Sex, health and habitat alter the community composition and assembly processes of symbiotic bacteria in captive frogs

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Abstract

Background

Frogs are critical economic animals essential to agricultural ecosystem equilibrium. Frog symbiotic microbes possess functions like elemental cycling and immune regulation, but little is known about how frog sex and health impact gut microbes. The relationship between frog habitat and soil microbes is unclear. We aimed to determine how frog sex, health and habitat influence symbiotic microbes and community assembly. To provide guidance for sustainable frog farming and conservation. We studied gut contents and symbiotic soils of frogs from a farm with Meningitis-like infectious diseases (MID).

Results

We used 16S rRNA sequencing to analyze gut microbes among frog sex and health. We also compared symbiotic microbes in frog aggregation soils, native soils, and soybean soils on the farm. Frog sex and health strongly impacted gut microbe β-diversity and taxonomy. Healthy frogs had better gut microbial metabolism. Symbiotic network analysis showed healthy female frogs were more complex than males, but diseased males were more complex than females. Male frog gut microbe assembly was primarily deterministic, but female assembly was stochastic. Stochastic dispersal limitation predominated gut microbe assembly in frogs of different health. Deterministic processes most strongly influenced soybean soil symbionts. Pathogens, nitrogen respiration/fixation were enriched in soybean soil. Sulfur respiration and hydrocarbon degradation were highest in aggregation soil.

Conclusion

Frog gut symbionts showed major differences in network structure and assembly based on sex and health. Disease reduced gut symbiont metabolic function. Diverse symbiotic soils, especially soybean soils, could benefit frog farming. Our findings provide insight into the negative impacts of disease and other factors on frog symbiotic microbes. This could aid development of sustainable frog farming practices.

1. Introduction

In recent years, the study of symbiotic microbial communities in amphibians, particularly frog gut microbiota, has gained interest due to their potential impacts on host health and ecological interactions. Frogs have evolved in environments surrounded by bacteria, forming highly complex symbiotic relationships [1]. Gut microbes play vital roles in host nutrient metabolism, disease resistance, immunity, and overall health by participating in nutrient metabolism and pathogen defense [2, 3]. The black-spotted frog (*Pelophylax nigromaculata*), an amphibian from the order Anura and genus Pelophylax, is crucial for maintaining agricultural ecosystem balance [4]. However, human activities and climate change have

raised concerns about the survival of *P. nigromaculata* [5]. Factors such as life stage, sex, diet, habitat conditions, seasonal variation, and host genetics influence gut microbiota composition and diversity [6–8]. For example, habitat degradation and anthropogenic disturbances can alter gut microbial community structure, potentially affecting amphibian health [9]. Symbiotic bacterial community assembly is crucial for host adaptation to changing environments [10]. Jin Zhou found that urbanization increased the stochasticity of microbial communities in frogs and reduced their ecological stability [11]. Seasonal shifts led to decreased frog microbial network complexity, while deterministic processes increased bacterial assembly from summer to fall [12]. Investigating these factors is essential for understanding the ecological role of symbiotic microbes in amphibians and informing conservation strategies.

Sex plays a pivotal role in shaping the composition of gut microbiota [13], yet the underlying mechanisms remain elusive. Meijer et al. investigated the presence of bacterial communities, such as *Alistipes* and *Rikenella*, in germ-free male mice, which proliferated in the absence of innate immune defenses. Upon transfer to germ-free female mice, these bacterial communities induced weight loss and inflammation [14]. Furthermore, Markle et al. demonstrated that male mice exhibited increased testosterone levels, promoting the growth of specific gut bacteria that protect against the development of type 1 diabetes [15]. In amphibian populations, females tend to be larger than males. According to the optimal foraging theory, larger frogs are likely to consume larger prey, consequently affecting their gut microbiota [16]. Research on frogs has indicated that although bacterial diversity did not significantly differ between sexes, community composition below the class level could reflect sex differences, particularly concerning Enterobacteriales, Enterobacteriaceae, and Peptostreptococcaceae [17]. These studies have elucidated the influence of sex on the composition and potential functionality of frog gut microbiota. However, limited research exists on the relationship between sex and gut microbial communities in black-spotted frogs, particularly regarding the complex interactions and assembly processes within these communities.

Amphibian health has gained prominence in recent years, given their vulnerability to environmental changes and emerging diseases. The gut microbiota serves as an essential immune organ in amphibians [18]. Kamada et al. identified two primary strategies through which gut microbiota helps hosts resist pathogen invasion: competing for limited nutrients and modulating host immune responses [19]. Kruger et al. discovered that the variation in Brazilian frog skin microbiota depended on host species and sampling location, with no differences observed between Bd-infected and healthy individuals [20]. This suggests that bacterial composition reflects host species and environmental factors, rather than Bd infection among sampled species. Beneficial gut symbiotic bacteria, such as *Janthinobacterium* [21] and *Akkermansia* [22], actively promote amphibian resistance to foreign pathogens. Meningitis-like infectious diseases (MID), also known as frog cataract and tarsal maggot, may be related to changes in gut microbial communities, but research remains limited [23]. Wengang Li reported that, compared to their healthy counterparts, infected bullfrogs with MID had higher oral and intestinal microbial richness, evenness, and abundance. In the diseased group, the abundance of Elizabethkingia increased while lactococci decreased [23]. Despite these advancements, knowledge about the response of frog symbiotic microbiota to health conditions remains limited.

Soil microorganisms play a crucial role in the soil environment, participating in processes such as mineralization of organic matter, formation and decomposition of humus, and transformation of nutrient elements [24]. Various frog habitats exist in breeding farms, including native soil, soybean soil, and frog-inhabited soil, necessitating the investigation of the relationship between soil microbes and frogs. Studies have shown that soybean cultivation has a more pronounced effect on the composition of rhizobia in agricultural soils compared to native soils, thereby reducing the complexity of microbial community interactions [25] Elly proposed that during the natural restoration process of fallow agricultural land, soil biotic community composition changes, networks contract, and carbon sequestration efficiency increases significantly [26]. To reduce the use of fertilizers and pesticides, the rice-frog ecosystem has emerged; researchers found that rice-frog (RF) cultivation significantly enriched the rhizosphere microbial communities of *Sandaracinaceae*, *Anaerolineaceae*, and *Candidatus Nitrososphaera*, which may be involved in improving nutrient cycling and promoting plant growth [27]. However, research on the response of soil bacteria in different land use types to the introduction of frogs remains scarce.

In this study, we focused on *P. nigromaculatus* from intensive frog farms in southern China, where severe MID infections are prevalent. We analyzed the effects of sex and health status on gut microbial communities using 16S rRNA amplicon sequencing. Additionally, we collected samples from frog-aggregation soil (AS), native soil (NS), and soybean soil (SS) in the farms to investigate variations in soil microbiota. We aimed to address three questions: (1) whether frog sex influences gut microbiota; (2) the variations in gut microbial communities and potential metabolic functions related to frog health status; and (3) the responses of soil bacterial communities to frog habitats, as well as the bacterial network structure and community assembly patterns. Research on frog microbiomes is crucial for maintaining host health and conserving amphibian habitats. This study has the potential to refine frog farming practices, improve the health of farmed frogs, and provide a basis for intensive frog rearing. Moreover, it may contribute to the promotion of effective, sustainable amphibian conservation strategies.

2. Materials and Methods

2.1. Gut and soil samples collection

Sixteen frogs (*P. nigromaculata*), consisting of eight females and eight males, were collected from Xuanzhou District, Anhui Province, South China (30°50'8.4"N, 118°36'9"E) in February 2020 (Supplementary Fig. S1). Each frog was individually placed in a plastic container and transported to the research laboratory for further analysis. To prevent bacterial contamination of samples, forceps and scissors were sterilised using an autoclave and a strong UV light source before the frogs were sacrificed. Following the procedure described by Mashoof et al. [28], the frogs were first rinsed with tap water and then rinsed with sterile water, then intestinal contents were collected from the stomach (excluding the stomach) to the anal intestinal contents after frog euthanasia. The euthanasia protocol was as follows: first, gauze was spread evenly in a glass drying chamber, followed by the placement of a cotton ball soaked in a The neck of each frog was then bent to expose the occipital foramen, whereupon a needle

was inserted. Before processing, the euthanised frogs were checked for cessation of heartbeat to confirm their death. Before processing, the euthanised frogs were checked for the cessation of heartbeat to confirm their death. Intestinal content samples were individually placed in sterilised EP tubes and stored at -80°C for later analysis.

At necropsy, some frogs were found to be infected with MID and were classified as unhealthy. Based on sex and health status, they were labelled MH (male healthy), FH (female healthy), MNH (male unhealthy) and FNH (female unhealthy). To investigate the effect of sex on gut microbiota, nine male (M) and nine female (F) individuals were compared. To evaluate the effect of health status on gut microbiota, infected individuals were assigned to the NH group and uninfected individuals were assigned to the H group, regardless of sex. Skeletochronology revealed that all frogs were two years old [29].

To investigate the adaptation of frogs to external habitats, three soil types were selected from the Xuancheng area as described previously. Soil samples were collected from the top layer (0–20 cm) after removing the surface grass. Native soil (NS) was collected from loose soil near the frog breeding site, where fewer frogs were present and active. frog-aggregation soil (AS) was collected from the bottom of the tray where frogs prefer to congregate, characterized as consistently moist, dark, and isolated from other environmental influences. Finally, soybean soil (SS) was collected from areas where soybean plants are grown, providing shelter and food for the frogs and creating a unique habitat. Each of these three soil types contained three replicates (using the multi-point mixed sampling method) [30], for a total of nine samples. For each soil type, approximately 10g of soil was collected from each control pool and immersed in 50ml of LifeGuard solution. All samples were stored at -20°C until required.

2.2. Total microbial DNA extraction, PCR amplification and Illumina sequencing

According to the manufacturer's instructions, Omega's Environmental DNA Extraction Kit was used to extract microbial DNA from frog gut and soil samples. The universal primer combination F338 (5'-ACTCCTACGGGAGGCAGCA-3') and R806 (5'-GGACTACVSGGGTATCTAAT-3') amplified the V3-V4 regions of the 16S rRNA gene [31]. PCR thermocycling consisted of 95°C for 5 minutes, 30 cycles of 30 seconds at 95°C, 50°C and 72°C, and a final extension at 72°C for 5 minutes. Prior to ligation of Illumina barcodes and adaptors, PCR products were purified using the Omega e.Z.N.A. TM CyclePure Kit, measured and aggregated in equimolar proportions. The libraries were sequenced according to the Illumina MiSeq instructions.

2.3. Microbiome bioinformatics and statistical analysis

QIIME2 (version QIIME2-2022.2) was employed to process the raw sequence data [32]. Paired reads (2 × 250 bp paired-end mode) from HiSeq4000 platforms were demultiplexed, filtered using vsearch, and subjected to quality control as follows: sequences with a length of 200 bp or an average quality score of 25 were eliminated, and ambiguous bases were not permitted. High-quality reads (> 97% identity) were clustered into operational taxonomic units (ASVs) using vsearch cluster-features-de-novo [33]. Samples

were rarefied to the same sequence depth (33,957 bacterial sequences per sample), and clustered feature tables were further filtered using QIIME2 feature-table filter-features (0.001%) [34]. Taxonomy was assigned to ASVs using the Silva v138 database and the Naive Bayes classifier [35]. After removing chloroplast and mitochondrial sequences, the final dataset comprised 1,214 ASVs for further analysis. The NCBI Sequence Read Archive (SRA) accession number for the genomic sequencing data is PRJNA415122.

2.4. Co-occurrence network analysis Measures of diversity and community structure

We utilized R v4.1.3.1 for data analysis and visualization. We assessed group differences through α diversity indices (Chao1, Shannon, Simpson, and Phylogenetic Diversity) using One-way ANOVA and Tukey HSD tests ($p < 0.05$) [36]. The β diversity was evaluated using PERMANOVA, ANOSIM (with 999 permutations), and Nonmetric multidimensional scaling (NMDS), as implemented in the R 'vegan' package [37]. To investigate the abundance of phyla and genera in amphibian intestine and soil bacteria, we generated bar charts and CIRCOS plots [38]. At the genus level, we performed Wilcoxon signed-rank tests with Bonferroni corrections between F and M samples, H and NH; samples grouped by frog habitats (AS, NS, SS) were analyzed using the Kruskal-Wallis rank sum test.

We constructed co-occurrence networks of gut bacteria using the 'WGCNA' R package, based on Spearman's correlation coefficients ($r > |0.9|$, $p < 0.01$) [39]. For soil bacterial networks, we identified significant associations ($r > |0.9|$, $P < 0.01$) among 50 major genera using Spearman's correlation tests. We visualized these networks and calculated their properties using Gephi 0.92 software [40]. We evaluated variations in network structure between groups by measuring the number of nodes, number of edges, average degree, degree centralization, graph density, graph modularity, and betweenness centralization. We identified the putative role of each node using two network topological features, intra-module connectivity (Zi) and inter-module connectivity (Pi): network hubs ($Zi > 8$ and $Pi > 0.62$) [41]. Keystone taxa, or network hubs, are species that may help maintain microbial community structure [42].

We then used PICRUSt to predict the microbial functions of the frog gut samples [43]. We used the MetaCyc databases and seeded them with 16s RNA gene sequences to generate a bar graph showing microbial functional profiles. We detected significant differences in MetaCyc pathways (level 2) among amphibian symbionts using an equal variance t-test [44]. In additionally, we performed functional annotation of soil bacteria using the "functional annotation of prokaryotic taxa" (FAPROTAX) program [45], which allowed for a comprehensive understanding of the functional roles of these bacterial communities.

2.5. The Calculation of Community Assembly Process

We assessed the assembly processes of bacterial communities in the samples by calculating the Beta Nearest Taxon Index (βNTI). Utilizing the 'comdist' function available in Phylocom v4.2 as part of the 'picante' package, we determined the β-mean nearest taxon distance (βMNTD) deviation from the null

model through βNTI values. Based on the findings by Stegen et al. [46], when |βNTI| exceeded 2, deterministic processes were the primary drivers of the microbial community. In contrast, when βNTI values were situated between − 2 and + 2, stochastic processes predominantly shaped the microbial community structure [47]. To evaluate pairwise microbial community turnover and further characterize assembly processes, we applied the Raup-Crick metric (RCbray). The results indicated that community assembly was subject to the influence of dispersal limitation (|βNTI| < 2 and RCbray > + 0.95) and homogenizing dispersal (|βNTI| < 2 and RCbray < -0.95) [48].

3. Results

3.1. Sequencing depth, alpha and Beta diversity of captive frogs and soil microbiota

The effect of sex and health status on the gut microbiota composition in captive frog samples was investigated. A 16S rRNA microbial data set consisting of 1018,720 filtered high-quality sequences was generated, with an average of 63670 ± 6,868 sequences per frog sample (Additional file 2). A total of 1,214 microbial species (ASVs) were identified in the gut communities based on > 97% sequence similarity, with an average length of 437 bp per sequence.

In terms of microbial α-diversity, no significant differences in total microbial diversity were observed among the four frog groups at the level of species richness, Shannon index, and phylogenetic diversity (Fig. S2). Furthermore, there was also no significant difference in richness and Shannon index among all frogs or between female and male frogs (t-test, $p > 0.05$) (Fig. 1a-b).

However, when comparing α-diversity differences between host habitats and the gut, we found that soil microbes exhibited higher diversity (Fig. 1c-d), which could be attributed to the combined influence of host species and external abiotic environmental factors. In contrast, a total of 4,461 ASVs were classified in soil bacteria. We also analyzed alpha indices for total soil bacteria in different frog habitats and found significant differences only in Chao1 values between groups. Community richness (Chao1) of soil bacteria in different frog habitats indicated that NS was significantly higher (t-test, $p = 0.01$) than AS and SS.

The NMDS plot (Fig. 2a) showed that the bacterial communities segregated significantly (ANOSIM: Bray-Curtis, $r = 0.650$, $P = 0.031$) into two major groups, the F-group and the M-group. According to the Bray-Curtis dissimilarity matrix (ANOSIM: Bray-Curtis, $r = 0.740$, $P = 0.044$) (Fig. 2b), the gut microbiota composition of captive amphibians from the H and NH groups was significantly different. In addition, soil samples were separated and significant differences in bacterial composition were observed between frog habitats ($R = 0.942$, $P = 0.005$, Fig. 2c-d). It clearly demonstrates the variation in bacterial diversity across frog gut samples, while in frog-aggregation soils.

3.2. Compositional and Distributional Patterns of Gut Bacteria, habitat soils related with frogs

Taxonomic assignment analysis revealed 10 phyla in captive frogs, and the most abundant phyla in all frogs were Firmicutes, Bacteroidetes, Firmicutes, and Proteobacteria (**Fig. S3**). At the phylum level, there were no significant differences in relative abundance between sexes or health status groups ($P > 0.05$). Among the habitat soils, the CIRCOS plots showed that 14 phyla were dominant (> 1%), including Proteobacteria, Bacteroidetes, Acidobacteria, Firmicutes, Nitrospirae, Saccharibacteria, Verrucomicrobia, Actinobacteria, Chloroflexi. Further comparison showed that Firmicutes was the most abundant in NS, Ignavibacteriae was much more abundant in AS than in other soils, and Actinobacteria occupied the highest relative abundance in SS. However, there was no significant difference between the soil microbiota at the phylum level (Fig. 3c).

At the genus level, frog gut bacteria contained 21 dominant genera, and Bacteroides (10.6%) was the most dominant bacterial genus, followed by *Citrobacter* (6.6%), *Parabacteroides* (5.3%), and *Akkermansia* (4.90%) (Fig. S4). Furthermore, our result showed that *Ruminococcaceae* was significantly enriched in the female group, whereas Laribacter was significantly enriched in the male group (Kruskal-Wallis rank sum test, $P < 0.05$; Fig. 3a) among the top 100 genera. Five genera were significantly different between two health statuses, with *Parabacteroides* significantly higher in NH than in H, whereas *Odoribacter* and *Akkermansia* were significantly higher in H than in NH (Fig. 3b). Among the habitat soils, there were 18 genera among all 223 identified genera that differed significantly among soil groups, among which *Bacillus, Nitrosospira*, and *Geobacter* were among the dominant genera (Fig. S5), with Bacillus being significantly higher in NS than in other groups. *Geobacter*, *Sphingobacterium, Bradyrhizobium, and Moheibacter* were significantly higher in relative abundance in SS than in other soils (Fig. 3d).

3.3. Potential Cooccurrence patterns of bacterial community in frog gut and habitat soils

To determine the interactions of frog gut microbes across sex and health status, we constructed four networks (Fig. 4), focusing on large modules in networks with at least 10 nodes through modularity. The diverse topological features of the four networks indicated that the co-occurrence patterns of microbes differed considerably across sexes and health states. The total number of nodes varied slightly (from 180 to 196), but the total number of links ranged from 440 (MH) to 1251 (MNH) (Table S1). Moreover, the number of nodes, total links, network density, average degree, and number of modules were strikingly similar in both female frog types, with their network structures also closely related, exhibiting relatively high complexity and modularity (0.84–0.897). In male frogs, however, the disease group displayed much higher topological parameters than the healthy group, and their microbial communities exhibited the most complex networks with the highest relative modularity values (0.911) and modules (12); MH had the simplest of all networks and the lowest degree of modularity (0.817), with only four large modules. Surprisingly, we found that among frogs of the same health status, network complexity differed significantly between females and males, with females being more complex than males in the H group and males being more complex than females in the NH group.

To further investigate the interactions between bacterial communities in different frog habitat soils, we constructed a Spearman correlation-based network for the top 50 genera (Fig. 5) and analyzed the

fundamental topological properties of the networks (Table S2). AS had the highest number of nodes, total connections, positive connections, and average degree of network density among all soils, with values of 50, 472, 51.27%, 0.401, and 19.265, respectively, indicating that the bacterial network of AS was the most complex and the positive associations between genera were intense. The lowest number of total connections, negative connections, and network density were found in NS soils, indicating that NS had the simplest networks. Further modularity analysis revealed the same significant modules for all three soils, with SS having the highest modularity (0.558) in all networks. In the AS network, *Ignavibacterium* (p__Ignavibacteriae) and *Sphingomonas* (p__Proteobacteria) were detected as the most critical taxa (strongest interaction). In the NS network, *g_Nitrosospira* (p__Nitrospirae) and *Bacillus* (p__Firmicutes) were similarly designated as key taxa in most genera in the third module, but they were much less closely related than in other soil network structures. The relatively more critical taxa in SS are *Geobacter* (p__Proteobacteria), *Sphingomonas* (p__Proteobacteria), *Sphingobacterium* (p__Bacteroidetes), and *Bradyrhizobium* (p__Proteobacteria). Additionally, we found that these key taxa were primarily significantly and positively correlated with other genera.

3.4. Prediction of bacterial metabolic function Variation based on PICRUSt and FAPROTAX

As analyses above demonstrated that gut microbiota was differentiated by sex and health status, we investigated whether these gut bacteria function differentially on metabolism or physiology of frogs. We were able to assign 417 out of 1,214 bacterial ASVs (34.35%) predicted by PICRUSt. Furthermore, we predicted 42 functional groups in MetaCyc at the second level, with functional bacteria of Vitamin Biosynthesis, Amino Acid Biosynthesis, Nucleoside and Nucleotide Biosynthesis, Fatty Acid and Lipid Biosynthesis, and Carbohydrate Biosynthesis being dominant in the gut of frogs. The relative abundances of Carboxylate Degradation, Pentose Phosphate Pathways, and Glycan Degradation were significantly higher in the M group than the F group, while Photosynthesis was significantly higher in the F group than the M group (Fig. 6a). Considering the health state of the frog, Carbohydrate Biosynthesis, Secondary Metabolite Biosynthesis, C1 Compound Utilization and Assimilation, and Nucleic Acid Processing had significantly higher relative abundances in H than the NH group, but Nucleoside and Nucleotide Degradation and Carboxylate Degradation were significantly lower than the NH group (Fig. 6b).

FAPROTAX enables the analysis of biogeochemical cycling processes in environmental samples like soil, particularly for the functional annotation prediction of elemental cycles such as carbon, phosphorus, sulfur, and nitrogen. 50 major function groups were obtained based on the FAPROTAX tool, and only 1588 ASVs were identified as known functions, representing 35.6% of the total ASVs (4461). There were 11 functions that differed significantly between soil habitats, for example, human_pathogens_all, nitrogen_respiration, and nitrate_respiration. Most notably, human_pathogens_all, nitrogen_respiration, nitrate_respiration, nitrogen_fixation, and iron_respiration were significantly enriched in SS compared to the others. On the other hand, sulfur_respiration, hydrocarbon_degradation, methylotrophy, and

dark_sulfur oxidation were significantly higher in AS than in the others. Interestingly, chloroplasts were the only function found in NS that was significantly greater than in other frog-inhabiting soils (Fig. 6c).

3.5. Bacterial community assembly patterns in frog gut and soils

To further explore the relative contribution of stochastic and deterministic processes to bacterial community aggregation, bNTI was calculated based on OTU abundance and its phylogenetic distance. The average bNTI value (3.15) in MNH was remarkably higher than the others, indicating that the deterministic processes are more important for community assembly than the stochastic processes in MNH, the other frog groups had the opposite result (Fig. 7a). The average bNTI value (2.17) in the M group was significantly higher than in the F group (0.21) (Fig. 7b), and the RCbray values showed that heterogeneous selection and dispersal limitation are equally important in the deterministic processes in the M group. All bNTI values in the F group were lower than 2 and higher than − 2, and all RCbray values were higher than 0.95, indicating that heterogeneous selection of stochastic processes determines the assembly of gut bacteria in the F group (Fig. 7c). However, there was no significant difference in the average bNTI between H and NH groups, and the stochastic processes dominate in the assembly of the bacterial community (Fig. 7d). Furthermore, the majority (91.7%) of H group belonged to dispersal limitation in community assembly, and dispersal limitation (58.3%) was also slightly more important than heterogeneous selection (41.7) in NH group (Fig. 7e). The value of the niche width index for frog-associated soil bacteria was NS > SS > AS (Fig. 7f), suggesting that deterministic mechanisms had a greater influence on community assembly in SS.

4. Discussion

4.1 Effects of sex on the Gut Microbiota of frogs

In this study, we collected frogs of different sexes from the same habitat and with similar dietary composition in southern China to explore the relationship between gut microbial communities and their host. In terms of alpha diversity, we did not find any significant differences in bacterial diversity and abundance indices between sexes in frog intestinal samples. Studies on the influence of sex on frog gut microbial diversity are scarce. Zhuo Chen's research on the gut microbiota of three species of montane stream salamanders found no significant differences in microbial community diversity among the three frog species [49]. Yilin Shu's study revealed no differences in microbial community diversity between male and female healthy Odorrana tormota intestines [17], suggesting that gut alpha diversity exhibits strong stability in frogs.

However, beta diversity analysis indicated that bacterial composition in frog groups was significantly different between sexes. Previous studies have consistently shown that factors such as frog species [49], sex of Chinese Odorrana tormota [17], and developmental stage [6] significantly affect gut bacterial composition. 16S rRNA sequencing and metagenomic studies confirmed that Firmicutes, Bacteroidetes,

and Proteobacteria are enriched in various frog gut microbiomes [50, 51], with no significant differences in relative abundance between sexes. Our findings indicate that Firmicutes, Bacteroidetes, Verrucomicrobia, and Proteobacteria are dominant phyla in frog gastrointestinal tracts with no differences in abundance. These results are consistent with previous findings that animals living in similar environments and under similar predation conditions tend to have similar microbial taxa at higher taxonomic levels [52]. Current research suggests that significant differences in gut microbial composition between sexes are observed at some lower taxonomic levels [53]. These differences may be due to subtle variations in predation between the sexes [15, 54]. At the genus level, we found that frog intestines had a significantly higher abundance of *Ruminococcaceae* in the female group, while *Laribacter* was significantly enriched in the male group. *Alistipes*, a Gram-negative bacterium, may have protective effects against liver fibrosis, cancer immunotherapy, and cardiovascular diseases, and is also associated with colorectal cancer and depression [15]. Yilin Shu's study found that *Robinsoniella* was significantly more abundant in female frogs than in males [17]; however, *Alistipes* was more abundant in males, potentially benefiting frog health. Differences in a few bacterial taxa may be due to the subtle variations in predation between sexes.

Network complexity plays a crucial role in understanding microbial interactions [55]. We found that bacterial co-occurrence networks among frogs of different sexes were similar; however, in healthy frogs, female microbial network complexity and modularity were higher than in males. Francis constructed interaction networks (symbiosis and competition) of tree frog gut microbiota, such as the symbiotic relationship between Garvieae and Corynebacterium variabile [56]. Our research suggests that frog sex does not significantly influence intestinal bacterial co-occurrence networks; however, differences in health status can alter co-occurrence networks between sexes. We found that the complexity of female networks was higher than in males in healthy frogs, possibly due to the regulation of symbiotic and competitive relationships among microbial communities. Moreover, a study by Liangliang and colleagues found that seasonal changes reduced the complexity of frog (skin and gut) microbiota networks from summer to autumn [12]. A previous study showed that high network complexity implies the need for a more stable microbial network to withstand harmful bacterial interference from the environment [57]. Consequently, microbial networks in healthy female frogs may be better equipped to resist environmental disturbances compared to males.

We discovered that sex influences the community assembly mechanisms of intestinal bacteria in frogs. Overall, deterministic processes were the dominant factors driving the assembly of symbiotic bacterial communities in male frogs. However, heterogeneous selection (selection caused by varying conditions) of stochastic processes determines the assembly of intestinal bacteria in the female group, leading to higher phylogenetic composition variations. There is a strong connection between bacterial phylogeny and function, so functional predictions can provide useful insights for the vast uncultured microbial communities obtained from amplicon sequencing [58]. Based on PICRUSt, the most abundant functions in Level 2 MetaCyc metabolic pathways include Vitamin Biosynthesis and Amino Acid Biosynthesis, mainly associated with biosynthesis. Existing research comparing the gut microbiota of three frog species found that the most abundant gene functions within these communities are primarily related to

metabolism, specifically amino acid metabolism, carbohydrate metabolism, and metabolism of cofactors and vitamins [49]. This finding differs significantly from our results. Additionally, Yilin Shu's research indicates that the COG functional profile of frog gut metagenomes reveals a rich array of carbohydrate transport and metabolic pathways [17]. Interestingly, our study discovered that Glycan Degradation is more abundant in male frogs compared to females, which is related to carbohydrate metabolism. Thus, it can be inferred that the gene function differences in frog gut microbiota may be associated with dietary habits caused by sex differences.

4.2 Changes in gut bacterial communities in Response to health status of frogs

In humans, there have been numerous reports on the association between diseases and gut microbiota. For example, non-alcoholic fatty liver disease (NAFLD) reduces the diversity of gut microbiota, both in terms of α and β diversity [59]. Consequently, gut microbiota may reflect the immune system status and overall health of the host species [60]. Wengang's study revealed that the richness, evenness, and abundance of microbial communities in the oral cavity and intestines of diseased bullfrogs were significantly higher than those in healthy bullfrogs. Furthermore, the abundance of Elizabethkingia markedly increased, while that of Lactococcus significantly decreased [23].

In this study, we examined the association between cataract infection and gut microbiota diversity and composition in frogs. Similarly, we found no significant differences in the richness and diversity of gut microbiota among frogs with different health statuses. Regarding microbial composition, we found that *Parabacteroides* was significantly enriched in the NH group, while *Odoribacter* and *Akkermansia* were significantly enriched in the H group. Yilin Shu's research found that the phylum Actinobacteria was more abundant in infected individuals, and the genus *Akkermansia* was more abundant in healthy frogs [60]. This is consistent with our findings, indicating that diseases lead to an increase in these taxa. The presence of Odoribacter is closely related to host health and participates in the metabolism of carbohydrates, lipids, and amino acids. Its abundance may be altered in some obesity and inflammatory bowel diseases [61]. Therefore, we can infer that cataract disease may also suppress Odoribacter, which helps maintain the balance of gut microbial communities.

Stochastic processes dominate the assembly of bacterial communities in frogs with different health statuses, primarily influenced by dispersal limitation. These results suggest that these frogs have a lower dispersal rate, so disease may restrict the spread of symbiotic bacterial communities. Heterogeneous selection is the second factor after dispersal. Thus, varying environmental selection pressures in amphibians (different infection levels) may cause significant differences in frog gut community assembly to adapt to different environmental factors or selection pressures [62]. Wengang's study suggests that pathogen (MID) infection may cause a decline in host immune function [23]. Consistently, our findings reveal that the majority of gut microbiota functions and metabolism (especially Carbohydrate Biosynthesis and Secondary Metabolite Biosynthesis) are significantly stronger in healthy frogs compared to diseased ones.

4.3 Environmental Effects on Soil Bacterial Community Composition in Frog Habitats

In the above section, we investigated bacterial diversity in frog intestines; however, soil microbiota, which also share a symbiotic relationship with frogs, are also worth exploring. First, we found that the number of ASVs for soil bacteria in frog habitats was twice that of gut bacteria, suggesting that the diversity and richness of soil bacteria is significantly greater than that of gut bacteria. Among different habitats, bacterial community richness was significantly higher in NS than in AS and SS, but soil bacterial diversity did not differ significantly. Xiaomei Yi et al. conducted the first comprehensive study on the structure and function of soil microbial communities in rice field (RF) and showed that RF significantly increased the diversity and richness of bacterial and fungal communities [27]. This may be due to the increase in frog feces with increasing cultivation time, which favors the growth of various microorganisms. Studies on soybean soil have also reported that bacterial species richness is significantly higher in agricultural soil than in native soil, but diversity does not differ significantly between the two soil types [25]. In our study, we reached a conclusion contrary to the two aforementioned studies, possibly because the intense activity of frogs in HS and SS suppressed soil bacterial richness.

Further research indicated that the type of frog habitat had a significant effect on soil bacterial β-diversity. Interestingly, a study by Pérez-Jaramillo et al. showed that there were significant differences between soybean agricultural soils and native soils [25]. We found that the dominant phyla of bacteria in frog habitat soil were mainly Proteobacteria, Bacteroidetes, Acidobacteria, and Chloroflexi, among which Firmicutes were most abundant in NS; Ignavibacteriae were much higher in AS than in other soils. However, their differences at the phylum level were not significant. The research of Xiaomei Yi et al. found that Proteobacteria, Acidobacteria, and Chloroflexi were the dominant bacterial communities in rice-frog cultivation (RF) soil, and the specific bacterial taxa enriched in RF played an indispensable role in organic matter decomposition and soil C, N, and P transformation processes [27]. They were also identified in our samples. In addition, research has shown that Acidobacteria have a higher relative abundance in native soil than in soybean agricultural soil [25], and in our study, Acidobacteria were found to be highest in SS (63.28%). Acidobacteria are generally considered to be oligotrophic, and the abundance of acidic bacterial species in soil [63], as well as their diversity in metabolic characteristics, make them a potentially important group in soil nutrient cycling [64]. This may be due to the fact that frog culture promotes the enrichment of Acidobacteria in soybean soil.

At the genus level, we observed significant differences in the dominant genera *Bacillus*, *Nitrosospira*, and *Geobacter*. Another study found that the core bacterial genera in agricultural soybean soil include *Rhizobium*, *Bradyrhizobium, Mesorhizobium*, and *Sphingomonas*, with a considerable portion comprised of nitrogen-fixing bacteria [25]. In our study, *Sphingomonas* and *Bradyrhizobium* were significantly higher in abundance in soybean soil compared to other soil types, suggesting that soybean soil in frog farming is rich in nitrogen-fixing bacteria.

Furthermore, we constructed bacterial ecological networks in frog habitat soils. This research revealed that the HS bacterial network structure was the most complex, with the tightest connections between bacteria. As the soil with the most active frog activity, this may be due to frog excreta and food providing a nutrient source for soil microbes. *Ignavibacterium* and *Sphingomonas* were detected as the most critical taxa (strongest interaction) in HS, with the stability and construction of the bacterial network structure primarily relying on them. In terms of bacterial community modularity, SS exhibited the most distinct pattern, while also possessing the highest number of key taxa, including *Geobacter, Sphingomonasa, Sphingobacterium, and Bradyrhizobium*. These key taxa displayed a significantly positive correlation with other genera. Previous research found that the interactions between bacterial taxa in native soil environments were more complex than those in soybean agricultural soil, but soybean soil favors the establishment of nutrient-rich organisms [25]. Based on these findings, we hypothesize that bacterial network modularity is shaped by soybean soil in frog farming, which in turn allows functionally relevant bacterial species to more easily establish themselves in soybean agricultural soil, such as *Geobacter, Sphingomonas, Sphingobacterium, and Bradyrhizobium.*

Bacteria play a crucial role in soil nutrient cycling, and their functions determine soil fertility and microbial vitality to some extent [65]. According to FAPROTAX, we predicted that 35.6% of ASVs possess potential ecological functions. Most functions in soybean soil (SS) were significantly higher than in the other two soil types, such as nitrogen_respiration, iron_respiration, sulfur_respiration, and nitrogen_fixation. Iron_respiration was notably higher in SS than in other soils, which might be closely related to the presence of abundant nitrogen-fixing bacteria in that environment. Furthermore, we found that many functions were significantly higher in HS than in the other two soil types, including hydrocarbon_degradation, dark_hydrogen_oxidation, methanotrophy, methylotrophy, and dark_sulfide_oxidation. HS provides favorable conditions for bacteria associated with dark_hydrogen_oxidation and dark_sulfide_oxidation due to its moist and light-avoiding environment. In contrast, NS areas lack interference from plants and animals such as frogs, resulting in generally lower functional microorganisms and weaker soil microbial metabolic activity compared to other soils. Regarding the assembly and ecological niche width of soil bacterial communities, we applied the neutral model to fit bacterial communities in different frog habitats. We found that the dispersal limitation of NS bacterial communities was much more severe. NS is considered to be heterogeneous and discontinuous soil for microbes [66]. In contrast, due to the influence of soybean plants (SS) or frog habitation (AS), the soil environment is more uniform. As a result, more ecological niches exist in NS soil. Additionally, deterministic mechanisms had a more significant impact on the community composition of SS, likely because deterministic processes have a more substantial influence on soybean soils with a narrower niche width.

5. Conclusions

Our study demonstrates that sex, health status, and habitat environment can significantly alter the community and assembly profile of symbiotic bacteria in captive frogs. Sex differences play a role in the

gut microbiota of frogs, with no significant differences in alpha diversity but distinct differences in beta diversity. Health status also affects the gut microbial communities, with cataract disease altering the abundance of specific bacterial taxa. Furthermore, the habitat environment has a significant impact on soil bacterial community composition in frog habitats. The type of habitat, whether it is natural soil, agricultural soil, or soil within frog enclosures, can influence bacterial diversity and the complexity of microbial interactions within the soil. Our research provides valuable insights into the factors shaping the microbial communities in frogs and their environments, which could be essential for understanding their ecological roles and potential implications for conservation efforts. Future studies should investigate the underlying mechanisms driving these differences, including host immune responses, environmental factors, and the roles of specific bacterial taxa in maintaining the health and well-being of frog populations.

Declarations

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Authors' contributions

HC, SL designed the study; SL, SI collected the samples and performed the experiments; SL, SH, SX and analyzed the data; SL, SI, SH and SX wrote the manuscript. HC, SL and SX review and editing. All authors read and approved the final manuscript.

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Conflicts of Interest

All authors state that they do not have known competing financial interests or personal relationships that may have an impact on the work reported in this article.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request. The raw amplicon data have been submitted to China National GeneBank DataBase, CNGBdb (accession number, PRJNA415122).

Competing interests

The authors declare that they have no competing interests.

Ethics approval and consent to participate

We confirm that the experimental protocol and the number of animals to be used were reviewed and approved by the Animal Welfare and Ethics Committee of Nanjing Agricultural University (Approval No. 101056). All animal experiments followed the International Council for Laboratory Animal Science (ICLAS) Ethical Guideline for Researchers, specific examples: 1 The use of animals in experimental research should be avoided wherever possible (Replacement). 2 When animal research is necessary, as few animals as possible should be used (Reduction). (3) The use of animals for necessary experimental research should be carried out to high quality standards, i.e. using optimal methods, in particular avoiding unnecessary pain (Refinement). The euthanasia of the frog was carried out in accordance with the American Veterinary Medical Association (AVMA) Guidelines for the Euthanasia of Animals (2020) to obtain microorganisms from the frog gut see S2.5 LABORATORY FISH, AMPHIBIANS, AND REPTILES for specific regulations.

Consent for publication

Not applicable.

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Figures

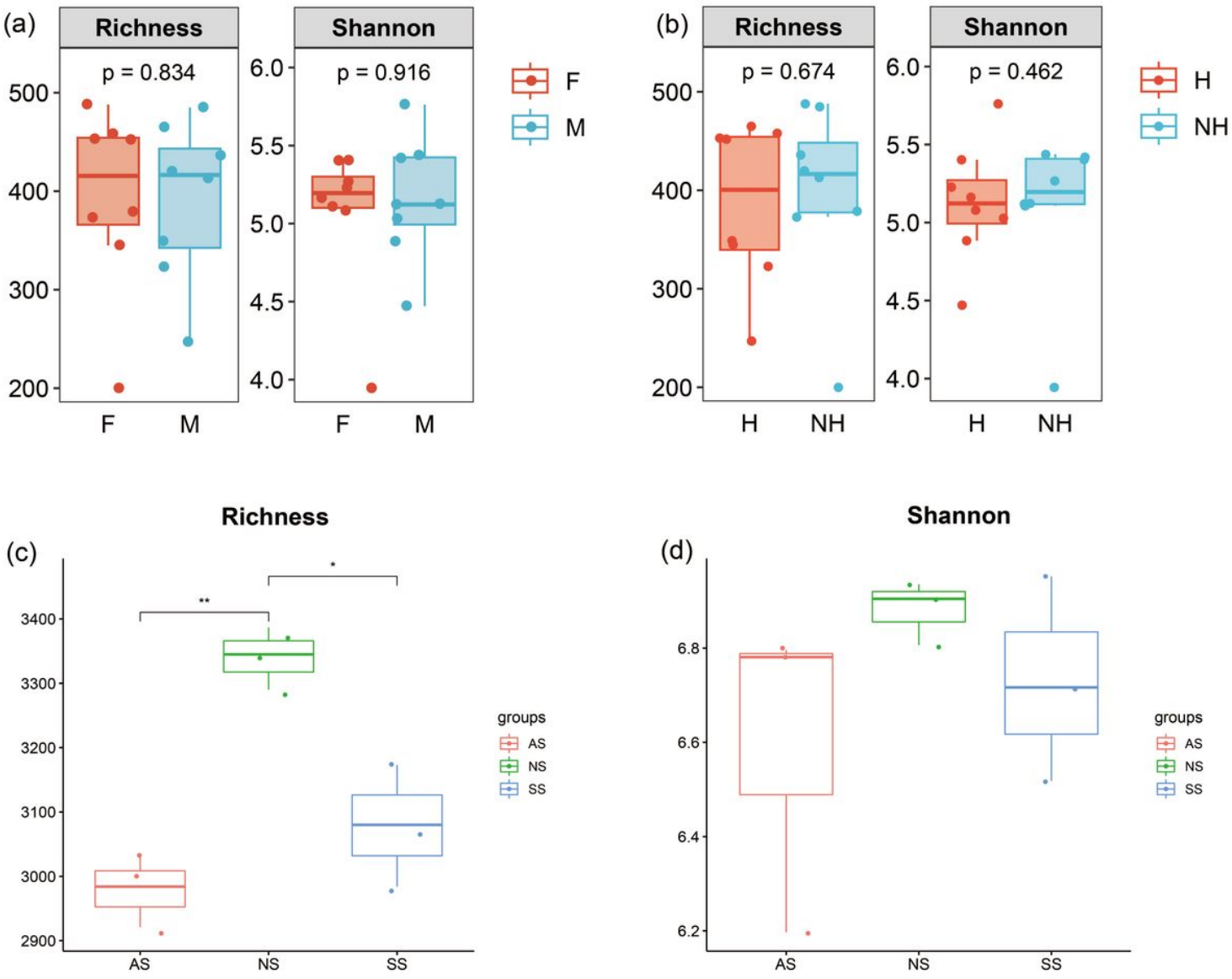

Figure 1

Comparative analysis of α-diversity in farmed frogs, including richness and Shannon index, between sexes (a) and health statuses (b). Richness (c) and Shannon index (d) were evaluated using t-tests among frog habitat soils (AS, NS, SS), with * and ** denoting significance at 0.05 and 0.01 levels, respectively.

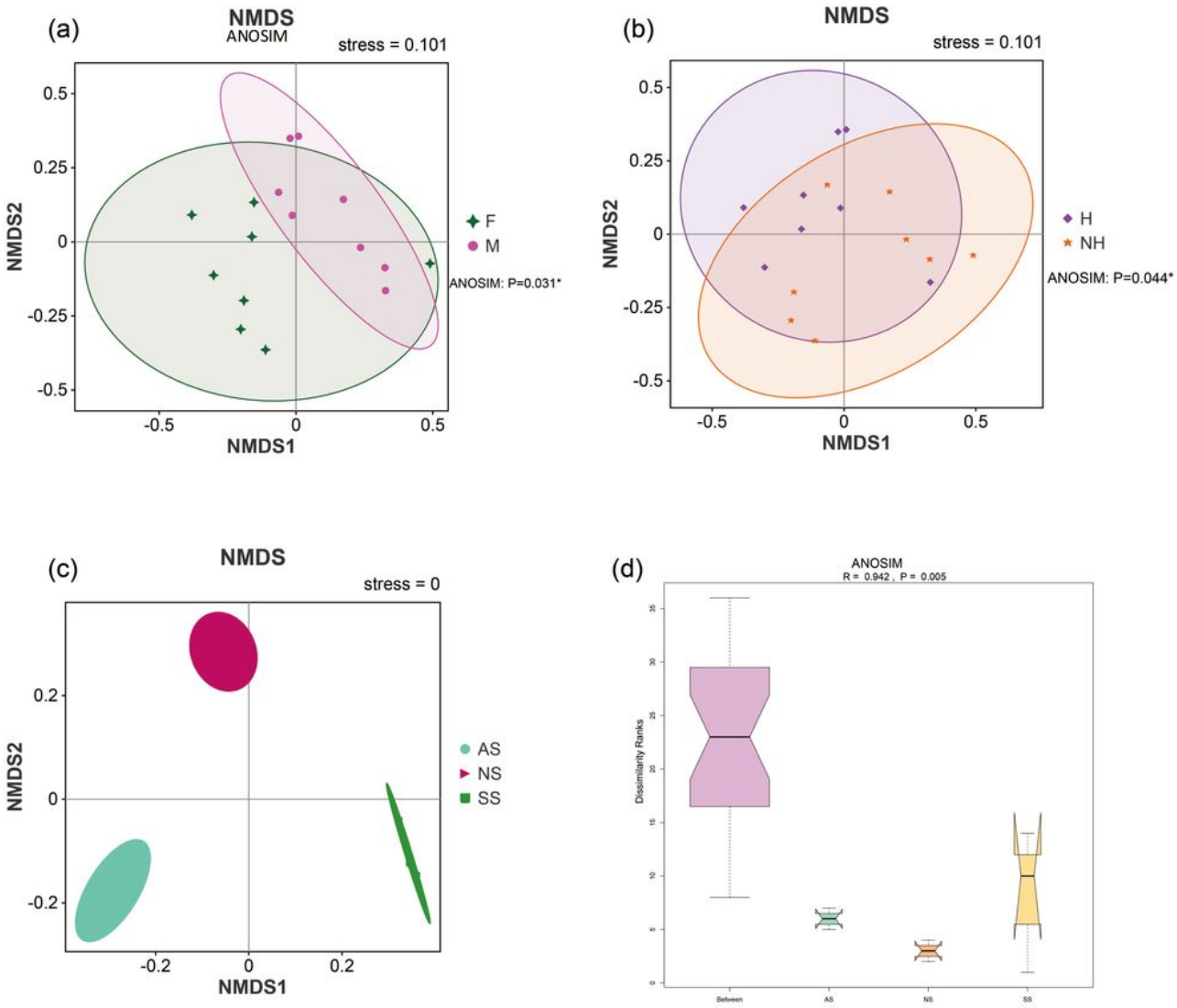

Figure 2

β-Diversity of Bacterial Community Structure in Frog Gut and Soil: (a) Nonmetric Multidimensional Scaling (NMDS) plot illustrating gut bacterial communities in frogs, comparing F (female) and M (male). (b) Comparison of gut microbiota in frogs between H (Healthy) and NH (Unhealthy). (c) Grouping of samples by frog habitats (AS, NS, SS), with each dot representing each sample. (d) Results displayed as relative variable importance (R2) and significance (p) calculated using PERMANOVA (Adonis).

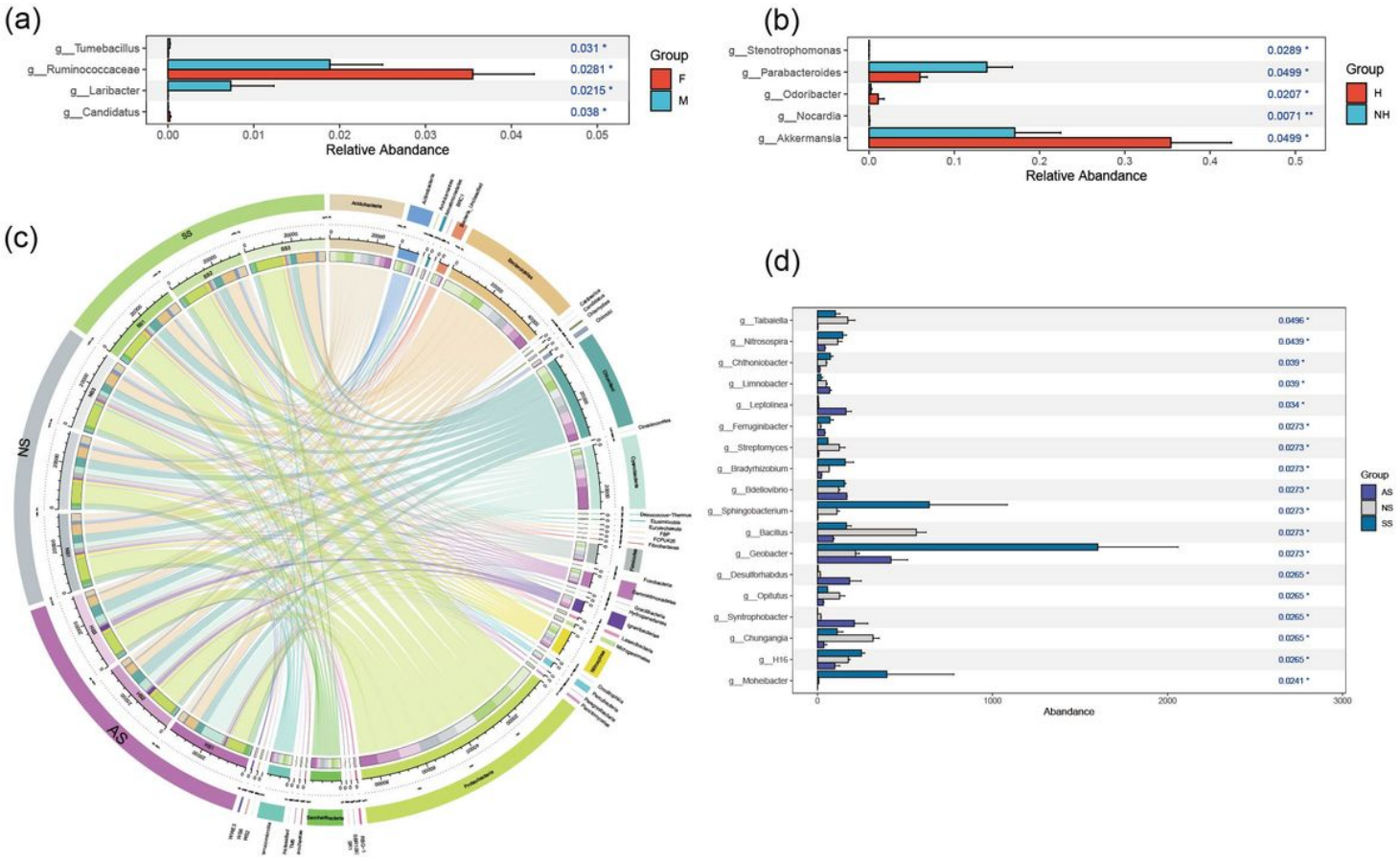

Figure 3

Differential Abundance of Bacterial Genera in Frog Guts and Soils: (a) Wilcoxon signed-rank tests with Bonferroni corrections were conducted between F (female) and M (male) frog samples at the genus level. (b) Comparison of gut bacterial communities in frogs between H (Healthy) and NH (Unhealthy). (c) CIRCOS plots depict the relative abundance of soil bacteria at the phylum level. (d) Samples grouped by frog habitats (AS, NS, SS) were analyzed using Kruskal-Wallis rank sum tests. Only differentially abundant genera are displayed, and asterisks indicate significant differences between groups.

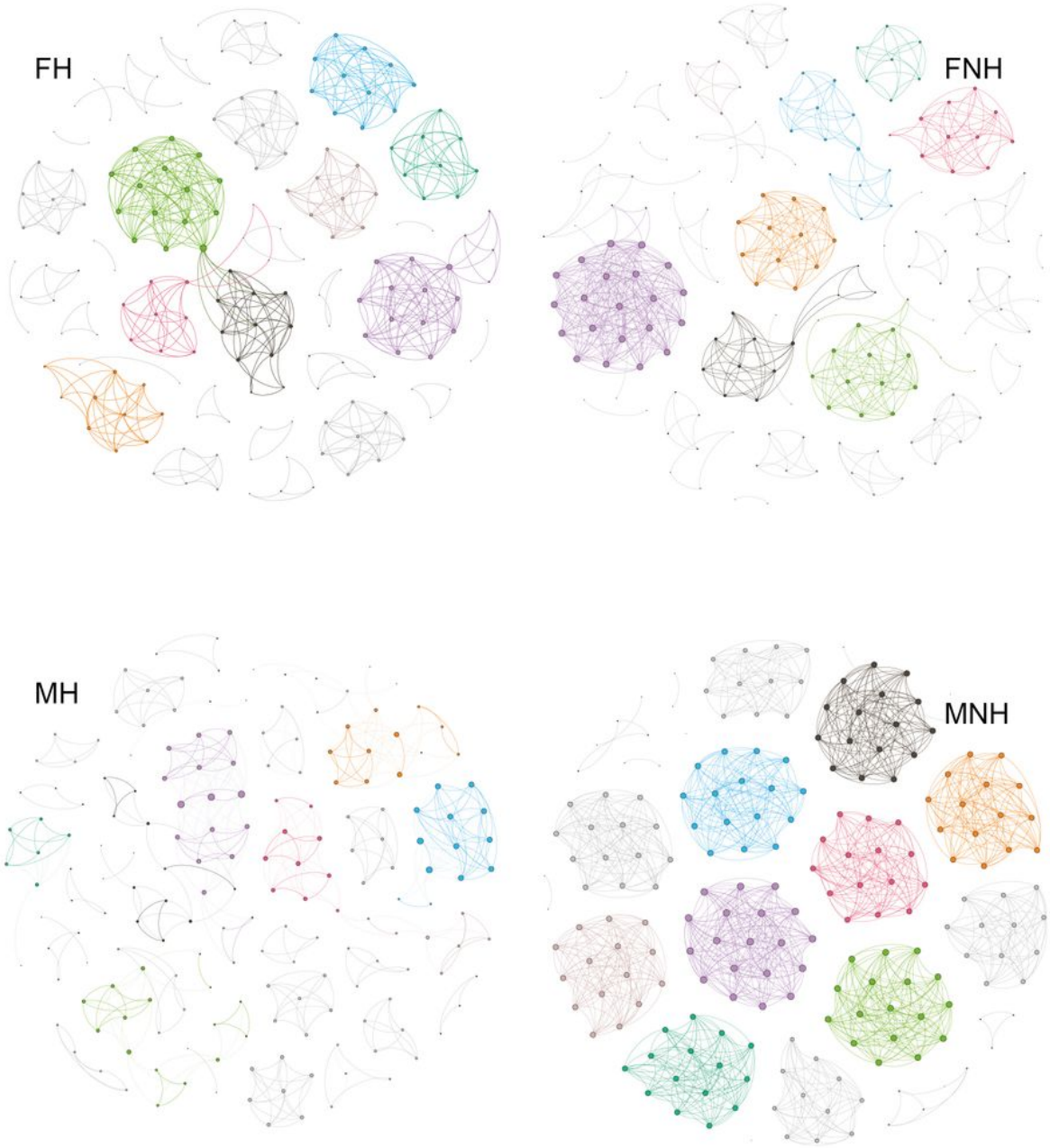

Figure 4

Co-occurrence Networks of Gut Bacterial Community: Networks are based on pairwise Spearman's correlations among abundant taxa (relative abundances of ASV > 0.01%). Each displayed connection has a correlation coefficient > |0.9| and a P value < 0.01. Node size is proportional to relative abundance. The network for frog gut samples with ASVs colored by modularity.

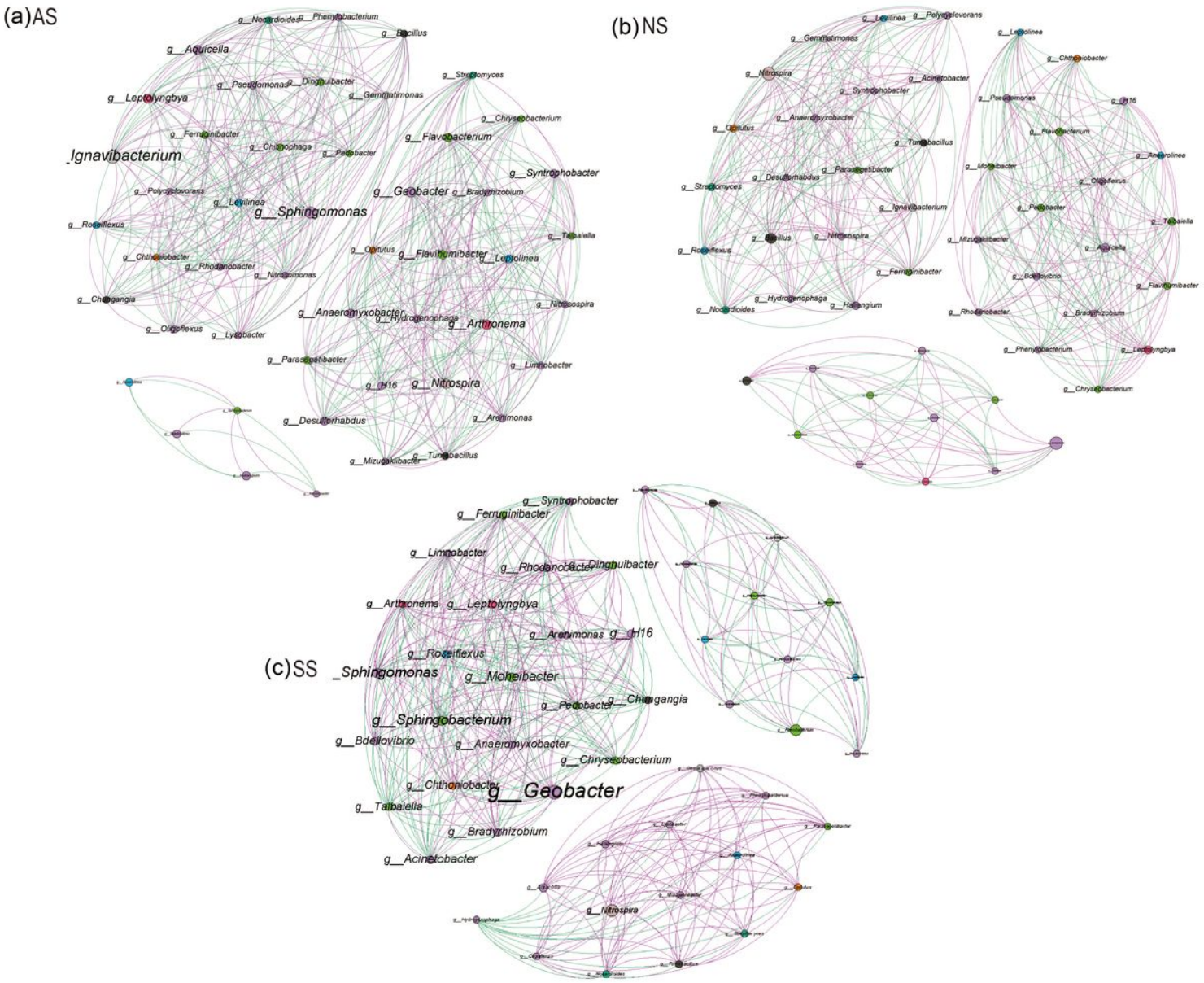

Figure 5

Bacterial Co-occurrence Networks in Frog Captive Fields: Networks are generated for AS, NS, and SS treatments based on correlation analysis. Connections represent strong (Spearman's P > 0.9) and significant (P value < 0.01) correlations for different treatments. Node names denote taxa at the bacterial genus level, with node size proportional to the number of links (degree) and colored by bacterial phyla. Networks consist of closely related bacterial modules to identify keystone taxa (module hubs), with node size proportional to relative abundance. Purple edges indicate positive interactions, while green edges signify negative interactions.

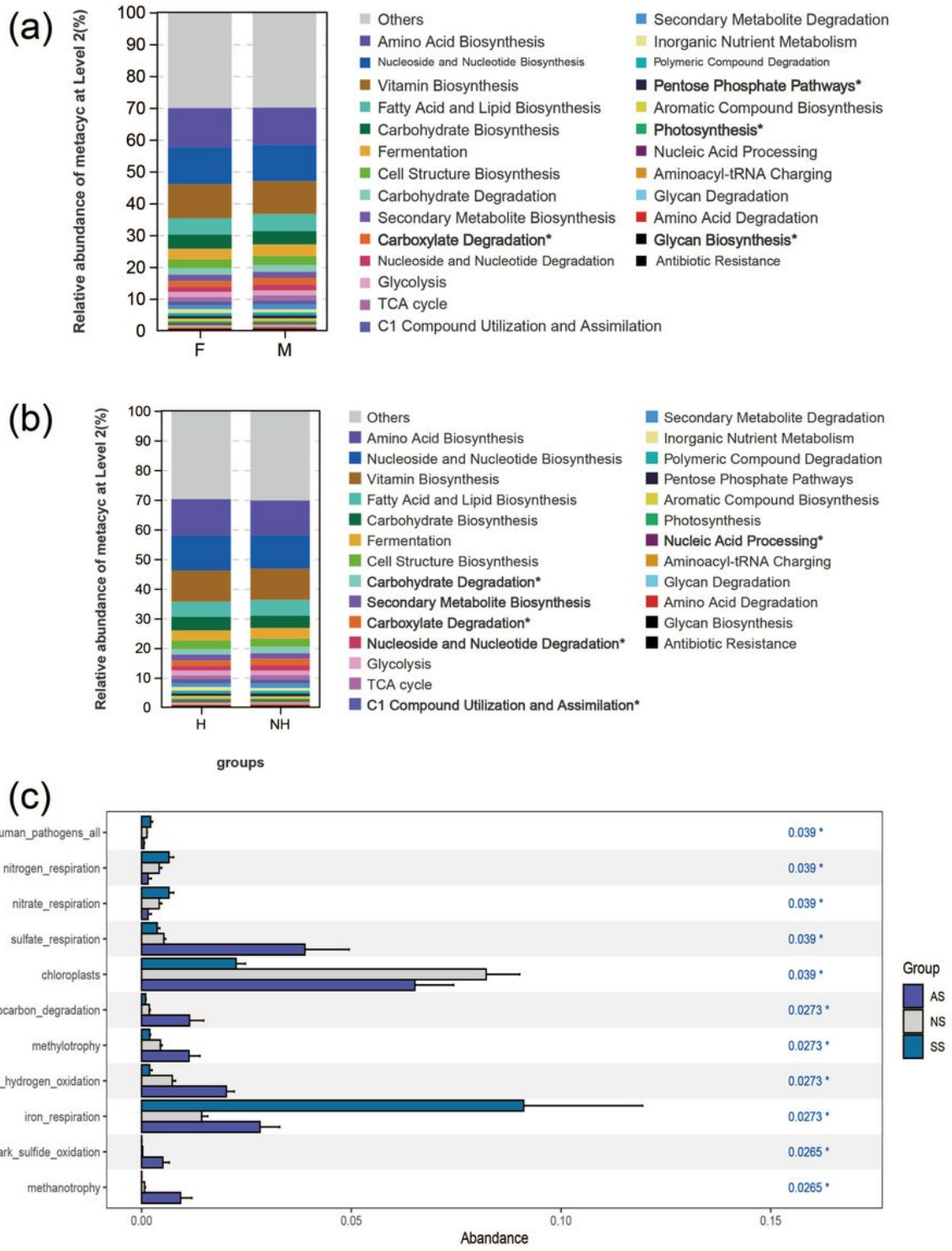

Figure 6

Predicted Gene Relative Abundances in 16S rRNA Amplicons: (a, b) Top 26 (> 0.01%) functional groups predicted by PICRUSt based on MetaCyc databases at the second level. (c) Bacterial functional groups from the FAPROTAX database to define habitat differences in soil microbiota using Kruskal-Wallis rank sum tests, with * indicating significance at 0.05 level.

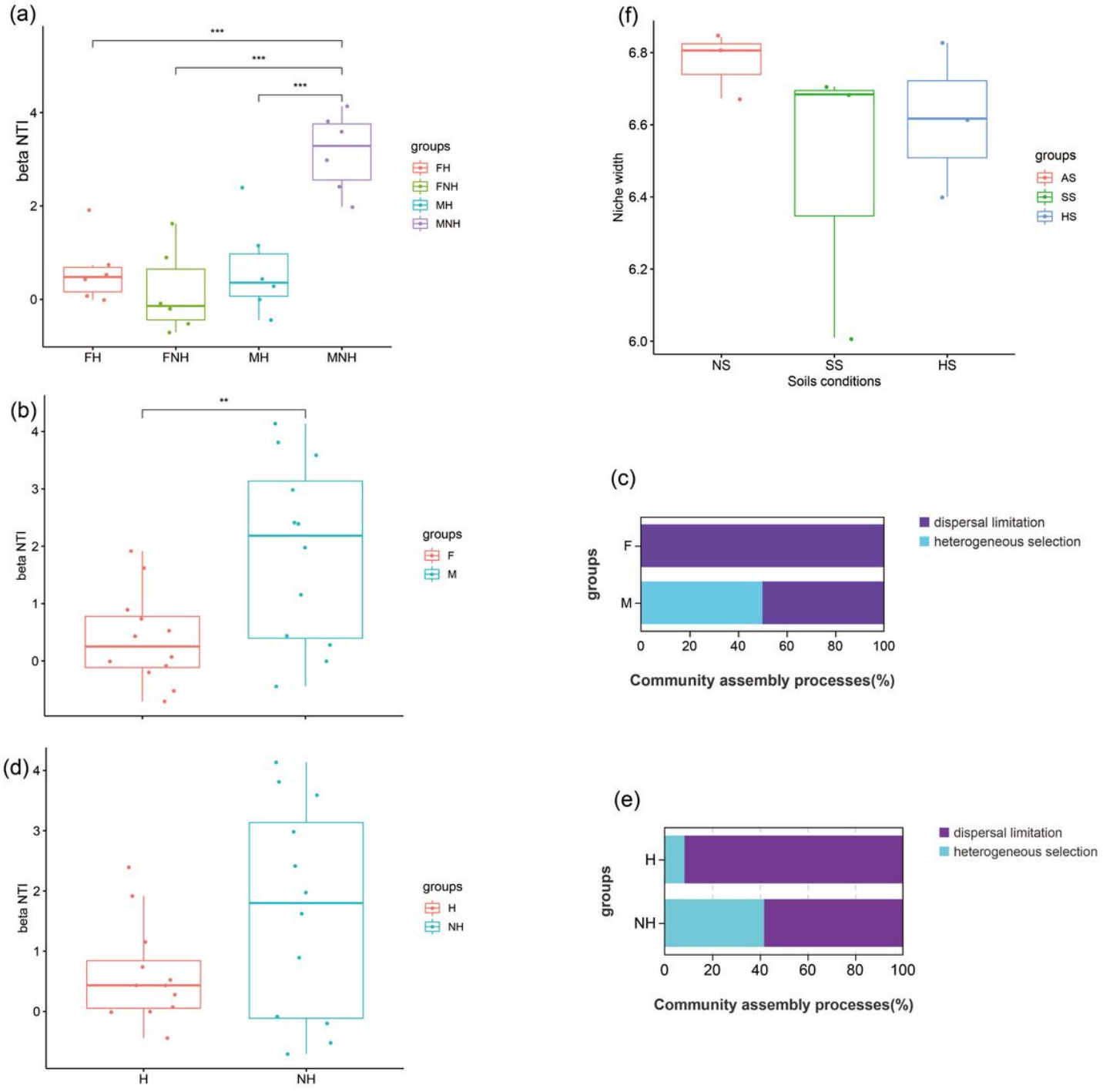

Figure 7

Bacterial Community Assembly Patterns: (a, b, d) b-Nearest Taxon Index (bNTI) of bacterial communities in all frogs, with horizontal dashed lines (bNTI values at 2 and -2) indicating significance thresholds. (c) Bray-Curtis-based Raup-Crick (RCbray) values of frog bacterial communities between F and M groups, with horizontal dashed lines at 0.95 and -0.95. Community assembly turnover is driven by various deterministic processes, including heterogeneous selection, and stochastic processes, including dispersal limitation by RCbray. (e) Comparisons between F and M groups, and H and NH groups. Boxplots depict the mean niche width (f) comparison of frog-associated soil bacteria.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Additionalfile1.doc
- Additionalfile2.xls