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Evaluation of gastric microbiome and metagenomic function in patients with intestinal metaplasia using 16S rRNA gene sequencing

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Abstract

Background: Despite recent advances in studies on the gastric microbiome, the role of the non-*Helicobacter pylori* gastric microbiome in gastric carcinogenesis remains unclear. We evaluated the characteristics of the gastric microbiome and metagenomic functions in patients with IM.

Methods: Participants were classified into six groups according to disease status (chronic superficial gastritis [CSG], intestinal metaplasia [IM], and cancer) and *H. pylori*- infection status (*H. pylori*-positive and *H. pylori*-negative). The gastric microbiome was analyzed in mucosal tissues at the gastric antrum by 16S rRNA gene sequencing. Moreover, we assessed the metagenome including the type IV secretion system (T4SS) gene, as T4SS proteins are essential for transferring CagA from *H. pylori*- into the human gastric epithelium.

Results: Among the 138 included patients, 48, 9, 23, 14, 12, and 32 were classified into the *H. pylori*-negative CSG, *H. pylori*-negative IM, *H. pylori*-negative cancer, *H. pylori*-positive CSG, *H. pylori*-positive IM, and *H. pylori*-positive cancer groups, respectively. Cyanobacteria were predominant in the *H. pylori*-negative CSG group compared to in the *H. pylori*-negative IM and *H. pylori*-negative cancer groups (*H. pylori*-negative CSG vs *H. pylori*-negative IM vs *H. pylori*-negative cancer: 14.0% vs 4.2% vs 0.04%, $P < 0.001$). In contrast, Rhizobiales were commonly observed in the *H. pylori*-negative IM group (*H. pylori*-negative CSG vs *H. pylori*-negative IM vs *H. pylori*-negative cancer: 1.9% vs 15.4% vs 2.8%, $P < 0.001$). The relative abundance of Rhizobiales increased as *H. pylori*-infected stomachs progressed from gastritis to IM. In the *H. pylori*-negative IM group, genes encoding T4SS were prevalent among the metagenome. Additionally, after *H. pylori*- eradication therapy, the gastric microbiome was similar to the microbiome observed after spontaneous clearance of *H. pylori*-.

Conclusions: The relative abundance of Rhizobiales was higher in patients with *H. pylori*-negative IM than in those with *H. pylori*-negative CSG or cancer. Additionally, T4SS genes were highly observed in the metagenome of patients with IM. Highly abundant T4SS proteins in these patients may promote gastric carcinogenesis.

Sang Kil Lee and Dong Soo Han contributed equally to this work as co-corresponding authors.

KEYWORDS
gastric cancer, *Helicobacter pylori*, intestinal metaplasia, microbiome

1 | INTRODUCTION

There are several unsolved aspects of *Helicobacter pylori* -related gastric carcinogenesis. First, *H. pylori*- often disappears spontaneously in elderly patients because of the progression of atrophic gastritis and intestinal metaplasia (IM),[1] while gastric cancer typically develops in elderly individuals rather than in younger individuals.[2] Although IM has been suggested as a precancerous lesion after the clearance of H. pylori, it is unknown whether IM itself causes gastric cancer development. Second, *H. pylori*- eradication therapy alone cannot completely prevent gastric cancer, particularly in patients with precancerous lesions, including IM.[3] These results indicate that specific changes that initiate gastric carcinogenesis occur in patients with IM.

Overgrowth of a bacterial species other than *H. pylori*- in patients with IM must be considered, as many human diseases are affected by the microbiome.[4] Previous studies reported that nitrate- and nitrite-reducing bacteria were identified in the achlorhydric stomach.[5,6] Additionally, the metagenome derived from intragastric bacteria other than *H. pylori*- should be evaluated. The type IV secretion system (T4SS) gene is particularly important because T4SS proteins are essential for transferring CagA from *H. pylori*- into the human gastric epithelium.[9] T4SS genes are present in various bacteria, including Rhizobiales, Legionellales, and Burkholderiales.[10] If T4SS genes are abundant in the stomachs of patients with intestinal metaplasia, horizontal gene transfer of T4SS genes between *H. pylori*- and other bacteria may be important. Horizontal gene transfer is an important adaptive force in evolution by contributing to metabolic, physiological, and ecological changes in most prokaryotes and some eukaryotes.[11]

Recently, there have been several attempts to determine the features of the gastric microbiome using next-generation sequencing. Previous studies demonstrated that the distribution of the gastric microbiome differs between patients with gastric cancer and those without gastric cancer.[12,13] Particularly, Coker et al[14] identified differences in bacterial interactions across stages of gastric carcinogenesis. However, the metagenome of the stomach has not been evaluated.[12,13] Additionally, previous studies mainly focused on the gastric microbiome in patients with gastric cancer rather than on precancerous lesions, such as IM. To better understand the role of the gastric microbiome in gastric carcinogenesis, it is necessary to analyze the metagenome in patients with IM. In this study, therefore, we aimed to evaluate the characteristics of gastric microbiomes and metagenomic functions in patients with IM. Therefore, we evaluated the characteristics of the gastric microbiome and metagenomic functions in patients with IM. We further analyzed whether there were differences in the gastric microbiome in patients with spontaneous clearance of *H. pylori*- and those with *H. pylori*- eradication via antibiotics.

2 | METHODS

2.1 | Study design

Asymptomatic volunteers or patients with dyspepsia who were scheduled for upper gastrointestinal endoscopy were recruited for this study. Exclusion criteria were as follows: (a) patients who took medications including proton-pump inhibitors, H_2 receptor antagonists, antacids, probiotics, mucosal protective agents, or antibiotics, within 3 months prior to enrollment, (b) patients with a history of gastric adenoma, carcinoma, or mucosa-associated lymphoid tissue lymphoma, and (c) patients who underwent gastrectomy.

Because *H. pylori*- is a predominant bacterial taxon in the gastric microbiome of patients with *H. pylori*- infection, patients were classified according to the histology of the gastric mucosa as well as *H. pylori*- infection status. After testing for *H. pylori*- infection and histologic examination of gastric tissue, patients who met one of the following criteria were enrolled and analyzed: (a) group A (*H. pylori*-negative chronic superficial gastritis [CSG]), patients with CSG and without *H. pylori*- infection, (b) group B (*H. pylori*-negative IM), patients with IM and without *H. pylori*- infection, (c) group C (*H. pylori*-negative cancer), patients with gastric cancer and without *H. pylori*- infection, (d) group D (*H. pylori*-positive CSG), patients with CSG and H. pylori infection, (e) group E (*H. pylori*-positive IM), patients with IM and H. pylori infection, and (f) group F *H. pylori*-positive cancer), patients with cancer and H. pylori infection. Group A represents patients without prior H. pylori infection, while Group B and C represent patients with prior infection and spontaneous clearance of H. pylori.

Microbial distribution was compared among the groups (*H. pylori*-negative CSG, *H. pylori*-negative IM, and *H. pylori*-negative cancer) using cladograms and principal component analysis plots. Linear discriminant analyses were performed to identify significant bacterial taxa in each group. We then further analyzed metagenomic functions using the phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt) method based on 16S rRNA gene sequences to identify the potential roles of non-H. pylori gastric microbiome components in gastric carcinogenesis.

The study protocol was approved by the Institutional Review Board on Human Subjects Research and Ethics Committees Hanyang University Guri Hospital, Korea. All research was performed in accordance with relevant guidelines and regulations. All patients provided written informed consent before enrollment. The study was registered at the International Clinical Trials Registry Platform, no. KCT0001602 (https://cris.nih.go.kr) on August 25, 2015.

2.2 | Tissue sampling and serologic testing

For microbiome analysis, four pieces of gastric mucosal tissue were obtained by endoscopic biopsy from the greater curvature side of the mid-antrum. Next, the rapid urease test was performed to evaluate *H. pylori*- infection status. One additional piece of mucosal tissue from the gastric antrum was biopsied for histologic examination of gastritis, IM, and cancer. All biopsy samples were obtained from nearly adjacent sites in the greater curvature side of the mid-antrum.

For serologic assessments of *H. pylori*- infection and atrophic gastritis, serum IgG anti-H. pylori antibody and pepsinogen I/II testing were evaluated by enzyme immunoassay and latex agglutination turbidimetric immunoassay, respectively.

2.3 | *H. pylori*- eradication therapy

In the *H. pylori*-positive CSG group, patients were administered *H. pylori*- eradication therapy with the standard triple regimen for 2 weeks (rabeprazole 20 mg, amoxicillin 1 g, clarithromycin 500 mg, twice daily). One month after eradication therapy, four pieces of gastric mucosal tissue in the greater curvature side of the antrum were endoscopically biopsied to analyze microbiome changes after eradication. *H. pylori*- eradication was confirmed by the[13] C-urea breath test. Patients administered H. pylori eradication therapy were further classified into successful eradication and failed eradication groups according to the results of H. pylori eradication therapy.

2.4 | Extraction of bacterial DNA

Extraction of bacterial DNA from mucosal biopsy samples was performed as previously described.[12] Briefly, 100 mg of frozen gastric mucosal tissues was suspended in 750 μL of sterile bacterial lysis buffer (200 mM NaCl, 100 mM EDTA [pH 8.0], 20 mM Tris base, 20 mg/mL lysozyme) and incubated at 37°C for 30 minutes. Next, 20 μL of proteinase K and 80 μL of 10% SDS were added to the mixture followed by incubation at 65°C for 30 minutes. Finally, bead beating was performed for 90 seconds at 6.9 g (PRECELLYS 24; Bertin Technologies, Le Bretonneux, France) after adding 300 mg of 0.1-mm zirconium beads (BioSpec Products, Bartlesville, OK) to finish homogenization. The homogenized mixture was cooled on ice and then centrifuged at 18.3 g for 5 minutes. Bacterial DNA was extracted from the supernatant by phenol/chloroform/iso-amyl alcohol (25:24:1) followed by chloroform/iso-amyl alcohol (24:1) and precipitated by absolute ethanol at −20°C for 1 hour. The precipitated DNA was suspended in DNase-free H_2O and cleaned using a DNA clean-up kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Isolated DNA was stored at −80°C until microbial characterization.

2.5 | 16S rRNA gene sequencing and analysis

Extracted gDNA was amplified using the following primers targeting the V3 to V4 regions of the 16S rRNA gene: forward, 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3′; reverse, 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3′. High-throughput sequencing was performed on an Illumina MiSeq system (Illumina, San Diego, CA) according to the manufacturer's instructions. Sequence data were processed using QIIME version 1.9.0.[15] Reads showing low quality, containing incorrect primer sequences, or containing more than one ambiguous base were excluded. The remaining reads were classified into groups based on their unique nucleotide barcodes. The read count was normalized to the corresponding copy number of 16S rRNA genes to reduce the potential bias of abundance estimation due to copy number variation.[16] Taxonomic composition from the phylum to the species levels and bacterial diversity for each sample were evaluated based on 97% similarity with the GreenGenes database (version 13.5), using QIIME. Principal component analysis of bacterial abundance was performed based on the Euclidean distance to compare the microbial communities of subgroups. Differential abundant features of relative abundance of bacterial taxa or metagenomic functions were identified using the Linear Discriminant Analysis Effect Size, which is an algorithm for high-dimensional biomarker discovery and explanation that identifies genomic features by characterizing differences under two or more biological conditions.[17]

DNA sequences obtained from this metagenomic project were deposited in the National Center for Biotechnology Information (NCBI) Short Read Archive under the Accession No. SRP109017.

In patients with *H. pylori*- infection, *cagA* positivity was determined by semi-quantitative PCR using the following primers[18]: forward, 5′-GATAACAGGCAAGCTTTTGAGG-3′; reverse, 5′-CTGCAAAAGATTGTTTGGCAGA-3′.

2.6 | Metagenome prediction

Functional composition of the microbiome was predicted using PICRUSt.[19] PICRUSt conducts evolutionary modeling to predict the metagenome from 16S rRNA gene data compared to a reference genome database.[19] Metagenome inference based on the PICRUSt method was performed using PICRUSt scripts (*normalize_by_copy_nubmer.py* and *predict_metagenomes.py*) with the GreenGenes database (version 13.5).

2.7 | Download of sequence data

To improve the comparability and statistical power, microbiome sequences in gastric cancer tissues were downloaded from the NCBI (https://www.ncbi.nlm.nih.gov) following literature review.[12,13,20] Inclusion criteria for microbiome sequences in the previous studies were as follows: (a) gastric microbiome derived from East Asian patients with gastric cancer and (b) known *H. pylori*- infection status. These sequence data were analyzed in the same manner as the sequence data.

2.8 | Statistical analysis

Continuous and categorical variables were described as the mean with standard deviation and number with proportion, respectively. In the linear discriminant analyses to detect the relative abundance of

TABLE 1 Baseline patient characteristics and microbiome reads of samples

Variable	HP(−) CSG	HP(−) IM	HP(−) cancer	HP(+) CSG	HP(+) IM	HP(+) cancer	*P*-value
N	48	9	23	14	12	32	
Age, y, mean ± SD	32.3 ± 11.0	67.4 ± 7.7	61.8 ± 10.9[a]	31.4 ± 8.8	60.9 ± 13.4	54.1 ± 12.5[a]	<0.001
Male, n (%)	23 (47.9)	4 (44.4)	14 (73.7)[a]	3 (21.4)	9 (75.0)	10 (66.7)[a]	0.022
Pepsinogen test							
Pepsinogen I, ng/mL, mean ± SD	54.7 ± 34.4	41.8 ± 16.9	NA	99.2 ± 56.4	64.0 ± 32.4	NA	0.001
Pepsinogen II, ng/mL, mean ± SD	12.0 ± 9.7	11.4 ± 4.8	NA	29.1 ± 15.4	25.5 ± 12.7	NA	<0.001
Pepsinogen I/II ratio, mean ± SD	4.9 ± 1.5	4.4 ± 2.3	4.7 ± 2.1[a]	5.3 ± 7.7	2.5 ± 0.9	3.3 ± 1.5[a]	0.156
Microbiome reads, mean ± SD							
Read count	9134 ± 5767	4542 ± 2983	1930 ± 2103	22430 ± 5881	14948 ± 5931	49849 ± 98572	0.003
OTU	292 ± 150	152 ± 101	81 ± 59	131 ± 58	179 ± 99	261 ± 364	0.001
Chao1 estimator	161.6 ± 58.8	95.2 ± 29.5	52.2 ± 29.6	85.2 ± 27.8	105.7 ± 32.9	105.9 ± 92.6	<0.001
Shannon's diversity index	3.84 ± 0.33	3.30 ± 0.46	1.74 ± 0.70	0.74 ± 0.41	1.74 ± 1.23	1.69 ± 0.96	<0.001
Simpson's diversity index	0.95 ± 0.03	0.93 ± 0.02	0.67 ± 0.19	0.22 ± 0.14	0.51 ± 0.35	0.60 ± 0.29	<0.001

CSG, chronic superficial gastritis; HP, *Helicobacter pylori*; IM, intestinal metaplasia; NA, not available; OTU, operational taxonomic unit; SD, standard deviation.

[a]Demographic data and pepsinogen I/II ratio values in the HP(−) cancer and HP(+) cancer groups were derived from 19 and 15 patients, respectively, in the study by Jo et al[13].

bacterial taxa and metagenomic function, statistical analysis was performed using the Kruskal-Wallis test with a significance level of 0.05. We used Bray-Curtis distance with Wilcoxon rank-sum test to analyze dissimilarity among groups. All statistical procedures were conducted using R (version 3.3.3; R Foundation for Statistical Computing, Vienna, Austria), except for linear discriminant analysis, which was performed using Galaxy, an open, web-based platform for computational biomedical research (https://huttenhower.sph.harvard.edu/galaxy).

3 | RESULTS

3.1 | Baseline characteristics and microbiome reads

Table 1 shows the baseline patient characteristics and microbiome reads. Among patients with CSG or IM, 48, 9, 14, and 12 were classified into the *H. pylori*-negative CSG, *H. pylori*-negative IM, *H. pylori*-positive CSG, and *H. pylori*-positive IM groups, respectively, according to H. pylori testing and histologic examination results. All microbiome sequences of patients with CSG or IM were obtained and analyzed in our institute.

We also enrolled one patient in the *H. pylori*-negative cancer group and two patients in the *H. pylori*-positive cancer group. To further analyze the differential characteristics of IM groups compared to cancer groups, we determined the microbiome sequences of 52 patients with gastric cancer from the NCBI (22 patients with *H. pylori*-negative cancer, and 30 patients with *H. pylori*-positive cancer; SRP038955,[12] GSE61493,[13] and SRP057951[20]). In total, sequence data from 138 patients were analyzed in this study.

3.2 | Relative bacterial abundance

Relative bacterial abundance at the phylum level is shown in Figure S1. The relative abundance of non-H. pylori Proteobacteria increased in the *H. pylori*-negative CSG group, followed by the *H. pylori*-negative IM group and *H. pylori*-negative cancer group. In patients with H. pylori, Proteobacteria (which mainly consisted of H. pylori) decreased in the *H. pylori*-positive CSG group, followed by the *H. pylori*-positive IM group and *H. pylori*-positive cancer group.

To compare the microbiome other than H. pylori with disease status (CSG vs IM vs cancer), we determined the relative bacterial abundance of the *H. pylori*-negative groups by using cladograms (Figure 1). Characteristically, the relative abundance of Cyanobacteria was high in the *H. pylori*-negative CSG group (14.0%) and relatively low in the *H. pylori*-negative IM group (4.2%). Cyanobacteria were rarely found in the *H. pylori*-negative cancer group (0.04%). In contrast, the relative abundance of Rhizobiales was distinctly high in the *H. pylori*-negative IM group (15.4%) and very low in the *H. pylori*-negative CSG and *H. pylori*-negative cancer groups (1.9% and 2.8%, respectively). Detailed data regarding relative bacterial abundance are presented in Appendix 1.

3.3 | Principal component analysis

The principal component analysis plot showed that the *H. pylori*-negative CSG group was distinct from the *H. pylori*-positive CSG, *H. pylori*-positive IM, and *H. pylori*-negative IM groups (Figure 2). A linear transition was identified in the *H. pylori*-positive CSG group,

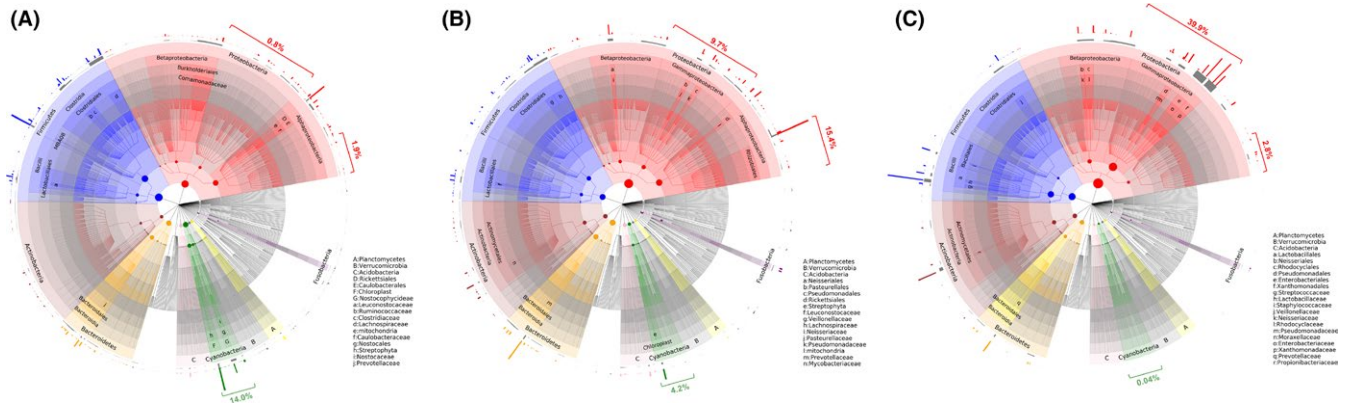

FIGURE 1 Cladograms for bacterial abundance in the HP-negative groups. (A) HP(−) CSG group, (B) HP(−) IM group, (C) HP(−) cancer group. Values represent the relative abundance of the specified bacterial taxa. Please refer to Appendix 1 for all detailed values of bacterial abundance. Clade marker size in the first and second inner circles represents the relative abundance of bacteria at the phylum and class levels, respectively. Height of the inner and outer rings represents the relative abundance of bacteria at the family and genus levels, respectively. CSG, chronic superficial gastritis; HP, *Helicobacter pylori*; IM, intestinal metaplasia

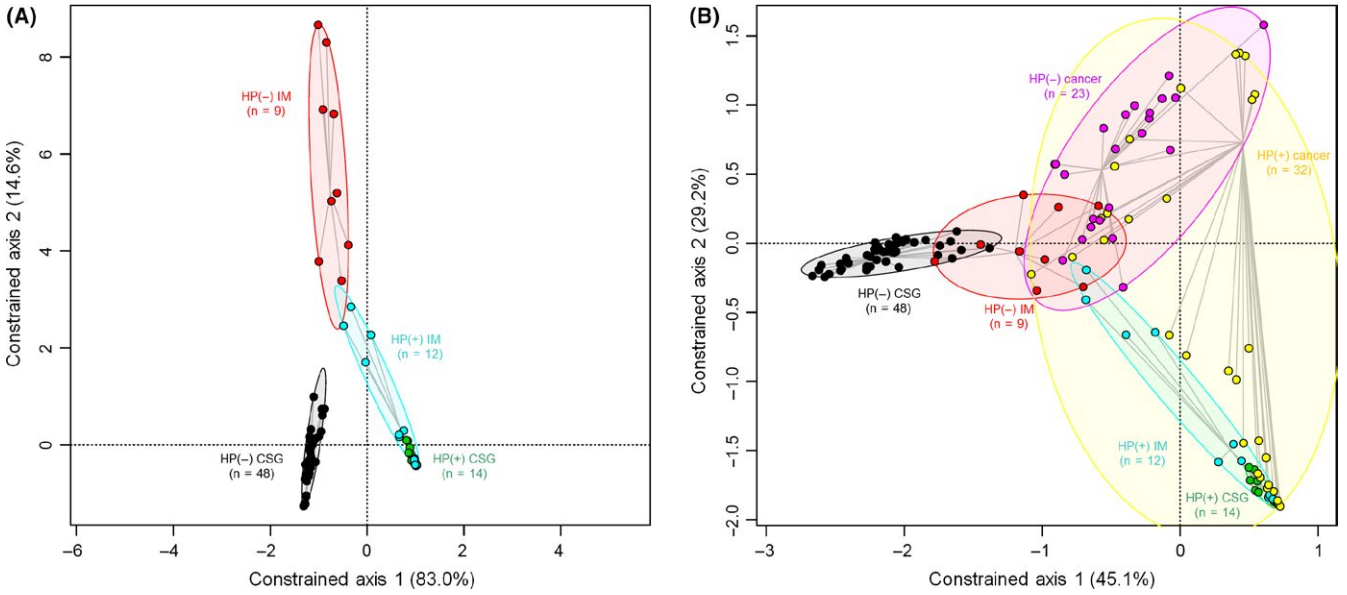

FIGURE 2 Principal component analysis plot. (A) CSG and IM groups, (B) All groups including cancer groups. A linear transition was identified in the HP(+) CSG group, followed by the HP(+) IM group and HP(−) IM group, as the relative abundance of HP decreased. Another linear transition was observed in the HP(−) CSG group, followed by the HP(−) IM group and HP(−) cancer group, mainly because of the decreased relative abundance of Cyanobacteria. CSG, chronic superficial gastritis; HP, *Helicobacter pylori*; IM, intestinal metaplasia

followed by the *H. pylori*-positive IM group and *H. pylori*-negative IM group as the relative abundance of H. pylori decreased.

3.4 | Linear discriminant analysis for relative bacterial abundance

Figure S2 shows the results of linear discriminant analysis for the relative bacterial abundance among *H. pylori*-negative groups. In the *H. pylori*-negative CSG group, the relative abundances of *Firmicutes* and Cyanobacteria were significantly higher compared to in other groups. The relative abundance of Rhizobiales was higher in the *H. pylori*-negative IM group than in the other two groups. Various bacterial taxa including Xanthomonadaceae, Streptococcaceae, Moraxellaceae, and Pseudomonadaceae were increased in the *H. pylori*-negative cancer group compared to their abundances in the *H. pylori*-negative CSG and *H. pylori*-negative IM groups.

3.5 | Metagenomic function

Metagenomic analysis was performed using 16S rRNA gene sequencing data and the PICRUSt method. Figure S3 shows the results of linear discriminant analysis for metagenomic function in the *H. pylori*-negative groups. Among the various functions that were

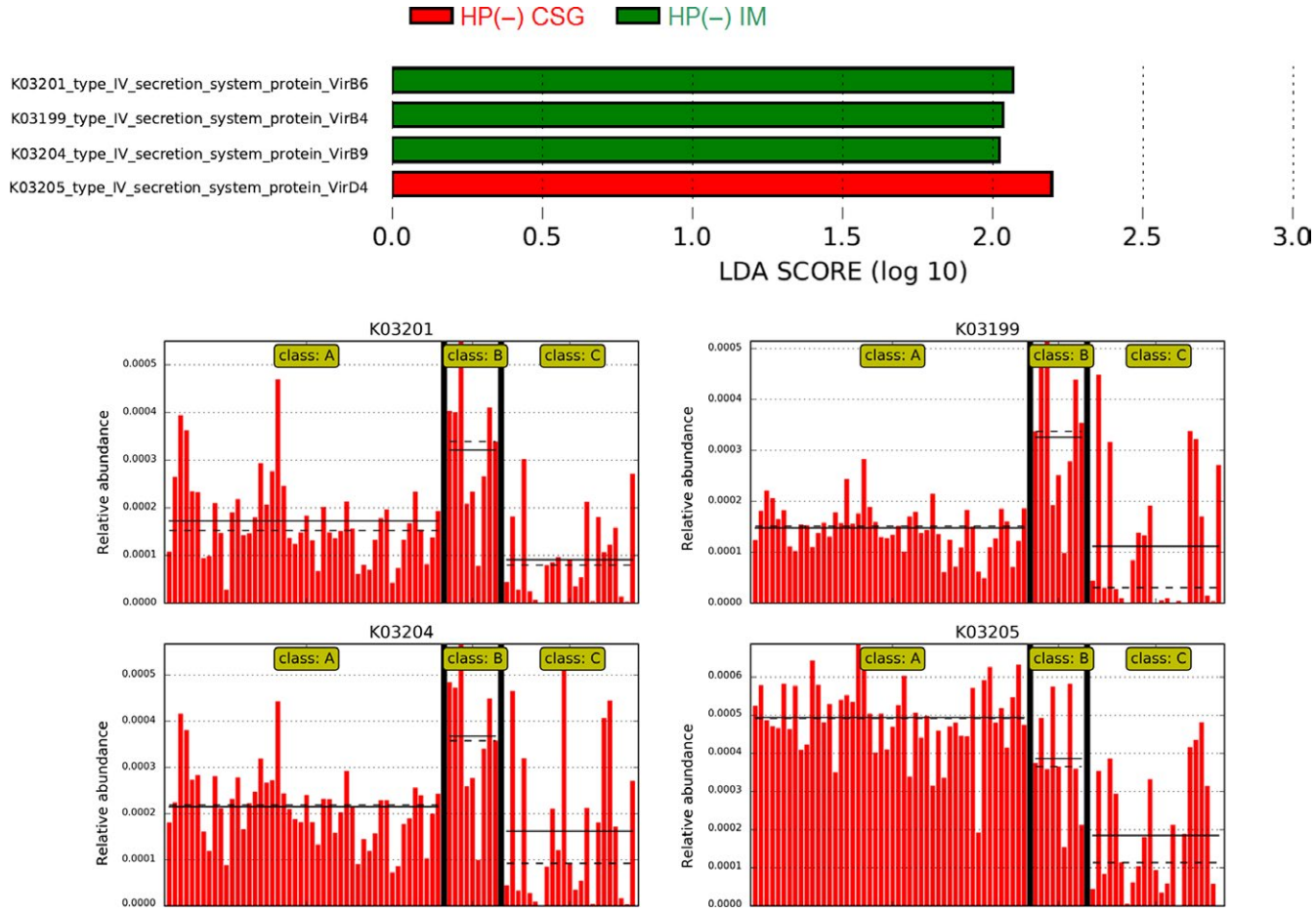

FIGURE 3 Highly identified genes encoding type IV secretion system protein subunits in the HP-negative CSG and IM groups. Class A, B, and C represent the HP(−) CSG, HP(−) IM, and HP(−) cancer groups, respectively. Red bar graphs represent the relative abundance of genes in each sample. Solid and dotted lines in the bar graphs represent the mean and median values of relative abundance, respectively, in each group. All demonstrated genes had significant difference among the groups ($P < 0.05$ by the Kruskal-Wallis test). CSG, chronic superficial gastritis; HP, *Helicobacter pylori*; IM, intestinal metaplasia; LDA, linear discriminant analysis

significantly higher in each group, we detected three genes, K03199, K03201, and K03204, which encode T4SS proteins VirB4, VirB6, and VirB9, respectively (Figure 3). These genes were detected at high rates in the *H. pylori*-negative CSG group compared to that in the *H. pylori*-negative CSG and *H. pylori*-negative cancer groups. Although the gene *VirD4* (K03205) encoding a T4SS protein was the most common in the *H. pylori*-negative CSG group, it was also commonly identified in the *H. pylori*-negative IM group compared to in the *H. pylori*-negative cancer group. Additionally, other genes encoding T4SS protein subunits, including K03197, K03198, K03200, K03202, K03203, K03195, and K03196, were generally higher in the *H. pylori*-negative IM group than in the *H. pylori*-negative CSG and *H. pylori*-negative cancer groups (Figure S4).

3.6 | Relationship between H. pylori and Rhizobiales

Figure 4 shows the relationship in the relative abundance between *H. pylori*- and other T4SS gene-contributing bacteria (Rhizobiales and Neisseriaceae) in the *H. pylori*-positive CSG, *H. pylori*-positive IM, and *H. pylori*-negative IM groups. The relative abundance of Rhizobiales and Neisseriaceae gradually increased as the abundance of *H. pylori*- decreased. Several samples, such as numbers 23-27 in Figure 4, showed a relatively high abundance of Rhizobiales and Neisseriaceae with a concurrent level of H. pylori abundance. In sample numbers 28-35, *H. pylori*- was rarely found, while Rhizobiales were commonly observed

3.7 | H. pylori eradication therapy in patients with CSG

Figure S5 shows the relative bacterial abundance at the phylum level before and after *H. pylori*+ eradication therapy in patients with CSG. Before eradication therapy, the relative abundance of Proteobacteria was >90% in all samples. As shown in the cladograms (Figure 5), the relative abundance of *Helicobacter* was 93.3%, while that of non-*H. pylori*- Proteobacteria was 2.9%. After eradication therapy, the relative abundance of Proteobacteria decreased; however, that of non-*H. pylori*- Proteobacteria increased (46.8%) in the successful eradication group. In contrast to the results in the successful eradication group, the relative abundance of Proteobacteria

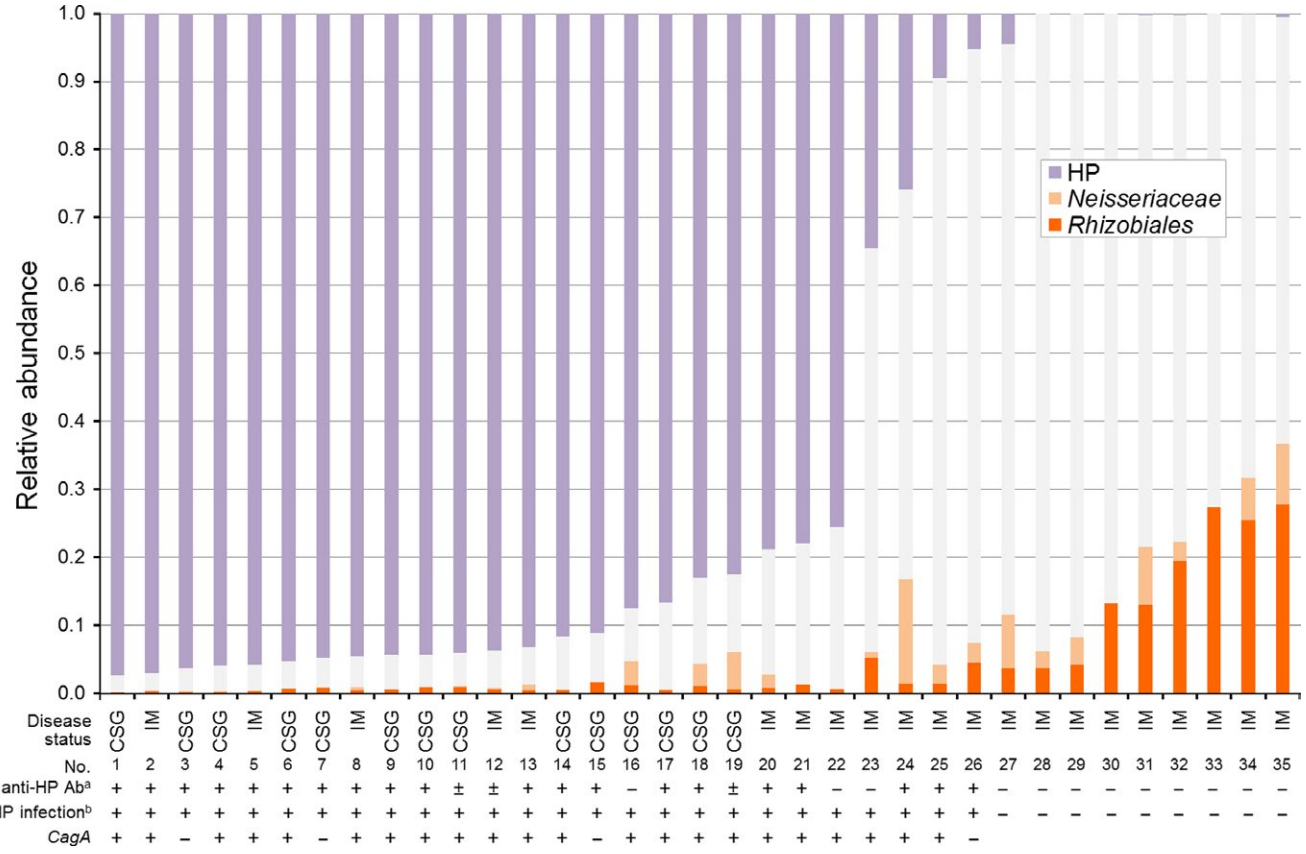

FIGURE 4 Relative abundance of HP, Rhizobiales, and Neisseriaceae in each sample of the HP(+) CSG, HP(+) IM, and HP(−) IM groups. The relative abundance of Rhizobiales and Neisseriaceae tended to gradually increase as the abundance of HP decreased. [a]Serum IgG anti-HP antibody was evaluated via enzyme immunoassay. [b]HP infection was determined based on conventional methods including rapid urease test, urea breath test, and histologic examination. CSG, chronic superficial gastritis; HP, *Helicobacter pylori*; IM, intestinal metaplasia

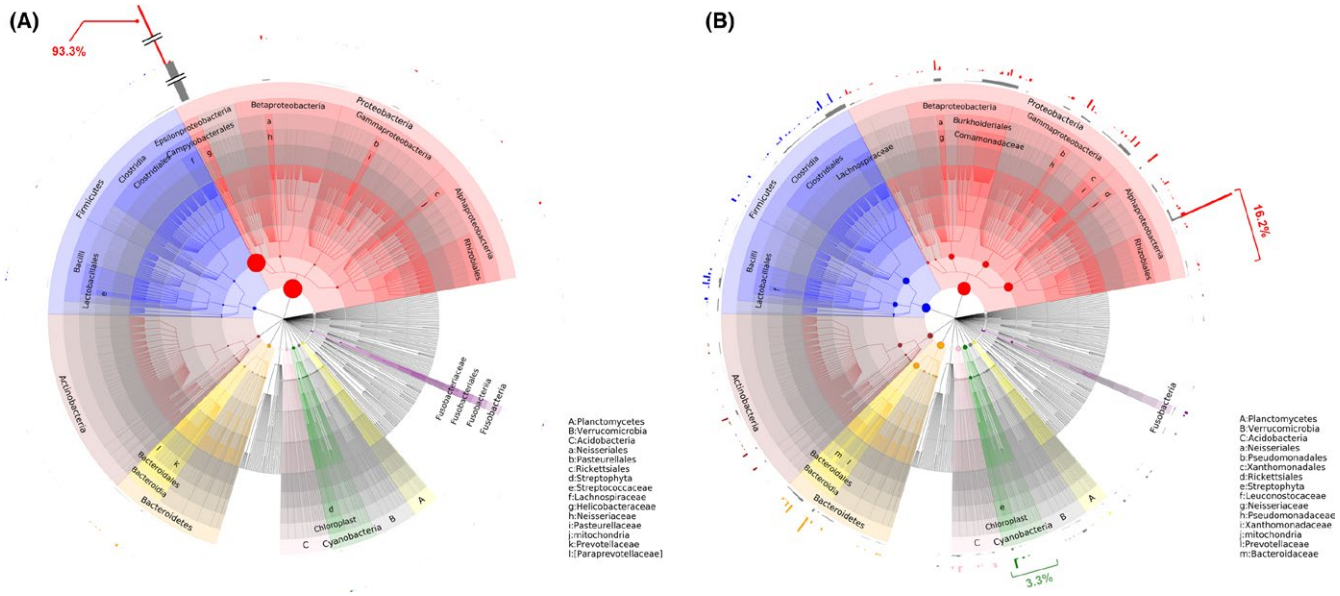

FIGURE 5 Cladograms for bacterial abundance before and after HP eradication in the HP(+) CSG group. (A) Before eradication in the HP(+) CSG group, (B) After eradication in the HP(+) CSG group. Clade marker size in the first and second inner circles represents the relative abundance of bacteria at the phylum and class levels, respectively. Height of the inner and outer ring represents the relative abundance of bacteria at the family and genus levels, respectively. CSG, chronic superficial gastritis; HP, *Helicobacter pylori*

was similar between before and after eradication therapy in the failed eradication group.

The distribution of bacterial abundance in the successful eradication group was similar to that in the *H. pylori*-negative IM group (Figure 5). The successful eradication group showed a high abundance of Rhizobiales (16.2%) and some Cyanobacteria (3.3%). Principal component analysis also revealed similar clustering between the successful eradication and *H. pylori*-negative IM groups (Figure S6). For quantitative assessment of group similarity, the Bray-Curtis distance between samples in the successful eradication and *H. pylori*-negative IM groups was plotted, as shown in Figure S7. Dissimilarity was even lower in samples between the successful eradication and *H. pylori*-negative IM groups than those within *H. pylori*-negative IM groups.

Finally, we analyzed the relative abundance of T4SS genes in the successful eradication group (Figure S8). The relative abundance of T4SS genes did not differ between the successful eradication and *H. pylori*-negative IM groups.

4 | DISCUSSION

In the present study, we identified differential bacterial taxa and metagenomics functions according to the histology of gastric mucosa. In the stomach of patients with IM, Rhizobiales was a commonly identified bacterial taxon. The relative abundance of Rhizobiales increased as *H. pylori*-infected stomachs progressed from gastritis to IM. We also found that genes encoding T4SS were prevalent in the metagenome of patients with IM.

T4SS proteins typically consist of 11 VirB proteins (VirB1-11) and the coupled protein (VirD4).[21] These proteins enable the secretion of CagA from the bacterial cytoplasm directly into the cytoplasm of gastric epithelial cells.[9] Next, membrane-tethered CagA initiates gastric carcinogenesis by recruiting a fraction of cytoplasmic SHP2 to the membrane and dissociating β-catenin from E-cadherin.[22] Based on our results, genes encoding T4SS proteins may be transferred from gastric bacteria including Rhizobiales and Neisseriaceae to H. pylori in patients with IM, facilitating gastric carcinogenesis.

Numerous studies have shown that horizontal gene transfer may occur between bacteria, including *H. pylori*-.[23,24] Particularly, genes from *H. pylori*- may be lost or gained during the progression from atrophic gastritis to gastric adenocarcinoma in the same patient.[24] Additionally, several plasticity zones are present in the H. pylori genome and show high genetic variation.[25] They generally contain complete sets of genes required to produce T4SS protein, as well as genes encoding different DNA processing proteins.[24,26,27] A complete T4SS plasticity zone cluster is known to be a virulence factor that may be important for the colonization of *H. pylori*- and development of severe outcomes of infection with cagA-positive strains.[29] Additionally, genes encoding T4SS proteins can be transferred by conjugation between *Rhizobium radiobacter* and *Bartonella rattaustraliani*.[30] Network analysis of gene sharing among Proteobacterial genomes also revealed higher horizontal gene transfer rates at the phylum level than at the species level.[31] Although there is no direct evidence of horizontal gene transfer between *H. pylori*- and Rhizobiales, our results suggest that T4SS gene transfer occurs between *H. pylori*- and other microbiome components.

One possible hypothesis based on our results is that gastric carcinogenesis initiation requires *H. pylori*- and other bacteria such as Rhizobiales and Neisseriaceae. From this perspective, patients with *H. pylori*-predominant CSG are not regarded as being at a high risk for gastric cancer. However, as *H. pylori*-infected stomach tissue progressed from gastritis to IM, the abundance of gastric microbiome other than *H. pylori*-, including Rhizobiales and Neisseriaceae, increased. Therefore, patients with progressed IM who exhibit abundance of *H. pylori*- and other microbiome may have a high risk of gastric cancer development. If CagA translocation occurs in these patients, gastric cancer cannot be prevented even if *H. pylori*- is eradicated.

Additionally, we found that the gastric microbiome in the successful eradication group resembled that in the *H. pylori*-negative IM group, which is regarded as a high-risk group. The results indicate that the gastric microbiome composition did not depend on the cause of *H. pylori*- clearance (eradication by antibiotics vs spontaneous clearance after IM progression). However, a change in the Rhizobiales-abundant microbiome after *H. pylori*- eradication therapy does not reflect an increase in gastric cancer risk, as the source of CagA has been removed through *H. pylori*- eradication. Thus, the risk-lowering effect of *H. pylori*- eradication therapy was related to *H. pylori*- eradication itself rather than changes in gastric microbiome components besides *H. pylori*-.

Interestingly, Cyanobacteria were highly abundant in the *H. pylori*-negative CSG group. The relative abundance of Cyanobacteria decreased in the *H. pylori*-negative CSG group, followed by the *H. pylori*-negative IM group and *H. pylori*-negative cancer group. Cyanobacteria abundance was not recovered in patients with a history of *H. pylori*- infection, even after *H. pylori*- eradication. These findings indicate that the Cyanobacteria-abundant microbiome exerts a protective effect against *H. pylori*- infection. Several previous studies showed that polysaccharides derived from Cyanobacteria prevented *H. pylori*- attachment to the gastric mucosa.[32,33] Considering both the potentially preventive effect of Cyanobacteria for *H. pylori*- infection and relatively low abundance of Cyanobacteria in *H. pylori*-eradicated patients, the re-infection risk of *H. pylori*- should be considered for patients administered eradication therapy.

Our study demonstrated that bacterial taxa that affect gastric carcinogenesis according to our next-generation sequencing results. Previous studies did not identify Rhizobiales as a bacteria that contributes to gastric carcinogenesis.[12,13] There are two main reasons for these discrepant results between studies. First, we initially analyzed the microbiome in *H. pylori*-negative groups and HP-positive groups individually. Because *H. pylori*- is the most abundant taxa in *H. pylori*-infected stomachs, differences in other microbiome species may not be detected if *H. pylori*-positive and *H. pylori*-negative groups are analyzed together. After identifying Rhizobiales and Neisseriaceae as taxa that potentially contribute to gastric carcinogenesis, we analyzed the

relationship among these bacterial taxa in both *H. pylori*-positive and *H. pylori*-negative groups simultaneously. Second, we focused on the gastric microbiome in patients with IM, rather than with cancer, because gastric carcinogenesis initiation events such as CagA translocation may occur in this stage. Considering the bacterial driver-passenger model in carcinogenesis,[34] the abundance of various taxa increased in the *H. pylori*–negative cancer group, including Xanthomonadaceae, Streptococcaceae, Moraxellaceae, and Pseudomonadaceae, which may be a result, rather than a cause, of cancer development.

Although this is the first study involving 16S rRNA gene sequencing analysis to propose bacteria that contribute to gastric carcinogenesis, there were several limitations to our study. First, the sample size was relatively small in several groups such as the *H. pylori*-positive IM and *H. pylori*–negative IM groups. Therefore, only the *VirB4*, *VirB6*, and *VirB9* encoding the T4SS protein subunits showed significantly different abundances among groups. However, other T4SS subunit genes tended to increase in the *H. pylori*–negative IM group compared to in the *H. pylori*–negative CSG and *H. pylori*–negative cancer groups. Larger studies may confirm our results and identify additional metagenomic functions affecting gastric carcinogenesis. Second, most sequence data in the cancer group were downloaded from NCBI, although all samples in patients with CSG or IM were obtained from our institute. Different study settings and sample collections may result in bias. However, our study mainly focused on the gastric microbiome in patients with IM compared to those with CSG, rather than cancer. Additionally, 12 of 52 downloaded samples were deposited by our group in the NCBI in our previous study (SRP038955).[12] Another 34 samples were collected from a geographically adjacent hospital from our institute (GSE61493).[13] Third, we did not analyze negative control samples such as PCR-amplified ultrapure water without template DNA. Because contaminating DNA may impact the results obtained from samples containing a low microbial biomass, concurrent sequencing of negative control samples is advised.[35] However, negative controls were not analyzed our study. Nevertheless, contamination may not have a significant impact on our results, as the intestinal metaplasia cannot be regarded as a low microbial environment. Fourth, we did directly demonstrate that horizontal gene transfer occurred between *H. pylori*- and other bacteria including Rhizobiales and Niserriaceae in the current study. Although we hypothesized that T4SS gene transfer occurs between *H. pylori*- and other bacteria, horizontal gene transfer is more likely to occur among members of the same class than among those of different classes. Experimental studies are needed to validate the hypothesis derived from this study.

Despite these limitations, our study improves the understanding of the potential role of the gastric microbiome in gastric carcinogenesis. As *H. pylori*-infected stomachs progressed from gastritis to IM, the relative abundance of Rhizobiales increased. In the metagenome of patients with IM, T4SS genes, which are mainly derived from Rhizobiales and Neisseriaceae, were highly abundant. Additionally, the gastric microbiome after *H. pylori*- eradication therapy was similar to that observed after spontaneous clearance of *H. pylori*-.

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ETHICAL APPROVAL

The study data and informed consent were obtained in accordance with the Declaration of Helsinki and were approved by the Institutional Review Board of Hanyang University Guri Hospital.

Informed Consent: Informed consent or a substitute for informed consent was obtained from all patients examined in the study.

CONFLICT OF INTEREST

The authors disclose no potential conflict of interests.

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SUPPORTING INFORMATION

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