ORIGINAL ARTICLE

Phagotrophic protists can change microbial nitrogen conversion patterns during swine manure composting

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Abstract

Protists are important microorganisms involved in the transformation of nutrients, but little information is available regarding their roles in composting. This study investigated the changes in the protist community during composting and their relationships with the conversion of nitrogen. The protist community structure varied among the different composting phases, but the communities were similar in the cooling phase and at the end of the thermophilic phase. Phagotrophic protists such as Acantharea-Group-II_XX and Acantharea_XXX dominated in the composting process. The abundance of Acantharea-Group-II_XX increased by 12.8% in the thermophilic phase, whereas that of Acantharea_XXX increased by 74.5% and 275.3% during the cooling and maturation phases, respectively. The protist community was mainly affected by the NO_3^- and NO_2^- concentrations and the bacterial community, where NO_3^- and NO_2^- inhibited phagotrophic protists. Furthermore, compared with the nitrifying bacterial communities, the denitrifying bacterial communities were more conducive to the growth of Acantharea-Group-II_XX and Acantharea_XXX. Phagotrophic protists were key predators that regulated bacteria with denitrification functions.

Keywords Bacteria · Composting · Fungi · Nitrogen conversion · Protist

1 Introduction

Aerobic composting is a highly effective approach for treating livestock and poultry manure to render it harmless, where this biochemical process is mediated by microorganisms [1].

The microbial community during the composting process mainly comprises bacteria and fungi, which can degrade organic matter and synthesize humus, and convert organic nitrogen into a form that can be used by organisms during composting [2, 3]. These microbial processes greatly affect the quality and efficiency of composting [4].

Protists are small, soft-bodied organisms that can survive in all types of natural conditions [5], but they are difficult to preserve or they have few morphological characters that can be observed using conventional microscopy [6]. Protists are crucial for linking the interactions in microbial communities [7], where they may compete with and prey on bacteria and fungi [8]. Fungi and bacteria are consumed primarily by protists, which can control the functions, composition, and changes in the microbial community via predation [9, 10]. For example, Rosenberg et al. (2009) found that amoebae could decrease the abundances of bacteria, and some specific bacterial taxa disappeared 2 days after inoculation [9]. Bacteria and fungi are generally considered to play important roles as catalysts in nutrient cycling during composting, such as in the nitrogen cycle [11]. Previous studies have shown that protists

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increase ammonia volatilization in soil, mainly because of the higher C/N ratio of protists compared with bacteria [12], and their predation on bacteria may lead to the release of more NH_3 [13]. Nitrogen cycling can lead to the release of nitrogen in the form of NH_3 during composting, but ammonia can also be transformed into nitrate through nitrification and denitrification to improve the quality of the compost products. Nitrification and denitrification are the most important processes, and nitrifying and denitrifying bacteria that carry the *nxrA* and *nirS* genes play key roles during composting [14]. However, protists are often ignored as components of the environmental microbiome [12], and very little is known about the interactions among bacteria, fungi, and protists. Thus, the roles of the protist community members during composting remain unclear.

Protists have diverse functions, such as phagotrophs, bacterivores, parasites, omnivores, and mixotrophs, and these functional groups strengthen the microbial loop [15] because of their important roles in biochemical nutrient cycling [16]. Previous studies have mainly investigated the changes in the protist communities in soil [12, 17–19], but little information is available about the variations in the protist communities during composting. The succession of the protist community during composting has not been investigated in previous studies, and the protist community might change during different composting phases in a similar manner to bacterial and fungal communities, but further verification is required.

Microorganisms require suitable environmental conditions to function, and in a similar manner to bacteria and fungi, studies have shown the protist community can be affected by environmental factors (e.g., NO_3^- and pH [12]) in the soil. In contrast to the soil environment, the environment changes greatly during composting, and the main environmental factors that influence the protist community might be different. In addition, biotic factors such as bacteria and fungi will have mutual effects on the protist community, and they will change during the composting process. However, previous studies have not investigated the relationships between biotic factors and the succession of the protist community. Thus, it is not known how abiotic and biotic factors might affect the protist community, and how they could contribute to these changes. Therefore, it is necessary to explore the abiotic factors and biotic factors that might affect the protist community during composting.

Artificial temperature control was used in this study to simulate the conventional composting process. The protist, bacterial, and fungal communities were identified by high-throughput sequencing. This study aimed (1) to determine the composition of the protist community, (2) to assess the effects of various factors on the changes in the protist community, and (3) to determine the ecological interactions among the protist, bacterial, and fungal communities.

2 Materials and methods

2.1 Experimental design and physical and chemical properties

In this study, a mixture of swine manure (total organic carbon and nitrogen contents of 380.2 g kg^{-1} and 26 g kg^{-1}, respectively) and wheat straw (total organic carbon and nitrogen contents of 496.3 g kg^{-1} and 6.5 g kg^{-1}, respectively) was used to simulate the conventional composting process. The temperature was manually controlled over a period as follows: 20–55 °C (0–5 days, mesophilic phase), above 50 °C (6–16 days, thermophilic phase), 50–40 °C (17–21 days, cooling phase), 40–20 °C (22–35 days, maturity phase), and the post-decomposition phase at 20 °C (36–105 days). The volume of each plastic composting reactor was 500 mL (each reactor contained 150 g (dry weight) of the raw material). The C:N ratio and moisture content of the composting material were adjusted to 20:1 and 55%, respectively, as described in a previous study [14]. Samples were collected from three test reactors in the mesophilic phase (day 2), thermophilic phase (day 7 and 14), cooling phase (day 21), and maturity phase (day 35), and the three samples were mixed until they were homogeneous.

Fresh samples were suspended in water at 1:10 (w/w) to measure the pH and water-soluble carbon (WSC) contents. Fresh samples were suspended in 2 mol L^{-1} KCl at 1:50 (w/w) to measure the NO_3^-, NO_2^-, and NH_4^+ contents. The pH values were determined with a Thermo Orion 3-star pH-meter (San Diego, CA, USA), and WSC was tested using a TOC Analyzer (Elementar, Germany). NO_3^-, NO_2^-, and NH_4^+ were analyzed colorimetrically by flow injection analysis. Kjeldahl analysis was used to determine the total nitrogen (TN) content (for details of the data, please refer to the supplementary material and our previous study [14]).

2.2 DNA extraction and sequencing

The composting samples were pretreated by freezing and crushing, before DNA extraction using a Fast DNA Kit for Soil (MP Biomedicals, USA), as described in a previous study [14].

High-throughput sequencing was conducted using the Ion S5 XL platform with the eukaryotic 18S V9 universal primers 1389F (TTGTACACACCGCCC) and 1510R (CCTTCYGCAGGTTCACCTAC), and ITS5-1737F (GGAAGTAAA AGTCGTAACAAGG) and ITS2-2043R (GCTGCGTTCTTCAT SCGATGC) for fungi. The 16S V4 region bacterial primers 341F (CCTACGGGAGGC

AGCAG) and 518R (ATTACCGCGGCTGCTGG) were used for bacteria, and the Illumina HiSeq platform was employed for high-throughput sequencing. All sequencing was performed by Novogene (Beijing, China). The raw sequences were deposited in the NCBI Sequence Read Archive (SRA) database under accession number PRJNA681775.

2.3 Bioinformatics analysis

The low-quality parts of the eukaryote reads and chimera sequences were removed using Cutadapt (V1.9.1). Chimera sequences were then removed according to the barcode data. Comparisons were conducted with the species annotation database to detect chimera sequences (https://github.com/torognes/vsearch/), before finally removing the chimera sequences to obtain the final valid data (clean reads). After clustering all of the effective tags (Uparse v7.0.1001), the sequences were clustered at 97% identity by default clustering to obtain operational taxonomic units (OTUs). QIIME software (Version 1.9.1) and BLAST were used for species annotation, and the pr2_version_4.10 database for species annotation analysis [20, 21]. MUSCLE (Version 3.8.31) was employed for rapid multiple sequence alignment to determine the phylogenetic relationships among all representative OTUs. Finally, the data in each sample were normalized and the sample with the lowest amount of data was used as the standard. Based on previous studies [15, 17, 18, 22], the protist OTUs were divided into different functional groups according to their feeding patterns, i.e., phagotrophs, bacterivores, omnivores, saprotrophs, and parasites. The bioinformatics analyses of bacteria and fungi were based on previous studies [23, 24].

2.4 Statistical analysis

Principal component analysis was conducted based on Euclidean distances to explore differences in the structure of the protist community. Heatmaps of the protist community were generated using TBtools [25]. Random forest analysis (random Forest package in R 3.5.0) was performed to determine the key environmental factors (pH, NO_3^-, NO_2^-, WSC, temperature, total nitrogen, and NH_4^+) and biological factors (including fungal and bacterial communities) associated with differences in the functional groups of protists during composting. The importance of each abiotic and biotic factor was calculated based on the average value obtained using 5000 trees and according to the increase in the mean square error. Redundancy analysis (RDA) was conducted to analyze the positive and negative correlations between the relative abundances of the main protist functional groups in compost and the most important abiotic and biotic factors screened by random forest analysis (CANOCO 5.0). In

order to further investigate the bacterial community, the key paths and relative contributions of *nxrA*-harboring nitrifiers and *nirS*-harboring denitrifiers to the changes in the protist community were established with AMOS 22.0 to produce structural equation models (SEMs). Network analysis was conducted to obtain insights into the potential interactions among microbes in samples from different composting phases. In these analyses, 60 bacterial genera (divided functions), 60 fungal genera, and 60 protist genera (including six functional groups) were used to construct a network. A similarity matrix was calculated based on the Spearman's rank correlations. The Gephi platform 0.9.2 was used to visualize the network graphs.

3 Results and discussion

3.1 Protist community structure and composition

The differences in the protist communities among all samples were determined by principal component analysis during different composting phases (Fig. 1a). The protist communities in the samples collected on days 2, 7, and 35 differed significantly from those in the other phases, but the protist communities were similar on days 14 and 21 (Fig. 1a). Similar changes to those in the protist community structures occur among bacteria during composting [24], and a previous study showed that the degradation of organic matter was determined by the metabolic and enzyme activities of bacteria during the thermophilic phase [24]. The main components of WSC are simple sugars produced by the decomposition of complex organic matter [24], and WSC increased by 55% during the initial thermophilic phase in the present study (Fig. S1). Thus, protists may have been involved in promoting the decomposition of organic matter during the thermophilic phase.

The phagotroph functional group dominated the protist community during composting (Fig. 1b), and previous studies also found that this functional group was dominant in other environments [18, 26]. Acantharea-Group-II_XX was dominant during the mesophilic and thermophilic phases, where its abundance increased by 12.8% during the thermophilic phase but decreased by 84.4% at the end of composting. Murdock and Juniper (2019) showed that Acantharea-Group-II_XX is a member of Rhizaria [27], and it has the ability to resist high temperatures during composting [28], and thus, it may have contributed to the degradation of organic matter by bacteria during the thermophilic phase. *Stachyamoeba* and Acantharea_XXX dominated during the cooling and maturity phases, where the abundance of Acantharea_XXX increased by 74.5% and 275.3%, respectively, during these phases. A previous study showed that nitrification was stronger than denitrification in the late stage
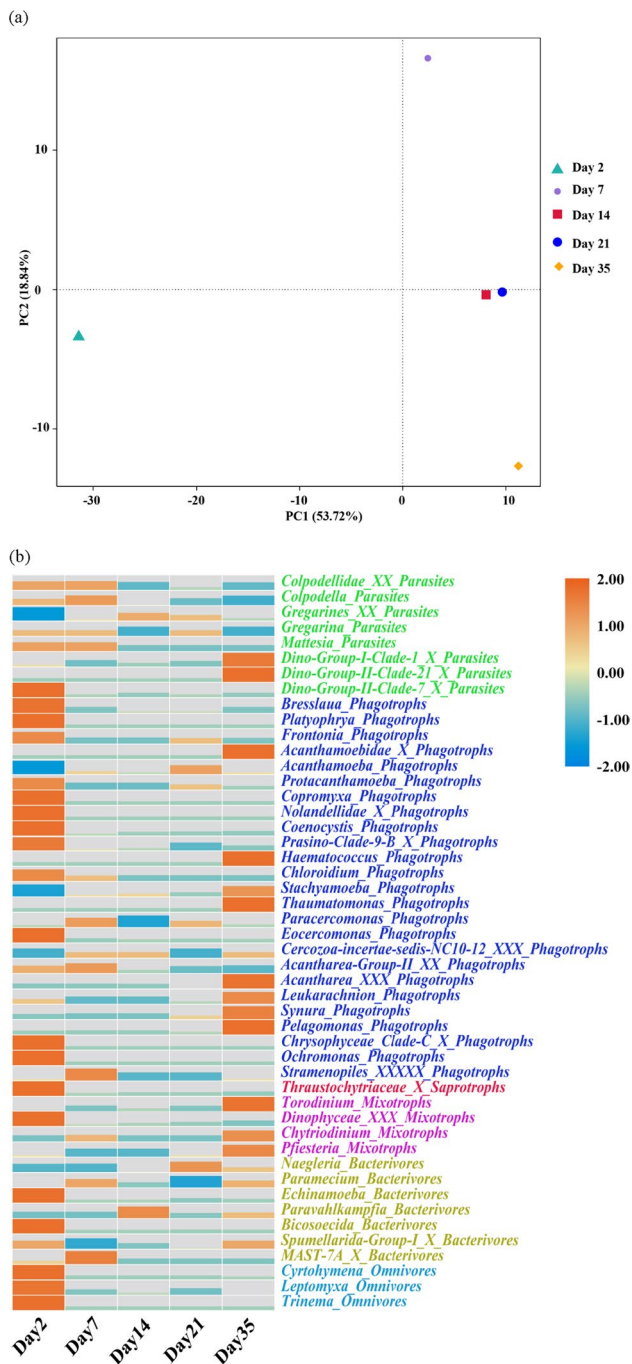
(a)

(b)

Fig. 1 **a** Principal component analysis of protist communities. **b** Protist community composition and functional groups during composting

of composting [14], and these conditions may have been closely related to the activity of this protist.

In addition, *Haematococcus*, *Thaumatomonas*, Gregarines_XX, *Chytriodinium*, and *Paravahlkampfia* were only found in the maturation phase, and these protists may reduce the abundances of pathogenic bacteria in composting products; e.g., *Thaumatomonas* has a significant predation

effect on MS2 phages [29, 30]. However, *Platyophrya*, Chrysophyceae_Clade-C_X, *Ochromonas*, and *Trinema* were only present in the initial stage of composting. Interestingly, *Frontonia*, *Protacanthamoeba*, and *Pfiesteria* disappeared in the thermophilic phase (Fig. 1b), and they may carry pathogenic factors; in particular, *Protacanthamoeba* is highly pathogenic [31]. Thus, phagotroph protists might have been involved in the transformation of the composting material, and the high temperature could have killed pathogenic protists.

3.2 Abiotic and biotic factors that affected changes in the protist community

According to the distribution and composition of the protist community, the environmental conditions and bacterial and fungal communities may have affected the protist community during composting. Random forest analysis showed that NO_2^- and NO_3^-, and the bacterial community were the most important factors ($P < 0.05$) to affect the protist community (Fig. 2a). A previous study showed that nitrate can inhibit phagotrophic protists when applied to the soil together with organic fertilizers [12]. NO_3^- and NO_2^- mainly had positive effects on omnivores and saprotrophs, whereas phagotrophs and parasites were negatively affected by NO_2^- and NO_3^- (Fig. 2b). In the maturity phase, the nitrate content was 1.2 times higher than that in the cooling phase (Fig. S1), thereby suggesting that a specific level of nitrate might have inhibited phagotrophic protists. In addition, the bacterial community composition explained 45.7% of the variation in the protist community, and the bacterial composition had positive effects on phagotrophs (Fig. 2b). Phagotrophic protists were dominant in terms of the number of species and their abundances in this study, which is consistent with previous results obtained in soils amended with organic fertilizer [18, 26]. Thus, protists may have interacted with bacteria involved in the transformation of NO_3^- and NO_2^-.

3.3 Effects of nitrifying/denitrifying bacterial communities on changes in the protist community

The conversion of NO_3^- and NO_2^- mainly involves nitrification and denitrification processes [32, 33], and there may be connections among nitrifiers, denitrifiers, and protists. Based on previous findings [14], *nxrA*-harboring nitrifiers, *nirS*-harboring denitrifiers, and the total bacterial community were analyzed to determine the relative contributions of bacteria associated with NO_3^- and NO_2^- conversion to the variations in the protist community (Fig. 3). The SEM results indicated that the overall bacterial community mainly affected protists through denitrifying bacteria. The protist community had significant positive effects on *nirS*-harboring
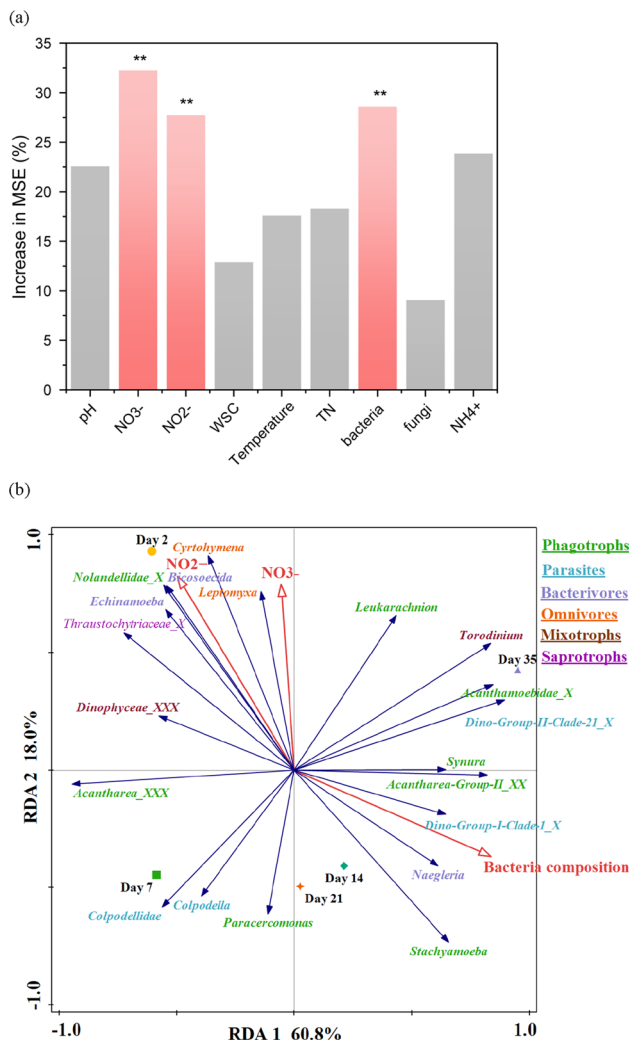
Fig. 2 **a** Random forest analysis based on the contributions of biotic and abiotic factors to the succession of the protist community. **b** Redundancy analysis results showing the effects of environmental factors on the succession of the protist community

denitrifiers ($P < 0.01$), but no significant negative effects on *nxrA*-harboring nitrifiers ($P > 0.05$). Positive correlations in SEMs indicate that two factors have mutually promoting effects and negative correlations denote the possibility of mutual use between two factors [34]. Thus, the denitrifying bacteria and protists may have mutually promoted growth, mainly because *nirS*-harboring denitrifiers could relieve the inhibition of nitrate. Indeed, the contribution of *nirS* to the conversion of NO_2^- mainly occurred in the early phase and that of *nxrA* in the maturity phase [14]. The NO_3^- content decreased in the early phases, but increased in the maturity phase. Therefore, the denitrifying bacterial communities were more conducive to the growth of protist communities compared with nitrifying bacteria.

3.4 Interactions among protists, bacteria, and fungi

The microbial network was constructed based on the bacteria, fungi, and protists to understand the ecological interactions among protists and other microorganisms. The results showed that the network formed three main modules, and some protists connected the bacterial and fungal groups (Fig. 4). Module 1 contained most of the bacteria (24 nodes), fungi (43 nodes), and protists (35 nodes), where the protists mainly belonged to phagotroph and bacterivore functional groups. *Thermomonas* and *Prevotella* were the most important bacteria, and *Neocosmospora*, *Fusarium*, and *Neonectria* were the most important fungi. Bacteria (such as *Luteimonas*, *Thalassospira*, and *Ethanoligenens*) and mixotroph protists (such as Dinophyceae_XXX) dominated module 2. Module 3 was dominated by bacteria such as *Bacillus* and bacterivore protists (such as *Echinamoeba*). Previous studies also found that protists had key roles in connecting bacterial and fungal communities in soil [12], groundwater [29], and extreme environments [27].
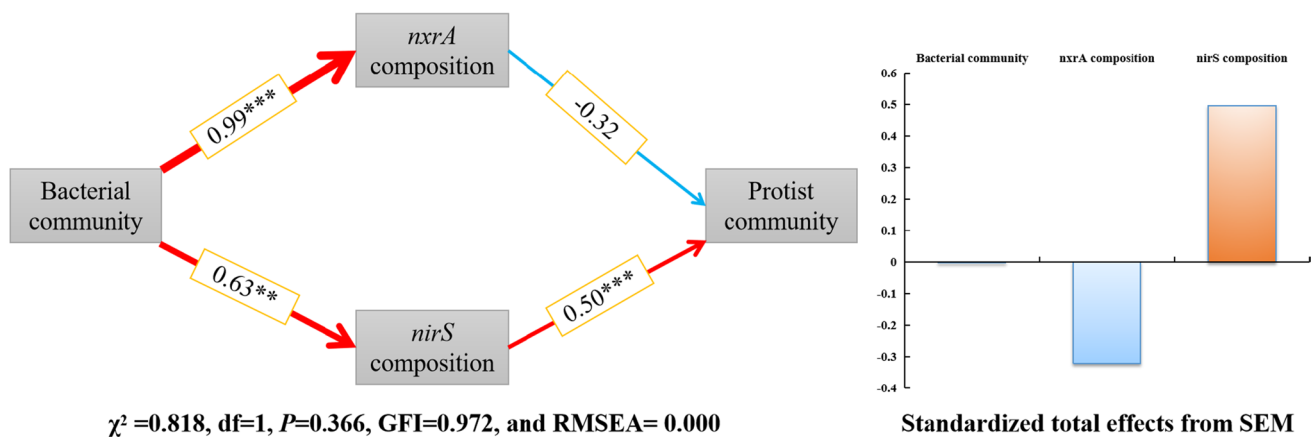

Fig. 3 Structural model equation analyses of the effects of *nxrA*- and *nirS*-harboring bacteria on protist communities
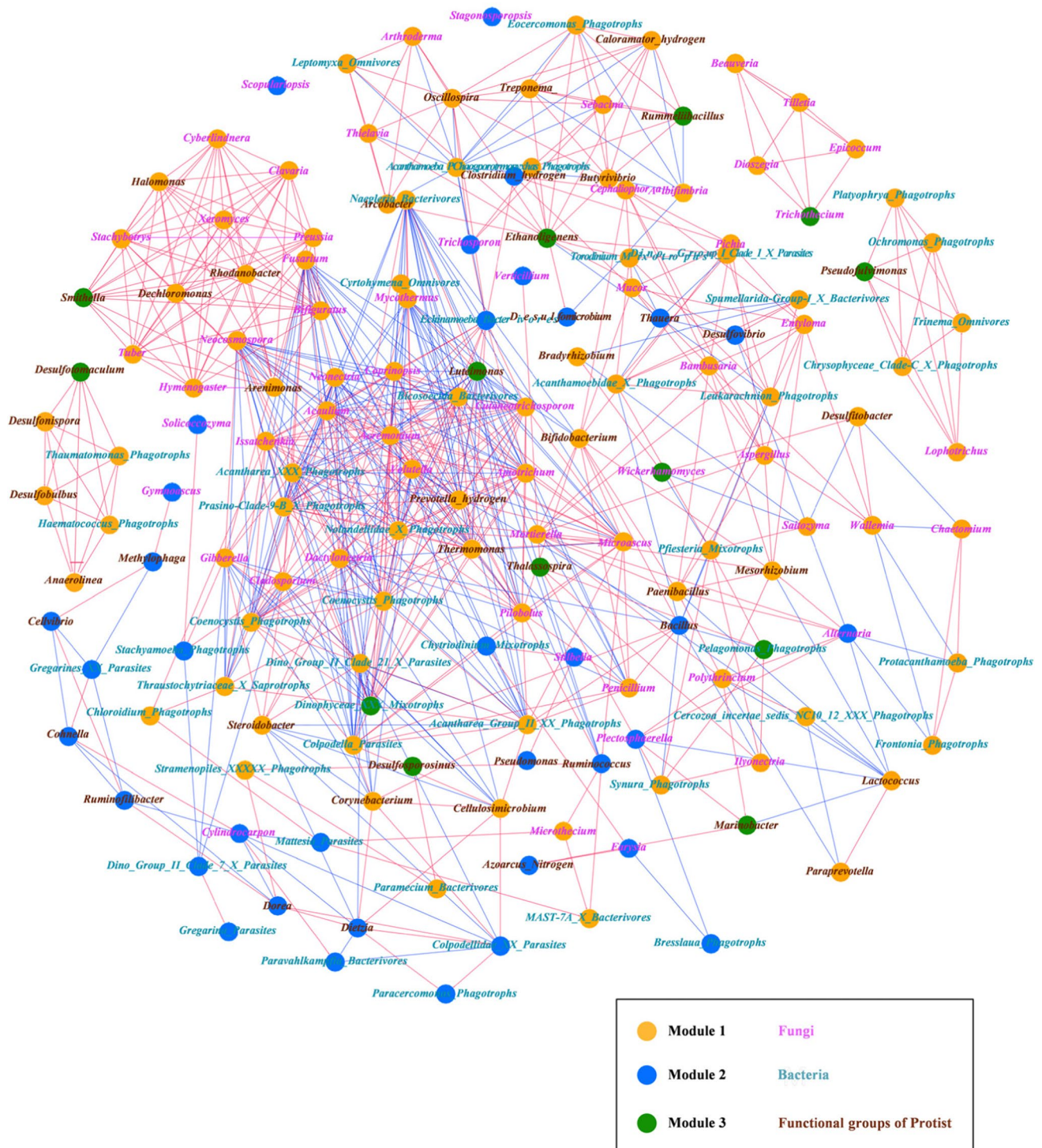
Fig. 4 Protists induced functional and dynamic hubs in the microbial community during composting. Significant positive correlations ($r > 0.90$) are represented by red lines and significant negative correlations ($r < -0.90$) by blue lines

Each module was generally associated with a specific range of functions, and module 1 was mainly important for maintaining phagotroph and bacterivore protist functional groups. Acantharea-Group-II_XX had positive interactions

with most other protists, but negative interactions with the main bacteria (such as *Steroidobacter*) and fungi (such as *Neocosmospora*, *Fusarium*, and *Neonectria*) in this module. Faust and Raes proposed that positive correlations may

indicate commensalism or mutualism, whereas negative correlations might be attributed to predation, competition, or unfair competition in an ecological network [35]. The results indicated that Acantharea-Group-II_XX was mainly reliant on predatory fungi for maintaining its activity during the mesophilic and thermophilic phases, and fungi exhibit greater tolerance of high temperature than bacteria [28, 36]. In addition, Fahrbach et al. found that *Steroidobacter* is an important host of *nirS* denitrification genes [37], thereby suggesting that Acantharea-Group-II_XX may prey on some denitrifying bacteria. However, Acantharea_XXX had negative interactions with various protists (including Acantharea-Group-II_XX, Prasino-Clade-9-B_X, and *Colpodella*), fungi, and bacteria (*Thermomonas* and *Prevotella*). Acantharea_XXX may have competitive or predatory relationships with many protists, fungi, and bacteria, which may explain the dominance of Acantharea_XXX in the cooling and maturation phases (Fig. 1b). Moreover, similar to Acantharea-Group-II_XX, Acantharea_XXX may prefer to prey on high temperature-tolerant denitrifying bacteria (such as *Thermomonas*).

Compared with module 1, the microbial relationships were simpler in modules 2 and 3. *Thalassospira* is a heterotrophic bacterial genus that can degrade organic matter [38], and Dinophyceae_XXX had positive correlations with protists and a negative correlation with *Thalassospira* in module 2. In module 3, *Echinamoeba* had positive correlations with other microorganisms, thereby suggesting that module 3 was associated with a bacterivore function. Modules 1, 2, and 3 were mainly connected by protists and bacteria, and protists were most closely related to bacteria with a denitrification function. For example, Prasino-Clade-9-B_X and Nolandellidae_X had negative interactions with *Luteimonas* (module 2), and *Echinamoeba* had negative interactions with *Thermomonas* (module 1) and *Oscillospira* (module 1). Previous studies also found that protists, especially phagotrophs, were key predators that promoted the cycling of nutrient elements and regulated the structure of the microbial community [5, 39]. In addition, some plant pathogens among fungi (such as *Fusarium* [40]) had negative correlations with the protists in each module, thereby demonstrating that protists had important roles in rendering the composting harmless.

4 Conclusion

In this study, the structure of the protist community in the cooling phase was similar to that at the end of the thermophilic phase, and the phagotrophic functional group dominated in different phases. Acantharea-Group-II_XX was dominant in the mesophilic and thermophilic phases, whereas Acantharea_XXX dominated the cooling and

maturation phases. Protists, bacteria, and fungi formed different hubs, and protists were most closely related to bacteria with denitrification functions. Denitrifying bacterial communities were more conducive to the growth of Acantharea-Group-II_XX and Acantharea_XXX, and phagotrophic protists were key predators that regulated the structure of bacteria with denitrification functions.

Author contribution Conceptualization: Yanan Yin; Methodology: Yanan Yin, Mengtong Li, Chao Yang; Data curation: Yanan Yin, Mengtong Li, Chao Yang, Xunzhang Hu; Writing — original draft preparation: Yanan Yin; Writing—review and editing: Yanan Yin, Wei Zheng, Manli Duan, Xiaochang Wang, Rong Chen; Funding acquisition: Yanan Yin, Rong Chen; Investigation: Mengtong Li, Chao Yang, Xunzhang Hu; Supervision: Xiaochang Wang, Rong Chen.

Declarations

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