**Relative enrichment of oral bacteria in feces denounces loss of gut commensals with implications to host health**

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**SUMMARY**

High percentages of oral bacteria in feces are typically considered abnormal and have been linked to intestinal disorders. But the association based on relative enrichment has two alternate explanations: ectopic oral bacteria may increase in absolute numbers in the gut and drive disease (the *driver* hypothesis) or, instead, gut commensals may decrease in absolute numbers and the relative rise in oral bacteria is simply an indicator (the *marker* hypothesis). Here we use experiments in mice and data from human patients to provide evidence that the *marker* hypothesis is true. We show that the depletion of gut bacteria by antibiotics increases the relative abundance of oral bacteria in the feces without increasing their absolute abundance. We propose, using mathematical ecology, that the *marker* hypothesis is achieved by balanced effects of direct antibiotic inhibition and indirect ecological release on the oral populations. This means that enrichments detected from compositional sequencing data of fecal samples quantify collateral damage to the gut commensal population, not the overgrowth of ectopic oral bacteria. Host disorders linked to high relative abundances oral bacteria in feces may result not from the expansion of ectopic bacteria but instead from the loss of commensals. Our results shift the paradigm of interpreting microbiome sequencing data with implications for intestinal disorders and microbiome therapies.

**INTRODUCTION**

For microbes, the distinct body sites of healthy people represent very different habitats and the compositions of the microbial communities that live in them are specific (Costello et al., 2009; The Human Microbiome Project Consortium, 2012). Some health disorders cause the translocation of bacteria between body sites and mixes the microbiome compositions (Imai et al., 2021). Bacteria bacteria typical of the oral microbiome can translocate to the lower gastrointestinal tract, and the finding of oral bacteria in feces has been linked to intestinal disorders such as inflammatory bowel disease (IBD) (Read et al., 2021) and colorectal cancer (CRC) (Komiya et al., 2019). Compared to the hematogenous route (oral-blood axis) (Abed et al., 2020), this enteral route (oral-gut axis) is a natural path (Jin et al., 2022; Kitamoto et al., 2020). An average person swallows ~1011 of oral bacteria cells per day (Segata et al., 2012) but oral bacteria remain rare in the healthy gut. Gastric acids, alkaline biles and antimicrobial peptides kill many of those, and the few survivors must overcome the commensal microbiome’s ability to resist colonization (Jin *et al.*, 2022). As a result, compositional sequencing data shows typically <2% of DNA from oral bacterial in the feces of healthy people (Rashidi et al., 2021; Schmidt et al., 2019).

Antibiotics, diet, aging, and gut inflammation can disrupt the oral-gut barrier and cause a relative enrichment oral bacteria in the feces (Kitamoto *et al.*, 2020). That enrichment has been linked to a variety of digestive diseases (e.g., Crohn’s disease (CD) (Gevers et al., 2014), ulcerative colitis (UC) (Schirmer et al., 2018), CRC (Kostic et al., 2013), liver cirrhosis (Qin et al., 2014)). However, nearly all those association come from compositional sequencing data, which gives relative abundances that—by itself—cannot determine if there was an expansion of oral bacteria in absolute numbers, and cannot revela the true cause of the disorder. Simple mathematics leads to two alternate explanations (Fig. 1): In the *driver* hypothesis, the relative abundance informs absolute abundance, and the relative enrichment reflects active expansion of oral bacterial population in the gut that then cause the disorder. By contrast in the *marker* hypothesis, the relative enrichment is driven by loss of gut commensals, and it is this loss that causes the disorder. Determining which hypothesis is true has implications for our understanding of human health and pathologies.

Here, we compare the *driver* and *marker* hypotheses by analyzing the dynamics of oral bacteria in feces in both mice and humans. We quantify the absolute abundances of oral bacteria in feces after antibiotic treatment to show, unequivocally, that their absolute numbers do not increase when their relative numbers do. Instead, the relative enrichment of oral bacteria in feces is due to a depleted gut bacterial population, which proves that the *maker*, not the *expansion*, hypothesis is true. This indicates that link between the relative enrichment of oral bacteria in feces and intestinal disorders is driven by the loss of gut commensals and their associated functions, a finding with implications for future microbiome-based treatments.

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**Fig. 1| The *Expansion* and *marker* hypotheses provide alternative mechanisms to explain the association between the enrichment of oral bacteria in feces and intestinal disorders.** Oral bacteria flow from the oral cavity to the gut where they encounter resistance to colonization by the gut commensals. The relative abundances of oral bacteria in feces can increase after treatment with certain antibiotics or with intestinal disorders. Simple mathematics shows that this enrichment has two possible mechanisms: The relative enrichment may be due to the increase in the absolute abundance of oral bacteria in the gut microbiome, which then drive the disorders (the *driver* hypothesis, in red). Alternatively, the relative enrichment may be due to the decrease in the absolute numbers of gut commensals, and it is this loss that drives the disorders (the *marker* hypothesis, in blue).

**RESULTS**

**Antibiotic treatment of mice depletes their gut commensals and raises percentage of oral bacteria in feces without raising absolute abundances**

Simple mathematics show that the enrichment of oral bacteria in feces could happen without a rise in their absolute abundances if the number of gut commensals decreases (Fig. 1). To show this empirically, we did an equally simple experiment: We treated 3 individually-housed mice with an antibiotic cocktail of ampicillin, vancomycin, and neomycin for a week (Fig. 2A), which we know from previous work impacts the gut microbiome (#cite relevant paper here, ask Ana#). We compared the bacterial compositions of the oral and fecal microbiomes on day 0, prior to the antibiotics, as well as the fecal microbiome on day 8. As expected, the initial microbiomes of the oral and fecal community were distinct, reflecting the different habitats (Fig. 2B). Surprisingly, after antibiotics, the fecal microbiome was more similar to the pre-treatment oral microbiome than the pre-treatment fecal microbiome (Fig. 2C; #can this be shown by PCoA?#). 10%-20% ASVs detected in the post-treatment feces (the majority from 6 members; Fig. S1) were only present in the pre-treatment oral samples and not in the fecal samples (Fig. 2D). We next quantified the abundance of ASVs typical of the oral cavity ( 1e-4 across pre-treatment oral samples and on average across pre-treatment fecal samples). Antibiotic treatment enriched the fecal abundances of these oral ASVs from ~0% to ~30% on average (Fig. 2E). A quantification of their absolute number shows, however, that the relative enrichment was not due to an increase in absolute abundances: the absolute abundances of oral bacteria actually decreased by half a log (Fig. 2F). Instead, we saw a much greater decrease in the absolute abundances of gut commensals is feces, which by ###-fold after antibiotic treatment.

The results from our simple experiment show that the enrichment of oral bacteria in feces can be caused by the loss of gut commensals, which supports the *marker* hypothesis. If this is true then we should be able to determine damage to the gut microbiota by quantifying the relative abundance of oral bacteria in feces. However, most mmicorbiome studies lack paired oral and gut samples, which would prevent us from applying the same paired-sample-analysis to quantify the oral fraction. To overcome the limitation, we developed a way to measure relative abundances of oral bacteria in feces. We compiled a reference set of 149 non-redundant, full-length 16S rRNA genes of culturable bacteria that are found in the oral bacteria of mice but not are rarely found in their gut (Fig. S2, Table S1, see STAR Methods). This database of reference oral bacteria from mice allows us to estimate the fraction of oral bacteria in mouse feces.

To validate this approach we analyzed published data from mice treated with an antibiotic cocktails of ampicillin, vancomycin, neomycin, and metronidazole for 5 days (Reese et al., 2018) (Fig. 2G). Consistent with our results, we saw that antibiotics enriched the feces in oral bacteria of three ASVs of *Lactobacillus*, *Proteus* and *Escherichia-Shigella* (Fig. 2H). In agreement with the *marker* hypothesis, their absolute abundances did not increase, but in fact decreased by ~10 fold on average. (Fig. 2I, left). The absolute abundances of gut bacteria decreased even more, by ### fold (Fig. 2I, right). Interestingly, the fraction of oral bacterial in feces showed a strong negative linearly correlation with the total bacterial loads on the log-log scale (Fig. 2J). This relationship is moderate to strong (Pearson’s r = -0.52, P = 3.5e-17). More importantly, the anti-correlation remained significant when we used the control group data alone (Fig. 2J, data in green, Pearson’s r = -0.25, P = 5.9e-3). This suggests that the fraction of oral bacteria in feces can indeed be used to determine the collateral damage of antibiotics on the gut bacterial populations, as well as to assess natural fluctuations in the gut bacterial populations even in untreated subjects.

We noted that 11% samples collected between day 1-6 (Fig. S3A) with low bacterial loads deviated from the trend line of the association and, according to the *marker* hypothesis, should have very high proportions of oral bacteria. We speculated that the dominant ASVs of these outliers (Fig. S3B) were orally derived but undetected by our inference approach. The omit of uncultured bacteria in the reference set is unlikely the major cause of the potential inference failure, because the proportions of uncultured bacteria based on taxonomic annotation are low to intermediate in these samples (Fig. S3C).

Another public dataset from bone-marrow-transplanted mice that received mono-antibiotic prophylaxis also supported the marker hypothesis (Staffas et al., 2018). In that study, ampicillin but not streptomycin, aztreonam, or vancomycin substantially reduced gut bacterial loads (Fig. S4A). The oral bacterial loads remained stable without active expansion throughout the experiments for all four antibiotics including ampicillin (Fig. S4B). The stable oral bacteria loads led to a strong log-log linear relationship between the oral bacterial fractions and total bacterial loads (Pearson’s r = -0.75, P = 5.5e-11) (Fig. S4C).

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**Fig. 2| Mice treated with antibiotics show that the loss of gut bacteria can lead to the enrichment of oral bacteria in feces without a rise in absolute numbers.** (A) A simple experiment with 3 mice (labeled as M1, M2, M3) treated with an antibiotic cocktail of ampicillin, vancomycin, and neomycin (AVN) for a week. The pre-treatment feces (fecal\_d0) and oral swabs (oral\_d0) were collected on day 0 and the post-treatment feces (fecal\_d8) were collected on day 8. (**B**) Microbiome compositions of all feces and oral swabs show very different oral and fecal microbiomes pre-antibiotics, but an enrichment of oral bacteria in feces post-antibiotics**.** (**C**) Compositional dissimilarity between post-treatment fecal samples and pre-treatment fecal or oral samples provides quantitative evidence. (**D**) Sources of ASVs in the post-treatment fecal samples. (**E,F**) Relative (E) and absolute (F) abundances of oral bacterial populations in the feces. (**G-J**) Reanalysis of a previous study (Reese *et al.*, 2018) leads to the same conclusion. (**G**) Experimental design. Mice were treated with either water (the control group; n=11) or antibiotic cocktails of ampicillin, vancomycin, neomycin, and metronidazole (AVNM) (the antibiotic group; n=10) for 5 days. (**H**) Compositional dynamics of oral bacterial ASVs in feces. (**I**) Absolute abundances of oral (left) and gut (right) bacteria in feces averaged across mice in the control or treatment group. Lines and dots: mean; shading: 95% confidence interval (CI). (**J**) The relationship between total bacterial loads and oral bacterial fractions indicates that the frction of oral bacteria in feces can be used to estimate the absolute amount of bacteria in the fecal microbiome, in antibiotic treated (orange) but also untreated subjects (green). Line: best linear fit; shading: 95% CI. Unit of bacterial load: 16S copies per gram of feces.

**Quantifying the fraction of oral bacteria in human feces**

Most studies of the human gut microbiome also lack paired oral samples. To quantify the oral fraction in the feces of humans , we followed a similar strategy as used in mice: we built a reference set of bacterial 16S rRNA gene sequences that typically colonize the human oral cavity (see STAR Methods). For this we leveraged the Human Microbiome Project (HMP) that have sequenced paired oral (multiple subsites)-gut (stool) microbiomes from 237 healthy volunteers (The Human Microbiome Project Consortium, 2012). Distinct bacterial communities were found inhabiting the two body sites of (Fig. 3A), which provides a pragmatic basis to define oral-typical ASVs based on their abundance and prevalence in healthy people. We called oral bacteria to those whose mean relative abundance and prevalence was greater than 0.01% and 5% respectively among all oral cavity samples, and no greater than the same cutoffs among all fecal samples (Fig. 3B,C). The rationale of filtering is that the more typical ASV of the oral cavity, the less likely it colonizes the gut due to niche specificity, and the more likely its presence in feces indicates oral-gut translocation.

The analysis revealed 178 unique oral ASVs (Table S2); the leading genera were *Prevotella* and *Streptococcus* (Fig. 3D). 219 out of 280 HMP fecal samples contain none of these oral ASVs, and their mean relative abundance is as low as 0.05% (Fig. 3E). This is consistent with previous findings that oral bacteria are rare in the feces of healthy people (Rashidi *et al.*, 2021; Schmidt *et al.*, 2019). To show that filtering is key to 16S rRNA-based inference of oral bacteria, we took an alternative approach by counting every single ASV in feces as of oral origin if it is found in any of paired oral cavity subsite samples. This naïve approach led to an average of 247 oral ASVs per HMP fecal sample and their mean relative abundance rises >300-fold to 15.6%. The severe overestimation suggests that the majority of shared ASVs are not indicators of oral-gut transmission but coincidences of closely related bacteria occupying both niches.

To validate this approach to quantify the abundance of oral bacteria in the gut of patients we used one of the few studis that has paired oral (saliva)-gut (stool) samples. The subjects in the study included IBD (CD and UC) and healthy controls (HC). We saw that the estimated oral bacterial fractions in feces was robust against variations of the filtering cutoffs used to generate the reference set of oral bacterial sequences (Fig. S5). Using the default reference set, we estimated that the total fractions of oral ASVs in the feces of IBD patients were averagely 4 times (CD: 4.2%, UC: 4.3%) as high as the mean fraction in the feces of HC (1.1%) (Fig. 3F), confirming the notion that IBD patients are enriched with oral bacteria in their gut. Among 99 fecal samples (HC:41, CD:16, UC:42) that contained at least one oral ASV, the proportion of oral ASVs that were also found in paired saliva samples is >70% in 87 samples (HC:36, CD:15, UC:36; Fig. 3G). Similarly, >70% of the total fractions of oral ASVs in 90 out of 99 fecal samples was contributed by those found in the paired saliva samples (HC:38, CD:15, UC:37; Fig. 3H). Both computational validations indicate that our inference approach is conservative and less prone to false-positive predictions (i.e., inferred oral ASVs absent from the oral cavity).

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**Figure 3. Data from healthy individuals reveals bacterial DNAs typically found in the human oral cavity but not the intestine.** (**A**) An overview of the 4,354 microbiome samples from the Human Microbiome Project (HMP). T-distributed stochastic neighbor embedding (tSNE) (Van der Maaten and Hinton, 2008) collapses all samples (dots) into distinct clusters based on their compositions. (**B, C**) The mean relative abundance (B) and prevalence (C) of ASVs (dots) among all HMP oral cavity samples (x axis) and the fecal samples (y axis). 178 ASVs were identified as oral-typical and highlighted in orange. (**D**) Genus-level distribution of the 178 oral-typical ASVs. (**E, F**) The total fractions of oral-typical ASVs in the feces of (E) HMP subjects (n=280) and (F) patients with inflammatory bowel disease as well as their own healthy controls (n=101). Each circle represents a fecal sample. HC: healthy control (n=43); CD: Crohn’s disease (n=16); UC: ulcerative colitis (n=42). (**G**) Proportions of fecal ASVs inferred as of oral origin that can also be found in paired saliva samples. (**H**) Total fractions of inferred oral ASVs that were also found in the paired saliva samples, divided by total fractions of all inferred oral ASVs in the feces. Data sources: panels A-E (The Human Microbiome Project Consortium, 2012); panels F-H (Imai *et al.*, 2021).

**DNA of oral bacteria increase in relative numbers in patient feces revealing damage to the gut microbiome**

We determined the relative abundances of oral bacteria in a large-scale microbiome dataset from adult patients receiving allogeneic hematopoietic cell transplantation (allo-HCT) (Liao et al., 2021; Yan et al., 2022). Allo-HCT us the only curative therapy for a variety of hematologic disorders including leukemias, lymphomas, and myeloma (Shono and van den Brink, 2018). Along with other related procedures (conditioning regime, antibiotic exposure, etc.), allo-HCT disrupts the gut microbiome compositions of the recipients, where *Enterococcus* and *Streptococcus* are the two most abundant genera that frequently dominate (>30% relative abundance of a single ASV; (Taur et al., 2012)) the intestinal bacterial communities (Fig. 4A, top) (Peled et al., 2020). Among the 10,433 samples from 1,276 patients, 901 were dominated by at least one oral ASV and the leading genera of these ASVs are *Streptococcus*, *Actinomyces*, and *Abiotrophia* (Fig. 4A, middle). The total bacterial loads of a subset of 3,126 samples showed that the fecal samples highly enriched with oral ASVs have lower bacterial biomass (Fig. 4A, bottom). We confirmed that the total fractions of oral ASVs detected in the feces of allo-HCT recipients are not correlated with sequencing depths (Fig. S6).

To further test whether the bacteria were indeed of oral origin, we used co-occurrence analysis (see STAR Methods) to identify combinations of oral ASVs that were simultaneously present in the feces more than expected by chance. The co-occurring oral ASVs, if existed, would indicate collective bacterial transmission from the oral cavity to the gut. We found 71 such oral-ASV groups of size from 2 to 5, among which 55 contain *Streptococcus* ASVs (Table S3). To identify the species of the *Streptococcus* ASVs, we extracted shotgun metagenomes from 19 samples which have at least 10% (measured by 16S amplicon sequencing) ASV\_8 (the most abundant *Streptococcus* ASV inferred as of oral origin) from the same allo-HCT cohort. We were able to recover 22 high-quality metagenome-assembled genomes of *Streptococcus spp.*, among which 15 were annotated as *S. thermophilus*—a probiotic species of lactic acid bacteria. To assess the viability of the *Streptococcus* genomes, we computed the ratio of metagenomic reads near the replication origin to the replication terminus (i.e., peak-to-trough (PTR) ratio) to estimate the simultaneous replication events averaged across the population ([Gao and Li, 2018](https://elifesciences.org/articles/45931#bib3); [Korem et al., 2015](https://elifesciences.org/articles/45931" \l "bib4)). We found that the averaged PTR ratio is 1.50 0.16 (Table S4), suggesting a possible mixture of 50% 2-fork and 50% single-fork cells. The PTR-based growth estimates indicated that these *Streptococcus* genomes generally had slow to intermediate growth at the time of sampling.

**Piperacillin/tazobactam depletes gut commensals and drives relative enrichment of oral bacteria in feces**

The allo-HCT recipients require antibiotics to prophylactically minimize the risk of developing infections before immune system reconstitution and/or to treat infections when they develop (Shono and van den Brink, 2018). For the cohort we used, the allo-HCT procedure usually started antibiotic prophylaxis within a week prior to transplantation. The timing of antibacterial antibiotic administration (Fig. 4B, top) corresponded well to the declined gut bacterial relative (Fig. 4B, middle) and absolute (Fig. 4B, bottom) abundances. Since the mean oral bacterial loads fluctuated around a stable average, the increased relative abundance of oral ASVs was mainly driven by the declined gut bacterial load. This supports the *marker* hypothesis and generates a negative correlation (Pearson’s r = -0.27, P = 2.2e-54) between the oral bacterial proportions and the total bacterial loads in feces (Fig. 4C). The inverse correlation of oral bacteria is in direct contrast to *Enterococcus* (Stein-Thoeringer et al., 2019) and *Candida spp.* (Rolling et al., 2021; Zhai et al., 2020) whose relative abundances in the gut informs their absolute quantities.

We next conducted a time-varying survival analysis to quantify the effects of the different antibiotics associated with enriched oral ASVs feces. Piperacillin/tazobactam (TZP, P=5.7e-7)—a combination of beta-lactam and beta-lactamase inhibitor—strongly increased the risk of domination by oral-derived bacteria (Fig. 4D). We confirmed this association in an independent cohort of pediatric allo-HCT recipients (Bekker et al., 2019), by observing that children who have received oral TZP had much higher relative abundance of oral bacteria in feces than those who have received oral polymyxin/neomycin (Fig. S7). According to the *marker* hypothesis, the positive association of TZP reflects the strong anaerobe-killing capacity of this drug (Morjaria et al., 2019). Other than TZP, orally (P=0.049) but not intravenously (P=0.58) administered vancomycin is associated with intestinal domination of oral bacteria, likely because the latter fails to reach the gut. Interestingly, quinolones (P=6.1e-4) reduced the risk and thus preserved the total bacterial loads.

(##talk about quinolones; I’ve added some text based on Tobias and Thierry’s e-mails. This may need more work.##). We also saw a negative association between quinolones and the fraction of oral bacteria in feces (Fig. 4D). Quinolones are widely used as prophylaxis for patients with neutropenia, active against *Pseudomonas* and many pathogenic Enterobactericeae. One explanation for the negative association could be a direct impact on oral communities by quinolones.

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**Figure 4. Oral bacteria in the feces of allo-HCT recipients increase in relative, but not absolute, numbers with procedures that cause collateral damage to the gut microbiota.** (**A**)Fecal microbiome compositions in all 10,433 fecal samples. The taxonomic composition (top), the calculated fractions of oral bacteria (middle), and total bacterial loads (bottom) aligned below each sample. (**B**) Antibiotic administration (top) and population dynamics of the relative (middle) and absolute (bottom) abundances (16S copies per gram feces) of oral and gut bacteria in the feces. Line and dots: mean; shading: 95% confidence interval (CI). (**C**) Negative association between proportions of oral bacteria in feces and total bacterial loads across 3,055 samples (dots) with loads no less than 1,000 16S copies per gram feces. Bar height: mean; red line: best linear fit; shading and bars: 95% CI. (**D**) Association between antibiotic exposure and intestinal domination of oral ASVs. Vertical line: hazard ratio; bar width: 95% CI. \*\*\*\*: P<0.0001; \*\*\*: P<0.001, \*: P<0.05. Chi-square test.

**Ecological mechanisms underlying the *marker* hypothesis**

To elucidate the mechanisms of the *marker* hypothesis, we developed a mathematical model of microibome ecology that considers two competing forces that regulate the oral bacterial load in the intestine (Fig. 5A): antibiotics can inhibit their growth but also relieve them from the competitive inhibitions from the gut commensals (i.e., ecological release effect). The kinetic equations of the model are shown below

Eq. (1)

Eq. (2)

Here and are the oral and gut bacterial loads respectively, is the rate of oral-gut transmission, is the rate constant of bacterial loss, and are the carrying capacities, and and are the maximum growth rates. The physiological values of these parameters (Table S5) were taken from the literature ( and ), predicted by theoretical modeling ( and ), or estimated by fitting human data ( and ). The antibiotic susceptibility of the two populations was described by and (the higher , the less susceptible) as free parameters. See Supplementary Text and Table S6 for basic model assumptions, analytical solutions, and parameter estimations.

The *marker* hypothesis states that antibiotics caused relative enrichment, but not absolute expansion, of oral bacterial population in feces. By simulating the model (Eq. 1-2) under constant antibiotic exposure, we identified the parameter ranges of and that correspond to the regime of the *marker* hypothesis. We found that the oral bacterial fractions (relative abundance) and loads (absolute abundance) can increase, remain nearly unchanged, or decrease, depending on the values of and (Fig. S8). However, the regime of the *marker* hypothesis (i.e., increased relative abundance but reduced absolute abundance) is limited to a narrow region of the parameter space (Fig. 5B, blue shading), where the two populations need to be both susceptible and have similar antibiotic susceptibility (Fig. 5B). In this scenario, the antibiotic-mediated growth inhibition and ecological release are generally balanced for the oral bacterial population. Though not observed in the data analysis of this study, our simulations suggest that antibiotics can increase both the relative and absolute abundances of oral bacterial populations in the gut (Fig. 5B, green). This condition requires that gut bacteria are more susceptible to antibiotics (), which can be met when the oral populations consist of multi-drug resistant bacteria.

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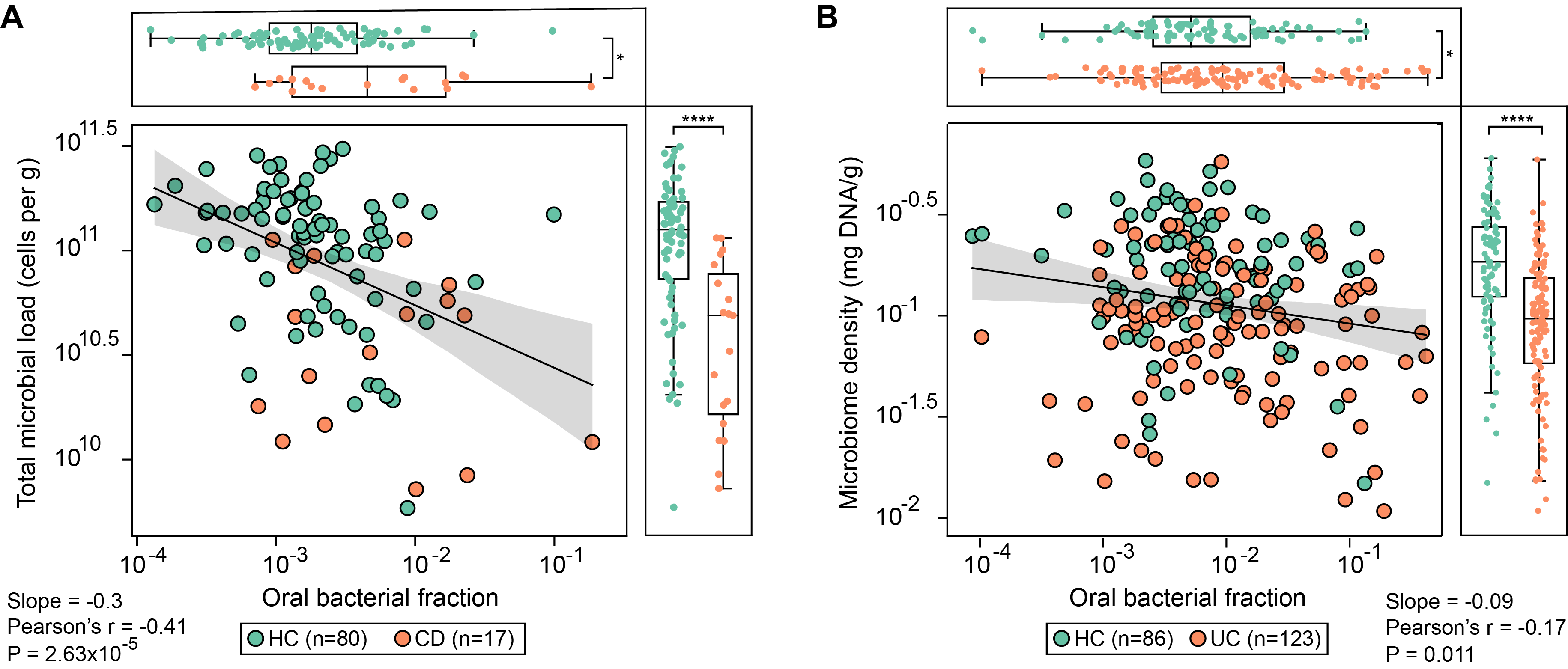
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**Fig. 5| Mathematical model of oral bacteria in the gut microbiome describes the shift from low-to-high relative numbers of oral bacteria in the feces after antibiotics.** (**A**) Microbiome ecology of oral bacterial growth in the gut. The oral bacterial load (absolute abundance) is regulated by two mechanisms in opposite directions: antibiotics directly inhibit oral bacterial growth but indirectly promote their growth by releasing their inhibitions from the gut bacteria. and quantify the inhibitory levels of antibiotics on the oral and gut bacterial populations respectively. (**B**) Distinct response patterns of oral bacteria to antibiotics. The patterns were distinguished by qualitative changes ( , increase; , decrease; , no change) in the time-averaged relative abundances (rel. abun.) and absolute abundances (abs. abun.).

**The *marker* hypothesis unifies competing microbiome biomarkers of IBD**

A damaged gut microbiota is a hallmark of intestinal disorders, but there are competing ways to determine damage. For example, different studies found associations between the relative enrichment of oral-associated bacterial species (Read *et al.*, 2021) and low absolute values of microbial loads (Contijoch et al., 2019; Vandeputte et al., 2017) in feces and IBD (both UC and CD). Since these associations were reported by separate studies, this raises a question: are they independent or related?

The *marker* hypothesis predicts a negative association between the two. Indeed, the fraction of oral bacteria and total bacterial loads in the feces of patients with CD (Pearson’s r = -0.41, P = 2.63e-5, Fig. 6A) and UC (Pearson’s r = -0.17, P = 0.011; Fig. 6B) were negatively correlated, and each feature was also significantly different between the patients and their healthy controls. For the UC cohort, the association became insignificant (P=0.32) after excluding patients with prior antibiotics or an unclear antibiotic history, due to the loss of significant differences of oral bacterial fractions between the patients and controls. Since oral bacteria can be significantly enriched in the feces of IBD patients who have not taken antibiotics weeks prior to study (Fig. S9), antibiotics can amplify the gut microbiome dysbiosis in IBD patients (Gevers *et al.*, 2014) and strengthen the negative relationships between the two biomarkers. In conclusion, both the fraction of oral bacteria and the total number of microbes in the gut could be indicating a loss of gut bacteria, with implications for IBD.



**Figure 4. The marker hypothesis unifies previous work showing associations between oral bacterial and low bacterial loads in the feces of patients with inflammatory bowel disease.** (**A**)Crohn’s disease (CD)(Vandeputte *et al.*, 2017)and (**B**)Ulcerative colitis (UC) (Contijoch *et al.*, 2019).Each circle represents a fecal sample. Lines: best linear fits; shading: 95% confidence intervals; HC: healthy control. \*\*\*\*P<0.0001; \*P<0.05; Welch's t-test.

**DISCUSSION**

Much of the human gut microbiome research focuses on amplicon-based profiling of microbiome composition, with a few notable exceptions (Contijoch *et al.*, 2019; Jian et al., 2020; Rao et al., 2021; Schluter et al., 2020; Vandeputte *et al.*, 2017; Vieira-Silva et al., 2019). Amplicon-based sequencing produces compositional data, which requires carefull interpretation. The absolute load of bacteria in the gut has received much less attention, but it is a fundamental parameter that impacts host-microbiome interactions (Contijoch *et al.*, 2019; Tang et al., 2019; Zarrinpar et al., 2018). For example, the total microbial load in the gut regulates the proportion of mucosal RORγt+ Treg cells in mice (Britton et al., 2020). Changes in gut microbial load do not strictly reflect changes in the biodiversity (Contijoch *et al.*, 2019), suggesting that the two metrics describe different aspects microbiome disfunction.

Cell-counting (e.g., flow cytometry) and molecular-based methods (e.g., qPCR, DNA mass) can measure absolute microbial abundances (Galazzo et al., 2020). These methods have their own limitations, but qPCR is arguably superior for quantifying of oral bacteria in feces thanks to the compatibility with the 16S rRNA sequencing (Jian et al., 2021). 16S sequencing and qPCR cannot distinguish live from dead (including free DNAs) cells, but their combination measures absolute abundances of live, not total (live and dead), oral bacteria (see Supplementary Text for justification). Technical biases introduced in the extraction, purification, and amplification steps of 16S sequencing are shared with qPCR, but not other approaches. Finaly, 16S qPCR measures bacterial loads, not toal microbial loads, which is compatible with 16S sequencing. Therefore, we exclusively used 16S qPCR-based quantification unless the data type is unavailable (e.g., Fig. 6).

Quantitative microbiome profiling bypasses compositionality effects and can thus calibrate the associations learned from relative microbiome profiling. One notable example is the negative association between *Bacteroides* and *Prevotella*, which is an outcome of relative microbiome analysis and disappears by taking absolute cell counts into accounts (Vandeputte *et al.*, 2017). Resonating with this finding, our study revealed another discrepancy between relative and absolute abundances: the relative enrichment of oral-derived bacteria in the gut does not reflect their active population expansion, but simply indicates a depleted gut microbiome state. Therefore, the various bacterial species (e.g., *Streptococcus spp.*, *Veillonella parvula*, *Fusobacterium nucleatum*) found enriched in the intestine of IBD patients (Read *et al.*, 2021) may not be biologically different; they could all be biomarkers of declined gut bacterial load (Duvallet et al., 2017). Despite low absolute amount of orally derived bacteria in the gut, they may have functional impacts on human health. For example, the orally translocated *Klebsiella spp.* causes Th1 cell expansion in the mouse gut (Atarashi et al., 2017). The functional characterization (e.g., metabolomics) of the depleted gut microbiome state with high relative abundance of oral bacteria certainly warrants further study in the future.

#New conclusion paragraph should return to the importance of the microbiome for human health, and the disorders associated with losing commensals. End by proposing that the oral fraction of bacteria in feces is an easy way to assess the size of the gut commensal population. This can open the way to new diagnostics and therapies to restore microbiome function. #

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**AUTHOR CONTRIBUTIONS**

Conceptualization, C.L., T.R. and J.B.X.; Mouse experiment: A.D.; Microbiome data processing: C.L. and H.L.; Microbiome data analysis, C.L.; Methodology, C.L.; Investigation, C.L. and T.R.; Writing – Original Draft, C.L., T.R., A.D., H.L.; Writing – Review and Editing, T.M.H., J.B.X., J.U.P., B.Z., L.D., and M.R.M.v.d.B.; Supervision, J.B.X. and T.M.H.

**DECLARATION OF INTERESTS**

J.U.P. reports research funding, intellectual property fees and travel reimbursement from Seres Therapeutics and consulting fees from DaVolterra, CSL Behring and from Maat Pharma. He has filed intellectual property applications related to the microbiome (reference nos. 62/843,849, 62/977,908 and 15/756,845). M.R.M.v.d.B. has received research support from Seres Therapeutics; he has consulted, received honorarium from or participated in advisory boards for Seres Therapeutics, WindMIL Therapeutics, Rheos, Frazier Healthcare Partners, Nektar Therapeutics, Notch Therapeutics, Forty Seven Inc., Priothera, Ceramedix, Lygenesis, Pluto Immunotherapeutics, Magenta Therapeutics, Merck & Co., Inc. and DKMS Medical Council (Board); and he has IP Licensing with Seres Therapeutics, Juno Therapeutics and stock options from Seres and Notch Therapeutics. T.M.H. has participated in a scientific advisory board for Boehringer-Ingelheim Inc.

**STAR METHODS**

**KEY RESOURCES TABLE**

|  |  |  |
| --- | --- | --- |
| **REAGENT or RESOURCE** | **SOURCE** | **IDENTIFIER** |
| **Biological samples** | | |
| Fecal and oral samples from mouse | This study | NA |
| **Chemicals** | | |
| Ampicillin |  | Cat# |
| Vancomycin |  | Cat# |
| Neomycin |  | Cat# |
| **Deposited Data** | | |
| This study | SRA | PRJNA873058 |
| Reese *et al.* (2018) | SRA | PRJEB26446 |
| Staffas *et al.* (2018) | SRA | PRJEB24887 |
| Theis et al. (2020) | SRA | PRJNA594727 |
| The Human Microbiome Project Consortium (2012) | Qiita | 1928 |
| Imai *et al.* (2021) | SRA | PRJNA684508,  PRJNA684584 |
| Pascal et al. (2017) | SRA | PRJNA422193 |
| Liao *et al.* (2021); Yan *et al.* (2022) | SRA | PRJNA394877, PRJNA607574, PRJNA606262, PRJNA548153, PRJNA545312 |
| Bekker *et al.* (2019) | SRA | PRJEB28845 |
| Vandeputte *et al.* (2017) | SRA | PRJEB21504 |
| Contijoch *et al.* (2019) | SRA | PRJNA413199 |
| **Experimental Models: Organisms/Strains** | | |
| Female C57BL6/J Mice | The Jackson Laboratory | JAX Cat# 000664; RRID: IMSR\_JAX:000664 |
| **Oligonucleotides** | | |
| Primer: 16S\_Forward\_563F 5’-AYTGGGYDTAAAGNG-3’ | Taur et al. (2018) | NA |
| Primer: 16S\_Reverse\_926R 5’-CCGTCAATTYHTTTRAGT-3’ | Taur *et al.* (2018) | NA |
| qPCR: 16S\_Forward\_27F 5’-AGAGTTTGATCMTGGCTCAG-3’ |  | NA |
| qPCR: 16S\_Reverse\_338R 5’TGCTGCCTCCCGTAGGAGT-3’ |  | NA |
| **Databases** | | |
| SILVA v138 | Quast et al. (2012) | www.arb-silva.de |
| Virtual Metabolic Human (VMH) | Magnúsdóttir et al. (2017) | www.vmh.life |
| The Mouse Gut Microbial Bank (mGMB) | Liu et al. (2020) | NA |
| The Mouse Intestinal Bacterial Collection (miBC) | Lagkouvardos et al. (2016) | NA |
| Mouse Oral Microbiome Database (MOMD) | Joseph et al. (2021) | momd.org |
| **Softwares** | | |
| Python v3.7.9 | Python | python.org |
| Scikit learn v0.24.0 | Pedregosa et al. (2011) | scikit-learn.org |
| Lifelines v0.25.6 | Davidson-Pilon (2019) | github.com/CamDavidsonPilon/lifelines |
| iRep v1.10 | Brown et al. (2016) | github.com/christophertbrown/iRep |
| DADA2 v1.20 | Callahan et al. (2016) | benjjneb.github.io/data2 |
| Cutadapt v3.4 | Martin (2011) | github.com/marcelm/cutadapt |
| Bhatt lab workflow | Siranosian et al. (2022) | github.com/bhattlab/bhattlab\_workflows |
| MEGAHIT v1.2.9 | Li et al. (2015) | github.com/voutcn/megahit |
| MetaBAT 2 v2.12.1 | Kang et al. (2019) | bitbucket.org/berkeleylab/metabat/src/master/ |
| CONCOCT v1.1.0 | Alneberg et al. (2014) | github.com/BinPro/CONCOCT |
| DAS Tool v1.1.2 | Sieber et al. (2018) | github.com/cmks/DAS\_Tool |
| Kraken2 v2.1.1 | Wood et al. (2019) | github.com/DerrickWood/kraken2 |
| QIIME2 v2021.8 | Bolyen et al. (2019) | qiime2.org |

**RESOURCE AVAILABILITY**

**Lead contact**

Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Joao B. Xavier (xavierj@mskcc.org)

**Materials Availability**

This study did not generate new unique reagents.

**Data and code availability**

* All raw sequences of the microbiome datasets analyzed in this study are public available with accession numbers listed in the key resource table.
* All processed data supporting the findings of this study are available within the article and its supplementary materials.
* Customized Python scripts for all analyses included in this study are available on Github (<https://github.com/liaochen1988/marker_hypothesis>).

METHOD DETAILS

**Mouse experiment setup**

We treated C57BL/6J female mice with cocktail of ampicillin (0.5g/l), vancomycin (0.5g/l) and neomycin (1g/l) for one week in drinking water. Antibiotics were changed once during the course of the treatment. Animals were single-housed in autoclaved cages. Autoclaved water supplemented with antibiotics and 5053 irradiated food was provided *ad libitum*. Fecal pellets were collected immediately before and one week after the initiation of antibiotic treatment. Oral swabs were collected as per (Abusleme et al., 2017) before the treatment was started. Briefly, mice were hand-held while sterile swab was introduced into mouth and swiped for at least 30 seconds. After, the swab was put into 150ul of TE, the tip was cut off so that the eppendorf can be closed. Samples were put immediately to dry ice. One negative control swab was taken by pulling out the swab from the pouch and swirling through air for at least 30 sec, after which it was put in TE and on dry ice. Fecal samples and oral swabs were kept at -80ºC until further processing.

**DNA extraction and sequencing**

Fecal DNA was extracted and 16S rRNA gene was amplified using the previously described protocol (Taur *et al.*, 2018). Illumina TruSeq Sample Preparation protocol was used for generating libraries, that were later quantified, normalized, and sequenced using MiSeq Reagent Kit V3. Oral DNA was extracted by using modified DNeasy Blood and Tissue Kit protocol as described in (Abusleme et al., 2017). After extraction 16S rRNA gene was amplified and KAPA LTP Library Preparation Kit was used to generate sequencing libraries that were later quantified, normalized and sequenced using MiSeq Reagent Kit V3.

**Quantitative PCR (qPCR) for determining bacterial load**

For assessing the bacterial load in fecal and oral samples, qPCR against standard curve was used to determine 16S rRNA copy number. For this purpose, the PowerUP qPCR Kit was used. Briefly, for each sample, 20μl PCR triplicates were prepared with each containing 2μl of the DNA used as template, 10μl of mix provided by the manufacturer, and 1μl of forward and reverse primers at the final concentration of 0.5μM (F- AGAGTTTGATCMTGGCTCAG; R- TGCTGCCTCCCGTAGGAGT). In order to complete the volume of the reaction, 6μl of water was added. A PCR product of the 16S rRNA gene from *Enterococcus faecium* ATCC 700221 strain was used for obtaining a standard curve by amplifying its 16S rRNA gene and purifying the product. The copy number of the PCR product was determined based on its concentration and 16S rRNA sequence. A standard curve was obtained by using 10-fold dilutions.

Cycling conditions of the qPCR were 50ºC for 2 minutes, 95ºC for 2 minutes, and 40 cycles of 95ºC for 15 seconds, 56ºC for 15 seconds and 72ºC for 60 seconds. By extrapolating results by looking the ones obtained from standard curve samples, the number of 16S rRNA genes was determined for each sample. The final number of 16S rRNA genes per 1g of fecal sample was calculated by multiplying the number of 16S rRNA molecules obtained by qPCR with DNA elution volume after DNA extraction and dividing this number with the weight of the fecal pellet from which DNA extraction was performed.

**Inference of oral-typical bacterial sequences**

We constructed two reference sets of 16S rRNA sequences, separately for humans (V4-V5 region) and mice (full length), to identify bacteria typically colonizing the oral cavity. A 16S rRNA sequence detected in human or mouse gut microbiome can be classified to be oral-typical or oral-atypical by exactly matching the sequence to the corresponding reference set. The 16S rRNA regions typical of the human oral cavity were selected by filtering the microbiome profiles of the Human Microbiome Project at the ASV (V4-V5 region of 16S rRNA gene) level. An ASV is oral-typical if (1) its relative abundance averaged across all oral cavity samples (including samples from all oral cavity subsites, the same below) is greater than 1e-4; and (2) its relative abundance averaged across all fecal samples is no greater than 1e-4; and (3) its prevalence across all oral cavity samples is greater than 0.05; and (4) its prevalence across all fecal samples is no greater than 0.05. Prevalence of an ASV was computed as the proportion of samples that contain the ASV at a relative abundance above 1e-3 (Machado et al., 2021). Similar thresholds of relative abundance (1e-3 at the species level) and prevalence (0.05) were used in a previous study to identify oral-typical species (not ASVs) from metagenomic profiles (Thomas et al., 2019).

Our approach of constructing the mouse reference set is visualized in Fig. S2. Due to a lack of large-scale microbiome datasets with paired oral and gut samples, we used the Mouse Oral Microbiome Database (MOMD) (Joseph *et al.*, 2021) as the starting point for gathering oral-typical sequences. MOMD has 164 full length rRNA sequences from cultured isolates. By reciprocal blast search (-perc\_identity 99 -qcov\_hsp\_perc 100 -ungapped) between MOMD and a combined collection of 344 cultured gut bacterial 16S rRNA sequences from the Mouse Gut Microbial Biobank (mGMB) (Liu *et al.*, 2020) and the Mouse Intestinal Bacterial Collection (miBC) (Lagkouvardos *et al.*, 2016), we found that 38 sequences are likely from common gut bacteria as well. Meanwhile, we profiled the paired oral (swab) and gut (distal colon) samples from a small set (n=11) of pregnant mice in a public study (Theis *et al.*, 2020) and identified 17 oral-typical ASVs by following conditions (1) and (2) defined for humans above. Only 5 of 17 ASVs were found in MOMD; by searching the nucleotide database in NCBI (National Center for Biotechnology Information), we obtained 29 full length mouse 16S rRNA sequences that cover 6 of the 12 missed oral-typical ASVs in full. Given these preliminary analyses, a filtered (and expanded) MOMD to be used for inference was constructed in three steps. First, 37 sequences that belong to either the set of 39 potential common gut bacterial sequences or the taxa that contains at least 3 sequences in the set, but not matched to the 17 oral-typical ASVs, were removed. Next, the 29 sequences matching the 17 oral-typical ASVs from the coverage test were added. Finally, 7 redundant sequences were found and removed if there exist longer sequences that fully contain them as parts.

**16S amplicon sequencing**

The >10,000 microbiome samples from the adult allo-HCT recipients were previously analyzed by an in-house processing pipeline and the ASV profiles were available in a recent compilation study (Liao et al., 2021). Briefly, reads were trimmed to the first 180 bp or the first point with a quality score Q<2, and removed if they contained ambiguous nucleotides (N) or if two or more errors were expected based on the quality of the trimmed reads. ASVs were identified using DADA2 (Divisive Amplicon Denoising Algorithm) (Callahan *et al.*, 2016) and classified by IDTaxa (Murali et al., 2018) and the SILVA v138 database (Quast *et al.*, 2012).

The demultiplexed and primer-trimmed HMP 16S sequences were downloaded from the Qitta repository (Gonzalez et al., 2018) and processed by QIIME (Quantitative Insights Into Microbial Ecology) 2 (Bolyen *et al.*, 2019) . DADA2 was used to denoise data and generate an ASV per sample counts table, using the QIIME denoise-pyro plugin (Bolyen *et al.*, 2019). Parameter --p-trunc-len 395 was used to remove low quality tails. Taxonomy classification of the representative ASV sequences was performed using the QIIME plugin “feature-classifier” (Bokulich et al., 2018) and the SILVA v138 database (Quast *et al.*, 2012). The classification took three steps. We first extracted the V3-V5 region of the SILVA reference sequences using the extract-reads method. Then we created a classifier by using the fit-classifier-naïve-bayes method with extracted reads and the SILVA reference taxonomy. Finally, we ran the classifier on the ASV sequences using the classify-sklearn method to get their taxonomy.

All other microbiome datasets used in this study were similarly processed using QIIME 2 (Bolyen *et al.*, 2019). When needed, primers were removed from demultiplexed short reads using the QIIME cutadapt plugin (Martin, 2011) with parameters “--p-error-rate 0.1” and “--p-overlap 3”. The trimmed reads were denoised using the QIIME dada2 plugin with truncation lengths determined by per-base quality scores to generate feature tables at the ASV level. Taxonomic classification was performed using the QIIME plugin “feature-classifier classify-sklearn” (Pedregosa *et al.*, 2011) against the SILVA 138 database (Quast *et al.*, 2012) at a cutoff of 80% and inferred to the lowest possible taxonomic level.

For each sample, we only kept bacterial ASVs and removed those whose taxonomy contains keywords “Chloroplast” or “Mitochondria”. We further discarded samples whose total sequencing depths are smaller than 1,000 reads.

**Shotgun metagenomic sequencing**

We adapted a recently published pipeline (Siranosian *et al.*, 2022) to assemble the contigs from short reads using MEGAHIT (Li *et al.*, 2015) and then bin the contigs into Metagenome-assembled genomes (MAGs) using two different methods: Metabat2 (Kang *et al.*, 2019) and CONCOCT (Alneberg *et al.*, 2014). The results were then aggregated to produce an optimized, non-redundant set of MAGs by DAS Tool (Sieber *et al.*, 2018). The high-quality MAGs (75% complete, 175 fragments/Mbp sequence, and 2% contamination) classified as *Streptococcus spp.* by Kraken2 (Wood *et al.*, 2019) were analyzed by iRep (Brown *et al.*, 2016). The iRep value of a MAG represents the average number of replication events over different subpopulations of the MAG weighted by their relative abundances.

**Co-occurrence analysis**

The algorithm for computing co-occurring bacterial communities is described in detail elsewhere (Machado et al., 2021). Briefly, it begins with ASV pairs and iteratively identifies co-occurring ASVs of larger combination sizes. The presence/absence of an ASV is determined by a relative abundance cutoff of 0.001. A combination of ASVs must satisfy the following criteria to be considered as co-occurring: (1) they must co-occur in at least 200 patients and 500 samples; (2) they must co-occur at least twice more than expected by chance, which is estimated by assuming each ASV is observed independently and its number of observation can be modeled by a binomial distribution; (3) the (false discovery rate) FDR-corrected *P*-values for the independent observation hypothesis must be less than 0.05.

**Cox’s proportional hazard model**

We used the Cox’s time-varying proportional hazard model to regress fecal domination (relative abundance > 30%) of any oral bacterial ASV as a microbial endpoint of interest against antibiotic administration as multivariable predictors. The exposure to each antibiotic takes a value of 1 on the day the antibiotic was administered and 0 otherwise. The time interval of allo-HCT recipients starts from 10 days prior to transplantation and ends by 40 days post-transplantation. Patients with less than 5 samples during the time interval were excluded. Rarely administered antibiotics that have been administered less than 10 times were also excluded.

**Quantification and statistical analysis**

The oral and gut bacterial loads in the intestine were computed by multiplying total bacterial loads (qPCR, flow cytometry, DNA mass) with relative abundance of oral-typical and oral-atypical ASVs respectively. By interpreting oral-atypical bacteria as gut bacterial members, we ignored bacterial transmission from other non-oral body sites to the gut. All statistical analysis, including linear regression, correlation between variables (Pearson’s and Spearman’s correlation), quantitation of microbiome similarity (Hamming and Bray-Curtis distances), and hypothesis testing (Kruskal-Wallis test and Welch’s t-test) were performed using python.

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