QUANTIFICATION OF THE BACTERIA ON HUMAN SKIN

by

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A THESIS

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Approved: _____

Brendan James Marc Bohannan

The skin is a primary interface for human-microbe interaction. Most studies are concerned with determining what bacterial taxa inhabit the skin, rather than the absolute quantities of bacteria inhabiting environmentally distinct dry, moist, and sebaceous skin habitat types. Additionally, few studies have used culture-independent methods to estimate bacterial abundance, and none yet published have determined what proportions of these communities are viable. Described here is the first study comparing colony counting, qPCR, and fluorescence microscopy for quantifying both viable and non-viable skin-associated bacteria. Data from colony counts and fluorescence microscopy showed that there are significantly more bacterial cells/cm^2 of skin sampled from sebaceous sites than from dry or moist sites, whereas qPCR showed no difference in the quantity of 16S amplicons between all three sample types. We found from fluorescence microscopy that the lowest cell viability occurred at sebaceous skin sites (6.78%) and the highest at moist sites (15.0%). Additionally, microscopy provided us with estimates of cell density (~10^7 cells/cm^2) that are similar to what has already been reported by other studies of the skin microbiome. These results provide foundational knowledge about the skin microbiome, with potential implications for the study of ecology and human health.

Acknowledgements

I would like to express my sincerest gratitude to all those who helped me complete this project, especially Ashley Bateman and Dr. Brendan Bohannan. Their mentorship has been unparalleled and I cherish the opportunity to have worked with such intelligent, kind people. I would also like to thank Dr. Timothy Williams for serving on my Thesis committee and being an excellent professor who helped hone my writing skills. I also appreciate all the feedback and technical assistance that has been provided to me by members of the Bohannan, Green, and Eisen labs at the University of Oregon. The following groups also played a monumental role in supporting my research: Oregon Undergraduate Researchers in SPUR, GrEBES, UROP, and UO Genomics Core. Finally, I would like to thank my family, especially my parents and sister, for their constant support and love.

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Quantification of the bacteria on human skin

Introduction

When you think about the word “ecology,” what comes to mind? Perhaps a scene of the quintessential African savannah: zebra and water buffalo drinking at a watering hole, while predatory crocodiles hide in the shallows? Alternatively, you may think about an endangered species, such as the Bornean Orangutan, because ecology and conservation biology are closely intertwined. While these are both perfectly valid interpretations of the word ecology, it is unlikely that you thought of the word “bacteria”; however, the interactions that bacteria have with each other, their environment, and other types of organisms are crucial for sustaining life as we know it.

While ecology can be broadly defined as the study of interactions between organisms and their environment, the science of microbial ecology encompasses how microorganisms, such as bacteria and viruses, assemble to form communities, and how these communities affect each other and their environment (Cain *et al.* 578, Madigan *et al*. 643). The study at hand will focus on the ecology of the bacterial populations of human skin, but first, it is worth reviewing what is already known about the bacteria inhabiting the skin (collectively termed the skin *microbiome* or *microbiota*) and their ecological role.

Background

Any individual comprises not only human cells, but also a variety of microscopic organisms, or microbes, which play a critical role in maintaining human health (Costello *et al.* 2012). Bacteria, viruses, protists, archaea, fungi, and other

eukaryotic microorganisms form the human microbiome, although out of these, bacteria are the best-studied with currently available methods (Ursell *et al.* 2012). While some pathogenic bacteria cause disease when they colonize humans, most bacteria associated with our bodies are commensal, meaning they have no measurable impact on us, or are mutualistic, meaning they confer some benefit (Madigan *et al.* 721). For example, bacteria in the intestines free up nutrients and/or energy from food that would otherwise be unavailable to us (Costello *et al.* 2012). In return for services such as this, we provide our resident bacteria with a place to live, feed, and reproduce.

It is important to understand that bacteria are not distributed uniformly throughout the human body – they show both numerical and compositional patterns of diversity. For example, the quantity and types of bacteria that live within the gut are distinct from those that inhabit the other relatively well-studied habitats such as the skin, vagina, and oral cavity. To illustrate this point further, approximately 98% of all bacteria residing in the gut fall within the phyla Firmicutes (64%), Bacteroidetes (23%), Proteobacteria (8%), or Actinobacteria (3%), and it is estimated that there are 10^{11}-10^{12} bacterial cells per gram of intestinal constituents (Madigan *et al.* 738-9). On the contrary, a survey of skin-associated bacterial communities determined that roughly 92.3% of all bacteria were related to the same four phyla mentioned above, but were present in different proportions: Actinobacteria (36.6%), Firmicutes (34.3%), Proteobacteria (11.9%) and Bacteroidetes (9.5%) (Costello *et al.* 2009).

In addition to the bacterial community variation that exists between body habitats, there is also be variation within a single habitat – the skin serves as an excellent example of this phenomenon. The total surface area of the skin is quite large

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(~1.5-2 m^2) and consists of microenvironments with distinct pH, moisture content, temperature, sebum content, and topography, among other factors (Oh *et al.* 2014). Multiple researchers have shown that the community composition varies by skin site, summarized in the following figure from Grice *et al*. (2011).

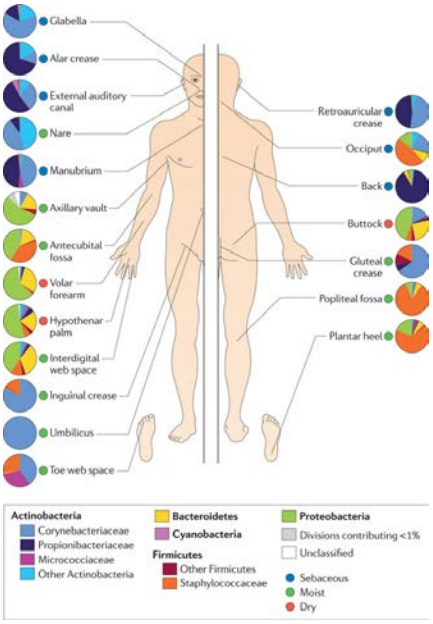

Figure 1. Microbial composition greatly depends on the sampled skin site.

The colors on this figure correspond to the major phylogenetic taxa that were found at each of the sites shown (dry, moist, and sebaceous). From Grice *et al*. 2011.

As shown in Figure 1, bacterial communities are generally more similar to each other if they inhabit the same skin habitat type. Particularly, dry skin sites such as the inner forearm are predominated by *β-proteobacteria* and *Flavobacteriales*; moist sites, such as the area behind the knee, are largely composed of *Corynebacteria* and *Staphylococci* species; and sebaceous sites, like the upper chest, have high proportions of *Propionibacteria* and *Staphylococci* species (Grice *et al*. 2009). These differences in

composition can be partly attributed to the physical characteristics of these skin sites. As the name suggests, sebaceous sites have a high density of sebum-producing glands, which favors the growth of lipophilic, anaerobic *Propionibacteria* species. Conversely, at moist sites there is high density of sudiferous (sweat) glands, and it is likely that *Staphylococci* present there use the urea in sweat as a source of nitrogen (Grice *et al.* 2011). However, while the physical characteristics of these habitats play a major role in determining what bacteria live there, other factors such as the host's genotype, immune defenses, and interactions with other individuals and environmental surfaces are important in shaping microbial community composition (Wilson 2005).

Up until now, we have primarily discussed how both abiotic and biotic factors may shape the bacterial composition of skin microenvironments, but it is likely that they influence the quantity of bacteria inhabiting a particular site as well. Studies concerned with enumerating the skin microbiota are fairly limited, and the ones that do exist mostly relied on culture-based methods, which are biased in the sense that only ~1% of all known bacterial species are cultivable in a laboratory setting (Grice *et al.* 2008). Still, culture-based estimates of skin-associated bacteria range from roughly 10^4 cells/cm^2 at dry sites to 10^7 cells/cm^2 at sebaceous sites, which implies that there is quantitative variation across skin types (Wilson 2005). Yet due to the inherent biases of culture-based methods, the actual number of bacteria inhabiting the skin is probably much greater than these estimates suggest.

Accurately and comprehensively quantifying the microbiota of human skin is a feat largely left unaccomplished. Providing insight into bacterial abundance of the skin is important because knowing how many bacteria occupy a habitat is critical for

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studying the ecological dynamics of colonization and dispersal, and may improve the efficacy of "microbial therapies" such as fecal transplants that some physicians use to treat illness. However, one study published by Gao *et al.* estimated the quantity of cutaneous bacteria using quantitative polymerase chain reaction (qPCR) of the bacterial gene encoding 16S rRNA, a culture-independent method. This is significant because, although qPCR tends to overestimate cell quantity (see Table 1 below), it likely provides a more accurate representation of how many bacteria inhabit the skin, when compared with colony counting. Across all the body sites surveyed, these researchers showed that the quantity of 16S amplicons (i.e., gene replicates) ranged from 10^3-10^7 copies, which falls within the range of cell quantity that culture-based studies have estimated. The greatest density of 16S amplicons were isolated from the axilla (armpits), a moist skin habitat, with 27,500 ± 1.51 copies/µL, while on the forehead (sebaceous), there were 346 ± 1.62 copies/µL, and at the forearm (dry), there were 776 ± 1.50 copies/µL (Gao *et al.* 2010). While this study provides useful baseline estimates of bacteria inhabiting different skin sites, there is still much left to find out about how cell quantity varies at distinct skin habitats.

It is crucial to note, however, that any variation we see in cell quantity between dry, moist, and sebaceous skin sites may vary based on the method of quantification used. We summarized some of the main benefits and limitations to qPCR, fluorescence microscopy, and colony counts in Table 1, but we will expand on some here. First, although qPCR is likely more accurate for estimating cell quantity than culture-based methods, it tends to overestimate the number of cells per sample, mainly because some species of bacteria contain more than one copy of the 16S gene. This bias merits that in

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the future, researchers correct their 16S amplicon data sets for 16S gene copy number (GCN) using bioinformatics (Angly *et al.* 2014). However, correction for GCN requires compositional data in addition to absolute abundance data, which puts additional constraints on researchers. Second, qPCR is limited in that 16S GCN only provides us with a proxy for bacterial cell quantity – probably the best way to be sure of the number of cells in a sample is to count them directly under a microscope. Third, qPCR primers may favorably bind to and facilitate amplification of DNA from certain taxa, skewing results away from the actual quantity of amplicons in a sample (Meisel *et al.* 2016). Finally, as far as we know, no studies have been published that give insight into what proportion of the human skin microbiota is active, dormant, or dead. This is relevant because even dead organisms play important ecological roles such nutrient cycling, providing habitat for other microbes, or preventing colonization of new microbes (Lennon and Jones 2011). Determining what proportions of the skin microbiome are active, dormant, or dead, in conjunction with compositional data, could reveal how many bacteria are functionally beneficial or harmful to human health. Many scientists employ fluorescent dyes during microscopy to ascertain the viability or metabolic activity of cells in a sample. This method can also be used to count cells directly; in fact, microscopy is one of the most commonly-used methods for enumerating bacteria and has been used to quantify bacteria sampled from many different environments. Moreover, bacteria can be counted directly using a microscope, whereas methods like qPCR and colony counting can only provide proxies for bacterial abundance. Therefore, it is likely that fluorescence microscopy will provide us with the most accurate estimation of the number of bacterial cells inhabiting the skin. However, counting

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bacteria with a microscope is very time consuming compared to qPCR and colony counting.

	Method of Quantification		
	qPCR	Fluorescence microscopy	Colony counts
Benefits	- 16S primers bind and facilitate amplification of virtually all bacterial DNA in sample - High-throughput - Relatively fast - Possible to correct total and relative abundance datasets for gene copy number	- Can directly count cells and photograph them for later reference - Cells can be stained to determine if they are alive or dead - Can make observations on cell morphologies and other characteristics of the sample - Computer programs are available to count cells	- Easy to perform - Can make observations on colony morphologies - Fast and inexpensive
Limitations	- Indirect method of quantification - Imperfect doubling - Primers may bind and amplify DNA of certain taxa preferentially - Expensive - Sometimes PCR doesn't work - qPCR cannot distinguish between DNA from live and dead cells	- Hand counting is time consuming - Computer programs may miscount cells - Difficult to view small or moving cells - Brightness (visibility) of the cells may be diminished based on limits of the dyes or properties of the cells	- Very few species are actually culturable in a laboratory setting - Species that are culturable may require special growth conditions

Table 1. Comparison of methods for quantifying bacteria.

Gaps in the current scientific literature regarding the skin microbiome have led us to ask the following questions: first, what are the relative merits of qPCR, colony counting, and fluorescence microscopy for quantifying the microbiota of human skin? Second, how do both the total number of bacteria and the proportion of live and dead bacteria vary by skin type (dry, moist, or sebaceous) and quantification method? We used both qPCR of 16S amplicons and colony counts to estimate total cell quantity, and fluorescence microscopy in tandem with viability stains to determine the relative proportions of live and dead bacteria at each skin type. We expect to see variation between estimates of cell abundance made from each method, with fluorescence microscopy providing estimates most similar to those that have been published in scientific literature regarding the skin microbiome. We hope that our data and

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evaluation of these methods will help future researchers appropriately design their experiments for quantifying any environmental or host-associated bacteria.

Methods

Participant criteria and demographics

Fourteen subjects (six female, eight male) participated in this study. Ages of the participants ranged from 20-31 years. All subjects were required to be free of any skin conditions, not immunocompromised, and to have not used antibiotics (topical or oral) six months prior to sampling. Twelve hours prior to sampling, the participants were asked to refrain from bathing, swimming, or applying anything, such as lotions or cosmetics, to their skin. All participants gave voluntary, informed consent. This protocol has been approved by the Institutional Review Board at the University of Oregon.

Standard sampling procedure

To sample a given skin site for bacteria, we moistened a nylon-flocked swab with sterile sampling solution (0.15 M NaCl, 0.1% Tween20) and flicked excess liquid off the swab tip. Then, a 16 cm^2 area of skin was sampled using a back-and-forth motion while rotating the swab for a period of ~30 seconds. Before further processing, the swab tip was stored briefly in its original container. We chose to use these swabs for sampling because they have been shown to be effective at both capturing and releasing bacteria (Panpradist *et al.* 2014, Probst *et al*. 2010). Furthermore, we believe that the quantity of bacteria captured by swabbing best represents the number of bacteria that would normally be transferred between individuals, within a single individual, or

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between individuals and their environment. It has been estimated from qPCR that these swabs capture ~10,000 cells/cm^2 of skin sampled, and although more invasive sampling methods (like scraping and punch biopsies) yield more 16S amplicons than swabbing, they identify virtually the same bacterial taxa (Grice *et al.* 2008).

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Elution of bacteria from the swab

In a laboratory setting, the "gold standard" for transferring bacteria from the swab tip to liquid solution is vortex mixing (Panpradist *et al.* 2014). After sampling, we used sterile scissors to cut off the swab tip into a tube containing 1 mL "vortex solution" (0.1% Tween20, 0.85% NaCl), then vortexed the tube on medium-high speed for 30 seconds. The tip was then transferred to another tube containing sterile vortex solution and vortexed again for 30 seconds. This process was repeated once more so that three tubes of vortex solution resulted, each containing an unknown quantity of bacteria. We used this protocol because from pilot experiments, we determined that three vortexes of the swab tip were sufficient to elute most of the sampled bacteria.

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Quantification of bacteria with colony counts, qPCR, and fluorescence microscopy

We developed a protocol that would allow us to quantify skin-associated bacteria using colony counts, qPCR, and fluorescence microscopy. Dry (inner forearm), moist (behind the knee), and sebaceous (upper chest) skin sites were sampled using the standard sampling and elution procedures previously described. Figure 2 diagrams our workflow, from sampling to sample processing and analysis with colony counting, qPCR, and fluorescence microscopy.

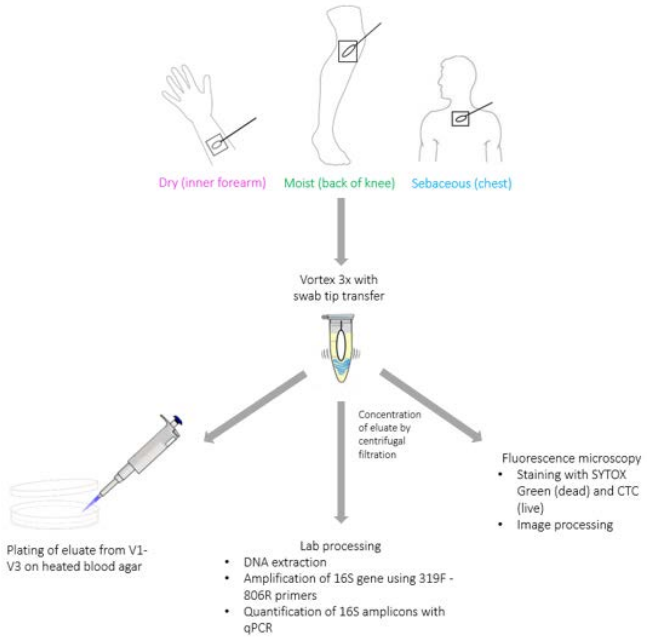

Figure 2. Methods schematic for the quantification of skin-associated bacteria.

Colony counts

For each swab, 100 µL of solution from each of the tubes after each vortex (vortexes 1-3) were plated separately on heated blood agar (HBA) plates. The plates were incubated at 37 °C for roughly 48 hours before counting colonies. Plates yielding greater than 200 colonies were counted using OpenCFU colony-counting software (Geissman 2013). Colony counts enabled us to estimate the number of colony-forming units (CFU) present per cm^2 of skin sampled.

Sample preparation for qPCR and fluorescence microscopy

After plating, the remaining solutions from vortexes 1-3 were combined in a centrifugal filter unit (Amicon Ultra-15, MWCO 30 kDa). The samples were

centrifuged at 4000 rpm for 4 minutes at 23 °C. Then, 100 µL of the pooled, concentrated filtrate was set aside and stored at -80 °C until later DNA extraction. The remaining filtrate for each sample was transferred to a microtube for staining. If less than 1 mL of filtrate remained, an appropriate volume of sterile 0.85% NaCl was added to the sample to bring it up to a volume of 1 mL.

qPCR

DNA was extracted from the entire liquid sample (100 µL) using the MoBio PowerSoil DNA Isolation Kit. Polymerase chain reaction (PCR) was performed on the genomic DNA using Illumina barcoded primers (319F-806R, V3-V4 hypervariable region of the 16S rRNA gene). The thermocycler protocol was as follows: 98 °C for 30 sec, [98 °C for 10 sec; 55 °C for 30 sec; 72 °C for 30 sec]35x, 72°C for 2 min, 4 °C final temperature. The PCR product was cleaned using the Ampure PCR purification protocol with Omega paramagnetic beads. The purified PCR product was diluted by a factor of 1000 before being quantified with the Kapa Biosystems Library Quantification Kit, using the manufacturer-suggested thermocycler protocol. Since our qPCR protocol contains two amplification steps, we could not confidently extrapolate estimates of cells/cm^2 of skin sampled from our data. Instead, we used qPCR to make relative comparisons between the data from dry, moist, and sebaceous sample types.

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Fluorescence microscopy

To each 1 mL cell suspension, 2 uL of SYTOX Green was added for a final dye concentration of 10 uM and incubated in the dark at room temperature for one hour. Next, 40 uL of 50 mM CTC (5-Cyano-2,3-di-(p-tolyl) tetrazolium chloride) was added to the cell suspension for a final concentration of 2 mM and incubated in the dark at

room temperature for 30 minutes. After incubation with the dyes, 10 uL of liquid sample was mixed and loaded into a hemocytometer slide and viewed with a Leica M205 FA dissecting microscope. The optical system for fluorescence observations included filter sets for viewing green fluorescent protein and DsRed (red fluorescent protein). For each sample, three to four images were taken, each at a different region of the smallest grid on the hemocytometer (squares 50 x 50 μm^2). All images were taken at 257X magnification (the highest possible magnification for our microscope) and processed using Leica Advanced Fluorescence software. Visible cells were scored manually in ImageJ.

Results and discussion

Colony counts

After 48 hours of incubation at 37 °C, we counted all visible colonies by hand, or with OpenCFU if there were more than 200 colonies per plate. Although we did observe interpersonal variability in the number of colony-forming units (CFUs) produced, shown below are examples that represent what many plates looked like for dry, moist, and sebaceous samples. Included in Figure 3 are photos that show how CFU quantity generally decreased after each successive vortex of the swab tip. Additionally, while not entirely apparent from the selected photos, we did observe that plates from sebaceous samples were generally dominated by small white colonies, while plates from dry or moist samples tended to have a higher proportion of yellow or pinkish colonies, in addition to white colonies. However, we did not attempt to identify taxa by colony morphology or any other methods in this study.

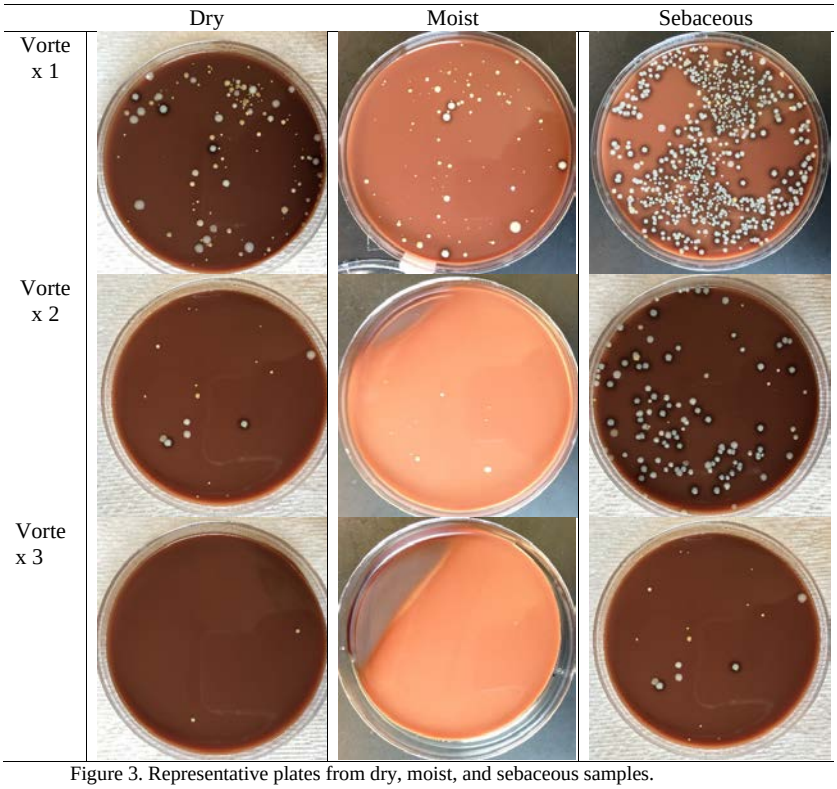

Figure 3. Representative plates from dry, moist, and sebaceous samples.

After swabbing the skin, bacterial samples were plated on HBA and incubated for 48 h at 37 °C before counting colonies. Vortexes 1, 2, and 3 show that, generally, the number of CFUs decreased after each successive vortex of the swab.

After counting colonies, we calculated the average number of CFUs per cm^2 of skin sampled for each skin type (dry, moist, or sebaceous). These results are presented in the Figure 4.

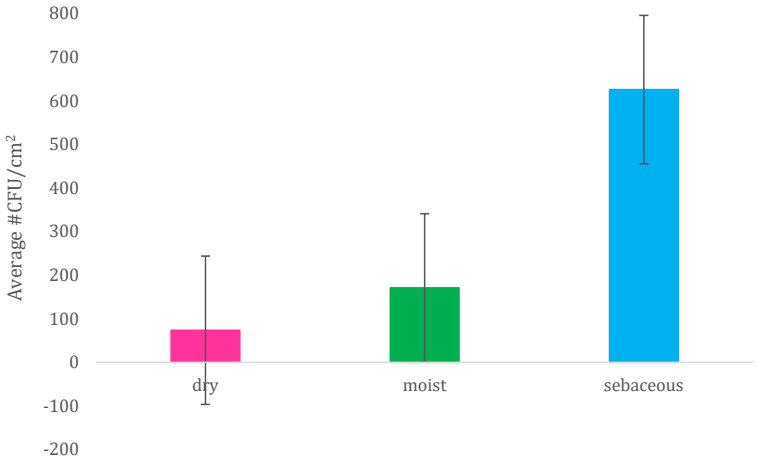

Figure 4. Estimates for the average number of CFUs per cm^2 of skin sampled.

Each bar represents the average number of CFUs per cm^2 of skin sampled. Estimates were made based on the number of colonies counted on each plate. Error bars represent the standard error. Numerical labels show the mean CFU/cm^2 for each skin type. No statistical significance was determined by one-way ANOVA ($F(2,39) = 1.68$, $p = 0.20$).

Despite the increased estimate for CFU/cm^2 for sebaceous skin sites, there were no statistically significant differences between group means as determined by one-way ANOVA. However, this pattern reflects what has been previously been reported about bacterial abundance in culture-based studies of the skin microbiome, and there are several possible reasons why we observed such differences in cell density. First, it may be true that more bacteria inhabit sebaceous skin sites that dry or moist sites. Second, there may be a higher proportion of viable, culturable bacteria at sebaceous sites than dry or moist sites, even if there are no differences in total bacterial abundance between sites. Third, it may be that the taxa that are prevalent at sebaceous sites are better suited to our culture conditions than those inhabiting dry or moist sites. Although we did not collect compositional data in this study, we did collect data on total bacterial abundance

and cell viability from our experiments with qPCR and fluorescence microscopy, respectively.

qPCR

We attempted to quantify total skin-associated bacteria by qPCR of 16S amplicons. It is important to note that qPCR theoretically quantifies all copies of the 16S gene present in a sample, including those that came from bacteria that were dead before sampling. However, imperfect doubling of amplicons and primer biases that differentially affect the amplification efficiency of bacterial taxa may have influenced our results (Meisel *et al.* 2016). Additionally, quantification of 16S amplicons is farther removed from the actual quantity of 16S gene copies present in the original sample, so we did not estimate the number of cells/cm^2 of skin sampled from qPCR data. Instead, we made relative comparisons between the data from dry, moist, and sebaceous sample types. These results are shown in Figure 5.

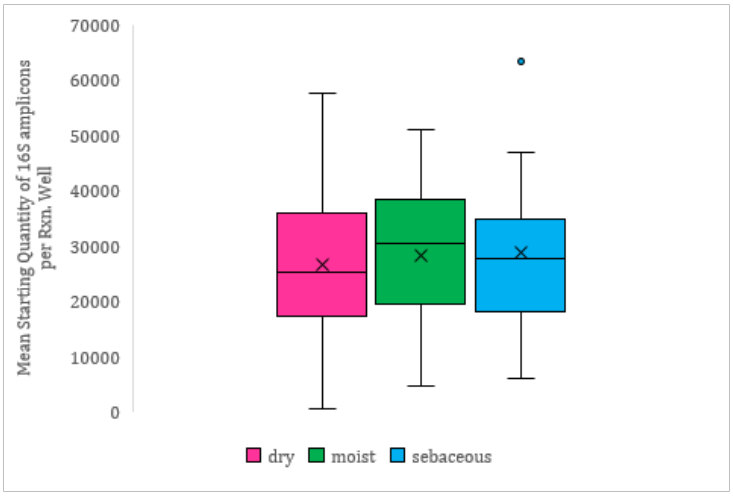

Figure 5. Mean starting quantities of 16S amplicons per reaction well.

No statistical significance was determined for between-group means by one-way ANOVA ($F(2,38) = 0.081$, $p = 0.92$).

Based on our results from colony counts, we might expect to see a greater quantity of 16S amplicons from sebaceous skin sites than from dry and moist sites. However, we did not observe a difference in amplicon quantity between each of our three sampling sites. This suggests that there is no difference in cell density between our three sampling sites, if we assume that each bacterium has one copy of the 16S gene. Therefore, it is more likely that the variation we observed between dry, moist, and sebaceous samples for colony counts was due to differences in cell viability and/or cultivability. Yet it is also possible that any differences in 16S gene abundance between our three sample types could have been attenuated by the qPCR protocol we used: two amplification steps and the PCR product clean-up step may have washed out any differences in amplicon quantity between sample sites ("Instructions…PCR Purification" 2016). We therefore recommend that future researchers wishing to

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quantify environmental bacteria use a standard qPCR protocol where genomic DNA, rather than 16S amplicons, is quantified. We believe results from this method are less likely to wash out differences between sample types and better facilitate estimation of the number of cells present in the original sample.

Researchers that aim to quantify skin-associated bacteria in the future may also want to consider collecting compositional data from their samples. This would help glean more information from qPCR because compositional data could be used to correct for differences in 16S GCN and determine which bacterial taxa are most prevalent at a particular skin site. While this is interesting from an ecological perspective, it is also pertinent to human health because a higher proportion of pathogenic taxa or low species diversity at a particular site may indicate a state of dysbiosis in the host, and could lead to conditions such as acne and atopic dermatitis (Naik *et al*. 2012).

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Fluorescence microscopy

Fluorescence microscopy was the only method we employed where we could directly count cells stained by SYTOX Green and CTC. SYTOX Green stains cells with decreased membrane permeability green, most of which are presumed to be dead, while CTC stains cells that are alive and actively undergoing respiration red. However, SYTOX Green may stain cells that are actively respiring but have a damaged cell membrane, while CTC may not stain dormant cells that have an intact membrane but are not actively respiring (Tashyreva *et al.* 2013). Similar to colony counts, there was apparent variability in the number of cells observed both between sites and subjects, nonetheless we observed a general pattern of cell density based on the skin site sampled. Representative photos taken on the microscope are shown in Figure 6.

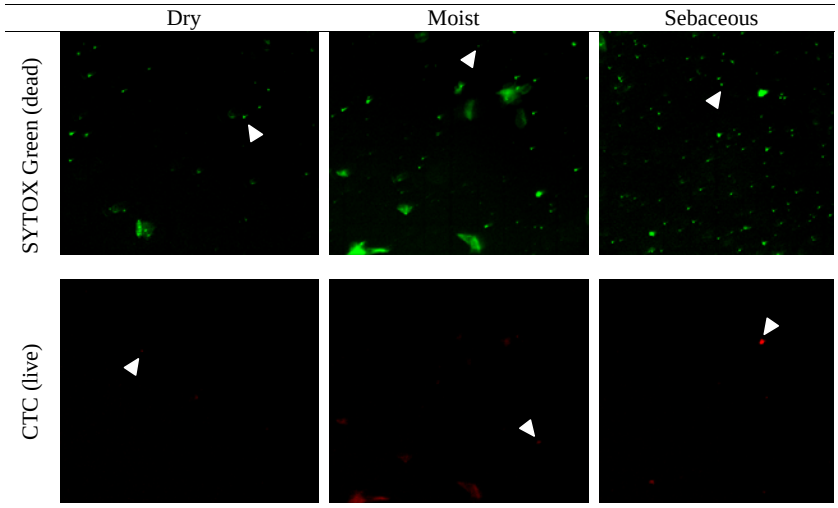

Figure 6. Fluorescence microscopy images.

Photos were taken at 257X magnification for each sample. Cells stained by the dyes were manually scored in ImageJ. Cells stained by SYTOX Green are presumed to be dead, while cells stained by CTC are presumed to be alive. White arrows denote particles that were assumed to be cells and counted. Labels below each column denote the number of cells counted in each photo.

After counting cells stained by SYTOX Green and CTC, we estimated the density of live and dead cells sampled from dry, moist, and sebaceous skin types. We calculated these estimates using dimensions of the hemocytometer and the area of skin sampled (Figure 7).

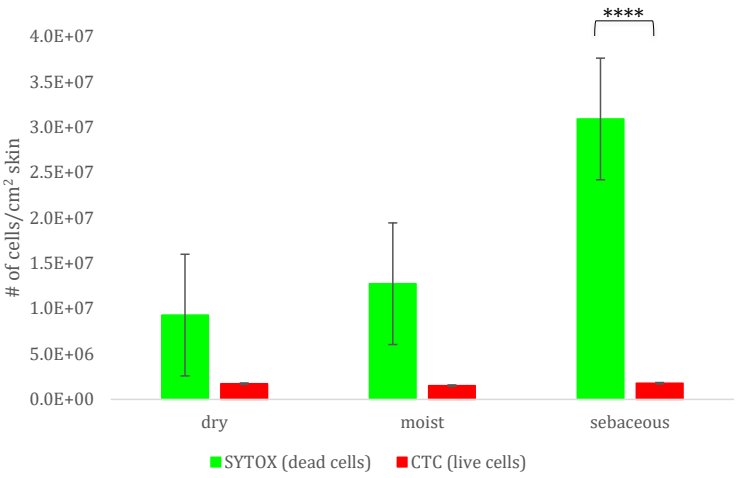

Figure 7. Estimated density of live and dead cells sampled from dry, moist, and sebaceous skin sites.

Estimates were made using the area of the smallest squares on the hemocytometer (50 x 50 μm^2), depth of the hemocytometer (0.1 mm), area of skin sampled (16 cm^2) and the number of cells counted per field of view. Significant difference was found between estimates of live and dead cells for sebaceous sites from multiple comparisons ANOVA, $p<<0.05$. Error bars represent the standard error.

From fluorescence microscopy, we determined that there were significantly more dead cells than live cells sample from sebaceous skin sites ($p<<0.05$). Interestingly, the quantity of live cells stayed roughly the same across all sample types. This is surprising because given data from colony counts and qPCR, we could have expected there to be a higher proportion of live cells at sebaceous sites. The opposite actually seems to be true – the lowest cell viability was observed for sebaceous sites, as shown in Figure 8.

Commented [AB24]: Make it clear that this is "proportional" viability, right? # of live cells out of total counted?

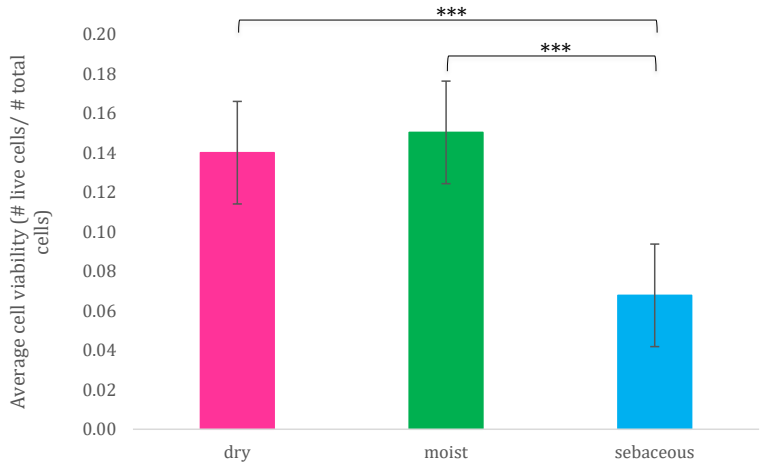

Figure 8. Average cell viability at dry, moist, and sebaceous skin types.

Cell viability was determined by dividing the number of live cells by the number of total cells (live + dead) for each sample. Error bars represent the standard error. Statistical significance for between-group means was determined by one-way ANOVA ($F(127,2) = 5.85$, $p = 0.0037$).

The differences in average cell viability were shown to be statistically significant by one-way ANOVA ($p = 0.0037$). This overall significance value was due to differences in average cell viability between dry and sebaceous ($p = 0.0046$) and moist and sebaceous ($p = 0.0075$) sites. These results provide foundational knowledge about the skin microbiome; the vast majority of cells seem to be dead, or not actively respiring, so perhaps skin-associated bacteria play a role in nutrient cycling or maintaining genetic diversity in addition to directly interacting with other bacteria or the human host (Lennon and Jones 2011). Moreover, the small proportion of viable cells could contain certain bacterial taxa that are most important for promoting or interfering with host health.

Comparison of methods

In this study, we sought to answer two main questions: first, what are the relative merits of colony counts, qPCR, and fluorescence microscopy for quantifying bacteria inhabiting dry, moist, and sebaceous skin sites? Second, how does the number of live and dead cells counted vary by skin type and method of quantification? After collecting numerical data from each method, we then attempted to estimate the number of bacterial cells per cm^2 of skin sampled (excluding qPCR). As we have shown, colony counts and fluorescence microscopy provided distinct estimates for the number of cells present per cm^2 of skin sampled. These differences are likely due to the inherent biases and limits of each method, previously summarized in Table 1.

In Figure 9, we compare the estimates of bacterial abundance from colony counts and fluorescence microscopy. As we hypothesized, fluorescence microscopy was the method that provided us with estimates of bacterial abundance closest to what has previously been reported for both culture-dependent and culture-independent methods, roughly 10^3-10^7 cells/cm^2 of skin sampled (Gao *et al.* 2011, Wilson 2005). This confirms the value of using direct cell counts to estimate the total number of cells inhabiting and environment. This method was also valuable because we used fluorescent dyes to estimate what proportions of the bacteria sampled were alive and dead. However, it is possible that some of the cells we sampled were dormant and not actively respiring, so they would not be stained by CTC, but also had an intact membrane, so they would not be stained by SYTOX Green, either. If we were to repeat this study, we would use DAPI in addition to SYTOX Green and CTC, because of its ability to stain the nucleic acids of all cells in sample, even those with an intact

membrane. This would allow us to not only make more accurate estimates of bacterial abundance on the skin, but also distinguish between the metabolically active, dormant, and dead cells in a sample, all of which may play important ecological roles in the skin microenvironment.

Commented [AB25]: And give us a more accurate estimate of the total number of bacterial cells on the skin.

From colony counts, we determined that there are roughly 34 CFU/cm^2 at dry skin sites, 71 CFU/cm^2 at moist skin sites, and 626 CFU/cm^2 at sebaceous skin sites. These estimates of cell abundance are lower than what has been previously been estimated from other culture-based studies, roughly 10^3-10^6 cells/cm^2 (Wilson 2005). However, in this study we only used a single type of media and invariable culture conditions to grow the sampled bacteria, and in most other culture-based studies multiple types of media and culture conditions are used to grow bacteria. It is also important to note that the colonies we counted represent only live, culturable bacteria, so the actual quantities of cells/cm^2 of skin are most likely higher because these would also include dead, dormant, or non-culturable cells.

In addition to the significant difference in quantification we found between colony count and fluorescence microscopy methods, we also determined from combined data from these methods that the total quantity of cells at sebaceous skin sites is significantly greater than those at moist (p = 0.012) and dry (p = 0.0061) sites. However, the only non-culture-based study we know of that quantified bacteria at different skin sites reported that moist skin (the axilla) harbors the most bacteria, followed by dry and sebaceous skin (Gao *et al*. 2010). This discrepancy could be due to several factors, including the distinct biases of qPCR, colony counts, and fluorescence microscopy for enumerating bacteria, and the different dry, moist, and sebaceous sites

sampled. More independent studies aiming to quantify the skin microbiota, especially those using fluorescence microscopy, are needed for us to corroborate our results.

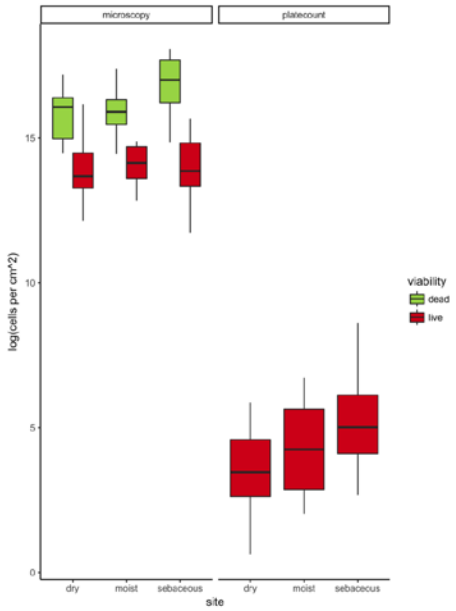

Figure 9. Comparison of cell density estimates made from colony counts and fluorescence microscopy.

Estimates for cells/cm^2 of skin sampled are shown for both fluorescence microscopy and colony count data. Estimates have been log transformed in order to represent these data on the same y-axis. Statistical significance between estimates made from colony counts and microscopy was determined from multiple comparisons ANOVA, $p << 0.05$. Combined data from these methods showed that abundance is greater at sebaceous sites than moist (p=0.012) and dry (p=0.0061) sites. Error bars on boxes represent upper and lower 95% confidence intervals.

qPCR is distinct from microscopy and colony counts because what we actually quantified were the 16S amplicons per sample. This is an indirect method of quantification and is more useful for making relative comparisons between samples, rather than estimating absolute cellular abundance. Still, from qPCR we did not observe

any significant differences in the number of 16S amplicons between sample types. As previously discussed, we may have achieved different results had we used a standard qPCR protocol that quantified 16S gene copies, rather than amplicons. This would have eliminated the additional amplification and clean-up steps that prevented us from making confident estimates of cellular density from qPCR data.

Conclusion

Understanding how the quantities of skin-associated bacteria vary across different body habitats is vital to understanding their ecology and their role in affecting human health – something as simple as a handshake has the potential to change the quantity and composition of our resident microbes. Knowing about the quantity of bacteria inhabiting with the human body may also be pertinent to the success of "microbial therapies," which are increasingly used by physicians to treat diseases such as those associated with C-section birth (Dominguez-Bello *et al.* 2016). The results from this study suggest that there is indeed variation in bacterial abundance between dry and sebaceous skin sites and between dry and moist skin sites, as determined by colony counts and fluorescence microscopy. We also found that each method yielded a distinct range of estimates for cell density at each skin site. With fluorescence microscopy, we could directly count cells labeled by CTC or SYTOX Green, and made estimates of cell abundance that are similar to what has already been reported about the skin microbiome. However, each of the methods we employed has its own set of benefits and limitations, which should be taken into consideration by future researchers interested in quantifying any environmental or host-associated microbiota.

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