SUPPLEMENTAL MATERIAL FOR:

"Antiseptic agents elicit short-term, personalized and body site-specific shifts in resident skin bacterial communities" by SanMiguel et al.

SUPPLEMENTAL RESULTS

Chlorhexidine retains free bacterial DNA at the skin surface

Given its proven efficacy against pathogenic microorganisms in hospital settings (Milstone et al., 2008), we were particularly struck that chlorhexidine did not elicit significant shifts in bacterial community membership or structure (**Fig. S7**). As chlorhexidine is known to cause allergic and dermatologic irritation in a subset of individuals (Opstrup et al., 2014), we hypothesized that acute treatment results in changes to the skin barrier that allows for enhanced binding of free DNA released from dead bacteria. 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 (Hon et al., 2008, Takahashi et al., 2014). 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. S8a**).

We furthered hypothesized that chemical properties inherent to chlorhexidine were 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, povidone-iodine, or chlorhexidine. We observed that chlorhexidine uniquely retained free bacterial DNA, 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. S8a, 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, mice treated with chlorhexidine retained more DNA at the skin surface compared to other treatment regimens (**Fig. S8b**). These experiments suggest that failure to detect differences in resident skin microbiota 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. We therefore focused additional investigations on water, alcohol, and povidone-iodine treatments only.

SUPPLEMENTAL METHODS

DNA retention. C57BL/6J mice were bred and maintained in specific pathogen free conditions at the University of Pennsylvania. All animal protocols were reviewed and approved by the University of Pennsylvania Institutional Animal Care and Use Committee. 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, povidone-iodine, or chlorhexidine for 1.5 minutes, similar to human experiments, and swabbed at 1hr and 6hr post-treatment. DNA was extracted using the Invitrogen PureLink kit, and *E. coli*-specific DNA was amplified using qPCR primers to the *ycct* gene (Clifford et al., 2012). Samples were compared to standard curves generated from known amounts of serially diluted *E. coli* DNA to calculate marker DNA concentrations.

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, povidone-iodine, 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.

Microbiome analysis. The datasets generated and analyzed during the current study are available in the NCBI Short Read Archive under BioProject: PRJNA395539. Sequences were preprocessed and quality filtered prior to analysis, including size filtering to 460-600 nucleotides. QIIME 1.7.0 was used for microbiome evaluation (Caporaso et al., 2010b). Because of the dataset size, sequences were clustered into OTUs with a modified open-reference OTU picking method. The reference set was generated by randomly subsampling 1% of sequences and performing *de novo* OTU picking by UClust (Edgar, 2010) with a 97% sequence-similarity threshold. Taxonomy was assigned to the most abundant representative sequence per OTU using the RDP classifier (Wang et al., 2007) with the Greengenes 97% sequence similarity database (DeSantis et al., 2006) specifying a confidence threshold of 80%. Sequences were aligned by PyNAST (Caporaso et al., 2010a), and chimeric sequences were removed using ChimeraSlayer (Haas et al., 2011). 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 (Team, 2016). In total, 71,167,526 16S rRNA gene reads were sequenced. Following quality control, the final study cohort represented 1,456 skin swab samples rarified to an even depth of 4,500 sequences per sample. For main analyses, samples in the Chlorhexidine treatment group (except for baseline samples) were removed prior to calculation of beta diversity metrics (removing 263 samples).

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 (Holmes et al., 2012). Samples were assigned to final community types based on posterior probabilities.

SUPPLEMENTAL REFERENCES

Caporaso JG, Bittinger K, Bushman FD, DeSantis TZ, Andersen GL, Knight R. PyNAST: a flexible tool for aligning sequences to a template alignment. Bioinformatics 2010a;26(2):266-7.

Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. QIIME allows analysis of high-throughput community sequencing data. Nat Methods 2010b;7(5):335-6.

Clifford RJ, Milillo M, Prestwood J, Quintero R, Zurawski DV, Kwak YI, et al. Detection of bacterial 16S rRNA and identification of four clinically important bacteria by real-time PCR. PLoS One 2012;7(11):e48558.

DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, et al. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Appl Environ Microbiol 2006;72(7):5069-72.

Edgar RC. Search and clustering orders of magnitude faster than BLAST. Bioinformatics 2010;26(19):2460-1.

Haas BJ, Gevers D, Earl AM, Feldgarden M, Ward DV, Giannoukos G, et al. Chimeric 16S rRNA sequence formation and detection in Sanger and 454-pyrosequenced PCR amplicons. Genome Res 2011;21(3):494-504.

Holmes I, Harris K, Quince C. Dirichlet multinomial mixtures: generative models for microbial metagenomics. PLoS One 2012;7(2):e30126.

Hon KL, Wong KY, Leung TF, Chow CM, Ng PC. Comparison of skin hydration evaluation sites and correlations among skin hydration, transepidermal water loss, SCORAD index, Nottingham Eczema Severity Score, and quality of life in patients with atopic dermatitis. Am J Clin Dermatol 2008;9(1):45-50.

Milstone AM, Passaretti CL, Perl TM. Chlorhexidine: expanding the armamentarium for infection control and prevention. Clin Infect Dis 2008;46(2):274-81.

Opstrup MS, Malling HJ, Kroigaard M, Mosbech H, Skov PS, Poulsen LK, et al. Standardized testing with chlorhexidine in perioperative allergy--a large single-centre evaluation. Allergy 2014;69(10):1390-6.

Takahashi H, Tsuji H, Minami-Hori M, Miyauchi Y, Iizuka H. Defective barrier function accompanied by structural changes of psoriatic stratum corneum. J Dermatol 2014;41(2):144-8.

Team RC. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria, URL https://www.R-project/org/. 2016.

Wang Q, Garrity GM, Tiedje JM, Cole JR. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol 2007;73(16):5261-7.

SUPPLEMENTAL FIGURES

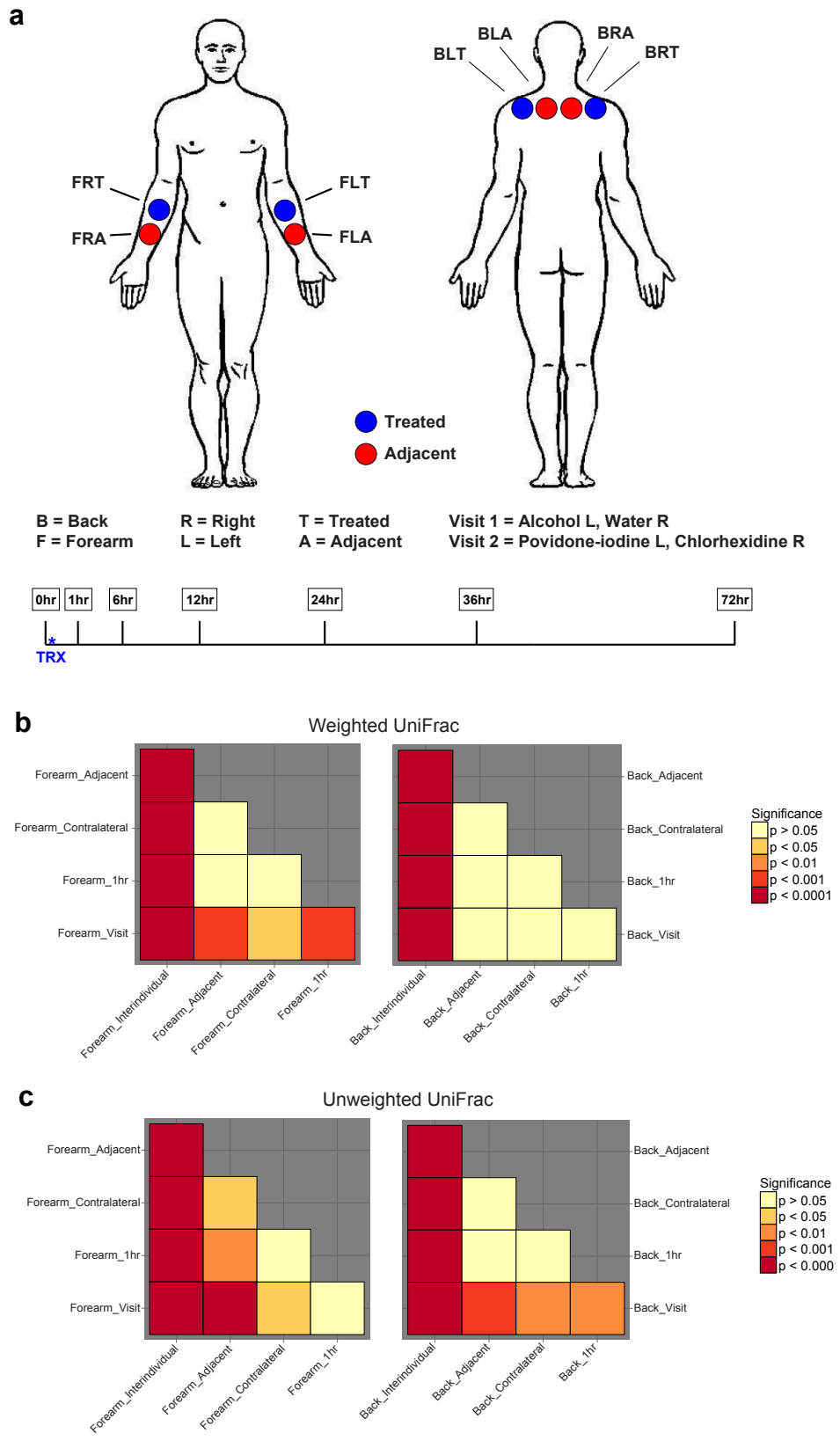

Fig. S1. Treatment regimen details and baseline community comparisons. (A) Diagram of sampling and treatment schedule for antiseptic study cohort. (B, C) Heat map of significances for weighted (B) and unweighted (C) UniFrac comparisons at the forearm and back for interpersonal, adjacent, contralateral, short-term (1hr), and long-term (visit) baseline community samplings.

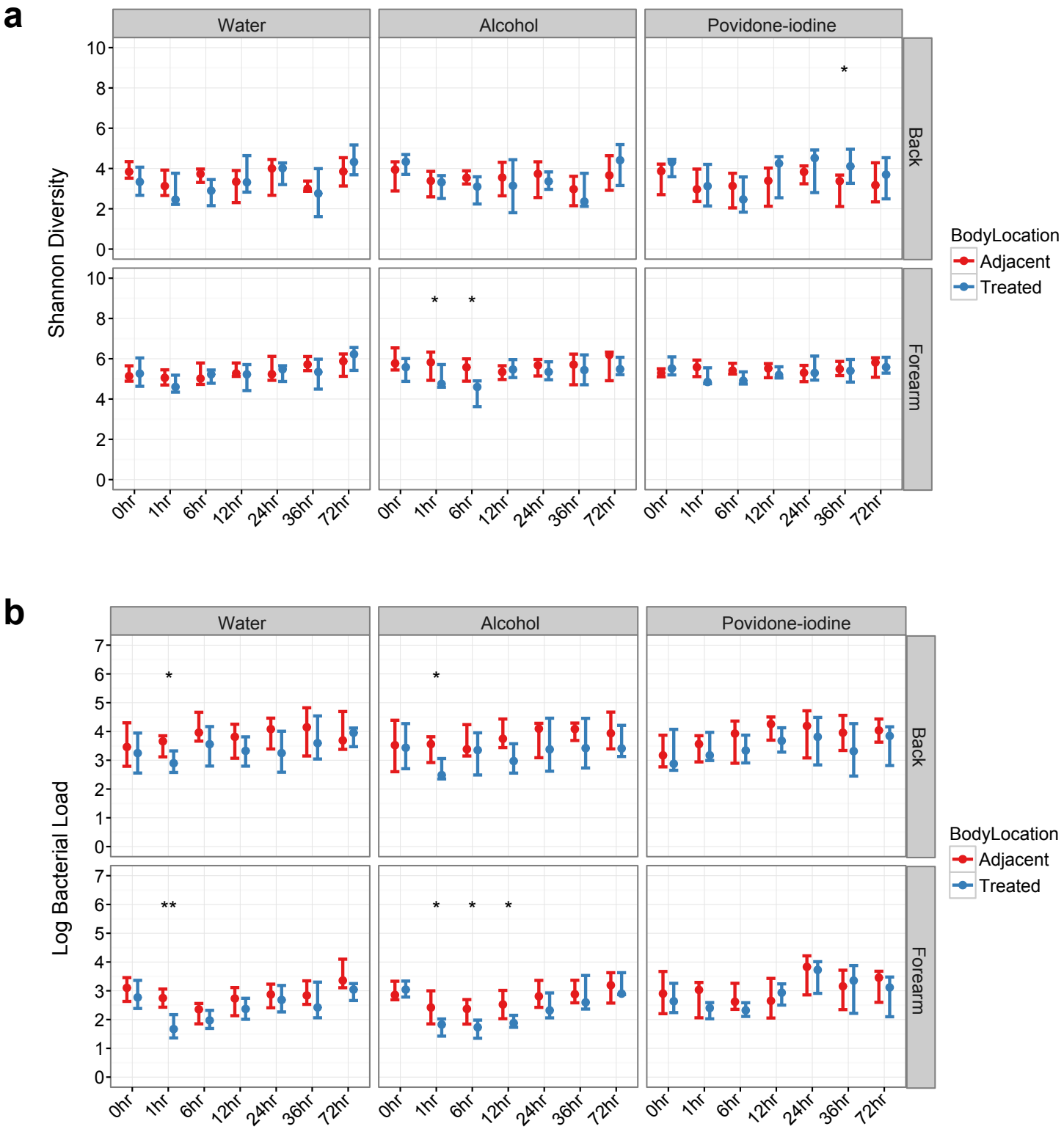

Fig. S2. Alpha diversity and bacterial load are decreased in response to certain treatment regimens. (A) Longitudinal comparisons of Shannon diversity for bacterial communities at adjacent and treated body sites of the back and forearm. (B) Bacterial load at the forearm and back for treated and adjacent 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).

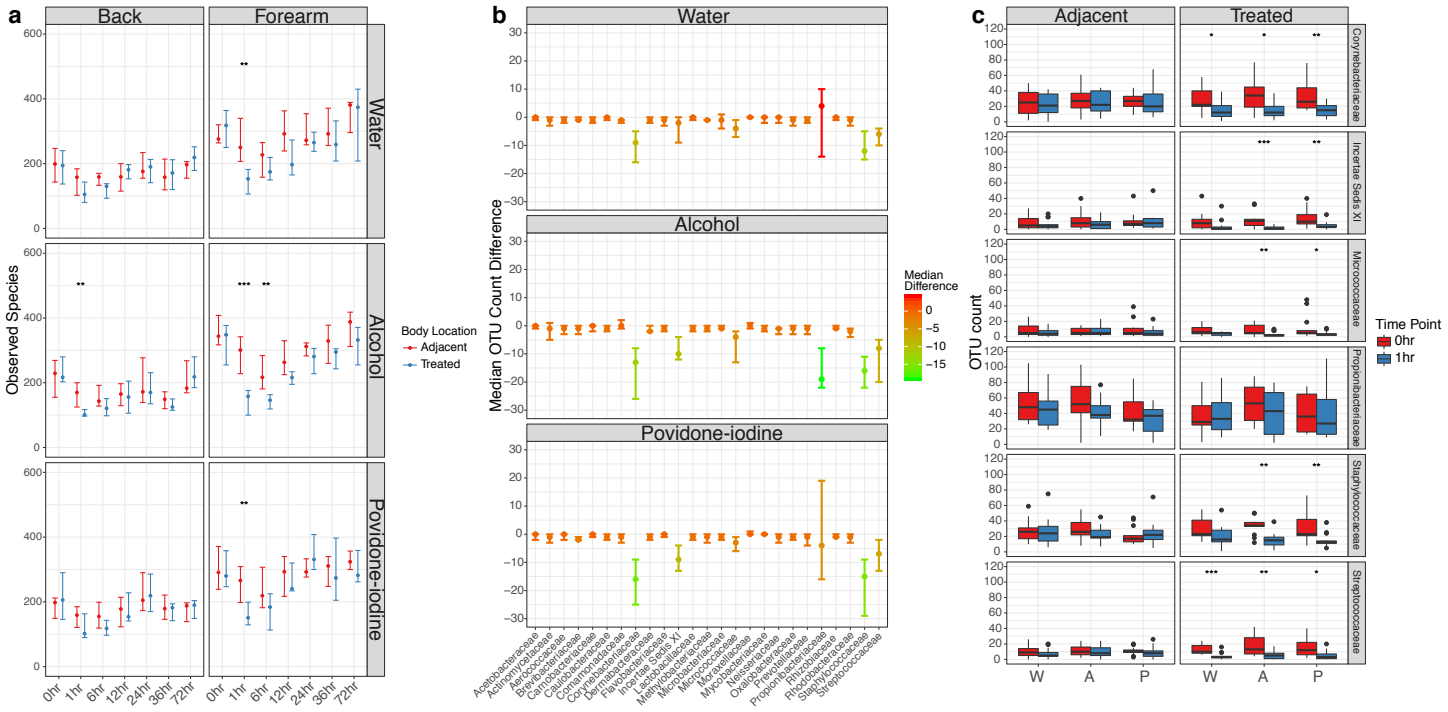

Fig. S3. Treatment elicits decreases in bacterial richness. (A) Longitudinal measurements of observed species for adjacent and treated body sites at the back and forearm. Data is presented by median points and interquartile regions. (B) Difference between OTU counts for the top 25 families at the back for baseline and 1hr post-treatment samples in response to water, alcohol, and povidone-iodine 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 for water (W), alcohol (A), and povidone-iodine (P) treatment between baseline and 1hr time points. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ by Wilcoxon rank sum test (Mann-Whitney U test).

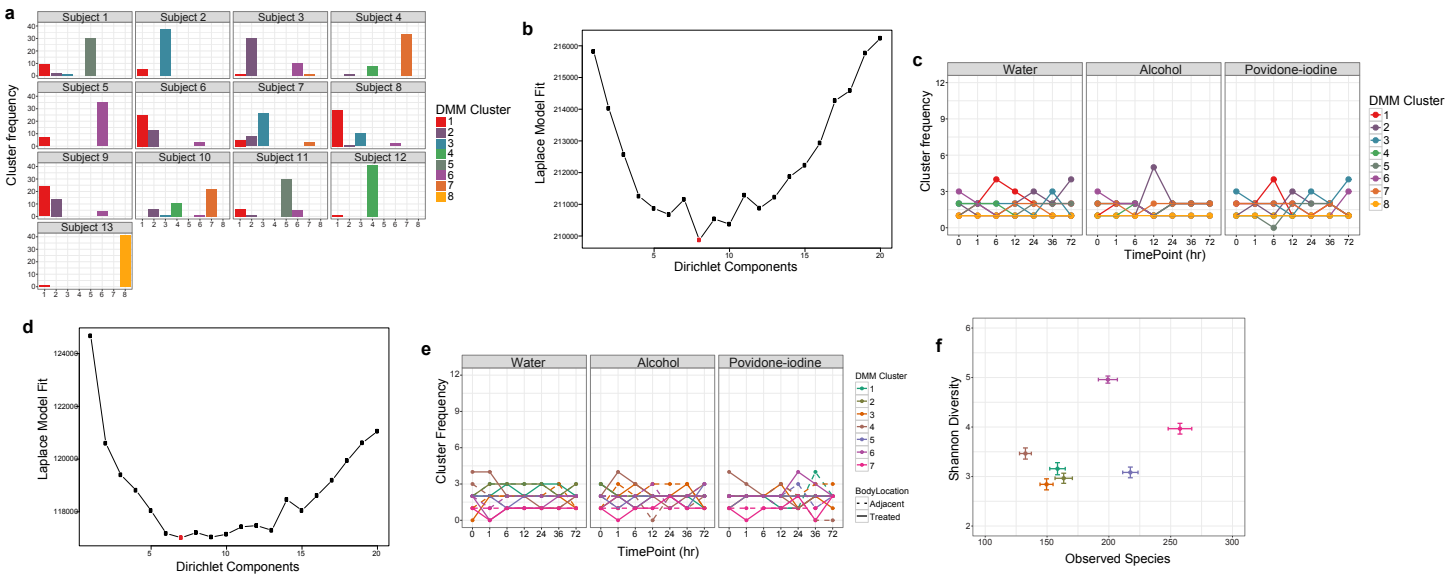

Fig. S4. Dirichlet multinomial mixture (DMM) model analysis for bacterial communities at the forearm and back. (A) Frequency of forearm DMM clusters by subject. (B) Laplace approximations for Dirichlet components of forearm communities. Global minimum is represented with a red point. (C) Frequencies of forearm DMM clusters at adjacent body sites over time. (D) Laplace approximations for Dirichlet components of back communities. (E) Longitudinal frequencies of DMM 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.

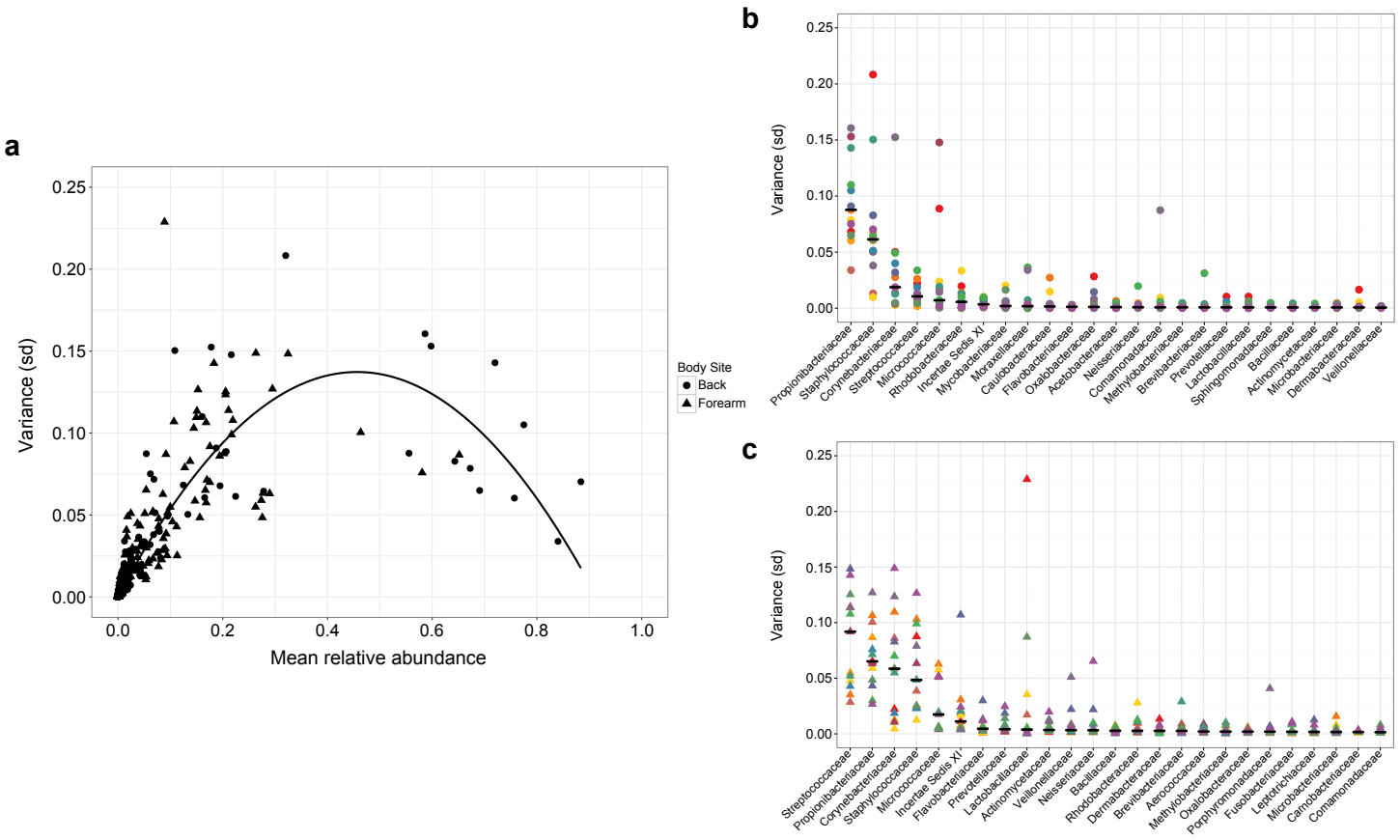

Fig. S5. Variance of bacterial taxa at baseline. (A) Relationship between the mean relative abundances of bacterial families at baseline and their variance as measured by standard deviation. Each point represents a different bacterial family in an individual subject, shaped by body site. Data was fitted 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.

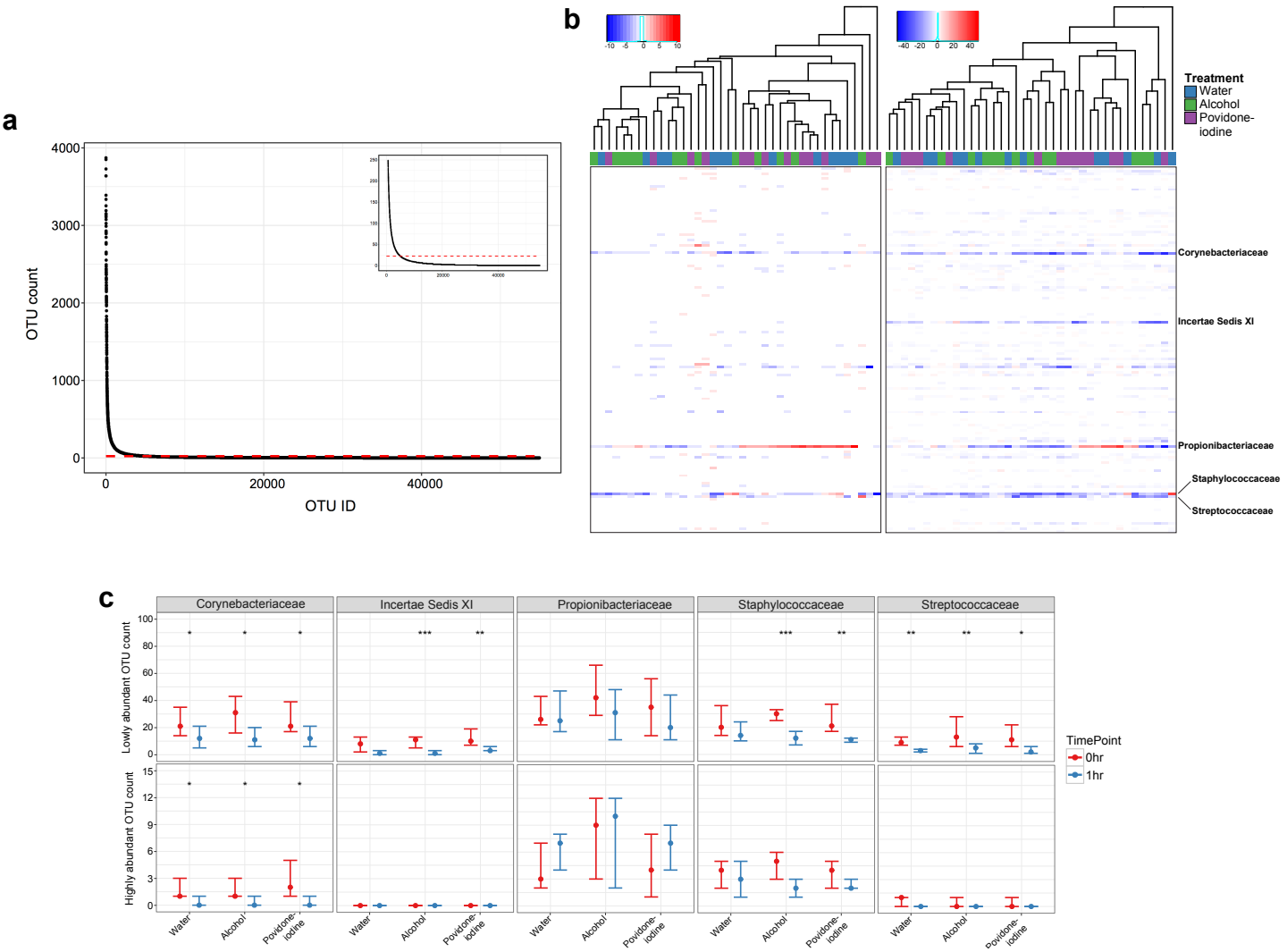

Fig. S6. Contribution of abundance to treatment-derived alterations in bacterial membership. (A) Sorted OTU abundances of all bacterial members in study cohort. Dashed red line represents 0.5% abundance threshold used to separate highly and lowly abundant OTUs. (B) Heat map of differences in bacterial family membership at the back between baseline and 1hr in subjects following treatment with water, alcohol, and povidone-iodine. 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 points and interquartile regions. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ by Wilcoxon rank sum test (Mann-Whitney U test).

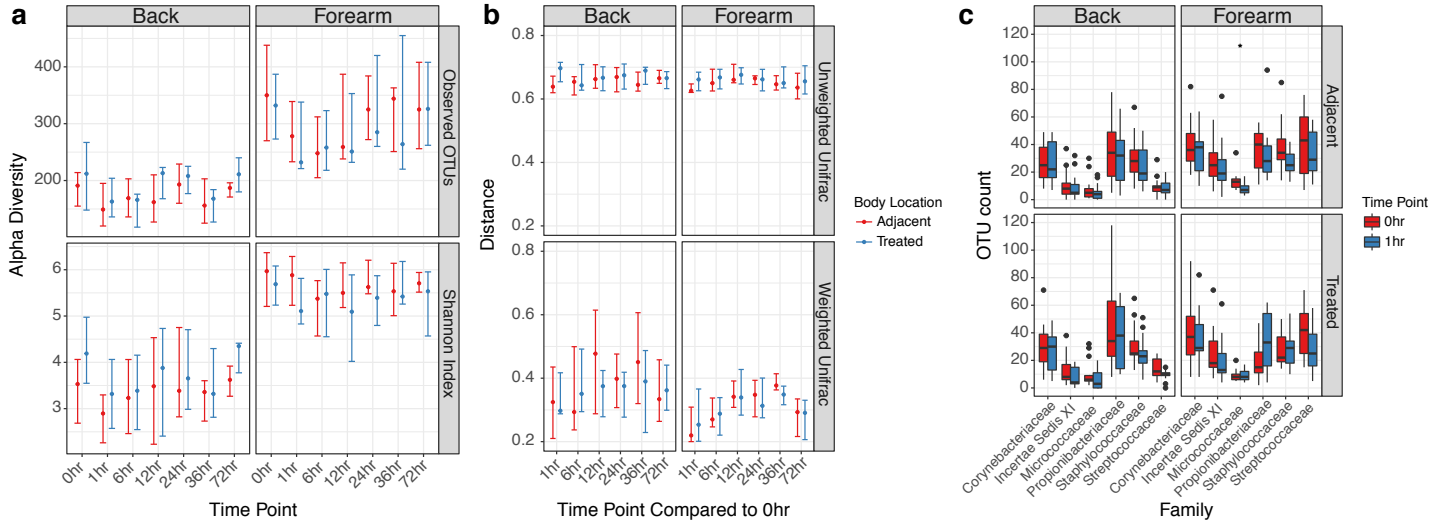

Fig. S7. Chlorhexidine treatment does not elicit bacterial shifts. (A) Longitudinal measurements of observed species and Shannon diversity for adjacent and chlorhexidine treated body sites at the back and forearm. Data is presented by median points and interquartile regions. (B) Weighted and Unweighted UniFrac distances of subjects' longitudinal time points compared to their individual baseline communities at chlorhexidine treated and adjacent body sites. Points represent the median of participants. Error bars designate interquartile regions. (C) Box and whisker plots of OTU richness at the back and forearm for major taxa at adjacent and chlorhexidine treated body sites between baseline and 1hr time points. * $P < 0.05$ by Wilcoxon rank sum test (Mann-Whitney U test).

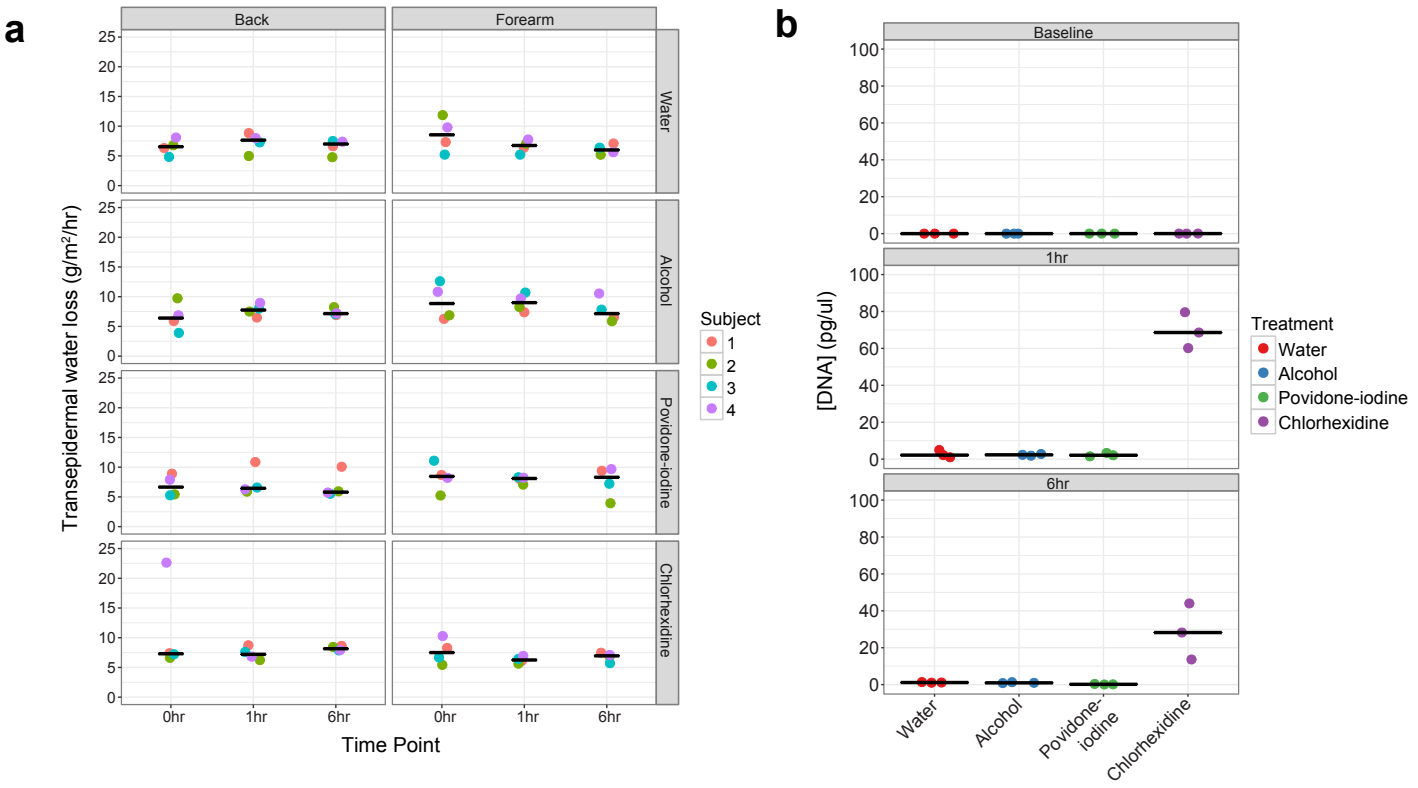

Fig. S8. Effect of chlorhexidine on skin integrity and bacterial DNA retention. (A) Transepidermal water loss (TEWL) of subjects at the back and forearm in response to treatment with water, alcohol, povidone-iodine, and chlorhexidine. Each point represents an individual subject. Black bars denote median. (B) Concentration of marker bacterial DNA at baseline and 1hr and 6hr post-treatment. Each point represents an individual mouse. Black bars denote median. Baseline refers to background concentrations of marker DNA prior to testing.