Impact of Helicobacter Pylori Eradication Therapy on Gastric Microbiome

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Research

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Impact of *Helicobacter pylori* eradication therapy on gastric microbiome

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Abstract

Background: *Helicobacter pylori* (Hp) eradication therapy has been used in clinical practice for many years. Yet, the effect of this therapy on existing gastric microflora has not been well understood. In this study, we explored the effect of eradication therapy on the microbial community in the stomach and the specific recovery after the successful eradication therapy.

Methods: Among the 89 included patients, 23, 17, 40, and 9 were enrolled into the Hp-negative, Hp-positive, Successful eradication, and Failed eradication groups, respectively. Four subgroups were further divided according to disease status (Hp-negative chronic gastritis [N-CG], Hp-negative atrophic gastritis [N-AG], successful-eradication chronic gastritis [SE-CG], and atrophic gastritis with successful eradication [SE-AG]). During the

endoscopic examination, one piece of gastric mucosa tissue was obtained from the lesser curvature side of the gastric antrum and gastric corpus, respectively. 16S rRNA gene sequencing was used to analyze the gastric mucosal microbiome.

Results: In Hp negative group, the gastric microbiota was dominated by five phyla: *Firmicutes*, *Proteobacteria*, *Actinobacteria*, *Bacteroidetes,* and *Fusobacteria*. Two Hp-related genera were selected as potential biomarkers: *Curvibacter* and *Acinetobacter*. After successfully eradicating Hp, the bacterial flora in the stomach recovered to a considerable extent, and the failure of eradication was almost unchanged compared with Hp positive subjects. SE-CG was characterized by an increase in *Firmicutes* taxa and a decrease in *Proteobacteria* taxa compared with N-CG. SE-AG was characterized by a decrease in *Firmicutes* relative to N-AG. Finally, no differences were found in pairwise comparisons of nitrate and nitrite reductase functions among the four subgroups.

Conclusions: After Hp infection, the diversity and relative abundance of gastric microflora were significantly decreased. Yet, gastric microbiota could be partially restored to the Hp-negative status after eradication; however, this effect was incomplete and might contribute to the long-term risks.

Key Words: 16S rRNA gene sequencing; Helicobacter pylori; Eradication therapy; Gastric microflora; Atrophic gastritis

1. Introduction

The gastrointestinal microecological balance has an important role in digestion, absorption, metabolism, immunity, and inhibition of pathogen colonization. The disorder of its structure or function can lead to many diseases. *Helicobacter pylori* (Hp) is the most important and most studied microorganism in the stomach. It has been associated with various gastrointestinal diseases, such as chronic gastritis, peptic ulcer, gastric mucosa-associated lymphoid tissue lymphoma, and gastric cancer. Numerous clinical studies have shown that Hp eradication reduces the incidence of gastric cancer, and this benefit becomes more pronounced with increasing age [1-3]. Currently, eradication therapy has been used in many regions to prevent the development of stomach cancer [4, 5].

The core of Hp eradication treatment is the acid-suppressive effect of PPIs and the bactericidal effect of antibiotics. Antibiotics have a direct and strong effect on the bacteria in the stomach [6]. The strong acid inhibitory effect of PPIs can sharply increase the stomach's pH value, thereby reducing gastric acid's effect on the removal of guest bacteria, which is not conducive to digestion and leads to various changes in substrate levels [7, 8]. Combined with existing research, the drug itself and Hp's elimination have a potential effect on the gastric flora [9, 10].

Through molecular methods, the main phyla detected in the stomach are *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, *Actinobacteria,* and *Fusobacteria*. The most abundant phyla after Hp infection in the stomach are *Proteobacteria*, *Firmicutes,* and *Actinobacteria* [11]. The gastric cancer model study of INS-GAS mice showed that the non-Hp flora could promote the occurrence of tumors [12, 13]. Other evidence in clinical research revealed that microbial diversity changes with the health of the gastric mucosal epithelium [14, 15]. Researches on the gastric cancer flora found that Niche-specific microbial networks may reflect the disease-specific microenvironment, and disease-associated bacteria can form a cooperative network, which additionally contributes to the disease [16, 17]. In addition, previous research has mainly focused on the gastric microbiome of patients with gastric cancers rather than precancerous lesions such as gastritis atrophy (AG). In this study, we analyzed the

influence of eradication treatment on gastric flora and evaluated patients' recovery with successful eradication under different mucosal states.

2. Methods

2.1 Patients and Samples

A total of 151 gastric biopsy tissues of different anatomical sites were obtained from 89 patients from The First Affiliated Hospital of Zhejiang Chinese Medical University, Hangzhou, China. Patients undergoing upper gastrointestinal endoscopy for physical examination or dyspepsia were included in this study. During the endoscopic examination, one piece of gastric mucosa tissue was obtained from the antrum's lesser curvature side and another piece from the lesser curvature side of the corpus. Each specimen was placed in a separate sterile cryopreservation tube. Another mucosal tissue of the gastric antrum was used for histological biopsy to assess gastric mucosa and Hp infection status.

Exclusion criteria were as follows: (1) patients who have taken proton pump inhibitors, H2 receptor antagonists or other antacids, probiotics, mucosal protective agents or antibiotics within recent four weeks; (2) history of gastric adenoma, gastric cancer, or mucosa-associated lymphoid tissue lymphoma; (3) patients who underwent gastrectomy; (4) patients who underwent Hp eradication therapy and were again Hp positive.

Current Hp infection was defined as a positive result from one of the following three tests: (1) C-urea breath test, (2) histologic examination, (3) Hp culture. Furthermore, according to some previous studies, those samples with < 1% of Hp relative abundance were excluded from the analysis to obtain higher representativeness [16, 18]. For patients with a history of eradication, we selected those with a completion time of one year. We combined past and current Hp infection status to confirm eradication situation. Only those whose gastric mucosa status was judged by endoscopy, which was further confirmed by pathological biopsy results, were classified into subgroups.

After confirming Hp infection status and Hp-eradication status, patients who met one of the following criteria were enrolled and analyzed: (a) group N (Hp-negative), (b) group P (Hp-positive), (c) group SE (Hp-successful eradication), (d) group FE (Hp-failed eradication). Then based on disease status, group N and group SE were divided into four subgroups: Hp-negative chronic gastritis [N-CG], Hp-negative gastritis atrophy [N-AG], successful-eradication chronic gastritis [SE-CG], and successful-eradication gastritis atrophy [SE-AG]).

The demographic details corresponding to all samples enrolled in the final analysis are compiled in **Table 1**.

Table 1. Summary of the study subjects' characteristics.

Characteristics	Age years mean±SD	Sex Female/Male
All patients (n=89)	52.73±14.05	45/44
Negative (n=23)	53.48±13.44	13/10
N-CG (n=14)	46.57±11.76	8/6
N-AG (n=6)	64.67±8.80	2/4
Positive (n=17)	41.24±12.62	9/8
Successful (n=40)	58.23±10.94	19/21
SE-CG (n=8)	49.25±9.91	7/1
SE-AG (n=29)	62.07±8.54	9/20
Failed (n=9)	48.11±17.77	4/5

2.2 Analysis and Testing Process

2.2.1 Extraction of Bacterial DNA

DNA from different samples was extracted using the E.Z.N.A.® Stool DNA Kit (D4015, Omega, Inc., USA) according to manufacturer ’s instructions. Polymerase chain reaction (PCR) was used to amplify the 16S rRNA V3-V4 region, using the 341F 5'-CCTACGGGNGGCWGCAG-3' and 805R 5'-GACTACHVGGGTATCTAATCC-3' primers. The 5' ends of the primers were tagged with specific barcodes per sample and sequencing

universal primers. Thermocycler settings were as follows: 98 °C for 30 seconds; 32cycles of denaturation at 98 °C for 10 seconds, annealing at 54 °C for 30 seconds, and extension at 72 °C for 45 seconds; final extension at 72 °C for 10 minutes. PCR amplification was performed in a total volume of 25 μL reaction mixture containing 2.5 μL of each primer, 12.5 μL PCR Premix, 25 ng of template DNA, and PCR-grade water to adjust the volume. The PCR products were confirmed via 2% agarose gel electrophoresis followed by purification with AMPure XT beads (Beckman Coulter Genomics, Danvers, MA, USA) and quantified by Qubit (Invitrogen, USA). When the amplicon pools were prepared for sequencing, Agilent 2100 Bioanalyzer (Agilent, USA) and the Library Quantification Kit for Illumina (Kapa Biosciences, Woburn, MA, USA) were used to assess the size and quantity of the amplicon library, respectively. The libraries were then sequenced on the NovaSeq PE250 platform.

2.2.2. Data Processing

Samples were sequenced on an Illumina NovaSeq platform according to the manufacturer's recommendations, provided by LC-Bio. Using unique barcode, paired-end reads were assigned to samples and truncated by cutting off the barcode and primer sequence. FLASH was used to merge these paired-end reads. Quality filtering on the raw reads was performed under specific filtering conditions to obtain high-quality clean tags according to the fqtrim (v0.94). Vsearch software (v2.3.4) was used to filter chimeric sequences. After dereplication using DADA2, the feature table and feature sequence were obtained. Next, we calculated alpha diversity and beta diversity by random normalization to the same sequences. Feature abundance was normalized using the relative abundance of each sample, according to SILVA (release 132) classifier. Alpha diversity and beta diversity were calculated by QIIME2. All diagrams were implemented using the R package (v3.5.0). The blast was used for sequence alignment, and the feature sequences were annotated with the SILVA database for each representative sequence.

2.2.3. Detection of Differential Taxa and Prediction of Metagenomic Functions

A linear discriminant analysis (LDA) and effect size (LEfSe) was performed to determine

important bacterial taxa in the comparison group.

Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2 (PICRUSt2) program (https://github.com/picrust/picrust2) was used to infer the metagenome functional content based on the microbial community profiles obtained from the 16S rRNA gene sequences. Predicted functional genes were categorized into Kyoto Encyclopedia of Genes and Genome (KEGG) orthology (KO).

2.3. Statistical Analysis

Quantitative variables were performed by the Mann-Whitney test. LEfSe analysis used a Kruskal-Wallis test and Wilcoxon test. Predicted KO functions were analyzed in STAMP using the two-group comparison with White's non-parametric t-test and corrected for multiple tests with Benjamini-Hochberg's false discovery rate. All p values were bilateral; a P-value of < 0.05 was considered statistically significant.

3. Results

3.1. Gastric antrum versus corpus mucosa

To evaluate alterations in the microbiota structure between the gastric antrum and corpus, we measured microbial alpha diversity and beta diversity. Alpha diversity showed a high degree of similarity (**Figure 1A**). Beta diversity revealed no significant differences between paired sample locations (ANOSIM R = -0.0131, P = 0.97, **Figure 2A**). Furthermore, LEfSe analysis (LDA>3.5) revealed no-positive results.

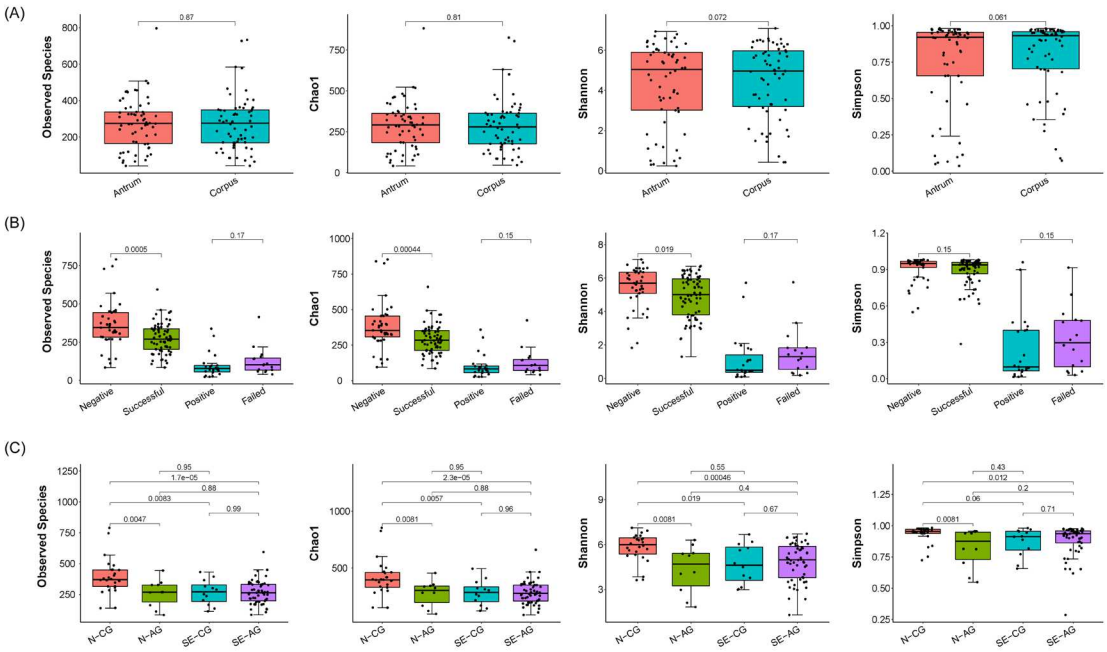


Figure 1. The Alpha diversity was evaluated and transformed into a box plot. The species diversity and complexity of the sample were analyzed by 4 indices, including Observed species, Chao1, Shannon index, and Simpson index. *(A) Boxplot in the gastric antrum and body mucosa groups.* ***(B)*** *Boxplot in 4 groups: Hp-Negative, Hp-Positive, Successful Eradication, and Failed Eradication.* ***(C)*** *Boxplot in 4 subgroups: N-CG, N-AG, SE-CG, and SE-AG. Statistical significance was determined by the Wilcoxon test.*

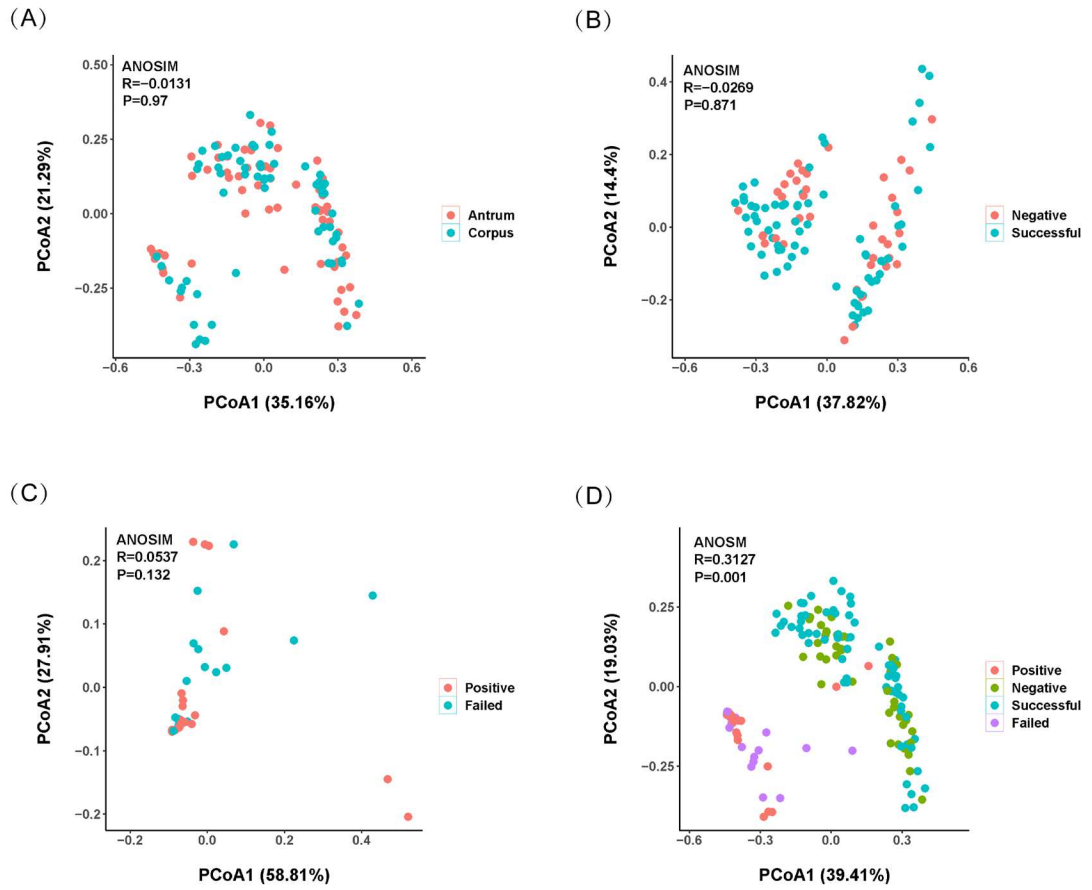


Figure 2. *Principal coordinate analysis (PCoA) plots in which samples were colored based on* ***(A)*** *paired sample location, and clinical grouping:* ***(B)*** *Hp-Negative vs. Successful-eradication;* ***(C)*** *Hp-Positive vs. Failed-eradication;* ***(D)*** *all 4 groups. ANOSIM, analysis of similarity.*

3.2. The Effect of Hp on gastric flora

In Hp negative group, the gastric microbiota was dominated by *Firmicutes* (32.95%), *Proteobacteria* (32.26%), *Actinobacteria* (11.80%), *Bacteroidetes* (8.32%), *Fusobacteria* (3.03%). At the same time, *Epsilonbacteraeota* (85.74%), *Proteobacteria* (4.31%), *Firmicutes* (3.21%), *Bacteroidetes* (2.72%), and *Actinobacteria* (1.49%) were the top five phyla in Hp positive group. According to the new classification standard, the original *Epsilonproteobacteria* (class level) is now assigned to *Epsilonbacteraeota* (phylum level), so *Helicobacter* (genus level) no longer belongs to *Proteobacteria* (phylum level)[19, 20]. Based on this change, we described *Epsilonbacteraeota* as the dominant phylum and *Helicobacter* as the dominant genus (both over 80%) in P and FE samples (**Figure 4A**).

To identify the potential biomarkers with Hp infection, we conducted LEfSe analysis between the paired groups (group N versus group P, group SE versus group FE). We selected the common bacteria at different levels through the Wilcoxon rank-sum test. *Campylobacteria*, *Campylobacterales, Helicobacteraceae,* and *Helicobacter* were predominant in class, order, family, and genus levels, respectively (**Figure 3A**). To show that relationships between disease-related taxa did not depend on differences observed in Helicobacter abundance, we performed a reanalysis subtracting *Helicobacter* readings from the data set. Most genera observed a significant decrease even if *Helicobacter* reads were removed from group P and group FE. In **Figure 3B**, group P and FE were relatively enriched for the confirmed genera *Curvibacter* and *Acinetobacter*. All potential biomarkers are shown in **Figures 3A** and **3B.**

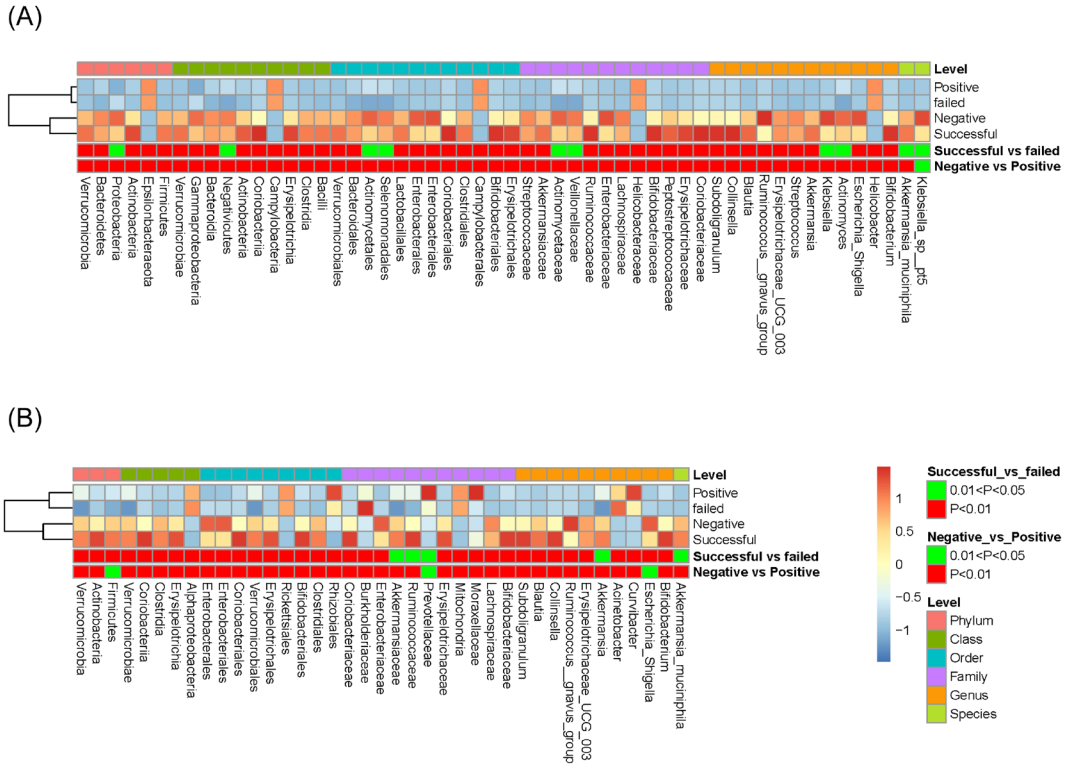
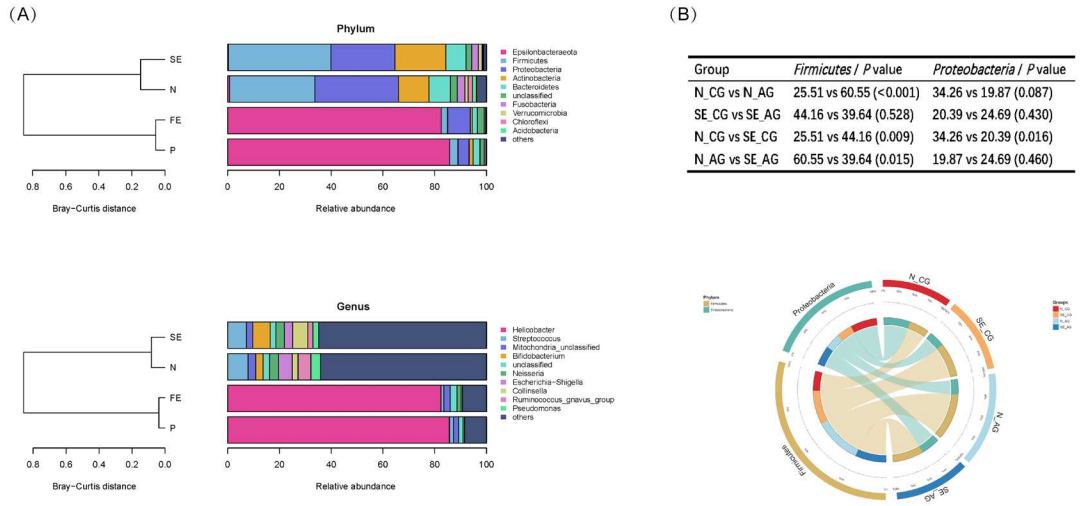


Figure 3. The Z-score is obtained by subtracting the average abundance and dividing by the standard deviation of all samples*. By converting the Z score into a heat map, the results of significant features (LDA score >3.5 and adjusted P < 0.1) were displayed, including Hp* ***(A)*** *and excluding Hp* ***(B)*** *related reads, P < 0.01 and P < 0.05 are marked in red and green, respectively.*

3.3. The Gastric Flora After Hp Eradication

First, we compared the gastric flora of N and SE samples and P and FE samples by analyzing alpha diversity and beta diversity. Despite the Simpson index having no statistical differences of N patients, a significant reduction of the observed species, Chao1, and Shannon index were found in SE patients (**Figure 1B**). No obvious differences were found between P and FE patients. Beta diversity analysis revealed no remarkable differences in microbial diversity between N and SE patients (ANOSIM R = -0.0269, P = 0.871, **Figure 2B**), as well as P and FE patients (ANOSIM R = 0.0537, P = 0.132, **Figure 2C**). When enrolling four groups together, we distinguished two pairs of groups from sample distribution (ANOSIM R = 0.3127, P = 0.001, **Figure 2D**). As the dominant phyla, the sum of the relative abundance of *Firmicutes* and *Proteobacteria* exceeded 60% in group N and group SE, and the main genera were *Streptococcus*, *Bifidobacterium*, *Escherichia-Shigella*, *Collinsella*, *Ruminococcus gnavus group*, *Neisseria*, *Pseudomonas,* and unclassified *Mitochondria* (**Figure 4A**). By comparing the bacterial composition between four groups, the bacterial composition of the group P and group FE were highly similar, as well as group N and group SE.

LEfSe analysis was used to identify the potential differentially of abundant bacterial taxa in group N and group SE. In the two groups, differences were found in 13 taxa (**Figure 5A**). Relative to N patients, SE patients exhibited preferential enrichment for *Actinobacteria*, whereas 12 bacterial taxa were preferentially depleted. Specifically, increased genera in group N included *Pseudomonas* and unclassified *Aminicenantales*. In group FE, no taxa were distinguished from group P. Overall, these results indicated that after eliminating Hp, the gastric bacterial flora could be partially restored. Lower relative abundance and richness, as mentioned in group SE, as well as reduced taxa implied that recovery might have some limits. No statistical differences were observed between group FE and group P, thus suggesting that the eradication treatment itself has little effect on the stomach flora.



Group	*Firmicutes* / *P* value	*Proteobacteria* / *P* value
N_CG vs N_AG	25.51 vs 60.55 (<0.001)	34.26 vs 19.87 (0.087)
SE_CG vs SE_AG	44.16 vs 39.64 (0.528)	20.39 vs 24.69 (0.430)
N_CG vs SE_CG	25.51 vs 44.16 (0.009)	34.26 vs 20.39 (0.016)
N_AG vs SE_AG	60.55 vs 39.64 (0.015)	19.87 vs 24.69 (0.460)

Figure 4. (A) *Top 10 relative abundance and Bray-Curtis distance of 4 groups (P, N, SE, and FE) were displayed in phylum level and genus level. Similar bacterial composition was observed between N and SE as well as P and FE. Bray-Curtis distances were used to determine the similarity of groups based on bacteria composition.* ***(B)*** *The average relative abundance of the two main phyla under different references in group N and group SE subgroups were compared, and the significance was calculated by the Mann-Whitney test (P<0.05). The average relative abundance was also shown by the Circos plot.*

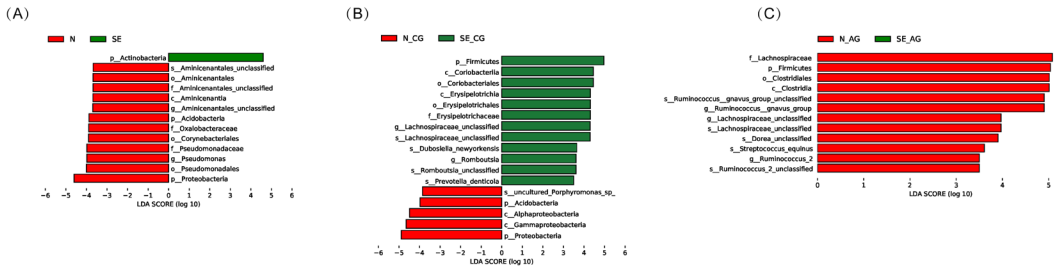


Figure 5. Association of specific microbiota taxa with the group of chronic gastritis and gastric carcinoma by LEfSe (LDA score >3.5, P<0.05). *We presented the results of the analysis between N and SE* ***(A)****, N-CG and SE-CG* ***(B)****, and N-AG and SE-AG* ***(C)****.*

3.4. The different Mucosal States are Related to Dysbacteriosis

In order to further explore the differences between CG and AG, we next analyzed four subgroups (N-CG, N-AG, SE-CG, and SE-AG). We found that N-CG had greater richness and diversity than the other three subgroups (except for Simpson's index comparing N-CG

and SE-CG, P=0.06), and there were no differences between the three subgroups (**Figure 1C**). The sum of the relative abundance of *Firmicutes* and *Proteobacteria* exceeded 50% in each subgroup. In group N, gastric mucosal atrophy showed an increase in *Firmicutes* (P<0.001, **Figure 4B**) and was accompanied by a relative decrease in *Proteobacteria* (without statistical significance, **Figure 4B**). However, the same trend was not observed in group SE (P=0.528, P=0.430, **Figure 4B**). Elevated levels of *Firmicutes* taxa: genera unclassified *Lachnospiraceae* and *Romboutsia* were detected in SE-CG patient samples, whereas *Proteobacteria* and *Acidobacteria* taxa were depleted in these samples (**Figure 5B**). Compared to N-AG, SE-AG mainly manifested as fewer *Firmicutes*, including genera *Ruminococcus gnavus group*, unclassified *Lachnospiraceae,* and *Ruminococcus 2* (**Figure 5C**). Overall, gastric microbial communities were different at high taxonomic levels when comparing chronic gastritis and atrophic gastritis separately after successful eradication, indicating that corresponding changes occurred at lower taxonomic levels as well.

3.5. Analysis of functional changes in the gastric microbiome.

To infer the metagenome functional content, we used the PICRUSt2 tool based on the microbial community profiles obtained from the 16S rRNA gene sequences. Differences in putative microbiome functionality and bacterial genera between the CG group and the AG group were identified via the LEfSe approach (LDA>3). In group N, 11 identified KEGG functions were different between N-CG and N-AG (**Figure 6A**), whereas no differential KEGG functions were found in group SE. We also evaluated the functionality of N-CG and SE-CG. As the results showed, the pathway involved in metabolism was overexpressed while the pathway involved in cell motility was inhibited in both N-AG and SE-CG relative to N-CG (**Figure 6B**). Additionally, we used correlation heatmaps to investigate the association between differential genera and KEGG pathways. Genera unclassified *Alphaproteobacteria* was positively correlated with cell motility, while genera unclassified *Lachnospiraceae* was negatively correlated with cell motility (**Figure 6A-B**). Interestingly, except for four genera (*Bifidobacterium*, *Bacillus*, unclassified *Aminicenantales*, and *Rhodococcus*), all negatively correlated genera are of the phylum *Firmicutes*, while all

positively correlated genera are of the phylum *Proteobacteria* (**Figure 6A-B**).

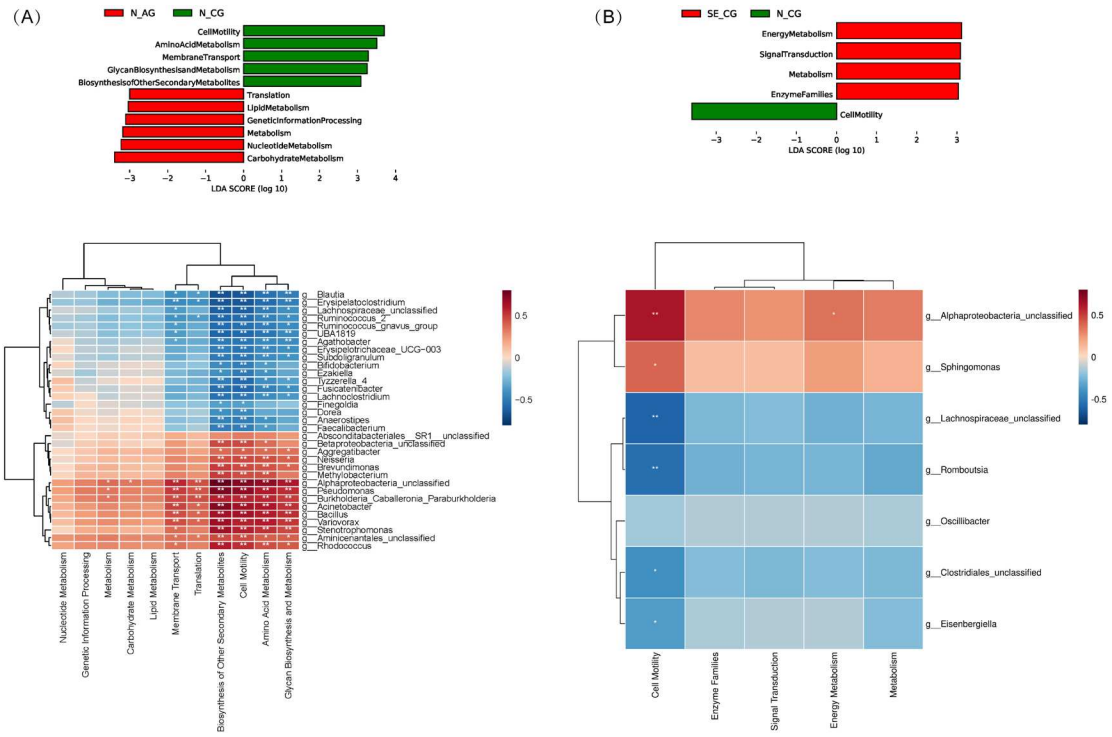


Figure 6. *Associations of microbiota with predicted KEGG functions evaluated by Spearman correlation coefficients between 33 genera and differential KEGG pathways in N-CG versus N-AG* ***(A)****, and between 7 genera and differential KEGG pathways in N-CG versus SE-CG* ***(B)****. KEGG, Kyoto encyclopedia of genes and genomes.*

The pathological changes from chronic gastritis, precancerous lesions to gastric cancer is a long process. Other non-Hp bacteria with specific functions are likely to be involved. The existing hypothesis is that nitrate-reducing bacterial species are associated with increased risks in gastric carcinoma [10]. Eradication therapy may have a potential impact on this function by changing the flora in the stomach. Therefore, we evaluated four subgroups and compared the results. Pairwise comparisons revealed that all nitrate and nitrite reductase functions had no significant differences (**Figure S1**).

4. Discussion

In the present study, we identified differential bacterial taxa and metagenomics functions before and after successful Hp's eradication. Based on our results, the bacterial composition between the paired gastric antrum and corpus was highly similar, which was consistent with previous results [21-23].

Previous research suggested that Hp had the greatest impact on gastric bacterial composition and diversity [21]. In this study, we found that the abundance and diversity of bacteria after Hp colonization were significantly lower than those of non-infected individuals. In addition, regardless of Hp infection, *Proteobacteria* was reported as the dominant bacterial group in the stomach [21, 24]. However, as the latest research no longer classifies Hp within *Proteobacteria*, *Epsilonbacteraeota* has become the most common phylum in Hp-infected patients [19, 20]. Despite this, identified genera *Curvibacter* and *Acinetobacter* that might be associated with Hp infection still belong to *Proteobacteria*. *Curvibacter,* which is a common part of oral microflora, is prevalent in patients with atherosclerotic plaques [25]. Earlier studies reported that atrophic gastritis was accompanied by a reduction in Hp colonization [26, 27]. Ofori-Darko *et al.* concluded that the OmpA-like protein from *Acinetobacter* spp. could stimulate the production of gastrin and IL-8 cytokine, which suggests they can cause gastritis or participate in the transformation towards atrophic gastritis [28]. Interestingly, *Acinetobacter* spp. might have a pathogenic role when Hp is decreased, which suggests they are not only participants but also activating factors.

The richness, diversity, and structure of the bacterial communities in the FE and P samples were highly similar. These results showed that once Hp abnormal occupied the stomach, the gastric flora was difficult to be disturbed. It also implied that Hp eradication drugs had a little long-term impact on the gastric flora. After successful eradication of Hp, the phylum and genus composition of the gastric flora could be restored to levels close to those of Hp-negative subjects, and the bacterial diversity index increased, which was consistent with previous reports [15, 21]. However, there was still a significant difference between the SE and N groups, revealing an outcome of limited recovery. We assumed these

differences might due to some irreversible changes after Hp colonization. To confirm this, we further evaluated whether gastric mucosal atrophy could affect the intragastric flora through four subgroups (N-CG, N-AG, SE-CG, SE-AG). The results showed that the richness and diversity of Hp-negative CG patients were significantly higher than those of the other subgroups, and no significant differences were observed among them (N-AG, SE-CG, and SE-AG). This further confirmed our hypothesis that regardless of gastric mucosal atrophy development, the gastric flora of patients with successful eradication was closer to N-AG. Hp often spontaneously disappears in elderly patients because of the progression of atrophic gastritis [29]. Thus, while Hp infection may initiate atrophic gastritis, it may not mediate the final transforming event. Additionally, other bacterial species may initiate these events. The bacterial driver could explain it–passenger model where Hp may initiate but not be a persisting factor during the long carcinogenic process [30]. It is possible that some irreversible changes occurred after Hp colonization. Moreover, the proportion of *Proteobacteria* and *Firmicutes* were similar in SE-CG and SE-AG groups. Thus, we speculated that Hp colonization probably accelerated the change in the flora of SE-CG patients to atrophied flora. From this perspective, patients with successful eradication still seem to be at a higher risk than the normal population.

Recent studies showed that in gastric carcinoma microbiota, increased nitrate reductase and nitrite reductase functions were considered as drivers of cancer development [17, 31, 32]. To assess this risk, we next addressed the functional features of the microbiota. However, our results did not reveal this trend in atrophy patients. We speculate that the risk of dysbacteriosis in this regard is relatively low due to the successful elimination of Hp.

To sum up, this article focused on the impact of eradication on the flora and assessment of the recovery of patients with successful eradication. Moreover, we described the effects of gastric mucosal atrophy on changes in gastric microbiota. Our study verified that in the presence of Hp, the gastric flora was quite stable and, therefore, difficult to alter by antibiotics and highly effective acid suppressants. Hp is the initiating factor and a key link in Correa's cascade [10]. Moreover, even when advanced precancerous lesions occur, the

successful removal of Hp is of great importance, especially in East Asia [33]. It seems that the risk of gastric cancer in patients with successful eradication has been greatly reduced, which has been confirmed by large-scale clinical research [34]. However, for those who already experienced precancerous lesions such as atrophy, the risk is still higher than in the normal aging stomach [35]. Consistent with this, our study reported that people who successfully eradicated Hp were closer to those with Hp-negative gastric mucosal atrophy, which represented a smaller bacterial community. Interestingly, this change may not have a profound impact.

This study has a few limitations. First, this was a single-center cross-sectional study with small sample size, especially considering those enrolled in group P and group FE. However, in this study, we implemented strict screening criteria and eliminated samples with < 1% of the Hp sequence in order to obtain higher representativeness. Secondly, we did not obtain mucosal samples from the same subject before and after Hp eradication treatment to achieve self-control. Third, the bacterial community is continuous and dynamic, so it was not possible to determine the causal relationship between these changes and different states. In addition, this study did not use PCR quantification techniques to quantify individual bacteria in different samples; thus, the analysis could only be based on the relative abundance of different bacteria. Therefore, further studies are still needed to verify and clarify the influence of eradication therapy and precancerous lesions on the gastric flora.

5. Conclusions

Hp eradication drug itself has little effect on gastric flora. After successful eradication, the gastric microenvironment could be partially restored to levels close to Hp-negative gastric mucosal atrophy patients. We also identified some biomarkers that may synergize with Hp to promote disease progression. The specific mechanisms and pathways underlying these changes will be explored in future research.

Authors' contributions

BL and LM conceived and designed the study project. LM, YZ, SW and LC did experiments and performed analysis. LM and YZ wrote the manuscript. All authors contributed at all stages and critically reviewed the content. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

Not yet uploaded to the database.

Consent for publication

Not applicable.

Ethics approval and consent to participate

The research protocol was approved by the Ethics Committee of The First Affiliated Hospital of Zhejiang Chinese Medical University. All studies were conducted in accordance with relevant guidelines and regulations. Ethics number: 2020-KL-107-02.

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References

1. Lee, Y.C., et al., *Association Between Helicobacter pylori Eradication and Gastric Cancer Incidence: A Systematic Review and Meta-analysis.* Gastroenterology, 2016. **150**(5): p. 1113-1124 e5.
2. Leung, W.K., et al., *Effects of Helicobacter pylori Treatment on Incidence of Gastric Cancer in Older Individuals.* Gastroenterology, 2018. **155**(1): p. 67-75.
3. Kumar, S., et al., *Risk Factors and Incidence of Gastric Cancer After Detection of Helicobacter pylori Infection: A Large Cohort Study.* Gastroenterology, 2020. **158**(3): p. 527-536.e7.
4. Malfertheiner, P., et al., *Management of Helicobacter pylori infection-the Maastricht V/Florence Consensus Report.* Gut, 2017. **66**(1): p. 6-30.
5. Sugano, K., et al., *Kyoto global consensus report on Helicobacter pylori gastritis.* Gut, 2015. **64**(9): p. 1353-67.
6. Rolig, A.S., et al., *The degree of Helicobacter pylori-triggered inflammation is manipulated by preinfection host microbiota.* Infect Immun, 2013. **81**(5): p. 1382-9.
7. Bruno, G., et al., *Proton pump inhibitors and dysbiosis: Current knowledge and aspects to be clarified.* World J Gastroenterol, 2019. **25**(22): p. 2706-2719.
8. Paroni Sterbini, F., et al., *Effects of Proton Pump Inhibitors on the Gastric Mucosa-Associated Microbiota in Dyspeptic Patients.* Appl Environ Microbiol, 2016. **82**(22): p. 6633-6644.
9. Li, L., et al., *The Effect of Helicobacter pylori Eradication on the Gastrointestinal Microbiota in Patients with Duodenal Ulcer.* J Gastrointestin Liver Dis, 2016. **25**(2): p. 139-46.
10. Correa, P., *Human gastric carcinogenesis: a multistep and multifactorial process--First American Cancer Society Award Lecture on Cancer Epidemiology and Prevention.* Cancer Res, 1992. **52**(24): p. 6735-40.
11. Hunt, R.H., et al., *The stomach in health and disease.* Gut, 2015. **64**(10): p. 1650-1668.
12. Lofgren, J.L., et al., *Lack of commensal flora in Helicobacter pylori-infected INS-GAS mice reduces gastritis and delays intraepithelial neoplasia.* Gastroenterology, 2011. **140**(1): p. 210-20.
13. Lertpiriyapong, K., et al., *Gastric colonisation with a restricted commensal microbiota replicates the promotion of neoplastic lesions by diverse intestinal microbiota in the Helicobacter pylori INS-GAS mouse model of gastric carcinogenesis.* Gut, 2014. **63**(1): p. 54-63.
14. Liu, X., et al., *Alterations of gastric mucosal microbiota across different stomach microhabitats in a cohort of 276 patients with gastric cancer.* EBioMedicine, 2019. **40**: p. 336-348.
15. Park, C.H., et al., *Evaluation of gastric microbiome and metagenomic function in patients with intestinal metaplasia using 16S rRNA gene sequencing.* Helicobacter, 2019. **24**(1): p. e12547.
16. Coker, O.O., et al., *Mucosal microbiome dysbiosis in gastric carcinogenesis.* Gut, 2018. **67**(6): p. 1024-1032.
17. Ferreira, R.M., et al., *Gastric microbial community profiling reveals a dysbiotic cancer-associated microbiota.* Gut, 2018. **67**(2): p. 226-236.

18. Kim, J., et al., *An Appropriate Cutoff Value for Determining the Colonization of Helicobacter pylori by the Pyrosequencing Method: Comparison with Conventional Methods.* Helicobacter, 2015. **20**(5): p. 370-80.
19. Waite, D.W., et al., *Erratum: Addendum: Comparative Genomic Analysis of the Class Epsilonproteobacteria and Proposed Reclassification to Epsilonbacteraeota (phyl. nov.).* Front Microbiol, 2018. **9**: p. 772.
20. Waite, D.W., et al., *Comparative Genomic Analysis of the Class Epsilonproteobacteria and Proposed Reclassification to Epsilonbacteraeota (phyl. nov.).* Front Microbiol, 2017. **8**: p. 682.
21. Li, T.H., et al., *Alterations in Gastric Microbiota After H. Pylori Eradication and in Different Histological Stages of Gastric Carcinogenesis.* Sci Rep, 2017. **7**: p. 44935.
22. Li, X.X., et al., *Bacterial microbiota profiling in gastritis without Helicobacter pylori infection or non-steroidal anti-inflammatory drug use.* PLoS One, 2009. **4**(11): p. e7985.
23. Bik, E.M., et al., *Molecular analysis of the bacterial microbiota in the human stomach.* Proc Natl Acad Sci U S A, 2006. **103**(3): p. 732-7.
24. Jo, H.J., et al., *Analysis of Gastric Microbiota by Pyrosequencing: Minor Role of Bacteria Other Than Helicobacter pylori in the Gastric Carcinogenesis.* Helicobacter, 2016. **21**(5): p. 364-74.
25. Ziganshina, E.E., et al., *Bacterial Communities Associated with Atherosclerotic Plaques from Russian Individuals with Atherosclerosis.* PLoS One, 2016. **11**(10): p. e0164836.
26. Kuipers, E.J., *Review article: exploring the link between Helicobacter pylori and gastric cancer.* Aliment Pharmacol Ther, 1999. **13 Suppl 1**: p. 3-11.
27. Farinati, F., et al., *Prevalence of Helicobacter pylori infection in patients with precancerous changes and gastric cancer.* Eur J Cancer Prev, 1993. **2**(4): p. 321-6.
28. Ofori-Darko, E., et al., *An OmpA-like protein from Acinetobacter spp. stimulates gastrin and interleukin-8 promoters.* Infect Immun, 2000. **68**(6): p. 3657-66.
29. Chen, S., et al., *The Prevalence of Helicobacter pylori Infection Decreases with Older Age in Atrophic Gastritis.* Gastroenterol Res Pract, 2013. **2013**: p. 494783.
30. Tjalsma, H., et al., *A bacterial driver-passenger model for colorectal cancer: beyond the usual suspects.* Nat Rev Microbiol, 2012. **10**(8): p. 575-82.
31. Chen, X.H., et al., *Mucosa-Associated Microbiota in Gastric Cancer Tissues Compared With Non-cancer Tissues.* Front Microbiol, 2019. **10**: p. 1261.
32. Wu, Z.F., et al., *A Comparison of Tumor-Associated and Non-Tumor-Associated Gastric Microbiota in Gastric Cancer Patients.* Dig Dis Sci, 2020.
33. Li, W.Q., et al., *Effects of Helicobacter pylori treatment on gastric cancer incidence and mortality in subgroups.* J Natl Cancer Inst, 2014. **106**(7).
34. Mera, R.M., et al., *Dynamics ofHelicobacter pyloriinfection as a determinant of progression of gastric precancerous lesions: 16-year follow-up of an eradication trial.* Gut, 2018. **67**(7): p. 1239-1246.
35. Wong, B.C., et al., *Helicobacter pylori eradication to prevent gastric cancer in a high-risk region of China: a randomized controlled trial.* Jama, 2004. **291**(2): p. 187-94.

Figures

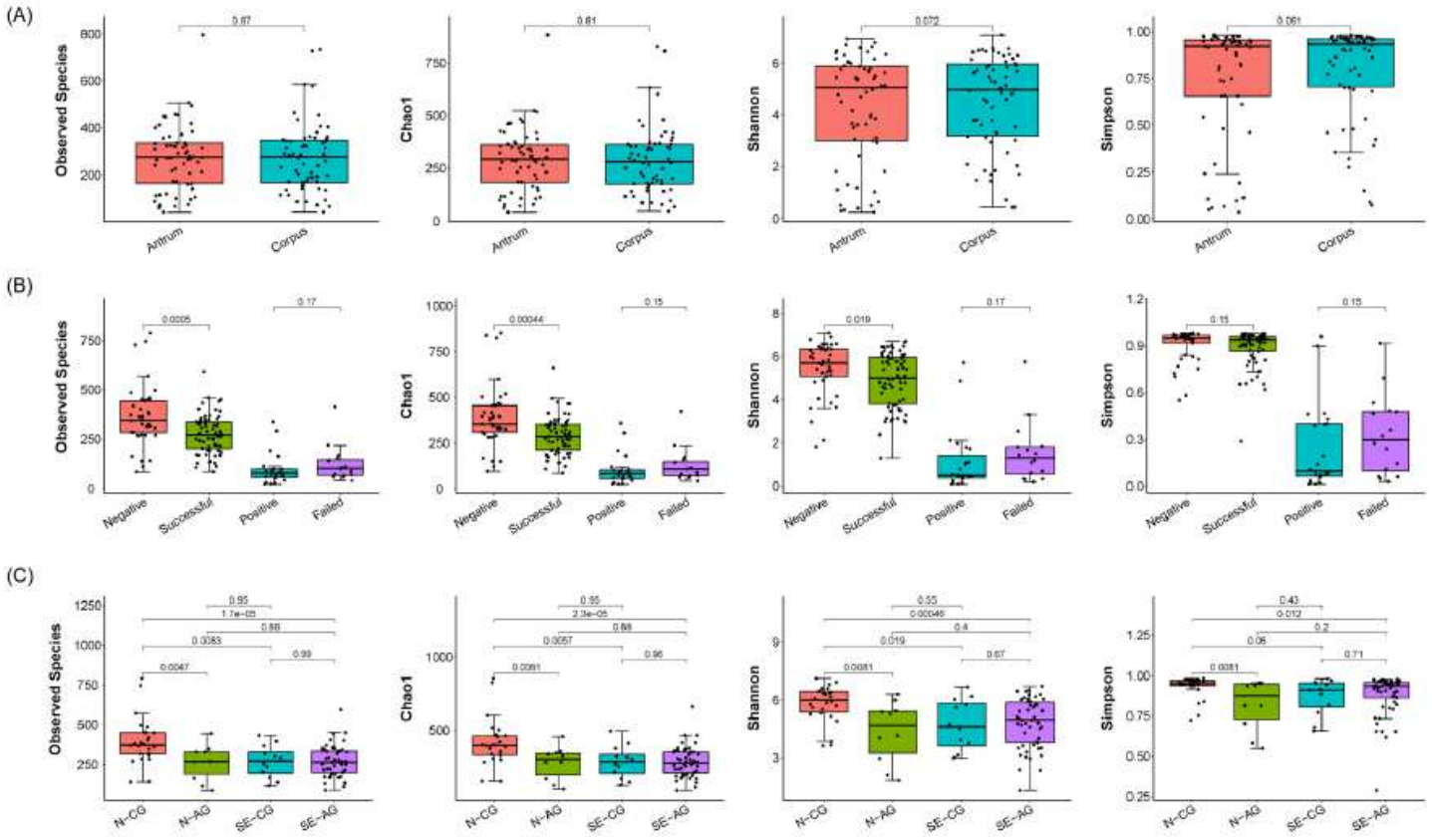


Figure 1

The Alpha diversity was evaluated and transformed into a box plot. The species diversity and complexity of the sample were analyzed by 4 indices, including Observed species, Chao1, Shannon index, and Simpson index. (A) Boxplot in the gastric antrum and body mucosa groups. (B) Boxplot in 4 groups: Hp-Negative, Hp-Positive, Successful Eradication, and Failed Eradication. (C) Boxplot in 4 subgroups: N-CG, N-AG, SE-CG, and SE-AG. Statistical significance was determined by the Wilcoxon test.

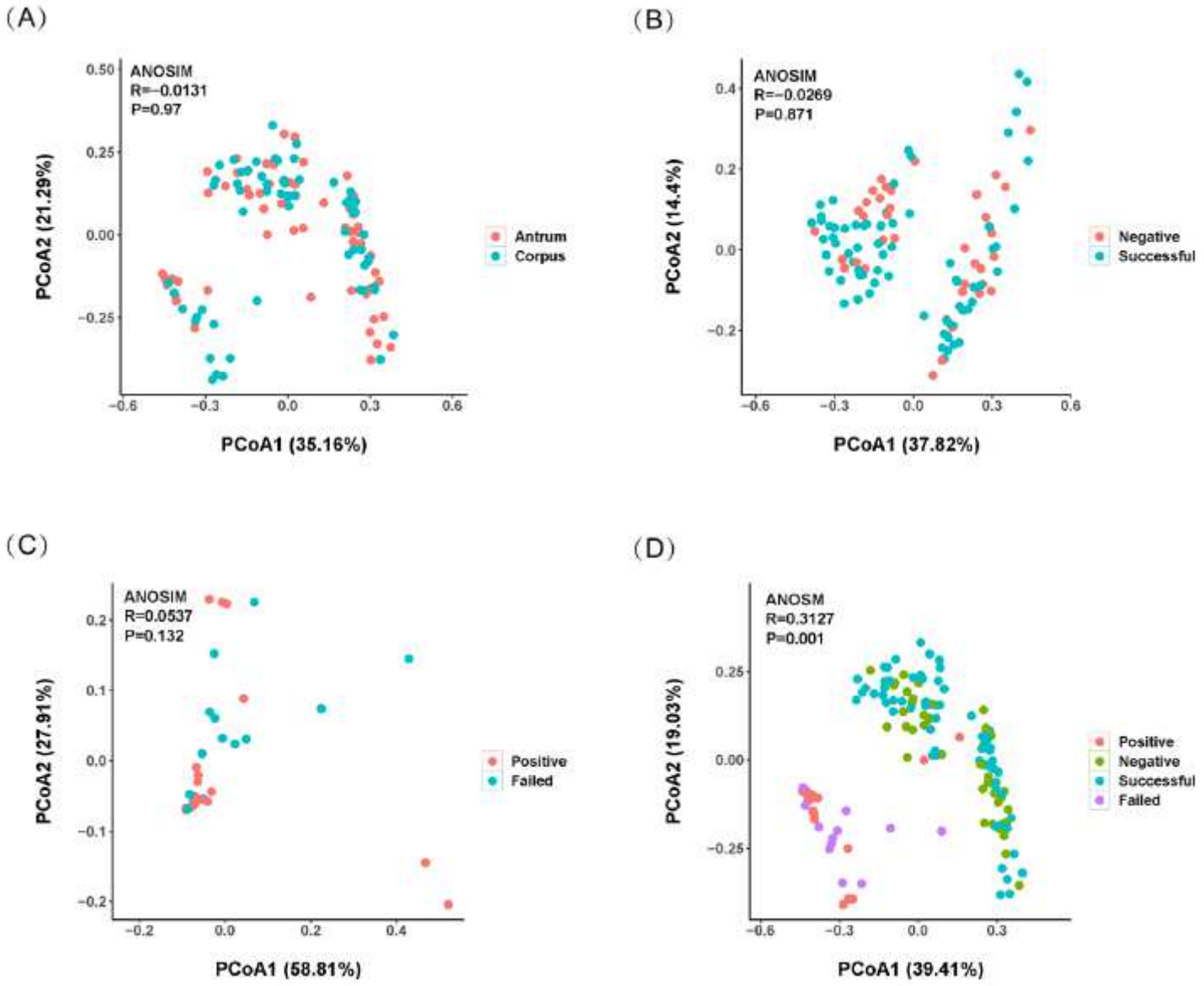


Figure 2

Principal coordinate analysis (PCoA) plots in which samples were colored based on (A) paired sample location, and clinical grouping: (B) Hp-Negative vs. Successful-eradication; (C) Hp-Positive vs. Failed-eradication; (D) all 4 groups. ANOSIM, analysis of similarity.

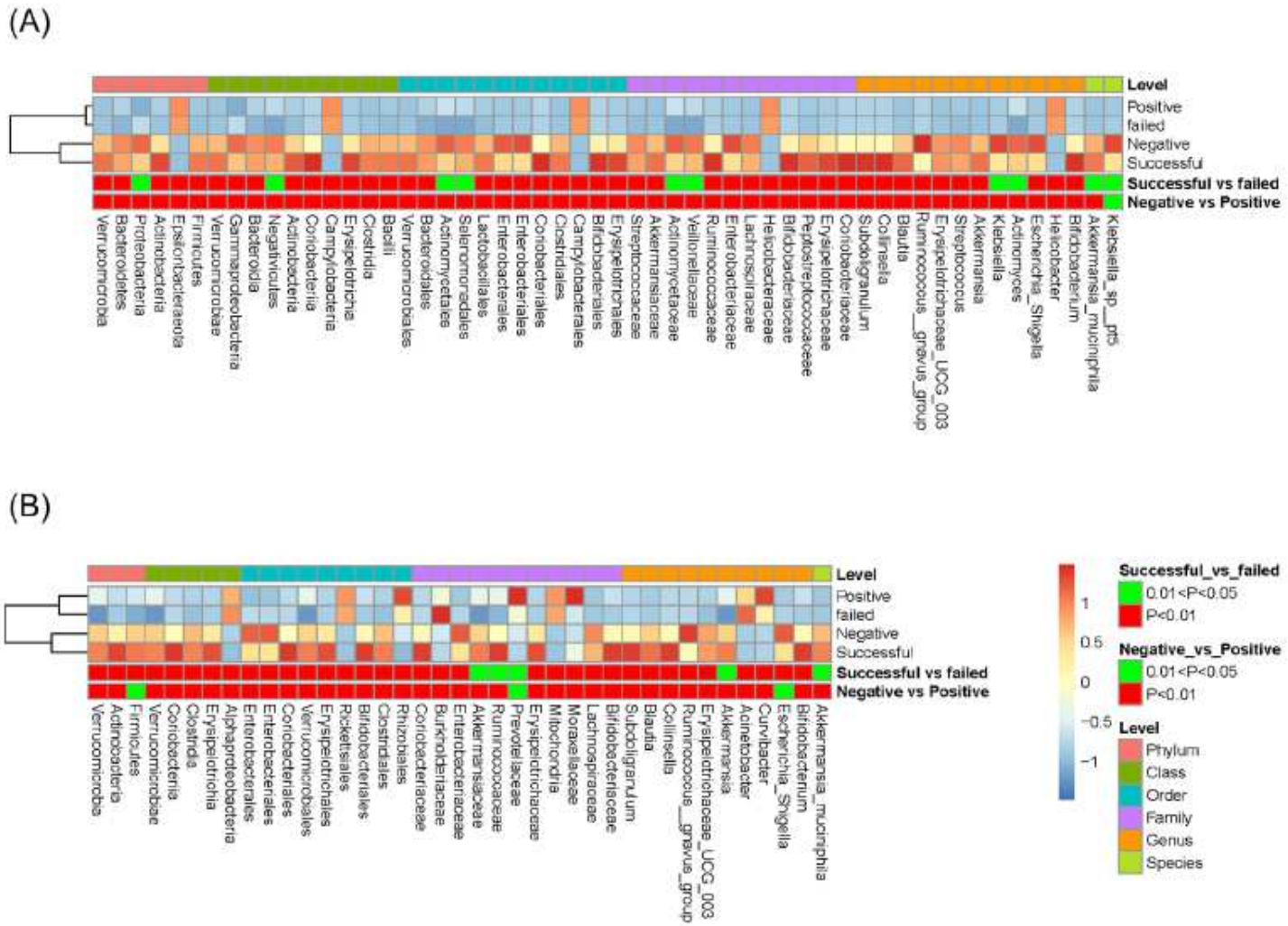
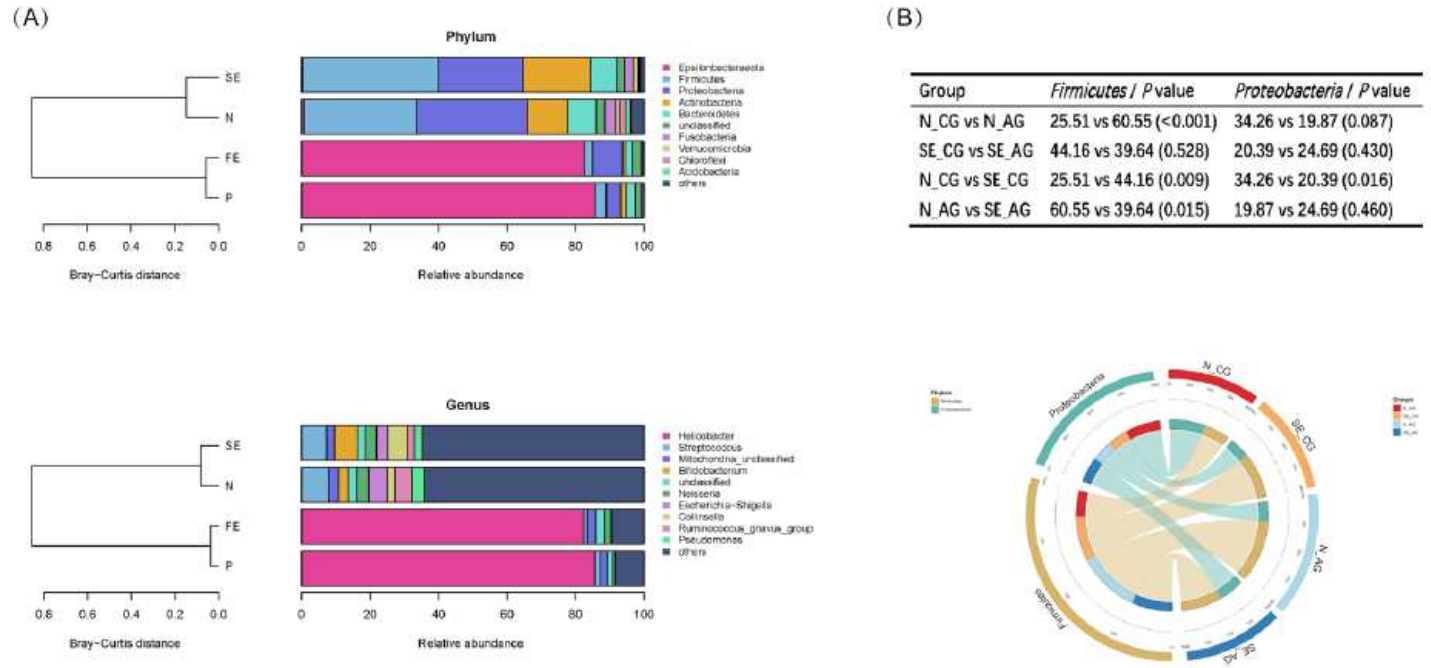


Figure 3

The Z-score is obtained by subtracting the average abundance and dividing by the standard deviation of all samples. By converting the Z score into a heat map, the results of significant features (LDA score >3.5 and adjusted P < 0.1) were displayed, including Hp (A) and excluding Hp (B) related reads, P < 0.01 and P < 0.05 are marked in red and green, respectively.



Group	*Firmicutes* / *P* value	*Proteobacteria* / *P* value
N_CG vs N_AG	25.51 vs 60.55 (<0.001)	34.26 vs 19.87 (0.087)
SE_CG vs SE_AG	44.16 vs 39.64 (0.528)	20.39 vs 24.69 (0.430)
N_CG vs SE_CG	25.51 vs 44.16 (0.009)	34.26 vs 20.39 (0.016)
N_AG vs SE_AG	60.55 vs 39.64 (0.015)	19.87 vs 24.69 (0.460)

Figure 4

(A) Top 10 relative abundance and Bray-Curtis distance of 4 groups (P, N, SE, and FE) were displayed in phylum level and genus level. Similar bacterial composition was observed between N and SE as well as P and FE. Bray-Curtis distances were used to determine the similarity of groups based on bacteria composition. (B) The average relative abundance of the two main phyla under different references in group N and group SE subgroups were compared, and the significance was calculated by the Mann-Whitney test (P<0.05). The average relative abundance was also shown by the Circos plot.

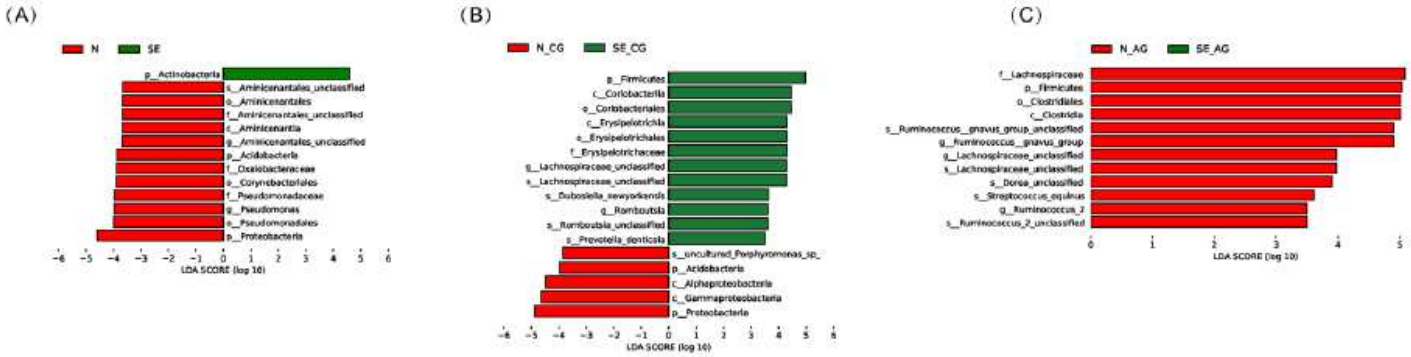


Figure 5

Association of specific microbiota taxa with the group of chronic gastritis and gastric carcinoma by LEfSe (LDA score >3.5, P<0.05). We presented the results of the analysis between N and SE (A), N-CG and SE-CG (B), and N-AG and SE-AG (C).

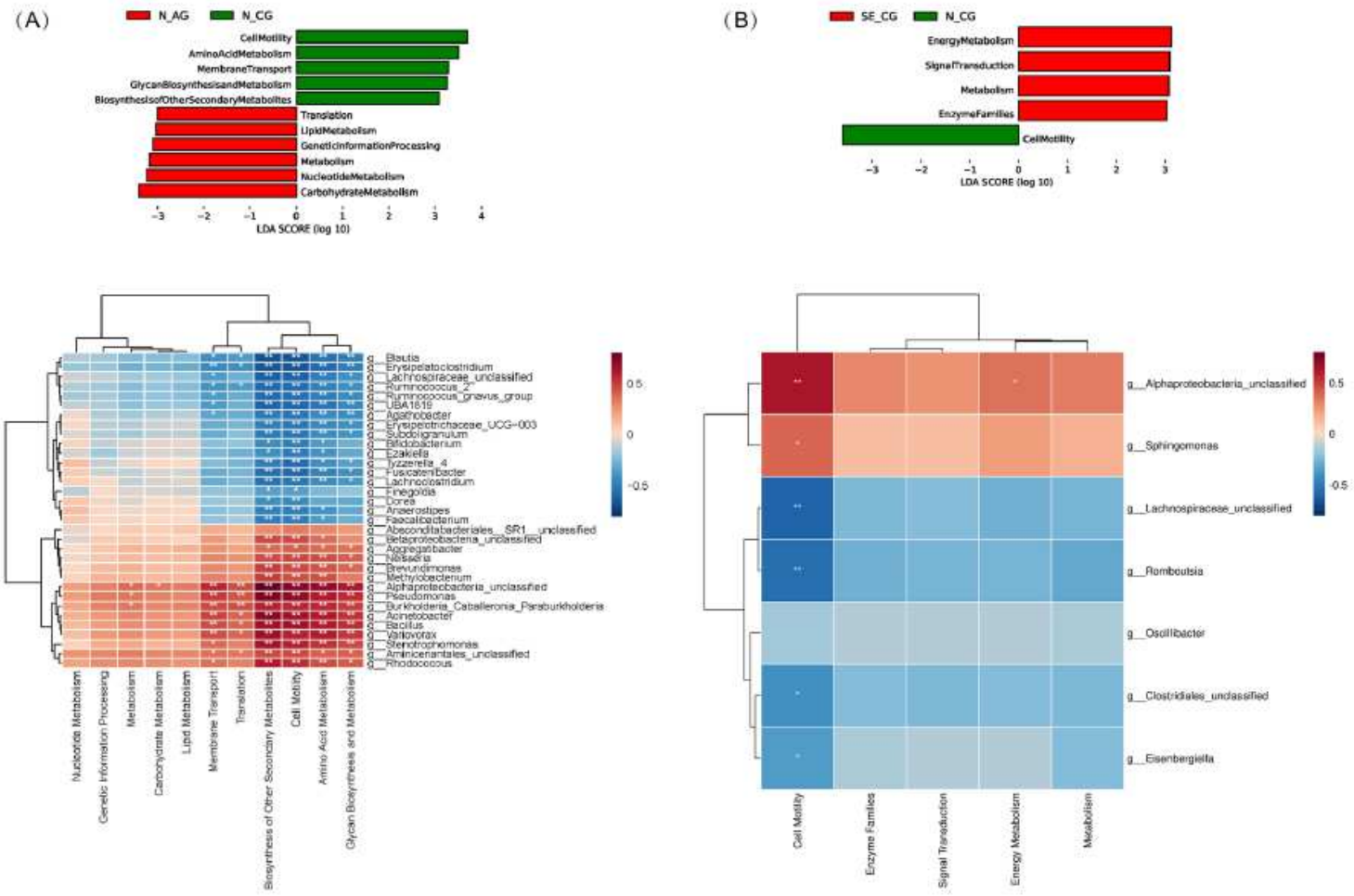


Figure 6

Associations of microbiota with predicted KEGG functions evaluated by Spearman correlation coefficients between 33 genera and differential KEGG pathways in N-CG versus N-AG (A), and between 7 genera and differential KEGG pathways in N-CG versus SE-CG (B). KEGG, Kyoto encyclopedia of genes and genomes.