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ORIGINAL PAPER

Biochar affects taxonomic and functional community composition of protists

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Abstract

Biochar-induced changes in microbial communities are exclusively derived from the studies on the soil bacterial and fungal communities, and we lack an understanding of how biochar can affect taxonomic and functional communities of protists. Here, the short-term effects of two biochars originating from rice husk and poultry litter (hereinafter referred to as RH and PL, respectively) on taxonomic and functional community compositions of protists in a rice rhizosphere were studied using high-throughput sequencing. Soil physicochemical properties were differentially affected by the RH and PL amendments. The relative abundance of *Stramenopiles*, mainly oomycetes (*Peronosporomycetes*), was increased in the RH-amended soil, which was correlated with the increased total pore volume and C/N ratio. In the PL amended soil, the relative abundances of *Amoebozoa*, *Alveolata*, and *Excavata* were increased, and those increases were correlated with the enhanced pH and nutrient conditions. Among functional groups, the relative abundance of phagotrophic protists increased by the PL amendment, while the relative abundance of plant pathogens was decreased by both the RH and PL amendments. Network analysis indicated that phagotrophs were the keystone group and were sensitive to the biochar amendments. The keystone taxa in each biochar treatment were different: *Cercozoa* (*Rhizaria*) in control, *Conosa* (*Amoebozoa*) in RH, and *Discoba* (*Excavata*) in PL. The impact of biochar on protist communities correlated with its physicochemical properties, which depends on the source material.

Keywords Protozoa · Biochar · Protists · High-throughput sequencing · Phagotrophs · Rhizosphere

Introduction

Soil microbial composition, which is fundamental for the ecosystem functioning and agricultural productivity, is often affected by agricultural managements (García-Delgado et al. 2019; Schmidt et al. 2019), especially by chemical and organic fertilisations (Lin et al. 2019; Yang et al. 2019; Zhao et al. 2019). The use of chemical fertilisers has several negative impacts on the environment, including the microbiome (Kamaa et al. 2011; Wang et al. 2011; Zhang et al. 2012). Organic fertilisers, on the other hand, are one of the core aspects of sustainable agriculture and climate-smart soils (Paustian et al. 2016). They ameliorate the negative impact of chemicals on the environment (Bastida et al. 2015; Jilani et al. 2007). Among them, biochar, a carbon-rich product of organic materials/wastes, has been receiving widespread attention as an organic soil amendment and considered to be one of the important materials for climate-smart soils (Paustian et al. 2016). Biochar amendment has been found to improve carbon sequestration, reduce greenhouse gas

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emission, ameliorate soil fertility and quality, and enhance agricultural productivity in soil (Mierzwa-Hersztek et al. 2018; Sheng and Zhu 2018; Sohi et al. 2010; Zheng et al. 2019).

Biochar amendment induces several changes in the soil physicochemical properties (Gul et al. 2015), which subsequently alters the community composition of micro (Lehmann et al. 2011) and macro-organisms (Kamau et al. 2019). Depending on the source of biochar, a subset of the microbial assemblage responds to the specific biochar properties (Hale et al. 2015; Kolton et al. 2011; Sheng and Zhu 2018; Wu et al. 2016). In general, effects of biochar amendment on the taxonomic composition of the microbial communities are often associated with changes in soil pH and nutrient contents (Dai et al. 2018; Hale et al. 2015), as well as the changes in physical properties of soil (Gul et al. 2015). In addition to the altered taxonomic composition of the soil microbiome, enhanced microbial functionality by biochar addition was also demonstrated. For instance, enhanced nitrous oxide reduction (Krause et al. 2018), nitrification (Prommer et al. 2014; Song et al. 2014), and methane oxidation (Reddy et al. 2014; Zhang et al. 2010) has been previously reported. The shifts in the soil microbial community composition were suggested to play roles to slow organic C turnover (Chen et al. 2018) and to reduce the mobility and bioavailability of heavy metals (Cheng et al. 2018). Biochar can influence the root colonisation and functioning of mycorrhizal fungi (Warnock et al. 2007). However, our knowledge of biochar–microbe interactions exclusively comes from bacteria and fungi, and we still lack an understanding of the responses of the taxonomically and functionally diverse communities of protists to biochar amendment. This gap limits our knowledge of the interaction between biochar and microbes, which is incomplete if we do not understand the effect of biochar on the largely unrevealed component of the microbiome, the protists.

Protists are one of the major groups of soil microorganisms, and they comprise a vast majority of the eukaryotes (Geisen et al. 2018). Protists are taxonomically diverse (Adl et al. 2018) with versatile functionality, which has an enormous impact on the microbiome and plant performance (Geisen et al. 2018). Therefore, the community composition of protists provides valuable information to understand the ecosystem functioning in a soil environment (Payne 2013). Phagotrophic protists are one of the main factors controlling bacterial and fungal communities in soil (Gao et al. 2019; Geisen 2016; Geisen et al. 2018). Nutrients immobilised in microbial biomass are released through the predation activity of phagotrophic protists, which accelerates nutrient turnover and increases the plant nutrient uptake (Bonkowski 2004; Clarholm 1985; Kuikman and Van Veen 1989). Furthermore, protists play important roles in nutrient cycling by organic matter degradation and carbon fixation (Jassey et al. 2015; Kramer et al. 2016). Economically important protist species include plant pathogens and parasites that have negative impacts on the soil microbiome and agricultural productivity (Latijnhouwers et al. 2003; Mahé et al. 2017).

Linking taxonomic profile to functionality has often been used in studies of bacteria and fungi and provides valuable information to understand ecosystem functioning (Langille et al. 2013). Assigning functionality to the taxonomy of protists (de Araujo et al. 2018; Dumack et al. 2019; Fiore-Donno et al. 2019; Guo et al. 2018; Mitra et al. 2016; Xiong et al. 2018) is underdeveloped compared to their bacterial and fungal counterparts. Taxonomically similar protists can play different roles from microbial predators to plant pathogens and parasites, and as well, organisms belonging to different taxonomic groups have evolved to similar trophic feeding modes (Geisen et al. 2018; Parry 2004). Therefore, studying not only taxonomy but also the potential functionality of protists would provide valuable information as has been suggested recently (Xiong et al. 2018) (Dumack et al. 2019).

Taken together, the diverse taxonomy and versatile functionality of protists reflect their importance in the soil ecosystem and for agricultural productivity. However, we are still in the infancy of understanding how environmental factors shape the protist communities. Soil protists are known to respond differently to the changes in the physicochemical properties of soils. For instance, the abundance of phagotrophic protists may increase with the increased nutrient conditions by application of organic fertilisers (Guo et al. 2018; Xiong et al. 2018), while oomycetes were controlled by the soil physical properties such as porosity, clay, and water contents (Rojas et al. 2017). Therefore, we hypothesised that the response of protist groups differs depending on the physical and chemical properties of biochar. To test this hypothesis, we applied two types of biochar: rice husk biochar (RH), which has relatively low nutrient content and high surface area, and poultry litter biochar (PL), which has relatively high nutrient content and low surface area and at two doses (2% and 4% (*w*/w)). We then studied the taxonomic and functional community compositions of rhizosphere protists in the early rice plant growth stage as affected by biochar amendments using high-throughput sequencing by using primers targeting the V9 region of the 18S rRNA gene (Amaral-Zettler et al. 2009). The taxonomic profiles were assigned to the potential functionalities of protists. The biochar effect on the correlations among taxonomic and functional groups of protists was then studied with a co-occurrence network analysis.

Materials and methods

Biochar production, soil samples, and rice seedlings

The raw materials of RH (*Oryza sativa* L.) and PL were obtained from a commercial enterprise in Çorum,

Turkey, and from the Research Plant of Agriculture Faculty, Ankara University, Ankara, Turkey, respectively. The raw materials were dried at 105 °C for 24 h, sieved through a 2-mm mesh, and stored at room temperature (25 °C) until pyrolysis. The raw materials were subjected to pyrolysis in an anaerobic pyrolysis chamber (Ankara University, Ankara, Turkey) at 300 °C with a heating rate of 10 °C min^{-1} for 120 min to obtain the biochars. To prevent the co-inoculation of microbes, the biochars were sterilised with three-time autoclaving at 121 °C for 60 min, as this was suggested to be the most efficient method for DNA-based molecular studies (Otte et al. 2018). Autoclaving may affect the physicochemical properties of the samples (Otte et al. 2018); however, the physicochemical properties of the biochars (Table 1) were analysed after the autoclave sterilisation.

The collection of soil samples and preparation of sterile rice (*Oryza sativa* L. Nipponbare) seedlings was performed as described previously (Asiloglu et al. 2020). The soil sample had the following characteristics: sand, 33.6%; silt, 47.0%; clay, 19.4%; TC, 1.6%; TN, 0.2%; pH, 5.0 (H_2O); CEC, 15 meq·100 g^{-1}. The sterile rice seedlings planted in agar media were grown for 26 days in a growth chamber at 24 °C with a day length of 16 h (250 μmol m^{-2} s^{-1}). Prior to the experiment, the agar particles that remained around the roots were gently washed-off with sterile ddH_2O.

Table 1 Physicochemical properties of the biochars

Physicochemical properties	RH	PL
pH	7.93 ± 0.12b	*10.1 ± 0.14a*
EC (dS m^{-1})	0.74 ± 0.06b	*4.67 ± 0.21a*
Ash (%)	33.8 ± 0.89a	33.8 ± 1.11a
OM (%)	66.1 ± 2.10a	66.2 ± 1.92a
BET surface area (m^2 g^{-1})	*12.8 ± 2.42a*	7.36 ± 1.62b
Bulk density	*0.20 ± 0.01a*	0.41 ± 0.02b
CEC (cmolc kg^{-1})	31.1 ± 3.22b	*48.4 ± 2.27a*
Total C (mg g^{-1})	208 ± 20.3b	*250 ± 32.6a*
Total N (mg g^{-1})	6.38 ± 0.82b	*29.3 ± 2.44a*
C/N	*32.6 ± 0.72a*	8.56 ± 0.29b
Available P (g kg^{-1})	0.85 ± 0.21b	*4.16 ± 0.18a*
K (g kg^{-1})	5.82 ± 0.25b	*34.6 ± 0.68a*
Mg (g kg^{-1})	0.53 ± 0.02b	0.57 ± 0.02a
Ca (g kg^{-1})	1.43 ± 0.03b	*2.79 ± 0.04a*
Na (mg kg^{-1})	0.15 ± 0.01b	*1.91 ± 0.08a*

RH rice husk biochar, *PL* poultry litter biochar

Different letters indicate significant difference ($p < 0.05$, ANOVA). Italicised numbers indicate a significant difference compared with control treatment

Experimental set-up and sampling

Centrifuge tubes (50 mL) were filled with 40 g ± 0.1 g of the paddy field soil and 2 or 4% (*w*/*w*) of either RH or PL with 3 replications per treatment. The control treatment was filled with only paddy field soil. As fertilizer, the Kasugai nutrient solution (per kg soil: 0.04 g $[NH_4]_2SO_4$, 0.02 g $Na_2HPO_4 \cdot 12H_2O$, 0.03 g KCl, 0.004 g $CaCl_2$, 0.006 g $MgCl_2 \cdot 2H_2O$, 0.005 g $FeCl_3$) was added to all microcosms. All microcosms were pre-incubated under submerged conditions. After 3 days of pre-incubation, one rice seedling was transplanted to each microcosm. The microcosms were covered with aluminium foil from the sides to block light penetration and watered daily to maintain submerged conditions during their 36 days of incubation in a growth chamber at 25/30 °C (day/night) with a day length of 16 h (250 μmol m^{-2} s^{-1}). The microcosms were destructively sampled after 36 days of incubation, and plant growth parameters were measured as described by Asiloglu et al. (2020). The rhizosphere samples were obtained as follows. First, the surface water of microcosms was removed, and the plants with soil were transferred from the microcosms into a 300-mL sterilised beaker. After the plant shoots were cut off, the rice roots, which densely grew in the microcosms, were cut (<5 mm) with sterilised scissors in the beaker and mixed with the soil. The mixture of the rice roots and soil were sampled for further analysis (hereafter called rhizosphere).

Physicochemical analyses of the biochars, the soil, and plant samples

The pH of biochars and the soil samples was measured in deionised water at a 1:2 (*w*/*w*) mass ratio using a pH meter (Mettler Toledo, FP20). The electrical conductivity (EC) of biochars was measured using an EC meter (Consort, C3010) as described by Rajkovich et al. (2012). The ash and organic matter contents of the biochar samples were determined by burning at 550 °C for 8 h. The surface area of the biochars and soil samples was analysed in a surface analyser (Nova, Quantachrome Instruments 77K, USA) with N_2 and was calculated by the Brunauer-Emmett-Teller (BET) equation (Brunauer et al. 1938). The biochar and the soil samples were degassed at 100 °C for 18 h prior to N_2 physisorption. The total C and N contents (TC and TN, respectively) in the biochar, soil, and plant shoot samples were analysed after drying at 105 °C for 24 h using an MT-700 Mark 2 CN analyser (Yanaco, Kyoto, Japan). The available P was extracted from the samples using 0.002 N H_2SO_4 and measured by the colourimetric method using a spectrophotometer (Shimadzu, UV-160A, Kyoto, Japan) with the Truog method (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).

Molecular analyses, bioinformatics, and statistical analyses

DNA was extracted from 0.5 g soil samples using ISOIL for Bead Beating (Nippon Gene, Tokyo, Japan) according to the manufacturer's instruction and eluted in 50 μL of TE buffer. The hypervariable V9 region of the 18S rRNA gene was amplified from the extracted DNA using the universal eukaryotic primers (1391F and EukBr) (Amaral-Zettler et al. 2009) tailed with Illumina barcoded adapters (Caporaso et al. 2012). Primary polymerase chain reaction (PCR) was performed as described previously (Murase et al. 2014). Purification of the PCR product, the second PCR, and the Illumina Miseq sequencing were performed as described previously (Asiloglu et al. 2020). Possible contamination of the DNA extraction kit was checked with negative control.

After sequencing, the primary analysis of raw FASTQ data was processed using the QIIME2 pipeline (version 2018.11, https://qiime2.org) (Bolyen et al. 2019) as described previously (Asiloglu et al. 2020). The taxonomic alignment was done as described elsewhere (Zhao et al. 2019). Briefly, DADA2 in QIIME2 has used to denoise the paired-end sequences into amplicon sequence variants (ASVs) (Callahan et al. 2016). QIIME2's q2-feature-classifier plugin was used for taxonomy assignment against the SILVA reference database (132 release) (Quast et al. 2012) at 99% OTUs. To obtain exclusive protist data, *Fungi, Metazoa, Plantae*, *Rhodophyta, Streptophyta, Opisthokonta,* and unclassified eukaryotes were removed from all samples (Qiime taxa filter-table/seq). To compare protist communities between the treatments, sequence read numbers were normalised to the minimum sequence number (10,000 reads) by random subsampling (Supplementary Fig. S3). Shannon index, Faith's phylogenetic distance, evenness, and observed ASVs were obtained. Non-metric multidimensional scaling (NMDS) analysis was performed based on the Bray–Curtis dissimilarity index to visualise the beta diversity dissimilarities at the genus level. To correlate the environmental parameters associated with the samples, we used the *envfit* function in the vegan package of R program version 3.6.1 (https://www.r-project.org/). Protist taxonomies were assigned to functional groups as described elsewhere (de Araujo et al. 2018; Xiong et al. 2018). Briefly, the taxonomic information at multiple taxonomic levels was used to match with the known functional roles of protists. Each protist taxon was separately categorised. The full list of the taxonomic information and the assigned functionalities is available in the Supplementary Table S5. To reveal the bioindicator species in each biochar-amended soil, a linear discriminant analysis effect size (LEfSe) method (Segata et al. 2011) was performed using the Galaxy server (http://huttenhower.sph.harvard.edu/galaxy/).

The microbial co-occurrence network was analysed as described previously (Williams et al. 2014) using pairwise correlation analysis of the taxonomic abundance matrices. The co-occurrence between all pairs of protists (using all samples) at the genus level within each treatment group was evaluated using the Spearman's correlation coefficient in R ($p < 0.05$). The significant positive ($r > 0.75$, $p < 0.05$) and negative ($r < -0.75$, $p < 0.05$) correlations were screened out, and co-occurrence networks were visualised in the Cytoscape software v. 3.7.2 (Shannon 2003). The node sizes indicate the mean taxonomic abundances of protists. The interactions between and within each functional or taxonomic group were calculated manually as follows. The edges directed from a functional or taxonomic group to the other groups were selected with special care not to include the connections that originated from the other groups. From the edge table menu of the Cytoscape, the connections were copied to an Excel sheet and counted, which gave us the number of connections from a taxonomic group to the other groups. Then, the selected edges were hidden on the Cytoscape, and the left connections gave us the number of connections within a functional or taxonomic group. The topological properties of the networks were calculated using the network analyser tool in the Cytoscape software. All statistical analyses were performed using the R program version 3.6.1 unless otherwise specified (https://www.r-project.org/) as described in Asiloglu et al. (2020) or as already described above.

Results

Physicochemical properties of biochar and soil and plant growth parameters

The RH and PL exhibited differential physicochemical characteristics (Table 1). The nutrient contents of PL, especially the C, N, P, and K, were higher than those of RH. The pH and EC were higher in PL, while RH was characterised by a higher surface area and C/N ratio. The soil physicochemical properties were affected (ANOVA, $p < 0.05$) by the biochar amendments, dose, and biochar × dose interactions (Table 2 and Supplementary Table S1). The soil pH was not affected by the RH application. The PL treatment increased the pH depending on the applied dose by 11.2% and 21.1% in the PL2 and PL4 treatments, respectively. The total pore volume was increased by 25.9% and 33.3% in the low (2%) and high (4%) doses of RH treatments. The total C content of the soil was increased by about 25% by the RH and PL amendments depending on the applied dose. Only the PL amendment increased the total N content of soil depending on the applied dose (29.0% and 66.6% increase), which caused a decrease in

Table 2 Impact of the biochar amendments on soil properties

Treatment	pH	Total pore volume ($cm^3\ g^{-1}$)	CEC (cmolc kg^{-1})	Total C (mg g^{-1})	Total N (mg g^{-1})	C/N ratio	Available P (mg kg^{-1})	K (mg kg^{-1})	Mg (mg kg^{-1})	Ca (g kg^{-1})	Na (mg kg^{-1})
Ctrl	4.88 ± 0.1 c	0.27 ± 0.01 b	24.4 ± 2.7 a	17.4 ± 0.5 d	1.65 ± 0.03 a	10.5 ± 0.1 c	47.30 ± 5.19 d	77.26 ± 49.37 b	273.25 ± 9.81 b	1.53 ± 4.64 a	181.55 ± 124.44 a
RH2	4.88 ± 0.1 c	*0.34 ± 0.01 a*	23.3 ± 4.1 a	*20.6 ± 0.5 c*	1.59 ± 0.08 a	*12.95 ± 0.4 b*	66.06 ± 4.97 cd	35.18 ± 6.72 b	288.43 ± 4.57 ab	1.20 ± 0.80 a	112.37 ± 42.81 a
RH4	4.90 ± 0.1 c	*0.36 ± 0.01 a*	23.4 ± 3.1 a	*22.2 ± 0.3 b*	1.64 ± 0.03 a	*13.5 ± 0.2 a*	*70.91 ± 7.16 c*	85.89 ± 2.98 b	275.30 ± 12.77 b	1.63 ± 0.07 a	89.55 ± 33.48 a
PL2	*5.43 ± 0.1 b*	0.28 ± 0.01 b	22.3 ± 4.2 a	*20.8 ± 0.4 c*	*2.13 ± 0.05 b*	*9.74 ± 0.1 d*	*109.69 ± 8.73 b*	207.4 ± 46.88 ab	*396.56 ± 15.12 a*	1.99 ± 0.28 a	137.92 ± 17.25 a
PL4	*5.91 ± 0.2 a*	0.30 ± 0.01 b	25.2 ± 2.7 a	*23.8 ± 0.4 a*	*2.75 ± 0.10 c*	*8.64 ± 0.2 e*	*174.14 ± 9.04 a*	*344.93 ± 138.59 a*	373.97 ± 90.36 ab	1.97 ± 0.33 a	161.25 ± 83.20 a

Ctrl control, *RH* rice husk biochar, *PL* poultry litter biochar, *2* and *4* percentage of added biochar (*w/w*)

Different letters indicate significant differences between the treatments ($p < 0.05$, Tukey's HSD). Italicised numbers indicate a significant difference compared with control treatment

the C/N ratio, while the C/N ratio of RH treatments increased. Available P was affected by biochars, applied dose, and biochar × dose interactions. The PL application increased K and Mg contents of the soil. The biochar application did not affect Ca and Na contents of the soil.

The PL amendment increased the rice plant growth and N and C uptake (ANOVA, $p < 0.05$), while the RH did not (Supplementary Fig. S1). The PL at both low (2%) and high (4%) doses increased the shoot biomass by about 30% (Supplementary Fig. S1A). Nitrogen uptake by the rice plants was 1.93 and 2.38 times higher in the lower and higher doses of PL, respectively than the control soil (Supplementary Fig. S1B). The PL increased the total C content in the rice shoots, but the applied dose did not affect (Supplementary Fig. S1C).

Taxonomic community composition of protists

In total, 908,403 quality sequences ($q > 30$) were obtained after the removal of chimeric, singleton, and doubleton sequences with 47,692–71,878 sequences obtained from each microcosm (mean frequency of 60,560). The sequences were assigned to 716 to 961 operational taxonomic units (OTUs). About 60% of the eukaryotic sequences were unclassified, and 4.66% of the sequences belonged to fungi (Supplementary Fig. S2). After removing the non-protist sequences, a total of 176,642 protist sequences were obtained with a mean frequency of 11,776 (total of 774 OTUs) ranging from 10,273 to 16,294. No significant differences were observed for the Shannon index, Faith's phylogenetic distance, evenness, and number of OTUs (Supplementary Table S2).

The biochars significantly affected the beta diversity of protists (PERMANOVA, $R^2 = 0.2705$, $p = 0.022$), while the effects of applied dose (PERMANOVA, $R^2 = 0.0871$, $p = 0.127$) and biochar × dose interaction (PERMANOVA, $R^2 = 0.0595$, $p = 0.394$) were not significant (Supplementary Table S3). The PL amendment significantly (PERMANOVA, $R^2 = 0.16325$, $p = 0.010$) affected the protist community composition, and the effect of RH amendment (PERMANOVA, $R^2 = 0.10725$, $p = 0.097$) was not significant (Supplementary Table S4). The NMDS analysis differentiated the protist community composition in the RH and PL treatments (Fig. 1). The protist community compositions of the control soil were clustered in the centre, and the communities in the RH and PL amended treatments were clustered in opposite directions based on the NMDS1 axis, while the NMDS2 axis showed no treatment effect. We found that impact of the PL on protist community composition correlated with increased pH ($p < 0.01$) and N ($p < 0.004$), P ($p < 0.02$), K ($p < 0.004$), and Mg ($p < 0.004$), while that of RH was correlated with increased total pore volume ($p < 0.03$) and C/N ratio ($p < 0.001$).

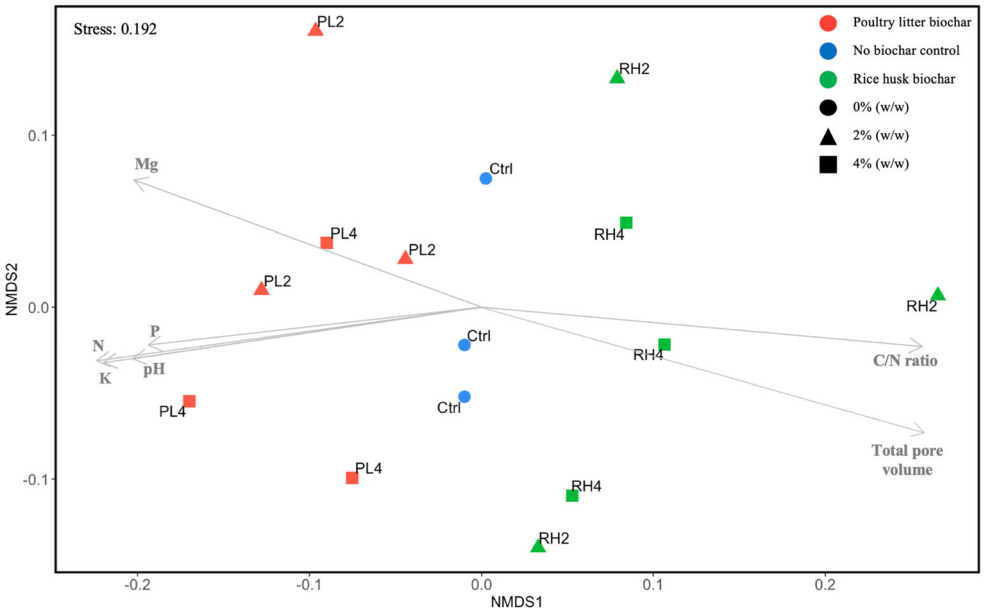


Fig. 1 Non-metric multidimensional scaling (NMDS) plots calculated based on the Bray–Curtis dissimilarity index of protist communities with significant correlations between protist community composition and soil physicochemical properties. Red colour, poultry litter biochar treatment (PL); blue colour, non-biochar control treatment (Ctrl); green colour, rice husk biochar treatment (RH); circle, control with no biochar addition; triangle, 2%(*w*/*w*); and square 4%(*w*/*w*) biochar treatments. Arrows indicate significant correlations among protist communities and environmental parameters ($p < 0.05$)

In general, *Stramenopiles* (41.1%) were the most dominant group in all treatments followed by *Archaeplastida* (19.4%), *Alveolata* (18.4%), *Rhizaria* (14.6%), *Amoebozoa* (4.4%), *Excavata* (2.1%), and *Haptophyta* (0.21%). Taxonomic super groups differed between the treatments. The PL and RH amendments differentially affected the taxonomic groups of protists (Fig. 2a). *Stramenopiles* tended to increase in the RH amendment, while *Alveolata*, *Rhizaria*, *Archaeplastida*, and *Amoebozoa* tended to increase the PL amendment. Compared with the control treatment, only *Stramenopiles* significantly increased in the RH2 treatment and decreased in the PL4 treatment. The relative abundances of *Stramenopiles*, *Alveolata*, *Rhizaria*, *Archaeplastida* and *Amoebozoa* were differentially affected by the PL and RH treatments. This was confirmed by the LEfSe analysis, in which significantly distinct protist groups at multiple taxonomic levels between the RH and PL treatments were characterised regardless of the applied doses (Fig. 3a and b). Both PL and RH affected several protist groups. LEfSe revealed that the biomarker protists in the RH amendment were *Stramenopiles* and *Peronosporomycetes*, while biomarker protist groups in the PL amendment were *Alveolata*, *Amoebozoa*, and *Excavata*. The LDA scores ranged from 3 to 5 (Fig. 3b). Total biomarker protists at multiple taxonomic levels detected by LEfSe were 31 for PL and 4 for RH treatments.

Functional community composition of protists

The assignment of the taxonomic profiles to their respective functionalities revealed the effects of biochar amendment on the potential functionalities of protists (Fig. 2b). In general, autotrophs were the most dominant group (26.4%), followed by phagotrophs (15.4%), omnivores (11.0%), plant pathogens (4.8%), and parasites (3.8%). We observed differences in the functional groups among the treatments (Fig. 2b). Compared with the control treatment, the addition of 2% and 4% of PL increased (ANOVA, $p < 0.05$) the relative abundance of phagotrophs by 47.4% and 113.7%, respectively. The relative abundance of plant pathogens was decreased (ANOVA, $p < 0.05$) by both RH and PL amendments. Compared with the control treatment, the decrease in the relative abundance of plant pathogens in the RH2, RH4, PL2, and PL4 treatments was 75.2%, 62.2%, 64.0%, and 48.0%, respectively.

The impacts of biochar treatments on the relative abundance of functional groups are shown for significantly affected taxa in Table 3 (ANOVA, $p < 0.05$) (A full list is available in the Supplementary Table S5). The relative abundance of several phagotrophs was increased in the PL2 (*Filamoeba*, *Echinamoeba*, and *Olygohymenophorea*) and PL4 treatments (*Filamoeba*, *Echinamoeba*, *Hartmanella*, *Heterolobosea*, *Naegleria*, *Colpodea*, and *Gymnophyrs*). The relative abundance of *Hartmanella* was decreased in both RH2 and RH4 treatments, and MAST (*Stramenopiles*) was decreased in only the RH4 treatment. The phagotrophic protist increased by the RH addition was only Soil_amoeba_AND16 in the RH4 treatment. Although no significant difference was observed for *Archaeplastida* and *Xanthophyceae* in RH and PL treatments compared with control treatment, *Archaeplastida* was significantly higher in the PL4 than the RH2, and *Xanthophyceae* was significantly lower in the PL4 than the RH2 and RH4. The relative abundance of *Diatomea* was increased in the PL4 treatment. Relative abundances of the plant pathogens, *Aphanomycetes* and *Phytium*, and the parasite *Apicomplexa* were decreased in both PL and RH treatments.

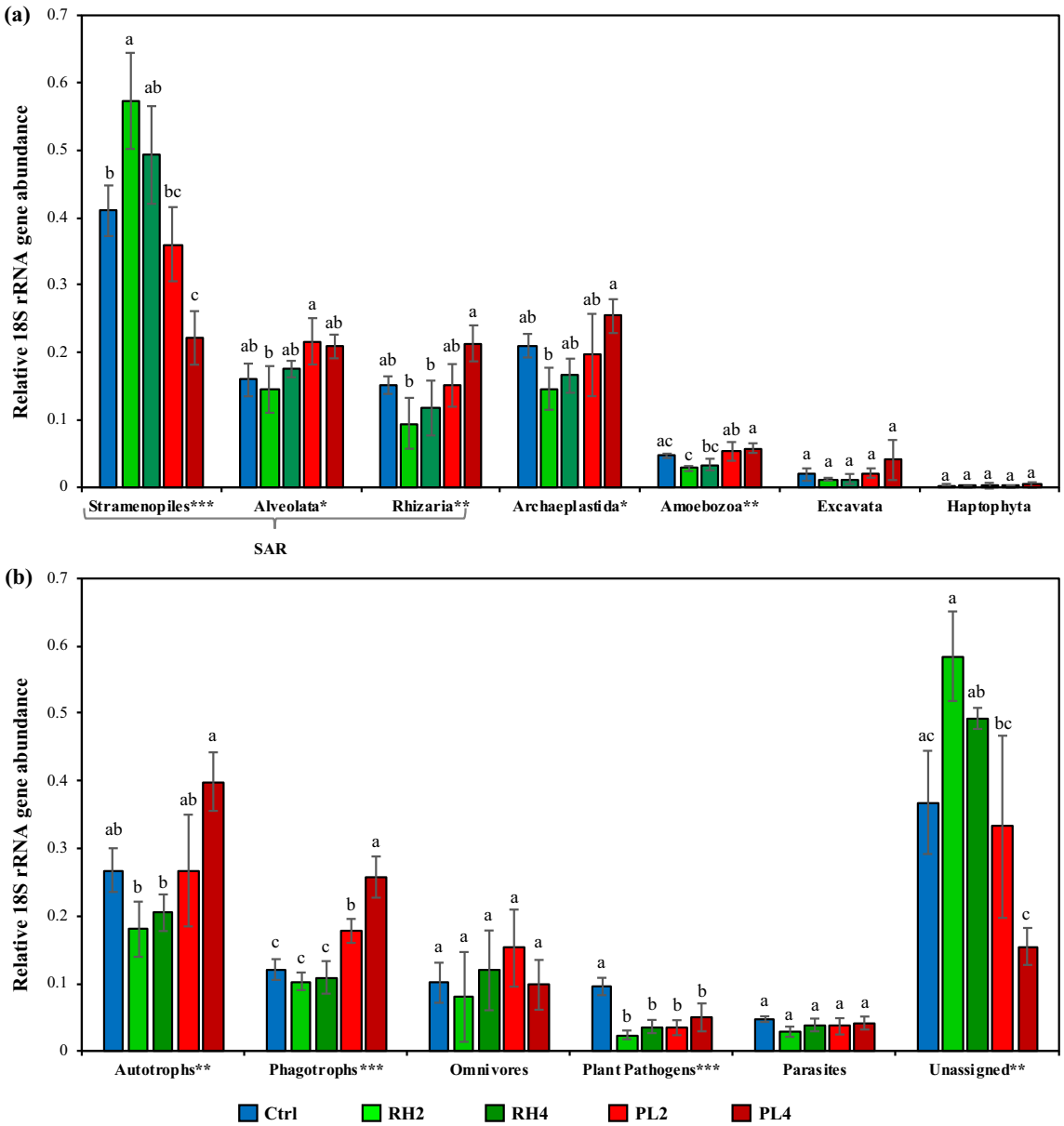


Fig. 2 Comparison of taxonomic supergroups (**a**) and functional groups (**b**) of protists. Bar plots showing the average percentage of protist abundances. Error bars represent standard deviations. Different letters indicate significant differences within each taxonomic or functional groups among the treatments (ANOVA). Asterisk after the names of the taxonomic or functional groups indicates the significant factor of one-way ANOVA results: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Blue colour, non-biochar control treatment (Ctrl); light green colour, 2%(*w*/*w*) rice husk biochar treatment (RH2); dark green colour, 4%(*w*/*w*) rice husk biochar treatment (RH4); light red colour, 2%(*w*/*w*) poultry litter biochar treatment (PL2); dark red colour, 4%(*w*/*w*) poultry litter biochar treatment (PL4). SAR represents a clade (the SAR supergroup) including Stramenopiles, Alveolates, and Rhizaria

Peronosporomycetes that could not be assigned to a functional group were increased in the RH treatment and decreased in the PL treatment; however, it was only significant between the RH2 and PL4 treatments.

Co-occurrence of protist communities

Co-occurrence analyses and network visualisations were performed to better understand the taxonomic relations of protists within the treatments. Furthermore, protist communities were sub-grouped to their functionalities to reveal the relationship within and between the functional groups (Fig. 4, Table 4, and Supplementary Table S6). The correlations between the taxonomic or functional groups were much higher than those within the taxonomic or functional groups (Table 4). We observed 62% more correlations within the functional groups than those within taxonomic groups. Compared with the control treatment, the number of nodes and abundance of the correlations were

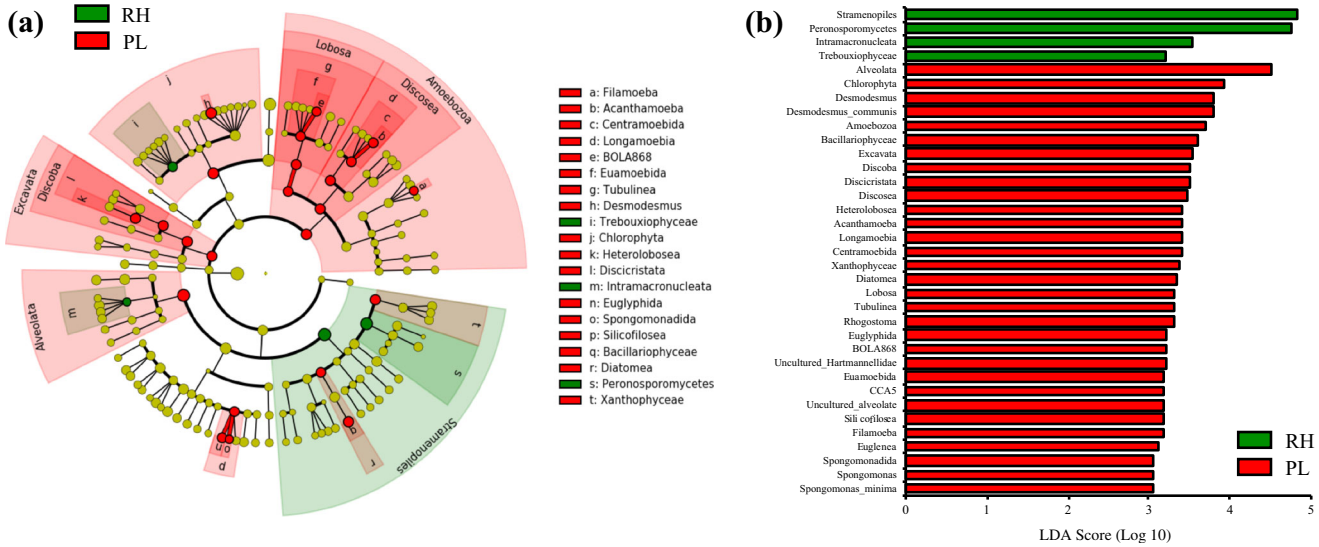


Fig. 3 A linear discriminant analysis effect size (LEfSe) method identifies the significantly different ($p < 0.05$, Kruskal–Wallis test, LDA score > 2.0) protists at multiple taxonomic levels by comparison of protist communities in the rice husk biochar and poultry litter biochar treatments. Cladograms are illustrating the taxonomic groups that explain the most variation among protist communities between rice husk biochar (RH) and poultry litter biochar (PL) treatments (**a**). Coloured dots represent the taxa with significantly different abundances between treatments, and from the centre outward, they represent the kingdom, phylum, class, order, family, and genus levels. The coloured shadows represent trends of the significantly differed taxa. Green colour rice husk biochar (RH); red colour, poultry litter biochar (PL). Histograms show the LDA scores of significant differences of protists (**b**). The words in the parentheses show the taxonomic level: *sg* supergroup, *p* phylum, *sp*. subphylum, *c* class, *o* order, *f* family, *g* genus, *s* species

increased in the RH2, RH4, and PL2 treatments, but were relatively less in the PL4 treatment. The proportion of negative correlations decreased upon treatment with both biochars.

To explore module–trait relationships, the protist network was clustered into modules (Supplementary Fig. S4). Phagotrophic protists dominated all of the modules regardless of the treatment. The dominance of plant pathogens decreased in all modules in the RH and PL treatments compared with the control.

Correlations within and between each functional and taxonomic group are shown in Supplementary Table S6. Correlations between different functional groups showed that phagotrophic protists had the most abundant positive and negative correlations with other functional groups. Correlations within the functional groups were more abundant in phagotrophs than the correlation within any other functional groups. Taxonomically, *Rhizaria* had the most abundant correlations with the other taxonomic groups, as well as with themselves. However, correlations within the *Amoebozoa* group and between the *Amoebozoa* group with the other taxonomic groups were more abundant than the other taxonomic groups in the RH treatment (Supplementary Table S6).

Discussion

Shifts in microbial communities caused by agricultural practices, especially fertilisers, have a direct impact on ecosystem functioning and agricultural productivity of soil, which makes it a necessity to understand how microbiome responds to agricultural practices. A great body of research has been conducted to understand the effect of biochar on microbial communities, as well as on their interactions (Graber et al. 2014; Lehmann et al. 2011; Zhu et al. 2017). Those studies provide essential knowledge of how biochar affects bacterial and fungal communities (Chen et al. 2019; Dai et al. 2018; Lehmann et al. 2011; Yu et al. 2018). This is the first study to reveal the effects of biochar amendment on taxonomic and functional groups of soil protists. The universal primers used in this study are not free of biases and likely lead to a significant underestimation of the true numbers of protistan taxa (Geisen et al. 2015, 2019; Jeon et al. 2008; Lentendu et al. 2014). However, in this study, we focused on the comparison of overall protist community composition as affected by biochar amendments, and our results indicate the effect of biochar amendment on the protist groups. We showed that protist communities are differentially affected by two biochars that originated from poultry litter and rice husk, indicating the importance of the source material of biochar for its impact on protists. Furthermore, the biochar influences the interactions among protists. Our study highlights the potential importance of biochar application for ecosystem functioning in the soil through its effect on the protist community composition.

Table 3 Relative abundance (%) of each taxon from the functional groups of protists

Functional groups	Taxonomic groups		Treatments					ANOVA results	
			Ctrl	RH2	RH4	PL2	PL4	Pr (>*F*)	*F* value
Phagotrophs	*Amoebozoa*	*Filamoeba***	0.09 ± 0.02 b	0.25 ± 0.11 ab	0.16 ± 0.01 b	*0.35 ± 0.06 a*	*0.35 ± 0.07 a*	2.3E-03	9.05
		Soil_amoeba_AND16***	0.00 ± 0.00 b	0.00 ± 0.00 b	*0.05 ± 0.02 a*	0.00 ± 0.00 b	0.00 ± 0.00 b	1.1E-04	19.36
		*Echinamoeba****	0.03 ± 0.02 c	0.06 ± 0.01 bc	0.08 ± 0.05 bc	*0.12 ± 0.02 b*	*0.36 ± 0.02 a*	3.1E-07	68.85
		*Hartmannella****	0.51 ± 0.10 b	*0.15 ± 0.03 c*	*0.20 ± 0.06 c*	0.68 ± 0.04 b	*0.98 ± 0.06 a*	9.5E-08	87.95
	Excavata	*Heterolobosea****	0.00 ± 0.00 b	0.11 ± 0.04 b	0.09 ± 0.06 b	0.14 ± 0.09 b	*0.60 ± 0.08 a*	2.4E-06	44.65
		*Naegleria****	0.30 ± 0.07 b	0.59 ± 0.33 b	0.39 ± 0.23 b	1.17 ± 0.28 b	*2.70 ± 0.82 a*	2.4E-04	16.03
	Alveolata	*Colpodea****	1.95 ± 0.32 b	3.32 ± 0.59 b	2.44 ± 0.29 b	3.02 ± 1.86 b	*7.18 ± 0.74 a*	3.9E-04	14.29
		*Oligohymenophorea **	0.07 ± 0.13 b	0.02 ± 0.03 b	0.49 ± 0.46 ab	*0.94 ± 0.04 a*	0.00 ± 0.00 b	1.1E-03	11.07
	Rhizaria	*Cercomonadidae**	3.46 ± 1.12 ab	1.66 ± 1.26 a	2.13 ± 0.93 ab	2.29 ± 0.85 ab	4.85 ± 1.05 b	2.5E-02	4.49
		*Gymnophrys****	0.44 ± 0.40 b	0.29 ± 0.08 b	0.31 ± 0.29 b	0.79 ± 0.22 b	*3.46 ± 0.28 a*	2.4E-07	72.71
	Stramenopiles	MAST**	1.68 ± 0.02 ab	1.37 ± 0.24 bc	*1.13 ± 0.24 c*	1.19 ± 0.20 bc	1.92 ± 0.21 a	3.1E-03	8.41
Autotrophs	*Archaeplastida*	Archaeplastida*	21.0 ± 1.74 ab	14.6 ± 3.12 b	16.5 ± 2.52 ab	19.6 ± 6.10 ab	25.4 ± 2.50 a	3.1E-02	4.15
	Stramenopiles	*Diatomea****	1.12 ± 0.73 b	0.73 ± 0.48 b	0.80 ± 0.52 b	2.35 ± 0.48 b	*9.01 ± 2.35 a*	2.2E-05	27.61
		*Xanthophyceae**	2.13 ± 0.22 ab	1.13 ± 0.17 a	1.21 ± 0.77 a	1.72 ± 0.55 ab	2.76 ± 0.71 b	2.2E-02	4.69
Plant pathogens	*Stramenopiles*	*Aphanomyces****	3.72 ± 0.62 a	*0.00 ± 0.00 b*	*0.57 ± 0.45 b*	*0.00 ± 0.00 b*	*0.05 ± 0.09 b*	4.0E-07	65.08
		*Pythium****	3.27 ± 0.14 a	*0.68 ± 0.22 b*	*0.79 ± 0.14 b*	*1.33 ± 0.36 b*	*1.14 ± 0.42 b*	3.0E-06	42.62
Parasites	*Alveolata*	*Apicomplexa***	3.60 ± 0.33 a	*2.02 ± 0.17 b*	*2.40 ± 0.28 b*	*2.24 ± 0.32 b*	2.70 ± 0.61 ab	3.4E-03	8.21
Unassigned	*Stramenopiles*	Peronosporomycetes***	26.1 ± 4.08 bc	*50 ± 9.27 a*	34.2 ± 2.99 b	20.8 ± 1.34 c	*2.23 ± 1.56 d*	4.1E-06	39.93

Ctrl control, *RH* rice husk biochar, *PL* poultry litter biochar, *2* and *4* applied biochar percentage (*w*/*w*)

Asterisks indicate significant codes: *** $p < 0.001$; ** $p < 0.01$, * $p < 0.05$. Different letters indicate significant differences between the treatments (Tukey's HSD)

Italicised numbers indicate significant differences compared with control treatment. The full list is available in Table S5

Biochar impact on the composition of protists

The differences in the protist community composition were explained by the opposite changes in the nutrient content of soils induced by biochars. The major protist group affected by the nutrient-rich biochar were the bacterial and fungal predators, phagotrophs. It is well known that biochar application favours bacteria and fungi by increasing soil nutrient availability (Lehmann et al. 2011; Mierzwa-Hersztek et al. 2018). Therefore, we suggest that the response of phagotrophs to the changes in the soil physicochemical properties was likely indirect, mediated by potential shifts in their microbial food sources. The relative abundance of Oomycetes (*Stramenopiles*) that are mainly controlled by the soil's physical properties such as porosity, clay, and water contents (Rojas et al. 2017), increased in the RH treatments, where soil porosity was enhanced. Therefore, we suggest that biochar-induced changes in the soil physiochemical properties have direct and indirect effects on the protist community composition depending on the source material.

There is a growing body of research showing that biochar application of soils is suppressing microbial pathogens (Graber et al. 2014), and our data on oomycetes support this evidence. Although the rice husk and poultry litter biochars tended to have opposite effects on protists, the only functional group that was affected similarly by

Table 4 Correlations and topological properties of the networks

Network properties	Treatments					
	All	Ctrl	RH2	RH4	PL2	PL4
Clustering coefficient	0.779	1	1	1	1	1
Number of nodes	147	41	60	52	50	37
Significant correlations	1812 (218)	269 (62)	577 (30)	433 (85)	408 (49)	212 (11)
Between FG	1355 (160)	206 (49)	449 (23)	303 (52)	308 (40)	157 (9)
Within FG	457 (58)	63 (13)	128 (7)	130 (33)	100 (9)	55 (2)
Between TG	1530 (176)	224 (47)	490 (25)	367 (71)	350 (39)	176 (9)
Within TG	282 (42)	45 (15)	87 (5)	66 (14)	58 (10)	36 (2)

"Between FG/TG" indicates the interaction of a functional or taxonomic group with the other groups, while "within FG/TG" indicates interaction within each group. Numbers indicate the total value of groups. For individual interactions of each group, see Table S6

All all of the treatments, *Ctrl* control, *RH* rice husk biochar, *PL* poultry litter biochar, *2* and *4* % (*w*/*w*) biochar dose, *FG* functional groups, *TG* taxonomic groups

The number in parentheses shows the significant negative interactions ($\rho < -0.75$, $p < 0.05$)

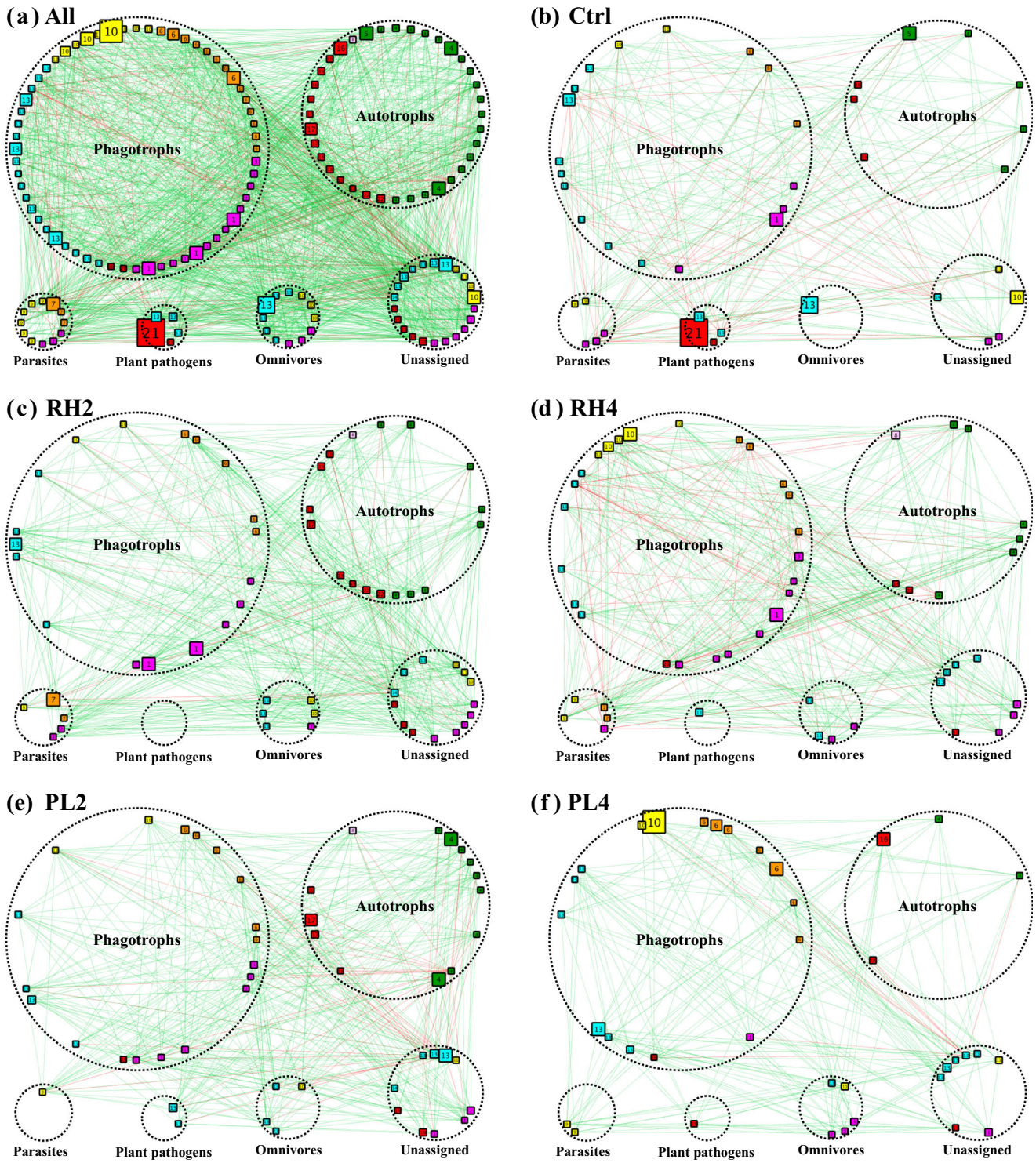
both biochars was plant pathogens, which is an intriguing part of our results (Fig. 2b). Thus, we assume that rather than changes in soil nutrients, pH, or physical properties, a different mechanism may control biochar–pathogen interaction. In line with our results, previously, four biochars that differed in physical and chemical properties showed strictly similar patterns to suppress plant pathogens including *Pythium* sp. (*Stramenopiles*) (Graber et al. 2014; Jaiswal et al. 2014). Although no direct evidence was shown in this study, it is likely that biochar-released organic compounds that can be photo/biotoxic or the volatile compounds, which have been traditionally used as pesticides (Orihashi et al. 2001; Yatagai et al. 2002), could act as pathogen inhibitors for protists.

Correlation among protists

Our approach to group protists based on functionality and analyse interactions within and between the functional groups provided better visualisation of the correlation among protists (Fig. 4). To the best of our knowledge, this is the first study showing a possible relationship within and between functional groups of protists. Among functional groups, most nodes and interactions were linked to phagotrophs (Fig. 4, Supplementary Fig. S4 and Supplementary Table S6), although they were not the most dominant group by the relative abundance. It has been reported that predator–prey interactions increase network stability and complexity (Allesina and Tang 2012), and phagotrophic protists are suggested to be the keystone taxa in the microbial networks (de Araujo et al. 2018; Xiong et al. 2018). This is no surprise as phagotrophic protists affect almost all microorganisms within their network. Microorganisms preyed by phagotrophs may be dramatically reduced below detection limit (Glücksman et al. 2010; Rosenberg et al. 2009). Other microorganisms that have not preyed (i.e., non-preferred or predation-resistant microbes) can benefit from the nutrients released from the protist-preyed microbial biomass (Bonkowski et al. 2000; Griffiths 1994) and/or gain a competitive advantage as a consequence of protist predation on their strong competitors (Bell et al. 2010; Flues et al. 2017; Jousset et al. 2008; Saleem et al. 2012). Therefore, our results suggest that microbial communities are likely to have a phagotroph-driven network.

Although the number of nodes and correlations of phagotrophs did not change by the biochar addition, the taxa having most nodes and correlations were linked to different phagotrophic groups in each biochar treatment: Cercozoa (*Rhizaria*) in control, Conosa (*Amoebozoa*) in RH, and Discoba (*Excavata*) in PL (Fig. 4 and Supplementary Table S6). Considering that different protists have distinct impacts on the bacterial community composition (Asiloglu et al. 2020; Rønn et al. 2002), the biochar-induced shift in the network and correlation among phagotrophic protists may have an indirect impact on the shifts in bacterial communities. Further studies on the biochar's effect on prey–predator

Fig. 4 Microbial co-occurrence networks based on the correlation analysis from taxonomic profiles of protist communities. The nodes were subgrouped based on their assigned functionality, and node sizes indicate the mean taxonomic abundances. Taxonomic supergroups were illustrated in different colours. Numeric labeling in the nodes represents taxonomic subgroups. Positive co-occurrence correlations (Spearman's $r > 0.75$, $p < 0.05$) were indicated with green-coloured edges, while negative co-occurrence correlations (Spearman's $r < -0.75$, $p < 0.05$) were indicated with red-coloured edges. *All*, indicates the whole community in all treatments; *Ctrl*, control treatment with no biochar addition; *RH2*, 2% rice husk biochar treatment; *RH4*, 4% rice husk biochar treatment; *PL2*, 2% poultry litter biochar treatment; *PL4*, 4% poultry litter biochar treatment



Amoebozoa
1. Conosa
2. Discosea
3. Lobosa

Archaeplastida
4. Chloroplastida
5. Rhodophyceae

Excavata
6. Discoba
7. Metamonada

Haptophyta
8. Prymnesiophyceae

Alveolata
9. Apicomplexa
10. Ciliophora
11. Dinoflagellata
12. Protalveolata

Rhizaria
13. Cercozoa
14. Foraminifera

Stramenopiles
15. Bicosoecida
16. Chrysophyceae
17. Diatomea
18. Eustigmatales
19. Hyphochytriales
20. MAST.12
21. Peronosporomycetes
22. Xanthophyceae

interaction would provide a better understanding of biochar-induced changes in soil microbiome.

Conclusion

In conclusion, both rice husk and poultry litter biochars altered the taxonomic and functional community composition of protists regardless of the applied doses. Our results indicated that the biochar effect is highly correlated with its physicochemical properties, especially pH, nutrients, and total pore volume. The network analysis indicated the importance of biochar on both protist–protist and prey–predator interactions. Overall, our results suggested that poultry litter biochar may contribute to ecosystem functioning, especially for C sequestration and plant growth through its impact on protist communities. It must be noted that our results and conclusions are based on the short-term impacts of the two biochars. We suggest that the potential effect of long-term biochar amendment on taxonomic and functional groups of protists should be further investigated.

Availability of data The raw sequence data obtained in this study have been deposited in the NCBI database under the BioProject ID PRJNA615322.

Authors' contributions RA conceived and designed the study, analysed the sequence data, performed bioinformatic and statistical analyses, interpreted the data, and prepared the manuscript. RA, BS, SOS, MOA, and PAB performed the laboratory works. KS, JM, OCT, and NH provided feedback and valuable comments. The authors read and approved the final manuscript.

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Compliance with ethical standards

Ethics approval Not applicable

Conflict of interest The authors declare they have no conflict of interest.

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