Original Article

Characterization of the gut phageome of Japanese patients with ulcerative colitis under endoscopic remission

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This study aimed to analyze the gut phageome in Japanese patients with ulcerative colitis (UC) in endoscopic remission. Fecal samples were collected from 35 UC patients and 22 healthy controls. The gut microbiome was analyzed using 16S rRNA amplicon sequencing, and the phageome was profiled through shotgun metagenomic sequencing. Compared to healthy controls, UC patients showed a significant reduction in phageome richness (observed species and Chao1 index). Principal coordinate analysis revealed a significant difference in beta-diversity between UC and healthy controls ($p = 0.001$). The abundance of temperate phages was higher in UC (15.2%) compared to healthy controls (5.9%), although this was not statistically significant ($p = 0.088$). Temperate phages associated with *Coprococcus* sp., *Bacteroides* sp. KFT8, and *Faecalibacterium prausnitzii*, as well as virulent phages associated with *Ruminococcus gnavus* and *Lactobacillus farciminis*, were increased in UC patients. Conversely, phages associated with *Thermosipho affectus*, *Bacteroides* sp. OF03-11BH, and *Odoribacter splanchnicus* were decreased in UC patients. Phages associated with the genera *Odoribacter* ($p = 0.0004$), *Ruminococcus* ($p = 0.009$), and *Veillonella* ($p = 0.013$) were significantly reduced in UC patients. The gut phageome of inactive UC patients exhibited notable alterations in viral composition compared to healthy controls. These results suggest that changes in the gut phageome might be involved in the pathogenesis of UC.

***Key Words*:** **bacteriophage, dysbiosis, Odoribacter splanchnicus phage**

Inflammatory bowel disease (IBD) including Crohn's disease (CD) and ulcerative colitis (UC) is a chronic inflammatory disorder of the gastrointestinal tract with an unknown etiology.[1] The incidence of IBD is increasing worldwide, particularly in newly industrialized countries with more westernized societies.[2] It is widely accepted that westernized diets and lifestyles modulate the gut microbiota, leading to an increased risk of IBD in genetically susceptible individuals.[3]

The gut microbiota is a complex ecosystem composed of billions of commensal microbes, including bacteria, viruses, fungi, archaea and others.[4] It plays a crucial role in maintaining human health by promoting gut barrier integrity, nutrient synthesis, modulation of immune response, and protection against invading pathogens. However, disruption of the gut microbiota, especially its bacterial components, can lead to abnormal immune responses in the mucosa and is significantly involved in the pathogenesis of a variety of diseases.[5,6] Numerous studies have shown that the gut microbiota in patients with IBD is characterized by an increased abundance of Proteobacteria and a decreased abundance of Firmicutes, especially butyrate-producing bacteria.[5,7] In contrast, although viruses are estimated to be nearly ten times more abundant than bacteria in the gut,[5] their involvement in pathophysiological conditions such as IBD is less well-studied compared to bacteria.

The human gut virome consists of both prokaryotic and eukaryotic viruses and maintains gut homeostasis through interactions with the gut microbial community.[8–10] Bacteriophages (phages), which infect prokaryotic cells (bacteria and archaea), account for approximately 90% of the gut virome. The remaining 10% consists of eukaryotic viruses that infect humans.[11] Phages can be classified into virulent and temperate phages based on their lifestyle.[12] Virulent phages (also known as lytic phages) replicate and proliferate within infected bacterial cells, leading to the release of viral particle through cell rupture (the lytic cycle).[11,13] In contrast, temperate phages (also known as lysogenic phages) undergo both the lysogenic and lytic cycles.[12] During the lysogenic cycle, a temperate phage integrates its genome into the host chromosome (as a prophage), and replicates its genome along with the host chromosome.[12] Under certain conditions, temperate phages can switch to the lytic cycle, resulting in the release of viral particles via the host cell lysis. These different processes significantly impact the gut microbiota and may be closely associated with human diseases.

In the gut virome of healthy individuals, phages from the order *Caudovirales* or the family *Microviridae* are predominant.[14,15] In patients with IBD, alterations in the gut virome have been reported in several studies, with recent reviews summarizing these findings.[4,16] The gut virome of IBD patients is generally characterized by a higher abundance of *Caudovirales* and a lower abundance of *Microviridae*,[4,16] although there is some variability across studies. There is ongoing debate about whether these changes in the gut virome play a significant role in the development of IBD.[4,16]

In this study, we investigated the gut virome of Japanese patients with UC, as it has not been thoroughly examined in this population before. The fecal microbiota during the active phase of UC is influenced by several factors, such as bleeding, antimicrobial proteins (including immunoglobulins and complements), antimicrobial peptides, and the washing force of diarrhea. To minimize the impact of these factors, we analyzed the gut virome of UC patients with endoscopic remission.

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Methods

Ethics. The study protocol received approval from the institutional review boards of Shiga University of Medical Science (approval number 2019-155). Written informed consent was obtained from all participants. The registration number to the University Hospital Medical Network Center (UMIN) is 000055410.

Patients and sample collection. Fecal samples were collected from 35 patients with endoscopically inactive UC and 22 healthy individuals. The median partial Mayo score was 0 (range 0 to 1),[5] and the median Mayo endoscopic sub score was 0 (0 to 1). No patients were undergoing treatment with antibiotics or probiotics. Patient characteristics are detailed in Table 1.

Bacterial DNA extraction and 16S rRNA amplicon sequencing. Extraction of bacterial DNA from fecal samples was carried out using Quick Gene DNA tissue kits (Kurabo, Osaka, Japan) as previously described.[17] 16S rRNA amplicon sequencing was performed using the MiSeq™ System (Illumina, San Diego, CA) according to established methods.[18]

16S rDNA-based taxonomic analysis. Sequence data were processed by QIIME2 (ver. 2024.5)[19] with the DADA2 plugin[20] for chimera checking and amplicon sequence variant definition. Taxonomic assignment was performed using the SILVA138 database.

Table 1. Demographic and basic characteristics of patients

	Healthy controls (n = 22)	UC (n = 35)
Female/Male	8/14	16/19
Age (years)	35 (31–46)	41 (32–48)
Smoking history		
Current/Former/Never	0/0/22	1/8/26
Partial Mayo score	—	0 (0–1)
Mayo endoscopic sub score	—	0 (0–1)
Disease type		
Proctitis/Left-sided colitis/Pancolitis	—	3/7/25
Medications		
5-ASA	—	27 (77.1%)
Prednisolone	—	1 (2.9%)
AZA/6-MP	—	13 (37.1%)
anti-TNFα antibodies	—	4 (11.4%)
anti-α4β7 integrin antibodies	—	3 (8.6%)

Values are reported as medians with interquartile ranges. 5-ASA, 5-aminosalicylic acid; AZA, azathioprine; 6-MP, 6-mercaptopurine.

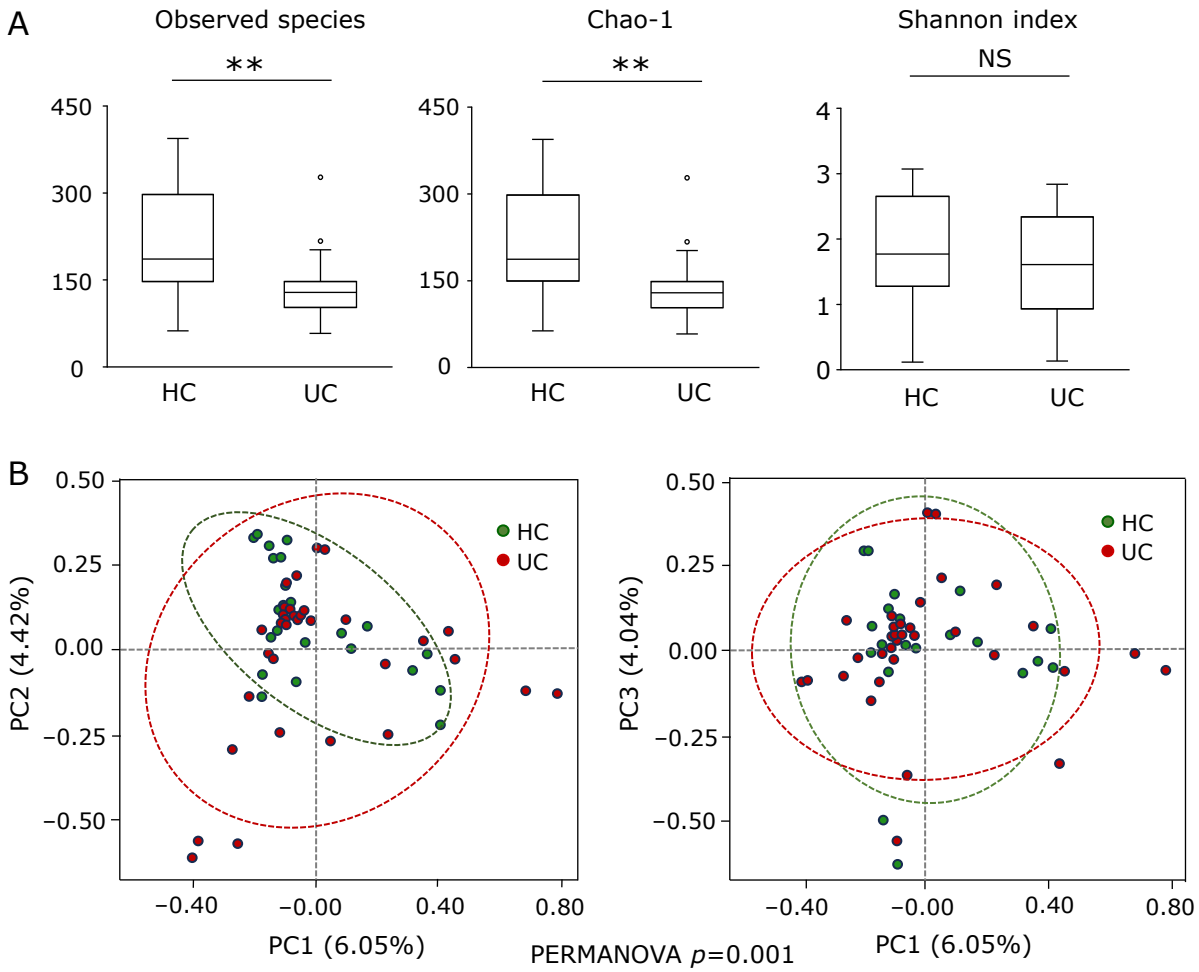

Fig. 1. Alterations in the diversity and structure of the gut virome in ulcerative colitis. (A) α-diversity of the gut virome of UC and HC. (B) β-diversity of the gut virome of UC and HC. The virome was analyzed and plotted using principal coordinate analysis (PCoA) based on the Bray–Curtis distance (PERMANOVA p = 0.001). Statistical significance was determined by Mann–Whitney U test. UC, ulcerative colitis; HC, healthy control; NS, not significant. **p<0.01.

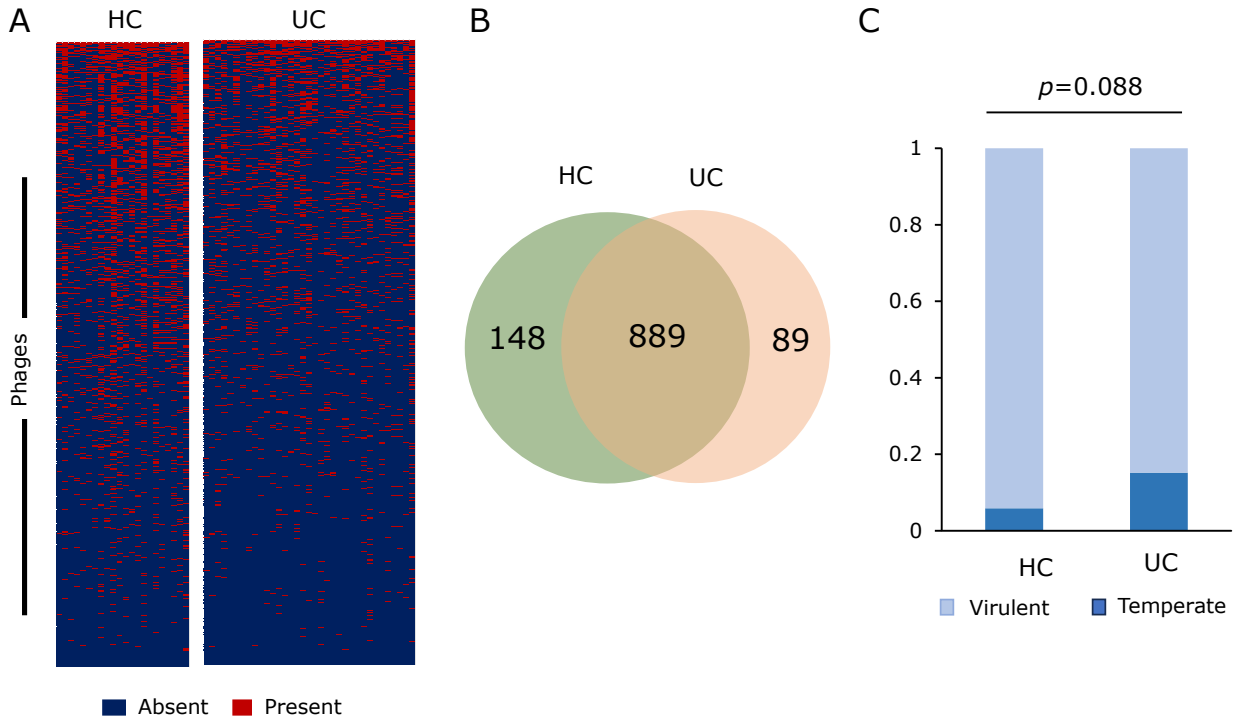

Fig. 2. Comparison of the gut virome between UC and HC. (A) Heat maps showing the presence or absence of bacteriophages in UC and HC. (B) Venn diagram of bacteriophages in UC patients and healthy controls. (C) Relative abundance of virulent and temperate phages in UC and healthy controls. Statistical significance was determined by *t* test. UC, ulcerative colitis; HC, healthy control.

Enrichment of virus-like particles (VLPs) and viral DNA sequencing. Enrichment of VLPs from fecal samples and viral DNA sequencing was performed according to the methods described in our previous report.[21] In this study, we used the NovaSeq X sequencing system (2 × 150 paired-end reads, NovaSeq X Series 1.5B Reagent Kit).

Virus DNA-based taxonomic analysis. The obtained sequences were first processed using SqueezeMeta (v1.6.2),[22] an automated metagenomic analysis pipeline. Briefly, *de novo* co-assembly was performed using metaSPAdes.[23] Gene prediction on the resultant contigs was carried out using Prodigal gene prediction software,[24] and homology searches of the genes were performed against the GenBank nr database using Diamond software,[25] Taxonomy for each contig was assigned by a custom script based on the LCA algorithm integrated into SqueezeMeta. To estimate the abundance of each contig in each sample, reads from each sample were mapped onto the contigs using Bowtie2,[26] and the raw number of reads mapped to each contig was extracted using BEDtools.[27]

Next, contigs assigned to viruses with lengths greater than 3,000 bases were extracted and analyzed using PhaBOX,[28] a server for identifying and characterizing phage contigs in metagenomic data. The identified phage contigs and their abundance in each sample were extracted, and this dataset was used as the phage distribution for subsequent analyses.

Statistical analyses. Alpha-diversity indices were calculated using the R "phyloseq" package[29] and analyzed with the Mann–Whitney *U* test. Beta-diversity was assessed using the UniFrac metric and Bray–Curtis dissimilarity, with statistical significance determined by permutational multivariate analysis of variance (PERMANOVA). MaAsLin2 (Multivariate Association with Linear Models) was employed to identify differentially enriched bacterial or viral taxa while adjusting for confounding factors.[30] Spearman's rank correlation coefficient was calculated to assess the relationship between the bacteriome and virome.

Results

Alteration in the diversity of gut virome in UC patients. We compared the fecal virome of 35 patients with UC to that of 22 healthy controls. Average clean paired reads were obtained from the enriched fecal VLP samples, with 2,663,349 reads for controls and 1,355,277 reads for UC patients. Compared to healthy controls, UC patients exhibited a significant decrease in phageome richness (observed species and Chao1) but not evenness (Shannon) (Fig. 1A)., indicating a decreased α-diversity (richness) in the gut phageome of UC patients. To evaluate the overall composition of the gut phageome, we utilized the β-diversity index derived from Bray–Curtis distances. Principal Coordinate Analysis (PCoA) demonstrated a significant distinction between UC patients and healthy controls (PERMANOVA $p = 0.001$) (Fig. 1B). These results suggest that the gut phageome profiles in UC patients are notably different from those in healthy individuals.

Changes in the gut phageome of UC patients. We conducted a more detailed analysis of the gut phageome. The presence-absence heat map of viral sequences revealed that each healthy control and each UC patient had a unique viral composition (Fig. 2A). Additionally, there were striking differences in the types of phage sequences observed between UC and control samples (Fig. 2B). Although many phage taxa were shared between the control and UC groups, each group also had its own unique phage taxa (present in both: 808; unique to controls: 143; unique to UC: 89) (Fig. 2B). As demonstrated in Fig. 2C, virulent phages were dominant in both controls and UC patients. The proportion of temperate phages tended to be higher in UC patients compared to healthy controls (healthy controls: 5.9%; UC: 15.2%, $p = 0.088$).

To understand the infective nature of phages, we classified them based on their host bacteria and their virulent or temperate characteristics. As shown in Fig. 3, MaAsLin2 analysis revealed

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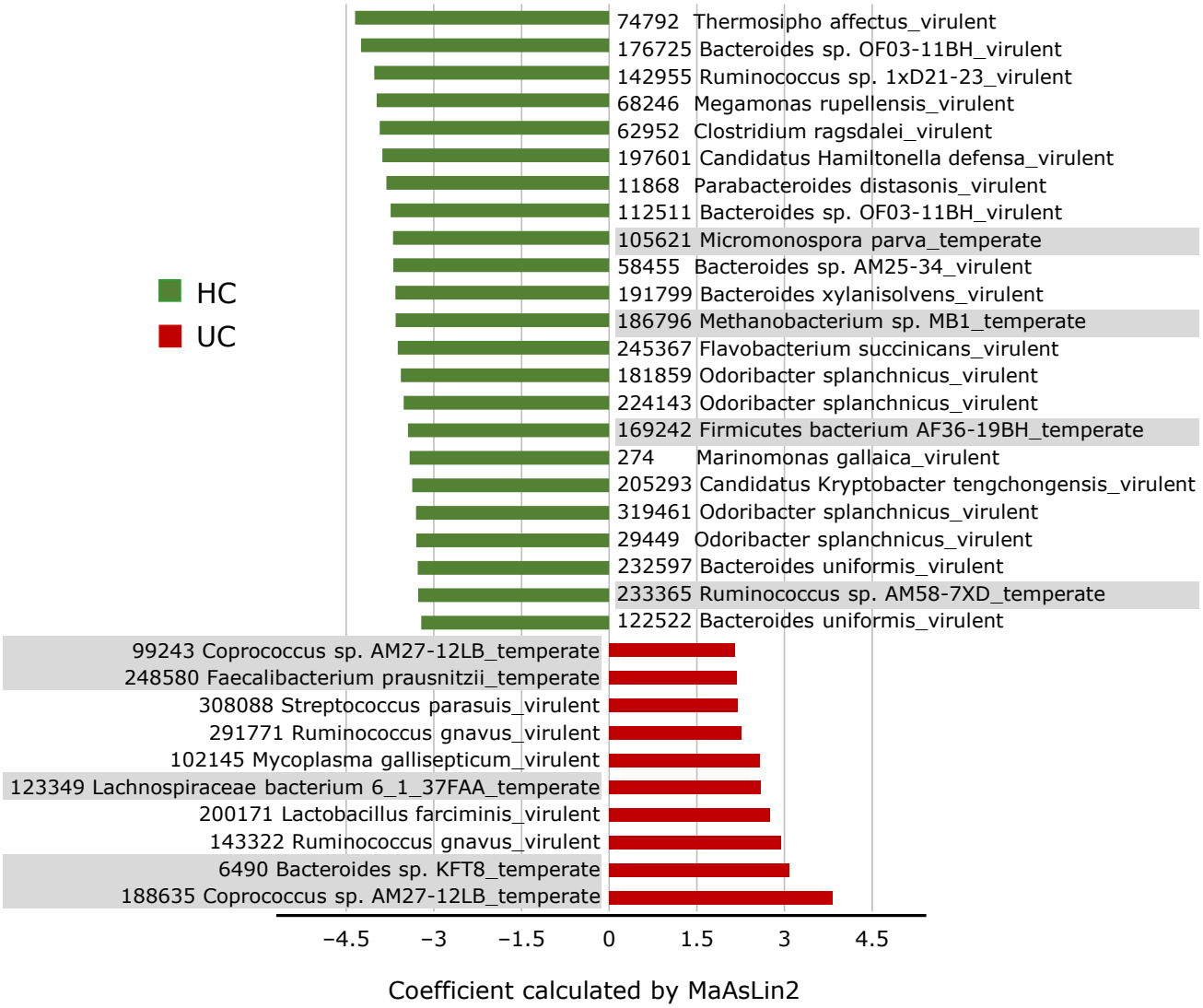

Fig. 3. Changes in bacteriophages in UC patients. Enriched viral taxa were identified through MaAsLin2 analysis.[30] We used the default settings with a *q* value threshold of 0.25 and a *p* value threshold of <0.05 to identify differentially abundant taxa between UC and HC. The x-axis represents the coefficient values calculated by MaAsLin2 analysis. Each phage name is preceded by the contig number. Temperate phages are indicated by shading. UC, ulcerative colitis; HC, healthy control.

that certain phages were found to be more prevalent in UC patients.[30] These include temperate Coprococcus sp. phages, temperate Bacteroides KTF8 phages, virulent Ruminococcus gnavus phages, virulent Lactobacillus farciminis phages, and temperate Faecalibacterium prausnitzii phages. Conversely, some phages were decreased in UC patients, including virulent Thermosipho affectus phages, virulent Bacteroides sp. OF03-11BH phages, virulent and temperate Ruminococcus sp. 1xD21-23 phages, and virulent Odoribacter splanchnicus phages.

To better understand these results, we grouped the phages by the genus of their host bacteria and analyzed the changes in UC patients (Fig. 4). In patients with UC, virulent phages associated with *Odoribacter*, *Ruminococcus*, *Veillonella*, *Bacillus*, *Bifidobacterium*, *Bacteroides*, and *Ruminococcus* were decreased. In contrast, there was an increase in temperate Faecalibacterium phage and virulent phages associated with *Xylanimonas*, *Fischerella*, *Prevotella*, and *Streptococcus*. Among these findings, the decrease in Odoribacter phages ($p = 0.0004$), Ruminococcus phages ($p = 0.009$), and Veillonella phages ($p = 0.013$) was statistically significant.

Changes in the gut bacteriome in UC patients. The gut bacteriome's richness, as assessed by observed species and Chao1, and its evenness, measured by Shannon, were significantly lower in UC patients than in healthy controls (Fig. 5A). Additionally, PCoA demonstrated a significant difference in the overall gut bacterial composition between patients and controls (PERMANOVA $p = 0.001$) (Fig. 5B). The gut bacteriome of UC patients was characterized by a decreased abundance of the genera *Fusicatenibacter*, *Akkermansia*, *Eubacterium*, *Alistipes*, and *Ruminococcus*, and an increased abundance of the genera or species including *Clostridium*, *Ruminococcus gnavus* (*R. gnavus*), and *Negativibacillus* (Fig. 5C).

Relationship between the gut bacteriome and phageome in UC and healthy controls. Many phages are capable of infecting and replicating in a variety of bacterial species.[31] To investigate the relationship between the gut phageome and bacteriome in UC patients and healthy controls, we used Spearman's correlation coefficient to assess the association between bacteria and phages. The number of correlations between the virome and bacteriome was lower in UC patients compared to healthy con-

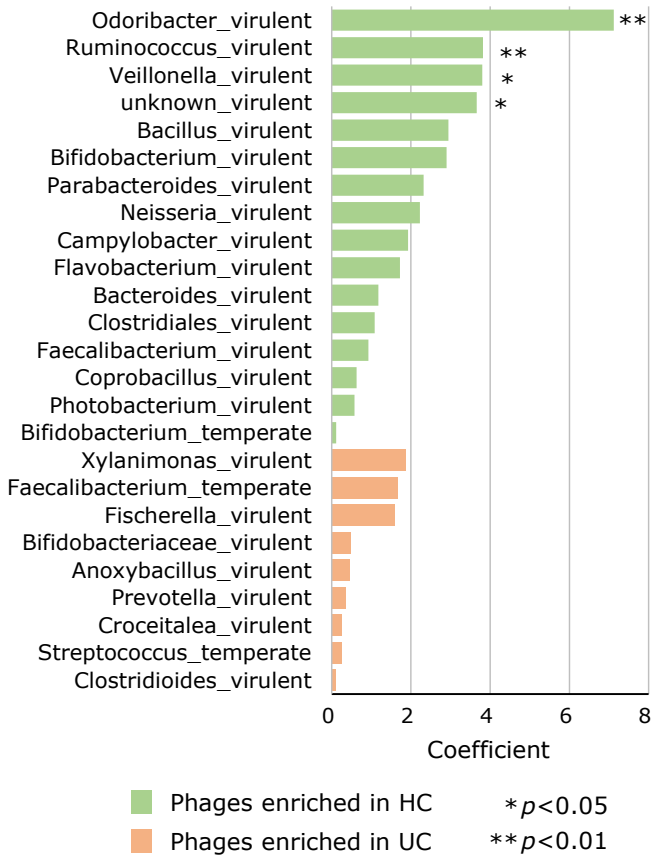

Fig. 4. Alteration of the abundance of phages categorized by host bacteria. Enriched viral taxa were identified through MaAsLin2 analysis.[30] The top 25 most abundant phages grouped by the host bacteria are shown. $^{**}p<0.01$, $^{*}p<0.05$.

trols (controls 84 vs UC 39) (Fig. 6). The correlations between the virulent *Ruminococcus* phage and their bacterial hosts were significantly stronger in the control group ($p<0.05$). Conversely, the correlations between the temperate Streptococcus phages and their bacterial hosts and virulent Parabacteroides phages and their bacterial hosts were significantly stronger in the UC patients ($p<0.05$). These results suggest that the homeostatic interactions between the bacterial and viral communities may be altered in UC.

Discussion

There is a growing body of research highlighting the crucial role of the gut microbiota in the pathophysiology of IBD.[7,9,32] While earlier studies have primarily concentrated on changes in the gut bacteriome, advanced metagenomic sequencing techniques have opened up new avenues to explore alterations in the gut virome across various diseases, including IBD.[9,33] Although it has been reported that Japanese individuals have a unique gut bacteriome,[34] there is limited knowledge about the gut phageome in both health and disease. In this study, we examined the gut phageome in conjunction with the gut bacteriome in Japanese patients with UC under endoscopic remission.

In this study, we found that the gut phageome in Japanese patients with UC is distinct from that of healthy controls, and this difference was associated with bacterial dysbiosis. These results suggest that dysbiosis involving interactions between phages and bacteria may play a role in the pathophysiology of UC. We also observed that both each UC patient and healthy individual exhibited unique viral composition pattern (Fig. 2A and B). This observation aligns with the findings of Reyes *et al.*,[35] who reported that the gut virome is unique to each individual, irrespective of genetic relationships. Similarly, Pérez-Brocal *et al.*[36] reported in their virome study of Crohn's disease that individual variability had a greater impact than the presence of the disease itself. Therefore, when considering changes in the gut phageome of UC patients, it is important to take into account the potential influence of individual variation.

Alpha-diversity (richness and evenness) of the gut bacteriome has been reported to be decreased in IBD patients,[7,13] and this was also confirmed in this study. On the other hand, there is no common observations regarding the α-diversity of the gut virome in IBD. Some reports showed an increase in α-diversity of the gut virome of IBD,[9] while others have found no difference compared to healthy individuals.[33] Recently, Zuo *et al.*[37] reported a decrease in both richness and evenness of the gut virome in UC patients. In this study, we detected a significant decrease in richness in the gut virome of UC, although there was no difference in evenness. Some aspects of these conflicting results may be attributed to variations in virome studies, such as differences in virus reference databases and methodologies for virus enrichment.[10]

We observed that the relative abundance of temperate phages appeared to increase in UC patients compared to healthy controls. It has been reported that a stable core of predominant virulent phages is associated with maintaining a healthy human gut, and that environmental stresses related to mucosal inflammation can trigger a reservoir of integrated prophages (lysogenic state) to enter the lytic cycle.[33] This process may be connected to the reduction in bacterial α-diversity observed in UC patients as observed in this study. Additionally, increased prophage induction and cell lysis could lead to higher levels of inflammatory bacterial debris, which in turn stimulates the local innate immune response.[38] Thus, the temperate phageome may respond to inflammatory stimuli, thereby altering the host bacterial population in IBD. Clooney *et al.*[33] proposed that a shift from lysogenic to lytic replication cycles is associated with an increase in the relative abundance of temperate phages in disease. Our observation in this study suggests that the conditions in the inflamed mucosa of UC patients, even in endoscopic remission, may not be able to maintain a stable core phageome.

It has been reported that many phages can infect and replicate in various bacterial hosts, and that different lineages within a single bacterial species can exhibit varying levels of resistance to a specific phage.[10] Consequently, establishing meaningful one-to-one correlations between phages and bacteria is challenging. In addition, progress has been made, but a significant portion of the sequencing data could not be assigned a virome database.[16] So, we grouped phages by host bacteria for further analyses. As a result, we found that the gut virome in UC patients differs markedly from that in healthy controls. This change in the virome was accompanied by the alterations of the bacteriome. The structural shift in the virome of UC patients was characterized by a decrease in virulent phages associated with *Odoribacter*, *Ruminococcus*, *Veillonella*, *Bacteroides*, *Bacillus*, and *Bifidobacterium*, and an increase in virulent Xylanimonas phages, temperate Faecalibacterium phages, and virulent Fischrella phages. In particular, the decrease in Odoribacter phages, Ruminococcus phages, and Veillonella phages were significant.

The decrease in virulent Odoribacter phages was accompanied by a reduced abundance of the genus *Odoribacter*. This might reflect cell lysis associated with the lytic effect of virulent Odoribacter phages targeting *Odoribacter splanchnicus*. *Odoribacter splanchnicus* has been reported to play an important role in protective immunity linked to Th17 immune responses.[39] It promotes mucosal healing during colitis by modulating regulatory T-cell responses and contributing to gut health. *Odoribacter*

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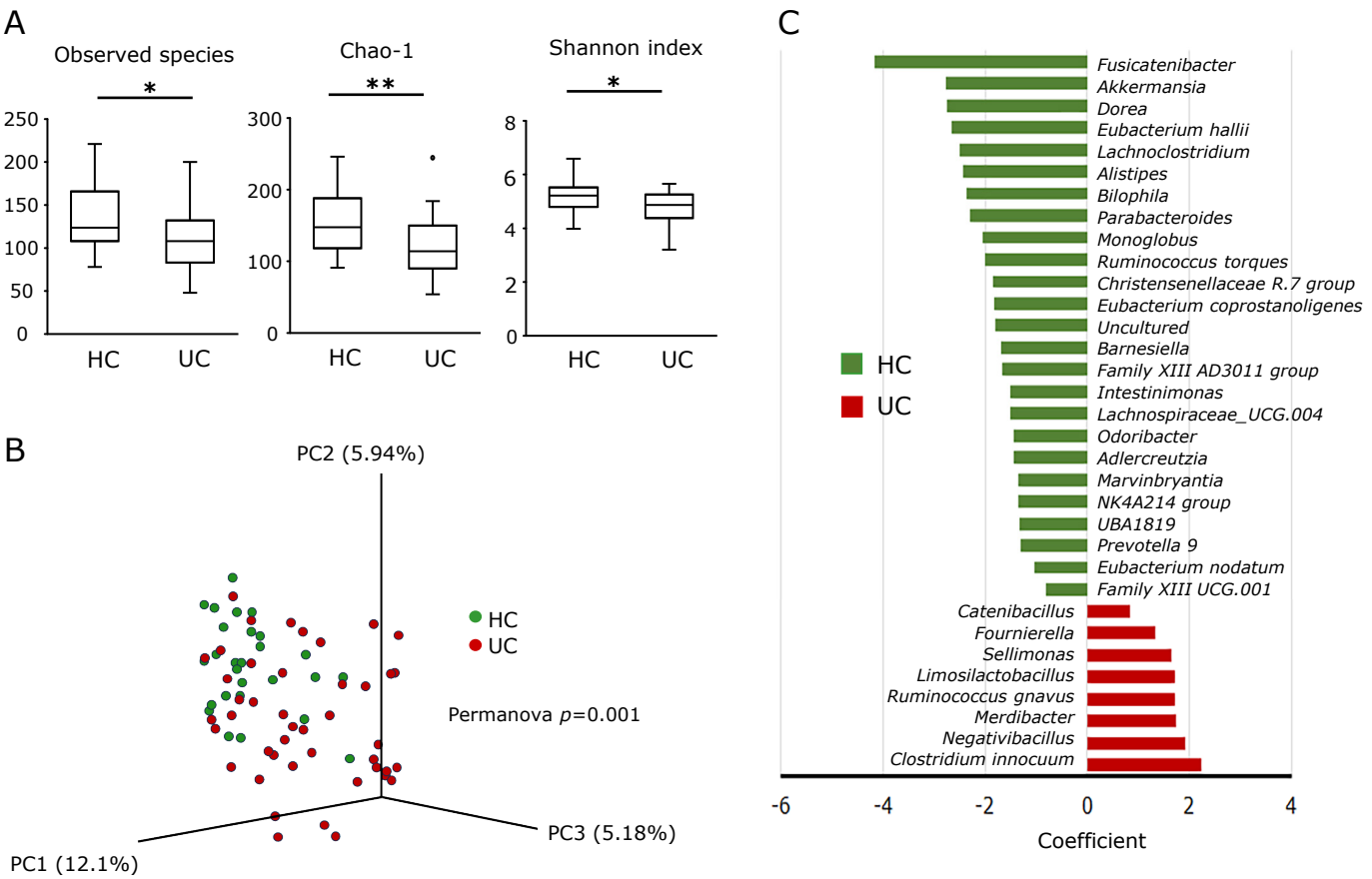

Fig. 5. Changes in the gut bacteriome of UC. (A) α-diversity of the gut bacteriome of UC and HC. $^{**}p<0.01$ by Mann–Whitney *U* test. (B) β-diversity of the gut bacteriome of UC and HC. The bacteriome of UC and HC was plotted using principal coordinate analysis (PCoA) based on the UniFrac distance (PERMANOVA $p = 0.001$). (C) Enriched bacteria in each group were identified through MaAsLin2 analysis.[30] UC, ulcerative colitis; HC, healthy control.

is a key bacterium that supports both metabolic and immune cell protection against colitis. Therefore, a significant decrease in the genus *Odoribacter* is considered indicative of reduced protective activity against mucosal inflammation.

As depicted in Fig. 3, virulent Ruminococcus gnavus phages engaged in patients with UC, and this was accompanied by an increased abundance of *R. gnavus* within the bacteriome. These results are consistent with recent findings by Buttimer *et al.*[40] *R. gnavus* is a gram-positive anaerobic bacterium commonly found in the gut microbiome of individuals, and recent literature categorizes it within the Lachnospiraceae family.[40] *R. gnavus* uses mucins as a nutrient source, which facilitates direct interaction with epithelial cells and luminal antigens, thereby promoting mucosal inflammation. Notably, *R. gnavus* is highly prevalent among patients with IBD,[7,40] and its growth often correlates with disease flare-ups.[7] These observations suggest that the proinflammatory environment driven by *R. gnavus* is associated with an increased abundance of virulent Ruminococcus gnavus phages.

There are some limitations in this study. First, the analysis was conducted on a small sample size, specifically limited to patients with UC. To fully understand the significance of alterations in the gut virome, a larger cohort that includes both CD and UC patients is necessary. Second, we enrolled patients with endoscopic remission, as bleeding and the secretion of anti-bacterial peptides/proteins during active disease significantly affect the gut bacteriome. To understand the changes in the virome under inflammatory conditions, it will also be necessary to examine patients under their active phase in the future.

In conclusion, this study represents the first report on the gut virome of Japanese patients with UC. The gut virome of inactive UC patients exhibits significant alterations in viral composition compared to healthy controls. These changes in the gut virome may contribute to the bacterial dysbiosis observed in UC patients. While establishing direct one-to-one correlations between phages and bacteria may be challenging, further research is required to explore the crucial interactions between the virome and bacteriome in the development of UC.

Author Contributions

All authors contributed to the study conception and design. Material preparation, performing experiments, data collection and analysis were performed by AO and TI. The first draft of the manuscript was written by RI and AA, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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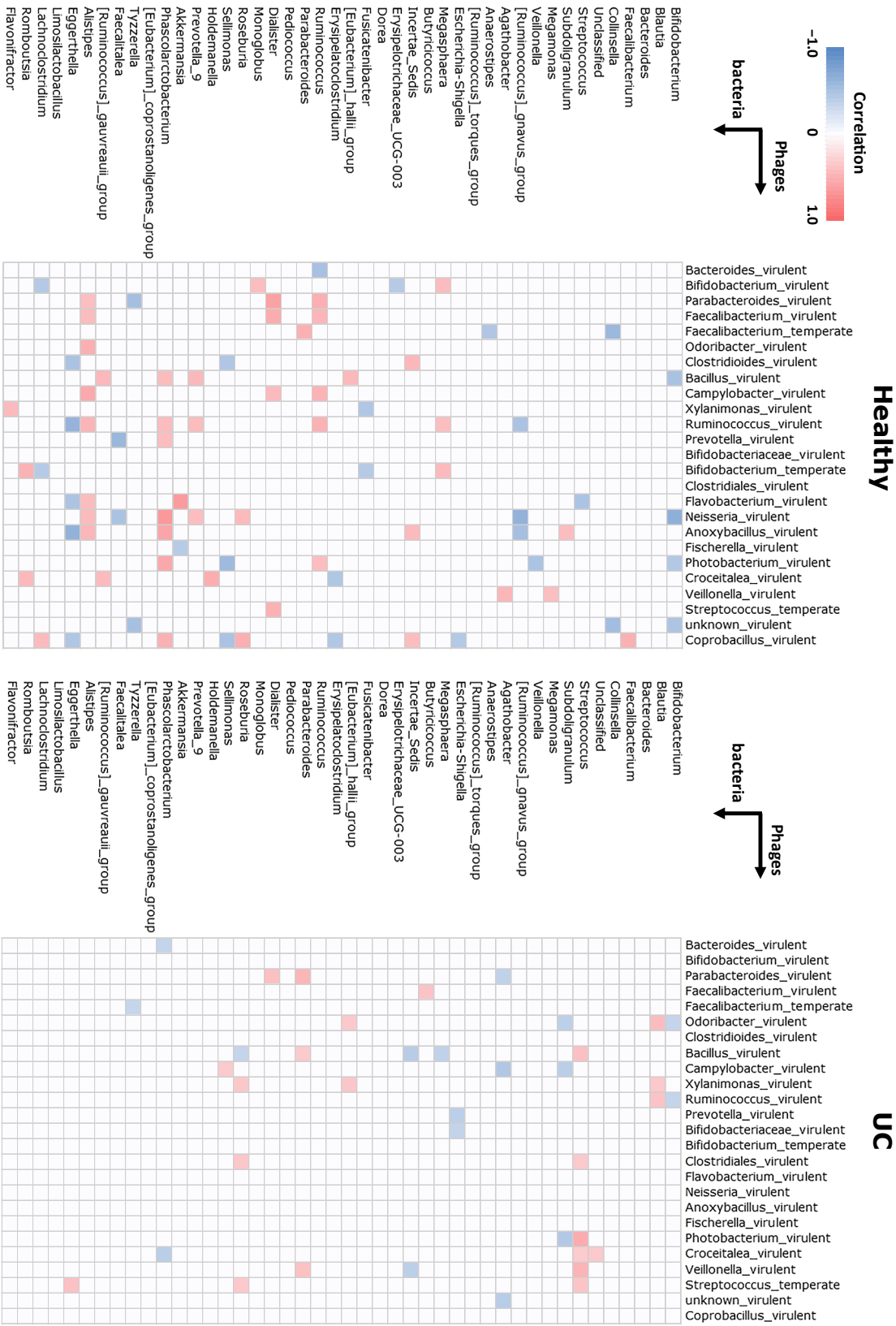

Fig. 6. Interactions between gut bacteriome and virome. The Spearman's rank correlation coefficient (ρ) was calculated to assess the relationship between the abundance of bacterial and viral taxa. Statistical significance was determined for all pairwise comparisons, with significant correlations indicated (p<0.05, ρ>0.4). The x-axis indicates virus taxa grouped by their host bacterium and the y-axis indicates bacteria taxa. UC, ulcerative colitis; HC, healthy controls.

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Conflict of Interest

AA receiving lecture fee from Takeda Pharmaceutical Co. Ltd., AbbVie GK, and Miyarisan Pharmaceutical Co. Ltd. All other authors declare that they have no conflict of interest in this study.

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