

Microbiome changes in patients with chronic heart failure with preserved ejection fraction correlate with fibrosis markers: Description of a Russian cohort

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Microbiome changes in patients with chronic heart failure with preserved ejection fraction correlate with fibrosis markers: Description of a Russian cohort

Short title: Microbiome changes and fibrosis development in CHF

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ABSTRACT

Background

Chronic heart failure (CHF) affects approximately 26 million people worldwide. Nearly half of CHF patients develop heart failure with preserved ejection fraction (HFpEF), which is associated with myocardial hypertrophy and fibrosis. Although chronic inflammation was suggested as a critical factor contributing to fibrosis development, a new hypothesis of CHF pathogenesis suggested that altered gut microbiota contributes to leaky gut phenotype development and promotes a systemic inflammatory state. CHF patients have an altered gut microbiome. However, the effect of gut microbiota on fibrosis development in HFpEF patients is not yet known. Thus, this clinical study involving HFpEF patients (n = 47) and healthy volunteers (n = 43) intended to identify the correlations between microbiota changes and fibrosis markers in HFpEF patients.

Methods

We used 16S rRNA metagenomic sequencing to identify the microbiota changes in HFpEF patients. Myocardial fibrosis was quantified using T1 myocardial mapping by using cardiac magnetic resonance. We also assessed the levels of microbial metabolites—trimethylamin N-oxide (TMAO) and short-chain fatty acids (SCFAs)—and measured bloodstream miRNAs and cytokines. The gut microbiome functions were simulated using PICRUSt algorithm.

Results

The gut microbial communities of HFpEF patients were markedly different from those of healthy individuals. The abundance of *Faecalibacterium*, *Prevotella*, and

Pseudomonas was significantly decreased, whereas that of *Lachnospirillum*, *Blautia*, *Haemophilus*, *Dorea*, *Peptococcus*, and *Tyzzrella* was increased in HFpEF patients. These changes could have affected TMAO metabolism and SCFA production: TMAO and hydroxypyruvate levels were significantly higher, whereas isovaleric, methylbutyric, and propionic acids were significantly lower in HFpEF patients than in healthy individuals. The simulation with PICRUST revealed that genes responsible for starch fermentation, SCFA production, and secondary bile acid metabolism were downregulated. Correlation analysis identified the involvement of microbiota changes and miRNAs 183-3p and 193b-3p.

Conclusions

Gut microbiome composition shifts in HFpEF patients impair biochemical functions, increase TMAO production, and decrease SCFA biosynthesis. The significant decrease in *Faecalibacterium* could have the most prominent effect on the host physiology. However, this needs to be determined by conducting experiments on animal models, because the mechanism by which the microbiota is associated with cardiac fibrosis development is not yet known.

Key Words: Chronic heart failure, myocardial hypertrophy, fibrosis, microbial metabolites, gut microbiome composition

Clinical Perspective

WHAT IS NEW?

- Patients with heart failure with preserved ejection fraction have depleted microbiome with impaired biochemical function.
- The most prominent effect on the host physiology could be caused by the significant decrease in species of the genus *Faecalibacterium*

WHAT ARE THE CLINICAL IMPLICATIONS?

- Our data support the previously suggested approach of microbiome treatment as one of the possible targets for the development of new therapeutics for patients with heart failure with preserved ejection fraction

Chronic heart failure (CHF) is a pathological condition characterized by ventricle remodeling and impaired heart function.¹ It affects approximately 26 million people worldwide, and the 5-year mortality rate after CHF diagnosis remains at approximately 50 %.^{2,3} Although the number of CHF patients has been estimated to increase between 2012 and 2030, there is still no effective treatment available. Considering that CHF care is extremely costly, new therapeutic approaches are urgently needed.⁴

CHF has two distinct phenotypes: heart failure (HF) with reduced ejection fraction and HF with preserved ejection fraction (HFpEF).⁵ These two phenotypes have completely different natural history, pathophysiological mechanisms, and underlying molecular processes^{5,6}. Clinical trials have thus far focused solely on HFpEF. Comorbidities such as arterial hypertension, diabetes mellitus, and obesity, which cause increased left ventricular load, are commonly observed in patients with HFpEF.^{7,8} Prolonged ventricular overload generates mechanic stimuli that together with neurohormonal signals promote structural protein production in cardiomyocytes, hypertrophic growth, and ventricle remodeling.¹ Myocardial hypertrophy is accompanied by fibrosis that develops as an adaptation to increased cardiomyocyte stiffness.^{9,10} Myofibroblasts are predominantly responsible for cardiac fibrosis.^{11,12} However, the origin of the cell types responsible for fibrosis development remains unknown; nonetheless, under hypertrophy, quiescent fibroblasts have been shown to be replaced by proliferative fibroblasts, which transform the myocardial matrix to abnormal structures.^{13,14} The emergence of pathological fibroblasts is governed by growth factors and inflammatory cytokines.^{15–18}

A recent study showed that the gut microbial community plays a pivotal role in CHF pathogenesis.¹⁹ A new hypothesis of CHF pathogenesis posits that the leaky gut phenotype

leads to the translocation of bacterial lipopolysaccharides to the bloodstream and promotes the systemic inflammatory state.^{20,21} Furthermore, the gut microbial community has been shown to produce numerous metabolites such as short-chain fatty acids (SCFAs), trimethylamine N-oxide (TMAO), and secondary bile acids, which are important metabolic regulators.²² Several clinical studies have identified differences in the microbiome composition of CHF patients and healthy individuals.^{23–25} However, how these microbiota changes affect cardiac fibrosis is not yet known.²⁶ To address these issues, we conducted a clinical trial involving HFpEF patients to identify the changes in the gut microbiome composition and the correlation of fibrosis markers with the microbial taxa.

METHODS

Data Availability

Source data are available at <https://github.com/NovoselovAL/CHF>. More data are available upon reasonable request.

Study Cohort

The present clinical trial focused on HFpEF; confounding effects of other diseases were avoided by including only HFpEF patients in the study cohort. HFpEF patients (n = 47) enrolled in this trial were in the age range of 18 to 80 years and had CHF symptoms, left ventricular ejection fraction (LVEF) of $\geq 50\%$, mitral inflow to annulus ratio (E/e') of ≥ 13 , and N-terminal probrain natriuretic peptide level of > 125 pg/ml. In addition, myocardial fibrosis was assessed using T1 myocardial mapping with cardiac magnetic resonance. The control group (n = 43) consisted of healthy individuals without CHF symptoms, in whom echocardiography with tissue Doppler study confirmed LVEF of $\geq 50\%$ and E/e' of < 8 . The following were the

exclusion criteria: class II obesity (BMI, ≥ 35 kg/m²), chronic kidney disease (GFR, < 30 ml/min/1.73 m² estimated using CKD-EPI), diabetes mellitus, smoking, history of myocardial infarction and allergic reaction to gadolinium contrast agents, chronic obstructive pulmonary disease, asthma, 2-week history of infection, cardiomyopathy, constrictive pericarditis, heart defect (including congenital heart conditions), autoimmune diseases, inflammatory bowel disease, cancer, 3-month history of probiotics or systemic glucocorticoid use, and pregnancy. The anthropometric parameters of the study groups are shown in Supplementary materials, S5. The following biomaterial was collected from study participants for microbiome research: blood samples (n = 90) and stool samples (n = 90). Stool samples were collected at the homes of participants. Any perturbation of gut microbiota was avoided by preserving the stool samples in care medium, the medium composition will be specify somewhere else. All patients read and signed the informed consent form. Ethics committee of each participating institute approved the study protocol. The study was conducted by the Federal State Institution “National Medical Research Center for Therapy and Preventive Medicine” of the Ministry of Healthcare of the Russian Federation in collaboration with the Center for Strategic Planning and Management of Medical and Biological Health Risks of the The Federal Medical-Biological Agency (FMBA). The study was approved by the Center for Strategic Planning and Management of Medical and Biological Health Risks institutional review board.

DNA Extraction, Library Preparation, and 16S rRNA Gene Sequencing

Service company “Genotek” (Russian Federation) extracted DNA from the fecal samples, prepared libraries, and conducted sequencing. In brief, DNA was extracted from the stool samples and subjected to 16S rRNA gene fragment amplification. Sequencing was conducted on an Illumina MiSeq system by using MiSeq Reagent Kit V2 Nano (PE 250), according to manufacturer’s protocol.

Bioinformatic Analysis

We applied the in-house automated pipeline for data processing. Quality check was conducted using FASTQC and MULTIQC tools. Low-quality reads were removed, and primers were trimmed using QIIME2 cutadapt. For chimera checking and amplicon sequence variant (ASV) picking, the algorithm QIIME2 DADA2 was used with the reference database HITdb v1.00.

The biochemical functions of gut microbiota were predicted by simulating metagenomes by using the PICRUST2- QIIME2 plug in.

Cytokine Measurement

The concentrations of TNF α , TGF- β 1, PICP, ST2, IL-6, IL-1 β , PIIINP, and GDF15 were determined using reagents from Cloud-Clone Inc. (USA), by using a sandwich enzyme-linked immunosorbent assay, according to manufacturer's protocol.

SCFA Measurement

Homogenized feces (3 g) were mixed with 30 ml of chloroform:methanol (2:1) solution. After the sample was dispersed in solution, 3.3 ml of deionized H₂O was added, and the mixture was vigorously shaken. The mixture was then placed into a funnel and left at +4°C for 3 h for phase separation. The clean lower phase was collected and dried on a rotary evaporator. The pellet was dissolved in 1 ml of hexane, followed by hydrolysis with 3 N methanolic-HCl (Supelco) at 90°C for 1 h. The samples were analyzed using GS-MS QP2010 Ultra (Shimadzu), under the following conditions: detector temperature, 200°C; detection range, 45 to 450 m/z. Chromatographic separation was achieved on capillary column MDN-5 (internal diameter, 30 μ m \times 0.25 mm; Supelco), with helium as the carrier gas. The flow rate was 35.6 cm/s (0.9 ml/min), and split ratio was 4:1.

RNA Extraction and cDNA Synthesis

Total RNA was extracted from the serum by using the TRIzol LS reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to manufacturer's protocol. In brief, 250 μ l of serum samples were homogenized in 750 μ l TRIzol reagent, and 200 μ l of chloroform was added to each sample. All samples were centrifuged at $12\ 000 \times g$ for 15 min at 4°C to separate the mixture. RNA in the aqueous phase was precipitated with 0.5 ml isopropyl alcohol and 1 μ l glycogen at room temperature for 10 min. After centrifugation, the pellets (i.e., RNA) were washed with 75 % ethanol. Air-dried RNA pellets were diluted in 100 μ l of RNA-free water.

Reverse transcription (RT) was performed using 5 μ l of total RNA in a total reaction volume of 15 μ l, by using the TaqMan™ MicroRNA Reverse Transcription Kit (Fermentas; Thermo Fisher Scientific, Inc.) and hsa-miR-specific RT primers. The sequences of the miRNAs assessed in this study—miR-1-3p, miR-21-3p, miR-29c-3p, miR-221-3p, miR-545-5p, miR-222-3p, miR-193b-3p, miR-193b-5p, miR-190a-3p, miR-183-3p, and miR-361-5p—are shown in Table III in the Data Supplement.

The RT reactions were conducted using the following program: 16°C for 30 min, followed by 42°C for 30 min, and termination by heating at 85°C for 5 min, by using 1 μ l MultiScribe™ Reverse Transcriptase.

Quantitative Polymerase Chain Reaction

Quantitative polymerase chain reaction (qPCR) was conducted using StepOnePlus (Applied Biosystems, USA) thermocycler. The qPCR contained 10 μ l PCR Master Mix 2 \times (TaqMan Universal PCR Master Mix with No AmpErase UNG), 1 μ l of the forward and reverse primers (TaqMan MicroRNA Assay, 20 \times), 7.67 μ l of RNase-free water, and 1.33 μ l of cDNA template

per reaction in a final volume of 20 μ l. The thermocycling conditions for qPCR were 95°C for 10 min, 40 cycles of 15 s at 94°C, and 60 s at 60°C.

Measurement of TMAO

Serum TMAO concentration was determined as previously described by Wu et al.²⁷ Serum samples were diluted in acetonitrile (1:10) and centrifuged at 16 000 g for 10 min (4°C), and supernatants were collected and analyzed using Faster-Performance Liquid Chromatography-Mass Spectrometry (UPLC-MS) method by using Agilent 6460C QQQ UPLC-MS/MS (Agilent, Santa Clara, California, USA). Chromatographic separation was performed using Waters ACQUITY BEH HILIC, 2 \times 100 mm column (Waters, USA). The flow rate of the mobile phase was 0.2 ml/min. The mobile phase consisted of (A) water containing a final concentration of 0.1 mmol/l formic acid and (B) acetonitrile containing a final concentration of 0.1 mmol/l formic acid (LC/MS grade). Gradient condition was 85 to 65 % B for 6 min, and the sample volume was 3 μ l. The MS detector conditions were as follows: MS ESI(+); selected reaction monitoring, 76 \rightarrow 59. An external standard C16:0, C18:0 fatty acids (Supelco, USA) was used for quantitative analysis.

Statistical Analysis

Data were analyzed using R software. Whether the relative abundances ASVs differed between the two groups was determined using *t*-test, with significance level of $P < 0.05$. Other metrics such as Shannon index, TMAO level, SCFA level, and miRNA content were evaluated using Mann–Whitney *U*-test. The associations between various parameters such as those between the relative abundances of ASV and SCFA levels, cytokine levels, and miRNA content were assessed using Kendall test with significance level of $P < 0.05$. Whether metagenomes differed between the two groups was evaluated using *t*-test, with significance level of $P < 0.05$. The source code of R scripts is available at <https://github.com/NovoselovAL/CHF>.

RESULTS

The sequencing of 90 samples resulted in a library of 10 42 844 high-quality reads, with mean sequencing depth of 11587 ± 3719 reads per sample. We identified 181 ASVs at the genus level. Microbial community richness was estimated using Shannon index and was found to be significantly low (Mann–Whitney *U*-test) in the samples of patients with CHF (Figure 1A). Similar results have been obtained in clinical studies in Germany, China, and Japan.^{23–25} Hierarchical clustering of the samples resulted in the grouping of the HF-pEF patients into several large groups. Each group had a unique pattern of microbiota (Figure 1B). Certain microbiota patterns have been associated with some disorder conditions.¹⁹ The microbiomes of patients with CHF were markedly different from those of healthy individuals. We found that the levels of *Faecalibacterium*, *Prevotella*, and *Pseudomonas* were significantly reduced (*t*-test) in the microbiome of patients with CHF (Figure 1C and 1D). Metagenomic research of human gut microbiomes has identified *Faecalibacterium* as the common member of the healthy gut microbiota, and its abundance was suggested as a hallmark for a healthy state.^{28,29} We found that the levels of *Lachnospirillum*, *Blautia*, *Haemophilus*, *Dorea*, *Peptococcus*, *Tyzzerella*, and *Peptostreptococcus* unclassified ASV2 had statistically significantly increased in patients with CHF (Figure 1C). The increase of *Lachnospirillum* and Peptostreptococcaceae species has been shown to be associated with several disorders such as acquired immune deficiency and cardiovascular diseases, respectively.^{26,30} Clinical studies have shown that certain bacterial species such as those belonging to Peptostreptococcaceae and *Faecalibacterium* are associated with the production of several critical microbial metabolites, for example, TMAO and SCFAs, and the abundance of these bacteria decreased in patients with CHF.^{25,31} Our results are in good agreement with those of previous studies. Considering that the changes in the abundance of

differential bacterial genera could lead to changes in the levels of SCFAs and TMAO, we measured their levels in the samples from the two groups. The following SCFAs were measured: butyric acid, hydroxide pyruvate, isobutyric acid, isovaleric acid, methylbutyric acid, propionic acid, and valeric acid. Patients with CHF had significantly high level of hydroxide pyruvate (Mann–Whitney *U*-test) and significantly low level of isovaleric, methylbutyric, and propionic acids (Figure 2A). Experiments on animal models have shown that propionic acid has antiinflammatory effects, and decrease of propionic acid in patients with CHF could be associated with chronic inflammation.³² The level of TMAO was significantly high in patients with HF-pEF (Figure 2B). High TMAO levels have been shown to be associated with chronic inflammation.³³ Therefore, we tested the level of cytokines. We detected significant (Mann–Whitney *U*-test) decrease in TNF- α and TGF- β 1 and increase in PICP (Figure 2C) in the serum of patients with HF-pEF. Furthermore, the level of TNF- α decreased, but not of IL-6 (Figure 2C). This difference in cytokine profile of the patients could be attributed to the difference in their treatment medications having antiinflammatory properties, such as aspirin or atorvastatin (Table II in the Data Supplement). The gut microbiota is known to influence the homeostasis of other organs through the production of fatty acids. For example, some studies have shown that certain SCFAs affect the expression of miRNAs.³⁴ Thus, we tested the levels of miRNAs in the plasma. Several clinical studies have revealed that the expression of miRNAs 221-3p and 361-5p significantly decreased in patients with CHF, and these miRNAs were suggested as the marker for CHF.³⁵ In this study, we detected statistically significant (Mann–Whitney *U*-test) decrease in the level of miRNAs 361-5p and 193b-3p in patients with HF-pEF and statistically significant increase in miRNA 183-3p (Figure 2D).

The changes in the composition of gut microbial community could interfere with the microbial biochemical functions and affect the fermentation of SCFAs and TMAO.³⁶ We run the

PICRUSt2 algorithm on our data to estimate the effect of HF-pEF on the gut microbiome biochemical capacity of patients. PICRUSt2 predicted the abundances for 10 500 genes in the gut metagenomes of patients with HF-pEF and healthy individuals. The comparison of metagenomes of healthy individuals and those of patients with HF-pEF revealed 365 downregulated and 75 upregulated genes (Figure I in the Data Supplement). Surprisingly, no significant difference was found in the level of genes responsible for TMAO production (Table I in the Data Supplement). We found statistically significant (*t*-test) decrease in certain genes responsible for starch, fatty acid, and secondary bile acid metabolism (Figure 3A). Decrease in the genes *baiI*, *baiF*, and *baiA* could have the most prominent impact on metabolism since these genes encode enzymes that play the most critical roles in the secondary bile acid biosynthesis pathway (Figure 3B). The effect of gut microbiome on the gut–heart axis was reported to be mediated via secondary bile acids, SCFAs, and the release of miRNAs into the bloodstream.³⁶ We tested the hypothesis whether the statistically significant difference in the level of certain cytokines, microbial metabolites (SCFAs and TMAO), and miRNA expression could be responsible for the gut microbiome changes. For this, we conducted correlation analysis (Kendall test) to identify statistically significant correlations between certain bacterial genera and cytokines, microbial metabolites, and miRNAs. We calculated the correlation coefficients between bacterial genera and changes in the microbial metabolites. We found that the increase in *Blautia* had statistically significant (*t*-test) positive correlation with the increase in hydroxide pyruvate. Decrease of *Pseudomonas* had a negative correlation with the decrease in isovaleric acid. Increase of *Dorea* had a positive correlation with valeric acid, which was used as a constant (Figure II in the Data Supplement). We found statistically significant correlations between increase in hydroxide pyruvate and bacterial genera *Clostridium* ASV1 and *Clostridiales* unclassified ASV2. The decrease in isovaleric acid level was associated with the genus *Ruminococcus* (Figure 4A). Our data indicated that significant changes in the

microbiome are associated with significant changes in SCFA levels in the gut. These experimental data are in good agreement with previously published data.^{19,23} We calculated the correlation coefficients between bacterial genera and cytokine level changes. The differential decrease in bacterial genus *Pseudomonas* had a strong positive correlation with the decrease in TGF β 1 in patients with CHF (Figure III in the Data Supplement). We found strong statistically significant negative correlation between the decrease of *Faecalibacterium* and increase of PICP. We also found strong positive correlation between the decrease of TGF- β and Lachnospiraceae unclassified ASV1 (Figure 4B). Bacteria of Lachnospiraceae have been suggested as a risk factor for cardiovascular disease.³⁷

We found strong statistically significant correlation between the abundances of different bacterial genera and changes in the level of certain miRNAs. Increase in *Dorea* had negative correlation with the increase of 193b-3p miRNA in patients with HF-pEF. Increase of *Tyzzerella* had positive correlation with the decrease of 183-3p miRNA in patients with HF-pEF (Figure IV in the Data Supplement). Altered level of miRNA 183-3p and 193b-3p is a diagnostic marker for HF-pEF.³⁸ Decrease of the bacterial genus *Prevotella* had a positive association with miRNA 1-3p and miRNA 221-3p (Figure 4C). This correlation is consistent with the results of previously published clinical studies, in which the anomalous level of *Prevotella* and miRNA 221-3p was shown to be associated with CHF.³⁹

DISCUSSION

Pressure-overload hypertrophy (POH) is typically considered as a cause of hypertrophic growth and myocardial fibrosis.^{10,40} The molecular mechanisms underlying myocardial fibrosis are yet not known; however, impaired fibroblast transdifferentiation to pathological hyperproliferative fibroblasts has been commonly observed in animal models.^{15,16} Pathological fibroblasts are

apoptosis resistant and rapidly replace normal stroma to the pathological matrix, resulting in myocardial stiffness and impaired relaxation.^{13,14}

Clinical studies indicate that impaired matrix turnover is reflected by the level of the procollagen type I carboxy-terminal propeptide (PICP) and has a strong correlation with fibrosis.¹⁰ The serum of HFpEF patients had an increased level of PICP.⁴¹ Our findings are in line with previously published data, which indicated high level of PICP in HFpEF patients (Figure 2C). We identified correlations between several bacterial ASVs and fibrosis markers (Table IV in the Data Supplement). Recently, the association of chronic inflammation with HFpEF pathogenesis has been postulated.⁴² Clinical studies indicate that patients with CHF have elevated levels of proinflammatory cytokines, particularly TNF- α and IL-6.⁴³ In our Russian cohort, TNF- α and IL-6 were not elevated (Figure 2C), probably because the patients had received treatment for HF-pEF. Inflammatory mediators play an important role in LV remodeling, including myocyte hypertrophy and fibrosis.⁴⁴ The activation of cardiac immune response is mediated through pathogen-associated molecular pattern (PAMP) or damage-associated molecular pattern (DAMP) receptors, which increase in response to heart tissue injury. PAMP receptors recognize both endogenous signals released by dying or injured myocardial cells, or exogenous signals, which are molecules derived from the gut microbiota.⁴⁵ The bacteria-derived pattern of lipopolysaccharides has been proposed as one of the causative agents promoting chronic inflammation.⁴⁶ Although the a causative link between gut microbiota and CHF remains illusive, numerous clinical studies have found alterations of gut microbiome in CHF patients.^{19,21,23-25} Published data indicate that microbial communities of CHF patients have depleted species richness.²³⁻²⁵ Our Russian cohort of HFpEF patients (Figure 1A) showed these common features with other studied cohorts. Several metabolites produced by the gut microbiota have been linked to CHF.¹⁹

We observed significantly low level of isovaleric, methylbutyric, and propionic acids in the gut of HFpEF patients (Figure 2A); however, whether this could contribute to CHF pathogenesis is not yet known. Recently, SCFAs have been mechanistically linked to hypertension, an important condition for CHF development.^{48,47} SCFAs alter blood pressure in the host via the FFAR3 and OR51E2 receptors.⁴⁷ Clinical studies have revealed a link between CHF and high level of TMAO in American and Norwegian cohorts.⁴⁸ Our observation is consistent with previously published data: TMAO was found to have significantly increased in the Russian cohort (Figure 2B). Experiments on animal models have shown that TMAO worsened cardiomyocyte contractility.⁴⁹ This finding suggests that TMAO may be one of the metabolites contributing to CHF. Host liver and gut microbiota produce TMAO, and evaluating the microbiome contribution to the net TMAO production is important.¹⁹ Analysis of 16S rRNA metagenomic data did not reveal any differences in bacterial genes responsible for TMAO metabolism (Table I in the Data Supplement); however, we found that the expression of certain genes responsible for secondary bile acid metabolism was significantly decreased (Figure 3A). Secondary bile acids are ligands of farnesoid-X-receptors, which mediate apoptosis in cardiomyocytes and contribute to myocardial injury.⁵⁰ The presence of fibrosis in HFpEF patients was also confirmed using magnetic resonance imaging studies (Table IV in the Data Supplement).

Taken together, our data suggest that HFpEF patients have depleted microbiome with impaired biochemical function. Increased level of TMAO and altered metabolism of secondary bile acids could be the environmental factors that interfere with intrinsic factors of CHF pathogenesis. The present data support the previously suggested approach of microbiome treatment as one of the possible targets for the development of new therapeutics for CHF.¹⁹

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Disclosures

None

SUPPLEMENTAL MATERIALS

Data Supplement Figures I–V

Data Supplement Tables I–IV

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Figure Legends

Figure 1

A, The gut microbiotas of heart failure with preserved ejection fraction (HF-pEF) patients share several common features. Patients with HF-pEF have low species diversity. **B**, The gut microbiotas of the HF-pEF patients clustered into several large groups. Grouping of the 28 most abundant bacterial genera is shown on the hit map. **C**, Changes in the amplicon sequence variant (ASV) abundances plotted on a volcano plot. Bacterial genera *Faecalibacterium*, *Prevotella* and *Pseudomonas* are less abundant, and *Lachnospirillum*, *Blautia*, *Haemophilus*, *Dorea*, *Peptococcus*, *Tyzzarella*, and Peptostreptococcaceae unclassified ASV2 are more abundant in patients with HF-pEF. **D**, The relative abundance of significantly different bacterial genera. *Faecalibacterium* and *Prevotella* are the most abundant differential bacterial species. * $p < 0.05$, *t*-test

Figure 2

A, The gut microbiotas of heart failure with preserved ejection fraction (HF-pEF) patients produce more hydroxide pyruvate and less of isovaleric, methylbutyric, and propionic acids. The levels of other short-chain fatty acids (SCFAs) did not differ between patients with HF-pEF and healthy individuals. **B**, The level of trimethylamin N-oxide (TMAO) was significantly high in HF-pEF patients. **C**, In HF-pEF patients, the levels of tumor necrosis factor (TNF)- α and transforming growth factor (TGF)- β 1 were decreased, and that of procollagen type 1 carboxy-terminal propeptide (PICP) was increased. The levels of other cytokines did not change significantly. **D**, In HF-pEF patients, the levels of miRNA 361-5p and 193b-3p decreased, and that of miRNA 183-3p increased. The levels of other miRNAs did not change significantly. * $p < 0.05$, Mann–Whitney *U*-test

Figure 3

The PICRUST2 annotation included 10 500 genes. **A**, We identified 440 genes that were differently expressed in the gut metagenomes of heart failure with preserved ejection fraction (HF-pEF) patients. Genes responsible for starch, fatty acid, and secondary bile acid metabolism were significantly decreased ($p < 0.05$, t -test) in HF-pEF patients. **B**, The *baiI*, *baiF*, and *baiA* genes responsible for the key steps in secondary bile acid biosynthesis, according to KEGG ontology.

Figure 4

A, Correlation analysis of bacterial genera and changes in microbial metabolites [short-chain fatty acids (SCFAs) and trimethylamin N-oxide (TMAO)] identified correlations between *Ruminococcus*, *Clostridium* ASV1, *Clostridiales* unclassified ASV2, and *Alistipes* and hydroxide pyruvate, isovalerianic acid, and TMAO. There were 15 common bacterial genera. **B**, Correlation analysis of bacterial genera and cytokines identified correlation between *Lachnospiraceae* unclassified ASV1, *Ruminococcus*, *Ruminococcus* unclassified ASV2, *Faecalibacterium*, *Clostridium* ASV1, *Bacteroides* ASV1, and *Alistipes* and GDF15, IL-1b, IL-6, PIIINP, ST2, TGF- β 1, and PICP. There were 15 common bacterial genera. **C**, Correlation analysis of bacterial genera and miRNA identified correlation between *Ruminococcus*, *Ruminococcus* unclassified ASV1, *Prevotella*, *Clostridiales* unclassified ASV2, and *Bacteroides* ASV1 and miRNA 1-3p, 190a-3p, 193b-3p, 193b-5p, 21-3p, 221-3p, 29c-3p, and 545-5p. There were 15 common bacterial genera. * $p < 0.05$, ** $p < 0.01$, Kendall test.

Figures

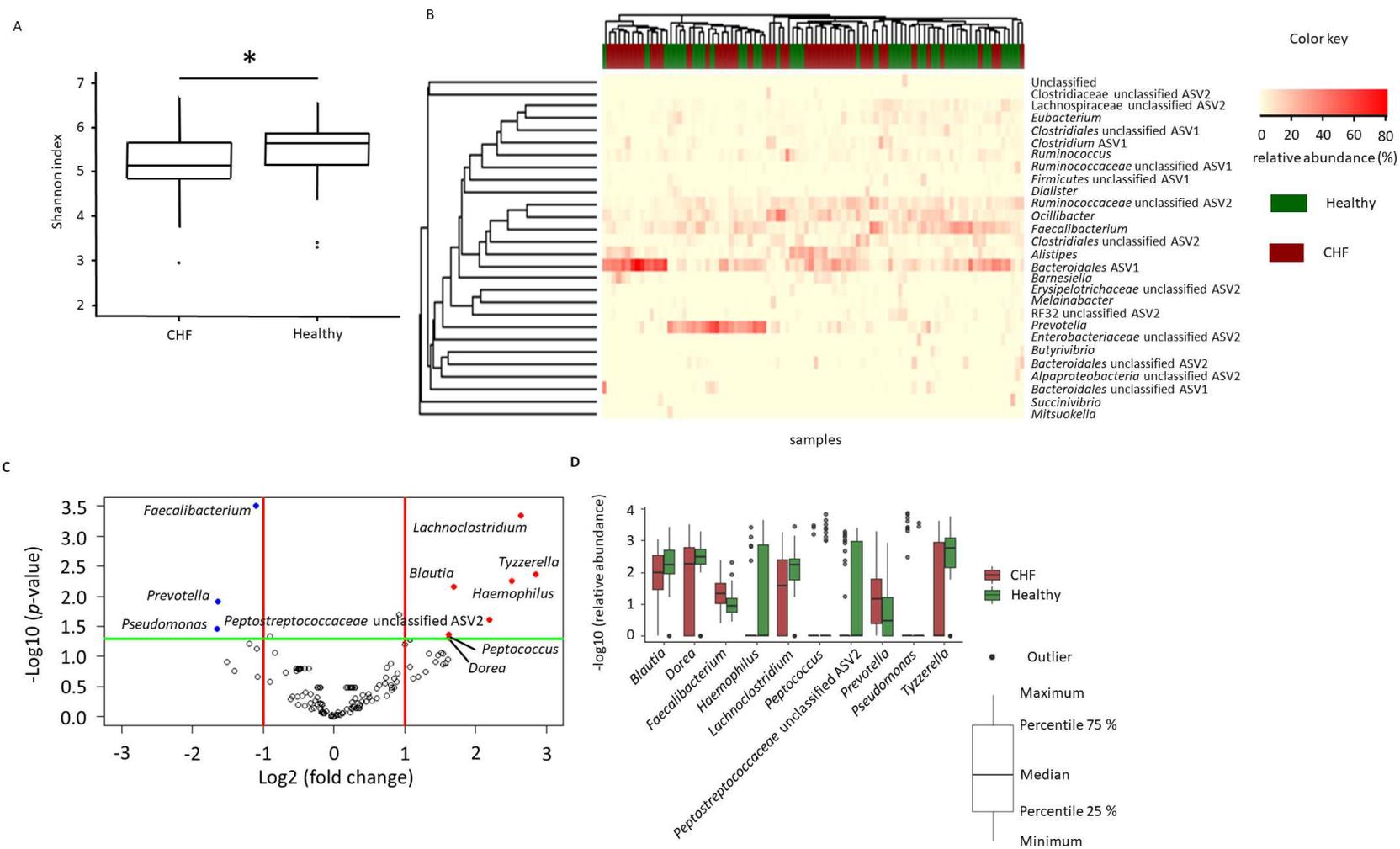


Figure 1

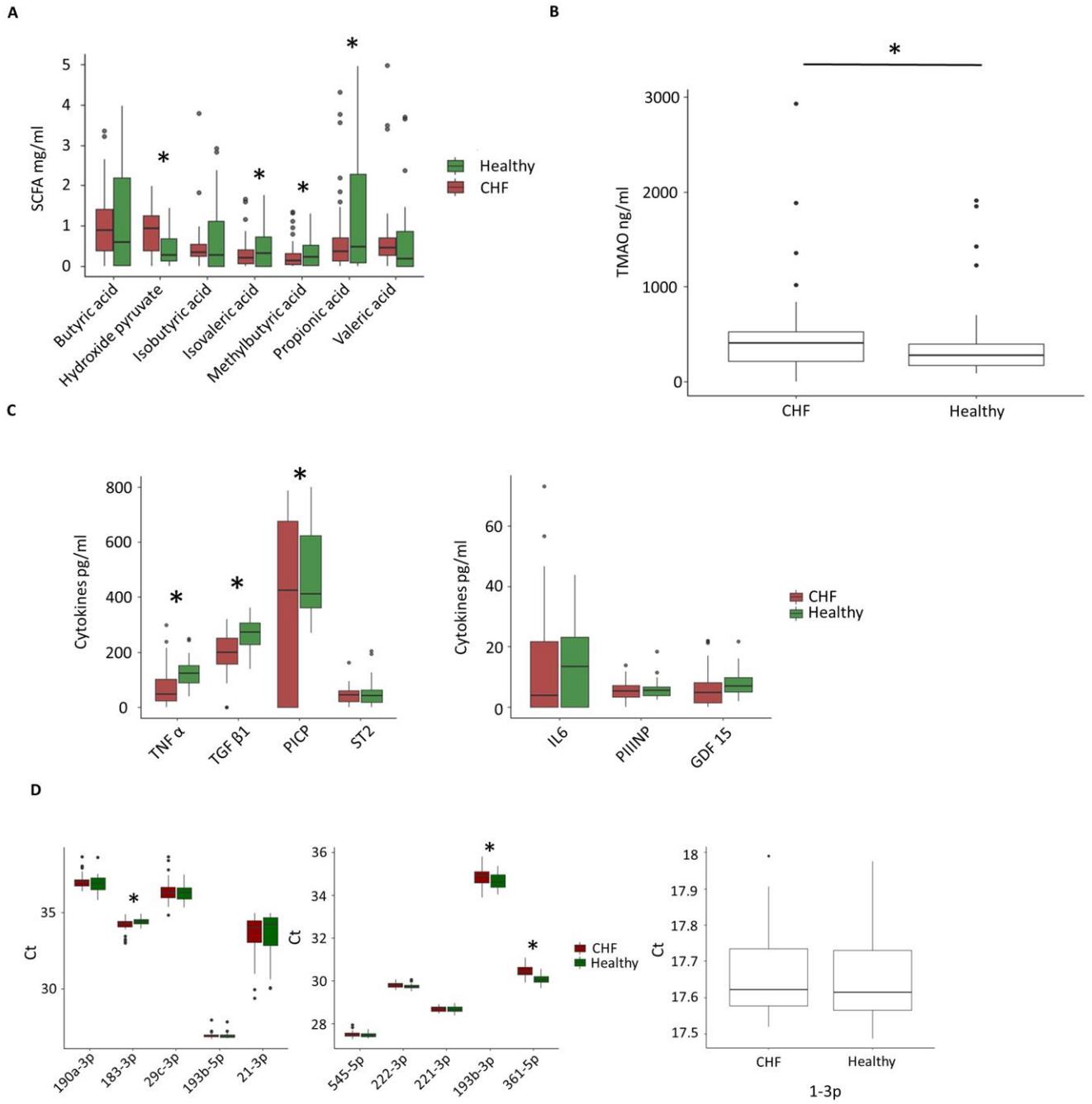


Figure 2

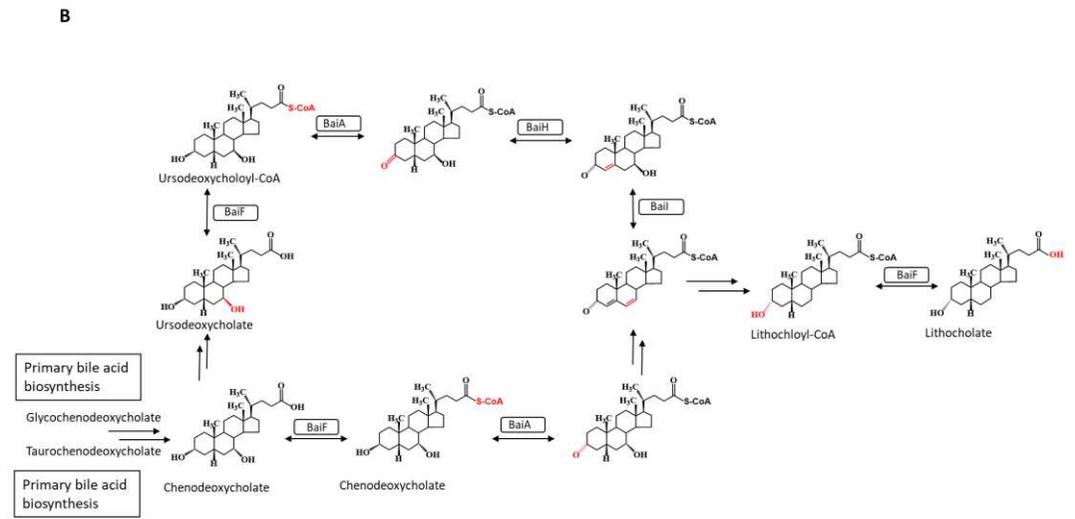
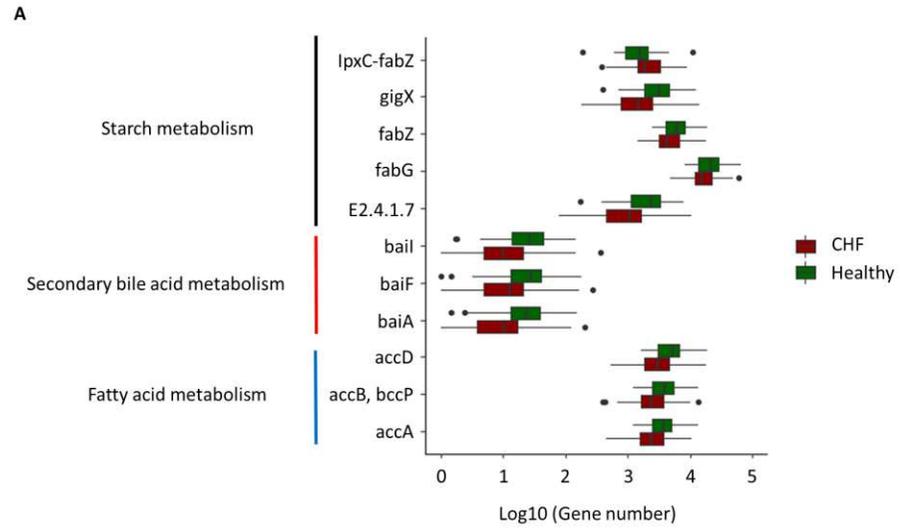


Figure 3

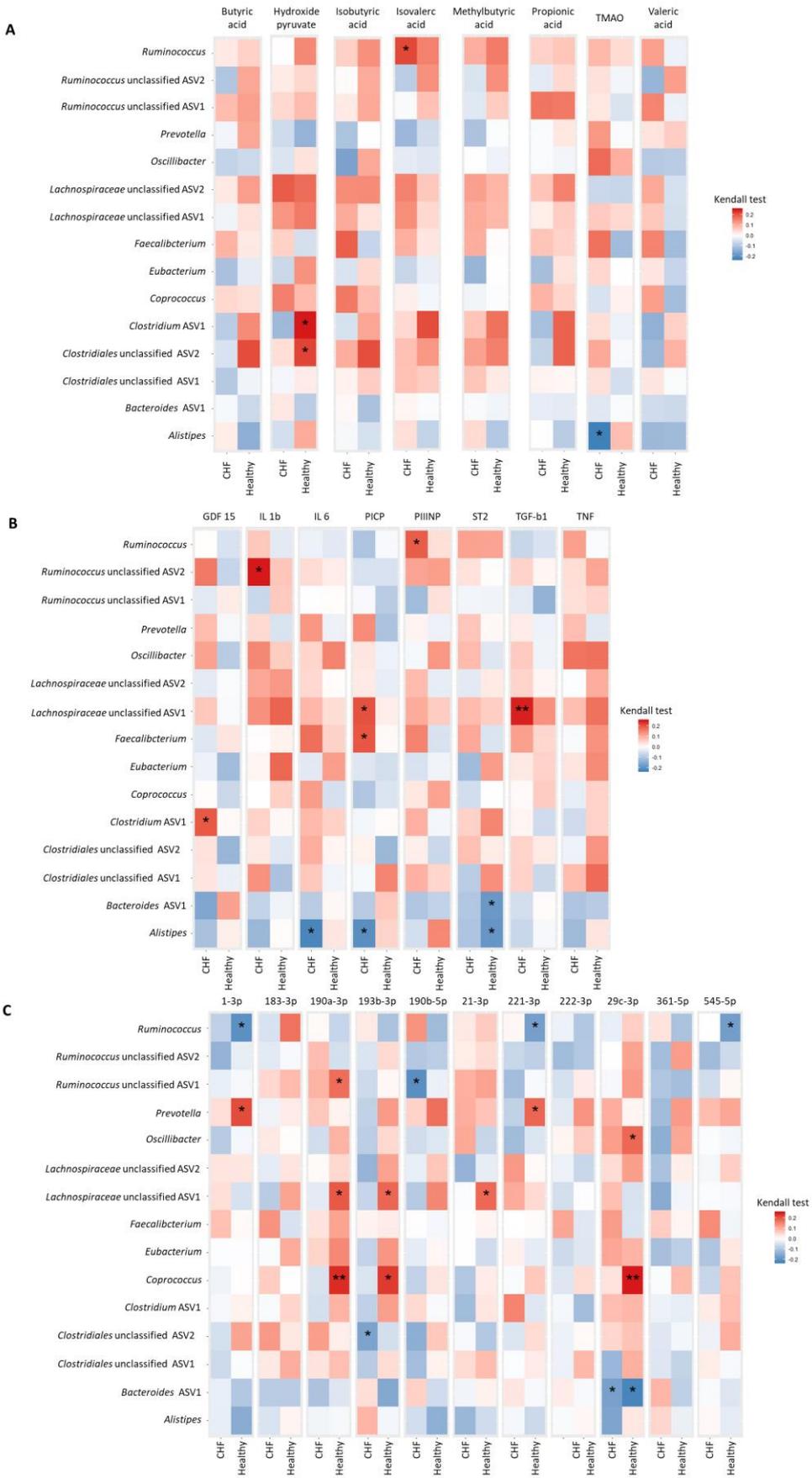


Figure 4

Figures

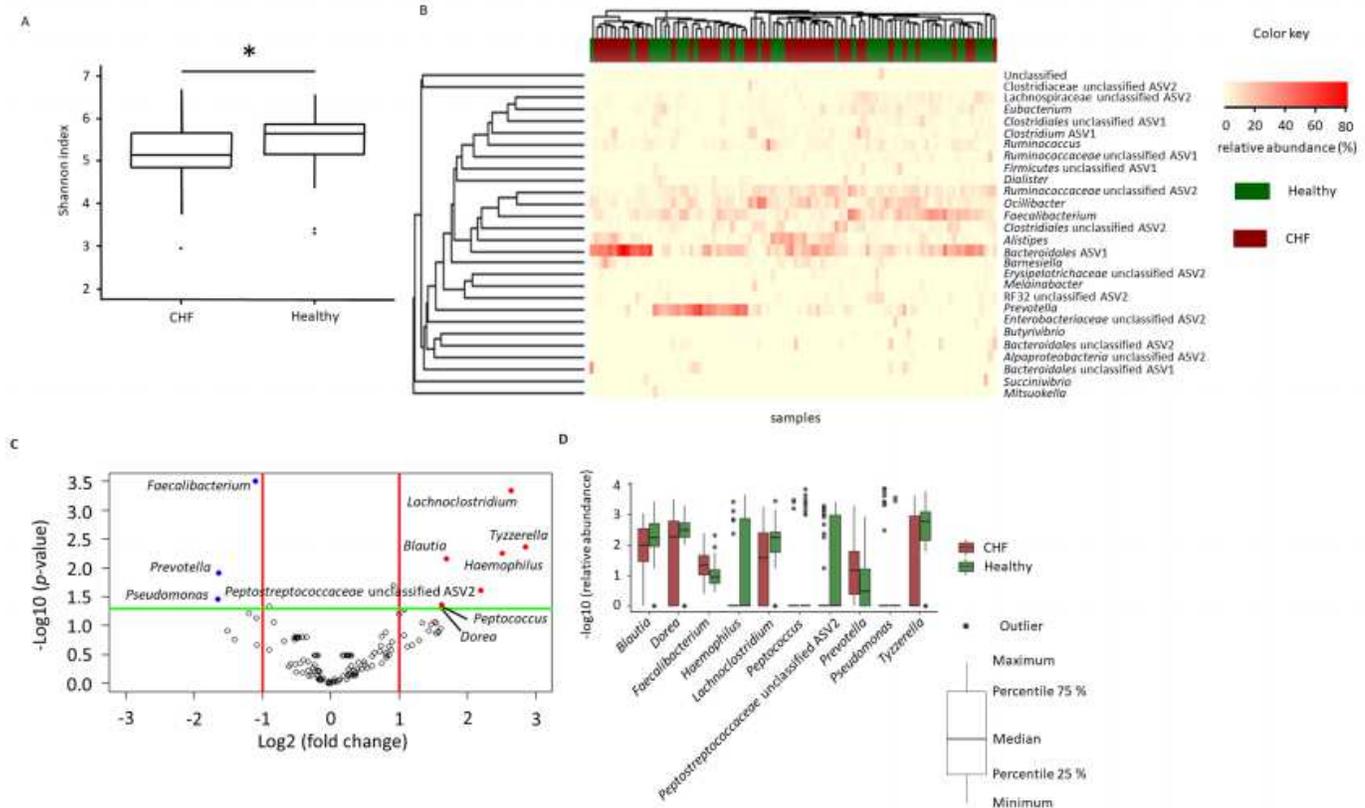


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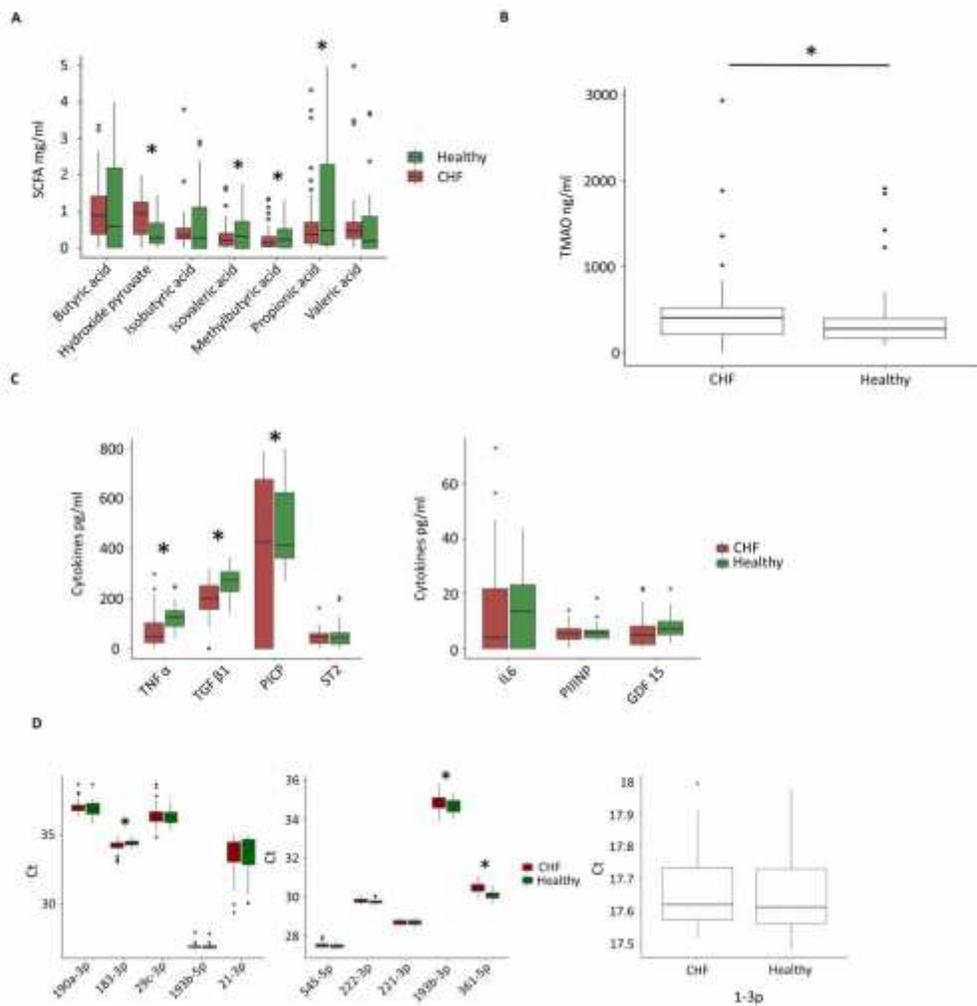


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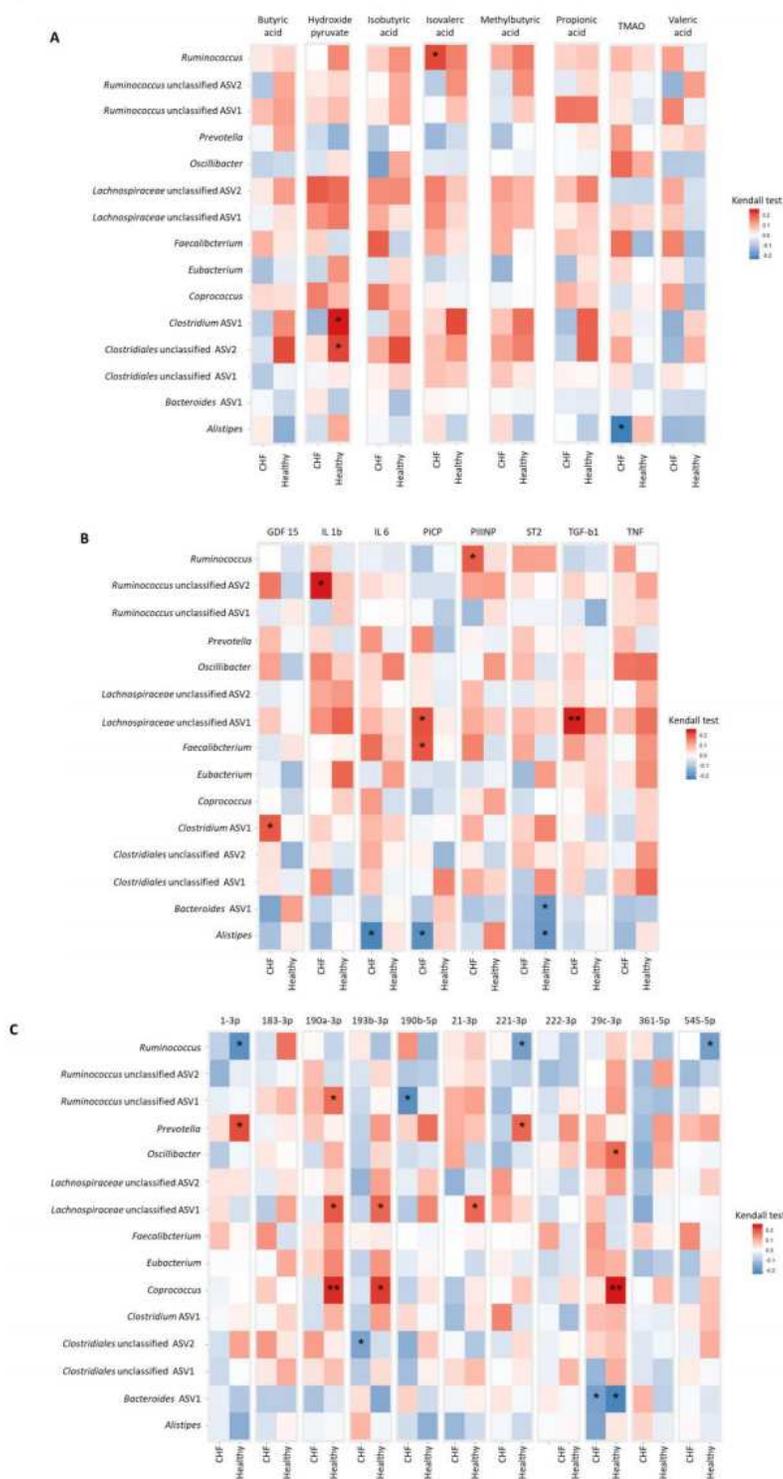


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A, Correlation analysis of bacterial genera and changes in microbial metabolites [short-chain fatty acids (SCFAs) and trimethylamin N-oxide (TMAO)] identified correlations between *Ruminococcus*, *Clostridium* ASV1, *Clostridiales* unclassified ASV2, and *Alistipes* and hydroxide pyruvate, isovaleric acid, and TMAO. There were 15 common bacterial genera. B, Correlation analysis of bacterial genera and cytokines identified correlation between *Lachnospiraceae* unclassified ASV1, *Ruminococcus*, *Ruminococcus*

unclassified ASV2, Faecalibacterium, Clostridium ASV1, Bacteroides ASV1, and Alistipes and GDF15, IL-1b, IL6, PIIINP, ST2, TGF- β 1, and PICP. There were 15 common bacterial genera. C, Correlation analysis of bacterial genera and miRNA identified correlation between Ruminococcus, Ruminococcus unclassified ASV1, Prevotella, Clostridiales unclassified ASV2, and Bacteroides ASV1 and miRNA 1-3p, 190a-3p, 193b-3p, 193b-5p, 21-3p, 221-3p, 29c-3p, and 545-5p. There were 15 common bacterial genera. * $p \leq 0.05$, ** $p \leq 0.01$, Kendall test.

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