The Healthcare Workers' Hand Microbiome: Community Structure and Dynamics

by

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Dedication

To my parents.

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Abbreviations

CFU	Colony-forming unit
HAI	Healthcare-associated Infection
HMP	Human Microbiome Project
ICU	Intensive Care Unit
MRSA	Methycilin-resistant *Staphylococcus aureus*
PCoA	Principal Coordinate Analysis
OTU	Operational Taxonomic Unit
QIIME	Quantitative Insights Into Microbial Ecology
SICU	Surgical Intensive Care Unit
spp	Species (pl)

Abstract

The complex microbial communities that inhabit our bodies, our microbiota, profoundly influence human health. Alterations in microbiota profiles occur as a result of host and environmental factors, and can lead to increased microorganism transmission, pathogen susceptibility, and disease. This interdisciplinary dissertation investigates hand microbiota dynamics over a 3-week period among 34 surgical intensive care unit healthcare workers (HCWs), while accounting for technical sources of variation. We also assess the role hand microbiota play in specific nosocomial pathogen carriage.

An accurate profile of the human microbiota requires assessment of the variation in biological patterns between individuals and within individuals over time, while considering technical variation due to (i) specimen collection method, (ii) DNA extraction technique, and (iii) sequencing. Analysis of the HCWs' hand microbiota showed that only sampling collection method appeared to have a significant impact on the observed microbial community structure. Samples collected using the glove-juice method showed that individuals' hands were slightly more similar to each other in microbial composition over time than between individuals. Using the swab method, however, samples from a single individual were no more similar to each other than to other individuals.

After HCW hand microbiota characterization, we assessed its role in nosocomial pathogen carriage. The proportion of pathogens detected using qPCR varied by collection visit: *Staphylococcus aureus* ranged from 41.2%-52.9%; *Enterococcus* spp. ranged from 52.9%-61.8%; *Candida albicans* ranged from 2.9%-8.8%; and, MRSA from 2.9%-5.9%. HCWs with lower microbiota diversity were more likely to carry a pathogen on their hands than those with higher diversity. HCWs took a self-administered questionnaire regarding

basic demographics, overall health, hand health, hand hygiene practices, and patient contact levels, to explore possible associations with pathogen carriage. Risk factors for pathogen carriage were pathogen specific. Hand microbiota may have mediated the relationship between hand hygiene and pathogen carriage; and, acted as effect modifiers in the relationships between a) age and *Enterococcus* spp. carriage, and b) work shift and *Staphylococcus aureus* carriage.

Understanding risk factors for pathogen carriage and its relationship to HCW hand microbiota has significant implications for pathogen transmission and hospital infection control policies.

Chapter 1: Introduction

Background

Infections acquired in intensive care units (ICU) are associated with significant morbidity and mortality. The burden of nosocomial infection varies, according to the hospital setting, the patient population, hospital length-of-stay, surveillance methods, reporting rates, and case definitions used (Doyle et al, 2011). According to the Centers for Disease Control and Prevention, approximately 1 out of every 20 hospitalized patients will contract a healthcare-associated infection (HAI). Others estimate up to 10% of admitted patients will acquire a nosocomial infection. These rates have once been reported to be 5-10 times higher in ICUs than among general ward patients (Trilla, 1994). Recently, a large, international study of the epidemiology of ICU infections reported a 51.4% prevalence among 13,796 adult patients at 1,265 participating ICUs from 75 countries (Vincent et al, 2009).

Among interventions geared at hospital infection control, hand hygiene remains the most important in reducing transmission. A review of studies from 1977 to 2008 looking at hand hygiene on the prevention of HAI showed that almost all report a temporal association between improved hand hygiene and reduced infection and cross-transmission rates (Allengranzi and Pittet, 2009). Nevertheless, even with high compliance, there is

much evidence pointing toward the insufficiency of hand hygiene in preventing HAI. One study which used transmission rates as the reference standard for evaluating indicators of infection control problems among 5 ICUs, showed no correlation between the incidence of transmission episodes and hand hygiene compliance (Eckmanns et al, 2006). An explanation of why increasing hand hygiene compliance is not the silver bullet against HAI is the increased rates of dermatitis among healthcare workers, acquired due to the extraordinary high frequency with which they wash their hands and use hand alcohol rubs. Their ensuing damaged hands often harbor more microorganisms than normal hands, because washing damaged hands is less efficient at reducing bacterial load (Larson, 2001). Damaged hands has also been shown to be associated with greater bacterial load with antimicrobial resistance, despite using just plain soap (Borges et al, 2007; Rocha et al, 2009). Poor hand health, however, may not be the only explanation for the observed increase in bacterial load. Perhaps, the inter-species interactions provided by the hand microbiota can also be held accountable. We do not know, for instance, enough about our skin microbiota to realize the full impact hand hygiene has on its balance, and consequently, on our potential to carry certain pathogens, and consequently transmit diseases. Clearly, control of transmission of hospital infections requires more than clean hands.

Overview

This dissertation work comprises of a detailed review (see chapter 2) of the skin microbiota and its association with its potential determinants of community structure, and ultimately health and disease, in addition to the results of a pilot study (chapters 3 and 4) called Healthy Hands Study, which implements the conceptual ideas from the review into

practice. The Healthy Hands Study is a longitudinal, short-termed, observational cohort study looking at the hand microbiota of healthcare workers (HCW) in a surgical intensive care unit. The HCWs' hand microbiome were collected and measured at three points in time, and time-invariant predictors of its variability and of pathogen carriage, were collected at baseline through a self-reported questionnaire. Additionally, HCWs were screened for four pathogens known to cause a high level of hospital infections worldwide. The relationship between the determinants of pathogen carriage, the hand microbiota, and pathogen carriage was investigated.

First, this dissertation describes the human skin microbiota, outlines the potential determining factors driving its variability, posits the likelihood of an association between the resulting microbial community structure on the skin with disease outcomes among individuals, and finally, presents some challenges and implications for studying the skin microbiota.

Second, it evaluates the biological variability of the skin microbiome of the hands of HCWs to consequently understand more accurately its impact in health, disease etiology, and microbial transmission. This dissertation investigates the dynamics, or the variation in biological patterns between individuals and within individuals over time, of the HCW hand microbiota after assessing the technical variation introduced due to (i) specimen collection, (ii) DNA extraction, and (iii) sequencing. These results are discussed in Chapter 3, which is entitled 'Hand Microbiome Dynamics among Healthcare Workers in a Surgical Intensive Care Unit'.

Third, upon assessing the dynamics of the HCW hand microbiome, this dissertation addresses its influence on nosocomial pathogen carriage. Among the most common ICU pathogens reported by the U.S. National Health Surveillance Network, are Staphylococci, Enterococci, and *Candida* (Doyle et al, 2011). HAI of methycillin-resistant *Staphylococcus aureus* (MRSA), while in decline in the United States, are still a major concern. A 6-year surveillance study from 173 ICUs across Latin American, Asia, Africa, and Europe, show MRSA bloodstream infection rates remain high (84.1%) (Doyle et al, 2011). These four nosocomial pathogens were therefore selected for screening the HCWs. Many factors, exogenous and endogenous, play a role in pathogen carriage upon exposure. The endogenous skin microbiota of the hand, is one of them. Through intra-specific ecological interactions, it is possible the hand microbiota can either mediate pathogen carriage or act as an effect modifier in the relationship between certain environmental factors and pathogen carriage. These potential roles the hand microbiota play in pathogen carriage are investigated in Chapter 4, entitled 'Healthcare Workers' Hand Microbiome at a Surgical Intensive Care Unit: Mediators or Effect Modifiers of Pathogen Carriage?'.

Lastly, this dissertation summarizes its key findings and addresses future directions for skin microbiome studies in public health, in its conclusions (see chapter 5).

Goal and Research Objectives

Overall, this dissertation uses laboratory techniques and ecological analyses within an epidemiological context, in order to characterize the dynamics of the microbial community structure of the hand microbiota of SICU nurses, and to assess the roles of the HCW hand microbiota in pathogen carriage.

Aim #1: To perform a literature examination of current and past research of human skin microbiota. To write a review that will summarize the skin microbiota, outline the potential determining factors driving its variability, posit the likelihood of an association between the resulting microbial community structure on the skin with disease outcomes among individuals, and finally, present some challenges and implications for studying the skin microbiome.

Aim #2: To assess the dynamics of skin microbial community structure within and between nurses over a three-week period, and to determine whether community structure varies by the current methods of specimen collection (i.e. glove juice and swabbing), and DNA extraction (i.e. enzyme cocktail and lysozyme only).

Hypothesis 1: The microbiome community structure on nurses' hands observed among sequencing replicates, using an Ion Torrent personal genome machine, is the same.

Hypothesis 2: The glove juice method of specimen collection captures greater microbial composition and diversity, likely to be more representative of the true hand microbiota, than the swabbing method. Moreover, the microbial community structure found on the nurses' hands is more similar within than between collection method.

Hypothesis 3: The diversity of the microbial community structure within individual nurses is lower than the diversity between all nurses. Additionally, the microbial community structure is not uniform within an individual nurse, nor across all nurses over time. The microbial community structure is more similar within individual nurses over time than between nurses.

Aim #3: To screen HCWs for known nosocomial pathogens via real-time qPCR, and determine whether (i) host demographic, behavioral, and environmental factors determine pathogen carriage, and whether (ii) hand microbiota is a mediator or an effect modifier in that relationship.

Hypothesis 1: Healthcare workers are not consistently colonized with known nosocomial pathogens, due to their high level of hand hygiene practice.

Hypothesis 2: Certain host demographic, behavioral, and environmental factors are associated with pathogen carriage among HCWs.

Hypothesis 3: Certain host demographic, behavioral, and environmental factors are associated with microbial community structure of the hand microbiota of HCWs.

Hypothesis 4: HCW hand microbiota is associated with certain pathogen carriage.

Public Health Significance

The advent of new technology has caused a fundamental shift in our ability to study native microbial communities, leading to substantial infectious disease epidemiology implications. Human microbiome studies, in general, give insight into whether changes in our communities of microbes can be correlated with changes in health. Studying the human skin microbiome, in particular, furthers our understanding of the microbial involvement in diseases and syndromes of the skin, their participation in transmission events, as well as advance therapeutic approaches, such as the implementation of pre- and probiotics.

Healthcare workers' hands are potential fomites carrying microorganisms between patients, causing healthcare-associated infections. Some hand microbiota are more or less resistant to the acquisition of certain nosocomial pathogens. Understanding the role of the hand microbiota in pathogen carriage and transmission is important to help identify effective hospital infection control policies.

Chapter 2: Skin Microbiota: Microbial Community Structure and its Potential Association with Health and Disease

Abstract

Skin, the largest human organ, is a complex and dynamic ecosystem inhabited by a multitude of microorganisms. Host demographics and genetics, human behavior, local and regional environmental characteristics, and transmission events may all potentially drive human skin microbiota variability, resulting in an alteration of microbial community structure. This alteration may have important consequences regarding health and disease outcomes among individuals. More specifically, certain diversity patterns of human microbiota may be predictive or diagnostic of disease. The purpose of this review is to briefly describe the skin microbiota, outline the potential determining factors driving its variability, posit the likelihood of an association between the resulting microbial community structure on the skin with disease outcomes among individuals, and finally, to present some challenges and implications for studying the skin microbiota.

Introduction

The skin is the largest human organ. As the skin is in direct contact with the environment, it is inhabited by and constantly exposed to microorganisms in the environment. The resident skin microbiota interacts with other microbes, with human cells, and with the human immune system in multiple ways that mediate risk of disease (Wilson, 2005; Wilson, 2008). The purpose of this review is to briefly describe the skin microbiota, outline the potential factors driving its variability, posit the likelihood of an association between the resulting microbial community structure on the skin with disease outcomes among individuals, and finally, to present some challenges and implications for studying the skin microbiota.

For many decades, researchers have been interested in defining the microbial inhabitants of human skin, focusing on descriptive features such as their association with infection (McBride et al, 1977), their stability over time (Evans, 1975), and their interactions with other microbes (Wright and Terry, 1981). Currently, our understanding of the human microbiota is undergoing a dramatic reassessment. The application of high-throughput DNA sequencing to the collection of individual genomes of microorganisms which normally inhabit the human body (the 'microbiome') (Peterson et al, 2009) enables characterization of microbial communities in addition to individual microbes. These new studies are using analytic methods from community ecology to describe the structure of the entire microbial community. Community ecology seeks to understand what determines the presence, abundance, and diversity of species in communities, focusing on the role of interactions among multiple species. We are just beginning to use ecological parameters to

explore the effects of microbial community structure on disease dynamics within a single host species.

Although there have been some initial reports characterizing the skin microbiota (Dekio et al, 2005; Fierer et al, 2008; Gao et al, 2007; Grice et al, 2008; Grice et al, 2009), most studies to date have focused on the gastrointestinal microbiota. In this system, the role of microbiota diversity in health and disease is unclear. For example, greater fungal richness and diversity were observed in 31 patients with Crohn's disease as well as 26 patients with ulcerative colitis compared to 47 controls (Ott et al, 2009). By contrast, among 3 patients with recurrent antibiotic-associated diarrhea due to *Clostridium difficile*, bacterial diversity was lower in the fecal microbiome compared to that found among 7 controls (Chang et al, 2008). The sample size of these initial studies are small, and the results do not give a clear picture of whether more or less microbial diversity in the gut is advantageous to the human host. Studies on other microbiota of clinical and general interest, including the oral, urogenital, and skin microbiota (McGuire et al, 2008), also do not show a consistent association between diversity and health and disease. It is still too early to predict whether certain microbial diversity patterns are good or bad, much less whether they cause disease. What is clear is that these patterns are highly complex and dynamic, and require ecological analytic approaches to characterize the microbial communities.

Skin is particularly interesting to study with an ecological approach because of the complexity of its ecosystem. It is composed of an intricate system of cell layers, nerves and glands, protecting the body against extreme environmental conditions, harmful chemicals

and pathogens. Keratinocytes, which form the outermost layer of cells on the skin, release antibacterial substances that help prevent infection. Skin also harbors a plethora of different groups of microorganisms that make up the human skin microbiota. Properly characterizing this microbiota has important clinical implications due to its interaction with other microorganisms that may play a role in human disease.

Most studies of skin microbiome have concentrated on characterizing the community structure of microbes inhabiting healthy human hosts or in examining "how particular bacteria become pathogenic" (Chiller et al, 2001; Cogen et al, 2007; Fierer et al, 2008; Gao et al, 2007; Grice et al, 2008; Grice et al, 2009). Though dermatological studies have long since shown associations between a number of skin infections and microbes (Masenga et al, 1990; McBride et al, 1977; Nakabayashi et al, 2000), most have been done using culture-based approaches. Aside from some studies comparing microbial composition between healthy adults and patients with psoriatic lesions (Gao et al, 2008), atopic dermatitis (Dekio et al, 2007), or acne (Bek-Thomsen et al, 2008), there is a surprising lack of literature evaluating potential associations of skin microbiota with health and disease, especially non-dermatological, systemic disease, using molecular approaches. In particular, the role of skin microbiota disturbance on the risk of infectious disease transmission, have not been explored.

Figure 2-1 describes our conceptual framework for understanding the interactions between skin microbiota, the human host and environment, and the resulting impact on human health outcomes. Significant and potentially harmful alterations of the skin microbial community structure may occur as a result of several factors, including (1) the

transmission (dispersal) of non-resident microorganisms into the microbiota, or the removal of dominant microorganisms from the microbiota, both resulting from direct human contact, (2) behavioral characteristics of the individual, such as handwashing practices, (3) local and regional environmental factors, such as the host skin condition and indoor settings, respectively, (4) host genetics, (5) and, host demographic characteristics. Behavioral and environmental characteristics, as well as host genetics and demographics, however, also all have their own direct effects on health outcomes, possibly by affecting host immunity. All of the driving factors included in the conceptual model interact to some degree, as noted by the two-directional arrows (Figure 2-1). For example, host demographics (e.g. gender) may interact with behavioral characteristics (e.g. cosmetic use) to influence the microbial community structure found on the hands.

It is generally accepted that host demographics and genetics, human behavior, certain environmental characteristics, and transmission events can all influence risk of disease. One question that has not been addressed, however, is to what extent these relationships are mediated through the microbial community present on the human body, specifically, the skin. Disturbance of skin microbiota, caused by the various driving factors listed in Figure 2-1, may influence the course of various disease states.

This review aims to summarize what is currently known about the skin microbiota, the methodological issues regarding how we have come to know it and what needs to be further explored (e.g. temporal dynamics), followed by a summary of each of the determining factors, shown in Figure 2-1, to be driving human skin microbiota variability.

Microbial Community Structure of Human Skin

Skin Microbiota

Humans are usually born from an essentially sterile environment (Hrncir et al, 2008; Stecher and Hardt, 2008), but quickly become colonized by microbes. Which microbes become established is primarily driven by the mode of delivery, with vaginally-delivered babies having a microbiota more similar to their mother's vaginal microbiota, and C-section babies having a microbiota more similar to their mother's skin microbiota (Dominguez-Bello et al, 2010). Bacteria and other microorganisms from the environment subsequently interact with the infant's epithelial cells leading to microbial colonization and co-existence. Eventually, an increasingly complex ecosystem forms, comprised of endogenous, or resident, and transient microorganisms (Tlaskalová-Hogenová et al, 2004). These include bacteria, viruses, fungi and protozoa. Humans harbor more microbial cells in their mucosal surfaces and skin than mammalian cells in the entire body (Foxman et al, 2008). While many of them are beneficial, commensal or neutral, some can still become pathogenic (Chiller et al, 2001). It remains to be demonstrated whether the potentially pathogenic members of the microbiota are kept in check by other resident microorganisms. Disruptions by antibiotics, handwashing or lotions may alter the microbial community enabling overgrowth by pathogenic members which then interact with the host causing disease. Additionally, it has been argued that what is considered solely a commensal or a pathogenic organism depends on the profile of the human immune system rather than "the inherent properties of the microbe" itself (Cogen et al, 2007).

The membership of the skin microbiota is quite diverse. A survey of twenty distinct skin sites of ten healthy volunteers using 16S rRNA gene phylotyping, identified 19 phyla

and 205 genera (Grice et al, 2009). Using broad-range 16S rRNA genes, PCR-based sequencing of randomly selected clones identified 8 phyla and 91 genera from the superficial volar forearms of six healthy subjects (Gao et al, 2007). In this study, *Actinobacteria*, *Firmicutes*, and *Proteobacteria* accounted for 94.6% of the clones. Using a pyrosequencing-based method, palmar surfaces of the hands of 51 healthy young adult volunteers were surveyed, and shown to harbor more than 25 phyla (Fierer et al, 2008). Of note, the same three phyla accounted for 94% of the sequences in this study. A more comprehensive "whole-body" survey, using a multiplexed barcoded pyrosequencing approach, of 27 body sites (including up to 18 different skin sites) among healthy adults, identified the same three phyla to account for over 82% of the sequences (Costello et al, 2009). According to a recent review of the cutaneous microbiota, "*Staphylococcus*, *Corynebacterium, Propionibacterium, Micrococcus, Streptococcus, Brevibacterium, Acinetobacterium*, and *Pseudomonas*" were named as human skin bacterial residents (Cogen et al, 2007). Many are now emerging as multidrug-resistant pathogens, such as *Staphylococcus aureus* and *Staphylococcus epidermidis*. (Marshall et al, 2008; Sommer et al, 2009). Better characterization of the human skin microbiota as "an antibiotic resistance reservoir" has tremendous clinical implications (Sommer et al, 2009).

Most viruses of eukaryotic organisms are not long-term residents on the skin but some "can proliferate within the living epidermis" (Kampf and Kramer, 2004). Only recently has the identification of two commensal viral groups, anelloviruses and GBV-C, alerted attention to the likelihood of a larger human virome (Delwart, 2007). The degree to which other viruses, such as the human papillomavirus and the Merkel cell polyomavirus are endogenous to the skin microbiota, is yet to be fully determined (Singh et al, 2009).

Viruses (e.g. hepatitis C virus, rhinovirus, adenovirus, and rotavirus) on the hands have been detected, however, usually as a result of transient hand carriage due to contamination or transmission events (Kampf and Kramer, 2004). Currently, these viral communities are not considered part of the human skin microbiota. The mycological and macroparasitic microbiota of healthy human skin is poorly characterized in comparison to its bacterial and viral counterparts, probably as a result of their rarity and asymptomatic nature (Cogen et al, 2007). Most fungal organisms belong to the genus *Malassezia*, formerly known as the yeast *Pityrosporum* (Paulino et al, 2006). Mites, such as *Demodex folliculorum*, are also "considered part of the normal" microbiota (Fredricks, 2001).

The presence of some microorganisms in the skin microbiota may have an effect on the growth of potential pathogens that may prompt various diseases, indicating the importance of interactions among species (McBride et al, 1977; Selwyn, 1975). For example, sealing certain skin abrasions with band-aids or other hermetic barriers may promote an overgrowth of potentially pathogenic anaerobes, causing a detrimental alteration of the microbiota. *S. aureus,* once believed to be a "transient colonizer during abnormal conditions", is now known to be a resident bacterium that somehow turns pathogenic upon disturbance of the individual’s skin microbiota (Fredricks, 2001; vanBelkum et al, 2009). Our growing knowledge of skin immunogenetics in the past few years has improved our understanding of the interactions among commensal and potentially pathogenic species (Bowcock and Woodson, 2004; Pivarcsi et al, 2005), however these relationships are not fully understood in terms of microbial communities.

Methodological Issues in Skin Microbiota Studies

Dependence on Sampling Methods and Laboratory Techniques

A representation of the microbiota found on human skin is only as accurate as the sampling methods used to harvest the microorganisms. While swabbing is the most convenient and innocuous of the three, it may not correctly estimate the true microbial diversity across all the skin layers. In comparison, skin scraping picks up more microorganisms per sampled area, but also picks up more skin cells. Punch biopsying, on the other hand, is thought to represent a more comprehensive microbiota since the technique samples across dermal layers (Grice et al, 2008). However, it is the most invasive and covers less surface area in comparison to other methods. Grice and colleagues have compared these sampling methods and concluded that all three yielded the same predominant phylum (i.e. *Proteobacteria*), and shared over 97% of all bacterial sequences; moreover, all three methods captured very similar bacterial community memberships and structures, as estimated by the high abundance-based Jaccard and Theta (θ) similarity indices, respectively (Grice et al, 2008). In terms of transmission, skin surface sampling of the hands, either by inserting them in a plastic bag filled with buffer solution or by swabbing them, may be the most informative since microbial transmission by humans occurs mostly via direct contact with other individuals and/or environmental surfaces. Estimates of microbial community structure vary by body site sampled. More exposed areas of the body may be composed of "higher proportions of transient microorganisms", in comparison to lesser exposed areas (Roth and James, 1988). Temperature, moisture level, and amount of sebaceous glands found on the skin vary by body location as well, and may affect where certain microorganisms are found (Grice et al, 2009; Roth and James, 1988).

What is known about human microbiota diversity also depends on the laboratory techniques (i.e. culture-dependent and culture-independent) used to characterize them. The classic approach to identify and quantify microorganisms from the environment has been to culture and differentiate them based on physiological and biochemical tests (Davies et al, 2001; Ogunseitan, 2005). However, culture-dependent methods do not accurately reflect the true bacterial community composition because of the selective properties of the growth media used. Culture-dependent techniques are costly and take time as a result of performing the necessary laboratory tests. After several passages, the microorganisms under study may even behave differently functionally and physiologically. Additionally, some microorganisms will not grow in the absence of others that could be required to provide optimal oxygen, pH, and/or osmotic pressure (Kaeberlein et al, 2002).

Microbial cultivation in the laboratory poorly assess species composition and function in environmental samples. It is generally assumed that "less than 10% of existing microbial diversity in [natural] ecosystems can be accounted for by cultivation" methods (Ogunseitan, 2005). Dekio and colleagues took swab-scrubbed forehead skin samples of five healthy volunteers and analyzed their microbiota using a culture-dependent and a culture-independent method, providing a direct comparison of the two characterization methods (Dekio et al, 2005). Analyses of 16S rRNA gene sequences obtained from the culture-independent method yielded an increased bacterial diversity compared with that derived from the culture methods (Dekio et al, 2005). Culture techniques, therefore, have major limitations in estimating species abundance in natural ecosystems.

Culture-independent microbial DNA-based approaches escape some of these limitations (Ogunseitan, 2005; Theron and Cloete, 2000). Some of these techniques, each having their own relative strengths and limitations, include: 16S rDNA sequencing, PCR and PCR-related techniques, nucleic acid hybridization techniques, polymorphism-based procedures, signature lipid biomarkers, protein profiles, and molecular microarray procedures. All bacteria contain the 16S rRNA gene, which encodes the small subunit of the RNA of the ribosome (i.e. the protein manufacturing machinery of all living cells). It encompasses highly conserved sequence domains interspersed with more variable regions. Identification of bacteria commonly uses the 16S rDNA sequence: conserved regions classify higher taxa, and variable regions differentiate between species. Different variable regions (V1-V9) of the 16S rRNA gene are targeted in different studies of the human skin microbiome, such as the V2 variable region (Costello et al, 2009; Dominguez-Bello et al, 2010; Fierer et al, 2008; Fierer et al, 2010) and the V1-V3 region (Dekio et al, 2005l Dekio et al, 2007). To date, there is no consensus of optimal variable region(s) to target for taxonomic assignment purposes at the genus level or below. Despite the fact that commonly used primers targeting these regions match most of the sequences in most databases, primer biases may still occur where certain phylotypes are missed, thereby generating biased community profiles (Hamady and Knight, 2009).

Though these robust techniques offer a much higher resolution to the characterization of complex microbial communities, they also possess several biases. Nucleic acid extraction and purification, for example, require proper microbial cell lysis and absence of enzymatic inhibitors. PCR techniques can generate artifacts such as chimeras, primer-dimers, and mutations that can lead to a false representation of microbial diversity,

and thus require specific and well-designed primers, appropriate nucleic acid starting quantities, and potential PCR elongation time troubleshooting. Another significant limitation to using culture-independent approaches is the inability of the techniques to identify viable microorganisms from samples, leaving unexplained whether the diversity obtained reflects true transients or residents of the skin, or whether they were simply dead contaminants retained on the skin.

The viral portion of the human metagenome, the virome, is more poorly characterized than the bacterial portion. Culture-based approaches suffer from the inability to replicate certain viruses *in vitro* and the difficulty in establishing viral antigenic/serological cross-reactivity (Delwart, 2007). Culture independent methods, such as shotgun library sequencing and high-throughput pyrosequencing, are also done, though the relatively few numbers of known viral sequences available make it difficult to identify viruses (Delwart, 2007).

Capturing Temporal Dynamics

Temporal patterns of microbial community structure can be extremely dynamic. Species composition can vary from one time point to another, with irregular cycles. Understanding these patterns is important for investigating associations between the human microbiome variability and health and disease, and ultimately, for determining whether a core human microbiome exists. Identifying a core microbiome for human skin would assist clinical applications, as diagnostic or prognostic factors may depend on recognizing significant deviations from the core. Changes in microbial community structures beyond what is expected over time may indicate an altered physiological state

conducive to disease. However, the existence of a core human skin microbiome remains to be determined because at present, the dynamic nature of the skin microbiota has not been adequately characterized.

To date, studies that have characterized the microbial profile of the skin through time, rarely involve more than a handful of intervals (Table 1). Overall, despite the fact that the time variation ranged from a couple hours to ten months in these studies, the skin microbiota was found to be relatively stable. However, these studies are not consistent with each other regarding the health of individuals sampled (e.g. healthy, psoriasis patients, dermatitis patients), the skin site sampled (e.g. facial skin, palmar surfaces), the sampling method (e.g. swab, scrape, scrub-swab), and the method of detection (e.g. pyrosequencing, RFLP analysis, clone libraries) used to characterize their microbiota. Any conclusions about the diversity and/or stability of microbial communities are highly dependent on these sampling issues as well as the taxonomic level analyzed. The inconsistencies between skin microbiota studies make it difficult to generalize results regarding temporal and spatial dynamics of human skin microbial communities.

To obtain a complete understanding of the temporal dynamics of the skin microbiota, it is necessary to capture the community structure at several time points. Figure 2-2 illustrates the difficulty in determining this dynamic profile. Panels A and B show how sampling (represented by the stars) at two or three different time points may not represent the true variability (represented by the curves) of the microbiota within an individual, and may lead to the conclusion that there is no variability (A) or that some external factor (e.g. treatment) may have contributed to the decrease in microbial diversity

in time (B). In reality, the full scope of the variability within an individual can only be determined when sampling is done at sufficient time points (Panel C). Only then, is it possible to speak of an average microbial community structure (represented by the line) per individual (Panel D). Another review recently stated that microbial communities have been thought of as stable because their "temporal variability is lower than inter-individual differences" (Dethlefsen et al, 2006). However, comparisons between the skin microbiota of an individual with another need to be done once the true variability within an individual is known, in order to establish any significant inter-individual differences. Interestingly, by using hierarchical distance-based metrics, Costello and colleagues have shown that human microbial communities cluster first by body site, followed by individuals then time (Costello et al, 2009).

To understand the dynamics of the microbial community structure processes, researchers examine the human microbiota over time and space, and the interconnectedness within and between individual hosts, respectively (Foxman et al, 2008). Skin microbial communities have been shown to display specific spatial patterns, with similar communities grouping together at left and right sides of the body, at regions close to the head, and at regions close to the arms (Costello et al, 2009). It has been suggested that, in general, skin sites in closer proximity appear to contain more similar microbial communities than other more distant skin sites (Fierer et al, 2008). Furthermore, certain bacterial phylotypes are shown to predominate moist, sebaceous, and dry skin regions differentially (Grice et al, 2009). Accounting for these spatial differences is important when attempting to capture temporal dynamics among and between individuals. While many studies tend to emphasize spatial and temporal distribution patterns of

microorganisms in a specified ecosystem, further explanations of how and why such patterns arise are still largely missing. Human skin microbiota diversity is thought to arise via the many factors depicted in Figure 2-1 and explored below. It remains to be seen whether any of such drivers of human microbiota diversity act to influence the development of health outcomes.

Driving Forces of Human Skin Microbiota Diversity

Transmission

Studies that have examined transmission of skin microorganisms often focus specifically on pathogenic microorganisms for the purpose of preventing infectious disease transmission, especially in healthcare settings. Unfortunately, few studies describe transmission of non-pathogenic microorganisms among healthy (and non-healthy) individuals. Additionally, the issues of whether there may be mutualistic relationships between pathogenic and commensal microorganisms that enhance transmission, or antagonistic relationships that minimize acquisition have not been fully assessed. As shown in Figure 2-1, the role of transmission in influencing the microbial community structure of resident human skin microbiota is very important.

Inter-species interactions can greatly influence the presence of microorganisms within a community. In the skin, for instance, *P. acnes* and *S. aureus* have been implicated in working synergistically to increasingly make worse skin lesions caused by one bacterium alone (Lo et al, 2010). Antagonistic interactions also occur, due to competition or predation (Little et al, 2008). For example, *S. aureus* and *S. epidermidis* are known to have competitive behaviors on the skin, which could be explained by the serine protease

Esp (Iwase et al, 2010) secreted by *S. epidermidis*, and possibly regulated by its agr pheromones (Otto et al, 2001). As discussed in detail by Chiller and colleagues, "bacteriocin and toxic metabolite production, induction of low reduction-oxidation potential, nutrient depletion, and inhibition of adherence and translocation" are just a few of the mechanisms used by bacteria that allow them to interact in the same community (Chiller et al, 2001). For example, bacteriocins, which are toxins produced by certain bacteria (e.g. *lactobacilli, propionibacteria*), are able to inhibit the growth of other, potentiallymore pathogenic bacteria (e.g. *staphylococci*) (Klaenhammer, 1993; Oh et al, 2006). Among those with damaged skin, certain bacteriocin producers proliferate and dominate the bacterial community (Roth and James, 1988). Novel bacteriocins are being identified at a growing pace (Martin-Visscher et al, 2008; Sawa et al, 2009; Tiwari and Srivastava, 2008). The bacteriocin nisin, from *Lactococcus lactis*, has been shown to reduce the clinical signs of mastitis, which is generally caused by a Staphylococcal infection (Fernandez et al, 2008). Indirect inter-species interactions also occur through the engagement of the host immune system (Chiller et al, 2001). Viral infection, for example, causes alterations on epithelial cell surface receptors (Roth and James, 1988).

Transmission via direct contact with other individuals or indirectly with fomites or water droplets found in the environment introduces transient microorganisms that may have the potential to alter the dynamics between resident microorganisms of the skin microbiota. Moreover, movement patterns of daily living may have an effect on microbiota, in its ability to enhance transmission probabilities. For instance, the number of people living in close contact with an individual and their networking patterns, the individual's

commuting practices, occupation and leisure pursuits, and interchanges with schools or child-care facilities all could influence the spread of skin microbes.

Host Demographic Characteristics

The microbial communities present on skin are determined by skin conditions, the host's hormonal status, age, gender, and ethnicity (Figure 2-1) (Fierer et al, 2008; Fredricks, 2001; Grice et al, 2009; Roth and James, 1988). In terms of skin conditions, overall the skin is cooler than core body temperature, and has a pH around 5, although it varies by body site (Chiller et al, 2001). Several molecules synthesized by the skin can contribute to skin surface conditions, which for the most part have the ability to discourage microbial growth. Even host gender shapes skin environment, thereby influencing what is able to colonize men and women. Women have been shown to have significantly greater bacterial diversity on their hands in comparison to men (Fierer et al, 2008). Though there have been reports of differences in carriage rates of microorganisms between races, there is still much to learn regarding the diversity of microbiota across a wide range of cultures and ethnicities (Evans et al, 1984; Mai and Draganov, 2009; Sultana et al, 2003). Explanations for age and gender related differences may include differences in hormones, sweat or sebum production, skin pH differences, and interactions with host behavior. For example, a plausible explanation for women having greater bacterial diversity on the skin of their hands may be that they likely have more contact with children, who commonly experience a high burden of common infectious diseases. Also, women may be more likely to use cosmetics, thereby altering the microbial community structure of their skin. A survey looking at potential associations between demographic information of neonatal intensive care unit nurses and the total microbial composition found on their hands,

showed that while age had a minimal effect, race was shown to be "a significant predictor of skin health" (Sultana et al, 2003). The authors note, however, the limited variation in age among the nurses surveyed.

Host Genetics

Apart from skin's structural cell layers and synthesis of molecules that influence microorganism proliferation, its immunological machinery may also play a role in skin microbial community structure. The innate immune system of the skin, now known to be important in regulating the microbiota at multiple epithelial surfaces, contains Langerhans cells, T lymphocytes, mast cells, and keratinocytes, which expresses Toll-like receptors and produces cytokines, chemokines, β-defensins, Rnase7, and other antimicrobial peptides (Pivarcsi et al, 2005). The skin-associated lymphoid tissue (SALT) has the ability to produce and secrete immunoglobulins, present antigens and activate T-cells, which can affect the composition of the microbial communities. Conversely, certain skin microbial residents are known to affect the host immune system. For instance, *S. epidermidis* has recently been shown to upregulate the expression of antimicrobial peptides in keratinocytes (Wanke et al, 2010). Immunogenetic components of the skin, such as the human leukocyte antigen (HLA) gene cluster, are shown to have associations with certain skin diseases, such as psoriasis (Bowcock and Woodson, 2004) and ashy dermatosis (Correa et al, 2007). It remains to be shown whether such associations are mediated by the microbial communities of the skin (Figure 2-1).

While genome-wide association studies (GWAS) have successfully identified many genetic variants to be associated with a number of human diseases, GWAS investigating

whether genetic variations in the human genome can influence microbiota composition are now emerging, owing in part to rapid advancements in sequencing technologies and bioinformatics. Commonly measured genetic variants include single nucleotide polymorphisms (SNP), non-SNP variants, and insertion-deletions (INDEL). Using a large murine intercross population, Benson and colleagues were able to show that host genotype does indeed explain some of the variation in the gut microbiota, controlling for environmental factors (Benson et al, 2010). Another murine model study looked for associations between the matriptase genetic variant, which led to filaggrin deficiency and atopic dermatitis phenotype, and skin microbiota (Scharschmidt et al, 2009). Scharschmidt and colleagues were able to show a significant alteration in the skin microbiota of these transgenic mice, in particular, a higher abundance of *Corynebacteria* and *Streptococci*, in comparison to their wild-type littermates (Scharschmidt et al, 2009).

Human Behavioral Characteristics

Behavioral factors such as the use of medications (e.g. antibiotics, steroids), hygiene practices (e.g. personal, domestic), and use of cosmetics (e.g. creams, lotions, emollients) have all been reported as having the ability to alter the microbial community structure of the skin (Figure 2-1) (Fierer et al, 2008; Fredricks, 2001; Grice et al, 2009; Larson, 2001; Larson et al, 2002; Roth and James, 1988). Other behavioral characteristics such as diet and nutrition, sun exposure, and smoking, are all considered contributing factors to skin and systemic health, however, their potential to influence the microbial community structure of skin has yet to be examined.

Hand washing has been long been considered to be the simplest and most effective method for controlling infectious diseases (Borges et al, 2007; Larson, 2001). Individuals' hands can be thought of as either fomites, by transiently carrying microorganisms, and/or as vectors, by harboring established, endogenous microorganisms that have the potential to be transmitted from one person to another. Despite the multitude of studies emphasizing the benefits of personal hygiene on reducing disease transmission by removing transients obtained by contamination (Aiello et al, 2008; Allengranzi and Pittet, 2009; Larson, 2001; Larson et al, 2004; Luby et al, 2005), the effects of hand washing on the microbial community structure of the hands is an area in need of more research. We still do not know the impact of hand washing on the longer term resident biota. However, such impacts have already been metaphorically equated to the disturbance caused by "hurricanes" and "forest wildfires" (Fredricks, 2001; Marris, 2009). Most reports of resulting microbial structure alterations rely on total bacterial colony-forming-units (CFU) in an attempt to explain disease causality. However, although hand washing is meant to remove transient microorganisms to either decrease self-inoculation (why we wash hands before eating) and/or transmission (why we wash hands after sneezing into them), researchers do not necessarily see a reduction in CFU counts after hand washing (Aiello et al, 2003). This may be a consequence of a microbial community structure disturbance whereby shedding of the skin reveals another layer of resident microorganisms. Realistically, diseases occur not just with an increase in bacterial loads, but also with an alteration in the microbiota of the individual and the resulting interaction with host immunity. Aside from reducing the number of transient microorganisms present on the skin, hand washing also has an impact on the skin condition itself, in altering the resistance

capacity of the stratum corneum (i.e. the electrical properties of the skin), lipids, transepidermal water loss, and pH, which could consequently affect the microbial community structure (Larson, 2001). Hand washing can be seen as a disturbance to the microbial community structure, possibly perturbing the existing trade-off between its microbial colonizers and competitors.

Individuals differ widely in their behavioral habits, which may have potentially meaningful consequences in altering the skin microbiota. Just in terms of hand hygiene alone, the frequency and duration of washes and type of soap product use (plain soap, antimicrobial soap, and/or alcohol sanitizers), can account for some of the variation in microbial community structure between individuals

Environmental Characteristics

Temperature, moisture, and exposure to ultraviolet radiation, are all known examples of environmental factors that can alter skin conditions and have the potential to influence the microbial community structure of the skin (Figure 2-1). For colonization to take place, microorganisms must adhere to a host by binding to specific receptors on the host epithelial cell, and have been shown to do so with varying affinities (Romero-Steiner et al, 1990). Though skin dryness may help to prevent the acquisition of certain transient microorganisms, consequent breaks on the skin surface may expose such receptors (Roth and James, 1988). Seasonality has been demonstrated in influencing diseases of the skin, likely as a result of microbiota alterations in response to climate changes (Jha and Gurung, 2006). Moreover, ultraviolet B (UVB) radiation, known to impact skin conditions, was observed to have disparate microbicidal effects on the skin microbiota (Dotterud et al,

2008). In particular, *S. aureus* appeared to be more sensitive to the radiation treatment than *S. epidermidis*.

Individuals are constantly being exposed to the microbial fluctuations of the indoor environment (Rintala et al, 2008). In addition, individuals differ widely in their occupational exposures (e.g. nurses, gardeners, teachers), which may also account for the variation between the community structures of their skin microbiota. For example, significant skin microbiota differences were observed between chronically ill outpatients and hospitalized inpatients, controlling for chronic illness as a potential confounder, which may indicate hospitalization as a potential driver of variability (Larson et al, 2000).

Just as the skin microbiota of humans harbor resident microorganisms, the physical environment (e.g. door handles, kitchen surfaces) surrounding the individual may be "reservoirs" for microbial colonization (Kagan et al, 2002). Even house dust has its own characteristic microbial composition (Maier et al, 2010; Rintala et al, 2008). Microbial communities within showerhead biofilms across the United States were found to contain opportunistic human pathogens (Feazal et al, 2009). These potential reservoirs likely increase the risk of microbial transmission, and thus the opportunity for disease. Concerns about the impact of environmental determinants of health are important, but their influence in altering the microbial community structure of skin microbiota, thereby resulting in adverse health outcomes, has not been sufficiently investigated. The fact remains that not much is known about the microbial composition of environmental settings (Feazel et al, 2009), nor how it influences the microbial community structure of skin.

The Impact of Microbial Community Structure on Health Outcomes

Underlying biological mechanisms explaining why an altered skin microbiota diversity may result in disease, thus explaining the arrow from 'species diversity / microbial community structure' to 'health outcomes' in Figure 2-1, include inflammation, absence of necessary members of the microbial community, and a decrease in microbial antagonistic interactions (Stecher and Hardt, 2008). Other possible mechanisms may consist of modifications to normal microbial signal transduction and quorum-sensing, resulting in cascades that may lead to damaging cellular changes in the host. Lateral gene transfer may allow skin microbiota to share functional roles, possibly eliminating redundant species and consequently impacting host health outcomes.

Given the many different ways in which the skin microbial community structure can be modified to potentially play a role in disease, it is clear that it is not the mere acquisition of a pathogen that causes disease, or hand washing that directly prevents disease, or that contaminated surfaces result in disease, or even that antibiotics eliminate disease. These events are all mediated by the resident skin microbial community structure of the individual. It is what happens to that microbial diversity that governs whether or not a host immune response is elicited, thereby establishing disease. In demonstrating that the skin microbiota is responsible for controlling cutaneous inflammatory responses, thereby protecting the host from unintended inflammatory diseases, Lai and colleagues provided evidence of a relationship between the microbiota, host immunity, and disease (Lai et al, 2009).

The microbial diversity present in and on humans is associated with several infectious and non-communicable diseases. Changes in resident microbial communities have been shown to be associated with skin conditions such as acne, atopic dermatitis, and psoriasis (Bek-Thomsen et al, 2008; Dekio et al, 2007; Gao et al, 2008). Even more broadly, The Human Microbiome Project has inspired exciting new studies demonstrating how changes in resident microbial communities play a role in disease, including antibiotic-associated diarrhea, bacterial vaginosis, human immunodeficiency virus, obesity and cardiovascular disease (Oakley et al, 2008; Ordovas and Mooser, 2006; Othman et al, 2008; Price et al, 2010; Young et al, 2008). Insights into the effects of resident microbial diversity of the human microbiota on health outcomes provide encouragement for further characterization of the skin microbiota.

Conclusions

Challenges in Assessing Multilevel Associations

Describing associations between the microbial community structure found on the skin and the health outcomes of individuals requires an integrative approach across several disciplines. Studying the human microbiota involves the fields of microbial ecology, population biology and microbiology. Further linking the skin microbiota to individual and population health outcomes also incorporates medicine, immunology, epidemiology and biostatistics. Thus, a comprehensive understanding of correlations between changes in the human microbiota and disease, with the consequent translation into public health benefits, requires an interdisciplinary endeavor.

Without an understanding of the normal range of microbial diversity within and between individual hosts, it is difficult to relate microbiota composition to disease status (Mai and Draganov, 2009). Further complication arises from recognizing that microbial diversity involves many levels: the microbial level (individual microbes as well as populations and communities of microbes), the individual level (host factors), and the human population level. Despite a number of ecological studies that assess population-level risk factors for disease, most epidemiological studies have traditionally looked at individual-level risk factors (Diez Roux and Aiello, 2005). Recently, however, it has become more apparent that focusing on the individual level does not account for other equally important health determinants such as the influence of social norms (a population level factor), like hygiene practices, on disease risk (Larson et al, 2004).

In any attempt to infer a causal association between human microbiota and disease, it is necessary to determine the risk of developing disease given the present microbial community on a host population. Therefore, another challenge, one effectively explained by Mai and Draganov, is the need for longitudinal studies with enough power to identify microbiota differences between groups despite the large variation that is likely to be observed within groups (Mai and Draganov, 2009).

Implications for Health

In this review, we have shown that skin, the largest organ of the human body, is normally colonized by a diverse community of microorganisms, some of which are potentially pathogenic under certain conditions. It is the continuing inter- and intra-species interactions of the microbial community, along with host immunity, that regulate

these conditions to avoid disease. An implication of this regulation is that when microbial community interactions are altered, certain microorganisms may become more easily dispersed and thus be more readily transmitted to another person or even oneself (i.e. autoinfection). Additionally, keeping the skin microbiota in check may allow the host immunity to be continually primed, so that in the event of disease onset, it is better equipped at controlling its progression.

We have also shown that the microbial community structure of the human skin is continuously influenced by microorganism dispersal, host behavioral characteristics, and the environment. These driving factors may lead to significant and potentially harmful alterations of the skin microbiota. The implication is that by manipulating the human skin microbiota community structure via its modifiable transmission-related, behavioral, and environmental pathways (Figure 2-1), disease could potentially be prevented or treated, especially given the recent advances in molecular technology. The most obvious example lies in the use of oral and topical probiotics, intended to limit the growth of pathogenic microorganisms while enhancing commensal ones (Krutmann, 2009; Ouwehand et al, 2003). Clinically, controlling the microbial community structure of the skin has the potential to decrease the rejection of viable skin grafts between individuals, as well as between different body locations within the same individual.

Identifying specific microbial community structure patterns of the human skin microbiota associated with disease will identify new potential intervention measures for improving health. It is anticipated that exploration of this new and different approach to human health will provide insights into disease etiology, management, and prevention.

Summary

Given the recent interest and technological advances in characterizing the human skin microbiota, it is important to learn whether certain diversity patterns or species composition of human microbiota are predictive or diagnostic of disease. A conceptual framework for understanding the interactions between skin microbiota, the human host and the environment is presented here in order to organize what host, dispersal, behavior, and environmental factors, or combination thereof, have the potential to drive the variability of the microbial community structure, thereby altering the skin microbiota diversity in such a way to cause disease.

Acknowledgements

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Figure 2-1. Conceptual framework of the driving forces behind the relationship between microbial species diversity / community structure of the human microbiota and health outcomes. Specific examples are shown as bullet points within each factor.

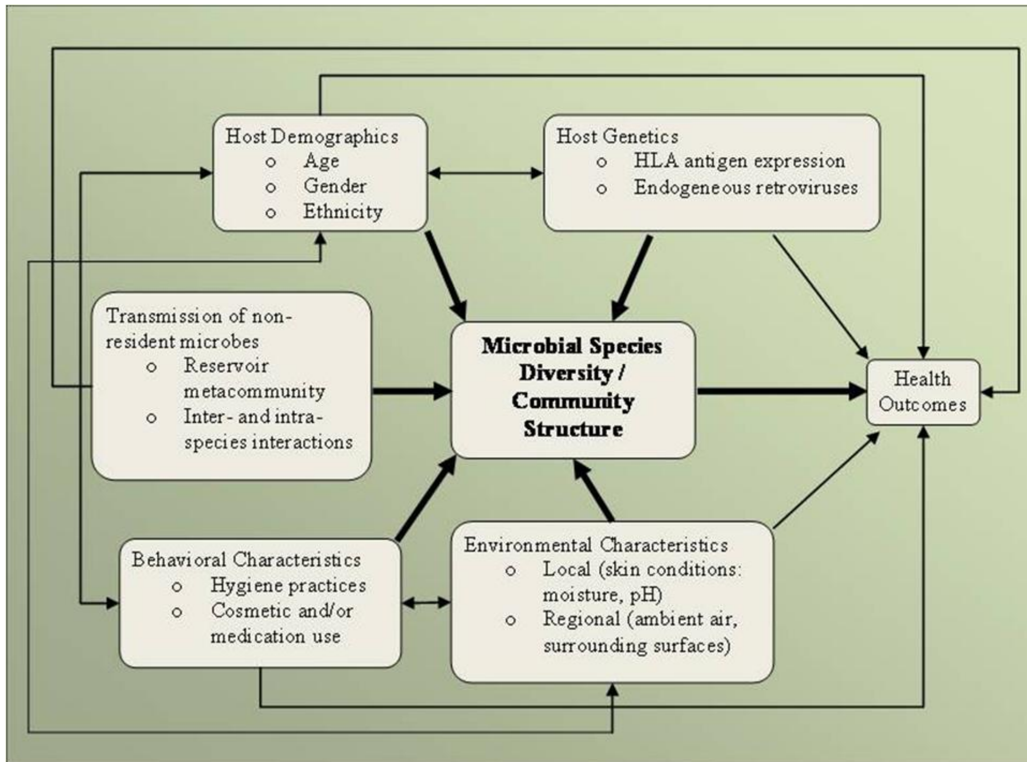

Figure 2-2. Schematic representations of the potential temporal variability of the human skin microbiome.

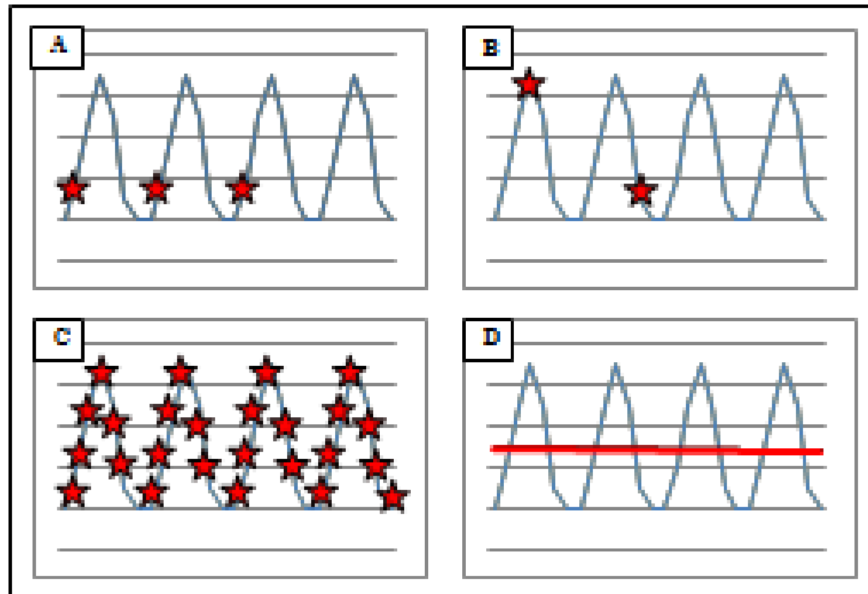

Table 2-1. A summary of selected skin microbiota studies that included temporal dynamics.

	Main Study Aim	Temporal Sampling Done
Costello et al, 2009 (Science)	Obtained an integrated view of the spatial and temporal distribution of the human microbiota from up to 27 sites in 7 to 9 healthy adults, using a multiplexed barcoded pyrosequencing approach.	Microbiota samples were donated on 17 and 18 June and 17 and 18 September 2008.
Dekio et al, 2007 (J Med Microbiol)	Compared the skin microbiota profiles in 13 patients with atopic dermatitis and 10 healthy controls, using terminal RFLP analysis of bacterial 16S rRNA genes.	Sampled 2 atopic dermatitis patients twice over 7 days.
Fierer et al, 2008 (PNAS)	Examined the palmar surfaces of the hands of 51 healthy young adult volunteers to characterize bacterial diversity and to assess its variability within and between individuals, using a novel pyrosequencing-based method.	Swabbed the palms of 4 men and 4 women every 2 h for a 6-h period after hand washing.
Gao et al, 2007 (PNAS)	Examined the diversity of the skin biota from the superficial volar forearms of 6 healthy subjects, using 16S rRNA genes PCR-based sequencing of randomly selected clones.	Re-sampled 4 of the 6 subjects 8 or 10 months later.
Grice et al, 2009 (Science)	Characterized the topographical and temporal diversity of the human skin microbiome from 20 diverse skin sites of 10 healthy volunteers, using 16S rRNA gene phylotyping.	Collected samples 4 to 6 months after initial visit from 5 of the 10 healthy volunteers.
Paulino et al, 2006 (J Clin Microbiol)	Used molecular methods to identify the fungal species present in 25 skin samples from 5 healthy subjects (flexor forearm) and 3 patients with psoriasis.	2 samples from each forearm of 2 healthy subjects, obtained 10 months apart; 2 samples from same lesion of 1 patient, obtained 6 months apart.

Chapter 3: Hand Microbiome Dynamics Among Healthcare Workers in a Surgical Intensive Care Unit

Abstract

We assess the dynamics of skin microbial community structure of 34 health care workers from a single surgical intensive care unit over a short (3 week) time period, whilst taking into account the variability introduced by specimen collection, DNA extraction, and sequencing. Sample collection took place at 3 different time points. Only sampling collection method appeared to have a significant impact on the observed hand microbial community structure among the healthcare workers. Analysis of samples collected using glove juice showed hands within individuals were slightly more similar in microbial composition over time than hands between individuals. This was not true for samples collected using swab, where samples from a single individual were no more similar to each other than those among other individuals, suggesting they were essentially independent.

Introduction

The human skin is made up of dermal layers, hairs, nerves, glands, and a complex ecosystem of microorganisms, the microbiota. Next-generation deep sequencing techniques have made characterization of the microbiota rapid and economically feasible, leading to a surge of studies. From these studies, including those funded by the first phase of The Human Microbiome Project (HMP), we are gaining an increasingly complete picture of the skin microbiota. However, obtaining an accurate profile of the skin microbiota requires an assessment of the variation in biological patterns between individuals and within individuals over time. This is challenging because true biological variation can be obscured by technical variation due to (i) specimen collection technique, (ii) DNA extraction methods, and (iii) sequencing.

Earlier studies suggest that the composition of hand microbiota varies widely. A study of the right and left hands of 51 healthy young adults found an average of 158 unique bacterial phylotypes per hand: only 17% were shared within individuals and 13% between individuals (Fierer et al, 2008). A high level of intra-personal variability in hand microbiota was also found by Caporaso and colleagues, who compared the right and left palms of two individuals over several months: the phylotypes present on each hand were not significantly correlated (at the species level) (Caporaso et al, 2011). However, despite recognizing that the way in which skin samples are collected can impact the diversity of the microbiota (Grice et al, 2008), that DNA is more easily extracted from gram negative than gram positive cells (Salazar and Oriana, 2007), and that sequencing introduces errors in terms of obtaining an accurate profile (Schloss et al, 2011), to our knowledge, no metagenomic study of the human skin microbiome has determined the effect that these

technical sources of variation have on the true biological variability of the skin microbiome. We address this gap in this paper, while characterizing the hand microbiome of healthcare workers (HCW).

Understanding the biological variability of the skin microbiome of the hands of HCWs is particularly important for gaining insight into the role of microbiota in pathogen resistance and susceptibility, and the potential for transmission to others, which occurs among HCWs despite their generally elevated hand hygiene efforts. In this study, we assess the dynamics of skin microbial community structure of 34 HCWs at a surgical intensive care unit over a short (3 week) time period, whilst taking into account the variability introduced by specimen collection techniques, DNA extraction methods, and sequencing. Specifically, we compared: 1) a swab versus glove juice technique, 2) DNA extraction by lysozyme only versus enzyme cocktail, and 3) sequencing one replicate versus another.

Methods

Study Population

Healthcare workers were recruited from the University of Michigan Hospital Surgical Intensive Care Unit (SICU). This is a 20-bed critical care unit that specializes in patient recovery after major post-operative procedures (e.g. transplants, aneurysm repairs, resections, vascular endarterectomies, and amputations) or those requiring extensive physiological monitoring. The SICU also accommodates patients from other surgical units (trauma-burn, neurosurgery, medical, and cardiovascular). To qualify for inclusion, volunteers had to be a healthcare worker working at the SICU, and not have received topical or systemic steroids or antibiotics for a period of 3 months before the start of the

study. Physicians were excluded from the study due to their high mobility. The study was presented at staff meetings and the first 35 HCWs who met eligibility criteria and gave written consent were included in the study. One HCW was lost to follow-up prior to sample collection leaving a total sample size of 34. The study took place July 5-28, 2011. The study protocol was reviewed and approved by the institutional review board of the University of Michigan (IRBMed #HUM00042622).

Sample Collection

To minimize sample cross-contamination the study recruiters donned a new pair of sterile gloves prior to each sample collection. Negative controls consisting only of buffer solution (20 mM Tris pH 8, 2 mM EDTA, and 1.2% Triton X-100) were collected and analyzed for each sampling. The palm, fingertip surfaces, and in-between the fingers of the participant's dominant hand were swabbed using sterile cotton-tipped swabs soaked in the buffer solution. Swabbing was performed in two perpendicular directions to ensure that the maximum surface area was represented in the sample. Immediately after swabbing, the participant's dominant hand was inserted into a sterile, polyethylene bag containing 50ml buffer solution (0.07 M PBS, 0.1% Tween-80) and massaged through the wall of the bag for 1 minute. The buffer solution, here termed glove-juice, was then collected. All samples were stored at -20°C until further processing.

DNA Extraction, Purification and Amplification

All swab samples and the pellet of 1 ml of all glove-juice samples were lysed using enzyme cocktail (mutanolysin @ 160U/ml, Rnase A @ 0.07mg/ml, lysostaphin @ 0.16 mg/ml, and lysozyme @ 7mg/ml) for 30 minutes at 37°C. A subset of ten glove-juice

samples from the first collection visit were lysed using only lysozyme @ 20 mg/mL (per manufacturer's recommendations) for 30 minutes at 37°C. The standard protocol for lysing gram-positive bacterial cell lysates of the PureLink Genomic DNA kit (Invitrogen Corp.; #K1820-02) was followed for all subsequent steps, with an additional incubation at 95°C for 2 minutes, prior to the addition of 96-100% ethanol to the lysates. Purified genomic DNA were re-suspended in 50 µl of PureLink Genomic Elution Buffer and stored at -80°C until sent for sequencing.

DNA was tested for PCR competency, using the following procedure. The primers L-V6 (5'-CAACGCGARGAACCTTACC-3') and R-V6 (5'-CAACACGAGCTGACGAC-3') were chosen to amplify the V6 hypervariable region of the 16S rRNA gene (Hummelen et al, 2011). After extraction, 1 uL of the purified genomic DNA was used as template for a 25 uL PCR reaction on a MyCycler Thermal Cycler (Bio-Rad Laboratories, Inc.). The following PCR reactions were used: 22.5 ul of Platinum Blue PCR SuperMix (Invitrogen Corp., #12580-023) 1 ul of 10 uM primer pair, and 0.5 ul of water. PCR conditions included: 94°C for 2 minutes; 30 cycles of [94°C for 30 seconds; 55°C for 30 seconds; 72°C for 30 seconds]; and hold at 4°C. A negative control including all ingredients but with water instead of DNA template was included alongside all test reactions. A constant volume aliquot of each PCR amplification product was run on a 1.5% agarose gel to determine PCR competency as well as the approximate amount of product. 10-20 ul of the purified genomic DNA were sent for sequencing at The London Regional Genomics Centre at the University of Western Ontario (London, ON, Canada).

DNA Preparation for Sequencing

The bacterial V6 rRNA region was amplified with the left-side primer CWACGCGARGAACCTTACC and the right-side primer ACRACACGAGCTGACGAC. These primer sequences are exact matches to >95% of the rRNA sequences from organisms identified in the human microbiome project (GBG, unpublished observations). The left-side primers contained the standard Ion Torrent (Ion Torrent Systems, Guilford, CT, USA) adapter and key sequence at their 5′ end (CCATCTCATCCCTGCGTGTCTCCGACTCAG). The right-side primer had the other standard Ion Torrent adapter sequence (CCTCTCTATGGGCAGTCGGTGAT) attached to its 5′ end. Amplification was performed for 25 cycles in 40 μl using the colorless GO-Taq hot start master mix (Promega; #M5133) according to the manufacturer's instructions with the following three-step temperature profile: 95°C, 55°C and 72°C for 1 minute each step. 5 μl of the resulting amplification were quantified using the QuBit broad-range double-stranded DNA fluorometric quantitation reagent (Invitrogen Corp.; #Q32854). Samples were pooled at approximately equal concentrations and purified using a Wizard PCR Clean-Up Kit (Promega; #A9285).

DNA Sequencing and Sequence Reads Filtering

Sequencing reactions were carried out on three Ion Torrent 316 platform chips, multiplexing up to 96 samples per run using the 200 bp sequencing reagent kit. Data from all runs were pooled. The sequence was provided in fastq format. All sequences were filtered according to the following criteria in order: exact match to the left-side primer including redundant positions in the primer, exact matches to the barcodes used, an exact match to the first six nucleotides of the right-side primer, and a length between the left-side and right-side primer of between 71 and 90 nucleotides. This length was chosen because it

encompasses the predicted amplicon product size from all human-associated bacterial organisms that have been cultured and sequenced as part of the HMP. Table 1 shows the number of raw and filtered reads obtained from each run. Run number 3 had the least number of sequences because of sub-optimal loading efficiency. However, as the reproducibility of the Ion Torrent platform for these types of analyses is excellent provided the number of reads per sample is greater than 1000 (Allen-Vercoe et al, 2012), this was not a concern.

Between 46 to 71% of the reads passed these filters; reads not passing the filters were not examined further. Reads were processed as previously described (Gloor et al, 2010) except that clustering with USEARCH was performed at 97% identity. Chimera detection was performed with UCHIME (version v5.2.32) using the de novo method (Edgar et al, 2011). Chimeric sequences in less than 0.05% in any sample (see below) were discarded. A table of counts for sequences grouped at 100% identical sequence unit (ISU) identity level were generated for each sample (Gloor et al, 2010), keeping all sequences that were represented in any sample at a frequency >0.5%. Reads that were never abundant in any sample (<0.5%) were discarded.

Taxonomic Classification

Classification of the sequences by either the Greengenes or RDP classifiers proved to be unreliable because of the short length of the V6 region. Classification of the sequences present in the count table was therefore performed using the RDP closest match option on the full-length, high-quality, isolated subset. The 20 best hits were identified, and the taxonomic classification of the best match and ties was collected. The classification of those

hits was adopted for all levels where the classification was identical across all best matches, otherwise the classification was marked as undefined. The taxonomic classification was added to the sequence count table and the data were presented in formats that could be accepted by QIIME 1.5.0 (Caporaso et al, 2010) as follows. Sequence alignments were built using Muscle (Edgar RC, 2004) and a neighbor-joining tree was generated by ClustalW2 (Larkin et al, 2007).

Statistics

Quantitative Insights into Microbial Ecology (QIIME, version 1.5.0), an open source software package for comparison and analysis of microbial communities, was used to process data from the Ion Torrent sequence reads. Analyses included removal of chloroplast sequences to the development of heatmaps, taxonomic summaries of communities, computing of alpha diversities, rarefaction curves, jackknifed bootstrapping of beta diversities, hierarchical clustering, principal coordinate analyses, distance histograms, and ANOSIM. Rarefied operational taxonomic unit (OTU) tables were generated to compute measures of alpha diversity. Metrics computed were Chao1, which estimates the species richness; observed species, which counts the number of unique OTUs in a sample; Shannon index, which estimates the species diversity; and PD_whole_tree, which is a phylogenetic distance metric. Rarefaction curves, showing the alpha diversity versus simulated sequencing effort, were generated.

To compare the bacterial communities between groups, beta diversity metrics were calculated based on the UniFrac algorithm, which measures the community similarity based on shared branch length on a phylogenetic tree (Lozupone and Knight, 2005). To

remove sample heterogeneity and standardize comparisons so that sequencing effort does not influence diversity estimates, the OTU tables were rarefied. Weighted UniFrac dissimilarity matrices of each comparison group formed the basis for the distance histograms, distance boxplots, principal coordinate analysis (PCoA), and hierarchical clustering. The distribution of distances (weighted UniFrac) within one group was displayed in a histogram, and overlayed with the distribution of distances between groups. Boxplots comparing distances within and between groups were generated from the sets of weighted UniFrac distance matrices. Jackknife bootstrapping was performed to estimate the uncertainty in the PCoA plots, by first creating distance matrices off of rarefied OTU tables, computing principal coordinates on each rarefied distance matrix, and comparing principal coordinates plots from each rarefied distance matrix. Unweighted pair group method with arithmetic mean (UPGMA), a type of hierarchical clustering technique that uses average linkage, was done to determine whether any clustering was formed per comparison group. To measure the robustness of this result to the sequencing effort, a jackknife bootstrapping analysis was performed where a smaller number of sequences were chosen at random from each sample and the resulting UPGMA tree from this subset was compared with the tree representing the entire data set.

Statistical comparisons using a paired t-test were made using the first principal components of the PCoA plots to assess significant differences between the comparison groups. Analysis of similarity (ANOSIM), which is a modified version of the Mantel Test based on a standardized rank of correlation between two distance matrices, was done to statistically test for significant differences between the comparison groups.

Results

We assessed the dynamics of skin microbial community structure of 34 healthcare workers at a surgical intensive care unit over a 3 week time period while considering the variability introduced by sampling collection method, DNA extraction method, and sequencing [Figure 3-1]. Thirty-four HCWs (and negative controls) were sampled at 3 different time points by swab and glove-juice, resulting in 105 specimens each [Supplemental Materials]. A negative control for each time point consisted of either a swab buffer or glove-juice buffer alone. DNA from all samples was extracted using the enzyme cocktail. Additionally, the first 10 specimens collected via glove-juice from the first visit were also extracted using lysozyme only, and sent for sequencing. The DNA from the first 10 specimens at each time point, collected from both swab and glove-juice samples, were divided into two and sent for sequencing. During analysis, DNA sequence identity level was kept at 100% so that true differences between microbial communities could be assessed in the several comparisons that follow. Moreover, all comparisons were made within the same OTU dataset without stratification, so as to control for the variability observed elsewhere. The mean number of sequencing reads assigned to the OTU table was 6,514 per sample (min=4, max=77,185).

Comparison of Sampling Collection Method

At each visit (n_v=3, where v=visit), samples (n_p=34, where p=participant) were first collected via swabs and immediately after, via glove-juice, totaling 102 samples per collection method. Following DNA extraction, these were sent for sequencing. Initial analyses, such as alpha diversity, and bootstrapped UPGMA tree, suggested that the differences between the two methods were small [Supplemental Materials]. The total

average number of unique phylotypes obtained by glove-juice and swab was 129 and 125, respectively ($t=1.32$, $p=0.19$). Further investigation, however, revealed some important differences. These included a histogram comparing weighted UniFrac distances, and PCoA plots performed with jackknife bootstrapping [Supplemental Materials]. The 2D and 3D plots of the PCoA show clear clusters per sampling collection method [Figure 3-2]. ANOSIM results indicate a statistically significant difference between the two sampling collection method sets of weighted UniFrac distance matrices ($R=-0.2649$, $p<0.001$) [Table 3-2]. A scatterplot of the first principal component of the PCoA comparing both sampling collection methods show most coordinates falling to the right of the expected line ($y=x$), indicating that the two sets are not equivalent ($t=10.51$, $p<0.001$) [Figure 3-3]. Boxplots show that the mean weighted UniFrac distance between the two sampling collection methods is higher than the mean weighted UniFrac distance within either of the two methods, indicating a meaningful difference between them [Figure 3-4]. Moreover, the mean weighted UniFrac distance within the samples collected via glove-juice was higher than the mean weighted UniFrac distance within the samples collected via swab, indicating a more variable community composition.

Comparison of DNA Extraction Technique

To test whether DNA extraction techniques influence microbial community structure, the DNA of the first 10 glove-juice samples from the first visit was extracted using two slightly different methods. One method used lysozyme (20 mg/ml) only; the other, an enzyme cocktail comprising of mutanolysin (60U/ml), Rnase A (0.07mg/ml), lysostaphin (0.16 mg/ml), and lysozyme (7mg/ml). Both sets were sent for sequencing. At first, differences between both DNA extraction techniques appeared to be meaningful.

While the relative phylum abundance appeared to vary similarly by HCW, samples with DNA extracted with the enzyme cocktail consisted mostly of Proteobacteria, whereas those extracted with lysozyme only consisted mostly of Firmicutes [Supplemental Materials]. Differences were also noted on a histogram comparing weighted UniFrac distances, as well as on a bootstrapped UPGMA tree [Supplemental Materials]. Boxplots show that the mean weighted UniFrac distance between the two DNA extraction techniques is slightly higher than the mean weighted UniFrac distance within either of the two techniques, indicating a meaningful difference between them [Figure 3-4]. Additionally, the mean weighted UniFrac distance within the cocktail set was slightly higher than the mean weighted UniFrac distance within the lysozyme set, indicating a more variable community composition. However, further analyses suggest there may not be significant differences in microbial community structure introduced by the different DNA extraction techniques. The 2D and 3D plots of the PCoA fail to show clear clusters per DNA extraction technique [Figure 3-2]. ANOSIM results indicate no statistically significant difference between the DNA extraction technique sets of weighted UniFrac distance matrices ($R=0.0901$, $p=0.067$) [Table 3-2]. Rarefaction curves of phylogenetic distance show that the average alpha diversity is equivalent for both extraction techniques; and, PCoA plots performed with jackknife bootstrapping do not show any clusters by DNA extraction technique [Supplemental Materials]. A scatterplot of the first principal component of the PCoA comparing both DNA extraction techniques show most coordinates falling around the expected line ($y=x$), also indicating that the two sets are equivalent ($t=-0.68$, $p=0.5047$) [Figure 3-3].

Comparison of Sequencing Replicates

Duplicate sets of the first 10 samples from each visit (n=30) were sent for sequencing. Sequencing replicates had similar relative abundances of taxa, and equivalent average alpha diversity, indicating a consistent sequencing effort [Supplemental Materials]. The 2D and 3D plots of the PCoA do not show any clustering by replicate set [Figure 3-2]. Other tests, including a histogram comparing weighted UniFrac distances, bootstrapped UPGMA tree, and PCoA plots performed with jackknife bootstrapping, found no significant differences between the replicates [Supplemental Materials]. A scatterplot of the first principal component of the PCoA comparing both replicate sets show most coordinates falling around the expected line (y=x), indicating that the two sets are equivalent (t=0.36, p=0.7536) [Figure 3-3]. ANOSIM results indicate no statistically significant difference between the two replicate sets of weighted UniFrac distance matrices (R=0.0122, p=0.326) [Table 3-2]. Boxplots show that weighted UniFrac distances within replicate sets were similar, and no different than between replicate set distances [Figure 3-4].

Comparison of Between Versus Within Healthcare Worker

To assess the biological variability of skin microbial community structure within and between HCWs, we sampled participants at three time points. The weighted UniFrac distribution of distances within HCWs are slightly shifted from the distribution of distances between HCWs [Supplemental Materials]. Moreover, the mean weighted UniFrac distance between HCWs is slightly higher than the mean weighted UniFrac distance within HCWs [Figure 3-4]. These results compare between and within HCWs over time, among glove-juice and swab samples combined. However, since significant differences were observed between samples collected via glove-juice and swab, the same comparisons were stratified

by sampling collection method. The difference in mean weighted UniFrac distances within and between HCWs by sampling collection method, as shown by the boxplots of weighted UniFrac distances, is more pronounced among the glove-juice samples, where the mean weighted UniFrac distance between HCWs is much higher than within HCWs [Figure 3-4]. Furthermore, PCoA of the HCWs were stratified by sampling collection method, showing more clustering by HCW among the glove-juice samples, indicating that HCWs' hand microbiota are more similar to their own hands over time than to other HCWs at the same time [Figure 3-5]. A two-sample t test comparing weighted UniFrac distances within versus between HCWs, found a significant difference among the samples collected via glove-juice ($t=5.35$, p-value <0.0001) but not swabs ($t=1.43$, p-value $=0.1516$).

Discussion

Analysis of the microbiome of 34 HCWs tested weekly over 3 weeks showed variability between and within HCWs that could not be attributed to technical variation introduced by sampling collection method, DNA extraction technique, and sequencing. Our key findings are these. First, using swab samples HCWs' hands were likely as similar in microbial composition to themselves as they were to the hands of other HCWs in the study. However, using samples from glove-juice, microbiota was slightly more similar within HCW than between HCW. This is consistent with the study of Caporaso and colleagues, who tested swab samples from hands of two individuals sampled at over 396 time points. That study found high variability within an individual across time, as measured in days, weeks and months; and, no significant correlation between the species-level taxa presence on the right palm compared to the left (Caporaso et al, 2011). Although each HCW cared for, on average, one to two patients, and were thus likely exposed to different microbes, it may be

that their high level of handwashing and use of alcohol gel were sufficient to remove from their palms whatever would differentiate one HCW from another in terms of the microbiota gathered from their patients. It is infection control policy to perform hand hygiene upon leaving a patient's room. The increased similarity between glove-juice samples within a HCW may reflect the larger surface area surveyed providing more opportunities for differences between individuals to arise. Ours is the first study, to our knowledge, that has compared two different methods that have been used in the hand hygiene literature for identifying bacterial counts and pathogens on the hands, for assessing overall microbial composition. Skin microbiome studies of the Human Microbiome Project (HMP) mostly use swabs to characterize the microbial communities of the skin; and, not much is known regarding skin microbiota dynamics. Hand hygiene studies in healthcare setting generally use the glove-juice method, mostly for obtaining microbial loads for culturing. A comparison of the two sampling collection methods -- research that is lacking in the literature -- and the dynamics observed in each, is meaningful for bridging the two research fields. Using a taxonomic classification at 100% sequence identity, based on glove-juice samples (but not swab samples), HCWs were slightly more similar to themselves than they were to other HCWs over a short time period. However, the microbial community structure of the hand microbiota within a HCW over 3 weeks was as variable as between HCWs, suggesting that - at least for swab specimens - each sample may be essentially considered as a unique sample.

Second, the findings obtained using glove-juice were somewhat different from those found using swab. Glove-juice involves inserting a participant's hand inside a sterile plastic bag containing a buffered medium and massaging the hand from the outside of the bag for

one minute. It is termed the 'gold standard' for infection control as it provides a thorough collection of transient microbial contamination as well as whole hand and nail microbiota (Banfield and Kerr, 2005). If transmission is presumed to arise solely from direct contact, swabbing may provide adequate representation of the microbiota present. However, if transmission is thought to arise both from direct contact and from shedding of skin cells, then sampling via glove-juice would give a more complete picture of the potential for transmitting both transient and colonizing microbiota. Of note, we detected a higher proportion of *Staphylococcus aureus* and *Pseudomonas* spp. with the glove-juice method than with the swab method [Figure 3-6]. The incidence of infections acquired by patients in an intensive care unit (ICU) is a great public health concern. A 2007 study of the prevalence of infection in 1265 ICUs from 75 countries found that in patients with positive isolates, the most common organisms were *Staphylococcus aureus* (20.5%) and *Pseudomonas* spp. (19.9%) (Vincent et al, 2009). Thus, the glove-juice method may provide a better representation of the organisms of interest for hospital infections.

Third, not all methods of DNA extraction, a required step in all metagenomic studies of microbiomes, are equivalent. This could impact the true representativeness of the metagenomic study and the generalizability of results between studies (Weaver, 2012). Extraction method affected the beta diversity observed, however this was not consistently demonstrated, probably due to the low sample size (n=10) we had for comparison. A recent study using six different DNA extraction techniques to compare the microbial profiles of 11 bacterial species and a mock community comprised of all these species found that none of the techniques were accurate in describing the composition of the mock community (Yuan et al, 2012). However, they determined that protocols using bead

beating and mutanolysin (25KU/ml) together, best represented the true microbial community structure. We used a lower concentration of mutanolysin (160U/ml) in our enzyme cocktail, however the cocktail also contained Rnase A, lysostaphin, and lysozyme.

Fourth, we obtained the same results for duplicate samples sequenced in different runs using the Ion Torrent Personal Genome Machine (PGM) technology. This is a relatively new technology that has not been extensively implemented in microbiome studies. To our knowledge, despite there being a few papers describing this new platform's performance (Rothberg et al, 2011; Loman et al, 2012; Quail et al, 2012; Liu et al, 2012), only one other metagenomic study of human microbiome has been published to date using this platform (Jünemann et al, 2012). This study is the first skin microbiome study to compare microbiome samples to themselves in order to assess technical variability introduced by the Ion Torrent PGM.

Hands, intrinsically, are constantly exposed to contaminants in the environment. Oversampling of transients picked up from the environment may have been more apparent among swab samples. Glove-juice samples were likely more representative of the HCWs' endogenous hand microbiota. Overall, there was a positive correlation between the microbial community structure observed from both sampling methods. This may suggest that the microbiota detected by the swabs is a subset of the microbiota detected by the glove-juice method. Further work is needed to establish whether the microbiota detected by swabs are indeed nested within the microbiota detected by glove-juice. A limitation of this study may be that our sample size, 34 participants sampled at three points in time, may still not be sufficient to accurately determine the short-term stability of hand microbiota.

Additional samples comparing DNA extraction techniques would also have proven beneficial. However, we feel that if there is an effect, it is small given that we were able to account for known sources of technical variability (e.g. sampling collection, DNA extraction technique, and sequencing). In addition, it would have been preferable to have had the HCWs perform the same hand hygiene protocol before sampling. However, the high frequency of overall hand hygiene per work shift reported among the participants do not suggest that keeping their handwashing and alcohol rub use constant would have prevented any meaningful confounding.

In conclusion, analyses of the microbiota found on HCWs' hands indicate that the dynamics of the microbial community structure is dependent on sample collection method. Using the glove-juice method, hands from within an individual were slightly more similar in microbial composition over time than between individuals. Using swab, samples from a single individual were no more similar to each other than those between individuals. Other sources of technical variation assessed, specifically DNA extraction techniques and sequencing, were not influential to the microbial community structures.

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Figure 3-1. Study Design Showing Levels of Comparisons of Hand Microbiota Samples Sent for Sequencing, from 34 Healthcare Workers at the University of Michigan Surgical Intensive Care Unit, July 5-28, 2011. Level A shows the comparison of within versus between HCWs (n_1=34, n_2=34, n_3=34); level B shows the comparison of sampling collection methods (n_{SW}=102, n_{GJ}=102); level C shows the comparison of sequencing replicates (n_1=30, n_2=30); and, level D shows the comparison of DNA extraction methods (n_C=10, n_L=10).

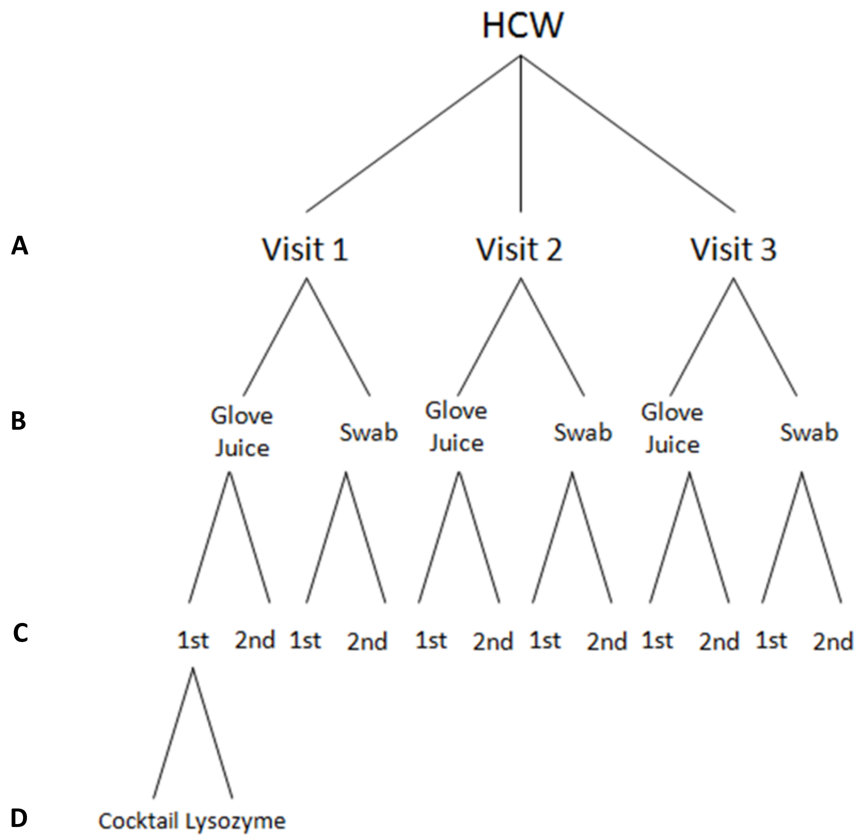


Figure 3-2. 2D and 3D Principal Coordinate Analysis (weighted UniFrac) of the Hand Microbiota from 34 Healthcare Workers at the University of Michigan Surgical Intensive Care Unit, July 5-28, 2011. Stratification by Sampling Collection Method (Panel A: Glove-Juice (red) and Swab (blue)), DNA Extraction Method (Panel B: Lysozyme (blue) and Cocktail (red)), and Sequencing Replicates (Panel C: Set #1 (blue) and Set #2 (red)).

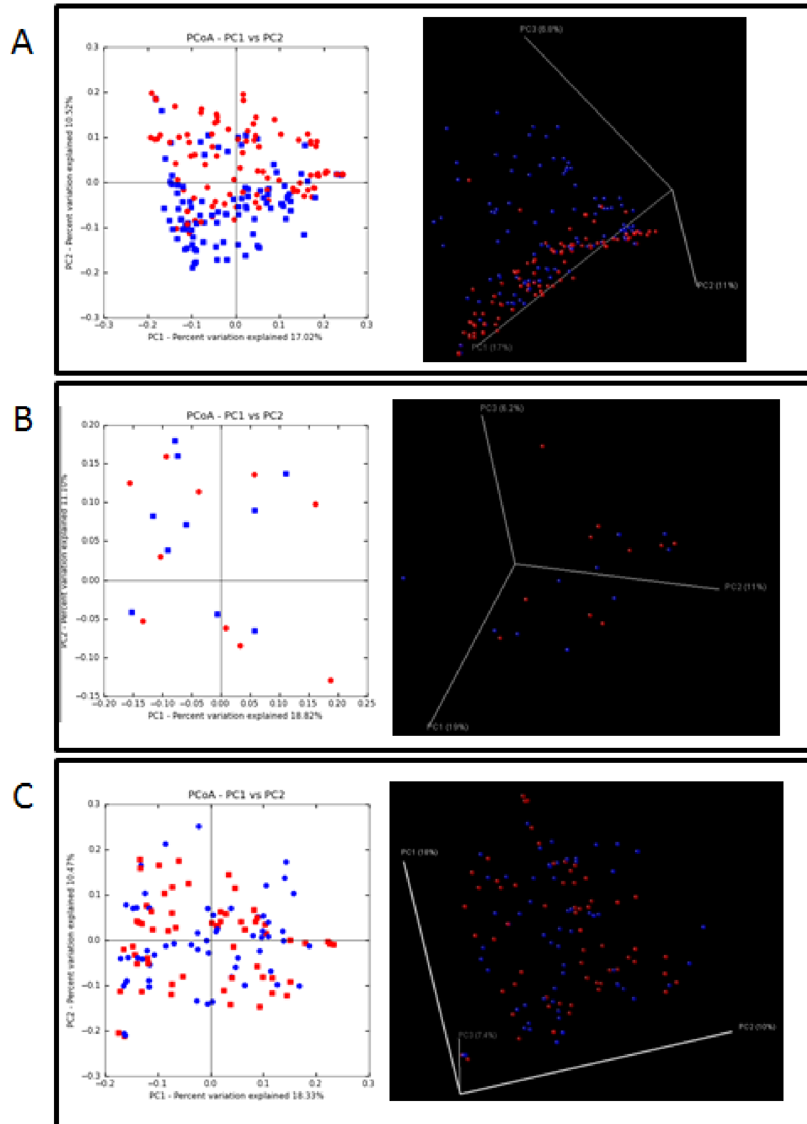


Figure 3-3. Scatterplots of the First Principal Components of the Principal Coordinate Analysis (weighted UniFrac) of the Hand Microbiota from 34 Healthcare Workers at the University of Michigan Surgical Intensive Care Unit, July 5-28, 2011. Stratification by Sampling Collection Method (Panel A: Glove-Juice (x-axis) and Swab (y-axis)), DNA Extraction Method (Panel B: Lysozyme (x-axis) and Cocktail (y-axis)), and Sequencing Replicates (Panel C: Set #1 (x-axis) and Set #2 (y-axis)).

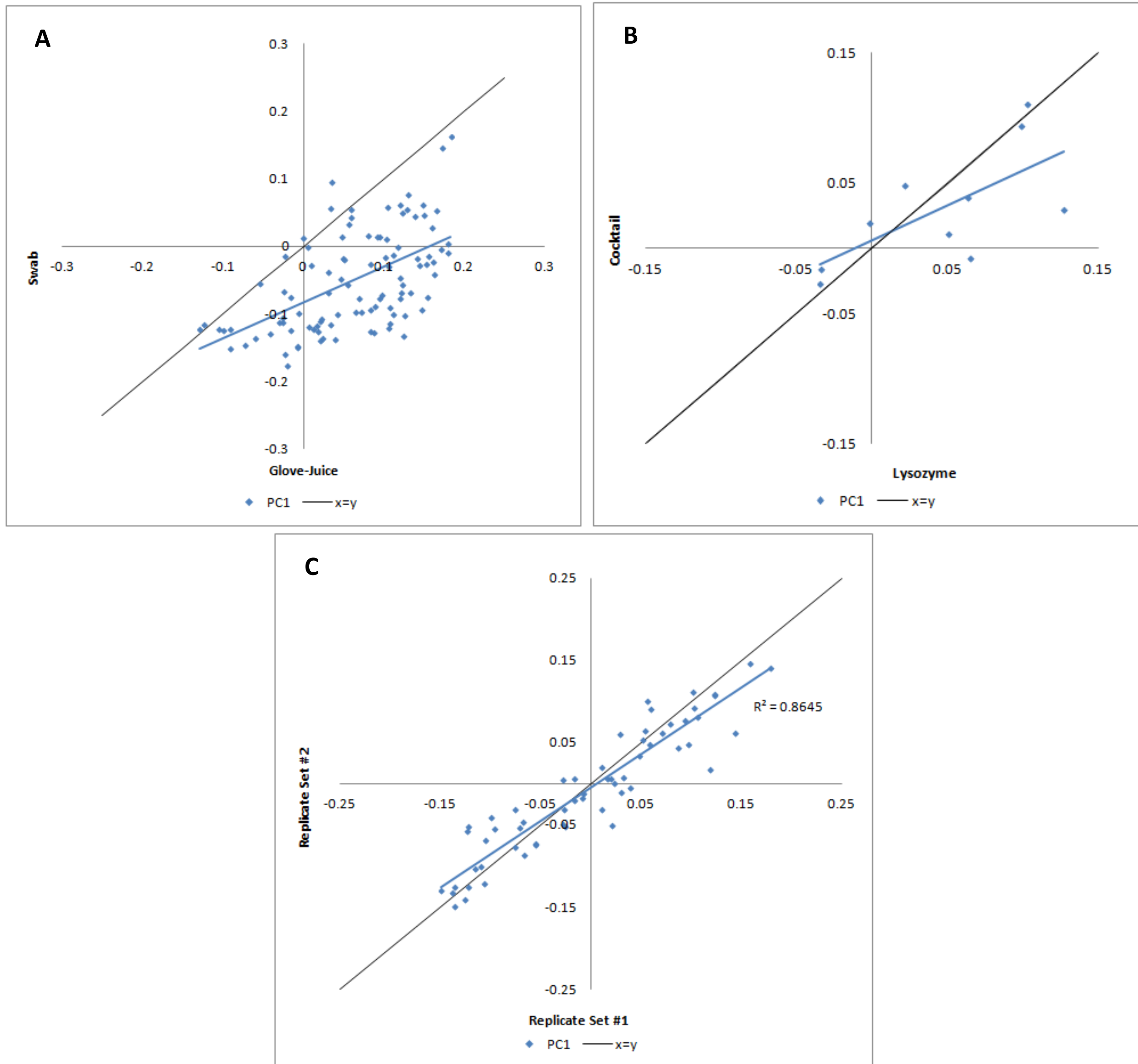


Table 3-1. Number of Raw and Processed Sequencing Reads per Ion Torrent Personal Genome Machine (PGM) Sequencing Run, Using 316 Chips, of 280 Samples of Hand Microbiota from 34 Healthcare Workers at the University of Michigan Surgical Intensive Care Unit, July 5-28, 2011.

Sequencing Run	Raw Sequence Reads	Processed Sequence Reads	Proportion of Processed/Raw Sequence Reads
1	2,787,276	1,292,855	0.464
2	3,160,031	2,132,925	0.675
3	903,240	643,015	0.712

Table 3-2. ANOSIM of the Hand Microbiota from 34 Healthcare Workers at the University of Michigan Surgical Intensive Care Unit, July 5-28, 2011, Comparing Sampling Collection Method (Glove-Juice and Swab), DNA Extraction Method (Lysozyme and Cocktail), and Sequencing Replicates (Set #1 and Set #2).

Group 1	Group 2	R Statistic	p-value
Glove-Juice	Swab	0.2649	<0.001
Lysozyme	Cocktail	0.0901	0.067
Replicate Set #1	Replicate Set #2	0.0122	0.326

Figure 3-4. Within and Between Weighted UniFrac Distances of the Hand Microbiota from 34 Healthcare Workers at the University of Michigan Surgical Intensive Care Unit, July 5-28, 2011. Stratification by Sampling Collection Method (Panel A: Glove-Juice and Swab), DNA Extraction Method (Panel B: Lysozyme and Cocktail), Sequencing Replicates (Panel C: Set #1 and Set #2), Healthcare Workers (Panel D: Within and Between), and Healthcare Workers by Sampling Collection Method (Panel E: Within and Between).

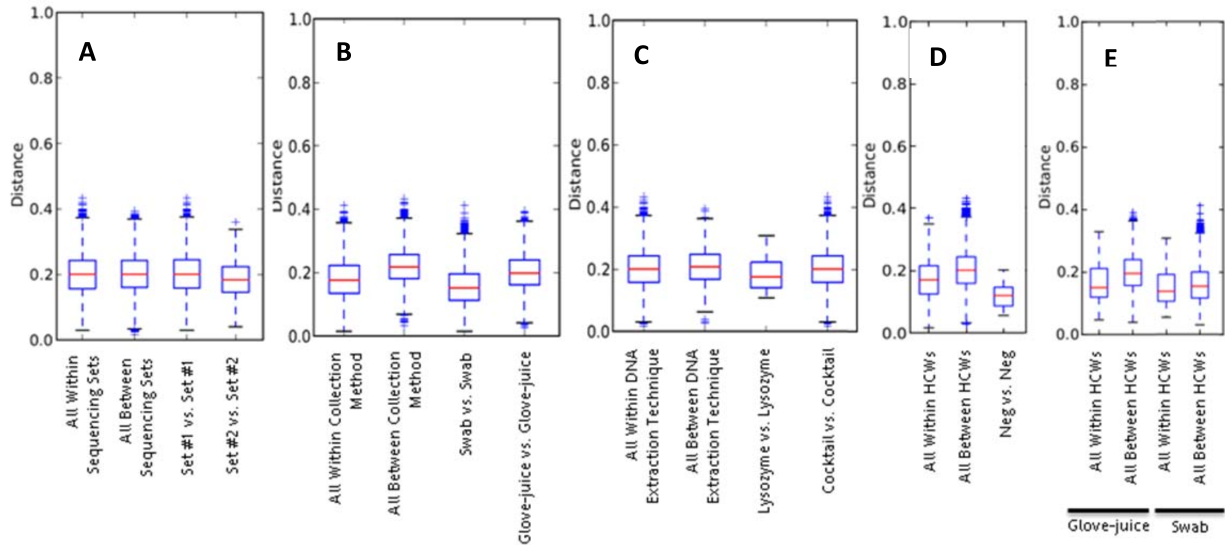


Figure 3-5. 2D and 3D Principal Coordinate Analysis (weighted UniFrac) of the Hand Microbiota from 34 Healthcare Workers at the University of Michigan Surgical Intensive Care Unit, July 5-28, 2011. Stratification by Sampling Collection Method (Glove-Juice and Swab).

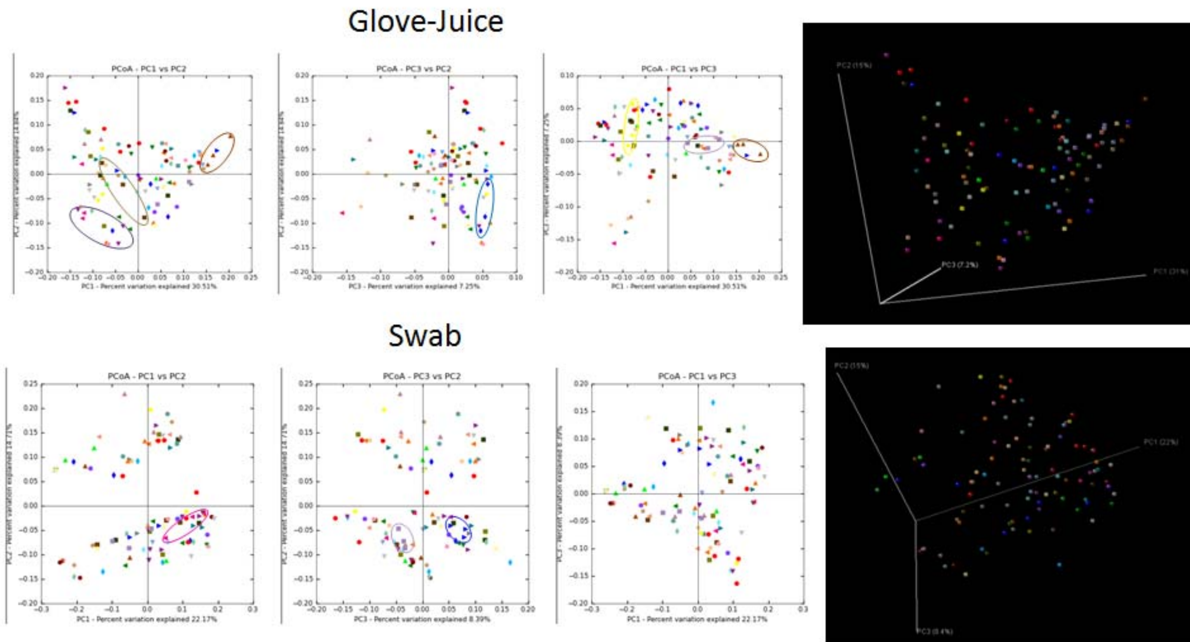
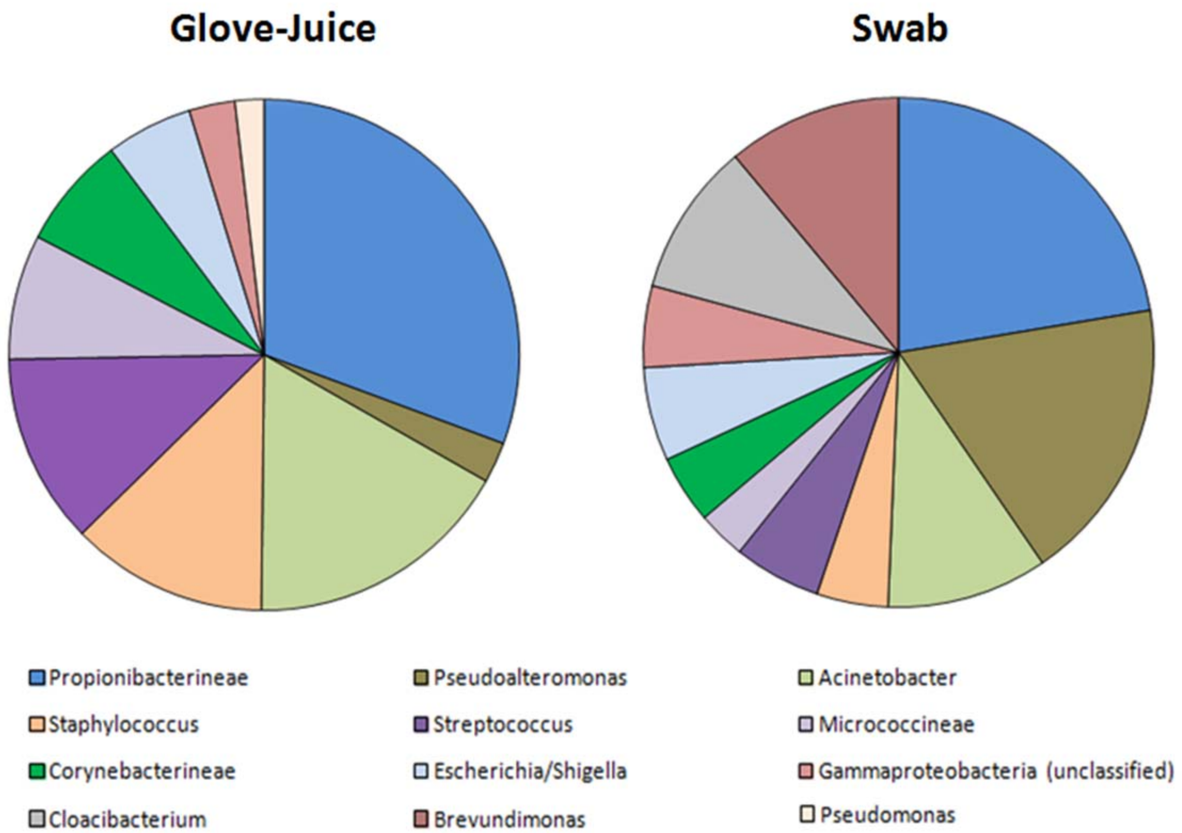


Figure 3-6. Relative Abundances of the Top 80% Most Abundant Taxa Detected per Sampling Method (Glove-Juice and Swab) of the Hand Microbiota from 34 Healthcare Workers at the University of Michigan Surgical Intensive Care Unit, July 5-28, 2011.



Chapter 4: The Role of the Surgical Intensive Care Unit Healthcare Workers' Hand Microbiota in Pathogen Carriage

Abstract

The human microbiome is known to play many roles in health and disease. The skin microbiome protect the skin from being infected by external microorganisms. In this study, we sought to determine whether the hand microbiota of 34 healthcare workers (HCW) in a surgical intensive care unit play a role in the relationship between potential demographic and behavioral risk factors for pathogen carriage, and pathogen carriage. We screened their dominant hands for four potential nosocomial pathogens, specifically *Staphylococcus aureus, Enterococcus* spp., methycillin-resistant *Staphylococcus aureus* (MRSA), and *Candida albicans*; and, evaluated age, hand hygiene, and work shift as significant risk factors for pathogen carriage. Risk factors for pathogen carriage were pathogen dependent. Additionally, HCW hand microbiota was associated pathogen carriage. The role of the hand microbiota in pathogen carriage has important implications. The hand microbiota community structure can not only act as a biomarker of pathogen carriage, but also be modified to enhance resistance to colonization by important nosocomial pathogens.

Introduction

Host intrinsic and extrinsic properties help to resist pathogen colonization and infection. Pathogen resistance mechanisms include the presence of barriers to colonization (e.g. hand hygiene), mechanisms that rapidly clear colonization and/or infection (e.g. host immunity), and, arguably, ecological relationships between the pathogen(s) and the host microbiota. In a clinical setting, healthcare workers (HCW) are continually exposed to pathogens, however, for the most part remain healthy. This pathogen resistance can be explained by their strict hand hygiene regimen, effective hospital infection control practices, the extent of their exposure, and inherently by their immunocompetency. It might also be explained by the composition of the microbial community present on their hands. Here, we aim to evaluate the risk factors for nosocomial pathogen carriage among HCWs in a surgical intensive care unit, and assess the role HCWs' hand microbiota play in the relationship between risk factors for pathogen carriage, and pathogen carriage (Figure 4-1).

Human skin is a complex ecosystem comprised of resident and transient microorganisms (Kampf and Kramer, 2004). The resident bacteria, viruses, fungi and protozoa, our microbiota, far outnumber human cells on and in our bodies. While the human microbiota have increasingly been shown to be associated with host health and disease, it is not yet clear whether they influence our capacity to carry or resist a pathogen. Human skin microbiota diversity is thought to arise via many factors such as the environment, host genetics, host demographics, and social/behavioral factors - some of which are themselves thought of as potential risk factors for pathogen carriage (Rosenthal

et al, 2011). Microbiota disruptions by hand hygiene regimens, for example, may impact the microbial community structure in ways that enhance pathogen resistance.

Understanding the association between pathogen carriage and factors such as host demographics like age, gender, and job title, behavioral factors like hand hygiene practices, host factors like overall health and health of their hands, and environmental factors like their level of patient contact, provides important insight into public health interventions in the clinical setting. The role of the hand microbiota in pathogen carriage can provide insight into the transmission potential of HCWs, and this has significant hospital infection control implications. We sought to assess whether HCW hand microbiota helped to potentially mediate or modify the effect of certain risk factors for pathogen carriage and carriage of *Staphylococcus aureus*, *Enterococcus* spp., MRSA, and *Candida albicans* among HCWs in a 20-bed surgical intensive care unit (Figure 4-1).

Methods

Study Overview

To determine whether HCWs' hand microbiota play a role in nosocomial pathogen carriage, we first assessed (i) potential risk factors for pathogen carriage, (ii) pathogen carriage, and (iii) the relationship between them. Next, we examined (iv) the relationship between the potential risk factors for pathogen carriage and the hand microbiota, and, (v) the relationship between the hand microbiota and pathogen carriage. These associations can be visualized in the conceptual framework in Figure 4-1. As illustrated, the hand microbiota is hypothesized to play several roles. One, it may act as a partial mediator in the relationship between host demographics, behavior, and environment, and pathogen

carriage. Two, it can potentially act as effect modifier of that same relationship. Or three, it can be completely unrelated to either the potential risk factors for pathogen carriage, and pathogen carriage.

Study Population

Healthcare workers were recruited from the University of Michigan Hospital Surgical Intensive Care Unit (SICU). This is a 20-bed critical care unit that specializes in patient recovery from major post-operative procedures (e.g. transplants, aneurysm repairs, resections, vascular endarterectomies, amputations) or those requiring extensive physiological monitoring. The SICU also accommodates patients from other surgical units (trauma-burn, neurosurgery, medical, and cardiovascular). To qualify for inclusion, volunteers had to be a healthcare worker working at the University of Michigan Hospital SICU, and not have received topical or systemic steroids or antibiotics for a period of 3 months before the start of the study. Physicians were excluded from the study due to their high mobility. The study was presented at staff meetings and the first 35 HCWs who met eligibility criteria and gave written consent were included in the study. One HCW was lost to follow-up prior to sample collection leaving a total sample size of 34. The study took place July 5-28, 2011. The study protocol was reviewed and approved by the institutional review board of the University of Michigan (IRBMed #HUM00042622).

Survey Instruments for Acquiring Potential Risk Factors Pathogen Carriage

At enrollment, study participants were given a self-administered questionnaire regarding basic demographics, overall health, hand health, hand hygiene practices, and levels of patient contact [Appendix A]. Questionnaire items were developed based on a

comprehensive literature review that identified elements important in shaping microbial community structure of healthcare worker hands (Rosenthal et al, 2011). Included was the Hand Skin Assessment (HSA), a self-rating scale used by the study participants to assess the current condition of the appearance, intactness, moisture content, and sensation of their hands. This scale, used extensively in other studies, has been used in previous studies of skin condition, and the scores correlate with other physiologic measures of skin damage (Larson et al, 1988; Larson et al, 1990; Berg M, 1991; Simion et al, 1993; Smit et al, 1992; Larson et al, 1997).

Upon completion of the questionnaire, a visual scoring of skin scale (VSS) was performed by the two trained data collectors (Cronbach's alpha = 0.7; ICC=0.59, 95%CI: 0.09-0.86), by visually inspecting the participant's dominant hand with a magnifying glass (30X magnification) for degrees of skin irritation. The possible range of scores indicating no observable scale or irritation of any kind to extensive cracking of skin surface, was 0 to 5, respectively. VSS scores are highly correlated with study participants' own ratings of the dryness of the skin of their hands, indicating good validity (Larson et al, 1997). Visual assessment is considered a cost-effective, practical, and accurate method of evaluating skin irritation (Farage et al, 2011; Larson et al, 1997).

Sample Collection

To minimize sample cross-contamination the study recruiters donned a new pair of sterile gloves prior to each sample collection. Negative controls consisting only of buffer solution (20 mM Tris pH 8, 2 mM EDTA, and 1.2% Triton X-100) were collected and analyzed for each sampling. The palm, fingertip surfaces, and in-between the fingers of the

participant's dominant hand were swabbed using cotton-tipped swabs soaked in the buffer solution. Swabbing was performed in two perpendicular directions to ensure that the maximum surface area was represented in the sample. Immediately after swabbing, the participant's dominant hand was inserted into a sterile, polyethylene bag containing 50ml buffer solution (0.07 M PBS, 0.1% Tween-80) and massaged through the wall of the bag for 1 minute. This is termed the glove-juice method. The buffer solution was then collected. All samples were stored at -20°C until further processing. Study participants were followed for 2 weeks, until three hand swab and glove-juice samples were obtained from each individual. HCWs were randomly sampled at the start, middle, and end of their shifts, and were not asked to wash their hands prior to collection, but were also not prevented from doing so. Although investigators did not observe the practices of all subjects throughout the study, subjects were visited on an unannounced, regular basis by investigators, usually at least once a day, during the data collection period (July 5-18, 2011).

DNA Extraction, Purification and Amplification

All swab samples and the pellet of 1 ml of all glove-juice samples were lysed using enzyme cocktail (mutanolysin @ 160U/ml, Rnase A @ 0.07mg/ml, lysostaphin @ 0.16 mg/ml, and lysozyme @ 7mg/ml) for 30 minutes at 37°C. The standard protocol for lysing gram-positive bacterial cell lysates of the PureLink Genomic DNA kit (Invitrogen Corp.; #K1820-02) was followed for all subsequent steps, with an additional incubation at 95°C for 2 minutes, prior to the addition of 96-100% ethanol to the lysates. Purified genomic DNA were re-suspended in 50 µl of PureLink Genomic Elution Buffer and stored at -80°C until sent for sequencing.

DNA was tested for PCR competency, using the following procedure. The primers L-V6 (5'-CAACGCGARGAACCTTACC-3') and R-V6 (5'-CAACACGAGCTGACGAC-3') were chosen to amplify the V6 hypervariable region of the 16S rRNA gene (Hummelen et al, 2011). After extraction, 1 uL of the purified genomic DNA was used as template for a 25 uL PCR reaction on a MyCycler Thermal Cycler (Bio-Rad Laboratories, Inc.). The following PCR reactions were used: 22.5 ul of Platinum Blue PCR SuperMix (Invitrogen Corp., #12580-023) 1 ul of 10 uM primer pair, and 0.5 ul of water. PCR conditions included: 94°C for 2 minutes; 30 cycles of [94°C for 30 seconds; 55°C for 30 seconds; 72°C for 30 seconds]; and hold at 4°C. A negative control including all ingredients but with water instead of DNA template was included alongside all test reactions. A constant volume aliquot of each PCR amplification product was run on a 1.5% agarose gel to determine PCR competency as well as the approximate amount of product. 10-20 ul of the purified genomic DNA were sent for sequencing at The London Regional Genomics Centre at the University of Western Ontario (London, ON, Canada). Further methodological information on DNA preparation for sequencing, sequencing and filtering, and taxonomic classification are provided (Supplemental Materials).

Real-time Quantitative PCR for Pathogen Carriage Detection

Relative abundances of four nosocomial pathogens were assessed among all glove-juice samples. These include *Enterococcus* spp., *Staphylococcus aureus*, methycillin-resistant *Staphylococcus aureus* (MRSA), and *Candida albicans*. These pathogens were selected based on the most prevalent ICU pathogens reported to the US National Healthcare Safety Network.

Primers sets for *Enterococcus* spp., *Staphylococcus aureus*, and MRSA were obtained from the literature, targeting the 16S rRNA gene, the nuc gene, and the single-locus mecA|orfx, respectively (Table 4-1). The primer set for *Candida albicans* was developed targeting the 18S rRNA gene.

We optimized real-time, quantitative PCR (qPCR) protocols using SYBR Green technology, namely SsoFast EvaGreen Supermix (Bio-Rad; #172-5200), on a CFX-96 thermocycler platform (Bio-Rad; #185-5195). Final optimal conditions are shown on Table 4-2. Standard curves from a 10-fold dilution series (10^8-10^2) were run using genomic DNA or cloned target DNA from the following positive controls, obtained from the University of Michigan's Clinical Microbiology and Virology Laboratories and the Molecular and Clinical Epidemiology Laboratory: *Enterococcus* spp. (ATCC# 29212), *S. aureus* (ATCC# 25923), MRSA (ATCC# 1026), and *C. albicans* (ATCC# MYA-2876).

Statistics

Four separate marginal models that accounted for repeated measures were fit to assess the association between the potential risk factors for pathogen carriage, and pathogen carriage. This type of model describes the fixed effects of covariates on the population mean response over the study period time. A backward selection model fitting strategy, where all covariates of interest were included, and then removed one at a time if found non-significant, was done to obtain mean predicted values of pathogen carriage of the HCW SICU population. The variance-covariance matrix of the random errors (i.e. R structure) was chosen by trying different R structures and comparing their model fit criteria. The structure that yielded the most parsimonious (lowest number of covariance

parameters) and lowest AIC and BIC, was selected. Most fixed effects that were non-significant, based on the Type 3 Tests, were removed from the model. Residual diagnostics were then evaluated (Supplemental Materials).

The UniFrac distance metric measures the difference between two groups in terms of the phylogenetic branch length that is unique to one group or the other. The branch lengths, in this case, are proportional to the number of base changes in the V6 16S rRNA gene. Because the relative abundance of different kinds of bacteria are critical for describing microbial community changes, the weighted UniFrac, which weights the branches based on abundance, was used. Distance plots are a way to compare microbiota samples from different categories and see which categories tend to have larger/smaller beta diversity than others. Beta-diversity metrics thus assess the differences between microbial communities. Associations between the HCWs' hand microbiota and pathogen carriage [Figure 4-1], were assessed by comparing the distribution of the weighted UniFrac distances among HCWs with *Enterococcus* spp. or *S. aureus* present with those without. Similarly, to investigate the association between the significant risk factors for pathogen carriage and the HCWs' hand microbiota [Figure 4-1], the distribution of weighted UniFrac distances between groups of HCWs that belonged to a certain category of a potential risk factor, were examined. A higher mean weighted UniFrac distance reflects a group of individuals with a more diverse microbial community. A wider distance distribution reflects a group of individuals that comprise highly diverse and less diverse microbial communities. According to previous analyses (see Chapter 2), HCWs sampled using swabs were determined to be statistically no more similar to themselves over the 3 time points than to other HCWs in the study, and therefore in this analysis, weighted UniFrac distances

were obtained from samples collected via swabs analyzed collectively. This analysis takes into account the microbial community structure of the microbiota as a whole.

Moreover, to confirm potential associations between the HCWs' hand microbiota and (i) risk factors for pathogen carriage, and (ii) pathogen carriage, a mixed model accounting for repeated measures was fit using the first principal components of the microbiota principal coordinate analyses as the outcome, and the risk factors for pathogen carriage, as well as levels of pathogen carriage, as the explanatory variables. The same previous model fitting strategy, variance-covariance matrix structure selection for the random errors, and residual diagnostics, were done.

To further evaluate the relationship between the HCWs' hand microbiota and the risk factors for pathogen carriage, correlations between individual OTUs within the microbiota and the risk factors for pathogen carriage, were assessed. Similarly, the relationship between the HCWs' hand microbiota and pathogen carriage was evaluated using correlations between individual OTUs within the microbiota and levels of pathogen carriage. These analyses do not compare the microbial community structures as a whole (e.g. beta diversities as shown via the weighted UniFrac distances) but instead each OTU within the community. Given the high degree of granularity of this analysis, samples were not assessed collectively. Instead, samples were analyzed one time point at a time. The correlation coefficients were then converted to a set of Z scores via Fisher transformation, and a normal quantile plot was done for each set (Supplemental Materials). This analysis takes into account the specific members of the microbial community structure of the microbiota.

Results

Survey Results

The self-reported questionnaire was designed to focus on a few potential risk factors of both pathogen carriage and hand microbiota. It included questions about host demographics, behavioral factors, host factors, and environmental factors. The 34 HCWs were mostly female (76.5%), Caucasian (91.1%) and born in the United States (91.1%). They averaged 34.5 years of age (range 20-59), and 7 (20.6%) had at least one child <5 years old living within their household. Twenty-four (70.6%) HCWs were RNs, 6 (17.6%) were Respiratory Specialists, and 4 (11.8%) were Nurse Technologists.

In terms of their hand hygiene practices, during a typical 12-hr work shift, about half (52.9%) HCWs washed their hands with soap and water 6-20 times, and 41.2% used alcohol rub >40 times. Two-thirds (61.8%) donned >40 pairs of gloves during a typical 12-hr work shift, mostly nitrile, powder-free (Figure 4-2). Washing hands with soap and water, using alcohol rub, and donning pairs of gloves were not correlated with each other. The majority (64.7%) used lotion or moisturizer on their hands 1-5 times per 12-hr work shift. Based on the 6-point visual scoring skin scale (VSS), only 6 (17.7%) HCWs had slightly scaly hands; the rest had either very slightly scaly or normal hands. Inter-rater agreement was good (Cronbach's alpha = 0.75) (Supplemental Materials). Based on the 7-point self-reported healthy skin assessment (HSA) scale, with 7 being the healthiest state, many HCWs reported a 6 on appearance (55.9%), intactness (38.2%), and moisture content (32.4%), and a 7 on sensation (64.7%) of their hands. In addition, most HCWs (97.1%) rated themselves in excellent or good overall health (Supplemental Materials).

Regarding the level of patient contact, most (58.8%) HCWs cared directly for an average of 1-2 patients per 12-hr work shift. The majority took vital signs (73.5%) and turned patients (55.9%) >10 times per 12-hr work shift. Tasks performed by HCWs 1-4 times per 12-hour work shift included blood draws (41.2%), dressing wounds (73.5%), caring for IVs, urinary catheters, endotracheal tubes, and/or drains (38.2%), performing a physical examination (55.9%), handling soiled bedpans (50.0%) and soiled linen (51.5%), and performing patient hygiene functions (61.8%) (Supplemental Materials).

qPCR Results

HCWs were sampled using swabs at the start, middle, or end of their 12-hr work shift. In an effort to ascertain the microbial load on HCWs' hands, HCWs were not asked to perform hand hygiene before sample collection. However, they were not prevented from doing so. In fact, most HCWs performed hand hygiene within 10 minutes before sample collection, ranging from immediately before to 160 minutes before (median of 10 minutes, mean of 24.5 minutes, std dev of 32.3 minutes). The proportion of potential pathogens detected using qPCR varied by collection visit: *S. aureus* ranged from 41.2% to 52.9%; *Enterococcus* spp. ranged from 52.9% to 61.8%; *C. albicans* ranged from 2.9% to 8.8% and MRSA ranged from 2.9% to 5.9% (Table 4-3). A closer look at whether these pathogens co-occurred, shows that *S. aureus* and *Enterococcus* spp. co-occurred the most frequently, ranging from 29.4% to 35.3% over the three collection visits (Supplemental Materials).

Risk Factors for Pathogen Carriage

The four mixed models, which looked at the fixed effects of the survey covariates on the mean level of pathogen detected, were as follows: $\text{Pathogen}_i = (\text{survey covariates})_i\beta + \varepsilon_i$,

where, $\varepsilon_i \sim N(0,R_i)$ and $Pathogen_i$ = [*S. aureus*, *Enterococcus* spp., MRSA, *C. albicans*]. The Toeplitz R structure was selected as the appropriate structure for the variances and covariances of the random errors (Supplemental Materials). It has homogenous variances and heterogeneous correlations between the elements. Since we have no reason to suppose that the error structure is changing over time, this R structure is suitable as it assumes no exponential decay (i.e. correlations among the errors do not decline exponentially with distance). Models predicting *C. albicans* carriage and MRSA carriage did not converge because there were not enough HCWs carrying these pathogens. Models predicting mean level of *S. aureus* carriage and *Enterococcus* spp. carriage were as follows: $S.\ aureus_i = (\text{alcohol rub})_i\beta + (\text{glove use})_i\beta + (\text{shift time})_i\beta + \varepsilon_i$, and $Enterococcus\ spp_i = (\text{age})_i\beta + (\text{handwashes})_i\beta + \varepsilon_i$. The outcome variable for both models were log transformed because they were not normally distributed. Also, this facilitated interpretation, since one unit increase in copy number is not biologically relevant.

According to the type 3 tests of fixed effects, the mixed model predicting levels of *S. aureus* detection on the HCWs' hands, shows that the frequency of alcohol rub use ($F = 7.86$, p-value= 0.0006), frequency of glove use ($F = 8.59$, p-value= 0.0007), and time within shift (e.g. start, middle or end of work shift) ($F = 4.28$, p-value= 0.0339), were statistically significantly associated with frequency of *S. aureus* carriage (Supplemental Materials). In comparison to not using any alcohol rub, HCWs who used it 1-5 times/work shift had a 20% decreased level of *S. aureus* ($p=0.03$) [Table 4-4]. This trend, however, was not statistically significant. Higher frequency of donning gloves during the 12-hr work shift was associated with a greater frequency of *S. aureus* on HCWs' hands [Table 4-4]. Sampling HCWs in the middle of their work shift was associated with a 43% lower amount of *S.*

aureus, in comparison to the start of their shift (p=0.01); sampling at the end was associated with an even lower amount, though this was not statistically significant [Table 4-4].

According to the type 3 tests of fixed effects, the mixed model predicting levels of *Enterococcus* spp. detection on the HCWs' hands, shows that frequency of handwashes (F=4.95, p-value= 0.0070) and age (F=29.24, p-value< 0.0001) were statistically significantly associated with frequency of *Enterococcus* spp. carriage. (Supplemental Materials). For every year increase in age, there was an approximate 9% greater frequency of *Enterococcus* spp. carriage (p<0.0001) [Table 4-4]. In terms of hand hygiene, washing hands with soap and water >40 times per 12-hr work shift resulted in a 9% lower frequency of *Enterococcus* spp. carriage (p=0.02) [Table 4-4].

Association Between Risk Factors for Pathogen Carriage and HCW Hand Microbiota

Swab samples from a single individual HCW were no more similar to each other over time than those between HCWs (MR, unpublished data), in terms of their hand microbiota profile. Consequently, associations assessed between HCW hand microbiota and potential risk factors for pathogen carriage, as well as pathogen carriage itself, were done so using swab samples. Of all potential risk factors for pathogen carriage, only hand hygiene (i.e. handwashing, alcohol rub use, and donning of gloves) was associated with HCW hand microbiota, as shown by the differences observed in weighted UniFrac distance distributions across different levels of hand hygiene [Figure 4-3]. HCWs who did not use alcohol rub had a wider distribution of weighted UniFrac distances than all other HCWs. However, the mean distance within all levels of alcohol rub use did not differ. This

association was different regarding handwashing. All frequencies of handwashing, except those who do so >40 times per 12-hr work shift, had similar and notably higher means. Washing hands >40 times per 12-hr work shift had a reduced mean distance of the microbial communities, in a way, a reduced overall diversity between those samples. Donning only 1-5 pairs of gloves per 12-hr work shift was associated with a slightly higher mean distance than other frequencies, however, donning gloves over 20 times was observed to have a wider distribution range. The distribution of weighted UniFrac distances by age and by time within work shift (e.g. start, middle, or end of work shift) did not differ, suggesting that there is no association between these risk factors for pathogen carriage, and the HCW hand microbiota.

The following mixed model predicting the first principal components of the hand microbiota principal coordinate analyses, by the fixed effects of the significant risk factors for pathogen carriage, as well as levels of pathogen carriage (log-transformed levels of *S. aureus* and *Enterococcus* spp.), resulted in no statistically significant associations: PC_i = (log *S. aureus*)$_i\beta$ + (log *Enterococcus* spp.)$_i\beta$ + (handwashes)$_i\beta$ + (alcohol rub)$_i\beta$ + (glove use)$_i\beta$ + (age)$_i\beta$ + (shift time)$_i\beta$ + ε_i (Supplemental Materials).

Association Between HCW Hand Microbiota and Pathogen Carriage

To assess the association between HCW hand microbiota and pathogen carriage, distributions of weighted UniFrac distances of the microbial communites among HCWs with pathogens detected (e.g. *S. aureus*, *Enterococcus* spp., MRSA, and *C. albicans*) were compared to those without. In general, the presence of a pathogen was associated with a lower mean weighted UniFrac distance [Figure 4-4]. Meaning, HCWs with a lower beta

diversity of their hand microbiota were more likely to have a pathogen present on their hands than those without. As previously mentioned, no statistically significant associations were observed between HCW hand microbiota and levels of pathogen carriage, when using the first principal components of the hand microbiota principal coordinate analyses as the outcome of interest in the mixed model (Supplemental Materials).

OTU Correlations with Risk Factors for Pathogen Carriage and with Levels of Pathogen Carriage

The comparison of the raw correlations with that of the Fisher transformed correlations, indicate that in terms of individual OTUs (as opposed to the microbial community structure as a whole), sampling time point collected matters (Supplemental Materials). For each sampling time, correlation coefficients higher than |0.6| were observed between levels of MRSA carriage and of *C. albicans* carriage, with the relative abundance of certain OTUs [Table 4-4]. In particular, increasing amounts of MRSA was positively correlated with increasing amounts of *Bifidobacteriaceae vaginalis.* Increasing amounts of *C. albicans* was positively correlated with increasing amounts of *Corynebactericeae, Micrococccineae mucilaginosa,* and *Methylobacterium*. Levels of *S. aureus* and of *Enterococcus* spp. on HCWs hands were not correlated with any particular OTU. Correlation coefficients higher than |0.6| were also observed between certain risk factors for pathogen carriage and the relative abundance of certain OTUs [Table 4-4]. Increasing the number of patients per HCW and having children <5y living with the same household, were positively correlated with particular OTUs. A negative correlation was observed, as expected, with increasing frequency of hand hygiene (alcohol rub use and glove use) and certain OTUs [Table 4-4].

Discussion

Analyses of the hands of 34 HCWs showed several key findings. First, the proportion of potential pathogens detected on their hands varied by collection visit, with *S. aureus* and *Enterococcus* spp. having relatively high proportions, in comparison to MRSA and *C. albicans*. Second, assessing various potential risk factors for pathogen carriage, including host demographics, hand hygiene practices, and level of patient contact, showed that significant predictors differed by specific pathogen carriage. Third, and perhaps most important, HCWs' hand microbiota play a meaningful role in the relationship between potential risk factors for pathogen carriage, and pathogen carriage.

Real-time, qPCR is a sensitive, effective, and valuable tool that can determine microbial counts on HCWs' hands. Using this technique, a Danish longitudinal study of *S. aureus* carriage on the hands of 20 HCWs showed that 45% of the participants were positive on all 10 days (Horn et al, 2007). Moreover, the average amount of *S. aureus* detected per hand was 2300. In our study, we found exactly the same proportion of positive HCWs over 3 weeks, with an average of 2642 *S. aureus* per hand. A molecular quantitation of the carriage of non-resistant *Enterococcus* spp. on HCWs' hands was not found in the literature. Although less than 10% of *E. faecalis* isolates from intensive care unit patients infected with Enterococci are resistant to vancomycin, 70% of the *E. faecium* isolates are resistant (Tenover and McDonald, 2005). Epidemiologic investigations of contamination of HCWs' hands with vancomycin-resistant Enterococci, have shown a range of 0 to 41% of hands positive (Hayden, 2000). In our study, we sought to determine the levels of *Enterococcus* spp., which may have included resistant and non-resistant bacteria. We found a relatively high prevalence of 62% over 3 weeks, with an average of 2135

Enterococcus spp. MRSA and *C. albicans* were detected at a much lower prevalence, at 3.9% and 5.9% positive over 3 weeks, respectively. In a recent study, the fingertips from 523 HCWs were sampled on 822 occasions, showing 38/822 (5%) positive (Creamer et al, 2010). An 8 month study of *C. albicans* carriage among patients and nursing staff at an intensive care unit in Kuwait showed that of the 90 swab samples taken from the hands of the nursing staff, 4 (4.4%) isolates were detected (Khan et al, 2003). Therefore, our findings are consistent with other studies among healthcare workers in the literature.

Significant risk factors for pathogen carriage depended on the pathogen. Frequency of alcohol rub use, frequency of glove use, and time within work shift were significantly associated with *S. aureus* carriage. In comparison to not using any alcohol rub, HCWs who used it 1-5 times/work shift had a 20% decreased level of *S. aureus*. A crossover effect happened, however, where using alcohol rub >5 times per 12-hr work shift was associated with higher frequency of *S. aureus* carriage. This may be explained by the effective killing or removal of bacterial species that compete with *S. aureus* occurred allowing them to flourish. Or, the ecosystem created on the HCWs' hands by the gloves resulted in a suitable environment (e.g. higher moisture, warmer temperatures) for the specific perpetuation of *S. aureus*. A 2004 review reports that a 30-second application of 70% ethanol achieved a 2.6-3.7 $\log_{10}$ unit reduction in *S. aureus* on artificially contaminated hands (Kampf and Kramer, 2004). However, the authors also indicate studies whereby an increase in microbial load happens after handwashing. Our observed crossover effect of alcohol rub use on the frequency of *S. aureus* carriage may possibly reflect the combined use of these two hand hygiene regimens performed by our study participants. Sampling HCWs in the middle of their work shift was associated with a 43% lower amount of *S. aureus*, in

comparison to the start of their shift. This association was not found in the literature; we suggest that it may reflect the cumulative effect of hand hygiene performed throughout their 12-hr shifts, or, the modifying effect of their hand microbiota throughout their shifts.

Other risk factors for pathogen carriage were significant for the carriage of *Enterococcus* spp. Unlike *S. aureus* carriage, *Enterococcus* spp. carriage was statistically significantly associated with the frequency of handwashing. However, this was only the case among HCWs who reported washing their hands >40 times per 12-hr work shift. Curiously, the use of alcohol rub was not associated with the frequency of *Enterococcus* spp. carriage, reflecting the differential effect of this type of hand hygiene on different bacterial species. For every year increase in age, there was an approximate 9% greater frequency of *Enterococcus* spp. carriage. This is likely explained by either the modifying effects of their hand microbiota, or the activities performed outside the SICU. All the tasks performed by HCWs in the SICU that were evaluated in this study were not associated with frequency of *Enterococcus* spp. carriage. Frequency of MRSA carriage and *C. albicans* carriage were likely too low to show any significant associations with any potential risk factors.

Frequency of alcohol rub use and glove use were both associated with both *S. aureus* carriage and with the HCWs' hand microbiota. Similarly, frequency of handwashing was associated with both *Enterococcus* spp. carriage and with the HCWs' hand microbiota. Hand microbiota was associated with the carriage of both pathogens. Therefore, the HCWs' hand microbiota may act as a partial mediator in the relationship between hand hygiene and pathogen carriage. Alternatively, time within work shift was associated with *S. aureus* carriage but not with the HCWs' hand microbiota. Similarly, age was associated with

Enterococcus spp. carriage but not with the HCWs' hand microbiota. Therefore, the hand microbiota may act as an effect modifier in the relationship between these risk factors and pathogen carriage. Results from the mixed model using the first principal component of the principal coordinate analysis of the HCWs' hand microbiota as the outcome, did not show any statistically significant associations to confirm the associations observed using the distribution of weighted UniFrac distances.

In terms of the specific bacterial community members of the HCWs' hand microbiota that were correlated with either the levels of pathogen carriage, or with potential risk factors for pathogen carriage, we first found that levels of *S. aureus* and of *Enterococcus* spp. on HCWs hands were not correlated with any particular OTU. This may be due to the ubiquity of these potential pathogens on the HCWs' hands. We observed a positive correlation between levels of MRSA and *Bifidobacteriaceae vaginalis*. This association is not very well characterized in the literature. There have been reports of heterosexual transmission of MRSA, but also of rare MRSA prevalence among sexually active adolescents (Cook et al, 2007; Handsfield HH, 2007; Huppert et al , 2011). *C. albicans* was positively correlated with *Corynebactericeae, Micrococccineae mucilaginosa,* and *Methylobacterium*. HCWs who had children <5y living within their household were positively associated with having *Acinetobacter* and *Veillonella* on their hands. Associations between children and presence of *Acinetobacter* are not uncommon (Andersson et al, 1999; Ege et al, 2012). Similarly, associations between children and presence of *Veillonella* have also been reported (Arif et al, 2008; Duytschaever et al, 2011). Positive correlations between an increase in the number of patients per HCW and *Vibrionaceae, Propionibacteriaceae, Delftia*, and *Acinetobacter*, were observed. *Delftia* is a genus of Comamonads, that have been

implicated in healthcare-associated infections (Kawamura et al, 2011; Preiswerk et al, 2011). *Propionibacteriaceae* are a common human skin commensal bacteria. And, *Acinetobacter* have been increasingly reported in the nosocomial infection literature (Doyle et al, 2011; Falagas et al, 2008). As expected, all correlations observed between levels of hand hygiene and levels of specific OTUs were negative.

These correlations are all biologically plausible according to the literature, however, further associative criteria need to be fulfilled in order to establish any causal relationships between these bacterial species and microbial community structure of HCWs' hand microbiota and pathogen carriage. For instance, none of the correlations observed were consistent across collection visits. Overall, both the HCWs' hand microbiota and certain risk factors for pathogen carriage, likely predict pathogen carriage. However, a limitation to this study remains the directionality of the association between pathogen carriage and HCWs' hand microbiota. While we concern ourselves with the pathogen carriage as an important public health outcome, it may be that the HCWs' hand microbiota is itself a result from the carriage of certain pathogens. This could also have meaningful health implications, with particular microbial community structures of the microbiota resulting in more harmful ecosystems than the presence of one pathogen alone.

A limitation to this study was the small sample size of HCW participants (n=34). While it yielded a very large amount of microbiota samples to analyze (n=102), in terms of the epidemiologic component of this study, 34 HCWs was likely an insufficient sample size for two of the four models of pathogen carriage to run. Additionally, the lack of consistency concerning the HCWs' hand hygiene practices before sample collection may have

contributed to some confounding in the results of the effect of their microbiota on pathogen carriage. Lastly, problems associated with multiple comparisons may have occurred, given the need to assess each relationship (e.g. HCW hand microbiota and pathogen carriage) [Figure 4-1] one at a time as a result of combining cross-disciplinary analytical techniques (i.e. microbial ecology and epimediology).

In conclusion, risk factors for pathogen carriage were pathogen dependent. Alcohol rub use, donning of gloves, and work shift were associated with *S. aureus* carriage. Age and handwashing, were associated with *Enterococcus* spp. carriage. HCW hand microbial community structure was associated with pathogen carriage. HCWs with a lower hand microbiota diversity were more likely to have a pathogen present on their hands. Ultimately, HCW hand microbiota may play a role in the relationship between certain risk factors for pathogen carriage, and pathogen carriage. It may act as a partial mediator in the relationship between hand hygiene and pathogen carriage. In addition, it may also act as an effect modifier in the relationship between some demographic factors and pathogen carriage. Understanding the different risk factors for pathogen carriage is important. But more importantly, we need to start thinking about the HCWs' hand microbiota in that relationship. The role of the hand microbiota in pathogen carriage can provide insight into the transmission potential of HCWs, and this has significant hospital infection control implications.

Acknowledgements

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Table 4-1. Primer Sets Used in the Real-time qPCR Assays of *Enterococcus* spp., *Staphylococcus aureus*, Methycillin-resistant *Staphylococcus aureus* (MRSA), and *Candida albicans*.

	Target	Primers (5' --> 3')	Reference
Staphylococcus aureus	nuc	GCGATTGATGGTGATACGGTT AGCCAAGCCTTGACGAACTAAAGC	Brakstad et al, 1992
MRSA	mecA|orfX	TATGATATGCTTCTCC AACGTTTAGGCCCATACACCA	Cuny and Witte, 2005
Enterococcus spp.*	16S	CCCTTATTGTTAGTTGCCATCATT ACTCGTTGTACTTCCCATTGT	Rintilla et al, 2004
Candida albicans	18S	GGATCGCTTTGACAATGG GCGGGTAGTCCTACCTGATTT	Developed in-house

* *Enterococcus faecalis , E. faecium, E. asini, E. saccharolyticus, E. casseliflavus, E. gallinarum, E. dispar, E. flavescens, E. hirae, E. durans, E. pseudoavium, E. raffinosus, E. avium, E. malodoratus, E. mundtii, E. azikeevi, E. canis, E. gilvus, E. haemoperoxidus, E. hermanniensis, E. moraviensis, E. pallens, E. phoeniculicola, E. villorum, E. rottae.*

Table 4-2. Real-time qPCR Conditions Used for *Enterococcus* spp., *Staphylococcus aureus*, methycillin-resistant *Staphylococcus aureus* (MRSA), and *Candida albicans* Assays, on a CFX-96 Thermocycler.

***Enterococcus* spp.**	***Staphylococcus aureus***
98C for 2min	98C for 2min
40 cycles of [98C for 1sec; 60C for 1sec]	40 cycles of [98C for 4sec; 60C for 4sec]
65C-95C (increment of 0.5C) for 5sec	65C-95C (increment of 0.5C) for 5sec
MRSA	***Candida albicans***
98C for 2min	98C for 2min
40 cycles of [98C for 2sec; 56C for 2sec]	40 cycles of [98C for 1sec; 63C for 1sec]
65C-95C (increment of 0.5C) for 5sec	65C-95C (increment of 0.5C) for 5sec

Figure 4-1. Conceptual Framework Describing the Role of the Hand Microbiota in the Relationship Between Potential Risk Factors for Pathogen Carriage and Pathogen Carriage, among Surgical Intensive Care Unit Healthcare Workers Participating in the Healthy Hands Study, July, 2011 (N=34).

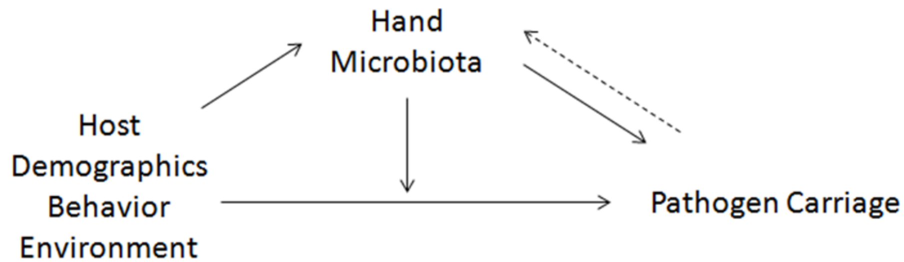


Figure 4-2. Frequency of Hand Hygiene Practices per 12-hour Work Shift among Surgical Intensive Care Unit Healthcare Workers Participating in the Healthy Hands Study, July, 2011 (N=34).

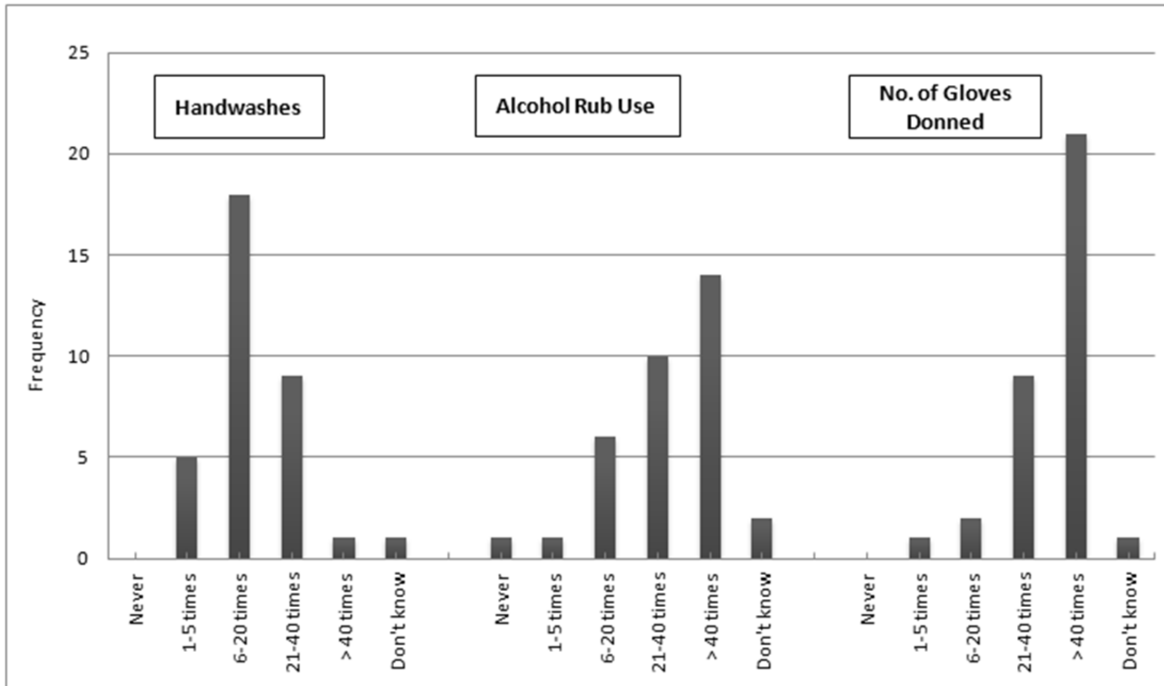


Table 4-3. Relative Abundances of Potential Pathogens Detected per Collection Visit on the Hands of Surgical Intensive Care Unit Healthcare Workers Participating in the Healthy Hands Study, July, 2011 (N=34).

Pathogen (targeted gene)	Collection Visit	Ɏ * Mean copies/ul	* Positive (%, n=34)
Staphylococcus aureus (nuc)	1	951.3	41.2
	2	6623.1	41.2
	3	351.4	52.9
Enterococcus spp. (16S)	1	1702.9	52.9
	2	2877.1	70.6
	3	1823.6	61.8
Candida albicans (18S)	1	663.8	8.8
	2	336.3	5.9
	3	142.7	2.9
MRSA (mecA/orfX)	1	173.5	2.9
	2	1405.8	5.9
	3	3763.5	2.9

Ɏ nuc (~1 copy / cell); Enterococcus (~5 16S rRNA copies / cell); C. albicans (~100 copies / cell).
** qPCR cut-off of 100 copies / ul defined as limit of qPCR detection used to identify a healthcare worker as positive.*

Table 4-4. Analysis Of Mixed Model Parameter Estimates, Accounting for Repeated Measures, Predicting Mean Level of *Staphylococcus aureus* and *Enterococcus* spp., as Measured by the log of Gene Copy Numbers Detected using qPCR, among Surgical Intensive Care Unit Healthcare Workers Participating in the Healthy Hands Study, July, 2011 (N=34).

	Staphylococcus aureus			***Enterococcus* spp.**		
Parameter	**Estimate**	**0.95 CL**	**p-value**	**Estimate**	**0.95 CL**	**p-value**
Intercept	3.063	(1.827, 4.299)	<.0001	2.779	(1.197, 4.360)	0.0012
Age				0.087	(0.0543, 0.121)	<.0001
Handwashes						
1-5 times	ref	ref	ref	ref	ref	ref
6-20 times	-0.045	(-0.626, 0.535)	0.87	0.462	(-0.513, 1.437)	0.34
21-40 times	-0.354	(-1.172, 0.464)	0.38	-0.493	(-1.555, 0.570)	0.35
>40 times	-0.125	(-0.847, 0.597)	0.72	-2.382	(-4.318, -0.446)	0.02
Alcohol Rub Use						
None	ref	ref	ref			
1-5 times	-1.546	(-2.892, -0.201)	0.03			
6-20 times	0.933	(0.207, 1.658)	0.01			
21-40 times	-0.017	(-0.713, 0.679)	0.96			
>40 times	0.115	(-0.694, 0.924)	0.77			
Pairs of Gloves Donned						
1-5 times	ref	ref	ref			
6-20 times	1.795	(0.272, 3.318)	0.02			
21-40 times	2.812	(1.592, 4.032)	0.0001			
>40 times	2.709	(1.384, 4.034)	0.0004			
Time within Shift						
start	ref	ref	ref			
mid	-0.833	(-1.443, -0.222)	0.01			
end	-0.366	(-1.279, 0.548)	0.41			

Note: ref=referent group; CL=confidence limits (p-value based on 95% level of significance).

Figure 4-3. Distributions of Weighted UniFrac Distances Within Categories of Survey Variables, of Surgical Intensive Care Unit Healthcare Workers Participating in the Healthy Hands Study, July, 2011 (N=34). Panel A: Frequency of Alcohol Rub Use; Panel B: Frequency of Handwashes; Panel C: Number of Gloves Donned; Panel D: Age; and, Panel E: Time Within Shift.

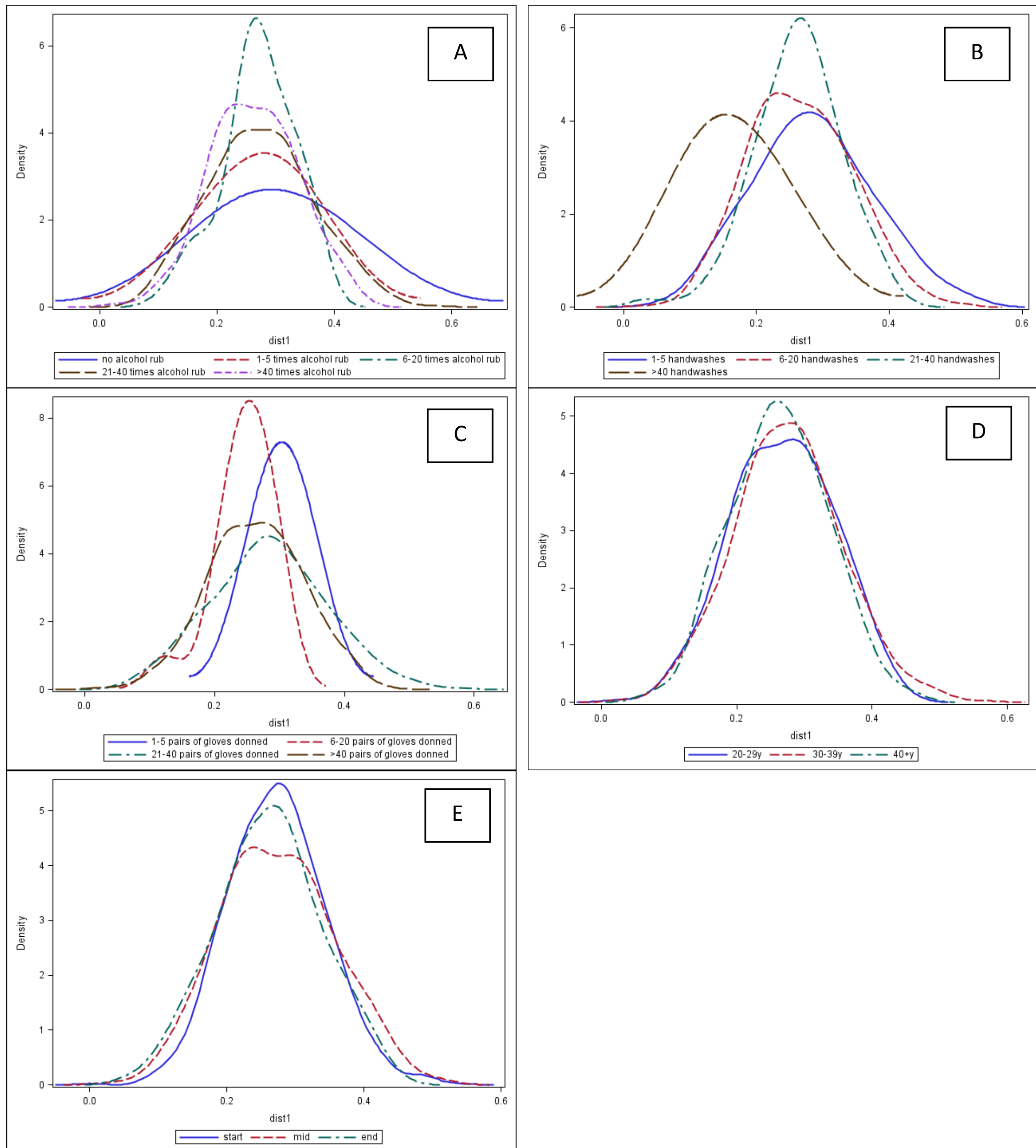


Figure 4-4. Distributions of Weighted UniFrac Distances Within Presence and Absence Categories of Potential Pathogens Detected among Surgical Intensive Care Unit Healthcare Workers Participating in the Healthy Hands Study, July, 2011 (N=34). Panel A: *Candida albicans*; Panel B: *Enterococcus* spp.; Panel C: Methycillin-resistant *Staphylococcus aureus* (MRSA); and, Panel D: *Staphylococcus aureus*.

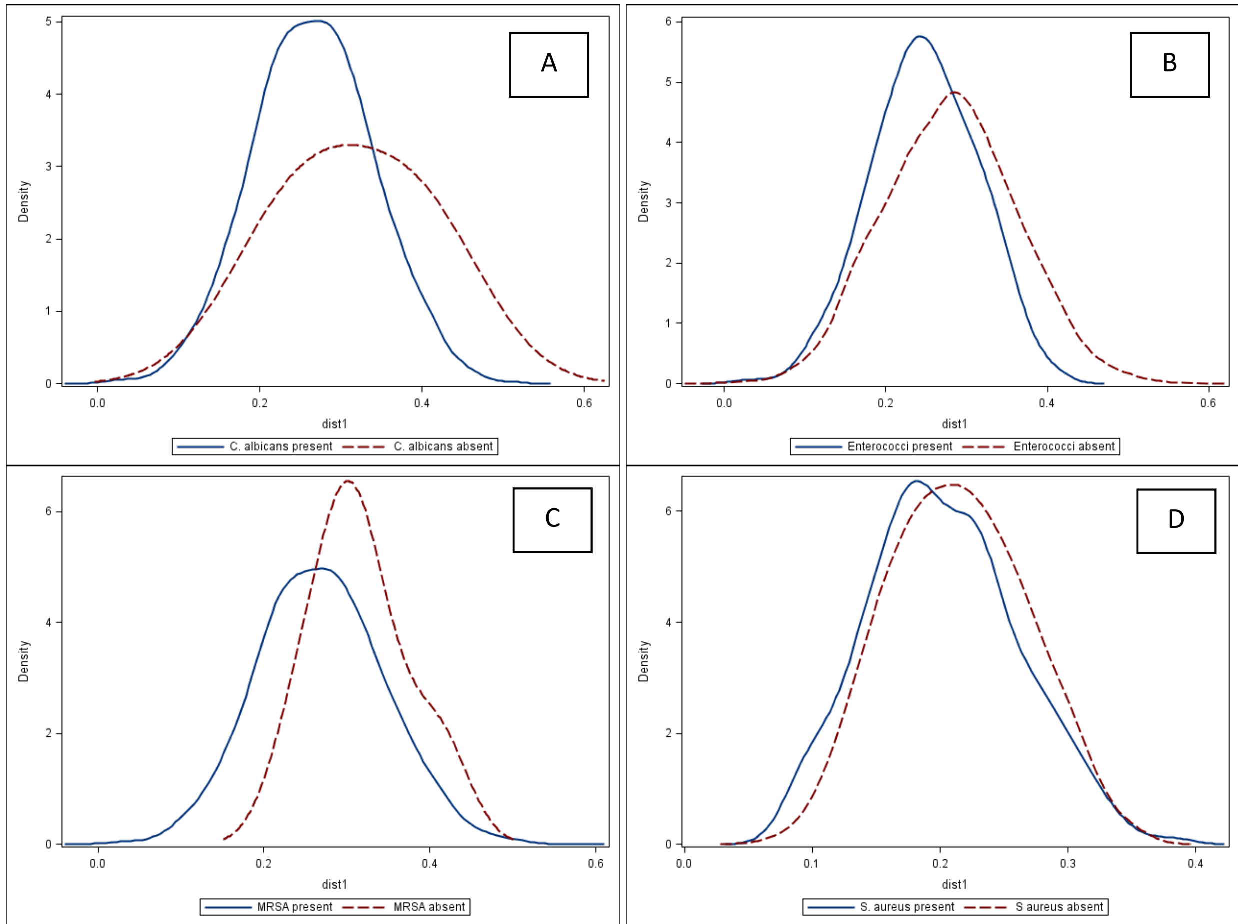


Table 4-5. List of Highly Correlated Operational Taxonomic Units (OTU) with Potential Pathogens Detected and with Potential Risk Factors for Pathogen Carriage, among Surgical Intensive Care Unit Healthcare Workers Participating in the Healthy Hands Study, July, 2011 (N=34).

Potential Pathogen	Correlation	OTU
Staphylococcus aureus	n/a	none
Enterococcus spp.	n/a	none
MRSA*	0.66	*Bifidobacteriaceae vaginalis*
Candida albicans	0.66 0.74 0.75	*Corynebacteriaceae* *Micrococccineae mucilaginosa* *Methylobacterium*
Potential Risk Factor	**Correlation**	**OTU**
Children <5y	0.63 0.75	*Acinetobacter* *Veillonella*
Alcohol Rub Use	-0.63 -0.66	*Neisseriaceae* *Conchiformibius*
Glove Use	-0.62 -0.63 -0.62	*Conchiformibius* *Proteobacteria* *Micrococcineae aeria*
Number of Patients	0.6 0.63 0.62 0.7	*Vibrionaceae* *Propionibacteriaceae* *Delftia* *Acinetobacter*

**MRSA=methycillin-resistant Staphylococcus aureus; n/a=not applicable.*

Chapter 5: Conclusions

Key Findings

As beautifully asked by Blaser, "How can we make sense of our microbial and metagenomic diversity and, importantly, use the information to improve the human condition?" (Blaser, 2010). It was the goal of this dissertation to shed insight into the HCW hand microbiota and its role in one aspect of public health within a healthcare setting, that of pathogen carriage.

This dissertation presents key findings that have several potentially important public health implications. From Chapter 2, a conceptual framework for understanding the interactions between skin microbiota, the human host, and the environment, was presented to help organize what host, dispersal, behavior, and environmental factors, or combination thereof, have the potential to drive the variability of the microbial community structure, thereby altering the skin microbiota community structure in such a way to cause disease. Chapters 3 and 4 followed this conceptual framework in their design. Chapter 2 also gave a detailed description of the very diverse human skin microbiota: *Actinobacteria*, *Firmicutes*, and *Proteobacteria*, consistently make up the majority of the endogenous bacteria. The concept of time in sampling for microbiota profiling accuracy was also discussed; microbial communities cluster first by body site, followed by individuals in time.

Finally, Chapter 2 presents some challenges and implications for studying the skin microbiota.

In Chapter 3, analyses of the microbiota found on HCWs' hands who participated in a pilot study called 'Healthy Hands Study', where 34 participants were sampled, indicate that the dynamics of the microbial community structure is dependent on sample collection method. Using the glove-juice method, hands from within an individual HCW were slightly more similar in microbial composition over time than between individual HCWs. Using swabs, samples from a single HCW in time were no more similar to each other than those between HCWs. In addition, the glove-juice method captured higher amounts of known hospital pathogens, such as *Streptococcus*, *Acinetobacter*, and *Pseudomonas*. Other sources of technical variation assessed, specifically DNA extraction techniques and sequencing, were not influential to the microbial community structures.

In Chapter 4, we learned the important influence the hand microbiota can have on a significant public health problem, nosocomial pathogen carriage among HCWs. We found that risk factors for pathogen carriage among HCWs were dependent on the specific pathogen present: frequency of alcohol rub use, frequency of glove use, and time within work shift (i.e. start, middle, or end) were associated with *Staphylococcus aureus* carriage; and, age and frequency of handwashing, were associated with *Enterococcus* spp. carriage. In addition, the HCW hand microbial community structure was shown to be associated with pathogen carriage: HCWs with a lower hand microbiota diversity were more likely to have a pathogen present on their hands. The HCW hand microbiota may act as a partial mediator in the relationship between hand hygiene and pathogen carriage; and

alternatively, that it may act as an effect modifier in the relationship between certain risk factors and pathogen carriage.

Strengths and Limitations

There are several strengths to this dissertation, most of which concern the design of the pilot study "Healthy Hands". The first, however, is the thorough review outlining the conceptual framework of how the skin microbiota is relevant to the epidemiological study of disease causation. Given the introduction of this concept (Chapter 2), we then used an interdisciplinary approach integrating ecological and laboratory analyses within an epidemiological context, to investigate hand microbiota dynamics among surgical intensive care unit healthcare workers while accounting for technical sources of variation (Chapter 3), and to assess the role hand microbiota play in specific pathogen carriage (Chapter 4). The advantages of taking an interdisciplinary approach to this dissertation are invaluable. Here, we were able to bridge a gap between fields of microbial ecology of the human microbiome and epidemiological practice. The significant implications of this bridge to the public health potential of human microbiome studies cannot be undermined. The design of the 'Healthy Hands' pilot study is another strength to this dissertation, because it accounted for the effect of time in the variation of hand microbiota in HCWs, and not just differences between individuals. The longitudinal sampling, albeit short in time frame, added a level of complexity that allowed us to determine the dynamics of the hand microbiota within and between study participants. Another advantage of 'Healthy Hands' is that the hand microbiota of a particular section of the population - HCWs - were evaluated. This offered direct public health implications. Chapter 3 is enriched with a plethora of comparisons, ensuring that the true biological dynamics of the hand microbiota

observed were done so accounting for technical sources of variation. No other study of the skin microbiome looked at the glove-juice method of sampling, which is a gold standard in the clinical and hand hygiene literature. In addition, this was the first metagenomic study of the human skin microbiome to use the third generation sequencing platform, 'Ion Torrent' PGM. In Chapter 4, we were successful to show how the hand microbiota of HCWs fit into the relationship between certain risk factors pathogen carriage and *S. aureus* carriage and *Enterococcus* spp. carriage. This chapter tied together the microbial ecology of the microbiota, the advantages of qPCR in the detection of pathogen carriage, the survey instrument response of every study participant (100% response rate), and advanced epidemiological methods.

Unfortunately, there were some limitations to this dissertation. The first is the natural limitations of any pilot study. Given the limited time and money available, we were only able to sample 34 HCWs at three points in time. This already yielded a very large amount of samples to study microbiome dynamics, however, when adding the epidemiological component, the sample size interfered with the ability of two of the four models of pathogen carriage, for instance, to converge. Additionally, the lack of consistency concerning the HCWs' hand hygiene practices before sample collection may have contributed to some confounding in the results of the effect of their microbiota on pathogen carriage. Another limitation was the low yield of bacterial DNA retrieved from sampling the skin. Considerable laboratory troubleshooting was done to understand and overcome this limitation, later discovered to be universal among skin microbiome studies. The consequent sequencing techniques implemented were PCR based, itself susceptible to certain limitations (e.g. artifacts, inhibitors, etc). Finally, the questionnaire used to assess

the determinants of pathogen carriage was self-reported, thus potentially suffering from bias, especially in terms of the frequency of hand hygiene practices.

Implications

Understanding the different risk factors for pathogen carriage is important. However, the role of the healthcare workers' hand microbiota in the relationship between risk factors for pathogen carriage and pathogen carriage, is even more important. For instance, it can provide insight into the transmission potential of healthcare workers, resulting in significant hospital infection control implications. Additionally, the hand microbiota community structure can ultimately act as a biomarker of pathogen carriage. That is, knowing how the structure changes in relation to pathogens will possibly inform infection control what to look for or what to expect given a certain community structure. In addition, the bacteria found on healthcare workers' hands might be modified to enhance resistance to colonization by important nosocomial pathogens. Biotechnology companies are already focusing on the idea that probiotics can be protected (or, encapsulated) and incorporated into alcohol rubs as a way to recolonize the hands with good bacteria after killing the bad. And finally, since the glove-juice method of sample collection was shown to capture higher amounts of known hospital pathogens, it may very well be the method of choice in hand hygiene studies in the healthcare setting.

Future Directions

It is our intention that this dissertation informs future researchers about (i) the need to account for technical sources of variation in their characterization of the human microbiota, (ii) the careful consideration of sampling method necessary to adequately

answer their question, and (iii) the utility of implementing the human microbiome into epidemiological studies to further elucidate health and disease, and transmission.

While this dissertation was able to test for several things, it was also very hypothesis generating. This dissertation opens several doors of opportunities for future studies. First, it would be informative to explore the directionality of the association between hand microbiota and pathogen carriage. This would allow us to better understand the role of the hand microbiota in the association between risk factors for pathogen carriage, and pathogen carriage. Second, it would be useful to investigate which members of the microbial communities are key in helping structure the hand microbiota as it responds to the presence of pathogens, or to hand hygiene practice. Third, eventually, it would be helpful to combine these metagenomic data with a functional assessment using transcriptomics, and metabolomics, for a more comprehensive view of the microbiota. Knowing simply what microorganisms are there, while an essential first step, does not fully explain the functions of the microbial community to host health. While Chapter 4 ends with a list of highly correlated OTUs with risk factors for pathogen carriage, as well as with specific pathogens themselves, we cannot be certain that the behavior of the microbial community lies solely on these few members. And last, but certainly not least, we need to involve more epidemiologists into the design and analyses of microbiome studies as they relate to human health and disease. Advances in the field of the human microbiome are happening quickly, from cheaper sequencing technology to more powerful yet user-friendly analytical tools. While this progress moves forward, it is imperative that more epidemiologists become involved into the design and analyses of microbiome studies as they relate to human health and disease. This, is a future direction that the public health

community must embrace. Characterizing human microbiota structure and function in well designed epidemiologic studies will improve our understanding of what makes one host susceptible to a certain pathogen over another, possibly leading to the discovery of diagnostic and prognostic markers.

Appendices

Appendix A - Healthy Hands Study Questionnaire

Study ID____________________
Today's date________________

Illustration by Paul Sahre and Loren Flaherty

University of Michigan, Ann Arbor, MI
Sponsored by Robert Wood Johnson Foundation Health & Society Scholars Small Grant Program

This questionnaire asks about factors which may or may not influence the amount and types of microorganisms that normally live on our hands. Results of the questionnaire will be used for research purposes only. The principal investigators for this study (Mariana Rosenthal, PhD student at the University of Michigan School of Public Health; Dr. Betsy Foxman, Professor at the University of Michigan School of Public Health; Dr. Allison Aiello, Associate Professor at the University of Michigan School of Public Health) will never know your name - only your study identification number. Your answers will not be linked to you and will not have any identifying information. Your responses will not be used in any way that would affect your work record or used against you in any way. If you have any questions about this study please call Mariana Rosenthal at (619)808-3992.

Thank you very much for your participation in our study. If you have any general comments on this survey, please put them here:

These first questions ask about your hand hygiene practices.

1. **During a typical work shift**, about how many times do you (please circle all that apply):

	never	1-5 times	6-20 times	21-40 times	> 40 times	don't know
1a. wash your hands with soap and water?	never [0]	1-5 times [1]	6-20 times [2]	21-40 times [3]	> 40 times [4]	don't know [8]
1b. clean your hands with alcohol rub?	never [0]	1-5 times [1]	6-20 times [2]	21-40 times [3]	> 40 times [4]	don't know [8]
1c. clean your hands with non-alcohol rub, hand sanitizer?	never [0]	1-5 times [1]	6-20 times [2]	21-40 times [3]	> 40 times [4]	don't know [8]
1d. don a pair of gloves?	never [0]	1-5 times [1]	6-20 times [2]	21-40 times [3]	> 40 times [4]	don't know [8]
1e. put lotion or moisturizer on your hands?	never [0]	1-5 times [1]	6-20 times [2]	21-40 times [3]	> 40 times [4]	don't know [8]

2. **During a non-work day at home**, about how many times **a day** do you (please circle all that apply):

	never	1-5 times	6-20 times	21-40 times	> 40 times	don't know
2a. wash your hands with soap and water?	never [0]	1-5 times [1]	6-20 times [2]	21-40 times [3]	> 40 times [4]	don't know [8]
2b. clean your hands with alcohol rub?	never [0]	1-5 times [1]	6-20 times [2]	21-40 times [3]	> 40 times [4]	don't know [8]
2c. clean your hands with non-alcohol rub, hand sanitizer?	never [0]	1-5 times [1]	6-20 times [2]	21-40 times [3]	> 40 times [4]	don't know [8]
2d. don a pair of gloves?	never [0]	1-5 times [1]	6-20 times [2]	21-40 times [3]	> 40 times [4]	don't know [8]
2e. put lotion or moisturizer on your hands?	never [0]	1-5 times [1]	6-20 times [2]	21-40 times [3]	> 40 times [4]	don't know [8]

3. During the **past week**, what type of gloves did you **most often** use? (please circle)

latex, powder-free [1] latex, powdered [2]

nitrile, powder-free [3] nitrile, powdered [4]

vinyl, powder-free [5] vinyl, powdered [6]

other [7], specify____

don't know [8]

These next few questions ask about your health.

4. During the past **6 months**, have you taken any:

4a. Oral antibiotics? (please circle) yes [1] no [0]

4b. If YES, when did you last take these? _mm_/_dd_/_yy_ [1] don't know [8]

4c. Topical antibiotics? (please circle) yes [1] no [0]

4d. If YES, when did you last take these? _mm_/_dd_/_yy_ [1] don't know [8]

4e. Oral steroids? (please circle) yes [1] no [0]

4f. If YES, when did you last take these? _mm_/_dd_/_yy_ [1] don't know [8]

4g. Topical steroids? (please circle) yes [1] no [0]

4h. If YES, when did you last take these? _mm_/_dd_/_yy_ [1] don't know [8]

5. **In general**, would you say your health is: (please circle)

excellent [4] good [3] fair [2] poor [1]

6. **Today**, do you have: (please circle all that apply)

6a. any chronic illnesses? yes [1] no [0]

6b. any infections? yes [1] no [0]

6c. any allergies? yes [1] no [0]

6d. irritant contact dermatitis? yes [1] no [0]

7. **In the last month**, have you had any of these symptoms: (please circle all that apply)

7a. Vomiting yes [1] no [0]

7b. Diarrhea yes [1] no [0]

7c. Fever yes [1] no [0]

7d. Runny Nose yes [1] no [0]

7e. Cough yes [1] no [0]

7f. Sore Throat yes [1] no [0]

7g. Skin Infection yes [1] no [0]

8. On a scale of 1-7, rate the current condition of the skin on your hands: (please circle)

8a. APPEARANCE 1 2 3 4 5 6 7
(abnormal) (normal)

8b. INTACTNESS 1 2 3 4 5 6 7
(many abrasions or fissures) (no abrasions or fissures)

8c. MOISTURE CONTENT 1 2 3 4 5 6 7
(extremely dry) (normal amount of moisture)

8d. SENSATION 1 2 3 4 5 6 7
(extreme itching, burning or soreness) (no itching, burning or soreness)

These next few questions ask about your level of patient contact.

9. How long have you been working as a nurse? _years_/_months_ [1] don't know [8]

10. How long have you been working in this SICU? _years_/_months_ [1] don't know [8]

11. What is your current shift schedule [# shifts per week(s)]?

shifts/_week(s)_ [1] don't know [8]

12. **On average**, how many patients do you **directly** care for during a shift? (please circle)

1-2 patients [1] 3-4 patients [2] 5-6 patients [3] 7-8 patients [4]

9-12 patients [5] 13-16 patients [6] > 16 patients [7] don't know [8]

13. How many times **during your last shift** have you (if this is the end of your shift please respond based on this shift): (please circle all that apply)

	never	1-4 times	5-9 times	> 10 times	don't know
13a. performed a physical examination on patients?	never [0]	1-4 times [1]	5-9 times [2]	> 10 times [3]	don't know [8]
13b. performed a wound dressing on patients?	never [0]	1-4 times [1]	5-9 times [2]	> 10 times [3]	don't know [8]
13c. performed a blood draw on patients?	never [0]	1-4 times [1]	5-9 times [2]	> 10 times [3]	don't know [8]
13d. bathed and/or performed another form of hygiene on patients?	never [0]	1-4 times [1]	5-9 times [2]	> 10 times [3]	don't know [8]
13e. used an intravascular catheter, urinary catheter, drain, and/or endotracheal tube, or cared for any of these sites on patients?	never [0]	1-4 times [1]	5-9 times [2]	> 10 times [3]	don't know [8]
13f. checked vital signs on patients?	never [0]	1-4 times [1]	5-9 times [2]	> 10 times [3]	don't know [8]
13g. touched or cleaned a soiled bed pan?	never [0]	1-4 times [1]	5-9 times [2]	> 10 times [3]	don't know [8]
13h. touched or cleaned a soiled sheet/linen/patient gown?	never [0]	1-4 times [1]	5-9 times [2]	> 10 times [3]	don't know [8]
13i. turned patients (to prevent skin sores)?	never [0]	1-4 times [1]	5-9 times [2]	> 10 times [3]	don't know [8]

These last questions ask about basic demographic information.

14. What is your gender? (please circle) Male [0] Female [1]

15. In what month and year were you born? _mm_/_yy_ [1]

16. What is your race/ethnicity? (please circle all that apply)

White [1] Hispanic [2] Black/African American [3] Native American [4]

Asian [5] Pacific Islander [6] Other [7], specify___________________________

17. What is your country of birth?

____ [1]

18. What is your job title?

____ [1]

19. Do you have any children < 5 years old living in your household? (please circle)

yes [1] no [0]

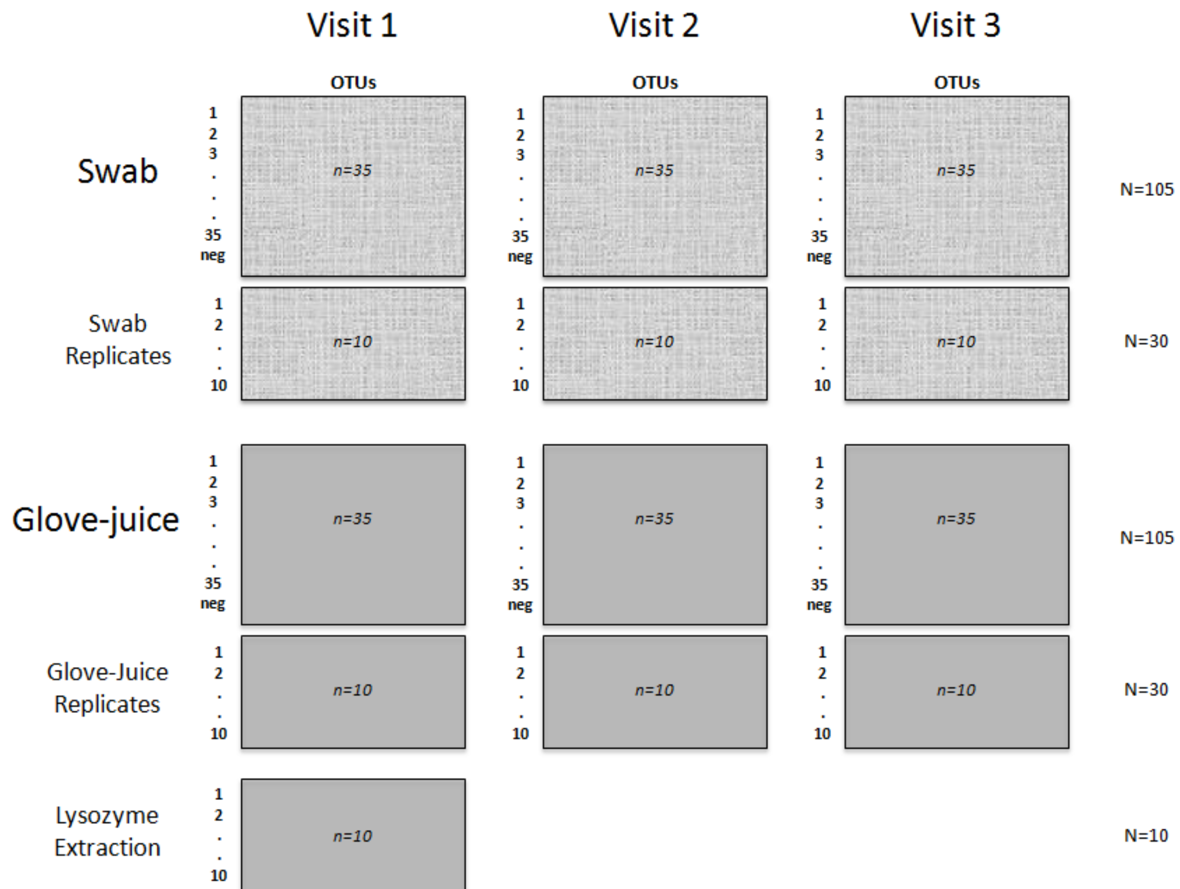
20. If you answered 'YES' for question 19, what is/are their age(s), and do they attend daycare, homecare, and/or pre-school? (please circle all that apply)

20a.	_mm_/_yy_ [1]	Daycare [2]	Homecare [3]	Pre-school [4]
20b.	_mm_/_yy_ [1]	Daycare [2]	Homecare [3]	Pre-school [4]
20c.	_mm_/_yy_ [1]	Daycare [2]	Homecare [3]	Pre-school [4]
20d.	_mm_/_yy_ [1]	Daycare [2]	Homecare [3]	Pre-school [4]
20e.	_mm_/_yy_ [1]	Daycare [2]	Homecare [3]	Pre-school [4]

Appendix B - Chapter 3 Supplementary Materials

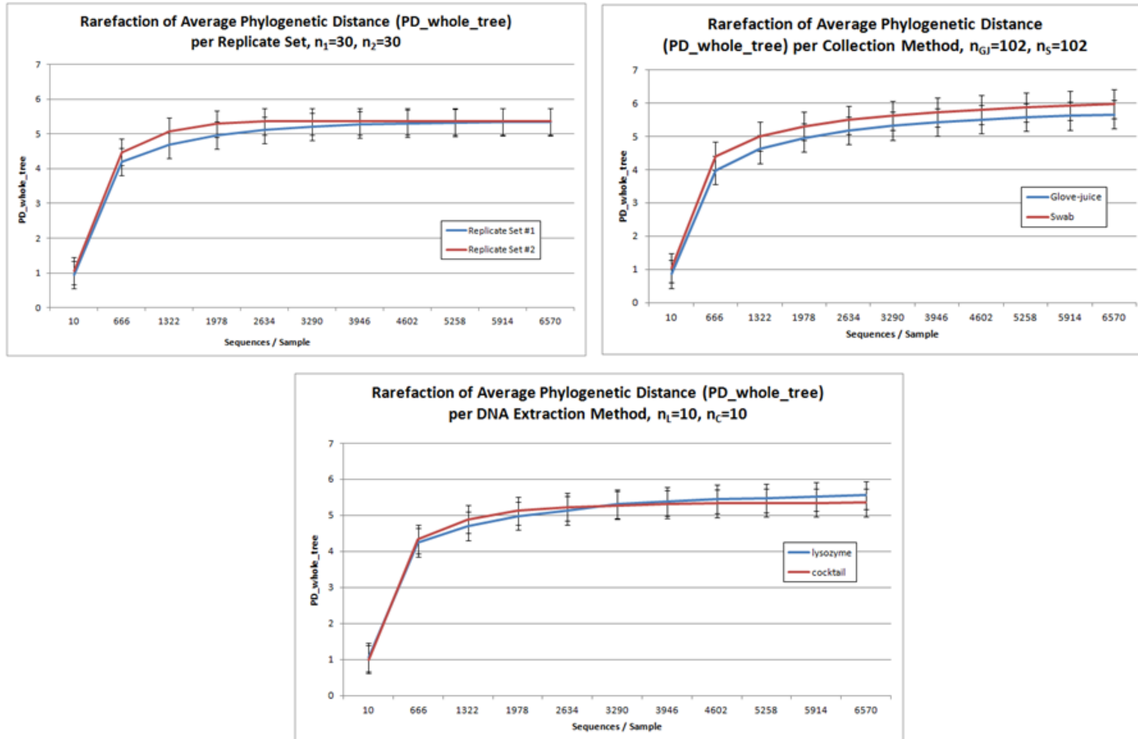
An overview of the organization of the hand microbiota samples collected in the study shows a total of 280 samples sent for sequencing [Supplemental Figure 1].

Figure 1. Organization of the 280 Hand Microbiota Samples from 34 Healthcare Workers at the University of Michigan Surgical Intensive Care Unit, July 5-28, 2011, Sent for Sequencing.



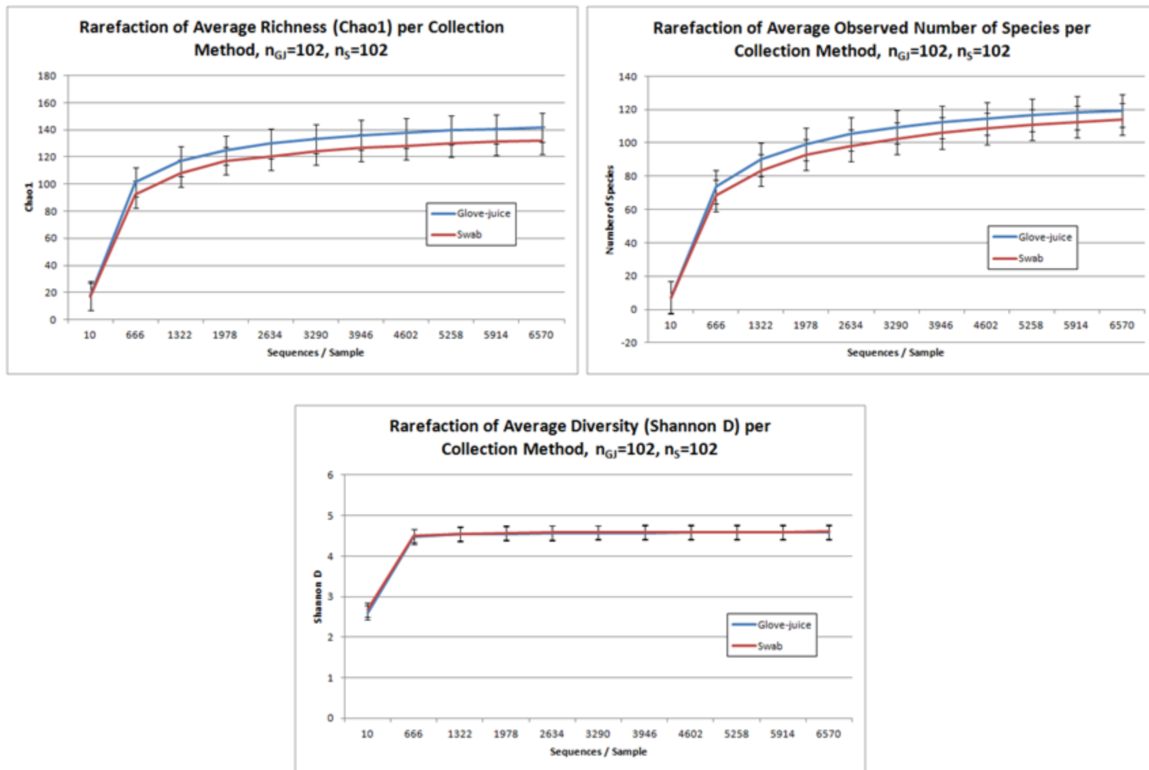
Rarefaction curves of phylogenetic distance show that the average alpha diversity is equivalent for both sets of sequencing replicates, and slightly yet not significantly different by collection method and DNA extraction technique [Supplemental Figure 2].

Figure 2. Rarefactions of Phylogenetic Distance (PD_whole_tree) between the Comparison Groups



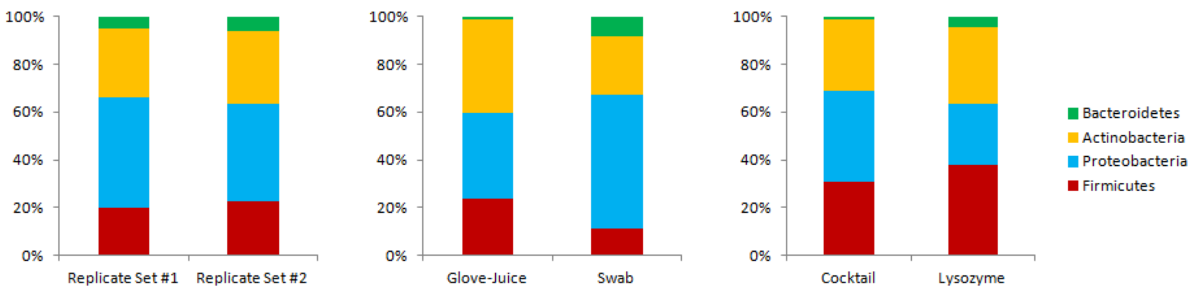
Measures of average species richness and number of observed species appear higher for samples collected via the glove-juice method, while the average species diversity seemed equal regardless of collection method [Supplemental Figure 3].

Figure 3. Rarefaction Curves of Alpha Diversities per Collection Method



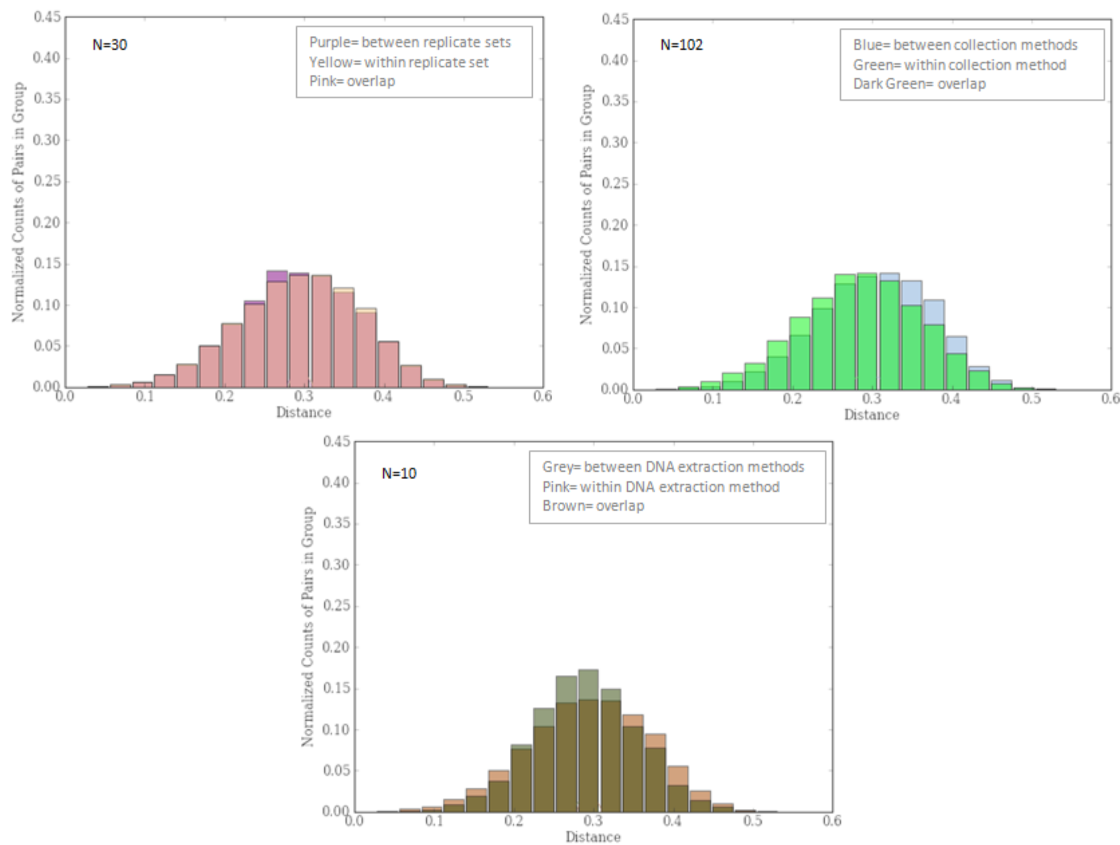
Grouping the OTUs by samples and different taxonomic levels shows that the replicates were composed of Proteobacteria (46.7% and 40.4%), Actinobacteria (28.9% and 30.5%), Firmicutes (19.7% and 22.4%), and Bacteroidetes (4.7% and 6.1%) in a similarly proportioned fashion [Supplemental Figure 4]. Glove-juice and swab samples were composed of Proteobacteria (35.9% and 56.2%), Actinobacteria (38.8% and 24.6%), Firmicutes (23.8% and 11.0%), and Bacteroidetes (1.4% and 8.0%), respectively [Supplemental Figure 4]. While the relative phylum abundance appeared to vary similarly by healthcare worker, samples collected via swab consisted predominantly of Proteobacteria, whereas those collected via glove-juice had a more even distribution among the top three phyla (Proteobacteria, Firmicutes, and Actinobacteria). Samples with DNA extracted using enzyme cocktail and those using lysozyme only were composed of Proteobacteria (38.2% and 25.5%), Firmicutes (30.6% and 37.6%), Actinobacteria (29.7% and 31.9%), and Bacteroidetes (1.5% and 4.5%), respectively.

Figure 4. Average Relative Phylum Abundance per Comparison Groups



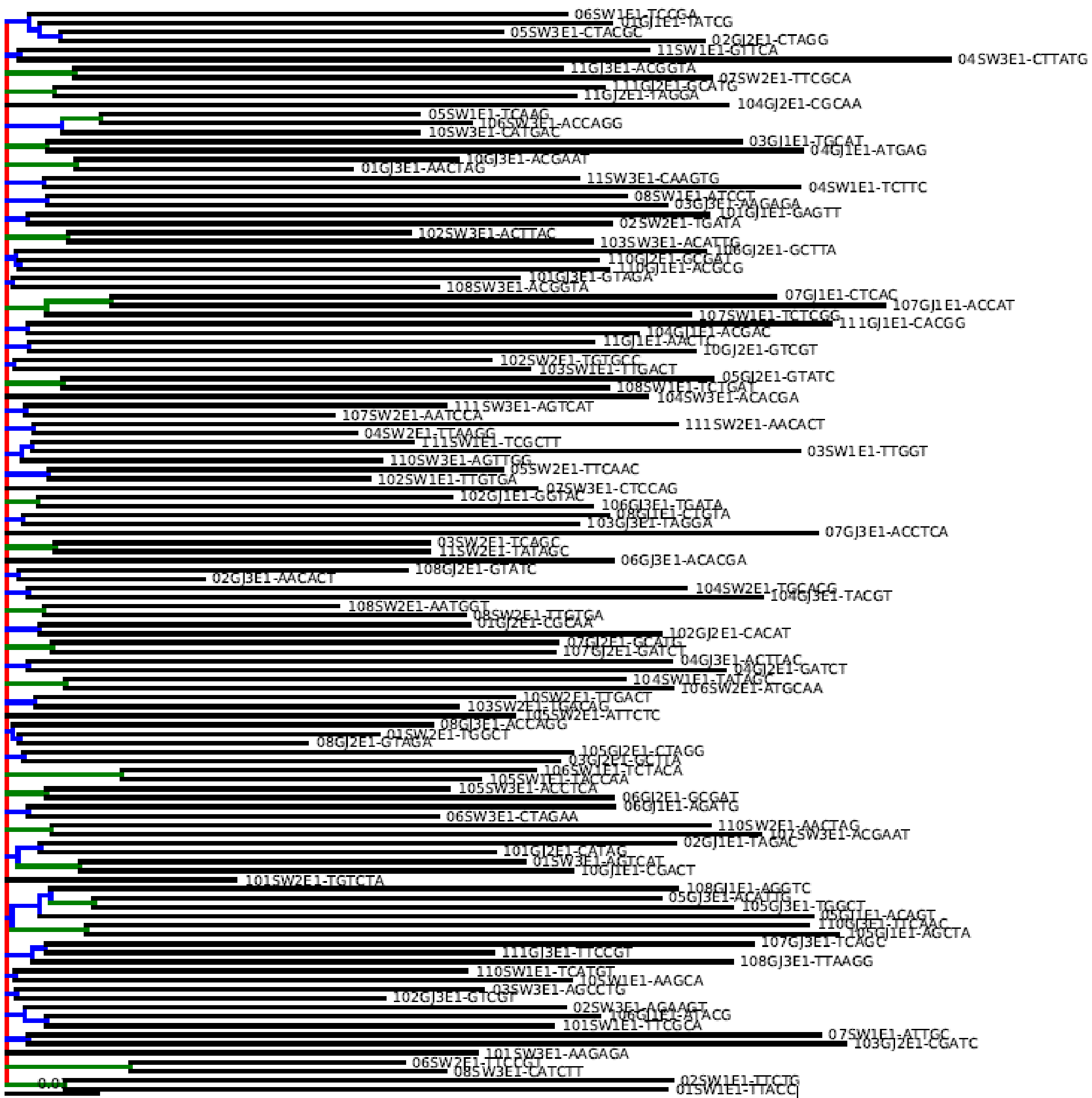
Distance (weighted unifrac) histograms comparing both sets of replicates showed that the distribution of distances within the replicate sets was not significantly different than the distribution of distances between the sets [Supplemental Figure 5]. The distribution of distances within sampling collection method was slightly shifted from the distribution of distances between sampling collection method. The distribution of distances within DNA extraction method was slightly different than the distribution of distances between them. Specifically, then mean distance between extraction methods was higher, and the two distributions were also shifted.

Figure 5. Distribution of Weighted Unifrac Distances Between and Within Each Comparison Group



Additionally, the bootstrapped UPGMA tree does not show any hierarchical clustering by replicate set [Supplemental Figure 6]. The bootstrapped tree is shown with the internal nodes colored red for 75-100% support, yellow for 50-75%, green for 25-50%, and blue for <25% support.

Figure 6. Bootstrapped UPGMA Tree of the Replicate Sets

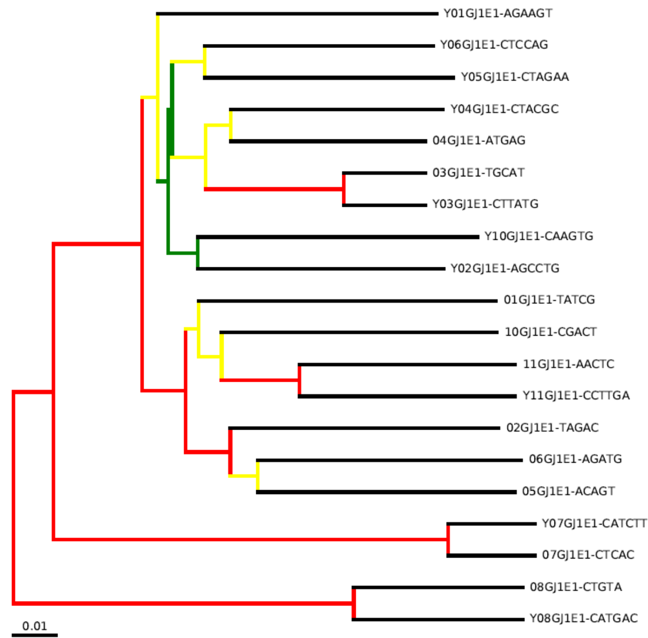


A bootstrapped UPGMA tree shows some hierarchical clustering by sampling collection methods [Supplemental Figure 7]. The bootstrapped tree is shown with the internal nodes colored red for 75-100% support, yellow for 50-75%, green for 25-50%, and blue for <25% support.

Figure 7. Bootstrapped UPGMA Tree of the Sampling Collection Methods

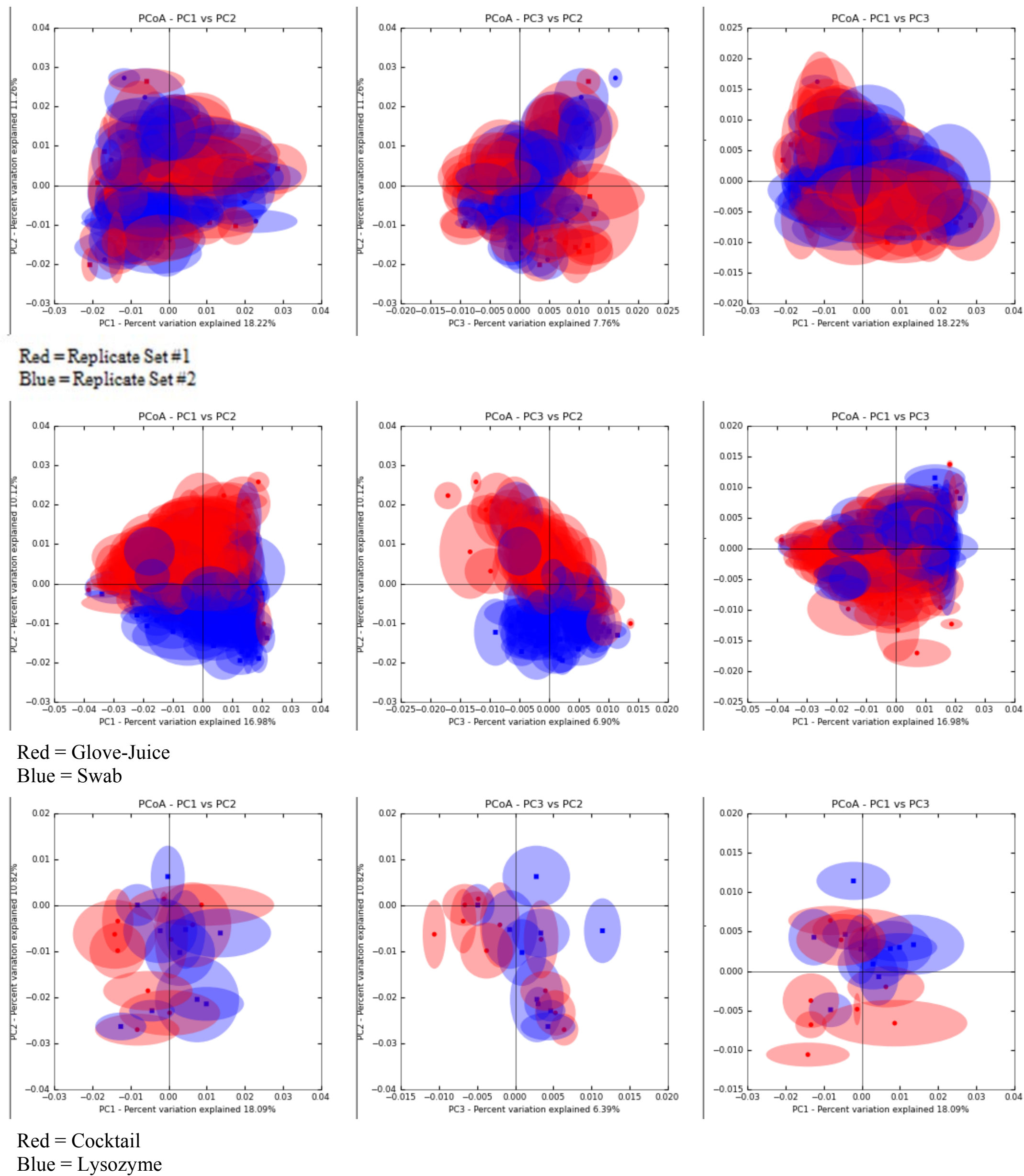
A bootstrapped UPGMA tree shows some hierarchical clustering by DNA extraction method [Supplemental Figure 8]. The bootstrapped tree is shown with the internal nodes colored red for 75-100% support, yellow for 50-75%, green for 25-50%, and blue for <25% support.

Figure 8. Bootstrapped UPGMA Tree of the DNA Extraction Methods



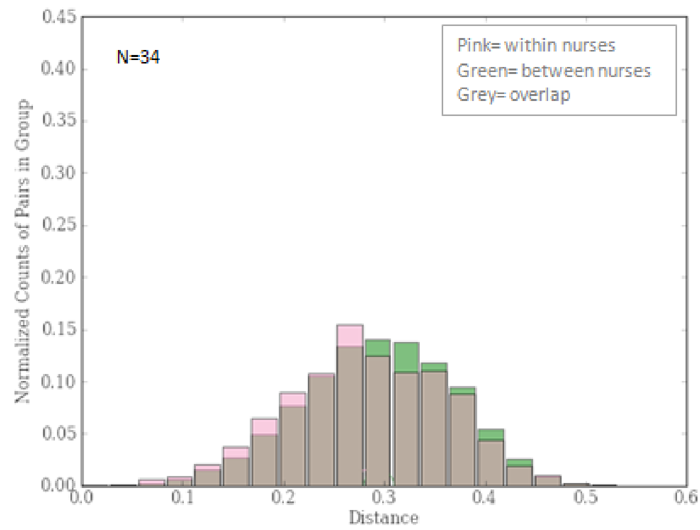
PCoA performed with jackknife bootstrapping shows considerable overlapping of both replicate sets as well as DNA extraction methods, but relative clustering by sampling collection method [Supplemental Figure 9].

Figure 9. 3D Jackknifed Principal Coordinate Analysis (weighted unifrac) per Replicate Set, Sampling Collection Method, and DNA Extraction Method.



Distance (weighted unifrac) histograms showed that the distribution of distances within healthcare workers are slightly shifted from the distribution of distances between healthcare workers [Supplemental Figure 10]. Specifically, distances between healthcare workers' appear, on average, greater than distances within.

Figure 10. Distribution of Weighted Unifrac Distances Between and Within Healthcare Workers



Appendix C - Chapter 4 Supplementary Materials

DNA Preparation for Sequencing

The bacterial V6 rRNA region was amplified with the left-side primer CWACGCGARGAACCTTACC and the right-side primer ACRACACGAGCTGACGAC. These primer sequences are exact matches to >95% of the rRNA sequences from organisms identified in the human microbiome project (GBG, unpublished observations). The left-side primers contained the standard Ion Torrent (Ion Torrent Systems, Guilford, CT, USA) adapter and key sequence at their 5′ end (CCATCTCATCCCTGCGTGTCTCCGACTCAG). The right-side primer had the other standard Ion Torrent adapter sequence (CCTCTCTATGGGCAGTCGGTGAT) attached to its 5′ end. Amplification was performed for 25 cycles in 40 μl using the colorless GO-Taq hot start master mix (Promega; #M5133) according to the manufacturer's instructions with the following three-step temperature profile: 95°C, 55°C and 72°C for 1 minute each step. 5 μl of the resulting amplification were quantified using the QuBit broad-range double-stranded DNA fluorometric quantitation reagent (Invitrogen Corp.; #Q32854). Samples were pooled at approximately equal concentrations and purified using a Wizard PCR Clean-Up Kit (Promega; #A9285).

DNA Sequencing and Sequence Reads Filtering

Sequencing reactions were carried out on three Ion Torrent 316 platform chips, multiplexing up to 96 samples per run using the 200 bp sequencing reagent kit. Data from

all runs were pooled. The sequence was provided in fastq format. All sequences were filtered according to the following criteria in order: exact match to the left-side primer including redundant positions in the primer, exact matches to the barcodes used, an exact match to the first six nucleotides of the right-side primer, and a length between the left-side and right-side primer of between 71 and 90 nucleotides. This length was chosen because it encompasses the predicted amplicon product size from all human-associated bacterial organisms that have been cultured and sequenced as part of the HMP. Table 1 shows the number of raw and filtered reads obtained from each run. Run number 3 had the least number of sequences because of sub-optimal loading efficiency. However, as the reproducibility of the Ion Torrent platform for these types of analyses is excellent provided the number of reads per sample is greater than 1000 (Petrof et al, in press at Microbiome), this was not a concern.

Table 1. Number of Raw and Processed Sequencing Reads per Ion Torrent Personal Genome Machine (PGM) Sequencing Run, Using 316 Chips, of 280 Samples of Hand Microbiota from 34 Healthcare Workers at the University of Michigan Surgical Intensive Care Unit, July 5-28, 2011.

Sequencing Run	Raw Sequence Reads	Processed Sequence Reads	Proportion of Processed/Raw Sequence Reads
1	2,787,276	1,292,855	0.464
2	3,160,031	2,132,925	0.675
3	903,240	643,015	0.712

Between 46 to 71% of the reads passed these filters; reads not passing the filters were not examined further. Reads were processed as previously described (Gloor et al, 2010) except that clustering with USEARCH was performed at 97% identity. Chimera detection was performed with UCHIME (version v5.2.32) using the de novo method (Edgar

et al, 2011). Chimeric sequences in less than 0.05% in any sample (see below) and were discarded. A table of counts for sequences grouped at 100% identical sequence unit (ISU) identity level were generated for each sample (Gloor et al, 2010), keeping all sequences that were represented in any sample at a frequency >0.5%. Reads that were never abundant in any sample (<0.5%) were grouped into the remainder and discarded.

Taxonomic classification

Classification of the sequences by either the Greengenes or RDP classifiers proved to be unreliable because of the short length of the V6 region. Classification of the sequences present in the count table was therefore performed using the RDP closest match option on the full-length, high-quality, isolated subset. The 20 best hits were identified, and the taxonomic classification of the best match and ties was collected. The classification of those hits was adopted for all levels where the classification was identical across all best matches, otherwise the classification was marked as undefined. The taxonomic classification was added to the sequence count table and the data were presented in formats that could be accepted by QIIME 1.5.0 (Caporaso et al, 2010) as follows. Sequence alignments were built using Muscle (Edgar RC, 2004) and a neighbor-joining tree was generated by ClustalW2 (Larkin et al, 2007).

Detection of Pathogen Co-occurrence

The proportion of nosocomial pathogens that co-occurred over the three collection visits were as follows:

Table 2. Proportion of Pathogen Co-Occurrence Among 34 Healthcare Workers at the University of Michigan Surgical Intensive Care Unit, July 5-28, 2011.

Proportion of Positive Pathogen Co-Occurrence (%, n=34)			
	Visit 1	Visit 2	Visit 3
S.aureus - *Enterococcus* spp.	29.4	44.1	35.3
S. aureus - *C. albicans*	5.9	2.9	2.9
S. aureus - MRSA	2.9	2.9	2.9
Enterococcus spp. - *C. albicans*	8.8	2.9	5.9
Enterococcus spp. - MRSA	2.9	2.9	5.9
C. albicans - MRSA	0	2.9	0

Raw Results of Survey Instruments

Table 3. Self-Reported Demographic Characteristics among 34 Healthcare Workers at the University of Michigan Surgical Intensive Care Unit, July 5-28, 2011.

Characteristics	N (%)
Gender (n=34)	
Male	8 (23.5)
Female	26 (76.5)
Age (n=34; mean=34.5; median=33)	
20-29 years	9 (26.5)
30-39 years	15 (44.1)
40-59 years	10 (29.4)
Race / Ethnicity	
White	31 (91.1)
Black / African American	1 (2.9)
Other	2 (5.9)
Country of Birth	
United States	31 (91.1)
Other	3 (8.8)
Job Title	
Nurse Aide	4 (11.8)
RN	24 (70.6)
Respiratory Specialist	6 (17.6)
Has children < 5 years old	7 (20.6)

Table 4. Self-Reported Hand Hygiene Practices among 34 Healthcare Workers at the University of Michigan Surgical Intensive Care Unit, July 5-28, 2011.

Hygiene Practices		Per work shift (N,%)	Per non-workday at home (N, %)
Soap and Water Handwashes			
	Never	0	0
	1-5 times	5 (14.7)	9 (26.5)
	6-20 times	18 (52.9)	24 (70.6)
	21-40 times	9 (26.5)	1 (2.9)
	More than 40 time	1 (2.9)	0
	Don't know	1 (2.9)	0
Alcohol Rub Handcleanings			
	Never	1 (2.9)	14 (41.2)
	1-5 times	1 (2.9)	16 (47.1)
	6-20 times	6 (17.7)	4 (11.8)
	21-40 times	10 (29.4)	0
	More than 40 time	14 (41.2)	0
	Don't know	2 (5.9)	0
Non-Alcohol Rub Handcleanings			
	Never	17 (53.1)	26 (78.8)
	1-5 times	4 (12.5)	4 (12.1)
	6-20 times	2 (6.3)	1 (3.0)
	21-40 times	1 (3.1)	0
	More than 40 time	3 (9.4)	0
	Don't know	5 (15.6)	2 (6.1)

Hygiene Practices		Per work shift (N,%)	Per non-workday at home (N, %)
Pairs of Gloves Donned			
	Never	0	29 (87.9)
	1-5 times	1 (2.9)	4 (12.1)
	6-20 times	2 (5.9)	0
	21-40 times	9 (26.5)	0
	More than 40 times	21 (61.8)	0
	Don't know	1 (2.9)	0
Used Hand Lotion / Moisturizer			
	Never	6 (17.7)	4 (12.1)
	1-5 times	22 (64.7)	25 (75.8)
	6-20 times	4 (11.8)	4 (12.1)
	21-40 times	0	0
	More than 40 times	0	0
	Don't know	2 (5.9)	0
Glove Type Most Often Used			
	Latex, powder-free	9 (27.3)	n/a
	Nitrile, powder-free	21 (63.6)	n/a
	Vinyl, powder-free	3 (9.1)	n/a

Table 5. Visual Scoring of Skin Scale (VSS) of 34 Healthcare Workers at the University of Michigan Surgical Intensive Care Unit, July 5-28, 2011.

VSS	N (%)
normal	12 (35.3)
very slightly scaly	16 (47.1)
slightly scaly	6 (17.7)
scaly	0
scaly to very scaly	0
very scaly	0

Note: Visually inspected the skin on the dominant hand with a magnifying glass. Cronbach's alpha = 0.75; ICC=0.59 (95%CI: 0.09, 0.86).

Table 6. Self-Reported Hand Skin Assessment (HSA) of 34 Healthcare Workers at the University of Michigan Surgical Intensive Care Unit, July 5-28, 2011.

	Current appearance* of skin on hands, N(%)	Current intactness# of skin on hands, N(%)	Current moisture content¤ of skin on hands, N(%)	Current sensationϒ of skin on hands, N(%)
1	0	0	0	0
2	0	1 (2.9)	0	0
3	1 (2.9)	1 (2.9)	4 (11.8)	1 (2.9)
4	2 (5.9)	3 (8.8)	9 (26.5)	2 (5.9)
5	2 (5.9)	5 (14.7)	7 (20.6)	2 (5.9)
6	19 (55.9)	13 (38.2)	11 (32.4)	7 (20.6)
7	10 (29.4)	11 (32.4)	3 (8.8)	22 (64.7)

** On a scale from 1 to 7, from abnormal (red, blotchy, rash) to normal (no redness, blotch, rash).*
 # On a scale from 1 to 7, from many abrasions or fissures to completely intact (no abrasions or fissures).
¤ On a scale from 1 to 7, from extremely dry to normal amount of moisture.
ϒ On a scale from 1 to 7, from extreme itching, burning, or soreness to no itching, burning, or soreness.

Table 7. Self-Reported Health Status of 34 Healthcare Workers at the University of Michigan Surgical Intensive Care Unit, July 5-28, 2011.

Characteristics	N (%)
Health Status	
Excellent	16 (47.1)
Good	17 (50)
Fair	1 (2.9)
Poor	0
Current	
Chronic Illness	3 (8.8)
Infections	1 (2.9)
Allergies	6 (17.7)
Irritant Contact Dermatitis	1 (2.9)
Within the last month	
Vomiting	1 (2.9)
Diarrhea	4 (11.8)
Fever	0
Runny Nose	3 (8.8)
Cough	2 (5.9)
Sore Throat	0
Skin Infections	1 (2.9)
In the past six months	
Oral Antibiotic	0
Topical Antibiotic	0
Oral Steroids	0
Topical Steroids	1 (2.9)

Table 8. Level of Patient Contact Attained During Last Work Shift, Reported by 34 Healthcare Workers at the University of Michigan Surgical Intensive Care Unit, July 5-28, 2011.

Patient Contact	N (%)
Performed Blood Draw	
Never	5 (14.7)
1-4 times	14 (41.2)
5-9 times	11 (32.4)
10 or more	4 (11.8)
Performed Wound Dressing	
Never	8 (23.5)
1-4 times	25 (73.5)
5-9 times	1 (2.9)
10 or more	0
Used (or cared for sites) IV, urinary catheters, drain, endotracheal tube	
Never	6 (17.6)
1-4 times	13 (38.2)
5-9 times	6 (17.6)
10 or more	9 (26.5)
Performed Physical Exam	
Never	5 (14.7)
1-4 times	19 (55.9)
5-9 times	7 (20.6)
10 or more	3 (8.8)
Turned a Patient	
Never	2 (5.9)
1-4 times	7 (20.6)
5-9 times	6 (17.6)
10 or more	19 (55.9)

Patient Contact	N (%)
Took Vital Signs	
Never	1 (2.9)
1-4 times	7 (20.6)
5-9 times	1 (2.9)
10 or more	25 (73.5)
Handled Soiled Bedpan	
Never	13 (38.2)
1-4 times	17 (50)
5-9 times	3 (8.8)
10 or more	1 (2.9)
Handled Soiled Linen	
Never	0
1-4 times	17 (51.5)
5-9 times	5 (15.2)
10 or more	11 (33.3)
Performed Hygiene Functions	
Never	5 (14.7)
1-4 times	21 (61.8)
5-9 times	6 (17.6)
10 or more	2 (5.9)
Avg. No. Patients Directly Cared For During a Shift	
1-2 patients	20 (58.8)
3-4 patients	4 (11.8)
5-6 patients	4 (11.8)
7-8 patients	1 (2.9)
9-12 patients	3 (8.8)
13-16 patients	2 (5.9)

Mixed Models Fitting Criteria

Table 9. Covariance Structure Comparisons of the *Staphylococcus aureus* Carriage Model.

Covariance Structure	Number of Covariance Parameters	AIC	BIC
Unstructured	6	1415.4	1424.5
Compound Symmetry	2	1442.9	1446.0
Compound Symmetry Heterogeneous*	4	1412.6	1421.0
Toeplitz	**3**	**1440.7**	**1445.3**
Toeplitz Heterogeneous*	5	1413.4	1421.0
Autoregressive*	4	1413.0	1419.1

*Convergence criteria met but final hessian is not positive definite.

The model with the Toeplitz(4) R structure was selected as the final model. Among those that had the convergence criteria fully met, it was the most parsimonious model (least parameterized), with the lowest AIC and BIC results. The final nuc model is:

$\log(nuc_i) = (HANDWASH)_i\beta + (ALCOHOL\ RUB)_i\beta + (GLOVE\ USE)_i\beta + (SHIFT)_i\beta + \varepsilon_i$

where, $\varepsilon_i \sim N(0,R_i)$

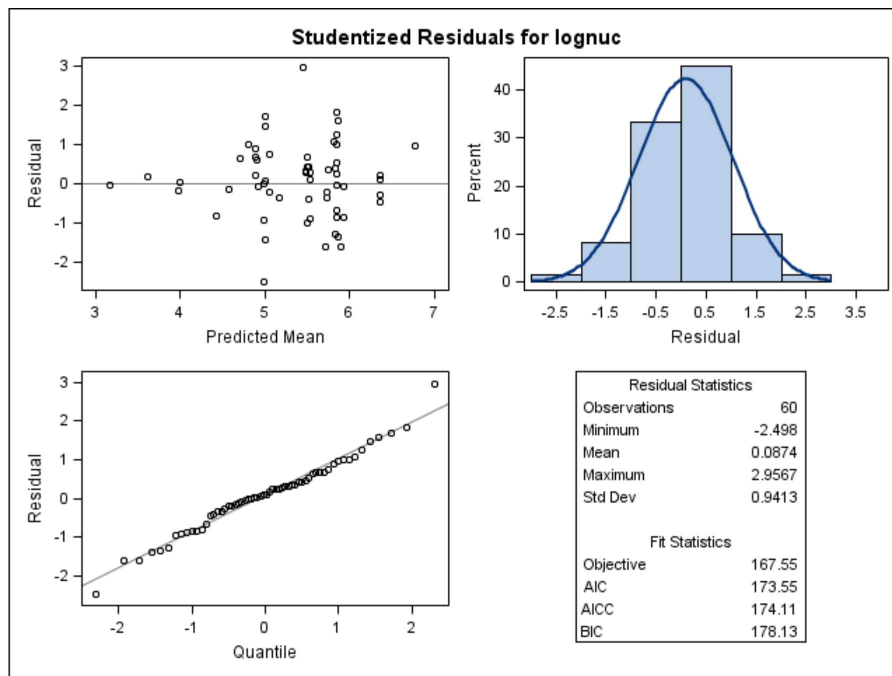
The type 3 tests of fixed effects, showing the statistical significance of each variable given all others listed in the model, are:

Table 10. Type III Tests of Fixed Effects of the *Staphylococcus aureus* Carriage Model.

Effect	Num DF	Den DF	F Value	Pr > F
HANDWASH	3	20	0.48	0.6982
ALCOHOL RUB	4	20	7.86	0.0006
GLOVE USE	3	20	8.59	0.0007
SHIFT	2	15	4.28	0.0339

The residual diagnostics performed were as follows:

Figure 1. Studentized Residuals Diagnostics for the *Staphylococcus aureus* model.



According to the plots, the variability of the standardized residuals appears similar across all predicted values (i.e. homogeneous). Also, the distribution of these residuals is normal (with very few outliers at both ends).

Table 11. Covariance Structure Comparisons of the *Enterococcus* spp. Carriage Model.

Covariance Structure	Number of Covariance Parameters	AIC	BIC
Unstructured	6	1643.6	1652.8
Compound Symmetry	2	1701.9	1704.9
Compound Symmetry Heterogeneous*	4	1643.5	1649.6
Toeplitz	**3**	**1692.4**	**1697.0**
Toeplitz Heterogeneous**	5	-	-
Autoregressive*	4	1640.1	1646.2

*Convergence criteria met but final hessian is not positive definite.
**Convergence criteria not met.

The model with the Toeplitz(4) R structure was selected as the final model. Among those that had the convergence criteria fully met, it was the most parsimonious model (least parameterized), with the lowest AIC and BIC results. The final *Enterococcus* spp. model is:

$\log(Enterococcus_i) = (\mathrm{HANDWASH})_i\beta + (\mathrm{AGE})_i\beta + \varepsilon_i$

where, $\varepsilon_i \sim N(0,R_i)$

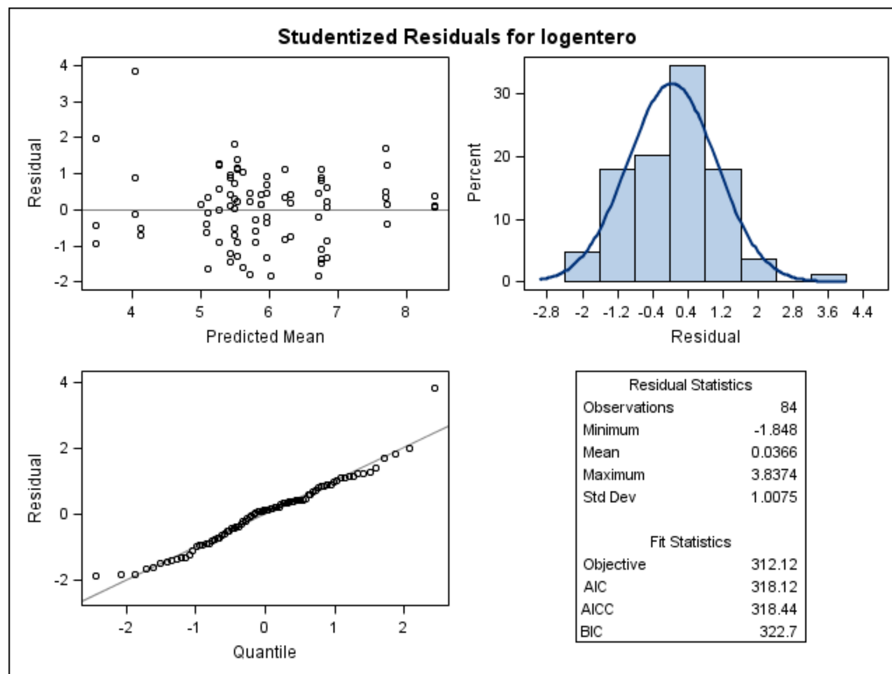
The type 3 tests of fixed effects, showing the statistical significance of each variable given all others listed in the model, are:

Table 12. Type III Tests of Fixed Effects of the *Enterococcus* spp. Carriage Model.

Effect	Num DF	Den DF	F Value	Pr > F
HANDWASH	3	28	4.95	0.0070
AGE	1	28	29.24	<.0001

The residual diagnostics performed were as follows:

Figure 2. Studentized Residuals Diagnostics for the *Enterococcus* spp. model.



According to the plots, the variability of the standardized residuals appears similar across all predicted values (i.e. homogeneous). Also, the distribution of these residuals is somewhat normal (with few outliers at both ends).

The following mixed model predicting the first principal components of the hand microbiota principal coordinate analyses, by the fixed effects of the significant risk factors

for pathogen carriage, as well as levels of pathogen carriage, resulted in no statistically significant associations:

$$PC_i = (\log \textit{S. aureus})_i\beta + (\log \textit{Enterococcus}\ \text{spp.})_i\beta + (\text{HANDWASH})_i\beta + (\text{ALCOHOL RUB})_i\beta + (\text{GLOVE USE})_i\beta + (\text{AGE})_i\beta + (\text{SHIFT})_i\beta + \varepsilon_i$$

where, $\varepsilon_i \sim N(0,R_i)$

The type 3 tests of fixed effects, showing the statistical significance of each variable given all others listed in the model, are:

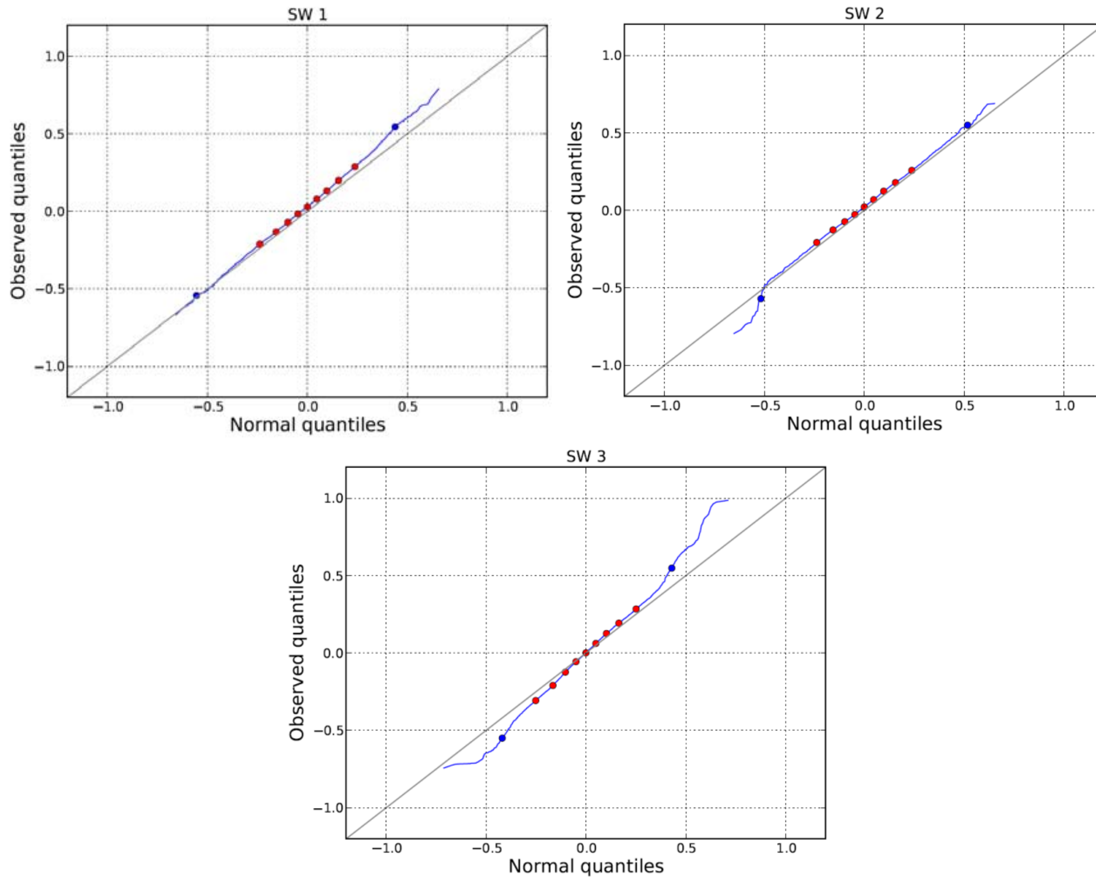
Table 13. Type III Tests of Fixed Effects of the First Principal Component of the Principal Coordinate Analysis of the Hand Microbiota Model.

Effect	Num DF	Den DF	F Value	Pr > F
log *S. aureus*	1	24	1.81	0.1905
log *Enterococcus* spp.	1	24	0.01	0.9145
HANDWASH	3	19	1.18	0.3452
ALCOHOL USE	4	19	1.66	0.2005
GLOVE USE	3	19	0.65	0.5939
AGE	1	19	0.12	0.7337
WORK SHIFT	2	15	2.01	0.1687

Comparison of OTU Correlations

The comparison of the raw correlations (between OTUs and potential pathogens and between OTUs and potential risk factors for pathogen carriage) with that of the Fisher transformed correlations in the following Q-Q plots indicate that in terms of individual OTUs (as opposed to the microbial community structure as a whole), swab sampling time point matters, especially concerning collection visit 3 where deviations in the end-points indicate higher than expected correlations.

Figure 3. Q-Q Plots Comparing the Distribution of Raw Correlation Coefficients with Fisher Transformed Correlation Coefficients, of the Associations Between Each Operational Taxonomic Unit (OTU) and Each Potential Pathogen and Risk Factor for Pathogen Carriage, by Swab Collection Visit.



Note: The red points indicate deciles; the blue points indicate +/- 0.5 correlation.

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