Non-Ischemic Heart Failure With Reduced Ejection Fraction Is Associated With Altered Intestinal Microbiota

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Background: Research suggests that heart failure with reduced ejection fraction (HFrEF) is a state of systemic inflammation that may be triggered by microbial products passing into the bloodstream through a compromised intestinal barrier. However, whether the intestinal microbiota exhibits dysbiosis in HFrEF patients is largely unknown.

Methods and Results: Twenty-eight non-ischemic HFrEF patients and 19 healthy controls were assessed by 16S rRNA analysis of bacterial DNA extracted from stool samples. After processing of sequencing data, bacteria were taxonomically classified, diversity indices were used to examine microbial ecology, and relative abundances of common core genera were compared between groups. Furthermore, we predicted gene carriage for bacterial metabolic pathways and inferred microbial interaction networks on multiple taxonomic levels. Bacterial communities of both groups were dominated by the Firmicutes and Bacteroidetes phyla. The most abundant genus in both groups was Bacteroides. Although α diversity did not differ between groups, ordination by β diversity metrics revealed a separation of the groups across components of variation. Streptococcus and Veillonella were enriched in the common core microbiota of patients, while SMB53 was depleted. Gene families in amino acid, carbohydrate, vitamin, and xenobiotic metabolism showed significant differences between groups. Interaction networks revealed a higher degree of correlations between bacteria in patients.

Conclusions: Non-ischemic HFrEF patients exhibited multidimensional differences in intestinal microbial communities compared with healthy subjects.

Key Words: 16S rRNA; Dysbiosis; Gut flora; Heart failure; Intestinal microbiota
been documented and, recently, HF was associated with both an increase in potentially pathogenic intestinal bacteria in an Italian cohort and a depletion of core intestinal microbiota in a population of north European patients.\(^\text{16-19}\)

By using 16S rRNA sequencing and predictive metagenomics, and by inferring microbial interaction networks, we explored possible compositional alterations of intestinal bacterial communities in Japanese patients with nonischemic HF with reduced ejection fraction (HFrEF), predicted their functional metabolic profiles, and assessed their correlation patterns.

**Methods**

**Study Design and Protocol**

This was a study recruiting consecutive patients with nonischemic HFrEF admitted to Osaka University Hospital from October 2015 to April 2017. Heart disease patients with no HF (termed “non-HF controls”) and healthy volunteers were also recruited as controls. HFrEF was defined as HF with EF <50% and diagnosis was made according to relative signs and symptoms, facilitated by echocardiographic measurements of the left ventricular EF (LVEF). Both chronic stable and acute HF/acute decompensated chronic HF patients were included in the final sample, representing, to the extent possible, a broad spectrum of the HF syndrome. Ischemic etiology for HFrEF patients was excluded based on coronary angiography (CAG) data. All subjects gave written informed consent to participate and the local ethics committee approved the study.

After being properly instructed, subjects gave a stool sample at their earliest convenience, which was collected by appointed staff with a feces tube from a commode specimen collector, immediately stored in a common house freezer until the appointed transporter arrived, and then carried in liquid nitrogen and stored at −80°C in a dedicated facility. The stool sampling day was considered as baseline for evaluations. Blood count, blood biochemistry, and various clinical and demographic data were collected from patient records or generated for healthy controls to explore differences between groups and correlations with bacterial community composition. Additionally, subjects underwent routine echocardiographic examination, as previously described.\(^\text{20}\)

**Study Population**

During the recruitment period, a total of 272 HF patients were admitted to Osaka University Hospital. Of them, 95 patients who either had HF with preserved EF (HFrEF) or HFrEF caused by ischemic heart disease were immediately excluded. Another 40 patients were excluded based on age (>70 years old), regardless of underlying disease. A further 109 patients were also excluded for a multitude of reasons, including denying consent, giving consent but failing to provide a sample in the designated commode specimen collector during their hospital stay, having a LV assist device implanted at the time of screening, and other specific exclusion criteria, chief among them the use of antibiotics and/or probiotics within 4 weeks prior to sampling (Figure 1, Table 1). Finally, 28 patients were included for analysis.

Eligibility for healthy controls was based on the exclusion criteria listed in Table 1; 20 healthy controls were voluntarily recruited, but 1 withdrew consent prior to sampling and was excluded, resulting in 19 healthy controls included in the study.

Non-HF controls were recruited by a similar process as used for the HFrEF patients to comparatively assess...
possible effects of age and heart disease-related medication on intestinal bacterial composition and came from a pool of patients scheduled for CAG, coronary computed tomography (CT) angiography, or catheter ablation during the recruitment period. Eligibility was based on the same exclusion criteria as for HFrEF patients, minus the coronary artery disease (CAD) criterion. All CAD patients had documented disease on CAG and/or coronary CT angiography. No patients with acute coronary syndrome were considered. Disease duration for non-HF controls reflected the time since documentation of the disorder with appropriate examination. A total of 18 non-HF controls were finally included in the analysis.

### Dietary Information

The nutritional intake of the HFrEF patients and non-HF controls is presented in Table 2. The intake of carbohydrates, lipids, and protein was significantly lower in HFrEF patients compared to non-HF controls. The intake of fiber was not significantly different among the groups. The energy intake was similar among the groups. The table also includes data on beta-blockers, ACE inhibitors or ARBs, and proton-pump inhibitors, indicating the proportion of patients in each group using these medications.

### Table 2. Baseline Characteristics

<table>
<thead>
<tr>
<th>Variables</th>
<th>HFrEF patients (n=28)</th>
<th>Non-HF controls (n=18)</th>
<th>Healthy controls (n=19)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Demographics</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, years</td>
<td>51±10.4*</td>
<td>57±9*</td>
<td>36±6</td>
</tr>
<tr>
<td>Males (%)</td>
<td>21 (75)</td>
<td>15 (83)</td>
<td>16 (84)</td>
</tr>
<tr>
<td><strong>Diagnosis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DCM 15 (54%)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>CAD 16 (89%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DHCM 5 (18%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arrhythmia 2 (11%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other 8 (28%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Disease duration, years</strong></td>
<td>3 (0.3–11.4)</td>
<td>1 (0.4–3.3)</td>
<td></td>
</tr>
<tr>
<td><strong>Clinical variables</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NYHA class III–IV (%)</td>
<td>17 (61)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td><strong>BMI, kg/m²</strong></td>
<td>21.7±3.4t</td>
<td>25.6±3.7t</td>
<td>21.8±1.9</td>
</tr>
<tr>
<td>Systolic BP, mmHg</td>
<td>95±13.1†</td>
<td>123±13</td>
<td>116±12</td>
</tr>
<tr>
<td>Diastolic BP, mmHg</td>
<td>59±8.8†</td>
<td>70±10</td>
<td>71±8</td>
</tr>
<tr>
<td><strong>Echocardiography</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>LVEF, %</td>
<td>25±9.4†</td>
<td>62±5</td>
<td>62±4</td>
</tr>
<tr>
<td>LVEDD/m², mm</td>
<td>41±8.1†</td>
<td>26±2</td>
<td>28±2</td>
</tr>
<tr>
<td>LVMI, g/m²</td>
<td>125±45.1†</td>
<td>81±16</td>
<td>69±10</td>
</tr>
<tr>
<td>E/A, m/s</td>
<td>1.75±0.97†</td>
<td>0.98±0.28#</td>
<td>1.82±0.44</td>
</tr>
<tr>
<td>E/e’ (average), cm/s</td>
<td>13±5.1†</td>
<td>9±3</td>
<td>6±1</td>
</tr>
<tr>
<td>RAP, mmHg</td>
<td>3 (3–8)</td>
<td>8 (3–8)</td>
<td>3 (3–8)</td>
</tr>
<tr>
<td><strong>Catheterization data</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RAP, mmHg</td>
<td>5 (3–9)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>CI (Fick), L/min/m²</td>
<td>2.6±0.7</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td><strong>Blood biochemistry</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>eGFR, mL/min/1.73 m²</td>
<td>64.4±25.2*</td>
<td>76.0±17.0</td>
<td>87.0±16.9</td>
</tr>
<tr>
<td>BNP, ng/L</td>
<td>375 (145–630)*±t</td>
<td>16 (8–36)*#</td>
<td>2 (2–6)</td>
</tr>
<tr>
<td>CRP, nmol/L</td>
<td>6.67 (3.81–35.24)*</td>
<td>3.81 (3.81–10.95)*</td>
<td>2.86 (1.90–3.81)</td>
</tr>
<tr>
<td><strong>Nutritional intake</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carbohydrates, g</td>
<td>260 (260–260)*</td>
<td>260 (260–288.4)*</td>
<td>292.9 (290.8–292.9)</td>
</tr>
<tr>
<td>Lipids, g</td>
<td>58 (58–58)*</td>
<td>58 (51.8–61.2)*</td>
<td>65.7 (64.7–65.7)</td>
</tr>
<tr>
<td>Protein, g</td>
<td>70 (70–70)*</td>
<td>70 (70–76.2)</td>
<td>74.8 (74.8–75.1)</td>
</tr>
<tr>
<td>Fiber, g</td>
<td>NA</td>
<td>NA</td>
<td>13.3 (13.3–13.3)</td>
</tr>
<tr>
<td>Energy, Kcal [MJoule]</td>
<td>1,800 (1,800–1,800)*</td>
<td>1,800 (1,750–2,171)†</td>
<td>2,161 (2,161–2,168)†</td>
</tr>
<tr>
<td><strong>Medication</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-blockers (%)</td>
<td>27 (96)†</td>
<td>9 (50)</td>
<td>NA</td>
</tr>
<tr>
<td>ACEI or ARB (%)</td>
<td>24 (86)†</td>
<td>10 (56)</td>
<td>NA</td>
</tr>
<tr>
<td>PPI (%)</td>
<td>16 (57)</td>
<td>14 (78)</td>
<td>NA</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± standard deviation for normally distributed variables, median and interquartile range for non-normally distributed variables, or number of patients and relative percentage of the group’s total in parentheses. *P<0.05 vs. healthy controls, †P<0.05 vs. non-HF controls. Because of missing data, HFrEF patients, non-HF controls, and healthy controls had the following number of subjects, respectively, regarding the following variables: BNP: n=28, n=18, n=17; CRP: n=28, n=17, n=17; E/A: n=21, n=17, n=19; E/e’: n=17, n=14; Estimated GFR: n=28, n=18, n=17; LVMI: n=28, n=17, n=19; RAP: n=28, n=11, n=19. Disease duration for non-HF controls was not compared with that of HFrEF patients, because the underlying conditions were unrelated. A, peak late diastolic transmitral flow velocity; ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin II receptor blocker; BMI, body mass index; BNP, B-type natriuretic peptide; BP, blood pressure; CAD, coronary artery disease; CI, cardiac index; CRP, C-reactive protein; DCM, dilated cardiomyopathy (idiopathic); DHCM, dilated phase of hypertrophic cardiomyopathy; E, peak early diastolic transmitral flow velocity; e’, tissue Doppler velocity of mitral annulus; eGFR, estimated glomerular filtration rate; HF, heart failure; HFrEF, heart failure with reduced ejection fraction; LVEDD, left ventricular end-diastolic diameter; LVMI, left ventricular mass index; NA, not applicable or not available; NYHA, New York Heart Association; PPI, proton-pump inhibitor; RAP, right atrial pressure.
Retrospective insight into dietary habits was provided through administration of a structured questionnaire, which assessed habitual intake in 21 food and drink categories. The answers for consumption fell into 4 categories of frequency (less than once a week/1–2 times a week/3–7 times a week/more than once a day) and were treated as ordinal variables.

controls was primarily estimated from dietary information available in hospital records. The nutritional intake for the healthy controls and a few other subjects sampled on their first day of hospital admission was based on the latest edition (2015) of the Japanese National Health and Nutrition Survey, which includes the age- and sex-adjusted daily average nutritional intake of the Japanese population (mhlw.go.jp/stf/houdou/0000142359). Subjects would be assigned estimated values included in the survey, according to their age and sex.
compared by Tukey’s range test when normally distributed and by the Steel-Dwass test when not, to control for multiple comparisons. Categorical variables were compared by Fisher’s exact test with post-hoc correction, as needed. Ordinal variables for dietary information were compared by the Wilcoxon rank-sum test.

Propensity scores were calculated with the R package matchIt.

Bacterial relative abundance data were compared between groups by the Wilcoxon rank-sum test, followed by false discovery rate (FDR) adjustment at the level of 0.05.

The within-subject α diversity of bacterial communities was assessed by richness (S), evenness (Shannon’s E), the Shannon index (H), the Chao 1 estimator, and the Simpson index (1/λ), and compared between groups by Welch’s t-test.

The between-subjects β diversity was assessed with the Bray-Curtis dissimilarity and the unweighted UniFrac distance metrics. The statistical significance of compositional differences between sets of samples was assessed by permutational multivariate analysis of variance (PERMANOVA) and analysis of similarities (ANOSIM).

Relationships between microbial data and host-related metadata were assessed by multivariate analysis (Supplementary Methods).

Functional metabolic profiles of bacterial communities were predicted with PICRUSt (phylogenetic investigation of communities by reconstruction of unobserved states).

DNA Extraction, 16S rRNA Sequencing, and Taxonomic Classification
DNA was extracted from stool samples using a DNeasy PowerSoil Kit (QIAGEN, Hilden, Germany). Libraries were prepared according to the Illumina 16S Metagenomic Sequencing Library Preparation Guide, with a primer set (27Fmod: 5′-AGR GTT TGA TCM TGG CTC AG-3′ and 338R: 5′-TGC TGC CTC CCG TAG GAG T-3′) targeting the V1–V2 regions of the 16S rRNA gene. 251-bp paired-end sequencing of the amplicons was performed on a MiSeq System (Illumina, San Diego, CA, USA), using the MiSeq 500-cycle v2 kit. Paired-end sequences were merged using PEAR (sco.h-its.org/exelixis/web/software/pear/). Merged reads were quality-trimmed with BBtrim (bbmap.sourceforge.net). Using random_sequence_sample.pl (ualberta.ca/~stothard/software.html), 20,000 reads per sample were randomly selected for further analysis. The processed sequences were clustered into operational taxonomic units (OTUs) defined at a 97% similarity cutoff using UCLUST version 1.2.22q. Representative sequences for each OTU were then classified taxonomically by RDP Classifier version 2.2, with the Greengenes 13_8 database. The bioinformatics pipeline QIIME version 1.9.1 was used as the informatics environment for processing of raw sequencing data.

Statistical Analysis
For baseline characteristics, continuous variables were compared by Tukey’s range test when normally distributed and by the Steel-Dwass test when not, to control for multiple comparisons. Categorical variables were compared by Fisher’s exact test with post-hoc correction, as needed. Ordinal variables for dietary information were compared by the Wilcoxon rank-sum test.

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Functional metabolic profiles of bacterial communities were predicted with PICRUSt (phylogenetic investigation of communities by reconstruction of unobserved states).
Altered Gut Flora in HF

Variation between groups was assessed by PERMANOVA. Microbial interaction networks were inferred by SPIEC-EASI (sparse inverse covariance estimation for ecological association inference), as detailed in Supplementary Methods. Network differences were assessed by measures of graph theory. The level of significance for all tests was set at $\alpha=0.05$ and a probability value of $P<0.05$ was considered to be statistically significant. The statistical software JMP Pro 13 (SAS, Cary, NC, USA), the statistical computing environment R (r-project.org), the statistical package Primer 7 (Quest Research Ltd, Auckland, New Zealand), the open-source bioinformatics pipeline QIIME version 1.9.1 (qiime.org), and Microsoft Office Excel 2013 (Microsoft Corp., Redmond, WA, USA) were used for database construction and data analysis.

Results

Subjects' Characteristics

The baseline characteristics of the HFrEF patients, non-HF controls, and healthy controls are shown in Table 2. The median disease duration for HFrEF patients was 3 years, and more than half were diagnosed with idiopathic dilated cardiomyopathy. Their mean LVEF was 25% and 61% were classified as NYHA class III or IV at baseline.

Dietary Data

The estimated daily median nutrient and calorie intake showed slight but statistically significant differences between groups (Table 2), but the general nutrient intake pattern was the same in all groups, with approximately two-thirds of intake provided by carbohydrates, one-sixth by protein, and one-sixth by lipids. The questionnaire-based assessment of dietary habits did not reveal significant differences in any of the major food categories (meat, seafood, dairy products, vegetables, and rice) between HFrEF patients and healthy controls after FDR adjustment, but showed that patients, when on their home, long-term diet, were consuming sweets, eggs, fast food, and coffee less frequently than healthy controls.

Taxonomic Classification of Intestinal Bacteria in HFrEF Patients and Healthy Controls

Intestinal bacteria with a relative abundance of at least 0.1% in any subject were classified into 13 phyla, 21 classes, 27 orders, 56 families, and 134 genera. The bacterial communities of both the patient and the healthy control group were dominated by the Firmicutes and Bacteroidetes phyla (Figure 2A). The third most abundant phylum was the Proteobacteria. These results were consistent with the findings of the Human Microbiome Project.
either group. The common core of both groups was characterized by 40 genera, 34 of which were shared (Figure 3A).

### Relative Abundance of Bacterial Genera

The most abundant genus for both HFrEF patients and healthy controls was *Bacteroides*, with a mean relative abundance of 30.5% and 24.2%, respectively.

**At the genus level, the patient group was characterized by 119 genera, 31 of each were absent from the healthy controls, while the healthy control group included 103 genera, 15 of which were absent from the patient group (Figure 3A, Table S1).** Whittaker plots revealed a similar pattern in evenness for both groups (Figure 3B).

To determine the presence of a common bacterial core, we looked for genera present in at least 50% of subjects in either group. The common core of both groups was characterized by 40 genera, 34 of which were shared (Figure 3A).

**Figure 6.** Metabolic pathway variation. (Top) Stacked bar chart of the 10 most abundant bacterial metabolic pathways in HFrEF patients and healthy controls, based on PICRUSt-predicted KO relative abundances. Their similar prevalence and even distribution are more than apparent, in stark contrast with the relative abundances of bacterial genera seen in Figure 2B. Beyond these pathways, however, the analysis revealed significant variation between patients and healthy controls. The scale of relative abundance was adjusted to reflect only the 10 most abundant pathways, excluding all others. Vertical bars represent individual samples. (Bottom) Principal Components Analysis ordination of KO relative abundances of all PICRUSt-predicted metabolic pathways in bacterial communities of HFrEF patients and healthy controls, showing a separation of samples by health status in the first 2 components of variation, which was verified by PERMANOVA. HFrEF, heart failure with reduced ejection fraction; KO, Kyoto Encyclopedia of Genes and Genomes (KEGG) Ortholog; PERMANOVA, permutable multivariate analysis of variance; PICRUSt, phylogenetic investigation of communities by reconstruction of unobserved states.
nance of Bacteroides was again consistent with previous findings.31

The 20 most abundant genera for patients and healthy controls accounted for 79.58% and 84.45%, respectively, of the total genera abundance in the 2 groups (Figure 2B, Table S2); 17 of these 20 genera (85%) were shared by both groups. A comparison of the relative abundances of the common core genera between the HFrEF patient and healthy control groups revealed that Streptococcus and Veillonella were enriched, while SMB53 was depleted in patients (adj. \(P=0.05\). Figure 4, Supplementary Results & Supplementary Discussion).

**Multivariate Analysis of Bacterial Community Composition and Host-Related Metadata**

Multivariate analysis using Spearman’s rank correlation and performing 9,999 permutations assessed how well the HFrEF and healthy host-related data variables described and performing 9,999 permutations assessed how well the Multivariate analysis using Spearman’s rank correlation and Host-Related Metadata

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**Microbial interaction Networks**

Networks of microbial associations, inferred by SPIEC-EASI, revealed differential correlation patterns in the HFrEF and healthy control groups (Supplementary Results & Supplementary Discussion, Figure S1).

**Comparison With Non-HF Controls**

To control for the differences in age, medication, and eGFR between HFrEF patients and healthy controls, we also analyzed available data from a total of 18 consecutive patients with CAD or arrhythmia that were not in HF status (non-HF controls). We calculated propensity scores and matched HFrEF patients and non-HF controls by age, eGFR, and \(\beta\)-blocker, angiotensin-converting enzyme inhibitor (ACEI)/angiotensin II receptor blocker (ARB), and proton-pump inhibitor (PPI) administration, on a 1:1 ratio (Table S7). The analysis resulted in a subset of 18 HFrEF patients and the whole group of 18 non-HF controls, who were matched for age, eGFR, ACEI/ARB, and PPI frequencies, and partially matched for \(\beta\)-blockers. Moreover, there were no differences in frequencies of consumption among the food categories between matched HFrEF patients and non-HF controls, and the nutrient intake patterns estimated from food records were the same. Alpha diversity indices were not different between groups (H, \(P=0.504\), Chao 1, \(P=0.613\), but PERMANOVA on Bray-Curtis similarities based on 4th root transformed relative abundances of the bacterial genera also uncovered the difference between groups (9,999 permutations, \(P=0.045\)). This indicated that even after adjustment for age, eGFR, and medication, there were still differences in \(\beta\) diversity between subjects with and without HF.

**Discussion**

In the present study, we employed 16S rRNA sequencing, a well-established, accurate, reliable, and reproducible method when properly used,32–34 to show that the intestinal bacterial communities of non-ischemic HFrEF patients differed from those in subjects without HF. This difference was based on a phylogenetic shift in community composition that extended to predicted information on metabolic pathways and differential network interactions of examined bacterial taxa.

**Microbial Dysbiosis**

Dysbiosis describes structurally and/or functionally altered states of microbiota as compared with homeostasis. To our
knowledge, only 2 other studies using 16S rRNA analysis of intestinal microbiota in HF patients have been indexed in PubMed, and no other predicting functional information or examining network interactions. As with previous reports, our study revealed microbial dysbiosis in HFrEF.

Microbial variation between our groups was primarily assessed with indices of α and β diversity. Whereas assessment of α diversity is roughly the equivalent of counting animal species in areas of a large forest, averaging results, and then comparing the results to those from a different forest (a type of univariate analysis), assessment of β diversity is roughly the equivalent of counting numbers of animal species in different areas of a forest, assessing what is common, and directly comparing the areas to each other and to areas of a different forest (a type of multivariate analysis). Beta diversity assessment was based on the presence or absence of taxa, as shown by the unweighted UniFrac distance metric, which importantly accounts for bacterial phylogenetic relationships among taxa and substantiates phylogenetic shifts. It also reflected taxa abundance, as shown by the Bray-Curtis dissimilarity. Common core microbiota displayed few and subtle differences between groups, suggesting that variation in key bacterial abundances is downplayed in HFrEF-related dysbiosis, and underlies the importance of a systems approach rather than a reductionist approach in addressing ecological variation, focusing on diversity and multivariate analysis rather than individual alterations.

Interestingly, Akkermansia, a common bacterial genus in the human colon, was absent from the healthy controls (Table S1). Although the universal presence of Akkermansia in all human hosts is not the case, it is also possible that this genus was present in at least some healthy controls, but in very low relative abundance, below the 0.1% threshold that was used for taxonomic classification in our study. At present, no causal effects of dysbiosis on HFrEF pathophysiology can be supported, although evidence suggests that microbial products are associated with HF mortality, justifying future options, such as fecal transplantations in laboratory animals, to examine functional effects of HFrEF-associated variation.

Correlations of Bacterial Composition With Host Metadata

Intriguingly, multivariate analysis of the relationship of host metadata and microbiotic data did not uncover significant correlations that would explain part or even the whole of the variation in bacterial communities. This is consistent with previous work, and implies that factors as yet undiscovered may play a role in shaping microbial communities in HFrEF patients, and that proxy variables of tissue hypoxia and congestion including LVEF, cardiac index, and right atrial pressure, may not be good correlates with bacterial abundance patterns.

Measurements of blood flow in the mesenteric arteries, as well as intestinal edema markers, such as bowel wall thickness, in ultrasound examinations, represent interesting tools for further examination of how host-related pathophysiology may be linked to 16S rRNA and metagenomic explorations of the intestinal microbiota.

Bacterial Metabolic Profiles

The metabolic functions of intestinal bacteria represent a major target in microbial research. Bacterial products, such as short-chain fatty acids and vitamins, affect the human host in multiple ways and bacterial metabolic functions may contribute to human health or predispose to disease. In the absence of shotgun sequencing data we used PICRUSt, a technique that adequately and cost-effectively predicts metagenomes (i.e., all genes from all organisms present in a sample) from 16S rRNA data and a reference genome database to evaluate the functional pathways of bacterial communities and assess their metabolic capabilities. The analysis is based on a complex algorithm that first computes gene content for each microorganism in a reference phylogenetic tree and then combines this result with the abundance of 16S rRNA genes of the microorganisms found in each sample, to arrive at the expected abundance of gene families in the bacterial community. In simple terms, the measured bacterial 16S rRNA gene abundances are transformed into predicted abundances of orthologous gene families attributed to different functional pathways. The final output is a table of gene counts per sample per predicted functional pathway, which can be standardized (i.e., divided by the total counts in each sample) to finally get the relative gene counts per sample for each of the predicted pathways. The predicted pathways are derived from the KEGG database, which can be used to link genes included in an organism’s genome to cellular metabolic pathways. This enables comparison of metabolic capacities between different organisms and different environments.

Although the present results should be considered suggestive, with an accuracy of approximately 85%, and do not constitute gene expression data, the discovered variation between patients and healthy controls hints at altered bacterial metabolic capacities in the gut of HFrEF patients. More specifically, the overall pattern revealed by PCA and verified by PERMANOVA provided evidence that HFrEF is associated with distinctions in relative gene abundances linked to bacterial metabolism, as compared with health. Metabolic pathway variation, of which the relative gene pool was significantly different in statistical analysis after FDR adjustment (Table S6), did not seem associated with marked adverse change that could affect host functions, with 1 notable exception: the depletion in patients of gene families associated with pantothenate synthesis. Pantothenate is the anion form of pantothentic acid (vitamin B5), an essential element of life across animal organisms, being a component of coenzyme A. As its Greek name implies, it is available in all sources of food; however, its content in food is reduced with food processing, freezing, cooking, and exposure to vinegar, the use of which is widespread in culinary preparations, especially in Japan. Although speculative, a diminished synthesis by intestinal bacteria in HFrEF may have implications for subjects already at risk for deficiency (e.g., the more elderly patients or patients with unbalanced dietary habits). Future work in this field may help uncover additional differences and verify metabolic distinctions in larger samples. With PICRUST providing evidence for bacterial metabolic pathway variation in HFrEF, further research with admittedly more expensive techniques of deeper sequencing levels may be reasonable.

Confounders of Variation

Age, ethnicity, renal function, and diet are known factors affecting intestinal microbiota composition and may confound results if not properly addressed. Subjects included in this study were all Japanese, belonged to an adult group aged 18–70 years, had preserved renal function,
and were consuming foods typically found in a common Japanese diet, with vegetarian diets being an exclusion criterion and estimated nutrient intake being similar in groups. Concerning age, especially, most research shows changes in intestinal microbial composition after a subject has passed the threshold of being elderly and much research shows that intestinal microbial communities remain relatively stable throughout adult life and before the stage of old age, if they are not seriously perturbed by, for example, antibiotic administration.1,4,31

Study Limitations
Limitations in this study include the small sample sizes, which reflect the strict exclusion criteria and sampling protocol to ensure sample quality and avoid contamination involved in 16S rRNA analysis, and the inherent inability to control for all confounders of variation in observational research. However, previous studies have included even smaller numbers of subjects and a comparison between groups of such sizes in HF and other disorders has previously yielded significant results.12,13,19 A further limitation of this study was the inability to match HFrEF and healthy controls by age, renal function, and medication. However, a comparison of HFrEF patients to age-, eGFR-, and medication-matched non-HF controls showed that differences in β diversity still remained between subjects with and without HF. Finally, sampling bias during recruitment cannot be completely excluded, particularly because patients who were admitted to the department of cardiovascular medicine but quickly transferred to other departments (e.g., department of cardiovascular surgery, intensive care unit) were generally not screened or sampled for logistic reasons.

Conclusions
We have found significant alterations in the intestinal bacterial communities of non-ischemic HFrEF patients on multiple dimensions of variation, including a basis of structure and extensions of metagenomic carriage of metabolic pathways and interactions of bacterial taxa. These findings may help lay the foundation for further research into HFrEF-related microbial dysbiosis.

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References
correlation patterns at the genus level between bacterial communities of HFrEF patients and healthy controls.

**Table S1.** Bacterial genera found only in HFrEF patients or only in healthy controls

**Table S2.** The 20 most abundant genera in HFrEF patients and healthy controls

**Table S3.** Similarity percentage (SIMPER) analysis of untransformed genus level Bray-Curtis similarities showing the top 9 genera contributing 50% of the average dissimilarity between HFrEF patients and healthy controls

**Table S4.** Classes of PICRUSt-predicted metabolic functions in sampled bacterial communities

**Table S5.** Ratio of PICRUSt-predicted counts of metabolism-related gene families by metabolic function and subject group

**Table S6.** KEGG metabolic pathways differentially abundant in HFrEF patients compared with healthy controls

**Table S7.** HFrEF patients and non-HF controls matched by age, eGFR, and medications, and summary of propensity score matching

**Table S8.** Significant differences in baseline characteristics of HFrEF patients in NYHA classes I–II and NYHA classes III–IV

Please find supplementary file(s);

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**Supplementary Files**

**Supplementary File 1**

**Supplementary Methods**

**Supplementary Results**

**Supplementary Discussion**

**Figure S1.** Microbial interaction networks showing different