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Top-down effects of protists are greater than bottom-up effects of fertilisers on the formation of bacterial communities in a paddy field soil

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ABSTRACT

Communities at any trophic level along the food chain are determined by simultaneous top-down (predators) and bottom-up (nutrients) effects; however, we still lack an understanding of this concept in the soil ecosystem. Here, we aimed to reveal the contributions of the top-down and bottom-up factors on the formation of paddy field bacterial communities. The position of an indigenous bacterial community at the trophic level was centred between soil nutrients (chemical and organic fertilisers [CF and OF, respectively], i.e., bottom-up factors) and bacterial predators (phagotrophic protists, i.e., top-down factors) in a paddy field soil. A 16S rRNA gene amplicon sequencing method was used to evaluate the top-down and bottom-up effects on the bacterial community composition. The results showed that the top-down effects of protists were greater than the bottom-up effects of the applied fertilisers on the formation of bacterial communities. The presence of protists caused the formation of a distinct bacterial community by affecting several bacterial species mainly belonging to *Proteobacteria* and *Bacteroidetes*. Among the bottom-up factors, OF significantly affected the bacterial beta diversity, while CF did not. The numbers of bioindicator genera that were associated with the top-down factors were 4.4 and 3.7 times higher than those associated with the bottom effects of CF and OF, respectively. Overall, we provided unique information on the importance of protists in regulating bacterial communities in paddy field soil, which is likely to affect bacterial activities and agricultural productivity.

1. Introduction

The regulation of microbial communities with biotic and abiotic factors has been a hot topic for decades. Ecological literature reveals two main concepts related to regulating communities: bottom-up and top-down (Elton 1927; Hairston et al., 1960; Leroux and Loreau 2015). The bottom-up concept refers to organisms being resource-limited, and resources shape the communities at each trophic level (Elton 1927). The top-down concept, on the other hand, refers to organisms being predator-regulated, and upper-level predators determine the communities of lower-level organisms (Hairston et al., 1960). Like all of the other organisms, the fate of the bacterial communities also depends on the bottom-up effects of resources (i.e., nutrients) and top-down effects of predators. The bacterial communities of marine ecosystems, which were initially thought to be controlled by bottom-up effects, have been shown to be top-down regulated with contributions from bottom-up factors (Weinbauer et al. 2003, 2007; Chow et al., 2014; Teira et al., 2019), while bottom-up factors play a larger role in freshwater ecosystems (Jardillier et al., 2005; Berdjeb et al., 2011). However, we still lack an understanding of this concept in the soil ecosystem. An understanding of the relative roles of the bottom-up and top-down factors is critical to better estimate the soil ecosystem dynamics and soil fertility.

Soil fertility, which mainly depends on the activities of soil bacterial communities (Dobermann et al., 2000; Kirk 2004), has far-reaching importance, particularly for rice production, which can be better explained with a Japanese proverb: *"Rice grows with soil fertility, while upland crops depend on fertilisation"*. Rice is one of the most important crops in the world, with flooded paddy fields accounting for over 85% of

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rice production. Unlike upland fields, paddy field characteristics change seasonally throughout the year with multiple drying-wetting cycles (Kirk 2004), which leads to the formation of distinct microbial communities (Abdallah et al., 2019; Ji et al., 2020). During the non-cultivated season in winter, paddy fields are not flooded and consist of upland field-like bacterial communities (Kirk 2004). When the soil is flooded just before the rice-growing season, the bacterial community composition shifts in order to adapt the flooded conditions, in other words, bacterial communities form a community with distinct members from those under non-flooded conditions (Zhou et al., 2014; Breidenbach et al., 2016; Lu et al., 2018). Fertiliser application (organic or chemical) follows flooded anoxic conditions and plays an important role in the formation of bacterial communities, as different types and amounts of fertilisers have distinct impacts on paddy field bacterial communities (Daquiado et al., 2016; Yang et al., 2019; Li et al., 2019). Therefore, the initial formation of the bacterial communities in the flooded paddy fields is variable depending on the bottom-up effects of the nutrients derived from the applied fertilisers. The main bottom-up effects of chemical fertilisers on soil bacterial communities correlate with the increase in inorganic nutrients such as N, P, and K (Geisseler and Scow 2014). Organic fertilisers, on the other hand, have profoundly different bottom-up effects on the bacterial composition than chemical fertilisers, mainly due to an increased amount of organic carbon and decomposable organic materials (Mader 2002).

Most bacterial predators are aquatic organisms, and thus, the top-down effects of the bacterial predators are mainly restricted to the water-filled pores in the soil ecosystem (Rutherford and Juma 1992). Once a paddy field is flooded, the water-filled pores are increased, which widens the bacterial predator domain and extends their predation efficiency (Hutner 1987; Rutherford and Juma 1992). Therefore, the bacterial communities in the flooded paddy fields are expected to be faced with more significant top-down effects than those in the fields with limited water-filled pores. Among the top-down predators, phagotrophic protists (hereafter referred to as “protists” for simplicity) are reported to be the major bacterial predators (Crotty et al., 2012; Trap et al., 2016). Protists are an integral part of the microbiota (Clarholm 1985; Bonkowski 2004) and one of the main eukaryotic groups in paddy fields (Murase et al., 2015; Asiloglu et al. 2015, 2021a; Asiloglu and Murase 2016, 2017). Protist predation alters the bacterial community structure (Rønn et al., 2002; Kreuzer et al., 2006; Flues et al., 2017) and stimulates bacterial activities in the soil ecosystem (Bonkowski and Brandt 2002; Hünninghaus et al., 2017; Gao et al., 2019), including paddy field soil (Murase et al., 2006; Murase and Frenzel 2007; Asiloglu et al. 2020, 2021b), and increases the growth of plants (Bonkowski 2004; Gao et al., 2019), including rice (*Oryza sativa* L.) (Kreuzer et al., 2006; Herdler et al., 2008; Asiloglu et al. 2020, 2021b). Rather than random patterns, protists selectively feed on bacteria at the genus (Singh 1941, 1942) and even at the species level (Murase and Frenzel 2008). Therefore, the bacterial species targeted by protists markedly decrease (Saleem et al., 2012), while the other bacterial species can benefit from protist predation through the nutrients released from the biomass of preyed upon bacteria and reduced competition (Moore et al., 2003; Jousset et al., 2008; Flues et al., 2017).

Population and composition of communities at any trophic level along the food chain are determined by simultaneous top-down and bottom-up effects; however, to date, the top-down and bottom-up effects on soil bacteria have been separately studied. Roles of fertilisers on controlling bacterial communities have been shown by many scientists in the last decades (Daquiado et al., 2016; Yang et al., 2019; Li et al., 2019). Therefore, we hypothesized that the bacterial communities in paddy field soil are mainly shaped by the impact of bottom-up fertilisers with the smaller influence of the top-down factors. In order to reveal the contributions of the top-down effects of protists and bottom-up effects of chemical and organic fertilisers on the formation of bacterial communities, we created a controlled laboratory environment in which the position of the bacteria at the trophic level is centred between soil nutrients (i.e., bottom-up) and bacterial predators (i.e., top-down). We used paddy field soil under gradationally decreasing redox potential to mimic bacterial formation in the initial flooded conditions of a paddy field. A 16S rRNA gene amplicon sequencing method was used to evaluate the top-down and bottom-up effects on an indigenous and exclusive (protist- and fungi-free) bacterial community that was obtained from paddy field soil. In conflict with what has been previously assumed, our results showed that rather than bottom-up factors (fertilisers), the top-down factors (bacterial grazers) had a more significant impact on the formation of bacterial communities in paddy field soil.

2. Materials and methods

2.1. Microorganisms, fertilisers and soil samples

We studied a mixture of four protists that were previously isolated from a paddy field (Asiloglu et al., 2020): *Vermamoeba vermiformis* (Amoebozoa; Tubulinea) (~20 μm), *Naegleria* sp. (Excavata, Heterolobosea) (~25 μm), *Colpoda steinii* (Alveolata; Ciliophora) (~30 μm), and *Heteromita globosa* (Rhizaria; Cercozoa) (~10 μm). Prior to the experiment, each protist species was separately grown for 2 weeks in sterile amoeba saline solution (Page, 1988) with autoclave-killed bacteria (approximately 10^7 cells mL^{-1} of *Escherichia coli* MG1655) to minimise the coinoculation of live bacteria. After the growth of protists, the cells were centrifuged at 1000 g for 5 min and washed with sterile water 3 times to separate the bacteria from protist cells and to eliminate the nutrients that came from the growth media. The presence of live bacteria in the protist cultures was checked with the following method: 500 μL of the washed protist culture was added to the 1% agar (Wako Pure Chemical Industries Ltd., Tokyo, Japan) - nutrient broth (Eiken Chemical Co. Ltd., Tochigi, Japan) media and checked for bacterial colonies for 7 days. The density of protists was determined by counting at × 200 and × 400 magnifications using an inverted microscope (Nikon Eclipse TE2000-S, Tokyo, Japan). An equal number of cysts of each protist species were added together and stored at 4 °C until used in the experiment. The preparation of protist- and fungi-free indigenous bacterial communities, which was realized by a filtration method (0.8 μm pore size mixed cellulose ester membrane filters [Advantec, Tokyo, Japan]), was performed as described previously (Asiloglu et al., 2020). After that, the filtered (0.8 μm) protist-free bacterial media (50 μL) was cultured in 96-well culture plates for one week, and the absence of protists was confirmed with an inverted microscope at × 100, × 200 and × 400 magnifications (Nikon Eclipse TE2000-S, Tokyo, Japan).

To mimic real paddy field conditions, we applied common types of chemical and organic fertilisers at field application doses generally used in paddy fields. As a chemical fertiliser (CF), a combination of 0.1 g kg^{-1} N as $(NH_4)_2SO_4$, 0.1 g kg^{-1} P_2O_5 as $CaH_4P_2O_8$ and 0.1 g kg^{-1} K_2O as KCl was applied. A mixture of cow manure and rice husk that was obtained from a commercially available product (Akagi Engei, Isesaki, Gunma, Japan) was applied as the organic fertiliser (OF). The amount of applied OF was calculated so that it would include the same amount of N as the CF. The nutrient content of the OF was as follows: C/N ratio, 15; nitrogen, 25 mg g^{-1}; phosphate, 34 mg g^{-1}; potassium, 31 mg g^{-1}. Soil samples were taken from a rice field under drained conditions at Shindori Station in the Field Center for Sustainable Agriculture and Forestry, Niigata University, Niigata, Japan (N37.86, E138.96) on July 7, 2019. The soil was air-dried, sieved (<2 mm), and then stored at 4 °C. The soil sample had the following characteristics: sand, 336 g kg^{-1}; silt, 470 g kg^{-1}; clay, 194 g kg^{-1}; total carbon (TC), 16 mg-C g^{-1}; total nitrogen (TN), 2 mg-N g^{-1}; pH, 5.0 (H_2O); CEC, 150 meq kg^{-1}. Before the experiment, the soil and the fertilisers were sterilised by autoclaving 3 times at 121 °C for 60 min.

2.2. The experimental setup, sampling and physicochemical analyses

The microcosms were established in sterile plastic tubes (volume:

100 mL) with 60 g of sterile paddy field soil and 40 mL of sterile H_2O. The 4 mL of the solution of protist- and fungi-free indigenous bacterial community was added to all microcosms. The microcosms (n = 72) were preincubated for one week under flooded conditions at 25 °C in the dark. This was done to obtain a stable bacterial community (10^8 cells g $soil^{-1}$) before the addition of top-down and bottom-up factors. The number of bacteria on day 0 of the experiment were counted with a plate method as follows: 0.5 g of soil was sampled from random microcosms (n = 9) and diluted with 4.5 mL of sterile H_2O. Appropriate dilutions of this suspension were spread on 1% agar (Wako Pure Chemical Industries Ltd., Tokyo, Japan) - nutrient broth (Eiken Chemical Co. Ltd., Tochigi, Japan) media. The plates were incubated for 7 days at 28 °C, and then bacterial colonies were enumerated. Following the preincubation, the protists (total 10^3 cells g $soil^{-1}$ [approximately 250 cells of each protist species g $soil^{-1}$]), CF, and OF and their factorial combinations were added to the microcosms as described in Table 1. Briefly, the following treatments with nine replications were prepared: Ctrl, control without protists (top-down) and fertilisers (bottom-up); CF, only chemical fertiliser; OF, only organic fertiliser; CF + OF, chemical and organic fertilisers (1:1 N ratio); P, only the mixture of the four protist isolates; P + CF, the protists and chemical fertiliser; P + OF, the protists and organic fertiliser; P + CF + OF, the protists and both the chemical and organic fertilisers. The microcosms were saturated with sterile H_2O and incubated for 21 days under the same conditions as the preincubation period.

The microcosms (n = 3) were destructively sampled 3, 7 and 21 days after the addition of bottom-up and top-down factors as follows: the surface water of the microcosms was removed, and the soil was mixed thoroughly. Immediately, 0.5 g of soil sample was placed into 2 mL DNA extraction tubes and stored at −80 °C until nucleic acid extraction. DNA was extracted using ISOIL for Bead Beating (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions and eluted in TE buffer (50 μL). The rest of the soil samples were stored at 4 °C for physicochemical analysis.

The pH of the soil samples was measured in deionized water at a 1:20 (w/v) mass ratio using a pH metre (Mettler Toledo, FP20). The TC and TN contents in the soil samples were analysed after drying at 105 °C for 24 h using an MT-700 Mark 2 CN analyser (Yanaco, Kyoto, Japan). Available P was extracted from 0.5 g of the soil samples with 0.002 N H_2SO_4 and then colourimetrically analysed by a spectrophotometer (SHIMADZU UV-160A, Japan) according to the Truog, 1930. Exchangeable forms of Ca, Mg, K, and Na in the biochars and soil samples were extracted according to Pansu and Gautheyrou (2006) with neutral 1 M ammonium acetate and measured using the polarised Zeeman atomic absorption spectrophotometer (Za3300, Hitachi High-Tech Ltd., Tokyo).

Table 1
Experimental set-up.

	Treatments							
	Ctrl	CF	OF	CF + OF	P	CF + P	OF + P	CF + OF + P
Indigenous bacterial community	O	O	O	O	O	O	O	O
Chemical fertilizer (CF)		O		O		O		O
Organic fertilizer (OF)			O	O			O	O
Protists (P)					O	O	O	O

*Indigenous bacterial community (108 cells g soil-1), the protist-free exclusive bacterial community obtained from the paddy field soil; chemical fertilizer, NPK; organic fertilizer, cow manure and rice husk; protists (103 cells g soil-1), the mixture of the four isolates.

2.3. Illumina library preparation, bioinformatics, and statistical analyses

Illumina library preparation and all bioinformatics procedures were performed as described previously (Asiloglu et al., 2020). Briefly, the V4 region of the 16S rRNA gene was amplified from the extracted DNA using universal primers (515F and 806R) tailed with Illumina barcoded adapters (Caporaso et al., 2012). After sequencing, the primary analysis of raw FASTQ data was processed using DADA2 in the QIIME2 pipeline (version 2018.11, https://qiime2.org) (Bolyen et al., 2019). All statistical analyses were performed using the R program version 3.6.1 (https://www.r-project.org/) as described in Asiloglu et al., (2020) unless otherwise specified. The rarefied sequences (depth: 10,000) were used to generate the dissimilarity matrices based on the Bray–Curtis distances using the *phyloseq* package at the genus level. The matrices were then used to calculate the permutational multivariate analysis of variance (PERMANOVA) with the *adonis* function in the vegan package. NMDS analysis was performed based on the Bray–Curtis dissimilarity index at the genus level using the *env* function in the vegan package to evaluate the correlations between bacterial community structure and soil chemical properties. In order to identify bioindicator bacterial taxa at multiple taxonomic levels, the linear discriminant analysis effect size (LEfSe) analysis (Segata et al., 2011) was performed using the Galaxy server (http://huttenhower.sph.harvard.edu/galaxy). A Venn diagram was constructed to verify the proportion of bacterial operational taxonomic units (OTUs) at the genus level that was unique and shared between treatments using Venny 2.1 (Oliveros 2007). Four-way ANOVA was used to evaluate the effects of top-down and bottom-up factors and time on soil physicochemical properties. Kruskal-Wallis test was applied for the effects of protists, chemical and organic fertilisers and time on the alpha diversity indexes. The raw FASTQ files obtained in this study for the MiSeq libraries have been deposited to the NCBI Sequence Read Archive (SRA) under BioProject accession number PRJNA673254.

3. Results

3.1. Soil chemical properties

The soil chemical properties shifted significantly over time with the addition of top-down and bottom-up factors (Fig. 1, Supplementary Fig. S1 and Table S1). The results of four-way ANOVA for the effects of protists, chemical and organic fertilisers, and time are summarised in Fig. 1 (see Supplementary Table S1 for full results). The soil redox potential (Eh) was significantly reduced with time, while the soil pH significantly increased (Fig. 1A and B, Supplementary Figs. S1A–B). The soil total C and C/N ratio and the available P were also shifted within the three weeks (Fig. 1C, E and F). The total C, C/N ratio, available P, and Na contents were significantly affected by both bottom-up factors (Fig. 1C, E, F and H). Among the bottom-up factors, only CF had a significant effect on the soil pH and Eh (Fig. 1A–B), while only OF had a significant effect on the total N and exchangeable K (Fig. 1D and G). The top-down effect of protists significantly changed the soil pH, available P and exchangeable K contents (Fig. 1B, F and G).

3.2. Top-down and bottom-up effects on bacterial diversity and community composition

In total, 3 071 605 demultiplexed sequences were obtained with a median frequency of 41 039. After quality filtering (q > 30) and removal of chimaeric, singleton and doubleton sequences, a total of 2 221 347 sequences were obtained with a median frequency of 29 667 sequences per sample, which were then assigned to 63 to 196 OTUs. The bacterial richness (observed OTUs) and diversity indexes (Faith's phylogenetic diversity and Shannon diversity) are shown in Supplementary Fig. S2, and the Kruskal-Wallis test results are summarised in Fig. 2 (see Supplementary Table S2 for full results). The bacterial alpha diversity and the observed OTUs significantly increased with time after submergence

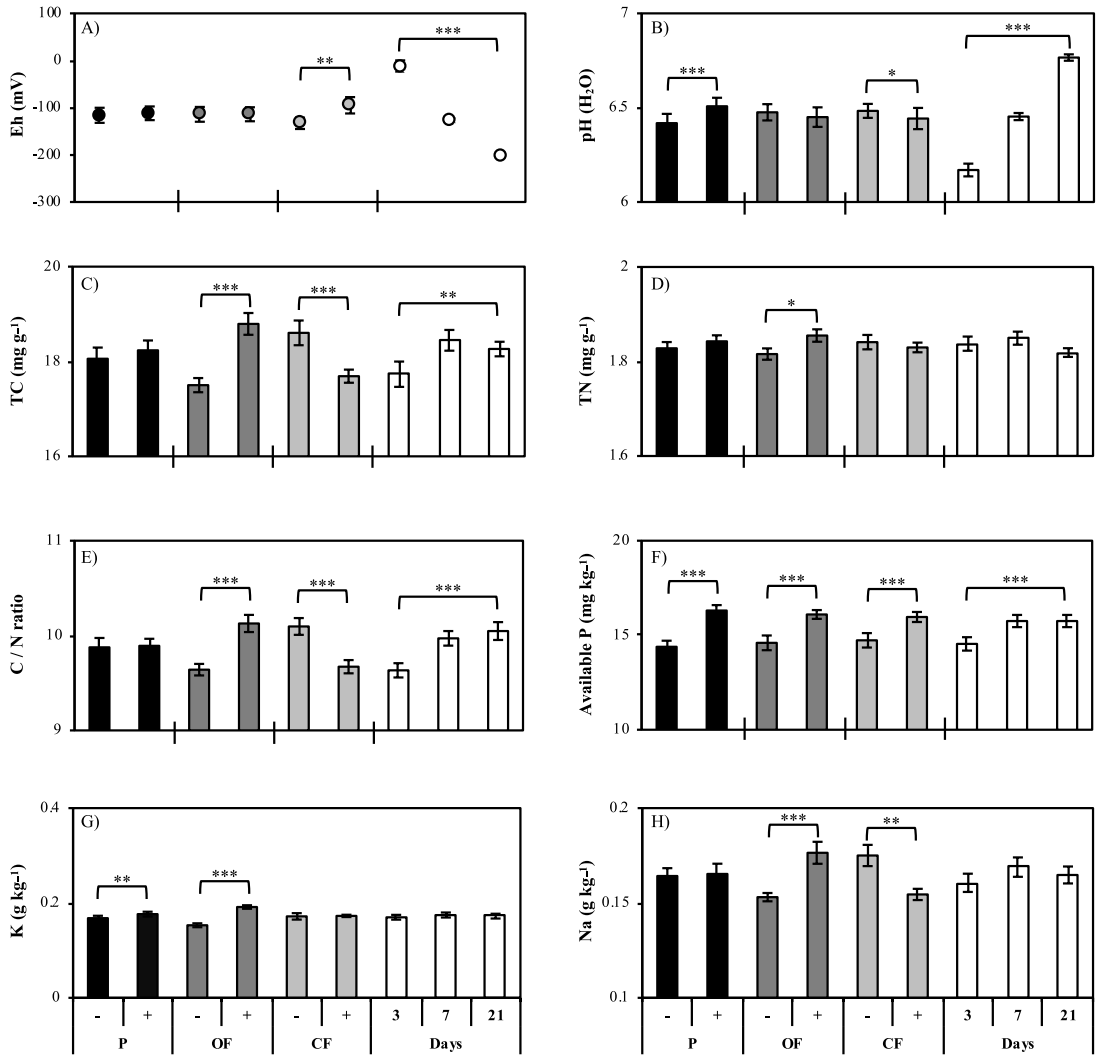


Fig. 1. The effects of top-down and bottom-up factors and time on soil physicochemical properties. P, protists (black bars); OF, organic fertiliser (dark grey bars); CF, chemical fertiliser (light grey bars); Days, days after incubation (white bars). Asterisks indicate the significance factor of the four-way ANOVA results (Days x P x CF x OF): *, $p < 0.05$; **, $p < 0.01$; ***; $p < 0.001$. Error bars represent standard deviations. For the detailed results, see Supplementary Fig. S1 and Table S1.

(Fig. 2). The top-down and bottom-up factors did not have a significant effect on either the observed OTUs or Shannon diversity (Fig. 2A–B). Faith's PD significantly increased in the protist treatments ($p < 0.05$), while the CF and OF treatments had no significant effect (Fig. 2C).

The bacterial beta diversity was shifted with time depending on the presence of top-down and bottom-up factors (Fig. 3), in which the top-down effects of protists (PERMANOVA, $R^2 = 0.03797$, $p = 0.001$) were greater than the bottom-up effects (Table 2). Among the bottom-up factors, OF (PERMANOVA, $R^2 = 0.01982$, $p = 0.013$) had a significant effect on bacterial beta diversity, while the effect of CF (PERMANOVA, $R^2 = 0.01446$, $p = 0.168$) was not significant. The bacterial beta diversity significantly changed with time (PERMANOVA, $R^2 = 0.10977$, $p = 0.001$). Although the CF did not have a significant effect on the bacterial beta diversity, the interaction of CF with protists had a significant effect (PERMANOVA, $R^2 = 0.01734$, $p = 0.037$) (Table 2). The other interactions among time, protists, CF, and OF at the 2nd, 3rd, and 4th order did not have a significant effect (Table 2). The presence of protists was significantly correlated with the shift in the beta diversity of bacterial communities ($p < 0.001$), and the NMDS analysis grouped bacterial communities into two groups based on the presence or absence of protists (Fig. 3). The bacterial communities were also grouped depending on the sampling time: the bacterial communities on day 21 were separated from those on days 3 and 7 (Fig. 3). The shifts in the bacterial community composition did not correlate with the soil nutrients. However, the increase in the soil pH and the decrease in the soil redox potential (Eh) with time were significantly ($p < 0.001$) correlated with the shift in the bacterial community composition (Fig. 3).

The differences in top-down and bottom-up effects on the bacterial community were illustrated by LEfSe analysis, which revealed the bioindicator groups of the bacterial communities at multiple taxonomic levels in the different treatments (Fig. 4 and Supplementary Table S3). The top-down effect of protists resulted in a higher number of bioindicator bacteria compared to the bottom-up effects of CF and OF (Fig. 3D). The number of bioindicator genera that were associated with the top-down factors was 4.4 and 3.7 times higher than those associated with the CF and OF. The bioindicator bacterial taxa mainly belonged to *Proteobacteria* and *Bacteroidetes* in both the presence and absence of protists (Fig. 4D). The bottom-up effects of CF and OF showed similar results (Fig. 4D), in which *Bacteroidetes*, *Proteobacteria*, and *Firmicutes* were affected.

3.3. Unique OTUs in the top-down and bottom-up treatments

A Venn diagram was created to understand the exclusive effects of

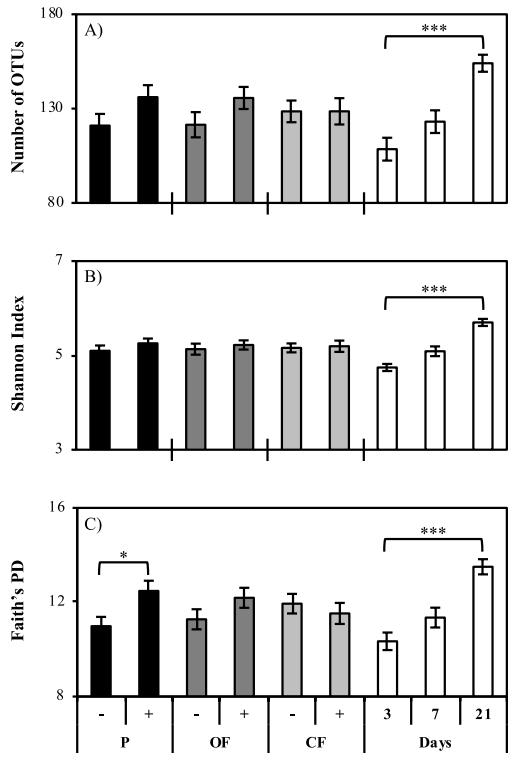


Fig. 2. The effects of top-down and bottom-up factors and time on the number of OTUs (A), Shannon index (B) and Faith's PD (C). P, protists (black bars); OF, organic fertiliser (dark grey bars); CF, chemical fertiliser (light grey bars); Days, days after incubation (white bars). Asterisks indicate the significance factor of the four-way ANOVA results (Days x P x CF x OF): *, $p < 0.05$; **, $p < 0.01$; ***; $p < 0.001$. Error bars represent standard deviations. For the detailed results, see Supplementary Fig. S2 and Table S2.

top-down and bottom-up factors on the bacterial communities (Fig. 5). To reveal the top-down effects of protists on the bacterial community composition, we compared the commonality and uniqueness of OTUs obtained from protist-inoculated and non-inoculated treatments (Fig. 5A). The results showed that 58.4% of the OTUs were common, and a total of 24.8% and 16.8% of the OTUs were specific to the protist and no-protist treatments, respectively (Fig. 5A). The composition of the unique OTUs in the absence and presence of protists showed similarity at the phylum level. The most dominant phylum of the unique OTUs was *Proteobacteria,* with 64% and 49% in the presence and absence of protists, respectively, followed by *Bacteroidetes,* with 26% and 31% in the presence and absence of protists, respectively. The presence of the bottom-up factors showed similar results, with 16.8% and 15.9% of unique OTUs specific to CF and OF, respectively (Fig. 5B and C). The composition of the unique OTUs was also similar for the CF and OF. Bacteroidetes was the main phylum, with 63% for both CF and OF, followed by *Proteobacteria* (27% for CF and 26% for OF). *Firmicutes* (43%) dominated the unique OTUs in the absence of CF followed by *Proteobacteria* (26%), while *Proteobacteria* (36%) and *Verrucomicrobia* (29%) were dominant in the absence of (Fig. 5C).

4. Discussion

The contribution of top-down and bottom-up factors to bacterial communities has been a hot topic for decades. Recent studies on marine ecosystems have recognised the irrefutable effects of top-down factors and challenged the conventional view that bacterial communities were primarily thought to be impacted by bottom-up factors (Weinbauer et al. 2003, 2007; Chow et al., 2014; Teira et al., 2019). Contrasting results have been obtained in freshwater ecosystems, where the effects of bottom-up factors on the bacterial communities are much stronger than the effects of the top-down factors (Jardillier et al., 2005; Berdjeb et al.,

Table 2
Permutational multivariate analysis of variance (PERMANOVA) results based on Bray-Curtis dissimilarities for the top-down and bottom-up effects on the bacterial community composition.

Interactions	Factors	F value	R^2	*p* value	SC
Single factor	**Protists**	**3.1044**	**0.03797**	**0.001**	***
	Time	**4.4868**	**0.10977**	**0.001**	***
	CF	1.182	0.01446	0.148	
	OF	**1.6203**	**0.01982**	**0.014**	*
2nd order interactions	Protists:Time	1.063	0.02601	0.267	
	Protists:CF	**1.4174**	**0.01734**	**0.037**	*
	Time:CF	1.0237	0.02504	0.384	
	Protists:OF	0.9354	0.01144	0.533	
	Time:OF	1.0325	0.02526	0.346	
	CF:OF	0.9749	0.01193	0.455	
3rd order interactions	Protists:Time:CF	1.1342	0.02775	0.135	
	Protists:Time:OF	1.0485	0.02565	0.315	
	Protists:CF:OF	1.0663	0.01304	0.277	
	Time:CF:OF	0.953	0.02332	0.591	
4th order interactions	Protists:Time:CF:OF	0.9823	0.02403	0.482	

Time, sampling time (days 3, 7 and 21); CF, chemical fertilizer; OF, organic fertiliser; SC, significance codes (***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$).

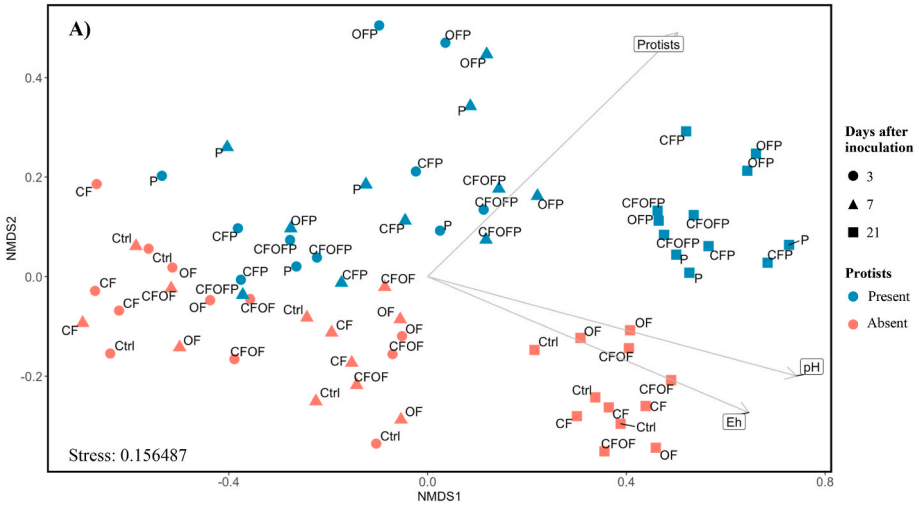
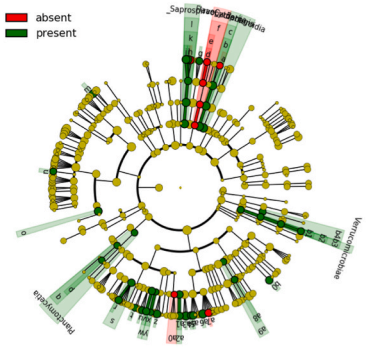


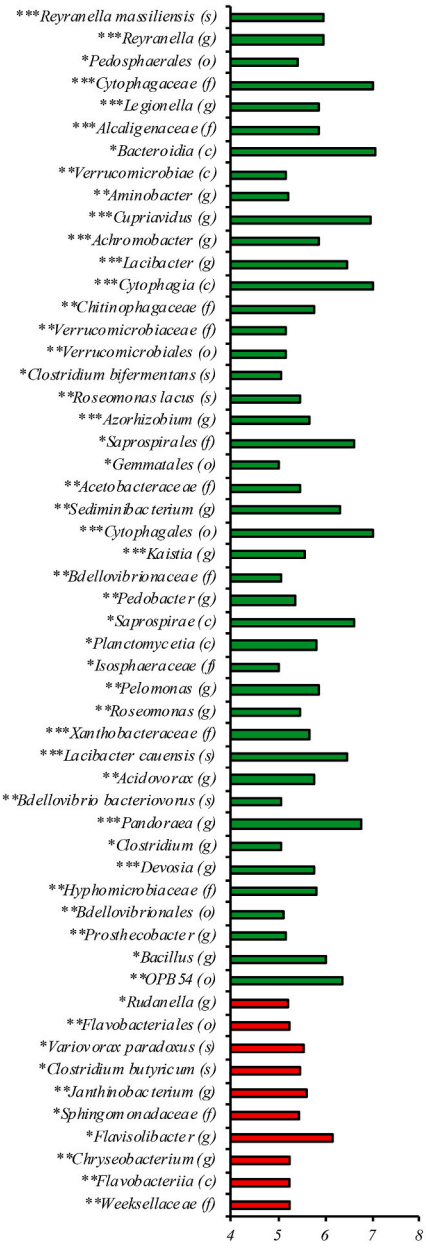
Fig. 3. Nonmetric multidimensional scaling (NMDS) plots calculated based on the Bray–Curtis dissimilarity index of bacterial communities (Stress: 0.1564) with significant correlations between community composition and soil physicochemical properties. Red colour, absence of protists; green colour, presence of protists; circle, day 3; triangle, day 7; and square, day 21. Ctrl, control; OF, organic fertiliser; CF, chemical fertiliser; P, protists. Arrows indicate significant correlations among the bacterial communities and environmental parameters ($p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Top-down effect

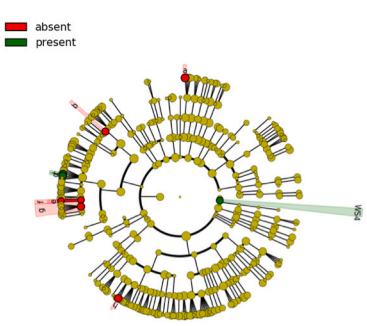
Bottom-up effect

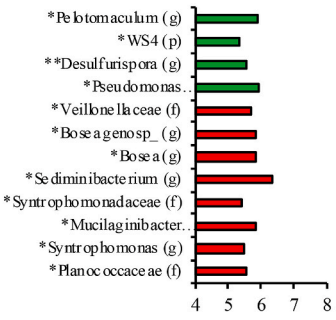
A) Protists



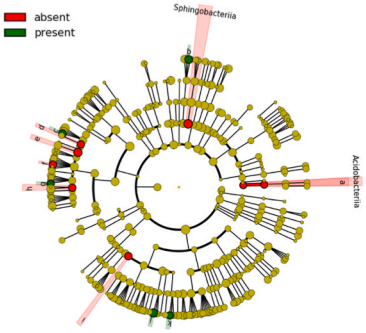


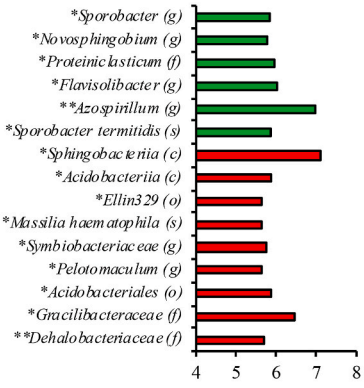
B) CF





C) OF





Number of significantly affected bacterial taxa

D)

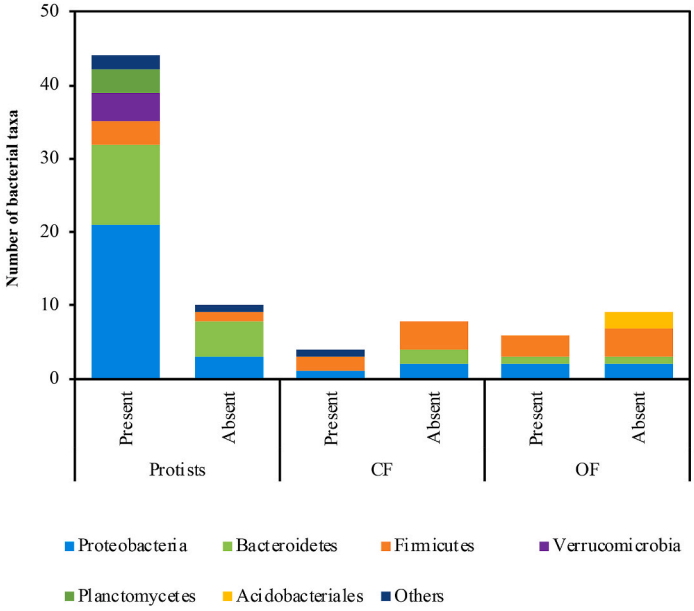


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Fig. 4. A linear discriminant analysis effect size (LEfSe) method identified the significantly different ($p < 0.05$, Kruskal-Wallis test, LDA score > 2.0) bacteria at multiple taxonomic levels by comparison of bacterial communities in the presence and absence of protists (A), the presence and absence of CF (B), and the presence and absence of (C). Cladograms illustrate the taxonomic groups that explain the most variation among bacterial communities. Coloured dots represent the taxa with significantly different abundances between treatments, and from the centre outward, they represent the kingdom, phylum, class, order, family, genus and species levels. The coloured shadows represent trends of the significantly different taxa. Histograms below the cladograms show the LDA scores for significant differences in bacteria. The words in the parentheses show the taxonomic level: p, phylum; c, class; o, order; f, family; g, genus; s, species. The asterisks before the names indicate the significance factor: *, $p < 0.05$; **, $p < 0.01$; ***; $p < 0.001$. The total number of significantly affected bacterial taxa is represented with a bar graph at the phylum level (D).

2011). Our study, the first to directly compare the top-down and bottom-up effects on bacterial community composition in a soil ecosystem, revealed that top-down predators significantly control the bacterial community structure regardless of whether bottom-up fertilisers are applied. In this study, we used autoclave-sterilised soil to study the bacterial community in the presence and absence of protists. The heat sterilization of soil enriches the available nutrients, including organic carbon, N, and P (Wolf and Skipper 2018), under which the bottom-up effect by fertilisation may have been underestimated compared to that of unsterilized soil. On the other hand, the top-down effect of predators was also limited under the enriched nutrient conditions (Weinbauer et al., 2003; Lenoir et al., 2007; Chow et al., 2014) Therefore, the top-down effects of protists may have also been slightly underestimated in this study. Nevertheless, our results indicated the importance of protists for regulating the formation of bacterial communities in paddy field soil.

4.1. The top-down effect on the bacterial community composition

In this study, the top-down effects of protists on the bacterial community composition were distinct and greater than the bottom-up effects of the fertilisers, which was in contract with our hypothesis. Soil water content is one of the critical factors affecting the activity of protists (Geisen et al., 2014). Following flooded conditions, protists can increase their domain and population by consuming bacteria that were previously unreachable under non-flooded conditions. Although most of the studies were performed with a single protist isolate, mainly amoeba (Bonkowski 2004), the enormous impact of protists on bacterial community composition has long been recognised (Gao et al., 2019). Compared to a single isolate, a mixed culture of protists is expected to feed on a wide variety of bacterial prey, leading to drastic changes in bacterial communities (Saleem et al., 2012). Our previous study showed that the effects of a mixture of the four protist isolates on paddy field bacterial community composition were greater than those of single isolates (Asiloglu et al., 2020). In this study, the use of four protist isolates that differ in taxonomy, feeding mode, cell size, and morphology should have better reflected the effects by impacting a wide variety of bacterial taxa. We assume that in the real paddy field environment, where protists are highly dominant and diverse (Murase et al., 2015; Asiloglu et al., 2015), the bacterial predators may have even more significant top-down effects controlling the formation of bacterial communities. Additionally, the densities of protists are likely to be higher in the natural conditions than the initially inoculated density (10^3 cells g soil^{-1}) in the current study, which is another important factor for the effects of protists on bacteria (Saleem et al., 2012). Further studies on protist-bacteria interactions are likely to provide a better understanding of the dynamics of bacterial communities in paddy fields.

The top-down effects of protists on the bacterial communities are generally explained by the changes within *Proteobacteria* and *Bacteroidetes* (Flues et al., 2017; Asiloglu et al., 2020), which is in line with our present results. Studies have suggested that bacterial species belonging to *Proteobacteria,* especially alpha- and beta-, and *Bacteroidetes* are preferred prey sources for protists due to their gram-negative status (Kreuzer et al., 2006; Murase et al., 2006; Rosenberg et al., 2009; Flues et al., 2017). However, many species of gram-negative *Proteobacteria* and *Bacteroidetes* can survive protist predation by several mechanisms, including intracellular resistance to digestion (Vaerewijck et al., 2014; Gong et al., 2016), high motility (Matz and Jürgens 2005), and biofilm production (Parry 2004; Huws et al., 2005; Matz and Kjelleberg 2005). Additionally, *Proteobacteria* and *Bacteroidetes* include many bacterial species with a fast-growing ability, which enables them to replace the cells lost to predation (Gurijala and Alexander 1990). Thus, while several members of *Proteobacteria* and *Bacteroidetes* can be preyed upon by protists, predation-resistant and fast-growing species can take advantage of protist predation, which explains the fluctuations within both phyla depending on the presence or absence of the protists.

Protist predation on bacteria often increases soil fertility and plant growth, which is linked with enhanced nutrient turnover and bacterial activities, such as N mineralization and IAA production (Clarholm 1985; Bonkowski and Brandt 2002; Bonkowski 2004; Gao et al., 2019). Previously, we showed that the presence of protists increased the abundance of plant growth-promoting bacteria (PGPR) in the rice rhizosphere (Asiloglu et al., 2020). Considering that many PGPR species belong to *Proteobacteria* and *Bacteroidetes*, protist-regulated bacterial community formation could be linked to high soil fertility in water-logged paddy fields.

4.2. The bottom-up effects of fertilisers on the bacterial community composition

The effects of organic and chemical fertilisers, which were applied at the field rate, were studied to evaluate the bottom-up effects; therefore, the potential bottom-up effects of the original soil nutrients were ignored in this study. Our results showed that the CF application had no significant bottom-up effect on the bacterial community composition. The effects of chemical fertilisers on soil bacterial communities usually correlate with the applied N. In this study, due to the high amount of N in the initial soil (1.9 mg g^{-1}), the total N content was not significantly affected by the CF and OF applications (0.1 mg N g soil^{-1}). Therefore, we did not observe any N-based effect of fertilisers on the bacterial communities. The OF application had a significant effect on the bacterial alpha and beta diversities. Previously, Daquiado et al., (2016) obtained similar results, in which the bacterial communities of unfertilised and chemically fertilised paddy soils were similar, while organic fertiliser application caused a shift in the bacterial communities. Similarly, Li et al., (2019) and Yu et al., (2019) showed that the long-term application of chemical fertilisers had only a small effect on the overall bacterial communities in paddy fields compared to the unfertilised fields.

Firmicutes and *Proteobacteria* were predominantly affected by bottom-up factors, and the CF- and OF-specific OTUs mainly belonged to *Bacteroidetes,* which is in line with previous studies in paddy field soils (Daquiado et al., 2016; Kumar et al., 2018). Previous studies have suggested that fertilisers, especially organic fertilisers, increase the abundance of copiotrophs, such as *Proteobacteria, Bacteroidetes*, and *Firmicutes* (Moreno-Espindola et al., 2018; Zhan et al., 2018). Several species in the *Proteobacteria*, *Bacteroidetes*, and *Firmicutes* phyla play roles in nutrient cycling; therefore, they are expected to respond well to the enriched bottom-up nutrients. Despite the similar bottom-up effects on bacteria at the phylum level, our results showed that CF and OF applications resulted in slightly different bacterial communities, indicating the importance of the source of the bottom-up effects. The OF application significantly increased the total C concentration. Previous studies showed that the increased soil C concentration due to the application of organic fertilisers was a dominant driving force for the spatial

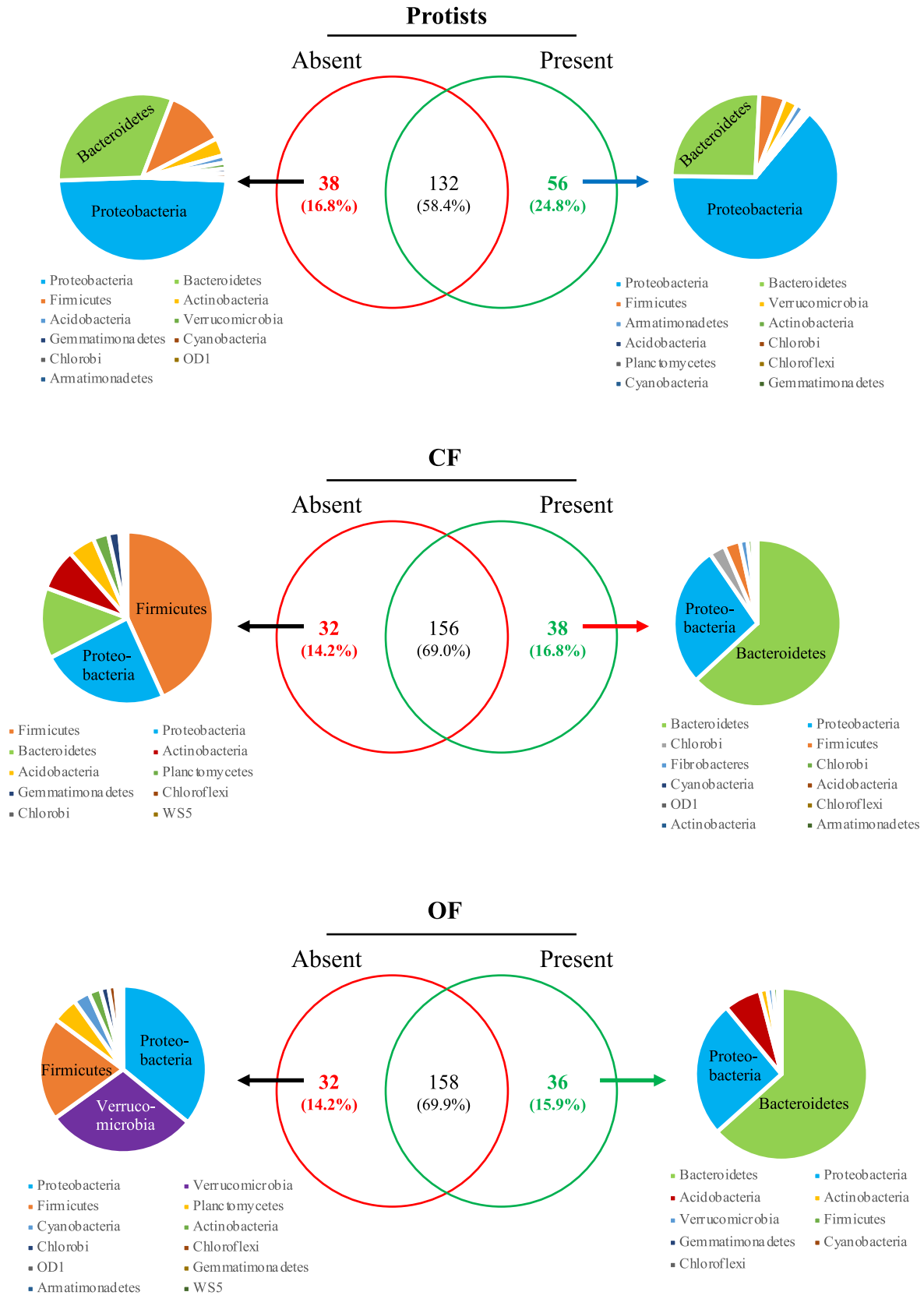

Fig. 5. Venn diagram illustrating the number and percentage of unique and shared bacterial OTUs at the genus level in the absence and presence of protists (A), chemical fertiliser (B) and organic fertiliser (C). The pie charts illustrate the average proportional distribution of the unique OTUs at the phylum level.

distribution of the microbial communities in paddy field soil (Briar et al., 2011; Jiang et al., 2013; Chen et al., 2015). Therefore, the significant bottom-up effect of on the alpha and beta diversities of bacteria in this study was likely to be organic C-based.

In the soil environment, fertilisers have profound effects on top-down grazers as well (Lentendu et al., 2014; Asiloglu et al., 2021a). For instance, Murase et al., (2015) showed that the long-term effect of fertilisers on microeukaryotic communities, including protists, was stronger than the effects of water management or seasonality in a paddy field. In fact, the fertilisers affect protist communities more than they affect bacterial communities (Zhao et al., 2019). Previously, we showed that biochar fertiliser application negatively affected the prey-predator interactions among protists and bacteria (Asiloglu et al., 2021b). Thus, the bottom-up effects of fertiliser application on protists, which could not be taken into consideration in this study, may add another layer of complexity to the response of bacteria to predator communities. Furthermore, we used only four protist species in this study. Since the diversity of soil protist in natural condition is much higher and the interaction between plants, protists, and bacteria is much more complex (Geisen et al., 2018), further studies focusing on the top-down and bottom-up factors in actual field conditions should provide a better understanding of the factors controlling bacterial community dynamics.

5. Conclusion

Here, we showed that the formation of bacterial communities following flooding conditions is mainly top-down and controlled by protists. The bottom-up effect on bacterial formation depended on the type of fertiliser. Overall, our results provide unique information on the importance of phagotrophic protists in regulating bacterial community formation in paddy field soil, which is most likely to affect bacterial activities and their resulting impacts on plant growth. Further research should reveal the activities of protist-regulated bacterial communities and how this could be translated to agricultural productivity.

Authors' contributions

RA conceived and designed the study, performed bioinformatics, and prepared and revised the manuscript. RA and NH performed statistical analyses and interpreted the data. KK performed sampling and molecular analyses, SOS performed chemical analyses, and BS performed soil redox potential analysis. KS, JM, and NH provided feedback and valuable comments. All authors read and approved the final manuscript.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.soilbio.2021.108186.

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