Understanding the Clinical Microbiome
Biological Engineering Thesis Proposal

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Contents

Abstract

Researchers and clinicians have long appreciated the contribution of microbes to human disease. Recently, many studies have moved from analyzing the effect of individual microbes on human health to studying the complex interactions between microbial communities and their human hosts, due in large part to developments in high throughput DNA sequencing. We are beginning to understand that microbial communities are involved in disease and are also integral to human health, but there remains no clear understanding on the precise relationships between human microbial communities and disease. Certain microbial communities, like those of the aerodigestive tract, remain drastically under-studied. On the other hand, the gut microbiome is extensively studied but lacks a synthesized understanding of the relationship between alterations in its microbes to disease in general and to specific diseases. This thesis will expand our understanding of the clinical relevance of the human microbiome by characterizing the understudied aerodigestive tract microbiome, synthesizing results from gut microbiome case-control studies across all studied disease states, and developing a tool to enable generalizable analysis of microbial communities. This work will identify promising candidates and approaches for follow-up mechanistic studies, and will move the field toward a more translationally-relevant understanding of the relationship between our microbes and our health.

1 Overall objectives and specific aims

1.1 Overall objectives

In spite of the recent increase in research on the human microbiome, there is not a clear consensus on the relationship between human microbial communities and disease. The microbiota of the aerodigestive tract is poorly studied, and we have little understanding of how the microbial communities in different aeordigest sites are related or affected by disease. In contrast, the gut microbiome has been extensively studied through many case-control studies. However, these studies have frequently yielded inconsistent or incomparable results. Existing meta-analyses have not extended to more than one or two diseases, and thus can not determine whether significant microbes are associated with specific diseases or with disease in general. Finally, there are no existing tools that can be used to extract general biological insights from groups of disease-associated microbes.

This thesis will expand our understanding of the clinical microbiome in three ways. First, I will characterize the relationships between the microbial communities of the aerodigestive tract and their associations with clinical factors, increasing our basic understanding of this under-studied system. Next, I will perform a meta-analysis of published case-control gut microbiome studies across many disease states, identifying consistent microbial markers of health and disease. Finally, I will curate groups of biologically related microbes, thus enabling enrichment analyses and generalizable interpretations of results from microbiome studies.

1.2 Specific Aims

Aim 1 Apply standard methods to identify microbial community characteristics associated with gastro-esophogeal reflux disease and aspiration.
1. Determine how lung, gastric, and throat microbial communities are related.
2. Identify clinical modulators of lung, gastric, and throat microbial communities.

Aim 2 Perform a meta-analysis of case-control gut microbiome studies to identify consistent microbial signatures within and across multiple diseases.
1. Compile and process publicly available case-control gut microbiome studies with a standardized method.
2. Determine whether certain microbes are consistently associated disease in general or with specific diseases.
3. Identify relationships between physiologically-related diseases by comparing their microbial characteristics.

Aim 3 Enable generalizable interpretations of microbiome analyses by assigning bacteria to groups with similar functions and known associations with disease.
1. Combine existing databases with targeted literature searches to define *microbe sets* based on known biological relationships.
2. Use machine-learning techniques to extract disease- associated *microbe sets* from datasets collected in Aim 2.
3. Develop these *microbe sets* into a collaborative tool for use in interpreting new microbiome studies.

2 Background and significance

Three to five pages

2.1 Biological background and significance

2.1.1 Aerodigestive tract

The aerodigestive tract consists of the upper gastrointestinal and respiratory tracts (REF). From an engineering perspective, the stomach, throat, and lungs can be thought of as different compartments connected by the esophagus and windpipe. (FIGURE) The mass transport between these compartments is regulated by complex physiological mechanisms. Swallowing guides material from the mouth to the stomach, but may dysfunction and allow material to enter the lungs. The esophageal sphincter usually prevents material from leaving the stomach, but in some diseases is dysfunctional. Finally, complex homeostatic mechanisms clear the lungs of foreign bodies and create a very selective environment for microbes in the lungs (REFS REFS REFS). Thus, while the throat, stomach, and lung "compartments" are physically connected, the amount of material, including bacteria, flowing between them is not readily apparent.

2.1.2 Gastro-esophogeal reflux disease and aspiration

Gastro-esophogeal reflux disease (GERD) is set of syndromes in which the reflux of stomach contents leads to troublesome symptoms or complications [1],[2] (FIGURE - Figure 2 of Vakil et al). The most common symptoms of GERD are regurgitation and heartburn, but the disease may also present asymptomatically[1],[2]. GERD can be diagnosed by demonstrating reflux of gastric contents (via pH and impedance monitoring), injury to the esophogus (via endoscopy), or based on symptoms alone[1].

Aspiration is the inhalation of foreign material such as food or gastric contents into the lungs [3]. Most aspiration events are unwitnessed without obvious outward signs or symptoms, and may involved large quantities of aspirated material or may be micro-aspiration events [3]. Aspiration resulting from swallowing dysfunction can be diagnosed with a Modified Barium Swallow test (MBS) [4]. In the MBS procedure, patients are observed videoradiographically as they swallow varying quantities and viscosities of food or liquid impregnated with barium, a contrast agent. The swallowing process is observed and abnormalities like aspiration of contents past the vocal chords can be diagnosed [5], [4]. However, the MBS test cannot be used to determine aspiration of gastric contents or episodes of micro- aspiration, which may also have clinical relevance [3],[6]. No validated clinical biomarkers exist to diagnose and define gastric and micro-aspiration events[6], but they are thought to play a role in causing or exacerbating many respiratory diseases [7],[8], [9]. Currently, microaspiration of gastric contents is studied by measuring the concentration of bile or pepsin in the lungs, but such assays are rarely validated against gold-standard methods and have not undergone clinical validation [9],[6]. Further complicating the issue is that many healthy patients have a baseline level of micro-aspiration (REF).

2.1.3 GERD, aspiration, and respiratory disease

GERD is associated with many respiratory diseases, but the precise link and mechanisms underlying these associations remains very unclear [9]. The prevalence of GERD in respiratory diseases has been estimated to be up to 90% for some diseases, and often presents without the common symptoms of heartburn or regurgitation [9]. Studies have shown that GERD is related to adverse outcomes after lung transplantation (REF) and reduced lung function in patients with cystic fibrosis [8]. Aspiration of stomach contents is thought to contribute to these adverse outcomes, either through the aspiration of bile triggering a change in the lung's environment and making it more favorable to colonization, or through direct aspiration of gastric bacteria leading to infection [8],[7]. However, because of the difficulties in diagnosing and studying microaspiration, aspiration of gastric contents, and GERD, precise causal links between reflux, aspiration, and lung disease have yet to be established [8],[9].

2.1.4 Microbiome of the aerodigestive tract

The microbiota of the human lungs and stomachs are among the least well-studied human-associated microbial communities. In fact, the lungs are classically thought to be sterile and free of bacteria in healthy people [10],[11]. Neither gastric nor lung sites were included in the Human Microbiome Project, leading to a dearth of studies and data on these important body sites (HMP REF). However, subsequent studies have found suggestions that aerodigestive tract sites are microbially related, and that these microbiomes are altered in certain disease states (REFS).

Few studies have examined the relationship between sites of the upper aerodigestive tract [11]. Bassis et al. found that oral microbial communities were more similar to both stomach and lung communities than nasal communities. However, these authors did not examine the inter-relationships between all sites - their driving hypothesis was that the mouth is the source of the downstream communities. In this work, we will take a broader view: which of the upper aerodigestive body sites exchange microbes, are these the same microbes across multiple patients, and are there patterns for these shared microbes?

2.1.5 Lower gastrointestinal tract: physiology and microbiome

The human gastrointestinal tract is integral to health and disease. Its primary function is to digest food and absorb nutrients, and it also plays important roles in many other aspects of physiology. For example, it is the largest secretor of serotonin and something else (blood flow??) (REF?!). The lower gastrointestinal tract is populated by a complex microbial community, which serves important functions in digesting and salvaging additional nutrients, interacting with the immune system, and protecting the gut from invasion by pathogenic organisms (REF).

We know that the microbial communities in our guts are unique to individuals (REF), modifiable on short- and long-term scales (HuGE REF), and integral to our overall health (REF). Fecal microbiota transplants have demonstrated the incredible causal ability of the microbiome to affect health in human patients. Germ-free mouse models have shown that microbes are necessary for healthy functioning, and specific animal models have allowed for

probing mechanistic understanding of host-microbial interactions (autism 440 ref, gordon mouse experiment ref).

Many case-control studies examining the association between gut microbial disease and putatively associated gut-related diseases exist, but there is little consensus among or synthesis of the existing research. A few meta-analyses have been recently undertaken to leverage multiple studies based on similar disease states. However, these studies have rarely found consistent signals and fail to account for the broad variety of disease states which are thought to be microbially-related (meta-analysis REFS). Thus, the current field of clinically-motivated microbiome science lacks a synthesized understanding of the relationship between gut microbial communities and disease states across the broad range of previously studied conditions.

2.1.6 Microbiome/disease associations of the lower gastrointestinal tract

The microbes in our guts provide essential functions to our well being. They help us harvest energy from the food we eat and digest otherwise undigestible fibers, train our immune system, break down xenobiotics and other foreign products, and release metabolites and hormones that provide chemical signals to our bodies. These signals can act locally in the gut and can also have larger systemic effects, for example by sending signals through the vagus nerve to the brain via the 'gut-brain axis' (REFS?!). Because of this complex interplay between host and microbes, many diseases have been hypothesized to have associations with the gut microbiome.

The relationship between the gut microbiome and obesity has been extensively studied in mouse models and human patients. These studies have found encouraging results and causal associations in mice but relatively little consensus in humans (REFS, incl jump, look at escobar paper for good conflicting refs). Related disorders like metabolic syndrome and diabetes have also been examined, with little consensus and speficic microbial markers (TODO: edit after reading papers!). Inflammatory bowel disease (IBD) is a chronic disease characterized by mucosal inflammation of the GI tract. Animal studies relating gut microbes, immune function, and IBD have pointed to an important role for bacteria in affecting the progression of IBD [12]. Recent 16S studies have focused on classifying IBD patients based on their fecal microbiota and on identifying discriminatory taxa in stool [?], [?], yielding mixed results. Colorectal cancer (CRC) has long been associated with the intestinal microbiome, with bacterial metabolites thought to contribute to the development of CRC and specific bacteria having been isolated from CRC tumors (zhao, xiang). 16S studies have analyzed disease associations with tumor- and lumen-associated microbiota, and have consistently found enrichment of the *Fusobacterium* genus in CRC patients. Diarrheal diseases caused by intestinal pathogens have also been extensively surveyed using 16S methods, especially in the context of *Clostridium difficile* infection (CDI) and related fecal microbiota transplants (REFS). Finally, diseases like rheumatoid arthritis, autism, Parkinson's, HIV, MHE, and others have also been examined for microbial associations, though these fields remain relatively unexplored (REFS).

2.2 Analytical background and significance

2.2.1 Data generation and challenges

The advent of high throughput next generation sequencing has enabled culture-independent analyses of complex microbial communities. Briefly, DNA is extracted from biological samples of interest and a portion of the universal bacterial 16S region is amplified. Data is sequenced using a variety of available technologies such as 454 Pyrosequencing or, more recently, Illumina HiSeq or MiSeq (REFS). The resulting reads are quality-controlled and processed into Operational Taxonomic Units (OTUs), clusters of similar sequences which serve as proxies for bacterial species. OTUs can be assigned bacterial taxonomies using a variety of methods, for example by mapping them to annotated reference genomes or using Bayesian inference trained on a reference set of annotated bacteria (GG and RDP REFS).

The resulting datasets are often very high-dimensional, with hundreds of OTUs present in a given cohort which may only have tens of samples. The data is also incredibly sparse: only very few OTUs tend to be present in many of the samples, and most entries in the data matrix are zeros. Furthermore, technical artifacts such as DNA extraction and PCR amplification biases are known issues that lead to strong batch effects across different studies. Different data processing, quality filtering, and name-assignment methods also lead to strong batch effects. For example, different taxonomy databases contain different microbes or conflicting names for the same bacteria, making it difficult to compare even published, annotated results across studies. These issues may be major contributors to the lack of consensus on the role of the microbiome in disease, in spite of the availability of many studies.

2.2.2 Current analytical approaches for 16S analyses

While there exist no established standards for processing or analyzing 16S data, most studies take similar approaches to gleaning insight from case-control cohorts. Alpha diversity, the diversity of species within each sample, is usually compared for case and control groups. Many studies have found slight indications that alpha diversity is higher in healthy patients (REF). Beta diversity, the diversity between samples, is also frequently compared to understand whether samples within the control or case group are more similar to each other than they are to the other group. Finally, most studies perform univariate non-parametric tests on the abundance of OTUs to find bacteria significantly associated with disease or health. These analyses suffer from high-dimenstionality coupled with low power (few samples) - after multiple hypothesis testing corrections, many studies yield no significant results (REF).

Existing meta-analyses of microbiome datasets often focus on healthy patients or on just one or two diseases [13], [14]. These meta-analyses have had mixed results. In some cases like IBD, strong and consistent signals can be found across studies and in other cases. A meta- analysis of obesity studies found no clear taxonomic signal, and a difference in alpha diversity that was significant but likely too small to be biologically relevant[14]. Another study found no clear results in obesity datasets but did identify a strong signal associated with IBD across multiple datasets [13]. Every meta-analysis performed on 16S data has observed strong batch effects between studies and noted the need for large sample sizes to extract any meaningful signal [14], [13],[15], [16].

Additionally, many of the most successful existing meta-analyses combine vastly different

types of microbial communities and non-case- control experimental designs. The positive results from these studies are not particularly biologically insightful: it is a much easier task to differentiate vastly different communities (like the skin vs. the gut) than it is to differentiate subtleties contributing to health and disease (like the inflamed gut vs. the healthy gut) [15].

2.2.3 Databases and tools for annotations

Few analytical tools exist to motivate biological hypotheses from the results of high-throughput case-control microbiome studies. Although studies often yield some significant OTU-disease associations, assigning biological interpretations to such results remains challenging. For example, the importance of short-chain fatty acid (SCFA) producers is well-accepted in IBD, and may play in a role in many other diseases as well (REFS). However, directly identifying enrichment of functionally-related bacteria (such as SCFA-producing bacteria) is not currently possible. Instead, researchers face the task of interpreting lists of individual OTUs into common biological themes or functions on their own [17].

Enrichment analysis is a powerful way to identify biologically meaningful patterns in high-dimensional data. Enrichment analysis is widely used in RNA expression studies and has been proposed for use in metabolomics studies ([17], [18]. In Gene Set Enrichment Analysis (GSEA), genes are first ranked by differential expression between two conditions. Then, *a priori*-defined groups of related genes are analzed for over- or under-representation at either end of the ranked list. Rather than asking whether individual genes are correlated with a phenotype, GSEA allows for the identification of groups of genes which change together. This allows for identification of significant phenotypes where individual genes do not exhibit large enough changes to reach significance on their own. It also enables more direct biological interpretation, since the gene sets are defined *a priori* based on biological knowledge.

GSEA relies on the existence of curated gene sets, which has no existing analog (*i.e.*, microbe sets) in microbial databases. In GSEA, genes were grouped into gene sets based on their common pathways, functions, locations in the chromosome, and associations with disease. Similar annotations exist in some microbial databases, but none of these databases or tools have been used to define groups of related microbes. ImG contains approximately 10,000 annotated bacterial genomes, but the annotations are not fully complete and do not span all categories of possible interest [19]. SourceTracker can be used to label microbial communities according to their environmental source, but requires input training sets with each use in order to learn and make the classifications [20]. Finally, bioinformatic tools have been developed that can infer functional content (PICRUST) [21] or metabolic pathways (HUMANN) [22] from 16S data. Again, these tools are dataset-specific and have not been generalized to define biologically related groups of organisms.

3 Research design and methods

Six to eight pages

3.1 Aim 1: Aerodigestive microbiota associated with GERD and aspiration

As discussed previously, patients with aerodigestive disorders like aspiration and GERD are at a higher risk for respiratory infections. We hypothesize that microbial communities in the aerodigestive tract share and exchange certain members which then contribute to infections, and that clinical factors like reflux or aspiration disease change the amount of bacterial exchange between aerodigestive sites.

Our patient cohort consists of 261 patients recruited by Rachel Rosen (M.D., GI/Nutrition) at Boston Children's

Sites	N
gastric, throat, & BAL	87
gastric & throat	45
gastric & BAL	34
BAL & throat	9

Table 1: Samples in study

Hospital for multiple studies over the course of the past 6 years. Multiple samples were taken from patients: throat swabs, gastric fluid, and broncho-alveolar lavages (BAL) (Table 1). 125 patients were monitored for full-column GERD and 112 patients were tested for aspiration. Overall, this cohort represents the largest existing human aerodigestive microbiome dataset.

3.1.1 Exchange of microbes between lung, gastric, and throat communities

To understand the microbial exchange between sites in the aerodigestive tract, we must first identify which microbes are being exchanged and then quantify their how "shared" they are across the sites. If a microbe is being exchanged between sites, we would expect that if we see more of it in one site, then we will also see more of it in the other. Thus, we define a microbe as exchanged between two sites if the Spearman correlation of its abundance in both sites is greater than 0.5. To calculate this correlation, we consider only patients who have the microbe present in both sites (blue dots in 1). We quantify the extent of exchange by asking how many of the total patients have the microbe present in both sites. In other words, p_s is the percentage of patients who are exchanging that microbe across the two sites. This definition of exchange, which depends on correlated abundances, is superior to simple co-occurence since there is significant overlap in the members of the three aeordigestive communities[11], [10].

To determine how lung, gastric, and throat microbial communities are related, we will calculate p_s for each of the site combinations. We will then see if there are apparent phylogenetic relationships between the exchanged microbes. We expect to find significantly more exchange between the throat and stomach, and very little exchange between the throat and lungs.

3.1.2 Clinical modulators of lung, gastric, and throat microbial communities

Once we quantify microbial exchange within the aerodigestive tract, we can begin to ask which clinical factors modulate how much exchange occurs between sites. We will calculate p_s for all a-priori defined exchanged microbes (Section 3.1.1), stratified by the clinical factor. Furthermore, we will investigate whether the total abundance of exchanged microbes in the different sites and beta diversity community similarities differ between our case and control patient groups.
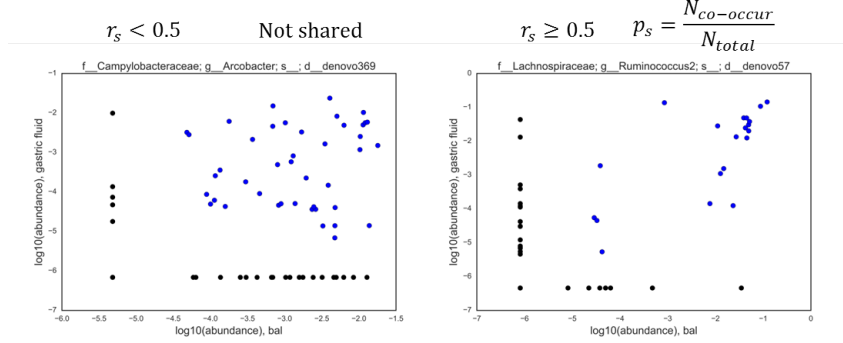
Figure 1: Calculating p_s: if the abundance of a microbe when it is present in both sites is correlated, then we consider it exchanged across those sites (blue dots). p_s is then calculated as the percentage of patients who have the microbe present in both sites (blue dots divided by total dots). Example of microbes which are (A) not exchanged and (B) exchanged between stomach and lungs.

Our first hypothesis is that aspirators will have a stronger connection between their throat and lungs. Additionally, because many of these patients have GERD, we also expect a slight increase in the connection between the stomach and lungs. Our dataset has 48 patients with abnormal MBS test results (Aspirators) and 63 patients with normal results.

Our next hypothesis is that patients with more severe GERD will have more sharing between the stomach and lung communities. Because are interested in GERD that may modulate the stomach-lung connection, we define "severe GERD" as reflux in which more than 50% of events are full-column reflux events. With this definition, we have 99 patients with severe GERD and 26 patients without. In addition to the binary severe/not severe comparison, we may regress each of the continuous measures of GERD severity onto the abundance of microbes in each patient's lung and stomach communities. We will also investigate how PPIs modulate the connection between aerodigestive sites, since PPIs are often prescribed for respiratory disease but have unclear clinical impact. The dataset has 114 patients on PPIs and 85 patients not on PPIs.

3.1.3   Additional considerations

One factor to consider when drawing conclusions from the p_s metric is that because of the low bacterial biomass in the gastric and lung sites, it is possible that some microbes which are "exchanged" across these sites are simply both being seeded by the environment. However, if these microbes are phylogenetically related or they are known members of the gastric or lung communities, this would indicate that the OTUs are being selected for by the environment and are relevant community members.

This work does not address the direction of microbial exchange between aerodigestive sites, nor does it directly link increased microbial exchange with adverse outcomes like respiratory infections. Follow up studies focus on patients who develop respiratory infections, or patients who frequently have GERD- or aspiration-associated respiratory infections.

Finally, an important caveat to note when discussing the human lung and gastric micro-

biomes in general is that our basic understanding of these communities in healthy humans is still quite rudimentary. For example, the extent to which lung microbial communities are composed of surviving and replicating microbes versus transient members is unknown. However, because we do see similarities in microbiota across patients, we can conclude that at least some selection is happening in these sites, and even if these communities are not thriving they may still be physiologically relevant.

3.2 Aim 2: Meta-analysis of gut microbiome studies

By combining results from existing gut microbiome case-control studies, we can move the field toward a consolidated understanding of consistent microbial markers of gut-related diseases. We hypothesize that certain bacteria will often be associated with disease, and that some of these bacteria will be associated with many different types of diseases while others will be unique to one or two conditions. Additionally, we hypothesize that microbial signatures of health and disease will be more similar in similar diseases (i.e. diabetes and obesity vs. diabetes and autism).

3.2.1 Compile and process gut microbiome datasets

To perform a meta-analysis, we need to collect a comprehensive selection of 16S gut microbiome case-control studies. We will identify these studies through a targeted literature search. See TABLE for exclusion and inclusion criteria for studies to be considered.

We will process these datasets using a standardized in-house pipeline developed by Thomas Gurry, a post-doc in the Alm lab. We will start with the rawest available data - in most cases, these will be fastq files but for some studies we will begin from quality-filtered fasta files. Sequences will be quality and length trimmed, clustered at 100% similarity, and assigned Latin taxonomic names using the RDP classifier. Samples with fewer than 100 reads will be removed from consideration. OTUs with fewer than 10 reads or which are present in less than 1% of samples will be removed. More stringent quality filtering may be considered in order to reduce noise in the dataset.

Because studies which sequence different 16S regions will have different sequences corresponding to the same bacteria, we can not used sequence-based open-reference approaches to compare OTUs across studies. After assigning Latin names based on OTUs within each study, we will collapse OTUs to the genus level and compare these across studies.

3.2.2 Identify microbial markers of disease

Once we have processed all datasets in a standardized way, our first goal is to identify microbial markers of general health and disease. For each study, we will compare gross summaries of the microbial communities (i.e. alpha diversity, Bacteroides/Firmicutes ratio) between cases and controls. Then, we will look for specific microbes associated with health or disease using univariate non-parametric statistics to compare abundances of microbes in cases and controls. To identify microbes which are *consistently* associated with health or disease, we will combine the results from all studies using the weighted Z-test, a weighted method
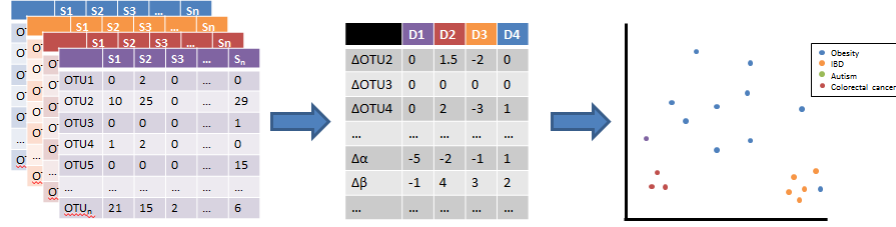
Figure 2: Defining microbial signatures

used in meta-analyses for combining p-values [23]. This will yield an overall significance for each microbe, incorporating the results from all case-control studies.

Next, we aim to identify microbes which are consistently associated with *specific* diseases. For diseases which have more than 3 studies, we will perform the same meta-analysis as above with only the studies of that disease. There are many interesting possible outcomes from this analysis. First, we could find bacteria which are not associated with disease in general but which *are* associated with that specific disease. We could also find bacteria which are significant markers of disease in general and that specific disease, but whose direction of change may differ in the two cases. For example, a bacteria could be significantly higher in Parkinson's patients but significantly lower in all other disease states. The microbes we identify with this analysis may be very interesting candidates for biomarkers or mechanistic follow-up studies.

3.2.3   Compare results between studies for related diseases

Our next hypothesis is that similar diseases will have similar signatures of dysbiosis. For example, we expect that metabolic diseases like obesity and diabetes will have more similar microbiota changes than they will to diarrheal diseases like *Clostridium difficile* infection or enteric diarrhea. We will summarize each dataset with one vector indicating its "microbial signature". This signature will be based on number and identities of microbes significantly associated with the cases and the direction of change of these microbes relative to the controls. Depending on the results from Section 3.2.2, we may also include factors like differences in alpha diversity or Bacteroides/Firmicutes ratios. Then, we will investigate which datasets cluster together in this "signature space". (Fig. 2)

If a disease has a strong impact on or association with the gut microbiome, then we would expect its signatures from multiple studies to cluster very tightly together. If this is the case, we can extract the bacterial features which contribute the most to this tight clustering - these will then be most likely to be associated with that specific disease, and would be good candidates for further mechanistic explorations. On the other hand, if datasets of the same disease or similar conditions do not have similar microbial signatures, this may indicate that the microbiome is not inherently implicated or affected by the disease. In this case, any signal that we see in the gut microbiome is likely driven by other non-disease effects, which are not necessarily the same across studies. Finally, if we find different diseases with similar underlying causes (i.e. inflammation) clustering near each other, then perhaps this would

indicate that the microbiome is affected or involved with the underlying cause rather than the specific diseases. Such insights could help us design better experiments to follow up on mechanism or causal relationships.

3.2.4 Additional considerations

It is possible that we struggle to find bacteria consistently associated with diseases because of technical batch effects, even where we expect to find a clear signal (i.e. in diseases which have had clear results from experimental or mechanistic studies (REFS)). Developing robust methods to overcome technical batch effects in 16S studies is not within the scope of this work. However, if standard meta-analysis methods fail, we can try some naive correction methods like a linear correction for the principal components which correlate closely with technical artifacts like read depth (FIGURE?). We could also consider using a phylogenetics-based approach rather than taxonomically-assigned closed-reference OTUs. We could identify associations with disease using open-referenced OTUs and then compare their phylogenetic relationships across all studies. Finally, we could approach our meta-analysis from a functional point of view by using tools like PiCRUST or HUMANN to assign functionality to our observed taxonomies [21], [22].

3.3 Aim 3: Assigning bacteria to groups with similar functions and disease associations

In this aim, we will curate biologically-motivated *microbe sets* to enable easier interpretation of results from 16S microbiome analyses. By defining groups of related microbes *a priori*, we will enable enrichment analyses similar to GSEA (2.2.3, [17]) for microbiome data. Enrichment analyses will allow for better biological understanding of individual studies' results as well as more consistent comparisons of results across individual studies in the literature.

3.3.1 Define microbe sets based on known biological relationships

Our first task is to curate and define *microbe sets* for use in enrichment analyses of microbiome datasets. We will perform an extensive literature search to identify existing databases with microbial annotations, with the goal of combining these databases and filling them out where they are missing annotations. We will begin with ImG which has approximately 10,000 annotated microbial genomes. About half of these genomes are human-associated bacteria, and half of those have annotations for categories like disease association, sporulation, body site habitat, and gram staining. We will extract the 16S sequences for all the annotated human-associated microbes in this dataset, build a phylogenetic tree, and determine whether they adequately span the phylogenetic diversity we expect to find in the human gut. If certain key clades are missing from these bacteria, we will manually include them from literature searches and NCBI queries.

In collaboration with Ilana Brito at Cornell, we will apply a combination of literature mining and bioinformatics approaches to fill out the missing annotations in the databases and to add our own fields of interest 2. We may also pursue unsupervised text mining of literature results to infer trait- associations for well-studied bacteria [24]. As we populate

Category	Approach
Pathogens	Targeted literature search, literature mining, & databases
Body sites	Literature mining, machine learning on HMP data
Environmental associations	Literature mining, machine learning on EMP data
Growth rate	Inference from 16S sequences in datasets from Aim 2 and HMP
Obesity-associated	Targeted literature search & machine learning on Aim 2 datasets
Inflammation-associated	Targeted literature search & machine learning on Aim 2 datasets
Miscellaneous functions (acid-tolerant, mucus-degrading, etc)	Targeted literature search, unsupervised PiCRUST clustering, genome mining

Table 2: Possible approaches to define microbe sets of interest

Microbe set association	Classification task
General health/disease	All healthy vs. all disease
Diarrhea	CDI, EDD, IBS-D vs. controls
Neurological	Autism, Parkinson's vs. controls
Immune system	Rheumatoid arthritis, allergy, Crohn's disease, Graft-versus-host disease vs. controls
Liver	NASH, MHE (all hepatic diseases) vs. controls
Metabolic syndrome	T1D, T2D, obesity, metabolic syndrome vs. controls

Table 3: Classification tasks to identify groups of phenotype- associated microbes

the our microbe annotations, we will also collect 16S sequences to put build a tree and apply appropriate inference models to infer the missing traits of leaf organisms when possible.

3.3.2 Extract disease-associated microbe sets from datasets in Aim 2

We will leverage the datasets collected in Aim 2 to extract additional disease-associated groups of microbes. We will define groups of microbes based on those that distinguished diseases or broader phenotypes in (Section 3.2.3). We will also pursue machine-learning driven approaches to identify novel disease- or phenotype-associated *microbe sets*. We will define *microbe sets* based on the most discriminating features of various comparisons, as described in Table 3.

3.3.3 Develop collaborative tool for interpreting microbiome studies

We will make the microbe set annotations available to researchers for further annotation and development. Through our literature searches, we will pay attention to the databases researchers have found most useful and strive to package our annotations in an easy-to-use

format. We will likely begin with one very large text file containing all of the microbes, 16S sequences, and metadata that we have gathered.

We will also package our *microbe set* annotations into a tool that researchers can use to interpret the results of their 16S studies. Our software will take as input an OTU table with taxonomically assigned microbes and disease-state labels for samples. It will perform enrichment analysis on the OTU table and return the results to researchers, similar to the Broad's GSEA tool [17].

All of this work will be done using public open-source tools like GitHub to encourage collaboration and dissemination of our findings.

3.3.4 Additional considerations

Developing a database of microbial annotations is a daunting task due to the vast diversity and complexity of microbes. We recognize the inherent difficulty of this task, and do not expect to produce a fully comprehensive database. However, because our annotations are intended to serve as a tool for biological interpretations and hypothesis generation, even a partially-complete database will be extremely valuable in reducing the number of false-negative results in case- control studies and also providing coherent biological interpretations of existing results. We also recognize that our work will be just the beginning of systematic grouping of phenotypically-associated microbes, and so we will ensure that the format of the database we develop is easily accessible and modifiable by other researchers.

This work will be the beginning of what will hopefully become a new approach to interpreting 16S datasets - moving the field from asking simply "what's different?" toward a more critical interpretation of "why are things different?"

4 Preliminary studies

Three to four pages

4.1 Aim 1

4.1.1 Microbiome community exchange

Using our definition of p_s (3.1.1), we identified over 100 OTUs exchanged across sites of the aerodigestive tract. As expected, the majority of these were exchanged between the throat and stomach. Interestingly, the stomach and lungs also had a significant amount of microbial exchange, and these communities were almost as similar to each other as the throat and stomach communities were. These findings support the hypothesis that frequent microaspiration of gastric contents into the lungs is occurring. While certain bacteria may be preferentially exchanged between the lungs and stomach, the lower number of exchanged microbes as compared to the throat and lungs, combined with the similar similarity of communities, indicates that much of the exchange may be more stochastic, and not selecting for specific community members across many patients. Finally, we observed a decreasing trend in number of exchange microbes across throat-stomach, lung-stomach, and throat-lung sites, respectively, for all phyla except Proteobacteria. More Proteobacteria were exchanged

Dataset ID	Diseases	Median reads/sample	Year	Platform	Region

Table 4: Datasets

between lungs and stomach than throat and stomach. Proteobacteria are known aerobes, and so may be preferentially selected for colonization in the lungs after microaspiration from the stomach.

4.1.2 Modulators of community exchange

We observed a distinct difference in the amount of exchange between the throat and lungs of aspirators versus patients with normal MBS results. We looked at the previously-defined microbes that were exchanged between the throat and lungs and re-caculated p_s, the percent of patients who have that microbe present in both sites, for the aspirator and non-aspirator groups separately. 25% of aspirators shared these microbes across their throats and lungs, while only 13% of non-aspirators did. Additionally, the throat and lung communities were significantly more similar in aspirating patients than non-aspirators. These results indicate that a consequence of abnormal swallowing dysfunction is likely a seeding of the lungs with oral bacteria. Interestingly, the stomach and lung communities of aspirating patients were slightly more similar to each other than in non-aspirators. By definition, aspirating patients are unable to protect their airways from foreign material - while most apparent in swallowing, such dysfunction may also enable more material to move between the stomach and lungs as well.

The effect of reflux on the stomach-lung connection was less striking but still apparent. Specifically, the stomach and lung communities were more similar to each other in patients with very frequent full-column reflux. However, here was no significant difference in the extent of microbial exchange between the stomach and lungs. In other words, the previously-defined stomach-lung exchanged microbes were as likely to be found in both sites in patients with frequent full-column reflux than in patients with less severe reflux. This may indicate a more stochastic seeding of the lungs from the stomach rather than reproducible exchange of the same microbes across patients. In this case, stomach microbes are not necessarily growing in the lungs but nonetheless do have measurable impact on the lung's microbial community.

4.2 Aim 2

4.2.1 Collecting and reprocessing 16S case-control datasets

Our extensive literature review has so far identified 54 suitable case-control 16S datasets. 29 datasets and their associated metadata have been downloaded and processed through our in-house pipeline. Characteristics of these datasets are shown in Table 4

4.2.2 Identify general patterns of health and diseases

We first summarized overall community structure using Shannon's alpha diversity index (SDI). We observed significant batch effects across studies, likely due to the differences in

sequencing depth. After standardizing SDI within studies and combining similar disease states, we observed little difference in overall community structure between cases and controls. An exception to this was seen in diarrheal diseases (*Clostridium difficile* infection and enteric diarrheal disease), in which alpha diversity was significantly lower in the cases.

We next performed univariate comparisons at the genus level for abundance in cases vs. controls for each study. This analysis revealed a similar result: diarrheal diseases showed striking shifts in many microbes, while other diseases had less obvious patterns of dysbiosis. We observed that many bacteria seemed to have relatively consistent shifts between cases and controls across many different diseases **??**. This supports the hypothesis that there is a general signature for disease, in other words, that sick people have altered microbiomes. In light of this finding, identifying microbial shifts that are unique to individual diseases will be crucial to finding specific biomarkers for diagnostic purposes and to motivate mechanistic investigations.

5   Gud werds

We hypothesize that there is a clinically-relevant exchange of bacteria within the aerodigestive tract that may be altered in certain disease states.

Another important consideration in this work may be if study-associated effects are larger than biological effects. For example, when we compare 'microbial signatures' across datasets, it's possible that the largest signal driving dataset clustering is sequencer or 16S region sequenced, rather than disease state. There are many approaches we could take to correct for such batch effects:

1. Subtracting the principal components corresponding to the technical artifacts.
2. Build a model that accounts for these technical artifacts by including them as factors in the model.
3. Non-parametric correction, like sample- or OTU-wise quantile normalization, using controls in each study as the reference distribution.

This work will improve our understanding of the clinical relevance of the human microbiome and will also provide new approaches and tools for analyzing future studies.

We will summarize each dataset's microbial communities methods commonly used in the literature: univariate non-parameteric statistical tests on relative abundances, alpha and beta diversity in different types of patients, and ratios of Firmicutes to Bacteroides in healthy vs. disease patients. It is generally thought that low alpha diversity is a marker of dysbiosis (REF), and that while most people have a Firmicutes/ Bacteroides ratio of (XXX), in certain diseases this ratio may be different (REF). (MAYBE BACKGROUND?) By analyzing each study in the same way from raw data, we can reduce the study-wise batch effects and increase our ability to identify general trends in the gut microbiome in health and disease. We will identify consistent markers of disease by using standard meta-analysis methods, comparing the effect sizes and directionality of bacteria across studies, and using Fisher's method to determine overall significant of a microbe (REFS).

"Thus, humans are super organisms: http://science.sciencemag.org/content/312/5778/1355"

References

[1] N. Vakil, S.V. Van Zanten, P. Kahrilas, J. Dent, and R. Jones. The montreal definition and classification of gastroesophageal reflux disease: a global evidence-based consensus. *The American journal of gastroenterology*, 101(8):1900–1920, 2006. doi: doi:10.1111/j.1572-0241.2006.00630.x. URL `http://dx.doi.org/10.1111/j.1572-0241.2006.00630.x`.

[2] J. Dent, H.B. El-Serag, M. Wallander, and S. Johansson. Epidemiology of gastro-oesophageal reflux disease: a systematic review. *Gut*, 54(5):710–717, 2005. doi: 10.1136/gut.2004.051821. URL `http://dx.doi.org/10.1136/gut.2004.051821`.

[3] K. Raghavendran, J. Nemzek, L.M. Napolitano, and P.R. Knight. Aspiration-induced lung injury. 39(4):818–826, 2011. doi: 10.1097/CCM.0b013e31820a856b. URL `http://dx.doi.org/10.1097/CCM.0b013e31820a856b`.

[4] B. Martin-Harris and B. Jones. The videofluorographic swallowing study. *Physical medicine and rehabilitation clinics of North America*, 19(4):769–778, 2008. doi: 10.1016/j.pmr.2008.06.004. URL `http://dx.doi.org/10.1016/j.pmr.2008.06.004`.

[5] B. Martin-Harris, J.A. Logemann, S. McMahon, M. Schleicher, and J. Sandidge. Clinical utility of the modified barium swallow. *Dysphagia*, 15(3):136–141, 2000. doi: 10.1007/s004550010015. URL `http://dx.doi.org/10.1007/s004550010015`.

[6] A. Lee, E. Festic, P.K. Park, K. Raghavendran, O. Dabbagh, A. Adesanya, O. Gajic, and R.R. Bartz. Characteristics and outcomes of patients hospitalized following pulmonary aspiration. *Chest*, 146(4):899–907, 2014. doi: 10.1378/chest.13-3028. URL `http://dx.doi.org/10.1378/chest.13-3028`.

[7] F.J. Reen, D.F. Woods, Mooij, M.J., M.N. Chrinn, D. Mullane, L. Zhou, J. Quille, D. Fitzpatrick, J.D. Glennon, G.P. McGlacken, and C. Adams. Aspirated bile: a major host trigger modulating respiratory pathogen colonisation in cystic fibrosis patients. *European Journal of Clinical Microbiology & Infectious Diseases*, 33(10):1763–1771, 2014. doi: doi:10.1007/s10096-014-2133-8. URL `http://dx.doi.org/10.1007/s10096-014-2133-8`.

[8] H. Al-Momani, A. Perry, C.J. Stewart, R. Jones, A. Krishnan, Robertson, A.G., S. Bourke, S. Doe, S.P. Cummings, A. Anderson, and T. Forrest. Microbiological profiles of sputum and gastric juice aspirates in cystic fibrosis patients. *Scientific Reports*, 6, 2016. doi: doi:10.1038/srep26985. URL `http://dx.doi.org/10.1038/srep26985`.

[9] L. A. Houghton, A. S. Lee, H. Badri, K. R. DeVault, and J. A. Smith. Respiratory disease and the oesophagus: reflux, reflexes and microaspiration. *Nature Reviews Gastroenterology & Hepatology*, 13(8):445–460, 2016. doi: 10.1038/nrgastro.2016.91. URL `http://dx.doi.org/10.1038/nrgastro.2016.91`.

[10] E.S. Charlson, K. Bittinger, A.R. Haas, A.S. Fitzgerald, I. Frank, A. Yadav, F.D. Bushman, and R.G. Collman. Topographical continuity of bacterial populations in

the healthy human respiratory tract. *American journal of respiratory and critical care medicine*, 184(8):957–963, 2011. doi: 10.1164/rccm.201104-0655OC. URL `http://dx.doi.org/10.1164/rccm.201104-0655OC`.

[11] C.M. Bassis, J.R. Erb-Downward, R.P. Dickson, C.M. Freeman, T.M. Schmidt, V.B. Young, J.M. Beck, J.L. Curtis, and G.B. Huffnagle. Analysis of the upper respiratory tract microbiotas as the source of the lung and gastric microbiotas in healthy individuals. *MBio*, 6(2):e00037–15, 2015. doi: 10.1128/mBio.00037-15. URL `http://dx.doi.org/10.1128/mBio.00037-15`.

[12] C.P. Tamboli, C. Neut, P. Desreumaux, and J.F. Colombel. Dysbiosis in inflammatory bowel disease. *Gut*, (1):1–4, 2004. doi: 10.1136/gut.53.1.1. URL `http://dx.doi.org/10.1136/gut.53.1.1`.

[13] Walters W., Xu Z., and Knight R. Meta-analyses of human gut microbes associated with obesity and ibd. *FEBS Letters*, 588:4223–4233, 2014. doi: 10.1016/j.febslet.2014.09.039. URL `http://dx.doi.org/10.1016/j.febslet.2014.09.039`.

[14] Sze M.A. and Schloss P.D. Looking for a signal in the noise: revisiting obesity and the microbiome. *mBio*, 7(4):e01018–16, 2016. doi: 10.1128/mBio.01018-16. URL `http://dx.doi.org/10.1128/mBio.01018-16`.

[15] D. Knights, E. Costello, and R. Knight. Supervised classification of the human microbiota. *FEMS Microbiology Reviews*, 35:343–359, 2010. doi: 10.1111/j.1574-6976.2010.00251.x. URL `http://dx.doi.org/10.1111/j.1574-6976.2010.00251.x`.

[16] C.A. Lozupone, J. Stombaugh, A. Gonzalez, G. Ackermann, D. Wendel, Y. Vzquez-Baeza, J.K. Jansson, J.I. Gordon, and R. Knight. Meta-analyses of studies of the human microbiota. *Genome research*, 23(10):1704–1714, 2013. doi: 10.1101/gr.151803.112. URL `http://dx.doi.org/10.1101/gr.151803.112`.

[17] A. Subramanian, P. Tamayo, V.K. Mootha, S. Mukherjee, B.L. Ebert, M.A. Gillette, A. Paulovich, S.L. Pomeroy, T.R. Golub, E.S. Lander, and J. Mesirov. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci USA*, 102(43):15545–15550, 2005. doi: 10.1073/pnas.0506580102. URL `http://dx.doi.org/10.1073/pnas.0506580102`.

[18] J. Xia and D. Wishart. MSEA: a web-based tool to identify biologically meaningful patterns in quantitative metabolomic data. *Nucleic Acids Res*, 38:W71–W77, 2010. doi: 10.1093/nar/gkq329. URL `http://dx.doi.org/10.1093/nar/gkq329`.

[19] V.M. Markowitz, I.M.A. Chen, K. Palaniappan, K. Chu, E. Szeto, M. Pillay, A. Ratner, J. Huang, T. Woyke, M. Huntemann, and I. Anderson. IMG 4 version of the integrated microbial genomes comparative analysis system. *Nucleic acids research*, page gkt963, 2013. doi: 10.1093/nar/gkt963. URL `http://dx.doi.org/10.1093/nar/gkt963`.

[20] D. Knights, J. Kuczynski, E.S. Charlson, J. Zaneveld, M.C. Mozer, R.G. Collman, F.D. Bushman, R. Knight, and S.T. Kelley. Bayesian community-wide culture-independent microbial source tracking. *Nature methods*, 8(9):761–763, 2011. doi: 10.1038/nmeth.1650. URL http://dx.doi.org/10.1038/nmeth.1650.

[21] M.G. Langille, J. Zaneveld, J.G. Caporaso, D. McDonald, D. Knights, J.A. Reyes, J.C. Clemente, D.E. Burkepile, Thurber, R.L.V., R. Knight, and R.G. Beiko. Predictive functional profiling of microbial communities using 16s rRNA marker gene sequences. *Nature biotechnology*, 31(9):814–821, 2013. doi: 10.1038/nbt.2676. URL http://dx.doi.org/10.1038/nbt.2676.

[22] S. Abubucker, N. Segata, J. Goll, A.M. Schubert, J. Izard, B.L. Cantarel, B. Rodriguez-Mueller, J. Zucker, M. Thiagarajan, B. Henrissat, and O. White. Metabolic reconstruction for metagenomic data and its application to the human microbiome. *PLoS Comput Biol*, 8(6):e1002358, 2012. doi: 10.1371/journal.pcbi.1002358. URL http://dx.doi.org/10.1371/journal.pcbi.1002358.

[23] D.V. Zaykin. Optimally weighted Ztest is a powerful method for combining probabilities in metaanalysis. *Journal of evolutionary biology*, 24(8):1836–1841, 2011. doi: 10.1111/j.1420-9101.2011.02297.x. URL http://dx.doi.org/10.1111/j.1420-9101.2011.02297.x.

[24] J.O. Korbel, T. Doerks, L.J. Jensen, C. Perez-Iratxeta, S. Kaczanowski, S.D. Hooper, M.A. Andrade, and P. Bork. Systematic association of genes to phenotypes by genome and literature mining. *PLoS Biol*, 3(5):e134, 2005. doi: 10.1371/journal.pbio.0030134. URL http://dx.doi.org/10.1371/journal.pbio.0030134.