

Heart failure is associated with depletion of core intestinal microbiota

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Abstract

Aims In spite of current medical treatment approaches, mortality of chronic heart failure (HF) remains high and novel treatment modalities are thus urgently needed. A recent theory proposes a possible impact of the intestinal microbiome on the incidence and clinical course of heart failure. This study sought to systematically investigate, if there are specific changes of the intestinal microbiome in heart failure patients.

Methods and results The intestinal microbiome of 20 patients with heart failure with reduced ejection fraction due to ischemic or dilated cardiomyopathy was investigated by applying high-throughput sequencing of the bacterial 16S rRNA gene. Microbial profiles were compared to those of matched controls in which heart failure was ruled out by clinical assessment and NT-proBNP serum levels ($n=20$). According to the Shannon diversity index (which measures the intra-individual alpha-diversity) based on the distribution of operational taxonomic units (OTUs), HF cases showed a nominally significantly lower diversity index compared to controls ($P_{\text{nom.}} = 0.01$), and testing for genera abundance showed a tendency towards a decreased alpha diversity of HF patients. Beta-diversity measures (inter-individual diversity) revealed a highly significant separation of HF cases and controls, (e.g. $P_{\text{weighted UniFrac}} = 0.004$). Assessing the individual abundance of core measurable microbiota (CMM), a significant decrease of *Coriobacteriaceae*, *Erysipelotrichaceae* and *Ruminococcaceae* was observed on the family level. In line with that, *Blautia*, *Collinsella*, uncl. *Erysipelotrichaceae* and uncl. *Ruminococcaceae* showed a significant decrease in HF cases compared to controls on the genus level.

Conclusions Heart failure patients showed a significantly decreased diversity of the intestinal microbiome as well as a downregulation of key intestinal bacterial groups. Our data point to an altered intestinal microbiome as a potential player in the pathogenesis and progression of heart failure.

Keywords Heart failure; Gut microbiome; Microbiota; 16S; Diversity

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Introduction

In spite of modern treatment options, chronic heart failure (HF) remains associated with poor prognosis and high mortality, exceeding 50% in 5 years.¹ Moreover, its increasing prevalence leads to huge economic burden, making HF a major

challenge of the future.² Therefore, deeper insights into the pathophysiology of the disease are urgently needed, since these efforts might give rise to new disease concepts and ultimately novel therapeutic prospects.

In this context, it has been proposed that heart failure should not be regarded solely as a cardiac disease but rather

a systemic multi-organ failure.³ This view might even be extended by the concept of the holobiont, defining a host and all of its symbiotic microorganisms as a target of analytic and therapeutic interventions. With regard to heart failure, potential interactions between the human host and gut microbiota have been discussed. Dysbiosis of gut bacteria communities has been proposed to be a pathogenic factor in several diseases, e.g. type 2 diabetes.⁴ Recent publications suggest that intestinal dysbiosis may also play an important role in the pathogenesis of heart failure, putting forward a “gut hypothesis” of heart failure.⁵

Recently, it has been reported that HF is associated with disrupted intestinal epithelial function, likely as a consequence of reduced intestinal perfusion and ischemia.^{6,7} Bowel wall thickness and epithelial permeability increase, whereas absorptive function decreases.⁸ It has been proposed that intestinal bacteria and/or endotoxins such as lipopolysaccharides (LPS) translocate to the systemic circulation.⁹ High LPS concentrations in hepatic veins of heart failure patients are consistent with intestinal translocation processes of gut microbes.¹⁰ LPS levels in the blood directly correlate with systemic inflammation in decompensated HF patients and decrease after recompensation.⁶

It is well known that HF is associated with a chronic state of inflammation,¹¹ which might be induced or aggravated by this pathomechanism and thereby indirectly affect cardiomyocyte function.

Of note, an augmented intestinal juxtamucosal bacterial biofilm has been reported in patients with HF, correlating with an enhanced immunoglobulin A-antilipopolysaccharide response.^{7,8} In patients with stable HF, intestinal overgrowth of pathogenic bacteria like *Campylobacter*, *Shigella*, *Salmonella*, and *Yersinia*, as well as *Candida* species has been observed.¹²

The present study aims at a first comprehensive description of the intestinal bacterial profile in patients with acute decompensated or stable HF. Gut microbiome data from a community-based sample served as comparison group. Intra- and inter-individual analysis of bacterial diversity was performed based on high-resolution 16S rDNA sequencing.

Materials and methods

Study population

Twenty patients with HF due to frequent etiologies like ischemic cardiomyopathy (ICMP) and dilated cardiomyopathy (DCM) were studied. All participants had a highly reduced left ventricular ejection fraction (LVEF \leq 35%). Seventy percent were in an acute state of cardiac decompensation and 30% in a stable state of HF. Demographic and clinical patients' characteristics are shown in *Table 1*. Patients received drug treatment according to current HF treatment guidelines,

Table 1 Characteristics of HF patients and controls

	HF Patients (n=20)	Controls (n=20)	P-value
Age, y	65 \pm 3,17	65 \pm 3,07	0,941
Men/women	11/9	11/9	1,000
BMI, kg/m ²	29,7 \pm 1,44	29,1 \pm 1,33	0,768
Smoking habits			0,733
never	11	11	
in the past	7	7	
current	2	2	
HF aetiology	ICMP 55% DCM 45%		
LVEF, %	22,3 \pm 2,85	NM	
CRP, mg/L	11,1 \pm 2,06	4,22 \pm 1,12 (n=15)	0,006
NT-proBNP, ng/L	6564,5 \pm 1187,23	109,2 \pm 45,91 (n=17)	<0,001
NYHA class			
I	1		
II	4		
III	6		
IV	9		
Medication			
Beta-blockers	80%	15%	<0,001
Diuretics	70%	35%	0,027
ACE-inh/ARBs	70%	35%	0,027
Ald-ags	40%	15%	0,081
Comorbidities			
DM type II	35%	15%	0,152
HTN	70%	40%	0,059

ACE-inh, angiotensin-converting-enzyme inhibitors; ARBs, angiotensin receptor blockers; Ald-ags, aldosterone antagonists; BMI, body mass index; CRP, C-reactive protein; DCM, dilated cardiomyopathy; DM, diabetes mellitus; HF, heart failure; HTN, arterial hypertension; ICMP, ischemic cardiomyopathy; LVEF, left ventricular ejection fraction; NM, not measured; NT-proBNP, N-terminal of the prohormone brain natriuretic peptide; NYHA, New York Heart Association; SEM, standard error of mean; NM, not measured. Values are % or mean \pm SEM.

including usage of angiotensin converting enzyme (ACE) inhibitors, beta-blockers, diuretic agents, aldosterone antagonists and antiarrhythmic/heart rate (HR) modulating agents as appropriate. These agents were considered in statistical analysis as they were sorted and examined in groups defined by their mechanism of action. Further medication was classified into groups defined by their indication. Three participants did not receive any drugs at the time of inclusion.

Exclusion criteria included acute infection, gastrointestinal diseases or malabsorptive disorders, antibiotic or probiotic treatments within the previous three months and cancer. Any other diseases like respiratory disease, apoplexia cerebri and migraine that could possibly influence the intestinal microbiome, were not significantly prevalent in both groups. All patients consumed a mixed European diet. To minimize confounding effects of hospital surrounding, diet and contact to other patients, we collected faecal samples within the first 24 hours after admission to hospital.

The control group consisted of twenty case-matched individuals (matched in terms of age, gender, body mass index (BMI) and smoking status) from a community-based sample, free of any heart and/or gastrointestinal disease as well as of cancer. According to the recent ESC guidelines on the diagnosis of heart failure,¹³ we defined the control group on the

basis of prior diagnoses, the absence of HF related clinical signs and symptoms as well as NT-proBNP measurements. Any medication of subjects from the control group has been considered in statistical analysis. Individuals were obtained from the PopGen biobank.^{14,15}

The study was performed at the University Medical Centre Schleswig-Holstein, Campus Kiel, Germany after approval by the local ethics committee. All study participants gave their written, informed consent.

Study protocol and clinical assessment

In HF patients, body height and weight, systolic and diastolic blood pressure and body temperature were measured by clinical staff and obtained from medical records. Blood samples were obtained by nurses or physicians. In the control sample, blood pressure, height and weight were determined by trained study nurses following a standardized protocol.

Body mass index (BMI) was calculated as weight/height.² A standardised survey on pre-existing illnesses, dietary habits and several lifestyle factors was completed by all patients and controls and a standardised interview was performed. In HF patients, cardiac function was evaluated by transthoracic Doppler echocardiography according to current guidelines. All imaging studies were judged by an experienced cardiologist unaware of the study protocol and archived digitally.

In HF patients, faecal samples were collected in standard stool collection tubes within the first 24 hours after admission to hospital and stored at -80°C until further processing. Control participants collected faecal samples at home in standard stool collection tubes. The samples were shipped immediately (within 24 hours) at RT and were also stored at -80°C until processing.

DNA extraction, 16S rDNA sequencing and quality control

DNA was extracted using the QIAcube and the QIAamp DNA stool kit (Qiagen) and a prior beat-beating step. Variable regions v1-v2 of the 16S rRNA gene were amplified using the primers described in Caporaso *et al.*¹⁶ PCR products were normalized using the SequelPrep Normalization Plate Kit (Life Technologies), pooled based on Qubit dsDNA BR Assay Kit measurements (Thermo Fisher) and sequenced on an Illumina MiSeq (2×300 bp). Demultiplexing was based on zero mismatches in the barcode sequences. Forward and reverse reads were merged using FLASH software allowing an overlap of the reads between 250 and 300 bp.¹⁷ Sequences with a sequences quality below 30 in less than 95% of the

nucleotides as well as chimeras were removed using UCHIME.¹⁸

Dataset generation

After quality control unique sequences from all samples were combined and *de novo* OTU picking was performed with Usearch¹⁹ (v7, Edgar). For each sample, 10 000 random sequences were picked to construct the OTU abundance table. The OTU sequences were aligned using Muscle^{20,21} and from the alignment a Maximum-Likelihood Phylogenetic Tree was constructed using a generally time-reversible model in FastTree.^{22,23} Using this tree and the OTU abundance table unweighted and weighted UniFrac²⁴ and phylogenetic diversity²⁵ were calculated using the *unifrac.unweighted*, *unifrac.weighted* and *phylo.diversity* function, respectively, in Mothur.²⁶ Further, these sequences were taxonomically annotated using the RDP classifier²⁷ and most recent Training Set provided on the RDP website (v14; <https://rdp.cme.msu.edu/>). The taxonomic information was used to construct abundance tables on different taxonomic levels from phylum to genus. Genera with a classification score lower than 0.8 were assigned to the corresponding group named 'unclassified [family]'.

Statistical analysis

Statistical analysis was carried out in R.²⁸ Alpha-diversity measures and Bray-Curtis dissimilarities were calculated using the respective functions of the VEGAN package²⁹ for R. When testing for differences in alpha-diversity, Wilcoxon rank sum test was used when value distribution deviated from normality, two samples T-test was used when not. Unconstrained MDS plots of beta diversity measures were generated using the *cmdscale* function in R. To test for differences in beta diversity, permutational MANOVA was performed using the *adonis* function of the VEGAN package with the option *sqrt.dist=T* when using abundance tables, but not when using UniFrac distances, and 10 000 permutations. The best fitting ordination model was calculated using db-RDA (*capscale*) and the respective distance matrices. AIC was used to select the best fitting model adding single a single explanatory variable to a model correcting for age, gender, BMI and smoking habits. F-statistics and P-values were calculated using *anova.cca* to compare the model with and without the added variable. Enrichments/depletions of taxonomic groups were calculated on a core-measurable microbiota (CMM). In the CMM we included all taxonomic groups having a mean abundance of at least 1% (0.5% for OTUs) in one of the groups. Additionally for OTUs, the OTU had to be present in at least 40% of the samples. Model fitting and

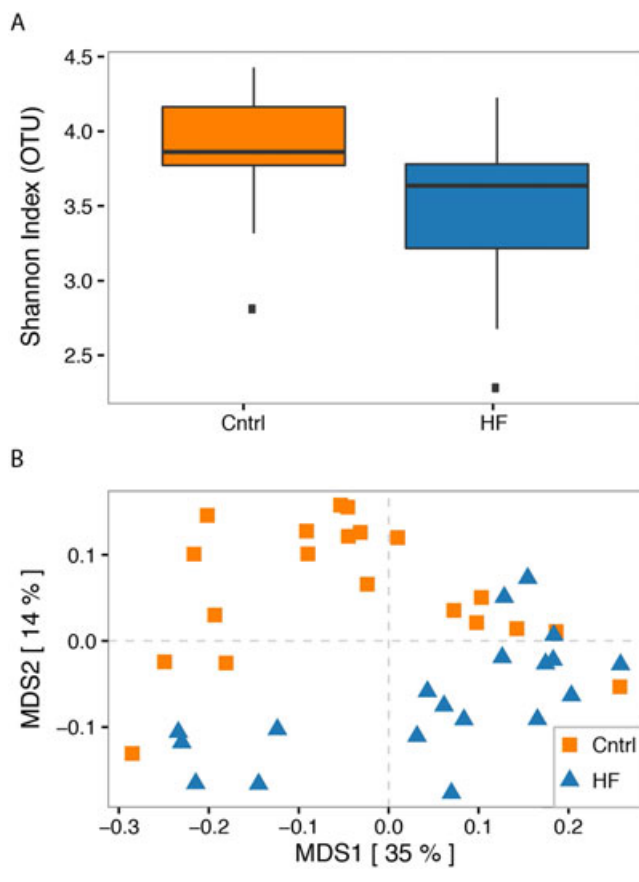
calculations were performed using the *manyglm* and *anova*. *manyglm* functions from the *mvabund* package,³⁰ using a negative binomial distribution. For the ANOVA, the number of permutations was set to 10 000 and permutation of residuals was used as resampling method, age, gender, BMI and smoking habits were used as covariates. Correction for multiple testing was done by applying Benjamini–Hochberg correction.³¹

Results

Differences between HF cases and controls with regard to alpha and beta diversity

With regard to alpha diversity, reflecting intra-individual bacterial variance, the Shannon diversity index based on OTU distribution showed a nominally significantly lower diversity index in HF cases compared to controls ($P_{\text{nom.}} = 0.01192$; *Figure 1A*). Indices based on genera abundance showed a tendency of decreased diversity as well, but these differences

Figure 1 (A) Alpha diversity: Shannon Index based on OTU abundance for controls and HF patients. (B) Beta diversity: PCoA plot of weighted UniFrac distances.



were not statistically significant upon correction for multiple testing (data not shown).

Assessment of beta diversity (PerMANOVA), a parameter which represents inter-individual variances, showed a significant separation of HF cases and controls after adjustment for gender, age, BMI and smoking habits on Bray-Curtis dissimilarity based on genera abundance (explained variance $R^2 = 5.5\%$, $P = 0.0124$), as well as OTU abundance ($R^2 = 3.9\%$, $P = 0.0099$) and in the weighted UniFrac analysis ($R^2 = 8.3\%$, $P = 0.0043$; *Figure 1B*). These results provide evidence for a shift in the community composition of the gut microbiota induced by heart failure. No significant differences were found using the unweighted UniFrac distance metric in this analysis. Of note, none of the additional explanatory variables included in the distance-based RDA yielded better results than the discrimination between HF cases and controls.

Figure 2 gives an overview of the abundances of the core-measurable microbiota (CMM) for the control group and the group of HF patients (*Fig. 2A*), as well as for each individual in each of the groups (*Fig. 2B*).

When looking at families, genera and OTUs contributing to the differences between HF cases and controls in a GLM-based model, *Coriobacteriaceae*, *Erysipelotrichaceae* and *Ruminococcaceae* showed a significant decrease in HF cases compared to controls (*Table 2*). Furthermore, *Blautia*, *Collinsella*, uncl. *Erysipelotrichaceae* and uncl. *Ruminococcaceae* showed a significant decrease in HF cases compared to controls on genus level. *Escherichia/Shigella* were enriched in HF cases, but this signal did not pass the correction for multiple testing (*Table 2*, *Figure 3*). The analysis on OTU basis revealed only nominally significant values in bacterial abundance, however reflecting the findings on genus and family level (*Table 2*, *Figure 3*).

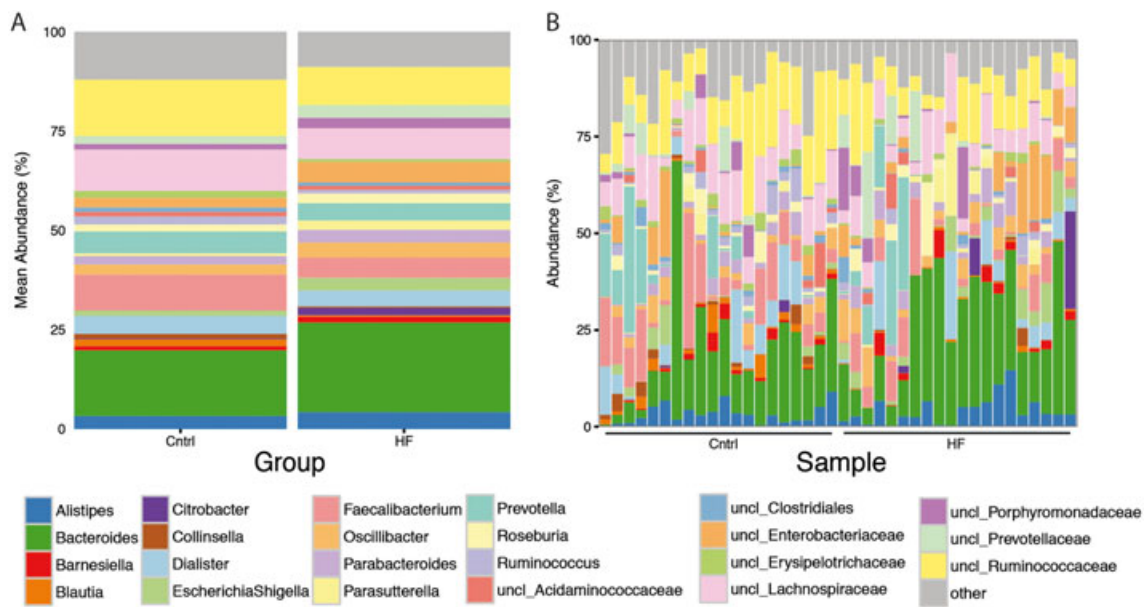
Differences in the stool microbiome within HF cases

To investigate whether certain variables are associated with inter-individual variation of the gut microbial community in HF, the PerMANOVA (*adonis*) approach was applied to the HF cases, testing all available clinical factors (gender, age, BMI, systolic and diastolic blood pressure, aetiology of HF, LVEF \leq or $>$ 20%, NYHA class, serum levels of NT-proBNP and CRP, medication and smoking habits) for significant association with genus and OTU composition. None of the variables showed a significant influence on the overall composition.

Discussion

In the present study, we sought to assess the composition and structure of the intestinal luminal microbiota in patients

Figure 2 Genus level abundances of CMM for controls and HF cases. (A) Mean relative abundances of the two groups. (B) Relative abundances for the individual samples.



with heart failure due to typical aetiologies like ICMP and DCM, using non-invasively obtainable faecal samples. Furthermore, we compared the stool microbiome of HF patients to a community-based control sample from the same geographical area, with each control being matched to a case, based on age, gender, body mass index, and smoking behaviour. The microbiome data of our control group were

consistent with available previous research on “normal” gut microbiome constellation.^{32,33}

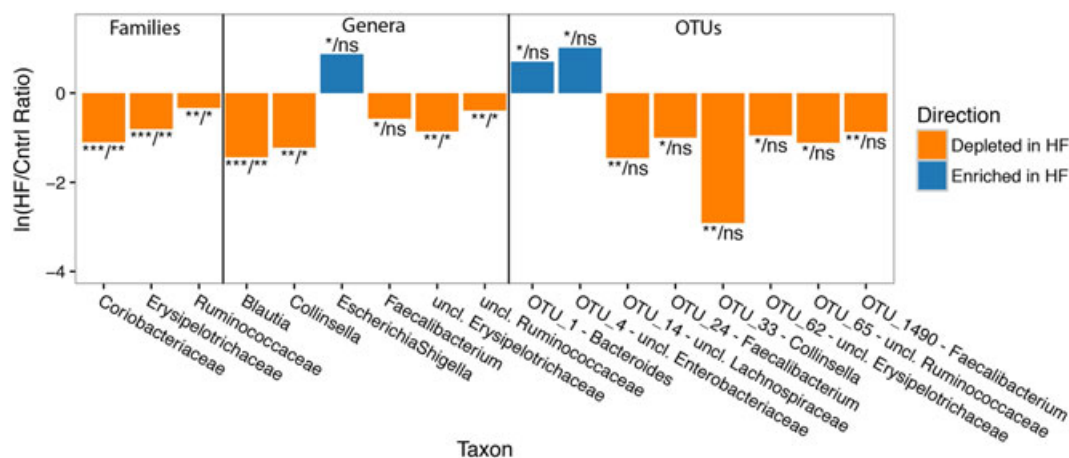
To our knowledge, this is the first systematic analysis of the intestinal bacterial microbiota of HF patients using high-throughput sequencing of bacterial 16S rRNA gene sequences. Our analysis revealed distinct differences in HF patients as compared to controls with respect to the abundance of

Table 2 Significant bacteria on family, genus and OTU level based on a GLM model

Bacteria	P-value	P _{adjust.}	% HF cases	% controls
Families				
<i>Coriobacteriaceae</i>	0.0002	0.0030	0.59	1.77
<i>Erysipelotrichaceae</i>	0.0006	0.0045	1.06	2.38
<i>Ruminococcaceae</i>	0.0054	0.0270	20.37	28.38
Genera				
<i>Blautia</i>	9.99e-05	0.0023	0.40	1.70
<i>Collinsella</i>	0.0082	0.0472	0.33	1.14
<i>Escherichia/Shigella</i>	0.0137	0.0630	3.19	1.34
<i>Faecalibacterium</i>	0.046	0.178	5.08	9.02
Uncl. <i>Erysipelotrichaceae</i>	0.0017	0.0196	0.72	1.71
Uncl. <i>Ruminococcaceae</i>	0.0081	0.0472	9.60	14.17
OTUs (no.)				
<i>Bacteroides</i> (1)	0.0430	0.2311	5.38	2.66
Uncl. <i>Enterobacteriaceae</i> (4)	0.0357	0.2311	2.74	0.99
<i>Lachnospiraceae</i> inc.	0.0031	0.0666	0.43	1.85
<i>Sedis</i> (14)				
<i>Faecalibacterium</i> (24)	0.0106	0.1040	0.87	2.36
<i>Collinsella</i> (33)	0.0021	0.0666	0.05	0.99
Uncl. <i>Erysipelotrichaceae</i> (62)	0.0121	0.1040	0.22	0.56
Uncl. <i>Ruminococcaceae</i> (65)	0.0411	0.2311	0.21	0.65
<i>Faecalibacterium</i> (1490)	0.0072	0.1032	0.90	2.15

(Number) corresponds to the OTU_number.

Figure 3 Taxonomic groups of CMM (family to OTU level) found with different abundances between HF cases and controls. ns: not significant ($P > 0.05$), *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$. P -values before and after correction for multiple testing are separated by the slash character.



taxonomic groups on the family to OTU level. These changes were also reflected in differences in beta diversity measures. In addition, a trend of reduced bacterial alpha diversity was observed in HF patients compared to controls.

Analysing intestinal bacterial profile, we could not detect a significantly enhanced abundance of particular (potentially pathogenic) bacteria on OTU level, questioning an infectious disease theory. Instead, observed shifts on family and genus level suggest functional adaptation to altered environmental conditions.

A recent study based on culture dependent methods demonstrated an intestinal enrichment of potential pathogenic bacteria, such as *Campylobacter*, *Shigella*, *Salmonella*, and *Yersinia enterocolitica*, and *Candida* species in HF patients.¹² In line with these results, we could demonstrate an increase of *Enterobacteriaceae* in HF patients, especially belonging to the *Escherichia/Shigella* cluster, using culture-independent high-throughput amplicon sequencing data. However, these results did either not meet the significance threshold (*Enterobacteriaceae*) or did not pass the correction for multiple testing (*Escherichia/Shigella*).

Rather than enrichment, we could show a significant decrease of other families and genera in HF patients. Our study illustrates changes in bacterial diversity mainly driven by significant depletion of the bacterial genera *Blautia* and *Collinsella*, as well as two unknown genera belonging to the families *Erysipelotrichaceae* and *Ruminococcaceae*. This constellation seems to be a specific attribute of heart failure, as there is no other disease entity reported to be accompanied by this alteration. It is conceivable that depletion of these genera contributes to HF pathogenesis, as recent findings from other inflammatory diseases suggest: For example, *Collinsella* bacteria have been reported to be associated with systemic atherosclerosis and type 2 diabetes mellitus (T2DM).^{34,35} Interestingly, this genus seems to be enriched

in patients suffering from atherosclerosis or T2DM, whereas we could demonstrate its depletion in HF patients. Remarkably, *Collinsella* was also downregulated in HF patients suffering from DM or ischemic heart disease. Thus, the downregulating effects of HF seem to outweigh opposing effects of these comorbidities in our patients. Therefore, we conclude that the depletion of *Collinsella* may be highly specific to HF. Moreover, recent research demonstrated that *Blautia* might be associated with anti-inflammatory mechanisms, as its intestinal abundance is associated with reduced death and improved overall survival in Graft-versus-Host-Disease.³⁶ Furthermore, a reduction in the butyrate-producing genus *Faecalibacterium* is observed in HF patients. *Faecalibacterium prausnitzii* was identified as an anti-inflammatory commensal and reduced abundance in species belonging to this genus were shown to have an adverse effect on intestinal permeability.^{37,38} These interesting findings outline that further in vitro studies need to be performed to further assess the functional role of these genera in heart failure.

Discussing possible confounders, multiple external factors (e.g. food intake, medical drug usage, environmental surrounding) and internal factors yield influence on the gut microbiome balance, possibly contributing to the observed effects. We intended to minimize the impact of dietary habits (e.g. high-fat diets) by excluding patients consuming unbalanced nutrition, although individual effects of nutrition cannot be excluded, as acute decompensated patients followed no standardized diet. Patients' medication has been considered in the statistical analyses.

Equally probable, internal changes due to HF may affect intestinal bacterial profile. For example, it is well known that HF is associated with a chronic state of inflammation^{11,39} and the intestinal microbiome might not only be addressed as a target of systemic inflammation, but also

as a trigger for this pathomechanism. Of note, this linkage has been proposed in several other inflammatory diseases such as rheumatoid arthritis.⁴⁰ Due to systemic inflammation, intestinal epithelial dysfunction and ultimately increased permeability possibly give rise to new immunogenic epitopes that induce increased autoantibody production and further activate inflammatory pathways, aggravating the primary inflammatory disease.⁴¹ It appears possible, that this concept of the intestine acting as a “percolator” of systemic inflammation, might also be functionally relevant in heart failure.

In addition to systemic inflammation, impaired fluid balance in HF patients might be important, worsening bowel perfusion, and causing hypoxemia and epithelial dysfunction. Effectively reduced bowel motility entails the intestinal content (bacteria and food components) to stay longer in the gut and thereby modifies bacterial supply of energy substrates possibly inducing a bacterial shift.⁴² To a certain degree, diuretic agents might modulate this effect. Since we could not exclude these agents in our study, as they are essential part of current treatment guidelines, we examined their effects on gut bacterial diversity, which did not yield a significant association between use of diuretics and composition of the gut microbiome.

All of these structural and functional changes may jointly affect the bacterial habitat (e.g. by oxygen availability, pH value, immunological competence), constraining dynamic adjustments of bacterial colonisation. Potential consequences might either be induced by the enrichment of pathogen effectors (e.g. bacteria, bacterial toxins, metabolic products, immune modulation) or the relative repression of beneficial bacteria in the compounded balance.

The current general opinion favours the concept that intestinal imbalance arises as a consequence of cardiac dysfunction. However, a functional relevance of an impaired and/or altered bacterial profile as a primary risk factor or early disease marker for the initial onset of heart failure seems to be conceivable as well. Of note, recent studies have shown that intestinal bacterial-dependent generated trimethylamine-N-oxide (TMAO) levels in blood are a prognostic factor for long-term mortality risk in HF patients independent of traditional risk factors and indices.^{43,44} In our study we focused on HF patients mostly in an acute state of decompensation. Future studies may assess if alterations of disease status during the progression of heart failure are mirrored by alterations of intestinal gut microbial composition, similar to e.g. Crohn’s disease.⁴⁵

Overall, significant decreases of bacterial families and genera indicate a complex multifactorially induced remodelling of the intestinal bacterial structure rather than an overgrowth of certain genera. Of note, this mechanism may not be a unique feature of heart failure but has been demonstrated in other widespread diseases as well, like diabetes and chronic kidney disease. However, the pattern of depleted

genera *Blautia* and *Collinsella*, as well as two unknown genera belonging to the families *Erysipelotrichaceae* and *Ruminococcaceae* seems to be HF specific and may even offer the basis for novel specific therapies in the future.

Conclusions

In this study, we could show that significant structural alterations of the intestinal bacterial microbiome can be found in HF patients. It seems possible that altered bacterial gut colonisation, most likely the depletion of distinct core intestinal microbiota, acts as a risk factor and disease marker for HF, enhancing disease progression in a vicious cycle. Future studies should focus on the pathomechanisms involved, e.g. altered inflammatory pathways.

Limitations

Our results are based on relatively small group sizes. Further largescale studies are necessary to prove our findings.

Furthermore, since the recent concept of advanced heart failure proposes a transition to multi-organ failure when reaching critical disease status,¹³ we cannot exclude that multiple factors occurring at this stage might superimpose the specific alterations we observed in heart failure patients, e.g. accompanying kidney failure, prolonged treatment on intensive care units, hospital acquired infections and repetitively required antibiotic therapy. Further longitudinal studies, that evaluate alterations of the intestinal microbiome during the course of disease, may help to better separate primary heart failure-specific alterations from these possible biases.

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Conflict of interest

None declared.

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