Dynamics of the dermatologic microbiome in military members.

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FINAL REPORT

October 2019

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REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
02-10-2019	Closeout	Feb 2017 – Oct 2019

4. TITLE AND SUBTITLE
Dynamics of the dermatologic microbiome in military members.

5a. CONTRACT NUMBER

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Gibbons, Thomas F.
Noe, Jody C.
Patterson, Andrew T.
Lenz, Brittany L.
Beachkofsky, Thomas M.

5d. PROJECT NUMBER
IRB #: FWH20170033H

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
59th MDW/ST
1100 Wilford Hall Loop, Bldg. 4554 / SA Lackland AFB, TX 78236

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Air Force Medical Support Agency (AFMSA)

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
DISTRIBUTION A. Approved for public release; distribution is unlimited.

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Background: New military members undergo a highly-regimented 7-week training course during which trainees live and work within the same group of approximately 50 subjects for nearly 24 hours a day. This environment allowed for the assessment of the impact of communal living on the collective skin microbiome. **Purpose:** The objective of this pilot study was to investigate dynamic changes of the skin microbiome in basic military trainees (BMT), in light of the unique environmental influences faced by this population. **Patients and methods:** We evaluated collective changes in the skin microbiome of normal healthy adult basic trainees in response to communal living and universal Group A *Streptococcus* prophylaxis with penicillin over the course of their [initial] 7-week training course. Samples from 10 flights of trainees were collected by swabbing their forearms and foreheads upon arrival at Lackland AFB for their training (week 0) which is prior to prophylaxis with penicillin, at the 4 week point, and at the conclusion of their 7-week course of basic military training. Three separate high-throughput sequencing platforms and three bioinformatic pipeline analysis tools were utilized to assess the data. **Results:** At all three time points we found that the top three bacterial genera identified were *Propionibacterium*, *Staphylococcus*, and *Corynebacterium*. We detected a community membership difference between the initial week 0 samples and the week 4 and 7 samples. A strong inverse correlation between *Propionibacterium* and *Staphylococcus* was noted with *Propionibacterium* being high at week 0 and much lower at weeks 4 and 7; conversely, *Staphylococcus* was low at week 0 and higher at weeks 4 and 7, this relationship was noted in both the individual and collective specimens. **Conclusion:** The collective dermatologic microbiome in the military trainee population examined exhibited a relative increase in *Staphylococcus* and *Corynebacterium* abundance coupled with a relative decrease in *Propionibacterium* abundance in this observational pilot study. Additional studies are needed to further assess the causal impact of communal living and widespread penicillin chemoprophylaxis.

15. SUBJECT TERMS
Metagenomics, 16S rRNA, prophylaxis, bacteria, penicillin

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			Andrew T. Patterson, MD
U	U	U	UU	22	**19b. TELEPHONE NUMBER** *(Include area code)* 210-292-8200

Standard Form 298 (Rev. 8/98)

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1.0 EXECUTIVE SUMMARY

This observational pilot study further characterized the dynamic temporal changes associated with the bacterial, fungal, and viral colonization of the skin (also known as the skin microbiome) in response to various external and environmental factors including communal living and universal Group A *Streptococcus* prophylaxis with penicillin in normal healthy adult basic trainees over the course of their 7 week training course.

2.0 INTRODUCTION

The skin microbiome contains a diverse microbial ecosystem that has yet to be fully characterized despite its wide-ranging potential linkages to host health and disease. The human body harbors numerous microbial communities consisting of bacteria, fungi, and viruses across various body surfaces including the skin, oral cavity, respiratory tract, gastrointestinal tract, and urogenital regions with a wide diversity of constituents depending on location. As our scientific capacity for genetic, transcriptomic, and microbial sequencing has increased, we have been able to better characterize several of these areas and their susceptibility to influence from external factors (such as colonic/gut flora's susceptibility to change based on antibiotic administration and subsequent attempts to regain homeostasis via probiotics, stool transplantation, and other means). The NIH recently launched the Human Microbiome Project (NIH Human Microbiome Project) in an attempt to better characterize the "normal" human microbiota with specific emphasis on its contributions to both healthy and disease states. Using metagenomic sequencing, researchers have been able to analyze and identify the complex taxonomy and composition of these microorganism environments on the body, whereas traditional bacterial culture mediums often allow growth of only the most hardy, easily cultivatable organisms from a site, and specific viral or fungal panels may report on only a handful of selected pathogens (Conlan et al. 2012).

The skin represents one of the most prominent body areas sustaining microorganism colonies that influence host health and disease in numerous ways that have yet to be fully delineated. Grice et al. (2009) documented distinct dermatologic microbiome niches based on location even within the same individual and effectively established a barometer for topographical and temporal diversity of the human skin microbiome among healthy individuals. Kong et al. (2012) further explored the relationship between dermatologic disease and skin microbiome when they examined a group of children affected by atopic dermatitis and identified statistically-significant alterations in their skin microbiome composition during flares and post-treatment with notable decreases in microbial diversity during flares, as well as an increase in *S. aureus* proportions. Significant decreases in community diversity have also been reported as hallmark signs of disease states by Oh et al. (2014). While this change in the proportion of the known pathogenic organism *S. aureus* could be reasonably assumed, the high-resolution metagenomic sequencing also identified a proportional increase in lesser considered skin bacterial factors in the form of *Streptococcus*, *Propionibacterium*, and *Corynebacterium* species during periods of disease improvement after topical corticosteroid therapy (Kong et al., 2012). This singular example of temporal diversity amongst the microbiota functions as an excellent demonstration of the presumed sensitivity of the dermatologic microbiome to outside influences. However, clearly additional longitudinal studies are needed to further outline the unique disease pathophysiology associated with the microbiomes of dermatologic conditions, such as atopic dermatitis, psoriasis, acne, and others.

This study sought to utilize the unique environmental influences faced by the BMT population to further demonstrate the dynamic changes of the skin microbiome that cannot be as reliably established outside this niche. Because of the highly-regimented nature of the 7 week BMT course and emphasis on working as an entire flight of approximately 50 subjects for nearly 24 hours a day, assessment of the impact of communal living on the collective skin microbiome was possible. We assessed and described this change by swabbing trainees upon arrival at Lackland AFB for their training as well as at the conclusion of their 7 week course. Additionally, while a few isolated studies such as Fanelli et al. (2011) have assessed the effects of systemic antibiotic therapy on targeted bacterial colonization (*S. aureus*) of the skin, to our knowledge prior to our study completion no one had yet assessed the universal impact of systemic antibiotics (in our setting penicillin administration) on the skin microbiome which we propose will serve as an extremely valuable baseline piece of knowledge as the worldwide use of antibiotics continues to rise. Blaser et al. (2016) further discusses how the use of the widespread use of these antibiotics may have meaningful ramifications for the non-pathogenic taxa of organisms on the skin that may function in not as-yet identified roles involving metabolism, anti-inflammatory capacities, and generalized antimicrobial properties. As our BMT population represents a rare instance where a group of relatively healthy subjects is exposed to a singular antibiotic at the same time, we were grateful for the opportunity to explore the ramifications of this universal prophylaxis on the collective cutaneous microbiome with a relatively low number of confounding variables compared to a similar study if conducted in a civilian population.

3.0 METHODS

3.1 Study Population

Ten groups (referred to as flights) of new BMT flights, 6 male and 4 female, encompassing an estimated 500 participating subjects were swabbed. Each flight was composed of between 30 and 50 trainees.

3.2 Sample collection

Subjects had sterile saline pre-moistened Copan Flocked Swabs (Copan, Murrieta, CA, USA; Cat# 501CS01) taken from the bilateral forearms and forehead at 3 time points – week 0, week 4, and week 7 of their training curriculum. Subjects with reported penicillin allergy were excluded from the study. Skin swabs were pooled, by flight and time point, into 5.5 mL of DNA/RNA Shield (Zymo Research Corp., Irvine, CA, USA; Cat# R1100-50) – described as “Collective” samples. Additionally, one flight had duplicate sterile swabs collected, for the collective samples above and the other for individual sample assessments – described as “Individual” samples. Individual samples were collected into 0.5 mL of DNA/RNA shield.

3.3 DNA extraction and purification

DNA was extracted from all collective and individual samples using the ZymoBIOMICS™ DNA Microprep Kit (Zymo Research Corp.; Cat# D4301). All manufacturer’s recommendations for low biomass samples were utilized with the following modifications for the collective

specimens only. Collective specimens were extracted in duplicate and the DNA combined to maximize final concentration. Purified genomic DNA was stored at ≤−20°C until used for library preparation.

3.4 Collective sample sequencing

The hypervariable regions of the rRNA gene were sequenced utilizing the Ion 16S Metagenomics kit (ThermoFisher, Waltham, MA, USA; Cat# A26216) following the manufacturer's protocol. Five microliters of swab-extracted DNA were amplified for each primer set. Amplicon libraries were prepared using the Ion Plus Fragment Library Kit (ThermoFisher, Cat# 4471252) and molecularly barcoded with IonXpress Barcode Adapters (ThermoFisher, Cat# 4471250) as described in the Metagenomics kit protocol. Amplified DNA was quantified with a Fragment Analyzer (Advanced Analytical, Ankeny, IA, USA) using their High Sensitivity NGS Fragment Analysis Kit (Cat# DNF-474-0500); 50 ng/µL of amplified DNA was used for barcode adaptor ligation for the pool samples. Barcoded libraries were quantified using the Fragment Analyzer using the High Sensitivity NGS Fragment Analysis Kit. Equimolar amounts of each library were used to seed an Ion PGM Hi-Q View OT2 (ThermoFisher, Cat# A29900) reaction and enriched using the Ion OneTouch ES system. Enriched, templated beads were loaded onto an Ion Torrent 318 V2 chip (ThermoFisher, Cat# 4488146) and sequenced using the Ion PGM Hi-Q View Sequencing kit (ThermoFisher, Cat# A30044).

3.5 Individual sample sequencing

The V3 and V4 hypervariable regions of the rRNA gene were tested as described in the Illumina (SanDiego, CA, USA) 16S Metagenomics Sequencing Library Protocol (15044223 B). The gene-specific primers used in this protocol target the 16S V3 and V4 regions, and they were selected from Klindworth et al. (2013).[9] Illumina adapter overhang nucleotide sequences (see underlined portion of primer) were added to the gene-specific sequences. The 16S rRNA gene forward primer was 5′-
TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG, and the reverse primer was 5′
GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC. The following modifications to the protocol were included: 7.5 µL of swab-extracted DNA was amplified with 2.5 µL each of 2 µM forward and reverse primers and KAPA HiFi HotStart Ready Mix (Roche, Indianapolis, Inc., USA, Cat# KK2602). The Nextera WT Indes Kit v2 Set A (Illumina, Cat# FC-131-2001) kit was used for molecular barcoding of the samples as described in the 16S Metagenomics Sequencing Library Protocol. Indexed libraries were quantified on the Fragment Analyzer using the High Sensitivity NGS Fragment Analysis Kit. Each sample was normalized to 1 nM, pooled, and denatured using 5 µL of pooled library and 5 µL of 0.2 N NaOH. PhiX control was denatured as described; 200 µL of 20 pM PhiX control and 400 µL of 5 pM diluted library were combined and used for loading on the MiSeq. This was the procedure utilized by CIRS. If you have a specific question on how this is done, please contact Dr. Thomas Gibbons at CIRS (thomas.gibbons.4@us.af.mil).

3.6 16S rRNA sequence data analysis

DNA sequences were processed using Ion Reporter Software 5.0, 16S Metagenomics workflow version 1.0. (ThermoFisher), BaseSpace Onsite 16S Metagenomics App version 1.0.1 (Illumina) and QIIME2-2018.4 (https://qiime2.org). Collective sample data analysis was performed using the Ion Reporter Software which leverages QIIME's open-source bioinformatics pipeline to produce diversity analyses and visualizations. Unaligned binary data files (Binary Alignment Map) generated by the Ion Torrent PGM were uploaded to an in-house Ion Reporter server (ThermoFisher) and analyzed using default settings. The genus level operational taxonomic unit (OTU) from the ION Reporter analysis of consensus reads was copied to an Excel sheet, where all results were normalized by converting OTUs for each sample to a percentage of the reads for a given sample. Any OTU with <0.1% reads from a given sample were excluded. Resulting OTUs for each sample were utilized to construct Venn diagrams and relative concentrations of specific phylotypes. Inverse Simpson's for alpha diversity was calculated by Ion Reporter as was Beta diversity principal-coordinate analysis (PCoA) showing the variation of microbiomes at specified time points. Individual sample data analysis was performed using the BaseSpace Onsite 16S Metagenomics App. The genus level OTU from the 16S Metagenomics App was imported into an Excel file, where the results were normalized by converting OTUs for each sample to a percentage of the reads from the sample. Any OTU with <0.1% reads from a given sample were excluded. Resulting OTUs for each sample were utilized to construct Venn diagrams and relative concentrations of specific phylotypes. Inverse Simpson's for alpha diversity was calculated from OTU data as reported by the 16S Metagenomics App. Beta diversity PCoA showing the variation of microbiomes at specified time points was produced with QIIME2. The raw sequencing data have been uploaded to the National Center for Biotechnology Information database under the BioProject SRA accession: PRJNA543597.

3.7 Metagenomic Sequencing

Successful RNA extraction, amplification, and sequencing performed on Hi-Seq 2500 and Ion Proton Next-Generation Sequencer instruments and analyzed for descriptive report of bacterial, fungal, and viral presence at Wright-Patterson AFB. Successful quantitative analysis of MRSA frequency amongst collective samples at all three time points performed by Wright-Patterson team.

4.0 MAJOR EVENTS/MILESTONES/SUCCESS

- IRB approval (to include ombudsman appointment/approval). February 17, 2017.
- First enrollment June 2017; 500 samples collected during June 2017; additional collections July 17 and August 17; samples from Weeks 0, 4 and 7 shipped and received successfully.
- Capt. Patterson and Dr. Noe presented poster "Dynamics of the Dermatologic Microbiome in U.S. Air Force Basic Training" at DoD Microbiome Symposium. April 17, 2017.

- Maj. Beachkofsky and Dr. Noe presented poster "Dynamics of the Dermatologic Microbiome in U.S. Air Force Basic Training" at MHSRS. August 17, 2017.
- Successful sequencing of pooled specimens for 7 of 10 trainee flights at all 3 time points for 16S bacterial rRNA genes at 59CRD (completed May 2018).
- Capt. Patterson presented poster "Dynamics of the dermatologic microbiome in military trainees in the setting of universal antibiotic prophylaxis and communal living" at the Fall 2018 Texas Dermatological Society Conference in Galveston, TX. September 21-22, 2018.
- Dr. Gibbons presented the attached Powerpoint presentation and abstract on behalf of the 59th team at the U.S. Army Medical Research and Materiel Command (USAMRMC) Systems Biology Collaboration Center workshop 'Integrative and Collaborative Biomedical Research for the 21st Century.' November 29-30, 2018.
- Successful sequencing of qualitative RNA signatures for viral, bacterial, and fungal elements from subject samples at 711th with quantitative sequencing of MRSA frequency shifts in the same subject groups over all three time points.
- Bacterial genera data presented at the 2019 American Academy of Dermatology Annual Meeting in Washington D.C. March 1-5, 2019.
- Publication of manuscript 30 August 2019 in *Clinical, Cosmetic and Investigational Dermatology.*

5.0 RISK ASSESSMENT

5.1 Risk Analysis

There were no known risks associated with this study. Skin swabbing (rubbing the skin with a tipped applicator) does not carry any risk of side effects or adverse events. As no PHI or individually-identifying information was collected, no risk of a confidentiality or privacy breach existed.

5.2 Technical Challenges

Communication barriers associated with any multi-site study were present during the study. An initial batch of trainee swabs was obtained at week 0 and those trainees were subsequently dropped from study participation secondary to specimen reservoir leakage during mail/transport. A national shortage of injectable penicillin delayed the start of sample collection for this project. Stock levels of injectable penicillin were anticipated to begin rising in the summer of 2017, and to be fully restored by FY17 Q4, per the manufacturer, but an earlier than expected delivery of penicillin enabled the start of sample collection in June 2017. Sample collection was completed in August 2017.

6.0 TRANSITION PLAN

6.1 Military Relevance

Military personnel are at an increased risk for skin and soft tissue infection development as a result of the unique environmental stressors faced during both combat and standard occupational

locales. Several prior studies have examined the increased propensity for skin and soft tissue infection (SSTI) in the basic military trainee (BMT) population with one study documenting nasal carriage of *Staphylococcus aureus* (*S. aureus*) and methicillin-resistant *S. aureus* (MRSA) associated with an increased rate of SSTI (Johnson et al. 2015). While it is reasonable to assume *S. aureus* and MRSA colonization play a significant role in development of SSTI in this group, an estimated 40-50% of the population (Fanelli et al., 2011), the large majority of which is healthy, have also been shown to be colonized by *S. aureus* suggesting a more complex interplay amongst both pathogenic and non-pathogenic microorganisms which defines the microbiome. Additional studies (Ellis et al., 2014) have examined various prophylactic hygiene regimens with an outcome measurement goal of reducing the incidence of infection, but no study has documented how the myriad of unique environmental variables encountered by the BMT population such as living in close quarters with the same group of peers for an extended period of time or the administration of universal prophylactic antibiotics influence the dermatologic microbiome. Determining how the skin flora of our BMT population responded to these stressors has established a baseline determination for the propensity these military members may have in developing dermatologic conditions such as SSTI, contact dermatitis, acne, and others once those at-risk microbiota skin signatures have been further elucidated. Further consideration of additional applications of non-invasive skin microbiome sampling also has AF-relevant implications for pre- and post-deployment member assessments to highlight location specific exposures that may have occurred, allowing for enhanced earlier recognition of potential microorganism hazards in the subclinical stage.

6.2 Transition Strategy

The clinical applications of this microbiome pilot study are broad, and future transitions into springboard studies are easily seen. We as researchers look forward to partnering with other organizations within the AF in applying the findings and methods established in our study to other areas of operational need in the midst of our numerous global expeditions. While the most natural initial follow-on cohort study would examine microbiome changes in penicillin-allergic BMT individuals receiving azithromycin in comparison to their BMT non-allergic peers, some other potential next step options include a pre- and post-deployment skin microbiome assessments to assess for local down-range flora in an attempt to preemptively prepare for emerging infections and outbreak sources and implementation of skin microbiome testing as a non-invasive genomic prognostic parameter of physical resiliency capacity prior to deploying airmen to hostile occupational and harsh environments.

7.0 RESULTS

7.1 Sequencing Results

Three separate high-throughput sequencing platforms (PGM, NextSeq, MiSeq) and three bioinformatic pipeline analysis tools (Ion Reporter, Base Space App, QIIME2) were utilized to assess the data. Using the PGM platform, next-generation sequencing (NGS) from 30 total

collective samples (3 time points from 10 flights) was conducted over two separate sequencing reactions. Three flights were ultimately excluded because a single time point did not produce results sufficient for analysis. Complete sequencing results from seven flights (21 collective samples) yielded a total of 8,352,499 raw sequences. Each collective sample had approximately 397,738 reads (range 201,876–749,856 reads) with an average read length of 255 base pairs (bp) (range 243–370 bp). From a single flight, 15 individual subjects, each with three individual weeks 0, 4, and 7 samples were processed for NGS on a single sequencing platform (MiSeq) reaction. Extraction of individual specimens yielded very low amounts of DNA and only 10 subjects yielded high-quality results at all 3 time points. A high-quality result was one in which rarefaction curves (data not shown) represented the total microbiota within each sample. These 30 individual samples produced 1,959,154 high-quality sequences after quality filtering. In total, each sample had approximately 65,305 reads (range 21,692–102,547 reads) with an average read length of 450 bp (range 299–479 bp). NGS from collective and individual specimens were also conducted on the NextSeq for comparative purposes.

7.2 Temporal microbiota phylotypes

The number of genera revealed by microbial profiling varied greatly with the collection method (pooled vs individual samples), sequencing platform and bioinformatics pipeline utilized. The difference was especially large prior to omitting reads in each specimen that represented <0.1% of the total mapped reads. Prior to omitting low reads on a per specimen basis, microbial profiling of the collective specimens with the PGM and Ion Reporter at the various time points revealed a total of 142 genera with an average of 37, 52, and 40 genera at the 0, 4, and 7 weeks collection times, respectively. When filtering for only genera that represent 0.01% of mapped reads for any given specimen, the total number of genera decreased to 98 with an average number of genera of 28, 39, and 29 at the same time points (weeks 0, 4, and 7). Further filtering for genera that produced a read representing at least 0.1% resulted in a total of 32 unique genera with weekly averages of 8, 12 and 9 (for weeks 0, 4, and 7, respectively). Microbial profiling of the individual specimens with MiSeq NGS platform and the QIIME2 analysis pipeline resulted in 261 total genera with an average of 50, 33 and 39 genera at the same time points in the 10 individual specimens. When utilizing the QIIME2 pipeline, we employed the QIIME2 dada2 denoise-paired option to denoise, dereplicate, and filter chimeras from the sequence data. Utilizing this step resulted in the same 261 genera after filtering for those that represent 0.01% of mapped reads. Further filtering to genera that produced a read representing at least 0.1% resulted in a total of 181 unique genera with weekly averages of 33, 24 and 29 (for weeks 0, 4, and 7, respectively). Collective and Individual specimens were also analyzed with the BaseSpace 16S app which produced much higher total genera identified as well as much higher genera at each of the 0, 4, and 7 week time points in the unfiltered and 0.01% filtered counts. Once the reads were filtered to only those at 0.1% or more the results became much closer (Figure 1) to those found with the PGM Ion Reporter and QIIME2 bioinformatics pipelines.

7.3 Temporal microbiota characterization

While more genera were identified in the individual specimens, the overall signatures for both the collective and individual specimens were similar and chiefly represented by three genera: *Propionibacterium*, *Staphylococcus* and *Corynebacterium*. In the individual specimens, the

species of *Propionibacterium* was *Propionibacterium acnes*, while *Staphylococcus* and *Corynebacterium* were only classified to the genus level; 33% of the reads classified by QIIME2 were *Staphylococcus*. Although these reads were not able to be confidently classified to the species level, we investigated the likelihood of these reads being *S. aureus* by comparing the top 10 reads to a reference strain of *S. aureus, Staphylococcus epidermidis* and *Staphylococcus hominis*. These top 10 reads comprised 85% of the *Staphylococcus* reads, and in all cases, *S. aureus* had the least amount of homology to the selected reference species (Figure 2). *Propionibacterium*, *Staphylococcus* and *Corynebacterium* genera comprise three of the seven genera most often found in other skin microbiome studies of similar sites.10 Heat maps representing the 32, 60, 122 and 181 identified phylotypes illustrate the similarities of both the collective and individual specimens regardless of the sequencing platform and bioinformatic pipeline utilized (Figures 3 and 4). The number of phylotypes present at each time point is shown in Venn diagrams for each specimen type and processing pipeline (Figure 5). A closer inspection of the genera identified at each of these specific time points was conducted for both the PGM/Ion Reporter (collective specimen analysis) and the MiSeq/QIIME2 (individual specimen analysis). While many phylotypes were noted only at a particular time point, none of the changes were consistently observed across multiple platforms (Figure 6).

7.4 Temporal microbiota diversity

When overall diversity was assessed using the inverse Simpson (invsimpson) index, the phylotypes showed little change in diversity overtime (Figure 7). While little change was noted in the alpha diversity at the weeks 0, 4 and 7 collection times, beta diversity analyses indicated a unique microbial composition of the week 0 specimens, this was more pronounced in the individual specimens. PCoA of the Bray–Curtis distance matrix revealed clear separation of the week 0 specimens from the weeks 4 and 7 specimens (Figure 8).

7.5 Microbial composition impact on *Staphylococcus* compared to *Propionibacterium*

To look for possible impacts of the overall microbiome composition on the relative amounts of *Staphylococcus*, we compared the abundance of the top three organisms, *Propionibacterium*, *Staphylococcus*, and *Corynebacterium* in both the PGM/Ion Reporter collective specimens and the MiSeq/QIIME2 individual specimens. When the abundance of *Staphylococcus* is plotted against the abundance of *Propionibacterium* a very strong inverse relationship is noted. *Propionibacterium* levels are relatively high at week 0 and lower at weeks 4 and 7 in both the collective and individual specimens while the opposite is noted with *Staphylococcus*. The coefficient of determination for this inverse relationship is r2=0.8581 for the collective specimens and r2=0.781 for the individual specimens (Figure 9).

7.6 Additional microbial composition comparisons

Abundance plots of *Staphylococcus* and *Corynebacterium* as well as *Corynebacterium* and *Propionibacterium* were also compared (Figure 10). A positive correlation of *Staphylococcus* and *Corynebacterium* was noted in the collective specimens but this was not noted in the individual specimens. A negative correlation of *Propionibacterium* and *Corynebacterium* was

noted in the collective specimens but again this was not noted at the same level in the individual specimens.

7.7 Collective versus individual sampling

To assess the impact of collective sampling, we compared the results from the one group that had both collective and individual samples. To minimize confounding variables for this comparison the number and amounts of phylotypes were both determined from the same NGS analyzer (NextSeq) and from the same BaseSpace 16S App. After filtering for genera that represent 0.1% of mapped reads or more, the total number of genera in the week 0 collective specimen was 20 while the number of genera in the 10 individual specimens that were part of the collective specimen was 89. A similar difference was noted in the week 7 collective specimen with 32 genera, while 61 genera were noted in the 10 individual specimens. With respect to the percent amount of the seven most common genera found in similar microbiome studies, the collective sample result (dashed line in Figure 11) was within 5% of the average individual result (solid line in Figure 11) for 12 of the 14 comparisons. The two outliers were the week 0 *Propionibacterium* with an 8% higher amount in the collective samples and the week 7 *Staphylococcus* specimens with a 16% higher amount in the individual samples. Heat map comparisons of the same data from these 7 genera also depict the impact of collective sampling (Figure 9). With respect to these genera, all 7 were detected at the 0.1% cutoff in at least one of the individual specimens, but when these specimens are pooled prior to analysis only 5 of 7 are detected at 0.1% or higher level. Both *Veillonella* and *Enhydrobacter* have collective results with <0.1% of the reads for the respective collective samples.

7.8 Descriptive viral, fungal, bacterial data with associated MRSA quantitation

Descriptive reports were provided by the Wright-Patterson AFB team listing bacterial, viral, and fungal species presence in the collective flights at Week 0, 4, and 7 time points from a descriptive fashion. MRSA presence was quantified and reported below (Figure 12). Significant increases in MRSA frequency were observed from Week 0 to Week 4 ($p = 0.0021$) and Week 0 to Week 7 ($p = 0.0013$) utilizing a two-tailed T-test.

8.0 DISCUSSION

The results of this study found similar composition patterns of bacteria in our cutaneous samples as those previously noted in other skin microbiome studies.[4,10,11] Although the same genera of bacteria were identified, importantly our study population did not show the temporal stability of the genera of the skin microbiome that has been previously noted in healthy individuals.[4] Instead, our population exhibited a rapid change in the collective microbiome composition from week 0

to weeks 4 and 7 time points as indicated in the beta diversity results. Presumably, a hallmark of this change was due at least in part to a strong negative correlation between *Staphylococcus* and *Propionibacterium*. The week 0 subjects' microbiome consisted of high levels of *Propionibacterium* and low levels of *Staphylococcus*, with the reverse occurring in the microbiota signatures at weeks 4 and 7. Per the Wright-Patterson team's analysis, a statistically significant increase in MRSA frequency was also observed from week 0 to weeks 4 and 7. It is possible that these changes in the microbiome are at least in part in response to the GAS chemoprophylaxis provided during week 0 of training. However, it is unknown whether these microbiome shifts may also occur in the absence of chemoprophylaxis with exposure to other factors such as communal living in a rigorous training environment. A similar correlation between *S. aureus* and *P. acnes* levels has been noted between lesional and non-lesional AD; the authors found a higher proportion of *S. aureus* relative to *P. acnes* in lesional skin compared to non-lesional skin[12]. In our population, it is unknown if this change in the skin microbiome, specifically the increase in *Staphylococcus* or the decrease in *Propionibacterium*, is likely to decrease or increase protection against the development of SSTIs. With this pilot study, the QIIME2 identification of *Staphylococcus* was limited to the genus level. There are competing mechanisms both for and against an increase in a pathogenic organism such as *S. aureus*. Although we were not able to fully identify the *Staphylococcus* to the species level, the alignment results of the most prominent *Staphylococcus* reads, which represented 85% of the *Staphylococcus*, showed more homology with *S. hominis* than *S. aureus*, suggesting there is likely not a large increase in *S. aureus*. *Propionibacterium* was identified to the species level, with *P. acnes* being the best fit. Depending on particular physiological and growth conditions, *P. acnes* has been shown to promote or inhibit the growth of *S. aureus*. Under certain conditions, *P. acnes* produces the small molecule coproporphyrin III, which promotes *S. aureus* aggregation and biofilm formation.[13] Under certain other conditions, *P. acnes* has been shown to inhibit *S. aureus* growth by producing propionic acid.[12]

Collective sampling did approximate the average microbiome of the aggregate of individual samples but with a loss in granularity. Fewer phylotypes were identified in the collective samples than in the corresponding individual specimens. While the results from the collective sample should not be interpreted as the average microbiome from the individuals in the sample, the collective samples did correspond well with the inverse correlation of *Propionibacterium* and *Staphylococcus* in this study. Future additional validation studies utilizing collective samples may prove to be a rapid cost-effective method to monitor for large shifts in the dermatologic microbiome in the BMT and other populations. There were instances where the collective results were contradictory to the individual samples. This was the case when examining the relationship of *Staphylococcus* and *Corynebacterium*, which highlights one of the limitations of collective sampling. While the cause of this observation is not fully understood, pooling of samples may result in fewer phylotypes represented than those actually present in certain individuals due to the threshold required for detection in sequencing analysis. This under representation of phylotypes may lead to erroneous results in some cases. Even with this limitation of collective sampling, the sample pooling approach has been successfully utilized in the past, and may prove to be a technique worth further investigation with respect to its utility in metagenomics screening of large populations. Screening samples for the presence of an agent is a procedure commonly used to reduce the cost of screening a large number of individuals for infectious diseases.[14] HIV

screening of pooled blood has been validated for both antibody screening and nucleic acid screening of blood with little loss in sensitivity or specificity.[15,16]

There are several limitations to our small, pilot study. Our analysis represents a mere 3 time points over the course of 7 weeks, providing only snapshots of changes in the microbiome evolution over that period. Our young, generally healthy study population may not reflect potential microbiome changes to the general population that would occur in similar circumstances. The study was performed in an isolated location (south Texas) and season (summer) which may not take into account humidity/weather/temperature influence on cutaneous microbial change. In addition, this study design did not control for incidental antibiotic administration that may have occurred during the course of training or variations in personal hygiene. Due to the large number of subjects, individuals were asked to self-swab specified sites to obtain samples. Skin swabs were obtained from easily accessible body sites on each individual (forehead/forearms), but they do not account for niche-specific changes to the axillae, groin, and other body areas. The microbiome of forehead and forearm was combined to one sample, and other studies have shown that there are differences in the microenvironments of these two sites.[10] While the genera level data trends are intriguing, we were unable to reach species-level classification using the 16S rRNA sequence reads we obtained with the approaches we employed. This taxonomic resolution limitation hampered precise identification of organisms, as well as detailed interpretation of data. We further note that this was a preliminary observational study; it was not designed to determine the causality of the observed changes in the collective skin microbiome.

In sum, the collective dermatologic microbiome in the military trainee population examined in this study exhibited a relative increase in *Staphylococcus* and *Corynebacterium* abundance coupled with a relative decrease in *Propionibacterium* abundance in this observational pilot study. Additional studies are needed to further assess the causal impact of communal living and widespread penicillin chemoprophylaxis. The cutaneous microbiome is dynamic in nature and involves a complex interplay between host and skin microbes as well as external influences. Targeted metagenomic sequencing may help elucidate the linkage between cutaneous dysbiosis and its clinical impact on dermatologic disease.

9.0 DELIVERABLES

9.1 Publications

Gibbons TF, Noe JC, Patterson AT, Lenz BL, Beachkofsky TM. Temporal shifts in the collective dermatologic microbiome of military trainees. Clinical, Cosmetic, and Investigational Dermatology 2019; 12: 625-637.

9.2 Presentations

Patterson AT, Lenz BL, Noe JC, Gibbons TF, Beachkofsky TM. Dynamics of the dermatologic microbiome in military trainees in the setting of universal antibiotic

prophylaxis and communal living. Podium presentation at the American Academy of Dermatology 2019 Annual Meeting, Residents and Fellows Symposium. Mar 2019.

Gibbons TF, Patterson AT, Lenz BL, Noe JC, Beachkofsky TM. Dynamics of the Dermatologic Microbiome in Military Members. Podium presentation for the 2018 U.S. Army Medical Research and Materiel Command (USAMRMC) Integrative and Collaborative Biomedical Research for the 21st Century Symposium. Fort Detrick, MD. Nov 2018.

Patterson AT, Lenz BL, Noe JC, Gibbons TF, Beachkofsky TM. Dynamics of the dermatologic microbiome in military trainees in the setting of universal antibiotic prophylaxis and communal living. Poster presentation at the 2018 Texas Dermatological Society Fall Meeting. Galveston, TX. Sep 2018.

Beachkofsky TM, Patterson AT, Webber BJ, Lenz BL, Brown PJ, Noe JC, et al. Dynamics of the Dermatologic Microbiome in U.S. Air Force Basic Training. Poster presentation at the 2017 Military Health System Research Symposium. Kissimmee, FL. Aug 2017.

Patterson AT, Beachkofsky TM, Webber BJ, Lenz BL, Brown PJ, Noe JC, et al. Dynamics of the Dermatologic Microbiome in U.S. Air Force Basic Training. Poster presentation at the 2017 Department of Defense Microbiome Symposium. Colorado Springs, CO. Apr 2017.

10.0 COST

This project received and executed $197,000.00 of FY16-18 funds.

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12.0 APPENDIX

12.1 Figures

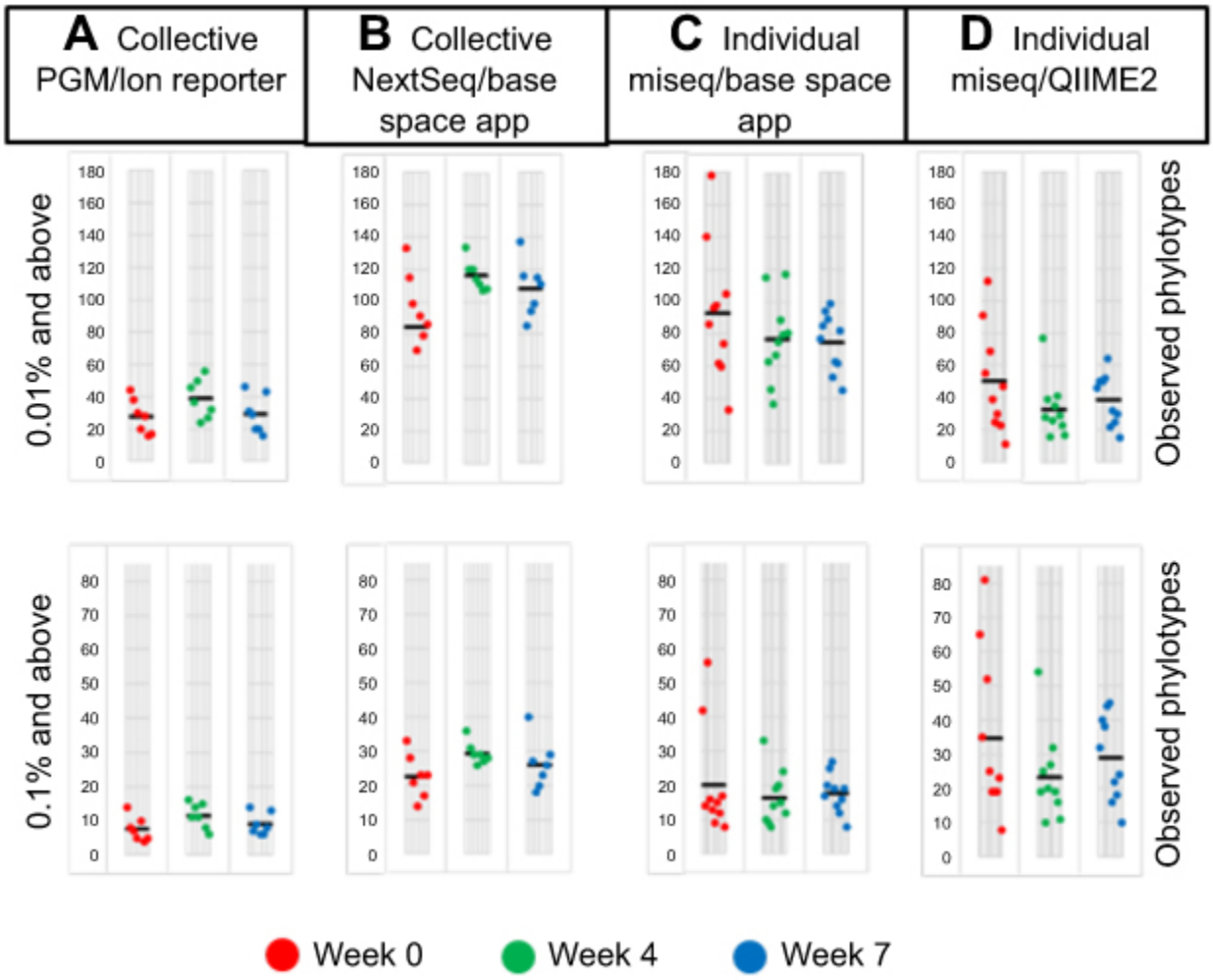

Figure 1 Number of phylotypes with 0.01% or 0.1% reads or more per sample. Each circle represents a single flight (**A** and **B**), or an individual sample (**C** and **D**). Mean values are indicated by a solid black line.

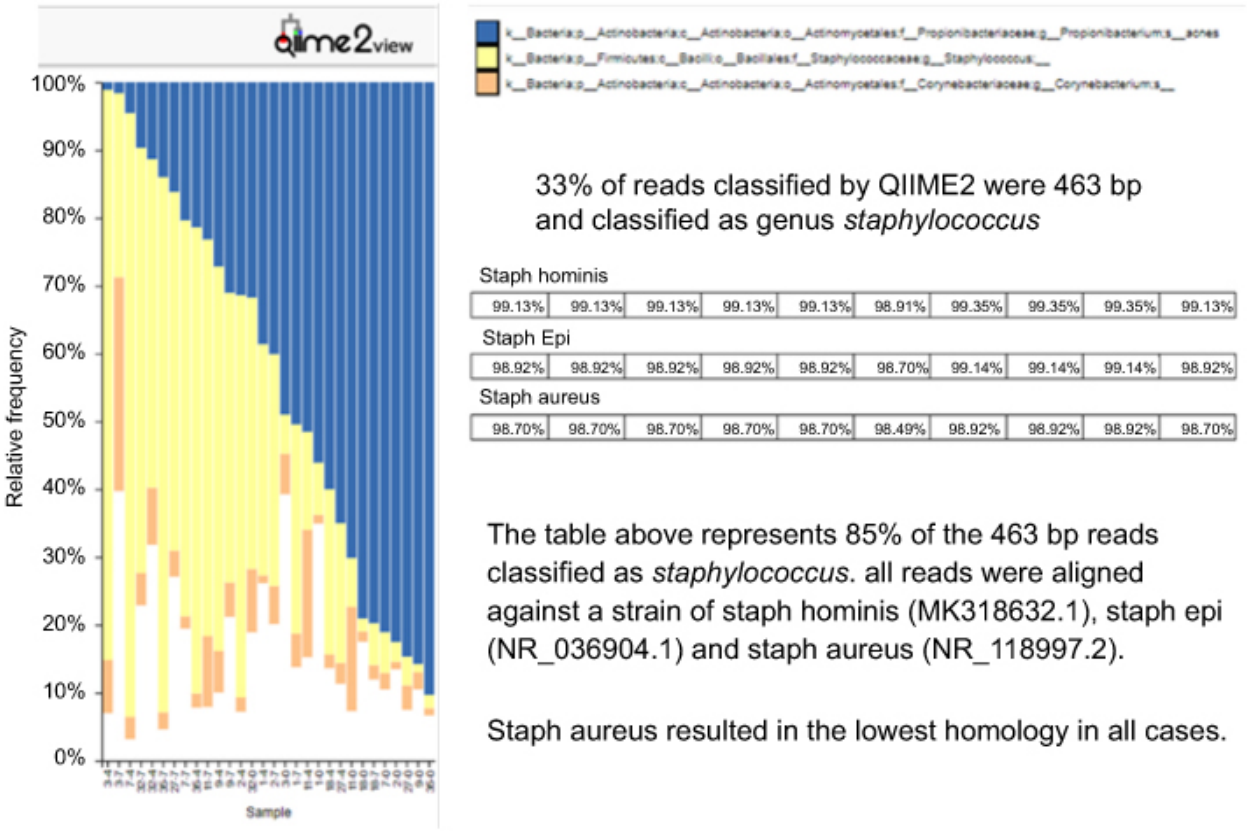

Figure 2 QIIME2 view of top three bacterial genera identified in individual specimens.
Only *Propionibacterium* was classified to the species level. The top 10 *Staphylococcus* reads were aligned against three species of *Staphylococcus*; Staph aureus gave the lowest homology.

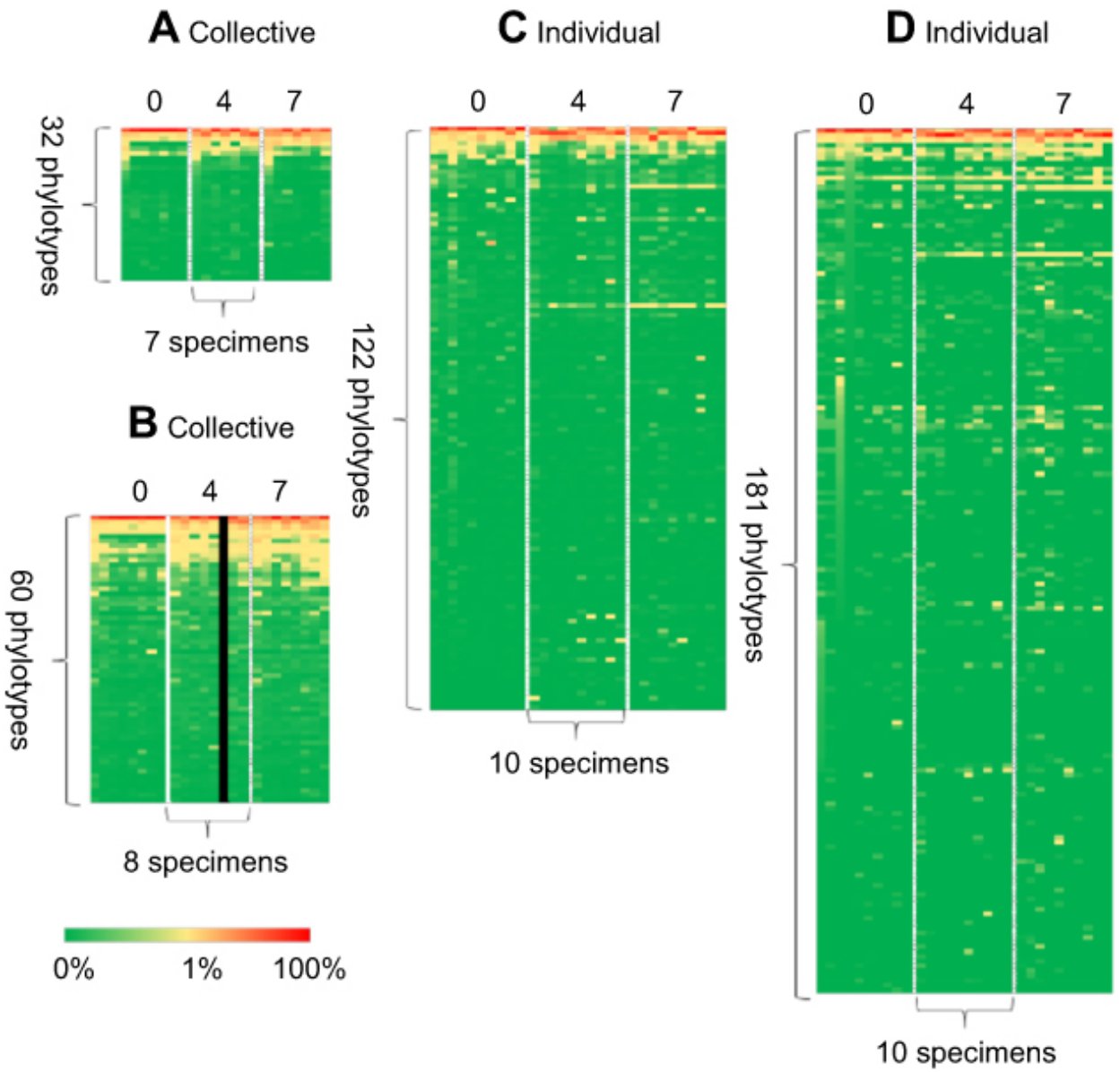

Figure 3 Heat maps showing the percentage distribution of phylotype levels at the 0, 4 and 7 weeks time points. Each row contains 7, 8 or 10 specimens. Each column contains 32, 60, 122 or 181 phylotypes. Percent abundance value of each genus is described by the color key: (**A**) PGM/Ion Reporter, (**B**) NextSeq/BaseSpace App, (**C**) MiSeq/BaseSpace App and (**D**) MiSeq/QIIME2.

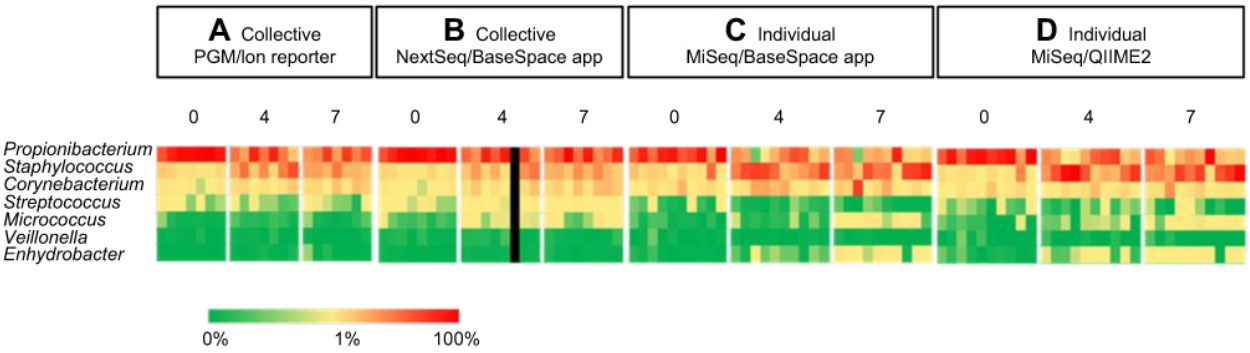

Figure 4 Select genus from heat maps. These 7 phylotypes represent the most common found in other skin microbiome studies of similar sites. Percent abundance value of each genus is described by the color key.

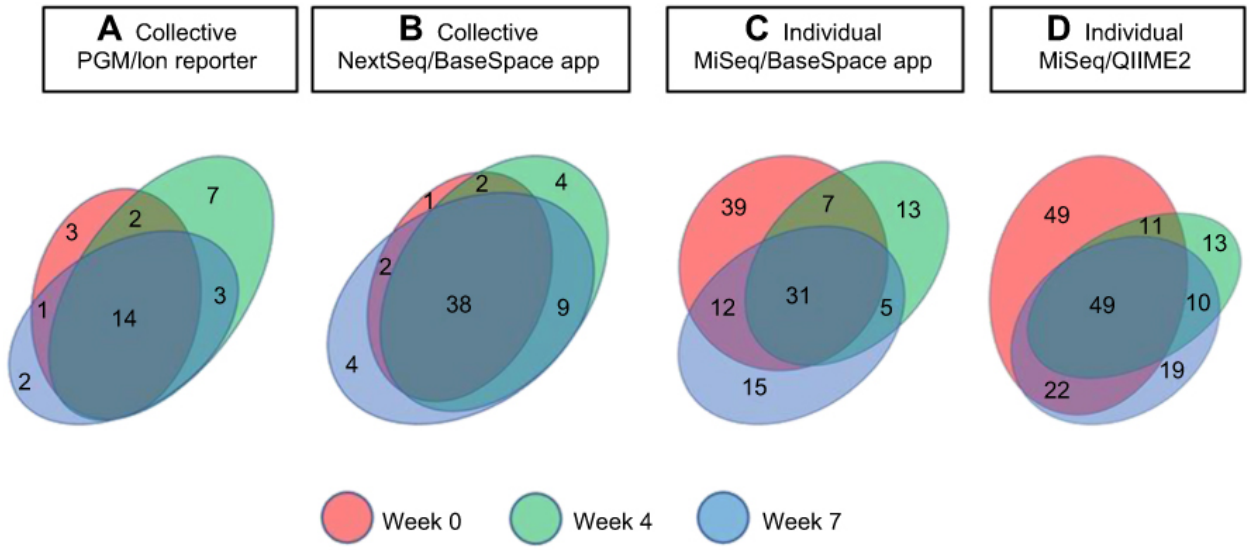

Figure 5 Phylotype distribution levels at different time points. Venn diagram showing the number of phylotypes at each time point for collective (**A, B**) and individual specimens (**C, D**). Values within overlapping circles represent phylotypes shared among different time points.

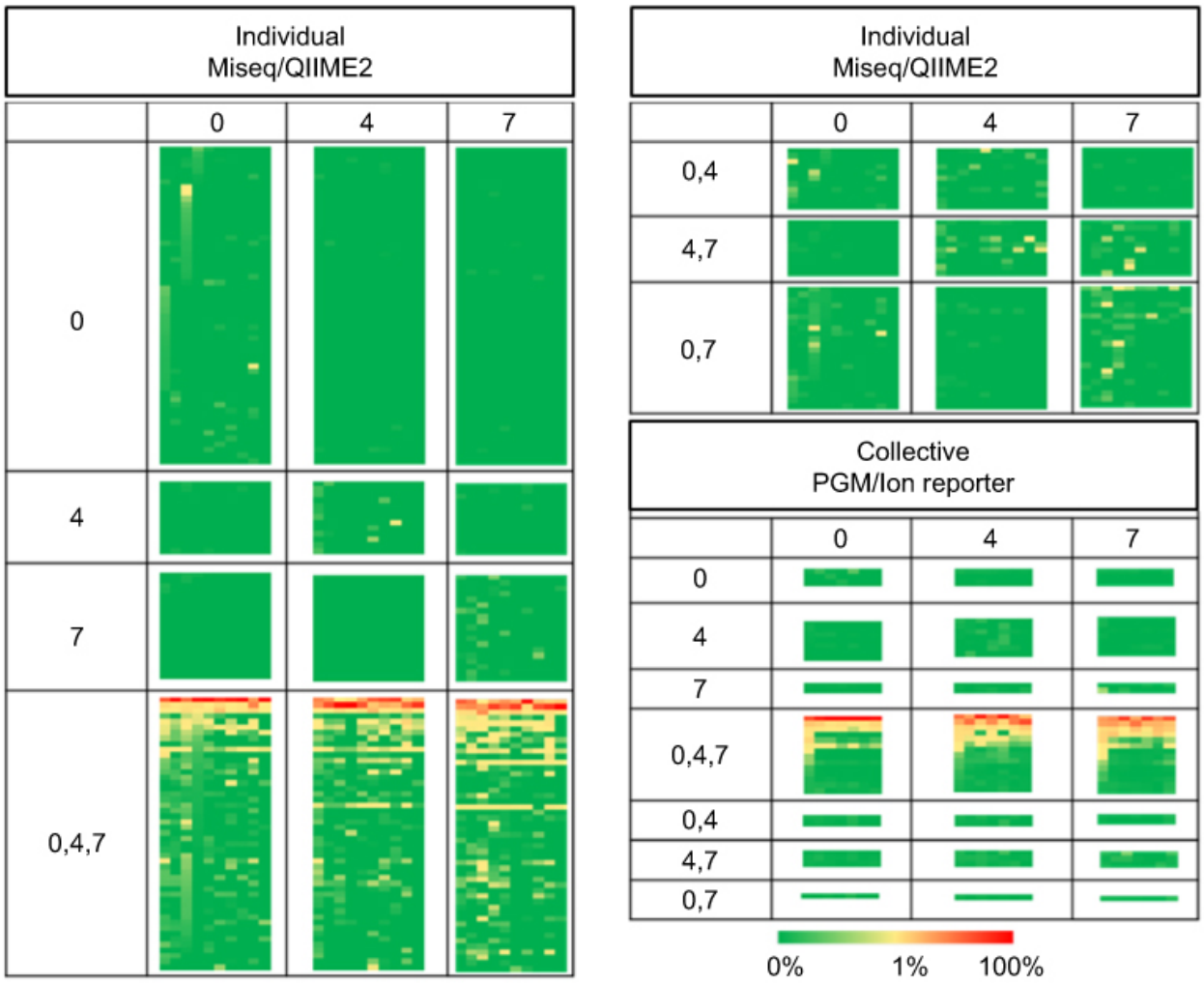

Figure 6 Heat maps of individual and collective samples. This is the same heat map represented in Figure 3A and D with the phylotypes divided into patterns of temporal occurrence illustrated by the relative Venn diagrams represented in Figure 5A and D.

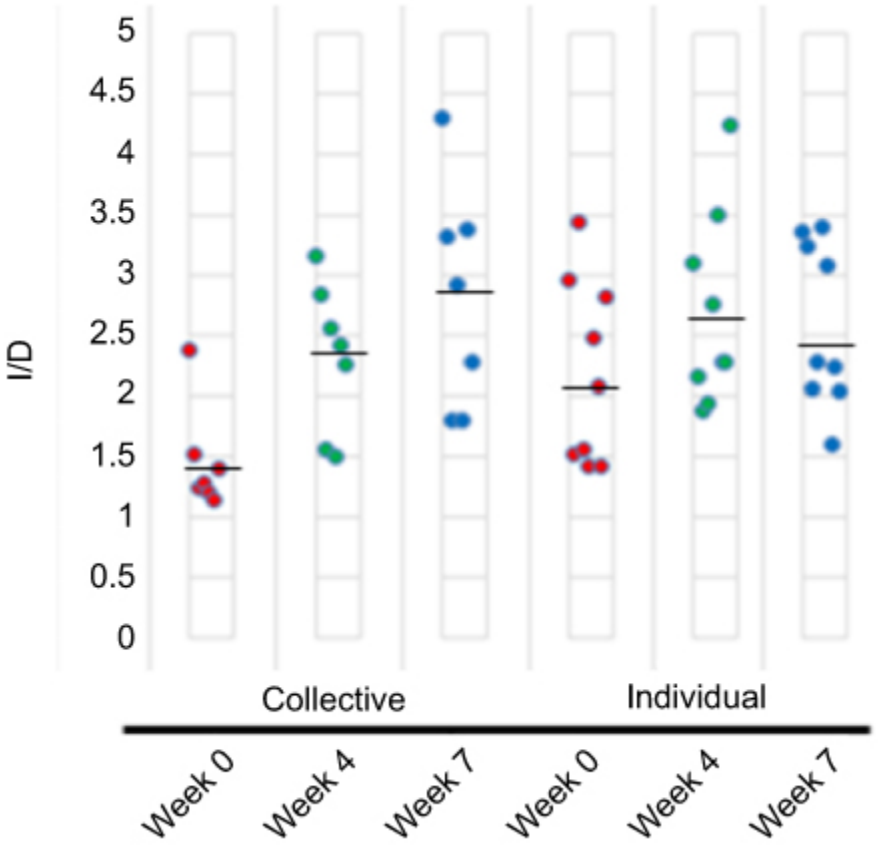

Figure 7 Sample diversity as determined using the invsimpon index (1/D). Each circle represents a single flight for collective sampling subject (PGM) or an individual for individual sampling (MiSeq) and the mean values are indicated by a solid black line. The higher the value, the more diverse the sample.

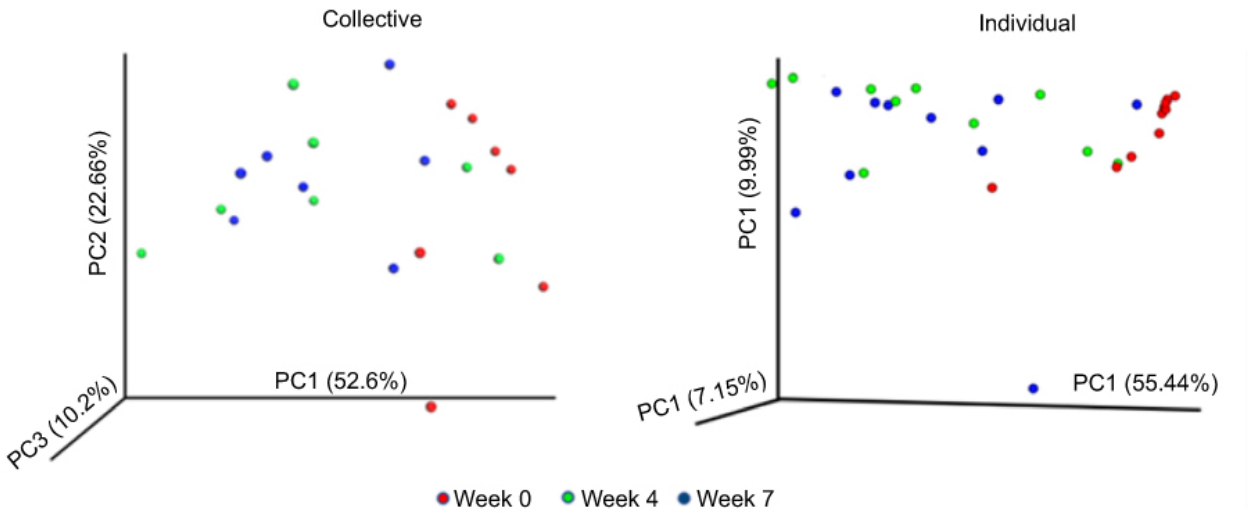

Figure 8 Principal-coordinate analysis (PCoA) showing the variation of microbiomes at time points as determined using the Bray–Curtis diversity calculator. Each colored symbol corresponds to an individual sample. The variation represented by each axis (PC1, PC2, or PC3) is shown in parentheses. (**A**) Collective samples (PGM) and (**B**) individual samples (MiSeq).

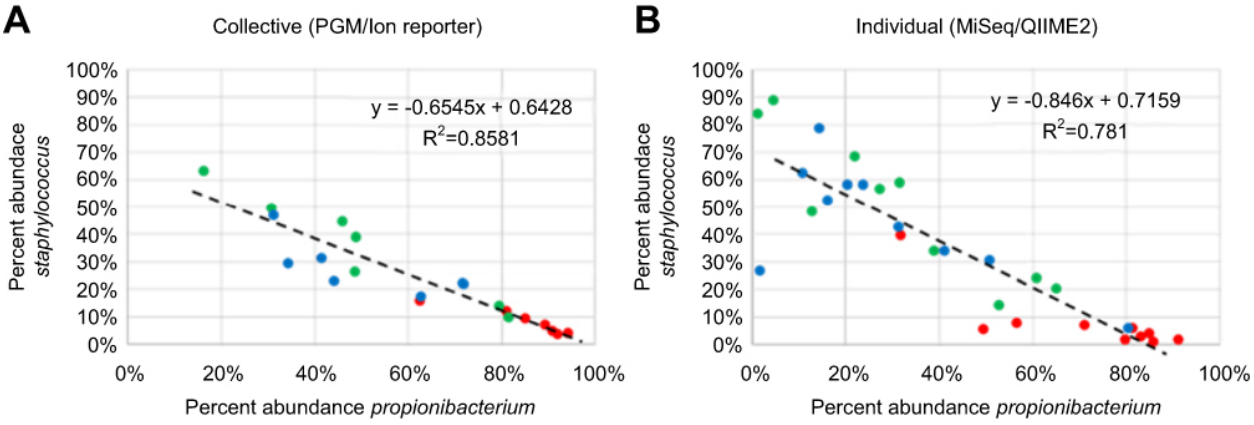

Figure 9 Percent abundance of *Propionibacterium* compared to abundance of *Staphylococcus* for week 0 (red), week 4 (green) and week 7 (blue). Each symbol corresponds to a collective ((**A**) PGM/Ion Reporter) or an individual ((**B**) MiSeq/QIIME2) sample. Line and inset equation represents the line of best fit [y=slope(**X**) + y intercept]. R^2 = coefficient of determination.

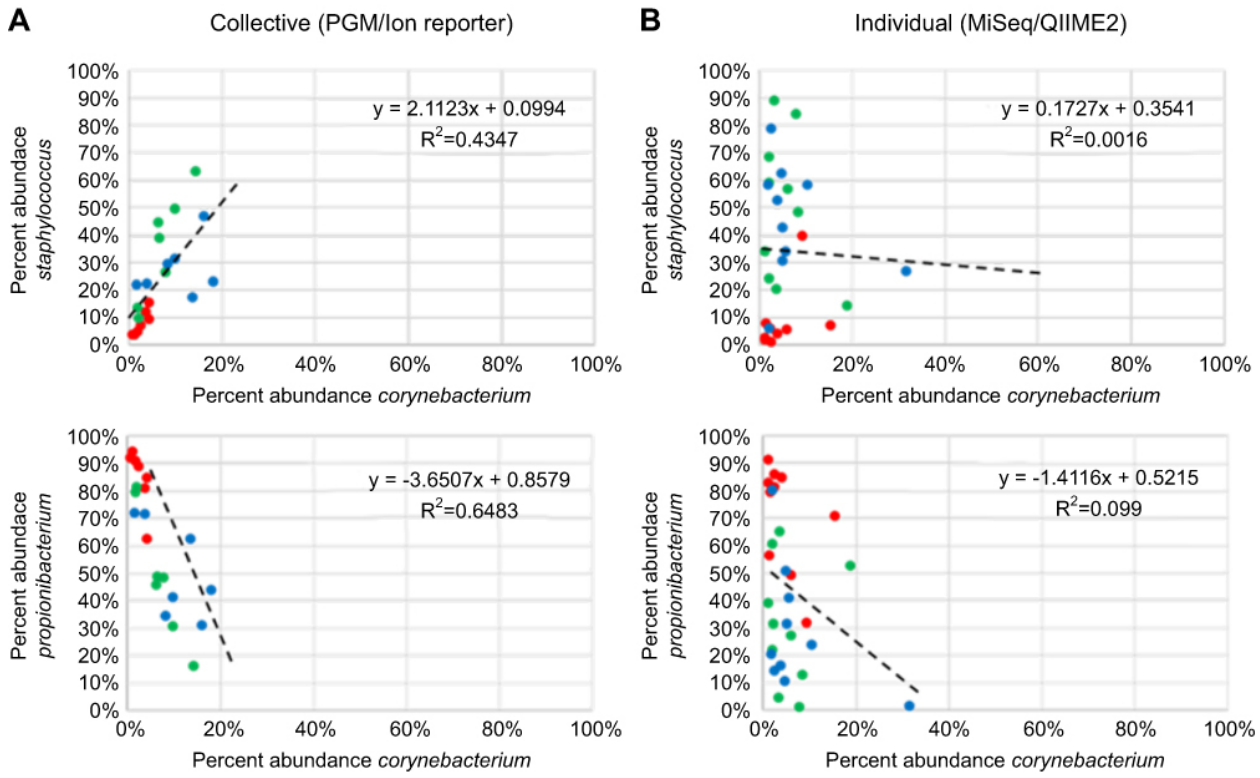

Figure 10 Percent abundance of *Staphylococcus* and *Corynebacterium* as well as *Propionibacterium* compared to *Corynebacterium* for week 0 (red), week 4 (green) and week 7 (blue). Symbols, line and inset equation represented as in Figure 9.

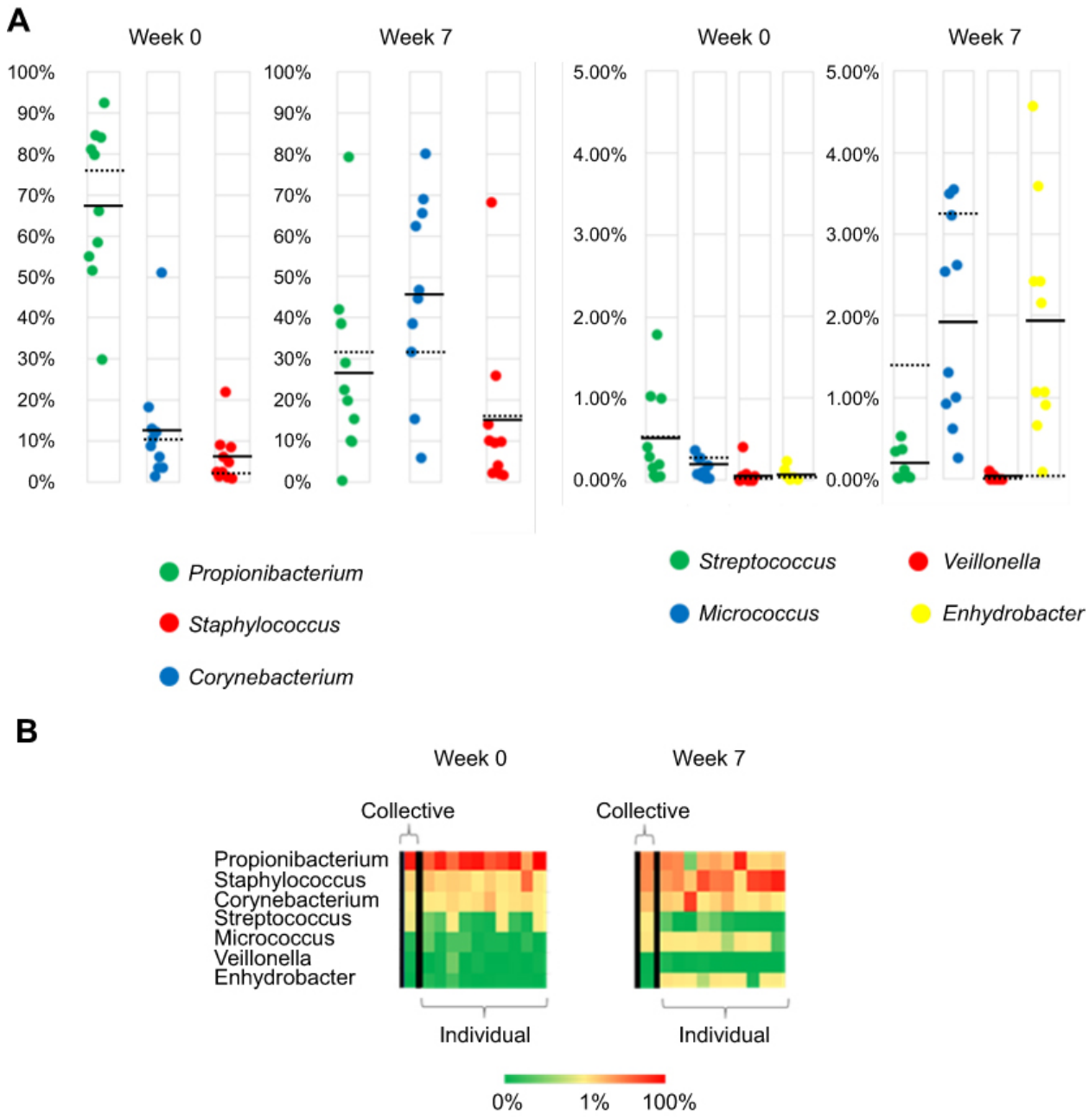

Figure 11 (**A**) Percent abundance of 7 phylotypes of bacteria at two collected time points as determined by BaseSpace 16S app for both individual and collective sampling. Each circle represents a single subject and the mean values are indicated by a solid black line. Dashed line is the value from collective sample. (**B**) Heat map of same 7 phylotypes with collective sample at left for both weeks 0 and 7.

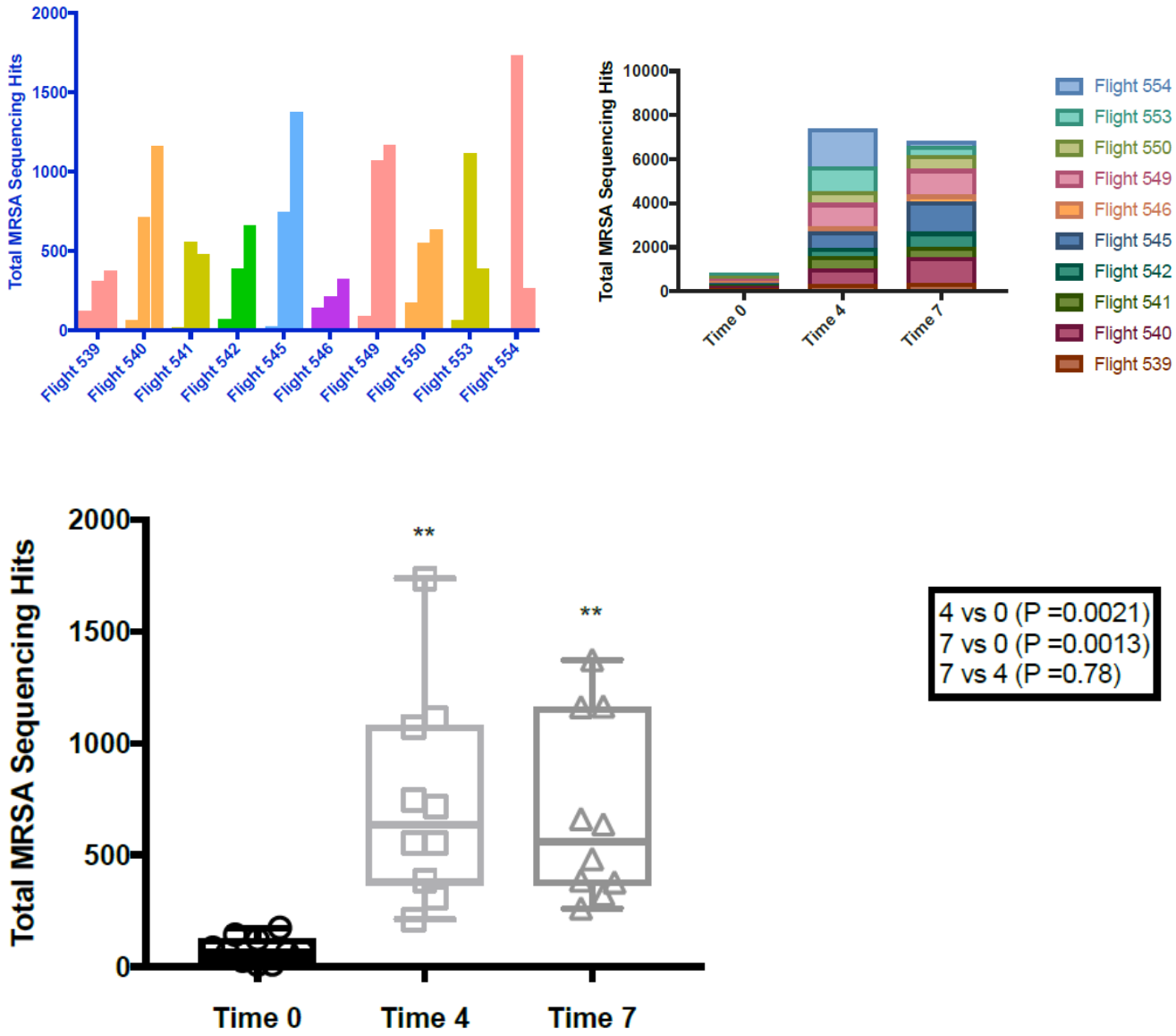

Figure 12 The upper left chart outlines MRSA hits at all 3 time points by flight. The upper right chart demonstrates the total MRSA hits by time points with individual flight data stacked vertically. The lower chart depicts statistical sequencing hits for MRSA at various time points 0, 4, and 7 Weeks with associated p values comparing the frequency between weeks (utilizing two-tailed T-test).