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# Do gut microbial communities differ in pediatric IBS and health?

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**Human gastrointestinal microbial communities are recognized as important determinants of the host health and disease status. We have recently examined the distal gut microbiota of two groups of children: healthy adolescents and those diagnosed with diarrhea-predominant irritable bowel syndrome (IBS). We have revealed the common core of phylotypes shared among all children, identified genera differentially abundant between two groups and surveyed possible relationships among intestinal microbial genera and phylotypes. In this article we explored the use of supervised and unsupervised ordination and classification methods to separate and classify child fecal samples based on their quantitative microbial profile. We observed sample separation according to the participant health status, and this separation could often be attributed to the abundance levels of several specific microbial genera. We also extended our original correlation network analysis of the relative abundances of bacterial genera across samples and determined possible association networks separately for healthy and IBS groups. Interestingly, the number of significant genus abundance associations was drastically lower among the IBS samples, which can potentially be attributed to the existence of multiple routes to microbiota disbalance in IBS or to the loss of microbial interactions during IBS development.**

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## Introduction

The human gut is rich in microbes, harboring approximately 100 trillion microbial cells.[1] This intestinal microbiota contains thousands of unique microbial phylotypes, with the vast majority of them known to be obligate anaerobes from bacterial phyla Firmicutes, Bacteroidetes and Actinobacteria. The gut microbiota is intimately involved in many host processes.[2] Microbes participate in carbohydrate degradation, modulation of dietary lipid uptake, production of certain vitamins and short-chain fatty acids, development and proper stimulation of the immune system, modulation of gut motility and protection of the host from pathogens.[3] At the same time, microbiota dysbiosis has been linked to a number of human disorders including irritable bowel syndrome (IBS), inflammatory bowel disease, obesity and colon cancer.[3] Among these, IBS is a functional bowel syndrome that has varied symptoms with no sign of visible mucosal damage or intestinal inflammation. Proposed causes of IBS include altered motor function, abnormal gas handling, acute bacterial gastroenteritis, food intolerance, increased intestinal permeability and gut motility, altered intestinal immune function, as well as bacterial overgrowth of small intestine (SIBO).[4,5] While none of the potential causes has yet emerged as the established determinant of IBS development, many of the symptoms of IBS are consistent with SIBO,[6] and the prevalence of SIBO was found to be higher in many previous investigations of IBS patients.[7-9] While the SIBO model of IBS development implicates intestinal microbiota as a major cause of this syndrome, the available studies of gut microbiota in IBS do not show a strong consensus as to which microbiota members might be responsible for the condition and whether specific

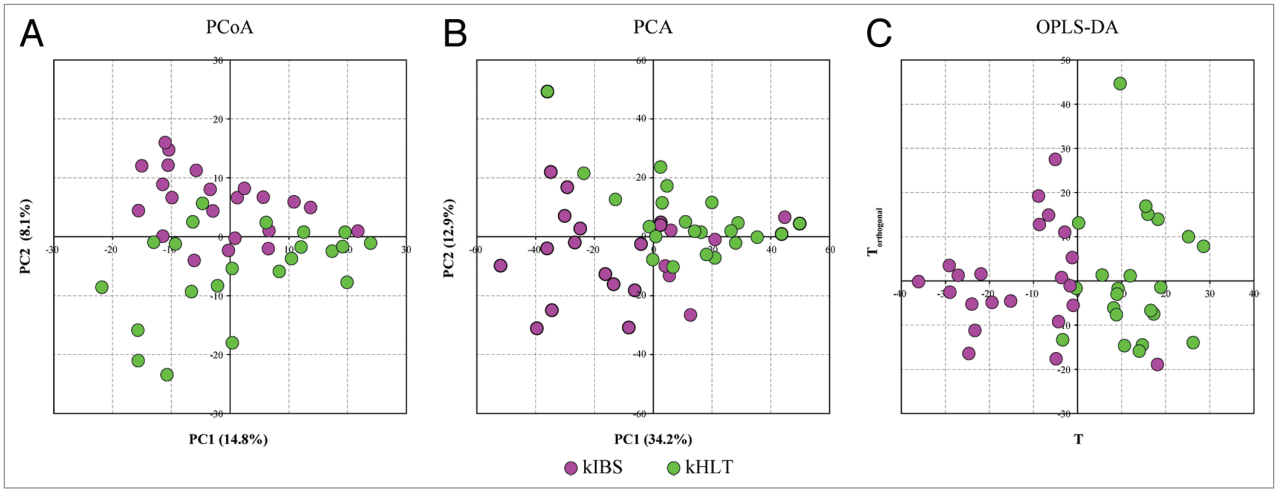


**Figure 1.** Sample separation based on the abundance profiles of gut microbiota members. (**A**) Sample separation was assessed by phylogenetic principal coordinates analysis of phylotype presence data (PCoA), (**B**) principal components analysis of Mahalanobis-scaled genus abundance data (PCA) and (**C**) the orthogonal projection to latent structures discriminant analysis of genus abundance data (OPLS-DA). kIBS, pre- and adolescent children diagnosed with diarrhea-predominant IBS; kHLT, healthy adolescents. In panels (**A**) and (**B**), the percent of data set variability explained by each principal component/coordinate is shown in parentheses in axis titles.

alterations to gut microbial communities take place in IBS patients is unclear.[10]

## Distal Gut Microbiota in Healthy and IBS Children

In a recent study by Rigsbee et al.,[11] we have explored the distal gut microbial communities in two groups of children: healthy pre- and adolescent volunteers (n = 22; average age: 12.6 y) and children newly diagnosed with diarrhea-predominant IBS (n = 22; average age: 13.2 y). A focus on adolescent children was justified by the higher prevalence of IBS in this population demographic.[12,13] Utilizing a combination of four molecular tools including phylogenetic Microbiota Array, 454-based pyrosequencing, fluorescent in situ hybridization and quantitative PCR, we aimed to determine whether the fecal microbiota of children with diarrhea-predominant IBS differed from that of healthy children. At the higher taxonomic level, similar distal gut microbiota composition was observed: both sample groups were dominated by bacterial phyla Firmicutes, Bacteroidetes and Actinobacteria, which cumulatively constituted 91% of overall microbiota composition on average. Community diversity and richness were also similar between the two sample groups. The only noticeable difference at the class level was a somewhat lower average abundance of Actinobacteria among IBS samples (designated kIBS) compared with the healthy group (designated kHLT), this was largely attributed to the lower prevalence of genus *Bifidobacterium* among IBS children. At the lower taxonomic level, however, a number of genera differentially abundant between kIBS and kHLT sample groups were identified. The most conspicuous examples included higher abundances of *Veillonella*, *Prevotella*, *Oxalobacter*, *Enterobacter* and *Parasporobacterium* in kIBS samples and higher abundances of *Verrucomicrobium*, *Fusibacter* and *Oxobacter* in kHLT samples.[11]

Utilizing the ability of the phylogenetic Microbiota Array, which was previously developed in our group,[14,15] to detect the presence of 775 different human-associated phylotypes in each sample, we also determined the child core microbiome. This set was defined as a group of phylotypes detected in all or almost all analyzed samples and was thought to represent microbiota members with important functional roles in the community or those with established host-microbial associations with gut mucosa. A combined core set of 55 phylotypes was identified. This core set was enriched in members of the genus *Ruminococcus* and also contained representatives from genera *Anaerostipes*, *Bacteroides*, *Clostridium*, *Dorea*, *Faecalibacterium*, *Lachnospira*, *Papillibacter*, *Roseburia*, *Streptococcus* and *Subdoligranulum*. Consistent with the concept of the importance of these core members in microbiota functionality, core microbes factored considerably in the overall microbiota composition;on average they contributed about 30% to the total microbiota abundance even though they only constituted 1/6th fraction of the total number of detected phylotypes in each sample on average. At the same time, core phylotypes varied less in their abundance among samples than other non-core phylotypes, which might be an indication of potential abundance restraints placed on the core members due to their prominent role in community structure and function.

## Sample Classification Based on Fecal Microbial Profiles

One benefit to distal gut microbiota profiling through the analysis of patient fecal material is the potential to clinically diagnose an intestinal disorder or syndrome based on an observed microbial population structure. Thus, we have now explored the use of several ordination and classification techniques to test our ability to separate and classify samples belonging to different analyzed groups (kIBS and kHLT).

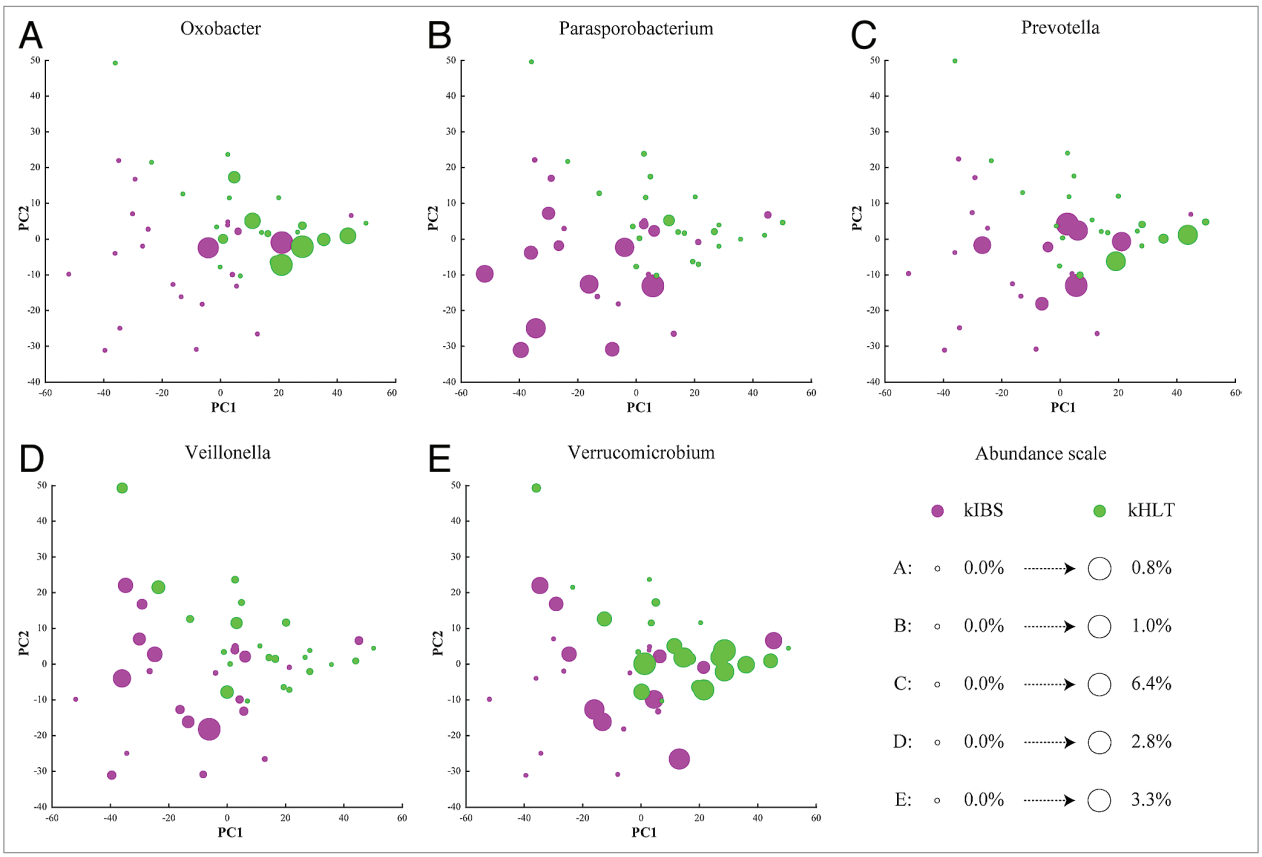


**Figure 2.** Associations between genus abundance and PCA sample clustering. All panels show PCA clustering of Mahalanobis-scaled genus abundance data (see **Fig. 1B**). Each panel corresponds to a specific genus as shown in the panel title, and the size of each sample spot is proportional to the relative abundance of that genus in that sample. Spot sizes were normalized within each genus. The ranges of abundances within each panel are shown in the legend.

Three different statistical approaches were used (**Fig. 1**): unsupervised principal components analysis (PCA) and phylogenetically-based principal coordinates analysis (PCoA) and supervised orthogonal projection to latent structures discriminant analysis (OPLS-DA). The human gut microbiota is recognized to have a strong inter-personal variability and is often considered to be unique to each individual.[16,17] In this situation, unsupervised methods such as PCA and PCoA, which do not use information about sample group assignment, can often find it difficult to separate different sample groups. This is because high inter-individual variability can easily mask small but consistent differences between microbiota profiles from different patient groups. Indeed, when we ran PCA analysis using Euclidean distance as a measure of sample composition similarity, the PCA visualization did not provide a clear separation of two groups (data not shown). Similarly, only a partial separation was evident in the PCoA analysis that used the UniFrac distance metric[18] to assess the similarity among microbial communities based on the phylogenetic distribution of detected phylotypes in each sample (**Fig. 1A**). The samples were spread along the principal coordinate 1 (PC1) axis—typically the axis of the highest data set variance—based on sample-to-sample variability, whereas the PC2 axis provided a separation according to health-disease status of the sample donor. One way to deal with high inter-sample variability within each group is to employ Mahalanobis scaling of abundance data prior to the dimensionality reduction step.[19,20] Mahalanobis scaling adjusts all values by the estimated variance within each group and thus can successfully reduce the effect of inter-personal variability on the data. Rerunning PCA on the Mahalanobis-scaled genus abundance data led to a significantly improved separation of samples from kIBS and kHLT groups (**Fig. 1B**). This separation was statistically significant as determined by a permutation test of the Davies-Bouldin index measure ($D_{DB}$ = 5.50, $\alpha$ = 0.0003 based on a randomized permutation test with 10,000 iterations).[21] In order to assess the potential contributions of different genera to the observed sample separation in the PCA space, the genus abundance in each sample was superimposed onto the two-dimensional PCA plot as shown in **Figure 2**. Localized differences in genus abundances across the PCA space were evident for *Oxobacter*, *Parasporobacterium* and *Prevotella*. In the case of *Verrucomicrobium* abundance, a cluster of samples that all lacked members of this genus was observed, while the preference for *Veillonella* to be more abundant

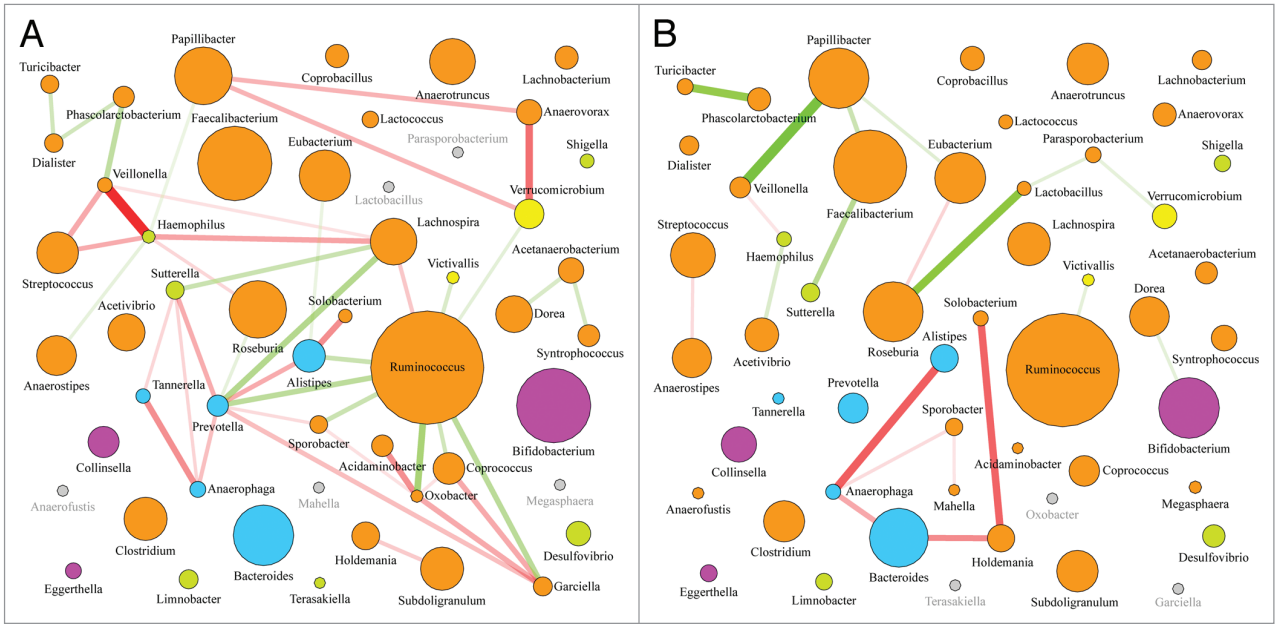


**Figure 3.** Correlations of genus abundances among healthy (**A**) and IBS (**B**) samples. Each node corresponds to an individual bacterial genus as shown. The correlations among genera are represented by lines connecting pairs of nodes; both the transparency and the width of each line are proportional to the absolute value of the Spearman rank correlation of the abundances of corresponding genera among all samples within that group. Positive relationships are designated by red, negative by green. Only genera with an average relative abundance of 0.1% and above are shown; and only relationships with at least 99% confidence are displayed. The size of the node is proportional to the square root of the average genus abundance among all samples within corresponding group. Genus assignment to a particular phylum is represented by the color of the node: Firmicutes are colored in orange, Bacteroidetes in blue, Actinobacteria in pink, Proteobacteria in light green and all other genera are colored in yellow. Gray nodes indicate genera with less than 0.1% average abundance in that group (healthy or IBS) but equal to or greater than 0.1% abundance in the other group. All nodes were positioned to minimize the number of intersections among edges.

among kIBS samples was also apparent (**Fig. 2**).

Because supervised classification methods take into consideration the known categorization (e.g., kIBS vs. kHLT) for each sample, they are often able to more effectively identify patterns that distinguish samples among groups. Accordingly, OPLS-DA analysis of the genus abundance data set revealed a statistically significant separation of samples between the kIBS and kHLT groups (**Fig. 1C**, $Q^2$ = 0.26, $R^2_Y$ = 0.59, model significance $\alpha$ = 0.0012 based on a permutation analysis with 10,000 iterations). The genera identified by OPLS-DA as statistically significantly contributing to the observed group separation included *Anaerostipes*, *Dorea*, *Papillibacter* and *Parasporobacterium*. As an alternative classification method we have also employed a Random Forest analysis that was recently utilized in a number of microbiota profiling studies.[22,23] The constructed model of group classification had 84.3% accuracy as assessed by the leave-one-out cross-validation. Among the genera identified by the Random Forest classifier as contributing the most to the group classification model were *Bryantella*, *Oxalobacter*, *Papillibacter* and *Parasporobacterium*.

Overall, we were able to achieve statistically significant separation and/or classification of the samples based on their IBS vs. healthy status, and we have identified a number of genera that contributed to the observed separations. While we are hesitant to recommend the use of these results in clinical diagnostics due to the modest sample size that was used in this study, our findings provide an excellent starting point for future exploration. Further validation can be provided by longitudinal analysis of differences in fecal microbiota between groups—in this case consistency of microbiota composition among multiple samples obtained from each individual over a period of time would present increased confidence of the observed separation of the healthy and IBS microbiota.

## Putative Associations among Microbial Members

The use of the phylogenetic Microbiota Array to profile microbial communities in kIBS and kHLT samples has allowed us to obtain quantitative estimates of relative abundances of different microbial genera in each sample. These data were used by Rigsbee et al. to calculate and construct a genus association network based on the non-parametric Spearman rank correlations calculated among genus abundances across all interrogated samples. Many associations were identified, and some of the observed correlations matched the experimentally available data from previous studies.[11] We have now computed such genus abundance correlations separately for the microbial communities of the healthy and IBS gut and further explored and compared the resulting association networks. **Figure 3** displays statistically significant relationships ($|R_s| \geq 0.54$; corresponds to test-wise error rate of 0.01 with n = 22)

among all genera with an average relative abundance above 0.1%. As is evident from the figure, healthy and IBS microbiota revealed a significant difference in their association patterns. Whereas 44 statistically significant associations were found in the kHLT data set, only 20 such relationships were statistically significant among kIBS samples (average network connectivity $k$ = 1.91 for kHLT and 0.83 for kIBS networks).[24] Only two relationships were found to be common to both the healthy and IBS networks: a positive association between *Hemophilus* and *Veillonella* and a negative association between *Ruminococcus* and *Victivalis*. The topology of the networks was also different: while several "hub" nodes were evident in the kHLT genus network [hub genera *Ruminococcus* ($k$ = 9), *Prevotella* ($k$ = 8) and *Hemophilus* ($k$ = 6)], only *Papillibacter* and *Anaerophaga* were connected to more than 2 other genera in the kIBS network ($k$ = 3 for both). Appropriately, the clustering coefficient (a measure of node interconnectivity[24]) was significantly higher for the kHLT network (0.19 and 0.00 for kHLT and kIBS networks, respectively; $\alpha$ = 0.0002 based on two-sample T-test).

Among the revealed statistically significant associations, healthy gut promoted more synergistic relationships (25 out of 44 connections positive), whereas IBS microbiota was dominated more by competitive exclusion manifested as negative associations (11 out of 20 connections negative). Caution should be exercised when interpreting negative associations in the relative abundance data, since mathematically negative relationships can be observed due to data compositionality (increase in the absolute abundance of one dominant member will lead to a decrease in relative abundances of many other members). An example of this can be observed in the associations of genus *Ruminococcus* with other genera; because of the high abundance of this genus among most samples, some of its revealed negative associations are likely explained by the compositional nature of the data.[25]

The strongest positive associations revealed in the healthy gut microbiome were between *Veillonella* and *Hemophilus* ($R_s$ = 0.89), between *Anaerovorax* and *Verrucomicrobium* ($R_s$ = 0.75) and between *Alistipes* and *Solobacterium* ($R_s$ = 0.70). In the IBS genus network, *Alistipes* with *Anaerophaga* ($R_s$ = 0.71) and *Holdemania* with *Solobacterium* ($R_s$ = 0.70) displayed the greatest positive correlations. We found several cases where a significant positive relationship between microbial genera in the healthy gut was lost or even reversed in the distal gut microbiomes from IBS participants. These included *Alistipes-Prevotella* ($R_s$ = 0.64 and $R_s$ = −0.19 in kHLT and kIBS networks, respectively), *Hemophilus-Roseburia* ($R_s$ = 0.57 and $R_s$ = −0.20, respectively), *Papillibacter-Verrucomicrobium* ($R_s$ = 0.63 and $R_s$ = −0.05, respectively) and *Prevotella-Anaerophaga* associations ($R_s$ = 0.61 and $R_s$ = −0.02, respectively). At the same time, new competitive relationships were formed in IBS microbiota between *Hemophilus* and *Acetivibrio* ($R_s$ = 0.23 and $R_s$ = −0.57 in kHLT and kIBS networks, respectively) and between *Phascolarctobacterium* and *Turicibacter* ($R_s$ = 0.46 and $R_s$ = −0.71, respectively).

A few recent studies have classified human gut microbiota into several enterotypes, with *Bacteroides* and *Prevotella* being keystone members of the two of them.[16,26,27] A negative correlation between levels of *Prevotella* and *Bacteroides* was often evident. In concordance with these findings, our association analysis revealed that both healthy and IBS gut displayed a negative relationship between levels of these two genera; however, it was not statistically significant ($R_s$ = −0.15 and $R_s$ = −0.22 in kHLT and kIBS networks, respectively). Interestingly, several positive associations were revealed among other members of phylum Bacteroidetes in both networks: in healthy intestine, these included *Tannerella-Anaerophaga*, *Anaerophaga-Prevotella* and *Prevotella-Alistipes*. In IBS samples, positive associations between *Alistipes* and *Anaerophaga* and between *Anaerophaga* and *Bacteroides* were found (**Fig. 3**).

Overall, the network analysis revealed a remarkable loss of genus associations among kIBS samples. While the observed differences in the association networks in health and IBS do not prove an underlying causal relationship between the disease and microbiota community structure, several speculations can be made. Epidemiologically, the loss of genus connectivity in IBS can potentially be attributed to the broad etiology of the syndrome that might encompass several pathophysiological conditions and several differently altered states of gut microbiota. Biologically, the lack of many positive associations among IBS microbiota members can indicate a loss of microbe-microbe interactions that play important roles in metabolic relationships among microbiota members and in the maintenance of microbiota homeostasis and its healthy status.[3] An increased number of negative associations between levels of several genera can be an indication of the higher competition for resources in IBS gut, potentially due to the loss of balanced metabolic interactions and a deficiency in metabolic processing of carbohydrate intermediates and fermentation products.

## Conclusions

- While human gut microbiota of each individual is unique, similar community properties and core members can be identified that are shared among all subjects.
- Our analyses of microbial interaction networks in IBS and healthy state indicate that microbe-microbe associations might be lost during IBS development. Alternatively, IBS can encompass multiple pathophysiological conditions with distinct disbalanced states of gut microbiota.
- Ordination and classification statistical approaches can be applied to microbiota abundance data to filter out inter-personal variability and to find any consistent differences between healthy and disease states of the intestinal microbiome.
- Such sample classification techniques can be employed in the future to assist clinicians in the diagnosis of gut dysfunction. Knowledge of microbiota structure can aid in the selection of the most appropriate therapy including individual patient-tailored probiotic and prebiotic supplementation options.

### Disclosure of Potential Conflicts of Interest

No potential conflict of interest was disclosed.


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