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## Observational Study

Effect of gastric microbiota on quadruple *Helicobacter pylori* eradication therapy containing bismuth

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## Abstract

## BACKGROUND

*Helicobacter pylori* (*H. pylori*) is an important pathogen that can cause a variety of diseases. Yet, full eradication of *H. pylori* remains a significant challenge in clinical practice. *H. pylori* and other microbial communities have complex interactions in the unique gastric microecological environment. However, it is not clear whether the interactions have any effect on the therapeutic effect of *H. pylori*.

## AIM

The aim was to investigate the characteristics of the gastric microbiota with *H. pylori* infection and the influence on the *H. pylori* eradication treatment.

## METHODS

Patients with *H. pylori* infection underwent gastroscopy and received treatment for eradication. The prescription included esomeprazole 20 mg bid, Livzon Dele 220 mg bid, amoxicillin 1000 mg bid, and clarithromycin 500 mg bid for 14 d. Patients who did not respond to treatment and failed eradication were compared with those who achieved eradication by 1:2 propensity matching. High-throughput sequencing of the gastric mucosal microbiota was performed, and the results were evaluated by alpha diversity analysis, beta diversity analysis, species correlation analysis, and metabolic pathway correlation analysis.

## RESULTS

The eradication rate of all the patients was 95.5% (171/179). Twenty-four patients were enrolled in the study after propensity-matched scoring. There were eight cases in the failure group (patients who did not respond well to therapy) and 16 cases in the success group. The majority phyla in the two groups were the same, and included Proteobacteria, Bacteroides, Firmicutes, Actinomycetes, and

additional data are available.

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Fusobacteria. The microbial diversity in the failure group had a decreasing trend ( $P = 0.092$ ) and the species abundance was significantly lower ( $P = 0.031$ ) compared with the success group. The high rate of *H. pylori* eradication was associated with *Rhodococcus*, *Lactobacillus*, and *Sphingomonas*, as they were significantly enriched in the successful group ( $P < 0.05$ ). *Veronococcus* and *Cilium* were enriched in the mucosa of chronic atrophic gastritis patients compared with chronic superficial gastritis patients ( $P = 0.0466$  and  $0.0122$ , respectively). In both study groups, *H. pylori* was negatively correlated with other bacterial genera. More bacterial genera were directly related to *H. pylori* in the successful group compared with the failure group.

## CONCLUSION

The effectiveness of quadruple *H. pylori* eradication therapy containing bismuth depended on gastric microbiota, and the high rate of *H. pylori* eradication was associated with the presence of *Rhodococcus*, *Lactobacillus*, and *Sphingomonas*.

**Key Words:** *Helicobacter pylori*; Eradication; Quadruple therapy; Influence factors; propensity matching; Gastric microbiota

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**Core Tip:** *Helicobacter pylori* (*H. pylori*) is an important pathogen that can cause a variety of diseases. Its eradication can be affected by many factors. In this study, we explored the effect of the gastric microbiota on quadruple *H. pylori* eradication therapy containing bismuth. The results indicated that quadruple *H. pylori* eradication therapy containing bismuth was affected by the gastric microbiota. A high rate of *H. pylori* eradication was associated with the presence of *Rhodococcus*, *Lactobacillus*, and *Sphingomonas*. Our findings may provide the basis for clinical treatment.

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## INTRODUCTION

The gastric mucosal environment was long thought to be sterile. Yet, the discovery of *Helicobacter pylori* (*H. pylori*) put an end to the traditional view of the sterile stomach [1]. Furthermore, the development of modern technology led to a deeper understanding of gastric microbiota. Numerous gastric microbiota have been discovered over the years by 16S rRNA sequencing[2]. *H. pylori* and other microbial communities have complex interactions in the unique gastric microecological environment. *H. pylori* can inhibit other microbial communities by inducing the production of cytokines and antimicrobial peptides[3]. On the contrary, other microbial communities can affect *H. pylori*. For example, *Streptococcus* can change the spiral form of *H. pylori* into a spherical shape and inhibit its growth[4].

*H. pylori* is an important pathogen associated with a variety of diseases[5-7] including gastric cancer[8]. About 50% of the global population is infected with *H. pylori*[9]. *H. pylori* infection is an infectious disease and a "screening-treatment" strategy has been recommended for the treatment of *H. pylori*. However, the eradication of *H. pylori* can be affected by many factors, including antibiotic resistance[10], medication compliance[11,12], virulence factors[13], and others. In this study, we investigated the characteristics of the gastric microbiota in patients with *H. pylori* infection and the effect of the gastric microbiota on the success of quadruple *H. pylori* eradication therapy containing bismuth.

## MATERIALS AND METHODS

### Participants

Patients diagnosed with *H. pylori* infection in the Gastroenterology Department of Peking University Third Hospital between were enrolled between July 2018 and July 2019. Patients who were (1) 18-70 years of age; (2) with *H. pylori* infection confirmed by gastroscopy and histopathology were eligible. Patients (1) with previous *H. pylori* eradication therapy; (2) using proton pump inhibitors, H<sub>2</sub> receptor blockers, bismuth, antibiotics, or other drugs that might affect the study results within 4 wk of inclusion; (3) with gastrointestinal tumors; (4) with a history of gastric or esophageal surgery; (5) with Zollinger-Ellison syndrome; (6) with abnormal liver or kidney function; (7) with severe cardiovascular, respiratory, blood, endocrine, neurological, or mental disease; (8) with allergy to a study drug; (9) were pregnant or lactating; and (10) or with histories of alcohol abuse or clinical conditions that might increase the risk of side effects were excluded.

### Methods

Before inclusion, all patients provided informed consent for clinical sample collection. A biopsy was taken from the antrum before *H. pylori* eradication treatment. A rapid urease test (RUT) was performed during the gastroscopy, and if the result was positive, gastric mucosa biopsies from the lesser curvature of antrum and corpus were collected, placed in a cryovial, and stored -80 °C. At the same time, mucosa specimens were collected from the gastric antrum and corpus were collected for histopathological examination and Warthin-Starry (WS) staining.

Patients diagnosed with *H. pylori* infection by positive RUT results and WS staining were treated with esomeprazole 20 mg bid, amoxicillin 1000 mg bid, clarithromycin 500 mg bid, Livzon Dele bismuth potassium citrate 220 mg bid for 14 d. A <sup>13</sup>C urease breath test (<sup>13</sup>C-UBT) was performed 8 wk after treatment, which was considered successful if the <sup>13</sup>C-UBT was negative.

Patients were divided into failure and the success groups after their treatment was completed. Patients who did not respond well to therapy were included in the failure group. The success group was evaluated by nearest-neighbor matching, which is a type of propensity score matching and paired with patients in the failure group who had a similar propensity index. The propensity index was estimated by the model so as to equalize the covariates between the two groups. Gender, age, body-mass index (BMI kg/m<sup>2</sup>), gastroscopy diagnosis, and background gastric mucosa were the covariables used to calculate the propensity values. Taking the sample size and matching quality, the allowable error was set to 0.1. The failure and success groups were matched at a ratio of 1:2. The gastric mucosa microbiota were assayed and compared according to the results of propensity score matching.

### Microbial diversity sequencing

The total DNA of the microbiota was extracted following the instructions with E.Z.N.A.<sup>®</sup> soil DNA kits (Omega Bio-Tek, Norcross, GA, United States) following the manufacturer's instructions, and the quality of DNA extraction was assayed by 1% agarose gel electrophoresis. DNA concentration and purity were determined by a NanoDrop 2000 spectrophotometer, and 338 F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') primers were used for PCR amplification of the V3-V4 region of the 16S rRNA gene. The PCR products were recovered on 2% agarose gels after combining the PCR products of the same sample. Recovered products were purified with AxyPrep DNA gel extraction kits (Axygen Biosciences, Union City, CA, United States) and assayed with a Quantus<sup>™</sup> Fluorometer (Promega, United States). The library was built with NEXTFLEX Rapid DNA-Seq Kits and sequenced with a Miseq PE300 platform (Illumina).

### Sequence data analysis

Fastp software was used for quality control of the original sequence, and Flash software was used for stitching. Usearch software (version 7.0, <http://drive5.com/uparse/>) was used to for operational taxonomic unit (OTU) clustering of sequences based on 97% similarity and elimination of chimeras. RDP classifier (<http://rdp.cme.msu.edu/>) was used to compare each sequence with the sequences included in Silva database (SSU132). The threshold was set to 70%, and the results of species classification annotations were obtained. The sample species composition was analyzed based on the annotation results.

The alpha diversity analysis (*i.e.* within-sample diversity) reflected species diversity, including Shannon, ace, and coverage indices. Beta diversity analysis (*i.e.* diversity between samples) or between-group differences in species composition was by principal coordinates analysis (PCoA). The Wilcoxon rank-sum test was used to compare the two groups. A *P* value of < 0.05 was considered significant. Correlation analysis of species was carried out by correlation heatmaps, network analysis, and metabolic pathway analysis. The relationship between the microbiota and the result of *H. pylori* eradication was preliminarily explained.

### Statistical analysis

SPSS 23.0 was used for data analysis. measurement data were reported as means  $\pm$  SD and compared by *t*-tests. Categorical data were compared by  $\chi^2$  tests *P* values of < 0.05 were considered statistically significant.

## RESULTS

### Basic information

Of the 179 enrolled patients, 171 responded well to therapy with successful *H. pylori* eradication. Eight patients failed treatment. The eradication therapy success rate was 95.5%. Propensity scoring resulted in matching eight failed cases with 16 successful cases. The results of gastroscopy revealed chronic gastritis in all patients; and after matching, there were no significant differences in the baseline characteristics between the two groups (Table 1).

A total of 1 204 878 reads and 1028 OTUs were obtained from 24 samples. The samples contained a mean of 50 203 reads and 191 OTUs. The reads in the failed eradication group (58 487) were significantly higher (*P* = 0.013) than those in the successful eradication group (46 061); the difference between the OTUs in the two groups was not significant (166 and 203, respectively, *P* = 0.719). The samples were randomly flattened according to the minimum number of sample sequences to avoid analysis deviation. A total of 980 OTUs were obtained, and each sample contained 30 043 reads. All samples were dominated by *H. pylori* at the genus level; *H. pylori* infection was pathologically confirmed.

### Analysis of microbiota composition and differences

The proportions of bacterial species in the failure and the success groups were evaluated by Good's species coverage index, which found that the difference between the two groups was not significant (*P* = 0.125). The coverage index in both groups was higher than 0.99 and confirmed that the test results covered most bacterial species in the gastric mucosa. Analysis of community composition showed that the gastric mucosa microbiota mainly contained Proteobacteria, Bacteroidetes, Firmicutes, Actinomycetes, and Fusobacteria, regardless of the study group. The abundance of Proteobacteria was higher in the failure group than in the success group, and that of Actinobacteria was lower (Table 2).

Alpha and beta diversity reflect differences in the microbial composition. The results of the Shannon index, which is one an indexes of alpha diversity, showed that the diversity in the failure group was reduced compared with the success group, but the difference was not significant (*P* = 0.092; Figure 1A). The Ace index showed that the species abundance was significantly lower in the failure group than in the success group (*P* = 0.031; Figure 1B). The dominant species in both study samples was *H. pylori*, but the microbiota composition differed at the genus level (Figure 2A). PCoA analysis of beta diversity resulted in a weighted UniFrac showing that the total diversity of the first two principal coordinates was 89.96% and the difference between the two groups was significant (ANOSIM, *P* = 0.048; Figure 2B). According to the unweighted UniFrac, the difference between the two groups was significant (ANOSIM, *P* = 0.001; Figure 2C). Binary Euclidean analysis, which was used to assess the difference in species composition, showed a significant difference between the two groups (ANOSIM, *P* = 0.001; Figure 2D). As shown in Figure 3, *H. pylori* was more abundance in the failure than in the success group, but the difference was not significant (Wilcoxon rank-sum test, *P* = 0.0809). *Rhodococcus*, *Lactobacillus*, and *Sphingomonas* were significantly enriched in the success group, (Wilcoxon rank-sum test, *P* < 0.05).

The flora composition of gastric mucosa that were histologically different was also analyzed. The abundance of *H. pylori* was higher in the mucosa of chronic superficial gastritis but the difference was not significant (Wilcoxon rank-sum test, *P* = 0.1179).

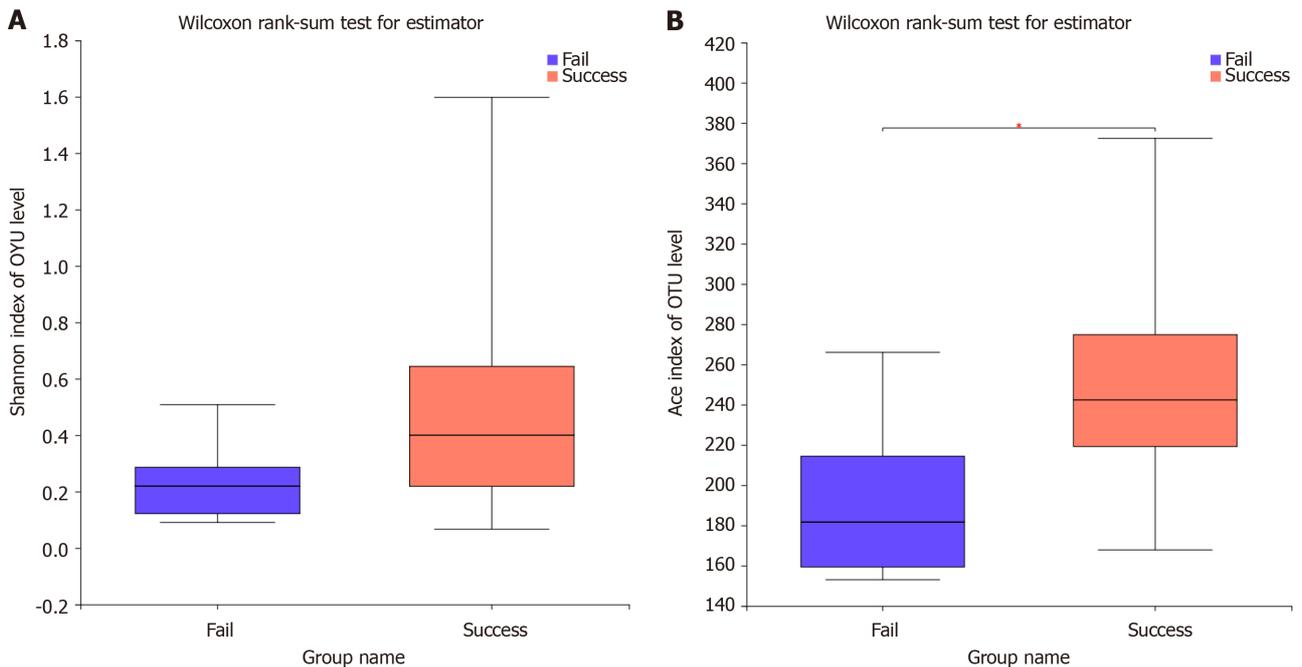
**Table 1** Baseline characteristics used in case matching of *Helicobacter pylori* eradication failure and success

		Failure group	Success group	P value
Sex	Male	7	14	1.000
	Female	1	2	
Age in yr, mean ± SD		40.13 ± 14.55	37.44 ± 6.42	0.532
BMI in kg/m <sup>2</sup> , mean ± SD		25.01 ± 5.32	25.20 ± 2.84	0.913
Background mucosa	Chronic superficial gastritis	6	13	1.000
	Chronic atrophic gastritis	2	3	

SD: Standard deviation.

**Table 2** Phylum-level differences of gastric mucosa microbiota

Phyla	Failure group, %	Success group, %	P value
Proteobacteria	98.26	94.60	0.0346
Bacteroidetes	0.60	1.23	0.4623
Firmicutes	0.52	1.23	0.2839
Actinomycetes	0.21	0.96	0.0016
Fusobacteria	0.11	0.27	0.6025

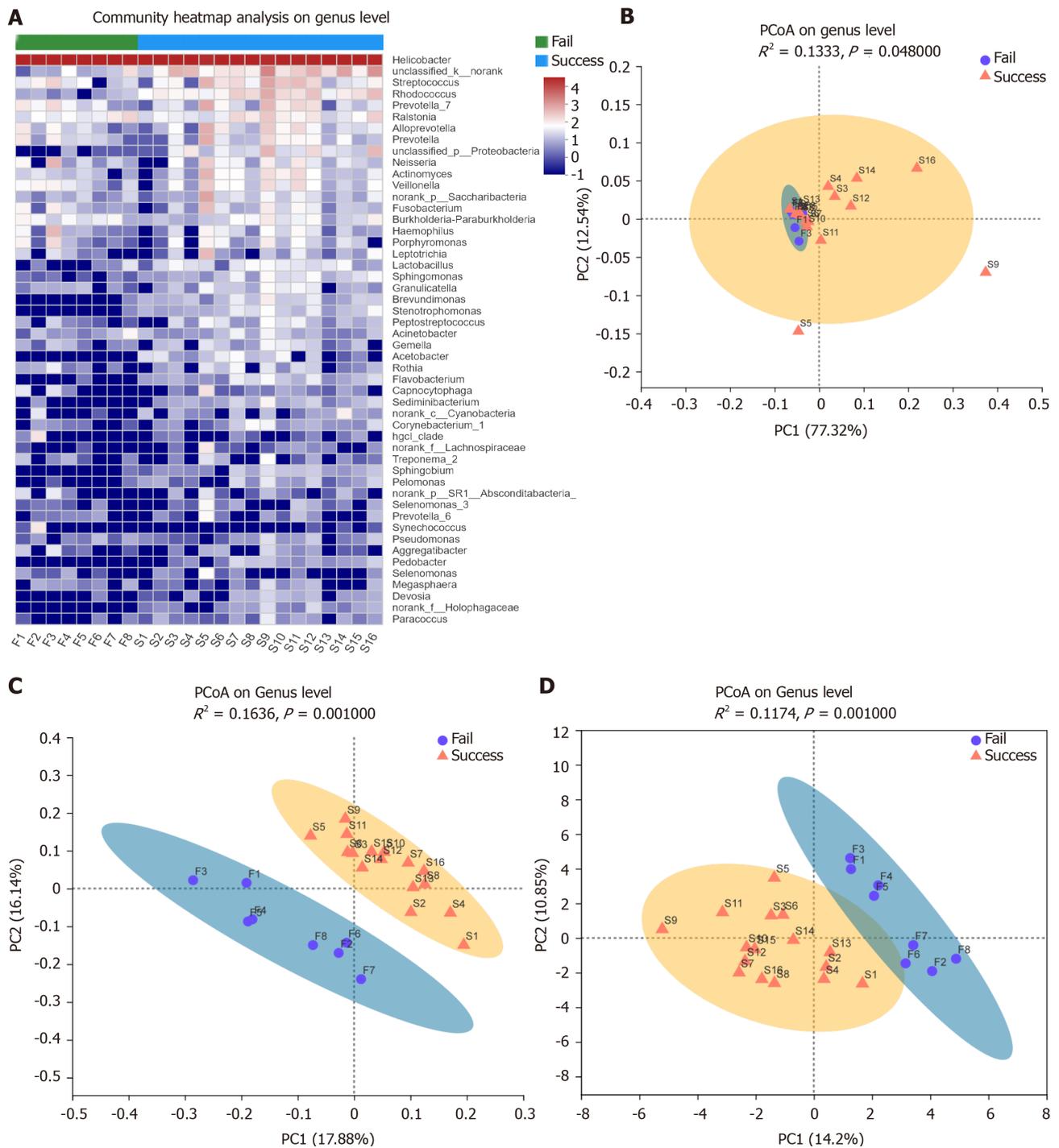


**Figure 1** Alpha diversity analysis. A: Shannon index; B: Ace index.

*Veronococcus* and *Cilium* were more enriched in the mucosa of chronic atrophic gastritis (Wilcoxon rank-sum test,  $P = 0.0466$  and  $0.0122$ ).

**Correlation analysis of microbiota**

Heatmap results of the species correlation analysis found that *H. pylori* was negatively correlated with other bacterial genera (Figure 4), *H. pylori* was negatively correlated with *Ralstonia* in the failure group (genus level) and negatively correlated with *Haemophilus*, *Prevotella*, *Streptococcus*, *Actinomycetes*, *Veillonella*, *Neisseria*, *Fusobacterium*, and *Leptotrichia* in the success group (genus level). The PICRUSt metabolic pathway



**Figure 2 Beta diversity analysis.** A: Heatmap; B: Weighted UniFrac; C: Unweighted UniFrac; D: Binary Euclidean. Points with different colors or shapes represent samples from different groups. The closer the two points are, the more similar the species composition is.

function prediction showed that the level two metabolic pathways in the two study groups were basically the same, mainly including carbohydrates, amino acids, energy, coenzyme factors, and vitamins.

## DISCUSSION

The composition of the gastric mucosa microbiota infected by *H. pylori* mainly included Proteobacteria, Bacteroidetes, Firmicutes, Actinobacteria, and Fusobacteria, which is consistent with the results reported by previous studies[14,15]. *H. pylori* infection can significantly reduce the diversity of gastric mucosal microbiota[16]. In this study, the Shannon index did not reveal a significant difference in the diversity of

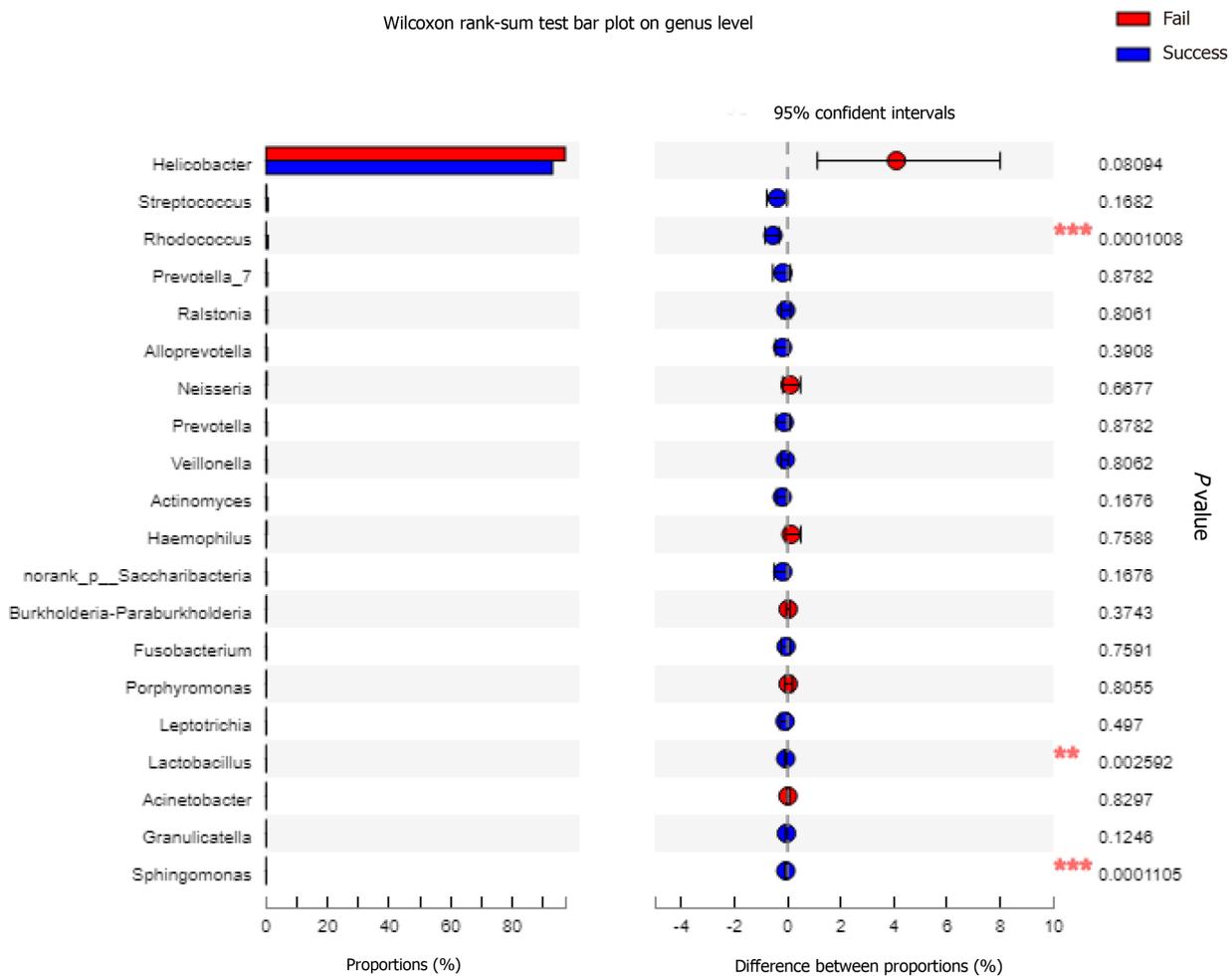
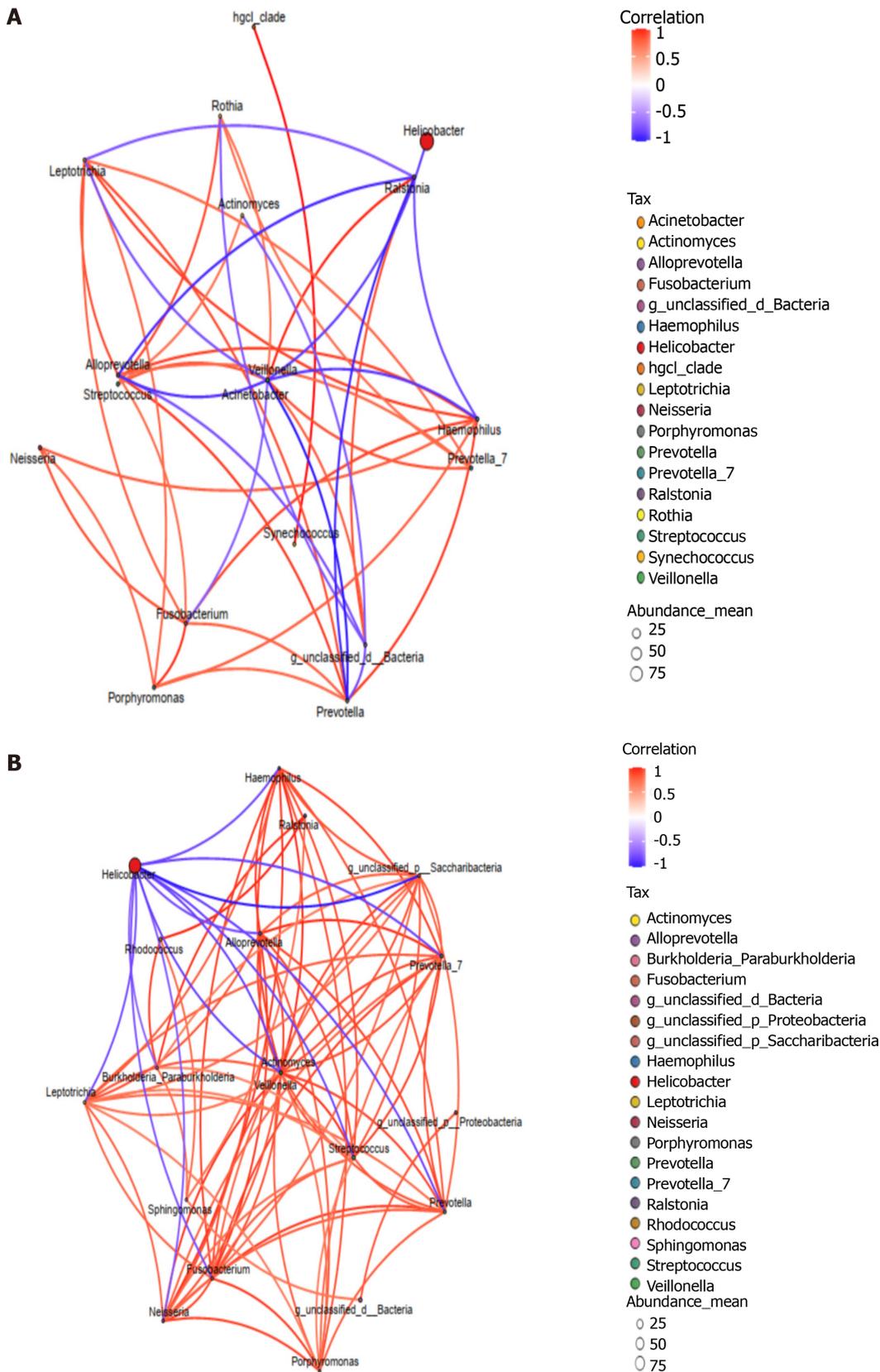


Figure 3 Genus-level differences between the success group and the failure group. The analysis includes the 20 most abundant genera.

the microbiota between the success and the failure group. However, the microbiota diversity in the failure group was lower, which suggests that eradication of *H. pylori* may be associated with the diversity of gastric mucosal microbiota.

The abundance of Proteobacteria was higher in the failure group. The genus level data suggests that the higher abundance of *H. pylori* in the eradication failure group might have caused an increased abundance of Proteobacteria. Although the difference in *H. pylori* abundance between the two groups was not significant, previous studies have shown that a higher abundance of *H. pylori* may reduce the effectiveness of empirical eradication therapy[17]. It has been reported that the abundance of *H. pylori* impacted only the result of traditional triple therapy[18]. Nevertheless, our results implied that the abundance of *H. pylori* had an impact on the eradication effect of quadruple therapy containing bismuth. In addition, 16s RNA may be more accurate than the <sup>13</sup>C/<sup>14</sup>C-UBT or histological evaluation for the determination of *H. pylori* abundance.

At the genus level, the gastric mucosa microbiota species diversity was similar in the two groups. However, PCoA analysis showed that there were differences in the species composition and abundance between the two groups. The Ace index confirmed the differences in abundance of the two groups, and statistical analysis confirmed that the success group was significantly enriched in *Rhodococcus*, *Lactobacillus*, and *Sphingomonas*. The reason for the increased abundance of Actinomycetes in the success group may be related to the enrichment of *Rhodococcus*, an opportunistic pathogen[19,20] that can be detected in the stool of healthy people. Thiamine is required for the growth of *Rhodococcus*[21] and is essential nutrient for the growth of *H. pylori*[22]. *H. pylori* is a thiamine auxotroph that lacks the gene that synthesizes thiamine[23]. Vitamin metabolism is one of the microbiota's main metabolic pathways predicted by PICRUST. Therefore, *Rhodococcus* may inhibit the growth of *H. pylori* through the acquisition of thiamine. *Lactobacilli*, which are beneficial bacteria, effectively improve *H. pylori* eradication if added to the prescription, especially in 7-d



**Figure 4 Single factor correlation network analysis including the 20 most abundant genera.** A: Failure group; B: Success group. Each circle represents a species. Red and blue line represent positive and negative correlations. The thickness of the lines represents the correlation coefficient.

and 14-d triple therapy[24]. Previous studies have shown that *Lactobacillus* is a strong antagonist of *H. pylori* by preventing colonization and growth[25,26] and inhibiting adhesion to and invasion of gastric epithelial cells. *Lactobacillus* even has an antagonistic effect on multidrug resistant *H. pylori*[27]. In this study, the abundance of

*Lactobacillus* was significantly higher in the success group than in the failure group, confirming the beneficial effect of *Lactobacillus* in the eradication of *H. pylori*. Moreover, previous studies found changes in *Sphingomonas* in a variety of diseases[28,29]. In this study, it had a significant negative correlation with *H. pylori* and was more abundant in the success group than in the failure group.

Analysis of the microbiota against different gastric mucosa backgrounds showed that the genera *Veronococcus* and *Cilium* were enriched in chronic atrophic gastritis. Previous studies have shown bacterial overgrowth in the gastric mucosa of pre-gastric cancer. *Veronococcus* and *Cilium* are enriched in gastric mucosa of gastric cancer[30]. *Veronococcus* can convert nitrate to nitrite, and the increased concentration of nitrite may promote gastric cancer[31]. In addition, *Veronococcus* is enriched in patients with oral cancer, colorectal cancer, and lung cancer[32-35]. A study conducted in Colombia showed that the gastric mucosal flora of a population at high risk of gastric cancer was significantly enriched in *Veronococcus* and *Cilium* but the rate of *H. pylori* infection was not increased. Therefore, the genus *Veronococcus* and *Cilium* may be factors promoting the occurrence of gastric cancer. Our results confirmed a trend in pre-gastric cancer.

A negative correlation was found between *H. pylori* and other microbiota, which was more significant in the success group than in the failure group. The negative correlation of *H. pylori* and other microbiota was associated with a positive correlation among the other bacterial communities. Co-inhibition of *H. pylori* by a variety of mutually-promoting microbiota may improve the effectiveness of *H. pylori* eradication. There was no difference in the abundance of mutually-promoting microbiota present in the two groups. The lack of correlation between mutually-promoting microbiota and *H. pylori* in the failure group may have been related to a difference in *H. pylori* strains and needs to be confirmed by further studies.

Differences in the gastric microbiota may have contributed to *H. pylori* eradication failure, because all samples were collected before *H. pylori* eradication. This study has some limitations. It had a relatively small sample size because of the high eradication rate. The result preliminarily showed an effect of the gastric microbiota on *H. pylori* eradication, but studies that have larger sample sizes are needed to confirm these findings. This study did not determine *H. pylori* resistance, but the results provide clinically significant guidance for empirical treatment.

## CONCLUSION

The effect of quadruple *H. pylori* eradication therapy containing bismuth depends on the gastric microbiota. A high rate of *H. pylori* eradication was associated with the presence of *Rhodococcus*, *Lactobacillus*, and *Sphingomonas*. The study identified gastric microbiota beneficial to *H. pylori* eradication and laid a foundation for further research on how the gastric microbiota influences *H. pylori* eradication. In addition, the study results may help to improve the eradication rate of *H. pylori* in the future.

## ARTICLE HIGHLIGHTS

### Research background

There are complex interactions between *Helicobacter pylori* (*H. pylori*) and other microbial communities in the gastric microecological environment. Yet, it remains unclear whether the interactions affect the eradication of *H. pylori*.

### Research motivation

The motivation was to explore the interaction between gastric microbiota and *H. pylori* and to determine the influence of gastric microbiota on the eradication of *H. pylori*.

### Research objectives

To investigate the characteristics of the gastric mucosa microbiota with *H. pylori* infection and the influence on *H. pylori* eradication treatment. This may help improve the eradication rate of *H. pylori* in the future.

### Research methods

Patients with *H. pylori* infection underwent gastroscopy and received treatment. Propensity matching analysis was conducted, including the number of patients who

did not respond to treatment. The gastric microbiota was assayed by high-throughput sequencing and subsequent analysis of alpha diversity, beta diversity, species correlations, and predicted metabolic pathways.

### Research results

The main phyla in the two groups were the same in the eight failure group patients who did not respond well to therapy and the 16 success group patients and included Proteobacteria, Bacteroides, Firmicutes, Actinomycetes, and Fusobacteria. The high rate of *H. pylori* eradication was associated with *Rhodococcus*, *Lactobacillus*, and *Sphingomonas*. *H. pylori* was negatively correlated with other bacterial genera, and more bacterial genera were directly related to *H. pylori* in the success group.

### Research conclusions

The effectiveness of quadruple *H. pylori* eradication therapy containing bismuth depended on the gastric microbiota. The high rate of *H. pylori* eradication was associated with *Rhodococcus*, *Lactobacillus*, and *Sphingomonas*.

### Research perspectives

This study laid a foundation for further research on the mechanism of the influence of the gastric microbiota on *H. pylori* eradication, which will help to improve the eradication rate of *H. pylori* in the future.

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