**Editor’s comments**:

**Comment #1**: “*I am enclosing the comments that the Referees have made on your paper. I am afraid their opinion is that the data are not decisive enough for the paper to be a strong candidate for publication in Cell Host & Microbe, and therefore it seems that the paper should be published elsewhere. I want to emphasize that this decision is not intended to imply a lack of interest on our part in either your work in particular or this field in general. We hope that you will continue to consider Cell Host & Microbe for future submissions when appropriate.*”  
**Author Reply**: We thank you and three anonymous referees for taking time to read our manuscript and provide constructive feedback. We have substantially improved the weakness on data and analysis approaches by

* Adding a conclusive mouse experiment that has collected paired oral-gut samples
* Reanalyzing two public mouse experiments with fecal samples only
* Refining our algorithm to identify oral taxa in human feces at the ASV level (it was at the genus level at the initial submission)

With these improvements, all data and analyses in the revised manuscript have pointed to the same, consistent conclusion: the relative enrichment of oral bacteria in the host feces does not reflect a corresponding rise in their absolute abundance. Instead, it indicates a depletion of gut-resident commensal bacteria.

Besides these major changes, we have addressed the other key concerns (e.g., restructure our manuscript to simplify the central message) and revised the manuscript based on the comments of the three referees. We also make sure that all data are available within the manuscript and its supplementary materials, and the computer codes are visible from the GitHub. Please see below for our detailed point-to-point responses.**Reply to Referee #1**   
  
**Comment #1**: “*The paper attempts to fill a critical knowledge gap of understanding the abundance of oral microbes in fecal samples during states of ectopic expansion associated with gut dysbiosis. The hypothesis is that oral microbes out compete or are able to survive in the gut during gut dysbiosis and can perturb additional disease and or prevent the normal gut flora from reestablishing itself in order to return to the healthy state.*

*The authors create a mathematical model for the presence of oral microbes in the gut. The model makes a few predictions about the population. Namely, oral microbes are present in fecal samples in a bimodal, increased percent of oral microbes in fecal samples occurs because of reduced gut microbial levels, and that absolute values of oral microbes are very stable.  
To test these predictions, they created a program to estimate the quantity of oral microbes within human samples based on the composition of the sample's microbial population. As they point out, most studies do not have matched oral and fecal samples so a way to estimate oral microbes is required.*

*They use the program to investigate several studies of gastrointestinal disease. Combined with 16S and metagenomic data they found support for the model predictions as well as the Marker hypothesis, that the correlation of a higher percentage oral microbes in feces in gastrointestinal disease occurs due to reduced gut microbes rather than disease being driven by oral microbes.*”

**Author reply**: We thank the referee for carefully reading our manuscript and summarizing the key points.

**Comment #2**: “*The first major issue is the taxonomic identification which seemed to be restricted to the genus level. It is well established that multiple species within a genera can be detected across human body sites as is evident in Figure 2A as both oral and skin level taxa cluster together however these are not the same species or the same strains as oral that are found on the skin. No study to date has really conclusively proven at the strain level that oral bacteria are indeed found in the human gut in healthy subjects. The reference to finding evidence for this model prediction in the fecal microbiota of healthy Western adults, "which show bimodal distributions of Prevotella species commonly found in the oral cavity (Lahti et al.,88 2014; Tett et al., 2021)" is a major issue given that there is an abundance of non-oral Prevotella in the healthy human gut. This underlies the flaw of the manuscript in that it does not distinguish between oral Prevotella species like denticola, nigrescens and gut prevotella species copri, histicola as an example and assigns Prevotella to only oral. They wrongly conclude that even healthy people can have significant proportions of oral bacteria in their feces.  
See: The Prevotella copri Complex Comprises Four Distinct Clades Underrepresented in Westernized Populations. Adrian Tett et al. Cell Host Microbe. 2019  
Vinod Kumar Gupta; Narendrakumar M Chaudhari; Suchismitha Iskepalli; Chitra Dutta (March 5, 2015). "Divergences in gene repertoire among the reference Prevotella genomes derived from distinct human body sites of human*”  
**Author reply**: Thanks for the comments and insights. We agree that genus level is insufficient to distinguish the body site origin for certain genera such as *Prevotella*. To overcome this limitation, we profiled the HMP 16S sequencing data at the ASV level and identified 178 ASV sequences typically found in the oral cavity but atypical for the gut based on their relative abundance and prevalence (see Methods at lines 508-522). We call them “oral-typical ASVs” (lines 183-186), which were then used to infer the body site origin of ASVs in any other (non-HMP) fecal samples at 100% sequence overlap. A similar approach was used before to identify oral-typical species (not ASV) from gut metagenomics (Thomas et al., Nature Medicine, 2019).

Using the inference approach, we found that 219 out of 280 HMP fecal samples contained none of the 178 oral-typical ASVs. Across all 280 fecal samples, the mean proportion of oral-typical ASVs is as low as 0.05% (Fig. 3D; lines 187-190). This is reassuring because it is generally accepted (though not fully conclusive) that oral bacterial sequences are rarely (typically <2%; Schmidt et al., Elife, 2019) found in the feces of healthy people.

The referee’s concern about the *Prevotella* is also valid. Among the 178 oral-typical ASVs, 24 belong to *Prevotella* (Fig. 3C). However, only 1 out of 280 HMP fecal samples contained any of the 24 oral-typical *Prevotella* ASVs. This clearly shows that the 24 oral-typical *Prevotella* ASVs are not gut-dwelling taxa. More importantly, we did not observe significant enrichment of *Prevotella* ASVs following antibiotic exposure in all mouse studies and human cohorts included in this study. Taken together, the classification of oral vs. gut *Prevotella* ASVs should not be a practical concern for our revised manuscript.

Finally, we have removed the model predictions about the bimodal distribution of oral species in the gut and the supporting reference since this result dilutes the main message.

Thomas, A. M., Manghi, P., Asnicar, F., Pasolli, E., Armanini, F., Zolfo, M., ... & Segata, N. (2019). Metagenomic analysis of colorectal cancer datasets identifies cross-cohort microbial diagnostic signatures and a link with choline degradation. Nature medicine, 25(4), 667-678.

Schmidt, T. S., Hayward, M. R., Coelho, L. P., Li, S. S., Costea, P. I., Voigt, A. Y., ... & Bork, P. (2019). Extensive transmission of microbes along the gastrointestinal tract. Elife, 8, e42693.

**Comment #3**: “*They also looked for co-occurrence of ASVs in the samples. However, again the assignment of ASVs to oral was based entirely on genus, rather than appearance of specific ASVs in oral and fecal samples. So, it is unclear what these results are really showing.*”

**Author reply**: We have performed the cooccurrence analysis at the ASV level in the revised manuscript. The results are described at lines 226-231.  
  
**Comment #4**: “*HMP and additional studies listed for source data provided species level assignments. I am concerned that the lower taxonomic assignments only at the genus level affected the results of their model and maybe why they are not included. However, this is not addressed in the current manuscript.*”

**Author reply**: Thanks for giving us an opportunity to explain why we focused on the genus level at the initial submission. Some microbiome studies reanalyzed in our study provide metagenomic data and species level assignments. But mostly do not and focused on 16S rRNA sequencing. 16S-based profiling may be annotated to species level for certain taxa, but it is generally accurate to the genus level. Most importantly, almost all microbiome studies with quantitative data (e.g., qPCR) are 16S-based. Therefore, we had a choice of estimating oral taxa at the ASV level or genus level. We chose genus level in the initial submission but the ASV feature table for the HMP dataset is not available for downloading. In the revised manuscript, we profiled the ASV-level compositions of HMP samples by ourselves and switched our analysis from the genus to the ASV level.  
  
**Comment #5**: “*A related issue is with the estimation of oral microbes. the centerpiece of the entire paper. The authors develop the program using data from the Human Microbiome Project (HMP). Unfortunately, the program was not tested for effectiveness. Figure 2D shows predictions for HMP, but that was the training dataset and so isn't a test of the program. They also compare their results to a Random Forest classifier and a Bayesian approach using SourceTracker2. Their results had reasonable correlation with these quantification approaches. But again, these models were trained on HMP and the results compared from analyzing HMP. While a means of estimating oral microbes is required because most studies do not have matched oral and fecal data, some studies should. To show the efficacy of the program the authors would ideally use matched data to establish a ground truth for the presence of oral microbes within fecal samples and compare these results with their estimates. At the very least, the authors need to test the program on data not used in the training set.*

*With that said the paper points out that there are few data sets that have paired gut and oral samples. However when trying to extrapolate to data without matched samples it would have been critical to include an evaluation of at least one matched dataset that could have been used to evaluate the models they developed and trained on unmatched sets.*”

**Author reply**: We thank the reviewer for raising this important concern. First, we added a mouse experiment where we have collected both paired oral and gut samples (lines 107-127). With known ground-truth, this experiment clearly shows that the Marker hypothesis is true, i.e., relative enrichment of oral bacteria in feces indicates reduced density of gut commensal bacteria.

For human microbiome studies that lack paired oral samples, we need to infer the body site origin of each bacterial taxa in fecal samples. As detailed in our reply to the Comment #2 of the Referee #1, we took a simple but conservative approach for identifying oral taxa. To test this approach, we analyzed an IBD (inflammatory bowel disease) cohort that included patients with Crohn’s disease (CD) and ulcerative colitis (UC) as well as their healthy controls (HC). Using the HMP dataset, we showed that identifying oral taxa simply by ASV sequence overlaps between paired oral and gut samples significantly overestimated oral-gut transfer events (lines 187-192). For this reason, we cannot use paired oral-gut samples to certainly know which fecal ASV has an oral origin.

Nevertheless, we can validate the conservativeness (false-positive rates) of our approach under the rationale that fecal ASVs, if transferred from the oral cavity, should be present in the oral cavity of the same persons. We detected oral ASVs in all CD and UC patients. As discussed in the Supplementary Text, their mean relative abundances are nearly 4 folds as high as that averaged over the HC group (4.2% and 4.3% vs. 1.1%). More importantly, >70% fecal ASVs inferred as of oral origin can be found in paired saliva samples in 87 out of 99 samples (a mixture of 41 HC, 16 CD, and 42 UC samples; Fig. S4C). This strongly suggests that the identified oral taxa are indeed transferred from the oral cavity. Since the HMP oral samples were sequenced at multiple subsites, it is understandable that not all predicted oral ASVs can be detected in saliva alone (a single subsite).

A conservative approach like ours may omit the detection of oral ASVs, but this theoretical concern does not seem to be practically important. In the gut microbiome of allogenic stem cell transplant recipients, the total percentage of oral ASVs in feces can reach >30% and sometimes nearly 100% (Fig. 4A). In IBD patients, the oral proportion is not that high but still exceeds 10% in certain patients (Fig. 5 and Fig. S10). We also varied the thresholds to determine oral-typical ASV sequences from the HMP dataset: different cutoff values may affect the identification of oral ASVs in certain samples of the IBD cohort but we did not observe a systematic change (Fig. S4A).

Taken together, our approach provides a conservative way to estimate the oral proportion of fecal samples in both healthy individuals and patients with dysbiotic microbiomes.

**Comment #6:** “*Additionally, Random Forest classifier and SourceTracker 2 are referred to as well-established microbial source tracking methods. There is little justification to develop a third method. Their estimates might be better than these methods, but without any demonstration of superior accuracy or explanation this alternative approach seems to lack support.”*

**Author reply:** The well-established approaches such as SourceTracker2, FEAST and Random Forest Classifier were typically used when potential source samples are provided. In our study, paired oral samples are unknown for certain human microbiome studies. To apply them, we may use fecal and oral microbiome samples from a large cohort of healthy people (e.g., HMP) as potential sources for each fecal sink sample. The total fractions of oral taxa in a target fecal sample can be calculated as the sum of proportion contributed by each HMP oral cavity source (for SourceTracker2 and FEAST) or as the sum of probability of each source from which the target sample comes (Random Forest Classifier). Regardless, they neither identify oral ASVs nor measure their total relative abundance in feces directly.

Additionally, there are a total of ~3,000 fecal and oral samples in the HMP dataset. If we use them all as sources, it becomes computationally very intensive to quantify oral proportions in a large microbiome dataset (e.g., our allo-HCT cohort has >10,000 samples): it takes ~71 hours and ~1.6 hours for SourceTracker2 and FEAST to run a single sink sample with 1,000 sources respectively (Shenhav et al., Nature Methods, 2019).

Taken together, the classical microbial source tracking algorithms may not appropriate to identify oral taxa and estimate their total abundances in the absence of paired oral samples. Instead, we were motivated by a recent study (Thomas et al., Nature Medicine, 2019) and decided to adopt a simple yet conservative approach. Please read our reply to Comment #2 and #5 of the Referee #1 for the details of our approach and its validations.

Shenhav, L., Thompson, M., Joseph, T. A., Briscoe, L., Furman, O., Bogumil, D., ... & Halperin, E. (2019). FEAST: fast expectation-maximization for microbial source tracking. Nature methods, 16(7), 627-632.

Thomas, A. M., Manghi, P., Asnicar, F., Pasolli, E., Armanini, F., Zolfo, M., ... & Segata, N. (2019). Metagenomic analysis of colorectal cancer datasets identifies cross-cohort microbial diagnostic signatures and a link with choline degradation. Nature medicine, 25(4), 667-678.  
  
**Comment #7**: “*More generally, the writing of the paper seems somewhat unfocused. For example. It is established that the percent of oral microbes in fecal samples is higher in the case of gastrointestinal disease. Thus, the main results of the paper are showing evidence for their mathematical model and for the Marker hypothesis. The primary support for these is shown in Figure 2B and C, out of A-I. They are almost lost in the clutter of plots. The paper needs a more organized approach to presenting the results.*”

**Author reply:** We thank the referee for this remark. With new data and analyses, we restructured our manuscript to center on the proof of the Marker hypothesis and its implications on host health. We believe that the current organization, as briefly described below, enables effective delivery of our central message:

* First, we demonstrated that the Marker hypothesis is true using mouse experiments.
* Next, we developed and validated an algorithm to identify oral taxa in human feces.
* Then, we applied this algorithm to study the associations of oral-gut transfer with antibiotics and human health in a large cohort of allogeneic stem cell transplant recipients.
* Finally, we elucidated the implication of the Marker hypothesis in linking competing biomarkers of IBD.

**Comment #8**: “*Overall the idea is interesting, but the data is a long way from supporting either hypothesis in the current form. Given these issues I cannot recommend publication of the paper in its current form.*”

**Author Reply:** We hope that our new mouse experiment, reanalysis of public mouse experiments, and ASV-level human microbiome analysis would clarify the issues. Please refer to our reply to Comment #2 and #5 of Referee #1 for details of these new data and analayses.

**Comment #9**: “*The issue of depth is a very important one. If depth of sequencing is low, and not capturing the diversity of a sample, it will appear to have lower diversity. The correlation of sequence depth with the presence of "oral" bacteria reads should be determined.*”

**Author reply**: We did not find any correlation between sequencing depth and the total relative abundance of oral bacteria in feces (Fig. S5). Since oral bacterial fraction is an indicator of total bacterial load, the lack of association is consistent with a previous report of no correlation between sequencing depth and total bacterial load (Vandeputte et al., 2017).

Vandeputte, D., Kathagen, G., D’hoe, K., Vieira-Silva, S., Valles-Colomer, M., Sabino, J., Wang, J., Tito, R.Y., De Commer, L., and Darzi, Y. (2017). Quantitative microbiome profiling links gut community variation to microbial load. Nature 551, 507-511.

**Comment #10**: “*Line 146. Shotgun sequencing does not produce ASV's, one can get strain information however this is not the same as an ASV. And the two cannot be linked unless there is a complete 100% overlap at the sequence level for the 16S rRNA gene to prove they are the same ASV.*”

**Author reply**: We agree with the referee that a metagenome-assembled genome (MAG) can only be linked to a ASV when the MAG’s 16S rRNA gene contains this ASV with 100% sequence match. We assembled 22 high-quality MAGs of *Streptococcus spp.*, among which 4 contains the target oral ASV (*Streptococcus* ASV\_8) that has dominated the intestine of allogeneic stem cell transplant recipients. Only the 4 MAGs were used in the downstream analysis (e.g., calculation of the peak-to-trough ratio). The reconstruction and analyses of *Streptococcus* MAGs were described at lines 232-244.

**Comment #11**: “*It is unclear if the differences across studies maybe be distorting the authors interpretation of their results. For example, it is unclear if the studies included in this meta-analysis were comparable at all. It is more likely that the different 16 studies were not performed using the same sequencing platform (Roche, Illumina, etc), similar primers, similar amplified regions of interest (V1-9), sequence depth, single or paired end, number of bp overlap, extraction/sequencing kit, etc. This is evident when the authors suggest they used the OTU or ASV designation provided by the original study. Thus it is likely clustering and divisive noise reducing strategies were used differently in the studies used to generate their model. This is not addressed in the current manuscript.*”

**Author reply**: We thank the referee for raising this point. Except for the cohort of allogenic stem cell transplantation recipients that have >10,000 samples, we reprocessed all mice and human microbiome samples using the same QIIME2-based pipeline (lines 543-572) in the revised manuscript. It also worth mentioning that we did not combine multiple heterogenous datasets in one analysis but used them individually, each at a time, to reduce the effects of potential biases.

**Comment #12**: “*The taxonomic assignments were also likely assessed with various versions of different reference databases, this is not addressed in the current manuscript.*”

**Author reply**: In the revised manuscript, all microbiome ASVs were taxonomically assigned using SILVA 138 (lines 543-572).

**Comment #13**: “*The authors include a random forest model with a maximum of 1000 trees. This is a vast under sampling of the data, whereas common 16S microbiome papers that apply this strategy have used a >5000 trees.*

[*https://microbiomejournal.biomedcentral.com/articles/10.1186/s40168-018-0568-3*](https://microbiomejournal.biomedcentral.com/articles/10.1186/s40168-018-0568-3) *a microbiome paper applying a similar technique using 10,000 trees”*

**Author reply**: We thank the referee for the paper. As explained in our reply to Comment #6 of the Referee #1, we no longer use Random Forest Classifier to identify oral taxa in host feces.

**Referee #2**

**Comment #1**: “*In their manuscript, "Oral bacteria in feces reflect loss of intestinal bacteria", Liao and colleagues develop a theoretical model and examine data from several studies, most notably a study involving 10,000 HCT patients, in order to answer the question of whether oral microbes bloom in the gut or whether other organisms are depleted. I commend the authors in looking at a wide range of datasets. This is an important question and the authors note that it would have implications for treatment of microbiome-associated disorders. The authors also note that the predominance of oral bacteria in patient cohorts, specifically those involving supragingival and subgingival plaque, have reduced survival rates.*

*The authors' argument rests on models, and the observations that oral species are common to all individuals' guts and that the 'gut-only' species are depleted under antibiotic treatment, as evidenced by relative abundance data and absolute abundance data (measured by qPCR and the weight of feces). I have several major concerns about these claims, which affect all subsequent observations.*”

**Author Reply**: We thank the referee for the insightful feedback.

**Comment #2**: “*The authors provide limited data that oral species are in fact in all individuals' guts. The lack of oral data on the HCT or any antibiotic treated cohort is a major weakness in the paper, but the authors could have propped up their modeling efforts by showing identical ASVs in paired HMP samples, or genome identities in paired HMP metagenomic samples. This is critical for their claims and important to show for both healthy individuals and individuals with dysbiotic microbiomes.*”

**Author Reply**: We agree with the referee that the lack of paired oral samples for the HCT cohort is a limitation. We have made two efforts to soften this limitation. First, we added a mouse experiment where we collected paired oral and gut samples before and after antibiotic treatment (lines 105-127). We showed that the post-treatment fecal samples are compositionally more similar to the pre-treatment oral swabs than the pre-treatment fecal samples (Fig. 2B,C). Quantitatively, the post-antibiotic feces contained 10%-20% ASVs that were exclusively found in the pre-antibiotic oral swabs and not found in the pre-antibiotic feces (Fig. 2D). This simple experiment clearly suggests oral-gut transfer of bacterial ASVs.

Second, we showed that our algorithm to detect oral ASVs in fecal samples without paired oral samples gives reasonable estimates of oral proportions. Notably, identifying oral taxa via 100% sequence overlap between paired oral and gut samples (regardless of how rare they are) can significantly overestimate the proportion of oral bacteria in feces (lines 187-192). Therefore, we focused on oral-typical ASVs, which were highly abundant/prevalent in the HMP oral cavity samples and lowly abundant/prevalent in the HMP fecal samples (lines 183-186 and lines 509-523). The oral-typical ASVs were rarely found in the feces of healthy individuals (lines 187-188), which is expected by definition and consistent with previous reports (Schmidt et al, 2019, Elife; Rashidi et al., 2021, PNAS). In a cohort of IBD patients with dysbiotic microbiome, the total fraction of oral-typical ASVs in the feces of patients with Crohn’s disease and ulcerative colitis are both nearly 4 times as high as the fraction averaged over their own healthy controls. This result suggests relative enrichment of oral ASVs in the gut of IBD patients, which is also consistent with the literature (Read et al., Nature Reviews Gastroenterology & Hepatology). All these tests support the use of ASVs typically colonizing the oral cavity of healthy individuals as reference sequences to infer oral taxa in any fecal samples.

Rashidi, A., Ebadi, M., Weisdorf, D. J., Costalonga, M., & Staley, C. (2021). No evidence for colonization of oral bacteria in the distal gut in healthy adults. Proceedings of the National Academy of Sciences, 118(42), e2114152118.

Schmidt, T. S., Hayward, M. R., Coelho, L. P., Li, S. S., Costea, P. I., Voigt, A. Y., ... & Bork, P. (2019). Extensive transmission of microbes along the gastrointestinal tract. Elife, 8, e42693.

Read, E., Curtis, M. A., & Neves, J. F. (2021). The role of oral bacteria in inflammatory bowel disease. Nature Reviews Gastroenterology & Hepatology, 18(10), 731-742.

**Comment #3:** “*The authors focus heavily on genera common to both oral and gut sites, yet grouping species at the genera-level is likely to lead to spurious inferences. The most obvious example is the Prevotella genus (see figure 1E, lower section and Figure S1). P. copri and to some extent P. ruminocola are the only Prevotella species commonly found in the gut, whereas the oral sites are dominated by species that diverge from the gut species, namely P. dentalis, P. melaninogenica, P. denticola and P. intermedia. These species have starkly distinct habitats and are rarely found in the other site, yet they would be grouped in this analysis. P. copri is also more prevalent in non-US cohorts, and may skew results from other cohorts imputed from the HMP data. Prevotella is the obvious case, but this issue likely affects additional genera.*”

**Author Reply**: We agree with the referee that genus level is insufficient to distinguish oral-transferred vs. gut-resident species. In the revised manuscript, we performed ASV-level analysis throughout the study. This comment is essentially the same as the Comment #2 of the Referee #1. Please refer our reply to that comment for details.  
  
**Comment #4:** “*Given that another major claim rests on abundance measurements that allow the researchers to calibrate their relative abundance data, it would have been nice to see more than one quantification of the stool amounts (another method besides qPCR).*”

**Author Reply**: Thanks for raising this point. Although all quantitation methods have their own limitations, we argue that qPCR is superior to the other molecular (DNA mass)- or cell (flow cytometry)-based approaches when used to estimate absolute numbers of oral bacteria in feces. This point has been discussed before in Jian et al., Frontiers in Cellular and Infection Microbiolgy, 2021: only qPCR is compatible with 16S rRNA sequencing due to shared technical biases introduced in 16S sequencing and its measurement of bacterial loads, not total microbial loads. Therefore, we exclusively used 16S qPCR-based quantification unless the data type is unavailable (e.g., Fig. 5). We discussed this point by adding a short paragraph in discussion (lines 376-389).

Jian, C., Salonen, A., & Korpela, K. (2021). Commentary: How to count our microbes? The effect of different quantitative microbiome profiling approaches. Frontiers in Cellular and Infection Microbiology, 11, 627910.

**Comment #5**: “*Although the writing is clear for the most part, there are places where the authors either assume knowledge of the reader or provide too little information for the author to make out what they did. For example: Line 148, the researchers mention peak-to-trough ratio. The authors assume the reader is familiar with this approach. Additionally, metagenomic sequencing is necessary for this analysis, but there was no preface of what metagenomic data they had (it was all 16S up until this point). It is also misleading as it seems to imply that this analysis was performed on the same samples from which the 16S data came from, but it is not clear if that was the case*.”

**Author Reply**: We thank the referee for thinking that the writing is mostly clear. The manuscript has been revised to improve clarity and effectiveness by adding relevant details and avoiding jargons as much as possible. Regarding the two specific questions, we have added additional descriptions of the peak-to-trough ratio method (lines 238-244) and explicitly mention that the metagenomics data was sequenced from the same patient cohort and samples where we have obtained their 16S data (lines 232-235).

**Comment #6**: “*Overall, the researchers should be more transparent when talking about predicted or imputed oral abundances, since the way it reads now, with so many datasets, it obscures the places in which the authors used actual data (HMP) versus predicted data (most everything else). This also goes for the validation of their predictions—it was difficult to determine what was the actual measured ASV amounts versus predicted via their method or SourceTracker etc.*”

**Author Reply**: We apologize for the confusion. In the current organization, we have an independent section where we established the method to infer oral ASVs in any fecal samples (lines 174-195). All human cohorts after this section were analyzed by applying this inference approach to predict oral ASVs in fecal samples without paired oral samples. We clarified this point at line 194-195.  
  
**Comment #7**: “*The authors' model and the text overall implies that migration of oral organisms to the gut microbiome is mainly through the alimentary canal. There is evidence that translocation happens through the blood stream (doi: 10.3389/fcimb.2020.00400).*”

**Author Reply**: We have mentioned the hematopoietic transmission route in the introduction (line 52).  
  
**Comment #8**: “*The github link was non-functional.*”

**Author Reply:** We apologize for not changing visibility of our repository to public before submission. The source code is now available at <https://github.com/liaochen1988/marker_hypothesis>.

**Referee #3**

**Comment #1**: “*This manuscript by Liao et al. tackles the open question of why increases of typically oral microbes are seen in gut microbiomes of diseased individuals. There are two open hypotheses, with analysis and data in this work supporting the "marker hypothesis". This hypothesis posits that these increases in oral bacteria are simply a marker of a diseased state, resulting from the loss gut taxa (vs. the oral taxa being the drivers of disease). An ecological model that supports this hypothesis is presented that predicts how the abundance of oral taxa in the gut would be affected by flow rates through the system, carrying capacity, and perturbations. Actual gut microbiome data in the form of 16S are then examined across multiple datasets to demonstrate predictions from the model. This manuscript addresses a pertinent question in the field using the mix of theory with data validation. There are many really well done, convincing analyses. The use of a variety of datasets and disease types and the inclusion of total microbial density were especially effective. That being said, there are several areas of concern I have that limit my enthusiasm for the conclusions as presented.*”

**Author Reply**: We thank the referee for the positive comments and constructive critiques to improve our paper.

**Comment #2**: “*My largest concern is over the accurate calling of "oral" taxa in the fecal dataset. Most of the taxa identified as "oral" are gut residents as well, including Prevotella, Veillonella, Actinomyces, etc. Prevotella for example are commonly found in the gut, and often make up large percentages of gut bacteria in non-Western populations. Furthermore, Prevotella collected from different body sites contain different genomic content, suggesting these aren't just translocations from one body site to another, but of evolution while residing in different body sites (*[*https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4359502/*](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4359502/)*). It's not clear what amount of each of these taxa are oral transplants vs. resident taxa in the gut. Classifying them purely as oral taxa will likely lead to misleading estimates of oral transfer, depending on the baseline occupancy of the gut strains of these taxa. Without a calibrated sense of how many of these "oral" bacteria are actually oral in origin, it's difficult to interpret the results of this manuscript.*

*What would clear this issue up would be having a set of paired oral and gut microbiome samples from the same individuals where the transfer of strains of oral origin could actually be quantified down to the strain level. The methods in this paper may very accurately correlate with the actual rate of transfer, but without the underlying experimental data showing that's true (vs. different strains in the oral and gut habitats), it's very hard to assess this. This would require further data collection, but is essential to demonstrate that "oral" taxa are being classified accurately.*”

**Author Reply**: We agree with the referee that some oral bacteria can colonize the gut as well, especially at the genus level (e.g., *Prevotella*). To increase the resolution of detecting oral-gut transmission events, we identified oral taxa at the ASV level in the revised manuscript. Using ASVs/OTUs to detect oral-gut transfer is not uncommon and has been adopted in many studies (e.g., Rashidi et al., PNAS, 2021; Coker et al., Gut, 2018; Hu et al., Genome Research, 2022). We also agree that strain-level analysis might further increase the resolution. But this strain-level analysis is currently prevented by the lack of public microbiome studies that have both quantitative data (e.g., qPCR) and paired oral-gut metagenomic samples. Therefore, the best resolution we can reach in the identification of oral taxa is by matching ASV sequences.

We first profiled the HMP dataset (healthy individuals) at the ASV level. On average, 15.6% fecal ASVs are present in at least one paired oral cavity sample from the same persons. Obviously and as the referee expected, this overestimates the oral-gut transfer because typically, the percentage of oral DNA sequences in fecal samples of healthy people is lower than 2% (Schmidt et al., Elife, 20019). We reduced this percentage substantially by being more conservative and focusing on “oral-typical ASVs”, under the rationale that the more typical ASV of the oral cavity, the less likely it colonizes the gut due to niche specificity, the more likely its presence in feces indicates oral-gut transfer.

We defined oral-typical ASVs as those whose relative abundance and prevalence are higher than certain cutoff values across all HMP oral samples but below the same cutoffs across all HMP fecal samples (lines 183-186 and lines 508-523). A similar idea to identify oral species (not ASVs) has been implemented before (Thomas et al., Nature Medicine, 2019). This filtering step to generate oral-typical ASVs results in reasonable estimates of oral proportions: 219 out of 280 HMP fecal samples contained no oral-typical ASVs and their average was 0.05% (>300-fold reduction compared to 15.6%). Other than the healthy gut microbiome, we further showed that our approach is conservative in identifying oral taxa in the feces of IBD patients: when a fecal ASV was identified as of oral origin, most times it is present in paired oral samples (Fig. S4).

On a final note, the referee’s concern over *Prevotella* has been addressed in our reply to Comment #2 of the Referee #1.

Rashidi, A., Ebadi, M., Weisdorf, D. J., Costalonga, M., & Staley, C. (2021). No evidence for colonization of oral bacteria in the distal gut in healthy adults. Proceedings of the National Academy of Sciences, 118(42), e2114152118.

Coker, O. O., Dai, Z., Nie, Y., Zhao, G., Cao, L., Nakatsu, G., ... & Yu, J. (2018). Mucosal microbiome dysbiosis in gastric carcinogenesis. Gut, 67(6), 1024-1032.

Hu, Y., Amir, A., Huang, X., Li, Y., Huang, S., Wolfe, E., ... & Xu, Z. Z. (2022). Diurnal and eating-associated microbial patterns revealed via high-frequency saliva sampling. Genome Research, 32(6), 1112-1123.

Schmidt, T. S., Hayward, M. R., Coelho, L. P., Li, S. S., Costea, P. I., Voigt, A. Y., ... & Bork, P. (2019). Extensive transmission of microbes along the gastrointestinal tract. Elife, 8, e42693.

Thomas, A. M., Manghi, P., Asnicar, F., Pasolli, E., Armanini, F., Zolfo, M., ... & Segata, N. (2019). Metagenomic analysis of colorectal cancer datasets identifies cross-cohort microbial diagnostic signatures and a link with choline degradation. Nature medicine, 25(4), 667-678.

**Comment #3:** “*There are several concerns with the methodology used to identify oral taxa as presented (NMF). a. There are large differences between the methodology proposed here (NMF) and RF and SourceTracker as indicated by the r > 0.6 between methods. The taxa defined as "oral" do not correlate well between methods (as demonstrated in Figure S1). It's not clear which method is potentially most accurate though, including the newly proposed method. A simulation analysis that demonstrates precision and recall of each method to distinguish source environments on known data would improve interpretability for the reader, providing evidence of effectiveness of each method.*”

**Author Reply:** Thanks for this comment. In the revised manuscript, we chose to replace NMF with a simple yet conservative approach to identify oral ASVs based on exact sequence match. Please refer to our replies to Comment #2 of the Referee #1, #2 and #3 for the details of this new algorithm. Please also refer to our reply to Comment #6 of the Referee #1 for explanation why classical microbial source tracking algorithms do not fit our purpose very well.

We agree with the reviewer that a simulation analysis with known ground truth may be one way to benchmark different approaches. However, gut microbiome ecology (e.g., microbial interactions and priority effects) driving the compositional changes after mixing of oral-transferred and gut-resident taxa has really challenged the accuracy of even well-established approaches such as Source Tracker, FEAST and Random Forest, as exemplified in this preprint (Wang et al., bioRxiv, 2022). Given the complexity of gut microbiome ecology, exploring how various ecological mechanisms affect the performance of different source tracing algorithms is beyond the scope of this paper and warrants a project on its own.

Wang, X. W., Wu, L., Dai, L., Yin, X., Zhang, T., Weiss, S. T., & Liu, Y. Y. (2022). Ecological Dynamics Imposes Fundamental Challenges in Microbial Source Tracking. bioRxiv.

**Comment #4:** “*b. For analyses like those presented in Fig. 3B, how are the abundances of gut and oral taxa being calculated? Is it from estimated proportions of gut and oral overall from each method, or was it by pulling the abundances of individual indicator taxa? Further clarification on how outputs are being used would be beneficial for the reader.*”

**Author Reply:** Throughout the study, the total abundances of oral and gut bacteria in feces were calculated by summing the abundances of individual ASVs inferred as of oral and gut origins respectively. We emphasized this point at both lines 135-136 (for oral taxa identification in mouse feces) and lines 192-194 (for oral taxa identification in human feces).

**Comment #5:** *“The github page with sourcecode is not accessible (perhaps set as private repo?)”*

**Author Reply:** We apologize for not changing visibility of our repository to public before submission. The source code is now available at <https://github.com/liaochen1988/marker_hypothesis>.

**Comment #6:** *“Page 19: Rarefaction to 100 sequences/sample doesn't seem like enough sequences for decent accuracy with SourceTracker.”*

**Author Reply:** As explained in our reply to Comment #3 of Referee #3, SourceTracker is no longer used for identification of oral bacteria in the revised manuscript.