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Protists modulate active bacterial community composition in paddy field soils

Mayu Fujino[1] · Kazuki Suzuki[2] · Naoki Harada[3] · Rasit Asiloglu[4]

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Abstract
Bacterial communities in the soil ecosystem including paddy fields are strongly controlled by the top-down force of predatory protists. However, the current knowledge on top-down regulation of bacterial communities exclusively comes from DNA-based molecular methods that have limitations to differentiate between alive and dead cells. Here, we compared DNA- and RNA-based high-throughput sequencing to estimate the effect of predatory protists on bacterial community composition. We studied the effect of three protist isolates on active (RNA-based) and total (DNA-based) bacterial community composition in two paddy field soils with different physicochemical properties. The main results are (1) protists control bacterial communities with a species-specific effect, (2) soil type is an important factor determining the outcome of the microbial trophic interactions, and (3) DNA-based methods are likely to overestimate the effect of protists for Firmicutes, while it may cause an underestimation of the protist-effect on Proteobacteria and Bacteroidota. Despite the over- and underestimation of specific bacterial taxa, both DNA- and RNA-based results provided similar patterns for the predatory effect of protists, which makes both DNA- and RNA-based methods powerful tools to evaluate trophic interactions. Nevertheless, the top-down regulation of paddy field bacterial communities in DNA-based studies should be carefully evaluated, especially for the bacterial taxa belonging to Proteobacteria and Firmicutes.

Keywords Bacterial community · Predatory protists · Trophic interaction · RNA-based · Paddy field soil · High-throughput sequencing

Introduction

Hundreds of species interact with each other and are frequently affected by external and internal factors, making the investigation of microbial ecology a challenging endeavor (Faust and Raes 2012). Inevitably, the study of microbial ecology heavily relies on molecular biological methods, especially rRNA gene fragments (DNA-based studies). The major advances driven by molecular technologies over the last two decades enabled us to understand the importance of trophic regulation of prey communities and functions by microbial predators (Gralka et al. 2020). Among microbial predators, protists—a diverse group of phagotrophic unicellular eukaryotes—are one of the major predators of bacteria (Geisen et al. 2018; Leander 2020). Although protists can show species-specific traits and strong prey selection patterns (Singh 1941; Singh 1942), in general, the predatory activities of protists alter the composition and functionalities of bacterial communities, positively affecting nutrient cycling and agricultural productivity (Bonkowski 2004; Gao et al. 2019; Thakur and Geisen 2019) including paddy fields (Asiloglu et al. 2020; Asiloglu et al. 2021b). The prey selection patterns of protists cause a drastic decrease in the populations of the preyed bacterial species (Saleem et al. 2012). Consequently, enhanced nutrient turnover and reduced bacteria-bacteria competition are the major outcomes of protist predation, which enables specific bacterial species to take advantage of trophic interactions to grow

✉ Rasit Asiloglu
asiloglu@agr.niigata-u.ac.jp

[1] Graduate School of Science and Technology, Niigata University, Niigata City, Niigata 950-2181, Japan

[2] Institute for Research Promotion, Niigata University, Niigata City, Niigata 950-2181, Japan

[3] Institute of Science and Technology, Niigata University, Niigata City, Niigata 950-2181, Japan

[4] Laboratory of Applied Protistology, Institute of Science and Technology, Niigata University, 8050, Ikarashi 2-no-cho, Nishi-ku, Niigata City, Niigata 950-2181, Japan

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better (Flues et al. 2017; Gao et al. 2019). Therefore, the top-down effects of predators on a bacterial community cause increased/decreased abundances of specific bacterial species, while some bacteria are not affected (Gao et al. 2019; Thakur and Geisen 2019). The high-throughput sequencing method, which has been increasingly applied to reveal microbial trophic interactions, provides an important platform to observe shifts in prey community composition (Flues et al. 2017; Asiloglu et al. 2020, 2021a, c). However, the current knowledge on top-down regulation of a microbial prey community and function exclusively comes from DNA-based molecular methods, which do not differentiate alive and dead cells (Cangelosi and Meschke 2014; Carini et al. 2017).

Indeed, studies showed that DNA-based amplicon sequencing may include relic DNA, meaning DNA in dead and resting cells and stabilized extracellular (Nannipieri et al. 2019; Pathan et al. 2021). The RNA degrades in minutes (Houseley and Tollervey 2009), and therefore, RNA-based studies (16S rRNA sequencing) are more reliable to differentiate alive and dead cells (Cangelosi and Meschke 2014), which enables us to demonstrate potentially alive (both dormant and metabolically active) cells (Li et al. 2017). Indeed, a comparison of DNA- and RNA-based methods showed that 16S rRNA sequencing is superior to the DNA-based method to exclude dead bacterial DNA (Li et al. 2017). Considering the fact that the death or survival of prey is the basis to evaluate top-down regulation, RNA-based studies should give the true picture of the trophic interactions by effectively differentiating the alive and dead organisms. However, to the best of our knowledge, the effects of microbial predators on a prey community have never been investigated in consideration of the presence of relic DNA. Our first hypothesis is that the relic DNA would obscure the effect of protists on bacterial community composition causing a misestimation of bacterial abundances.

It is well known that protists selectively feed on bacteria at the genus (Singh 1941; Singh 1942) and even at the species level (Murase and Frenzel 2008). Despite the strong selective predation of protists on bacteria, the environmental variables, which are far more complex in extremely heterogeneous soil ecosystems compared to those of marine and freshwater ecosystems, determine the prey-predator dynamics of protists feeding on specific bacteria. Therefore, soil types with different physicochemical properties should influence the trophic interactions between protists and bacteria (Erktan et al. 2020). However, still, less is known about the effect of protists on bacterial communities in different soil types. The second hypothesis of this study is that the effects of protists on bacterial community composition would differ depending on the soil type. Our previous studies showed that bacterial communities in the paddy field soil are strongly controlled by the top-down force of protists (Asiloglu et al. 2021b, 2021a), with a species-specific effect (Asiloglu et al. 2020). Here, we aimed to reveal the efficiency of DNA- and RNA-based high-throughput sequencing to estimate the effect of protists on bacterial community composition. We studied the effect of three protist species isolated from a paddy field soil on active (RNA-based) and total (DNA-based) bacterial community composition in two paddy field soil with different physicochemical properties. The bacterial community composition was analyzed with a high-throughput sequencing method.

Materials and methods

Soil samples and preparation of microorganisms

The grey lowland (Gl) soil was sampled from a paddy field on the 25th of March, 2021 at Shindori Station in the Field Center for Sustainable Agriculture and Forestry, Niigata University, Niigata, Japan (N 37.86, E 138.96). The andosol (As) was sampled from a paddy field on the 23rd of April, 2021 in Hata, Matsumoto City, Nagano, Japan (N 36.20, E 137.87). After sampling, soils were air-dried, sieved (<2 mm), and then stored at 4 °C. The physicochemical properties of the soils are shown in Table S1. Briefly, Gl had higher Mg, Na, and clay content, while As had higher pH, CEC, C, N, C/N ratio, K, and silt content.

In order to study the effect of protists on the bacterial community, we extracted indigenous bacterial community (protist-free) from each fresh soil as described previously (Asiloglu et al. 2021a) with minor modifications. Briefly, 200 g of soil was mixed with 300 mL of demineralized water and shaken at 170 rpm for 60 min. The soil particles were separated with a 500-µm sieve. Then, protists were excluded by sieving through a 1.2-µm pore-size mixed cellulose ester membrane filter (ADVANTEC, Tokyo, Japan). The 50 µL of each protist-free bacterial inoculum was cultured in 100 µL of the amoeba saline solution (Page 1988) at 25 °C in 96-well microtiter plates for 7 days to check for the absence of protists ($n = 96$). Three predatory protists used in this study represent the common soil protists, which were isolated previously from a paddy field soil (Asiloglu et al. 2020). These are the following treatments: an amoeba (treatment Amo), *Vermamoeba vermiformis* LAP1-2017 (Amoebozoa; Tubulinea; ~20 µm); a ciliate (treatment Cil), *Colpoda steinii* LAP2-2017 (Chromalveolata; Alveolata; ~30 µm); and a flagellate (treatment Fla), *Heteromita globosa* LAP3-2017 (Rhizaria; Cercozoa; ~10 µm). The isolation method and PCR analysis of the 18S rRNA gene from the isolates were described previously (Asiloglu et al. 2020). The sequence data have been submitted to DDBJ/EMBL/GenBank databases under accession numbers LC764480-LC764482. The

preparation of the axenically grown protist isolates was previously described by Asiloglu et al. (2020).

The experimental setup, sampling, and molecular analyses

Prior to the experiment, the soil samples were sterilized by autoclaving 3 times at 121 °C for 20 min. In total, we had 90 microcosms (2 soil types × 5 protists treatments [no protist control, each of three isolates and their equal combination] × 3 sampling times × 3 replications]). All of the microcosms (total light-shutoff 125 mL UG plastic jars, Tokyo, Japan) were filled with 40 g of sterile soil and the protist-free bacterial inoculum. Then, the microcosms were submerged with sterile ddH_2O and incubated for 1 week to allow bacteria to grow, where the number of bacteria in the microcosms reached 8×10^7 cells g $soil^{-1}$. Then, each protist isolate and their equal combination (treatment Mix) was added to the microcosms (total 10^3 cells g $soil^{-1}$). The protist-free control received the same amount of sterile ddH_2O. The microcosms were incubated in a growth chamber (LPH-220SP, Nippon Igaku Kiki Seisakusho, Osaka, Japan) at 24 °C under dark conditions.

The microcosms ($n = 3$) were destructively sampled on days 7, 21, and 35 by the methods previously described (Asiloglu et al. 2021a). Briefly, the surface water of the microcosms was removed, and the soil was mixed thoroughly. The 2 g of soil sample was placed into the 2-mL sterile tubes and immediately frozen in liquid nitrogen. The samples were then stored in a freezer at −80 °C until nucleic acid extraction.

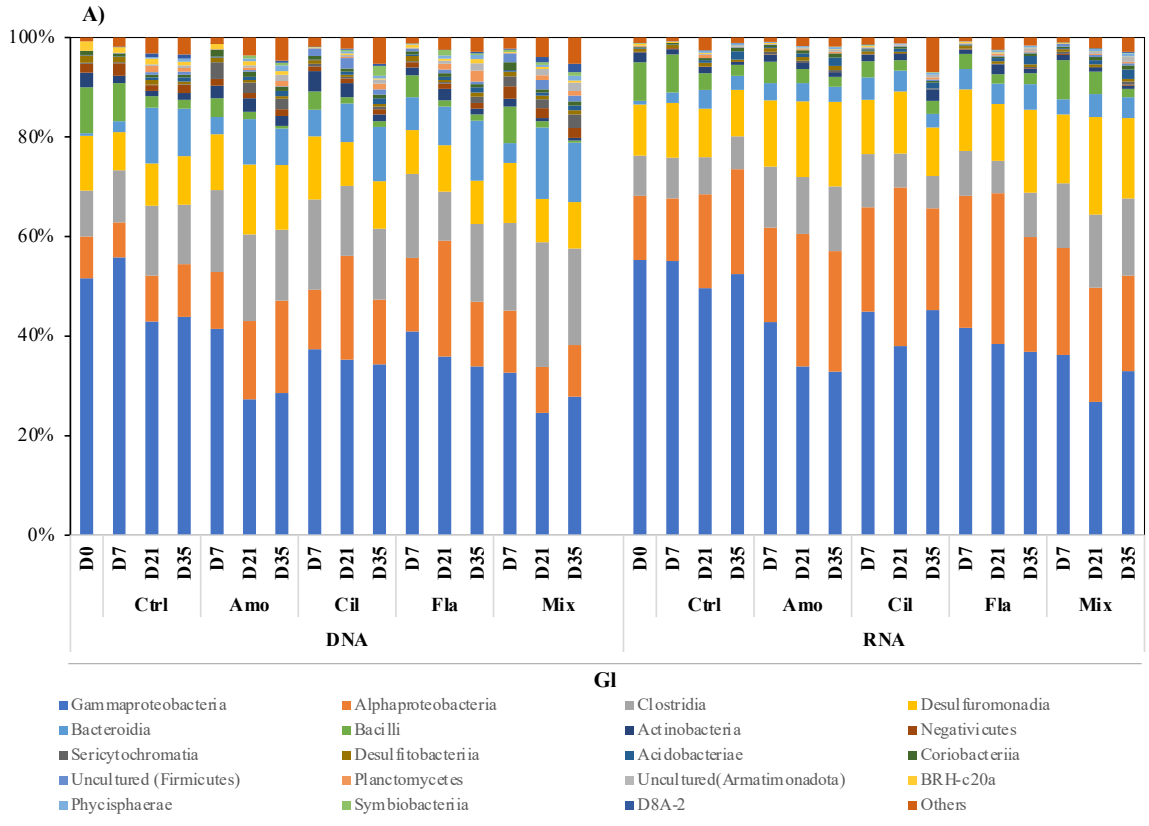
Soil nucleic acids were extracted using ISOIL for RNA Kit (NIPPON GENE, Tokyo, Japan). The extraction procedure was followed according to the instructions, and from the total 155 µL of the nucleic acid solution obtained, 55 µL was used for soil DNA analyses. Then, to the remaining 100 µL of the nucleic acid solution, 55 µL of TE was added and treated according to the DNase treatment protocol ISOIL for RNA (NIPPON GENE) to obtain RNA. The RNA was converted to cDNA using PrimeScript™ 1st Strand cDNA Synthesis Kit (Takara Bio Inc., Otsu, Japan) according to the provided cDNA synthesis protocol.

The number of soil protists was estimated using the most probable number (MPN) method as described by Asiloglu et al. (2020). Briefly, 5 g of the soil of day 35 samples was mixed with 45 mL of AMS solution on the sampling day to obtain ten times diluted soil solution. This mixture was used in a threefold dilution series as the final concentration of diluted soil solutions ranged from $3^1 \times 10$ to $3^8 \times 10$. Fifty microliters of the diluted suspensions ($n = 8$) was added with 100 µL food bacteria (*Escherichia coli* MG1655 (ATCC #47076); final concentration ~10^7 cells mL^{-1}) and cultured in 96-well microtiter plates at 25 °C in dark. After 1 week, the growth of amoeba, flagellates, and ciliates in the wells was observed at ×200 and ×400 magnifications using an inverted microscope (Nikon Eclipse TE2000-S, Tokyo, Japan). The bacterial growth was evaluated by amplification of DNA and cDNA with a real-time PCR using universal bacterial primers (515F and 806R). The PCR mixture and the program were described by Asiloglu et al. (2020) with a modification on annealing temperature (57 °C). The standard curve was generated with serial dilutions of a previously calculated copy number of 16S rRNA gene from *Escherichia coli* MG1655 as described by Lee et al. (2006).

Illumina library preparation and all bioinformatics procedures were performed as described previously (Asiloglu et al. 2021a). The V4 region of the 16S rRNA gene was amplified from the extracted DNA and cDNA using universal primers (515F and 806R) tailed with Illumina barcoded adapters (San Diego, CA). After purification with Agencourt XP Ampure Beads (Beckman Coulter Inc., Brea, CA), the PCR products were tagged with sequencing adapters using Nextera XT Index Kit (Illumina). Library samples were sequenced with MiSeq Reagent Kit V2 (Illumina) (2 × 300 paired-end reads).

Bioinformatics and statistical analyses

Sequencing data obtained with MiSeq were analyzed using QIIME2 (Bolyen et al. 2019) and were subjected to quality filtering, noise removal, paired-end linkage, and chimeric sequence removal (Bolyen et al. 2019). For paired-end linkage, forward reads of 180 bp and reverse reads of 140 bp were used. All of the bioinformatics and statistical analyses were conducted as described previously (Asiloglu et al. 2021a). Briefly, the primary analysis of raw FASTQ data was processed using DADA2 in the QIIME2 pipeline (version 2021.11, https://qiime2.org). DADA2 in QIIME2 has been used to denoise the paired-end sequences into amplicon sequence variants (ASVs) after random resampling. QIIME2's q2-feature-classifier plugin was used for taxonomy assignment against the latest SILVA reference database (138.1 release). The rarefied sequences (10,000 depth) 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. Non-metric multidimensional scaling (NMDS) analysis was performed based on the Bray–Curtis dissimilarity index to visualize the beta diversity dissimilarities at the genus level. In order to reveal the bacterial taxa that were significantly affected by protists, the linear discriminant analysis effect size (LEfSe) (Segata et al. 2011) was performed using the Galaxy server (http://huttenhower.sph.harvard.edu/galaxy). Firstly, the non-parametric factorial Kruskal-Wallis sum-rank test ($p < 0.05$) was conducted to detect features with significantly different abundances. After this step, linear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Bacteroidia
Bacilli
Actinobacteria
Negativicutes
Sericytochromatia
Desulfitobacteriia
Acidobacteriae
Coriobacteriia
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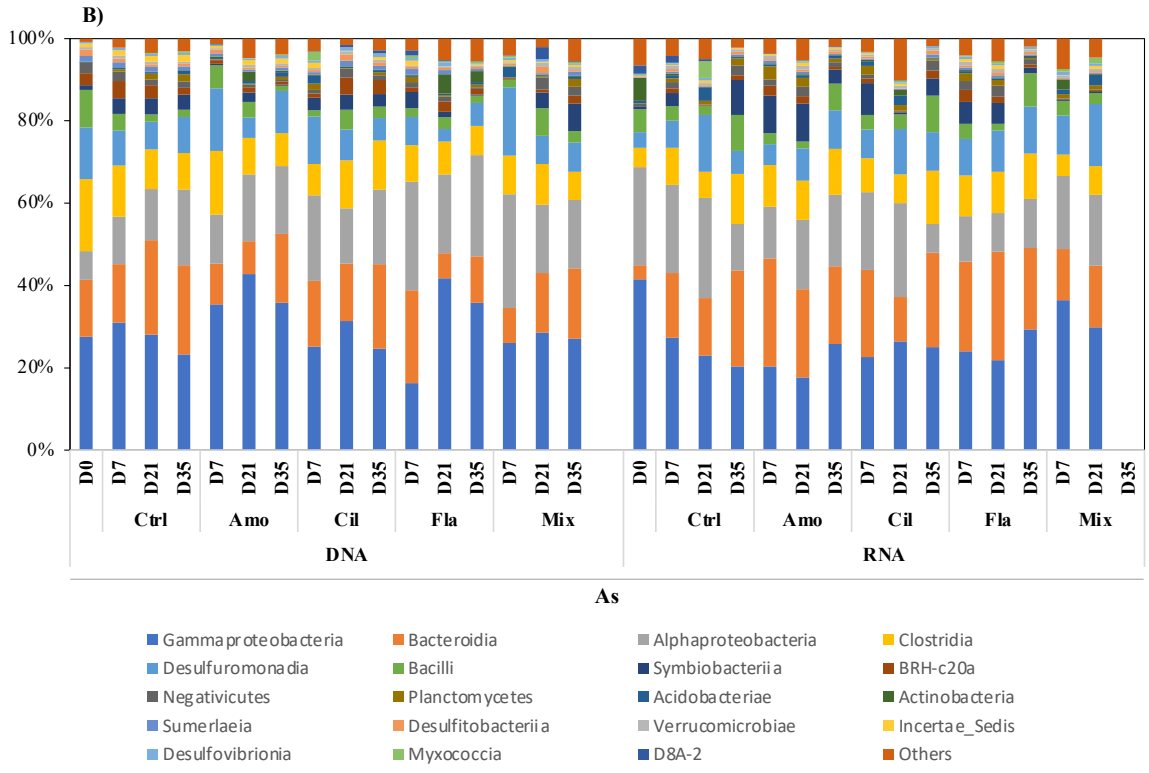
D8A-2
Others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Bacteroidia
Alphaproteobacteria
Clostridia
Desulfuromonadia
Bacilli
Symbiobacteriia
BRH-c20a
Negativicutes
Planctomycetes
Acidobacteriae
Actinobacteria
Sumerlaeia
Desulfitobacteriia
Verrucomicrobiae
Incertae_Sedis
Desulfovibrionia
Myxococcia
D8A-2
Others

◂**Fig. 1** Bacterial abundances for each treatment derived from amplicon sequencing at the class taxonomic level of the twenty most abundant groups for **A** Gl and **B** As communities. Ctrl, control with no protists; Amo, *Vermamoeba vermiformis*; Cil, *Colpoda steinii*; Fla, *Heteromita globosa*; Mix, a mixture of the three protist isolates; Gl, Grey lowland soil; As, Andosol; D represents incubation days

discriminant analysis (LDA), in which the logarithmic score was set to 2.0, was conducted to estimate the significant effect size of each differentially abundant feature, which shows the enriched and depleted bacterial taxa in the protist treatments compared to the control. Prior to conducting ANOVA analyses, the normality assumption of the data and homogeneity of variances within each group was confirmed ($p > 0.05$) using the Shapiro-Wilk test and the Bartlett test, respectively. All statistical analyses were performed using the R program version 4.1.2 (2021.11.01; https://www.r-project.org/) as described previously (Asiloglu et al. 2021a) unless otherwise specified. The raw FASTQ files obtained in this study for the MiSeq libraries have been deposited to the NCBI Sequence Read Archive (SRA) under accession number SUB13094008.

Results

Microbial growth and bacterial community composition

At the end of the experiment, the introduced protists (10^3 cells g^{-1} soil) successfully populated the microcosms (> 10^4 cells g^{-1} soil) in all protist-inoculated treatments, while the non-protist microcosms did not contain detectable levels of protists (Fig. S1). All three protist species were able to grow in combination with each other in the mix treatment. In both soils, ciliates were the less populated protists followed by amoeba (Fig. S1). Fla and mix treatments had the biggest values on the protist population (Fig. S1). The bacterial population was estimated with a qPCR method. The introduced bacteria successfully populated the microcosms in both soils (Fig. S2). The effect of incubation time and protists was shown in Table S2 for each soil type and each nucleic acid. Incubation day significantly affected the bacterial populations, while the effect of protists was only significant (p=0.048) in DNA treatments of As soil (Table S2).

A total of 2,835,342 quality sequences were obtained after chimeric and doubleton removal, with a median frequency of 14,767. Overall, the bacterial community composition in the samples mainly consisted of Gammaproteobacteria (33.3%), Alphaproteobacteria (16.5%), Clostridia (11.4%), Bacteroidia (11.2%), and Desulfuromonadia (10.3%) (Fig. 1). Data at the genus level (Fig. S3) showed that *Burkholderia* (13.2%) was the major microorganism followed by *Citrifermentans* (%7.7%), WCHB1-32 (uncultured Bacteriodetes; 5.6%), *Massilia* (4.9%), and *Azospirillum* (4.7%). The main microbial groups higher in relative abundance in Gl were Gammaproteobacteria, Alphaproteobacteria, Clostridia, and Desulfuromonadia, while Gammaproteobacteria, Bacteroidia, Alphaproteobacteria, and Clostridia had higher relative abundance in As. In both soils, the relative abundance of Gammaproteobacteria decreased with incubation time, while the relative abundance of Alphaproteobacteria and Bacteroidia increased during the 35 days of incubation. Overall, the relative abundances of bacteria belonging to Proteobacteria and Bacteroidota were higher in the active community, while those belonging to Firmicutes were higher in the total community.

The alpha diversity indexes are shown in Fig. 2 for Gl (Fig. 2A–C) and As (Fig. 2D–F). Bacterial alpha diversities were more significantly affected by protists in Gl than As (Table S3). In As, only nucleic acid affected the ASVs, while incubation time had a strong impact on Shannon and Faith's PD (Fig. 2A–C and Table S3). The effect of protists was significant for Shannon's index (Fig. 2B and Table S3). Shannon indexes were significantly affected by protists and incubation time in As, while nucleic acid significantly affected ASVs and Faith's PD (Fig. 2D–F and Table S3). The PERMANOVA analysis (Table 1) and the NMDS analysis (Fig. 3) showed that the bacterial community composition was significantly different in both soils. There was a significant difference between total (DNA-based) and active (RNA-based) communities (Table 1 and Fig. 3). Although the NMDS analyses mainly separated bacterial communities based on soil type and nucleic acid (Fig. 3), PERMANOVA analysis showed that the protists significantly altered bacterial community composition (Table 1). We further analyzed the data to better understand the importance of using nucleic acid on prey-predator interaction.

Protist effect on bacterial community estimated by DNA- and RNA-based methods

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 each protist in each soil type separately for the DNA- and RNA-based methods (Fig. 4). The details of the bacterial names and results of statistical analyses can be found in Supplementary material 2. Overall, the mix culture of protists had the greatest impact on bacteria at the genus level, followed by amoeba, flagellate, and ciliate (Fig. 4). All of the protist species and their mixture had a greater impact on bacterial communities in the Gl treatment, compared to those of As. However, the major factor affecting the outcome of prey-predator interaction was nucleic acid used in molecular

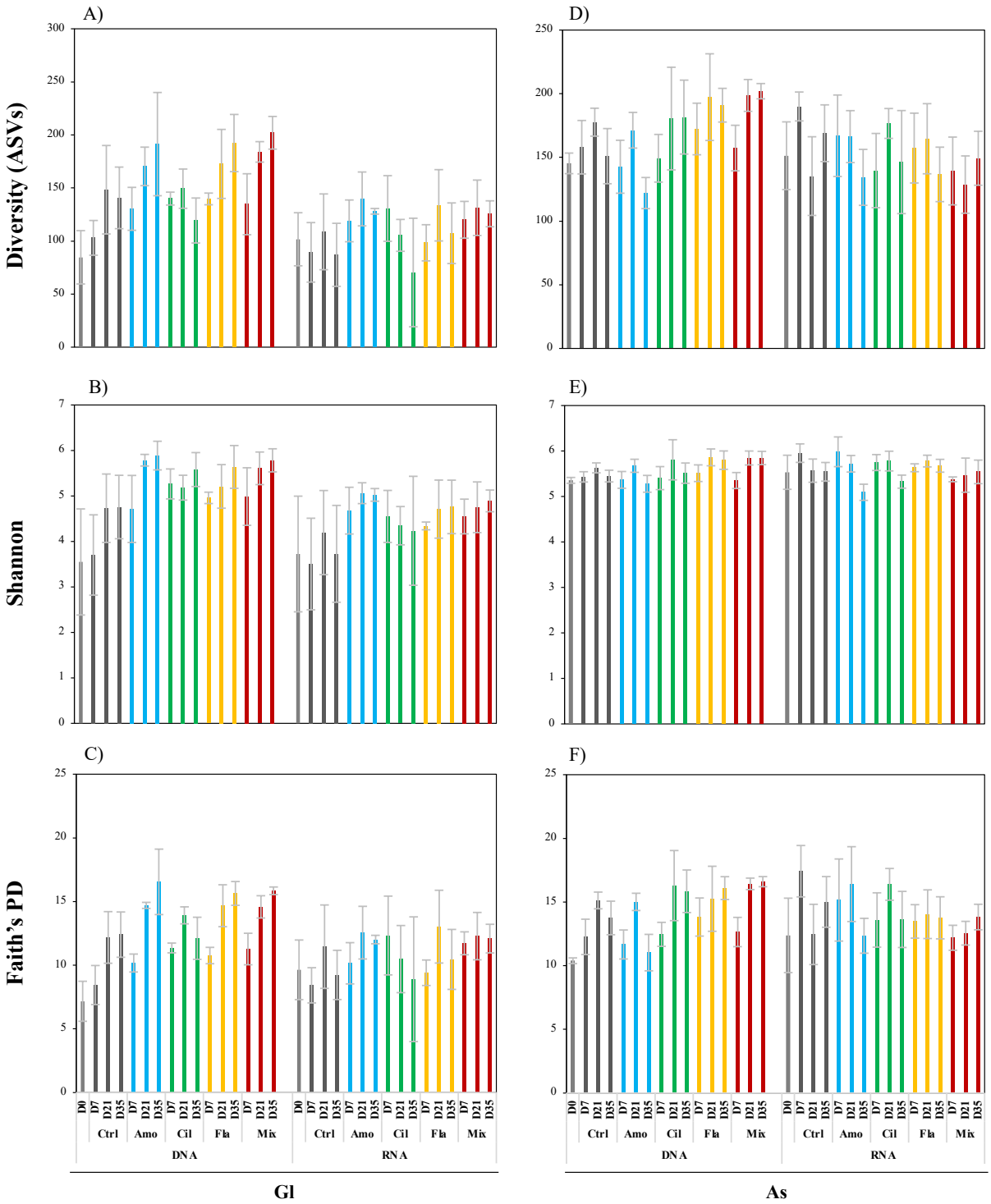

Fig. 2 The number of ASVs (**A** and **D**), Shannon's index (**B** and **E**), and Faith's PD (**C** and **F**) in Gl (**A**–**C**) and As (**D**–**F**) communities for each treatment. Ctrl, control with no protists (dark grey); Amo, *Vermamoeba vermiformis* (blue); Cil, *Colpoda steinii* (green); Fla, *Heteromita globosa* (yellow); Mix, a mixture of the three protist isolates (red); Gl, grey lowland soil; As, andosol; D represents incubation days. Error bars represent standard deviations. For statistical analysis, please see Table S3

Table 1 Permutational multivariate analysis of variance (PERMANOVA) results based on the Bray–Curtis dissimilarities for the effects of soil type, protists, nucleotide, and incubation days on the bacterial community composition

Factors	Df	Sums of sqs.	Mean sqs.	F. model	R^2	*P* value
Soil type (*S*)	1	16.428	16.428	132.382	0.352	0.001***
Protist (*P*)	4	3.071	0.768	6.186	0.065	0.001***
Day (*D*)	3	3.022	1.008	8.118	0.064	0.001***
Nucleotide (*N*)	1	1.701	1.700	13.702	0.036	0.001***
$S \times P$	4	2.557	0.639	5.945	0.054	0.001***
$S \times D$	3	0.908	0.303	2.816	0.019	0.002**
$S \times N$	1	0.603	0.604	5.611	0.012	0.001***
$P \times D$	8	0.994	0.124	1.155	0.021	0.195
$P \times N$	4	0.456	0.114	1.061	0.011	0.319
$N \times D$	3	0.553	0.184	1.713	0.012	0.034*
$S \times P \times D$	8	0.981	0.123	1.141	0.021	0.236
$S \times P \times N$	4	0.267	0.067	0.621	0.005	0.964
$S \times N \times D$	3	0.292	0.098	0.907	0.006	0.545
$P \times D \times N$	8	0.554	0.069	0.644	0.012	0.989
$S \times P \times D \times N$	8	0.621	0.078	0.721	0.013	0.947
Residuals	126	13.551	0.108	0.291	–	–
Total	189	46.559	1	–	–	–

*p <0.05; **p >0.01; ***p <0.0001

analyses. On average, the effect of protists on bacteria at the genus level was estimated to be 60% higher by the DNA-based method compared to the RNA-based method (Fig. 4).

Classification of significantly enriched and depleted bacterial taxa at the phylum level by protists in DNA- and RNA-based results is shown in Fig. 5. Comparison of DNA- and RNA-based results showed that Firmicutes were the most overestimated taxa in the DNA-based results followed by Actinobacteriota. On the other hand, Proteobacteria and Bacteroidia were underestimated by the DNA-based method. As the effect of each protist was different on bacterial community composition, we further showed the under- and overestimated bacterial phyla for each protist isolate (Fig. S4).

For amoeba, consistent results with the overall results were obtained for enriched bacterial taxa, while ciliate treatment showed a difference in the Proteobacteria phylum, and flagellate treatment showed a difference in Firmicutes. Interestingly, no bacterial taxa belonging to Proteobacteria were depleted in the presence of amoeba, while Proteobacteria was one of the main depleted phyla in ciliate and flagellate treatments (Fig. S4). The effect of all protist isolates on Bacteriodia was underestimated by the DNA-based method for both enriched and depleted taxa.

Discussion

Here we studied the effect of predatory protists on bacterial communities in two paddy field soils with DNA- and RNA-based high-throughput sequencing methods. Although our results represent bacterial relative abundances, here we focused on the fluctuations within the bacterial community composition, which showed that protists differently affected bacterial community composition depending on the DNA- and RNA-based methods (Figs. 4 and 5), confirming our first hypothesis. In vitro studies do not necessarily represent natural conditions such as the density and composition of protist and bacterial communities; however, they are crucially important to understand the microbial trophic interactions, which are too complex to study under natural conditions. The protist species used in this study are commonly present in paddy field soils (Asiloglu et al. 2021c). Protists significantly altered bacterial communities in both soils, and different bacterial taxa were affected even by the same protist species (Figs. 4 and 5) indicating the importance of soil type for microbial trophic interactions, which confirms our second hypothesis. The importance of soil physicochemical properties on protist-bacteria interactions has been shown in previous studies (Ekelund and Rønn 1994). For instance, the efficiency of protist predation on bacteria can be affected by the clay particles (England et al. 1993; Erktan et al. 2020) and the amount of available nutrients (Kurm et al. 2019). In addition, the heat sterilization of soil enriches the available nutrients, including organic carbon, N, P, and microbial necromass (Wolf and Skipper 1994), which may have stimulated the growth of inoculated bacteria. However, in our study, the initial bacterial community in each soil was also different from each other. Therefore, we are far from concluding whether the differences in the soil physiochemical properties or the initial bacterial community caused the differences in the protist effect on the prey community composition. Future studies focusing on the effect of protists on a

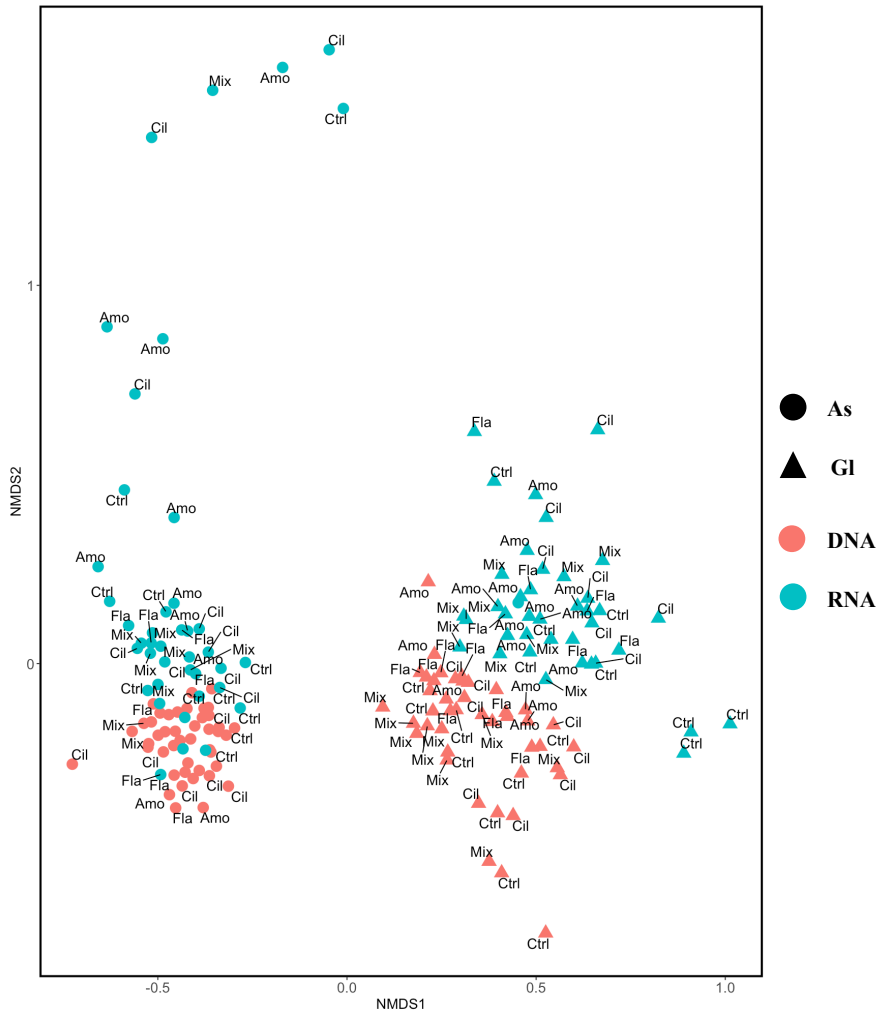

Fig. 3 Non-metric multidimensional scaling (NMDS) plots calculated based on the Bray–Curtis dissimilarity index of bacterial communities (stress: 0.1928). Red color, DNA-based communities; green color, RNA-based communities; circle, Andosol; triangle,grey lowland soil. Ctrl, control with no protists; Amo, *Vermamoeba vermiformis*; Cil, *Colpoda steinii*; Fla, *Heteromita globosa;* Mix, a mixture of the three protists isolates

bacterial community in different soil types could clarify the determining effect of the soil properties on the prey-predator interactions.

Our results showed that the total bacterial population was not affected by the presence of protists, except the results of the DNA-based method in the Andosol. Although protist predation significantly decreases the population of preyed bacterial species, protists release nutrients in their waste products, which can serve as a food source for several bacterial species (Gao et al. 2019). In addition, protist predation on bacteria may reduce competition among bacterial species (Flues et al. 2017). Therefore, losses in bacterial numbers of one taxon can be compensated by other bacterial species, which may explain our results. This can be further supported by the alpha diversity indexes which showed that the effect of protists on richness, and Shannon's index and Faith's PD were not negative, indicating that preyed bacterial species are likely to be replaced with predation-resistant species (Gao et al. 2019).

Selective feeding is one of the most important factors for the effect of predators on prey communities. We showed that the effect of protists on the paddy soil bacterial communities depended on the protist species, which is in line with previous studies (Asiloglu et al. 2020). The protists used in our study differ from each other by morphotype (a ciliate, a flagellate, and an amoeba), phylogeny, and size, which are known to differently affect prey-predator interactions (Gao et al. 2019; Leander 2020). Not only predators' traits, but also the characteristics of the bacterial species in a given community potentially influence the selective feeding behavior of protists including chemical cues, prey motility, prey size, prey biochemical composition, and cell surface characteristics (Matz and Kjelleberg 2005). Although many bacterial species have evolved to have effective survival

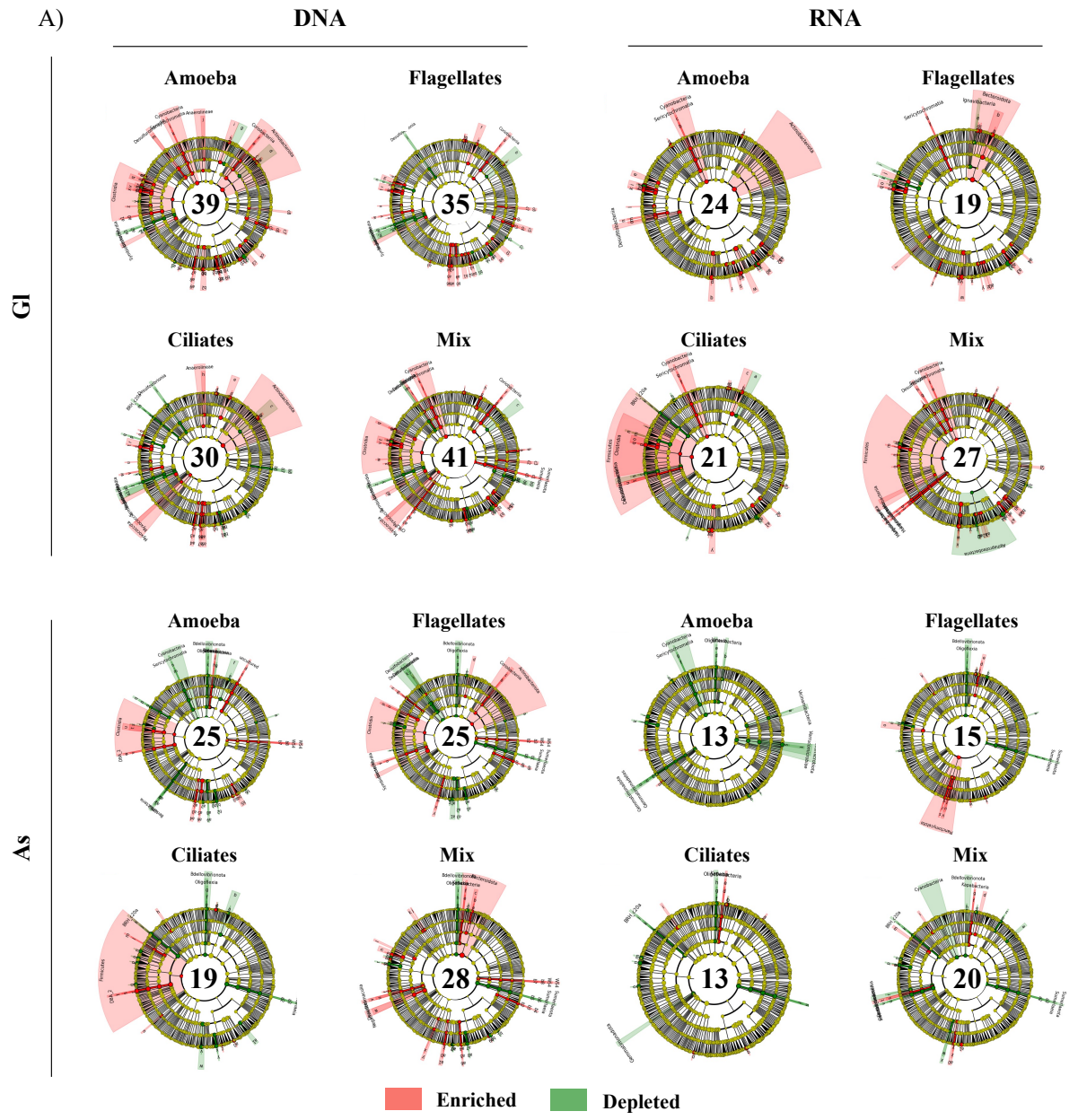

Fig. 4 A linear discriminant analysis effect size (LEfSe) method identified the significantly different ($p < 0.05$, Kruskal-Wallis test, LDA score > 2.0) bacteria at multiple taxonomic levels by comparison of bacterial communities in the presence and absence of protists in each soil type separately for the DNA- and RNA-based methods. Cladograms illustrate the taxonomic groups that explain the most variation among bacterial communities. From the center outward, they represent the kingdom, phylum, class, order, family, genus, and species levels. Colors represent the taxa with significantly different abundances between treatments: red, enriched bacterial taxa in the presence of protist isolates; green depleted bacterial taxa in the presence of protist isolates. The numbers in the center represents significantly affected bacterial taxa at the genus level. The details of the bacterial names and results of statistical analyses can be found in Supplementary material 2

mechanisms, the fate of bacterial survival may change depending on the present predator species. For instance, the efficiency of bacterial microcolony formation, a well-known survival mechanism (Matz and Kjelleberg 2005), changes depending on the feeding modes of protists (Bohme et al. 2009). Taken together, the traits of both bacteria and protists

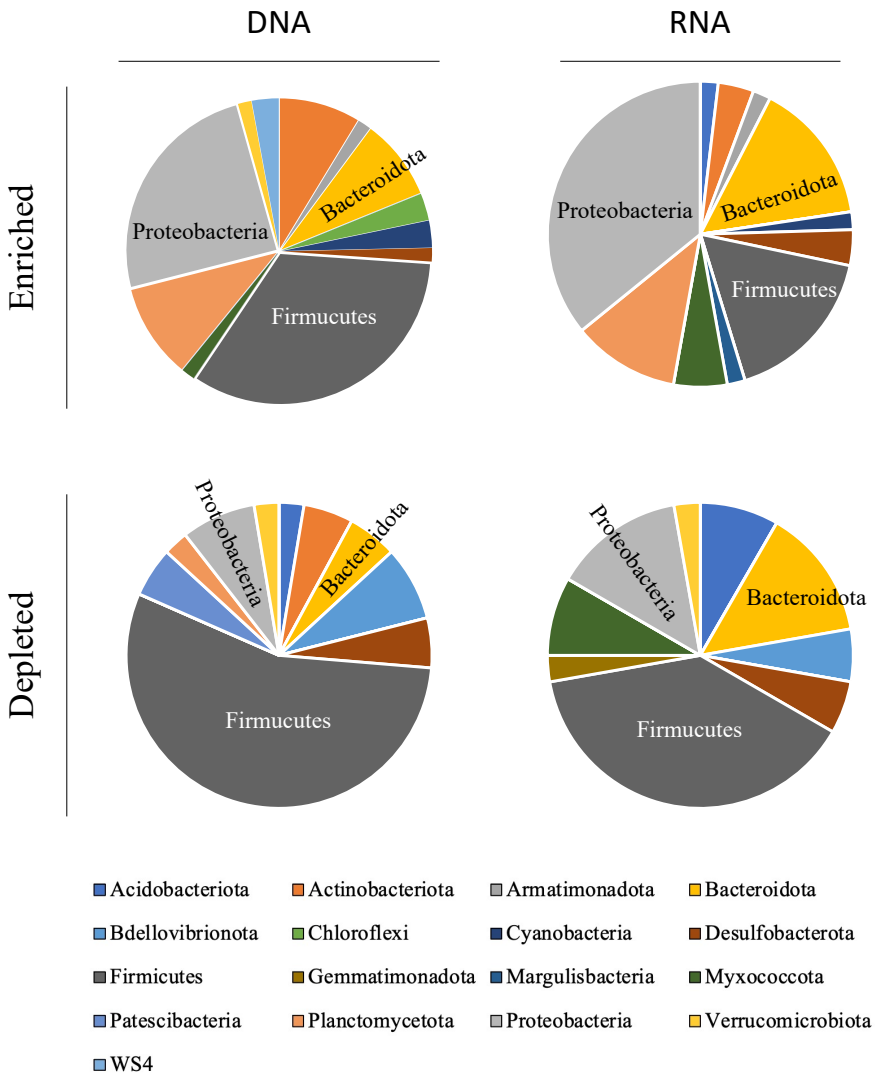

Fig. 5 Classification of significantly enriched and depleted bacterial taxa at the phylum level by protists in DNA- and RNA-based results. The results for each protist isolate are shown in Supplementary Figure S2

determine the species-specific interaction, which could explain the differences in the bacterial community composition among the protist treatments. It should be noted that the knowledge of selective feeding of protists mostly comes from controlled laboratory experiments, in which a few species of bacteria and protists are used, considering the fact that soil is an extremely complex and heterogenous environment and even a simple change in the population of a single bacterial species can result in unpredictable results for both protists and bacteria. For instance, Becks et al. (2005) showed that only a slight change in the bacterial numbers can end up with unpredictable results, which is defined as "chaos." Examples of such chaotic interactions in fragmented populations of protists and bacteria can occur on each grain of soil particles. Therefore, the overall effect of protists on bacterial communities can show both deterministic (e.g., selective feeding) and chaotic (e.g., the chance of an encounter between protist and bacterial species and bacterial competition) effects, and this can depend on soil physical properties (Erktan et al. 2020). Although protist-bacteria interaction in paddy field soil is well documented (Murase and Asiloglu, 2023), future studies on the effects of protists on bacterial communities under different paddy field soils would have the potential to shed light on the dynamics of deterministic/chaotic trophic interactions between protists and bacteria.

The bacterial community composition shifted with the incubation time. The top-down effects of protists on the bacterial communities were evaluated through a comparison of protist treatments with the control treatment in each specific sampling time. In general, the presence of protists alters the relative abundances of Firmicutes, Proteobacteria, and Bacteroidota (Murase et al. 2006; Flues et al. 2017; Asiloglu et al. 2020; Asiloglu et al. 2021a), which is in line with our results (Fig. 5). Bacterial species belonging to Proteobacteria and Bacteroidetes are preferred preys for protists (Murase et al. 2006; Krome et al. 2009; Flues et al. 2017). However, many Proteobacteria and Bacteroidota species 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 (Huws et al. 2005). Additionally, Firmicutes, Proteobacteria, and Bacteroidota include many bacterial species with a fast-growing ability, which enables them to replace the cells lost to predation (Gurijala and Alexander 1990; Kurm et al. 2019). Thus, while several members of Firmicutes, Proteobacteria, and Bacteroidota 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.

The major difference between DNA- and RNA-based methods for microbial community analyses is the differentiation of alive/dead cells (Cangelosi and Meschke 2014; Carini et al. 2017). Here we showed that the effect of protists on bacteria was much higher in the DNA-based results, indicating that relic DNA can influence the results of trophic interactions. In addition, we showed that DNA-based methods caused underestimations of Proteobacteria and Bacteroidota and an overestimation of Firmicutes. The detection of relic DNA from dead bacterial cells can be specific to bacterial properties. Bacterial cell wall type (i.e., Gram status) is an important factor in this matter (Nocker et al. 2006). For instance, a comprehensive study in various soil types showed that relic DNA from dead microorganisms caused an underestimation of bacterial taxa belonging to Proteobacteria and Bacteroidota, which are mainly composed of *gram*-negative cells (Carini et al. 2017). Relic DNA from gram-positive cells is likely to stay longer in soil (Carini et al. 2017), which is likely the main reason for the overestimation of Firmicutes in DNA-based methods.

Conclusion

Protists are one of the most important factors controlling bacterial communities in paddy field soil (Asiloglu et al. 2021a). In this study, we focused on the effect of three protist isolates on active and total bacterial community composition in two paddy field soils. Our study, the first to reveal the effect of protists on active bacterial taxa at the community level, revealed that (1) protists control bacterial communities with a species-specific effect, (2) soil type is an important factor determining the outcome of the microbial trophic interactions, and (3) DNA-based methods are likely to overestimate the effect of protists for Firmicutes, while it may cause an underestimation of the protist effect on Proteobacteria and Bacteroidota. Our previous DNA-based studies showed that protist-bacteria interaction enhances rice plant growth (Asiloglu et al. 2020, 2021b). However, we are still far from understanding the mechanisms of protist-enhanced plant growth. We suggest that the RNA-based methods could provide a better understanding of protist-rhizobacteria and protist-plant interactions.

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Author contribution RA conceived and designed the study and interpreted the data. RA and FM analyzed the sequence data, performed bioinformatic and statistical analyses, and prepared the manuscript. FM performed the experiments. KS and NH provided feedback and valuable comments. All authors read and approved the final manuscript.

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Availability of data All of the data obtained in this study are available upon reasonable request to RA.

Declarations

Ethics approval Not applicable.

Conflict of interest The authors declare no competing interests.

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