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Value and pitfalls of skin microbiome research in atopic diseases

Investigation of the bacteria-microenvironment interaction

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Graphical abstract

High quality next-generation sequencing data for analysis of the skin microbiome in the context of atopic dermatitis

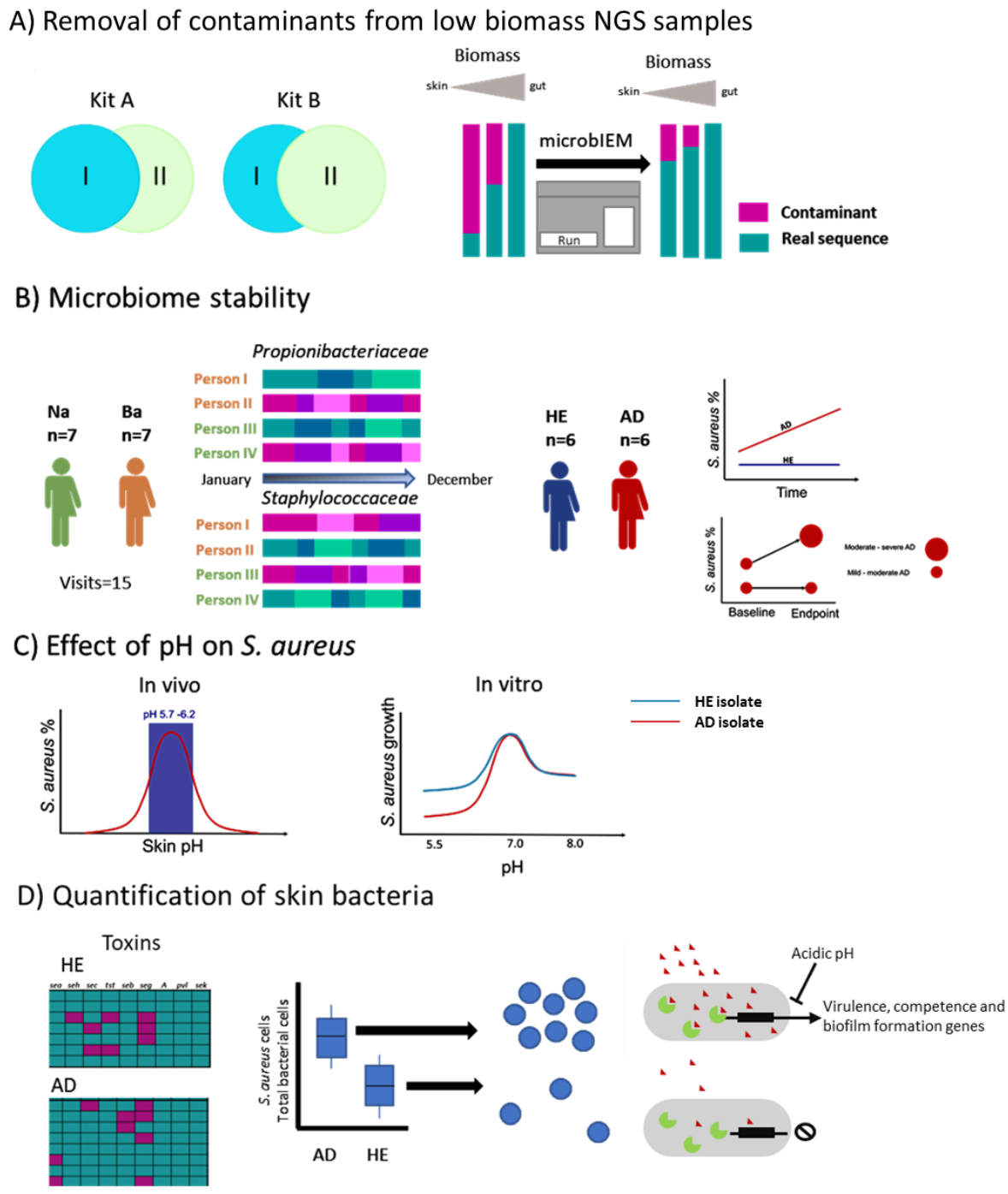

Figure 1 Graphical abstract

Overview of the different projects addressed within the scope of this thesis. Contaminants were identified and removed from next-generation sequencing data (A) in order to investigate skin microbiome stability in healthy and atopic individuals over the course of a year and upon pH challenge (B). *Staphylococcus aureus* pH behavior *in vivo* and *in vitro* (C), toxin patterns, and absolute bacterial and *S. aureus cell* numbers were determined and compared between healthy and atopic individuals (D), within the context of quorum sensing.

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Abstract

Background: In recent years, the prevalence of atopic diseases has been on the rise, especially in westernized countries. A skin barrier disruption as observed in atopic dermatitis (AD) is believed to be the entrance door for the subsequent development of allergic rhinitis (AR) and asthma, the so-called atopic march. Therefore, the skin barrier is an important leverage point in the fight against atopic diseases. The skin barrier defects in AD include mutations in skin barrier genes, higher skin pH and a dysbiosis in the skin microbiome, often involving high relative frequencies of the opportunistic bacterium *Staphylococcus aureus*. *S. aureus* is capable of producing toxins which further irritate AD skin. Interestingly, these factors are all highly interconnected. Mutations in the skin barrier gene filaggrin indirectly lead to an increase in skin pH towards a more neutral pH which is in turn the growth optimum of *S. aureus*. However, the causality between these factors has yet to be completely revealed. Skin microbiome analyses were previously hindered by the susceptibility of low biomass samples to contaminants.

Hypothesis and Aims: We hypothesized that the choice of the DNA extraction kit greatly impacts the results of skin microbiome analyses due to inherent kit contaminants. The primary aim of this thesis was the establishment of a user-friendly widely usable tool for high-quality skin microbiome data analysis by dataset specific removal of putative contaminants based on negative controls. This tool was subsequently used for the analysis of the short- and long-term stability of the skin microbiome in human studies. We hypothesized that the skin microbiome is influenced by the seasons due to varying temperature, humidity, and UV-exposure all of which influence the microenvironmental niche of the skin inhabitants. Furthermore, we considered that the skin microbiome can actively be transformed by applying acidic and basic emollients. It was thought that *S. aureus* is adapted to the microenvironment it is isolated from, such that *S. aureus* from healthy individuals can tolerate more acidic environments. Additionally, *S. aureus* isolates from AD patients were hypothesized to be more pathogenic as compared to isolates from healthy individuals. The pathogenicity of *S. aureus* is regulated via the quorum sensing system which is amongst other factors, activated at high cell density and neutral pH. Consequently, we hypothesized that not only the *S. aureus* relative frequency, but also the absolute cell numbers are increased and play a role in AD pathogenesis. Changing the skin microbiome or skin microenvironment, thus hindering *S. aureus* colonization could be a putative personalized treatment option in the context of AD.

Methods: Skin microbiome analyses were performed by 16S rRNA amplicon next-generation sequencing (NGS). The effect of different DNA extraction kits was evaluated by taking adjacent

skin swabs which were then extracted with two different kits but otherwise processed the same. The tool for contaminants removal was developed in R shiny, and filtering efficiency was tested by using a mock community with a defined bacterial composition. To evaluate the annual skin microbiome stability, skin swabs were taken at 15 visits throughout one year from seven non-allergic and seven birch-allergic participants. Furthermore, the skin microbiome of six healthy and six AD participants was challenged with an emollient of pH 5.5 on one bodyside and pH 8.5 on the other bodyside over eight weeks. Weekly sampling, physiological and physician's assessment of the skin were performed. *S. aureus* isolates were taken from the skin and nose of healthy and AD participants and cultivated at different pH levels to assess the adaptation of the isolates to different environments. Furthermore, the pathogenicity of the different strains was assessed via PCR of the most common *S. aureus* toxins. The absolute number of 16S copies as proxy for number of bacteria, *Staphylococcus* spp. and *S. aureus* cell counts were determined via qPCR and also via spike-in of a species alien to the human microbiome in NGS data.

Results: The data presented in this thesis clearly shows that the results of skin microbiome analysis are greatly influenced by the choice of the DNA extraction kit due to kit-inherent contaminants. The tool microbIEM was successfully developed for dataset specific removal of contaminants. In a human study we were able to show that the skin microbiome was stable over one year throughout the four seasons. Interestingly, two different 'epitypes' were observed irrespective of the health-status (non-allergic, birch allergic). These epitypes were either *Staphylococcaceae* or *Propionibacteriaceae* dominated and relatively stable over one year. Whereas the skin microbiome of healthy individuals remained stable upon emollient application, in AD patients the *S. aureus* relative and absolute abundance was found to increase between the skin pH levels of 5.7 and 6.2. Interestingly though, the emollient pH was not associated with changes of the skin microbiome. However, emollient pH induced low-level changes in the skin pH, especially in AD patients. Strikingly, the baseline abundance of *S. aureus* was predictive for worsening of AD severity. When comparing *S. aureus* isolates from healthy individuals and AD patients *in vitro*, it was found that isolates taken from healthy were more adapted to an acidic environment. Interestingly, no difference between the toxin patterns of isolates taken from healthy or AD individuals were seen.

Conclusion: In summary, the choice of DNA extraction kit and removal of contaminants are crucial for low biomass microbiome analysis as the skin. With microbIEM, we developed a user-friendly tool to remove dataset specific contaminants. Especially the healthy skin microbiome is stable and individual-specific whereas the skin microbiome of AD is less stable.

However, influencing the skin microbiome via the microenvironment by targeting the skin pH was not successful in this study. Since isolates from AD patients are less adapted to an acidic environment than those from healthy controls, it would still be a putative therapeutic target. Reducing the number of *S. aureus* cells inhabiting AD patients could inactivate the quorum sensing system and therefore also the pathogenicity of the isolates. Further functional analysis of the quorum sensing system inhibition should be performed in future as a putative treatment option.

Zusammenfassung

Hintergrund: In den letzten Jahren ist die Prävalenz von atopischen Erkrankungen vor allem, in westlichen Ländern gestiegen. Eine Barrierestörung, wie sie bei Neurodermitis beobachtet wird, gilt als Eintrittspforte für die nachfolgende Entwicklung von Heuschnupfen und Asthma, dem sogenannten atopischen Marsch. Folglich ist die Förderung einer intakten Hautbarriere ein wichtiger Ansatzpunkt im Kampf gegen atopische Erkrankungen. Die beobachteten Barrierestörungen bei Neurodermitis schließen genetische Defekte in Barriere Genen, einen höheren Haut pH-Wert und eine Dysbiose im Hautmikrobiom, insbesondere dem erhöhten Vorkommen von *Staphylococcus aureus* mit ein. *S. aureus* ist ein opportunistisches Bakterium, welches Toxine exprimieren kann, die wiederum die Haut von Neurodermitikern irritieren können. Interessanterweise sind diese Faktoren stark miteinander verknüpft. Mutationen in dem Barriere Gen Filaggrin führen indirekt zu einem höheren, neutralen Haut pH-Wert, welcher wiederum von *S. aureus* bevorzugt wird. Trotz des immensen Forschungsinteresses an Neurodermitis konnten die Kausalitäten zwischen den Faktoren noch nicht vollständig enthüllt werden. Im Gegensatz zur etablierten Stuhlmikrobiom Analyse werden Hautmikrobiomanalysen durch die Anfälligkeit von Proben mit wenig Ausgangsmaterial für Kontaminanten erschwert.

Hypothesen und Ziele: Es wurde vermutet, dass die Wahl des DNA-Extraktionskits die Ergebnisse von Hautmikrobiomanalysen durch Kit-spezifische Kontaminanten stark beeinflussen. Daher war ein primäres Ziel dieser Arbeit die Entwicklung eines benutzerfreundlichen Programms zur Datensatz spezifischen Entfernung von mutmaßlichen Kontaminanten basierend auf Negativkontrollen für qualitativ hochwertige Hautmikrobiomdaten. Dieses Programm wurde anschließend für die Analyse der Kurz- und Langzeitstabilität des Hautmikrobioms verwendet. Es wurde angenommen, dass das Hautmikrobiom durch die Jahreszeiten mit unterschiedlicher Temperatur, Luftfeuchtigkeit und UV-Strahlung das Mikroumfeld der Haut und deren Bewohner verändert. Außerdem wurde in

Betracht gezogen, dass das Hautmikrobiom durch das kontinuierliche Auftragen von sauren und basischen Cremes aktiv verändert werden kann. Des Weiteren stellten wir die Hypothese auf, dass *S. aureus* Isolate von Gesunden besser an saure pH Bedingungen angepasst sind. Zudem wurde eine höhere Pathogenität bei Isolaten von Neurodermitis Patienten vermutet. Da die Pathogenität unter anderem durch Quorum Sensing reguliert wird, vermuteten wir, dass nicht nur die relative Verteilung, sondern auch die absolute Besiedelung mit *S. aureus* bei Neurodermitis Patienten erhöht ist. Die Veränderung des Hautmikrobioms oder der Mikroumgebung zu *S. aureus* inhibierenden Bedingungen wäre ein möglicher personalisierter, therapeutischer Ansatz zur Behandlung von Neurodermitis.

Methoden: Hautmikrobiomanalysen wurden durch 16S rRNA Amplikon-Sequenzierung durchgeführt. Der Effekt von verschiedenen DNA Extraktionskits wurde durch die Probennahme an benachbarten Haustellen und DNA Extraktion mit zwei verschiedenen Kits aber ansonsten gleicher Verarbeitung evaluiert. Ein Programm zur Entfernung von Kontaminanten wurde in R shiny programmiert und mit Hilfe einer Mock community mit definierter Bakterienzusammensetzung getestet. Um die saisonalen Unterschiede des Hautmikrobioms zu bewerten wurden über ein Jahr verteilt an 15 Terminen von sieben Nicht-Allergikern und sieben Birken-Allergikern Proben genommen. Außerdem wurde versucht, das Hautmikrobiom von sechs Gesunden und sechs Neurodermitis Patienten aktiv mit Hilfe einer Creme mit pH 5.5 auf einer, und pH 8.5 auf der anderen Körperseite zu verändern, die zweimal täglich über acht Wochen aufgetragen wurde. Wöchentlich wurden Proben genommen und die Hautphysiologie und Schweregrad der Neurodermitis beurteilt. *S. aureus* Isolate wurden von Gesunden und Neurodermitis Patienten von der Haut und aus der Nase gewonnen und in Verschiedenen pH Bedingungen kultiviert. Zudem wurde die Pathogenität der Isolate durch PCR der geläufigen Toxine bestimmt. Die absolute Zahl der 16S Kopien als Annäherung an die Gesamtbakterienzahl, *Staphylococcus* spp. und *S. aureus* Zellen wurde via qPCR bestimmt. Zusätzlich wurde eine absolute Quantifizierung der NGS Daten durch den Zusatz von *Imtechella halotolerans* angestrebt, welcher nicht im menschlichen Mikrobiom vorkommt.

Ergebnisse: Die Daten dieser Arbeit zeigen eindeutig, dass Hautmikrobiom Ergebnisse aufgrund von Kit-spezifischen Kontaminanten stark durch die Wahl des DNA-Extraktionskits beeinflusst werden. Das entwickelte Programm microbIEM konnte erfolgreich für die Eliminierung von Datensatz-spezifischen Kontaminanten angewendet werden. Eine Studie zeigte, dass das Hautmikrobiom über die Jahreszeiten stabil bleibt. Interessanterweise konnten unabhängig des Gesundheitsstatus (Nicht-Allergiker, Birken-Allergiker) zwei verschiedene

‚Epitypen' identifiziert werden. Diese Epitypen waren entweder *Propionibacteriaceae* oder *Staphylococcaceae* dominiert und relativ stabil über die Zeit. Während das gesunde Hautmikrobiom trotz Creme Applikation stabil blieb, war das Hautmikrobiom von Neurodermitis Patienten instabil; das relative und absolute Vorkommen von *S. aureus* stieg über den Zeitverlauf im pH Bereich von pH 5.7 bis 6.2 an. Der pH der Creme hatte jedoch keinen Einfluss auf das Hautmikrobiom, während vor allem bei Neurodermitis Patienten eine geringfügige Verschiebung des pH-Wertes messbar war. Interessanterweise war das Vorkommen von *S. aureus* vor Behandlungsbeginn ein Prädiktor für die Verschlechterung des Schweregrades bei Neurodermitis Patienten. Isolate von Gesunden waren ebenfalls besser an eine saure Mikroumgebung angepasst. Interessanterweise war kein Unterschied in den Toxin Mustern zwischen Isolaten von Gesunden und Neurodermitikern erkennbar.

Fazit: Zusammenfassend ist die Wahl des DNA-Extraktionskits und das Entfernen von Kontaminanten essenziell für Mikrobiomanalysen mit wenig Ausgangsmaterial wie der Haut. microbIEM ist ein benutzerfreundliches Programm für Datensatz-spezifische Reduktion von Kontaminanten. Insbesondere in gesunder Haut ist das Mikrobiom stabil und Individuum spezifisch. Das Hautmikrobiom von Neurodermitikern ist weniger stabil, konnte jedoch in dieser Studie nicht über Mikroumgebung Haut pH verändert werden. Nichtsdestotrotz könnte dieser therapeutische Ansatz gelingen, da insbesondere *S. aureus* Isolate von Neurodermitikern weniger an saure pH Bedingungen angepasst sind. Die Reduktion von *S. aureus* Zellen, die Neurodermitiker kolonisieren, könnte ein Weg sein, das Quorum Sensing System und somit die Pathogenität zu inhibieren. Weitere funktionale Analysen in diesem Bereich sollen in naher Zukunft erfolgen.

Abbreviation

Abbreviation	Full term
Ac	Antecubital fossa
AD	atopic dermatitis
Agr	accessory gene regulator
AMP	antimicrobial peptides
AR	allergic rhinitis
ASV	amplicon sequence variants
AUC	area under the curve
Ba	birch-allergic
BSA	Bovine serum albumin
Cfu	colony forming units
Cq	quantity cycles
DC	dendritic cell
DNA	Deoxyribonucleic acid

dNTPs	Deoxynucleotide Triphosphates
Fem	Aminoacyltransferases
Filaggrin	filament aggregating protein
FLG	filaggrin
HE	healthy
IgE	Immunglobulin E
IL	Interleukin
LS	lesional
mecA	penicillin-binding protein 2
Na	non-allergic
NGS	next generation sequencing
NL	non-lesional
NMF	Natural moisturizing factors
OD	optical density
OTU	operational taxonomic unit
PAMPs	pathogen associated molecular patterns
PCA	pyrroliodone-5-carboxylic acid
PCR	polymerase chain reaction
pH	*potentia Hydrogenii*
PRR	pattern recognition receptor
Pvl	Panton-Valentine leukocidin
qPCR	quantitative PCR
QS	Quorum sensing
RT	room temperature
SA	superantigens
SB	stratum basale
SC	stratum corneum
SE (A-I)	staphylococcal enterotoxin (A-I)
SEl	staphylococcal enterotoxin like
SG	stratum granulosum
SS	stratum spinosum
TEWL	transepidermal waterloss
Th-1 cell	Type 1 T-helper cell
Th-2 cell	Type 2 T-helper cell
TLR	toll-like receptors
trans-UCA	trans-urocanic acid
TSST-1	toxic shock syndrome toxin1
TST-1	toxic shock syndrome toxin1
UV	ultraviolet
V	hypervariable regions
ZO-1	zona-occludin

1.0 Introduction

1.1 The atopic march as an epidemic

Atopic diseases are associated with high individual and social burdens and are currently on the rise. The most common atopic diseases include allergic rhinitis (AR), atopic eczema (atopic dermatitis, AD), asthma, and certain food allergies (Ring, 2006; Thomsen, 2015). The word "atopy" derived from the Greek word *atopos* which can be translated to "out of place" and refers to the disturbed immune system. These diseases are unified by an immunological hypersensitive reaction of Immunoglobulin E (IgE) or a local adaptive immune response by Type 2 T-helper 2 (Th-2) cells towards innoxious environmental substances, for example from pollen or food (Stephen J. Galli, Tsai, & Piliponsky, 2008; Kubo, Morita, Sugita, & Akdis, 2017). Repetitive exposure to the allergen can lead to chronic inflammation, characterized by innate and adaptive immune mediators which can result in functional and structural changes in the affected epithelium (Stephen J. Galli et al., 2008). AD in infancy is often the first disease in an atopic career, sequentially followed by other Th-2 mediated diseases as AR and asthma, also known as the atopic march (Figure 2) (Bantz, Zhu, & Zheng, 2014; Dharmage et al., 2014).

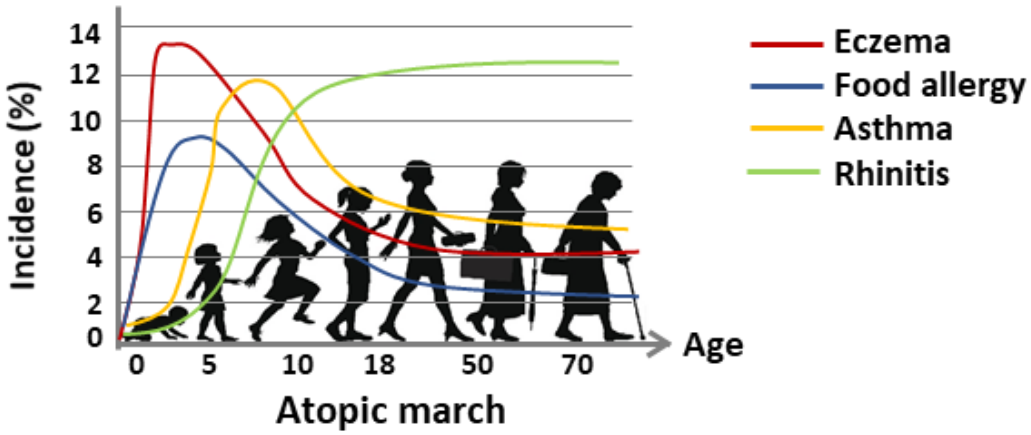


Figure 2 The atopic march

Schematic drawing of the atopic march model. Atopic dermatitis peaks in early infancy and opens the door for other atopic diseases as allergic rhinitis, asthma, and food allergies in adulthood, especially in severe atopic dermatitis cases in infancy. Figure adapted from (Davidson et al., 2019) and https://en.uit.no/forskning/forskningsgrupper/gruppe?p_document_id=345695 [01.09.2020].

In westernized countries, allergies are 20 times more prevalent than in low income countries (D. Strachan et al., 1997). The westernized lifestyle tremendously changed our environment, lifestyle, and diet, leading to more indoor activities and less exposure to environmental biodiversity (Lambrecht & Hammad, 2017; D. Strachan et al., 1997). Already in 1989, Strachan proposed the "hygiene hypothesis", stating a correlation between reduced household size and

hay fever incidence (D. P. Strachan, 1989). This hypothesis was further developed into the "biodiversity hypothesis" by Haahtela, according to which reduced macro-and micro diversity is correlated with reduced indigenous microbiome diversity (Haahtela et al., 2013; von Hertzen, Hanski, & Haahtela, 2011). Similarly, the "old friends' hypothesis" links the loss of symbiotic parasites and bacteria to the increase of allergic diseases (G. A. W. Rook, 2010; Graham A. W. Rook, Lowry, & Raison, 2013). The exposure to pathogens inducing the Type 1 T helper cells (Th-1) response is lost in the urbanized environment, resulting in a Th-1/Th-2 imbalance (Holgate & Broide, 2003; Romagnani, 2000, 2004). However, also other factors occurring alongside with the westernization accelerate susceptibility to allergic diseases. Among others, psychological stress, an unhealthy diet and a lack of physical exercises have been shown to induce a higher susceptibility to allergic diseases (Corbo et al., 2008; Dave, Xiang, Rehm, & Marshall, 2011; Ellwood et al., 2013; Hersoug & Linneberg, 2007; Reznik, 2013; R. J. Wright et al., 2004). Furthermore, high air pollution and climate change on the one hand increase the prevalence and severity of allergic diseases. On the other hand, these factors influence the airborne allergens by a longer pollination period resulting in shorter allergen-free seasons (D'Amato et al., 2015; Fotiou, Damialis, Krigas, Halley, & Vokou, 2011; Schiavoni, D'Amato, & Afferni, 2017; Ziello et al., 2012).

1.2 Atopic dermatitis – general aspects

AD is a chronic relapsing inflammatory skin disease which is currently on the rise worldwide. Predominantly, children are affected with a prevalence up to 15-20% in Europe, whereas the disease only persists to adulthood in 1-5% of the population (Asher et al., 2006; Barbarot et al., 2018). Typical AD symptoms are reoccurring reddened, dry, and itchy skin patches, typically on the folds of arm and knee and can even lead to sleep loss (Jeon et al., 2017). Due to the high personal and socioeconomic burden, AD is in the focus of current dermatologic research. However, the pathogenesis of AD involves a complex interplay between genetic, immunologic, and environmental factors, and is not yet fully understood. The multifactorial disease can be stratified into different endophenotypes, especially separating an extrinsic form with- and an intrinsic type without IgE manifestation (Novak & Bieber, 2003; Nutten, 2015; Tokura, 2010; Werfel et al., 2016). Furthermore, the extrinsic form is associated with loss-of-function mutations in skin barrier genes (Weidinger et al., 2007). There are multiple subgroups in between, which can be separated by the age of disease onset, the severity, ethnicity of the patients and certain genetic traits. However, a clear separation between the subgroups is lacking to date. The identification of individual or subgroup specific biomarkers is essential for the development of personalized medicine for the complex disease as until now AD is often treated

as one disease, neglecting the high numbers of non-responders to conventional treatments (Bieber et al., 2017; Breiteneder et al., 2019; Roesner, Werfel, & Heratizadeh, 2016; Werfel et al., 2016).

Even though the disease is multi-faceted, AD patients are unified by having corrupting barrier defects of the skin (Agrawal & Woodfolk, 2014). The perturbed skin barrier, especially in children with early onset, severe extrinsic AD is a strong risk factor of entering the atopic march (Schäfer et al., 1999; S. Weidinger et al., 2006).

1.3 Skin barrier

The skin is not only our largest organ but also our protective barrier against the environment (Swann, 2010). The skin barrier is a connected network consisting of the components skin microbiome and chemical, physical, immunologic and neurologic factors (Eyerich, Eyerich, Traidl-Hoffmann, & Biedermann, 2018; Kanwar, 2018). If any part of the skin barrier is disturbed, skin diseases as AD, and psoriasis can develop (Hellings & Steelant, 2020; Sano, 2015). In the following paragraph, the components of the healthy skin barrier are explained in more depth.

1.3.1 Physical barrier

Primarily, the skin is a physical barrier of defense consisting of barrier epithelial cells, protecting our body from the environment. The skin consists of three layers, the epidermis, dermis and hypodermis (Zaidi & Lanigan, 2010). The outermost layer of the skin is the epidermis, forming a 100 to 150 µm thick, waterproof barrier which can again be divided into four layers: the stratum basale (SB), stratum spinosum (SS), stratum granulosum (SG), and stratum corneum (SC) (Kanitakis, 2002; Menon, 2002). Keratinocytes represent the major cell type of the epidermis (Figure 3), and the keratinocyte stem cell is located in the basal layer where it is dividing. Keratinocytes are connected via transmembrane proteins like junction adhesion proteins, claudins and occludins (Niessen, 2007). These are connected in the inside of the cell with scaffolding proteins as zonula occludins 1-3 (ZO-1-3), MUPP1 and MAGI (Niessen, 2007). While aging, cells undergo a differentiation process called cornification, which results in terminally differentiated corneocytes with highly crosslinked N^{ε}-(γ-glutamyl)lysine bonds between structural proteins of the cornified envelope and accumulate specific lipids on the outside (Candi, Schmidt, & Melino, 2005; Eckhart, Lippens, Tschachler, & Declercq, 2013). The lipids are mainly ceramides, free fatty acids and cholesterol and prevent excessive waterloss and penetration of environmental compounds due to their hydrophobicity (Jungersted, Hellgren, Jemec, & Agner, 2008; van Smeden & Bouwstra, 2016). The corneocyte

itself mainly consists of the aggregated **fil**ament **agg**regating prote**in** (filaggrin) barrier protein, ensuring the mechanical integrity of the skin (Kezic & Jakasa, 2016). During the differentiation process, cells move up in the skin layers so that corneocytes form a physical barrier on the outside (Thakur, Batheja, Kaushik, & Michniak, 2009).

1.3.2 Chemical barrier

The chemical skin barrier consists of a moist and acid mantle. Its maintenance is crucial, as the healthy cutaneous microenvironment inhibits growth of pathogens and consequently ensures skin health (Eyerich et al., 2018). Natural moisturizing factors (NMF) such as amino acids, lipids, lactate, urea and electrolytes are found mainly in the SC (Ali & Yosipovitch, 2013; Verdier-Sévrain & Bonté, 2007). Interestingly, NMF are formed by and dependent on the degradation of the histidine-rich filaggrin protein (Rawlings & Harding, 2004). The cutaneous pH is the second pillar of the chemical skin barrier. Generally, the healthy skin pH is acidic. However, the skin pH differs between individuals, skin site and studies between pH 4 and 6 (Lambers, Piessens, Bloem, Pronk, & Finkel, 2006). Whereas low skin pH is associated with low scaling and high hydration levels, a higher skin pH is connected to skin barrier dysfunction and decreased SC integrity (Ali & Yosipovitch, 2013; J.-P. Hachem et al., 2003). Since pH regulates the activity of enzymes, the formation of lipids and cohesion proteins and antimicrobial activity, the maintenance of an acidic skin pH is crucial. Again, filaggrin degradation plays an important role in the maintenance of an acidic skin pH as the organic acids trans-urocanic acid (trans-UCA) and pyrroliodone-5-carboxylic acid (PCA) reduce the skin pH (McAleer & Irvine, 2013). Low skin pH is associated with higher skin microbiome diversity and enhanced growth of the commensal *Staphylococcus epidermidis* (Peter M. Elias, 2007; Korting, Hübner, Greiner, Hamm, & Braun-Falco, 1990).

1.3.3 Immune barrier

The immune system consists of the innate and the adaptive immune system and both play a role in the immune barrier of the skin. An important part of the innate immunity are antimicrobial peptides (AMPs) which are found ubiquitously and are produced among others by keratinocytes (Diamond, Beckloff, Weinberg, & Kisich, 2009; Herman & Herman, 2019). These small peptides are active against a variety of gram-positive and negative bacteria, fungi and viruses (Zhang & Gallo, 2016). Furthermore, pattern recognition receptors can recognize pathogen associated molecular patterns (PAMPs) and contribute to their elimination by activating microbicidal and pro-inflammatory responses. There are four subfamilies of pattern recognition receptor (PRR) including toll-like receptors (TLR), nucleotide-binding oligomerization domain

and leucine rich repeats, which all recognize different PAMPS as lipopolysaccharides from gram-negative and lipoteichoic acids from gram-positive bacteria (Amarante-Mendes et al., 2018; McInturff, Modlin, & Kim, 2005). Subsequently, the innate immune response leads to the activation of the pathogen specific adaptive immune response (Alberts B, 2002). The epidermis is mainly populated with specific dendritic cells (DCs), the Langerhans cells, whereas deeper cell layers of the dermis are occupied with DCs, macrophages and T-cells (Matejuk, 2018) (Figure 3). Langerhans cells (LS) promote a tolerogenic response in the absence of danger signal, whereas pathogens which reach the deeper layers promote an inflammatory response by DCs (Dubois et al., 1999; Matejuk, 2018; Shklovskaya et al., 2011). In combination with danger signals as *S. aureus* cell wall teichoic acid, LC can induce inflammatory responses through langerin (van Dalen et al., 2019). Mast cells in the upper dermis are also protecting from infections, but also contain histamine, typically known from allergies (S. J. Galli & Tsai, 2010). Th1, Th2 and Th17 cells are important effector cells in allergic and inflammatory skin diseases and protect from pathogenic microbes as *Staphylococcus aureus* (B. S. Kim, Wojno, & Artis, 2013).

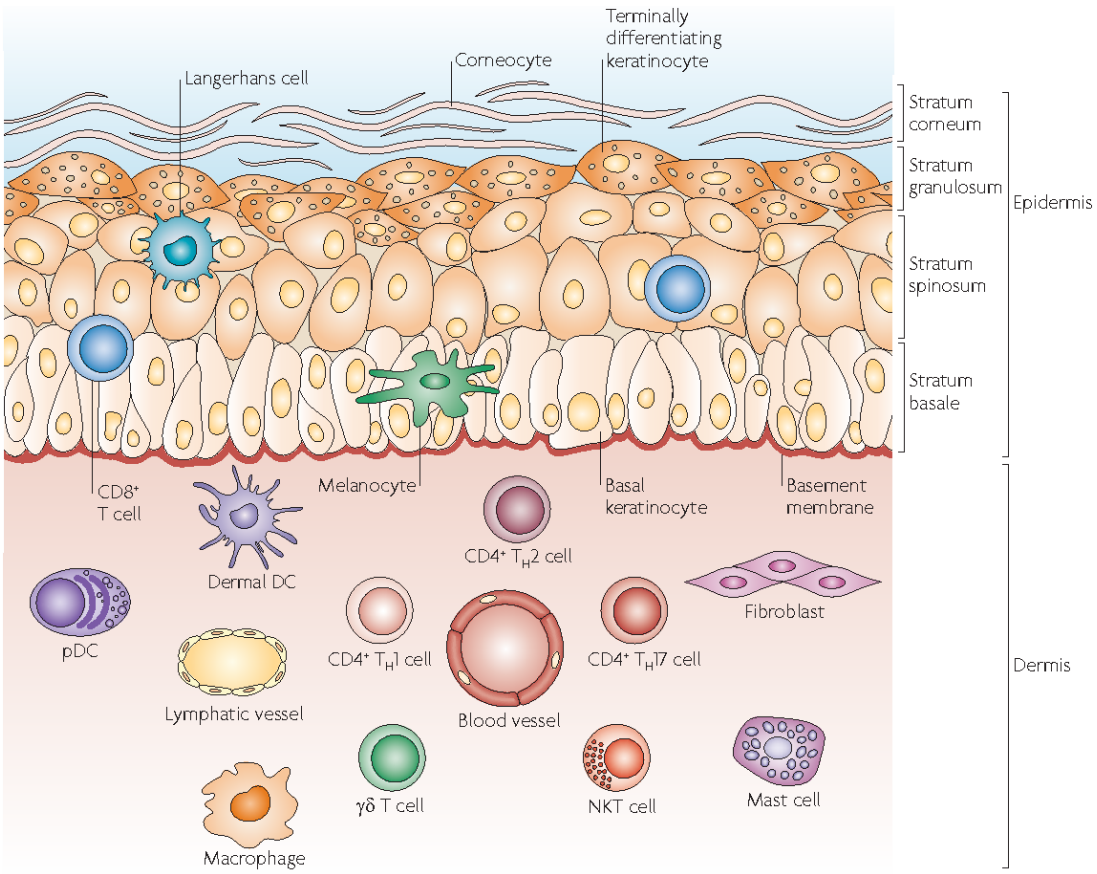


Figure 3 Skin structure and cellular components.
The healthy skin is a mechanical, and immunological barrier (Nestle, Di Meglio, Qin, & Nickoloff, 2009)

1.3.4 Microbiome

Bacteria co-inhabit the human body interfaces as gut, nose, mouth lung and skin in 1:1 ratio with human cells and contribute to our outermost line of defense - the skin (Khan, Petersen, & Shekhar, 2019; Panther & Jacob, 2015). Furthermore, the human skin is also colonized by fungi and viruses (Hannigan et al., 2015; Jo, Kennedy, & Kong, 2017; Seed, 2014). Symbiotic skin bacteria prevent the colonization with transient (pathogenic) organisms, educate the immune system and play an essential role in the lipid metabolism (Belkaid & Segre, 2014; Byrd, Belkaid, & Segre, 2018). As the skin is a remarkably diverse habitat, there is a site specificity of the microbial composition and function. Factors shaping the natural bacterial community include topography and temperature, but also skin pH, moist and sebum content (Costello et al., 2009; Findley et al., 2013; Grice et al., 2009; Oh et al., 2014). Typical inhabitants of the skin belong to the phyla Actinobacteria, Firmicutes, Bacteroidetes and Proteobacteria, whereby species of Actinobacteria are the most common on the skin (Grice & Segre, 2011). Whereas moist skin areas as the armpits are typically inhabited by *Staphylococcaceae* and *Corynebacteriaceae*, sebaceous skin areas as the forehead are commonly colonized by *Propionibacteriaceae* (Grice & Segre, 2011). However, the individual skin microbiome is shaped by intrinsic (e.g. age, gender) and extrinsic factors (e.g. hygiene habits, stress, environmental factors as UV radiation, moisture) (Dimitriu et al., 2019; Fredricks, 2001; Patra, Byrne, & Wolf, 2016; Schommer & Gallo, 2013). Interestingly, the skin microbiome of an individual is stable over time and site (Oh, Byrd, Park, Kong, & Segre, 2016). As all single species have an individual genetic setup, in the context of (skin) health, it is of importance which species and even which strains are inhabiting the human body. Commensal staphylococci as *Staphylococcus epidermidis*, *Staphylococcus hominis* and *Staphylococcus lugdunensis* for example, can induce the expression of protective AMPs in human keratinocytes and also produce AMPs themselves, which are active against the pathogenic *S. aureus* (Bitschar et al., 2019; Teruaki Nakatsuji et al., 2017; Wanke et al., 2011). *Cutibacterium acnes* is capable of triglyceride metabolism and therefore contributes to the maintenance of the skin pH (Gribbon, Cunliffe, & Holland, 1993; Platsidaki & Dessinioti, 2018). However, species as *S. aureus* can also express harmful toxins and disturb the skin barrier. Unsurprisingly, an altered skin microbiome, often accompanied with reduced diversity, is often associated with diseases as AD and psoriasis (Zeeuwen, Kleerebezem, Timmerman, & Schalkwijk, 2013).

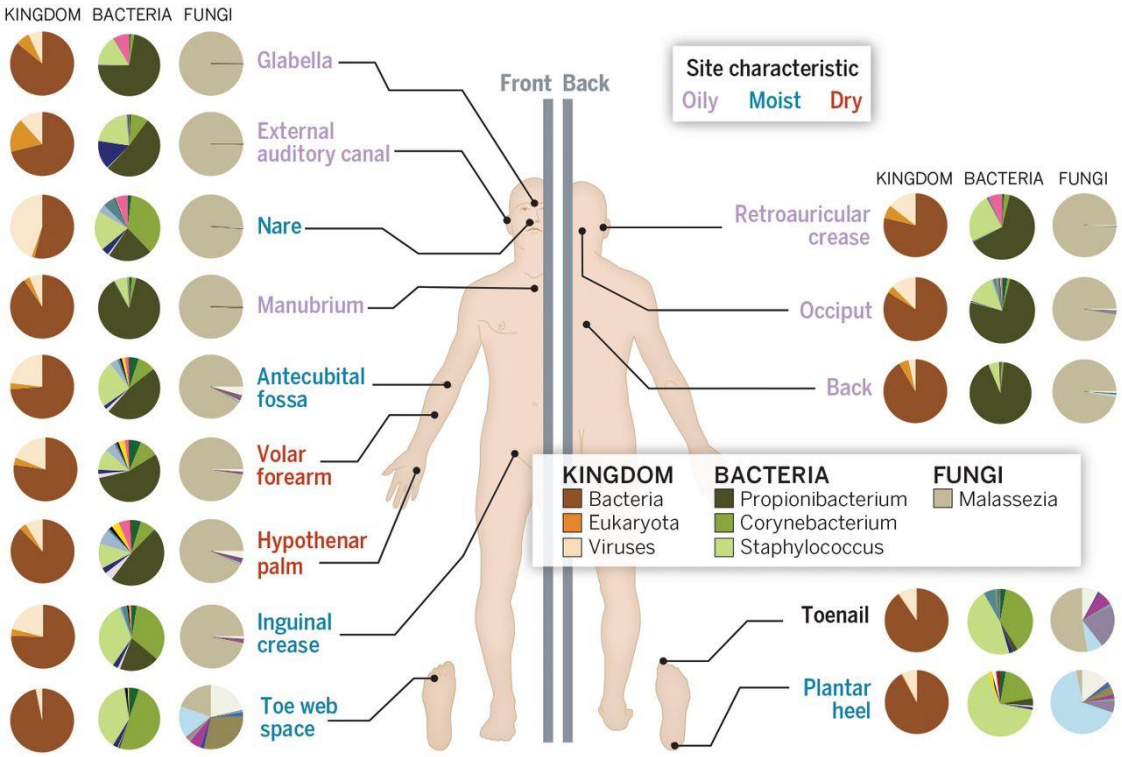


Figure 4 Typical skin inhabitants based on bodysite
The skin is inhabited by bacteria, fungi and viruses. The composition depends on the bodysite and site characteristics. Figure by (Belkaid & Segre, 2014)

1.4 Microenvironment in atopic dermatitis

1.4.1 Intrinsic factors of the microenvironment

As all components of the skin barrier are highly interconnected, a disturbance in one factor leads to subsequent effects in all other components. In AD, the connected network of the skin barrier is majorly impaired. The skin barrier is disrupted, leading to a higher transepidermal waterloss (TEWL), lower hydration and higher pH of the skin. Most remarkably, the skin microbiome is imbalanced, often due to an overgrowth of *S. aureus*, a pathogen which is able to express toxins and superantigens (SA) which in turn stimulate mast cell degranulation and lead to itch causative for aggrandized scratching (J. Kim, Kim, Ahn, & Leung, 2019; Nakamizo et al., 2015).

One key point in AD pathology is the barrier disruption. One known genetic risk factor of AD are mutations in the filaggrin protein, which is a filament binding barrier protein in epithelial cells contributing to the acidity and hydration of the skin. Four null mutations are associated with AD: R2447X, S3247X, R501X and 2282del4, where the last two are typically found in the Caucasian population (Greisenegger et al., 2010; Sandilands et al., 2006). FLG mutations are linked to severe AD starting in infancy and persisting into adulthood (Barker et al., 2007;

Rodríguez et al., 2009; Rupnik, Rijavec, & Korošec, 2015). Especially the extrinsic AD subtype which is characterized with high IgE levels and concomitant allergic sensitization is linked with FLG mutations (Stephan Weidinger et al., 2006). Due to the role of filaggrin in skin barrier integrity and as NMF, it is unsurprising that AD patients suffer from higher TEWL. On top, filaggrin is crucially involved in the maintenance of low skin pH in healthy individuals (McAleer & Irvine, 2013). In AD, skin pH is generally higher than in healthy control, especially in lesional skin (LS), where skin pH is even higher than in non-lesional (NL) skin of AD patients (Danby & Cork, 2018). Moreover, skin pH is associated with disease severity (Seidenari & Giusti, 1995). Also, especially patients with FLG mutation, show decreased levels of ceramides in LS and NL skin, due to pH dependent elevated serine protease activity reducing ceramide synthesis (P. M. Elias & Wakefield, 2014; Jungersted et al., 2010). However, FLG cannot be the only factor in AD, as it only affects 15- 20% of AD patients and not all people with FLG mutation develop AD (Palmer et al., 2006). AD patients had a combined mutation frequency of 12.6%, contrasting 1.9 % in healthy controls (González-Tarancón et al., 2020). Apart from FLG, also other tight junction abnormalities have been reported. Less Claudin1 has been found in NL skin of AD patients (Tokumasu et al., 2016). An inverse correlation between tight junctions transcription of Claudin 4, 5 and TJP1 and *S. aureus* and a positive correlation between tight junction expression and other *Staphylococcal* species has been described (Altunbulakli et al., 2018).

The innate and adaptive immune system in AD is disturbed on multiple levels, as nicely summarized by Werfel et al (Werfel et al., 2016). An imbalance of Th1, Th2, Tregs and IgE mediated hypersensitivity is described in AD (Roesner et al., 2016). Th2 cytokine expression of IL-4 and IL-13 are elevated in AD (Hamid, Boguniewicz, & Leung, 1994). However, also Th17 and Th22 are activated in AD, underlining the heterogenous and complex situation in the disease. The innate immune system in AD is characterized by reduced AMPs and recruitment of innate immune cells as well as genetic polymorphisms in PRR (Anna De Benedetto, Agnihothri, McGirt, Bankova, & Beck, 2009).

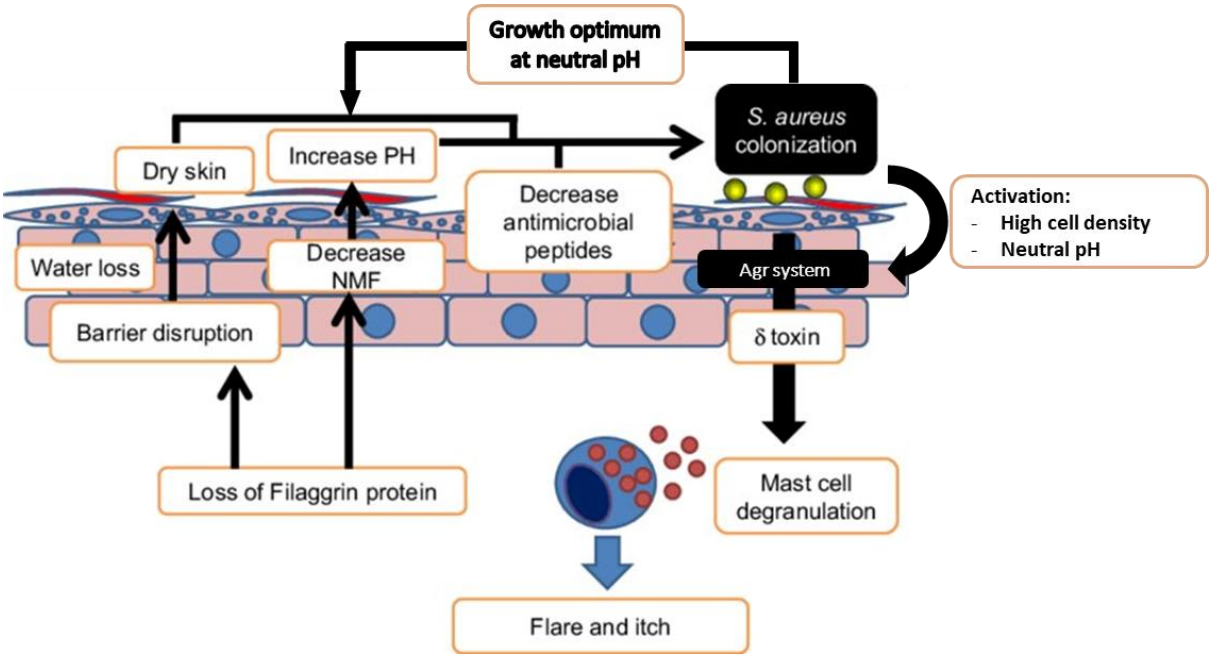


Figure 5 Microenvironment in atopic dermatitis
The skin microenvironment in atopic dermatitis is disturbed on multiple levels which are highly interconnected as depicted. Adapted from (Nakamizo et al., 2015)

1.4.2 *S. aureus* in the context of atopic dermatitis

Coherent with the impaired skin barrier and immune system, the skin microbiome is disturbed in AD. Also in the context of AD, a reduced microbiome diversity has been reported, especially during disease flares (Altunbulakli et al., 2018). The reduced diversity mainly occurs due to a microbial imbalance towards *S. aureus* overgrowth (Heidi H. Kong et al., 2012). On healthy skin, AMP producing and inducing commensals as *S. epidermidis*, *S. hominis* and *S. lugdunensis* outnumber *S. aureus*, balancing the skin microbiome (Teruaki Nakatsuji et al., 2017). Whereas only 5-30% of healthy individuals are inhabited by *S. aureus*, 60-100% of AD patients are colonized by *S. aureus* (Paller et al., 2019; Totté et al., 2016). In multiple ways, AD skin is predisposed to *S. aureus* colonization. Underlying barrier defects, scratching and proteases expressed by *S. aureus* exposes submerged fibronectin to which *S. aureus* binds (Cho, Strickland, Boguniewicz, & Leung, 2001). Furthermore, *S. aureus* binds stronger to deformed corneocytes which form due to lower filaggrin degradation products in AD (J. Kim et al., 2019). In AD, the expression of IL-37 and human β-defensin 2 (HBD-2), which have antimicrobial and anti *S. aureus* activity, is reduced (Ong et al., 2002). The increased skin pH in AD to neutral conditions is favorable for *S. aureus* growth (Korting et al., 1990; Valero et al., 2009). Furthermore, breakdown products of filaggrin, UCA and PCA reduce virulence of *S. aureus* as proteins required for immune evasion (e.g. protein A) and colonization (e.g. fibronectin binding protein A) are suppressed (Miajlovic, Fallon, Irvine, & Foster, 2010). The inflamed

environment with increased IL-4 was shown to be favorable for *S. aureus* binding in a mouse model (Cho, Strickland, Tomkinson, et al., 2001). Vice versa, *S. aureus* infiltration of deeper skin layers induced IL-4, IL-13, and IL-22 and was dependent on *S. aureus* viability and protease expression (T. Nakatsuji et al., 2016). *S. aureus* is a potentially pathogenic bacterial species, which is capable of expressing SA, proteases and toxins and the formation of biofilms. These virulence factors are often encoded on mobile genetic elements and therefore vary between strains (Otto, 2014). SA include membrane damaging and non-membrane damaging toxins as staphylococcal enterotoxin A- Y (SEA – SE/Y) and toxic shock syndrome toxin1 (TSST-1), and proteases (Fisher, Otto, & Cheung, 2018; Otto, 2014). *S. aureus* produced SA can initiate a vicious cycle by the induction of T-cells, IL-4 and IL-13 and increased specific SA IgE levels (Blicharz, Rudnicka, & Samochocki, 2019; Leung, 2005; T. Nakatsuji et al., 2016). Superantigen producing *S. aureus* strains were associated with more severe AD. However, other factors must also be involved as also in healthy individuals SA producing strains were found (Zollner et al., 2000). Furthermore, the ability to form biofilms was also associated with more severe AD (Enea Gino Di Domenico et al., 2019; T. Gonzalez et al., 2020). Interestingly, the expression of toxins is controlled by the quorum sensing system of the accessory gene regulator (Agr) in *S. aureus* (Wang & Muir, 2016). The quorum sensing system responds to environmental factors such as *S. aureus* cell density in the population, but also pH, linking the cutaneous environment and virulence of *S. aureus* (Weinrick et al., 2004). Acidic pH inhibits tsst1 expression but alkaline conditions reduce the expression of the toxin sec, underlining the complex role of pH in AD (Bergdoll, 1989; Regassa & Betley, 1992).

Summarizing, mutations in the filaggrin protein indirectly lead to an increased skin pH which in turn is favorable for *S. aureus* colonization. *S. aureus* further damages the sensitive skin barrier in AD through toxins, leading to pruritus and consequently scratching, which again boosts inflammation (Figure 5).

1.5 Potential therapeutics in AD

Typical basic therapies in AD rely highly on moisturizers and bathing therapies (Kowalska-Olędzka, Czarnecka, & Baran, 2019). However, avoidance strategies (aeroallergens, house dust -mite) and dietary interventions are also recommended (Wollenberg et al., 2018). In the acute phase of AD, topical corticosteroids and calcineurin inhibitors are often used as a topical anti-inflammatory measure. In severe AD, also systemic treatment, among others targeting IL-4/IL-13 pathways are used as summarized elsewhere (Simpson et al., 2017; Wollenberg et al., 2018).

As skin pH seems to play an important role in AD, skin pH has been subject of treatment over the years. Especially, diluted bleach baths have been used over the last years although its

efficiency is not scientifically proven (Chopra, Vakharia, Sacotte, & Silverberg, 2017; Sawada et al., 2019). Contrarily, in the Guidelines of care for the management of AD from 2015, a rather acidic basic skin care was advised, supporting the natural acidic mantle of the skin (Bieber et al., 2016; Eichenfield et al., 2014). Successful acidification of the skin was reported to improve barrier function, lipid procession and prevented AD in murine models (J. P. Hachem et al., 2010; Hatano et al., 2009; Kilic et al., 2019; H. J. Lee et al., 2014; Panther & Jacob, 2015).

More recent, the restoration of the natural skin microbiome was being elaborated, as the skin microbiome dysbiosis is apparent in AD (Blicharz et al., 2019). For example, skin microbiome transplants were suggested. The topical transplantation of *Roseomonas mucosa* to AD patients significantly improved disease severity and *S. aureus* burden in a phase I/II study (Myles et al., 2018).

1.6 Allergic rhinitis – general aspects

A disturbed epithelial barrier as observed in AD facilitates the entry and sensitization against potential allergens in mice and humans and can lead to AR and asthma (Kondo, Ichikawa, & Imokawa, 1998; Lack, Fox, Northstone, & Golding, 2003; Spergel et al., 1998). Allergens crossing the epithelial barrier are recognized by DCs and elicit a Th2 response via IL-4 and IL-13 which induce an immunoglobulin class-switch recombination in B-cells to IgE. Cross-linking of the Fc epsilon Receptor (FceRI) via IgE-antigen complexes in turn leads to mast cell degranulation releasing enzymes, cytokines and histamine in mucosal areas (Stephen J. Galli et al., 2008). The consequence are typical AR symptoms as sneezing, nasal pruritus and nasal discharge (Bousquet et al., 2008; Wheatley & Togias, 2015). AR can either be perennial against indoor allergens as house dust mite and pets or seasonal, typically against pollen as grass and birch pollen or molds (Bousquet et al., 2008; Sibbald & Rink, 1991). The prevalence of AR in Europe is 23%, of which 20% are strictly seasonal (Bauchau & Durham, 2004; Skoner, 2001). The most relevant allergenic pollen worldwide belongs to the family *Poaceae* (grass) followed by *Betulaceae* (alder, birch) in Northern and Central Europe (García-Mozo, 2017; Panzner, Vachová, Vítovcová, Brodská, & Vlas, 2014). Allergy to birch pollen often co-occurs with hazel and alder pollen due to a high homology between their major allergens. The birch pollen season depends on species, latitude, and annual weather, but typically stretches from early march to mid-may in Western Europe (Biedermann et al., 2019). Interestingly, also non-allergic individuals suffer from allergic-like symptoms upon real-life pollen exposure (Gökkaya et al., 2020)

1.7 Allergic rhinitis – microenvironment

AR affects the nasal and bronchial mucosa, which fulfills the tasks of a physical barrier, innate immune defense and clearance of inhaled particles by ciliary movements and mucus production by specialized goblet cells (Gohy, Hupin, Ladjemi, Hox, & Pilette, 2020). The mucus is the first line of defense, trapping potential allergens. In healthy individuals, the production is tightly regulated, whereas in AR and related diseases the mucus production is dysregulated (Martínez-Antón, Roca-Ferrer, & Mullol, 2006; Schuhl, 1995). In AR, similar to AD, underlying barrier defects were reported. Amongst others, decreased expression of ZO-1 and occludin in epithelial cells is associated with severe AR (Steelant et al., 2016). Epithelial leakiness results in a higher accessibility of allergens to submucosal compartments or even systematic circulation (Steelant, 2020). Furthermore, mast cell degranulation was shown to be facilitated in a setting of a disturbed nasal epithelium (Kortekaas Krohn et al., 2020). Interestingly, mast cell degranulation and Th2 mediated IL-4 and IL-13 release contribute to a disturbed barrier (Saatian et al., 2013; Steelant et al., 2018). On top, certain allergens are capable of cleaving the epithelial barrier themselves (Georas & Rezaee, 2014; Post et al., 2012).

Generally, immune dysfunctions in the innate immune system occurs in AR. Interestingly, a decreased expression of TLR-9, responsible for Th2 antagonizing Th1 differentiation was reported (Broide, 2009; Melvin, Nguyen, Lane, & Lin, 2011). An altered immunological setup and response in the nose can also lead to an altered nasal microbiome in AR. The healthy nasal microbiome is dominated by *Actinobacteria (Corynebacterium)* and *Firmicutes (Staphylococcus)* (Bassis, Tang, Young, & Pynnonen, 2014). Nasal microbiome studies are still in its infancy. However, a trend of increased *S. aureus* abundance in AR patients was reported in some studies. Contrasting results were found regarding differences in the bacterial diversity (Mahdavinia, Keshavarzian, Tobin, Landay, & Schleimer, 2016; Rajasekaran, B. V., & Sriraman, 2017). In one study, AR differ in Beta-diversity from healthy and the difference is not lost upon immunotherapy treatment although symptomatic improvements occur (Bender et al., 2020).

Summarizing, a defective barrier in AR include genetic, immunological and most likely microbial factors.

1.8 Pitfalls in skin microbiome analysis

The microbiome originally refers to all microorganisms of a habitat, including bacteria, archaea, lower and higher eukaryotes, and viruses. However, the term microbiome is nowadays often used to describe the collection of only bacterial genes found in a certain habitat (Marchesi &

Ravel, 2015). The invention of next generation sequencing (NGS) in a multiplex manner, enabled the intensive study of the microbiome based on the 16S rRNA until now. The 16S rRNA is a component of the 30S small subunit of the prokaryotic ribosome which is used for phylogenetic analysis due to its slow rate of evolution. It is 1500 base pairs (bp) long and consists constant and 9 hypervariable regions V1-V9 (Chakravorty, Helb, Burday, Connell, & Alland, 2007; Janda & Abbott, 2007). Due to a limited amplicon read length of 300 bp with NGS, most microbiome analysis are based on sequencing only parts of the 16S rRNA to date (Cruaud, Rasplus, Rodriguez, & Cruaud, 2017; Herzyk, 2014). Depending on the chosen region, species level annotation is possible (E. Gonzalez, Pitre, & Brereton, 2019; Walker et al., 2015). However, strains resolution cannot be performed by short amplicon 16S sequencing (Johnson et al., 2019). Deeper genetic and functional analysis can be revealed by metagenomics, genome-level characterization of communities in one habitat, which is therefore on the rise (Marchesi & Ravel, 2015; Ranganathan, 2019). The first commercially available sequencer in 2005, significantly reduced the sequencing time and effort and opened the field for non-experts performing microbiome research (S.-C. Park & Won, 2018; S. T. Park & Kim, 2016). Although microbiome analyses of the highly colonized gut are already performed for two decades, low biomass environments like the skin have not yet been extensively characterized. This is at least in part due to the need for more careful handling as these samples are more affected by contaminants. In contrast to 10^{11} cells/mL colon content, dry skin harbors only 10^{3} to 10^{6} cells/cm^{2}, increasing the risk for contaminants (Bibel & Lovell, 1976; J. E. Kim & Kim, 2019; Leyden, McGinley, Nordstrom, & Webster, 1987; Sender, Fuchs, & Milo, 2016). Consequently, it is important to develop and adhere to gold standards which need to be adapted to the investigated environment to ensure correctness and reproducibility of microbiome studies.

Generally, following aspects must be considered when performing skin microbiome studies: design, sampling and storage, sample processing and sequencing, and the bioinformatic analysis (H. H. Kong et al., 2017). Microbiome studies must be carefully designed as interindividual variations are encountered which require high sample sizes. Factors influencing the skin microbiome are gender, age, ethnicity, sampling location, and medication such as antibiotics or corticosteroids (Fierer, Hamady, Lauber, & Knight, 2008; Grice et al., 2009; H.-J. Kim et al., 2019; Li et al., 2019). Also, the sampling itself is a source for variation. When looking at skin microbiome sample collection dry and wet swabbing, tape stripping, scraping and biopsies are possible of which wet swabs are most commonly but not exclusively used. Although a similar microbiome composition has been found when comparing the methods,

sticking to one method per study is advisable (Grice et al., 2008; Ogai et al., 2018). A major source of variety between studies is the sample processing, especially the choice of a DNA extraction kit and the regions targeted for amplification. Particularly in low biomass environments as the skin, the choice of DNA extraction kits is of importance as endogenous contaminating bacterial sequences, the so-called "kitome", show up in the analysis. Typical contaminants include *Alcaligenes, Bradyrhizobium, Pseudomonas, Sphingomonas* and many more (de Goffau et al., 2018; Hornung, Zwittink, & Kuijper, 2019; Salter et al., 2014). Furthermore, kits especially developed for low background as the Qiagen UCP Pathogen Kit are recommended (D. Kim et al., 2017). Especially in low biomass samples, a variety of controls are essential for reliable results and include extraction and post extraction controls. Extraction controls refer to pipeline controls which are taken in parallel to sampling. Afterwards, the biological control passes through all necessary steps for NGS. Therefore, contaminants from each step should accumulate in the biological negative control. Post extraction controls are water controls added to the PCR to identify downstream contaminants (D. Kim et al., 2017). A lack of controls can lead to false results as illustrated in the case of multiple reports about low-abundance microbiome in the placenta which could retrospectively not be confirmed but turned out to be microbial contamination instead of true results (Aagaard et al., 2014; Lauder et al., 2016). Apart from careful and controlled experimental setups, the low-abundance microbiome analysis also faces some technical issues. DNA extraction Kits not only differ in their inherent microbiome, but also the lysis mode differs. Generally, mechanical lysis leads to better results compared to enzymatic or chemical lysis only (Yuan, Cohen, Ravel, Abdo, & Forney, 2012). Furthermore, the variable region chosen for 16S rRNA gene sequencing is another important factor to be considered depending on the sequenced habitat. For gut microbiome analysis, typically the variable regions V3 and V4 are sequenced whereas in skin microbiome analysis the variable regions V1-V3 are more suitable as typical skin inhabitants as *Staphylococcaceae* and *Propionibacteriaceae* can be discriminated down to species level based on the V1-V3 region (Meisel et al., 2016; Walker et al., 2015). However, not only the experimental part but also the bioinformatic processing of the genetic information is a source of bias (Sinha, Abnet, White, Knight, & Huttenhower, 2015). Following the sequencing step, the obtained sequences can be clustered by a fixed (typically 97%) similarity threshold into Operational Taxonomic Units (OTUs) by software like UCLUST, Mothur, and Uparse (Chen, Zhang, Cheng, Zhang, & Zhao, 2013; Edgar, 2010, 2013; Nguyen, Warnow, Pop, & White, 2016; Schloss et al., 2009). From each OTU a representative sequence is selected and annotated with databases like SILVA, RDP, Ezbiocloud or NCBI source (Cole et al., 2014; Nguyen et al., 2016; Quast et al., 2013;

Yoon et al., 2017). Alternatively, a clustering free approach with amplicon sequence variants (ASVs) can be chosen (Callahan, McMurdie, & Holmes, 2017; Tikhonov, Leach, & Wingreen, 2015). The ASV approach by DADA2 showed more real biological variants and less spurious sequences than other methods (Callahan et al., 2016). The sequences should pass quality control for PCR and sequencing errors and chimeras. Furthermore, especially in low biomass samples, contaminants should be removed. To remove contaminations two observations can be consulted: 1) contaminating sequences correlate inversely with total DNA 2) contaminating DNA is present in negative controls. Available pipelines for contaminant exclusion include R-packages like Decontam, but also pipelines in combining NGS results with quantification of sequences via qPCR (Davis, Proctor, Holmes, Relman, & Callahan, 2018; Karstens et al., 2019; Lazarevic, Gaïa, Girard, & Schrenzel, 2016; Salter et al., 2014).

Another limitation of NGS is that only relative frequencies and not absolute cell numbers are obtained, which might also be an important factor in skin diseases as AD (Reiger, Traidl-Hoffmann, & Neumann, 2020).

1.9 Study Hypothesis and Aim

The skin microbiome is in the focus of allergy and AD research as it opens avenues for therapeutic intervention, diagnostic and predictive tools. However, technical issues, especially contaminants, hamper the research on this topic and hinder the comparability between skin microbiome studies. Therefore, the first aim of this thesis was the development of a user-friendly tool for tailored removal of contaminants especially but not only for low biomass skin samples to reveal the true microbial inhabitants on the skin. The successful bioinformatic contaminants removal will contribute to a gold standard and reliable skin microbiome results which will be comparable between studies conducted at different laboratories with different wet-lab pipelines including DNA extraction.

Contradicting published data, we hypothesized that the skin microbiome is influenceable by extrinsic, environmental factors as different bacterial species have varying requirements and optima of the microenvironment. We hypothesized, that the seasons with fluctuating temperature, humidity and UV radiation are an influencing factor on the long-term stability of the microbiome. Furthermore, we considered an active challenge of the skin microbiome with emollient of different pH levels as a factor influencing the skin microbiome. If the microbiome is shapeable by extrinsic factors and is not only regulated by intrinsic factors, it is a putative personalized therapeutic approach in AD treatment.

As the opportunistic skin inhabitant *S. aureus* is exposed to different environmental conditions in AD and healthy, we hypothesized that *S. aureus* isolates from healthy are more adapted to acidic conditions. Furthermore, we hypothesized that the genetic features of isolates from AD patients are more pathogenic compared to strains from healthy. As the pathogenicity including toxin expression is regulated by the quorum sensing system of *S. aureus*, which is amongst other regulated by pH and number of bacterial cells, we assumed that not only the relative frequency but also total cell number is increased in AD.

2.0 Material and Methods

2.1 Material

2.1.1 Instruments

Table 1 Instruments

Instrument	Supplier
Biodrop	BioDrop UK Ltd, Cambridge, UK
Biological Safety Cabinets, class II	Thermo Scientific, Göttingen, Germany
Biometra T-advanced Thermocycler	Analytik Jena, Göttingen, Germany
BioPhotometer D30®	Eppendorf, Gießen, Germany
Centrifuge 5417R	Eppendorf, Heidelberg, Germany
Centrifuge 5804R	Eppendorf, Tuttlingen, Germany
CFX384 Real-Time PCR Detection System	Bio-Rad, U.S.A, California
CO2 Incubator CB160	Binder, Tuttlingen, Germany
Corneometer® CM825	Courage + Khazaka electronic GmbH, Köln, Germany
D30 BioPhotometer	Eppendorf, Männedorf, Switzerland
Gel iX Imager	Intas, U.S.A, California
Illumina MiSeq® platform	Illumina Inc., California, USA
Integra Vacusafe Vacuum pump	QIAGEN, Usmate, Italy
LGex 3410 (Freezer (-20°C))	LiebHerr MEDline, Feldkirchen, Germany
Magnetic stirrer 505-20000-00	Heidolph, Sulzemoos, Germany
Microwave	Severin , Wessling-Berzdord, Germany
Minitron Incubation shaker	Infors HT, Montigny Le Bretonneux, France
New Classic MS Precision balance	Mettler Toledo, Waltham, America
PCR Thermocycler Biometra TADvanced	Analytik-Jena AG, Jena, Germany
PowerPacTM Basic Power Supply (Gel electrophoresis)	Bio-Ra, Wessling-Berzdord, Germany
Precellys Evolution Tissue Homogenizer	Bertin Technologies, Sundern, Germany
QUINTIX 3102-1S Scale	Sartorius, Germantown, America
SeqStudio Genetic Analyzer	Thermo Fisher, Germany, Jena
SevenCompact pH meter S210	Mettler-Toledo GmbH, Gießen, Germany
Skin-pH-Meter PH905	Courage + Khazaka electronic GmbH, Köln, Germany
Spark Reader	Tecan, Crailsheim, Germany
SW22 Water bath	Julabo , Hamburg, Germany
Systec DX-65 (Autoclave)	Systec, Wessling-Berzdord, Germany
Tewameter® TM300	Courage + Khazaka electronic GmbH, Köln, Germany

ThermoMixer C (Heating block)	Eppendorf, Schwabach, Germany
Ultra-freezers series UF V (Freezer (-80°C))	Binder GmbH, Bulle, Swiss
ZX3 Advanced Vortex Mixer	VELP Scientifica, Seelbach , Germany

2.1.2 Consumables

Table 2 Consumables

Consumable	Supplier
96-well plate	Fisher Scientific GmbH, Schwerte, Germany
Inocculation loop sterile, 10 µl	Carl Roth, Karlsruhe, Germany
Microbank storage vial beads	Pro-Lab Diagnostics, Richmond Hill, ON, Canada
Optifit Tips	Sartorius, Göttingen, Germany
PCR foil MicroAmp	Applied Biosystems (Life technologies) , Carlsbad, CA, U.S.A.
PCR tubes	Eppendorf, Hamburg, Germany
qPCR plates 384 well	Thermo Fisher Scientific, Schwerte, Germany
Reaction tubes (0.5; 1.5; 2 ml)	Sarstedt, Hamburg, Germany
Reaction tubes (15 ml; 50 ml)	Sarstedt, Nürnbrecht, Germany
Safety SpaceTM Filter Tips	Sartorius, Göttingen
Screw Cap Micro Tubes	Sarstedt, Nümbrecht, Germany
Sigma Dry Swab Peel Pouch	MWE, Wiltshire, England
Sterile filter device (250 ml; 500 ml)	Sarstedt, Nürnbrecht, Germany
Syringe filter units (0.22; 0.45 µm)	Merck Millipore, Darmstadt, Germany
Tape strip	
Tissue culture flask (25; 75 and 175 cm2	Greiner bio-one, Frickenhausen, Germany
Tissue culture plates (96; 48; 24; 12 and 6 well)	Corning Incorporated (Falcon), Tewksbury MA, USA
Transwell®plates with 0.4 µm Pore Polyester Membrane Insert	Corning Incorporated (Falcon), Tewksbury MA, USA
Tubes and Domed Caps, strips of 8	Thermo Scientific, Waltham, U.S.A.

2.1.3 Kits and reagents

Table 3 Kits

Kit	Supplier
QIAamp UCP Pathogen Mini Kit (50)	Qiagen, Germantown, USA
RTP® Pathogen Kit	STRATEC Molecular GmbH, Berlin, Germany
RNeasy kit	Qiagen, Hilden, Germany

Table 4 Reagents

Reagent	Supplier
Acetic Acid 1M	Merck, Darmstadt, Germany
BSA	Sigma Aldrich, St. Louise, America
DEPC Treated Water	Ambion, Naugatuck, America
DMSO	NewEngland Biolabs, Frankfurt , Germany

dNTPs	Thermo Fisher Scientific, Vilnius, Lithuania
DX reagent	Qiagen, Hilden, Germany
EDTA	Sigma Aldrich, St. Louise, America
Ethanol absolute	VWR, Bruchsal, Germany
HDGreen Plus DNA stain	Intas, Göttingen, Germany
iScript Advanced cDNA Synthesis Kit	Thermo Fisher Scientific, Vilnius, Lithuania
iTaq Universal SYBR Green Supermix	Bio-Rad, California, USA
lysostaphin (0.1 mg/mL)	Sigma Alderich, Missouri, USA
MiSeq® Reagent Kit v3 600 cycles	Illumina Inc., California, USA
peqGOLD Universal Agarose	PEQLAB Biotechnologie GmbH, Erlangen, Germany
PerfeCTa Multiplex qPCR ToughMix	Quantabio, Beverly, USA
PhiX control library spike-in	Illumina Inc., California, USA
Proteinase K (10 mg/mL)	AppliChem GmbH, Darmstadt, Germany
purple gel loading dye	New England Biolabs Massachusetts, USA
Q5 Hot Start High-Fidelity DNA polymerase	New England Biolabs, Massachusetts, USA
Q5 polymerase and reaction mix	New England Biolabs, Massachusetts, USA
RNAprotect Bacteria Reagent	Qiagen, Hilden, Germany
silica/zirconium beads (100 µm diameter)	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Stool Stabilizer Solution	Invitek Molecular, Berlin, Germany
TE buffer (10 mM Tris·Cl, 1 mM EDTA, pH 8.0)	Promega, Wisconson, USA
Tris Base 2M	Sigma Aldrich, St. Louise, America
Universal Agarose	VWR, Bruchsal, Germany

Table 5 Medium Microbiology

Medium for microbiology was autoclaved for 20 min at 120°C and stored at room temperature.

Medium	**Supplier**
Mannit-NaCl-Agar	Carl Roth, Karlsruhe, Germany
Colombia agar with sheep blood	Thermo Fisher Scientific Inc., Massachusetts, USA
LB agar	
Trypton/Peptone from Casein 2 g	Carl Roth, Karlsruhe, Germany
Yeast extract 1 g	Carl Roth, Karlsruhe, Germany
NaCl 1 g	Carl Roth, Karlsruhe, Germany

Table 6 Medium Cell culture

Medium for cell culture was sterile filtered and stored at 4°C.

Medium	**Supplier**
Keratinocyte Growth Medium 2 (KGM2)	PromoCell, Heidelberg, Germany
3T3 medium	
Ingredient	**Final concentration**
DMEM high Glucose	395 ml
FCS	100ml (20%)
Penicillin-Streptomycin	5mL of 10000U/mL (100 U/ml)

Table 7 Software

Software	Supplier
Bioinformatics & Evolutionary Genomics – Venn Diagrams	online [2016-2020]
Eulerr (Venn diagram)	online [2020]
Gel iX Imager and GeldDoc software	Intas, Germany, Göttingen
GraphPad Prism 8.4.3	GraphPad Software, Inc, San Diego, California, USA
Illumina Sequence Analysis Viewer (SAV) v 2.4.5	Illumina Inc., California, USA
Illumina software MiSeq® Reporter (MSR) v 2.5.1.3	Illumina Inc., California, USA
IMNGS	online [2016-2020]
microbIEM 0.41	Environmental medicine, UNIKA-T Augsburg
NEB Tm calculator v. 1.12.0	New England Biolabs Inc., Massachusetts, USA
Spark Control Method Editor	Tecan, Männedorf, Switzerland

2.2 Preparation of a mock community to compare DNA extraction kits

2.2.1 Choice of strains

In order to test DNA extraction kits for low input material samples as skin microbiome samples, a defined mock community of fifteen strains kindly provided from the ZIEL strain collection was prepared (Table 8). This mock community contained typical skin inhabitants of the skin as *C. acnes* and members of the *Staphylococcaceae* family.

Table 8: Mock community strains

# ZIEL strain	Species	Growth	°C	Medium	Desired cell number
G9391	*Acinetobacter baumannii*	Aerob	32	LB	1.00E+08
G6352	*Bacillus cereus*	Aerob	32	LB	1.00E+08
G3260	*Bacillus subtilis*	Aerob	32	LB	1.00E+08
G9497	*Clostridium beijerinckii*	Anaerob	36	LB	1.00E+08
G9776	*Enterococcus faecalis*	Aerob	32	LB	1.00E+08
G8985	*Escherichia coli*	Aerob	32	LB	1.00E+07
G657	*Lactobacillus reuteri*	Anaerob	36	Blood agar	1.00E+07
G1702	*Listeria monocytogenes*	Aerob	32	LB	1.00E+07
G9716	*Cutibacterium acnes**	Anaerob	36	LB	1.00E+07
G9034	*Pseudomonas aeruginosa*	Aerob	32	LB	1.00E+07
G9703	*Staphylococcus aureus*	Aerob	32	LB	1.00E+06
G9342	*Staphylococcus epidermidis*	Aerob	32	LB	1.00E+06

G9343	*Staphylococcus hominis*	Aerob	32	LB	1.00E+06
G3649	*Streptococcus mutans*	Aerob	32	LB	1.00E+06
G4622	*Streptococcus pneumoniae*	Anaerob	36	LB	1.00E+06

* Previously *Propionibacterium acnes*

2.2.2 Cultivation of mock community

Aerobic and anaerobic bacteria were included in the mock community. Each strain was cultivated in the appropriate medium, temperature, and oxygen supply at each of the following steps (Table 8). To obtain pure cultures, the frozen strains from ZIEL were streaked out with an inoculation loop on cultivation plates and grown overnight. A single colony was picked to inoculate 3 mL of medium. After 6h, 10 µL of the pre-culture were transferred to flasks (baffled flask if oxygen required for growth) containing either 100, 250 or 500 mL of the respective medium depending on their growth capacity. Bacteria were cultivated overnight. The next morning, the content of the (baffled) flasks was aliquoted to 50 mL Falcon tubes and spun down 10 min at 3000 x g at room temperature (RT). The supernatant was discarded, and the pellet resuspended in 2 mL medium. Respective pellets from the same strain were pooled in 15 mL Falcon tubes. The pellet was washed twice more as described. The culture was aliquoted into one part to be frozen at -80°C and one part to determine the cell number.

2.2.3 Determination of cell number

To ascertain the cell number, a dilution series was prepared, and the optical density (OD) measured. To approximate the cell number, a measured OD_{600} of 1 was equated with 10^9 cells. Subsequently, 50 µL of the dilutions expecting 1, 10, 100 and 1000 cells/plate were plated with a Drigalski spatula. When colonies were grown, the colony forming units (cfu) were counted on the plates. Subsequently, the cell number was calculated for each strain. Lastly, the cell numbers were adjusted to the scheme described in (Table 8).

2.3 Studies

2.3.1 Panel study

2.3.1.1 Study design

The panel study is a comprehensive study conducted at the Chair and Institute of Environmental medicine (IEM). Described below are only parts which are relevant for this thesis. Further samples and information were collected as described in detail in the doctoral thesis of Denise Rauer (IEM) and Mehmet Gökkaya (IEM) and published in (Gökkaya et al., 2020).

For this study, 10 non-allergic (Na) and 8 AR patients sensitized against birch pollen (Birch-allergic, Ba) were included and monitored to assess immune parameters and the human microbiome stability over one year. The panel study was carried out from November 2015 to October 2016 in the region of Augsburg with monthly intervals out of season and biweekly intervals in the birch pollen season (Visit 6 to 8; April). At the 15 visits, skin swabs for microbiome analysis were taken from the skin (Antecubital fossa, Ac) and the head (Figure 6). Approval of the study was given by the local ethics committee of the Technical University of Munich (internal code 19/15) and complied with guidelines of the Declaration of Helsinki. Study participants were only enrolled after written informed consent.

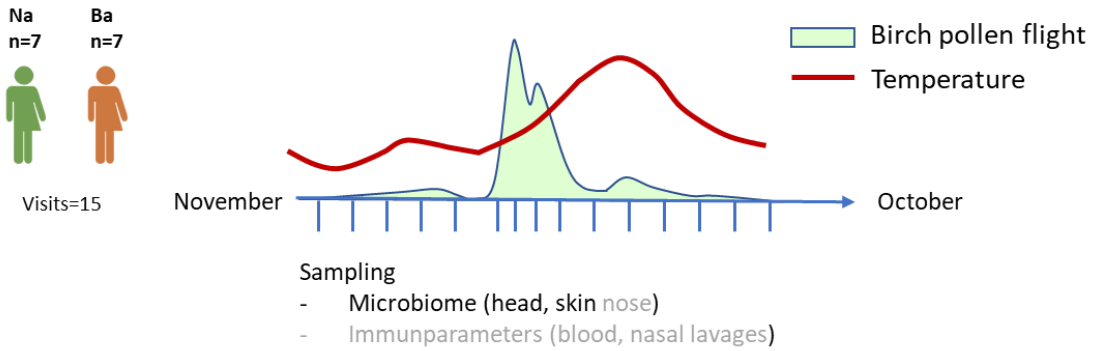


Figure 6 Study design Panel study
For the panel study, 7 healthy non-allergic and 7 birch-allergic individuals were included and samples were taken from November to October the following year with monthly visits out of pollen season and biweekly visits within the main birch pollen season (April), a total of 15 visits. Skin, head and nose microbiome samples were taken every visits. Other samples for immune-parameters were taken and a symptom diary was completed daily as described elsewhere and were not used for this project (Gökkaya et al., 2020).

2.3.1.2 Inclusion/exclusion criteria

Suitability of Ba patients for the study was determined via anamnesis by an otolaryngologist who excluded other forms of rhinitis than seasonal AR. However, Ba patient Ba 3 was excluded retrospectively as no seasonal symptoms were observed. Inclusion criteria for Na individuals were negative skin prick tests and specific aeroallergen IgE levels below detection limit of ImmunoCAP. Out of ten included Na participants, two had to be excluded due to noncompliance (Na 2, 4). Due to low reads generated via NGS after quality control and filtering, Na 1 had to be excluded from microbiome analysis.

2.3.1.3 Microbiome sampling

Skin microbiome samples were taken either dry, or by rubbing pre-wetted (0.9% NaCl) skin swabs 30 times for 20 sec in an area of 2x2 cm^2 in the Ac and an area of 5-10 cm at the head (vertex) for 10 times. Skin swabs were stored in 500 µL Stool DNA Stabilizer solution at -80 °C until further processing.

2.3.2 BampH study

2.3.2.1 Study design

The study was designed in double-blinded intra-individual fashion to assess the impact of pH on the skin microbiome. Therefore, 6 AD participants and 6 healthy (HE) controls applied the same emollient with either acidic or alkaline pH (pH 5.5 or 8.5, respectively) on a randomized body side twice daily over 8 weeks (Figure 7). The study was conducted from October to December 2017 (Four HE: IDs 3-6, four AD: IDs 11-14) and from April to June 2018 (Two HE: IDs 1,2, two AD: IDs 15,16). The study was approved by the local ethics committee of the Technical University of Munich (187/17S) and participants signed informed consent statements before study begin.

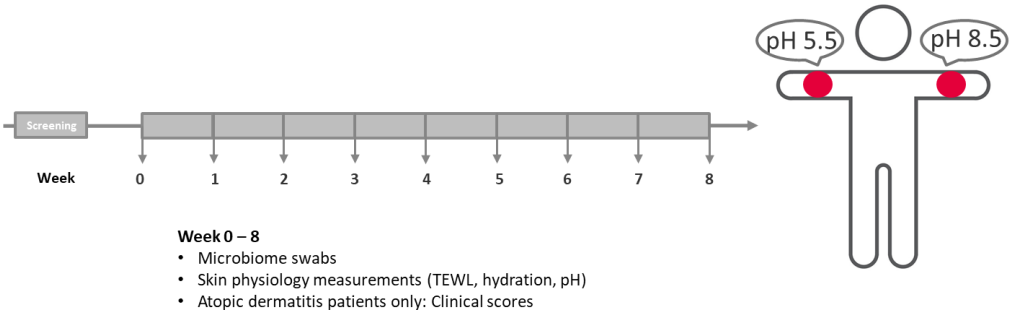


Figure 7 Study design BampH study
For the BampH study, 6 healthy individuals and 6 atopic dermatitis patients put an emollient with pH 5.5 and an emollient with pH 8.5 twice daily on opposite bodysides for 8 weeks. Weekly, samples were taken for skin microbiome analysis and skin physiology and AD severity were assessed.

2.3.2.2 Inclusion and exclusion criteria

To exclude a hypersensitivity reaction against the study emollient, an epicutanous patch test was performed. Reddening and papulation was analyzed after 48h and a skin reaction lead to exclusion. Prior to the study, participants had to restrain 14 days from antibiotic treatment and 7 days from application of steroids or calcineurin inhibitors within the region of interest or systemic use. During the study, these restrictions were continued. Therefore, only patients with mild to moderate AD (moderate SCORAD <40 (min=12.8, max= 37.6, average= 29.4) with comparable lesion severity on both body sides were included. Body regions included were: 2x dorsal lower leg, 2x antecubital areas, 1x volar upper arm, 1x volar shoulder. Accordingly, the body region was chosen in HE. Study groups were matched in age and gender.

2.3.2.3 Application of study emollient

The study emollient was provided by Sebapharma GmbH & Co. KG. During the study period, participants restrained from using other skin care products in the body area. With a fresh glove,

the according study emollients were applied on the previously determined study are of 10x15 cm. On average, 4.7 mg/cm^2 were applied in both study groups and treatment arms.

2.3.2.4 Physiologic measurements and sampling

Prior to physiological measurements and sampling, participants restrained from showering for 12 h and last emollient application had to be 3 h ago. Before measurements, an acclimatization period of 20 min was required. Measurements were taken at a mean RT of 22.2°C and mean humidity of 46.6g/m³. For transepidermal water loss (TEWL), hydration and skin pH determination, the mean of three measurements with Tewameter® TM300, Corneometer® CM825 and Skin-pH-Meter PH905 by Courage + Khazaka electronic GmbH were used. General AD disease severity was assessed by objective SCORAD (Oranje), and EASI (Leshem, Hajar, Hanifin, & Simpson, 2015; Tofte et al., 1998) at study begin and end. At each visit, the local disease severity of the test region was evaluated from missing (0) to severe (3) for the criteria reddening, edema/papulation, excoriation, oozing/crusts, lichenification and subjective itching. Skin microbiome samples were collected by 20 times dry swabbing of a field of 2x2cm with a skin swab and were subsequently stored in 500 µL Stool DNA Stabilizer solution at -80 °C.

2.4 Microbiome sampling via tape-stripping

To compare the results from swabbing and tape-stripping, also other studies were included. Dry swabbing was done as described in 2.3.2.4 Physiologic measurements and sampling. Tape-stripping was performed using a tape strip with a diameter of 22 mm.

2.5 DNA extraction

2.5.1 Spike-in of alien species

As an internal calibrator of DNA extraction efficiency, microbiome samples were spiked with 2000 cells of *Imtechella halotolerans,* a species alien to the human body. The cells were kindly provided by ZYMO RESEARCH EUROPE GMBH, Freiburg, Germany.

2.5.2 DNA extraction of microbiome samples

2.5.2.1 Single tube QIAamp UCP Pathogen kit

Routinely, for DNA extraction for microbiome analyses, the QIAamp UCP Pathogen kit was used according to manufacturer's instructions with the following deviations. Frozen skin swabs in screw cap tubes containing 500 mg of 100µm diameter zirconia-silica beads and 500 µL Stool stabilizing solution were thawed and 650 µL of ATL buffer containing 4.3 uL DX buffer were added. Cell lysis was performed using Precellys Evolution for 90 seconds twice with 15 seconds break. The supernatant was then further processed according to the manufacturer's

instruction. The DNA was eluted twice with 2x 50 µL elution buffer and frozen at -20°C until further processing.

2.5.2.2 RTP® Pathogen Kit

The RTP Pathogen kit was used according to manufacturer's instructions. The DNA was frozen at -20°C until further processing.

2.6 Next generation sequencing

2.6.1 Target amplification

For sequencing of the variable region V1- V3, the target amplicon was amplified using the primers 27F-YM (5 -AGAGTTTGATYMTGGCTCAG-3) and 534R (5 -ATTACCGCGGCTGCTGG-3) in polymerase chain reaction (PCR) as shown in (Table 9). In a second PCR step, two barcodes were added (Table 10).

Table 9 Preparation of first polymerase chain reaction step: Amplification of target region

Reagent	Volume in µl
Q5 buffer	5.00
Q5 enhancer	5.00
dNTPs	0.50
Primer 27F-YM (5µM)	1.25
Primer 534R (5µM)	1.25
Q5 Polymerase	0.25
Water	6.75
Sample	2

°C	Time	
98° C	1 min	
98° C	10 sec	
59° C	20 sec	25 Cycles
72° C	15 sec	
72° C	2 min	
4° C	∞	

Table 10 Preparation of second polymerase chain reaction step: Attachment of barcode and adaptor

Reagent	Volume in µl
Q5 buffer	10.00
Q5 enhancer	10.00
dNTPs	1.00
Forward primer*	0.3125
Reverse primer*	2.5
Q5 Polymerase	0.5
Water	23.6875
1st PCR step product	2

°C	Time	
98° C	40 sec	
98° C	20 sec	
55° C	40 sec	8 Cycles
72° C	40 sec	
72° C	2 min	
4° C	∞	

* Unique combinations of forward and reverse barcodes

2.6.2 Preparation for Next generation sequencing

Subsequently, the generated indexed amplicons were purified using AMPure XP beads (Beckman Coulter, Fullerton, Calif) with a bead/DNA ratio of 0.7:1 (vol/vol), according to the manufacturer's instructions. The purified amplicons were quantified with the fluorescent dye-

based Qubit® dsDNA HS Assay Kit (Invitrogen) and all samples were pooled equimolarly. The library was purified with AMPure XP as described before. To ensure DNA integrity and peak distribution, the High Sensitivity DNA LabChip Kit (Agilent Technologies) was used on the 2100 Bioanalyzer (Agilent Technologies). For single stranded DNA, the library was denatured with NaOH.

2.6.3 Next generation sequencing

The 16S rRNA sequencing was performed on an Illumina MiSeq® platform (Illumina Inc.) with the 2x300 bp pair-end reads (MiSeq® Reagent Kit v3 600 cycles: Illumina Inc.). The final library concentration was 8 pM and 20% PhiX control library spike-in (Illumina Inc.). For imaging, data processing and evaluation, the Illumina software MiSeq® Reporter (MSR) v 2.5.1.3 on the MiSeq® system and the Illumina Sequence Analysis Viewer (SAV) v 2.4.5 were chosen.

2.6.4 Bioinformatic clustering, annotation and quality control

Clustering of operational taxonomic units was performed either with CLC Genomics Workbench 11.0.1 and its microbial genomics module or with IMNGS. Optimized species annotation was performed by using the algorithm of Bhattacharyya et al 2020 (Bhattacharyya M., 2019). Sequencing was carried out either at the core facility at ZIEL or at IMGM.

2.7 Quantification of genes in microbiome samples

Absolute quantification of 16S rRNA genes, *Staphylococcus* spp., *S. aureus* and *Imtechella halotolerans* cells were determined by qPCR Taq MAN assays using the primer and probes as displayed in Table 11. For detection of number of cells, the 16S rRNA gene was chosen which has multiple and differing copies per cell depending on the species and therefore only gives a rough estimation. For *Staphylococcus* sp., the unique gene tuf was chosen, for *S. aureus* the unique nuc gene and for *I. halotolerans* the unique nhaC gene. As template, 5 µL of 100 µL eluted DNA was used. A standard curve was created with the multiplex UNIKAT Panel v2plasmid containing one copy of the respective genes. Reactions were performed in 10µl final volume using the PerfeCTa Multiplex qPCR ToughMix with a 100nM concentration for each primer and probe in the multiplex setup. After a 2 minute denaturation-activation step at 95°C, 45 cycles were performed with a denaturation step of 15 seconds at 95°C and an annealing-elongation step of 60 seconds at 60°C in a CFX384 Real Time System. The quantity cycles (Cq) determined as average of independent triplicates.

Table 11 Primers and probes used for absolute quantification of bacteria

Target	Primer	Sequence	Citation
Eubacteria (16S rRNA gene)	RT-16S Eubac FW	TGGAGCATGTGGTTTAATTCGA	(Yang et al., 2002)
	RT-16S Eubac Rev	TGCGGGACTTAACCCAACA	(Yang et al., 2002)
	RT-16S Eubac Probe	Cy5-CACGAGCTGACGACARCCATGCA-BHQ2	(Yang et al., 2002)
Staphylococcus ssp. (tuf gene)	RT-Tuf 2Fw	DCAAATGGACGGMGSTATCT	Genetic ID
	RT-Tuf 2Rev	WGCTGGHACACCAACGTTACG	Genetic ID
	RT-Tuf 2Probe	Hex-ATGCCACAAACTCGT-MGB	Genetic ID
Staphylococcus aureus (nuc gene)	RT-Nuc Fw	GTTGCTTAGTGTTAACTTTAGTTGTA	(Kilic, Muldrew, Tang, & Basustaoglu, 2010)
	RT-Nuc Rev	AATGTCGCAGGTTCTTTATGTAATTT	(Kilic et al., 2010)
	RT-Nuc Probe	FAM-AAGTCTAAGTAGCTCAGCAAATGCA-BHQ1	(Kilic et al., 2010)
Imtechella. Halotolerans (nhaC gene)	RT-NhaC Fw	CGGCTTGTGTGGTTATTTGTTC	Genetic ID
	RT-NhaC Rev	CCGGCCGTCATACCTAAAGA	Genetic ID
	RT-NhaC Probe	ROX-TAGCTGGACCACATCAGCAACCGTAGG-BHQ2	Genetic ID

2.8 In vitro analysis of *Staphylococcus aureus*

2.8.1 Strain collection

To compare *S. aureus* isolates from healthy individuals and AD patients, a strain collection was established at the IEM, referred to as IEM strain collection. Nose and skin swabs from the participants of the multiple studies were streaked onto high salt Staphylococci-selective Mannitol agar plates. Due to a pH indicator, *S. aureus* colonies appear yellow as they have the ability to metabolize Mannitol resulting in acidification of the medium, whereas coagulase negative Staphylococci (CoNs) appear pink on the plate. From each original plate, purification streaks of five CoNs and five *S. aureus* isolates were streaked on a fresh Mannitol agar plate. Subsequently, a single colony was re-streaked on a blood agar plate and the plate was sent to the University Clinic Augsburg for identification by MALDI-TOF analysis. Each strain was saved by inoculation of Microbank storage vial beads with the respective colonies from the plate according to manufacturer's instructions. The strains were stored at -80°C.

2.8.2 Cultivation of *S. aureus*

S. aureus strains were routinely cultivated by sterilely inoculating one bead of the Microbank vials in 3 mL liquid LB medium. Cultivation was conducted at 32°C and 250 rpm. Furthermore, *S. aureus* strains were cultivated on either LB, Mannitol or blood agar solid plates,

2.8.3 Growth curves

Pre-cultures of the respective *S. aureus* strains were prepared as described before in liquid LB medium (pH 7) and incubated for 6h at 32°C and 180 rpm. Afterwards, the optical density at 600 nm wavelength (OD_{600}) of the respective strains was determined in a 1:10 dilution with a D30 BioPhotometer. Subsequently, the OD_{600} was adjusted to 0.01 in buffered medium with either pH 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0. Then, 150 µL of each strain and pH were transferred to a 96-well plate as technical triplicates and one blank for each pH medium. Outer wells were filled with water. The changes in OD were recorded by a Spark Reader every 10 minutes for 16h at constant 32°C (Table 12). For data analysis, the mean of the technical triplicates and following the mean of biological duplicates was calculated.

Table 12 Settings of Spark reader for growth curve measurement

Absorbance parameter	**Setting**	**Shaking parameter**	**Setting**
Measurement	OD_{600}	Mode	Double orbital
Wavelength	600 nm	Duration	Continuous
Number of flashed	10	Amplitide	1.5 mm
Settle time	50 ms	Frequency	180 rom

2.8.4 DNA extraction

For subsequent PCR reactions, overnight cultures of the respective strains were cultivated as described previously. Subsequently, 100 µL of the liquid culture was centrifuged for 10 min at 5000 x g at RT. The supernatant was discarded, and the pellet resuspended with 100 µL lysis buffer containing Sodium dodecyl sulfate. This mixture was then incubated for 10 min at 95°C in ThermoMixer C heat block. Afterwards, the samples were centrifuged for 10 min at 10,000 x g at RT to remove cell debris and the supernatant containing the DNA was transferred to a fresh tube. The DNA concentration was determined using a Biodrop and the DNA was stored at -20 °C until further use.

2.8.5 Toxin multiplex PCR

To determine the presence of a toxin in the genome of an uncharacterized *S. aureus* isolate, a multiplex PCR reaction addressing nine toxins and two *S. aureus* specific genes was performed. The tested toxins were the Staphylococcal enterotoxins A, B, C, G, H, and K (*sea*, *seb*, *sec*, *seg*, *seh*, and *sek*, respectively), toxic shock syndrome toxin-1 (*tst*), Panton-Valentine-Leukocidin (*pvl*), and the methicillin resistance determinant penicillin-binding protein 2‘ (*mecA*). Furthermore, the *S. aureus* specific genes encoding for the Aminoacyltransferases FemA and FemB were included in the panel to confirm that these isolates are *S. aureus* strains. Three

different panels were designed, primer mix 1 containing *sea*, *sec*, *seh*, and *tst*, primer mix 2 containing *seb*, *seg*, *mecA* and *pvl* and primer mix 3 containing *sek*, *femA* and *femB*. The PCR was performed with the Q5 Hot Start High-Fidelity DNA polymerase with GC Enhancer according to manufacturer's description with 0.5 µM of each primer, 200 µM dNTPs and 1µL template. To each experiment, a no template control and a respective positive control were added. Thermocycling conditions were run according to manufacturer's recommendations for the Q5 Hot-start High-fidelity polymerase with varying annealing temperatures for the reaction mixes (Mix 1 and Mix 2 at 58 °C, Mix 3 at 54°C).

Table 13 Primer for toxin PCR

Primer name	Description	Nucleotide Sequence (5' - 3')	Amplicon size (bp)
femB1	forward primer for *femB*	TTACAGAGTTAACTGTTACC	
FemB2	reverse primer for *femB*	ATACAAATCCAGCACGCTCT	651
SEB-1	forward primer for *seb*	TCGCATCAAACTGACAAACG	
SEB-4	reverse primer for *seb*	GCAGGTACTCTATAAGTGCCTGC	477
SEC-3	forward primer for *sec*	CTCAAGAACTAGACATAAAAGCTAGG	
SEC-4	reverse primer for *sec*	TCAAAATCGGATTAACATTATCC	271
SEA-3	forward primer for *sea*	CCTTTGGAAACGGTTAAAACG	
SEA-4	reverse primer for *sea*	TCTGAACCTTCCCATCAAAAAC	127
SEG-1	forward primer for *seg*	AAGTAGACATTTTTGGCGTTCC	
SEG-2	reverse primer for *seg*	AGAACCATCAAACTCGTATAGC	287
SEH-1	forward primer for *seh*	GTCTATATGGAGGTACAACACT	
SEH-2	reverse primer for *seh*	GACCTTTACTTATTTCGCTGTC	213
femA1	forward primer for *femA*	AAAAAAGCACATAACAAGCG	
FemA2	reverse primer for *femB*	GATAAAGAAGAAACCAGCAG	134
TST-3	forward primer for *tsst-1*	AAGCCCTTTGTTGCTTGCG	
TST-6	reverse primer for *tsst-1*	ATCGAACTTTGGCCCATACTTT	447
SEK-1	forward primer for *sek*	TAGGTGTCTCTAATAATGCCA	
SEK-2	reverse primer for *sek*	TAGATATTCGTTAGTAGCTG	293
mecA P4	forward primer for *mecA*	TCCAGATTACAACTTCACCAGG	
mecA P7	reverse primer for *mecA*	CCACTTCATATCTTGTAACG	162
pvl-F	forward primer for *pvl*	GCTGGACAAAACTTCTTGGAATAT	
pvl-R	reverse primer for *pvl*	GATAGGACACCAATAAATTCTGGATTG	85

2.8.6 Visualization of polymerase chain product via gel electrophoresis

To visualize the results of the PCR reactions, a 2% agarose gel containing 0.00005 % HDGreen Plus DNA stain was prepared. For each sample, 5 µL were mixed with 1 µL purple gel loading dye and loaded onto the gel. Gel electrophoresis was then carried out at 100 Volts for 90 minutes. The gel was then visualized with a Gel iX Imager and GeldDoc software.

2.9 Microbe-host interaction

2.9.1 Monolayer cultivation of keratinocytes

For monolayer cultivation, either primary keratinocytes or the cell line NTERT were taken. Per well, 40,000 keratinocytes were seeded into a 24-well plate in 500 µL KGM2 medium (NTERT: supplemented with G418) and incubated for approximately 48 h at 37 °C and 5 % CO_2 or until confluent.

2.9.2 Stimulation of monolayer keratinocytes

The monolayer of keratinocytes was stimulated using 10^5 bacteria in 350 µL KGM2 per well. As control, only medium, 100 ng IL-17, or 0.1 m EDTA per well were used. Supernatant was frozen for ELISA at -20°C. Cells were frozen in 200 µL 1x Laemmli buffer for Western blot.

2.9.3 Western blot of skin barrier proteins

Samples in 1x Laemmli buffer were heated for 5 min at 95°C, afterwards, samples were vortexed and briefly centrifuged. Ready to use 4-20 % gel Mini Protean TGX TM from Biorad were loaded with 10 µL marker and 20 µL sample in running buffer and run at 100 volts until the bands reached the bottom. Proteins were transferred on membrane in transfer buffer for 90 min at 100 Volt. The membrane was incubated for 10 min in Ponceau red to visualize bound proteins. The membrane was blocked for 30 min in 10 mL TBS-T containing 5 % BSA at RT. A 1:200 dilution of Claudin-4 antibody or 1:100 dilution of ZO-1 primary antibody was prepared and incubated overnight at 4°C. Secondary antibody was prepared on the next day. For claudin-4, a 1:5000 dilution of goat-anti-mouse antibody IgG1 plus HRP (Santa Cruz) was prepared whereas for ZO-1 a 1:5000 dilution of goat anti rabbit HRP (sc2030 Santa Cruz) was prepared. The secondary antibody was incubated for 2 h at RT on a roller laboratory shaker. The membrane was then washed with TBS-T for 1 h at room temperature on a shaker. ECL Prime WB detection (GE Healthcare, RPN 2232) was used for detection. After 5 min incubation in the dark, Intas software was used for scanning of membranes.

2.9.4 Cultivation of a 3D skin model

Approximately 10 days before starting the 3D skin model, primary fibroblasts were seeded in 3T3 medium in one T75 flask and primary keratinocytes in KGM2 medium in one T125 flask. Medium was changed every other day and the cells were split once to obtain enough cells. The collagen matrix was prepared by adding 845 µL 10x HBSS to 6760 µL collagen G drop by drop. To neutralize the solution, 83.2 µL 1M sterile NaOH was added until a color change to red was observed. The solution was stored at 4°C until the fibroblasts were prepared. For fibroblast preparation, the cells were washed once with PBS and were subsequently trypsinized

with 1 mL 0.25% trypsin. The reaction was stopped with 3 mL 3T3 medium. Fibroblasts were spun down for 10 min at 1200 rpm. Fibroblasts were resuspended in FCS and the cell number was adjusted. Per well, either 75,000, 50,000 or 25,000 fibroblasts in 65 µL FCs were gently mixed gently with the collagen (585 µL/well), avoiding bubbles. Per well, 650 µL of the mix were added to the insert of a Corning CoStar Transwell plate. The mixture was incubated for 2h at 37°C in the drying cabinet. Afterwards, 500 µL KGM2 medium were added at the apical and 1500 µL at the basolateral side of the insert. The fibroblasts in the collagen matrix were then incubated at 37°C and 5% CO_2 overnight. The following day, keratinocytes were added on top. The keratinocytes were washed, trypsinized and spun down as the fibroblasts. Thereafter, the apical medium of the inserts was removed and 250,000 keratinocytes in 500 µL KGM2 medium were added instead on top of the inserts. Then, the cells were incubated at 37°C at 5% CO_2. If cells adhered to the insert the next day, they were gently detached from the insert with a yellow pipette tip. On day 4, the apical medium was removed. The medium in the basolateral compartment was replaced with KGM2 medium without BPE and Epinephrin but supplemented with ascorbic acid, BSA and Calcium. The medium was then exchanged every other day. After 7 days, the model is ready for stimulation.

2.9.5 Haematoxylin and eosin staining

The H&E staining of the 3D skin model embedded in paraffin were prepared according to the standard procedure of the pathology at the Uniklinikum Augsburg.

2.10 Statistical analysis

Gaussian distribution of the data was tested via Shapiro-Wilk test. Statistical significance of differences of continuous variable results of two unpaired groups (HE against AD, Na against Ba, microenvironment (dry, moist sebaceous), tape-stripping vs swabbing) without Gaussian distribution was assessed by the nonparametric Mann-Whitney test, while differences between paired groups (Kit differences extraction from one person, emollient application pH 5.5 vs pH 8.5) was examined with the paired Wilcoxon non-parametric test. In case of more than two groups, Kruskal-Wallis test was performed with Dunn's test for multiple comparison correction. As stated in the figures, either mean (standard deviation) or median (min, max) are shown. Correlation between continuous variables were analyzed with the nonparametric Spearman rank test. P-values were considered as significant at the alpha-error two-tailed level of $P< 0.05$. Statistical analysis and plots were either created in R version 3.6.3 or GraphPad Prism version 8.00 for Windows, GraphPad Software, La Jolla California USA.

3.0 Results

3.1 The effect of contaminants in low biomass samples

3.1.1 Higher number of contaminants in low biomass samples

In order to confirm the susceptibility of low biomass samples to contaminants in NGS, a dilution series from 10^9 to 10^2 total cells of a mock community consisting of 15 known strains was prepared in triplicates and the V1-V3 region was sequenced via NGS.

In high biomass samples, > 95 % of reads (10^7 total cells) or even > 98 % of reads (10^8 and 10^9 total cells) belonged to the species of the mock community (Figure 8). Reduction of input material resulted in an increase of contaminants, defined as species not originally included in the mock community. With 10^6 cells input material, still 68.9 %, and in 10^5 only 24.3 % could be attributed to mock species. Lower input material (10^2 to 10^4) yielded only < 4 % of reads from the mock community.

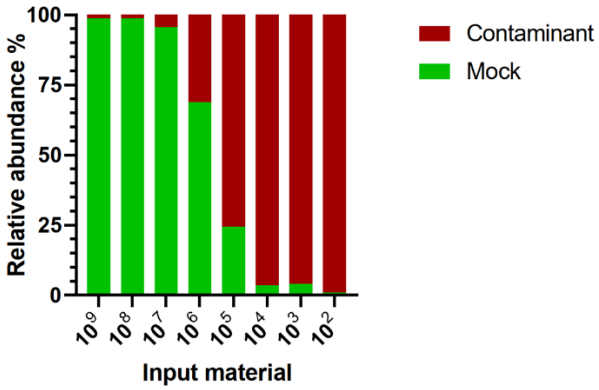


Figure 8 Susceptibility of low biomass samples for contamination
Low biomass samples are more susceptible for contamination in next-generation sequencing approaches. A dilution series from 10^9 to 10^2 total cells of a mock community consisting of 15 strains was prepared in triplicates and sequenced via NGS. The mean relative abundance of reads from the mock community (green) and other reads defined as contaminants (red) are shown. Adapted from Bachelor Thesis of Veronika Erhart, IEM, 2020.

3.1.2 Microbiome sample clustering according to DNA extraction kits

Low biomass samples are prone to contamination in NGS. To understand the impact of different DNA extraction kits on NGS results of low biomass skin samples, skin swabs from adjacent skin sites were taken from two different people. The DNA was then extracted with the DNA extraction kits RTP® Pathogen Kit (STRATEC Molecular GmbH, Berlin) (Kit A) and QIAamp® UCP Pathogen Mini (QIAGEN N.V., Netherlands, Venlo) (Kit B) but were otherwise processed the same.

When looking at a dendrogram of similarity, samples cluster significantly according to the DNA extraction kit and not the individuals they were taken from (p-value < 0.001), showing that the

DNA extraction kit has a higher influence on the sequencing than the biological difference between the individuals (Figure 9 A). Whereas in Kit B the different people still cluster together, in Kit A separation between the individuals is impossible (Figure 9 A). Despite a shared core microbiome between both kits of 20 families there are 16 unique families in Kit A and 6 unique families in Kit B (Figure 9 B). Furthermore, the abundance of typical skin inhabitants as *Propionibacteriaceae* and *Staphylococcaceae* is significantly different between the DNA extraction kits (p-value <0.001) (Figure 9 C). Looking at genus level, especially in Kit A, the two individuals appear similar whereas in Kit B they appear to be more diverse (Figure 9 C).

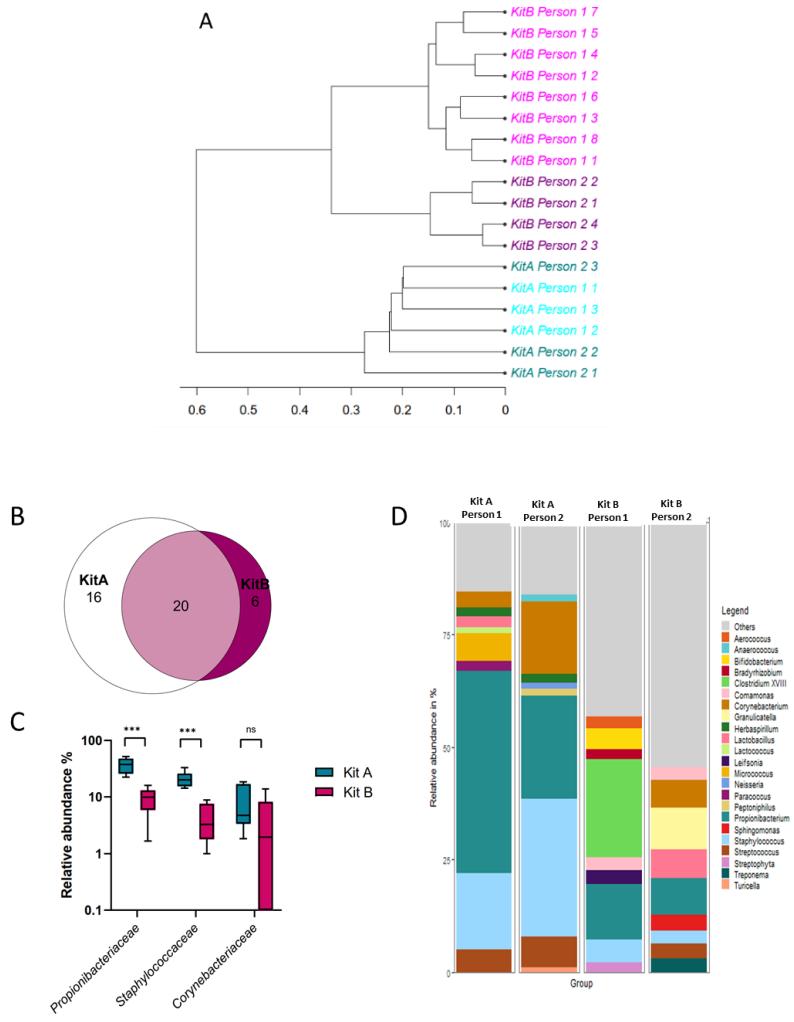


Figure 9 Effect of DNA extraction kits on NGS results in low biomass samples.
The choice of the DNA extraction kit majorly impairs the next-generation sequencing result in skin microbiome analysis. Skin swabs from adjacent skin sites were taken from two people and were extracted with either Stratec (Kit A, n= 3 per individual) or Qiagen (Kit B, n=8 person A, n=4 person B). The samples cluster according to DNA extraction kit instead of people (A). A core microbiome between the kits and unique families per kit could be identified (B). The abundance of typical bacterial skin inhabitants differs (C). The relative abundance of the top 10 genus separated according to kit and person. *** p < 0.001 (Multiple T-Test).

3.1.3 Contaminants in NGS

To better understand and evaluate the results, a list of typical contaminants was prepared based on a literature search. Included were 10 publications dealing with contaminations in NGS (Barton, Taylor, Lubbers, & Pemberton, 2006; Ducarmon, Hornung, Geelen, Kuijper, & Zwittink, 2020; Eisenhofer et al., 2019; Glassing, Dowd, Galandiuk, Davis, & Chiodini, 2016; Grahn, Olofsson, Ellnebo-Svedlund, Monstein, & Jonasson, 2003; Lauder et al., 2016; Laurence, Hatzis, & Brash, 2014; Salter et al., 2014; Tanner, Goebel, Dojka, & Pace, 1998; Weyrich et al., 2019). In total, 114 contaminants were identified on genus-level of which most belong to the phylum *Proteobacteria* followed by *Actinobacteria*. As frequent contaminants (found in 7 or 8 out of 8 paper) *Propionibacterium*, *Escherichia*, *Pseudomonas* and *Stenotrophomonas* were identified (Table 14). However, the sampling location has to be considered, as some of the contaminants listed are derived from the skin as for example *Propionibacterium*, *Staphylococcus* and *Corynebacterium*.

Table 14 Typical contaminants from literature

Contaminants found in literature were divided into frequency levels of how often they appeared. Low frequency 1-2, moderate frequency 3-4, high frequency > 5. Adapted from Bachelor Thesis of Veronika Erhart, IEM, 2020.

Phylum	Frequency	Genus
Actinobacteria	low	*Aeromicrobium, Arthrobacter, Atopobium , Beutenbergia, Brevibacterium, Curtobacterium, Dietzia, Geodermatophilus, Janibacter, Kocuria, Microbacterium, Micrococcus, Microlunatus, Patulibacter, Rhodococcus Rothia, Tsukamurella*
	moderate	*Actinomyces, Corynebacterium*
	high	***Propionibacterium***
Bacteroidetes	low	*Capnocytophaga, Dyadobacter, Hydrotalea, Niastella , Olivibacter, Pedobacter, Porphyromonas, Sediminibacterium , Wautersiella*
	moderate	*Chryseobacterium, Prevotella, Flavobacterium*
Deinococcus-Thermus	low	*Deinococcus*
Firmicutes	low	*Abiotrophia, Anaerococcus , Brevibacillus, Brochothrix, Clostridium, Coprococcus , Dialister, Facklamia, Geobacillus , Megasphaera,* ***Staphylococcus****, Veillonella*
	moderate	*Bacillus , Enterococcus, Lactobacillus , Paenibacillus, Streptococcus*
Fusobacteria	moderate	*Fusobacterium , Leptotrichia*
Proteobacteria	low	*Achromobacter, Afipia, Aquabacterium, Asticcacaulis, Aurantimonas, Azoarcus, Azospira, Beijerinckia, Bosea, Brevundimonas, Caulobacter, Craurococcus, Curvibacter, Devosia, Duganella Enterobacter, Hoeflea, Kingella , Leptothrix, Limnobacter, Mesorhizobium, Methylophilus , Methyloversatilis, Neisseria, Nevskia , Novosphingobium, Ochrobactrum, Oxalobacter, Paracoccus, Pedomicrobium Polaromonas, Pseudoxanthomonas, Psychrobacter, Roseomonas, Schlegelella, Sphingobium, Sphingopyxis, Sulfuritalea, Undibacterium , Variovorax, Xanthomonas*
	moderate	*Acidovorax, Acinetobacter, Bradyrhizobium, Burkholderia, Comamonas, Cupriavidus , Delftia, Enhydrobacter , Heaemophilus, Herbaspirillum, Janthinobacterium, Massilia, Methylobacterium, Pelomonas, Phyllobacterium Ralstonia, Rhizobium, Sphingomonas*
	high	*Escherichia, Pseudomonas, Stenotrophomonas*

3.1.4 Development of a new contaminant filtering tool

Therefore, each dataset must be checked individually for contaminants. Due to a lack of satisfying tools for contaminant removal at that time [2017], we developed microbIEM, a basic filtering and analysis tool with a graphical interface and dynamic sample selection and grouping based on metadata. The programming in R shiny was carried out by Dr. rer. Nat. Thomas Nussbaumer. As input, either an annotated OTU or ASV table and a mapping file with individual meta data are required. In the mapping file, samples must be marked as technical or biological negative control, positive control, or sample, respectively. Technical negative controls refer to PCR water controls and serve as control to rule out major contaminations whereas biological negative control are pipeline controls passing all necessary steps for NGS parallel to the real samples.

Filtering of the dataset in microbIEM is carried out in multiple steps (Figure 10 A). First, samples are excluded based on sequence depth; the user can determine the minimal number of reads required per sample. Subsequently, taxonomic units, either ASVs or OTUs are filtered based on minimum reads per taxonomic unit, allowing e.g. the removal of singletons or doubletons from the dataset. Furthermore, microbIEM gives the option to remove taxonomic units with low relative frequency. Following, the user has the opportunity to use technical and/or biological negative controls separately, to remove dataset-specific contaminants (Figure 10 B). Therefore, the ratio between mean frequency of the taxonomic unit in the negative controls and the mean frequency of the taxonomic unit in the samples is considered. For example, if a user wants to exclude all taxonomic units which are two times more abundant in the negative controls than in the samples, all taxonomic units with a ratio higher than 2 are excluded. Furthermore, the span of the taxonomic unit in the samples and negative controls can be considered. Although default settings are given, the tool allows individual change of all settings by the user. The impact of the filtering steps is visualized graphically as bar chart for the number of lost reads, correlation between number of reads and number of taxonomic units and as correlation between number of reads before and after filtering (Figure 10 C).

After the filtering procedure, basic analysis can be performed including α-diversity, β-diversity, and taxonomic analyses. These analyses are based on the R-scripts RHEA (Lagkouvardos, Fischer, Kumar, & Clavel, 2017). Conveniently, microbIEM has a graphical user interface and allows dynamic sample selection for quick and easy analyses feasible for non-bioinformaticians.

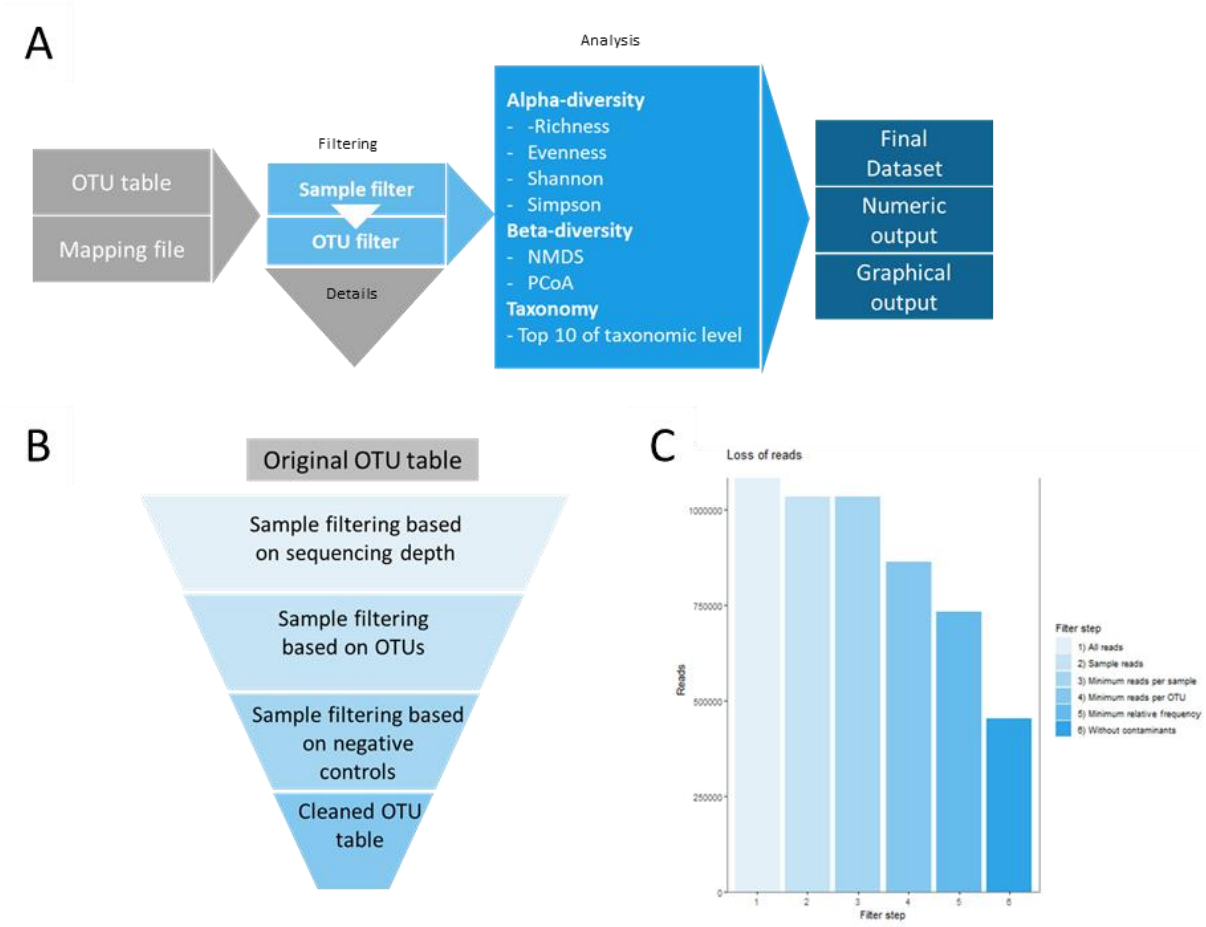


Figure 10 MicrobIEM workflow.
MicrobIEM combines quality and filtering steps with basic analysis in a dynamic matter (A). Filtering of samples is based sequencing depth whereas OTUs are filtered based on negative controls (B). Graphics depict the effect of the chosen filtering criteria, e.g. the loss of reads (C).

Comparing microbIEM to established pipelines as QIIME, Mothur, RHEA, and Decontam, microbIEM combines the advantages of all pipelines. The tool has a graphical user interface,and allows filtering of the input dataset as well as basic microbiome analysis with dynamical sample selection.

3.1.5 Contaminants in a mock dilution dataset

To test whether microbIEM can successfully remove contaminations, the mock community dataset with a dilution series from 10^9 to 10^2 in triplicates was used. The samples were sequenced twice. Once a major contamination with *Pseudomonas* spp. was introduced in the downstream processing PCR step (now referred to as "contaminated dataset") and had a mean of 10,440 reads in the PCR control. This was circumvented in the second sequencing run ("resequenced dataset") where the PCR control only displayed less than 100 reads. However, also in the resequenced dataset, contaminants acquired while sampling, DNA extraction and handling were observed (Figure 8)

The introduced *Pseudomonas* contamination in the contaminated dataset consisted of three major OTUs, annotated as *Pseudomonas poae* (OTU 1), *Pseudomonas migulae* (OTU 160) and *Pseudomonas veronii* (OTU 423), which was also reflected by the negative controls (pipeline control and PCR water control). Whereas the contamination only made up 1.6 % in total in the sample with high input material (10^9), this steadily increased to 20.6 % in 10^8, 49.5 % in 10^7, 88.8 % in 10^6 and > 90 % in 10^5 to 10^2 cells starting input material, showing once more the impact of contaminants on low input samples (Figure 11).

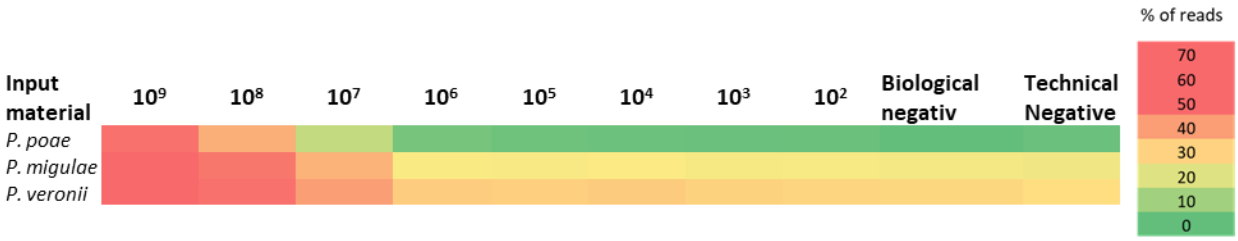


Figure 11 Impact of introduced contaminant on low and high biomass samples
Contaminations introduced post-DNA extraction majorly impacts low biomass samples. A dilution series from 10^9 to 10^2 total cells of a mock community consisting of 15 strains was prepared in triplicates and sequenced via NGS. A major contamination consisting of three OTUs was introduced in the downstream process after DNA extraction. The severity of contamination from the respective OTU is shown. Red = severe (70%), green= no contamination (0%). Shown is the mean of the triplicates.

The resequenced negative control was more diverse (Figure 12). 3.68 % of the pipeline control were mock species, 96.09 % contaminating OTUs. The most abundant contaminating OTUs were *Methylobacterium persicinum* (20.04 %), *Delftia acidovorans* (17.19 %), *Stenotrophomonas maltophilia* (14.87 %), *Bradyrhizobium denitrificans* (8.07 %) and *Achromobacter xylosoxidans* (7.24 %). These also appeared in a decreasing extent in the 10^3, and 10^6 datasets.

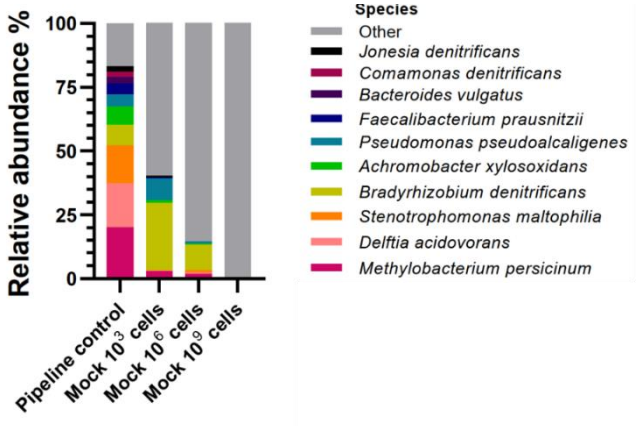


Figure 12 Top 10 contaminants in pipeline control and samples

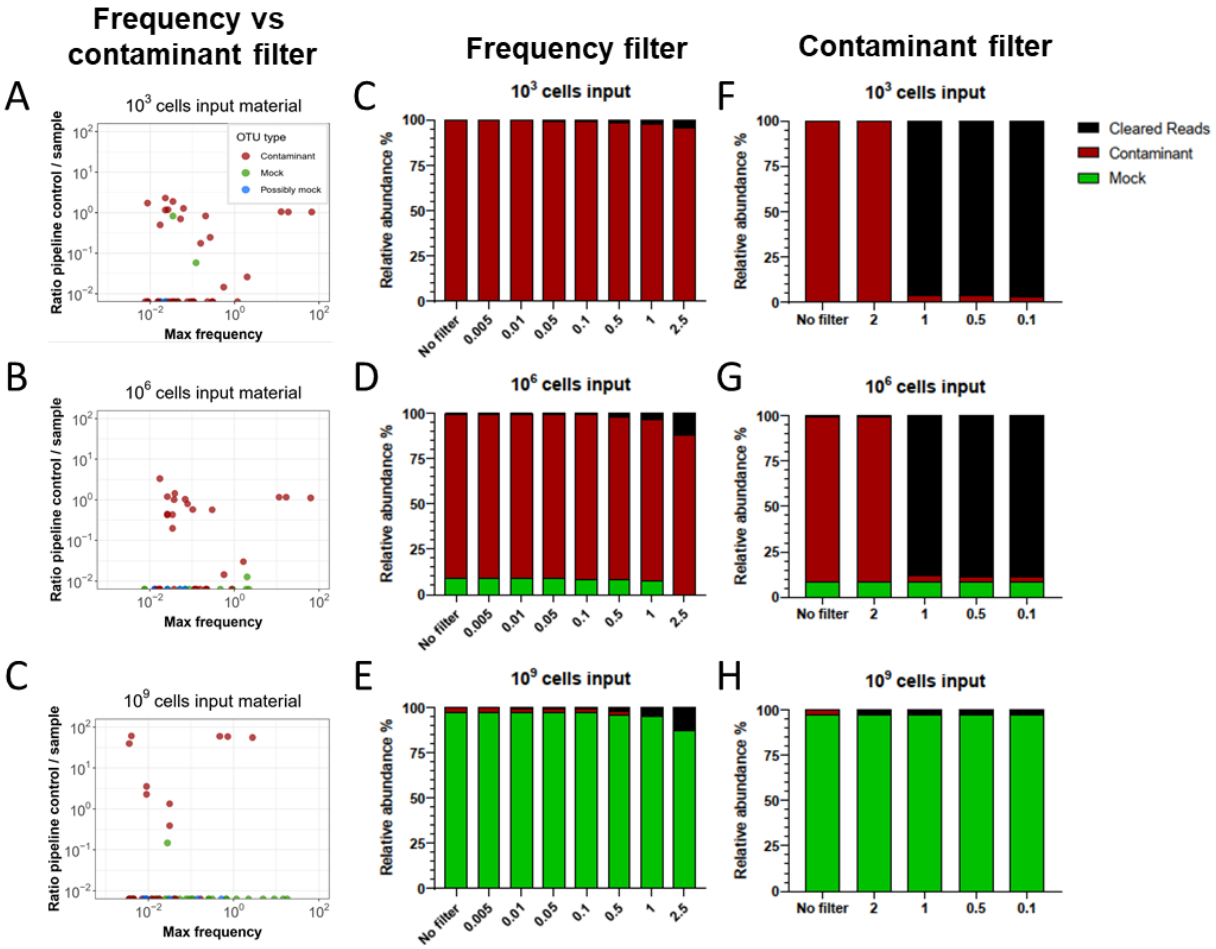
Contaminants detected in the pipeline control also appear in real samples. A mock dilution with 15 strains was diluted from 10^9 total cell to 10^2 cells in triplicates. Shown are the top 10 contaminants at species level of the pipeline control and the same species in the 10^3, 10^6 and 10^9 datasets with no major contaminant introduced in the downstream process after DNA extraction (resequenced dataset). Shown is the mean of triplicates.

3.1.6 Filtering with microbIEM

To further test the filtering efficiency of microbIEM, two different filters were compared using the contaminated and the resequenced dataset. Compared were a frequency filter, eliminating all OTUs which did not reach a certain minimum frequency in any sample and the ratio of mean frequency of the OTU in the sample divided by the mean frequency of the OTU in the pipeline control. OTUs were defined as mock, when the species annotation was one of the included mock species. If genus was correct but species different or not available, this OTU was defined as possibly mock. All other were labeled contaminant. The analysis was done for the dilutions 10^3, 10^6, and 10^9 cells input material.

3.1.6.1 Filtering of contaminated dataset

In the contaminated dataset it becomes apparent that the three contaminating OTUs have a higher frequency than all other OTUs in 10^3 and 10^6 input material dataset and still a high frequency in the 10^9 datasets (Figure 13 A-C). Therefore, the frequency cutoff failed to remove the contamination. In all dilution, the frequency cutoff also removed mock OTUs whereas it failed to remove the majority of contaminating sequences (Figure 13 A-F). Contrastingly, the contaminant specific filter based on the pipeline control efficiently removed the three major contaminating OTUs (Figure 13 G-I). However, the required cutoff also depends on the amount of input material. The calculated ratio of mean frequency sample divided by mean frequency pipeline control was 1.06 for 10^3 cells, 1.18 for 10^6 cells and 72.27 for 10^9 cells to remove all three major contaminants (Figure 13 J). With 10^3 cells input material, the contaminant frequency could be reduced from 99.8 % to below 4 % using a ratio stricter than 1 whereas the mock OTUs remained stable. The same pattern was seen in 10^6 cells where the percentage of contaminants could be reduced from 91.2 % to < 3 % with a filter ratio between pipeline control and sample of 1 or stricter whereas no mock OTUs were removed. In the 10^9 dataset, contaminants were reduced from 2.5 % to < 0.8 % whereas the percentage of mock OTUs remained stable (Figure 13 G-I).



	Mean frequency OTU in pipeline control/ mean frequency OTU in sample		
Contaminant	**10^3**	**10^6**	**10^9**
P. poae	1.06	1.18	72.27
P. migulae	1.08	1.22	79.08
P. veronii	1.09	1.20	75.08

Figure 13 Removal of contaminants from a contaminated dataset with frequency and contaminants filter

Removal of contaminants and not real sequences is more efficient using a contaminant specific filter than using a frequency cut-off in a post-DNA-extraction contaminated dataset. A mock dilution with 15 strains was diluted from 10^9 total cell to 10^2 cells in triplicates and sequenced via NGS. After the DNA extraction process, a major contamination was introduced. The removal efficiency of the minimum frequency filter and the pipeline control filter were compared 10^3, 10^6 and 10^9 datasets. Shown is the maximum frequency filter against the ratio of mean frequency of the OTU in the pipeline control divided by the mean frequency of the OTU in the sample for the dataset 10^3, 10^6, 10^9 (A-C). Green=mock OTU, red=contaminant (all OTUs which were not mock species), blue=possibly contaminants (OTUs which could only be annotated to genus level or could be missannotated). The relative frequency of mock OTUs (green), contaminants (red) and cleared frequencies (black) using a minimum frequency filter (D-F) or the pipeline control filter (G-I). The ratio of the mean frequency of the OTU in the biological control divided by the mean frequency of the OTU in the sample depends on the input material (J).

3.1.6.2 Filtering of resequenced dataset

A similar pattern was observed in the resequenced dataset (Figure 14). However, less contaminating sequences could be cleared. The maximum cleared in 10^3 was 19.6 %, and in 10^6 it was 14.9 %, without dramatically reducing the percentage of mock OTUs. In the dataset

with 10^9 cells input material, only 1 % of OTUs were contaminating sequences, which could neither be filtered completely with a frequency filter (reduction 0.4 %) nor the pipeline control filter (reduction 0.1 %). Looking at the top 10 contaminating OTUs from the resequenced negative control, 9 appeared in 10^3, 6 in 10^6 and 2 in 10^9 cells dataset. Using the pipeline control filter, most of these contaminants could be eliminated. In the 10^3 dataset 7 out of 9 (remaining: *Bradyrhizobium denitrificans*, *Pseudomonas pseudoalcaligenes*), in the 10^6 cells dataset 5 out of 6, and 2 out of 2 in the 10^9 cells dataset.

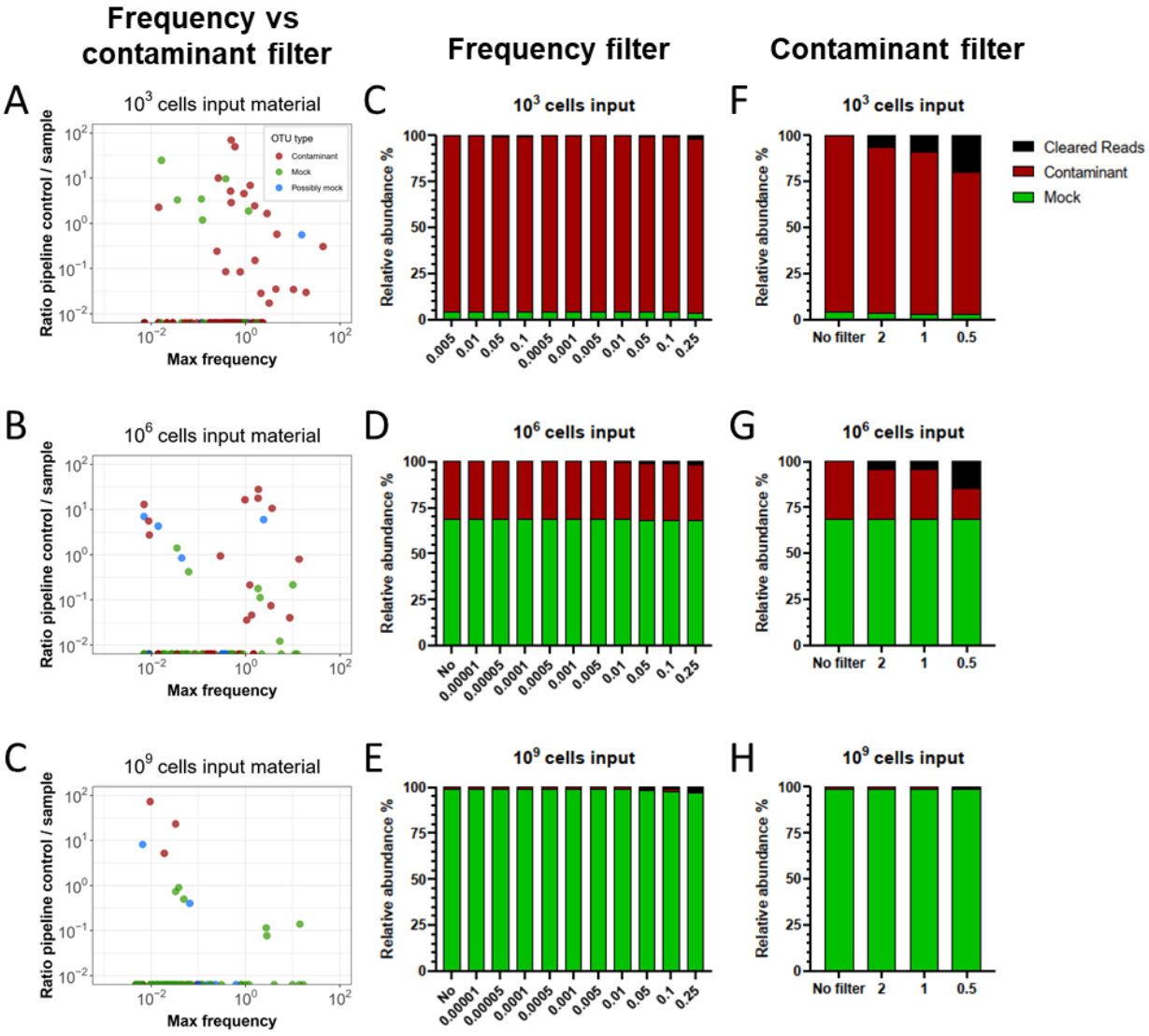


Figure 14 Removal of contaminants from a dataset with frequency and contaminants filter
Removal of contaminants and not real sequences is more efficient using a contaminant specific filter than using a frequency cut-off. A mock dilution with 15 strains was diluted from 10^9 total cell to 10^2 cells in triplicates and sequenced via NGS. The removal efficiency of contaminants of the minimum frequency filter and the pipeline control filter were compared for the 10^3, 10^6 and 10^9 cells datasets. Shown is the maximum frequency filter against the ratio of mean frequency of the OTU in the pipeline control divided by the mean frequency of the OTU in the sample for the dataset 10^3, 10^6, 10^9 (A-C). Green=mock OTU, red=contaminant (all OTUs which were not mock species), blue=possibly contaminants (OTUs which could only be annotated to genus level or could be miss annotated). The relative frequency of mock OTUs (green), contaminants (red) and cleared frequencies (black) using a minimum frequency filter (D-F) or the pipeline control filter (G-I).

3.2 Skin microbiome stability

3.2.1 Annual microbiome stability

To investigate the annual stability of the microbiome, 7 birch allergic (Ba) and 7 non-allergic (Na) people were recruited and sampled over one year. Samples were taken from the head and the skin of the Ac.

Comparing skin and head samples, the bacterial richness was significantly higher in skin samples (p-value < 0.001) (Figure 15A). Ba had a significantly higher bacterial richness in skin samples than Na (p-value<0.0001) but significantly lower Simpson Diversity (p-value<0.01) (Figure 15 B, C). The head samples consisted on average of 86.11 % in Ba and 81.44 % in Na of *Propionibacteriaceae* over the visits with low fluctuations except for visit 11 in Na (Figure 16A, B). Contrastingly, skin samples were more diverse (Figure 16C, D).

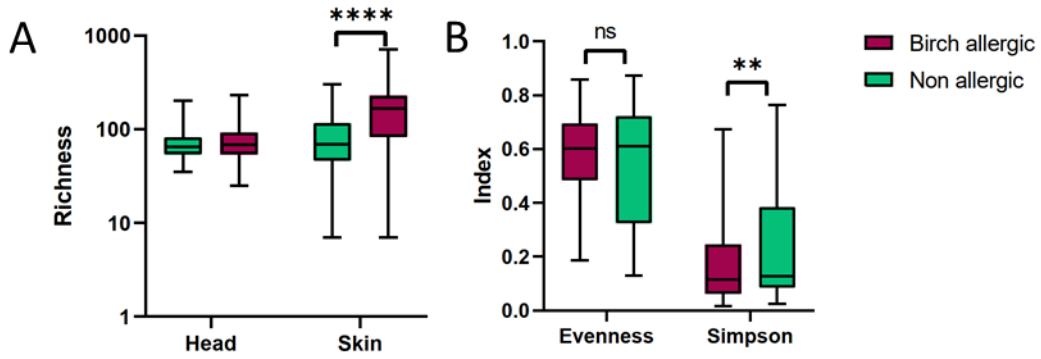


Figure 15 Alpha diversity of skin and head samples
The richness of head samples (Head, n=174) and volar forearm samples (Skin, n=173) are compared (A). The richness (B), evenness and Simpson diversity index are compared in skin samples between birch allergic (n=87) and non-allergic (n=86). n.s= not significant, **p-value 0.01, ****p-value 0.0001)

The most abundant families in the Ac in the Na and Ba group over all visits were *Propionibacteriaceae* (mean: Ba=29.99 %, Na= 22.59 %, p-value>0.05) and *Staphylococcaceae* (mean: Ba=23.06 %, Na=32.31 %, p-value>0.001) followed by *Corynebacteriaceae* (mean: Ba=8..13 %, Na=7.32 %, n.s.)(Supplement 1). There was no significant difference in the top 10 bacterial families over the visits.

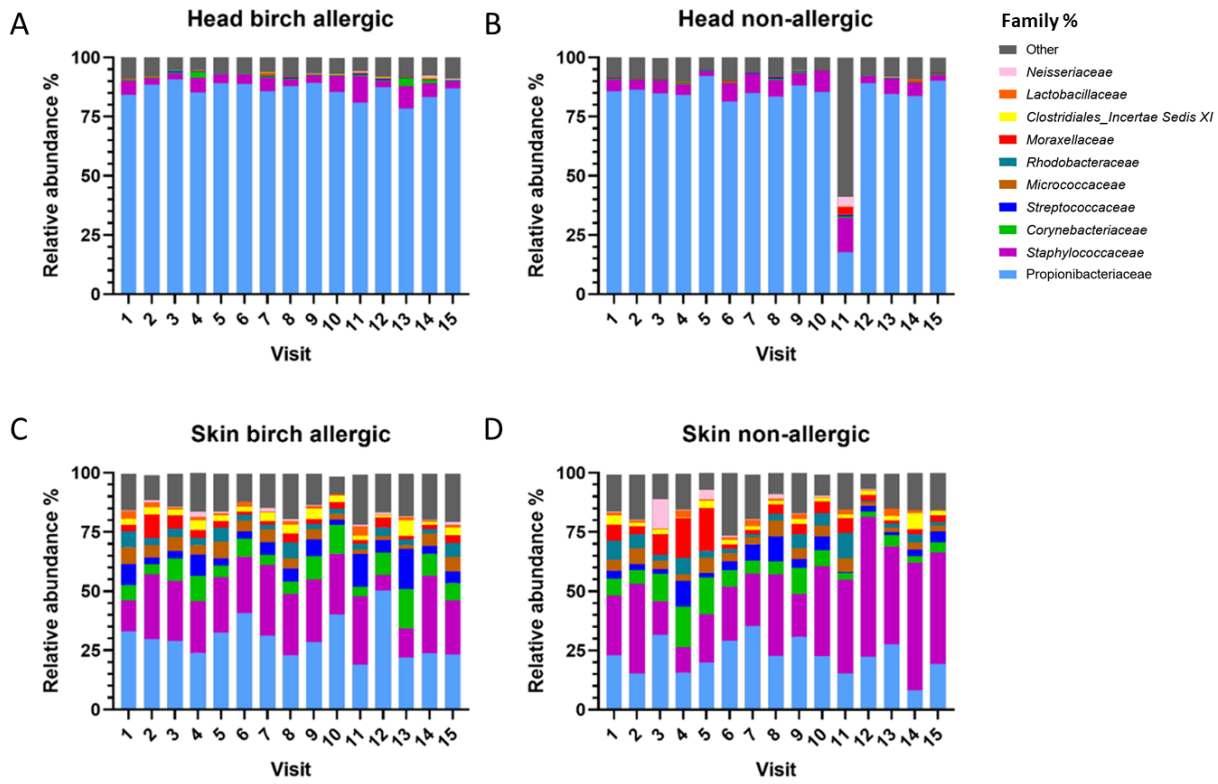


Figure 16 Top 10 bacterial families in head and skin samples over the visits.
Major differences between skin and head samples at family level. The mean of the top 10 bacterial families over the visits are shown for the head samples from birch allergic (n=7) (A), and non-allergic (n=7) (B) and for skin samples from birch allergic (n=7) (C) and non-allergic (n=7) participants (D). Not all participants were available at each visit (Supplement 2).

Due to the high standard deviation, it was necessary to look at individual levels (Figure 17 A-D). Interestingly, 2 out of 7 participants in the Na as well as Ba group were dominated by *Staphylococcaceae* over the majority of the visits (relative frequency > 50 % in 78.5 % of the samples). The other individuals were dominated by *Propionibacteriaceae*. Based on this observation, individuals were separated into the epitype *Staphylococcaceae* (Epitype S) and epitype *Propionibacteriaceae* (Epitype P). Although the abundance fluctuated over the year, the epitype remained stable over the year. The Epitype S and Epitype P differ significantly in the top 10 families *Staphylococcaceae*, *Propionibacteriaceae*, *Micrococcaceae*, and *Lactobacillaceae* (p-value<0.0001), and *Moraxellaceae*, *Rhodobacteraceae*, *Corynebacteriaceae*, *Streptococcaceae* and *Clostridiales Incertae Sedis XI* (p-value<0.001) (Figure 17 E). The Epitype S had 6 times higher abundance of *Staphylococcaceae* and 2 times higher abundance of *Streptococcaceae*, all other families were underrepresented in the Epitype S compared to Epitype P.

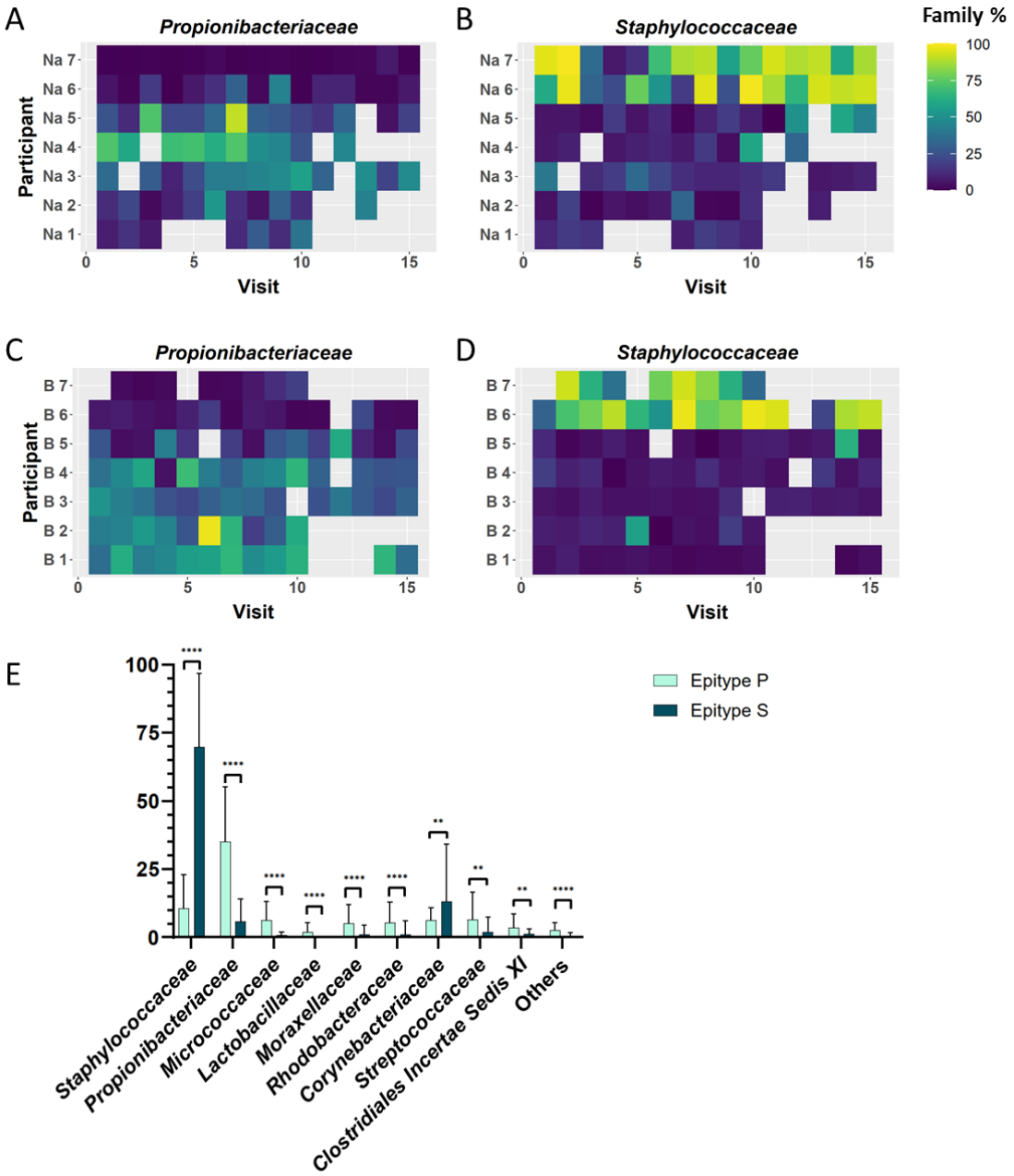


Figure 17 Propionibacteriaceae and Staphylococcaceae abundance at individual level of Non-allergics and Birch-allergics over the visits

Epitypes of indivudals are relatively stable in skin samples over one year. Shown are the individual abundance per participants of the two most abundant families *Propionibacteriaceae* and *Staphylococcaceae* over the visits separated by birch allergic (A, B) and non-allergic (C, D) in a heatmap. Darkblue = abundance 0 %, yellow= abundance 100 %. Based on dominating bacterial family, participants were separated into the *Staphylococcaceae* epitype S and the *Propionibacteriaceae* dominated epitype P. The differences between Epitype S and Epitype P for the top 10 families are shown (E). ** p-value < 0.01, **** p-value < 0.0001.

Looking at the species distribution within the groups Na and Ba, the family *Propionibacteriaceae* was dominated by *P. acnes* (now *C. acnes*) with an average of 99.9%. Contrarily, the *Staphylococcaceae* family was more diverse and were dominated by *Staphylococcus hominis* (Mean: Na=60.36 &, Ba=39.59%, p-value<0.0001) and

Staphylococcus epidermidis (mean: Na=26.86 %, Ba=41.57%, p-value>0.0001). There were no significant changes over the visits at species level in the Ba or Na group (Figure 18).

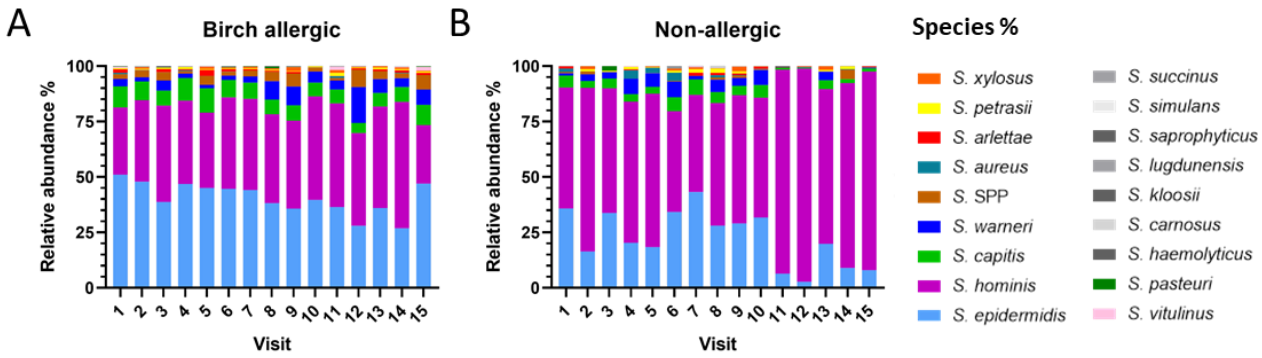


Figure 18 Staphylococci species in the Antecubital fossa of Non-allergics and Birch-allergics
S. hominis and *S. epidermidis* are the most abundant species of the *Staphylococcaceae* family in non-allergics and birch-allergics. The mean of the top 10 *Staphylococcus* spp. species abundance over the visits are shown for the skin samples from the antecubital fossa of birch allergic (n=7) (A), and non-allergic (n=7) (B). Not all participants were available at each visit (Supplement 2).

At individuum level, three out of four individuals belonging to the Epitype S were dominated by *S. hominis* (63.4% of samples more than 90% in Epitype S, contrasting 7.1% in Epitype P) whereas the other individuals were populated with higher numbers of *S. epidermidis* (Figure 19).

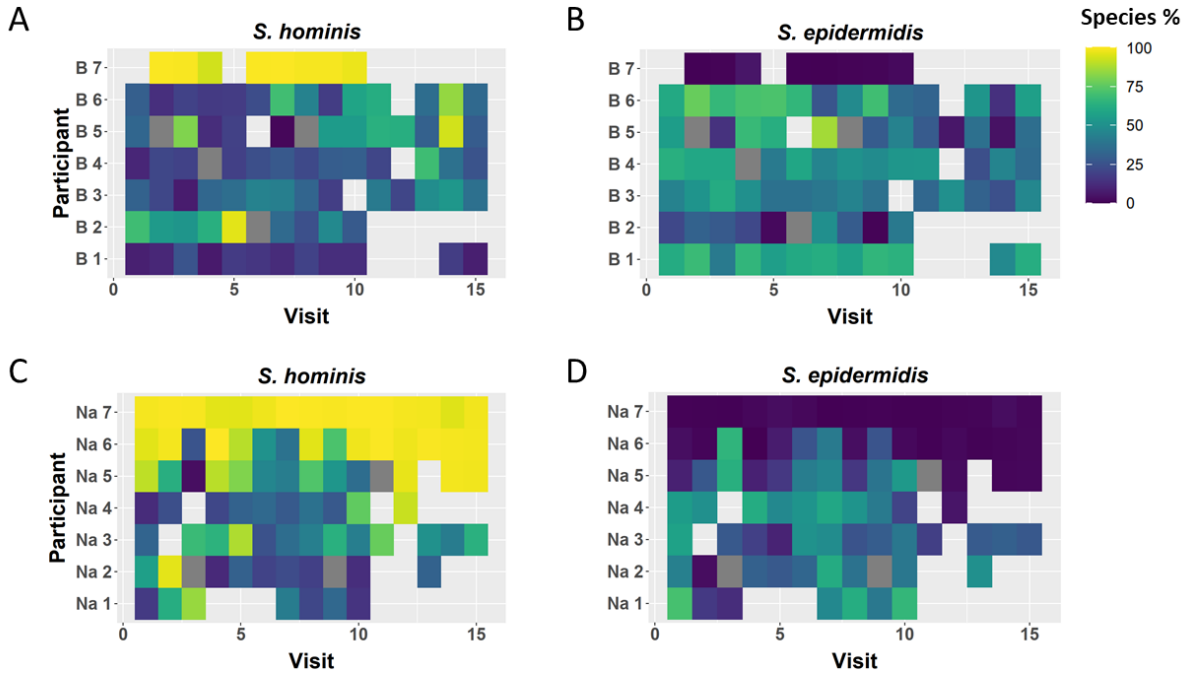


Figure 19 S. epidermidis and S. hominis abundance at individual level of Non-allergics and Birch-allergics over the visits
Whereas individuals of the epitype *Staphylococcaceae* are dominated by *S. hominis*, individuals of the epitype *Propionibacteriaceae* are dominated by *S. epidermidis*. Shown are the individual abundance per participants of the two most abundant *Staphylococcus* spp. *S. hominis* and *S. epidermidis* over the visits separated by birch allergic (A) and non-allergic (B) in a heatmap. Darkblue = abundance 0 %, yellow= abundance 100 %.

In accordance, in β-diversity analysis samples cluster according to participants whereas the visits do not separate, underlining again inter-individual differences and a high microbiome stability (Figure 20).

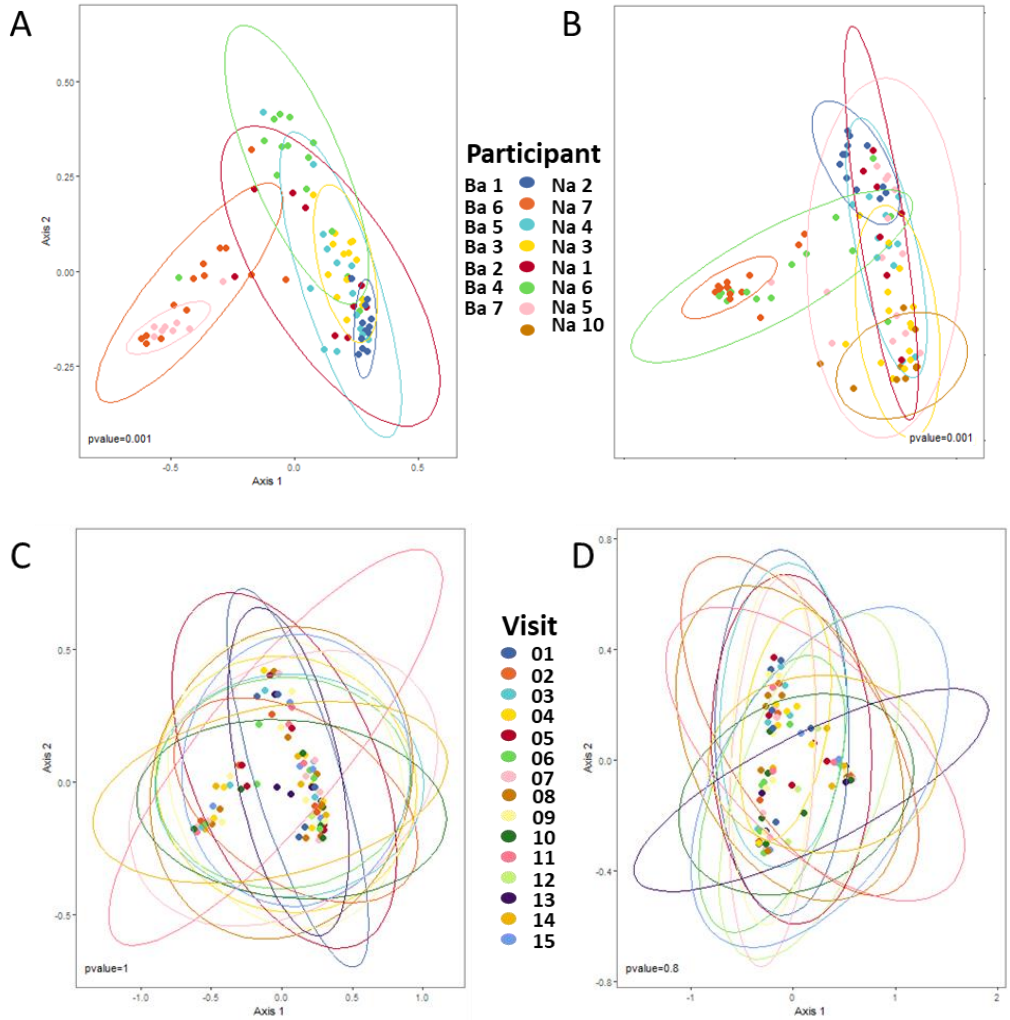


Figure 20 β-diversity clustering of participants and visits
The samples cluster according to participants in birch-allergic (A) and bon-allergic (B) participants but not visit 1 to 15 in birch-allergic (C) and non-allergic (D) participants.

3.2.2 Skin microbiome stability upon emollient application with different pH levels

To investigate the skin microbiome stability upon challenging with an emollient of either pH 5.5 or pH 8.5, a study was performed over eight weeks with weekly sampling for skin microbiome analysis and physiological and clinical measurements. The acidic skin pH is part of the healthy skin barrier and is known to be disturbed in atopic eczema patients. Therefore, six atopic eczema patients and six healthy controls were included. On one bodyside the emollient with pH 5.5 and on the other bodyside the emollient with pH 8.5 was applied (Hülpüsch et al., 2020).

The chapter was published as Hülpüsch, C, Tremmel, K, Hammel, G, et al. Skin pH–dependent *Staphylococcus aureus* abundance as predictor for increasing atopic dermatitis severity. Allergy. 2020; 00: 1– 11. https://doi.org/10.1111/all.14461 (Hülpüsch et al., 2020)

3.2.2.1 Skin microbiome

Before treatment, no differences between the bodysides were detected in any group in the skin microbiome parameters α-diversity and β-diversity or at taxonomy level. Looking at the global skin microbiome, the samples formed two clusters in PcoA analysis, HE and AD samples. There was no separation between the emollient treatments in both groups at any timepoint (Figure 21). (Hülpüsch et al., 2020)

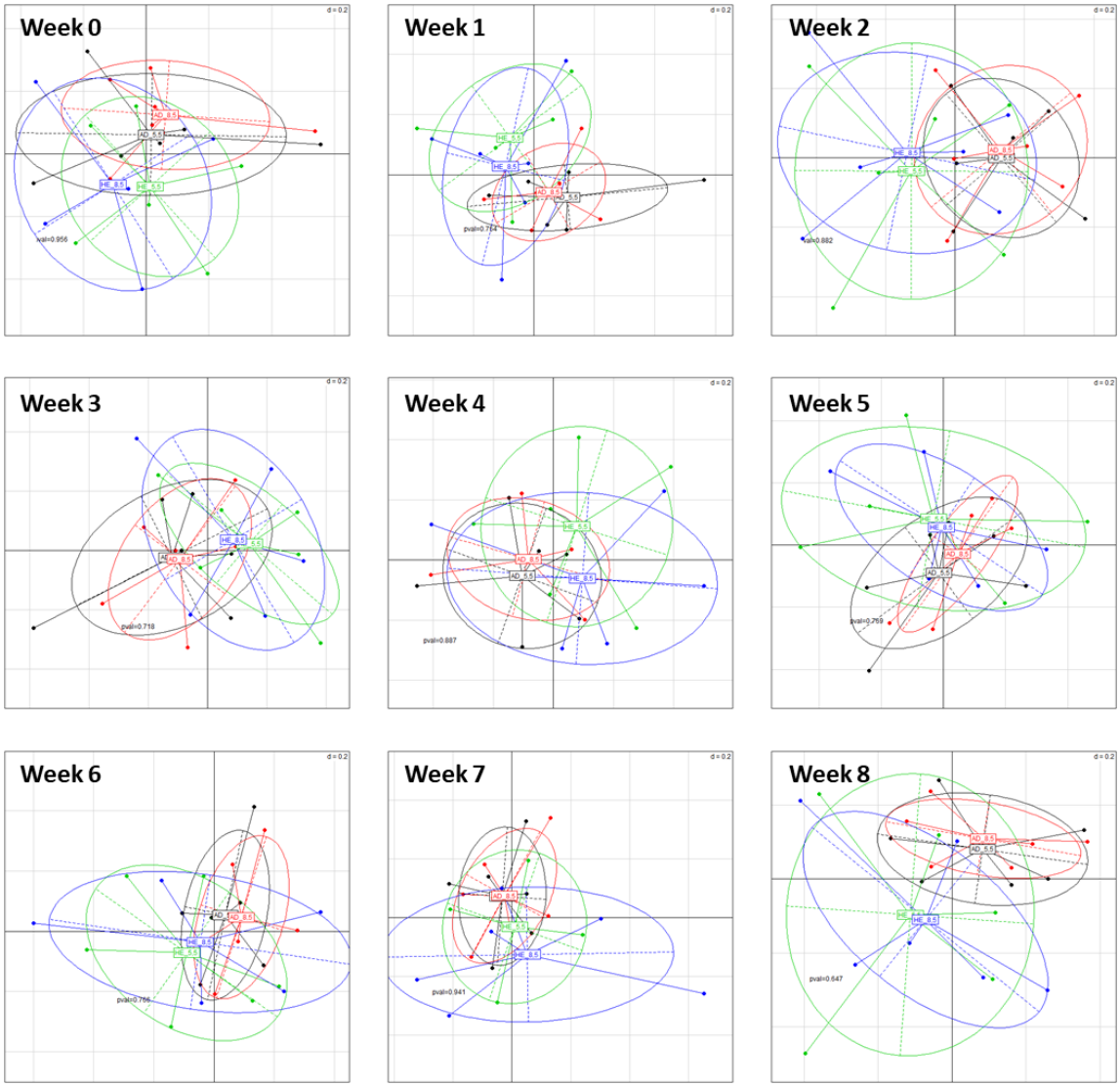


Figure 21 Global microbiome diversity at each visit for healthy and AD and pH 5.5 and pH 8.5 treatment

Samples cluster according to health status but not treatment. The β-diversity of six atopic dermatitis and 6 healthy controls treated with either pH 5.5 or pH 8.5 is shown for baseline without treatment and eight visits (Visit 0 to 9), each one week apart. Green = healthy, emollient pH 5.5; blue = healthy, emollient pH 8.5; black = atopic dermatitis, emollient pH 5.5; red = atopic dermatitis, emollient pH 8.5. HE = healthy, AD = atopic dermatitis.

Since changes in skin microbiome were independent of the emollient pH, data from the 2 body sides were combined for analyses of differences between AD and HE groups and longitudinal analysis. Comparing the HE and AD group, the richness was significantly higher in AD in week 2 and 5 (Figure 22 A), and evenness significantly lower at week 2 and 8 (p-value>0.05) (Figure 22 B). Robust differences between the HE and AD group at taxonomy level were significantly higher abundance of *S. aureus* on species level and lower abundance of *Micrococcaceae* on family level in AD (p-value<0.05). Over time, the skin microbiome of HE was stable, no consistent changes were observed. Contrastingly, in AD richness increased significantly at all timepoints except for week 3 whereas evenness remained stable (Figure 22 A, B). In accordance, *S. aureus* abundance significantly increased at all timepoints except for week 3 whereas *Lactobacillaceae* levels significantly decreased at all timepoints except for week 1 and 6 (p-value<0.05). Looking at individual AD patients, not all increased in *S. aureus* abundance (Figure 22 F). However, the results are qualitatively the same also when analyzed separately for each of the emollient applications. For longitudinal analysis, the mean of all measured physiological, clinical and microbiome parameters of early (week 0-2), mid (week 3-5) and late (week 6-8) phase of the study was calculated for samples of each body side separately to reduce fluctuations in the data. (Hülpüsch et al., 2020)

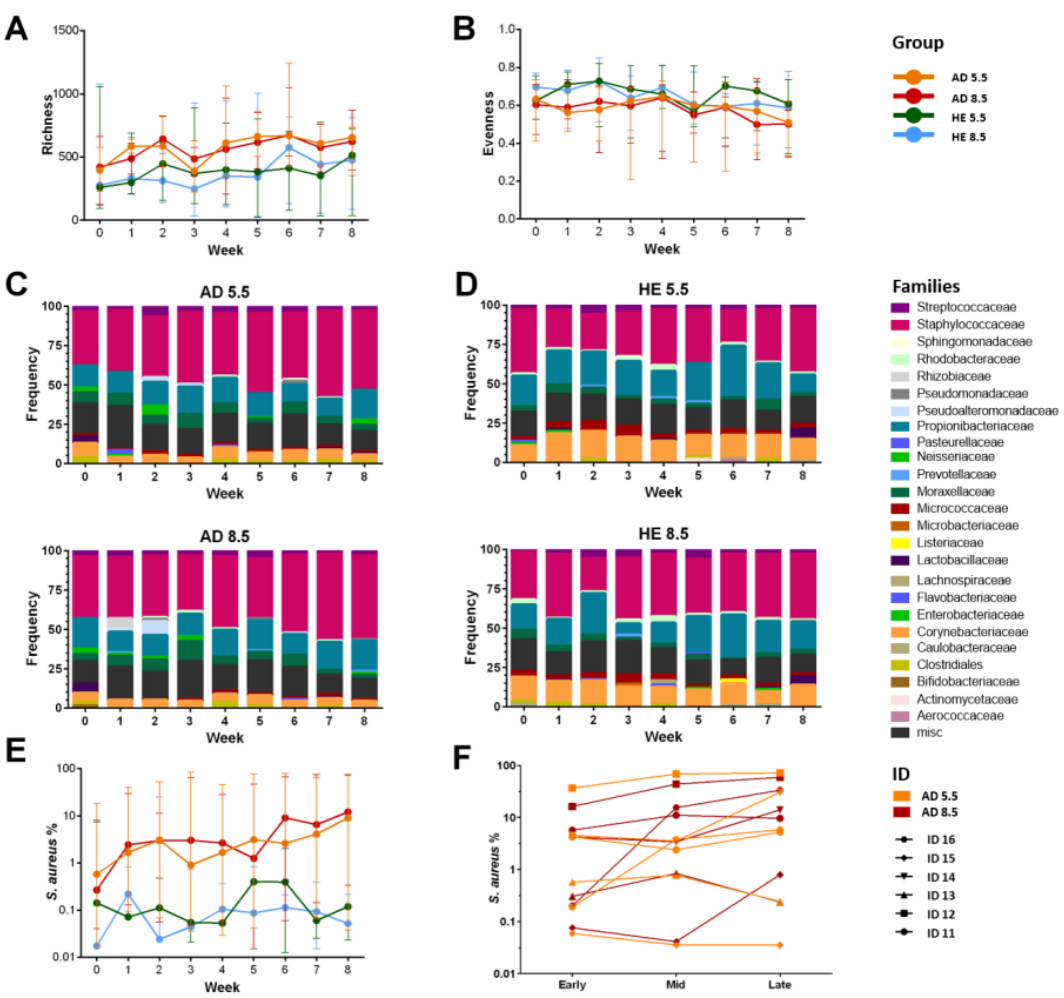


Figure 22 Skin microbiome analysis over the visits
Whereas the skin microbiome of healthy is relatively stable, the skin microbiome of atopic dermatitis patients is less stable, particularly *S. aureus* abundance increases over time. The median of richness (A) evenness (B) and *S. aureus* abundance (E) are shown for healthy control treated with emollient pH 5.5 (green), healthy control treated with emollient pH 8.5 (blue), atopic dermatitis patient treated with emollient pH 5.5 (orange) and atopic dermatitis patient treated with pH 8.5 (red). The median of the top 10 bacterial families are shown for atopic dermatitis patients with emollient pH 5.5 treated and emollient pH 8.5 treated (C) and for healthy controls emollient pH 5.5 and pH 8.5 treated (D). The individual *S. aureus* abundance per atopic dermatitis patient per arm is shown (F). Orange= atopic dermatitis patient emollient pH 5.5 treated, red=atopic dermatitis patient emollient pH 8.5 treated. HE = healthy (n=6), AD = atopic dermatitis (n=6) (Hülpüsch et al., 2020).

3.2.2.2 Disease severity

The local disease severity of AD, local SCORAD, was not affected by the emollient pH at any time point (Figure 23 A). The general severity score objective SCORAD increased in 5 out of 6 patients, EASI scores in 4 out of 6 (Figure 23 B, C) (Hülpüsch et al., 2020).

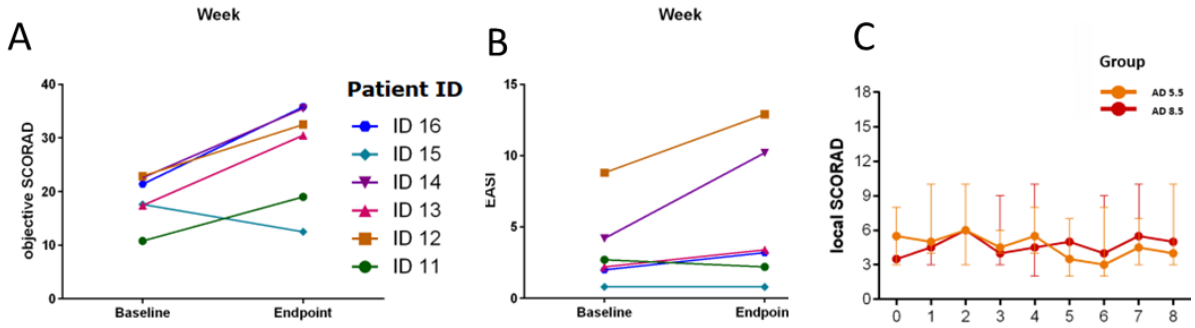


Figure 23 Disease severity in atopic dermatitis
The disease severity objective SCORAD (A) and EASI (B) of atopic dermatitis patients at baseline and endpoint are shown per individual. The median local SCORAD from visit 0 to 8 are shown for pH emollient pH 5.5 treated (orange) and emollient pH 8.5 treated (red). AD= Atopic dermatitis (n=6), HE=healthy (n=6). (Hülpüsch et al., 2020)

3.2.2.3 Connecting disease severity with *S. aureus*

In the early phase of the study, generally lower abundance of *S. aureus* was not correlated with local disease severity. However, in the mid and late phase of the study with increasing *S. aureus* levels, a strong correlation between *S. aureus* and disease severity was observed (R=0.79, p-value<0.01) (Figure 24 A-C). (Hülpüsch et al., 2020)

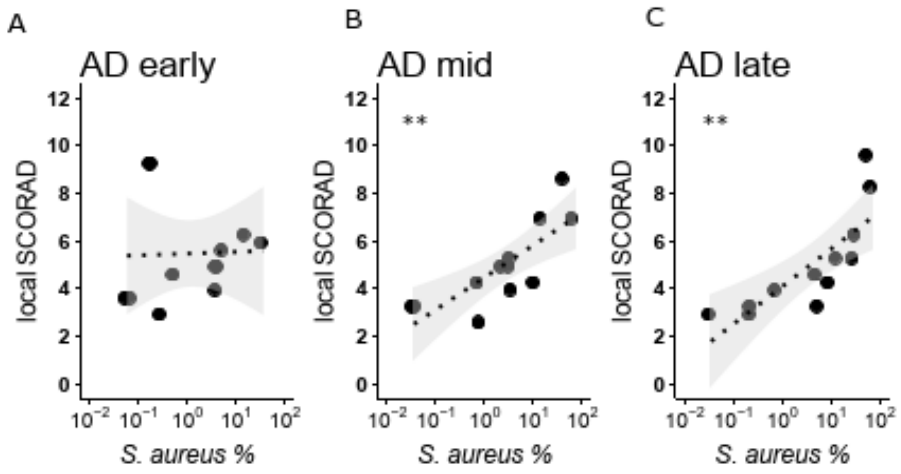


Figure 24 Correlation between S. aureus abundance and atopic dermatitis severity
In mid and late phase of the study, *S. aureus* abundance AD local severity are strongly correlated. The correlation of mean local SCORAD and mean *S. aureus* abundance in % are shown for early (week 0 to 2), mid (week 3 to 5), and late (week 6 to 8) phase of the study. **= p-value < 0.01. (Hülpüsch et al., 2020)

Interestingly, high baseline *S. aureus* was predictive for disease worsening at endpoint for change in local SCORAD (r=0.59, p-value<0.05) and change in EASI (r=0.64, p-value<0.05) but not change in objective SCORAD (Figure 25 A-D). (Hülpüsch et al., 2020)

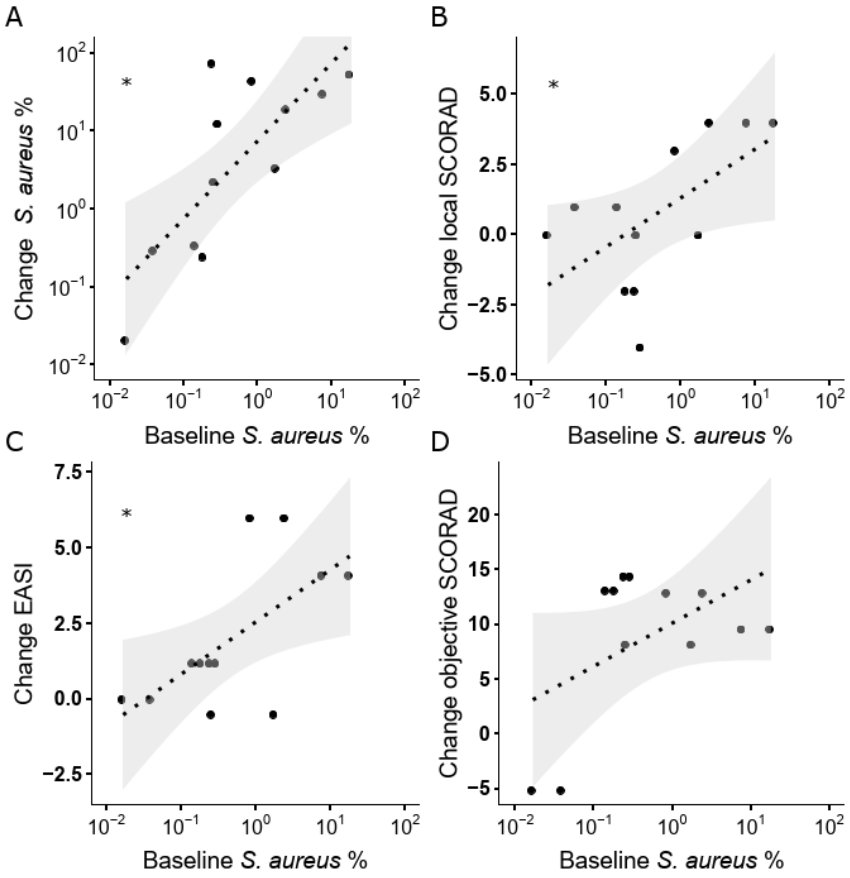


Figure 25 S. aureus as predictor for atopic dermatitis worsening Baseline *S. aureus* is a predictor for change of *S. aureus* abundance (A), change of local SCORAD (B), change of EASI (C) but not change of objective SCORAD, each time change from baseline to endpoint. *= p-value < 0.05. (Hülpüsch et al., 2020)

3.2.2.4 Skin physiology

At baseline, both bodysides of the HE and AD group revealed comparable skin pH, TEWL and hydration (Figure 26 A, B, C, respectively). Comparing AD and HE individuals over the study period, the TEWL was significantly higher in AD at all visits (median AD= 27.4, HE= 9.3; p-value 0.001) and the hydration lower in 6 out of 9 visits (median AD=27.8, HE 46.6; p-value<0.05). There was no difference in skin pH at any time point. The emollient pH had a significant effect on the skin pH of AD patients at week 2, 5 and 7, whereas on HE only a significant difference was observed at week 6 (p-value<0.05). However, looking at the absolute skin pH levels, the skin pH remained rather stable even though an emollient of pH 8.5 was applied. The median pH of the pH 8.5 emollient treated skin was 6.05 in HE and 6.04 in AD compared to pH 5.99 in HE and pH 5.81 in AD at the pH 5.5 emollient treated bodyside. (Hülpüsch et al., 2020)

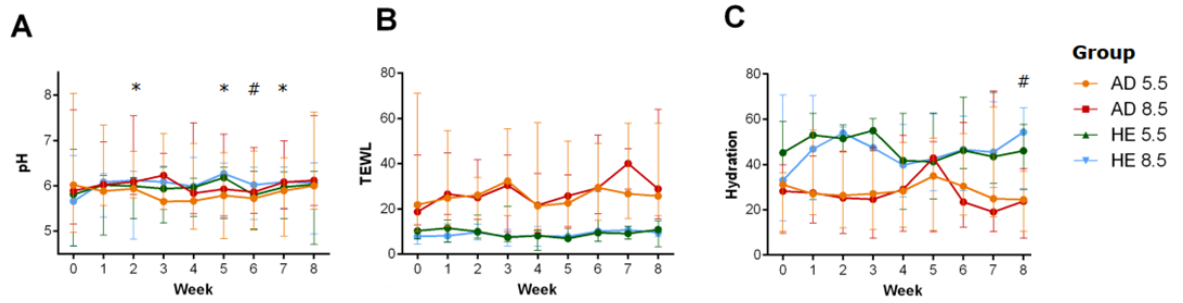


Figure 26 Skin physiology of healthy and atopic dermatitis patients over 8 weeks
The emollient pH slightly alters the skin pH especially in AD but not any other physiological parameters. Shown are the median pH (A), TEWL (B), and Hydration (C) for 6 AD patients and 6 healthy controls upon application with either acidic pH 5.5 or basic pH 8.5 emollient over eight weeks. Orange= AD pH 5.5, red= AD pH 8.5, green= HE pH 5.5, blue = HE pH 8.5. (Hülpüsch et al., 2020)

3.2.2.5 Connecting skin pH with skin physiology and AD severity

The skin physiology parameters are inter-correlated. In particular, the influence of pH on the other factors was of interest. Skin pH was positively correlated with TEWL at early, mid, and late phase of the study R>0.6, p< 0.5 (Figure 27 A-C). Contrastingly, there was a trend for an inverse association between skin pH and skin hydration at the late phase of the study with the same trend visible for early and mid-phase (R>0.5, p-value<0.1) (Figure 27 D-F). No correlation was visible between skin pH and local SCORAD (Figure 27 G-I). (Hülpüsch et al., 2020)

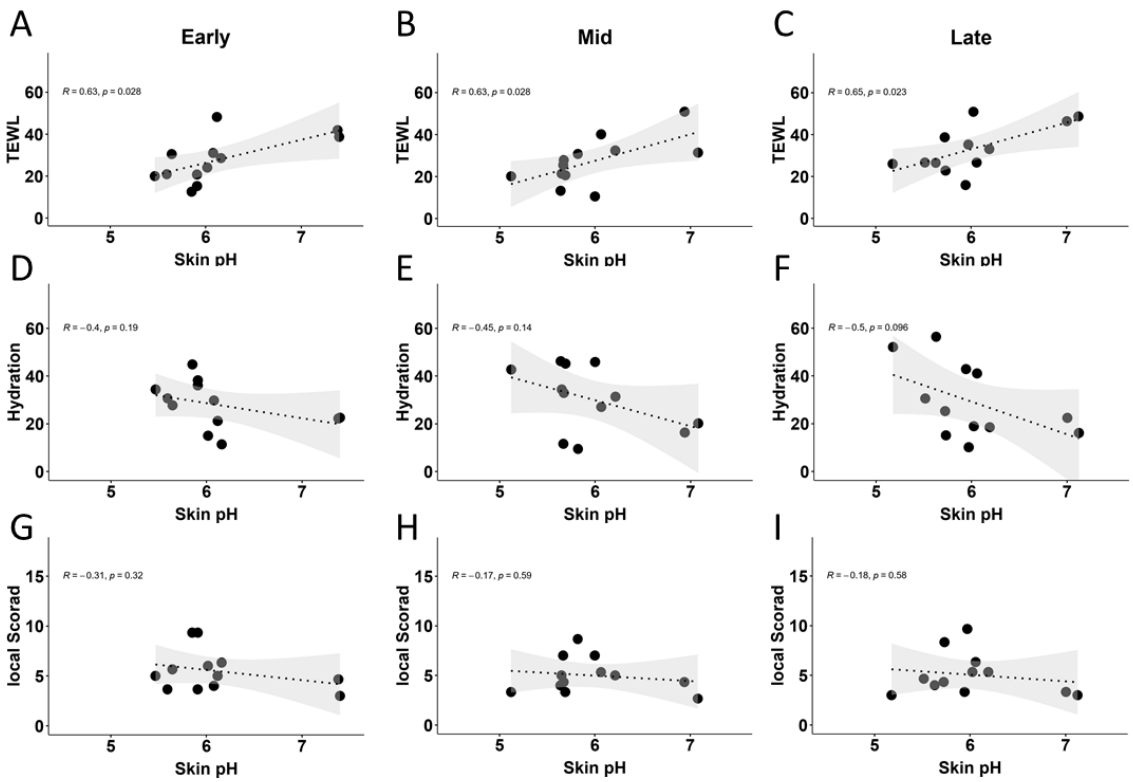


Figure 27 Correlation between pH and skin physiology parameters and local AD severity
Skin pH positively correlates with TEWL and there is a negative association with hydration. The correlation between skin pH and TEWL (A-C), hydration (D-F) and local SCORAD (G-I) are shown for early (mean week 0 to 2), mid (mean week 3 to 5), and late (mean week 6 to 8) phase of the study. * p-value < 0.05, + p-value < 0.1. (Hülpüsch et al., 2020)

3.2.2.6 Connecting skin physiology with *S. aureus*

In AD, higher *S. aureus* levels than 10 % were only found in a pH range from 5.7 to 6.2 in early mid and late phase of the study (Figure 28 A-C). Only in this pH range, a strong increase of *S. aureus* was observed over time with an association of higher local SCORAD. Lower or higher skin pH was associated with lower *S. aureus* frequencies. Low hydration level was correlated in the early and associated in the mid and late phase of the study with higher *S. aureus* levels (Figure 28 D-F). Contrastingly, TEWL was not associated with *S. aureus* in any phase of the study (Figure 28 G-I). (Hülpüsch et al., 2020)

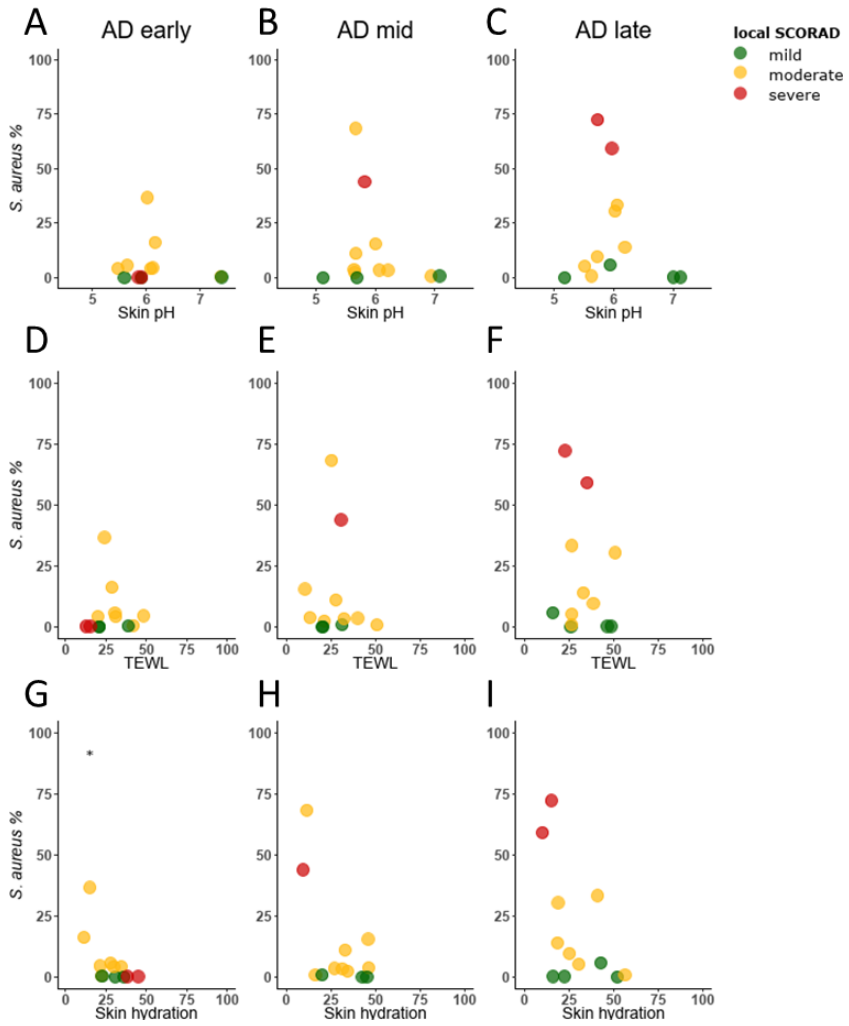


Figure 28 Connection of skin physiology and S. aureus abundance

Skin pH of 5.7-6.2 and low hydration are associated with high *S. aureus* abundance. *S. aureus* abundance is shown as function of skin pH (A-C), TEWL (D-F) and skin hydration (G-I) at early (mean week 0-2), mid (mean of week 3-5) and late (mean of week 6-8) phase of the study. Colors correspond to local SCORAD values (green = mild (<5), yellow = moderate (5-8), red = severe (>8). Significant correlations between the physiological parameters and *S. aureus* are marked with * ($p < 0.05$). (Hülpüsch et al., 2020)

3.3 Characterization of *S. aureus* isolates from HE and AD individuals

3.3.1 Establishment of a bacterial strain collection

To gain a deeper knowledge about the differences of *S. aureus* strains, a bacterial strain collection with bacterial strains isolated from either the skin (AD; NL or LS) or the nose (AD, HE) was established. From each swab, an original streak-out on Mannit agar was used to distinguish between *S. aureus* and CoNs. From each plate, if possible, five *S. aureus* and five CoNs colonies were picked, purified, and identified via MALDI-TOF. This way, a bacterial strain collection of 328 *S. aureus* strains and 164 CoNs could be build up (19.07.2020).

3.3.2 Characterization of *S. aureus* strains in different pH environments

The skin environment of AD patients is characterized by a higher skin pH in rather neutral pH conditions compared to the acidic skin pH 5 in HE individuals. Naturally, *S. aureus* prefers neutral pH conditions. To understand, why there is an increase of relative abundance of *S. aureus* in AD patients, we investigated whether *S. aureus* isolates from AD are more adapted to this microenvironment. Furthermore, we hypothesized that isolates from HE are more adapted to an acidic environment. Therefore, isolates from HE and AD individuals were cultivated for 16 hours until stationary phase in buffered liquid LB medium at either pH 5.5, 6.0, 6.5, 7.0, 7.5 or 8.0, so the pH range found on HE and AD skin.

Looking at the growth curve of all strains together, it is apparent that in all pH conditions, there is a decrease in growth rate at an OD of 0.5, which could be a diauxic shift. However, at pH 6.0, this shift is especially long Figure 29A. For further analyses, the area under the curve (AUC) was calculated for each strain. The maximum growth appeared at pH 7.0, with no significantly reduced growth at pH 7.5, and pH 6.5, whereas pH 5.5, 6.0 and 8.0 showed significantly reduced growth and 8.0 ($p<0.0001$). The lowest growth overall was observed at pH 5.5 (Figure 29 B). This pattern was true for strains from HE nose, AD nose and AD skin samples (Figure 29 C).

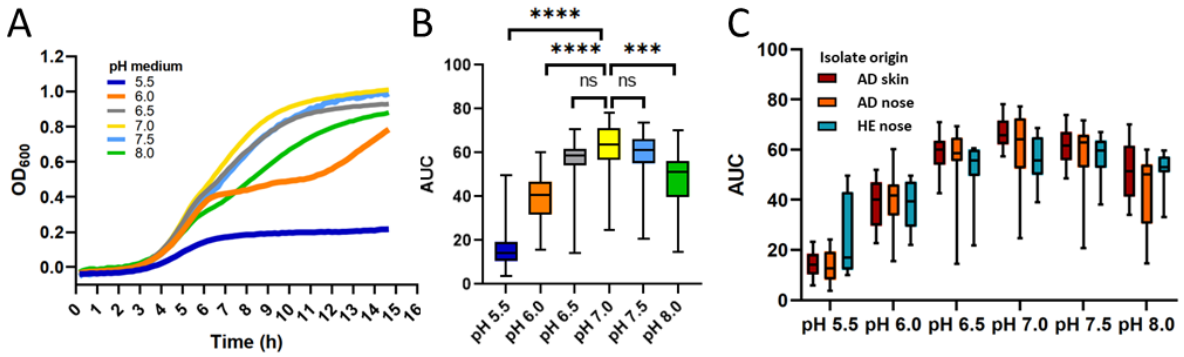


Figure 29 Growth behavior of S. aureus isolates in different pH conditions
Growth optimum of *S. aureus* isolates is at pH 6.5 to 7.5. Shown is the overlay of all growth curves for *S. aureus* isolates at pH 5.5 to 8.0 in 0.5 steps, revealing a diauxic shift at OD_{600} of 0.5 (A). The area under the curve was calculated for all isolates for the growth conditions pH 5.5 to pH 8.0 together (B) and separated by health status (atopic dermatitis, healthy) and location (skin, nose). AD=atopic dermatitis, HE=healthy, AUC=area under the curve. Significant differences between the area under the curve are marked with n.s.=not significant, *** ($p < 0.001$), ****($p<0.0001$). Figure 29A adapted from the Master Thesis of Katherine Wald.

For each strain, the individual change of the AUC to the AUC at pH 7 where maximum growth was observed was calculated. Comparing AD samples taken from the nose and skin, there were no significant differences between AUC change to AUC at pH 7 of AD nose and AD skin isolates at any pH condition (Figure 30 A). Therefore, the samples were treated as one group AD in further analysis. AD isolates had a significantly higher negative AUC change compared to pH 7 at pH 5.5 (p-value 0.01) and pH 6.0 (p-value 0.05), hinting towards a better adaption of HE isolates to an acidic microenvironment. At the more neutral pH 6.5, 7.5 and 8.0 there was no significant difference observed between HE and AD isolates (Figure 30 B).

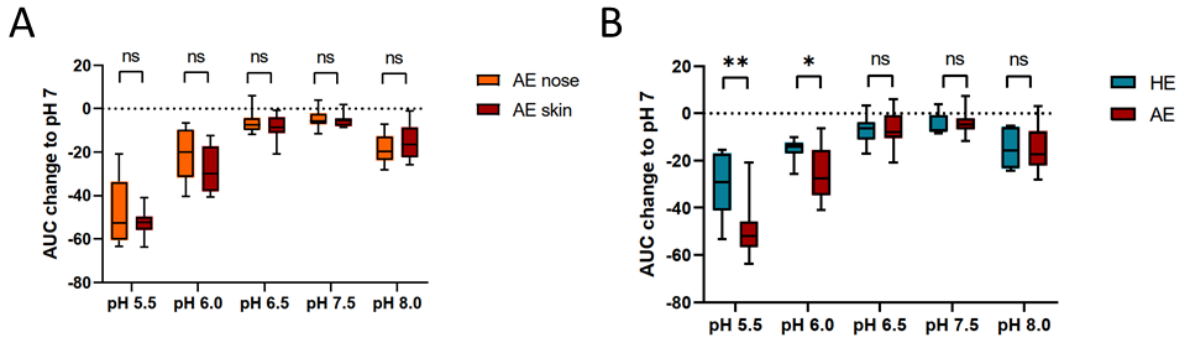


Figure 30 Adaptation of strains from healthy individuals and atopic dermatitis patients to an acidic microenvironment
S. aureus strains from healthy individuals are better adapted to an acidic environment than strains from atopic dermatitis patients. No significant difference the area under the curve between strains from atopic dermatitis patients from the nose (orange) or the skin (red) were identified at pH 5.5 to pH 8.0 in 0.5 steps (A). Therefore, nose and skin isolates were taken together (red) and the area under the curve was compared to the one of strains from healthy individuals (blue) at pH 5.5 to pH 8.0 in 0.5 pH steps (B). Significant differences between the area under the curve between healthy and atopic dermatitis *S. aureus* isolates are marked with n.s.=not significant, * ($p < 0.05$), **($p<0.01$).

3.3.3 Characterization of toxin patterns of *S. aureus* strains

To identify, whether *S. aureus* isolates from HE and AD individuals differ in their pathogenicity, the isolates were tested for the Staphylococci enterotoxin sea, seb, sec, seg, seh, *sek* and *tst* as well as *pvl* and *mecA* via a multiplex PCR. For each individuum, only one isolate was chosen per nose and skin region.

From 39 tested isolates, 17 had no toxins (43.59 %), 9 had 1 toxin (23.08 %), 11 had 2 toxins (28.2 %) and 2 had 3 toxins (5.13 %). The most abundant toxin was seg which was present in 21 strains, followed by *sec* (6 strains). Interestingly, *sea*, *seb*, and *sek* were only detected in AD isolates but not HE. The genes *seb* (3 isolates) and *sek* (1 isolate) only occurred in isolates from patients with severe AD (SCORAD < 40) and not at all in HE. No other differences between HE and AD could be determined with this number of isolates (Figure 31).

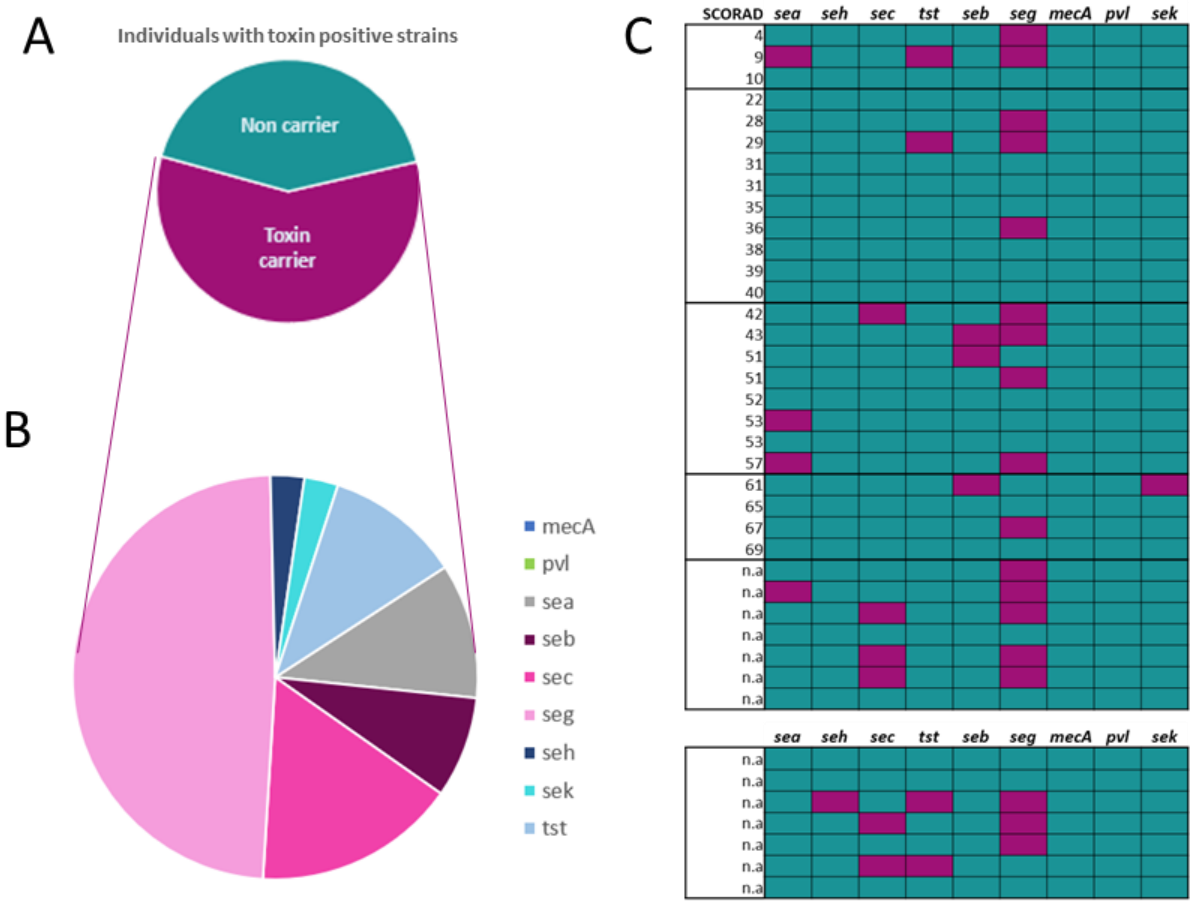


Figure 31 Toxin pattern of S. aureus isolates from healthy individuals and atopic dermatitis patients

No striking difference in toxin pattern were observed between *S. aureus* isolates from healthy and atopic dermatitis individuals. The % of patients which carry a strain with any toxin and no toxin are shown in A. The percentage of toxins which were observed in toxin positive strains are shown in B. However, one strain could carry multiple toxins as shown in C. Each line represents one strain, each row one toxin. Violet=positive, turquois=negative. The SCORAD is listed in the first column.

3.4 Quantification of skin bacteria

As the agr system which regulates virulence in *S. aureus* is among others regulated by cell number, not only the change in distribution but also the absolute cell numbers are important in the context of AD. Therefore, we developed two concepts for absolute quantification: 1) spike-in of bacterial species alien to the human microbiome (*Imtechella halotolerans*) as calibrator in NGS results and 2) a multiplex qPCR specific for 16S rRNA copies as proxy for total bacterial cells, a unique *Staphylococcaceae* gene (*tuf*) and a unique *S. aureus* gene (*nuc*). On top, a qPCR primer-probe combination specific for *I. halotolerans* (*nhaC*) was developed. The genes for *Staphylococcaceae*, *S. aureus* and *I. halotolerans* have only one copy in the respective genomes so that each detected copy refers to one cell.

To find the ideal number of *I. halotolerans* cells for spike-in, a pilot experiment was conducted, where 1000, 2000 and 4000 cells were spiked into skin samples from HE and AD individuals before extraction with the standard DNA extraction protocol. Via qPCR, in HE for 1000, 2000 and 4000 cells on average 1695, 2142 and 5443 cells were detected. In AD, 3466, 4090 and 7457 cells, respectively. This was from 0.2 to 0.47 % of the 16S copies in AD and 7.17 to 12.27 % in HE. In NGS, the detected frequencies were between 0.21 to 0.35 in AD and 3.65 to 6.83 in AD. For further experiments, 2000 *I. halotolerans* cells were chosen for spike-in (Supplement 3).

In an experiment with 360 AD patients and 2000 cells of spike-in, *I. halotolerans* was not detected in 105 samples (30,0 % of the samples). On average, 350 cells were detected (Figure 32 A). The number of detected *I. halotolerans* cells was not correlated with total 16S copy numbers (Figure 32 B). Via NGS, in only 5 out of 350 samples (1.43 %) *I. halotolerans* could not be detected (Figure 32 C). In NGS, *I. halotolerans* frequency was 1.15 %. There was no correlation with total reads (Figure 32 D).

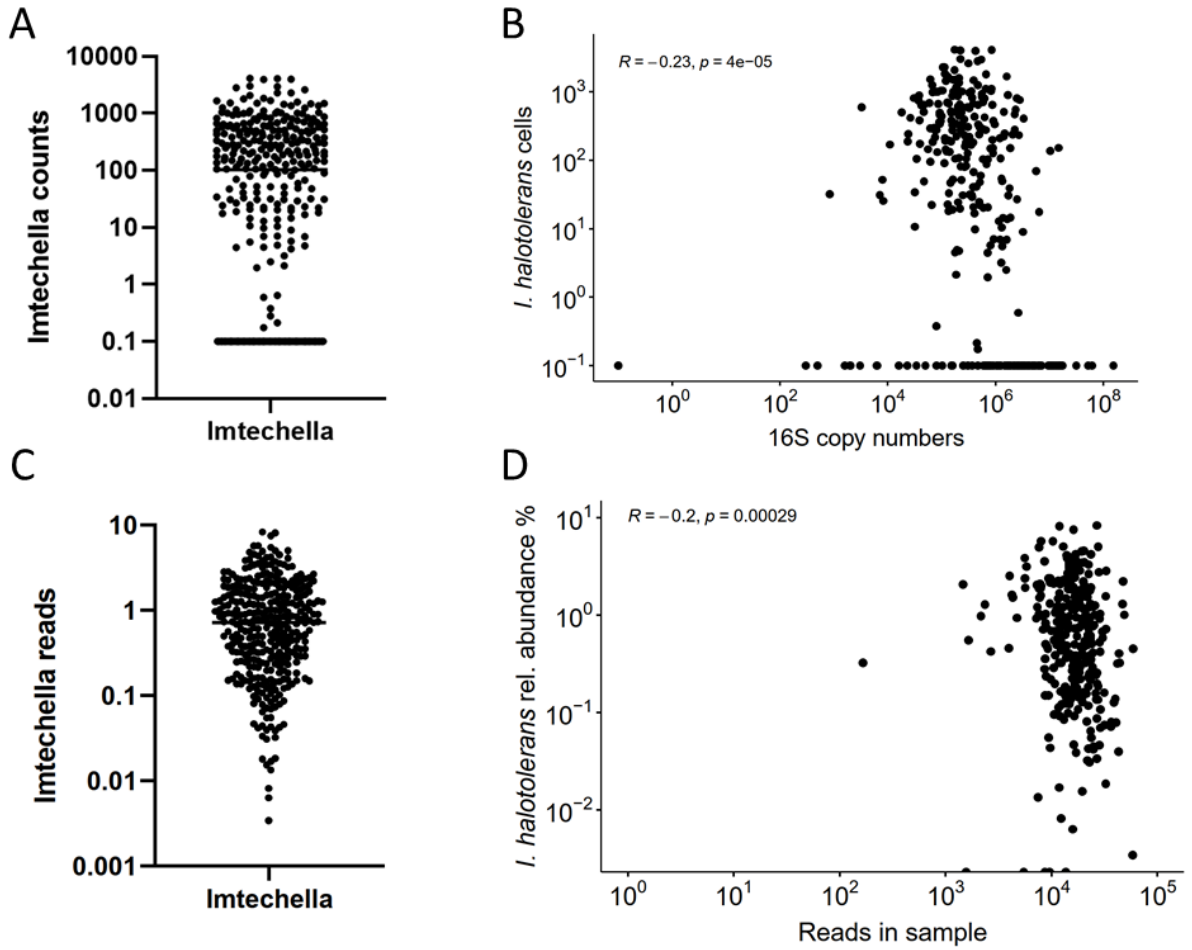


Figure 32 Absolute quantification via spike-in with Imtechella halotolerans
For absolute quantification, 2000 cells of *I. halotolerans* were spiked-in to 375 skin microbiome samples. The cells were detected via qPCR (A). The number of detected *I. halotolerans* cells were independent from observed total 16S copies (B). Furthermore, the relative frequency of *I. halotolerans* in each sample in next generation sequencing results is depicted (C). The frequency of *I. halotolerans* was independent from total reads (D).

Next, the impact of the established sampling methods for skin microbiome analyses, tape stripping and swabbing on the amount of recovered biomaterial (Figure 33). However, the comparison was not from one person (paired); instead, data from studies with either tape stripping or swabbing was compared. Compared were samples from HE, AD NL skin and LS skin. In HE, AD NL and AD LS, swabbing yielded significantly more 16S rRNA copies than tape-stripping (mean swab=10^6 cells, mean tape strip 10^4 cells, p-value<0.0001) (Figure 33 A). In accordance, significantly more *S. aureus* cells were detected in all three groups (Figure 33 B).

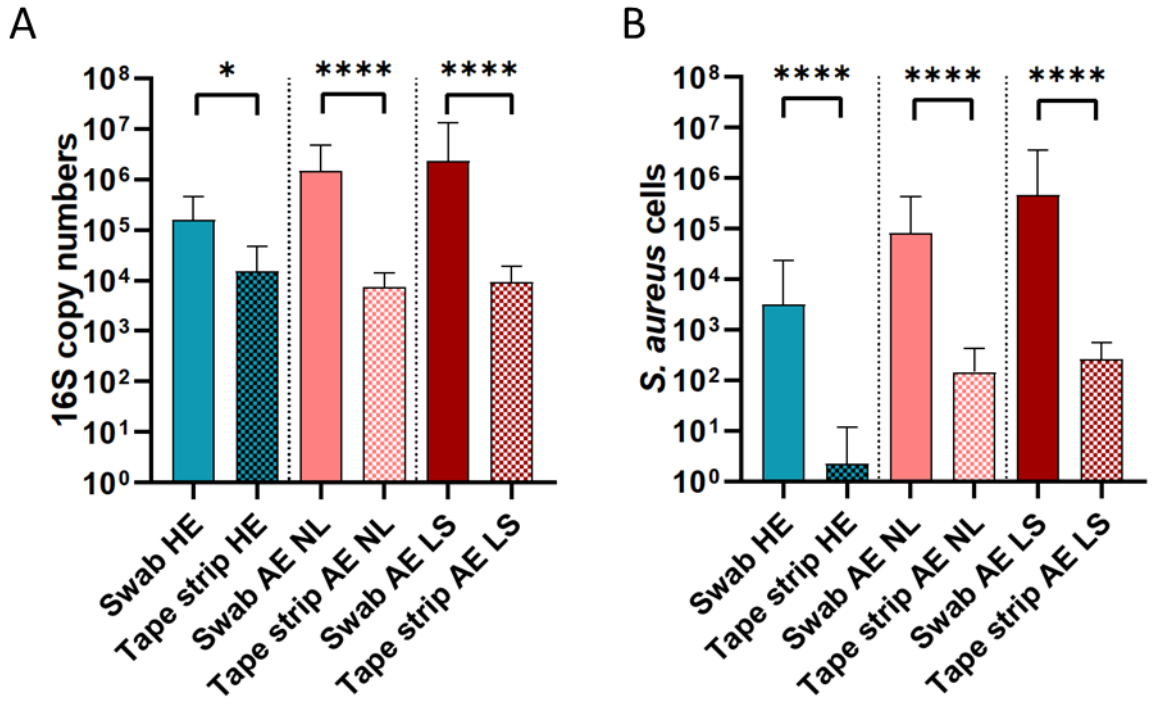


Figure 33 Difference in biomass obtained from the skin via tape stripping and swabbing
More biomass was obtained via swabbing compared to tape-stripping. The number of 16S copies (A) and *S. aureus* cells (B) per sampling method (swabbing=clean, tape-stripping=dotted) are depicted for healthy (blue), atopic dermatitis non lesional (pink), atopic dermatitis lesional (red). HE=healthy, AD=atopic dermatitis, NL=non-lesional, LS=lesional. Significant differences between the sampling techniques are marked with * ($p < 0.05$), ****($p<0.0001$).

Via swabbing, significantly less 16S rRNA copies were recovered from HE compared to AD NL and AD LS (HE=$1.62x10^5$, AD NL=$1.50x10^6$, AD LS=$3.27x10^6$, p-value 0.0001) (Figure 34 A). There was no difference between AD NL and AD LS. From AD LS samples, significantly more *S. aureus* cells were recovered compared to HE and AD NL (HE=$3.77x10^2$, AD NL= $8.43x10^4$, AD LS=$7.16x10^5$, p-value 0.001) (Figure 34 B). The difference between HE and AD NL was not significant. Contrastingly, no differences in 16S copy numbers were observed when the samples were taken via tape-stripping between any of the groups (Figure 34 C). Significantly more *S. aureus* cells were detected in AD NL and AD LS samples compared to HE (p-vlaue<0.0001) (Figure 34 D).

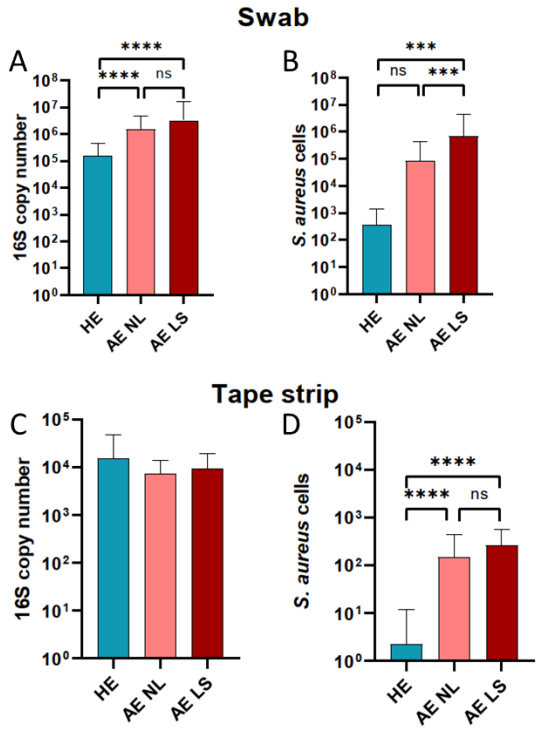


Figure 34 Biological differences between HE skin, AD NL and AD LS

Whereas swabbing reveals that healthy carry significantly less bacteria than atopic dermatitis patients, this is not seen in tape-stripping as the limit of capacity is reached at 10^4 16S copies. The difference in detected 16S. copies (A) and *S. aureus* cells (B) is shown for swabbing and tape-stripping (C,D). Blue=healthy, pink=atopic dermatitis non-lesional, red= atopic dermatitis lesional. HE=healthy, AD=atopic dermatitis, NL=non-lesional, LS=lesional. Biological significant differences are marked with n.s= non-significant, *** ($p < 0.001$), **** ($p>0.0001$).

To evaluate, if the number of bacteria detected as 16S rRNA copies and *S. aureus* cells remain stable over a period of time, the samples from the study with the pH emollient challenge were quantified (2.3.2 BampH study).

Interestingly, no significant difference in total 16S copies or *S. aureus* numbers were detectable between the bodysides at any timepoint (Figure 35 A-D). Therefore, the bodyside was not considered for further analysis. Interestingly, in the HE group a higher individuum dependent variation of 16S copies was observed. However, in both, the bacterial load per patient remained stable over time (Figure 35 E, F). No robust difference was found in 16S copies between HE (median=$7.5x10^4$, min=$1.46x10^2$, max=$1.62x10^7$) and AD individuals (median=$4.59x10^5$, min=$2.27x10^3$, max=$5.02x10^6$) in this study. Whereas the 16S copy number was stable over time in AD patients, in HE there were significant differences to baseline in week 1,2, 6 and 7 (p-value < 0.05). Contrastingly, significantly more *S. aureus* cell were detected in AD in all weeks except for week 3 (p-value <0.05). Median *S. aureus* load of all samples in AD was $8.6x10^2$ cells (min= <detection limit, max=$6.06x10^5$) and $6.8x10^1$ cells in HE (min=<detection limit, max=$2.25x10^5$). The general *S. aureus* load in AD increased significantly compared to baseline in all weeks except for week 3. In HE, *S. aureus* load was fluctuating and is significantly different to baseline in week 1, 3 and 5. Looking at individuals *S. aureus* levels, 2 out of 6 AD patients and 4 out of 6 HE individuals do not carry considerable numbers of *S. aureus* during the course of the study (Figure 35 C,D). In both, 16S copies and *S. aureus* abundance, HE 1 was identified as outlier. As this participant was the oldest in the cohort, the

effect of age was further investigated and people older than 60 were identified as outlier in the HE and AD group. When excluding HE 1 and AD 12 from the analysis, there is a significant difference between HE and AD individuals in 16S rRNA copies as shown before (median HE= $5.24x10^4$, AD median= $4.41x10^5$, p-value<0.0001).

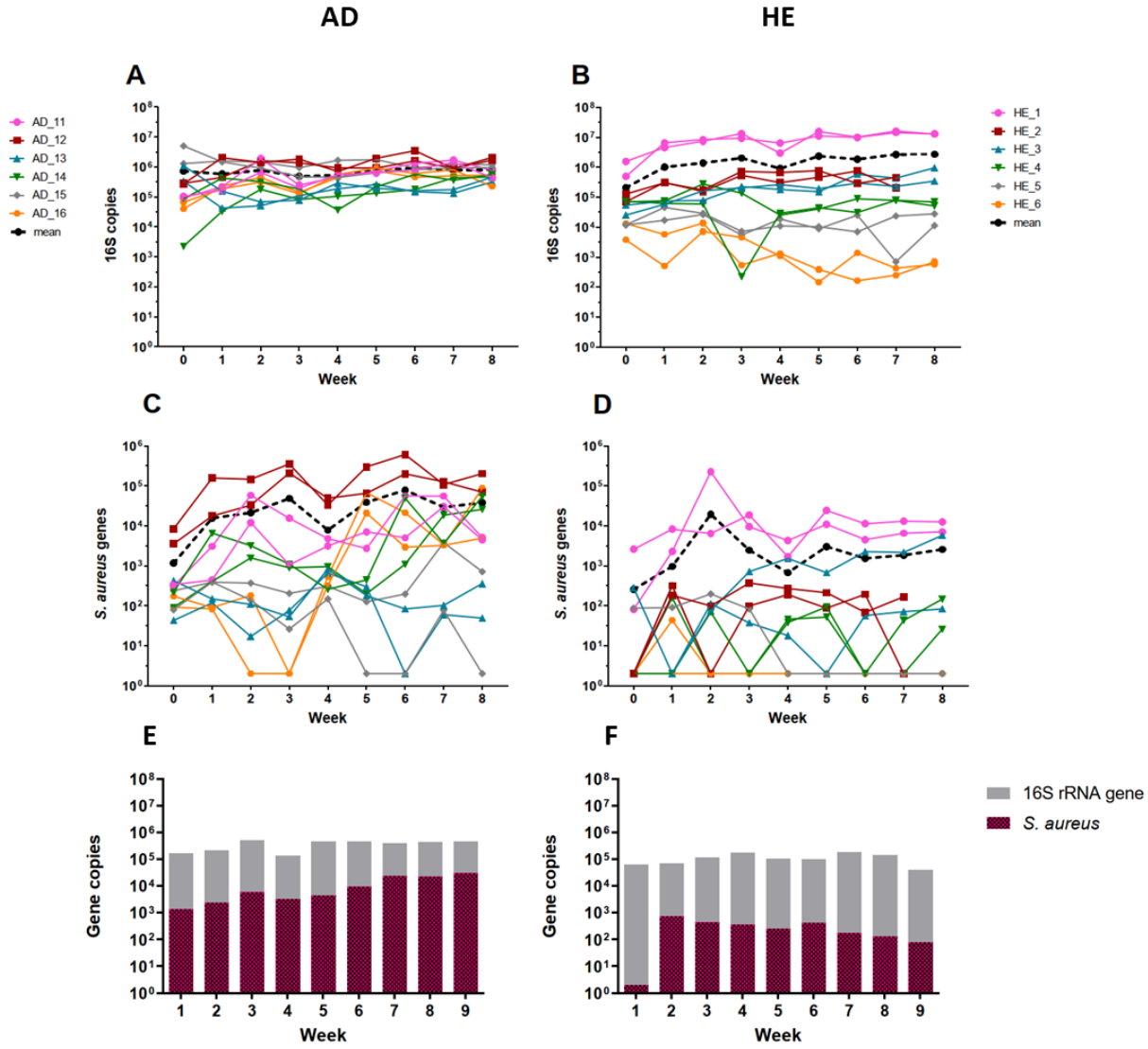


Figure 35 Longitudinal analysis of 16S copy number and S. aureus cell number of both bodysides of atopic dermatitis patients and healthy individuals
The 16S copy number is stable over eight weeks in atopic dermatitis patients and healthy individuals and left and right bodyside are very similar. The 16S copy number is depicted for eight weeks for both bodysides of six atopic dermatitis patients (A) and six healthy individuals (B). Also *S. aureus* cells are depicted for atopic dermatitis patients (C) and healthy (D). The median 16S copy number (grey) and *S. aureus* cell number (pink) of the group of atopic dermatitis (E) and healthy (F) individuals is shown.

Next, the impact of different microenvironments was investigated. Therefore, body areas were stratified into dry, moist, and sebaceous body sites according to Grice and Segre 2011 and Altmeyers Enzyklopädie. For unallocated locations, a dermatologist was involved.

In HE, AD NL and AD LS, most bacterial 16S copies were found in moist skin areas. In HE, least bacterial 16S copies were recovered from sebaceous areas compared to dry (p-value 0.0001) and moist (p-value 0.001) areas. In AD NL areas, the difference between moist and dry areas (p-value 0.01) and moist and sebaceous areas (p-value 0.05) was significant. In AD LS areas, only the difference between detected 16S copies between moist and dry areas was significant (p-value 0.001) (Figure 36 A-C) .The number of *Staphylococcaceae* spp. cells was only significantly different in AD NL areas, where moist areas were most populated by Staphylococci with significantly less cells in dry and sebaceous skin areas (p-value 0.01) (Figure 36 D-F). For *S. aureus* cells, the difference was significant only in HE, with more cells detected in moist compared to dry and sebaceous areas (p-value 0.05). However, also in AD NL and AD LS areas, there is a trend for low bacterial cells detected in sebaceous areas (Figure 36 G-I).

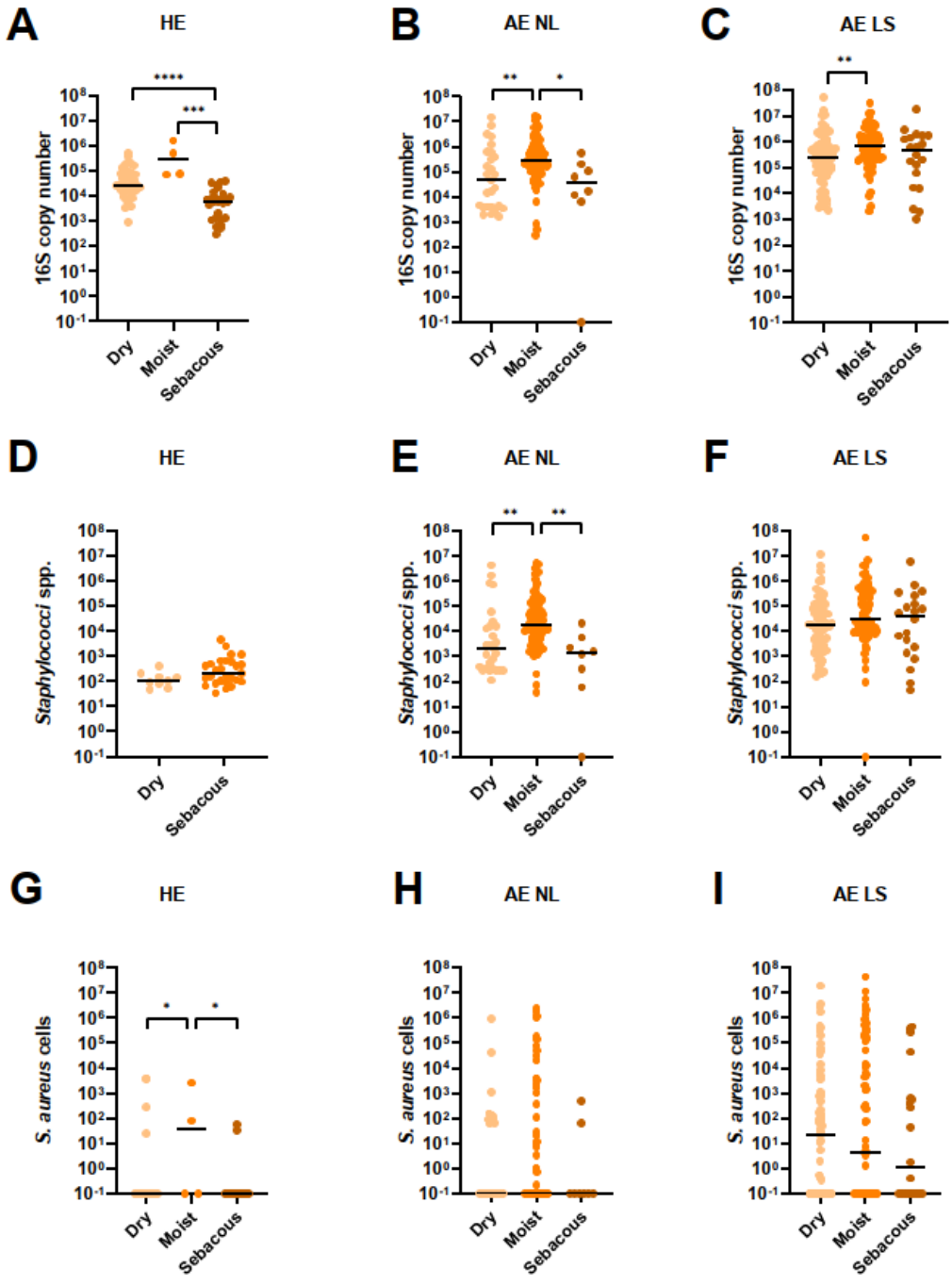


Figure 36 The effect of the microenvironment on 16S copy number, Staphylococcaceae and S. aureus cells

Moist environment has a tendency for a higher bacterial colonization. Shown are the 16S copy numbers for healthy (A), atopic dermatitis non-lesional (B) and atopic dermatitis lesional (C) for dry, moist, and sebaceous microenvironments. Furthermore, number of *Staphylococcaceae* for healthy (D), atopic dermatitis non-lesional (E) and atopic dermatitis lesional (F) for dry, moist, and sebaceous microenvironments and *S. aureus* numbers for healthy (G), atopic dermatitis non-lesional (H) and atopic dermatitis lesional (I) for dry, moist and sebaceous microenvironments. HE=healthy, AD=atopic dermatitis, NL=non-lesional, LS=lesional. Significant differences between microenvironments are marked with * ($p < 0.05$), ** ($p>0.01$), *** ($p<0.001$), **** ($p>0.0001$).

To understand whether the higher number of bacterial cells measured as 16S rRNA copies is due to an overgrowth of *S. aureus* or a generally higher bacterial load, the number of measured 16S rRNA copies were correlated with the number of *S. aureus* cells (Figure 37). The more *S. aureus* cells were detected, the more 16S rRNA copies were detected. Interestingly, the plot could be separated into two parts. In the samples with low *S. aureus* cells ($<10^4$) and low 16S copies ($<10^4$), no correlation was observed. However, when more than 10^4 *S. aureus* cells or more than 10^4 16S copies were detected, there was a strong correlation (r=0.88, p-value<0.0001), hinting towards an overgrowth of *S. aureus* and not only a change in distribution of bacterial species.

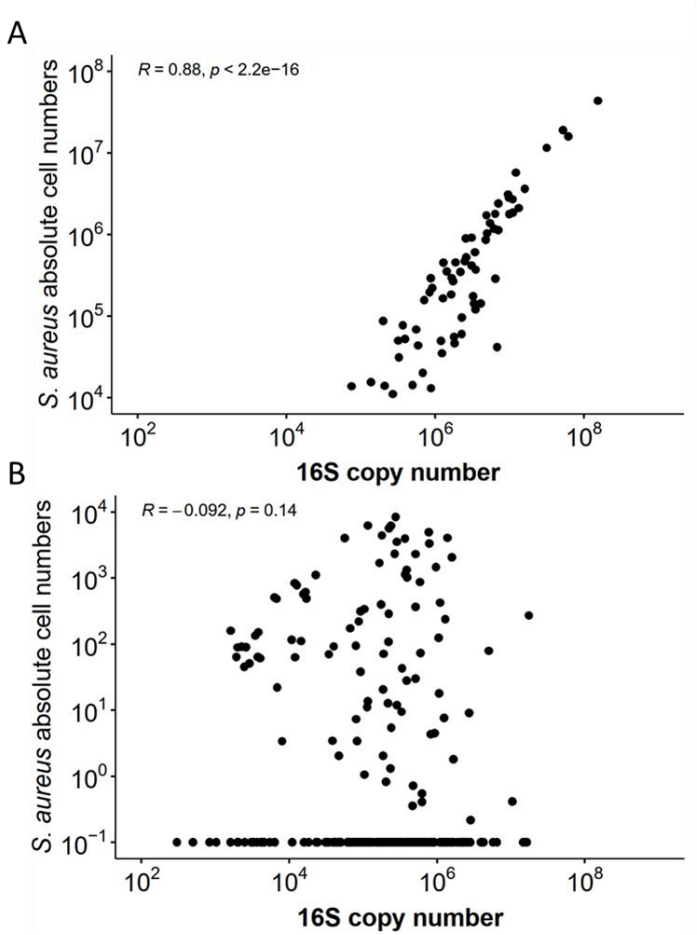


Figure 37 Overgrowth of S. aureus in AD

S. aureus abundance drives high 16S copy numbers hinting towards an overgrowth of *S. aureus*. Shown is the correlation between *S. aureus* cells and 16S copy numbers as representative of bacterial cells for high *S. aureus* cell number >10^4) (A) and low *S. aureus* cell numbers ($<10^4$) (B) in atopic dermatitis patients. Shown are the correlation coefficient R and the p-value.

To investigate whether the AD disease severity is driven by the distribution of *S. aureus* within the bacterial community or by absolute *S. aureus* cell numbers, both were correlated with SCORAD (Figure 38 A, B). Both showed a correlation (rel. frequency r=0.38, absolute cell numbers r= 0.35, both p-value<0.0001). Interestingly, with both methods it becomes clear, that there is an AD population with severe AD (SCORAD>40) and a relative *S. aureus* frequency <1 % or > 100 *S. aureus* cells. Contrarily, there was also a population with high *S. aureus*

abundance (rel. freq. >1 % or > 100 *S. aureus* cells). This hints towards the differences either in AD subtype or in the *S. aureus* strains.

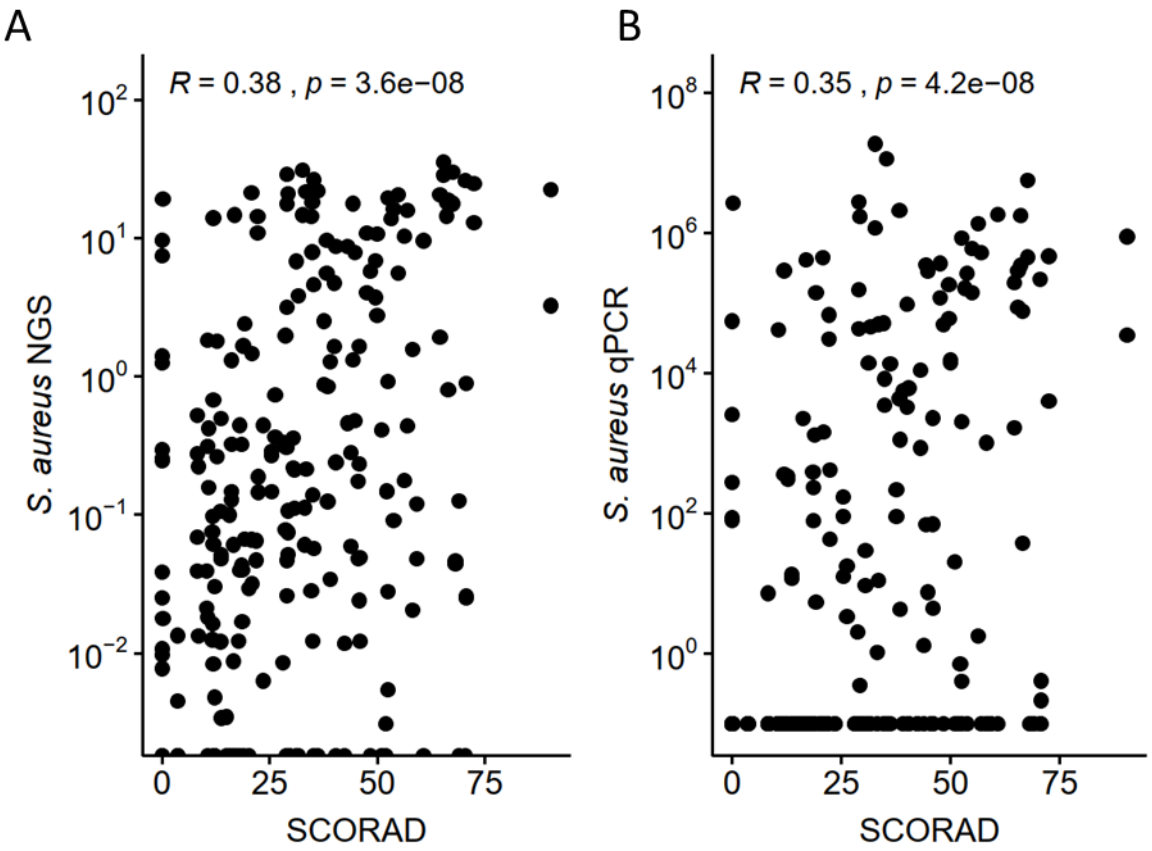


Figure 38 Correlation between S. aureus frequency and cell numbers with AD severity
S. aureus cell number and *S. aureus* frequency are associated with atopic dermatitis severity score SCORAD. Shown are the correlation between *S. aureus* relative frequency detected by next generation sequencing and SCORAD (A) and *S. aureus* cell number detected via qPCR and SCORAD (B) in atopic dermatitis patients. The correlation coefficient and the p-value are stated in the figure.

To verify results obtained via qPCR, the qPCR data was combined with NGS results. Interestingly, there was a strong correlation between cells detected via qPCR and NGS for *S. aureus* (r=0.9, p-value<0.0001) (Figure 39 A), *Staphylococcaceae* spp. (r=0.71, p-value<0.0001) (Figure 39 A) and also number of 16S rRNA copies and number of total reads in NGS (r=0.59,p-value<0.0001) (Figure 39 C).

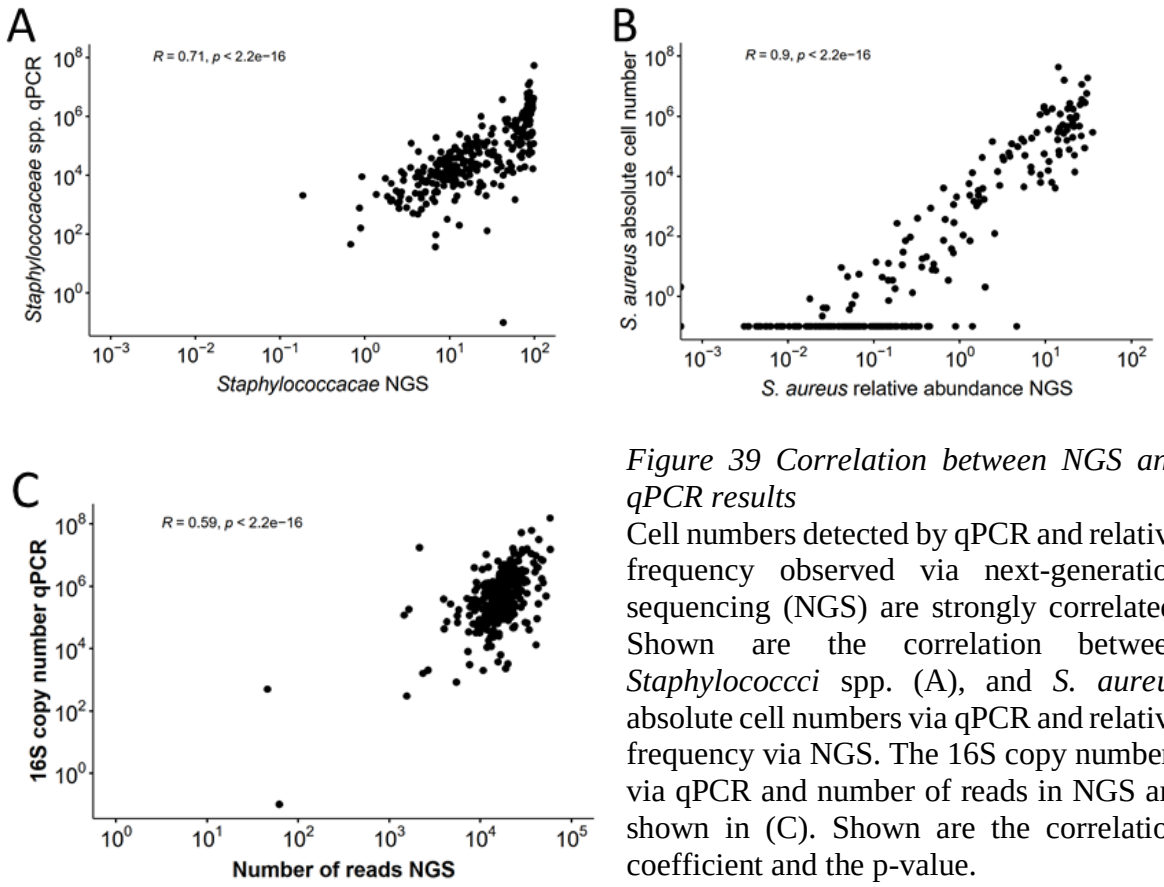


Figure 39 Correlation between NGS and qPCR results
Cell numbers detected by qPCR and relative frequency observed via next-generation sequencing (NGS) are strongly correlated. Shown are the correlation between *Staphylococcci* spp. (A), and *S. aureus* absolute cell numbers via qPCR and relative frequency via NGS. The 16S copy numbers via qPCR and number of reads in NGS are shown in (C). Shown are the correlation coefficient and the p-value.

3.5 Outlook: investigation of the microbe-host interaction

As previously described, *Staphylococcus* spp. seem to be associated with human skin barrier genes. To test this hypothesis, different *Staphylococcus* spp. isolates were used to stimulate a monolayer and air-liquid interface models with *S. aureus*. Visually, after 6h of stimulation, keratinocytes stimulated with *S. hominis* were still healthy, whereas cells with *S. aureus* isolate 30 or MW2 started to go into apoptosis. However, in a Western blot, no difference in the barrier protein Claudin 4 and TJP-1 were detected, neither in monolayer, nor in air liquid interface models (Supplement 1).

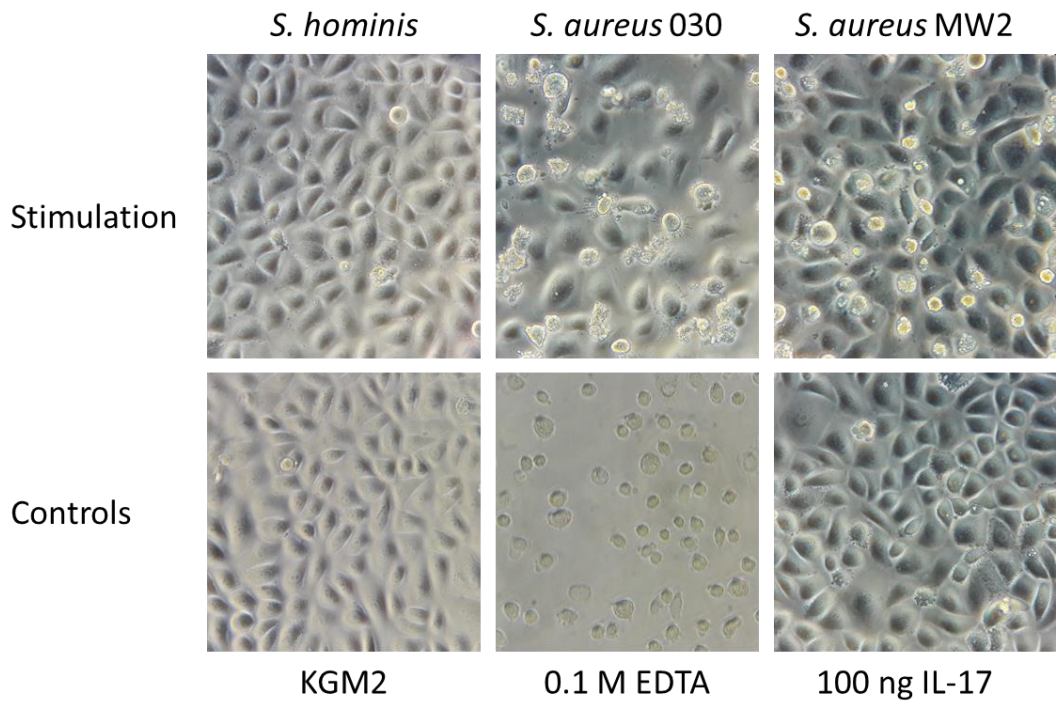


Figure 40 Transfer to the human system

Keratinocytes tolerate *S. hominis* but not *S. aureus* stimulation for 6 hours. Shown are representative pictures of a monolayer of NTERT keratinocytes stimulated for 6h hours with either *S. hominis*, *S. aureus* isolate #30, *S. aureus* MW2, or with control only medium, 0.1 M EDTA or 100 ng IL-17.

Furthermore, a 3D skin model was established, consisting of primary human fibroblasts in a collagen G matrix and primary keratinocytes on top CoStar plate with insert. Tested were different amounts of primary fibroblasts. From 25,000 to 75,000 primary fibroblasts, the models developed nicely, whereas higher cell numbers resulted in contraction of the skin equivalent. Furthermore, 0.1 M calcium concentration was required for a barrier formation of the keratinocytes

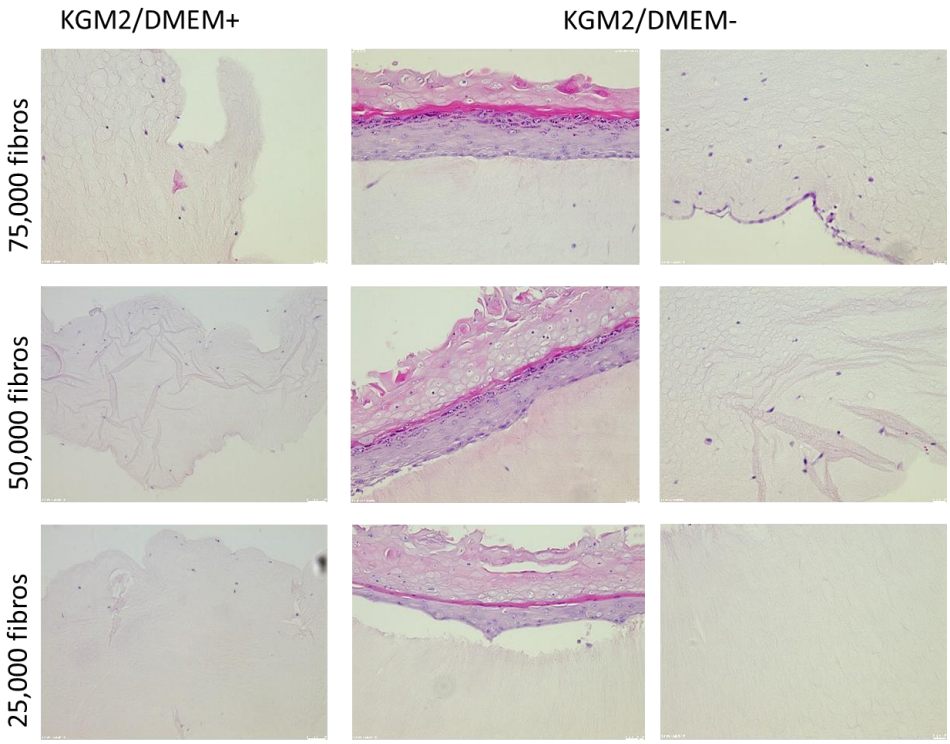


Figure 41 3D skin model

H&E staining of a 3D skin model with 25.000, 50,000 and 75,000 fibroblasts in a collagen matrix and primary keratinocytes in the upper layer.

4.0 Discussion

4.1 Context of this work

Atopic diseases are a major concern in our modern world since they developed epidemic characteristics nowadays (Thomsen, 2015). The prevalence is 20 times higher in westernized than in developing countries (Lambrecht & Hammad, 2017; D. Strachan et al., 1997). Certain genetic traits are a risk factor for atopic diseases. However, there is increasing evidence, that atopic diseases not only have a genetic but also an environmental component (Gilles et al., 2018). One example is that the prevalence in western and eastern Germany was significantly different directly after the reunification but reached similar plateaus only 30 years later, ruling out the possibility of a strict genetic disease (Bergmann, Heinrich, & Niemann, 2016; Hermann-Kunz, 1999). The western lifestyle, which is characterized by a diet poor of fibers but rich in saturated fats and sweeteners, a lack of physical activity, more time spend indoors and change in culture, is one of the factors hold responsible for this dramatic development. These factors are associated with a higher risk of obesity and low grade chronic inflammation (Cordain et al., 2005; Eckersley, 2005; Lambrecht & Hammad, 2017; Ruiz-Núñez, Pruimboom, Dijck-Brouwer, & Muskiet, 2013). Moreover, in our modern world the exposure to viruses, bacteria and helminths is reduced which already was associated with a higher prevalence of asthma,

eczema and urticaria in 1976 (Gerrard, Geddes, Reggin, Gerrard, & Horne, 1976). Non-lower tract infections are a protective factor from allergic sensitization and asthma (Illi et al., 2001). According to Rook and Haahtela, the loss of our "old friends", symbiotic relationships with bacteria and parasite, is responsible for the increasing prevalence in atopic diseases (Haahtela et al., 2013; Graham A. W. Rook et al., 2013). This coincides with study results, that atopic diseases are concomitant with a reduced diversity of the skin and gut microbiome (J. E. Kim & Kim, 2019). The skin microbiome was reported to show a lower diversity and is dominated by *S. aureus*, especially during disease flare (Paller et al., 2019). Also the gut microbiome of infants with AD was associated with a low diversity and low numbers of *Bifidobacterium*, *Bacteroides*, *Akkermansia*, and *Faecalibacterium* (Abrahamsson et al., 2012; Fujimura et al., 2016). Furthermore, high numbers of butyrate-producing bacteria and a high diversity was reported to correlate with more mild AD symptoms (Nylund et al., 2015). Interestingly, these two environments are linked via the skin-gut axis for example by short chain fatty acids which are end products of dietary fiber fermentation in the gut and are able to influence the skin microbiome composition (Salem, Ramser, Isham, & Ghannoum, 2018). Whereas gut microbiome analyses are well established, skin microbiome analyses are more challenging due to the comparably low level of bacterial colonization and thus higher risk for contamination.

4.2 Impact of contaminants in low biomass samples

The impact of contaminants on low biomass samples was well established over the last years (Salter et al., 2014). The general concept that low biomass samples are more susceptible could be confirmed with a dilution series of a mock community from 10^9 to 10^2 cells, showing a stepwise increase of contaminants from less than 1 % in samples with 10^9 cells input material to more than 96 % when only 10^4 or less bacterial cells were used as input material. Whereas high input samples were significantly different from controls, low input samples resembled the negative controls in a study by Kim et al 2017 (D. Kim et al., 2017). A total of 63 kit specific contaminants were shared among samples and negative controls in a study by Salter et al 2014. As this can even lead to false biological conclusions, more attention has to be drawn to this matter (D. Kim et al., 2017; Salter et al., 2014). As the skin only harbors 10^3 to 10^6 bacterial cells per cm^2, these samples are susceptible to contaminations acquired during any of the necessary steps for NGS, starting from sampling, DNA extraction, and PCR. To understand the effect of the choice of a DNA extraction Kit on the results of skin samples, swabs from adjacent skin sites were taken from two different individuals. Subsequently, the DNA was extracted either with a Stratec DNA extraction kit or with a DNA extraction Kit from Qiagen which was designed to have little inherent contaminants and therefore less background noise according to

the manufacturer. Significant differences in the abundance of typical skin inhabitants at family level were observed. Therefore, it is essential to stick to one DNA extraction kit during one study (Goodrich et al., 2014; H. H. Kong et al., 2017). Meta-analysis of studies performed at different institutes are for the same reason not possible. Hence, a universal protocol used internationally for skin microbiome analysis would be required. Interestingly, the samples clustered according to DNA extraction kit instead of individuals, completely covering any biological differences. Whereas in the Qiagen Kit the individuals were separable, this was impossible in the Stratec DNA extraction Kit. While in the Qiagen Kit only 6 unique genera appeared, the Stratec Kit had 16 unique genera which were not shared between both extraction kits, most likely coming from the extraction instead of the skin environment. Looking at the top 10 genera from both Kits and both individuals, the two individuals seem to be more similar when DNA was extracted with the Stratec Kit then with the Qiagen Kit. Similar results were obtained by Kim et al 2017 (D. Kim et al., 2017). Even batch effects of Kits from one supplier were reported (Leek et al., 2010). In other settings as stool microbiome analysis, the DNA extraction Kit does not strongly cover the biological differences (Rubin et al., 2014; Wagner Mackenzie, Waite, & Taylor, 2015). Therefore, especially for low biomass samples it is advisable to use DNA extraction Kits specifically designed for extracting low biomass samples (D. Kim et al., 2017). Among the observed genera in the top 10 of both Kits, typical contaminants described in literature were found as *Bradyrhizobium* and *Sphingomonas*. However, extensive literature search revealed that also typical skin inhabitants as *Staphyolococci* and *Propionibacterium* are frequently named as contaminants (Glassing et al., 2016; Salter et al., 2014; Weiss et al., 2014). However, human skin is a likely source of contamination during sample processing. In skin microbiome research, it is consequently difficult to discriminate between contaminating and real sequences. Consequently, the urge for dataset specific removal of contaminants evolved. At this point, no adequate tools were available for this purpose, so that a tool was developed for contamination removal and basic microbiome analysis. As microbiome analyses are more and more performed by non-bioinformaticians, the tool was created with a graphical interface with R Shiny. Multiple sequential steps clear the dataset of under sequenced samples or infrequent and contaminating OTUs. All steps have default values; however, the operator can dynamically change the parameters. The filtering frequency is solely based on the maximum frequency of one OTU which must be reached in at least one sample. The contaminant specific filter uses either the PCR or the pipeline control and calculates the ratio between the mean frequency of the OTU in the respective negative control divided by the mean frequency of the OTU in the sample.

The contaminated dataset, the PCR water control contained 100 times more reads than in the resequenced dataset. Especially in the resequenced dataset, the PCR control also contained mock species. The low number of overall reads in the PCR control resulted in a high frequency of the mock species, leading to erroneous exclusion of true sample OTUs based on the PCR control. Indeed, depending on the Illumina platform, in 0-10 % of the sequenced data index hopping occurs, leading to sample OTUs in the negative controls, although this effect should be mitigated by quality control of Illumina reads (Hornung et al., 2019; Sinha et al., 2017; E. S. Wright & Vetsigian, 2016). Therefore, PCR controls should be used as a quality control to check for major contaminants. Contrastingly, the pipeline control which contains all contaminants gathered at any step of the process was more successful in eliminating contaminating OTUs in the contaminated and the resequenced dataset. In the contaminated dataset, almost all contaminating OTUs could be cleared using the pipeline control filter, whereas the frequency filter was inefficient due to the high frequency of the contaminating OTUs. Furthermore, the frequency filter also eliminated mock OTUs, which remained when applying the pipeline control filter. This was also true for the resequenced dataset, even though the contaminant reduction was less efficient (20 % of contaminants in 10^3, 48 % of contaminants in 10^6, 10^9 in total only 1 % contaminants which could not be eliminated). Therefore, microbIEM should be put into perspective with other decontamination tools which were published recently. Other filtering tools are based either on i) taxonomy, ii) OTUs in negative controls, ii) relative abundance, iv) inverse correlation with DNA concentration, and v) based on defined contamination sources (Karstens et al., 2019). Each filter must be reviewed critically and has inherent flaws. Solely filtering based on a list of determined contaminants neglects the different environments, where *Staphylococci* are frequent contaminants from the skin but are true when looking at skin samples. Therefore, a carefully curated separate list of contaminating taxonomic units would be required for each specific environment. Furthermore, miss annotations are problematic in this setting (Sheik et al., 2018). Eliminating all sequences found in the negative control is precariously due to the possibility of cross contamination from the sample to the negative control. Only deleting low frequency sequences was not successful in our study. In low biomass samples with high fractions of contaminants, this approach is not advised (Davis et al., 2018). However, for environments like the gut with a high biomass, this approach might be reasonable. The R package Decontam considers the inverse correlation between DNA amount and contaminating OTU, assuming that the amount of contaminating OTUs remains the same in all samples whereas the sample DNA varies, resulting in a lower frequency of the contaminating sequence in samples with high initial DNA amount. This

additional information can be obtained via fluorescent measurement or qPCR and is required for the library preparation. Another option is to track from the contamination source by SourceTracker (Karstens et al., 2019; Scott, Rose, Jenkins, Farrah, & Lukasik, 2002). Furthermore, NGS results can be adjusted by qPCR information to correct for amount of input material and removal of contaminants (Bittinger et al., 2014; Lazarevic et al., 2016).

Summarizing, contaminants are problematic in low biomass setting. The increasing number of publications on this topic are promising that awareness of this challenge is rising. However, the vast majority of researchers does not yet address contaminants in skin sequencing datasets. MicrobIEM adds another option to clear datasets from contaminants. Conveniently, microbIEM does not require additional information apart from the OTU/ASV table and metadata file. As microbIEM has a graphical user interface, no programming knowledge is required. We hope to contribute to tackling this challenge.

Furthermore, it stands out by basic microbiome analysis with dynamic sample selection, allowing to gain a fast overview over the dataset.

4.3 Annual skin microbiome stability

After developing a tool for reliable removal of contaminants from skin microbiome datasets, the stability of the head and skin microbiome of the Ac over one year was investigated in healthy and birch allergic individuals.

The head and skin microbiome were significantly different, independent of the health status of the individuals. The skin had a higher richness than the head. Whereas the head was dominated by 80 % by members of the *Propionibacteriaceae*, the skin was more diverse, harboring mostly *Propionibacteriaceae*, *Staphylococcaceae* and *Corynebacteriaceae*. The differences observed in the microenvironment are in line with literature. Sebaceous body areas as the scalp are known to be inhabited by *Propionibacteriaceae* (Grice & Segre, 2011). In a study comparing the scalp microbiome of 140 Indian woman with and without dandruff showed that *P. acnes* (*now C. acnes*) and *S. epidermidis* were the most abundant bacterial species with *C. acnes* being associated with a healthy scalp (Saxena et al., 2018). However, in this study, *C. acnes* abundance only reached up to 30 %. Looking at the group, neither the skin nor the head microbiome of Ba and healthy individuals changed over time. Shared community membership and structure over time in the skin microbiome was already reported in 2009 by Grice et al and confirmed by others (Brandwein et al., 2018; Grice et al., 2009; Oh et al., 2016). However, in previous studies only up to three subsequent timepoints after either weeks or month were taken, so that fluctuations occurring seasonally might not be revealed contrasting to our study with 15

visits within one year. Also other environments as saliva and stool were reported to have a temporal stable microbiome (Cameron, Huws, Hegarty, Smith, & Mur, 2015; Rajilić-Stojanović, Heilig, Tims, Zoetendal, & de Vos, 2013). Interestingly, in both groups, healthy and Ba, two individuals respectively had a high relative abundance of *Staphylococcaceae*, whereas the other five per group had higher relative abundance of *Propionibacteriaceae*. Strikingly, although fluctuations occurred over time, the general "Epitype" remained either Epitype *Propionibacteriaceae* (Epitype P) or Epitype *Staphylococcaceae* (Epitype S). The concept of different enterotypes was already published in 2011 for different clusters of gut microbiome groups, which could not be explained by the body mass index, age, gender or ethnicity (Arumugam et al., 2011). It was suggested that functional analysis might be important and that different enterotypes might react differently to drugs. Later, the existence of strict enterotypes was mitigated and rather smooth gradients between enterotypes were described, striking that the enterotype, though promising, should not be the only parameter to look at. Different methods varied in their efficiency to detect clusters (Costea et al., 2018; Koren et al., 2013). However, enterotypes were also reported from other environments as anterior nares, four skin sites and the vagina (Ravel et al., 2011; Zhou et al., 2014). The different enterotypes were dominated by either *Sporacetigenium, Staphylococcus, Ralstonia, Propionibacterium, Corynebacterium, Streptophyta, Streptococcus* or *Haemophilus*. The most abundant enterotypes were *Propionibacterium* or *Corynebacterium* dominated. Males had a prevalence for the *Propionibacterium* dominated enterotype, likely due to higher collagen, sebum content and more sweat, providing nutrient for the slower growing genera. (Rosenthal, Goldberg, Aiello, Larson, & Foxman, 2011; Zhou et al., 2014). These enterotypes were mostly stable over two visits. Our data adds that the entero- or epitype also remains stable throughout one year. The epitype S was strongly dominated by *S. hominis* in three out of four individuals. Even at species level, the abundance was quite stable over one year.

Skin microbiome samples from Ba and Na formed separate clusters and Ba had a higher richness and lower Simpson diversity index. Generally, diseased microbiome is often associated with a decreased diversity. Low gut microbiome diversity is associated with the development of atopic diseases in children (Durack & Lynch, 2019). In parallel to the skin, the nasal microbiome and immune parameters were monitored over the year within the described study and are discussed in the Doctoral thesis of Denise Rauer, M.Sc. and Mehmet Gökkaya, Dipl. Ing., respectively. From this study, already biomarkers for symptom prediction in Ba and non-allergics under natural pollen exposure were published (Gökkaya et al., 2020).

4.4 Skin microbiome stability in HE but not AD

After validating that the skin microbiome is stable over one year in healthy and Ba individuals, we investigated the stability of the skin microbiome over eight weeks upon challenging with and emollient with either acidic pH 5.5 or basic pH 8.5 in the context of AD. In parallel, the skin physiology and the disease severity were examined. Six AD patients and six healthy controls put the emollient twice daily with pH 5.5 on one arm and pH 8.5 on the other arm. The complex interplay between pH, *S. aureus* and AD severity was examined to test whether i) the skin microbiome is influenced by the application of an emollient, ii) the skin pH can be altered with an emollient, iii) an acidic skin emollient supports physiological barrier function and inhibits *S. aureus*

The skin microbiome of AD showed higher abundance of *S. aureus* and lower abundance of *Micrococcaceae* as described previously (Paller et al., 2019; Wongpiyabovorn et al., 2019). The skin microbiome of healthy remained stable over eight weeks. Neither the alpha diversity nor the top 10 families and species were altered robustly over time, showing that not even challenging the microbiome with an emollient with acidic or basic pH affects a healthy microbial community on the skin. Similarly, the skin microbiome of healthy volunteers showed higher inter-individual than temporal microbiome differences under dead sea climatotherapy while the mycobiome did not remain stable (Brandwein et al., 2018). Unlike the healthy microbiome, the skin microbiome in AD changed over time. Richness increased as previously described upon emollient application (Bouslimani et al., 2019; Wallen-Russell, 2019). In parallel, the family *Lactobacillaceae* was reduced over the study period. *Lactobacillaceae* harbor probiotic strains which are even considered for AD treatment (Navarro-López et al., 2018; Rather et al., 2016). Furthermore, *S. aureus* significantly increased over time, which might be due to the fact that AD patients restrained from using additional medication and changed their daily skin care habits. As previously described by others, *S. aureus* was associated with AD severity (Heidi H. Kong et al., 2012). *S. aureus* is capable of expressing SA and enterotoxins, which can lead to an inflammation cascade further damaging the skin barrier and worsening of AD symptoms (J. Kim et al., 2019; Nakamizo et al., 2015; T. Nakatsuji et al., 2016) (Leung, 2005). Moreover, high abundance of *S. aureus* at baseline was strongly correlated with the worsening of AD severity and increasing *S. aureus* abundance as previously hypothesized by Reiger et al., 2020 (Reiger et al., 2020). This might hint into the direction that *S. aureus* abundance precedes a disease flare. Similarly, it was observed in a children cohort, that high *S. aureus* abundance at 3 months preceded AD development in these children (Williams & Gallo, 2017).

Comparing healthy individuals and AD patients, AD patients had a higher TEWL and lower hydration than healthy individuals, showing again the damaged skin barrier in AD. The maintenance of an acidic skin pH is important for skin barrier integrity and influences the activity of proteases, bacterial toxins and antibacterial agents (Danby & Cork, 2018; Eyerich et al., 2018; Kuo, Shen, Shen, & Cheng, 2020; Panther & Jacob, 2015). Whereas the skin pH of healthy individuals is reported to be rather acidic between pH 4 to 6, the pH is increased in NL and even higher in LS AD skin (Lambers et al., 2006). However, in our cohort no difference in skin pH was observed at baseline. This might be due to the fact that compared to literature, the healthy participants in our study had a relatively high skin pH (pH 5.7 at baseline). Furthermore, skin pH increase in AD is linked with disease severity, which was according to the inclusion criteria only mild to moderate as patients had to constrain for eight weeks from any further medication (Seidenari & Giusti, 1995). Also, the reported differences in skin pH are not high, therefore it might be difficult to replicate in a study with low number of participants. Interestingly, *S. aureus* has a growth optimum at pH 7 whereas growth is inhibited in acidic conditions (Contreras & Zaritzky, 1999; Stewart et al., 2002; Whiting, Sackitey, Calderone, Morely, & Phillips, 1996). In this cohort, high frequencies of *S. aureus* were only observed in AD between a skin pH of 5.7 and 6.2. However, other factors must be considered as high and low counts of *S. aureus* were found alongside. These factors can be microenvironmental as dryness, osmolarity or genetic factors as FLG mutations. Interestingly, a higher adhesion of *S. aureus* to corneocytes which are deformed due to FLG mutations was found. On top, FLG mutation lead to less NMF and a subsequently higher pH which again enhances corneocyte adhesion (J. Kim et al., 2019; Mempel et al., 1998). Another consequence of less NMF is dry skin which is less protected from invading *S. aureus* strains and an increase of oxygen in the skin. Invading *S. aureus* cells compete with keratinocytes for glucose and oxygen and lead to local hypoxia, which induces a metabolic switch in keratinocytes. Subsequently, a reduction in lactate production leads to less acidification in an *S. aureus* infection setting (Wickersham et al., 2017). This might contribute to the link between increased pH with *S. aureus* colonization.

Due to the connection between pH, *S. aureus* and AD or AD severity, the regulation of the skin pH is discussed as a putative treatment option (Ali & Yosipovitch, 2013; Heidi H. Kong et al., 2012). Bleach bathes were widely used as treatment options, although the efficiency in reducing AD severity and *S. aureus* loads is not scientifically proven (Chopra et al., 2017; Sawada et al., 2019). However, in the "Guidelines of care for management of AD", a rather acidic skin care is advised (Eichenfield et al., 2014). No difference due to the emollient pH was seen in TEWL, hydration and local SCORAD. Interestingly, especially in AD patients, a temporary difference

in skin pH depending on the treatment was observed, hinting towards a reduced buffering capacity of AD skin. Considering that common skin care products have a rather basic pH, this might be of importance (Ali & Yosipovitch, 2013). However, the differences in pH between the bodysides, though treated with extreme pH emollients, were still very small. The amount of the applied emollient was monitored by weighing and met the predefined quantities, so that compliance did not seem to be a problem in our study. Likewise, previous studies also showed that a sustainable reduction of skin pH with apple cider vinegar was not possible. Also the barrier function did not improve (Luu et al., 2019). However, in mouse models the acidification of the stratum corneum improved or even prevented murine AD (Hatano et al., 2009; N. R. Lee et al., 2016). In this study, skin pH and hydration in AD patients was inversely associated in early mid, and late phase of the study whereas pH and TEWL correlated positively at early, mid, and late phase of the study, confirming positive effects of lower skin pH on the skin barrier. However, the local SCORAD was not correlated with the skin pH, only with *S. aureus* abundance.

In conclusion, the skin microbiome cannot be altered upon challenging with emollients with acidic or basic pH. Interestingly, the skin microbiome of healthy is stable, whereas the skin microbiome in AD was susceptible for change, most notably in *S. aureus*. *S. aureus* abundance was not only correlated with AD severity but even high *S. aureus* abundance at baseline was even predictive for AD worsening. As high *S. aureus* abundance was only observed between pH 5.7 and 6.2, management of skin pH seems to be important in AD. The skin pH particularly in AD was slightly altered by the pH of the applied emollient, however these changes were only minimal. As low skin pH was associated with higher skin hydration, lower TEWL and low *S. aureus* abundance, a more effective method to reduce skin pH should be considered for future studies. The utilization of an even more acidic emollient might be a solution.

4.5 Adaptation to an acidic environment of *S. aureus* isolates from healthy individuals

To investigate the adaptation to different pH conditions of strains from healthy and AD individuals, isolates were cultivated in pH conditions from 5.5 to 8.0 in 0.5 pH steps.

Ideal growth conditions for isolates independent from source (healthy or AD, nose and skin) were around pH 7, as previously reported (Contreras & Zaritzky, 1999; Stewart et al., 2002; Whiting et al., 1996). Around this pH, a bell shape was observed. pH 6.5 and 7.5 did not grow significantly worse, whereas at pH 5.5, 6.0 and 8.0 growth was significantly reduced. The least growth was detected at pH 5.5, which is an expected pH on healthy skin (Lambers et al., 2006).

Intriguingly, *S. aureus* from healthy individuals were more adapted to acidic conditions than isolates from AD patients.

4.6 Toxin pattern in healthy and atopic individuals

In the context of AD and AD severity, Staphylococcal enterotoxins (SE), Staphylococcal enterotoxin-like (SEl) and TSST-1 play an important role (Na, Roh, Kim, Tamang, & Lee, 2012; Schlievert, Case, Strandberg, Abrams, & Leung, 2008). To compare the pathogenicity of *S. aureus* isolates from healthy individuals and AD patients, *S. aureus* isolates were screened for the genes that code for Staphylococcal Enterotoxin-A, -B, -C, -G, and -H (*sea, seb, sec, seg,* and *seh*, respectively), Staphylococcal Enterotoxin-like protein K (*selk*), toxic shock syndrome toxin-1 (*tst*), Panton-Valentine leukocidin (*pvl*), and the methicillin resistance determinant penicillin-binding protein 2‘ (*mecA*).

SE show an emetic capacity whereas SEl do not have an emetic ability (Argudín, Mendoza, & Rodicio, 2010). SE and SEl toxins consists of 22 exoproteins which exacerbate inflammation via T-cell proliferation and cytokine release (Argudín et al., 2010). Despite the similarity between the SEs, SEls and TSST-1, each superantigen interacts differently with MHC II molecules and has unique activation patterns (Argudín et al., 2010; Larkin, Carman, Krakauer, & Stiles, 2009). All superantigen genes are located on accessory genetic elements which are mobile and can be distributed between isolates via gene transfer (Larkin et al., 2009). *S. aureus* pathogenic islands are extremely common 15 to 17 kb mobile islands which usually carry one or more SA highly transferred across strains. Toxin genes like *seb*, *sec*, *selk* and *tst* have been identifies at staphylococcal pathogenicity islands (SaPIs) (Novick & Subedi, 2007). In our study, most isolates had 1 or 2 toxins. Sequencing the different SaPI revealed that *seb* and *selk* occur together on SaPI3 whereas *sec* and *tst* were found together on SaPIm1/n1occur (Novick & Subedi, 2007). However, in our study only one case per combination was found among the 39 isolates. In the tested isolates from our biobank, SEG was the most abundant *S. aureus* toxin with 54 %, irrespective of the origin (AD, healthy). SEG was discovered in 1998, after the characterization of SEA-SEE and SEH (Munson, Tremaine, Betley, & Welch, 1998). Interestingly, SEG was already identified as the most common toxin in healthy nasal *S. aureus* carrier with 57 % coding for this toxin (Jarraud et al., 2001). So far, SE were mainly investigated in the context of food poisoning. In a study with 30 AD patients, 54 % were colonized with superantigen producing strains, most frequently with SEB, SEC and TSST-1 coding strains (Nada, Gomaa, Elakhras, Wasfy, & Baker, 2012).Especially SEA and SEB were also correlated with high SCORAD and IL-4 levels in AD (Seiti Yamada Yoshikawa et al., 2019). Specific IgE antibodies to SEA and SEB were stronger correlated to disease severity than total IgE levels

(Bunikowski et al., 1999). In our isolates, *sea*, *seb* and *sek* were only detected in isolates from AD patients, *seb* and *sek* only in strains isolated from patients with a severe SCORAD. SEB is known to induce IL-31 which causes severe pruritus (Dillon et al., 2004). Nonetheless, this must be confirmed in a larger set of isolates.

No differences between the abundance of toxins between strains from healthy participants and AD patients were detected. Therefore, the question arises whether these toxins are induced in the environment. *S. aureus* virulence factors are regulated by the quorum sensing agr system which was first described by Peng et al 1988 and consists of four different types, the agr system I-IV (Peng, Novick, Kreiswirth, Kornblum, & Schlievert, 1988). Toxin expression is induced at conditions with neutral pH and high cell density, both of which are given in the setting of AD skin (Jenul & Horswill, 2018; Regassa & Betley, 1992; Tan, Li, Jiang, Hu, & Li, 2018). Consequently, it is possible in healthy individuals no toxins are expressed whereas with rising cell numbers in AD, and the disturbance of the acid mantle and skin barrier, *S. aureus* starts to express toxins and starts a viscous cycle with increasing toxin expression, pruritus induced by IL-31, itch and further barrier disruption. To investigate this further, a reliable assay to determine toxin expression at different settings would be required in future as the house keeping gene was not stably expressed in our assay.

The equal abundance of toxins in healthy and AD individuals must be confirmed in a larger cohort. If *S. aureus* isolates do not differ between healthy and AD individuals but the toxin expression is downregulated in an AD environment, quorum sensing control would be a putative new therapeutic option for AD as suggested by Tan et al 2018 (Baldry et al., 2018; Tan et al., 2018; Williams et al., 2019). Complementary to the PCR and growth experiments, the strains will be whole genome sequenced in the future to gain even more knowledge about the differences at isolate level between strains from AD patients and healthy controls.

4.7 Quantification of bacteria

Especially in the context of quorum sensing regulating virulence factors of *S. aureus*, not only the relative frequency distribution but also the absolute cell numbers are important in the context of skin diseases as AD. Therefore, NGS results might not reveal all the relevant information. This conclusion was already drawn by others, showing that reduced microbial abundance in Crohn's disease patients stool was a key factor for separating from healthy stool samples, as well as a higher count for IgG-binding gut bacteria (Harmsen, Pouwels, Funke, Bos, & Dijkstra, 2012; Vandeputte et al., 2017). To analyze genuine host-microbiome interactions, it might be necessary to change from bacterial frequencies to cell counts (Vandeputte et al.,

2017). Longitudinal stool microbiome analysis revealed distinct non-ambiguous abundance profiles of bacterial taxa which were comparable across studies. Furthermore, an increase in relative frequency is not necessarily linked to an increase in absolute cell numbers, showing the importance of combining absolute and frequency information (Props et al., 2017). This shows that a combination of both approaches might reveal more information than only quantities or relative frequencies.

Traditional cultivation of bacteria for absolute cell numbers have its drawbacks as not all bacterial species can be cultured (Dethlefsen, McFall-Ngai, & Relman, 2007). This is not the case in relevant bacterial species in AD. However, cultivation it is not always feasible when samples are collected in study centers without lab access. To assess the absolute bacterial cell numbers, two approaches were taken, i) establishment of a multiplex qPCR assay to detect 16S copy numbers, *Staphylococcaceae* cells, and *S. aureus* cells and ii) a spike-in approach with a species alien to the human body as calibrator of NGS results.

The approach of spiking-in three alien species in stool samples as calibrator was already successfully performed, showing changes in absolute bacterial load and frequency distribution after antibiotic treatment and radio-chemotherapeutic conditioning (Stämmler et al., 2016). Nevertheless, the number of spike-in bacterial cells must be adapted to the sampling location due to varying numbers of expected bacterial cell load. Although a pilot study indicated that 2000 cells of *I. halotolerans* are ideal for skin samples, this could not be confirmed in a larger study. Via qPCR, only in 29 % of the samples, *I. halotolerans* could be detected, whereas via NGS, only 1 % of the samples had undetectable amounts of *I. halotolerans*. This could be due to the fact that more input material was used for NGS than qPCR, increasing the likelihood of having an *I. halotolerans* sequence in the sample volume. The mean of detected *I. halotolerans* cells was 350. Neither in the qPCR results not in the sequencing was a correlation between *I. halotolerans* and total 16S copies or total reads, respectively.

The most common sampling techniques for skin microbiome analysis are swabbing and tape-stripping, because of the low invasiveness of these techniques (H. H. Kong et al., 2017). Via tape-stripping reproducibly high amounts of biomass are obtained (Chng et al., 2016). As on skin only low numbers of bacterial cells are found, it is important to choose a method with which the highest possible number of bacteria are sampled. To ascertain which method is more suitable for this purpose, a meta-analysis across studies where tape stripping or swabbing was sued was performed. In all studies, healthy, AD NL and AD LS were compared. Significantly more 16S copies and *S. aureus* cells were counted with swabbing compared to tape strip in

healthy, AD NL and AD LS. Whereas via swabbing it was apparent that healthy had significantly less bacteria than AD NL and AD LS skin, this biological difference was lost when looking at tape stripping samples. It seems like the maximum capacity of one tape strip is 10^4 16S copies, whereas via swab up to 10^7 16S copies were detected. Consequently, biological differences in bacterial abundance, which can be estimated by 16S copy number must be analyzed using swabbing.

Comparing the bodysides, the 16S copy number was very similar as already described in literature (Zhan Gao, Perez-Perez, Chen, & Blaser, 2010). Whereas all AD individuals had high 16S copies, in HE this was individuum specific and remained stable over time as also reported for the gut microbiome (Props et al., 2017). Interestingly, one of the individuals from the healthy cohort had high 16S copy numbers and *S. aureus* counts and was more similar to individuals with AD. Therefore, it would be interesting to investigate further, whether this person has a history of AD or if AD will be diagnosed in a follow up investigation. Furthermore, this person was the oldest in the cohort (age 68). It is known that the skin and skin microbiome is altered at higher age (H.-J. Kim et al., 2019; Varani et al., 2006).

Comparing dry, moist and sebaceous environments, there is a trend in HE, AD NL and AD LS that the moist environment is highest colonized with bacteria with 10^5 to 10^6 16S copies. In another study, the axilla which is also a moist environment had the highest 16S copy numbers with 10^6 copies per 2x2 cm^2, which is the same as used in our study. Counts in sebaceous sites were lower and dry sites the lowest (Zhan Gao et al., 2010). Furthermore, Gao et al analyzed the abundance of the main genera found on skin, *Corynebacterium*, *Streptococcus*, *Staphylococcus*, and *Propionibacterium* and one fungal genus (*Malassezia*) which should be included in a follow up study (Z. Gao, Tseng, Pei, & Blaser, 2007; Paulino, Tseng, Strober, & Blaser, 2006).

To investigate whether only the distribution between the microbial community changed or *S. aureus* actually overgrew, the number of *S. aureus* cells was correlated with 16S copy numbers. Interestingly, at *S. aureus* numbers higher than 10^4 cells, there is a strong correlation between 16S copy numbers and *S. aureus* cells, hinting towards an overgrowth of *S. aureus* and not only a change in distribution within the bacterial community. *S. aureus* is capable of biofilm formation. In a biofilm, bacterial cells form sessile communities embedded in extracellular polymeric substance matrix (Donlan & Costerton, 2002). Apart from better nutrient sequestration, a biofilm is advantageous as it facilitates the escape from host and synthetic clearance mechanisms (Archer et al., 2011; Beveridge, Makin, Kadurugamuwa, & Li, 1997;

Enea Gino Di Domenico et al., 2019; Lewis, 2010). Protected by the biofilm, *S. aureus* can alter the environment towards favorable inflamed conditions (e.g. increase of pH). Superantigen and toxin expression results in IL-4, IL-5, and IL-13 expression, inhibiting AMP release and induce Th2 inflammation which combined with biofilm formation can result in apoptosis of keratinocytes (Wan & Chen, 2020). Altogether, this could form an environment which enables an overgrowth of *S. aureus*. Furthermore, biofilm formation strength is correlated with AD severity (Allen et al., 2014; E. G. Di Domenico et al., 2018).

The correlation between *S. aureus* and AD disease severity is well established (Heidi H. Kong et al., 2012). The correlation was equally strong between disease severity and *S. aureus* absolute cell numbers and *S. aureus* frequency, which is not surprising as *S. aureus* absolute numbers are strongly correlating with relative frequency observed in NGS. However, there are also patients with low SCORAD and high *S. aureus* and patients with high SCORAD, but low *S. aureus* counts and frequencies. This could be explained by various factors as the virulence factors of *S. aureus* as toxins, and biofilm formation, highlighting again the importance for individual *S. aureus* isolate characterization.

4.8 Transfer to the human system

AD is a barrier defect disease. AD patients have less ZO-1 and Claudin-1 proteins in biopsies (A. De Benedetto et al., 2011; Yuki, Tobiishi, Kusaka-Kikushima, Ota, & Tokura, 2016). *S. aureus* was previously described to be inversely correlated with the expression of the barrier gene Claudin 4 and TJP-1 in a human study (Altunbulakli et al., 2018). Also in vitro, *S. aureus* was shown to downregulate TJ proteins (Ohnemus et al., 2008). Therefore, the effect of different *S. aureus* isolates on the skin barrier genes was assessed in a monolayer of keratinocytes stimulated with a coagulase negative *S. hominis*, one *S. aureus* isolate and a Methicillin resistant strain of *S. aureus*, MW2. Whereas the stimulation with *S. hominis* did not negatively affect the keratinocytes, the cells died when stimulated with any *S. aureus*. However, on Western blot level, no significant differences were visible on the level of Claudin 4 and ZO-1. However, immunohistochemistry might be a better approach for looking at barrier damage in this system. Furthermore, a 3D skin model containing fibroblasts in a collagen matrix and keratinocytes on top was established to test the effect of the different *S. aureus* strains on the skin barrier genes in the future.

4.9 Targeting the skin microbiome as therapy

Especially the healthy skin microbiome seems to be stable against general influences as the season or pH. Although *S. aureus* abundance increased over an eight-week period in AD

patients, this could not be influenced by an acidic or basic emollient. As in vitro *S. aureus* from AD patients is less adapted to an acidic environment, the drastic and sustainable reduction of skin pH could still be a putative treatment approach. However, this must be done carefully, as this can also lead to skin irritations (Luu et al., 2019). As no difference in toxin patterns between isolates found on healthy individuals and AD patients were observed, the expression of the toxins is of interest. Toxins are regulated by the agr quorum sensing system, which is activated at high cell densities and inhibited at acidic pH. Therefore, a specific reduction of *S. aureus* cells with AMPs are putative treatment options (Teruaki Nakatsuji et al., 2017; Newstead, Varjonen, Nuttall, & Paterson, 2020). AMPs which are active against *S. aureus* are often expressed by Coagulase negative Staphylococci. Strikingly, CoNS expressing these AMPs are high in healthy but low in AD patients (Teruaki Nakatsuji et al., 2017). Therefore, bacteria which produce antimicrobials can be used to control *S. aureus* colonization and thereby AD severity (Teruaki Nakatsuji & Gallo, 2019). Also, CoNS as *Staphylococcus caprae* were reported to inhibit the *agr*-mediated quorum sensing of *S. aureus*, reduce toxin expression and thereby improved *S. aureus* infections and lesions in AD (Paharik et al., 2017; Williams et al., 2019). Thus, not only removal of *S. aureus* but also inhibition of the pathogenicity improves AD.

5.0 Conclusion

Due to the high personal, psychosocial, and economic burden of atopic diseases, this field of study is of high research interest. Especially in AD, the skin microbiome plays an important role. Thus, it has been in the focus of attention in recent years and is increasingly performed by non-bioinformaticians. Low biomass samples such as those from the skin are highly susceptible to contaminants. Even the choice of the DNA extraction kit can strongly influence the sequencing results due to kit-inherent contaminants, thus thwarting the possibility to compare studies performed according to different pipelines. Consequently, user friendly and efficient tools for contaminant removal from microbiome datasets are required. The pipeline control-based removal of contaminants with microbIEM was more efficient to remove contaminants than frequency-based contaminant removal.

The skin microbiome of healthy and birch-allergic participants is stable over one year with monthly screening. Individual ‘epitypes’ are not influenced strongly by the exposure to different seasons. Furthermore, weekly sampling for two months confirmed these results in healthy individuals even upon exposure to an acidic and basic emollient. The skin microbiome in AD is less stable over the same period of time. Strong increase in *S. aureus* abundance

appeared alongside increasing AD severity. Intriguingly, *S. aureus* abundance at baseline is a predictor for worsening of AD symptoms. Although the emollient pH had no influence on the skin microbiome, the skin pH restricted *S. aureus* growth between pH 5.7 and 6.2. Specifically in AD patients, the skin pH was slightly altered due to the emollient. Therefore, a more drastic approach could lead to an acidification of the skin in AD. Since it was found that *S. aureus* isolates from AD skin are less adapted to an acidic environment this approach still seems promising.

As no striking difference in the toxin patterns between isolates from healthy and AD individuals were found, the quorum sensing-mediated expression of these toxins should be further investigated. Quorum sensing is regulated by environmental stimuli such as pH and cell density. Interestingly, AD patients are generally more highly colonized with bacteria than healthy individuals. Furthermore, more *S. aureus* cells were found on AD skin. The known increase in *S. aureus* frequency is not only a shift in distribution within the skin microbiome, but also an overgrowth of *S. aureus*. Future research should be dedicated to either specifically reducing *S. aureus* numbers, inhibiting the quorum sensing directly, or by environmental stimuli, such as pH, to tackle the burden of AD disease.

In summary, the data acquired within the scope of this thesis displays both the need and the solution for a user-friendly tool for contaminant removal from low-biomass NGS data. High quality skin microbiome data showed highly stable individual-specific microbiome patterns in healthy participants contrasting to an instable microbiome in AD with increasing *S. aureus* relative and absolute abundance. *S. aureus* is a marker for worsening of AD severity and its growth is limited by pH *in vivo* and *in vitro* and is therefore a putative leverage point for therapy in AD.

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Table of posters and talks

Oral presentation

Conference	Title	Authors
ADF Conference 2017, Göttingen, Germany, 09–11 March 2017	Factors influencing the skin microbiota and its effect on the skin barrier function and the local immune system in the context of allergic and atopic patients	**Hülpüsch C**, Reiger M, Neumann AU, Traidl-Hoffmann C.
ADF Conference 2019, Munich, Germany, 13-16 March 2019	Effect of skin neutral pH versus basic emollient application on skin microbiome and physiology in atopic dermatitis patients and healthy controls	**Hülpüsch, C**, Tremmel, K, Hammel, G, Bhattacharyya, M, de Tomassi, A, Nussbaumer, T, Neumann, AU, Reiger, M, Traidl-Hoffmann, C
Mainzer Allergie Kongress 2019, Mainz, Germany, 29.-30. March 2019	Impact of emollient application on microbiome stability and skin homeostasis in atopic eczema patients and healthy controls	**Hülpüsch C**, Tremmel K, Nussbaumer T, Bhattacharyya M, De Tomassi A, Schwierzeck V, Hammel G, Neumann AU, Reiger M, Traidl-Hoffmann C

Poster presentation

Conference	Title	**Authors**
Wissenschaftstag Klinikum Augsburg, Augsburg, Germany, 30. November 2017	Skin microbiome analysis is affected by choice of DNA extraction kit	**Claudia Hülpüsch**, Matthias Reiger, Avidan U. Neumann, Claudia Traidl-Hoffmann
ADF Conference 2018, Zurich, Switzerland, 07- 10 March 2018	Skin microbiome analysis is affected by choice of DNA extraction kit	C. Hülpüsch; M. Reiger; T. Nussbaumer; A. U. Neumann; C. Traidl-Hoffmann
ISDS Conference 2018, Vienna, Austria	Experimental and computational analysis of contaminants in skin microbiome research	**Hülpüsch C**, Nussbaumer T, Schwierzeck V, Reiger M, Traidl-Hoffmann C, Neumann AU
Wissenschaftstag Klinikum Augsburg, Augsburg, Germany, 06 December 2018	Experimental and computational analysis of contaminants in skin microbiome research	Hülpüsch C, Nussbaumer T, Schwierzeck V, Reiger M, Traidl-Hoffmann C, Neumann AU
ADF Conference 2019, Munich, Germany, 13-16 March 2019	Effect of skin neutral pH versus - basic emollient application on - skin microbiome and physiology in atopic dermatitis patients and healthy controls	**C. Hülpüsch**; K. Tremmel; T. Nussbaumer; M. Bhattacharyya; A. De Tomassi; V. Schwierzeck; G. Hammel; A. U. Neumann; M. Reiger; C. Traidl-Hoffmann
EAACI Congress 2019, Lisbon, Portugal, 01 - 05 June 2019	Microbiome stability and skin physiology in atopic eczema patients and healthy controls upon application of emollients with different pH	**Hülpüsch C**, Tremmel K, Nussbaumer T, Bhattacharyya M, De Tomassi A, Schwierzeck V, Hammel G, Neumann AU, Reiger M, Traidl-Hoffmann C
2. Neurodermitis Symposium, Augsburg, Deutschland, 19th July 2019	Microbiome stability and skin physiology in healthy and atopic individuals upon application of emollients with different pH	**Hülpüsch C**, Tremmel K, Nussbaumer T, Bhattacharyya M, De Tomassi A, Schwierzeck V, Hammel G, Neumann AU, Reiger M, Traidl-Hoffmann C

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Supplement

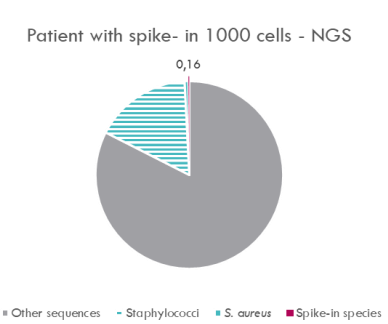
Supplement 1 Minimum, maximum, range, mean, standard deviation over all visits for MHl Ba and Na per bacterial family

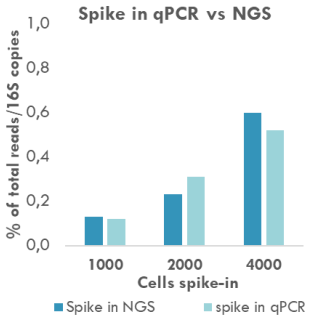
MHl_Ba										
	Propionibacteriaceae	*Staphylococcaceae*	*Corynebacteriaceae*	*Streptococcaceae*	*Micrococcaceae*	*Rhodobacteraceae*	*Moraxellaceae*	*Clostridiales_Incertae Sedis XI*	*Lactobacillaceae*	*Other*
Number of values	15	15	15	15	15	15	15	15	15	15
Minimum	18.96	6.55	4.199	2.247	1.678	2.023	1.301	1.008	0.09513	7.4
Maximum	50.25	32.8	16.61	16.94	7.202	6.756	10.05	6.68	4.013	21.2
Range	31.29	26.25	12.41	14.69	5.524	4.732	8.749	5.672	3.918	13.8
Mean	29.99	23.06	8.131	6.26	4.492	3.612	3.216	2.981	1.449	15.6
Std. Deviation	8.476	7.156	3.459	4.307	1.835	1.759	2.255	1.402	1.024	3.836
Std. Error of Mean	2.189	1.848	0.8932	1.112	0.4737	0.4542	0.5822	0.3619	0.2644	0.9906
MHl Na										
	Propionibacteriaceae	*Staphylococcaceae*	*Corynebacteriaceae*	*Streptococcaceae*	*Micrococcaceae*	*Rhodobacteraceae*	*Moraxellaceae*	*Clostridiales_Incertae Sedis XI*	*Lactobacillaceae*	*Other*
Number of values	15	15	15	15	15	15	15	15	15	15
Minimum	8.015	10.8	2.355	0.4562	1.636	0.6475	1.446	0.2998	0.3313	6.52
Maximum	35.34	58.96	17.05	10.89	6.733	10.72	17.97	6.795	3.132	25.15
Range	27.33	48.16	14.69	10.43	5.097	10.07	16.52	6.495	2.801	18.63
Mean	22.59	32.31	7.322	4.178	4.085	4.107	5.708	2.214	1.408	13.67
Std. Deviation	7.381	14.62	4.54	3.143	1.704	2.912	5.175	1.513	0.9756	5.32
Std. Error of Mean	1.906	3.775	1.172	0.8115	0.44	0.752	1.336	0.3907	0.2519	1.374

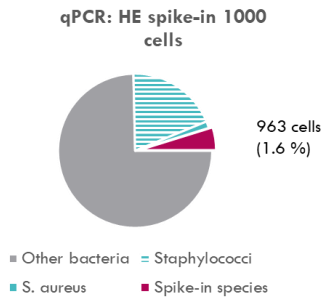
Supplement 2 Overview over available samples (white=available, grey=not available)

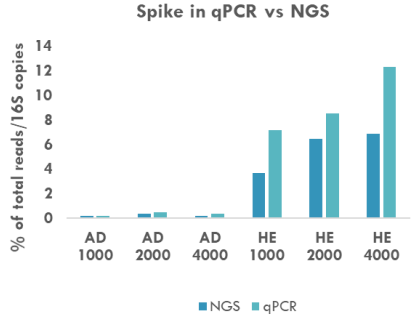
Particpant	Visit 1	Visit 2	Visit 3	Visit 4	Visit 5	Visit 6	Visit 7	Visit 8	Visit 9	Visit 10	Visit 11	Visit 12	Visit 13	Visit 14	Visit 15
PAB_b_1															
PAB_b_2															
PAB_b_3															
PAB_b_4															
PAB_b_5															
PAB_b_6															
PAB_b_7															
PAB_Na_3															
PAB_Na_5															
PAB_Na_6															
PAB_Na_7															
PAB_Na_8															
PAB_Na_9															
PAB_Na_10															

Supplement 3 Pilot study spike-in of different numbers of I. halotolerans cells for quantification of next-generation sequencing data









Supplement 4 Detection of ZO-1 and Claudin 4 via Western blot

Monolayer primary keratinocytes were stimulated for 1, 3 or 6h with 10^5 *S. aureus* MW2 cells either with or without IL-4 and IL-13 co-stimulation. ZO-1 and Claudin 4 were visualized via Western Blot as described, Beta-actin was used as house-keeping gene.