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Distinct Denitrifying Phenotypes of Predominant Bacteria Modulate Nitrous Oxide Metabolism in Two Typical Cropland Soils

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Abstract

Denitrifying nitrous oxide (N_2O) emissions in agroecosystems result from variations in microbial composition and soil properties. However, the microbial mechanisms of differential N_2O emissions in agricultural soils are less understood. In this study, microcosm experiments using two main types of Chinese cropland soil were conducted with different supplements of nitrate and glucose to simulate the varying nitrogen and carbon conditions. The results show that N_2O accumulation in black soil (BF) was significantly higher than that in fluvo-aquic soil (FF) independent of nitrogen and carbon. The abundance of most denitrifying genes was significantly higher in FF, but the ratios of genes responsible for N_2O production (*nirS* and *nirK*) to the gene responsible for N_2O reduction (*nosZ*) did not significantly differ between the two soils. However, the soils showed obvious discrepancies in denitrifying bacterial communities, with a higher abundance of N_2O-generating bacteria in BF and a higher abundance of N_2O-reducing bacteria in FF. High accumulation of N_2O was verified by the bacterial isolates of *Rhodanobacter* predominated in BF due to a lack of N_2O reduction capacity. The dominance of *Castellaniella* and others in FF led to a rapid reduction in N_2O and thus less N_2O accumulation, as demonstrated when the corresponding isolate was inoculated into the studied soils. Therefore, the different phenotypes of N_2O metabolism of the distinct denitrifiers predominantly colonized the two soils, causing differing N_2O accumulation. This knowledge would help to develop a strategy for mitigating N_2O emissions in agricultural soils by regulating the phenotypes of N_2O metabolism.

Keywords Agricultural soil · Denitrification · Denitrifying genes · Bacterial community · Nitrous oxide

Introduction

The nitrogen cycle and the associated microbes play important roles in the sustainability of ecosystems [1]. N_2O has drawn much attention as an important ozone-depleting substance [2] and has global warming potential approximately 265 times greater than that of CO_2 [3]. Agricultural soils are a major source (60%) of anthropogenic nitrous oxide (N_2O) emissions globally [4]. Denitrification is a major source of N_2O in agricultural soil and the main process responsible for N loss to the atmosphere [5]. Denitrification, the stepwise reduction of nitrate to N_2, involves four reduction steps. The enzymes catalyzing the four steps are encoded by the *narG* (nitrate reductase), *nirK* or *nirS* (nitrite reductase), *norB* (nitric oxide reductase, responsible for N_2O generation), and *nosZ* (N_2O reductase) genes [6]. Microorganisms with higher catalytic activity for N_2O reduction than for N_2O generation could mitigate N_2O emissions by acting as N_2O sinks. In contrast, those having stronger activity for N_2O generation but less N_2O reduction would act as N_2O sources [7].

Rates of N_2O emission from agroecosystems are often explained by variations in soil properties and are strongly influenced by the abundance and diversity of denitrifying microbes [8, 9]. Some studies have focused on how environmental factors such as fertilization [10], carbon substrate availability [11], and pH [12] affect denitrifying

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microorganisms, which further trigger the production of N_2O from soil. An increasing number of investigations have addressed microbial processes related to N_2O emissions from soil. To date, the correlations between denitrifying genes and N_2O flux are inconsistent for different soils. For example, Yang et al. [13] found the abundance of the *narG*, *nirS*, and *nirK* genes to be correlated with annual N_2O emissions in intensively managed calcareous soil. However, several studies indicated that denitrifier gene abundance was not the main factor influencing N_2O emissions [14, 15]. Instead, some studies show a linkage between the taxonomic composition of soil denitrifying bacteria in farmland soil and the denitrification rate or N_2O production [16, 17].

Microbial communities in different soils are structured over the long term by distal controls, which include both environmental factors and biotic interactions [18, 19]. Black soil and fluvo-aquic soil are among the most widely distributed farmland soils in China. Black soil is mainly distributed in Northeast China and is considered one of the most fertile soils [20]. The North China Plain lies in the alluvial plain [21] and is dominated by fluvo-aquic soil. This region is characterized by low levels of soil organic carbon, poor soil structure, and high pH [22]. It is noteworthy that both of the abovementioned soils are now being irrigated with the application of a large amount of fertilizer N with the aim of obtaining higher yields, which has led to substantial total N_2O emissions in the two regions [21, 23]. Compared to the 1990–2003 levels, the annual growth rate of N_2O emissions from farmland in China decreased significantly from 2004 to 2014, but the rate in Heilongjiang Province, where black soil is widely distributed, showed a significant increase [24]. Zhang et al. [25] found that *nosZ* gene abundance is the only biological factor that could explain the higher N_2O accumulation in black soil than fluvo-aquic soil. However, this study did not pay attention to the effects of the composition of denitrifying bacteria in the two soils. It is necessary to further unravel the underlying microbial mechanisms causing differences in N_2O accumulation in these two types of soil to establish an effective means to mitigate the N_2O emissions resulting from agricultural activity.

We hypothesize that the varied communities contain denitrifiers with different metabolic phenotypes, which shape the distinct denitrification processes in different soils. To test this hypothesis, in this study, we performed comparative studies with microcosm experiments using black soil and fluvo-aquic soil under three controlled incubation conditions with different amounts of nitrate and glucose. We used a robotized incubation system to continuously measure N_2O and N_2 fluxes. Furthermore, we investigated the structure of the total bacterial and denitrifying bacterial communities and quantified key denitrifying genes. Moreover, we isolated the predominant denitrifying bacteria from both soils and verified their denitrification properties. As a result, this study explored the microbial mechanisms for different accumulations of N_2O in the two main types of agricultural soils in China.

Materials and Methods

Soil Used for Experiments

Two types of soil samples were used for incubation experiments in this study. Black soil (BF) and fluvo-aquic soil (FF) were collected from cropland in long-term experimental fields in Northeast China and the North China Plain, respectively. Details of the sample information are shown in Table 1. Samples of each type of soil were taken from five different points at depths ranging from 0 to 20 cm and mixed as the final sample. All samples were placed in black plastic bags and stored at 4 ℃ before use. Soil samples were sieved (2 mm) to remove stones and coarse roots prior to incubation experiments.

Experimental Design and Microcosm Incubation

Twenty grams (dry weight) of soil samples each in 120-mL serum vials was adjusted to 70% water holding capacity and underwent CK (blank control), N250 (with an initial nitrate content of 250 mg/kg), and N250 + G (with 250 mg/kg nitrate plus 1000 mg/kg glucose) treatments.

The triplicate vials containing soil samples were sealed with airtight butyl-rubber septa and aluminum crimp caps.

Table 1 Physicochemical properties in black soil (BF) and fluvo-aquic soil (FF)

	BF	FF
Latitude and longitude	43°18′ N,124°14′ E	40°18′ N,116°10′ E
Cropping system	Maize	Wheat, maize
Manure input	N: 234 kg N/ha	Winter wheat 300 kg N/ha
	P_2O_5: 108 kg/ha	Summer corn 260 kg N/ha
	K_2O:108 kg/ha	
Nitrogen management	Compound fertilizer	Conventional fertilization
pH	7.9 ± 0.1	8.3 ± 0.1
Water content (%)	18.90 ± 0.04	10.17 ± 0.06
WHC (%)	45.26 ± 1.50	41.34 ± 0.08
DOC (mg/kg)	26.95 ± 1.11	18.08 ± 0.96
NO_2^- (mg/kg)	0	0
NO_3^- (mg/kg)	13.67 ± 0.12	26.11 ± 0.47
NH_4^+ (mg/kg)	4.94 ± 5.73	0.26 ± 0.29

Values represent the mean ± standard deviation ($n = 3$)

WHC, water holding capacity; *DOC*, dissolved organic carbon

The headspace of the serum vials was alternately evacuated and refilled with high purity helium (99.999%) four times to create a completely anaerobic environment. All vials were incubated at 25 ℃ for 7 days. The headspace of serum vials was sampled every 4 h and analyzed for N_2O and N_2 concentrations in a robotized incubation system as described by Molstad [26]. To characterize the ratio of N_2O in a mixture of nitrogen elements, we calculated an N_2O production index (expressed as $N_2O/(N_2O+N_2)$) as described in previous literature [12]. Soil pH, dissolved organic carbon (DOC), nitrate-nitrogen, ammonium-nitrogen, nitrite-nitrogen, and soil water content were detected (see Supplementary Material for details).

DNA Extraction and Quantification of Denitrifying Genes

DNA was extracted from 0.3 g of frozen soil as described previously [27, 28]. DNA from soil samples was used as template for quantitative amplification (qPCR) of 16S rRNA genes and *narG*, *nirK*, *nirS*, and *nosZ* genes, which were performed on a Light Cycler 96 system (Roche, Basel, Switzerland). Detailed PCR conditions were performed as previously described [13].

Community Analysis by 16S rRNA Gene Sequencing

A library of V3-V4 regions of the 16S rRNA gene amplicons was constructed for sequencing on the MiSeq platform (Illumina Inc., USA). Quality control and further processing of sequences were performed as described in the previous literature [29]. Representative operational taxonomy units (OTUs) were selected by UPARSE's default [30]. In addition, reference-based chimera detection was performed using UCHIME [31] against the RDP classifier training database (v9) [32]. Finally, the OTU table was generated by mapping quality-filtered reads to the obtained OTUs with the Usearch [33] global alignment algorithm at a 97% cutoff. Representative sequences for each OTU were submitted to the online RDP classifier (RDP database version 2.10) to determine the phylogeny with a cutoff of 80%. The alpha and beta diversity were analyzed using the QIIME platform (version 1.8) [34].

The functional gene compositions were predicted based on 16S rRNA gene sequences by using PICRUSt analysis [35]. Variation significance among the groups was conducted with multivariate analysis of variance (MANOVA) in MATLAB 2018a (MathWorks Inc., USA). Linear discriminant analysis effect size (LEfSe) analysis was performed using parameters of $p < 0.05$ and LDA score 3.5 [36]. The significant differences in all measured data in the soil samples were analyzed with two-way ANOVA.

Isolation and Identification of Denitrifying Bacteria

Three grams of BF or FF from the N250 + G treatment was diluted 1:10 in 0.85% saline. Each of five duplicate plates was spread individually by sequentially diluting soil suspensions on 1/10 TSA medium (Merck, Germany) and incubated in parallel under aerobic and anaerobic conditions at 28 °C. All single, well-spaced colonies were selected and purified, aiming at obtaining as large a variation of bacterial isolates as possible. Enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) was conducted for the genotyping of all isolates [37]. Representative strains for different ERIC fingerprints were selected for 16S rRNA gene amplification using universal eubacterial primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGYTACCTTGTTACGACTT-3′) and sequencing to identify the taxonomy.

Measurement of the Denitrification Function of Bacterial Isolates

The vials containing 30 mL of medium were inoculated with bacterial isolates and anaerobically incubated. The nitrogen transformation during denitrification was measured. Specifically, the N_2O and N_2 produced from bacterial liquid cultures were measured using the robotized incubation system [38]. Based on the results of denitrifying gene prediction, 0.505 g L^{-1} KNO_3 and 0.1 g L^{-1} $NaNO_2$ were used as electron acceptors in the medium for isolates of *Castellaniella* and *Rhodanobacter*, respectively.

Castellaniella sp. OFA38 cultures in TSB medium were centrifugally collected, suspended in Ringer solution (sodium chloride 9 g, potassium chloride 0.4 g, anhydrous calcium chloride 0.25 g in 1 L pure water), and added to BF or FF with 10^7 cells/g soil. The N_2O and N_2 from the soil were measured using the robotized incubation system.

Results

Physicochemical Properties of Soil Samples

The soils of BF and FF had different physicochemical properties, as shown in Table 1. The values of water content, water hold capacity (WHC), and DOC and ammonium content were higher in BF than in FF, but the pH and nitrate content were lower in BF than in FF. Most nitrate in the samples of the N250 group remained at the end of anaerobic incubation (Table S1), and this group showed a decrease in pH. In contrast, supplementation with glucose resulted in an approximately complete reduction in nitrate and increased pH in both soils. There was no accumulation of nitrite except for a low level in the N250 group of BF. In addition, after

incubation, the ammonium content in BF was higher than that in FF regardless of the conditions.

Gas Kinetics During Anaerobic Incubation

The N_2O concentrations in the vials of the control group (CK) for both soils were very low throughout the incubation period. However, their N_2O kinetics showed a similar trend, with the N_2O concentration slowly peaking and then decreasing during the incubation period. No obvious NO_2 accumulation in FF was observed in CK after 64 h (Fig. 1a). In the N250 group, the N_2O in BF constantly increased during the incubation period, while it slightly increased early on but gradually declined after 96 h of incubation in FF (Fig. 1b). In the N250 + G group, N_2O accumulation in BF increased drastically before 80 h and thereafter quickly decreased. However, much less N_2O accumulated in FF and gradually decreased after 112 h (Fig. 1c).

The difference in the accumulation area under the N_2O dynamic curve in BF and FF was significant ($p < 0.037$, two-way ANOVA) after 96 h in all treatments. The total N_2O accumulation area in BF was 2.3–6.2 times higher than that in FF depending on the treatments (Fig. 1d–f).

The accumulation of N_2 production by BF and FF in the CK and N250 groups increased linearly during the incubation period (Fig. 1g, h). In CK, there was no difference in N_2 accumulation between these two types of soil, while in the N250 and N250 + G groups, N_2 accumulation in FF was higher than that in BF (Fig. 1g–i).

In addition, the area under the dynamic curve of $N_2O + N_2$ produced in BF and FF increased over time throughout the incubation period (Fig. S1a–c). The $N_2O/(N_2O + N_2)$ ratio of each type of soil was significantly stimulated by

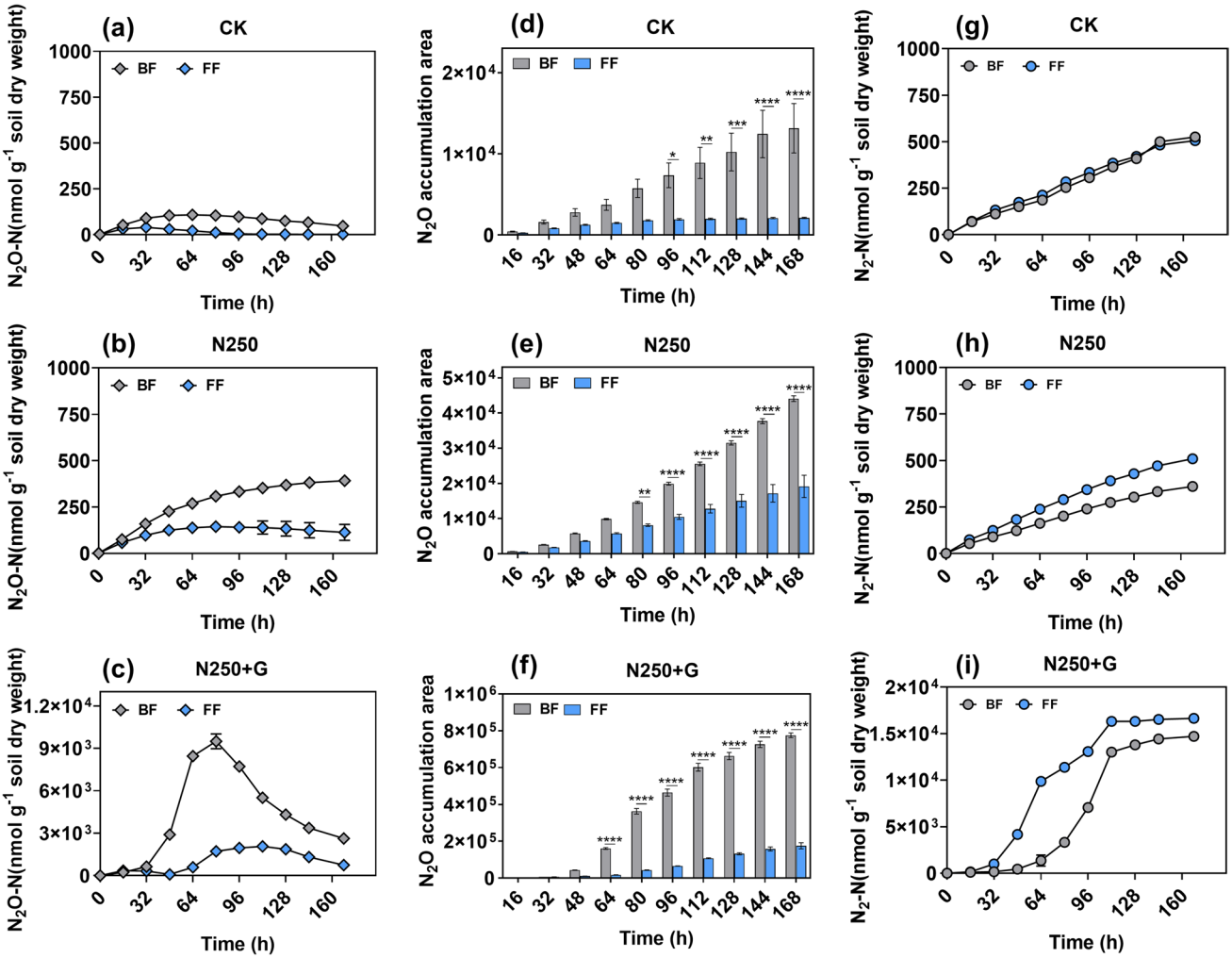

Fig. 1 Kinetics of N_2O and N_2 during anaerobic incubation. Kinetics of N_2O (**a**–**c**), peak area of N_2O accumulation (**d**–**f**), and kinetics of N_2 (**g**–**i**) in three different treatments. Bars indicate means, and error bars indicate the SEM. Differences in the peak area of N_2O accumulation between BF and FF were calculated via a two-way ANOVA. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$

nitrate addition, whereas it declined with glucose addition (Fig. S1d).

In general, N_2O accumulation and $N_2O/(N_2O+N_2)$ in BF were consistently higher than those in FF regardless of the initial levels of nitrate and glucose.

Quantity of Denitrifying and 16S rRNA Genes

The copy numbers of the 16S rRNA (Fig. 2a), *nirK* (Fig. 2c), and *nosZ* (Fig. 2d) genes were significantly higher in FF than in BF in the CK and N250 + G groups. These three genes were also higher in FF in the N250 group, although the difference was not significant ($p > 0.05$). The copy numbers of the *narG* and *nirS* genes (Fig. 2b, c) were significantly higher in FF than in BF for all treatments. The ratio of *narG*/*nosZ* (Fig. 2e) was not significantly different in BF and FF, except for the N250 + G group. The cumulative values of *nirK*/*nosZ* and *nirS*/*nosZ* (Fig. 2f) and ratios of *narG*, *nirS*, and *nosZ* to 16S rRNA (Fig. S2a, c, d) showed no significant differences in BF and FF in all treatments (see Supplementary Material for details).

Variations in Microbial Community Structures

In 18 samples, a total of 364,452 high-quality 16S rRNA gene sequences were clustered into 6536 representative OTUs. There was no significant difference in the Shannon index between BF and FF (Fig. S3a). The trajectory of the community structure in the three-dimensional PCoA plot based on the Bray–Curtis distance showed divergence between BF and FF and the influence of nitrogen and carbon addition on the bacterial communities (Fig. 3a). The MANOVA test results confirmed that the structures of the bacterial communities of the two soils were significantly separated (****$p < 0.0001$, MANOVA test). The community structures of the CK and N250 groups were more similar (Fig. 3b) but significantly different from those of the N250 + G group within the same type of soil (***$p < 0.001$, ****$p < 0.0001$, MANOVA test).

There were nine dominant phyla, including Actinobacteria, Proteobacteria, Acidobacteria, Bacteroidetes, Chloroflexi, Firmicutes, Gemmatimonadetes, Planctomycetes, and Nitrospirae, and 12.1 ~ 13.6% of reads were unclassified

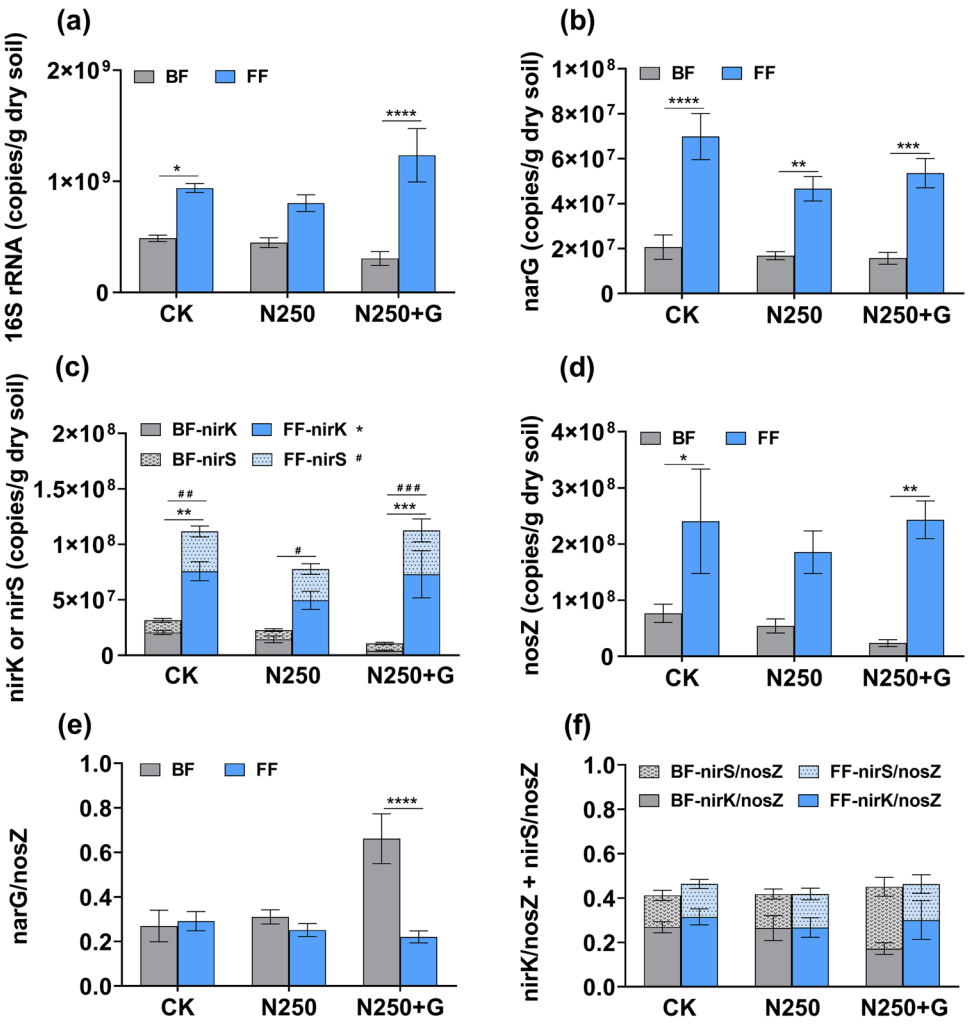

Fig. 2 Quantity of denitrifying genes and ratios of denitrifying functional genes to *nosZ*. 16S rRNA (**a**), *narG* (**b**), *nirK* or *nirS* (**c**), *nosZ* (**d**), *narG*/*nosZ* (**e**), and *nirK*/*nosZ* + *nirS*/*nosZ* (**f**). Bars indicate means, and error bars indicate the SEM. Differences in the ratios were calculated via a two-way ANOVA. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. # represents the significant difference in the abundance of *nirS* between BF and FF. #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$. The *nirK*/*nosZ* + *nirS*/*nosZ* represent the cumulative value of *nirK*/*nosZ* and *nirS*/*nosZ*

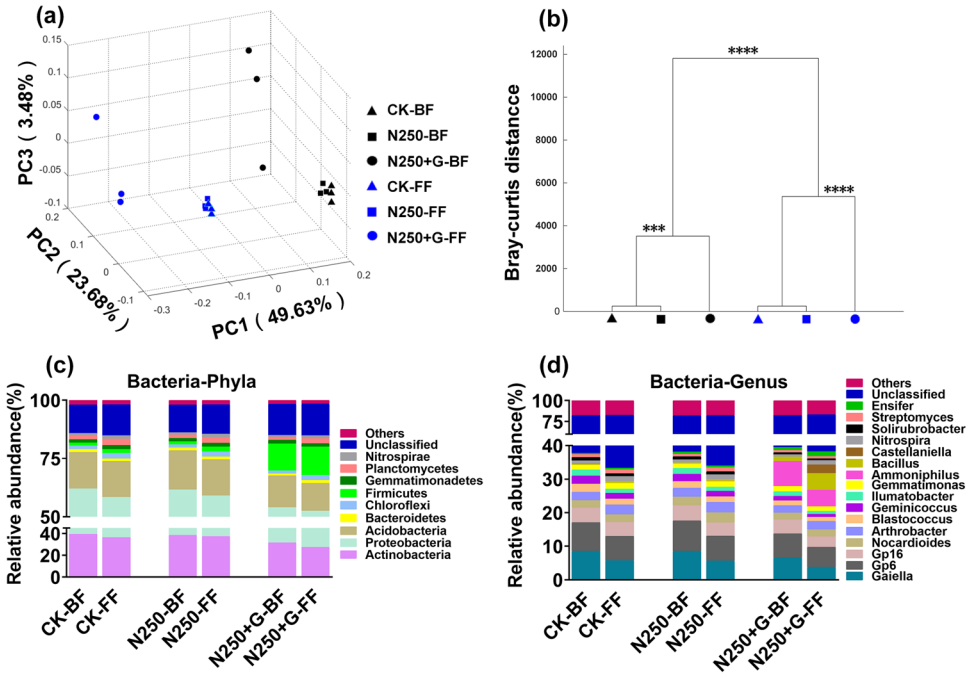

Fig. 3 Comparison of microbial community structure and composition in soils with different treatments. Tri-dimensional PCoA plot of the microbial community based on the Bray–Curtis distance (**a**), and clustering based on Bray–Curtis distance calculated with a MANOVA test, $***p < 0.001$, $****p < 0.0001$ (**b**). Distribution of dominant taxa in BF and FF at the phylum level (**c**) and at the genus level (**d**)

bacteria (Fig. 3c). Notably, glucose specifically enriched bacteria belonging to Firmicutes.

The dominant genera (relative abundance ≥ 1%) in BF and FF were approximately 37.6 ~ 39.5% and 33.3 ~ 38.2%, respectively, including *Gaiella*, *Gp6*, *Gp16*, *Nocardioides*, *Arthrobacter*, *Blastococcus*, *Geminicoccus*, *Ilumatobacter*, *Gemmatimonas*, *Ammoniphilus*, *Bacillus*, *Castellaniella*, *Nitrospira*, *Solirubrobacter*, *Streptomyces*, and *Ensifer* (Fig. 3d). The relative abundance of these genera in each type of soil was influenced by nitrogen and carbon sources. In particular, when supplemented with nitrate and glucose, *Ammoniphilus* was enriched in both soils, while *Castellaniella* and *Bacillus* were more enriched in FF than in BF.

Prediction of Functional Denitrifiers

Out of 6536 OTUs identified in all samples, 1197 and 732 were predicted to contain the *norB* and *nosZ* genes, respectively. The Shannon index for *norB*-containing denitrifying bacteria in BF was significantly higher than that in FF in the presence of sufficient carbon and nitrogen, although that in the CK and N250 groups was not significantly different (Fig. S3b). However, the Shannon diversity of *nosZ* in FF was significantly higher than that in BF in all treatments (Fig. S3c). The Bray–Curtis distance showed that the community structures of *norB*- (Fig. S4a, b) or *nosZ*- (Fig. S4e, f) containing bacteria in BF and FF were significantly different. The beta diversity plot demonstrated that the community structures of *norB*- or *nosZ*-containing bacteria of the CK and N250 groups were significantly different from those of the N250 + G group within the same type of soil (Fig. S4b, f) ($**p < 0.01$, $***p < 0.001$, MANOVA test).

norB-Containing Bacteria in Two Types of Soils

The *norB*-containing OTUs were affiliated with 3 dominant phyla, namely, Acidobacteria, Actinobacteria, and Proteobacteria, as well as other rarer phyla (relative abundance of phylum < 1%) (Fig. S4c). The *norB*-containing OTUs were affiliated with 12 dominant *norB*-containing genera (Fig. S4d). Among these genera, 7 lacked the *nosZ* gene. Five of these 7 genera, *Candidatus Solibacter*, *Dokdonella*, *Kaistobacter*, *Lysobacter*, and *Phyllobacterium*, were more abundant in BF than in FF. Another two genera, *Thermomonas* and *Castellaniella*, were more abundant in FF than in BF only in the N250 + G group. Five genera were predicted to contain both the *norB* and nosZ genes. Among them, *Azospirillum*, *Dechloromonas*, *Devosia*, and *Rhodonobacter* were more abundant in BF than in FF.

At the OTU level, only OTU114930 of *Azospirillum*, which was enriched in BF in N250 + G, showed a negative correlation with the $N_2O/(N_2O + N_2)$ ratio, while 21 OTUs enriched in BF (especially the OTUs affiliated with *Kaistobacter* and *Rhodonobacter*) showed a positive correlation with the $N_2O/(N_2O + N_2)$ ratio (Fig. 4a). In contrast, 17 OTUs enriched in FF showed a negative correlation with the $N_2O/(N_2O + N_2)$ ratio (see Supplementary Material for more details).

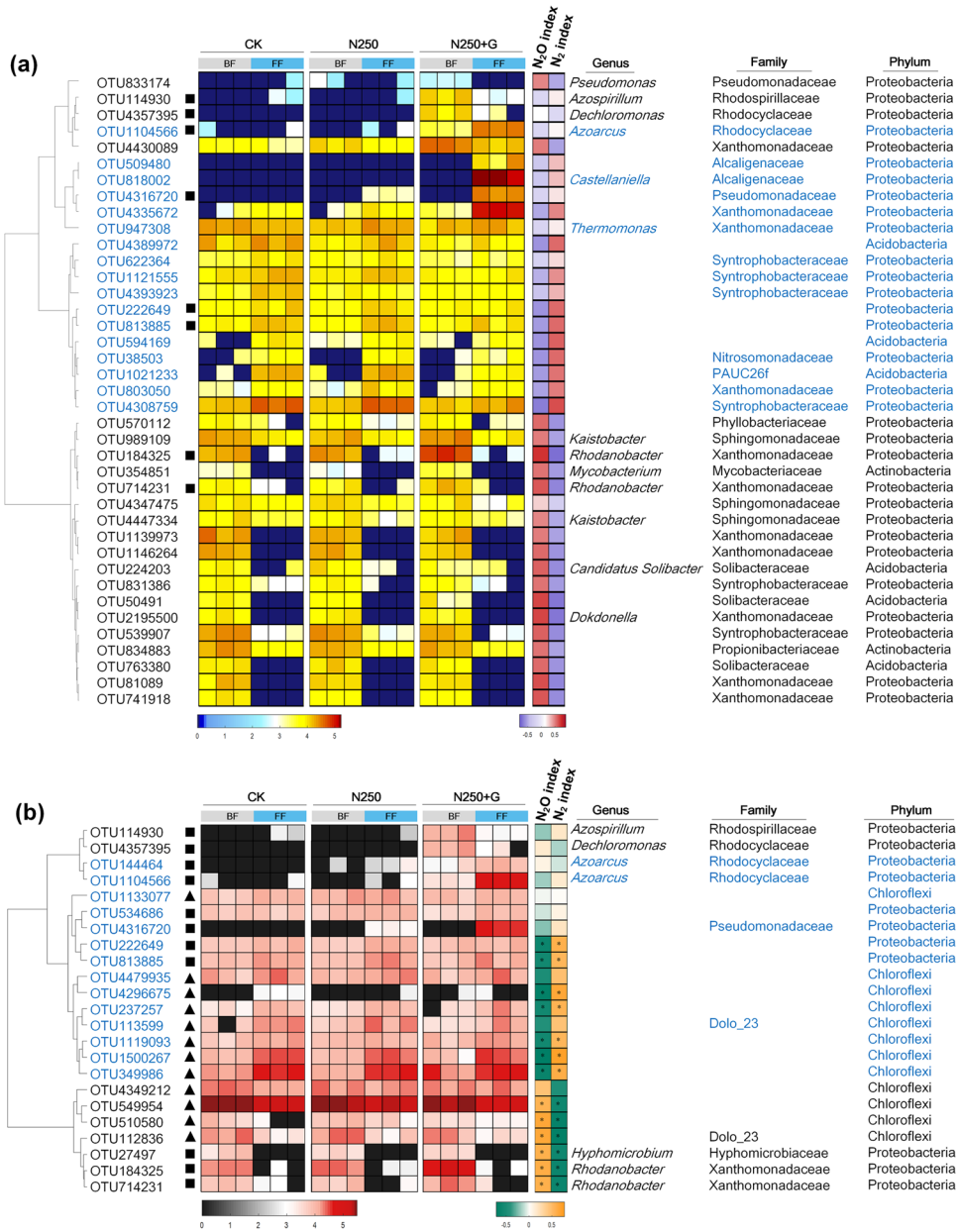

Fig. 4 Heatmap of key denitrifiers identified for discriminating BF and FF. Heatmap of the 39 key OTUs containing *norB* (**a**). Heatmap of the 23 key OTUs containing *nosZ* (**b**). OTUs marked with "■" represent those containing both *norB* and *nosZ*, whereas OTUs marked with "▲" represent OTUs containing *nosZ* but lacking *norB*. OTUs without marks represent OTUs containing *norB* but lacking *nosZ*. The colors of the spots in the left panel represent the relative abundance (log-transformed) of the OTUs in each sample, while those in the right panel denote the *R*-value of Spearman's correlation

nosZ-Containing Bacteria in Two Types of Soils

The *nosZ*-containing OTUs were affiliated with Bacteroidetes, Chloroflexi, Proteobacteria, Verrucomicrobia, and some rare phyla (Fig. S4g). Out of 7 dominant genera, *Caldilinea* and *Opitutus* containing *nosZ* but lacking *norB* and *Azoarcus* containing both *nosZ* and *norB* were more abundant in FF than in BF when sufficient carbon and nitrogen were provided (Fig. S4h).

At the OTU level, two OTUs of *Rhodanobacter*, an OTU of *Hyphomicrobium*, and three OTUs of *Chloroflexi* were enriched in BF and significantly positively correlated with the $N_2O/(N_2O+N_2)$ ratio (Fig. 4b), while several OTUs of Chloroflexi and Proteobacteria were enriched in FF and significantly negatively correlated with the $N_2O/(N_2O+N_2)$ ratio.

Denitrification Functions of Representative Bacterial Isolates

Among the 437 isolates from BF, 25 were identified as bacteria belonging to *Rhodanobacter*. These isolates were clustered into 18 strains by ERIC typing. Fifteen of these strains had 100% 16S rRNA gene sequence similarity with the predominant *Rhodanobacter* OTU in the BF bacterial community, while the remaining 3 strains had high sequence similarity with another much less abundant *Rhodanobacter* OTU (Table S2). Seventy-two of the 456 isolates obtained

from FF belonged to *Castellaniella* and were clustered into 8 different strains by ERIC typing. All of these strains had 100% 16S rRNA gene sequence similarity with the predominant *Castellaniella* OTU in the FF bacterial community (Table S3).

The denitrifying functions of eighteen representative isolates of *Rhodanobacter* and eight representative isolates of *Castellaniella* were measured. The results showed that most isolates of *Rhodanobacter*, which correspond to the most abundant OTU in the BF, were only capable of reducing nitrite, rather than reducing nitrate, to produce N_2O without any further reduction, resulting in the obvious accumulation of N_2O (Fig. 5a). Only a slight reduction in N_2O was present in the culture of 2 isolates of *Rhodanobacter*, which corresponds to a less abundant OTU in the BF (Fig. 5a). However, all representative isolates of *Castellaniella* effectively reduced nitrate to N_2 without significant accumulation of N_2O (Fig. 5b).

Reducing Soil N_2O Generation by Augmenting *Castellaniella* Isolate.

Castellaniella sp. OFA38, one of the 8 different strains of *Castellaniella* isolated from FF, rapidly reduced N_2O to N_2 and efficiently reduced N_2O emissions in the microcosm experiment (Fig. 6). During the incubation period of 64 h, the N_2O accumulation area in inoculated BF decreased by 97.5% compared to that without inoculation (Fig. 6a). Since much less N_2O accumulated in the uninoculated FF, inoculation with *Castellaniella* sp. OFA38 in this soil showed only a 26.4% lower accumulation of N_2O (Fig. 6b).

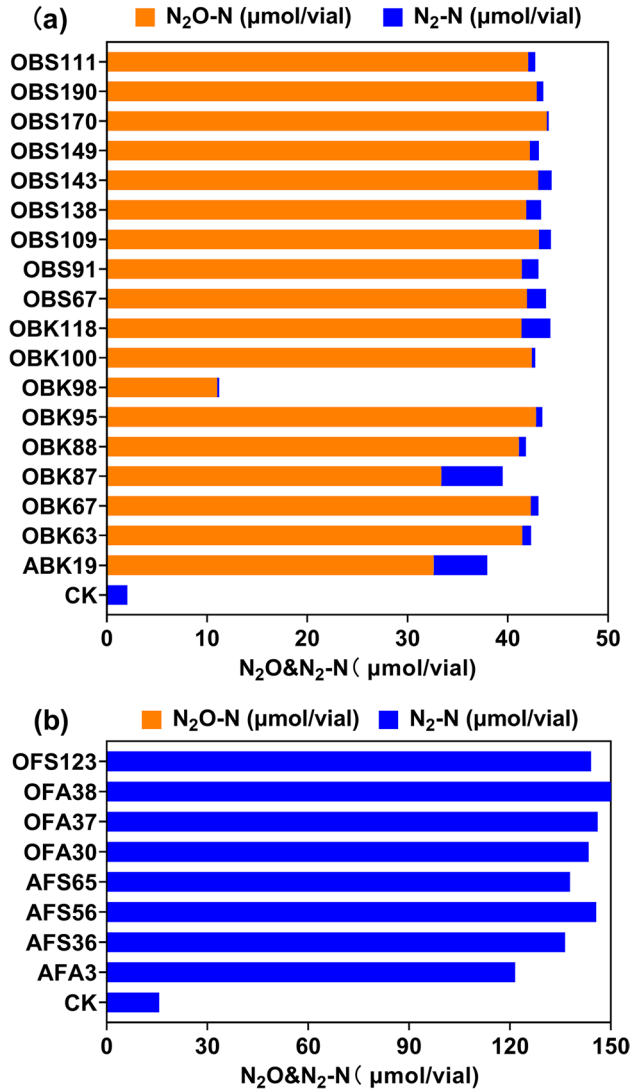

Fig. 5 Denitrification functions of selected isolates. The N_2O and N_2 production during incubation of eighteen strains of *Rhodanobacter* isolated from BF (**a**) and eight strains of *Castellaniella* isolated from FF (**b**)

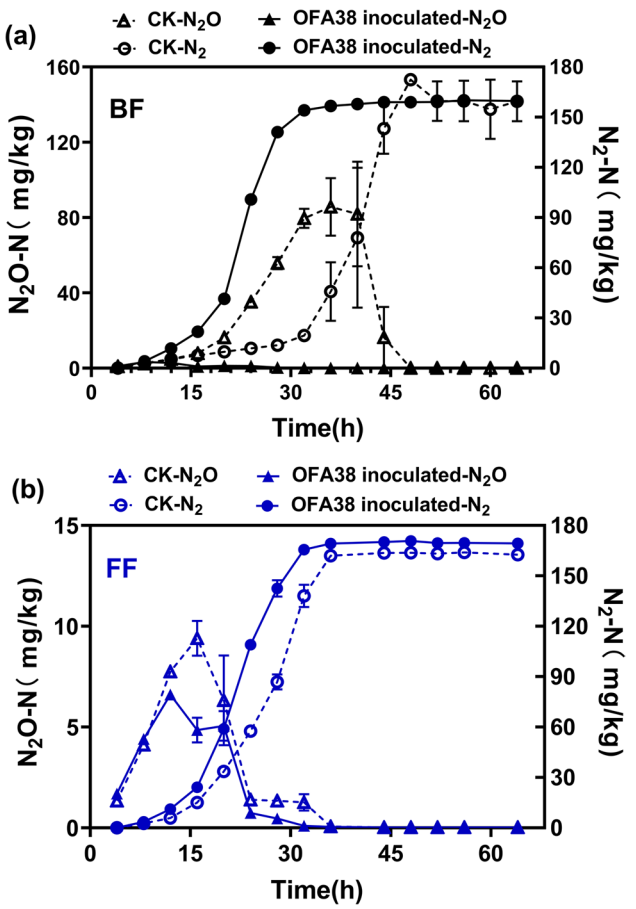

Fig. 6 Effects of *Castellaniella* sp. OFA38 on N_2O emissions in the microcosm experiment. N_2O and N_2 measured in the vials of BF (**a**) and FF (**b**) during anaerobic incubation

Discussion

Effect of Nitrate and Glucose on Bacterial Communities

The physicochemical properties shaped the unique bacterial communities in different soils. For example, chronic N inputs influenced soil microorganisms and denitrifying communities [18, 39]. However, the temporary addition of nitrate did not significantly change the microbial community structure in either soil in this study. This result is probably because short-term denitrification metabolism could not change the biomass of the corresponding bacteria in the soil.

However, many studies have shown that soil carbon availability, as the most profound factor, affects the distinct variation in the structure of the soil microbial community [40, 41]. Consistent with a previous study, glucose amendment in this study significantly increased the relative abundance of copiotrophic organisms, such as Firmicutes (*Ammoniphilus* and *Bacillus*) in two types of soil and Proteobacteria (*Castellaniella*) in FF, which tend to be found in nutrient-rich environments [42].

Effect of Soil Nitrogen and Carbon on N_2O Accumulation

The nitrate in BF and FF was almost completely reduced to N_2O and N_2 over 7 days of incubation in the N250 + G group, which is consistent with a pulse emission of N_2O produced due to strong denitrification immediately after fertilization and irrigation in the field [43] and which shows that both C and N might be limiting factors of denitrification activity in these two types of soils in terms of their denitrification potential [44]. N_2O accumulation peaked before 7 days in all treatments. In this study, the short period 7-day measurement would demonstrate the denitrification activity of two soils with their original microbial community. Moreover, measuring denitrification under different carbon and nitrogen conditions would help fully elucidate the relationship between the soil microbiome and N_2O emissions.

Many studies indicate the effects of nitrogen and carbon on denitrification. In terms of each type of soil, the amount of N_2O produced in the N250 group was higher than that in the CK group because pulses of nitrate may have a substantial and immediate impact on N_2O emission without a prior change in denitrifying community structure [45]. A few previous studies have shown that the reducing power for nitrogen reduction is generated from carbon oxidative catabolism [46, 47]. Therefore, the accumulation of N_2O during denitrification was influenced by carbon and nitrogen availability [48]. As expected in this study, much more N_2O accumulated when glucose was added, although the reduction to N_2 was also enhanced. Glucose, as a simple substrate, can be easily utilized by microorganisms and increases soil microbial activity, leading to nitrate depletion and an increased consumption of N_2O [49].

Nevertheless, nitrate and DOC levels were not direct proximal factors determining the difference in N_2O accumulation between BF and FF. Relative to BF, FF showed a higher efficiency in consuming N_2O under all nitrogen and carbon conditions used in this study.

Effect of Denitrifying Gene Abundance on N_2O Accumulation

Previous studies demonstrated that N_2O flux correlated with the quantity of denitrifying genes [50, 51]. For example, one study found that a strong correlation exists between *nirS* gene abundance and potential N_2O emissions [52].

Our findings indicate that the quantity of the denitrifying genes *narG*, *nirK*, *nirS*, and *nosZ* was generally higher in FF, which implies that the potential for the production and reduction of N_2O in FF was stronger than that in BF. Nevertheless, the lack of differences in the ratios of nitrous oxide producing genes to reducing gene, such as the cumulative value of *nirK*/*nosZ* and *nirS*/*nosZ*, between the two types of soil does not explain the consistently higher levels of accumulated N_2O in BF than in FF. However, higher rates of both the generation and reduction of N_2O do not necessarily result in higher N_2O accumulation because N_2O emissions should be the result of a net balance between generation and consumption [51]. A study on dazomet-fumigated FF [15] indicated that N_2O emissions were significantly correlated with NH_4^+, dissolved amino acids, and microbial biomass nitrogen, but uncorrelated with functional gene abundance. Another study on differently fertilized soils in a vegetation greenhouse elucidated that the difference in N_2O emissions between the soils was not because of the cumulative nitrate content or the quantity of denitrifying gene transcripts [17]. However, previous studies did not address the importance of the balance of nitrous oxide metabolism between the generation and reduction.

Effect of Denitrifier Composition on N_2O Accumulation

Studies have revealed that N_2O emissions are anchored in the taxonomic composition of denitrifier communities [17, 52]. For example, one study indicated that the taxonomic composition of *nirK*- and *nirS*-type denitrifier communities influenced the denitrifier enzyme activity of the soils [53]. Liu et al. [38] found eight *Thauera* strains divided into two

distinct denitrification regulatory phenotypes with different N_2O accumulation. Specific denitrification activity differs among individual strains. Therefore, it provides a good explanation for the different denitrifier communities having varied denitrification activities.

In this study, N_2O-generating bacteria containing *norB* but lacking *nosZ* were found at much higher levels in BF than in FF. This may be a reason for the high levels of accumulated N_2O in BF. In addition, microbial diversity also plays a role in N_2O emissions [54]. In this study, a higher diversity of *nosZ* but a lower diversity of *norB* were found in FF than in BF, as shown by the Shannon index. This may be beneficial for N_2O reduction in FF due to the presence of more diverse highly efficient N_2O-reducing bacteria in FF.

Rhodanobacter had been reported to be the most abundant genus among all denitrifiers in a chronic fertilization experiment performed in a black agricultural soil field [55]. The unique physicochemical properties of fertilized black soil probably establish a niche for *Rhodanobacter*, as was observed for BF in this study. A strong correlation between N_2O accumulation and the abundance of *Rhodanobacter* OTUs has been reported for conventionally fertilized soil [17]. In addition, three denitrifiers belonging to *Rhodanobacter* isolated from acidic soil strongly accumulated N_2O under denitrifying conditions [7].

Bacteria from *Castellaniella* were also identified as key denitrifying bacteria in this study. The *Castellaniella* bacterium was reported to effectively reduce nitrate to gaseous nitrogen in sewage [56]. However, the N_2O production and reduction capacities of *Castellaniella* in the soil environment have not been reported. In this study, we report the first observation of highly efficient N_2O-reducing bacteria of *Castellaniella* in farmland soil.

N_2O Metabolism of Isolates Corresponding to the Key Bacteria in Soil

PICRUST prediction provides clues to finding denitrifying microorganisms, but the actual function of the bacteria must be determined by function verification. In this study, we isolated bacteria from the two soils by culturomics and screened denitrifying isolates belonging to the predominant genera for physiological function measurements. Among 18 representative *Rhodanobacter* isolates, only 2 isolates corresponding to the less abundant OTU of *Rhodanobacter* slightly reduced N_2O, and the remaining isolates could not reduce N_2O. Considering that high proportions of denitrifiers belonged to *Rhodanobacter* in BF, the results of their denitrifying function well explained the reason for the strong N_2O accumulation in BF. Meanwhile, the highly efficient reduction of N_2O in eight isolates of *Castellaniella* obtained from FF provided evidence that the predominant denitrifying bacteria of *Castellaniella* contributed to the efficient reduction of N_2O in FF. In addition, the isolates of *Castellaniella* sp. OFA38 rapidly and efficiently reduced N_2O emissions when inoculated into BF, exhibiting great potential of this bacterium to mitigate N_2O emissions from various farmland soils.

Conclusions

This study demonstrates that two types of typical Chinese agricultural soils show different N_2O accumulation patterns during anaerobic incubation, with BF accumulating more N_2O. Investigations of the microbial mechanism show that the difference in N_2O accumulation is not due to the differences in nitrate and DOC contents or the difference in the quantity of denitrifying genes in soils. The different compositions of denitrifiers in the two types of soil could be the main reason for the distinct N_2O metabolism. As proven by isolates, bacteria of the most abundant members of *Rhodanobacter* in BF were only capable of reducing nitrite to N_2O and accumulating N_2O. However, bacterial isolates of *Castellaniella* enriched in FF showed a capacity for the rapid reduction of N_2O to N_2. The findings of this study provide new insights into strategies for mitigating N_2O emissions in different agricultural soils by regulating the composition of the denitrifier community via bioaugmentation to enhance the N_2O sink in the soil.

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Author Contribution Q.W. and X.Z. conceived and designed the study; Q.W., M. J., S.Y., J.L., X.W., and B.L. designed the methodology; Q.W. and S.Y. collected the data and performed the data analysis; X.J. and B.L. were involved in the discussion of results; Q.W. wrote the first draft of the manuscript. Q.W. and X.Z. contributed to revisions. All authors contributed to the drafts and gave the final approval for publication.

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Data Availability The raw Illumina sequence data generated in this study have been deposited to the GenBank Sequence Read Archive (SRA) database in the National Center for Biotechnology Information (NCBI) under the accession number PRJNA755188. 16S rRNA gene sequences for selected predominant bacterial strains were deposited in GenBank under accession numbers MZ824722–MZ824747.

Declarations

Conflict of Interest The authors declare no competing interests.

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