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# Effects of deer-exclusion fences on soil microbial communities through understory environmental changes in a cool temperate deciduous forest in Southern Japan

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## ABSTRACT

The increasing deer population has significantly altered the forest ecosystems. Deer browsing affects not only plant species diversity and composition but also other organisms indirectly, along with soil properties. However, the effects of deer grazing on the soil microbial community and the underlying mechanisms have not been well understood. To assess these effects, we compared the understory environment and soil microbial community inside and outside fences at Mt. Shiraga, where severe soil environmental degradation has occurred due to sika deer grazing. We selected 20 *Fagus* trees inhabiting areas within and outside the fences with similar topological features. Eleven environmental variables and both soil prokaryotic and fungal communities were compared between samples inside and outside the fences. The area inside the fence had significantly higher dwarf bamboo density and soil carbon (C) and nitrogen (N) content, whereas soils outside the fences had higher pH and bulk density. The diversity index of the fungal community, in terms of the number of amplicon sequence variants, inside the fence was higher than that outside, whereas that of the prokaryotic community did not differ between fences. Both prokaryotic and fungal communities differed between inside and outside fences. The prokaryotic community changed with the soil C/N ratio, and the relative abundance of oligotrophic bacteria increased with decreasing soil C/N ratio. The fungal community also changed with soil pH and dwarf bamboo density, with the relative abundances of symbiotrophic fungi and ectomycorrhizae increasing with increasing soil pH. Comparative analyses between inside and outside fence samples might provide information on soil microbial community changes with the changes in soil properties after deer grazing.

## 1. Introduction

The increasing population of deer (*Cervus* sp.) has significantly altered the forest environment (Takatsuki, 2009). Deer grazing leads to the loss of understory vegetation and damage to upper canopy trees (Takatsuki and Gorai, 1994; Beguin et al., 2011). Grazing affects not only plant diversity and composition but also soil properties and inhabiting organisms, such as insects, indirectly (Furusawa et al., 2003; Sakai et al., 2012; Harada et al., 2020; Nakahama et al., 2020; Ohira et al., 2022; Gomi et al., 2022). Fences are an effective means to protect vegetation from deer (Miller et al., 2017). Inside fences, the understory vegetation remains intact, thereby preventing soil and litter movements

compared to areas outside fences (Furusawa et al., 2003). Although studies on vegetation cover affecting soil environmental properties, such as litter mass, soil bulk density (Yanagi et al., 2008; Harada et al., 2020), and microbial biomass (Furusawa et al., 2005), have been conducted, studies on the soil microbial community in response to fence installation are scarce (Kadowaki et al., 2023).

Soil microbial communities are closely linked to vegetation and soil properties. Removal of understory layers modifies these community compositions (Kong et al., 2017). In *Fagus* tree-dominated forests in Japan, dwarf bamboo often thickens and competes with other plants in the roots (Tripathi et al., 2005; Yanagawa et al., 2023). One species, *Sasa senanensis*, has mutualistic relationships with arbuscular mycorrhizae

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fungi (Fukuchi et al., 2011), while another, *Sasa kurilensis*, affects the soil microbial community, increasing the relative abundance of Pezizaceae (Ascomycota), which are bamboo-associated mycorrhizal fungi (Kong et al., 2017). Soil properties, such as nutrients and bulk density, also affect soil fungal and bacterial communities (Zhang et al., 2019a; Lan et al., 2022; Sui et al., 2023). Soil bacterial communities change along nutrient gradients, with nutrient-poor soil favoring oligotrophic bacteria (Zhang et al., 2019a; Lan et al., 2022). Soil nitrogen content positively relates to fungal richness (Sui et al., 2023), with ectomycorrhizal fungi decreasing in biomass and enzyme activity with increasing nitrogen availability (Jørgensen et al., 2024). Differences in community changes related to vegetation and soil properties may exist between inside and outside fences, where natural vegetation, including dwarf bamboo, and surface soils have been protected by deer fences.

Long-term grazing can induce irreversible changes in plant species compositions and productivity (White, 2012; Nagaike et al., 2014), reaching a tipping point beyond which ecosystems cannot revert to their original state (Lenton, 2013; Dakos et al., 2019). Continuous deer grazing detaches soil surface layers, exposing them to direct raindrops and freeze–thaw processes, leading to soil erosion (Gomi et al., 2022). Severe erosion results in increased soil bulk density, reducing seedling survivability in disturbed forest areas (Tsujino and Yumoto, 2004; Yanagi et al., 2008). Although previous studies have proposed these processes (Ohira et al., 2022; Gomi et al., 2022), the underlying mechanisms, including changes in soil environment and microbial communities, remain poorly understood. Comparative analysis of soil ecosystems between grazed and fenced sites can deepen our understanding of soil disturbances post deer grazing and identify thresholds for reverting to the original vegetation.

In the Kyushu Mountains, another dwarf bamboo species, *Sasamorpha borealis*, covers the natural forest understory. Since the 1980 s, Japanese deer (*Cervus nippon* Temminck, 1836) have grazed the understory, leaving behind some unpalatable grasses and plants. With annual rainfall exceeding 3000 mm, the disappearance of understory layers and O horizon has led to severe soil erosion (Abe et al., 2022; Katayama et al., 2023). Previous studies have noted declines in soil organic matter content and shifts in the soil microbial community toward symbiotrophic-poor and saprotrophic-enriched states due to erosion (Katayama et al., 2023; Chen et al., 2024). In certain areas, deer fences have been installed to protect vegetation. However outside these fences, there is an increase in surface soil exposure, posing risks of soil environmental changes, which diminish soil ecological functions and exacerbate erosion. Comparisons of soil environment and microbial community inside and outside of fences can help elucidate the effects of deer fences on soil and ecosystems, providing fundamental insights into soil ecosystem dynamics during deer overgrazing.

To investigate the impact of deer fences on soil ecosystems in mountainous regions, we compared the forest understory environment and soil microbial communities both inside and outside the deer fences. We hypothesized that deer grazing and the resulting soil environmental changes would influence the diversity, composition, and functions of soil prokaryotes and fungi. Subsequently, we examined the effects of deer fences on forest soil ecosystems in areas affected by severe deer grazing.

## 2. Materials and methods

### 2.1. Study site

Our study was conducted in the Mt. Shiraga area [32°09'N, 130°55'E, 1250 m a.s.l.], situated in the southern-central part of Kumamoto Prefecture, Japan, where the annual mean precipitation exceeds 2800 mm (Environmental Agency, Japan, 1988). The natural vegetation above 1,000-m altitude comprises a cool temperate deciduous broadleaved forest consisting of deciduous broadleaved trees (*Fagus crenata* [Fagaceae], *Carpinus japonica* [Betulaceae], and *Acer*

*sieboldianum* [Sapindaceae]) and evergreen conifers (*Tsuga sieboldii* [Pinaceae], and *Abies firma* [Pinaceae]) in the upper tree layers, with dwarf bamboo (*S. borealis*) in the understory (Environmental Agency, Japan, 1988). Other deciduous broadleaved trees (*Pterocarya rhoifolia* [Juglandaceae] and *Hydrangea paniculata* [Hydrangeaceae]) are also frequently observed (Environmental Agency, Japan, 1988). Vegetation cover has decreased due to sika deer overgrazing, leading to surface soil erosion in some areas, including our study site (Kyushu Regional Forest Office, 2009; Ministry of Environment, Japan, 2011; Katayama et al., 2023). In certain forest areas, there is an increase in the number of plants unpalatable to deer, such as *Dennstaedtia scabra* (Dennstaedtiaceae), *Illicium anisatum* (Schisandraceae), *Skimmia japonica* (Rutaceae), and *Symplocos myrtacea* (Symplocaceae) (Ministry of Environment, Japan, 2011).

Because *Fagus* trees represent almost the southernmost populations in Japan and are surrounded by nearly pristine vegetation, the Environmental Agency of Japan designated the region as a Nature Conservation Area in 1980 (Environmental Agency, Japan, 1988), and the Forest Agency of Japan designated the forest areas as Conservation Forests in 1993 (Kyushu Regional Forest Office, 2023). Since 2005, both public and private sectors have constructed deer fences to protect *Fagus* trees and understory vegetation (Ministry of Environment, Japan, 2011). By 2009, plants inside the deer fences, such as dwarf bamboo and *Fagus* trees, were showing signs of recovery (Ministry of Environment, Japan, 2011). Although dwarf bamboo remained within some fences, other vegetation, such as grasses and shrubs, was observed in other fences, potentially breached by deer. Exact records on maintaining each fence were unavailable. We classified fences on with thick distributions of dwarf bamboo as undisturbed fences and selected them to test the fence effect for the study design outlined below (Fig. 1).



Fig. 1. A photograph of one of the sampling points around the summit of Mt. Shiraga. The foreground depicts the area outside the fence, whereas the background shows the area inside the fence. Inside the fence, dense growth of dwarf bamboo, reaching approximately 2.0 m in height, is visible.



## 2.2. Environmental properties and microbial communities inside and outside deer fences

To compare environmental properties between inside and outside deer fence areas, we followed the methods outlined by [Katayama et al. \(2023\)](#) (Table 1). In July 2022, we selected 10 deer fences established between 2007 and 2011 where thick growth of dwarf bamboo remained. Subsequently, we identified *Fagus* trees within and outside these fences, situated in similar topographies (Fig. 1). In total, 20 trees were designated as target trees and sampling points (Appendix 1). We measured the diameter at breast height (DBH) of each *Fagus* tree using steel measures. Leaf area index (LAI) and canopy openness were quantified by capturing fish-eye images of the understory canopy and forest floor beneath the *Fagus* tree canopies using a theta-V camera (Ricoh, Japan). The image files were analyzed using Gap Light Analyzer ver. 2.0 ([Frazer et al., 1999](#)) for LAI and CanopOn2 software (<http://takenaka-akio.org/etc/canopon2/>) for canopy openness. Stem densities of dwarf bamboo were determined by setting 50 cm × 50 cm frames 1–2 m apart from each *Fagus* tree and counting bamboo stems. Slopes around the plots were measured using a clinometer (TANDEM 360 R DG, Suunto, Finland). The mass of the soil O horizon was measured by placing 20 cm × 20 cm frames under the canopy of each *Fagus* tree, collecting the O layers (litter and humus), drying them in an oven at 72°C for 72 h, and weighing them to 0.1 g. Soil bulk density was determined by collecting soil A layers under each *Fagus* tree canopy using a 100-cc syringe, drying them in an oven at 108°C for 72 h, and weighing them to 0.1 mg. Additionally, soil samples were collected for chemical and microbial community analyses from the same layers. Soil samples for chemical analysis were sieved using a 2-mm mesh, and soil carbon (C) and nitrogen (N) contents (%) were measured using a CN coder (MT-700, Anatek Yanaco, Kyoto, Japan). Soil pH (H<sub>2</sub>O) was measured by extracting soils with water at a 1:2.5 (w/w) ratio and measuring pH using a glass electrode (Horiba, Kyoto, Japan).

Soil samples for the microbial community analysis were freeze-dried using a freeze dryer (VD-250R, Taitec, Saitama, Japan) and ground using a multibead shocker (Yasui Kikai Corporation, Osaka, Japan) at 1500 rpm for 2 min. After adding Lysis Solution F (Nippon Gene, Tokyo, Japan), the samples were incubated at 65°C for 10 min. The solutions were then centrifuged at 12,000 ×g for 2 min, and the supernatant was transferred to another tube. Purification Solution F (Nippon Gene, Tokyo, Japan) was added to the solutions, followed by vortexing, and centrifuging at 12,000 ×g for 15 min, and the supernatant was transferred to new tubes. DNA was purified using an automated DNA purification system, MPure-12, and the MPure Bacterial DNA Extraction Kit (MP Biomedicals, Santa Ana, US-CA), with 10% PVPP solutions added. Extracted DNA was then amplified for prokaryotic 16 S rRNA using the primer set 16 S (341f-805r) and for fungal internal transcribed spacer

(ITS) regions using the ITS1 primer set (ITS1-F\_KYO1-ITS2\_KYO2; [Toju et al., 2012](#)) with KOD-Fx Neo (Toyobo, Japan). The library was constructed following kit instructions and sequenced using the MiSeq system and MiSeq Reagent Kit v3 (Illumina, San Diego, US-CA) at the Bioengineering Lab. Co., Ltd. (Kanagawa, Japan). In total, 1,072,712 paired-end reads were obtained in prokaryotes (average 53,636; range 45,699–60,224) and 998,405 paired-end reads in fungi (average 49,920; range 34,648–71,252) (Table S1). Sequencing quality was assessed using FASTX-Toolkit (ver. 0.0.14), and sequences under Q20 quality were discarded using sickle (ver. 1.33). Paired-end reads were merged using FLASH (ver. 1.2.11). Chimera and noise were removed using the dada2 plugin in Qiime2 (ver. 2022.2), and the sequences of the amplicon sequence variant (ASV) were exported with the read count (Table S1). Finally, 679,180 filtered reads were obtained in prokaryotes (average 33,959; range 28,131–38,914) and 776,099 reads in fungi (average 38,805; range 23,685–59,834) (Table S1). Phylogenetic estimations of prokaryotes and fungi were conducted using a feature-classifier plugin with references of SILVA (ver. 132) and UNITE (ver. 8.3), respectively, at a 97% threshold. Functional estimations of prokaryotes and fungi were performed using MetaCyc in PiCrust2 ([Douglas et al., 2020](#)) and FunGuild ([Nguyen et al., 2016](#)), respectively.

## 2.3. Statistical analysis

To evaluate the difference in the 11 measured variables (tree, soil, and environmental properties) between inside and outside the fences, we conducted t-tests or Welch's t-tests for all variables. Additionally, to assess the correlation among the measured variables, Pearson's product-moment correlation coefficients were calculated for all paired variables.

To rarefy the read counts of microbial community data, all samples were resampled based on the lowest number of read counts: 28,131 for prokaryotes and 23,685 for fungi (Table S1, Fig. S1). Subsequently, four alpha diversity indices (number of ASVs and Shannon, Simpson, and inverse Simpson indices) were calculated using the rarefied read count data. Each alpha diversity index was then compared between inside and outside the fences using t-tests or Welch's t-tests.

To assess compositional differences in microbial communities inside and outside the deer fences, the Bray–Curtis similarity index was calculated for all combinations of samples using rarefied read count data. The significance of fence effects on microbial communities was evaluated using Permutational Multivariate Analysis of Variance (PERMANOVA) with 20,000 permutations. Additionally, we conducted nonmetric multidimensional scaling (NMDS) based on the Bray–Curtis similarity index to explore relationships among samples. For prokaryotic communities we used a dimensionality parameter (k) of 2, whereas for fungal communities, k was set to 3, ensuring stress values below 0.2 for

**Table 1**  
Scores of each property inside and outside the deer fences with t-test results.

Measurement items	Inside		Outside		p-value		
Tree properties							
Canopy openness (%)	46.8	±	9.2	49.5	±	7.3	0.845
LAI (m <sup>2</sup> m <sup>-2</sup> )	1.4	±	0.5	1.5	±	0.7	0.357
Tree DBH (cm)	49.0	±	9.3	34.1	±	11.0	<b>0.004</b> **
Soil properties							
Dwarf bamboo density (no. m <sup>-2</sup> )	154.0	±	67.4	0.0	±	0.0	< <b>0.001</b> ***
O horizon mass (g m <sup>-2</sup> )	1180.8	±	611.6	661.4	±	420.8	<b>0.042</b> *
pH	3.40	±	0.10	3.50	±	0.10	<b>0.046</b> *
C content (%)	14.5	±	5.9	9.5	±	3.1	<b>0.034</b> *
N content (%)	1.09	±	0.35	0.80	±	0.22	<b>0.041</b> *
C/N ratio	13.0	±	1.2	11.8	±	0.8	<b>0.016</b> *
Bulk density (g cm <sup>-3</sup> )	0.25	±	0.11	0.41	±	0.20	<b>0.039</b> *
Environmental properties							
Slope (°)	14.80	±	8.87	13.90	±	9.70	0.831

Scores are presented as means and standard deviations (SD)

P-values: [blank] > 0.10, < 0.1, \* < 0.05, \*\* < 0.01, and \*\*\* < 0.001.

each analysis. Significant variables identified by t-tests (tree DBH, dwarf bamboo density, O horizon mass, pH, soil C and N contents, soil C/N ratio, and bulk density) were used to analyze the effects of environmental variables on the microbial community through envfit functions. To analyze the relationships between microbial taxonomic or functional groups and significant environmental variables, linear model (LM) analyses were conducted. To prevent multicollinearity, environmental variables were selected based on  $R^2$  values, significance of the envfit test, and correlation coefficients. Specifically, for the prokaryotic community analysis, we selected the C/N ratio as the explanatory variable. For the fungal community analysis, we chose dwarf bamboo density and soil pH as explanatory variables (for details, see Sections 3.2 and 3.3). LMs were constructed with response variables representing read counts of microbial taxonomic categories (phylum and order levels) and functional categories estimated using PiCrust2 for the prokaryotic community, and read counts of phylum and order levels and functional categories of trophic modes and guilds for the fungal community. Significance of explanatory variables was tested via Type-II ANOVA. Additionally, response variables were tested between inside and outside the fences using t-tests or Welch's t-tests, and consistency of results between ANOVA and t-tests was assessed.

Statistical analyses were conducted using R version 4.1.2 (R Core Team, 2022) and additional packages, including car (Fox and Weisberg, 2019), lme4 (Bates et al., 2015), vegan (Oksanen et al., 2022), and vegan3d (Oksanen et al., 2023).

### 3. Results

#### 3.1. Environmental differences between inside and outside deer fences

Most of the measured variables differed between inside and outside the fences (Table 1, Appendix 1). Dwarf bamboo density, O horizon mass, soil C and N contents, and soil C/N ratio were significantly higher inside the fences than outside (t-test:  $p < 0.05$ ). In contrast, soil pH and bulk density were higher outside the fences (t-test:  $p < 0.05$ ). Tree DBH was also larger inside the fences than outside (t-test:  $p < 0.05$ ); however, the LAI of *Fagus* trees was not different between inside and outside the fences (t-test:  $p = 0.986$ ).

#### 3.2. Differences in soil prokaryotic communities and functions between inside and outside fences

Amplicon sequencing of prokaryotic 16 S rRNA V3–V4 regions confirmed the presence of 34 phyla spanning 2 domains (archaea and bacteria) (Fig. S2a, Table S2). The top five phyla, constituting approximately 90% of total read counts, were as follows: Proteobacteria (average 39.5%; range 37.8%–40.6%), Acidobacteria (24.2%; 23.0%–24.8%), Actinobacteria (12.7%; 11.5%–13.7%), Planctomycetes (7.9%; 7.5%–8.3%), and Chloroflexi (4.3%; 3.1%–5.9%) (Fig. S2a, Table S2). However, diversity indices for prokaryotes did not significantly differ between inside and outside the fences, although average scores inside were slightly higher than those outside (Table 2, t-test:  $p > 0.10$ ).

**Table 2**

Prokaryotic and fungal alpha diversity inside and outside deer fences along with t-test results.

	Diversity indices	Inside		Outside		p-value
Prokaryotes	Number of ASVs	1196.0	± 86.4	1188.8	± 117.9	0.878
	Shannon	6.51	± 0.09	6.47	± 0.12	0.396
	Simpson	0.99692	± 0.00077	0.99655	± 0.00072	0.276
	Inverse Simpson	339.6	± 66.8	300.4	± 58.7	0.180
Fungi	Number of ASVs	239.8	± 62.1	153.7	± 49.4	<b>0.003</b> **
	Shannon	4.15	± 0.68	3.83	± 0.67	0.304
	Simpson	0.93290	± 0.06293	0.93347	± 0.05073	0.983
	Inverse Simpson	33.8	± 27.3	26.3	± 21.1	0.503

Scores are presented as means and standard deviations (SD)

P-values: [blank]  $> 0.10$ ,  $< 0.1$ , \*  $< 0.05$ , \*\*  $< 0.01$ , and \*\*\*  $< 0.001$ .

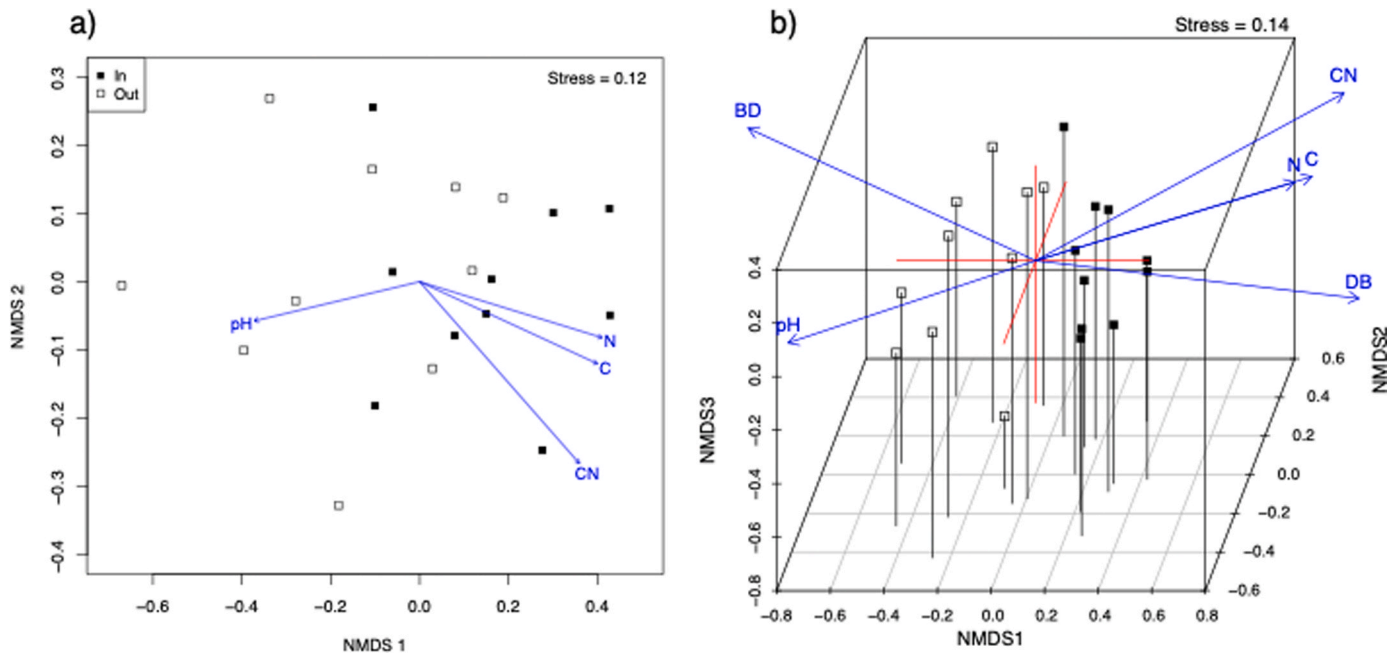
Based on the PERMANOVA test utilizing Bray–Curtis indices, significant differences in prokaryotic communities were observed between fence demarcations (PERMANOVA:  $F_{1,18} = 1.754$ ,  $p = 0.014$ ). The NMDS plot depicting prokaryotes (Fig. 2a) revealed a predominant clustering of inside-fence samples on the right side and outside-fence samples on the left side; however, several inside-fence samples were interspersed among the outside-fence samples. envfit analysis indicated that pH, soil C and N contents, and soil C/N ratio had significant effects (envfit:  $p < 0.01$ , Table 3). Among these variables, soil C/N ratio exhibited the highest  $R^2$  value ( $R^2 = 0.774$ ) and demonstrated trends similar to those of soil C and N contents, effectively distinguishing samples inside and outside the fenced area. Correlation coefficients among the four variables indicated strong associations ( $|r| > 0.6$ ) with statistical significance ( $p < 0.01$ , Table S3). Consequently, we used the soil C/N ratio as the explanatory variable for subsequent LM analysis.

Among the 34 prokaryote taxa examined, Acidobacteria (LM: coefficient =  $-384.312$ ,  $p < 0.05$ , Fig. S3a) and Chloroflexi (LM: coefficient =  $-546.471$ ,  $p < 0.001$ , Fig. 3a) exhibited negative associations with the soil C/N ratio (Table S2), whereas it had significantly positive effects on the relative abundances of Actinobacteria (LM: coefficient =  $501.048$ ,  $p < 0.01$ , Fig. S3b), Chlamydiae (LM: coefficient =  $52.571$ ,  $p < 0.001$ ), and Cyanobacteria (LM: coefficient =  $14.315$ ,  $p < 0.05$ ), with marginally significant effects on Dependitiae (LM: coefficient =  $14.016$ ,  $p < 0.10$ ). In the t-test, significant differences were observed in the relative abundances of Chlamydiae (t-test:  $p < 0.10$ ) and Chloroflexi (t-test:  $p < 0.05$ ) between samples inside and outside the fences, whereas no significant differences were observed for Acidobacteria, Actinobacteria, Cyanobacteria, and Dependitiae (t-test:  $p > 0.10$ ). Conversely, Elusimicrobia, Planctomycetes, and WS4 exhibited significant differences between samples inside and outside the fences in the t-test (Table S2).

PiCrust2 estimated 418 prokaryotic-related pathways from the samples, with certain pathways exhibiting variations based on fence demarcations and soil C/N ratio (Table S4). Twenty-two functions were significantly more abundant inside the fences than outside (t-test:  $p < 0.05$ ) and demonstrated significant increases with the soil C/N ratio (Tables 4 and S4, LM:  $p < 0.05$ ), predominantly involving disaccharide (sucrose and lactose) degradation and sucrose and starch biosynthesis processes. Conversely, three functions showed opposite trends, being more abundant in outside-fence samples: coenzyme B biosynthesis, coenzyme M biosynthesis, and anaerobic processes (Tables 4 and S4, t-test:  $p < 0.05$ , LM:  $p < 0.05$ ).

#### 3.3. Differences in soil fungal communities and functions between inside and outside fences

In fungal community analysis (Fig. S2b), 11 phyla were identified. Among these, Ascomycota and Basidiomycota collectively accounted for over 70% of the total read counts, followed by Mortierellomycota (average 3.4%), Rozellomycota (average 1.7%), and Chytridiomycota (average 1.3%) (Table S5). The remaining eight phyla each contributed less than 1% of the total read counts. ASVs inside the fences significantly outnumbered those outside (Table 2, t-test:  $p < 0.01$ ). However,



**Fig. 2.** Nonmetrical multidimensional scaling plot of two microbiomes based on Bray–Curtis indices and significant environmental variables detected using the envfit function (Table 3,  $p < 0.10$ ); a) prokaryotes and b) fungi; filled squares represent samples inside the fences, whereas open squares represent samples outside the fences. C: soil C content, N: soil N content, CN: soil C/N ratio, DB: dwarf bamboo density, BD: soil bulk density.

**Table 3**

Effects of measured variables on prokaryotic and fungal communities analyzed using the envfit function in NMDS analysis.

Variables	Prokaryotes				Fungi				
	NMDS1	NMDS2	R <sup>2</sup>	p-value	NMDS1	NMDS2	NMDS3	R <sup>2</sup>	p-value
Tree DBH	0.748	0.664	0.184	0.175	0.969	-0.163	-0.185	0.257	0.184
Dwarf bamboo density	0.961	-0.278	0.230	0.112	0.980	0.084	-0.178	0.519	<b>0.009</b> **
O horizon mass	0.190	-0.982	0.031	0.769	0.790	0.614	-0.005	0.287	0.128
Soil pH	-0.988	-0.153	0.549	<b>0.002</b>	** -0.827	0.309	-0.469	0.558	<b>0.004</b> **
Soil C content	0.958	-0.287	0.676	< <b>0.001</b>	*** 0.932	-0.102	0.349	0.468	<b>0.012</b> *
Soil N content	0.981	-0.196	0.680	< <b>0.001</b>	*** 0.900	-0.189	0.393	0.468	<b>0.013</b> *
Soil C/N ratio	0.804	-0.595	0.774	< <b>0.001</b>	*** 0.904	0.188	0.385	0.521	<b>0.008</b> **
Soil bulk density	-0.785	0.620	0.089	0.450	-0.926	0.067	0.371	0.502	<b>0.010</b> **

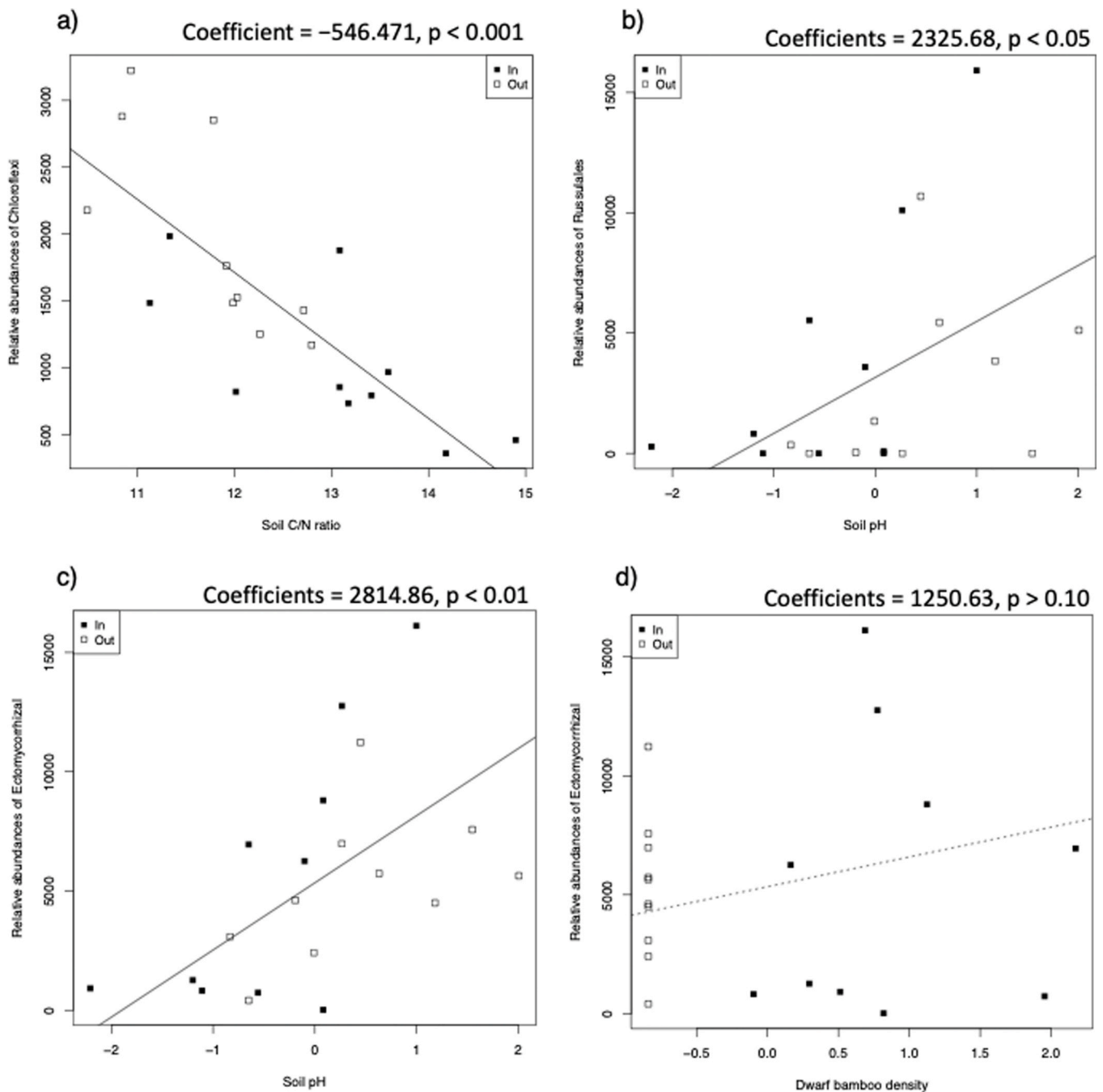
P-values: [blank] > 0.10, < 0.1, \* < 0.05, \*\* < 0.01, and \*\*\* < 0.001.

diversity indices (Shannon, Simpson, and inverse Simpson) showed no significant differences based on fence demarcations (t-test:  $p > 0.10$ ).

Fungal NMDS plotting and PERMANOVA analysis revealed a significant difference in fungal communities between samples inside and outside the fences (PERMANOVA:  $F_{1,18} = 1.3039$ ,  $p < 0.001$ ). Inside-fence samples predominantly clustered on the right side of the plot (Fig. 2b). Environmental variables, except tree DBH and O horizon mass, significantly influenced fungal community composition (envfit:  $p < 0.05$ , Table 3). Among these variables, soil pH exhibited the highest R<sup>2</sup> value ( $R^2 = 0.558$ ), inversely correlating with the NMDS1 axis. Soil pH was significantly correlated with soil C and N contents, C/N ratio, and bulk density, with arrow directions either opposite or similar ( $p < 0.05$ , Table S3). Dwarf bamboo density showed different directions with no significant correlations with soil pH but displayed significant negative correlations with bulk density ( $p < 0.05$ , Table S3). Dwarf bamboo density had the third-highest R<sup>2</sup> value ( $R^2 = 0.519$ ), correlating with the NMDS1 axis, suggesting an impact of fence presence. Samples roughly segregated into two groups along two axes: 1) along soil pH, outside-fence samples with high pH and low soil N and C contents, and inside-and outside-fence mixed samples with low pH and high soil N and C contents were distributed; 2) along dwarf bamboo density, both inside-and outside-fence samples were distributed, potentially indicating an association with dwarf bamboo protection provided by deer fences. For

subsequent LM analysis, we used dwarf bamboo density and soil pH as explanatory variables.

Ascomycota and Basidiomycota collectively accounted for over 30% of the total read counts, with no significant differences observed between the fenced and unfenced areas (Table S5, t-test:  $p > 0.10$ ) and no discernible effect of dwarf bamboo density (LM:  $p > 0.10$ ). However, Ascomycota exhibited a negative association with soil pH (LM: coefficient = -1817.71,  $p < 0.01$ ), whereas Basidiomycota exhibited a positive association with soil pH (LM: coefficient = 2475.19,  $p < 0.05$ ). The relative abundance of Mucoromycota varied between the fenced and unfenced areas with marginal significance (t-test:  $p < 0.10$ ). Rozellomycota did not exhibit significant differences between the fenced and unfenced areas (t-test:  $p > 0.10$ ) but displayed a negative association with soil pH (LM: coefficient = -223.47,  $p < 0.01$ , Fig. S3c). At the fungal order level, several prevalent taxa (constituting more than 1% of total read counts) were correlated with soil pH, negatively impacting the relative abundances of Hypocreales (LM: coefficient = -604.10,  $p < 0.001$ ) and Rozellomycota unidentified (LM: coefficient = -145.49,  $p < 0.01$ ) and positively influencing Russulales (LM: coefficient = 2325.68,  $p < 0.05$ , Fig. 3b). Dwarf bamboo density significantly influenced one order in Ascomycota and two orders in Basidiomycota (LM:  $p < 0.05$ ). Nevertheless, the relative abundances of most taxa showed no difference between the fenced and unfenced areas (Table S6, t-test:  $p > 0.10$ ).



**Fig. 3.** Relationships between environmental variables and the relative abundances of prokaryote and fungal taxa and guilds; a) relationship between soil C/N ratio and the relative abundance of Chloroflexi; b) relationships of soil pH with the relative abundances of Russulales and c) ectomycorrhizal fungi; d) relationship between the dwarf bamboo density and the relative abundance of ectomycorrhizal fungi; filled and open squares represent samples inside and outside fences, respectively. The solid and dotted lines indicate significant and nonsignificant effects of explanatory variables, respectively (see also Tables S5–S8).

The fungal functional groups, characterized by trophic mode and guilds as estimated using FunGuild, were influenced by either fence presence or environmental variables (Table S7). Although the relative abundances of fungal trophic modes did not differ significantly between fenced and unfenced areas ( $t$ -test:  $p > 0.10$ ), soil pH positively affected symbiotrophic fungi (LM: coefficient = 2842.31,  $p < 0.01$ , Fig. S3d). Dwarf bamboo density also showed positive effects on symbiotrophic fungi, although the effects were statistically marginal (LM: coefficient = 1677.83,  $p < 0.10$ ). Regarding fungal guilds, most showed no significant differences between fenced and unfenced areas (Table S8,  $t$ -test:  $p > 0.10$ ). However, the symbiotrophic-related guild, ectomycorrhizal was positively affected by soil pH (LM: coefficient = 2814.86,  $p < 0.01$ ,

Fig. 3c), whereas endophytes were negatively affected by it (LM: coefficient =  $-207.95$ ,  $p < 0.01$ ). The multifunctional guild—animal pathogen—clavicipitaceous endophyte—fungal parasite, which represented a fungal family in the order Hypocreales—was negatively affected by soil pH (LM: coefficient =  $-189.60$ ,  $p < 0.05$ ). Dwarf bamboo density did not significantly affect dominant guilds such as ectomycorrhizae (LM: coefficient = 1250.63,  $p > 0.10$ , Fig. 3d) but negatively affected endophytes (LM: coefficient =  $-122.79$ ,  $p < 0.10$ ).

#### 4. Discussion

We conducted a comparative analysis of understory environments,



**Table 4**

Average relative abundances of prokaryotic functions estimated using BioCyc along with t-test and linear model results.

BioCyc ID	Descriptions	Relative abundances		Significances of t-test	Coefficients	
		Inside	Outside		Soil C/N ratio	
<b>Abundant inside fence</b>						
FOLSYN-PWY	superpathway of tetrahydrofolate biosynthesis and salvage	18650.2	17178.7	*	902.5	**
GLCMANNANAUT-PWY	superpathway of N-acetylglucosamine, N-acetylmannosamine and N-acetylneuraminic acid degradation	789.0	529.8	*	150.6	***
GLUCOSE1PMETAB-PWY	glucose and glucose-1-phosphate degradation	1613.5	1176.4	*	227.9	**
LACTOSECAT-PWY	lactose degradation I	27.5	5.8	**	9.1	**
PWY-5181	toluene degradation III (aerobic) (via p-cresol)	2421.7	1925.9	*	207.1	*
PWY-5384	sucrose degradation IV (sucrose phosphorylase)	1567.0	1150.2	*	217.6	**
PWY-5509	adenosylcobalamin biosynthesis from adenosylcobinamide-GDP I	6560.8	5692.6	*	381.7	*
PWY-5531	3,8-divinyl-chlorophyllide a biosynthesis II (anaerobic)	500.7	254.5	*	125.2	**
PWY-5747	2-methylcitrate cycle II	2146.3	1589.2	**	246.8	**
PWY-621	sucrose degradation III (sucrose invertase)	1574.2	1157.6	*	217.9	**
PWY-622	starch biosynthesis	1938.5	1443.3	*	300.5	***
PWY-6269	superpathway of adenosylcobalamin salvage from cobinamide II	6519.4	5648.1	*	378.9	*
PWY-6507	4-deoxy-L-threo-hex-4-enopyranuronate degradation	2852.0	2316.9	*	282.4	*
PWY-6612	superpathway of tetrahydrofolate biosynthesis	15798.7	14280.8	*	916.3	**
PWY-6891	thiazole component of thiamine diphosphate biosynthesis II	389.9	239.0	**	85.6	***
PWY-6895	superpathway of thiamine diphosphate biosynthesis II	1850.7	1156.8	**	392.4	***
PWY-7084	nitrifier denitrification	172.2	34.6	***	44.6	**
PWY-7159	3,8-divinyl-chlorophyllide a biosynthesis III (aerobic, light independent)	500.7	254.5	*	125.2	**
PWY-7347	sucrose biosynthesis III	798.7	581.6	***	64.3	*
PWY0-1479	tRNA processing	8980.4	7204.8	**	907.5	***
PWY0-42	2-methylcitrate cycle I	2447.9	1843.4	**	259.6	**
SUCSYN-PWY	sucrose biosynthesis I (from photosynthesis)	2033.3	1494.0	***	189.1	**
<b>Abundant outside fence</b>						
P241-PWY	coenzyme B biosynthesis	361.2	643.9	*	-173.9	***
P261-PWY	coenzyme M biosynthesis I	818.9	1144.1	*	-185.8	***
PWY-7046	4-coumarate degradation (anaerobic)	517.1	789.6	*	-186.5	***

The full list is shown in Table S4.

P-values: [blank] &gt; 0.10, &lt; 0.1, \* &lt; 0.05, \*\* &lt; 0.01, and \*\*\* &lt; 0.001.

soil prokaryotes, and fungal communities between areas inside and outside deer fences. Consistent with our hypothesis, protecting vegetation within deer fences prevented a decrease in soil fungal diversity. However, prokaryote diversity did not exhibit differences between areas inside and outside the fences. Variations in prokaryote and fungal communities and their functionalities between areas inside and outside the fences can be attributed to changes in soil environmental factors, particularly the soil nutrient composition, associated with deer grazing. The following sections discuss the differences in understory environments between areas inside and outside the fences (4.1) and the associations of understory vegetation with prokaryotic (4.2) and fungal diversity, communities, and functionalities (4.3). Finally, we explore the potential impacts of changes in microbial communities following deer grazing.

#### 4.1. Effects of deer fences on the understory environment

Comparing environmental variables between areas inside and outside the fences, it was found that dwarf bamboo density, O horizon mass, soil C and N contents, and soil C/N ratio inside the fences exceeded those outside. Conversely, soil pH and bulk density outside the fences exceeded those inside (Table 1). The installation of deer fences effectively prevented browsing by deer, thereby conserving dwarf bamboo and leaf litter and maintaining a soil environment rich in organic matter. Outside the fences, soil compaction, likely caused by rain, deer, or human activity, was observed. These environmental changes outside the fences are consistent with findings from previous studies (Yanagi et al., 2008; Ohira et al., 2022; Katayama et al., 2023), which have reported increased soil hardness or bulk density and decreased soil organic matter content following vegetation loss due to deer grazing. With the disappearance of the understory, soil and litter movement escalate, potentially leading to soil erosion outside the fences (Furusawa et al., 2003). By preserving the understory vegetation, the installation of deer fences reduces the likelihood of subsequent soil erosion. Thus, the anticipated

effects of installing fences have been validated in the study area of Mt. Shiraga.

#### 4.2. Effects of deer fences and accompanying soil environmental changes on the soil prokaryote community

The community structures of soil prokaryotes were influenced by the presence of fences (PERMANOVA  $p = 0.014$ ), with associated soil environmental changes impacting the overall soil prokaryote community. However, the diversity indices of prokaryote communities did not differ significantly between areas inside and outside the fences. In our analyses, environmental variables affecting the prokaryote community included soil pH, and soil C and N contents, and C/N ratio, with soil C/N ratio used as a representative. Along with soil C/N ratio, the relative abundances of Chloroflexi and Acidobacteria decreased, whereas those of Actinobacteria increased. Chloroflexi and Acidobacteria, known as oligotrophic bacteria, are typically prevalent in deeper soils (Zhang et al., 2019a; Koner et al., 2022; Lan et al., 2022), whereas Actinobacteria play crucial roles in soil nutrient cycling, such as C and N (Zhang et al., 2019b). In severely eroded areas near our target trees, Chloroflexi increased with organic matter depletion, whereas Actinobacteria decreased with root exposures, an indicator of soil erosion (Chen et al., 2024). Significantly higher proportions of Chloroflexi were observed outside the fences (Fig. 3a, Table S2), suggesting potential soil and nutrient runoff due to understory grazing, leading to changes in prokaryotic communities associated with nutrient-poor soil environments. Additionally, functionalities varied between areas inside and outside the fences (Tables 4 and S4), with sucrose degradation and biosynthesis processes exhibiting higher ratios inside the fences. As soil nutrient availability decreases outside the fences, not only prokaryote taxa but also functionalities related to nutrient processes may decline. The study areas around Mt. Shiraga receive over 2800 mm of annual precipitation (Environmental Agency, Japan, 1988), leading to frequent soil nutrient runoff, which could induce changes in prokaryotic



communities and functionalities. Conservation of understory vegetation through fence installation in such areas has impacted prokaryotic communities and functionalities, following soil environmental changes.

#### 4.3. Effects of deer fences and accompanying understory environmental changes on soil fungal communities

The fungal community was significantly influenced by the presence of fences in terms of the number of ASVs, community composition, and functionalities. Inside the fences, there was a higher number of ASVs compared to outside, suggesting that fence installation not only conserved understory vegetation but also promoted fungal species richness. Soil pH typically exhibits a unimodal relationship with fungal richness, peaking at pH 5 (Tedersoo et al., 2020). Soil pH inside fences was lower than that outside (Table 1), suggesting that diversity scores within the fence should be lower compared to those outside. A previous study also reported a positive relationship between fungal richness and soil nutrient levels (Sui et al., 2023). Samples inside fences exhibited low pH and high soil organic matter contents (Table 1). Consequently, the result of fungal alpha diversity within fences, as indicated by the number of ASVs, might be influenced by organic matter content, potentially explaining why the diversity score within the fence was higher than that outside. The fungal NMDS plot also indicated distinguishable effects of fences on microbial communities (PERMANOVA  $p < 0.001$ ). However, differences caused by the fences were not observed in most phylum and order taxa and functions (t-test  $p > 0.10$ , Tables S5–S8); instead, relative abundances of taxa and guilds changed in response to environmental variables, particularly soil pH, rather than dwarf bamboo density or the presence of the fence itself. Soil pH was significantly negatively correlated with soil C and N contents and C/N ratio. The relative abundances of the symbiotrophic trophic mode and the ectomycorrhizal guild increased significantly with soil pH (Fig. 3c, and S3d). These results may be influenced by the response of specific ectomycorrhizal taxa, such as the fungal order Russulales, which also increased in frequency along with pH, although other ectomycorrhizal taxa, such as Helotiales (Ascomycota) and Athellales (Basidiomycota), displayed opposite trends (Table S6). Ectomycorrhizal fungi, which supply nitrogen to host trees, may decrease in biomass and enzymes with increasing N availability (Jørgensen et al., 2024). However, responses to soil pH  $< 4.0$  in acidic soils have been scarcely reported. Moreover, the response to soil pH differs among taxa (Aggangan et al., 1996; Thomson et al., 1996; Ge et al., 2017). For instance, no significant relationships between pH (4.6–6.6) and inoculation rates have been reported in Agaricales and Boletales, whereas the frequency of Pezizales is higher at higher pH (4.5–7.5). The response of Russulales to low soil pH (3.21–3.67) could not be clarified in the present study. However, changes in soil pH and related variables, such as C and N contents, attributable to deer grazing might alter the frequencies of this taxon and ectomycorrhizal fungi. Nevertheless, dwarf bamboo density did not affect the relative abundance of ectomycorrhizal fungi (Fig. 3d). These taxa may respond to soil environmental changes induced by fence installation rather than understory dwarf bamboo density. In the same areas, severely soil-eroded areas are distributed outside fences, and the relative abundances of symbiotic fungi, including ectomycorrhizal fungi, have declined in such areas (Chen et al., 2024). The observed proportional changes in fungi, consistent with the findings of previous studies, may indicate a declining process in the soil microbial community due to deer grazing and subsequent soil erosion outside fences.

The relative abundance of the fungal phylum Rozellomycota decreased significantly with increasing soil pH. Rozellomycota fungi are often found in cool temperate regions, and certain taxa, such as GS11, are known to inhabit soils with low pH (Tedersoo et al., 2017), consistent with the findings of this study (Table S6). Regarding other taxa, the relative abundance of the fungal order Hypocreales (Ascomycota) and the guild of animal pathogen–Clavicipitaceous endophyte–fungal parasite significantly decreased with increasing soil pH. Previous reports

have indicated that the relative abundance of Hypocreales increases with pH ranging from 4 to 9 (Rousk et al., 2010). However, limited studies have investigated the responses of this taxon to low soil pH.

#### 4.4. Within-site differences in the microbial community and future studies for soil microbial community changes during deer grazing

The preceding sections have discussed the proportional changes in microbial taxa and functions associated with the installation of deer fences. Although changes along environmental variables have been identified, direct effects of the fences on most taxa and functions have not been observed. Significant environmental variables, such as soil C/N ratio and pH, were broadly distributed across both inside- and outside-fence samples (Fig. 3 and S3). In particular, soil pH scored significantly lower inside-fence samples (average 3.40) than outside-fence samples (average 3.50), although average scores of soil pH inside and outside fences differed by 0.10. In addition, samples inside fences ranged from 3.21 to 3.56, while those outside ranged from 3.36 to 3.67. These variations within the sites may contribute to the nonsignificant differences in the microbial taxa observed between areas inside and outside the fences. Sample variability encompasses environmental factors influenced by fence installation as well as inherent location characteristics, such as slope direction, temperature fluctuations, and water flows from upper slopes. Differences in soil microbial communities are often attributed to environmental variables and geographical distances (Xiang et al., 2019; Li et al., 2020, 2023). In our study sites, samples were collected inside and outside fences along mountain ridges. Considering other microtopological features may provide further insights into understory environmental changes accompanying alterations in microbial communities.

The present study underscores differences in microbial communities between areas inside and outside fences. Deer grazing and subsequent changes in soil environment reduced fungal diversity but had no significant impact on prokaryote diversity. Environmental alterations associated with deer grazing modify both prokaryote and fungal communities, with areas outside fences experiencing understory removal and soil runoff. Our findings rigorously consider these two effects of environmental changes. During deer grazing in regions with high rainfall, understory vegetation such as dwarf bamboo is consumed, leading to a thinning of the O horizon. Consequently, surface soil exposure alters physicochemical properties of the soil, with declining soil nutrients such as C and N and increasing soil bulk density (Ohira et al., 2022; Gomi et al., 2022). Previous studies at the same site indicated that in severely soil-eroded areas, the final stages of the soil microbial community exhibit low microbial abundance and diversity (Katayama et al., 2023; Chen et al., 2024). Chen et al., (2024) noted shifts in the soil microbial community with severe soil erosion, with an increase in the relative abundances of pathotroph fungi and a decrease in that of ectomycorrhizal fungi with declining organic matter content. As some samples outside the fences showed similar relative abundances of ectomycorrhizal fungi to those inside fences (Fig. 3c), these samples might represent an intermediate stage of deer grazing and soil runoff in this region. This suggests that these sampling locations could potentially revert to vegetation conditions similar to those inside fences. Indeed, in several fenced areas, surface soil exposure was observed, and vegetation consisted of sparsely distributed moss, grasses, or shrubs, rather than reverting to vegetation dominated by *Fagus*; this phenomenon has been observed in fenced areas after long-term deer grazing (Nagaike et al., 2014; Harada et al., 2020; Gomi et al., 2022). The underlying mechanisms of this phenomenon reportedly involve seed source limitations and degraded soil conditions in long-term grazed areas (Harada et al., 2020). However, our results indicate that changes in soil microbial communities associated with soil property changes, especially mycorrhizal fungi, may also contribute to this phenomenon. Therefore, differences in vegetation and soil properties between areas inside and outside fences may indicate an ecological tipping point for deer grazing,

beyond which the forest ecosystem cannot revert to its original vegetation due to changes in soil properties and microbial communities. By focusing on identified soil environmental variables (i.e., soil pH and C/N ratio), it may be possible to identify locations where vegetation could be reversed or unreversed.

Our results only reflect the differences in dwarf bamboo and accompanying soil environmental changes between areas inside and outside fences. Future studies should explore the relationships between vegetation types within deer fences, and changes in microbial communities to elucidate the comprehensive effects of deer grazing and fence installation. Furthermore, deer fences have been in place at our study site since 2005, with ongoing installation efforts. Given the decline in *Fagus* tree growth in the study areas and the risk of trees mortality (Abe et al., 2024), continuous monitoring of soil environments will be essential for the conservation of *Fagus* trees and mountain vegetation in this region.

### CRediT authorship contribution statement

**Yuji Tokumoto:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation. **Yuki Sakurai:** Methodology, Investigation, Data curation. **Hayato Abe:** Writing – review & editing, Methodology, Investigation, Data curation. **Ayumi Katayama:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization.

### Declaration of Competing Interest

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

### Data availability

All the raw sequence data of the prokaryotic 16S rRNA and eukaryotic 18S rRNA genes were submitted in the Sequence Read Archive of DDBJ database under the accession number DRA016987 and DRA016988, respectively. Environmental data are within Supporting Information files.

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### Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.foreco.2024.121993](https://doi.org/10.1016/j.foreco.2024.121993).

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