**Cross-Body Site Microbial Interactions Influence the Human Plasma Metabolome**

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**Supplementary Information**

<https://docs.google.com/document/d/1zGXYuzBaabxb3EEbujq0sk1Vd2HlHhBx7WZ58U7rLLE/edit?usp=drivesdk>

**Supplementary Data**

[Supplementary Table](https://drive.google.com/drive/folders/1qw2fSVURxb2Q0GV-67n7Deb8Q5NigedI?usp=drive_link)

**Abstract**

The human microbiome profoundly influences host plasma metabolome and health, yet most studies have focused on the gut microbiome in isolation. Here, we provide a comprehensive analysis of how microbiomes from multiple body sites selectively and jointly influence the human plasma metabolome. Using data from three independent human cohorts (n = 435 in total), we demonstrate that microbiomes from all body sites can influence the human plasma metabolome (814 annotated metabolites). Together, these microbiomes explained 33.25% of the inter-individual variation of plasma metabolome, with the gut microbiome contributing the most (18.04%), followed by the oral (13.35%), skin (9.07%), and nasal (5.82%) microbiomes. Notably, our findings reveal that microbial composition does not necessarily predict metabolic function. For instance, despite distinct microbial composition, oral and gut microbiomes exhibit similar associations with circulating metabolites. Machine learning and mediation models uncover widespread cooperative and synergistic microbial interactions across body sites, particularly along the oral-gut axis. This axis demonstrates cross-body site microbial crosstalk and sequential metabolic processing that jointly regulate circulating metabolites, including indole derivatives and carboxylic acids. This oral-gut microbiome-metabolome axis is further amplified in insulin resistance (IR), linking enhanced microbial cooperation to metabolic dysregulation. Overall, our results establish the systemic and interactive nature of microbiome-metabolome relationships, highlighting the importance of integrating spatially distributed microbial ecosystems to fully understand host metabolic regulation and diseases.

**Introduction**

The human microbiome, composed of trillions of microorganisms residing across diverse body sites, is increasingly recognized as one of the central regulators of host physiology and metabolism[1–3](https://www.zotero.org/google-docs/?x0fEHn). Among these microbial communities, the gut microbiome has been most extensively studied, with substantial evidence linking it to circulating metabolites and systemic metabolic phenotypes such as obesity, IR, and inflammation[4–6](https://www.zotero.org/google-docs/?u6zVGC). These insights, along with other studies, have established the gut microbiome as a significant contributor to host metabolic homeostasis[7,8](https://www.zotero.org/google-docs/?WzxlGX). However, the human body harbors other large and ecologically distinct microbial ecosystems, such as those in the oral cavity, nasal cavity, and on the skin, that are less well characterized in terms of their contributions to host systemic metabolism.

Emerging evidence suggests that these non-gut microbiomes may also influence host physiology beyond their local environments[9–11](https://www.zotero.org/google-docs/?OGrP1g). For example, oral and nasal microbiomes have been implicated in respiratory diseases and cardiovascular risk[12,13](https://www.zotero.org/google-docs/?TYAk3w), and skin microbes are known to participate in immune modulation[14](https://www.zotero.org/google-docs/?AFndEz). Despite this, the influence of microbiomes from these non-gut sites on the human plasma metabolome and their subsequent effects on human health remain poorly understood. It also remains unclear whether microbial communities from different body sites act independently or interactively to influence systemic metabolic profiles.

This gap is particularly important given the growing recognition that microbial ecosystems across the human body sites may not function in isolation[15,16](https://www.zotero.org/google-docs/?s4rdQH). Anatomically connected sites, such as the oral cavity and gut, engage in microbial “crosstalk,” potentially enabling cooperative or sequential processing of dietary and host-derived substrates[17](https://www.zotero.org/google-docs/?PJTyxN). For instance, dietary tryptophan may be metabolized into indole derivatives by the oral microbiome and further transformed by the gut microbiome into circulating metabolites with immunomodulatory properties[18,19](https://www.zotero.org/google-docs/?vnq5qs). While previous studies have suggested that the oral microbiome can influence gut microbial composition or function[20,21](https://www.zotero.org/google-docs/?NrimKv), the potential for coordinated microbial metabolism across body sites remains largely unproven.

To address these knowledge gaps, we conducted an integrative multi-omics study to systematically characterize how microbiomes from multiple body sites influence the human plasma metabolome. We analyzed data from three independent human cohorts, including one discovery cohort with matched microbiome data from the gut, oral cavity, nasal cavity, and skin, along with paired plasma metabolomics data[22–24](https://www.zotero.org/google-docs/?C2gNuI) . Two additional validation cohorts with gut and/or oral microbiome data were used to confirm key findings[25,26](https://www.zotero.org/google-docs/?h8uV8j). Using machine learning approaches, including gradient-boosted decision trees (GBDT), combined with metabolite variance decomposition, cross-body site microbial interaction modeling, and mediation analysis, we quantitatively assessed the individual and joint contributions of microbiomes from different body sites to circulating metabolite levels.

We hypothesized that microbiomes from different body sites, particularly the oral and gut microbiomes, cooperatively regulate the plasma metabolome via synergistic and potentially sequential metabolic processes. Specifically, we sought to answer the following questions: (1) Which microbiomes, by body site, contribute most strongly to inter-individual variation of the plasma metabolome? (2) Do microbiomes from different body sites interact synergistically to co-regulate specific circulating metabolites? (3) Can directional microbial processing across body sites, particularly from the oral to the gut, be demonstrated via mediation modeling? (4) What are the broader biological implications of multiple body site microbial cooperation for systemic host metabolism and metabolic disease? By addressing these questions, our study provides new insights into the systemic and cooperative roles of the human microbiome across multiple body sites, advancing the understanding of how host-microbiome interactions extend beyond individual body sites to collectively influence human metabolic health.

**Results**

**Microbiomes from Different Body Sites Show Selective and Non-Compositional Influences on the Plasma Metabolome**

To investigate the relationship between the microbiomes from multiple body sites and human plasma metabolome, we analyzed data from three independent human cohorts with varying levels of multi-omics resolution (**Methods**). The discovery cohort included 108 participants with matched microbiome data from four body sites, namely the gut, oral cavity, nasal cavity, and skin, along with paired plasma metabolome data[22–24](https://www.zotero.org/google-docs/?LsAGWb) (**Fig. 1a**). Validation cohort 1 included 200 participants with gut and oral microbiome and plasma metabolome data[25](https://www.zotero.org/google-docs/?sRBJDm), while validation cohort 2 included 127 participants with gut microbiome and plasma metabolome data[26](https://www.zotero.org/google-docs/?GbIBQv). The demographic characteristics of the discovery cohort and validation cohort 2 are summarized in **Extended Data Fig. 1a,b**, and provided in **Supplementary Table 1**.

In the discovery cohort, 814 circulating metabolites were detected using LC-MS/MS across diverse metabolite classes (**Fig. 1b**). The most prevalent categories included carboxylic acids and derivatives (166, 20.39%), fatty acyls (115, 14.13%), and glycerophospholipids (64, 7.86%) (**Methods** and **Supplementary Table 2**). Notably, a subset of these metabolites, spanning multiple chemical classes, was identified as microbiota-derived, indicating their production or co-metabolism by microbiota (**Methods**). Although they represented a smaller fraction of total metabolites (78/814, 9.58%), these microbiota-derived metabolites were distributed across various classes including carboxylic acids (n = 15), benzene and substituted derivatives (n = 13), indoles and derivatives (n = 11), and phenolic compounds (n = 5) (**Fig. 1b** and **Extended Data Fig. 1c**). These findings are consistent with previous studies highlighting the widespread metabolic influence of microbial communities on circulating metabolites[27,28](https://www.zotero.org/google-docs/?8gMr55). With this comprehensive metabolomic landscape established, we next examined how microbiomes from different body sites relate to these circulating metabolites.

We assessed the intrinsic compositional similarity of microbiomes from multiple body sites. Non-metric multidimensional scaling (NMDS) based on microbial community profiles (relative abundance at genus level; **Methods**) revealed that skin and nasal microbiomes clustered closely, indicating similar overall microbial composition structures. In contrast, oral and gut microbiomes formed distinct and well-separated clusters (**Fig. 1c**), which are consistent with previous studies[29](https://www.zotero.org/google-docs/?4JkdHz). To characterize these patterns further, we defined core microbiota at the genus level using relative abundance and prevalence thresholds (**Methods**). As expected, the results showed that the skin and nasal microbiome have very similar core microbiota, and are both predominantly composed of *Staphylococcus*, *Cutibacterium*, and *Corynebacterium*[30,31](https://www.zotero.org/google-docs/?vGJo8U)(**Fig. 1d**). In contrast, the gut microbiome was enriched for *Phocaeicola*, *Bacteroides*, and *Faecalibacterium*[32,33](https://www.zotero.org/google-docs/?194Lgt), while the oral microbiome was dominated by *Prevotella*, *Neisseria*, and *Veillonella*[34–36](https://www.zotero.org/google-docs/?HZaqJ2)(**Fig. 1d**). These findings are consistent with previous studies describing the spatial compartmentalization and ecological differentiation of the human microbiome across different body sites[37](https://www.zotero.org/google-docs/?QhqPLY).

Given the distinct microbial composition from different body sites, we next assessed their overall associations with the human plasma metabolome. Previous studies have demonstrated associations between gut microbiome diversity and plasma metabolome[7,38](https://www.zotero.org/google-docs/?oEuowN), but it remains unclear whether microbiomes from other body sites exhibit similar associations with the plasma metabolome. To this end, we computed Spearman correlations between microbiome alpha diversity (Shannon index) from each body site and each circulating metabolite (**Methods** and **Supplementary Table 3**). As expected, multiple known microbiota-derived metabolites, including phenylacetylglutamine (PAGln)[39](https://www.zotero.org/google-docs/?blIgvs), 5-hydroxyhexanoic acid[40](https://www.zotero.org/google-docs/?0ngVtY), p-cresol sulfate[41](https://www.zotero.org/google-docs/?meAzYo), and p-hydroxyphenylacetic acid[42](https://www.zotero.org/google-docs/?IAKg0C), showed significant positive correlations with gut microbiome alpha diversity (**Fig. 1e** and **Supplementary Table 3**; FDR-adjusted P < 0.05).

Interestingly, we also observed body site-specific patterns between the microbiome and circulating metabolites (**Supplementary Table 3**). For example, glycerophospholipid metabolites are positively correlated with skin microbiome alpha diversity but negatively with nasal microbiome diversity (**Fig. 1e**), even though the skin and nasal microbiomes have similar microbial composition (**Fig. 1c,d**). This result suggested that the function of the microbiome may be closely related to its ecological niche[43](https://www.zotero.org/google-docs/?uh6FSN). Overall, microbiota-derived metabolites exhibited significantly stronger absolute correlations with microbiome alpha diversity across all body sites than non-microbiota-derived metabolites (mean absolute rho: 0.31 *vs.* 0.26; Wilcoxon test, P < 0.01; **Fig. 1f**). Additionally, the strength of associations with circulating metabolites varied microbiomes by body site, with gut microbiome consistently showing the strongest overall correlations with circulating metabolites, followed by oral, skin, and nasal microbiomes (Wilcoxon test, P < 0.01; **Supplementary Table 3; Fig. 1g**).

Lastly, we examined whether the metabolite class distributions of significantly associated metabolites differed across microbiomes from different body sites. The metabolite classes associated with oral and gut microbiome diversity were highly similar (**Fig. 1h** and **Extended Data Fig. 1d**; Chi-squared test, P > 0.05), both enriched in fatty acyls and carboxylic acid derivatives (**Fig. 1h**). In contrast, circulating metabolites associated with skin and nasal microbiomes showed greater heterogeneity (**Fig. 1h** and **Extended Data Fig. 1d**).

Overall, while microbiomes from different body sites exhibit clear spatial distinctions in their microbial composition (**Fig. 1c,d**), these microbial compositional patterns do not directly reflect their metabolic function, namely, their impact on the plasma metabolome. We found that the metabolic associations of each microbiome are highly selective and body site-specific, with skin and nasal microbiomes showing divergent correlations with key metabolite classes despite their similarity of microbial composition. These findings suggest that similar microbial communities may exert distinct metabolic effects depending on their anatomical context, highlighting the importance of integrating functional data (metabolome) to fully understand host-microbiome interactions beyond the microbiome alone.

**Gut Microbiome Dominates in Influencing Plasma Metabolome Compared to Other Body Sites**

Having established that microbiomes from multiple body sites are associated with the human plasma metabolome above, we next sought to quantify the extent of this influence and identify which body sites exert the most significant effects. To this end, we calculated the proportion of inter-individual variance in the plasma metabolome (814 annotated metabolites) explained by each body site microbiome (**Methods**). The results revealed that the microbiomes explained 33.25% of the inter-individual variation in plasma metabolome, with the gut microbiome contributing the most (18.04%), followed by the oral (13.35%), skin (9.07%), and nasal (5.82%) microbiomes (**Fig. 2a**). The magnitude of influence observed for the gut microbiome is consistent with a previous study reporting that gut microbial features explained approximately 12.8% of the variance in the plasma metabolome in a Dutch cohort[8](https://www.zotero.org/google-docs/?xZGXge). However, few previous studies have quantified the contributions of microbiomes beyond the gut, making our findings for microbiomes from other body sites particularly novel and informative. Interestingly, we found that combining the microbiome data from four sites can significantly improve the explanatory power of metabolome variation (33.25%, **Fig. 2a**). To understand which specific microbial taxa drive these body site-specific contributions, we performed detailed correlation analyses between individual genera and metabolite profiles.

As expected, microbiomes from all body sites explained a greater proportion of variance in microbiota-derived metabolites than in the overall metabolites: 37.22 for all microbiome, 25.12% for the gut, 15.21% for the oral, 13.22% for the skin, and 9.12% for the nasal microbiome (**Fig. 2a**). These results reinforce the well-established role of microbial communities in producing or modifying specific metabolites, which is well studied in gut microbiomes[7,8,44](https://www.zotero.org/google-docs/?fUeiZ7). More importantly, these results also suggest that microbiomes from all body sites exert systemic plasma metabolic influence through additional pathways beyond direct metabolite secretion (**Fig. 2a**). These may include host-microbiota co-metabolism[45](https://www.zotero.org/google-docs/?vKJW7v), regulation of epithelial and metabolic signaling pathways[46](https://www.zotero.org/google-docs/?3ypzSw), and immune-mediated mechanisms[47](https://www.zotero.org/google-docs/?bzNGo5), all of which contribute to influencing the broader plasma metabolome.

Next, to identify which specific microbial taxa contribute most strongly to plasma metabolome variation, we performed principal component analysis (PCA) on plasma metabolome and overlaid genus-level microbial taxa from each body site (**Fig. 2b** and **Extended Data Fig. 2d**; **Methods**). Among these, *Oscillibacter* and *Phocaeicola* in the gut microbiome were most strongly associated with the primary axes of variation (PC1 and PC2) (**Fig. 2b**), indicating that these two genera play central roles in influencing human plasma metabolome and contribute substantially to inter-individual variability[48](https://www.zotero.org/google-docs/?1IBvBb).

We further examined these associations by calculating Spearman correlations between the relative abundance of each genus and the abundance of each circulating metabolite (**Fig. 2c** and **Extended Data Fig. 2a**), as well as between each genus and plasma metabolome PC1 (**Fig. 2d** and **Supplementary Table 4**; **Methods**). As expected, the gut microbiome exhibited the highest number of significant correlations with circulating metabolites (n = 427 metabolites; FDR-adjusted P < 0.05), followed by oral (n = 243), nasal (n = 233), and skin (n = 189) microbiomes (**Extended Data Fig. 2a**), which are almost consistent with the overall variance explained by each body site (**Fig. 2a**). To identify the most influential genera for each body site, we summarized the genera with the largest number of significantly associated metabolites (**Extended Data Fig. 2b**). Notably, *Oscillibacter* and *Phocaeicola* again emerged as the top genera in terms of breadth of associations, further supporting their key role in influencing the plasma metabolome (**Extended Data Fig. 2b**). Specifically, both genera showed the strongest correlations with PC1 of the plasma metabolome (**Fig. 2b**), reinforcing their dominant contribution to the variation of the plasma metabolome. These results are consistent with previous studies. For example, a large-scale analysis identified *Oscillibacter* as one of the most metabolically influential genera across 930 blood metabolites[49](https://www.zotero.org/google-docs/?fjr3Ne). Similarly, *Phocaeicola* (previously classified under *Bacteroides*) has been linked to bile acid metabolism and amino acid metabolism in human plasma, further supporting its functional relevance in host-gut microbial metabolic interactions[50](https://www.zotero.org/google-docs/?pZygco).

To validate these associations, we tested the key gut genus-metabolite pairs in validation cohort 2, and most associations were reproducible (89 out of 100), supporting the robustness of our findings (**Extended Data Fig. 2d** and **Methods**). Interestingly, *Oscillibacter* and *Phocaeicola* from the gut microbiome showed opposing correlations with PC1 of the plasma metabolome (**Fig. 2b,e,f**), prompting us to investigate their specific metabolite associations further (**Methods**). As expected, *Oscillibacter* was positively correlated with a wide array of microbiota-derived metabolites, while *Phocaeicola* showed negative correlations with the same set, including PAGln, p-hydroxyphenylacetic acid, p-cresol sulfate, phenol sulfate, p-cresol glucuronide, and 4-hydroxybenzoic acid-4-O-sulfate (**Fig. 2g**). This antagonistic relationship between *Oscillibacter* and *Phocaeicola* has also been found in the Framingham cohort[48](https://www.zotero.org/google-docs/?nu6ja8).

In summary, these results demonstrate that among microbiomes from multiple body sites, the gut microbiome exerts the most pronounced influence on the human plasma metabolome, both in terms of overall variance explained and number of metabolite associations. While oral, skin, and nasal microbiomes also contribute meaningfully, the gut microbiome remains dominant, largely driven by the metabolic impact of genera such as *Oscillibacter* and *Phocaeicola*. Together, these findings underscore the central role of the gut microbiome in influencing the plasma metabolome and establish a framework for cross-body site microbiome-metabolome integration. While the gut microbiome showed the strongest overall influence, our analyses revealed that many metabolites were associated with microbiomes from multiple body sites. This observation prompted us to investigate whether these associations reflect independent or cooperative microbial influences.

**Microbiome from Multiple Body Sites Cooperatively Influences Circulating Metabolites**

We have observed above that many circulating metabolites were significantly associated not only with genera from a single body site, but also with genera originating from multiple body sites (**Fig. 2c**, **Extended Data Fig. 2a**). Such as, indole and indole derivatives, which have significant correlations with oral and gut microbiomes. This result is consistent with previous studies, which show that indole and indole derivatives can be influenced by the microbiome from the oral[51](https://www.zotero.org/google-docs/?Jg3apU) and gut[52,53](https://www.zotero.org/google-docs/?SrResU). This prompted us to investigate whether microbiomes from different body sites could jointly influence individual circulating metabolites. To this end, we employed both Lasso regression and gradient-boosted decision trees (GBDT) to model the contribution of microbiota from multiple body sites on each circulating metabolite[44,54](https://www.zotero.org/google-docs/?sWcbqU)(**Methods**). Lasso was used for its strength in variable selection and handling collinearity, while GBDT was chosen for its ability to capture complex, non-linear relationships and interactions. Upon comparing model performance, GBDT consistently outperformed Lasso across all four body site microbiomes in terms of fitting accuracy (**Extended Data Fig. 3a**). As a result, we used the **GBDT** model for all subsequent variance decomposition analyses. Using the superior GBDT approach, we systematically identified metabolites influenced by single versus multiple body site microbiomes.

We first identified 380 (46.68%) circulating metabolites whose inter-individual variation could be explained by microbiomes from at least one body site (adjusted R2 > 5%), with 256 (31.45%) of these exhibiting adjusted R2 values greater than10% based on GBDT modeling (**Fig. 3a** and **Supplementary Table 5**; **Methods**). To evaluate the relative influence of each type of microbiome, we classified these 256 metabolites according to the microbiome that contributed the largest share of explained variance, categorizing them as gut, oral, skin, or nasal-dominated metabolites (**Fig. 3a**). Specifically, 165 metabolites were dominated by the gut microbiome, followed by 35, 30, and 26 metabolites for the oral, nasal, and skin microbiomes, respectively. This distribution is mostly consistent with our above findings, further supporting the conclusion that the gut microbiome has the most substantial impact on the plasma metabolome among all body sites' microbiomes examined.

As expected, several metabolites that showed strong associations with *Oscillibacter* and *Phocaeicola*, such as p-cresol and PAGln, were significantly influenced and well predicted by the gut microbiome in the GBDT model (**Fig. 3b, c**). To assess the robustness of these predictions, we applied the GBDT model to validation cohort 2 (**Methods**). Approximately one-third of themetabolites (30 out of 111, 27.0%) could be successfully predicted (adjusted R2 > 10%; **Extended Data Fig. 3b**), with the best-predicted metabolites largely overlapping with previously identified microbiota-derived metabolites (**Extended Data Fig. 3c**). These results suggest that gut microbiome-metabolite associations, particularly for microbiota-derived metabolites, are reproducible across independent cohorts. Interestingly, several metabolites, including Piperidine, Phenylalanylphenylalanine, O-Succinyl-L-homoserine, Edetic acid, Asymmetric dimethylarginine, and Aminoisobutyric acid, were influenced by microbiomes from all four body sites simultaneously (**Fig. 3a** and **Supplementary Table 5**).

We subsequently performed metabolite class enrichment analysis to examine whether microbiomes from different body sites preferentially influenced specific classes of circulating metabolites (**Supplementary Table 6** and **Methods**). The gut microbiome showed significant enrichment for influencing metabolites classified as benzene and substituted derivatives (*e.g.*, Hippuric acid; hypergeometric test, P < 0.05) and organooxygen metabolites (*e.g.*, p-cresol glucuronide; P < 0.05) (**Fig. 3d**). This observation is consistent with previous studies that the gut microbiome plays a central role in the metabolism of aromatic amino acids, which often give rise to benzene derivatives and organooxygen metabolites[55,56](https://www.zotero.org/google-docs/?3cypoV). In contrast, the oral microbiome showed a strong enrichment for indoles and their derivatives (*e.g.*, indole, P < 0.05) (**Fig. 3d**). This aligns with the known role of oral microbiome in degrading tryptophan to produce indole, and skatole, which contribute to oral malodor and local inflammation[57](https://www.zotero.org/google-docs/?wFAlv6). Both the skin and nasal microbiomes showed significant enrichment for influencing carboxylic acids and their derivatives (*e.g.*, Malonic acid, P < 0.05) (**Fig. 3d**).

We further performed enrichment analysis on metabolites jointly influenced by different body site microbiomes (**Fig. 3e** and **Supplementary Table 6**). Notably, metabolites jointly influenced by the oral and gut microbiomes were significantly enriched for amino acid-related pathways, particularly those involving tryptophan, histidine, and arginine metabolism (**Fig. 3e**). This observation reflects the complementary microbial processing occurring across these body sites. We hypothesize that tryptophan could undergo partial degradation to indole or kynurenine in the oral cavity, followed by further microbial transformation into indoleacetic acid or indole propionate in the gut by taxa such as *Bacteroides* and *Clostridium spp.*, potentially enabling cross-body site metabolic cooperation[57,58](https://www.zotero.org/google-docs/?oD2YNA).

Given that many circulating metabolites were found to be jointly influenced by microbiomes from multiple body sites, we next investigated which body site pairs exhibited the greatest overlap in their metabolic influences. We found that the gut and oral microbiomes shared the largest number of jointly influenced metabolites, with 100 overlapping features (**Fig. 3f,g**). This observation aligns with our above results (**Fig. 1h**) and previous studies describing functional and microbial communication between the oral and gut ecosystems, commonly referred to as the oral-gut axis[16,59](https://www.zotero.org/google-docs/?f5xWHf). Among the metabolites co-regulated by the oral and gut microbiomes were several previously noted examples, including Piperidine, O-Succinyl-L-homoserine, and aminoisobutyric acid, as well as key aromatic amino acid derivatives such as tryptophan, indole, indoleacetic acid, and indolelactic acid[58](https://www.zotero.org/google-docs/?wVjGzC)(**Fig. 3h**). Notably, these jointly influenced metabolites also tended to exhibit higher explained variance overall, further supporting the coordinated and potentially synergistic roles of the oral and gut microbiome in influencing plasma metabolic profiles (**Fig. 3h**).

Together, these findings reveal that the human plasma metabolome is influenced not only by individual body site microbiomes but also through cooperative influences across body sites, particularly between the oral and gut microbiomes. The strong overlap in metabolite regulation by these two body sites suggests a coordinated oral-gut microbiome-metabolome axis. The strong overlap in metabolite regulation between oral and gut microbiomes, particularly for amino acid-related pathways, suggested potential synergistic interactions. We therefore developed models to explicitly test for cross-body site microbial cooperation.

**Oral and Gut Microbiome Synergistically Regulate Circulating Metabolites Through Cross-Body Site Microbial Interactions**

Given the strong joint influence of the oral and gut microbiomes on circulating metabolites observed above (**Fig. 3f-h**), we next sought to quantitatively assess whether these two body site microbiomes interact synergistically to regulate circulating metabolites. To this end, we constructed a joint GBDT model incorporating both oral and gut microbiomes, explicitly including interaction terms between taxa from the two body sites (**Fig. 4a** and **Methods**). To identify the specific taxa driving these interactions, we compiled all significant cross-body site relationships. As expected, we found that for 97% of the metabolites (97/100), the adjusted R2 values of the joint model exceeded the sum of those from the individual oral- and gut-only models (**Fig. 4b** and **Supplementary Table 7**), indicating synergistic effects between them. Furthermore, the number of significant interaction terms in the joint model was comparable to the gut-only model but significantly greater than that of the oral-only model (Wilcoxon test, P < 0.001; **Extended Data Fig. 4a,b**). Importantly, the contribution of interaction features in the joint model far exceeded those from either body site alone, with particularly high contributions observed for metabolites involved in histidine, arginine, and lysine (**Fig. 4a**). This modeling approach revealed extensive synergistic effects. Together, these results reveal that the oral and gut microbiomes do not act independently but instead interact extensively to modulate many circulating metabolites, underscoring the importance of oral-gut microbiome cooperation in regulating circulating metabolites.

To gain deeper insight into how the oral and gut microbiomes interact to influence circulating metabolites, we next investigated which specific microbial taxa (genera) participate most prominently in these oral-gut microbial interactions. We systematically compiled all genera involved in significant interaction terms across the GBDT models (**Methods** and **Supplementary Table 8**). In the gut microbiome,*Bifidobacterium*emerged as the genus most frequently involved in interaction terms, contributing to the regulation of 23 circulating metabolites (**Fig. 4c**). In the oral microbiome, *Enterococcus* was the most prominent genus, participating in interactions associated with 29 metabolites (**Fig. 4c**). We then examined the metabolite classes of the metabolites most strongly influenced by these genera-specific interactions. Notably, carboxylic acids and derivatives constituted the dominant class affected by both *Bifidobacterium* and *Enterococcus*, accounting for 56.52% and 48.28% of their respective associated metabolites (**Fig. 4c** and **Supplementary Table 8**). These findings suggest that cross-body site microbial interactions between oral and gut converge on shared metabolic pathways, particularly those involved in carboxylic acid metabolism, highlighting the biochemical coordination between anatomically distant microbial communities in modulating systemic host metabolism.

To illustrate how oral and gut microbiomes can jointly influence circulating metabolites through cross-body site microbial interactions, we examined two representative examples involving *Enterococcus* from the oral microbiome and *Paraprevotella* from the gut microbiome. These two genera exhibited coordinated effects on the levels of tryptophan and its derivative, 5-hydroxy-L-tryptophan (5-HTP), in circulation (**Fig. 4d,e**). Specifically, the relative abundance of *Enterococcus* from the oral microbiome modulated both the strength and direction of the correlation between *Paraprevotella* from the gut microbiome and these metabolites. At low levels of *Enterococcus*, *Paraprevotella* showed a strong positive correlation with tryptophan and 5-HTP, suggesting it played a primary role in their biosynthesis or regulation. However, as *Enterococcus* abundance increased, the influence of *Paraprevotella* diminished, eventually becoming neutral or even negative (**Fig. 4d,e**). This interaction pattern implies a competitive or antagonistic relationship between the two genera at higher *Enterococcus* levels, potentially through substrate competition, inhibitory metabolite production, or host-mediated responses. Both *Enterococcus* and *Paraprevotella* have been implicated in microbial tryptophan metabolism pathways, including decarboxylation and transamination processes[60,61](https://www.zotero.org/google-docs/?HlikAW), supporting the plausibility of such cross-body site metabolic modulation. These findings exemplify how microbial dynamics across the oral and gut can converge to regulate key metabolic pathways systemically, particularly those involving amino acid-derived neuromodulatory and immunomodulatory compounds. These synergistic interactions raised the question of whether there might be directional relationships between oral and gut microbiomes in their influence on circulating metabolites, potentially reflecting the anatomical flow from the oral cavity to the gut.

**Oral-Gut Microbiome-Metabolome Axis Mediates Coordinated Metabolism of Circulating Metabolites**

Building on our finding that the human plasma metabolome is influenced by cooperative influences across body sites, particularly between the oral and gut microbiomes, we next investigated whether directed mediating effects exist between these two microbial communities in influencing circulating metabolites. Previous studies have suggested that the oral-gut axis may facilitate sequential co-metabolism of dietary substrates, where compounds initially modified by oral microbiota are further processed by gut microbiota[62](https://www.zotero.org/google-docs/?5vh5dB). To examine this possibility, we constructed bidirectional mediation models to test two hypotheses: (1) an oral-gut-metabolite pathway, where the oral microbiome modifies substrates that are subsequently metabolized by the gut microbiome, and (2) a gut-oral-metabolite pathway, where the direction of influence is reversed (**Methods**).

In the discovery cohort, we identified 48 significant oral-gut-metabolite mediation relationships, involving 26 oral genera, 15 gut genera, and 11 circulating metabolites (**Fig. 5a** and **Supplementary Table 9**). To assess the robustness of these findings, we applied the same modeling framework to validation cohort 1 and found that the majority of the oral-gut mediation effects were reproducible (33 out 48; **Extended Data Fig. 5a**). Importantly, consistent with our hypothesis that microbial processing flows from the oral to the gut microbiome, the number of significant mediation effects in the oral-gut-metabolite direction far exceeded those in the gut-oral-metabolite direction in both the discovery cohort (48 *vs.* 13) and validation cohort 1 (4,122 *vs.* 1,018; species level, **Methods**) (**Fig. 5b**). These findings support the existence of a directional oral-gut microbiome-metabolome axis that mediates coordinated metabolic transformation of some host circulating metabolites.

Within the oral-gut-metabolite mediation network, we found that the majority of mediation relationships involved the gut microbiome genera *Oscillibacter* and *Phocaeicola* serving as key mediators (**Fig. 5a-e** and **Supplementary Table 9**). The metabolites influenced through these pathways were predominantly of microbial origin, highlighting the importance of microbiota-derived metabolites in systemic circulation. To illustrate these oral-gut cross-site interactions, we selected representative examples. Notably, *Dialister* may modulate *Oscillibacter* activity involved in tyrosine fermentation and p-cresol production (**Fig. 5c**), while *Lancefieldella*, possibly through modulation of phenylalanine flux, may enhance *Oscillibacter*-linked PAGln biosynthesis (**Fig. 5d**), which was confirmed in validation cohort 1 (**Extended Data Fig. 5a-c**). Similarly, *Granulicatella* and *Aminipila* may alter the gut’s capacity to process xenobiotics such as paracetamol via *Phocaeicola* and *Coraliomargarita*, respectively (**Fig. 5e,f**). The actual mechanism by which the oral and gut microbiomes influence circulating metabolites requires further investigation through additional experimentation. In summary, these findings support the concept of an oral-gut-metabolite axis and suggest that cross-body site microbial cross-talk contributes to host systemic metabolism.

**Oral-Gut Microbiome-Metabolome Axis Is Altered in Insulin Resistance**

Emerging evidence suggests that the oral-gut axis plays a pivotal role in the pathogenesis of metabolic disorders, including type 2 diabetes (T2D) and IR[63,64](https://www.zotero.org/google-docs/?Q2ZokT). Previous studies have shown that translocation of periodontal pathogens to the gut can exacerbate glucose intolerance and promote systemic inflammation[65](https://www.zotero.org/google-docs/?FbgM7t). However, whether this axis influences disease via modulation of host metabolism remains incompletely understood. Given our earlier findings that the oral and gut microbiomes jointly regulate circulating metabolites, forming an oral-gut microbiome-metabolome axis, we next investigated how this axis differs between insulin-sensitive (IS) and IR individuals.

We first assessed whether the gut and oral microbiomes differ in microbial composition between the IS and IR groups. As expected, NMDS analysis revealed that the oral and gut microbiomes were clearly distinct from each other (**Fig. 6a**), in line with the above result (**Fig. 1c**). Within each body site, the gut microbiome exhibited a clear separation between IR and IS individuals, while the oral microbiome showed no significant differentiation (**Fig. 6a and Extended Data Fig. 6a-b**). This pattern aligns with previous studies that consistently report gut microbiome alterations in IR, while findings for the oral microbiome are more variable[63,66](https://www.zotero.org/google-docs/?GEvIha).

To evaluate whether oral-gut microbial interactions are altered in IR, we compared the number of oral-gut interaction terms in GBDT models across all circulating metabolites (**Methods**). Remarkably, the IR group exhibited a significantly greater number of interaction terms than the IS group (**Fig. 6b**; Wilcoxon test, P < 0.001), suggesting enhanced microbial crosstalk between the oral and gut. We further performed mediation analysis separately in each group to examine directional influence (**Methods**). Consistent with the interaction analysis, the IR group exhibited a markedly higher number of significant oral-gut-metabolite mediation pathways compared to the IS group (68 *vs.* 24; **Fig. 6c**). These findings collectively indicate that the oral-gut microbiome-metabolome axis exhibits elevated functional activity in IR individuals.

To identify key circulating metabolites differentially regulated by the oral-gut axis in IR versus IS individuals, we compared the variance explained (adjusted R2 values) from GBDT models between the two groups (**Methods**). We identified 136 metabolites with significant intergroup differences, of which 103 were more strongly associated with both oral and gut microbiota in the IR group (**Fig. 6d** and **Supplementary Table 10**). These metabolites included central components of energy metabolism such as sucrose, D-fructose, glutamic acid, and D-ribose, pointing to widespread disruptions in metabolic homeostasis (**Fig. 6e,f**). In addition, multiple metabolites involved in aromatic amino acid metabolism, including L-tyrosine, phenylalanine, 4-hydroxyphenylpyruvic acid, and kynurenic acid, were significantly elevated or more strongly influenced by the oral-gut axis in IR individuals. Elevated levels of aromatic amino acids have been implicated in the development of IR, potentially via activation of the mTOR pathway and inhibition of downstream insulin signaling cascades[67](https://www.zotero.org/google-docs/?e86QoM). Alterations were also observed in histidine metabolism, with metabolites such as imidazolelactic acid and methylimidazoleacetic acid showing increased association with the oral-gut microbiome in IR individuals (**Fig. 6e,f**). Histidine plays key roles in modulating inflammation, scavenging reactive oxygen species, and maintaining redox balance[68](https://www.zotero.org/google-docs/?RZwEue). Disruptions in histidine metabolism may impair glucose homeostasis via effects on histamine biosynthesis or microbial composition in the gut[69](https://www.zotero.org/google-docs/?mLY5j7).

Taken together, these results reveal that the oral-gut microbiome-metabolome axis becomes functionally reprogrammed in IR individuals, exhibiting stronger microbial interactions and greater influence over key circulating metabolites. This shift particularly affects pathways related to energy metabolism and aromatic and histidine amino acid metabolism, suggesting a mechanistic link between cross-body site microbial crosstalk and metabolic dysregulation in IR. These findings highlight the oral-gut microbiome-metabolome axis as a potential contributor to the pathophysiology of metabolic diseases and a promising target for future microbiome-metabolome-based interventions. Collectively, these results reveal that the oral-gut microbiome-metabolome axis undergoes functional reprogramming in IR, with enhanced microbial cooperation potentially contributing to metabolic dysregulation.

In summary, our multi-cohort analysis reveals that microbiomes from multiple body sites cooperatively influence the human plasma metabolome, with particularly strong synergistic interactions between oral and gut microbiomes that are amplified in IR. These findings challenge the gut-centric view of microbiome-metabolome interactions and highlight the importance of considering the human microbiome as an interconnected, multi-site ecosystem.

**Discussion**

In this study, we provide a comprehensive and integrative analysis of how microbiomes from multiple body sites, the gut, oral cavity, skin, and nasal cavity, influence the human plasma metabolome. While the key role of the gut microbiome in regulating host metabolism is well-established[8,44,70](https://www.zotero.org/google-docs/?kWO3nG), our work expands this understanding by quantitatively revealing contributions from non-gut body sites, particularly the oral microbiome.

The skin and nasal microbiomes, though less influential in magnitude compared to gut and oral, also exhibited distinct metabolic associations, suggesting body site-specific roles in host systemic metabolism. Notably, we uncovered that microbial composition alone is not predictive of functional metabolic impact. For example, the skin and nasal microbiomes share highly similar microbial composition but exhibit divergent metabolic associations, while the compositionally distinct oral and gut microbiomes influence overlapping classes of metabolites (**Fig. 1c,d,h**). We speculate that this characteristic may be caused by the different microenvironments of the bacteria's ecological niches. There are significant differences in temperature, humidity, ultraviolet intensity, and available nutrients between the nasal cavity and the skin. In addition, the types and metabolic characteristics of the host's epithelial cells are also different, all of which may cause the metabolic functions of the same bacteria in different ecological niches to differentiate. These findings highlight the functional divergence and specialization of microbiomes across different body sites and underscore the necessity of incorporating metabolomic data to capture microbiome-host interactions more completely.

We found that microbiomes from all four body sites contribute to the overall inter-individual variation of the plasma metabolome. The gut microbiome accounted for the largest proportion of overall explained variance of plasma metabolome (18.04%), followed by the oral (13.35%), skin (9.07%), and nasal (5.82%) microbiomes (**Fig. 2a**). Our results also reveal a pattern of systemic and cooperative metabolic regulation, wherein specific circulating metabolites are influenced by microbiomes from multiple body sites (**Fig. 3a**). Through GBDT modeling, we demonstrate that cooperative contributions from oral and gut microbiomes account for the variance in many circulating metabolites (**Fig. 3f-h**). The observed joint influence on metabolites such as indole derivatives, carboxylic acids, and aromatic amino acid metabolites reflects biochemical cooperation of microbiomes from multiple body sites. For instance, indole and its derivatives, commonly implicated in gut-brain and gut-immune signaling[71](https://www.zotero.org/google-docs/?Qn41EW), were jointly associated with genera from both oral and gut microbiomes, suggesting that these spatially distinct microbial communities may collaborate along the same metabolic pathways.

Mechanistically, our modeling uncovered extensive cross-body site microbial interactions, particularly between the oral and gut microbiomes. GBDT models incorporating interaction terms between genera revealed that the combined influence of oral and gut microbiomes on metabolite variance exceeds the sum of their independent effects for the vast majority of circulating metabolites, indicating strong synergistic regulation (**Fig. 4a,b**). Key genera from each site, such as *Bifidobacterium* (gut) and *Enterococcus* (oral), emerged as central hubs in these interactions, jointly modulating important metabolic pathways, including those involving carboxylic acids and amino acid derivatives. Mediation analyses further supported the notion of functional interdependence, revealing directional relationships where oral microbiota influence metabolite levels through downstream modulation by gut taxa (**Fig. 5**). These findings suggest the existence of cross-body site microbial processing chains that operate sequentially to regulate host metabolic homeostasis.

Importantly, we found that the oral-gut microbiome-metabolome axis is functionally altered in individuals with IR. Compared to IS individuals, IR subjects exhibited stronger oral-gut microbial interactions and a greater number of significant mediation relationships (**Fig. 6b-c**). These enhanced microbial crosstalk patterns were accompanied by increased regulation of metabolites implicated in energy metabolism, aromatic amino acid pathways (*e.g.*, tyrosine, phenylalanine), and histidine metabolism (**Fig. 6e,f**). These metabolic alterations are mechanistically linked to impaired insulin signaling and redox imbalance in IR[72](https://www.zotero.org/google-docs/?uow6vM), suggesting that the reprogramming of oral-gut microbial cooperation may contribute to the metabolic dysregulation observed in early metabolic disorders. Our findings thus implicate the oral-gut axis not merely as a passive bystander but as an active participant in the pathogenesis of metabolic disorders.

Collectively, our work establishes the oral-gut microbiome-metabolome axis as a fundamental conduit of metabolic communication of microbiomes across multiple body sites. These findings argue for a systemic perspective on host-microbiome interactions, moving beyond the traditional gut-centric paradigm. Given the extensive cross-body site microbial cooperation revealed here, interventions targeting a single body site microbiome, such as the gut or oral cavity, may exert far-reaching effects on host metabolism. For example, many studies have found that oral care and intervention can help diabetic patients control blood sugar[73](https://www.zotero.org/google-docs/?VnQRZ8), which is of great significance for the development of microbiome-based treatment and diagnostic methods, and emphasizes the need to consider microbiota from multiple body parts when designing systemic metabolic interventions.

Our study presents certain limitations. First, although the discovery cohort included microbiomes from four body sites, the two validation cohorts lacked complete multi-body site sampling, limiting replication of some cross-body site findings in the study. In addition, the sample sizes of the three groups were relatively small. Although we set more stringent variable screening and parameter settings when constructing the GBDT model, it may still not be possible to completely avoid model overfitting, so a larger cohort is needed to further confirm our findings. Second, microbiome resolution was restricted to the genus level due to 16S rRNA sequencing. Deeper insights into strain-level functions and microbial gene content would benefit from the use of shotgun metagenomics or metatranscriptomics in the future. Third, our metabolite annotation in the metabolomics analysis, based on MS/MS spectra, likely missed a fraction of low-abundance or metabolic features without MS/MS spectra, and future work incorporating new metabolite annotation strategies could expand the metabolic landscape analyzed. Additionally, while our modeling approaches suggest potential mechanistic pathways, they remain correlative. Functional validation via isotope tracing, microbial knockouts, and intervention using animal models will be critical for establishing causal links in the future.

In conclusion, our study reveals that microbiomes from multiple body sites exert selective and cooperative influence on the human plasma metabolome. By elucidating the synergistic interactions and metabolic coordination between oral and gut microbiomes, we uncover a previously underappreciated axis of cross-body site microbial communication that influences host metabolic physiology. These findings underscore the importance of integrating microbiome data from multiple body sites and metabolome data to better understand the systemic nature of host-microbiome interactions and their role in human health and disease.

**Methods**

**Study design and human cohort**

All three human cohorts' multi-omics data were obtained from previous studies. The detailed information about them could be found in the publications published by Zhou *et al.*[24](https://www.zotero.org/google-docs/?UsPNkK), Shen *et al.*[22](https://www.zotero.org/google-docs/?B9r2aB), Saar Shoer *et al.*[25](https://www.zotero.org/google-docs/?3nTu8H), and Wu *et al.* [26](https://www.zotero.org/google-docs/?UfETdc). Brief details of the sample collection and data processing are provided below.

**Participant recruitment**

*Discovery cohort.*This cohort consisted of 108 participants enrolled under IRB 23602, approved by the Stanford University Institutional Review Board. All participants provided written informed consent and agreed to the publication of potentially identifiable information. Ethical standards were upheld throughout the study. Participants underwent a one-time modified insulin suppression test at enrollment to quantify insulin-mediated glucose uptake. Insulin sensitivity was assessed using steady-state plasma glucose (SSPG) levels: individuals with SSPG < 150 mg/dL were classified as insulin sensitive, while those with SSPG ≥ 150 mg/dL were considered insulin resistant. SSPG data were unavailable for 38 participants. Fasting plasma glucose (FPG) was also measured at baseline for 69 participants, identifying two cases of diabetes (FPG > 126 mg/dL). Hemoglobin A1C (HbA1C) was assessed during each visit; HbA1C ≥ 6.5% indicated diabetes, and four additional cases were identified during follow-up. Exclusion criteria included anemia, kidney disease, cardiovascular disease, cancer, chronic inflammation, psychiatric conditions, and history of bariatric surgery or liposuction. BMI was measured at enrollment. No compensation was provided to participants.

*Validation cohort 1.* Participants were recruited in Israel between January 2017 and January 2019, with final follow-up in March 2020. Eligibility required meeting two pre-diabetic criteria as per the 2010 American Diabetes Association (ADA) guidelines: (1) fasting plasma glucose (FPG) between 100-125 mg/dL (5.6-6.9 mmol/L) and (2) HbA1c between 5.7-6.5% (39-48 mmol/mol). Additional criteria included age 18-65 years and daily use of a smartphone application for dietary logging. Exclusion criteria encompassed use of diabetes or weight loss medications, antibiotic use within three months before enrollment, chronic diseases, or chronic medication use affecting glucose or energy metabolism. Volunteers self-identified as pre-diabetic via a dedicated website and were screened through a questionnaire followed by an in-person screening visit at AMC Medical Center Laboratory. For further methodological details, see Ben-Yacov et al. Participants received no compensation.

*Validation cohort 2.* This single-blinded, parallel-design clinical trial was conducted in Singapore from November 2020 to July 2021 and registered at ClinicalTrials.gov (NCT04745702). Ethics approval was obtained from the National Healthcare Group Domain Specific Review Board (Ref: C/2019/00997). All participants provided written informed consent. Eligible participants were prediabetic Chinese men and women aged 45-75 years with a BMI between 19.5 and 32.0 kg/m². Prediabetes was defined based on ADA criteria as having one or more of the following: (1) fasting blood glucose > 5.5 mmol/L and < 7.0 mmol/L, (2) oral glucose tolerance test (OGTT) value ≥ 7.8 mmol/L and ≤ 11.0 mmol/L, or (3) HbA1c ≥ 5.7% and ≤ 6.4%. Major exclusion criteria included smoking, food allergies or dietary restrictions, sustained hypertension (> 160/95 mmHg), and a history of metabolic, cardiovascular, hepatic, renal, or thyroid disorders, as well as recent weight loss. Recruitment was conducted via newspaper advertisements, word of mouth, and a local research database. Screening involved completion of a Health and Lifestyle Questionnaire, anthropometric assessments, and biochemical measurements, including HbA1c (DCA Vantage, Siemens), fasting glucose/OGTT (HemoCue 201, Radiometer), height (Seca 217), and weight (Tanita BC-418). The 2-hour OGTT was performed using a 75g glucose solution. Participants were reimbursed for travel and time.

**Sample collection and processing**

*Discovery Cohort.*Biological samples were collected longitudinally from four body sites: stool, skin (forehead), oral cavity (buccal mucosa), and nasal cavity. Sampling occurred approximately every 3 months over up to six years, with additional collections during acute events (*e.g.*, respiratory infections, vaccination, or antibiotic use). All participants were instructed to avoid topical antibiotics and oral antiseptics for at least 24 hours before collection. Samples were self-collected or obtained by trained staff and immediately stored at −80°C until processing. DNA was extracted using standardized protocols, followed by 16S rRNA gene sequencing targeting the V4 region to resolve amplicon sequence variants (ASVs). Plasma samples were collected concurrently by venipuncture after an overnight fast and used for steady-state plasma glucose (SSPG), HbA1c, fasting glucose (FG), and multi-omics assays (proteomics, metabolomics, lipidomics, cytokines). Quality control included technical replicates and negative extraction controls.

*Validation Cohort 1*. Microbiome samples were collected at baseline and after the 6-month intervention period. Stool samples were self-collected at home using standardized kits and shipped under controlled temperature conditions. Oral microbiome samples were obtained via subgingival plaque swabs using sterile dental curettes by trained dental professionals. All samples were stored at −80°C before DNA extraction. Microbial composition was analyzed via whole-metagenome shotgun sequencing. Concurrently, venous blood samples were collected in serum-separating tubes (SST), centrifuged, and aliquoted for metabolomic and cytokine profiling.

*Validation Cohort 2*.At the screening and baseline enrollment visits, participants underwent standardized sample collection after an overnight fast (≥ 10 hours). Blood samples were obtained via venipuncture for measurement of fasting plasma glucose (FG), oral glucose tolerance test (OGTT) with 75g glucose load, and HbA1c levels. Glucose assays were performed using HemoCue® 201 (Radiometer, Denmark), and HbA1c was assessed using the DCA Vantage Analyzer (Siemens, USA). Anthropometric measurements included body weight (Tanita BC-418), height (Seca 217), and BMI calculation. While no microbiome samples were collected in this cohort, the clinical data supported metabolic phenotyping for insulin sensitivity status. All biological materials were processed within 2 hours of collection and stored at −80 °C for further analysis. Instruments were regularly calibrated, and staff adhered to standardized protocols.

**Microbiome data acquisition and processing**

*Discovery cohort.*Microbial DNA was extracted from stool, skin, oral, and nasal samples using the Qiagen PowerSoil DNA Isolation Kit, with mechanical bead-beating to ensure broad lysis of microbial cells. The V4 region of the 16S rRNA gene was PCR-amplified using barcoded primers and sequenced on the Illumina MiSeq platform (2 × 250 bp). Raw sequences were demultiplexed, quality-filtered, and processed using the DADA2 pipeline to generate amplicon sequence variants (ASVs). Taxonomy was assigned via the SILVA reference database (v138).

*Validation cohort 1.*Whole-metagenome shotgun sequencing was conducted on DNA isolated from stool and subgingival plaque samples. DNA extraction followed by mechanical and enzymatic lysis using the ZymoBIOMICS DNA Miniprep Kit. Sequencing libraries were prepared using the Nextera XT kit and sequenced on the Illumina HiSeq platform (2 × 150 bp). Human reads were filtered out by alignment to the hg38 reference genome using Bowtie2. Taxonomic profiling was performed using MetaPhlAn3, and functional pathway analysis was conducted using HUMAnN3. Data were normalized to counts per million (CPM), and low-prevalence features were excluded. Both taxonomic and functional data were arcsine square root transformed before analysis. Diversity indices and inter-group differences were assessed using non-parametric statistical tests.

*Validation cohort 2.* Stool microbiome profiling was performed on DNA extracted from 450 μL stool aliquots preserved in DNA/RNA Shield (Zymo Research). Samples underwent enzymatic pretreatment with lysozyme, Proteinase K, and RNase A at 37 °C and 60 °C. Homogenization was performed twice using Lysing Matrix E tubes at 6 m/s for 40 s. Supernatants were subjected to further RNase A digestion, followed by phenol: chloroform extraction (1:1) and purification using the Maxwell® 16 FFS Nucleic Acid Extraction Kit (Promega). Sequencing libraries were prepared without PCR amplification and subjected to Illumina NovaSeq 6000 paired-end shotgun metagenomic sequencing.

**Plasma metabolome data acquisition and processing**

*Discovery cohort.* Untargeted metabolomic profiling was conducted on fasting plasma samples using liquid chromatography-mass spectrometry (LC-MS) with both hydrophilic interaction chromatography (HILIC) and reverse-phase liquid chromatography (RPLC) separation strategies. Metabolites were extracted using a ternary solvent system comprising acetone, acetonitrile, and methanol (1:1:1). Dried extracts were reconstituted in a 1:1 methanol: water solution before analysis. HILIC and RPLC separations were performed on Thermo Q Exactive Plus mass spectrometers in full MS scan mode under both positive and negative ionization modes. A ZIC-HILIC column was used for HILIC (mobile phase: 10 mM ammonium acetate in 50:50 and 95:5 acetonitrile: water), while RPLC used a Zorbax SBaq column with 0.06% acetic acid in water and methanol as the mobile phase. MS/MS spectra were acquired from pooled quality control (QC) samples for feature annotation. Data processing was performed using Progenesis QI software. Features detected in blanks or lacking linearity across dilutions were removed. Only features present in at least 33% of the samples were retained. Missing values were imputed using the k-nearest neighbors (kNN) algorithm. Metabolite class (HMDB.Class) and source information were annotated based on the Human Metabolome Database (HMDB)[74](https://www.zotero.org/google-docs/?OxMAkJ).

*Validation cohort 1.* Untargeted metabolomic profiling was performed by Metabolon Inc. on serum samples using high-resolution LC-MS, with measurements taken on March 20, 2020. Metabolites were quantified based on normalized area counts. Quality control, batch correction, and metabolite identification were performed using Metabolon’s proprietary pipelines. The final processed dataset contained 1,095 metabolite features across 312 samples. Principal component analysis (PCA) was applied, and two principal components were removed during preprocessing. Outlier features (e.g., halogenated molecules such as “3-bromo-5-chloro-2,6-dihydroxybenzoic acid”) were flagged for caution. Final data were log-transformed for downstream analyses. Cytokine profiling in this cohort was performed independently using Olink’s proximity extension assay (PEA) and is not included in the metabolomics dataset.

*Validation cohort 2.* Targeted plasma metabolomics was performed using ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS) on a Waters ACQUITY UPLC-Xevo TQ-S system. Sample preparation followed the N300 metabolomics kit protocol (Novogene, Beijing, China). Metabolites were separated on ACQUITY BEH C18 columns using an 18-minute linear gradient with eluent A (0.1% formic acid in water) and eluent B (acetonitrile: IPA = 70:30). The mass spectrometer operated in both positive and negative electrospray ionization modes. Multiple reaction monitoring (MRM) was used for metabolite detection, with compound identification based on Q1/Q3 transitions, retention time (RT), declustering potential (DP), and collision energy (CE). Quantification was performed using internal standard calibration curves. Raw data were processed using MassLynx v4.1 for peak integration and correction. Values below the detection limit were imputed using KNN.

**Statistics and reproducibility**

All data processing, statistical analyses, and visualizations were performed using R (<https://www.r-project.org/>) in RStudio. A full list of R packages used is provided in the **Supplementary Note**. Multiple comparisons were corrected using False Discovery Rate (FDR). Figure icons were sourced from iconfont.cn and are used under the MIT license for non-commercial purposes (<https://pub.dev/packages/iconfont/license>).

*Discovery cohort.* This is a longitudinal study. To create the ‘cross-sectional’ dataset, we extracted summary information from the longitudinal data. Specifically, the mean intensity of each molecule was calculated for each participant across all time points. Similarly, each participant’s age was represented by the average age at the time of all sample collections. Oral and gut microbiome data were preprocessed before analysis to ensure consistency and reduce bias. First, microbial features were aggregated to the genus level, and only genera present in at least 10% of samples were retained to remove low-prevalence taxa. Relative abundance transformation was then applied to account for differences in sequencing depth across samples. The data were standardized using Z-score transformation (subtracting the mean and dividing by the standard deviation) to normalize feature scales. To control for potential confounding effects, a linear model was used to adjust for BMI, sex, IRIS group, and ethnicity. The resulting adjusted expression matrices were used for downstream analyses, ensuring comparability across participants and reducing systematic bias.

*Validation cohort 1.* Oral and gut microbiome compositional species and pathways data, serum metabolites and cytokines went through the same processing steps which included: (1) Log 10 transformation (2) Robust standardization using median and standard deviation calculated over 90% of the central distribution (3) Outliers clipping to five standard deviations from the mean (4) Features filtering out, if they existed in less than 20 samples (5) Missing values imputation with feature’s minimal value only if there was a value in participant’s complementary sample (pre- or post- intervention) (6) Batch correction if one of the first five principal components (PCs) explained at least 5% of the data’s variance and was significantly associated with a batch (P < 0.05, Mann-Whitney U test), in a positive case it was inversely transformed and reduced from the data. Since this study only provided standardized species-level feature tables, the standardized species-level characteristic tables were also used in the subsequent model validation process.

*Validation cohort 2* Gut metagenomic and plasma metabolomic data from the FBIP cohort were preprocessed to ensure consistency and quality for downstream analyses. Species-level microbial abundance profiles were aggregated to the genus level by summing species within each genus.

**Non-metric multidimensional scaling (NMDS)**

NMDS was used to visualize differences in microbiome composition. Bray-Curtis dissimilarity was first calculated using genus-level relative abundance data with the phyloseq::distance function. The resulting distance matrix was subjected to NMDS ordination via the ordinate function from the *phyloseq* package.

**Core microbiota identification**

To identify core microbiota across four body sites, the gut, oral cavity, skin, and nasal cavity, we performed a series of data processing and filtering steps based on relative abundance and prevalence thresholds. Microbiome profiling data were aggregated at the genus level using the summarize\_variables() function from the *microbiomedataset* package[75](https://www.zotero.org/google-docs/?rVErKL), summing intensities by genus. To avoid sequencing contamination and the increase of false positive rate of analysis due to rare species, only genera present in more than 10% of samples per site were retained to remove extremely rare taxa, and all remaining data were transformed into relative abundances via transform2relative\_intensity(). After preprocessing, genus-level abundance tables from the four sites were merged. To ensure taxonomic consistency across datasets, a unified genus list was constructed, and zero values were imputed where taxa were missing from specific datasets. Sample metadata were appended to track subject identity and anatomical site of origin. These data were then used to construct a phyloseq object for downstream analysis. Core genera were identified independently within each body site using the core\_members() function from the *microbiome* package. A taxon was considered part of the core microbiota if it exceeded a detection threshold of 0.1% relative abundance in at least 50% of samples (prevalence threshold) within the respective site. If more than 10 core genera were detected in a site, the top 10 most abundant were retained for consistency in visualization.

To assess the robustness of core taxa across detection and prevalence thresholds, we used the plot\_core() function to generate heatmaps, displaying the proportion of samples in which each genus was detected across varying detection thresholds (0.0001 to 0.2) and prevalence levels (5% to 100%).

**Distance matrix-based variance estimation**

To evaluate the overall impact of microbiomes from different body sites on the plasma metabolome, we first preprocessed the microbiome data collected from four sites: the gut, the oral cavity, the nasal cavity, and the skin. For each body site, microbial abundance data at the genus level were filtered to retain genera present in at least 10% of the samples.

For the plasma metabolomics dataset, metabolites were log2-transformed and similarly adjusted using linear modeling to correct for the same covariates. A subset of microbiota-derived metabolites was also identified based on HMDB annotations and analyzed separately.

To quantify the explanatory power of each site-specific microbiome on the plasma metabolome, we used Permutational Multivariate Analysis of Variance (PERMANOVA) via the adonis2 function from the *vegan* R package. Euclidean distances were computed for metabolomic profiles, and PERMANOVA models were constructed with individual microbial genera as predictors. Genera significantly associated with metabolomic variation (P < 0.05) were then included in a final multivariate model to calculate the cumulative explained variance (R2).

This procedure was performed separately for the full metabolomic dataset and the subset of microbial-origin metabolites, allowing comparison of site-specific microbiome influence on both overall and microbially related metabolic profiles.

**Principal Component Analysis (PCA)**

PCA was conducted using the *massstat* package (v1.0.6) on the normalized, log2-transformed, and covariate-adjusted metabolite intensities. Before PCA, metabolite data were adjusted for potential confounding factors (BMI, sex, and ethnicity) using linear models. To visually integrate microbiome and metabolome data, we created a PCA biplot incorporating bacterial contribution vectors. The top five bacterial genera with the strongest correlations to metabolome PC1 were selected for this analysis. The environmental fitting approach (envfit function from the *vegan* package) was employed to project bacterial influences onto the metabolome PCA space. The contribution of each genus to the metabolome principal components was calculated based on 999 permutations. Bacterial contribution vectors were scaled according to their correlation strength and overlaid on the PCA plot. The direction and length of these vectors indicate the strength and direction of the relationship between each bacterial genus and the metabolomic profile represented by PC1 and PC2. This visualization approach allows for the interpretation of how variations in specific bacterial taxa may relate to the overall metabolic landscape.

**Gradient Boosting Decision Tree (GBDT) model**

To evaluate the gut microbiome's explanatory capacity for plasma metabolite levels, we developed individualized GBDT models for each metabolite. These models utilized genus-level gut microbial abundances as predictors and the corresponding metabolite's relative concentration across samples as the response variable. Feature selection was performed by calculating Spearman correlation coefficients between each metabolite and all microbial genera. We retained only genera meeting dual criteria: (1) absolute correlation coefficient ≥ 0.3, and (2) P < 0.05. The GBDT models were parameterized following extensive hyperparameter optimization. We selected 50 trees based on convergence analysis, showing that model performance plateaued beyond this number while avoiding overfitting in our sample size. The learning rate of 0.01 was selected to ensure stable convergence while maintaining model interpretability. The minimum of 8 observations per terminal node was set at approximately 7.5% of our sample size (8/108) to prevent overfitting to individual samples while allowing sufficient model flexibility. These parameters were validated through permutation testing, which confirmed that our models captured genuine biological signals rather than noise. To ensure robust performance assessment, we implemented a bootstrap resampling approach with 100 iterations. Each iteration incorporated 10-fold cross-validation on the training set with the coefficient of determination (R²) as the performance metric. The explanatory power for each metabolite was quantified by the mean R² value and its corresponding 95% confidence interval across bootstrap replicates. Statistical significance of the explanatory power was evaluated using a one-sample t-test comparing the mean R² against zero. The comprehensive results included average R² values per metabolite, counts of selected microbial features, feature-specific correlation statistics, and complete R² distributions across bootstrap iterations.

**Correlation network between microbiota and metabolites**

To explore the associations between microbial taxa and plasma metabolites, we constructed correlation networks separately for gut, oral, skin, and nasal microbiomes. For each body site, microbial abundance data were first aggregated at the genus level using the summarize\_variables() function from the *microbiomedataset* package. Genera present in less than 10% of samples were excluded. Abundances were then transformed to relative intensities and standardized. Spearman correlation coefficients were computed between each genus and each plasma metabolite using the cor.test () function. Only associations with an absolute correlation coefficient ≥ 0.3 and a P < 0.05 were retained as significant. The resulting significant microbiota-metabolite associations were integrated into a network using the *igraph* and *ggraph* packages. Nodes represented genera or metabolites, while edges denoted significant correlations. Node types (microbe or metabolite) and sample source (body site) were annotated. Network topologies, including degree, betweenness, closeness, and eigenvector centralities, were calculated to identify key nodes. The top genera (by degree) from each site and the top shared metabolites influenced by microbes from multiple sites were highlighted. Visualization was performed using a stress layout to improve readability and cluster separation in the network graph.

**LASSO regression model**

We used LASSO regression to assess associations between gut microbial genera and plasma metabolites. A nested 5-fold cross-validation was used: the inner loop selected the optimal regularization parameter (λ), scaled by 1.5, and the outer loop evaluated model performance using R² and RMSE. For each metabolite, 100 permutations of the outcome variable were conducted to generate a null distribution of R² values, from which empirical P values were calculated. FDR correction was applied, and metabolites with FDR-adjusted P < 0.05 were considered significant.

**Verification of the GBDT model in the validation cohort**

After training the GBDT model in the discovery cohort, we validated the model on validation cohort 2. Specifically, we applied the trained model to predict metabolite levels in individuals from the validation cohort 2 using their corresponding input features. We then compared the predicted metabolite levels to the observed values in the validation cohort 2 by computing Pearson correlation coefficients. A metabolite was considered successfully predicted if the correlation between predicted and observed values was statistically significant (P < 0.05). This approach allowed us to assess the model’s predictive power and generalizability across cohorts.

**Metabolite class enrichment**

To explore the biochemical characteristics of microbiome-associated metabolites, we performed metabolite class enrichment analysis across four body sites (gut, oral cavity, skin, and nasal cavity). Metabolite importance was derived from GBDT models previously trained to predict microbial genus profiles based on plasma metabolomics data. For each site, the R2 was used to rank metabolite predictive power.

We filtered metabolites with R2 > 5% in each site as significant microbiome-associated metabolites. To identify overrepresented metabolite classes among these significant metabolites, we performed class enrichment analysis using a custom function based on the hypergeometric test. The analysis leveraged metabolite class annotations curated from the Human Metabolome Database (HMDB), focusing on the following manually selected categories: Benzene and substituted derivatives, Carboxylic acids and derivatives, Fatty Acyls, Glycerophospholipids, Indoles and derivatives, Organooxygen compounds, and Steroids and steroid derivatives.

For each metabolite class, the number of significant metabolites observed was compared to the number expected by chance, given the total number of metabolites in that class and across all classes. Fold enrichment and enrichment P values were calculated using the hypergeometric distribution.

**Metabolomics pathway enrichment**

We used the HMDB pathway database from the *metpath* package[76,77](https://www.zotero.org/google-docs/?qwINnK). To ensure consistency and biological relevance, only pathways containing metabolites present in the study's annotation table were retained. The function enrich\_hmdb was then applied to each body site's selected metabolites.

**GBDT with interaction factors**

To capture potential synergistic or antagonistic relationships between gut and oral microbiota, we generated interaction features representing relationships between microbes from different body sites. For computational efficiency, the most informative gut and oral features were pre-selected based on a combination of variance and mean abundance. Interaction features were created using a multiplication approach, calculated as the product of gut and oral microbial abundances. To minimize spurious interactions, only feature pairs with a Spearman correlation coefficient above 0.2 were used to generate interaction features. For each metabolite, a final GBDT model was trained on the entire dataset to assess feature importance. The relative contribution of gut microbes, oral microbes, and their interaction features was quantified and compared. Special attention was given to analyzing which interaction features contributed substantially to metabolite prediction, providing insights into potential cross-talk between oral and gut microbial communities.

**Bi-directional mediation analysis**

Bidirectional mediation analysis was conducted to explore the potential causal relationships among oral microbiota, gut microbiota, and host plasma metabolites. First, Spearman correlations were calculated between oral microbiota and metabolites, as well as between gut microbiota and metabolites, to identify significantly associated pairs (P < 0.05). Correlations between oral and gut microbial features were then computed to detect potential microbial interactions. Based on these associations, two mediation directions were evaluated: (1) oral microbiota-gut microbiota-metabolite, and (2) gut microbiota-oral microbiota-metabolite. For each direction, mediation analysis was performed using the R package *mediation*, applying linear models and nonparametric bootstrapping (100 simulations) to estimate the average causal mediation effect (ACME), its P value, and the proportion mediated. Significant mediation paths (ACME P < 0.05) were retained, and P values were adjusted for multiple testing using the FDR method.

**Key metabolites differentially regulated by the oral-gut axis in IR *vs.* IS individuals**

To identify key metabolites differentially regulated by the oral-gut microbiome axis in IR versus IS individuals, we built GBDT models to predict microbial genus profiles (from both oral and gut sites) based on plasma metabolite levels. These models were trained separately within the IR and IS groups, enabling group-specific estimation of metabolite-microbiome association strength, quantified by the R2. We then compared metabolite-level R2 values between the two groups. For each metabolite, the difference in R2 (IR minus IS) was calculated; metabolites with FDR-adjusted P < 0.05 and absolute R2 differences > 10% were considered significantly differentially regulated by the oral-gut axis between IR and IS individuals.

**Code availability**

This study utilized R (<https://www.r-project.org/>) and its associated packages for statistical analysis and data processing. All code used for data processing, analysis, and visualization in this study can be accessed at <https://github.com/jaspershen-lab/triplem_project>.

**Data availability**

All microbiome and metabolomics data analyzed in this study were obtained from the previously published studies. All source data supporting the findings are available as **Supplementary Data**.

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**Author contributions**

X.S. conceived the study and provided overall supervision. J.Z. and X.S. analyzed the data. J.Z. and X.S. generated figures. X.S. and J.Z. wrote the manuscript. All authors reviewed and contributed to the final manuscript.

**Competing interests**

M.P.S. is a cofounder, scientific advisor, and shareholder of Filtricine, Iollo, January AI, Marble Therapeutics, Next Thought AI, Personalis, Protos Biologics, Qbio, RTHM, and SensOmics. M.P.S. is a scientific advisor and equity holder of Abbratech, Applied Cognition, Enovone, M3 Helium, and Onza. M.P.S. is a scientific advisor and stock option holder of Jupiter Therapeutics, Mitrix, Neuvivo, Sigil Biosciences, WndrHLTH, and Yuvan Research. M.P.S. is a cofounder and stock option holder of Crosshair Therapeutics. M.P.S. is an investor in and scientific advisor of R42 and Swaza. M.P.S. is an investor in Repair Biotechnologies. M.P.S. is a cofounder, shareholder, and director of Exposomics, Fodsel, and InVu Health. M.P.S. is a cofounder and equity holder of Mirvie, NiMo Therapeutics, and Orange Street Ventures. The other authors declare no competing interests.

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