General discussion

MAIN FINDINGS OF THIS THESIS

The aim of this thesis was to characterize the microbial composition of the skin, nose and gut in patients with mild to severe atopic dermatitis (AD) We focused on *S. aureus* and the humoral immune response towards this bacterium, and we designed a clinical study to test the effect of a new endolysin-based therapy that specifically targets *S. aureus* in AD. In the following paragraphs we discuss the main findings of this thesis.

The skin and nasal microbiome are associated with AD severity

In Chapter 2 of this thesis we found an association between the microbial composition of both the nose and skin and AD severity (R^2 = 2.6%; p=0.017 and R^2 =7%; p=0.004). The skin microbiome has been associated with AD severity before in children.[1] However, as far as we know we were the first to evaluate both the skin and nasal microbiome of children in a multivariate model, adjusting for covariates including age, use of antibiotics and the location of sample collection on the skin. We found that staphylococci highly contribute to the association with AD severity in both the nose and skin. Although we could not differentiate between the different staphylococcal species, our results are in line with studies that showed higher density of *S. aureus* in more severe AD (Chapter 4).[1,2] In an additional analysis to characterize *S. aureus,* we found that children with severe AD were positive for *S. aureus* on lesional skin more often than children with mild AD (58% vs 39%). However, this difference was not significant. In the nose, next to staphylococci, also *Moraxella* were positively associated with AD severity. Although the presence of this species in the nose has not been associated with AD severity before, different studies have found that *Moraxella* is associated with asthma and asthma development.[3,4] We retrieved information on the diagnosis of asthma or bronchial hyperactivity (28% of the children) from patient records, and the diagnosis did not influence our results when adjusting for it. For some species, a decreased abundance contributed to the association between the microbiome and AD severity in our study, for example *Dolosigranulum* in the nose and *Streptococcus* on the skin. High abundance of *Dolosigranulum* was suggested to be beneficial for respiratory health in a study of Biesbroek *et al*.[5] *Streptococcus* was observed before in lower relative abundance in skin lesions compared to nonlesional skin in young patients.[6] Although the cross-sectional design of our study precludes evaluating cause-event relationships, our results indicate that both the nasal and skin microbiome might play a role in the severity of inflammation in pediatric AD. Staphylococcal species seem important drivers for the association between both skin and nasal microbiome and AD severity. Prospective and controlled cohort studies are needed to validate our results and determine which species contribute to (or protect for) AD and its severity.

The skin and nose in AD harbor distinct microbial communities, but correlations exist between the two niches.

In Chapter 2 we found that microbial communities in the skin and nose are significantly different from each other (R^2=26.8, p<0.001). This is in line with studies in healthy subjects showing that each body site is characterized by its own microbial community harboring dominant signature taxa.[7] Nevertheless, some species such as staphylococci, were present in both niches in our patients. However, where most skin samples were dominated by staphyloccoci, only a few nasal samples showed dominance of staphylococcal species. Looking at abundance patterns of species in the nose compared with (other species) on the skin, we found that many species in the nose and skin showed similar patterns of species abundance. Staphylococci on the skin formed an exception as they were negatively correlated with many other species in the nose and skin. The exact meaning of these relations should be further explored as different biological and/or immunological mechanisms might underlie our observation. A possible underlying mechanism might be cross-transmission of bacteria between the nose and skin.[8-10] Prospective large cohort studies should further evaluate the presence of cross-transmission between the nasal and skin microbiome, as it might be of relevance for determining treatment strategies for AD. Furthermore, it might help to determine if a diagnosis of AD influences the persistence of nasal carriage of *S. aureus,* a risk factor for infections with the bacterium.[11-13]

The gut microbiome differs between children with AD, with and without a food allergy

Severe AD is associated with food allergy.[14] Although several studies have shown associations between the intestinal microbiome and development of atopic diseases, the link between intestinal microbiota and food allergy has rarely been studied.[15-17] In Chapter 3 of this thesis we described the gut microbiome in a group of 82 children with mild to severe AD. Of these children, 20 were diagnosed with food allergy mainly for peanut and cow's milk. We aimed to identify gut microbial characteristics associated with food allergy, using the gold standard for diagnosing food allergy (double blind placebo controlled food challenge), which has rarely been done before. Six bacterial species from the gut microbiome were identified that when combined discriminate between children with and without food allergy: *Bifidobacterium breve, Bifidobacterium pseudocatenulatum, Bifidobacterium adolescentis, Escherichia coli, Faecalibacterium prausnitzii* and *Akkermansia muciniphila (*AUC 0.83, sensitivity 0.77, specificity 0.80). Our pilot results are based on a small cross-sectional study and should be confirmed in future prospective studies and further adjusted for confounders, such as diet and AD severity. The exact mechanisms through which the intestinal microbiome influences food allergy are not elucidated yet and it is not clear whether a change in microbiome precedes

or follows the development of food allergy. A possible mechanism is disruption of the gut microbiome that alters the gut epithelial integrity, thereby increasing the risk of allergic sensitization through direct uptake of allergens, a hypothesis that is mainly based on animal studies.[17] Another possible mechanism could be the immune stimulatory capacity of gut microbes (via secretion of molecules).[18-20] In our study, the children with a food allergy were also found to have higher thymus and activation-regulated chemokine (TARC) levels, a serum biomarker for AD severity, suggesting increased AD severity compared to the non-allergic group.[21,22] This raises the possibility that the six bacterial species also correlate with AD severity. Furthermore, we cannot exclude that elimination diets in the food allergic group have led to changes in the gut microbial composition. However, one third of the children in the non-food allergic group also reported an elimination diet for a specific food, which makes it unlikely that our findings are solely attributed to differences in diets. Our results need to be confirmed in larger studies that enable stratification on specific food allergies and adjustment for AD severity and dietary factors.

Patients with AD have an increased risk of colonization with *S. aureus*

In Chapter 4 we quantified the prevalence of *S. aureus* in AD patients compared to controls and concluded that patients with AD are significantly more likely to carry *S. aureus* than healthy controls on both the lesional and nonlesional skin. *S. aureus* was identified on lesional skin in 70% of the patients, with a higher prevalence of *S. aureus* in patients with severe AD and in patients under 18 years old. We showed that heterogeneity among the included studies was substantial, ranging from 63% to 88% for the pooled outcomes, which can be partly explained by the quality of the studies and disease severity of the included patients. We also detected publication bias causing an overestimation of the pooled outcomes. Although different studies calculated prevalence rates of *S. aureus* in AD, with this meta-analysis we were the first to systematically summarize the data of the different studies. Our meta-analysis indicates the importance of *S. aureus* in AD, not only in skin lesions but also in nonlesional skin and the nose. A positive association between AD and the presence of *S. aureus* both on the skin and nose suggest a possible role of the bacterium in aggravation of AD inflammation and encourages further research into exact mechanisms.

***S. aureus* evokes an IgE based immune response in a subgroup of patients with AD**

In Chapter 5 of this thesis we described the first systematic review that summarizes data on antibody prevalence against *S. aureus* in AD. In a pooled analysis we found that IgE against SEA and SEB (two staphylococcal superantigens) is present significantly more often in patients with AD compared to healthy controls. Pooled analysis of IgE

against TSST-1 included only two studies and showed the same trend (not statistically significant). Pooled prevalence estimates of antistaphylococcal IgE patients were 33% for SEA, 35% for SEB, 14% for SEC, 5% for SED and 16% for TSST-1. Data on other antigens were insufficient for a pooled analysis. The observed high heterogeneity in the pooled analysis could be partly explained by the variety in IgE detection methods used. We did not find a difference in IgE levels between children and adults. Other variables such as AD severity and treatment are also likely to contribute to the observed heterogeneity, but could not be explored as this information was not available for the included studies. The increased IgE in patients compared to controls indicates that *S. aureus* might stimulate AD inflammation via IgE mediated mechanisms, such as mast-cell degranulation, suggesting a role of *S. aureus* as an allergen. It is unclear whether the increased IgE is the result of increased skin barrier permeability in AD, predominance of SEB and SEA carrying strains in AD skin and/or an inappropriate immune response towards the bacterium. It is probably a combination of these factors. Notably, only a subgroup of patients show elevated IgE, suggesting that only a part of the AD patients reacts in an IgE dependent matter towards *S. aureus*. On the other hand, only 14% and 24% of the *S. aureus* isolates carry the genes to express SEA and SEB, so a group of AD patients might not have encountered the allergen yet.[23] Further research is needed to clarify the clinical relevance of IgE responses against *S. aureus* in patients. Our results also indicate a lack of studies that evaluate immune responses other than the IgE mediated response against staphylococcal superantigens.

Children with AD develop an IgG dependent immune response against *S. aureus* and the bacterium might use immune-modulatory antigens to persist on the skin in AD

As outlined in Chapter 5, most research has focused on IgE humoral responses towards a limited panel of *S. aureus* antigens. As IgG is known for its involvement in the neutralization and elimination of microbes, we profiled IgG antibodies against 55 *S. aureus* antigens in two cohorts of children with AD (Chapter 6). Our results showed that the children are exposed to a wide range of antigens and develop an IgG mediated humoral immune response towards them. In one of the cohorts, the IgG response against antigens with mainly immune-modulatory functions, (e.g. Leukotoxin (Luk) D and E) was associated with the severity of the AD. The exact pathophysiological mechanism that explains this finding is unclear, although it can be argued that children with more severe AD might have an altered immune response against staphylococcal antigens. The association could also be a reflection of the higher *S. aureus* load on the skin of children with more severe AD, that IgG tries to counteract. However, in the latter case one would expect increased IgG against all *S. aureus* antigens rather than a subset. On the other hand, *S. aureus* might express more immune-modulatory antigens, which may lead to a more se-

vere AD phenotype. By down regulating the immune system locally, *S. aureus* can more easily maintain its colonization on the skin, which can cause chronic inflammation in AD, characterized by Th1 cells in the skin. *In vitro* studies showed that activation of dentritic cells by *S. aureus* lipoteichoic acid and the Th2 cytokine IL-4 (combined) can result in enhanced Th1 and Th17 priming.[24,25] In acute AD inflammation, where IL-4 is present, additional presence of *S. aureus* on the skin can cause a state of persistent and chronic AD. The exact role of *S. aureus* specific IgG in the inflammatory response in AD should be further investigated. A study of Meulenbroek *et al*. found that the presence of IgG affects the binding of food allergen-IgG complexes to B-cells. This mechanism was also seen for birch pollen, suggesting a role for IgG also in the allergic response.[26] Determination of IgG subtypes combined with IgE can help clarify the role of IgG in AD, as different IgG subtypes might have different characteristics relating to other AD phenotypes. For example in AD, tolerance to cow's milk was associated with increased IgG4 in combination with low specific IgE.[27] A control group of children is needed to investigate the normal range of IgG antibodies in children without AD. Nevertheless, the results of our study shed light onto the IgG mediated immune response to *S. aureus* in children with AD and highlight the relevance of other antigens (adhesins and immune modulators) next to the often studied superantigens. Further studies, including IgG subtype responses, need to be conducted to validate our results and will help us understand how microbes interact with the immune system and possibly induce inflammation.

Techniques for skin microbiome research: scrub results in higher collection of bacterial and fungal DNA compared to swab

As mentioned in the introduction of this thesis, investigating the skin microbiome imposes some challenges. For example, the low biomass of microbial DNA on the skin requires adequate sampling that produces sufficient yield for reliable analysis. In Chapter 7 we compared dry nylon-flocked eSwabs versus a scrub method to collect skin microbial samples. These eSwabs are thought to enhance bacterial absorption and release compared to traditional cotton or rayon swabs, which could be attributed to the flocked structure of the swab.[28] The scrub sample was collected by placing a sterile sampling ring on the skin and adding sterile wash fluid. After scrubbing the skin within the ring with a sterile swab, the fluid was collected and analyzed. [29,30] We showed with quantitative qPCR that scrubs result in significantly higher amounts of total bacterial DNA compared to the swab. However, with 16S rRNA sequencing we showed that both methods identify the major genera equally well. The potential of the scrub method to increase bacterial discovery rates was described earlier, but based on culture methods.[31] Fungal DNA was found more often in scrub samples compared to swabs (36% versus 9%). Increased yields of bacterial and fungal DNA make scrubs the preferred method, especially for characterizing fungi, rare microorganisms and for low biomass areas of

the skin. However, dry eSwabs are more user-friendly and can be applied on all skin sites. Also, eSwabs identify the dominant sequences of the microbiome equally well as scrubs, at least in the sites sampled in our study (mainly the antecubital folds). Therefore, the swabs can be considered for large scale studies and for body sites that are difficult to sample with the scrub. A comparison of the eSwab and the scrub with premoistened cotton swabs, a method that is commonly used now for microbiome analysis, might further help identify the preferred method for microbial analysis.

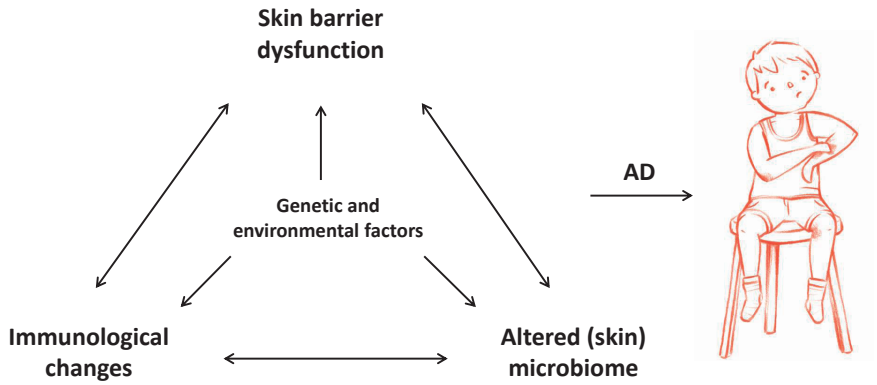
PLACE OF THE MICROBIOME IN THE ATOPIC DERMATITIS DISEASE MODEL

A recent review on atopic dermatitis describes two main pathophysiological landmarks in AD, namely (1) abnormalities of the skin barrier and (2) changes in the immune response, with a close interaction between skin barrier biology and immune mechanisms. *S. aureus* is shortly mentioned as a possible contributor to AD exacerbations and chronic inflammation.[32] We propose a model for AD pathophysiology that includes a third main component, namely alterations in the microbiome (figure 1). This model incorporates several findings of this thesis that include an association between microbial composition and AD severity (Chapter 2 and 4) and the interaction of *S. aureus* with the immune system (Chapter 5 and 6).

The model in figure 1 shows three main components: the skin barrier, the immune system and the (skin) microbiome. Together they contribute to a balanced ecosystem which is important for a healthy skin, influenced by genes and environment (including environmental microbes). Bidirectional interactions between the components contribute to the mechanical and immunological barrier function of the skin. A healthy microbiome strengthens the mechanical skin barrier as commensals occupy space that could otherwise be colonized by pathogens. Microbes such as *S. epidermidis* support the local immune system by producing antimicrobial peptides that suppress *S. aureus*.[33,34] Inflammation in AD is probably the result of a disbalance in this skin ecosystem, resulting in an altered barrier, immunological changes and an altered skin microbiome (figure 1). The alterations in all three components together contribute to AD etiology. The initial trigger that causes the disbalanced ecosystem can affect each of the components, which might result in a vicious circle where the components constantly aggravate each other, leading to a situation of chronic inflammation. It is likely that the contribution of each of the three components to the inflammation differs per patient and even within patients over time.

The model in figure 1 includes microbiome alterations as a basis for AD pathogenesis, and questions the relevance of the debate on whether alterations in the microbiome are cause or consequence of AD. It is likely that microbial dysbiosis and *S. aureus* overgrowth

Figure 1. Disease model of AD including the (skin) microbiome. *(Illustration by Marloes van Loon)*



are not the primary events that cause (flares in) AD. A pre-existent barrier defect and immunological changes are probably a prerequisite for *S. aureus* to overgrow. The skin barrier defect in AD provides a suitable environment for *S. aureus* to grow and allows contact with immune cells in the skin. Also, presence of Th2 cells is needed for the production of IgE against *S. aureus* antigens.

Once present on the skin, *S. aureus* can induce inflammation via different mechanisms, namely excretion of virulence factors and the activation of both IgG and IgE mediated immune pathways. Via these mechanisms *S. aureus* has the ability to further aggravate inflammation or induce flares, even if the microbial dysbiosis and overgrowth is primarily caused by other factors, such as a skin barrier defect. Theoretically, this makes the microbiome a possible target for treatment of AD (flares). Although we know that it is theoretically possible, evidence that proves that *S. aureus* is actually aggravating the inflammation during a flare is still scarce. Large prospective cohort studies are needed that further clarify the role of the microbiome in AD, including *S. aureus,* with sampling specifically around disease flares. The above mentioned 'cause or consequence' discussion is also outlined in a letter published by our group in the British Journal of Dermatology (Totté *et al*. 2017).[35]

CLINICAL IMPLICATIONS OF THE RESULTS PRESENTED IN THIS THESIS

The skin microbiome as a therapeutic target in AD

Currently, there is no place for antimicrobial therapy in the treatment of AD without signs of infection (fever, high staphylococcal load or clinically infected AD (impetiginization of the lesions)).[36] Based on our model for AD pathogenesis (figure 1), we hypothesize that a

combined treatment strategy targeting all three components, including the alterations in the microbiome, could optimize the treatment of AD. To determine the right strategy to target the microbiome, we need to know which alterations in the microbiome are associated with AD and AD flares. The established association between *S. aureus* colonization and AD, makes *S. aureus* the first target of interest (Chapter 4). In Chapter 2 of this thesis we identified other microorganisms that were associated with AD severity. Their role as potential treatment targets, next to *S. aureus,* in AD should be further explored. The added value of antistaphylococcal therapy in AD without symptoms of infection could not be confirmed before in a published review of clinical studies.[37] However, the studies that were assessed in this review included approaches that have been tested on their ability to treat an exacerbation, in trials with relatively short follow-up periods, not showing whether the treatment can provide long-term control of the disease activity. We hypothesize that long-term modulation of the microbiome may be needed to maintain a stable and balanced skin microbial composition. This can be argued as *S. aureus,* combined with a Th2 cell acute inflammatory environment, can contribute to a state of persistent AD.[24] Antistaphylococcal treatment could temporarily reduce the 'pressure' of *S. aureus* and relieve symptoms, but after stopping the treatment the bacterium is likely to regrow quickly. Especially, in the presence of an underlying genetic barrier effect in part of the AD patients, which might facilitate colonization with *S. aureus*.[38] Studies did describe before that mutations in the gene encoding filaggrin, an important protein for skin barrier homeostasis, were associated with the microbial composition in nonlesional AD skin.[10] Also gram-positive anaerobe cocci were found underrepresented in the microbiome filaggrin-deficient human skin, which was speculated to favour growth of *S. aureus*.[39]

Results of two more recent intervention trials that reported significant improvement of disease severity in non-infected AD after two and three months of therapy with antistaphylococcal therapy (bleach baths) support the hypothesis that long-term treatment is necessary for better disease control.[16,40-42] We conclude that long-term studies are needed to determine if treatment that aims to restore microbial alterations might have a place in the treatment of non-infected AD.

Strategies for long term modulation of the skin microbiome

Currently available treatment strategies to reduce *S. aureus*, include antibiotics, bleach baths and Povidon-iodine (Betadine) scrubs. These agents have a broad-spectrum activity, indicating that they also affect beneficial microbes on the skin and other body sites.[43] In addition, long-term treatment with antibiotics is undesirable as it can induce *S. aureus* resistance to antibiotics, causing more severe disease, prolonged hospitalization and increased mortality.[44-46] New treatment strategies that specifically target only the microbe of interest and that allow long-term treatment is therefore urgently needed. New tar-

geted strategies have been developed, including anti-*S. aureus* vaccines (unsuccessful in clinical testing until now) and topical endolysin-based treatment against *S. aureus* that recently became available for clinical use.[38,47] Next to reducing the load of certain target species, alterations in the behavior of pathogens changing from virulent to commensal have been studied. In an *in vitro* study, *S. aureus* was found to change from virulent to commensal when exposed to the commensal *Corynebacterium striatum*.[48] A third option to reduce the overgrowth of *S. aureus* includes topical probiotic strategies that involve application of species that inhibit growth of *S. aureus*, for example *S. epidermidis* and *S. hominis.*[33]

Next to therapy directed to *S. aureus,* treatment with an emollient has also been found to reduce AD symptoms with subsequent reduction of *Staphylococcus* species load.[49] Also, emollient treatment from birth was considered an effective approach for atopic dermatitis prevention in a group of high-risk infants.[50] These results make treatment with an emollient an important first step to optimize the skin microbial composition. The precise mechanisms through which emollients have beneficial effects are still poorly understood and need further exploration, but they are probably related to improved barrier integrity.[32] Furthermore, Czarnowicki *et al.* identified alterations in the expression of antimicrobial peptides with the application of petrolatum, an over-the-counter moisturizer.[51]

Influence of extra-cutaneous microbial niches

In Chapter 4 we found an increased risk for *S. aureus* colonization in the nose of AD patients compared to healthy controls. These results raise the question whether the nose should be included when applying topical therapy to modulate the skin microbiome. Current American treatment guidelines also recommend combined intranasal mupirocin and bleach baths for infections in AD.[52] Studies that evaluate the effect of new topical antimicrobials should incorporate measurements of extra-cutaneous microbial niches. This will help determine whether a certain extra-cutaneous microbial profile influences treatment effects. In general, niches that can be carriage sites are the nares, the oropharynx, the axillae, groin, the perineum, and the vagina.[53]

The microbiome and diagnostics

In current clinical practice, swabs of AD lesions are rarely collected when the lesion does not show signs of clinical infection. In this thesis we discussed that *S. aureus* on the skin and in the nose likely plays a role in aggravating inflammation in non-infected AD as well, particular in severe AD. This raises the question whether collection of skin and nasal swabs to determine *S. aureus* load in non-infected AD should be considered standard practice. Having information about the presence or absence of *S. aureus* can help determining whether targeting the microbiome should be considered as part of the treatment

strategy, next to skin barrier and anti-inflammatory treatment. Chapter 6 of this thesis supports that *S. aureus* might also contribute to development of chronic AD lesions, also previously suggested by Biedermann *et al*.[24] These results should encourage clinicians to also consider *S. aureus* culture in chronic dermatitis where the role of *S. aureus* seems less obvious compared to the more fierce red and oozing acute lesions. Future studies should investigate how *S. aureus* behaves on/in skin with chronic dermatitis compared to skin with acute inflammation.

Although the data are very preliminary, the results of Chapter 3 suggest possibilities to use the microbial composition as a diagnostic tool. Our pilot data indicated that microbial patterns in the gut are associated with food allergy in children with AD and that it is possible to distinguish non-food allergic children from food allergic children using fecal samples. The use of fecal samples would allow for a simple and cheap method to distinguish children without a food allergy from children with a food allergy as a first step in the diagnostic process, as the current gold standard for diagnosis of food allergy, a double blind placebo controlled food challenge, is (time) invasive, costly and can be difficult in young children. As discussed in Chapter 3, this was a pilot study and the findings need to be validated in other (clinical) studies, including adjustment for important confounders.

Design for an intervention study to target atopic dermatitis - anti-*S. aureus* endolysins

When the first targeted anti-*S. aureus* treatment strategy, based on endolysins, became available on the market, we decided to study its effect in a clinical setting and were the first to report on this.[47,54] The endolysin Staphefekt SA.100™ (Staphefekt) is an engineered chimeric endolysin that specifically lyses the cell membrane of *S. aureus* via endopeptidase and putative amidase activities. Incorporated in a cetamacrogol based cream, the endolysin is registered as a medical device class 1 in Europe and available for topical application. In vitro studies tested the activity of Staphefekt in phosphate buffered saline against 28 clinical strains of MSSA, 8 strains of MRSA, and four other staphylococcal strains (*S. epidermidis, S. hominis, S. haemolyticus* and *S. lugdunensis)*.[47] These results indicate that Staphefekt kills different types of *S. aureus* including methicillin resistant strains (mean reduction in OD of 58%), while causing little harm to the four other staphylococci (mean reduction in OD of 4.3%). The effect on other commensal species needs further investigation.

We tested Staphefekt in a study of 3 cases with *S. aureus*-related skin conditions, folliculitis and impetiginized dermatitis, and found that Staphefekt led to a reduction of clinical symptoms (Totté et al. CR in Dermatology 2017).[54] Bacterial resistance to Staphefekt was not found in our case study during 4 months of treatment (assessed in one of the three patients). We hypothesized that targeting *S. aureus* with Staphefekt can

restore the microbial balance, which might relieve AD inflammation or even prevent flares and reduce the use of (topical) steroids. The low risk of resistance should enable us to study the effect of long-term microbiome modulation in non-infected AD. Also, the targeted mechanism of action enables us to study specifically the role of *S. aureus* in AD pathogenesis. In Chapter 8 we described a protocol to carry-out a randomized controlled trial to evaluate the effect of a three months antistaphylococcal therapy with Staphefekt. The clinical trial started in 2016 and data collection just finished at the time of writing this thesis.

In the trial design, we incorporated the knowledge gained from the studies in this thesis. For example, only patients with moderate to severe AD were included, as they have more overgrowth of *S. aureus* and are therefore more likely to benefit from the anti-microbial therapy (Chapter 4). Also, we choose to collect additional scrubs of the skin for optimal collection of microbial biomass and resolution for sequencing (Chapter 7). The decision to test the efficacy of the endolysin directly in patients, has its pros and cons. The AD skin is subject to many factors that influence microbial growth due to interaction with the skin barrier and host immune system and its direct exposure to the environment. This hampers measuring the exact effect of Staphefekt on *S. aureus* growth. An experimental *in vitro* set up, or a model using the human nares, would enable more stable conditions and a better estimation of the direct effect of the endolysin on the bacterium. However, the patient-based setting and pragmatic approach in which we compared the add-on of the endolysin to standard care with standard care alone, gives results that are directly relevant to practice and will help making decision about treatment options. The relevance of microbial outcomes is hard to interpret without being able to relate them to clinical outcomes. For example, we do not know yet whether a complete elimination of *S. aureus* needs to be achieved for clinical improvement, or if decreasing the bacterial load will also be sufficient. Based on our case series, where we found a clinical improvement while *S. aureus* could still be cultured from the skin, we hypothesize that a reduction of *S. aureus* can already initiate rebalancing of the microbial dysbiosis. The extent of that reduction is difficult to determine and probably differs per patient and disease episode, skin barrier status and host-immune factors. We expect that the trial will increase our understanding about how antimicrobial therapies affect the microbiome and how this affects diseases states.

METHODOLOGICAL CONSIDERATIONS

In part 1 and 2 of this thesis we used two pediatric AD patient cohorts to characterize the microbiome, with a focus on staphylococci, and the antibody response against it. To investigate how specific the results are for AD inflammation, healthy control groups and

patients with other chronic inflammatory skin diseases, such as psoriasis, are needed. However, our conclusion focusses on AD severity in well characterized cohorts of children with AD. The lack of controls does not influence this particular comparison. Another limitation might be the cross-sectional design of our studies which does not allow the investigation of cause–effect relationships between the microbiome and AD severity and between anti-*S. aureus* IgG levels and AD severity. Furthermore, we conducted our cohort studies in academic centers and our results might not be representative for the general AD population.

In our microbiome studies, we determined the bacterial microbiome by sequencing of the 16S rRNA gene. We amplified the V4 variable region of the gene. This region was often used in studies characterizing the gut microbiome. In more recent studies it was shown that sequencing of this region does not allow classification of staphylococci at the species level, which is important when characterizing the skin microbiome. To further specify staphylococci in our studies, we performed additional qPCR on *S. aureus* and S. epidermidis. Because we were not able to identify staphylococcal species or even strains, we might have missed existing correlations between staphylococcal species and AD severity. Additionally, the V4 region in combination with the reverse primer that we used (806R) is known for poor coverage of Propionibacteria. Of note, in our samples Propionibacterium were indeed low in presence in both the skin and nasal samples, while they are described as part of the healthy microbial communities.[55] However, the low Propionibacteria can still be a true reflection of the AD lesions as staphylococci are known to overgrow other species. Thereby, we sampled the antecubital folds which are known for a low abundance of Propionibacteria that prefer more sebaceous environments.[56] Current studies recommend amplification of the V1-V3 region or the use of a modified V4 primer for skin microbiome research. This results in better coverage of Propionibacterium and better classification of S. epidermidis. Classifying S. aureus remains difficult with current 16S rRNA sequencing approaches. [57,58] It should be noted that amplification of long regions (such as V1-3) also imposes some challenges with regard to generating good quality sequences. New recommendations for sequencing will be taken forward for analyzing samples of the clinical trial (Chapter 8).

FUTURE PERSPECTIVES

Aims for future studies

This thesis and previously published literature have shown the complexity of the microbial ecosystem and its interaction with the skin barrier and immune system. It also shows the relevance of the microbiome in AD pathogenesis and encourages further research on the topic as many aspects still need to be elucidated, such as 1) which microbes are

implied in AD pathogenesis, 2) by which mechanisms do they interact with the skin barrier and immune system and cause inflammation and 3) what is the effect of microbial modulation on clinical disease.

In this thesis we confirmed the role of *S. aureus* in AD and we identified other microorganisms that might be involved in AD inflammation. Future studies should confirm the associations found in this thesis and determine associations on the species level. A comparison of microbial communities between patients and healthy controls is crucial to generate hypotheses around possible drivers of AD disease. Explorative research to identify microorganisms of relevance, should not only focus on bacteria but also on other microorganisms including fungi and yeasts. Chapter 3 of this thesis highlights the relevance of looking at the effects of groups of bacteria when performing this kind of studies, instead of single species. Furthermore, the influence of communities on other body sites apart from the skin should be part of the focus. Once species are identified, experimental *in vitro* models and animal challenge models could help to understand by which mechanisms bacteria influence inflammation and interact within the skin barrier and local immune system. Although there is great interest in new omics techniques, culturing of species of interest from humans remains very important to perform these experiments. With respect to *S. aureus,* it would be particularly interesting to study which mechanisms or triggers change the behavior of *S. aureus* into a disease causing pathogen, as we know the bacteria can also colonize the skin and mucosa of healthy individuals.[13] As *in vitro* and animal models lack generalizability to the complex skin ecosystem *in vivo*, additional patient-based studies are needed to further clarify the role of microbial dysbiosis in AD and untangle interactions between the microbiome and the host skin barrier and immune system. The still developing AD and immune-system makes the pediatric population of interest for studying these interactions. After identifying mechanisms that are of possible relevance in AD inflammation, well-designed clinical trials are needed to determine the added value of new treatment strategies.

Longitudinal studies will allow the investigation of cause-effect relationships between AD, the microbiome, and the humoral immune response. Longitudinal population based studies from birth to disease development are needed to clarify the role of the microbiome in AD development. Furthermore, it might be important to sample at multiple time points during a child's development. Some studies suggest that the microbiome might only influence the development of AD in certain 'critical' timeframes during infancy.[59-61] As evidence that proves that *S. aureus* is truly aggravating the inflammation is still scarce, longitudinal sampling around the period of a disease flare is also still needed to identify whether changes in the microbiome and increased *Staphylococcus* levels precede flares. This would support a contribution of the microbiome in the onset of flares.

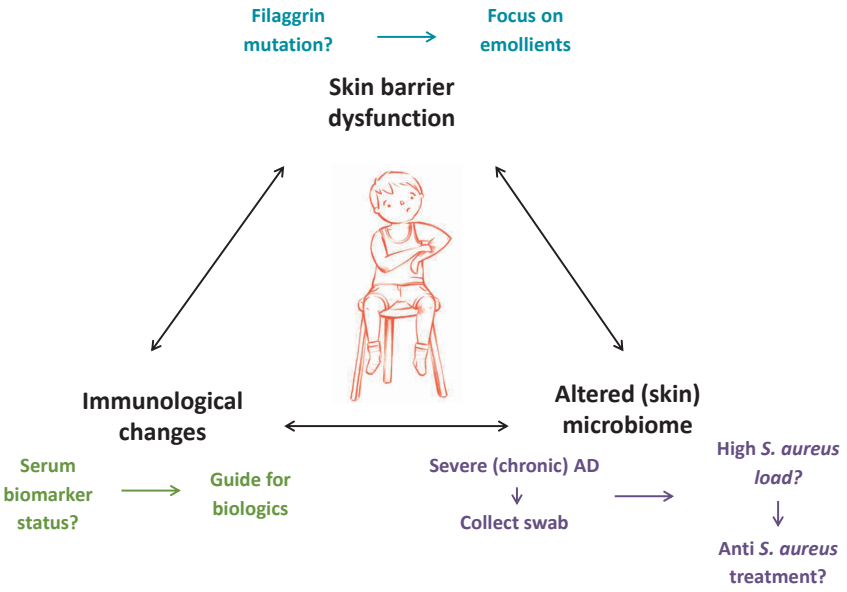
Techniques and standardization

A complex ecosystem requires complex analysis techniques to enable analysis of all species and their interactions with each other and the host. The upcoming omics techniques already incorporate a broader perspective of the microbiome. Eventually, these new techniques that allow functional analysis next to association analysis, will be the key to a better understanding of the relations between microbes and the host immune system, the skin barrier and the other microbes and antimicrobial peptides. Especially in AD research, where it is crucial to specify staphylococci at the species level, metagenomics sequencing has advantages as it provides sufficient resolution to differentiate species and even strains.[62] This has been proven difficult with different 16S rRNA sequencing approaches, but seems important as studies showed that functional differences of staphylococcal strains exist and probably contribute to the complexity of AD.[2,57] However, due to the rapid development of the field, methods are not standardized yet. In general, differences in study population, sample preparation, sequencing methodology and use of bioinformatics tools including reference databases probably cause variation in outcomes. Specifically in skin microbiome research, we have to consider the unique features of the skin. The low biomass (compared to for example the gut and nose), the site specific microenvironments, the distinct local immune system and the high risk of contamination require specific approaches. Standardized methods and bioinformatics protocols are needed to gain more robust results. Transparency and sharing of sequencing and bioinformatics methodology in international databases would help standardize microbiome research and improvement of methods.

Personalized treatment: identifying phenotypes for atopic dermatitis

The diverse symptoms, different ages of onset, varying natural disease courses and comorbidities illustrate the heterogeneity of AD. Due to this heterogeneity, it can be expected that patients do not respond equally well to standardized 'one size fits all' treatment regimens. Also, systemic treatment and upcoming biologics are costly. Therefore, it is important to decide which patient will benefit from which type of treatment. Characterizing patients based on biomarkers of genetic, immunological and microbiological origin (the three components of figure 1), will support clinical decision-making and lead to a more personalized treatment of AD. By measuring specific biomarkers related to these three components, a balanced treatment strategy with more or less focus on certain components can be designed. Likely a mix of multiple biomarkers will be needed to define subpopulations and predict treatment response (figure 2).[63] Some research has been done on characterization of barrier and immunological biomarkers. Skin barrier integrity can be measured via filaggrin mutation status amongst others.[64] In case of a genetic defect, focus should be on treatment with emollients to enhance the barrier function of the skin. A recent study among patients with moderate to severe AD already

Figure 2. possible biomarkers of interest for research on AD treatment personalization. *(Illustration by Marloes van Loon)*



identified distinct clusters of patients based on serum biomarker profiles, illustrating biological differences between patients, which will help guide the use of biologics.[65] With respect to the microbial component, it is also likely that the role of the microbiome in AD pathogenesis differs within subsets of patients. We found that *S. aureus* was not dominating the lesional skin in all children and every child with severe AD had a unique skin microbial composition (Chapter 2). Treatment targeting the microbiome might therefore not be preferred for all AD patients at all times. Patients with severe AD are more likely to benefit from anti- *S. aureus* treatment, while in mild AD a skin (and nasal) swab can be collected to guide decisions.

Next to determining biomarkers to guide AD treatment, it is also important to identify biomarkers in young patients that predict the development towards severe disease. We know that early treatment of high risk patients has been shown to prevent AD.[50] Currently, family history is often used to identify high risk patients.[66] Also the filaggrin mutation status can be used as a biomarker, because a mutation in this gene strongly predicts a worse prognosis with more severe AD and atopic comorbidities.[64,67]

It should be clear that first attempts have been made towards personalizing treatment for AD. However, further characterization of patients and development of biomarkers is needed. Future cohort studies and trials should incorporate an endotyping approach in their design, including assessment of the epidermal, immunological and microbial bio-

markers, such as filaggrin mutation status and *S. aureus* presence, and sufficient power to eventually determine which type of patients will benefit from which therapy.[63]

FINAL CONCLUSION

This thesis shows the relevance of the microbiome, in particular *S. aureus*, in the pathogenesis of AD. *S. aureus* seems to evoke immune responses through different mechanisms, as a directly stimulating antigen and as an allergen. We found that next to *S. aureus,* other microbes on the skin and also microbial communities in the nose might be involved in AD inflammation. Our results may contribute to the development of treatment strategies that target the microbiome in AD. Further prospective cohort studies and experimental research are needed to clarify the role of the microbiome in AD and its role in AD treatment. As the role of the microbiome likely differs between patients, probably as a result of various genetic and environmental factors, further stratification of patients is needed to better guide therapeutic approaches. Eventually this will lead to more personalized treatment of AD.

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