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ORIGINAL ARTICLE

eDNA metabarcoding analysis reveals the consequence of creating ecosystem-scale refugia from deer grazing for the soil microbial communities

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Abstract

Ungulate overbrowsing is a growing problem in forests worldwide due to its prolonged and pervasive impact on plant biodiversity and ecosystem functioning. It has been shown that overbrowsing not only reduces plant species diversity and biomass (i.e., direct effects) but also causes a loss of associated trophic levels that could potentially feedback to influence plant community structure (i.e., indirect effects). One of the primary pathways of such indirect effects that have not been fully examined is the impact of overbrowsing on soil microorganisms. Recent studies have shown that soil microorganisms maintain vegetation diversity and drive succession, so it is of critical importance to understand how soil microbial communities might be affected by or protected from the deer impact. To assess the consequence of creating artificial grazing refugia on the structure and composition of soil microbial communities, we compared the distribution and abundance of soil microbial taxa (bacteria, archaea, fungi) at the fenced versus unfenced control sites in the context of a catchment-scale field

experiment in Japan. The eDNA metabarcoding analysis of soil microbial communities showed that the numbers of archaea and basidiomycetes fungal species were greater in the fenced site than in the control, while no such pattern was found for bacteria and ascomycetes fungi. Despite the lack of significant influence of the fence treatment on taxonomic composition in the soil fungal communities, their functional guild composition was influenced by the fenced treatment, with significant changes in the abundance of animal pathogens. Thus, although the effect of fencing on soil microbial communities is characterized by complex responses that vary from taxon to taxon, our work suggests that creating ecosystem-scale refugia from deer overgrazing might help sustain certain, if not all, taxa of soil microbial communities.

KEYWORDS
animal pathogen, archaea, Ascomycota, bacteria, Basidiomycota, deer fence

1 | INTRODUCTION

The impact of deer (and ungulate herbivores in general) on terrestrial ecosystems can have significant direct and indirect effects on forest structure and dynamics, productivity, and ecosystem function (Côté et al., 2004; Kaji et al., 2022). Deer overgrazing directly reduces the abundance and diversity of plant species (Kaji et al., 2022), and alters the outcome of plant species coexistence by modifying the dominance of certain plant species (Augustine & McNaughton, 1998; Nishizawa et al., 2016). Furthermore, when vegetation is removed by deer overbrowsing, not only the diversity of plant species itself but also that of associated trophic levels (e.g., arthropod herbivores, pollinators, and other animals) are disrupted (Kato & Okuyama, 2004; Sakata & Yamasaki, 2015). The loss of the associated biota could feedback to accelerate secondary plant species loss or retard vegetation recovery (i.e., indirect effects; Pires et al., 2020). Thus, an improved understanding of deer's impact on terrestrial ecosystems requires assessing direct effects on plant communities as well as indirect effects mediated by the associated biota.

Recent studies have shown that soil microorganisms play an important role in forest tree community assembly including succession and coexistence (Kadowaki et al., 2018; van der Heijden et al., 2018; Van der Putten et al., 2013), so assessing the effects of deer foraging on soil microbial communities must be urgently addressed. Of significant concern is whether deer overbrowsing may eliminate certain microbial species from the soil microbial communities, including those that play key roles in vegetation growth and regeneration (Eom et al., 2001). Theory suggests that the loss of mutualistic fungi such as mycorrhizal fungi might slow vegetation recovery, while the loss of pathogens reduces plant diversity by allowing certain plant species (i.e., those that escape from pathogen-induced mortality) to become dominant (Bever et al., 1997; Van der Putten et al., 2013). Despite the potential functional importance of soil microorganisms (van der Heijden et al., 2018), little is understood about how deer overbrowsing might modulate the structure and function of soil microbial communities.

One of the most common approaches to investigating the impact of deer overbrowsing is to establish fenced areas where the deer population is excluded and observe the process of ecosystem recovery after the establishment of fencing (Fujiki & Sakata, 2021; Tanentzap et al., 2012; van Klink et al., 2016). While the deer-proof fencing approach has been implemented worldwide, the size of the fencing area has been a matter of dispute (reviewed by Kaji et al., 2022). When the fencing covers a small area (e.g., 10–1000 m^2), vegetation structure may be necessarily oversimplified and susceptible to edge effects (Kaji et al., 2022; van Klink et al., 2016), raising questions about whether the observed fence effects represent a natural recovery. Notably, small-scale fence studies also cannot capture the indirect effects mediated by the associated biota, particularly when the primary pathways of such indirect effects involve processes that manifest at large spatial scales (such as dispersal) and/or at long time scales (Mysterud, 2006). Installing large-scale fenced areas offers the promise of accounting for the components of diversity that are regulated by spatial processes. This may be particularly relevant for soil microorganisms, a diverse group of organisms for which dispersal (spatial processes) and abiotic filtering (and hence environmental heterogeneity) may be key determinants of community structure (Kadowaki et al., 2014; Li et al., 2020; Matsuoka et al., 2016). Large-scale ecosystem-level experiments provide important insights into ecosystem conservation (Kaji et al., 2022; Scheffer et al., 2001) and hence can effectively advance our understanding of the consequence of creating artificial refugia for the soil microbial communities.

This study combines a large-scale deer fence experiment with environmental DNA metabarcoding analysis to assess the impact of creating artificial deer refugia on soil microbial communities in a cool-temperate forest ecosystem in Japan. In recent years, analysis of soil microbiota has become routine in experimental ecology and

provides insights into taxon-specific responses to the experimental treatment (Kadowaki et al., 2018; Tatsumi et al., 2021). We established deer-proof fencing at a scale that encompasses the entire catchment (as a natural unit of the ecosystem) adjacent to a control catchment (Fukushima et al., 2014; Sakai et al., 2012). By collecting soil samples from the fenced and control sites, we investigated the diversity and composition of microbial communities and tested for differences in diversity and taxonomic composition between the two sites. Our target microbiota comprises bacteria, archaea, and fungi (Ascomycota and Basidiomycota), all being functionally important taxa for forest ecosystems (Baldrian, 2017). It is expected that vegetation cover and plant diversity in the fenced site could foster microbial diversity that might be absent in the control site, and some microbial taxa could be more responsive to the presence versus absence of understory vegetation cover and diversity than other taxa. We posit the following hypothesis: soil in the deer fence site will have a higher species richness of bacteria, archaea, and fungi than the control site, and the fencing treatment will alter the taxonomic and functional composition of microbial communities, including pathogenic and mutualistic microbes.

2 | MATERIALS AND METHODS

2.1 | Study site

The study site comprises a cool-temperate natural forest at the Ashiu Forest Research Station, Field Science Education and Research Center, Kyoto University located in the uppermost part of the Yura River, northern Kyoto, Japan (35°32′ N, 135°75′ E, altitude 355–959 m). The forest canopy consists mainly of Japanese cedar (*Cryptomeria japonica* var. *radicans*) in the upper to middle part of the slope, Japanese beech (*Fagus crenata*) in the lower part of the slope, and *Aesculus turbinata* and *Pterocarya rhoifolia* in the valley area (Sakaguchi et al., 2008, 2012). In the study area, sika deer (*Cervus nippon*) density drastically increased in the 2000s and peaked in 2010 (Mizuki et al., 2020). Since the end of the 1990s, the degradation of vegetation by deer browsing has become extensive and profound, resulting in the significant loss of understory vegetation (Fujiki & Takayanagi, 2008; Fukuda & Takayanagi, 2008).

2.2 | Experimental design

The large-scale fence experiment was initiated in 2006 when the urgent need to defend vegetation from deer overabundance was unfolding (Mizuki et al., 2020; Sakaguchi et al., 2012). In the experiment, a large deer fence encompassing the entire catchment (13 ha) was established and maintained to observe ecosystem recovery, and an unfenced, adjacent catchment (19 ha) was set as a control site accessible to the deer population. The catchment-scale fence has been maintained for 15 years, minimizing the number of deer entering the fence (Fukushima et al., 2014). For the design and structure of the guard fence, readers may refer to Sakai et al. (2012).

2.3 | Soil sampling

In 2021 summer, soils in the valley, where the grazing refugia effect via fencing was thought to be more pronounced than those in the ridge (Fukushima et al. online resource, https://www.jstage.jst.go.jp/article/pronatura/29/0/29_1/_article/-char/ja/), were studied. The understory vegetation inside the fences comprised a taxonomically rich array of groups, including herbaceous, fern (e.g., *Polystichum* sp.), and woody plants (e.g., wingnut *Pterocarya rhoifolia*). Outside the fence, the understory vegetation was almost nonexistent, with only a few species of nonpreferred ferns sparsely thriving.

Topsoil was sampled at 2–3 m intervals along the valley transects in each catchment site. At each sampling point, after removing the litter layer, a soil core sampler (DIK-1601, Daiki) was used to collect a total of 24 undisturbed soil core samples per site at a depth of 5 cm. This gave a total of 48 soil core samples in this study. To limit cross-contamination among samples and represent their spatial variability in microbial species composition within each site, a new core tube was used at each sampling point, put in a separate plastic bag, sealed, and immediately stored in the refrigerator until analyses. To further minimize cross-contamination among samples, 3 g of substrate soil was subsampled from the interior of the collected core using a sterilized spatula, dried in a plastic bag loaded with silica gel beads, and then used for DNA extraction.

2.4 | DNA extraction

DNA was extracted and purified using an ISOIL Beads Beating kit (Nippon Gene Co. Ltd., Tokyo) with a few modifications to the original protocol by the manufacturer. First, 0.50 g of soil sample was dispensed into the supplied 2 mL tube and was added with two types of Lysis Buffer (BB and 20S). The sample was pulverized using a Multi-Beads Shocker (Yasui Kikai, Osaka, JAPAN) at 2500 rpm for 2 min, and then centrifuged (10,500 rpm, 2 min). The supernatant was taken to a new tube containing Purification Solution and mixed thoroughly. The solution was added with chloroform, vortexed for 15 s, and centrifuged again (10,500 rpm, 15 min, ambient temperature). The supernatant was transferred to a new tube, with Precipitation solution added, and was centrifuged (10,500 rpm, 30 min, 4°C). The supernatant was discarded, and then the tube was washed with wash solution, mixed, and centrifuged (10,500 rpm, 10 min, 4°C). After discarding the supernatant, 70% ethanol and Ethachinmate were added, vortexed, and

centrifuged (10,500 rpm, 10 min, 4°C). The precipitated DNA was air-dried, dissolved with TE buffer, and stored at −20°C until further processing.

2.5 | PCR, sequencing, bioinformatics

We used a two-step PCR approach for the library preparation for MiSeq sequencing. The first-round PCR (first PCR) was conducted to amplify the 16S rRNA V4 regions and the ITS1 region using primers specific to prokaryotes (bacteria and archaea) (515F and 806rR; Apprill et al., 2015; Caporaso et al., 2011) and fungi (ITS1-F-KYO1 and ITS2-KYO2; Toju et al., 2012, 2014), respectively. For the 16S rRNA region PCR was performed using KAPA HiFi HS (Nippon Genetics, Tokyo) with the temperature profile of 35 cycles at 98°C for 20 s (denaturation), 65°C for 15 s (annealing of primers), and 72°C for 30 s (extension), and a final extension at 72°C for 6 min. On the other hand, for ITS1 region, PCR was performed using KAPA HiFi HS (Nippon Genetics, Tokyo) with the temperature profile of 35 cycles at 98°C for 20 s (denaturation), 52°C for 15 s (annealing of primers), and 72°C for 30 s (extension) and a final extension at 72°C for 6 min. The PCR products were purified using the AMPureXP Kit (Beckman Coulter, Inc., Brea, CA) to remove primer dimers (shorter than 200 bp).

We performed an additional experiment to test the effects of changing the annealing temperature in first PCR. Two samples were randomly selected from the fenced and control sites of the 16S samples, and both samples were subject to PCR using the two different levels of the annealing temperature, 50 and 60°C (note the experiment above was done with 65°C). Sequencing the soil samples using different annealing temperatures helps examine how soil microbial diversity is affected by specific protocols (see Figure S1).

The second-round PCR (second PCR) was carried out to append indices for different samples for sequencing with MiSeq. PCR was performed using KAPA HiFi HS with the temperature profile of 35 cycles at 98°C for 20 s (denaturation), 65°C for 15 s (annealing of primers), and 72°C for 30 s (extension), and a final extension at 72°C for 6 min. The 16S and ITS1 libraries were subject to the second PCR with different concentrations of template DNA. The second PCR products were then pooled for each of the 16S rRNA and fungal ITS1 regions after a purification step using the AMPureXP Kit. To further remove primer dimers, we performed electrophoresis using E-gel (Invitrogen™, E-Gel™ SizeSelect™ II Agarose Gels, 2%) and excised bands of desired DNA size from the gel. A screening using a Bioanalyzer showed that the excised bps of 16S and ITS were 439 bp and 419 bp, respectively. The two samples were mixed to equal molar concentrations and purified again using AMPureXP such that the ratio of AMPureXP reagent to the pooled library was set to 0.5 (v/v). The DNA library was paired-end sequenced (2 × 150 bp) on the MiSeq platform (Illumina, San Diego, CA, USA) using the MiSeq Reagent Kit (Illumina).

2.6 | Bioinformatics

Amplicon sequencing data were analyzed in R using "DADA2" (Callahan et al., 2016) and "phyloseq" (McMurdie & Holmes, 2013). Prior to getting started with the bioinformatics pipeline in DADA2, we used the "cutadapt" tool to remove primers from the data and ensured that our data set comprises demultiplexed, paired-end fastq files. After inspecting read-quality profiles, sequences were filtered using the default setting (error rate = 1.0×10^8) and then trimmed under the truncation parameters ("truncLen") being set to 200 for forward and 150 for reverse sequences, respectively, to remove low-quality tails. DADA2's core algorithm infers unique biological variants (hereafter amplicon sequence variant, or ASV) using a denoising algorithm based on an error model of MiSeq amplicon sequencing. We applied the core sample inference algorithm to the trimmed and filtered data using "dada" function, and then forward and reverse reads were merged to obtain the full denoised sequences (the "mergePairs" function in the package DADA2). After the removal of the chimeras (the "removeBimeraDenovo" function), we performed taxonomic assignment. Each ASV was taxonomically assigned using the naive Bayesian classifier algorithm (the "assignTaxonomy" function in the package DADA2); in so doing, the RDP classifier reference database (Wang et al., 2007), and the UNITE database (Nilsson et al., 2018) were used as training data sets with known taxonomy for bacteria and archaea, and for fungi, respectively. We used a method to make species-level assignments based on exact matching by implementing the "addSpecies" function to the bacteria and archaea ASVs. Finally, we performed functional guild assignment to fungal ASVs using the FUNGuild database (Nguyen et al., 2016); by applying the "funguild_assign" function in the "FUNGuildR" package to each of the genus-assigned ASVs (Nguyen et al., 2016), functional guilds were assigned to as many ASVs as possible in order to ensure a comparison of the overall patterns of fungal guild composition between the two sites.

2.7 | Statistical analysis

Prior to statistical analysis, we conducted rarefaction to account for variability in sequencing completeness among samples, rather than standardizing samples by an equal number of sequencing reads (Chase et al., 2018). Specifically, we employed coverage-based rarefaction (coverage = 0.999 representing the rarefaction slope being 0.001 based on "rareslope" function) (Chao & Jost, 2012), and created a rarefied matrix according to the same level of sequencing completeness. This approach is firmly anchored in sampling theory (Chao & Jost, 2012) and is particularly useful when comparing diversity between samples of unequal completeness; for example, when different samples cross their rarefaction curves, potentially affecting the conclusion drawn from the diversity estimate (Chase et al., 2018). We confirmed that most samples attained some

asymptotic level of sequencing reads (Figure S2), and after excluding samples that did not meet this completeness criterion (coverage = 0.999), the remaining 31 samples (fenced:control = 15:15) and 33 samples (fenced:control = 17:16) were used for 16S (bacteria/archaea) and ITS (fungi) data in community ecology analysis.

We counted the richness of ASVs (the total number of ASVs as a measure of microbial diversity) of bacteria, archaea, and fungi (Basidiomycota and Ascomycota) for each sample, and compared the richness between the fenced versus control sites. To do this, the abundance of ASVs in a rarefied sample was analyzed as the response variable with and treatment (categorical, two levels including fenced versus control) as the predictor (explanatory variable), using a generalized linear model ("glm") based on Poisson distribution with the log link function. The nomenclature for the generalized linear model, therefore, follows: glm(richnessAVS ~ site, family = "poisson").

Next, to compare taxonomic composition among samples between sites, we sorted all the sequence reads associated with certain taxons at the genus level in each sample, and used the sorted sample-by-genus matrix for multivariate analysis. The reason for this coarse-level sorting before the analysis involves that the high-resolution characterization of ASV (amplicon sequence variant) of DADA2 yielded unnecessarily high heterogeneity of ASV composition within each site (with few common ASVs across samples), making the community matrix not amenable to comparison across samples. The reason for using the genus-by-sample matrix involves that the genus level allows for ecologically meaningful inference of site occurrences because congeneric ASVs tend to share common ecological characteristics both for bacteria and fungi.

To visualize the site difference in microbial composition at the genus level (i.e., reads were grouped and summed at the genus level within each sample), we performed nonmetric multidimensional scaling ("metaMDS" function in vegan) for each of the three taxa separately, that is, bacteria and archaea (combined), Ascomycota (ascomycetes fungi) and Basidiomycota (basidiomycetes fungi). Note that the decision to combine archaea with bacteria for this ordination analysis resulted from the insufficient number of archaea-specific ASVs to achieve a statistically meaningful ordination. The Bray–Curtis dissimilarity index was used to quantify compositional dissimilarity. We assessed whether the two sites harbored different taxonomic compositions using permutational multivariate analysis of variance PERMANOVA ("adonis2" function in the package "vegan"; Oksanen et al., 2022). To ensure that the significance in PERMANOVA arises from the different average composition rather than a difference in site-level heterogeneity in multivariate dispersion, we performed an additional test of Permutational Multivariate Analysis of Dispersion (PERMDISP) using "betadisper" function if necessary.

To further delve into what microbes specifically are responsible for the observed site difference in taxonomic composition, we applied the CLAM test (Chazdon et al., 2011) to the genus-by-sample matrix using "clamtest" function in the package "vegan" (Oksanen et al., 2022). Here, the CLAM test uses a multinomial model based on the estimated relative occurrences (counts of presences of ASVs) in two habitats (control versus fenced). It minimizes bias due to differences in sampling intensities between two habitat types as well as bias due to insufficient sampling within each habitat. The method permits a robust statistical classification of habitat specialists and generalists, without excluding rare species a priori (Chazdon et al., 2011). Based on a user-defined specialization threshold (2/3 in our case), the model classifies taxa (genera) into one of four groups: (1) generalists (occurring in both control and fenced sites), (2) taxa that occur specifically at the control site, (3) taxa that occur specifically at the fenced site, and (4) too rare to classify with confidence. We analyzed the top 20 most abundant genera of Bacteria and Archaea (combined), Ascomycota, and Basidiomycota, respectively.

Finally, we examined the functional guild composition of the soil fungal communities between the two sites. Each of the genus-assigned taxa was assigned its functional guild using the FUNGuild database, and the guild-by-sample matrix was created and subject to the same set of statistical analyses (NMDS, PERMANOVA, and the CLAM test).

All statistical analyses were performed using R ver. 4.2.3 software, and the minimum level of significance was set at $p = 0.05$.

3 | RESULTS

3.1 | Richness

A total of 19,344,302 reads were obtained, including 9,285,324 and 10,058,978 reads for bacteria (16S) and fungi (ITS) data, respectively. The sample mean of the raw sequence data was 224,933 reads (SD = 176,617). After trimming, filtering, and denoising, we obtained 1,701,600 and 1,279,386 reads for bacteria (16S) and fungi (ITS) data, respectively. The sample-by-ASV matrix (in which the row represents the sample, the column represents the ASV, and each entry counts represents the number of sequencing reads), and the results of the taxonomic assignment are provided in Data S1–S4. We performed downstream analysis using the rarefied data set to account for variability in sequencing completeness rather than standardizing samples by sequencing reads.

The number of ASVs was assessed using a generalized linear model based on Poisson distribution, and the difference between the fenced and control sites was statistically tested (Figure 1). The numbers of ASVs of bacteria and Ascomycetes fungi were not significantly different between the fenced and control sites (bacteria, Poisson regression, $z = 1.251$, $p = 0.211$ N.S.; Ascomycetes fungi, $z = -1.526$, $p = 0.127$, N.S.; Figure 1a,c). The fenced site had significantly more archaea ASVs ($z = 2.131$, $p = 0.033$; Figure 1b) and Basidiomycetes ASVs ($z = 2.564$, $p = 0.01$; Figure 1d) than the control site.

3.2 | Taxonomic composition

A nonmetric multidimensional scaling (NMDS) plot visualized differences between samples in microbial composition (beta diversity) at the genus level between the fenced site and the control site (Figure 2). The results of PERMANOVA based on the Bray–Curtis

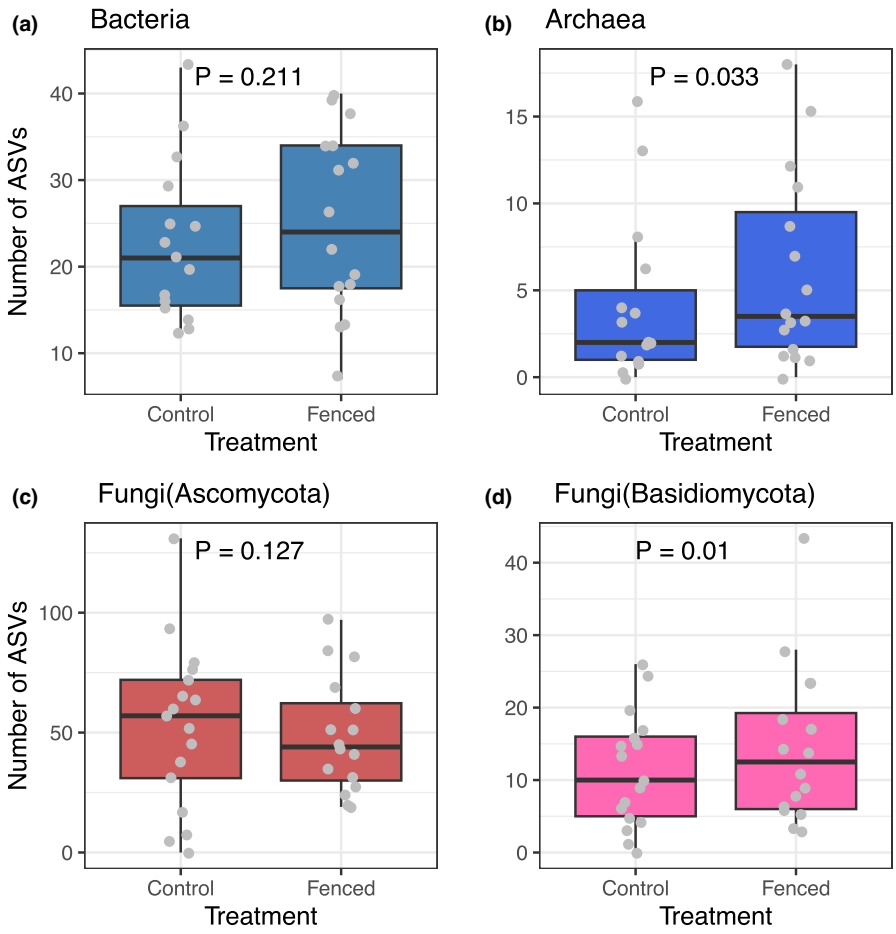

FIGURE 1 Boxplots of the number (richness) of ASVs (amplicon sequence variants) for the control versus fenced sites for (a) bacteria, (b) archaea, (c) fungi (Ascomycota), and (d) fungi (Basidiomycota). A significant difference in the number of ASVs was examined based on the results of the Poisson regression model.

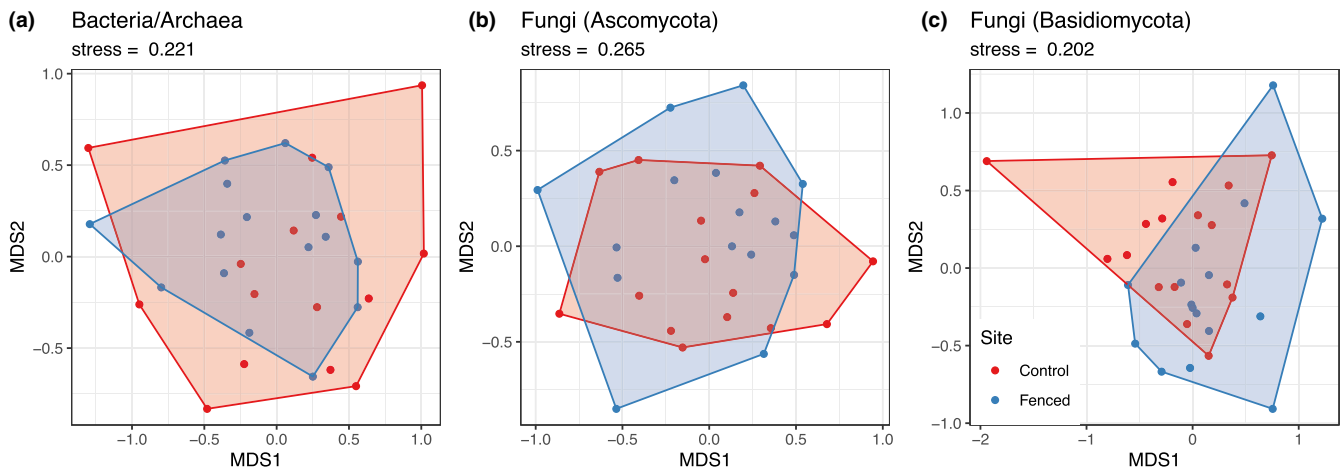

FIGURE 2 Nonmetric multidimensional scaling plot illustrating the differences in the (order-level) taxonomic composition between the fenced versus control sites for (a) bacteria/archaea and (b) ascomycetes fungi (Ascomycota), and (c) basidiomycetes fungi (Basidiomycota).

dissimilarity index showed that none of the microbial groups (bacteria and archaea, Ascomycota and Basidiomycota) differed significantly in taxonomic composition between the fenced versus control sites (Table 1). To complement the (genus-level) taxonomic composition analysis, we compared the top 20 most abundant taxonomic genera in terms of the number of occurrences (presences) of a focal taxon (note that we excluded unassigned ASVs from counting presences at the genus-level taxon sorting). The dominant bacteria genera were HSB OF53-F07 (belonging to the phylum: Chloroflexi), *Udaeobacter* (phylum: Verrucomicrobia), *Pseudolabrys* (order: Hyphomicrobiales), *Nitrosotalea* (phylum: Nitrososphaerota), and *Acidothermus* (order: Acidothermales) were classified as generalist occurring on both sites with a lack of site preference; and the other bacteria/archaea genera were too rare to classify site preference (Figure 3a). The dominant Ascomycota genus *Archaeorhizomyces* was identified as occurring more frequently in the fenced samples than in the control

TABLE 1 Results of PERMANOVA (permutational multivariate analysis of variance) testing for the site difference (fenced vs. control) in the composition of microbial communities, (a) bacteria, (b) archaea, (c) ascomycetes fungi, and (d) basidiomycetes fungi.

	df	SS	R^2	*F*	*p*
(a) Bacteria and archaea					
Site	1	0.131	0.016	0.4578	0.916
Residual	29	8.284	0.984		
Total	30	8.415	1.000		
(b) Fungi (Ascomycota)					
Site	1	0.400	0.039	1.060	0.345
Residual	26	9.814	0.961		
Total	27	10.214	1.000		
(c) Fungi (Basidiomycota)					
Site	1	0.443	0.047	1.384	0.138
Residual	28	8.958	0.953		
Total	29	9.401	1.000		

Note: Note that samples not containing focal taxonomically assigned taxa were excluded from analysis, leading to different degrees of freedom for different microbial groups (from a to c).

samples, while *Beauveria* exhibited significant specificity to the control site. *Trichoderma*, *Chaetosphaeria*, *Penicillium*, etc. did not show any tendency to occur more frequently in either site (Figure 3b). The CLAM test showed that four Basidiomycota genera (*Solicoccozyma*, *Saitozyma*, *Sebacina*, *Ganoderma*) were dominant and occurred in both sites (Figure 3c). The relative abundance of genera appeared to be different although not statistically significant; for example, *Hygrocybe* and *Tausonia* seemed to occur more frequently in the fenced versus control site, while *Hyphodontia* showed an opposite trend.

3.3 | Fungal guild composition

The result of PERMANOVA showed significant differentiation in the functional guild structure between the fenced and control samples (Table 2). The nonmetric multidimensional scaling plot showed the difference in the centroid in the guild composition (Figure 4a), and the back-to-back barplot with the CLAM test provided a more detailed view of the guild composition difference (Figure 4b). According to the CLAM test, most of the functional guilds were identified as generalist occurring in both sites, but animal pathogen was the only guild that exhibited a significant deviation from random, predominantly occurring in the control site (Figure 4b).

4 | DISCUSSION

Although previous studies suggest that the strong foraging pressure by deer might have persistent impacts on soil properties (Fukushima et al., 2014) and can mediate feedback between plant growth and soil environments (Ohira et al., 2022), much less insights have been gained regarding the consequence of creating deer grazing refugia on the soil microbial communities. Using microbial eDNA metabarcoding, our study demonstrates that the effect of fencing on soil microbial communities is characterized by complex responses that vary from taxon to taxon. Our results suggest that the numbers of archaea and basidiomycetes fungal species were greater in the fenced site than in the control, while no such pattern was found for bacteria and ascomycetes fungi. Despite the lack of significant influence of the fence treatment on taxonomic composition in the soil fungal communities (Table 1, Figure 2), their functional guild composition was influenced by the fenced treatment, with significant changes in the abundance of animal pathogens (Figure 4). In what follows, we discuss the consequence of building deer fences for the structure and function of soil microbial communities and highlight avenues for future research.

The most striking difference between the two sites in our long-term experiment is the presence or absence of understory vegetation (Fukushima et al., 2014), and the logical question is: can any sign of this understory effect be seen in the results of the microbial communities? Some archaea catalyze the oxidation of ammonium (NH_4^+) to nitrite (NO_2^-), which is subsequently oxidized to NO_3^-, the bioavailable form of N that plants utilize (Isobe et al., 2011). Given their propensity to associate with understory vegetation and their ability to retain NO_3^- in soil (Fukushima et al., 2014), the greater diversity of archaea in the fenced site versus the control site (Figure 1b) might represent the possibility that ecosystem-scale refugia from deer overbrowsing might help sustain the extant archaeal community. Nevertheless, further work is needed to test the possibility that archaea tend to be lost earlier than the other microbial taxa in the control site under deer grazing pressure; for example, archaea species might be more vulnerable than other bacterial species to soil erosion that often occurs as a result of understory vegetation loss.

The most abundant group of fungi in our soil samples, Ascomycota comprises ecologically diverse species that play key ecosystem functions (e.g., decomposers, pathogens, mycorrhizal fungi). Inspection of the top 20 most abundant genera suggests that some of them were likely to be members of the cosmopolitan and dominant soil fungal taxa (Egidi et al., 2019). We found that (i) more Ascomycota ASVs were detected at the control site than at the fenced site but the difference was not statistically significant (Figure 1b), (ii) no differences in taxonomic composition were found for Ascomycota (Table 1b), (iii) many of Ascomycota genera occurred equally frequently in the fenced site as in the control site ("generalist" taxa; Figure 3b), and (iv) however, two genera *Archaeorhizomyces* and *Beuveria* exhibited some level of site specificity (to fenced and control, respectively).

In contrast, our results showed that the number of ASVs of basidiomycetes fungi was significantly greater in the fenced site than in the control site. The back-to-back barplot in Figure 3c showed apparently different patterns of the relative abundance of basidiomycetes genera. Inspection of the top 20 most abundant genera suggests that only four genera were identified as generalist occurring in both sites and other genera were too rare to assign their site preference. These observations led us to reason that the differences in the number of Basidiomycetes ASVs might arise from the lack of

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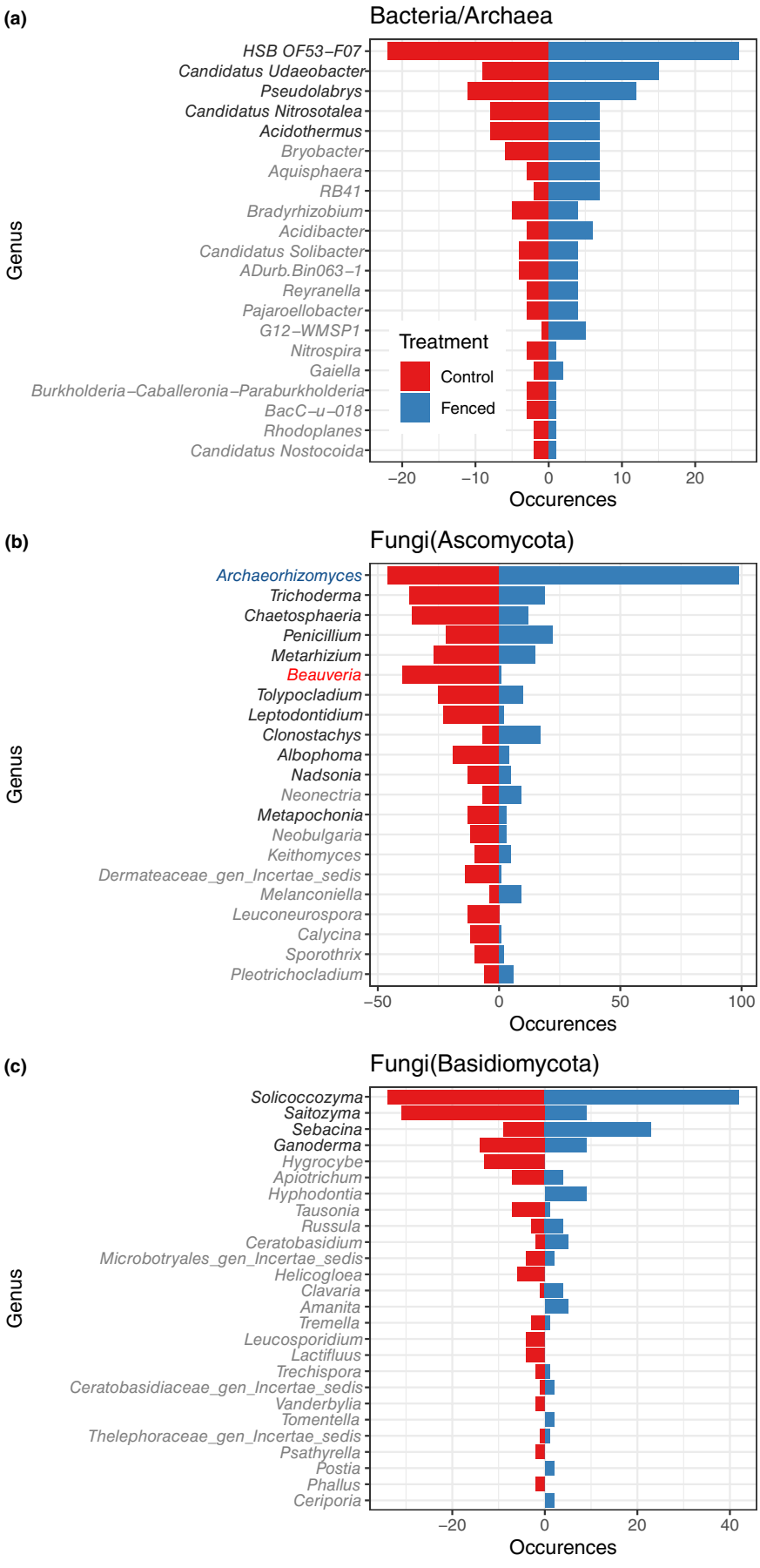

FIGURE 3 Back-to-back bar plots of the top 20 most abundant (and taxon-assigned) genera of (a) Bacteria/Archaea, (b) Ascomycota, and (c) Basidiomycota in terms of the number of presences (counts) of ASV sorted at the genus level. The genus names follow the color codes specified by the results of the CLAM test; black letters, generalist genera that occur both at the control and fenced sites; gray letters, genera that were too rare to classify site specificity; blue letters, genera that were more frequently represented at the fenced site than at the control site; and red letters, genera that were more frequently represented at the control site than at the fenced site.

"rare" taxa in the fenced site (Figure 3c). What makes such "rare" Basidiomycota more pronounced in the fenced versus the control habitat? One possibility is that basidiomycetes fungi might have stronger species-specific relationships with plants than ascomycetes fungi (e.g., mycorrhizal fungi *Amanita*, etc.), and hence be more likely to be influenced by differences in plant species composition between the fenced and control sites. Thus, some fungal groups might respond to changes in plant species composition due to the presence of fencing, and unraveling the responses to those vegetation parameters will be a subject of future research.

We found that the fencing treatment significantly influenced the fungal guild composition but not the taxonomic composition of the soil fungal communities (Table 2, Figure 4). One interesting yet unexpected pattern involves more abundant animal pathogens in the control versus fenced sites. We propose that the potential access of deer in the control site might result in more fecal pellets and urines deposited compared to the fenced site, thereby facilitating some pathogenic Ascomycota species that had been suppressed at the fenced site (such as *Beuveria*). In addition, more plant pathogens were found in the control site than in the fenced site (Figure 4b). Thus, it is concluded that the deer fencing treatment might contribute to reducing pathogen prevalence.

It should be noted, however, that ASV richness is highly variable among primer sets, sampling effort, bioinformatic pipelines and other variables (Dopheide et al., 2019; Grey et al., 2018; Porter et al., 2019). Given that some of our results are statistically marginal, we should be careful in drawing conclusions about the pattern of ASV richness between the fenced and control sites, particularly, archaea and basidiomycetes richness. Future studies are needed to confirm the generality and robustness of this fence treatment with an improved study design that accounts for the inherent spatial heterogeneity of soil microbial communities.

The present work highlights that some of the components of the soil microbial community may be more strongly affected by the fencing treatment than others, with possible consequences for the functioning of the forest soil ecosystems. The less affected taxa might be able to survive even without understory vegetation as long as the adult trees that make up the canopy remain intact (providing litterfall and root exudates), while the more affected taxa might be dependent on the presence of understorey vegetation environment. Future studies should examine that our finding about the taxon-specific response to the fencing treatment is general and holds even when one considers the temporal comparison of the soil microbial communities (before and after the installation). Particularly, the underlying environmental factors explaining the recovery or resilience of soil microbial communities should be clarified. Several studies suggest that after chronic herbivory, the recovery rate of understory vegetation and its function can be slow even with decreasing grazing pressure (Ohira et al., 2022; Tanentzap et al., 2012). Tatsumi et al. (2021) showed that deer exclosure established in restoration sites (following agricultural abandonment) had no detectable effect on soil fungal communities, suggesting the potentially slow re-assembly of soil fungal communities. Because eDNA analysis does not necessarily indicate the functioning of the living soil microbiota (for example, relict DNA; Carini et al., 2016), explicit consideration of the temporal scale for studying soil microbiota will be the next challenge to better understand the efficacy of employing deer fence as a measure to protect forest ecosystems.

TABLE 2 Results of PERMANOVA (permutational multivariate analysis of variance) testing for the site difference (fenced vs. control) in the functional guild composition of soil fungal communities.

Factors	*df*	SS	R^2	*F*	*p*
Site	1	0.388	0.063	1.762	0.049*
Residual	26	5.733	0.937		
Total	27	6.121	1.000		

*Denotes statistical significance ($p < 0.05$).

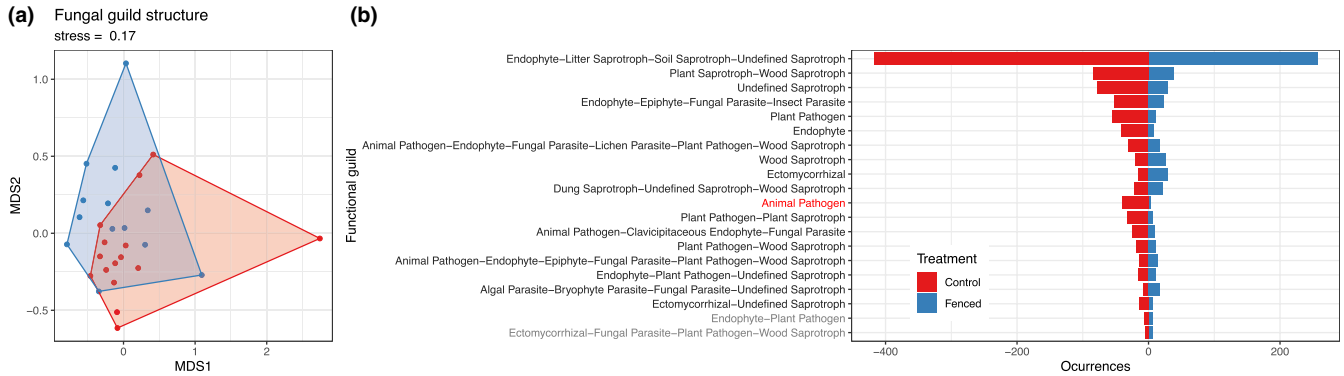

FIGURE 4 Results of functional guild composition analysis for the soil fungal communities. (a) Nonmetric multidimensional scaling plot illustrating the differences in the guild composition between the fenced versus control sites. (b) Back-to-back bar plot of the top 20 most abundant (and guild-assigned) taxa in terms of the number of presences (counts) of ASV sorted at the guild level. The guild names defined by the FUNGuild database follow the color codes specified by the results of CLAM test; black letters, generalist guilds that occur both at the control and fenced sites, gray letters guilds that were too rare to classify site specificity; and red letters, guilds that were more frequently represented at the control site than at the fenced site.

AUTHOR CONTRIBUTIONS

KK, FK, and IIM designed the study; KK, MH, NN, and YK performed soil sampling; MH and NN performed molecular analysis; KF, SS, MI, DF, MS, AT, MY, NT, DT KN, and KM established the site (with the help from DT KN, KM); KK, SM, TY, KF, and MH interpreted the results; KK wrote the first draft and all the co-authors contributed to revision.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Raw sequence data are deposited under the accession BioProject PRJDB16295 and are also available from the corresponding author upon reasonable request. The data that supports the finding of the study are available in the supporting information.

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SUPPORTING INFORMATION

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