Title
Persistent Airway Microbiota in HIV-Positive Pneumonia Patients Recieving Antibiotics

Author
Huang, Janmei D

Publication Date
2011-04-01

Persistent Airway Microbiota in

HIV-Positive Pneumonia Patients Receiving Antibiotics

By

Janmei Delphine Huang

A thesis submitted in partial satisfaction of the

requirements for the degree of

Master of Science in

Health and Medical Sciences

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Dr. John Balmes, Co-Chair
Dr. Susan Lynch, Co-Chair
Dr. John Swartzberg
Dr. Mary Firestone
Dr. Laurence Huang

Spring 2011

The thesis of Janmei Delphine Huang, entitled "**Persistent Airway Microbiota in HIV-Positive Pneumonia Patients Receiving Antibiotics,**" is approved:

Chair _____ Date 1/21/11

Chair _____ Date 1/25/2011

_____ Date Jan 19, 2011

_____ Date 1/19/11

Lawrence Huang MD Date 1/20/11

University of California, Berkeley

The thesis of Janmei Delphine Huang, entitled "**Persistent Airway Microbiota in HIV-Positive Pneumonia Patients Receiving Antibiotics,**" is approved:

Chair		Date	1/21/11
Chair		Date	1/25/2011
	Mary K. Firestone	Date	Jan 19, 2011
		Date	1/19/11
	Lawrence Huang MD	Date	1/20/11

University of California, Berkeley

Acknowledgements

I would like to thank Susan Lynch and Laurence Huang for providing me with the opportunity to work with them. Their patience, mentorship and support provided me with invaluable experience and lessons in properly designing and conducting good research. They have challenged me to think intellectually and write critically. I feel honored to have worked closely with such wonderful mentors and experts in the field, who have helped me in so many ways to realize who I am and what I hope to achieve. Shoko Iwai has spent numerous hours patiently answering all my questions, teaching me basic laboratory techniques and statistical analysis. She is a wonderful teacher and role model for me. Serena Fong helped to problem-solve through any challenges and has been a supportive colleague. John Swartzberg and John Balmes advised me through the entire thesis process, even when I felt the obstacles were too great. They were always there to listen to me and give me guidance. Mary Firestone for editing and reading my thesis. I would like to thank the JMP faculty and staff for the love and support. I would like to acknowledge classmates, my friends, and my family for being there for me.

Paper #1: Literature Review of the Human Microbiome

Delphine Huang[1], John Swartzberg,[1] John Balmes[1], Laurence Huang[2], Mary Firestone[3], Susan V. Lynch[4]

[1] UC Berkeley- UC San Francisco Joint Medical Program, 570 University Hall #1190 Berkeley, CA
[2] AIDS Chest Clinic, San Francisco General Hospital, University of California, 995 Potrero Avenue, San Francisco, CA
[3]UC Berkeley, Department of Environmental Sciences, Policy and Management, 32 Hilgard Hall, Berkeley, CA
[4] Colitis and Crohn's Disease Center, Division of Gastroenterology, Department of Medicine, University of California San Francisco, 513 Parnassus Ave., S363

Introduction

The human body is teeming with more than 100 trillion microbes that co-exist and operate within highly variable and diverse polymicrobial ecosystems or "microbiomes" across different surfaces of the body. It is estimated that the number of microbes that live inside and on humans, outnumbers human cells by 10 to 1. Over human evolution, these microbes have evolved at the same time, developing symbiotic relationships. The interaction between two species, known as symbiosis, comes in various forms. Some microbes are parasites, and their presence is harmful to the host. Other microbes are commensals where they gain benefits through their relationship with the host, but the host is neither helped nor harmed. The last type of symbiotism involves a mutualistic partnership, where both microbes and host benefit. While previously assumed to play a relatively unimportant role as transient passer-bys or non-contributory commensals, research suggests that the native microbiota is critical in human development and normal physiology. These microbial residents serve as key components in maintaining our health. Many of these organisms have developed mutualistic relationships over time with humans. While their survival depends on the host, these microbes also provide a wide array of critical and beneficial services for humans, including participating in immune development, nutrition processing and other metabolic functions.[1-4] This intertwined host-and-native microbiota partnership has garnered much scientific interest, as it has become apparent that the human microbiome has an essential role in maintaining human health. The National Institutes of Health and the E.U Metagenomics of the Human Intestinal Tract have sponsored a collaborative effort called the International Human Microbiome Consortium, to explore and catalog the human microbiota.[2] These scientific inquiries stem from questions about the particular role of

microbial communities in humans, and about how shifts in the microbial balance may result in healthy and disease states.[5]

The complex interplay between the triad of the host, the microbiota and the environment appears to have important consequences in the maintenance of human homeostasis. While many diseases can be tied to one causative agent, results from microbial ecology studies have shown that the local microbial community may play an important role in a pathogen's ability to colonize. Aberrations in the composition or function of the gut microbiota have been associated with inflammatory stimulation and development of a number of disorders including allergy [6], chronic obstructive lung disease (COPD) [7], cancer [6], cystic fibrosis [8], inflammatory bowel disease (IBD) [3], and obesity.[9] Simultaneously, the host and the local immune environment are both dynamically interacting and adapting to the microbiota to maintain healthy-promoting organisms, while eliminating pathogenic ones. Investigating how disturbances to any arm of the triad may shift the microbial balance and shepherd disease pathogenesis will provide clues about how to better approach disease management and therapy.

Tremendous advances in culture-independent technologies have made it possible to characterize the genetic and functional composition of the human microbiome in detail. This paper will first discuss the application of these technologies in identifying members of these mixed microbial communities. Next, the paper will provide the framework for the role of microbiota in healthy individuals. It has been hypothesized that healthy individuals share a group of similar microbes or "functional core" that are critical for

maintaining humans in a healthy state. Various studies have provided clues to the mechanisms for microbial acquisition and how its' establishment may induce protective measures against the invasion of pathogens. Furthermore, a variety of different theories for microbe-microbe communications and host-microbe interactions, have been posited. Understanding the symbiotic relationship between microbe, its community, and the host will clarify how shifting community dynamics can predispose one's risk to developing disease. This paper will also explore the role of community composition and microbial diversity in maintaining homeostasis. When there is a loss of diversity, this may contribute to altered, unstable microbial communities. Microbial studies hypothesize that certain collective consortia of bacteria and their interactions may increase an individual's risk for disease development. Lastly, the paper will explore the potential therapies that artificially manipulate the microbiota in order to restore microbial balance. Approaching possible therapies through this perspective shifts the paradigm for therapeutic targets from one that traditionally focused on pathogen elimination to innovative strategies that support the addition of healthy-promoting microbes. These new therapies hold promise for treating many acute and chronic diseases.

Tools for Studying Microbiota

Analysis of Phylogenetic Profiles

Culture-based approaches have traditionally been the gold standard for clinical microbial identification and diagnosis, but they are limited in cases of complex, multi-species infections.[10] Moreover, the majority of bacteria are non-cultivable, fastidious, or impossible to detect by culture-based approaches.[10, 11] For example, cultures collected

during respiratory infections of cystic fibrosis (CF) patients experiencing respiratory infections, identified *Pseudomonas aeruginosa*, *Burkholderia cepacia* complex, *Staphylococcus aureus*, and *Haemophilus influenzae* as the primary pathogens. However, culture-independent approaches indicated that this was not the case, and that non-cultureable species represented nearly 80% of the five most common species.[12]

In contrast to culture-based techniques, many novel molecular approaches avoid the need for *in vitro* cultivation and are capable of distinguishing many different organisms within an entire population. The technological advances that heralded the genomics revolution have radically improved in precision, providing the field of microbial ecology with the ability to move beyond the examination of a single species to the interrogation of entire communities, their collective genomes and functional profiles.[11, 13] Some of the popular culture-independent approaches are based on sequence polymorphisms in genes ubiquitous to each kingdom, such as the 16S ribosomal RNA (16S rRNA) gene for bacteria or the 18S-28S interspacer region for fungal identification.[10, 14] The 16S rRNA gene is an excellent example of a bacterial biomarker that can be used to classify species and assign phylogeny. It is encoded by all known bacterial species, but is not present in eukaryotes.[13] This allows for selective amplification of only bacterial community members from mixed communities (e.g. clinical samples with mammalian DNA present). While there are regions of sequence conservation that permit the use of universal primers, the 16S rRNA gene also possesses regions of sequence variability, which permits phylogenetic classification.[10, 11]

Clone libraries

The traditional method that uses 16S rRNA is clone libraries, where polymerase chain reaction (PCR)-amplified 16S rRNA gene fragments from mixed-culture samples are separated by inserting the DNA sequence-of-interest into a vector, which then acts as a vehicle for transferring genetic material into *E. coli* to be cloned (Figure 1). These products are then sequenced and compared to 16S rRNA databases. If no sequence is perfectly matched, it can be assumed that a new species is identified. Although this technique obtains comparatively longer lengths (~700bp) of DNA, which can more easily facilitate species identification, it only provides information for the most dominant species. Furthermore, this method can be very labor and time-intensive.

Next-generation sequencing

Recently, next-generation sequencing (NGS) has been introduced as an alternative method to clone libraries. NGS permits for community profiling analysis and sequencing of the 16S rRNA. It skips the use of clones and goes directly to sequencing by separating each DNA fragments to a single special bead for amplification (Figure 2). By exploiting the use of shorter fragments (200-400 bp), these cost-effective and rapid automated systems allow for hundreds of thousands of fragments to be sequenced in parallel. However, the relatively short read lengths can pose difficulty in differentiating between closely related species since similar DNA sequences may cross-hybridize overlapping DNA sections. In addition, NGS misses rare species, if there is insufficient sequence representation.

High-throughput arrays

The third popular option is the use of high-throughput phylogenetic arrays. Phylogenetic arrays permit in-depth semi-quantitative screening of large, complex pools of microbial nucleic acids. PCR-amplified 16S rRNA sequences bind to pre-determined, but unique probes. Using fluorescence intensity as a marker for a matched sequence, profiles of mixed composition are obtained (Figure 3). Since hybridization is independent of the amount of organism present, this technique is highly sensitive for low-abundance species. However, in comparison to clone libraries and NGS, this design relies on *a priori* knowledge of the bacterial sequence and previously determined probe sets. Furthermore, this technique may be subjected to cross-hybridization, leading to false positives. In these cases, these methods can be used in conjunction or validated through other techniques, such as qPCR and sequencing to verify the presence of organisms.

Microbial community analysis primarily uses three measures to characterize the mixed population: richness, evenness and diversity. Species richness is a measure of the total number of distinct species present in the community. Species evenness provides information on the relative abundance of community members within a community.[11] It determines if there is equality in numbers across the individual species, or if one species has significantly more members.[15] These two metrics are then used to calculate diversity. Diversity incorporates both evenness and richness together to describe the number of categories that can be differentiated and the number of each organism in

each category.[16] Several different diversity indices are used and have been described elsewhere.[16]

Functional Profiling Tools

The latest direction in microbiology is metagenomics, the study of the pan-genome of microbes in a community.[17, 18] The newest technologies not only have the capability to discern species-specific 16S rRNA sequences, but also can tease out the genetic makeup and functions of these organisms. One of the most commonly-used approaches is done by shotgun sequencing, where randomly shredded short DNA fragments are cloned into universal cloning vectors, then sequenced and re-assembled [19, 20] This approach is currently reserved for simple communities because of the potential for wrongly mismatched sequences. Furthermore, this technique is limited because it only provides static information and cannot distinguish between highly metabolically active, senescent or dead bacteria.

Since only a small percentage of the genome is functionally important, researchers are incorporating other methods that focus on the activated genes and their functional products, such as mRNA, proteins and metabolites. By using mRNA, researchers can pinpoint genes that are being actively expressed. The initial technique was to convert mRNA to cDNA using reverse-transcriptase PCR (RT-PCR), but this method only allowed for a small number of genes to be quantified at one time. Replacing this, the field of metatranscriptomics has opened promising avenues for the study of collective gene expression profiles of the whole community. While each of these technologies

warrants their own detailed description, in general, serial analysis of gene expression (SAGE), chip-based reverse transcript PCR, NGS, and direct RNA sequencing (DRS), permit for high-throughput, parallel identification of RNA molecules. Despite these advances, RNA-based methods are hindered by several challenges because bacterial mRNA tends not to be stable and is frequently eclipsed by the presence of the more abundant ribosomal RNA (rRNA), leading to extremely low yield.

Furthermore, mRNA is an intermediary to proteins, which are the true effectors. Since any post-translational modifications will not be captured using RNA techniques, the next step is to pinpoint the essential proteins. Proteins and enzymes represent the final products of DNA transcription, and they are the true representatives of the active components of cellular metabolic pathways. The study of proteins and enzymes is being addressed by technologies that incorporate metaproteomics and metabolomics analysis. Metaproteomics provides a global analysis of the protein profile of a community. Several strategies include two-dimensional polyacrylamide gel electrophoresis (PAGE) and fluorescence gel electrophoresis, which can separate proteins according to their isoelectric point and mass. Other approaches are gel-free profiling procedures and use isotope-tagging. Metabolomics profiles the metabolic products produced and can provide detailed information on the overall community's actions or dynamic responses to environmental modulation. Techniques employed are special gas chromatography and capillary electrophoresis mass spectrometry, as well as nuclear magnetic resonance spectroscopy. While these approaches are in their

nascent states and have not yet been perfected, the potential for deciphering how the genotype translates into the phenotype may be best represented by these techniques.[21]

Novel approaches to microbial community ecology through the fields of these various 'omics,' have made it possible for researchers to intimately explore the different levels of the cascade from genes to proteins to metabolic products (Figure 4). This has enabled researchers to not only identify key pathogens from a pool of microbes but also, begin to clarify various functional components and how they are involved in the metabolism and regulation of members of the community. This is done without presupposing which genes should be targeted. Moreover, these designs allow for comprehensive analysis of microbial adaptations due to external pressures and changes in expression patterns during diseased states.[22] While no single analysis will completely suffice in unraveling the complexities of a niche's microbiota, an integrated approach using various techniques, provides a clearer picture of the system's complex dynamics.[21] These new tools are redefining the study of microbial ecosystems, and inevitably changing the way we view and understand the human microbiome and its function.[18, 23, 24]

The Microbiome in Healthy Individuals

Common Functional Core Microbiome and Microbial Acquisition

The study of phylogenetics is the study of evolutionary relatedness among a various groups of organisms. Libraries of catalogued DNA sequences of known microbes allow

researchers to search for their target, and also identify new species. These organisms are grouped together based on their DNA sequence homology. A group that shares a certain degree of evolutionary relatedness, can be put together in a phyla, which is then related to other phyla using the hierarchy of biological classification with the eight major taxonomic ranks (Figure 5).

One fundamental question is whether or not there is a human microbiome that all humans share.[2, 23] Although different bodily niches may vary in the specific phylogenetic composition of their microbiota, four phyla dominate the healthy human body (Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria) and represent a common functional core [4] (Figure 6). Thus, at the phylum level, the human microbiome is phylogenetically similar and shallow across individuals, consisting of a small subset of all known bacterial and archeal phyla. However, moving down the hierarchical classification from the phylum level to the species level, the distribution begins to radiate like a fan. There is immense inter-individual variation, providing an individual uniqueness that can almost be equated to "one's own fingerprint" [4] (Figure 7).

The distribution of conserved phyla is suggestive that human evolution has applied a selective pressure on the human microbiota, specifically supporting only a subset of the majority of known microbes. Furthermore, the human microbiota has stabilized across populations. It is resistant and resilient to fluctuating and disruptive processes. It is hypothesized that the presence of multiple species that are each capable of performing the same essential functions are responsible for this stability. This theory called the

"insurance hypothesis", postulates that functional redundancy reduces the need for "keystone species" (defined as species with a central role in the system and whose loss would cause a dramatic change in processes and diversity).[25] By having organisms share metabolic responsibility, the community is able to respond and adapt to environment disturbances, even if large numbers of microbes are eliminated. Experiments where the native mouse's diverse gut microbiota was replaced by a *Bacteroidetes* mono-species, show that despite not having a full set of bacteria, the few genomes shared a considerable degree of redundancy in their ability to process plant polysaccharides.[25] It is theorized that microbes are able to adjust their metabolic targets and make up for any deficiencies.

While individual uniqueness and variability makes it difficult to make gross generalizations about the human microbiome, patterns do emerge across bodily niches, individuals and populations.[4] Evidence suggests that the functional core shows greater similarities between family members than unrelated persons.[4] Some theories suggest that we may be able to trace the evolutionary roots of the human microbiome and imagine how large societal influences (e. g. migration, industrialization or epidemics), acted as selective pressures, propagating only specific human microbiomes to future generations.[4] For example, certain diseases (such as tuberculosis and cholera) could not have survived in sparse human societies, yet some microbial evolutionalists, like Les Defthelsen argues that urbanization and global travel have made it easier for pathogen transmission.[4] How these dynamics influence the microbiota has not been explored, but it is likely to have an impact on human disease development.

We can theorize that the introduction to certain organisms shapes the initial microbial colonization and is involved in the establishment of a proper immune system.[6] Recent research is interested in the initial microbiota acquisition from mother to child, especially in the context of the development of early childhood allergies. The inoculating microbial community, as well as the subsequent order of the early colonization patterns appears to strongly influence the adult gut microbiome later in life.[26] This may be determined as early as *in utero*. Maternal exposure to environmental pathogens or antibiotics appears to set the stage.[3] Ege and colleagues speculated that exposure to farm environments, rich with microbial components during pregnancy may be associated with the development of atopic sensitization and up-regulated receptors of innate immune system in the children.[27] These findings are supported by other studies that demonstrated that mothers exposed to farms during pregnancy have children with lowered Th2 cytokine secretions (which is a characteristic of allergic response), and subsequently, less likelihood of development asthma or allergies. These findings support the "hygiene hypothesis" proposed by David Strachan, which postulates that underexposure to allergens and infectious agents, results in inappropriate immune development and increased susceptibility to allergic diseases.[28] Epidemiological studies further support this hypothesis, demonstrating that contact with animals has a contributory role in the microbial composition and provides protection against allergic disease development. [3, 29]

Moreover, the type of early exposure appears important. Children who were vaginally delivered at home, contained higher numbers of beneficial health-promoting bacteria, like *Bifidobacteria* and *Bacteroides* species in their gut and possessed distinct gastrointestinal microbiota from that of Caesarean-section (C-section) infants. [3, 26, 30] A study by Thavagnanam and colleagues suggest that children born by C-section are associated with a 20% increased risk of developing asthma. [31] Whether atopic disease susceptibility is due to poor immune development or lack of exposure to maternal microbiota, remains unclear.

Throughout early infancy, there are temporal fluctuations in bacterial communities and a distinct order of colonization.[3, 4] Aberration in colonization patterns can lead to deviant immune development. Perturbations, for example by antibiotics or by specific bacteria, of the gastrointestinal microbiota at 3 weeks up to the first year of life were associated with increased risk of developing allergies.[26] The Copenhagen Prospective Study on Asthma (COPSAC) carried out an extensive, longitudinal study where asymptomatic infants, whose throats were colonized by *S. pneumoniae, M. catarrhalis, H. influenzae*, or some combination of these organisms within the first month of life, had a two to four-fold increase in the risk of developing wheezing, increased blood eosinophil counts and total IgE, and eventually, developing asthma.[32] Therefore, children may be born with the proper microbiota, but because of certain exposures (or the lack of specific exposures) become predisposed to developing allergies. How these events shape the human microbiome and subsequently adult microbiota composition is subject to considerable research.

Microbe-Microbe Interactions

Before delving more into how aberrant microbiomes are associated with diseases, establishing the relationship between co-existing microbes will explain how organisms gain access to a particular niche that is already filled with other microbes. The overall composition of the microbiota and its' collective survival depends on the cooperative and/or competitive nature of the microbe-microbe interactions.[33] Traditional ecological theory teaches that in head-to-head competition, the most-fit variants tend to be retained by natural selection. While this may be initially advantageous for the dominant species, there can be detrimental repercussions, including the rapid depletion of resources. In the microbial world, where co-existence is typically in a fixed area, microorganisms that have adapted to use different resources or metabolic by-products of other microbes as their energy source to avoid this problem.[25, 34] For example, in the gut, *Bifidobacterium* degrades indigestible polysaccharides and releases lactate as waste. This fermentation efficiency is enhanced by *Eubacterium hallii*, which converts lactate to butyrate. Finally, the human host recycles the butyrate for energy.[35] This efficient process minimizes outright competition and benefits all members of the gut microbiome.

Microbes interact with each other by using extensive cell-to-cell communication signals. Bacteria secrete molecules that trigger neighboring organisms to express certain genes and virulence factors, which ultimately influences microbial behavior and affects overall population density.[36-38] For example, native streptococcal strains in the oral cavity are highly effective in secreting metabolites that inhibit pathogenic colonization.[5]

Interspecies communications are dynamic and multidirectional. Animal studies show that *P. aeruginosa* and native oral flora produce several signaling systems.[36, 38] This finding is of particular interest because *P. aeruginosa* is the organism most commonly implicated in acute lung infections of CF patients. Its presence is not only contributory to the development of infection, but also has a role in altering the native bacterial community composition.[8, 33, 39, 40] *P. aeruginosa* infections result in increased airway secretions and reduced levels of oxygen, providing a more favorable environment for anaerobes.[40] At the same time, the oral flora bacteria, which has previously been thought of as a non-contributing factor in infection, now appears to express high metabolic activity and modulate the virulence of *P. aeruginosa* through secreted molecules.[38] Rat models show that when the airways are instilled with *P. aeruginosa* and oropharyngeal microbiota, more airway damage occurs than with instillation of *P. aeruginosa* alone. [33, 38] These findings are associated with dramatically reduced host survival rates. [8] Similar hypothesis have been proposed for the mechanisms of other diseases, such as Fournier gangrene. It has been proposed that synergistic effects between microbes and their resultant enzymes end up propagating the spread of this polyorganism infection. [41] The consequences of these communication patterns are only recently been explored though it is becoming more apparent that global interactions within a community impact the development of disease.

Host-Microbiota Interactions

It is well-understood that the human microbiome and its host have co-evolved dependency over time, where microbes provide important metabolic functions such as

immune development and nutrient provisions in exchange for safe harboring. However, recent studies have revealed that the microbiome has a greater impact on the host physiology than previously appreciated (Figure 8).

IBD studies show morphological differences in germ-free animals when compared to conventionally-reared animals. Germ-free mice have demonstrated defects in intestinal villi and abnormal cecum enlargement. Furthermore, germ-free mice exhibit slowed small intestinal peristalsis, which can be reversed through the introduction of normal gut flora. This suggests that bacterial signals influence the intestinal functions. (Hill) Another good example has been demonstrated in mouse experiments on examining obesity. The microbiome of the obese *(ob/ob)* mouse's had increased expression of microbial genes, which are important for importing and metabolizing otherwise indigestible polysaccharides into short-chain fatty acids. This provides one explanation for the obese phenotype of these animals. Furthermore, the transfer of the gut microbiota from either obese (*ob/ob*) or lean *(+/+)* mice into germ-free wild-type mice transformed the wild-type's phenotype to reflect the source's degree of adiposity.[9, 42, 43]

This evidence underscores the involvement of the microbiome in determining impact the host phenotype -- but this relationship is bi-directional. As much as the resident microbiota plays a role in the host's state of health, the host's characteristics can shift microbial behavior. Modifications to environment resources such as a change to high-fat/high-sugar diets are associated in altered microbial composition and metabolism.[3, 43] There is a significant shift from Bacteroidetes to Firmicutes, as well as a reduction in

Bifidobacteria, in obese patients.[43, 44] These alterations are associated with increased microbial energy harvest from an abundance of carbon sources and fermentation of simple sugars, suggesting that the host's diet can restructure microbial milieu. These recent findings suggest a dynamic relationship between the host and its microbiota that has the capacity to influence states of health and disease.

Immune System and the Microbiota

This relationship is likely very important in human immunity. The native microbiota is a key protective barrier against incoming pathogens by providing colonization resistance. Furthermore, the microbiota is critical for developing the immune system. Underexposure to microbiota reduces the conversion of CD4 T cells to other regulatory T cells that appears essential for building proper immunity and maintaining T cell homeostasis. For example, mice studies report that Th17 cells appear in the intestine only after colonization with commensal microbiota (e. g. Cytophaga-Flovobacteria-Bacteroides), and their differentiation is inhibited when the mice were treated with selective antibiotics.[45] Microbial studies are only just beginning to evaluate the regulation of specific microorganisms on the immune cells. A polysaccharide produced by a gut commensal, *Bacteriodes fragilis,* has a critical role in T cell balance. Deficiency in this polysaccharide leads to improper release of proinflammatory cytokines and increased susceptibility to *Helicobacter hepaticus*-induced colitis.[46] Other animal models of IBD have shown that germ-free mice have reduced and underdeveloped intestinal lymphathic tissues. It is hypothesized that selective bacterial colonization restores the lymphoid structure through signals, as studies show that peptidoglycan from gram-

negative bacteria is both necessary and sufficient induce intestinal lymphoid follicle formation in the intestines through innate immune recognition of bacterial-derived signals.[6] The notion that the native microbiota is involved in immune development has been established, but our knowledge of the details behind microbial signaling in proper immune development remains fragmentary.

The immune system must create a favorable environment for its native microbiota, while at the same time, distinguishing and suppressing harmful species. Intestinal dendritic cells sample the bacteria in Peyer's patches using innate pattern-recognition receptors, and selectively induce T cell differentiation for only foreign antigens.[6] Other immune components appear important in shaping the microbiota composition. Secretory IgA (sIgA) is not only a critical antibody defense that prevents bacterial translocation from mucosal surfaces into the bloodstream, but studies suggest that sIgA is also involved in maintaining homeostasis of the gut microbiota. While loss of sIgA results in colonization of atypical bacteria in the gut, reconstitution of sIgA promotes recovery of normal gut microbiota.[47] Furthermore, sIgA may simultaneously facilitate beneficial microbial growth on the epithelial surface. While it has been established that sIgA is a key factor in agglutinating bacteria, which curbs translocation, *in vitro* data postulates that this same mechanism sequesters commensal species and promotes the growth of normal enteric bacteria.[48] Theories about the impact of early microbial colonization patterns and fetal sIgA development provide some good hypotheses about how the immune system can differentiate between beneficial bacteria and pathogens. One idea is that as soon as weaning ceases, maternal sIgA levels fall. This provides a window for bacterial

diversification to occur in the gut, before fetal sIgA begins its own development.[49] This would permit time for tolerance, as the native microbiota are established as part of the human body prior to activation of the newborn's immune system. When there is a loss of this tolerance, altered immune response to the microbiota may propagate inflammation and act as a potential trigger for disease pathogenesis.[6]

Diversity, disturbances, diseases

The purpose of studying the human microbiome lies behind the theory that certain composition of the human microbiota will critically influence healthy and diseased states.[4, 25] As discussed above, functional redundancy maintains homeostasis and resilience against perturbations. A number of studies to date have demonstrated that various human niches exhibit a diverse and temporally stable microbial consortia that are distinct from those detected in disease states, such as obesity [44], CF, and IBD.[3] The concept of dysbiosis, the condition of having microbial imbalance, suggests that changes in the microbiota composition predispose one to getting disease. [6] In the gut, key bacterial species from the Firmicutes and Bacteriodes phyla are essential for maintaining homeostasis. The loss of these organisms in IBD patients is accompanied by dominance of pathogenic organisms. A significant reduction in microbial diversity and loss of key functional groups consequently leads to the development of a chronic, over-stimulated inflammatory response by the host.[3, 50]

Similar findings are seen in antibiotic use, which results in a reduction of bacterial flora diversity that, among other things, decreases interspecies competition, allowing for the overgrowth of more pathogenic organisms. This has been well-documented for *Clostridium difficile* infections, where the bacteria thrive after local microbiota have been destroyed by antibiotics.[51] Furthermore, successive losses in diversity result in permanent alterations. Successive courses of antibiotic administrations in CF patients were associated with species replacement and homogenous community composition. Older CF patients showed less improvement in lung function despite antimicrobial therapy compared to their younger counterparts, suggesting that chronic antibiotic use may serve as a high-pressure perturbation that contributes to the development of resistant, phylogenetically-similar microbial consortia. [52] The association between loss of diversity and disease severity is demonstrated by another study of patients with ventilator-associated pneumonia requiring antibiotics therapy. The reduction in diversity and the subsequent overgrowth of *P. aeruginosa* were highly associated with the severity of pneumonia and the risk of death. Interestingly, ceased usage of antibiotics resulted in the return of most, but not all microorganisms. Furthermore, resistant strains persisted for years after antibiotic administration. These theories postulate that the microbiota can act as a reservoir for resistant strains.[53-55] This may explain why repeated use of antibiotics is associated worsening lung function and poor recovery in these patients over time.

More specifically, the beneficial value of diversity is not just dependent on the number of species, but relies on the specific key members involved. The presence of particular

bacteria within a microbiota may create an environment that is more favorable for pathogenic colonization.[50] Klepac et al. showed that the breadth of phylogenetic diversity was largest with communities lacking *P. aeruginosa* and smallest within communities with *P. aeruginosa*.[33] Furthermore, differences in microbial composition may be associated with disease development. A study of mice by Ley et al. demonstrated that while obese mice (ob/ob) had similar cecal microbiota composition to that of their wild-type or heterozygous siblings, there were differences in the relative abundance of specific phyla.[25] This suggests that the specific pattern of microbial arrangement influences clinical symptoms. Germ-free mice that had been inoculated with only a small number of local commensals (low community complexity) were more susceptible than conventional mice (high community complexity) to colonization by *Salmonella enterica* and subsequent development of enterocolitis. 16S rRNA sequence analysis revealed that increased microbiota complexity was correlated with increased resistance against *S. enterica* infection. Most interestingly, invaders were most successful in colonization when introduced into a community harboring close relatives, suggesting that the abundance of similar phylotypes influences infectivity.[56] Given that many pathogens are closely related to nonpathogenic symbionts that are part of the normal microbiota, this may explain how pathogens are able to avoid immune detection and invade.

Medical Applications

Deliberately altering the human physiology by altering the human microbiota has intriguing health-promoting potential. Elie Metchnikoff pioneered this idea through their initial work that demonstrated that certain microbes could be used to modify the gut flora and alter the course of disease.[57, 58] The field of using health-promoting bacteria or "probiotics" is highly debated, as researchers question whether addition of these commensal species may help to quell inflammatory processes. Administration of bacteria such as specific strains of *Lactobacilli* and *Bifidobacteria* appears effective in changing the existing microbiota to be more protective against pathogen invasion. [59]

Cluster analysis of IBD patients who received probiotics demonstrated that the microbiota tended to be more evenly distributed. Furthermore, there was a reduction in pathogenic organisms. This may be associated with improved functional stability of the microbial consortia, since probiotics not only changes the microbial composition, but also subsequently downregulates inflammatory processes that are the hallmark of chronic intestinal diseases.[3, 60] For example, the introduction of *Lactobacillus* induces IL-10 production by dendritic cells, inhibits Th1 cell generation, and abolishes TNF-alpha stimulation, while stimulating regulatory T cells. The consummation of all those actions prevents the development of experimental colitis in mice. [61] This initial research has demonstrated additive synergism with the administration of mixed probiotics, which appears to invoke a more robust activation of T regulatory cells than the usage of mono-species. [56, 60]

Though outside the scope of this review, emerging evidence suggests that the use of microbiome manipulations may have applications in the management of disease-carrying vectors. An interesting study by Dong and colleagues demonstrated that by perturbing the native gut microbiota of mosquitoes, the natural vector for *Plasmodium falciparum* (one of causative agents of malaria), there was decreased susceptibility of the host to this pathogen.[62] Altering the cyclical disease pathogenesis through microbiome manipulation of the vector, offers potentially innovative strategies for treating many infectious diseases.

Conclusion

Each niche in the body contains complex bacterial communities that provide benefits for the host. In addition to out-competing pathogenic bacteria, the human microbiome provides a protective secondary barrier and participates in energy metabolism, it is also actively involved in immune regulation and metabolism homeostasis. Disruption to the native microbiota appears to not only change the microbial composition, but also enhances susceptibility of the human host to acute and chronic diseases. With the advent of new culture-independent technologies, we have the tools to profile the members of microbiota in various body niches and elucidate their function during healthy and diseased states. Many of these organisms have not been previously implicated in disease, but given the complex interactions of these polymicrobial communities, it is most likely that they are involved in positive and negative feedback of pathogens and host response. Understanding these behaviors will help to elucidate therapeutic targets and offer new strategies for tackling polymicrobial infections.

Figure 1: Simplified explanation the construction of clone libraries: Polymerase chain reaction (PCR)-amplified 16S rRNA gene fragments from mixed-culture samples are separated by inserting the DNA sequence-of-interest into a vector, which then acts as a vehicle for transferring genetic material into *E. coli* to be cloned. These products are then sequenced and compared to 16S rRNA databases. [63]

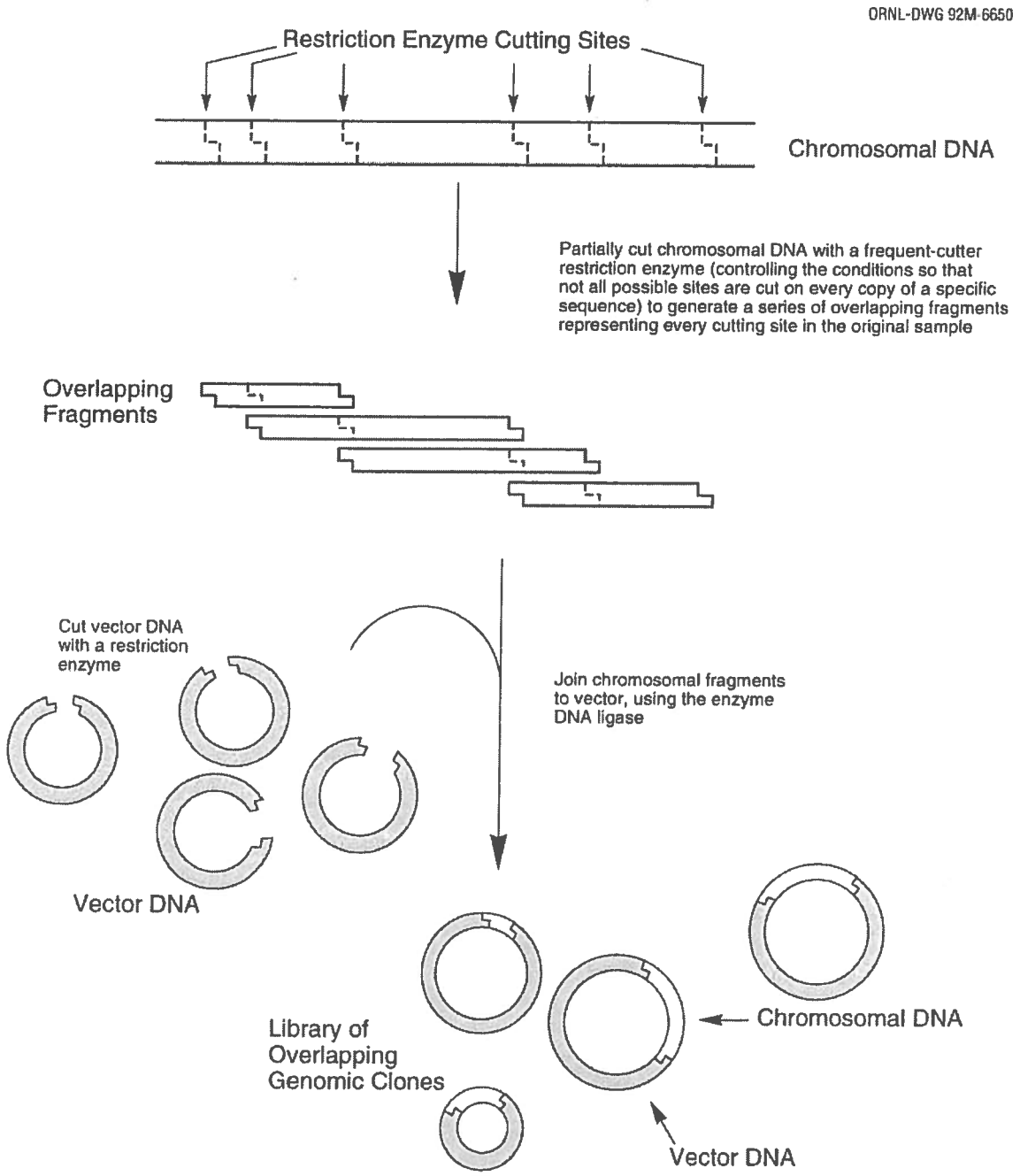


Figure 2: Next generation sequencing: PCR-amplified 16S rRNA gene fragments from mixed-culture sample are separated by inserting the DNA sequence-of-interest into a vector, which then acts a vehicle for transferring genetic material into *E. coli* to be cloned.[64]

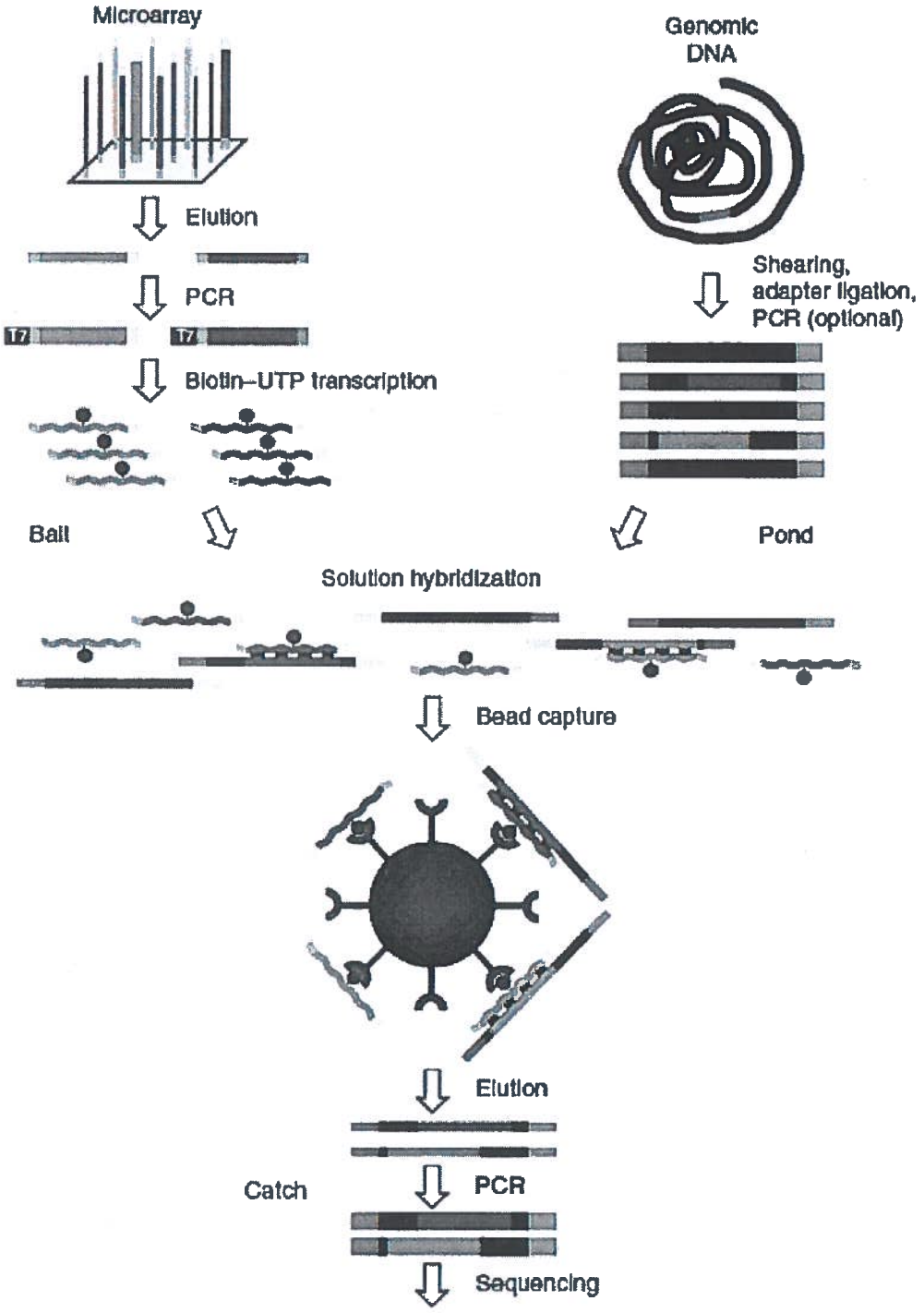
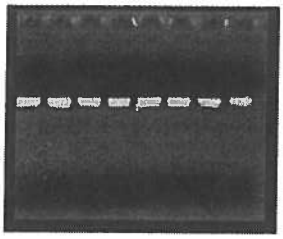


Figure 3:PhyloChip, a type of high-throughput phylogenetic array. 16S rRNA is amplified, hybridized and fluorescent-tagged on a chip with specific primers for microbes.

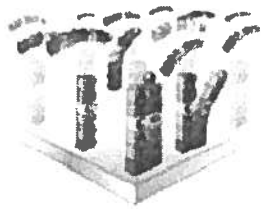
Culture-independent 16S rRNA PhyloChip analysis

Extracted total DNA from a bacterial community



16S rRNA gene is amplified using universal primers in 12 replicate reactions across a gradient of annealing temperatures

Amplicon pool fragmented, biotin labeled and hybridized to PhyloChip



Fluorescently labeled 16S rRNA fragments hybridize to complementary probes on the array surface.

Microarray stained, washed and scanned

Fluorescence data analyzed and microbes identified

Figure 4. Different points of application for the various 'omics'[21]

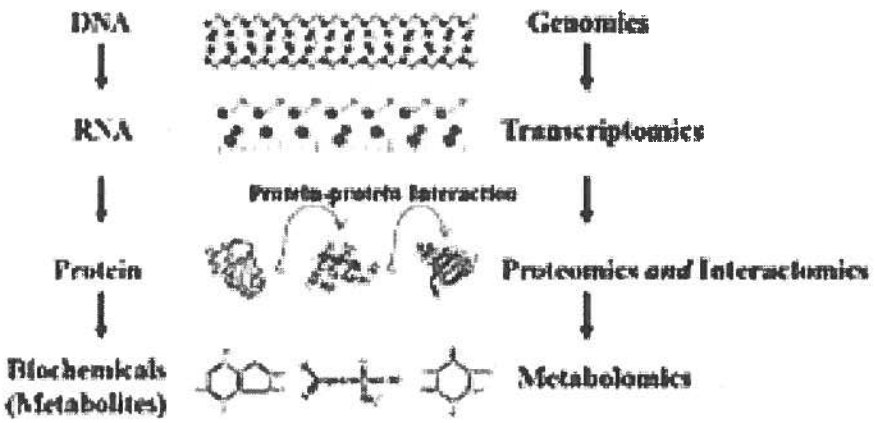


Fig. 1. Schematic diagram of various 'omics' technologies targeting different layers of cellular information.

Figure 5. Hierarchical taxonomic rank

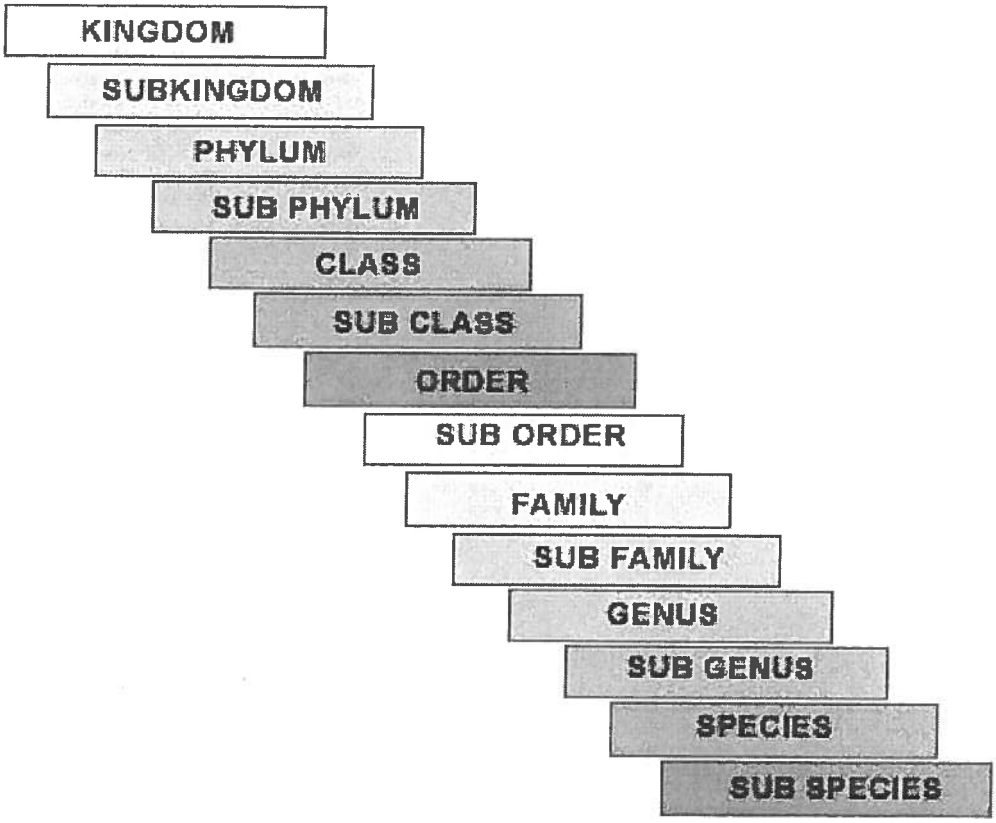


Figure 6. Distribution of the human microbiome [4]

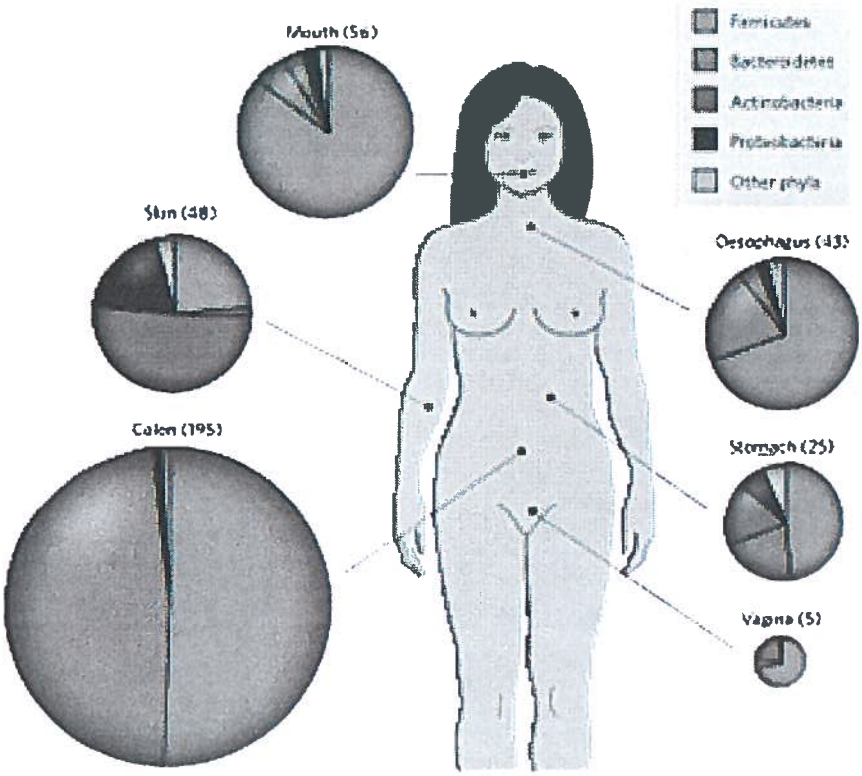


Figure 1 | Site-specific distributions of bacterial phyla in healthy humans. The area of the chart for each site represents the average number of distinct phylotypes (approximate species-level taxa, based on 16S rRNA gene-sequence analysis) per individual. (The mean number of phylotypes per individual is shown in parentheses; 3–11 individuals were studied per habitat.) The coloured wedges represent the proportion of phylotypes belonging to different phyla. More than 50 bacteria phyla exist, but human microbial communities are overwhelmingly dominated by the 4 that are shown. The relative abundance of these phyla at most sites tends to be consistent across individuals: for example, in almost all humans studied so far, Bacteroidetes and Firmicutes predominate in the colon. By contrast, the composition of the vaginal microbiota is more variable; most women have a preponderance of Firmicutes with few other representatives, whereas a minority of women have a preponderance of Actinobacteria with few other representatives. An estimated 20–80% of human-associated phylotypes (depending on habitat) are thought to have eluded cultivation so far. Data taken from refs 1–7.

Figure 7. Diversity of human microbiome [4]

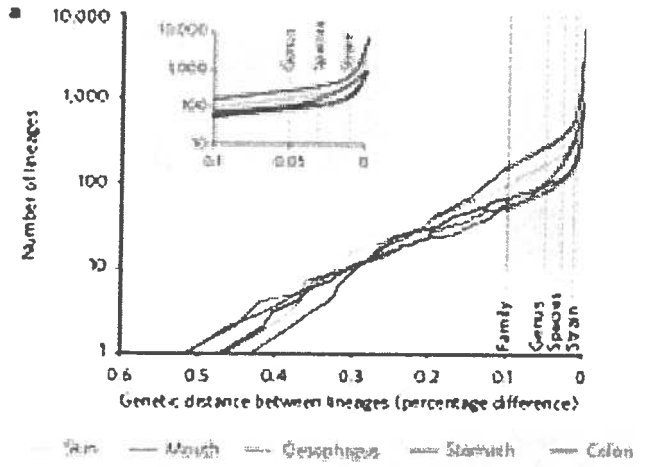


Figure 2 | Patterns of human-associated microbial diversity. a, Lineage-by-distance analysis of 16S rRNA gene-sequence data from human microbial communities in specific habitats. The *x* axis shows the percentage difference threshold (Olsen correction), over 1,241 unambiguously aligned positions of near full-length 16S rRNA gene sequences, for delineating separate lineages. The *y* axis shows the number of distinct lineages that exist at the distance threshold. If speciation and extinction occur with constant probabilities as 16S rRNA gene sequences diverge, this would result in an exponentially increasing number of lineages with diminishing evolutionary distances between them (a straight line on a semi logarithmic plot). Such a pattern seems to hold from the phylum level (largest distances between lineages) to approximately the species level. However, relative to this trend, all sites have an excess of recently diverged lineages. The excess lineages accumulate in the range of 16S rRNA gene divergence that is typically associated with species and strains. The inset depicts a portion of the same data at a larger scale. Samples were taken from 3–11 individuals, depending on the site. Data taken from refs 1–5. **b,** When displayed as a dendrogram, 16S rRNA gene-based patterns of microbial diversity in soil and aquatic environments generally resemble the tree shape on the left, with new branches arising at all distances from the root. Patterns of diversity in vertebrate-associated communities resemble the tree shape on the right, with few branches arising close to the root and many branches arising close to the branch tips.

Figure 8. Proposed function of microbiome in health and disease [6]

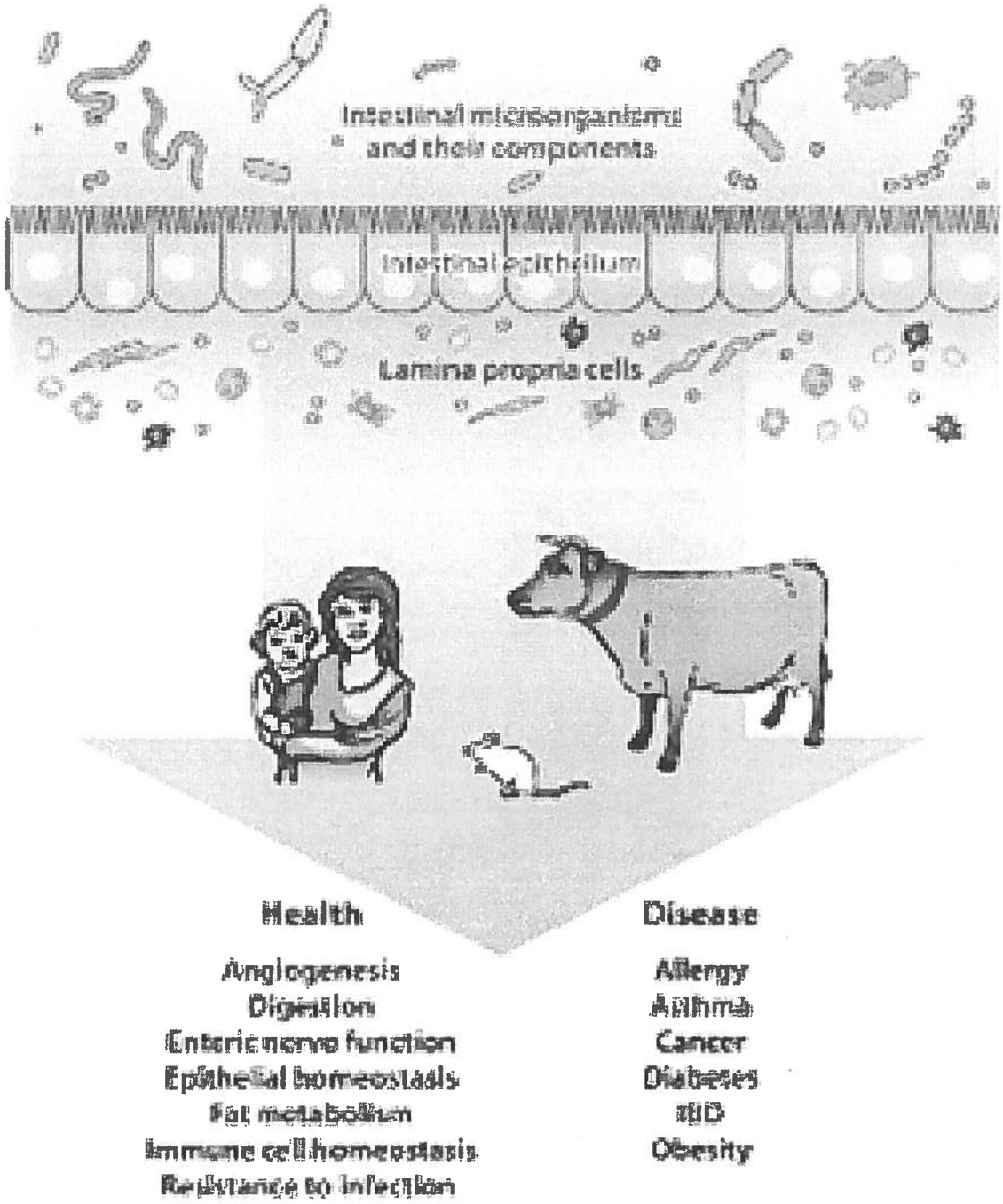


Figure 1

Intestinal bacteria in mammalian health and disease. Schematic of the known influences of intestinal bacteria on normal mammalian physiology and inflammatory disease states.

Paper #2: Persistent airway microbiota in HIV-positive pneumonia patients receiving antibiotics.

Delphine Huang[1,2], Shoko Iwai,[1] Laurence Huang[2*], Susan V. Lynch[1*]

[1] Colitis and Crohn's Disease Center, Division of Gastroenterology, Department of Medicine, University of California San Francisco, 513 Parnassus Ave., S363
[2] AIDS Chest Clinic, San Francisco General Hospital, University of California, 995 Potrero Avenue, San Francisco, CA 94110.

Abstract

RATIONALE: We have previously demonstrated the presence, despite antimicrobial treatment, of mixed species bacterial communities in the airways of non-HIV infected patients with pneumonia. HIV-positive patients are more likely to experience severe respiratory infections, however little is known about the composition of the HIV-airway microbiome.

METHODS: Fifteen HIV-positive patients, receiving antibiotic treatment for pneumonia, underwent bronchoalveolar lavage; an aliquot was used for airway microbiota analysis. A tongue scraping and oropharyngeal wash collected immediately prior to bronchoscopy was used for oral bacterial community profiling. Amplified 16S rRNA was hybridized to the 16S rRNA PhyloChip to profile bacterial communities. Non-metric multidimensional scaling was used to examine (dis)similarity in community structure across all samples. Wilcoxon rank sum test or t-test was performed to determine differences in bacterial community metrics and taxonomic abundance between groups. Clinical variables, including age, prior pneumonia, CD4 cell count, and antibiotic administration, were evaluated.

RESULTS: Despite being on antimicrobial therapies, over 1,600 bacterial taxa, representing 152 distinct families were detected in HIV-positive airway samples; many of which had not previously been associated with airway diseases. Comparable numbers of detected taxa were found in both oral and airway niches indicating that airway microbiota of HIV-infected patients were as rich as that of the oral cavity.

However, comparative analysis demonstrated that the oral microbiota were distinct from that of the airway and characterized by species known to inhabit this niche e.g. *Porphyromonas* spp., *Prevotella* spp. In comparison, the airway microbiota were primarily characterized by members of the Proteobacteria. In addition to examining the taxa that distinguished these niches, 22 taxa were identified at both sites, including several HIV-associated pathogens, suggesting the presence of a core community of bacteria associated with HIV-positive patients. Although airway microbial community structure and HIV-associated clinical variables exhibited no significant relationships, number of days on antibiotics and use of particular antibiotics approached significance ($p = 0.0989 - 0.1299$). In larger cohorts, those variables may represent significant influences on the microbiota. Comparative analysis of our HIV-infected population with a comparable HIV-negative control group with pneumonia receiving antibiotics, revealed that the HIV-positive cohort exhibited greater airway community richness, evenness and diversity, suggesting that immunosuppression results in increased bacterial colonization of HIV-airways.

CONCLUSIONS: The persistence of certain bacteria, despite the administration of antibiotics specifically used for lung infections, may explain the poor long-term outcomes and recurrence of acute respiratory infection in HIV-positive patients with pneumonia.

Introduction

Despite advances in antimicrobial therapies, acute bacterial respiratory infections are the leading cause of morbidity and mortality in HIV-positive patients. Bacterial pneumonias in this population tend to occur more frequently with complications (eg. bacteremia) and permanent decline in pulmonary function compared to CD4 matched controls[65]. *Streptococcus pneumoniae, Haemophilus influenzae*, and *Staphylococcus aureus* are the most commonly isolated bacteria found in HIV-positive populations with acute pneumonia[65]; however, a growing body of lung microbiome literature suggests that even in the presence of a dominant pulmonary organism, multiple other species exist in the airways of patients with underlying predisposition to airway disease [7, 33, 39, 52, 66] Early evidence suggests that these microbes, which were previously assumed to have an unimportant role, may in fact influence disease pathogenesis.[38, 40] Furthermore, while our understanding about the role of diversity in microbiota is still rudimentary, there appears to be distinctly different microbiota in states of health and disease.[2, 3, 50]. While healthy subjects exhibit low if any bacterial burden in the airways,[67] non-HIV patients with pneumonia exhibit relatively diverse airway bacterial communities despite antimicrobial treatment.[68] Given these findings, we suspect that unique HIV/AIDS characteristics, such as seropositivity and immunosuppression, may modify the microbiota in acute bacterial pneumonia. Identification of bacterial community members in this patient population will provide the first steps towards understanding the fundamental dynamics of the HIV airway microbiome.

Capturing the diversity of the microbiota in a specific host niche provides valuable insights into the compositional and putative functional shifts associated with host health status. [4, 11, 25]. To detect these species, including those that are fastidious or non-culturable, it is necessary to use culture-independent approaches. Molecular methods for microbial identification are primarily based on sequence polymorphisms in biomarker genes that are ubiquitous to all members of that kingdom e.g. the bacterial 16S ribosomal RNA (16S rRNA) gene or the fungal Internal Transcribed Spacer region. The advent of biomarker-based culture-independent, molecular phylogenetic profiling approaches have permitted more detailed assessment of mixed-species consortia and have identified specific families or species responsible for particular host immune phenotypes or associated with disease state and severity. [3, 9, 10, 69]

In this initial study, we characterize the bacteria in paired oral and airway samples from 15 hospitalized HIV-positive patients with acute respiratory infection receiving antibiotics for their pulmonary episode. Using a phylogenetic array, the 16S rRNA PhyloChip, a high-density microarray that detects approximately 8,500 bacterial taxa [defined as groups of bacteria sharing at least 97% 16S rRNA sequence homology [13]], we examined array-reported niche specificity of the persistent oral and airway microbiota in these patients. Though our cohort was relatively small, we also examined whether members of the airway bacterial community correlated with clinical variables. Finally, to examine the impact of HIV-associated immunosupression on the airway microbiota, we conducted a comparison of airway microbiota in HIV-positive and HIV-negative patients with pneumonia receiving active antibiotic therapy. [68] Given the high incidence of

bacterial pneumonia and severe respiratory infections in HIV/AIDS population, determining the airway microbiota of HIV-positive patients with acute pneumonia could identify potential pathogens. As recurrent infection or relapse are characteristic of these patients [65], determining the airway community members that persist despite therapeutic interventions would provide invaluable insights into the efficacy of current treatments. To our knowledge, this is the first study to characterize the HIV oral and airway microbiota during acute respiratory infection and demonstrate a distinct and diverse antibiotic-insensitive airway assemblage associated with HIV-infected patients.

Materials and Methods

Sample collection

The Committee on Human Research at UCSF approved all study protocols, and all patients provided written, informed consent. HIV-infected patients admitted to San Francisco General Hospital (University of San Francisco, California) for acute pneumonia who were undergoing bronchoscopy were approached for consent. A total of 15-paired samples of oropharyngeal wash (oral samples) and bronchoalveolar lavage (airway samples) were used for this study. Oropharyngeal rinses were used to decontaminate the mouth prior to airway sampling and to provide a sample for oral microbiota analysis. Patients first rinsed with 10 ml of Periogard (0.12% chlorhexidine gluconate) followed by a gargle using 10 ml of sterile 0.9% saline (NaCl) for 60 seconds. Both rinses were combined for subsequent oral microbiota analysis. Airway samples were collected from a subsegment of the right middle lobe by instilling five 50

ml aliquots of 0.9% sterile saline and removal by suction. Clinical data were recorded in a secure database, including patient demographics, risk behavior, CD4 cell count, viral load, antibiotic and steroid administration, and clinical laboratory microbiological data. A comparative analysis of our HIV-infected population with a comparable and previously analyzed HIV-negative control group with pneumonia receiving antibiotics was done.

DNA extraction, 16S rRNA gene amplification and PhyloChip processing

Bacterial genomic DNA from airway and oral samples was extracted using the AllPrep DNA/RNA extraction kit (Qiagen, Hilden, Germany). Cell pellet was centrifuged and lysed in Buffer RLT Plus. Supernatant was transferred to the DNA column provided by the kit. The 16S rRNA gene was amplified across a gradient of annealing temperatures (48°C – 58°C) using universal 16S rRNA primers 27F and 1492R to maximize the diversity recovered.[7, 52, 60] After 30 cycles of amplification, PCR products were pooled, gel-purified and 500 ng of the purified products per sample were processed as previously reported. [13, 70] A negative control reaction was processed in parallel.

Statistical analyses

Normalization

PhyloChip analysis consisted of transforming fluorescent intensity (FI) from the chip to numerical equivalents for each of the represented operational taxonomic unit (OTUs; total = 8916 for PhyloChip G2). Data sets were filtered by applying relatively conservative detection and quantification criteria for each taxon as previously described.

[13] Briefly, each taxon has probe-pairs that consist of a perfectly matched probe and a mismatched probe (containing a mismatch at the 13th nucleotide), which act as a control for cross-hybridization. Cross-hybridization may occur when a partially complementary single-stranded DNA sequence anneals to the target probe generating a false positive fluorescence signal. Probe-pairs are scored as positive if they meet the following criteria: 1) the FI of the perfectly matched probe is at least 1.3 times greater than that of the mismatched probe, 2) the value of differences in intensity between perfectly matched probe and mismatched probe is 130 times greater than the squared noise for that array and 3) the positive fraction (pf) of the probe pairs for each taxon (a minimum of 11, a median of 24 probe-pairs per taxon) is ≥90%.

Prior to statistical analyses, the FI values were normalized to the standard control spike-in and averaged overall array intensity to adjust for variability and differences in chips or reagents. Normalization allows for multiple samples to be compared. Outliers were identified as arrays that exhibited > 2 times the standard deviation from the mean total fluorescence intensity. All samples were within 2 times the standard deviation. Data was log-transformed prior to statistical analyses.

Richness, Evenness Diversity Analysis

Microbial communities can be grossly described using richness, evenness and diversity indices. Species richness is a measure of the total number of distinct species present in the community. Evenness provides information on the relative abundance of community

members within a community. These two metrics were used to calculate Shannon diversity indices for each sample.

Community statistical analyses were performed in the R environment, using the ecological community analysis package *vegan.* (www.R-project.org) Boxplots were used to illustrate richness, evenness and diversity distribution and variation across sample groups (HIV-positive oral versus airway and HIV positive versus HIV-negative airway). Due to skewed distribution and unequal variation, Wilcoxon rank sum tests were used to calculate significance.

Two-tailed Welch's t-test (p-value) was used to examine significant increases or decreases in taxon relative abundance across groups using the *multtest* package. Data was corrected for multiple comparisons by calculating false discovery rate using q-values. [71] False discovery rate control is used in multiple hypotheses testing to check for the expected proportion of false predictions within a set of predictions (false positives error) and to verify that the findings are truly significant. Taxa were considered significant if $p \leq 0.05$ and $q \leq 0.05$. Venn diagrams demonstrating the number of shared and unique taxa in each group, were constructed for visual representation, though not to scale.

An iTOL phylogenetic tree was generated from Newick format nearest neighbor interchange (NNI) tree using FastTree software and annotated using the Interactive Tree of Life.[72] Nearest taxon index (NTI), Net relatedness index (NRI) and their

respective p-values were calculated using the function, *picante,* to determine the extent of phylogenetic clustering based on the mean branch length distance between members in a given community. NRI describes the phylogenetic relatedness at the upper levels of classification e.g. phylum level, while NTI describes relatedness at the lower level of classification e.g. genera level.

Non-metric multidimensional scaling (NMDS), was used to determine community (dis)-similarity based on cumulative ecological distances between samples based on aBray-Curtis dissimilarity matrix (*vegdist* function). In addition, hierarchical cluster analysis (HCA), another exploratory analysis tool that maps bacterial community relatedness, was used. Like NMDS, HCA permits visualization of phylogenetically similar and dissimilar samples using a dendrogram.

HIV-associated and pneumonia-associated clinical variables were examined. Multivariate analysis of these clinical factors was determined using the function, *envfit.* Comparison of the microbial composition of HIV-positive patients for the following groups was conducted: young age versus old age, HAART versus no HAART usage, low CD4 count versus high CD4 count, vancomycin/cefepime therapy versus no vancomycin/cefepime therapy, 2-4 days on antibiotics versus 7-9 days on antibiotics, and steroid use versus no steroid use. Since there were unequal numbers in each comparison, we generated multiple combinations and tested for consistency between trials. We performed Welch's t-testing to identify taxa exhibiting significantly increased or decreased relative abundance between treatment groups.

Results

Persistent airway and oral microbiota of HIV-infected patients

Despite antimicrobial therapy for pneumonia, a total of 1,654 array-reported bacterial taxa representing 41 phyla and 152 distinct families based on Greengenes taxonomy [Hugenholtz classification [73]], were detected in at least one of the 15 airway samples analyzed. This was comparable to the number of taxa detected in the oral samples of these patients (1,754 taxa, representing 42 phyla and 153 families; Figure 1). For all samples analyzed, gross metrics of community composition were calculated. Mean community richness was 625±368 and 502±372 in airway and oral samples, respectively, and comparative t-testing demonstrated that no significant difference in community richness existed between these two niches. (Figure 2a) Similarly, a comparison of airway and oral community evenness and Shannon diversity measures also demonstrated no significant difference in these community metrics between these two sites (Figure 2b and 2c).

Comparative analysis of the airway and oral microbiota of HIV-positive patients

Hierarchical cluster analysis was used to determine whether the oral and airway microbiota [74, 75]represented extensive shared community membership or distinct niche-specific communities, Of the 15 paired samples analyzed, seven exhibited relatively short phylogenetic distance between their respective airway and oral microbiota, indicating a high degree of microbiota similarity in these patients at these distinct anatomical sites (Figure 3, highlighted in gray). The remaining eight paired samples appeared phylogenetically distinct. Four patients in our cohort were treated with ART, of

which three subjects shared the closest phylogenetic relatedness between their airway and oral microbiota. (Figure 3, highlighted by *)

We examined taxonomic differentials between the airway and oral microbiota to determine which taxa were present in significantly higher abundance at each site. In the oral cavity, members of phyla characteristically associated with this niche such as the Bacteroides (Prevotellaceae and Porphyromonadaceae), Firmicutes (Streptococcaceae) and TM7 (associated with subgingival plaque) as well as members of relatively newly described phyla e.g. OP10 (Figure 4), were detected in significantly higher abundance compared to airway samples. In comparison, taxa exhibiting significantly higher relative abundance in the airways, primarily belonged to the main divisions of the Proteobacteria and included members of the Alcaligeneaceae, Oxalobacteriaceae, Procabacteriaceae, Rhodocyclaceae, Alteromonadaceae, Enterobacteriaceae, Coxiellaceae, Oceanospirillaceae, Pasteurellaceae and Pseudomonadaceae. In addition to the Proteobacteria, other phyla represented in significantly higher relative abundance in the airways of these patients included the Actinobacteria (Gordoniaceae) and Firmicutes (Acholeplasmataceae).

In addition to examining taxa that distinguished these niches, we also determined those community members that were common to both sites in all patients. (Figure 5) Twenty-two taxa belonging to the phyla Actinobacteria, Bacteroidetes, Chloroflexi, Cyanobacteria, Firmicutes and Natronoanaerobium fulfilled these criteria. Some of the more notable representative species detected included *Streptococcus bovis* (Lancefield

group D Streptococci) and *Chryseobacterium* species. These opportunistic pathogens have previously been associated with infections of HIV and immunosuppressed patient populations. [76-79]These results suggest that despite inter-personal variation in the resident microbiota, these taxa represent core species common to airway and oral microbiota of our HIV-positive cohort that persist despite antimicrobial therapy and putatively contribute to opportunistic infection of these patients.

Relationships between airway microbiota and clinical variables of HIV-positive patients

Although the microbiota of the airway was most likely influenced by on-going antibiotic administration, we examined clinical variables for potential associations with the airway microbiota. Variables examined included: age, CD4 cell count, HIV RNA level, ART and antibiotics administration, days on antibiotics prior to sampling (Table 1). Other variables included were prednisone administration, smoking history, alcohol history, current drug use, prior pneumonia status, structural lung disease, and microbial cultures (*Pneumocystic jiroveci, Cytomegalovirus*, and *Mycobacterium avium* Complex). (Supplement Table 1 and 2) Fittings of the variables to NMDS showed no significant ($p \leq 0.05$) relationships to airway microbiota. Comparative t-testing (between young age (27-37 years old) and old age (46-50 years old), low CD4 count (2-6 cells/mm^3) and high CD4 count (101-305 cells/mm^3), low viral load ($<10^4$ copies) and high viral load (405002 – 500001 copies), 2-4 days on antibiotics and 7-9 days on antibiotics, and not on ART and on ART) showed no significant relationships ($p \leq 0.05$) with any of the measured variables and the airway microbiota composition.

Number of days on antibiotics and use of particular antibiotics did approach significance, suggesting that in larger cohorts, these variables may represent significant influences on the airway microbiota. Comparative t-testing showed significantly ($p \leq 0.05$) higher abundance of Proteobacteria in early stage (2-4 days), which then shifted to higher abundance of Firmicutes observed in 7-9 days of the antibiotic treatment. This suggests that there are members of the Firmicutes that persist despite a week of antibiotics. However, calculated q-values were very high (>0.6), suggesting that these taxa may represent random events, due to interpersonal microbiota heterogeneity and low sample numbers.

Comparison of HIV-positive and HIV-negative airway microbiota during acute pulmonary exacerbation

Airway microbiota of our HIV-positive patients were compared to those of a cohort of HIV-negative patients with pneumonia (n = 5), who had also received comparable standard of care antibiotic therapy (for 5 – 15 days) for their pulmonary episode. These samples had been previously analyzed under identical conditions using the PhyloChip.[68] Five HIV-positive patients who received antibiotics for similar lengths of time (6-9 days) and represented the most immunosuppressed patients in our cohort (CD4 cell counts = 2-45), were selected for analysis (SFGH111, SFGH121, SFGH161, SFGH241, and SFGH291). Relatively higher numbers of taxa were detected in our HIV-infected airway samples despite antimicrobial therapy compared to the HIV-negative controls. Calculated diversity measurements demonstrated a trend towards higher richness, evenness and Shannon diversity in HIV-positive compared to HIV-negative airway

samples, although these findings did not reach significance (Figure 5a, 5b, and 5c). Hierarchical clustering analysis suggested relative clustering of HIV positive and HIV negative subjects. (Figure 6) There was a clustering of 4 HIV-positive BAL samples, though some HIV negative samples also shared similar communities. Hierarchical cluster analysis is used as exploratory and visual data to distinguish whether there are any gross distinctions between samples. Because of the variability in individual microbiomes and the small sample size, these results may have been skewed may have skewed results and warrants further investigation

Of the 838 taxa detected in HIV-negative patients, 784 taxa (93.6%) were also present in the airways of HIV-positive patients. However, while only 54 taxa were unique to HIV-negative subjects, a total of 735 taxa were present exclusively in HIV-positive patients (Figure 7). A more precise comparative analysis of the taxa characterizing these airway microbiota revealed that compared to HIV-negative patients, who primarily were characterized by significantly higher relative abundance of members of the Proteobacteria (*Pseudomonas aeruginosa*) and some Bacteroidetes ($p \leq 0.05$; $q \leq 0.05$), HIV-positive patients exhibited significant ($p \leq 0.05$; $q \leq 0.05$) increases in taxa belonging to a large number of distinct phyla (Figure 8). These included members of the Actinobacteria, Bacteroidetes, Chloroflexi, and Cyanobacteria as well as members of the Bacteroidetes (including family Prevotellaceae) and Firmicutes (including family Clostridiaceae). Although our numbers are small, initial data suggests that distinct persistent and substantially more diverse microbiota exist in the airways of patients with HIV-associated immunosuppression.

Discussion

Following the introduction of ART, the prevalence of HIV-associated pneumonias, such as *Pneumocystis jivoreci* pneumonia, has declined over the past decade, however a co-incident rise in the number of HIV-positive patients presenting with bacterial pneumonias has occurred during this period.[80] More recently, culture-independent studies have demonstrated that bacterial pneumonias are typically associated with mixed microbial communities with a much greater diversity of species present than was previously appreciated. [68]A large number of chronic inflammatory airway diseases have also been shown to possess a polymicrobial component [7, 8, 33, 39, 66] suggesting that the composition of the airway microbiome may play a significant role in airway disease status and progression. While limited by small sample size and the lack of significance found in our tests, this is the first study to profile the airway microbiota in HIV–positive patients with acute pneumonia.

Human microbiome studies have recently demonstrated that microbiota dysbiosis is characteristic of a variety of human diseases (eg. obesity, IBD, COPD). [3, 10, 42, 52] HIV-associated immunodeficiency combined with other factors, such as chronic administration of various drugs, (e.g. HAART, antimicrobials and steroids) likely has a profound impact on the indigenous airway microbiota and potentially could promote colonization by unusual mixed microbial infections. Using a high resolution, culture-independent phylogenetic array, the 16S rRNA PhyloChip, we profiled airway and oral bacterial communities of HIV-infected patients, on active antibiotic treatment, who were

clinically diagnosed with pneumonia. A comparison of oral versus airway HIV-infected microbiota revealed that while the oral niche primarily contained taxa from the Bacteroides and Firmucutes families, airway microbiota included taxa from Proteobacteria, Actinobacteria and Firmicutes. These findings support other studies that have demonstrated that despite inter-personal variation, distinct microbial communities exist at specific human host sites. [81, 82] Given that the bronchoscopy must pass through the oral cavity to enter airway, contamination of the airway is possible. Patients were given Periogard prior to bronchoscopy to minimize contamination. Furthermore, detection of characteristic community members in significantly higher abundance in each niche suggests that the airway samples collected were not heavily contaminated with oral secretions, and that characteristics of the particular niche (eg. mucosal surface physiology, immune responses, etc.) may act as a selective pressure on the local microbiota.[81] Healthy subjects exhibit a low rate of 16S rRNA positivity (~30%) and insubstantial bacterial burden in their lower airways. [67] In comparison, our HIV-positive cohort, who exhibited severe immunosupression (9 patients with CD4 counts < 100 cells/mm^3) had, despite antimicrobial administration, high diversity in this niche.

We examined the data to determine those species that were common to both the oral and airway sites. Of the 22 taxa fulfilling this criterion, species of note included *Streptococcus bovis* and *Chryseobacterium* species. Both are opportunistic pathogens that have previously been associated with HIV-positive and immunosuppressed patient populations. [75, 77-79, 83, 84] This suggests that the oral community may act as a reservoir for specific species capable of infecting the lower airways in HIV infected patients. [85, 86]

While the DNA-based phylogenetic tools used in this study provided no information about the viability and functionality of these species, the prominence of these particular opportunistic pathogens in both niches suggest that they may contribute to pathogenic processes at these sites. In other studies, we have previously noted that within 24 hours of antimicrobial administration to cystic fibrosis patients, bacterial richness decreased approximately 10-fold as detected by the PhyloChip (Lynch, unpublished data). This suggests that DNA turnover is relatively rapid in the airways of pulmonary disease patients, and that the taxa detected during antimicrobial administration in our HIV-positive patients largely represent the viable portion of the community. Future studies integrating techniques, such as mRNA and protein profiling will provide information on the relative contribution of these and other members of the HIV-infected patient microbiota at these sites.

HCA demonstrated that 3 patients who were receiving ART exhibited phylogenetically similar oral and airway microbiota, suggesting that ART, in addition to reducing HIV viral load, may also influence the composition of HIV-associated microbiota. In a study of HIV-associated periodonitis, De Souza Goncalves and colleagues reported that ART impacts the subgingival microbiota. HIV-positive subjects on ART had less prevalence of several typical oral bacteria in the subgingival biofilm compared with HIV-negative controls. However, atypical species such as *Helicobacter pylori*, *Enterococcus faecalis*, and *P. aeruginosa* were found in the HIV-positive patients receiving ART. [87, 88]An independent study by Navezesh *et al.* on salivary microbiota also reported that HIV-positive patients on ART had more pathogenic organisms and a distinct microbiota

compared to those not receiving the therapy. Despite examining various mediators, including immunosupression levels, ART was the only significant factor in that study. [89] It is well-known that ART improves immune function by inhibiting the HIV virus and preserving CD4 T cells; collectively, the data suggests that ART administration may act as a selective pressure on the local bacterial community actively shifting the microbial composition in HIV-infected patients.

Patients who were on 2-4 days of antibiotics were characterized by significantly higher abundances of Proteobacteria in their airways, while those who received 7-9 days of antibiotics tended to exhibit higher relative abundance of taxa belonging to the phylum, Firmicutes. Days on antibiotics and use of particular antibiotics approached significance, suggesting that if a larger population was studied, these variables may reach significance. Interestingly, there were no common taxa found for patients that were on vancomycin and cefepime. Vancomycin is generally reserved for patients who are very ill or have complex infections. Patients that were on vancomycin may harbor a different airway microbiota from the other subjects due to the severity of their disease or because of vancomycin's properties. Moreover, these findings may have been confounded by both study numbers and immune status, as three out of the four patients had a CD4 count < 50 cells/mm.^{3} These patients were also on cefepime, which is also an antibiotic used primarily for severe nosocomial pneumonia caused by multi-drug resistant microorganisms (e.g. *P. aeruginosa*). The lack of common taxa despite several rounds of different permutations of clinical samples, is suggestive of a highly disorganized and variable microbiota among these particular patients. Ideally a comparison to HIV-

positive patients that have not yet been administered any antibiotics and thus have an unadulterated microbiota that are representative of HIV infection and acute pneumonia, would add to our understanding of the impact of HIV infection on oral and airway microbiota. However, in a clinical setting, this tends to be difficult to obtain without jeopardizing patient care.

Our findings suggest that despite interpersonal variation, a core of microbial species persists in the HIV-infected airway despite antibiotic administration. Antibiotics not only target key pathogens, but also wreak collateral damage on the host's native microbiota, thus it is not surprising that antibiotics can shift the entire composition. Antibiotics are suspected of decreasing interspecies competition and subsequently encouraging overgrowth of pathogenic organisms. Events like this have been well-documented in *Clostridium difficile* infections; this species thrives following depletion of the local microbiota. [51] Understanding how antimicrobials change the microbial composition, and which organisms persist can help to clarify the current knowledge surrounding drug-microbial interactions and resistance. Of interest, studies have shown that while most of the local microbiota return to normal after discontinuation of antibiotics, pathogenic organisms still linger even after significant time has passed. [4, 53-55] This is concerning since it indicates that antibiotic use may have a permanent legacy effect on microbiota composition, the impact on which on human health is currently unknown.

The microbiota of HIV positive subjects were clustered separately from HIV negative controls despite antibiotic administration in both cohorts. This implies that there may be

distinct patterns of colonization distinguishing the two populations. However, we also had HIV negative samples that shared overlapping communities. In addition, HIV-infected patients harbored a large number of additional taxa compared to the HIV-negative cohort. We hypothesize that HIV-mediated immunodeficiency and/or related therapies received by this patient population contributed to this phenomenon. Some of prevalent taxa detected in our HIV-positive cohort included known respiratory pathogens, such as *P. aeruginosa* and *H. influenzae*. Although our numbers were small and our findings did not reach significance, it is possible that seropositivity may be associated with altered bacterial composition. Studies of dental microbes have demonstrated that HIV-positive subjects have a higher abundance and a peculiar distribution of pathogenic bacteria and fungi that are not found in HIV-negative patients. [90] Microbiome studies of different bodily sites (eg. vagina, saliva and GI) of HIV-positive subjects have described unusual bacteria as well. [91-93]

Despite the small sample size and thus lack of statistical power in this study, other studies suggest that immunosupression may be closely connected with the host's microbiota. CD4 T cells are critically important in their role as the primary activators for multiple branches of the immune system. Infection and depletion of these cells by the HIV virus result in dysfunctional immunity with knock-on effects on microbiota composition. Initial studies have found associations between deficient immune functioning and altered composition of microbiota. It is hypothesized that regulatory T cell development and native microbiota have concurrent protective functions that are highly co-dependent. The loss of one component ensues deficiencies in the other. In the

gut, commensal microbiota are shown to control CD4 differentiation in HIV positive individuals. [94-96]Underexposure to microbiota reduces conversion of CD4 T cells to other regulatory T cells that appear essential for building proper immunity and maintaining T cell homeostasis. Furthermore, subsequent abnormal activation of T cells and release of proinflammatory cytokines results in damage to the protective mucosal barrier. [95] Increased intestinal permeability and mucosal damage may permit translocation of specific microbes that are then able to further incite chronic immune activation. Abnormal chronic immune activation represents one of the main factors associated with HIV disease progression. [93, 96]. Mouse models demonstrate that immunodeficient mice are incapable of modulating the relative abundance of certain bacterial strains in the gut. [47] Studies of the subgingival microbiota observed that HIV-positive individuals had higher prevalence of periodontal pathogens and this was correlated with degree of immunosupression. [92] Whether or not these findings that microbial dysbiosis and its subsequent inappropriate activation of the immune system can be applicable to the HIV lung warrants further investigations.

Given the evidence that viral infection, especially by influenza, has a propensity to incite subsequent bacterial superinfection, [97-99] it is plausible that the HIV virus itself, may impact the microbiota directly. Several studies have demonstrated that the virulence of a dominant pathogen is up-regulated and enhanced by certain other members of the microbial community[38], implying that the same effect may occur between HIV virus and the microbiota. Co-occurrence of pathogens with commensal oropharyngeal microbes has been shown to increase the virulence profile and increase lung injury in a rat model

of airway infection. [38, 40] How the HIV virus itself may influence microbial composition remains obscure. The synergistic impact of HIV virus, local microbial community and the preferential deletion of resident CD4 T cells within a particular niche, is likely additive with complex processes occurring in parallel.

Distinct patterns of bacterial colonization associated with acute pneumonias in HIV-positive patients raise questions regarding the triadic relationship between the local microbiota, the impaired host immune defenses and the HIV virus itself. Many researchers are curious if microbial manipulation through the use of probiotics may be a novel therapeutic approach, especially as recent studies have shown restoration of organ function with medical microbial reconstitution in the gut and lungs. [60, 61, 100-103] If restoring native microbiota could interrupt pathogenesis of HIV-related infections, this could potentially change current strategies. Understanding how HIV infection is involved in shaping the microbial landscape, will provide information about how to tackle the collective mechanisms contributing to HIV-associated airway diseases, as well as, expose new methods to treating these polymicrobial infections.

Figures

Figure 1: Phylogenetic tree of bacterial communities that were detected in samples. Phyla are separated by colors. Outer Ring. Oral samples; Inner Ring. Airway samples

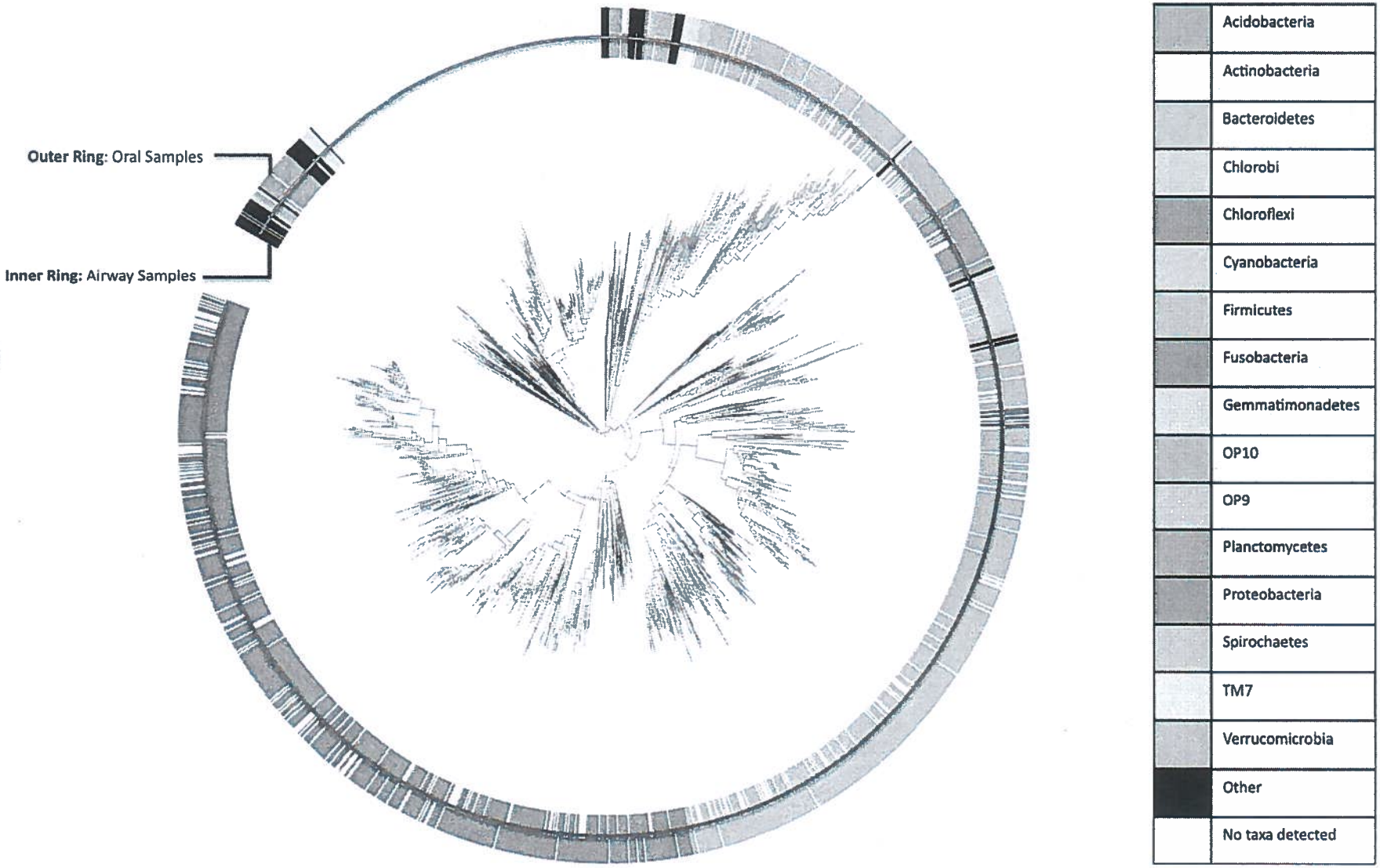
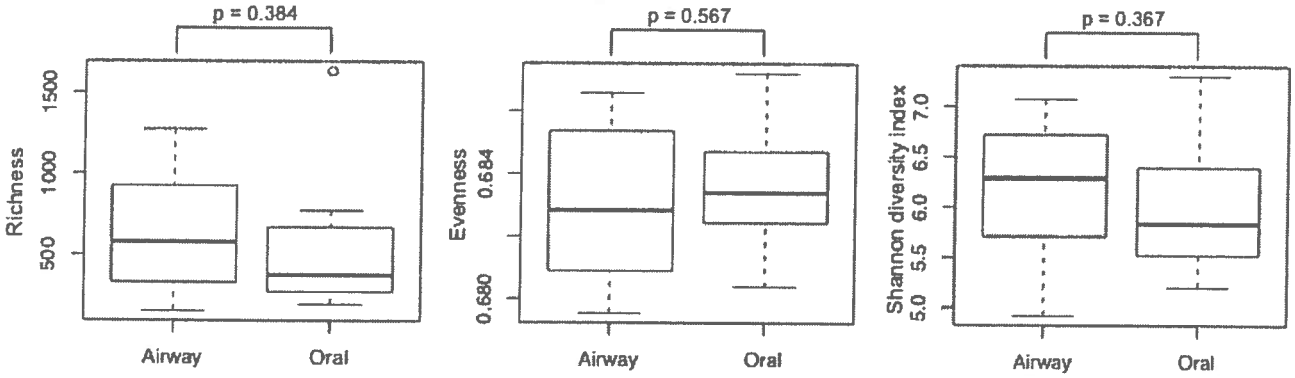


Figure 2: Comparative analysis of community richness, evenness and Shannon diversity measurements between airway and oral samples



2a 2b 2c

Figure 3: Hierarchical cluster analysis of oral and airway samples. Patients on ART indicated by * and patients with closely phylogenetic relatedness of their airway and oral samples are indicated in gray.

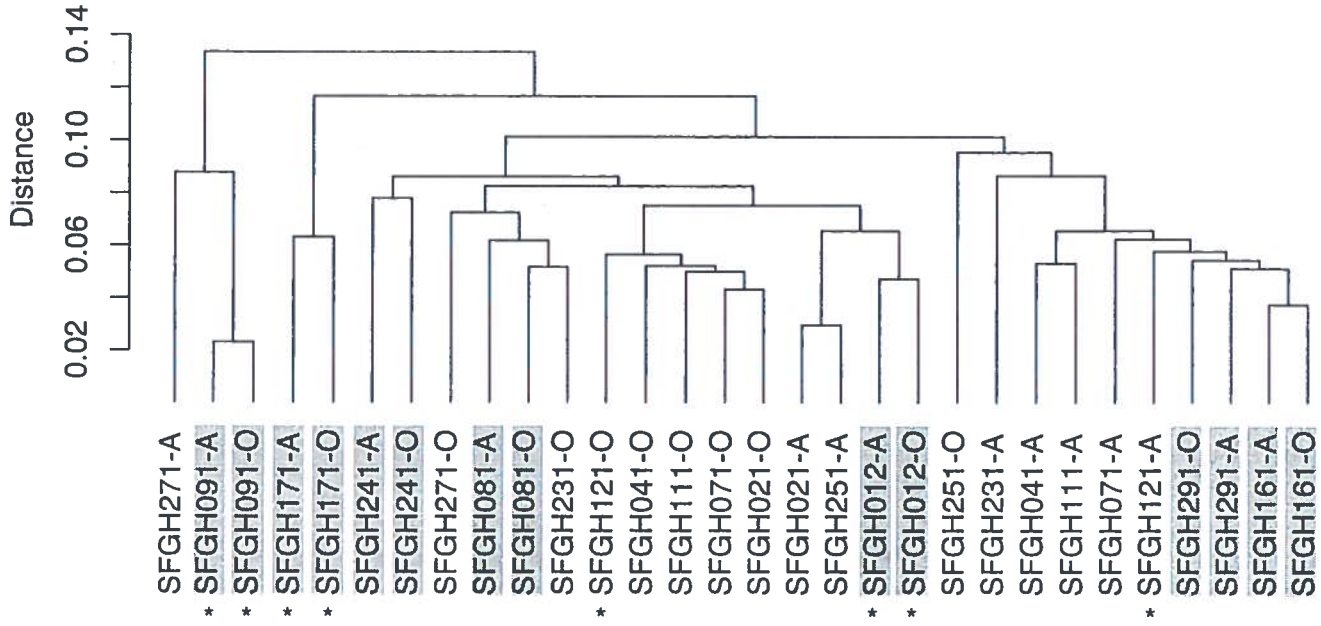


Figure 4: Comparison by significant taxa between airway and oral samples. Significant ($p \leq 0.05$) changes in taxon mean relative fluorescence intensity values reported for oral and airway samples. Red bars indicate greatest change of fluorescence intensity (≥1,000). Background is colored based on phylum

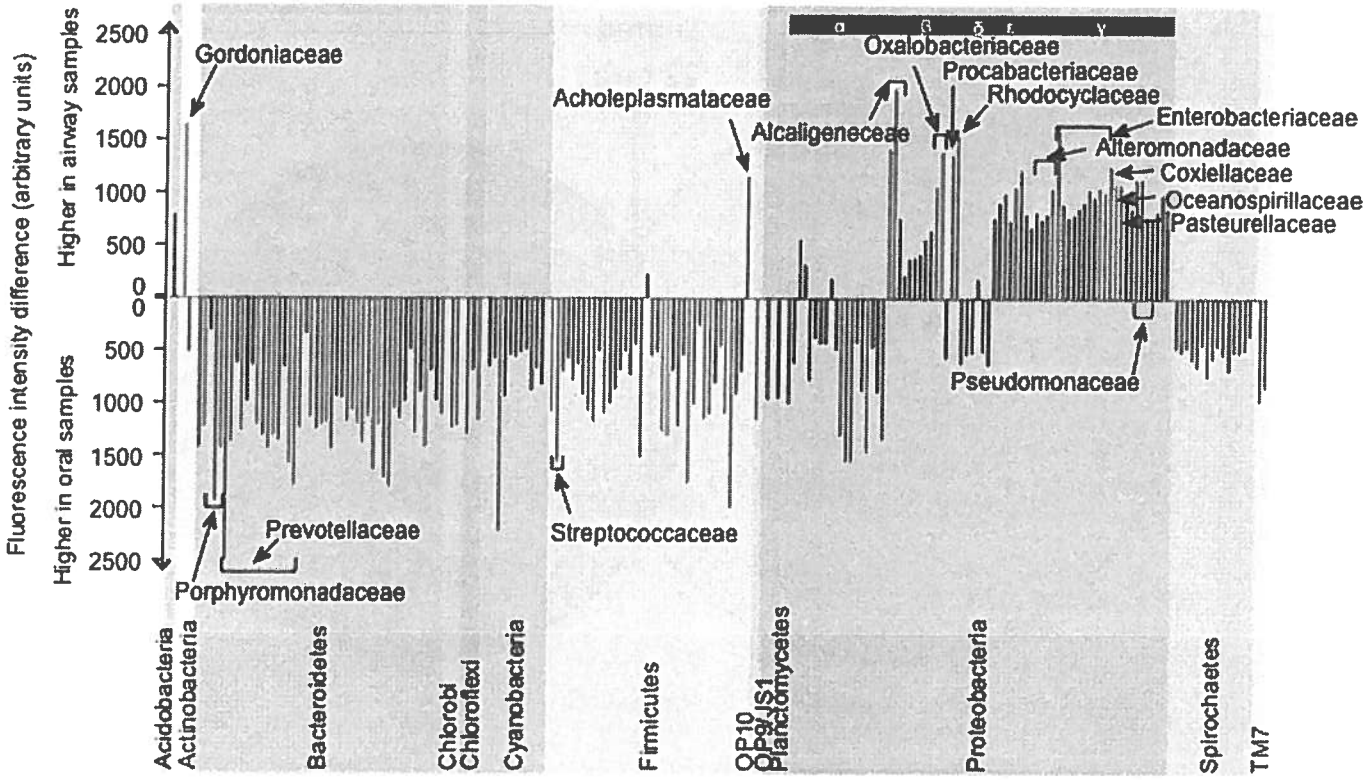


Figure 5. Number of taxa detected common or exclusive to airway (white circle) and oral (gray circle) samples in HIV-positive patient samples.

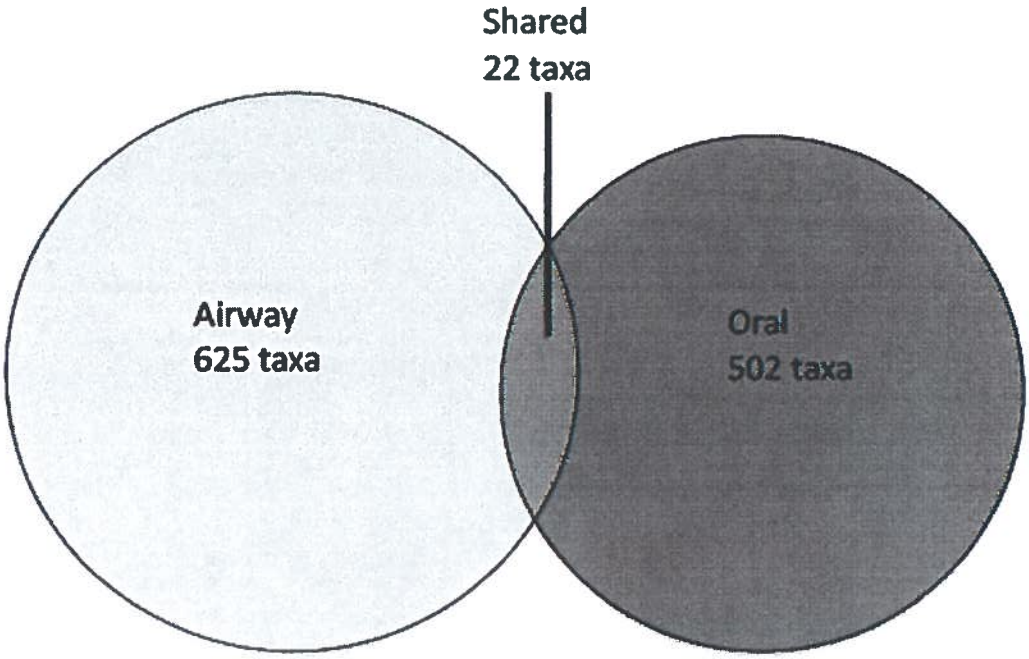


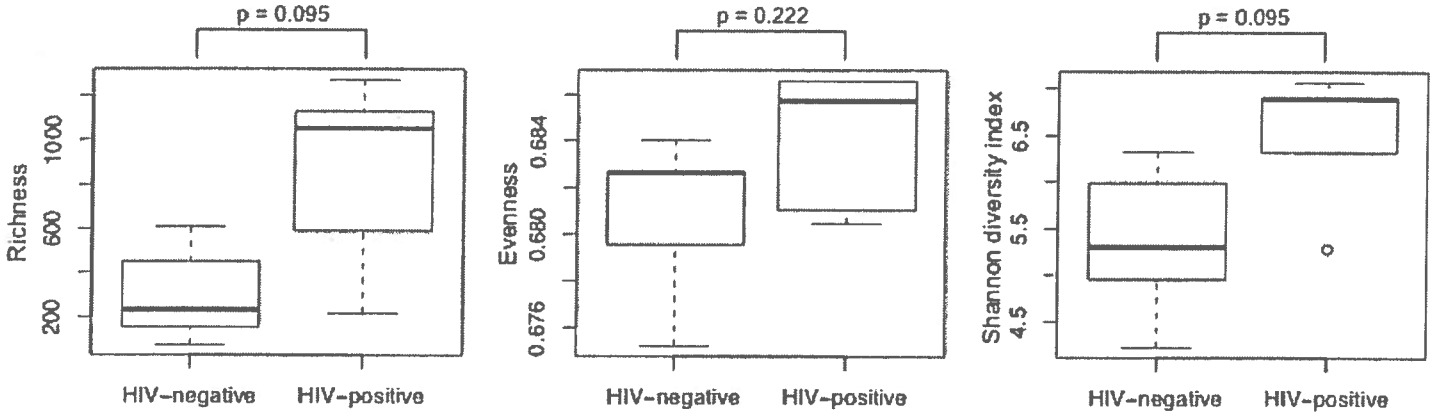
Table 1. Clinical characteristics of samples

ID	Age	Gender	CD4 Cell Count*	HIV RNA Level†	ART‡	Antimicrobial Therapy	Days on Antimicrobial Therapy Prior to Sampling§
HIV-positive patients admitted to this study							
SFGH012	47	F	101	74	+	Atovaquone, clarithromycin, ethambutol, vancomycin	6
						Cefepime	4
						Clindamycin, primaquine	3
SFGH021	43	M	49	500001	-	Clindamycin, primaquine, septra	STOP
						Doxycycline	5
						Azithromycin, clindamycin, primaquine	4
SFGH041	39	M	38	405002	-	Ceftriaxone, doxycycline, septra	4
						Azithromycin	2
SFGH071	60	M	305	72851	-	Ceftriaxone, doxycycline, septra	START
SFGH081	42	M	145	408204	-	Ceftriaxone, doxycycline	5
						Clindamycin, primaquine	NR‖
SFGH091	31	M	36	429	+	Septra	2
SFGH111	36	M	2	80732	-	Ceftriaxone, doxycycline, septra	6
						Vancomycin	2
SFGH121	33	M	28	39	+	Dapsone, ethambutol	6
						Vancomycin	5
						Piperacillin	2
						Azithromycin, cefepime, clarithromycin, clindamycin,	NR
SFGH161	27	F	45	277614	-	Ceftriaxone, doxycycline	7
						Clindamycin, primaquine	6
SFGH171	50	F	2	24507	-	Posaconazole	37
						Azithromycin, septra	NR
SFGH231	37	M	178	705753	-	Ceftriaxone, doxycycline, septra	4
SFGH241	48	M	24	20557	-	Levaquin	9
						Azithromycin, cefepime, septra, vancomycin,	4
SFGH251	50	F	29	84815	-	Ethambutol, isoniazid, pyrazinamide, rifampin	STOP
						Doxycycline, septra,	3
						Azithromycin	NR
SFGH271	46	M	6	336899	-	Ceftriaxone, doxycycline	4
						Clindamycin, fluconazole, primaquine	3
SFGH291	45	M	5	69885	-	Ceftriaxone, doxycycline	8
						Septra	5
						Azithromycin	4

HIV-negative patients admitted to Flanagan *et al.*'s study [68]							
NEG1049[¶]	57	F	NR	NR	-	Cefazolin, fluconazole, levofloxacin	NDR[**]
						Piperacillin-tazobactam	NDR
NEG1150	79	M	NR	NR	-	Antifungal, cefazolin, ceftazidime, ciprofloxacin	NDR
						Piperacillin-tazobactam, vancomycin	NDR
NEG1900[¶]	54	F	NR	NR	-	Ciprofloxacin	NDR
NEG1578[¶]	55	M	NR	NR	-	Piperacillin-tazobactam, vancomycin	NDR
NEG1523[¶]	85	F	NR	NR	-	Ciprofloxacin, clindamycin, piperacillin-tazobactam	NDR
						Vancomycin	NDR

[*] The day CD4 count was measured varies from 103 days to 3 days ago of the sampling date.
[†] The day HIV RNA level was measured varies from 155 days ago to one day after the sampling date.
[‡] +, under administration; -, not under administration.
[§] STOP, stopped prior to collection; START, started prior to collection
[‖] NR, no record
[¶] ID correlates to "patient number"-"(sample number)" from Flanagan *et al.* [68]
[**] No duration recorded, though treated within 4-17 days prior to sampling.

Figure 5: Comparative analysis of community richness, evenness and Shannon diversity measurements between HIV-positive and HIV-negative subjects.



5a 5b 5c

Figure 6: Hierarchical cluster analysis of HIV positive (gray box) and HIV negative subjects

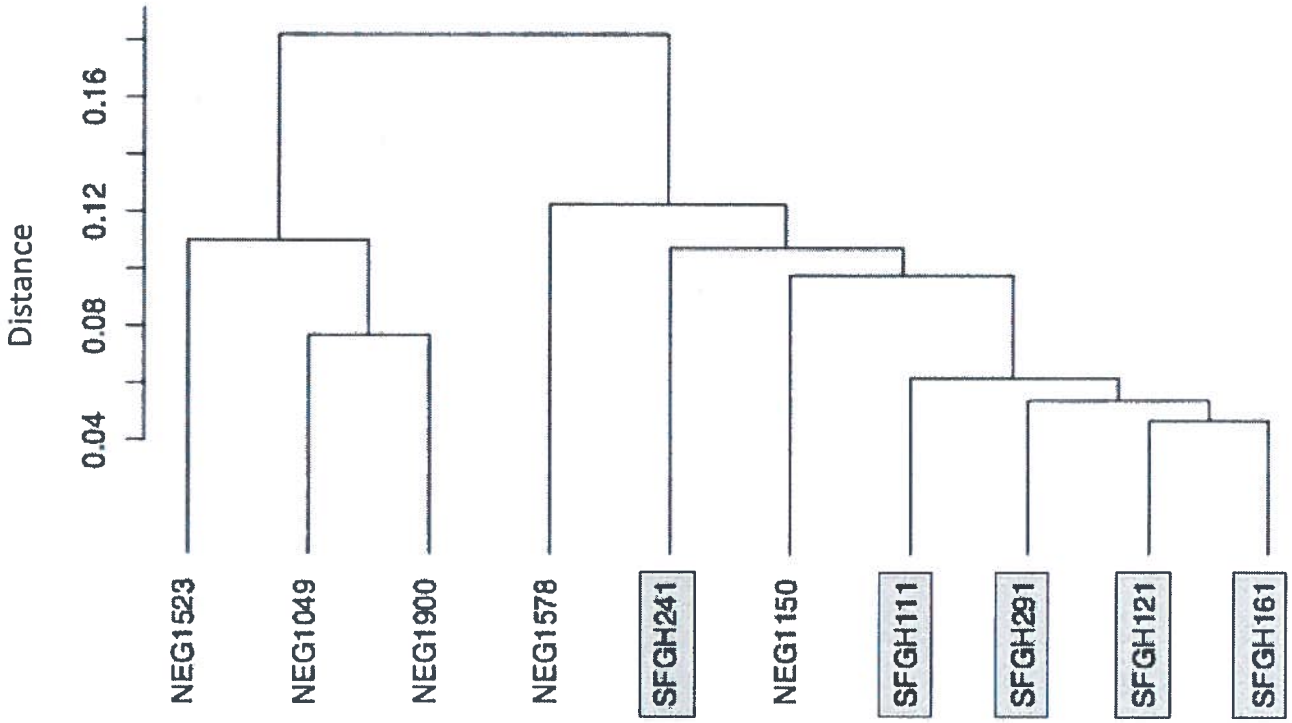


Figure 7: Number of taxa detected common or exclusive to HIV-infected (white circle) and HIV-negative (gray circle) patients.

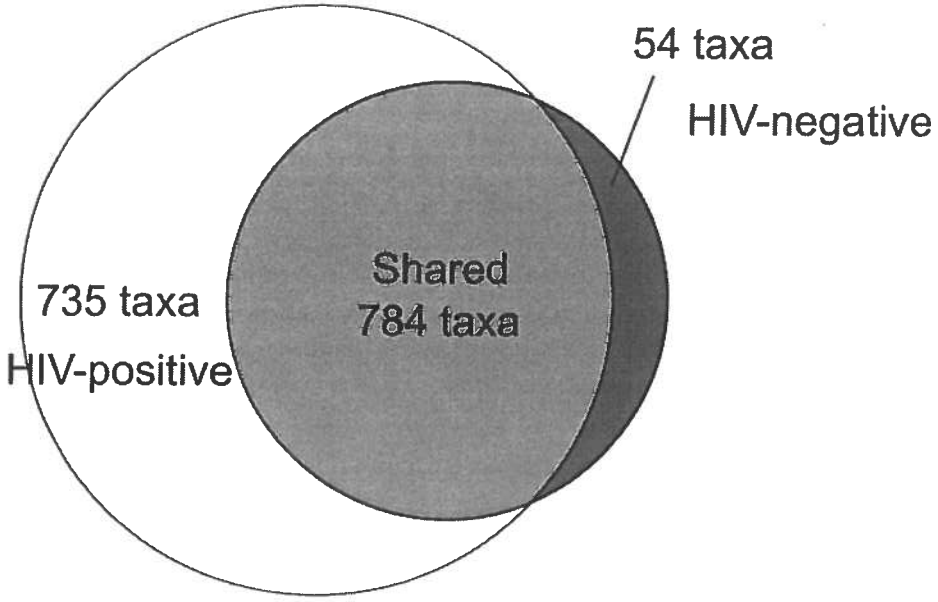
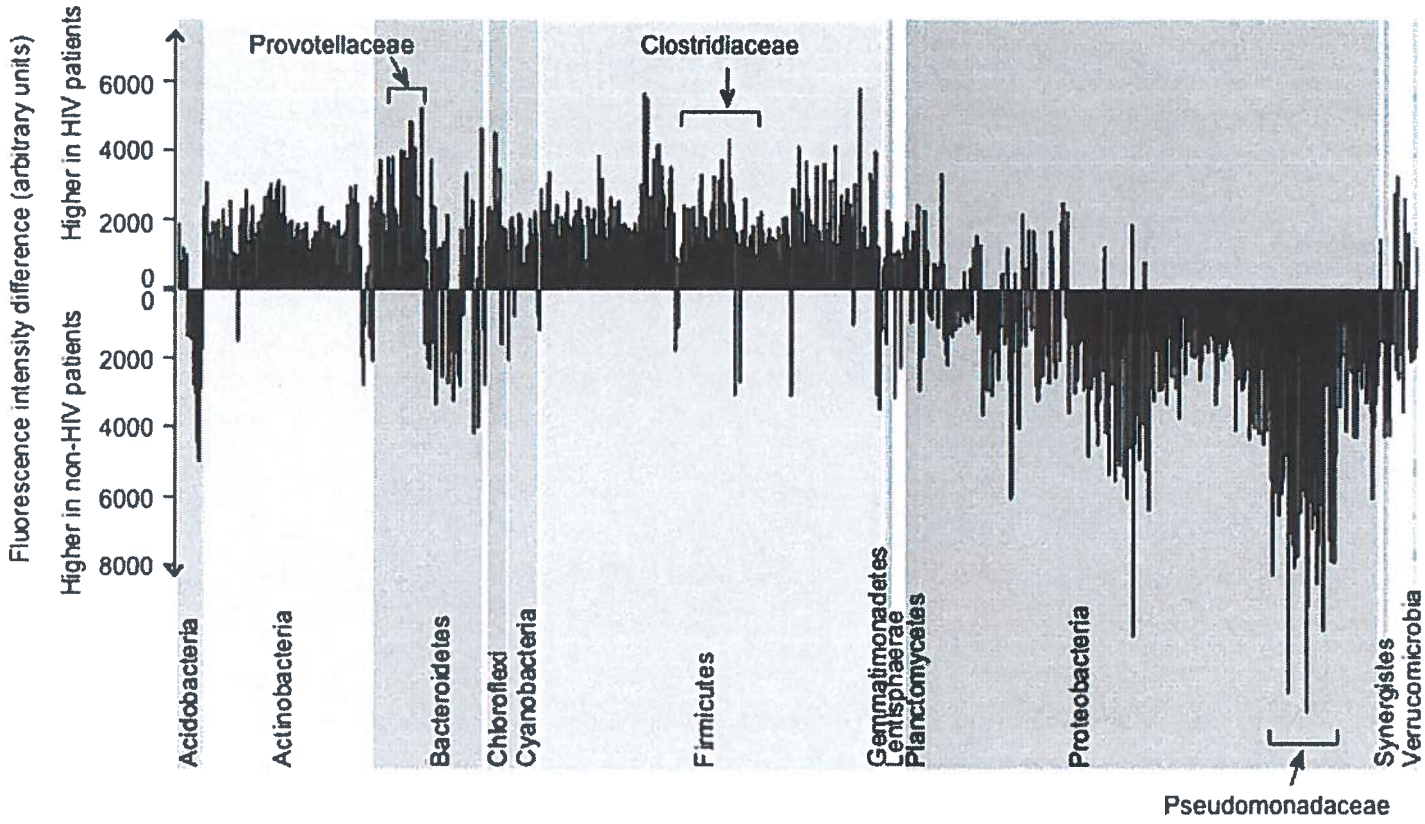


Figure 8: A comparison of significantly ($p \leq 0.05$) different mean taxonomic relative fluorescence intensity values between HIV-positive and HIV-negative samples identifies taxa characteristic of each group. Background is colored based on phylum.



References

1. Zoetendal EG, Vaughan EE, de Vos WM. A microbial world within us. Mol Microbiol. 2006; **59**(6): 1639-50.
2. Turnbaugh PJ, Ley RE, Hamady M, Fraser-Liggett CM, Knight R, Gordon JI. The human microbiome project. Nature. 2007; **449**(7164): 804-10.
3. Fujimura KE, Slusher NA, Cabana MD, Lynch SV. Role of the gut microbiota in defining human health. Expert Rev Anti Infect Ther. 2010; **8**(4): 435-54.
4. Dethlefsen L, McFall-Ngai M, Relman DA. An ecological and evolutionary perspective on human-microbe mutualism and disease. Nature. 2007; **449**(7164): 811-8.
5. Jenkinson HF, Lamont RJ. Oral microbial communities in sickness and in health. Trends Microbiol. 2005; **13**(12): 589-95.
6. Hill DA, Artis D. Intestinal bacteria and the regulation of immune cell homeostasis. Annu Rev Immunol. **28**: 623-67.
7. Huang YJ, Kim E, Cox MJ, Brodie EL, Brown R, Wiener-Kronish JP, et al. A persistent and diverse airway microbiota present during chronic obstructive pulmonary disease exacerbations. Omics. **14**(1): 9-59.
8. Rogers GB, Hart CA, Mason JR, Hughes M, Walshaw MJ, Bruce KD. Bacterial diversity in cases of lung infection in cystic fibrosis patients: 16S ribosomal DNA (rDNA) length heterogeneity PCR and 16S rDNA terminal restriction fragment length polymorphism profiling. J Clin Microbiol. 2003; **41**(8): 3548-58.
9. Turnbaugh PJ, Gordon JI. The core gut microbiome, energy balance and obesity. J Physiol. 2009; **587**(Pt 17): 4153-8.
10. Relman DA. New technologies, human-microbe interactions, and the search for previously unrecognized pathogens. J Infect Dis. 2002; **186 Suppl 2**: S254-8.
11. Rogers GB, Carroll MP, Bruce KD. Studying bacterial infections through culture-independent approaches. J Med Microbiol. 2009; **58**(Pt 11): 1401-18.
12. Heijerman H. Infection and inflammation in cystic fibrosis: a short review. J Cyst Fibros. 2005; **4 Suppl 2**: 3-5.
13. DeSantis TZ, Brodie EL, Moberg JP, Zubieta IX, Piceno YM, Andersen GL. High-density universal 16S rRNA microarray analysis reveals broader diversity than typical clone library when sampling the environment. Microb Ecol. 2007; **53**(3): 371-83.
14. Schutte UM, Abdo Z, Bent SJ, Shyu C, Williams CJ, Pierson JD, et al. Advances in the use of terminal restriction fragment length polymorphism (T-RFLP) analysis of 16S rRNA genes to characterize microbial communities. Appl Microbiol Biotechnol. 2008; **80**(3): 365-80.
15. Wittebolle L, Marzorati M, Clement L, Balloi A, Daffonchio D, Heylen K, et al. Initial community evenness favours functionality under selective stress. Nature. 2009; **458**(7238): 623-6.
16. Kindt R, Coe R. Tree diversity analysis : A manual and software for common statistical methods for echological and biodiversity studies. Nairobi: World Agroforestry Cenre. 2005.
17. Turnbaugh PJ, Gordon JI. An invitation to the marriage of metagenomics and metabolomics. Cell. 2008; **134**(5): 708-13.

18. Medini D, Serruto D, Parkhill J, Relman DA, Donati C, Moxon R, et al. Microbiology in the post-genomic era. Nat Rev Microbiol. 2008; **6**(6): 419-30.
19. Staden R. Automation of the computer handling of gel reading data produced by the shotgun method of DNA sequencing. Nucleic Acids Res. 1982; **10**(15): 4731-51.
20. Anderson S. Shotgun DNA sequencing using cloned DNase I-generated fragments. Nucleic Acids Res. 1981; **9**(13): 3015-27.
21. Zhang W, Li F, Nie L. Integrating multiple 'omics' analysis for microbial biology: application and methodologies. Microbiology. 2010; **156**(Pt 2): 287-301.
22. Moran M. Metatranscriptomics: Eavesdropping on Complex Microbial Communities. Microbe. 2009; **4**(7): 329-35.
23. Hamady M, Knight R. Microbial community profiling for human microbiome projects: Tools, techniques, and challenges. Genome Res. 2009; **19**(7): 1141-52.
24. Hugenholtz P, Tyson GW. Microbiology: metagenomics. Nature. 2008; **455**(7212): 481-3.
25. Ley RE, Peterson DA, Gordon JI. Ecological and evolutionary forces shaping microbial diversity in the human intestine. Cell. 2006; **124**(4): 837-48.
26. Penders J, Thijs C, Vink C, Stelma FF, Snijders B, Kummeling I, et al. Factors influencing the composition of the intestinal microbiota in early infancy. Pediatrics. 2006; **118**(2): 511-21.
27. Ege MJ, Bieli C, Frei R, van Strien RT, Riedler J, Ublagger E, et al. Prenatal farm exposure is related to the expression of receptors of the innate immunity and to atopic sensitization in school-age children. J Allergy Clin Immunol. 2006; **117**(4): 817-23.
28. Strachan DP. Hay fever, hygiene, and household size. BMJ. 1989; **299**(6710): 1259-60.
29. von Mutius E. Of attraction and rejection--asthma and the microbial world. N Engl J Med. 2007; **357**(15): 1545-7.
30. Dominguez-Bello MG, Costello EK, Contreras M, Magris M, Hidalgo G, Fierer N, et al. Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. Proc Natl Acad Sci U S A. 2010; **107**(26): 11971-5.
31. Thavagnanam S, Fleming J, Bromley A, Shields MD, Cardwell CR. A meta-analysis of the association between Caesarean section and childhood asthma. Clin Exp Allergy. 2008; **38**(4): 629-33.
32. Bisgaard H, Hermansen MN, Buchvald F, Loland L, Halkjaer LB, Bonnelykke K, et al. Childhood asthma after bacterial colonization of the airway in neonates. N Engl J Med. 2007; **357**(15): 1487-95.
33. Klepac-Ceraj V, Lemon KP, Martin TR, Allgaier M, Kembel SW, Knapp AA, et al. Relationship between cystic fibrosis respiratory tract bacterial communities and age, genotype, antibiotics and Pseudomonas aeruginosa. Environ Microbiol. 2010; **12**(5): 1293-303.
34. Read AF, Taylor LH. The ecology of genetically diverse infections. Science. 2001; **292**(5519): 1099-102.
35. Flint HJ, Duncan SH, Scott KP, Louis P. Interactions and competition within the microbial community of the human colon: links between diet and health. Environ Microbiol. 2007; **9**(5): 1101-11.
36. Juhas M, Eberl L, Tummler B. Quorum sensing: the power of cooperation in the world of Pseudomonas. Environ Microbiol. 2005; **7**(4): 459-71.

37. Ramsey MM, Whiteley M. Polymicrobial interactions stimulate resistance to host innate immunity through metabolite perception. Proc Natl Acad Sci U S A. 2009; **106**(5): 1578-83.
38. Duan K, Dammel C, Stein J, Rabin H, Surette MG. Modulation of Pseudomonas aeruginosa gene expression by host microflora through interspecies communication. Mol Microbiol. 2003; **50**(5): 1477-91.
39. Rogers GB, Carroll MP, Serisier DJ, Hockey PM, Kehagia V, Jones GR, et al. Bacterial activity in cystic fibrosis lung infections. Respir Res. 2005; **6**: 49.
40. Tunney MM, Field TR, Moriarty TF, Patrick S, Doering G, Muhlebach MS, et al. Detection of anaerobic bacteria in high numbers in sputum from patients with cystic fibrosis. Am J Respir Crit Care Med. 2008; **177**(9): 995-1001.
41. Ecker KW, Derouet H, Omlor G, Mast GJ. [Fournier's gangrene]. Chirurg. 1993; **64**(1): 58-62.
42. Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI. An obesity-associated gut microbiome with increased capacity for energy harvest. Nature. 2006; **444**(7122): 1027-31.
43. Turnbaugh PJ, Backhed F, Fulton L, Gordon JI. Diet-induced obesity is linked to marked but reversible alterations in the mouse distal gut microbiome. Cell Host Microbe. 2008; **3**(4): 213-23.
44. Turnbaugh PJ, Hamady M, Yatsunenko T, Cantarel BL, Duncan A, Ley RE, et al. A core gut microbiome in obese and lean twins. Nature. 2009; **457**(7228): 480-4.
45. Ivanov, II, Frutos Rde L, Manel N, Yoshinaga K, Rifkin DB, Sartor RB, et al. Specific microbiota direct the differentiation of IL-17-producing T-helper cells in the mucosa of the small intestine. Cell Host Microbe. 2008; **4**(4): 337-49.
46. Mazmanian SK, Liu CH, Tzianabos AO, Kasper DL. An immunomodulatory molecule of symbiotic bacteria directs maturation of the host immune system. Cell. 2005; **122**(1): 107-18.
47. Suzuki K, Meek B, Doi Y, Muramatsu M, Chiba T, Honjo T, et al. Aberrant expansion of segmented filamentous bacteria in IgA-deficient gut. Proc Natl Acad Sci U S A. 2004; **101**(7): 1981-6.
48. Bollinger RR, Everett ML, Wahl SD, Lee YH, Orndorff PE, Parker W. Secretory IgA and mucin-mediated biofilm formation by environmental strains of Escherichia coli: role of type 1 pili. Mol Immunol. 2006; **43**(4): 378-87.
49. Inoue R, Otsuka M, Ushida K. Development of intestinal microbiota in mice and its possible interaction with the evolution of luminal IgA in the intestine. Exp Anim. 2005; **54**(5): 437-45.
50. Frank DN, St Amand AL, Feldman RA, Boedeker EC, Harpaz N, Pace NR. Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. Proc Natl Acad Sci U S A. 2007; **104**(34): 13780-5.
51. Rohde CL, Bartolini V, Jones N. The use of probiotics in the prevention and treatment of antibiotic-associated diarrhea with special interest in Clostridium difficile-associated diarrhea. Nutr Clin Pract. 2009; **24**(1): 33-40.
52. Cox MJ, Allgaier M, Taylor B, Baek MS, Huang YJ, Daly RA, et al. Airway microbiota and pathogen abundance in age-stratified cystic fibrosis patients. PLoS One. **5**(6): e11044.

53. Lofmark S, Jernberg C, Jansson JK, Edlund C. Clindamycin-induced enrichment and long-term persistence of resistant Bacteroides spp. and resistance genes. J Antimicrob Chemother. 2006; **58**(6): 1160-7.
54. Sjolund M, Tano E, Blaser MJ, Andersson DI, Engstrand L. Persistence of resistant Staphylococcus epidermidis after single course of clarithromycin. Emerg Infect Dis. 2005; **11**(9): 1389-93.
55. Sjolund M, Wreiber K, Andersson DI, Blaser MJ, Engstrand L. Long-term persistence of resistant Enterococcus species after antibiotics to eradicate Helicobacter pylori. Ann Intern Med. 2003; **139**(6): 483-7.
56. Stecher B, Chaffron S, Kappeli R, Hapfelmeier S, Freedrich S, Weber TC, et al. Like will to like: abundances of closely related species can predict susceptibility to intestinal colonization by pathogenic and commensal bacteria. PLoS Pathog. 2010; **6**(1): e1000711.
57. Gordon S. Elie Metchnikoff: father of natural immunity. Eur J Immunol. 2008; **38**(12): 3257-64.
58. Kaufmann SH. Immunology's foundation: the 100-year anniversary of the Nobel Prize to Paul Ehrlich and Elie Metchnikoff. Nat Immunol. 2008; **9**(7): 705-12.
59. Doron SI, Hibberd PL, Gorbach SL. Probiotics for prevention of antibiotic-associated diarrhea. J Clin Gastroenterol. 2008; **42 Suppl 2**: S58-63.
60. Cox MJ, Huang YJ, Fujimura KE, Liu JT, McKean M, Boushey HA, et al. Lactobacillus casei abundance is associated with profound shifts in the infant gut microbiome. PLoS One. 2010; **5**(1): e8745.
61. Resta-Lenert SC, Barrett KE. Modulation of intestinal barrier properties by probiotics: role in reversing colitis. Ann N Y Acad Sci. 2009; **1165**: 175-82.
62. Dong Y, Manfredini F, Dimopoulos G. Implication of the mosquito midgut microbiota in the defense against malaria parasites. PLoS Pathog. 2009; **5**(5): e1000423.
63. Mapping and Sequencing the Human Genome. Human genome project foundation.
64. Hybrid Selection of Targets for Next-Generation Sequencing. Agilent Technologies.
65. Lucas G. Bacterial pulmonary infections in HIV-infected patients. Up-to-date. 2009.
66. Hilty M, Burke C, Pedro H, Cardenas P, Bush A, Bossley C, et al. Disordered microbial communities in asthmatic airways. PLoS One. 2010; **5**(1): e8578.
67. Huang YJ, Nelson CE, Brodie EL, DeSantis TZ, Back MS, Woyke T, et al. Airway Microbiota and Bronchial Hyperesponsiveness in Patients with Sub-optimally Cotrolled Asthma. Journal of Allergy and Clinical Immunology.
68. Flanagan JL, Brodie EL, Weng L, Lynch SV, Garcia O, Brown R, et al. Loss of bacterial diversity during antibiotic treatment of intubated patients colonized with Pseudomonas aeruginosa. J Clin Microbiol. 2007; **45**(6): 1954-62.
69. Turnbaugh PJ, Ridaura VK, Faith JJ, Rey FE, Knight R, Gordon JI. The effect of diet on the human gut microbiome: a metagenomic analysis in humanized gnotobiotic mice. Sci Transl Med. 2009; **1**(6): 6ra14.
70. Brodie EL, Desantis TZ, Joyner DC, Baek SM, Larsen JT, Andersen GL, et al. Application of a high-density oligonucleotide microarray approach to study bacterial

population dynamics during uranium reduction and reoxidation. Appl Environ Microbiol. 2006; **72**(9): 6288-98.
71. Storey JD. The Positive False Discovery Rate: A Bayesian Intepretation and the q-value. The Annals of Statistics. 2003; **31**(6): 2013-35.
72. Letunic I, Bork P. Interactive Tree Of Life (iTOL): an online tool for phylogenetic tree display and annotation. Bioinformatics. 2007; **23**(1): 127-8.
73. DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, et al. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Appl Environ Microbiol. 2006; **72**(7): 5069-72.
74. de Silva T, Raychaudhuri M, Poulton M. HIV infection associated with Strongyloides stercoralis colitis resulting in Streptococcus bovis bacteraemia and meningitis. Sex Transm Infect. 2005; **81**(3): 276-7.
75. Jain AK, Agarwal SK, el-Sadr W. Streptococcus bovis bacteremia and meningitis associated with Strongyloides stercoralis colitis in a patient infected with human immunodeficiency virus. Clin Infect Dis. 1994; **18**(2): 253-4.
76. Manfredi R, Nanetti A, Ferri M, Calza L, Tadolini M, Chiodo F. Streptococcus bovis bacteremia in patients infected with the human immunodeficiency virus: case reports and literature review. Eur J Clin Microbiol Infect Dis. 1999; **18**(2): 148-50.
77. Chiu CH, Waddingdon M, Greenberg D, Schreckenberger PC, Carnahan AM. Atypical Chryseobacterium meningosepticum and meningitis and sepsis in newborns and the immunocompromised, Taiwan. Emerg Infect Dis. 2000; **6**(5): 481-6.
78. Bloch KC, Nadarajah R, Jacobs R. Chryseobacterium meningosepticum: an emerging pathogen among immunocompromised adults. Report of 6 cases and literature review. Medicine (Baltimore). 1997; **76**(1): 30-41.
79. Squier C, Yu VL, Stout JE. Waterborne Nosocomial Infections. Curr Infect Dis Rep. 2000; **2**(6): 490-6.
80. Fei M, Huang L. HIV-Associated Pneumonias

. The body. 2008; (Winter).
81. Lemon KP, Klepac-Ceraj V, Schiffer HK, Brodie EL, Lynch SV, Kolter R. Comparative analyses of the bacterial microbiota of the human nostril and oropharynx. MBio. 2010; **1**(3).
82. Peterson J, Garges S, Giovanni M, McInnes P, Wang L, Schloss JA, et al. The NIH Human Microbiome Project. Genome Res. 2009; **19**(12): 2317-23.
83. Getaneh A, Medhin G, Shimelis T. Cryptosporidium and Strongyloides stercoralis infections among people with and without HIV infection and efficiency of diagnostic methods for Strongyloides in Yirgalem Hospital, southern Ethiopia. BMC Res Notes. 2010; **3**: 90.
84. Manfredi R, Tadolini M, Calza L, Chiodo F. [Streptococcus bovis as an opportunistic pathogen during advanced HIV disease]. Infez Med. 1999; **7**(3): 187-91.
85. Torres A, El-Ebiary M, Soler N, Monton C, Fabregas N, Hernandez C. Stomach as a source of colonization of the respiratory tract during mechanical ventilation: association with ventilator-associated pneumonia. Eur Respir J. 1996; **9**(8): 1729-35.
86. Torres A, el-Ebiary M, Soler N, Monton C, Gonzalez J, Puig de la Bellacasa J. The role of the gastric reservoir in ventilator-associated pneumonia. Clin Intensive Care. 1995; **6**(4): 174-80.

87. de Souza Goncalves L, Ferreira SM, Silva A, Jr., Villoria GE, Costinha LH, Souto R, et al. Association of T CD4 lymphocyte levels and subgingival microbiota of chronic periodontitis in HIV-infected Brazilians under HAART. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2004; **97**(2): 196-203.
88. de Souza Goncalves L, Souto R, Colombo AP. Detection of Helicobacter pylori, Enterococcus faecalis, and Pseudomonas aeruginosa in the subgingival biofilm of HIV-infected subjects undergoing HAART with chronic periodontitis. Eur J Clin Microbiol Infect Dis. 2009; **28**(11): 1335-42.
89. Navazesh M, Mulligan R, Pogoda J, Greenspan D, Alves M, Phelan J, et al. The effect of HAART on salivary microbiota in the Women's Interagency HIV Study (WIHS). Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2005; **100**(6): 701-8.
90. Aas JA, Barbuto SM, Alpagot T, Olsen I, Dewhirst FE, Paster BJ. Subgingival plaque microbiota in HIV positive patients. J Clin Periodontol. 2007; **34**(3): 189-95.
91. Spear GT, Sikaroodi M, Zariffard MR, Landay AL, French AL, Gillevet PM. Comparison of the diversity of the vaginal microbiota in HIV-infected and HIV-uninfected women with or without bacterial vaginosis. J Infect Dis. 2008; **198**(8): 1131-40.
92. Silva-Boghossian C, Castro GF, Teles RP, De Souza IP, Colombo AP. Salivary microbiota of HIV-positive children and its correlation with HIV status, oral diseases, and total secretory IgA. Int J Paediatr Dent. 2008; **18**(3): 205-16.
93. Gori A, Tincati C, Rizzardini G, Torti C, Quirino T, Haarman M, et al. Early impairment of gut function and gut flora supporting a role for alteration of gastrointestinal mucosa in human immunodeficiency virus pathogenesis. J Clin Microbiol. 2008; **46**(2): 757-8.
94. Round JL, Mazmanian SK. Inducible Foxp3+ regulatory T-cell development by a commensal bacterium of the intestinal microbiota. Proc Natl Acad Sci U S A. 2010; **107**(27): 12204-9.
95. Ivanov, II, Atarashi K, Manel N, Brodie EL, Shima T, Karaoz U, et al. Induction of intestinal Th17 cells by segmented filamentous bacteria. Cell. 2009; **139**(3): 485-98.
96. Brenchley JM, Price DA, Schacker TW, Asher TE, Silvestri G, Rao S, et al. Microbial translocation is a cause of systemic immune activation in chronic HIV infection. Nat Med. 2006; **12**(12): 1365-71.
97. Hament JM, Kimpen JL, Fleer A, Wolfs TF. Respiratory viral infection predisposing for bacterial disease: a concise review. FEMS Immunol Med Microbiol. 1999; **26**(3-4): 189-95.
98. Hartshorn KL. New look at an old problem: bacterial superinfection after influenza. Am J Pathol. 2010; **176**(2): 536-9.
99. Peltola VT, McCullers JA. Respiratory viruses predisposing to bacterial infections: role of neuraminidase. Pediatr Infect Dis J. 2004; **23**(1 Suppl): S87-97.
100. Koning CJ, Jonkers D, Smidt H, Rombouts F, Pennings HJ, Wouters E, et al. The effect of a multispecies probiotic on the composition of the faecal microbiota and bowel habits in chronic obstructive pulmonary disease patients treated with antibiotics. Br J Nutr. 2010; **103**(10): 1452-60.
101. Resta-Lenert S, Barrett KE. Live probiotics protect intestinal epithelial cells from the effects of infection with enteroinvasive Escherichia coli (EIEC). Gut. 2003; **52**(7): 988-97.

102. Servin AL, Coconnier MH. Adhesion of probiotic strains to the intestinal mucosa and interaction with pathogens. Best Pract Res Clin Gastroenterol. 2003; **17**(5): 741-54.
103. Rauch M, Lynch SV. Probiotic Manupulation of the Gastrointestinal Microbiota. Gut Microbe. 2010; **1**(5).