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Association between bacteria other than *Helicobacter pylori* and the risk of gastric cancer

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Abstract

Background: The gastric microbiota, including *Helicobacter pylori* (HP), has a remarkable role in gastric cancer (GC) occurrence. Evidence for the role of non-HP bacteria in GC risk is limited. We aimed to observe the association between bacteria other than HP and risk of GC in a Korean population.

Methods: In this study, 268 GC cases and 288 healthy controls were included. Demographic data and total energy intake data were collected using a general questionnaire and a semiquantitative food frequency questionnaire, respectively. 16S rRNA gene sequencing was performed using DNA extracted from gastric biopsy samples.

Results: Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria, and non-HP Proteobacteria were the five main phyla in the gastric environment. The five phyla were negatively related to the relative abundance of Helicobacter species (all $p < 0.001$). The Shannon index, richness, and Pilou-evenness were negatively correlated with Helicobacter species (all $p < 0.001$), while the microbial dysbiosis index was positively correlated with Helicobacter species ($p < 0.001$). Participants with a higher relative abundance of Actinobacteria species showed a significantly increased risk of GC (OR: 3.16, 95% CI = 1.92–5.19, p-trend<0.001). The non-HP microbiota composition among the four groups (HP+cases, HP- cases, HP+controls, and HP- controls) was significantly different (ANOSIM R = 0.10, $p = 0.001$).

Conclusion: Other than HP, several bacterial species might be associated with GC risk. HP status and GC status could determine the differences in microbial compositions. Further large prospective studies are warranted to confirm our findings.

KEYWORDS
association, gastric cancer, gastric microbiome, non-*Helicobacter pylori*

1 | INTRODUCTION

In 2020, Global Cancer Observatory (GLOBOCAN) estimated that the age-standardized incidence rates of gastric cancer (GC) for males and females were 39.7 and 17.6 per 100,000, respectively.[1] In 2018, the Korean Central Cancer Registry reported that the age-adjusted incidence rates of GC for all registrants, males, and females were 30.4, 44.3, and 18.3 per 100,000, respectively.[2] Recent epidemiological studies have focused on the association between the gastric microbiome and GC risk because many gastric microbiota constituents other than *Helicobacter pylori* (HP) have a pivotal role in gastric carcinogenesis.

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Due to high acid production, the stomach was initially considered a sterile organ where no microorganisms could survive. This belief was changed after the discovery of HP, which is a strong risk factor for GC,[3-5] and it was suspected that HP is the only bacterium that can survive in the gastric environment.[6] The recent development of technology such as 16S rRNA gene sequencing analysis has provided a clear picture of the bacterial community present in the human stomach in addition to HP.[7-9] The majority of studies related to gastric microbiota identified Proteobacteria, Actinobacteria, Firmicutes, Bacteroidetes, and Fusobacteria as the main dominant phyla in the gastric environment.[7,8] In the premalignant stages, HP dominates the gastric microbiota, and it is difficult to identify differences in other bacterial taxa between cancer and noncancer tissues in the same patient.[7] However, only 1–2% of HP-infected individuals develop GC where its pathogenic mechanisms are still unclear.[10] The involvement of gastric microbiota in the link between HP and gastric carcinogenesis has been reported.[11,12] A condition such as chronic atrophic gastritis, where gastric acidity is decreased, facilitates the colonization of other bacteria that can produce reactive oxygen and nitrogen species to modulate the inflammatory response.[13,14]

The imbalance of the microbial community present in the gastric environment is conducive to the development of GC.[15] HP and other gastric microbiota can induce the activation of Toll-like receptors to stimulate gastric carcinogenesis. Initially, HP can increase the activation of Toll-like receptors, and this is followed by the involvement of other gastric microbiota.[16] Moreover, HP facilitates the production of N-nitroso compounds (NOCs) that increase the risk of GC.[17] In addition, other urease-producing bacteria and non-HP nitrate-reducing bacteria are also involved.[8]

To develop novel preventive and treatment strategies, the identification of non-HP bacteria in GC carcinogenesis is necessary and helps differentiate which bacteria are causative of GC. Thus, the aim of our study was to observe the associations between gastric microbiota and gastric carcinogenesis, focusing on bacteria other than HP in a Korean population.

2 | METHODS

2.1 | Study population

Participants were recruited at the National Cancer Center Hospital in Korea between March 2011 and December 2014. Individuals who had been histologically confirmed as early GC patients within the preceding three months at the Center for Gastric Cancer were included in the case group. Early GC was defined as an invasive carcinoma confined to the mucosa and/or submucosa, regardless of lymph node metastasis status. Patients diagnosed with diabetes mellitus, a history of cancer within the past five years, advanced GC, or severe systemic or mental disease, as well as women who were pregnant or breastfeeding, were excluded. The control group was selected from individuals undergoing health screening examinations at the Center for Cancer Prevention and Detection at the same hospital. Individuals with a history of cancer, diabetes mellitus, gastric ulcers, and HP treatment in the control group were excluded. In total, 1727 participants were recruited (1227 controls and 500 cases), and 1671 individuals provided data through a semiquantitative food frequency questionnaire (SQFFQ) and a self-administered questionnaire. Individuals with a total energy intake of <500 kcal or ≥4000 kcal (n = 15) were excluded because of the reliability of the data. Of the 1656 participants remaining, the control and case groups were frequency-matched by age (within five years) and sex at a ratio of 2:1 (controls: cases). The sample included 1245 participants comprising 830 controls and 415 cases. Of this group, 556 participants, 268 patients and 288 controls (men, 353; women, 203), were selected for the final analysis based on the availability of the 16S rRNA gene sequencing data. This study was approved by the Institutional Review Board of the National Cancer Center [IRB Number: NCCNCS-11–438]. Written informed consent was obtained from all participants.

2.2 | Data collection

Five gastric mucosa biopsy samples were collected from each study participant following the Sydney system after endoscopy and examination of the stomach. For the 16S rRNA gene sequencing, a biopsy sample from the greater curvature of the corpus at least 3 cm away from each tumor was collected from each GC patient, and a biopsy sample from the greater curvature of the corpus was collected from each control. The HP infection status was determined by a rapid urease test, a serological test, and histological evaluation. Regarding the rapid urease test, one biopsy sample was taken from the greater curvature of the corpus. Four biopsy samples were collected from the lesser curvature of the corpus and antrum for histological evaluation. The HP status was determined via Wright-Giemsa staining of the biopsy specimens by a pathologist who specialized in GC. A current infection was defined as at least one positive rapid urease test result or histological evaluation of four biopsy sites.[18] Participants were asked to complete a self-administered questionnaire. Demographic, lifestyle, physical activity, and medical history data were collected from the participants. Total energy intake was obtained from the semiquantitative food frequency questionnaire (SQFFQ), which has been previously reported as a reliable and valid questionnaire.[19]

2.3 | DNA extraction

DNA was extracted from the biopsy samples using a MagAttract DNA Blood M48 kit (Qiagen, Hilden, Germany) and BioRobot M48 automatic extraction equipment (Qiagen), according to the manufacturers' instructions.

2.4 | 16S rRNA gene sequencing

Input gDNA (12.5 ng) was amplified with 16S rRNA gene V3-V4 primers, and a subsequent limited cycle amplification step was performed to add multiplexing indices and Illumina sequencing adapters. The final products were normalized and pooled using PicoGreen, and the library sizes were verified using a LabChip GX HT DNA High Sensitivity Kit (PerkinElmer, Massachusetts, USA). Then, we sequenced the samples using the MiSeq™ platform (Illumina, San Diego, USA). Each sequenced sample was prepared according to Illumina 16S rRNA gene Metagenomic Sequencing Library protocols. DNA quantification and quality were measured by PicoGreen and Nanodrop analyses, respectively. The 16S rRNA genes were amplified using 16S rRNA gene V3-V4 primers for the 288 control samples and 268 GC patient samples. The 16S rRNA gene V3-V4 primer sequences were as follows:

16S rRNA gene Amplicon PCR Forward Primer,

5′ TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACG GGNGGCWGCAG 3′

16S rRNA gene Amplicon PCR Reverse Primer,

5′ GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTA CHVGGGTATCTAATCC 3′

The paired-end FASTQ files that had already been demultiplexed were imported to create QIIME2 artifact files. After removing the barcodes/adaptors using Cutadapt, the DADA2 pipeline was applied to remove noisy reads, dereplicate sequences, cluster sequences, and remove chimeras using QIIME v2.2019.7.[20] An amplicon sequence variant table was obtained as the end product. Taxonomic abundance was counted with the Ezbio database.[21] Host mitochondria and chloroplasts, archaea, eukaryotes, and unassigned reads were filtered before calculating relative abundance. The microbial composition was normalized using the values calculated from the taxonomic abundance count divided by the number of preprocessed reads for each sample to obtain the relative abundance.

2.5 | Statistical analysis

2.5.1 | Descriptive statistics

To compare the demographic and lifestyle characteristics between the cases and controls, the chi-square test and Student's *t* test were performed for categorical variables and continuous variables, respectively. Comparisons of the continuous parameters based on the relative abundance of microbial species were performed by the Mann-Whitney U test and Kruskal-Wallis test. *p*-values were adjusted by applying the false discovery rate (FDR) for multiple testing. Spearman rank correlation was applied to calculate the correlation coefficients. Detailed methods for the calculation of microbial diversity measures, including the Shannon index, evenness, richness, Pilou-evenness, and microbial dysbiosis index (MDI), can be found in our previous publication.[22] Briefly, the MDI was derived as follows. The Compositionality Corrected by REenormalization and PEermutation (CCREPE) method was applied to the relative abundance values of 73 genera. Four matrices (*p*-values, Z-stat values, NC score, and false discovery rate (FDR) corrected Q values) were obtained based on the CCREPE analysis. The sub-correlation matrix of the NC score was extracted according to the following two criteria: FDR corrected Q values <0.05 and pairs of genera NC scores |>0.30|. Finally, 64 genera were selected for further analysis. The fold changes of selected genera were calculated by dividing the mean abundance in the cases by that of the controls to identify the genera that were increased in GC (fold change>1) and decreased in GC (fold change<1). The MDI was calculated as the log of [total abundance in genera increased in GC] over [total abundance in genera decreased in GC].

2.5.2 | Association between gastric microbiome and GC risk

The relative abundance of the candidate taxa was categorized into tertiles based on the relative abundance in the control group. The group with the lowest relative abundance was used as the reference group. The odds ratios (ORs) and 95% confidence intervals (CIs) were estimated using unconditional logistic regression models. The median values of relative abundance in each tertile category were used as continuous variables to test for trends. The OR estimates were calculated for the crude model (model I) and model II. Model II was adjusted for age, sex, family history of GC, smoking, alcohol consumption, education, regular exercise, occupation, monthly income, and total energy intake. The interaction between selected bacterial taxa and HP in relation to GC was tested using logistic regression models via likelihood ratio tests. The relative abundances of HP and five other candidate phyla were divided into two groups (low and high) based on the median relative abundance of the control group. All analyses were performed using SAS version 9.4 software (SAS Inc., Cary, NC, USA) and R version 3.5.1 (The R Foundation for Statistical Computing, Vienna, Austria).

2.5.3 | Principal coordinate analysis (PCoA)

PCoA was performed on Bray-Curtis and Jaccard distance measures based on the relative abundance table for the selected bacterial species level by using the R package "vegan." Sample clustering in beta diversity analysis was tested using analysis of similarity (ANOSIM). The statistical significance of the observed R was assessed by 10^4 permutations.

3 | RESULTS

3.1 | General characteristics of the study population

The participants with GC apparently had a family history of GC ($p = 0.003$), were involved in less regular activity ($p < 0.001$), and had lower educational status ($p < 0.001$), lower employment rates

($p = 0.037$), and lower monthly income ($p < 0.001$) than control subjects. The GC patients consumed higher total energy than the controls ($p < 0.001$). Controls had a significantly higher relative abundance of the Actinobacteria phylum than GC patients ($p < 0.001$). Regarding diversity measures, the Shannon index ($p = 0.030$) and Pilou-evenness ($p = 0.004$) were significantly lower among GC cases, whereas richness was significantly lower among healthy controls ($p = 0.009$) (Table S1).

3.2 | Comparison of mean percent of relative abundance

The five main phyla were selected, namely Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria, and non-HP Proteobacteria. After adjustment for the FDR, the abundances of *Actinobacter JVOJ* ($p = 0.012$), *Actinobacter KV797954* ($p < 0.001$), and *Actinobacter KV808398* ($p = 0.018$) species belonging to the Actinobacteria phylum were significantly higher in healthy controls, whereas the abundance of the *Cutibacterium acnes* ($p < 0.001$) species was significantly higher in GC cases. Regarding the Bacteroidetes phylum, the abundance of *Prevotella nigrescens* ($p = 0.042$) was significantly lower in GC cases, while that of *Bacteroides plebeius* ($p = 0.004$) was significantly lower in controls. For the Firmicutes phylum, *Firmicute GL732452* ($p = 0.013$), *Streptococcus vestibularis* ($p = 0.002$), and *Peptostreptococcus stomatis* ($p = 0.002$) were significantly less abundant in GC cases. In the non-HP Proteobacteria phylum, the abundance of *Lautropia mirabilis* ($p = 0.014$) was significantly higher in healthy controls, whereas *Ochrobactrum pseudogrignonense* ($p = 0.048$) and *Proteobacteria NEFZ* ($p = 0.002$) were significantly more abundant in GC patients (Table 1).

A comparison of the mean percent of the relative abundance of bacterial species for the five selected phyla based on HP(-) controls, HP(-) cases, HP(+) controls, and HP(+) cases is presented in Table S2. *Actinobacteria JDFH* was highly abundant in HP(-) controls ($p < 0.001$), whereas *Cutibacterium acnes* was highly enriched in HP(+) cases ($p < 0.001$). In the Bacteroides phylum, *Bacteroides plebeius* ($p < 0.001$), *Porphyromonas endodentalis* ($p < 0.001$), *Porphyromonas gingivalis* ($p = 0.029$), *Porphyromonas pasteri* ($p < 0.001$), *Alloprevotella rava* ($p < 0.001$), and *Alloprevotella tannerae* ($p < 0.001$) were more abundant in the HP(-) controls than in the other three groups. Among the Firmicutes phyla, *Gemella taiwanensis* ($p < 0.001$), *Streptococcus NCVM* ($p < 0.001$), *Streptococcus vestibularis* ($p < 0.001$), *Megasphaera micronuciformis* ($p < 0.001$), and *Veillonella atypica* ($p < 0.001$) were significantly highly enriched in the HP(-) controls. Among the Fusobacteria phyla, *Fusobacterium nucleatum* ($p < 0.001$), *Fusobacterium PEQX* ($p < 0.001$), and *Leptotrichia honkongensis* ($p < 0.001$) were more abundant in the HP(-) controls than in the other three groups. Regarding non-Helicobacter Proteobacteria, *Bosea vaviloviae* ($p = 0.001$), *Delftia acidovorans* ($p < 0.001$), *Delftia lacustris* ($p < 0.001$), *Lautropia mirabilis* ($p < 0.001$), *Neisseria elongata* ($p < 0.001$), *Neisseria perflava* ($p < 0.001$), and *Nisseria subflava* ($p < 0.001$) were significantly highly abundant in HP(-) controls.

3.3 | Relationship between selected phyla and diversity measures

The relative abundances of non-HP Proteobacteria, Actinobacteria, Bacteroidetes, Firmicutes, and Fusobacteria were significantly inversely correlated with the relative abundance of Helicobacter species. Additionally, the Shannon index, richness, and Pilou-evenness indices were significantly inversely correlated with the relative abundance of Helicobacter species. Interestingly, the MDI was significantly positively correlated with the relative abundance of Helicobacter species for the total population, GC cases, and control populations (Table 2). In contrast, the relative abundance of non-Helicobacter Proteobacteria was significantly positively correlated with the Shannon index ($p < 0.001$), richness ($p < 0.001$), and Pilou-evenness ($p < 0.001$), whereas it was negatively significantly correlated with the MDI ($p < 0.001$) (Table S3).

3.4 | Association between selected phyla and gastric cancer risk

We observed that the participants who were in the third tertile of relative abundance of Actinobacteria species showed a significantly higher risk of GC than those who were in the lowest tertile (OR: 3.16, 95% CI = 1.92–5.19, p-trend<0.001) (Table 3).

3.5 | Interaction between selected phyla and *Helicobacter pylori*

Although we did not observe significant interactions, those who were in the category of high relative abundance of Actinobacteria species and HP had a significantly increased risk of GC (OR: 18.63, 95% CI = 5.87–59.18). Moreover, participants who had a high relative abundance of Firmicutes and HP had a significantly increased risk of GC (OR: 8.75, 95% CI = 2.01–38.04) (Table 4).

3.6 | Beta diversity analysis of the microbial composition

A PCoA plot based on the relative abundance of species belonging to five selected main phyla with the Bray-Curtis distance measure is presented in Figure 1. The 2-D plot of the first two principal coordinates shows a significant divergence between the HP statuses. The first two principal coordinates accounted for 18.3% of the total diversity of microbial composition. The non-HP microbiota composition of the four groups was significantly different in the HP-positive and HP-negative groups (ANOSIM R = 0.576, $p = 0.001$) (Figure 1). The Jaccard distance measure also showed similar results (ANOSIM R = 0.576, $p = 0.001$) (Figure S1).

A PCoA plot based on the relative abundance of species belonging to five selected main phyla with the Bray-Curtis distance

TABLE 1 Comparison of mean percent of relative abundance between GC cases and controls

Phylum	Species	Mean (%) ± SD		*p*-value*	FDR adjusted *p*-values
		Controls ($n = 288$)	Cases ($n = 268$)		
Actinobacteria	*Actinobacter JVOJ*	0.012 ± 0.047	0.008 ± 0.026	0.005	0.012
	Actinobacter KV797954	0.02 ± 0.19	0.01 ± 0.03	<0.001	<0.001
	Actinobacter KV808398	0.04 ± 0.23	0.03 ± 0.08	0.013	0.018
	Actinobacter KV831974	0.02 ± 0.11	0.01 ± 0.04	0.393	0.393
	Cutibacterium acnes	0.002 ± 0.008	0.007 ± 0.018	<0.001	<0.001
	Actinobacter JDFH	0.013 ± 0.06	0.007 ± 0.02	0.058	0.068
Bacteroidetes	*Bacteroides plebeius*	0.007 ± 0.04	0.008 ± 0.01	<0.001	0.004
	Bacteroides ADCM	0.005 ± 0.03	0.001 ± 0.006	0.122	0.466
	Bacteroides KI259256	0.009 ± 0.06	0.005 ± 0.02	0.911	0.977
	Porphyromonas endodontalis	0.10 ± 0.38	0.05 ± 0.15	0.695	0.834
	Porphyromonas gingivalis	0.09 ± 0.50	0.03 ± 0.19	0.062	0.326
	Porphyromonas pasteri	0.32 ± 1.02	0.16 ± 0.42	0.792	0.899
	Bacteroides CP017038	0.003 ± 0.01	0.004 ± 0.03	0.431	0.724
	Tannerella forsythia	0.009 ± 0.05	0.004 ± 0.02	0.244	0.539
	Alloprevotella rava	0.11 ± 0.44	0.05 ± 0.23	0.479	0.740
	Alloprevotella tannerae	0.14 ± 0.62	0.05 ± 0.17	0.688	0.834
	Bacteroides FJ976422	0.13 ± 0.67	0.05 ± 0.17	0.077	0.359
	Bacteroides LT608321	0.11 ± 0.56	0.07 ± 0.39	0.579	0.785
	Bacteroides PAC001345	0.28 ± 1.06	0.20 ± 0.66	0.452	0.730
	Bacteroides AF385509	0.01 ± 0.06	0.005 ± 0.02	0.506	0.740
	Bacteroides AM419982	0.009 ± 0.05	0.003 ± 0.012	0.235	0.539
	Bacteroides AM420032	0.021 ± 0.08	0.009 ± 0.05	0.211	0.539
	Bacteroides AY005065	0.02 ± 0.18	0.01 ± 0.04	0.296	0.592
	Bacteroides AZHT	0.02 ± 0.11	0.006 ± 0.03	0.677	0.834
	Bacteroides CP003667	0.09 ± 0.42	0.03 ± 0.13	0.010	0.105
	Bacteroides HE999470	0.007 ± 0.039	0.004 ± 0.021	0.930	0.977
	Bacteroides KI259591	0.02 ± 0.11	0.004 ± 0.02	0.061	0.326
	Bacteroides PAC001346	0.25 ± 0.99	0.11 ± 0.39	0.411	0.719
	Prevotella aurantiaca	0.02 ± 0.14	0.005 ± 0.02	0.004	0.056
	Prevotella baroniae	0.01 ± 0.07	0.002 ± 0.007	0.511	0.740
	Prevotella denticola	0.009 ± 0.05	0.003 ± 0.01	0.675	0.834
	Prevotella histicola	0.33 ± 1.87	0.16 ± 0.75	0.244	0.539
	Prevotella intermedia	0.10 ± 0.46	0.03 ± 0.14	0.023	0.193
	Prevotella jejuni	0.28 ± 1.09	0.14 ± 0.66	0.106	0.445
	Prevotella melaninogenica	0.71 ± 2.21	0.32 ± 1.00	0.038	0.266
	Prevotella nanceiensis	0.04 ± 0.15	0.04 ± 0.16	0.572	0.785
	Prevotella nigrescens	0.08 ± 0.62	0.01 ± 0.05	0.002	0.042
	Prevotella oris	0.04 ± 0.19	0.02 ± 0.05	1.000	1.000
	Prevotella pallens	0.40 ± 1.45	0.20 ± 0.80	0.189	0.539
	Prevotella pleuritidis	0.008 ± 0.06	0.002 ± 0.008	0.313	0.597
	Prevotella salivae	0.11 ± 0.38	0.07 ± 0.30	0.995	1.000
	Prevotella shahii	0.02 ± 0.11	0.007 ± 0.04	0.175	0.539
	Bacteroides DQ241813	0.02 ± 0.06	0.01 ± 0.06	0.781	0.899

(Continues)

TABLE 1 (Continued)

Phylum	Species	Mean (%) ± SD		p-value*	FDR adjusted p-values
		Controls (n = 288)	Cases (n = 268)		
	Bacteroides JQ448119	0.008 ± 0.03	0.009 ± 0.05	0.198	0.539
	Capnocytophaga gingivalis	0.02 ± 0.24	0.005 ± 0.02	0.892	0.977
	Capnocytophaga granulosa	0.03 ± 0.16	0.009 ± 0.03	0.372	0.679
	Capnocytophaga leadbetteri	0.03 ± 0.16	0.02 ± 0.11	0.229	0.539
	Capnocytophaga sputigena	0.02 ± 0.11	0.008 ± 0.04	0.262	0.550
Firmicutes	*Firmicute AY642552*	0.006 ± 0.02	0.007 ± 0.01	0.075	0.231
	Anaerobacillus macyae	0.001 ± 0.005	0.002 ± 0.007	0.008	0.064
	Gemella morbillorum	0.004 ± 0.02	0.009 ± 0.06	0.994	0.999
	Gemella sanguinis	0.007 ± 0.03	0.01 ± 0.04	0.219	0.438
	Gemella taiwanensis	0.10 ± 0.69	0.07 ± 0.40	0.044	0.160
	Firmicute JVNU	0.03 ± 0.12	0.03 ± 0.07	0.019	0.109
	Firmicute CP006776	0.02 ± 0.08	0.02 ± 0.06	0.136	0.360
	Firmicute GL732452	0.07 ± 0.24	0.06 ± 0.19	0.001	0.013
	Firmicute GL890994	0.004 ± 0.02	0.006 ± 0.03	0.345	0.569
	Firmicute KB373315	0.02 ± 0.06	0.02 ± 0.07	0.850	0.895
	Firmicute KQ969067	0.02 ± 0.05	0.01 ± 0.03	0.006	0.060
	Streptococcus NCVM	0.75 ± 2.87	0.78 ± 2.45	0.019	0.109
	Streptococcus vestibularis	0.08 ± 0.65	0.05 ± 0.22	<0.001	0.002
	Firmicute GQ422712	0.005 ± 0.03	0.002 ± 0.009	0.671	0.818
	Firmicute PAC001351	0.006 ± 0.03	0.004 ± 0.02	0.513	0.684
	Catonella morbi	0.02 ± 0.07	0.008 ± 0.03	0.403	0.613
	Stomatobaculum longum	0.009 ± 0.04	0.004 ± 0.02	0.356	0.569
	Oribacterium asaccharolyticum	0.009 ± 0.04	0.003 ± 0.02	0.717	0.819
	Oribacterium sinus	0.008 ± 0.03	0.005 ± 0.01	0.171	0.360
	Eubacterium sulci	0.008 ± 0.03	0.005 ± 0.02	0.320	0.556
	Filifactor alocis	0.02 ± 0.09	0.006 ± 0.02	0.068	0.227
	Peptostreptococcus stomatis	0.009 ± 0.04	0.008 ± 0.03	<0.001	0.002
	Firmicute PAC001339	0.013 ± 0.08	0.004 ± 0.02	0.695	0.818
	Firmicute PAC001348	0.01 ± 0.06	0.005 ± 0.02	0.170	0.360
	Solobacterium moorei	0.009 ± 0.03	0.008 ± 0.03	0.101	0.288
	Firmicute CP012071	0.03 ± 0.11	0.02 ± 0.07	0.414	0.613
	Selenomonas sputigena	0.01 ± 0.05	0.008 ± 0.04	0.816	0.882
	Dialister invisus	0.005 ± 0.02	0.003 ± 0.01	0.028	0.140
	Dialister pneumosintes	0.01 ± 0.07	0.003 ± 0.01	0.266	0.484
	Megasphaera micronuciformis	0.04 ± 0.19	0.04 ± 0.16	0.042	0.160
	Firmicute AFUJ	0.02 ± 0.12	0.005 ± 0.03	0.596	0.769
	Firmicute PAC001353	0.02 ± 0.11	0.007 ± 0.03	0.034	0.151
	Veillonella atypica	0.31 ± 1.14	0.18 ± 0.80	0.742	0.824
	Veillonella dispar	0.30 ± 0.11	0.17 ± 0.57	0.249	0.474
	Veillonella rogosae	0.07 ± 0.22	0.06 ± 0.16	0.499	0.684
	Veillonella tobetsuensis	0.04 ± 0.17	0.03 ± 0.11	0.475	0.679

(Continues)

TABLE 1 (Continued)

Phylum	Species	Mean (%) ± SD		p-value*	FDR adjusted p-values
		Controls (n = 288)	Cases (n = 268)		
	Parvimonas micra	0.003 ± 0.01	0.003 ± 0.01	0.146	0.360
Fusobacteria	*Fusobacterium canifelinum*	0.03 ± 0.16	0.02 ± 0.08	0.855	0.914
	Fusobacterium nucleatum	0.15 ± 0.54	0.05 ± 0.11	0.075	0.410
	Fusobacterium PEQX	0.31 ± 0.98	0.17 ± 0.44	0.191	0.478
	Fusobacterium FJ976402	0.01 ± 0.05	0.008 ± 0.03	0.898	0.914
	Fusobacterium KI272869	0.02 ± 0.14	0.02 ± 0.07	0.082	0.410
	Leptotrichia hongkongensis	0.005 ± 0.02	0.005 ± 0.02	0.293	0.586
	Fusobacterium PAC001344	0.02 ± 0.13	0.02 ± 0.16	0.184	0.478
	Fusobacterium PAC001350	0.13 ± 0.60	0.05 ± 0.19	0.890	0.914
	Fusobacterium PAC001356	0.03 ± 0.11	0.01 ± 0.07	0.914	0.914
	Fusobacterium AJ289183	0.01 ± 0.05	0.003 ± 0.01	0.457	0.762
Non-HP Proteobacteria	*Brevundimonas albigilva*	0.002 ± 0.005	0.002 ± 0.004	0.226	0.428
	Bosea vaviloviae	0.03 ± 0.03	0.02 ± 0.02	0.612	0.847
	AXAI	0.007 ± 0.01	0.007 ± 0.01	0.018	0.076
	Ochrobactrum pseudogrignonense	0.006 ± 0.01	0.007 ± 0.01	0.008	0.048
	Taonella mepensis	0.001 ± 0.003	0.001 ± 0.004	0.623	0.847
	JRKM	0.003 ± 0.008	0.004 ± 0.008	0.103	0.285
	Delftia acidovorans	0.03 ± 0.05	0.03 ± 0.04	0.001	0.012
	Delftia lacustris	0.04 ± 0.04	0.03 ± 0.03	0.547	0.821
	Diaphorobacter polyhydroxybutyra	0.001 ± 0.005	0.003 ± 0.008	0.002	0.014
	OCMW	0.08 ± 0.10	0.08 ± 0.08	0.017	0.076
	Lautropia mirabilis	0.03 ± 0.17	0.006 ± 0.02	0.002	0.014
	JYOB	0.08 ± 0.09	0.08 ± 0.07	0.057	0.205
	Kingella denitrificans	0.005 ± 0.02	0.004 ± 0.02	0.831	0.870
	Neisseria elongate	0.02 ± 0.12	0.03 ± 0.09	0.314	0.538
	Neisseria flava	0.09 ± 0.38	0.14 ± 0.61	0.870	0.870
	Neisseria oralis	0.007 ± 0.04	0.005 ± 0.02	0.507	0.794
	Neisseria perflava	1.05 ± 4.61	0.47 ± 1.84	0.635	0.847
	Neisseria subflava	0.66 ± 2.56	0.49 ± 1.49	0.183	0.366
	Campylobacter showae	0.012 ± 0.05	0.006 ± 0.02	0.128	0.315
	JH414887	0.08 ± 0.41	0.02 ± 0.08	0.178	0.366
	Cardiobacterium hominis	0.002 ± 0.02	0.002 ± 0.008	0.073	0.228
	Shigella flexneri	0.003 ± 0.03	0.003 ± 0.009	<0.001	0.002
	Actinobacillus minor	0.05 ± 0.23	0.07 ± 0.28	0.285	0.513
	Actinobacillus porcinus	0.01 ± 0.06	0.02 ± 0.13	0.773	0.863
	Aggregatibacter aphrophilus	0.03 ± 0.21	0.02 ± 0.08	0.760	0.863
	Aggregatibacter segnis	0.03 ± 0.16	0.03 ± 0.18	0.762	0.863
	Haemophilus parahaemolyticus	0.88 ± 3.07	0.87 ± 3.61	0.673	0.863
	Haemophilus quentini	0.10 ± 0.41	0.078 ± 0.28	0.791	0.863
	Haemophilus sputorum	0.03 ± 0.16	0.02 ± 0.09	0.852	0.870

(Continues)

TABLE 1 (Continued)

Phylum	Species	Mean (%) ± SD		p-value*	FDR adjusted p-values
		Controls (n = 288)	Cases (n = 268)		
	JH591066	0.13 ± 0.98	0.08 ± 0.37	0.755	0.863
	JUTE	0.51 ± 1.25	0.37 ± 0.84	0.140	0.315
	KV838018	0.11 ± 0.36	0.08 ± 0.24	0.076	0.228
	NEFZ	0.006 ± 0.02	0.01 ± 0.08	<0.001	0.002

Note: *p-values were obtained using Mann-Whitney U test.
Abbreviation: FDR, False discovery rate.

TABLE 2 Correlation between relative abundance of Helicobacter species among selected phyla and diversity measures

Total population	Rel.abundance of Helicobacter species
Rel. abundance of Non-Helicobacter Proteobacteria species	R = −0.834, $p < 0.001$
Rel.abundance of Actinobacteria species	R = −0.627, $p < 0.001$
Rel.abundance of Bacteroidetes species	R = −0.883, $p < 0.001$
Rel.abundance of Firmicutes species	R = −0.716, $p < 0.001$
Rel.abundance of Fusobacteria species	R = −0.780, $p < 0.001$
Microbial dysbiosis index (MDI)	R = 0.868, $p < 0.001$
Shannon index	R = −0.938, $p < 0.001$
Richness	R = −0.577, $p < 0.001$
Evenness	R = −0.045, $p = 0.290$
Pilou-evenness	R = −0.926, $p < 0.001$
GC cases	Rel.abundance of Helicobacter species
Rel. abundance of Non-Helicobacter Proteobacteria species	R = −0.783, $p < 0.001$
Rel.abundance of Actinobacteria species	R = −0.647, $p < 0.001$
Rel.abundance of Bacteroidetes species	R = −0.790, $p < 0.001$
Rel.abundance of Firmicutes species	R = −0.778, $p < 0.001$
Rel.abundance of Fusobacteria species	R = −0.711, $p < 0.001$
Microbial dysbiosis index (MDI)	R = 0.790, $p < 0.001$
Shannon index	R = −0.880, $p < 0.001$
Richness	R = −0.537, $p < 0.001$
Evenness	R = −0.008, $p = 0.898$
Pilou-evenness	R = −0.870, $p < 0.001$
Controls	Rel.abundance of Helicobacter species
Rel. abundance of Non-Helicobacter Proteobacteria species	R = −0.857, $p < 0.001$
Rel.abundance of Actinobacteria species	R = −0.637, $p < 0.001$
Rel.abundance of Bacteroidetes species	R = −0.923, $p < 0.001$
Rel.abundance of Firmicutes species	R = −0.721, $p < 0.001$
Rel.abundance of Fusobacteria species	R = −0.815, $p < 0.001$
Microbial dysbiosis index (MDI)	R = 0.905, $p < 0.001$
Shannon index	R = −0.967, $p < 0.001$
Richness	R = −0.635, $p < 0.001$
Evenness	R = −0.060, $p = 0.309$
Pilou-evenness	R = −0.950, $p < 0.001$

measure is presented in Figure 2. The 2-D plot of the first two principal coordinates shows a marked divergence between the HP status and GC status. The first two principal coordinates accounted for 18.3% of the total diversity of microbial composition. The non-HP microbiota composition of the four groups was significantly different from that of the four groups (ANOSIM R = 0.10, $p = 0.001$). The

TABLE 3 Association between selected phyla and GC risk

	No.of controls	No.of cases	Model I OR (95% CI)	Model II OR (95% CI)
Actinobacteria species				
<0.0000761	96(33.3)	42(15.7)	1.00	1.00
0.0000761–0.0003455	95(33.0)	78(29.1)	1.88(1.17–3.00)	1.96(1.17–3.31)
>0.0003455	97(33.7)	148(55.2)	3.48(2.24–5.43)	3.16(1.92–5.19)
p-trend			<0.001	<0.001
Bacteroidetes species				
<0.002511289	95(33.0)	101(37.7)	1.00	1.00
0.002511289–0.010917	97(33.7)	81(30.2)	0.79(0.52–1.18)	0.81(0.51–1.28)
>0.010917	96(33.3)	86(32.1)	0.84(0.56–1.26)	0.73(0.46–1.17)
p-trend			0.602	0.252
Firmicutes species				
<0.001619615	95(33.0)	75(28.0)	1.00	1.00
0.001619615–0.007862165	96(33.3)	100(37.3)	1.32(0.87–1.99)	1.36(0.85–2.16)
>0.007862165	97(33.7)	93(34.7)	1.21(0.80–1.84)	0.95(0.59–1.53)
p-trend			0.669	0.395
Fusobacteria species				
<0.000475638	95(33.0)	105(39.2)	1.00	1.00
0.000475638–0.001839759	96(33.3)	69(25.8)	0.65(0.43–0.98)	0.67(0.41–1.07)
>0.001839759	97(33.7)	94(35.1)	0.88(0.59–1.30)	0.84(0.54–1.33)
p-trend			0.914	0.894
Non-HP Proteobacteria species				
<0.004894718	95(33.0)	111(41.4)	1.00	1.00
0.004894718–0.014834	96(33.3)	66(24.6)	0.59(0.39–0.89)	0.57(0.35–0.91)
>0.014834	97(33.7)	91(34.0)	0.80(0.54–1.19)	0.70(0.44–1.11)
p-trend			0.755	0.401

Note: Model I: crude model. Model II: adjusted for age, sex, family history of GC, smoking, alcohol consumption, education, regular exercise, occupation, monthly income, total energy intake.

Jaccard distance measures also showed similar results (ANOSIM $R = 0.10$, $p = 0.001$) (Figure S2).

4 | DISCUSSION

In this study, we primarily focused on the role of non-HP bacteria in the risk of GC in a Korean population. We selected five main phyla present in the gastric environment, namely Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria, and non-HP Proteobacteria. All five selected phyla were significantly negatively correlated with the relative abundance of Helicobacter species for the total population, GC cases, and controls. Interestingly, microbial diversity measures viz. the Shannon index, and Pilou-evenness were significantly negatively correlated with the relative abundance of Helicobacter species, while the MDI was significantly positively correlated with Helicobacter species. There were no significant interactions between HP and other candidate phyla in the risk of GC. Based on permutational multivariate analysis of variance (PERMANOVA), microbial compositions were significantly different based on HP status and HP status within GC cases and controls.

A study on the gastric microbiota of 10 subjects without HP infection concluded that Firmicutes was the most dominant phylum, followed by Proteobacteria, Bacteroidetes, Actinobacteria, and Fusobacteria.[23] This finding was similar to our results, in which we found five similar main phyla that were highly abundant in our study population, although the order of abundance was different. Some previous studies have reported that HP can influence changes in gastric microbial composition.[24,25] We found that the microbial composition was significantly different between HP-positive and HP-negative individuals in our study. A study profiled the gastric microbiota based on HP status using biopsy samples collected from 31 patients with GC. Their results revealed that the relative abundances of the *Bradyhizobiaceae*, *Caulobacteraceae*, *Lactobacillaceae*, and *Burkholderiaceae* families were significantly greater among patients who are negative for HP infection while the relative abundance of family *Helicobacteraceae* was significantly higher in patients who are positive for HP.[26]

TABLE 4 Interaction between selected phyla and *Helicobacter pylori* in the risk of GC

	H. pylori < 0.978 [Low]		*H. pylori* ≥ 0.978 [High]		
	Low	High	Low	High	*p*-interaction
Actinobacteria species	<0.0002	≥0.0002	<0.0002	≥0.0002	
No. Controls/Cases	26/5	118/121	118/60	26/82	
Crude OR	1.00(ref)	5.33(1.98–14.35)	2.64(0.97–7.23)	16.40(5.72–47.03)	0.794
Model I OR	1.00(ref)	4.69(1.60–13.74)	2.74(0.92–8.13)	18.63(5.87–59.18)	0.558
Bacteroidetes species	<0.006	≥0.006	<0.006	≥0.006	
No. Controls/Cases	16/18	128/108	128/131	16/11	
Crude OR	1.00(ref)	0.75(0.37–1.54)	0.91(0.45–1.86)	0.61(0.22–1.70)	0.841
Model I OR	1.00(ref)	0.68(0.31–1.49)	0.98(0.45–2.14)	0.71(0.24–2.16)	0.906
Firmicutes species	<0.003	≥0.003	<0.003	≥0.003	
No. Controls/Cases	17/3	127/123	127/113	17/29	
Crude OR	1.00(ref)	5.49(1.57–19.19)	5.04(1.44–17.65)	9.66(2.47–37.86)	0.121
Model I OR	1.00(ref)	4.48(1.18–16.96)	4.92(1.30–18.64)	8.75(2.01–38.04)	0.213
Fusobacteria species	<0.001	≥0.001	<0.001	≥0.001	
No. Controls/Cases	19/15	125/111	125/123	19/19	
Crude OR	1.00(ref)	1.13(0.55–2.32)	1.25(0.61–2.56)	1.27(0.50–3.21)	0.841
Model I OR	1.00(ref)	1.35(0.60–3.04)	1.73(0.76–3.91)	1.67(0.57–4.86)	0.566
Non-HP Proteobacteria species	<0.008	≥0.008	<0.008	≥0.008	
No. Controls/Cases	15/11	129/115	129/133	15/9	
Crude OR	1.00(ref)	1.22(0.54–2.75)	1.41(0.62–3.18)	0.82(0.26–2.55)	0.220
Model I OR	1.00(ref)	1.52(0.60–3.92)	2.05(0.80–5.25)	1.11(0.31–4.02)	0.131

Note: Model I: adjusted for age, sex, family history of GC, smoking, alcohol consumption, education, regular exercise, occupation, monthly income, total energy intake.

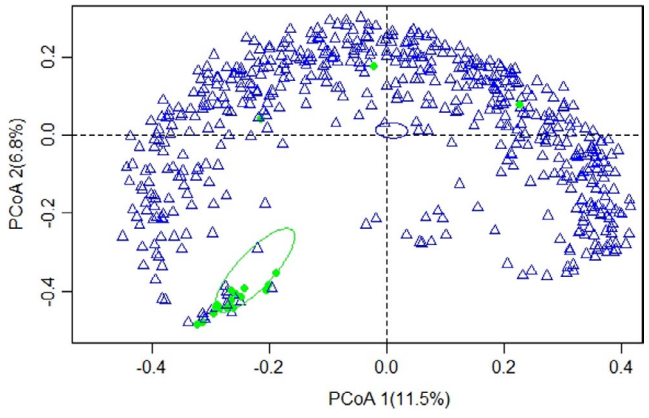

FIGURE 1 Principal coordinate analysis (PCoA) plot of the Bray-Curtis distance depending on the HP status. Blue: HP(+), green: (HP(-). The blue, and green ellipses represent where 95% of data belong to the HP(+), and HP(-) groups, respectively

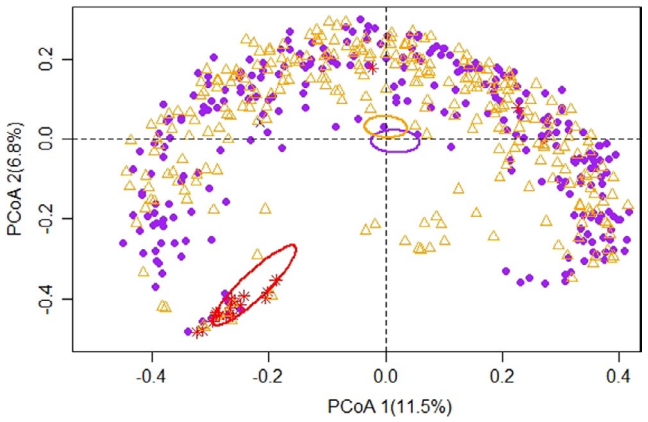

FIGURE 2 Principal coordinate analysis (PCoA) plot of the Bray-Curtis distance depending on the HP and GC status. Blue: HP(-) cases, red: HP(-) controls, purple: HP(+) controls, and orange: HP(+) cases. The blue, red, purple, and orange ellipses represent where 95% of data belong to the HP(-) cases, HP(-) controls, HP(+) controls, and HP(+) cases, respectively

Interestingly, we observed that *Peptostreptococcus stomatis* was highly enriched in healthy controls compared with GC cases. Although this bacterium has been identified as a commensal bacterium, it was reported that *P. stomatis* was associated with gastric tumors.[27] The relative abundance of *Cutibacterium acnes*, which is formally known as *Propionibacterium acnes*, was significantly higher in GC cases than in controls. In our previous study that reported an association between the relative abundance of gastric microbiota and GC risk, *P. acnes* was relatively highly abundant in GC cases.[28] *C. acnes*, which can be primarily found in the skin microbiome, has been reported to be present in the gastric microbiome.[29] Furthermore, *C. acnes* can cause lymphocytic gastritis, leading to the production of proinflammatory

cytokines such as IL-15, which can stimulate the process of gastric carcinogenesis.[30] *P. acnes* was found to be more abundant in men with prostate carcinoma, and experimental results revealed that *P. acnes* has the capacity to modulate the secretion of IL-6 and IL-8, which are suggested to play an important role in the development of different types of cancer, including prostate cancer.[31] We also found that the relative abundance of *Prevotella nigrescens* was significantly higher in healthy controls. A previous study reported that *P. nigrescens* seems to be more frequently recovered from healthy gingivae as a commensal bacterium.[32]

Porphyromonas endodentalis, *Porphyromonas gingivalis*, and *Porphyromonas pasteri* bacterial species were identified from those who had untreated necrotic pulp (primary infection), although there is a paucity of data on their roles in GC.[33] In our study, we found that the relative abundance of those three species was higher in controls who were HP(-). *Veillonella atypica* was also observed as a highly abundant bacterium in HP(-) healthy controls. Although it is an oral microbe, a previous study reported that *Veillonella* was associated with an increased risk of cardia cancer.[34] *Fusobacterium nucleatum* was also highly enriched in HP(-) controls in our study. However, previous evidence reported that *F. nucleatum* is a pathogenic bacterium that increases GC risk.[35,36] Neisseria species appear to be early colonizers of the oral cavity, and *Neisseria subflava* and *Neisseria perflava* have been identified as nonpathogenic bacteria in the human oral cavity.[37] We found that these two Neisseria species were relatively highly enriched in HP(-) controls.

We used the relative abundance of the microbiome data, which are compositional in nature. The relative abundance of a taxon in a clinical sample is the fraction of the taxon observed in the feature table relative to the sum of all observed taxa corresponding to the sample in the feature table, while the absolute abundance refers to the unobservable actual abundance in a unit volume of an ecosystem.[38] The compositional nature of the microbiome data comes from the fact that a correction must be made for different samples having different numbers of sequences while the total absolute abundance of all bacteria in each sample is unknown.[39] This issue is important in the differential abundance analysis of microbiome data and for the interpretations of the results.

The relative abundances of Actinobacteria, Bacteriodetes, Firmicutes, Fusobacteria, and non-HP Proteobacteria were negatively associated with the relative abundance of Helicobacter species in our study. This could be explained by the bacterial succession mechanism due to pH changes in the stomach environment.[36] Additionally, bacterial diversity measures such as the Shannon index and Pilou-evenness were negatively associated with the relative abundance of Helicobacter species. It has been found that individuals who have HP-negative status show more diversity of the microbial community in the stomach.[36] The relative abundance of Helicobacter species was positively associated with MDI in our study. In our previous study, we found that MDI was associated with an increased risk of GC.[22] At the genus level, *Rhodococcus* has been identified as one of the genera enriched in GC,[40] although this genus is very rare in our study population. Many studies have found that the flora of GC patients has changed, and the significantly changed flora in the cancer tissue at different levels of classification have been identified.[41,42]

Participants who were in the third tertile of relative abundance of the Actinobacteria phylum showed a significantly higher risk of GC than those who were in the lowest tertile. Furthermore, we did not identify significant interactions between selected phyla and the relative abundance of Helicobacter species. However, those who had a high relative abundance of Actinobacteria and Firmicutes with a high relative abundance of Helicobacter species showed a significantly increased risk of GC. There is overwhelming evidence supporting the notion that HP plays a vital role in GC, while few studies have identified the mechanisms of non-HP bacteria that also play an important role in the development of GC.[43,44]

Several possible biological mechanisms related to non-HP bacteria in the risk of GC have been reported. They can promote GC by inducing inflammatory responses by producing redox proteins in the human body, which results in the presence of several diseases, including GC.[34,45] Non-HP bacteria can promote GC by influencing the function of immune cells in the tumor microenvironment. A recent study found correlations between gastric microbiota and immune cells: The number of BDCA2+ plasmacytoid dendritic cells was positively correlated with the abundance of *Stenotrophomonas*, and the number of Foxp3+ regulatory T cells was positively correlated with the abundance of *Selenomonas* in the microenvironment of GC. They concluded that these immune cells may be modulated by the changed microbiota, which participates in the formation of an immunosuppressive microenvironment.[46] Non-HP bacteria can promote GC through the production of ion metabolites, specifically reactive oxygen species (ROS) and NOCs that are associated with the risk of GC.[11,47,48]

Our study has several strengths. First, we included a relatively large sample size to observe the associations and interaction effects with increased statistical power. Second, we considered several possible confounding variables that are potential risk factors for the gastric microbiome and GC risk. There are potential limitations associated with our study. First, selection and recall biases need to be considered due to the case-control nature of the study. Second, due to the case-control study design, the associations observed for the non-HP microbiome and GC risk may not be causal.

In conclusion, non-HP bacteria may play a pivotal role in GC development. The five main phyla present in the stomach, namely Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria, and non-HP Proteobacteria, have a negative relationship with Helicobacter species. Microbial diversity measures were also negatively associated with Helicobacter species abundance. The microbial composition between GC cases and controls differed based on HP-positive and HP-negative status. This finding may indicate the specific roles of non-HP bacteria in GC development in a Korean population.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

MG, IJC, YIK, and JK designed the study. JL, IJC, YIK, and JK collected the data and conducted the study. MG and JL performed the statistical analysis. MG drafted the paper. IJC, YIK, and JK provided critical review of the manuscript. JK had primary responsibility for the final content. All authors read and approved the final manuscript.

SEQUENCE DATA

These sequence data have been submitted to the GenBank databases under accession number KEQH00000000.

DATA AVAILABILITY STATEMENT

Data are available from the corresponding author upon request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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