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Impact Of Topical Antimicrobial Treatments On Skin Bacterial Communities

Abstract

Skin is our primary interface to the outside world, representing a diverse habitat with a multitude of folds, invaginations, and appendages. While each of these structures is essential to host cutaneous function, they also serve as unique ecological niches that can support an array of microbial inhabitants. Together, these microorganisms constitute the skin microbiome, an assemblage of bacteria, fungi, and viruses with the potential to influence cutaneous biology. While a number of studies have described the importance of these residents to immune function and development, none to date have assessed their dynamics in response to antimicrobial stress, nor the impact of these perturbations on host cutaneous defense. Rather the majority of work in this regard has focused on a subset of microorganisms studied in isolation. Herein, we present the impact of topical antibiotics and antiseptics on skin bacterial communities, and describe their potential to shape cutaneous interactions. Using mice as a model system, we show that antibiotics can elicit a distinct shift in skin inhabitants characterized by decreases in diversity and domination by previously minor contributors. By contrast, we report a relatively modest impact of antiseptics on skin bacteria, largely preserving inhabitant structure at the community-level. Despite these differences, we show a significant decrease in Staphylococcus residents regardless of treatment, a subset of inhabitants which we also found to influence colonization by the skin pathogen Staphylococcus aureus. To determine the relevance of these findings in human systems, we further treated thirteen subjects with antiseptics at the forearm and back. Similar to mouse experiments, we observed a relatively minor effect of these treatments on bacterial inhabitants at the population-level. However, when controlling for factors such as interindividual differences and body-site specificity, we observed a more significant impact, governed in large part by decreases in lowly abundant members of the skin microbiota. We also found bacterial identity to be a key contributor to this effect, with certain skin taxa exhibiting more robust shifts than others. In all, these results underscore the ability of antimicrobial drugs to alter skin bacterial residence, and outline the importance of these inhabitants to host cutaneous defense.

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Elizabeth A. Grice

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IMPACT OF TOPICAL ANTIMICROBIAL TREATMENTS ON SKIN BACTERIAL COMMUNITIES

Adam Jason SanMiguel

A DISSERTATION

in

Cell and Molecular Biology

Presented to the Faculties of the University of Pennsylvania

in

Partial Fulfillment of the Requirements for the

Degree of Doctor of Philosophy

2017

Supervisor of Dissertation

____

Elizabeth A. Grice Ph.D.
Assistant Professor in Dermatology

Graduate Group Chairperson

____

Daniel S. Kessler, Ph.D.
Associate Professor of Cell and Developmental Biology

Dissertation Committee

Frederic D. Bushman, Ph.D.
William M. Measey Professor in Microbiology

Ronald G. Collman, M.D.
Professor of Medicine

Mark Goulian, Ph.D.
Professor of Biology

Sarah A. Tishkoff, Ph.D.
David and Lyn Silfen University Professor

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DEDICATION

To my mentor Elizabeth Grice and the other members of Club Grice, who have contributed greatly to my successes and helped me to overcome my far more frequent encounters with failure.

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ABSTRACT

IMPACT OF TOPICAL ANTIMICROBIAL TREATMENTS ON SKIN BACTERIAL COMMUNITIES

Adam Jason SanMiguel

Elizabeth Anne Grice, Ph.D.

Skin is our primary interface to the outside world, representing a diverse habitat with a multitude of folds, invaginations, and appendages. While each of these structures is essential to host cutaneous function, they also serve as unique ecological niches that can support an array of microbial inhabitants. Together, these microorganisms constitute the skin microbiome, an assemblage of bacteria, fungi, and viruses with the potential to influence cutaneous biology. While a number of studies have described the importance of these residents to immune function and development, none to date have assessed their dynamics in response to antimicrobial stress, nor the impact of these perturbations on host cutaneous defense. Rather the majority of work in this regard has focused on a subset of microorganisms studied in isolation. Herein, we present the impact of topical antibiotics and antiseptics on skin bacterial communities, and describe their potential to shape cutaneous interactions. Using mice as a model system, we show that antibiotics can elicit a distinct shift in skin inhabitants characterized by decreases in diversity and domination by previously minor contributors. By

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Chapter 1 – Introduction to antimicrobial drugs and the skin microbiome

1.1 Historical perspective

Studies of the skin microbiome have, throughout history, become inextricably linked with a desire to remove said inhabitants. This is perhaps best exemplified by early reports describing the utility of hygiene and antiseptics under Ignaz Semmelweis and Joseph Lister in the mid-1800s[1,2]. These studies described the potential for antibacterial compounds to reduce infection and improve surgical mortality rates, a figure well above 10% in developed countries at the time[3]. While initially controversial, antiseptic methods were ultimately accepted by the medical community, and heralded as a proficient means to reduce the levels of skin bacterial residents with the potential to cause disease[4-7].

As time progressed, researchers built upon these studies with a focus on chemicals displaying the greatest potential for skin sterilization[8,9], hoping to improve upon the findings of Semmelweis and Lister. Eventually, however, two factors became increasingly apparent in the fight against infection. First, that no technique was capable of complete and consistent sterilization of the skin[10,11]. And second, that many of the surviving microorganisms were also known residents of the skin, eliciting little detriment in the overall outcome of patients[12,13]. This newfound understanding led to a fundamental shift in the field of skin bacteriology, and, paired with the advent of narrow spectrum antibiotics, resulted in experiments focused on

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the disruption of specific pathogenic microorganisms rather than sterilization of entire cutaneous populations[14,15].

Since this time, a wealth of literature has described the impact of antibiotics and antiseptics on infectious bacteria, underscoring the importance of these drugs to human health and disease[16-18]. However, this focus has also led to constraints in our knowledge of resident bacterial response. Indeed, although antimicrobial drugs are commonly applied directly to the skin, few studies have assessed their impact beyond that of pathogenic microorganisms[19]. A reliance on culture-based systems has further complicated this question, with a true survey of antimicrobial effects being all but impossible without prior knowledge of resident growth requirements[20]. This has resulted in an over-simplification of the field, and the presumption that effects on easily cultured skin residents can be applied to other cutaneous inhabitants[21]. No study to date has adequately verified this assertion, however, leading to conflicting views amongst researchers and physicians alike.

1.2 New technologies to answer an old question

In light of these shortcomings, we and others have begun to apply less-biased methods to the identification of skin bacterial residents. Specifically, the introduction of sequencing-based technologies has enabled the study of nearly all bacterial inhabitants, rather than limiting researchers to microorganisms with well-established, culture-based protocols[22]. These approaches also represent a more high-throughput method for bacterial community identification, exhibiting a

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significant advance on previous strategies which required an abundance of selective medias and agars to differentiate between inhabitants[23].

Sequencing-based technologies generally fall within two broad categories: marker-based and whole genome[24]. As its name would suggest, marker-based methods rely on specific genes found throughout the bacterial kingdom to describe community membership. Ideally, these genes contain stretches of both conserved and hypervariable sequences. This then allows for the design of primers to conserved areas, and the use of nearby hypervariable regions to distinguish amongst residents[25,26]. The best example of such markers is the 16S rRNA gene, found ubiquitously in bacteria[27]. This gene contains nine hypervariable regions, each representing the opportunity to resolve differences in bacterial membership under a variety of conditions. We and others have described the utility of the V1-V3 hypervariable regions for human skin bacterial identification[28]. However, other regions can also be helpful depending upon the environment to be sampled[29].

Despite these advantages, marker-based analyses are not without limitations. Specifically, species- and strain-level information can be difficult to extract when comparing residents with high proportions of sequence homology. To compensate for this restriction, whole genome shotgun sequencing is often employed to acquire longer stretches of information[30]. While more expensive than marker-based approaches, this technique allows for higher levels of resolution when attempting to distinguish between closely related bacterial inhabitants[31]. This method is also

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useful in metagenomic analysis, a type of examination which describes both the composition and functional potential of a given community[32]. Cost can still represent a prohibitive variable when making multiple comparisons, however. As such, both resolution and price must be weighed appropriately, prior to investigation, to maximize the breadth and depth of one's analyses.

1.3 Study outline and rationale

With this information in mind, we applied sequencing-based methods to more completely evaluate the impact of topical antimicrobial drugs on skin bacterial residence. As our experiments represent the first attempts at said endeavors, marker-based 16S rRNA gene sequencing was employed for community analyses. This enabled us to widen the breadth of our comparisons, and to assess the longitudinal dynamics of skin residents in response to multiple treatment regimens.

To control for the greatest number of variables, we began our tests in the murine model system. SKH-1 hairless mice were treated at the dorsum with a triple antibiotic ointment (Bacitracin, Neomycin, and Polymyxin B) or the narrow spectrum antibiotic Mupirocin. Samples were then collected longitudinally to assess changes in the skin microbiota over time. Antibiotic treated mice were compared to those receiving vehicle ointments to control for non-antibacterial effects. A separate cohort was also treated with water or the common clinical antiseptics alcohol (80% ethanol) and povidone-iodine (Betadine) to compare mechanistically distinct antimicrobial interventions.

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During these investigations, we observed a conserved ability of all treatments to disrupt *Staphylococcus* skin residence. Because members of this genus have been shown to influence colonization by the skin pathogen *Staphylococcus aureus*[33,34], we also used our system to interrogate the role of antimicrobial drugs in skin colonization resistance. Colonization resistance represents a means for resident species to defend a host against more pathogenic microorganisms[35]. Moreover, in the gastrointestinal tract, antibiotic treatment has been shown to increase host susceptibility to infection through alterations in resident communities[36]. We show that the skin microbiota functions in a similar manner, with multiple *Staphylococcus* residents representing potential *S. aureus* competitors at the skin surface.

As a validation of these experiments, our final assessment included an investigation of antiseptics and human skin bacterial residents. Previous studies have defined these inhabitants as exhibiting high levels of both inter- and intrapersonal variability[37,38]. This includes a distinct stratification of communities by cutaneous biogeograpy[39]. To account for these variables, thirteen subjects were treated identically at the back and forearm, body sites with significant topographical distinction[40]. In all, these studies enabled us to evaluate the resilience of human bacterial residents at the population-level, and together with our murine experiments, expound the importance of key variables to skin bacterial community response.

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Chapter 2 - Interactions between host factors and the skin microbiome

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2.1 Abstract

The skin is colonized by an assemblage of microorganisms which, for the most part, peacefully coexist with their hosts. In some cases, these communities also provide vital functions to cutaneous health through the modulation of host factors. Recent studies have illuminated the role of anatomical skin site, gender, age, and the immune system in shaping the cutaneous ecosystem. Alterations to microbial communities have also been associated with, and likely contribute to, a number of cutaneous disorders. This review focuses on the host factors that shape and maintain skin microbial communities, and the reciprocal role of microbes in modulating skin immunity. A greater understanding of these interactions is critical to elucidating the forces that shape cutaneous populations and their contributions to skin homeostasis. This knowledge can also inform the tendency of perturbations to predispose and/or bring about certain skin disorders.

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2.2 Introduction

The skin is our primary interface to the external environment, supporting the growth of commensal microorganisms while impeding invasion by more pathogenic species. Culture-independent techniques that employ sequencing of marker genes, such as the bacterial-specific 16S ribosomal RNA (rRNA) gene, have begun to elucidate the community characteristics of these cutaneous microorganisms. In addition, these analyses have been used to inform elements of intrapersonal and interpersonal variability, as well as longitudinal dynamics of skin microbial communities. These studies have also led to investigations into the importance of host-microbe interactions, and their ability to shape the identity and composition of commensal relationships. This review will highlight these determinants as they pertain to a number of host factors. It will also address the role of microbiome-host interactions in certain skin disorders. While numerous microorganisms are thought to colonize the skin surface, we will emphasize the contribution of bacterial and fungal inhabitants. However, it is important to note that viruses, mites, and archaea are all capable of influencing residential populations of the skin.

2.3 Cutaneous architecture and biochemistry

To fully appreciate the microbial diversity of the skin, one must first understand the complex architecture and environment of this organ. As a critical barrier to the outside world, human skin is essential for activities such as thermoregulation, gas exchange, and hydration [1]. It also represents one of the body's largest and most exposed organs with approximately 1.8 m^2 of total surface area. The biogeography

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of the skin includes a number of planes, folds, and invaginations, each capable of maintaining a unique microenvironment. For this reason, microbial communities above the cool, desiccating skin surface often differ greatly from those found within shielded pores and follicles [2]. Different skin sites can also contribute to microbial heterogeneity through the production of various lipid- and water-based solutions. These determinants then work in concert with additional host factors and the external environment to shape an individual's core microbiome.

2.3.1 Skin strata

Human skin consists of two main layers: the epidermis and the dermis (Figure 1). As the most superficial layer, the epidermis contributes the majority of barrier functions while the dermis provides a structural framework made of fibrous and connective tissues. Underlying these strata is a layer of subcutaneous fat, which is critical for the protection of deeper tissues and bones.

As a continually self-renewing epithelium, the epidermis can be subdivided into four main strata, characterized by cells at varying stages of development (Figure 1). The bottommost layer, the stratum basale, contains a single layer of undifferentiated stem cells that rest upon the epidermal basement membrane [3]. All keratinocytes originate from these basal cells, and they are essential for the regeneration of keratinocytes lost to terminal differentiation and desquamation [4]. During asymmetric cell division, these progenitor cells produce a subset of daughter cells that exit the cell cycle and separate from the basement membrane to form the

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stratum spinosum. In this layer, immature keratinocytes are characterized by abundant calcium-dependent desmosomes, which promote intercellular adhesion and resistance to mechanical stress [5]. As these cells continue to develop, they also flatten and initiate the formation of lamellar bodies and keratin filaments to support overall skin structure [5].

Upon further maturation, keratinocytes progress upwards to populate the stratum granulosum, so-named for the presence of prominent keratohyalin granules. These vesicles contain filaggrin, keratin filaments, loricrin, and involucrin – all necessary components for the hydration and structure of mature epidermal tissue [5]. Keratinocytes of the stratum granulosum are also held together by a number of extracellular tight junction proteins including claudins and occludins, which are essential to epidermal barrier function [6]. During the terminal stages of differentiation, cells of the granular layer compress and anucleate to form the stratum corneum. At this stage, keratinocytes then become known as corneocytes for their highly cornified cellular envelopes. These protein-enriched cells are also held together by keratins, corneodesmosomes, and a lipid-enriched extracellular matrix to provide a strong physical barrier that is resistant to mechanical stress, UV damage, and permeation [7].

2.3.2 Appendages

In addition to these strata, the skin is also characterized by a number of appendages that can extend beyond the epidermis into the dermis. These include sebaceous

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glands, hair follicles, and sweat glands. Sebaceous glands specialize in the secretion of sebum, an oily, lipid-rich substance that provides skin flexibility and waterproofing. Most sebaceous glands are also connected to hair follicles to form pilosebaceous units that concentrate on the face and upper body [8]. Pilosebaceous follicles support an array of niche-specific microorganisms that can thrive in anoxic environments rich in sebum-derived lipids [9]. These lipids can then be metabolized into free fatty acids by bacterial commensals, which contribute to the acidic pH of the skin [10]. Importantly, while the number and distribution of sebaceous glands remains relatively constant throughout life, their size and activity fluctuates widely depending on age and hormone levels [8]. It is thus not surprising that puberty marks a defining period in skin development characterized by the elevated production of sebum and sebum-related products, as well as the subsequent growth of lipophilic skin microbial inhabitants [11].

Sweat glands, another critical appendage of the epidermis, can be divided into two major types: apocrine and eccrine. Like sebaceous glands, apocrine sweat glands release oily secretions into upper hair follicles and are especially active during puberty. Apocrine sweat is composed of a milieu of proteins, lipids and steroids [12]. Apocrine glands are also more sparsely distributed, often localized to especially pileous regions such as the axillae and perineum [12].

Eccrine sweat glands, in contrast, are widely distributed throughout the body with high concentrations at the forehead, axillae, palms, and soles [13]. They are also the

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only gland with direct access to the skin surface, and as such, continuously bathe the epidermis in a water- and salt-based sweat solution. These secretions are critical to thermoregulation and hydration, and also contribute to the relatively acidic pH of skin surfaces.

In all, the dissemination and activity of epidermal appendages provide essential roles for the human body. By creating habitats with unique levels of moisture, pH and nutrients, they also represent specialized niches that can promote the growth of distinct microbial communities. This then contributes to the unique stratification of bacterial populations at skin sites throughout the body.

2.4 Host factors and the skin microbiota

2.4.1 Topographical variability

The site-specificity of the skin microbiota has been borne out in multiple experiments analyzing unique topographical locations of the skin (Figure 2). For example, a study of 20 distinct body sites representing sebaceous, moist, and dry physiological environments found that *Propionibacterium* and *Staphylococcus* species dominated sebaceous skin sites including the face and upper body [14]. By contrast *Corynebacterium*, β-Proteobacteria, and *Staphylococcus* were the major genera at moist sites such as the axilla, antecubital fossae (inner elbow), and popliteal fossae (inner knee). Dry sites including the forearm and buttock were found to be more variable, supporting the growth of numerous phylotypes including β-Proteobacteria, *Corynebacterium*, and Flavobacteriales.

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Upon more in-depth analyses, it was revealed that the sites richest in bacterial operational taxonomic units (OTUs; a sequence-level proxy for designating species) were often dry regions such as the forearm, while sebaceous sites including the upper back and retroauricular crease (behind the ear) were home to fewer bacterial phylotypes. In addition, sebaceous regions were consistently lower in bacterial evenness as measured by the relative distribution of sequences among OTUs. Interpersonal variation (differences between individuals) was found to be greater than intrapersonal variation (differences within individuals) over time. This suggests that individuality and body site physiology are both strong determinants of bacterial community membership and structure.

Similarly to above, Costello et al. observed that temporal intrapersonal variability was less pronounced than interpersonal variability between individuals [15]. These studies also confirmed that spatial intrapersonal variability (e.g. variability in microbiomes of distinct body sites such as forehead, arm, and umbilicus) was even greater than interpersonal variability at the same skin site. As such, although individual microbial populations of the skin are often more similar to themselves in regard to symmetry and time, these likenesses appear to breakdown when comparing separate biogeographic regions.

In accordance with Grice et al., this group also found high levels of *Propionibacterium* at sebaceous sites on the face, and greater diversity at areas such as the popliteal fossa, forearm, and palm. Moreover, it was shown that the variation

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of these sites remained relatively constant over time, as the palm and forearm were both consistently more diverse than the forehead at four separate collection periods.

The influence of body site in regard to overall community structure was also tested by inoculating bacteria from foreign sites onto new areas of the skin. These studies observed a relative flexibility in forearm community membership, while the forehead microbiota rapidly returned to a population resembling its native state. This suggests that host factors may vary in their ability to promote bacterial colonization, especially at sebaceous sites with strong environmental biases.

Whereas these studies sought to compare multiple body sites, additional research has focused on individual skin regions. These studies largely complement one another, providing greater insight into the contribution of topography to skin microbial communities. For example, studies performed on the human forearm have illustrated relatively high degrees of bacterial diversity, although this population is consistently dominated by *Propionibacterium, Corynebacterium, Staphylococcus, Streptococcus,* and *Acinetobacter* [14-17]. While these major taxa appear throughout the literature, however, it appears that their relative contributions to the forearm community can fluctuate greatly.

The same can be said of the palmar region, which is frequently exposed to new surfaces and environments - while major phylotypes such as *Propionibacterium,*

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Streptococcaceae, and Staphylococcaceae are consistently observed, a great amount of variability exists in regard to their absolute numbers and proportions [14,15,18,19]. Thus it appears that certain exposed regions including the palm and forearm are less restricted in overall community membership and highly susceptible to temporal variability.

By contrast, other regions, including those with high sebaceous gland activity, are much more exclusive. For example, the forehead harbors fewer bacterial species and is largely dominated by *Propionibacterium* [14,15,17,20]. This observation is congruent among multiple studies, and as such, represents a relatively consistent trend. Whether this effect is inherent to the lipid-rich environment of the forehead, or whether *Propionibacterium* can successfully restrict membership alone is currently unknown. Regardless, this region appears largely invariant compared to more diverse sites of the skin, and thus represents a more stable overall community structure.

While compelling, the stratification illustrated by certain dry and sebaceous sites is by no means absolute, as multiple sites of the skin are characterized by intermediate diversity with both dominant and transient taxa [14,15]. Therefore, further research will be necessary to determine the role of intrinsic host factors and extrinsic microbial traits as they pertain to skin bacterial communities.

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Recent studies have also begun to elucidate the topographical diversity of fungal communities on human skin [21]. Specifically, it was shown that *Malassezia* predominated at core body and arm sites, but that discrete signatures could be observed at the species level. For example, the face was dominated by *Malassezia restricta* while the back, occiput (back of neck), and inguinal crease (groin) were all characterized by higher levels of *Malassezia globosa*.

In contrast to these areas, regions of the foot such as the plantar heel, toenail, and toe-web space were all defined by significantly greater amounts of fungal diversity. While *Malassezia* was still detected in all samples, subjects were also colonized by relatively high proportions of *Aspergillus* and *Epicoccum*. Interestingly, regional localization was found to be the strongest determinant of fungal community membership as feet, arms, the head, and torso all formed distinct communities regardless of physiological environment. This suggests that while bacterial populations are subject to factors such as sebum content and hydration, fungal communities are more flexible in resource utilization, a less surprising realization given their pronounced evolutionary differences.

2.4.2 Gender

The contribution of gender to skin microbial diversity likely arises as a downstream effect of male and female steroid production [13]. For example, it is thought that androgen expression and identity are both critical to sex-defined differences in skin thickness [22,23]. Males also exhibit increased levels of sebaceous and sweat gland

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activity compared to females, a trait that strongly contributes to differences in skin surface biochemistries [24,25]. Even the presence or absence of body hair could presumably result in alternative microenvironments with the potential to support the growth of niche-specific microorganisms. Interestingly, mixed results have been observed in regard to gender and pH. While some studies have detected a more acidic pH in female skin, others have demonstrated no differences [26-29]. This suggests that variation in male and female physiologies have the potential to influence microbial communities, but that certain factors likely contribute to skin habitats more strongly than others.

With this in mind, a recent study that sampled the palmar regions of male and female undergraduate students observed significantly different bacterial communities on the skin surface in regard to gender [18]. While no taxa were specific to either sex, there were marked differences in the relative abundances of numerous bacterial groups. For example, *Propionibacterium* and *Corynebacterium* were 37% and 80% more abundant in men, respectively, along with a trend towards higher levels of *Staphylococcus*. By contrast, Enterobacteriales, Moraxellaceae, Lactobacillaceae, and Pseudomonadaceae were all over 150% more abundant in females. Women were also found to harbor significantly greater levels of alpha diversity, a metric that defines "within" sample diversity and is often measured by numbers of OTUs, their evenness, and their degree of phylogenetic difference.

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In contrast to these results, a study of healthy Chinese undergraduates showed no significant differences between the palmar bacterial communities of men and women [19]. However, higher relative abundances of distinct taxa such as *Propionibacterium*, *Corynebacterium,* and *Staphylococcus* were once again observed in male subjects while *Lactobacillus* was over-represented in females. Interestingly, *Enhydrobacter* and *Deinococcus* also made up a large portion of female hand communities, while Fierer, et al, found no such contribution in either sex. This suggests that geographical or cultural aspects may also play a large role in diversifying skin microbial communities, a concept that has been supported by a number of additional reports as well [30,31].

In a study comparing the skin microbiota at varying developmental stages, males and females between the ages of 2 and 40 were swabbed at the antecubital and popliteal fossae, the volar forearm, and the nares [32]. In all, no significant differences were observed between the bacterial communities of males and females regardless of age group. Moreover, a study comparing the levels of *Propionibacterium* and coagulase negative *Staphylococcus* in middle-aged men and women found no significant differences at the forehead, cheek, upper chest, or back [33]. However, it was found that males harbored greater total amounts of the fungi *Malassezia.*

Studies have also examined the human axilla, upper buttock, forehead, and forearm as potential sites of gender variability. Interestingly, the bacterial communities of

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the axillary vault were found to stratify into two main groups, those colonized predominantly by *Staphylococcus* and those with high relative abundances of *Corynebacterium* [34]. While not absolute, female subjects were generally found within the *Staphylococcus* cluster whereas males were more often associated with the *Corynebacterium* cluster. Analysis of the upper buttock also exhibited a strong effect of gender with males illustrating relatively high proportions of *Corynebacterium*, *Dermacoccus, Streptococcus*, and *Finegoldia* while females displayed elevated levels of *Lactobacillus*, *Propionibacterium*, *Staphylococcus*, and *Enhydrobacter* [35]. Despite these distinctions, there were no significant differences between genders when taking the entire microbial community into account, suggesting that individualized signatures were still the best indicators of variability.

On the forehead, males and females were found to harbor differences in overall bacterial diversity [17]. However, when accounting for the use of make-up, significant variability between these groups was no longer detected. In contrast, microbial diversity of the forearm was significantly different between men and women at both the genus and species level.

In all, it appears that gender may contribute to microbial community structure, but that the importance of this factor likely varies in a site-dependent manner. As male and female physiology differs throughout the body, it is not surprising that the contribution of gender to microbial communities is also inconstant. More detailed studies will be necessary to determine the importance of potential driving factors,

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as no studies to date have measured microbial populations and biochemical signatures in concert.

2.4.3 Age

The human skin begins to develop *in utero* during the first trimester of gestation, and by 34 weeks, a well-defined stratum corneum has formed [36]. In the weeks leading up to delivery, the epidermis further matures, and begins to resemble a competent adult-like barrier by week 40 [36]. Upon birth, the skin undergoes a number of rapid changes as it acclimates to a dry, gaseous climate very much at odds with its former aqueous environment. During this time, the skin is characterized by quantal bursts of improved barrier function that persist for multiple weeks postnatal delivery [38]. Development then continues during the first year, after which point infant skin begins to resemble that of mature adults [39].

During maturation, infant skin is defined by a thin layer of corneocytes that are, on average, much smaller than adult corneocytes [40]. In addition, infant skin contains lower lipid content resulting in an epidermal barrier with higher water levels and increased permeability [39,41]. Neonates are also born with a relatively alkaline skin pH that remains less acidic than adult skin for the first two years of life [42].

All of these developmental features likely contribute to the differences seen between adult and infant bacterial communities. For example, *Staphylococcus* species, which are known to predominate at moist body sites on the adult

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epidermis, have been found at significantly higher levels on neonatal skin. In fact, a recent study of the infant microbiota observed that *Staphylococcus* and *Streptococcus* species could account for up to 40% of skin bacterial populations during the first six months of life, before giving way to a more diverse community [43]. Interestingly, site-specificity also began to appear within the first few months of life. *Staphylococcus, Streptococcus, Corynebacterium,* and *Propionibacterium* were all found to predominate at the arm and forehead of infant skin while the buttock was colonized by both gut- and skin-associated taxa such as *Clostridium*, *Staphylococcus*, *Streptococcus,* and *Ruminococcus.* This suggests that as the skin matures, it becomes more adept at influencing resident bacterial communities at certain body sites.

Additional experiments have also examined the route of delivery as a direct contributor to the human skin microbiota [44]. These analyses have shown that vaginally born neonates harbor skin bacterial communities very similar to those found in the vagina. This includes an abundance of both *Lactobacillus* and *Prevotella*. In contrast, babies born by Cesarean section were colonized by common skin residents such as *Acinetobacter,* Bacillales, Micrococcineae, and *Staphylococcus.* Interestingly, this study also found that babies born through conventional methods displayed skin bacterial communities most similar to their mother's microbiota, while babies born by Cesarean section were no more similar to their own mother than any other subject. As such, while an initial vertical transmission of the bacterial microbiota existed in vaginally delivered neonates, no such transmission occurred

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in babies delivered by Cesarean section. Rather it appears that incidental exposures, likely provided by hospital staff and environmental surfaces, were the greatest contributors to microbial communities in these subjects.

While the initial inhabitants of infant skin can vary greatly depending upon age and delivery mode, their microbiomes appear to stabilize over time, reaching an adult-like community at sexual maturity. A study employing Tanner staging to distinguish between children and adults found that the microbiota of subjects within Tanner stages 1, 2, and 3 segregated significantly from that of individuals at stages 4 and 5 [32]. Similarly to above, it was also shown that higher levels of Proteobacteria and Firmicute*s* such as Streptococcaceae distinguished the microbiota of younger cohorts, while adolescents/post-adolescents were dominated by *Propionibacterium* and *Corynebacterium*. This particular result corresponds well with the developmental milestones reached at higher Tanner stages including elevated hormone levels and increased sebaceous gland activity, as both factors promote the growth of more lipophilic microorganisms [45].

Interestingly, it has also been shown that the common fungal commensal *Malassezia* colonizes neonate skin during the birthing process [46]. At day 0 following delivery, *Malassezia* DNA was successfully detected in 24 of 27 subjects, and by day 30 approximately 10^4 residents were estimated by qPCR. While the specific distribution of *Malassezia* residents differed greatly in newborns compared to their mothers, these rates stabilized to a level very near that of adulthood by day 30.

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Overall, these results suggest that the skin and its microbial inhabitants develop together over time. While the physiological and biochemical attributes of the skin contribute a great deal to microbial diversity, this niche also represents a blank slate with the potential to accommodate a vast array of microbial organisms. For this reason, further research will be necessary to fully elucidate the dynamic nature of age-related succession.

It may also be necessary to revise the long-held belief that most fetuses develop in a sterile environment. Recent evidence suggests that bacteria can be reproducibly isolated from newborn meconium and umbilical cords of healthy, full-term neonates [47,48]. *Enterococcus faecium* has also been isolated from newborn meconium and amniotic fluid following oral inoculation of pregnant mice, and fluorescent *in situ* hybridization can be used to visualize 16S rRNA-containing species deep within human fetal membranes [47-49]. A recent study of the placental microbiome also reported a diverse community of bacterial species characterized by increased levels of Proteobacteria [50]. In addition, both Gram-positive and Gram-negative intracellular bacteria have been detected in over a quarter of placental basal plate samples [51]. These findings are in stark contrast to the notion that newborns are not exposed to microorganisms until birth, and these microbes could contribute to the initial inoculum present on newborn epidermis.

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2.4.4 Immune system

The host immune system and the skin microbiota are in constant communication as each works to establish a steady equilibrium. This is not surprising given the intimate contact made between the two. In fact, it is thought that as many as 10^7 bacteria/cm^2 colonize the epidermis at any given time [52]. Although the vast majority of these microorganisms inhabit the stratum corneum, recent evidence has shown that bacterial species may also reside within deeper layers of the epidermis and dermis [35,53]. For this reason, it is essential for hosts to control the cutaneous immune response, and tailor it to a given threat, as persistent activation against resident skin bacteria could lead to chronic inflammatory disorders.

To perform this function, the skin is equipped with a number of professional innate and adaptive immune cells including multiple dendritic and T cell subsets (Figure 3). Keratinocytes also provide support through the expression of Toll- and Nod-like receptors and the secretion of antimicrobial peptides, proinflammatory cytokines, and chemokines [54]. Even melanocytes can assist in the overall immune response by recognizing and responding to specific foreign antigens [55].

While all of these cells play a crucial role in epidermal barrier function, Langerhans cells (LCs) are thought to act as the key initiators of cutaneous immunity by sampling the upper strata for microbial antigens and presenting these peptides to adaptive immune cells [56,57]. However, the exact role of these specific dendritic cells has recently come into question, as many of the tasks previously attributed to

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LCs, such as cross-presentation, may actually be performed *in vivo* by a separate subset of myeloid cells known as dermal dendritic cells [58,59]. Regardless of subtype, it appears that dendritic cells are crucial to mediating the initial response to barrier disruption. Upon antigen uptake, these cells travel to cutaneous draining lymph nodes where they present foreign peptides to naïve T cells. These T cells then become activated and imprinted with skin-specific homing markers such as cutaneous leukocyte antigen (CLA), CCR4, CCR8, and CCR10 [60-63]. The ligands for these receptors are expressed at low levels during steady state, but they can be upregulated during inflammation, allowing for the recruitment of effector T cells to the skin epithelium. Upon antigen clearance, these mature T cells differentiate into resident or effector memory T cell subsets. Resident memory $CD8^{+}$ T cells are then thought to remain within the epidermis while effector memory $CD4^{+}$ T cells traffic to more distal sites of the skin [64,65].

While this pathway has been established in response to infection, less information exists in regard to the skin's response to commensal microorganisms. Specifically, it is currently unclear how the immune system can differentiate between pathogenic and non-pathogenic species, especially when considering the close proximity of keratinocytes, melanocytes, and LCs to conserved microbial antigens. A recent paper sheds some light on this debate by suggesting that LCs may perform separate roles depending on the state of epidermal tissue [66]. This group found that upon insult, resident LCs were crucial for the activation of resident memory T cells. However, at steady state, these cells promoted a homeostatic balance through the activation and

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preservation of regulatory T cells. While it is proposed that these regulatory T cells are important for the maintenance of self-tolerance, this process could also regulate the host immune response to resident skin microorganisms and inhibit excess inflammation.

With this in mind, various groups have explored the direct interactions of skin inhabitants with keratinocytes and the immune system. For example, the common skin commensal bacterium *Staphylococcus epidermidis* has been found to activate TLR2 signaling and the production of antimicrobial peptides and proinflammatory cytokines, augmenting the immune response to both group A *Streptococcus* and HPV infection [67-69]. The TLR2 ligand lipotechoic acid has also been shown to reduce TLR3-mediated inflammation in keratinocytes and promote the induction of cathelicidin-producing mast cells [70,71]. Interestingly, this effect does not appear to extend to macrophages, dendritic cells, or mouse endothelial cells, as exposure in these cell types results in an inflammatory response that is equal to or greater than that of epidermal keratinocytes. Therefore, a division of labor may exist within the cutaneous epithelium in which only certain cells can promote inflammation, a finding supported by the differential expression of Toll-like receptors at distinct layers of the epidermis [72].

Our lab and others have also focused on the relationship between host immunity and skin bacterial residents in order to identify key members of this host-microbe interaction network. By treating mice with a C5aR antagonist, we have shown that

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disruptions to the complement pathway can lead to significant changes in skin community structure including an increase in Actinobacteria and a decrease in Firmicutes [73]. We also observed a significant decrease in bacterial diversity (defined as the number of OTUs and their evenness), upon treatment, as well as a reduction in the overall number of bacterial OTUs. In addition, the expression of antimicrobial peptides, cytokines, chemokines, cell adhesion molecules, and pattern recognition receptors were all reduced in antagonist-treated mice, along with decreased levels of immune cell infiltration. This suggests that complement proteins may act to induce and/or maintain stable levels of these effectors, and that alterations to this balance can significantly shape skin microbial populations. The expression of complement genes in the skin of germ-free and conventionally-raised mice were also compared to determine the importance of bacterial stimulation to complement gene expression. In the absence of bacterial colonization, we observed significantly lower expression of over 30 genes related to complement activation and binding, indicating that both the skin and its resident microorganisms are capable of influencing the identity of their respective interaction partners.

The ability of the immune system to shape bacterial communities has also been observed by comparing the skin microbiota of healthy and immunocompromised mice [74]. Here, it was found that healthy mice were colonized by an abundance of Proteobacteria including *Acinetobacter*, *Escherichia*/*Shigella*, and *Acidovorax* while immunodeficient mice were dominated by Firmicutes, especially those of the *Staphylococcus* genus. This difference was borne out in diversity metrics as well,

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with healthy mice displaying a significantly greater degree of variation when compared to immunodeficient mice.

Importantly, a recent study of humans with primary immunodeficiencies (PIDs) shows that this effect is not limited to murine models [75]. PID patients were defined by increases in microbial permissiveness to atypical microorganisms such as the opportunistic pathogen *Serratia marcescens*. Depending on the specific PID, patients were also characterized by decreases in site specificity, interpersonal variation, and longitudinal stability, suggesting a generalized dysbiosis caused by alterations to the host immune response. Paradoxically, these changes did not result in significant alterations to microbial diversity, however, indicating that site-specific restraints in humans may still control overall community structure.

Work has also compared the adaptive immune systems of germ-free (GF) and specific-pathogen free (SPF) mice to determine the importance of commensal bacteria to cutaneous immunity [76]. This study found that skin bacterial residents influence T cell number and function, as GF mice had higher levels of Foxp3^{+} regulatory T cells and lower amounts of the cytokines IFN-γ and IL-17A. Importantly, this effect on IL-17A could be rescued by monocolonization with the skin commensal bacterium *Staphylococcus epidermidis.* These results were also extended to infection by the parasite *Leishmania major*. In this model, GF mice were unable to mount a robust immune response to *L. major* while monoassociation with *S. epidermidis* could restore protection in an IL-17A-dependent manner. IL-1α

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expression was essential for this response, as neutralization of this cytokine impaired the restoration of IL-17A signaling. As such, it appears that IL-1 signaling pathways are enhanced by the skin microbiota, and that this response can promote overall skin immune fitness.

A more recent report supports this finding by confirming the ability of T cells to shape skin bacterial communities [77]. Adoptive transfer of T cells from WT mice into *Rag1*$^{-/-}$ mice resulted in the rapid proliferation of both CD4^{+} and CD8^{+} T cells within skin draining lymph nodes, consistent with a memory immune response to skin bacterial antigens. The number of live bacteria and 16S rRNA bacterial sequences were also higher in *Rag1*$^{-/-}$ compared to WT mice, and the transfer of T cells from WT to immunodeficient mice resulted in a steady decline of these markers. This response was abrogated in the absence of IL-17A and IFN-γ, while B cell deficient mice mirrored WT phenotypes, suggesting that certain T cell profiles are essential for the recognition and control of skin bacterial residents.

In all, these results indicate that the immune system and skin microbiota are in constant communication, and that each is necessary to promote homeostasis at the skin surface. However, these interactions appear to vary greatly depending on the specific immune cell subset and signaling pathway, and perhaps even the conditions in which mice are housed. Indeed one group recently reported no differences between the skin microbiota of healthy and immunocompromised mice, although variation is readily detectable when comparing the mice within different

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experimental groups [78]. As such, further research will be necessary to describe the intimate relationship between hosts and bacterial inhabitants, and to determine the key players of this particular host-microbe interaction network.

2.5 Host-microbiome interactions in cutaneous disease

Many cutaneous disorders are caused by, or associated with, overt microbial infection. Here we focus on three of these disorders: acne vulgaris, psoriasis and atopic dermatitis. While complex in etiology, these conditions are thought to involve both microbial and host components. In addition, studies of these diseases have included deep sequencing approaches as a means to elucidate the contribution of skin microbial communities to disease pathology. As such, these disorders represent a model system to study the interactions of host factors and bacterial residents as they pertain to disruptions in skin homeostasis.

2.5.1 Acne

Acne vulgaris is one of the most prevalent skin diseases in the world, representing a financial burden of over 3 billion dollars per year in the United States alone [79]. Despite this figure and studies showing that acne can affect approximately 80% of adolescents and young adults [80], relatively little is known in regard to the events underlying this disorder. In particular, it remains unclear whether: (i) comedone formation is the cause or effect of inflammation in pilosebaceous follicles, (ii) which immune cells and cytokines drive the overall inflammatory response, and (iii) the specific role of skin microbial residents such as *Propionibacterium acnes*.

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Over the past decade, a number of groups have begun to address these questions, outlining a multifactorial process driven, in large part, by increases in androgen production during puberty. This increase in hormone signaling activates sebaceous gland activity and induces epithelial hyperproliferation and keratinization [81]. These changes can then promote the colonization and growth of *Propionibacterium acnes,* and contribute to the chronic inflammation seen in affected pilosebaceous follicles.

Multiple *in vitro* studies have demonstrated the ability of *P. acnes* to increase the expression of key inflammatory cytokines such as IL-1α, IL-1β, IL-6, IL-8, IL-12, and TNF-α by human sebocytes, keratinocytes, and monocytes [82-84]. The presence of infiltrating $CD4^+$ T cells has also been observed by a number of groups, suggesting that the recruitment of these cells could promote inflammation within acne lesions [85-87].

Recently, a number of independent reports confirmed the ability of *P. acnes* to upregulate the production of IL-1β through the activation of the NLRP3 inflammasome [88-90]. Higher expression levels of NLRP3 and caspase-1 were observed in the areas surrounding acne lesions and both markers co-localized with infiltrating tissue macrophages [88,90]. Mice challenged with *P. acnes* also showed increased expression of caspase-1 and IL-1β, while NLRP3 knockout mice displayed a significant decrease in these inflammatory markers [89,90]. In sebocytes, this activity was dependent upon reactive oxygen species and *P. acnes* protease activity,

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while monocytes required bacterial uptake, potassium efflux, and reactive oxygen species [88-90]. This information, coupled with studies showing increased expression of TLR-2 on acne-localized macrophages [83], suggests a mechanism by which monocytes are recruited to early acne lesions, and then activated by *P. acnes* to induce a more robust inflammatory response.

Recent studies have also demonstrated the ability of *P. acnes* to stimulate Th17 differentiation and activity. These reports have shown that IL-17-expressing cells often localize to affected pilosebaceous follicles and are elicited by the production of IL-1β, IL-6, and TGF-β [91]. In addition, *P. acnes*-reactive Th17 cells were isolated from the blood of acne patients at higher frequencies than those of healthy subjects [92]. Two commonly employed dermatologic acne treatments, all-trans retinoic acid and 1,25-dihydroxyvitamin D3, were also found to downregulate *P. acnes*-induced IL-17 mRNA and protein expression [91]. Together these results suggest that $CD4^+$ Th17 cells may be key mediators of the chronic inflammation found within moderate-to-severe acne lesions, and that modulation of these cells could resolve certain aspects of *P. acnes*-induced pathology.

While convincing, these results do not address the fact that *P. acnes* is a common skin inhabitant regardless of acne phenotype. Rather, reconciliation with this observation has come in the form of more detailed experiments describing the specific localization and genetic-signatures of individual *P. acnes* clones. These studies have shown that pilosebaceous follicles are more frequently colonized by *P.*

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acnes in affected, compared to unaffected, individuals [93,94]. This bacterium is also found more commonly as macrocolonies within acne lesions in contrast to the sparse distributions that typically attach to the outer surface of the epidermis in healthy individuals [93,94]. Interestingly, within these follicles, multiple strains of *P. acnes* have been observed, but only certain strains, such as subtype IA, are associated with acne vulgaris [94-97]. A recent study utilizing 16S rRNA gene sequencing of *P. acnes* populations confirmed this finding by isolating certain subtypes of *P. acnes* from acne patients more frequently than others [98]. Interestingly, this group also reported a specific phylotype of *P. acnes* that associated more commonly with healthy subjects compared to acne patients, underscoring the importance of strain-specific profiles in *P. acnes* pathogenesis.

Overall, it appears that androgen-induced increases in sebum production during puberty may promote *P. acnes* colonization, but that this effect is not necessarily emblematic of disease. Rather, the growth of specific *P. acnes* strains may be required for acne lesions to develop into fully mature papules and pustules. Indeed, studies have reported a differential immune response in sebocytes and keratinocytes when exposed to alternative strains of *P. acnes*, a characteristic that could explain the ubiquity of *P. acnes* in both affected and unaffected individuals [82,99].

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2.5.2 Psoriasis

Psoriasis is a common inflammatory disease affecting approximately 2-3% of the world's population [100]. While multiple phenotypes exist, this condition is often characterized by well-demarcated erythematous plaques, resulting from chronic inflammation and the hyperproliferation of keratinocytes [101]. At onset, an initial inflammatory event is thought to precede plaque formation and induce the production of numerous proinflammatory cytokines. Further inflammation is then promoted by $CD4^+$ Th1, Th17, and Th22 cells leading to distinct changes in skin architecture [102]. These include the thickening of epidermal cell layers, elongation of epidermal rete ridges, hypogranulosis, and parakeratosis [103].

Genome-wide association studies have largely supported these phenotypic observations with most identified defects belonging to the IL-23/Th17 axis, NF-κB pathway, and epidermal differentiation complex [104-106]. However, the major genetic determinant of psoriasis is found within the *HLA-Cw0602** allele of the MHC class I molecule, HLA-C [107]. Mutations within this locus are thought to account for approximately 60% of all psoriasis cases suggesting that $CD8^+$ T cells may also play a major role in disease pathogenesis [108].

Although a number of pharmaceutical drugs are currently available to mediate the inflammatory nature of psoriasis, little is known in regard to the source of this inflammation. Physical trauma (Koebner's phenomenon) and infection have both been associated with the induction of psoriatic flares [109,110]. This is supported

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by the observation that surgical procedures and streptococcal throat infections often precede lesion formation [103,111-113]. However, no study to date has identified an antigen capable of eliciting a complete psoriatic phenotype in healthy skin, despite links between superantigens and certain streptococcal surface proteins [114-116]. It is interesting to note that while infection of the throat with streptococcal species is the best-studied site of proclivity, *Streptococcus* is also a common resident of the skin [14-16]. As such, physical trauma and infection need not be mutually exclusive events if injury results in the presentation of streptococcal-associated (or alternative bacterial) antigens.

In this vein, a number of groups have attempted to characterize the microbiota of psoriasis plaques in search of inflammatory antigens and disease-associated microbial signatures. The first of these found an overabundance of Firmicute*s* in psoriasis skin compared to uninvolved skin, while Actinobacteria were significantly underrepresented at affected skin sites [117]. Psoriasis plaque communities were also more diverse than unaffected skin with elevated *Streptococcus*/*Propionibacterium* ratios. Unfortunately, this particular analysis employed an unmatched study design, raising the possibility that observed differences could also be due to variation between microbial communities at distinct topographical sites.

To address this concern, more recent studies have employed a matched control design that compares identical unaffected/affected skin sites. The first utilized skin

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biopsies to study microbial populations on the trunk, arms, and legs of affected individuals [118]. This group found no differences in alpha or beta diversity between psoriatic and normal skin. Moreover, when taking body site into account, no differences were observed between Firmicutes or Actinobacteria species at the trunk or limbs. Proteobacteria were found to be significantly greater in trunk psoriasis samples compared to the control group, however this result was not significant when comparing the legs and arms of psoriasis subjects to controls. Similar to above, the ratio of *Streptococcus*/*Propionibacterium* was elevated in the psoriasis group with respect to controls, but this result was largely due to the absence of *Propionibacterium* in a number of psoriasis samples, rather than significant fluctuations in streptococcal species.

More recently, Alekseyenko et al. compared swabs of psoriasis lesions to unaffected skin sites and demographically-matched controls [119]. While trending towards decreased alpha diversity, no significant differences in this metric were detected between lesions, unaffected sites, or control samples at the OTU level. There were also no differences in the relative abundances of Firmicutes or Actinobacteria. Notably, Proteobacteria were found at significantly higher levels in unaffected skin, in contrast to the abovementioned study. Plaque specimens also displayed the greatest intragroup diversity while unaffected skin from psoriasis patients was more similar to control skin. This suggests that psoriasis plaques may be more permissive to alternative phylotypes, while unaffected skin may retain its ability to influence microbial populations.

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In all, these studies indicate that skin bacterial communities from affected subjects may be shifted in a modest, but significant manner. Given the intrapersonal variability of the microbiota at sites with disease predilection, it is also possible that stochastic differences between subjects are masking additional, more subtle trends. For this reason, longitudinal comparisons of subjects may prove more valuable as a means to survey the skin over time and monitor each individual with respect to his/her unique microbial community. This is especially important when considering disorders such as psoriasis, in which alterations to the microbiota appear less pronounced.

2.5.3 Atopic Dermatitis

Atopic dermatitis (AD) is a chronic inflammatory skin disease that affects 10-20% of the childhood population [120]. This condition initially appears as an eczematous rash with pruritis and erythema, but during later stages of disease these lesions can mature into lichenified plaques [121]. AD also predisposes individuals to increased prevalence of asthma, allergic rhinitis, and food allergies - a condition known as the "atopic march" [122]. Unlike psoriasis, AD is a $CD4^+$ Th2-mediated disorder with IL-4, IL-5, and IL-13 driving initial inflammatory events [123-126]. Upon sensitization, epidermal cells secrete pro-inflammatory cytokines such as thymic stromal lymphopoietin (TSLP), IL-25, and IL-33 [127-129]. This response then promotes a Th2-specific immune response which can lead to elevated infiltration by mast cells, eosinophils, and allergen-specific IgE [130-132].

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Similar to the aforementioned conditions, the underlying cause of AD pathology also remains unclear. Although both immune dysfunction and epidermal abnormalities have been implicated by GWAS analyses, loci associated with cutaneous barrier function have been associated most strongly with the disease, specifically mutations in the filament-aggregating protein, filaggrin [133]. Filaggrin is a major structural protein of the epidermis that aligns keratin filaments and contributes to the contractile strength of the stratum corneum [134]. Over time, filaggrin is also broken down into natural moisturizing factors and amino acid derivatives to assist in the hydration and acidification of the stratum corneum [135]. As such, this protein represents an essential member of the epidermal differentiation complex.

Because of the strong association between *FLG* mutations and AD, it is generally thought that disruptions to the epidermal barrier predispose the skin to allergen sensitization and immune dysfunction. However, this alteration in structure cannot fully explain the development of AD, as approximately 40% of patients with *FLG* mutations often fail to develop the characteristic lesions seen in affected individuals [136]. *FLG* expression can also be downregulated in patients with wildtype *FLG* alleles, suggesting that filaggrin levels and activity could be affected by peripheral means [137]. Indeed, exposure to the cytokines IL-4 and IL-13 can reduce expression of *FLG*, suggesting an alternative model in a subset of individuals whereby immune dysregulation could portend epidermal barrier abnormalities [138].

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Interestingly, a number of studies also suggest that AD can promote colonization of the skin by *Staphylococcus aureus*. While *S. aureus* is a rather infrequent inhabitant of extranasal body sites in healthy individuals, it has been shown to colonize >80% of patients with AD [139-141]. In support of this, a recent study utilizing 16S rRNA gene sequencing found that *Staphylococcus* species, specifically *S. aureus* and *S. epidermidis*, dominated atopic lesions, while the common skin residents *Corynebacterium*, *Streptococcus*, and *Propionibacterium* were all significantly reduced [142]. The relative abundances of *S. aureus* were also correlated with AD disease severity, similarly to previous reports, indicating an increased propensity for *S. aureus* to colonize AD lesions [140,141,143].

This increase in colonization has been hypothesized to occur for a number of reasons including a rise in the availability of *S. aureus* binding receptors, decreases in the expression of antimicrobial peptides (AMPs), and elevated levels of IL-4 expression. In this regard, the lack of an intact stratum corneum in AD skin could expose extracellular matrix proteins to the surface and promote *S. aureus* colonization. Indeed, *S. aureus* adherence to the skin is reduced following preincubation with fibrinogen or fibronectin, and *S. aureus* strains lacking fibrinogen- and fibronectin-binding proteins illustrate significantly impaired binding to AD skin [144,145]. The cytokine IL-4 has also been shown to upregulate the production of fibronectin by dermal fibroblasts while binding of *S. aureus* to the skin is significantly impaired in IL-4 knockout mice [144,146].

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Unfortunately, the importance of antimicrobial peptides to *S. aureus* colonization remains unclear. It was initially thought that reduced expression of AMPs in atopic skin could eliminate a key barrier to *S. aureus* colonization. In support of this, numerous studies have reported decreased expression of AMPs in AD-affected skin compared to that of psoriatic lesions [147-149]. However, more recent data comparing the levels of antimicrobial peptides in AD skin to that of unaffected controls has shown increased expression of multiple AMPs including RNase 7, psoriasin, hBD-2, hBD-3, and LL-37 [150,151]. Therefore, the previously ascribed reduction of AMPs in AD skin may be due more to the upregulation of these genes in psoriatic skin, rather than their decreased production in atopic individuals.

In all, it appears that both barrier disruptions and improper immune activation contribute to lesions in AD patients. While the underlying cause of inflammation remains unclear, it is likely that this determinant involves a combination of genetic and environmental factors. Notwithstanding, AD pathology consistently leads to shifts in skin microbial communities including an increase in staphylococcal species such as *S. aureus*. While this observation is a satisfying explanation for the increased prevalence of *S. aureus* infections in AD patients, it is perhaps more striking that this rate is not higher [152]. *S. aureus* levels have been found to reach 10^7 CFU/cm^2 in uninfected individuals [139,140], indicating that affected subjects may retain the ability to limit *S. aureus* pathogenesis despite a number of immune abnormalities. As such, a compartmentalized response in AD patients may exist, similarly to that seen in the gut, whereby atopic lesions can unintentionally promote the growth of *S.*

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aureus at the skin surface while simultaneously opposing infection of the underlying tissues.

2.6 Concluding remarks

Advances in sequencing technology have enhanced our ability to characterize cutaneous microbial communities in a more precise and accurate manner, and as a result, our knowledge regarding host-microbe interactions in skin health and disease is steadily increasing. As these insights are deepened and developed, a major challenge will be to translate this knowledge into strategies that improve skin health and cutaneous diagnostic techniques. Future analyses employing shotgun metagenomics and metabolomics are essential to this goal, as we work towards a better comprehension of skin microbial population dynamics. Indeed a recent study of the skin microbiome utilizing metagenomic approaches has contributed greatly to our understanding of skin bacterial communities (153). Studies such as these are crucial to our perception of cutaneous microorganisms and can inform future experimental approaches. Only following these initial characterizations can we hope to truly appreciate the dysbiotic states associated with disease, and only then can we strive to successfully elucidate the importance of microbial inhabitants to hominal equilibria.

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2.7 Acknowledgements

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2.8 Figures

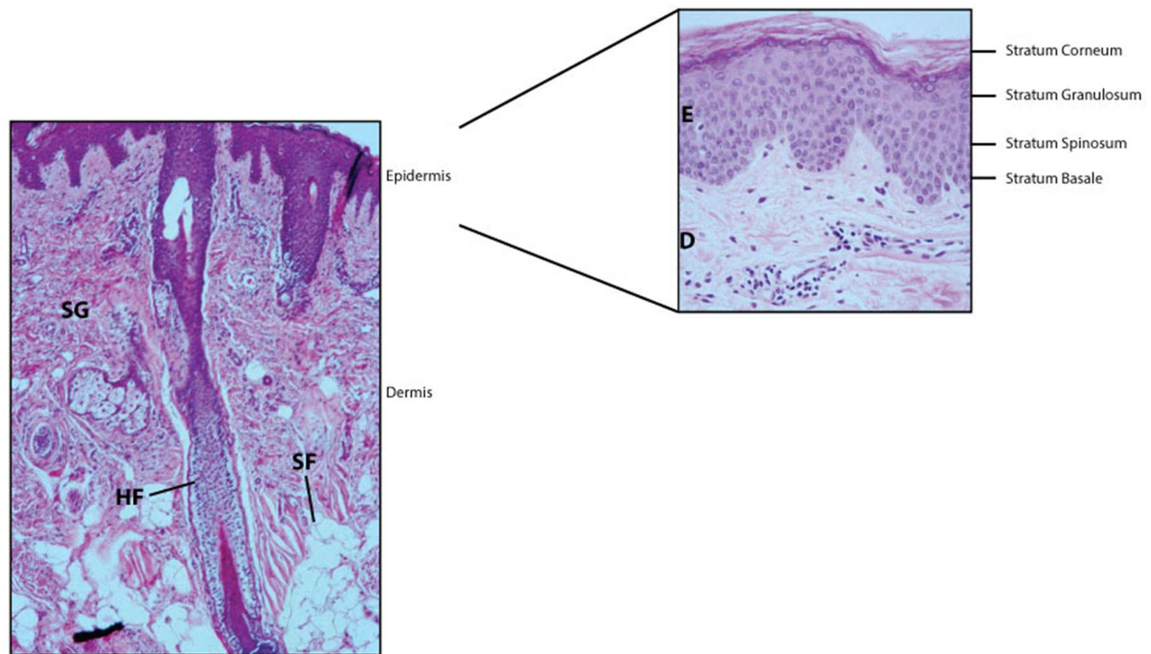

Figure 1 Skin structure and morphology. The skin can be divided into two main layers, the epidermis (E) and dermis (D), and underlying regions of subcutaneous fat (SF). Hair follicles (HF) extend from the skin surface into the dermis and are often associated with sebaceous glands (SG). The epidermis contains distinct layers of keratinocytes at varying stages of development. Basal stem cells are found at the stratum basale while daughter cells mature to populate the stratum spinosum, stratum granulosum, and upon terminal differentiation, the stratum corneum.

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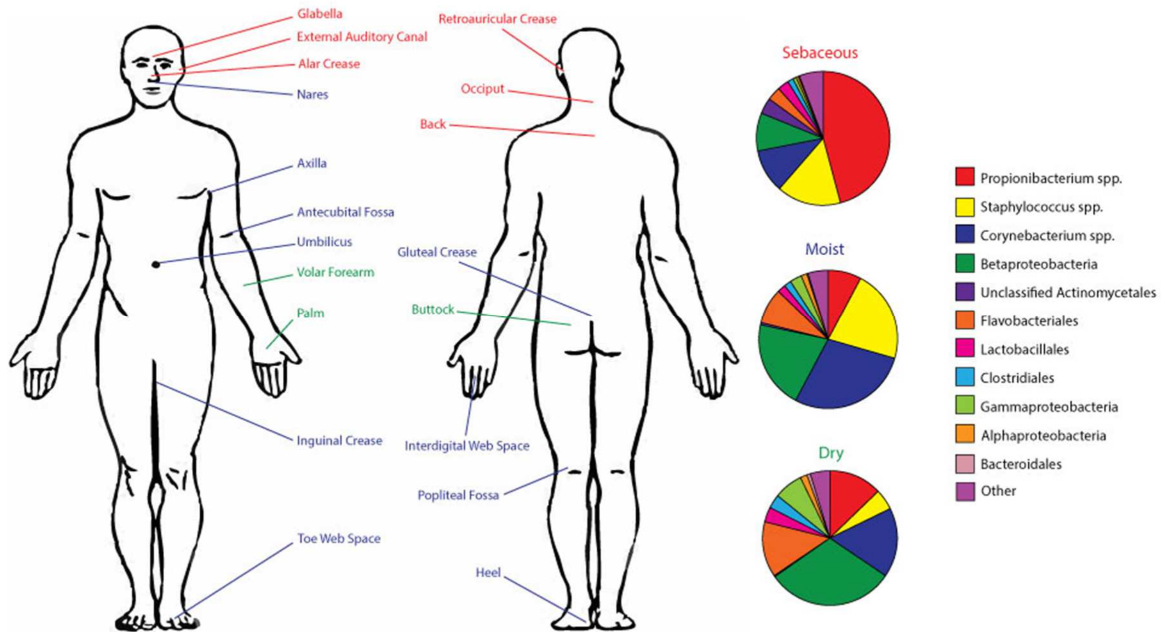

Figure 2 Regional variation of skin microbial communities. The cutaneous microbiota varies according to body site and is strongly influenced by differences in cutaneous physiological environments. Each pie chart represents the mean bacterial community of a given biogeographic region. Sebaceous (red), moist (blue), and dry (green) regions are highlighted. Data from Grice et al. [13].

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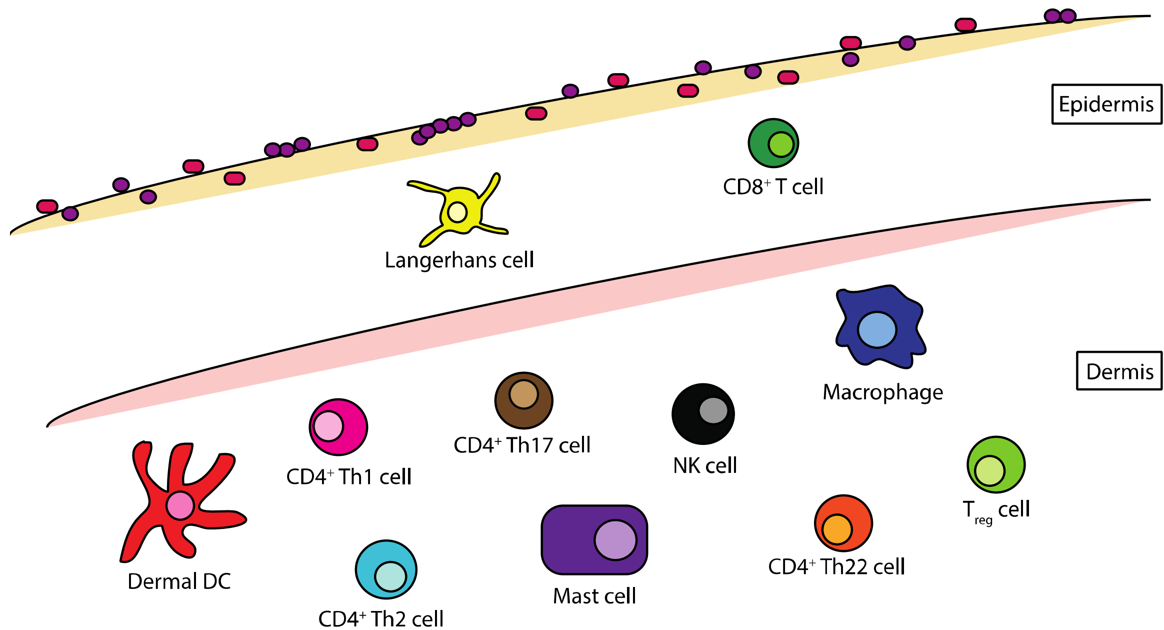

Figure 3 Major skin immune cell subsets. Human skin is characterized by an array of innate and adaptive immune cells. In the epidermis, this includes Langerhans dendritic cells and CD8+ T cells. The dermis is home to a more varied population of innate dermal dendritic cells, NK cells, and mast cells, as well as adaptive CD4+ Th1, Th2, Th17, and Th22 cells.

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CHAPTER 3 - Topical antimicrobial treatments can elicit shifts to resident skin bacterial communities and reduce colonization by *Staphylococcus aureus* competitors

The contents of this chapter are under review for publication with the authors: SanMiguel AJ, Meisel JS, Horwinski J, Zheng Q, Grice EA.

3.1 Abstract

The skin microbiome is a complex ecosystem with important implications for cutaneous health and disease. Topical antibiotics and antiseptics are often employed to preserve the balance of this population, and inhibit colonization by more pathogenic bacteria. Despite their widespread use, however, the impact of these interventions on broader microbial communities remains poorly understood. Here we report the longitudinal effects of topical antibiotics and antiseptics on skin bacterial communities and their role in *Staphylococcus aureus* colonization resistance. In response to antibiotics, cutaneous populations exhibited an immediate shift in bacterial residents, an effect that persisted for multiple days post-treatment. By contrast, antiseptics elicited only minor changes to skin bacterial populations, with few changes to the underlying microbiota. While variable in scope, both antibiotics and antiseptics were found to decrease colonization by commensal *Staphylococcus* spp. by sequencing- and culture-based methods, an effect which was highly dependent on baseline levels of *Staphylococcus*. Because *Staphylococcus*

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residents have been shown to compete with the skin pathogen *S. aureus*, we also tested whether treatment could influence *S. aureus* levels at the skin surface. We found that treated mice were more susceptible to exogenous association with *S. aureus*, and that precolonization with the same *Staphylococcus* residents that were previously disrupted by treatment could reduce *S. aureus* levels by over 100-fold. In all, this study indicates that antimicrobial drugs can alter skin bacterial residents, and that these alterations can have critical implications for cutaneous host defense.

3.2 Introduction

Antimicrobial drugs are commonly employed to inhibit the growth of pathogenic microorganisms. However, these interventions are rarely narrow in spectrum, instead acting on a range of bacterial species in our commensal microbiota (1). A number of studies have elucidated this effect in gut microbial populations, describing a dramatic reorganization of resident communities (2). This includes decreased bacterial diversity, and outgrowth by previously minor contributors (3-5). Importantly, these alterations can persist for months to years post-treatment (6-8), and also affect a number of host functions including metabolism, immunity, and transcriptional regulation (9, 10).

Despite these findings, few studies have assessed the impact of antimicrobial drugs at alternative body sites such as the skin. Rather the majority of research at this site has been devoted to a subset of easily cultured microorganisms studied in isolation (11). This includes minimum inhibitory concentration tests of pathogenic skin

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bacteria, as well as exogenous colonization studies in which non-resident, test microorganisms are applied to the skin prior to treatment (12). While these results are often applied more broadly, their main purpose is to inform the effect of antimicrobial drugs on transient, infectious bacteria, rather than more stable members of the community (13). As such, few studies have truly assessed the impact of antimicrobial drugs on inhabitant cutaneous populations. This dearth of research is especially notable given the frequency with which humans disrupt skin bacterial communities in both clinical and non-clinical settings. Indeed the intent of most antiseptics is to sterilize the skin by employing agents with non-specific mechanisms of action (14), with little regard for their effect on the resident microbiota.

While culture-independent surveys have recently illuminated the complexity of the skin microbiota (15-17), its necessity for normal function and disease remains unclear. One postulated function includes a role in colonization resistance, whereby members of the commensal microbiota could protect the host from infection by opportunistic and pathogenic skin microorganisms (18). This particular process has been well-documented in the gut. Here numerous studies have highlighted the ability of bacterial residents to impair colonization by pathogenic bacteria through immune activation, nutrient exclusion, and the production of toxic metabolites (19). Antibiotics have also been shown to shift the resident microbiota, and render hosts more susceptible to certain pathogenic bacteria (20). This includes studies of the sporulating bacterium *Clostridium difficile*, which can recur repeatedly in response

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to antibiotic treatment, but can also be controlled in most patients following the administration of fecal material from healthy, unaffected donors (21-23). Importantly, this particular effect is not isolated to *C. difficile*, as a number of bacterial pathogens including vancomycin-resistant *Enterococcus* and *Salmonella enterica* have been shown to exploit newly available niches in response to treatment as well (24-26).

Similar to the gut, recent studies have begun to assess the potential for skin microorganisms to play a role in colonization resistance. This includes defense against *Staphylococcus aureus* by unique strains of *S. epidermidis* (27), *S. lugdunesis* (28), and most recently *S. hominis* (29). Here, it was found that certain individuals are colonized by host-specific *Staphylococcus* strains with the ability to alter *S. aureus* colonization patterns. While these studies also suggest that a removal of resident bacteria with antimicrobial agents could promote *S. aureus* colonization, no study to date has assessed this hypothesis in detail. Indeed, the long-term impact of topical antimicrobial drugs on skin bacterial communities, and their ability to alter colonization patterns by *S. aureus* competitors, remains largely unknown.

Here we report this missing link by assessing the effect of antibiotics and antiseptics on the resident skin microbiota through a comparative time-series analysis. We report a differential impact of treatment on skin bacterial inhabitants, with the greatest disturbances elicited by a broad-spectrum triple antibiotic cocktail of bacitracin, neomycin, and polymyxin B. By contrast, we report a relatively muted

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effect of antiseptics, with only modest alterations to overall bacterial community structure. Despite these differences, we identified a conserved decrease in the levels of *Staphylococcus* residents regardless of treatment, a result that was strongly influenced by baseline levels of *Staphylococcus*.

Because commensal *Staphylococcus* spp. have been shown to impair colonization by the skin pathogen *Staphylococcus aureus*, we further evaluated this antimicrobial effect in the context of *S. aureus* colonization resistance. We show that treatment can promote exogenous association with *S. aureus*, and that the same *Staphylococcus* residents disrupted by treatment are also capable of *S. aureus* competition, decreasing *S. aureus* levels by over 100-fold in precolonization experiments. In all, our results demonstrate that antimicrobial drugs can elicit long-term shifts in skin bacterial communities, and that treatment with these agents has key implications for host susceptibility to pathogens such as *S. aureus*.

3.3 Results

3.3.1 Topical antibiotic treatment alters skin bacterial residents

To assess the impact of topical antibiotics on the skin microbiota, we began by treating the dorsal skin of SKH-1 hairless mice twice daily for one week with the narrow spectrum antibiotic mupirocin; a broad spectrum triple antibiotic ointment (TAO: bacitracin, neomycin, polymyxin B); or their respective vehicles, polyethylene glycol (PEG) and petrolatum (Fig. S1a). In all, antibiotics led to durable changes in skin bacterial residents, with populations forming three distinct clusters (I – III) and

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four sub-clusters (III_{A-D}) (Fig. 1a). Interestingly, Clusters I and III_{A} were composed largely of baseline and early time point samples high in *Staphylococcus*, while treatment with antibiotics led to sustained decreases in *Staphylococcus* (Fig. S1b) and alternative clustering patterns. Cluster II, by contrast, was composed almost entirely of TAO-treated mice, a group that exhibited significant increases in Enterobacteriaceae, Porphyromadaceae, and Ruminococcaceae, as well as significant decreases in Lachnospiracea*e* and certain taxa classified more generally within the Clostridiales family (Fig. 1b-d). This distinction led to a marked absence of TAO-treated mice from Clusters III_{B-D}, and, similar to *Staphylococcus*, was sustained for multiple weeks post-treatment.

Unlike TAO-treated mice, those administered mupirocin displayed community shifts largely in line with those treated with the vehicle PEG. Indeed while these mice exhibited significant increases in *Alistipes* and decreases in *Oscillibacter* and *Staphylococcus* (Fig. S1b, S1c), these minor changes were not enough to elicit separate clustering patterns amongst the two treatment groups. These particular changes also displayed similar kinetics to bacterial taxa in TAO-treated mice, i.e. immediate increases and sustained post-treatment effects, underscoring the difficulties faced by skin communities when attempting to re-acclimate upon treatment cessation.

Analysis of bacterial burden revealed a contrasting effect of antibiotics on absolute abundance, with only mupirocin leading to the characteristic decreases often

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associated with antibiotic treatment (Fig. S1d). TAO treatment, by contrast, resulted in increased bacterial burden suggesting that the elevated levels of Enterobacteriaceae and Porphyromonadaceae were due not just to increases in relative proportions, but also overall numbers.

3.3.2 Topical antibiotics shift bacterial community structure

To better quantify these results at the community-level, we next evaluated the diversity of bacterial populations over time. Similar to taxonomic analyses, we observed a relative stability in untreated mice and those treated with PEG, mupirocin, and petrolatum when testing alpha diversity metrics such as Shannon diversity, which takes into account the richness and evenness of taxa (Fig. 2a). By contrast, those treated with TAO exhibited an immediate and significant decrease in diversity starting after a single day (d1) of treatment, an effect that was maintained for greater than one week post-treatment. This was also recapitulated when evaluating community similarity by the weighted UniFrac metric, which assesses population differences based on abundance and phylogeny. When comparing each mouse to their baseline (d0) samples, we observed significantly greater differences within the TAO-treated group compared to vehicle-treated mice, a trend not shared by those administered mupirocin (Fig. 2b). Additional visualization of these samples by principle coordinates analysis further confirmed these results, as distinct clustering patterns were observed when comparing TAO-treated mice to other treatment groups (Fig. 2c).

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Previously, others have shown similarities in the functional composition of a population despite differences in community membership and structure (30). To evaluate whether antibiotic treatment could lead to changes in the functional potential of skin inhabitants, we also utilized the PICRUSt software package (31) to infer metagenomic content of our populations. Specifically, PICRUSt analysis focuses on chromosomally-encoded, conserved differences amongst species as a method to approximate functional disparities. We found that treatment with antibiotics and vehicles led to a number of significant differences in genes associated with metabolism, signaling, transport, and biosynthesis, among others (Fig. S2). As such, the potential exists that by shifting the residents of the cutaneous microbiota, treatment may shift the functional capabilities of these populations as well.

3.3.3 Antiseptic treatment elicits only modest changes to skin bacterial community structure

Following our tests with antibiotic regimens, we next endeavored to evaluate the impact of antiseptics, a more promiscuous class of antimicrobials, on the skin microbiome. We reasoned that these topical interventions should provide an even greater impetus for community disruption due to their indiscriminate mechanisms and proven efficacy in clinical settings (14). To evaluate this hypothesis, we treated mice with the common clinical antiseptics alcohol (80% ethanol) or povidone-iodine (10%), and compared this to mice treated with water or untreated controls (Fig. S3a). Surprisingly, we observed no clustering of mice in response to treatment when taking into account major taxonomic groups (Fig 3a). Furthermore, when comparing

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the relative abundances of individual taxa, we detected no significant differences among treated mice and untreated controls (Table S1). To evaluate whether subtle differences could contribute to a disruption at the population level, we also tested the diversity of communities in response to treatment. Similar to our taxonomic analyses, we found that antiseptic treatment resulted in no significant differences to Shannon Diversity (Fig. 3b), nor could we detect significant clustering by treatment using beta diversity metrics such as weighted UniFrac (Fig. 3c). To assess whether we had missed decreases in absolute abundance by focusing our analyses on the relative proportions of taxa, we also tested the impact of treatment on the bacterial load of communities. Once again, we observed no significant differences between treated and untreated mice (Fig. 3d), underscoring the stability of cutaneous bacterial communities in response to antiseptic treatment.

As this result was particularly surprising, we further compared bacterial phylotypes at baseline to their d1 counterparts. This allowed us to evaluate whether treatment could shift populations in a conserved manner, thus explaining the modest effects seen between regimens at d1 post-treatment. However, when comparing the abundances of major taxonomic groups, we once again observed relatively few changes from d0 to d1 in response to treatment. Only *Staphylococcus* differed significantly, and only in response to alcohol treatment (Table S2). Interestingly, this effect was strongly dependent upon starting communities, as mice with higher baseline levels of *Staphylococcus* were more strongly disrupted than those with lower baseline levels, regardless of treatment (Fig S3b.). In all, this indicates that

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antiseptics elicit a more muted response in skin bacterial populations, but that their effects may be dependent upon starting communities.

3.3.4 Culture-based studies recapitulate sequence analyses of skin microbiota dynamics

Our finding that most antiseptics elicited only minor changes to the resident skin microbiota was particularly surprising given the wealth of data describing their benefit in clinical settings. To address this discrepancy, we next sought to validate our findings using culturable skin inhabitants. Specifically, *Staphylococcus* was chosen as a proxy because of its established response to topical antimicrobials in the clinic and its importance to human health. These bacteria were also the only inhabitants to vary in response to both antibiotics and antiseptics in our sequencing experiments, and thus represented the best opportunity to verify our results in a culture setting.

Because our antiseptic experiments exhibited an antibacterial effect dependent upon baseline communities, we began by designing a system to control *Staphylococcus* levels in murine populations. Specifically, we observed that mice housed in cages changed once per week displayed significant elevation in *Staphylococcus* levels (high *Staphylococcus*; HS) compared to those changed more frequently (low *Staphylococcus*; LS) (Fig 4a). When controlled over time, this effect could be maintained for multiple weeks and had the potential for reversibility, as mice swapped from frequent to infrequent cage changes rapidly converted to the

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alternate phenotype. Cage change frequency and monitoring thus presented the opportunity to maintain *Staphylococcus* at distinct levels prior to treatment.

To evaluate the impact of antimicrobial drugs on culturable *Staphylococcus*, we began by housing mice in cages with frequent or infrequent changes, and then treating with PEG, mupirocin, petrolatum, or TAO. Similar to sequencing experiments, antibiotic treatment led to a significant decrease in *Staphylococcus* starting at d1 post-treatment regardless of starting community, although this effect was more pronounced in LS mice (Fig. 4b,c). Interestingly, while we also observed a gradual decrease of *Staphylococcus* in response to PEG treatment, petrolatum-treated LS mice displayed increased *Staphyloccocus* colonization at early time points, and elevated levels of *Staphylococcus* compared to untreated controls in HS mice. Because our sequencing results revealed similar decreases in *Staphylococcus* in response to treatment with antibiotics, but not petrolatum, this represents a reproducible mechanism in multiple testing protocols.

To assess this effect in the context of antiseptics, a separate cohort of HS and LS mice were next treated with water, alcohol, or povidone-iodine, and compared to untreated controls. Unlike those treated with antibiotics, no significant differences in *Staphylococcus* were observed in LS mice following treatment with water, alcohol, or povidone-iodine compared to baseline colonization (Fig. 4d). Moreover, while HS mice were significantly decreased in *Staphylococcus* following treatment, untreated mice with a single cage change exhibited an almost identical reduction in

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colonization, confirming that a change in environment can also have significant impacts on bacterial communities (Fig. 4e). In all, these experiments indicate that antibiotics and antiseptics have distinct effects on skin bacterial residents, and that the magnitude of this response can vary depending upon starting communities.

3.3.5 Antimicrobial drugs reduce colonization by *Staphylococcus aureus* competitors

After confirming our sequencing results with culture experiments, we next endeavored to explore the ramifications of cutaneous bacterial community disruption. As previous studies have suggested a role for the skin microbiota, and specifically resident *Staphylococcus* spp., in *S. aureus* colonization resistance (27-29), we chose this particular commensal-pathogen pair for further analysis. We were particularly attracted by the ability of antimicrobial drugs to shift communities for multiple days post-treatment, suggesting a window in which *S. aureus* could access the skin unencumbered by competing residents or antimicrobial drugs. As alcohol was found to have relatively minor effects on skin bacterial residents, with the exception of *Staphylococcus* spp., we first tested whether treatment with this antiseptic could promote *S. aureus* association. Specifically, mice were treated with alcohol, similarly to previous experiments, and then exogenously associated with *S. aureus* one day post-treatment. As hypothesized, we observed a slight, but significant, increase in *S. aureus* levels in treated mice compared to untreated controls, indicating a reduction in colonization resistance in response to treatment (Fig. 5a).

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Because this effect could also be the result of additional factors including previously unidentified microbial inhabitants, we next profiled individual *Staphylococcus* isolates that were reduced by antimicrobial treatment in our previous experiments. We reasoned that if these bacteria were the true source of colonization resistance, than adding them back to the skin should reduce *S. aureus* association in kind. Following phenotypic analysis and full-length 16S rRNA gene sequencing, we isolated five unique resident *Staphylococcus* genotypes – AS9, AS10, AS11, AS12, and AS17. Comparing these to reference sequences within the Ribosomal Database Project (RDP) (32), we identified four distinct species and two strain level variants: *S. epidermidis* (AS9), *S. xylosus* (AS10, AS11), *S. nepalensis* (AS12), and *S. lentus* (AS17) (Fig. 5b). Interestingly, while each of these bacteria fell within the *Staphylococcus* genus, they also had considerable genomic variability within the 16S rRNA gene region, suggesting a relative permissivity at the skin surface for these particular taxa (Fig. S4).

To assess the colonization potential of each isolate, we next compared their growth dynamics under various conditions. When comparing growth in enriched media, we observed distinct differences amongst isolates, with AS17 *S. lentus* and AS10 *S. xylosus* displaying the most robust expansion kinetics (Fig. 5b). By contrast, AS9 *S. epidermidis* appeared to replicate the slowest and exhibited the most gradual exponential curve. AS11 *S. xylosus* and AS12 *S. nepalensis* both displayed intermediate growth patterns. To further evaluate colonization potential, we also applied each *Staphylococcus* isolate to murine dorsa every other day for 1 week to

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promote monocolonization. Despite variable growth dynamics *in vitro*, all isolates colonized mice to an equal titer *in vivo*, suggesting conserved, undefined factors to promote colonization at the skin surface (Fig. 5c).

As each of these isolates displayed notable colonization when added to murine hosts, we further tested all five to see whether they could also represent potential *S. aureus* competitors. To evaluate the ability of each isolate to restrict *S. aureus* colonization, we precolonized mice with each *Staphylococcus* resident prior to *S. aureus* challenge one day later. While isolates exhibited varying levels of competition, all resulted in significant decreases to *S. aureus* association compared to uncolonized mice (Fig. 5d). Indeed most mice exhibited greater than 10-fold reductions in *S. aureus*, and many, including those precolonized with *S. epidermidis*, were capable of decreasing *S. aureus* by levels greater than 100-fold. In all, this shows that skin bacterial residents can compete with *S. aureus* at the skin surface, and that their removal can impact *S. aureus* colonization potential.

3.4 Discussion

Given the expansive use of topical antibiotics and antiseptics, it is somewhat surprising that longitudinal studies to evaluate their effects on a community-wide scale are not more common. Here we report that antimicrobial drugs can elicit significant changes to skin bacterial community membership and structure, albeit to varying degrees. We also demonstrate that these alterations can have important

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consequences for colonization resistance and the skin pathogen *Staphylococcus aureus*.

Previous work has focused extensively on antibiotics and the gut microbiota. These studies have highlighted the ability of antimicrobials to disrupt bacterial communities and the consequences of these drugs on host physiology (33). One such example includes the elimination of colonization resistance leading to increased susceptibility to bacterial infections (34). By altering the structure of bacterial populations in the gut, antibiotics can shift the balance in favor of more infectious microorganisms (19). *Clostridium difficile* is perhaps the best-studied representation of this effect (35). However, additional pathogens such as vancomycin-resistant *Enterococcus* and *Salmonella enterica* can also exploit newly available niches and cause disease (36, 37). As a result, the true question has transcended beyond whether or not antimicrobial drugs can promote pathogenicity, to how best to mediate these unintended consequences.

The first step in such ventures is the elucidation of antimicrobial effects on a community-wide scale. While studies of the gut have been vital to this endeavor, we present the skin as an additional body site worthy of consideration. In our investigations, triple antibiotic ointment (TAO) was found to provoke the greatest response in microbial residence, with a significant decrease in bacterial diversity and domination by previously minor contributors. While these changes originated as a result of treatment-specific effects, they often endured, and in some cases were

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enhanced, following treatment cessation. This indicates that disrupted resident skin bacteria must undergo multiple levels of succession prior to community stabilization, similar to the gut (38).

In accordance with their mechanisms of action, we also found the overall effect of mupirocin to be relatively minor compared to that of TAO. While TAO led to profound increases in bacteria from multiple families including Enterobacteriaceae and Porphyromonadaceae, mupirocin produced relatively minor shifts in less abundant taxa such as *Alistipes* and *Oscillibacter*. This finding is particularly notable as certain members of the Enterobacteriaceae and Porphyromonadaceae families have known intrinsic resistance mechanisms against TAO components such as polymyxin B (39, 40), a result that could also explain the increase in overall bacterial load seen in mice following TAO administration.

Perhaps most surprisingly, we also report a relatively muted impact of antiseptics on the skin microbiota, with alcohol and povidone-iodine both failing to shift baseline communities in a significant manner. While it is tempting to explain this finding as an inability of 16S rRNA gene sequencing to distinguish between live and dead bacteria, we find this conclusion unlikely in the context of our studies and those before us. Indeed, our ability to detect differences in TAO-treated mice within one day of treatment provides strong evidence to the contrary. Others have also reported a similar community response to both decolonization protocols (41) and

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mild and antibacterial soaps (42), confirming the stability of cutaneous populations in response to certain acute stressors.

With this in mind, it is important to note that multiple studies have also shown a reduction of certain culturable skin inhabitants in response to antisepsis. This includes residents from the commonly studied genus *Staphylococcus*, often chosen for its ease of use in culture-based experiments (43, 44). In line with these findings, we also observed a decrease in *Staphylococcus* residents in our sequencing and culture studies. However, we note that because this bacterium was only one member of the larger community, this decline did not lead to shifts in overall population structure.

Interestingly, *Staphylococcus* residents also exhibited baseline-dependent dynamics in response to antiseptic treatment during our sequencing experiments. Specifically, we observed that mice with high levels of *Staphylococcus* responded more readily to treatment than mice with low levels of colonization. This suggested a nuanced impact of antiseptics on certain bacterial inhabitants, whereby treatment effects could vary depending upon starting communities. To verify this hypothesis, we developed a system in which *Staphylococcus* could be tested for antimicrobial susceptibility at both high and low colonization levels. As anticipated, we found the efficacy of antiseptics to be highly dependent upon baseline communities. Mice with low levels of *Staphylococcus* at baseline (LS) exhibited little to no decline in *Staphylococcus*, while mice with high levels (HS) were reduced by approximately

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100-fold. Importantly, we observed a similar effect in control HS mice, suggesting that higher levels of *Staphylococcus* are less stable in general, and thus represent atypical colonization. By contrast, the inability of antiseptics to reduce *Staphylococcus* in LS mice indicates a relative stability in this community, and a population capable of resisting the short-term stressors of antisepsis. We believe these studies have important implications for antimicrobial efficacy, particularly in the case of human skin, as humans are likely exposed to a greater number of transient microorganisms compared to laboratory mice housed in more controlled environments (45).

When comparing antibiotic and antiseptic treatments, we observed that a standard course of antibiotics was more capable of community disruption than that of acute antisepsis. While these are the most commonly employed regimens in the clinic, further research should also evaluate the effects of long-term antiseptic treatments on the skin microbiota as well as other delivery mechanisms. Indeed the potential exists that consistent exposure to antiseptics through alternative means may have a more significant impact on skin inhabitants due to increased contact time or bioavailability. This is especially important when considering the rise of decolonization practices in the clinic, a procedure employing multiday, prophylactic antibiotic and antiseptic treatments to remove resident *Staphylococcus* species (46, 47). While these methods efficiently remove endogenous *S. aureus* from the nares and extranasal body sites, they likely alter the underlying skin microbiota in kind. Without proper re-colonization, these interventions could feasibly elicit long-term

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shifts to the skin microbiota, similar to our experiments, and promote infection by more dangerous hospital- and community-acquired pathogens (48-50).

To assess this very possibility, we investigated the potential of treatment to promote *S. aureus* colonization at the skin surface in our mouse model. In response to treatment, we observed a significant increase in *S. aureus* levels compared to untreated controls following exogenous association, suggesting an increase in cutaneous permissivity. As previous studies have illustrated the role of certain *Staphylococcus* spp. to compete with *S. aureus* for colonization (27-29), we proceeded by testing the ability of murine *Staphylococcus* isolates to compete with *S. aureus*. Specifically, we chose *Staphylococcus* residents that were disrupted by antibiotic and antiseptic treatment in our previous experiments for further analysis. This allowed us to determine whether these particular bacterial residents were responsible for the decrease in colonization resistance, and to confirm the ability of antimicrobial drugs to alter communities with the potential for *S. aureus* competition. Importantly, we found that all isolates were capable of protecting against *S. aureus* association, with a number of mice exhibiting reductions in *S. aureus* levels by over 100-fold. These results support the notion that antimicrobial drugs can impact *S. aureus* colonization resistance, and argue for enhanced stewardship in the context of post-treatment recovery.

In all, we describe the importance of antimicrobial drugs to skin bacterial community dynamics. By detecting unique changes in the microbiota in response to

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topical antibiotics and antiseptics, we present the skin as a body site capable of reproducible disruptions and fluctuations in colonization resistance. For this reason and others, we further advocate for the judicious use of antibiotics and antiseptics, as well as increased monitoring of bacterial populations, in order to combat the unintentional consequences which can proceed cutaneous perturbations.

3.5 Materials and Methods

3.5.1 Mice

Six-week-old female SKH-1 immunocompetent hairless mice were purchased from Charles River and acclimated for at least two weeks prior to testing. Throughout experimentation, mice were housed on ALPHA-Dri bedding and given ad libitum access to autoclaved food and water. Mice treated with the same antimicrobial drug or exogenous *Staphylococcus* strains were housed together to avoid mixing, and at least two cages were used per condition to assess caging effects. All cages were changed three to four times per week during the course of a study unless otherwise noted. All mouse procedures were performed under protocols approved by the University of Pennsylvania Institutional Animal Care and Use Committee.

3.5.2 Antimicrobial treatment

For experiments involving antibiotics, mice were treated on the dorsum with mupirocin (2% in polyethylene glycol) or a triple antibiotic ointment (Bacitracin 400U, Neomycin 3.5mg, Polymyxin B 5,000U in petrolatum) every 12 hours for 7 days. To control for any vehicle-specific affects, the control ointments for each

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antibiotic were also used: polyethylene glycol (PEG 400, PEG 3350) and petrolatum. For experiments involving antiseptics, mice were treated on the dorsum with UltraPure water (MoBio), alcohol (80% ethanol), or povidone-iodine (Betadine, 10%) every eight hours, three times total. At least three cages of three mice each were used for all conditions to evaluate caging effects.

3.5.3 Bacterial DNA isolation and 16S rRNA gene sequencing and qPCR

Cutaneous swabs were collected at baseline, during, and following treatment from mouse dorsa and stored at −20 °C. Bacterial DNA was extracted as described previously (51). Briefly, Ready-Lyse Lysozyme solution (Epicentre), bead beating, and heat shock at 65 °C were used to lyse cells. The Invitrogen PureLink kit was used for DNA extraction. During our testing, the V4 region of the 16S rRNA gene was found to better approximate murine skin communities compared to V1V3. PCR and sequencing of the V4 region was thus performed using 150-bp paired end chemistry and barcoded primers (515F, 806R) on the Illumina MiSeq platform. Accuprime High Fidelity Taq polymerase was used for PCR cycling conditions: 94 °C for 3 min; followed by 35 cycles of 94 °C for 45 sec, 50 °C for 60 sec, 72 °C for 90 sec; and ending with 72 °C for 10 min. For bacterial load comparisons, 16S rRNA genes were amplified by qPCR using Fast SYBR Green Master Mix (Fisher Scientific) and the optimized primers 533F, 902R. Samples were compared to standard curves generated from known concentrations of serially diluted bacterial DNA to calculate burden.

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3.5.4 Microbiome analysis

Sequencing data was processed using QIIME 1.7.0 (52). Briefly, sequences were *de novo* clustered into OTUs based on 97% similarity by UClust (53), and taxonomy was assigned to the most abundant representative sequence per cluster using the RDP classifier (54). Sequences were aligned by PyNAST (55), and chimeric sequences were removed using ChimeraSlayer (56) along with those identified as Unclassified, Bacteria;Other, or Cyanobacteria. Singletons were also removed in addition to any OTU found at greater than 1% abundance in at least 50% of control samples to eliminate potential contaminating sequences. All antiseptics, antibiotics, and vehicles were similarly sequenced and evaluated for possible contaminating sequences. All samples were rarified to 5,000 sequences, and samples below this cut-off were removed from downstream analyses. Alpha and beta diversity matrices were calculated in QIIME, and statistical analysis and visualization were performed in the R statistical computing environment (57). Heat maps were constructed by condensing all OTUs above 0.1% to the top 30 taxonomic identifications. The PICRUSt bioinformatics software package was used to infer functional content of bacterial communities (31).

3.5.5 Caging effects

Mice were housed three per cage, three cages per group, and cages were randomly assigned to be changed every other day (frequently) or once per week (infrequently) for four weeks. Swabs were taken every seven days prior to changes of the infrequent group, and cultured for *Staphylococcus* residents on Mannitol Salt

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Agar (acumedia) overnight at 37 °C. At d28, mice from each cohort were reassigned to the alternate group, and swabbed for an additional four weeks to evaluate normalization.

3.5.6 Antimicrobials and alternate *Staphylococcus* communities

Mice were assigned to frequent or infrequent cage changes prior to treatment to generate low *Staphylococcus* and high *Staphylococcus* communities respectively, and treated as described above. During experimentation, all cages were changed on a frequent schedule with untreated mice representing controls. Swabs were taken at baseline, d1, d4, and d7 for antibiotic-treated mice, and at baseline and 4 hours post-treatment for antiseptic-treated mice. Samples were cultured on MSA overnight at 37 °C to enumerate *Staphylococcus* numbers.

3.5.7 *Staphylococcus* isolation, sequencing, and phylogenetic tree

To obtain a more complete profile of our *Staphylococcus* isolates, phenotypically distinct *Staphylococcus* colonies were picked from MSA plates following culture from murine dorsa prior to and following antimicrobial treatment. DNA was extracted from colonies as described above, and DNA was PCR-amplified using full-length 16S rRNA gene primers (27F, 1492R). The primary PCR conditions used were 98 °C for 3 min; 35 cycles of 95 °C for 45 sec, 56 °C for 60 sec, 72 °C for 90 sec; and 72 °C for 10 min. Full-length 16S rRNA gene sequencing was performed by Sanger sequencing, and resident *Staphylococcus* isolates were compared to known *Staphylococcus* 16S rRNA genes downloaded from the RDP database (32).

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Phylogenetic trees were generated by FastTree (58) and visualized in FigTree v1.4.3.

3.5.8 Growth curves

Staphylococcus isolates were grown at 37 °C in liquid Luria Broth (Fisher Scientific) for 12 hours shaking at 300 rpm. Samples were taken every hour and optical density was determined at OD_{600} using the BioTek Synergy HT plate reader.

3.5.9 Exogenous *Staphylococcus* colonization and *S. aureus* competition

Staphylococcus isolates were grown overnight in liquid Luria Broth (Fisher Scientific) at 37 °C and 300rpm. On the following day, isolates were subcultured and incubated to achieve log growth, and resuspended in PBS to acquire 10^8 CFU/ml inoculums. Titers were validated by culture and optical density measurements at OD_{600}. Two cages of three mice each were monoassociated at the dorsum with 200ul of *Staphylococcus* isolate inoculum using a sterile swab. Application of *Staphylococcus* suspensions were repeated every other day over the course of one week for a total of four applications. Mice were then swabbed one day post-association, and cultured on MSA overnight at 37 °C for CFU enumeration. *S. aureus* 502A with selective streptomycin resistance was chosen for *S. aureus* competition studies because of its proven efficiency in skin colonization and potential for pathogenicity (59, 60). *S. aureus* was grown similarly to *Staphylococcus* isolates and applied one day post-treatment or one day post-monoassociation with individual *Staphylococcus* isolates. Control mice were administered PBS only. Mice were then

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swabbed the following day for *S. aureus*, and cultured on LB agar with streptomycin for selective CFU enumeration.

3.6 Accession numbers

16S rRNA sequence reads have been deposited in the NCBI Short Read Archive under BioProject ID: PRJNA383404

3.7 Acknowledgements

We thank Penn Next Generation Sequencing Core for sequencing support, the Penn Medicine Academic Computing Services for computing support, Dr. Jeffrey Weiser for the kind gift of *S. aureus* strain 502A engineered with streptomycin resistance, and members of the Grice laboratory for their underlying contributions. Funding for this work was provided by the National Institutes of Health, National Institute of Arthritis, Musculoskeletal, and Skin Diseases (R00AR060873 and R01AR066663 to EAG). AJS is supported by a Department of Defense National Defense Science and Engineering Graduate fellowship. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health or the Department of Defense.

3.8 Competing interests

The authors declare no competing financial interests.

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3.9 Figures

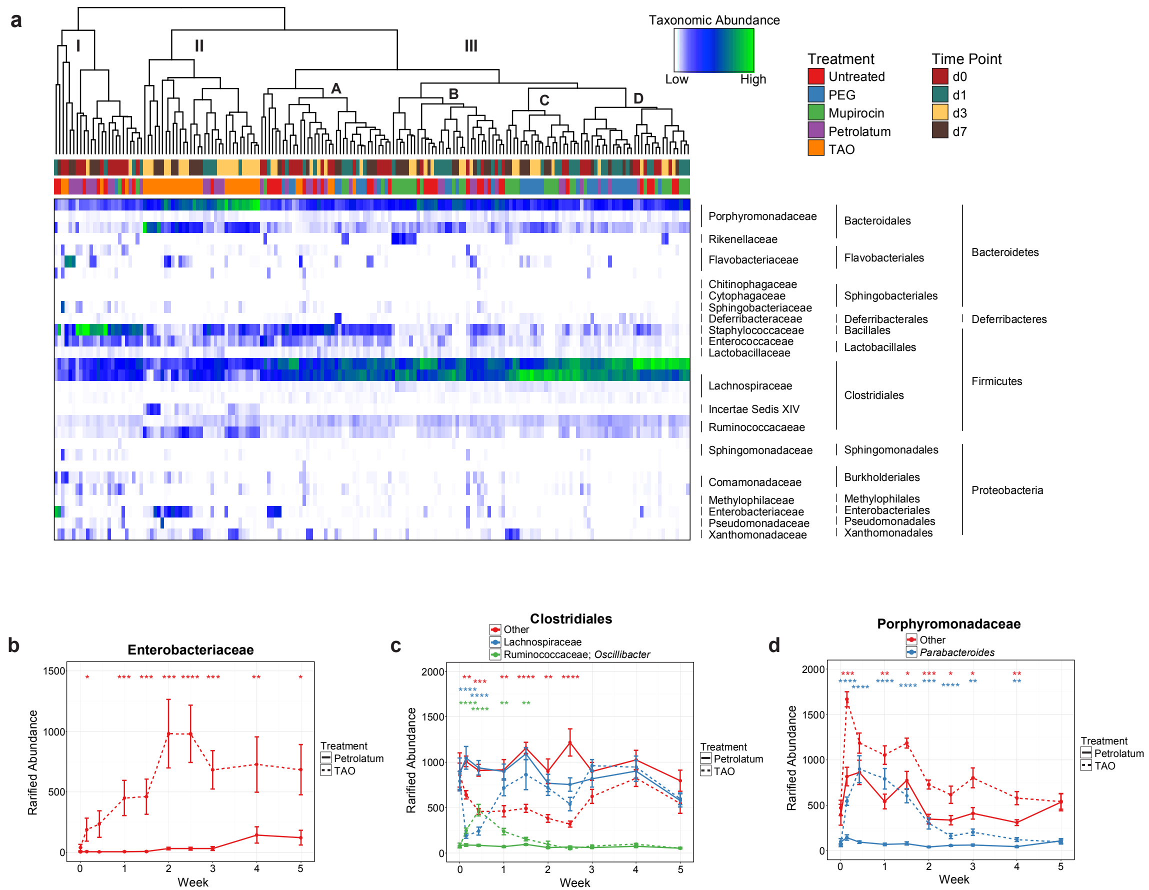

Figure 1 Topical antibiotics induce long-term shifts to skin microbial residents. (a) Heat map of rarified abundances for the 30 most common phylotypes on murine skin in response to treatment with polyethylene glycol (PEG), mupirocin, petrolatum, or triple antibiotic ointment (TAO). Dendrograms represent hierarchical clustering of Euclidean distances using complete agglomeration. Horizontal bars above the graph designate treatment and time point features for individual mice. (b-d) Breakdown and longitudinal analysis of rarified abundances for Enterobacteriaceae (b), Clostridiales (c), and Porphyromonadaceae (d). Data are

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presented as mean ± s.e.m. Statistical significance was determined at each time point by Wilcoxon rank sum test (Mann Whitney U test). $*P < 0.05$, $** P < 0.01$, $*** P < 0.001$, $**** P < 0.0001$.

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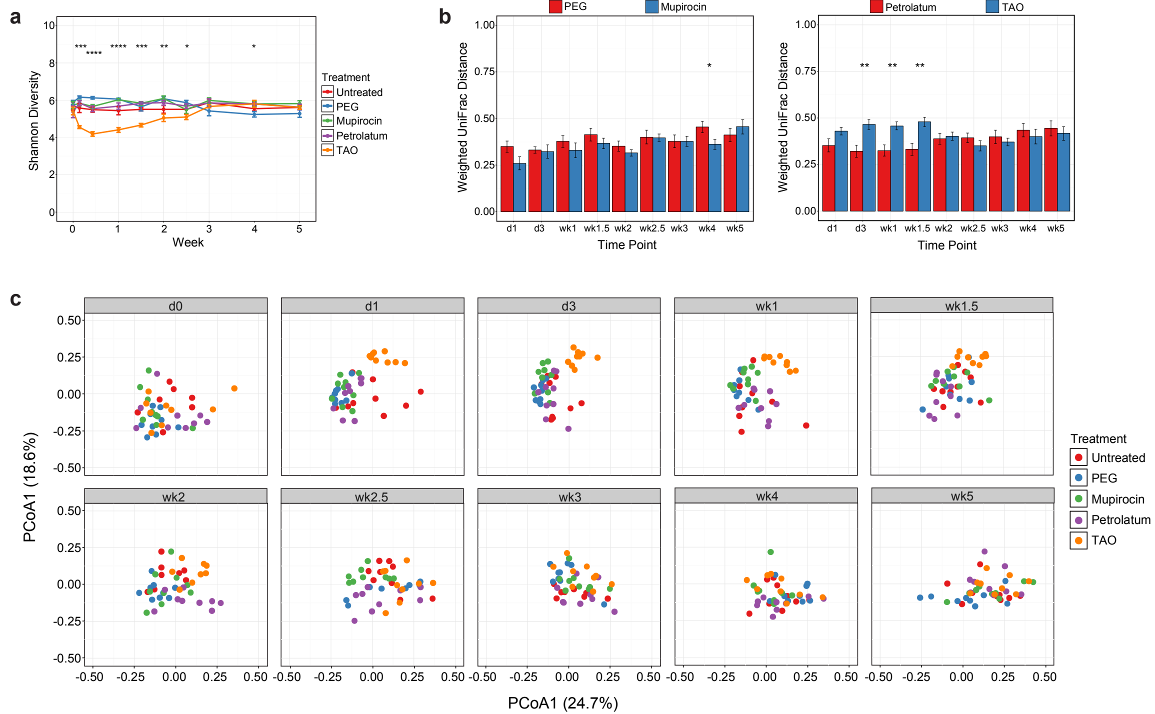

Figure 2 Triple antibiotic ointment alters skin bacterial diversity. (a) Shannon diversity measurements of murine bacterial communities following treatment with antibiotics and vehicles over time. (b) Weighted UniFrac distances comparing longitudinal time points to baseline communities of bacterial residents in treated and untreated mice. (c) Principal coordinates analysis of weighted UniFrac distances for murine bacterial communities over time. Data are presented as mean ± s.e.m. Statistical significance was determined at each time point by Kruskal-Wallis rank sum test (a) or Wilcoxon rank sum test (Mann Whitney U test) (b). *$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

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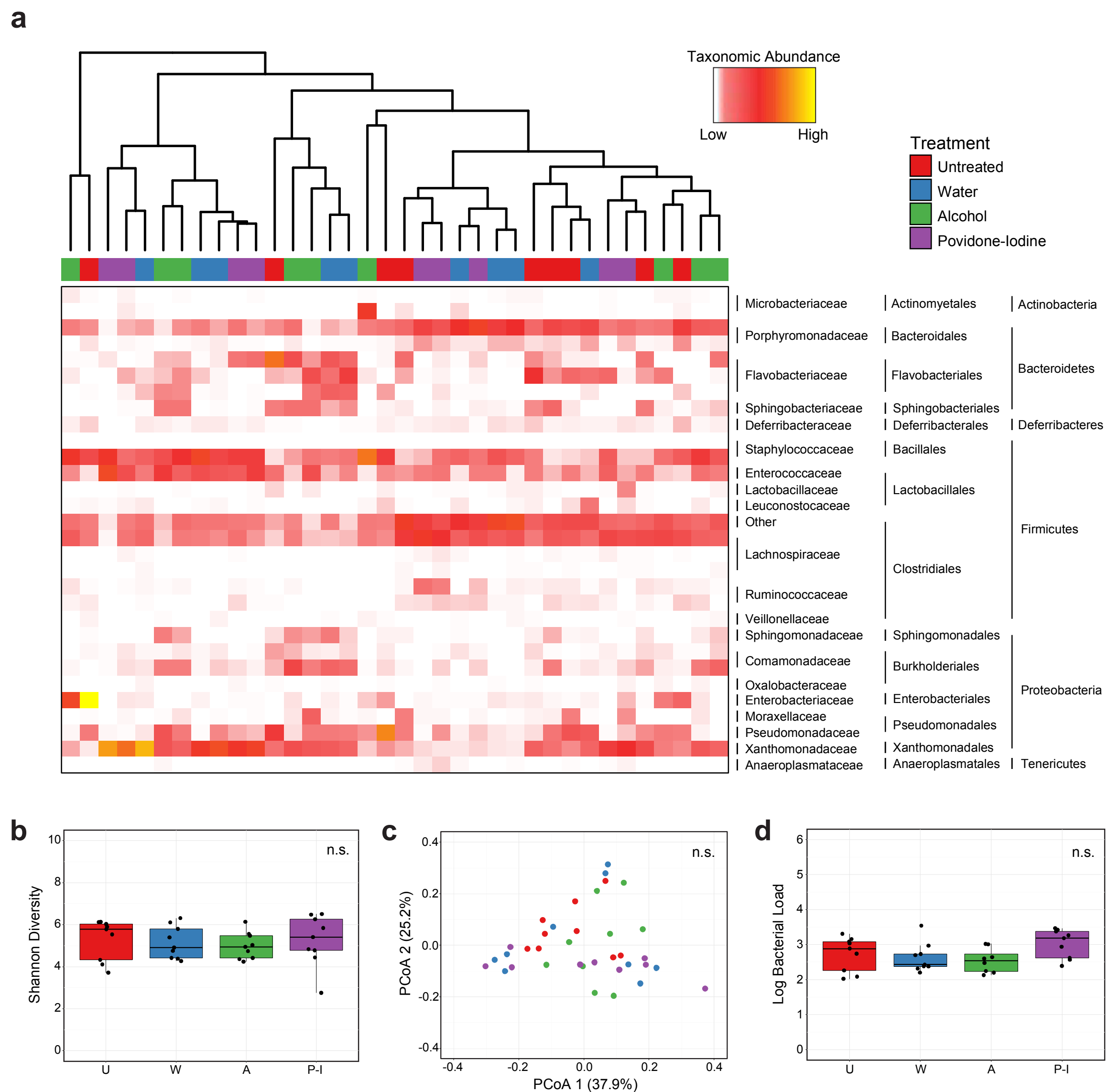

Figure 3 Antiseptic treatment induces only minor changes to skin microbial communities. (a) Heat map of rarified abundances for the 30 most common phylotypes on murine skin following treatment with water, alcohol, or povidone-iodine. Dendrograms represent hierarchical clustering of Euclidean distances using complete agglomeration. Horizontal bar above the graph designates treatment for individual mice. (b) Shannon diversity of murine bacterial communities in response to treatment. (c) Weighted UniFrac principle coordinates analysis representing

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differences in murine bacterial populations following treatment. (d) Bacterial load comparison of treated and untreated mice calculated by 16S rRNA gene content at the skin surface. Untreated (U), water (W), alcohol (A), povidone-iodine (P-I). Treatments were compared by Kruskal-Wallis rank sum test (b, d) or the adonis statistical test for community similarity (c).

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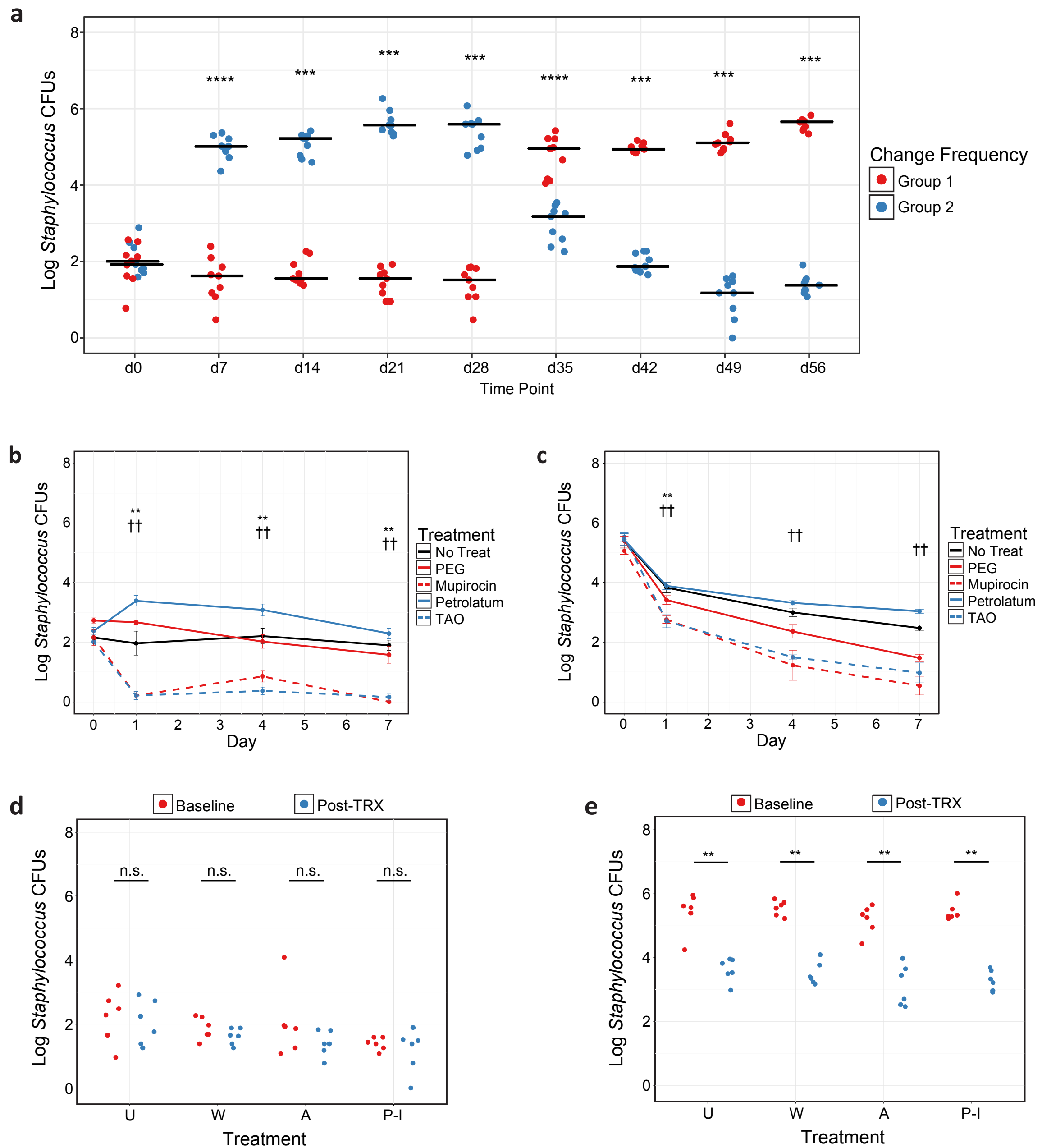

Figure 4 Antimicrobial treatment alters resident *Staphylococcus* colonization in a baseline-dependent manner. (a) Murine resident *Staphylococcus* colony forming units (CFUs) in response to cage change frequency over time. Group 1 mice were changed every other day and Group 2 mice were changed once per week at the start. Groups were switched to the alternate regimen at d28. Data are presented with

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median bars. (b, c) Murine resident *Staphylococcus* CFUs in response to antibiotic treatment starting at low (b) or high (c) baseline levels. Statistical comparisons were made between polyethylene glycol (PEG) and mupirocin (*) or petrolatum and triple antibiotic ointment (TAO) (†). Data are presented as mean ± s.e.m. (d, e) Murine resident *Staphylococcus* CFUs in response to antiseptic treatment starting at low (d) or high (e) baseline levels. Untreated (U), water (W), alcohol (A), povidone-iodine (P-I). Statistical significance was determined by Wilcoxon rank-sum test (Mann Whitney U test). *$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

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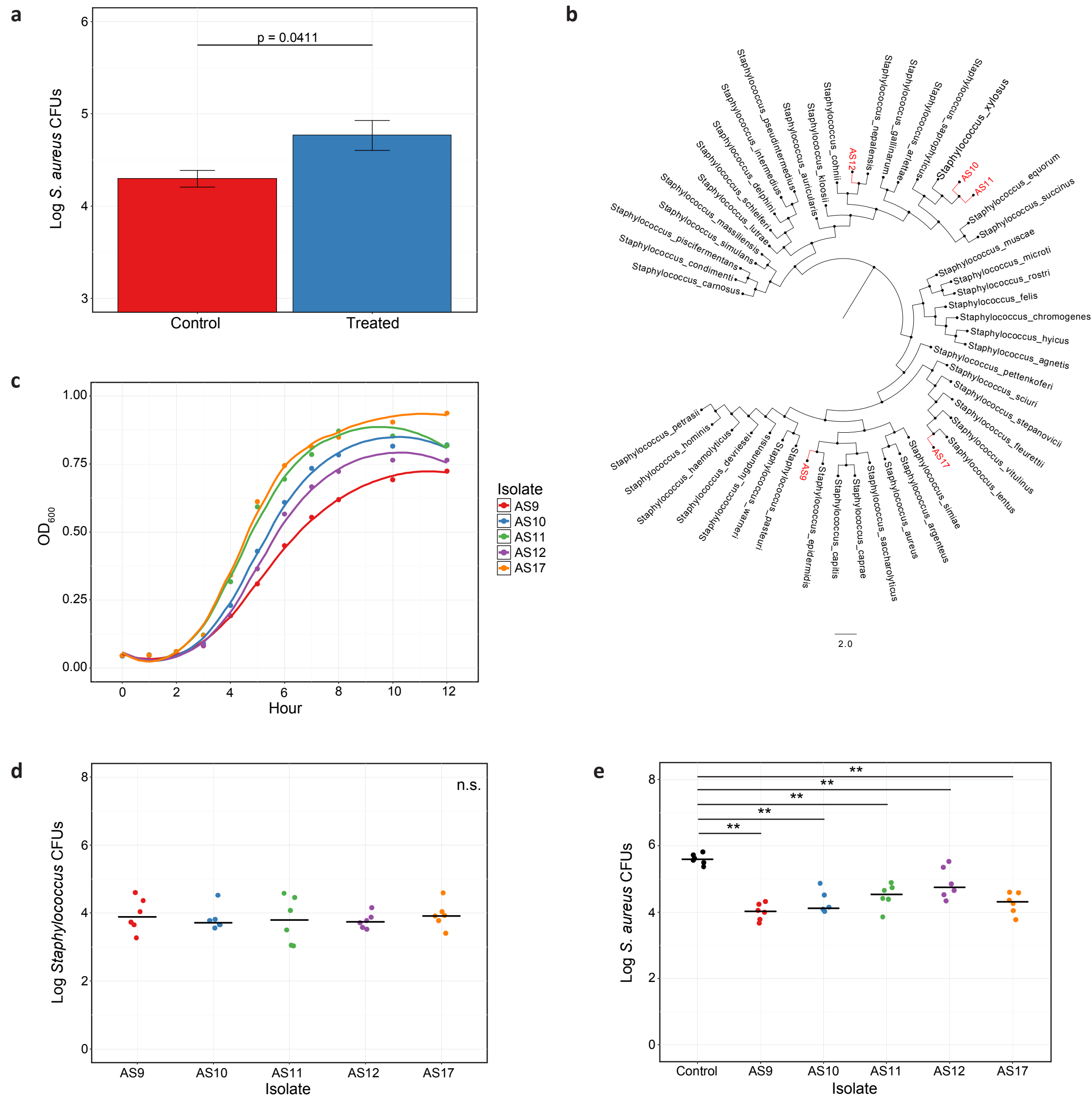

Figure 5 Resident *Staphylococcus* can reduce colonization by *Staphylococcus aureus*. (a) *Staphylococcus aureus* colony forming units (CFUs) following exogenous administration in mice pretreated with alcohol or untreated controls. (b) Phylogenetic tree of 16S rRNA gene diversity using approximate-maximum-likelihood to compare murine *Staphylococcus* residents (red) to known *Staphylococcus* isolates from the RDP database (black). (c) Growth curve analysis of

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resident *Staphylococcus* isolates at Optical Density 600 (OD_{600}). (d) Enumeration of *Staphylococcus* isolate CFUs following exogenous administration to mouse dorsum. (e) *S. aureus* CFU levels following precolonization of mouse dorsum with resident *Staphylococcus* isolates. Data are presented as mean ± s.e.m (a) or with median bars (d, e). Statistical significance was determined by Wilcoxon rank-sum test (Mann Whitney U test). *$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

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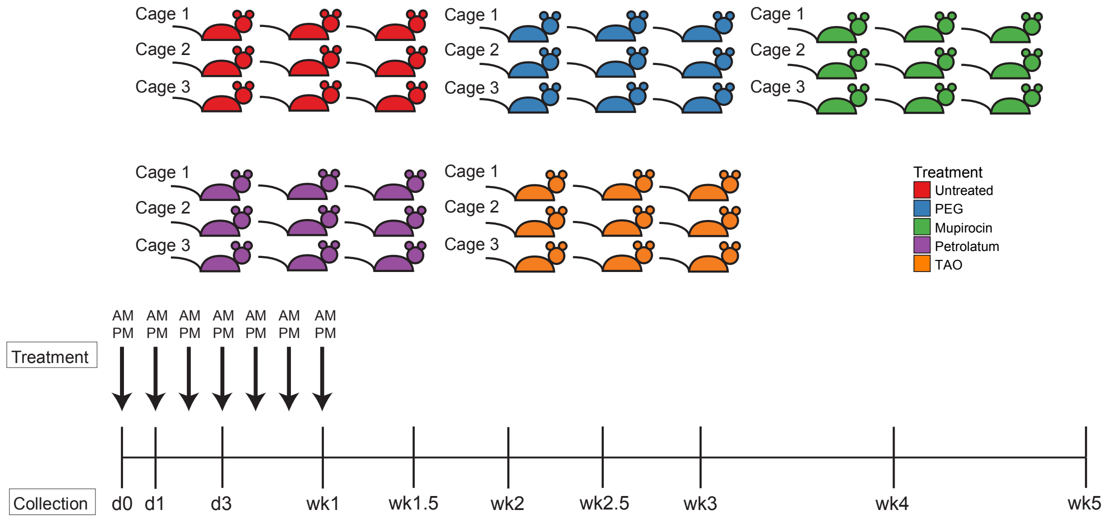
3.10 Supplemental Figures

a



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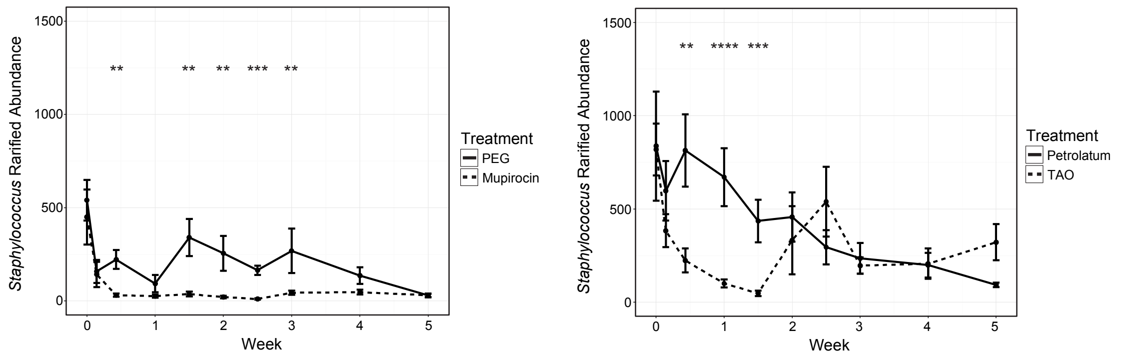

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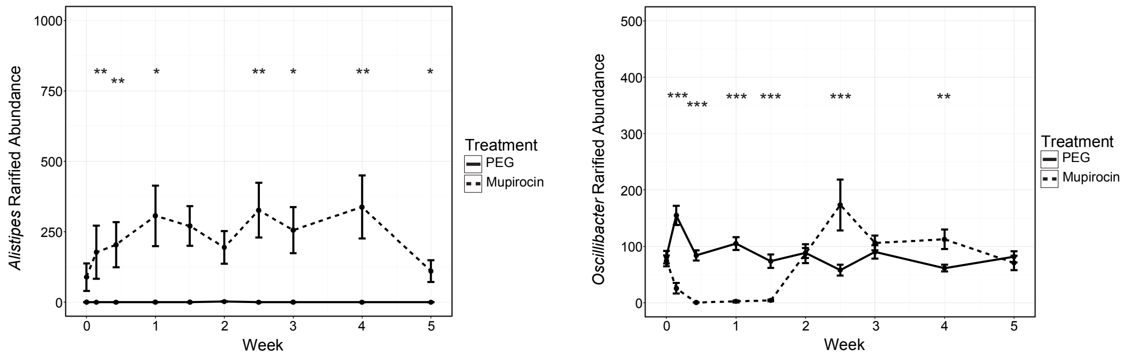

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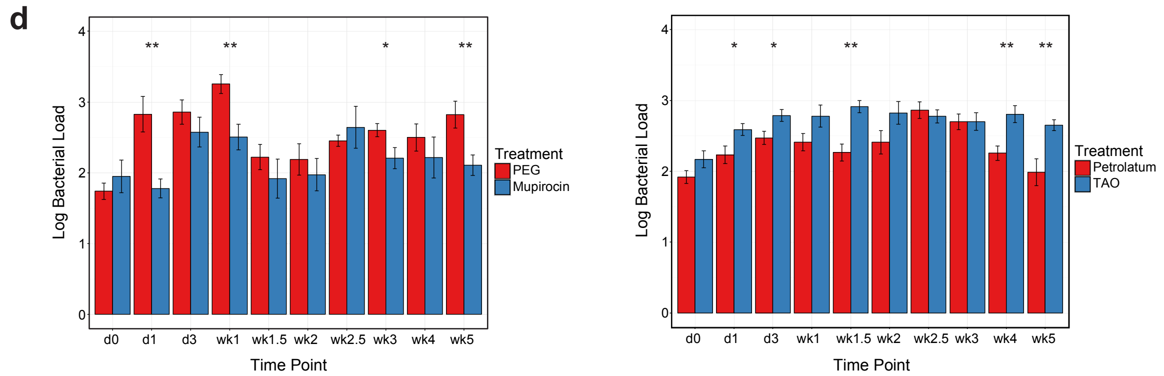

Supplemental Figure 1 Long-term alterations of select bacterial taxa in response to topical antibiotic treatment. (a) Schematic diagram of antibiotic treatments and sample collection regimen in mice administered polyethylene glycol (PEG), mupirocin, petrolatum, or triple antibiotic ointment (TAO). Treatment cohorts consisted of nine mice total, three cages of three mice each. Treatment occurred every 12 hours for one week, and mice were followed for 4 weeks post-treatment. (b,c) Longitudinal rarified abundances of (b) *Staphylococcus* and (c) *Alistipes* and *Oscillibacter*. (d) Variations in bacterial burden over time in response to treatment. Data are presented as mean ± s.e.m. Statistical significance was determined at each time point by Wilcoxon rank sum test (Mann Whitney U test). *$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

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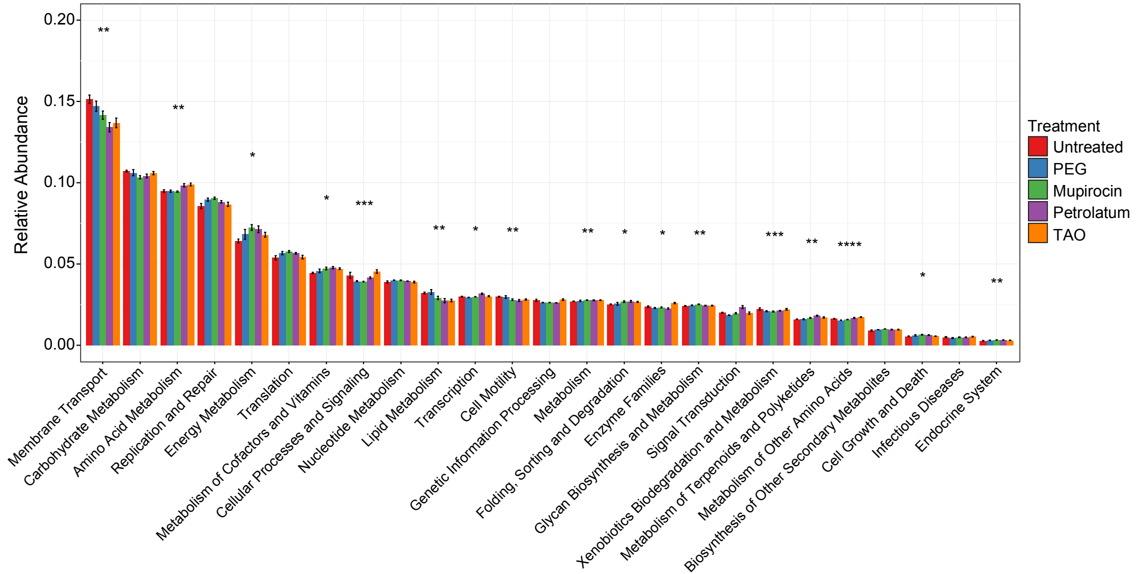

Supplemental Figure 2 Changes to functional potential of bacterial communities in response to topical antibiotic treatment. Relative abundances of major KEGG Pathways calculated by PICRUSt analysis of bacterial populations following one week of treatment with polyethylene glycol (PEG), mupirocin, petrolatum, or triple antibiotic ointment (TAO). Data are presented as mean ± s.e.m. Statistical significance was determined for major pathway designations by Kruskal-Wallis rank sum test and FDR correction. *$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

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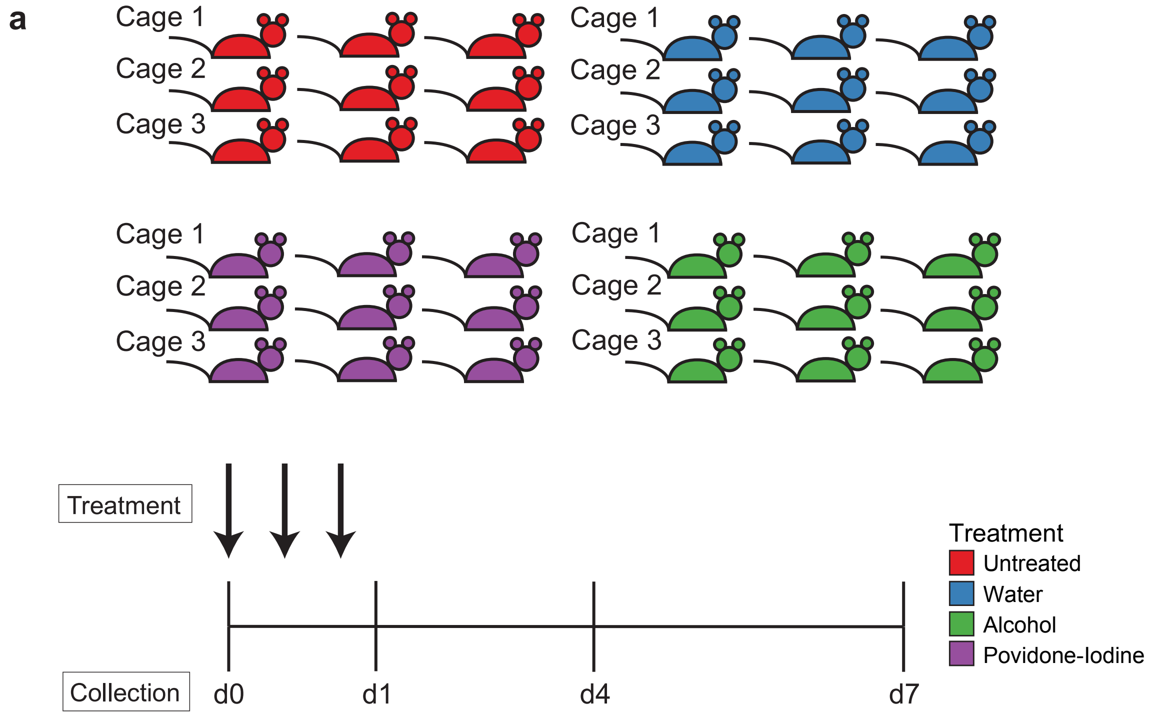

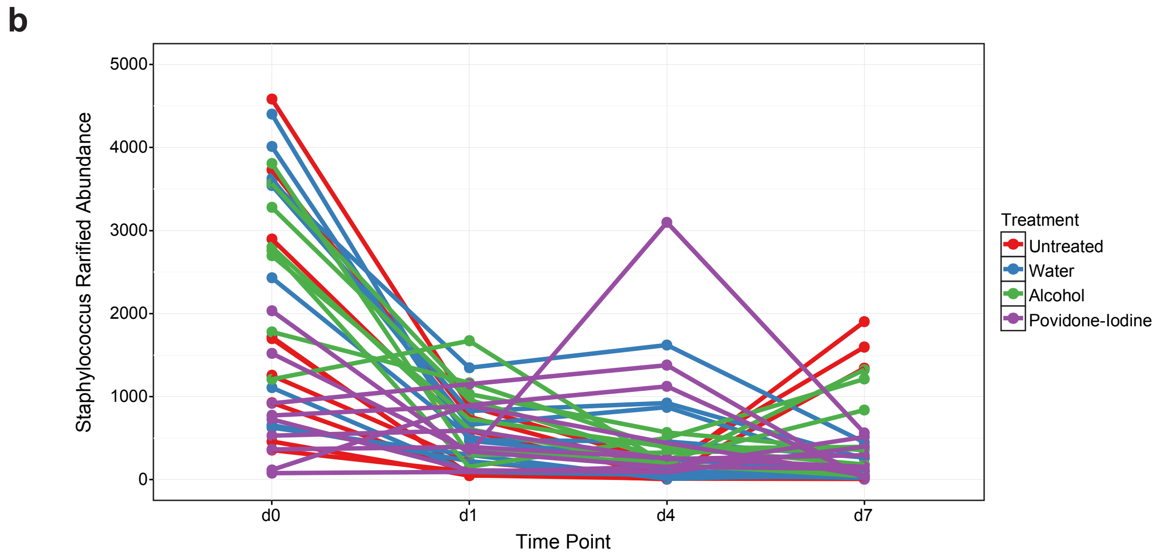

Supplemental Figure 3 Effects of antiseptics on skin bacterial inhabitants. (a) Model of antiseptic treatments and sample collection in mice treated with water, alcohol, or povidone-iodine. Treatment cohorts consisted of nine mice total, three cages of three mice each. Mice received three treatments total, one every 8 hours,

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and were followed for one-week post treatment. (b) Rarified Staphylococcus abundance in response to treatment over time.

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X non conserved
X ≥50% conserved

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Supplemental Figure 4 Resident *Staphylococcus* isolate 16S rRNA gene sequences. Comparison of Sanger sequenced murine resident *Staphylococcus* 16S rRNA genes. Consensus sequence logo is notated above isolate sequences. Nucleotides with greater than 50% conserved identity are depicted in blue. Non-conserved nucleotides are unhighlighted.

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3.11 Supplemental Tables

Supplemental Table 1 Taxonomic comparisons of treatment groups at d1 post-treatment. Significances calculated with FDR-corrected Kruskal-Wallis tests.

Lineage	FDR-corrected p-value
Actinobacteria; Actinobacteria; Actinomycetales; Microbacteriaceae	0.9263883
Actinobacteria; Actinobacteria; Actinomycetales; Micrococcaceae; Kocuria	0.6638857
Bacteroidetes; Bacteroidia; Bacteroidales; Porphyromonadaceae	0.6638857
Bacteroidetes; Bacteroidia; Bacteroidales; Porphyromonadaceae; Parabacteroides	0.4254647
Bacteroidetes; Flavobacteria; Flavobacteriales; Flavobacteriaceae; Chryseobacterium	0.6638857
Bacteroidetes; Flavobacteria; Flavobacteriales; Flavobacteriaceae; Elizabethkingia	0.6638857
Bacteroidetes; Flavobacteria; Flavobacteriales; Flavobacteriaceae; Flavobacterium	0.6486878
Bacteroidetes; Sphingobacteria; Sphingobacteriales; Sphingobacteriaceae; Sphingobacterium	0.1687431
Deferribacteres; Deferribacteres; Deferribacterales; Deferribacteraceae; Mucispirillum	0.395196
Firmicutes; Bacilli; Bacillales; Staphylococcaceae; Jeotgalicoccus	0.6638857
Firmicutes; Bacilli; Bacillales; Staphylococcaceae; Staphylococcus	0.3879636
Firmicutes; Bacilli; Lactobacillales; Enterococcaceae; Enterococcus	0.3879636
Firmicutes; Bacilli; Lactobacillales; Lactobacillaceae; Lactobacillus	0.6638857
Firmicutes; Bacilli; Lactobacillales; Leuconostocaceae; Weissella	0.6486878
Firmicutes; Clostridia; Clostridiales	0.6638857
Firmicutes; Clostridia; Clostridiales; Lachnospiraceae	0.6486878
Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Dorea	0.3827328
Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Johnsonella	0.649452
Firmicutes; Clostridia; Clostridiales; Ruminococcaceae	0.4254647
Firmicutes; Clostridia; Clostridiales; Ruminococcaceae; Oscillibacter	0.4254647
Firmicutes; Clostridia; Clostridiales; Veillonellaceae; Veillonella	0.6486878
Proteobacteria; Alphaproteobacteria; Sphingomonadales; Sphingomonadaceae; Novosphingobium	0.1687431
Proteobacteria; Betaproteobacteria; Burkholderiales; Comamonadaceae	0.6486878
Proteobacteria; Betaproteobacteria; Burkholderiales; Comamonadaceae; Comamonas	0.6486878
Proteobacteria; Betaproteobacteria; Burkholderiales; Oxalobacteraceae; Massilia	0.8707155
Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae	0.7751558
Proteobacteria; Gammaproteobacteria; Pseudomonadales; Moraxellaceae; Acinetobacter	0.6638857
Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas	0.1687431
Proteobacteria; Gammaproteobacteria; Xanthomonadales; Xanthomonadaceae; Stenotrophomonas	0.6486878
Tenericutes; Mollicutes; Anaeroplasmatales; Anaeroplasmataceae; Anaeroplasma	0.6486878

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Supplemental Table 2 Taxonomic comparisons of lineages at d0 and d1 post-treatment. Significances calculated with FDR-corrected Wilcoxon rank sum tests.

Treatment	Lineage	FDR-corrected p-value
Betadine	Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas	1
Betadine	Bacteroidetes; Bacteroidia; Bacteroidales; Porphyromonadaceae	1
Betadine	Firmicutes; Bacilli; Bacillales; Staphylococcaceae; Staphylococcus	1
Betadine	Firmicutes; Clostridia; Clostridiales	1
Betadine	Firmicutes; Clostridia; Clostridiales; Lachnospiraceae	1
Betadine	Proteobacteria; Gammaproteobacteria; Xanthomonadales; Xanthomonadaceae; Stenotrophomonas	1
Betadine	Firmicutes; Bacilli; Lactobacillales; Enterococcaceae; Enterococcus	1
Betadine	Bacteroidetes; Flavobacteria; Flavobacteriales; Flavobacteriaceae; Elizabethkingia	1
Betadine	Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae	1
Betadine	Firmicutes; Clostridia; Clostridiales; Ruminococcaceae	1
Betadine	Bacteroidetes; Bacteroidia; Bacteroidales; Porphyromonadaceae; Parabacteroides	1
Betadine	Bacteroidetes; Flavobacteria; Flavobacteriales; Flavobacteriaceae; Flavobacterium	1
Betadine	Deferribacteres; Deferribacteres; Deferribacterales; Deferribacteraceae; Mucispirillum	1
Betadine	Bacteroidetes; Sphingobacteria; Sphingobacteriales; Sphingobacteriaceae; Sphingobacterium	1
Betadine	Bacteroidetes; Flavobacteria; Flavobacteriales; Flavobacteriaceae; Chryseobacterium	1
Betadine	Proteobacteria; Betaproteobacteria; Burkholderiales; Comamonadaceae; Comamonas	1
Betadine	Firmicutes; Clostridia; Clostridiales; Ruminococcaceae; Oscillibacter	1
Betadine	Firmicutes; Bacilli; Lactobacillales; Leuconostocaceae; Weissella	1
Betadine	Proteobacteria; Betaproteobacteria; Burkholderiales; Comamonadaceae	1
Betadine	Firmicutes; Bacilli; Bacillales; Staphylococcaceae; Jeotgalicoccus	NA
Betadine	Proteobacteria; Alphaproteobacteria; Sphingomonadales; Sphingomonadaceae; Novosphingobium	1
Betadine	Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Dorea	1
Betadine	Firmicutes; Bacilli; Lactobacillales; Lactobacillaceae; Lactobacillus	1
Betadine	Proteobacteria; Gammaproteobacteria; Pseudomonadales; Moraxellaceae; Acinetobacter	1
Betadine	Tenericutes; Mollicutes; Anaeroplasmatales; Anaeroplasmataceae; Anaeroplasma	1
Betadine	Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Johnsonella	1
Betadine	Actinobacteria; Actinobacteria; Actinomycetales; Micrococcaceae; Kocuria	1
Betadine	Actinobacteria; Actinobacteria; Actinomycetales; Microbacteriaceae	1
Betadine	Firmicutes; Clostridia; Clostridiales; Veillonellaceae; Veillonella	1

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Betadine	Proteobacteria; Betaproteobacteria; Burkholderiales; Oxalobacteraceae; Massilia	1
Ethanol	Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas	1
Ethanol	Bacteroidetes; Bacteroidia; Bacteroidales; Porphyromonadaceae	1
Ethanol	Firmicutes; Bacilli; Bacillales; Staphylococcaceae; Staphylococcus	0.019744961
Ethanol	Firmicutes; Clostridia; Clostridiales	1
Ethanol	Firmicutes; Clostridia; Clostridiales; Lachnospiraceae	1
Ethanol	Proteobacteria; Gammaproteobacteria; Xanthomonadales; Xanthomonadaceae; Stenotrophomonas	1
Ethanol	Firmicutes; Bacilli; Lactobacillales; Enterococcaceae; Enterococcus	1
Ethanol	Bacteroidetes; Flavobacteria; Flavobacteriales; Flavobacteriaceae; Elizabethkingia	1
Ethanol	Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae	1
Ethanol	Firmicutes; Clostridia; Clostridiales; Ruminococcaceae	1
Ethanol	Bacteroidetes; Bacteroidia; Bacteroidales; Porphyromonadaceae; Parabacteroides	1
Ethanol	Bacteroidetes; Flavobacteria; Flavobacteriales; Flavobacteriaceae; Flavobacterium	1
Ethanol	Deferribacteres; Deferribacteres; Deferribacterales; Deferribacteraceae; Mucispirillum	1
Ethanol	Bacteroidetes; Sphingobacteria; Sphingobacteriales; Sphingobacteriaceae; Sphingobacterium	1
Ethanol	Bacteroidetes; Flavobacteria; Flavobacteriales; Flavobacteriaceae; Chryseobacterium	1
Ethanol	Proteobacteria; Betaproteobacteria; Burkholderiales; Comamonadaceae; Comamonas	1
Ethanol	Firmicutes; Clostridia; Clostridiales; Ruminococcaceae; Oscillibacter	1
Ethanol	Firmicutes; Bacilli; Lactobacillales; Leuconostocaceae; Weissella	1
Ethanol	Proteobacteria; Betaproteobacteria; Burkholderiales; Comamonadaceae	1
Ethanol	Firmicutes; Bacilli; Bacillales; Staphylococcaceae; Jeotgalicoccus	1
Ethanol	Proteobacteria; Alphaproteobacteria; Sphingomonadales; Sphingomonadaceae; Novosphingobium	1
Ethanol	Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Dorea	1
Ethanol	Firmicutes; Bacilli; Lactobacillales; Lactobacillaceae; Lactobacillus	1
Ethanol	Proteobacteria; Gammaproteobacteria; Pseudomonadales; Moraxellaceae; Acinetobacter	1
Ethanol	Tenericutes; Mollicutes; Anaeroplasmatales; Anaeroplasmataceae; Anaeroplasma	1
Ethanol	Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Johnsonella	1
Ethanol	Actinobacteria; Actinobacteria; Actinomycetales; Micrococcaceae; Kocuria	1
Ethanol	Actinobacteria; Actinobacteria; Actinomycetales; Microbacteriaceae	1
Ethanol	Firmicutes; Clostridia; Clostridiales; Veillonellaceae; Veillonella	1
Ethanol	Proteobacteria; Betaproteobacteria; Burkholderiales; Oxalobacteraceae; Massilia	1
No Treat	Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas	0.932949404
No Treat	Bacteroidetes; Bacteroidia; Bacteroidales; Porphyromonadaceae	1
No Treat	Firmicutes; Bacilli; Bacillales; Staphylococcaceae; Staphylococcus	0.148087207

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No Treat	Firmicutes; Clostridia; Clostridiales	1
No Treat	Firmicutes; Clostridia; Clostridiales; Lachnospiraceae	1
No Treat	Proteobacteria; Gammaproteobacteria; Xanthomonadales; Xanthomonadaceae; Stenotrophomonas	1
No Treat	Firmicutes; Bacilli; Lactobacillales; Enterococcaceae; Enterococcus	1
No Treat	Bacteroidetes; Flavobacteria; Flavobacteriales; Flavobacteriaceae; Elizabethkingia	1
No Treat	Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae	1
No Treat	Firmicutes; Clostridia; Clostridiales; Ruminococcaceae	1
No Treat	Bacteroidetes; Bacteroidia; Bacteroidales; Porphyromonadaceae; Parabacteroides	1
No Treat	Bacteroidetes; Flavobacteria; Flavobacteriales; Flavobacteriaceae; Flavobacterium	1
No Treat	Deferribacteres; Deferribacteres; Deferribacterales; Deferribacteraceae; Mucispirillum	1
No Treat	Bacteroidetes; Sphingobacteria; Sphingobacteriales; Sphingobacteriaceae; Sphingobacterium	0.388151273
No Treat	Bacteroidetes; Flavobacteria; Flavobacteriales; Flavobacteriaceae; Chryseobacterium	1
No Treat	Proteobacteria; Betaproteobacteria; Burkholderiales; Comamonadaceae; Comamonas	0.289846607
No Treat	Firmicutes; Clostridia; Clostridiales; Ruminococcaceae; Oscillibacter	1
No Treat	Firmicutes; Bacilli; Lactobacillales; Leuconostocaceae; Weissella	1
No Treat	Proteobacteria; Betaproteobacteria; Burkholderiales; Comamonadaceae	1
No Treat	Firmicutes; Bacilli; Bacillales; Staphylococcaceae; Jeotgalicoccus	1
No Treat	Proteobacteria; Alphaproteobacteria; Sphingomonadales; Sphingomonadaceae; Novosphingobium	0.070091195
No Treat	Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Dorea	1
No Treat	Firmicutes; Bacilli; Lactobacillales; Lactobacillaceae; Lactobacillus	1
No Treat	Proteobacteria; Gammaproteobacteria; Pseudomonadales; Moraxellaceae; Acinetobacter	1
No Treat	Tenericutes; Mollicutes; Anaeroplasmatales; Anaeroplasmataceae; Anaeroplasma	1
No Treat	Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Johnsonella	1
No Treat	Actinobacteria; Actinobacteria; Actinomycetales; Micrococcaceae; Kocuria	1
No Treat	Actinobacteria; Actinobacteria; Actinomycetales; Microbacteriaceae	1
No Treat	Firmicutes; Clostridia; Clostridiales; Veillonellaceae; Veillonella	1
No Treat	Proteobacteria; Betaproteobacteria; Burkholderiales; Oxalobacteraceae; Massilia	1
Water	Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas	1
Water	Bacteroidetes; Bacteroidia; Bacteroidales; Porphyromonadaceae	1
Water	Firmicutes; Bacilli; Bacillales; Staphylococcaceae; Staphylococcus	0.22213081
Water	Firmicutes; Clostridia; Clostridiales	1
Water	Firmicutes; Clostridia; Clostridiales; Lachnospiraceae	1
Water	Proteobacteria; Gammaproteobacteria; Xanthomonadales; Xanthomonadaceae; Stenotrophomonas	1
Water	Firmicutes; Bacilli; Lactobacillales; Enterococcaceae; Enterococcus	1

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Water	Bacteroidetes; Flavobacteria; Flavobacteriales; Flavobacteriaceae; Elizabethkingia	1
Water	Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae	1
Water	Firmicutes; Clostridia; Clostridiales; Ruminococcaceae	1
Water	Bacteroidetes; Bacteroidia; Bacteroidales; Porphyromonadaceae; Parabacteroides	1
Water	Bacteroidetes; Flavobacteria; Flavobacteriales; Flavobacteriaceae; Flavobacterium	1
Water	Deferribacteres; Deferribacteres; Deferribacterales; Deferribacteraceae; Mucispirillum	1
Water	Bacteroidetes; Sphingobacteria; Sphingobacteriales; Sphingobacteriaceae; Sphingobacterium	1
Water	Bacteroidetes; Flavobacteria; Flavobacteriales; Flavobacteriaceae; Chryseobacterium	1
Water	Proteobacteria; Betaproteobacteria; Burkholderiales; Comamonadaceae; Comamonas	1
Water	Firmicutes; Clostridia; Clostridiales; Ruminococcaceae; Oscillibacter	1
Water	Firmicutes; Bacilli; Lactobacillales; Leuconostocaceae; Weissella	1
Water	Proteobacteria; Betaproteobacteria; Burkholderiales; Comamonadaceae	1
Water	Firmicutes; Bacilli; Bacillales; Staphylococcaceae; Jeotgalicoccus	NA
Water	Proteobacteria; Alphaproteobacteria; Sphingomonadales; Sphingomonadaceae; Novosphingobium	1
Water	Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Dorea	1
Water	Firmicutes; Bacilli; Lactobacillales; Lactobacillaceae; Lactobacillus	1
Water	Proteobacteria; Gammaproteobacteria; Pseudomonadales; Moraxellaceae; Acinetobacter	1
Water	Tenericutes; Mollicutes; Anaeroplasmatales; Anaeroplasmataceae; Anaeroplasma	1
Water	Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Johnsonella	1
Water	Actinobacteria; Actinobacteria; Actinomycetales; Micrococcaceae; Kocuria	1
Water	Actinobacteria; Actinobacteria; Actinomycetales; Microbacteriaceae	1
Water	Firmicutes; Clostridia; Clostridiales; Veillonellaceae; Veillonella	1
Water	Proteobacteria; Betaproteobacteria; Burkholderiales; Oxalobacteraceae; Massilia	1

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Chapter 4 - Topical treatment interventions elicit personalized and site-specific shifts in human skin bacterial communities

The contents of this chapter are prepared for submission with the authors:
SanMiguel AJ, Meisel JS, Horwinski J, Zheng Q, Bradley CW, Grice EA.

4.1 Abstract

The skin microbiome represents a significant contributor to cutaneous health and disease. This includes its roles in immune tolerance and defense against pathogenic microorganisms. Despite these critical functions, the impact of topical interventions meant to disrupt these communities remains poorly understood. In this study, we present the effects of three clinically-relevant antiseptics, alcohol, povidone-iodine (Betadine), and chlorhexidine, on cutaneous bacterial populations. We illustrate a proficiency of these treatments in altering skin bacterial communities, a result which was highly dependent on interpersonal and body site-specific signatures. We also show that the magnitude of this response can be influenced by both the identity and relative abundances of bacterial inhabitants. By comparing the effects of antiseptic regimens, we highlight the importance of antibacterial activity and mechanical clearance to treatment disruption. We also demonstrate the potential for pre-treatment communities to inform post-treatment response. In all, these results further our understanding of treatment-derived perturbations to the skin microbiota, and establish the ability of topical interventions to influence skin bacterial dynamics.

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4.2 Introduction

Skin represents a unique habitat, colonized by an equally unique set of microorganisms (1). Previous studies have analyzed these residents in-depth, describing a stable community distinguished by both inter- and intrapersonal differences (2, 3). This includes the skin's ability to select for microbial residents at distinct biogeographic regions, each representing a niche with selective pressures that can influence cutaneous microbial inhabitance (4). A number of studies have also tested the importance of these residents to human health, underscoring their ability to educate the immune system and protect against pathogenic skin microorganisms (5-8). Together, these studies have highlighted the importance of the skin microbiota, and outlined its role in host cutaneous defense.

In light of these findings, it is important to note that humans are constantly working to disrupt skin microbial communities in both clinical and non-clinical settings (9-12). While antimicrobial agents are largely employed to reduce infection by pathogenic microorganisms (13-15), these treatments can also act on resident cutaneous species (16-18). This is especially true for antiseptics, a group of antimicrobial agents used specifically for their indiscriminate mechanisms of action (19, 20). Antiseptics are a mainstay of modern medicine, but have also infiltrated our daily lives in the form of gels, wipes, and sprays designed to sterilize host cutaneous surfaces (21-23). As the significance of skin resident microorganisms becomes increasingly apparent, assessing the impact of these treatments on the colonization dynamics of skin inhabitance becomes of equal importance. Indeed, we

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and others have recently expounded the potential for altered skin bacterial communities to impact colonization by *Staphylococcus aureus*, while additional reports have identified their importance in cutaneous diseases such as atopic dermatitis (24-26). These studies have highlighted the significance of skin microbial residents, and necessitated further research into treatment-derived perturbations.

To expand our knowledge in this regard, we present the first study to date of antiseptics on human skin bacterial populations using sequencing-based approaches. We show that treatment elicits a significant impact on skin communities that is both personalized and body site-specific. We also show that certain microorganisms are more likely to be perturbed than others, with both abundance and bacterial identity representing key predictors of this response. Upon deeper analysis, water was found to elicit a similar shift in bacterial communities compared to alcohol and povidone-iodine (Betadine), indicating a conserved effect of these interventions based on mechanical disruption. By contrast, we observed a minimal impact of chlorhexidine treatment on skin residents, an effect likely influenced by the ability of this antiseptic to kill, but not necessarily remove, bacterial markers. In all, these results further our understanding of bacterial dynamics at the skin surface, and outline the potential for topical treatments to disrupt skin bacterial residence.

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4.3 Results

Thirteen subjects, six females and seven males, were recruited to evaluate the effects of antiseptics on the skin microbiome. Treatments were applied to the volar forearm and the upper back to evaluate alternate skin microenvironments (dry and sebaceous, respectively), and each subject received identical treatments to control for interpersonal variability. Subjects received water and alcohol (80% ethanol) on contralateral body sites during their first series of visits, and povidone-iodine (Betadine) and chlorhexidine during their second series of visits. Swab specimens to analyze the microbiota were collected at baseline, prior to treatment, and post-treatment for 72 hours to assess longitudinal dynamics. Treated body sites were also accompanied by adjacent, untreated control sites, while visits were separated by at least two weeks to allow for microbial equilibration. Specific treatment topography, timing, and subject demographics are provided in Fig. S1a and Table S1. In total, 71,167,526 16S rRNA gene reads (hypervariable regions 1-3) were sequenced. Following quality control and filtering, the final study cohort represented 1,456 samples rarified to an even depth of 4,500 sequences per sample.

4.3.1 Baseline characteristics of study cohort

To validate our methods, we started by characterizing the baseline communities of our study cohort. As previously reported (2, 4), we identified a strong impact of biogeography on the skin microbiota. Back communities were largely dominated by Propionibacteriaceae and Staphylococcaceae (Fig. 1a). By contrast, forearm communities were more permissive, hosting increased proportions of additional

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taxa such as Streptococcaceae and Corynebacteriaceae, amongst others. Reflecting these community compositions, alpha diversity was significantly different between body sites, with the forearm exhibiting increased Shannon diversity, observed species, and equitability compared to the back (Fig. 1b). These metrics also highlighted the importance of interpersonal variability, as data points showed consistent grouping by individual when assessing diversity at both body sites. When comparing these communities at the population-level, prominent clustering of subjects and body sites was observed by both weighted and unweighted UniFrac metrics (Fig. 1c). Comparisons of baseline communities also identified interpersonal variability and site-specificity as the most significant contributors to variation, followed by time and body symmetry respectively (Fig. S1b, c). In all, these results confirm previous work, and highlight the unique nature of resident skin bacterial communities.

4.3.2 Treatment elicits personalized shifts to skin bacterial community structure

To begin our investigation of antiseptics and the skin microbiota, we compared baseline resident populations to communities at 1hr post-treatment. Using weighted UniFrac and principal coordinates analysis, we observed minimal clustering of samples in response to treatment at the forearm and back, with none eliciting a significant shift in bacterial population structure (Fig. 2a). Because interpersonal differences were the strongest contributors to variability at baseline, and could thus mask more subtle effects of our treatments, we also compared subjects' post-

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treatment communities to their baseline controls. Using this method we detected a significant effect of both water and alcohol at the forearm for at least 6 hours post-treatment, suggesting a personalized effect of these treatments on population structure (Fig. 2b). Indeed, while both treatments caused a more robust shift than that seen in adjacent controls, neither could promote bacterial communities to a state outside that of the broader study cohort (Fig. 2c). Comparisons of Shannon diversity and bacterial burden also confirmed these effects with alcohol eliciting significant decreases in diversity, and both water and alcohol decreasing overall bacterial load (Fig. S2a, b).

To determine the specific taxa responsible for this shift, we next focused our analysis on the most abundant taxa seen at the forearm. Specifically, Corynebacteriaceae, Propionibacteriaceae, Streptococcaceae, and Staphylococcaceae were chosen, as they represented a mean relative abundance of approximately 70% in baseline samples. Similar to community level analyses, most taxa were not significantly altered despite consistent changes by these taxa in the majority of subjects in our study cohort (Fig. 2d). Indeed, only Streptococcaceae was significantly decreased in response to treatment at the forearm, although both Propionibacteriaceae and Staphylococcaceae were also disrupted in nearly all subjects. In all, these data suggest that certain treatments can elicit changes to skin bacterial communities, but that this effect is often masked by interpersonal variability.

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4.3.3 Treatment results in decreases to skin bacterial membership

We next investigated whether treatment could elicit more significant changes to skin bacterial membership by unweighted metrics, which are agnostic to the relative proportions of bacterial taxa. In contrast to weighted comparisons, these tests revealed a prominent shift in bacterial communities following treatment at both the forearm and back (Fig. 3a). Moreover, when comparing treated communities to their baseline controls, both the back and forearm were significantly disrupted by water, alcohol, and Betadine compared to adjacent controls (Fig. 3b). To evaluate the cause of this shift, we analyzed the effect of treatment on the total number of observed species. We found that changes to community membership were largely driven by a decrease in bacterial richness, with water, alcohol, and Betadine all significantly reducing the number of observed species compared to adjacent controls (Fig. S3a).

To further investigate these results, we also tested the effect of treatment on the membership of individual bacterial families. We found that Corynebacteriaceae, Incertae Sedis XI, Micrococcaceae, Staphylococcaceae, and Streptococcaceae were the most prominently disrupted taxa at both the forearm and back (Fig. 3c, Fig. S3b). Moreover, when comparing the richness of these taxa at treated and adjacent body sites, we found that each of these families were significantly decreased at treated, but not untreated, areas of the skin (Fig. 3d, Fig. S3c). Interestingly, this effect did not extend to all highly abundant families, as Propionibacteriaceae remained largely unchanged regardless of treatment or body site. This suggests that certain bacteria

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may be less susceptible than others when assessing treatment-derived alterations to bacterial membership.

4.3.4 Chlorhexidine retains free bacterial DNA at the skin surface

During these initial tests, we were particularly struck by the inability of chlorhexidine to elicit a significant shift in bacterial community membership or structure. This was especially surprising given its proven efficacy against pathogenic microorganisms in hospital settings (27). As chlorhexidine is known for its ability to cause allergic and dermatologic irritation in a subset of individuals (28), we wondered whether acute treatment could result in cutaneous changes that would allow for better binding of free DNA from dead bacteria. This would then explain our inability to detect changes in the skin microbiota following chlorhexidine application. To test this hypothesis, we evaluated a subset of our subjects for alterations in skin barrier function by transepidermal water loss (TEWL) in response to treatment. We reasoned that if chlorhexidine were to alter the skin, making it more likely to bind free DNA, we should observe an increase in TEWL similar to that seen in patients with atopic dermatitis and other dermatologic conditions (29, 30). Upon testing, however, we found no significant differences in TEWL when comparing treatments to each other, or to baseline controls at 1hr and 6hr post-treatment (Fig. S4a). This suggests that acute treatment with chlorhexidine does not uniquely alter the integrity of the skin barrier.

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Although we have previously shown that treatment with antibiotic ointment, a particularly adherent substance, has a minimal effect on the retention of dead bacterial DNA (24), we still wondered whether chemical properties inherent to chlorhexidine could be responsible for its lack of observed effect. To evaluate this question, we applied marker bacterial DNA to the skin of mouse dorsa, and tested its persistence following treatment with water, alcohol, Betadine, or chlorhexidine. Surprisingly, we observed a unique ability of chlorhexidine to retain free bacterial DNA at the skin surface, with the total amount of marker bacterial DNA exceeding that of other treatment regimens at 1hr post-treatment by over 10-fold on average (Fig. S4a, b). To test whether this effect could persist for multiple hours post-treatment, we also evaluated the quantity of DNA at 6hr post-treatment. Similar to 1hr time points, we found that mice treated with chlorhexidine retained more DNA at the skin surface compared to other regimens at this time point as well (Fig. S4c). These experiments suggest that our inability to detect differences following chlorhexidine treatment were likely due to a unique ability of this antiseptic to bind bacterial DNA to the skin surface, and not necessarily a deficiency in antibacterial activity.

4.3.5 Treatment elicits convergence at distinct community types in a site-specific manner

Because distinct chemical properties of chlorhexidine could represent a confounding factor in our experiments, we next focused our investigations on water, alcohol, and Betadine treatments only. Specifically, we tested whether an

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unsupervised approach to community analyses could identify a conserved microbial signature in post-treatment populations. Dirichlet multinomial mixture (DMM) modeling utilizes probability distributions to establish a prior of metacommunities (31). Clusters can then be generated based on the similarity of a sample to a given metacommunity. Using this approach, DMM models identified 8 distinct clusters at the forearm, with individual subjects often being dominated by a single community type (Fig. S5a, b). Despite these interpersonal differences, however, we observed a prominent convergence at DMM cluster 1 in response to all treatments, an effect that was not observed at adjacent body sites (Fig. 4a, Fig. S5c). DMM cluster 1 was differentiated by decreased bacterial diversity, specifically richness (Fig. 4b), confirming our finding that treatment can significantly disrupt bacterial membership. This particular cluster also displayed fewer taxon-specific indications, suggesting a normalization of bacterial residents in response to treatment (Fig. 4c). In contrast to the forearm, back communities did not converge on a single community type following treatment (Fig. S5d, e). However, we did observe a slight increase in low diversity clusters in certain instances (Fig. S5f). In all, these data verify that treatment can elicit reproducible changes to skin bacterial communities, but also underscore the importance of body site to calculations of resident stability.

4.3.6 Highly abundant bacterial families are the greatest contributors to treatment-derived changes in skin bacterial communities

Our initial analyses suggested that certain bacterial taxa were disrupted more significantly than others, an effect which could lead to the increased frequency of

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DMM cluster 1 we observed immediately post-treatment. To assess this hypothesis, we next tested characteristics shown to influence variation in untreated settings. We reasoned that the most variable taxa in the absence of treatment were also the most likely to change in response to topical intervention. As previous analyses have identified intermediately abundant taxa as the most susceptible to temporal fluctuation (32), we started by assessing the baseline variance of these taxa in our study cohort. Specifically, we compared the variance of bacterial residents at adjacent, contralateral, and temporally-controlled body sites to their mean relative abundances. Similar to previous data, we observed a distinct second-order, power-law relationship in skin bacterial residents, with intermediately abundant members varying the most in untreated, baseline communities (Fig. S6a).

To test which taxa were specifically responsible for these shifts, we assessed baseline variance at the family level for each subject at the forearm and back. We found that Propionibacteriaceae, Streptococcaceae, Staphylococcaceae, Corynebacteriaceae, Micrococcaceae, and Incertae Sedis XI constituted the most variable groups in baseline communities (Fig. S6b, c). Interestingly, rather than representing only intermediately abundant taxa, however, these families were often the most abundant residents in our study cohort, and also the most likely to vary in response to treatment. To investigate this discrepancy more directly, we again compared the variance of baseline taxa to their mean relative abundances, but this time we further controlled for both interindividual differences and body site-specificity. While we had previously observed a second-order relationship when

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aggregating subjects and body sites, stratification resulted in a more nuanced effect, with the variance of taxa frequently plateauing when plotted against their mean relative abundances (Fig. 5a). Indeed, top taxonomic groups were often found to exhibit both the greatest levels of variance and the greatest mean relative abundances, especially in the case of Propionibacteriaceae. Together, these results suggest that intermediately abundant skin bacteria are the most likely to fluctuate at higher levels of comparison, but that predominant taxa are more variable when assessing personalized biogeographic regions.

Following these analyses, we next tested whether taxonomic variation at baseline could be used as an indicator of post-treatment effects. Specifically, we compared the baseline variance of bacterial families to their mean response following water, alcohol, and Betadine treatments. We found that the taxa most likely to vary in the absence of treatment were indeed the most likely to be disrupted by topical intervention, with decreases in the relative proportions of most taxa being offset by increases in Propionibacteriaceae (Fig. 5b). Importantly, we also observed that interpersonal variability was a strong contributor to this trend, as subjects with low variation of a given bacterial family were also less likely to exhibit shifts by those members following treatment. This trend was recapitulated when comparing the mean relative abundances of taxa to their mean treatment response as well. Once again, the greatest differences were observed within the Propionibacteriaceae family, which was both the most abundant bacterial family and the most likely to increase following treatment (Fig. 5c). In all, these results indicate that both

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abundance and variation in untreated controls can inform treatment-derived effects, but that bacterial identity is also an important variable when measuring overall community response.

4.3.7 Body site specificity informs fluctuations of the most abundant bacterial taxa

During these analyses, we noted that, unlike other taxa, Propionibacteriaceae increased in relative abundance following treatment of the back. We also found that a subset of subjects exhibited similar dynamics when Staphylococcaceae was their most abundant taxon. Because we observed a decrease in bacterial load following treatment in our previous analyses, these increases in relative proportions were unlikely to represent increases in absolute abundance. However, they did suggest a personalized response in which the most abundant taxon per subject was also the most likely to persist following treatment. To test this hypothesis, we compared the levels of each subject's most abundant taxon at baseline to its mean relative abundance following water, alcohol, and Betadine treatment. We found that in all cases but one, the most abundant taxon at the back increased in relative proportions following treatment regardless of identity, indicating a distinct competitive advantage (Fig. 5d). To assess whether this effect was specific to the back, we also examined the most abundant residents at the forearm. Unlike the back, only three subjects displayed taxa at this body site with greater than 50% relative abundance. Despite these varying properties, however, we still observed an increase in the relative proportions of Propionibacteriaceae in multiple subjects following

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treatment, although this effect was not absolute (Fig. 5e). Interestingly, this trend did not extend to all skin residents, as Corynebacteriaceae, Staphylococcaceae, and Streptococcocaceae all decreased in abundance at the forearm, regardless of status. These results verify that abundance can be used to predict treatment effects, but also highlight the importance of body site to these outcomes.

4.3.8 Lowly abundant members of predominant bacterial families are the most likely to vary in response to treatment

Our previous investigations outlined the importance of abundance and bacterial identity when assessing the effects of water and antiseptic stress. To build on this finding, we further asked whether relative abundance could be used to predict the fluctuations of all taxa, rather than just the most prevalent taxon per subject. Indeed, the mere fact that certain bacterial families were found stably at the skin surface, regardless of subject, suggested a degree of competitive advantage for a subset of residents. To assess this hypothesis, we began by partitioning OTUs into highly or lowly abundant groups based on an abundance threshold of 0.5% - a value chosen from the inflection point of OTU counts at baseline (Fig. S7a). We then investigated alterations to the membership of these bacteria in response to treatment. In all, we observed a significant decrease in the number of lowly abundant OTUs following treatment at both the forearm and back (Fig. 6a), an effect due in large part to decreases in Corynebacteriaceae, Incertae Sedis XI, Staphylococcaceae, and Streptococcaceae (Fig. 6b, c Fig. S7b, c). By contrast, when evaluating highly abundant OTUs, only Streptococcaceae at the forearm and Corynebacteriaceae at

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the back were significantly reduced, a result which did not significantly decrease the total number of highly abundant OTUs. Similar to previous results, we also observed no significant differences in the membership of Propionibacteriaceae, regardless of abundance or body site. These findings confirm that bacterial identity represents a critical factor when evaluating skin resident stability, and underscores the importance of abundance to predictions of treatment response.

4.4 Discussion

The skin microbiota has proven essential to numerous functions in cutaneous health and disease (5-8). However, few studies have assessed our ability to disrupt these communities, or their dynamics following antimicrobial stress. Herein, we present the impact of topical antiseptics on human skin bacterial populations, and outline the importance of key variables to overall community response.

When evaluating treatments at a comparative level, we found water, alcohol, and Betadine to have similar effects on skin bacterial residents. Rather than highlighting the antibacterial nature of alcohol and Betadine, these results appear to underscore the generalized qualities of certain topical interventions, namely their ability to reduce inhabitance by mechanical cleansing (19). This result has been particularly well-established in culture-based systems. Here, reports have outlined the ability of certain topical treatments to both kill, and remove, pathogenic microorganisms, with each feature playing an important role in infection control (33, 34). Mild, non-antibacterial soaps are also used with the sole purpose of clearance, further

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emphasizing the importance of this mechanism to skin hygiene and community disruption (35, 36).

Unlike alcohol and Betadine, chlorhexidine was found to elicit only minor shifts in skin bacterial residence. Given the ability of this antiseptic to reduce infections in clinical settings (37), this suggested that chlorhexidine might work in a unique manner to kill, but not remove, bacterial markers from the skin surface. To test this hypothesis, we applied free bacterial DNA to mouse dorsa, and treated with water, alcohol, Betadine, and chlorhexidine. We found that chlorhexidine was uniquely proficient at retaining bacterial DNA at the skin surface, with significantly greater levels of DNA at both 1hr and 6hr post-treatment. Further research will be necessary to elucidate the precise mechanism by which chlorhexidine achieves this feat. However, it is interesting to note that chlorhexidine is a cationic molecule, distinguished by an ability to bind the epidermal surface for multiple hours post-treatment (38). As such, the potential exists that this antiseptic may retain bacterial DNA through a dual interaction with skin keratinocytes and the negatively charged DNA backbone.

While no study to date has investigated the impact of antiseptics on the human skin microbiota by sequencing, others have assessed the effects of hand-sanitizers and soaps (39, 40). These studies have largely supported culture-based tests, outlining the importance of conserved mechanisms to topical treatment response. For example, a recent study by Zapka, et al. found that water and hand washing elicited

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similar alterations to the skin microbiota as alcohol-based hand sanitizers in most tests (39). We have also observed similar findings in murine skin communities, with water reducing levels of colonizing *Staphylococcus* in a similar manner to 80% ethanol and Betadine (24). A recent comparison of mild and antibacterial soaps has confirmed these results as well, showing minimal differences when comparing their impact on the colonizing levels of *S. epidermidis* (40).

In addition to these findings, each of the abovementioned studies observed a relatively minor impact of treatment on skin bacterial communities. Given these results, it is perhaps unsurprising that we initially observed only modest differences in response to antiseptic stress. Only after controlling for personalization and body site-specificity could we observe the true impact of our treatment regimens. These results are further underscored by our finding that treatment often elicited the strongest effects in low-level inhabitants. Indeed, highly abundant species likely exist at a given skin niche due to an ability to resist both host-derived and acute external stressors. As the skin is often colonized by particular strains with temporal stability for years at a time (32, 41), this outlines a system by which multiple taxa can exist on the skin surface, but only a subset is uniquely adapted for long-term colonization. These observations pair well with previous, culture-based analyses. Here, highly abundant skin residents have been shown to persist in response to various treatments while transient, low-level bacteria often represent a less stable group of community inhabitants (42-44).

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In addition to abundance, we also found that bacterial identity could influence treatment response, with lowly abundant species from top taxa often being more significantly disrupted than other skin residents. This finding underscores the ecological advantages seen in bacterial families such as Propionibacteriaceae, Staphylococcocaceae, Streptococcaceae, and Corynebacteriaceae. The prevalence of these taxa at baseline in most subjects highlights their ability to utilize conserved resources at and within the skin surface (45). Upon the introduction of treatment-derived stressors, however, a generalized selective advantage is no longer enough, leading to the persistence of only the most resilient and well-adapted members of each group.

Treatment-derived alterations were also observed to be dependent upon body site, with the back representing a more stable habitat than the forearm. However, when assessing the richness of predominant bacterial families, the back and forearm were both found to be susceptible to a loss of lowly abundant OTUs. This finding emphasizes the reproducibility of disruption in low level inhabitants, and illustrates the conservation of certain outcomes at distinct biogeographic regions. Interestingly, this result did not extend to all major taxa, as members of the Propionibacteriaceae family persisted regardless of body site. We believe this particular effect could be due to an inherent resilience of Propionibacteriaceae, or an increased abundance at deeper, newly exposed layers of the skin. Regardless, the persistence of this taxon likely represents a unique opportunity to thrive in the post-treatment setting.

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In all, this study furthers our understanding of skin bacterial dynamics and elucidates the effect of topical treatments on cutaneous resident populations. While we observed a similar impact of water and certain antiseptics, we note that our studies were designed to assess the totality of skin residents in healthy individuals. As such, we caution against the application of these findings to clinical settings in which the dynamics of pathogen and commensal are highly skewed. Indeed, previous studies have described, in-depth, the utility of antiseptics in these particular environments (46-48). As our study assesses only the effect of acute stressors, we also advocate for further research into long-term treatment regimens. The potential exists that more lasting perturbations may elicit even greater shifts to skin bacterial communities, an important consideration when evaluating the nexus of host-microbial interactions.

4.5 Materials and Methods

4.5.1 Human subjects and sample collection. Thirteen healthy subjects aged 23-30 (median:27, 6 females) and without chronic skin disorders were recruited to participate in a controlled skin antiseptic study (Table S1). To be eligible for participation, subjects were required to be greater than 21 years of age, and could not have taken oral or topical antibiotics within 6 months of their first visit. Subjects were swabbed at baseline and then administered one of four treatments for 1.5 minutes. Each participant received water (UltraPure Distilled Water, Invitrogen) and alcohol (80% ethanol) on contralateral forearm or back body sites during their first visit series, and povidone-iodine (Betadine, 10% povidone-iodine)) and

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chlorhexidine (chlorhexidine-gluconate 4%) during their second visit series (Fig. S1a). Visit series were separated by at least two weeks to allow for microbial equilibration. Following treatment, subjects were swabbed at 1hr, 6hr, 12hr, 24hr, 36hr, and 72hr post-treatment at both treated and adjacent body sites. Swabbed regions were delineated by a skin marker to ensure that the same body site was swabbed at longitudinal time points. Subjects were instructed to refrain from showering for at least 12 hours prior to each time point. Protocols were approved by the Institutional Review Board of the University of Pennsylvania, and written informed consent was obtained for all study participants prior to sampling.

4.5.2 Transepidermal water loss. Transepidermal water loss was measured in a subset of four subjects using a Tewameter TM300 (Courage+Khazaka, Cologne, Germany) according to the manufacturer's instructions. Briefly, subjects were equilibrated for at least 10 minutes prior to testing. Noninvasive probes were then pressed to the skin at baseline, 1hr, and 6hr post-treatment with water, alcohol, Betadine, or chlorhexidine to measure changes in skin epidermal barrier function. Each process was repeated at both the forearm and back to assess differences by body site.

4.5.3 Bacterial DNA isolation, 16S rRNA gene sequencing, and qPCR. Bacterial DNA was extracted as described previously (49) using the Invitrogen PureLink kit. PCR and sequencing of the V1V3 hypervariable region was performed using 300-bp paired end chemistry and barcoded primers (27F, 534R) on the Illumina MiSeq

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platform. Accuprime High Fidelity Taq polymerase was used for PCR cycling conditions: 94 °C for 3 min; 35 cycles of 94 °C for 45 sec, 50 °C for 60 sec, 72 °C for 90 sec; 72 °C for 10 min. For bacterial load comparisons, 16S rRNA genes were amplified by qPCR using Fast SYBR Green Master Mix (Fisher Scientific) and the optimized primers 533F, 902R. Samples were compared to standard curves generated from known concentrations of serially diluted bacterial DNA to calculate burden.

4.5.4 Microbiome analysis. Sequences were preprocessed and quality filtered prior to analysis, and QIIME 1.7.0 was used for microbiome evaluation (50). Briefly, sequences were *de novo* clustered into OTUs based on 97% similarity by UClust (51), and taxonomy was assigned to the most abundant representative sequence per cluster using the RDP classifier (52). Sequences were aligned by PyNAST (53), and chimeric sequences were removed using ChimeraSlayer (54). Sequences with calls to Unclassified, Bacteria;Other, or Cyanobacteria were removed in addition to singletons. Antiseptics and negative controls were similarly sequenced and analyzed for possible contaminating sequences, with no OTUs being found at consistently high levels. All samples were rarified to 4,500 sequences, and samples below this cut-off were removed from downstream analyses. Alpha and beta diversity matrices and taxonomy tables were formulated in QIIME. Statistical analysis and visualization were performed in the R statistical computing environment (55).

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4.5.5 Dirichlet multinomial mixture models. Subsampled OTU counts were aggregated at the highest level of taxonomic classification. Samples were separated by body site and spurious taxa in less than 1% of samples were removed. Clusters were generated separately on forearm and back samples using the R package Dirichlet Multinomial (v1.14.0), and community types for each body site were calculated based on absolute minima from Dirichlet components and Laplace approximations of model evidence (31). Samples were assigned to final community types based on posterior probabilities.

4.5.6 DNA retention. C57BL/6 mice were bred and maintained in specific pathogen free conditions at the University of Pennsylvania. Eight to fifteen week old males and females were randomized to control for differences in age and gender, and each mouse was housed singly to avoid cross-contamination. Mice were shaved at the dorsum and acclimated for at least 2 days prior to experimentation. 5-6 ng/ul of extracted *Escherichia coli* DNA was applied to mouse dorsa and permitted to dry for 1hr prior to treatment. Mice were then administered water, alcohol, Betadine, or chlorhexidine for 1.5 minutes, similar to human experiments, and swabbed at 1hr and 6hr post-treatment. Following sample collection, DNA was extracted using the Invitrogen PureLink kit, and *E. coli*-specific DNA was amplified using qPCR primers to the *ycct* gene (56). Samples were compared to standard curves generated from known amounts of serially diluted *E. coli* DNA to calculate marker DNA concentrations.

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4.6 Accession Numbers

16S rRNA sequence reads have been deposited in the NCBI Short Read Archive under BioProject ID: XXXXXXXXXXX

4.7 Acknowledgements

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4.8 Competing interests

The authors declare no competing financial interests.

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4.9 Figures

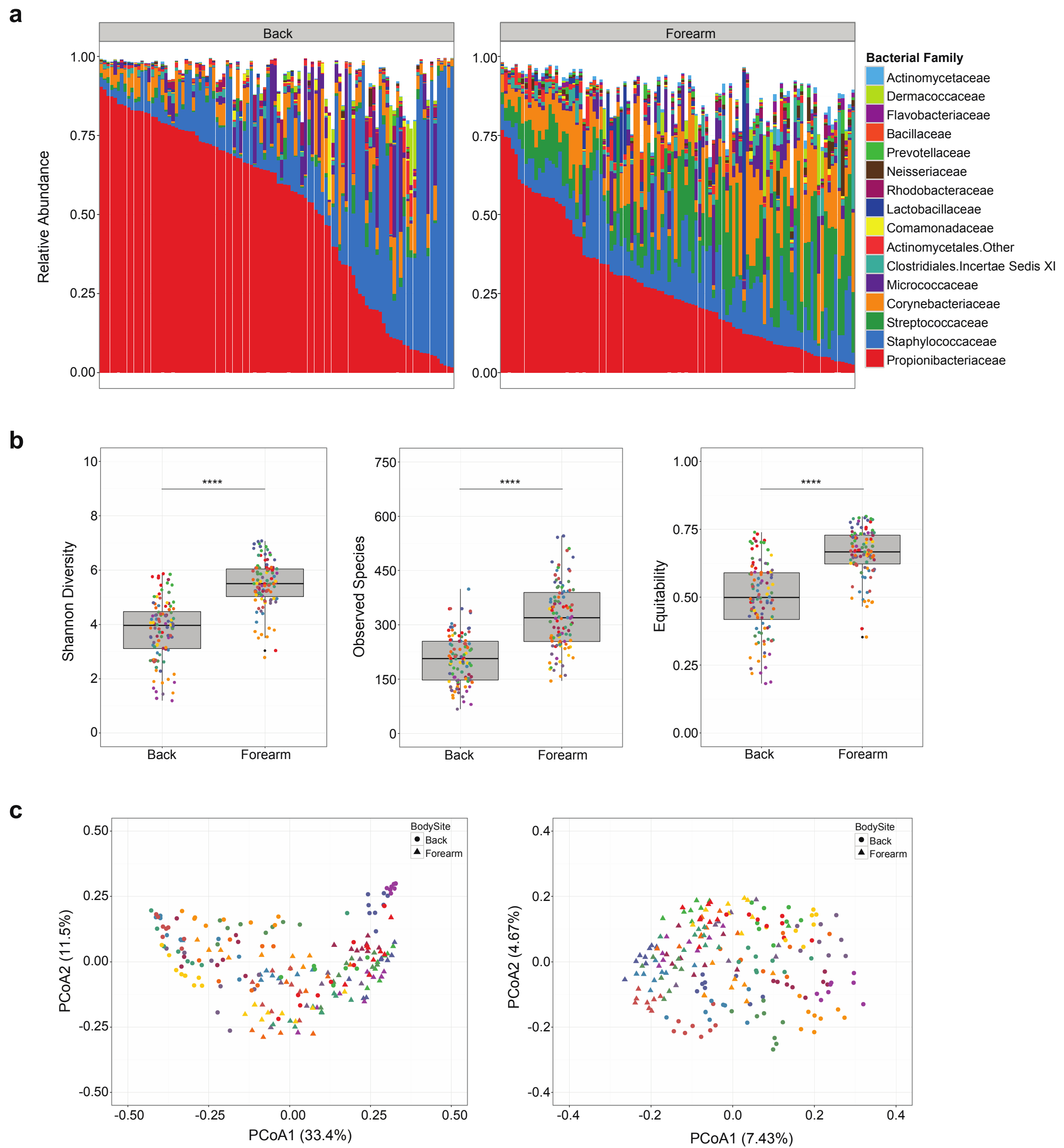

Figure 1 Skin bacterial communities exhibit site-specificity and interpersonal variability at baseline. (a) Family-level relative abundances of baseline communities for subjects at the forearm and back. Each bar represents an individual sample with eight samples per subject based on controls at adjacent and contralateral body sites for each visit series. (b) Alpha diversity of baseline communities at the forearm and

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back. Shannon diversity, observed species, and equitability are illustrated separately. Each point is colored by subject. (c) Weighted (left) and unweighted (right) UniFrac principal coordinates analyses of baseline samples. Each point is colored by subject and shaped by body site. **** $P < 0.0001$ by Wilcoxon rank sum test (Mann-Whitney U test).

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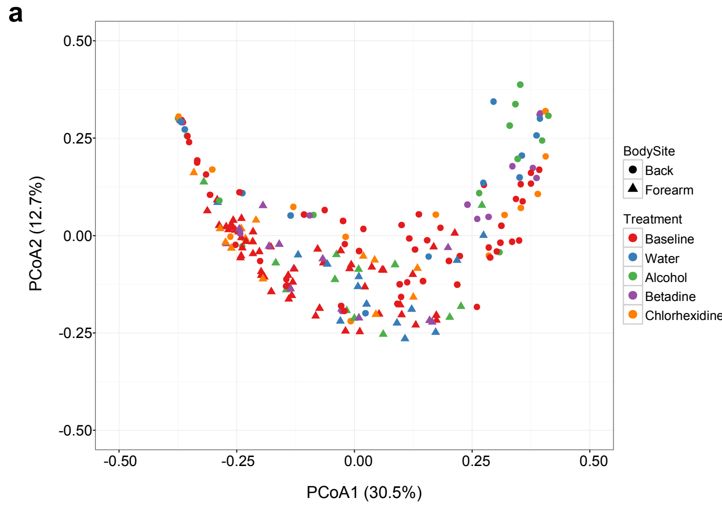

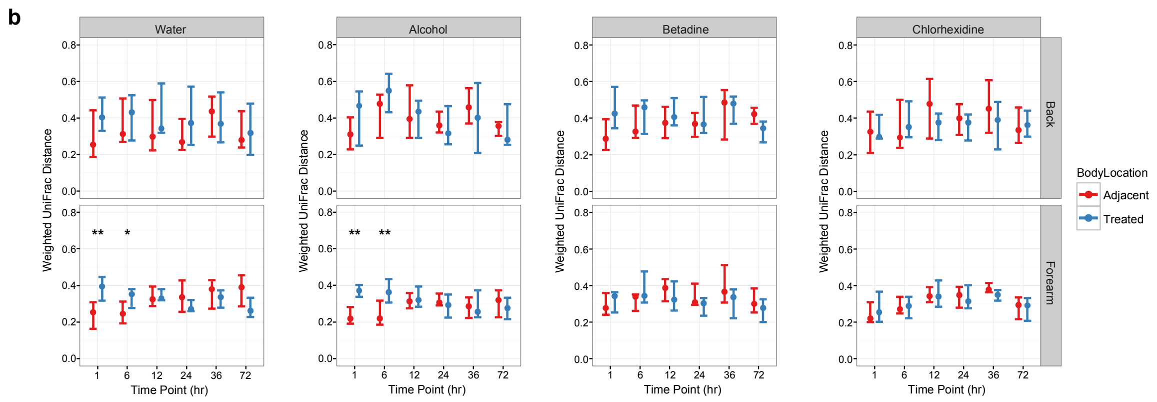

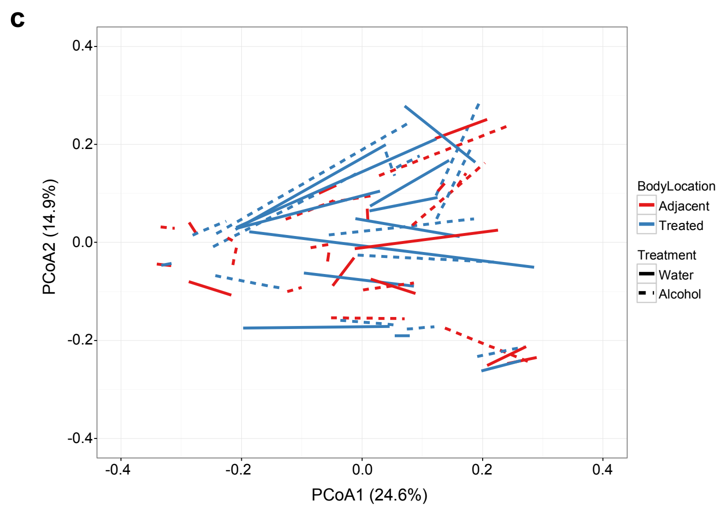

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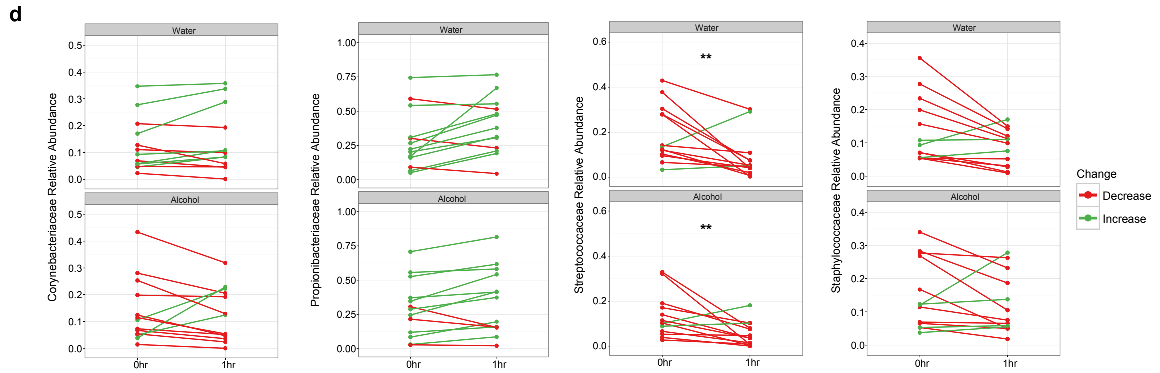

Figure 2 Treatment elicits personalized shifts in weighted comparisons of skin bacterial populations. (a) Principal coordinates analysis of weighted UniFrac distances for treated body sites at baseline and 1hr post-treatment. Each point represents a single sample, colored by treatment and shaped by body site. (b) Weighted UniFrac distances of subjects' longitudinal time points compared to their individual baseline communities at treated and adjacent body sites. Points represent the median of participants. Error bars designate interquartile regions. (c) Subanalysis of weighted UniFrac distances visualized by principal coordinates analysis in subjects treated with water and alcohol at the forearm. Lines connect baseline and 1hr post-treatment samples for individual subjects, and line types designate treatment regimen. Line colors refer to treated body sites or their respective adjacent controls. (d) Comparison of relative abundances for the top 4 taxa at baseline and 1hr post-treatment with water or alcohol. Each line represents an individual subject colored by an increase or decrease in relative abundance following treatment. * $P < 0.05$, ** $P < 0.01$ by Wilcoxon rank sum test (Mann-Whitney U test).

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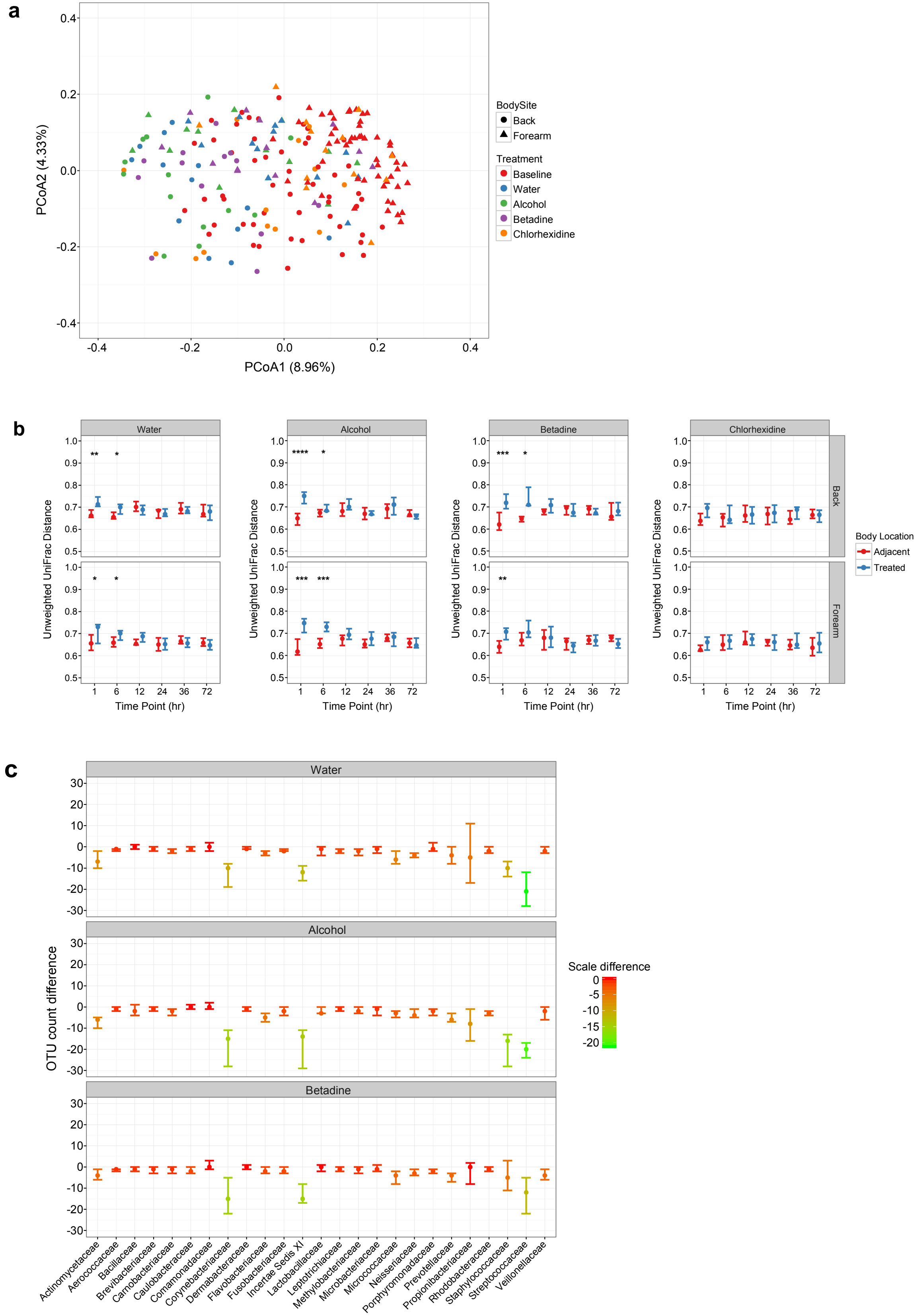

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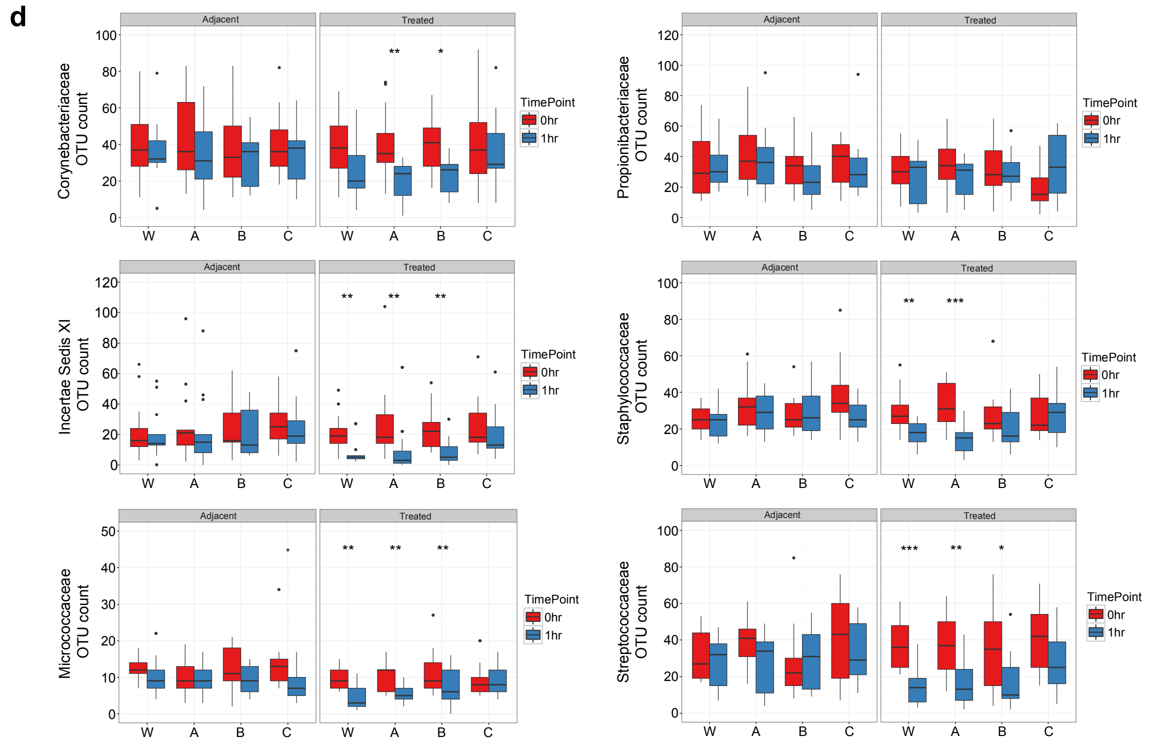

Figure 3 Treatment results in distinct alterations to skin bacterial residents by unweighted metrics. (a) Visualization of unweighted UniFrac distances by principal coordinates analysis for treated body sites at baseline and 1hr post-treatment. Each point represents a single sample, colored by treatment and shaped by body site. (b) Comparison of unweighted UniFrac distances for baseline and post-treatment communities in response to treatment at the forearm and back. Points represent the median of participants. Error bars designate interquartile regions. (c) Difference between OTU counts for the top 25 families at the forearm for baseline and 1hr post-treatment samples in response to water, alcohol, and Betadine treatment. Points represent the median of participants and are colored by scaled differences in total count. Error bars designate interquartile regions. (d) Box and whisker plots of OTU counts for major taxa at adjacent and treated body sites of the forearm

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between baseline and 1hr time points. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ by Wilcoxon rank sum test (Mann-Whitney U test).

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Figure 4 Dirichlet multinomial modeling identifies convergence at distinct forearm community types following treatment. (a) Longitudinal frequencies of DMM clusters in response to treatment with water, alcohol, and Betadine. (b) Shannon diversity and observed species counts of individual DMM clusters. Data are presented as mean ± s.e.m. (c) Heat map of square root counts for the top bacterial taxa contributing to cluster identity. Dark bars correspond to greater counts.

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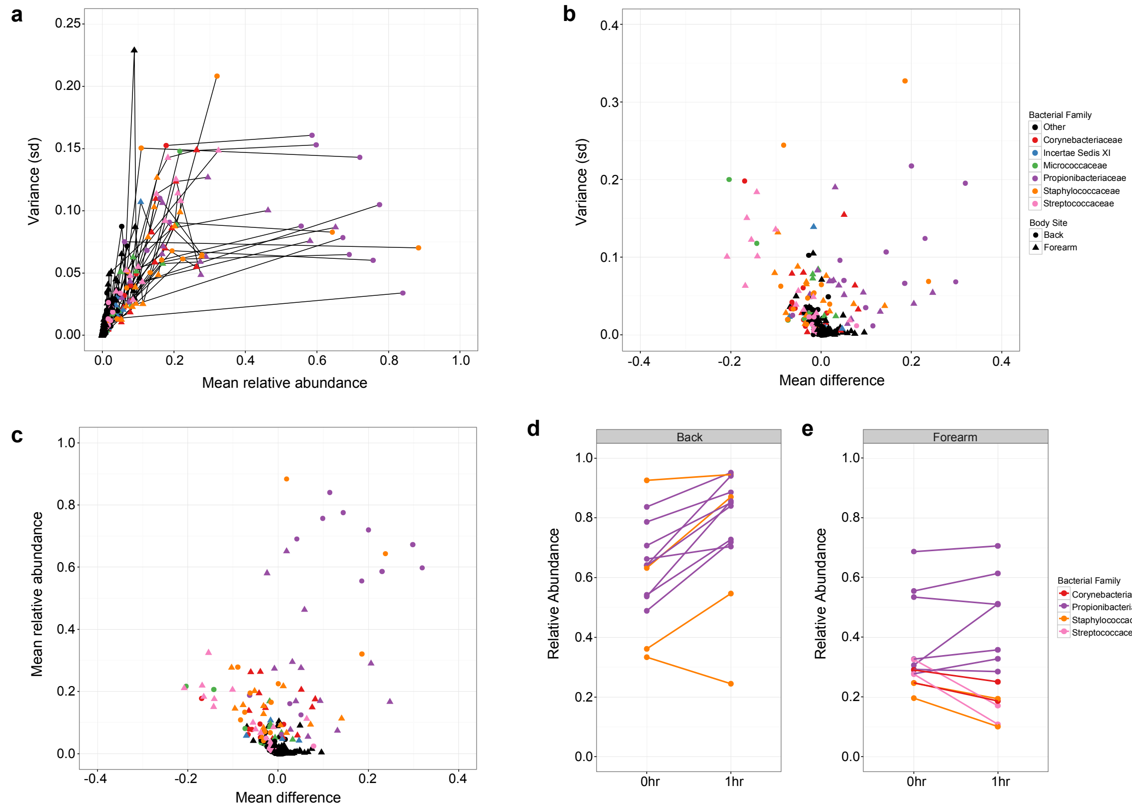

Figure 5 Baseline variance and abundance are indicators of treatment-derived alterations to the skin microbiota. (a) Family-level comparison of the baseline variances (standard deviation) and mean relative abundances for subjects at the forearm and back. Each point represents the values for bacterial families of an individual subject, shaped by body site and colored by family. Lines connect families of an individual subject and body site. "Other" designations refer to any bacterial family different from the listed members (b) Baseline variance of bacterial families plotted against their mean treatment effect in response to water, alcohol, and Betadine treatment at the forearm and back. (c) Mean relative abundance of bacterial families at baseline compared to mean treatment effects at the forearm and back. (D, E) Mean difference in relative abundance of the most dominant taxon

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per subject following treatment at the back (d) and forearm (e). Each point represents a single subject colored by bacterial family identity.

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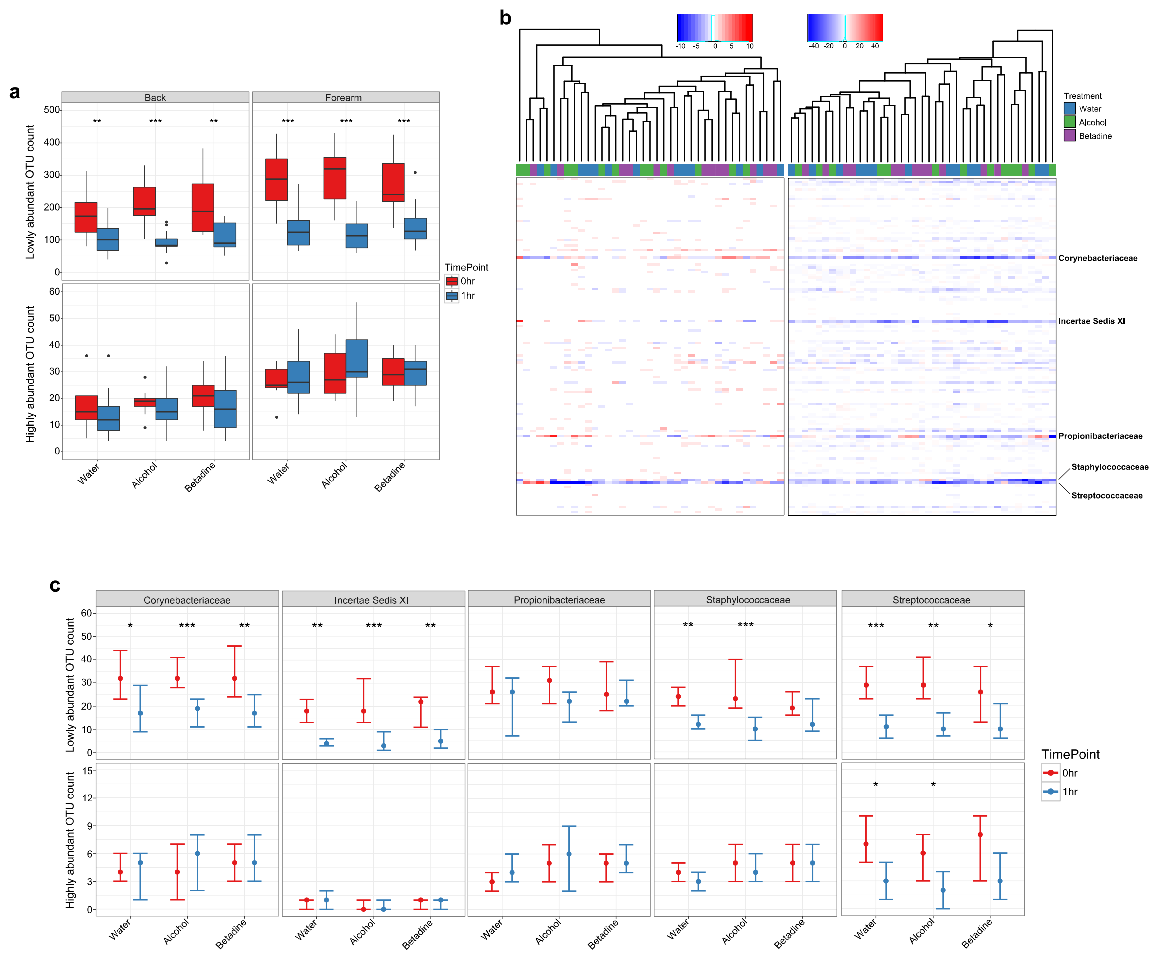

Figure 6 Lowly abundant members of prominent taxa are the greatest contributors to treatment effects at the skin surface. (a) Box and whisker plots of lowly and highly abundant OTU counts as defined by a 0.5% relative abundance threshold following treatment at the forearm and back. (b) Heat map of differences in forearm OTU counts between baseline and 1hr post treatment with water and antiseptics. Each column represents the difference measured for a single subject and treatment, and each row represents a bacterial family. Samples are clustered by the Unweighted Pair Group Method with Arithmetic means (UPGMA). Color-coded bars above the graph designate treatments for each sample. (c) Comparison of lowly and

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highly abundant OTU counts at the forearm in major taxonomic families at baseline and 1hr post-treatment. Points represent the median of the study cohort. Error bars designate interquartile regions. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ by Wilcoxon rank sum test (Mann-Whitney U test).

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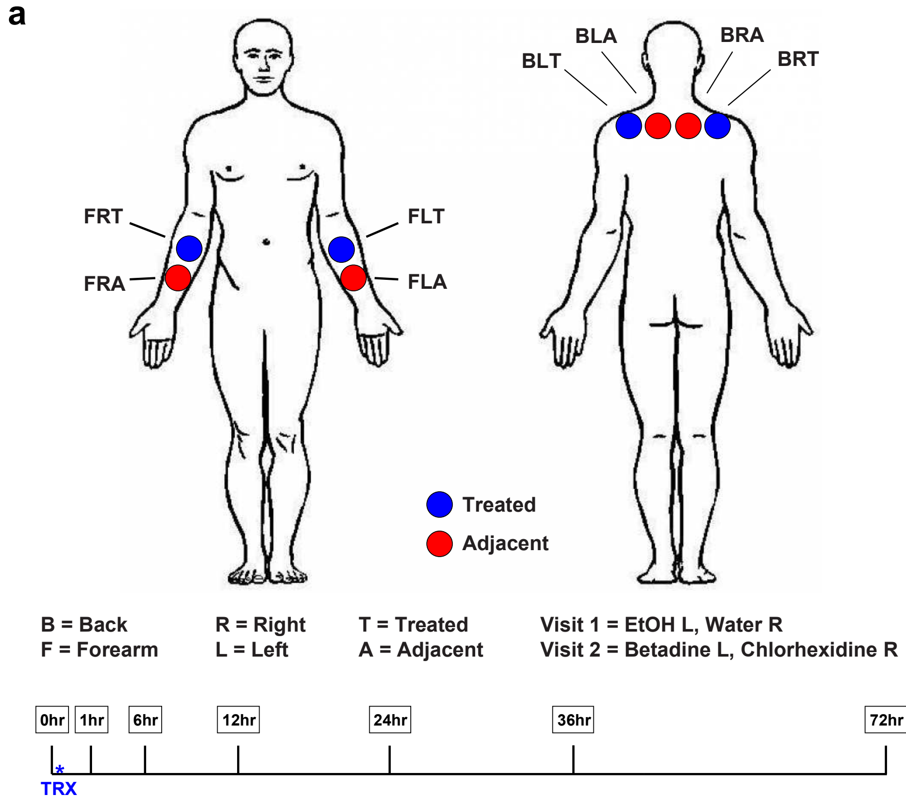
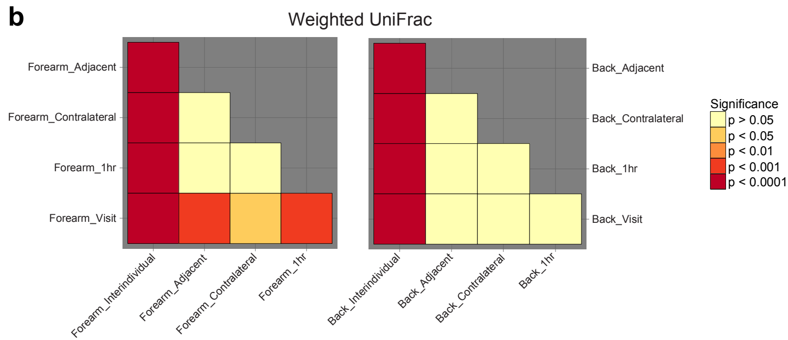
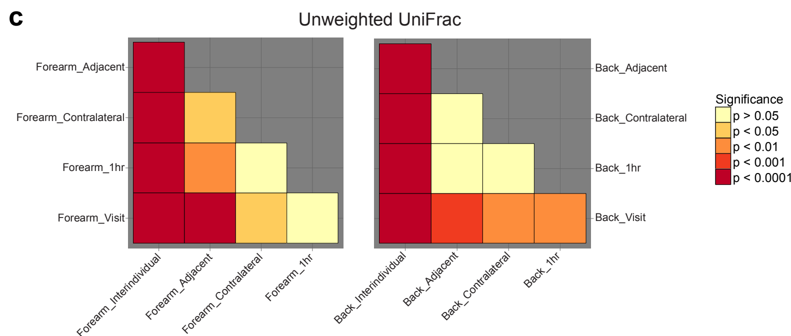
4.10 Supplemental figures







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Supplemental Figure 1 Treatment regimen details and baseline community comparisons. (a) Diagram of sampling and treatment schedule for antiseptic study cohort. (b, c) Heat map of significances for weighted (b) and unweighted (c) UniFrac comparisons at the forearm and back for interpersonal, adjacent, contralateral, short-term (1hr), and long-term (visit) baseline community samplings.

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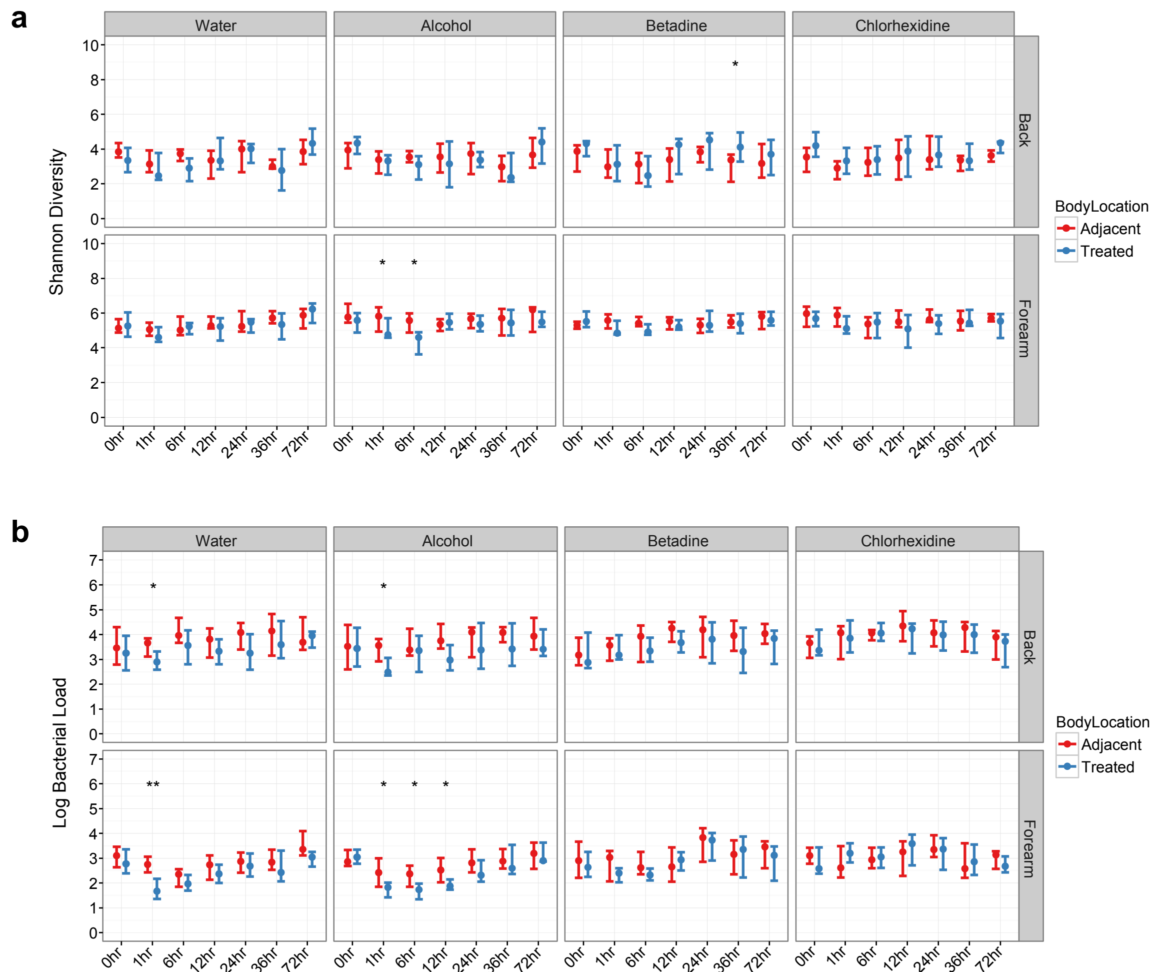

Supplemental Figure 2 Alpha diversity and bacterial load are decreased in response to certain treatment regimens. (a) Longitudinal comparisons of Shannon diversity for bacterial communities at adjacent and treated body sites of the back and forearm. (b) Bacterial load at the forearm and back for treated and adjacent body sites over time. Data is presented by median points and interquartile regions. * P < 0.05, ** P < 0.01 by Wilcoxon rank sum test (Mann-Whitney U test).

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a

Back
Forearm
Water
Alcohol
Betadine
Chlorhexidine
Observed Species
0hr 1hr 6hr 12hr 24hr 36hr 72hr
BodyLocation
Adjacent
Treated

b

Water
Alcohol
Betadine
Median OTU count difference
Median Difference
Acetobacteraceae Actinomycetaceae Aerococcaceae Brevibacteriaceae Carnobacteriaceae Caulobacteraceae Comamonadaceae Corynebacteriaceae Dermabacteraceae Flavobacteriaceae Incertae Sedis XI Lactobacillaceae Methylobacteriaceae Microbacteriaceae Micrococcaceae Moraxellaceae Mycobacteriaceae Neisseriaceae Oxalobacteraceae Prevotellaceae Propionibacteriaceae Rhizobiaceae Rhodobacteraceae Staphylococcaceae Streptococcaceae

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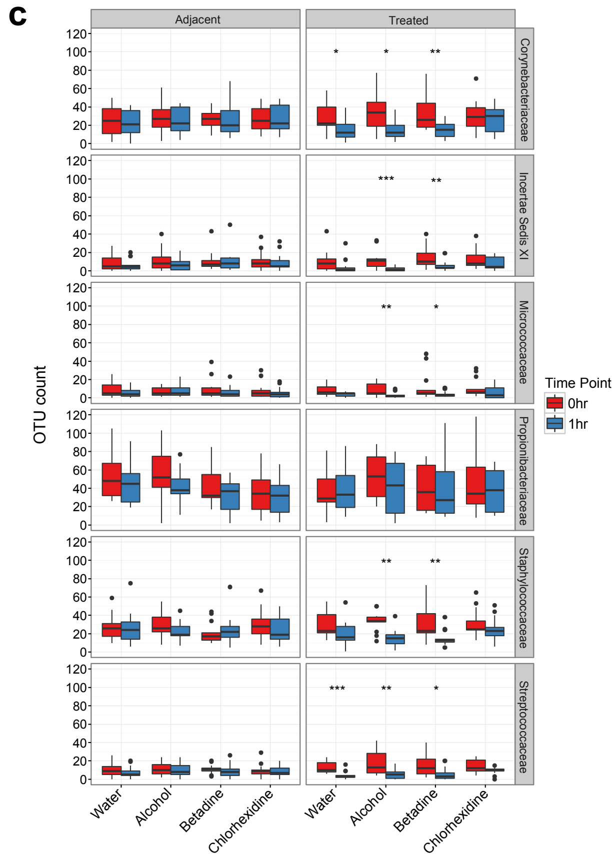

Supplemental Figure 3 Treatment elicits decreases in bacterial richness. (a) Longitudinal measurements of observed species for adjacent and treated body sites at the back and forearm. Data is presented by median points and interquartile regions. (b) Difference between OTU counts for the top 25 families at the back for baseline and 1hr post-treatment samples in response to water, alcohol, and Betadine treatment. Points represent the median of participants and are colored by the scaled difference in total count. Error bars designate interquartile regions. (c) Box and whisker plots of OTU richness at the back for major taxa at adjacent and treated body sites between baseline and 1hr time points. * P < 0.05, ** P < 0.01, *** P < 0.001 by Wilcoxon rank sum test (Mann-Whitney U test).

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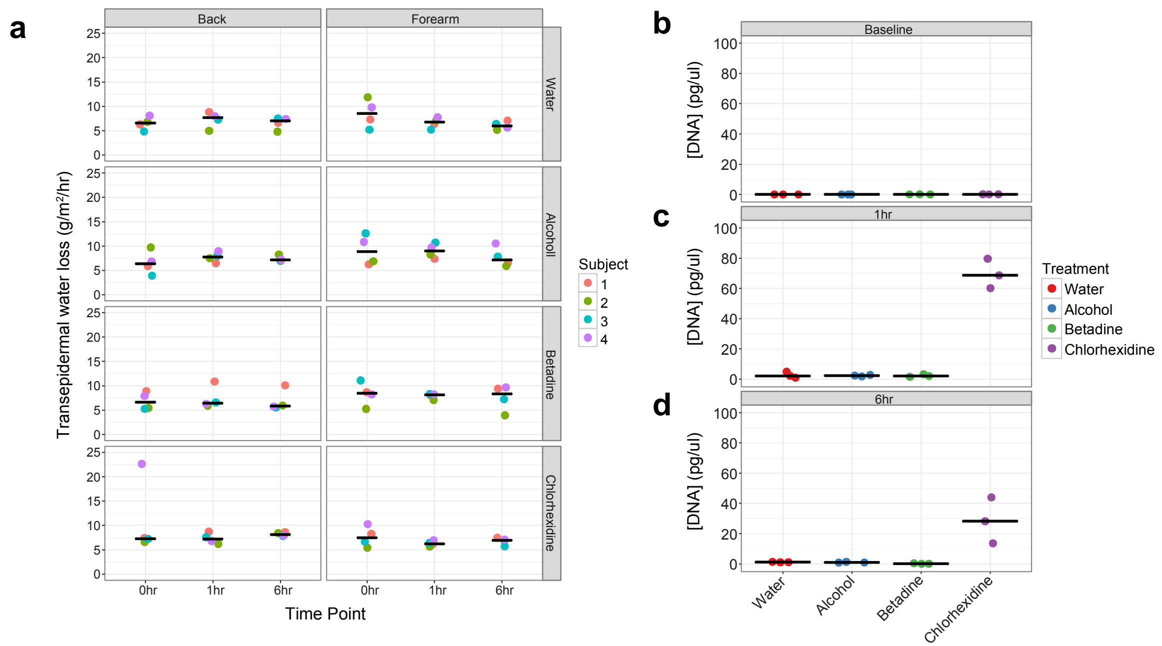

Supplemental Figure 4 Effect of chlorhexidine on skin integrity and bacterial DNA retention. (a) Transepidermal water loss (TEWL) of subjects at the back and forearm in response to treatment with water, alcohol, betadine, and chlorhexidine. Each point represents an individual subject. Black bars denote median. (b-d) Concentration of marker bacterial DNA at (b) baseline, (c) 1hr, and (c) 6hr post-treatment. Each point represents an individual mouse. Black bars denote median. Baseline refers to background concentrations of marker DNA prior to testing.

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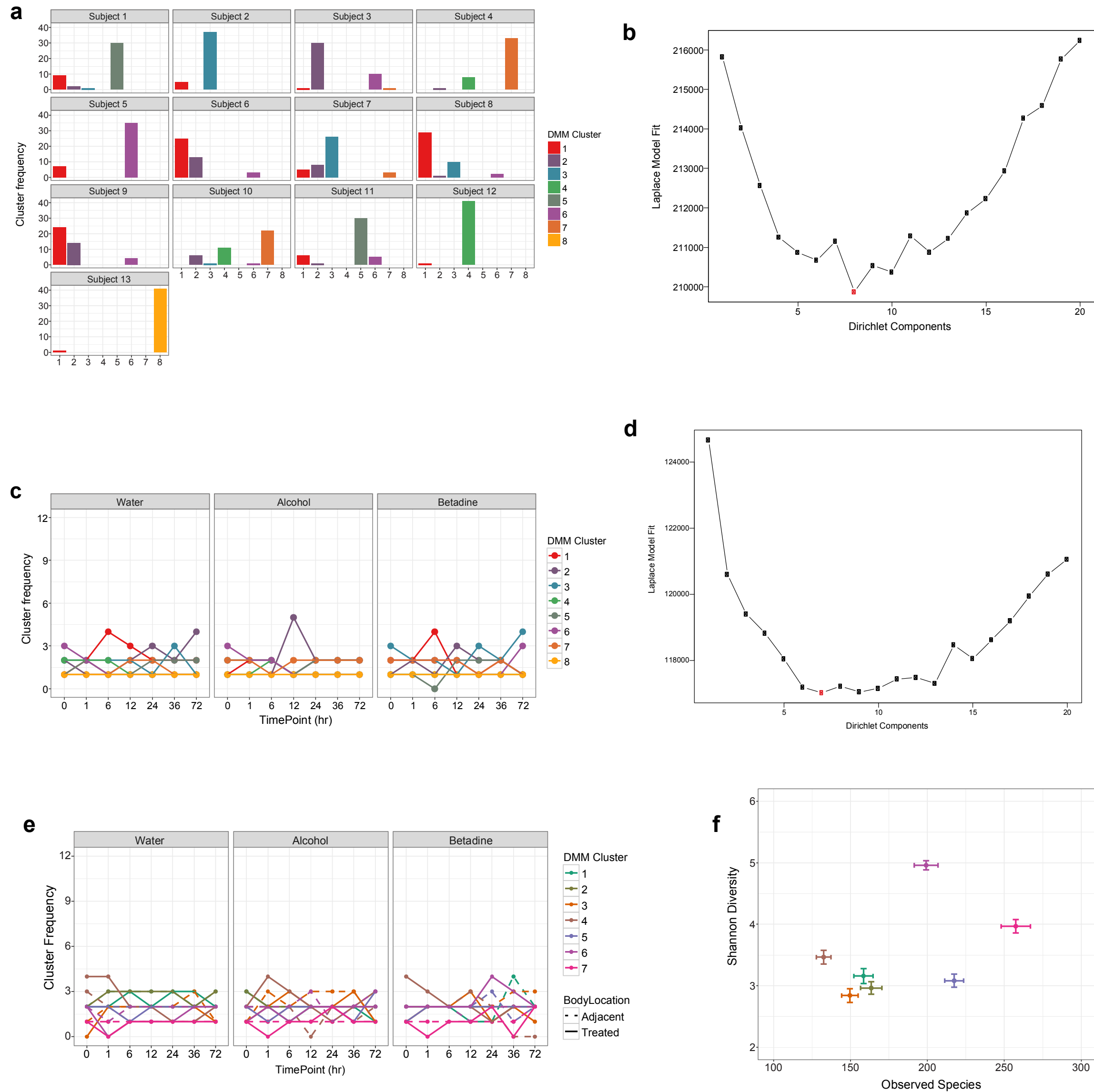

Supplemental Figure 5 Dirichlet multinomial mixture (DMM) model analysis for bacterial communities at the forearm and back. (a) Frequency of forearm DMM clusters by subject. (b) Laplace approximations for Dirichlet components of forearm communities. Global minimum is represented with a red point. (c) Frequencies of forearm DMM clusters at adjacent body sites over time. (d) Laplace approximations for Dirichlet components of back communities. (e) Longitudinal frequencies of DMM

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clusters at the back for adjacent and treated body sites. (f) Shannon diversity and observed species counts of individual DMM clusters at the back. Data are presented as mean ± s.e.m.

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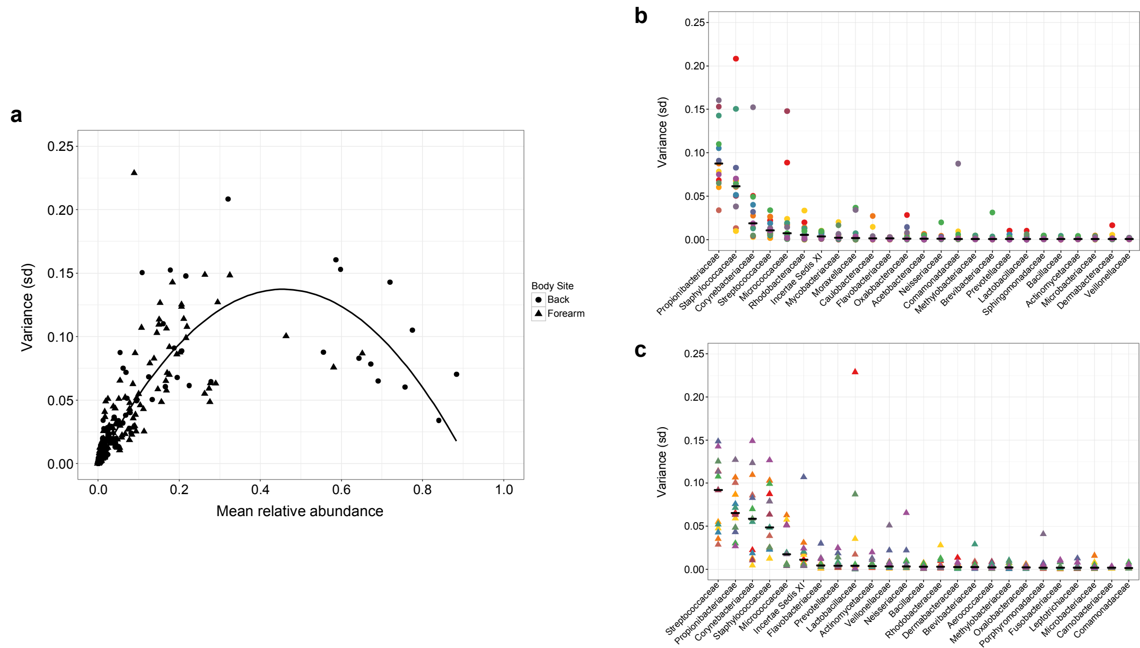

Supplemental Figure 6 Variance of bacterial taxa at baseline. (a) Relationship between the mean relative abundances of bacterial families at baseline and their variance as measured by standard deviation. Each point represents a different bacterial family in an individual subject, shaped by body site. Data was fitted with a second-order curve to approximate taxonomic distributions. (b, c) Baseline variance of top 25 bacterial families at the back (b) and forearm (c). Points are colored by subject, and shaped by body site. Black bars represent median variance.

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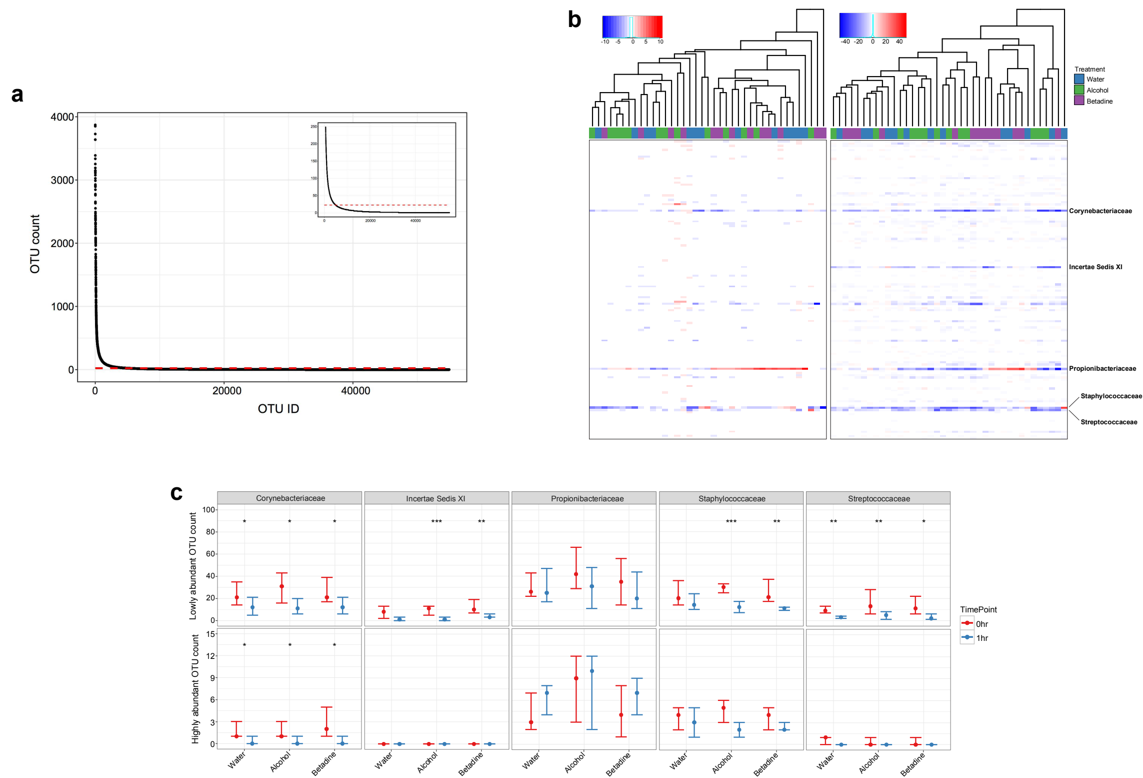

Supplemental Figure 7 Contribution of abundance to treatment-derived alterations in bacterial membership. (a) Sorted OTU abundances of all bacterial members in study cohort. Dashed red line represents 0.5% abundance threshold used to separate highly and lowly abundant OTUs. (b) Heat map of differences in bacterial family membership at the back between baseline and 1hr in subjects following treatment with water, alcohol, and Betadine. Each column represents the sample of an individual subject, and each row represents a bacterial family. Samples are clustered by the Unweighted Pair Group Method with Arithmetic means (UPGMA). Color-coded bars above the graph designate treatments for each sample. (c) Comparison of lowly and highly abundant OTU counts at the back in major taxonomic families at baseline and 1hr post-treatment. Data is presented by median

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points and interquartile regions. * P < 0.05, ** P < 0.01, *** P < 0.001 by Wilcoxon rank sum test (Mann-Whitney U test).

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Chapter 5 - Conclusions and future directions

5.1 Conclusions and future directions

The results presented herein outline the impact of topical antimicrobial treatments on skin bacterial inhabitance. In our experience, a number of conflicting opinions exist in regard to skin bacterial dynamics. For example, it is not uncommon to question a group of researchers and find that some believe the skin microbiota exists as a stable resident community, while others assert its vulnerability to even minor perturbations. As is often the case in scientific endeavors, it appears as though the truth is likely complicated by circumstance, with neither view representing an adequate means to describe the intricacies of our cutaneous cohabitants.

In support of this concept, we found antiseptics to elicit varying effects in skin bacterial residence. When assessing the impact of treatment on human subjects, we observed a reproducible decrease in lowly abundant bacteria. By contrast, highly abundant residents remained relatively stable over time. As highly abundant residents comprised the majority of cutaneous populations, community structure was largely preserved in response to treatment. However, changes to lowly abundant residents did represent the opportunity to identify personalized alterations in residence, shifts which were largely dependent on baseline populations. This result was also seen in murine studies in which housing conditions could influence baseline *Staphylococcus* levels, and the subsequent

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magnitude of antimicrobial response. We did not observe a significant reduction in lowly abundant residents in these particular experiments, however, an effect we hypothesize may be due to mice being housed in clean environments with reduced exposure to transient bacteria.

The ability of highly abundant residents to resist acute antiseptic stress is perhaps not altogether surprising. Indeed, when considering the dynamics of a community, it appears most likely that highly abundant taxa have attained this status by more than mere coincidence. Our results suggest that highly abundant bacteria represent a group of inhabitants uniquely adapted to an individual, while lowly abundant bacteria are more akin to transient passengers with a reduced capacity for persistence. If true, acute treatment-derived alterations may enable researchers to better interrogate host-microbial relationships, as they represent an opportunity to distinguish between long and short-term inhabitance.

We find this proposition particularly attractive given the observation that certain taxonomic groups were more easily disrupted than others in our studies, specifically lowly abundant residents of top bacterial families. This points to the potential of the skin to select for specific classes of bacteria, while only the most well-adapted of each group may colonize stably over time. Indeed, without the competitive advantages of highly abundant taxa, lowly abundant residents are likely ill-equipped to survive most perturbations, resulting in their decreased residence in the post-treatment setting.

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With this in mind, our investigations educe two important questions. First, why some taxonomic families are better equipped than others to withstand acute stressors? And second, why certain members of these families are more capable of long-term colonization than others? The answer to each question is likely the result of both host- and microbial-derived features, with each contributing an important role in skin bacterial residence.

We present *Propionibacterium* as a particularly salient example in this regard. As a lipophilic member of the skin microbiota, these residents thrive on the oily secretions produced by human sebaceous glands[1]. This, paired with their anaerobic preferences, results in a distinct localization of *Propionibacterium* to cutaneous structures with low levels of oxygen and high sebaceous gland activity[2]. During our experiments, both highly and lowly abundant members of this genus were capable of resisting treatment-derived alterations, leading to the possibility that *Propionibacterium* may be uniquely adapted to resist antibacterial perturbation. However, we find it equally likely that simply shielding themselves within cutaneous appendages could also contribute to this resident's overall stability.

With this in mind, we further note that *Propionibacterium acnes* strains have shown a wide array of stratification throughout the human population[3]. Traditionally thought of as the causative agent in acne vulgaris, certain strains of *P. acnes* are considered to be more pathogenic than others[4]. Certain individuals are colonized by these strains more readily than others as well[5]. This suggests that hosts play an

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important role in the selection of their *P. acnes* strains, with each clone exhibiting unique competitive advantages over non-resident strains. Despite myriad treatments to combat this bacterium and reduce the overall number of *P. acnes* inhabitants, acne vulgaris remains a prevalent disorder in the developed world[6]. Based on our experiments, we would hypothesize that *P. acnes* clones may persist due to a unique ability to withstand acute interventions, and that certain treatments may even exacerbate this condition if they present resilient *P. acnes* strains with newly accessible niches.

Similar dynamics can also be seen with the skin pathogen *Staphylococcus aureus*. As the most prominent cause of skin and soft tissue infections, *S. aureus* can establish long-term residence in a subset of individuals[7,8]. Host-specific strains can then cause a number of complications during surgery or other unplanned breaches of the skin[9]. To combat this effect, decolonization strategies have been advanced as a means to eliminate inhabitant *S. aureus*[10]. However, many of these studies have illustrated no significant reductions in overall infection[11]. We believe this phenomenon may occur as the result of *S. aureus* colonization's dual nature in a host, with the ability to both precipitate infection while simultaneously protecting against it depending upon the circumstance.

To support this hypothesis, we note that while decolonization can reduce *S. aureus* inhabitance, subsequent surgical infections can be accompanied by more severe complications[12]. This happens as the result of infection by non-resident *S. aureus*, a

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result we propose may occur because of a loss in colonization resistance. Generally, bacterial infections within a hospital setting are caused by microorganisms with greater levels of resistance and increased virulence[13,14]. As such, the removal of colonizing *S. aureus* acquired in the community-setting could explain infection by these more harmful, hospital-associated pathogens.

This principle has been well-established in the gastrointestinal tract. Here, *Clostridium difficile*, vancomycin-resistant *Enterococcus*, and *Salmonella enterica* are all capable of causing disease with increased frequency following antibiotic intervention[15]. Moreover, in the case of *C. difficile*, the administration of fecal material with potentially protective bacteria can lead to a resolution of disease[16]. These results indicate that antimicrobial treatment in the gut can reduce resident competition in a host, and increase susceptibility to infection and disease.

To test the importance of this mechanism at the skin surface, we treated mice with antimicrobial drugs and assessed their impact on the skin microbiota. We observed a number of alterations in skin resident populations, including a conserved decrease in *Staphylococcus* membership regardless of treatment. As these experiments included the culture of pre- and post-treatment communities, we were able to identify the specific *Staphylococcus* residents disrupted by treatment. We could then pre-colonize mice with these same residents to evaluate their potential to compete with *S. aureus* at the skin surface. As hypothesized, each resident was found to

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reduce association with *S. aureus*, underscoring the potential for the skin microbiota to exhibit colonization resistance features.

While others have illustrated a similar ability of skin residents to compete with *S. aureus*[17,18], our study represents the first to establish the role of antimicrobial drugs in this process. These results support the notion that a reduction in resident *S. aureus* may impact colonization resistance and infection in human patients as well. Further research is necessary to more completely evaluate this hypothesis, however. This includes an investigation of additional factors that can influence *S. aureus* residence, as well as studies outlining the importance of the human microbiota in this process.

Because our studies only assess the impact of antimicrobial drugs on healthy skin, in the absence of infection or wounding, additional research should also examine the influence of these variables as they pertain to colonization resistance. The utility of antibiotics and antiseptics to improve patient outcomes has been expounded at-length in these particular environments. However, little information exists in regard to the susceptibility of patients to infection in the post-treatment setting. Our work suggests that a window of susceptibility may exist during this time due to an inability of communities to re-stabilize for multiple weeks post-treatment. If this period includes impaired recolonization by protective skin residents, the potential exists that antimicrobial treatments could promote infection by new pathogens as well as recurrent strains.

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Fortunately, the skin represents a body site with unique advantages to study these principles, namely an ability to apply competing residents directly to a site of interest. While less harmful bacteria can be taken orally to compete against pathogenic inhabitants of the gut, there is no guarantee that these bacteria will be able to access a particular niche. Indeed, many experiments have outlined the difficulties encountered by exogenously administered bacteria when attempting to establish long-term colonization of the gut[19-21]. As colonization is the first step in the majority of interference pathways, a deficiency at this stage understandably complicates the study of bacterial competition.

Unlike the gut, skin represents an easy to access biological surface for colonization resistance studies. Indeed, one can apply varying concentrations of bacteria directly to the skin surface, and shift the equilibrium amongst any number of bacterial inhabitants. We provide one such example of these advantages in our own experiments. However, one could imagine the utility of these approaches when testing additional skin residents and pathogens for competition and colonization potential. We suggest that future experiments employ these methods to further establish the mechanisms behind our particular findings, and extend these observations to additional models.

Finally, we note that antimicrobial treatments varied in their ability to alter skin bacterial residence. As previously mentioned, the antiseptics alcohol and povidone-iodine elicited relatively minor changes to bacterial population structure. Antibiotic

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treatment, by contrast, resulted in disruptions that were both immediate and durable, with triple antibiotic ointment (TAO) shifting communities for multiple weeks post-treatment. TAO treatment also led to the outgrowth of previously minor contributors, underscoring the potential for alternative community states to predominate following antibiotic treatment.

These results represent a number of opportunities for future investigation, with a subset mentioned herein. First, we recognize that antibiotic effects were largely agent-dependent, with TAO eliciting a more significant response than Mupirocin. This suggests that broad spectrum antibiotics have a greater impact on skin bacterial communities than those which target specific bacterial taxa. While this is to be expected, subsequent work should also explore the ramifications of narrow spectrum antibiotics that target more abundant skin residents. Indeed, the bacterial genera most disrupted by Mupirocin, *Staphylococcus* and *Streptococcus*, were found at relatively low levels in appropriately housed mice. As such, their removal would not necessarily be expected to result in large shifts to overall community structure.

Second, we note that our antibiotic studies were performed only in murine hosts. Consequently, the impact of antibiotics on human skin residents remains largely unknown. If these drugs are capable of disrupting the human skin microbiota in a similar manner to mice, this could have a number of important implications for host cutaneous biology. Studies at alternate body sites have previously expounded the importance of bacterial residents to immune function and development[22]. Future

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investigations should thus employ measurements of host gene expression and immune cell populations, in order to more completely characterize the consequences of skin bacterial perturbation.

Third, because our experiments focused on topical antimicrobial treatments, the impact of oral antibiotics remains largely unknown. While oral antibiotics have the potential to disrupt both gut and skin communities, no study to date has rigorously explored the interplay between these systems. This would be especially compelling in the case of antibiotics available in both oral and topical formulations. Indeed, the potential exists that different routes of administration could have important implications for skin and gut bacterial inhabitance alike.

As a final note, we recognize that our studies were performed using 16S rRNA gene sequencing for bacterial identification. While this was necessary to maximize the breadth of our comparisons, our studies have now outlined the optimal parameters for these types of analyses. Future work can thus build upon these findings by employing whole metagenome shotgun sequencing as a means to evaluate the effects of antimicrobial drugs on the functional potential of skin communities, and with strain-level resolution. These investigations will also be useful in determining the impact of antibiotics and antiseptics on non-bacterial residents. Indeed, while we have largely framed the question of antimicrobial effects as a “simple” matter of bacterial dynamics, fungal and viral populations are undoubtedly affected in kind.

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In all, our studies outline the potential for antibiotics and antiseptics to alter skin bacterial residence. However, future research will be essential to a more complete understanding of this response. Our data underscore the potential for said endeavors to inform unique mechanisms of cutaneous health and disease, and it is our hope that studies such as these will result in a more prudent approach to antimicrobial use in clinical settings and beyond.

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