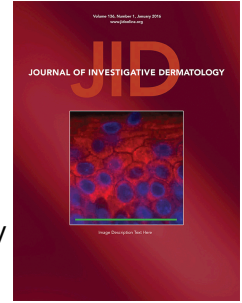# Journal Pre-proof

Bacterial microbiota composition in hidradenitis suppurativa differs per skin layer

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Bacterial microbiota composition in hidradenitis suppurativa differs per skin layer

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Abbreviations used: ASV; amplicon sequence variant, HS; hidradenitis suppurativa.

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Dear editor,

Hidradenitis Suppurativa is a chronic, inflammatory skin disease in which a shift in skin microbiota composition plays a role in the initiation and/or maintenance of the disease.(Mintoff et al. 2021; van Straalen et al. 2022; Wark and Cains 2021) Differences across studies regarding the relevant bacteria in HS persist due to the use of different sampling techniques (swabs, scrapings, or biopsies). This affects the comparability of current HS microbiome studies.(Nakatsuji et al. 2013; Prast-Nielsen et al. 2019) Therefore, in this study we analysed the microbiota composition captured at different depths of HS lesional skin using swabs, superficial, and deep biopsies in different disease stages.

In total, 96 samples were collected from skin of 32 HS patients (Hurley 1; n=10, Hurley II; n=12, and Hurley III; n=10) collected during routine surgery. The institutional review board of the Erasmus University Medical Center allows the use of surgical discard tissue for research purposes under an opt-out principle, therefore no additional informed consent was required for this study. Fifteen patients were sampled in the groin, 13 in the axilla, and four in other areas (buttock n=3, and neck n=1). The mean BMI was 29.1 kg/m^2 (±6.3) and 78% (25/32 patients) were current smokers. Detailed patient characteristics, sample collection, methods, and statistical analyses can be found in the Supplemental Table 1.

After quality control, 585 amplicon sequence variants (ASVs) were identified in 84 remaining samples (swabs; n=32, superficial biopsies; n=28, deep biopsies; n=24). After adjusting for age, BMI, smoking status, anatomical location, and Hurley stage, both superficial and deep biopsies were significantly associated with a decreased richness (p<0.001 and p<0.001) and Shannon-diversity (p<0.001 and p=0.03) when compared with swabs. In addition, adjusted for all other factors, Hurley stage III was associated with a decreased Chao1 index (p=0.02). These results show that the deeper layers of the skin, captured by biopsies, carry

specific microbiota niches colonized by a limited subset of bacteria compared to the external epidermis.

The 585 ASVs remaining after filtering belonged to Actinobacteria (147), Bacteroidetes (251), Firmicutes (184), Fusobacteria (7) and Proteobacteria (15) (Supplemental Figure 1). While prominent in HS swabs, Firmicutes and Bacteroidetes were found to be significantly less abundant in deep biopsies, respectively p=0.020 and p=0.027, Figure 1A-B, Supplemental Table 2. This was accompanied by an increase in Proteobacteria (p<0.001) in biopsies. Multivariable analysis showed that sampling technique explained 14% of the overall microbiota composition at phylum level, whereas Hurley stage and BMI accounted for 3% (p<0.001) and 2% (p<0.001) respectively. Of note, there was an increased relative abundance of Proteobacteria from swabs to superficial to deep biopsies in all Hurley stages, while the relative abundance of this phylum was low in Hurley stage III regardless of sampling technique (Figure 1C-D, Supplemental Figure 2). Both Hurley stages II and III showed a prominent decrease in Bacteroidetes from swabs to deep biopsies, where Hurley stage I showed an increase in abundance.

In line with previous studies, *Prevotella, Corynebacterium_1*, *Ezakiella*, *Porphyromonas,* and *Peptoniphilus* together accounting for approximately 80% of the total relative abundance, were the most common bacteria found in swabs (Figure 2A).(Schell et al. 2021) *Prevotella* , *Ezakiella*, *Porphyromonas,* and *Peptoniphilus* were also consistently found among the top 10 most abundant genera of superficial and deep biopsies which is consistent with previous data from HS tunnel content.(Ring et al. 2017) Univariable analysis revealed that relative abundance of *Corynebacterium_1*, *Peptonipihilus*, *Porphyromonas*, and *Finegoldia*, was significantly higher in swabs versus deep biopsies in contrast to *Pelomonas* and *Sphingomonas* which were increased in deep biopsies. (Supplemental Table 3, Figure 2B). Only, *Corynebacterium_1*, *Peptonipihilus*, and *Pelomonas*, remained significant after

multiple testing. *Corynebacterium_1* and *Peptonipihilus* have consistently been found to be enriched in HS lesional skin.(Schell et al. 2021) The fact that *Pelomonas* has not been found in HS before could be due to its existence deep in the dermis which is not captured with more superficial sampling techniques.(Bay et al. 2020; Schell et al. 2021) Multivariate analysis (permANOVA) revealed that sampling technique explained 9% of the genera variation (p<0.001), followed by Hurley severity (4%; p<0.001), and BMI (3%, p<0.001). When splitting the samples per Hurley stage *Peptonipihilus* was found to be primarily present in swabs from Hurley stage I and to a much lesser extent in Hurley stages II-III (Figure 2C). In contrast, Hurley II-III swabs showed the greatest contribution of *Corynebacterium_1*. Hurley stage III samples showed a higher relative abundance of *Prevotella* compared with Hurley stage I-II samples.

Enrichment analysis of Kyoto Encyclopedia of Genes and Genomes (KEGG) using PICRUSt2 (Douglas et al., 2020), adjusted for multiple testing, identified 'polycyclic aromatic hydrocarbon degradation' (ko00624, p=0.014), and 'benzoate degradation' (ko00362, p=0.038) pathways to be significantly enriched in deep biopsies compared with swabs, Figure 2D.

In this study we demonstrate that significantly different microbiome results are found when analysing different Hurley stages with different sampling technique. In line with previous studies, we identified *Prevotella* and *Porphyromonas* to be the most prominent genera in HS lesional skin, with the highest abundance in Hurley III samples.(Schell et al. 2021; Wark and Cains 2021) The relative abundance of *Corynebacterium_1, and Staphyloccocus,* which are main components of skin swabs, decreased significantly in deep biopsies. In contrast, *Porphyromonas* and *Prevotella_6* increased in deep biopsies relative to

4

swabs. Superficial biopsies seemed to have an microbiota composition between swabs and deep biopsies.

Studies of *Prevotella* and *Porphyromonas* in HS-associated diseases demonstrate that they can drive Th17 immune responses, leading to an upregulation of key HS cytokines (e.g., IL-23A, IL-17, and IL-1), and can stimulate epithelial cells to promote neutrophil recruitment through IL-8 and IL-6 secretion.(Larsen 2017; van Straalen et al. 2022) Potentially, this microbiota driven effect could be an understudied factor in the poorer clinical response of Hurley stage III patients to adalimumab treatment.(Kimball et al. 2016) As such, combining antibiotics with biologics might be needed to achieve higher response rates.

Using enrichment analysis of KEGG pathways we found not previously described enrichment of pathways associated with xenobiotic metabolism in deep biopsies.(Ring et al. 2017; Schneider et al. 2020) These pathways have previously been found in the skin microbiota of individuals exposed polycyclic aromatic hydrocarbons, which can be found in pollution and tobacco smoke.(Leung et al. 2020) Enrichment of these pathways could be driven the higher number of smokers in our study (78.1% vs 67% and 45.5%) or be a result of our deep sampling technique.(Ring et al. 2017; Schneider et al. 2020)

A major strength of our study is that we used the recommended hypervariable regions for skin microbiota studies (V1-V3) in contrast to many previous studies.(Mintoff et al. 2021; Wark and Cains 2021) Limitations include the relatively small sample size and the absence of healthy control skin samples.

In conclusion, our data demonstrates a marked shift at both phylum and genus level between swabs and deep biopsies, further diversified by different patterns in different Hurley stages. Therefore, we recommend that in future HS microbiota studies both swabs and (deep) biopsy samples should be assessed in HS patients and analyses should be stratified for Hurley stages.

DATA AVAILABILITY

Raw fastq files containing the 16S RNA microbiome data were uploaded to the Mendeley Data and Digital Commons Data and will be publicly available on January 2024. The data can be downloaded from 10.17632/knwhjtwb46.

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CONFLICT OF INTEREST STATEMENT

CW, CBA, and RK report no conflicts of interest. LMP: Consultant at Centogene GmbH. EPP: Consultant, speaker, principal investigator or received grants from AbbVie, Amgen, Biogen, Celgene, Eli Lilly, Janssen-Cilag, Novartis, Pfizer, and UCB. KRvS: Consultant / received honoraria from Novartis, UCB, Boehringer-Ingelheim.

AUTHOR CONTRIBUTION STATEMENT

Conceptualization: EPP; Data Curation: LMP; Formal Analysis: LMP; Investigation: CBA, RK, KRvS; Methodology: RK, EPP; Resources: RK; Visualization: LMP, KRvS; Writing – Original Draft: CBA, KRvS; Writing – Review & Editing: LMP, CW, CBA, RK, EPP, KRvS

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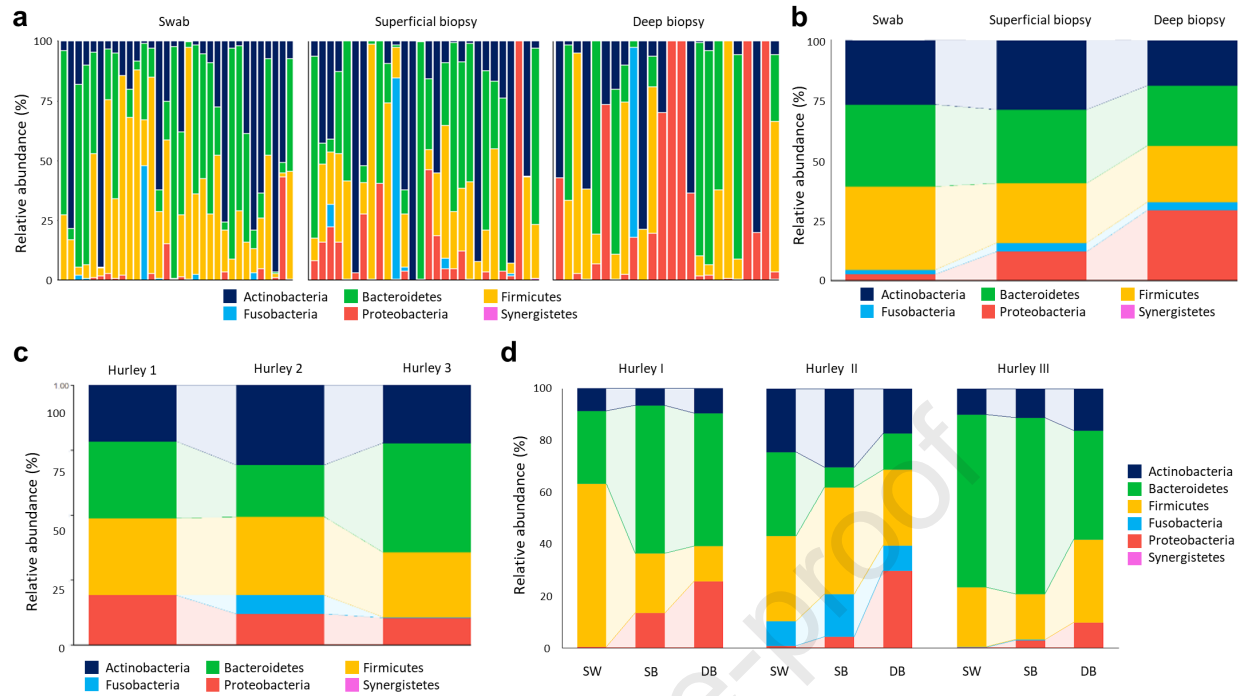
FIGURE LEGENDS

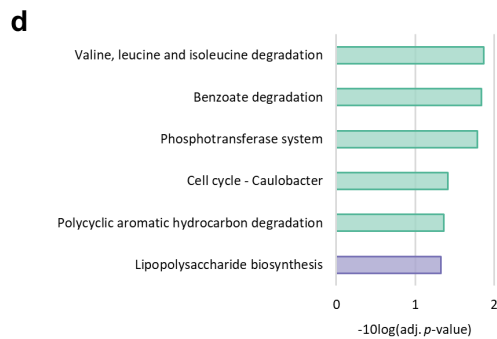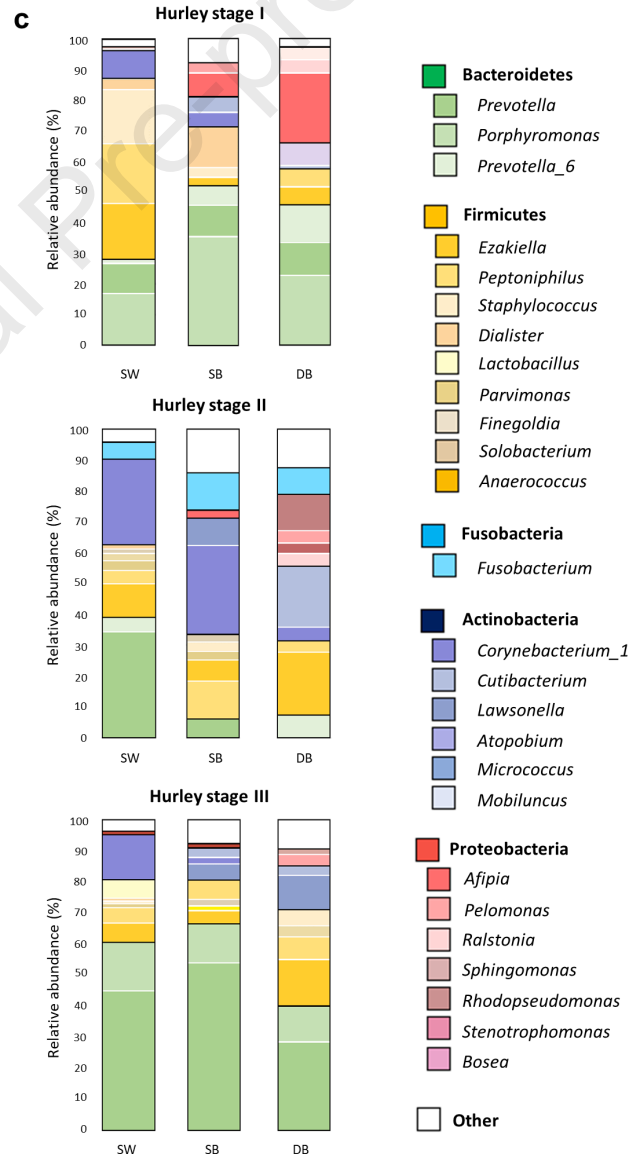**Figure 1. Phylum differences per sampling technique and Hurley stage..**

(A) Bar chart showing the relative abundance of each phylum per sample stratified for sampling technique. (B) Bar chart depicting the relative abundance of each phylum per sampling technique. (C) Bar chart showing the relative abundance of each phylum in each sample stratified per Hurley stage. (D) Bar chart showing the phylum composition per Hurley stage, stratified for sampling technique.

Figure 2. Genera differences per sampling technique and Hurley stage.

(A) Sunburst graphs showing the relative abundance of the top 10 genera stratified per sampling technique. (B) Bar charts depicting the relative abundance of the genera found to differ significantly between swab and dep biopsies, superficial biopsies not analysed. (C) Bar charts showing the relative abundance of the top 10 genera for each Hurley stage stratified for sampling technique. SW; swab, SB; superficial biopsy, DB; deep biopsy. (D) Bar chart showing the enriched KEGG pathways in swabs versus deep biopsies (purple), and in deep biopsies versus swabs (green).

a

Swab | Superficial biopsy | Deep biopsy

- **Bacteroidetes**
  - *Prevotella*
  - *Porphyromonas*
- **Firmicutes**
  - *Ezakiella*
  - *Peptoniphilus*
  - *Staphylococcus*
  - *Dialister*
  - *Lactobacillus*
- **Actinobacteria**
  - *Corynebacterium_1*
  - *Cutibacterium*
  - *Lawsonella*
- **Proteobacteria**
  - *Afipia*
  - *Pelomonas*
- **Fusobacteria**
  - *Fusobacterium*
- **Synergistetes**
- **Unknown**
- Other **phyla**/genera not among top 10

b

c

Hurley stage I

- **Bacteroidetes**
  - *Prevotella*
  - *Porphyromonas*
  - *Prevotella_6*
- **Firmicutes**
  - *Ezakiella*
  - *Peptoniphilus*
  - *Staphylococcus*
  - *Dialister*
  - *Lactobacillus*
  - *Parvimonas*
  - *Finegoldia*
  - *Solobacterium*
  - *Anaerococcus*
- **Fusobacteria**
  - *Fusobacterium*
- **Actinobacteria**
  - *Corynebacterium_1*
  - *Cutibacterium*
  - *Lawsonella*
  - *Atopobium*
  - *Micrococcus*
  - *Mobiluncus*
- **Proteobacteria**
  - *Afipia*
  - *Pelomonas*
  - *Ralstonia*
  - *Sphingomonas*
  - *Rhodopseudomonas*
  - *Stenotrophomonas*
  - *Bosea*
- **Other**

Hurley stage II

Hurley stage III

d

- Valine, leucine and isoleucine degradation
- Benzoate degradation
- Phosphotransferase system
- Cell cycle - Caulobacter
- Polycyclic aromatic hydrocarbon degradation
- Lipopolysaccharide biosynthesis

-10log(adj. *p*-value)

Swabs | Superficial | Deep

SUPPLEMENTAL MATERIAL

MATERIAL AND METHODS

Study samples

A swab, superficial biopsy, and deep biopsy were collected from a single lesion before the start of routine surgery in 32 patients with hidradenitis suppurativa at the Department of Dermatology of the Erasmus University Medical Center in Rotterdam, The Netherlands, between January and September 2018

Inclusion criteria were adult patients with physician diagnoses hidradenitis suppurativa undergoing routine excision of active inflamed lesions at the Erasmus University Medical Center between January and September 2018. Exclusion criteria were: oral and topical antibiotic use for at least two weeks prior to surgery, or immunosuppressive or immunomodulatory therapies including biologics for at least three months prior to surgery. The Erasmus University Medical Center has an opt-out principle for the use of surgical discard for research. As such this study required no required no additional informed consent.

Sample collection and processing

One swab, one superficial biopsy, and one deep biopsy were taken sequentially from the exact same location. Swabs were collected using a pre-moistened (sterile NaCl 0,9%) cotton swabs which were rubbed on the skin for 30 seconds. Subsequently, a superficial 4-mm punch biopsy was taken from the same lesion as the swab. The deep biopsy was taken from the skin opening formed by the first superficial biopsy, obtaining the deeper dermal tissue exposed by the superficial biopsy. The skin swabs and biopsies were stored in empty 1.5 mL sterile tubes and snap-frozen in liquid nitrogen. All samples were stored at -80°C until further analysis.

DNA extraction

DNA was extracted using the DNA Extraction Kit on the Arrow pipetting instrument (DiaSorin S.P.A., Saluggia, Italy). Swabs and biopsies were treated with DNA Pretreatment Buffer 2 and Proteinase K for 30 minutes at 56°C. Subsequent DNA isolation was performed in the Arrow instrument in batches of 12 samples per run according to the manufacturer's protocol. DNA concentration was measured using the Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific, Waltham, MA). All extracted DNA was stored at -20°C.

16S rRNA Gene Polymerase Chain Reaction Amplification and Sequencing

The V1 to V3 variable regions of the 16S rRNA gene were amplified using the 27F-519R primer pair and dual indexing as previously described (Fadrosh et al., 2014). The pools were purified using Agencourt AMPure XP (Beckman Coulter Life Science, Indianapolis, IN) and the quantity of the pool was assessed using the Quant-iT PicoGreen dsDNA Assay Kit. PhiX Control v3 library (Illumina Inc., San Diego, CA) was spiked into (~10%) the pool prior to sequencing on an Illumina MiSeq sequencer (MiSeq Reagent Kit v3, 2 x 300 bp). Amplicons were normalized and pooled in one batch. Details of the procedure of amplicon purification are presented in the supplementary information. All samples were sequenced in one batch. Negative controls were added to control for contamination.

Bioinformatic analysis

Raw reads from Illumina MiSeq were de-multiplexed and trimmed following an in house bioinformatic pipeline (see Sanders et al for details) (Callahan et al., 2017, Sanders et al., 2021) Trimmed FASTQ files were further processed using the DADA2 pipeline. (Callahan et al., 2017) Quality filtering was performed in DADA2 using the following criteria: trim=0, maxEE=c(2,2), truncQ=2, rm.phix=TRUE. Filtered reads were run through the DADA2

Amplicon Sequence Variant (ASV) assignment and ASVs were assigned a taxonomy from the SILVA version 132 rRNA database (Quast et al., 2012) using the RDP naïve Bayesian classifier.(Wang et al., 2007). Data tables were combined into a phyloseq object using Phyloseq.(McMurdie and Holmes 2013)

Quality control of the microbiome data

Data quality control was done on the microbiome data. We first filtered out obvious contaminant phyla (*Planctomycetes, Epsilonbacteraeota, Cyanobacteria, Chloroflexi, Ambiguous taxa, BRC1, WPS-2, FBP and Deinococcus-Thermus*). Next, we included bacteria that were present in at least 5% of the dataset. Further, we included samples with at least 100 counts.

Statistical analysis

Baseline characteristics were reported using frequency (percentage) for all used categorical variables. To compare the profile of bacteria within the different layers of the skin, the alpha (α)-diversity was calculated using Chao1 and Shannon diversity. Chao1 α-diversity is an estimation of the richness of the sample, while Shannon α -diversity gives an estimate of the relative distribution. Differences in Chao1 and Shannon diversity between sampling procedure were tested using linear mixed models to accommodate the paired nature of the data (different sampling techniques from the same patient) and the p-values were adjusted for pair-wise comparisons. We used the package nlme (Pinheiro and Bates, 2000) and function lme using Chao1 and Shannon-diversity as outcomes and sampling as predictor and adjusting for disease severity (Hurley stages), age sex, smoking status, anatomical location and BMI. After this analysis we only carried for further analysis the variables that were statistically significant.

Before the formal statistical testing we used normalised the count data using the centered-log ratio approach to account for unequal library sizes and compositionality of the data. This data transformation is used to analyze data derived from Next Generation Sequencing (NGS) since the data is expected to be compositional (data conveys only relative information since the total abundance is unknown). (Gloor et al., 2017, Gloor et al., 2016) For this we first imputed zeros assuming that any ASV observed in more than one sample could appear in another sample if sequenced with infinite depth, which can be modelled using Bayesian-Multiplicative replacement of count zeros.(Palarea-Albaladejo and Martín-Fernández, 2015). Next, permutation multivariate analysis of variance (PermANOVA) (Kelly et al., 2015) was used to test for differences in microbiome composition and adjusted for BMI, severity (Hurley stage I, II and III) and anatomical region of sampling (ST) using id as stratifying variable to account for correlated data. This was done at both phylum and genus level. For the latter we agglomerated the ASVs into genus level using functions from the phyloseq package in R. In total, we analysed 41 genera.

Next, compositional data analysis (CoDA) was performed to test for differential abundance between specific amplicon sequence variants (ASVs) using the ALDEx2 package.(Fernandes et al., 2013) . For this analysis we focussed on the differential composition between swabs and deep biopsies using paired t-test and correcting for multiple testing. Significant p-values were defined with a threshold of p-value=0.05 (Fernandes et al., 2013) Nominal p-values were adjusted using a Benjamini-Hochberg correction.
All the packages were incorporated in the Rv.3.5.2 (Eggshell Igloo version).

Lastly, we carried out pathway analysis of the 16S RNA sequences to predict functional pathways from the ASVs (bacterial pathways) using PICRUSt2 (Douglas et al., 2020) with parameter defaults. We focused on the KEGG pathways because previous studies used this dataset for *in silico* functional pathway analysis. We used reads corresponding to

584 ASVs selected after filtering. Next, we tested for differences in the pathway abundances between swabs and deep biopsies using paired t-test. The latter analysis was done using ALDEx2, since this are also compositional data.

RESULTS

Description the microbiome

A total of 96 samples were analysed from 32 HS patients. The total number of reads was 1007659 with a median count of 9068 (range 257-22629) and an average number of 10282 reads per sample. The proportion of singletons was 29%. After quality control 585 ASV remained for analysis.

SUPPLEMENTARY TABLES

Supplemental Table 1. Patient characteristics

	HS patients *(n=32)*	
Sex		
Female, *n (%)*	16	(50.0)
Age, *mean ± SD*	37.3	±12.5
BMI, *mean ± SD*	29.1	±6.3
Smoking status		
Current smokers, *n (%)*	25	(78.1)
Hurley Stage		
Hurley stage I, *n (%)*	10	(31.3)
Hurley stage II, *n (%)*	12	(37.5)
Hurley stage III, *n (%)*	10	(31.3)
Sample location		
Axilla, *n (%)*	13	(46.6))
Groin, *n (%)*	15	(46.9)
Other*, *n (%)*	4	(12.4)

HS; hidradenitis Suppurativa, SD; standard deviation, BMI; body mass index. *Other includes neck (n=1) and buttocks (n=3).

Supplemental Table 2. Multivariate analysis reveals sampling technique and Hurley stage significantly influence diversity indices

	Chao1 index			Shannon diversity index		
	Effect size	**(SE)**	**P-value**	**Effect size**	**(SE)**	**P-value**
Hurley severity						
Hurley I		*Reference*			*Reference*	
Hurley II	22.3	(16.5)	0.18	0.29	(0.27)	0.30
Hurley III	33.8	(15.68)	**0.02**	0.30	(0.26)	0.25
BMI	-0.3	(1.09)	0.77	-0.01	(0.02)	0.44
Age	0.46	(0.54)	0.39	0.01	(0.01)	0.13
Smoking status						
Non-smoker		*Reference*			*Reference*	
Smoker	8.19	(16.11)	0.61	0.17	(0.27)	0.53
Site Location						
Axilla		*Reference*			*Reference*	
Groin	-8.79	(14.47)	0.54	-0.08	(0.24)	0.71
Other	16.10	(22.13)	0.47	-0.01	(0.36)	0.92
Sampling technique						
Swab		*Reference*			*Reference*	
Superficial biopsy	-38.16	(9.42)	**<0.001**	-0.32	(0.15)	**<0.001**
Deep biopsy	-55.23	(9.96)	**<0.001**	-0.72	(0.15)	**0.03**

SE; standard error, BMI; Body mass index

Supplemental Table 3. Paired analysis at genus level between deep biopsies and swabs

Genus	Welsh test	
	P-value *raw*	**P-value** *adj.*[1]
Corynebacterium_1	**0.001**	**0.008**
Pelomonas	**0.002**	**0.018**
Peptoniphilus	**0.002**	**0.023**
Porphyromonas	**0.011**	0.060
Finegoldia	**0.023**	0.086
Sphingomonas	**0.046**	0.130
Anaerococcus	0.051	0.139
Cutibacterium	0.063	0.161
Ezakiella	0.069	0.190
Escherichia/Shigella	0.120	0.228
Prevotella_6	0.101	0.228

[1] Benjamini-Hochberg adjusted P-value.

SUPPLEMENTAL FIGURES

Supplemental Figure 1. Prevalence of amplicon sequence variants

Scatter plot depicting the prevalence of major phyla as a fraction of the samples over their total

abundance.

Supplemental Figure 2. Phylum composition of each sample stratified for Hurley stage

Bar chart of the relative abundance of each phylum stratified for Hurley stage.

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