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Searching for a Consensus Among Inflammatory Bowel Disease Studies: A Systematic Meta-Analysis

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Abstract

Background: Numerous studies have examined the gut microbial ecology of patients with Crohn's disease (CD) and ulcerative colitis, but inflammatory bowel disease–associated taxa and ecological effect sizes are not consistent between studies.

Methods: We systematically searched PubMed and Google Scholar and performed a meta-analysis of 13 studies to analyze how variables such as sample type (stool, biopsy, and lavage) affect results in inflammatory bowel disease gut microbiome studies, using uniform bioinformatic methods for all primary data.

Results: Reduced alpha diversity was a consistent feature of both CD and ulcerative colitis but was more pronounced in CD. Disease contributed significantly variation in beta diversity in most studies, but effect size varied, and the effect of sample type was greater than the effect of disease. *Fusobacterium* was the genus most consistently associated with CD, but disease-associated genera were mostly inconsistent between studies. Stool studies had lower heterogeneity than biopsy studies, especially for CD.

Conclusions: Our results indicate that sample type variation is an important contributor to study variability that should be carefully considered during study design, and stool is likely superior to biopsy for CD studies due to its lower heterogeneity.

Lay Summary

To assess reproducibility in inflammatory bowel disease microbiome research, we performed a meta-analysis of 13 inflammatory bowel disease studies, measuring effects of disease and sample type. Crohn's disease studies were more heterogeneous than ulcerative colitis studies, and sample type variation was a major contributor to inconsistency.

Keywords: IBD, UC, CD, meta-analysis, alpha diversity, beta diversity, microbiome, sequencing

Introduction

Inflammatory bowel disease (IBD) is a lifelong condition that affects more than 0.3% of the population of North America, Oceania, and numerous European countries,[1] and its prevalence continues to rise globally.[2] IBD includes Crohn's disease (CD) and ulcerative colitis (UC); both can cause debilitating and chronic relapsing inflammation, either limited to the colon (UC) or potentially throughout the digestive tract (CD). Although the exact cause of IBD is not known, it is theorized that it is due to an interplay of microbial, genetic, and environmental factors.[3-6] However, the reported effects of IBD on overall gut ecology,[7,8] as well as the specific microbes implicated in IBD.[9,10] are highly variable between studies. With few exceptions,[11-13] the mechanistic contributions of most species to IBD are poorly understood.

Overwhelming evidence suggests that the microbiome is necessary for the pathogenesis of IBD.[14-20] It is widely believed,[21-26] but not universally accepted,[10,27] that the number of taxa in the gut microbiome of IBD patients are reduced compared with non-IBD control subjects. Other widely reported findings include greater variation within microbiome structure (beta diversity) in IBD patients compared with control subjects,[8,28] and differences in taxonomic composition in both UC and CD patients.[10,29-31] Study design elements such as small sample size,[32,33] choice of samples used,[34] and patient clinical heterogeneity[35-37] can all contribute to the difficulty of determining which microbial features are generally associated with IBD.

Disagreements within scientific literature may be resolved by using larger datasets[38-40] or meta-analysis.[41-43] While some meta-analyses have examined the effect of IBD on specific species[44,45] or ecological aspects of the gut microbiome, such as the impact of probiotics and fecal microbiota transplantation,[46,47] very few have comprehensively or systematically examined the microbial ecology of IBD within the human gut.[48,49]

The inconsistency in ecological effects and effect sizes reported across IBD studies is problematic, as it hinders the progression of IBD understanding. Thus, we conducted a meta-analysis of the CD- and UC-associated gut microbiome. We analyzed taxonomic differences and alpha and beta diversity to determine ecological effects and effect sizes. To the best of our knowledge, this project represents the most up-to-date and largest systematic meta-analysis on the IBD gut

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Key messages

It is known that inflammatory bowel disease (IBD) affects the gut microbiome, but the species affected are often inconsistent between studies.
To assess reproducibility in IBD microbiome research, we performed a meta-analysis of 13 IBD studies, measuring the effects of disease and sample type.
Here, we show that Crohn's disease studies are more heterogeneous than ulcerative colitis studies, and sample type variation is a major contributor to inconsistency because biopsy microbiomes are more heterogeneous than stool samples.
The results of this study will improve patient care by showing how to minimize heterogeneity during sample collection for future clinical studies.

microbiome using 16S ribosomal RNA (rRNA) sequencing data.[50,51]

Methods

Search Strategy

We conducted a systematic search for 16S rRNA-based studies comparing CD or UC patients with non-IBD control subjects. The PubMed and Google Scholar databases were used to search relevant articles from 2012 to 2020. We selected these dates due to lack of large IBD studies analyzing 16S rRNA sequencing data prior to 2012. We used an advanced PubMed search using the key words "Inflammatory Bowel Disease" and "16S" and the following filters: "human" for species, "English" for languages, and "Abstract" for text availability. Review articles were excluded. This yielded 190 results. The initial search keywords used for Google Scholar were "Inflammatory Bowel Disease," "16S," "illumina," "human," "Ulcerative Colitis," and "Crohn's Disease." However, because this project required publicly available sequences and metadata, the search was narrowed down by adding the following sequence database project accession codes: "SRA," "bioproject," "bioprojects," "ENA," "DDBJ," and "EBI." Both PubMed and Google searches were repeated with the replacement of "illumina" with "454." This yielded 435 results. To screen for data availability, the Web organization "Crossref"[52] was used to match article titles from these searches with DOI. The R packages "EBImage,"[53] "metagear,"[54] and "pdftools"[55] were used to retrieve and screen PDF copies of the articles. Studies were first screened based on information in the titles and abstracts to confirm that they were not fecal microbiome therapy studies and that there were at least 50 human participants. We excluded studies that included microbiota-altering factors such as fecal microbiota transplantation. The majority of selected studies had excluded participants that were undertaking a course of antibiotics, some studies included samples with antibiotic use. The full article text was then read to confirm that the studies used either Illumina or Roche 454 amplicon sequencing of the 16S rRNA gene and that sequencing data and metadata were publicly available. If study metadata were adequate for analysis (eg, included sample of origin, host codenames, and time points for each sample in longitudinal studies), samples were downloaded (Figure 1A). Thirteen studies met these criteria (Table 1).

Downloading and Processing Sequencing Data

Sequencing data from each study was retrieved from the Sequence Read Archive (SRA) database at the National Center for Biotechnology Information using the fasterq-dump tool. DADA2[65] was used for quality control, chimera detection, identification of amplicon sequence variants, and taxonomic assignment, using the authors' recommended parameters for Illumina or 454 data as applicable. For each study, raw sequences were trimmed based on inspection of quality control plots. Taxonomy was assigned using the SILVA v132 reference database. Each study was imported into a phyloseq object using the "phyloseq" R package.[66] Accompanying metadata were imported from the SRA database.

The authors of the Kim et al[60] study analyzed data originally collected by Eun et al[67] but only the former study was retrieved by our systematic search strategy; thus, this dataset is referred to as Kim et al.

Correction for Pseudoreplication

Several of the datasets analyzed in this study contained multiple samples from the same individual (for example, due to longitudinal study design) and thus data were corrected for pseudoreplication prior to downstream analysis as follows.

Both Gevers et al[30] and Morgan et al[10] included multiple cohorts within their studies. Gevers et al predominantly used the RISK (the pediatric RISK stratification study) cohort, but also used samples from the PRISM (the Prospective Registry in IBD Study at MGH [Massachusetts Geneneral Hospital]) cohort that were included in Morgan et al[10] study (PRISM cohort). Therefore, PRISM cohort samples were excluded from the Gevers et al[30] dataset.

Lloyd-Price et al[61] sequenced multiple samples from the same individuals in their study, both for temporal (time) and spatial (area) comparison. To avoid pseudoreplication, the first collected ileal CD sample or rectal UC were compared with the first collected ileal and rectal control samples, respectively, for analysis. Ileal samples were selected because a significant portion of CD patients are affected in the small intestine, especially in the ileum.[68] Rectal samples were used for analyzing UC because most UC patients had biopsy samples taken from the rectum. We standardized biopsy location within studies when it was possible to do so to minimize heterogeneity and conserve sample numbers, selecting only ileal biopsies within the longitudinal data collected by Lloyd-Price et al.[61] However, this was not possible for some CD biopsy datasets, as they included a mixture of both ileal and colonic samples but had incomplete metadata for a more thorough selection of samples. Thus, a combination of samples from different gastrointestinal locations were included. and in many cases, biopsy location may not reflect the location of active disease.

Halfvarson et al,[58] another longitudinal study, collected 1 to 10 stool samples every 3 months on different dates for a 2-year period. The earliest sample was chosen from each subject for use in this analysis. This method of selection was also used for samples from longitudinal studies of Forbes et al,[57] Braun et al,[56] and Schirmer et al.[64]

The Gevers et al[30] study used both stool and biopsy samples but were uploaded to the SRA database separately, and thus stool and biopsy samples from the same individual could

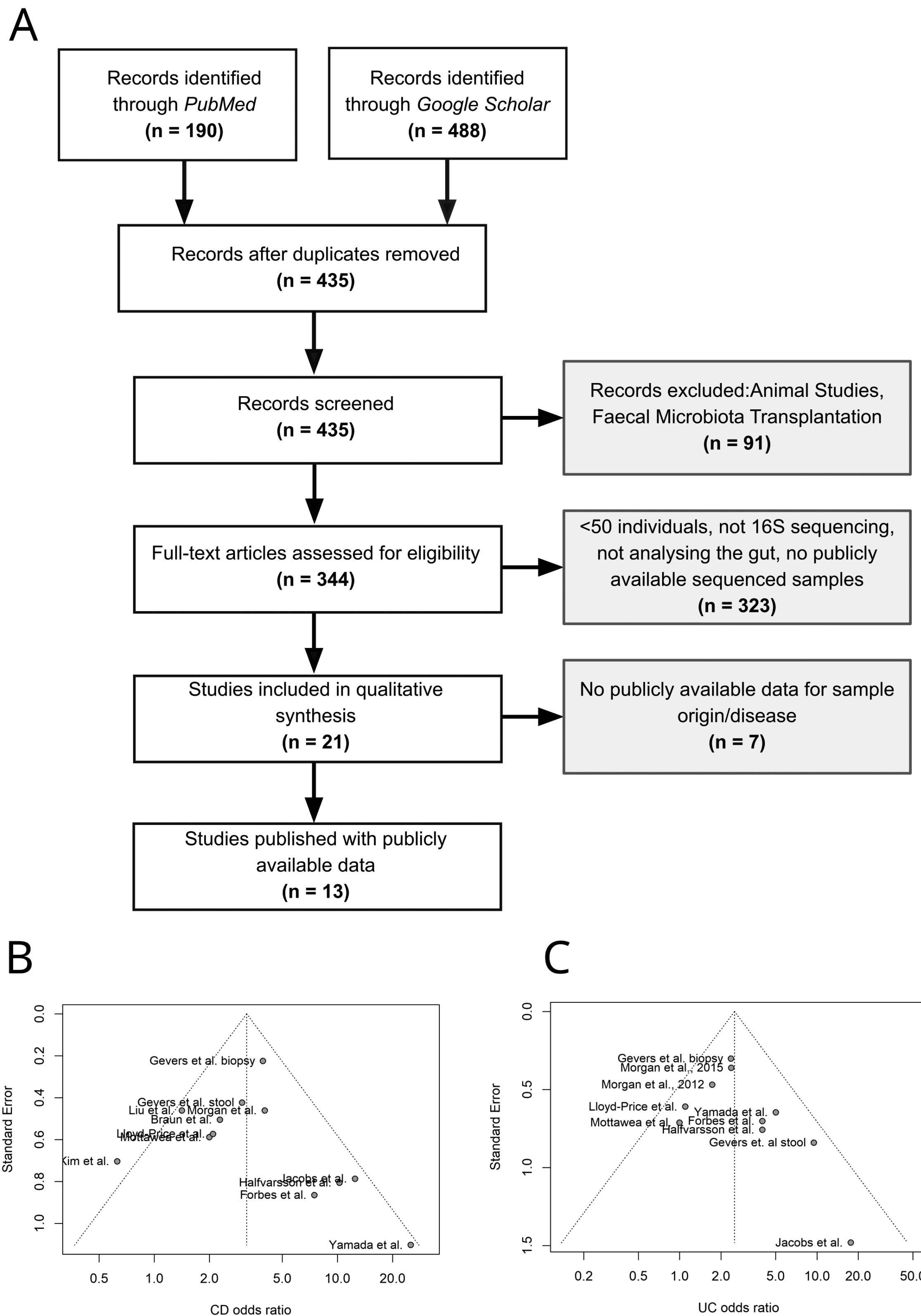

Figure 1. PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) diagram and bias funnel plot. **A,** PRISMA flow chart of study selection process. **B,** Bias funnel plot of odds ratios for Crohn's disease (CD) samples to have below-average observed richness. Egger's regression test $P > .05$ **C,** Bias funnel plot of odds ratios for ulcerative colitis (UC) samples to have below-average observed richness. Egger's regression test $P > .05$. We acknowledge the work of all authors of each study; however, we removed "et al" in figures for simplicity in visualization.

Table 1. Summary of Sequencing, Sample Numbers, and Type for the Studies Selected for This Meta-Analysis

Study	Country	Sequencing Method	Hypervariable Region	Sample Type	Total Samples for Analysis in this Project	UC	CD	Non-IBD
Braun et al[56]	Israel	Illumina	V4	Stool	83	NA	61	22
Forbes et al[57]	Canada	Illumina	V4	Stool	62	19	20	23
Gevers et al[30]	United States	Illumina	V4	Biopsy	424	66	214	144
Gevers et al[30]	United States	Illumina	V4	Stool	260	23	211	26
Halfvarson et al[58]	Sweden	Illumina	V4	Stool	118	60	49	9
Jacobs et al[59]	United States	Illumina	V4	Stool	90	10	26	54
Kim et al[60]	South Korea	454	V1-V3	Stool and biopsy	55	NA	45	10
Lloyd-Price et al[61]	United States	Illumina	V4	Biopsy	70	18	30	22
Liu et al[26]	United States	Illumina	V4	Biopsy	82	NA	35	47
Morgan et al[10]	United States	454	V3-V5	Stool and biopsy	201	69	107	25
Morgan et al[62]	United States	Illumina	V4	Biopsy	182	144	NA	38
Mottawea et al[63]	United States	Illumina Hiseq	V6	lavage	72	15	37	20
Schirmer et al[64]	United States and Canada	Illumina	V4	Stool and biopsy	405	405[a]	NA	NA
Yamada et al[25]	Japan	Illumina	V3-V4	Stool	72	28	21	23

Abbreviations: CD, Crohn's disease; IBD, inflammatory bowel disease; NA, not applicable; UC, ulcerative colitis.
[a]Control subjects are UC samples from patients in remission in this study.

not be consistently and reliably identified with the provided metadata. Because of this, these samples were analyzed as 2 datasets: Gevers et al stool and Gevers et al biopsy.

Data Analysis

Some downloaded samples contained so many low-quality reads (quality score of <20) that they did not pass the DADA2 quality control filtering process, as there were insufficient reads remaining for analysis. In some cases, this resulted in smaller datasets used for analysis than in the original studies.

All datasets were analyzed using R (R Foundation for Statistical Computing) version 4.1.1.[69] The packages "phyloseq,"[66] "vegan,"[70] and "DESeq2"[71] were used to analyze alpha diversity, beta diversity, and taxonomic changes.

Alpha Diversity

For comparisons of observed alpha diversity, each study was rarefied to exclude the 5% of samples with the lowest number of reads, with the aim of balancing conservation of sample numbers as well as maximizing each study's alpha diversity estimates. For each study, after rarefaction, observed richness was compared between IBD subjects and control subjects. In this study, we used observed richness because Shannon is influenced by both number and distribution of species and is therefore less readily interpretable. Owing to differences in methods and sequencing depth between studies, observed richness could not be directly compared. Therefore, we used odds ratios (ORs) to compare the difference in observed richness in cases and control subjects, as previously described by Sze and Schloss,[72] using the R package "metabin."[73] Specifically, the proportion of cases and control subjects with alpha diversity greater than the median of healthy control subjects was summarized as a 2-way contingency table (Supplementary Figure 1). For each study, the ORs were summarized as forest plots. Heterogeneity was also calculated using the Mantel-Haenszel test.

Beta Diversity

Beta diversity was measured using the Bray-Curtis distance. The PERMANOVA (permutational multivariate analysis of variance), betadisper, and anosim tests in the vegan[70] package were used to test the data for beta diversity effects in association with IBD and sample type.

Identifying Taxa Differences

Taxa with significant differences in genus counts between disease groups and non-IBD control subjects were identified using the Wald test ($P < .05$) in the DESeq2 package, with Benjamini-Hochberg false discovery correction ($Q < 0.1$). To maximize clinical relevance of results (ie, common, abundant taxa), unrarefied data were glommed to the genus level, and only genera with a detected relative abundance of at least 3% in each dataset were included in DESeq2 analysis. Differentially abundant taxa were summarized as log2 fold changes between groups for comparison between datasets.

Control Group

All studies contained groups of samples obtained from IBD patients and either healthy or non-IBD control participants, except for the Schirmer et al[64] study (Supplementary Table 3). The Schirmer et al[64] study included samples from UC patients with active and inactive disease and used the latter as the control group. In this study, to reduce confounding factors, the Schirmer et al[64] dataset was only used for the analysis of sample type (stool and biopsy) in association with UC beta diversity and was excluded from any analysis of UC compared with non-IBD control subjects.

In this study the term "non-IBD control subjects" is interchangeable with "healthy control subjects," as many participants in the included datasets were not necessarily healthy but had their samples retrieved during medical examinations of suspected IBD (thus, they were clinically symptomatic in some way but not diagnosed with IBD).

Publication Bias

To detect publication bias, funnel plots were used to visualize the relationship between the OR of each study (x-axis) and SE (y-axis). Each OR described the probability of the CD or UC samples in each study having below-average observed richness. Egger's regression test was used to confirm or disprove publication bias.

With the exceptions of trimming parameters used for each dataset, DADA2 parameters optimized for 454 or Illumina, and rarefaction threshold, bioinformatic methods used in this project were consistent for all studies for the meta-analysis. All R and Bash scripts used for analysis, as well as processed phyloseq objects, can be found at the following link: https://gitlab.com/abdla136/meta_analysis_IBD.

Ethical Considerations

All data used for this study were previously published and is publicly available from the SRA.

Results

Study Selection

Published studies that used 16S rRNA analysis to study the IBD microbiome and had publicly available data and metadata were systematically selected as described in the Methods. Study selection followed the guidelines of PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) (Figure 1A).[74] A total of 13 studies (represented as 14 datasets) met these criteria (Table 1). Not all studies could be included in all comparisons in this paper due to limited metadata.

Uniform bioinformatic methods were used for analysis across all datasets. Funnel plots comparing the ORs of IBD patients having below-average observed richness and SE of all studies were symmetrical, indicating a lack of overall study bias (Egger's test $P > .05$ for CD and UC) (Figure 1B, 1C).

Lower Observed Alpha Diversity Is a Feature of Both CD and UC Microbiomes

For each study, we calculated the OR for CD and UC patients having lower observed richness than the median of non-IBD control subjects (hereafter referred to as "low richness"), as previously described by Sze and Schloss.[72] IBD patients were approximately 2 to 3 times more likely than healthy control subjects to have low richness ($P < .0001$) (CD: OR, 3.20; 95% confidence interval [CI], 2.09-4.88; UC: OR, 2.51; 95% CI, 1.73-3.64) (Figure 2A, 2B). Study heterogeneity was low for

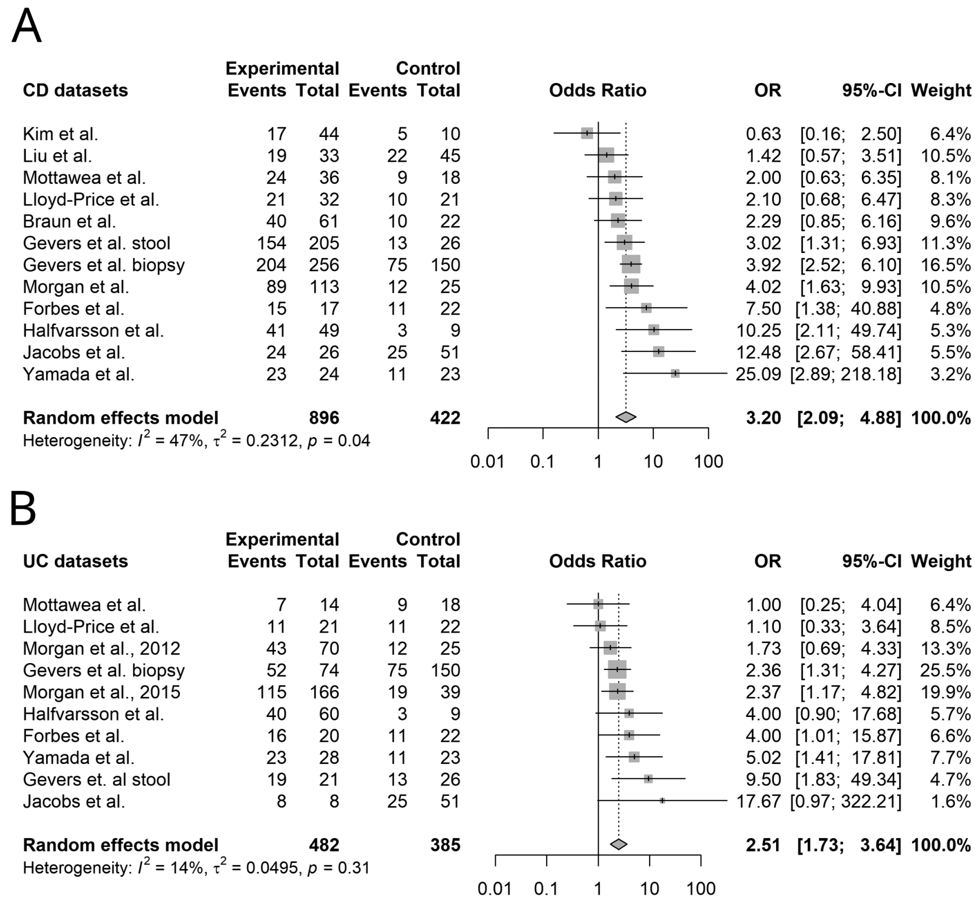

Figure 2. Odds ratios (ORs) for below-average observed richness in the gut microbiome of inflammatory bowel diseasepatients: **A,** Crohn's disease (CD) patients; and **B,** ulcerative colitis (UC) patients. We acknowledge the work of all authors of each study; however, we removed "et al" in figures for simplicity in visualization. CI, confidence interval.

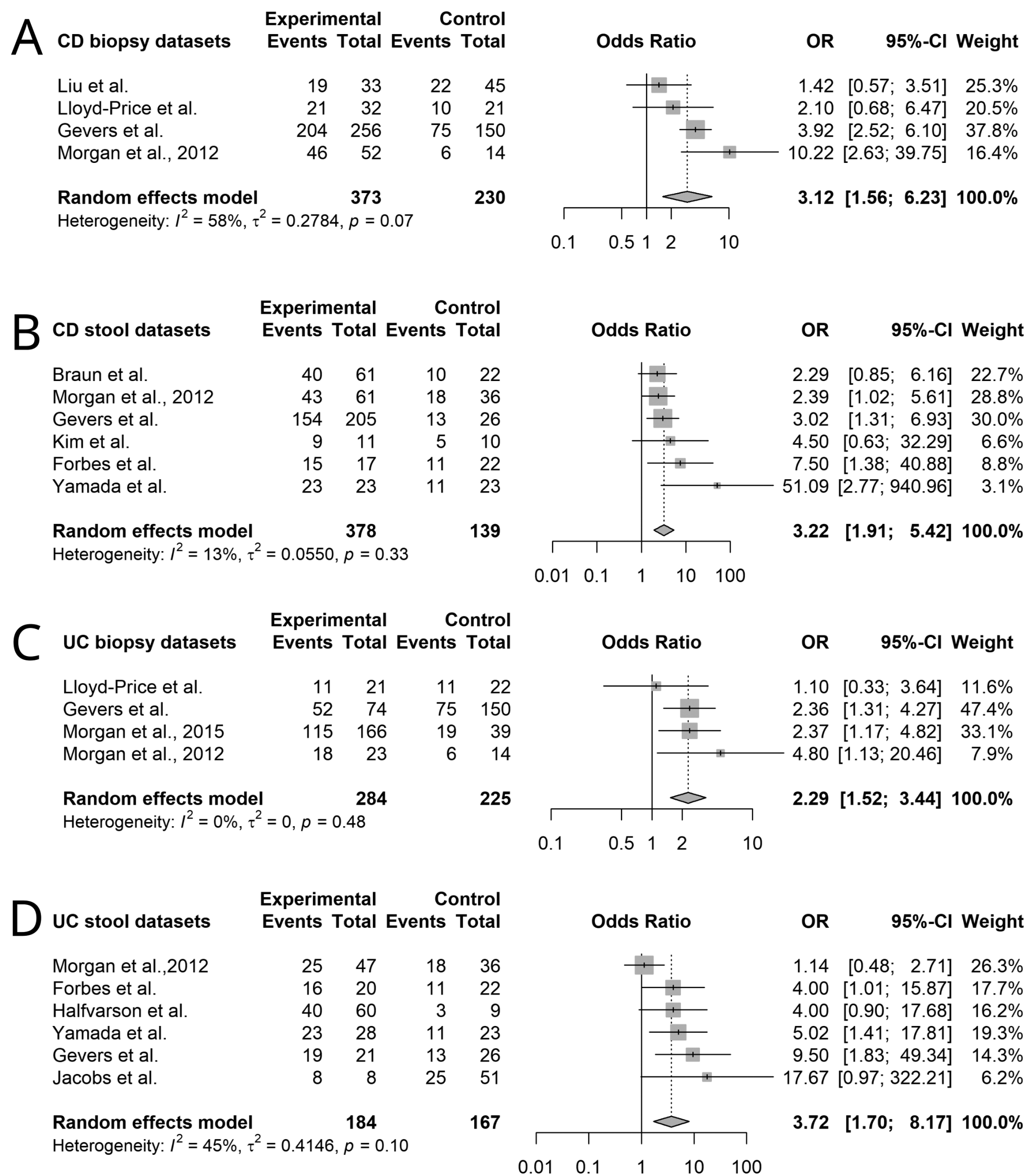

Figure 3. The relationship between odds ratios (ORs) for low richness and sample type: A, Crohn's disease (CD) biopsy; **B,** CD stool; **C,** ulcerative colitis (UC) biopsy; **D,** UC stool. We acknowledge the work of all authors of each study; however, we removed "et al" in figures for simplicity in visualization. CI, confidence interval.

both CD and UC (I^2 < 50%) (Figure 2). Therefore, individuals with both CD and UC were more likely to have low richness than individuals without IBD.

Stool Has Greater Sensitivity Than Biopsy for Detection of IBD-Associated Decreases in Alpha Diversity

The CD- and UC-associated decrease in richness was observed in both stool (CD: OR, 3.22 [1.91-5.42]; UC: OR, 3.72; 95% CI, 1.70-8.17; both P < .05) and biopsy (CD: OR, 3.12; 95% CI, 1.56-6.23; UC: OR, 2.29; 95% CI, 1.52-3.44; both P < .05) (Figure 3A-3D) samples. However, variation was higher within studies using biopsy samples for both CD and UC. Furthermore, there was high study heterogeneity within CD studies that use biopsy samples (I^2 > 50%; P < .05) (Figure 3A). Interstudy differences in biopsy location, extent of disease, medication, patient age, and previous surgery may have all contributed to this heterogeneity. These results indicate that although both CD and UC patients are more likely than non-IBD control subjects to have low richness, and it is detectable in both sample types, stool samples may yield more consistent results than biopsy samples. Furthermore, the heterogeneity observed within CD biopsy samples means that it is questionable whether this effect can be generalized to all populations.

The Loss of Alpha Diversity Is Greater in CD Than in UC

Having observed that both CD and UC patients were at a higher risk than control subjects of having low richness, we next calculated each study's effect size (using Cohen *d*) to estimate the

number of taxa lost in association with CD and UC. Every CD study had at least a small effect size, and *d* was medium or large for most CD studies (mean *d* = 0.8 [range, 0.2-2.0]) (Table 2). For all CD stool studies, *d* was medium or large, corresponding to a loss of up to 58% of taxa. The lone exception was the Kim et al dataset, which showed a CD-associated gain of 9% of taxa (Table 2). The range of Cohen's *d* for UC studies was wider; effect sizes ranged from negligible to large (mean *d* = -0.6 [range, 0.0-0.9]) (Table 2). A clear distinction was observed between UC stool studies and UC biopsy and combination studies. Cohen's *d* for UC stool studies was medium to large (mean *d* = 0.84 [range, ≥0.6-0.9]) (Table 2). In contrast, with the exception of the Gevers et al biopsy dataset, Cohen's *d* for UC biopsy and sample combination studies was negligible to small (*d* = 0.0-0.4) (Table 2. However, average taxa loss was comparable among stool and biopsy UC studies (5%-29% and 8%-19%, respectively) (Table 2). These results indicate that CD has a larger effect on the observed alpha diversity of the microbiome on average than UC; however, the number of taxa lost in UC patients is more consistent.

Table 2. Comparison of Observed Richness for CD vs Control Subjects and UC vs Control Subjects: Study Effect Sizes

Sample Type	Dataset	CD			UC		
		Cohen's *d*	Effect Size	Percentage Observed Richness Change	Cohen's *d*	Effect Size	Percentage Observed Richness Change
Lavage	Mottawea et al	-0.4	Small	14% loss	-0.2	Medium	21% loss
Stool and biopsy combination	Kim et al	0.2	Small	9% gain			
	Morgan et al 2012	-0.6	Medium	19% loss	-0.3	Small	11% loss
Stool	Forbes et al	-1.7	Large	52% loss	-0.9	Large	22% loss
	Yamada et al	-2.0	Large	38% loss	-1.0	Large	35% loss
	Jacobs et al	-1.2	Large	39% loss	-0.7	Medium	5% loss
	Halfvarson et al	-1.2	Large	58% loss	-0.7	Large	22% loss
	Braun et al	-0.6	Medium	9% loss			
	Gevers et al stool	-0.5	Medium	25% loss	-0.8	Large	29% loss
Biopsy	Lloyd-Price et al	-0.6	Medium	15% loss	0.04	Negligible	8% loss
	Liu et al	-0.1	Small	6% loss			
	Gevers et al biopsy	-0.9	Large	37% loss	-0.6	Medium	17% loss
	Morgan et al 2015				-0.5	Medium	19% loss

Effect size is measured as Cohen's *d* (large effect >0.8, medium effect >0.5, small effect >0.2). Positive and negative scale indicate opposite directions of change. A negative effect size corresponds to a disease-associated loss of taxa, while a positive effect size corresponds to a disease-associated gain of taxa (ie, if the Cohen's *d* is negative, then the mean observed richness percent in the disease group is lower than the mean of the control group).
Abbreviations: CD, Crohn's disease; UC, ulcerative colitis.

Table 3. The Effects of Disease and Sample Type on Community Structure

Sample Type	Dataset	Disease		Sample Type	
		R^2	*P* Value	R^2	*P* Value
Lavage	Mottawea et al	3.6%	NS		
Stool and biopsy combination	Kim et al	3.6%	<.05	8.5%	<.001
	Morgan et al 2012	2.9%	<.001	9.1%	<.001
	Schirmer et al			8.3%	<.001
Stool	Forbes et al	10.1%	<.001		
	Yamada et al	12.9%	<.001		
	Jacobs et al	5.4%	<.001		
	Halfvarson et al	4.9%	<.001		
	Braun et al	2.1%	<.001		
	Gevers et al stool	0.6%	NS		
Biopsy	Lloyd-Price et al	1.8%	NS		
	Liu et al	3.1%	<.01		
	Gevers et al biopsy	1.5%	<.001		
	Morgan et al 2015	2.3%	<.01		

PERMANOVA analysis is based on Bray-Curtis distance. Empty cells represent unavailable metadata.
Abbreviation: NS, not significant..

Microbiome Structure Is Altered With Disease and Sample Types

To determine the effects of disease and sample type on overall community structure (beta diversity), we used PERMANOVA based on Bray-Curtis distance (Table 3). Disease accounted for 1.5% to 12.9% of variation in community structure and significantly affected beta diversity in 10 of 13 analyzed datasets (PERMANOVA) ($P < .05$) (Table 3). The effect of disease could not be measured in the Schirmer et al dataset because the study design did not include healthy control subjects. The influence of sample type was measured within studies containing both stool and biopsy samples. Sample type explained 8.3% to 9.1% of community variance (PERMANOVA) ($P < .05$) (Table 3) in all 3 datasets. These results support previous claims that both UC and CD significantly impact the gut microbiome community structure; however, they also indicate that sample type contributes to community variation and could be a major contributor to inconsistencies in results across studies.

CD- and UC-Associated Taxonomic Changes Are Inconsistent But Some Changes May Be Specific to Sample Type

Previous literature has associated CD and UC with distinctive taxonomic changes at both the phylum and genus levels.[75-77] Among the most widely reported are decreases in *Faecalibacterium prausnitzii*[75,78,79] and increases in *Escherichia coli*.[45,80-83] We used DESeq2 to test which genera were significantly associated with disease in each study (Figures 4, 5, and 6; Supplementary Tables 1 and 2).

The most consistent IBD-associated taxonomic changes were *Erysipelaoclostridium* and *Tyzzerella_4*, both of which were more abundant in both CD and UC samples. *Veilonella, Enterococcus*, *Eggerthella*, and *Hungatella* were also positively CD and UC associated in multiple studies, while the Lachnospiraceae_NK4A136_group and *Prevotella_9* were negatively associated with both diseases. *Haemophilus* was consistently more abundant in UC biopsy than control samples. Most genera were not consistently associated with disease across UC studies, but in general, Proteobacteria and Actinobacteria tended to increase with UC, Bacteroidetes tended decrease, and Firmicutes were inconsistent.

Increases in many proteobacteria were associated with CD, but unexpectedly, a significant increase in *Escherichia* was seen in only 1 study (Figures 4 and 5). *Faecalibacterium* did not change in most datasets, and the direction of change within CD was not consistent. *Fusobacterium* was the genus most consistently and specifically associated with CD. Firmicutes were variably affected by disease. Facultative anaerobes such as *Streptococcus* and *Enterococcus* were the most consistently increased in CD samples, specifically in stool. Multiple CD studies showed increases in *Blautia*, *Flavonifractor*, and *Veilonella*. Many studies showed decreases in Lachnospiraceae and Ruminococcaceae, although the affected genera varied between studies. This trend was observed both in CD and UC studies but was more consistent in CD. *Lachnospira* was the genus most consistently decreased consistently across most CD datasets. For UC, the number of genera associated with disease in more than 1 study was comparable for stool and biopsy samples (7 and 8 genera, respectively). In contrast, for CD, 19 genera were disease-associated in more than 1 study for stool samples, but only 1 genus was disease-associated in multiple biopsy studies (Figures 4, 5, and 6). This result indicates that for CD, stool samples may be more consistent than biopsy samples for associating taxa with disease.

Discussion

We have performed a thorough, systematic literature search for 16S rRNA sequencing-based studies of the IBD microbiome, and a meta-analysis of the 13 studies that met our inclusion criteria. To the best of our knowledge, this is the largest systematic meta-analysis of the ecology of the IBD gut microbiome to date. We examined the effects of disease and sample type on alpha diversity, beta diversity, and taxonomic composition, examining both effect sizes and consistency between studies. This study confirmed that a decrease in observed richness relative to healthy control subjects was consistent for both UC and CD and showed that this effect is slightly stronger and less heterogeneous in stool samples. Beta diversity was consistently affected by disease (UC and CD) and sample type (stool and biopsy); effect size varied but was more consistent within stool samples than within biopsies. Taxonomic changes were inconsistent across studies.

Biopsy Location Heterogeneity Adds Uncertainty to Alpha and Beta Diversity Measurements and Should Be Considered During Study Design

Observed richness and Shannon entropy are 2 of the most widely used metrics for measuring alpha diversity. Richness calculations are heavily influenced by bioinformatic methods,[84] as well as by other aspects of study design, such as sequencing technology,[85] sequencing depth,[86,87] and primer choice.[88] We have controlled for this as much as possible in 2 ways. First, we reprocessed primary data from all studies using uniform bioinformatic methods, minimizing this as a source of bias. Furthermore, to control for the influence other technical issues, we used case vs control ORs for comparing richness. This presumes that for each study, technical issues affect cases and control subjects equally. In agreement with many existing studies,[8,21,23,24] gut richness was significantly reduced in both UC and CD patients. However, the 95% CI for CD biopsy samples was the widest of all sample types. Although biopsies represent the microbiome of the specific gut site from which they were collected more accurately than fecal samples,[89,90] biopsy location was heterogeneous both within[30,61] and between[26,30] CD studies, as Morgan et al, Lloyd-Price et al,[61] and Gevers et al[30] mixed biopsy types, some from different locations of the gastrointestinal tract as well as sites with active and inactive disease[26,30]

Some of the CD studies included in this analysis had both ileal and colonic samples.[10,30] In contrast, the locations of UC biopsy samples were more homogeneous, and the 95% CIs were narrower. It is possible that higher data heterogeneity within biopsy studies has impacted the conflicting results found in the literature regarding richness in CD.[10,63] Alternatively, the magnitude of the effect of CD on the gut microbiome may vary more than that of UC. This result suggests that uniform biopsy location and standardization of disease activity would improve data resolution in microbiome studies. Previous studies[91,92] have found greater richness in fecal samples than in biopsy samples. Our results, however, suggest that fecal samples may be more consistent than

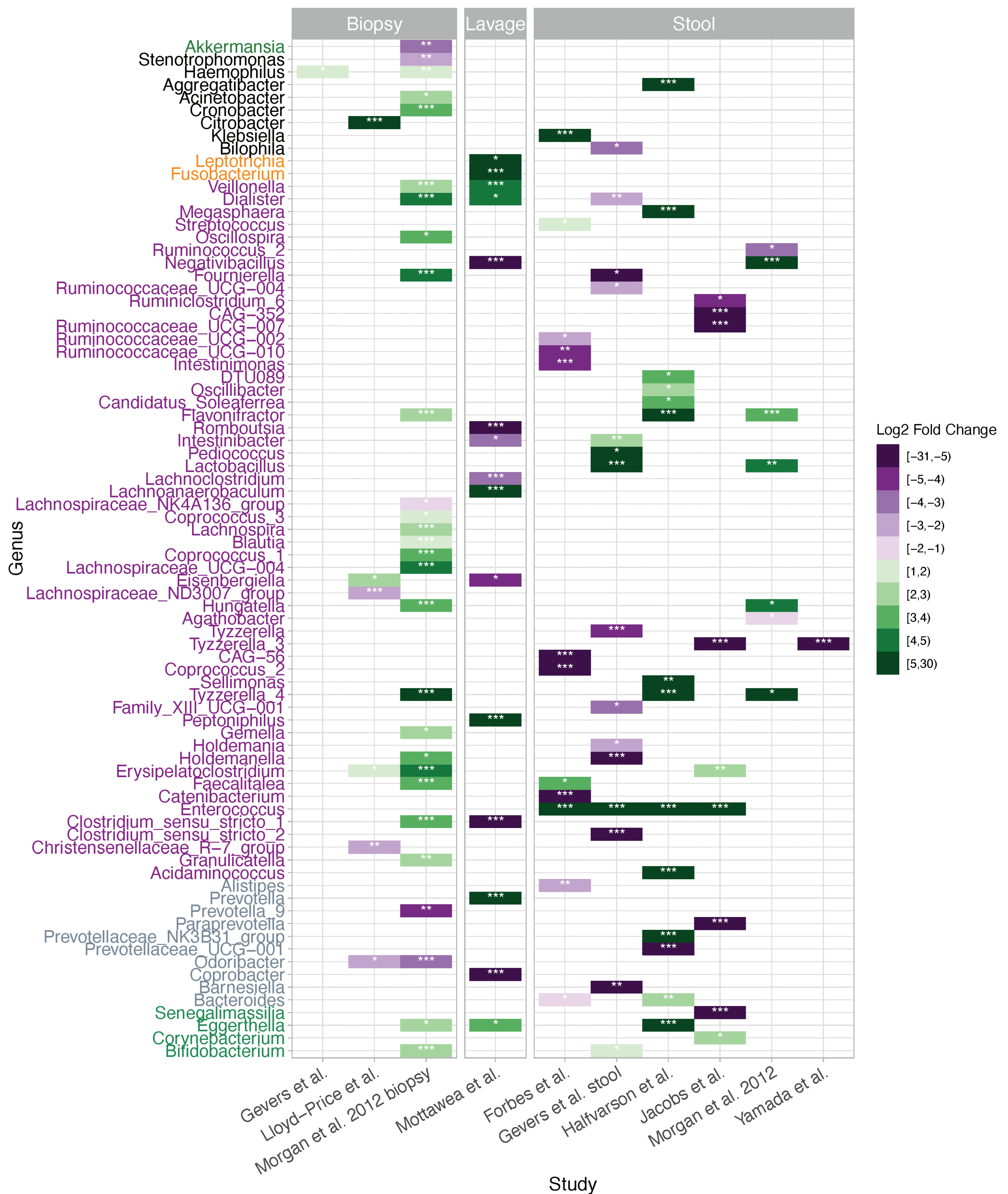

Figure 4. Log2 fold changes in the abundance of genera in ulcerative colitis samples compared with control subjects. Ulcerative colitis vs non–inflammatory bowel disease. The y-axis colors pastel green, gray, magenta, orange, black, and lime green indicate phyla Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria, Proteobacteria, and Verrucomicrobia, respectively. *, **, and *** denote $P < .05$, $P < .01$, and $P < .001$, respectively. Genera are organized by family within phyla (See online version for color figure)..

biopsy samples for studying alpha diversity within the IBD microbiome.

Our analysis of beta diversity showed that both UC and CD affected the overall community structure of the gut microbiome. The Bray-Curtis distance was used in this study because it compares samples based on both the taxa present and differences in their proportions. The relationship between beta diversity and IBD has been researched extensively,[10,30,61,64]

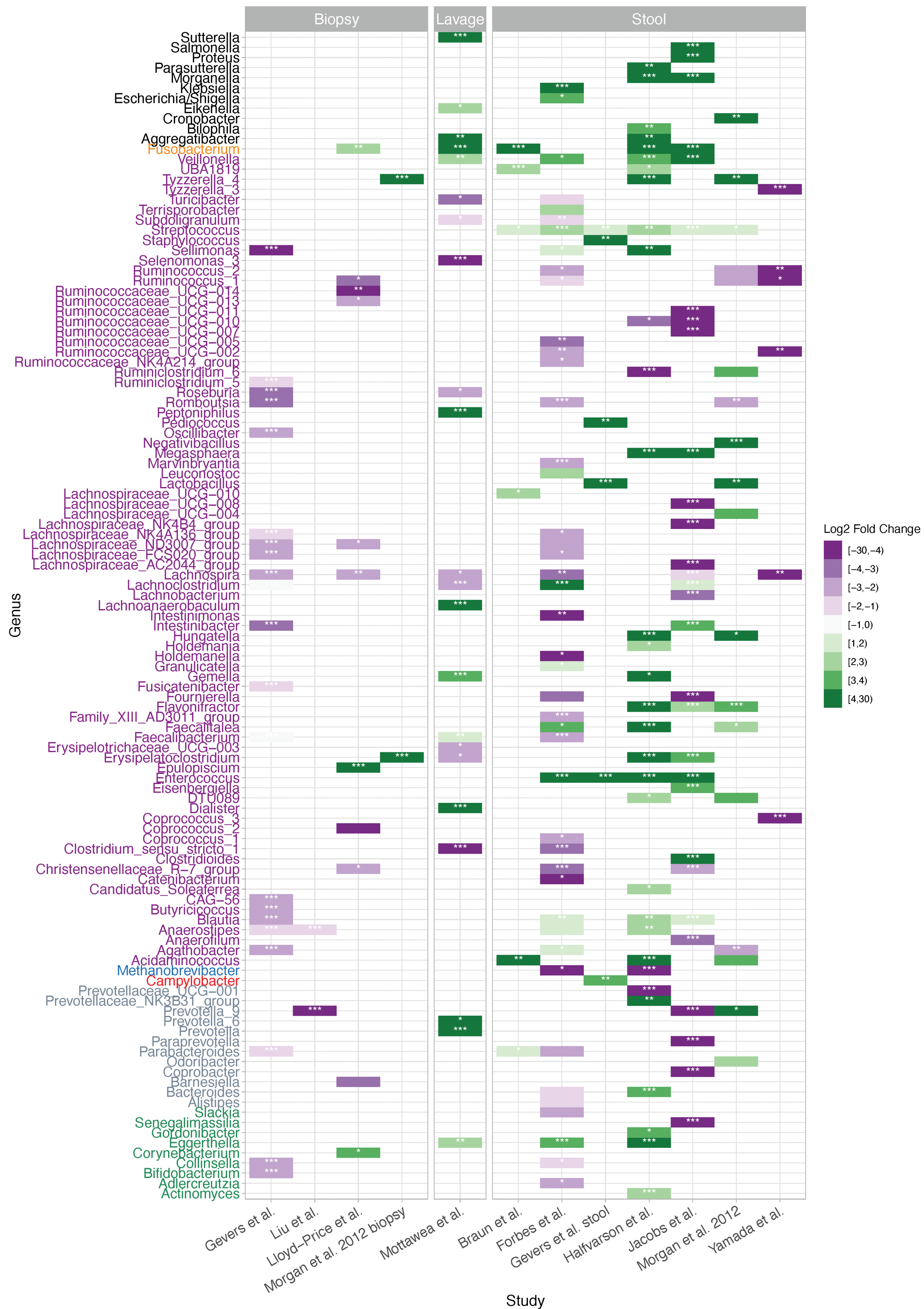

Figure 5. Log2 fold changes in the abundance of genera in Crohn's disease samples compared with control subjects. Crohn's disease vs non–inflammatory bowel disease. The y-axis colors green, gray, blue, magenta, orange, and black indicate phyla Actinobacteria, Bacteroidetes, Euryarchaeota, Firmicutes, Fusobacteria, and Proteobacteria, respectively. *, **, and *** denote $P < .05$, $P < .01$, and $P < .001$, respectively. Genera are organized by family within phyla (See online version for color figure).

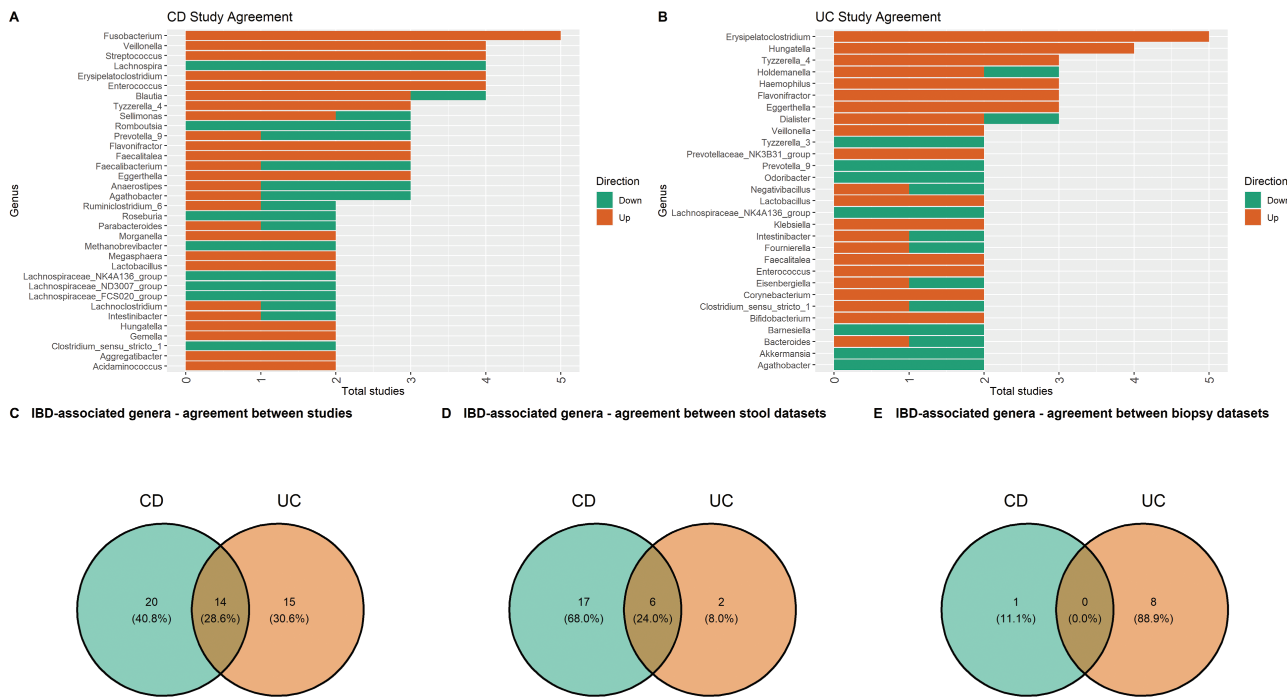

Figure 6. Inflammatory bowel disease–associated taxa: interstudy agreement. All disease-associated taxa identified by DESeq2 ($Q < .2$, Benjamini-Hochberg false discovery correction) in at least 2 (A) Crohn's disease (CD) or (B) ulcerative colitis (UC) studies are shown. Color corresponds to direction of inflammatory bowel disease–associated change. C, Total numbers of CD- and UC-associated genera in at least 2 studies (See online version for color figure).

but the significance and effect size vary. In this analysis, disease significantly impacted beta diversity in most studies, but the effect size varied widely, and was typically smaller than the effect of sample type. Gevers et al[30] and Morgan et al[10] previously reported significant variation in community structure due to different sample types (stool and biopsy). The mean beta diversity effect size observed in stool samples was greater than that of biopsy samples. Again, location heterogeneity within biopsy samples is a likely contributor. This indicates that stool may be superior to biopsy for study of disease-associated beta diversity. Alternatively, these results may highlight the susceptibility of microbiome changes with sample handling, as the collection of stool samples is more likely to be mishandled by participants at home than the collection of biopsy samples by health professionals. However, many of the included studies have implied that participants were informed of ideal stool sample storage conditions to adhere to (Supplementary Table 3).

As mentioned previously, we were unable to measure the effect of sample type in the Gevers et al[30] study because stool and biopsy samples were uploaded to the SRA database separately; thus, this study was analyzed as 2 datasets of different sample type: Gevers et al stool and Gevers et al biopsy. As observed in other datasets, microbiome community variation associated with sample type exceeded variation associated with disease. All 3 mixed-sample datasets showed that more than 7% of the total community variation was due to the use of different sample types. This should be carefully considered during study design due to its substantial impact on study results.

Our results for beta diversity indicate that CD and UC patients have gut microbiomes that differ significantly from non-IBD individuals, but the size of this effect varies. In addition to sample type, many other factors may influence beta diversity. For example, extent of inflammation,[9,25,58,80,93-95] previous IBD-associated surgery,[58,93] medication use,[30,94,95] and disease location (and hence biopsy location)[7,10,30,58,79] are all known to affect the IBD gut microbiome and may be particularly problematic in small studies with high heterogeneity.

Fusobacterium and Enterococcus Are Most Consistently Associated With IBD

IBD is widely reported to change the taxonomic composition of the gut microbiome,[10,29,30] but these changes may be either cause or consequence of IBD. We used DESeq2 to quantify taxonomic changes associated with UC and CD. Many studies have examined IBD-associated microbiota at the phylum level. As found in previous studies, proteobacteria were generally more abundant in IBD samples, and many Firmicutes were less abundant.[96-98] Although previous studies have reported CD- and UC-associated decreases in Bacteroidetes,[21,44] our analysis found that fewer Bacteroidetes than Firmicutes were affected by disease. Increased levels of *Lachnospira* have been associated with successful anti-tumor necrosis factor therapy outcomes in CD patients.[99,100] We observed reduced levels of *Lachnospira* within the Gevers et al[30] dataset, which comprised new onset IBD, and within Lloyd-Price et al,[61] in which we used the first time point from new onset IBD. Thus, *Lachnospira* may be a potential marker genus for active disease and inflammation. *Faecalibacterium prausnitzii* and *Roseburia* are 2 genera consistently linked to UC and CD.[78,79,101-104] Reduced levels of these butyrate-producing genera have been associated with a higher risk of postoperative recurrence of ileal CD, and dysbiosis that contribute to inflammation and possibly pathogenicity in UC

and CD patients.[101,103] However, the extent of reduction has been shown to depend on disease status (active or in remission),[105] which may be a reason we did not observe consistent reductions in these genera. Unfortunately, most studies did not include this metadata, so it was not possible to test in this analysis.

Fusobacterium and *Enteroccocus* were consistently enriched in IBD patients across numerous studies. *Fusobacterium* was mostly associated only with CD, while *Enterococcus* was associated with both CD and UC. *Fusobacterium* has been shown to cause mucosal inflammation and colonic ulcers in mice,[106] and previous studies have reported a positive correlation between levels of *Fusobacterium* and severity of IBD.[107] *Fusobacterium* adheres to a wide variety of immune cell types.[108,109] It is a potent immune stimulator and can invade human epithelial cells.[108] It is not known whether *Fusobacterium*'s invasive ability contributes directly to the pathogenesis of CD development or elevated levels simply reflect opportunistic growth.

In this study, high levels of *Enterococcus* were only observed in stool samples, and *Enterococcus* was more consistently enriched in CD. Enterococci may be more enriched in CD due to their relatively high tolerance for bile salt in comparison with other commensals.[110-113] Bile salts are potent antimicrobial agents[114] released into the small intestine and mostly reabsorbed into the bloodstream in the terminal ileum,[115] so colonic concentrations are much lower. Reabsorption efficiency is impaired when the ileum is damaged due to disease or surgery,[116] but this process is specific to CD and may also to the changes in alpha and beta diversity associated with CD. *Enterococci* also have high levels of intrinsic antibiotic resistance.[117,118] Because they are facultative anaerobes,[119] they may become abundant in patients treated with metronidazole[62,120] due to reduced competition. Metronidazole is widely used in treatment of both CD and UC.[121-123] Again, it is unclear whether Enterococcus contributes to IBD pathogenesis or is an opportunistic bystander.

Application of Updated Bioinformatic Methods May Increase the Resolution of Historic Datasets

In some cases, our reanalysis of datasets does not concur with the original studies. For example, in 4 cases we found a significant difference in alpha diversity that was not reported by the study authors.[10,26,58,63] It is likely that this is due to differences in bioinformatic methods, which affect alpha diversity results of 16S rRNA amplicon sequencing.[124] Many studies that found no significant difference in alpha diversity between control subjects and disease groups used Qiime 1.0,[26,63] which has previously been shown to yield higher estimates of alpha diversity than many newer, more stringent methods.[124,125] This may have decreased the effect size so that differences between cases and control subjects could not be reliably detected. In this study, we used DADA2[65] for primary data processing, which may improve the accuracy of alpha diversity estimates due to improved denoising.[85,126]

Another discrepancy between this study and the original studies is inconsistent detection of IBD-associated taxa such as *Faecalibacterium* and *Roseburia*. These calculations are influenced by choice of OTU AND ASV method, as previously discussed, as well as the taxonomic database used. In addition, the choice of tool and tool parameters used for differential abundance analysis impacts results.[127] Some of the original studies used multivariate analysis (analyzing taxonomic changes in association with more than 1 factor, such as antibiotic use or disease location),[10,56] or examined specific subpopulations. For example, Halfvarson et al[58] found significant levels of *Faecalibacterium* when specifically comparing ileal CD samples from patients that had undergone ileocecal resection surgery with those that had not and non-IBD control subjects.[58] OWING to lack of consistent metadata necessary for more nuanced analysis, our study only compares diseased and healthy control samples. Any combination of these factors could contribute to differences between our results and those of the authors of the original studies.

This analysis confirmed a consistent effect from IBD on alpha and beta diversity, estimated effect sizes, and included the largest and most recently published datasets with publicly available data and metadata. Our results show that sample type is likely to contribute to inconsistencies in IBD microbiome results observed in the literature and highlight the importance of careful consideration of this during study design. One limitation of our study is that many samples were lost due to removal of multiple samples from the same individuals in longitudinal datasets. Another limitation is that we could not directly compare biopsy with stool samples within Gevers et al,[30] one of the largest IBD datasets, due to inadequate metadata. Finally, we could not analyze the impact of important clinical factors such as inflammation, biopsy location, and medication, also due to the lack of metadata. We highly recommend that when making data available, study authors include metadata such as patient disease, disease location, disease activity, biopsy location, and medication, as this information can be used in future analysis of the IBD gut microbiome. We also urge authors to ensure that the timing of sample collection is clear within longitudinal studies, and that multiple samples from the same individual are clearly documented within their metadata.

Conclusions

Although we found few consistent taxonomic differences across UC and CD datasets, our results show that IBD has a consistent effect on ecosystem alpha and beta diversity and suggest that stool type may be superior to biopsy in representing IBD-associated dysbiosis due to decreased heterogeneity. We further demonstrate the benefit of applying updated bioinformatic methods to historic data.

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Conflicts of Interest

None declared.

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