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PhD Thesis

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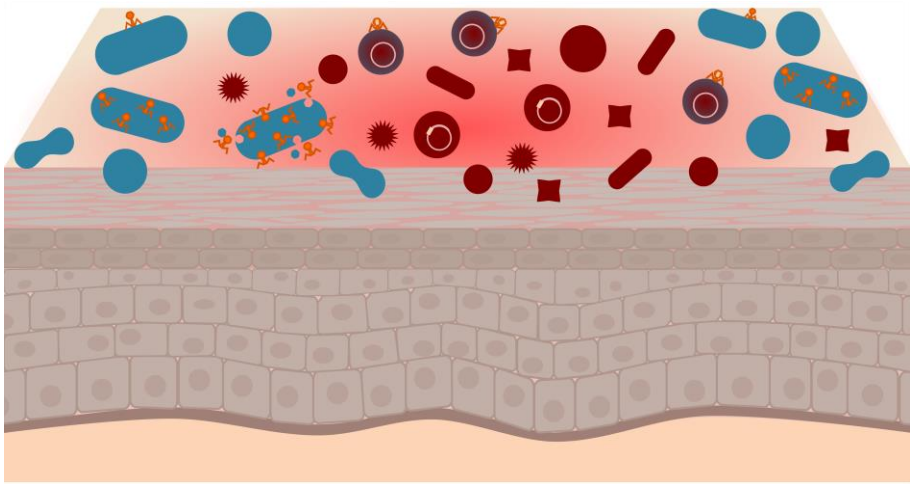
The skin microbiome in atopic dermatitis



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The skin microbiome in atopic dermatitis

PhD Thesis
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An ongoing prospective randomized clinical study entitled “Changes in the normal and atopic dermatitis skin microbiome in relation to environmental exposures”, characterizing the effect of a moisturizer, the fragrance farnesol and farnesol as an ingredient in a moisturizer on the microbiome of normal and atopic skin (appendix I)

Preface

This thesis is based on research carried out at the National Allergy Research Centre, the Department of Dermatology and Allergy, Copenhagen University Hospital Herlev and Gentofte, and Department of Microbiology, Tumor and Cell Biology, Karolinska Institute, Stockholm between 2015 and 2020. This project was funded by the Danish Environmental Protection Agency and received financial support from the Aage Bang's Foundation, Inge and Asker Larsen's Foundation, A.P. Møller Foundation and internal financial support from the Hospital, all gratefully acknowledged.

First, I would like to thank all participants providing material for the scientific work presented here.
I am grateful to my principal supervisor Professor Jeanne Duus Johansen for giving me the opportunity, trust, and guidance in initiating work on a new field in house and grow as a project leader and scientist. I have always met an open door to Jeanne's office and even though her desk was cluttered, there has always been room for an extra pile of documents. The working conditions and atmosphere on the National Allergy Research Centre have been friendly, supportive and inspiring with a lot of competent collaborating people throughout my stay. A special thanks to Anne Marie Topp and Lene Toft-Jensen for helping me with project logistics and keeping a cheerful mood. Anna Bünning Olsson for superb graphical support and Julie Sølberg for humorous and professional inputs. During this project, I have also become a mother to two girls and have looked forward to coming back after each leave. For this I would like to thank all former and present colleagues. I am thankful to my co-supervisor Lone Skov for introducing me to the dermatological clinic and her scientific and practical guidance. I thank Lars Engstrand and his group of skilled molecular biologists and bioinformaticians in Sweden for fruitful cooperation.

Finally, I would like to thank my husband and "fourth supervisor" Eik Dybboe Bjerre for his willingness to discuss every matter and together with my parents and parents-in-law being supportive in the entire process. My children Elin and Kaisa, thank you for bringing joy, laughter and perspective. In the final phase of this PhD, the corona virus came along complicating everyday life and resulted in a slimmer version of the thesis than initially planned. Hopefully the reader appreciates this.

Rie Dybboe Bjerre,
Copenhagen, April 2021

Abbreviations

AD: Atopic dermatitis

S. aureus: *Staphylococcus aureus*

UV: Ultraviolet

SC: Stratum corneum

FFA: Free fatty acid

AMP: Antimicrobial peptide

IgE: Immunoglobulin E

C. acnes: Cutibacterium acnes

M. globosa: Malassezia globosa

MRSA: Methicillin-resistant *Staphylococcus aureus*

TLR2: Toll-like receptor 2

Th2: T-helper cell 2

FLG: Filaggrin gene

NGS: Next generation sequencing

PCR: Polymerase chain reaction

bp: Base pair

OTU: Operational taxonomic unit

NCBI: National center for biotechnology information

MGS: Metagenomic species

SCORAD: Scoring atopic dermatitis

EASI: Eczema area and severity index

HECSI: Hand eczema severity index

ET: Exfoliative toxin

M. luteus: *Micrococcus luteus*

M. osloensis: *Moraxella osloensis*

[P.] humerusii: [Propionibacterium] humerusii

R. mucosa: Roseomonas mucosa

Table of contents

Summary

The human skin is colonized by a variety of microorganisms, interacting with the host and modulating immunity. In atopic dermatitis (AD), the pathogenic bacterium *Staphylococcus aureus* expand, worsening disease. Technological advances now allow characterizing whole communities of microbiota and enhance our knowledge on imbalanced microbial compositions, dysbiosis. The overall aim was to gather knowledge on the skin microbiome and its role in AD, establish methodology and investigate the skin microbiome, including bacteria, fungi and virus, across multiple skin sites in patients with AD.

In an initial systematic review, we identified AD skin to have low bacterial diversity, most pronounced at lesional sites, with higher relative abundance of *S. aureus* and *S. epidermidis* and lower abundances of *Cutibacterium* and *Malassezia*. A causal role of dysbiosis in dermatitis was indicated. We furthermore identified great variability in methodological approaches, likely affecting the outcomes. In comparing sampling strategies in our methodological study, eSwabs were preferable. Reducing human DNA in vitro before shotgun metagenomic sequencing did not skew microbial populations, however, may have caused low success of library preparation in the case-control study. The case-control study comprised 10 adult AD patients and 5 skin-healthy age- and sex-matched controls sampled on 14 non-overlapping skin areas. The microbial compositions differed clearly between AD and control, most pronounced at the flexures and neck. Besides from the phenotype found in our systematic review, AD skin was characterized by higher relative abundances of *Moraxella osloensis* and *Micrococcus luteus* and lower relative abundances of *S. hominis* and *C. acnes*. The AD-virome had increased abundances of *Propionibacterium phages, PHL041* and *PHL092*, and *Staphylococcus epidermidis phages, CNPH82* and *PH15*. Higher absolute abundances of *Staphylococcus* phages, *Ipla5* and *Ipla7*, in lesional AD skin. We found great subject specificity in skin microbiomes, including strains of *S. aureus*.

In conclusion, robust and standardized methodologies for investigating the skin microbiome is still warranted and we would recommend dealing with human DNA computationally, post metagenomic sequencing. Dysbiosis in AD involve both the bacteriome, mycobiome and virome. The results imply a key role of the skin microbiome in AD where phages support the bacterial dysbiosis, potentially by lysing commensals and providing *S. aureus* and *S. epidermidis* with virulence genes.

Dansk resumé

Menneskets hud er massivt koloniseret af mikroorganismer, som interagerer med værtens celler og modulerer immunsystemet. Ved atopisk eksem er der høj forekomst af den patogene bakterie *Staphylococcus aureus* (*S. aureus*), som kan virke forværrende på eksem. Nye teknologiske metoder gør det nu muligt at karakterisere hele samfund af mikroorganismer og øge vores viden om ubalance i sammensætningen af mikroorganismer, dysbiosis. Det overordnede formål med denne afhandling var at samle viden om hudmikrobiomet og dets rolle ved atopisk eksem, etablere metode til at undersøge hudmikrobiomet; bakterier, svampe og virus, på adskillige hudområder hos patienter med atopisk eksem.

I en indledende systematisk litteraturgennemgang identificerede vi, at der hos personer med atopisk eksem var lavere diversitet af bakterier i huden, særligt på læsionel hud, med højere forekomst af *S. aureus* og *S. epidermidis* og lavere forekomst af *Malassezia* og *Cutibacterium*. Dysbiose så ud til at kunne forårsage eksem. Vi identificerede ydermere stor variation i metodologi, hvilket højst sandsynligt påvirkede resultaterne af undersøgelserne.

I vores metodestudie sammenlignede vi to måder at tage prøver fra huden og fandt, at eSwabs var at foretrække. Vi reducerede humant DNA før shotgun metagenomisk sekventering, hvilket ikke påvirkede sammensætningen af mikroorganismer, men det kan have forårsaget at mange af vores prøver ikke kunne opbygge biblioteker til sekventering i vores case-control studie. I case-control studiet tog vi prøver fra 14 ikke-overlappende hudområder fra 10 patienter med atopisk eksem og 5 hudraske alders- og kønsmatchede kontroller. Der var markant forskel på sammensætning af mikroorganismer på huden mellem patienterne med atopisk eksem og de hudraske, særligt på halsen, i albuebøjninger og knæhaser. Vi genfandt de karakteristika for hudmikrobiomet ved atopisk eksem, som vi fandt i litteraturgennemgangen, og så også øget forekomst af bakterierne *Moraxella osloensis* og *Micrococcus luteus* og mindre forekomst af *S. hominis* og *C. acnes*. Viromet ved atopisk eksem var karakteriseret ved øget mængde af to *Propionibacterium phager*, *PHL041* og *PHL092*, samt *S. epidermidis phagerne CNPH82* og *PH15*. Læsionel hud havde større mængde af *Staphylococcus* phagerne *Ipla5* og *Ipla7*. I alle studier var der store individuelle forskelle i hudmikrobiomet, også i stammer af *S. aureus*.

Vi konkluderer, at der stadig mangler robuste og standardiserede metoder til at undersøge hudmikrobiomet og vi anbefaler at håndtere det humane DNA efter metagenomisk sekventering. Dysbiosen i hudmikrobiomet ved atopisk eksem omfatter både bakteriomet, mykobiomet og viromet. Resultaterne indikerer, at hudmikrobiomet har en stor rolle ved atopisk eksem og at phager understøtter dysbiosen, sandsynligvis ved at lysere fredelige bakterier og give *S. aureus* og *S. epidermidis* virulens gener.

1. Introduction

Trillions of bacteria, archaea, fungi, viruses and small arthropods colonize the human skin. It has long been recognized that the microbiota is linked to human health and disease, often by focusing on pathogens involved in inflammatory conditions[1]. Atopic dermatitis (AD) is one such condition characterized by a disturbed skin microbiota with overgrowth of *Staphylococcus aureus* (*S. aureus*) compromising the abundance of other potential beneficial microorganisms. By studying genetic material from microbiota (the microbiome), recent methodological advances have enabled us to characterize whole skin microbial communities more thoroughly than ever before. This has reopened a wealth of questions, including which microbes are present on the skin surface in different conditions, how they maintain health or contribute to disease and how dermatological practices alter the communities. The main focus of the work for this PhD thesis was to examine the skin microbiome in AD using different methodological approaches.

1.1 The skin

The human skin, lung and gut are considered the largest organs in the body. Historically, the skin has been described as a dense mechanical 2 m^2 barrier protecting us from dangerous substances in our surrounding. Recently, this view has evolved and nowadays it is regarded as an active app. 25 m^2 organ[2] comprising a variety of chemical mediators and cells – structural, immune and microbial[3] - all important in regulating water loss, moisture and body temperature, transmitting sensations, protecting us from UV light, chemicals and pathogens. The commensal microbiome prevents pathogens from establishing residence by physically occupying the space but the extent to which the commensals provide other benefits is still an open research field. In this section, the skin is examined as a habitat for microbial colonization.

1.1.1 Physical and chemical skin characterization

The skin is divided in two sections: 1) The inner dermis composed of connective tissue, capillary and lymphatic vessels and 2) the outer epidermis primarily consisting of keratinocytes (Figure 1). The epidermis is a self-renewing organ with a turnover time of approximately 1 month in humans[4]. Epidermal stem cells in the basal layer (*stratum basale*) continuously divide and move upward while differentiating resulting in 4-5 distinguishable layers: *Stratum basale, stratum spinosum*, *stratum granulosum, stratum lucidum (*in thick skin of palms and soles[5]) and *stratum corneum* (SC). In the granular layer, cells produce and store lipids with antimicrobial activity[6,7] and proteins (including filament aggregating protein, filaggrin), which are involved in later packing of the cytoskeleton when the keratinocytes collapse to flat corneocytes in the SC. The SC comprises 15-30 layers of corneocytes and the structure resembles bricks (corneocytes) and mortar (lipids – ceramides, cholesterol, free fatty acids (FFAs))[8], well fulfilling the barrier function of keeping microorganisms out of our internal bodily milieu. This wall-like structure of the SC also retains water, and central to this function is natural moisturizing factor (NMF) residing in the

corneocytes and consisting of amino acid breakdown products from filaggrin[9], lactic acid, urea, citrate and sugars[10] (Figure 1). Filaggrin breakdown products also contribute in upholding a slightly acidic pH[11], which typically is in the range of 4-6 in intact skin.

The surface of SC is perturbed by openings from hair follicles and glands (Figure 1). The hair follicle exudes lipid-rich sebum manufactured from an attached sebaceous gland – forming the pilosebaceous unit. Eccrine sweat glands release saline water and apocrine sweat glands secrete a viscous, lipid-rich product also containing protein, sugar, and ammonia into hair follicles when they are hormonally stimulated (first in puberty)[12].

1.1.2 Immunological skin characterization

A variety of both innate and adaptive immune cells populate the dermis and epidermis. Furthermore, most non-immune cells sense danger signals via pattern recognition receptors and produce cytokines upon activation, involved in local immune responses[7]. Here follows a brief description of selected cells and molecules functioning in protection from pathogenic microbial invasion.

In the epidermis, the main immune active cells are keratinocytes, Langerhans cells (dendritic cells) and the adaptive tissue-resident memory T cells (Trms)[7] (Figure 1). Keratinocytes produce antimicrobial peptides (AMPs) – capable of disrupting bacterial membranes and implicated in modulating host immune responses, e.g. by recruiting different immune cells[7]. Langerhans cells detect intruding antigens and can migrate to draining lymph nodes and present them, whereby they initiate immune responses or promote tolerance to self-antigens. However, they also regulate adaptive immune responses locally in the epidermis[13].

In the dermis, a broader set of immune cells take residence[3] (Figure 1) and many produce AMPs[7]. Mast cells, eosinophils and natural killer cells are granular cells rich in cytotoxic proteins released upon stimulation, including recognition of microbial pathogens[7].

Dermal dendritic cells and macrophages engulf intruders; however, most often act differently hereafter. Dermal dendritic cells migrate to lymph nodes. Macrophages produce cytokines affecting the immune response evoked, i.e. which CD4+ T helper cells gets activated[7]. Th1 cells produce interferon-γ and IL-12 in response to intracellular infections, recruiting phagocytotic cells. Th2 cells produce a range of cytokines in response to helminth infections, recruiting eosinophils and affecting B-cell differentiation and antibody production in attempt to expulse the parasite. Th17 cells produce IL-17 recruiting granulocytes, playing a role in defense against extracellular bacteria and fungi[14].

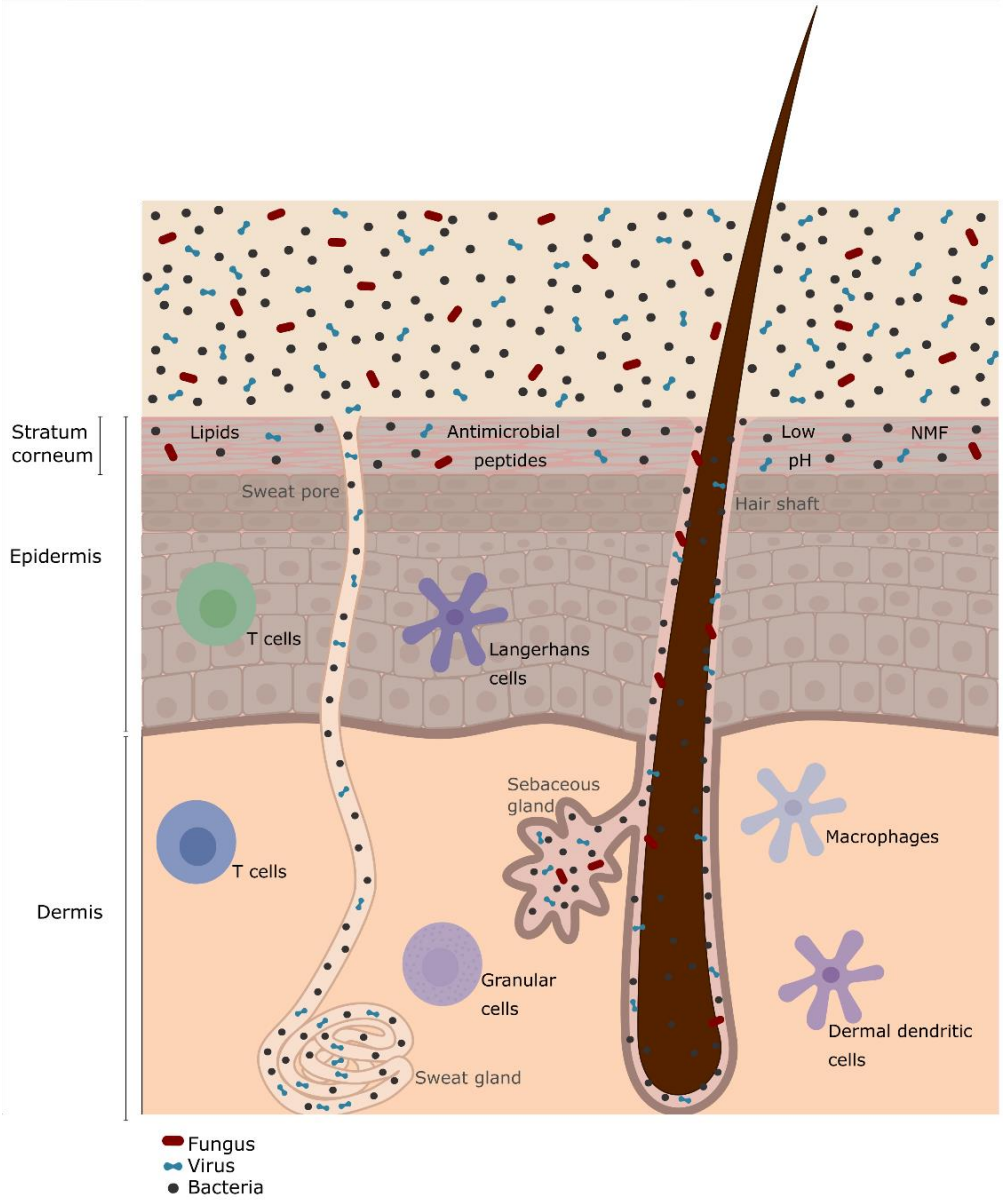

Figure 1: Sections and components of the skin barrier.
Abbreviation: NMF, Natural Moisturizing Factor.

1.1.3 Skin topography

The skin environment varies across the body with zones being composed differently regarding skin thickness, densities of hair follicles and glands[12]. Some regions of the skin are higher in temperature and humidity due to partial occlusion. Immune cells in the skin may also be differently distributed, e.g. with more Trms on sites being more exposed to antigens and mast cells being found most numerous in the arms and legs[7]. Furthermore, topographical variation in chemicals in the skin can appear due to extrinsic factors being selectively used on certain skin sites, e.g. hygienics or cosmetics. Some sites are also more exposed to UV light than others.

1.2 Microbial inhabitants of the skin

The epidermal surface of the skin might seem hostile for microorganisms with an acidic pH and the constant shed of corneocytes. In utero, fetal skin is nearly sterile, but colonization occurs immediately after birth when the skin is exposed to the outside environment. Vaginally delivered infants acquire bacterial communities resembling their mother's vaginal microbial community, whereas cesarean section infants harbor bacterial communities more similar to those found on their mother's skin surface[15]. These initial communities are transient, however, it is not known exactly how long time this pattern persists and whether there are associated long term effects. In establishing the microbial communities on skin, antenatal (e.g. maternal diet), perinatal (e.g. delivery mode, early bathing) and postnatal (e.g. animal contact) factors are all thought to play a role[16]. At 2 months of age, the amount of *Lactobacillales* is high. During childhood the diversity of bacteria increases. Reaching puberty, colonization by *Actinobacteria*, including *Cutibacterium acnes (C. acnes)*, increases and the microbial communities on skin resemble that of adults[17]. It is estimated that about 1 billion bacteria inhabit a typical square centimeter of skin, covering the skin surface and extending down into the appendages and glands[18]. The exact number of microorganisms per square centimeter has been a subject of research for many years. Mary Marples depicted the density of bacterial populations and certain microorganisms on homunculi and it was already apparent approximately 50 years ago that the load of microbial inhabitants varied between individuals and across topographical habitats with "*the sparsely inhabited desert of the forearm*" and "*the heavily populated tropical forest of the axilla*"[19]. Furthermore, it was generally agreed that densest bacterial populations were found on the face, neck, axilla and groin. Whereas the trunk and upper arms were more sparsely populated and the microbial community on feet and between toes were large and diverse[19].

Mary Marples and other prominent scientists used culture-based approaches to describe microbial inhabitants on the skin[20]. Though they were limited by providing specific growth conditions, landmark findings were made. They found the bacterial domain to dominate skin. Few fungal members were found and were mainly species of *Malassezia,* except from fungus growing between the toes. The *Malassezia* species seemed to be dependent on lipids from the host and could under some circumstances be involved in skin infections[21].

Viruses living on the skin could not be studied due to methodological limitations[19].

Members of the healthy skin bacterial communities were found to include *S. epidermidis*, and the genera *Corynebacterium*, *Propionibacterium* (today *Cutibacterium*), *Brevibacterium* and *Micrococcus*[20]. Bacteria were found by microscopy to be located on the skin surface and beneath superficial cells of SC. Openings of hair follicles contained numerous microorganisms, often associated with sebum[22]. A more recent study on the location of skin bacteria using tape stripping found 85% of the total bacterial load within the first 6 corneocyte layers and 25% localized within the hair follicles[23]. It has long been realized that *Propionibacterium* thrive in the anoxic environment in pilosebaceous units, utilizing the lipids[24].

1.3 Microbial community analysis

Investigating microorganisms is a field with a backbone of technological advances. With the building and use of microscopes magnifying objects from 25-250-fold, microorganisms were discovered between 1665 and 1678[25]. In the 19th century it was established that they could cause disease[26], but later in the same century, the bacteria *Escherichia coli* was found to be present in the intestinal microbiota of both healthy children and children with diarrhea[27]. In subsequent decades, bacteria were discovered throughout the human body, including the skin, by use of culture-based approaches selecting for single microorganisms that thrive in artificial growth conditions. As many organisms have narrow windows of growth, much of our knowledge of bacterial physiology stem from a small subset of easily cultivated bacteria[28]. It is estimated that >90% of microbial species cannot be readily cultured[29]. Approaching the end of the 20th century, the term “microbiome” appeared in the literature, describing whole microbial communities and their activity[30]. Original approaches for investigating the microbiome targeted marker genes for upregulation by polymerase chain reaction (PCR). In prokaryotes the ~1500 bp 16S component of the small subunit (30S) of the ribosomal RNA (rRNA) gene is a great marker gene, as is consists of both conserved and variable regions. The conserved regions allow universal amplification and the variable makes discrimination between specific bacteria possible. It was first used in 1977[31] and still preferred today. Originally, the gene was PCR amplified and products (amplicons) were separated in electrophoretic devices and banding patterns used to compare changes in taxonomic compositions, even though different organisms could give rise to identical bands[28]. To assign taxonomy to the bands, DNA from high abundance bands was selected and cloned into a vector, transformed into bacteria, plated and then colonies were picked for Sanger sequencing[32]. As each colony represented one 16S rRNA gene, this approach could typically analyze 10-100 sequences per sample[32], providing a larger view of the bacterial diversity than before, but still not capturing the rare biosphere.
The rise of Next Generation Sequencing (NGS) in 2006 changed the field dramatically. Millions of DNA fragments could now be sequenced simultaneously directly from amplified pools of 16S rRNA genes, at a fraction of the cost[32]. The first NGS platform used was 454 pyrosequencing[33], followed by Illumina[32], which now generates the vast majority of the world’s sequencing data.

In amplifying the DNA fragments, two sets of constructs are ligated, flanking the fragments and being complementary to flowcell oligos (Figure 2). These constructs also contain sample-specific barcodes, allowing many samples to be run together (multiplexing), and sequencing primer binding sites. When fragments attach to the flow cell, a complimentary strand covalently bound to the flowcell is synthesized. The original strand is washed away, leaving out fragment copies in a mixture of orientations. 1000 copies of each fragment are generated by bridge amplification, where DNA polymerases create a reverse strand, both strands are released, straightened and form new bridges for amplification resulting in clonal clusters of both reverse and forward strands.

For sequencing, Illumina uses a “sequence by synthesis” approach (Figure 2): One of the two adaptor constructs are cut off ensuring that the primers attach to the same end and copies are sequenced in the same direction. Fluorescently labelled nucleotides, with different colors for different bases, are added and during each sequencing cycle, one fluorophore attach to the growing strands. Laser excites the fluorophores and an optic scanner collects the light emission from each clonal cluster. The cycle is repeated to create the desired read lengths (n bases). For paired end sequencing the templates are stripped, bridge amplification again results in clusters with different orientations and then the other adaptor construct is cut off and sequencing performed again.

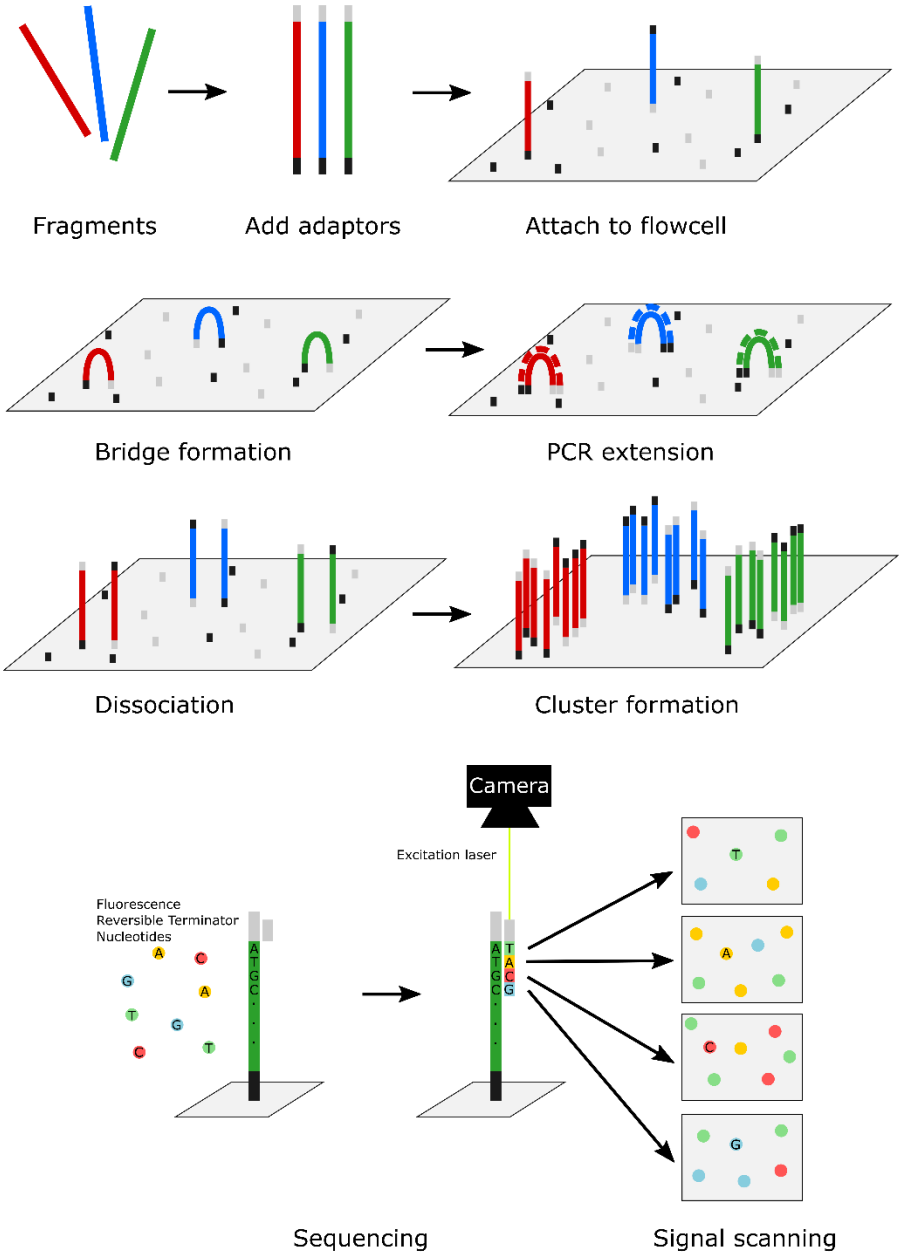

Figure 2: Outline of workflow in Illumina sequencing
The figure is adapted from Illumina.com (“An introduction to Next-Generation Sequencing Technology”)

In 2005, a single sequencing run generated one gigabase of data, which increased to 1.8 terabases in 2014, increasing read depth. Given a limited budget, one wishes to reach the minimum amount of reads to uncover the full richness of the samples studied. In fulfilling this, a standard number is around 20,000 reads per sample[34].

The initial sequence reads are then processed and non-biological sequences, e.g. primer and adaptor sequences, removed. Next, sequences cluster according to similarity in Operational Taxonomic Units (OTUs)[28] (Figure 3), which can be done by different approaches[28]. Using Usearch/Usparse, each sequence is compared to a "centroid": Sequences are sorted by abundance, and the first sequence is considered a centroid. The second sequence is compared to the first, and is either incorporated into the same cluster, if they are sufficiently similar (threshold is typically 97% or 99%) or becomes a centroid for the second cluster. This process is repeated until all samples have been assigned. Sequencing data are often summarized as a table of read counts per OTU per sample. As the total number of reads may vary greatly between multiple samples, a normalization of data is necessary for comparing relative abundances. This can be done by simply dividing the counts by the total count of the sample[28]. Finally, OTUs are assigned a taxonomic classification at the genus level, and species level when possible, using alignment-based methods and a reference database[35].

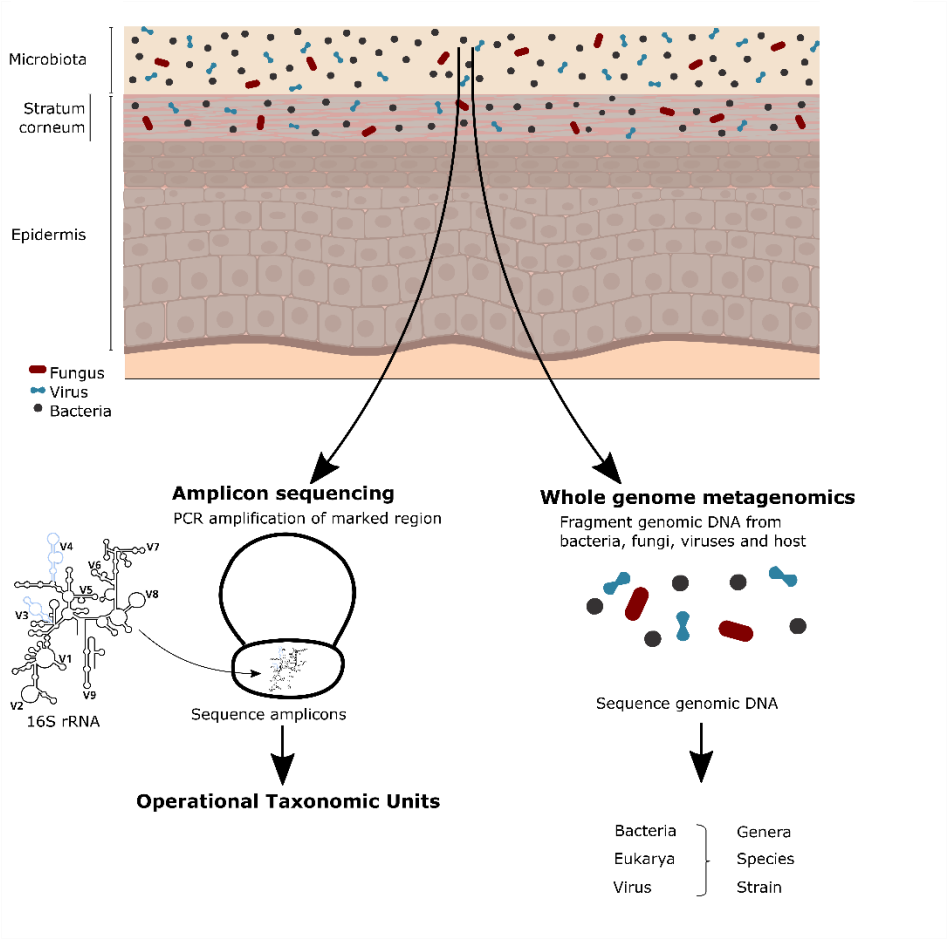

Figure 3: 16S rRNA gene versus shotgun metagenomics sequencing

Though the 16S rRNA gene is widely used as a biological fingerprint for bacterial species, also in skin microbiome surveys, there are some limitations. These include the unilateral focus on bacteria and archaea and risk of skewing the population structure due to errors and bias during PCR resulting in overamplification of some rRNA genes (well described in Hugerth et al., 2017[28]). Over the past few years, the pace of advance in microbial ecology has increased further and today whole genome shotgun sequencing is increasingly being used in skin microbiome studies. In this method, DNA from a sample is fragmented randomly followed by NGS, generating primer-free sequences from all DNA material in a sample, allowing for domain and virus abundances to be inferred (Figure 3). The resolution is also high enough for species and even strain level classification. However, as the human genome is app. 1000 times larger than bacterial genomes, reads from human DNA constitute a great amount of the total reads. Handling of the human DNA can be done by removing it in vitro before sequencing or computationally post sequencing. Assembling genes and genomes from this data is also complicated[32] and both reference-based and reference-free methods can be applied. Current computational approaches to classify metagenomic data mostly apply reference genomes from cultivated microbes. However, such genomes represent only a fraction of the species and viruses present in samples.
In this thesis we used the National Center for Biotechnology Information's (NCBI) Reference Sequence Database[36] to assign taxonomy by either blasting sequence reads or mapping sequence reads to a gene catalog with 234 metagenomic species (MGS) with co-abundant genes[37], where the catalog has been blasted to NCBI.

1.3.1 Alpha- and beta-diversity

When characterizing microbial communities, the diversity is a crucial parameter and refers broadly to the complexity of the microbial composition in a community.
Alpha diversity is generally understood as the diversity within a single sample. Alpha diversity can be determined as the richness of species in a sample, simply by counting the number of different OTUs or MGS's in a sample. As it most often requires too big a sampling effort, or simply is impossible, to identify every single taxon in a sample, different techniques are developed to account for this incompleteness[28]. The most common approach for microbial studies is nonparametric estimation based on mark-release-recapture statistics for estimating the size of animal populations. These approaches consider the proportion of species that have been observed before ("recaptured") and those observed only once. In a very diverse community, the probability that species will be observed more than once will be low whereas in a uniform community, the probability that a species will be observed more than once will be higher[38]. The Chao1 estimate of richness is based on this principle.
In addition to the number of species in a sample, the evenness of their distribution is also relevant. For instance, the evenness is high in a sample of 10 OTUs where each compose 10%. Shannon's entropy index combines species richness and evenness. It gives a low score to a community dominated by few species (even though the total number of species is high), and high score to communities where many different species have relatively high or similar abundances.

It is worth noting, that both the Chao1 and Shannon metrics give an equal weight to each OTU or MGS, whereby a community composed of 10 species from a single genus and a community composed of 10 species from 10 different phyla are given the same diversity[28]. To avoid this effect, other researchers prefer the phylogenetically informed Unifrac measures[39].

Beta-diversity refers to dissimilarity between samples, the degree to which two samples are different. A true distance metric is always positive and must fulfill these assumptions; The distance between a point and itself is 0, the distance between A and B is identical to the distance between B and A and the sum of the distance between A and B and between B and C is not lesser than the distance between A and C. The last assumption, known as the triangular inequality, fails for many dissimilarity indexes, e.g. if all three points are in a line with B in the middle.
The Bray-Curtis dissimilarity is a widely used metric in microbial ecology to quantify the difference between samples. As it fails the triangle inequality axiom described above, it is not a true distance and therefore referred to as a dissimilarity measure. It is assumed that the data are taken from the same physical size, area or volume because the dissimilarity is computed on relative abundances, so a higher overall abundance of OTUs or MGS's at one site is treated as part of a difference between two samples[28]. This measure provides values between 0 (samples identical) and 1 (samples completely dissimilar).

In evaluating community dissimilarity, it is also common in microbial ecology to use correlation coefficients such as Pearson's product moment or Spearman's rank correlation, where the influence of noise is minimized using ranks. Most often visualizations of scatter plots are shown, from where the coefficients are derived and can be seen as the degree to which the scatter deviates from a straight diagonal line[28].

1.4 Skin microbiome characteristics

Genomic approaches characterizing microorganisms in samples from the presence of their genes (the microbiome) have revealed great diversity in microbial skin inhabitants and increased our understanding of community structures and dynamics of coexistence. Genomic studies find too, that the bacterial domain dominates on skin and over 1000 different species have been identified[40], whereas fungi and virus contribute with relatively few species[41]. Bacterial community structures are associated with sebaceous (dominated by *Cutibacterium*), moist (dominated by *Staphylococcus* and *Corynebacterium spp.*) and dry (diverse bacterial representation) body sites[18]. Surprisingly, the Gram negative Proteobacteria have been found in relatively high abundances on dry skin sites and some moist[18]. Gram-negative organisms have previously been regarded as rare contaminants on skin.

On strain level, some species are likely more dependent on skin site. This is demonstrated by Oh and colleagues finding *S. epidermidis* strains to be more site driven with diminished inter-individual variation, whereas *C. acnes* strains are more individual- than site-specific[41]. The fungal microbiome comprises about 80 species[40]. *Malassezia* species dominate the core body and arms[42], and the feet have low fungal representation (approximately 0.5% of total kingdom abundance)[41], but exhibit great diversity[42]. At species-level resolution, fungal colonization is more dependent on body site than individual subject, e.g. with *M. globosa* dominating on back and a combination of *M. restricta*, *M. globosa* and *M. sympodialis* in nares, antecubital fossa, volar forearms, and palms[42].
The viral microbiome (virome) is still not well characterized. It exhibits more diversity[43] than the fungal community and is more dependent on individuality[41,44]. Individuals with sites where vira "bloom" (representing up to 96% kingdom diversity), *Cutibacterium* or *Staphylococcus* phages and/or human viral pathogens (including Merkel cell polyomavirus) dominate[41,45].

The microorganisms in the skin are considered belonging to two groups: 1) Transient, persisting in hours to days or 2) resident, being found routinely on skin and often regarded as neither harmful nor beneficial (commensal) to the host or beneficial (symbionts)[20]. Longitudinal samples collected over 1-2 months and 1-2.5 years found the skin microbial communities to be largely stable over time with higher similarity in community structure within individuals over time than between individuals. The skin maintained a core of microorganisms, down to strain level, rather than loosing and reacquiring from the environment[44]. In line, the same microbiome comes back after using cosmetics and washing[46,47]. However, the temporal stability is low on feet compared to all other body sites, which might be explained by transient presence of fungi in the environment[48]. The stability is a prerequisite for clinical studies exploring alterations in disease states and in relation to other factors.

1.5 Functions of the skin microorganisms

The cutaneous microenvironments provide substrates for microbial existence and at the same time constrains which microorganism can thrive. This is not only reflected in taxonomy, but also in the functional potential of microbial communities, where sebaceous sites are distinguished by overrepresentation of glycolysis, likely driven by *C. acnes* and *M. restricta*, whereas dry sites are characterized by presence of citrate cycle components[41]. Spread of some antibiotic resistance genes also show relations to site-specific microenvironment[41].
Our coexistence with microorganisms goes beyond the ecological relationship. As our immune system matures in early life, the cross talk with microbial inhabitants is thought to be key in shaping our immune system and responses to microorganisms. The skin microorganisms directly inhibit pathogen growth by occupying the bodily space and producing AMPs. Furthermore, the skin commensal communities modulate host innate and adaptive immunity (Figure 4). Here, examples of associations between the skin microbiome and host immunity are outlined.

1.5.1 Skin microbial communities contribute to host defense

Colonization of skin by pathogens is associated with low relative abundance of commensal strains[49]. Commensals protect us from pathogens by various mechanisms. *C. acnes* and *S. epidermidis* are well known and abundant commensal microorganisms occupying the skin niches. *C. acnes* ferments glycerol, a metabolite from the skin, into short chain fatty acids resulting in suppression of methicillin-resistant *S. aureus* (MRSA) growth in vitro by lowering the intracellular pH[50,51] (Figure 4). As *Malassezia* also convert lipids of the skin into FFAs, it might likewise be implicated in cutaneous defense[52].

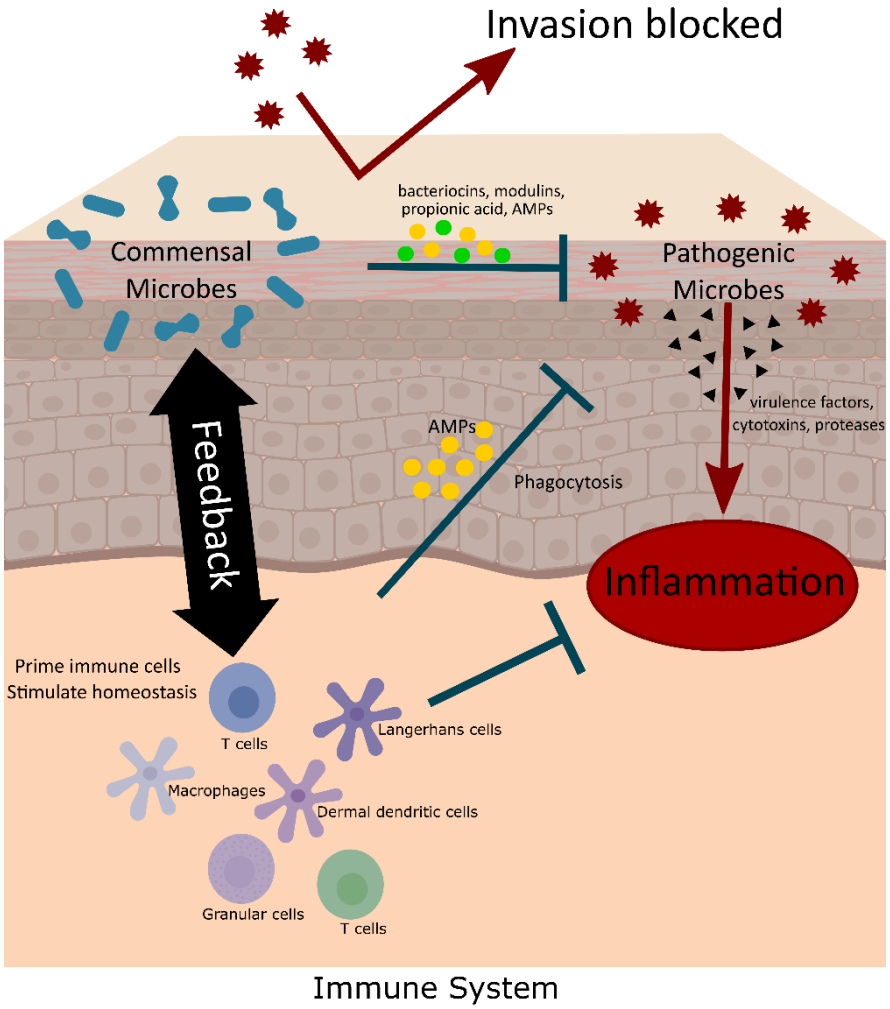

Figure 4: Mechanisms by which skin commensals contribute to host defence
The figure is adapted from Yu et al.[53]
Abbreviations: NMF, Natural Moisturizing Factor, AMPs: Antimicrobial Peptides

S. epidermidis possess several weapons influencing pathogens (Figure 4): Production molecules with antimicrobial actions, including AMPs[54], phenolsoluble modulins[55] and proteases[56]. *S. epidermidis* also manipulates the host's immune response in numerous ways, e.g. stimulation of Toll-like receptor 2 (TLR2) resulting in increased production of AMPs by keratinocytes[57]. TLR2 activation by lipoteichoic acid from the cell wall of *S.*

epidermidis are also reported to influence host mast cells, increasing viral immunity[58], and improve barrier function by increasing tight junctions in cultured keratinocytes[59].

The host's adaptive immunity is also affected by *S. epidermidis*. Colonization in early development generates regulatory T cells enabling a stable commensalism between the bacteria and host without eliciting immune responses[60]. Colonization with *S. epidermidis* is important for increasing the number of CD8+ T cells and IL17 production, improving protection against epicutaneous infection with the fungal pathogen *Candida albicans* in adulthood[61]. It seems that induction of IL-17 is a relatively normal response when the skin encounters a commensal bacterium. Exposure of commensals from the human (*Corynebacterium pseudodiphtheriticum, C. acnes* and *S. aureus*) and murine (*S. xylosus, S. lentus, Rothia nasimurium* and *S. epidermidis 42E03*) skin microbiota to specific pathogen free mice (already having a diverse skin microbiome), showed that six out of eight bacteria increased the number of IL-17 producing T cells[61].
Acinetobacter species have also been reported to influence adaptive immunity by controlling the local inflammatory milieu. In healthy subjects associations have been reported between skin colonization by *Acinetobacter* species and expression of anti-inflammatory molecules including IL-10 by peripheral blood mononuclear cells – an association lacking in atopic subjects[62].
The last example of a skin microorganism affecting adaptive immunity is when commensal *S. aureus* protects against MRSA by inducing antibody production against α-hemolysin[63], an important toxin in *S. aureus* virulence, reviewed in Divyakolu et al.[64].

The immune system appears to be the primary site of communication between microbiome and host. Though this section has focused on one-way communication from the microorganism to the host, the immune system also controls the microbial populations and their effects. The importance of cross-communication is exemplified by Naik et al., showing that mice with a deficiency of certain dermal dendritic cells fail to develop CD8+ T cell responses (IL-17 expression) when encountering *S. epidermidis*[65]. Thus, cooperation between skin resident dendritic cells promotes and tunes responses to *S. epidermidis*.

1.6 Atopic dermatitis

AD is a complex skin disease characterized by dry and itchy skin with chronic or recurrent acute dermatitis which can occur at any body site, but typically with symmetrical affected cheeks, scalp, extensor sides of the extremities in infants, flexural aspects of joints in schoolchildren and a varied representation in adults including hands and neck (Figure 5)[66].

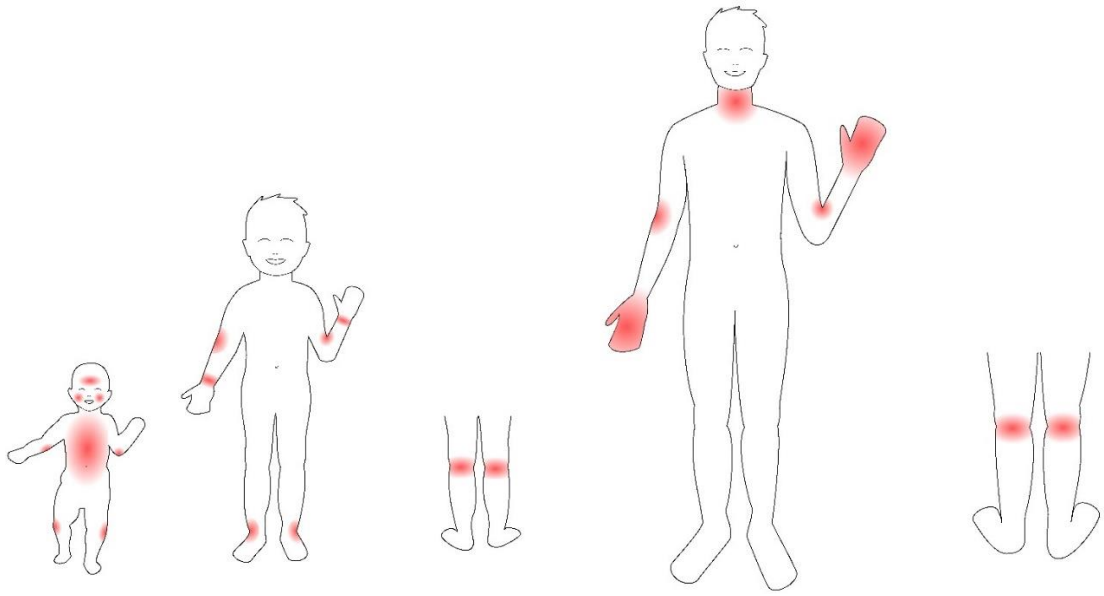

Figure 5: Locations of atopic dermatitis in infancy, child- and adulthood

It is a heterogenous disease and the clinical manifestation is wide, some patients experience minimal flexural involvement or eczema limited to hands, other have eczema affecting almost the entire bodily surface. In developed countries, the prevalence is about 20% in children and 5% in adults. AD is originally regarded a childhood disease and 80% have an onset before 6 years of age[67]. Persistence into adulthood and late-onset disease is common[68], but the proportions are uncertain. Other features commonly related to AD include a personal and/or family history of atopic diseases (AD, asthma, allergic rhinitis and food allergies), high total or allergen-specific serum IgE concentrations and the presence of generalized skin dryness. The risk of having other inflammatory diseases, such as arthritis, is also increased[67]. AD has substantial effects on patients also mentally with diminished self-esteem, sleep deprivation and poor performance at school and work[66].

The etiopathogenesis of AD is complex, multifactorial and unclear. The strongest risk factor for AD is a family history of atopic diseases and genome-wide association studies have identified several AD susceptibility loci critical to innate immunity, Th2-mediated inflammation, and skin barrier function, highlighting the importance of these pathways[69].
Two hypotheses of etiopathogenesis have been proposed[70] where the first “inside-to-outside” theory considers the primary defect in the immune system causing excessive IgE sensitization and inflammation, leading to a secondary dysfunctional skin barrier. AD has been considered a Th2 disease for a long time, but in recent years many players of the immune system have been implicated, depending on disease stage[71], patient age[72], and ethnic background[73].
The second is the “outside-to-inside” theory, considering the primary defect to reside in the skin barrier causing increased allergen and pathogen penetration leading secondarily to higher IgE-sensitization and inflammation[74]. Supporting this second theory, skin barrier breakages are key to avoid in AD. Skin barrier function is greatly dependent on the structure

and composition of the SC. Loss of function mutations in the filaggrin gene (FLG), found in approximately one-third of European AD patients compared to 10% in the background population, is the strongest risk factor for AD, leading to decreased aggregation of keratin filaments, reduction in maturation and excretion of lamellar bodies, lower expression of tight junctions and amount of NMF, and fewer acidic metabolites leading to increased pH. Mutations in FLG are not the only cause of decreased filaggrin expression, factors such as irritant exposure and an inflammatory cytokine milieu can also lower the expression in the skin of AD patients[71]. However, more than 50% of individuals carrying mutations in do not develop AD, indicating that mutations are not sufficient for developing AD[67].
Food allergy is particularly present in infants and children with moderate-severe AD. As they grow older some allergies resolve and others persist, while the sensitization pattern simultaneously shifts towards aeroallergens[66]. Exposure to aeroallergens can cause AD flareups. Some adult AD patients experience worsening of eczematous lesions on areas not covered by clothing after being exposed to aeroallergens such as grass pollen, suggesting that these aeroallergens directly penetrate the impaired skin barrier and trigger the immune system[75].

1.6.1 Treatment

AD management is dependent on disease severity and comorbidities. Typically, interventions are aimed at avoiding relevant triggers, improving the skin barrier, normalizing skin dysbiosis and reducing inflammation[67]. Topically applied emollients are a part of standard prevention and treatment. To dampen inflammation, topically applied corticosteroids are used as first-line treatment, but other topically applied anti-inflammatory agents (e.g. calcineurininhibitors) are also available. When topical treatment fails to control the disease, phototherapy and/or systemic immunosuppressants can be considered. A clearer picture of the molecular biology of the dysregulated immune system has led to the emergence of new drugs, such as the biologic immunomodulating monoclonal antibody Dupilumab blocking the IL-4 receptor[67].
As AD has a high degree of clinical heterogeneity, researchers and clinicians are interested in finding approaches to stratify patient groups to optimize treatment strategies and components such as onset of disease, malfunctioning molecules and the skin microbiome are being used[76-78]. Targeting microbial dysbiosis is receiving increasing attention[79].

1.6.2 Dysbiosis in atopic dermatitis

The AD skin is more prone to infections and microbial dysbiosis is a hallmark of the disease. *S. aureus* commonly colonize AD skin and cause secondary infections. This has been recognized for a long time[1]. A recent meta-analysis of 95 culture-based reports finds prevalence percentages of *S. aureus* carriage of 70% in lesional skin and 39% at non-lesional sites, and 62% of the nares[80]. The colonization frequency is correlated with disease severity[80,81], suggesting an active involvement of the bacteria during flares. Also, worsening of AD severity after stopping usual treatment is correlated to initial abundance of *S. aureus*[82]. *S. aureus* is nearly absent from healthy skin, however, up to 30% of the healthy population

carries *S. aureus* in their nares[83]. Differences among *S. aureus* strains found in unaffected healthy carriers and from patients with AD are found, with clonal complex 1 strains frequently found in AD[84]. The overgrowth of *S. aureus* in AD seems to result from numerous factors both related to the host, e.g. elevated pH[85] and reduced levels of certain AMPs[86], resulting in higher permissiveness of *S. aureus* growth and the bacteria having multiple mechanisms increasing the binding to AD skin[87].
In AD, several nonbacterial infections can also occur, often concomitantly with *S. aureus* infection. The viruses herpes simplex and molluscum contagiosum are commonly observed in AD[66,88]. Fungal colonization is also thought to play a role in the pathogenesis of AD, especially in a subset of patients with head and neck dermatitis having higher rates of sensitization to *Malassezia* compared to healthy controls[89]. In 1983, Clemmensen and Hjorth reported that antifungal therapy improve the clinical severity in these patients[90], however in a more recent systematic review evaluating the effect of antifungal therapy five of eight studies found beneficial effects compared with placebo or standard therapy and three none[89].

After the rise of NGS, studies of the skin microbiomes in AD have revealed even further dysbiosis. Most studies sample the flexures[91,92-94] and sequence the 16S rRNA gene of bacteria, showing reduced bacterial diversity in AD[92,95,96]. There is a gap in knowledge about microbial compositions at other body sites. Furthermore, shotgun data of whole metagenomes are emerging, providing better resolution of data and enable analyses of microbiota from different domains, however not much is known about the virome in AD.

In human infants, changes in the gut microbiota have been implicated in the development of asthma[97]. The associations are not as clear when it comes to skin, and it is debated whether the dysbiotic changes in AD are directly causative of disease or merely a consequence of a disturbed skin barrier. Although animal models cannot fully mimic the human disease state, using model organisms shed light on this fundamental issue.

2. Objectives

The overall objective of this PhD thesis was to establish methodology and examine the human skin microbiome in AD.

In more detail, the aims of this thesis were:

Aim I: To identify and synthesize research to describe the skin microbiome in AD and address whether there is a causal role of dysbiosis in dermatitis by including animal studies and evaluating effects of treatment (Manuscript I).

Aim II: To establish methodology for skin microbiome analyses focusing on sampling technique and DNA extraction (Manuscript II).

Aim III: To thoroughly characterize the skin microbiome, including bacteria, fungi and virus, at different body sites in AD and address effects of lesional state and *S. aureus* strain colonization (Manuscript III).

3. Results and manuscripts

This section summarizes the key findings of the manuscripts included in this thesis. The original papers are included after each summary.

Manuscript I: The role of the skin microbiome in atopic dermatitis: a systematic review

Of 5735 texts screened, 12 human studies and 6 animal studies, of which most (17 of 18) applied amplicon sequencing, were included and we found an AD skin microbiome profile characterized by:

- Low bacterial diversity, especially at lesional sites.
- Higher relative abundance of *S. aureus*, *S. epidermidis* and non-*Malassezia* fungi.
- Lower relative abundance of *Propionibacterium* [*Cutibacterium*] and *Malassezia,* along with other genera: *Streptococcus, Acinetobacter, Corynebacterium* and *Prevotella*.

Other findings:

- One animal study indicated that dysbiosis was a driving factor in dermatitis.
- Different methodologies applied might have affected the outcomes.
- Ongoing studies investigated the effect of different treatments.

SYSTEMATIC REVIEW

BJD
British Journal of Dermatology

The role of the skin microbiome in atopic dermatitis: a systematic review*

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Summary

Dysbiosis is a hallmark of atopic dermatitis (AD). The composition of skin microbiome communities and the causality of dysbiosis in eczema have not been well established. The objective of this review is to describe the skin microbiome profile in AD and address whether there is a causal relationship between dysbiosis and AD. The protocol is registered in PROSPERO (CRD42016035813). We searched PubMed, Embase, Scopus and ClinicalTrials.gov for primary research studies applying culture-independent analysis on the microbiome on AD skin of humans and animal models. Two authors independently screened the full text of studies for eligibility and assessed risk of bias. Because of heterogeneity no quantitative synthesis was done. Of 5735 texts, 32 met the inclusion criteria (17 published: 11 human and six animal studies). The studies varied in quality and applied different methodology. The skin in AD had low bacterial diversity (lowest at dermatitis-involved sites) and three studies showed depletion of *Malassezia* spp. and high non-*Malassezia* fungal diversity. The relative abundance of *Staphylococcus aureus* and *Staphylococcus epidermidis* were elevated and other genera were reduced, including *Propionibacterium*. A mouse study indicated that dysbiosis is a driving factor in eczema pathogenesis. The data are not sufficiently robust for good characterization; however, dysbiosis in AD not only implicates *Staphylococcus* spp., but also microbes such as *Propionibacterium* and *Malassezia*. A causal role of dysbiosis in eczema in mice should encourage future studies to investigate if this also applies to humans. Other important aspects are temporal dynamics and the influence of methodology on microbiome data.

What's already known about this topic?

- Dysbiosis is a hallmark of atopic dermatitis (AD): *Staphylococcus aureus* colonization is frequent and affects disease severity adversely.
- Recent availability of culture-independent methods to profile microorganisms has enabled studies of whole microbial communities and their role in dermatitis.

What does this study add?

- AD skin has low bacterial diversity, high non-*Malassezia* fungal diversity, high abundance of *S. aureus* and *Staphylococcus epidermidis* and reduced abundances of other genera.
- An animal study indicates that dysbiosis is a driving factor in eczema.
- More data are warranted for better characterization of the role of the microbiome in AD and the influence of methodological approaches needs to be resolved.

Recent availability of culture-independent methods to profile microorganisms and study microbial communities has increased our understanding of the microbiome and its impact in health and disease. Much research has focused on the gut microbiome, where findings demonstrate associations between dysbiosis and diseases such as diabetes and asthma.[1] The number of skin microbiome studies is rising. The skin is composed of a variety of niches selecting for colonization by specific microorganisms.[2] Host factors, for example sex, age and environmental exposures,[3–6] also affect the niches and microbiome communities and it is becoming increasingly apparent that the skin microbiome, in turn, influences vital functions in the host, such as immunity and colonization by pathogenic microorganisms.[5]

Atopic dermatitis (AD) is a chronic skin disease affecting up to 20% of children; it is less prevalent in adults.[7] Depending on age, it manifests at different sites with dry, itchy skin and relapsing eczema: in infants the cheeks are typically the first place to be affected, in toddlers it is the extensor aspects of joints, in older children it is flexure aspects of joints, and it has a varied presentation in adults. AD is characterized by immune dysregulation predisposing to IgE production.[8] Conventional culture-based work has established that dysbiosis is also a hallmark:[9] 70% of lesional and 39% of nonlesional skin sites are colonized by *Staphylococcus aureus*,[10] which adversely affects disease severity. Fungi are also implicated.[11] Conventional culturing fails to grow about 80% of bacterial species.[12] By applying culture-independent molecular methodology, dysbiosis is broadly described and the relative amount of present microbes becomes evident, which is also true for microbes not present. Although skin dysbiosis and the microbiome are anticipated to have an important role in the development of treatments, there has been no systematic review of the skin microbiome profile in AD. AD is a multifactorial disease, but the gene–environment interactions leading to the development of AD are not fully understood. Controversy remains with regard to distinguishing between primary events leading *to* AD and secondary events resulting *from* AD.[13] Whether the skin microbiome is a primary factor in AD pathogenesis is uncertain.

This systematic review provides an overview of the AD skin microbiome profile. It is questioned if causal relationships between the skin microbiota and disease exist. To elaborate on this, animal studies are also included and the effect of treatment of AD on the microbiome is evaluated. A discussion of future directions in AD microbiome research is included.

Materials and methods

Complete literature search methods, risk of bias and data extraction were specified in advance and documented in a protocol registered in PROSPERO (CRD42016035813).

A systematic literature search, conducted on 21 October 2016, was performed in PubMed, Embase, Scopus and ClinicalTrials.gov using search terms from the categories 'skin', 'microbiome' and 'AD' – without language or date limitations. After an initial screen of titles and abstracts, two authors independently screened full texts for eligibility. Primary research studies (observational and interventional) were included if they applied culture-independent methods and whole-community analyses to characterize the AD skin microbiome of humans and animal models. Studies were excluded if they did not present data, included an incorrect study population, investigated the microbiome of other body sites or investigated selected microbial taxonomic units. Duplicate studies were excluded. Disagreements on eligibility were resolved by contacting the authors of the original studies.

Two authors assessed the risk of bias. For human studies we used the Cochrane Collaboration's tool for randomized controlled trials (RCTs) and adjusted Newcastle–Ottawa Scales for analytical nonrandomized case–control studies and cohort studies without a control group. For the animal studies we used adjusted versions of the Systematic Review Centre for Laboratory Animal Experimentation's (SYRCLE) risk-of-bias tool.[14] The highest-quality evidence received the greatest emphasis.

Two authors collected study characteristics and relative abundances of microbial taxonomic units (when > 1% *and* significant differences were found compared with a reference).

The criteria for study inclusion allowed for heterogeneity in study population, design and methods; therefore, no quantitative synthesis was carried out.

Results

In total, 5735 studies were identified (Fig. 1). After review of the titles and abstracts, the full texts of 90 were screened. Based on the selection criteria, 32 records were included – 17 were published (Table S1; see Supporting Information) and 15 were ongoing (Table S2; see Supporting Information). The published studies were examined in the most detail.

Human studies

Study description

Eleven human studies were identified (Table S1; see Supporting Information): two RCTs, seven case–control studies and two cohort studies. The studies included 355 patients with AD. Oh *et al.* also included patients with primary immunodeficiencies ($n = 41$), characterized by AD-like eczema.[15] Their data were regarded to be from an AD population. The age of the participants ranged from 2 months to 62 years and both sexes were included in 10 of the 11 studies.[15–22] AD was clinically characterized by SCOring of Atopic Dermatitis (SCORAD) in eight of the studies;[15–19,21–23] Eczema Area and Severity Index in one;[24] and Rajka and Langeland in one.[4] Patients with mild AD were included in two studies;[16,20] moderate AD in nine;[4,15–20,23,24] and patients with severe AD in seven.[4,15–17,19,20,24,25] Only one study distinguished microbiome composition according to disease severity.[20] The skin microbiomes of anatomically defined skin area(s) were investigated in nine of the 11 studies.[4,15,17,19–21,23,25] Other studies compared affected with nonaffected skin sites.[4,16,18,23,24]

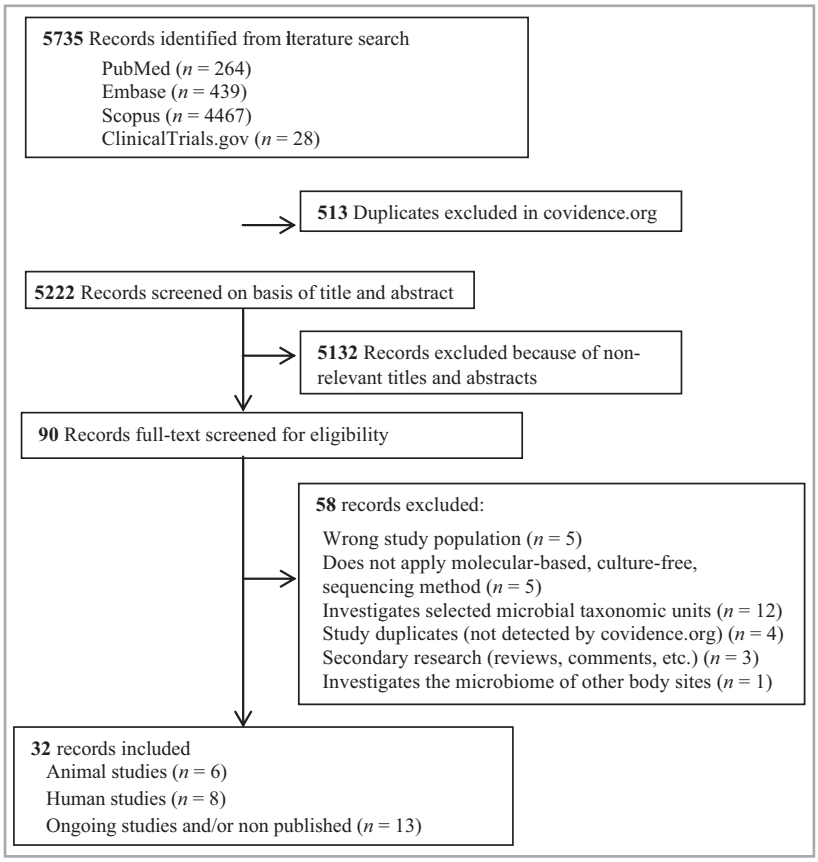

Fig 1. Flow diagram of study selection.

Well-defined criteria for treatment allowed before and during the studies were provided in 10 of the 11 studies.[4,15,16,18–25]

Methodology

The primary sampling technique of skin was swabs. No biopsies were taken. Different protocols were used for DNA extraction. One study applied a metagenomic sequencing approach profiling all microbes.[25] For bacterial microbiome analyses 10 of 11 studies used 16S rRNA sequencing,[4,15–19,21–24] applying either broad-range 16S gene primers,[17,19] or targeting hypervariable region 1–3 (V1–V3);[4,15,21,22] V3;[16] V4;[24] V1–V2;[18] or V2–V3.[23] The fungal microbiome was characterized in two studies,[15,20] using either the internal transcriber spacer sequence and 18S rDNA as targets for amplification,[15] or the D1/D2 hypervariable region of the 28S rDNA gene.[20] The number of polymerase chain reaction (PCR) cycles varied from 30 to 35 to 40.[16–18,20,23,24] Five studies did not provide information on the number of PCR cycles.[4,15,19,21,22] Relative abundances of microbial taxonomic units were provided in percentages in seven of the 11 studies;[4,15–17,19,20,25] in five studies estimations were made from readings of figures.[18,21–24] Taxonomic classification was performed either at the genus level;[15,17–19,21,24] the family level;[22,23] or the species level.[4,17,20,25] More studies included additional species-level identification of *Staphylococcus* spp. or only *S. aureus*.[15,18,19,21,22] The study by Bourrain *et al.* only identified *S. aureus* vs. diversified microbiota.[16]

Risk of bias

The quality of the human studies varied (Table S3; see Supporting Information). We rated them as very good [score of 9; one RCT was rated low with regard to risk of bias (n = 4)], good (score 7–8; n = 4), fair (total score 5; n = 2) and poor (a poorly reported RCT; n = 1). The main reason for downgrading the quality of the 'fair' studies was either lack of information on cases and controls,[20] or missing a proper, nonexposed control population.[18]

Atopic dermatitis skin microbiome profile

Staphylococcus aureus was abundant on AD skin compared with control skin,[4,15,19] and correlated positively to disease severity (Table S4; see Supporting Information).[15] Affected skin sites were dominated more by *S. aureus* than unaffected sites,[4,15,16,26] especially inflamed areas (vs. xerotic)[16] – and during a flare the abundance increased dramatically in untreated patients.[19] Besides *S. aureus*, other species from the *Staphylococcus* genus were increased on involved sites.[24] These included *S. epidermidis* and *S. haemolyticus*.[15,19,26]

The bacterial diversity on AD skin was low compared with control skin,[15,19] and reduced during a flare.[19] Reductions in species from the genera *Streptococcus*, *Propionibacterium*,[4] *Acinetobacter*, *Corynebacterium* and *Prevotella* were found – not solely attributed to an increase in *S. aureus*.[19] *Propionibacterium acnes* was also found less frequently on

facial AD skin than on control skin,[17] and was inversely correlated to disease severity.[15] Interestingly, although *Corynebacterium* decreased during AD flares,[19] it was increased in the antecubital flexure of patients with primary immunodeficiency.[15] After a flare, the species that were reduced increased in relative abundance.[19]

The fungal microbiome showed, overall, that patients with AD had depleted numbers of *Malassezia* spp.[25] but enrichment of *M. dermatis* and more diverse non-*Malassezia* spp. than healthy controls.[15,20,25] These included *Aspergillus*,[15] *Candida albicans* and *Cryptococcus diffluens*.[20]

Effect of treatment on the skin microbiome in atopic dermatitis

Compared with no treatment, intermittent treatment decreased the predominance of *S. aureus* and loss of bacterial diversity during a flare (Table S3; see Supporting Information),[19] with no improvement in SCORAD (data in original paper by Kong *et al.*).[19] In contrast, Oh *et al.* found no such treatment-associated shifts in bacterial community diversity.[15]

One study evaluated the effect of dilute bleach baths and found that 10 days of baths improved SCORAD and number of lesional sites colonized by *S. aureus*.[16] Another study evaluated the effect of topical corticosteroid treatment alone or in combination with dilute bleach baths. Both treatments improved the clinical eczema representation and suppressed *Staphylococcus* spp. on lesional and nonlesional sites, with the authors concluding that there was no effect of the additional dilute bleach baths.[24]

Emollient usage improved SCORAD and resulted in minor changes in the microbiome: 28 days of emollient use did not induce changes in the genus-level microflora at unknown skin site(s), but *S. aureus* increased in the nonemollient control group.[21] Eighty-four days of emollient use on affected and unaffected sites improved SCORAD in 26 of 36 individuals.[26] These 26 individuals had less relative abundance of *Staphylococcus* spp. and significantly more *Stenotrophomonas*, which also was inversely correlated to disease severity.[15] However, *Stenotrophomonas maltophilia* was found in the facial skin of patients with AD but not in controls.[17]

Association between dysbiosis and atopic dermatitis

Two-month-old infants who were later diagnosed with AD and had affected skin at the age of 12 months demonstrated a significantly lower number of commensal *Staphylococcus* spp. in their antecubital fossae than children with unaffected skin at the age of 12 months.[22] These data suggest that cutaneous dysbiosis might play a role in initiation of AD and that exposure to commensal staphylococci during early infancy might be important.

Animal studies

We included six animal studies (four noninterventional, two interventional), either with dogs with AD or mouse models (Table S1; see Supporting Information). Four studies sampled the skin by swabbing and two by biopsies.[27–32] Different DNA extraction protocols were used. The bacterial microbiome was analysed in five of the six studies by 16S rRNA sequencing.[27,29–32] The fungal microbiome was characterized in one study.[28] No information on the number of PCR amplification cycles were given in four of the six studies.[27–30]

Relative abundances of microbial taxonomic units were provided in percentages in three of the studies,[27,28,32] and estimated from readings of figures in three studies.[29–31] Taxonomic classification was performed either at the family-level,[29,32] with additional analysis of *Staphylococcus* spp. in one study;[29] at the genus-level;[27,28,31] or at the phylum-level with species-level identification of *Staphylococcus* and *Corynebacterium*.[30]

The animal studies were mostly unclear with regard to risk of bias due to poor reporting (Table S3; see Supporting Information), which is common for animal studies.[14]

Animal atopic dermatitis skin microbiome profile and effect of treatment

Like humans, dogs with AD and Adam17-deficient mice had decreased bacterial diversity;[27,29,33] increased abundance of *Staphylococcus* spp.;[29] and *S. aureus* at the onset of eczematous inflammation (Table S5; see Supporting Information).[33] *Corynebacterium* spp. were also found to be increased.[29,31,33]

Antimicrobial treatment of dogs presenting AD lesions decreased the clinical eczema score and reduced transepidermal water loss (TEWL).[29] No difference was found in skin pH. Furthermore, bacterial diversity normalized with decreased relative abundance of *Staphylococcus* spp.

Causality between dysbiosis and atopic dermatitis

In Adam17-deficient mice a prescreening of microbial composition was used to target systemic antimicrobial therapy.[33] Therapy resulted in decreased clinical scores and TEWL along with decreased relative abundance of the targeted species, *S. aureus* and *Corynebacterium bovis*, and increased bacterial diversity. Withdrawal of treatment dissipated the improvements in diversity. Eczema and dysbiosis reappeared after 2 weeks, as shown in a crossover design, where systemic antibiotics were shown to protect the Adam17-deficent mice from developing eczema and losing microbiome diversity. These data suggest a causal relationship between dysbiosis and AD in an animal model.

Discussion

In this systematic review we have demonstrated that AD skin in humans is characterized by low bacterial diversity and high non-*Malassezia* fungal diversity. On involved skin the bacterial diversity is even lower. The relative abundance of both *S. aureus* and *S. epidermidis* is elevated and the abundance of *Propionibacterium* is reduced, along with other genera (*Streptococcus*, *Acinetobacter*, *Corynebacterium* and *Prevotella*). A birth cohort study indicated that

the absence of early colonization with commensal staphylococci might precede AD presentation, and an animal study indicated that dysbiosis was a driving factor in the pathogenesis of eczema. In interpreting these data, it should be emphasized that they were drawn from few studies with substantial heterogeneity and varied quality. Many of the included studies (15 of 17) analysed the microbiome using 16S rRNA sequencing, and even though the 16S rRNA gene is widely accepted as a biological fingerprint for bacterial species, there are some limitations. Some bacterial species have multiple copies of 16S rRNA genes, which may lead to an artificial over-representation in the data.[34] In addition, technical aspects may also introduce uncertainty; these include sampling technique, DNA extraction and sequencing protocol.[35–37] For instance classification accuracy varies with the specific region of the 16S rRNA gene chosen to be sequenced.[37] The limitations to 16S surveys have made the newer approach of whole-metagenome shotgun sequencing attractive. This method allows for analysis of the entire gene content of the microbial population, catch most species and may sequence deeply enough to identify strains.[37] This is crucial when it comes to understanding the physiological implications of a modified microbiome. Only one of 17 studies applied this method. Sequences obtained have short read lengths and many have no representation within databases. Therefore the different methodologies applied in the included studies likely affect outcome in microbiome composition and underline the importance of transparency in methodological approach. Not all of the included studies provided enough information on each methodological step. This shows a need for guidelines on good reporting of microbiome studies.

A common criticism of using DNA-based technology to identify microbial communities is that DNA from dead and viable microorganisms are not distinguished. In the future, attempts to reduce DNA from dead microorganisms or performing RNA (cDNA)-based community analysis may help to minimize the detection of dead microorganisms. Such approaches would also contribute to the enlightenment of potential interplays and communication between hosts and the microbiome, for example in processes such as eczematous inflammation. Studies are moving from describing the microbiome to focusing on interactions by implicating RNA, protein and/or metabolite data. A study by Fyhrquist *et al.* showed a positive correlation between the relative abundance of skin *Acinetobacter* spp. and expression of anti-inflammatory molecules among healthy subjects, which was not present in atopic individuals.[38] In the study by Kong *et al.*,[19] the relative abundance of *Acinetobacter* increased postflare, which supports a potential anti-inflammatory role of *Acinetobacter* in AD.

Ongoing studies are investigating the effect of age and treatment of AD (Table S2; see Supporting Information). The finding of *Corynebacterium* being reduced in AD flares but increased in the antecubital flexure of patients with primary immunodeficiency suggest that underlying genetics may affect the microbiome. Mutations in the gene encoding the protein filament aggregating protein (filaggrin) leading to a functional absence of the protein predisposes individuals to developing atopic eczema, an increase in stratum corneum pH, and an increase in susceptibility to recurrent bacterial skin infection among patients with AD.[39–41] Filaggrin deficiency in ichthyosis vulgaris is associated with a low abundance of proteolytic Gram-positive anaerobic cocci, which are shown to be better at inducing expression of antimicrobial peptides in cultured keratinocytes.[42] This could be a mechanism favouring the growth of *S. aureus* or infection. A trend for lower bacterial diversity in one control and two AD filaggrin-null mutation subjects was seen in the study by Chng *et al.*[25] However, the role of filaggrin on the skin microbiome in AD is not known and none of the studies included in this review could elaborate thoroughly on this.

In line with the *S. aureus* data presented in this review, a recent meta-analysis showed that patients with AD were more likely to be colonized with *S. aureus* than healthy controls, with higher odds ratios (OR) for lesional skin [OR 19·74, 95% confidence interval (CI) 10·88–35·81] than for nonlesional skin (OR 7·77, 95% CI 3·82–15·82).[10] With *S. aureus* being more abundant on nonlesional skin, this suggests that the skin is susceptible to pathogen colonization and at risk of progressing toward a diseased state. This indicates that antistaphylococcal treatment could be beneficial. However, a systematic review by Bath-Hextall *et al.* showed that reducing the number of *S. aureus* in people with uninfected eczema did not result in reduced disease activity.[43] Targeting specific *S. aureus* strains could potentially improve the outcome of antistaphylococcal treatment. This is supported by the finding of a single nucleotide polymorphism in a staphylococcal lipase gene being preferentially hosted in AD.[25] However, targeting other bacteria might also be beneficial. An idea of a critical window early in life during which exposure to certain microbes is important for the development of the immune system and allergic diseases has arisen and is supported by studies showing reduced microbial diversity in the gut before the development of atopy.[1,44,45] Further, tolerance to the skin commensal *S. epidermidis* is preferentially established in neonatal life in mice.[46] Current data are limited and it is difficult to evaluate whether the cutaneous microbiome plays a role in the initiation of AD.[22] By hypothesizing that dysbiosis precedes AD flares and severity, studies are currently investigating prevention and treatment targeting dysbiosis. Moisturizers are key in AD management, to restore and preserve skin barrier integrity. One RCT showed that emollient therapy from birth in babies at high risk of AD enhanced the skin barrier and reduced the relative risk of AD incidence by 50% after 6 months.[47] An ongoing study by Glatz (Table S2; see Supporting Information) is investigating if shifts in the skin microbiome are associated with this improvement. Preliminary data show that preventative emollient usage lowers pH, does not change TEWL and increases the number of bacterial taxonomic units and *Streptococcus* spp.[48] As *Streptococcus* was reduced during flares but increased in the abscense of commensal staphylococci in infants before AD presentation,[19,22] future studies should investigate the role of *Streptococci* spp. in AD. *Stenotrophomonas* spp. may also have an important role in restoration of the skin microbiome.[18]

Another approach to manipulating the skin microbiome is by adding beneficial bacteria to moisturizers. One RCT showed that cream containing 5% lysate of the nonpathogenic Proteobacteria *Vitreoscilla filiformis* significantly improved SCORAD, TEWL, the patient's assessment of itch and loss of sleep compared with placebo.[49,50] Ongoing studies by Gallo and colleagues apply the same principle (Table S2; see Supporting Information): in an attempt to decrease *S. aureus* colonization in AD skin, they are isolating beneficial *Staphylococcal* species from the patients themselves and placing them in a moisturizer and applying the moisturizer to the subjects' own arms.

To utilize the microbiome in prevention and treatment strategies of AD, more data from human studies are needed on the skin microbiome dynamics related to clinical measures, temporal resolution and how different factors modify the microbial abundances to be able to predict responses in the microbiome to perturbations. Good speciation and strain-level identification in combination with RNA, protein and metabolite data would strengthen such data and provide valuable insights.

While the microbiome is increasingly drawing attention as a possible target in the prevention and treatment of AD, new methodological approaches have not yet brought us far in understanding the impact of dysbiosis in AD. Staphylococcal species are key players in worsening of AD, and may also be important in the establishment of the disease. Other microbes such as *Propionibacterium*, *Streptococcus*, *Acinetobacter* and *Malassezia* have been found to be implicated in AD dysbiosis. However, robust data are missing on the influence of methodological procedures, characteristics of the microbiome structure related to temporal dynamics, clinical measures and factors altering the microbiome.

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References

1 Arrieta MC, Stiemsma LT, Dimitriu PA *et al.* Early infancy microbial and metabolic alterations affect risk of childhood asthma. *Sci Transl Med* 2015; **7**:307ra152.
2 Grice EA, Kong HH, Conlan S *et al.* Topographical and temporal diversity of the human skin microbiome. *Science* 2009; **324**:1190–2.
3 Fierer N, Hamady M, Lauber CL, Knight R. The influence of sex, handedness, and washing on the diversity of hand surface bacteria. *Proc Natl Acad Sci U S A* 2008; **105**:17994–9.
4 Shi B, Bangayan NJ, Curd E *et al.* The skin microbiome is different in pediatric versus adult atopic dermatitis. *J Allergy Clin Immunol* 2016; **138**:1233–6.
5 Rosenthal M, Aiello A, Larson E *et al.* Healthcare workers' hand microbiome may mediate carriage of hospital pathogens. *Pathogens.* 2013; **3**:1–13.
6 Callewaert C, Hutapea P, Van de Wiele T, Boon N. Deodorants and antiperspirants affect the axillary bacterial community. *Arch Dermatol Res* 2014; **306**:701–10.
7 Carroll CL, Balkrishnan R, Feldman SR *et al.* The burden of atopic dermatitis: impact on the patient, family, and society. *Pediatr Dermatol* 2005; **22**:192–9.
8 Akdis CA, Akdis M, Bieber T *et al.* Diagnosis and treatment of atopic dermatitis in children and adults: European Academy of Allergology and Clinical Immunology/American Academy of Allergy, Asthma and Immunology/PRACTALL Consensus Report. *Allergy* 2006; **61**:969–87.
9 Leyden JJ, Marples RR, Kligman AM. *Staphylococcus aureus* in the lesions of atopic dermatitis. *Br J Dermatol* 1974; **90**:525–30.
10 Totte JE, van der Feltz WT, Hennekam M *et al.* Prevalence and odds of *Staphylococcus aureus* carriage in atopic dermatitis: a systematic review and meta-analysis. *Br J Dermatol* 2016; **175**:687–95.
11 Darabi K, Hostetler SG, Bechtel MA, Zirwas M. The role of *Malassezia* in atopic dermatitis affecting the head and neck of adults. *J Am Acad Dermatol* 2009; **60**:125–36.
12 Ward DM, Weller R, Bateson MM. 16S rRNA sequences reveal numerous uncultured microorganisms in a natural community. *Nature* 1990; **345**:63–5.
13 Bieber T. Mechanisms of disease: atopic dermatitis. *N Engl J Med* 2008; **358**:1483–94.
14 Zeng X, Zhang Y, Kwong JS *et al.* The methodological quality assessment tools for preclinical and clinical studies, systematic review and meta-analysis, and clinical practice guideline: a systematic review. *J Evid Based Med* 2015; **8**:2–10.
15 Oh J, Freeman AF, Park M *et al.* The altered landscape of the human skin microbiome in patients with primary immunodeficiencies. *Genome Res* 2013; **23**:2103–14.
16 Bourrain M, Ribet V, Calvez A *et al.* Balance between beneficial microflora and *Staphylococcus aureus* colonisation: *in vivo* evaluation in patients with atopic dermatitis during hydrotherapy. *Eur J Dermatol* 2013; **23**:786–94.
17 Dekio I, Sakamoto M, Hayashi H *et al.* Characterization of skin microbiota in patients with atopic dermatitis and in normal subjects using 16S rRNA gene-based comprehensive analysis. *J Med Microbiol* 2007; **56**:1675–83.
18 Seite S, Bieber T. Barrier function and microbiotic dysbiosis in atopic dermatitis. *Clin Cosmet Investig Dermatol* 2015; **8**:479–83.
19 Kong HDH, Oh J, Deming C *et al.* Temporal shifts in the skin microbiome associated with disease flares and treatment in children with atopic dermatitis. *Genome Res* 2012; **22**:850–9.
20 Zhang E, Tanaka T, Tajima M *et al.* Characterization of the skin fungal microbiota in patients with atopic dermatitis and in healthy subjects. *Microbiol Immunol* 2011; **55**:625–32.
21 Bianchi P, Theunis J, Casas C *et al.* Effects of a new emollient-based treatment on skin microflora balance and barrier function in children with mild atopic dermatitis. *Pediatr Dermatol* 2016; **33**:165–71.
22 Kennedy EA, Connolly J, Hourihane JO *et al.* Skin microbiome before development of atopic dermatitis: early colonization with commensal staphylococci at 2 months is associated with a lower risk of atopic dermatitis at 1 year. *J Allergy Clin Immunol* 2017; **139**:166–72.
23 Drago L, De Grandi R, Altomare G *et al.* Skin microbiota of first cousins affected by psoriasis and atopic dermatitis. *Clin Mol Allergy* 2016; **14**:2.
24 Gonzalez ME, Schaffer JV, Orlow SJ *et al.* Cutaneous microbiome effects of fluticasone propionate cream and adjunctive bleach baths in childhood atopic dermatitis. *J Am Acad Dermatol* 2016; **75**:481–93.
25 Chng KR, Tay ASL, Li C *et al.* Whole metagenome profiling reveals skin microbiome-dependent susceptibility to atopic dermatitis flare. *Nat Microbiol* 2016; **1**:16106.

26 Seite S, Flores GE, Henley JB *et al.* Microbiome of affected and unaffected skin of patients with atopic dermatitis before and after emollient treatment. *J Drugs Dermatol* 2014; **13**:1365–72.
27 Rodrigues Hoffmann A, Patterson AP, Diesel A *et al.* The skin microbiome in healthy and allergic dogs. *PLoS One* 2014; **9**: e83197.
28 Meason-Smith C, Diesel A, Patterson AP *et al.* What is living on your dog's skin? Characterization of the canine cutaneous mycobiota and fungal dysbiosis in canine allergic dermatitis. *FEMS Microbiol Ecol* 2015; **91**:pii:fiv139.
29 Bradley CW, Morris DO, Rankin SC *et al.* Longitudinal evaluation of the skin microbiome and association with microenvironment and treatment in canine atopic dermatitis. *J Invest Dermatol* 2016; **136**:1182–90.
30 Kobayashi T, Glatz M, Horiuchi K *et al.* Dysbiosis and *Staphyloccus aureus* colonization drives inflammation in atopic dermatitis. *Immunity* 2015; **42**:756–66.
31 Scharschmidt TC, List K, Grice EA *et al.* Matriptase-deficient mice exhibit ichthyotic skin with a selective shift in skin microbiota. *J Invest Dermatol* 2009; **129**:2435–42.
32 Kubica M, Hildebrand F, Brinkman BM *et al.* The skin microbiome of caspase-14-deficient mice shows mild dysbiosis. *Exp Dermatol* 2014; **23**:561–7.
33 Kobayashi T, Glatz M, Horiuchi K *et al.* Dysbiosis and *Staphylococcus aureus* colonization drives inflammation in atopic dermatitis. *Immunity* 2015; **42**:756–66.
34 Pei AY, Oberdorf WE, Nossa CW *et al.* Diversity of 16S rRNA genes within individual prokaryotic genomes. *Appl Environ Microbiol* 2010; **76**:3886–97.
35 Grice EA, Kong HH, Renaud G *et al.* A diversity profile of the human skin microbiota. *Genome Res* 2008; **18**:1043–50.
36 Willner D, Daly J, Whiley D *et al.* Comparison of DNA extraction methods for microbial community profiling with an application to pediatric bronchoalveolar lavage samples. *PLoS One* 2012; **7**: e34605.
37 Meisel JS, Hannigan GD, Tyldsley AS *et al.* Skin microbiome surveys are strongly influenced by experimental design. *J Invest Dermatol* 2016; **136**:947–56.
38 Fyhrquist N, Ruokolainen L, Suomalainen A *et al.* *Acinetobacter* species in the skin microbiota protect against allergic sensitization and inflammation. *J Allergy Clin Immunol* 2014; **134**:1301–9.e11
39 Nomura T, Akiyama M, Sandilands A *et al.* Prevalent and rare mutations in the gene encoding filaggrin in Japanese patients with ichthyosis vulgaris and atopic dermatitis. *J Invest Dermatol* 2009; **129**:1302–5.
40 Gruber R, Elias PM, Crumrine D *et al.* Filaggrin genotype in ichthyosis vulgaris predicts abnormalities in epidermal structure and function. *Am J Pathol* 2011; **178**:2252–63.
41 Cai SCS, Chen H, Koh WP *et al.* Filaggrin mutations are associated with recurrent skin infection in Singaporean Chinese patients with atopic dermatitis. *Br J Dermatol* 2012; **166**:200–3.
42 Zeeuwen PL, Ederveen TH, van der Krieken DA *et al.* Gram-positive anaerobe cocci (GPAC) are underrepresented in the microbiome of filaggrin-deficient human skin. *J Allergy Clin Immunol* 2017; **139**:1368–71.
43 Bath-Hextall FJ, Birnie AJ, Ravenscroft JC, Williams HC. Interventions to reduce *Staphylococcus aureus* in the management of atopic eczema: an updated Cochrane review. *Br J Dermatol* 2010; **163**:12–26.
44 Abrahamsson TR, Jakobsson HE, Andersson AF *et al.* Low diversity of the gut microbiota in infants with atopic eczema. *J Allergy Clin Immunol* 2012; **129**:434–U244.
45 Abrahamsson TR, Jakobsson HE, Andersson AF *et al.* Low gut microbiota diversity in early infancy precedes asthma at school age. *Clin Exp Allergy* 2014; **44**:842–50.
46 Scharschmidt TC, Vasquez KS, Truong HA *et al.* A wave of regulatory T cells into neonatal skin mediates tolerance to commensal microbes. *Immunity* 2015; **43**:1011–21.
47 Simpson EL, Chalmers JR, Hanifin JM *et al.* Emollient enhancement of the skin barrier from birth offers effective atopic dermatitis prevention. *J Allergy Clin Immunol* 2014; **134**:818–23.
48 Glatz M, Polley EC, Simpson EL, Kong HH. Emollient therapy alters skin barrier and microbes in infants at risk for developing atopic dermatitis. *J Invest Dermatol* 2015; **135**:S31.
49 Gueniche A, Knaudt B, Schuck E *et al.* Effects of nonpathogenic gram-negative bacterium *Vitreoscilla filiformis* lysate on atopic dermatitis: a prospective, randomized, double-blind, placebo-controlled clinical study. *Br J Dermatol* 2008; **159**:1357–63.
50 Barthow C, Wickens K, Stanley T *et al.* The Probiotics in Pregnancy Study (PiP Study): rationale and design of a double-blind randomised controlled trial to improve maternal health during pregnancy and prevent infant eczema and allergy. *BMC Pregnancy Childbirth* 2016; **16**:133.

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Table S1. Characteristics of the published studies included in the review.

Table S2. Studies in progress (registered and/or unpublished) investigating the skin microbiome in atopic dermatitis.

Table S3. Review authors' scores of risk of bias of included studies using the Cochrane Collaboration's risk of bias tool; an adjusted Newcastle–Ottawa Scale (NOS) for case–control studies; and cohort studies where points (*) are assigned for no biases and an adjusted Systematic Review Centre for Laboratory Animal Experimentation (SYRCLE) tool for noninterventional (six entries) and interventional (eight entries) animal studies.

Table S4. Summary of relative abundance (%) of microorganisms found on skin, and clinical and physiological outcomes (human studies).

Table S5. Summary of relative abundance (%) of microorganisms found on skin, and clinical and physiological outcomes in animal studies.

Video S1. Author video.

	First author	Sampling	Area sampled	Setting	Study population	Treatment	N	Study type	Sample #	Method Extraction and sequencing	Physiological and clinical measures
Human studies	Bourrain [16]	Swab	5 cm^2 **I**nflam., **N**on-lesional and **X**erotic sites Body site: NA (dry, moist, seb.)	France	Mild-severe AD 18-40 years Mixed sex	Before and during the study: 1 wk: No use of top. steroids. 2 wk: No use of top. or oral immunomodulators, antibiotics, antiseptics or antifungal	25	Prospective cohort study: 18 days of hydrotherapy	4	DNeasy Blood and Tissue kit (Qiagen) 16S rRNA (V3), 30 PCR cycles Diversified microflora or *S. aureus* abundant	SCORAD
	Dekio[17]	Swab-scrub	4.9 cm^2 facial skin	Japan	Mod.-severe AD, Healthy ctrl 19-54 years Mixed sex	Not specified	13 AD 10 Ctrl	Case-control	1	Extraction buffer, glass beads 16S rRNA (V1-V9), 40 PCR cycles, Terminal Restriction Fragment Length Polymorphism	SCORAD
	Flores [18]	Swab	1 cm^2 of affected and nearby unaffected skin Body site: Diverse	France, Slovakia and USA, California and Colorado	Mod. AD 3-39 years Mixed sex	During the study: Instucted not to use other emollients or drugs, incl. corticotherapy and antibiotherapy	49	Prospective cohort study: 84 days emollient treatment	2	MoBio PowerSoil DNA isolation kit 16S rRNA (V1-V2), 35 PCR cycles, 454-pyrosequencing	SCORAD, erythema, dryness, desquamation
	Kong [19]	Swab	Antecubital and popliteal creases, volar forearms, nares	USA, Maryland	Mod.-severe AD Healthy ctrl 2-15 years Mixed sex	"No": No top. for 1 wk, no oral antibiotics for 4 wk prior to sampling. "Intermittent": Top. in the prev. 1 wk and/or oral antibiotics in the prev. 4 wk	12 AD 11 Ctrl	Case-control: Baseline-flare-post flare Treatment (No N=7, intermittent N=5)	3	Lysis buffer and lysozyme, bead-beated, Invitrogen PureLink Genomic DNA kit 16S rRNA (V1-V8), Sanger sequencing	SCORAD
	Oh [15]	Swab and scrape	4 cm^2. Nares, retroauricular crease, antecubital fossa, volar forearm	USA, Maryland	PID patients (2-37 y) with 1) **H**yper IgE, 2) **W**iskott-Aldrich, 3) **D**OCK8 deficiency. Mod.-severe AD (2-17 y). Healthy ctrl (2-40 y). Mixed sex	Only data on PID patients: 22/25 H patients got antifungals and/or antibiotics. 6/10 W patients got antibiotics. 4/6 D patients got antibiotics	41 PID: 25H, 10W, 6D. 13 AD 49 Ctrl	Case-control	1	Lysis buffer and lysozyme, bead-beated, Invitrogen PureLink Genomic DNA kit 16S rRNA (V1-V3) 18SF and ITS1 (Only H patients) Sanger and 454 Sequencing	SCORAD
	Zhang [20]	Strip (x3)	63 cm^2 facial skin	Japan	Mild, mod., severe AD Healthy ctrl Mixed sex	Intermittent medium/strong top. steroids. No systemic or top. antibiotics or antifungals	3+3+3 AD 10 Ctrl	Case-control	1	Lysing solution, ethanol precipitation 28S rDNA (D1/D2), 30 PCR cycles, Sanger (3730x)	
	Bianchi [21]	Scratch	Right antecubital fossa (unaffected)	France, Italy	Children with mild AD 1-4 years Mixed sex	No immunosuppressant's a month before. Systemic antibiotics, probiotics or anti-inflammatory treatment 2 wk before, local top. a wk and no cream 48 h before	55	RCT: 28 days of 1) hygiene product or 2) hygiene product + emollient	2	QIAamp DNA Investigator Kit 16S rRNA (V1-V3), 454-pyrosequencing	SCORAD TEWL
	Drago [23]	Scrape	Behind the ear (lesional + non-lesional)	Italy	3 first cousins: mod. AD, mod. psoriasis, healthy ctrl 50 y Males	No pharmacological therapy or probiotics 1 month before sampling. Restricted on lifestyle, diet, sexual activity, personal care	1 AD 1 Ctrl	Case-control	1	Geneaid Genomic DNA Mini Kit (tissue) 16S rRNA (V2-V3), 30 PCR cycles Torrent PGM	SCORAD
	Kennedy [22]	Swab	Antecubital and popliteal fossae, nasal tip, cheek	Ireland USA, Maryland	AD and healthy ctrl infants from the Cork BASELINE Birth Study Mixed sex	Emollient usage in 6/10 AD infants and 2/10 healthy. No differences in bathing frequency or antibiotic usage	10 AD (4 affected at 12 months of age) 10 Ctrl	Case-control from a prospective birth cohort study. Swabbed at months 2, 6 and 12 and alsoclinical assessed at 24 months of age	3	Epicentre MasterPure Kit, bead-beated, Invitrogen PureLink Genomic DNA Kit, 16S rRNA (V1-V3), 454-pyrosequencing (GS FLX)	SCORAD (month 24). Filaggrin genotype (no mutations)
	Chng [25]	Tape-strip	Antecubital fossae	Singa-pore	Singaporean Chinese population, non-flare AD	Only restricted from using antibiotics	19 AD 15 Ctrl	Case-control	1	Qiagen EZ1 DNA Tissue Kit, Shotgun whole-metagenome sequencing	Filaggrin genotype (mutations in 2 AD, 1 Ctrl)

					> 18 years Mixed sex						TEWL, pH
	Gonzalez[24]	Swab	3 lesional (2 representative + the worst) and 1 contralateral or adjacent non-lesional site. Ctrl at 4 sites with AD predilection	USA, New York	Mod.-severe AD Healthy ctrl 3 months – 5 years Mixed sex	Excluded if overt infection, concurrent chronic skin disorders or use of antibiotics, systemic or top. corticosteroids or calcineurin inhibitors in the prior 2 wk	21 AD 14 Ctrl	RCT: 4 wk treatment of 1) top. corticosteroid (plus water baths) or 2) top. corticosteroid plus bleach baths	2	MoBio PowerSoil DNA Isolation kit 16S rRNA (V4), 35 PCR cycles, Illumina MiSeq	Hanifin and Rajka, EASI
	Shi [4]	Swab	25 cm^2 lesional and adjacent non-lesional skin on volar forearm	USA, Cali-fornia	Mod-severe AD Healthy ctrl 2-12, 13-17 and 18-62 years Mixed sex	Excluded if temp > 38.5 Prior sampling: 20 days: No phototherapy or immunosuppressant's 1 wk: No antibiotics, topicals, bleach baths 24 h: No creams/lotions, bathes.	128 AD 68 Ctrl	Case-control: Comparison among age groups	1	QIAamp DNA micro kit incl. bead beating. 16S rRNA (V1-V3) Illumina MiSeq	Rajka-Langeland
Animal studies	Rodrigues Hoffmann [27]	Swab	**A**xilla, **g**roin, **n**asal, skin **in**-between digits	USA, Texas	Allergic dogs (6, 5 with AD) Healthy ctrl dogs	No syst. antibiotics 30 d prior to sampling. 3 got glucocorticoids or cyclosporine, 3 got allergen-specific immunotherapy	6 A 12 Ctrl	Case-control	1	MoBio Power Soil DNA isolation kit 16S rRNA (V1-V3), 454-pyrosequencing	
	Meason-Smith [28]	Swab	**A**xilla, **g**roin, **n**asal, skin **in**-between digits, ear canal, lumbar	USA, Texas	Allergic dogs (8, 6 with AD) Healthy ctrl dogs 1.5-11 y Mixed sex	No syst. antibiotics or antifungals in the allergic dogs 1 month prior sampling (6 in the healthy). Top. allowed	8 A 10 Ctrl	Case-control	1	MoBio Power Soil DNA Isolation kit Internal Transcribed Spacer region (1F and 4R), Illumina MiSeq	
	Bradley [29]	Swab	Axilla, groin, pinna, mouth	USA, Pennsyl-vania	AD dogs with active lesions Healthy control dogs	4 used antibiotics within 45 d before. Targeted antimicrobial therapy in the interventional period	14 AD 16 Ctrl	Prospective cohort study: Flare-post therapy-post conclusion	3	Lysozyme, bead-beating, protein precipitation, Genomic DNA Isolation Kit (Life Tech) 16S rRNA (V1-V3) Illumina MiSeq	Clinical scoring, TEWL, pH
	Kobayashi [33]	Swab	Cheek	JapanUSA, Maryland and Minnesota	Disintegrin and metalloproteinase 17 deficient mice in Sox9-tissue, incl. epidermidis ($AD17^{fl/fl}Sox9\text{-}^{Cre}$).		A) 3 WT 3 AD17 B) 8 WT 8 AD17 C)12 AD17	A) Tanner stage B) Antibiotics targeting *S. aureus* and *C. bovis* C) Crossover D) Characteristics	A) 7 B) 3 C) 2 D) 1	Incubated in lysis buffer and lysozyme, (maybe bead-beated), Invitrogen PureLink Genomic DNA kit. 16S rRNA (V1-V3), 454-pyrosequencing	TEWL
	Kubica [32]	Punch biopsy (4 mm)	Ear	Belgium	Caspase-14 (involved in filaggrin degradation) knock out hairless mice		5 WT 4 KO	Animal study, case-control	1	QIAamp DNA Stool Mini Kit, 16S rRNA (V3-V5), 25 PCR cycles, 454-pyrosequencing	
	Schar-schmidt [31]	Biopsy	Ear flexure	USA, Maryland	Ichthyotic model: Matripase (degrades profilaggrin) deficient Mice, 1% of WT levels ($St14^{hypo/-}$)		3 WT + 3 $St14^{hypo/-}$	Animal study, case-control	1	DNAeasy kit (Qiagen), protocol for Gram-pos. bacteria (incl. bead-beating) 16S rRNA (V1-V8), 23 PCR cycles, Sanger	

Table S1: Characteristics of included published studies. Inflam.: Inflammatory. NA: Not available. Seb.: Sebaceous. NIH: National Institutes of Health. AD: Atopic dermatitis. Mod.: Moderate. Wk: Week. Top.: Topical. Prev.: Previous. PID: Primary Immunodeficiency. H: Hyper-IgE. W: Wiskott-Aldrich. D: DOCK8 deficiency. Syst.: Systemic. H: Hours. D: Days. EGFR: Epidermal Growth Factor Receptor. AD17: $AD17^{fl/fl}Sox9\text{-}^{Cre}$ (AD17=ADAM17, a metallopeptidase involved in epidermal barrier integrity). WT: Wild Type. KO: Knock Out. $St14^{hypo/-}$: Mice with one null and one hypomorphic allele of "Suppressor of tumorigenicity 14", matriptase = a serine protease. Ctrl: Control. A: Allergic. AD: Atopic dermatitis. RCT: Randomised Controlled Trial. *S. aureus: Staphylococcus aureus. C. bovis: Corynebacterium bovis*. PCR: Polymerase Chain Reaction. rRNA: ribosomal RNA. ITS1: Internal Transcribed Spacer region 1. V3: Variable region 3 of the 16S rRNA gene. Gram-pos.: Gram-positive. SCORAD: SCORing Atopic Dermatitis. TEWL: TransEpidermal Water Loss. EASI: Eczema Area and Severity Index.

Author(s)	Title	Sample method	Area sampled	Setting	Study population	N	Study type	Sample #	Method	Physiological and clinical measures
Barnes & Beck	Registry for the AD Research network	Swab		NIAID	AD, AD+Eczema Herpeticum (*S. aureus* pos. and neg.), AD+Eczema Vaccinatum, Non-atopic	2600	Multi-center clinical registry study. Determination of genetic markers associated with susceptibility of AD patients to infections. Database for future studies			
Beck	Bleach Bath Treatment of Adults With AD			NIH, Roches-ter	Mod.-severe AD 18-65 years Mixed gender	40	Prospective cohort study: Baseline – after diluted bleach baths twice a week for 12 weeks	2		TEWL, itch, skin permeability, transepithelial electrical resistance
Blaser & Gonzalez	The cutaneous microbiota in AD changes with top. corticosteroid and bleach bath treatment	Swab	Lesional Non-lesional Ctrl	NYU	AD 0.25-5 years Healthy ctrl	21 AD 14 Ctrl	Randomised, placebo-controlled, single-blinded trial. 4 weeks of treatment (top. corticosteroid twice daily alone or + twice weekly bleach baths)	2	16S rRNA	
Carlsen	Preventing AD and ALLergies in Children (PreventADALL)			Oslo, Østfold, Stock-holm	New-born babies	5200	A multi-national prospective birth cohort study with a factorial designed RCT of 1) skin care 0-9 months and 2) early food induction by 3-4 months. Thereafter only observation			6-12-36-48 months, annually: AD and food allergy
Gallo	Evaluation of the Kinetic Properties of an Autologous Microbiome Transplant in Adult AD Patients	Swab	Arms	San Diego	AD patients (*S. aureus* positive, not methillin-resistant *S. aureus*)	1-2) 10 3) 14	Prospective cohort studies: 1) Bacteria-containing moisturiser 2) Moisturiser containing the subject's own antimicrobial Staphylococcal species 3) 15 days moisturiser vs. moisturiser+own antimicrobial Staphylococcal bacteria	1) 4 2) 1-2 3) 4		Survival of transplanted bacteria, adverse reactions
	Validation of the Short-term Antimicrobial Action of Transplanted Bacteria						Each arm is treated 24 hours with moisturiser vs. moisturiser+own antimicrobial Staphylococcal bacteria			
	Examination of Whether Host Preconditioning Modifies Short-term Transplant Survival						One arm is pretreated with soap and alcohol to monitor if this increase survival of the transplanted bacteria			
Glatz	Emollient therapy alters skin barrier and microbes in infants at risk for developing AD	Swab	Cheek, dorsal and volar forearm	Substudy (UK, USA (42))	Newborns at risk of AD	19	Cross-sectional study from a RCT starting at birth: 6 months emollient or ctrl	1 (after)	16S rRNA	Skin pH, TEWL, water capacitance
Kong & Osgood	Effects of Treatment on AD			NCI	Healthy adults 18-50 years. AD 2-50 years.		Intervention: Randomisation to antimicrobial treatment study groups (and placebo)			
Mukherjee	Disease-specific changes in skin bacteriome and mycobiome in AD patients	Swab	Affected, Unaffected	Ohio	AD patients Allergic contact dermatitis Post-pubertal	13	Case control	1		
Nakatsuji & Gallo	The innate immune defect in AD includes a deficiency in antimicrobial activity produced by the skin microbiome	Swab	Lesional Non-lesional	San Diego	AD patients Healthy ctrl Mice		Case control and animal studies		16S rRNA Sanger	
Oetjen & Kim	Commensal microbiota-responsive basophils promote AD-associated itch				Mice (with cutaneous basophilia, pruritus)		Animal study			IgE, cytokines
Shi (& Gallo)	The skin microbiome Differs with Age in AD	Swabs	Lesional and non-lesional, volar forearm	Los Angeles	AD patients and healthy ctrl in two age groups: 2-12 and 13-62	128 AD 68 Ctrl	Case control		16S rRNA	

Table S2. Studies in progress (registered and/or unpublished) investigating the skin microbiome in atopic dermatitis.
NIAID: National Institutes of Allergy and Infectious Diseases. AD: Atopic dermatitis. Pos.: Positive. Neg.: Negative. NIH: National Institutes of Health. Mod.: Moderate. Top.: Topical. Ctrl: Control. NYU: New York University. Ctrl: Control. RCT: Randomised Controlled Trial. *S. aureus: Staphylococcus aureus.* UK: United Kingdom. USA: United States of America. NCI: National Cancer Institute

(a): Cochrane Collaboration's tool for assessing risk of bias in randomised controlled trials			
Study	**Domain**	**Review authors judgement**	**Support for judgement**
Bianchi [21]	Random sequence generation	Unclear	No information
	Allocation concealment	Unclear	No information
	Blinding of participants and personnel	Unclear	Patients not blinded, but no information on personnel.
	Blinding of outcome assessment	Unclear	No information
	Incomplete outcome data	Low risk	Reason given for one exclusion
	Selective reporting	Unclear	No study protocol available
	Other sources of bias	Unclear	Insufficient rationale: No sample size calculation. Objective is given but no clear hypothesis. No specified setting
Gonzalez [24]	Random sequence generation	Low risk	Shuffling envelopes
	Allocation concealment	Low risk	Numbered containers
	Blinding of participants and personnel	Low risk	Participants (incl. parents) and clinical personnel blinded
	Blinding of outcome assessment	Low risk	Investigators, data analysts, and sequences blinded to treatment until unblinding was necessary for comparative data analysis after ended experiment
	Incomplete outcome data	Low risk	Reasons for missing outcome data and balanced across intervention groups
	Selective reporting	Unclear	No study protocol available
	Other sources of bias	Low risk	The study appears to be free of other sources of bias

(b): Newcastle-Ottawa Scale for assessing quality of case-control studies				
Studies	**Selection** Definition and selection of cases and controls (max=4*)	**Comparability** of cases and controls (max=2*)	**Exposure** Blinding, same method, rel. abundances as outcome, complete data (max=4*)	**Total** (max=10*)
Dekio [17]	***	**	**	7
Kong [19]	***(*) (4/11 healthy children have fam. history of AD)	**	***	9
Oh [15]	***	**	***	8
Zhang [20]	*	*	***	5
Drago [23]	****	**	**	8
Kennedy [22]	****	**	***	9
Chng [25]	***	**	***	8
Shi [4]	****	**	***	9

(c): Newcastle-Ottawa Scale for assessing quality of cohort studies				
Studies	**Selection** True and/or somewhat representatives of AD, ascertainment of exposure, outcome at baseline (max=4*)	**Comparability** +/- treatment of matched skin areas, controlling for additional factors (max=2*)	**Outcome** Blinding, time to follow-up, complete follow-up, bias due to missing follow-ups (max=4*)	**Total** (max=10*)
Bourrain [16]	***	*	****	9
Flores [26]	**	*	**	5

(d): Adjusted SYRCLE's tool for assessing risk of bias in animal studies							
Type of bias	**Domain**	**Scharschmidt [31]**	**Kubica [32]**	**Rodrigues Hoffmann [27]**	**Meason-Smith [28]**	**Kobayashi [33]**	**Bradley [29]**
Selection bias	**Group similarity (sex, age)**	Low risk	Low risk	High risk	High risk	Low risk	High risk
Performance bias	**Random housing**	Unclear	Low risk	Low risk	Unclear	Unclear	Unclear
Detection bias	**Blinding**	High risk	Unclear	Unclear	Unclear	Unclear	High risk
Detection bias	**Blinding of outcome assessor**	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear
Attrition bias	**Incomplete outcome data**	Unclear	Unclear	Low risk	Low risk	Unclear	Low risk
Reporting bias	**Selective outcome reporting**	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear
Biases associated with interventional studies	**Allocation**					Unclear	Low risk
	Baseline characteristics					B) Unclear C) Low risk	Low risk

Table S3: Review authors scores of risk of bias of included studies using the Cochrane Collaboration's risk of bias tool (a), an adjusted Newcastle-Ottawa Scale for case-control studies (b) and cohort studies (c) where points (*) are assigned for no biases and an adjusted SYRCLE's tool for non-interventional (6 entries) and interventional (8 entries) animal studies (d).

Phylum	Family	Genus or species	Bourrain [16] #	De-kio [17]	Flores [26]	Kong [19]	Oh [15]	Zhang [20]	Bianchi [21] ¤	Drago [23]	Kennedy [22]	Chng [25]	Gonzalez [24]	Shi [4]
			Baseline/D1 D10 D18 **X I N**	¤ C AD	Pre-tr Post-tr U A Resp Non U+A U+A	Antecub & popl C B F P I N	Antecub Volar forearm C PID AD H W D	AD C M Mo S	D0 D28 C E	C AD U A	Antecub M2 U A M12	C AD	Baseline Post-tr **C Tr+bl Tr-bl** **L N L N**	C AD L/N Ch Teen-Adu
Firmicutes	Staphylococca-ceae									33 32 33	50 7			
		Staphylococcus		75 54	17 33* 15 52	16 35 31 90 20	11 28* 7 35*47* 6 18* 7 11 8*					9 7	12 62 31 58 34 12 24*6* 24* 40	6 9 37/21*38/29*
		S. aureus	36 52/56*16 48 44 24 24 28 20		1 8	1 17 15 65* 6	0 10* 1 8		28 28 NA 28 (6.5x↑)*					0 1 24/13*20/13*
		S. capitis				2 2 6 1 3					3 0			
		S. epidermidis			11 19	9 7 7 20* 8	5 13* 2 7*				27 4			1 4 9/4 11/9
		S. hominis				3 5 2 2 2								2 2 2/2 4/4
		S. haemolyticus					0 1* 0 1*							
		S. cohnii									19 1			
	Alicyclobacilla-ceae				5 4 6 4									
	Streptococca-ceae										5 24			
		Streptococcus		80 54	8 7 6 5	15* 2 14*						1 3*	16 9 8 7 9 20 13 8 13 8	26 14 20/26* 10/11
		S. mitis										1 2*		
	Neisseriaceae	*Neisseria*												2 1 2/2 1/1
	Veillonellaceae	*Veillonella*												2 2 1.5/2* 1/1
Proteo-bacteria	Rhodobactera-ceae									1 1 1				
	Moraxellaceae	*Acinetobacter*			2 1.2 2 1	1 0 3*							7 3 3 3 3 11 3 3 11 4	1 1 1/1 1/1
	Xanthomonada-ceae	*Stenotropho-monas*			7 1.5*									
		S. maltophilia		0* 38										
	Alcaligenaceae	*Alcaligenes xylosoxidans*		0 7										
	Enterobacteria-ceae	*Serratia marcescens*					0 8* 3 0 0 0 8* 3 0 0							
Actino-bacteria	Dietziaceae	*Dietzia maris*		80* 15										
	Propionibacteria-ceae									18 14 15	5 13			
		Propioni-bacterium			11 8 11 5	6 1 12*	24 11 26 9 7					56 67		13 28 5/5 11/14
		P. acnes		75* 35										13 28 4/5 10/13
	Corynebacteria-ceae									3 2 1	2 4			
		Coryne-bacterium			4 3 8 3	9* 1 9*	9 17* 14 15 5 8 18 9 17 5					6 2		3 8 3/4* 8/11*
	Dermacocca-ceae											1 0.5*		
	Actinomycetaceae	*Rothia*												2 2 2/2 1/1

Bacte-roidetes		*Prevotella*			1 2	1 0 1								3 3 2/2 2/2
Asco-mycota	Trichocomaceae	*Aspergillus*					1 4* 0 7*							
	Saccharomyce-tales	*Candida*					0 0.4* 0 0.3*							
		C. albicans						1 2 3 0						
	Davidiellaceae	*Cladosporium*						5 5 6 3						
	Capnodiales	*Toxicoclado-sporium irritans*						2 2 1 0						
Basidio-mycota	Malasseziaceae											2 1*		
		Malassezia					96* 71 96* 67	69(all) 79						
		M. restricta						48 49 34 59						
		M. globosa						15 16 27 14				19 10*		
		M. dermatis										2 8*		
	Tremellaceae	*Cryptococcus diffluens*						2 1 3 1						
Shannon diversity (- *S. aureus*)					A=6.0; U=6.3 Pre-post: ΔU=0.2 ΔA=0.08	3.4 2.8 2.7 0.7* 3.4 *2.8 2.5 2.5 1.5* 2.8*	2.8 2.4 2.7 2.3 2.1* 2.8 2.7 2.9 2.8 2.7 No treatment effect in P at any site		2 2 2 2		NS	1.7 1.4	Improved with no differences between tr-groups. Inversely corr. to EASI	
SCORAD			Reduction at D18 (30.8±7.2 to 20.0±10.2)	36	Δ = -12. 78% sites had ↓severity	NA 21.8 42.1 18.1	0 22 6 11 28		10 12 8* 6*	32	Not relevant			
EASI													Improved with no differences between tr-groups	
Spearman corr.							To SCORAD, inverse: *Stenot.*, *P. acnes*, *Neisseria*, *Streptococcus*. Pos.: *S. aureus*					Inverse: *P. acnes* and *S. epidermidis*. *Dermacoccus* and *S. aureus*		
TEWL (g/m^2/hour)									D28: No change in C, 34% ↓ in E			No diff		
Skin pH												No diff		

Table S4: Summary of relative abundance (in percent) of microorganisms found on skin, clinical and physiological outcomes, human studies. Taxonomic units with % relative abundance ≤ 1 are not included in this table.
#: Percentage of total amount of samples dominated by *S. aureus* (vs. diversified microbiota)
¤: Percentage of individuals in the study population with a specific microorganism (in percent)
X: Xerotic. I: Inflammatory. N: Non-lesional. L: Lesional site. .D1: Day 1. M2: Month 2. AD: Atopic Dermatitis. Antecub: Antecubital Fossa. Popl: Popliteal region. C: Control. Tr: Treatment. U: Unaffected. A: Affected. Resp: Responders. B: Before. F: Flare. I: Intermittent (treatment). N: No (treatment). P: Post flare. NA: Not available. PID: Primary Immunodeficiency. H: Hyper-IgE. W: Wiskott-Aldrich. D: DOCK8 deficiency. M: Mild. Mo: Moderate. S: Severe. E: Emollient (group). Ch: Children. Teenagers-Adults. SCORAD: SCORing Atopic Dermatitis. EASI: Eczema Area and Severity Index. TEWL: TransEpidermal Water Loss. * Indicate statistical differences found in the original papers.

Phylum	Family	Genus or species	Meason-Smith [28] C/A # Diff. between C and A, all sites Ax G I N E L	Rodrigues Hoffmann [27] C/A Ax G I N	Kobayashi [33] Time (wk) after birth / Mechanism WT/AD17 2 4 6 8 10 12 14	Antibiotic treatment AD17 −AB/+AB 2 4 8	Antibiotic treatment WT −AB/+AB 2 4 8	Crossover, AD17 -AB→+AB 10 13	Crossover, AD17 +AB→-AB 10 13	Bradley [29] C/AD Flare Ax G P	Bradley [29] C/AD Post TR Ax G P	Bradley [29] C/AD Followup Ax G P	Kubica [32] WT/KO	Scharschmidt [31] WT/St14hypo/−
Firmicutes														2/9*
		Other than *Streptococcus* + *Staphylococcus*			82 53 76 60 46 41 36 83 15 10 3 1 0 7	82 13 0 94 35 48	86 41 49 92 50 48	0 33	38 0					
	Class: Bacilli												5/2*	
	Order: Bacillales												2/8	
	Bacillaceae	*Bacillus*		3 1 1 0 4 2 0* 0										
	Staphylococcaceae												92/77*	
	Staphylococcaceae	*Staphylococcus*		1 1 1 0 0 3 1 0						3 12 4 33*43*45*	6 11 6 12 9 17	4 11 6 6 9 20	50/52	
		S. aureus			0 0 0 0 0 0 0 0 12 8 49 45 30 25	0 16 39 0 1 2	0 0 0 0 0 0	23 5	5 17	Across all skin sites: Flare: 10/10	Post TR: 1/7	P: 11/5		
		S. lugdunensis								Across all skin sites: Flare: 20/2	Post TR: 18/10	P: 20/12		
		S. pseudintermedius								Across all skin sites: Flare: 52/82	Post TR: 62/68	P: 52/68		
		S. lentus			3 5 2 2 0 0 0 4 1 0 0 0 0 0	0 1 0 0 1 0	0 0 0 0 0 0	0 4	3 1					
		Other than *aureus and lentus*			0 0 0 0 0 0 0 1 2 4 0 0 1 0	0 0 0 0 0 0	0 0 0 0 4 0	0 2	6 3					
	Alicyclobacillaceae	*Alicyclobacillus*		0 0 0 0 1 1 2 0										
	Streptococcaceae	Streptococcus			0 1 0 16 3 10 11 0 4 0 2 1 1 0	1 2 0 1 1 0	0 10 5 0 1 0	1 1	5 2	3 10 4 3 2 3	4 8 7 4 5 6	4 4 5 5 10 5		0/6*
Proteobacteria					4 16 3 3 8 3 3 1 5 2 1 0 0 0	3 4 0 3 19 2	3 14 9 3 6 5	0 12	16 0					97/75*
	Class: Beta-proteobacteria	Dominated by *Janthinobacterium*												35/31
	Neisseriaceae	*Conchiformibius*								2 6 3 0 1 1	4 7 3 1 2 1	2 7 2 3 4 4		
	Class: Gamma-proteobacteria	Dominated by *pseudomonas*												48/33
	Pasteurellaceae									3 3 4 2 1 1	2 2 2 3 1 2	3 2 2 2 2 2		
	Rhodobacteraceae	*Rubellimicrobium*		1 0 0 0 1 1 0* 0										
	Ralstoniaceae	*Ralstonia*		4 2 7 17 0 0* 0*0*										
	Sphingomonadaceae	*Sphingomonas*		0 1 0 0 3 2 0 0										
	Xanthomonadaceae												1/3*	
		Stenotrophomonas											0/4*	
Actinobacteria														2/13*
	Propionibacteriaceae	*Propionibacterium*								5 1 6 3 1 2	3 1 7 3 1 3	2 1 3 6 1 5		

	Corynebacteriaceae	*Coryonebacterium*				3 6 4 6 12*11	4 3 4 7 14 11	3 3 4 6 9 8		0 13*
		C. bovis			0 0 0 0 0 0 0 0 0 12 26 51 67 54	0 50 59 0 15 5	0 0 0 0 0 3	74 15 7 13			
		C. mastitidis			0 0 0 0 0 0 0 0 51 63 15 0 0 0	0 9 1 0 7 1	0 0 0 0 0 0	0 1 1 0			
		Other than *bovis, mast., jeikeium, tuberculostearicum*			2 11 2 11 9 11 19 0 0 0 0 0 0 0	0 0 0 1 8 12	0 16 7 0 12 47	0 6 7 0			
Bacteroidetes					6 9 3 13 29 28 25 6 6 2 0 0 0 5	7 2 0 6 16 6	6 16 26 6 25 8	0 17 4 0			
	Porphyromonadaceae	*Porphyromonas*							7 8 6 3* 3 1*	5 8 7 3 3 4	9 10 6 4 5 4		
	Flavobacteriaceae								1 1 1 1 0 0	1 1 1 4 2 1	2 2 1 2 1 1	0 6*	
Tenericutes	Mycoplasmataceae	*Mycoplasma*		0 0 0 0 2* 0 0 0									
Ascomycota	Pleosporaceae	*Alternaria*	30 28 22 33 23 32 20 20 26 30 11 22										
		Epicoccum	5 5 9 11 7 16 10 9 10 10 14 15										
	Davidiellaceae	*Cladosporium*	33 31 37 16 17 16 35 37 30 16 36 22										
	Saccharomycetales	*Candida*	# 0 1 0 0 0 0 0 4 0 0 0 0										
	Clavicipitaceae	*Claviceps*	# 1 1 0 0 0 1 0 4 0 0 0 0										
	Nectriaceae	*Fusarium*	1 2 3 0 2 0 2 5 2 0 0 2										
Basidiomycota	Malasseziales	*Malassezia*	1 1 0 5 1 1 1 2 2 0 2 0										
	Wallemiaceae	*Wallemia*	# 0 0 4 14 9 1 0 0 0 12 22 5										
	OTHER		# 2 4 5 0 7 4 5 1 2 5 12 2										
Shannon Diversity			All sites: $\frac{1.9}{1.7}$. Only diff in ear (C>A)	AGI: N: 6 2.9 5.4 2		2.3 1.3*0.8* 2.2 3 2.5	2 3.5 3.3 1.9 3 2.5		8 7 8 6*6 3*	8 8 7.5 7.5 7 7.5	8.5 7.5 8 7.5 6.5 7.5		
Chao1 richness				432 100 168* 40*									
Struc. similarity (θ)													WT ≠ KO
TEWL					5 6 8 NA 8 NA 9 7*23*27*NA 34*NA 40*	7 19 NA 7 12*NA	30 13*10 37 9 18		14 10 9 26 14 14	13 11 13 14 14 14	16 10 14 17 10 13		
Clinical score						NA 20 37 NA 12*8*		38 15* 5 43	0 22.0	0 14.9	0 15.9		
Antimicrobial activity												KO> WT	

Table S5: Summary of relative abundance (in percent) of microorganisms found on skin, clinical and physiological outcomes, animal studies. Taxonomic units with % relative abundance ≤ 1 are not included in this table. C: Control. A: Allergic. Ax: Axilla. G: Groin. I: In-between digits. N: Nasal. E: Ear Canal. L:lumbar. Wk: week. WT: Wild Type. AD: Atopic dermatitis. AD17: AD17$^{fl/fl}$Sox9-^{Cre}. AB: Antibiotics. EGFR: Epidermal Growth Factor Receptor. P: Pinna. KO: Knock Out. St14$^{hypo/-}$: Mice with one null and one hypomorphic allele of "Suppressor of tumorigenicity 14", matriptase = a serine protease. * Indicate statistical differences found in the original paper.

Manuscript II: Effects of sampling strategy and DNA extraction on human skin microbiome investigations

Using 16S rRNA gene sequencing and shotgun metagenomics on a subset of the samples (12/165) we found:

- Overlap of 99.3% of DNA sequences comparing the sampling strategies eSwab and scrapes of human skin.
- Higher consistency using eSwabs.
- Success rate of library preparation applying 12 different commercial DNA extraction kits ranging from 39% to 100%.
- Different microbial communities captured applying 12 different commercial DNA extraction kits.
- Clustering first by skin site (including nasal samples), then subject and finally by extraction kit.
- Reduction of the human DNA using one kit from app. 90% to 57% in nasal samples.
- Reduction in human DNA did neither lower the success of 16S rRNA gene library preparation nor caused taxonomic bias.

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Effects of sampling strategy and DNA extraction on human skin microbiome investigations

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The human skin is colonized by a wide array of microorganisms playing a role in skin disorders. Studying the skin microbiome provides unique obstacles such as low microbial biomass. The objective of this study was to establish methodology for skin microbiome analyses, focusing on sampling technique and DNA extraction. Skin swabs and scrapes were collected from 9 healthy adult subjects, and DNA extracted using 12 commercial kits. All 165 samples were sequenced using the 16S rRNA gene. Comparing the populations captured by eSwabs and scrapes, 99.3% of sequences overlapped. Using eSwabs yielded higher consistency. The success rate of library preparation applying different DNA extraction kits ranged from 39% to 100%. Some kits had higher Shannon alpha-diversity. Metagenomic shotgun analyses were performed on a subset of samples (N = 12). These data indicate that a reduction of human DNA from 90% to 57% is feasible without lowering the success of 16S rRNA library preparation and without introducing taxonomic bias. Using swabs is a reliable technique to investigate the skin microbiome. DNA extraction methodology is crucial for success of sequencing and adds a substantial amount of variation in microbiome analyses. Reduction of host DNA is recommended for interventional studies applying metagenomics.

The human skin is colonized by millions of bacteria, fungi and vira composing the skin microbiome. It has long been recognized that microbes are important players in skin diseases. Recently the relationship between host and skin microbes has experienced a renaissance of research activity after the rise of high-throughput DNA sequencing in 2006[1] and subsequently increased access and continued decrease in cost. Most protocols for characterization of human microbial communities have been developed for gut microbiome studies. The skin harbors completely different niches and unique methodological challenges such as high contamination risk[2] mainly due to low microbial biomass combined with a risk of adding contaminating microbes during handling of samples, e.g. from extraction reagents[3]. Difficulties in acquiring sufficient bacterial DNA for microbiome analysis is also an obvious challenge in skin microbiome studies[4,5].

The most common approach to characterize the skin microbiome is amplicon sequencing of the small subunit ribosomal RNA (16S rRNA in prokaryotes) gene. This gene is ideal for community fingerprinting since it is highly conserved and possesses conserved and variable regions. Groundbreaking studies of the skin microbiome have been made with amplicon sequencing. Grice and colleagues have shown that physiologically comparable skin sites (sebaceous, moist and dry) harbor similar bacterial communities and that sampling with swabs, scrapes and punch biopsies captures the same dominant microbial components[6,7]. Optimal methodologies for conducting skin microbiome research have received increased focus (reviewed in Kong *et al.*[8]) and it is becoming apparent that multiple steps in the analyses pipeline influence the results. Concerning sampling strategy different methodologies differ not only in sampling depth and discomfort but also in biomass yield and human DNA contribution. Compared to swabs, scrapes potentially increase the biomass collected which is useful in studies with rare taxa[9]. When isolating DNA for sequencing, most scientists conducting skin microbiome research use commercial kits relying on protocols which often differ in their strategy for disrupting bacterial cells. Enzymatic treatment, thermal disruption and/or mechanical lysis are commonly used. Bead size and material are likely to influence and select certain microbial populations.

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Over the past few years, whole metagenomic shotgun sequencing has become a common method for assembling genomes. This method has increased resolution and higher internal consistency[10] compared to 16S rRNA gene sequencing and can identify bacteria to strain-level. Shotgun sequencing captures all the genetic material in a sample without a targeted amplicon step, allowing comparisons on kingdom abundances. One such study of the skin has shown that site specificity also applies to fungi, but not to eukaryotic vira[11]. The same study showed that healthy adults maintain their skin communities for up to two years. However, since the human genome is about one thousand times larger than bacterial genomes, the total DNA pool can easily become dominated by host DNA, which might obscure small microbial differences e.g. induced in interventional studies. Depleting host DNA in samples for shotgun sequencing may be a crucial approach to improve data and different approaches have recently been compared in saliva samples[12].

While more thorough investigations of the effect of sampling procedures have been conducted for samples with high bacterial load and low amount of human DNA[13,14], a comparable effort focusing on the skin microbiome is lacking. With a very large amount of human DNA and a particular microbiome composition, this environment presents different challenges. Here we present a comparison of two sampling strategies and 12 commercially available DNA extraction kits for investigating the skin and nasal microbiome. We amplified the 16S rRNA gene and sequenced hypervariable regions 3–4. Nasal samples were also sequenced with shotgun metagenomics to evaluate the usefulness of host DNA depletion applied in two kits by use of lysis of human cells and subsequent addition of nucleases.

Results

Success of 16S rRNA gene sequencing varied with DNA extraction method. Nine healthy volunteers (5 women, 4 men), aged 26 to 64 years, were included in the study. In total, 220 samples were collected, including 144 skin samples from eSwabs and scrapes (Fig. S1, Table S1), 36 nasal eSwabs, 16 *E. coli* positive controls, and 24 negative controls containing either preservation medium from eSwabs or buffer from the kits. All 220 samples were analysed with 16S rRNA gene sequencing. Quality filtering removed samples with loading mistakes or less than 5000 reads, leaving 165 samples including 137 skin and nares samples used for figures. In total, 4,017,433 reads were produced in the 137 samples. The minimal number of reads in a sample was 11,360 and the median was 27,947. Furthermore, operational taxonomic units (OTUs) present in less than 10% of the samples were removed, leaving 4814 OTUs in the 137 samples (Table S2, a complete OTU table).

We applied 12 different DNA extraction kits (Table 1) with different success rates of library preparation (Table 2). Kits number 2, 10, 11 and 12 performed poorly with rates of successful libraries ranging from 39–79%. Success in library preparation was overall independent of DNA concentrations in the samples (Table 2). However, the worst performing kit, number 11, with a success rate of 39%, also had a very low average DNA concentration of 0.09 ng/µl.

The eSwab is a preferable method. We used eSwabs collecting material from the surface of the skin and scrapes collecting skin cells and microbes from the outermost part of the epidermis. There were no differences in concentrations of isolated DNA or success of library preparation between eSwabs and scrapes (data not shown). Furthermore, of the total 4814 OTUs, 4325 (89.8%) were identified with both sampling methods and these OTUs represented 3,989,311 sequence reads of the total 4,017,433 reads (99.3%) (Fig. 1a). Unique OTUs were identified using both eSwabs and scrapes. To further evaluate potential differences between microbial populations, Shannon alpha-diversity and Chao1 richness were compared (Fig. 1b). Differences between eSwabs and scrapes were found in Shannon alpha-diversity (mean scrape: 3.2; mean swab: 3.8; unadjusted p-value: 0.04), and in Chao1 richness, with lower richness in scrapes (mean scrape: 743; mean swab: 1180; unadjusted p-value: 0.00008). Notably, scrapes captured more *Pseudomonas* than eSwabs (Fig. S2). Redoing this analysis after removing all Pseudomonadales, this conclusion still holds for the richness, albeit with a higher p-value (Shannon's p-value: 0.08; Chao1: $p = 0.016$). The eSwab seems to be a more consistent method than scrapes, with better Pearson's product-moment coefficients at all taxonomic levels compared to scrapes (Figs. 1c and S3–5). Since the nare samples were only collected by eSwabs, we also made the scatter plots without those samples to rule out that inclusion of those made the scrapes seem worse off (Fig. 1c). Excluding Pseudomonadales improves Pearson's r at all taxonomic levels, but scrapes still perform consistently worse than swabs.

DNA extraction method captured different microbial communities. The spread observed on the scatter plots (Figs. 1 and S3–5) suggests that different extraction methods capture somewhat different communities since each pair of samples on the plots comes from the same skin site in the same individual but were extracted with different kits. If the performance of all kits were the same, the points would be close to the $y = x$ diagonal. The 12 different DNA extraction kits applied (Table 1) had some influence on Shannon alpha-diversity and Chao1 richness. Especially kit number 8 differs from most of the other kits, with high Shannon alpha-diversity and Chao1 richness (Fig. 2). Kit number 7 had this tendency as well. Notably, both kit number 7 and 8 applied a 3-mm stainless steel ball and bead beating in a Tissuelyser for mechanical disruption of bacterial cell walls and membranes (Table 1). However, kit number 10 also applied this bead beating protocol and did not yield higher Shannon alpha-diversity or Chao1 richness. Kit number 4 and 5 (same kits with different lot numbers, Table 1) had low Shannon-diversity and Chao1 richness (Fig. 2) and were dominated by Enterobacteriales (Fig. 3c), which might indicate contamination. Kits 5 and 6 also presented a large relative abundance of Pseudomonadales, also suggesting kit contamination. Our negative controls show that the main background is Pseudomonas (average 72% in all kit negative controls) and E. coli is present in most negative controls as well (average 3%) (Fig. S6). Two kits, number 8 and 10, have diverse profiles in their negative controls. Kit number 10 has a large representation (17%) of Burkholderia.

	DNA extraction kit	Kit number in this study	Storage of samples	Removal of human DNA	Thermal disruption	Chemical disruption	Mechanical disruption, bead beat	Binding to column	Washing	Elution	Storage temp.
MO BIO	BiOstic Bacteremia DNA Isolation Kit	1	−80 °C	no	70 °C	yes (no proteinase k)	MicroBead 	yes	yes	30 µL solution CB5	−80
	Microbial DNA Isolation Kit	2	−80 °C	no	4 °C	yes (no proteinase K)	MicroBead 	yes	yes	30 µL solution MD5	−20
	PowerLyzer UltraClean Microbial DNA Isolation Kit	3	−80 °C	no	4 °C	yes (no proteinase k)	Glass MicroBead 	yes	yes	30 µL solution MD5	−20
	UltraClean Tissue & Cells DNA Isolation Kit (Old LOT)	4	−20 °C	no	No	yes (high salt, proteinase K)	Dry Beads 	yes	yes	30 µL solution TD3 (no salt)	−80
	UltraClean Tissue & Cells DNA Isolation Kit (New LOT)	5	−20 °C	no	No	yes (high salt, proteinase K)	Dry Beads 	yes	yes	30 µL solution TD3 (no salt)	−80
	PowerSoil DNA isolation kit	6	−80 °C	no	4 °C	yes (no proteinase k)	PowerBeads + solution 	yes	yes	30 µL solution C6	−80
Epicentre	MasterPure yeast DNA purification kit (+PureLink Genomic DNA kit)	7	−80 °C	no	65 °C	yes (+lysozyme, no proteinase K)	3 mm stainless steel 	yes	yes	30 µL Milipore DNase free water	−20
QIAGEN	QIAamp DNA Investigator Kit	8	2–8 °C (max 48 hours)	no	56 °C	yes	3 mm stainless steel 	yes	yes	30 µL buffer ATE	−20
	QIAamp DNA Microbiome Kit	9	2–8 °C (max 48 hours)	yes (benzonase)	56 °C	yes	Large 	yes	yes	50 µL buffer AVE	−20
Invitrogen	PureLink Genomic DNA Kit	10	−80 °C	no	55 °C	yes (+lysozyme)	3 mm stainless steel 	yes	yes	35 µL elution buffer	−20
	PureLink Microbiome DNA purification	11	−80 °C	no	65 °C	yes (proteinase k)	specialized beads 	yes	yes	30 µL S6	−20
Molzym	MolYsis™ Complete5 (Ultra-deep Microbiome prep kit)	12	−20 °C	yes (MolDNase B)	37 °C −>56 °C −>70 °C	yes (+2−mercaptoethanol)	no	yes	yes	40 µL deionized water (70 °C)	−20

Table 1. Specifications of the used DNA extraction kits.

The heatmap (Fig. 3a) of Bray-Curtis dissimilarities did not indicate clustering according to DNA extraction kit. The subject had more influence on clustering than kit (Figs. 3a and 4).

Variation by skin location. Evaluation of beta-diversity using Bray-Curtis Dissimilarity reveals clustering by skin site (Fig. 3a) first and then by subject. Whether samples were taken from the antecubital and popliteal fossae or volar forearm had a relatively minor impact on Shannon-alpha Diversity, Chao1 richness (Fig. 3b) and the composition of the skin microbiome (Fig. 3c). Nasal samples differed significantly from skin samples, with lower Shannon-alpha Diversity, Chao1 richness and a microbiome dominated by *Staphylococcus* (primarily *S. epidermidis*) and *Corynebacterium*. The skin also contained *Staphylococcus* and *Corynebacterium* (Fig. S2). The experimental procedures used here did not allow us to assess the impact of Cutibacterium (see Discussion for details).

Reduction of host DNA content for shotgun sequencing. To investigate the efficacy of host DNA removal, one nasal sample from each of the 12 DNA extraction kits were also shotgun sequenced (Table S3). The samples were from subjects 2 (kit number 1, 2, 4 and 5), 5 (kit number 3, 6, 10 and 11) and 8 (kit number 7, 8, 9 and 12). A detailed description of the sequencing procedure and basic sequencing data quality measures is available in the Methods section.

Kit number 9 reduced the percent of human DNA from app. 90% to 57% (Table 2). Kit number 12 did not succeed in depleting or reducing human DNA. Importantly, using kit number 9 does not seem to introduce taxonomic biases as the sample clusters with samples from other kits (Fig. 4a) and the microbial profile is similar to samples from other kits.

Kit number	DNA concentration (ng/µl) total kit average	DNA concentration (ng/µl) Skin samples average	DNA concentration (ng/µl) Nares samples average	DNA concentration (ng/µl) E. Coli samples average	Successful libraries (16S)	% human DNA (shotgun)
1	1.99	0.05	12.17	3.03	100% (15/15)	90.1
2	0.67	0.02	3.76	0.34	67% (8/12)	90.3
3	0.52	0.01	0.91	6.85	94% (16/17)	85.3
4	0.51	0.00	3.30	NA	100% (13/13)	91.3
5	0.65	0.00	1.13	1.89	100% (16/16)	91.0
6	0.41	0.03	4.34	0.37	100% (12/12)	91.1
7	1.26	0.02	1.72	17.28	95% (17/18)	89.4
8	1.22	0.99	6.24	NA	95% (17/18)	89.8
9	0.09	0.01	0.06	0.68	95% (17/18)	57.4
10	1.95	0.03	8.69	8.68	78% (14/18)	91.7
11	0.09	0.02	0.27	0.60	39% (7/18)	88.8
12	0.39	0.04	0.72	2.36	79% (15/19)	89.6

Table 2. DNA concentrations after extraction, success rate of libraries prepared for 16S rRNA gene sequencing and percent of human DNA in samples from the nares. Success of libraries for 16S rRNA sequencing was evaluated based on 194 samples in total, 26 samples affected by loading errors were excluded. Percent of human DNA in samples were found from 12 samples from the nares which were shotgun sequenced.

The sample from kit number 7 gave the most distinct microbial profile (Fig. 4c) with a predominance of *Cutibacterium acnes* and unclassified *Escherichia* (Fig. 4a), where the latter might indicate contamination. As with 16S, the other samples were dominated by *Staphylococcus* and *Corynebacterium*. The sample from kit number 8 clusters with the one from kit number 7 and lacks a high abundance of *Corynebacterium propinquum* and *C.- accolens* (Fig. 4a).

The proportion of viral DNA (Fig. 4b) was influenced by the subject sampled. All samples from subject 2 contained viral DNA. Additionally, viral DNA was found in the sample extracted with kit number 8 from subject 8.

Discussion

We compared sampling of skin using eSwabs and scrapes with subsequent 16S ribosomal RNA gene sequencing. A very large overlap was found, both in OTU identified and in OTU counts, indicating that these methods can be used interchangeably. This is in line with data from Chng *et al.* comparing a modified cup scrub, swab and tape-strip[10] data from Ogai *et al.* comparing swabs and tape strips[15] and data from Grice *et al.* comparing swabs, scrapes and punch biopsies[7]. Grice *et al.* argue that microbiota from swabs and scrapes represent a history of skin differentiation, implying that the microorganisms from deeper layers transit to the surface with differentiating skin cells. With this perspective, the outermost microbiome (live or dead) can very well indicate which processes and physiological roles the microorganism deeper in the skin[16] may have. However, this might be too much of a simplification, as we also identify unique OTUs using each sampling method and a difference in Chao1 richness. A study applying repeated tape stripping for removal of the stratum corneum layers also show some significant differences in microbial composition between superficial and deeper layers of the stratum corneum with an increase in the relative abundance of Firmicutes (*Staphylococcus*) in the deeper layers and a decrease of Actinobacteria (*Cutibacterium*)[17]. We do not find such striking differences in microbial composition between samples of the outermost skin taken with eSwabs and scrapes going deeper in the stratum corneum. This could be due to differences in methodology, as our scrapes also capture the outermost microbiota which swabbing of specific layers after sequential tape stripping does not, but it might also be an artefact of the primers used, which do not appropriately amplify *Cutibacterium*. When sampling superficially by swabs one might overlook specific microbiota and potential interactions between microbiota and live human cells deeper in the skin. However, in our hands, data collected by eSwabs were more consistent. This technique is also less invasive and therefore more useful for certain purposes.

Many samples contained trace amounts of chloroplasts. Two of them, however, (kit 10, subject 4 and 6, flexure) contained large numbers of chloroplasts (15–25%). Since they do not consistently appear in all of kit 10's samples, we suspect that these subjects may have considerable physical contact with plants in their everyday life or made regular use of plant-based cosmetics.

Choice of DNA extraction kit affects the observed microbial profiles, but not more than inter-individual variation. It is difficult to assess which kit comes closest to the biological truth and a limitation of this study is a lack of a proper mock community as positive control. However, kit number 4 and 5 seemed to be dominated by contaminating bacteria and are not recommended for examination of the skin microbiome.

Other factors one should consider when choosing a protocol for DNA extraction is success in sequencing and convenience of usage in a specific setting. Kits number 2, 10, 11 and 12 performed poorly with rates of successful libraries for 16S rRNA gene sequencing ranging from 39–79%. We would avoid these kits. When taking samples from patients in the clinic it is of priority that they can be stored immediately. Kit number 12 had considerable hands-on time before a storage is possible. In general, less total hands-on time is also preferable.

Kit number 9 reduced the content of human DNA from nasal samples from 90% to 57%. We were worried that adding a nuclease for reduction of host DNA would destroy free microbial DNA as well and skew the picture of microbial communities compared to extraction kits without this step. Fortunately, no taxonomic skewing was observed in 16S

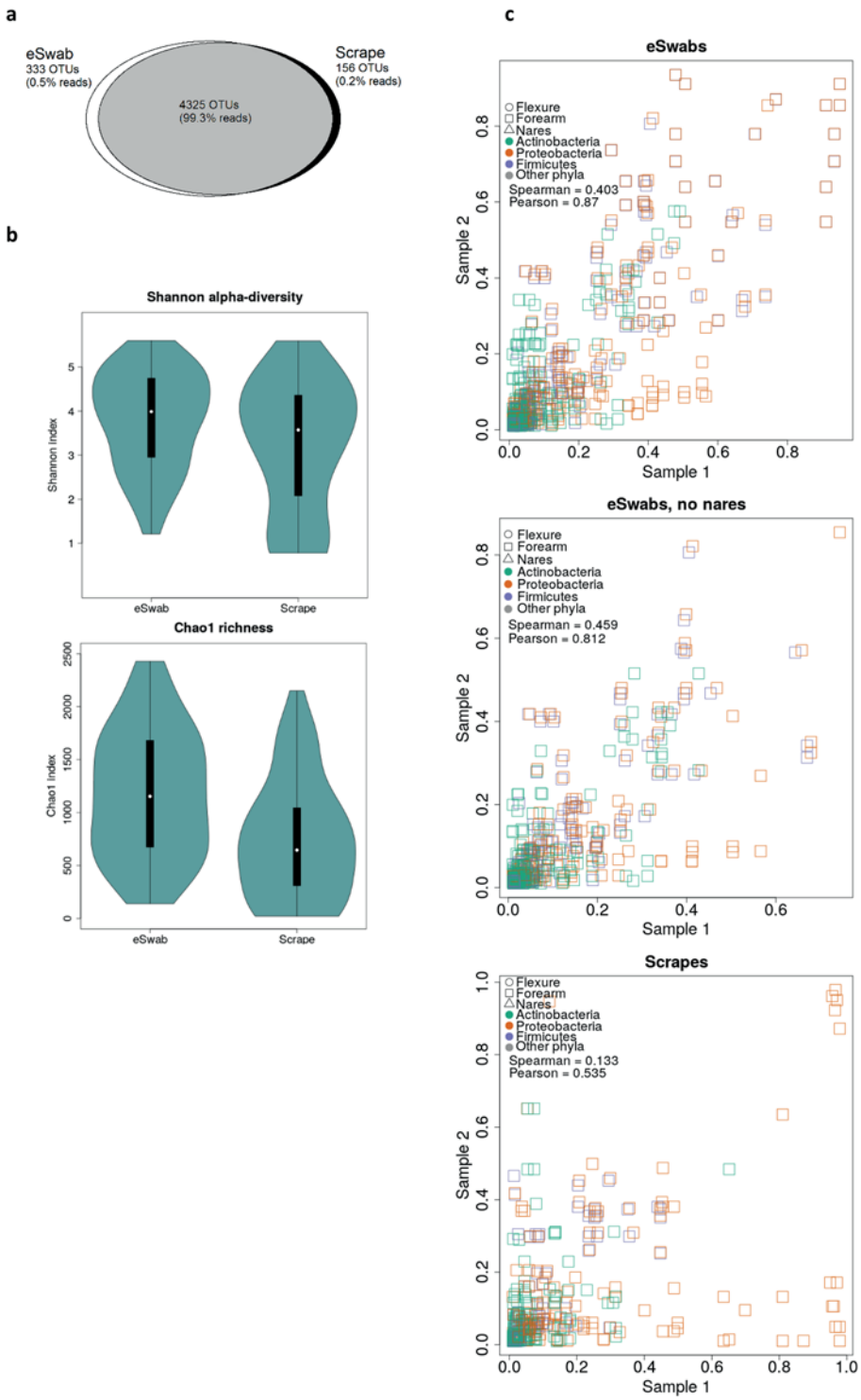
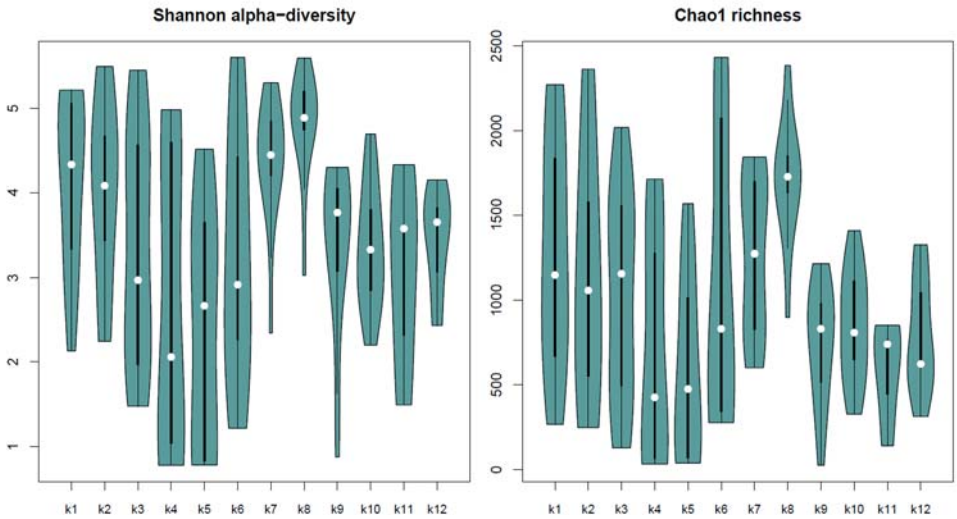


Figure 1. Comparison of skin sampling method. (**a**) A Venn diagram illustrating overlap of OTUs with ≥98% similarity and percent of sequence reads overlapping in parenthesis. (**b**) Violin plots illustrating Shannon alpha-diversity and Chao1 richness according to sampling method. (**c**) Scatter plots comparing the proportion of reads from a pair of samples from the same clade at the genus taxonomic level. Each sample is a pair of samples from the same skin site in the same individual, extracted with different kits. Pearson's product moment and Spearman's rank correlation were calculated for each plot.

or shotgun data. The good correspondence between the 16S and shotgun taxonomy profile using this kit also demonstrates that reagent contamination after DNA extraction was not an issue here. Another concern was that reduction of total DNA in the samples would increase the risk of failure in library preparation, as the DNA concentrations in skin

a



b

Shannon	k2	k3	k4	k5	k6	k7	k8	k9	k10	k11	k12
k1	0.871	0.773	0.871	0.773	0.773	0.980	0.773	0.773	0.753	0.773	0.773
k2		0.773	0.980	0.773	0.857	0.773	0.753	0.773	0.773	0.773	0.773
k3			0.773	0.980	0.980	0.753	0.171	0.980	0.846	0.773	0.980
k4				0.773	0.773	0.980	0.773	0.773	0.773	0.773	0.773
k5					0.980	0.726	0.171	0.846	0.857	0.773	0.980
k6						0.773	0.539	0.980	0.773	0.773	0.980
k7							0.753	0.171	0.171	0.386	0.171
k8								0.101	0.101	0.191	0.101
k9									0.773	0.773	0.773
k10										0.773	0.773
k11											0.773

Chao1	k2	k3	k4	k5	k6	k7	k8	k9	k10	k11	k12
k1	1	1	1	1	1	1	0.937	0.937	0.937	0.937	0.937
k2		1	1	1	1	1	0.937	1	1	1	1
k3			1	1	1	0.937	0.148	1	1	1	1
k4				1	1	1	0.479	0.937	0.937	0.937	0.937
k5					1	0.937	0.148	1	1	1	1
k6						1	0.937	1	1	1	1
k7							0.479	0.532	0.532	0.532	0.479
k8								***0.019***	***0.019***	0.148	***0.019***
k9									1	0.937	1
k10										1	1
k11											1

Figure 2. Influence of DNA extraction kit on microbiome diversity and richness. (**a**) Violin plots illustrating Shannon alpha-diversity and Chao1 richness according to DNA extraction kit. (**b**) Tables with p-values from Kruskal-Wallis-tests corrected for multiple testing by the Benjamini-Hochberg procedure, bold and underlined when statistical significance.

swabs generally are low, around 5 ng in total. Again, this was not a problem. Had it been a problem it would probably be advisable to sequence deeper to discover the effects of interventions rather than to reduce human DNA.

One drawback of using kit number 9 was that the total hands-on time was substantial. One recently published study compared this specific kit (Qiagen QIAamp DNA Microbiome Kit) with other methods of depleting host DNA in saliva[12]. It was found that treatment involving osmotic lysis of human cells and subsequent treatment with propidium monoazide is very efficient in removing host-derived sequences with a small taxonomic bias compared to untreated samples. Furthermore, this treatment is much cheaper than using the kit and requires fewer washing steps and less hand-on time[12]. Future studies should test this treatment on skin samples.

Whether samples were taken from popliteal and antecubital flexures or volar forearms had no impact on Shannon-alpha Diversity, Chao1 richness (Fig. 3b) or the composition of the skin microbiome (Fig. 3c). This is contradictory to the pioneer work performed by Grice *et al.*[6] and Findley *et al.*[9] showing that moist, dry and sebaceous areas have distinct microbial profiles. This is a general picture, and the actual differences found in these studies between the specific volar forearm and flexures are modest. Also, factors such as body composition and posture, clothing and weather can affect the moistness of the flexures. A dry flexure might be relatively similar to a volar forearm. Nasal samples differed significantly from skin samples with lower Shannon-alpha Diversity,

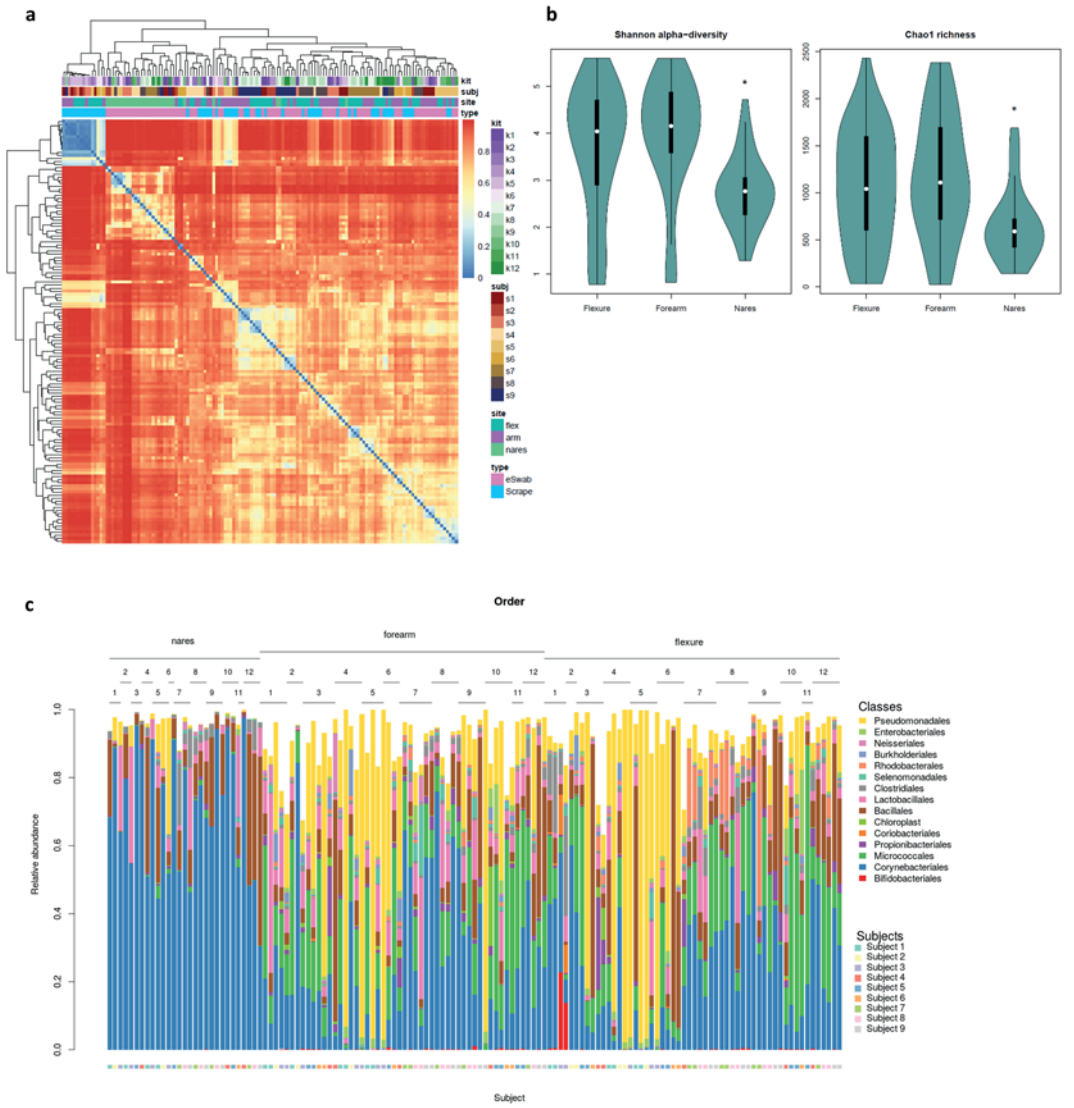


Figure 3. Variation by skin site. (**a**) A heatmap of Bray-Curtis distances between samples, with metadata plotted on the axis above and color code to the right. 0 indicates that samples share the same OTU and 1 that they are totally different. (**b**) Violin plots illustrating Shannon alpha-diversity and Chao1 richness according to skin site, * when statistical significance in a Kruskal-Wallis-test corrected for multiple testing by the Benjamini-Hochberg procedure ($p < 0.05$). (**c**) Bar charts depicting relative abundances of bacteria at the order taxonomic level. Samples are sorted by skin site and number of kit used is assigned above the charts. Individual subject numbers is indicated by the colour bar at the bottom of the figure.

Chao1 richness and a microbiome dominated by *Staphylococcus* (primarily *S. epidermidis*) and *Corynebacterium*. Choosing to investigate more distinct skin areas or including more areas and using metagenomics would possibly enable us to see a general pattern in differences between moist, dry and sebaceous areas.

We interpret the spread on the scatter plots as extraction methods capturing different communities. However, as we sampled non-overlapping skin areas, local differences in communities might also contribute to this spread. There are known variations in transepidermal water loss within the volar forearm, with higher values near the wrist compared to other sites of the forearm[18]. Also, recent studies show that sebum and hydration levels are predictors of microbiome composition[19] and that the specific composition of epidermal lipids strongly affects bacterial colonization[20]. It is however not possible to circumvent this issue when comparing multiple factors, as in our study.

In addition to false negatives, DNA extraction kits can contribute with false positives, especially in environments with relatively low bacterial abundance, as the human skin. Indeed, kit 4 and 5, which had the lowest DNA extraction yield for skin samples (Table 2), also had the highest amount of Pseudomonadaceae. This family has been described to be abundant in human skin before[7], but this observation has not been reproduced. Furthermore, Pseudomonadaceae have been found in high abundance in the "kit-ome", i.e. the background of bacterial DNA present in DNA extraction kits and PCR reagents[2]. No thermal disruption was applied in kit number 4 and 5 (Table 1) which could result in the low amounts of isolated microbial DNA from skin samples and higher amplification of contaminating DNA. However, our main conclusions hold even when excluding all Pseudomonadales and Enterobacteriales.

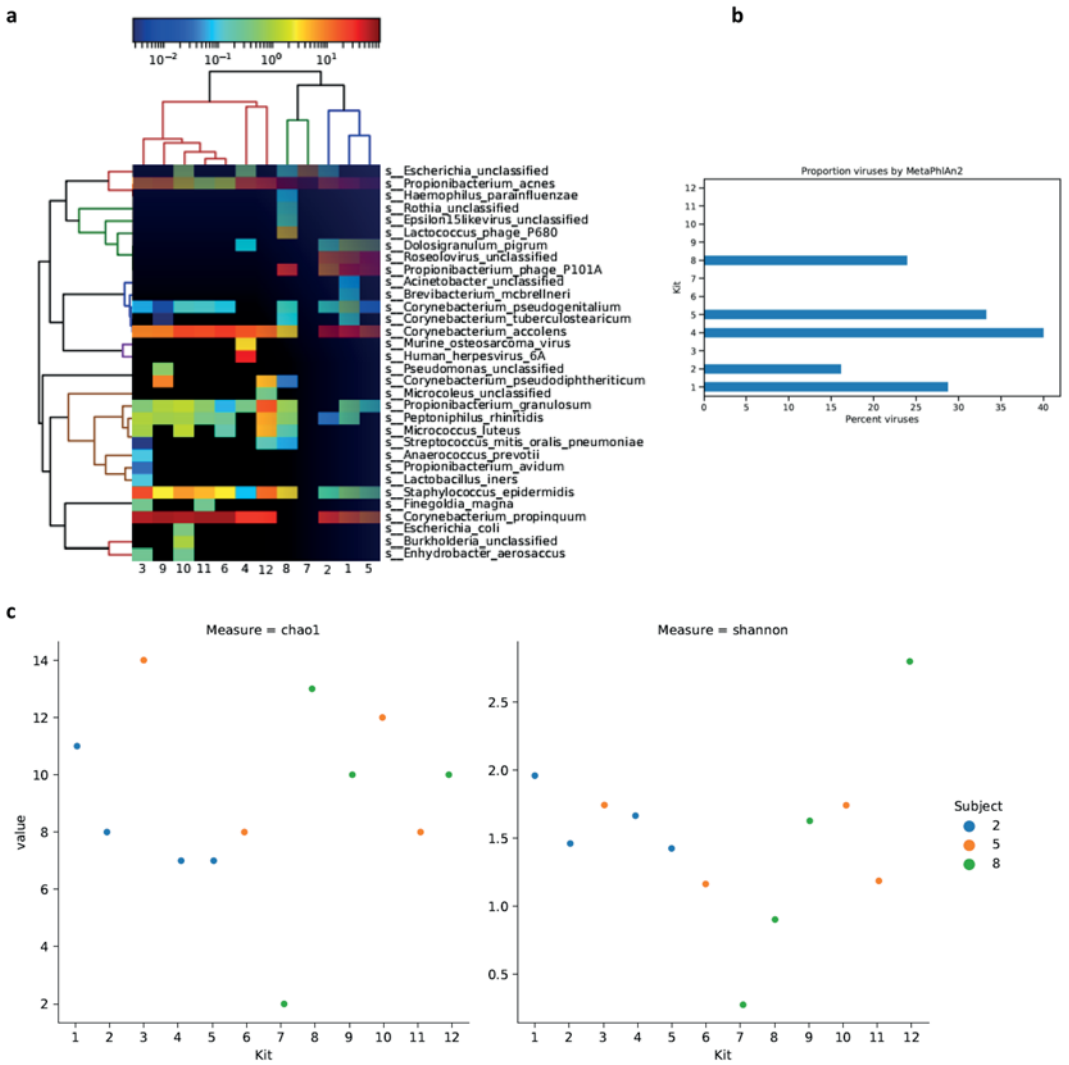


Figure 4. Reduction of host DNA does not influence microbial communities. (**a**) A MetaPhlAn2 clustered heatmap showing the distribution of microbes in the 12 samples, each representing one nasal sample from the kits applied (Table S2). Kit number is annotated along the x-axis and detected species-level names on the y-axis on the right side. (**b**) Percent viral DNA (x-axis) in the samples from each kit (y-axis). (**c**) Scatter plots illustrating Shannon alpha-diversity and Chao1 richness.

After sampling and DNA extraction, another major source of bias in amplicon-based studies is primer choice. The primer pair used here was suboptimal for skin microbiome studies, since it specifically excludes Cutibacterium, as evidenced by this clade being found in shotgun, but not in amplicon samples. However, since this bias was kept constant for all samples investigated, they can still be compared. Still, future studies on the human skin microbiome will benefit from using a shotgun approach when possible (see e.g.[10]), or another primer pair for the 16S region. In this case, two approaches are possible, either selecting a different region of this gene[21,22] or simply modifying the reverse primer to amplify the V4 region of Cutibacterium spp.[23].

Conclusion

Swabs and scrapes can be used interchangeably to investigate the skin microbiome. Swabs may be preferable as they are more consistent and less invasive. DNA extraction methodology is crucial for success of sequencing and adds a substantial amount of variation in microbiome analyses. However, clustering of data was more influenced by subject than kit. Using the QIAamp DNA Microbiome Kit from Qiagen, host DNA is reduced without introducing taxonomic biases, which is recommended for interventional studies applying metagenomics.

Methods

The study was approved by the ethics committee of the Capital Region of Denmark (H-16020971). All participants signed a written informed consent form prior inclusion and any sampling. All methods were performed in accordance with relevant guidelines and regulations.

Study participants. Nine healthy Caucasian volunteers were recruited from Hospital office staff in September 2016. Inclusion criteria were age 18 or older. Exclusion criteria were current or previous eczema, pregnancy, breastfeeding, scar tissue on sampling areas, active infections and use of antibiotics or probiotics within the past four weeks. The volunteers were instructed not to shower, use chlorinated pools, sauna, steam bath, sun tanning and topicals (e.g. moisturizers) two days before sampling at the Department of Dermatology and Allergy at Herlev and Gentofte Hospital.

Sampling. Skin samples were collected from non-overlapping areas on the dry volar forearms and moist antecubital and popliteal fossae. The fossae were defined as the region from the flexure + / − 4 cm and the volar forearm as starting after the antecubital fossa to 4 cm from the wrist. One side of the body was randomized to sampling with eSwabs (8 samples in total) and the other with scrape (8 samples in total). Four nasal eSwabs were collected from each subject as well, giving a total of 20 samples from each subject (Fig. 1S and Table S1). For sample collection, no prior cleaning or preparation of the skin surface was done. A fresh pair of gloves were worn for each sample. The flocked swab was premoistened in either preservation medium or enzymatic lysis buffer. A timer was set at 30 sec. for rubbing the skin area. Superficial skin scrapings were obtained by taking 20 strokes in different directions at the skin with a disposable scalpel. Sample material was placed into 2 ml LoBind Eppendorf tubes containing buffer from the kit (according to manufacturer).

E. coli ATCC 8739–0483E7 Epower pellets (SSI Diagnostica, CFU per pellet approximately 5×10^7) were suspended (according to manufacturer) in either preservation medium, buffer from the kit or enzymatic lysis buffer. Samples were either stored at −20 °C, −80 °C or processed immediately, according to DNA extraction protocol.

DNA extraction. DNA was extracted using 12 different commercial kits (kit number 4 and 5 were similar, but had different lot numbers) according to manufactures' protocols: 1. BiOstic Bacteremia DNA Isolation Kit (MO BIO, lot no.: BC16C25), 2. Microbial DNA Isolation Kit (MO BIO, lot no.: U16E2), 3. PowerLyzer UltraClean Microbial DNA Isolation Kit (MO BIO, lot no.: PL16C29), 4. UltraClean Tissue & Cells DNA Isolation Kit (MO BIO, lot no.: U15I14), 5. UltraClean Tissue & Cells DNA Isolation Kit (MO BIO, lot no.: U16D18), 6. PowerSoil DNA Isolation kit (MO BIO, lot no.: PS16C29), 7. MasterPure yeast DNA purification kit (Epicentre, lot no.: 0020027874), 8. QIAamp DNA Investigator Kit (QIAGEN, lot no.: 154018987), 9. QIAamp DNA Microbiome Kit (QIAGEN, lot no.: 154026306), 10. PureLink Genomic DNA Kit (Invitrogen, lot no.: 17462207), 11. PureLink Microbiome DNA purification (Invitrogen, lot. No.: 1761498), 12. MolYsis Complete5 Ultra-deep Microbiome prep kit (Molzym, lot no.: S22qKG020025). These kits were chosen because they were applied in published skin microbiome studies and/or recommended by the manufacturer to be useful for skin microbiome analysis. The combinations of kits, locations and subjects are described in Fig. S1 and Table S1.

16S rRNA gene sequencing. DNA concentration was determined with Quant-iT ds DNA broad range kit (Thermo Fisher Scientific, Waltham, MA, USA). The V3-V4 hypervariable region of the 16S rRNA gene was amplified using universal primers F341 and R805[24]. The PCR was performed according to the 2-step PCR protocol as described in Hugerth *et al.* 2018 with 23 µL of DNA solution as input[25]. The product was then cleaned with AMPure XP beads (Beckman Coulter, Brea, CA, USA) before being submitted to a 13 cycle barcoding reaction with Nextera XT index kit V2 (Illumina, San Diego, CA, USA) according to instructions from the manufacturer. The amplicons were sequenced on Illumina's MiSeq platform with 2 × 300 bp reads and a cutoff value of 5000 reads was applied. A blank (negative) PCR control was amplified and sequenced with each plate.

Operational taxonomic unit picking. After amplicon sequencing, we used Cutadapt v.1.16[26] to remove read pairs not carrying both primers or with an average Phred score < 15. Read pairs were then merged using Vsearch v.2.6.2[27] and excluding non-merging reads, merged pairs containing any ambiguous bases, with more than 3 expected errors over the full length or with a length <380 bp or >520 bp. We then used the unoise algorithm from Usearch v.10.0.240[28] to denoise reads. For quantification, all merged reads were mapped back to the centroid sequences requiring at least 98% identity over the full length of the query. To differentiate between *S. aureus* and *S. epidermidis* an additional run was made with 99.8% identity clustering. Taxonomy was assigned based on the SILVA database v128[29] using the algorithm described by Hugerth *et al.* (2018). Plant-chloroplast and mitochondrial OTUs were removed. All calculations on 16S rRNA gene data are OTU-based.

Metagenomic sequencing and analysis. Shotgun metagenomic sequencing was performed on 12 nasal samples (Table S3, accession number table). Sequencing libraries were constructed using Rubicon ThruPLEX DNA-seq, with an average fragment size of 365 base pairs (min: 304, max: 441, stdev: 42.2). Clustering was done by 'cBot' and samples were sequenced on an Illumina HiSeq2500 (HiSeq Control Software 2.2.58/RTA 1.18.64) with a 2 × 126 setup using 'HiSeq SBS Kit v4' chemistry. The Bcl to FastQ conversion was performed using bcl2fastq_v2.19.1.403 from the CASAVA software suite. The samples were sequenced in 2 lanes with 259 and 261 million clusters each, respectively, producing an average of 40.8 million reads (min: 30.8, max: 55.2, stdev: 8.1) with on average 93.0 of bases with Q-scores greater than 30. Metagenomic sequencing data was analyzed using StaG-mwc[30,31] version 0.2.0-dev. Reads were preprocessed with BBDuk[32] 37.99 using the default settings defined in StaG-mwc. Host contamination was removed with BBMap[32] v37.99 by mapping reads to a masked version of hg19 (http://seqanswers.com/forums/showthread.php?t=42552) using the default settings defined in StaG-mwc. Taxonomic profiling was performed using MetaPhlAn2[33] v2.7.7 using the default settings defined in StaG-mwc, with the addition of '-t rel_ab_w_read_stats' to produce estimated read counts per taxa used for downstream calculations.

Statistical analyses. Intra-sample diversity and richness were calculated using Shannon's entropy and Chao1 richness, respectively. Inter-sample diversity was estimated as Bray-Curtis divergence. Pairwise comparisons were performed with the Kruskal-Wallis test. All pairwise statistical comparisons were corrected for multiple

testing using the Benjamini-Hochberg procedure unless otherwise stated. All calculations for 16S data were performed in R v.3.4.3, with the additional packages Vegan v.2.4-5[34], Fossil v.0.3.7[35] and Vioplot v.0.2 (an R package based on the original work by Hintze *et al.*[36]). Calculations and visualizations of shotgun metagenomic data was performed in a Jupyter notebook (Jupyter v4.4.0) using SciKit-bio v0.5.4, matplotlib v3.0.0[37], seaborn v0.9.0[38], pandas v0.22.0[39] in Python v3.6.6.

Data availability

The sequence files and metadata for each sample in this study is publicly available at NCBI (Submission number: SUB4053477). Code used for the analysis of amplicon sequencing data is available at this repository: https://github.com/ctmrbio/Amplicon_workflows. The workflow used for the analysis of the shotgun metagenomics data is available at this repository (version 0.2.0-dev): https://github.com/boulund/stag-mwc (commit: ea3781d). Jupyter notebooks used to produce plots are available at https://doi.org/10.6084/m9.figshare.8319842.v1.

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References

1. Sogin, M. L. *et al.* Microbial diversity in the deep sea and the underexplored "rare biosphere". *Proceedings of the National Academy of Sciences* **103**, 12115–12120 (2006).
2. Salter, S. J. *et al.* Reagent and laboratory contamination can critically impact sequence-based microbiome analyses. *BMC biology* **12**, 87, https://doi.org/10.1186/s12915-014-0087-z (2014).
3. de Goffau, M. C. *et al.* Recognizing the reagent microbiome. *Nat Microbiol* **3**, 851–853, https://doi.org/10.1038/s41564-018-0202-y (2018).
4. Hannigan, G. D. *et al.* Culture-Independent Pilot Study of Microbiota Colonizing Open Fractures and Association with Severity, Mechanism, Location, and Complication from Presentation to Early Outpatient Follow-Up. *J. Orthop. Res.* **32**, 597–605, https://doi.org/10.1002/jor.22578 (2014).
5. Altunbulakli, C. *et al.* Relations between epidermal barrier dysregulation and Staphylococcus species-dominated microbiome dysbiosis in patients with atopic dermatitis. *J Allergy Clin Immunol*, https://doi.org/10.1016/j.jaci.2018.07.005 (2018).
6. Grice, E. A. *et al.* Topographical and temporal diversity of the human skin microbiome. *Science* **324**, 1190–1192 (2009).
7. Grice, E. A. *et al.* A diversity profile of the human skin microbiota. *Genome Res.* **18**, 1043–1050, https://doi.org/10.1101/gr.075549.107 (2008).
8. Kong, H. H. *et al.* Performing Skin Microbiome Research: A Method to the Madness. *J Invest Dermatol* **137**, 561–568, https://doi.org/10.1016/j.jid.2016.10.033 (2017).
9. Findley, K. *et al.* Topographic diversity of fungal and bacterial communities in human skin. *Nature* **498**, 367–370, https://doi.org/10.1038/nature12171 (2013).
10. Chng, K. R. *et al.* Whole metagenome profiling reveals skin microbiome-dependent susceptibility to atopic dermatitis flare. *Nature Microbiology* **1**, https://doi.org/10.1038/nmicrobiol.2016.106 (2016).
11. Oh, J., Byrd, A. L., Park, M., Kong, H. H. & Segre, J. A. Temporal Stability of the Human Skin Microbiome. *Cell* **165**, 854–866, https://doi.org/10.1016/j.cell.2016.04.008 (2016).
12. Marotz, C. A. *et al.* Improving saliva shotgun metagenomics by chemical host DNA depletion. *Microbiome* **6**, 42, https://doi.org/10.1186/s40168-018-0426-3 (2018).
13. Costea, P. I. *et al.* Towards standards for human fecal sample processing in metagenomic studies. *Nature biotechnology* **35**, 1069–1076, https://doi.org/10.1038/nbt.3960 (2017).
14. Sinha, R. *et al.* Assessment of variation in microbial community amplicon sequencing by the Microbiome Quality Control (MBQC) project consortium. *Nature biotechnology* **35**, 1077–1086, https://doi.org/10.1038/nbt.3981 (2017).
15. Ogai, K. *et al.* A Comparison of Techniques for Collecting Skin Microbiome Samples: Swabbing Versus Tape-Stripping. *Front Microbiol* **9**, 2362, https://doi.org/10.3389/fmicb.2018.02362 (2018).
16. Nakatsuji, T. *et al.* The microbiome extends to subepidermal compartments of normal skin. *Nature Communications* **4**, https://doi.org/10.1038/ncomms2441 (2013).
17. Zeeuwen, P. L. J. M. *et al.* Microbiome dynamics of human epidermis following skin barrier disruption. *Genome Biol.* **13**, https://doi.org/10.1186/gb-2012-13-11-r101 (2012)
18. Tupker, R. A. *et al.* Guidelines on sodium lauryl sulfate (SLS) exposure tests. A report from the Standardization Group of the European Society of Contact Dermatitis. *Contact Dermatitis* **37**, 53–69 (1997).
19. Mukherjee, S. *et al.* Sebum and Hydration Levels in Specific Regions of Human Face Significantly Predict the Nature and Diversity of Facial Skin Microbiome. *Sci Rep* **6**, 36062, https://doi.org/10.1038/srep36062 (2016).
20. Baurecht, H. *et al.* Epidermal lipid composition, barrier integrity, and eczematous inflammation are associated with skin microbiome configuration. *J Allergy Clin Immunol* **141**, 1668–1676.e1616, https://doi.org/10.1016/j.jaci.2018.01.019 (2018).
21. Meisel, J. S. *et al.* Skin Microbiome Surveys Are Strongly Influenced by Experimental Design. *J Invest Dermatol* **136**, 947–956, https://doi.org/10.1016/j.jid.2016.01.016 (2016).
22. Kuczynski, J. *et al.* Experimental and analytical tools for studying the human microbiome. *Nature reviews. Genetics* **13**, 47–58, https://doi.org/10.1038/nrg3129 (2011).
23. Zeeuwen, P. *et al.* Reply to Meisel et al. *J Invest Dermatol* **137**, 961–962, https://doi.org/10.1016/j.jid.2016.11.013 (2017).
24. Hugerth, L. W. *et al.* DegePrime, a program for degenerate primer design for broad-taxonomic-range PCR in microbial ecology studies. *Appl Environ Microbiol* **80**, 5116–5123, https://doi.org/10.1128/aem.01403-14 (2014).
25. Hugerth, L. W. *et al.* A comprehensive automated pipeline for human microbiome sampling, 16S rRNA gene sequencing and bioinformatics processing. *bioRxiv*, 286526. Preprint at, https://www.biorxiv.org/content/biorxiv/early/2018/03/21/286526.full.pdf (2018).
26. Martin, M. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet. journal* **17**, 10–12 (2011).
27. Rognes, T., Flouri, T., Nichols, B., Quince, C. & Mahé, F. VSEARCH: a versatile open source tool for metagenomics. *PeerJ* **4**, e2584 (2016).
28. Edgar, R. C. UNOISE2: improved error-correction for Illumina 16S and ITS amplicon sequencing. *BioRxiv*, 081257. Preprint at, https://www.biorxiv.org/content/biorxiv/early/2016/10/15/081257.full.pdf (2016).
29. Quast, C. *et al.* The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic acids research* **41**, D590–D596 (2012).
30. Boulund, F. StaG-mwc v0.3.0-beta. *Zenodo*, https://doi.org/10.5281/zenodo.1483891 (2018).
31. Boulund, F. & Olsson, L. StaG Metagenomic Workflow Collaboration (mwc). *GitHub*, https://github.com/ctmrbio/stag-mwc (2018).
32. Bushnell, B. BBMap: a fast, accurate, splice-aware aligner. Lawrence Berkeley National Lab.(LBNL), Berkeley, CA (United States). Poster at, https://jgi.doe.gov/wp-content/uploads/2013/11/BB_User-Meeting-2014-poster-FINAL.pdf (2014).

33. Truong, D. T. *et al*. MetaPhlAn2 for enhanced metagenomic taxonomic profiling. *Nature methods* **12**, 902 (2015).
34. Oksanen, J. *et al*. vegan: Community Ecology Package. R package version 1.17-2. *R Development Core Team. R: A language and environment for statistical computing. Vienna: R Foundation for Statistical Computing* (2010).
35. Vavrek, M. J. Fossil: palaeoecological and palaeogeographical analysis tools. *Palaeontologia Electronica* **14**, 16 (2011).
36. Hintze, J. L. & Nelson, R. D. Violin plots: a box plot-density trace synergism. *The American Statistician* **52**, 181–184 (1998).
37. Hunter, J. D. Matplotlib: A 2D graphics environment. *Computing in science & engineering* **9**, 90 (2007).
38. Waskom, M. *et al*. mwaskom/seaborn: v0.9.0. *Zenodo*, https://doi.org/10.5281/zenodo.1313201 (2018).
39. McKinney, W. pandas: a foundational Python library for data analysis and statistics. *Python for High Performance and Scientific Computing* **14** (2011).

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Author contributions
R.D.B. and J.D.J. designed the experiment. R.D.B. collected samples, extracted DNA and wrote the manuscript with input from all authors. M.S. made libraries for amplicon sequencing. L.W.H., F.B., R.D.B. and L.E. analyzed, interpreted and visualized data. All authors read and approved the final manuscript.

Competing interests
All authors declare that they have no financial competing interests. J.D.J. is head of the cosmetic counsel (unpaid position) advisory to the Danish Environmental Protection Agency.

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Electronic Supplementary Material

This supplementary material has been provided by the authors to give readers additional information about their work.

Supplement to: Rie Dybboe Bjerre[1*], Luisa Warchavchik Hugerth[2], Fredrik Boulund[2], Maike Seifert[2], Jeanne Duus Johansen[1], Lars Engstrand[2]. Effects of sampling strategy and DNA extraction on human skin microbiome investigations. Scientific Reports.

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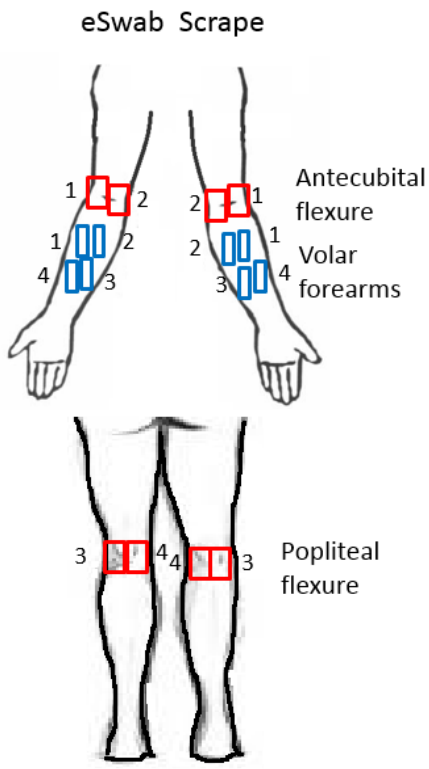

Figure S1. Skin sampling. Skin samples were collected from non-overlapping areas (illustrated with boxes) on the dry volar forearms and the moist antecubital and popliteal fossae. Each fossa was divided in two and volar forearm in four as illustrated. Kit number is exemplified with the numbers next to the boxes.
One side of the body was randomized to sampling with eSwabs (8 samples in total) and the other with scrape (8 samples in total). Four nasal eSwabs were collected from each subject as well, giving a total of 20 samples from each subject.

Sample	eSwab or scrape	Kit no.											
		5	4	1	2	3	6	11	10	12	9	8	7
Neg. Ctrl.	eSwab	1	4	7	10	73	76	79	82	145	148	151	154
Neg. Ctrl.	Scrape	2	5	8	11	74	77	80	83	146	149	152	155
Extraction Ctrl., E. coli		3e	6e	9	12	75	78	81	84	147e	150e	153	156
		3s	6s							147s	150s		
Nare	eSwab	17	22	27	32	89	94	99	104	161	166	171	176
		37	42	47	52	109	114	119	124	181	186	191	196
		57	62	67	72	129	134	139	144	201	206	211	216
Flexure, moist	eSwab	13	18	23	28	85	90	95	100	157	162	167	172
		33	38	43	48	105	110	115	120	177	182	187	192
		53	58	63	68	125	130	135	140	197	202	207	212
	Scrape	15	20	25	30	87	92	97	102	159	164	169	174
		35	40	45	50	107	112	117	122	179	184	189	194
		55	60	65	70	127	132	137	142	199	204	209	214
Volar forearm, dry	eSwab	14	19	24	29	86	91	96	101	158	163	168	173
		34	39	44	49	106	111	116	121	178	183	188	193
		54	59	64	69	126	131	136	141	198	203	208	213
	Scrape	16	21	26	31	88	93	98	103	160	165	170	175
		36	41	46	51	108	113	118	123	180	185	190	195
		56	61	66	71	128	133	138	143	200	205	210	215

Subject 1
Subject 2
Subject 3
Subject 4
Subject 5
Subject 6
Subject 7
Subject 8
Subject 9

Table S1: Metadata table. Each extraction kit was tested on samples from three subjects (colour). The letter "e" after some samples refers to suspension of E. Coli in preservation buffer from the eSwab and the letter "s" refers to suspension of E. Coli in kit buffer.

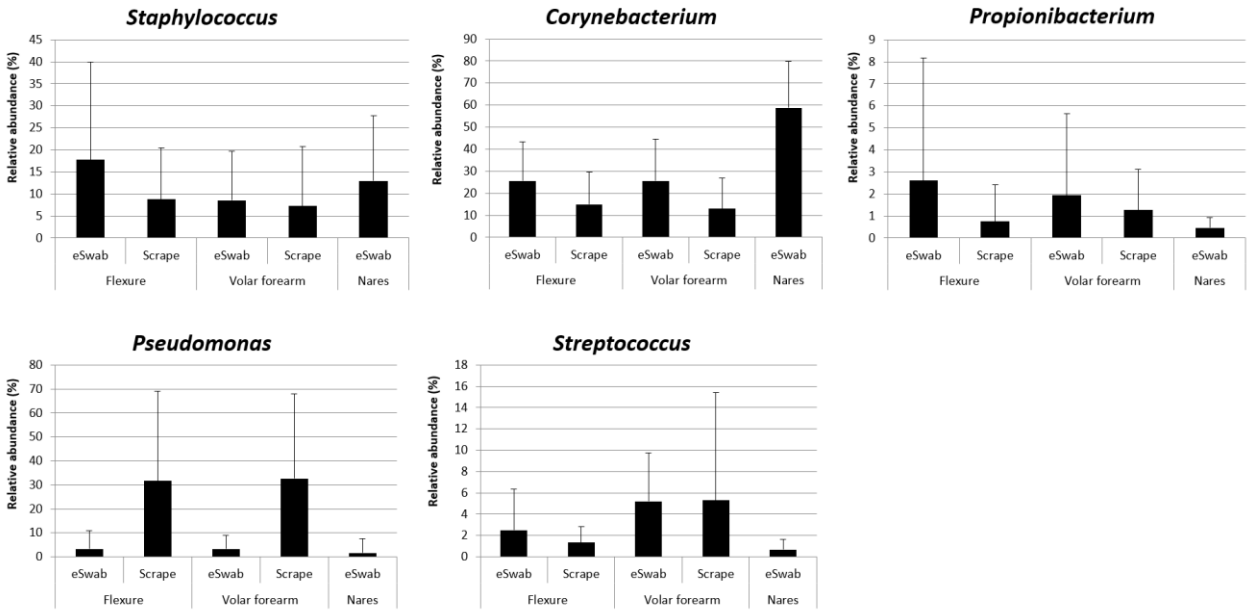

Figure S2. Relative abundances of common bacterial residents in skin. Bar charts with standard deviations depicting relative abundances of selected bacterial genera. Divided according to skin site (flexure, volar forearm, nares) and sampling method (eSwab, scrape). Pseudomonas is most likely contamination.

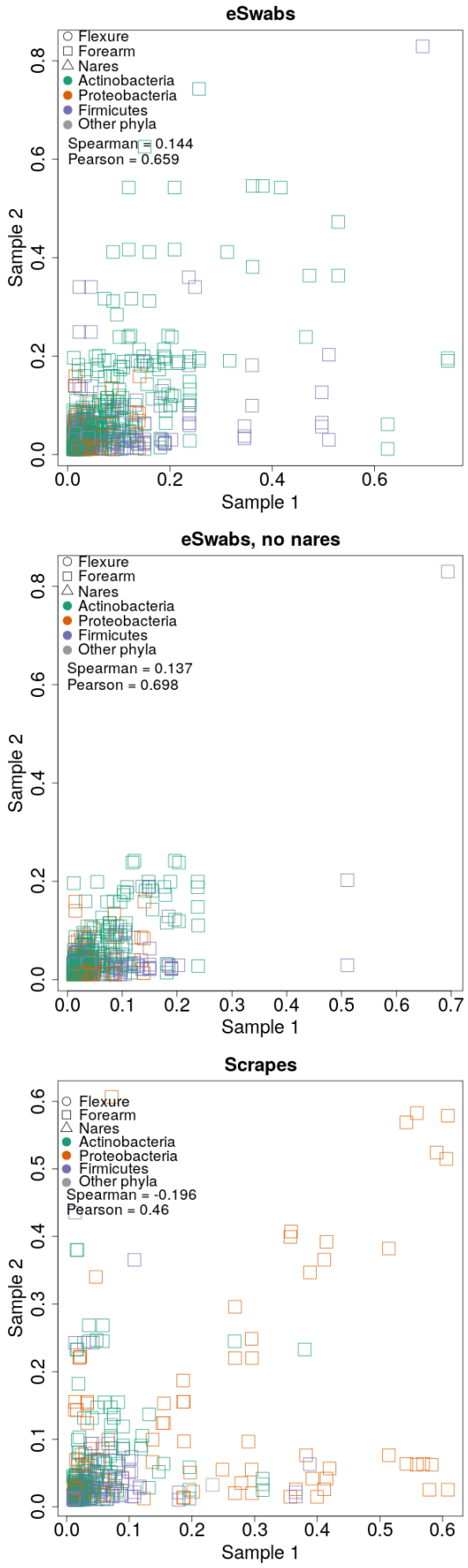

Figure S3: Community dissimilarities at OTU level. Scatter plots comparing the proportion of reads from a pair of samples from the same clade at the OTU taxonomic level. Each sample is a pair of samples from the same skin site in the same individual, extracted with different kits. Pearson's product moment and Spearman's rank correlation were calculated for each plot.

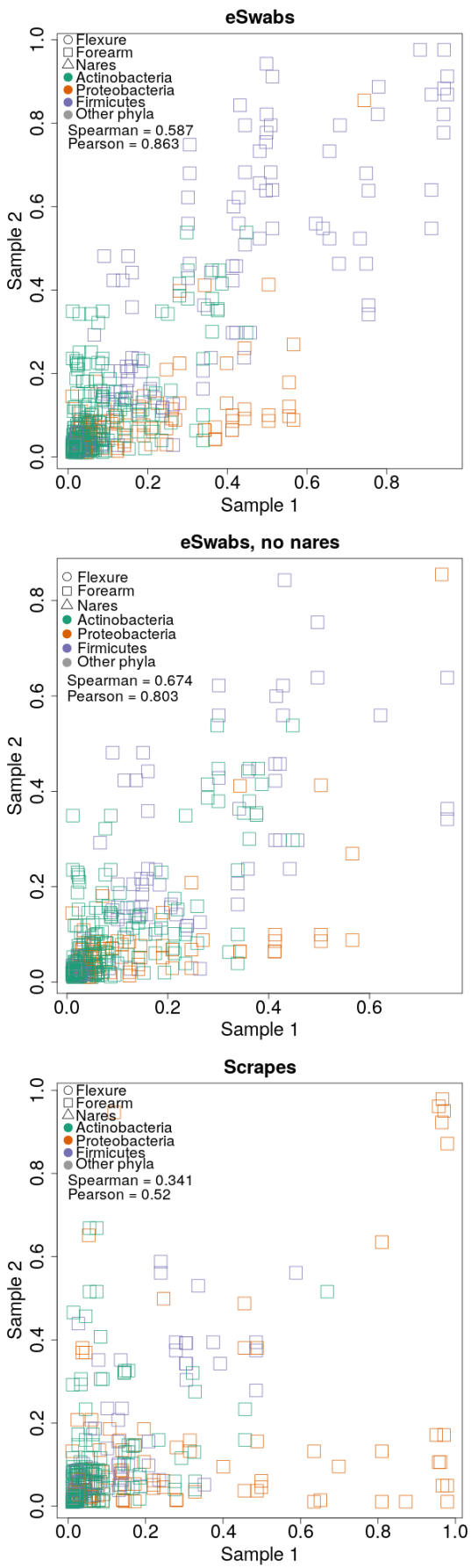

Figure S4: Community dissimilarities at family level. Scatter plots comparing the proportion of reads from a pair of samples from the same clade at the family taxonomic level. Each sample is a pair of samples from the same skin site in the same individual, extracted with different kits. Pearson's product moment and Spearman's rank correlation were calculated for each plot.

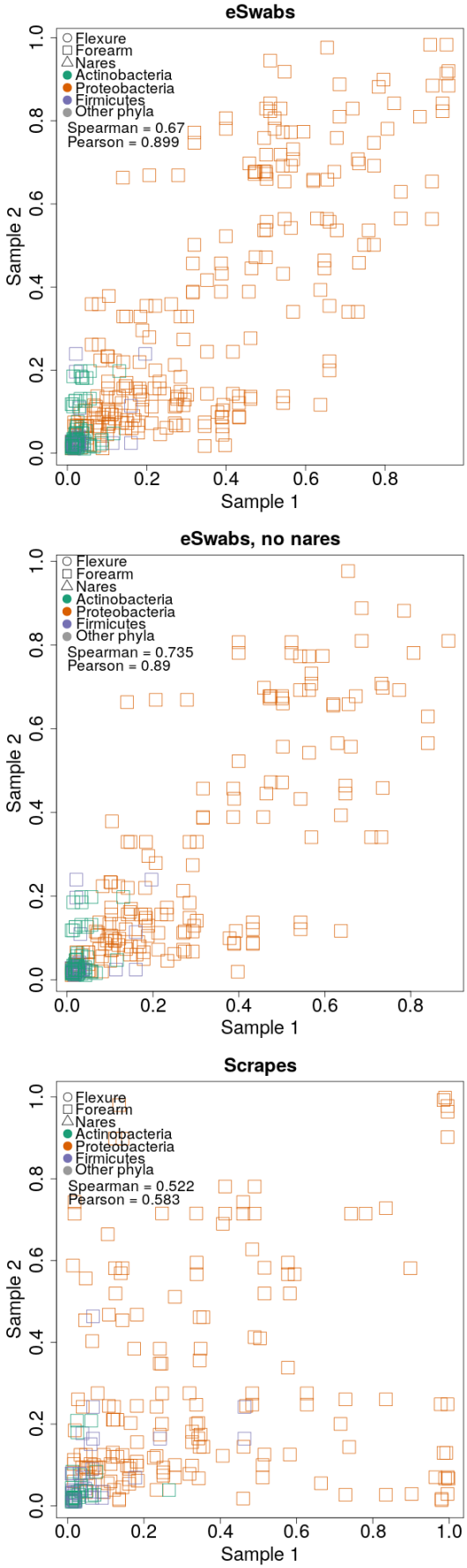

Figure S5: Community dissimilarities at class level. Scatter plots comparing the proportion of reads from a pair of samples from the same clade at the class taxonomic level. Each sample is a pair of samples from the same skin site in the same individual, extracted with different kits. Pearson's product moment and Spearman's rank correlation were calculated for each plot.

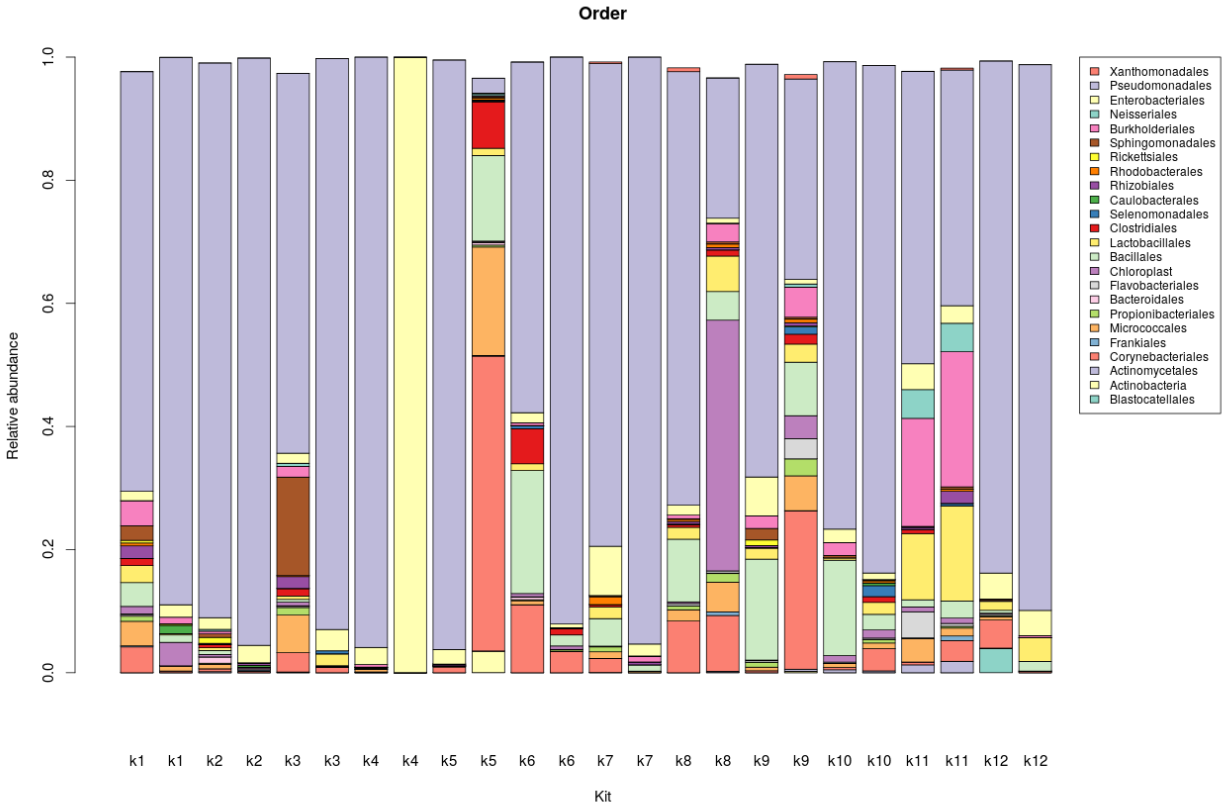

Figure S6: Relative abundances in negative controls. Bar charts depicting relative abundances of bacteria at the order taxonomic level in our negative control samples from each kit.

Accession	Sample name
SRR7294131	Neg ctrl eSwab k5
SRR7294130	Neg ctrl Scrape k5
SRR7294133	Ecoli ctrl eSwab k5
SRR7294132	Ecoli ctrl Scrape k5
SRR7294127	Neg ctrl eSwab k4
SRR7294126	Neg ctrl eSwab k1
SRR7294129	Neg ctrl Scrape k1
SRR7294128	Ecoli ctrl k1
SRR7294135	Ecoli ctrl k2
SRR7294134	eSwab Antecubital Flexure moist s1 k5
SRR7294163	eSwab Volar Forearm dry s1 k5
SRR7294162	Scrape Antecubital Flexure moist s1 k5
SRR7294161	Scrape Volar Forearm dry s1 k5
SRR7294160	eSwab Nare s1 k5
SRR7294159	eSwab Antecubital Flexure moist s1 k4
SRR7294158	eSwab Volar Forearm dry s1 k4
SRR7294157	Scrape Antecubital Flexure moist s1 k4
SRR7294156	Scrape Volar Forearm dry s1 k4
SRR7294165	eSwab Nare s1 k4
SRR7294164	eSwab Popliteal Flexure moist s1 k1
SRR7294038	eSwab Volar Forearm dry s1 k1
SRR7294039	Scrape Popliteal Flexure moist s1 k1
SRR7294036	Scrape Volar Forearm dry s1 k1
SRR7294037	eSwab Nare s1 k1
SRR7294034	eSwab Volar Forearm dry s1 k2
SRR7294035	eSwab Nare s1 k2
SRR7294032	Scrape Antecubital Flexure moist s2 k5
SRR7294033	eSwab Nare s2 k5
SRR7294030	eSwab Antecubital Flexure moist s2 k4
SRR7294031	eSwab Volar Forearm dry s2 k4
SRR7294196	eSwab Antecubital Flexure moist s2 k4
SRR7294195	Scrape Volar Forearm dry s2 k1
SRR7294198	eSwab Nare s2 k1
SRR7294197	eSwab Popliteal Flexure moist s2 k2
SRR7294192	eSwab Volar Forearm dry s2 k2
SRR7294191	Scrape Popliteal Flexure moist s2 k2
SRR7294194	eSwab Antecubital Flexure moist s3 k5
SRR7294193	eSwab Volar Forearm dry s3 k5
SRR7294190	Scrape Antecubital Flexure moist s3 k5
SRR7294189	Scrape Volar Forearm dry s3 k5
SRR7294102	eSwab Nare s3 k5
SRR7294103	eSwab Volar Forearm dry s3 k4
SRR7294104	Scrape Antecubital Flexure moist s3 k4
SRR7294105	Scrape Volar Forearm dry s3 k4
SRR7294098	eSwab Nare s3 k4
SRR7294099	eSwab Popliteal Flexure moist s3 k1
SRR7294100	eSwab Volar Forearm dry s3 k1
SRR7294101	Scrape Popliteal Flexure moist s3 k1
SRR7294106	Scrape Volar Forearm dry s3 k1
SRR7294107	eSwab Nare s3 k1
SRR7294068	eSwab Volar Forearm dry s3 k2
SRR7294067	Scrape Popliteal Flexure moist s3 k2
SRR7294066	Scrape Volar Forearm dry s3 k2
SRR7294065	eSwab Nare s3 k2
SRR7294072	Neg ctrl eSwab k3
SRR7294071	Neg ctrl Scrape k3
SRR7294070	Ecoli ctrl k3
SRR7294069	Neg ctrl eSwab k6
SRR7294063	Neg ctrl Scrape k6
SRR7294062	Ecoli ctrl k6
SRR7294138	Neg ctrl eSwab k11
SRR7294139	Neg ctrl Scrape k11
SRR7294136	Ecoli ctrl k11
SRR7294137	Neg ctrl eSwab k10
SRR7294142	Neg ctrl Scrape k10
SRR7294143	Ecoli ctrl k10
SRR7294140	eSwab Antecubital Flexure moist s4 k3
SRR7294141	eSwab Volar Forearm dry s4 k3
SRR7294144	Scrape Antecubital Flexure moist s4 k3
SRR7294145	Scrape Volar Forearm dry s4 k3
SRR7294109	eSwab Nare s4 k3
SRR7294108	eSwab Antecubital Flexure moist s4 k6
SRR7294111	Scrape Volar Forearm dry s4 k6
SRR7294110	eSwab Popliteal Flexure moist s4 k11
SRR7294113	eSwab Volar Forearm dry s4 k11
SRR7294112	Scrape Popliteal Flexure moist s4 k11
SRR7294115	Scrape Volar Forearm dry s4 k11
SRR7294114	eSwab Nare s4 k11
SRR7294117	eSwab Popliteal Flexure moist s4 k10
SRR7294116	eSwab Volar Forearm dry s4 k10
SRR7294064	Scrape Popliteal Flexure moist s4 k10
SRR7294097	Scrape Volar Forearm dry s4 k10
SRR7294073	eSwab Nare s4 k10
SRR7294074	eSwab Antecubital Flexure moist s5 k3
SRR7294075	eSwab Volar Forearm dry s5 k3
SRR7294121	Scrape Antecubital Flexure moist s5 k3
SRR7294205	Scrape Volar Forearm dry s5 k3
SRR7294086	eSwab Nare s5 k3
SRR7294040	eSwab Antecubital Flexure moist s5 k6
SRR7294041	Scrape Antecubital Flexure moist s5 k6
SRR7294125	Scrape Volar Forearm dry s5 k6
SRR7294124	eSwab Nare s5 k6
SRR7294123	eSwab Volar Forearm dry s5 k11
SRR7294122	Scrape Popliteal Flexure moist s5 k11

SRR7294120	Scrape Volar Forearm dry s5 k11
SRR7294119	eSwab Popliteal Flexure moist s5 k10
SRR7294118	eSwab Volar Forearm dry s5 k10
SRR7294180	Scrape Popliteal Flexure moist s5 k10
SRR7294169	Scrape Volar Forearm dry s5 k10
SRR7294168	eSwab Nare s5 k10
SRR7294154	eSwab Antecubital Flexure moist s6 k3
SRR7294155	eSwab Volar Forearm dry s6 k3
SRR7294152	Scrape Volar Forearm dry s6 k3
SRR7294153	eSwab Antecubital Flexure moist s6 k6
SRR7294150	eSwab Volar Forearm dry s6 k6
SRR7294151	Scrape Antecubital Flexure moist s6 k6
SRR7294148	eSwab Volar Forearm dry s6 k10
SRR7294149	Scrape Popliteal Flexure moist s6 k10
SRR7294146	Scrape Volar Forearm dry s6 k10
SRR7294147	eSwab Nare s6 k10
SRR7294175	Neg ctrl eSwab k12
SRR7294174	Neg ctrl Scrape k12
SRR7294177	Ecoli ctrl Scrape k12
SRR7294176	Neg ctrl eSwab k9
SRR7294171	Neg ctrl Scrape k9
SRR7294170	Ecoli ctrl eSwab k9
SRR7294173	Ecoli ctrl Scrape k9
SRR7294172	Neg ctrl eSwab k8
SRR7294167	Neg ctrl Scrape k8
SRR7294166	Ecoli ctrl k8
SRR7294056	Neg ctrl eSwab k7
SRR7294057	Neg ctrl Scrape k7
SRR7294058	eSwab Volar Forearm dry s7 k12
SRR7294059	Scrape Antecubital Flexure moist s7 k12
SRR7294052	eSwab Nare s7 k12
SRR7294053	eSwab Antecubital Flexure moist s7 k9
SRR7294054	Scrape Antecubital Flexure moist s7 k9
SRR7294055	Scrape Volar Forearm dry s7 k9
SRR7294060	eSwab Nare s7 k9
SRR7294061	eSwab Popliteal Flexure moist s7 k8
SRR7294090	eSwab Volar Forearm dry s7 k8
SRR7294089	Scrape Popliteal Flexure moist s7 k8
SRR7294088	eSwab Nare s7 k8
SRR7294087	eSwab Popliteal Flexure moist s7 k7
SRR7294094	eSwab Volar Forearm dry s7 k7
SRR7294093	Scrape Popliteal Flexure moist s7 k7
SRR7294092	Scrape Volar Forearm dry s7 k7
SRR7294091	eSwab Nare s7 k7
SRR7294096	eSwab Antecubital Flexure moist s8 k12
SRR7294095	Scrape Antecubital Flexure moist s8 k12
SRR7294044	Scrape Volar Forearm dry s8 k12
SRR7294045	eSwab Nare s8 k12
SRR7294042	eSwab Antecubital Flexure moist s8 k9
SRR7294043	eSwab Volar Forearm dry s8 k9
SRR7294048	Scrape Antecubital Flexure moist s8 k9
SRR7294049	Scrape Volar Forearm dry s8 k9
SRR7294046	eSwab Nare s8 k9
SRR7294047	eSwab Popliteal Flexure moist s8 k8
SRR7294050	eSwab Volar Forearm dry s8 k8
SRR7294051	Scrape Popliteal Flexure moist s8 k8
SRR7294077	Scrape Volar Forearm dry s8 k8
SRR7294076	eSwab Nare s8 k8
SRR7294079	eSwab Popliteal Flexure moist s8 k7
SRR7294078	eSwab Volar Forearm dry s8 k7
SRR7294081	Scrape Popliteal Flexure moist s8 k7
SRR7294080	Scrape Volar Forearm dry s8 k7
SRR7294083	eSwab Nare s8 k7
SRR7294082	eSwab Antecubital Flexure moist s9 k12
SRR7294085	eSwab Volar Forearm dry s9 k12
SRR7294084	Scrape Antecubital Flexure moist s9 k12
SRR7294181	Scrape Volar Forearm dry s9 k12
SRR7294182	eSwab Nare s9 k12
SRR7294183	eSwab Antecubital Flexure moist s9 k9
SRR7294184	eSwab Volar Forearm dry s9 k9
SRR7294185	Scrape Antecubital Flexure moist s9 k9
SRR7294186	Scrape Volar Forearm dry s9 k9
SRR7294187	eSwab Popliteal Flexure moist s9 k8
SRR7294188	eSwab Volar Forearm dry s9 k8
SRR7294178	Scrape Popliteal Flexure moist s9 k8
SRR7294179	Scrape Volar Forearm dry s9 k8
SRR7294204	eSwab Nare s9 k8
SRR7294203	eSwab Popliteal Flexure moist s9 k7
SRR7294202	eSwab Volar Forearm dry s9 k7
SRR7294201	Scrape Popliteal Flexure moist s9 k7
SRR7294200	Scrape Volar Forearm dry s9 k7
SRR7294199	eSwab Nare s9 k7
SRR9696275	Shotgun metagenomics of eSwab Nare s2 k5
SRR9696276	Shotgun metagenomics of eSwab Nare s2 k4
SRR9696277	Shotgun metagenomics of eSwab Nare s5 k3
SRR9696278	Shotgun metagenomics of eSwab Nare s5 k6
SRR9696284	Shotgun metagenomics of eSwab Nare s8 k12
SRR9696283	Shotgun metagenomics of eSwab Nare s8 k9
SRR9696281	Shotgun metagenomics of eSwab Nare s2 k1
SRR9696282	Shotgun metagenomics of eSwab Nare s2 k2
SRR9696273	Shotgun metagenomics of eSwab Nare s5 k11
SRR9696274	Shotgun metagenomics of eSwab Nare s5 k10
SRR9696279	Shotgun metagenomics of eSwab Nare s8 k8
SRR9696280	Shotgun metagenomics of eSwab Nare s8 k7

Table S3: Accession number table. Sample name and SRA identifier.

Manuscript III: Skin dysbiosis in atopic dermatitis is site-specific and involves the bacteriome, mycobiome and virome

Using shotgun metagenomics to characterize the skin microbiome profile at 14 non-overlapping skin sites on 10 patients with AD and 5 skin-healthy controls, we found:

- Success of library preparation to be related to control (87%, 61 of 70) versus AD (45%, 32 of 71 in lesional samples and 39%, 27 of 69 in non-lesional) and factors such as subject and skin site.
- Clear differences in microbial compositions of 121 samples between AD and skin-healthy controls.
- Most pronounced AD signature on the flexures and neck but also on hands and arms, while feet, periorbital and perioral areas were more similar.
- Lower alpha-diversity in flexures and a dominance of the genus *Staphylococcus*, especially *S. aureus* accompanied by *S. epidermidis* in lesions.
- Association between severe AD and *S. aureus* colonization (r = 0.63, P=0.00013).
- An absence of *Malassezia* species on the neck and flexures in AD.
- The skin virome to be dominated by [*Propionibacterium]* phages and *Staphylococcus* phages in both healthy controls and AD – with increased abundances of *Propionibacterium phages, PHL041* and *PHL092*, and *Staphylococcus epidermidis phages, CNPH82* and *PH15,* in AD.
- Higher absolute abundances of *Staphylococcus phages*, *Ipla5* and *Ipla7*, in lesional AD skin.
- Lower relative abundances of *S. hominis* and *C. acnes* when *S. aureus* was highly abundant.
- Higher relative abundances of *Moraxella osloensis* and *Micrococcus luteus* in AD.
- *S. aureus* strains to be subject specific and both similar and dissimilar to the ones in the nares.

Skin dysbiosis in atopic dermatitis is site-specific and involves the bacteriome, mycobiome and virome

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Abstract

Background: Microbial dysbiosis on the skin is a hallmark of AD, however most microbiome studies focus on bacteria in the flexures and the microbial composition at other body sites have not been studied systematically.
Objectives: The aim of the study is to characterize the skin microbiome, including bacteria, fungi and virus, at different body sites in relation to AD, lesional state, and *S. aureus* colonization, and to test whether the nares could be a reservoir for *S. aureus* strain colonization.
Methods: Using shotgun metagenomics we characterized microbial compositions of 121 samples from 14 well defined skin sites from 10 patients with AD and 5 healthy controls.
Results: We found clear differences in microbial composition between AD and controls at multiple skin sites, most pronounced on the flexures and neck. The flexures exhibited lower alpha-diversity and were colonized by *S. aureus*, accompanied by *S. epidermidis* in lesions. *Malassezia* species were absent on the neck in AD. The virome mostly constituted *Propionibacterium* and *Staphylococcus* phages, with increased abundance of *Propionibacterium phages PHL041* and *PHL092* and *Staphylococcus epidermidis phages CNPH82* and *PH15* in AD. In lesional samples, both the genus *Staphylococcus* and *Staphylococcus phages* were more abundant. *S. aureus* abundance was higher across all skin sites except from the feet. In samples where *S. aureus* was highly abundant, lower abundances of *S. hominis* and *Cutibacterium acnes* were observed. *Moraxella osloensis* and *Micrococcus luteus* were more abundant in AD. By single nucleotide variant analysis of *S. aureus* we found strains to be subject specific and both similar and dissimilar to the ones in the nares.
Conclusions: Our data indicate a global and site-specific dysbiosis in AD and indicate that interactions between bacteria, fungus and virus contribute to skin dysbiosis in AD. When defining targeted treatment clinicians should both consider the individual and skin site.

Key words: Atopic dermatitis, skin microbiome, dysbiosis

Background

AD is a complex skin disease with a lifetime prevalence of 15-20% in developed countries[1]. AD is characterized by epidermal barrier dysfunction, immune dysregulation and microbial dysbiosis. On healthy human skin, the most abundant bacterial genera are *Cutibacterium*, *Staphylococcus* and *Corynebacterium* with marked topographical diversity[2]. Based on conventional culture-based studies, it has long been recognized that *S. aureus* is highly abundant in AD[3], colonizing 70% of lesional and 39% of non-lesional sites, and 62% of the nares samples[4]. *S. aureus* colonization adversely affect disease severity[5]. In recent years, skin microbiomes in AD have been studied in a variety of conditions[5-8,9,10]. Most studies are based on sequencing the 16S rRNA gene of bacteria. Applying this method, bacterial diversity has been shown to be lower in AD[5,7,11] and *S. epidermidis* abundant[5,12]. Therapy increases diversity[9] and the abundances of *Streptococcus*, *Cutibacterium* and *Corynebacterium*[5]. By applying shotgun sequencing of whole metagenomes a better taxonomical resolution is achieved and all domains can be analysed. Studies applying this method in AD are emerging[8,10,13,14] and describe specific *S. aureus* strains in severe AD[8], perturbations in the eukaryotic community[13], and define AD subgroups[14].

There is growing evidence of a key role of the microbiome in the pathogenesis of AD[15]. This is supported by studies showing that microbiome dysbiosis can precede AD in early childhood[16,17]. Though there might be a critical window for establishing a healthy microbiome and immune tolerance toward it in early childhood[18], studies applying topical commensals to re-establish a healthy microbiome in AD show promising results[19-21]. However, benefits of using commensals have been reported to be dependent on skin site, for instance with a treating effect of transplanting *Roseomonas mucosa* in the antecubital flexure of AD patients but no effect on hands[19]. In general, most microbiome studies in AD focus on the body flexures but do not address microbial composition at other body sites. Furthermore, the virome in AD has not been investigated. Here, we present a case-control study applying shotgun metagenomics to characterize the skin microbiome of AD patients at different body sites.

Results

Samples from 5 healthy controls (3 women, 2 men), aged 27-63 and 10 patients with AD (7 women and 3 men), aged 24-62 years, were included in this study. Mean SCORAD for patients with AD was 30.8 (Table 1). Of 212 samples (including *E. coli* and buffer controls), 91 samples were of insufficient DNA quality and/or amount for sequencing (Table 1). Success of library preparation in lesional samples were 45% (32/71), 39% (27/69) in non-lesional and 87% (61/70) in controls. Other factors influencing success of library preparation were related to subject and skin site (Table 1). Initially, data were described according to the 14 skin sites sampled. When analyzing the effect of lesions, the 14 skin sites were pooled, with a minimum number of 5 samples per group.

								Skin site | sample number when available, if lesional (LS)													
ID	**Age**	**Sex**	**SCORAD**	**HECSI**	**FLG**	**Co-morbidities**	**Treatment**	**Nasal**	**Peri-orbital**	**Perioral**	**Neck**	**Upper inner arms**	**Antecubital flexures**	**Volar forearms**	**Dorsum of hands**	**Palmar hands**	**Between fingers**	**Popliteal flexures**	**Dorsum of feet**	**Arches of feet**	**Between toes**
AD1	62	M	29.4	13	UN	Asthma	Methotrexat	2.1	2.2	2.3 (LS)	- (LS)	-	- (LS)	- (LS)	- (LS)	2.9 (LS)	2.10 (LS)	-	2.12 (LS)	-	2.14
AD2	41	F	68.3	11	UN	No	No (Nizoral occasionally)	3.1	3.2 (LS)	3.3 (LS)	3.4 (LS)	3.5 (LS)	3.6 (LS)	3.7 (LS)	3.8 (LS)	3.9	3.10 (LS)	3.11 (LS)	- (LS)	-	3.14
AD3	49	F	16.7	4	Mut	Hay fever, CD	Elocon (No Nizoral)	4.1	4.2	4.3	4.4	-	4.6	-	- (LS)	-	4.10 (LS)	-	-	-	4.14
AD4	42	F	19.7	5	WT	Asthma, hay fever, CD	Elocon (Nizoral in year 2018, likely after study participation)	5.1	5.2 (LS)	5.3 (LS)	5.4 (LS)	-	-	- (LS)	-	5.9 (LS)	- (LS)	-	- (LS)	-	5.14 (LS)
AD5	38	F	29.9	9	Mut	Asthma, hay fever, CD, FA	Dermovat	7.1	-	7.3	-	-	-	-	- (LS)	- (LS)	- (LS)	-	- (LS)	- (LS)	- (LS)
AD6	24	M	24.8	15	UN	No	Elocon	8.1	-	8.3	-	-	- (LS)	-	- (LS)	- (LS)	- (LS)	-	-	-	8.14 (LS)
AD7	59	M	27.3	14	Mut	No	No	-	- (LS)	- (LS)	- (LS)	- (LS)	- (LS)	- (LS)	- (LS)	-	-	- (LS)	- (LS)	-	-
AD8	53	F	24.9	6	UN	CD	Cellcept, betnovat	12.1	- (LS)	- (LS)	- (LS)	-	- (LS)	- (LS)	- (LS)	-	- (LS)	-	-	-	12.14
AD9	56	F	20.7	7	Mut	Asthma, hay fever, CD	Dupilumab (No Nizoral)	18.1	-	18.3	18.4	18.5 (LS)	18.6 (LS)	- (LS)	- (LS)	18.9	18.10 (LS)	18.11	18.12 (LS)	18.13 (LS)	18.14
AD10	46	F	45.9	16	NA	Asthma, hay fever	Imurel (No Nizoral)	20.1 (LS)	20.2 (LS)	20.3 (LS)	20.4 (LS)	- (LS)	20.6 (LS)	20.7 (LS)	- (LS)	20.9	20.10 (LS)	-	-	-	20.14
C5	27	M	0	0	NA	No	No	15.1	15.2	15.3	15.4	-	15.6	-	15.8	15.9	15.10	15.11	15.12	15.13	15.14
C6	63	M	0	0	NA	No	No	16.1	16.2	16.3	16.4	16.5	16.6	-	16.8	16.9	16.10	16.11	16.12	16.13	16.14
C8	42	F	0	0	NA	No	No	19.1	19.2	19.3	19.4	-	19.6	19.7	-	-	19.10	-	19.12	19.13	19.14
C9	60	F	0	0	NA	CD	No	21.1	21.2	21.3	21.4	21.5	21.6	21.7	-	21.9	21.10	21.11	21.12	21.13	21.14
C10	50	F	0	0	NA	No	No	22.1	22.2	22.3	22.4	22.5	22.6	22.7	22.8	-	22.10	22.11	22.12	22.13	22.14

Table 1: Characterization of the study population and samples.

The 14 skin areas sampled are listed in the top of the table and in detail include: The neck (the anterior triangle), and bilaterally from the anterior nares, periorbital and perioral areas, antecubital and popliteal flexures (midline +/- 5 cm), upper inner arms (starting after the flexural area ending before the armpit, before presence of hair follicles from the armpit), volar forearms (starting after the antecubital fossae to 4 cm from the wrist), dorsum of the hands and feet (from wrist to joints of the digits), the web spaces between the fingers and toes, palmar hands (from wrist to joints of the digits), and arches of the feet . Abbreviations: AD, atopic dermatitis; C, control; M, male, F, female; SCORAD, Severity Scoring Atopic Dermatitis; HECSI, Hand Eczema Severity Index; FLG, filaggrin gene; UN, unknown; CD, contact dermatitis; FA, food allergy; WT, wildtype; Mut, mutation; NA, not applicable; LS, lesional skin.

Beta diversity revealed characteristic AD skin sites

Subject explained the majority of the explained microbial variance (PERMANOVA test; R^2 = 19%; P = 0.0001), however, the overall skin microbial composition differed significantly between AD and controls (PERMANOVA test; R^2 = 6%, P = 0.0001). As visualized on the principal coordinate analysis (PCoA, Fig. 1), samples from the hands and arms, flexures and neck showed the clearest separation according to control or AD (Fig. S1). The lowest separation was observed for perioral and periorbital samples.

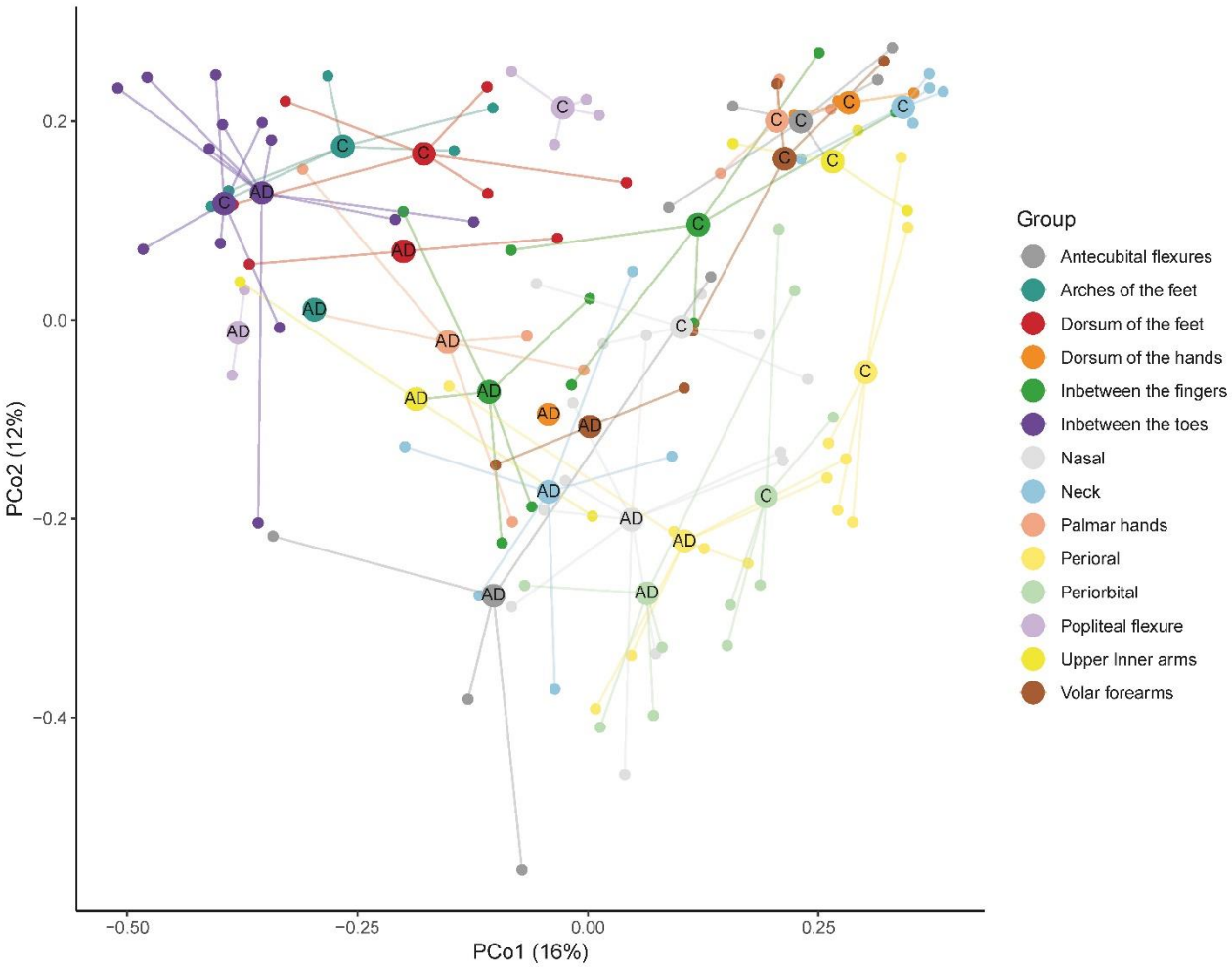

Figure 1: Characteristic AD skin sites.

Principal coordinate analysis plot based on Bray-Curtis distances between healthy control and AD samples within each skin site. Centroids represent the arithmetic mean position of the points belonging to the specific category. Samples from the hands, arms, and flexures separate according to AD, whereas feet, periorbital and perioral areas do not.

Alpha diversity and bacterial species in AD and healthy controls

Initial exploration of differences in the microbiome composition showed lower bacterial alpha-diversity at the flexures in AD (Fig. S2). The flexures in AD were dominated by the genus *Staphylococcus,* mostly the species *S. epidermidis*, and *S. aureus* (Fig. 2 and S3).

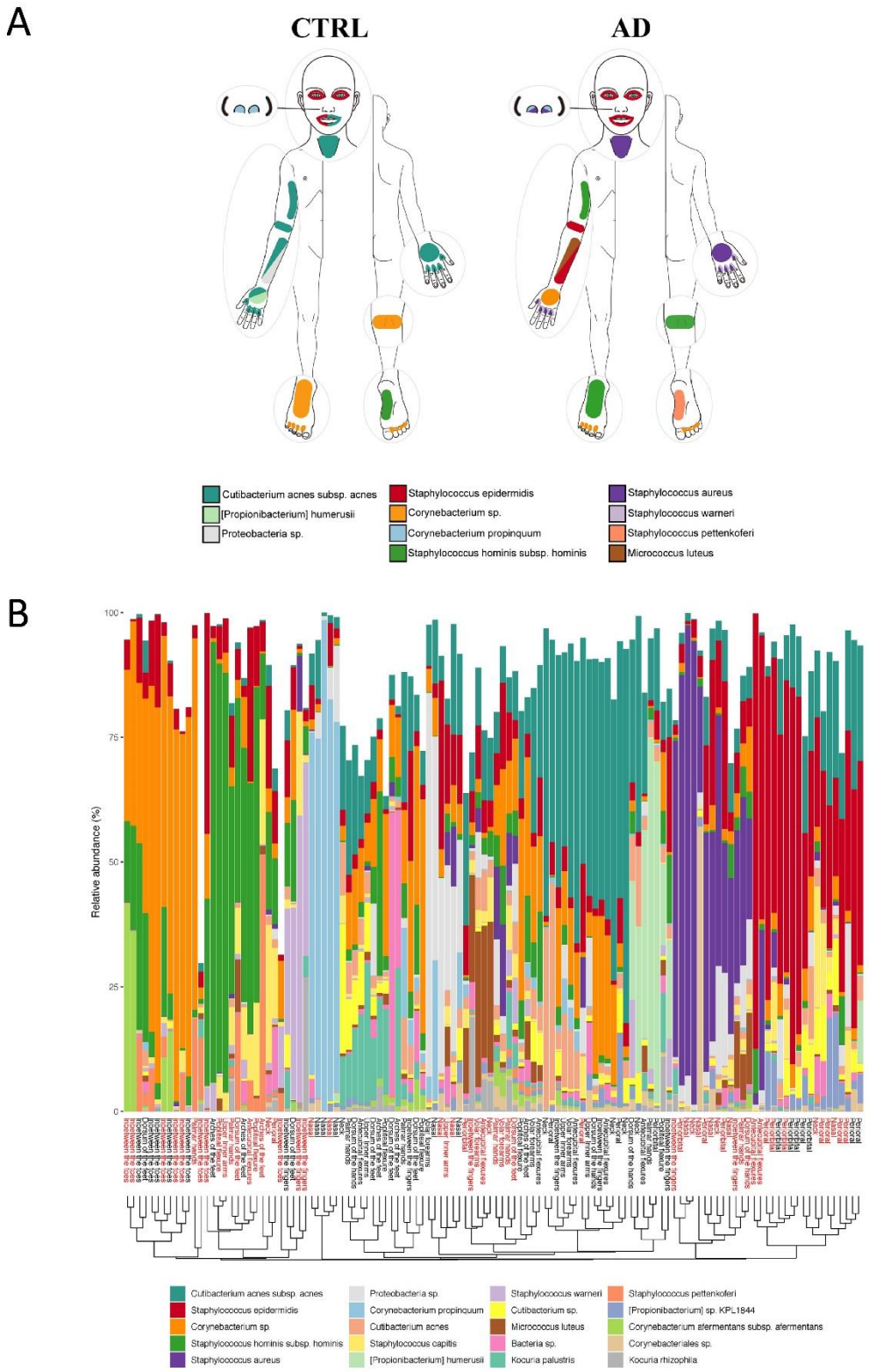

Figure 2: Bacterial species at different skin sites in healthy controls and patients with AD.

A, Illustration of the most predominant bacterial species at the 14 non-overlapping skin areas investigated: Two species are depicted at one skin site when the second most abundant specie was within 5% in total rel. abundance compared to the most predominant. **B**, Stacked bar plots of the relative abundances of the 20 bacterial taxa with highest average abundances across all samples, arranged according to similarity (Bray-Curtis). Skin site is stated in red for AD and black for healthy control samples.

S. aureus was low or undetected in control samples but present at most skin sites among AD patients and occasionally dominated the community (Fig. S3). Individual differences were also seen in *S. aureus* colonization, where AD10 was highly colonized across all skin sites (except from the feet, Fig. S4). Other species more abundant in AD included *Micrococcus luteus, S. epidermidis, S. saccharolyticus,* S. *lugdunensis, Moraxella osloensis* and *Rothia sp. ND6WE1A* (Fig. 2 and Table 2). On the contrary species higher in abundance in controls include *Cutibacterium acnes, [Propionibacterium] humerusii, Corynebacterium sp.* and *Corynebacterium singular* (Fig. 2, S3 and Table 2).

Feet were dominated by *Corynebacterium sp.* (Fig. S3). The nares were dominated by *C. propinquum and Proteobacteria sp.*, except from those dominated by *S. aureus* in AD subjects (Fig. 2).

	Pos. individuals (> 0% at one site)/total			Mean relative abundance (%)																													
				Nasal		Periorbital		Perioral		Neck		Upper inner arms		Antecubital flexures		Volar forearms		Dorsum of hands		Palmar hands		Between fingers		Popliteal flexures		Dorsum of feet		Arches of feet		Between toes	
Species	**C**	**AD**	**Subjects with site(s) ≥1%**	**C**	**AD**	**C**	**AD**	**C**	**AD**	**C**	**AD**	**C**	**AD**	**C**	**AD**	**C**	**AD**	**C**	**AD**	**C**	**AD**	**C**	**AD**	**C**	**AD**	**C**	**AD**	**C**	**AD**	**C**	**AD**	**C**	**AD**
Moraxella osloensis	5/5	9/9	AD2, AD3, AD4, AD8, AD9	0.0	0.1	0.0	0.2	0.0	0.1	0.0	0.5	0.2	1.6	0.1	0.4	0.1	1.5	0.1	1.2	0.1	0.8	0.1	2.7	0.2	0.6	0.2	0.4	0.2	0.1	0.0	2.4
Paracoccus yeei	5/5	7/9	AD3	0.0	0.0	0.0	2.0	0.0	0.2	0.0	0.2	0.2	0.1	0.2	1.5	0.0	0.3	0.3	0.1	0.0	0.0	0.0	2.4	0.2	0.0	0.0	0.1	0.0	0.0	0.0	0.6
Rothia sp. ND6WE1A	0/5	3/9	AD9	0.0	0.2	0.0	0.0	0.0	0.8	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.0	0.5	0.0	1.1	0.0	0.1	0.0	0.1	0.0	0.0
Malassezia globosa	5/5	8/9	C5, C6, C8, C9, C10, AD2, AD6	0.0	0.4	0.1	0.2	0.5	0.3	2.0	0.2	0.4	0.1	1.3	0.1	1.6	0.2	0.3	0.0	0.3	0.2	0.2	0.1	0.1	0.0	0.1	0.2	0.1	0.0	0.0	0.0
Malasseziales sp.	5/5	9/9	C5, C6, C8, C9, C10, AD6, AD8	0.0	0.0	0.1	0.0	0.0	0.0	1.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.2	0.1	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Staphylococcus saccharolyticus	1/5	4/9	AD2, AD3	0.0	0.0	0.0	3.9	0.0	0.2	0.0	3.4	0.1	0.0	0.0	1.1	0.0	0.7	0.1	0.0	0.0	0.0	0.0	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Staphylococcus lugdunensis	2/5	7/9	AD9	0.0	2.6	0.0	0.0	0.0	0.2	0.0	0.8	0.0	0.2	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.1	0.0	0.1	0.0	0.3	0.0	0.1	0.0	0.0
Staphylococcus cohnii	4/5	4/9	C9, C10	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.6	0.1	2.5	0.0	0.0	0.0
Staphylococcus pasteuri	5/5	8/9	C5, C6, AD1, AD2, AD3, AD9	0.0	0.1	0.1	0.3	0.0	0.3	0.1	0.7	0.2	0.0	0.2	0.5	0.1	0.4	0.5	0.1	0.8	1.0	2.6	2.8	0.2	0.0	0.3	0.5	0.1	0.1	0.0	0.1
Staphylococcus haemolyticus	5/5	7/9	C5, C9, C10, AD1, AD9, AD10	0.0	0.1	0.0	0.1	0.0	0.2	0.0	0.3	0.1	0.5	0.1	1.1	0.0	0.2	0.5	0.3	0.4	0.6	0.3	0.2	0.8	0.2	1.7	2.6	0.6	1.0	0.1	8.1
Corynebacterium singulare	4/5	4/9	C6	0.1	0.0	0.2	0.0	0.0	0.0	0.1	0.0	0.3	0.1	0.4	0.0	0.0	0.0	0.6	0.0	1.7	0.0	1.2	0.0	1.3	0.0	0.4	0.1	0.4	0.0	0.0	0.0
Corynebacterium appendicis	2/5	1/9	C8	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.6	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.1	0.0	0.3	0.0	1.9	0.0	0.0	0.0
Corynebacterium jeikeium	5/5	3/9	C9, AD8	0.0	0.0	0.0	0.2	0.0	0.1	0.0	0.1	0.2	0.1	0.3	0.0	0.1	0.3	0.1	0.4	0.6	0.3	0.4	0.1	1.6	0.0	0.4	0.5	0.2	0.0	0.1	1.7
Corynebacterium simulans	4/5	6/9	C5, C6, AD8	0.1	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.1	0.0	0.0	0.0	0.4	0.0	0.1	0.0	0.0	0.0	0.5	0.2
Streptococcus thermophilus	5/5	8/9	C6, C9, AD4	0.1	0.0	1.9	0.1	1.1	0.3	0.1	0.1	1.0	0.2	1.9	0.0	0.3	0.2	0.8	0.1	1.5	0.2	0.5	0.1	0.4	0.0	0.4	0.0	0.2	0.0	0.0	0.0
Streptococcus gordonii	5/5	8/9	C6, C8, AD1, AD10	0.0	0.0	0.0	0.3	1.0	0.8	0.2	0.1	0.2	0.0	0.1	0.1	0.4	0.2	0.1	0.1	0.2	0.6	0.1	0.7	0.0	0.0	0.1	0.1	0.0	0.0	0.0	0.0
Streptococcus oralis	4/5	8/9	C6, C10, AD4, AD9, AD10	0.0	0.0	0.0	0.2	0.3	1.0	0.0	0.1	0.5	0.1	0.0	0.1	0.1	0.2	0.0	0.1	0.0	0.5	0.0	0.9	0.0	0.0	0.3	0.1	0.0	0.0	0.0	0.0
Kocuria marina	5/5	8/9	C5, C6, AD9	0.3	0.0	0.8	0.2	0.7	0.1	1.9	0.1	3.2	0.0	2.5	0.2	0.4	0.3	2.5	0.0	2.0	0.3	0.7	0.3	3.1	0.0	1.5	0.1	2.1	0.0	0.0	0.3
Kocuria palustris	5/5	9/9	AD1, AD4, AD9, AD10, C5, C6, C8, C9	0.1	0.1	0.4	0.3	0.2	0.4	2.0	0.2	9.5	0.1	4.5	0.1	0.6	0.4	5.0	0.3	4.2	3.2	2.0	2.3	7.5	0.0	3.2	2.5	9.0	0.0	0.1	0.5
Kocuria sp. WRN011	5/5	5/9	C5, C6, AD1, AD9, AD10	0.2	0.0	0.1	0.3	0.1	1.1	0.0	0.1	0.6	0.0	0.5	0.1	0.3	0.1	0.9	0.0	0.8	1.0	0.7	0.3	0.7	0.0	0.5	0.3	0.5	0.0	0.1	0.2
Cutibacterium avidum	5/5	7/9	C8, AD1, AD8	1.0	0.6	0.1	0.0	0.0	0.1	0.0	0.0	0.1	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
[Propionibacterium] namnetense	5/5	8/9	C5, C10, AD4, AD6, AD10	0.1	0.0	0.3	0.6	0.2	0.6	0.5	0.3	0.4	0.0	1.0	0.0	0.3	0.2	0.6	0.1	0.8	0.4	1.0	0.1	0.4	0.0	0.1	0.1	0.0	0.0	0.0	0.0
Veillonella parvula	4/5	8/9	C6, C8, AD1, AD3, AD6, AD10	0.0	0.0	0.0	0.3	0.5	0.9	0.0	0.1	0.1	0.1	0.0	0.1	0.6	0.2	0.2	0.0	0.1	0.7	0.1	1.8	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0
Gardnerella vaginalis	3/5	3/9	C8, AD2	0.0	0.0	0.0	0.2	0.0	0.2	0.0	0.0	0.0	0.4	0.1	0.0	0.1	0.1	0.0	3.1	0.0	0.6	0.2	2.6	0.0	0.4	0.0	0.0	0.0	0.0	0.0	0.0

Table 2: Low abundant species and their presence in control and atopic dermatitis subjects at different skin sites.

The table represent low abundant species (with at least 3 samples with ≥ 1% relative abundance and not among the 20 taxa with highest average abundance across all samples) with characteristic distribution according to control versus atopic dermatitis. Abbreviations: AD, atopic dermatitis; C, control.

Changes in the mycobiome associated with AD

The bacterial domain dominated the samples of both control and AD. However, fungi were highly present at the neck of controls but not in subjects with AD (Table 2). *Malassezia globosa* was present in relative abundance ranging from 0.9-2.1% at antecubital flexures and 0.1-3,4% at the neck of healthy controls, whereas it was almost absent in AD antecubital flexures (0-0.3%) and neck (0-0.8%). The same pattern was observed for *Malasseziales sp.* (Table 2).

Changes in the virome associated with AD

The number of viral reads were comparable to fungus and the *E. Coli* control had very few viral reads compared to all the skin samples (Table S2). Both absolute and relative (Fig. S5) abundance of virus (top15) were strongly dependent on the individual. Overall, *Propionibacterium* (now *Cutibacterium*) *phages* and *Staphylococcus phages* dominated the skin of both healthy controls and AD (Fig. 3). Distinct skin site related patterns appeared with more *Propionibacterium phage PHL041* in the nares and more *Staphylococcus phages* on feet (Fig. S6). In AD we found increased abundances of *Propionibacterium phages, PHL041* and *PHL092*, and *Staphylococcus epidermidis phages, CNPH82* and *PH15*[25] (Fig. 3) – not driven by subject or skin site (Fig. S7B). In lesional skin, Staphylococcus phages expanded (Fig. 3), including *phages Ipla5* and *Ipla7* (Fig. S7). It is also noteworthy, that the patient AD10 with extensively *S. aureus* colonization also has higher abundances of the *Stahylococcus aureus phage phiETA* (Fig. S8).

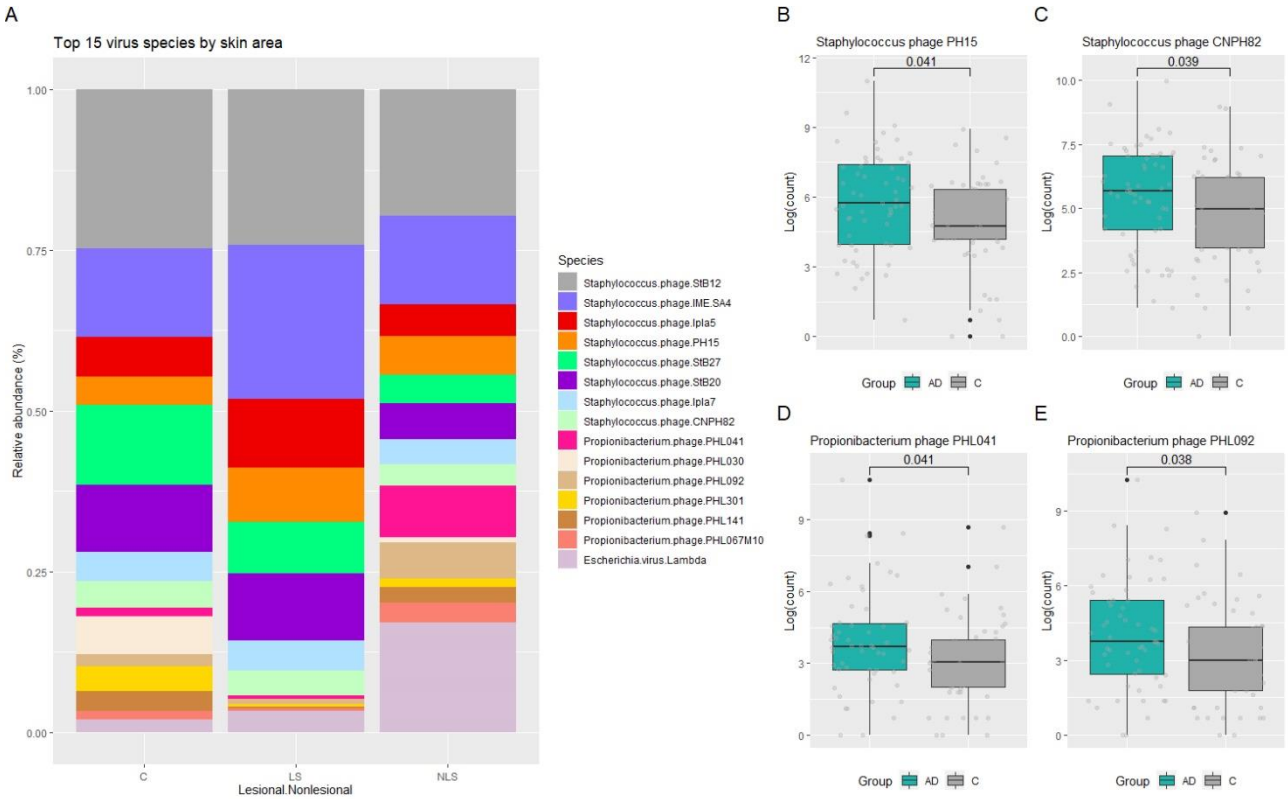

Figure 3: The virome in control and atopic dermatitis.

A, The bar plots represent the relative abundances of the 15 most predominant viruses and show domination of Propionibacterium and Staphylococcal phages. **B-E**, Box plots of the absolute abundances of phages with significant differences between AD and control.

Lesional state and *S. aureus* presence

We observed control samples grouping together while AD samples cluster further apart from each other (Fig. 1). Lesional state explained this pattern (Fig. S9A) as lesional sample composition was significantly different from control samples (PERMANOVA test; R^2 = 7%, P=0.0001), again with a large impact of subject on the microbial composition (R^2=22%, P=0.0001). However, testing whether the lesional versus non-lesional state explained microbial composition variance did not achieve statistical significance.

In lesional samples, severe AD was associated with higher *S. aureus* colonization (r = 0.63, P=0.00013), not seen in non-lesional (r = 0.28, P=0.15) (Fig. S9B). *S. aureus* colonization were higher across all skin sites except from the feet in lesional samples (Fig. 4). When *S. aureus* colonization was high, the relative abundance of *S. hominis* and *C. acnes* were lower (Fig. 4).

In the AD flexures, bacterial diversity (Shannon diversity) was lowest at lesional sites and *S. epidermidis* colonization seemed to accompany *S. aureus* dominance, not however at other sites (Fig. 4).

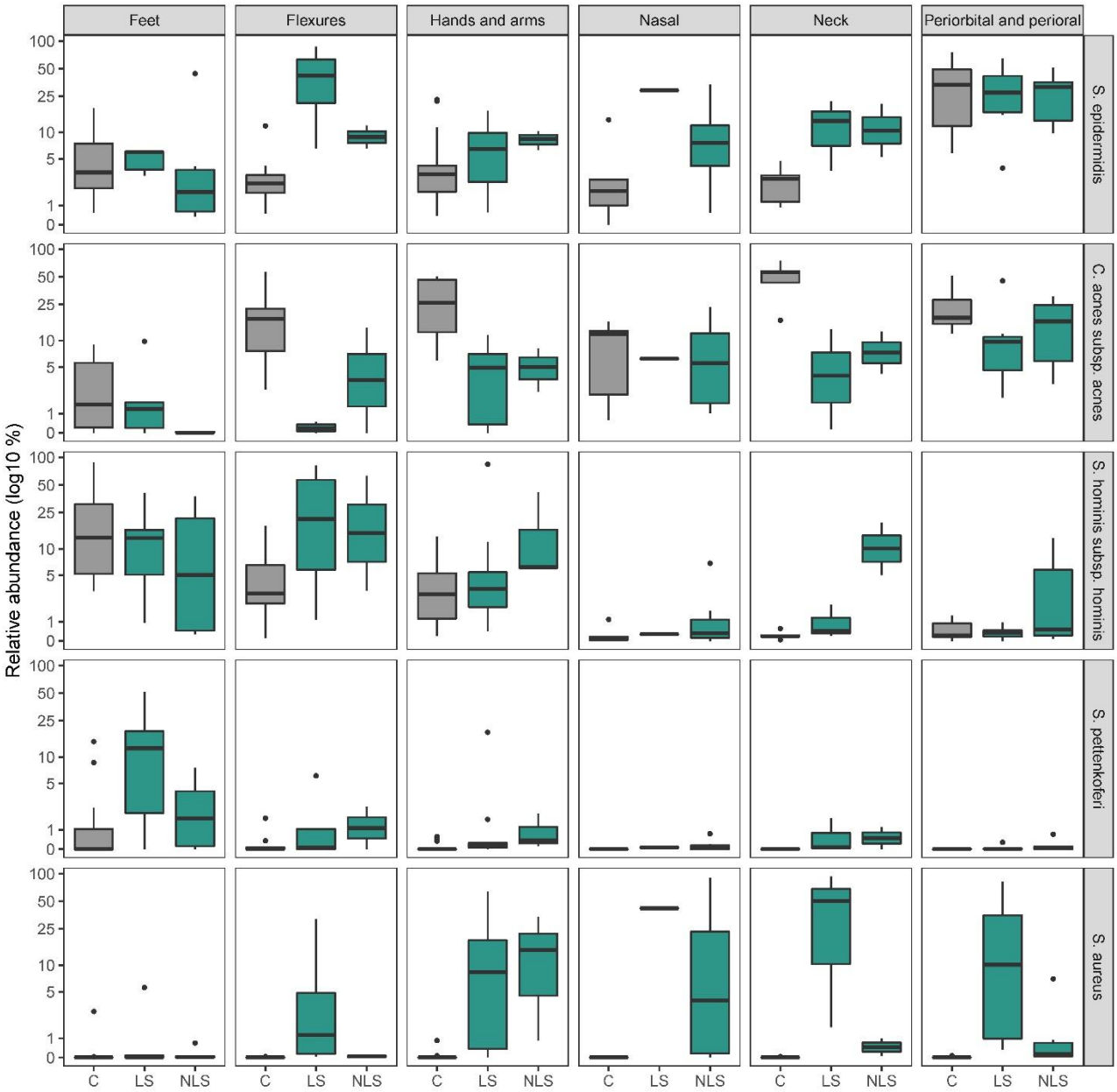

Figure 4: Relative abundances of Staphylococcal species and *Cutibacterium acnes.*

Boxplots of mean log10 transformed relative abundances of Staphylococcal species and *Cutibacterium acnes* grouped according to healthy control and lesional status within each grouped skin site.

S. aureus strain colonization

In total, 42 samples (of 121) had enough *S. aureu*s coverage for single nucleotide variation (SNV) analysis, which were mostly lesional (Fig. S10). In general, the *S. aureus* strains from the same subject exhibited high similarity and lesional samples from three different AD subjects (AD2, 3 and 4) clustered together in the top branch of the tree (Fig. 5A), suggesting that the strains could be lesion and subject-specific and that different *S. aureus* strains may be implied in AD.

In analyzing the nares as reservoir of recurrent *S. aureus* infections, it could be expected that the SNVs from the nares would be similar to the ones in lesional skin, at least at the skin nearby the

nose (perioral, periorbital) and at the hands (touching the nares). However, our analysis indicated no such specific pattern (Fig. 5B).

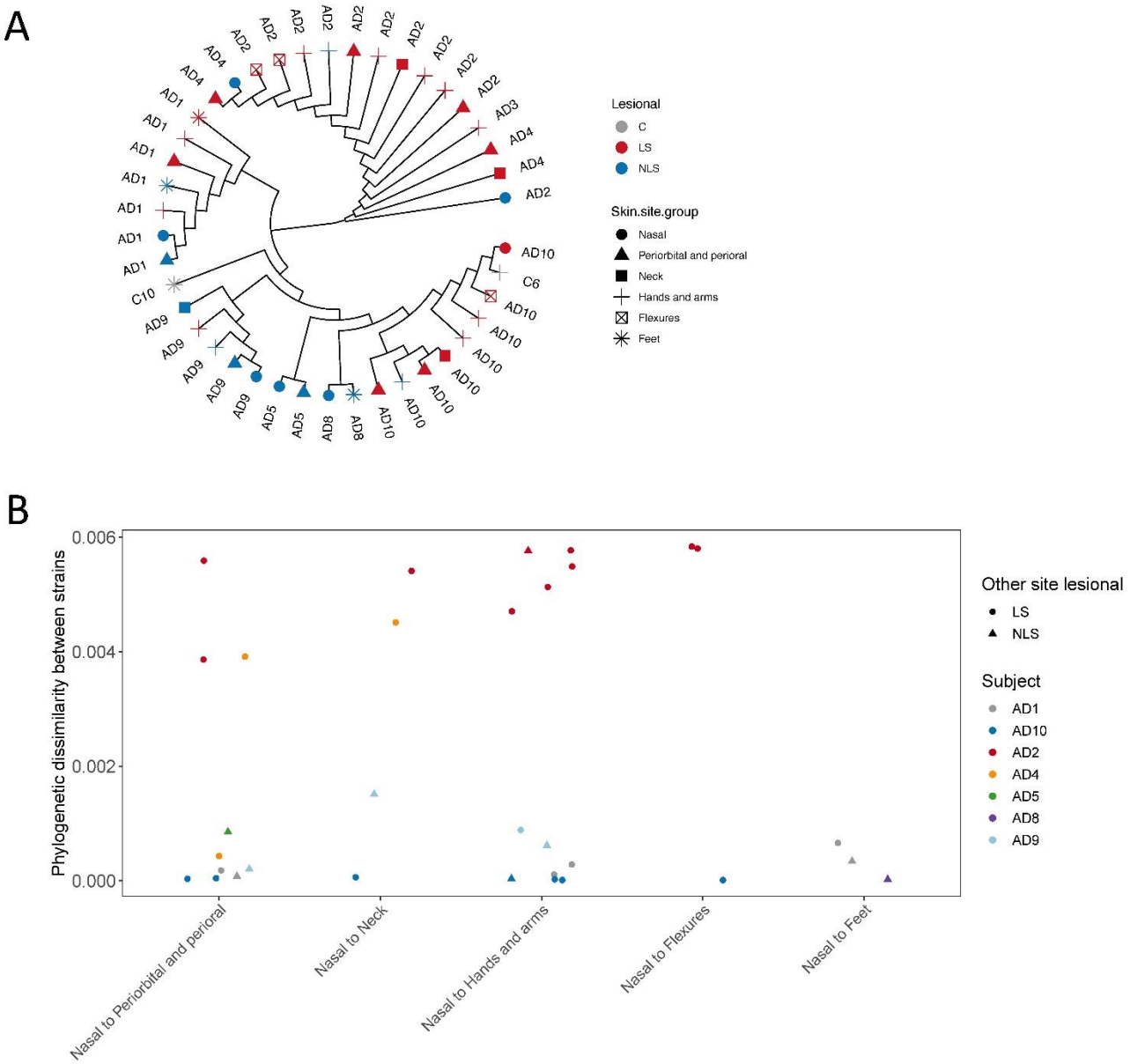

Figure 5: Single Nucleotide Variance analysis of *S. aureus.*

The analysis was based on 3317 SNVs detected from 100 signature genes of *S. aureus* MGS.skin0051p. We detected on average 1286 SNVs per sample ranging from 313 to 2268. **A**, A phylogenetic tree based on *S. aureus* SNV alignments coloured and shaped by disease state and skin site. The top branch is enriched in lesional samples. **B**, Phylogenetic distances between nasal and other skin site samples. *S. aureus* strains are mostly subject specific.

Discussion

In this study we demonstrated a global skin dysbiosis in AD at flexures, neck, hands and arms, while skin from the feet, periorbital and perioral areas were more similar to healthy controls. Most skin microbiome studies in AD have solely been focusing on flexures and bacteria, finding marked

AD-specific microbial signatures [26,5,12,14,27]. Our data are in line with findings from Baurecht and colleagues[28] showing microbial dysbiosis in AD across four skin sites (antecubital flexure, forehead, extensor- and volar forearm).
In our study, AD flexure samples had significantly lower alpha-diversity and was dominated by *Staphylococcus species,* mostly *S. aureus* and *S. epidermidis*, as previously reported in the flare condition by Byrd et al.[8]. In lesional samples, the increased abundances of the *Staphylococcus epidermidis phages CNPH82* and PH15 likely contribute to conversion from commensalism to pathogenicity of *S. epidermidis*, as most of the phage's gene content are lysogenic[25]. Likewise, S. aureus might in one AD patient, *S. aureus* might exploit the phage phi-ETA for acquiring the virulent gene encoding exfoliative toxin (ET)[29]. These potential associations between bacteria and viruses in AD are important findings. Interestingly, we also found lower relative abundances of *C. acnes* and *[P.] humerusii* in AD and a higher colonization of *Propionibacterium phages*, *PHL041* and *PHL092*, which might contribute to poorer growth conditions for *Cutibacterium* ([*Propionibacterium*]). *[P.] humerusii*[30] is a common inhabitant of the pilosebaceous unit[31], but to our knowledge this is the first study to report a difference in abundance in control versus skin disease. *C. acnes* has previously been reported to be reduced in AD skin[14,32,33]. It is a lipophilic bacteria, and altered sources of fatty acid substrates in AD skin[28,34] might also restrict its growth. *C. acnes* ferments glycerol into short-chain fatty acids, including propionic acid, which can inhibit growth of *S. aureus*[42].
M. luteus was more abundant particularly in two AD subjects and may indicate a certain AD dermotype, as recently suggested[14]. *M. luteus* has the capability to augment proliferation of virulence of *S. aureus*[36]. A new important finding of this study is a potential association between *Moraxella osloensis* and AD. *Moraxella* species are part of the human skin microbiota[37] and *M. osloensis* is a rare causative organism of human infections[38-42]. It may therefore be relevant to investigate further whether *M. osloensis* is an active player in AD.

No study has yet characterized the skin microbiome of the anterior triangle of the neck in AD, which is colonized with high amount of Staphylococcal species, but interestingly, also characterized by a lack of *Malassezia* species. *Malassezia* is a genus of lipophilic yeasts and comprises the most common fungi on healthy human skin[43]. The role of *Malassezia* in AD is debated. It is often attributed a pathogenic role. Especially in a subset of AD patients with symptoms predominating on the head and neck. However, despite that numerous studies have attempted to show a difference in frequency of *Malassezia* skin colonization in AD patients, there is no such evidence (reviewed by Glatz et al.[43] and Tsakok et al. [44]). As some randomized controlled studies report beneficial effects of anti-fungal treatment[44], we asked the patients whether they have used antifungal treatment (Table 1) and 2/5 might have used Nizoral shampoo around study participation, which could explain some lack of *Malassezia* in AD. However, two recent microbiome studies indicate a lack of *Malassezia* in AD too[45,46] – with one of the studies conducted in an AD prone population, with past AD episodes[45], thus not expected to use

antifungal treatment. Poor growth conditions in dry AD skin and absence of *C. acnes* providing substrates for *Malassezia* could restrict the growth.

Variability in beta-diversity within AD sites are higher than in controls, which we ascribe differences in lesional state. Other endogenous and exogenous factors might also explain larger variability in AD samples. Clinically the disease shows great patient to patient variability, and effort are being put into defining endotypes of the disease [10,14,47,48]. It was recently reported that lesional AD skin is characterized by larger inter- and intra-patient microbiome variability than non-lesional skin[49]. The inter-patient variability mainly originated from *S. aureus* abundance.
Here, lesional samples were characterized by higher *S. aureus* colonization across all skin sites, except from the feet. We find that high abundance of *S. aureus* was accompanied by lower relative abundances of *S. hominis,* which is in line with data from Baurecht et al. showing decreased *S. hominis* at four AD skin sites[28]. Nakatsuji et al. reports that AD patients lack strains of coagulase-negative *Staphylococcus (including S. hominis* strains) producing antimicrobial peptides against *S. aureus*[21], which can explain their opposing presence in the skin microbiome. In a previous study, reintroducing antimicrobial coagulase negative strains to human subjects with AD decreased *S. aureus* colonization[21]. Other studies have also succeeded in treating AD with microorganisms[19,20], indicating that microbiome transplants could be a promising strategy in AD management and highlighting the clinical relevance of finding skin site-specific species. Our data furthermore indicate, that it is highly relevant to investigate both bacteria, fungi and virus for understanding skin dysbiosis. Using phages for targeting microbial dysbiosis in AD yields potential, which is supported by the specificity of phages[50]. Phage-derived endolysins have been used to target *S. aureus* specifically, however not in AD patients[51].

Strengths and limitations

Published skin shotgun sequencing data from AD is sparse and having 121 samples successfully analyzed is a large number. However, a substantial number of samples failed sequencing due to insufficient biomass, making it difficult to evaluate the influence of all relevant factors. The low biomass is a known challenge[6,52]. We included AD patients in systemic treatment, which may affect the microbiome. However, even though the patients using topicals were instructed not to apply it 48 hours before, we did not found differences in microbial composition between AD patients in topical versus systemic treatment (PERMANOVA, R^2=4%, P=0.98). Another limitation is the use of DNA to characterize skin microbiota as we cannot assess if the microbes are dead or alive or metabolically active. Furthermore, we cannot not detect RNA viruses. Reference databases lack annotation for some organisms, which is the case of *Malassezia restricta* in this study. Studies combining microbiome and transcriptome data in AD are emerging[6,10] and in general, future studies would benefit from integrating omics data in capturing the flow of information underlying disease states in AD.

Conclusion

Though the microbial dysbiosis in AD is global, some sites are more affected than others. In our study, the flexures and neck showed marked taxonomical changes compared to healthy control. The flexures with lower alpha-diversity and high *S. aureus* abundance and high abundance of *S. epidermidis* in lesions, while at the neck *Malassezia* species were not detected. *S. aureus* colonization was observed across all lesional skin sites except the feet. In general, the *S. aureus* strains were highly similar within subjects both between lesional and non-lesional samples, indicating that more *S. aureus* strains are involved in AD. *S. aureus* may outgrow the coagulase negative *S. hominis* and *C. acnes.* Furthermore, phages targeted [*Propionibacterium]* and virulent phages such as *Staphylococcus phi-ETA phage* might support the growth of *S. aureus. M. luteus and M. osloensis* are more abundant in AD and may be active players in the disease.

Methods

Study participants

Samples from 10 adult patients with current atopic- and hand dermatitis and 5 healthy age and sex matched controls were enrolled from March to July 2018. All patients were recruited from the Department of Dermatology and Allergy at Herlev and Gentofte Hospital, Denmark. AD had been diagnosed by a physician and confirmed by the UK Working Party Diagnostic Criteria[22] at inclusion. Patients were characterized by demographic data, treatment, co-morbidities, FLG mutations (R501X, 2282del4, and R2447X) when available in their medical records and disease severity assessed by Severity Scoring of Atopic Dermatitis (SCORAD) and Hand Eczema Severity Index (HECSI) (Table 1). Exclusion criteria included active infections, use of antibiotics or probiotics within the past four weeks and for healthy volunteers a history of eczema. Two days before sampling, subjects were instructed not to shower or use topicals.

Sampling, DNA extraction and sequencing

Skin samples were collected using eSwabs from non-overlapping areas on 14 sites (Table 1) as described previously[23]. When eczema was present, the area affected, and morphology were described (Table S1).

DNA was extracted using QIAamp DNA Microbiome Kit (QIAGEN, lot no.: 154026306) according to manufactures' protocol. The DNA was randomly sheared into fragments of around 350 basepairs. Library preparation was performed with the NEBNext Ultra II Library Prep Kit for Illumina (New England Biolabs). Paired-end sequencing (2 x 150 basepair) was performed on an Illumina platform and generated an average of 36 million read pairs per sample.

Preprocessing of sequencing data and mapping reads to the gene catalog

For analyses of the bacteriome and mycobiome, adaptor removal from raw FASTQ files was performed using KneadData (v. 0.6.1) and Trimmomatic. Trimmed reads shorter than 100 bases were discarded. PCR/optical duplicates were removed using samtools (v. 1.6). Host reads mapping to the human reference genome GRCh38 (with Bowtie2 v. 0.0.3.2) were excluded. Read pairs in

which both reads passed filtering were retained and mapped using BWA mem (v 0.7.16a) to a reference gene catalogue built by Clinical Microbiomics from shotgun sequencing data from 1972 skin microbiome samples, containing 4.4 million non-redundant genes and 234 skin-associated metagenomic species (MGS, v3.0)[24]. To taxonomically annotate the MGSs, all the catalog genes has been blasted to the NCBI RefSeq genome database (2019-02-18). A MGS was considered detected if read pairs were mapped to at least three of its 100 signature genes. Normalization was done to the effective gene length and then to sum 100%, resulting in a relative abundance estimate of each MGS.
For analyses of the virome, quality processed FASTQ files (AdaptorRemoval-2.1.3) were assigned taxonomic labels using the database Kraken 2.

Ultrahigh-resolution phylogenetic profiling
For all samples in which *S. aureus* (MGS.skin0051p) was detected, we extracted the reads aligning to 100 signature genes of MGS.skin0051p and used the BCFtools (v. 1.6) multiallelic genotype caller to summarize the counts of each base observed in each position (requiring: sequencing depth ≥ 5 and $\geq 80\%$ major allele fraction and filtering to remove indels and SNVs near indels). Samples with at least 40% of the positions with a called base were retained for further analysis.
Maximum-Likelihood phylogenetic trees with pairwise distances were inferred using IQ-TREE (v. 1.6) based on the alignment of the Single Nucleotide Variants (SNV) considered from the 100 signature genes for MGS.skin0051p. By using ModelFinder Plus we selected the best substitution model estimated separately for each gene. This resulted in phylogenetic trees where each branch represents the most dominant *S. aureus* strain in a given sample. The phylogenetic distances matrix was constructed from all pairwise tree-branch length distances between any two samples in the tree (i.e. patristic pairwise distances).

Statistical analyses
Beta-diversity was estimated by Bray-Curtis dissimilarity among samples and alpha-diversity using Shannon's index, both measures were based on MGS abundances. Permutational multivariate analysis of variance (PERMANOVA) was used to assess the effects of disease (AD vs Control) or lesional state (Control, Lesional and Non-lesional), considering a nested model of disease within skin area and adjusting for subject variability. Pearson correlations were calculated between AD severity scores and *S. aureus* abundance. Wilcoxon signed-rank test was used to compare viral abundances between two groups. Outliers in box plots were defined by the interquartile range rule. Visualizations and statistics were conducted in R (R core team, version 4.0.4, http://www.R-project.org/), where we also used the application Pavian for gathering Kraken reports.

Declarations
Ethical approval and consent: This study was approved by the ethics committee of the Capital Region of Denmark (H-16020971) and all participants gave informed consent.

Availability of data: The datasets generated and analysed during the study are not publicly available due to privacy of the participants but are available from the corresponding author upon reasonable request.
Competing interests: None.
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Authors' contributions:
RDB, JDJ and LS initiated the study. LS and RDB included subjects, sampled and did clinical scorings. RDB extracted DNA. JBH and AP sequenced the samples and performed analyses of the bacteriome and mycobiome. JS and RDB analysed the virome. RDB wrote the main manuscript. All authors read and approved the final manuscript.
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References

1 Deckers, I. A. *et al.* Investigating international time trends in the incidence and prevalence of atopic eczema 1990-2010: a systematic review of epidemiological studies. *PLoS One* **7**, e39803, doi:10.1371/journal.pone.0039803 (2012).
2 Grice, E. A. *et al.* Topographical and temporal diversity of the human skin microbiome. *Science* **324**, 1190-1192 (2009).
3 Leyden, J. J., Marples, R. R. & Kligman, A. M. Staphylococcus aureus in the lesions of atopic dermatitis. *The British journal of dermatology* **90**, 525-530 (1974).
4 Totte, J. E. *et al.* Prevalence and odds of Staphylococcus aureus carriage in atopic dermatitis: a systematic review and meta-analysis. *The British journal of dermatology*, doi:10.1111/bjd.14566 (2016).
5 Kong, H. D. H. *et al.* Temporal shifts in the skin microbiome associated with disease flares and treatment in children with atopic dermatitis. *Genome Res.* **22**, 850-859, doi:10.1101/gr.131029.111 (2012).
6 Altunbulakli, C. *et al.* Relations between epidermal barrier dysregulation and Staphylococcus species-dominated microbiome dysbiosis in patients with atopic dermatitis. *J Allergy Clin Immunol*, doi:10.1016/j.jaci.2018.07.005 (2018).
7 Clausen, M. L. *et al.* Association of Disease Severity With Skin Microbiome and Filaggrin Gene Mutations in Adult Atopic Dermatitis. *JAMA Dermatol* **154**, 293-300, doi:10.1001/jamadermatol.2017.5440 (2018).
8 Byrd, A. L. *et al.* Staphylococcus aureus and Staphylococcus epidermidis strain diversity underlying pediatric atopic dermatitis. *Sci Transl Med* **9**, doi:10.1126/scitranslmed.aal4651 (2017).
9 Callewaert, C. *et al.* IL-4Ralpha Blockade by Dupilumab Decreases Staphylococcus aureus Colonization and Increases Microbial Diversity in Atopic Dermatitis. *J Invest Dermatol* **140**, 191-202.e197, doi:10.1016/j.jid.2019.05.024 (2020).
10 Leung, D. Y. M. *et al.* The nonlesional skin surface distinguishes atopic dermatitis with food allergy as a unique endotype. *Sci Transl Med* **11**, doi:10.1126/scitranslmed.aav2685 (2019).
11 Song, L. *et al.* Cheek Microbial Communities Vary in Young Children with Atopic Dermatitis in China. *Dermatology*, 1-10, doi:10.1159/000502469 (2019).
12 Totte, J. E. E. *et al.* Nasal and skin microbiomes are associated with disease severity in paediatric atopic dermatitis. *The British journal of dermatology* **181**, 796-804, doi:10.1111/bjd.17755 (2019).

13 Chng, K. R. *et al.* Whole-metagenome profiling reveals distinct population and functional differences in skin microbiomes of individuals predisposed to atopic dermatitis. *British Journal of Dermatology* **174**, e49 (2016).
14 Tay, A. S. L. *et al.* Atopic dermatitis microbiomes stratify into ecological dermotypes enabling microbial virulence and disease severity. *J. Allergy Clin. Immunol.*, doi:10.1016/j.jaci.2020.09.031 (2020).
15 Bjerre, R. D., Bandier, J., Skov, L., Engstrand, L. & Johansen, J. D. The role of the skin microbiome in atopic dermatitis: a systematic review. *The British journal of dermatology* **177**, 1272-1278, doi:10.1111/bjd.15390 (2017).
16 Meylan, P. *et al.* Skin Colonization by Staphylococcus aureus Precedes the Clinical Diagnosis of Atopic Dermatitis in Infancy. *J Invest Dermatol* **137**, 2497-2504, doi:10.1016/j.jid.2017.07.834 (2017).
17 Kennedy, E. A. *et al.* Skin microbiome alterations in infants before clinical atopic dermatitis. *J. Invest. Dermatol.* **136**, S84 (2016).
18 Leech, J. M. *et al.* Toxin-Triggered Interleukin-1 Receptor Signaling Enables Early-Life Discrimination of Pathogenic versus Commensal Skin Bacteria. *Cell host & microbe* **26**, 795-809.e795, doi:10.1016/j.chom.2019.10.007 (2019).
19 Myles, I. A. *et al.* First-in-human topical microbiome transplantation with Roseomonas mucosa for atopic dermatitis. *JCI insight* **3**, doi:10.1172/jci.insight.120608 (2018).
20 Gueniche, A. *et al.* Effects of nonpathogenic gram-negative bacterium Vitreoscilla filiformis lysate on atopic dermatitis: A prospective, randomized, double-blind, placebo-controlled clinical study. *British Journal of Dermatology* **159**, 1357-1363 (2008).
21 Nakatsuji, T. *et al.* Antimicrobials from human skin commensal bacteria protect against Staphylococcus aureus and are deficient in atopic dermatitis. *Sci Transl Med* **9**, doi:10.1126/scitranslmed.aah4680 (2017).
22 Williams, H. C. *et al.* The U.K. Working Party's Diagnostic Criteria for Atopic Dermatitis. I. Derivation of a minimum set of discriminators for atopic dermatitis. *The British journal of dermatology* **131**, 383-396 (1994).
23 Bjerre, R. D. *et al.* Effects of sampling strategy and DNA extraction on human skin microbiome investigations. *Sci Rep* **9**, 17287, doi:10.1038/s41598-019-53599-z (2019).
24 Nielsen, H. B. *et al.* Identification and assembly of genomes and genetic elements in complex metagenomic samples without using reference genomes. *Nature biotechnology* **32**, 822-828 (2014).
25 Daniel, A., Bonnen, P. E. & Fischetti, V. A. First complete genome sequence of two Staphylococcus epidermidis bacteriophages. *J Bacteriol* **189**, 2086-2100, doi:10.1128/jb.01637-06 (2007).
26 Kennedy, E. A. *et al.* Skin microbiome before development of atopic dermatitis: Early colonization with commensal staphylococci at 2 months is associated with a lower risk of atopic dermatitis at 1 year. *J Allergy Clin Immunol* **139**, 166-172, doi:10.1016/j.jaci.2016.07.029 (2017).
27 Oh, J. *et al.* The altered landscape of the human skin microbiome in patients with primary immunodeficiencies. *Genome Res.* **23**, 2103-2114, doi:10.1101/gr.159467.113 (2013).
28 Baurecht, H. *et al.* Epidermal lipid composition, barrier integrity, and eczematous inflammation are associated with skin microbiome configuration. *J Allergy Clin Immunol* **141**, 1668-1676.e1616, doi:10.1016/j.jaci.2018.01.019 (2018).
29 Yamaguchi, T. *et al.* Phage conversion of exfoliative toxin A production in Staphylococcus aureus. *Molecular microbiology* **38**, 694-705, doi:10.1046/j.1365-2958.2000.02169.x (2000).
30 Butler-Wu, S. M., Sengupta, D. J., Kittichotirat, W., Matsen, F. A., 3rd & Bumgarner, R. E. Genome sequence of a novel species, Propionibacterium humerusii. *J Bacteriol* **193**, 3678, doi:10.1128/jb.05036-11 (2011).
31 Fitz-Gibbon, S. *et al.* Propionibacterium acnes Strain Populations in the Human Skin Microbiome Associated with Acne. *J. Invest. Dermatol.* **133**, 2152-2160, doi:10.1038/jid.2013.21 (2013).

32 Dekio, I. *et al.* Characterization of skin microbiota in patients with atopic dermatitis and in normal subjects using 16S rRNA gene-based comprehensive analysis. *Journal of Medical Microbiology* **56**, 1675-1683 (2007).
33 Fyhrquist, N. *et al.* Microbe-host interplay in atopic dermatitis and psoriasis. *Nat Commun* **10**, 4703, doi:10.1038/s41467-019-12253-y (2019).
34 van Smeden, J. & Bouwstra, J. A. Stratum Corneum Lipids: Their Role for the Skin Barrier Function in Healthy Subjects and Atopic Dermatitis Patients. *Curr Probl Dermatol* **49**, 8-26, doi:10.1159/000441540 (2016).
35 Wang, Y. *et al.* Propionic acid and its esterified derivative suppress the growth of methicillin-resistant Staphylococcus aureus USA300. *Benef Microbes* **5**, 161-168, doi:10.3920/bm2013.0031 (2014).
36 Boldock, E. *et al.* Human skin commensals augment Staphylococcus aureus pathogenesis. *Nat Microbiol* **3**, 881-890, doi:10.1038/s41564-018-0198-3 (2018).
37 Lim, J. Y. *et al.* Complete Genome Sequences of Three Moraxella osloensis Strains Isolated from Human Skin. *Genome Announc* **6**, e01509-01517, doi:10.1128/genomeA.01509-17 (2018).
38 Roh, K. H. *et al.* Three cases of Moraxella osloensis meningitis: a difficult experience in species identification and determination of clinical significance. *Journal of Korean medical science* **25**, 501-504, doi:10.3346/jkms.2010.25.3.501 (2010).
39 Alkhatib, N. J., Younis, M. H., Alobaidi, A. S. & Shaath, N. M. An unusual osteomyelitis caused by Moraxella osloensis: A case report. *Int J Surg Case Rep* **41**, 146-149, doi:10.1016/j.ijscr.2017.10.022 (2017).
40 Han, X. Y. & Tarrand, J. J. Moraxella osloensis blood and catheter infections during anticancer chemotherapy: clinical and microbiologic studies of 10 cases. *American journal of clinical pathology* **121**, 581-587, doi:10.1309/qbb3-avcm-gwa3-k1xk (2004).
41 Buchman, A. L., Pickett, M. J., Mann, L. & Ament, M. E. Central venous catheter infection caused by Moraxella osloensis in a patient receiving home parenteral nutrition. *Diagn Microbiol Infect Dis* **17**, 163-166, doi:10.1016/0732-8893(93)90028-6 (1993).
42 Sifri, C. D. *et al.* Moraxella osloensis bacteremia in a kidney transplant recipient. *Transplant international : official journal of the European Society for Organ Transplantation* **21**, 1011-1013, doi:10.1111/j.1432-2277.2008.00727.x (2008).
43 Glatz, M., Bosshard, P. P., Hoetzenecker, W. & Schmid-Grendelmeier, P. The role of malassezia spp in atopic dermatitis. *Journal of Clinical Medicine* **4**, 1217-1228 (2015).
44 Tsakok, T., Schulenburg, H., Smith, C., Till, S. & Flohr, C. The Role of Yeast in Atopic Dermatitis Revisited: a Critical Appraisal. *Current Dermatology Reports* **4**, 228-240, doi:10.1007/s13671-015-0123-4 (2015).
45 Chng, K. R. *et al.* Whole metagenome profiling reveals skin microbiome-dependent susceptibility to atopic dermatitis flare. *Nat Microbiol* **1**, 16106, doi:10.1038/nmicrobiol.2016.106 (2016).
46 Moosbrugger-Martinz, V. *et al.* Initial Evidence of Distinguishable Bacterial and Fungal Dysbiosis in the Skin of Patients with Atopic Dermatitis or Netherton Syndrome. *J Invest Dermatol* **141**, 114-123, doi:10.1016/j.jid.2020.05.102 (2021).
47 Paternoster, L. *et al.* Identification of atopic dermatitis subgroups in children from 2 longitudinal birth cohorts. *J Allergy Clin Immunol* **141**, 964-971, doi:10.1016/j.jaci.2017.09.044 (2018).
48 Dyjack, N. *et al.* Minimally invasive skin tape strip RNA sequencing identifies novel characteristics of the type 2-high atopic dermatitis disease endotype. *J Allergy Clin Immunol* **141**, 1298-1309, doi:10.1016/j.jaci.2017.10.046 (2018).
49 van den Munckhof, E. H. A. *et al.* Inter- and Intra-patient Variability Over Time of Lesional Skin Microbiota in Adult Patients with Atopic Dermatitis. *Acta Derm Venereol* **100**, adv00018, doi:10.2340/00015555-3373 (2020).
50 Shimamori, Y. *et al.* Staphylococcal Phage in Combination with Staphylococcus Epidermidis as a Potential Treatment for Staphylococcus Aureus-Associated Atopic Dermatitis and Suppressor of Phage-Resistant Mutants. *Viruses* **13**, doi:10.3390/v13010007 (2020).

51 Tham, E. H., Koh, E., Common, J. E. A. & Hwang, I. Y. Biotherapeutic Approaches in Atopic Dermatitis. *Biotechnology journal* **15**, e1900322, doi:10.1002/biot.201900322 (2020)

52 Hannigan, G. D. *et al.* Culture-Independent Pilot Study of Microbiota Colonizing Open Fractures and Association with Severity, Mechanism, Location, and Complication from Presentation to Early Outpatient Follow-Up. *J. Orthop. Res.* **32**, 597-605, doi:10.1002/jor.22578 (2014).

Electronic Supplementary Material

This supplementary material has been provided by the authors to give readers additional information about their work.

Supplement to: RD Bjerre[1*], JB Holm[2], A Palleja[2], J Sølberg[1], L Skov[3], JD Johansen[1]. Skin dysbiosis in atopic dermatitis is site-specific and involves the bacteriome, mycobiome and virome

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Sample no	Area affected (%)				Morphology					
	1-25	26-50	51-75	76-100	Erythema	Edema	Lichenification	Excoriation	Dryness	Fissures
2.3	x				x		x			
2.9	x				x	x	x		x	
2.10			x		x				x	
2.12	x				x		x			
3.2			x		x	x	x			
3.3				x	x		x		x	X
3.4		x			x					
3.5		x			x	x				
3.6		x			x			x		
3.7			x		x	x				
3.8		x			x	x				X
3.10	x				x					
3.11	x				x	x				
4.10	x				x				x	
5.2	x						x		x	
5.3	x						x		x	
5.4	x								x	
5.9	x				x	x			x	
5.14	x								x	
8.14	x				x		x		x	
18.5	x				x		x		x	
18.6	x				x					
18.10	x								x	
18.12	x						x		x	
18.13	x								x	
20.1	x				x		x	x		
20.2		x			x	x	x	x		
20.3		x			x		x	x		
20.4		x			x	x	x	x		
20.6		x			x	x	x		x	
20.7		x			x	x	x		x	
20.10	x				x				x	x

Table S1: Clinical description of lesional samples

Name	Number of raw reads	Classified reads	Chordate reads	Artificial reads	Unclassified reads	Microbial reads	Bacterial reads	Viral reads	Fungal reads	Protozoan reads
12_1	34,753,836	28,198,542	24,976,131	0	6,555,294	3,205,694	3,155,241	31,058	13,322	618
12_14	33,616,810	13,776,661	1,874,953	0	19,840,149	11,892,013	11,868,644	4,781	8,502	893
15_1	33,079,474	31,303,958	30,572,543	0	1,775,516	728,274	722,770	2,551	719	23
15_10	31,736,784	25,124,146	798,785	0	6,612,638	24,292,741	24,262,644	18,770	6,737	508
15_11	35,105,856	10,439,252	2,240,949	0	24,666,604	8,187,196	8,053,729	6,164	80,659	11,584
15_12	35,334,183	20,674,228	1,320,907	0	14,659,955	19,330,334	19,270,559	38,172	7,353	642
15_13	34,359,322	15,876,778	1,314,218	0	18,482,544	14,478,927	14,374,081	92,748	4,918	759
15_14	38,942,945	15,927,422	888,088	0	23,015,523	15,037,151	15,021,844	8,733	670	17
15_2	40,259,627	35,107,669	6,798,702	0	5,151,958	28,303,610	28,266,456	7,680	22,382	1,032
15_3	36,956,489	29,494,768	905,050	0	7,461,721	28,577,288	28,522,268	31,566	17,593	734
15_4	35,251,880	33,315,543	626,445	0	1,936,337	32,687,682	32,662,831	15,739	6,669	470
15_6	34,404,603	28,628,005	22,068,641	0	5,776,598	6,556,699	6,526,999	2,132	19,035	1,550
15_8	36,411,453	29,719,204	1,306,032	0	6,692,249	28,402,679	28,367,768	14,318	15,169	382
15_9	35,878,274	23,516,373	1,110,549	0	12,361,901	22,398,866	22,362,478	5,068	18,769	1,357
16_1	44,960,603	38,322,198	35,025,167	0	6,638,405	3,294,675	3,237,399	50,531	2,961	101
16_10	36,106,417	25,104,739	1,125,386	0	11,001,678	23,973,757	23,911,998	25,317	12,131	925
16_11	28,109,218	14,463,450	1,886,690	0	13,645,768	12,573,344	12,542,104	5,959	7,946	976
16_12	28,855,445	25,906,190	24,156,891	0	2,949,255	1,747,139	1,734,907	5,531	3,005	532
16_13	29,452,983	14,127,616	2,354,935	0	15,325,367	11,765,718	11,646,649	92,998	11,617	1,870
16_14	30,833,782	12,311,509	88,432	0	18,522,273	12,218,640	12,183,632	24,668	2,953	187
16_2	35,242,369	33,518,248	29,394,470	0	1,724,121	4,121,771	4,107,851	6,083	4,850	403
16_3	36,157,002	24,934,671	2,431,088	0	11,222,331	22,498,481	22,428,852	22,599	38,855	936
16_4	32,977,866	18,567,235	1,072,721	0	14,410,631	17,491,722	17,416,662	7,622	54,348	1,563
16_5	37,385,543	18,876,603	3,411,333	0	18,508,940	15,456,685	15,402,761	8,175	28,610	4,748
16_6	37,161,468	19,517,085	2,122,472	0	17,644,383	17,389,218	17,325,450	8,230	40,049	1,458
16_8	36,163,331	20,698,129	2,639,160	0	15,465,202	18,055,856	18,002,290	11,057	25,761	2,169
16_9	32,812,655	20,336,429	2,134,028	0	12,476,226	18,192,966	18,126,439	12,580	35,873	1,118
18_1	35,061,165	31,020,064	24,822,838	0	4,041,101	6,174,226	6,123,980	16,730	14,839	3,181
18_10	32,738,950	20,776,056	724,035	0	11,962,894	19,020,216	18,967,860	36,061	7,705	2,333
18_11	38,689,600	21,718,575	249,300	0	16,971,025	21,444,400	21,315,052	115,155	4,246	167
18_12	38,993,913	22,419,690	1,709,350	0	16,574,223	20,691,120	20,607,504	60,987	8,940	673
18_13	41,058,934	32,816,543	403,643	0	8,242,391	32,397,894	32,346,877	35,988	8,746	230
18_14	37,304,178	16,842,206	39,337	0	20,461,972	16,779,506	16,737,154	35,691	2,973	22
18_3	37,112,802	26,674,467	7,265,733	0	10,438,335	19,386,084	19,345,419	16,861	14,454	1,853
18_4	37,450,852	21,740,647	3,260,393	0	15,710,205	12,644,136	12,427,633	154,436	25,533	9,949
18_5	35,825,511	16,182,570	185,161	0	19,642,941	15,967,797	15,826,872	133,703	2,210	39
18_6	33,648,399	22,258,936	494,649	0	11,389,463	21,723,180	21,629,824	82,907	4,414	325
18_9	37,692,855	17,919,480	1,613,309	0	19,773,375	16,280,112	16,245,598	16,345	6,982	184
19_1	38,883,613	16,205,896	8,490,258	0	22,677,717	7,711,398	7,563,722	140,644	806	21
19_10	31,380,810	20,624,531	712,637	0	10,756,279	19,900,221	19,848,883	13,257	16,989	733
19_12	33,783,174	11,812,078	1,115,998	0	21,971,096	10,681,158	10,568,048	31,054	51,480	4,379
19_13	33,140,806	11,828,623	841,431	0	21,312,183	10,973,263	10,853,953	22,926	54,132	8,179
19_14	32,501,485	17,630,355	53,235	0	14,871,130	17,561,788	17,476,192	68,647	9,210	1,076
19_2	31,853,495	26,172,247	3,765,012	0	5,681,248	22,393,463	22,337,373	35,525	14,915	825
19_3	31,680,029	25,750,570	352,050	0	5,929,459	25,385,973	25,322,848	29,725	28,810	697
19_4	32,259,329	25,021,697	380,736	0	7,237,632	24,637,789	24,564,771	9,705	50,701	2,519
19_6	30,688,119	11,757,598	1,147,257	0	18,930,521	10,602,456	10,478,016	40,943	52,726	6,214
19_7	35,336,542	15,380,628	1,297,990	0	19,955,914	14,069,705	13,932,399	22,164	80,864	6,043

Name	Number of raw reads	Classified reads	Chordate reads	Artificial reads	Unclassified reads	Microbial reads	Bacterial reads	Viral reads	Fungal reads	Protozoan reads
2_1	27,527,627	9,448,844	1,545,095	0	18,078,783	7,901,005	7,632,323	243,074	19,079	375
2_10	35,415,497	22,661,804	2,629,865	0	12,753,693	19,981,319	19,817,644	60,507	47,567	28,779
2_12	32,009,915	29,856,773	27,703,550	0	2,153,142	2,133,002	2,104,495	7,589	8,392	632
2_14	34,322,699	13,188,567	186,019	0	21,134,132	12,995,874	12,971,275	13,668	3,319	58
2_2	27,404,207	24,378,047	16,588,946	0	3,026,160	7,778,188	7,739,289	31,386	4,770	157
2_3	30,389,533	21,503,844	1,674,965	0	8,885,689	19,800,552	19,712,259	71,378	11,985	290
2_9	35,371,327	18,863,394	6,162,508	0	16,507,933	12,634,179	12,523,191	37,165	34,317	4,620
20_1	35,934,902	29,459,180	20,085,845	0	6,475,722	9,333,335	9,166,497	158,375	3,560	154
20_10	42,675,239	30,395,195	5,431,775	0	12,280,044	24,847,112	23,657,253	1,180,220	3,888	53
20_14	26,106,458	10,208,533	176,716	0	15,897,925	10,024,580	9,969,543	47,838	1,567	49
20_2	33,263,286	30,713,421	19,921,898	0	2,549,865	10,690,836	10,657,879	19,680	8,011	916
20_3	30,976,332	26,483,177	13,298,145	0	4,493,155	13,123,089	13,071,689	41,502	4,350	87
20_4	35,998,482	34,639,233	12,804,656	0	1,359,249	21,593,549	21,560,260	27,072	3,046	96
20_6	43,796,833	40,237,949	17,032,609	0	3,558,884	23,118,593	22,733,472	368,041	4,044	76
20_7	30,386,598	16,664,407	11,738,049	0	13,722,191	4,664,700	4,437,138	130,786	19,486	3,994
20_9	35,158,448	27,007,072	22,412,868	0	8,151,376	4,578,430	4,435,263	118,550	9,423	1,297
21_1	33,068,476	16,656,511	12,574,709	0	16,411,965	4,079,240	4,070,188	4,901	888	17
21_10	31,292,502	17,384,178	1,926,986	0	13,908,324	15,444,568	15,372,473	31,524	21,132	3,281
21_11	34,789,652	16,101,579	7,314,008	0	18,688,073	8,780,857	8,719,965	10,623	26,031	3,590
21_12	32,518,922	7,600,499	1,014,673	0	24,918,423	6,567,210	6,470,899	5,695	50,850	7,415
21_13	35,384,651	7,749,714	447,844	0	27,634,937	7,292,876	7,176,499	4,787	62,961	9,568
21_14	34,443,388	14,911,422	30,742	0	19,531,966	14,873,832	14,828,442	15,397	20,563	697
21_2	30,770,576	29,639,021	27,162,799	0	1,131,555	2,473,367	2,446,644	21,832	2,685	247
21_3	33,494,669	27,833,965	1,840,933	0	5,660,704	25,986,845	25,712,015	233,824	33,481	1,103
21_4	30,121,958	25,664,071	380,186	0	4,457,887	25,282,312	25,243,941	3,501	28,608	775
21_5	52,498,578	38,580,078	23,741,902	0	13,918,500	14,812,925	14,737,150	21,625	21,009	538
21_6	31,933,899	16,856,559	3,374,243	0	15,077,340	13,467,217	13,410,846	6,827	26,387	3,921
21_7	38,726,699	24,035,350	6,973,184	0	14,691,349	17,045,694	16,970,108	11,467	28,367	2,414
21_9	36,818,792	15,625,940	6,819,149	0	21,192,852	8,792,583	8,694,703	11,052	36,929	5,658
22_1	34,090,278	31,780,774	31,159,565	0	2,309,504	620,029	613,262	3,819	1,100	287
22_10	31,781,514	21,139,333	4,042,060	0	10,642,181	17,085,076	16,993,127	47,225	31,240	2,728
22_11	38,743,122	19,262,781	10,120,414	0	19,480,341	9,130,723	8,979,072	42,789	82,257	3,755
22_12	33,028,052	6,316,369	698,150	0	26,711,683	5,612,461	5,495,550	3,406	71,121	8,135
22_13	35,587,999	8,408,611	2,024,633	0	27,179,388	6,376,148	6,126,611	30,453	187,081	6,169
22_14	37,418,951	17,554,705	76,840	0	19,864,246	17,472,768	17,412,892	38,012	9,635	1,514
22_2	35,887,688	32,374,380	4,729,751	0	3,513,308	27,613,145	27,478,532	116,403	14,198	1,146
22_3	28,171,831	21,633,136	3,666,087	0	6,538,695	17,963,608	17,328,916	603,407	22,578	1,369
22_4	32,853,111	19,150,694	2,128,257	0	13,702,417	17,019,722	16,790,343	102,458	100,723	4,934
22_5	35,827,086	34,358,999	31,597,306	0	1,468,087	2,758,967	2,683,381	68,523	4,300	836
22_6	39,580,911	24,371,670	10,907,226	0	15,209,241	13,457,688	13,330,177	18,001	77,137	8,663
22_7	34,733,677	34,441,795	34,275,100	0	291,882	165,894	163,263	854	807	63
22_8	27,869,265	18,457,838	9,046,653	0	9,411,427	9,405,684	9,341,645	12,664	28,172	1,011
23_1	54,732,100	54,667,349	149,931	0	64,751	54,463,071	54,447,234	46	9,877	626
3_1	28,248,705	22,400,441	14,168,528	0	5,848,264	8,210,440	8,074,204	98,568	26,876	764
3_10	36,062,684	23,280,593	13,782,980	0	12,782,091	9,419,087	9,346,104	14,506	34,435	2,253
3_11	37,889,147	22,562,347	7,646,337	0	15,326,800	14,847,698	14,544,744	286,668	5,631	93
3_14	40,908,660	13,839,463	465,360	0	27,069,197	13,359,946	13,323,507	23,418	5,500	7
3_2	35,970,744	34,116,866	31,778,198	0	1,853,878	2,321,595	2,305,593	3,679	7,329	510
3_3	37,765,727	36,747,126	30,866,375	0	1,018,601	5,869,820	5,822,607	6,000	38,873	182
3_4	33,250,994	31,631,167	29,714,880	0	1,619,827	1,888,408	1,875,952	4,542	4,308	406

Name	Number of raw reads	Classified reads	Chordate reads	Artificial reads	Unclassified reads	Microbial reads	Bacterial reads	Viral reads	Fungal reads	Protozoan reads
3_5	31,151,567	29,616,158	28,254,934	0	1,535,409	1,347,588	1,333,057	6,541	3,753	218
3_6	39,471,099	37,988,212	21,470,357	0	1,482,887	16,494,261	16,464,939	24,884	2,556	27
3_7	34,123,898	25,052,953	19,763,998	0	9,070,945	5,255,855	5,137,062	10,043	95,889	1,163
3_8	34,226,228	29,666,807	25,696,234	0	4,559,421	3,936,848	3,914,283	9,348	6,545	201
3_9	32,984,202	28,487,861	25,153,572	0	4,496,341	3,301,037	3,257,325	6,702	28,438	744
4_1	38,946,148	33,947,665	32,565,939	0	4,998,483	1,379,379	1,373,484	2,932	758	19
4_10	30,004,901	18,344,937	3,582,279	0	11,659,964	14,740,652	14,662,890	39,918	19,660	859
4_14	39,163,988	27,109,743	69,492	0	12,054,245	27,027,438	26,894,814	128,631	1,328	77
4_2	33,921,428	24,148,234	9,034,616	0	9,773,194	15,065,161	14,985,782	22,840	32,543	1,367
4_3	42,015,358	34,006,630	2,943,901	0	8,008,728	31,044,522	30,320,667	714,837	4,507	192
4_4	37,380,685	23,688,624	4,938,877	0	13,692,061	18,706,128	18,660,673	21,895	14,277	532
4_6	37,048,448	24,244,397	9,979,657	0	12,804,051	14,190,867	14,077,770	25,236	13,828	789
5_1	34,795,491	34,662,540	29,911,138	0	132,951	4,628,695	4,578,459	48,404	234	69
5_14	35,356,022	15,278,425	1,333,164	0	20,077,597	13,919,169	13,891,483	6,698	7,128	703
5_2	30,633,433	27,365,040	15,248,270	0	3,268,393	12,087,173	12,044,355	32,008	6,268	910
5_3	35,151,891	27,050,437	722,073	0	8,101,454	26,315,455	26,211,904	98,545	2,124	112
5_4	30,607,218	21,665,633	4,630,730	0	8,941,585	16,981,288	16,827,280	124,345	16,666	2,844
5_9	38,780,712	16,389,433	78,879	0	22,391,279	16,301,580	16,266,530	26,132	1,176	150
7_1	30,945,078	30,089,151	29,164,909	0	855,927	922,784	910,873	10,298	717	126
7_3	34,204,709	25,892,290	3,260,528	0	8,312,419	22,625,539	22,567,775	38,392	13,780	1,187
8_1	54,301,715	53,390,023	52,443,674	0	911,692	944,814	920,141	8,297	9,971	578
8_14	45,926,012	19,088,972	497,432	0	26,837,040	18,579,732	18,456,408	114,418	1,835	33
8_3	34,835,147	24,069,638	10,657,739	0	10,765,509	13,380,719	13,277,313	62,939	31,782	1,180

Table S2: Classification report from Kraken 2

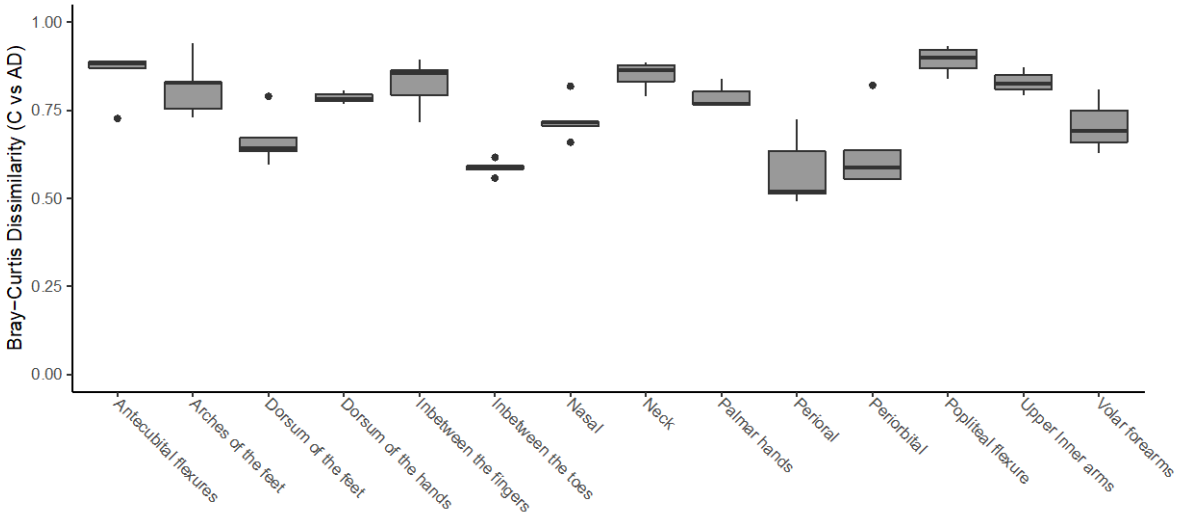


Figure S1: Bray-Curtis Dissimilarity between controls and patients with AD

Skin sites are shown at the x-axis.

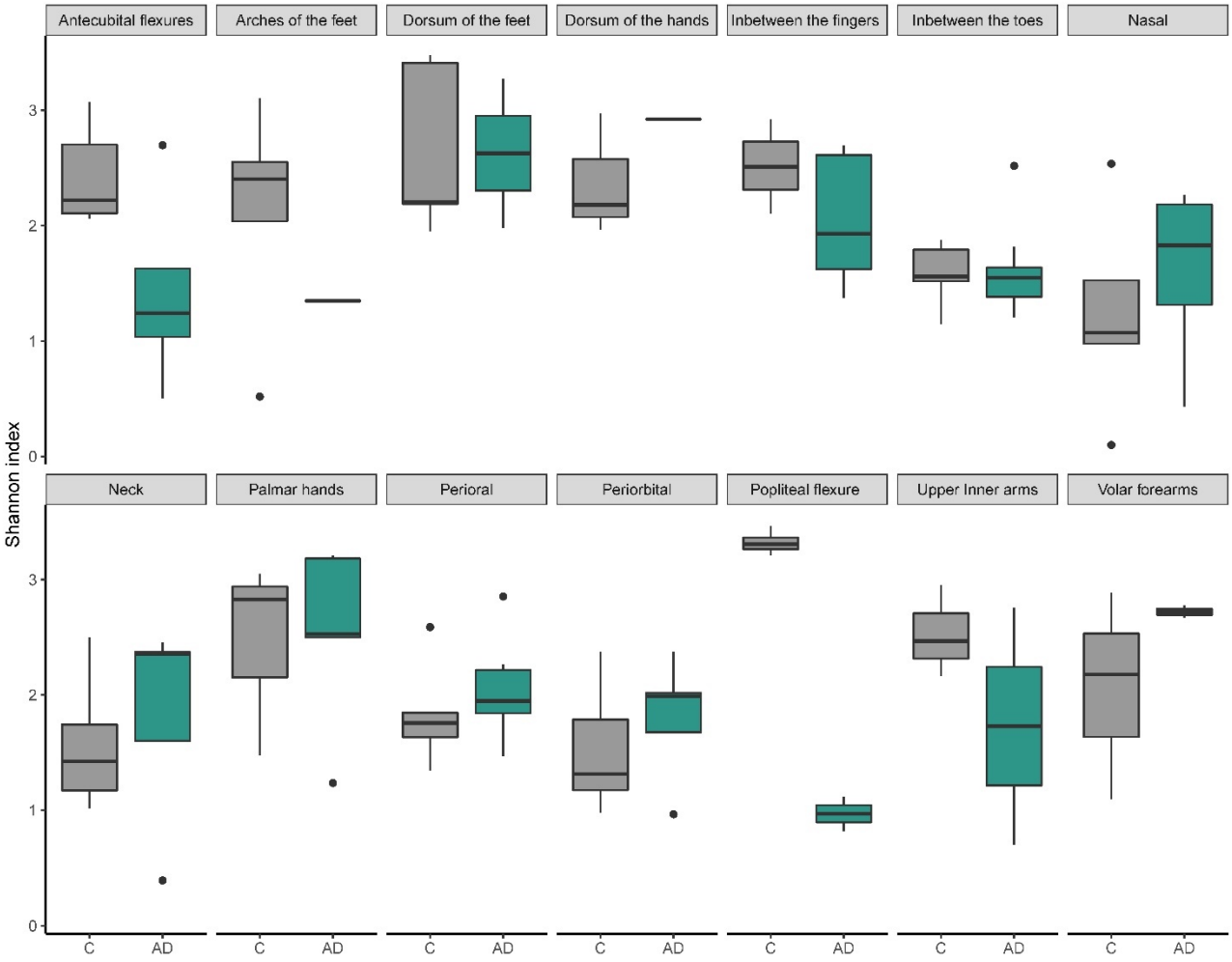


Figure S2: Lower bacterial diversity at the flexures in AD.

Boxplots of Shannon diversity grouped according to healthy control (C) and AD samples within each skin site.

Figure S3: Stacked bar plots of relative abundances of bacterial species at different skin sites in healthy controls and patients with AD. Sample numbers are shown at the x-axis where the first number refers to the subject (inclusion number) and the second the skin site sampled. The figure shows the 20 taxa with highest average abundance across all samples. The taxa are sorted from most abundant to least abundant.

Figure S4: Stacked par plots of relative abundances of bacterial species arranged according to individual. Skin sites are shown at the x-axis. The figure shows the 20 taxa with highest average abundance across all samples. The taxa are sorted from most abundant to least abundant.

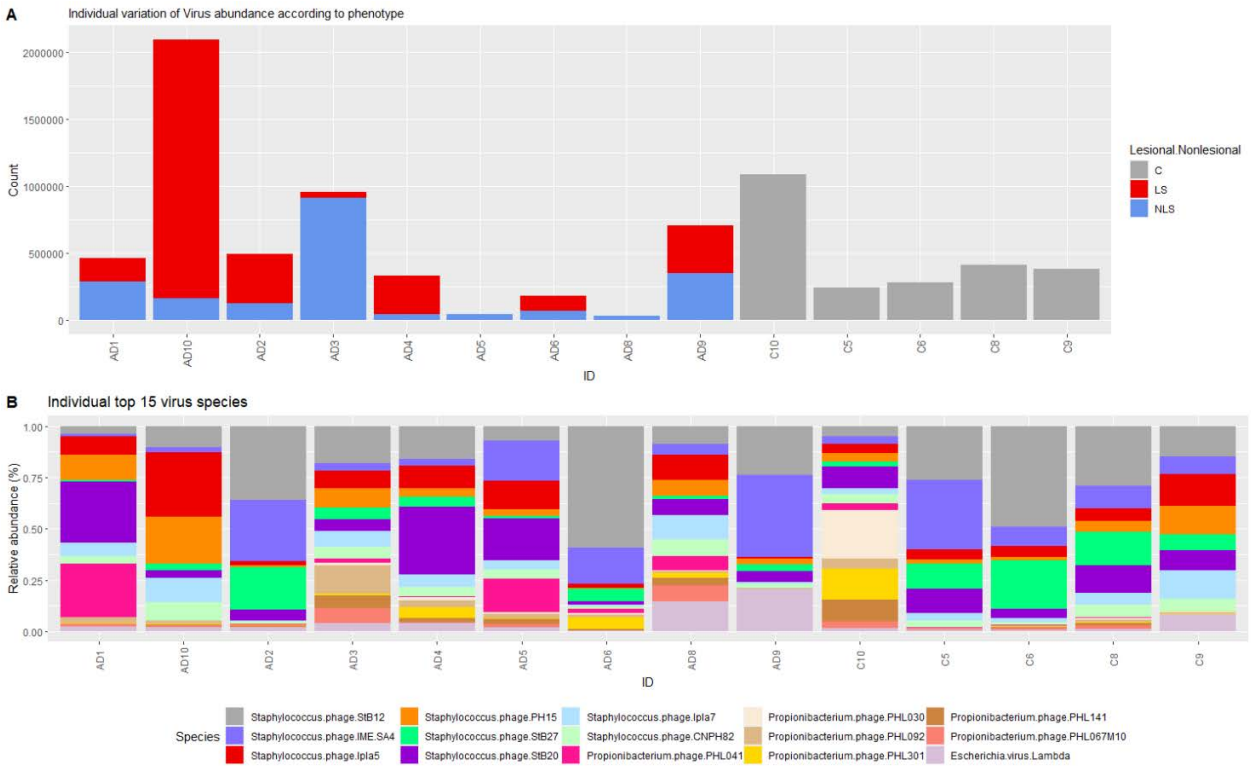


Figure S5: Individual variation in total and relative viral abundances

Individuals are shown on the x-axis. The absolute number of reads (A) are divided according to lesional state (LS: Lesional, NLS: Nonlesional, C: Control). Figure B) shows the 15 viruses with highest relative abundance across all samples.

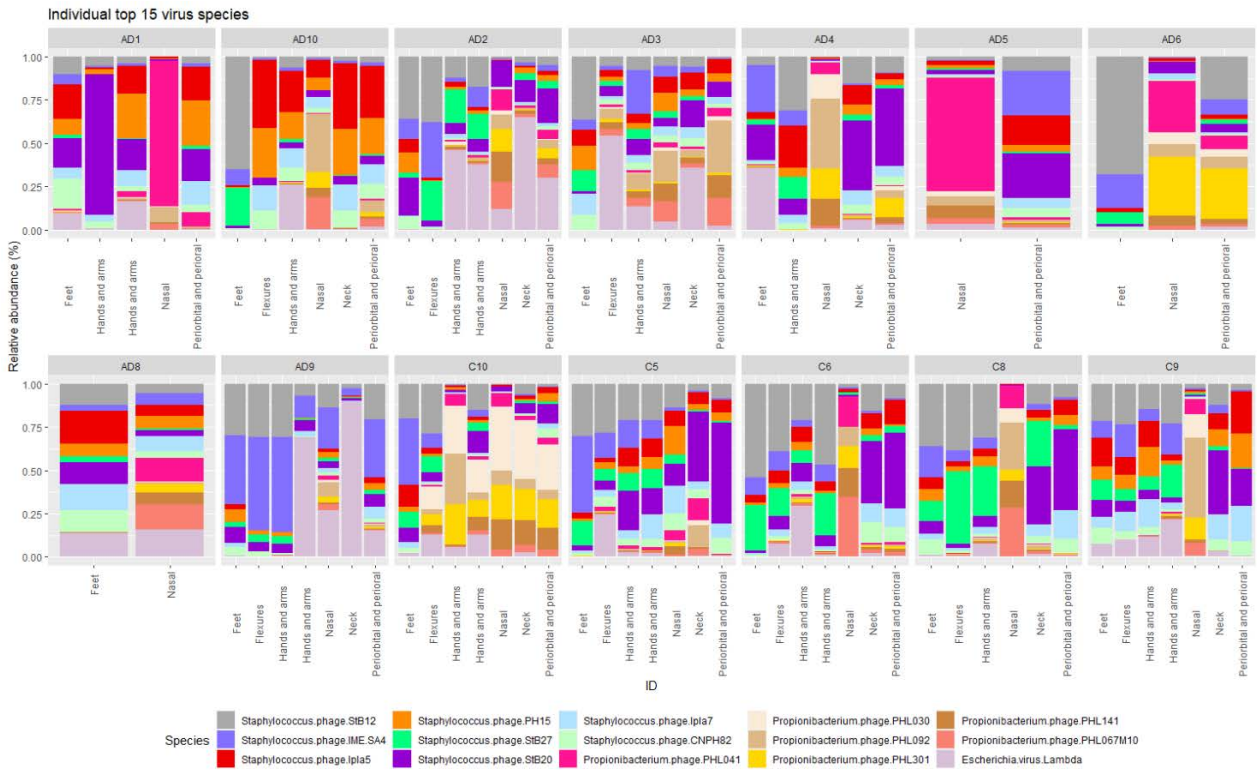
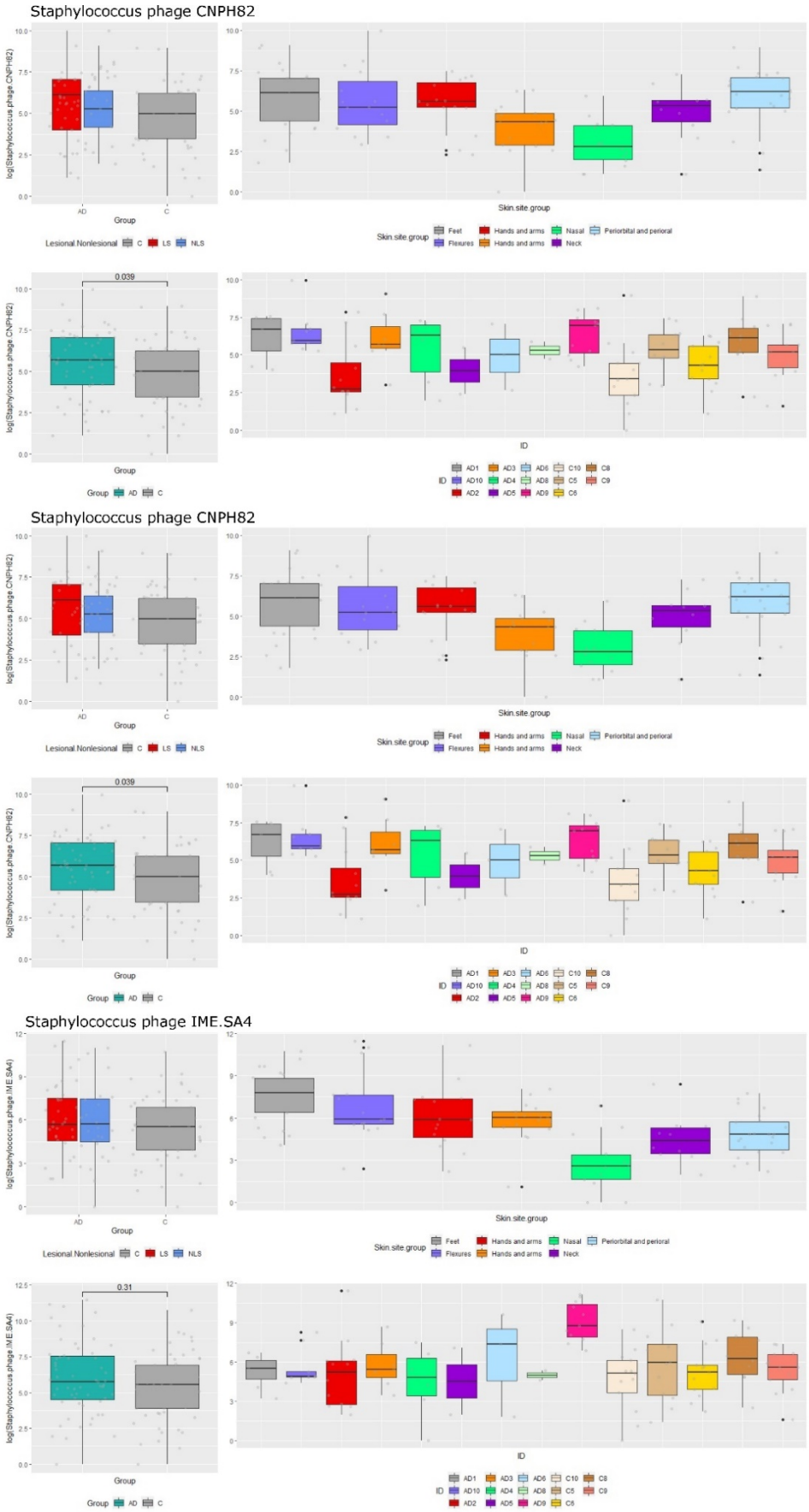


Figure S6: Stacked par plots of relative abundances of viruses grouped according to individual.

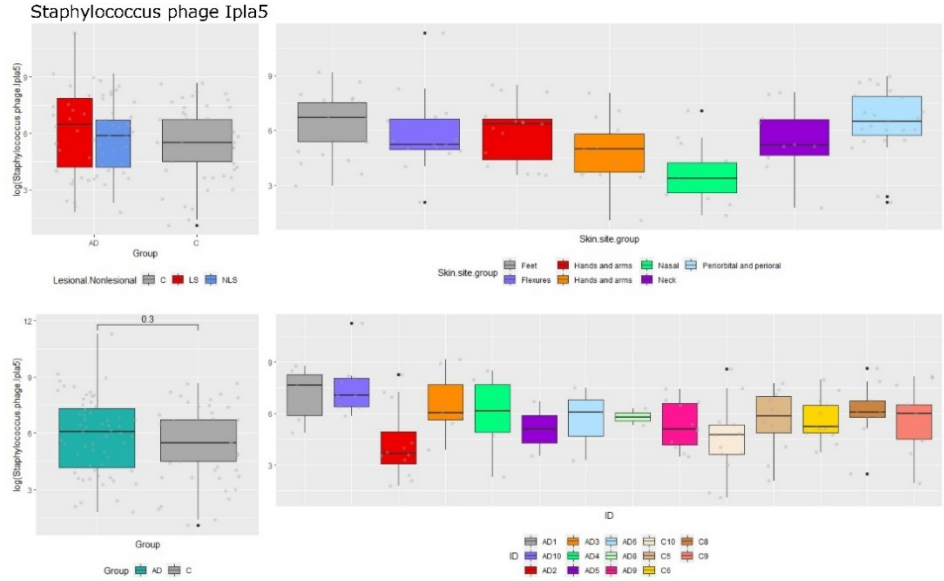
Skin groups are shown at the x-axis. The figure shows the 15 viruses with highest relative abundance across all samples.

A.

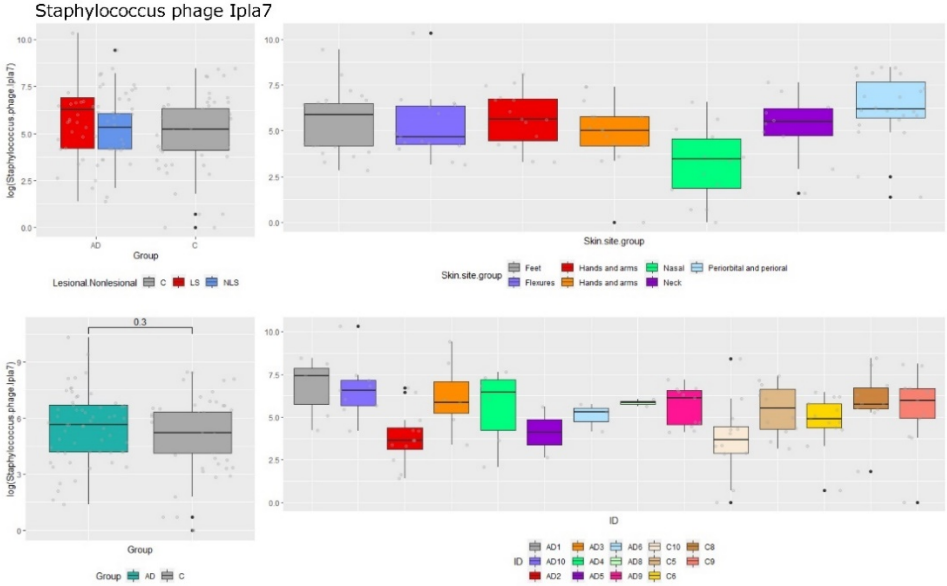

Staphylococcus phage CNPH82
log(Staphylococcus phage CNPH82)
Group
AD
C
Lesional.Nonlesional
C
LS
NLS
Skin.site.group
Feet
Hands and arms
Nasal
Periorbital and perioral
Flexures
Hands and arms
Neck
0.039
ID
AD1
AD3
AD6
C10
C8
AD10
AD4
AD8
C5
C9
AD2
AD5
AD9
C6
Staphylococcus phage CNPH82
Staphylococcus phage IME.SA4
log(Staphylococcus phage IME.SA4)
0.31

A. (continued)

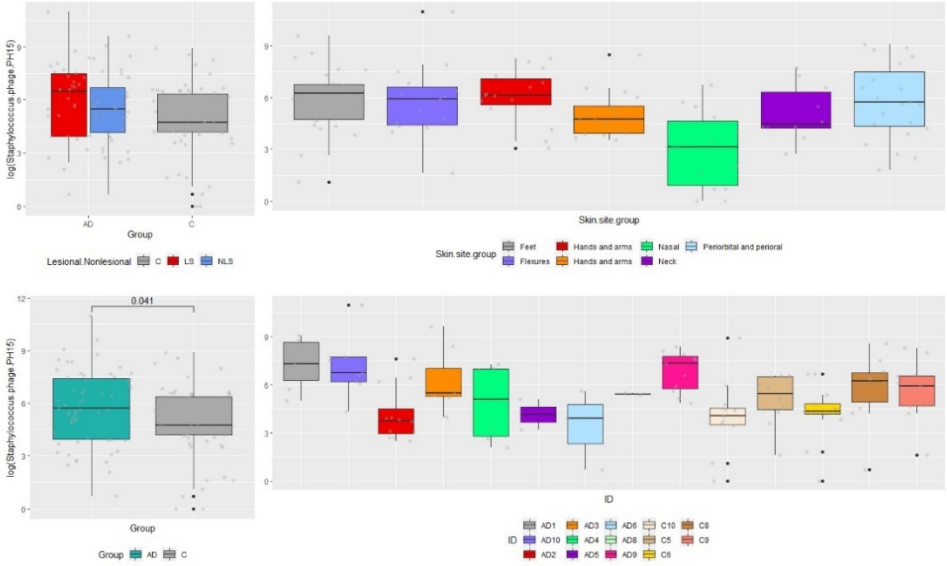
Staphylococcus phage Ipla5



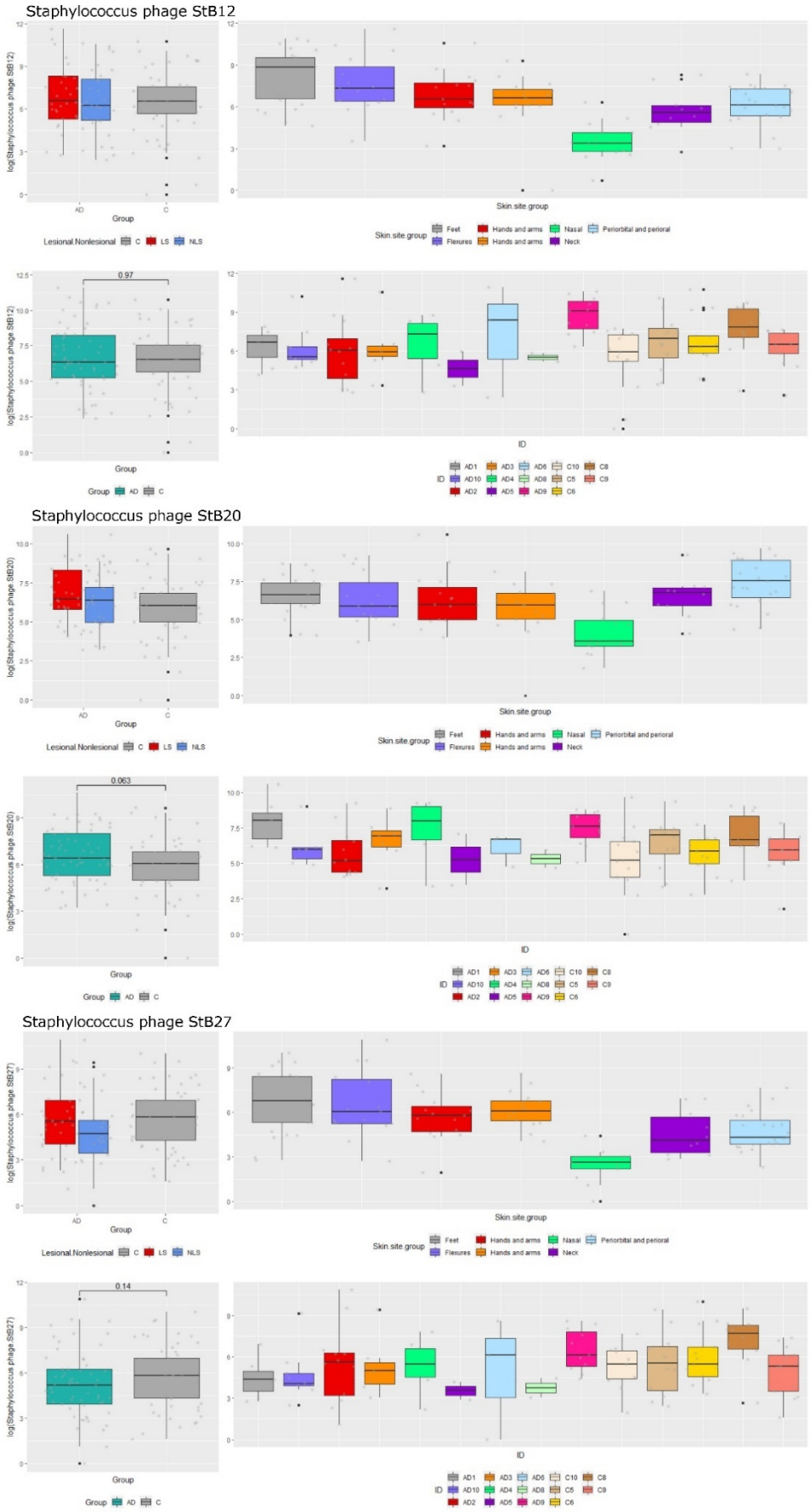
Staphylococcus phage Ipla7



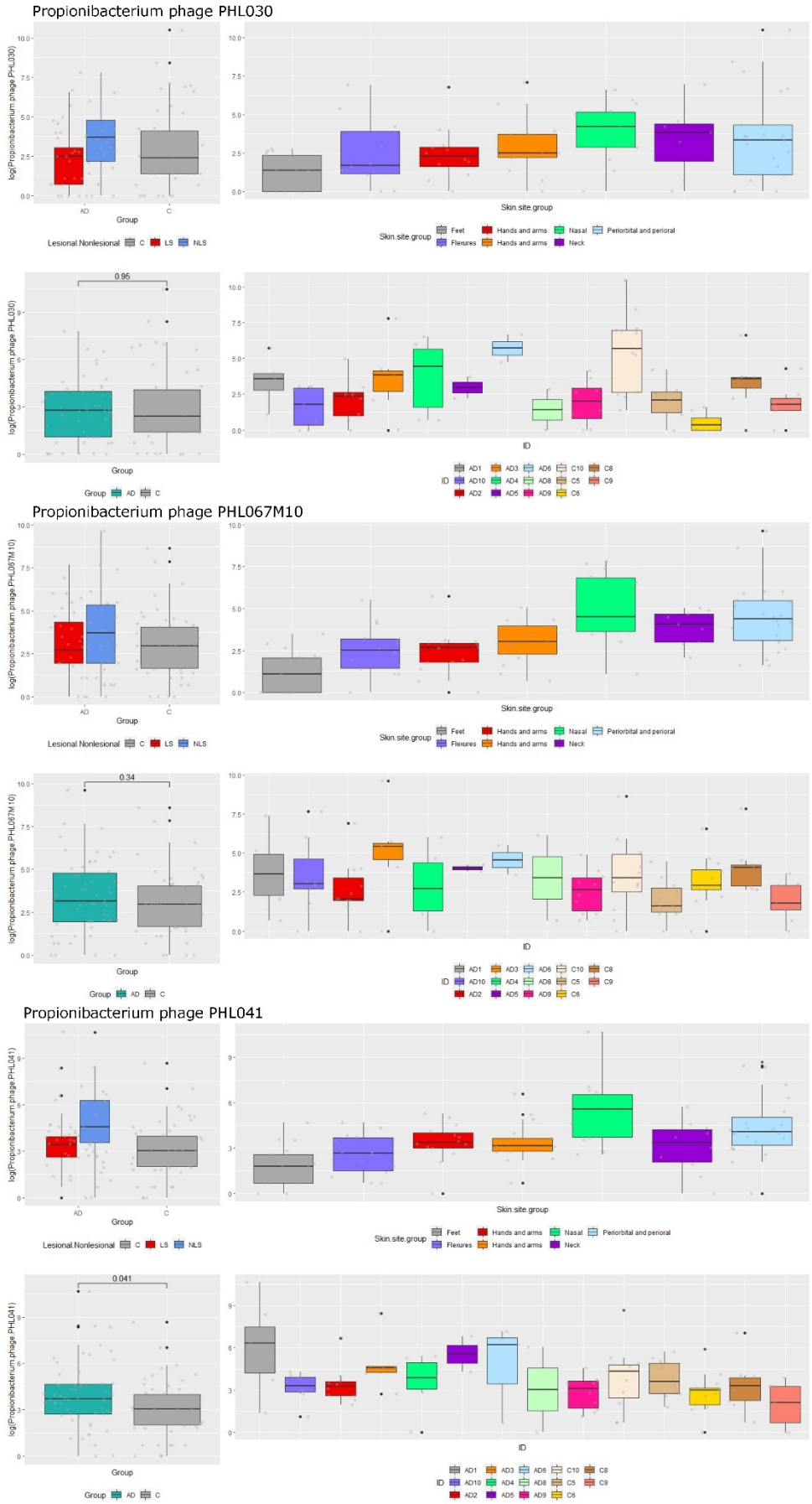
Staphylococcus phage PH15



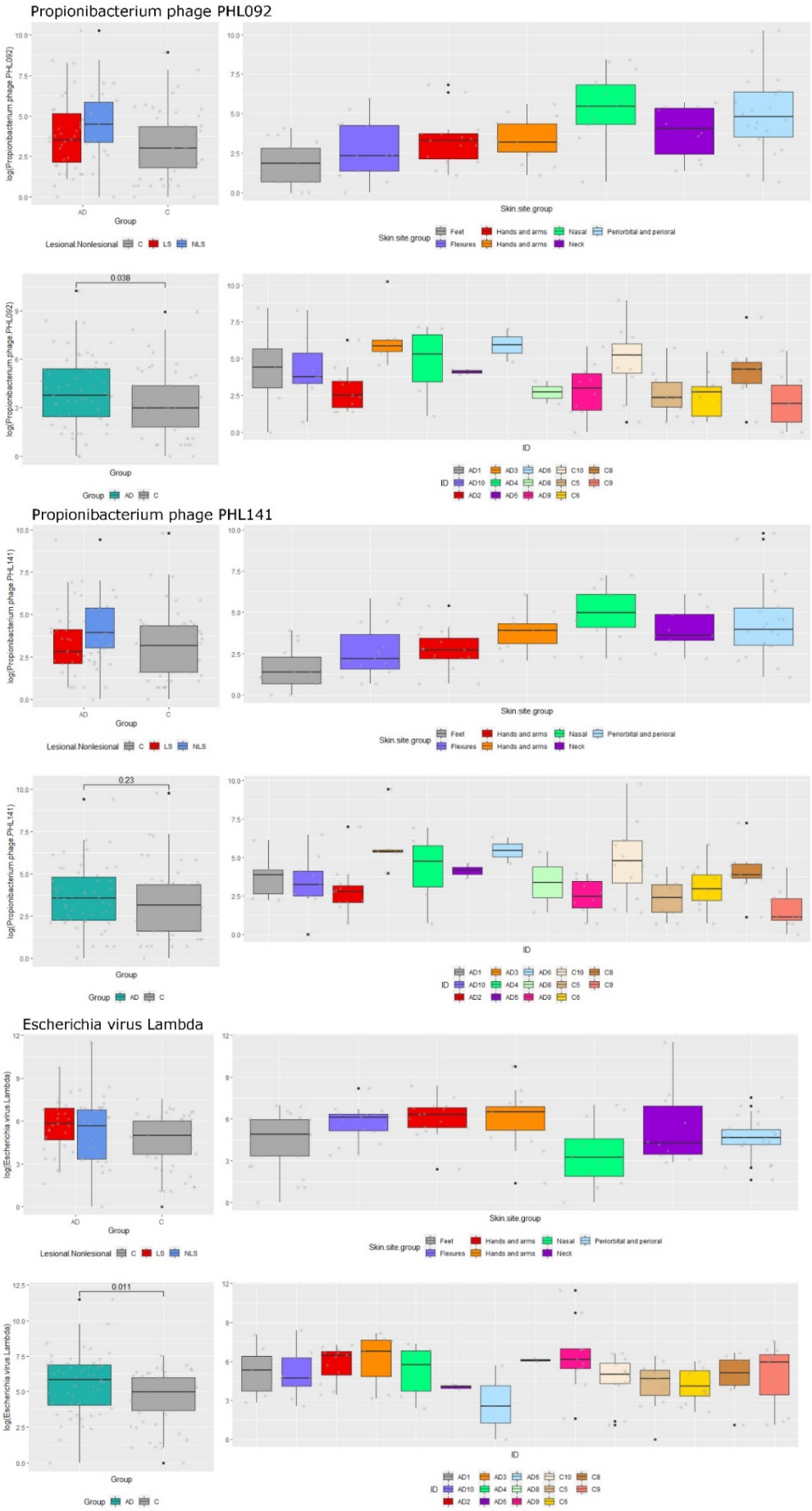
A. (continued)


Staphylococcus phage StB12
log(Staphylococcus phage StB12)
Group
AD
C
Lesional.Nonlesional
C
LS
NLS
Skin.site.group
Feet
Hands and arms
Nasal
Periorbital and perioral
Flexures
Hands and arms
Neck
0.97
ID
AD1
AD3
AD6
C10
C8
AD10
AD4
AD8
C5
C9
AD2
AD5
AD9
C6
Staphylococcus phage StB20
log(Staphylococcus phage StB20)
0.063
Staphylococcus phage StB27
log(Staphylococcus phage StB27)
0.14

B.


Propionibacterium phage PHL030
log(Propionibacterium phage PHL030)
Group
AD
C
Lesional.Nonlesional
C
LS
NLS
Skin.site.group
Feet
Hands and arms
Nasal
Periorbital and perioral
Flexures
Hands and arms
Neck
0.95
ID
AD1
AD3
AD6
C10
C8
AD10
AD4
AD8
C5
C9
AD2
AD5
AD9
C6
Propionibacterium phage PHL067M10
log(Propionibacterium phage PHL067M10)
0.34
Propionibacterium phage PHL041
log(Propionibacterium phage PHL041)
0.041

B. (continued)



C.

Figure S7: Boxplots of the top 15 most abundant viruses

The absolute abundances (counts) of A) *Staphylococcus phages*, B) *Propionibacterium phages* and C) *Escherichia lambda phage* are grouped according healthy control (C) and AD, including lesional state (LS:Lesional, NLS:Nonlesional, C:Control) to the left. To the right groupings are according to skin site group and individual.

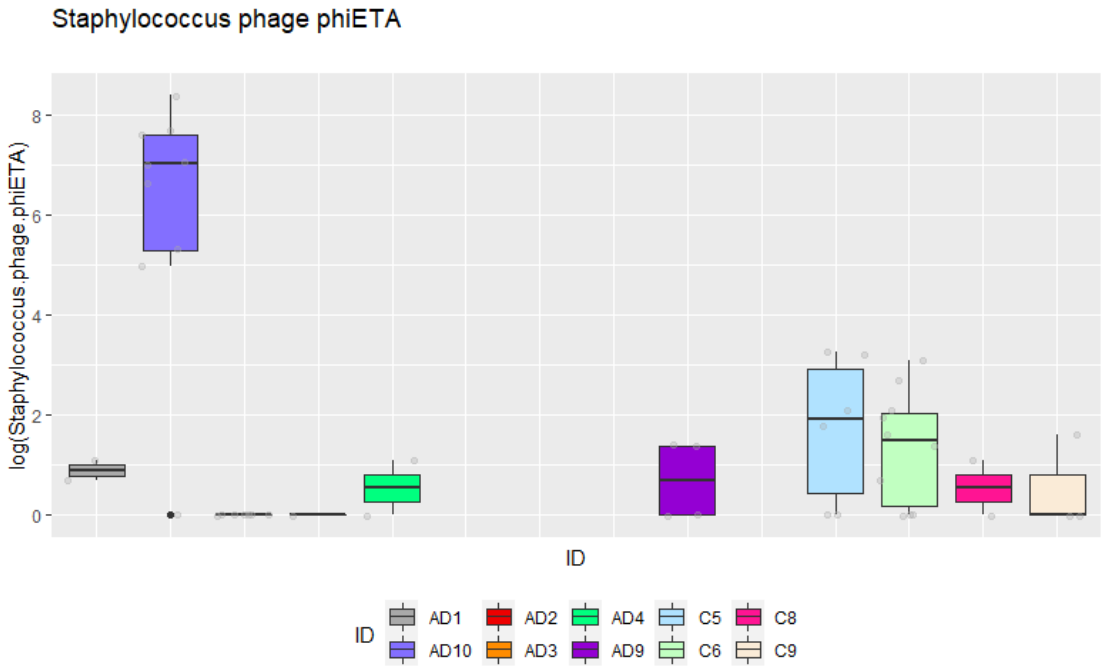
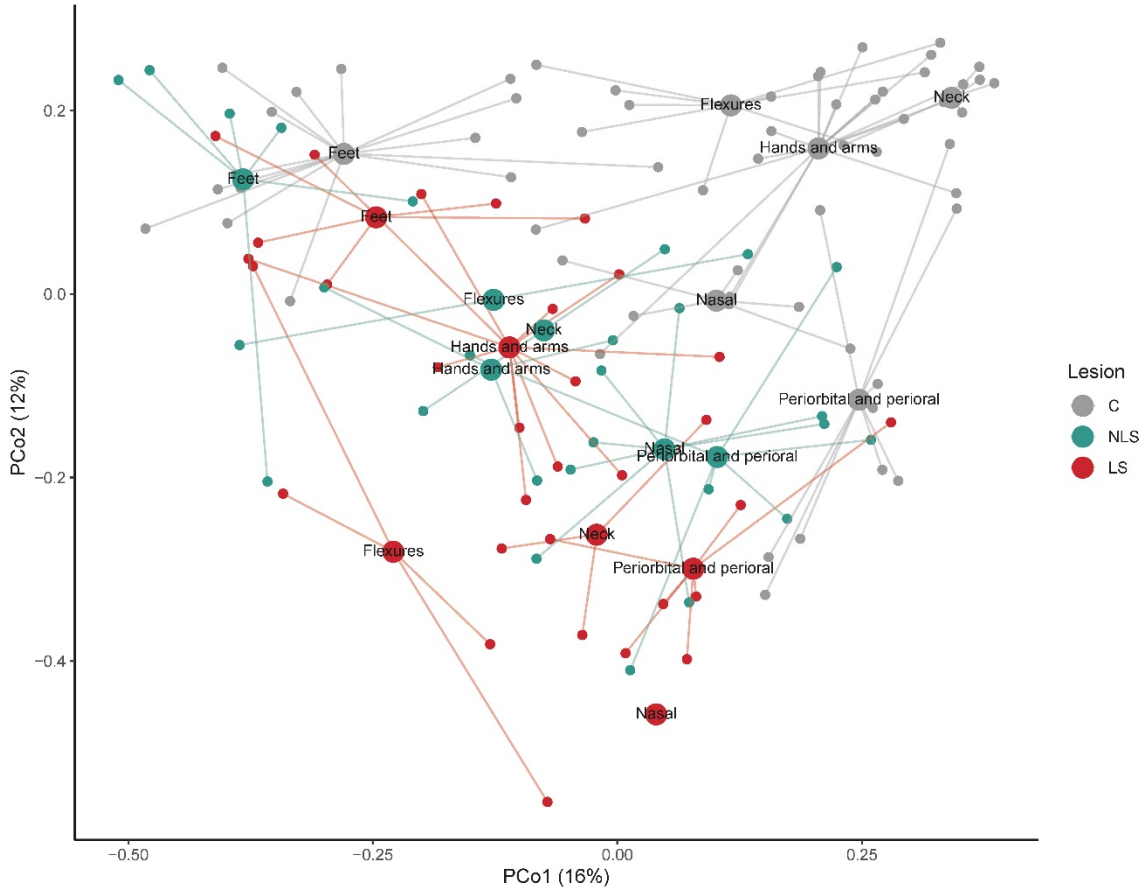


Figure S8: Boxplot of the absolute abundance of *Staphylococcus phage phiETA*

Grouped according to individual.

A)



B)

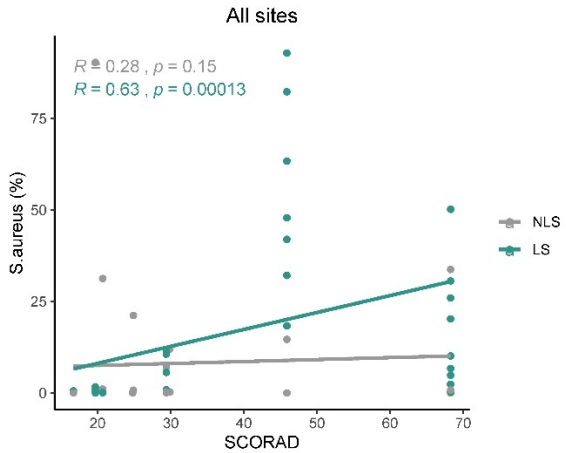


Figure S9: Lesional state, effects on clustering according to skin site and *S. aureus* colonization

The 6 skin area groups are: Feet (arches of the feet, dorsum of the feet and between the toes; C N=15, AD N=11), flexures (antecubital and popliteal flexures; C N=9, AD N=6), hands and arms (palmar and dorsum of the hands, between the fingers, volar forearms and upper inner arms; C N=17, AD N=15), Nasal (C N=5, AD N=9), neck (C N=5, AD N=5), and periorbital and perioral (C N=10, AD N=13).

A, Principal coordinate analysis based on Bray-Curtis distances between samples. Centroids represent the arithmetic mean position of the points belonging to the specific category.

B, Proportion *Staphylococcus aureus* versus SCORAD for all skin sites of AD patients according to lesional state, Pearson partial correlation.

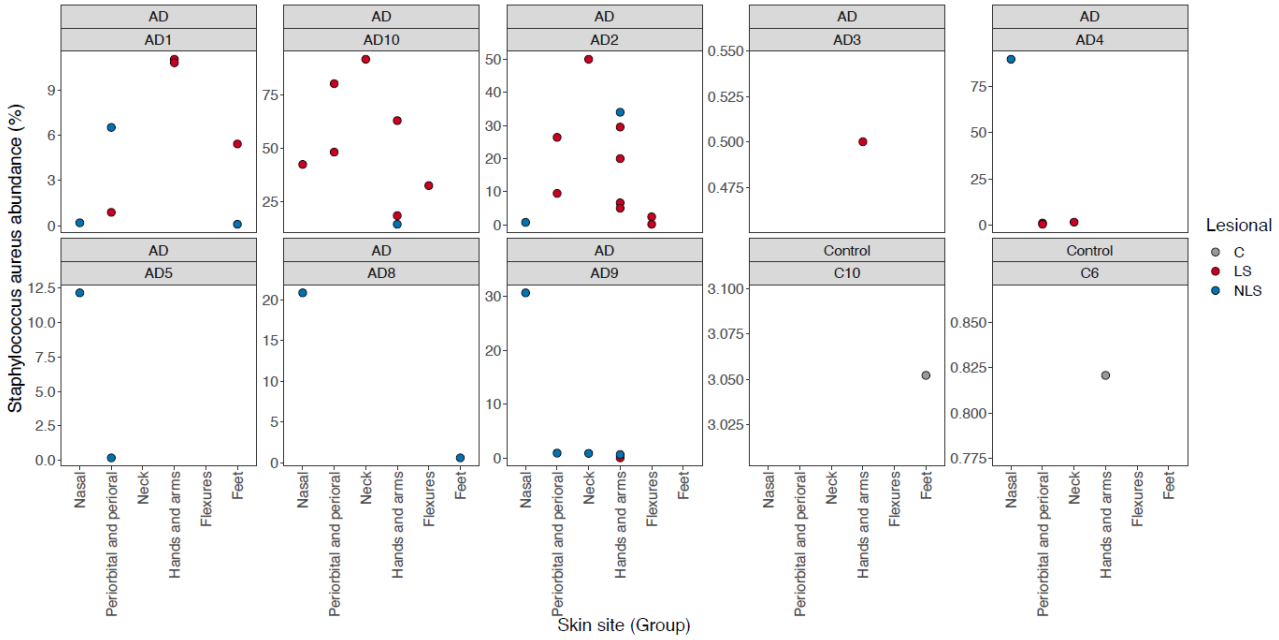


Figure S10: Samples with *S. aureus* detected with enough SNV coverage (N=42)

Skin site is shown at the x-axis, *S. aureus* abundance on the y-axis coloured according to disease state.

4. Considerations and comments on methodology

The studies presented were conducted at the Department of Dermatology and Allergy, National Allergy Research Centre, Herlev-Gentofte Hospital between 2015 and 2020.
The thesis consists of one systematic review (Manuscript I) and two original experimental studies (Manuscript II and III).

The following section is an elaboration on the methodology and considerations not covered or only briefly in the manuscripts.

4.1 Limitations in using DNA to describe microbial communities

Limitations in using DNA to describe microbial communities include that it cannot be assessed if the microbes are dead or alive or metabolically active. Furthermore, it is not possible to analyze genomes, which are not present in the reference databases, making their usefulness limited, and the databases are generally sparse in fungal and viral genomes. To gain insight into these uncharacterized taxa, researchers are developing reference-free methods for de novo segregation of metagenomic data into specific biological entities[37], however, it remains an unresolved problem. Other approaches include describing the functional potential of the sequenced material[41], e.g. in programs such as MEGAN (MEtaGenome ANalyzer). This is crucial and may reveal that highly variable communities in different individuals are functional redundant[98]. When analyzing DNA, functional analyses are more indirect compared to analyses of RNA or protein material. Studies combining microbiome and transcriptome data in AD are emerging[99-102] and in general, future studies would benefit from integrating omics data in capturing the multiple levels of information underlying disease states.

4.2 Manuscript I

The primary outcome for this systematic review was the relative abundances of microbiota. We chose this to focus on microbial communities instead of single taxons, e.g. *S. aureus* dominance in relation to AD, which is the focus of numerous primary studies and literature reviews. But conducting the study, it became clear that choices of methodology for finding microbial communities greatly affected this outcome, e.g. PCR primers, as the same community amplified with different primer pairs will give different microbial profiles, since different linages may evolve at different rates in each variable region of their marker gene[28]. Methodologies are not assessed by standard tools like the study quality, but we choose to lay forward some details on sampling, extraction and sequencing for each study in supplementary table 1. Furthermore, assessing risk of bias of animal studies using SYRCLE's risk of bias tool were difficult and we had to judge many entries as "unclear risk of bias" due to poor reporting on experimental details. This is a common issue for animal studies[103].
Reflecting on these challenges and the aim of this knowledge synthesis, where we did not address the feasibility, appropriateness, meaningfulness or effectiveness of a certain

treatment or practice[104], but investigated the role of the skin microbiome in AD, it could have been relevant to conduct this study as a scoping review instead of a systematic review. Furthermore, the research field is rapidly evolving using lots of new techniques producing heterogeneous and complex data, and with lots of new papers emerging frequently. This underlines that a scoping review could be a valuable alternative. A scoping review does not aim at producing a synthesized answer to a precise question (as a systematic review), but rather to provide an overview of the evidence and usually also ongoing research. Therefore, the assessment of methodological limitations or risk of bias is generally not performed. It should not be confused with traditional literature reviews though, as scoping reviews are conducted with an *a priori* protocol, a systematic and exhaustive information search, include multiple reviewers and extract and present data in a structured manner – all aiming at being transparent and reproducible about a topic or issue. Hence, the topic of this manuscript could have remained the same, but the analysis could have been open to many measures.

4.3 Manuscript II

Collecting skin samples for microbiome characterization can be done by a variety of methods, including biopsies, scrapes, tape-stripping, cub scrubs, swabs. Great overlap in microbial communities have been demonstrated using different methods[105,106], suggesting that study design, population and other factors are essential when choosing a sample strategy. We prioritized non-invasiveness and used scrapes and eSwabs.

As for storage and DNA extraction, we used commercial kits dictating the protocols. In general, the extraction method relied on the principles (Figure 6): 1) Lysis of cells mechanically, chemically and/or thermally, 2) binding the negatively charged DNA to a positively charged membrane under a high-salt condition, 3) washing contaminants and impurities away and 4) eluting DNA from the membrane. Many variations and modifications exist, potentially both affecting the amount of DNA successfully extracted and which microorganism efficiently have their cell walls and membranes disrupted[28]. Furthermore, samples from skin with low microbial densities are more affected by contaminating DNA from surroundings, e.g. added during handling of samples, generating spurious reads[107,108].

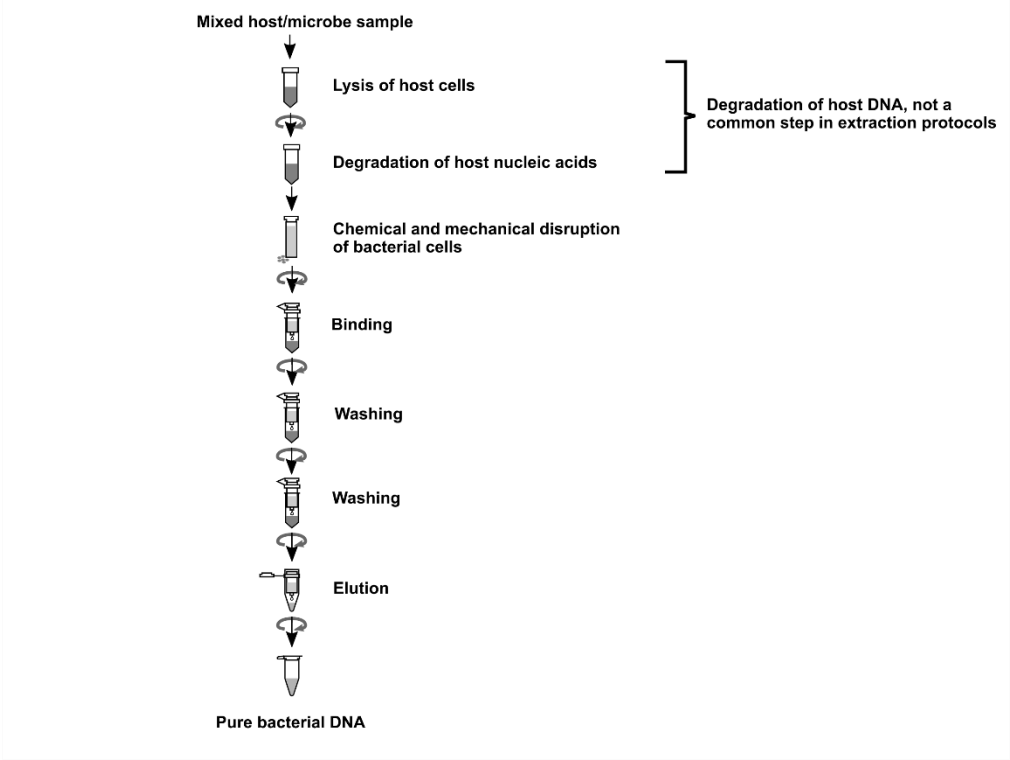


Figure 6: DNA extraction
Adapted from QIAGEN (QIAamp DNA Microbiome Kit Handbook)

We chose to focus on DNA extraction kits in this study, as the main obstacle in our pilot phase was to obtain enough DNA from skin samples for microbiome investigations – a known challenge in this field[99,109]. We generally got the same concentrations from skin samples as from negative swab controls (around 0.17 ng/µL). The study setup was rather complex, as we wanted to evaluate many kits and two different sampling strategies at specific skin sites. It would have been preferable if all kits were tested on the same persons, at the same skin site, e.g. by reducing the amount of kits included.
To evaluate microbial contributions from surroundings we used empty negative control samples. Initially, we wanted to evaluate DNA extraction efficacy, but since we made choices making such evaluation of poorer quality, we did not put much emphasize on it in the paper. These choices included not sampling equal sized (cm^2) skin areas per skin location, as we wanted to collect as much DNA as possible and having a single species (*E. coli)* as positive control. It could have been much better to include a mock community as some kits may be better suited for extracting certain microbes (e.g. Gram negative versus positive) and the challenge is to extract DNA from multiple organisms in correct proportions, without losing one. A mock community would also have enabled us to evaluate the kits' performance in capturing a known microbial community. In capturing the "biological truth" from skin samples, we made an unfortunate choice of primers using the V3-V4 combination 4341F/805R to amplify the 16S rRNA gene which poorly amplifies *Cutibacterium*, common inhabitants of the skin[110].

Other methodological decisions in this study relied too on data from our pilot phase, e.g. findings that premoistening the flocked swab in preservation medium from the e-swab resulted in higher relative abundances of skin bacteria compared to common contaminating bacteria compared to dry swabs and premoistening in phosphate buffered saline. After learning from the literature, that a strategy used to deal with the challenge of having enough DNA from skin samples was to increase the number of PCR cycles for 16S sequencing[111], we tried to compare 25 and 30 PCR cycles, and found that 30 cycles generally expanded the bacteria already present in high relative abundance and thus chose to use 25 cycles.

4.4 Manuscript III

Knowing that the skin microbiome composition may be affected by a variety of factors, such as hygiene[47,112,113] and skin care products[46], we instructed subjects not to shower, use chlorinated pools, sauna, steam bath, sun tanning and topicals locally on test areas two days before sampling. As compounds from deodorant usage still can be detected in the armpits 1-3 weeks following the last day of use[46], two days was a rather short washout period. However, we did not aim at taking subjects out of their surroundings and acknowledge too that the skin is affected differently by chemicals from e.g. mattresses, furniture and clothes. In line with this, we did not chose to exclude patients with AD in systemic immunomodulating treatment, despite knowing that treatment could affect the microbiome[92,114] and potentially ended up causing the observed lower success of sequencing in AD subjects compared to controls. We did test for an effect of systemic versus topical treatment and did not found one. Untreated AD is seen very rarely in the out-patient clinic at the Hospital, from where we recruited, as the patients typically consult their general physician initially and start treatment before coming to the Hospital. Also, many patients have been treated for AD since childhood. When the patients had been FLG genotyped for the three most common loss-of-function mutations in the Northern European population (R501X, 2282del4 and R2447X), we noted the information from their medical records. As research indicate that FLG status may be relevant for microbiome compositions in AD[96,115], it would have been a better strategy to test all subjects included, also skin-healthy controls. For assessing the severity of AD, the tool SCORing Atopic Dermatitis (SCORAD) was chosen. This is a validated tool, holds a high inter-rater reliability and correlates well with both objective assessments such as Eczema Area and Severity Index (EASI) and also the Dermatology Life Quality Index[116] and importantly, though EASI is broader in adoption, SCORAD is the most common tool applied in existing literature on the skin microbiome in AD. SCORAD is a composite score integrating both the surface area of involvement in AD and the severity of six clinical signs on a 4-point scale (erythema, edema/papulation, oozing/crusting, excoriation, lichenification, and dryness), and furthermore patient-reported pruritus and sleep loss[117]. EASI covers only clinical signs and does not cover oozing/crusting and dryness of unaffected skin. These differences result in a complex relationship between EASI and SCORAD[118], and scores cannot be directly translated without caution.

Hand eczema was an inclusion criterion in this study, as AD is often manifested on the hands of adults[119] and the microbiome here was poorly characterized. We used the hand eczema

severity index (HECSI) for assessing severity. This is a validated tool[120], however, not developed to atopic hand eczema specifically. Hand eczema is a multifactorial condition and apart from a manifestation of AD often developed after repeated or prolonged contact to irritants and/or allergens[121].

To circumvent variation introduced by differences in handedness[113], we sampled bilateral areas of the body. As the microbiome also might be affected by hydration levels[122], we chose to ask the participants when they last moisturized the test areas and also which type of cream they used (lipid content). We also chose to exclude participants with scar tissue on test areas and at lesional sites, the area affected (4 categories: 1-25%, 26-50%, 51-75%, 76-100%) and morphology (erythema, edema, lichenification, scratch marks, dryness, fissures) were described. Unfortunately, as we had a lot of missing samples, this detailed information could not be used. Several other potential confounding factors on microbiome compositions exist. As we experienced difficulties in obtaining enough microbial DNA from skin samples from especially the hands and arms, it would have been relevant to ask to the subjects about time since last hand wash or use of disinfectants e.g. in connection with a toilet visit. Differences in microbiome compositions between men and women have been reported[113], these differences might both be due to biological factors, such as different effects of sex hormones on secretion of body fluids, but studies have also shown that frequent handwashing is more prevalent among women[123], indicating that behavioural factors also could introduce bias. We enrolled subjects from March to July and thus experienced seasonal variations in the weather. Knowing that season influence levels of NMF in skin[124], it would have been most optimal to enrol subjects in the same season. This was however not possible for practical reasons.

With regards to analytical methods used, we chose to remove host DNA in samples (Figure 6) to increase sequencing depth, however, reducing the total DNA contents in samples likely caused more samples to fail library preparation. A better strategy could be to keep the human DNA in the samples and sequence deeper.
For explorative investigations, we chose to pool samples into groups representing larger body areas, to have at least 5 samples representing a skin site. However, it is well known, that the skin microbiome is highly dependent on specific characteristics at the skin site[18,122]. Despite of the high amount of missing samples, having 121 samples successfully analyzed with the expensive shotgun sequencing and metagenomics is a large number. The methodology is being increasingly used and six other studies have applied it in patients with active AD[78,100,101,125-127] at the time of writing.

5. Discussion

5.1 Investigating the skin microbiome

With our systematic review (manuscript I) we identified great variability in methodological approaches used in the 18 studies included. As we furthermore struggled in obtaining enough microbial material from skin samples for microbiome investigations in our pilot phase (a known challenge in the field[99,109,127]), we established methodology for investigating the skin microbiome by comparing two different sampling strategies and 12 different DNA extraction kits (manuscript II). We found that swabs and scrapes could be used almost interchangeably, which is in line with data from other studies comparing different skin sampling methodologies[105,106,128]. The non-overlapping reads from eSwabs and scrapes constituted 0.7%. We believed the difference was due to differences in sample depth and associated changing living conditions for microorganisms. A study applying swabbing of specific layers of the SC after repeated tape stripping found decrease in the relative abundance of Actinobacteria (*Cutibacterium*) and increase in the relative abundance of Firmicutes (represented by *Staphylococcus*) in the deeper layers of the SC[111]. Another study comparing swabs and biopsies also found enriched abundance of the Firmicutes order Clostridiales in biopsies[129]. As these are obligate anaerobic, oxygen availability in the different skin layers might shape the composition of microbiota. Though we did not observe differences in OTUs and chao1 richness between eSwabs and scrapes, this could be due to differences in sampling techniques as our scrapes also sample the outermost microbial material which swabbing of sequential tape strips does not. Also, we could not amplify *Cutibacterium* appropriately in our study.

Some studies indicate that the microbiome of the deeper layers of the skin is the core skin microbiome. As the surface skin microbiome 14 days after complete removal of the SC by tapestripping was more similar to the deeper SC layers than the surface layers[111]. Also, bacterial DNA has been found in the dermis and subcutaneous fat[130]. Grice and colleagues speculate that bacteria from the deeper layers may transit to the surface of the skin with differentiating skin cells[106]. However, colonization from the surroundings may also occur. With specific developed habitats of the skin, certain microorganism may be selected for colonization and growth which would also explain stability of the skin microbiome.

Taken together, superficial sampling by swabs might cause one to overlook specific species. It is likely that moistening the swab before sampling enables catching some material from hair follicles, glands and perhaps also from layers of the SC below the outermost. This could explain our initial difficulties in obtaining enough DNA from dry swabs. Another reason for using the eSwab is the consistency, which we found to be higher than using scrapes, and non-invasiveness. Especially when sampling eczematous skin, scraping with a scalpel can easily induce damage and bleeding. An alternative approach is tape-stripping, which is also relatively non-invasive and enable sampling of the deeper SC.

Out of 12 DNA extraction kits, we would avoid using 8 due to poor rates of library formation, remarkably higher alpha-diversity or domination by contaminating bacteria. One of the remaining 4 kits managed to reduce the host DNA from app. 90% to 57% in one nasal sample without inducing taxonomic skewing. The proportion of host and bacterial cells varies greatly in different samples and between samples sites[131], but since the human genome is app. 1000 times larger than bacterial genomes, human DNA can easily drown out microbial reads. Therefore, it could be an effective strategy to reduce host DNA, especially when investigating potential changes in the microbiome in interventional studies. However, the hands-on time using this kit was substantial and a drawback with increased risk of introducing sample contamination. Other approaches of reducing host DNA may be preferable[131]. Furthermore, we experienced in manuscript III that using this kit for DNA extraction, 43% of the skin samples (91 out of 212) failed library preparation, indicating that handling of human DNA post sequencing could be preferable.

Though the extraction kits captured somewhat different microbial communities, samples cluster more by skin site and subject. The strong individual-specificity in skin bacteria have been reported numerous times[111,44,113], from other human bodily habitats as well[98,132] and we also found it in manuscript III. Host-specific factors could both be genetic, e.g. sex[113] and filaggrin[115], and environmental, for instance washing[112,113], cosmetics[47,133,134] and use of medication[135]. Some factors have been investigated for their implication on the skin microbiome, but a great deal of knowledge is lacking on this area. We are currently looking into effects of a fragrance compound with known antimicrobial action and moisturizer usage with and without the fragrance added as ingredient on the microbiome of normal and AD skin (appendix I).

5.2 Skin dysbiosis in atopic dermatitis

With our systematic review (manuscript I) we identified an AD associated skin dysbiosis characterized by low bacterial diversity, especially at lesional sites. This finding has been reproduced by others since[126] and we also saw it in manuscript III. In general, we found lesional state to affect microbial community composition across most skin sites. The AD related dysbiosis described in manuscript I was based on samples from either unknown bodily locations or the face or arms, most frequently the antecubital fossae.

In manuscript III we demonstrated that not only the flexures, but also the neck, have significantly different microbial communities in patients with AD. The hands and arms also showed distinct AD associated patterns. Global microbial dysbiosis in AD including the arms and forehead has been reported elsewhere[115] and it has furthermore been indicated that certain anatomical sites might be more prone to have an AD-like microbiome[102]. In our study, we also identified skin sites being more similar in healthy controls and AD, including the feet, periorbital and perioral areas. The feet may be more affected by similar external factors, e.g. footwear. Other studies also find the skin microbiome of the feet to be significantly different from the rest of the skin, with diverse fungi colonization[35] and low stability compared to other skin areas[44]. In this regard it is however interesting that the hand microbiome is relatively

stable[44] despite of being exposed to numerous different external factors. We expected to see greater differences between AD and control on the facial areas. Maybe fluids from the mouth and eyes constitute a major common exposure on these specific habitats. Another explanation could be that facial areas with increased abundances of sebaceous glands selected for microorganisms being less AD-like.
A purpose of this study was to describe the hand microbiome in AD. Unfortunately, many of the samples from the hands failed sequencing and we chose to pool the samples with those from the volar forearm and upper inner arms. An ongoing study from our research group is now looking further into biomarkers, including the skin microbiome, of irritant, allergic and AD hand eczema.

In our systematic review (manuscript I), we warranted robust data on the influence of clinical measures on the skin microbiome. Meanwhile, around 41 papers on the human skin microbiome in AD have been published and more studies have focused on filaggrin. As we missed information about filaggrin genotype from all subjects in manuscript III, we could not assess if mutations in this gene affected the skin microbiome. The newer studies indicated that persons with filaggrin deficiency have overall microbial compositions resembling AD-patterns rather than healthy controls[115]. In patients with AD, structural differences in skin microbiomes associated with filaggrin has been found specifically in nonlesional skin[96]. Patients with AD and ichthyosis vulgaris, FLG null mutations and noninflamed dry skin, both have expansion of *Staphylococcus*, suggesting that overgrowth might correlate with the severity of epidermal barrier impairment rather than inflammation[126] .

5.2.1 Microbiota enriched in AD and microbial interactions

In manuscript III we found that the flexures were dominated by *S. aureus* and *S. epidermidis* (Figure 7), the latter especially at lesional sites. This is in line with metagenomic data from Byrd and colleagues[125]. In general, we found *S. aureus* to be present at most skin sites among AD patients and occasionally dominated the community. Furthermore, we identified an association between severe AD and *S. aureus* colonization, which has been established before[125]. Interestingly, two studies found *S. epidermidis* to predominate skin of AD patients with less severe disease[125,126], suggesting that *S. epidermidis* overcolonization indicate some kind of “permissiveness” in the skin to Staphylococcal colonization. Furthermore *S. epidermidis* versus *S. aureus* overcolonization might serve as marker of disease severity. Biologically, these species likely compete as they produce AMPs targeted each other[136] and specific conditions then allow *S. aureus* to expand. It has been reported that certain strains of *S. aureus* bloom in flaring skin and persist after the flare condition in lower relative abundance[125]. We identified a subject-specificity of *S. aureus* strains, indicating that different strains could be involved in the disease.
An interesting finding in manuscript III was the increased abundance of *Propionibacterium* and *Staphylococcus epidermidis* phages in AD. Bacteriophages are viruses that infect specific bacteria. After infection the phage either induce a lytic cycle, where the phage uses the bacterium to produce lots of phages and then kill it, or a lysogenic cycle where DNA from

the phage is inserted into the bacterial chromosome whereby the bacteria can acquire virulence determinants. The literature on the phages we have identified is sparse, but it is likely, that they contributed to drive pathogenicity both by lysis of *Cutibacterium* and introducing virulence genes in *S. epidermidis*[137] and *S. aureus*. In line, it is noteworthy, that one AD patient was extensively colonized by *S. aureus* (Figure S4 in the supporting information to manuscript III) and have higher abundances of the *Stahylococcus aureus phages phiETA* (Figure S8 in the supporting information), which possesses the ability to spread the virulence gene encoding exfoliative toxin (ET) to *S. aureus*[138], which could contribute to worsen the disease (Figure 7).

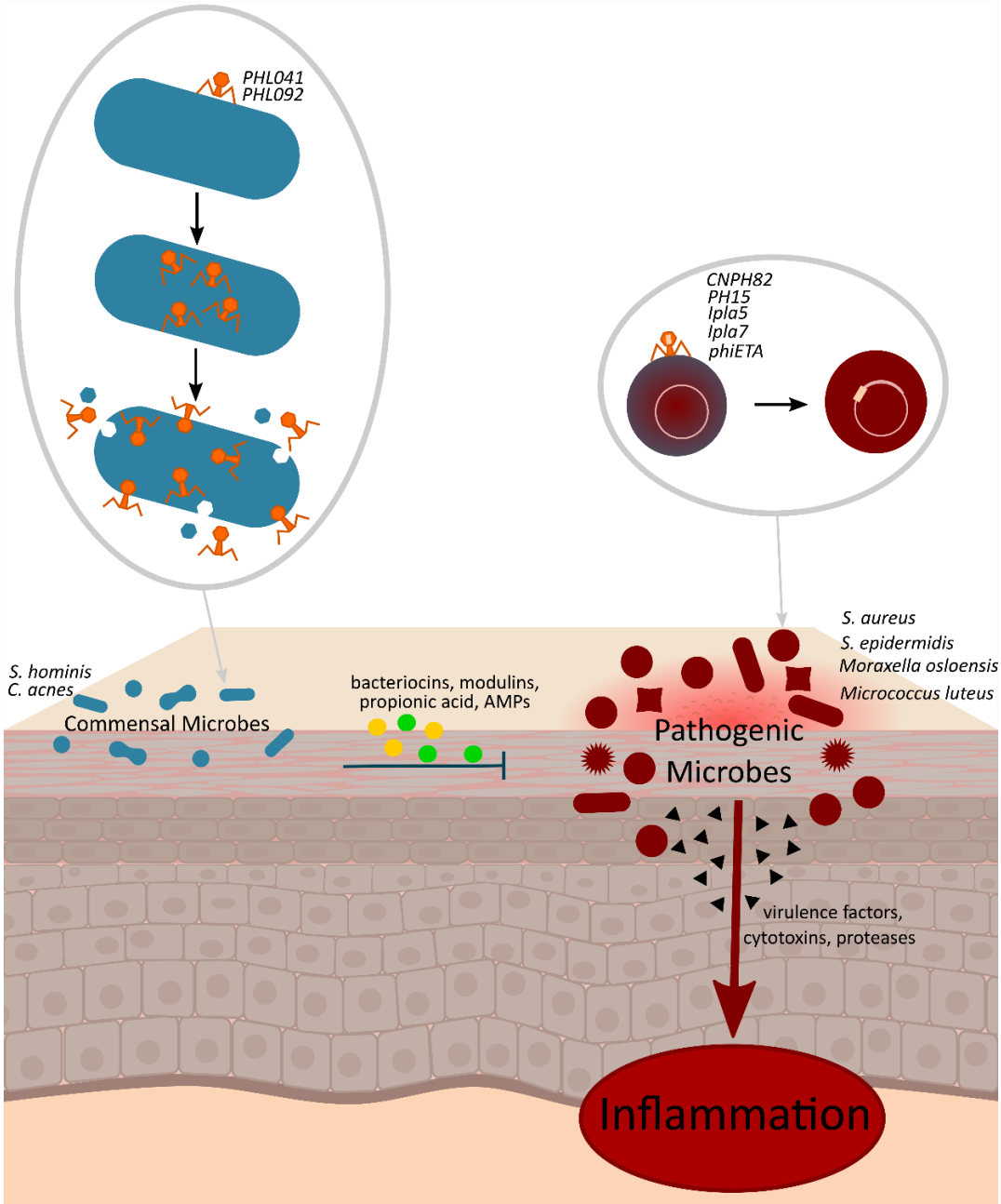


Figure 7: Skin dysbiosis in AD

Proposed mechanisms by which phages contributes to bacterial dysbiosis and pathogenesis

Other bacteria associated with AD included *Micrococcus luteus, Moraxella osloensis* (Figure 7), *S. saccharolyticus,* S. *lugdunensis* and *Rothia sp.* ND6WE1A*.* Another metagenomic study has also identified *S. capitis* to be increased in relative abundance in AD[78]*. M. luteus* can augment proliferation of *S. aureus*[139] and recently this bacteria has been associated to a certain AD dermotype also characterized by severe disease and itch, frequent flares, reduced bacterial richness[78]. The other AD associated species might also be relevant to investigate further for a role in AD – especially *M. osloensis* being a rare causative organisms of human infections[140-144].

5.2.2 Microbiota reduced in AD and microbial interactions

In manuscript III, the high relative abundance of *S. aureus* was accompanied by lower relative abundances of *S. hominis* (Figure 7)*.* A reduced relative abundance of *S. hominis* in AD is also reported elsewhere[54,115,127]. Their opposing presence in the skin microbiome is likely explained by regulatory factors; AD patients have previously been reported to lack coagulase-negative *Staphylococcus strains* (including *S. hominis* strains) producing AMPs against *S. aureus*[54]*.*

We also found *C. acnes* to be significantly reduced in AD patients compared to healthy controls (Figure 7) and furthermore *[Propionibacterium] humerusii* to be associated to a healthy control skin microbiome. Species within the *Propionibacterium* genus are currently being reclassified, placing the cutaneous propionibacteria in the *Cutibacterium* genus[145]. *[P.] humerusii* was described in 2011 during a project aiming at sequencing clinical isolates of *C. acnes*[146]. *[P.] humerusii* has been found to be a common inhabitant of the pilosebaceous unit[147], but to our knowledge this is the first study to report a difference in abundance in control versus skin disease. *C. acnes* has previously been reported to be reduced in AD compared to control[127] at forehead skin[148] and at the upper/lower back, posterior thigh or buttocks[101], but also to be less common in severe AD[95], at lesional skin with increased *S. aureus* colonization[149] – and more common at non-lesional AD skin[150] and a tendency for increase post flares[125]. *C. acnes* ferments glycerol from skin into short-chain fatty acids, including propionic acid. Propionic acid can inhibit growth of *S. aureus*[50]. Therefore, it seems reasonable that *C. acnes* contributes to protect against AD flares.
Fatty acid substrates coming from *C. acnes'* metabolization also feed *Malassezia,* the most common fungi on healthy human skin[151], and we find a lack of *Malassezia* in AD, especially at the neck (Figure 8). Hence, we believe that poor growth conditions in dry AD skin restrict the growth of microbiota on different trophic levels. A newly published paper explore inter-domain relationships between bacteria and fungi in AD by correlation analyses and found positive correlations between Staphylococcal species and the fungi *Cladosporium spp*. and *Debaryomyces hansenii*, both which might provide *Staphylococcus* with substrates[126]. Little research into inter-domain relationships in the skin microbiome exist and details on coexisting bacteria, fungi and virus might shed light on the establishment of skin dysbiosis in AD and other skin diseases.

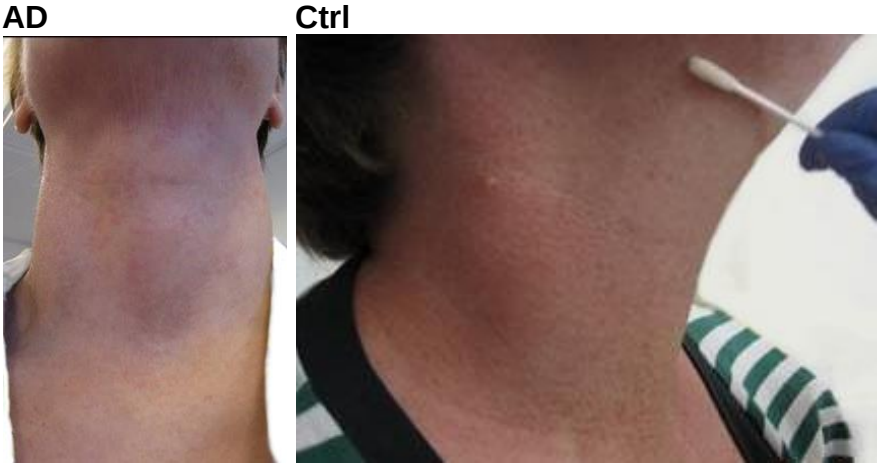


Figure 8: eSwabs from the anterior triangle of the neck

Other species associated with a control skin microbiome included *Corynebacterium sp.* and *Corynebacterium singular.* Other studies have identified species such as *Dermacoccus* and *Methylobacterium*[78] to be reduced in AD too. Anaerobic species such as *Finegoldia* have also been identified to be reduced in AD[101], likely caused by the broken barrier and increased oxygenation. In general, there is a lack of consistency between microbiome studies when the microbiota is less abundant – and there are conflicting data about *Corynebacteria* colonization in AD. *Corynebacterium* has been reported to be reduced in abundance in AD[101,127], during an AD flare[152] and to be positively associated with high levels of long-chain unsaturated fatty acids[115], where AD skin typically is characterized by higher levels of a-hydroxy ceramides[153]. *Corynebacteria* are lipophilic bacteria lacking fatty acid synthase and requiring an exogenic source of FFAs. However, *Corynebacteria* have also been reported to expand on dry ichtyotic skin[126] and also *C. bovis* and *C. mastitidis* emerged at the onset of dermatitis in an AD mouse model, where the former drove inflammation[154]. More knowledge on the specific species and strains of *Corynebacteria* and their metabolism might resolve these apparently conflicting data.

5.3 Is skin dysbiosis in AD a driving factor in the disease?

In manuscript I, we questioned if skin dysbiosis is a primary factor in AD development and included animal studies to elaborate on this. Although 6 animal studies were included, 4/6 studies were case-control studies either in dogs or different rodent models. Only one animal study investigated causative mechanisms[155]. Mice deficient in a disintegrin and metalloproteinase 17 developed dermatitis with dysbiosis where *S. aureus* and C*orynebacterium spp.* dominated. Targeting these bacteria with antibiotics both prevented onset and suppressed active inflammation. Furthermore, inoculation with these bacteria accelerated the inflammation. However, many findings in animals are difficult to translate to humans. When we published manuscript I, one longitudinal study in humans linked a lower colonization with commensal *Staphylococcus* species in infants with later AD diagnosis[156], addressing a key question in evaluating if dysbiosis is cause or consequence, which is if dysbiosis precede onset or worsening of AD. Furthermore, two studies on prophylactic

emollient usage in high risk babies reported reduced incidence of AD at 6 and 8 months with regular application of emollients from birth[157,158]. It was hypothesized that the intervention prevented dysbiosis and an ongoing study evaluated the skin microbiomes from a subset of the babies. This study has now been published and found that emollient usage in the high-risk infants reduced skin pH and increased proportions of commensals, indicating that the microbiome in fact could mediate the protective effects[159]. Meanwhile two studies with more participants found no effect of prophylactic emollient therapy against AD development in high-risk infants[160,161], reducing the optimism on this intervention.

A second highly relevant question to pose is if changing an AD characteristic dysbiosis in humans to a “healthy state” also improve disease. For years, one approach has been to target the microbial dysbiosis in AD by eradicating S*. aureus* with anti-staphylococcal antibiotic treatments. However, this does not improve disease significantly compared with non-antimicrobial agents[162] and furthermore, using antimicrobial therapy may promote acquisition of antibiotic resistance. New studies have emerged in recent years, applying topical commensals in re-establishing a “healthy” microbiome in AD showing promising results. E.g. reintroducing selected coagulase negative Staphylococcal strains, with antimicrobial activity against *S. aureus* in culture, to AD subjects decreased *S. aureus* colonization[54]. Adding the Gram negative Proteobacteria *Vitreoscilla filiformis* to a moisturizer significantly improved AD with decreased clinical scoring, itch and better sleep compared to placebo[163]. Mechanistically, this was shown to be driven by induction of IL-10, resulting in reduced IFN-γ production and T cell proliferation[164]. Another study has succeeded in using the gram negative *Roseomonas mucosa* obtained from healthy donors and transplanted to AD subjects. These subjects displayed improved disease severity, reduced need for topical steroids and lower *S. aureus* colonization[165]. Skin improvements and colonization by *R. mucosa* was later shown to persist for up to 8 months and it was proposed that *R. mucosa* initiate epithelial repair pathways by producing of activating sphingolipids[166]. However, the effects were dependent on skin site, with improvements in the antecubital fossae and no effects on hands[165]. Though transplantation and manipulation of microbiomes could be a promising strategy, much more need to be learned. Our data from manuscript III furthermore indicated, that elucidating the role of the virome in AD could provide useful knowledge on the skin dysbiosis and open potential treatment strategies.

6. Conclusions

Methodologically, we identified great variability in approaches used for skin sampling and downstream microbiome analysis. As large amounts of keratinocytes DNA interfere with bacterial, fungal and viral DNA we tried to reduce the human DNA in vitro before sequencing. Initially, this was done in a nasal sample showing promising results with no substantial skewing of microbial communities compared to samples without reduction in human DNA. However, in our subsequent case-control study, 43% of the skin samples (91 out of 212) failed library preparation, fragilizing our data. Paradoxically, as library preparation depends on the total DNA content in samples, keeping the human DNA might increase the success rate and for future studies we would deal with the human DNA computationally, post sequencing.

The skin was colonized by both bacteria, fungi and virus, especially phages, with great individual variability. Altered communities were found in AD, with some sites being more affected than others. The flexures and neck showed marked taxonomically changes with high Staphylococcal colonization and an absence of *Malassezia* species. *S. aureus* was highly abundant in AD and our data suggest more mechanisms potentially supporting its growth, including interplay with phages.
There is growing evidence of a key role of the skin microbiome in AD pathogenesis.

7. Future perspectives

As dysbiosis plays a key role in AD, it is obvious to investigate the skin microbiome as target for treatment. Research into the gut microbiome is more advanced and interestingly a link between gut and skin health appears. Altered gut microbiomes have been found in skin diseases such as acne[167], rosacea[168] and AD[169,170] and a meta-analysis found that targeting the gut microbiome in children with AD with probiotics, strains of *Lactobacillus,* improved the clinical scoring of the skin (SCORAD)[171]. The gut microbiome should not, however, be regarded as the master governor of the skin microbiome. Treating healthy human skin with narrow band ultraviolet light increased both serum vitamin D and the diversity of gut bacteria[172], implying bidirectional interactive mechanisms between skin and gut. Most research indicate that microorganisms and their metabolites exert their action on systemic immunity (reviewed in Forbes et al.[173]). The skin is however more easily assessed than the gut and therefore represent an excellent organ for manipulating the microbiome and evaluate potential improvements of disease. In recent years, studies trying to manipulate the AD skin microbiome have been published, showing promising results[54,165,174]. Also, in healthy subjects, augmentation with *S. epidermidis* obtained from own skin has been shown to improve skin moisture[175]. An interesting approach is to use genetic engineered skin commensals associated with a healthy skin microbiome profile, such as *S. epidermidis* or *C. acnes*, to produce and secrete active biotherapeutics lacking in AD, such as filaggrin or components of NMF (discussed in a recent review by Callewaert[176]). Our study points at another interesting field for exploration; the virome. Phages already colonize our skin; they are highly specific[177] and can easily be manipulated to carry certain genes for bacterial lysis or other functions. One major challenge in manipulating the skin microbiome with live microbiota is to make them stable on skin. The host microbiota cannot fully be removed which will lead to competition and need for repeated application[176].

Studies indicate that there might be an early life critical window for establishing a healthy microbiome[60,97]. As colonization with commensal Staphylococci in the antecubital fossae in infants was associated with decreased incidence of AD, in some cases, at 1 year[156], it can be speculated that an early effort to shape or re-shape the microbiome might be a solid strategy to reduce need for treatment later in life. Especially in infants or toddlers with either high risk of developing AD, detected skin dysbiosis in infancy or early life diagnosed AD. Epidemiological studies have both identified environmental factors associated with higher and lower risk of developing childhood AD[178,179]. Living in rural areas lower the risk of developing childhood AD, which might be explained by exposure to increased biodiversity in the environment[17,180]. Little is known about the direct associations between environmental factors, skin microbiome and AD from interventional studies. We therefore initiated a prospective clinical study on this area (appendix I). In this study we have included adults, but with a critical window in mind, it would also be interesting to investigate the impact of specific environmental factors on the composition and stability of the skin microbiome in different stages of life.

8. References

1 Leyden, J. J., Marples, R. R. & Kligman, A. M. Staphylococcus aureus in the lesions of atopic dermatitis. *The British journal of dermatology* **90**, 525-530 (1974).

2 Gallo, R. L. Human Skin Is the Largest Epithelial Surface for Interaction with Microbes. *The Journal of investigative dermatology* **137**, 1213-1214, doi:10.1016/j.jid.2016.11.045 (2017).

3 Eyerich, S., Eyerich, K., Traidl-Hoffmann, C. & Biedermann, T. Cutaneous Barriers and Skin Immunity: Differentiating A Connected Network. *Trends Immunol* **39**, 315-327, doi:10.1016/j.it.2018.02.004 (2018).

4 Houben, E., De Paepe, K. & Rogiers, V. A keratinocyte's course of life. *Skin Pharmacol Physiol* **20**, 122-132, doi:10.1159/000098163 (2007).

5 McGrath, J., Eady, R. & Pope, F. Anatomy and organization of human skin. *Rook's textbook of dermatology* **10**, 9781444317633 (2004).

6 Debra Jan, B., Raza, A. & Henry, R. S. Antimicrobial Activity of Sphingosines. *J. Invest. Dermatol.* **98**, 269, doi:10.1111/1523-1747.ep12497842 (1992).

7 Nguyen, A. V. & Soulika, A. M. The Dynamics of the Skin's Immune System. *Int J Mol Sci* **20**, doi:10.3390/ijms20081811 (2019).

8 Nemes, Z. & Steinert, P. M. Bricks and mortar of the epidermal barrier. *Experimental & molecular medicine* **31**, 5-19, doi:10.1038/emm.1999.2 (1999).

9 McLean, W. H. Filaggrin failure - from ichthyosis vulgaris to atopic eczema and beyond. *The British journal of dermatology* **175 Suppl 2**, 4-7, doi:10.1111/bjd.14997 (2016).

10 Rawlings, A. V. & Harding, C. R. Moisturization and skin barrier function. *Dermatol Ther* **17 Suppl 1**, 43-48, doi:10.1111/j.1396-0296.2004.04s1005.x (2004).

11 Parra, J. L. & Paye, M. EEMCO guidance for the in vivo assessment of skin surface pH. *Skin Pharmacology and Applied Skin Physiology* **16**, 188-202, doi:10.1159/000069756 (2003).

12 Baker, L. B. Physiology of sweat gland function: The roles of sweating and sweat composition in human health. *Temperature (Austin)* **6**, 211-259, doi:10.1080/23328940.2019.1632145 (2019).

13 Igyarto, B. Z. & Kaplan, D. H. The evolving function of Langerhans cells in adaptive skin immunity. *Immunology and cell biology* **88**, 361-365, doi:10.1038/icb.2010.24 (2010).

14 Salimi, M. & Ogg, G. Innate lymphoid cells and the skin. *BMC dermatology* **14**, 18-18, doi:10.1186/1471-5945-14-18 (2014).

15 Dominguez-Bello, M. G. *et al.* Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. *Proc. Natl. Acad. Sci. U. S. A.* **107**, 11971-11975, doi:10.1073/pnas.1002601107 (2010).

16 Prescott, S. L. *et al.* The skin microbiome: impact of modern environments on skin ecology, barrier integrity, and systemic immune programming. *The World Allergy Organization journal* **10**, 29, doi:10.1186/s40413-017-0160-5 (2017).

17 Lehtimaki, J. *et al.* Patterns in the skin microbiota differ in children and teenagers between rural and urban environments. *Sci Rep* **7**, 45651, doi:10.1038/srep45651 (2017).

18 Grice, E. A. *et al.* Topographical and temporal diversity of the human skin microbiome. *Science* **324**, 1190-1192 (2009).

19 Marples, M. J. Life on the human skin. *Scientific American* **220**, 108-115, doi:10.1038/scientificamerican0169-108 (1969).

20 Kong, H. H. & Segre, J. A. Skin microbiome: Looking back to move forward. *J. Invest. Dermatol.* **132**, 933-939, doi:10.1038/jid.2011.417 (2012).

21 Acton, H. W. & Panja, G. Seborrhoeic dermatitis or pityriasis capitis: a lesion caused by the Malassezia ovale. *The Indian Medical Gazette* **62**, 603 (1927).

22 Montes, L. F. & Wilborn, W. H. Anatomical Location of Normal Skin Flora. *Archives of Dermatology* **101**, 145-159, doi:10.1001/archderm.1970.04000020015004 (1970).

23 Lange-Asschenfeldt, B. *et al.* Distribution of bacteria in the epidermal layers and hair follicles of the human skin. *Skin Pharmacol Physiol* **24**, 305-311, doi:10.1159/000328728 (2011).
24 Leeming, J. P., Holland, K. T. & Cunliffe, W. J. The microbial ecology of pilosebaceous units isolated from human skin. *Journal of general microbiology* **130**, 803-807, doi:10.1099/00221287-130-4-803 (1984).
25 Gest, H. The discovery of microorganisms by Robert Hooke and Antoni van Leeuwenhoek, Fellows of The Royal Society. *Notes and Records of the Royal Society* **58**, 187-201, doi:10.1098/rsnr.2004.0055 (2004).
26 Koch, R. In Xth International Congress of Medicine, Berlin. (1890).
27 Shulman, S. T., Friedmann, H. C. & Sims, R. H. Theodor Escherich: The First Pediatric Infectious Diseases Physician? *Clin. Infect. Dis.* **45**, 1025-1029, doi:10.1086/521946 (2007).
28 Hugerth, L. W. & Andersson, A. F. Analysing microbial community composition through amplicon sequencing: from sampling to hypothesis testing. *Frontiers in Microbiology* **8**, 1561 (2017).
29 Ranjan, R., Rani, A., Metwally, A., McGee, H. S. & Perkins, D. L. Analysis of the microbiome: Advantages of whole genome shotgun versus 16S amplicon sequencing. *Biochemical and biophysical research communications* **469**, 967-977, doi:10.1016/j.bbrc.2015.12.083 (2016).
30 Prescott, S. L. History of medicine: Origin of the term microbiome and why it matters. *Human Microbiome Journal* **4**, 24-25, doi:10.1016/j.humic.2017.05.004 (2017).
31 Woese, C. R. & Fox, G. E. Phylogenetic Structure of the Prokaryotic Domain: The Primary Kingdoms. *Proc. Natl. Acad. Sci. U. S. A.* **74**, 5088-5090, doi:10.1073/pnas.74.11.5088 (1977).
32 Nossa, C., Yi-Wei, T. & Zhiheng, P. Pearls and pitfalls of genomics-based microbiome analysis. *Emerging Microbes & Infections* **1**, e45, doi:10.1038/emi.2012.41 (2012).
33 Sogin, M. L. *et al.* Microbial diversity in the deep sea and the underexplored “rare biosphere”. *Proceedings of the National Academy of Sciences* **103**, 12115-12120 (2006).
34 Hugerth, L. W. *et al.* A comprehensive automated pipeline for human microbiome sampling, 16S rRNA gene sequencing and bioinformatics processing. *bioRxiv*, 286526 (2018).
35 Byrd, A. L., Belkaid, Y. & Segre, J. A. The human skin microbiome. *Nat. Rev. Microbiol.* **16**, 143 (2018).
36 O'Leary, N. A. *et al.* Reference sequence (RefSeq) database at NCBI: current status, taxonomic expansion, and functional annotation. *Nucleic Acids Res* **44**, D733-745, doi:10.1093/nar/gkv1189 (2016).
37 Nielsen, H. B. *et al.* Identification and assembly of genomes and genetic elements in complex metagenomic samples without using reference genomes. *Nature biotechnology* **32**, 822-828 (2014).
38 Hughes, J. B., Hellmann, J. J., Ricketts, T. H. & Bohannan, B. J. Counting the uncountable: statistical approaches to estimating microbial diversity. *Applied and environmental microbiology* **67**, 4399-4406, doi:10.1128/aem.67.10.4399-4406.2001 (2001).
39 Lozupone, C. & Knight, R. UniFrac: a new phylogenetic method for comparing microbial communities. *Applied and environmental microbiology* **71**, 8228-8235, doi:10.1128/AEM.71.12.8228-8235.2005 (2005).
40 Grice, E. A. & Segre, J. A. The skin microbiome. *Nat. Rev. Microbiol.* **9**, 244-253, doi:10.1038/nrmicro2537 (2011).
41 Oh, J. *et al.* Biogeography and individuality shape function in the human skin metagenome. *Nature* **514**, 59-64, doi:10.1038/nature13786 (2014).
42 Findley, K. *et al.* Topographic diversity of fungal and bacterial communities in human skin. *Nature* **498**, 367-370, doi:10.1038/nature12171 (2013).
43 Hannigan, G. D. *et al.* Evolutionary and functional implications of hypervariable loci within the skin virome. *PeerJ* **5**, e2959, doi:10.7717/peerj.2959 (2017).
44 Oh, J., Byrd, A. L., Park, M., Kong, H. H. & Segre, J. A. Temporal Stability of the Human Skin Microbiome. *Cell* **165**, 854-866, doi:10.1016/j.cell.2016.04.008 (2016).

45 Moore, P. S. & Chang, Y. Common Commensal Cancer Viruses.(Pearls)(Report). *PLoS Pathogens* **13**, e1006078, doi:10.1371/journal.ppat.1006078 (2017).
46 Bouslimani, A. *et al.* The impact of skin care products on skin chemistry and microbiome dynamics. *BMC biology* **17**, 47, doi:10.1186/s12915-019-0660-6 (2019).
47 Callewaert, C., Hutapea, P., Van de Wiele, T. & Boon, N. Deodorants and antiperspirants affect the axillary bacterial community. *Arch. Dermatol. Res.* **306**, 701-710, doi:10.1007/s00403-014-1487-1 (2014).
48 Allyson, L. B., Yasmine, B. & Julia, A. S. The human skin microbiome. *Nat. Rev. Microbiol.* **16**, doi:10.1038/nrmicro.2017.157 (2018).
49 Shi, B., Leung, D. Y. M., Taylor, P. A. & Li, H. Methicillin-Resistant Staphylococcus aureus Colonization Is Associated with Decreased Skin Commensal Bacteria in Atopic Dermatitis. *J. Invest. Dermatol.* **138**, 1668-1671, doi:10.1016/j.jid.2018.01.022 (2018).
50 Wang, Y. *et al.* Propionic acid and its esterified derivative suppress the growth of methicillin-resistant Staphylococcus aureus USA300. *Benef Microbes* **5**, 161-168, doi:10.3920/bm2013.0031 (2014).
51 Shu, M. *et al.* Fermentation of Propionibacterium acnes, a Commensal Bacterium in the Human Skin Microbiome, as Skin Probiotics against Methicillin-Resistant Staphylococcus aureus. *PLoS One* **8**, doi:10.1371/journal.pone.0055380 (2013).
52 Grice, E. A. & Dawson, T. L. Host–microbe interactions: Malassezia and human skin. *Current Opinion in Microbiology* **40**, 81-87, doi:10.1016/j.mib.2017.10.024 (2017).
53 Yu, Y., Dunaway, S., Champer, J., Kim, J. & Alikhan, A. Changing our microbiome: probiotics in dermatology. *The British journal of dermatology* **182**, 39-46, doi:10.1111/bjd.18088 (2020).
54 Nakatsuji, T. *et al.* Antimicrobials from human skin commensal bacteria protect against Staphylococcus aureus and are deficient in atopic dermatitis. *Sci Transl Med* **9**, doi:10.1126/scitranslmed.aah4680 (2017).
55 Anna, L. C. *et al.* Selective Antimicrobial Action Is Provided by Phenol-Soluble Modulins Derived from Staphylococcus epidermidis, a Normal Resident of the Skin. *J. Invest. Dermatol.* **130**, 192, doi:10.1038/jid.2009.243 (2010).
56 Sugimoto, S. *et al.* Staphylococcus epidermidis Esp degrades specific proteins associated with Staphylococcus aureus biofilm formation and host-pathogen interaction. *Journal of bacteriology* **195**, 1645-1655, doi:10.1128/JB.01672-12 (2013).
57 Lai, Y. *et al.* Activation of TLR2 by a small molecule produced by staphylococcus epidermidis increases antimicrobial defense against bacterial skin infections. *J. Invest. Dermatol.* **130**, 2211-2221, doi:10.1038/jid.2010.123 (2010).
58 Wang, Z., MacLeod, D. T. & Di Nardo, A. Commensal bacteria lipoteichoic acid increases skin mast cell antimicrobial activity against vaccinia viruses. *Journal of Immunology* **189**, 1551-1558, doi:10.4049/jimmunol.1200471 (2012).
59 Yuki, T. *et al.* Activation of TLR2 enhances tight junction barrier in epidermal keratinocytes. *Journal of immunology (Baltimore, Md. : 1950)* **187**, 3230-3237, doi:10.4049/jimmunol.1100058 (2011).
60 Scharschmidt, T. C. *et al.* A Wave of Regulatory T Cells into Neonatal Skin Mediates Tolerance to Commensal Microbes. *Immunity* **43**, 1011-1021, doi:10.1016/j.immuni.2015.10.016 (2015).
61 Naik, S. *et al.* Commensal-dendritic-cell interaction specifies a unique protective skin immune signature. *Nature* **520**, 104-108, doi:10.1038/nature14052 (2015).
62 Fyhrquist, N. *et al.* Acinetobacter species in the skin microbiota protect against allergic sensitization and inflammation. *J Allergy Clin Immunol* **134**, 1301-1309 e1311, doi:10.1016/j.jaci.2014.07.059 (2014).
63 Yang, J.-J. *et al.* Commensal Provokes Immunity to Protect against Skin Infection of Methicillin-Resistant. *International journal of molecular sciences* **19**, doi:10.3390/ijms19051290 (2018).

64 Divyakolu, S., Chikkala, R., Ratnakar, K. S. & Sritharan, V. Hemolysins of Staphylococcus aureus—An Update on Their Biology, Role in Pathogenesis and as Targets for Anti-Virulence Therapy. *Advances in Infectious Diseases* **09**, 80-104, doi:10.4236/aid.2019.92007 (2019).
65 Naik, S. *et al.* Commensal–dendritic-cell interaction specifies a unique protective skin immune signature. *Nature* **520**, 104-108 (2015).
66 Weidinger, S. & Novak, N. Atopic dermatitis. *The Lancet* **387**, 1109-1122, doi:10.1016/S0140-6736(15)00149-X (2016).
67 Weidinger, S., Beck, L. A., Bieber, T., Kabashima, K. & Irvine, A. D. Atopic dermatitis. *Nature reviews. Disease primers* **4**, 1, doi:10.1038/s41572-018-0001-z (2018).
68 Abuabara, K., Yu, A. M., Okhovat, J. P., Allen, I. E. & Langan, S. M. The prevalence of atopic dermatitis beyond childhood: A systematic review and meta-analysis of longitudinal studies. *Allergy* **73**, 696-704, doi:10.1111/all.13320 (2018).
69 Lyons, J. J., Milner, J. D. & Stone, K. D. Atopic Dermatitis in Children. *Immunology and Allergy Clinics of North America* **35**, 161-183, doi:10.1016/j.iac.2014.09.008 (2015).
70 Bieber, T. Mechanisms of disease: Atopic dermatitis. *N. Engl. J. Med.* **358**, 1483-1494, doi:10.1056/NEJMra074081 (2008).
71 Grobe, W., Bieber, T. & Novak, N. Pathophysiology of atopic dermatitis. *JDDG: Journal der Deutschen Dermatologischen Gesellschaft* **17**, 433-440, doi:10.1111/ddg.13819 (2019).
72 Esaki, H. *et al.* Early-onset pediatric atopic dermatitis is TH2 but also TH17 polarized in skin. *The Journal of Allergy and Clinical Immunology* **138**, 1639-1651, doi:10.1016/j.jaci.2016.07.013 (2016).
73 Chan, T. C. *et al.* Atopic dermatitis in Chinese patients shows TH2/TH17 skewing with psoriasiform features. *The Journal of Allergy and Clinical Immunology* **142**, 1013-1017, doi:10.1016/j.jaci.2018.06.016 (2018).
74 Bieber, T. & Prölss, J. in *Clinical and Basic Immunodermatology* 193-206 (2008).
75 Werfel, T. *et al.* Exacerbation of atopic dermatitis on grass pollen exposure in an environmental challenge chamber. *The Journal of Allergy and Clinical Immunology* **136**, 96-103.e109, doi:10.1016/j.jaci.2015.04.015 (2015).
76 Thijs, J. L. *et al.* Moving toward endotypes in atopic dermatitis: Identification of patient clusters based on serum biomarker analysis. *The Journal of Allergy and Clinical Immunology* **140**, 730-737, doi:10.1016/j.jaci.2017.03.023 (2017).
77 Paternoster, L. *et al.* Identification of atopic dermatitis subgroups in children from 2 longitudinal birth cohorts. *J Allergy Clin Immunol* **141**, 964-971, doi:10.1016/j.jaci.2017.09.044 (2018).
78 Tay, A. S. L. *et al.* Atopic dermatitis microbiomes stratify into ecological dermotypes enabling microbial virulence and disease severity. *J. Allergy Clin. Immunol.*, doi:10.1016/j.jaci.2020.09.031 (2020).
79 Tham, E. H., Koh, E., Common, J. E. A. & Hwang, I. Y. Biotherapeutic Approaches in Atopic Dermatitis. *Biotechnology journal* **15**, e1900322, doi:10.1002/biot.201900322 (2020).
80 Totte, J. E. *et al.* Prevalence and odds of Staphylococcus aureus carriage in atopic dermatitis: a systematic review and meta-analysis. *The British journal of dermatology*, doi:10.1111/bjd.14566 (2016).
81 Tauber, M. *et al.* Staphylococcus aureus density on lesional and nonlesional skin is strongly associated with disease severity in atopic dermatitis. *J. Allergy Clin. Immunol.* **137**, 1272-1274.e1273, doi:10.1016/j.jaci.2015.07.052 (2016).
82 Hülpüsch, C. *et al.* Skin pH–dependent Staphylococcus aureus abundance as predictor for increasing atopic dermatitis severity. *Allergy (Copenhagen)*, doi:10.1111/all.14461 (2020).
83 Sakr, A., Brégeon, F., Mège, J.-L., Rolain, J.-M. & Blin, O. Staphylococcus aureus Nasal Colonization: An Update on Mechanisms, Epidemiology, Risk Factors, and Subsequent Infections. *Frontiers in microbiology* **9**, 2419-2419, doi:10.3389/fmicb.2018.02419 (2018).

84 Iwamoto, K., Moriwaki, M., Miyake, R. & Hide, M. Staphylococcus aureus in atopic dermatitis: Strain-specific cell wall proteins and skin immunity. *Allergology International* **68**, 309-315, doi:10.1016/j.alit.2019.02.006 (2019).
85 Miajlovic, H., Fallon, P. G., Irvine, A. D. & Foster, T. J. Effect of filaggrin breakdown products on growth of and protein expression by Staphylococcus aureus. *J. Allergy Clin. Immunol.* **126**, 1184-U1182, doi:10.1016/j.jaci.2010.09.015 (2010).
86 Hata, T. R. *et al.* History of eczema herpeticum is associated with the inability to induce human β-defensin (HBD)-2, HBD-3 and cathelicidin in the skin of patients with atopic dermatitis. *British Journal of Dermatology* **163**, 659-661, doi:10.1111/j.1365-2133.2010.09892.x (2010).
87 Paller, A. S. *et al.* The microbiome in patients with atopic dermatitis. *The Journal of Allergy and Clinical Immunology* **143**, 26-35, doi:10.1016/j.jaci.2018.11.015 (2019).
88 Alexander, H. *et al.* The role of bacterial skin infections in atopic dermatitis: expert statement and review from the International Eczema Council Skin Infection Group. *The British journal of dermatology*, doi:10.1111/bjd.18643 (2019).
89 Tsakok, T., Schulenburg, H., Smith, C., Till, S. & Flohr, C. The Role of Yeast in Atopic Dermatitis Revisited: a Critical Appraisal. *Current Dermatology Reports* **4**, 228-240, doi:10.1007/s13671-015-0123-4 (2015).
90 Clemmensen, O. Treatment of dermatitis of the neck with Ketoconazole in patients with type I sensitivity to pityrosporum orbiclare. *Semin. Dermatol.* **2**, 26-29 (1983).
91 Kennedy, E. A. *et al.* Skin microbiome before development of atopic dermatitis: Early colonization with commensal staphylococci at 2 months is associated with a lower risk of atopic dermatitis at 1 year. *J Allergy Clin Immunol* **139**, 166-172, doi:10.1016/j.jaci.2016.07.029 (2017).
92 Kong, H. D. H. *et al.* Temporal shifts in the skin microbiome associated with disease flares and treatment in children with atopic dermatitis. *Genome Res.* **22**, 850-859, doi:10.1101/gr.131029.111 (2012).
93 Oh, J. *et al.* The altered landscape of the human skin microbiome in patients with primary immunodeficiencies. *Genome Res.* **23**, 2103-2114, doi:10.1101/gr.159467.113 (2013).
94 Totte, J. E. E. *et al.* Nasal and skin microbiomes are associated with disease severity in paediatric atopic dermatitis. *The British journal of dermatology* **181**, 796-804, doi:10.1111/bjd.17755 (2019).
95 Song, L. *et al.* Cheek Microbial Communities Vary in Young Children with Atopic Dermatitis in China. *Dermatology*, 1-10, doi:10.1159/000502469 (2019).
96 Clausen, M. L. *et al.* Association of Disease Severity With Skin Microbiome and Filaggrin Gene Mutations in Adult Atopic Dermatitis. *JAMA Dermatol* **154**, 293-300, doi:10.1001/jamadermatol.2017.5440 (2018).
97 Arrieta, M. C. *et al.* Early infancy microbial and metabolic alterations affect risk of childhood asthma. *Sci Transl Med* **7**, 307ra152, doi:10.1126/scitranslmed.aab2271 (2015).
98 Turnbaugh, P. J. *et al.* The human microbiome project. *Nature* **449**, 804-810, doi:10.1038/nature06244 (2007).
99 Altunbulakli, C. *et al.* Relations between epidermal barrier dysregulation and Staphylococcus species-dominated microbiome dysbiosis in patients with atopic dermatitis. *J Allergy Clin Immunol*, doi:10.1016/j.jaci.2018.07.005 (2018).
100 Leung, D. Y. M. *et al.* The nonlesional skin surface distinguishes atopic dermatitis with food allergy as a unique endotype. *Sci Transl Med* **11**, doi:10.1126/scitranslmed.aav2685 (2019).
101 Fyhrquist, N. *et al.* Microbe-host interplay in atopic dermatitis and psoriasis. *Nat Commun* **10**, 4703, doi:10.1038/s41467-019-12253-y (2019).
102 Ottman, N. *et al.* Microbial and transcriptional differences elucidate atopic dermatitis heterogeneity across skin sites. *Allergy*, doi:10.1111/all.14606 (2020).
103 Hooijmans, C. R. *et al.* SYRCLE's risk of bias tool for animal studies. *BMC Medical Research Methodology* **14**, 43, doi:10.1186/1471-2288-14-43 (2014).

104 Munn, Z. *et al.* Systematic review or scoping review? Guidance for authors when choosing between a systematic or scoping review approach. *BMC Medical Research Methodology* **18**, 143, doi:10.1186/s12874-018-0611-x (2018).
105 Chng, K. R. *et al.* Whole-metagenome profiling reveals distinct population and functional differences in skin microbiomes of individuals predisposed to atopic dermatitis. *British Journal of Dermatology* **174**, e49 (2016).
106 Grice, E. A. *et al.* A diversity profile of the human skin microbiota. *Genome Res.* **18**, 1043-1050, doi:10.1101/gr.075549.107 (2008).
107 Salter, S. J. *et al.* Reagent and laboratory contamination can critically impact sequence-based microbiome analyses. *BMC biology* **12**, 87, doi:10.1186/s12915-014-0087-z (2014).
108 de Goffau, M. C. *et al.* Recognizing the reagent microbiome. *Nat Microbiol* **3**, 851-853, doi:10.1038/s41564-018-0202-y (2018).
109 Hannigan, G. D. *et al.* Culture-Independent Pilot Study of Microbiota Colonizing Open Fractures and Association with Severity, Mechanism, Location, and Complication from Presentation to Early Outpatient Follow-Up. *J. Orthop. Res.* **32**, 597-605, doi:10.1002/jor.22578 (2014).
110 Kuczynski, J. *et al.* Experimental and analytical tools for studying the human microbiome. *Nature Reviews Genetics* **13**, 47-58 (2012).
111 Zeeuwen, P. *et al.* Microbiome dynamics of human epidermis following skin barrier disruption. *Genome Biol.* **13**, 18, doi:10.1186/gb-2012-13-11-r101 (2012).
112 Rosenthal, M., Aiello, A., Larson, E., Chenoweth, C. & Foxman, B. Healthcare workers' hand microbiome may mediate carriage of hospital pathogens. *Pathogens* **3**, 1-13, doi:10.3390/pathogens3010001 (2013).
113 Fierer, N., Hamady, M., Lauber, C. L. & Knight, R. The influence of sex, handedness, and washing on the diversity of hand surface bacteria. *Proc. Natl. Acad. Sci. U. S. A.* **105**, 17994-17999, doi:10.1073/pnas.0807920105 (2008).
114 Callewaert, C. *et al.* IL-4Ralpha Blockade by Dupilumab Decreases Staphylococcus aureus Colonization and Increases Microbial Diversity in Atopic Dermatitis. *J Invest Dermatol* **140**, 191-202.e197, doi:10.1016/j.jid.2019.05.024 (2020).
115 Baurecht, H. *et al.* Epidermal lipid composition, barrier integrity, and eczematous inflammation are associated with skin microbiome configuration. *J Allergy Clin Immunol* **141**, 1668-1676.e1616, doi:10.1016/j.jaci.2018.01.019 (2018).
116 Gooderham, M. J. *et al.* Approach to the Assessment and Management of Adult Patients With Atopic Dermatitis: A Consensus Document. Section II: Tools for Assessing the Severity of Atopic Dermatitis. *J Cutan Med Surg* **22**, 10s-16s, doi:10.1177/1203475418803628 (2018).
117 Severity scoring of atopic dermatitis: the SCORAD index. Consensus Report of the European Task Force on Atopic Dermatitis. *Dermatology* **186**, 23-31, doi:10.1159/000247298 (1993).
118 Chopra, R. *et al.* Relationship between EASI and SCORAD severity assessments for atopic dermatitis. *The Journal of allergy and clinical immunology* **140**, 1708-1710.e1701, doi:10.1016/j.jaci.2017.04.052 (2017).
119 Simpson, E. L., Thompson, M. M. & Hanifin, J. M. Prevalence and Morphology of Hand Eczema in Patients with Atopic Dermatitis. *Dermatitis* **17**, 123-127, doi:10.2310/6620.2006.06005 (2006).
120 Held, E., Skoet, R., Johansen, J. D. & Agner, T. The hand eczema severity index (HECSI): a scoring system for clinical assessment of hand eczema. A study of inter- and intraobserver reliability. *The British journal of dermatology* **152**, 302-307, doi:10.1111/j.1365-2133.2004.06305.x (2005).
121 Diepgen, T. L. *et al.* Hand eczema classification: a cross-sectional, multicentre study of the aetiology and morphology of hand eczema. *British journal of dermatology (1951)* **160**, 353-358, doi:10.1111/j.1365-2133.2008.08907.x (2009).
122 Mukherjee, S. *et al.* Sebum and Hydration Levels in Specific Regions of Human Face Significantly Predict the Nature and Diversity of Facial Skin Microbiome. *Sci Rep* **6**, 36062, doi:10.1038/srep36062 (2016).

123 Mollerup, A., Veien, N. K. & Johansen, J. D. An analysis of gender differences in patients with hand eczema - everyday exposures, severity, and consequences. *Contact Dermatitis* **71**, 21-30, doi:10.1111/cod.12206 (2014).
124 Engebretsen, K. A. *et al.* Changes in filaggrin degradation products and corneocyte surface texture by season. *The British journal of dermatology* **178**, 1143-1150, doi:10.1111/bjd.16150 (2018).
125 Byrd, A. L. *et al.* Staphylococcus aureus and Staphylococcus epidermidis strain diversity underlying pediatric atopic dermatitis. *Sci Transl Med* **9**, doi:10.1126/scitranslmed.aal4651 (2017).
126 Moosbrugger-Martinz, V. *et al.* Initial Evidence of Distinguishable Bacterial and Fungal Dysbiosis in the Skin of Patients with Atopic Dermatitis or Netherton Syndrome. *J Invest Dermatol* **141**, 114-123, doi:10.1016/j.jid.2020.05.102 (2021).
127 Nath, S. *et al.* Dysbiotic Lesional Microbiome With Filaggrin Missense Variants Associate With Atopic Dermatitis in India. *Front Cell Infect Microbiol* **10**, 570423-570423, doi:10.3389/fcimb.2020.570423 (2020).
128 Ogai, K. *et al.* A Comparison of Techniques for Collecting Skin Microbiome Samples: Swabbing Versus Tape-Stripping. *Front Microbiol* **9**, 2362, doi:10.3389/fmicb.2018.02362 (2018).
129 Prast-Nielsen, S. *et al.* Investigation of the skin microbiome: swabs vs. biopsies. *The British journal of dermatology* **181**, 572-579, doi:10.1111/bjd.17691 (2019).
130 Nakatsuji, T. *et al.* The microbiome extends to subepidermal compartments of normal skin. *Nature Communications* **4**, doi:10.1038/ncomms2441 (2013).
131 Marotz, C. A. *et al.* Improving saliva shotgun metagenomics by chemical host DNA depletion. *Microbiome* **6**, 42, doi:10.1186/s40168-018-0426-3 (2018).
132 Zhou, X. *et al.* Differences in the composition of vaginal microbial communities found in healthy Caucasian and black women. *The ISME journal* **1**, 121-133, doi:10.1038/ismej.2007.12 (2007).
133 Glatz, M., Polley, E. C., Simpson, E. L. & Kong, H. H. Emollient therapy alters skin barrier and microbes in infants at risk for developing atopic dermatitis. *J. Invest. Dermatol.* **135**, S31 (2015).
134 Urban, J. *et al.* The effect of habitual and experimental antiperspirant and deodorant product use on the armpit microbiome. *PeerJ* **4**, e1605, doi:10.7717/peerj.1605 (2016).
135 Kong, H. H. *et al.* Temporal shifts in the skin microbiome associated with atopic dermatitis disease flares and treatment. *J. Invest. Dermatol.* **132**, S113 (2012).
136 Chen, Y. E., Fischbach, M. A. & Belkaid, Y. Skin microbiota-host interactions. *Nature* **553**, 427-436, doi:10.1038/nature25177 (2018).
137 Daniel, A., Bonnen, P. E. & Fischetti, V. A. First complete genome sequence of two Staphylococcus epidermidis bacteriophages. *J Bacteriol* **189**, 2086-2100, doi:10.1128/jb.01637-06 (2007).
138 Yamaguchi, T. *et al.* Phage conversion of exfoliative toxin A production in Staphylococcus aureus. *Molecular microbiology* **38**, 694-705, doi:10.1046/j.1365-2958.2000.02169.x (2000).
139 Boldock, E. *et al.* Human skin commensals augment Staphylococcus aureus pathogenesis. *Nat Microbiol* **3**, 881-890, doi:10.1038/s41564-018-0198-3 (2018).
140 Roh, K. H. *et al.* Three cases of Moraxella osloensis meningitis: a difficult experience in species identification and determination of clinical significance. *Journal of Korean medical science* **25**, 501-504, doi:10.3346/jkms.2010.25.3.501 (2010).
141 Alkhatib, N. J., Younis, M. H., Alobaidi, A. S. & Shaath, N. M. An unusual osteomyelitis caused by Moraxella osloensis: A case report. *Int J Surg Case Rep* **41**, 146-149, doi:10.1016/j.ijscr.2017.10.022 (2017).
142 Han, X. Y. & Tarrand, J. J. Moraxella osloensis blood and catheter infections during anticancer chemotherapy: clinical and microbiologic studies of 10 cases. *American journal of clinical pathology* **121**, 581-587, doi:10.1309/qbb3-avcm-gwa3-k1xk (2004).
143 Buchman, A. L., Pickett, M. J., Mann, L. & Ament, M. E. Central venous catheter infection caused by Moraxella osloensis in a patient receiving home parenteral nutrition. *Diagn Microbiol Infect Dis* **17**, 163-166, doi:10.1016/0732-8893(93)90028-6 (1993).

144 Sifri, C. D. *et al.* Moraxella osloensis bacteremia in a kidney transplant recipient. *Transplant international : official journal of the European Society for Organ Transplantation* **21**, 1011-1013, doi:10.1111/j.1432-2277.2008.00727.x (2008).
145 Scholz, C. F. P. & Kilian, M. The natural history of cutaneous propionibacteria, and reclassification of selected species within the genus Propionibacterium to the proposed novel genera Acidipropionibacterium gen. nov., Cutibacterium gen. nov. and Pseudopropionibacterium gen. nov. *Int J Syst Evol Microbiol* **66**, 4422-4432, doi:10.1099/ijsem.0.001367 (2016).
146 Butler-Wu, S. M., Sengupta, D. J., Kittichotirat, W., Matsen, F. A., 3rd & Bumgarner, R. E. Genome sequence of a novel species, Propionibacterium humerusii. *J Bacteriol* **193**, 3678, doi:10.1128/jb.05036-11 (2011).
147 Fitz-Gibbon, S. *et al.* Propionibacterium acnes Strain Populations in the Human Skin Microbiome Associated with Acne. *J. Invest. Dermatol.* **133**, 2152-2160, doi:10.1038/jid.2013.21 (2013).
148 Dekio, I. *et al.* Characterization of skin microbiota in patients with atopic dermatitis and in normal subjects using 16S rRNA gene-based comprehensive analysis. *Journal of Medical Microbiology* **56**, 1675-1683 (2007).
149 Francuzik, W., Franke, K., Schumann, R. R., Heine, G. & Worm, M. Propionibacterium acnes Abundance Correlates Inversely with Staphylococcus aureus: Data from Atopic Dermatitis Skin Microbiome. *Acta Derm Venereol* **98**, 490-495, doi:10.2340/00015555-2896 (2018).
150 Kwon, S. *et al.* Changes in Lesional and Non-lesional Skin Microbiome During Treatment of Atopic Dermatitis. *Acta Derm Venereol* **99**, 284-290, doi:10.2340/00015555-3089 (2019).
151 Glatz, M., Bosshard, P. P., Hoetzenecker, W. & Schmid-Grendelmeier, P. The role of malassezia spp in atopic dermatitis. *Journal of Clinical Medicine* **4**, 1217-1228 (2015).
152 Kong, H. H. *et al.* Temporal shifts in the skin microbiome associated with disease flares and treatment in children with atopic dermatitis. *Genome Res.* **22**, 850-859 (2012).
153 van Smeden, J. & Bouwstra, J. A. Stratum Corneum Lipids: Their Role for the Skin Barrier Function in Healthy Subjects and Atopic Dermatitis Patients. *Curr Probl Dermatol* **49**, 8-26, doi:10.1159/000441540 (2016).
154 Kobayashi, T. *et al.* Dysbiosis and Staphylococcus aureus Colonization Drives Inflammation in Atopic Dermatitis. *Immunity* **42**, 756-766, doi:10.1016/j.immuni.2015.03.014 (2015).
155 Kobayashi, T. *et al.* Dysbiosis and Staphyloccus aureus Colonization Drives Inflammation in Atopic Dermatitis. *Immunity* **42**, 756-766, doi:10.1016/j.immuni.2015.03.014 (2015).
156 Kennedy, E. A. *et al.* Skin microbiome before development of atopic dermatitis: Early colonization with commensal staphylococci at 2 months is associated with a lower risk of atopic dermatitis at 1 year. *J Allergy Clin Immunol*, doi:10.1016/j.jaci.2016.07.029 (2016).
157 Simpson, E. L. *et al.* Emollient enhancement of the skin barrier from birth offers effective atopic dermatitis prevention. *J Allergy Clin Immunol* **134**, 818-823, doi:10.1016/j.jaci.2014.08.005 (2014).
158 Horimukai, K. *et al.* Application of moisturizer to neonates prevents development of atopic dermatitis. *J Allergy Clin Immunol* **134**, 824-830 e826, doi:10.1016/j.jaci.2014.07.060 (2014).
159 Glatz, M. *et al.* Emollient use alters skin barrier and microbes in infants at risk for developing atopic dermatitis. *PLoS One* **13**, e0192443, doi:10.1371/journal.pone.0192443 (2018).
160 Lødrup Carlsen, K. C. *et al.* Preventing Atopic Dermatitis and ALLergies in Children-the PreventADALL study. *Allergy* **73**, 2063-2070, doi:10.1111/all.13468 (2018).
161 Chalmers, J. R. *et al.* Daily emollient during infancy for prevention of eczema: the BEEP randomised controlled trial. *Lancet* **395**, 962-972, doi:10.1016/s0140-6736(19)32984-8 (2020).
162 Bath-Hextall, F. J., Birnie, A. J., Ravenscroft, J. C. & Williams, H. C. Interventions to reduce Staphylococcus aureus in the management of atopic eczema: an updated Cochrane review. *British Journal of Dermatology* **163**, 12-26, doi:10.1111/j.1365-2133.2010.09743.x (2010).
163 Gueniche, A. *et al.* Effects of nonpathogenic gram-negative bacterium Vitreoscilla filiformis lysate on atopic dermatitis: A prospective, randomized, double-blind, placebo-controlled clinical study. *British Journal of Dermatology* **159**, 1357-1363, doi:10.1111/j.1365-2133.2008.08836.x (2008).

164 Volz, T. *et al.* Nonpathogenic bacteria alleviating atopic dermatitis inflammation induce IL-10-producing dendritic cells and regulatory Tr1 cells. *J. Invest. Dermatol.* **134**, 96-104, doi:10.1038/jid.2013.291 (2014).
165 Myles, I. A. *et al.* First-in-human topical microbiome transplantation with Roseomonas mucosa for atopic dermatitis. *JCI insight* **3**, doi:10.1172/jci.insight.120608 (2018).
166 Myles, I. A. *et al.* Therapeutic responses to Roseomonas mucosa in atopic dermatitis may involve lipid-mediated TNF-related epithelial repair. *Sci Transl Med* **12**, doi:10.1126/scitranslmed.aaz8631 (2020).
167 Deng, Y. *et al.* Patients with Acne Vulgaris Have a Distinct Gut Microbiota in Comparison with Healthy Controls. *Acta Derm Venereol* **98**, 783-790, doi:10.2340/00015555-2968 (2018).
168 Nam, J. H. *et al.* Rosacea and its association with enteral microbiota in Korean females. *Exp Dermatol* **27**, 37-42, doi:10.1111/exd.13398 (2018).
169 Abrahamsson, T. R. *et al.* Low diversity of the gut microbiota in infants with atopic eczema. *J. Allergy Clin. Immunol.* **129**, 434-U244, doi:10.1016/j.jaci.2011.10.025 (2012).
170 Watanabe, S. *et al.* Differences in fecal microflora between patients with atopic dermatitis and healthy control subjects. *J. Allergy Clin. Immunol.* **111**, 587-591 (2003).
171 Huang, R. *et al.* Probiotics for the Treatment of Atopic Dermatitis in Children: A Systematic Review and Meta-Analysis of Randomized Controlled Trials. *Front Cell Infect Microbiol* **7**, 392-392, doi:10.3389/fcimb.2017.00392 (2017).
172 Bosman, E. S., Albert, A. Y., Lui, H., Dutz, J. P. & Vallance, B. A. Skin Exposure to Narrow Band Ultraviolet (UVB) Light Modulates the Human Intestinal Microbiome. *Frontiers in microbiology* **10**, 2410-2410, doi:10.3389/fmicb.2019.02410 (2019).
173 Forbes, J. D., Van Domselaar, G. & Bernstein, C. N. The gut microbiota in immune-mediated inflammatory diseases. *Frontiers in Microbiology* **7**, doi:10.3389/fmicb.2016.01081 (2016).
174 Gueniche, A. *et al.* Effects of nonpathogenic gram-negative bacterium Vitreoscilla filiformis lysate on atopic dermatitis: A prospective, randomized, double-blind, placebo-controlled clinical study. *British Journal of Dermatology* **159**, 1357-1363 (2008).
175 Nodake, Y. *et al.* Pilot study on novel skin care method by augmentation with Staphylococcus epidermidis, an autologous skin microbe--A blinded randomized clinical trial. *J Dermatol Sci* **79**, 119-126, doi:10.1016/j.jdermsci.2015.05.001 (2015).
176 Callewaert, C., Knödlseder, N., Karoglan, A., Güell, M. & Paetzold, B. Skin microbiome transplantation and manipulation: Current state of the art. *Computational and structural biotechnology journal* **19**, 624-631, doi:10.1016/j.csbj.2021.01.001 (2021).
177 Shimamori, Y. *et al.* Staphylococcal Phage in Combination with Staphylococcus Epidermidis as a Potential Treatment for Staphylococcus Aureus-Associated Atopic Dermatitis and Suppressor of Phage-Resistant Mutants. *Viruses* **13**, doi:10.3390/v13010007 (2020).
178 Thorsteinsdottir, S. *et al.* Domestic dog exposure at birth reduces the incidence of atopic dermatitis. *Allergy* **71**, 1736-1744, doi:10.1111/all.12980 (2016).
179 Schram, M. E. *et al.* Is there a rural/urban gradient in the prevalence of eczema? A systematic review. *The British journal of dermatology* **162**, 964-973, doi:10.1111/j.1365-2133.2010.09689.x (2010).
180 Hanski, I. *et al.* Environmental biodiversity, human microbiota, and allergy are interrelated. *Proc. Natl. Acad. Sci. U. S. A.* **109**, 8334-8339, doi:10.1073/pnas.1205624109 (2012).
181 Brussow, H. Turning the inside out: The microbiology of atopic dermatitis. *Environ Microbiol*, doi:10.1111/1462-2920.13050 (2015).
182 Lee, H. J. *et al.* Effects of cosmetics on the skin microbiome of facial cheeks with different hydration levels. *Microbiologyopen*, doi:10.1002/mbo3.557 (2017).
183 Masako, K. *et al.* A novel method to control the balance of skin microflora - Part 2. A study to assess the effect of a cream containing farnesol and xylitol on atopic dry skin. *J. Dermatol. Sci.* **38**, 207-213, doi:10.1016/j.jdermsci.2005.01.003 (2005).

184 Williams, H. C. *et al.* The U.K. Working Party's Diagnostic Criteria for Atopic Dermatitis. I. Derivation of a minimum set of discriminators for atopic dermatitis. *The British journal of dermatology* **131**, 383-396 (1994).
185 Meldgaard, M. *et al.* A novel multiplex analysis of filaggrin polymorphisms: a universally applicable method for genotyping. *Clin Chim Acta* **413**, 1488-1492, doi:10.1016/j.cca.2012.06.014 (2012).
186 Bjerre, R. D. *et al.* Effects of sampling strategy and DNA extraction on human skin microbiome investigations. *Sci Rep* **9**, 17287, doi:10.1038/s41598-019-53599-z (2019).

Appendix I: Unpublished study

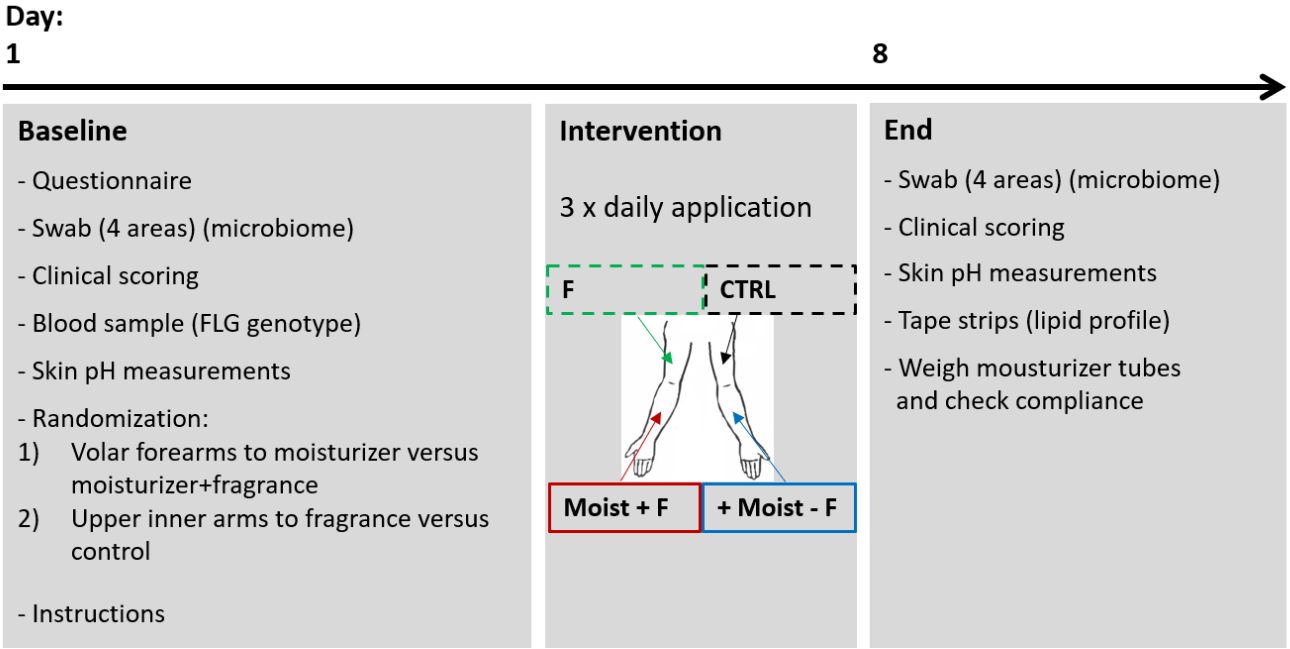
Running title: Changes in the normal and atopic dermatitis skin microbiome in relation to environmental exposures

Introduction and objectives

Atopic dermatitis (AD) is a common disease affecting 20% of children and adults causing major disability. It is caused by genetic and/or environmental factors. In the environment, contact with irritants increases the risk of dermatitis. Hallmarks of AD include a disturbed epidermal-barrier function culminating in dry skin, relapsing skin inflammation and microbial skin dysbiosis with extensively *S. aureus* colonization, which adversely affects disease severity[181]. Using next generation sequencing, this dysbiosis has been shown to be more extensive, involving the bacteriome, mycobiome and virome (manuscript III), and the microbiome is anticipated an important role in future diagnostics and treatment of skin diseases. However, interventional studies are warranted.

Moisturizers are used as standard treatment of dermatitis and are also used by many healthy individuals. Using moisturizers has been shown to increase hydration level in the skin alongside with effecting the microbiome; increased bacterial diversity, relative abundance of the genus *Ralstonia* and enrich metabolic functions related to lipid metabolism, replication and repair as well as other categories[182]. Sebum and hydration levels are predictors of microbiome composition[122] and the specific composition of epidermal lipids strongly affects bacterial colonization[115]. Furthermore, the ingredients of the specific moisturizers are important for shaping the skin as habitat for microbial growth[47]. A study by Myles and colleagues showed that a select number of emollients had differences in growth inhibition of cultured *S. aureus* and the gram negative bacteria *Roseomonas mucosa*, which has a favorable effect on severity of AD[165]. This indicates that variation in topical products applied could exacerbate dermatitis conditions, e.g. by creating a microenvironment more favorable for *S. aureus* growth.

Fragrance ingredients are frequently used in moisturizers. Some plant derived fragrance ingredients, such as farnesol, are known to have antimicrobial actions. Farnesol has previously been shown to inhibit growth of *S. aureus* selectively[183]. Using farnesol as a model compound of fragrances with antimicrobial action, we have conducted a clinical prospective within-subject study, where bilateral symmetrical areas on subject's arms were randomized to interventions (Figure of study setup below), to test effects on the whole skin microbiome and how addition of farnesol to a moisturizer impacts the microbiome on healthy and AD skin.



Study setup

Methods

This study was approved by the ethics committee of the Capital Region of Denmark (H-18058392) and all participants gave informed consent.

Study participants and intervention

From September 2020 to XXXX (still running at the time of writing), we enrolled a Caucasian population with AD (N=15) with current dermatitis and a history starting in childhood and healthy age and sex matched controls (N=15). AD had been diagnosed by a physician and confirmed by the UK Working Party Diagnostic Criteria[184] at inclusion. All subjects met at the Hospital twice (Figure of study setup). At the first visit, they were characterized by demographic data, treatment, co-morbidities, FLG mutations (R501X, 2282del4, and R2447X) and exposures from the environment, e.g. application of skin care products. Four 5x10 cm^2 areas on their arms were randomized to moisturizer (Doublebase Gel), moisturizer+farnesol (0.1%), farnesol (0.1%) or control; the moisturization areas by concealed and double-blinded randomization. The four areas were clinically assessed, swabs were taken for microbiome analyses and pH measured. The subjects were instructed to apply a thin covering layer of 0.2 mL moisturizer (-/+ farnesol) and 50 µL farnesol three times daily for one week, with a farnesol exposure of 2.6 $mg/cm^2/day$. At the final visit, tape strips were taken for analyses of skin lipids. Cases with AD were furthermore assessed by Eczema Area and Severity Index (EASI). Exclusion criteria included fragrance allergy, active infections, use of antibiotics, probiotics and fungicides within the past four weeks and for healthy volunteers a history of eczema. 7 days prior entering the study and during the study period, subjects were instructed not to use chlorinated pools, sauna etc. and avoid fragrance

and topicals on their arms. When entering the study, subjects were instructed to replace soaps and shampoos with provided fragrance-free products and avoid all exposures on test areas not related to the study.

Sampling, DNA extraction and sequencing

Skin pH were measured with the Mettler-Toledo Seven2Go pH/mV meter S2 with a surface-probe, using the mean value of triplet measurements. From blood samples, genomic DNA was purified and typed for the FLG loss-of-function mutations R501X, 2282del4 and R2447X as described previously[185]. Skin samples were collected using eSwabs as described previously[186]. DNA was extracted using DNeasy PowerSoil Pro Kit (QIAGEN) according to manufactures' protocol.

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