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Microbial metabolites associated in stool and left ventricle of heart failure patients revealed by meta-analysis

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Heart Failure (HF) impacts approximately 64 million people globally. While overall incidence of HF is relatively stable across countries, the overall number of HF patients is increasing due to aging populations. Many articles examine the microbiome in HF, however, studies from humans have not been analyzed systematically. The aim of this meta-analysis is to bridge this gap by analyzing previously published data on human HF patients with untargeted metabolomics to understand whether microbially-mediated metabolites are consistently important for HF status. A systematic survey of the literature identified 708 articles discussing HF, the microbiome, and metabolomics. Of these, 82 were primary studies of HF patients, 61 studied human adults, 23 included an untargeted metabolomics measure, and 3 studies had data that was usable and publicly accessible. These studies include a GCMS study from stool, NMR of saliva and exhaled breath condensate, and LCMS from left ventricle of HF patients undergoing transplantation and unused donor hearts. Significant differences were observed from PCA between HF and controls for stool and left ventricle, but not saliva or EBC samples. OPLS-DA was conducted for stool and ventricle samples, and further revealed significant group differences. Univariate testing with FDR correction revealed 8 significant microbially-relevant metabolites ($p < 0.005$ after correction), most notably asparagine from left ventricle and 2-methylbutyryl carnitine from stool. Though there is much discussion of the microbiome in health outcomes in HF, there is limited research from human populations. Some microbial co-metabolites from both stool and heart were significantly associated with HF.

Keywords Heart failure, Metabolomics, Microbiome, Meta-analysis, Bioinformatics

Heart failure is a leading cause of death globally with rising incidence[1]. Heart failure is a disease in which the heart cannot successfully pump blood through the body, which can ultimately result in death. Though recent advances in novel therapies have increased survival for heart failure patients[2], the overall 3 year survival rate is still low[3]. Further, the improvements in therapies have increased the number of individuals living with chronic heart failure, which in turn increases the need for better therapies and prevention of heart failure alongside the increased demand from aging populations. One hopeful domain for new therapies has been the gut microbiome, as the microbes living in the gastrointestinal tract are thought to contribute towards disease etiology.

The human gut microbiome has been implicated in nearly every aspect of human health[4–6]. This is unsurprising as humans have evolved with microbes throughout our entire evolutionary history, which has allowed the human body to outsource certain key functions, like immune priming[7] or digestion[8], to these evolutionary partners. In the context of human health, microbiota, or the microbes living inside a person, present a potentially easily modifiable target for new disease therapies as the microbiota is functionally stable but malleable over the human lifespan[9]. For heart failure specifically, the metabolites produced by gut microbes can be protective in the context of adverse cardiovascular events, such as butyrate[10,11], or risk factors, such as Trimethylamine, or TMA[12].

As sequencing and metabolomics technology has advanced, much evidence has been generated from our group and others showing that the gut microbiome can indeed be a protective or risk factor for HF through

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modulation of metabolites[12–14], though the evidence from human patients has not yet been systematically combined. In part, it is difficult to combine results across different metabolomics studies, as different studies will use different analytical methods, different heart failure categories, and different bioinformatics methods when analyzing their data. Further, though this topic is widely discussed with many review papers published on the topic, few empirical studies have actually been conducted with human patients with heart failure, as heart failure is still a relatively complex clinical event that is much simpler to study in animal models than in people.

The goal of this study is to examine all the evidence of microbially-mediated metabolites in heart failure patients from previously published studies which implement untargeted metabolomic methods of any biospecimen type. While certain microbially relevant metabolites (such as short chain fatty acids) are difficult to capture from untargeted methods, it is entirely possible that many important bacterially-mediated metabolites are missed from the targeted methods used for the most well-documented microbial metabolites. This study will apply the same analytical methods to previously published data with the same filtering parameters, which will make understanding the relationship between microbially-mediated metabolites in heart failure patients across studies and sample types easier, and in turn generate a more holistic understanding of the relevance of the microbiota in heart failure.

Results

Systematic review

Of 708 papers identified by systematic review, 82 were studies of heart failure; 61 of those included human adults; 23 of those papers included an untargeted metabolomics measure; and only 3 of those 23 papers had publicly available data that was properly uploaded to a data repository (Supplemental Fig. 1). A total of 162 patient samples were included for analysis in this study (HF = 111, control = 51).

Principal component analysis and ortholog partial least squares discriminant analysis

PCA plots and significance testing revealed that metabolites from stool (D1) and left ventricle (D3) of heart failure patients are significantly different than metabolites from the same tissues of controls (PCA p value < 0.01, high OPLS-DA R2Y and Q2Y; Fig. 1/Table 1). While D2 EBC samples did not show significant group differences from PCA, potentially due to small sample size. D2 saliva samples had uneven group dispersion (within-group variance) and did not meet the assumptions for PCA.

OPLS-DA on D1 and D3 both revealed stable models, as indicated by high R2Y and Q2Y values, with high prediction (Q2Y) and low error (RMSEE). Overall, each model captured 22.9% and 31.2% of the variance in the data as indicated by R2X for D1 and D3, respectively. This, together with PCA results, indicates there are significant metabolite differences in the stool and left ventricle of heart failure patients compared to controls, but not in saliva or EBC.

Differential metabolite abundance analysis

Univariate testing with false discovery rate corrections show significant metabolites in D1 and D3 (Fig. 2), but no metabolites were significant in D2 saliva or EBC after FDR correction. Overall, a total of 66 metabolites were significant from univariate testing after FDR correction. Of these, 8 metabolites are either solely synthesized by bacteria or related to both bacterial and human metabolism (e.g. Asparagine from D1 stool). Two of these metabolites are related to ketone body synthesis through acetyl-CoA metabolism (Alpha-hydroxyisobutyric acid and 2-Methylbutyryl Carnitine), which is previously found to increase risk of thrombosis in a sick population[15]. Other bacterial metabolites like asparagine have similarly been implicated in cardiovascular health previously[16,17], though it is not clear if this asparagine is from bacterial or human metabolism. Only three metabolites were significantly differentially abundant in both stool and left ventricle samples of heart failure patients–serotonin, N-carbamoyl-aspartate, and thymidine (serotonin and N-carbamoyl-aspartate adjusted $p < 0.01$; thymidine adjusted $p < 0.05$; Table 2).

Pathway analysis revealed that the top 5 KEGG pathways implicated in heart failure are more relevant to human and not bacterial metabolism, so they are not discussed here (Table 3). Other metabolites significant but not relevant to microbial metabolism are not discussed in this paper, but included in the accompanying coding repo on GitHub so other researchers interested in heart failure can explore metabolites significant for this clinical population across measurement and sample types.

Discussion

The human microbiome has been widely discussed as important for cardiovascular health and recovery after adverse cardiac events, though actual human studies of patients with heart failure are limited. This is potentially unsurprising, as the integration of microbiome and metabolomics measures into clinical studies is a relatively recent transition in research. However, the proportion of review studies ($n = 98$) to empirically based studies ($n = 23$) is quite surprising. Nonetheless, three empirical research studies (out of 708 screened) were conducted that included a measure of untargeted metabolomics and have data properly deposited on public repositories. The objective of this study was to apply a unified data analysis approach to previously published data from heart failure patients in order to understand what microbially-relevant metabolites are consistently associated with heart failure.

We found that there were significant differences in metabolites from the stool and left ventricle of the heart from heart failure patients compared to controls, however, no significant differences from saliva or exhaled breath condensate (EBC) between HF and control. The study with saliva and EBC samples had a much smaller sample size ($N = 23$), so it is not clear if differences may exist in the saliva or EBC for heart failure patients in a larger sample. Nonetheless, of 66 significant metabolites identified from univariate testing with FDR

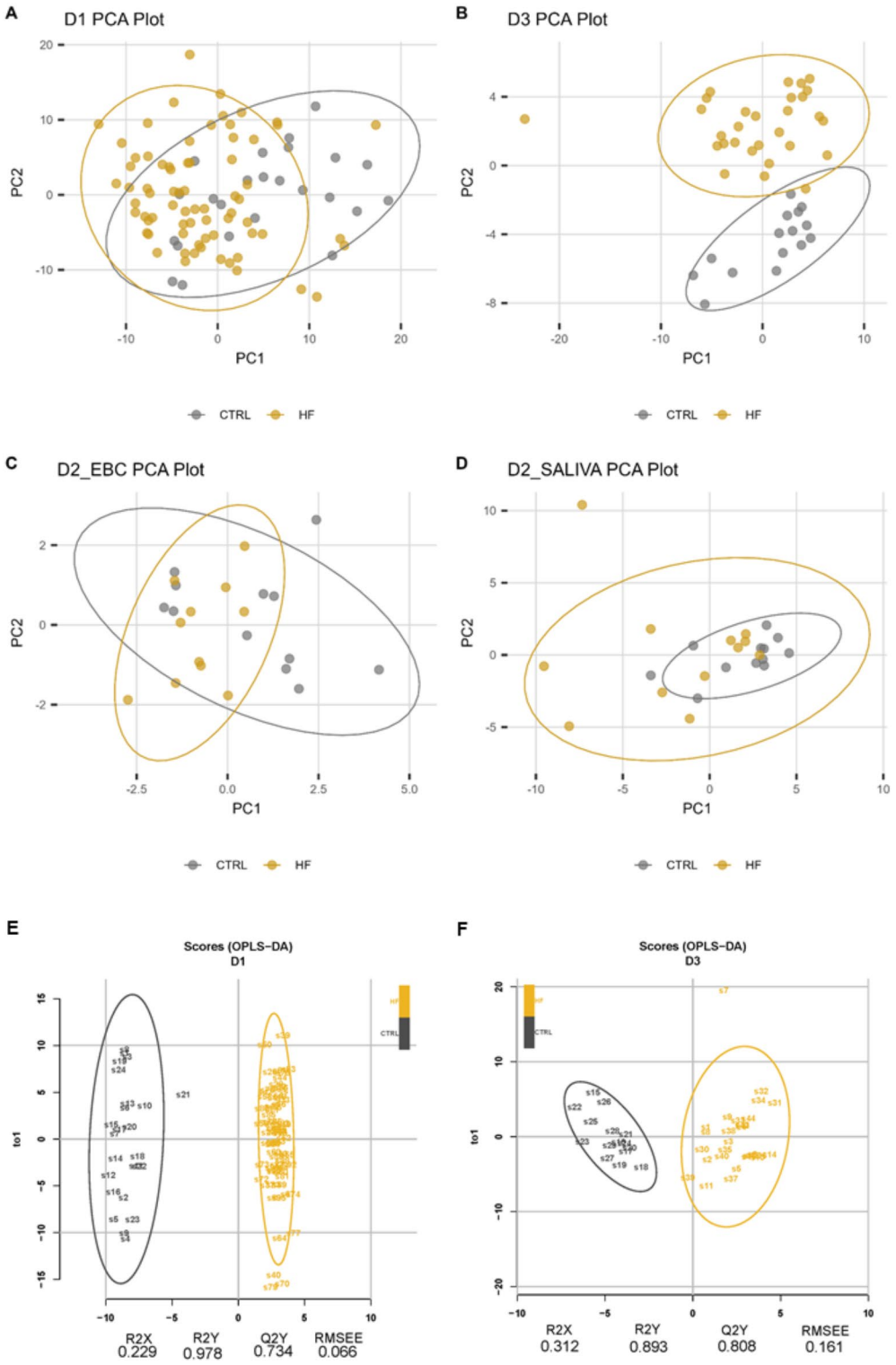

Fig. 1. Dimension reduction: PCA and OPLS-DA results. Principal component analysis for each study labeled by heart failure status. D1 stool (**A**) and D3 left ventricle (**B**) both display significant group differences as calculated by PCAtest(). D2 EBC (**C**) and saliva (**D**) do not display significant group differences, though PC1 is significant for D2 EBC (likely due to collinearity in metabolic pathways). D2 saliva PCA shows uneven group dispersion, so no further conclusions from D2 saliva PCA can be made. OPLS-DA plots for D1 (**E**) and D3 (**F**) show clear separation between heart failure conditions. R2X represents the fraction of variance explained by the metabolites, while R2Y and Q2Y represent the stability of the model. Q2Y is specifically the predictive performance of the model on all permutations of the model, with higher values indicating better model performance and stability across permutations. RMSEE is the root mean squared error of estimation where lower values show better model performance. OPLS-DA is not conducted for D2 samples as there are no significant group differences from the PCA analysis.

Study	*P* value	Number of significant axes	Percent variance explained by significant axes
D1	$P<0.001$	20	62.1%
D2_saliva	(assumptions violated)	3	66.6%
D2_EBC	$P<0.001$ (PC1 only)	1	34.1%
D3	$P<0.001$	5	52.9%

Table 1. PCA statistics. PCA revealed significant differences between group structure for D1 and D3 (observed psi and phi greater than null, p value < 0.01). While D2 EBC samples had a single significant principal component, this is likely due to confounding, such as from multicollinearity among metabolic pathways, rather than meaningful group differences, as significance was only in one dimension (PC1). Further, D2 saliva samples had uneven beta dispersion across groups (within-group variance), violating the PCA assumptions of multivariate homogeneity of group dispersions, so no conclusions of significance can be drawn from D2 PCA results. We are reporting the p-values here for the sake of replicability.

correction, 8 of these metabolites are potential bacterial metabolites–most are human and bacterial metabolites. Most of these metabolites have been previously implicated in cardiovascular health outside of the original studies the datasets came from (Alpha-hydroxyisobutyric acid[18,19]; 2-Methylbutyryl Carnitine[15]; Asparagine[20]; tetrahydrobiopterin[21]; 7-methylguanine[22]; Cadaverine[23]), though minimal mechanistic work has been published with any of these metabolites, particularly in the context of the human microbiome. KEGG pathway analysis revealed that human-specific pathways from significant metabolites are the most predominant, which is not surprising, but may emphasize the importance of thinking about the effect size of microbial influence on heart failure development or recovery.

Most original studies of the role of the microbiome in heart failure focus on the role of 3-indolepropionic acid, or IPA, or short chain fatty acids as as they are microbial metabolites with clear links to diet and cardiovascular health. However, these most discussed microbial metabolites require special experimental preparation, isotope labeling, or mass spectrometry settings due to their physical and biochemical properties, and are difficult to detect from untargetted metabolomic methods[24–26]. Little attention is paid to these other microbial metabolites that are more easily detectable by untargetted metabolomics. Future work trying to understand the influence of the microbiome on heart failure and cardiovascular health can follow up on these bacterial metabolites which have been repeatedly found to be up/downregulated in heart failure patients. Many of these metabolites are also implicated in metabolic syndrome[23,27,28]. There is strong comorbidity between heart failure and metabolic syndrome[29,30], though not all patients with heart failure have metabolic syndrome and vice versa. It will be increasingly important to understand the role of these significant metabolites in metabolic disease and subsequent adverse cardiovascular events, particularly in the context of the microbiome, as the microbiota in the gastrointestinal tract process dietary foods into metabolites in a way that may be relevant to the development or progression of metabolic syndrome[31,32]. As these microbially-mediated metabolites have been found to be significant for heart failure or cardiovascular events more broadly in multiple studies, it is worthwhile for future research to further the therapeutic potential or detrimental nature of these metabolites.

The most significant KEGG pathway from D1 stool samples was the biosynthesis of unsaturated fatty acids. Previous research of human stool has found that gut microbiomes cluster by saturated fatty acid and polyunsaturated fatty acids and are relevant to steatosis[33], and that dietary long chain unsaturated fatty acids can promote obesity in a microbe-dependent manner[34]. Nucleotide metabolism was the second most significant pathway for both stool and left ventricle samples, possibly due to a shift from aerobic to anaerobic glycolysis as previously reported in congestive heart failure patients[35]. cGMP-PKG signaling pathway and cAMP signaling pathway are related to ligands that bind PKG and PKA respectively, and significant for both stool and left ventricle samples. This significance is attributed almost entirely to adenosine and serotonin, of which adenosine is upregulated in the left ventricle of heart failure patients, and serotonin is downregulated stool and left ventricle of heart failure patients. These broad signaling pathways don't reveal much information beyond disrupted cell signaling without follow-up animal models, which may be an opportunity for future researchers.

One limitation of this study is the lack of control for sex or age. It is known that there can be age and sex differences in heart failure outcomes and relevant metabolites[36,37], but as two of the three studies did not include patient sex in their publicly available metadata file, and no study provided patient age, we are unable to control for it evenly in our analysis. All studies use age and sex-matched controls, which are included in this analysis, though we cannot account for these factors specifically in the present analysis. An additional limitation of this meta-analysis is the relatively small sample size for a meta-analysis. This is largely due to inaccessible data accompanying publications. This also highlights a need for consistent, open-access data when participants consent to having their data publicly available. Nonetheless, this study highlights the relevance of the microbially produced metabolites in people living with heart failure. These metabolites present an opportunity for future work to confirm mechanistic pathways to identify potential disease mechanisms and test if modulation of the microbiota, and subsequently those metabolic pathways, can influence disease outcomes.

This study was able to implement a late-stage integration analysis for untargetted metabolomic data from heart failure patients with the same statistical methods across multiple datasets, as opposed to conducting an analysis on the statistics reported from multiple studies as is the traditional meta-analysis model. This is beneficial as the same statistical analysis and filtering methods when applied to different sets of data make the connections between significant results across studies more straightforward, whereas the original papers

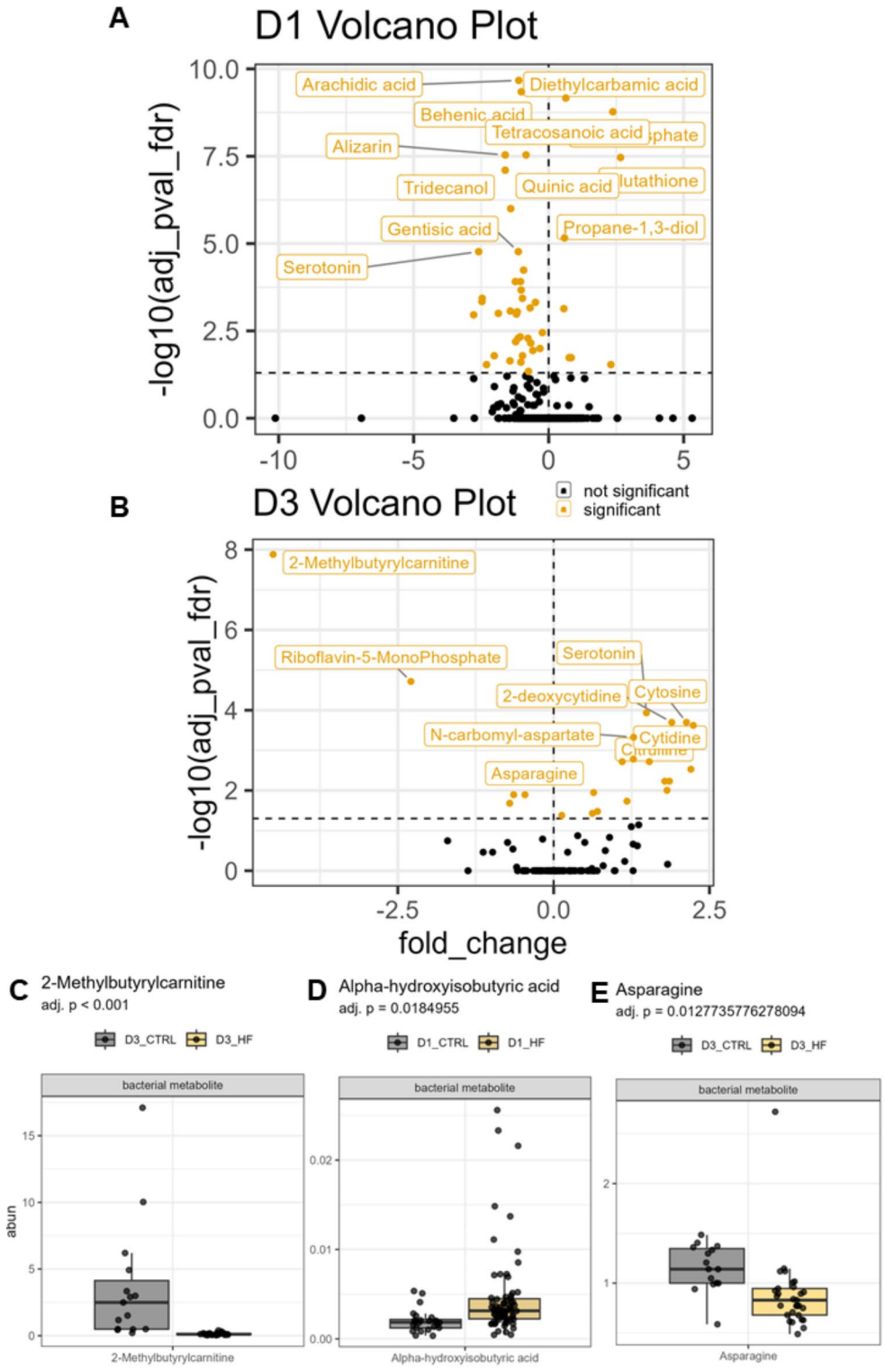

Fig. 2. Differential abundance of significant metabolites. Differential abundance volcano plots of the log fold change in metabolite abundance for D1 from stool (**A**) and D3 left ventricle (**B**). Negative changes indicate metabolites that are higher in control samples than heart failure samples (left of the vertical dotted line), while positive fold changes indicate metabolites significantly more abundant in heart failure samples. Golden colored dots indicate which metabolites were significant after FDR correction ($p < 0.05$, specific p values in Table 2). Bacterially-relevant metabolites discussed in the manuscript are shown in their reported abundance in heart failure versus control samples (**C**–**E**).

Metabolite	studyID	adj_pval_fdr	Direction
2-Methylbutyryl Carnitine (possible bacterial metabolite)	D3	0.000000013152880392570300000	Downregulated in HF
4a-carbinolamine tetrahydrobiopterin (possible bacterial metabolite)	D1	0.045687360000000000000000000	Downregulated in D1 HF
7-methylguanine (possible bacterial metabolite)	D1	0.029272880000000000000000000	Upregulated in D1 HF
Alpha-hydroxyisobutyric acid (possible bacterial metabolite)	D1	0.018495500000000000000000000	Upregulated in D1 HF
Asparagine (possible bacterial metabolite)	D3	0.012773577627809400000000000	Downregulated in D3 HF
Tridecanol (possible bacterial metabolite)	D1	0.000000079240350000000000000	Downregulated in D1 HF
Cadaverine (possible bacterial metabolite)	D1	0.000994724100000000000000000	Downregulated in D1 HF
Erythrose (possible bacterial metabolite)	D1	0.000482173000000000000000000	Downregulated in D1 HF

Table 2. Univariate statistics for significant metabolites with FDR correction. Table provides FDR-adjusted P values from univariate analysis for the significant metabolites relevant to bacterial metabolism, direction of relationship (up or down regulated in HF patients), and which study the metabolite was significant in (D1 stool or D3 left ventricle).

D1 stool				
KEGG.id	**Entry.type**	**KEGG.name**	**p.score**	**StudyID**
hsa01040	Pathway	Biosynthesis of unsaturated fatty acids	8.34E−05	D1
hsa01232	Pathway	Nucleotide metabolism	0.002083187464	D1
hsa04022	Pathway	cGMP-PKG signaling pathway	8.34E−05	D1
hsa04024	Pathway	cAMP signaling pathway	0.01608202051	D1
hsa04151	Pathway	PI3K-Akt signaling pathway	0.002083187464	D1
D3 left ventricle				
KEGG.id	**Entry.type**	**KEGG.name**	***p*.score**	**StudyID**
hsa00240	Pathway	Pyrimidine metabolism	0.002083187464	D3
hsa01232	Pathway	Nucleotide metabolism	8.34E−05	D3
hsa02010	Pathway	ABC transporters	0.004083020755	D3
hsa04022	Pathway	cGMP-PKG signaling pathway	0.002083187464	D3
hsa04024	Pathway	cAMP signaling pathway	0.01208235392	D3

Table 3. Top 5 KEGG pathways. This table provides the top 5 KEGG pathways based on significant metabolite abundances. KEGG labels were assigned by MetaboAnalyst and pathway analysis is carried out by FELLA() in R.

implemented different types of models and heart failure subgroupings that could make it difficult to understand the relevance of results between studies with the traditional meta-analysis approach. We found that, overall, the most reported on microbial metabolites in heart failure reviews (IPA, SCFAs) are not detected in traditional untargetted metabolomics (and therefore not included in this study), but that 8 other bacterial metabolites are found to be significant across the stool and heart tissue of heart failure patients. These metabolites present the opportunity for future work to confirm mechanisms and create a more cohesive understanding of the role of the microbiome in people with heart failure.

Methods

Systematic survey of literature

We conducted a systematic survey of all literature published prior to April 1, 2024 in PubMed and Web of Science and on metabolomics repositories MetaboLights and MASSIVE. The following search terms were used for PubMed and Web of Science search: ((metabolomics) OR (metabolite) OR (metabolic) OR (LCMS) or (LC-MS) OR (liquid chromatography mass spectrometry) OR (GC-MS) OR (GCMS) OR (gas chromatography mass spectrometry) OR (NMR) OR (Nuclear magnetic resonance spectroscopy)) AND ((Heart failure with preserved ejection fraction) OR (heart failure)) AND ((human) OR (person) OR (patient) OR (patients) OR (Homo sapiens)) AND ((microbe) OR (microbiome) OR (microbial) OR (microbially) OR (microbiota) OR (gut dysbiosis) OR (microbes) OR (holobiont)). For searching on metabolomics repositories, entries were searched with the following: Description="heart failure"; Sample Type = "Homo sapiens".

Papers were included if they met the following criteria: (1) an observational study of human adults (18 years of age or older) diagnosed with heart failure (not other cardiovascular diagnoses that puts one at risk for heart failure); (2) collected an untargeted metabolomics measure (e.g., GCMS, LCMS, NMR, etc.) from a biological sample (e.g. plasma or stool); (3) made their data publicly available without undue burden (e.g. "data available upon reasonable request") and in an unusable format (e.g. not raw data with no internal controls data), and (4) the main body of the text is in English. Papers were excluded if they were not in English, not specifically examining heart failure, studied children or nonhuman mammals, conducted a targeted metabolomics method,

or did not have publicly available data. We identified a total of 708 citations from PubMed (681), Web of Science (11), MassIVE (10), and Metabolomics Workbench (6). Of these 708 papers/studies in public repositories, 662 were in English with a PubMed ID and not duplicated in the citation plus repository search; 82 were studies of heart failure; 61 of those included human adults; 23 of those papers included an untargeted metabolomics measure; and only 3 of those 23 papers had publicly available data. A schematic is provided in Supplemental Fig. 1 highlighting the papers that passed each qualification check. All citations were screened by two reviewers for qualification criteria. Any discordance between screeners' records was resolved through discussion between screeners, and overall discordance was low (~ 2–5% for reviewer pairs).

The resulting three studies which qualified for inclusion are as follows: **D1** is a study from Huang et al., 2023[14] examining stool metabolites with GCMS from 96 heart failure patients with/without depression and controls. Heart failure was diagnosed via the 2016 ESC guideline criteria for the diagnosis of heart failure, and integration of at least one of the following: elevated natriuretic peptide levels, objective evidence of cardiogenic pulmonary or body circulation stasis, including imaging (e.g., chest radiograph and echocardiogram), or resting or stress hemodynamic monitoring (e.g., right heart catheter and pulmonary artery catheter). **D2** comes from the Metabolites Repository and does not have an affiliated publication[38]. This cohort comprises of 11 stable heart failure patients and 12 control patients who provided exhaled breath condensate (EBC) and saliva sent for NMR. Heart failure was diagnosed as classic systolic HF who are admitted to St Mary's Hospital with decompensated disease. Control subjects are age and gender matched controls. **D3** is a study from Li et al. 2020[36] of left ventricle (LV) metabolites from LCMS for 51 heart failure patients undergoing heart transplantation and donor hearts that could not be used due to patient-donor mismatch, transportation issues, and other reasons. Fifty-six donor hearts are age matched and represent even gender spread.

Meta-analysis

Processed data files were downloaded from their respective repository. All samples had heart failure status available in metadata files, though only one study included patient sex and no studies included patient age. As such, all samples were included but only heart failure status is examined in subsequent analysis without correcting for sex or age (though all studies specify using sex- and age- matched controls). Abundance data on the repositories was already processed and normalized, so no further transformation (e.g. log transformation or uv scaling) was done. As each metabolomics study examines a different biospecimen tissue with a different metabolomics method, a late integration approach is used for analysis where studies are analyzed for significance separately and then their final predictions are combined[39]. Broadly, first differences between heart failure or control patients were tested for significance using PCA, and further dimension reduction (OPLS-DA) was used to confirm group differences and test which features contribute to group differences. For studies with significant group differences from PCA and OPLS-DA, univariate significance testing with FDR correction was conducted. Significant metabolites (adjusted p value < 0.05) were included in a metabolomic pathway analysis. Further details on the execution of each step is discussed below.

For dimension reduction on already normalized abundance tables, metabolites that are present in fewer than 20% of samples per group (heart failure status) within their respective study for D1 and D3 or fewer than 5 samples for D2 were excluded from further analysis. Principal component analysis (PCA) was used to assess correlation structure of the data as a whole with R package PCAtest() (100 bootstraps 100 permutations)[40]. Univariate testing is conducted with a two-tailed Mann-Whitney U-Test with function wicox_test()[41] and false discovery rate for multiple test corrections with p.adjust()[42] (Table 2). Metabolites significant after FDR correction were assigned to KEGG pathways[43–45] with MetaboAnalyst[46] and underwent pathway analysis with FELLA()[47]. Results of the top 5 KEGG pathways are in Table 3.

Ortholog partial least squares discriminant analysis (OPLS-DA) was conducted only for studies with significant group differences in PCA to examine what features contribute the most to group differences (D1 and D3) with R package ropls()[48]. OPLS-DA is inclined to overfit models to data, as discussed in[49], and the risk of overfitting results is reduced by only conducting OPLS-DA for data with significant group differences as revealed by PCA. VIPs from the OPLS-DA are not reported as VIPs are not useful for OPLS modeling of a single response (i.e. heart failure status). Further results of specific metabolites from OPLS-DA are not reported as univariate testing with stringent FDR correction and p-value filtering more appropriate for biomarker analysis[50,51].

Data availability

All data is previously published and publicly available. D1 data is available from MetaboLights under Project ID MTBLS8183. D2 saliva and EBC data are available from Metabolomics Workbench under Project ID PR000430. D3 data are available from Metabolomics Workbench under Project ID ST001364. The code for all analyses accompanying this paper has been uploaded to github at https://github.com/ewissel/HF_Metabolomics_MetaAnalysis.

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Author contributions

E.F.W. , H.Y.C., K.H.W., Y.C.L., and K.U. all screened the manuscripts and data repositories for inclusion in analysis. E.F.W. analyzed the data. The entire authorship team discussed interpretation of the results. An initial draft of this manuscript was written by E.F.W. and revised by the entire authorship team. P.H. provided supervision and funding in addition to interpreting results and manuscript revisions. All authors approve of the final manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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