



ORIGINAL ARTICLE

Clinical Mechanisms in Allergic Disease

The influence of treatment in alpine and moderate maritime climate on the composition of the skin microbiome in patients with difficult to treat atopic dermatitis

Minke M.F. van Mierlo¹ | Joan E.E. Totté¹ | Karin B. Fieten^{2,3,4} |
Tim J. van den Broek⁵ | Frank H.J. Schuren⁵ | Luba M. Pardo¹ | Suzanne G.M.A. Pasmans^{1,2}

¹Department of Dermatology-Center of Paediatric Dermatology, Sophia Children's Hospital, Erasmus MC University Medical Center Rotterdam, Rotterdam, The Netherlands

²Department of Dermatology and Allergology, University Medical Center, Utrecht, The Netherlands

³Dutch Asthma Center Davos, Davos, Switzerland

⁴Swiss Institute of Allergy and Asthma Research (SIAF), University of Zürich, Davos, Switzerland

⁵Microbiology and Systems Biology, TNO Zeist, The Netherlands

Correspondence

Minke van Mierlo, Department of Dermatology, Erasmus MC University Medical Center Rotterdam, Dr. Molewaterplein 40, 3015 GD Rotterdam, The Netherlands.
Email: m.vanmierlo@erasmusmc.nl

Suzanne Pasmans, Department of Dermatology-Center of Paediatric Dermatology, Sophia Children's Hospital, Erasmus MC University Medical Center Rotterdam, Sp-1540, Wyttemaweg 80, 3015 CN Rotterdam, The Netherlands.
Email: s.pasmans@erasmusmc.nl

Funding information

Micreos Human Health, the Netherlands; Merem Dutch Asthma Center Davos; European Allergy and Asthma Center Davos; patient support group "Vereniging Nederland Davos"

Abstract

Background: The skin microbiome, characterized by an overgrowth of *Staphylococcus aureus*, plays an important role in the pathogenesis of atopic dermatitis (AD). Multidisciplinary treatment in alpine climate is known for its positive effect on disease severity in children with AD and can result in a different immune response compared with moderate maritime climate. However, the effect on the composition of the skin microbiome in AD is unknown.

Objective: To determine the effect of treatment in alpine climate and moderate maritime climate on the microbiome for lesional and non-lesional skin in children with difficult to treat AD.

Results: Alpine climate treatment led to a significant change in the microbiota on lesional skin, whereas no significant change was found after moderate maritime climate. On both lesional and non-lesional skin, we observed a significant increase in Shannon diversity and a significant decrease in both *Staphylococcus* abundance and *S aureus* load after alpine climate treatment. The decrease in *S aureus* was significantly larger on lesional skin following alpine climate treatment compared with moderate maritime climate treatment. *Staphylococcus epidermidis* load was stable over time.

Conclusions and clinical relevance: Alpine climate treatment leads to significant changes in the composition of the skin microbiome in children with AD, mainly caused by a reduction in the *Staphylococcus* genus. This study shows new perspectives in the potential mode of action for therapies in AD.

KEYWORDS

atopic dermatitis, dermatology, microbiome, paediatrics, staphylococcus

1 | INTRODUCTION

Atopic dermatitis (AD) is a chronic, relapsing skin disease with a prevalence of 10%-20% in West-European children.¹ AD is characterized by immunological changes, skin barrier dysfunction and

changes in the microbial composition of the skin, influenced by genetics and environmental factors. While the skin barrier and the immune system were the most important factors in AD aetiology, the role of the skin microbiome recently gained more attention due to development of new molecular methods.^{2,3} The skin

microbiome is in constant interaction with the skin barrier and immune system, reinforcing the process of inflammation.⁴⁻⁶ The skin in AD is characterized by an overgrowth of *Staphylococcus aureus* and analysis of the skin microbiome in different disease states of AD showed a correlation between the abundance of *S aureus* and disease severity, with a higher load during disease flares.⁷ Also, a reduced diversity of other bacteria on the skin was found.⁶⁻⁸

Atopic dermatitis treatment involves epidermal barrier repair using emollients, anti-inflammatory therapy using corticosteroids and trigger avoidance. In cases of severe (or infected) AD, systemic treatment and antimicrobial therapy is used.^{9,10} Alpine climate therapy has been used in patients with asthma and/or AD for decades. Alpine climate is characterized by lower exposure to allergens and pollution and an increased ultraviolet radiation (UV-R).¹¹⁻¹³ Previous research, evaluating the effect of alpine climate treatment in patients with AD, showed improvement in disease activity and a reduced use of topical corticosteroids.^{13,14} The rationale of alpine climate therapy is mainly based on trigger avoidance and dampening the immune response. A recent study exploring the underlying immunological effects of alpine climate therapy found a significant reduction in blood eosinophils and an increase in circulating memory B cells, CD8 + T cells and Th2 cells which reflected a reduction in disease severity.¹⁵⁻¹⁷

It is known that climate factors could influence the skin microbiome. A study performed in healthy individuals within different humidity and temperature conditions showed an effect on the quantities of bacteria on the skin.¹⁸ Furthermore, UV-R can modulate the skin microbiome by causing direct microbial DNA damage and by affecting the immune system.¹⁹⁻²²

Evaluation of alpine climate treatment has mainly focused on the immune system. The effect of alpine climate on the skin microbiome is still unclear. Identification of the skin microbiome and factors influencing the microbial composition might help in developing treatment strategies that improve disease severity by targeting the microbiome. Therefore, the aim of this study was to assess the effect of alpine climate treatment on the skin microbiome in children with difficult to treat AD in a randomized controlled trial (RCT), comparing 6 weeks of alpine climate treatment with treatment in moderate maritime climate.²³

2 | METHODS

2.1 | Study design

This study is incorporated in the DAVOS trial, a pragmatic RCT including children with difficult to treat AD. The trial is registered at Current Controlled Trials (ISRCTN88136485). The detailed study protocol and primary outcomes have been published previously.^{14,23} Briefly, Dutch children and adolescents were randomized to a six-week personalized integrative multidisciplinary (PIM) treatment in either a clinic in the Swiss alps at 1560 metres (intervention, alpine climate group) or an outpatient treatment programme in the Netherlands in moderate maritime climate (control, moderate maritime climate group). Patients were assessed before the start of

treatment (time-point T0) and within 72 hours after the end of the six-week treatment (time-point T1). All study assessments were performed in the Netherlands. Study procedures were reviewed and approved by the Medical Ethics Committee of the University Medical Center Utrecht, the Netherlands (reference 09-192/K). This study involves secondary outcomes of this trial.

2.2 | Participants

Dutch children between 8 and 18 years old, with difficult to treat AD were eligible for participation in the study. We defined difficult to treat as use of at least a class three topical corticosteroid and not being able to step down, or current use of systemic immunosuppressive treatment, or repeated treatment with potent topical corticosteroids or systemic immunosuppressive treatment, or a history of use of systemic treatment, or a significant impact of AD on the child's or the families quality of life, or seemingly unresponsive to conventional therapy according to current guidelines.¹⁴ All patients and if needed, their parents provided written informed consent. Demographic data were extracted from questionnaires and the electronic patient files. Microbiome samples were obtained prior to and after the end of the six-week treatment in the Wilhelmina Children's Hospital, the Netherlands.

2.3 | Microbial samples

Microbial samples were collected from the lesional and non-lesional skin. Samples taken from the lesional skin were preferably taken from the antecubital fold or the popliteal fold. Non-lesional skin samples were taken from the volar arm if possible. We used sterile cotton swabs soaked in sterile NaCl 0.9%. Skin samples were collected by rubbing the skin for 30 seconds. All samples were stored at -80°C until further processing.

2.4 | DNA isolation and qPCR

For DNA isolation, phenol extraction and magnetic beads were used (Agowa mag Mini DNA isolation kit; LCG). First, 150 uL from the sample was added to 350 uL lysis buffer, 500 uL phenol (Tris pH 8) and 500 uL 0,1 mm zirconium beads. This mixture was mechanically disrupted with a beadbeater (Biospec products) twice for 2 minutes, followed by centrifuging for 10 minutes at 1690 RCF to separate the aqueous and phenolic phases. The aqueous phase was purified using AGOWA mag Mini DNA isolation kit. The bacterial DNA concentration measured after DNA extraction was performed using universal 16S qPCR (16S-uni-I-F (5'-CGA AAG CGT GGG GAG CAA A-3'), 16S-uni-I-R (5'-GTT CGT ACT CCC CAG GCG G-3'), 16S-uni-I MGB Taqman[®] probe (5'-ATT AGA TAC CCT GGT AGT CCA -3') with FAM[™] label). *Staphylococcus aureus* and *S epidermidis* load were quantified using multiplex quantitative (q)PCR with the following combination of primers and probes: 16S-S.aur-F1 (5'-GCG AAG AAC CTT ACC AAA TCT TG-3'), 16S-S.aur-R1 (5'-TGC ACC ACC TGT CAC TTT GTC-3'), 16S-S.aur MGB Taqman[®] probe (5'-CAT CCT TTG ACA ACT CT-3') with NED[™] label. 16S-S.epi-R1 (CAT GCA CCA CCT

GTC ACT CTG T) and the 16S-S.epi MGB Taqman probe (CCT CTG ACC CCT CTA G) with VIC label. Forty cycles of qPCR were performed. The DNA concentration was reported as log₁₀ transformed, femtogram per microlitre (fg/uL) in this paper. Detailed information about the DNA concentrations before log transformation are noted in Table S1.

2.5 | 16S rRNA sequencing and taxonomic classification

Microbiome analysis was performed with massively sequencing of the 16S rRNA gene using V4 hypervariable region on the Illumina MiSeq sequencer (Illumina). Barcoded DNA fragments spanning the V4 hypervariable region were amplified with a standardizing level of template DNA (1 ng). This was used to prevent over-amplification. Amplicons, generated using adapted primers F515 and R806 (using 30 PCR cycles), were bidirectionally sequenced using the MiSeq system.²⁴ Samples containing insufficient amounts of DNA did not result in usable sequence data and were therefore omitted. Pre-processing and classification of sequences were performed using the Mothur V.1.31.1 software platform. To assign taxonomic names, the Ribosomal Database Project (RDP) Classifier was used.²⁵ Technical performance was checked by using standardized mock communities. Negative control samples of the lysis buffer did not show signs of contamination. A genus table with raw read counts was generated for downstream analysis.

2.6 | Statistical analysis

Our statistical analysis was performed in patients with available data at both time-points per outcome. Shannon diversity index was calculated at genus level on non-subsampled unfiltered data. For further analysis of the microbiome, we used non-subsampled genus tables and excluded genera with a relative abundance lower than 0.0001. Prior to ordination analysis, the filtered genus tables were square-root transformed with subsequent application of Wisconsin double standardization. To visualize bacterial community compositions, Bray-Curtis distance-based multidimensional scaling (MDS) was used. Permutational multivariable analysis of variance (PERMANOVA) was used to determine significant changes in microbiota. To assess whether the change in microbiota was significantly different between both treatment groups, we used the covariates “time-point” (T0 or T1) and “treatment group” (alpine climate or moderate maritime climate) as interaction terms in this model. If any statistically significant difference was detected, we obtained PERMANOVA coefficients to determine which genera contributed most to this change.²⁶

To detect changes in relative abundance within the 10 most abundant genera over time, we performed univariate analysis using a negative binomial generalized linear model.²⁷ In-depth analysis was performed on *S aureus* and *S epidermidis* abundance by comparing log₁₀ transformed concentrations (fg/uL). Undetectable DNA concentrations were noted as equal to zero and referred to as negative. A linear mixed-effect model with post hoc analysis was used

to assess the changes in *S aureus* and *S epidermidis*. The differences between both treatment groups were assessed by calculating the interaction between covariates “time-point:treatment group”. This statistical model was also applied to Shannon diversity index.

Statistical analysis was performed in SPSS (version 21) and R software (version 3.5.1). linear mixed-effect models were performed using “lme” and “lme4” package. Post hoc analysis was performed with “multcomp” package and corrected for multiple testing.^{28,29} We used the packages “ape” and “vegan” for MDS and PERMANOVA, respectively.³⁰ In this model, we accounted for repeated measurements using the “strata” argument. The changes in relative abundance for the 10 most abundant genera were analysed using package “DESeq2”.²⁷ “ggplot2” was used for visualization.³¹ A P-value of $\leq .05$ was considered statistically significant.

3 | RESULTS

3.1 | Study subjects

A total of 84 patients were randomized of whom 79 patients started the intervention. Two patients from the moderate maritime climate group did not complete intervention and sequencing data and qPCR data were missing for two other patients in this group. This resulted in 75 patients in our study: 38 patients in the alpine climate group and 37 patients in the moderate maritime climate group (Figure 1). Demographic data, comorbidities and disease severity were not significantly different between both groups at the start of intervention (Table 1). The median severity score at T0 was 39.0 (IQR 18.7-59.3) for the alpine climate group and 40.8 (IQR 22.2-52.8) for the moderate maritime climate group.

3.2 | Sample characteristics

Lesional and non-lesional skin samples were collected from all 75 patients in this study at both time-points and were analysed using qPCR techniques. Sufficient amount of DNA to perform 16S rRNA sequencing was available at both time-points in 49 patients for lesional skin and 45 patients for non-lesional skin (Figure S1). Patient characteristics of missing data did not differ from the study group. A total of 1 603 092 sequences (median 9600; IQR 3516-26420) were obtained from the 98 lesional samples and 1 949 477 sequences (median 28 780; IQR 6007-347885) from the 90 non-lesional samples. All sequences belonged to 603 genera. After filtering, 213 genera remained. The 10 most abundant genera on lesional and non-lesional skin, before and after both treatment regimens, are shown in Figure S2. *Staphylococcus* was predominant in all groups followed by the *Corynebacterium* genus and *Streptococcus* genus.

3.3 | The effect of alpine climate treatment on the microbial composition

We visualized the microbiota separated for lesional and non-lesional skin (Figure 2, Figure S3). Before the start of treatment, the microbial

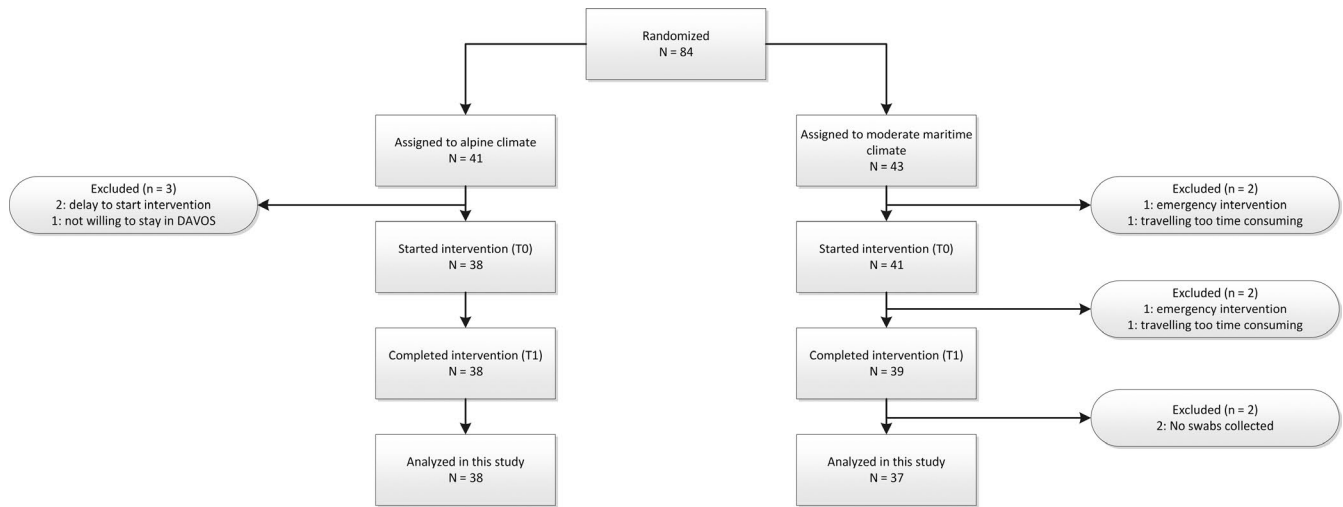


FIGURE 1 Flowchart of study participants

composition did not differ significantly between both treatment groups (PERMANOVA: lesional skin: $R^2 = .025$, $P = .25$, non-lesional skin: $R^2 = .025$, $P = .22$). After 6 weeks of alpine climate treatment, a significant shift in microbiota was observed on lesional skin (PERMANOVA: $R^2 = .035$, $P = .01$), whereas no significant change occurred after treatment in moderate maritime climate (PERMANOVA: $R^2 = .011$, $P = .81$). The observed change on lesional skin after alpine climate was mainly driven by *Staphylococcus* genus with a coefficient which was 2.7 times larger than for other genera (Figure S4). The interaction “time-point:treatment group” was not significant, indicating that the change in microbiota was not significantly different affected by treatment protocol ($P = .19$). The microbiota on non-lesional skin did not change significantly following either treatment (Figure S3).

Compared with baseline, Shannon diversity index was significantly increased after alpine climate treatment on both lesional and non-lesional skin ($P < .01$ and $P = .02$ respectively). This could not be observed after moderate maritime climate ($P = .26$ and $P = .70$, respectively). Moreover, the change in Shannon index was not significantly different between both treatment groups (lesional skin $P = .26$ and non-lesional skin $P = .07$; Figure 3).

3.4 | Effect of alpine climate treatment on the abundance of the 10 most abundant genera

The *Staphylococcus* genus showed a significant reduction in the group treated in alpine climate on both lesional and non-lesional skin (both $P < .01$). In the moderate maritime climate group, a significant reduction in *Staphylococcus* genus was found on lesional skin ($P < .01$). The other 10 most abundant genera, as noted in Figure S2, were not significantly affected by both treatment regimens (Figure S5).

3.5 | Effect of alpine climate treatment on species within the *Staphylococcus* genus

To get more insight in the *Staphylococcus* genus, additional qPCR was performed to identify *S aureus* and *S epidermidis*. For all participants,

qPCR data was available and included in our analysis ($n = 75$). In the total study population, 57 (76.0%) patients were positive for *S aureus* on lesional skin at T0 (alpine climate group: $n = 29$ [76.3%] and moderate maritime climate group: $n = 28$ [75.7%], Table 1). At T1, 40 (53.3%) patients remained positive on lesional skin (alpine climate group: $n = 16$ [42.1%] and moderate maritime climate group: $n = 24$ [64.9%]). These percentages were lower on non-lesional skin (T0: $n = 19$ [50.0%] and $n = 18$ [48.6%] for alpine climate and moderate maritime climate, respectively, T1: $n = 10$ [26.3%] and $n = 14$ [37.8%], respectively). After 6 weeks of treatment, the decrease in *S aureus* load on lesional skin was significantly different in patients treated in alpine climate compared with moderate maritime climate ($P = .02$; Figure 4). After alpine climate treatment *S aureus* reduced from a median of 2.6 fg/uL log₁₀ (IQR 0.1-3.4) at T0 to 0.0 fg/uL log₁₀ (IQR 0.0-0.9) at T1 ($P < .01$), due to the proportion of patients with undetectable *S aureus* concentration at T1. In the moderate maritime climate group, *S aureus* decreased from a median of 2.0 fg/uL log₁₀ (IQR 0.3-3.0) to 1.1 fg/uL log₁₀ (IQR 0.0-2.7; $P = .11$). On non-lesional skin, a significant drop in *S aureus* load was observed after treatment in alpine climate with a median of 0.4 fg/uL log₁₀ (IQR 0.0-2.1) at T0 and 0.0 fg/uL log₁₀ (IQR 0.0-1.0) at T1 ($P < .01$; Figure S6). *Staphylococcus epidermidis* was positive in 71 (94.7%) patients in the total study group on both lesional and non-lesional skin at T0, compared with 72 (96%) and 74 (98.7%) for lesional and non-lesional skin at T1 (Table 1). *Staphylococcus epidermidis* load was not affected by either treatment protocol (Figure 4 and Figure S6).

4 | DISCUSSION

This study showed that alpine climate treatment affects the microbiome on both lesional and non-lesional skin in children with difficult to treat AD. We found a significant change in the overall skin microbiome on lesional skin after 6 weeks of alpine climate treatment, whereas no significant change was observed after moderate maritime climate treatment. Moreover, a significant change was observed on both lesional and non-lesional skin in Shannon diversity

TABLE 1 Baseline characteristics

	Alpine climate group (n = 38)	Moderate maritime climate group (n = 37)
Sex (female), n(%)	17 (44.7)	19 (51.4)
Age, mean ± SD	13.1 ± 2.5	12.8 ± 2.4
Age of AD onset <6 mo, n(%)	6 (15.8)	5 (13.5)
Asthma ^a , n(%)	25 (65.8)	29 (78.4)
Rhinitis ^b , n(%)	34 (89.5)	32 (86.5)
Food allergy ^c , n(%)	26 (68.4)	26 (70.3)
SA-EASI, median (IQR)	39.0 (18.7-59.3)	40.8 (22.2-52.8)
Topical corticosteroids ^d , n(%)		
None	1 (2.6)	1 (2.7)
Moderate	1 (2.6)	3 (8.1)
Potent	35 (92.1)	30 (81.1)
Very potent	1 (2.6)	3 (8.1)
Systemic medication, n(%)		
Prednisone	3 (7.9)	-
Cyclosporine	1 (2.6)	4 (10.8)
Oral antibiotics, n(%)	-	1 (2.7)
Positive for <i>S aureus</i> , n(%) ^e		
Lesional skin	29 (76.3)	28 (75.7)
Non-lesional skin	19 (50.0)	18 (48.6)
Positive for <i>S epidermidis</i> , n(%) ^e		
Lesional skin	35 (92.1)	36 (97.3)
Non-lesional skin	36 (94.7)	35 (94.6)

Abbreviations: AD, atopic dermatitis; SA-EASI, Self-Administered Eczema Area and Severity Index; SD, standard deviation.

^aAsthma was diagnosed based on spirometry reversibility testing and Methacholine Challenge Test.

^bRhinitis was diagnosed based on assessment by a paediatrician.

^cFood allergy was defined as a positive double-blind placebo-controlled food challenge (DBPCFC) or convincing clinical history (a reported Type I allergic reaction with acute symptoms within 2 h after ingestion of the food) in combination with sensitization to the specific food allergen.

^dUK potency system used.

^eDetermined using qPCR methods.

index, *Staphylococcus* abundance and *S aureus* load in particular. The reduction in *S aureus* load was significantly larger when compared to moderate maritime climate. For the group treated in moderate maritime climate, only a significant reduction on lesional skin for the *Staphylococcus* genus was found.

This is the first study describing the effect of alpine climate treatment on the skin microbiome in patients with AD. Alpine climate offers favourable features for patients with AD, including lower exposure to allergens and pollution and an increased UV-R.^{11,12} Moreover, children treated in alpine climate were separated from their parents and intensively monitored by the multidisciplinary treatment team. This treatment setting in alpine climate has beneficial effects on disease severity and was shown to affect the immune

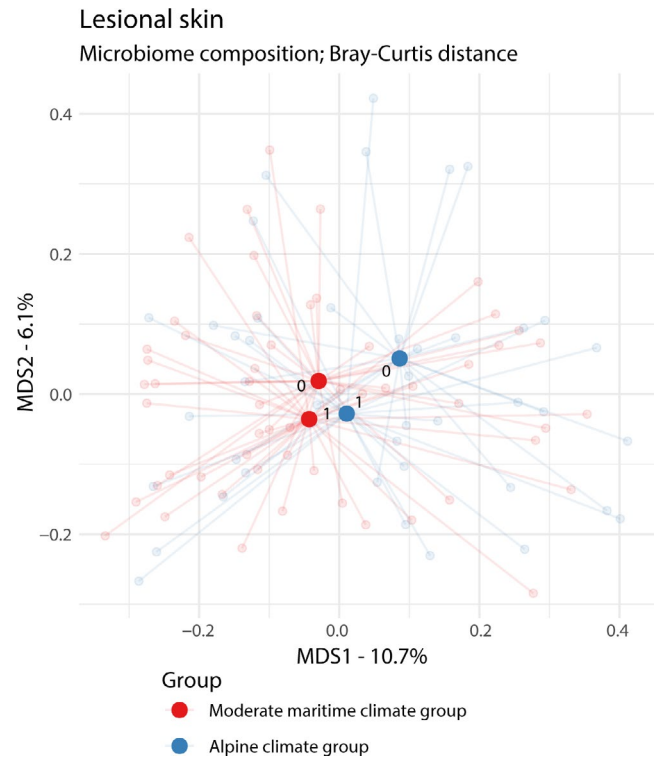


FIGURE 2 Bray-Curtis distance-based multi-dimensional scaling plot showing the microbiota of the lesional skin samples before and after the six-week intervention period in alpine climate and moderate maritime climate. Statistical analysis was performed on 21 patients in the alpine climate treatment group and 28 patients in moderate maritime climate. For ordination analysis, genus tables were standardized using square-root transformation with subsequent application of Wisconsin double standardization. The change in microbiota was significant for patients treated in alpine climate (PERMANOVA: $R^2 = .035$, $P = .01$). No significant change was observed after moderate maritime climate treatment (PERMANOVA: $R^2 = .011$, $P = .81$). MDS 1: dimension 1, representing 10.7% of total variation. MDS 2: dimension 2, representing 6.1% of total variation. T0: before the start of the intervention period. T1: after 6 wk of treatment

response (blood eosinophils, memory B cells, CD8+ T cells and Th2 cells).^{15,19,32} Besides effects on the immune system, studies also proposed that geographical variability, with variation in UV-R, can influence the skin barrier and the microbiome.^{2,33} Although this study shows a change in the skin microbiome after alpine climate treatment, we cannot prove that the observed effect is directly caused by the alpine climate or through the effect on the immune system or treatment setting.

After alpine climate treatment, we found a significant increase in Shannon diversity index and a significant reduction in the quantity of *S aureus* on both lesional and non-lesional skin. Moreover, the decrease in *S aureus* load on lesional skin was significantly different from the maritime climate group ($P = .02$). In a previous paper, describing the effectiveness of alpine climate treatment in this study population, a significantly larger decrease in disease severity was observed following alpine climate treatment than maritime climate

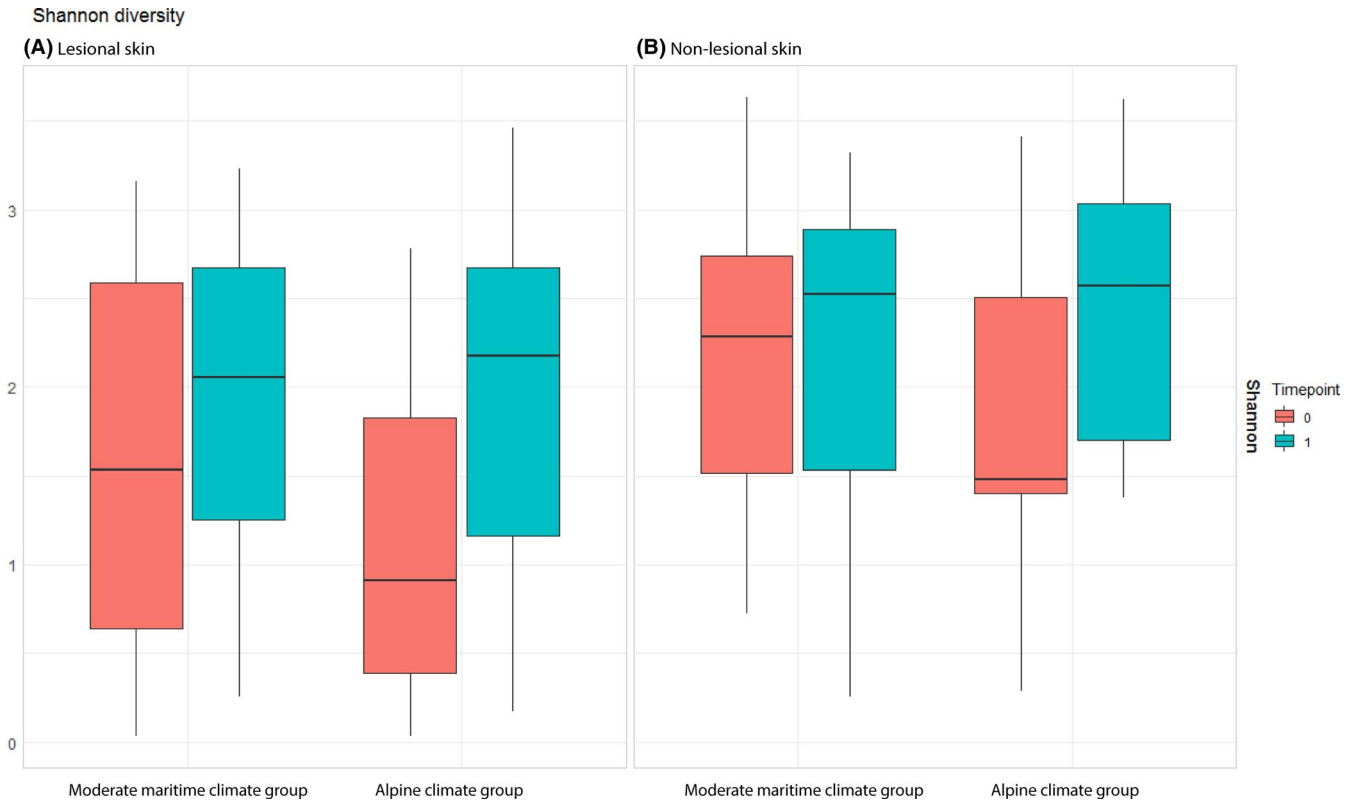


FIGURE 3 Shannon diversity index of skin samples before and after the six-week intervention period in alpine climate and moderate maritime climate. Shannon diversity index represents the number of different genera (richness) and how even they are distributed (evenness). The boxes represent the 25th percentile, median and 75th percentile. Statistical analysis was performed using a linear mixed-effect model with post hoc analysis. Time-point 0: before the start of the intervention period. Time-point 1: after 6 wk of treatment. A, Lesional skin samples. Statistical analysis was performed on 21 patients in the alpine climate treatment group and 28 patients in moderate maritime climate group. Compared with baseline, significant differences in Shannon diversity index were found after treatment in alpine climate ($P < .01$). No significant change was observed after moderate maritime climate ($P = .09$) or between both treatment groups ($P = .26$). B, Non-lesional skin samples. Statistical analysis was performed on 19 patients in the alpine climate treatment group and 26 patients in moderate maritime climate group. Compared with baseline, significant differences in Shannon diversity index were found after treatment in alpine climate ($P = .02$). No significant change was observed after moderate maritime climate ($P = .70$) or between both treatment groups ($P = .07$)

treatment ($P < .01$). The SA-EASI score decreased from a median of 39.0 (IQR 18.7-59.3) to 2.6 (IQR 0.3-6.2) and 40.8 (IQR 22.2-52.8) to 12.0 (IQR 3.6-22.1) after six-week treatment in alpine climate and moderate maritime climate, respectively.¹⁴ Literature shows a positive correlation between the abundance of *Staphylococcus*, in particular *S aureus*, and disease severity in patients with AD.^{6-8,34} A decrease in disease severity also leads to higher bacterial diversity.⁷ It is likely that our results are affected by the differences in disease severity. However, it is unknown whether the *S aureus* abundance is a result or a cause of changes in disease severity. More studies with frequent sampling around flares are needed to answer this question.

Compared with lesional skin, we were not able to detect a change in the microbiota on non-lesional skin after alpine climate treatment. However, the changes in Shannon diversity index, *Staphylococcus* abundance and *S aureus* after alpine climate treatment were significant for both lesional and non-lesional skin. Non-lesional skin in AD is known to differ from both lesional AD skin and healthy skin.^{35,36} This can be explained by the impaired skin barrier in patients with AD, which also affects non-lesional skin and makes it more susceptible to penetration of allergens and bacteria than healthy skin.³⁷ A

previous study investigating the effect of topical corticosteroids and bleach baths in patients with AD also showed a significant change in microbial composition after treatment on lesional skin, but not for non-lesional skin.⁸ This is in line with our findings. A possible explanation can be the higher diversity and lower *Staphylococcus* abundance, compared with lesional skin, making changes more subtle. Another explanation can be the lesser impact of disease severity and thus inflammation on this skin. Non-lesional skin might tell us more about the effect of climate than lesional skin which is more subject to the secondary effects of inflammation.

In this study, both lesional and non-lesional skin in AD patients were dominated by the *Staphylococcus* genus. These results support previous literature describing an excess of *Staphylococcus*, and more specific of *S aureus*, in the skin microbiome of patients with AD.^{3,7,36} Other abundant genera included *Streptococcus* (known to be more present in children with AD) and *Corynebacterium* (common in healthy skin microbiome).^{3,35,38} Looking in more depth at the *Staphylococcus* genus, the prevalence of *S aureus* at the start of intervention was 76.0% and 49.3% for lesional and non-lesional skin, respectively. These percentages are slightly higher than described in

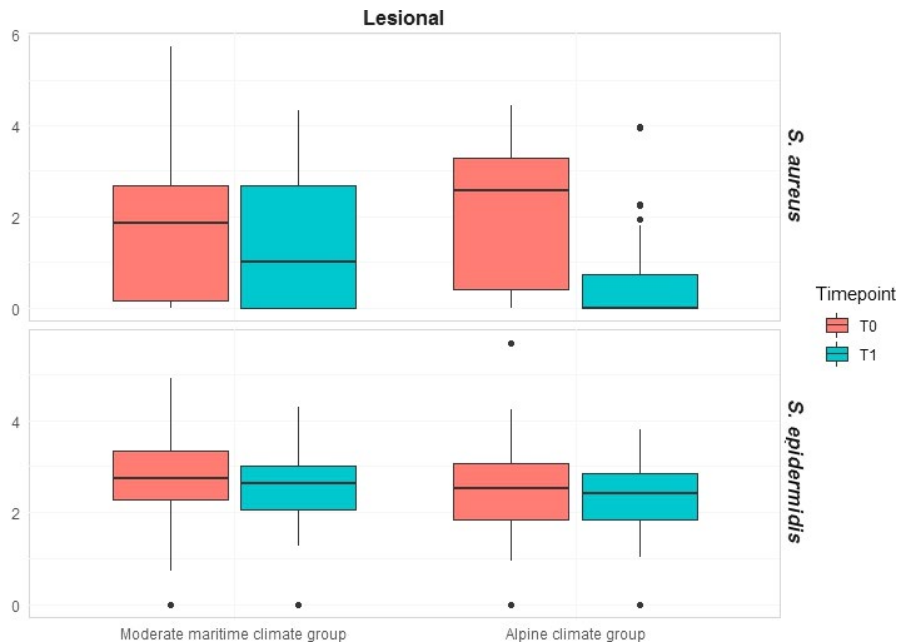


FIGURE 4 Quantitative PCR results of the lesional skin samples for *Staphylococcus aureus* and *S. epidermidis* before and after the six-week intervention period in alpine climate and moderate maritime climate. The results for *S. aureus* and *S. epidermidis* load are shown using log₁₀ transformed data. Statistical analysis was performed on 38 patients in the alpine climate treatment group and 37 patients in moderate maritime climate group. A significant difference in *S. aureus* load was found after alpine climate treatment ($P < .01$). *Staphylococcus epidermidis* was stable during the treatment period. The decrease in *S. aureus* load was significantly larger after alpine climate compared with moderate maritime climate ($P = .02$). The boxes represent the 25th percentile, median and 75th percentile. Dots represent individual samples. Statistical analysis was performed using a linear mixed-effect model with post hoc analysis. Time-point 0: before the start of the intervention period. Time-point 1: after six weeks of treatment

a recent meta-analysis on this subject and might be explained by our inclusion criteria, selecting patients with moderate to severe AD.³⁹ Although patients showed a significant reduction in disease severity in this study, a large proportion remained positive for *S. aureus* on the skin (lesional T0: 76,0%, T1: 53,3% and non-lesional T0: 49,3%, T1: 32,0%). These results suggest that AD symptoms are not only associated with the presence or absence of *S. aureus*, but more importantly with the total *S. aureus* load on the skin.

We did not find a significant change in *S. epidermidis* load in our study. The role of *S. epidermidis* in the pathogenesis of AD is still unclear and literature on this subject is conflicting.^{7,40,41} Due to the inhibitory effect of *S. epidermidis* on *S. aureus*, by the production of bacteriocins, serine protease Esp and phenol-soluble modulins, some correlation may be expected.⁴²⁻⁴⁴ In a previous study, an increase in *S. epidermidis* was found during disease flare in patients with AD.⁷ In our study, the quantities of *S. epidermidis* were stable over time despite a drop in *S. aureus* load and severity following treatment. It is possible that *S. epidermidis* is elevated in the acute stadium (flares) as a compensatory mechanism to control *S. aureus*, but in a chronic stadium these levels normalized.

This study has a pragmatic design, which makes it hard to assess what contributed most to the observed outcomes. Characteristic of the alpine climate group, besides the unique aspects of this climate, was the supervision leading to optimal treatment compliance. It might be that due to supervision, the application of topical corticosteroids was more adequately and frequent in this group

and could have affected disease severity and the skin microbiome. Moreover, this study describes secondary outcomes of this trial and medication use was not applied as exclusion criteria. Previous studies show an effect of medication use on the skin microbiome. However, it was not possible to discontinue medication use in this group of patients with difficult to treat, moderate to severe disease.^{8,45} During this study, the use of medication was carefully monitored and showed no significant differences between both treatment groups. Results for patients using systemic medication at T0 did not deviate from the rest of the study population (data not shown). Moreover, the proportion of patients using topical and/or systemic medication during the intervention was stable (Table S2).

A limitation of this study was the use of the V4 hypervariable region for sequencing. With this variable region it is not possible to properly detect the *Propionibacterium* or to classify the *Staphylococci* at species level. To overcome this problem for the *Staphylococci*, we determined *S. aureus* and *S. epidermidis* abundance with qPCR methods. Since the body sites which were mainly sampled for this study are usually low or devoid of *Propionibacterium* this should only have had a minor to negligible effect on the data presented here.²

This study encourages to perform explanatory studies with a similar treatment setting in both climates to confirm the effect of climate conditions alone on the skin microbiome. Including samples from the skin of healthy subjects in future studies might give us more

information about the differences in the skin microbiome between AD and controls and assess if a decrease in disease severity (which was observed at T1) leads to a microbiome more comparable to healthy subjects. Furthermore, it would be interesting to assess if residents of moderate maritime climate and alpine climate have distinct microbiota.

In conclusion, 6 weeks of treatment in the alpine climate, affects the skin microbiome in children with difficult to treat AD. In contrast to moderate maritime climate, alpine climate treatment caused a significant change in the microbiota on lesional skin. In addition, we found a significant increase in Shannon diversity index and a significant decrease in abundance of the *Staphylococcus* genus and *S aureus* load on both lesional and non-lesional skin. This study shows new perspectives in the potential mode of action for therapies in patients with AD and encourages further investigation of skin microbiome modulating therapies.

ACKNOWLEDGEMENT

The authors are very grateful to the children and their parents for participating in the study and to J. Beutler for assistance in collection of the samples. We thank the Dutch patient support group VMCE for endorsing the study. We want to acknowledge all dermatologists for enabling patients to participate in our study by referring them to our hospital. This study was supported by unrestricted grants from the European Allergy and Asthma Center Davos, the Merem Dutch Asthma Center Davos, the patient support group "Vereniging Nederland Davos" and Microcos Human Health, the Netherlands.

CONFLICT OF INTEREST

This study was supported by unrestricted grants from the European Allergy and Asthma Center Davos, the Merem Dutch Asthma Center Davos, the patient support group "Vereniging Nederland Davos" and Microcos Human Health, The Netherlands. These parties were not involved in analysis of the data or the writing of this paper.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Minke van Mierlo  <https://orcid.org/0000-0001-5397-7382>

Joan Totté  <https://orcid.org/0000-0002-3231-4720>

Karin Berthine Fieten  <https://orcid.org/0000-0003-3790-7581>

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: van Mierlo M, Totté J, Fieten KB, et al. The influence of treatment in alpine and moderate maritime climate on the composition of the skin microbiome in patients with difficult to treat atopic dermatitis. *Clin Exp Allergy*. 2019;49:1437-1445. <https://doi.org/10.1111/cea.13492>