report the higher AP activity in pig manure treatment compared to straw incorporation.

Such a discrepancy likely originated from variations in soil properties, crop types, and manure application rates. Notably, Zheng et al. (2020) report that while a low application rate of animal manure may suppress AP activity, a higher rate could potentially enhance it, though this effect was non-significant.

4.2 Soil *cbbL*-harboring bacterial community was changed by organic fertilization

Organic fertilizers bring in abundant and diverse organic substrates into the soil, promoting the proliferation of copiotrophic microorganisms while competitively excluding oligotrophic species (Leff et al. 2015). Given the versatile metabolic strategies of the *cbbL*-harboring microorganisms, such organic substrates' input inevitably alters their community traits. In this study, organic amendments consistently reduced the Shannon index of *cbbL*-harboring community (Fig. 3). This phenomenon might be attributed to the higher proportion of facultative autotrophs, which grew faster in substrate-rich environments and subsequently suppressed the obligate autotrophs, even leading to their disappearance in the soil. It was obvious that the plant residue-derived organic fertilizers (GM and RS) were more effective than the pig manure in altering the *cbbL*-harboring community structure. This difference likely stemmed from the higher nutrient availability in pig manure, which substantially modified the facultative-to-obligate autotroph ratio. This assumption was further supported by the decline in unique OTUs in the amended soils (Fig. S3).

Changes led by the organic amendments were also manifested in the *cbbL*-harboring taxonomic distribution. The biomarkers in the control were mainly affiliated with *Acidithiobacillus* and *Bradyrhizobium* (Fig. 4), suggesting that these taxa were suppressed by the organic amendments. *Acidithiobacillus*, as an obligate acidophile, can fix CO_2 as its sole C source under a low pH environment (Holanda et al. 2016). Some *Bradyrhizobium* strains possess both *cbbL* and *nifH* (nitrogenase iron protein) genes, enabling dual metabolic capabilities: (1) chemolithoautotrophic growth using alternative energy sources (e.g., H_2, Fe^{2+}), and (2) heterotrophic N-fixation in symbiosis with legumes (Liu et al. 2022; Wang et al. 2021b). These physiological traits enable their adaptation to oligotrophic conditions but make them susceptible to suppression in nutrient-rich environments (Li et al. 2020). Those patterns were further supported by the significant negative correlations between those biomarkers and soil physicochemical parameters, e.g., SOC, TN, TP, AvailN, and AvailP (Fig. 5).

The influence of organic amendments exhibited treatment-specific patterns on the *cbbL*-harboring bacterial community. In the plant residue-derived treatments, RS significantly enriched biomarkers from *Rhizobium, Sulfuricaulis, and Methylotenera*, while GM selectively enhanced *Afipia* (Fig. 4). These taxa exhibit diverse metabolic strategies. *Sulfuricaulis* can assimilate CO_2 by utilizing energy from the oxidation of inorganic sulfur compounds (Kojima et al. 2016). *Methylotenera* exhibits versatile metabolism, including methylamine utilization for C, N, and energy (Kalyuzhnaya et al. 2010). The co-enrichment of *Sulfuricaulis* and *Methylotenera* in RS was likely attributed to the high sulfur content in rice straw (Chivenge et al. 2020), which created a niche opportunity for sulfur-adapted taxa (Wei et al. 2025). The absence of significant correlations between these biomarkers and the measured physicochemical properties (Fig. 5) is understandable, given that the contents of sulfur- and methylamine-related compounds were not quantified in this study. The GM treatment specifically enriched *Afipia*, a metabolically versatile genus capable of both nitrification and degradation of recalcitrant organic compounds (Moosvi et al. 2005). This enrichment likely reflected the distinctive properties of Chinese milk vetch (*Astragalus sinicus*) as a green manure, which provided readily mineralizable organic N substrates that selectively favor nitrifying bacteria like *Afipia*. The PM treatment was characterized by *Methyloferula* and *Pseudonocardiales* as key biomarkers. Generally, the PM treatment had a greater potential than the straw returning treatment in increasing the emission of CH_4 in paddy soil (Takakai et al. 2020), which might provide more substrates for CH_4 oxidation by *Methyloferula*. *Pseudonocardiales* (Actinobacteria) demonstrate remarkable metabolic versatility, functioning as both obligate and facultative autotrophs capable of CO_2 fixation while simultaneously utilizing labile and recalcitrant C sources (Franco and Labeda 2014). Additionally, many strains within the order *Pseudonocardiales* are known for their ability to produce antibiotics. Such metabolic flexibility, combined with their potential antibiotic resistance, allows them to thrive in PM treatment, which typically contained abundant labile C and residual antibiotics such as tetracyclines and sulfonamides.

4.3 Factors shaping the *cbbL*-harboring bacterial community

Among the soil physicochemical properties, only pH and SOC showed substantial impacts on the *cbbL*-harboring bacterial community traits (Fig. 6). Soil pH could influence the *cbbL*-harboring microorganisms in several ways. First, it changed the ecological niches for microorganisms. Some biomarkers, such as *Acidithiobacillus,* prefer acidic environments. As the organic amendments generally enhanced the soil pH in our study, which would suppress the growth of those lineages, and subsequently the whole community.

Second, soil pH modulates the dissolved concentration and ionization of CO_2 in the soil, thus affecting the CO_2 and HCO_3^- utilization by *cbbL*-harboring autotrophs (Zheng et al. 2022).

For the soil enzyme activities, the C- and N-acquisition enzymes exhibited significant negative impacts on the *cbbL*-harboring bacterial community, either the Shannon index or the community composition, depending on the enzymes. This might stem from the fact that a large proportion of the organic materials served as the direct C- and N-sources for microorganisms in the organic amendments, and the C- and N-acquisition enzymes are responsible for breaking down those materials. The diverse small-molecular substrates derived from this process would boost soil heterotrophic respiration (Ning et al. 2023). The enhanced heterotrophic activity would have a competitive advantage over autotrophic CO_2-fixing microorganisms (Nemergut et al. 2007), and thus the decreased Shannon index or composition of *cbbL*-harboring bacterial community. In support, Zhou et al. (2024a) report the negative association between these enzymes and *cbbL* gene abundance in the straw-amended soil. An opposite trend in the enzyme activities was that the P-acquisition enzyme positively influenced the *cbbL*-harboring bacterial community. P is essential in the Calvin cycle as a constituent of key intermediates like sedoheptulose-7-phosphate and D-fructose 1,6-bisphosphate. The limitation in P source would constrain CO_2 fixation in Calvin-cycle-dependent autotrophs (Zheng et al. 2022), which explains the significant positive influence of phosphatase activity on the *cbbL*-harboring bacterial community. Although soil available P also exhibited a positive association with this community, the effect was non-significant. This discrepancy may arise from the fact that the available P pool reflects the balance between competing plant and microbial sinks, as well as inputs from organic matter mineralization, a process partly controlled by phosphatase activity (Margalef et al. 2017). Consequently, phosphatase activity showed a stronger direct association with soil microbial community (including *cbbL*-harboring microorganisms) than did available P concentration.

5 Conclusions

In summary, our findings demonstrated that the long-term organic amendments significantly improved the soil physicochemical properties by increasing soil pH, SOC, TN, TP, etc. The soil C-, N-, and P- acquisition enzyme activities responded variably to the organic amendments. The organic amendments did not significantly change the *cbbL* gene abundance, however, they reduced the Shannon index and shifted the composition of the *cbbL*-harboring community. Those effects were particularly pronounced under the plant residue-derived amendments (GM and RS). The amendment-specific biomarkers were detected, including *Afipia* in GM, *Pseudonocardia* in PM, as well as *Methylotenera* and *Sulfuricaulis* in RS. PLS-PM analysis further revealed that soil pH, SOC, soil C- and N-acquisition enzyme activities negatively influenced the *cbbL*-harboring community, whereas P-acquisition enzyme activity had a positive effect. Taken together, our results revealed the key physicochemical and enzymatic drivers governing the *cbbL*-harboring bacterial community, advancing our mechanistic understanding of its assembly.

There were several limitations that should be acknowledged. First, our findings were based on a single sampling time point, which might not fully reflect the dynamic changes in soil physicochemical properties, enzyme activities, and *cbbL*-harboring bacterial community, particularly in paddy soil that is frequently subjected to wet-dry transitions. Future studies incorporating multiple sampling points throughout the rice growth season would provide a more comprehensive understanding of these temporal variations. Additionally, although we identified biomarkers under different organic amendments, direct evidence of microbial CO_2 fixation would require advanced approaches such as DNA-based stable isotope probing (DNA-SIP) combined with multi-omics (metagenomics/transcriptomics). Applying these methodologies is expected to provide deeper insights into the regulatory mechanisms governing CO_2 fixation in agricultural soil ecosystems.

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Data Availability Data are available upon request to the first author.

Declarations

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.

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