Responses to Reviewer Critiques of Yu et al (MCO2-2023-0122).

Preamble: *We thank the editors and the four expert reviewers for the productive and insightful critiques concerning our manuscript entitled "Intratumoral Leptotrichia is a novel microbial marker for favorable clinical outcomes in head and neck cancer patients". Those comments are all valuable and very helpful for revising and improving our paper. We have performed extensive analysis and revised our manuscript designed to appropriately address the concerns raised by the reviewers. We believe these additions have strengthened the manuscript, and hope it proves acceptable for publication in MedComm.*

The referees' comments are copied below, *followed by our responses,* revised parts were marked in red in the manuscript.

Reviewers' Comments to Author:

Reviewer #1:
The short manuscript investigated the comparison of microbiome composition in early and late stages of HNSCC and identified Leptotrichia as a potential marker for discriminating early and late stages of the HNSCC. The study is interesting and valuable for HNSCC. The manuscript was well written and clearly organized. The reviewer has some minor concerns about the study.
1. In Figure 1E, the study showed some taxonomy units had higher abundance in early-stage patients than that in late-stage. Are there any taxonomy units which show opposite pattern?
Response: *We thank the reviewer for carefully reviewing. In Figure 1E, we showed all taxonomy units that were significantly different between early-stage patients and late-stage patients, and all the altered taxonomy units showed the same trend (higher abundance in early-stage patients.)*

2 Still in Figure 1E, in which way the authors measure the abundance of microbiome? Relative abundance or absolute abundance?
Response: *We thank the reviewer for carefully reviewing. The relative abundance of the microbiome was measured in Figure 1E. And we added a precise description in the revised manuscript.*
Changes in the text: *Line 105, marked in red.*

3 The number of samples used in the analysis, and the relevant accession numbers should be provided.
Response: We thank the reviewer for providing helpful suggestions. There were a total of 153 patients (including T12 stage samples: N = 61, T34 stage samples: N = 92, and Normal samples: N=22) included in this study from the TCGA database. The additional validation cohort included 15 patients (each patient had a tumor sample). We have added

such detail in the revised version of our manuscript and added the labels of sample numbers in the new Figure 1.

Changes in the text: *Lines 75-76, Line 139, marked in red.*

4 The methods used in analysis of the microbiome, survival analysis and statistical analysis should be described in supplementary materials.

Response: Statistical methods used to analyze the microbiome were described and revised in the "Biostatistical analysis" part of supplementary methods. Prognosis data of the TCGA cohort were acquired as described in the "TCGA patient cohort" part of supplementary methods. Kaplan–Meier curves were plotted for survival distributions with SPSS version 23.0 (IBM Corporation, Armonk, NY, USA), which was added in Line 34 in supplementary methods. And beyond that, we are also concerned that supplementary materials may be distorted in the submission system or downloading process, so we also uploaded all the figures and supplementary materials to GitHub (https://github.com/Jora1991/Intratumoral_Leptotrichia_HNSCC) for easy access from the reviewers.

Changes in the text: *Lines 24-25, 34 in supplementary methods, marked in red.*

5 The method of FISH used in the study should be described in supplementary materials, or the authors may cite related references.

Response: We thank the reviewer for carefully reviewing and providing helpful suggestions.

The method of FISH was described in the "Fluorescence in situ hybridization (FISH)" part in supplementary methods. And beyond that, we are also concerned that supplementary materials may be distorted in the submission system or downloading process, so we also uploaded all the figures and supplementary materials to GitHub (https://github.com/Jora1991/Intratumoral_Leptotrichia_HNSCC) for easy access from the reviewers.

6 Which gene was used when quantifying the relative expression level of Leptotrichia using qPCR?

Response: We thank the reviewer for the suggestion. Gene ABC transporter of Leptotrichia was used in PCR, which was added in Line 46 in supplementary methods.

Changes in the text: *Line 46 in supplementary methods, marked in red.*

7. L124, Figure 2A should be Figure 1F

Response: We thank the reviewer for carefully reviewing, and we are sorry for the clerical mistake. The correction has been made in Line 131 in the revised manuscript.

Changes in the text: *Line 131, marked in red.*

Reviewer #2:

the logic of the present paper was confusing. the microbial in HNSCC patients could be

different, due to the tumor stage, nutritional states, medication, etc. the variation of the microbial between different patients should be the results, but not the reason for the prognosis. the patients of early stages gain better results than those of the advanced ones, but not due to the differences of the microbial. So I could not suggest its publication.

Response: We thank the reviewer for raising this concern and providing helpful suggestions. We hope we could explain and clarify this issue. In the present study, We found that the microbial composition of early-stage HNSCC tumor tissues was distinct from that of advanced-stage HNSCC tumor tissues. Leptotrichia was significantly increased in early-stage patients compared to advanced-stage patients. To decrease the influence of confounding factors, we also performed a multivariate analysis based on basic characteristics and survival data of the TCGA cohort (Table below). Leptothichia was found to be an independent factor of overall survival (OS) and was correlated with better OS.

Multivariate Cox Regression Survival Analyses in TCGA cohort

Variate	*P value*	*Hazard Ratio*	*95% confidence interval*
Leptotrichia	***0.001***	*0.370*	*0.210-0.653*
Age	*0.601*	*1.102*	*0.766-1.584*
Gender	*0.485*	*1.214*	*0.704-2.092*
Smoke	*0.566*	*1.182*	*0.668-2.093*
Alcohol	*0.554*	*1.175*	*0.688-2.008*
Race	*0.307*	*0.715*	*0.376-1.361*
Stage	*0.918*	*1.029*	*0.595-1.780*
Tumor site	*0.253*	*0.825*	*0.593-1.148*

In summary, the relationship between microbiome and stage/clinical outcomes has not been proven to be causal in this study. But other excellent microbial studies demonstrated that microbial factors, independent of the genomic composition of the tumor, may determine tumor behavior and patient outcomes [1-5].

The aim of the manuscript type of "Letter to the editor" in this journal is to provide a rapid and concise report of a novel discovery that is brief in nature and should be of general interest to the field of biomedicine. We believe our study did reveal some useful discoveries and conclusions, which showed a potential rationale for further validation and mechanism studies. Hopefully, we can be accepted to publish this letter and extend these initial findings in our future studies.

References:

1. Riquelme E, Zhang Y, Zhang L, et al. Tumor Microbiome Diversity and Composition Influence Pancreatic Cancer Outcomes. Cell. 2019;178(4):795-806 e712.

2. Gopalakrishnan V, Spencer CN, Nezi L, et al. Gut microbiome modulates response to anti-PD-1 immunotherapy in melanoma patients. Science. 2018;359(6371):97-103.

3. Matson V, Fessler J, Bao R, et al. The commensal microbiome is associated with anti-PD-1 efficacy in metastatic melanoma patients. Science. 2018;359(6371):104-108.

4. Routy B, Le Chatelier E, Derosa L, et al. Gut microbiome influences efficacy of PD-1-based immunotherapy against epithelial tumors. Science. 2018;359(6371):91-97.
5. Qiao H, Tan XR, Li H, et al. Association of Intratumoral Microbiota With Prognosis in Patients With Nasopharyngeal Carcinoma From 2 Hospitals in China. JAMA Oncol. 2022;8(9):1301-1309.

Reviewer #3:
This study investigated microbial differences between early stage (T1-2) and advanced-stage (T3-4) HNSCC patients. They found Leptotrichia was associated with better overall survival, and verified by further experimental validation in an independent HNSCC cohort.
1. As the composition of microbiome is quite different in oral cavity, oropharynx, hypopharynx and laryngeal, especially oral microbiome is frequently influenced by periodontal microorganisms. I suggest to analysis the composition of microbiome in different location of head and neck.
Response: We thank the reviewer for carefully reviewing and providing helpful suggestions.
As suggested, we performed microbial analyses based on the tumor site (new Supplementary Table 2). Besides, as far as we know, it is relatively common to treat the head and neck regions as a whole in many excellent microbiome studies [6,7]. We agreed that the composition of the microbiome is different in various sites of the head and neck cancers, and further study with more HNSCC patients will be conducted to compare the microbial composition in different locations of the head and neck between early-stage and advanced-stage patients. And beyond that, we are also concerned that supplementary materials may be distorted in the submission system or downloading process, so we also uploaded all the figures and supplementary materials to GitHub (https://github.com/Jora1991/Intratumoral_Leptotrichia_HNSCC) for easy access from the reviewers.

Changes in the text: *Supplementary Table 2 has been added.*

2. The authors mentioned that microbial alpha-diversity was significantly decreased in T34 samples compared to normal samples and in T34 samples compared to T12 samples. The authors were suggested to discuss the reason.
Response: We thank the reviewer for providing helpful suggestions. Some discussions were added to the revised manuscript as follows. Mucosal sites in the head and neck region harbor a site-specific microbiome, which has an essential role in maintaining health and homeostasis [8]. High microbial diversity is considered to be a sign of health [9]. In head and neck malignancies, the local microbiota composition and biomass loads change considerably, and the microbiome is characterized by an overgrowth of diverse bacteria, such as Fusobacterium, Prevotella, Veillonella, etc [10]. Therefore, it is reasonable that alpha-diversity was significantly decreased in T34 samples compared to normal samples

and in T34 samples compared to T12 samples.
Changes in the text: *Lines 87-93, marked in red.*

3. Patients with Leptotrichia-positive samples had significantly prolonged OS compared with those with Leptotrichia-negative samples (P=0.0005) using univariate Cox proportional hazard models (Figure 2A). I don't see the figure 2A in the figure section.
Response: We thank the reviewer for carefully reviewing. Figure 2A in Line 131 should be Figure 1F, and we are sorry for the clerical mistake. The correction has been made in the revised manuscript.
Changes in the text: *Line 131, marked in red.*

4. In figure 1J, it suggested to compare the relative expression level of Leptotrichia. in the tumor area and normal area. And what the patients' characteristic is like? How many patients were oral cancer, how many were others?
Response: We thank the reviewer for providing this helpful suggestion. There were a total of 15 patients in the validation cohort, who were all diagnosed with laryngeal squamous cell carcinoma. We have performed PCR in tumor samples and paired normal samples, and the results are shown in the revised version of Figure 1J.
Changes: *Line 144-149, Line 257 in text, marked in red.*

Reviewer #4:
This paper reported a study on microbial effect on tumor behavior. The authors discovered the distinct microbial differences between early-stag (T1-2) and advanced-stage (T3-4) HNSCC patients. Moreover, they found that Leptotrichia was significantly increased in early-stage patients , and related to the difference of overall survival in the HNSCC cohort. Overall the findings are interesting and may potentially profit the microbiota-based cancer therapies. Here are my concerns.
1)I missed some discussion on the possible biological mechanism of the protective value of Leptotrichia in the HNSCC tumor.
Response: We thank the reviewer for raising this concern and providing helpful suggestions. As far as we know, the role of Leptotrichia in HNSCC has rarely been reported in the literature. Therefore, we added some discussion on possible mechanisms of the protective value of Leptotrichia based on their function in the immune response.
The genus Leptotrichia consists of slow-growing, non-motile facultative anaerobic/anaerobic Gram-negative rods that reside in the oral cavity, the genitourinary and intestinal tract [11]. Leptotrichia species were traditionally considered non-pathogenic but have recently been considered as opportunistic causes of human disease [12,13]. Many types of Gram-negative bacteria secrete LPS that stimulates the immune system, including Leptotrichia. A study by Langfeldt et al. found that Leptotrichia was able to trigger the transcription level of proinflammatory interleukin (IL)-1β, IL-6, IL-8, and IL-10 in epithelial cells [14]. These proinflammatory cytokines can induce the host's cellular immune response. As is known, tumor development is intricately linked with the immune system [15],

and the host cellular immune response triggered by Leptotrichia might improve anti-tumor immunity and determine tumor behavior. (As there are limitations of 10 references, these discussions were simplified and added to the manuscript.)

Changes in the text: *Lines 160-162, marked in red.*

2)Fig. 1 showed the significant microbial differences between early-stage (T1-2) and advanced-stage (T3-4) HNSCC patients, however, did the authors eliminate any other reasons that may raise the differences? I missed the detail of the methods for data processing.

Response: We thank the reviewer for raising this important concern and providing helpful suggestions. We hope we could explain and clarify this issue. Clinical profiles of patients in the TCGA cohort were made into a table (new Supplementary Table 1). It is shown that there were no differences regarding age, gender, smoking, drinking, or race between early-stage and advanced-stage HNSCC patients in this study. We agreed that other factors could also raise differences in microbial composition, and further study with more HNSCC patients will be conducted to reduce the influence of confounding factors.

The detail of the methods for data processing was described in the "Biostatistical analysis" part in Supplementary Methods. And beyond that, we are also concerned that supplementary materials may be distorted in the submission system or downloading process, so we also uploaded all the figures and supplementary materials to GitHub (https://github.com/Jora1991/Intratumoral_Leptotrichia_HNSCC) for easy access from the reviewers.

3)It will be more convincing if there are more evidences of microbial effects between early-stage (T1-2) and advanced-stage (T3-4) HNSCC patients.

Response: We thank the reviewer for providing helpful suggestions. In the present study, We found that the microbial composition of early-stage HNSCC tumor tissues was distinct from that of advanced-stage HNSCC tumor tissues. Leptotrichia was significantly increased in early-stage patients compared to advanced-stage patients. To decrease the influence of confounding factors, we also performed a multivariate analysis based on basic characteristics and survival data of the TCGA cohort (Table below). Leptothichia was found to be an independent factor of overall survival (OS) and was correlated with better OS.

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In summary, the relationship between microbiome and stage/clinical outcomes has not been proven to be causal in this study. But other excellent microbial studies demonstrated that microbial host factors, independent of the genomic composition of the tumor, may determine tumor behavior and patient outcomes [1-5].
We believe our work (in the format of the "Letter to the editor") did reveal some useful discoveries and conclusions, which showed a potential rationale for further validation and mechanism studies. Hopefully, we can be accepted to publish this letter and extend these initial findings in our future studies.

4)"The" should be "the" on page 4 line 74.
Response: We thank the reviewer for carefully reviewing. We have made such corrections in the revised manuscript.
Changes in the text: *Lines 73-74, marked in red.*

Editor decision:
1.Please provide the APPROVAL NUMBER for the clinical experiment in Ethic approval （if available.
Response: We thank the editor for providing helpful suggestions. The ethics approval ID number has been added to the "Ethics approval and consent to participate" part.

2.Please note that ALL authors and their contributions should be mentioned.
Response: We have made such corrections in the revised manuscript in the "Author Contributions" part.

3.Typically speaking，"Letter" type of manuscript is about 1200 words. Please expand the content in main text to about 1200 words for Letter.
Response: After revision of the manuscript, there are now a total of 1189 words in the revised manuscript.

4.Please reduce the reference within 10 and revise the format of references according to the attached Endnote file.
Response: We thank the editor for this helpful suggestion. We have reduced the number of references to 10 and revised the format in the new manuscript.

5.Typically speaking, there should be no more than 10 authors and no more than 3 institutions for letter type articles. Please make appropriate modifications. Maybe merging the different departments from the same large institution.
Response: We thank the editor for this helpful suggestion. In the revised manuscript, we have reduced to six authors who made essential contributions to this study and worked in four institutions.

6.Please revise all figures;

1) The figure size should be 170 mm wide and not higher than 210 mm.

2) The optimum font size in figures is 8~10 pt (at least 6 pt, only under special circumstances), and no lines should be thinner than 0.25 pt (0.09 mm). Label for each panel should be 12 pt, bold uppercase (A, B, C, etc.) and placed at the top-left corner.

3) All figures should be of high resolution (at least 300 dpi) and high-definition.

Response: We have made such revisions in the new Figure.

7.Please delete the abbreviation list and define an abbreviation when it first appears in the manuscript. If certain abbreviation first appears in figure or table, please define it in corresponding legend.

Response: We have made such revisions in the revised manuscript.

References:

1. Riquelme E, Zhang Y, Zhang L, et al. Tumor Microbiome Diversity and Composition Influence Pancreatic Cancer Outcomes. *Cell*. 2019;178(4):795–806 e712.
2. Gopalakrishnan V, Spencer CN, Nezi L, et al. Gut microbiome modulates response to anti–PD–1 immunotherapy in melanoma patients. *Science*. 2018;359(6371):97–103.
3. Matson V, Fessler J, Bao R, et al. The commensal microbiome is associated with anti–PD–1 efficacy in metastatic melanoma patients. *Science*. 2018;359(6371):104–108.
4. Routy B, Le Chatelier E, Derosa L, et al. Gut microbiome influences efficacy of PD–1–based immunotherapy against epithelial tumors. *Science*. 2018;359(6371):91–97.
5. Qiao H, Tan XR, Li H, et al. Association of Intratumoral Microbiota With Prognosis in Patients With Nasopharyngeal Carcinoma From 2 Hospitals in China. *JAMA Oncol*. 2022;8(9):1301–1309.
6. Hayes RB, Ahn J, Fan X, et al. Association of Oral Microbiome With Risk for Incident Head and Neck Squamous Cell Cancer. *JAMA Oncol*. 2018;4(3):358–365.
7. Dohlman AB, Arguijo Mendoza D, Ding S, et al. The cancer microbiome atlas: a pan–cancer comparative analysis to distinguish tissue–resident microbiota from contaminants. *Cell Host Microbe*. 2021;29(2):281–298 e285.
8. Kim YK, Kwon EJ, Yu Y, et al. Microbial and molecular differences according to the location of head and neck cancers. *Cancer Cell Int*. 2022;22(1):135.
9. Human Microbiome Project C. Structure, function and diversity of the healthy human microbiome. *Nature*. 2012;486(7402):207–214.
10. Burcher KM, Burcher JT, Inscore L, Bloomer CH, Furdui CM, Porosnicu M. A Review of the Role of Oral Microbiome in the Development, Detection, and Management of Head and Neck Squamous Cell Cancers. *Cancers (Basel)*. 2022;14(17).
11. Eribe ERK, Paster BJ, Caugant DA, et al. Genetic diversity of Leptotrichia and description of Leptotrichia goodfellowii sp. nov., Leptotrichia hofstadii sp. nov., Leptotrichia shahii sp. nov. and Leptotrichia wadei sp. nov. *Int J Syst Evol Microbiol*. 2004;54(Pt 2):583–592.
12. Couturier MR, Slechta ES, Goulston C, Fisher MA, Hanson KE. Leptotrichia bacteremia in patients receiving high–dose chemotherapy. *J Clin Microbiol*. 2012;50(4):1228–1232.
13. Sabater Cabrera C, Fernandez Blazquez A, Garcia Carus E. Bacteremia due to Leptotrichia trevisanii after an allogeneic bone marrow transplant. *Enferm Infecc Microbiol Clin*.

2017;35(6):389—390.

14. Langfeldt D, Neulinger SC, Stiesch M, et al. Health— and disease—associated species clusters in complex natural biofilms determine the innate immune response in oral epithelial cells during biofilm maturation. *FEMS Microbiol Lett*. 2014;360(2):137—143.

15. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. 2011;144(5):646—674.