Effects of *Bacillus subtilis* and *Saccharomyces cerevisiae* inoculation on soil bacterial community and rice yield under combined irrigation with reclaimed and fresh water

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Abstract: In order to save fresh water and reduce soil salt accumulation, reclaimed water-fresh water combined irrigation, i.e, irrigation with reclaimed water for 50 d and then with fresh water till harvest, was used in rice planting. *Bacillus subtilis* and *Saccharomyces cerevisiae* were inoculated into the soil at the end of reclaimed water irrigation. The inoculation weight per pot of these microorganisms was as follows: 0 g and 0 g (J0), 5 g and 0 g (J1), 3.75 g and 1.25 g (J2), 2.5 g and 2.5 g (J3), 1.25 g and 3.75 g (J4), and 0 g and 5 g (J5), respectively. Treatment using reclaimed water in the whole stage was used as the control (CK). The plant height, tiller, physical and chemical properties of the soil, and soil bacterial diversity were measured. It was found that the plant height of rice was increased significantly by J1-J5 treatments. The dry weight of rice root, stem, and panicle and the 1000-grain weight increased significantly, while the leaf dry weight decreased. Microorganism inoculation significantly increased the nutrient absorption capacity of the crops. J1, J2, and J4 treatments significantly increased the amount of nitrate-nitrogen, ammonium nitrogen, available phosphorus, and available potassium, while J3, J4, and J5 treatments increased the soil organic matter, and microbial inoculation significantly decreased the EC of soil. J4 treatment induced the largest reduction in EC, and microorganisms treatments increased soil pH. Bacterial function prediction based on the KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway indicated that soil metabolic function was not significantly disturbed by the treatments. Organic matter and pH are the two main factors affecting the structure of the bacterial community in soil. 3.75 g of *B. subtilis* and 1.25 g of *S. cerevisiae* per pot is the best inoculation ratio.

Keywords: *Bacillus subtilis*, *Saccharomyces cerevisiae*, rice, reclaimed water, soil
DOI: 10.25165/j.ijabe.20221503.6423

Citation: Lu H F, Qi X B, Li P, Qiao D M, Shafeeq-u-R, Bai F F, et al. Effects of *Bacillus subtilis* and *Saccharomyces cerevisiae* inoculation on soil bacterial community and rice yield under combined irrigation with reclaimed and fresh water. Int J Agric & Biol Eng, 2022; 15(3): 33–46.

1 Introduction

Rice is the most widely planted crop in China and one of the most important food staples[1]. Among crops, rice consumes the most water[2], accounting for about 50% of China's agricultural water consumption[3]. A large area of paddy field is found along the middle and lower reaches of the Yellow River, which has a strong demand for water resources. However, the water resources in this area are not rich enough, which leads to many farmers giving up on the practice of rice cultivation.

Reclaimed water is an important means to resolve the short-term problem of water resources, and has already been applied in many countries[4-6]. At present, reclaimed water drip irrigation is being used in Beijing, China, to alleviate local water shortages[7,8]. The reclaimed water contains nitrogen (N), potassium (K), and other nutrients needed by crops[9,10], as the reclaimed water is used for irrigation, the amount of fertilizer can be reduced[11,12]. However, reclaimed water irrigation has a number of disadvantages, including its high salt content[13], which results in soil salinization[10] and poor growth in several crops, including halophyte, cotton, and date-palm[14-16]. There is currently no active or effective solution to address this problem.

In recent years, microorganisms are becoming widely used in agricultural production, including *Bacillus subtilis* (*B. subtilis*)[17], *Serratia odorifera*[18], and Arbuscular mycorrhizae (AM) fungi[19]. Microbial compound fertilizer, produced by combining microorganisms with fertilizer, is currently also being applied in

Received date: 2021-08-21 **Accepted date:** 2021-11-28
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agricultural production. The inoculation of two or more kinds of microorganisms can boost the crop growth promoting effects of fertilizer. For example, effective microorganisms are widely used in agriculture and water environment control. *B. subtilis* (AUBS1) was found to increase the activities of phenylalanine ammonia-lyase (PAL) and peroxidase (PO) and an accumulation of pathogenesis-related (PR) protein in rice leaves[20], while inoculation with *Pseudomonas putida* and *Pseudomonas fluorescens* or *Bacillus strains* had positive effects on root nodule, enzyme production, and plant growth[21]. However, the research on the mixed inoculation of the bacteria and fungi into the soil is relatively deficient, and mixed inoculation of different microorganisms may also have adverse effects.

Some rice varieties are able to acclimatize to salt stress. Previous studies have found that the OVP1 gene enhances salt stress tolerance in rice cultivars[22]. By contrast, rice is tolerant to sodicity[23], the reclamation of soda alkali soil in coastal areas[24]. Most of the farmland soils in the North China Plain are alkaline[25], and planting rice is good for local soil improvement. However, reclaimed water irrigation will increase the salt content of the soil, which will lead to changes in the soil environment, affecting rice growth. Rice has been found to have a threshold tolerance for salt, where a salt soil content of over 0.15%, inhibits rice growth and development[26].

Since reclaimed water irrigation increases the soil salt content, measures must be taken to improve the salt tolerance of rice to promote growth and maintain high yields under reclaimed water irrigation systems. The simultaneous inoculation of endophytic and rhizospheric bacteria has been found to increase soil nitrogen[27]. Many bacteria and fungi are found in soil, and as the structure of the bacterial community changes, the fungal community also changes[28,29]. Thus, inoculating a plant growth promoting bacteria (PGPB) and a plant growth promoting fungus (PGPF) into the soil at the same time increases the capacity for improving the soil environment. For example, a mixture of AM fungus and *B. subtilis* was previously found to increase the yield of geranium[30]. Another study found that *B. subtilis* secretes exopolysaccharides and iron carriers, which inhibit the movement of toxic ions, help to maintain the ion balance, promote the movement of water in plant tissues, and inhibit the growth of pathogenic microorganisms[31], *B. subtilis* mf497446 can promote plant growth on Cd contaminated soil[17], while *C. railenensis* (native maize rhizosphere yeasts) could promote AM fungal root colonization without P fertilization[32] and can promote the production of chlorophyll[33]. Inoculating soils with both microorganisms simultaneously can enhance these advantages and promote the growth of plants under otherwise adverse conditions.

Treated wastewater (TWW) was found to exhibit an increased bacterial abundance, particularly G^+ bacteria[34], while long-term TWW irrigation has been found to increase the metabolic activities of microorganisms in soils[35]. Water saving irrigation can also change the proportion of bacteria in soils[36], such as alternate moderate wetting and drying irrigation, which was found to increase the proportion of Aerobacter, nitrifying bacteria, phosphate, and potassium solubilizing bacteria (which participate in the nutrient cycling). Zhang et al.[37] found that mild water stress is another water management technique that can increase the diversity and richness of soil microorganisms in greenhouse grape soils, as well as save water resources.

Although there is still no large-scale application of reclaimed water to irrigate rice in the current period, its water supply may not meet the field demand when fully irrigated with reclaimed water. Therefore, reclaimed water can be used as one of the irrigation water sources. Meanwhile, short-term irrigation with reclaimed water would restrain rice to an extent, but once irrigation with fresh water is resumed, the soil salt would no longer increase, based on this, reclaimed water-fresh water combined irrigation was put forward. In view of the role of microorganisms in improving soil properties, *B. subtilis* and *Saccharomyces cerevisiae* (*S. cerevisiae*) were inoculated into the soil of rice cultivars at various ratios to analyze the growth and development of rice and the physical indicators of soil. The aim of this study is to provide a theoretical basis for the safe utilization of reclaimed water by studying suitable microbial application schemes of *B. subtilis* and *S. cerevisiae*, and characterizing the relationship between the community structure of soil bacteria and the physical indicators of soil.

2 Materials and methods

2.1 Site description

The experiments were carried out in the greenhouse (20 m×50 m), equipped with solar-shading screens, a heater, and a wet-curtain-fan-cooling system[38], at the Agricultural Soil and Water Environment Field Scientific Observation and Experiment Station of the Chinese Academy of Agricultural Sciences. The test site is located in Xinxiang City, Henan Province, China, where the annual average temperature is 14.1°C, the frost-free period is 210 d, the mean sunshine times is 2398.8 h, the average annual precipitation is 589 mm, and the average annual evaporation is 2000 mm. The temperature and humidity in the greenhouse during the experiment period are shown in Figure A1.

2.2 Experimental materials

The experimental rice variety was “Wugeng 519”. The plastic buckets used had bottom diameters of 20.5 cm, upper diameters of 25 cm, and depths of 28.5 cm. The test soil was a sandy loam, which was obtained from a wheat field near the test station. Total nitrogen (TN), Available phosphorus (AP), available potassium (AK), organic matter (OM), Na^+, K^+, EC, and pH were 0.96 mg/g, 0.12 mg/g, 0.18 mg/g, 21.05 mg/g, 0.26 mg/g, 0.034 mg/g, 510 μs·cm^{-1}, and 8.94, respectively. *B. subtilis* and *S. cerevisiae* were cultured by Shandong Sukehan Bioengineering Co., Ltd., at a concentration of 20 billion CFU/g. The properties of the water quality are described in Table 1.

Table 1 Water quality indices

Water source	NO_3^--N /mg·L^{-1}	NH_4^+-N /mg·L^{-1}	pH	EC /μS·cm^{-1}	K^+ /mg·L^{-1}	Na^+ /mg·L^{-1}
Reclaimed water	21.72±0.11	11.02±1.24	7.68±0.21	1411±45	7.92±1.14	126.95±15.68
Tap water	11.17±0.15	0.83±0.02	8.762±0.35	259±20	2.80±0.21	16.26±1.34

2.3 Experimental design

The experiment used reclaimed water - fresh water (in this experiment tap water was used as fresh water) combined irrigation. The soil moisture was determined by weighing the pots using an electronic scale (20 kg) daily at 8:00 am. Each pot contained 11 kg of dry soil, with saturated moisture content (by mass) in the soil of 38.92%. The urea, potassium sulfate, and potassium dihydrogen phosphate concentrations were 2.5, 1.0, and 3.0 g, respectively.

The experiments were carried out between May and October 2018. The day of transplanting was considered the first day of the rice growth period, denoted as S1. The seedbed was prepared and soaked on May 3, seeded on May 5, loaded on June 9, soaked on

June 12, and transplanted on June 14 (S1, rice growth stage day 1). There were three points in each pot, distributed in a triangle, with 2 plants in each point, harvested on October 18 (S127, rice growth stage day 127). Based on the results of previous researches[20,39,40], five combinations of *B. subtilis* and *S. cerevisiae* were used as the treatments (wt/wt): 5 g and 0 g, 3.75 g and 1.25 g, 2.5 g and 2.5 g, 1.25 g and 3.75 g, and 0 and 5 g, denoted as J1, J2, J3, J4, J5, respectively (Table 2). Based on the result that soil OM, TN, TP, and EC were increased after irrigated with reclaimed water for 20-60 d[41], and increasing the irrigation time of reclaimed water will cause salt stress to crops[16], in this experiment, rice was only irrigated with reclaimed water for 50 d (S11-S61, tillering stage and jointing stage), then *B. subtilis* and *S. cerevisiae* were mixed in tap water and used to irrigate the soil at S61. Tap water was used to irrigate J1, J2, J3, J4, and J5 treatments at S61-S127. Treatments with no *B. subtilis* and *S. cerevisiae* application in the case of the reclaimed water (control, CK) and tap water (J0) were also established. Each treatment had three replicates. Table 2 provides a list of treatment conditions.

Table 2 Amounts of *Bacillus subtilis* (BS) and Saccharomyces *cerevisiae* (SC), irrigation methods, and water sources of each treatment

Treatment	S1-S10	S11-S60	S61-S127	BS and SC amount
CK	All clean tap water -flooded*	All reclaimed water-controlled irrigation	Reclaimed water-controlled irrigation	0
J0	All clean tap water -flooded*	All reclaimed water-controlled irrigation	Clean tap water-controlled irrigation	0
J1				BS 5 g
J2				BS 3.75 g + SC 1.25 g
J3				BS 2.5 g + SC 2.5 g
J4				BS 1.25 g + SC 3.75 g
J5				SC 5 g

Note: * rice seedling survival and growth were significantly reduced under salinity stress[42], so clean water was used within 10 d after transplanting. S1-S127 represent rice growth stages 1 to 127 in 2018 starting from the day of transplantation to harvest. At 60 d after transplanting, *Bacillus subtilis* (BS) and *Saccharomyces cerevisiae* (SC) were mixed in water proportionally and then irrigated into the soil. Controlled irrigation: no water layer will be established in other growth periods except for the 0-50 mm water layer after transplanting for 10 d; the upper limit of soil water control in the root layer is the saturated water content, and the lower limit is 60%-80%.

2.4 Test index and analysis method

At S127, each pot was divided into three layers (0-5 cm, 5-15 cm, and 15-25 cm), and two soil samples were taken from each layer. One was put in the shade and air dried naturally, and the other was stored in the fridge (–4°C) as fresh soil. The three layers of fresh soil were mixed evenly and placed into 4 mL sterile centrifuge tubes with the same mass, and then stored in a refrigerator at –80°C. The nitrate nitrogen (NO_3^--N) and ammonium nitrogen (NH_4^+-N) in the soil were determined using AA3 flow analyzer (Brown Rupee Pte Ltd.), and the pH was measured using Lei-ci PHSJ-6L (INESA Scientific Instrument Co., Ltd), the soil electrical conductivity (EC) was measured using Lei-ci DDB-303A (INESA Scientific Instrument Co., Ltd), the AP and AK were determined using the method of Lu[43], and the Na^+ and K^+ were measured by flame photometry. The average value of the three replications for each layer was used as the one-pot data for correlation analysis.

At S71, S80, S90, S104, and S124, the plant height (the vertical distance between the soil surface and the highest leaf) and the number of tillers of rice in each pot were measured. After soil sampling, the soil was washed slowly with fresh water to avoid root damage. The roots, stems, leaves, and spikes were separated and placed in an oven at 105°C for half an hour and then dried at 80°C to a constant weight. The yield was measured separately in each basin and repeated four times. The following series of measurements were performed: 1000-grain weight, the length of ear, the number of grains per panicle, the mass of a single panicle, the number of full grains, and the number of withered grains.

The structure of the soil microbial community was analyzed using a high-throughput sequencing platform (Shanghai Majorbio Bio-pharm Technology Co., Ltd., China). All extracted DNA samples were stored at −80°C. The 16S sequence primers were denoted 338factcctaggggcgagcag and 806rggactachvggtwtctaat. For 16S functional prediction analysis, the operational taxonomic units (OTUs) of the sample were standardized using PICRUSt to eliminate the interference of the copy component in the genome of the species. Then, information on clusters of orthologous groups (COGs) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) was obtained, and the abundance was calculated according to the Greengene ID corresponding to each sequence.

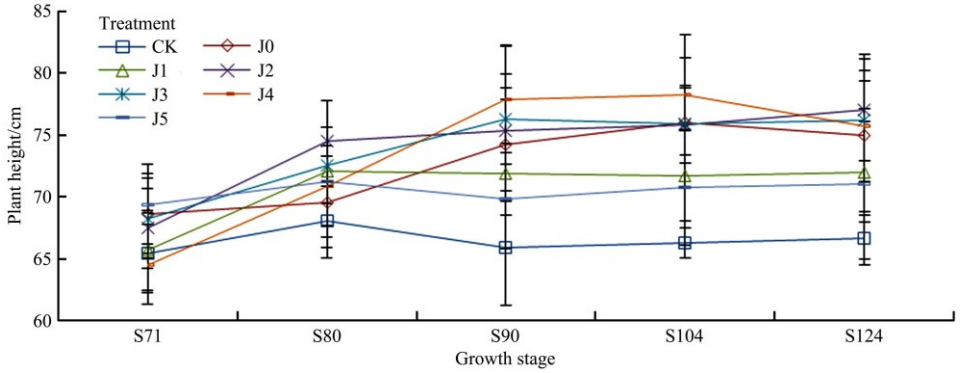
The figures were illustrated using MS Excel 2010 (Microsoft Corp, Redmond, WA, USA). Analysis of variance and correlation was performed using IBM SPSS Statistics 19.0 (IBM Corp., Armonk, NY), and Redundancy Discrimination Analysis (RDA) was used to detect the distribution of the bacterial community in relation to environmental explanatory variables using CANOCO 5.0 (Microcomputer Power, Ithaca, NY, USA).

3 Results

3.1 Plant height and tiller number

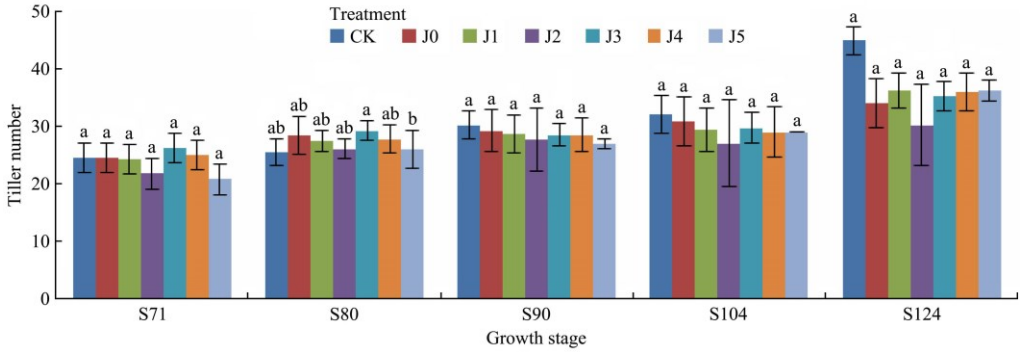
The heights of rice inoculated with microorganisms are shown in Figure 1, and the results of variance analysis are listed in Table A1. At S71 (10 d after microorganism treatment), the plant height of J0, J2, J3, and J5 was higher than that of CK, while J4 was lower than that of CK; however, the differences were not significant. At S80, the plant height of J0-J5 is higher than that of CK, while the plant height of J1-J5 was higher than that of J0; however, the difference between treatments was not significant. At S90-S124, the plant height of J0, J2, J3, and J4 treatment was significantly higher than that of CK, while that of J1 and J5 treated-plants was also higher than that of CK; however, the difference was not significant. At S90, the plant height of J0, J2, J3, and J4 was significantly higher than that of CK by 12.70%, 14.45%, 15.78%, and 18.17%, respectively (p<0.05). The plant height of J4 at S90-S104 was the highest but dropped at S124, which was still higher than J0.

The rice tiller numbers of plants grown in soil treated with the microorganism combinations are shown in Figure 2. Compared to CK, at S80, the tiller number after J0-J5 treatment was higher than that of CK, J3 had a significant difference from J5. At S90 and S104, the tiller number after J0-J5 treatment was lower than that of CK; however, this difference was also not significant. At S124, the tiller number after J0-J5 treatment was lower than that of CK treatment (19.44%-32.78%), where treatments with J0, J2, and J3 were significantly different from CK. Compared to S104, the tiller number of CK at S124 increased by 40.61%, and more ineffective tillers were produced. The tiller number of rice increased slightly within 20 d after soil inoculation. Compared to treatment without microorganisms, the tiller number of rice did not increase significantly by the end of growth.



Treatments: reclaimed water with no *B.subtilis* and *S. cerevisiae* (CK), tap water with no *B.subtilis* and *S. cerevisiae* (J0), tap water with *B.subtilis* 5g and *S. cerevisiae* 0 g (J1), tap water with *B.subtilis* 3.75 g and *S. cerevisiae* 1.25 g (J2), tap water with *B.subtilis* 2.5 g and *S. cerevisiae* 2.5g (J3), tap water with *B.subtilis* 1.25 g and *S. cerevisiae* 3.75 g (J4), tap water with *B.subtilis* 0 g and *S. cerevisiae* 5 g (J5). S71 represents rice growth stage day 71 starting from transplanting day and harvest, the rest are similar.

Figure 1 Changes in the height of rice plants treated with different microorganisms



Note: Different lowercase letters in the same stage represent significant differences among treatments (p<0.05).

Figure 2 Changes in the rice tillers treated with different microorganisms

3.2 Dry weight and yield

The dry matter and yield composition of the rice after treatment with microorganisms are shown in Table 3. At S127, the root and stem dry weight after J0-J5 treatment were higher than those of CK. J4 showed the largest increase, which was significantly higher than CK by 39.88% and 45.74% (p<0.05), respectively. The leaf dry weight after J0-J5 treatment was lower than that of CK by 8.36%-16.83%, however, these differences were not significant, which may be due to the higher proportion of dry matter transfer from leaves to grains in the later stage compared to CK. The difference in spike dry weight between the J0-J5 treatments and CK was significant and was increased by 3.33-5.27 times, with J2 treatment showing the largest increase.

Compared with CK, the weight of a single spike (Table 3) dry weight after J0-J5 treatment increased by 0.86-2.48 times. Except for J5 treatment, all treatments showed significant differences compared to CK. The ear length after J0-J5 treatment increased significantly (12.84-27.02%; p<0.05). Moreover, the number of filled grains after J0-J5 treatment increased by 4.71, 5.61, 5.94, 4.61, 5.82, and 1.64 times, respectively. The number of shriveled grains after J0 and J1 treatment decreased by 13.69% and 2.23%, respectively. By contrast, treatment with J2-J5 increased the number of shriveled grains and was higher than CK; however, the differences were not significant. J0-J5 treatment increased the weight of filled grain by 2.44-8.33 times, and showed significant differences with CK. Shriveled grain weight after J0-J5 treatment was higher than that in CK, however, the difference was not significant. Notably, J0-J5 treatment significantly increased 1000-grain weight by 45.28%-61.32%. Compared with CK, the root/shoot ratio after J0-J5 treatment was decreased, although the difference was small. Under controlled irrigation conditions, the use of reclaimed water irrigation in the early stage, followed by fresh water irrigation and treatment with microorganisms in the later stage significantly increased the dry matter quality of a single spike, the number of full grains, the quality of full grains, and the quality of 1000 grains. The results of the J1, J2, and J4 treatments were the most marked, while the J5 treatment was poor.

3.3 Soil physical indicators

After J0 treatment, soil NO_3^--N was reduced by 2.67 mg/kg compared to CK. Meanwhile, treatment with J1, J2, J3, and J4 significantly reduced NO_3^--N, by 80.36%, 82.54%, 58.17%, and 53.33%, respectively. The effect of J1 and J2 treatments were lower than J0, while treatment with J5 was higher than CK. The levels of NH_4^+-N after J3 treatment were significantly higher than after treatment with J1, J2, J4, and J5; however, there was no significant difference with CK. The J1-J5 treatments reduced the AP and AK more than CK and J0 treatments; however, there was no significant difference between the treatments. Compared with CK and J0 treatment, the organic mass after J3 treatment increased by 19.99% and 35.82% respectively, while that of J1 and J2 was significantly lower than that of CK. J2-J5 treatment significantly increased the soil pH. Compared with CK, J0-J5 treatment significantly reduced soil EC and Na^+. Compared with J0, J1-J5 further decreased Na^+, among which J4 treatment resulted in the largest decrease in EC, while the J1 and J2 treatments resulted in a larger decrease in Na^+. The levels of K^+ after J0-J5 treatment also decreased, with J5 treatment resulting in the largest decrease.

3.4 Soil bacterial diversity and OTUs

The soil diversity indexes of Sobs, Shannon, Simpson, Ace, Chao, and Coverage of each treatment at harvest time are shown in Table 5. Except for J0 treatment, the Sobs and Chao indexes of

the other treatments were lower than CK. The Shannon index after the J0, J1, J3, and J5 treatments was lower than CK. The Ace index after J0, J1, J2, and J3 treatment was higher than CK. However, the diversity indexes showed no significant differences between J0-J5 and CK. The OTU overlapping Venn diagrams for each soil treatment are shown in Figure A2.

Table 3 Rice dry weight and yield at S127 after application of microorganisms

Treatment	Dry weight/g					Spike length /cm (spike number per pot)	Filled grain number per spike	Shriveled grain number per spike	Filled grain weight per spike/g	Shriveled grain weight per spike/g	1000 grain weight/g	Root/ shoot ratio
	Root	Stem	Leaf	All spike	Single spike							
CK	4.73±0.44^{b}	12.86±1.69^{b}	9.00±0.41^{a}	1.04±0.24^{c}	0.18±0.07^{b}	10.23±1.70^{b} (15.3)	3.50±3.82^{b}	44.75±12.12^{a}	0.05±0.06^{c}	0.10±0.04^{a}	10.91±4.68^{b}	0.21±0.020^{a}
J0	5.07±0.99^{ab}	15.18±0.6^{b}	8.08±1.11^{a}	5.18±0.69^{ab}	0.48±0.10^{a}	11.54±1.02^{a} (17.3)	20.00±11.45^{a}	38.63±18.42^{a}	0.33±0.19^{ab}	0.12±0.07^{a}	15.89±2.03^{a}	0.18±0.037^{a}
J1	5.27±0.32^{ab}	16.13±2.85^{ab}	7.59±3.51^{a}	4.58±1.26^{b}	0.60±0.49^{a}	12.89±1.35^{a} (20.3)	23.13±17.92^{a}	43.75±20.49^{a}	0.39±0.28^{ab}	0.12±0.06^{a}	17.43±2.37^{a}	0.19±0.019^{a}
J2	5.20±0.70^{ab}	14.39±2.09^{b}	7.54±1.16^{a}	6.53±1.42^{a}	0.64±0.32^{a}	12.99±1.29^{a} (20.8)	24.38±17.54^{a}	50.38±9.74^{a}	0.44±0.33^{a}	0.14±0.04^{a}	17.60±2.76^{a}	0.18±0.020^{a}
J3	4.77±0.85^{b}	16.12±1.33^{ab}	7.49±1.13^{a}	5.32±1.01^{b}	0.54±0.32^{a}	12.88±1.23^{a} (21.0)	19.63±15.18^{a}	50.38±12.95^{a}	0.35±0.33^{ab}	0.13±0.04^{a}	15.87±3.28^{a}	0.17±0.027^{a}
J4	6.62±2.15^{a}	18.74±3.19^{a}	8.11±1.01^{a}	5.20±1.41^{ab}	0.58±0.26^{a}	12.81±1.06^{a} (22.0)	23.88±15.83^{a}	46.00±20.56^{a}	0.41±0.27^{ab}	0.13±0.08^{a}	16.85±1.99^{a}	0.20±0.046^{a}
J5	4.89±0.82^{b}	15.97±1.01^{ab}	8.25±0.64^{a}	4.51±1.58^{b}	0.34±0.10^{ab}	12.59±1.30^{a} (19.3)	9.25±7.15^{ab}	49.88±15.50^{a}	0.16±0.12^{bc}	0.14±0.04^{a}	15.85±3.53^{a}	0.18±0.027^{a}

Note: Different lowercase letters in the same column represent significant differences among treatments (p<0.05).

Table 4 Soil physical indicators at S127 after application of microorganisms

Indicators	CK	J0	J1	J2	J3	J4	J5
NO_3^--N/mg·kg^{-1}	19.65±6.09^{a}	16.98±6.27^{ab}	3.86±1.50^{c}	3.43±2.07^{c}	8.22±3.42^{bc}	9.17±2.42^{bc}	21.09±8.94^{a}
NH_4^+-N/mg·kg^{-1}	1.11±0.63^{ab}	1.41±1.02^{ab}	0.7±0.15^{b}	1.02±0.24^{ab}	2.25±1.36^{a}	0.80±0.03^{b}	0.86±0.09^{b}
Available phosphorus/mg·kg^{-1}	0.20±0.07^{a}	0.22±0.07^{a}	0.16±0.11^{a}	0.11±0.06^{a}	0.13±0.04^{a}	0.12±0.02^{a}	0.18±0.03^{a}
Available potassium/mg·kg^{-1}	0.27±0.04^{a}	0.26±0.04^{a}	0.22±0.02^{a}	0.24±0.04^{a}	0.24±0.02^{a}	0.23±0.03^{a}	0.21±0.07^{a}
Organic matter/mg·kg^{-1}	21.51±0.24^{b}	19.00±0.35^{bcd}	17.25±0.77^{cd}	16.52±0.90^{d}	25.81±0.82^{a}	23.16±3.30^{ab}	21.00±4.66^{bc}
pH	8.88±0.04^{cd}	8.86±0.01^{d}	8.98±0.09^{bc}	9.13±0.06^{a}	9.10±0.03^{a}	9.07±0.06^{ab}	9.07±0.08^{ab}
EC/μS·cm^{-1}	1373.33±149.07^{a}	838.89±56.59^{b}	742.78±45.51^{bc}	724.56±66.52^{bc}	765.78±214.98^{bc}	565.22±135.59^{c}	671.33±149.37^{bc}
Na^+/mg·kg^{-1}	0.54±0.04^{a}	0.35±0.05^{b}	0.25±0.09^{bc}	0.23±0.06^{c}	0.35±0.04^{b}	0.32±0.03^{bc}	0.31±0.06^{bc}
K^+/mg·kg^{-1}	0.049±0.004^{a}	0.044±0.008^{ab}	0.038±0.007^{ab}	0.036±0.01^{ab}	0.032±0.005^{ab}	0.036±0.010^{ab}	0.030±0.016^{b}

Note: Different lowercase letters in the same line represent significant differences among treatments (p<0.05).

Table 5 Diversity index of bacterial community in soil treated with microorganisms

Treatment	Sobs	Shannon	Simpson	Ace	Chao	Coverage
CK	2566.67±56.57^{a}	6.77±0.041^{a}	0.0026±0.00021^{a}	3418.03±135.06^{a}	3396.91±170.7^{a}	0.96±0.0022^{a}
J0	2571.00±49.87^{a}	6.75±0.059^{a}	0.0027±0.00023^{a}	3462.14±65.33^{a}	3433.75±81.9^{a}	0.96±0.0012^{a}
J1	2521.33±59.37^{a}	6.72±0.05^{a}	0.0028±0.00038^{a}	3374.12±129.09^{a}	3353.89±114.36^{a}	0.96±0.0019^{a}
J2	2541.00±48.75^{a}	6.77±0.025^{a}	0.0026±0.00018^{a}	3373.34±151.6^{a}	3346.38±107.5^{a}	0.96±0.0025^{a}
J3	2480.00±19.92^{a}	6.72±0.055^{a}	0.0028±0.00035^{a}	3284.90±1.79^{a}	3284.12±28.84^{a}	0.96±0.0005^{a}
J4	2513.33±71.82^{a}	6.78±0.028^{a}	0.0024±0.00012^{a}	3317.96±125.23^{a}	3302.16±129.47^{a}	0.96±0.002^{a}
J5	2494.33±64.75^{a}	6.74±0.049^{a}	0.0027±0.00013^{a}	3276.08±24.08^{a}	3251.84±29.27^{a}	0.96±0.0009^{a}

Note: Different lowercase letters in the same column represent significant differences among treatments (p<0.05).

3.5 Composition of soil bacterial community

The distribution of the bacterial species in the soil at the phylum level is shown in Figure 3.

As shown in Figure 4, the bacterial composition of the soil after the different treatments was mainly comprised of *Proteobacteria*, *Actinobacteria*, *Acidobacteria*, and *Chlorofloxi*, where *Proteobacteria* accounted for over 1/4 of the total. In addition to the J3 treatment, the abundance of *Proteobacteria* in soil after the other treatments was higher than that in CK, with J0 treatment resulting in the largest increase; however, there were no significant differences between the treatments. Compared with CK, the J0, J3, and J5 treatments reduced the ratio of *Actinobacteria*, while the other treatments increased this ratio, where the J5 treatment significantly increased the ratio compared to CK and the J1, J2, and J4 treatments. After J0 and J3 treatment, the abundance of *Chloroflexi* was lower than that of CK, which induced by J2 and J3 treatments were significantly higher than CK, J0, and J1. The abundance of *Firmicutes* in J5 treatment was significantly different from CK and the J1, J2, J3, and J4 treatments, and the effect of J2 treatment was significantly lower than that of J0 treatment. The abundance *Gemmatimondetes* and *Bacteroidetes* after J3 treatment was significantly lower than J0 treatment. The abundance of *Tectomicrobia* was significantly lower after J1, J2, and J3 treatment compared to J0 treatment.

The distributions of bacterial species in the treated soil at the class level are shown in Figure 4. The main horizontal bacterial groups in the soil classes after each treatment were *Actinobacteria*, *Acidobacteria*, and *Alphatroteobacteria*, accounting for more than 10%. Compared with CK, the abundance of *Actinobacteria* in soils treated with J0, J3, and J5 decreased by 4.76%, 1.89%, and 24.27%, respectively, with significant differences between the J5 treatment and CK, J1, J2, and J4. The abundance of *Alphaproteobacteria* in the soil after J3 treatment decreased by 10.42%, but increased after the other treatments. The abundance of *Deltaproteobacteria* after J5 treatment was significantly higher compared to J1 treatment. The abundance of *Bacilli* in the soil after J0 and J5 treatment increased by 6.74% and 45.91%, respectively, compared to a 10.18%-38.92% decrease after J1-J4 treatment; The abundance was significantly higher after J5 treatment compared to CK and the J1, J2, J3, and J4 treatments, and significantly higher after J0 treatment compared to J1 and J2 treatment. The abundance of *Gemmatimonadetes* after J3 treatment was significantly lower compared to CK, but significantly higher after the other treatments compared to CK. The abundance of

KD4-96 in the soil after J0, J4, and J5 treatment decreased by 9.68%, 1.84%, and 17.51% respectively, while that after J1-J3 treatment increased; The abundance in the soil after J3 treatment was significantly different from that after J0 and J5 treatment.

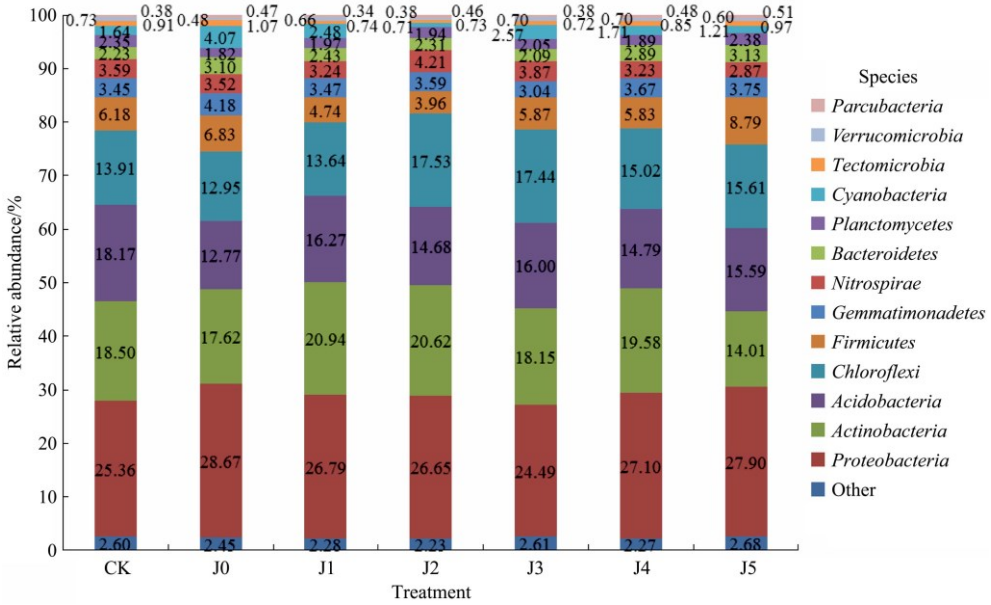


Figure 3 Changes in bacterial species composition in the soil at the phylum level

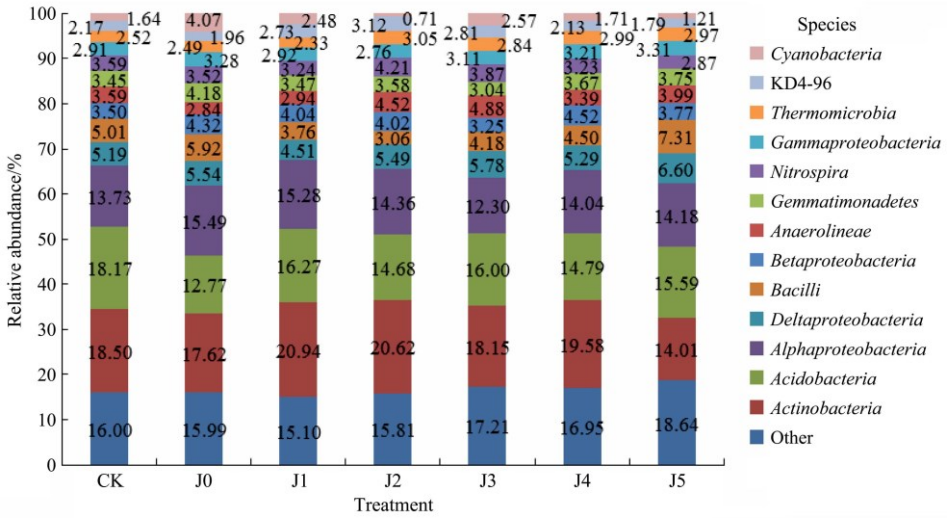


Figure 4 Changes in bacterial species composition in soil at the class level

Coordinate analysis was performed by calculating the Bray Curtis distance of the soil bacterial community structure (Figure 5). The use of different proportions of bacteria was found to have a significant impact on the structure of the bacterial community. PC1 and PC2 explained 15.82% and 14.06% of the total variance, respectively. The distribution of the bacterial community in soils after J1 treatment was clustered in the lower right quadrant, while soils treated with the other combinations were found in two or three quadrants. The J1, J2, and J3 treatments were found in the positive quadrants of PC1, while J5 treatment was found in the negative quadrants. The discreteness of the J0, J4, and J5 treatments was relatively large, while that of J1 was the smallest.

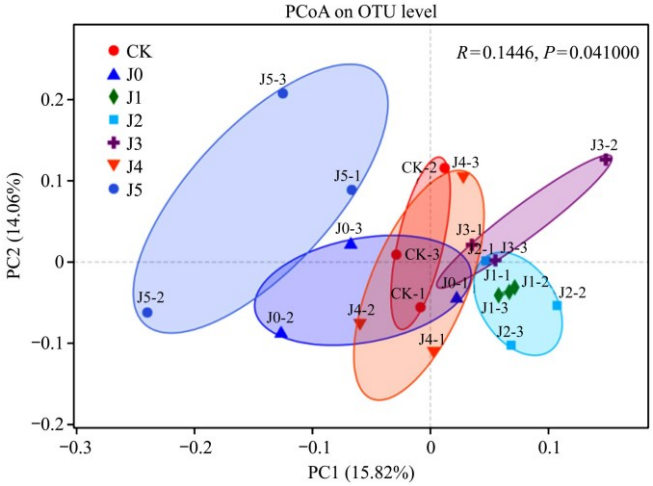


Figure 5 PCoA of bacterial community structure in soil after different treatments

4 Discussion

4.1 Microorganisms promote rice growth and yield

Water and nutrient management have a substantial influence on rice yield[44], where the grain yield of rice plants highly relies on the number of spike-bearing tillers produced by each plant, filled grains, grain weight[45], and the number of effective tillers[46,47]. In this study, the plant height increased significantly when the soils were inoculated with *B. subtilis* and *S. cerevisiae*. *B. subtilis* secretes metabolites that promote plant growth and prevent

pathogen infection[48], which enhances the activities of antioxidant enzymes in rice leaves[20]. Similarly, *S. cerevisiae* increases the supply of phosphorus (P) and provided abundant hormones and minerals for use by the plants[49]. Moreover, the application of microbial agents increased the absorption of nutrients by plants[50], where inoculation with phosphate-solubilizing bacteria increased the plant height and rice biomass. However, the results of the different treatments were not consistent. J4 treatment resulted in the fastest growth rate (plant height), due to its higher chlorophyll content (Unpublished data, 2.52, 2.01, 2.38, 3.51, 2.72, 3.16, 3.55 mg/kg for CK, J0-J5, respectively), more photosynthetic products, and more developed root system than the other treatments. Treatment with *B. subtilis* alone or in a high proportion (75%) strongly promoted plant height and dry matter accumulation but had no obvious effect on tiller number. The tiller number of the rice plants after treatment with *B. subtilis* and *S. cerevisiae* at the time of harvest was less than that of CK. For CK, using reclaimed water for irrigation in the whole growth period led to an increase in the soil N, P, and K content[51], which promoted rice tillering. However, these were ineffective tillers (the number of total tillers (Figure 2) minus panicles (Table 3)), possibly due to the salt and alkali stress during the reproductive growth period which significantly affects the process of young spike differentiation[52].

PGPB can mitigate the adverse effects of salt stress, which hinders the growth and development of rice plants, through the mediation of phytohormone (ethylene) and reactive oxygen species (ROS) accumulation, maintaining ion homeostasis, improving photosynthetic capacity, and enhancing stress-responsive genes expression[53]. Rice seeds inoculated with individual isolates and different *Bacilli* consortia showed significantly improved growth parameters[54]. In this study, the dry weight of the root, stem, and leaf, the spike length, the filled grain number, and the weight of plants grown in soil inoculated with *B. subtilis* and *S. cerevisiae* were higher than that in CK. This is due to the fact that the salinity of the inoculated soils does not increase after the restoration of fresh water irrigation (Table 4), while the soil salinity in CK continues to increase (Table 4) and the net photosynthesis decreases[55]. With an increased salinity, the rice yield and stem weight decreased significantly[56]. Moreover, the application of bacterial agents can improve the resistance of crops to salt stress[57,58], Busari[59] also confirmed the fact that alternating between wetting and drying irrigation using anaerobic baffled reactor (ABR) effluent domestic sewage (The reclaimed water used in this experiment also comes from domestic sewage) decreased plant height, leave area index (LAI), and the number of filled grains per panicle, while increasing the number of panicles per m^2, the number of tillers per plant, the number of filled grains per m^2, and the grain yield. The dry weight of the roots after treatment with J1, J2, and J3 was higher than in J0. The dry filled grain number, 1000-grain weight of stems, and panicles after J2 and J4 treatment were higher than after J0 treatment, indicating that the application of microorganisms significantly promoted the growth and development of rice. This may be due to a reduction in the soil EC (Table 4) and the increased supply of nutrients[60]. Similar results were also obtained by Cavite et al.[61] when using *Acidovorax delafieldii* which promoted rice growth. However, the dry matter weight of the leaves was lower than that in CK. This may be due to the higher amount of photosynthate transferred from the leaves to grains compared to from the stems. Combined with the dry matter and yield index, the optimal treatment was 5 g of *B. subtilis* per pot (J1). This result is similar to that reported by Fajaruddin et al.[62], who used 12 mL of liquid silica and 6 g/pot of *Bacillus* sp.

However, a previous study found that if the salt content of reclaimed water was different, the role of microorganisms in improving rice physiology would change[63]; In addition, temperature, sowing time, irrigation method, and rice variety would affect the promotion of microorganisms on rice. Therefore, it is necessary to study different varieties of rice and irrigation methods using reclaimed water.

4.2 Microorganisms affect soil physical indicators

After the restoration of fresh water irrigation, the soil content of NO_3^--N, NH_4^+-N, AP, AK, and OM changed little compared to irrigation using reclaimed water. Although the NO_3^--N and OM content decreased only slightly, the content of EC and Na^+ decreased significantly, which indicated that reclaimed water irrigation increased the soil nutrient supply[64], but also increased salt stress, as previously found by Ayoub[65] and Asiloglu[66]. The reason for the high nitrate content of CK is that saline soil generally has low nitrogen (N) availability and restricted N uptake[67]. Yeast has the ability to dissolve phosphorus[68], and it is generally believed that phosphorus absorption efficiency of crops was improved[69]. In this experiment, soik P content decreased significantly during harvesting. Microbial treatment at the end of the jointing stage increased the soil P content in a short period of time. Thus, rice growth and development were strengthened, and a large amount of nutrient was absorbed, which led to the decreased soil P levels during harvesting. However, the changes in the soil P levels were insufficient in this study, it is necessary to study the change of insoluble phosphate in soil. The pH is the major limiting factor for rice production in saline-sodic soils, where the impact of salinity and alkalinity superimposed results in a greater decrease in plant growth, as well as grain yield, shoot weight, 1000-grain weight, and panicle number of rice[70]. Although the pH value of the soil was higher after bacterial treatment (increase $\leq$ 0.25 units) (Table 4), the salt content decreased greatly and did not inhibit the growth of rice. When *B. subtilis* was applied at 5 g and 3.75 g, the levels of NO_3^--N, NH_4^+-N, AP, AK, OM, EC, Na^+, and K^+ in the soil all decreased. This is due to *B. subtilis* inducing the rice root system and improving the absorption of nutrients[62], as well as accelerating the development of the roots, stems, and leaves (Figure 1 and Table 3). The nutrient consumption in the soil after inoculating with bacilli consortia was high[54]. Thus, inoculation with *Bacilli* consortia improved the growth parameters of rice[61], similar to Plant Growth-Promoting Rhizobacteria (*Acidovorax delafieldii*), which increases the absorption of N, P, and K.

When the amount of *S. cerevisiae* inoculated was high (2.5, 3.75, or 5 g), OM increased, while EC and K^+ decreased. With 1.25 g of *B. subtilis* and 3.75 g of *S. cerevisiae*, inoculation of the soil resulted in a large decline in the levels of NO_3^--N, NH_4^+-N, AP, and AK. The largest decline was observed for EC, indicating that the appropriate proportion of 1.25 g of *B. subtilis* and 3.75 g of *S. cerevisiae* is key to promoting plant growth. Yeast is considered a rich source of plant hormones, vitamins, enzymes, amino acids, and minerals[49] and can use the decomposition products provided by other microorganisms[71,72]. These two microorganisms produced indole compounds, siderophore, and ACC deaminase[61,73], and increased the activity of ROS-quenching enzymes[74,75]. The NO_3^--N content in soil treated with J3, J4, and J5 was higher than that in soils treated with J1 and J2. In particular, the NO_3^--N content in soil treated with J0 increased by nearly 25% compared to CK, indicating that *S. cerevisiae* can accelerate the mineralization

of soil nitrogen. The high levels of available nitrogen (AN) are the reason why yeast is able to increase the chlorophyll content of leaves[76]. Rice is widely considered to be sensitive to soil salinity[42,77], and the results of this study demonstrate that the use of microbial agents is a feasible strategy to reduce soil salt stress.

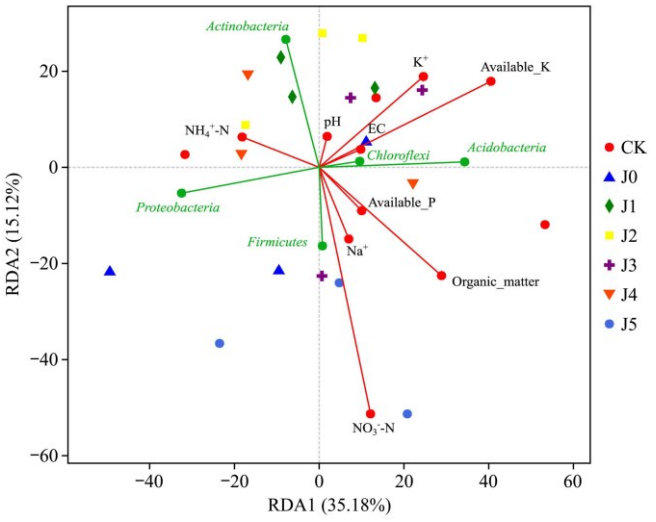
4.3 Bacteria affect soil nutrient supply

Actinobacteria and *Proteobacteria* have clear competitive advantages for niches under nutrient-rich conditions[78]. Similarly, *Acidobacteria* is known to degrade plant residue multimers[79]. *Proteobacteria*, *Actinobacteria*, *Acidobacteria*, and *Curvularia* were found to be the main bacterial phyla in rice plant soils after the different treatments, in agreement with the results reported by Maguire et al.[29]. In contrast, Xu et al.[80] found that the top four most abundant phyla were *Proteobacteria*, *Chloroflexi*, *Bacteriodetes*, and *Firmicutes*. The differences in the proportions of the latter three bacteria are most likely due to differences in the types of irrigation systems and soil types used. Compared with CK, J5 treatment significantly reduced the proportion of *Actinobacteria* and *Firmicutes*, while J3 treatment significantly reduced the proportion of *Actinomycetes* and *Bacteroidetes*. The content of AN and AP in soil was lower than that in CK, and the reduction of nutrients was not conducive to the reproduction of dominant bacteria. However, treatment with J2 and J3 significantly increased the proportion of *Chloromycetes* in the soil, which was conducive to the degradation of carbon in the soil. Compared with the soil subjected to no microorganisms treatment, J1, J2, and J3 treatment significantly reduced the proportion of *Tectomicrobia* due to the lower amounts of NO_3^--N and AP in the soil. N and P are known to be important factors affecting bacterial reproduction[81]. Furthermore, the proportions of *Actinomycetes* in soils treated with J5 and *Alphaproteobacteria* in soils treated with J3 were lower than in CK.

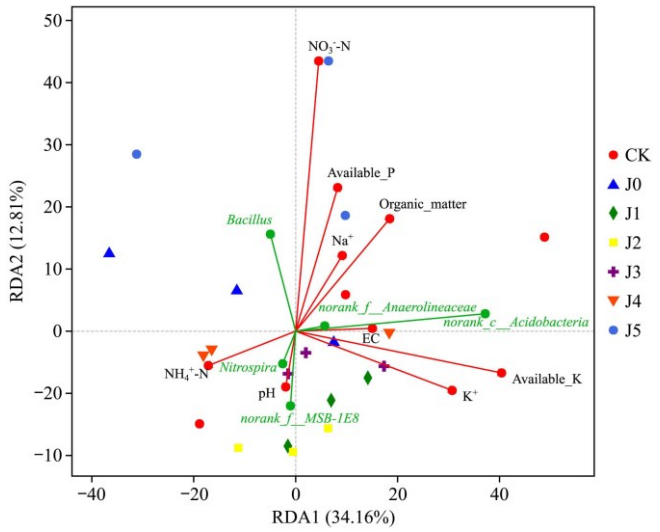
Compared with *B. subtilis* alone, treatment with *S. cerevisiae* alone increased the proportion of *Proteus* and *Bacillus* in the soil since *S. cerevisiae* increases the organic quality of soil. A rich carbon source is one of the important conditions for microbial reproduction[82]. Compared with no microorganism treatment (J0), the proportion of *Bacillus* was found to decrease in soils treated with J1 and J2 due to the lower organic quality. The soils treated with J1 and J2 resulted in plants with more developed roots, which leads to more oxygen consumption[83]. This condition was not conducive to the reproduction of this kind of aerobic bacteria. Compared to CK, the changes in the structure of the bacterial community in the soil treated with J1, J2, and J5 were greater than those associated with J0 treatment (Figure 5), indicating that the bacterial community structure could be changed through the use of microbial agents. The relative abundance of bacteria in the soil at the genus level (Figure A3) was different between all treatments, however, the overall change was not very obvious during harvest, and the bacterial diversity was not significantly changed. This may be due to the reduction in soil nutrients and an increased pH at the end of the growth period, which limits the multiplies of some bacteria[82]. Generally, the suitable pH for microorganisms in soil is 6.5-7.5. The activity of microorganisms can be seriously inhibited in overly acidic or alkaline environments, which affects the transformation and supply of nitrogen and other nutrients. Notably, the bacterial community structure at the different growth stages of rice is quite different[84]. In this study, competition among the different bacteria colonies was fierce, and bacterial function changed greatly in a short period of time. However, the bacterial community tended to gradually stabilize over time. Moreover, the abundance of bacteria obtained by the sequencing of the amplicons does not necessarily represent the number of bacteria[66]. In order to accurately analyze the effects of changes in the bacterial structure of soil on the physical and chemical properties of soil and rice growth, quantitative PCR and metagenomic detection are needed. Moreover, the total nitrogen and total phosphorus of the soil system are closely related to the dominant species of *Actinomycetes*, *Acidobacteria*, and *Bacteroides*[85], which are equally noteworthy.

The restoration of fresh water irrigation and the application of microorganisms did not significantly improve the functional abundance of metabolic function, however, differences were observed between treatments using different proportions of microorganisms (Figure A4). Compared with the single application of *S. cerevisiae*, *B. subtilis* alone significantly enhanced the metabolic function, metabolism, genetic information, and enzyme family function abundance. Compared with the treatment without microorganisms, the 1:1 combined application of *B. subtilis* and *S. cerevisiae* significantly reduced the cell process, signal function, and signal transduction function. There was little difference in the bacterial metabolic function between the different treatments. Microbial diversity and community structure are buffered against declines in their functioning as a high species diversity provides greater guarantees that some microbes will remain functioning even if others fail[86]. It can be inferred that applying microorganisms did not significantly affect the metabolic function of bacteria, because the soil has a strong buffer capacity.

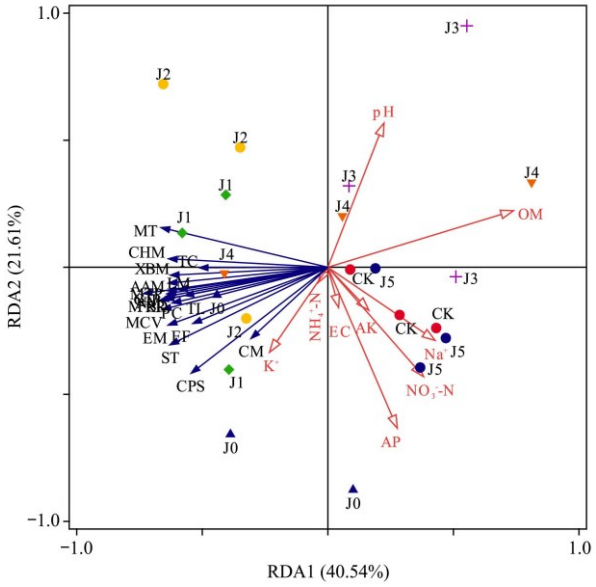
The soil microbial community is very sensitive to changes in the soil microenvironment (pH, EC, nutrients, water)[87,88]. RDA analysis showed that NO_3^--N (Phylum level: $R^2 = 0.4998$; $p = 0.004$; Genus level: $R^2 = 0.5519$; $p = 0.003$) and AK (Phylum level:$R^2 = 0.3209$; $p = 0.024$; Genus level: $R^2 = 0.3708$; $p = 0.015$) were the main factors causing changes in the soil bacterial community (Figure 6), which is consistent with Zhu et al.[89] Moreover, the soil OM (22.3%, p=0.018) was the main factor affecting changes in bacterial metabolic function (Figure 6c), which played a key role in the structuring of microbial communities, including revegetation[90]. Soil OM also provides nutrients and the capacity for microbial activity, as well as improving the soil properties and buffer capacity. Guo et al.[91] found that the microbial functional categories (including the carbohydrate and energy metabolism, and biodegradation and metabolism of xenobiotics) were correlated with soil OM and TN. The pH is a secondary factor influencing



a. Phylum level



b. Genus level



c. Soil bacterial composition

Figure 6 RDA analysis of soil bacterial composition (Top 5) and soil physical index

the structure of the bacterial community, affecting the concentration of various ions in the soil and the effectiveness of various elements on plants. Maguire et al.[29] also reported that soil pH is one of the factors responsible for changes in the bacterial community.

5 Conclusions

Microorganisms treatments increased the tiller number of rice in a short time (within 20 d), which was not significantly increased at the end of growth, while the rate of ear formation was increased. *B. subtilis* and *S. cerevisiae* also provided beneficial effects, increasing the dry weight of the rice roots, stems (J4 treatment induced the largest increase), and ear (J2 treatment induced the largest increase). *B. subtilis* and *S. cerevisiae* also promoted rice yield by changing the nutrient supply in the soil and reducing the soil's electrical conductivity. Subsequent changes in the physical and chemical properties of the soil resulted in significant changes to the bacterial abundance of *Actinomycetes*, *Firmicutes*, *Curvularia*, *Proteobacteria*, *Bacillus*, and *Nitrospira*, but did not significantly change the diversity of soil-native bacteria. After irrigation with reclaimed water for 50 d, fresh water irrigation reduced the amount of salt entering the soil, *B. subtilis* and *S. cerevisiae* helped to alleviate salt stress to rice and improve yield. In this respect, 3.75 g of *B. subtilis* and 1.25 g *of S. cerevisiae* per pot were found to be the best combination.

Acknowledgements

This work was financially by the Natural Science Foundation of Henan Province (Grant No. 212300410309), Program of the National Natural Science Foundation of China (Grant No. 51679241, 51879268), Key Research and Development and Promotion Projects of Henan Province (Grant No. 212102110237), the Agricultural Science and Technology Innovation Program (CAAS-ASTIP-FIRI-03), the Chinese Academy of Agricultural Sciences and Central Public-interest Scientific Institution Basal Research Fund (Farmland Irrigation Research Institute, CAAS, FIRI202001-06).

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Appendix

Table A1 Results of variance analysis of rice plant height treated with different microorganisms

Treatment	S71	S80	S90	S104	S124
CK	a	a	c	b	c
J0	a	a	ab	a	ab
J1	a	a	abc	ab	ab
J2	a	a	ab	a	a
J3	a	a	a	a	a
J4	a	a	a	a	ab
J5	a	a	bc	ab	bc

Note: Different lowercase letters in the same column represent significant differences among treatments (p<0.05).

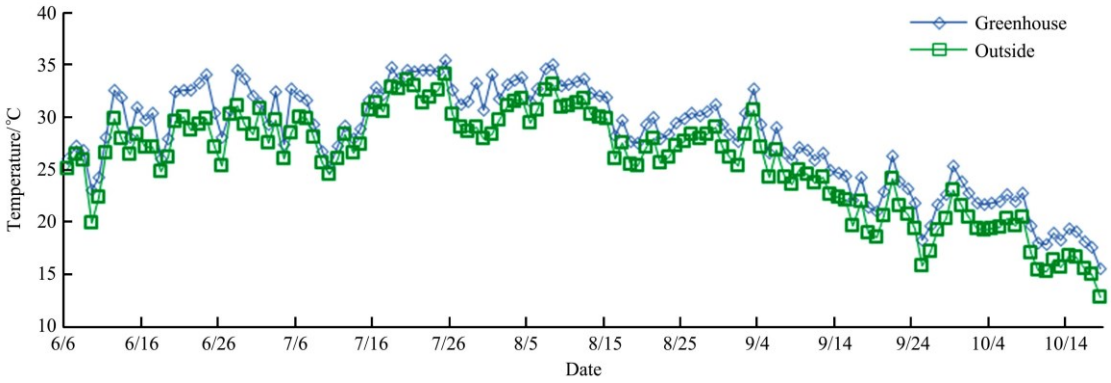
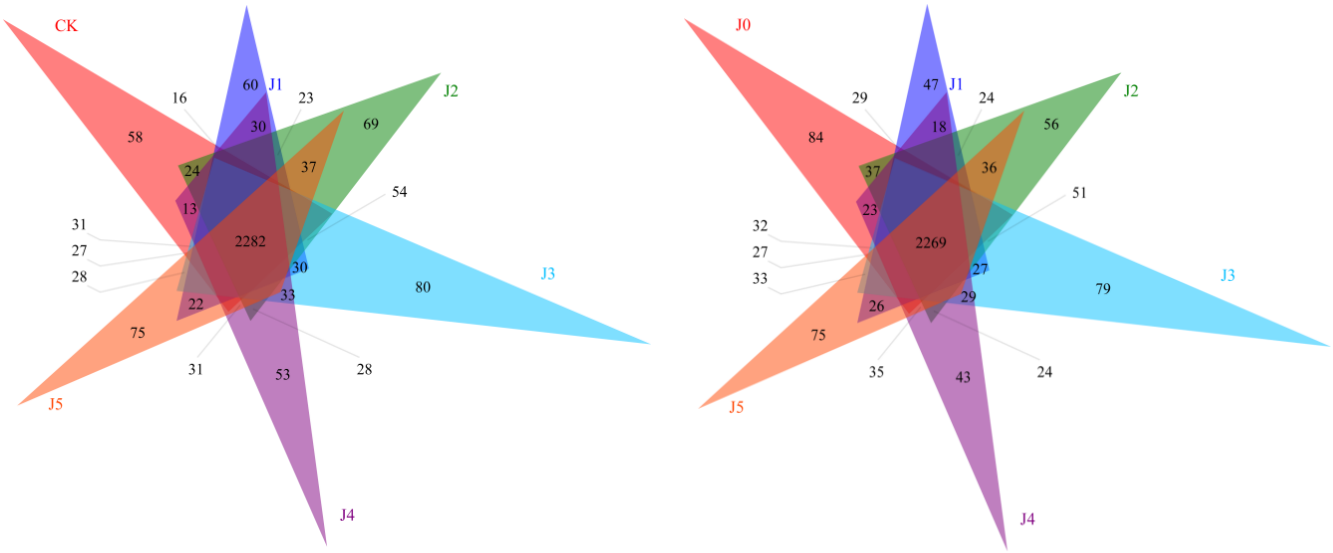


Figure A1 Temperature and humidity in the greenhouse during the experiment period



a. CK and J1-J5 b. J0 and J1-J5

Figure A2 OTU overlapping Venn diagrams of the treated soils

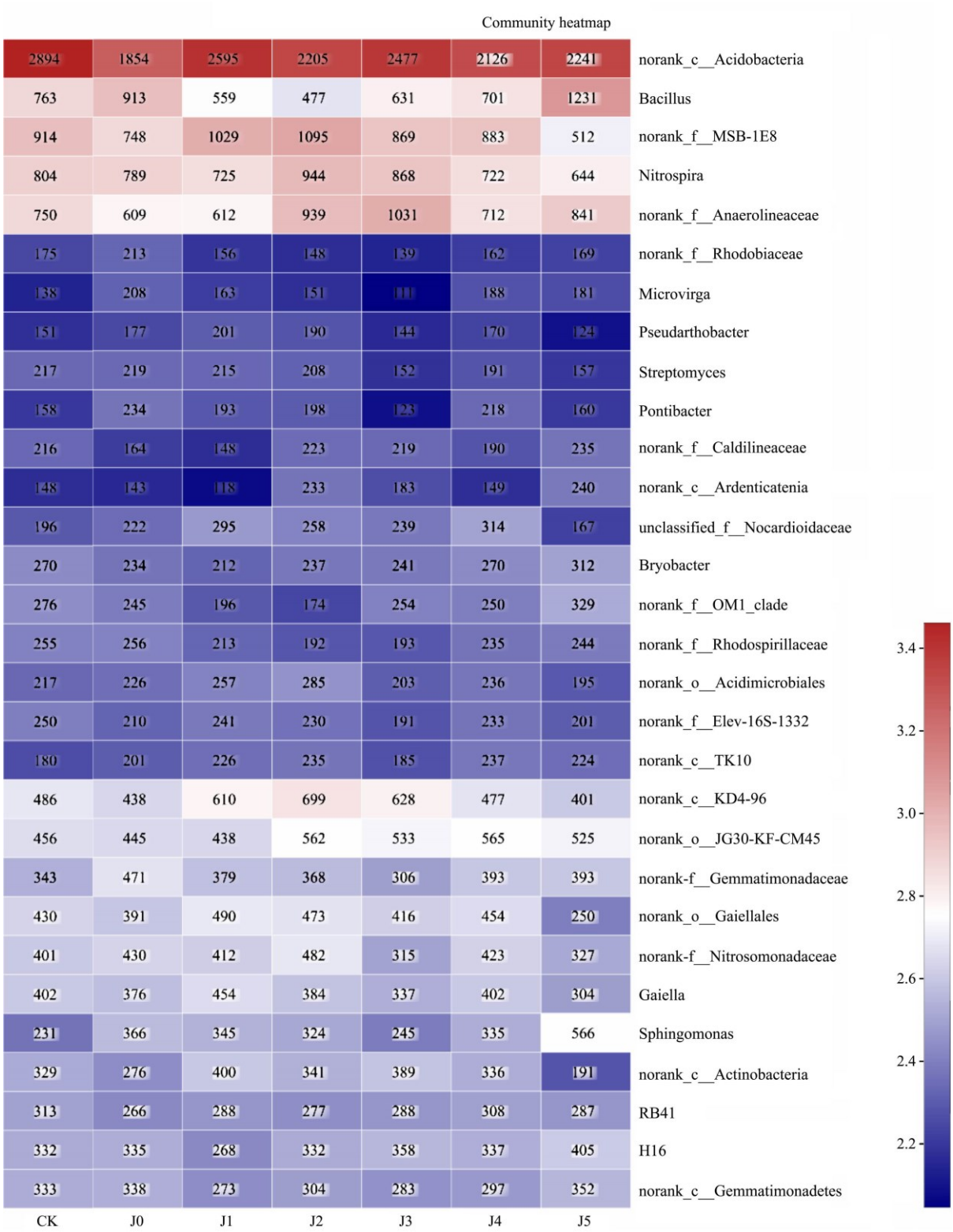


Figure A3 Relative abundance of bacteria in soil at genus level

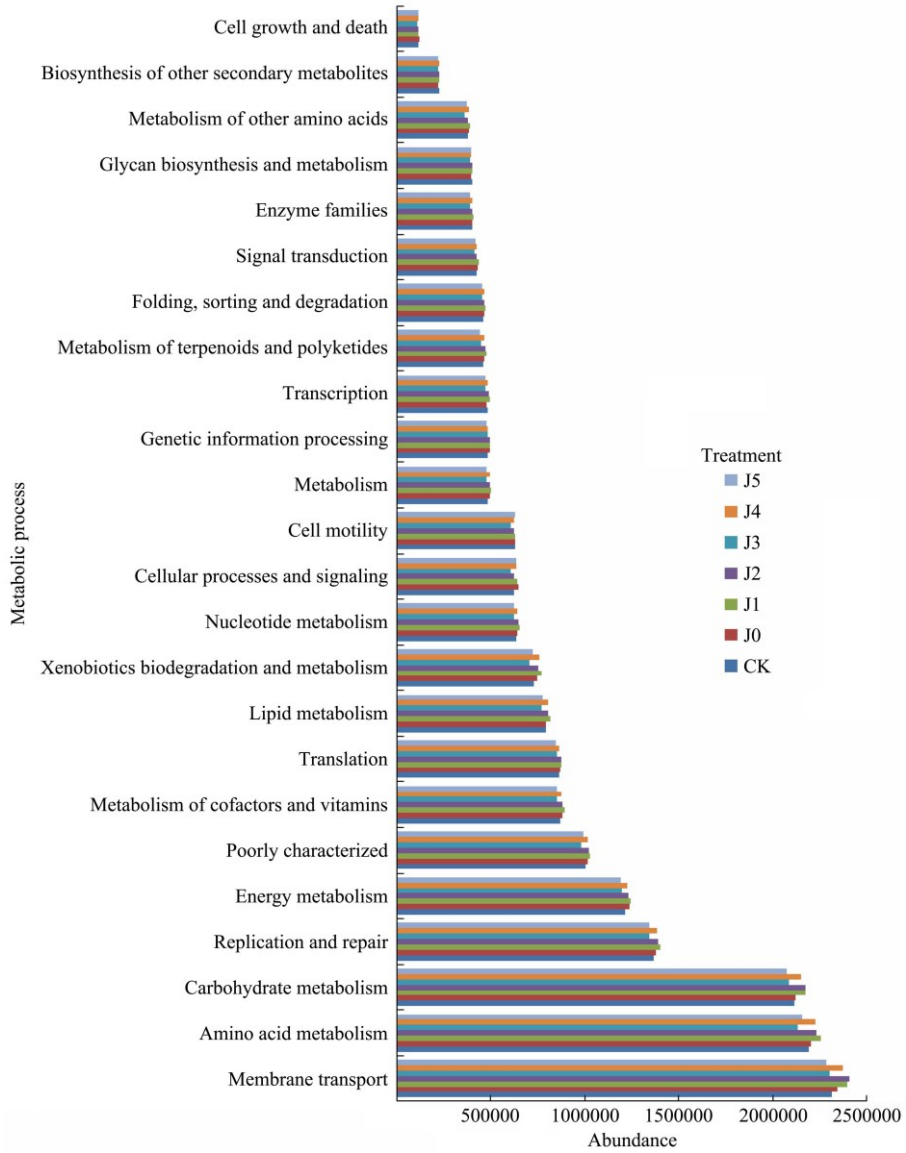


Figure A4 Abundance of KEGG metabolic pathway