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Gastric microbiota in patients with *Helicobacter pylori*-negative gastric MALT lymphoma

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Abstract

To investigate the mucosal microbiota in the stomach of patients with *Helicobacter pylori*-negative mucosa-associated lymphoid tissue (MALT) lymphoma by means of metagenomic analysis.

Although some gastric MALT lymphomas are associated with the presence of *H. pylori*, other gastric MALT lymphomas occur independently of *H. pylori* infection. The pathogenesis of *H. pylori*-negative MALT lymphoma remains unclear.

Mucosal biopsy specimens were collected from the gastric body from 33 MALT lymphoma patients with gastric lesions, including both *H. pylori*-infection naïve patients and posteradication patients, as well as 27 control participants without *H. pylori* infection or cancer. Subsequently, the samples were subjected to 16S rRNA gene sequencing. Quantitative insights into microbial ecology, linear discriminant analysis effect size, and phylogenetic investigation of communities by reconstruction of unobserved states softwares were used to analyze the participants' microbiota.

H. pylori-negative MALT lymphoma patients had significantly lower alpha diversity ($P = .04$), compared with control participants. Significant differences were evident in the microbial composition ($P = .04$), as determined by comparison of beta diversity between the 2 groups. Taxonomic composition analysis indicated that the genera *Burkholderia* and *Sphingomonas* were significantly more abundant in MALT lymphoma patients, while the genera *Prevotella* and *Veillonella* were less abundant. Functional microbiota prediction showed that the predicted gene pathways "replication and repair," "translation," and "nucleotide metabolism" were downregulated in MALT lymphoma patients.

H. pylori-negative MALT lymphoma patients exhibited altered gastric mucosal microbial compositions, suggesting that altered microbiota might be involved in the pathogenesis of *H. pylori*-negative MALT lymphoma.

Abbreviations: LEfSe = linear discriminant analysis effect size, MALT = mucosa-associated lymphoid tissue, PPIs = proton pump inhibitors, QIIME = quantitative insights into microbial ecology.

Keywords: dysbiosis, metagenomics, mucosa-associated lymphoid tissue lymphoma, mucosal microbiome, stomach

1. Introduction

Mucosa-associated lymphoid tissue (MALT) lymphoma is a low-grade malignant lymphoma that arises in the MALT of extranodal organs such as the gastrointestinal tract, lungs, salivary glands, thyroid, liver, and skin. The stomach is the organ most frequently affected by MALT lymphoma; it is involved in 20% to 40% of all extranodal lymphomas and 40% to 50% of primary gastric malignant lymphomas.[1] *Helicobacter pylori* infection is a known etiological agent of this disease, such that approximately 90% of affected patients exhibit *H. pylori* infection; moreover, *H. pylori* eradication results in remission in 70% to 80% of affected patients.[2] However, some gastric MALT lymphomas occur independently of *H. pylori* infection. Furthermore, some MALT lymphoma patients do not respond to *H. pylori* eradication therapy, while others respond to antibiotics despite the absence of *H. pylori*.[3] For patients with *H. pylori*-negative MALT lymphoma, the possibility of bacterial infections other than *H. pylori* has been suggested. Among *Helicobacter* spp., *Helicobacter heilmannii*[4] and *Helicobacter suis*[5] are reportedly associated with gastric MALT lymphoma. *Campylobacter jejuni* in the duodenum and jejunum, *Borrelia burgdorferi* in the skin, and *Chlamydia psittaci* in the ocular appendages are also potentially associated with MALT lymphoma.[6]

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The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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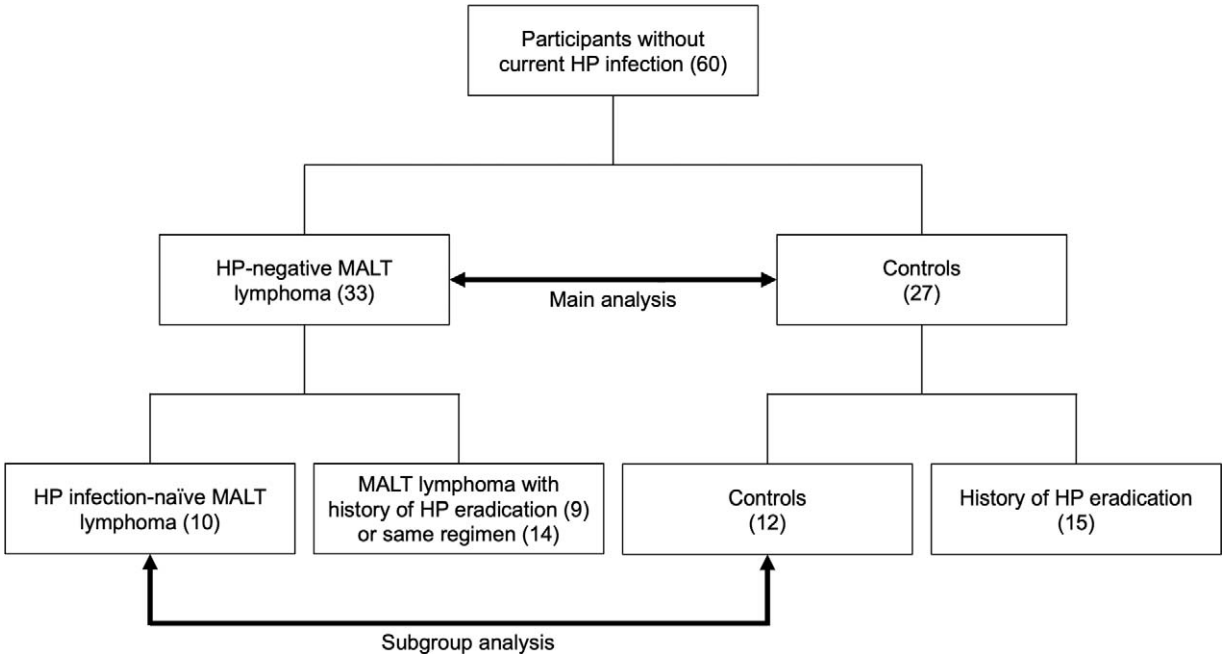


Figure 1. Schematic of participant selection and analysis. All participants were not currently infected with *H. pylori* (HP). They were divided into 2 groups: *H. pylori*-negative gastric MALT lymphoma (n = 33) and controls (n = 27). *H. pylori*-infection naïve patients were defined as those who had no signs of current or prior infection with *H. pylori*. In the *H. pylori*-infection naïve group, 10 patients had not been treated with antibiotics mimicking eradication therapy. MALT = mucosa-associated lymphoid tissue mucosa-associated lymphoid tissue.

Recently, high-throughput sequencing and metagenomic analysis have revealed that the intestinal microbiota may be involved in the pathogenesis of various diseases.[7,8] Although the importance of microbiota in the stomach has been unclear (with the exception of *H. pylori*), gastric cancer patients have been reported to exhibit a dysbiotic microbial community.[9] To the best of our knowledge, there have been no studies of the microbiota in patients with gastric MALT lymphoma. In this study, we investigated whether bacteria other than *H. pylori* may be involved in the pathogenesis of MALT lymphoma. We compared the composition of the gastric mucosal microbiota between patients with *H. pylori*-negative gastric MALT lymphoma and control participants.

2. Materials and methods

2.1. Participants

This cross-sectional study was conducted at Kyushu University Hospital from August 2017 to July 2019, with the primary endpoint of identifying bacteria other than *H. pylori* that may be involved in the pathogenesis of *H. pylori*-negative MALT lymphoma. Sixty participants were enrolled in the study; this number of participants was determined on the basis of our previous results.[8] An a priori power analysis using G*Power 3.1 (Heinrich Heine University, Duesseldorf, Germany)[10] revealed that a sample size of 58 should be sufficient to detect a similar congruency effect in our experiment. Accordingly, the study was designed with 60 overall participants. All control participants ("controls") had no *H. pylori* infection or cancer. Furthermore, patients with a history of malignant lymphoma, inflammatory bowel disease, gastrointestinal cancer, or gastrectomy were excluded from the study. The Lugano International Conference Classification was used to determine the clinical stage, while prognostic factors for MALT lymphoma[11] were used to stratify patients according to the risk of MALT lymphoma. Biopsy specimens of gastric mucosa were used to evaluate *Apoptosis*

Table 1

Participants' clinical characteristics.

	Patients with MALT lymphoma (n = 33)	Controls (n = 27)
Sex (male/female)	13/20	10/17
Age (years, median [IQR])	58 (49–64)	65 (57–71)
Clinical stage (Lugano International Conference Classification)		
I	32	–
II	0	–
IV	1	–
API2-MALT1 fusion	5	–
MALT lymphoma International Prognostic Index		
Low	29	–
Intermediate	3	–
High	1	–
Concomitant drugs		
Proton pump inhibitor	6	3
Probiotics	0	0
Lymphoma treatment		
Untreated	10	–
Eradication therapy for *Helicobacter pylori*	9	–
Antibiotics	14	–
Helicobacter pylori infection		
Naïve	24	12
Posteradication	9	15

Data shown as numbers of patients, except where indicated.
API2 = apoptosis inhibitor 2, IQR = interquartile range, MALT = mucosa-associated lymphoid tissue.

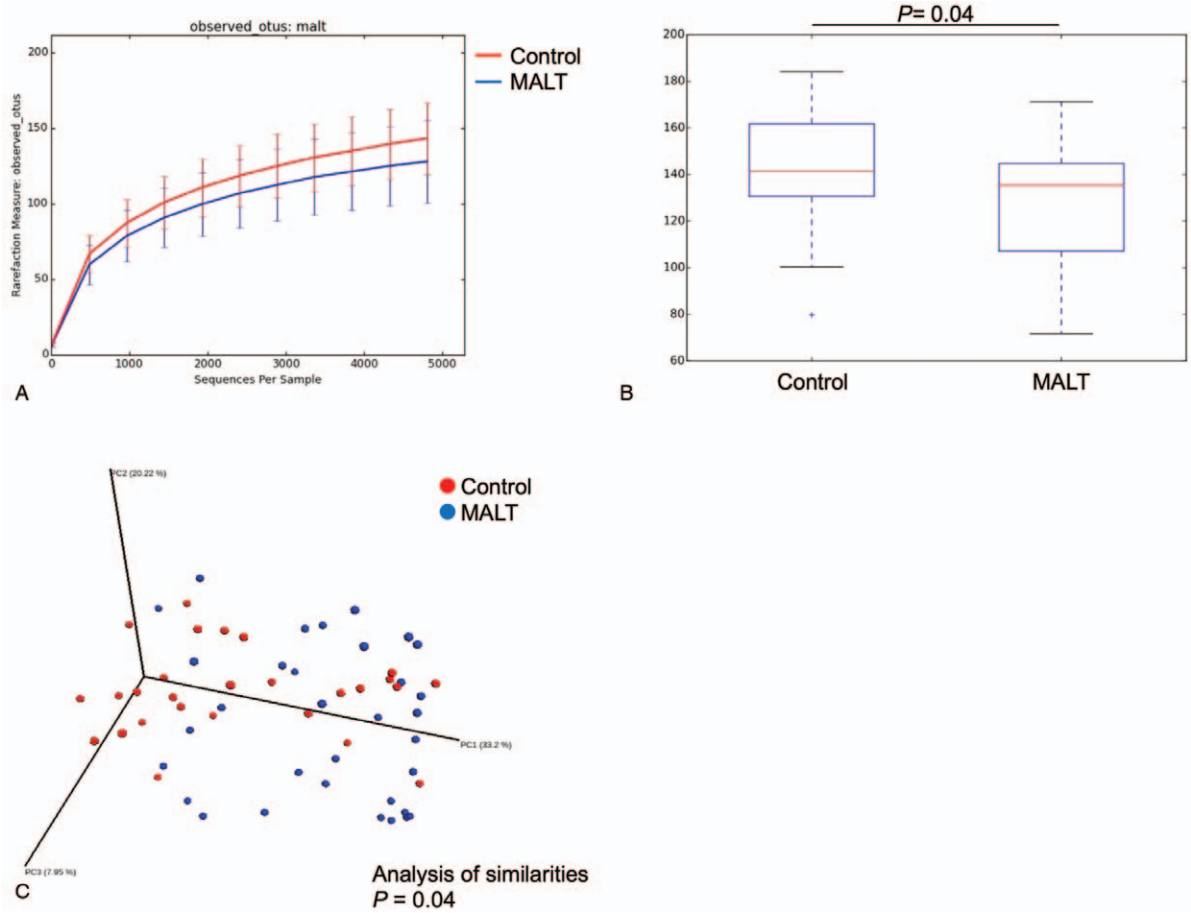


Figure 2. Comparisons of alpha and beta diversities between *H. pylori*-negative gastric MALT lymphoma patients and controls. (A) Rarefaction curves showing observed species in MALT lymphoma patients (n = 33) and controls (n = 27) at various sequencing depths. (B) Box plots showing alpha diversities at higher sequencing depths. Alpha diversity was significantly lower in MALT lymphoma patients than in controls ($P = .04$). (C) Principal coordinates analysis with weighted UniFrac beta diversity metrics showing significantly different microbial composition between MALT lymphoma patients and controls ($P = .04$). MALT = mucosa-associated lymphoid tissue mucosa-associated lymphoid tissue.

inhibitor 2-MALT gene translocation by means of the fluorescence in situ hybridization method. *H. pylori* infection was assessed by both serum antibody and urea breath test analyses; a serum antibody level of ≤3 U/mL and a negative urea breath test result were considered to indicate the absence of *H. pylori*. If at least one of the tests had positive results, the patient was considered *H. pylori*-positive; if both tests had negative results, the patient was considered *H. pylori*-negative. Successful eradication of *H. pylori* was recorded following a negative urea breath test result after eradication therapy. As treatment for MALT lymphoma, *H. pylori*-positive patients received *H. pylori* eradication therapy. The regimen of *H. pylori* eradication therapy was triple therapy including a proton pump inhibitor or vonoprazan (20 mg), amoxicillin (750 mg), and clarithromycin (200 or 400 mg).[12] All participants were not currently infected with *H. pylori*. Among the 33 patients with *H. pylori*-negative gastric MALT lymphoma, 24 were *H. pylori*-infection naïve patients who had no signs of current or prior infection with *H. pylori*, while 9 were posteradication patients (Fig. 1). Among the 24 *H. pylori*-infection naïve patients, 14 received the antimicrobial regimen used for eradication therapy, without any therapeutic effect concerning MALT lymphoma (Fig. 1). Specimens were collected from *H. pylori*-infection naïve patients for at least 6 months after antibiotic treatment. The use of probiotics, antimicrobials, and proton pump inhibitors (PPIs) within 2 months of specimen collection was investigated.

2.2. Sample collection and DNA extraction

The gastric mucosa was washed with tap water containing a dimethicone solution under esophagogastroduodenoscopy. Subsequently, biopsy specimens were collected from the greater curvature of the gastric body from both MALT patients and controls by using disposable biopsy forceps (Olympus, Tokyo, Japan). Controls volunteered to provide biopsy samples from mucosal sites without signs of atrophy or neoplastic lesions. Mucosal biopsy specimens were stored at −80°C until DNA extraction. Mucosal bacterial DNA was extracted from biopsy specimens using NucleoSpin Tissue XS (Macherey-Nagel, Düren, Germany) and a Tissue raiser (Qiagen Inc., Valencia, CA) with 0.1-mm beads vibrating at a speed of 25 times per second for 1 minute.

2.3. 16S rRNA gene amplification and sequencing

16S rRNA gene sequencing was conducted as follows. Briefly, the extracted bacterial DNA was used as the template to amplify the V4 region of each 16S rRNA gene with the primer pair 515F/

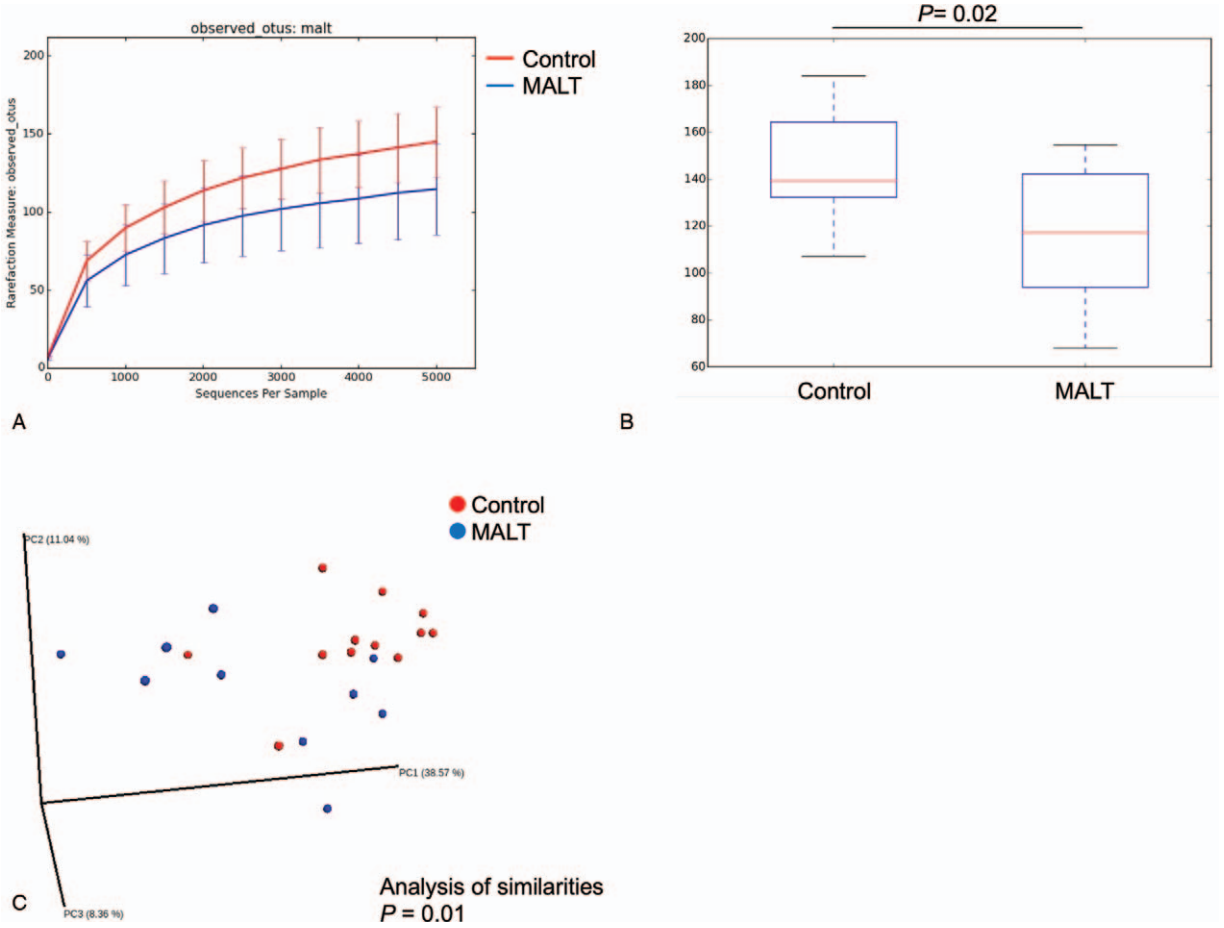


Figure 3. Comparisons of alpha and beta diversities between *H. pylori*-infection naïve gastric MALT lymphoma patients and controls. (A) Rarefaction curves showing observed species in *H. pylori*-infection naïve MALT lymphoma patients (n = 10) and controls (n = 12) at various sequencing depths. (B) Box plots showing alpha diversities at higher sequencing depths. Alpha diversity was significantly lower in the MALT lymphoma group than in the control group ($P = .02$). (C) Principal coordinates analysis showing significantly different microbial population composition between MALT lymphoma patients and controls ($P = .01$). MALT = mucosa-associated lymphoid tissue mucosa-associated lymphoid tissue.

806R, including the Illumina Flowcell adapter sequence.[7] The reverse primers also contained a 12-base barcode sequence. Paired-end sequencing of the polymerase chain reaction amplicons was performed on the Illumina MiSeq platform (Illumina Inc., San Diego, CA) using custom primers.

2.4. Processing of sequence reads and statistical analysis

Sequencing data obtained by MiSeq were subjected to demultiplexing and quality filtering using quantitative insights into microbial ecology (QIIME) software, version 1.9.1.[13] The open-reference strategy was employed to pick operational taxonomic units, using UCLUST at a minimum sequence identity of 97% against Greengenes 13_818 to cluster the pre-processed sequences into operational taxonomic units; these units were defined by intrinsic phenotypic similarities that constitute candidate taxa. Reads without hits in the reference database were randomly subsampled and clustered de novo. Chimera Slayer, a component of QIIME, was used to remove chimeric sequences. Alpha diversity (i.e., the species richness of microbes in a single sample) was calculated in QIIME using observed species diversity indices. *P* values for group comparisons were determined using a non-parametric two-sample *t* test and the default number (999) of Monte Carlo permutations. Beta diversity (i.e., the difference in microbial communities between samples) was calculated using phylogenetically informed weighted UniFrac distances and visualized using principal coordinates analysis. The statistical significance of the distance between the 2 groups was calculated using permutation multivariate analysis of variance. To identify bacteria that significantly differed between groups, taxonomic summaries were reformatted and input into linear discriminant analysis effect size (LEfSe) analyses.[14] In the LEfSe settings, the non-parametric factorial Kruskal–Wallis rank-sum test ($\alpha = 0.05$) was used to detect taxa with significant differential abundances. The threshold logarithmic linear discriminant analysis score for discriminative features was set at 2.0. If multiple varieties with different ranks showed significance within the same taxon, the lowest-ranked varieties were considered responsible. Significant taxa were used to make taxonomic cladograms illustrating differences between sample classes in the Galaxy framework.[15] Based on the Kyoto Encyclopedia of Genes and Genomes database, the predicted functional profiles of the microbial communities were inferred from the 16S rRNA dataset obtained in this analysis; these inferences were made using phylogenetic

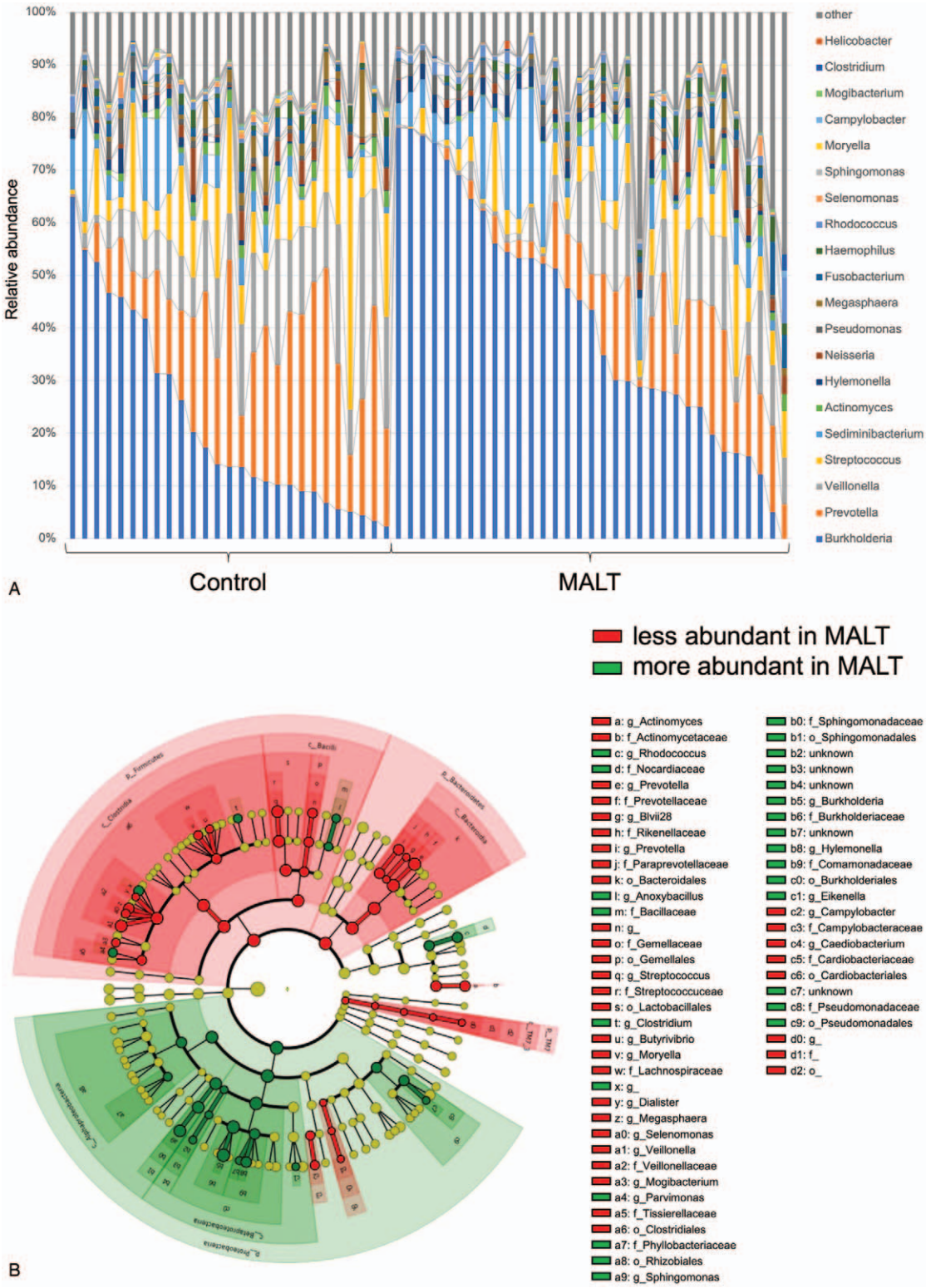


Figure 4. Bacterial composition at the genus level in MALT lymphoma patients and controls. (A) Bar graphs showing relative abundance at the genus level. (B) Cladogram showing discriminative classification between MALT lymphoma patients and controls based on LEfSe and linear discriminant analysis (LDA). (C) LDA scores showing significant differences in the microbiota. In MALT lymphoma patients, the genera *Burkholderia* and *Sphingomonas* were more abundant (green), compared with controls, while the genera *Prevotella* and *Veillonella* were less abundant (red). (D) Bar graphs showing relative abundances of *Helicobacter* spp. Bar graphs of *H. pylori*-infected patients are shown for reference. LEfSe = linear discriminant analysis effect size, MALT = mucosa-associated lymphoid tissue mucosa-associated lymphoid tissue.

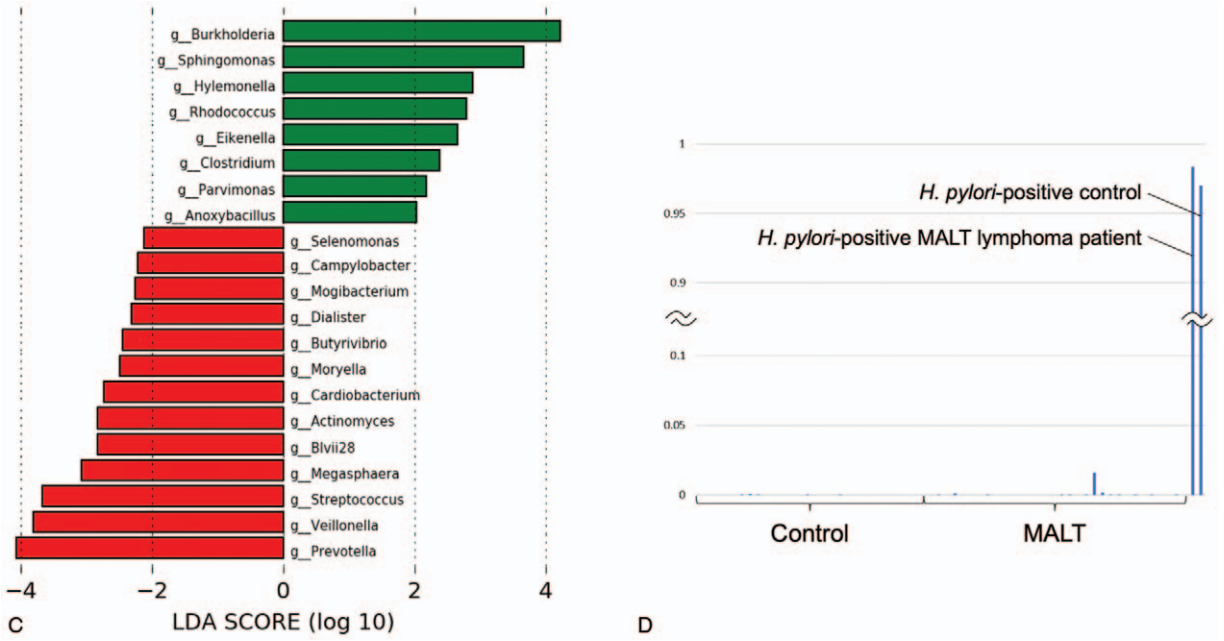


Figure 4. (Continued).

investigation of communities by reconstruction of unobserved states software[16] and analyzed using statistical analysis of taxonomic and functional profiles software.[17]

2.5. Ethics statement

This study was conducted in accordance with the tenets of the Declaration of Helsinki. Written informed consent was obtained from all participants and the protocol was approved by the Ethics Committee of Kyushu University (approval number: 29-246).

3. Results

Gastric mucosal samples were collected from 60 participants, comprising 33 *H. pylori*-negative gastric MALT lymphoma patients and 27 controls. Table 1 and Figure 1 show the clinical characteristics of the participants. All MALT lymphoma patients had MALT lymphoma with gastric lesions. Twenty-four patients were *H. pylori*-infection naïve, while 9 patients had undergone successful *H. pylori* eradication and their lymphoma had not responded. Furthermore, the *H. pylori*-infection naïve patients included 10 who received no treatment and 14 who did not respond to antibiotic treatment. Among the 27 controls, 12 were *H. pylori*-infection naïve and 15 had undergone *H. pylori* eradication.

3.1. Comparison of alpha and beta diversities

In total, 1,905,986 quality-filtered sequences were obtained through 16S rRNA gene sequencing, with a mean of 31,766 ± 21,350 sequences per sample. The alpha diversity (i.e., the observed species index) was significantly lower in MALT lymphoma patients than in controls ($P = .04$), indicating lower mucosal microbiota diversity in MALT lymphoma patients than in controls (Fig. 2A, B). For calculation of beta diversity, the weighted UniFrac distance was used to compare overall microbial composition between MALT lymphoma patients and controls. Weighted principal coordinates analysis indicated that the microbial compositions were significantly different between the 2 groups ($P = .04$; Fig. 2C).

In an analysis limited to *H. pylori*-infection naïve participants, the alpha diversity was also significantly lower in MALT lymphoma patients than in controls ($P = .02$; Fig. 3A, B). Beta diversity analysis indicated that the microbial compositions were significantly different between the 2 groups ($P = .01$; Fig. 3C).

3.2. Comparison of taxonomic compositions

The results of taxonomic composition analysis are shown in Figure 4. The genera *Burkholderia*, *Prevotella*, *Veillonella*, and *Streptococcus* were abundant in stomach mucosa (Fig. 4A). LEfSe analysis revealed that the genera *Burkholderia* and *Sphingomonas* were more abundant in MALT lymphoma patients than in controls. Conversely, the genera *Prevotella* and *Veillonella* were less abundant in MALT lymphoma patients than in controls (Fig. 4B, C). Although the genus *Helicobacter* was detected in MALT lymphoma patients and controls, the abundance of this genus was negligible in both groups, compared with the abundance in reference data from patients who had *H. pylori*-positive samples (Fig. 4D).

Figure 5 shows the results of taxonomic composition analysis limited to *H. pylori*-infection naïve participants. The genera *Burkholderia* and *Sphingomonas* were more abundant in MALT lymphoma patients than in controls, while the genera *Prevotella* and *Veillonella* were less abundant in MALT lymphoma patients (Fig. 5B, C).

3.3. Functional microbiota prediction

Functional microbiota prediction revealed that the abundances of microbiota gene pathways significantly differed between MALT

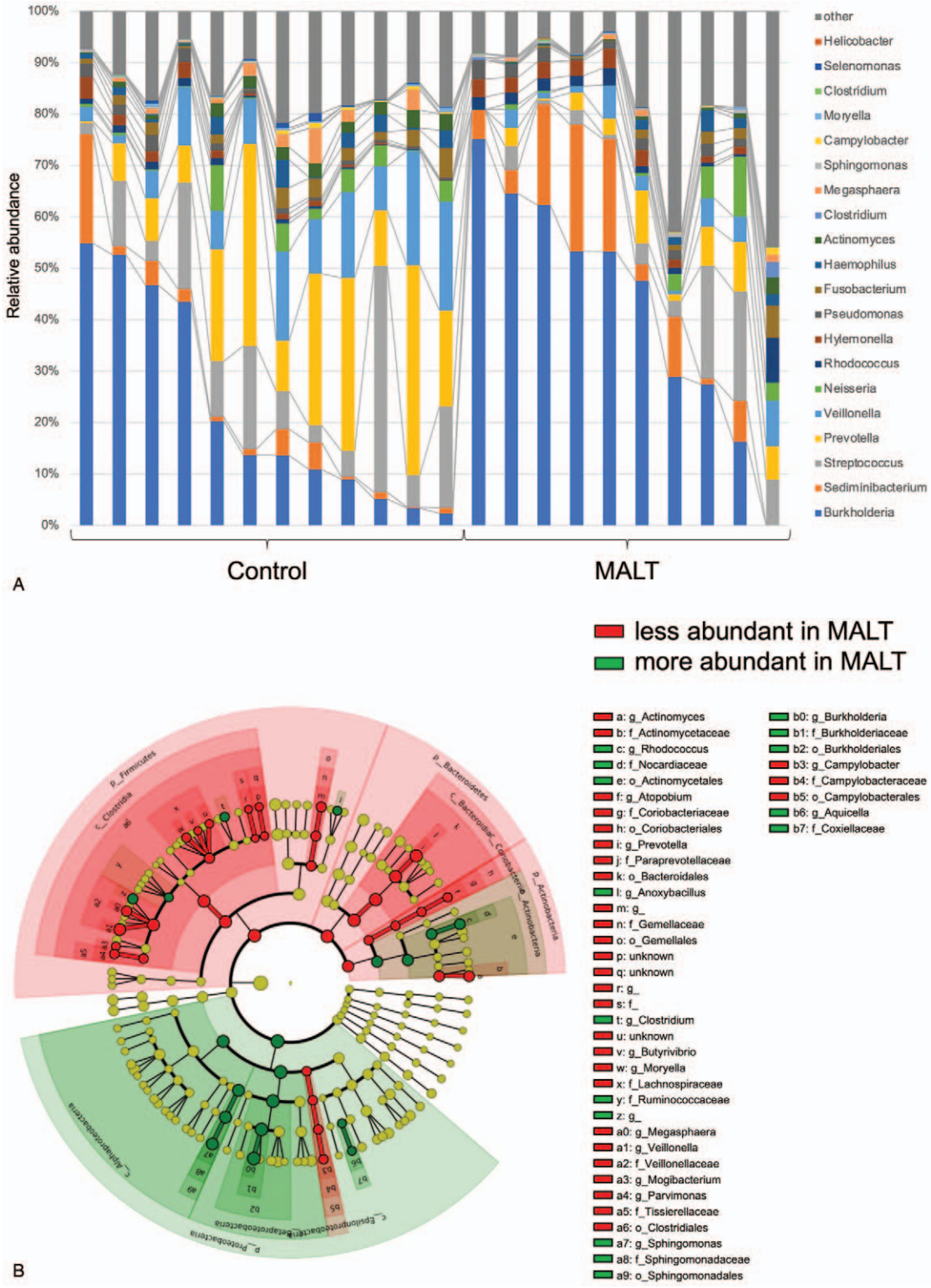


Figure 5. Bacterial composition at the genus level in *H. pylori*-infection naïve MALT lymphoma patients. (A) Bar graphs showing relative abundances at the genus level in *H. pylori*-infection naïve MALT lymphoma patients (n = 10) and controls (n = 12). (B) Cladogram showing taxa that discriminated between MALT lymphoma patients and controls. (C) LDA scores showing that the genera *Burkholderia* and *Sphingomonas* were more abundant (green), while the genera *Prevotella* and *Veillonella* were less abundant (red), in MALT lymphoma patients than in controls. LDA = linear discriminant analysis, MALT = mucosa-associated lymphoid tissue mucosa-associated lymphoid tissue.

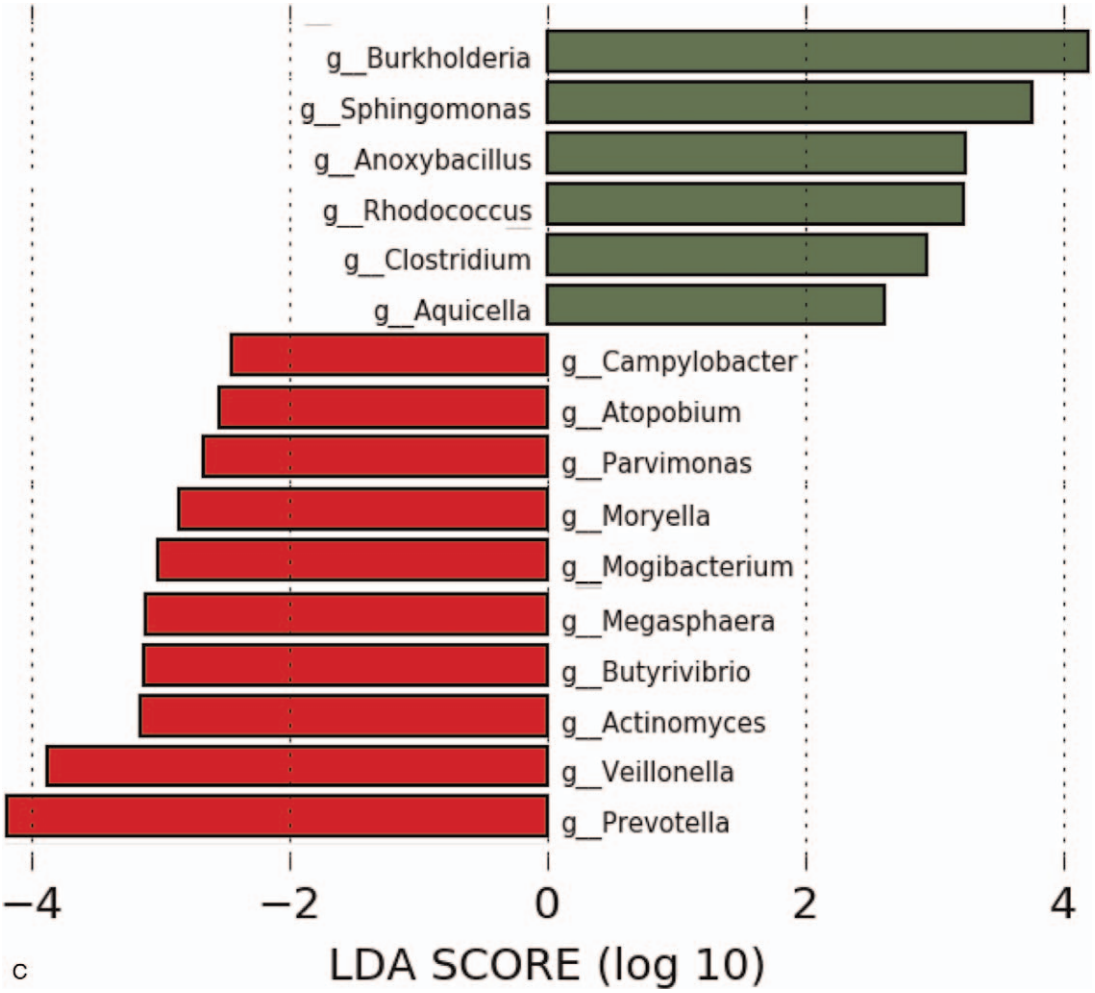


Figure 5. (Continued).

lymphoma patients and controls, following correction for multiple comparisons. The 2 groups differed in the predicted pathways of "replication and repair," "translation," and "nucleotide metabolism," suggesting that microbiota may affect gastric MALT lymphoma pathogenesis ($P < .05$; Fig. 6A). In an analysis limited to *H. pylori*-infection naïve participants, the predicted pathways common to the overall and subgroup analyses were "replication and repair," "translation," and "nucleotide metabolism" ($P < .05$; Fig. 6B).

4. Discussion

In the present study, we investigated gastric microbiota in *H. pylori*-negative gastric MALT lymphoma patients using metagenomic analysis. To the best of our knowledge, this is the first study to examine the microbiota of these patients. We demonstrated that the gastric mucosal flora in *H. pylori*-negative gastric MALT lymphoma patients had significantly reduced diversity, compared with that of controls. The composition of gastric microbiota considerably differed between *H. pylori*-negative gastric MALT lymphoma patients and controls. These results suggested that bacterial homeostasis in the gastric mucosa was disrupted in patients with *H. pylori*-negative gastric MALT lymphoma. In our previous study, we found that microbial dysbiosis in the duodenum was associated with gastrointestinal follicular lymphoma.[8] Thus, dysbioses in the gastrointestinal tract may contribute to the onset of gastrointestinal lymphoma.

In this study, the genera *Burkholderia* and *Sphingomonas* were more abundant in MALT lymphoma patients than in controls. *Burkholderia* can be an opportunistic pathogen[18]; lectins from *Burkholderia* have been reported to promote the growth of follicular lymphoma.[19]*Burkholderia* expresses a protein with the MviN domain. Importantly, both *H. pylori* and *H. suis* express proteins with the MviN domain; both species have been associated with MALT lymphoma.[20] The MviN domain-containing protein in *Burkholderia* may be involved in the pathogenesis of MALT lymphoma. In animal models, intragastric exposure to *Sphingomonas* has been shown to activate natural killer cells and alter systemic immunity.[21] Therefore, the genera *Burkholderia* and *Sphingomonas* may promote the development of MALT lymphoma.

In contrast, we found that the genera *Prevotella* and *Veillonella* were less abundant in MALT lymphoma patients than in controls. *Prevotella* is endemic to the human oral cavity and is known to be protective against inflammation. There are fewer members of the *Prevotella* genus in patients with inflammatory

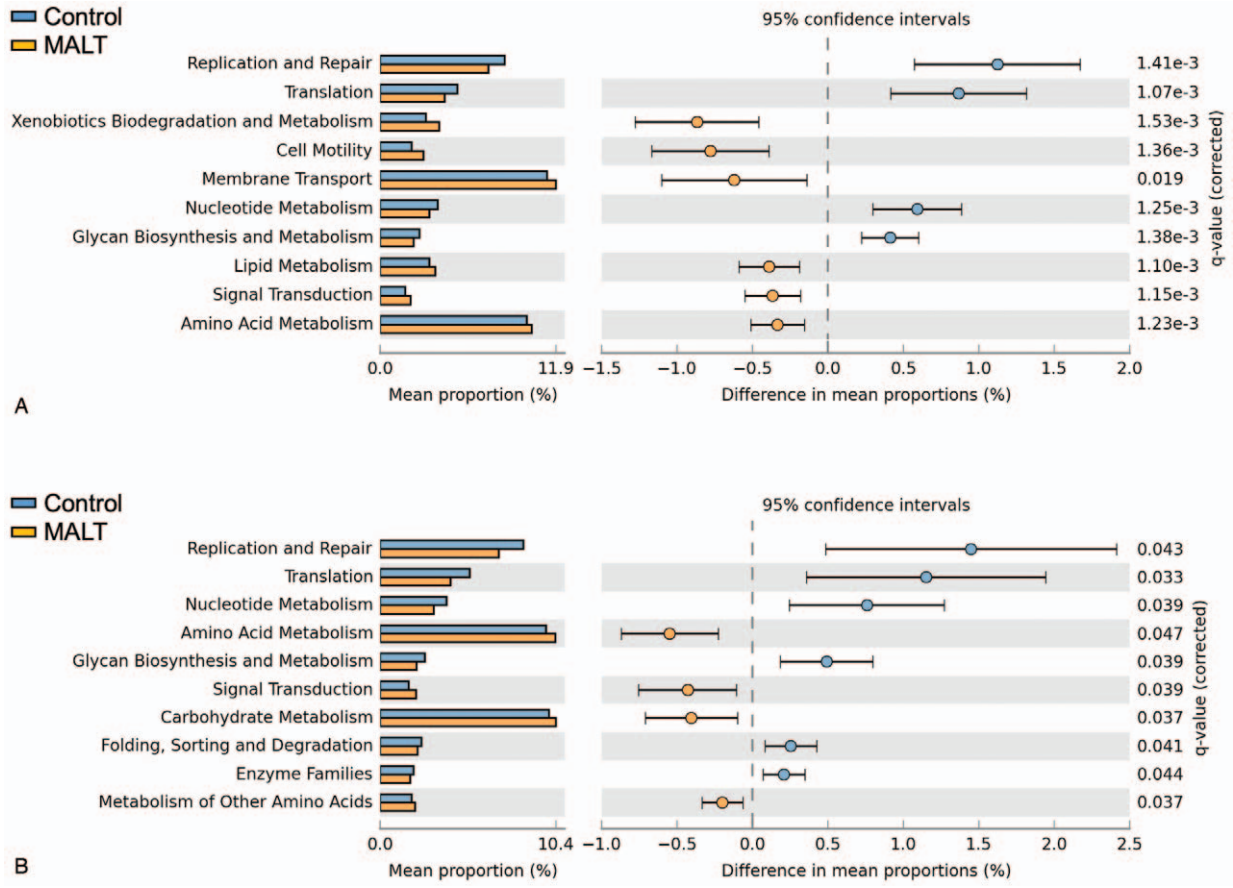


Figure 6. Comparison of the relative abundances of functional pathways in microbiota between MALT lymphoma patients and controls. The figures show 10 representative microbiota gene pathways that have been identified. (A) The microbiota gene pathways differed between *H. pylori*-negative MALT lymphoma patients and controls ($P < .05$). (B) In an analysis limited to *H. pylori*-infection naïve participants, the top 10 pathways differed between MALT lymphoma patients and controls ($P < .05$). MALT = mucosa-associated lymphoid tissue mucosa-associated lymphoid tissue.

bowel diseases, such as Crohn disease and ulcerative colitis.[7,22] *Veillonella* has been reported to promote the reduction of inflammation and subsequent improvement of athlete performance through its consumption of lactate, a metabolite associated with fatigue.[23] In the gastric mucosa, attenuated intestinal protection due to *Prevotella* and *Veillonella* reduction may contribute to the development of MALT lymphoma. In the present study, *Helicobacter* spp. were detected in gastric mucosal specimens from patients who had negative findings in *H. pylori* infection analyses (i.e., both serum antibody and urea breath test results). Other bacteria in the *Helicobacter* genus have been proposed to contribute to MALT lymphoma.[5,20] However, negligible amounts of these species were detected in the present study.

Functional microbiota prediction showed differences in the abundances of predicted gene pathways between *H. pylori*-negative gastric MALT lymphoma patients and controls. The predicted gene pathways "replication and repair," "translation," and "nucleotide metabolism" were downregulated in MALT lymphoma patients. In patients with non-Hodgkin lymphoma, including MALT lymphoma, variants related to DNA repair are known to exist; these genetic variants are involved in the development of lymphoma.[24] Abnormal expression patterns of genes involved in transcription, repair, and translation may be involved in the development of MALT lymphoma. Nucleotide metabolism is an important pathway for DNA replication and RNA synthesis. Nucleotides are involved in lymphoma proliferation[25] and tumor immunity.[26] Therefore, abnormalities in nucleotide metabolism may contribute to the pathogenesis of MALT lymphoma. These functional depressions are also involved in DNA metabolism; notably, non-metabolized bacterial DNA is known to induce inflammation[27] and chronic inflammation may contribute to the development of MALT lymphoma.

This study had some limitations. First, because of the small sample size, variations in microbiota among participants in each group may have impacted the results. The gastrointestinal tract is the most common site of epithelial malignancies; gastrointestinal malignant lymphoma constitutes 1% to 8% of all gastrointestinal malignancies.[28] Furthermore, *H. pylori*-infection naïve patients constitute 10% of all patients with gastric MALT lymphoma,[2] which may have contributed to the low number of patients included in this study. Therefore, our observations must be validated in a larger cohort study. Second, it was unclear whether microbiota differences between patients with gastric MALT lymphoma and controls were etiological factors or consequences of disease. These relationships require confirmation by eradication therapy, as administered for patients with *H. pylori*

infection. Third, some participants were taking PPIs in this study. Several studies have reported that PPIs alter the gastric microbiota[29,30]; thus, PPIs may have affected the results in the present study. However, when the analysis was limited to *H. pylori*-naïve patients who were not taking PPIs, the overall results remained consistent in terms of bacterial diversity and microbiota composition; therefore, the effects of PPIs were presumably minimal in this study. Fourth, patients with *H. pylori*-positive MALT lymphoma were not included in this study because this study was designed to analyze the gastric microbiota in *H. pylori*-negative lymphoma patients. The inclusion of *H. pylori*-positive MALT lymphoma patients in a future study will provide greater clarity concerning the roles of gastric microbiota in MALT lymphoma.

In conclusion, obvious changes in microbial composition were found in the gastric mucosa of *H. pylori*-negative gastric MALT lymphoma patients, suggesting that these changes might play important roles in the development of gastric MALT lymphoma. These results may aid in elucidating factors other than *H. pylori* that contribute to the onset of gastric MALT lymphoma, as well as in the development of new therapies for affected patients.

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Author contributions

Takahide Tanaka, Takehiro Torisu, and Takanari Kitazono designed this study. Takahide Tanaka, Yuichi Matsuno, Junji Umeno, Keisuke Kawasaki, Shin Fujioka, and Yuta Fuyuno collected the mucosal biopsies. Takahide Tanaka, Atsushi Hirano, and Hiroki Shibata performed the experiments. Takahide Tanaka, Takehiro Torisu, Motohiro Esaki, and Takanari Kitazono drafted the manuscript.

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Validation: Takahide Tanaka.
Visualization: Takahide Tanaka.
Writing – original draft: Takahide Tanaka.
Writing – review & editing: Yuichi Matsuno, Takehiro Torisu, Motohiro Esaki.

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