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ORIGINAL RESEARCH

The Gut Microbiome of Heart Failure With Preserved Ejection Fraction

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BACKGROUND: Risk factors for heart failure with preserved ejection fraction (HFpEF) include hypertension, age, sex, and obesity. Emerging evidence suggests that the gut microbiota independently contributes to each one of these risk factors, potentially mediated via gut microbial-derived metabolites such as short-chain fatty acids. In this study, we determined whether the gut microbiota were associated with HFpEF and its risk factors.

METHODS AND RESULTS: We recruited 26 patients with HFpEF and 67 control participants from 2 independent communities. Patients with HFpEF were diagnosed by exercise right heart catheterization. We assessed the gut microbiome by bacterial 16S rRNA sequencing and food intake by the food frequency questionnaire. There was a significant difference in α-diversity (eg, number of microbes) and β-diversity (eg, type and abundance of microbes) between both cohorts of controls and patients with HFpEF (P=0.001). We did not find an association between β-diversity and specific demographic or hemodynamic parameters or risk factors for HFpEF. The Firmicutes to Bacteroidetes ratio, a commonly used marker of gut dysbiosis, was lower, but not significantly so (P=0.093), in the patients with HFpEF. Compared with controls, the gut microbiome of patients with HFpEF was depleted of bacteria that are short-chain fatty acid producers. Consistent with this, participants with HFpEF consumed less dietary fiber (17.6±7.7 versus 23.2±8.8 g/day; P=0.016).

CONCLUSIONS: We demonstrate key changes in the gut microbiota in patients with HFpEF, including the depletion of bacteria that generate metabolites known to be important for cardiovascular homeostasis. Further studies are required to validate the role of these gut microbiota and metabolites in the pathophysiology of HFpEF.

Key Words: 16S ■ heart failure with preserved ejection fraction ■ microbiome ■ microbiota ■ short-chain fatty acids

Recognition of the role of the microbiota in the human intestinal tract in the development and severity of cardiovascular disease is progressively increasing.[1] Recent research has highlighted roles for the gut microbiota in regulating hypertension; atherosclerosis; thrombotic events, including acute myocardial infarction and stroke; vascular inflammation; and heart failure.[1–3]

Mechanisms through which the gut microbiota modify the development of cardiovascular disease are manifold,[1] ranging from translocation of bacteria and their wall products into the circulation with inflammatory activation[4] to modulation of metabolites with both beneficial and harmful influences on the development of cardiovascular disease. Harmful products include the metabolism of phosphatidylcholine to trimethylamine *N*-oxide,[5] which promotes atherosclerosis and thrombosis and is associated with cardiovascular events.[6] Beneficial products include the production of short-chain fatty acids (SCFAs), which can improve blood pressure, inflammation, and myocardial repair and are closely related to dietary fiber composition.[7–9]

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CLINICAL PERSPECTIVE

What Is New?

- Well hemodynamically characterized patients with heart failure with preserved ejection fraction had a gut microbiome composition which differed substantially from controls without significant medical history independent of confounding factors, with significant reductions of microbial taxa capable of producing short-chain fatty acids.

What Are the Clinical Implications?

- These data suggest that the gut microbiome could play a role in the development or severity of heart failure with preserved ejection fraction and provide potential mechanistic insights given that short-chain fatty acids have established roles in hypertension, cardiac and renal fibrosis, immune function, and systemic inflammation.
- This highlights a possible role for dietary modulation and/or short-chain fatty acid supplementation in mitigating the development and severity of heart failure with preserved ejection fraction, which would benefit from further investigation.

Nonstandard Abbreviations and Acronyms

HFpEF	heart failure with preserved ejection fraction
HFrEF	heart failure with reduced ejection fraction
OTU	observed taxonomic unit
SCFA	short-chain fatty acid

Studies investigating the composition and role of the gut microbiota in heart failure with reduced ejection fraction (HFrEF) have identified a reduction in gut microbial diversity[2] with greater quantities of pathogenic bacterial taxa,[10] circulating bacterial wall compounds with inflammatory activation,[11] and higher levels of trimethylamine *N*-oxide, which correlated with poorer clinical outcomes.[3] By contrast, whether the gut microbiota of patients with heart failure with preserved ejection fraction (HFpEF) is different and how it contributes to the development of HFpEF is not known.

Our previous studies have identified an interaction between the gut microbiota, blood pressure, and related cardiac and renal fibrosis prevented by SCFAs in experimental models.[8,12] Given the fundamental role of hypertension and cardiac fibrosis in the pathogenesis of HFpEF, we evaluated the composition of the gut microbiota in patients with HFpEF with a particular focus on changes in the representation of key metabolite-producing bacteria and their association with relevant demographic and hemodynamic features.

METHODS

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Study Population

This study complied with the Declaration of Helsinki and was approved by the human ethics research committee of Alfred Hospital, Melbourne, Australia. All participants gave informed consent and were recruited between August 2017 and January 2020. The HFpEF cohort included patients referred to the Department of Cardiology, Alfred Hospital for invasive hemodynamic assessment of exertional dyspnea with features suggestive of HFpEF but inconclusive noninvasive investigations for the diagnosis of HFpEF in a similar manner to published algorithms[13] or those undergoing exercise right heart catheterization to assess for eligibility for a concurrent clinical trial. Confirmation of the diagnosis was based on a resting pulmonary capillary wedge pressure ≥15 mm Hg or exercise pulmonary capillary wedge pressure ≥25 mm Hg and a left ventricular ejection fraction >50% according to the recognized diagnostic criteria for HFpEF. Patients were excluded if they had more than mild valvular stenosis or regurgitation, evidence of significant pulmonary disease on lung function testing or pulmonary imaging, chronic pulmonary emboli, hypertrophic cardiomyopathy, or previous heart transplantation. Patients with HFpEF and controls were excluded if they had taken antibiotics or probiotics within the past 3 months.

Because the HFpEF cohort comprised patients from both metropolitan and regional areas, they were compared with 2 independent control cohorts from metropolitan Melbourne ("metropolitan controls") and a regional area, Shepparton, near Melbourne ("regional controls"). Inclusion criteria included being 40 to 70 years of age and either sex and having a body mass index (BMI) 19 to 30.5 kg/m^2 and not taking antihypertensive medications. Exclusion criteria for controls were having any type of gastrointestinal disease (including a history of intestinal surgery, inflammatory bowel disease, celiac disease, lactose intolerance, chronic pancreatitis, or other malabsorption disorder) or having type 1 or 2 diabetes mellitus or chronic kidney disease.

Patients with HFpEF were asked to complete a questionnaire at the time of admission for exercise right heart catheterization detailing anthropometric

data, medications, comorbidities, and usual physical activity levels. Controls completed similar questionnaires. Medication and comorbidity data for patients with HFpEF were verified against their electronic medical record, whereas comorbidity and medication data for controls were self-reported.

Food Frequency Questionnaire

The dietary intake during a period of 12 months was assessed in both groups by the Dietary Questionnaire for Epidemiological Studies version 3.2, a self-administered and validated semiquantitative food frequency questionnaire developed by the Cancer Council Victoria that reflects the dietary intake of the Australian population.[14] The food frequency questionnaire assesses the consumption and frequency of 140 foods and beverages during the previous 12 months. Dietary intake estimates of 98 nutrients were derived from 2 Australian databases, AUSNUT 2007 and NUTTAB 2010.

Participants' diet quality was measured by comparing their intake (according to age and sex) with the Dietary Guidelines for Australian recommendations using the Australian Dietary Guideline Index 2013.[15] The Australian Dietary Guideline Index 2013 includes 10 components in total: 6 core food components reflecting adequacy, quality, and variety of intake within the Australian Dietary Guideline core food groups (vegetables, fruit, grains, lean meats and alternatives, and dairy and alternatives), 1 reflecting adequacy of fluid intake, and 3 noncore food groups (unsaturated spreads or oils, discretionary items, and alcohol) reflecting compliance with guidelines to moderate or limit intake.

Exercise Right Heart Catheterization Protocol

Exercise right heart catheterization was performed using supine cycle ergometry as previously reported by us.[16] All measurements and exercises were performed in an unfasted state together with regular medications. A 7-F Swan-Ganz catheter was inserted through the brachial or internal jugular vein with the patient under local anesthesia. End-expiratory measurements were taken from the right atrium, right ventricle, pulmonary artery, and pulmonary capillary wedge pressure position. Pulmonary capillary wedge pressure position was confirmed by identification of the appropriate pressure waveform and by biochemical demonstration of an arterialized blood gas sample when required. Cardiac output was calculated using thermodilution, and the average of 3 measurements were taken. Measurements recorded noninvasively included heart rate, systemic blood pressure, and arterial oxygen saturation by pulse oximetry. Noninvasive and invasive measurements were taken at rest and at 3-minute intervals during exercise until the patients reached their peak tolerated workload. An important feature of this approach is the application of a weight-corrected workload protocol, consisting of an initial workload of 0.3 W/kg with a rise to 0.6 W/kg and then 0.9 W/kg every 3 minutes and to 1 W/kg after 12 minutes. Exercise duration was limited by patient symptoms (dyspnea or fatigue). Participants were instructed to maintain a cycle cadence of 60 rpm during exercise. Mixed venous blood gas measurements were taken from the pulmonary artery at rest and at peak exercise.

Invasive hemodynamic data are presented as raw values or indexed to body surface area as appropriate. The potential impact of differences in work capacity was accounted for by indexing key parameters to workload as reported by us and others.[16]

Fecal DNA Extraction and Sequencing

Stool samples were collected by participants at home in tubes containing RNAlater (Thermo Scientific), which was previously shown to preserve bacterial DNA.[17] The self-reported time of stool collection and stool consistency, according to the Bristol stool chart, were recorded. Tubes were brought to the clinics immediately or stored at –20°C for <24 hours and then brought to the hospital or clinic, where they were stored at –80°C until further processing. DNA was extracted using the DNeasy PowerSoil DNA isolation kit (Qiagen). The V4–V5 region of the bacterial 16S rRNA was amplified by polymerase chain reaction using 20 ng of DNA, Platinum Hot Start PCR master mix (ThermoFisher Scientific), 515F and 926R primers (Bioneer), and methods previously described in a Veriti Thermal Cycler (ThermoFisher Scientific). The quantity of the polymerase chain reaction product was assessed in a Qubit (ThermoFisher Scientific), and 240 ng of polymerase chain reaction product per sample were pooled and cleaned using the PureLink PCR Purification kit (ThermoFisher Scientific). The product was then sequenced in an Illumina MiSeq sequencer (300 bp paired-end reads). This study followed recently published guidelines for gut microbiome studies.[18]

Microbiome Bioinformatic Analyses

All samples were analyzed using the same QIIME2[19] workflow. Raw reads from FASTQ files were checked for quality and trimmed at 241 and 220 bases for R1 and R2, respectively. Paired-end reads then were denoised, a process including merging, dereplicating, and removing chimeric reads using the QIIME2 DADA2 plugin through q2-DADA2.[20] This resulted in a total of 5 832 240 reads across 94 samples, with varying degrees of depth in each sample (Figure S1). Samples were rarefied to 28 500 reads, losing 1 sample because of an insufficient read count. A pretrained naïve Bayes classifier targeted toward the 16S V4

region using the GreenGenes database (version 13_8; 99% observed taxonomic unit [OTU] taxonomy) was then used to assign taxonomic data to each amplicon sequence variant (via q2-feature-classifier). We used Calypso to perform unsupervised hierarchical clustering analysis based on the microbiome data using the top 100 most abundant taxa.[21]

To assess statistical significance, we used Jaccard, Bray-Curtis dissimilarity, and unweighted and weighted UniFrac distances to cluster the data in principal coordinate analyses based on different parameters (eg, controls versus HFpEF) using the QIIME2 plugin q2-diversity. All β-diversity metrics gave very similar results between groups. α-Diversity was measured using the OTUs, Shannon and Chao1 indexes. To test the significance of the separation between groups, we used the PERMANOVA test on diversity metrics using default pseudo-*F* distribution settings through QIIME2. The *q* values were false discovery rate corrected using the Benjamini–Hochberg method for multiple testing; $q<0.05$ was considered significant. Linear discriminant analysis effect size was used to identify differentially abundant taxa between patients with HFpEF and controls, with a specified effect size cutoff of 3.0. The main findings from the linear discriminant analysis were validated by area under the curve analysis in Calypso. To identify co-occurring and mutual exclusive bacteria present in patients with HFpEF and controls, we performed a network analysis in Calypso using Spearman correlation and including the 100 most abundant taxa.

Statistical Analysis

Data are presented as mean±SD, and the Student's *t* test was used for comparison of numerical variables. Categorical variables were compared using the chi-square test for independence. Correlations between hemodynamic and abundance of specific microbial taxa were performed using the Spearman method. A 2-tailed $P<0.05$ was considered statistically significant. For linear regression analysis on principal coordinate analysis differences, a dendrogram was produced using the "hclust" function in R, forming 2 clusters. Predictors of cluster assignment were then assessed with an ANCOVA analysis to identify significant contributors to β-diversity based on principal coordinate analysis distances. This analysis and the comparison of dietary data used 1 combined control group of both regional and metropolitan controls. Analyses of differences in α-diversity and β-diversity, along with comparisons of microbial taxa, were performed between patients with HFpEF and both control cohorts separately to validate the results in a separate cohort in a different geographic location. Statistical analyses of hemodynamic data were performed using R version 3.5.1 software (R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Clinical Parameters

A total of 26 patients with invasively diagnosed HFpEF and 67 controls were recruited (39 from metropolitan Melbourne and 28 from regional Victoria). Characteristics of patients in the HFpEF and control cohorts are outlined in Table 1 and Table S1. Patients with HFpEF were significantly older than controls and had a greater BMI. Patients with HFpEF had a higher prevalence of hypertension and atrial arrhythmias in particular and a greater prevalence of all recorded comorbidities. Few controls took regular medications, which was significantly different from patients with HFpEF. All patients with HFpEF were New York Heart Association functional class II–III. The majority of patients with HFpEF did no vigorous or moderate exercise (73%), whereas 75% of controls did vigorous and 82% did moderate exercise.

Patients with HFpEF had hemodynamic and echocardiographic impairments as outlined in Table S2. The majority of patients had relatively normal resting hemodynamics, which changed substantially with exercise, consistent with the physiology of HFpEF.[22] Resting echocardiography demonstrated mild elevations in left atrial volume index, without significant abnormalities in right ventricular systolic pressure, mean E/e', or left ventricular mass index, with an increased left ventricular mass index present in 21% of patients with HFpEF only.

Table 1. Demographics of Patients With HFpEF and Controls

Characteristic	HFpEF, n=26	Metropolitan Controls, n=39	*P* Value	Regional Controls, n=28	*P* Value
Age, y	68±7.5	58.3±7.9	<0.001	61±6	<0.001
Female sex	20 (77)	17 (44)	0.02	19 (68)	0.66
BMI, kg/m^2	32.8±5.8	25.1±2.9	<0.001	25.3±2.5	<0.001
BNP, ng/L	113.2±96	N/A	N/A	NA	NA
Systolic blood pressure	143.7±19.1	131.2±16.7	0.007	120±12.3	<0.001

Data are provided as mean±SD or number (percentage). BMI indicates body mass index; HFpEF, heart failure with preserved ejection fraction; and N/A, not available.

Dietary Results

All patients with HFpEF were asked to complete the dietary questionnaire, with a completion rate of 73%. Dietary data were available for all controls. We first assessed dietary fiber as an important macronutrient that affects the gut microbiota.[8] Patients with HFpEF had significantly lower total dietary fiber intake than controls (17.6±7.7 versus 23.2±8.8 g/day; P=0.016). We then examined the Australian Dietary Guideline Index 2013 score as a marker of diet quality and found that patients with HFpEF had lower scores than controls (51.7±8.5 versus 59.7±7.5; P<0.001), indicative of lower overall dietary quality. There was no interaction between dietary fiber or dietary quality indexes and hemodynamic or echocardiographic variables in the HFpEF cohort.

Gut Microbiome

As shown in Figure 1 and Figure S2, the gut microbiome composition of HFpEF differed significantly from that of controls as evaluated by β-diversity (P=0.001). This is also visualized with hierarchical clustering (Figure 2) demonstrating the separation of patients with HFpEF and controls in diversity. Differences in β-diversity between HFpEF and controls were independent of differences in BMI, age, sex, hypertension, dietary score, and fiber intake (Table 2).

We next investigated for the presence of key taxonomic patterns that are known to be associated with altered gut microbiota composition. The ratio of Firmicutes to Bacteroidetes, a commonly used marker of gut dysbiosis, tended to be lower in patients with HFpEF compared with controls (mean±SEM, 1.11±0.026 versus 1.48±0.026), although this did not reach statistical significance (P=0.093; Figure S3). There were, however, significant differences in the abundance of specific bacterial populations between patients with HFpEF and controls (Figure 3). Patients with HFpEF had a depletion of bacteria known to be SCFA producers,[23] particularly *Ruminococcus* (Figure S4). This finding was validated by other tests, including an area under the curve of 0.99 (95% CI, 0.96–1; false discovery rate corrected, q<0.001; Table S3). Indeed, the sparse partial least squares discriminant analysis showed that *Ruminococcus* was a driver for the difference between HFpEF and controls (Figure S5). *Ruminococcus* abundance also increased with higher fiber intake and showed inverse relationships with female sex, hypertension, BMI, and unintuitively dietary score (Figure S6). The depletion of *Ruminococcus* in patients with HFpEF appeared independent of BMI, age, and hypertension, whereas female sex, lower fiber intake, and higher dietary score were likely independently associated with depletion of *Ruminococcus,* although the skewed distribution of these data introduce inaccuracies to the estimates in this analysis (presented in Table S4). There were no statistically significant associations between the abundance of specific taxa and hemodynamic or echocardiographic parameters within the HFpEF cohort; however, this analysis was limited by the small sample size. We further explored these changes in bacterial taxa in a network analysis, which takes into consideration the whole microbial community. This again confirmed that HFpEF and controls had co-occurring and mutually exclusive bacteria (Figure S7).

α-Diversity (a measure of variability within each sample) was evaluated using the Shannon diversity

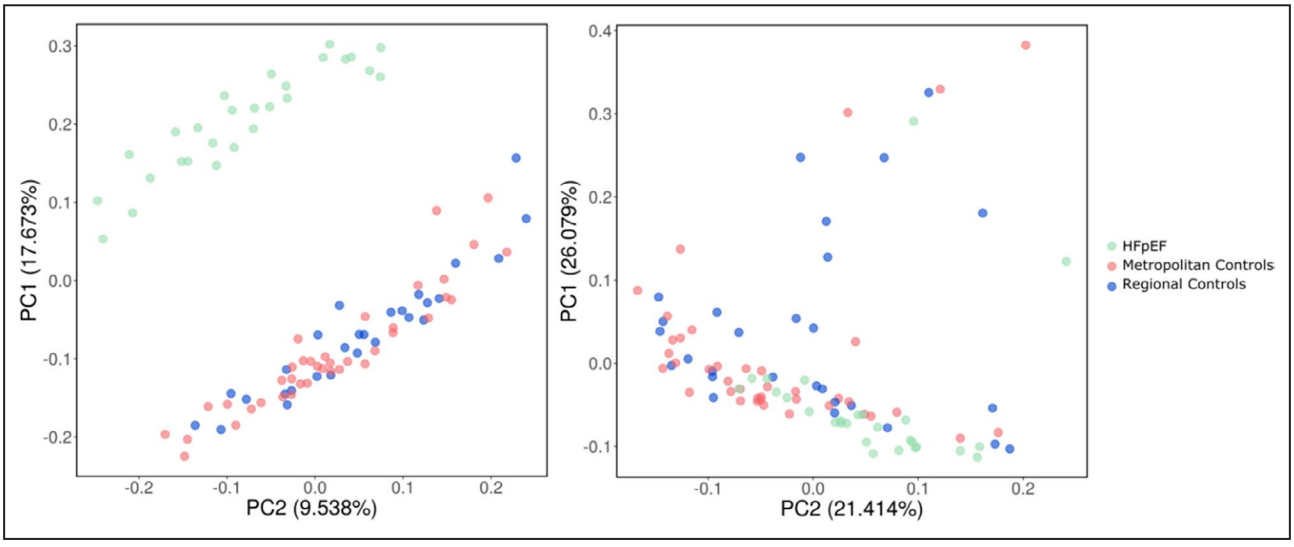

Figure 1. β-Diversity differed significantly in patients with HFpEF and controls.
There was a significant difference (P=0.001) between patients with HFpEF and both control cohorts in β-diversity (showing unweighted UniFrac principal coordinate analysis plot). This difference persisted when comparing weighted UniFrac plots (P=0.004). HFpEF indicates heart failure with preserved ejection fraction. PC1, principal component 1; PC2, principal component 2.

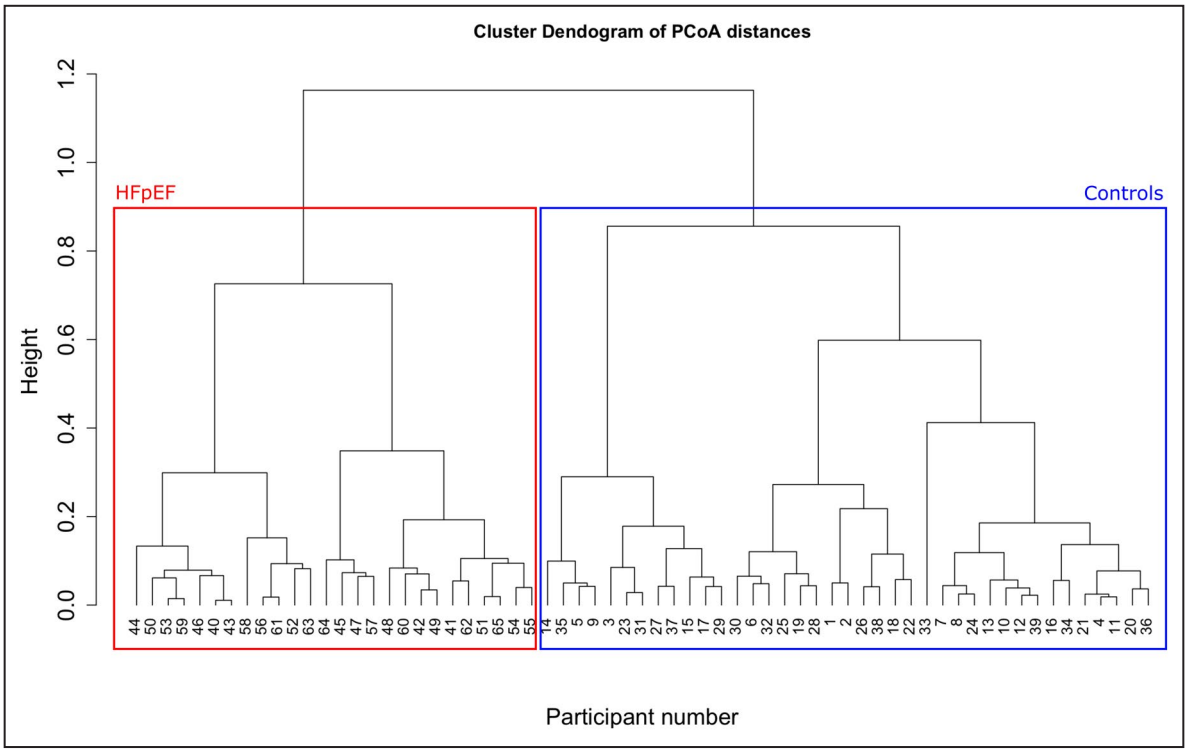

Figure 2. Patients with HFpEF and controls clustered separately when analyzed according to components of the principal coordinate analysis.
Hierarchial clustering of principal coordinate analysis distances in HFpEF and controls. For the full legend for this figure, refer to Figure S6. HFpEF indicates heart failure with preserved ejection fraction.

index, Chao1 index, and OTUs, represented in Figure 4. There were significant differences between patients with HFpEF and metropolitan controls in both Chao1 and OTU measures of α-diversity, but no statistically significant differences in Shannon diversity.

DISCUSSION

Given the link between hypertension and obesity with HFpEF together with experimental data indicating a role for the gut microbiota in cardiovascular homeostasis, we hypothesized that the gut microbiota influenced by diet are important factors in HFpEF pathogenesis. In this study, we determined the differences in the gut microbiota between patients with hemodynamically confirmed HFpEF and 2 independent control cohorts. Our key finding is that there are profound changes in the gut microbiota of patients with HFpEF that are independent of BMI, age, hypertension, diet (including fiber intake), and sex, and this finding was replicated when compared with both control cohorts. Patients with HFpEF also had lower α-diversity than metropolitan controls (city matched) across multiple indexes, particularly those relevant to richness (ie, number of taxa) such as number of OTUs and Chao1. Relative to metropolitan and regional controls, patients with HFpEF had a depletion of the SCFA-producing *Ruminococcus*, which was a driver for the differences

Table 2. Multiple Regression Analysis for Cluster Assignment According to Principal Coordinate Analysis Measures of β-Diversity in Patients With HFpEF and Controls

Parameter	β	95% CI	SEM	*t* Value	*P* Value
BMI	−1.78	−6.38 to 63.7	1.34	−1.33	0.18
Age	−0.06	−4.47 to 0.91	0.17	−0.37	0.71
Sex (male)	2.91	−3.69 to 9.52	3.28	0.89	0.37
Hypertension (normotensive)	9.14	−6.99 to 25.27	8.01	1.14	0.25
Dietary score	0.45	−0.22 to 1.13	0.34	1.35	0.18
Fiber intake	−0.13	−0.34 to 0.08	0.10	−1.28	0.20

BMI indicates body mass index; and HFpEF, heart failure with preserved ejection fraction.

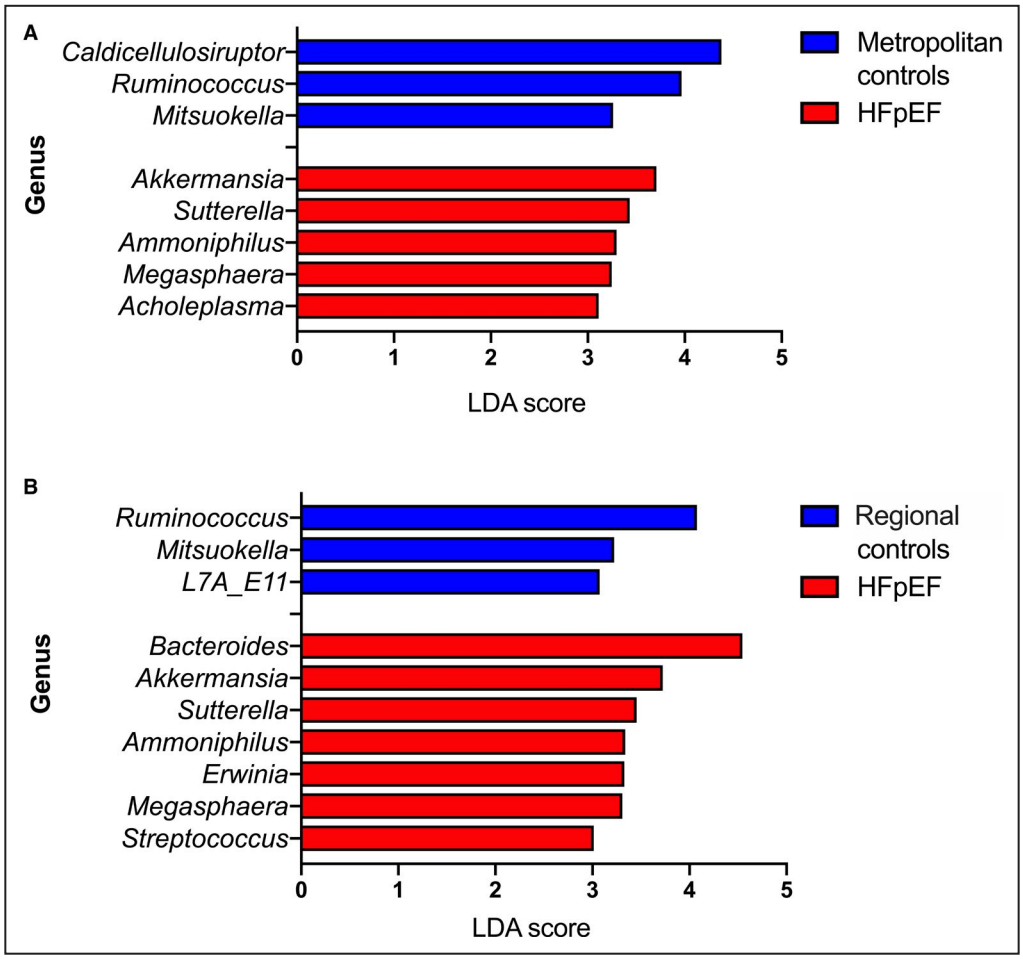

Figure 3. Taxonomic differences in patients with HFpEF and controls.
Differences in the abundance of specific taxa in (**A**) patients with HFpEF compared with metropolitan controls and (**B**) patients with HFpEF compared with regional controls. Taxa classifiable to the genus level are shown, with a complete analysis at the observed taxonomic unit level represented in Figure S8. Differences in abundance were calculated using LDA, with a cutoff score of 3. HFpEF indicates heart failure with preserved ejection fraction; and LDA, linear discriminant analysis.

observed between HFpEF and controls, with an area under the curve of 0.99. Consistent with this, patients with HFpEF had lower dietary fiber intake and overall dietary quality index than controls, suggesting that their dietary patterns could be an initial factor contributing to changes in the gut microbiota, which in turn are associated with HFpEF.

It has been well established that gut microbiota generate SCFAs through the anaerobic fermentation of dietary fiber and that these metabolites play roles in lipid metabolism, neurogenesis, gut inflammation, and glucose homeostasis.[1] Factors common in a Western lifestyle, such as low-fiber diets rich in processed foods and a lack of exercise, can affect the gut microbiota including the species and proportions of bacteria and may, therefore, affect the ability of the gut microbiome to ferment fiber to SCFAs.[7] Conversely, high-fiber diets increase populations of SCFA-producing bacteria and circulating SCFA levels,[7] and accordingly diets high in fiber are associated with lower blood pressure in humans. Importantly, we have recently shown that a lack of prebiotic fiber intake is not only associated with cardiovascular disease but also can lead to gut microbiota that on their own can drive an increase in blood pressure, cardiac hypertrophy, higher B-type natriuretic peptide, and fibrosis in experimental models.[8] This can be attenuated with the administration of SCFAs even when combined with a diet that lacks fiber.[9] Similarly, SCFAs attenuate hypertension, cardiac hypertrophy and fibrosis, arrhythmias, and atherosclerosis in murine models, with underlying immune-modulatory mechanisms.[24] Consistent with the present study, the gut microbiota of patients who are hypertensive and patients who are prehypertensive have been found to have lower gene richness and diversity and particularly lower SCFA-producing bacteria compared with

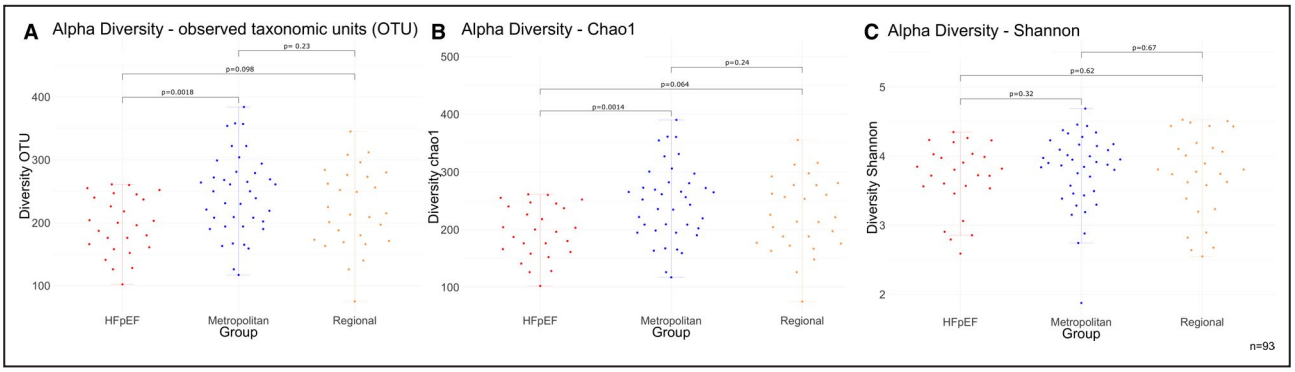

Figure 4. α-Diversity in patients with HFpEF and metropolitan and regional controls.
α-Diversity was measured using the (**A**) OTUs, (**B**) Chao1 index, and (**C**) Shannon index, demonstrating significant differences in α-diversity between patients with HFpEF and metropolitan, but not regional, controls. HFpEF indicates heart failure with preserved ejection fraction; and OTUs, observed taxonomic units.

controls, and lower plasma SCFA levels have been found in patients who are hypertensive.[25]

SCFA-producing bacteria, depleted in patients with HFpEF in the present study, have been associated with HFrEF previously. *Ruminococcus, Erisipelotrichaeceae*, and *Blautia* were depleted in patients with heart failure in a study of 20 patients with HFrEF and matched controls whose β-diversity was also strikingly different.[2] A prior study investigating the role of the gut microbiome in HFrEF has similarly found reduced microbial richness in patients compared with controls, with a depletion of *Lachnospiraceae*, which are SCFA producers, in patients with HFrEF. *Lachnospiraceae* correlated negatively with soluble CD25, a marker of T cell activation and hence inflammation, a finding that was more pronounced in patients meeting adverse clinical end points.[26] The comparable findings of differences in diversity of the microbiome and reduction of SCFA-producing bacteria among patients with heart failure in these studies and ours builds on the evidence that the gut microbiota, and in particular specific microbial signatures with the depletion of key bacterial taxa, may play a role in the pathogenesis and severity of heart failure, regardless of the phenotype.

The interplay between SCFAs, hypertension, and immune activation point to a number of possible mechanisms through which the depletion of SCFA-producing gut microbiota could lead to the development and exacerbation of HFpEF. A prevailing unifying hypothesis for HFpEF is that comorbidities drive a systemic inflammatory state that leads to coronary microvascular inflammation, reduced nitric oxide availability, and downstream myocardial hypertrophy, fibrosis, and subsequent diastolic dysfunction.[27] HFpEF is associated with immune dysregulation, with alterations to the activity and ratios of helper versus regulatory T cells.[28] A depletion of SCFAs resulting from alterations to the gut microbiota promotes this process at multiple stages: contribution to obesity and insulin resistance,[29] diabetes mellitus with impacts on diabetic control,[30] interplay with physical inactivity,[31] and aforementioned roles in hypertension[8,9]; increased levels of regulatory T cells with fiber and SCFA administration[9]; enhanced systemic inflammation and endothelial dysfunction without SCFAs[32]; and regulation of downstream effects on myocardial tissue, with SCFAs protecting against the development of myocardial hypertrophy and fibrosis.[8,9,24] Other mechanisms through which the microbiota may be associated with heart failure include reduced intestinal wall integrity as a result of reduced cardiac output and oxygen delivery and venous congestion, resulting in translocation of bacteria into the circulation, fueling systemic inflammation.[4] Because HFpEF remains a disease without effective therapies, partly because of its heterogenous nature, fiber and the gut microbiome and specifically SCFAs could prove as potential targets in the prevention and treatment of all patients with HFpEF.

The findings of distinct gut microbiota profiles based on β-diversity, along with differential microbial taxa with less SCFA-producing bacteria in patients with HFpEF, may have been modified by the presence of confounding factors. The control group differed from the HFpEF cohort in terms of age, sex, BMI, and systolic blood pressure. All of these factors can contribute to differences in the gut microbiota.[7,33,34] However, β-diversity analysis remained different between HFpEF and controls independent of these factors. Furthermore, few controls were on medications compared with patients with HFpEF, and because medications can have an effect on the gut microbiome,[35] this could have contributed to the differences seen. The striking differences in β-diversity between patients with HFpEF and the control groups was independent of dietary quality and fiber intake, suggesting that although diet is an important modifier of the gut microbiota, the changes seen

in HFpEF are far beyond that explained by poor diet alone.

Several possible confounders are active in the *Ruminococcus* abundance analysis, with significant interactions between *Ruminococcus* and sex, hypertension, BMI, dietary score, and fiber intake. *Ruminococcus* abundance was affected by fiber intake, consistent with dietary fiber promoting SCFA-producing bacteria. Interestingly, a higher overall dietary quality score was associated with lower *Ruminococcus* abundance, suggesting that fiber is of central importance above other dietary components. *Ruminococcus* abundance had a negative relationship with female sex, which may point to a lower SCFA-producing capacity in the female sex[36] and could be a contributing factor to sex differences in HFpEF epidemiology.[37] Although *Ruminococcus* abundance discriminated clearly between patients with HFpEF and controls, this very low abundance of *Ruminococcus* in patients with HFpEF raised inaccuracies in the analysis of independence from confounders and may question the biological plausibility of this finding. Furthermore, medications were not included in this analysis and may have contributed to differences in *Ruminococcus* abundance between patients with HFpEF and controls; although angiotensin-converting enzyme inhibitors, taken by 50% of patients with HFpEF, have actually been associated with increased *Ruminococcus* abundance.[38] This finding therefore raises the possibility of a mechanistic link between the gut microbiome and HFpEF but requires further validation in larger cohorts. Furthermore, studies on the effect of the manipulation of the gut microbiome on hemodynamic parameters and HFpEF progression would be valuable in further exploring this association.

We acknowledge that this study had inherent limitations. The cohort of patients with HFpEF was relatively small; however, these patients were very well phenotyped, including the incorporation of invasive exercise hemodynamics, which is considered the gold standard diagnostic approach. When compared with 2 independent cohorts, the observation in HFpEF was found to be consistent. Blood samples were not taken for all participants with HFpEF, and therefore an analysis of circulating SCFAs or trimethylamine *N*-oxide levels was not possible. There is inherent variability in gut microbiome sequencing data; however, to minimize this, all HFpEF samples were sequenced in the same run using the same method. Several data points were self-reported, including comorbidities in control groups and dietary questionnaire data. Although the HFpEF cohort was well phenotyped for heart function, the control cohort had limited clinical data available as it would be challenging to recruit a cohort of similar size with invasive cardiac catheter studies.

CONCLUSIONS

Our findings highlight substantial differences in the gut microbiota between patients with HFpEF and controls, with differences in the abundance of microbial taxa that may provide mechanistic insights. Importantly, these differences were independent of confounding factors such as age and the presence of hypertension, validated across 2 independent control cohorts. This suggests that the gut microbiota and its metabolites, specifically SCFAs, may be a target for the prevention and possibly treatment of HFpEF in the future.

ARTICLE INFORMATION

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Disclosures

None.

Supplementary Material

Tables S1–S4
Figures S1–S8

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SUPPLEMENTAL MATERIAL

Table S1. Comorbidities and medications in HFpEF patients and control cohorts.

		HFpEF (n=26)	Controls, metro (n=39)	*P*-value	Controls, regional (n=28)	*P*-value
Comorbidities (%)						
	Hypertension	69	38	0.03	18	<0.01
	Atrial arrhythmia	69	3	<0.01	5	<0.01
	Diabetes mellitus	15	0	<0.01	0	<0.01
	IHD	27	0	<0.01	0	<0.01
	COPD[2]	8	0	<0.01	0	<0.01
Medications (%)						
	ACEi/ARB	50	0	<0.01	0	<0.01
	Beta-blocker	44	0	<0.01	0	<0.01
	MRA	24	0	<0.01	0	<0.01
	CCB	28	0	<0.01	0	<0.01
	Loop diuretic	16	0	<0.01	0	<0.01
	Aspirin	40	3	<0.001	0	<0.01
	Oral anticoagulant	56	0	<0.01	0	<0.01
	Statin	64	5	<0.001	11	<0.01

ACEi/ARB, angiotensin converting enzyme inhibitors/angiotensin receptor blocker; CCB, calcium channel blocker; COPD, chronic obstructive pulmonary disease; HFpEF, heart failure with preserved ejection fraction; IHD, ischaemic heart disease; MRA, mineralocorticoid receptor antagonist.

Table S2. Invasive haemodynamic and echocardiographic data for the HFpEF cohort.

RHC data		
	Right atrial pressure, mmHg	6.6 ± 3.6
	Rest mean pulmonary artery pressure, mmHg	19.9 ± 4.3
	Exercise mean pulmonary artery pressure, mmHg	41.1 ± 7
	PCWP at rest, mmHg	11.9 ± 3.6
	PCWP at exercise, mmHg	28.3 ± 7.2
	ΔPCWP, mmHg	16.2 ± 6.6
	Rest CI, mL/min/m^2	2.7 ± 0.6
	Exercise CI, mL/min/m^2	4.9 ± 1.6
	ΔCI, mL/min/m^2	2.2 ± 1.3
	Systemic vascular resistance at rest, mmHg/L/min	18.3 ± 5.6
	Pulmonary vascular resistance at rest, mmHg/L/min	1.6 ± 0.7
Echocardiography data		
	Ejection fraction, %	60.5 ± 5.1
	Left atrial volume index, mL/m^2	42.7 ± 22.1
	E/e' mean	11.4 ± 3.8
	Left ventricular mass index, g/m^2	85.7 ± 20.4

CI, cardiac index; PCWP, pulmonary capillary wedge pressure; LVMI, left ventricular mass index. Abnormal LVMI is LVMI ≥95g/m^2 for women and ≥115g/m^2 for men.

Table S3. Area under the curve analysis of the main taxa that were identified in the linear discriminant analysis.

Taxa	P-value	FDR	AUC (CI)	Odds Ratio (CI) HFpEF/Healthy
p__Firmicutes__g__Ruminococcus	3.5E-12	1.3E-10	0.99 (0.96-1)	0.02 (0-0.12)
p__Firmicutes__g__Ammoniphilus__s__oxalaticus	1.1E-10	2.7E-09	0.89 (0.81-0.97)	90.25 (15.44-1745.02)
p__Verrucomicrobia__g__Akkermansia__s__muciniphila	0.000034	0.00017	0.8 (0.69-0.91)	19.92 (4.76-138.47)
p__Firmicutes__g__Megasphaera	0.000082	0.00034	0.79 (0.67-0.9)	11.67 (3.81-40.77)
p__Proteobacteria__g__Sutterella	0.006	0.014	0.7 (0.56-0.84)	4.92 (1.67-15.77)
p__Firmicutes__g__Streptococcus	0.0091	0.02	0.69 (0.56-0.82)	4.17 (1.47-12.53)
p__Proteobacteria__g__Erwinia	0.015	0.032	0.67 (0.54-0.8)	4.67 (1.45-17.01)
p__Firmicutes__g__Mitsuokella	0.027	0.052	0.66 (0.52-0.8)	0.28 (0.1-0.77)

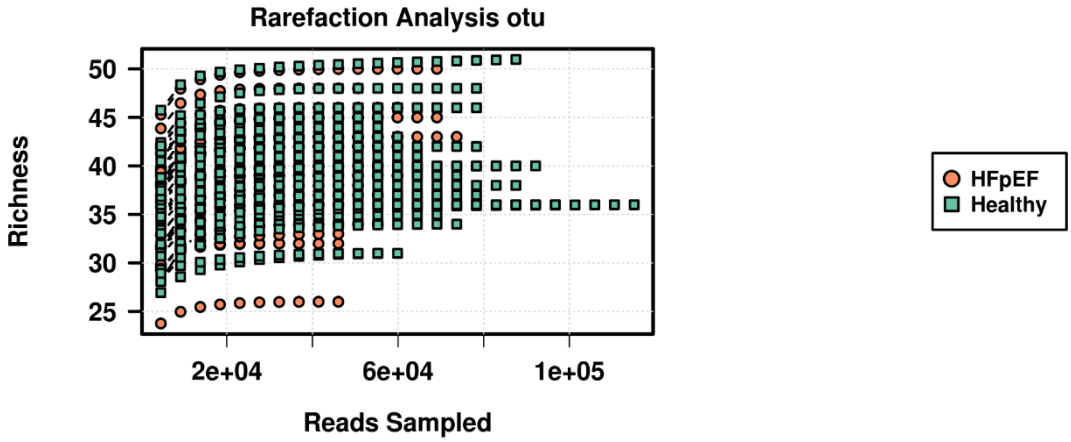
AUC, area under the curve; CI, confidence interval; FDR, false discovery rate; HFpEF, heart failure with preserved ejection fraction; OTU, observed taxonomic unit.

Table S4. Multiple regression analysis for *Ruminococcus* abundance in HFpEF patients and controls.

Parameter	**β**	**95% CI**	**SEM**	**t value**	***p* value**
BMI	68.4	-24.6 - 161.4	46.2	1.48	0.15
Age	-52.5	-113.9 - 9	30.5	-1.72	0.09
Sex (Male)	2166.9	994.4 - 3339.5	582.2	3.72	<0.001
Hypertension (Normotensive)	948	-86.1 - 1982.1	513.4	1.85	0.071
Dietary score	-99.7	-175.2 - -24.2	37.5	-2.67	0.01
Fibre intake	134.56	73 - 196.1	30.5	4.4	<0.001

BMI, body mass index; CI, confidence interval; HFpEF, heart failure with preserved ejection fraction; SEM, standard error of the mean.

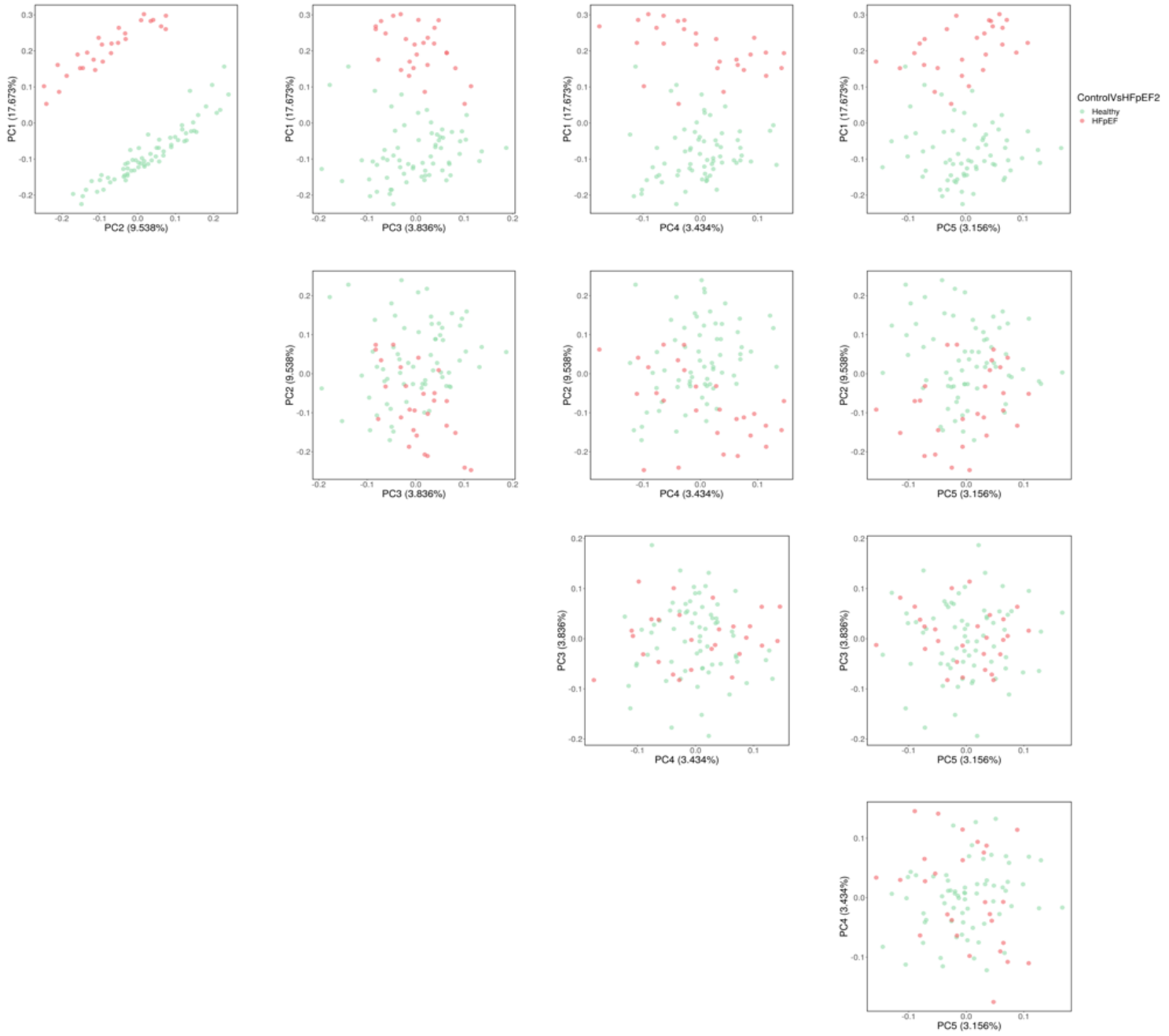
Figure S1. Rarefaction of samples based on microbiome sequencing depth.



HFpEF, heart failure with preserved ejection fraction.

Figure S2. Principal coordinate (PC) analysis plots for PC1 to 5.

Unweighted Unifrac



Weighted Unifrac

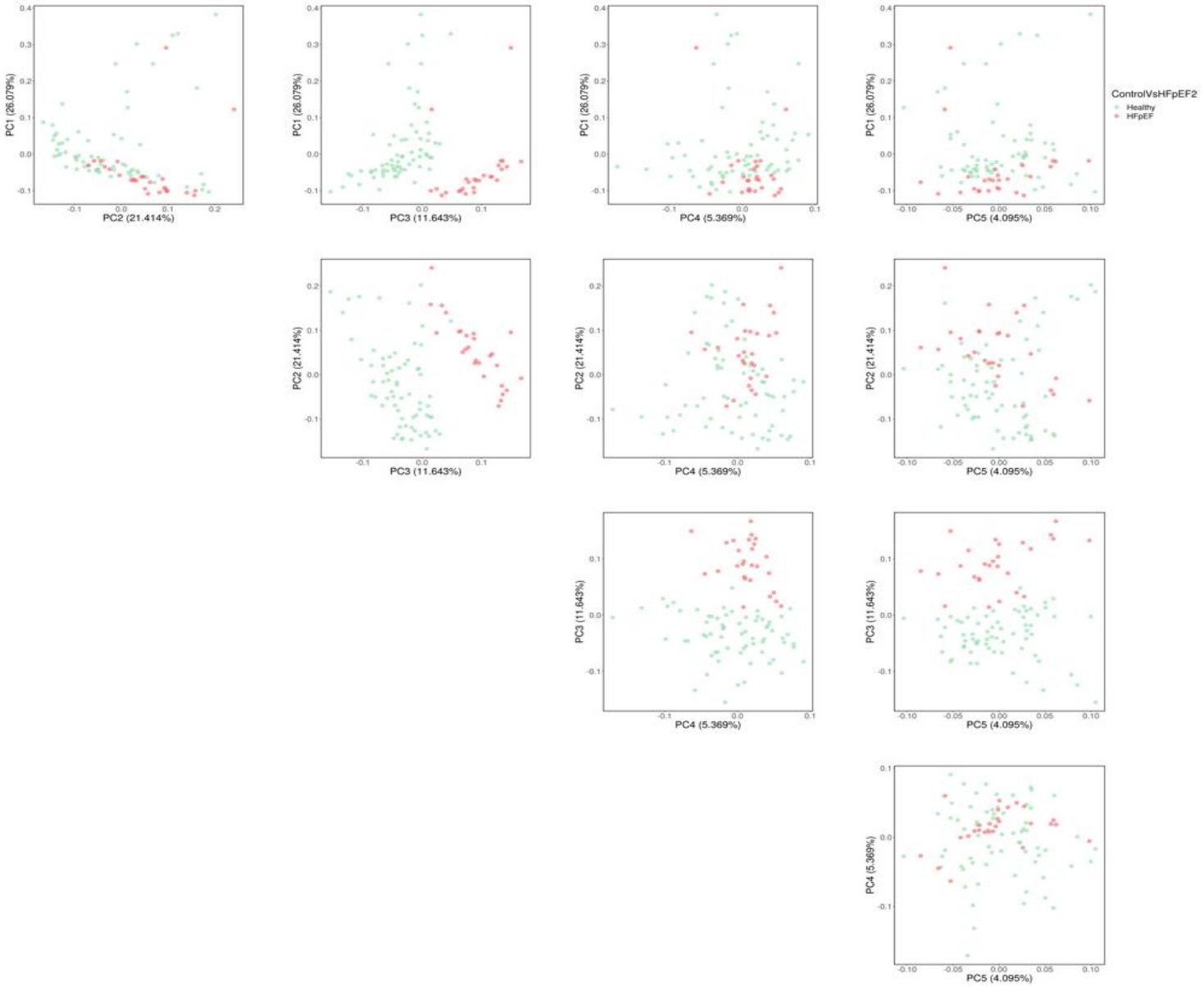
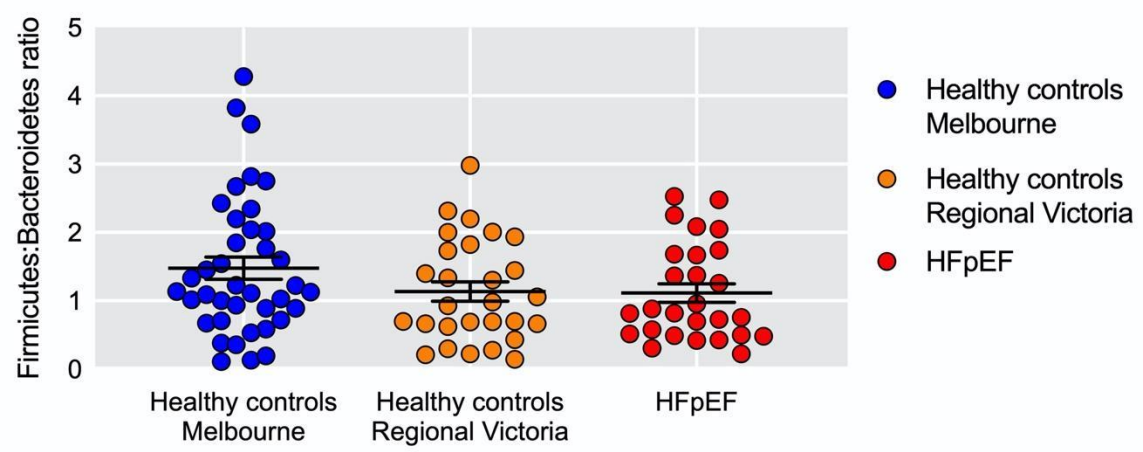
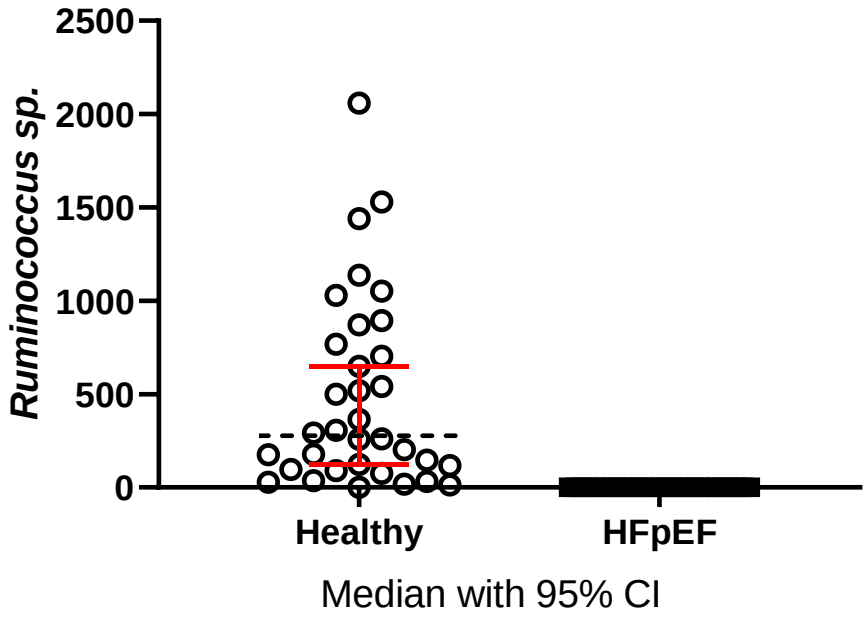


Figure S3. Firmicutes:Bacteroidetes ratio in HFpEF patients and metropolitan and regional controls.



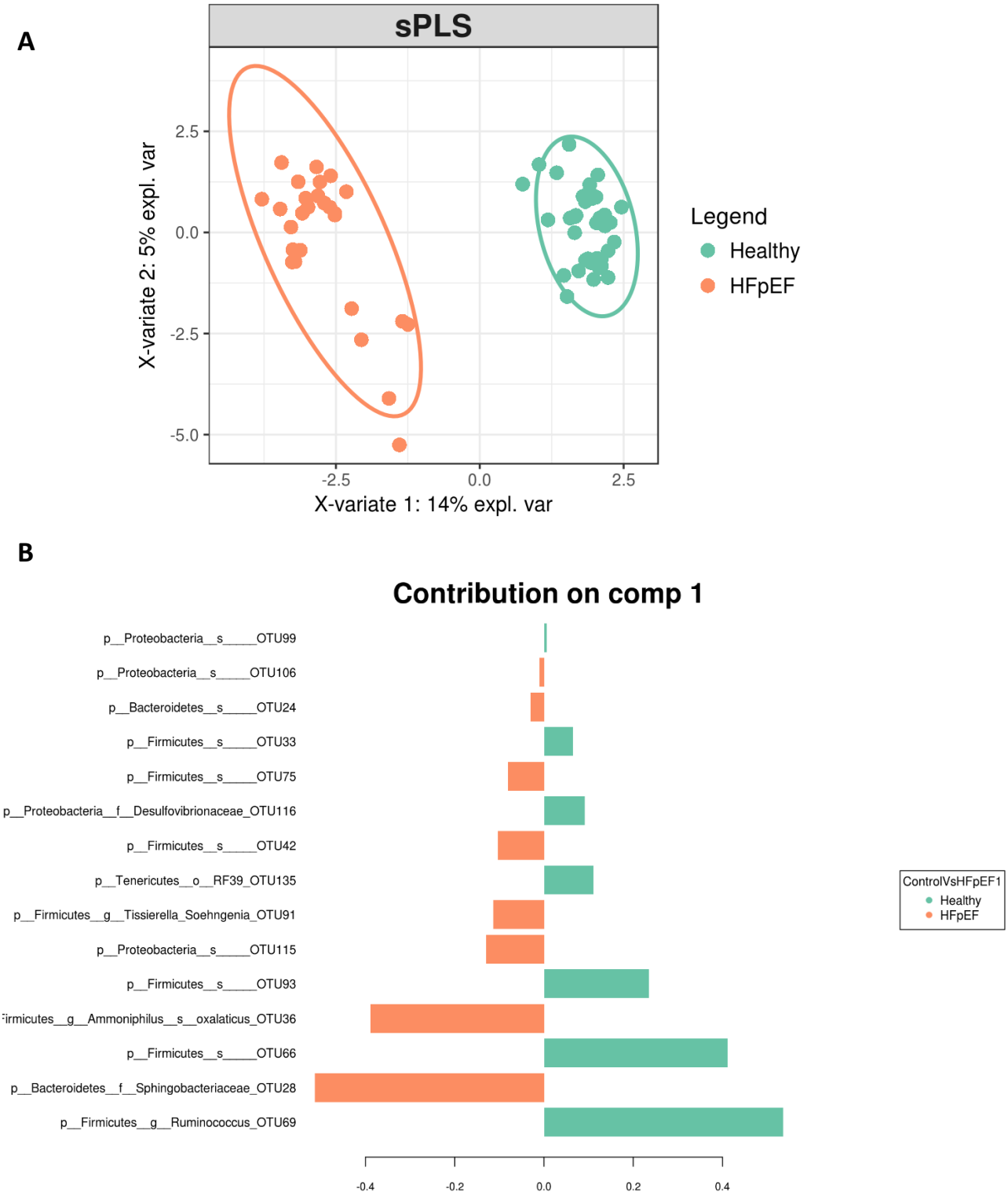
This ratio was not significantly different between HFpEF patients and controls overall (P=0.093) nor individual control cohorts (P=0.1144 for metropolitan controls, *P*=0.912 for regional controls.

Figure S4. Validation that HFpEF patients have lower levels of *Ruminococcus* compared to healthy controls.



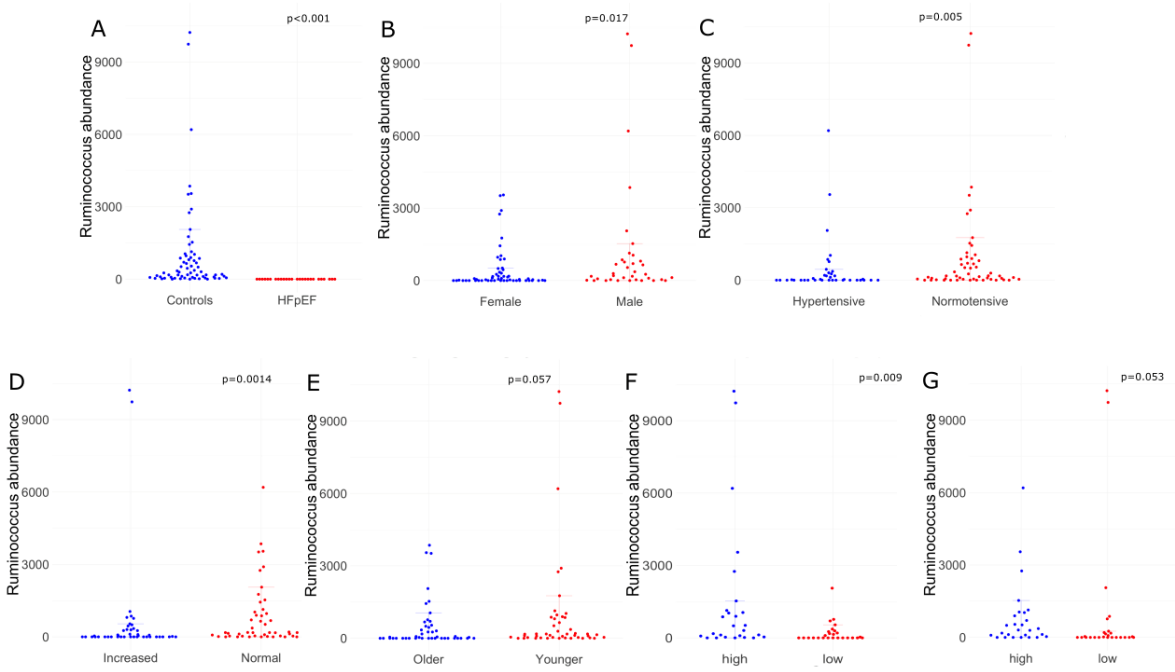
HFpEF, heart failure with preserved ejection fraction.

Figure S5. Sparse partial least squares discriminant analysis confirming differences in ß diversity and highlighting *Ruminococcus* as a driver for these differences.



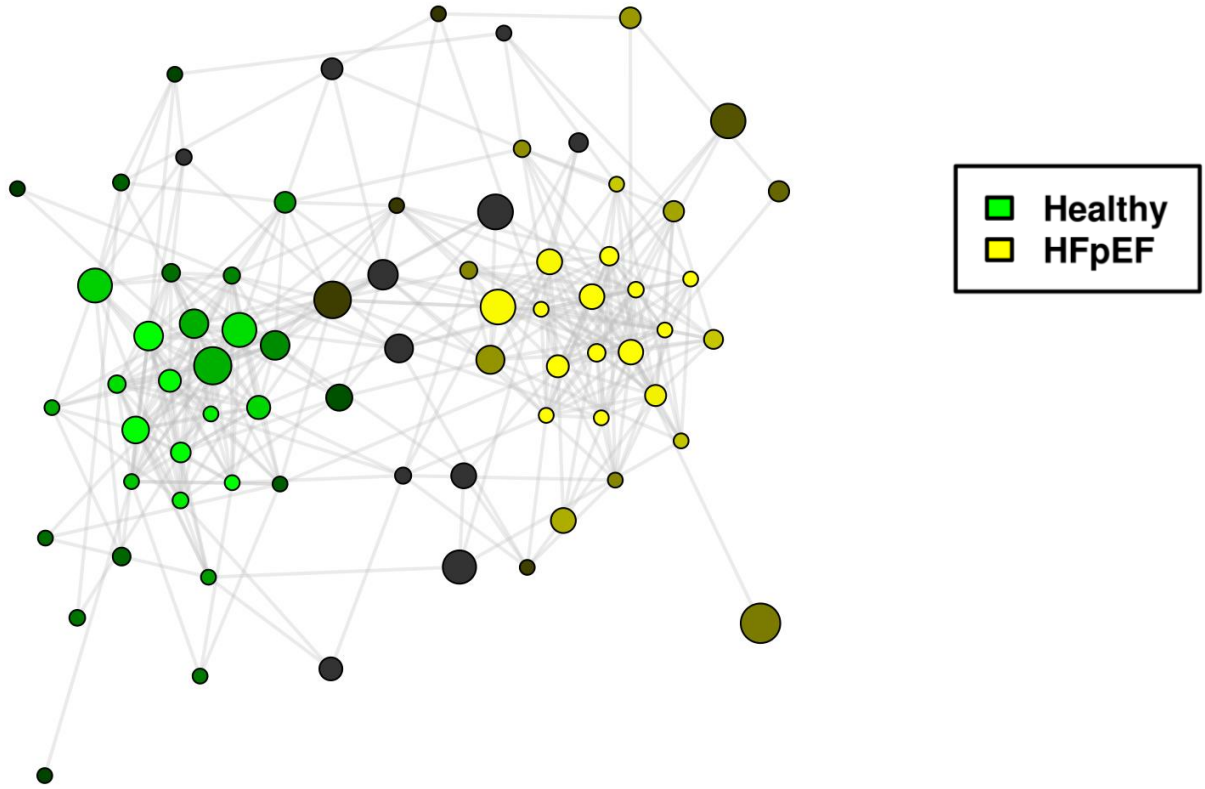
(A), Sparse Partial Least Squares Discriminant Analysis (sPLS-DA) validating the principal coordinate analysis plot findings in Figure 1. (B), Breakdown of the contribution of each bacterium to the differences observed in the sPLS-DA, supporting that *Ruminococcus* has an important contribution to the differences between groups.

Figure S6. The relationship between Ruminococcus abundance and HFpEF vs. controls, along with possible confounders.



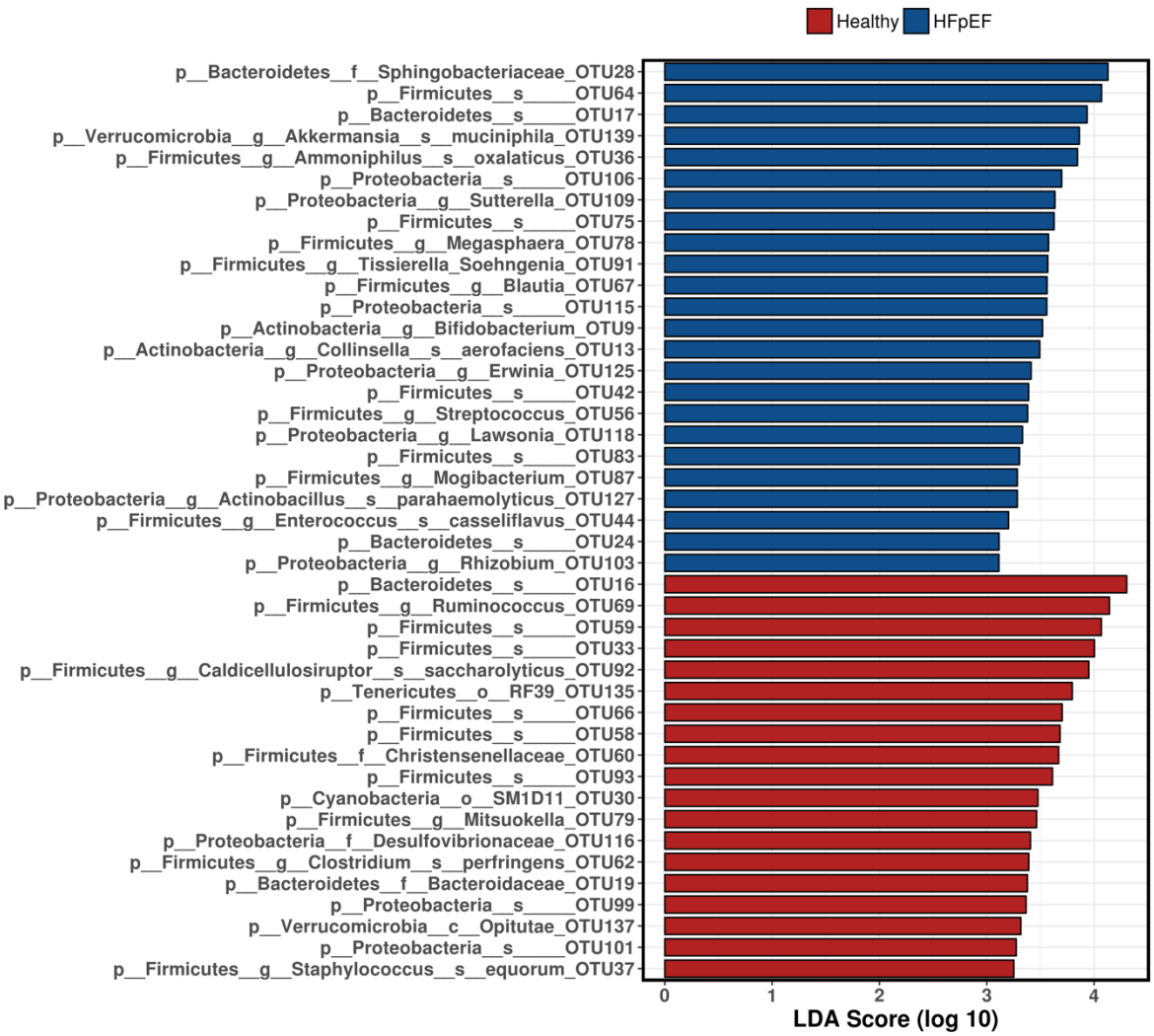
Ruminococcus abundance according to: A, HFpEF patients vs. controls; B, sex; C, presence of hypertension; D, BMI category; E, age category; F, fiber category; G, dietary score. Categories were determined according to the median value given data were not normally distributed.

Figure S7. Network analysis of co-occurring and mutually exclusive bacteria showing that communities of bacteria in HFpEF or healthy controls are different.



Each node represents a taxon, and the node size is relative to the taxa abundance.

Figure S8. Linear discriminant analysis Effect size for HFpEF patients and controls at the OTU level.



HFpEF, heart failure with preserved ejection fraction; LDA, linear discriminant analysis; OTU, observed taxonomic unit.