Dear Editor,

Alterations in the human microbiome are closely related to various hepatobiliary diseases. Gut microbial dysbiosis has been found in patients with cholangiocarcinoma (CCA) [1]. However, the characteristics of oral microbiome in patients with CCA have not been studied.

Herein, a total of 272 saliva samples were prospectively collected. After the exclusion process, salivary samples from 74 patients with CCA, 150 healthy controls (HC) and 35 patients with hepatocellular carcinoma (HCC) were ultimately used for further analysis (Additional file 1). In the discovery phase, we characterized the CCA-associated microbiome and constructed a diagnostic model with 50 CCA patients and 100 HCs. Then, in the validation phase, the diagnostic model was validated by the other 24 CCA patients and 50 HCs. Finally, 35 HCC patients were used to evaluate the ability of the diagnostic model to distinguish intrahepatic cholangiocarcinoma (ICC) from HCC (Additional file 2: Fig. S1; Additional file 3: Table S1).

Compared with HC group, the platelets were significantly decreased, and liver function indices were worse in CCA group (Additional file 3: Tables S2, S3). The diversity analysis showed that the \( \alpha \)-diversity and the abundance of rare species were significantly increased in CCA group than those in HC group (Fig. 1a; Additional file 2: Fig. S2a–c; Additional file 3: Tables S4, S5). The principal co-ordinates analysis (PCoA) (Fig. 1b) and nonmetric multidimensional scaling (NMDS) analysis (Additional file 2: Fig. S2d) indicated that the overall oral microbial composition was different between the two groups. Furthermore, a Venn diagram illustrated that 34 operational taxonomy units (OTUs) were exclusive to the CCA group (Fig. 1c). Subsequently, a heatmap based on the relative abundance of OTUs that had significant differences between the two groups showed that 6 OTUs including OTU17 (Halomonas), OTU74 (Pelagibacterium), OTU136 (Prevotella), OTU139 (Prevotella), OTU13 (Peptostreptococcus), and OTU18 ([Eubacterium]_nodatum group) were depleted in CCA group, and 60 OTUs, such as OTU30 (Alloprevotella), OTU61 (Prevotella) and OTU75 (Alloprevotella), OTU29 (Neisseria) and OTU119 (Eikenella) were enriched in the CCA group compared with the HC group (Additional file 2: Fig. S3; Additional file 3: Table S6, S7).

Then, we found that the compositions of the dominant species composition of the CCA and HC groups were similar (Fig. S2e–f). At the phylum level, 8 phyla which consisted of Cyanobacteria, Spirochaetota, Campilobacterota, Fusobacteriota, Firmicutes, Synergistota, Desulfovibacterota and Chloroflexi were significantly increased in the CCA group, and 3 phyla, covering Actinobacteriota, Bacteroidota and unclassified Bacteria were enriched in the HC group (Fig. 1d; Additional file 3: Tables S8, S9). Moreover, at the genus level, 36 genera were identified as the genera with significant differences between the two group than those in HC group (Fig. 1a; Additional file 2: Fig. S2a–c; Additional file 3: Tables S4, S5). The principal co-ordinates analysis (PCoA) (Fig. 1b) and nonmetric multidimensional scaling (NMDS) analysis (Additional file 2: Fig. S2d) indicated that the overall oral microbial composition was different between the two groups. Furthermore, a Venn diagram illustrated that 34 operational taxonomy units (OTUs) were exclusive to the CCA group (Fig. 1c). Subsequently, a heatmap based on the relative abundance of OTUs that had significant differences between the two groups showed that 6 OTUs including OTU17 (Halomonas), OTU74 (Pelagibacterium), OTU136 (Prevotella), OTU139 (Prevotella), OTU13 (Peptostreptococcus), and OTU18 ([Eubacterium]_nodatum group) were depleted in CCA group, and 60 OTUs, such as OTU30 (Alloprevotella), OTU61 (Prevotella) and OTU75 (Alloprevotella), OTU29 (Neisseria) and OTU119 (Eikenella) were enriched in the CCA group compared with the HC group (Additional file 2: Fig. S3; Additional file 3: Table S6, S7).

Then, we found that the compositions of the dominant species composition of the CCA and HC groups were similar (Fig. S2e–f). At the phylum level, 8 phyla which consisted of Cyanobacteria, Spirochaetota, Campilobacterota, Fusobacteriota, Firmicutes, Synergistota, Desulfovibacterota and Chloroflexi were significantly increased in the CCA group, and 3 phyla, covering Actinobacteriota, Bacteroidota and unclassified Bacteria were enriched in the HC group (Fig. 1d; Additional file 3: Tables S8, S9). Moreover, at the genus level, 36 genera were identified as the genera with significant differences between the two
groups \((P<0.05)\), the top 10 with the highest abundance were displayed in Fig. 1e, among which *Streptococcus*, *Veillonella*, *Haemophilus*, *Leptotrichia*, *Granulicatella*, *Capnocytophaga* and *Alloprevotella* were enriched in the CCA group, and *Rothia*, *Actinomyces* and *Peptostreptococcus* were enriched in the HC group. The phylogenetic characteristics and gene function of oral microbial communities were displayed in Additional file 2: Figs. S4–S5 and Additional file 3: Tables S12–S14. Correlations between the microbiome and clinical characteristics were shown in Additional file 2: Fig. S6 and Additional file 3: Tables S15, S16).

The oral microbiome is used as a diagnostic biomarker in many diseases. However, the diagnostic potential of the oral microbiome for CCA has not been evaluated. Herein, we constructed a diagnostic model that could specifically identify CCA based on the oral microbiome. The fivefold cross-validation showed that the 3 OTU markers [(OTU20 (*Lautropia*), OTU30 (*Alloprevotella*) and OTU51 (*Actinomyces*)] were selected as the optimal marker set based on the discovery cohort (Additional file 2: Fig. S7a, b). We calculated the probability of disease (POD) index for each sample. In the discovery phase, the POD index was significantly increased in the CCA group (Additional file 2: Fig. S7c; Additional file 3: Table S17). The POD index achieved an AUC value of 0.9922 (Fig. S7d). To verify the diagnostic potential of the oral microbiome, the POD value was also significantly increased in the validation phase (Additional file 2: Fig. S7e; Additional file 3: Table S18) with a high AUC value of 0.9808.
Microbial dysbiosis has been reported in different parts of the human body in patients with CCA (Additional file 3: Table S20) [1–5]. Increased Prevotella was identified in the oral, gut and bile microbiome of patients with CCA [2, 5]. In addition, increased Actinomyces has been found in the gut and bile microbiome in CCA [1, 5]. However, in this study, the abundance of Actinomyces in oral cavity showed a significant decrease in CCA patients versus healthy individuals. Interactions between different human microbiomes in CCA patients need further research in the future. This study described the characteristics of the oral microbiome in CCA patients and reported the successful establishment of a diagnostic model of oral microbial markers for CCA. Moreover, oral microbiota-targeted biomarkers could serve as efficient and noninvasive diagnostic tools for CCA.

Abbreviations
AUC: Area under the curve; CCA: Cholangiocarcinoma; CI: Confidence interval; HC: Healthy control; HCC: Hepatocellular carcinoma; ICC: Intrahepatic cholangiocarcinoma; LDA: Linear discriminant analysis; nMDS: Nonmetric multidimensional scaling; OTUs: Operational taxonomy units; PCoA: Principal coordinate analysis; POD: Probability of disease.

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s40779-022-00423-x.

Acknowledgements
We thank all the generous volunteer subjects who enrolled in the study.

Authors’ contributions
ZGR and ZJY designed the study. BCR, GZZ and TR collected clinical samples. YWZ extracted the bacterial DNA. HYR and CL performed MiSeq sequencing testing. BCR and CL analyzed the data. BCR and ZGR wrote the manuscript. All authors read and approved the final manuscript.

Funding
This study was sponsored by grants from the National Natural Science Foundation of China (L2004121, 82070643, and U1104164), the Research Project of Jinan Microecological Biomedicine Shandong Laboratory (JNL-2022015B and JNL-2022001A), and the National Key Research and Development Program of China (2018YFC0200500).

Availability of data and materials
The raw Illumina read data for all samples were available through the European Nucleotide Archive (ENA) at the European Bioinformatics Institute (EBI) under accession number PRJNA846868.

Declarations
Ethics approval and consent to participate
Ethical approval for this study was granted by the Ethics Committee of the First Affiliated Hospital of Zhengzhou University (2021-KY-G7160-003). Written informed consent was collected from each participant.

Consent for publication
Not applicable.

Competing interests
All authors declare that they have no competing interests.

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Received: 5 February 2022 Accepted: 24 October 2022
Published online: 08 November 2022
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