**RESEARCH ARTICLE**

# Decreased diversity of salivary microbiome in patients with stable decompensated cirrhosis

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**Abstract**

**Background:** In the setting of the oral-gut-liver axis, microbiome dysbiosis has been associated with decompensated cirrhosis progression. However, little is known on salivary microbiome profiles in stable decompensated patients.

**Methods:** We studied patients with stable decompensated cirrhosis (n =28) and matched healthy controls (n =26). There were five patients (17.8 %) with hepatocellular carcinoma (HCC). Microbiomes of the 54 salivary samples were profiled through next-generation sequencing of the 16S-rRNA region in bacteria.

**Results:** The two study groups (patients and controls) did not differ significantly concerning their baseline characteristics. The most abundant phyla were *Firmicutes, Bacteroidetes, Proteobacteria,* and *Fusobacteria*. Proposed dysbiosis ratio *Firmicutes/Bacteroidetes* was lower in patients than in controls (range: 0.05-2.54 vs. 0.28-2.18, p =0.4), showing no statistical significance. Phylum *Deinococcus-Thermus* was detected only in controls, while Phylum *Planctomycetes* only in patients. A-diversity analysis indicated low diversity of salivary microbiome in decompensated patients and patients with HCC, who presented specific discriminative taxa. On principal coordinate analysis (PCoA), the patients' and controls' salivary microbiomes clustered apart, suggesting differences in community composition (PERMANOVA test, p =0.008). Boruta wrapper algorithm selected the most representative genera to classify controls and patients (area under the curve =0.815).

**Conclusions:** Patients with stable decompensated cirrhosis of various etiology and history of complications have decreased diversity of their salivary microbiome. PCoA and Boruta algorithm may represent useful tools to discriminate the salivary microbiome in patients with decompensation. Further studies are needed to establish the utility of salivary microbiome analysis, which is easier obtained than fecal, in decompensated cirrhosis. HIPPOKRATIA 2020, 24(4): 157-165

**Keywords:** Decompensated cirrhosis, salivary microbiome, bacterial diversity, dysbiosis

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## Introduction

Liver cirrhosis is acknowledged as an increasing cause of morbidity and mortality worldwide[1]. Patients with compensated cirrhosis may remain so for many years[2], while survival for patients with decompensated cirrhosis is much lower[3]. Understanding the natural history of cirrhosis and the pathophysiology of disease progression, marked by complications, could help improve patients' management[4,5]. Recently, new insights arise through applying advanced molecular techniques for the study of the human microbiome in the pathogenesis of liver cirrhosis and the development of decompensating complications[6].

At least during the preceding decade, it has been reported that the gut flora contributes to the pathogenesis of cirrhosis' complications[7]. More specifically, altered microbiome or the so-called dysbiosis that occurs in the gut of patients with cirrhosis contributes to hepatic encephalopathy. It leads to bacteria translocation, thus developing spontaneous bacterial peritonitis (SBP), bacteremia, and sepsis[8]. Preliminary data in advanced liver disease came from studies that analyzed the human microbiome in stool samples and confirmed that its composition differs significantly in cirrhotics compared to healthy controls[9,10]. Bajaj et al introduced the ratio of "good vs. bad" taxa abundance termed the cirrhosis dysbiosis ratio (CDR)[11].

Recently, microbiome dysbiosis was found in both stool and saliva in cirrhotic patients with hepatic encephalopathy[12]. Specific salivary microbial signatures have been certified in patients with minimal hepatic encephalopathy, reflecting their cognitive function[13]. Existing studies focused on decompensated patients with hepatic encephalopathy, but similar dysbiosis in saliva might likely be present in those with other complications of cirrhosis.

Moreover, Qin et al had explained the role of the oral-gut-liver axis in the pathogenesis of cirrhosis. The study supported that most of the changes in the gut microbiome composition observed in cirrhosis were probably due to a massive invasion by bacteria originating from the mouth[14]. Accordingly, oral dysbiosis was shown in hepatitis B virus (HBV)-induced liver cirrhosis[15] and in cirrhotic patients with hepatocellular carcinoma (HCC)[16].

To our knowledge, the human microbiome has never been studied in salivary samples of patients with liver cirrhosis of diverse etiology and history of different etiology of decompensation. So, we analyzed the salivary microbiome in cirrhotics compared to that of healthy controls. Our efforts were to establish a distinct microbiome profile in patients with stable decompensated cirrhosis.

**Materials and methods**

We prospectively studied all consecutive patients with stable decompensated cirrhosis presented for pre-liver transplantation (LT) evaluation in our Hepatology Department during the first semester of 2017. Definition for decompensated cirrhosis included a history of ascites, variceal bleeding, or encephalopathy in patients with known cirrhosis. Patients enrolled were stable concerning their chronic liver disease: i.e., they had no decompensating event during the preceding month before admission. Moreover, for those with alcoholic liver disease, we required six months of abstinence before inclusion in the study. Patients underwent a detailed clinical evaluation, laboratory measurements, and radiological exams to exclude those suffering from clinical or subclinical infection. We obtained information regarding medication administered for their liver disease, and we excluded from the study those who received steroids, antibiotics, or rifaximin during the preceding 12 weeks. We evaluated the severity of their liver disease by estimating the Model for End-Stage Liver Disease (MELD)[17] and Child-Pugh (CTP)[18] scores. Moreover, detailed demographic characteristics were recorded for every patient.

Ultimately, we screened and studied 28 consecutive patients with stable decompensated cirrhosis of different etiology with complete demographic and laboratory data. Their features at baseline were recorded, especially the history of the decompensating event. Every patient included in the study signed an informed consent form before enrolment.

We also enrolled 26 matched healthy volunteers as controls for the study. Inclusion and exclusion criteria for controls were the same as for patients, excluding those who used antibiotics, probiotics, or corticosteroids within 12 weeks before enrolment or those who had a history of alcohol consumption or dental disease. We designed matching the comparison group on restrictive criteria regarding age, sex, ethnicity, and other possible confounding factors such as smoking to enhance statistical power. Healthy volunteers from our department, health care workers, or administrative staff consisted of the pool from which we selected control subjects to undergo matching with patients. We evaluated each participant's interest, assessed variables pertinent to the matching criteria for the study, including sex, date of birth, and smoking status. Before recruitment, we confirmed willingness to participate, and an appointment was scheduled for sampling, following the same procedure as for patients. All healthy volunteers gave verbal consent before enrolment.

The study protocol was approved by the Institutional Review Board and the Ethical Committee of Aristotle University of Thessaloniki (decision No 20, date: 22/1/2019). Its design conformed to the ethical guidelines of the 1964 Declaration of Helsinki and its later amendments.

The methodology used has been previously published by Gioula et al[19] and is briefly described below. Saliva sample collection was followed by total genomic DNA extraction. Then, the construction of DNA libraries followed, their concentration was determined to perform template preparation and next-generation sequencing. After that, 16s RNA sequence data were pre-processed. A total of 8,067,695 raw reads were generated, processed, and clustered into operational taxonomic units (OTUs) at 97 % similarity. 8,212 OTUs were available for further analysis. The above procedure was also described in detail by Zorba et al[20].

Statistical analysis of the microbiome data was performed with the open-source R programming language 3.3.1v while the vegan 2.4.2v[21] and phyloseq 1.19.1v[22] R packages were imported. We explored the relative abundances of bacterial taxa at phylum, family, genus, and species level with Kruskal-Wallis non-parametric test, bar plots, heatmap plots, and Venn diagrams. The term abundance refers to the number of reads for each family converted to a scale from 0 to 1. Thus, abundances for each family are expressed as a percentage of the total reads for each group.

Richness was evaluated, and the Shannon a-diversity index[23] was calculated to explore the microbial diversity within each sample and detect differences in a-diversity among patients and controls. We implemented the linear discriminant analysis (LDA) Effect Size (LEfSe)[24] method to detect differentially abundant taxa, with threshold LDA value 2.0 that described differences between controls and patients. Subgroups of patients were also explored, patients were categorized according to the presence of HCC.

To consider b-diversity between the samples, we calculated the Bray-Curtis dissimilarity matrix and applied principal coordinate analysis (PCoA). We used PCoA to

detect dissimilarities, where principal coordinate axes resulted from eigenvectors standardized by dividing the square root of their corresponding eigenvalue. It revealed taxa that counted more in total variance and used for pattern visualization. OTUs did not appear more than five times in more than 25 % of the subjects were filtered out. To confirm significant differences between patients and controls and between the subgroups of patients, we implemented permutational multivariate analysis of variance (PERMANOVA)[25] with 1,000 permutations together with PERMutational analysis of multivariate DISPersion (PERMDISP)[26,27] to strengthen the results of the PERMANOVA test further.

Boruta feature selection algorithm with a maximum number of 1,500 runs was implemented, using Boruta 5.2.0v[28] package at the genus level. We removed those taxa not seen more than five times in at least 25 % of the samples. In addition, the Random Forest[29] classification algorithm was used with randomForest 4.6.12v[30] package to examine the predictive power of selected genera and their contribution to the separation of controls and patients.

We implemented the Chi-square test to examine differences between patients' and controls' gender. Also, the independent samples t-test examined differences between patients' and controls' age. The area under the receiver operating characteristic (ROC) curve was utilized to evaluate the discriminative ability of the Boruta algorithm to classify patients with decompensated cirrhosis[31]. A p value <0.05 was considered statistically significant. The above-mentioned statistical analysis was conducted using the IBM SPSS Statistics for Windows, Version 25.0 (IBM Corp., Armonk, NY, USA).

**Results**

We studied 28 stable decompensated patients (20 males, age 53.1 ± 11.5 years). Baseline features are reported in Table 1. Chronic viral hepatitis B or C was the cause of cirrhosis in 39 % of the patients. The mean value of the MELD score was 12 ± 3 and the median value of the CTP score was 7 (range: 5-11). There were 16 patients with MELD score <12, and nine patients were classified as CTP class A. Five patients (17.8 %) had HCC, while six patients (21.4 %) had a history of hepatic encephalopathy. Moreover, we analyzed 26 matched healthy controls (18 males, age 51.8 ± 10.4 years). The two groups did not differ significantly regarding their baseline characteristics, age, and gender (Table 1).

*Alterations in the composition of the salivary microbiome*

In the present study, 8,067,695 total raw reads were obtained. Exploration of relative abundance identified 14 phyla and 113 families in all 54 study subjects, 28 patients, and 26 controls. Thirteen phyla and 95 families were identified in controls, and 13 phyla and 103 families in patients. In total, the most abundant phyla detected were *Firmicutes* (38.43 %) and *Bacteroidetes* (29.88 %), followed by *Proteobacteria* (21.17 %), *Fusobacteria* (4.73 %), A*ctinobacteria* (3.30 %), and *Tenericutes* (1.09 %). The remaining eight phyla S*pirochaetes, Chloroflexi, Verrucomicrobia, Synergistetes, Nitrospirae, Deinococcus-Thermus, Cyanobacteria,* and *Planctomycetes*, contributed fewer than 2.0 % to the total abundance. Details about total abundance and the number of families for each phylum in controls and patients are depicted in Table 2. Phylum *Deinococcus-Thermus* was detected only in controls, while Phylum *Planctomycetes* only in patients.

**Table 1:** Baseline characteristics of the 28 decompensated patients and 26 healthy controls included in the study.

| Variable | Patients n =28 | Controls n =26 | p value |
|---|---|---|---|
| **Age (mean ± SD, years)** | 53.1 ± 11.5 | 51.8 ± 10.4 | 0.65 |
| **Gender, male, n (%)** | 20 (71) | 18 (69) | 0.86 |
| **Etiology of cirrhosis, n (%)** | | | |
| Viral hepatitis | 11 (39) | | |
| Alcohol | 11 (39) | | |
| Other | 6 (22) | | |
| **MELD score (mean ± SD)** | 12 ± 3 | | |
| MELD score <12, n (%) | 16 (57) | | |
| MELD score ≥12, n (%) | 12 (43) | | |
| **CTP score (median, range)** | 7 (5-11) | | |
| CTP class A, n (%) | 9 (32) | | |
| CTP class B/C, n (%) | 19 (68) | | |
| **History of complications** | | | |
| SBP, n (%) | 3 (10.7) | | |
| GI Bleeding, n (%) | 10 (35.7) | | |
| Encephalopathy, n (%) | 6 (21.4) | | |
| **HCC, n (%)** | 5 (17.8) | | |

n: number, SD: standard deviation, MELD: Model for End-stage Liver Disease, CTP: Child-Pugh score, SBP: spontaneous bacterial peritonitis, GI: gastro-intestinal, HCC: Hepatocellular carcinoma.

**Table 2:** Total abundance and number of families for each phylum in patients and controls. Percentages are expressed in percent of total reads.

| | Patients | | Controls | |
|---|---|---|---|---|
| **Phyla** | **#Families** | **Total Abundance (%)** | **#Families** | **Total Abundance (%)** |
| *Firmicutes* | 23 | 40.86 % | 23 | 36.12 % |
| *Bacteroidetes* | 12 | 27.69 % | 10 | 31.96 % |
| *Proteobacteria* | 40 | 20.28 % | 37 | 22 % |
| *Actinobacteria* | 11 | 4.83 % | 10 | 1.86 % |
| *Fusobacteria* | 2 | 4.22 % | 2 | 5.21 % |
| *Tenericutes* | 4 | 1.14 % | 3 | 1.04 % |
| *Spirochaetes* | 1 | 0.56 % | 1 | 1.39 % |
| *Chloroflexi* | 4 | 0.31 % | 4 | 0.35 % |
| *Verrucomicrobia* | 1 | 0.08 % | 1 | 0.01 % |
| *Synergistetes* | 2 | 0.02 % | 1 | 0.06 % |
| *Nitrospirae* | 1 | 0.002 % | 1 | 0.003 % |
| *Cyanobacteria* | 1 | 0.001 % | 1 | 0.002 % |
| *Planctomycetes* | 1 | 0.001 % | | |
| *Deinococcus-Thermus* | | | 1 | 0.003 % |

We calculated the proposed *Firmicutes/Bacteroidetes* ratio[15] in our healthy controls and patients. *Firmicutes* were increased in patients, while *Bacteroidetes* in controls. The ratio was lower in patients than in controls, but this difference was not significant [range: (0.05-2.54) vs. (0.28-2.18), p =0.4].

Regarding family level, 85 families were identified both in patients and controls: 18 families were unique in patients and 10 families in controls. The five most abundant families detected both in controls and patients, with no statistical difference in relative abundance, were *Streptococcaceae* (11.4 % vs. 20 %, respectively, p =0.29) and *Veillonellaceae* (7.47 % vs. 7.70 %, respectively, p =0.5) in phylum *Firmicutes*, *Prevotellaceae* (19.46 % vs. 24.61 %, respectively, p =0.15) and *Porphyromonadaceae* (6.35 % vs. 6.11 %, respectively, p =0.56) in phylum *Bacteroidetes,* and *Pasteurellaceae* (8.96 % vs. 8.41 %, respectively, p =0.37) in phylum *Proteobacteria.* Among the 20 most abundant families in controls and patients, family *Micrococcaceae* in phylum *Actinobacteria* was detected to be more abundant in patients than in controls (3.45 % vs. 0.61 %, p =0.024). On the contrary, families *Clostridiaceae* (4.86 % vs. 2.69 %, p =0.004) and *Clostridiales Family XIII Incertae Sedis* (1.71 % vs. 1.26 %, p =0.0006) in phylum *Firmicutes*, *Sphingomonadaceae* (1.65 % vs. 0.71 %, p =0.001), *Enterobacteriaceae* (1.10 % vs. 0.00041 %, p =0.004), and *Oxalobacteraceae* (1.28 % vs. 0.91 %, p =0.024) in phylum *Proteobacteria*, and *Spirochaetaceae* (1.39 % vs. 0.56 %, p =0.025) in phylum *Spirochaetes,* were more abundant in controls.

*Exploration of bacterial diversity*

*a-diversity in controls versus patients*

We calculated the Shannon index to explore a-diversity and used the non-parametric Kruskal-Wallis test to compare these indexes between patients and controls. Results demonstrated that the level of diversity of the salivary microbiome was low in patients; comparison with that of healthy controls gave no statistical significance (p =0.27).

Moreover, we used the LEfSe method to identify the specific bacterial taxa in the salivary microbiome composition and resulted in 53 differentially abundant taxa that contributed to differences between patients and controls (Figure 1). Thirty-one taxa were significantly increased in abundance in controls, and 22 taxa were significantly increased in patients. Families *Coriobacteriaceae (Actinobacteria), Prevotellaceae (Bacteroidetes)*, and *Erysipelotrichaceae (Firmicutes)* were found in more abundance in controls, whereas families *Micrococcaceae (Actinobacteria), Lactobacillaceae (Firmicutes)*, and *Leptotrichiaceae* (*Fusobacteria*) in patients. Of the total genera detected in the salivary microbiome samples, seven genera were differentially abundant in controls and three in patients. The heatmap plot depicted the correlations between patients and controls, and the abundance of selected genera represented in their microbiome samples (Figure 2). Differentially abundant species of *Prevotella* have been detected both in controls (*Prevotella pallens, Prevotella shahii)* and patients (*Prevotella loescheii, Prevotella micans, Prevotella oris, Prevotella saccharolytica, Prevotella sp*). Moreover, four species of genus *Streptococcus* were found more abundant in patients (*Streptococcus sp, Streptococcus intermedius, Streptococcus mutans, Streptococcus tigurinus*), according to the LEfSe method.

LEfSe analysis found ten differentially abundant gen-

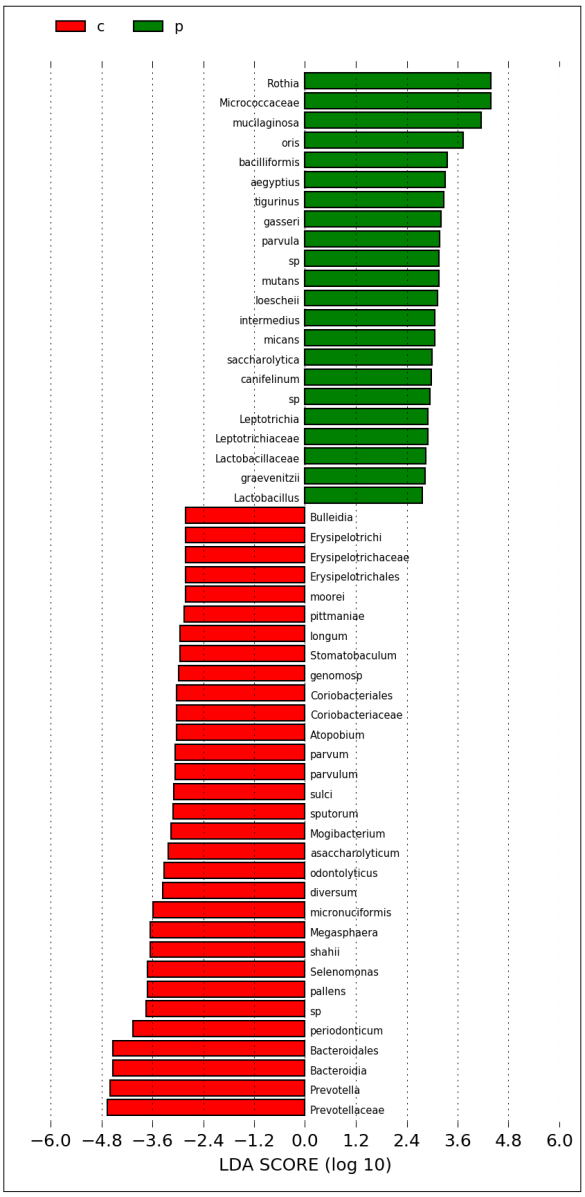


**Figure 1:** Depiction of the 53 differentially abundant taxa that contributed to differences between patients and controls, according to the linear discriminant analysis effect size (LEfSe) analysis.

era, which explained 44.9 % (Principal Coordinate-PC1) and 30.1 % (PC2) of the total variation. PCoA was applied for finding those taxa that were measured more in total variance and for pattern visualization. Genera more abundant in controls were clustered together. In contrast, genera *Rothia, Lactobacillus*, and *Leptotrichia* that were more abundant in patients, tend to be removed, contributed thus to the differentiation between controls and patients.

*Exploration of a-diversity in patients with HCC*

There were five patients with HCC, and we looked for bacterial taxonomic differences in their microbiome.

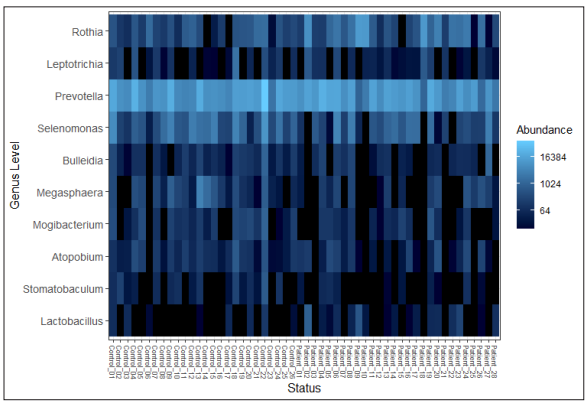


**Figure 2:** Heatmap indicating the genus-level changes in healthy controls and patients. The legends below the heatmap represent each participant. The relative abundance of the bacteria in each genus is indicated by a gradient of color from light blue (low abundance) to black (high abundance).

Shannon index suggested lower diversity in patients with HCC but without statistically significant differences (p =0.26). LefSe analysis revealed discriminative taxa with the highest mean in patients without HCC; *Gemella* genus and *Granulicatella elegans* in phylum *Firmicutes*, and *Haemophilus parainfluenzae* in phylum *Proteobacteria*. On the other hand, there were discriminative taxa with the highest mean in patients with HCC; genera *Lactococcus* and *Veillonella* in phylum *Firmicutes*, and species *Tobetsuensis, Paracaseia* and *Fermentum* in genus *Lactobacillus*, *Massilia namucuonensis* in phylum *Proteobacteria*, and genus *Treponema* in phylum *Spirochaetes*. Moreover, family *Geodermatophilaceae* and species *Scardovia wiggsiae*, *Blastococcus saxobsidens*, *Corynebacterium argentoratense*, and *Prevotella bivia* in family *Actinobacteria*. The Venn diagram performed at the genus level showed 44 common genera in patients (with or without HCC) and controls. Still, there were two unique genera in patients with HCC, 12 unique genera in patients without HCC, and 30 unique genera in controls (Figure 3).

*Exploration of b-diversity*

PCoA showing different clustering of patients' and controls' salivary microbiome is displayed in Figure 4. Moreover, the diverse composition of the bacteria population between controls and patients was confirmed by the PERMANOVA test (p =0.008). That differences were not attributed to dispersions of the samples within the different groups (p =0.32). Species that contributed to the first two PCs with statistically significantly different abundance in the two groups are presented in Table 3.

*Predictive Model Building*

We also performed a sensitivity analysis to select the most representative genera able to classify controls and patients. We executed the Boruta wrapper algorithm and selected genera *Rothia, Atopobium, Serratia, Butyrivibri*,

*laceae* were among those that significantly contributed to the differentiation of patients' and controls' salivary microbiomes. Thus, these family members seem to play either beneficial or pathogenetic roles in the evolution of hepatic diseases[39]. We also found that patients had a significantly higher abundance of *Micrococcaceae* than controls (p =0.024), a finding related to systemic inflammation in decompensated patients[12,40]. These interesting results summarize known changes previously found primarily in the gut microbiome of patients with advanced liver disease[35] and establish the significance of salivary microbiome in general, in decompensated cirrhosis with the advantage of a more accessible patient's sample.

Notably, at the family level, we managed to detect significant differences in controls' salivary microbiome, which presented higher abundances of families *Clostridiaceae* (p =0.004), *Clostridiales Family XIII Incertae Sedis* (p =0.0006), *Oxalobacteraceae* (p =0.024), and *Sphingomonadaceae* (p =0.001), compared to patients. This predominance of autochthonous taxa in controls is consistent with the previously known reduction in stools, sigmoid colon mucosa, serum, and saliva of patients with cirrhosis[41]. Principally, reduction of autochthonous to non-autochthonous taxa has been associated with the development of cirrhosis-related complications[33]. Similar results regarding autochthonous taxa have been described in the saliva of cirrhotics with a previous history of hepatic encephalopathy[12,13]. Our study confirmed these findings in patients with stable disease, though with a history of any decompensation.

Moreover, the a-diversity analysis showed that our patients had decreased salivary microbiome diversity, expressing low richness and evenness of detected taxa. Reduced bacterial diversity has been detected in salivary and gut microbiota in patients with advanced liver disease[15,35,42] and is considered a marker of dysbiosis[43]. More specifically, the LEfSe method detected differentially abundant taxa that contributed to differences between patients and controls.

Further analysis in our patients with HCC presented lower bacterial diversity. Although our results were based on the analysis of only five salivary samples, LEfSe analysis revealed that there are discriminative taxa; 12 unique genera in patients without HCC, two in patients with HCC, and 30 unique genera in controls. Nevertheless, to date, knowledge is equivocal, as Lu et al[16] suggested that microbiome diversity of tongue coat in patients with HCC is significantly increased. At the same time, Zeng et al[44] presented lower diversity in fecal samples.

Notably, our patients could be clearly separated according to their tested salivary microbiome composition as determined using PCoA plotting. This shows that the patients' salivary microbiome had pronounced differences compared to healthy controls, confirming that specific bacterial taxa may characterize decompensated cirrhosis' salivary microbiome[14].

For the first time, we applied the Boruta package[28] to deal with the available overlarge data obtained from salivary microbiome analysis. We detected a small feature set of bacterial taxa (possibly minimal) through this classification algorithm, allowing the best possible classification results for our patients. Although the ROC curve [area under the curve (AUC): 0.815] suggested this algorithm is an excellent discriminative model, there were patients classified as controls (false negatives), and thus, we cannot rely solely on this model for separating patients. The detected genera enhance the results of our previous analysis and imply the utility of such classification algorithms in decompensated cirrhosis using salivary microbiome.

Our study has limitations, as it is a single-center study that included a small number of patients and controls, so larger samples would allow reaching more robust and significant conclusions. We acknowledge that small cohorts may lead to statistical errors type II, meaning be underpowered to detect existing differences. In addition, we analyzed only salivary samples and no fecal or serum samples, in order to look for associations and functional implications. However, the oral cavity is more accessible than the gut, and salivary sampling is easier than fecal. Accordingly, it seems preferable to estimate salivary as a reflection of the gut microbiome based on the notion of bacteria translocation from the oral cavity to the gut[14]. Future studies will clarify the validity and utility of our findings in stable decompensated cirrhosis. In an ongoing study, it would be worthy of recording patients' outcomes and whether they develop further complications, too.

In conclusion, our study provides novel data to support that patients with decompensated cirrhosis have a distinct salivary microbiome, supposing pathogenic implications. There were prominent differences in patients compared to controls, adding to the existing knowledge for cirrhotic saliva. Our specificity was that we analyzed all patients regardless of previous history and stage of decompensation cirrhosis. Patients presented salivary microbiome dysbiosis characterized by decreased *Firmicutes/Bacteroidetes* ratio and bacterial diversity overall. Specific bacterial features were searched to distinguish patients, and the Boruta algorithm was applied for the first time in such samples and provided innovative data. Accordingly, dysbiosis exists in the salivary microbiome of patients with stable decompensated cirrhosis, and further studies will clarify its therapeutic and prognostic perspectives.

**Conflict of interest**

Authors declare no potential conflicts of interest.

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