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

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Running Head: Topical antimicrobial drugs and the skin microbiome

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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* 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.

47 **Introduction**

48 Antimicrobial drugs are commonly employed to inhibit the growth of pathogenic
49 microorganisms. However, these interventions are rarely narrow in spectrum, instead
50 acting on a range of bacterial species in our commensal microbiota (1). A number of studies
51 have elucidated this effect in gut microbial populations, describing a dramatic
52 reorganization of resident communities (2). This includes decreased bacterial diversity,
53 and outgrowth by previously minor contributors (3-5). Importantly, these alterations can
54 persist for months to years post-treatment (6-8), and also affect a number of host functions
55 including metabolism, immunity, and transcriptional regulation (9, 10).
56
57 Despite these findings, few studies have assessed the impact of antimicrobial drugs at
58 alternative body sites such as the skin. Rather the majority of research at this site has been
59 devoted to a subset of easily cultured microorganisms studied in isolation (11). This
60 includes minimum inhibitory concentration tests of pathogenic skin bacteria, as well as
61 exogenous colonization studies in which non-resident, test microorganisms are applied to
62 the skin prior to treatment (12). While these results are often applied more broadly, their
63 main purpose is to inform the effect of antimicrobial drugs on transient, infectious bacteria,
64 rather than more stable members of the community (13). As such, few studies have truly
65 assessed the impact of antimicrobial drugs on inhabitant cutaneous populations. This
66 dearth of research is especially notable given the frequency with which humans disrupt
67 skin bacterial communities in both clinical and non-clinical settings. Indeed the intent of
68 most antiseptics is to sterilize the skin by employing agents with non-specific mechanisms
69 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 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

93 in detail. Indeed, the long-term impact of topical antimicrobial drugs on skin bacterial
94 communities, and their ability to alter colonization patterns by *S. aureus* competitors,
95 remains largely unknown.
96
97 Here we report this missing link by assessing the effect of antibiotics and antiseptics on the
98 resident skin microbiota through a comparative time-series analysis. We report a
99 differential impact of treatment on skin bacterial inhabitants, with the greatest
100 disturbances elicited by a broad-spectrum triple antibiotic cocktail of bacitracin, neomycin,
101 and polymyxin B. By contrast, we report a relatively muted effect of antiseptics, with only
102 modest alterations to overall bacterial community structure. Despite these differences, we
103 identified a conserved decrease in the levels of *Staphylococcus* residents regardless of
104 treatment, a result that was strongly influenced by baseline levels of *Staphylococcus*.
105
106 Because commensal *Staphylococcus* spp. have been shown to impair colonization by the
107 skin pathogen *Staphylococcus aureus*, we further evaluated this antimicrobial effect in the
108 context of *S. aureus* colonization resistance. We show that treatment can promote
109 exogenous association with *S. aureus*, and that the same *Staphylococcus* residents disrupted
110 by treatment are also capable of *S. aureus* competition, decreasing *S. aureus* levels by over
111 100-fold in precolonization experiments. In all, our results demonstrate that antimicrobial
112 drugs can elicit long-term shifts in skin bacterial communities, and that treatment with
113 these agents has key implications for host susceptibility to pathogens such as *S. aureus*.
114
115

116 **Results**

117 **Topical antibiotic treatment alters skin bacterial residents**

118 To assess the impact of topical antibiotics on the skin microbiota, we began by treating the
119 dorsal skin of SKH-1 hairless mice twice daily for one week with the narrow spectrum
120 antibiotic mupirocin; a broad spectrum triple antibiotic ointment (TAO: bacitracin,
121 neomycin, polymyxin B); or their respective vehicles, polyethylene glycol (PEG) and
122 petrolatum (Fig. S1a). These particular antibiotics were chosen for their range of activities,
123 as well as their extensive use as both therapeutic and prophylactic agents in both clinical
124 and non-clinical settings (30). In all, antibiotics led to durable changes in skin bacterial
125 residents, with populations forming three distinct clusters (I – III) and four sub-clusters
126 ($III_{A\text{-}D}$) (Fig. 1a). Interestingly, Clusters I and III_A were composed largely of baseline and
127 early time point samples high in *Staphylococcus*, while treatment with antibiotics led to
128 sustained decreases in *Staphylococcus* (Fig. S1b) and alternative clustering patterns.
129 Cluster II, by contrast, was composed almost entirely of TAO-treated mice, a group that
130 exhibited significant increases in Enterobacteriaceae, Porphyromadaceae, and
131 Ruminococcaceae, as well as significant decreases in Lachnospiracea*e* and certain taxa
132 classified more generally within the Clostridiales family (Fig. 1b-d). This distinction led to a
133 marked absence of TAO-treated mice from Clusters $III_{B\text{-}D}$, and, similar to *Staphylococcus*,
134 was sustained for multiple weeks post-treatment.
135
136 Unlike TAO-treated mice, those administered mupirocin displayed community shifts
137 largely in line with those treated with the vehicle PEG. Indeed while these mice exhibited
138 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, including immediate increases in rarified abundance 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 as well. While mupirocin led to the characteristic decreases often associated with antibiotic treatment, TAO treatment resulted in increases in bacterial load at numerous time points as measured by 16S rRNA gene qPCR (Fig. S1d). These findings further highlight the impact of antibiotic treatment on skin communities, and suggest that the changes elicited by TAO may also be due to increases in the overall numbers of certain bacteria, and not just their relative proportions.

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).

Previously, others have shown similarities in the functional composition of a population despite differences in community membership and structure (31). To evaluate whether antibiotic treatment could lead to changes in the functional potential of skin inhabitants, we also utilized the PICRUSt software package (32) 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 predicted to be 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.

Antimicrobial Agents and Chemotherapy

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 antisepsis when taking into account major taxonomic groups at even the earliest d1 post-treatment time point (Fig 3a). Furthermore, when comparing the relative abundances of individual taxa following treatment, 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 at d1 post-treatment (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), further underscoring the stability of cutaneous bacterial communities in response to antiseptic treatment.

As this result was particularly surprising, we also 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 antiseptics elicit a more muted response in skin bacterial populations, but that their effects may be dependent upon starting communities.

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.

229 Because our antiseptic experiments exhibited an antibacterial effect dependent upon
230 baseline communities, we began by designing a system to control *Staphylococcus* levels in
231 murine populations. Specifically, we observed that mice housed in cages changed once per
232 week displayed significant elevation in *Staphylococcus* levels (high *Staphylococcus*; HS)
233 compared to those changed more frequently (low *Staphylococcus*; LS) (Fig 4a). When
234 controlled over time, this effect could be maintained for multiple weeks and had the
235 potential for reversibility, as mice swapped from frequent to infrequent cage changes
236 rapidly converted to the alternate phenotype. Cage change frequency and monitoring thus
237 presented the opportunity to maintain *Staphylococcus* at distinct levels prior to treatment.
238
239 To evaluate the impact of antimicrobial drugs on culturable *Staphylococcus*, we began by
240 housing mice in cages with frequent or infrequent changes, and then treating with PEG,
241 mupirocin, petrolatum, or TAO. Similar to sequencing experiments, antibiotic treatment led
242 to a significant decrease in *Staphylococcus* starting at d1 post-treatment regardless of
243 starting community, although this effect was more pronounced in LS mice (Fig. 4b,c).
244 Interestingly, while we also observed a gradual decrease of *Staphylococcus* in response to
245 PEG treatment, petrolatum-treated LS mice displayed increased *Staphyloccocus*
246 colonization at early time points, and elevated levels of *Staphylococcus* compared to
247 untreated controls in HS mice. Because our sequencing results revealed similar decreases
248 in *Staphylococcus* in response to treatment with antibiotics, but not petrolatum, this
249 represents a reproducible mechanism in multiple testing protocols.
250

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 at d1 post-treatment (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 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.

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).

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, then 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) (33), 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. 5c). By contrast, AS9 *S. epidermidis*

297 appeared to replicate the slowest and exhibited the most gradual exponential curve. AS11
298 *S. xylosus* and AS12 *S. nepalensis* both displayed intermediate growth patterns. To further
299 evaluate colonization potential, we assessed the ability of these isolates to colonize murine
300 dorsa *in vivo*. Specifically, mice were housed in frequently changed cages to reduce
301 endogenous *Staphylococcus*, and then epicutaneously inoculated every other day for 1
302 week to promote association with individual *Staphylococcus* isolates. Despite variable
303 growth dynamics *in vitro*, all isolates colonized mice to an equal titer *in vivo*, suggesting
304 conserved, undefined factors to promote colonization at the skin surface (Fig. 5d).
305
306 As each of these isolates displayed notable colonization when added to murine hosts, we
307 further tested all five to see whether they could also represent potential *S. aureus*
308 competitors. To evaluate the ability of each isolate to restrict *S. aureus* colonization, we
309 precolonized mice with each *Staphylococcus* resident, similar to above, and then challenged
310 with *S. aureus* one day later. While isolates exhibited varying levels of competition, all
311 resulted in significant decreases to *S. aureus* association compared to uncolonized mice
312 (Fig. 5e). Indeed most mice exhibited greater than 10-fold reductions in *S. aureus*, and
313 many, including those precolonized with *S. epidermidis*, were capable of decreasing *S.*
314 *aureus* by levels greater than 100-fold. In all, this shows that skin bacterial residents can
315 compete with *S. aureus* at the skin surface, and that their removal can impact *S. aureus*
316 colonization potential.
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320

321 **Discussion**

322 Given the expansive use of topical antibiotics and antiseptics, it is somewhat surprising that
323 longitudinal studies to evaluate their effects on a community-wide scale are not more
324 common. Here we report that antimicrobial drugs can elicit significant changes to skin
325 bacterial community membership and structure, albeit to varying degrees. We also
326 demonstrate that these alterations can have important consequences for colonization
327 resistance and the skin pathogen *Staphylococcus aureus*.

328

329 Previous work has focused extensively on antibiotics and the gut microbiota. These studies
330 have highlighted the ability of antimicrobials to disrupt bacterial communities and the
331 consequences of these drugs on host physiology (34). One such example includes the
332 elimination of colonization resistance leading to increased susceptibility to bacterial
333 infections (35). By altering the structure of bacterial populations in the gut, antibiotics can
334 shift the balance in favor of more infectious microorganisms (19). *Clostridium difficile* is
335 perhaps the best-studied representation of this effect (36). However, additional pathogens
336 such as vancomycin-resistant *Enterococcus* and *Salmonella enterica* can also exploit newly
337 available niches and cause disease (37, 38). As a result, the true question has transcended
338 beyond whether or not antimicrobial drugs can promote pathogenicity, to how best to
339 mediate these unintended consequences.

340

341 The first step in such ventures is the elucidation of antimicrobial effects on a community-
342 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 enhanced, following treatment cessation. This indicates that disrupted resident skin bacteria may also undergo multiple levels of succession prior to community stabilization, similar to the gut (39).

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 (40, 41). This could also explain the increase in overall bacterial load seen in mice following TAO administration, as certain bacteria may thrive when given access to a newly available cutaneous niche.

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

366 ability to detect differences in TAO-treated mice within one day of treatment provides
367 strong evidence to the contrary. Others have also reported a similar community response
368 to both decolonization protocols (42) and mild and antibacterial soaps (43), further
369 validating this assertion.
370
371 Rapid repopulation of the skin could also explain our perceived lack of effect in response to
372 antiseptic stress. However, as our study and those before us employed relatively early post-
373 treatment samplings, we find it unlikely that residents could re-colonize the skin in such a
374 short period of time. Indeed, many of the bacteria observed in our experiments have been
375 shown to exhibit particularly slow growth dynamics in previous examinations (44, 45).
376 Repopulation is likely shaped by both the magnitude of change and the environment,
377 however. As such, future work will be necessary to establish a more complete
378 understanding of this process as it relates to skin bacterial dynamics.
379
380 With this in mind, it is important to note that multiple studies have shown a reduction of
381 certain culturable skin inhabitants in response to antisepsis. This includes residents from
382 the commonly studied genus *Staphylococcus*, often chosen for its ease of use in culture-
383 based experiments (46, 47). In line with these findings, we also observed a decrease in
384 *Staphylococcus* residents in our sequencing and culture studies. However, we note that
385 because this bacterium was only one member of the larger community, this decline did not
386 lead to shifts in overall population structure. As such, we hypothesize that the true utility of
387 antiseptics may lie in their ability to disrupt a particular subset of microorganisms at the
388 skin surface, while leaving the underlying community relatively unchanged.

389

390 Interestingly, *Staphylococcus* residents also exhibited distinct baseline-dependent
391 dynamics in response to antiseptic treatment during our sequencing experiments.
392 Specifically, we observed that mice with high levels of *Staphylococcus* responded more
393 readily to treatment than mice with low levels of colonization. This suggested a nuanced
394 impact of antiseptics on certain bacterial inhabitants, whereby treatment effects could vary
395 depending upon starting communities. To verify this hypothesis, we developed a system in
396 which *Staphylococcus* could be tested for antimicrobial susceptibility at both high and low
397 colonization levels. As anticipated, we found the efficacy of antiseptics to be highly
398 dependent upon baseline communities. Mice with low levels of *Staphylococcus* at baseline
399 (LS) exhibited little to no decline in *Staphylococcus*, while mice with high levels (HS) were
400 reduced by approximately 100-fold. Importantly, we observed a similar effect in control HS
401 mice, suggesting that higher levels of *Staphylococcus* are less stable in general, and thus
402 represent atypical colonization. By contrast, the inability of antiseptics to reduce
403 *Staphylococcus* in LS mice indicates a relative stability in this community, and a population
404 capable of resisting the short-term stressors of antisepsis. We believe these studies have
405 important implications for antimicrobial efficacy, particularly in the case of human skin, as
406 humans are likely exposed to a greater number of transient microorganisms compared to
407 laboratory mice housed in more controlled environments (48).
408
409 When comparing antibiotic and antiseptic treatments, we observed that a standard course
410 of antibiotics was more capable of community disruption than that of acute antisepsis.
411 While these are the most commonly employed regimens in the clinic, further research

412 should also evaluate the effects of long-term antiseptic treatments on the skin microbiota
413 as well as other delivery mechanisms. Indeed the potential exists that consistent exposure
414 to antiseptics through alternative means may have a more significant impact on skin
415 inhabitants due to increased contact time or bioavailability. This is especially important
416 when considering the rise of decolonization practices in the clinic, a procedure employing
417 multiday, prophylactic antibiotic and antiseptic treatments to remove resident
418 *Staphylococcus* species (49, 50). While these methods efficiently remove endogenous *S.*
419 *aureus* from the nares and extranasal body sites, they likely alter the underlying skin
420 microbiota in kind. Without proper re-colonization, these interventions could feasibly elicit
421 long-term shifts to the skin microbiota, similar to our experiments, and promote infection
422 by more dangerous hospital- and community-acquired pathogens (51-53).
423
424 To assess this very possibility, we investigated the potential of treatment to promote *S.*
425 *aureus* colonization at the skin surface in our mouse model. In response to treatment, we
426 observed a significant increase in *S. aureus* levels compared to untreated controls following
427 exogenous association, suggesting an increase in cutaneous permissivity. As previous
428 studies have illustrated the role of certain *Staphylococcus* spp. to compete with *S. aureus* for
429 colonization (27-29), we proceeded by testing the ability of murine *Staphylococcus* isolates
430 to compete with *S. aureus*. Specifically, we chose *Staphylococcus* residents that were
431 disrupted by antibiotic and antiseptic treatment in our previous experiments for further
432 analysis. This allowed us to determine whether these particular bacterial residents were
433 responsible for the decrease in colonization resistance, and to confirm the ability of
434 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 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.

458

459 **Materials and Methods**

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

469

470 **Antimicrobial treatment and sample collection.** For experiments involving antibiotics,
471 mice were treated every 12 hours for 7 days on the dorsum with mupirocin (2% in
472 polyethylene glycol), a triple antibiotic ointment (Bacitracin 400U, Neomycin 3.5mg,
473 Polymyxin B 5,000U in petrolatum), or their respective vehicles polyethylene glycol (PEG
474 400, PEG 3350) and petrolatum. Mice were swabbed longitudinally as described in Fig. S1a,
475 with collections occurring prior to morning applications during treatment to minimize
476 experimental disruptions. For experiments involving antiseptics, mice were treated on the
477 dorsum with UltraPure water (MoBio), alcohol (80% ethanol), or povidone-iodine
478 (Betadine, 10%) every eight hours, three times total. Mice were swabbed as described in
479 Fig. S3a, with d1 collections occurring 4hr after the final treatment. At least three cages of
480 three mice each were used for all conditions to evaluate caging effects. All treatments were

481 applied with sterile, UV-irradiated cotton swabs (CVS, Beauty 360), and samples were
482 collected with sterile foam tipped applicators (Puritan). A standard topical inoculum of
483 approximately 150ul per mouse was utilized for both antibiotic and antiseptic experiments.
484 All swabs were stored at −20 °C prior to extraction.
485
486 **Bacterial DNA isolation and 16S rRNA gene sequencing and qPCR.** Bacterial DNA was
487 extracted as described previously (54). Briefly, Ready-Lyse Lysozyme solution (Epicentre),
488 bead beating, and heat shock at 65 °C were used to lyse cells. The Invitrogen PureLink kit
489 was used for DNA extraction. During our testing, the V4 region of the 16S rRNA gene was
490 found to better approximate murine skin communities compared to V1V3. PCR and
491 sequencing of the V4 region was thus performed using 150-bp paired end chemistry and
492 the barcoded primers 515F: 5' GTGCCAGCMGCCGCGGTAA 3' and 806R: 5'
493 GGACTACHVGGGTWTCTAAT 3' (55) on the Illumina MiSeq platform. Accuprime High
494 Fidelity Taq polymerase was used for PCR cycling conditions: 94 °C for 3 min; followed by
495 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
496 min. For bacterial load comparisons, 16S rRNA genes were amplified by qPCR using Fast
497 SYBR Green Master Mix (Fisher Scientific) and the qPCR optimized primers 533F: 5'
498 GTGCCAGCAGCCGCGGTAA 3' and 902R: 5' GTCAATTCITTTGAGTTTYARYC 3'. Samples were
499 compared to standard curves generated from known concentrations of serially diluted
500 bacterial DNA to calculate burden.
501
502 **Microbiome analysis.** Raw sequences were assembled, demultiplexed, and trimmed to
503 yield 24,026,791 total high-quality V4 reads. Sequences were then further processed using

QIIME 1.7.0 prior to downstream analyses (56). Briefly, sequences were *de novo* clustered into OTUs based on 97% similarity by UClust (57), and taxonomy was assigned to the most abundant representative sequence per cluster using the RDP classifier (58). Sequences were aligned by PyNAST (59), and chimeric sequences were removed using ChimeraSlayer (60) 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 kit and environmental 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/sample corresponding to an average Good's coverage of 0.95/sample, 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 (61). 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 (32).

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 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.

527

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

535

536 ***Staphylococcus* isolation, sequencing, and phylogenetic tree.** To obtain a more
537 complete profile of our *Staphylococcus* isolates, phenotypically distinct *Staphylococcus*
538 colonies were picked from MSA plates following culture from murine dorsa prior to and
539 following antimicrobial treatment. DNA was extracted from colonies as described above,
540 and DNA was PCR-amplified using full-length 16S rRNA gene primers (27F, 1492R). The
541 primary PCR conditions used were 98 °C for 3 min; 35 cycles of 95 °C for 45 sec, 56 °C for
542 60 sec, 72 °C for 90 sec; and 72 °C for 10 min. Full-length 16S rRNA gene sequencing was
543 performed by Sanger sequencing, and resident *Staphylococcus* isolates were compared to
544 known *Staphylococcus* 16S rRNA genes downloaded from the RDP database (33).
545 Phylogenetic trees were generated by FastTree (62) and visualized in FigTree v1.4.3.

546

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

550

551 **Exogenous *Staphylococcus* colonization and *S. aureus* competition.** *Staphylococcus*
552 isolates were grown overnight in liquid Luria Broth (Fisher Scientific) at 37 °C and 300rpm.
553 On the following day, isolates were subcultured and incubated to achieve log growth, and
554 resuspended in PBS to acquire 10^8 CFU/ml inoculums. Titers were validated by culture and
555 optical density measurements at OD_{600}. Two cages of three mice each were housed in
556 frequently changed cages to reduce levels of endogenous *Staphylococcus*, and
557 monoassociated at the dorsum with 200ul of *Staphylococcus* isolate inoculum using a
558 sterile swab. Application of *Staphylococcus* suspensions were repeated every other day
559 over the course of one week for a total of four applications. Mice were then swabbed one
560 day following the fourth application, and cultured on MSA overnight at 37 °C for CFU
561 enumeration. *S. aureus* 502A with selective streptomycin resistance was chosen for *S.*
562 *aureus* competition studies because of its proven efficiency in skin colonization and
563 potential for pathogenicity (63, 64). *S. aureus* was grown similarly to *Staphylococcus*
564 isolates and applied one day post-treatment or one day post-monoassociation with
565 individual *Staphylococcus* isolates. Control mice were administered PBS only. Mice were
566 then swabbed the following day for *S. aureus*, and cultured on LB agar with streptomycin
567 for selective CFU enumeration.

568

569 **Accession numbers.** 16S rRNA sequence reads have been deposited in the NCBI Short
570 Read Archive under BioProject ID: PRJNA383404. Sequences of *Staphylococcus* isolates
571 have been deposited in GenBank under accession numbers MF286534-MF286538.

572

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770
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Competing interests. The authors declare no competing financial interests.

Figure Legends.

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 presented as individual mice (a) or mean ± s.e.m (b-d). 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.

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) Prinicipal coordinates analysis of weighted UniFrac distances for murine bacterial communities over time. Data are presented as mean ± s.e.m (a, b) or individual mice (c). 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.

Figure 3 | Antiseptic treatment does not significantly alter skin bacterial community structure.

(a) Heat map of rarified abundances for the 30 most common phylotypes on murine skin following treatment with water, alcohol, or povidone-iodine at d1 post-treatment. 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 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).

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 as individual mice with 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. Data are presented as individual mice at baseline and d1 post-treatment. 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.

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 resident *Staphylococcus* isolates at Optical Density 600 (OD600). (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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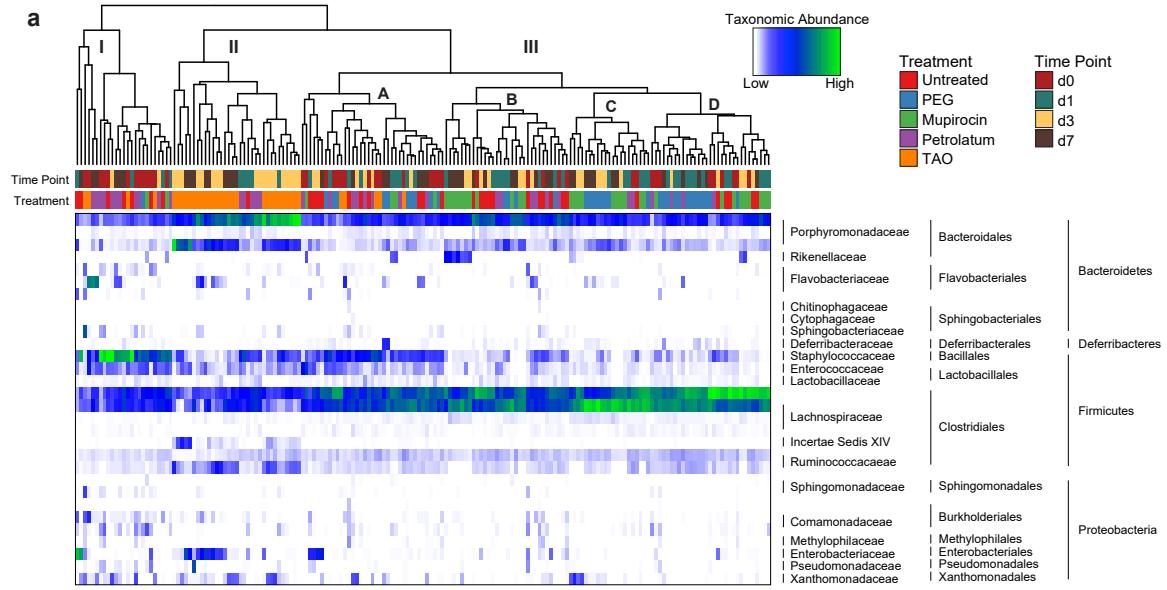

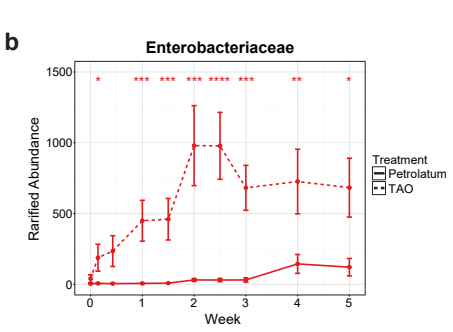

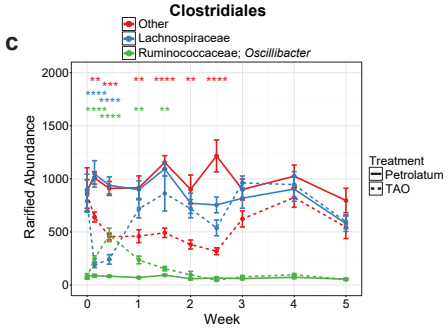

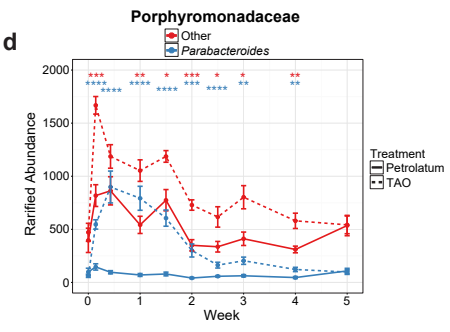

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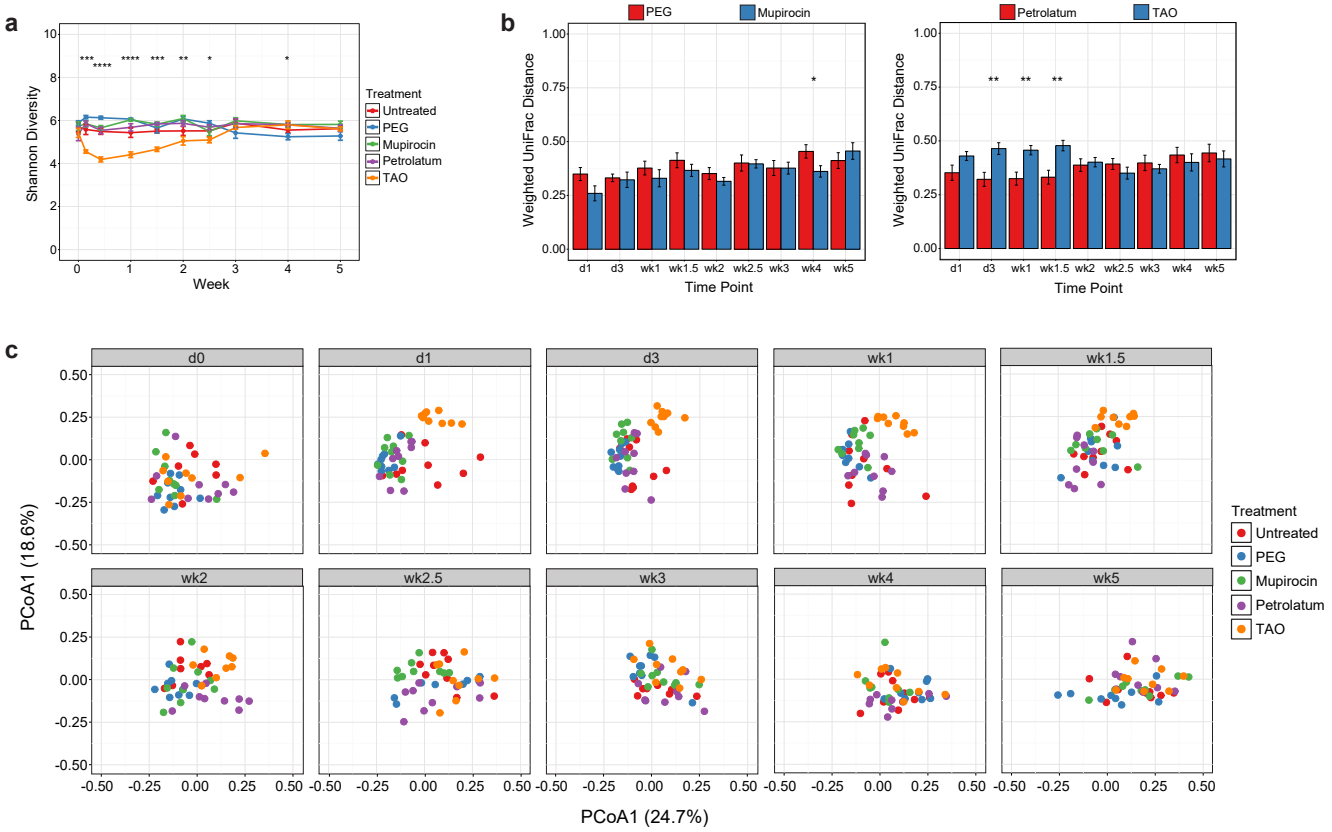
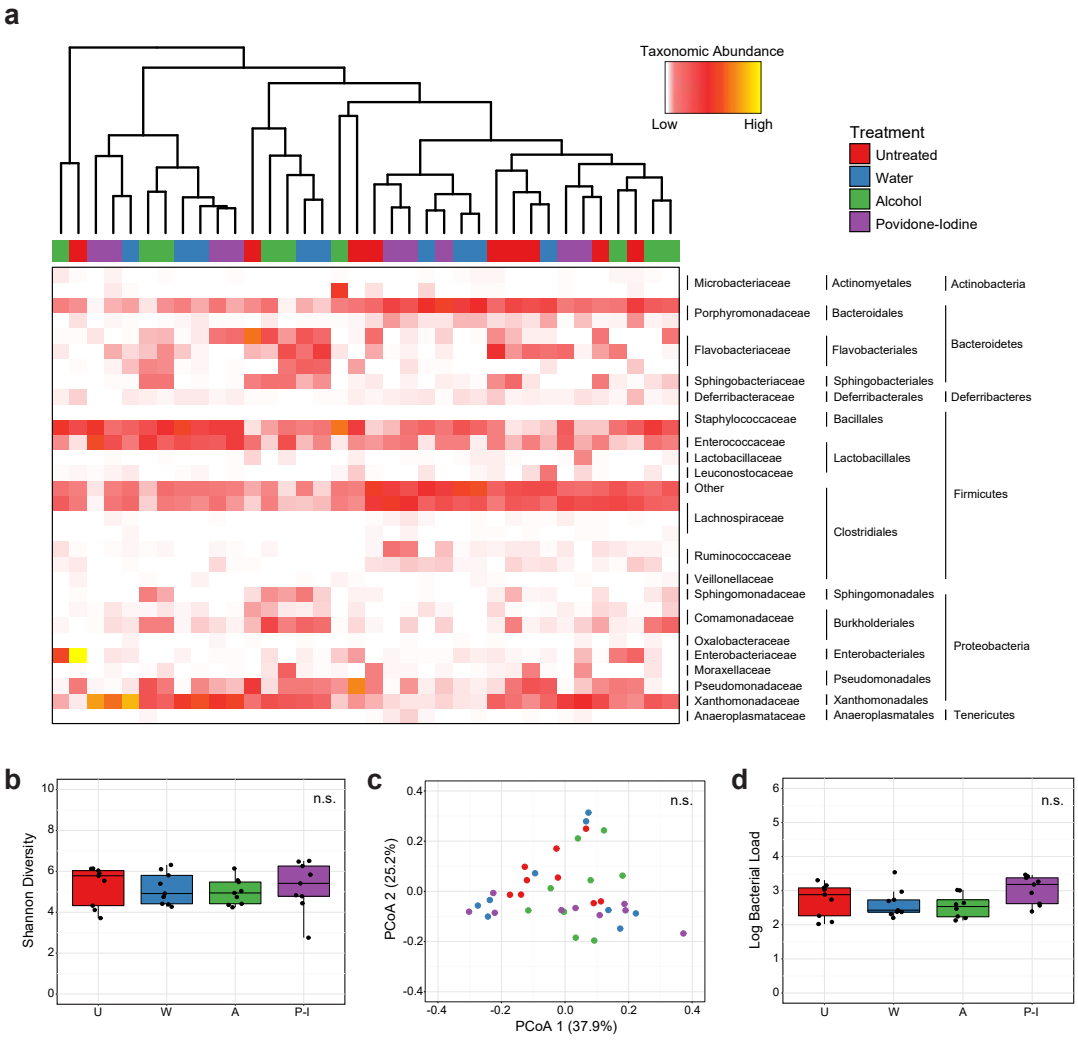


Antimicrobial Agents and Chemotherapy



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