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ORIGINAL ARTICLE
Heart Failure

Gut Microbiome and Plasma Microbiome-Related Metabolites in Patients With Decompensated and Compensated Heart Failure

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Background: Gut microbiome composition or circulating microbiome-related metabolites in patients with heart failure (HF) have not been investigated at different time points (i.e., in the decompensated (Decomp) and compensated (Comp) phases).

Methods and Results: We prospectively enrolled 22 patients admitted for HF and 11 age-, sex-, and comorbidity-matched hospitalized control subjects without a history of HF. Gut flora and plasma microbiome-related metabolites were evaluated by amplicon sequencing of the bacterial 16S ribosomal RNA gene and capillary electrophoresis time-of-flight mass spectrometry, respectively. HF patients were evaluated in both the Decomp and Comp phases during hospitalization. The phylum Actinobacteria was enriched in HF patients compared with control subjects. At the genus level, *Bifiodobacterium* was abundant while *Megamonas* was depleted in HF patients. Meanwhile, plasma concentration of trimethylamine *N*-oxide (TMAO), a gut microbiome-derived metabolite, was increased in HF patients (Decomp HF vs. control, P=0.003; Comp HF vs. control, P=0.004). A correlation analysis revealed positive correlations between the abundance of the genus *Escherichia*/*Shigella* and levels of TMAO and indoxyl sulfate (IS, a microbe-dependent uremic toxin) in Comp HF (TMAO: r=0.62, P=0.002; IS: r=0.63, P=0.002). *Escherichia*/*Shigella* was more abundant in Decomp than in Comp HF (P=0.030).

Conclusions: Our results suggest that gut microbiome composition and microbiome-related metabolites are altered in HF patients.

Key Words: Gut microbiome; Heart failure; Metabolites

Chronic heart failure (HF) is highly prevalent and has a poor prognosis, making it a significant healthcare burden worldwide.[1] The pathophysiology of HF involves multiple organ systems connected via neurohormonal pathways and pro-inflammatory cytokines.[2] Chronic HF is characterized by morphological and functional changes in both the small and large intestines, as well as altered gut microbiome composition resulting from inflammation.[3] These features are aggravated in chronic HF patients in the decompensated state, which may be attributable to intestinal ischemia and/or congestion.[4,5]

Editorial p30

The gut microbiome contributes to the regulation of various host metabolic pathways.[6] For instance, the gut-derived metabolite, trimethylamine *N*-oxide (TMAO), has been directly linked to adverse cardiovascular events and all-cause mortality,[7] and an elevated level of circulating TMAO serves as a prognostic biomarker in both chronic and acute HF, independent of traditional risk factors and B-type natriuretic peptide (BNP) level.[8,9] Preclinical studies

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have also suggested that microbe-generated metabolites, such as indoxyl sulfate (IS), a protein-bound uremic toxin, play an important role in the pathogenesis of HF.[10] Short-chain fatty acids (SCFA i.e., butyrate, propionate, acetate, and lactate) have several beneficial physiological effects on the host, and previous studies have demonstrated an association between changes in SCFA-producing bacteria and metabolic disorders such as obesity and diabetes mellitus.[6]

Recent investigations of gut microbial composition in patients with HF have revealed a disturbance of the gut microbiome relative to healthy or community-based controls with different comorbidities, medication, and diet from HF patients.[11,12] The cause–effect relationship of gut microbiome composition and HF pathophysiology remains unclear. The present study reports an analysis of gut microbiome and plasma-related metabolites in patients with decompensated HF (Decomp HF) and the same patients after compensation (Comp HF).

Methods

Study Population and Clinical Assessment

Between October 2016 and April 2017, 22 HF patients were prospectively enrolled who were admitted to Kobe University Hospital for de novo acute decompensated HF or acute worsening of chronic HF. A diagnosis of HF was made based on modified Framingham criteria.[13] We also recruited 11 age-, sex-, and comorbidity-matched control subjects with HF risk factors but no history of HF who were hospitalized at the same hospital. All patients provided written informed consent prior to study enrollment. Patients underwent standardized evaluation including detailed medical history (comorbid conditions and medication), physical examination, blood chemistry, 12-lead electrocardiogram, chest X-ray, and echocardiography. We also investigated gut microbiome composition by high-throughput amplicon sequencing of the bacterial 16S ribosomal (r)RNA gene, as well as plasma microbiome-related metabolites with a capillary electrophoresis time-of-flight mass spectrometry (CE-TOFMS) analysis; these were performed at 2 different time points during hospitalization (i.e., decompensated and compensated phases) in HF patients. The presence of an increase in symptoms of HF, such as breathlessness, fluid retention, and/or fatigue that requires additional and immediate therapeutic intervention, was regarded as decompensated state. HF was regarded as compensated if all of the following conditions were fulfilled: improvement of HF symptoms; amelioration of edema and pulmonary rales; withdrawal of intravenous agents such as diuretics and inotropes; and optimization of oral diuretics. We defined the decompensated phase as the period from hospital admission to compensation of HF, and the compensated phase as the period from compensation of HF to hospital discharge. The average length of fecal sampling of HF patients from the decompensated phase to the compensated phase was 11 days. HF with reduced ejection fraction (HFrEF) and HF with preserved EF (HFpEF) were defined as a left ventricular ejection fraction (LVEF) <40% and ≥50%, respectively.[14] There were 12 HFrEF and 10 HFpEF patients in the HF group. Of 11 control subjects, 2 were admitted for the treatment of type 2 diabetes in the Division of Diabetes and Endocrinology, and the remaining 9 control subjects were admitted in the Division of Cardiovascular Medicine (6 patients for catheter ablation of atrial fibrillation, 2 patients for elective diagnostic coronary angiography, and 1 patient for symptomatic sick sinus syndrome requiring permanent pacemaker implantation). Fecal samples of controls were collected before catheterization or pacemaker implantation.

Patients with acute coronary syndrome, renal failure (serum creatinine levels >3.0 mg/dL at the time of admission), active infectious diseases, malignancy, autoimmune disorders, inflammatory or malabsorptive intestinal diseases, history of enterectomy, or hepatic diseases such as infection of hepatitis and liver cirrhosis were excluded. Patients who had undergone antibiotic or steroid treatment within 1 month before admission and during hospitalization were also excluded. The presence of hypertension, diabetes, and dyslipidemia was recorded according to relevant guidelines.[15]

The study protocol complied with tenets of the Declaration of Helsinki and was approved by the Ethics Committee of Kobe University (approval no. 160072) and registered with the UMIN Clinical Trials Registry (trial registration no. UMIN000022414, URL: http://www.umin.ac.jp/ctr/).

DNA Extraction From Fecal Samples

Fecal samples were collected at Kobe University Hospital from HF patients and control subjects on the hospital diet. The samples were immediately stored at −20°C and flash frozen in liquid nitrogen, and then transferred to a −80°C freezer within 12 h. DNA was extracted from fecal samples at Nihon Gene Research Laboratories according to a previously established procedure.[16]

16S rRNA Gene Amplification and Sequencing

Parts of the 16S rRNA genes were PCR-amplified using our non-degenerate universal primer set of 342F and 806R. More detailed information on the primer set and PCR conditions is available elsewhere.[17] After addition of the sequencing adapters, the amplicons were sequenced using Illumina Miseq platform (Illumina Inc., San Diego, CA, USA) according to the manufacturer's protocol at Takara Bio Inc. To make the bacterial composition matrix, we used USEARCH 10.0.240.[18] Our previous protocol was also used to select high-quality 16S rRNA gene amplicon sequences generated using Trimmomatic 0.33[19] with the parameters 'LEADING:17 TRAILING:17 AVGQUAL:25 MINLEN:100'. The remaining reads were processed using the -fastq_mergepairs command of USEARCH, with default parameters. Next, we removed sequences without the primer region using TagCleaner 0.16,[20] with parameters '-tag5 CTACGGGGGGCAGCAG -mm5 3 -tag3 AGATACCCCGGTAGTCC -mm3 3 -nomatch 3'. After removal of the primer, sequences with N were removed using an in-house python script. To remove PhiX reads, we used the -filter_phix command of USEARCH. Finally, we removed short sequences using the USEARCH command -sort_by_length, with the parameter '-minseqlength 300'. Finally, we generated operational taxonomic unit (OTU) tables using UPARSE algorithms (-fastx_unique command, and otu_cluster command with the parameter '-minsize 1').[21] The representative sequences of each OTU were annotated to bacterial genus using RDP Classifier 2.12, with a confidence value ≥0.5.[22] Moreover, we annotated each representative sequence of each OTU to the reference database silva Living Tree Project 128[23] using blastn command of BLAST+ 2.6.0, with the identity threshold

≥97% and coverage ≥80%.

Predictive Functional Profiling of Gut Microbial Communities Based on 16S rRNA Gene Sequences

OTUs were constructed using the raw data reads from fecal samples. To join 2 paired-end reads, we used fastq-join software with default options. The chimeric sequences were deleted with USEARCH 6.1.544_i86.[24] OTUs at the 97% similarity threshold were selected with the Greengenes database in the QIIME 1.8.0 pipeline.[25] Analysis of gut microbial trimethylamine (TMA) lyase (CutC/D) and tryptophanase gene abundances based on Kyoto Encyclopedia of Genes and Genomes (KEGG)[26] and Clusters of Orthologous Groups (COG) databases[27] was performed using PICRUSt 1.1.1.[28]

Metabolite Extraction and CE-TOFMS Analysis

Peripheral venous blood samples were collected in tubes containing EDTA-2Na after overnight fasting and immediately centrifuged at 1,200 g and 4°C for 10 min to obtain plasma, which was stored at −80°C until CE-TOFMS analysis by Human Metabolome Technologies.[29] CE-TOFMS analysis was carried out using an Agilent capillary electrophoresis system (Agilent Technologies, Waldbronn, Germany). The systems were connected by a fused silica capillary (50 μm i.d.×80 cm total length) with commercial

Table. Characteristics of HF Patients

	Control (n=11)	Decomp HF (n=22)	Comp HF (n=22)	P value		
				Control vs. Decomp HF	Control vs. Comp HF	Decomp HF vs. Comp HF
Age, years	72±7	72±18		0.955		
Male sex	6 (55%)	14 (64%)		0.614		
NYHA class II		5 (23%)				
NYHA class III		8 (36%)				
NYHA class IV		9 (41%)				
Peripheral edema		16 (73%)				
Body mass index, kg/m²	24.4±3.1	25.8±7.1	23.6±5.9	0.522	0.701	<0.001
Smoking	3 (27%)	11 (50%)		0.278		
Vitals						
Heart rate, beats/min	73.4±11.4	87.3±27.8	69.0±9.7	0.123	0.265	0.006
SBP, mmHg	133.3±13.1	135.2±26.6	113.2±13.2	0.821	<0.001	<0.001
DBP, mmHg	68.8±8.1	80.4±24.9	60.5±10.2	0.146	0.024	0.003
LVEF, %	63±4	42±17		<0.001		
HFrEF		12 (55%)				
HFpEF		10 (45%)				
Primary cause of HF						
Ischemic heart disease		2 (9%)				
Dilated cardiomyopathy		7 (32%)				
Hypertension		4 (18%)				
Valvular disease		5 (23%)				
Others		4 (18%)				
Comorbidities						
Hypertension	9 (82%)	21 (95%)		0.252		
Diabetes mellitus	5 (45%)	8 (36%)		0.614		
Dyslipidemia	6 (55%)	9 (41%)		0.458		
Atrial fibrillation	6 (55%)	15 (68%)		0.443		
Medication						
ACEi and/or ARB	5 (45%)	9 (41%)	15 (68%)	0.803	0.208	0.069
β-blocker	4 (36%)	13 (59%)	20 (91%)	0.218	0.002	0.034
Calcium-channel blocker	3 (27%)	9 (41%)	10 (45%)	0.703	0.456	0.864
Aldosterone receptor antagonist	0 (0%)	3 (14%)	9 (41%)	0.534	0.015	0.088
Diuretics (loop or thiazide)	2 (18%)	13 (59%)	21 (95%)	0.034	<0.001	0.009
Statin	5 (45%)	4 (18%)	4 (18%)	0.121	0.121	>0.999
PPI/H_2 blocker	9 (82%)	15 (68%)	17 (77%)	0.681	>0.999	0.498
Antidiabetic agent (including insulin therapy)	4 (36%)	4 (18%)	5 (23%)	0.392	0.438	>0.999
Antiarrhythmic	0 (0%)	4 (18%)	4 (18%)	0.276	0.276	>0.999
Nitrate	1 (9%)	2 (9%)	2 (9%)	>0.999	>0.999	>0.999
Antiplatelet	2 (18%)	4 (18%)	5 (23%)	>0.999	>0.999	>0.999
Anticoagulant agent	6 (55%)	15 (68%)	18 (82%)	0.443	0.121	0.488

(**Table** continued the next page.)

	Control (n=11)	Decomp HF (n=22)	Comp HF (n=22)	P value		
				Control vs. Decomp HF	Control vs. Comp HF	Decomp HF vs. Comp HF
Biochemical measures						
BUN, mg/dL	20.0 (19.3–23.6)	19.8 (15.8–33.8)	21.4 (16.3–34.3)	0.829	0.313	0.211
Creatinine, mg/dL	1.0±0.3	1.2±0.4	1.2±0.5	0.123	0.109	0.359
eGFR, mL/min/1.73 m^2	54.0±11.7	48.8±19.4	49.0±22.5	0.426	0.496	0.948
Sodium, mEq/L	139.9±1.4	139.3±2.4	138.4±2.9	0.419	0.115	0.221
Potassium, mEq/L	4.2±0.5	4.1±0.4	4.2±0.4	0.785	0.715	0.349
Hemoglobin, g/dL	13.3±1.4	12.2±2.3	12.1±2.5	0.156	0.155	0.671
Total bilirubin, mg/dL	1.0±0.3	0.9±0.4	0.8±0.3	0.626	0.077	0.128
hs-CRP, mg/dL	0.04 (0.03–0.06)	0.30 (0.14–0.67)	0.46 (0.06–1.51)	<0.001	0.001	0.818
Albumin, g/dL	3.7±0.3	3.4±0.5	3.2±0.5	0.072	0.009	0.050
HbA1c, %	6.1 (5.7–6.8)	6.0 (5.7–6.4)		0.703		
Total cholesterol, mg/dL	192.1±31.0	171.5±34.6		0.126		
HDL-cholesterol, mg/dL	53.3±10.0	52.1±16.1		0.800		
LDL-cholesterol, mg/dL	118.1±31.3	103.1±26.6		0.185		
Triglycerides, mg/dL	157.7±70.8	92.7±31.6		0.002		
BNP, pg/mL	53 (23–109)	445 (328–763)	284 (152–378)	<0.001	<0.001	<0.001

Binary data are presented as number (%); continuous variables are presented as mean (SD) or median (interquartile range). ACEi, angiotensin-converting enzyme inhibitor; ARB, angiotensin II receptor blocker; BNP, B-type natriuretic peptide; BUN, blood urea nitrogen; Comp HF, compensated heart failure; DBP, diastolic blood pressure; Decomp HF, decompensated heart failure; eGFR, estimated glomerular filtration rate; HbA1c, hemoglobin A1c; HDL, high-density lipoprotein; HF, heart failure; HFpEF, heart failure with preserved ejection fraction; HFrEF, heart failure with reduced ejection fraction; hs-CRP, high-sensitivity C-reactive protein; LDL, low-density lipoprotein; LVEF, left ventricular ejection fraction; NYHA, New York Heart Association; PPI, proton pump inhibitor; SBP, systolic blood pressure.

electrophoresis buffer (H3301-1001 and H3302-1021 for cation and anion analyses, respectively; Human Metabolome Technologies) as the electrolyte. The spectrometer was scanned from *m*/*z* 50 to 1,000.[29] The peaks were extracted using MasterHands automatic integration software version 2.17.1.11 (Keio University, Tsuruoka, Yamagata, Japan) to obtain the peak information including *m*/*z*, peak area, and migration time.[30] The areas of the annotated peaks were then normalized based on internal standard levels and sample amounts in order to obtain the relative level of each metabolite. With the exception of lactate, we were unable to detect SCFA in the circulation. Absolute quantification of choline, TMAO, tryptophan, and IS among detected gut microbiome-related metabolites was performed using single-point calibration.

Statistical Analysis

Data are expressed as mean±SD or SEM for normally distributed data and as the median (25–75th percentiles) for non-normally distributed data. Comparisons between groups were carried out using the t-test or Wilcoxon rank-test for normally and non-normally distributed data, respectively. Comparison between control subjects and HF patients was carried out using an unpaired test. Decomp HF was compared with Comp HF using the paired test. Categorical variables were compared between groups with the χ^2 test or Fisher's exact test as appropriate. The Shannon-Wiener index was calculated to estimate microbial α diversity. We analyzed gut microbiomes exhibiting at least 0.1% mean abundance in all samples to determine between-group differences in composition. Principal coordinate analysis was used to determine the microbial profile of each group. We examined the association between 2 parameters using the Pearson or Spearman correlation test. Statistical analyses were carried out using R software v.3.1.0 (http://www.r-project.org/), Prism v.7.0 (GraphPad Inc., San Diego, CA, USA), and JMP10 (SAS Institute, Cary, NC, USA). All statistical analyses were two-sided. A P value <0.05 was considered significant. In the analysis of relative abundance of gut microbiome, q values were calculated with the Benjamini-Hochberg method to adjust P values for multiple comparisons.

Results

Baseline Patient Characteristics

Characteristics of the control subjects and HF patients are shown in **Table**. The patients were similarly distributed in terms of age, sex, and renal function. There were no differences between the control and HF patients with respect to the prevalence of comorbidities such as hypertension, diabetes mellitus, dyslipidemia, and atrial fibrillation. Diuretic (loop and/or thiazide) use and high-sensitivity C-reactive protein (hs-CRP) and BNP levels were higher whereas LVEF and triglyceride levels were lower in HF patients than in control subjects. The characteristics of patients with HFrEF and HFpEF are shown in **Supplementary Table 1** and **Supplementary Table 2**, respectively.

Gut Microbiome Composition in HF Patients and Control Subjects

We analyzed the abundance of 16S rRNA reads at the phylum and genus levels. Intra-individual gut microbial variance was evaluated according to the Shannon diversity index. The α diversity of the genus-level gut microbiome

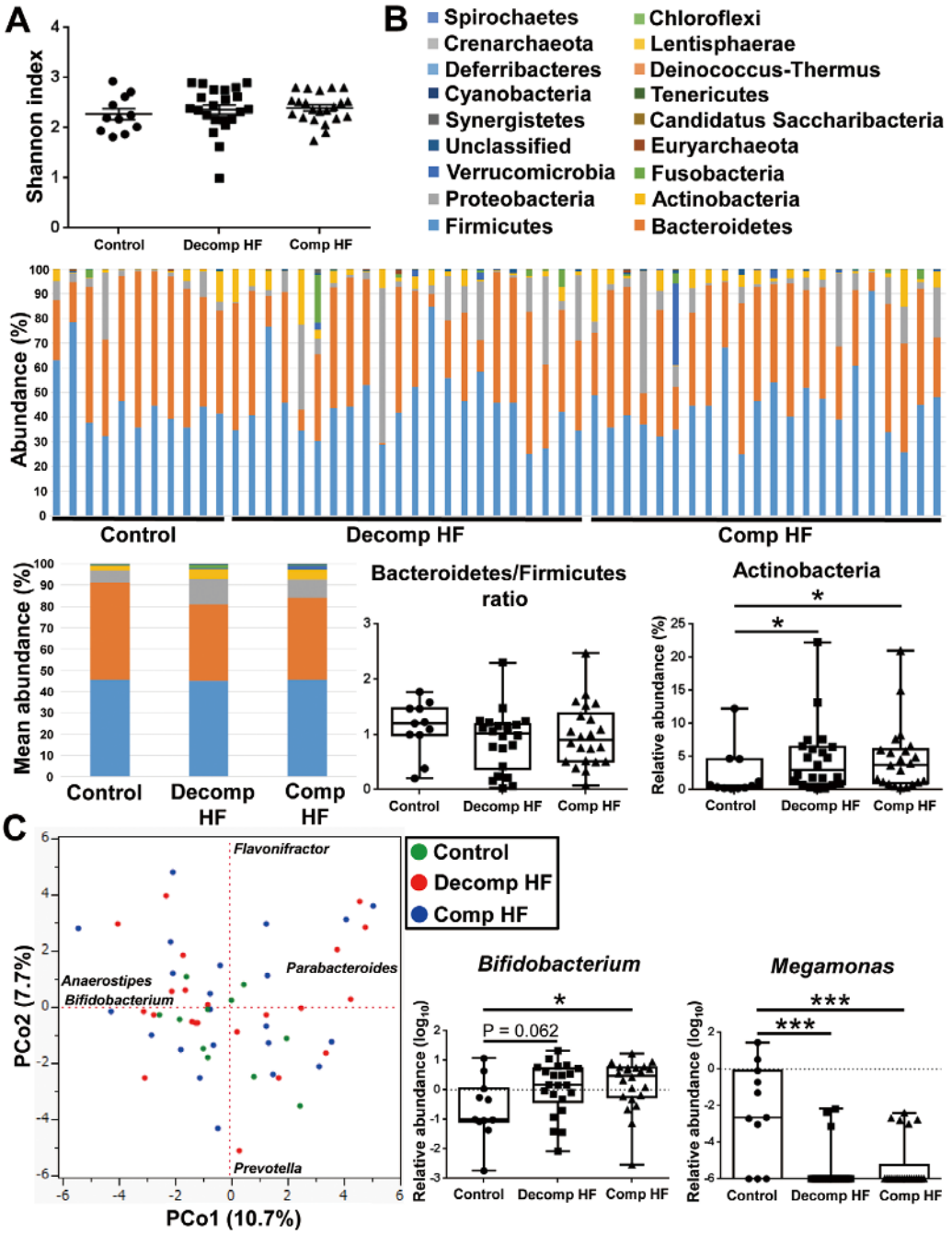

Figure 1. Gut microbiome composition in heart failure (HF) patients and controls. (**A**) Shannon index of the genus-level gut microbiome in control subjects and patients with decompensated and compensated HF (Decomp and Comp HF, respectively). Values are mean±SEM. (**B**) Phylum level. (**C**) Principal coordinate analysis plot of genus-level gut microbiome in control subjects (green) and Decomp HF (red) and Comp HF (blue) patients. The relative abundances of the *Bifidobacterium* and *Megamonas* genera are shown. In the box-and-whisker plot, middle line represents median value, box indicates interquartile range (25–75th percentiles), and range bars show maximum and minimum. *P<0.05; ***P<0.001.

was comparable between control subjects and HF patients (control: 2.27±0.37; Decomp HF: 2.36±0.46; Comp HF: 2.40±0.29) (**Figure 1A**). **Figure 1B** shows the taxonomic assignment at the phylum level for each individual within each group, as well as the mean abundance. Among the 4 major phyla (i.e., Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria), Actinobacteria was enriched in HF patients (control: 0.62 [0.25–4.66]%; Decomp HF: 2.99 [0.87–6.51]%; Comp HF: 3.80 [1.05–6.14]%; Decomp HF vs. control, P=0.035, q=0.190; Comp HF vs. control, P=0.026, q=0.156). However, the relative abundance of Actinobacteria was unaffected by short-term treatment

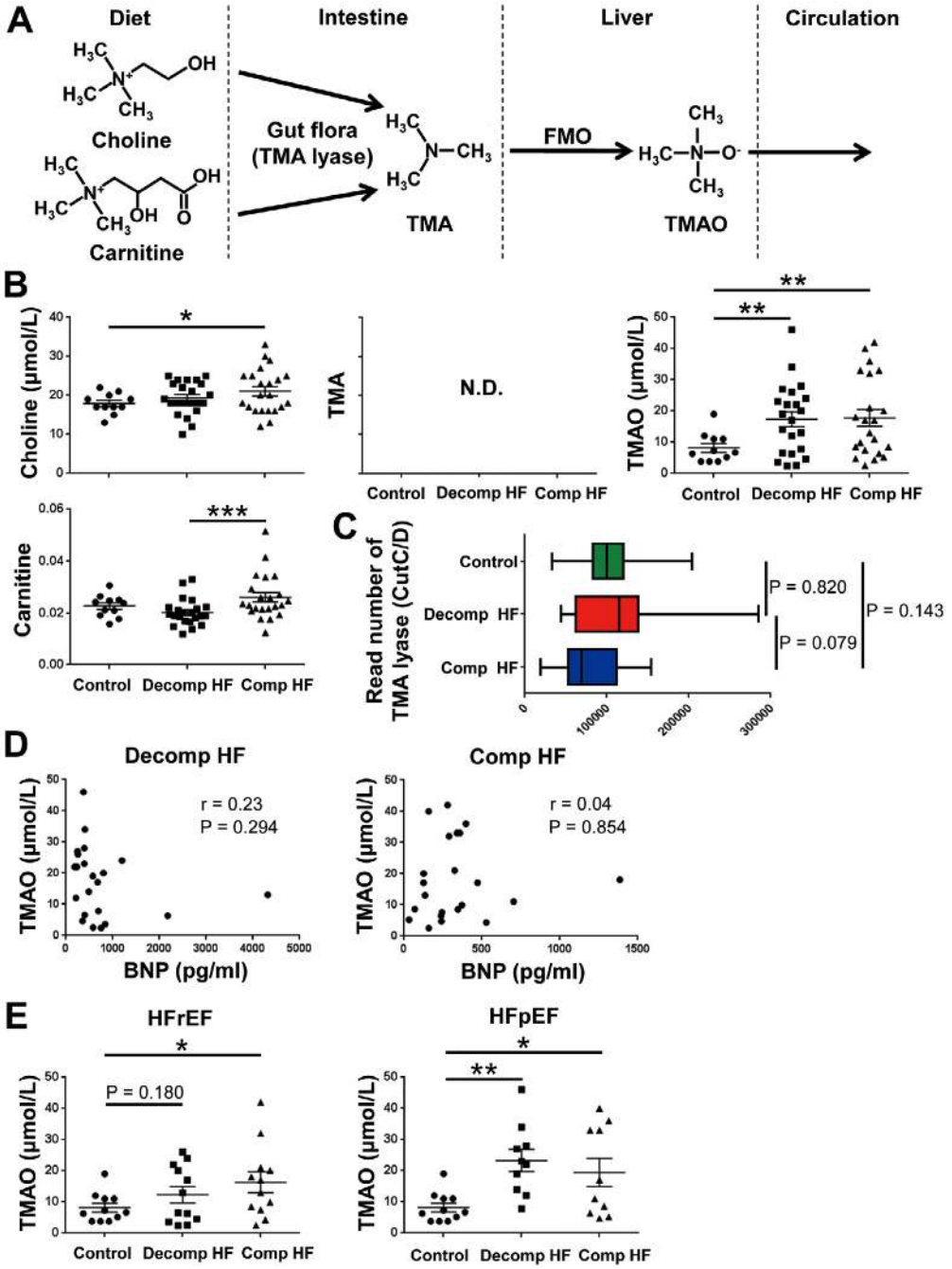

Figure 2. Trimethylamine (TMA)/trimethylamine *N*-oxide (TMAO) metabolic pathway. (**A**) Schematic illustration of TMA/TMAO metabolic pathway. (**B**) Plasma levels of TMAO-related metabolites. (**C**) Microbial TMA lyase (CutC/D) gene abundance. (**D**) Relationship between circulating B-type natriuretic peptide (BNP) and TMAO levels in patients with heart failure (HF). (**E**) TMAO concentrations in HF with reduced ejection fraction (HFrEF) and HF with preserved EF (HFpEF). Data in (**B**) and (**E**) are expressed as mean±SEM. In the box-and-whisker plot, middle line represents median value, box indicates interquartile range (25–75th percentiles), and range bars show maximum and minimum. *P<0.05, **P<0.01, ***P<0.001. Comp HF, compensated heart failure; Decomp HF, decompensated heart failure; FMO, flavin monooxygenase; N.D., not detected.

for HF. There were no significant differences in the Bacteroidetes/Firmicutes ratio among groups. The principal coordinate analysis scatter plot in **Figure 1C** shows each genus represented in the gut microbiome. At the genus level, *Bifidobacterium* was enriched in Comp HF as compared with control subjects (3.10 [0.56–5.56]% vs. 0.10 [0.09–1.10]%, P=0.017, q=0.437), whereas *Megamonas* was less abundant in Decomp HF and Comp HF than in the control group (Decomp HF vs. control, P<0.001, q=0.010; Comp HF vs. control, P<0.001, q=0.047) (**Figure 1C**).

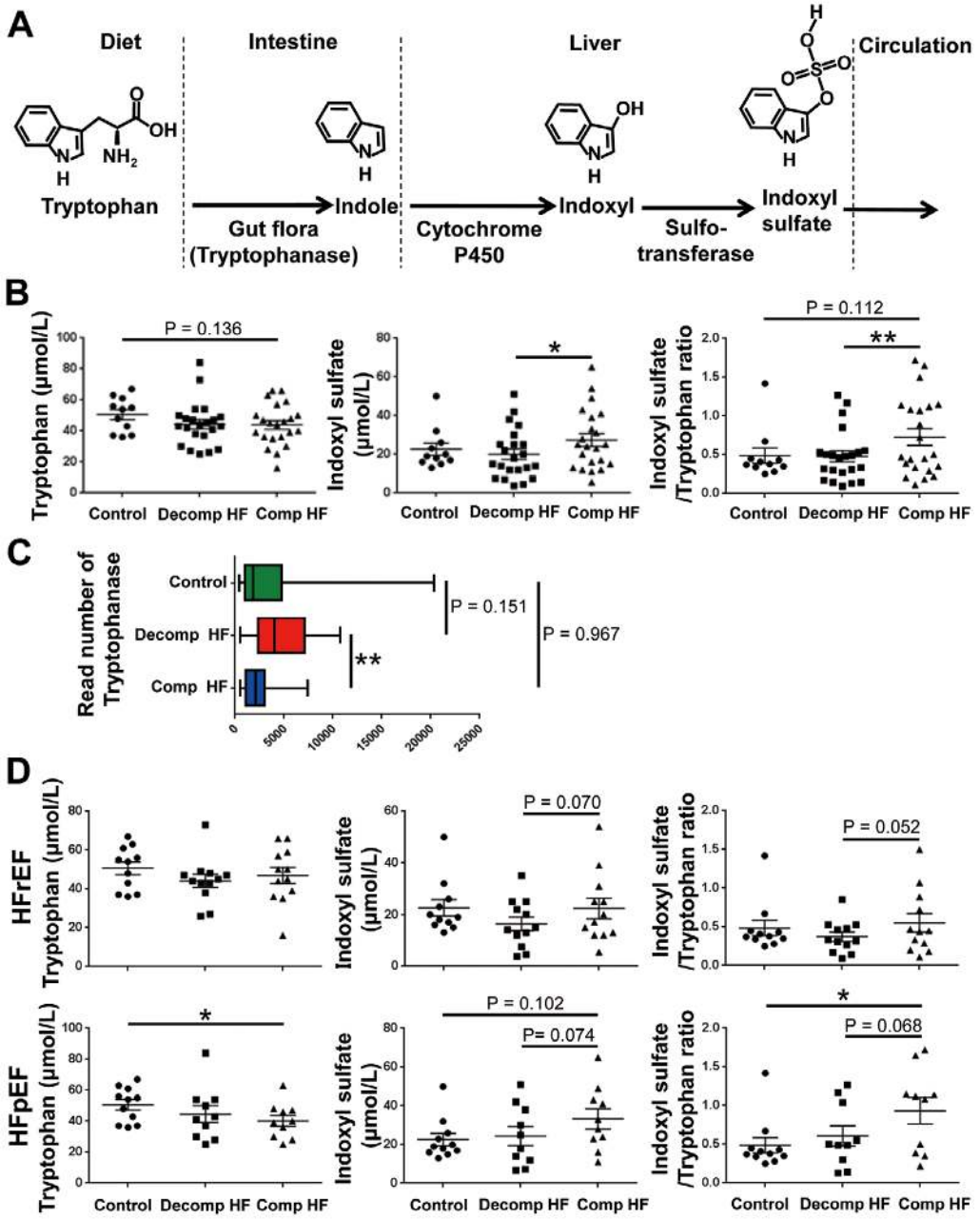

Figure 3. Tryptophan/indoxyl sulfate metabolic pathway. (**A**) Schematic illustration of tryptophan/indoxyl sulfate metabolic pathway. (**B**) Plasma concentrations of tryptophan and indoxyl sulfate, and indoxyl sulfate/tryptophan ratio. (**C**) Abundance of microbial tryptophanase gene. (**D**) Indoxyl sulfate-related metabolites in heart failure with reduced ejection fraction (HFrEF) and heart failure with preserved ejection fraction (HFpEF). Data in (**B**) and (**D**) are expressed as mean±SEM. In the box-and-whisker plot, middle line represents median value, box indicates interquartile range (25–75th percentiles), and range bars show maximum and minimum. *P<0.05, **P<0.01. Comp HF, compensated heart failure; Decomp HF, decompensated heart failure.

Microbiome data at the phylum and genus levels are shown in **Supplementary Table 3** and **Supplementary Table 4**, respectively. **Supplementary Figure 1** and **Supplementary Figure 2** reveal gut microbiome composition and proton pump inhibitor (PPI)-associated genera in PPI/H_2 blocker users vs. non-users.

Plasma Levels of TMAO-Related Metabolites and Microbial TMA Lyases

Decomp HF and Comp HF had higher plasma concentrations of TMAO than control subjects (control: 8.2±4.7 μmol/L; Decomp HF: 17.3±11.7 μmol/L; Comp HF: 17.7±12.6 μmol/L; Decomp HF vs. control, P=0.003; Comp HF vs. control, P=0.004). The concentration of the TMAO precursor choline was higher in Comp HF than in

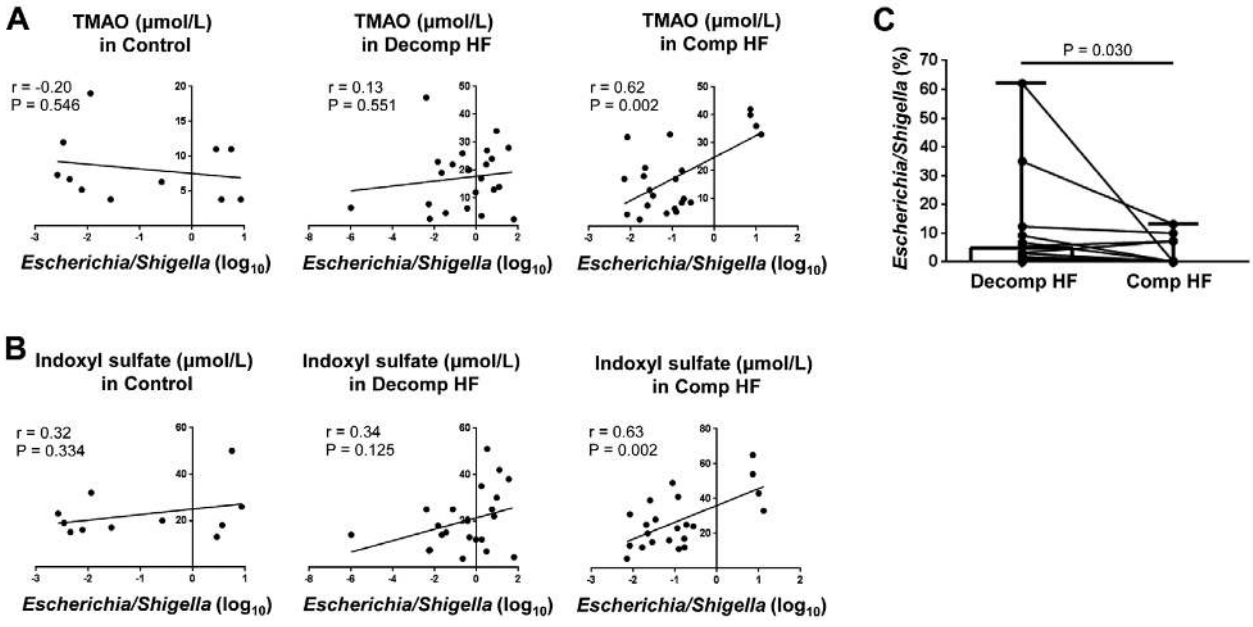

Figure 4. Genus *Escherichia/Shigella* and gut microbiome-derived metabolites. (**A**) Relationship between relative abundance of the genus *Escherichia/Shigella* and plasma trimethylamine *N*-oxide (TMAO) concentration. (**B**) Relationship between relative abundance of the genus *Escherichia/Shigella* and plasma indoxyl sulfate concentration. (**C**) Relative abundance of the genus *Escherichia/Shigella* in decompensated and compensated heart failure (Decomp HF and Comp HF, respectively). In the box-and-whisker plot, box indicates interquartile range (25–75th percentiles) and range bars show maximum and minimum.

control subjects (21.1±5.6 μmol/L vs. 17.9±2.6 μmol/L, P=0.039), while that of carnitine, another TMAO precursor, was decreased in Decomp HF as compared with Comp HF (2.0E-02±5.2E-03 vs. 2.6E-02±8.7E-03, P<0.001) (**Figure 2A**,**B**). We found that Decomp HF tended to have a higher gene abundance of microbial TMA lyase (CutC/D) than Comp HF (**Figure 2C**). There was no correlation between BNP and TMAO concentrations (**Figure 2D**). Importantly, TMAO levels were elevated in compensated HFrEF and HFpEF (**Figure 2E**).

Plasma Levels of IS-Related Metabolites and Microbial Tryptophanases

Decomp HF had a lower IS concentration and lower ratio of IS to tryptophan (the dietary precursor of indole) than Comp HF (IS 19.9±12.8 μmol/L vs. 27.4±15.6 μmol/L, P=0.010; IS/tryptophan ratio 0.5±0.3 vs. 0.7±0.5, P=0.007, respectively) (**Figure 3A**,**B**). Our analysis using PICRUSt revealed a higher gene abundance of microbial tryptophanase in Decomp than in Comp HF (**Figure 3C**), with the latter exhibiting a lower tryptophan concentration and higher IS/tryptophan ratio than control subjects, although the differences were not statistically significant (tryptophan 43.9±13.3 μmol/L vs. 50.6±11.1 μmol/L, P=0.136; IS/tryptophan ratio 0.7±0.5 vs. 0.5±0.3, P=0.112, respectively). The subgroup analysis showed that patients with compensated HFpEF had lower concentrations of tryptophan and a higher IS/tryptophan ratio than controls (tryptophan 40.2±11.4 μmol/L vs. 50.6±11.1 μmol/L, P=0.047; IS/tryptophan ratio 0.9±0.5 vs. 0.5±0.3, P=0.039, respectively) (**Figure 3D**).

Correlation Analyses Between Gut Microbiome and Plasma Microbiome-Derived Metabolites

To clarify the association between gut microbiome composition and the plasma microbiome-derived metabolites in HF, fold differences in the relative abundance of each bacterium at the genus level between Comp HF and control subjects or between Decomp HF and Comp HF were calculated (**Supplementary Figures 3**,**4**, respectively). We analyzed the correlations between bacterial genera (P<0.05 and $\log_2$[fold difference] ≤−0.5, or P<0.05 and $\log_2$[fold difference] ≥0.5) and microbime-derived metabolites (**Supplementary Figures 3**,**4**). There were no correlations between TMAO level and the abundance of *Bifidobacterium* and *Megamonas* genera; however, an inverse correlation tended to be observed between IS level and *Bifidobacterium* abundance in Comp HF (r=−0.36, P=0.099, q=0.198) (**Supplementary Figure 5**). We also found positive correlations between the genus *Escherichia*/*Shigella* and TMAO and IS levels in Comp HF (r=0.62, P=0.002, q=0.002 and r=0.63, P=0.002, q=0.002, respectively) (**Figure 4A**,**B**). Interestingly, *Escherichia*/*Shigella* was more abundant in Decomp than in Comp HF (0.70 [0.02–5.83]% vs. 0.10 [0.02–0.21]%, P=0.030) (**Figure 4C**).

Discussion

This study analyzed the relationship between the gut microbiome and plasma-related metabolites in patients with HF, including those in the decompensated and compensated phases. We observed changes in gut microbiome-related metabolites as well as correlations between gut microbes and levels of harmful metabolites in HF patients.

Gut microbiome composition varies with age, diet, medication, ethnicity, and feces sample preparation method (stool collection procedure and storage conditions).[31,32] Unlike previous reports,[11,12,33] we tried to minimize these confounds by recruiting age-, sex-, and comorbidity-matched control subjects whose hospital diet, medical drug use, and fecal sample preparation were similar to the HF patients. Additionally, the patients had not had antibiotic treatment within the 1-month period before admission or during hospitalization. We observed a trend of an enrichment of the phylum Actinobacteria and the genus *Bifidobacterium*, and a reduced abundance of the genus *Megamonas* in HF patients relative to controls by 16S rRNA gene amplicon sequencing. These results are in agreement with a recent study showing that *Bifidobacterium* was abundant in HF patients compared with healthy controls.[11] *Bifidobacterium* has various physiological effects, including decreasing the levels of unfavorable bacteria (e.g., *Escherichia coli*), modulating host immunity, and improving the gut environment by reducing fecal ammonia concentration and pH.[34] A previous study also showed that *Megamonas* can generate propionate and acetate from glucose in vitro.[35] Importantly, patients with Behcet's disease, a systemic inflammatory disorder, exhibit similar changes in gut microbiome composition, including an increase in *Bifidobacterium* and decrease in *Megamonas* abundance.[36]

TMAO is generated by hepatic flavin monooxygenase from TMA, which is formed by gut microbial TMA lyases from dietary phosphatidylcholine, choline, and carnitine.[37] Clinical and experimental evidence suggests potential roles for choline and TMAO in the pathogenesis of HF.[8,9,38] Bacteria such as *Escherichia fergusonii*, *Clostridium sporogenes*, and *Proteus penneri* that produce TMA from choline in vitro have been detected in the human gut.[39] Moreover, the Bacteroidetes-to-Firmicutes ratio was found to be decreased in healthy high-TMAO-producing young men after dietary intake of TMA precursors, indicating that TMAO production differs according to gut microbial function.[40] In our study, plasma TMAO concentrations were elevated in Decomp and Comp HF, and the plasma choline level was also increased in the latter group. Circulating levels of TMAO and its precursors are influenced by the degree of renal dysfunction.[41] Although we cannot fully exclude the effect of renal function on plasma concentrations of these metabolites, estimated glomerular filtration rates were comparable among the control, Decomp HF, and Comp HF groups in the present study. Using PICRUSt to analyze microbial function, we observed an increasing trend of TMA lyase (CutC/D) gene abundance in Decomp HF compared with Comp HF, implying that certain bacteria harboring TMA lyases may become enriched in the decompensated phase of HF as compared with the stable state. However, it is unknown whether or to what extent an elevated plasma TMAO level is attributable to gut microbiome composition in HF patients. Hepatic expression level of flavin monooxygenase was shown to be linked to plasma TMAO concentration in mice and humans.[37] Therefore, measuring the TMA concentration in feces and flavin monooxygenase expression in liver could clarify these points.

IS is the most widely studied protein-bound uremic solute because of its harmful effects on the cardiovascular system. The IS level is related to inflammation, endothelial dysfunction, oxidative stress, and impaired LV diastolic function, and so on.[10] IS is generated by gut microbe tryptophanases that convert dietary tryptophan into indole,[42] which is then converted to indoxyl and IS in the liver by the sequential actions of cytochrome P450 enzymes and sulfotransferase 1A1. We noted an upregulation of microbial tryptophanases and a reduction in plasma IS concentration in Decomp HF as compared with Comp HF, and speculate that impaired intestinal epithelial absorption during progressive HF heavily influences the circulating levels of these metabolites.[3,4]

It was previously reported that HF patients exhibit intestinal overgrowth of pathogenic bacteria including *Shigella*, *Campylobacter*, and *Salmonella* according to HF severity.[5] Consistent with these findings, we showed that the genus *Escherichia/Shigella* was more abundant in the decompensated than in the compensated phase of HF in the same patient, as determined by non-culture-based methods. Intriguingly, the abundance of the *Escherichia/Shigella* cluster, comprising TMA- and indole-producing microbes harboring TMA lyases and tryptophanases,[26,27] positively correlated with circulating TMAO and IS levels. Thus, there is a vicious circle of deterioration of intestinal conditions in Decomp HF. In addition to morphological and functional changes in the intestine, gut microbiome composition and function underlie the pathophysiology of HF. On the other hand, there may be an inverse correlation between the abundance of the genus *Bifidobacterium* and the plasma IS level. Oral intake of *Bifidobacterium* in a gastro-resistant seamless capsule form has been reported to reduce blood IS accumulation in hemodialysis patients, presumably by restoring a favorable intestinal environment and suppressing bacteria such as *Escherichia coli*.[43] Taking into account the heart-kidney interrelationship underlying HF pathophysiology, we propose that *Bifidobacterium* has protective effects against IS in HF patients.

Diuretics can modulate bowel perfusion and the gut environment, including epithelial function and pH, and are an essential aspect of HF treatment. However, a previous report has demonstrated no association between diuretic use and gut microbiome composition in HF patients.[12] Among PPI-associated genera,[44] *Streptococcus* was enriched in Comp HF patients with PPI use compared with those not using PPI.

Although morbidity and mortality in patients with HFrEF have declined, there are no therapies to date that can improve the outcome of HFpEF.[14] Notably, HFpEF patients had an increased plasma TMAO concentration and a presumably higher IS concentration than control subjects. These findings indicated that microbe-associated metabolites and the gut microbiome itself may be associated with the pathophysiology of HF, including HFpEF.

Study Limitations

Firstly, this was a single-center study with a small patient population; therefore, additional larger studies are warranted to verify our observations. Secondly, given the study design, it remains unclear whether changes in gut microbiome composition precede the onset of HF. We also did not collect any information on patients' diet before admission and cannot exclude the influence of diet or probiotics on gut microbiome. Besides, medication needs to be considered as a confounder when interpreting the results. Finally, a $P<0.05$ was considered significant in the analysis of relative abundance of gut microbiome and type I errors can be of concern in this study.

Conclusions

Our results indicated that gut microbiome composition and microbiome-related metabolites were altered in HF patients. In addition, we detected possible correlations between specific bacterial genera and circulating levels of harmful metabolites such as TMAO and IS.

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Disclosures

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Supplementary Files

Please find supplementary file(s);
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