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Analysis Of Microbiomic Changes Induced By Uric Acid Lowering Therapy In Gout Patient And Asymptomatic Hyperuricemic Status

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Analysis Of Microbiomic Changes Induced By Uric Acid Lowering Therapy In Gout Patient And Asymptomatic Hyperuricemic Status

Directed by Professor Min-Chan Park

The Doctoral Dissertation
submitted to the Department of Medicine,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree
of Doctor of Philosophy in Medical Science

Hye Won Kim

December 2021

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I am forever indebted to my parents for giving me the opportunities and experiences that have made me who I am. They selflessly encouraged me to explore new directions in life and seek my own destiny. This journey would not have been possible if not for them, and I dedicate this milestone to them.

I would also like to express my gratitude to my beloved husband and my son, who are always there for me and give me courage.

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I would also like to express my sincere gratitude to all those who have loved and encouraged me, although I have not been able to mention them in space. I will do my best to become a competent being that can contribute to humanity and our neighbors.

Lastly, I thank God for guiding me in every step of my life.

Hye Won Kim, 2021

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ABSTRACT

Analysis Of Microbiomic Changes Induced By Uric Acid Lowering Therapy In Gout Patient And Asymptomatic Hyperuricemic Status

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(Directed by Professor Min-Chan Park)

Background: Intestinal microbiome display various structures in individual and disease groups, and functionally contribute to the formation of the immune system through interaction with humans. Likewise, patients with gout have also been shown to have a distinguished intestinal microbiome as compared to healthy controls. However, until now there have been no studies investigating changes in the intestinal microbiome, in a scope of occurrence of gouty arthritis at the edge of asymptomatic hyperuricemia and recovery of the pathological microbiome of gouty arthritis after appropriate uric acid lowering treatment (ULT).

Objectives: Here, we aimed to elucidate the differences in microbiota composition between gout patients and asymptomatic hyperuricemic patients and determine the effect of uric acid-lowering treatment (ULT) on the gut microbiome.

Methods: Stool samples from asymptomatic hyperuricemic patients (asHU, n = 8) and three groups of gout patients, i.e., acute gout patients before ULT (0ULT, n = 14), the same acute gout patients after 30-day ULT (30ULT, n = 9), and chronic gout patients after ≥ 6-month ULT (cULT, n = 18) were collected and analyzed using 16S rRNA gene-based pyrosequencing. The composition of

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microbial taxonomy and communities, species diversity, and relationships among microbial communities were elucidated by bioinformatic analysis.

Results: Gout patients showed less diverse gut microbiota than asHU patients. The microbiota of the asHU group exhibited a higher *Firmicutes*-to-*Bacteroidetes* (F/B) ratio and lower *Prevotella-to-Bacteroides* (P/B) ratio than the gout group; significantly, the F/B ratio increased in gout patients after ULT. Moreover, a balanced enterotype populated asHU patients compared to gout patients. Notable taxa presented significantly differed proportions: *Prevotella copri, Odoribacter splanchnicus, were* enriched in gout patients whereas, in the asHU patients, *Streptococcus salivarius, S.parasanguinis, S.sinensis, Enterococcus durans, Anaerostipes hardrus, Bifidobacterium catenulatum, B.breve, B.bifidum, Weissella, Lactobacillus plantarum, L.reuteri, L.murinus,* and *L.fermentum* were enriched indicating protective microbiome against acute gout attack or chronic gout development despite existing hyperuricemia. *Bifidobacteriales_uc, Lactobacillus helveticus group, L. plantarum group, Streptococcaceae_uc, EF400193_s, EF403944_s* and *KI392030_s* were significantly increased after ULT indicating restoration of microbial taxa.

Conclusion: We found that microbial composition differs between asHU and gout patients. The differential gut microbiota in asHU patients may protect against gout development, whereas that in gout patients may have a role in gout provocation. ULT in gout patients altered the gut microbiota and may help alleviate gout pathology and mitigate gout progression.

Key words : gout, asymptomatic hyperuricemia, gut microbiome, uric acid-lowering therapy

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I. INTRODUCTION

Hyperuricemia is a strong risk factor for gout and exerts its pathological effects in a non-linear concentration-dependent manner.[1] However, not all patients with hyperuricemia develop clinical gout, and only one-third of patients show progression to gout. In contrast, some patients with gout remain normouricemic. [2, 3]

In patients with hyperuricemia, several factors can trigger a gout flare. The deposition of monosodium urate crystals in connective tissues is believed to be a trigger [4]; however, 76% of asymptomatic hyperuricemia (asHU) patients do not have monosodium urate crystal deposition.[3] In patients with gout, a flared inflammatory response is often triggered after a heavy meal or alcohol consumption. Studies have reported that some dietary lipids or alcohol consumption can directly trigger gout flares via the activation of the NALP3 inflammasome by binding to Toll-like receptors. [5, 6]

The role of the intestines in uric acid regulation has been investigated. Although the kidneys are responsible for most uric acid excretion, the intestines contribute to 25% of uric acid excretion, which is further enhanced during renal dysfunction.[7] ATP-binding cassette, subfamily G2 is one of the major urate secretion transporter involved in uric acid excretion from the intestines.[8] In hyperuricemia, the extra-renal excretion of abundant serum uric acid creates an intestinal environment rich in uric acid content.[9]

These findings have raised the possibility that the intestinal microbiota are affected by high serum uric acid levels.[10] Indeed, a remarkable study showed that the gut microbiota are altered in patients with gout compared with healthy controls, and that gout patients have a distinct gut microbial signature compared with healthy individuals.[11] Another study reported that some metabolites present in the fecal samples of gout patients are involved in uric acid excretion, purine metabolism, and the inflammatory response.[12] The

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positive association between gout-related metabolites and microbial taxa in patients with gout suggests that a high uric acid concentration and uric acid metabolites interact with the gut microbiota. Thus, the gut microbiota in gout patients may play a role in gout pathophysiology and serve as a new target for the diagnosis and treatment of gout.[10]

However, the aforementioned studies were conducted on the assumption that patients with asHU and gout have comparable microbiota composition; thus, gout development in the hyperuricemia condition has not been explained.

Hence, in this study, we aimed to investigate whether patients with asHU and gout have differential gut microbiota signatures. Additionally, we investigated whether uric acid-lowering therapy (ULT) can change the microbiota composition.

II. MATERIALS AND METHODS

1. Survey of population ecological information

A total of 40 patients were enrolled to this study. The study cohort comprises four groups; asymptomatic hyperuricemic patients (asHU, n = 8) and three groups of gout patients, i.e. a group of acute gout patients before ULT (0ULT, n = 14), a group of matched patients after the 30-day-ULT (30ULT, n = 9) and a group of chronic gout patients having the ≥ 6-month-ULT (cULT, n = 18). Patients with asymptomatic hyperuricemia included who visited health screening center or outpatient clinic, incidentally found to have hyperuricemia without previous acute gout attack. Diagnosis of asymptomatic hyperuricemia was confirmed when serum urate concentration is elevated level > 8.0 mg/dL. All patients with gout fulfilled the American College of Rheumatology

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(ACR)/European League Against Rheumatism (EULAR) gout classification criteria.[13] All participants met the following criteria: 1) age older than 19 years, 2) no previous antibiotics use within one month, 3) active systemic infectious diseases were excluded from the study. The following questions was checked for each of the patients; Each patient's sex, age, duration of gout, medication dose, comorbidities such as diabetes, hypertension or chronic kidney disease. The study protocol was approved by the ethics committee of Gangnam Severance hospital (IRB protocol 2016-0124-001), and written informed consent was obtained from each patient.

2. Sample Preparation and experiment

A. Stool and serum collection

Before stool sample collection, all patients were required to submit a diary with details of food intake during the previous 3 days, although no restriction was imposed on their diet except for antibiotic use. Stool samples (5–10 g) were collected in the morning before breakfast and stored at −20 °C until further processing. Serum samples were collected on the day of the clinic visit.

B. Fecal DNA isolation and pyrosequencing

The extraction of bacterial DNA was performed using FastDNA™ SPIN Kit for Soil (MP Biomedical, Santa Ana, CA, USA). The quality of the extracted DNA was evaluated by performing 0.8% agarose gel electrophoresis, and the DNA was quantified using a NanoDrop® spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). All DNA samples were stored at −20 °C until further processing. Polymerase chain reaction (PCR) was performed using extracted DNA with primers targeting the V3−V4 regions of the 16S rRNA gene. For bacterial amplification, the primers 341F

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(5′-TCGTCGGCAGCGTC-AGATGTGTATAAGAGACAG-CCTACGGGNGGCWGCAG-3′) and 805R (5′-GTCTCGTGGGCTCGG-AGATGTGTATAAGAGACAG-GACTACHVGGGTATCTAATCC-3′) were used. The amplifications were carried out under the following conditions: initial denaturation at 95 °C for 3min, followed by 25 cycles of denaturation at 95 °C for 30 sec, primer annealing at 55 °C for 30 sec, and extension at 72 °C for 30 sec, with a final elongation at 72 °C for 5 min. Then, secondary amplification for attaching the Illumina NexTera barcode was performed with i5 forward primer (5′-AATGATACGGCGACCACCGAGATCTACAC-XXXXXXXX-TCGTCGGCAGCGTC-3′; X indicates the barcode region) and i7 reverse primer (5′-CAAGCAGAAGACGGCATACGAGAT-XXXXXXXX-AGTCTCGTGGGCTCGG-3′). Mixed amplicons were pooled, and sequencing was performed at ChunLab, Inc. (Seoul, Korea) on an Illumina MiSeq Sequencing System (Illumina, San Diego, CA, USA) according to the manufacturer's instructions.

C. Pyrosequencing sequence processing and bioinformatics analysis

To improve data quality, low quality (<Q25) reads were filtered using the Trimmomatic (V0.32) read trimming tool (Usadel Lab, Aachen, Germany) and paired-end sequence data were merged using PANDAseq.[14] Primers were then trimmed with ChunLab's in-house program at a similarity cut-off of 0.8. Non-specific amplicons encoding non-16S rRNA genes were detected using the HMMER hmmsearch program (EMBL-EBI, Hinxton, Cambridge, UK) and were not considered for further sequence analysis. Sequences were denoised using DUDE-Seq and non-redundant reads were extracted using the UCLUST clustering method.[15] Bacterial taxonomic assignments were performed based on the EzBioCloud database using USEARCH (8.1.1861_i86linux32) followed by more precise pairwise alignment.[15] UCHIME [16] and the non-chimeric 16S rRNA

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database from EzBioCloud were used to detect chimera on reads with <97% similarity. Reads that were not identified at the species level (with <97% similarity) in the EzBioCloud database were compiled, and UCLUST5 was used to perform de novo clustering to generate operational taxonomic units (OTUs). Finally, OTUs with single reads (singletons) were omitted from further analysis. The bacterial taxonomic composition was evaluated from the phylum to the species level.

D. Quantitative PCR

Bacterial biomarkers discriminating patients before and after ULT were identified from sequencing analysis and further confirmed using quantitative PCR (qPCR). Quantification of specific taxa before and after ULT was performed using qPCR (a LightCycle® 480 system [Roche Diagnostics, Basel, Switzerland]) with LightCycler® FastStart DNA Master SYBR Green I (Roche). The primers used in the study were newly designed to target homologous sequences in the 16S rDNA of each bacteria to quantify the amount of bacteria in each sample

3. Statistical analysis

All statistical analyses were performed using R software (version 4.1.0; R Foundation for Statistical Computing, Vienna, Austria). Baseline characteristics of four groups of patients were compared using one-way analysis of variance. Categorical variables were compared using χ^2 statistics. Alpha diversity was assessed using the Shannon index (evenness and richness), valid reads, and the number of observed species (OTUs). Beta diversity was assessed using a principal component (PCoA) score plot based on Bray-Curtis dissimilarity metrics, canonical correspondence, or redundancy analyses. The Wilcoxon rank-sum test was used to compare the taxa abundance ratio in the

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asHU group with that in the total gout group. To discover biomarkers with statistical differences among groups, the Kruskal-Wallis rank test with a *P*-value of 0.05 was first used to compare differential microbiota abundance between groups. Then, a linear discriminant analysis (LDA) was performed to evaluate the influence of biomarkers on significantly different microbial groups based on LDA scores. Linear discriminant analysis Effect Size (LEfSe) was used to determine potential markers among different groups. Pattern search function of the MicrobiomeAnalyst, a web-based tool was used to compare abundance patterns in the dataset.[17] Nonparametric Spearman's test was used to test the correlation between microbial taxa and serum uric acid level. Moreover, Tax4Fun2, an R package,[18] was used for the prediction of functional profiles from 16S rRNA gene sequences. Pathway analysis were conducted using Kyoto Encyclopedia of Genes and Genomes (KEGG) database.

III. RESULTS

1. Characteristics of the patients with gout and asymptomatic hyperuricemia

Table 1. Characteristics of patients with asymptomatic hyperuricemia and gout

	Asymptomatic Hyperuricemia (asHU) (n = 8)	Acute gout (0ULT*) (n = 14)	Chronic gout (cULT) (n = 18)	*p* value	*Post hoc p* value (Acute vs Chronic gout)	*Post hoc p* value (asHU vs total gout)
Male, n (%)	8 (100.0%)	14 (100.0%)	18 (100.0%)			

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Age in years	56.5 ± 12.9	49.7 ± 15.8	50.0 ± 17.4	0.413	0.962	0.298
Height (cm)	171.5 ± 4.9	172.8 ± 4.8	174.6 ± 7.9	0.23	0.456	0.364
Body weight (Kg)	76.0 ± 13.8	74.2 ± 8.5	80.9 ± 14.9	0.263	0.158	0.697
BMI (kg/m^2)	25.7 ± 3.5	24.8 ± 2.4	26.6 ± 5.2	0.473	0.23	0.939
18.5-23	1 (12.5%)	3 (23.1%)	4 (23.5%)	0.443	0.233	0.638
≥23	4 (50.0%)	5 (38.5%)	3 (17.6%)			
≥25	2 (25.0%)	5 (38.5%)	6 (35.3%)			
≥30	1 (12.5%)	0 (0.0%)	4 (23.5%)			
Comorbidities						
Hypertension, n (%)	0 (0.0%)	4 (28.6%)	7 (38.9%)	0.122	0.815	0.08
Dyslipidemia, n (%)	0 (0.0%)	3 (21.4%)	7 (38.9%)	0.1	0.501	0.165
Diabetes, n (%)	0 (0.0%)	0 (0.0%)	1 (5.6%)	0.534	0.999	0.999
Chronic kidney disease, n (%)	0 (0.0%)	1 (7.1%)	13 (72.2%)	0.001	0.001	0.034
Risk factors for gout						

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Protein/alcohol intake	2 (25.0%)	10 (71.4%)	8 (44.4%)	0.091	0.243	0.236
Family history of gout	0 (0.0%)	2 (14.3%)	1 (5.6%)	0.433	0.819	0.881
Disease duration of gout	NA	1.0 ± 1.4	41.4 ± 36.8	NA	0.001	NA
Laboratory findings						
Total bilirubin (mg/dL)	0.6 ± 0.2	0.8 ± 0.3	0.8 ± 0.2	0.11	0.878	0.036
AST (IU/L)	33.6 ± 14.3	26.9 ± 8.5	29.3 ± 10.9	0.523	0.49	0.216
ALT (IU/L)	26.9 ± 5.2	28.6 ± 17.3	31.8 ± 20.9	0.477	0.649	0.363
ALP (IU/L)	79.1 ± 21.9	74.9 ± 18.5	80.1 ± 31.9	0.827	0.592	0.896
Triglyceride (mg/dL)	285.5 ± 57.7	266.4 ± 127.5	220.0 ± 134.9	0.303	0.443	0.515
Glucose (mg/dL)	100.0 ± 6.8	97.5 ± 14.4	107.7 ± 23.2	0.232	0.206	0.403
LDL-cholesterol (mg/dL)	135.1 ± 17.6	134.0 ± 37.7	132.1 ± 32.4	0.866	0.907	0.909
HbA1c (%)	5.5 ± 0.3	5.5 ± 0.5	5.6 ± 0.1	0.694	0.673	0.886
ESR (mm/hr)	12.7 ± 13.5	24.7 ± 26.8	16.6 ± 12.7	0.66	0.538	0.335
CRP (mg/L)	1.5 ± 1.3	9.1 ± 14.6	4.5 ± 5.4	0.471	0.518	0.078

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BUN (mg/dL)	13.8 ± 3.5	17.3 ± 7.3	17.5 ± 6.0	0.202	0.925	0.134
Uric acid (mg/dL)	8.2 ± 1.3	8.8 ± 1.6	6.0 ± 2.7	0.006	0.002	0.156
Creatinine(mg/dL)	0.9 ± 0.1	1.1 ± 0.3	1.1 ± 0.2	0.068	0.784	0.001
Creatinine clearance (ml/min/1.73 m²)	92.8 ± 12.9	83.5 ± 26.2	80.3 ± 22.9	0.22	0.718	0.220
ULT						
Allopurinol, n (%)	0 (0.0%)	5 (45.5%)	2 (11.1%)	0.001	0.099	0.001
Febuxostat, n (%)	0 (0.0%)	6 (54.5%)	16 (88.9%)			

Data are shown in mean ± SD. *Only baseline characteristics before ULT in acute gout patients were collected.

BMI: body mass index; AST: aspartate aminotransferase; ALT: alanine aminotransferase; TG: triglyceride; LDL: Low-density lipoprotein; BUN: blood urea nitrogen; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; ULT: uric acid-lowering treatment; NA: Not applicable; 0ULT: acute gout patients before ULT; cULT: chronic gout patients after >6-month ULT.

The characteristics of enrolled patients are described in Table 1. All patients were male and the mean ages were comparable between groups. Gout patients had a statistically higher incidences of hypertension and chronic kidney disease compared to asHU patients. Serum creatinine concentration was significantly higher and there was a non-significant trend for elevated C-reactive protein in patients with gout than in asHU patients, indicating the presence of

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comorbid conditions with inflammatory burden in the gout patients compared to asHU patients. However, there were no statistical differences in age, alcohol or protein consumption, family history, body mass index between asHU and gout patients. Disease duration of gout were significantly shorter in the acute gout patients compared to chronic gout patients (1.0 vs. 41.4 months, $p<0.001$) reflecting that patients with early and advanced phase of gout were enrolled. There were no statistical differences in body mass index, comorbidities except higher prevalence of chronic kidney disease (7.1% vs. 72.2%, $p=0.001$) between acute and chronic gout patients. Serum uric acid levels were elevated in patients with acute gout before ULT and in patients with asHU than in patients with chronic gout who received ULT.

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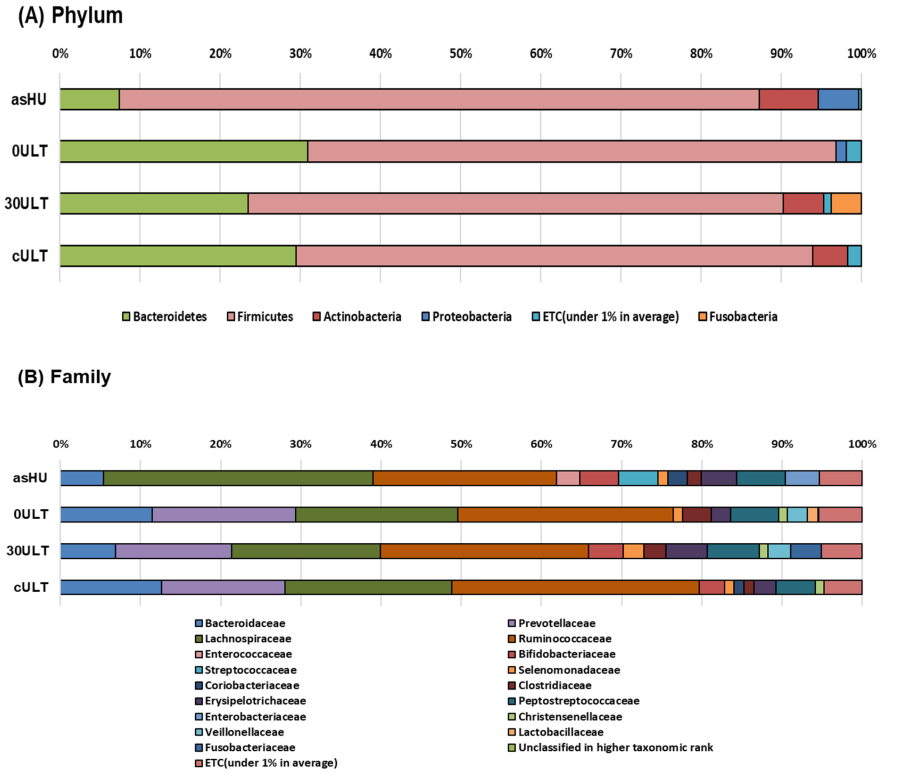
2. *Comparison of gut microbiota between asHU and gout patient*



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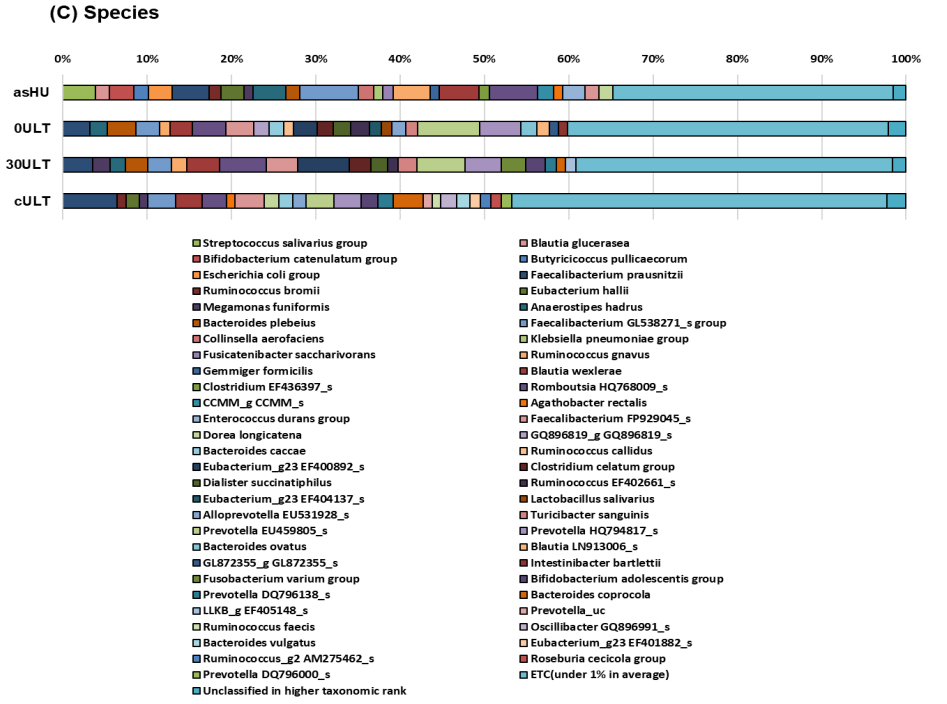

Figure 1. Averaged toxonomic compositions in the bacterial phyla, families and species. Variations were apparent between asymptomatic hyperuricemia patients and gout patients

Variations in the bacterial phyla and family profiles were apparent between asHU group and gout group (Figure 1.).

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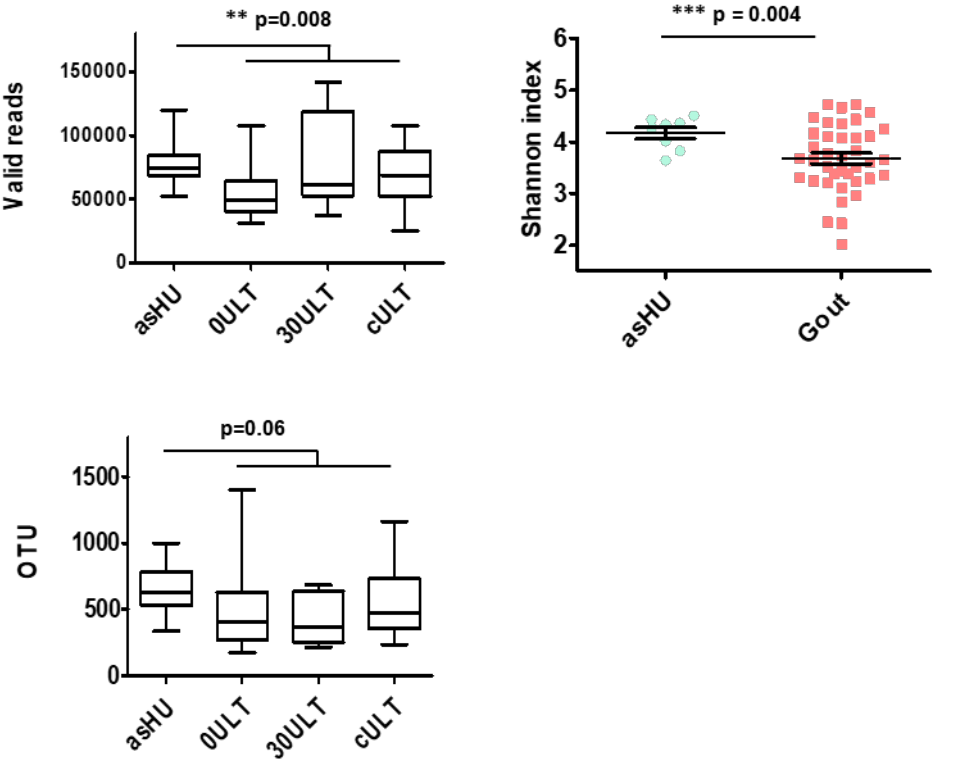

Figure 2. The alpha diversity assessed by the valid reads, number of observed species (OTU), Shannon diversity indices among the groups.

Species richness, measured as the number of valid reads and OTUs, were statistically different between the asHU and whole gout groups (Figure 2.). OTUs and valid reads showed a trend toward notably reduced microbiota diversity in acute gout patients compared with chronic gout patients, and gout patients showed more reduced microbiota diversity than asHU patients, indicating that the gut microbiome is different between asHU and gout patients. In gout patients, ULT may partially restore the microbiota composition, although the differences were not statistically significant.

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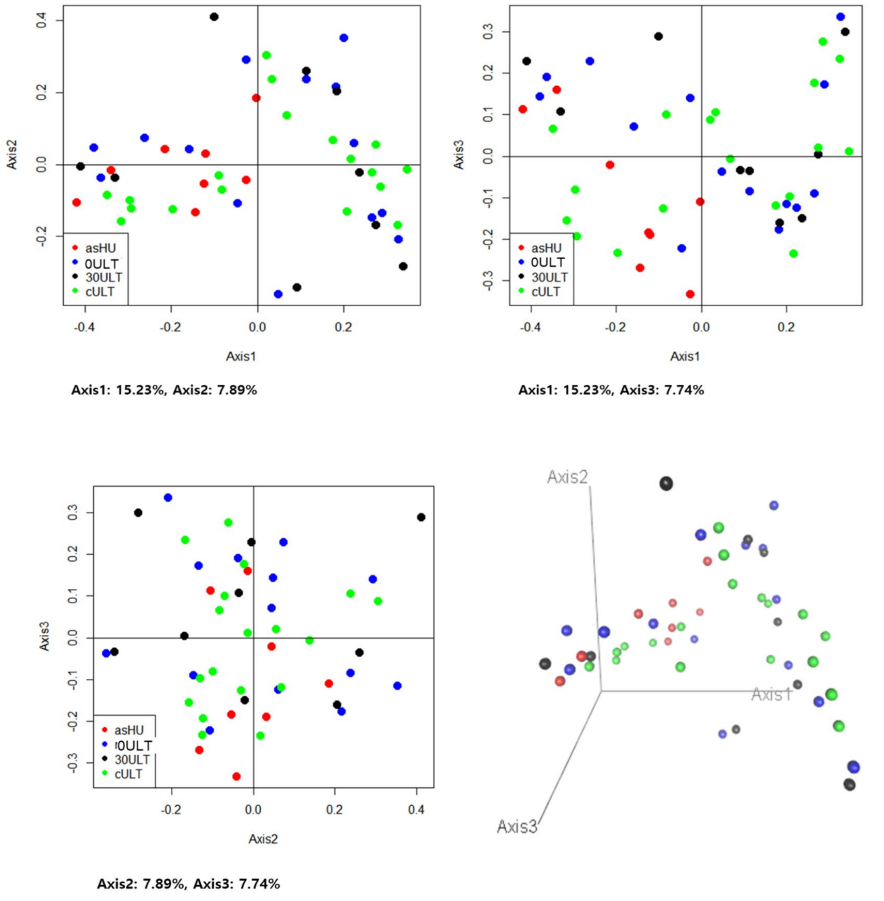

Figure 3. A principal component (PCoA) Score plot based on Bray-Curtis dissimilarity metrics for all participants. Each point represents the composition of the intestinal microbiota of each individual. ULT; uric acid lowering treatment, asHU; asymptomatic hyperuricemic patients, 0ULT; acute gout patients before ULT, 30ULT; acute gout patients after the 30-day-ULT, cULT; chronic gout patients having the more than 6-month-ULT

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A PCoA score plot showed that gut microbiota of asHU and gout patients were separated (permutational multivariate analysis of variance F-value: 3.536; R^2: 0.014; P <0.05), indicating statistically significant community differences between asHU and gout patients.

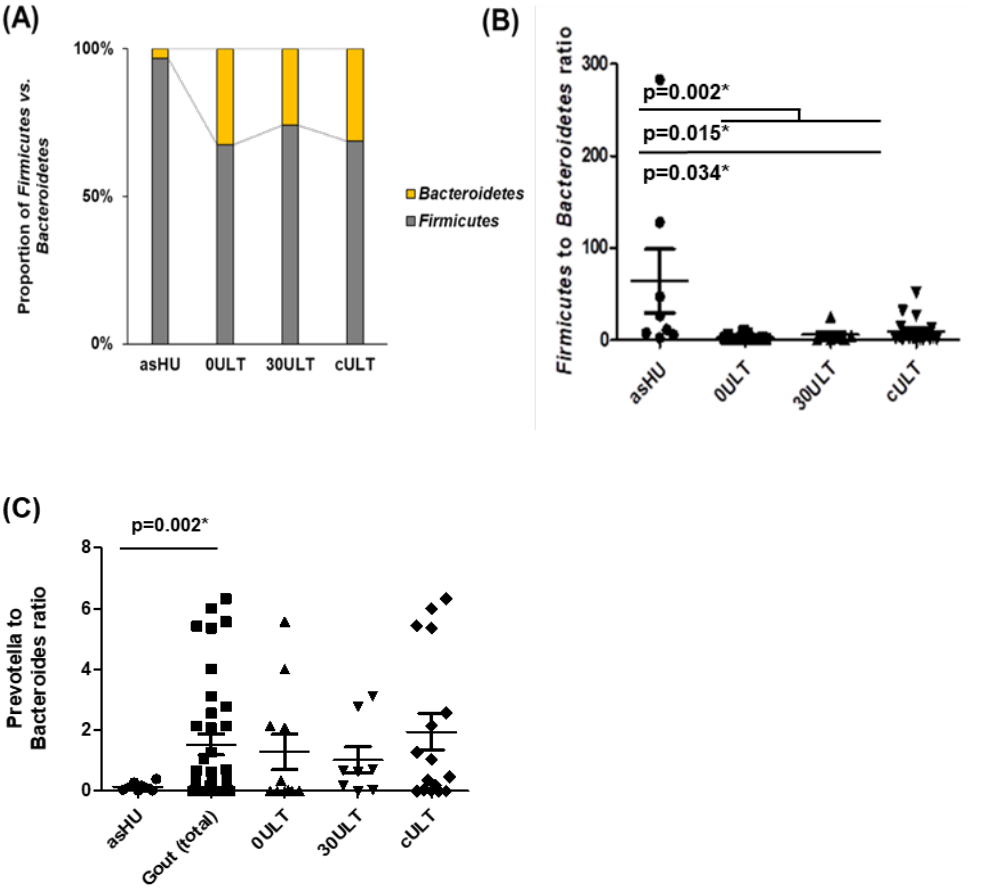

Figure 4. Bacterial family analysis. (A) Proportion of *Firmicutes vs. Bacteroidetes*, (B) *Firmicutes-to-Bacteroidetes* (*Firmicutes* (%)/

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***Bacteroidetes* (%)) at the phylum level, (C) *Prevotella-to-Bacteroides* (P/B) ratio from the genus level.**

To detect the microbial groups with significantly different compositions in different patient groups, we first analyzed the ratio of *Firmicutes (%)* to *Bacteroidetes (%)* (F/B) at the phylum level and *Prevotella (%)* to *Bacteroides (%)* (P/B) ratio at the genus level. Our results showed that the relative abundance of *Firmicutes* was higher in the asHU group than in any gout group (Figure 4A.). The F/B ratio at the phylum level increased after ULT (Fig. 2B). The F/B ratio was significantly higher in asHU patients than in total gout patients (p=0.002, Wilcoxon rank sum test). The taxonomic composition at the genus level revealed that the P/B ratio was significantly lower in asHU patients than in gout patients (p=0.002, Wilcoxon rank sum test, Figure 4C.). Moreover, asHU patients had a significantly low proportion of *Bacteroidetes* and a high proportion of *Firmicutes* compared with gout patients (p<0.001, p<0.01, Wilcoxon rank sum test, respectively)

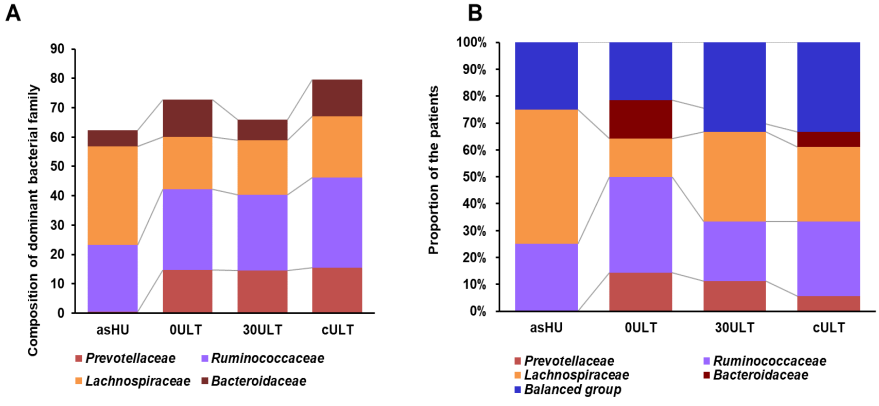

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Figure 5. Enterotype analysis. (A) Composition of dominant bacterial families in gout and asymptomatic hyperuricemia patients. (B) Proportion of patients with dominant taxa group.

At the family level, the proportion of *Prevotellaceae* and *Bacteroidaceae* was lower, whereas that of *Lachnospiraceae* was higher, in asHU patients than in gout patients (Figure 5A.). Next, the patients were grouped into harboring different enterotypes based on dominant bacteria clusters: *Prevotellaceae*-, *Lachnospiraceae*-, *Rumiococcaceae*-, and *Bacteroidaceae*-dominant enterotypes and balanced enterotype (with no particular dominant microbiota) (Figure 5B.). Balanced enterotype populated asHU patients compared to 0ULT patients, whereas the proportion of the *Bacteroidaceae*-dominant enterotype was absent in asHU. After 30-day ULT, the number of patients with *Bacteroidaceae*-dominant enterotype was lower than that in the 0ULT group; however, these changes reverted in the cULT group, indicating that although ULT induces changes in the intestinal microbiome, it does not maintain these changes over a longer duration.

Based on the results of the Kruskal-Wallis test, we identified a significantly different taxa composition in the gut microbiome between asHU and gout patients (p<0.05) (Table 2). Next, bacterial taxa that were differentially represented between groups and had an LDA score of >2 were further analyzed (Figure 6.). LEfSe analysis confirmed that *Prevotella copri* and *Odoribacter splanchnicus* were enriched in gout patients. In the asHU group, *Streptococcus salivarius*, *S. parasanguinis*, *S. sinensis*, *Enterococcus durans*, *Anaerostipes hadrus*, *Bifidobacterium catenulatum*, *B. breve*, *B. bifidum*, *Lactobacillus plantarum*, *L. reuteri*, *L. murinus*, and *L. fermentum* were enriched.

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Table 2. Significantly different selected taxa between asHU and gout patients based on Kruscal-Wallis test (p<0.05) asHU : asymptomatic hyperuricemia; FDR: False discovery rate.

Patient category	Taxonomy	*p* value	FDR-adjusted *p* value
Favor gout	*Prevotella copri*	0.00000	0.00910
	Odoribacter splanchnicus	0.02621	0.25409
	Enterococcus faecalis	0.00186	0.14676
	Ruminococcus faecis	0.04098	0.37171
	Megamonas funiformis	0.04826	0.41895
	Brevibacterium iodinum group	0.01912	0.23345
	Enterococcus casseliflavus group	0.00075	0.11027
Favor asHU	*Streptococcus salivarius group*	0.00035	0.11027
	Bifidobacterium breve	0.00037	0.11027
	Lactobacillus murinus	0.00039	0.11027
	Streptococcus sinensis group	0.00101	0.11027
	Alkalilimnicola ehrlichii	0.00122	0.11027
	Escherichia hermannii group	0.00122	0.11027
	Pseudomonas flavescens group	0.0016	0.13122
	Haemophilus parainfluenzae group	0.00219	0.16827
	Lactobacillus plantarum group	0.00231	0.17559
	Streptococcus parasanguinis group	0.00273	0.19733

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Dorea formicigenerans	0.00335	0.23345
Coprococcus comes	0.00362	0.23345
Enterococcus durans group	0.00367	0.23345
Anaerostipes hadrus	0.00738	0.23345
Blautia coccoides group	0.0086	0.23345
Dorea longicatena	0.00863	0.23345
Lactobacillus reuteri group	0.01181	0.23345
Veillonella dispar	0.01416	0.23345
Clostridium ramosum	0.01753	0.23345
Collinsella aerofaciens	0.01768	0.23345
Escherichia coli group	0.02295	0.23345
Lactobacillus fermentum	0.02479	0.2442
Holdemanella biformis	0.02752	0.26547
Bifidobacterium catenulatum group	0.02795	0.26918
Bifidobacterium bifidum	0.04824	0.41895

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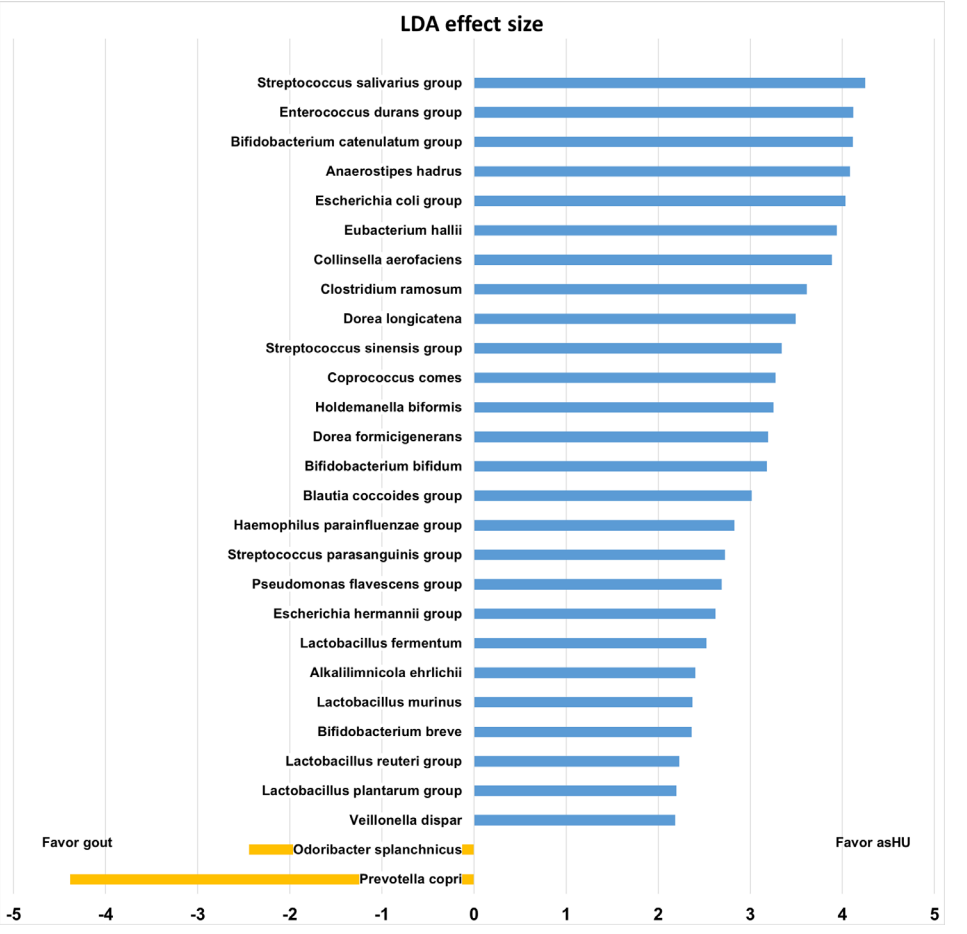

Figure 6. Linear discriminant analysis(LEfSe). Bacterial taxa that were differentially represented between asHU and gout patients, with statistical level of significance according to linear discriminant analysis (LDA score > 2). LDA Effect Size plot of taxonomic biomarkers identified within fecal samples of asHU and gout patients. Left bars are indicative of enrichment within samples of gout patients, whereas right bars are indicative of enrichment within samples of asHU patients.

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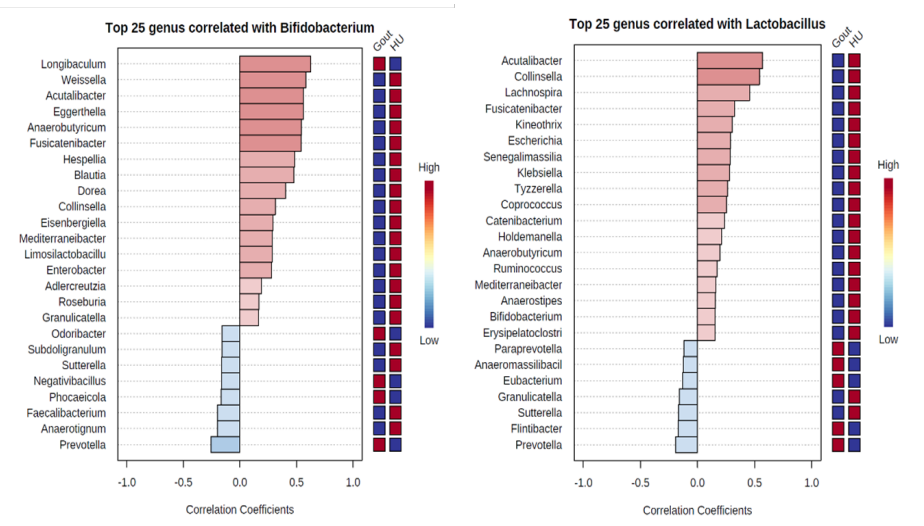

Figure 7. Co-clustering patterns of *Lactobacillus spp.* and *Bifidobacterium spp.*

We identified abundance patterns based on correlation analysis with specific taxon. The microbial clusters of *Lactobacillus spp*. and *Bifidobacterium spp*. are presented in Figure. 7. Interestingly, *Lactobacillus spp*. showed a co-occurrence pattern with *Bifidobacterium spp*., suggesting symbiotic interactions. On the other hand, both *Lactobacillus spp*. and *Bifidobacterium spp.* had negative co-occurrence patterns with *P.copri.* Notably, nonparametric Spearman correlation showed a negative correlation between serum uric acid level and the proportion of *P. copri* (Figure 8.).

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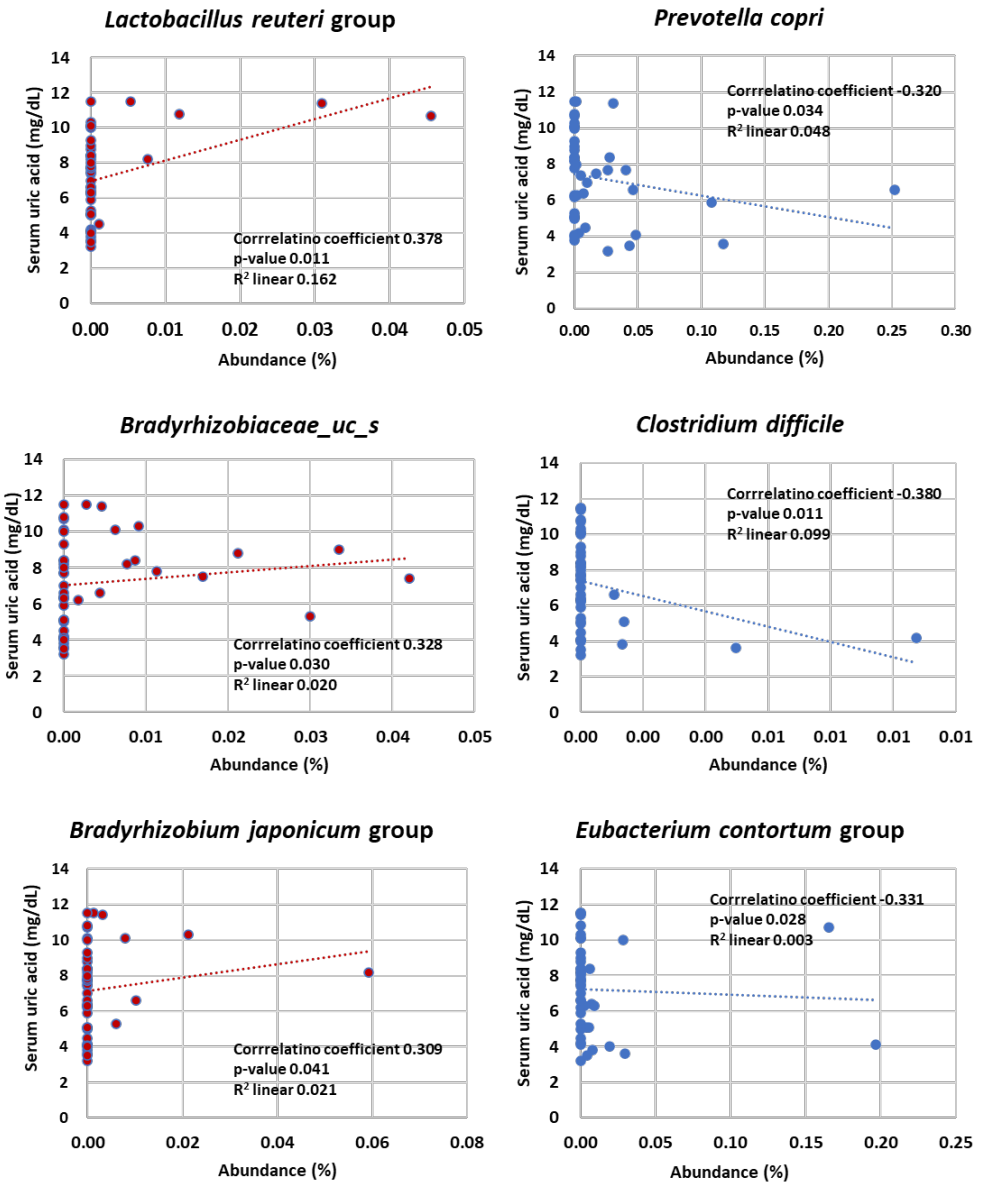

Figure 8. Nonparametric spearman correlation between serum uric acid and microbiota.

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3. *Comparison of gut microbiota before and after uric acid lowering treatment in acute gout patient*

Next, we assessed whether ULT can modulate microbiota composition in patients with acute gout. Minimal changes were observed in alpha diversity before and after the intervention in acute gout patients (Figure 1.), indicating that the microbiota composition did not change. However, beta diversity analysis with canonical correspondence ($P < 0.05$) and redundancy analyses ($P < 0.05$) showed that the composition of the gut microbiome before and after 30-day ULT was significantly different (Figure 9.).

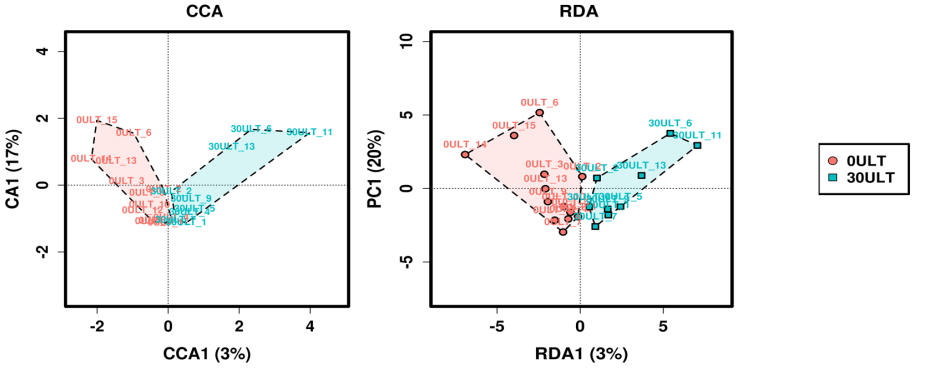

Figure 9. Beta diversity analysis using canonical correspondence analysis and redundancy analysis for acute gout patients before and after ULT. Each point represents the composition of the intestinal microbiota of each individual. ULT; uric acid lowering treatment, 0ULT; acute gout patients before ULT, 30ULT; acute gout patients after the 30-day-ULT, CCA; Canonical correspondence analysis, RDA; redundancy analysis.

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Table 3. Significantly different selected taxa before (0ULT) and after 30days of ULT (30ULT) based Kruscal-Wallis test (p<0.05)

Patient category	Taxon name	*p* value	FDR-adjusted *p* value
Favor 0ULT	*Arthrobacter globiformis group*	0.02908	0.02948
	Bradyrhizobium_uc	0.00156	0.00156
	Sphingomonas_uc	0.00451	0.00454
	Clostridium_g18: AM275423_s	0.01192	0.01204
Favor 30ULT	*Lachnospiraceae: AJ518873_g: EF400193_s*	0.04027	0.04108
	Eubacterium_g: EF403944_s	0.02908	0.02958
	Lactobacillus helveticus group	0.02908	0.02957
	Lactobacillus plantarum group	0.02908	0.02959
	Oxalobacter: KI392030_s	0.02908	0.02959

FDR: false discovery rate.

Significant taxonomic changes were observed in the fecal samples of patients before ULT and after 30-day ULT (P <0.05) based on the Kruskal-Wallis (Table 3). The proportion of *L. helveticus* and *L. plantarum* was significantly higher in the 30ULT group, whereas that of *Bradyrhizobium* (taxon id: *Bradyrhizobium*_uc) was higher in the 0ULT group. The results were consistent

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with qPCR analysis, which showed that the proportion of *Bradyrhizobium* was significantly higher in acute gout patients who experienced acute gout attacks and never received ULT, and this proportion decreased after ULT (Table 4).

Table 4. Changes in microbiome before and after ULT by patient confirmed using Quantitative PCR. *Bradyrhizobium_uc* were significantly higher in acute gout patients who had been recently experienced acute gout attack and never exposed to ULT and consistently decreased after ULT. ULT; uric acid lowering therapy.

	by Absolute values									by Proportion								
Patient	**1**	**2**	**4**	**5**	**6**	**7**	**9**	**11**	**13**	**1**	**2**	**4**	**5**	**6**	**7**	**9**	**11**	**13**
EU459805_s	+	+	0	-	-	+	-	+	+	+	+	0	-	-	+	-	+	+
Prevotella_uc	-	+	+	-	+	+	-	+	-	-	+	-	-	+	-	-	+	-
Bacteroides_uc	+	-	+	+	-	+	-	+	-	+	-	+	+	-	+	-	+	-
Dialister succinatiphilus	+	-	-	+	0	+	0	0	0	+	-	-	+	0	+	0	0	0
GL872355_s	-	+	0	0	+	0	-	-	+	-	+	0	0	-	0	-	-	+
EU462041_s	+	-	0	0	0	+	-	+	0	+	-	0	0	0	+	-	+	0
Prevotellaceae_uc_s	-	+	-	-	-	+	-	+	+	-	+	-	-	-	-	-	+	-
Bacteroidaceae_uc_s	+	+	+	+	-	+	-	+	-	+	+	+	+	-	+	-	+	-
EF405071_s	0	+	-	+	0	+	+	+	0	0	+	-	+	0	+	+	+	0
DQ824928_s	0	0	+	0	0	0	+	0	0	0	0	-	0	0	0	+	0	0
Dialister_uc	-	-	+	+	0	+	+	0	0	-	-	+	+	0	-	+	0	0
Agathobacter_uc	0	-	0	+	0	0	+	0	0	0	-	0	+	0	0	+	0	0
Ruminococcus_g2_uc	+	+	0	+	0	-	-	0	0	+	+	0	+	0	-	-	0	0
Clostridiales_uc_s	0	-	-	-	+	+	-	+	+	-	-	-	-	-	-	-	-	+
PAC000196_s	0	-	+	+	0	0	0	+	0	0	-	+	+	0	0	0	-	0
FJ510897_s	0	0	0	0	+	+	0	+	0	-	0	0	0	-	+	0	+	0
Clostridium_g24_uc	0	0	0	0	+	0	-	+	+	0	0	0	0	+	0	-	+	+
Veillonellaceae_uc_s	-	-	+	+	0	+	+	0	+	-	-	+	+	0	-	+	0	-
Bacteroidales_uc_s	-	-	+	-	-	+	-	+	+	-	-	+	-	-	+	-	+	-
AB009176_g_uc	0	0	0	0	0	+	0	0	0	0	0	0	0	0	+	0	0	0
EF402847_s	-	0	+	+	0	+	-	0	0	-	0	+	+	0	+	-	0	0
DQ905770_s	0	+	+	+	0	+	+	0	0	0	+	+	+	0	+	+	0	0
Butyricimonas virosa	0	-	+	+	0	0	+	+	0	0	-	+	+	0	0	+	+	0
Christensenella_uc	+	0	+	-	0	+	-	0	0	+	0	+	-	0	+	-	0	0
Leuconostocaceae_uc_s	0	0	0	+	0	+	+	-	-	0	0	0	+	0	+	+	-	-
FJ880520_s	0	+	0	0	+	0	0	+	0	0	+	0	0	-	0	0	-	0
FJ680757_s	0	-	0	0	0	0	+	0	0	0	-	0	0	0	0	+	0	0
HQ770262_s	0	-	0	0	0	0	0	+	0	0	-	0	0	0	0	0	+	0
Lactobacillus casei group	+	+	0	0	0	0	+	+	+	+	+	0	0	0	0	+	+	+
Mitsuokella jalaludinii	+	-	0	0	0	0	0	0	0	-	-	0	0	0	0	0	0	0
JPZU_g_uc	0	0	0	0	0	0	-	+	0	0	0	0	0	0	0	-	+	0
Paraburkholderia kururiensis	0	0	-	0	0	0	0	+	+	0	0	-	0	0	0	0	+	+
EU776475_s	0	-	0	+	-	0	-	+	0	0	-	0	+	-	0	-	+	0
HQ816544_s	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	-
Bradyrhizobiaceae_uc_s	-	-	-	-	-	0	+	0	-	-	-	-	-	-	0	+	0	-
Actinomyces viscosus group	+	0	0	0	0	0	0	+	0	+	0	0	0	0	0	0	-	0
Anaerofustis_uc	-	0	-	0	0	+	0	0	0	-	0	-	0	0	-	0	0	0
Rhodospirillaceae_uc_s	+	0	-	0	0	0	-	0	-	+	0	-	0	0	0	-	0	-
Bradyrhizobium_uc	-	0	0	-	0	0	-	0	-	-	0	0	-	0	0	-	0	-
AM713401_s	+	0	-	0	0	-	0	0	0	+	0	-	+	0	-	0	0	0
Burkholderiales_uc_s	-	0	0	0	+	+	+	0	-	-	0	0	0	+	-	+	0	-
Natronincola_f_uc_s	0	0	0	0	+	+	0	+	+	0	0	0	0	+	+	0	+	+
Gemmata_f_uc_s	0	0	0	0	0	0	0	0	-	0	0	0	0	0	0	+	0	-

0; no detection before/after ULT; +: more detection after ULT; - : less detection after ULT.

Consistent "+" results (0 is ignored) is marked in green, and "-" is marked in scarlet.

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Furthermore, nonparametric Spearman correlation test showed a significant correlation between serum uric acid level and the proportion of *Bradyrhizobium* (Figure 8). Using LEfSe, the unique taxa that were differentially present in acute gout patients before and after ULT were identified in Figure 10.

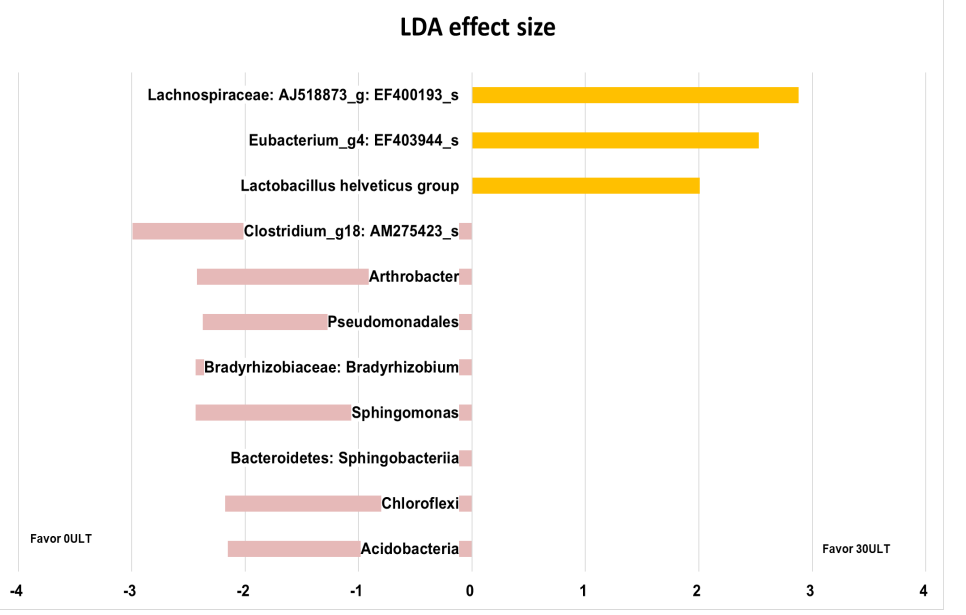

Figure 10. LDA Effect Size (LEfSe) plot of taxonomic biomarkers identified within fecal samples of acute gout patients before and after ULT. Bacterial taxa that were differentially represented between groups, with statistical level of significance according to linear discriminant analysis (LDA score > 2). Left bars are indicative of enrichment within samples of acute gout patients before ULT, whereas right bars are indicative of enrichment within samples of acute gout patients after 30days of ULT.

Pathway analysis using KEGG database revealed that among significantly predicted genes “Apoptosis,” “Lysosome,” Rheumatoid arthritis,” and “Osteoclast differentiation” genes were over-represented in gout patients

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compared with asHU patients (Figure 11A.). Furthermore, the pathway related to the “Biosynthesis of unsaturated fatty acids” was more abundant in the after 30-day treatment group than in the before-treatment group (Figure 11B.).

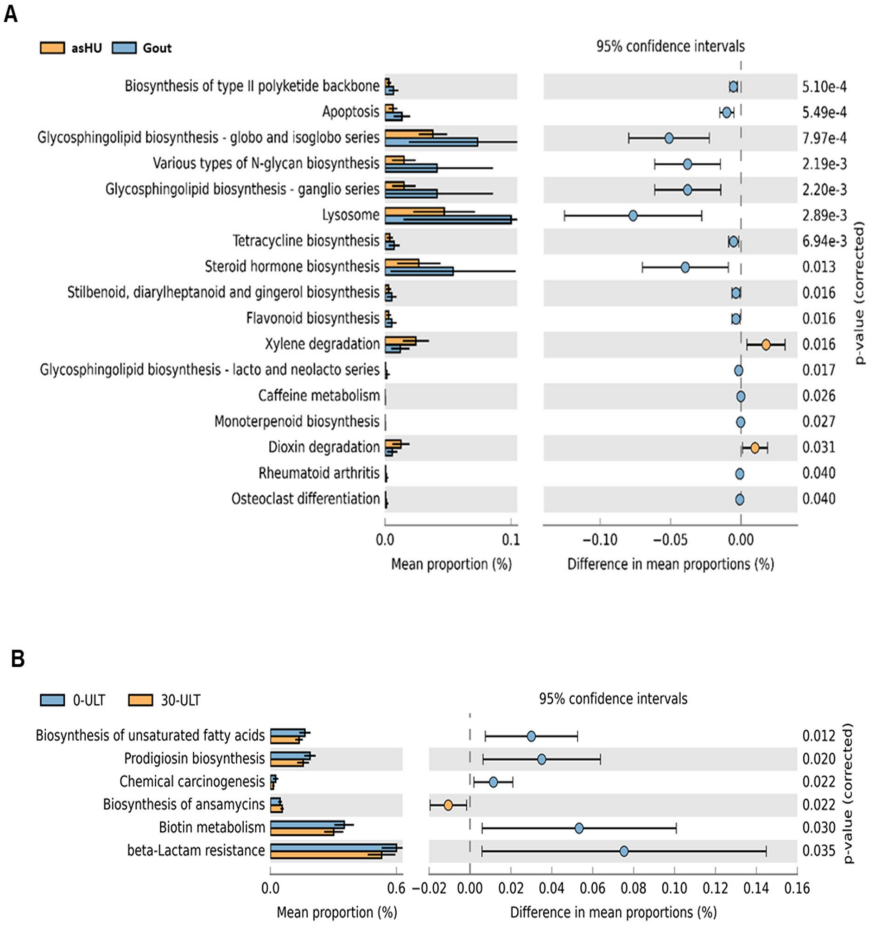

Figure 11. Comparison of predicted KEGG pathway in A. asHU and gout patients and in B. before and after ULT treatment. An extended error bar plot for the comparison of asHU vs gout patients. Only functions with $p < 0.05$ are shown.

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IV. DISCUSSION

Here, we determined the microbial markers that were enriched or depleted in the asHU and gout groups at different taxonomy levels. Based on the abundance of microbiota, we identified a gout and asHU classifier that could prevent or provoke clinical gout. Subsequently, microbiome transformation was identified that may functionally affected by ULT. Overall, we found that gut microbiota composition differs significantly between asHU and gout patients and propose microbial markers that may influence the restoration of microbiota composition. The intestinal tract playing an important role in lowering uric acid and altered gut microbiome in gout patients have highlighted new insights in the pathogenesis and diagnosis of gout.[9, 11] We observed that the F/B ratio was significantly higher in asHU patients than in acute gout and chronic gout patients; this ratio increased in gout patients after ULT, indicating that the gut microbiota composition was restored during ULT. Additionally, the gut microbiota in asHU patients had a lower P/B ratio but was more diverse. The F/B ratio has been suggested as an index of gut microbiome health and is associated with obesity, insulin resistance, dyslipidemia, and other related diseases; it is also used as a biomarker for obesity-associated phenotype,[19, 20] whereas the P/B ratio predicts body weight and fat-loss success.[21] Therefore, higher F/B and lower P/B ratios in asHU patients than in gout patients indicated that the microbiome of asHU patients is associated with metabolic diseases, whereas that of gout patients is associated with an inflammatory or immune-related disease. Studies have shown that gout is more likely an autoinflammatory disease than a metabolic syndrome. For example, a Guangzhou study analyzing the gut microbiome function and metabolome suggested that gout is more similar to rheumatoid arthritis and ankylosing spondylitis than to obesity and type-2 diabetes.[22] Another previous study reported that the number of

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Faecalibacterium prausnitzii, which exerts anti-inflammatory effects, is lower in gout patients than in healthy controls, whereas that of *Bacteroides caccae*, which induces inflammation, is higher in gout patients.[11] However, previous studies were conducted on the assumption that patients with asHU and gout have comparable microbiota composition; thus, gout development in the hyperuricemia condition has not been explained. Our findings revealed previously unknown differences in gut microbiota composition between asHU and gout patients and will provide insights for future research in this field.

Collectively, the results of previous studies and our present study indicate that the gut microbiota in asHU patients have anti-inflammatory properties and participate in uric acid processing. Some probiotic *Lactobacillus* strains can reduce serum uric acid levels and prevent renal changes and hypertension caused by hyperuricemia.[23-26] *L. paracasei* suppresses NLRP3 inflammasome activation and inflammatory stress-induced caspase-1 activation by either promoting interleukin-10 production [27] or inhibiting interleukin-1β secretion.[28] Considering that NLRP inflammasome activation triggers acute gouty arthritis,[5] the lactic acid bacteria may suppress gout development owing to their inhibitory effects on inflammasome activation. Therefore, the increased abundance of lactic acid bacteria after ULT may be attributed to the preventive and therapeutic effects observed against gout. Moreover, an increase in the proportion of *Bifidobacterium* in the gut microbiota of mice exerts beneficial effects in high-fat-diet-induced diabetes by improving glucose tolerance and glucose-induced insulin secretion and reducing inflammation development.[29] S*treptococcus salivarius*,[30] *Anaerostipes hadrus,* [31] and *Enterococcus durans* [32] have also been shown to have anti-inflammatory properties. Thus, the abundance of these bacteria could play a protective role against gout development.

Meanwhile, gout-enriched microbiota can be a trigger or may increase

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the probability of gout development when implemented to the gut of healthy individual or asymptomatic hyperuricemia patients. One animal study showed that healthy control rats become hyperuricemic when they received fecal transplant from hyperuricemic rats.[33] The changes of gut microbiome along with metabolites such as decreased short chain fatty acid may increase the possibility of gout disease in the susceptible individuals.[34] Gut-dwelling *Prevotella copri* have been associated the pathogenesis of rheumatoid arthritis especially in pre-clinical stages.[35] A putative role for P. *copri* in the pathogenesis of rheumatoid arthritis is *Prevotella copri* 27-kD protein (*Pc*-p27) associated immune response.[36] Given the distinct pathophysiologic features of rheumatoid arthritis and gout, it is not likely *P. copri* causes joint inflammation through same mechanism in both diseases. Instead, presence of abundance of *P. copri* may reflect subclinical gut inflammation and gingival inflammation. Periodontal *Prevotella intermedia*, different strain of *Prevotella* species that has been implicated to cause periodontitis was found to be significantly more abundant in gout patients compared to healthy controls.[37] Likewise, *P. copri* may lead to subclinical gastrointestinal inflammation and increased permeability allows pathogens to enter the circulatory system inducing immune responses such as inflammasome activation.[38] *Bradyrhizobium* is a symbiotic abundant in roots of many legumes, that allow the plant to fix nitrous which is associated with purine metabolism and may increase serum uric acid when present in the host. *Bradyrhizobium elkanni* produced uric acid from purine patchy containing xanthine dehydrogenase enzyme.[39] Our findings showing high abundance of *Bradyrhizobium* in acute gout patients and decreased significantly after 30 days of treatment support the potential to trigger acute gout attack of these microbiome, whether it is metabolite or increased serum uric acid is unknown. Until now, we did not know which trigger factor contributes to acute gout attack in patients with existing hyperuricemia. Considering the

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microorganisms we found, and the functional aspects suggested by previous studies, the presence of these gout enriched microbiota could be associated with gouty arthritis.

In our study, microbiota diversity was high among asHU patients, low among acute gout patients, and restored among chronic gout patients. Moreover, acute gout patients harbored a low proportion of balanced enterotype, and an increased proportion of specific strain-dominant type, suggesting that a balanced gut microbiota is important in addition to the presence of specific bacteria. Decreased microbiota diversity may be a trigger for an acute gout attack in hyperuricemic patients. The lack of microbial diversity in terms of taxa diversity and microbial gene richness is related to trigger, relapse, or treatment response in various diseases.[40, 41] The overall gut microbiota composition explains gut health better than changes in specific bacterial species.[42] Hence, future studies should focus on restoring the gut microbiota in gout patients to those observed in healthy individuals, notably by improving the gut microbiota diversity.

Results obtained in our study underline the link between gut microbiota and hyperuricemia and propose several considerations. Globally, the incidence of hyperuricemia is steadily increasing, possibly due to ingestion of protein dense food, sedentary occupation, antibiotics exposure and industrialized life style that are also associated with altered gut microbiota.[40] Moreover, ULT holds its limitation owing to poor compliance to taking daily medications and systemic side effects such as skin eruption, and there are contentious argues that ULT do not guarantee against gout attacks or prevention of end organ damages.[43] Our findings on the difference of microbial composition between asHU and gout patients throw valuable impression at the preventional stages of gout. Fecal transplantation or probiotics using collection of beneficial microbiotas enable preventing gout in at-risk patients, thereby saving money,

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time and injured quality of life owing to unnecessary treatment. Blood uric acid level, unreliable serum marker that cannot delineate gout and asymptomatic hyperuricemia patients can be replaced by a commercialized personal microbiome analysis as diagnostic purpose and biomarker of the treatment effect.

Our results suggest that disturbed microbiome by the dominant increase of "pathogens" with flora disequilibrium may provoke the risk of gout whereas the abundance of "probiont" may protect patients with asHU to develop gout. The limitations of ULT include poor patient adherence to medication and systemic adverse effects, and it does not guarantee protection against gout attacks or end-organ damages.[43] Our findings revealing the differences in microbiota composition between asHU and gout patients have implications for gout prevention, diagnosis, and monitoring of gout pathology. The effort to modify gut microbiota using probiont may prevent the gout development for at-risk patients at its occult stages and mitigate activity of overt gout. Fecal transplantation or probiotics using beneficial microbiota may prevent gout in at-risk patients. Moreover, blood uric acid level, which is an unreliable serum marker as it does not differentiate gout and asHU, can be replaced by a commercialized personal microbiome analysis.

This study included Korean patients with gout and asHU, and there is a possibility of ethnic or geographical influences on differential composition of the gut microbiome. Earlier studies have investigated lifestyle, dietary, and uncharacterized differences collectively resulting in the gut microbiota variation within an ethnicity.[22, 44] Therefore, diet and lifestyle of Koreans could have affected the microbiome of gout patients and asHU patients in our results. However, our gout patients shared the characteristic altered microbial taxa which were previously announced from different countries as microbial features of higher abundance of *Prevotella* and *Bacteroides* in gout patients.[11, 12, 22, 45]

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Our study has some limitations. We included limited samples only from men, and their diet was not strictly controlled. Future studies should include larger patient cohorts including both sexes. Moreover, both allopurinol and febuxostat were used as ULT, and the impact of medications on the microbiome was not investigated. This should be explored in future investigations.

V. CONCLUSION

In conclusion, gout and asHU patients harbor gut microbiota of different compositions, and specific taxa present in the gut microbiome of gout patients may play a role in provoking or preventing gout development. Further assessment to uncover the relationship between specific microbes and gout development and pathogenesis, as well as functional analysis related to uric acid processing and inflammation in the intestines, should be conducted.

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ABSTRACT(IN KOREAN)

통풍환자의 요산강하치료로 인한 장내 미생물군집의 변화

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배경: 장내 미생물군집은 개인 및 질병군에서 다양한 구조를 나타내며, 인간과의 상호작용을 통해 면역체계 형성에 기능적으로 기여한다. 통풍 환자는 건강한 대조군과 다른 장내 미생물군집을 보인다. 그러나 지금까지 적절한 요산강하치료 후 통풍 관절염의 병적 미생물군집의 회복을 관찰하거나, 무증상 고요산혈증의 경계에서 통풍 관절염의 발병을 촉발하는 장내 미생물군집의 특징을 조사한 연구는 없었다.

목적: 한국인 급성, 만성 통풍 환자와 무증상 고요산혈증 환자의 장내 미생물군집 차이를 밝히고, 급성 통풍 환자의 요산강하치료 전후 미생물군집의 차이를 알아보고자 하였다.

방법: 무증상 고요산혈증 환자(asHU, n = 8) 및 3 개의 통풍 환자 그룹, 즉 요산강하치료 전 급성 통풍 환자(0ULT, n = 14), 30 일 치료 후 통풍 환자 (30ULT, n = 9) 및 6 개월 이상 요산강하치료를 받은 만성 통풍 환자(cULT, n = 18)의 대변 시료를 수집하고 16S rRNA 유전자 기반 파이로시퀀싱을 수행하였다. 장내 미생물군집의 계통발생 유형을 분류한 뒤 종의 다양성 확인, 미생물군집의 조성 확인, 환자 그룹간 미생물군집의 변화를 확인하는 생물 정보 분석을 수행하였다

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결과: 통풍 환자의 장내 미생물군집은 무증상 고요산혈증환자와 뚜렷이 구분되었으며, 다양성이 감소하였다. 문 수준에서 *Firmicutes(%)/Bacteroidetes(%)*의 비율은 급성 통풍 및 만성 통풍에 비해 무증상 고요산혈증환자에서 유의하게 높았으며 요산강하치료 후에 증가하여 요산강하치료에 의해 미생물군집의 병리학적 구조가 회복되었음을 나타낸다. 네가지 enterotype 중, 무증상 고요산혈증환자는 balanced enterotype 이 다른 그룹의 환자에 비해 많았고, *Bacteroidaceae* enterotype 은 적었다. 통풍환자에서 요산 강하치료를 받은 급성기이후 Bacteroidaceae enterotype 이 감소하였다가, 만성기 이후에 다시 회복되어, 요산 강하 치료로 인한 미생물 군집의 변화가 유지되지 않음을 시사하였다. 통풍환자에서 *Provotella copri, Odoribacter splanchnicus,* 무증상 고요산혈증에서 *Streptococcus salivarius, Streptococcus parasanguinis, Streptococcus sinensis, Enterococcus durans, B, Anaerostipes hardrus, Bifidobacterium catenulatum, Bifodobacterium breve, Bifidobacterium bifidum, Lactobacillus plantarum, Lactobacillus reuteri, Lactobacillus murinus 및 Lactobacillus fermentum 가 증가하여, 통풍의 위험 균주* 및 유익 균주 존재의 가능성을 시사하였다. *Bifidobacteriales_uc, Lactobacillus helveticus, Lactobacillus plantarum, Streptococcaceae_uc, EF400193_s, EF403944_s* 및 *KI392030_s* 는 요산강하치료 이후 증가된 균주로 치료효과를 보여주었다.

결론: 이 연구는 특정 장내 미생물군집이 무증상 고요산혈증에서 통풍의 발병을 예방할 수 있으며 통풍 환자의 장내 미생물군집이 요산강하치료에 의해 변화됨을 제시한다.

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핵심되는 말 : 통풍, 무증상 고요산혈증, 장내 미생물군집, 요산강하치료