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RESEARCH ARTICLE

# Effects of integrated rice-frog farming on soil bacterial community composition

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## ABSTRACT

Rice-fish integrated farming model has become one of the main directions for sustainable agricultural development due to its good ecological and environmental effects and ability to produce green rice (*Oryza sativa* L.) that meets the market's food safety needs. Its study characterizes soil bacterial community structure in this model for the improvement of planting and breeding technology and realization of ecological regulation. The community structure of soil microorganisms was compared between two models of rice-frog (black-spotted pond frog; *Pelophylax nigromaculatus* Hallowell, 1861) co-cropping (DW) and rice monoculture (DD) at tillering stage, full heading stage and maturity stage, to accumulate data for research on ecology of rice-frog integrated farming and provide a theoretical basis for optimization of this production technology. Main bacterial phyla occurring in rhizosphere soil of rice fields in both models under three stages were *Proteobacteria*, *Chloroflexi*, *Acidobacteriota*, *Bacteroidota*, *Firmicutes*, *Actinobacteriota*, *Nitrospirota* and *Desulfobacterota*. Compared to DD, DW group had more *Actinobacteriota* in the dominant phyla at maturity and less *Desulfobacterota* throughout the growth period. The main dominant genera were *norank_f_Anaerolineaceae*, *norank_f_norank_o_norank_c_Thermodesulfovibrionia*, *norank_f_norank_o_SBR1031*, *norank_f_norank_o_Vicinamibacterales*, *unclassified_k_norank_d_Bacteria* and *Thiobacillus*. There were five categories with significant difference, *norank_f_Anaerolineaceae*, *norank_f_norank_o_SBR1031*, *norank_f__norank_o_Vicinamibacterales*, *Thiobacillus* and *unclassified_k_norank_d_Bacteria*; all were higher in relative abundance in DW than in DD group. It indicates that rice-frog crop model promotes the growth of these five types of bacteria, which have a boosting effect on rice growth. The bacterial Chao index and Shannon index of rhizosphere soil of rice field increased after introduction of frog in DW group. The two models showed less variation in soil bacterial community structure in root system from full heading to maturity stage, and co-cropping model showed less variation compared to rice monoculture.

**Key words:** *Oryza sativa*, *Pelophylax nigromaculatus*, relative abundance, rice-frog farming, soil bacterial, tiller stage.

## INTRODUCTION

Soil microorganisms, as the main participants of the ecosystem processes, actively participate in all aspects of the C and N cycle, such as C and N mineralization, nitrification, denitrification and other processes (Edwards et al., 2015). And through its own metabolism and turnover, it promotes nutrient cycling and plant effectiveness, becoming an important source of effective soil nutrients. Soil microbial diversity and function are the most sensitive indicators of soil quality (Zak et al., 1994). For the study of soil microbial community structure, the high-throughput sequencing technology used obtains a large number of gene sequences and can detect non-culturable and trace microorganisms, which has become an important tool for studying the diversity of microbial communities (Han et al., 2016; Zhang et al., 2016). Rhizosphere is the direct interface of material and energy conversion between plant roots and soil (Prashar et al., 2014; Zhang et al., 2018), rhizosphere microorganisms are closely related to plant health, pest and disease defense and yield (Li et al., 2007). Some studies have shown that each plant has a specific rhizosphere microbiome (Zhang et al., 2017a), and the rhizosphere soil microbial diversity of different varieties of oleander differed (Zhang et al., 2017b). The rice (*Oryza sativa* L.) variety used in this experiment is giant rice, and it is important to study the composition and diversity of the rhizosphere soil microbial community of giant rice to ensure the production of giant rice and promote the development of giant rice industry.

Frog is a native amphibian in rice field, and introducing frog into rice field is a more ecological and natural rice planting mode. Many studies have shown that the introduction of frogs can effectively reduce the populations of rice pests such as rice lice, and improve the nutrient status of the soil (Chang et al., 2021). To a certain extent, it increases soil microbial biomass and soil dehydrogenase, peroxidase, acid phosphatase and other soil enzymes, thus promoting rice growth and seed yield of rice. In addition, the rice-frog crop model also has the potential to reduce greenhouse gas $CH_4$ emissions. Studies have shown that a variety of paddy ecological farming models have the ability to influence the structure of soil microbial communities, the soil microbial population and microbial biomass C in the rice-duck crop model were significantly increased (Dexin et al., 2005). And the integrated rice-loach farming has certain influence on soil microbial community structure (Zhao et al., 2021). Little research has been reported on the changes in soil microbial community structure of giant rice paddies with rice growth. This experiment compared the diversity characteristics of soil bacteria in the rhizosphere under two modes of rice-frog crop and paddy monoculture, and explored the dynamic changes of soil microbial community structure and composition in the rhizosphere of rice under the two cropping modes at different periods of rice growth, in order to reveal the internal relationship among frog metabolism, rice growth and soil microbial community structure.

## MATERIALS AND METHODS

### Experimental field description

The experimental site is located in Renshou City (29°51′15.58″ N, 104°12′41.56″ E), Sichuan Province, China. This area belongs to the central subtropical humid monsoon climate zone with an average annual temperature of 16.8 °C, an average annual rainfall of 1153.7 mm, an average annual number of rainfall days of 159 d, and an annual frost-free period of about 310 d.

The experimental field is the first year of implemented rice (*Oryza sativa* L.)-frog integrated farming. The experiment was designed with two treatments, the rice-frog integrated farming group (DW) and the rice monoculture group (DD), with three replicates of each treatment (six fields), each with an area of 667 $m^2$ and a 1.5 m wide ridge between the fields. Each rice-frog integrated field is surrounded by 1 m wide food table, of which the inner side is a 1 m wide and 50 cm deep ring ditch, and the central rice planting area of about 450 $m^2$. Each field is surrounded by an anti-escape net, which covers food table with one side 30 cm below the ring ditch and the other side 1 m above the food table. The rice, giant rice 'Jufeng No. 5', was transplanted on 20 May with a plant spacing of 30 cm × 30 cm. Rice harvesting was carried out on 29 September.

The breed of frog is the black-spotted pond frog (*Pelophylax nigromaculatus* Hallowell, 1861). About 100 000 tadpole fries were placed in each field on 5 April, and 2.5 ± 0.5 kg powdered forage was fed to each field every day, and the survival rate of tadpoles was about 30%. Tadpoles begin to metamorphose in mid to late May. During the metamorphosis period, tadpoles rely on the absorption of the tail as a source of nutrition and do not need to feed. After metamorphosis into juvenile frogs, feed small pellets with 40% animal protein according to 2%-3% of the frog's body weight. When they grow into adult frogs after 40 d, large pellets were fed with 38% animal protein according to 2%-3% of their body weight in the morning and evening. Fertilizer was not applied to rice-frog integrated fields, while rice monoculture fields were fertilized with base fertilizer and topdressing. The first time before rice transplanting, 450 kg $ha^{-1}$ potassium sulfate type compound fertilizer (N:P:K = 24%:9%:18%) was applied, and the second time 40 d after rice transplanting, 90 kg ha$^{-1}$ were applied.

### Soil sampling and measurements

Soil samples were collected on 1st April (before planting and raising), 27 June (tiller stage), 27 August (full heading stage) and 27 September (maturity stage) in 2021 respectively. In the tiller stage, DW group is recorded as aa1 and DD group is recorded as aa2. In the full heading stage, DW group is recorded as aa3 and DD group is aa4. In the maturity stage, DW group is recorded as aa5 and DD group is aa6. The five-point sampling method was used to select five points in the four corners and center of each paddy field, taking soil around the root system at 10-20 cm depth, then mixing the soil from the five points into one sample, with sample numbers DW1, DW2, DW3 and DD1, DD2, DD3. Samples were taken back to the laboratory in 5 mL centrifuge tubes and stored at -80 °C for microbial diversity analysis. In addition, take five points of soil from 10 to 20 cm and mix well, remove plant and animal residues, weeds and stone impurities and then use for soil physical and chemical property testing. Soil physical and chemical property, including soil pH, organic matter (OM), total N (TN), total P (TP), total K (TK), available P (AP), available K (AK) and alkali hydrolyzed N (AN), were measured with experimental methods according to Bao (2000).

### Soil DNA extraction, PCR amplification and sequencing

The soil samples were weighed (0.3 g), extracted with the Rapid Soil Genomic DNA Extraction Kit (Sangon Biotech, Shanghai, China) and detected by 1% agarose gel electrophoresis, then stored at -20 °C in a refrigerator. The common primers 515F (5′-GTGCCAGCMGC-CGCGG-3′) and 907R (5′-CCGTCAATTCMTTTRAGTTT-3′) were used to amplify the high variant region V3 ~ V4 of 16S rRNA gene. Each sample was repeated three times, after which the PCR products were recovered by gel cutting using the AxyPrep DNA Gel Recovery Kit (Axygen Scientific, Union City, California, USA), eluted with Tris_HCl; detected by 2% agarose electrophoresis. The PCR products were detected and quantified by QuantiFluor, -ST Blue Fluorescence Quantification System (Promega Corporation, Madison, Wisconsin, USA), after the concentrations of each sample were normalized, mixed and sequenced. The sequencing platform was the MiSeq PE300 platform from Illumina (San Diego, California, USA).

### Data processing and analysis

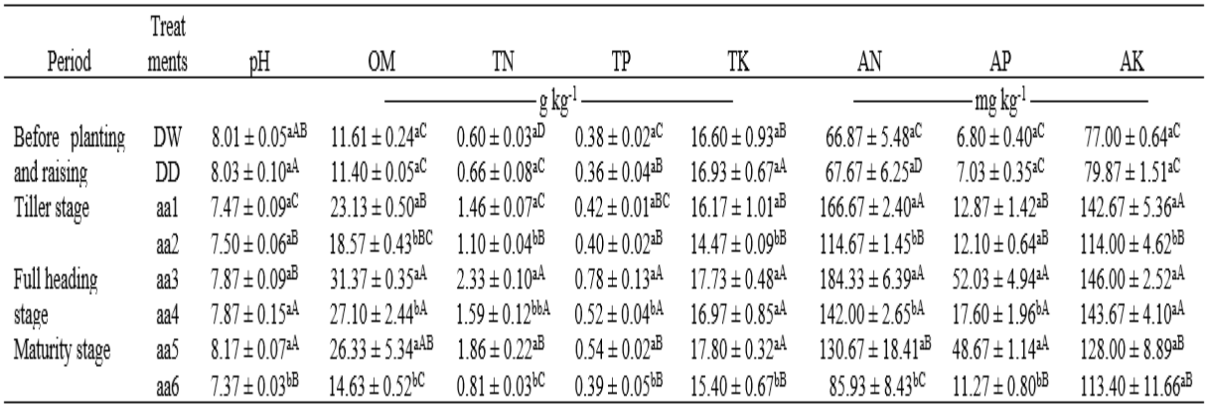
After 16S rRNA MiSeq sequencing, the quality of reads was quality-controlled filtered by using the software Trimmomatic and FLASH, and data filtering was completed after the operation process of removing low-quality bases and splice contamination sequences to obtain high-quality target sequences that can be used for subsequent analysis. The follow-up bioinformatics operations were done using USEARCH and mothur softwares, the source of the comparison data was Silva’s bacterial database, and the data statistics and graphing were done using R language. The differences in soil physicochemical property data, microbial diversity index data and relative abundance of major genera in the paddy fields under the two models were analyzed by one-way ANOVA and Duncan method using SPSS 16.0 software (IBM, Armonk, New York, USA), and $P < 0.05$ indicated significant differences.

## RESULTS AND DISCUSSION

### Physical and chemical properties

There was nonsignificant difference ($P > 0.05$) in the physical and chemical properties of the soil between the two modes before the start of the experiment (Table 1). Compared with the DD group, the DW group had significantly higher soil total N (TN) and alkaline N (AN) content throughout the growth period, higher soil total P (TP) and available P (AP) content at the full heading and maturity stages, and higher soil available (AK) content at the tiller stage. From the tillering stage to the full heading stage, soil pH, organic matter (OM), total N (TN) and total P (TP) increased significantly in both groups, and from the full heading stage to the maturity stage, soil TN and TP, alkaline N content decreased significantly in both groups.

**Table 1.** Soil properties of paddy field in two models. The same lowercase letters mean nonsignificant differences ($P > 0.05$) in this indicator between the two modes during the same period, while different lowercase letters mean significant differences ($P < 0.05$) between two modes. The same uppercase letters mean nonsignificant differences ($P > 0.05$) in the same mode at different times, while different uppercase letters mean significant differences ($P < 0.05$) in the same mode at different times. OM: Organic matter; TN: total N; TP: total P; TK: total K; AN: alkali hydrolyzed N; AP: available P; AK: available K; DW: rice-frog co-cropping; DD: rice monoculture; aa1, aa3, aa5: average values of DW group of three fields for tillering, full heading and maturity stages, respectively; aa2, aa4, aa6: average values of DD group of three fields for tillering, full heading and maturity stages, respectively.



| Period | Treatments | pH | OM | TN | TP | TK | AN | AP | AK |
|---|---|---|---|---|---|---|---|---|---|
| | | | g kg$^{-1}$ | | | | mg kg$^{-1}$ | | |
| Before planting and raising | DW | $8.01 \pm 0.05^{aAB}$ | $11.61 \pm 0.24^{aC}$ | $0.60 \pm 0.03^{aD}$ | $0.38 \pm 0.02^{aC}$ | $16.60 \pm 0.93^{aB}$ | $66.87 \pm 5.48^{aC}$ | $6.80 \pm 0.40^{aC}$ | $77.00 \pm 0.64^{aC}$ |
| | DD | $8.03 \pm 0.10^{aA}$ | $11.40 \pm 0.05^{aC}$ | $0.66 \pm 0.08^{aC}$ | $0.36 \pm 0.04^{aB}$ | $16.93 \pm 0.67^{aA}$ | $67.67 \pm 6.25^{aD}$ | $7.03 \pm 0.35^{aC}$ | $79.87 \pm 1.51^{aC}$ |
| Tiller stage | aa1 | $7.47 \pm 0.09^{aC}$ | $23.13 \pm 0.50^{aB}$ | $1.46 \pm 0.07^{aC}$ | $0.42 \pm 0.01^{aBC}$ | $16.17 \pm 1.01^{aB}$ | $166.67 \pm 2.40^{aA}$ | $12.87 \pm 1.42^{aB}$ | $142.67 \pm 5.36^{aA}$ |
| | aa2 | $7.50 \pm 0.06^{aB}$ | $18.57 \pm 0.43^{bBC}$ | $1.10 \pm 0.04^{bB}$ | $0.40 \pm 0.02^{aB}$ | $14.47 \pm 0.09^{bB}$ | $114.67 \pm 1.45^{bB}$ | $12.10 \pm 0.64^{aB}$ | $114.00 \pm 4.62^{bB}$ |
| Full heading stage | aa3 | $7.87 \pm 0.09^{aB}$ | $31.37 \pm 0.35^{aA}$ | $2.33 \pm 0.10^{aA}$ | $0.78 \pm 0.13^{aA}$ | $17.73 \pm 0.48^{aA}$ | $184.33 \pm 6.39^{aA}$ | $52.03 \pm 4.94^{aA}$ | $146.00 \pm 2.52^{aA}$ |
| | aa4 | $7.87 \pm 0.15^{aA}$ | $27.10 \pm 2.44^{bA}$ | $1.59 \pm 0.12^{bbA}$ | $0.52 \pm 0.04^{bA}$ | $16.97 \pm 0.85^{aA}$ | $142.00 \pm 2.65^{bA}$ | $17.60 \pm 1.96^{bA}$ | $143.67 \pm 4.10^{aA}$ |
| Maturity stage | aa5 | $8.17 \pm 0.07^{aA}$ | $26.33 \pm 5.34^{aAB}$ | $1.86 \pm 0.22^{aB}$ | $0.54 \pm 0.02^{aB}$ | $17.80 \pm 0.32^{aA}$ | $130.67 \pm 18.41^{aB}$ | $48.67 \pm 1.14^{aA}$ | $128.00 \pm 8.89^{aB}$ |
| | aa6 | $7.37 \pm 0.03^{bB}$ | $14.63 \pm 0.52^{bC}$ | $0.81 \pm 0.03^{bC}$ | $0.39 \pm 0.05^{bB}$ | $15.40 \pm 0.67^{bB}$ | $85.93 \pm 8.43^{bC}$ | $11.27 \pm 0.80^{bB}$ | $113.40 \pm 11.66^{aB}$ |

## Overview of MiSeq sequencing and diversity analysis

The average total sequence numbers obtained from MiSeq sequencing of rice-frog co-cropping and rice monoculture root soils were $4.21 \times 10^5 \pm 6.27 \times 10^3$ and $3.66 \times 10^5 \pm 1.04 \times 10^4$ at tiller stage, $2.44 \times 10^5 \pm 1.84 \times 10^4$ and $2.21 \times 10^5 \pm 9.32 \times 10^3$ at full heading stage, $2.62 \times 10^5 \pm 2.44 \times 10^4$ and $2.00 \times 10^5 \pm 1.41 \times 10^4$ at maturity stage. After removing the primers, ambiguous bases and sequences with chimeras, the total effective sequence number finally obtained was 5 938 318, with sequence lengths ranging from 360 to 400 and average fragment length of 376 bp. At the operational taxonomic unit (OTU) classification level of 97% similarity, the sequencing coverage reached about 98%, reflecting that the sequencing results were sufficiently representative of the real situation of the microorganisms in the samples. In addition, to ensure the same sequencing depth of the samples used, we selected 172 155 (i.e., the one with the lowest number of valid sequences among all samples) valid sequences from each sample for normalization and used them for the next data analysis.

The results of soil microbial diversity analysis of rice roots under two modes showed that the Chao index and Shannon index of soil bacteria in the root system of the rice-frog co-cropping mode were significantly higher than the rice monoculture mode in all periods ($P < 0.05$) (Figure 1). The Chao index and Shannon index of soil bacteria in both models were highest at the tiller stage and significantly higher than those at the full heading stage and maturity stage ($P < 0.05$), and there was nonsignificant change in the two groups from the full heading stage to maturity stage. It indicates that farmed frogs can increase the diversity and richness of soil microorganisms in paddy fields, and the changes of soil microorganisms in paddy fields are more obvious at the tiller stage.

Among the 22 086 OTUs obtained at 97% similarity, 4612 OTUs were shared by six groups, accounting for 20.88% of the total OTUs, indicating that there were some differences in the composition of microbial species among the groups (Figure 2). The OTU species specific to the DW group were higher than those in the DD group in all three periods, indicating that frog farming can increase soil microbial richness in rice fields. Both the DW group and the DD group had the most unique OTU species at the tiller stage, accounting for 13.15% and 8.35%, respectively. The number of unique OTU species in both groups decreased sharply at the full heading stage, and did not change much at the maturity stage.

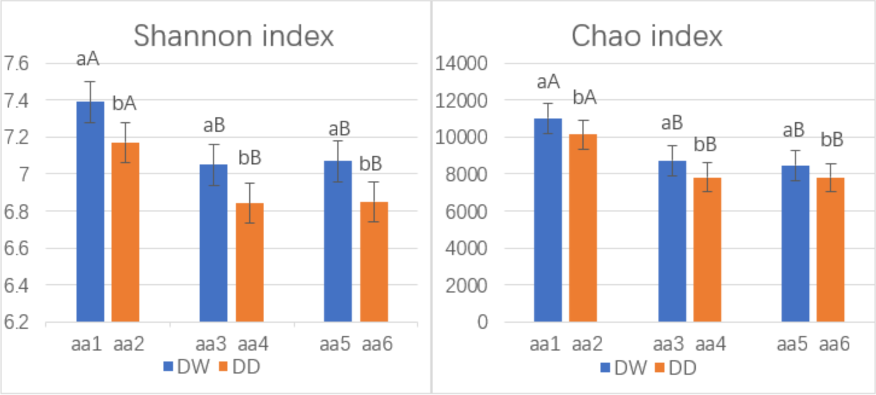


**Figure 1.** Diversity index of rhizosphere soils in paddy field in two models. The same lowercase letters mean nonsignificant differences (P > 0.05) in this indicator between the two modes during the same period, while different lowercase letters mean significant differences (P < 0.05) between two modes. The same uppercase letters mean nonsignificant differences (P > 0.05) in the same mode at different times, while different uppercase letters mean significant differences (P < 0.05) in the same mode at different times. DW: Rice-frog co-cropping; DD: rice monoculture; aa1, aa3, aa5: average values of DW group of three fields for tillering, full heading and maturity stages, respectively; aa2, aa4, aa6: average values of DD group for tillering, full heading and maturity stages, respectively.

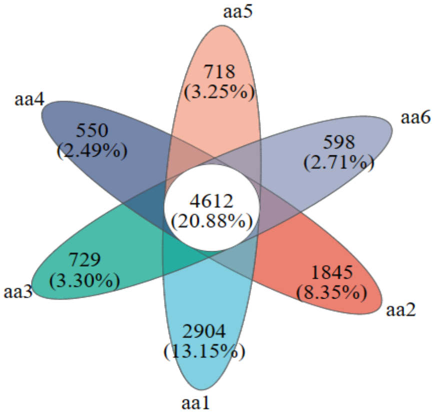


**Figure 2.** The statistical result of operational taxonomic unit (OTU) of samples aa1, aa2, aa3, aa4, aa5 and aa6. aa1, aa3, aa5: Average values of rice-frog co-cropping group of three fields for tillering, full heading and maturity stages, respectively; aa2, aa4, aa6: average values of rice monoculture group of three fields for tillering, full heading and maturity stages, respectively.

### Analysis of bacterial community structure in rhizosphere soil of different patterns

In terms of microbial composition, the dominant phylum (relative abundance > 5%) contained in the rice rhizosphere soil was relatively similar in the two models (Figure 3). The dominant phylum shared by the two groups at the tiller stage were *Proteobacteria*, *Chloroflexi*, *Acidobacteriota*, *Bacteroidota*, *Firmicutes*, and *Actinobacteriota*, and the DD group with more *Desulfobacterota*, accounting for 77.86% and 77.35% of the total sequence numbers. The dominant phylum in the DW group at the full heading stage were *Proteobacteria*, *Chloroflexi*, *Acidobacteriota*, *Bacteroidota* and *Nitrospirota*, in the DD group were *Proteobacteria*, *Chloroflexi*, *Acidobacteriota*, *Bacteroidota* and *Nitrospirota*. The dominant phylum in the DW group at maturity was *Proteobacteria*, *Chloroflexi*, *Acidobacteriota*, *Bacteroidota*, *Nitrospirota*, *Desulfobacterota*. *Actinobacteriota*, *Nitrospirota*, and in the DD group were *Proteobacteria*, *Chloroflexi*, *Acidobacteriota*, *Bacteroidota*, *Nitrospirota*, and *Desulfobacterota*. *Proteobacteria* was the highest relative abundance phylum among all samples at tiller and full heading stage with an average relative abundance of 22.40% (highest 25.78% ± 1.56% [aa1] and lowest 19.13% ± 1.75% [aa2]). The highest relative abundance of the two treatment groups at maturity became *Chloroflexi*, with 22.13% ± 1.89% of *Chloroflexi* in the DW group and 22.99% ± 1.69% of *Chloroflexi* in the DD group. From the results, it is clear that *Firmicutes* and *Actinobacteriota* decreased and *Nitrospirota* increased with the growth of rice.

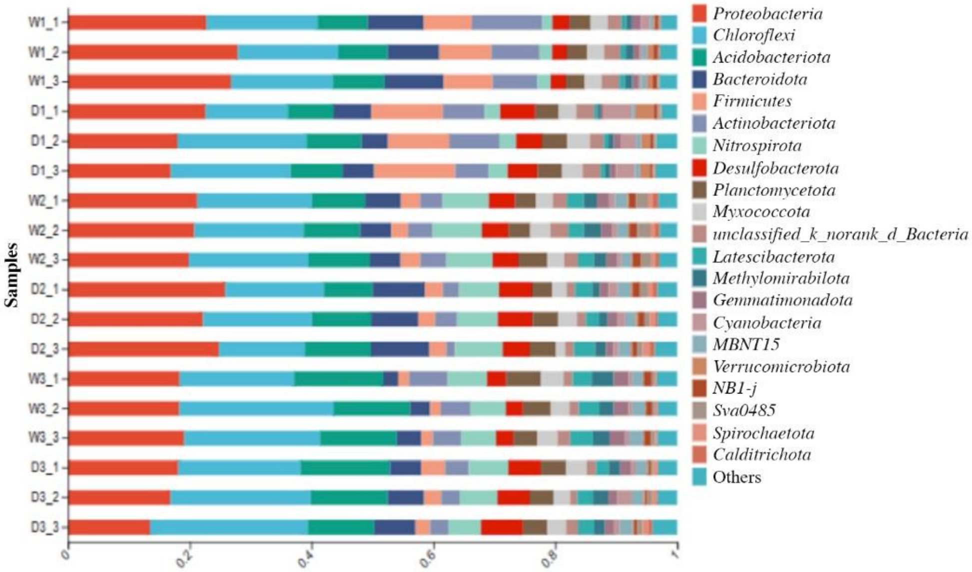


**Figure 3.** Percentages of the major phyla of rhizosphere soils in paddy field in two models.

Comparison of the relative abundance of 16S rRNAs sequences in the eight major phyla showed that there were large differences in different samples of each group. The relative abundance of *Actinobacteriota* was higher in the rice-frog co-crop field than in the rice monoculture field at all periods, and was significant ($P < 0.05$) at the tiller and maturity stages. In contrast, the relative abundance of *Desulfobacterota* in rice-frog co-cropped fields was lower than that in rice monoculture fields, and was significant ($P < 0.05$) at tiller and maturity stages. From the rice growing stage, the relative abundance of *Firmicutes* and *Actinobacteriota* was significantly lower and *Nitrospirota* was higher ($P < 0.05$).

## Genus level analysis

At the genus level, a large proportion of the sequences was not classified (41.11%-55.96%). As with other MiSeq sequencing results, many special genera (e.g., genera followed by unclassified) appeared in this sequencing, and Albertsen et al. (2012) speculated that this category of unculturable genera may contain bacteria that have a significant role in the physicochemical properties of the soil and in the growth process of rice. Among the 1787 taxonomic units of bacteria obtained at the genus level, 954 taxonomic units coexisted in two treatment groups, and they contained 52.10% of the total sequences, with only a small fraction of the sequences in a single treatment group (Table 2). The top 55 microbial populations in each sample category were ranked (Figure 4).

*Norank_f__Anaerolineaceae* was the genus with the highest average relative abundance among soils, with 400 OTUs assigned to this genus in the OTU classification, and its relative abundances in aa1-aa6 were 5.01% ± 0.22%, 4.92% ± 0.34%, 6.47% ± 0.13%, 4.96% ± 0.31%, 8.06% ± 0.53%, and 6.32% ± 0.93% respectively. The relative abundance of the bacterium in the root system of the rice-frog co-crop soil was higher than that of the rice monoculture, and it was significant ($P < 0.05$) at the tiller and full heading stages. From the growth period of rice, the relative abundance of this bacterium was significantly higher from the tiller stage to the maturity stage. *Norank_f_norank_o_norank_c_Thermodesulfovibrionia* and *norank_f_norank_o_SBR1031* are common groups of bacteria in the soil (Duan et al., 2009). Similarly, in this study, these two groups were also the genera with higher relative abundance among all samples. The relative abundance of *norank_f_norank_o_norank_c_Thermodesulfovibrionia* in the DW group was higher at the full heading stage and lower at the tiller and maturity stages compared to the DD group. The relative abundance of *norank_f_norank_o_SBR1031* in the DW group was higher at tiller and full heading stage, significant at full heading stage ($P < 0.05$), and no difference at maturity stage.

**Table 2.** Percentages of rhizosphere soils common genera's corresponding sequences and the number of unique genera in two models. DW: Rice-frog co-cropping; DD: rice monoculture; aa1, aa3, aa5: average values of DW group of three fields for tillering, full heading and maturity stages, respectively; aa2, aa4, aa6: average values of DD group of three fields for tillering, full heading and maturity stages, respectively.

| | Numbers of genera | Percentage in common genera | Numbers of unique genera |
|---|---|---|---|
| aa1 | 1565 | 60.9 | 75 |
| aa2 | 1552 | 61.5 | 57 |
| aa3 | 1373 | 69.5 | 15 |
| aa4 | 1348 | 70.8 | 10 |
| aa5 | 1274 | 74.9 | 10 |
| aa6 | 1251 | 76.3 | 7 |

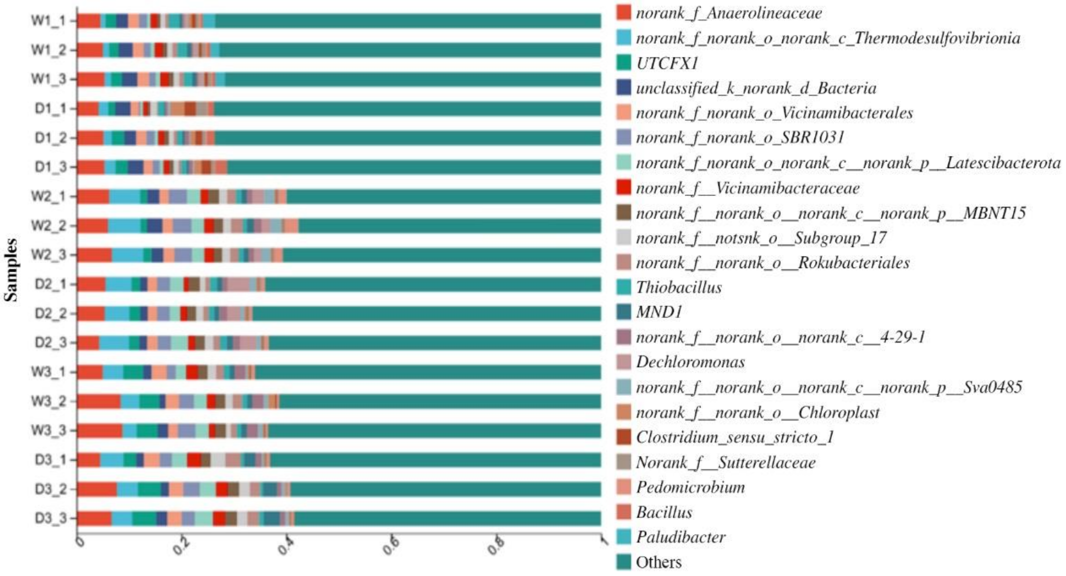


**Figure 4.** Percentages of the major genera of rhizosphere soils in paddy field in two models.

*Norank_f_norank_o_Vicinamibacterales* is another dominant genus, a group of microorganisms containing pit genes encoding low-affinity P transporters (Huber and Overmann, 2015). In the present study, 100 OTUs were retrieved for this genus. The relative abundance of soil *norank_f_norank_o_Vicinamibacterales* in the root system of the DW group was higher than that of the DD group in all three periods and was significant ($P < 0.05$) at tiller and maturity stages.

In addition to the above four categories, both *unclassified_k_norank_d_Bacteria* and *Thiobacillus* also had high relative abundance in the paddy soil. The relative abundance of *unclassified_k_norank_d_Bacteria* was significantly higher ($P < 0.05$) than that of the DD group at the full heading stage, but there was nonsignificant difference at the tiller and maturity stages. The relative abundance of *Thiobacillus* in the co-crop field was higher than that in the monocrop field at the tiller and maturity stages and was significant ($P < 0.05$) at the tiller stage. From the rice growing period, the relative abundance of unclassified*_k_norank_d_Bacteria* was significantly lower ($P < 0.05$) in the co-cropped field from the full heading stage to maturity, and there was nonsignificant change in the monocropped field. *Thiobacillus* did not change significantly.

In order to understand the relationship between major genera and changes in soil physicochemical properties in rice fields, the top 15 genera in terms of relative abundance in the soil and soil environmental factors were analyzed using correlation heatmap (Figure 5). The results showed that *norank_f_norank_o_norank_c_Thermodesulfovibrionia* was significantly and positively correlated with pH, OM, and AP ($P < 0.05$). *Norank_f_norank_o_SBR1031* was significantly and positively correlated with AN ($P < 0.05$). *Norank_f_Anaerolineaceae* was negatively correlated with pH, OM, and AP, and *Thiobacillus* was negatively correlated with pH and AP, but not significantly.

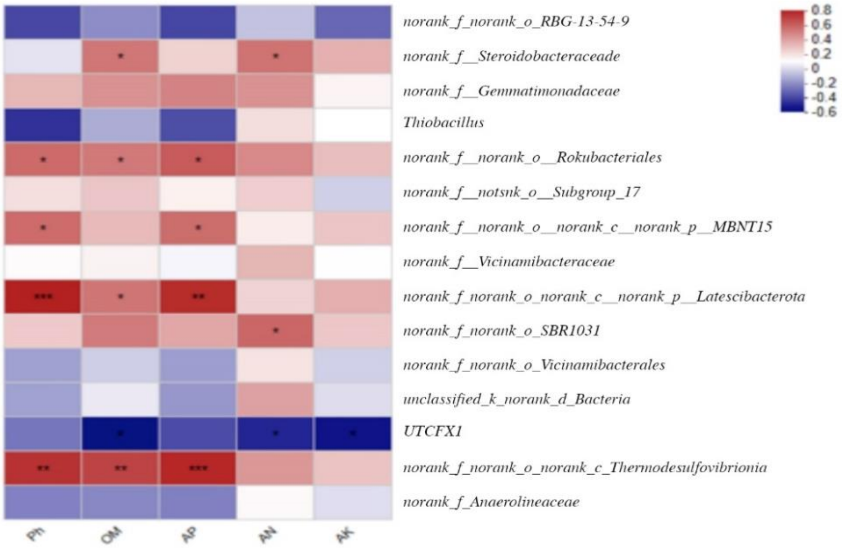


**Figure 5.** Heatmap of top 15 soil abundance genera and environmental factors. Note: X-axis and Y-axis are environmental factors and species, respectively, and the correlation R-value and P-value were obtained by calculation. r-Values are shown in different colors in the figure, and P-values are marked with * if they are less than 0.05. the right-hand legend shows the color intervals of different R-values; $*0.01 < P \leq 0.05$, $**0.001 < P \leq 0.01$, $***P \leq 0.001$.

### Similarity analysis based on bacterial communities

Clustering analysis of the microbial communities in the root soil showed that the bacterial communities in the three replicate groups under the same treatment were clustered together, indicating that the samples had good reproducibility (Figure 6). At the same time, the rice-frog co-cropping pattern was in the same branch as the rice monoculture pattern at the tiller stage, and the two patterns were in the same branch at the full heading stage and the maturity stage, indicating that the bacterial community structure changed less from the full heading stage to the maturity stage. Non-metric multidimensional scaling (NMDS) analysis of the bacterial community structure in the two modes showed that the differences in soil bacterial community structure in the root system were greater at the tiller and maturity stages and less at the full heading stage (Figure 7). From the rice growing period, the bacterial community was more concentrated in the rice-frog co-cropping model compared with the rice monocropping model, indicating that the soil bacterial community structure was more stable in the rice-frog co-cropping model.

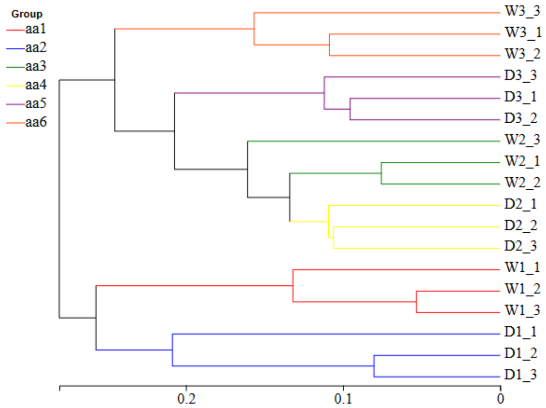


**Figure 6.** Hierarchical clustering tree of the 18 different soil bacterial community on operational taxonomic unit (OTU) level. The length between branches represents the distance between samples, and different groups are presented in different colors. DW: Rice-frog co-cropping; DD: rice monoculture; aa1, aa3, aa5: average values of rice-frog co-cropping group of three fields for tillering, full heading and maturity stages, respectively; aa2, aa4, aa6: average values of rice monoculture group of three fields for tillering, full heading and maturity stages, respectively; W1-1, W1-2, W1-3: DW group of three fields at tillering stage, W2-1, W2-2, W2-3: DW group of three fields at full heading stage, W3-1, W3-2, W3-3: DW group of three fields at maturity stage. DD group is the same as it.

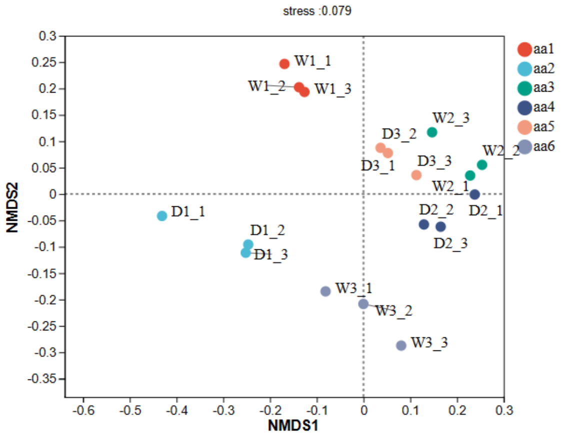
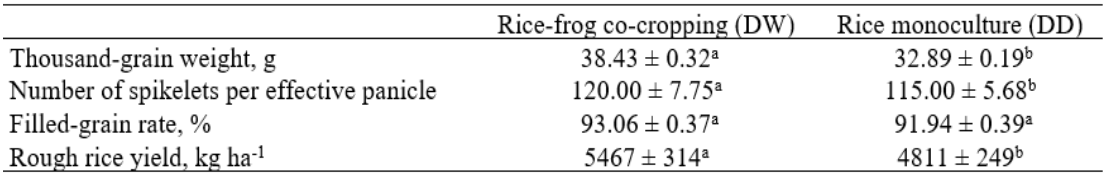


**Figure 7.** Non-metric multidimensional scaling (NMDS) of the 18 different soil bacterial community on operational taxonomic unit (OTU) level. Points of different colors or shapes represent samples of different groupings. The closer the points of two samples are, the more similar the species composition of the two samples is. The horizontal and vertical coordinates indicate the relative distance, which has no practical significance. stress: test the merit of NMDS analysis results. It is usually considered that when stress < 0.2 can be represented by the two-dimensional point plot of NMDS, and its graph has some interpretative meaning; when stress < 0.1, it can be considered a good ranking. DW: Rice-frog co-cropping; DD: rice monoculture; aa1, aa3, aa5: average values of DW group of three fields for tillering, full heading and maturity stages, respectively; aa2, aa4, aa6: average values of DD group of three fields for tillering, full heading and maturity stages, respectively; W1-1, W1-2, W1-3: DW group of three fields at tillering stage, W2-1, W2-2, W2-3: DW group of three fields at full heading stage, W3-1, W3-2, W3-3: DW group of three fields at maturity stage. DD group is the same as it.

## Rice yield

The various indicators of rice yield in the DW group were higher than those in the DD group (Table 3). There were significant differences ($P < 0.05$) in the three indicators of rice thousand grain weight, effective panicle number, and rice yield, and the DW group increased rice yield by about 13.6% compared to the DD group. After the introduction of the black spotted frog, the frog's feces, residual bait, and metabolic waste can continuously supplement the nutrients required for rice growth, promote rice growth, make the grains fuller, and increase the number of effective panicles, thereby increasing the yield of rice per unit area.

**Table 3.** Data of rice yield related factors under two modes.



| | Rice-frog co-cropping (DW) | Rice monoculture (DD) |
|---|---|---|
| Thousand-grain weight, g | $38.43 \pm 0.32^a$ | $32.89 \pm 0.19^b$ |
| Number of spikelets per effective panicle | $120.00 \pm 7.75^a$ | $115.00 \pm 5.68^b$ |
| Filled-grain rate, % | $93.06 \pm 0.37^a$ | $91.94 \pm 0.39^a$ |
| Rough rice yield, kg $ha^{-1}$ | $5467 \pm 314^a$ | $4811 \pm 249^b$ |

In this study, it was found that the difference in farming patterns not only caused changes in the physicochemical properties of paddy soils, but also had a significant effect on the soil microorganisms in the rhizosphere of paddy fields. In contrast, root soil bacteria could be more closely related to the growth of rice (Dexin et al., 2005). One of the most important ecological principles underlying rice-fishery integrated farming is the mutually beneficial symbiotic relationship between rice and fishery (Dexin et al., 2005). In the rice-fishery symbiosis, the most benefit is naturally the ecological benefit of aquatic animals in removing insects, weeding and providing nutrients to rice, in contrast to the indirect effect of aquatic animal introduction on soil bacterial communities, which has not been much studied. As an important component of species diversity in the rice-frog ecosystem, bacterial communities can regulate nutrient cycling, organic matter degradation and energy flow in the ecosystem. It even plays a significant role in maintaining the diversity of plants and animals in the rice-frog symbiotic ecosystem as well as the stability of the ecological structure (Kosolapov et al., 2004; Martins et al., 2011). This study showed that in the rice-frog symbiosis system, the TN, TP, AN, and AP of soil in rice-frog co-cropped fields were significantly elevated due to the residual bait and feces and excretion of frogs, and the increase in nutrients inevitably provided sufficient nutrients for the growth of microorganisms in them. At the same time, microbial growth is related to the C:N ratio (Xu et al., 2013). A significant increase in TN content in rice-frog co-cropped fields will certainly decrease its C:N ratio, which will have an impact on the microbial community composition. In addition, the activities of frogs also disturb the soil, which can change the permeability of the soil and enhance the exchange of gas between the root soil and the outside world, and these may also have an impact on the soil microbial composition of the rice-frog symbiosis system (Shangkun et al., 2015).

## Dominant phylum analysis

From the available results, the microbial diversity of paddy soils was rich, with a total of 20 139 OTUs obtained at 97% similarity between the two models in the three periods, and microbial populations of 1787 genera in 68 phyla were detected at the taxonomic level of phylum and genus. At the taxonomic level of the phylum, the dominant phylum occurring in each sample in each period were *Proteobacteria*, *Chloroflexi*, *Acidobacteriota*, *Bacteroidota*, *Firmicutes*, *Actinobacteriota*, *Nitrospirota*, and this is slightly different from the previous studies (Jangid et al., 2008). The dominant phylum in this study did not have *Firmicutes*, which can play a role in plant disease control by inducing plant immune responses, suggesting that giant rice is more resistant to diseases compared to common rice (Hashmi and Bindschedler, 2020). Compared to the DD group, the DW group had more *Actinobacteriota* in the dominant phyla at maturity and less *Desulfobacterota* throughout the growth period. The reason is that the soil sulfate content increased after fertilization in the rice monoculture model, and *Desulfobacterota* can reduce sulfate to mono sulfur or $H_2S$

and reduce soil sulfate content (Chen et al., 2009; Kuever et al., 2015). A certain amount of $H_2S$ can promote root growth, but too much will cause root rot. Due to the accumulation of soil sulfate in the soil after fertilizer application leading to soil acidification, the results of physicochemical properties showed that soil pH was significantly higher in the rice-frog co-cropping pattern than in the rice monoculture pattern at maturity. *Actinobacteriota* can promote the decomposition of plant and animal manure and debris, etc. (Greening et al., 2022). During the maturity period, the nutrients of fertilizer are used in rice monoculture fields, and there is no supplementary nutrient, while the manure of frogs in rice-frog co-culture fields needs to be decomposed, so the abundance of *Actinobacteriota* is higher than that of monoculture fields.

From the rice growing period, *Proteobacteria* were dominant at tiller and full heading stages, while *Chloroflexi* had the highest abundance at maturity. *Proteobacteria* include alpha-transforming bacteria that can perform N fixation and symbiosis with plants, and can regulate major soil nutrients to promote rice growth (Mukhopadhya et al., 2012). The N uptake of rice before the full heading stage exceeded 80% of the N uptake during the whole period (Yu et al., 2020), so the content of *Proteobacteria* was higher during the tiller and full heading stages. As for *Chloroflexi*, it is a class of anaerobic bacteria that use $CO_2$ as the main C source, and the reduced nutrient elements give it a higher competitive advantage and provide it with a very suitable environment to survive, so its relative abundance is high (Garrity et al., 2001). The relative abundance of *Firmicutes* and *Actinobacteriota* decreased significantly and the relative abundance of *Nitrospirota* increased significantly from the tiller stage to the full heading stage, and the changes from the full heading stage to the maturity stage were nonsignificant. The rice grows vigorously during the tillering period, *Firmicutes* plays an important role in rice disease control, *Actinobacteriota* can promote the decomposition of animal remains and feces, etc., to provide nutrients for rice growth, and their relative abundance is high to ensure normal growth of rice. The growth of rice slowed down during the full heading stage, and their relative abundance was low. When N content is high at the full heading stage, *Nitrospirota* was involved in the N cycle, its relative abundance increased (Umezawa et al., 2020).

## Dominant genus analysis

Among the major dominant genera, there were five groups with significant differences, namely *norank_f_Anaerolineaceae*, *norank_f_norank_o_SBR1031*, *norank_f_norank_o_Vicinamibacterales*, *Thiobacillus*, and *unclassified_k_norank_d_Bacteria*, all with higher relative abundance in the rice-frog co-crop group than in the rice monoculture model. *Norank_f_Anaerolineaceae* was the genus with the highest relative abundance among the samples, involved in the degradation of organic compounds (Liu et al., 2020). And rice growth requires nutrients such as N from the soil, the bacterium needs to decompose frog manure for rice uptake and use, and fertilization in the rice monoculture group can significantly increase the soil C content in many forms and soil nutrient content. The N applied to the soil will directly supply the nutrient growth of rice without the need to obtain N source by degrading organic matter, therefore, its relative abundance was higher in the rice-frog co-crop group than in the rice monoculture group. The high relative abundance of *norank_f_norank_o_SBR1031*, which plays an important role in nitrification, indicates that the farmed frogs enriched the paddy soil with bacteria with high ammonia removal function and effectively promoted the N cycle in the rice-frog breeding process (Chang et al., 2020). Combined with the heatmap, this genus also showed a significant positive correlation with soil AN content. *Norank_f_norank_o_Vicinamibacterales* is a genus of bacteria containing a pit gene encoding a low-affinity P transporter, its high relative abundance indicates that cultured frogs effectively contribute to the P cycle in rice-frog integrated farming, providing the necessary P fertilizer for rice growth. The *unclassified_k_norank_d_Bacteria* are unrecorded denitrifying bacteria, capable of converting nitrate into ammonia and N under anoxic conditions, and are a key part of the N cycle, eliminating the toxic effects on organisms due to nitrate accumulation (Sun et al., 2022). Thiobacillus can oxidize sulfide ($H_2S$, $S_2O_3^{2-}$) or elemental S to sulfuric acid, and the S absorbed and utilized by rice is mainly sulfate (Leduc and Ferroni, 1994). Sulfur and N metabolism in rice is very closely related, and S deficiency will disrupt normal protein metabolism and hinder protein synthesis. *Thiobacillus* is negatively correlated with pH as shown by the heatmap. After the introduction of farmed animals, the activities of feeding and fecal excretion, to a certain extent, affect the uninterrupted fertilization, resulting in an increase in the content of nutrients such as soil organic matter and N, P and K, as well as supplementing trace elements required by rice, such as S (Wang et al., 2011). The results showed that the introduction of frog changed the soil physicochemical properties and increased the content of some beneficial bacteria genera in the rice field (Figures 3, 4), such as nitrifying and denitrifying bacteria that play an important role in the ecosystem. Able to meet the nutrients required for rice growth without fertilization.

Biodiversity is an important factor in evaluating the structural characteristics and stability of a community, and higher biodiversity is more likely to ensure the functional redundancy of an ecosystem, thus maintaining the stability of the ecosystem (Yachi and Loreau, 1999). In summary, the bacterial composition and community structure in paddy soils could be well investigated by MiSeq sequencing technology. Chao index and Shannon index of rhizosphere soil microorganisms were enhanced after frog farming in rice fields. The increased abundance of organic matter-degrading and nitrifying bacteria in the soil of the rice-frog co-crop group promoted the efficiency of soil N cycling, thus ensuring that the organic matter and inorganic N pollution load in the soil was within a manageable range. This study was conducted to reveal the mechanisms of changes in soil fertilization capacity and physicochemical properties of a co-cropping complex ecosystem after the introduction of farmed animals into a rice field from a microbial perspective.

## CONCLUSIONS

The study reveals that the soil properties and bacterial community composition in rice-frog field was significantly different with that in rice monoculture. Significant differences of soil bacterial communities were also observed at both phylum level and genus level among different treatments. Soil properties, such as alkali hydrolyzed N and available K, play an important role in the differentiation of soil bacterial composition in rice-frog field. Therefore, it also increases rice yield.

### Author contributions

Conceptualization: H.T., Y.J. Methodology: H.T., Y.J. Software: Y.J. Validation: Y.J. Formal analysis: Y.J., Y.Z. Investigation: Y.J., Y.Z., H.T. Resources: H.T. Data curation: Y.J. Writing original draft: Y.J., T.H. Project administration: Y.Z., H.T. All co-authors reviewed the final version and approved the manuscript before submission.

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