Spatial variations in the microbial community structure and diversity of the human foot is associated with the production of odorous volatiles

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Key words; isovaleric acid; microbiomics; odour; GC/MS

Running title; Microbial ecology of the foot and its relationship to odour

Abstract

The human foot provides an ideal environment for the colonization and growth of bacteria and subsequently is a body site associated with the liberation of odour. This study aimed to enumerate and spatially map bacterial populations resident across the foot to understand any association with odour production. Culture based analysis confirmed that Staphylococci were present in higher numbers than aerobic corynebacteria and Gram positive aerobic cocci, with all species being present at much higher levels on the plantar sites compared to dorsal sites. Microbiomic analysis supported these findings demonstrating that *Staphylococcus* spp. were dominant across different foot sites and comprised almost the entire bacterial population on the plantar surface. The levels of volatile fatty acids, including the key foot odour compound isovaleric acid, that contribute to foot odour were significantly increased at the plantar skin site compared to the dorsal surface. The fact that isovaleric acid was not detected on the dorsal surface, but was present on the plantar surface is probably attributable to the high numbers of *Staphylococcus* spp. residing at this site. Variations in the spatial distribution of these microbes appear to be responsible for the localised production of odour across the foot.

Introduction

The commensal microbial flora of human skin represents a diverse and dynamic ecosystem which has evolved to colonise virtually every available habitat both on and within the integumentary system (Malcolm & Hughes, 1980; Roth & James, 1988). This microbial flora varies in number from <10 to $>10^7$ micro-organisms per square cm (Bojar & Holland, 2002; Marshall *et al*., 1988). These variations are a consequence of the different environmental, topographical and biochemical conditions encountered between different skin sites and are also a reflection of person-to-person variability (Costello *et al*., 2009; Leeming *et al*., 1984). These factors also play a major role in determining the bacterial diversity of the microbial communities that reside at the different body sites (Grice & Segre, 2011). It has been well documented that body sites exhibiting increased temperatures and humidity (i.e. axilla, perineum and foot) support the highest bacterial populations compared to other body sites (Costello *et al*., 2009; Findley *et al*., 2013; Grice *et al*., 2009). These sites have been shown to be preferentially colonised by *Staphylococcus* and *Corynebacterium* spp. (Bojar & Holland, 2002; Taylor *et al.*, 2003) and display reduced levels of bacterial diversity than drier less moist sites (Gao *et al*., 2007). These bacteria are also the key microbes responsible for the biotransformation of odourless glandular secretions into volatile odorous compounds (James *et al*., 2013; Natsch *et al*., 2006; Troccaz *et al*., 2008).

The human foot provides an ideal environment for the colonization and growth of bacteria, affording high levels of humidity and a rich supply of nutrients. Previous culture and genomic based studies have demonstrated that *Staphylococcus* is the most dominant organism present on the plantar surface of the foot, with *Corynebacterium* also providing a significant contribution. *Micrococcus*, *Propionibacterium*, *Betaproteobacterium* and *Brevibacterium* species also make up a significant contribution to the overall foot microflora in some subjects (Costello *et al.*, 2009; Findley *et al.*, 2013; Grice *et al.*, 2009; Marshall *et al*., 1987). The spatial distribution of these bacterial populations across the foot has not been studied in any great detail. Eccrine sweat glands are the primary source of foot

secretions and are present on both the plantar and dorsal surface, secreting a primarily aqueous solution containing inorganic electrolytes, free amino acids, lactic acid and urea (Quinton *et al*., 1999). Unlike most areas of the body, the plantar surface of the feet have no sebaceous glands and also lack apocrine glands which are associated with odour production in the axilla. One of the chief consequences of this high microbial load is the production of an unpleasant odour which has a distinct cheesy/acidic note (James *et al*., 2013). Previous work has demonstrated that this odour emanates from the bacterial biotransformation of branched-chain amino acids, such as leucine and valine into volatile fatty acids (VFA's), in particular isovaleric acid (Ara *et al*., 2006; Kanda *et al*., 1990). The branched-chain amino acids are liberated *via* the degradation of the exfoliated layers of the stratum corneum under the influence of extra-cellular bacterial proteases (Holland *et al*., 1990). Alternatively, they originate as a soluble component of the secreted eccrine sweat (Ara *et al*., 2006; Harker & Harding, 2012). The key causative bacteria responsible for this biotransformation on the foot are *Staphylococcus* spp., but some species of *Brevibacterium*, *Micrococcus* and *Kytococcus* are biochemically competent at converting branched-chain amino acids to VFA's (Ara *et al*., 2006; James *et al*., 2013).

Although the key chemical components of foot odour have been identified, in the majority of the published studies the analytical approaches adopted did not directly sample the headspace above the foot. In most instances the odorous volatile components were identified through either chemical modification of liquid extracts obtained from the foot; or by incubating liquid extracts from the foot with foot derived bacterial isolates (Ara *et al*., 2006; Caroprese *et al*., 2009; James *et al*., 2013; Kanda *et al*., 1990). The application of such methods does not fully resolve the question of the exact chemical identity of the volatile components responsible for the generation of foot odour. In this study we sought to enumerate and spatially map the key bacteria resident across the foot. We then obtained diversity profiles of the foot microflora at two different sites to investigate how this influenced the VFA profiles across the dorsal and plantar surfaces under controlled conditions.

Materials & Methods

Subjects

These studies were conducted in accordance with the ethical principles of Good Clinical Practice and the Declaration of Helsinki. Ethics committee approval was sought before commencement of the studies and all subjects gave written informed consent. Subjects recruited for the various studies were aged between 21 to 65 years old and in good general health (BMI 19.8 to 35.0).

Microbiological sampling, isolation and enumeration of bacteria

Sixteen subjects (8 females and 8 males) were microbiologically sampled by the standard Williamson and Kligman technique (Williamson & Kligman, 1965) from the foot surface 24 hrs after undertaking a controlled foot wash. Subjects refrained from washing their feet or applying any foot products for the intervening 24 hrs prior to microbiological sampling. They were requested to wear test centre supplied socks for the duration of the study. The microbiological sampling procedure was performed eight times on each foot on adjacent areas with four samples taken from the plantar surface and four samples from the dorsal surface of the foot (for exact locations see Fig. S1). The four dorsal sites sampled comprised of two adjacent sites on the forefoot nearest to the toe web space in line with the first and third toe clefts, and 2 sites closely located further up the dorsal surface one near to the hind-foot and one closer to the mid-foot labelled as sites A, B, C and D, respectively. Similarly, four sites were sampled on the plantar surface two adjacent sites one on the ball of the big toe and one from the plantar heel and an adjacent site towards the mid-foot plantar arch labelled as sites E, F, G and H, respectively. Samples were taken by standardised scrubbing using a sterile sampling fluid (75 mM sodium phosphate buffer, 0.1 % Triton X-100, pH 7.9).

Collected samples were plated on three different types of media; a, aerobic coryneform medium with phosphomycin (ACP) (Taylor *et al*., 2003) for the enumeration of aerobic *Corynebacterium* spp.; b, *Staphylococcus* selective medium (SS) (Marshall *et al*., 1987; Schleifer & Kloos, 1975) for the enumeration of aerobic *Staphylococcus* spp.; c, fastidious anaerobic agar containing novobiocin (FaNov) (46 mg mL^{-1} Fastidious anaerobe agar (LabM), 3 mg mL^{-1} yeast extract, 5 mg mL^{-1} Tween 80, 50 µl mL^{-1} defibrinated horse blood (Oxoid), 10 µg mL^{-1} Novobiocin (Oxoid)) for the enumeration of Gram-positive anaerobic cocci (GPAC). This medium is designed to facilitate the growth of GPAC microbes and inhibit the growth of facultative anaerobic staphylococci, anaerobic corynebacteria and anaerobic propionibacteria. Samples were spiral plated with either neat inoculums or diluted 10^{-2} (Don Whitley Scientific) onto each selective medium and incubated at 37 ^{o}C for 2 days for the aerobic bacteria. The anaerobic bacteria were incubated at 37 ^{o}C for 7 days in an anaerobic chamber supplied with a gas mix of 80 % (v/v) nitrogen, 10 % (v/v) carbon dioxide and 10 % (v/v) hydrogen. After incubation, plates were selected for each medium type with an estimated count of between 30 to 300 colonies. The plates were enumerated using the Protocol2 colony counter (Synbiosis) and data presented as colony forming units per millilitre sample (cfu mL^{-1}).

Statistical analysis

Data from the Protocol2 was converted to log colony forming units per square centimetre of skin (log_{10} cfu cm^{-2} skin). Data was analysed using the statistical package JMP using the 'Fit Model'. The minimum detectable number of bacteria for the method used is 5 cfu mL^{-1} (which equates to 0.72 log_{10} cfu cm^{-2}). In cases where no bacterial colonies were recovered values provided are 0.42 log_{10} cfu cm^{-2} generated from an artificially provided value of 2.5 cfu ml^{-1} bacteria.

Microbiomic sampling, DNA extraction, sample preparation and sequence processing

Ten subjects (5 females and 5 males) were microbiologically sampled by the standard Williamson and Kligman technique (Williamson & Kligman, 1965) from the foot surface 24 hrs after undertaking a controlled foot wash. Subjects refrained from washing their feet or applying any foot products for the intervening 24 hrs prior to microbiological sampling. They were requested to wear test centre supplied socks for the duration of the study. The microbiomics sampling procedure was performed twice times on either the left or right foot with one sample taken from the plantar surface (site E) and the other sample from the dorsal surface (site B) (Fig. S1).The DNA extraction, PCR amplification and subsequent analysis was performed as previously published (Harker *et al*., 2014) with certain alterations to the protocol. Twenty buffer scrub samples (50 mM Tris-HCl + 0.1 % v/v Triton-X100, pH 7.9) were concentrated by centrifugation at 13,200 rpm for 20 min, the supernatant was removed, and the cells resuspended in 500 µL Tris-EDTA (TE) buffer (10 mM Tris-HCl; 1 mM EDTA, pH 7.4), in a Pathogen Lysis L tube (Qiagen). A negative control sample of the same buffer used for sampling was included in the extraction and PCR amplification steps. Cell lysis of the buffer samples was carried out by adding 3 µL Epicentre® Ready-Lyse lysozyme (250 U/µL) and incubating the cell suspension with agitation of 300 rpm at 37 ^{o}C for 18 h, followed by bead beating at 6.5 m/s for 2 x 45 seconds, with 5 minutes rest between bursts (on ice), using the FastPrep 24 machine (MPBiomedicals). Samples were placed on the QIAsymphony DNA extraction robot and DNA extracted as per the manufacturer's instructions using the Virus/Pathogen DNA extraction kit (Qiagen).

Three primers pairs (27f/338r, 8f/1391r & 515f/1061r) targeting different regions of the 16S rRNA gene were evaluated *in-silico* using the open-source software tool PrimerProspector v1.0.1 (Walters *et al*., 2011) and the Silva database. The primer pair 515f/1061r predicted 100 % taxonomic coverage across 23,441 bacterial sequences including the phyla Actinobacteria (most dominant reported genera: *Corynebacterium and Propionibacterium*) and Firmicutes (majorly represented by *Staphylococcus* spp.), and was

178 selected for this study (Fig S2). The forward primer contained the 454 Life Sciences adaptor
179 A, the primer key “TCAG”, a unique 12 base pairs (bp) error-correcting Golay code (Hamady
180 *et al*., 2008), was used to tag each PCR product, and the bacterial primer 515f (Marcille *et*
181 *al*., 2002; Meyer *et al*., 2004)]. The reverse primer contained the 454 Life Sciences adaptor
182 B, the primer key “TCAG”, and the bacterial primer 1061r (Andersson *et al*., 2008).
183 Quantitative PCR (qPCR) amplification was carried out on each sample to quantify the DNA
184 and to determine the number of PCR cycles required to reach the sub-plateau phase of
185 amplification. It has been reported that elevated cycle number increases the potential for the
186 generation of chimeras, which can result in the overestimation of microbial community
187 diversity (Farrelly *et al*., 1995; Gonzalez *et al*., 2005). Reactions consisted of 2 µL (5 µM) of
188 each forward and reverse primer, 10 µL of Rotor-Gene SYBR Green Mastermix (Qiagen), 3
189 µL of template DNA and 3 µL molecular grade water (Qiagen). Samples were amplified
190 using the following parameters: 95 ^{o}C for 5 min, 40 cycles of; 95 ^{o}C for 45 s, 65 ^{o}C for 30 s,
191 and 72 ^{o}C for 90 s, with a final extension of 10 min at 72 ^{o}C. To minimize PCR amplification
192 bias, samples were grouped by the cycle number required to reach the late exponential
193 phase of the amplification curve, as determined by the qPCR step, resulting in 4 PCR runs of
194 between 18 to 37 cycles.
195 The 16S rRNA amplification consisted of 2 µL (5 µM) of each forward and reverse
196 primer, 10 µL of HotStarTaq Plus Mastermix (Qiagen), 3 µL of template DNA and 3 µL
197 molecular grade water (Qiagen). The samples were amplified in triplicate using the following
198 parameters: 95 ^{o}C for 5 min, either 18, 23, 30 or 37 cycles of; 95 ^{o}C for 45 s, 65 ^{o}C for 30 s,
199 and 72 ^{o}C for 90 s, with a final extension of 10 min at 72 ^{o}C. The replicate PCR amplicons
200 from each sample were pooled and purified using the Qiaquick PCR purification kit with the
201 QIAcube robot (Qiagen) as per the manufacturer’s instructions. The DNA of each sample
202 was quantified by using Quant-IT Broad Range dsDNA kit (Invitrogen), and a composite
203 sample for pyrosequencing was prepared by pooling equal amounts of PCR amplicon from
204 each sample. The QIAgility liquid handing robot (Qiagen) was used to prepare samples for
205 PCR, pooling and DNA quantification.

206 The composite sample was sent to the Centre for Genomics Research (CGR) at
207 Liverpool University for pyrosequencing on the Genome Sequencer FLX (Roche/454 Life
208 Sciences). The pooled amplicons were checked on an Agilent Bioanalyzer DNA HS chip for
209 the absence of primer-dimers, and quantitated using the Qubit® dsDNA HS Assay Kit, with
210 the Qubit® 2.0 Fluorometer (Invitrogen). The amplicon library with an average size of 640 bp
211 was diluted to 1×10^7 molecules/µL. Emulsion PCR was set up according to Roche's
212 recommendations. Following emulsion PCR enrichment, beads produced were deposited
213 into 2-region gasket format wells of a Titanium Series PicoTiterPlate device. 454
214 Sequencing was performed using the GS FLX Titanium Sequencing Kit XLR70, on the GS
215 FLX instrument according to the manufacturer's recommendations (Roche/454 Life
216 Sciences, Branford, CT). Image analysis, shotgun signal processing, and base calling were
217 performed using the supplied system software (version 2.6). The Standard Flowgram
218 Format (sff) files output from base calling were employed in subsequent analysis.
219 The resulting flowgrams were processed using the AmpliconNoise algorithm (Quince *et al.,*
220 2011), to remove inherent noise in the data which has been shown previously to drastically
221 over-estimate true levels of α-diversity (Quince *et al*., 2009). Chimeras were removed using
222 the AmpliconNoise associated Perseus algorithm, and the resulting denoised sequences
223 were clustered into Operational Taxonomic Units (OTUs) at the 3 % level. OTUs were
224 taxonomically assigned using the Ribosomal Database Project (RDP) Classifier version 2.2
225 (Wang *et al*., 2007) accessed via *QIIME PIPELINE version 1.5.0* (Quantitative Insights into
226 Microbial Ecology), an open source software package for analysis of microbial communities
227 (Caporaso *et al*., 2010)..
228
229 **Microbial Diversity**
230 α-diversity was measured using Renyi Entropy, which employs a generalisation of a number
231 of information theoretic measures. This allows the estimation of diversity across abundance
232 levels from rare species to the most abundant. One community can be regarded as more
233 diverse than the other if its Renyi diversity is higher across all abundance levels (Mecklin,

2004). β-diversity between communities was measured using the weighted UniFrac distance and illustrated on a two dimensional plot produced from a principal coordinate analysis performed in QIIME (Caporaso *et al*., 2010). The mean β-diversity was confirmed using permutation ANOVA (Anderson, 2001).

Comparative Testing

A comparison of taxonomic differences in the mean relative abundance of taxa can be modelled using a Multinomial sampling model. However, due to the very sparse nature of the data which may lead to over dispersion, a Dirichlet-Multinomial sampling model was used to provide a better fit to the data. The statistical hypothesis testing was performed using the vegan: Community Ecology Package (Vegan: Community Ecology Package. R package version 2.0-10. http://CRAN.R-project.org/package=vegan).

Data Visualisation

The primary visualisation technique employed involves using colour coded tables, commonly known as heatmaps. They provide a visual overview of distribution within various data analysis contexts. Here, an in-house heatmap was used to analyse the differences and similarities of sample groups in the context of microbial distribution within samples, based on the relative abundance of microbes. This includes microbes that comprise 97% of the total microbial population represented by their taxonomic classification and ordered based on relative sample abundances with the most abundant microbes at the top. Samples were ordered by clustering using the Bray-Curtis dissimilarity matrix. To highlight subtler differences in low and medium abundance cells, a logarithmically scaled colouring was used where black represent cells with no abundance, blue to light yellow represent cells with low to medium abundance and light yellow to red represent cells with medium to high abundance.

Nucleotide Sequence Accession Number

The raw sequences reported in this paper have been deposited in the NCBI BioProject database, Accession no. PRJNA263008.

Volatile organic compound (VOC) analysis

Ten subjects (4 female and 6 male) undertook a controlled wash of each foot and were provided with a clean pair of socks. Subjects refrained from washing their feet or applying any foot products, after 24 hrs subjects were provided with another clean pair of socks to wear, such that test centre supplied socks were worn continuously 48 hrs prior to sampling. On the day of sampling participants were instructed to fast and avoid drinking anything other than water until the samples were taken. Conditioned polydimethylsilicone (PDMS) membrane patches (Goodfellow Ltd, Cambridge, P/N Si303045), were placed onto the dorsal (Site B) and plantar (Site E) surfaces of the foot (Fig. S1), an unscented cotton wool pad (that had previously been conditioned by vacuum polishing at 75 ^{o}C wrapped in foil for 12 hours) was placed on top of the skin patch and then secured in place using microporous tape. Patches remained on the foot for 30 min whereupon they were removed with a clean pair of tweezers and placed back in the corresponding 1 cm Tenax® thermal desorption tube (Markes International). The tube fittings were then tightened and the tube was stored at 4 ^{o}C prior to gas chromatography-mass spectrometry (GC-MS) analysis. Field blanks were taken by placing a conditioned patch between 2 cotton wool pads and sealing them together with microporous tape. This was left in the room while sampling took place for 30 min before returning it to a thermal desorption tube and storing at 4 ^{o}C.

Skin patch thermal desorption tube preparation

All samples were collected using PDMS skin patches (Goodfellow Ltd, Cambridge, P/N Si303045), tubes and patches were prepared using the method as previously described (Martin *et al*., 2012; Riazanskaia *et al*., 2008). The skin patches were subject to 3

consecutive methanol washes, with 10 min in an ultra-sonic bath at 30 ^{o}C for each wash. After cleaning the patches were conditioned at 185 ^{o}C and a pressure <1 mbar for no less than 15 hrs. Once conditioned the tubes were immediately capped and stored in an airtight box at 4 ^{o}C. Prior to use the skin sampling-patches were thermally desorbed to establish that any residues of VOC's within them were at acceptable levels and to further "polish" the patch prior to sampling. Each tube was spiked with 0.2 µL of 20 ng µL^{-1} toluene-D8 solution (4 ng total loading) while skin sampling took place. This was accomplished using a Markes International tube loading device operating at 100 mL^{-1} min for 1 min.

Thermal desorption GC-MS

All analyses were performed using a VG Trio 1 mass spectrometer combined with a HP5890 GC system and a Markes UNITY 2 thermal desorption unit. Samples were analysed using the thermal desorption method as previously reported (Martin *et al*.,2012). The resolution conditions and mass spectrometer parameters are summarised in supplementary Table S1. A sulphur cold trap was utilised in the Markes UNITY 2(PN: U-T6SUL-2S) for the duration of this experiment to give enhanced retention of low Mw volatile fatty acids (VFA's). Eight *m/z* ratios were monitored during sample analysis. Specific retention times of VOC's of interest are provided in supplementary Table S2 alongside the respective target ion m/z ratios used for detection of each of these individual metabolites.

Quantification of target VOC's was facilitated by the construction of calibration curves of authentic standards of the reference compounds. Standard graphs were obtained for 9 of the VFAs acetic, 2-methylbutyric, 4-methylvaleric, butyric, hexanoic, isobutyric, isovaleric, propionic and valeric acid, respectively. Standards were prepared as v/v mixtures in acetonitrile, at a range between 156.25 pg/µL to 10 ng/µL. Tubes used in quantification were firstly spiked with 4 ng of toluene-D_8 and then 1 µL of the standard solution was spiked directly on to the skin patch prior to thermal desorption GC-MS analysis. The linearity of

each calibration curve was determined by plotting the peak area of each compound at a minimum of four different concentrations *vs*. detector response.

The lower limit of quantification is defined as the lowest concentration on the calibration curve with an acceptable accuracy (relative error, RE) within ± 20 % and a precision (relative standard deviation, RSD) below 20 %. The standard calibration curves for each VOC were linear over the concentration range of 312.5 pg/µL to 5 ng/µL (r^2 ≥ 0.99 to 0.97). Dilution of the VOC's to 156.25 pg/µL was shown to be below the experimental limit of detection for all 10 VFA's measured.

Statistical analysis

Quantitative data were expressed as means with standard deviations. Statistical comparisons were performed using the two-sided paired t-test. A *P* value < 0.05 was considered significant.

Results

Microbiological mapping of commensal bacteria on the foot

The feet of 8 female and 8 male panellists were sampled 24 hours after a controlled wash from 8 different sites on each foot. Bacterial enumeration *via* culture dependent methods of samples obtained from different sites on the foot, revealed significant differences in the numbers of commensal bacteria observed on each media between the dorsal and plantar surfaces of the foot as presented (Fig. 1). Plantar sites E and F (the ball of the foot) had significantly higher mean log counts than G and H (the heel of the foot). However, in the case of GPAC sites F and G were not significantly different from each other. There was a significant difference between site A (dorsal, near the big toe) and the other dorsal sites for staphylococci (p=0.004), for corynebacteria these differences were non-significant (p=0.07). No significant differences existed between the other dorsal sites for any organism. Sites G

and H (plantar heel) exhibited significantly higher bacterial numbers than all the dorsal sites A-D ($p<0.0001$). The difference was most pronounced for staphylococci and then corynebacteria but it was also significant for the anaerobes ($p<0.0001$). *Staphylococcus* spp. bacteria were isolated in high numbers from all panellists whereas aerobic corynebacteria and GPAC were much lower in number and were not isolated from a large proportion of samples. The culture based data demonstrates the relative numbers of each organism within the bacterial population of the foot with *Staphylococcus* spp. making the largest contribution to the microbial community, with the next largest contribution made by *Corynebacterium* spp. and the smallest contribution being made by the GPAC organisms (Fig. 1).

Figure 2 depicts contour plots of the bacterial enumeration data across all the sites for each media type and coloured by gender. To illustrate, dense red indicates $\log_{10}$ counts that are exhibited by a high proportion of the male population, e.g. for total staphylococci, site E (plantar, ball of foot) for the male sample has high numbers of subjects whose $\log_{10}$ counts are ~6-7. Although the adjusted mean log counts were slightly higher for the female samples than for the males, for all organisms at all sites these differences were not statistically significant across gender. The only exception was for GPAC numbers at sites E and F demonstrating significantly greater $\log_{10}$ cfu cm^{-2} in female subjects. Other than this one example there are distinct areas of overlap within the bacterial enumerations on each media type at each site between males and females.

Microbiomic analysis of the human foot

We obtained 201,136 raw sequences of which 16,898 sequences were not assignable to a specific sample due to errors in barcodes or primer sequence. A further 41,699 sequences were removed during additional denoising and chimera checks, leaving 142,539 sequences for downstream analysis with an average length of 392 base pairs (min 220, max 400). The number of sequences for each sample varied from 306 to 65805 (Table S3). No sequences

368 were obtained from the negative control as insufficient DNA was generated from PCR to
369 warrant sequencing. Figure 3 illustrates the individual (and mean Fig S3) relative
370 abundance of the major bacterial genera obtained from either the dorsal or plantar surfaces
371 of the foot. This data indicates an increase in the relative diversity of the dorsal surface
372 compared to the homogenous nature of the plantar surface, with *Staphylococcus* spp. the
373 only statistically significantly genus enriched at the plantar surface and making up 98.6% of
374 the total plantar skin microbiome. On the dorsal surface *Staphylococcus* spp. are still the
375 dominant organisms but to a much lesser degree (26.9%) compared to the plantar surface,
376 with *Corynebacterium* spp. (12.1%) the second most abundant organisms, indicating that the
377 remaining ~60% of bacteria present represent a diverse range of cutaneous commensals.
378 Twleve genera were significantly enriched at the dorsal sites with a relative abundance of
379 between 0.10 to 12.10%, including *Corynebacterium* (12.1%), *Enhydrobacter* (6.5%),
380 *Chryseobacterium* (6.0%) and *Micrococccus* (5.5%) and the GPACs, *Peptinophilus* (0.6%)
381 and *Finegoldia* (0.5%). *Kytococcus* and *Brevibacterium*, genera previously implicated in foot
382 malodour were present in low relative abundance at both plantar (0.10%, 0.22%,
383 respectively) and dorsal (0.02%, 0.06%, respectively) sites. Table S4 provides a list of the
384 genera showing evidence of statistically significant differences between the mean relative
385 abundance on the dorsal and plantar surfaces.
386 The heatmap visualisation of classified genera reveals cluster patterns based on the
387 genera present at the plantar and dorsal sites (Fig. 4). Samples were seen to cluster into
388 two groups, based on the Bray-Curtis distance, indicating a high degree of dissimilarity.
389 Most samples clustered according to their site, however the plantar sample from subject 8
390 clustered with the dorsal samples (highlighted in green), indicating that this sample shares a
391 more similar microbial community structure with this site . The ability to measure the
392 diversity within a site (α-diversity) allows a greater understanding of the differences in
393 bacterial populations at those sample sites. The α-diversity was examined by generating
394 diversity profiles using Renyi entropy (Mecklin, 2004). Figure S4 compares the α-diversity of

the dorsal and plantar sites of the foot confirming that diversity at the dorsal site is significantly greater than the plantar across all levels of taxonomic abundance. Analysis of the diversity between the bacterial communities of different sites (β-diversity) shows clustering of the plantar samples with the exception of the plantar sample from subject 8, which clusters with the dorsal samples (Fig. S5) and confirms the previous observations (Fig. S4). Analysis of these weighted UniFrac distances using permutation ANOVA revealed a statistically significant difference in mean diversity between plantar and dorsal sites ($Pr(>F) = < 0.001$). No evidence of a difference was observed for gender ($Pr(>F) = 0.56$. Due to uneven read distribution among two samples (3P & 6P) these were removed from a subsequent analysis. Removal of these samples did not cause a significant shift in community α or β-diversity measurement (Fig. S4 & Fig. S5).

Analysis of volatile fatty acids by thermal desorption-GC-MS of skin patches sampling different sites on the foot

The volatile fatty acid quantification from the dorsal and plantar surfaces from 10 subjects are provided in Table 1. This data demonstrates significant increases of specific volatiles symptomatic of foot odour on the plantar surface when compared with the lower concentrations detected from the dorsal region i.e. acetic, butyric and valeric acid. Even more pronounced is the complete absence of the highly odiferous volatile isovaleric acid from the dorsal surface, whereas in contrast this key foot odorant is readily detected on the plantar surface. This data indicates VOC's responsible for foot malodour to be more prevalent and abundant on the sole of the foot especially in the case of isovaleric acid which was never observed in samples obtained from the dorsal surface. Figure 5 illustrates representative chromatograms of PDMS skin patches derived from the dorsal and plantar surfaces of the same male volunteer (subject 5), showing increases in hexanoic and valeric acid and the appearance of isovaleric acid in the skin patch from the plantar sample compared to the dorsal sample.

Discussion

The results obtained in the present study are similar to those reported previously. Staphylococci were isolated from every foot at almost every site. In total, staphylococci were prevalent in 93.3% of all foot samples. Aerobic corynebacteria were isolated from 86.9% of all samples. The prevalence of bacteria was lower from dorsal surfaces than plantar surfaces for all bacterial types. Staphylococci were present in 96.8% plantar samples compared to 89.6% dorsal samples. Corynebacteria were isolated from 95.3% plantar samples and 78.4% dorsal samples. GPAC were isolated from 90.6% plantar sample and only 68.3% dorsal samples. The prevalence of staphylococci and aerobic corynebacteria was 100% and 98.3% from the dorsal and 100% and 96.6%, respectively in the population sampled previously (Marshall *et al.*, 1987). GPAC's were isolated from 79.8% of all samples, which is considerably higher than previously reported levels of 6.7% from both plantar and dorsal surfaces (Marshall *et al*., 1987).

Staphylococci were present in higher numbers than aerobic corynebacteria and GPAC. The population density of staphylococci ranged from 4 to 5 $\log_{10}$ cfu/cm^2 on the plantar sites and was approximately 2.0 $\log_{10}$ cfu/cm^2 on the dorsal surface of the foot, similar to those reported previously. The mean population densities of staphylococci from a study involving 30 males and 30 females were 5.48 and 2.54 $\log_{10}$ cfu/cm^2 for the plantar and dorsal surfaces respectively (Marshall *et al*., 1987). In another study of 19 males, the mean population density of staphylococci from the plantar surface was 5.94 $\log_{10}$ cfu/cm^2, of the 19 feet studied, 17 had microflora dominated by staphylococci (Marshall *et al*., 1988). Similarly, the counts obtained for aerobic corynebacteria were also higher from the plantar sites (2.5 to 4.0 $\log_{10}$ cfu/cm^2) than the dorsal sites (1.5 to 2.0 $\log_{10}$ cfu/cm^2). Again, this reflects previous data where a study of 30 males and 30 females recovered 4.21 $\log_{10}$

448 cfu/cm^2 corynebacteria from the plantar and 2.4 $\log_{10}$ cfu/cm^2 corynebacteria from the dorsal
449 surfaces (Marshall *et al*., 1987). A study of 19 males also yielded a mean bacterial density
450 from the plantar surface of 4.92 $\log_{10}$ cfu/cm^2 corynebacteria (Marshall *et al*., 1988). The
451 mean counts for GPAC were 1.0 and 1.0 to 2.0 $\log_{10}$ cfu/cm^2 for the dorsal and plantar
452 surfaces, respectively. Previous work demonstrated higher counts for GPACs on the dorsal
453 than the plantar surface but these were in the range of 0.86 to 2.25 $\log_{10}$ cfu/cm^2 (Marshall *et*
454 *al*., 1987). The inter-toe web space is reported to sustain the highest microbial populations
455 on the foot, however, due to the absence of a suitable sampling method (i.e. ability to
456 sample bacteria per unit area) this site was not evaluated in the present study.
457 The dominance of *Staphylococcus* spp. across different foot sites was also
458 supported by the microbiomic analysis, which highlighted the fact that staphylococci
459 comprised almost the entire bacterial population on the plantar surface. This rather extreme
460 finding has not been reported previously across an entire study population but has been
461 noted in isolated individuals in previous studies (Costello *et al*., 2009; Findley *et al*., 2013;
462 Grice *et al*., 2009). This finding is potentially a consequence of the stringent subject
463 restrictions imposed during the course of the study, resulting in a very homologous microbial
464 composition residing on the plantar surface. This is a novel finding as previous studies
465 investigating the microbial composition of the plantar surface of the foot have not included
466 such clear instructions for subject adherence, in terms of bathing and the type of foot wear to
467 be worn throughout the course of the study. This has resulted in much higher levels of
468 bacterial diversity being reported on the plantar surface (Costello *et al*., 2009; Findley *et al*.,
469 2013; Grice *et al*., 2009). This imposition of a relatively uniform external environment across
470 subjects indicates that host factors do not contribute significantly to increasing bacterial
471 diversity at this site (Grice & Segre, 2011).
472 In general genomic approaches to characterize skin bacteria have revealed a much
473 greater diversity of organisms than that revealed by culture-based methods (Grice & Segre,
474 2011). However, genomic approaches to characterize skin bacteria on the foot have not
475 drastically changed our understanding of the microbiota of this site. The available

476 microbiomic data available for feet demonstrates that *Staphylococcus* is the most dominant
477 organism present on the plantar surface of the foot with *Corynebacterium* spp. also providing
478 a significant contribution (Costello *et al*., 2009; Findley *et al*., 2013; Grice *et al*., 2009). Data
479 from the present study supports the former but not the latter conclusion. The inter toe web
480 space has been shown to display a relatively low bacterial diversity, being dominated by
481 *Staphylococcus* and/or *Corynebacterium* spp. with *Betaproteobacteria* making a significant
482 contribution in some individuals (Findley *et al*., 2013; Grice *et al*., 2009).
483 This is the first study to directly compare the bacterial diversity of the plantar and
484 dorsal surfaces. Although the prevailing environmental conditions on both surfaces can be
485 considered similar i.e. high levels of moisture and high temperature, these surfaces support
486 vastly different microbial populations both in terms of composition and number. The dorsal
487 surface of the foot is known to produce more than twice as much sweat as the plantar
488 surface,(Fogarty *et al*., 2007; Smith & Havenith, 2011; Taylor *et al*., 2006) whereas plantar
489 skin displays a thickened stratum corneum, (Kim *et al*., 2010) a reduced lipid content,
490 (Egelrud & Lundstrom, 1991)contains one of the highest sweat gland densities found on the
491 human body (620 ± 120 glands cm^{-2}) (Szabo, 1962) and is subject to increased mechanical
492 stress (Kim *et al*., 2010). Which if any of these factors is responsible for the stark
493 differences in microbial numbers and diversity between these two sites remains to be
494 established, although superficially not drastically different, the intrinsic subtleties between
495 these two sites appear to be responsible for causing radical differences in the skin
496 microbiota at these two sites. Of particular importance may be the low levels of extracellular
497 lipids available to sustain the growth of Corynebacteria spp. Many *Corynebacterium* spp.
498 are known to be lipid-dependent as they lack the necessary fatty acid synthase gene
499 essential for lipid biosynthesis, rendering these microbes dependent on their host for a
500 supply of lipids to sustain growth (Tauch *et al*., 2005). Therefore, a lack of available lipid on
501 the plantar surface may be one reason why these bacteria are unable to effectively colonise
502 this site, particularly since this bacterium is unable to utilize glucose or acetate as its sole
503 carbon source.

Foot malodour has a distinct cheesy, acidic note that has been attributed to VFA's, and, in particular, to isovaleric acid (Ara *et al*., 2006; Caroprese *et al*., 2009). *Staphylococcus* spp. isolated from the human foot have been shown to convert branched aliphatic amino acids, such as L-leucine, to short-chain methyl-branched VFA's, including isovaleric acid (James *et al*., 2013). Isovaleric acid was detected in the plantar skin patches of 8 subjects but was not detected in measurable quantities in any of the dorsal samples. The fact that isovaleric acid was not detected on any skin patches obtained from the dorsal surface, but was present on the plantar surface is almost certainly attributable to the high numbers of *Staphylococcus* spp. residing at this site. Although staphylococci were present on the dorsal surface it would appear that there were insufficient numbers to produce detectable levels of isovaleric acid. The levels of other VFA's that contribute to foot odour (Ara *et al*., 2006; Caroprese *et al*., 2009; Kanda *et al*., 1990) were also significantly increased on the plantar skin patches compared to those obtained from the dorsal surface, indicating that staphylococci may play a major role in the generation of these acids also. The skin patch data supports the notion that high numbers of bacteria, in particular *Staphylococcus* spp., are implicated in the production of foot odour. Variations in the spatial distribution of these microbes appear to be responsible for the localised production of odour across the foot, a notion put forward previously but until now not verified (James *et al*., 2013).

One particular bacterium that has been linked with the generation of foot malodour is *Kytococcus sedentarius* (Marshall *et al*., 1988). Strains of *K. sedentarius* isolated from normal feet are capable of efficiently degrading callus, which is mainly composed of keratin protein, into soluble peptides and free amino acids, which may then serve as direct substrates for malodour formation (Holland *et al*., 1990). The data obtained in the current study suggests that *K. sedentarius* is not particularly prevalent on the plantar surface of the foot (20%) and that the generation of VFA's responsible for foot odour are not dependent on its presence. Whether or not this bacterium is associated with extremes of foot odour is open to question, what is clear though is that the generation of appreciable levels of odorous

VFA's can be accomplished in its absence. The absence of large numbers of certain bacteria implicated in the production of foot odour such as *K. sedentarius*, *Brevibacterium* and *Micrococcus* is in agreement with previous studies that have investigated the foot microbiome (Grice *et al*., 2009; Findley *et al*., 2013) and is not considered to be an experimental artefact given the extensive taxonomic coverage of the approach adopted.

The intensity of foot malodour would appear to be associated with an increase in the total microbial load, along with elevated skin pH and minor pitting of the stratum corneum (Marshall *et al*., 1988). Elevated hydration levels, as a consequence of the use of occlusive footwear and/or high environmental temperatures, lead to an increase in microbial numbers, particularly *Staphylococcus* spp. Under these conditions the combination of increasing levels of eccrine sweat (Harker & Harding, 2012) and callus-degrading activity by the foot microbiota (Holland *et al*., 1990), leads to the liberation of high levels of peptides and amino acids which, in turn, serve as substrates for the production of malodour. Due to differences in the spatial distribution of the microbial population across the foot the plantar surface is a major site of malodour production. Given the high microbial load and propensity of *Staphylococcus* spp. to colonise this site it is also expected that that the inter-toe web space is an important site for malodour production on the foot.

Further studies should seek to establish more direct relationships between the foot microbiota and the generation of odorous volatiles. Current sampling techniques prevent the direct sampling of the same skin sites for both microbial and volatile analysis. The development of more discreet and/or less invasive sampling strategies should remedy this issue and facilitate the direct statistical comparison of microbial and VOC datasets. It is clear from the current work that high numbers of *Staphylococcus* spp. are correlated with the production of isovaleric acid and increases in the levels of other odorous acids. Nevertheless, parallel integrated sampling would facilitate greater statistical scrutiny of such datasets and potentially help identify new causative organisms involved in foot odour production.

Acknowledgements

The authors would like to thank Dr. Gordon James and Dr Barry Murphy for critical review of this manuscript. We also thank Prof. Neil Hall and the Centre for Genomics Research (CGR), Liverpool, UK for help with pyrosequencing of the bacterial samples. Finally, we thank all subjects for their cooperation and participation in this study.

Conflict of Interest

The authors declare no conflicts of interest

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689 Williamson P & Kligman AM (1965) A new method for the quantitative investigation of
690 cutaneous bacteria. *J Invest Dermatol* **45:** 498-503.**Figures and Legends**
691

692 **Figure 1.** Mapping of bacteria across the foot at sites A to H. Bars represent mean bacterial enumeration on
693 three different media; a.SS, *Staphylococcus* selective medium; b. ACP, aerobic *Corynebacterium* spp.; c. FaNov,
694 fastidious anaerobe agar for the enumeration of Gram-positive anaerobic cocci. N = 16 subjects and left and
695 right so each bar is indicative of 32 data points. Coloured by foot area, blue = dorsal, red = plantar. Letters
696 above the bars refer to those skin sites displaying statistically significant differences in bacterial numbers
697 compared to the skin site represented by that bar.
698

699 **Figure 2**. Contour plot displaying differences in numbers of bacteria across sites A to H on the foot between
700 males and females. Bacterial enumeration data from three media ; a.SS, *Staphylococcus* selective medium; b.
701 ACP, aerobic *Corynebacterium* spp.; c. FaNov, fastidious anaerobe agar for the enumeration of Gram-positive
702 anaerobic cocci. N = 16 subjects (8 males and 8 females), left and right foot sampled so each site has 32 data
703 points. Contour plot coloured by gender and overlaid contours, blue = female, red = male.
704

705 **Figure 3.** Interpersonal variation in bacterial community composition for the 13 most predominant genera
706 observed across the dorsal and plantar skin sites.

Figure 4. Heat map representation of the prevalence of bacterial genera at plantar and dorsal sites. Colours reflect the relative abundance of genera from low (black) through blue and yellow to red (high). The genera form the rows sorted by abundance, and samples form the columns and are clustered based on the Bray-Curtis dissimilarity matrix.

Figure 5. Example of a selected ion (*m/z* 60) chromatogram obtained from GC/MS analysis of a PDMS skin patch of the A dorsal, and B. plantar surfaces of the foot of the same subject. The fatty acids identified are; 1 = acetic acid, 2 = butyric acid, 3 = isovaleric acid, 4 = valeric acid, 5 = hexanoic acid.

Table 1. Quantification of volatile fatty acids from the dorsal and plantar surface of the foot and the respective p values comparing both sites (n=10).

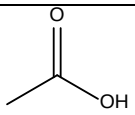
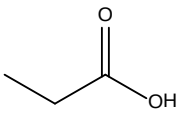
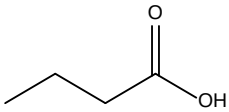
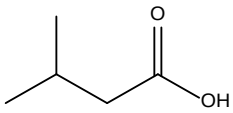
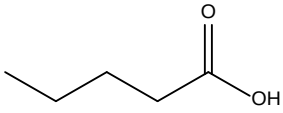
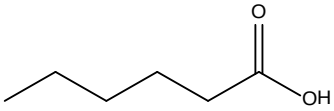
VFA	Dorsal Amount (ng) ± Standard deviation	Plantar Amount (ng) ± Standard deviation	t-test p-value
 Acetic acid	4.309 (± 1.302)	8.359 (± 3.742)	0.013
 Propionic acid	1.376 (± 0.446)	1.830 (± 0.655)	0.189
 Butyric acid	1.591 (± 0.934)	2.370 (± 0.878)	0.152
 Isovaleric acid	0 (± 0)	0.310 (± 0.256)	0.012
 Valeric acid	1.216 (± 0.584)	2.173 (± 0.84)	0.022
 Hexanoic acid	3.952 (± 2.862)	10.049 (± 4.282)	0.003

Table S1 : Thermal desorber and GC-MS conditions

Parameter	**Setting**
Markes International	**Unity 2 Thermal Desorption Conditions**
Primary desorption flow	50 $cm^3 min^{-1}$
Primary desorption temperature	180^oC
Primary desorption split	Splitless
Primary desorption time	5.0 min
Cold trap	Markes International UK general purpose hydrophobic cold trap (Part No U-T2GPH-2S)
Cold trap low temperature	-10^oC
Secondary desorption flow	1 $cm^3 min^{-1}$
Secondary desorption temperature	300^oC
Secondary desorption split	Splitless
Secondary desorption time	5.0 min
	Gas Chromatograph Conditions
Column	DB5 MS 60 m x 0.25 mm x 0.25 µm
Column flow	2 ml/min^{-1}
Carrier gas	Helium
Temperature program	40^oC initial, hold 0.0 min 5^oC min^{-1} to 300^oC, hold 8.0 min
Total run time	60 min
	Mass Spectrometer Conditions
Scan type	TIC or SIM (see table 3 for SIM target details)
Mass range	40 to 445 m/z
Scan time	0.45 secs
Ionisation	EI+
Total run time	60 min (140min)
Source Temperature	200ºC
Interface Temperature	250ºC

Table S2: Volatile fatty acid GC/MS retention times using the separation conditions as described and their respective target ions in selected ion chromatogram mode.

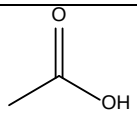
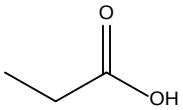
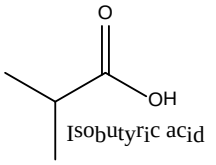
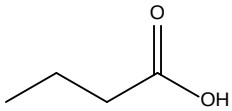
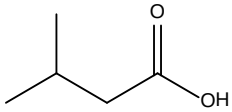
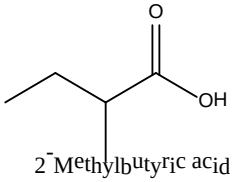
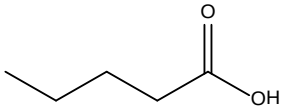
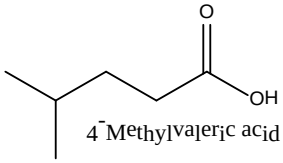
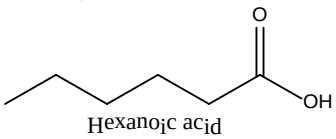
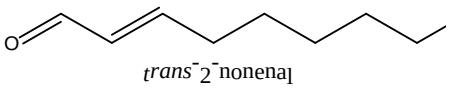
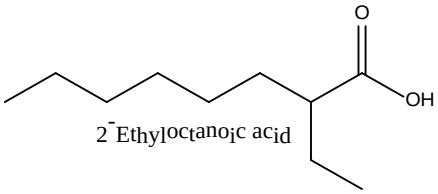
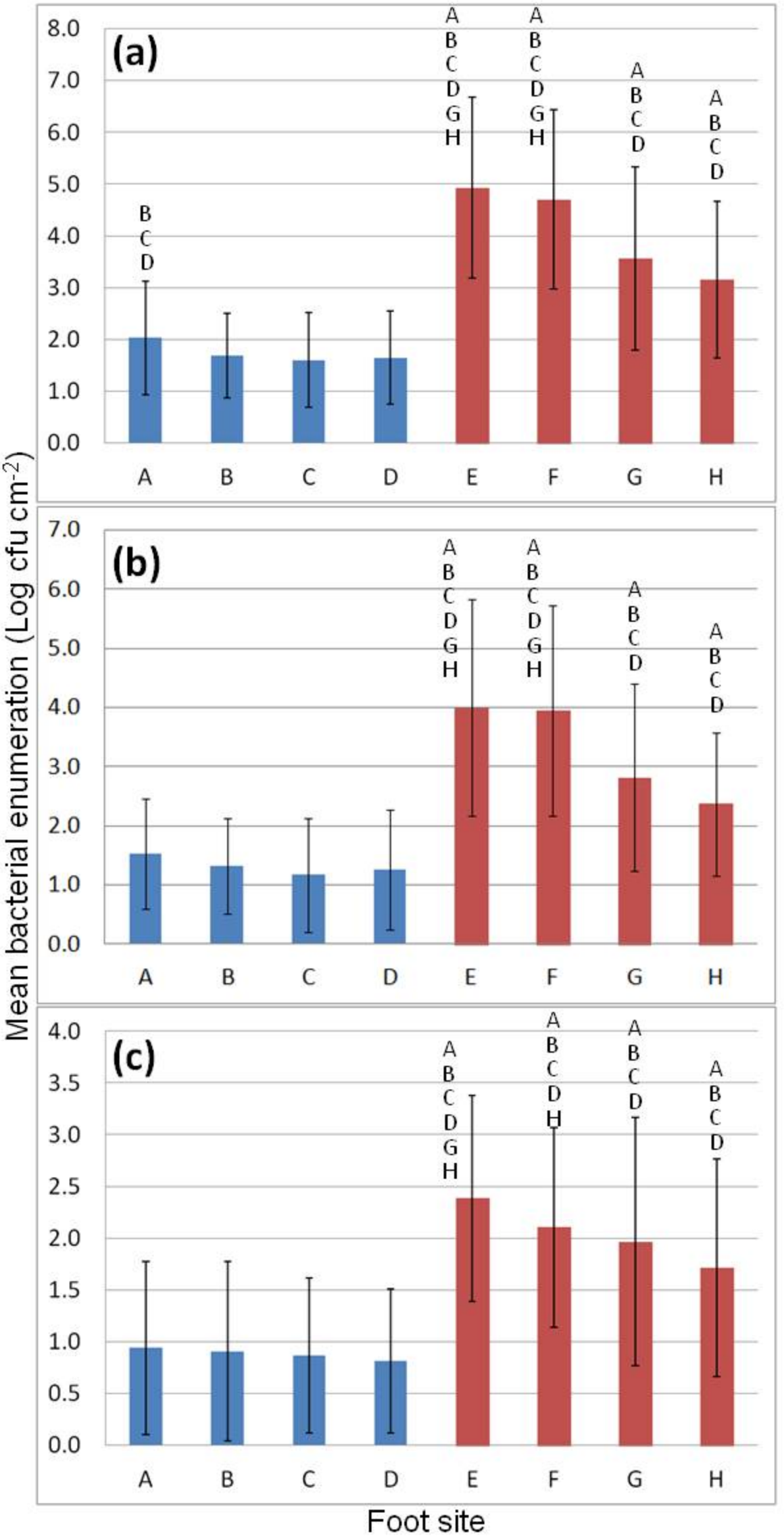
VFA	Retention time (minutes)	Target ion mass in SIC mode (*m/z*)
 Acetic acid	3.72	60
 Propionic acid	4.57	74
 Isobutyric acid	5.23	73
 Butyric acid	5.94	60
 Isovaleric acid	7.12	60
 2-Methylbutyric acid	7.41	74
 Valeric acid	8.25	60
 4-Methylvaleric acid	9.99	74
 Hexanoic acid	10.89	60
 *trans*-2-nonenal	16.04	83
 2-Ethyloctanoic acid	21.38	73

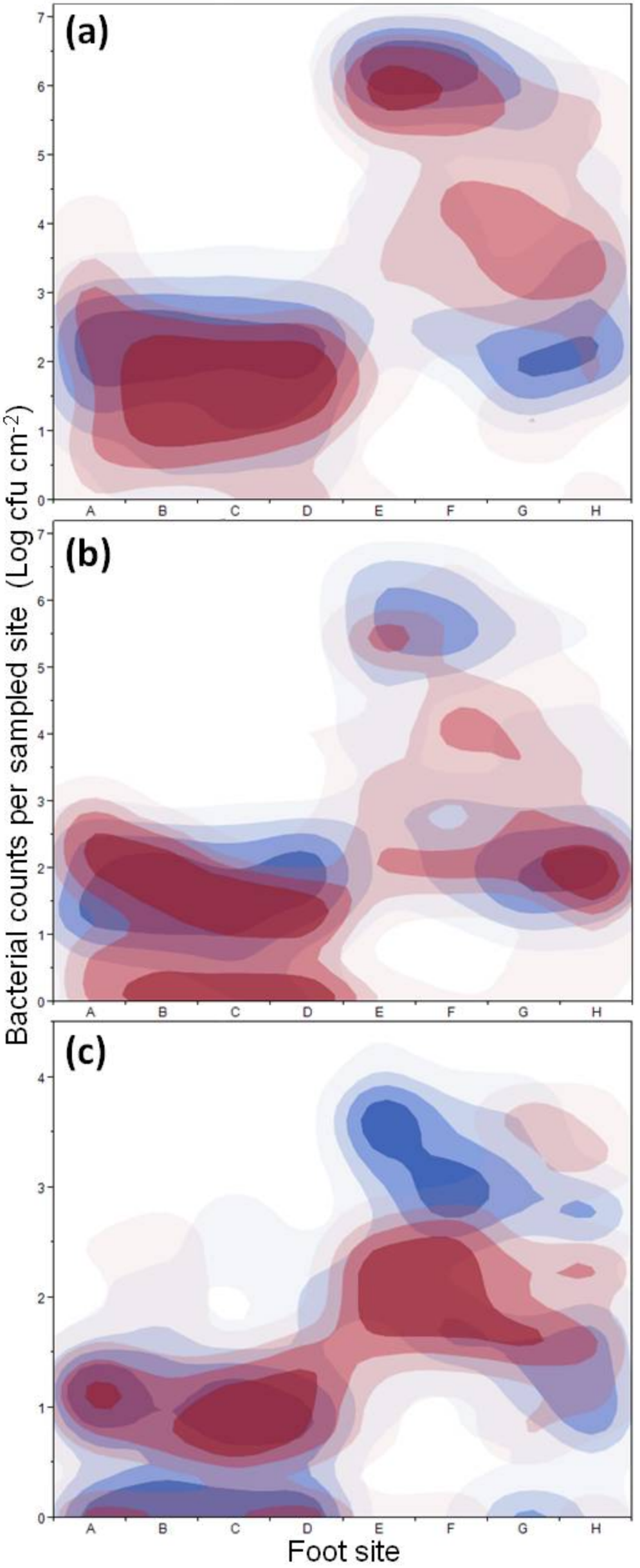
Table S3: Sequence and OTU distribution by individual sample. The table illustrates the effect of barcode & primer sequence errors (1), denoising and chimera removal (2) on the sequence data and indicates the number of OTUs obtained per sample. Data here shows 10 subjects (Sample ID) 1 to 10 together with the corresponding site sampled (D, Dorsal; P, Plantar). Total OTUs are all observations found in the individual samples.

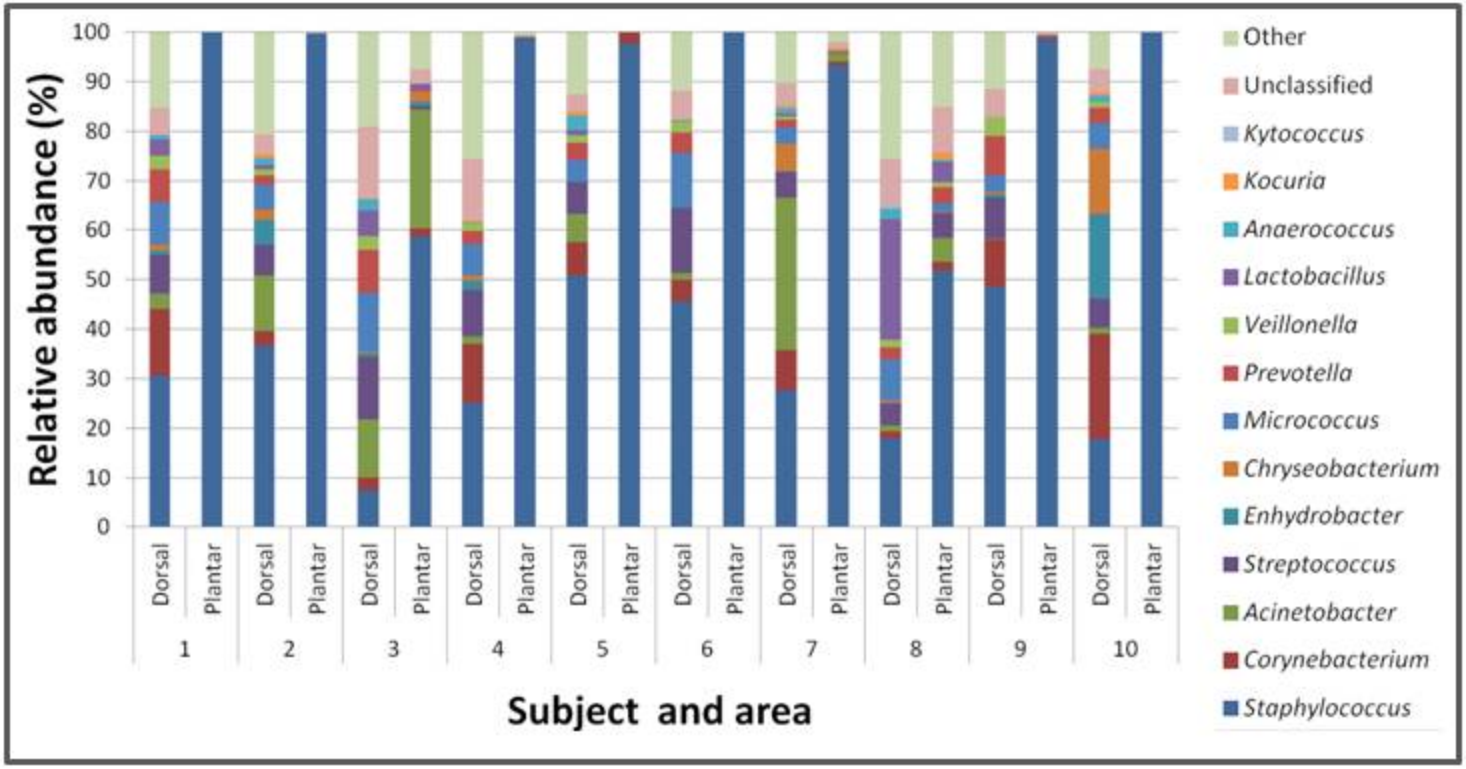
Sample	Assignable Raw Sequences (1)	Processed Sequences (2)	OTUs
1D	1030	944	82
1P	3462	3162	3
2D	2771	2583	169
2P	7800	7262	13
3D	2166	1997	124
3P	336	306	36
4D	5299	4906	212
4P	9329	8509	58
5D	1382	1307	91
5P	7581	6982	3
6D	2692	2453	95
6P	101019	65805	1
7D	8900	8296	98
7P	3610	3327	60
8D	1751	1615	131
8P	1884	1785	134
9D	4148	3764	114
9P	1542	1432	15
10D	15249	13993	244
10P	2287	2111	2
Total	**184238**	**142539**	**681**

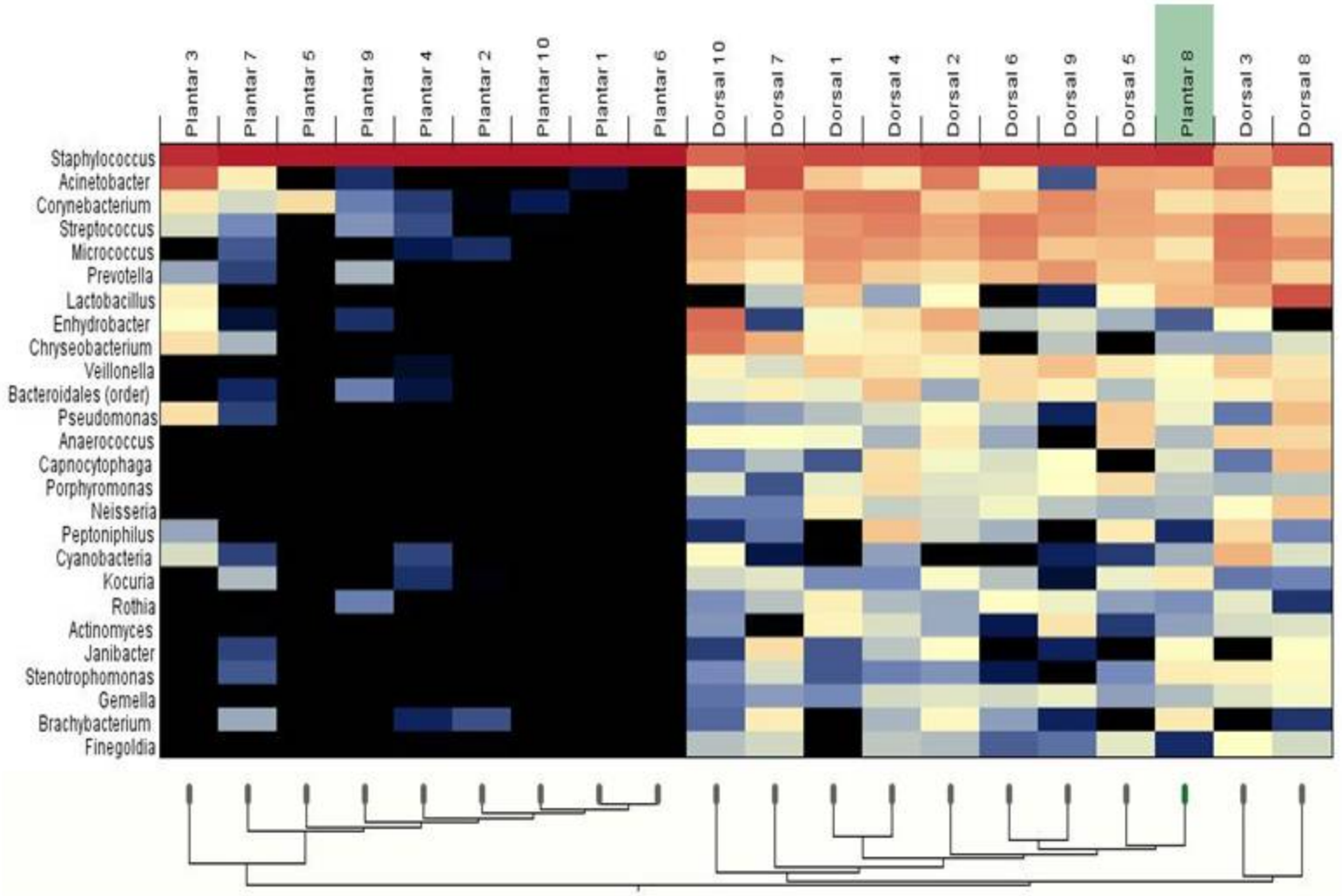
Table S4: Comparative testing using the Dirichlet-multinomial model. Genera showing evidence of a statistically significant difference between the mean relative abundance on the dorsal and plantar surfaces at the 95% confidence level.

Genus	Dorsal	Plantar	p-value	q value
Staphylococcus	0.26908	0.98605	0.0010	0.0020
Corynebacterium	0.12064	0.00228	0.0029	0.0047
Enhydrobacter	0.06548	0.00004	<.0001	<.0001
Chryseobacterium	0.05998	0.0002	0.0015	0.0026
Micrococcus	0.05512	0.00042	0.0106	0.0139
Peptoniphilus	0.00596	0.00001	0.0477	0.0490
Finegoldia	0.00464	0.00001	0.0006	0.0014
Fusobacterium	0.00323	0.00001	<.0001	<.0001
Paracoccus	0.0021	0.00001	0.0126	0.0150
Spirosoma	0.00191	0.00001	<.0001	<.0001
Aerococcus	0.00073	0.00001	0.0001	0.0003
Adhaeribacter	0.00071	0.00001	0.00001	<.0001
Arthrobacter	0.00017	0.00001	0.0204	0.0225


(a)
(b)
(c)
Mean bacterial enumeration (Log cfu cm-2)
Foot site
A
B
C
D
E
F
G
H
B C D
A B C D G H
A B C D
A B C D


(a)
(b)
(c)
Bacterial counts per sampled site (Log cfu cm-2)
Foot site
A
B
C
D
E
F
G
H
0
1
2
3
4
5
6
7


Relative abundance (%)
100
90
80
70
60
50
40
30
20
10
0
Dorsal
Plantar
1
2
3
4
5
6
7
8
9
10
Subject and area
Other
Unclassified
Kytococcus
Kocuria
Anaerococcus
Lactobacillus
Veillonella
Prevotella
Micrococcus
Chryseobacterium
Enhydrobacter
Streptococcus
Acinetobacter
Corynebacterium
Staphylococcus


Plantar 3
Plantar 7
Plantar 5
Plantar 9
Plantar 4
Plantar 2
Plantar 10
Plantar 1
Plantar 6
Dorsal 10
Dorsal 7
Dorsal 1
Dorsal 4
Dorsal 2
Dorsal 6
Dorsal 9
Dorsal 5
Plantar 8
Dorsal 3
Dorsal 8
Staphylococcus
Acinetobacter
Corynebacterium
Streptococcus
Micrococcus
Prevotella
Lactobacillus
Enhydrobacter
Chryseobacterium
Veillonella
Bacteroidales (order)
Pseudomonas
Anaerococcus
Capnocytophaga
Porphyromonas
Neisseria
Peptoniphilus
Cyanobacteria
Kocuria
Rothia
Actinomyces
Janibacter
Stenotrophomonas
Gemella
Brachybacterium
Finegoldia

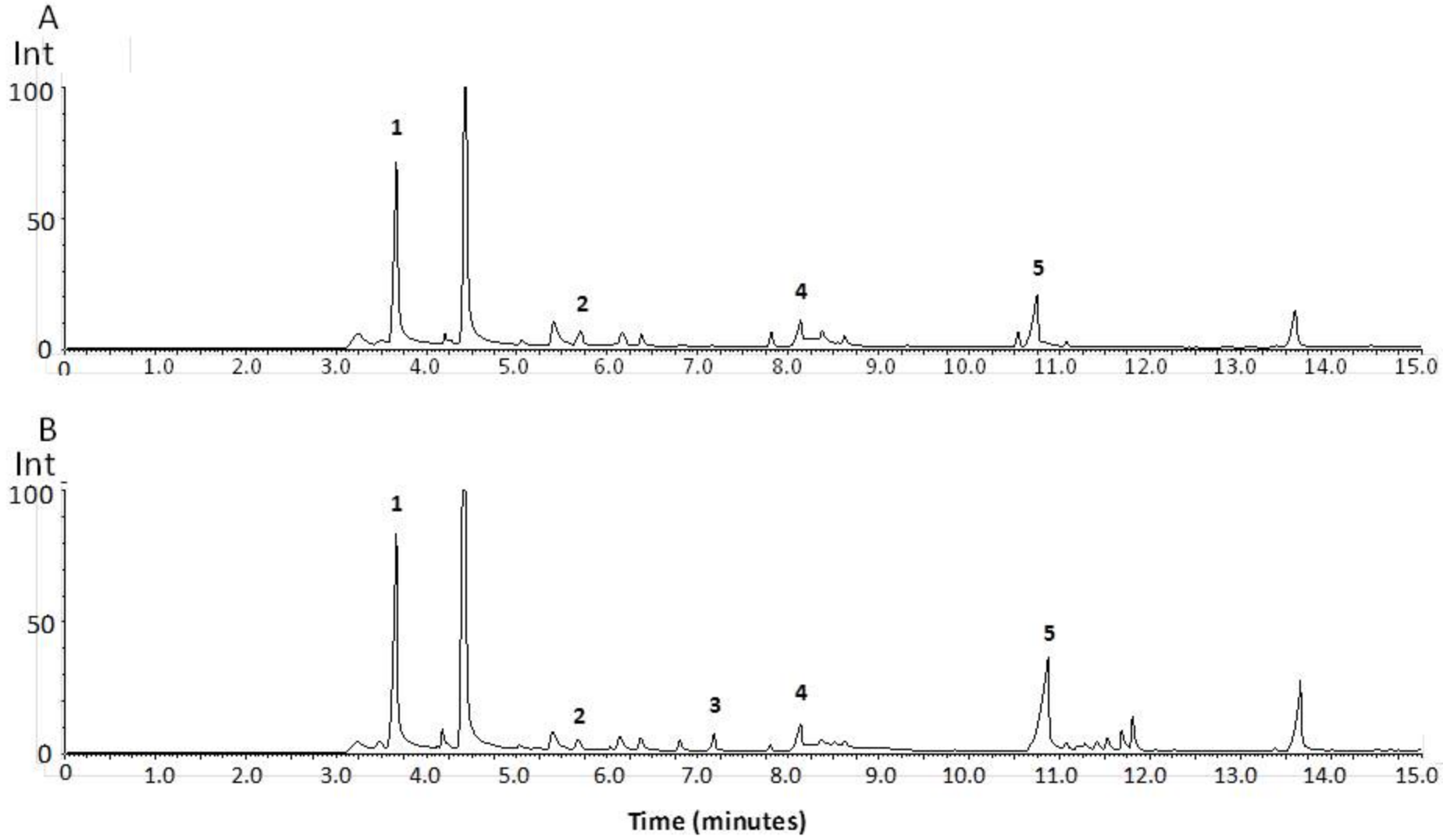

A
Int
100
50
0
1
2
4
5
0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10.0
11.0
12.0
13.0
14.0
15.0
B
Int
100
50
0
1
2
3
4
5
0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10.0
11.0
12.0
13.0
14.0
15.0
Time (minutes)

Figure S1. Diagrammatic representation of the 8 sampling sites selected for bacterial enumeration *via* culture based methods.

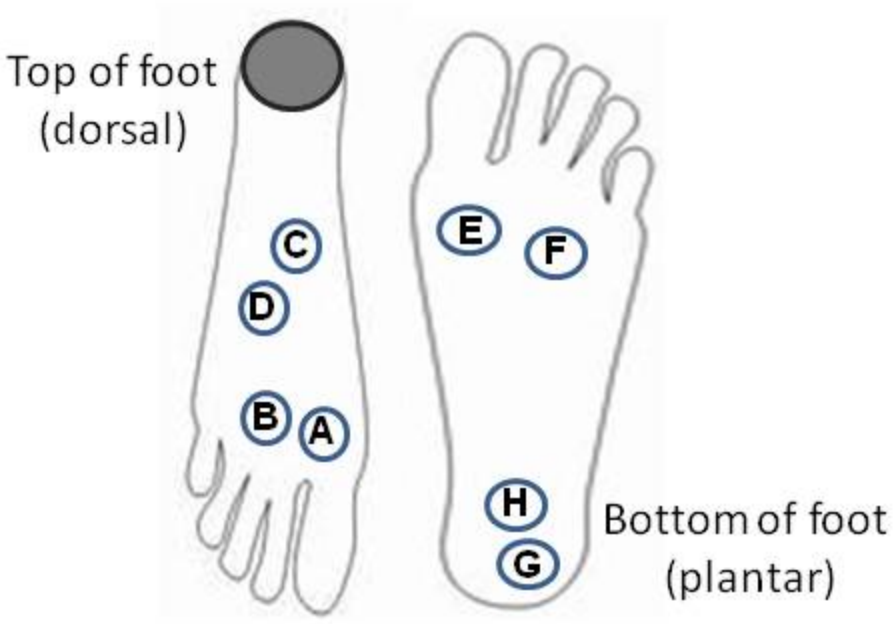


Figure S2. Predicted taxonomic coverage of primer pairs targeting different regions (V1 – V6) of the 16S rRNA gene using PrimerProspector v1.0.1. The phyla highlighted in red include genera commonly associated with the skin microbiome. The 515f/1061r primer pair are predicted to provide broader phylum coverage and were selected for this study.

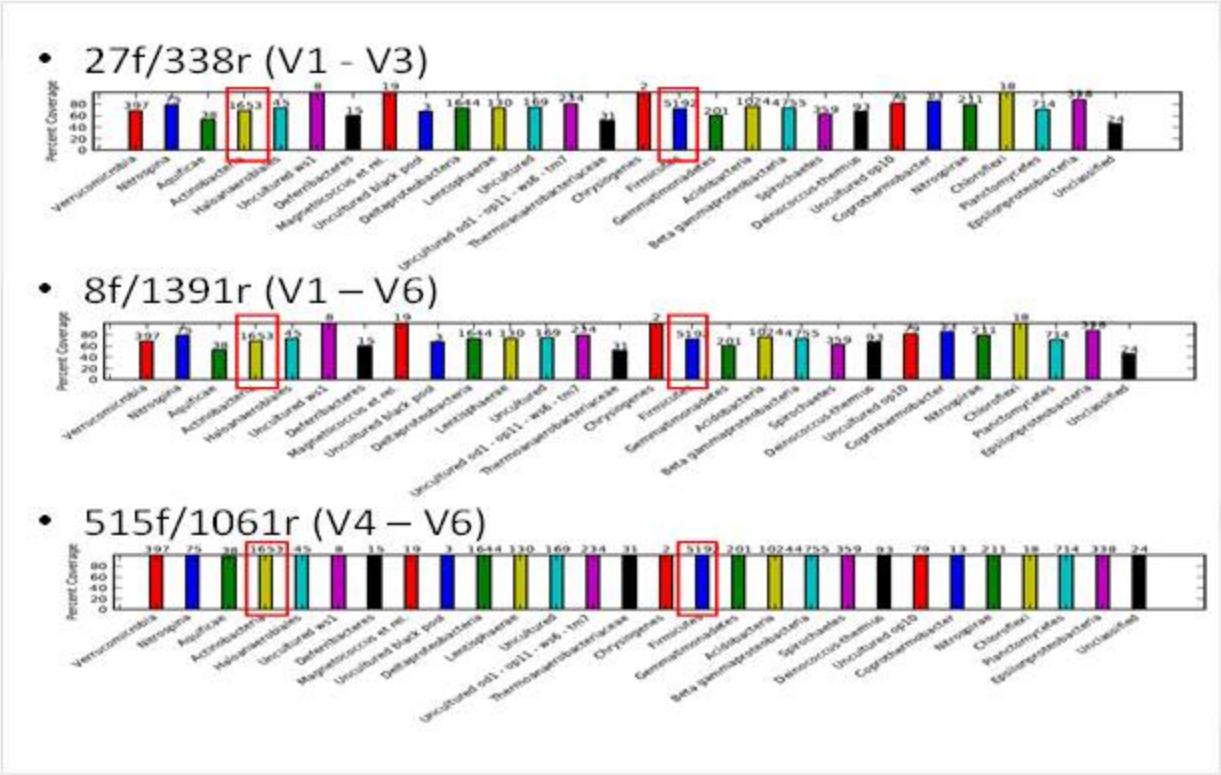


Figure S3. Mean relative abundance of the bacterial genera observed across the dorsal and plantar surfaces of all subjects.

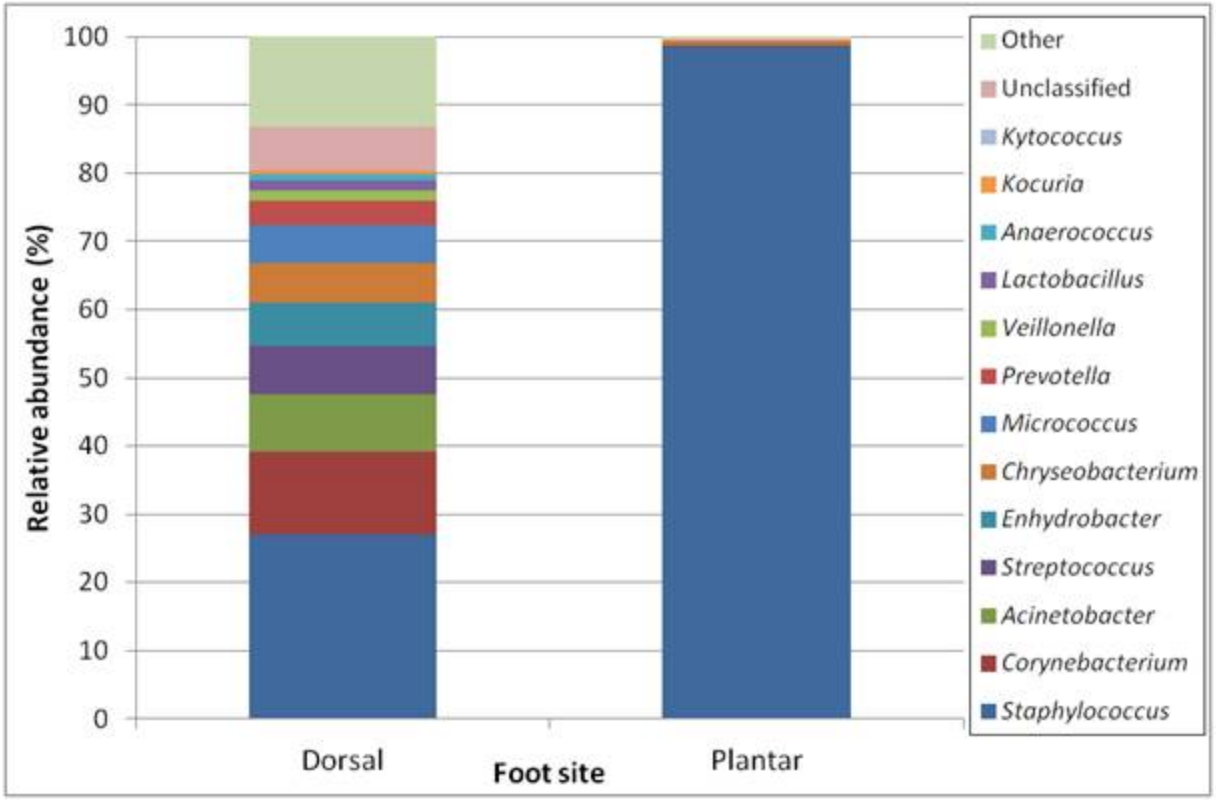
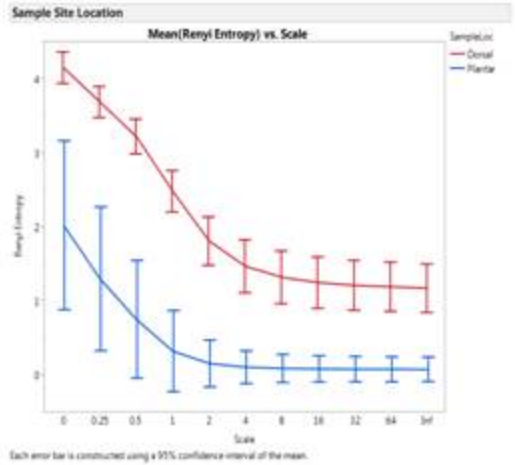
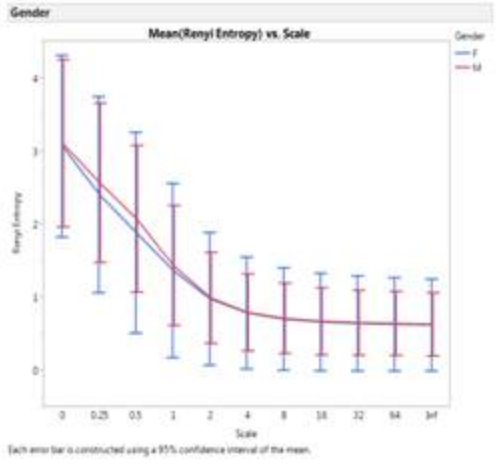


Figure S4: α diversity plots generated using Renyi Entropy calculations. This allows the estimation of diversity across abundance levels from rare species (0) to the most abundant (Inf). Significant differences in diversity are observed where there is a clear separation of the error bars which represent 95% confidence intervals. A. Comparison of the two sample sites, dorsal (red) and plantar (blue); B. Comparisons between gender, female (blue) and male (red); D. Comparison of the two sample sites, dorsal (red) and plantar (blue) excluding samples 3P & 6P; Comparisons between gender, female (blue) and male (red) excluding samples 3P & 6P.

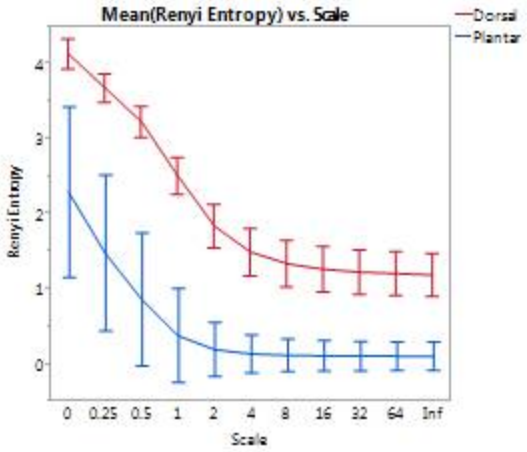
A.



B.



C.



D.

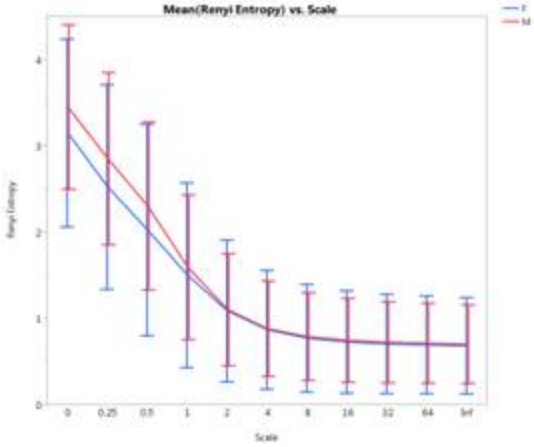


Figure 5S: β-diversity plots generated using weighted UniFrac distances. A. Comparison of the two sample sites, dorsal (red) and plantar (blue); B. Comparisons between gender, female (red) and male (blue). The mean β-diversity was tested using permutation ANOVA and revealed a statistically significant difference by foot site (Pr(>F)=<0.001) but not by gender (Pr(>F) = 0.56). C Comparison of the two sample sites, dorsal (red) and plantar (blue) excluding samples 3P & 6P; D. Comparisons between gender, female (red) and male (blue) excluding samples 3P & 6P. The mean β-diversity was tested using permutation ANOVA and revealed a statistically significant difference by foot site (Pr(>F)=<0.001) but not by gender (Pr(>F) = 0.62).