CLINICAL REPORT

Propionibacterium acnes Abundance Correlates Inversely with *Staphylococcus aureus*: Data from Atopic Dermatitis Skin Microbiome

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The microbiome may influence disease severity in atopic dermatitis. The skin of atopic dermatitis patients and healthy individuals was sampled in a standardized manner and the microbial composition analysed using next-generation sequencing. Optical density measurements were used to investigate bacterial growth under defined conditions *in vitro*. Lesional skin from patients with atopic dermatitis had a higher abundance of *Staphylococcus aureus* and reduced quantities of *Propionibacterium acnes* and *Lawsonella clevelandensis* compared with non-lesional skin. The abundance of *P. acnes* correlated negatively with that of *S. aureus* ($\rho = -0.6501$, $p < 0.0001$). Fermentation products of *P. acnes* inhibited the growth of *S. aureus* and *S. epidermidis*. Serum from patients with atopic dermatitis inhibited the growth of *S. aureus* to a greater extent than did serum from healthy individuals. These results suggest that selective modification of the skin microbiome could potentially be used as a therapeutic strategy in atopic dermatitis.

Key words: P. acnes; atopic dermatitis; *S. aureus*; microbiota, high-throughput nucleotide sequencing; skin; eczema.

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Atopic dermatitis (AD) is a chronic, pruritic inflammatory skin disease with an estimated prevalence of 10–20% among children in Western countries and 2–3% among adults (1). A genetically determined defect in epidermal function is thought to be the initial mechanism in the pathogenesis of AD (2). This barrier defect results in increased water loss, xerosis and reactivity of immune cells to exogenous antigens, such as pollen and microbial flora. The ensuing immune reaction in the skin leads to pruritus and scratching, which further promote inflammation. Lesional overgrowth with *Staphylococcus aureus* has been described in several reports. *S. aureus* is a well-known pathogen that can increase disease severity (3) and has been found to be more frequent in patients with AD than in healthy individuals (4).

The Human Microbiome Project described the composition of the healthy skin microflora (5), and skin microbiome dysregulation has been reported previously in patients with AD (6, 7). Mechanisms that either promote or depend on changes to the microbiome are currently of high scientific interest. It has been shown that the commensal skin microbiome impacts on local and systemic immunity (8). The lesional overgrowth of *S. aureus* correlates with AD flare-ups (9). *S. epidermidis*, which is considered a harmless skin commensal, promotes an innate immune response (10, 11) and, moreover, inhibits the growth of *S. aureus* and *Propionibacterium acnes* (12).

The skin microbiome in healthy individuals and patients with AD with lesions in various anatomical sites was studied using an unbiased approach in order to determine whether a common form of microbial dysregulation is present in patients with AD. By analysing how skin bacteria interact with each other, the aim of this study is to elucidate the underlying mechanisms of AD and provide a basis for the development of novel therapeutic strategies for this disease.

METHODS

Patient recruitment and sampling

Ethical approval for this single-centre research study was provided by the Charité Ethical Committee (EA1/194/14). After obtaining written informed consent, patients with AD and healthy individuals (7 in each group) were included in the study. All procedures were performed in accordance with the Declaration of Helsinki (13). The participants were recruited from the outpatient clinic of the Department of Dermatology Venereology and Allergology. The inclusion criteria for the AD group (modified from (9)) comprised a diagnosis of child-onset AD according to the criteria of Hanifin & Rajka (14); abstaining from the use of topical corticosteroids, skin disinfectants or antibiotics for at least 4 weeks; and avoiding skin washing for 12 h prior to sampling. All participants were male, between 18 and 60 years of age, and were included after a thorough examination at the Comprehensive Allergy Center Charité.

The control group was composed of non-atopic, healthy volunteers with no personal or family history of allergic rhinitis, allergic asthma, or atopic dermatitis, with negative standard prick test results, and normal serum IgE concentrations. To allow for naïve microbial skin colonization, volunteers were excluded if they had used skin disinfectants, topical corticosteroids or antibiotics in the 4 weeks prior to sampling.

Sampling was performed according to the protocol by Oh (15). However, we enlarged the sampling area to increase DNA concentrations in the final samples. Catch-All swabs (Epicentre, Madison, WI, USA) were moisturized with DNAse-free water and used in 3 strictly defined locations (antecubital fossa 40 cm^2, interscapular region 40 cm^2 and retroauricular region 10 cm^2). DNA was extracted using the PowerSoil DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA), quantified using a BioPhotometer (Eppendorf AG, Hamburg, Germany) and confirmed by conventional PCR (using 341F: CCTACgggAggCAgCAg

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and 785R: TgACTACAAgggTATCTAATCC primers, TIB MOLBIOL GmbH, Berlin, Germany).

Next-generation sequencing (NGS)

The bacterial 16S ribosomal RNA genes were amplified by PCR using the 341F-785R primers covering the 3rd to 4th hypervariable regions (16). Sequencing was performed on a miSeq Illumina platform (LCG Berlin Lab, Berlin, Germany). The Bioconductor Workflow (17) was used to analyse the microbiome data. Briefly, raw reads were taken of the forward and reverse strands and those that did not meet the quality threshold were filtered out (more than 3 low-quality reads in the forward strands and more than 6 low-quality reads in the reverse strands). Low-quality reads were determined based on the quality scores incorporated into the FASTA files from the Illumina sequencer. The reads were subsequently de-replicated and nearly all sequencing errors were removed using the Divisive Amplicon Denoising Algorithm (dada2) (18). Next, after merging both strands and eliminating chimeras, we obtained a high-quality database and assigned taxonomies to it.

Functional microbiology studies

S. aureus and *S. epidermidis* were cultured in 96-well plates under standard conditions (200 µl lysogeny broth medium (LB medium, Sigma-Aldrich, Saint Louis, MO, USA) at 37°C in a shaker-incubator, 150 rotations per minute). Microbial growth was measured as the optical density over time (OD_{600nm} MRX II, Dynex, Chantilly, VA, USA). The influence of *P. acnes* on *S. aureus* was determined using supernatants from 5 clones of *P. acnes* (provided by the Institute for Microbiology and Hygiene, Charité Universitätsmedizin Berlin, Germany) cultured for 5 days in the following conditions: LB medium, with or without the addition of 0.01% glycerol, at 37°C in anaerobic conditions (BD Gaspack, New Jersey, USA) in a shaker-incubator at 150 rpm. Colonies were centrifuged at 5,000 rpm for 30 min. *S. aureus* was then cultured in fresh LB medium with or without the addition of *P. acnes* supernatant at increasing concentrations (0%, 6.25%, 12.5%, 25%, 50%, and 100%). The impact of propionic acid on the growth of *S. aureus* was analysed in cultures treated with increasing concentrations of propionic acid (Sigma-Aldrich, Saint Louis, MO, USA), using OD measurements.

Statistical analysis

Statistical analysis was performed using the "phyloseq" (19) and "microbiome" (20) packages for the R Statistical Platform (21). Differences in the relative abundances of specific bacteria were analysed using a Mann–Whitney *U* test or, if 3 groups were compared, a Kruskal–Wallis test with a Nemenyi *post-hoc* test and Holm's correction. Pairwise correlations of microbial abundances were estimated using Spearman's method. Growth curves were evaluated using the "growthcurver" (22) package by comparing their mean area under the curve (AUC) values using pairwise Mann–Whitney *U* tests with Holm's correction to account for multiple comparisons. To measure the diversity of the microbial communities the Shannon Diversity Index was applied.

RESULTS

Sample characteristics

Both groups of patients were comparable regarding age and sex (mean ± standard deviation (SD) age: AD 35 ± 9.64 and control 29 ± 6.08 years). Topical treatment with corticosteroids was stopped 4 weeks prior to sampling in the AD patient group. The median (interquartile range [IQR]) Eczema Area and Severity Index (EASI) of the patients was 29 (12.7), the median (IQR) Dermatology Life Quality Index (DLQI) was 13 (24), and the mean ± SD body surface area (BSA) was 31.9 ± 16.7%.

P. acnes *colonization is reduced in patients with atopic dermatitis.*

The 5 most abundant species (among a total of 56) identified from the 3 anatomical sites were *P. acnes*, *S. epidermidis*, *S. aureus*, *P. pseudogenitalium*, and a recently described representative of Corynebacterineae: *Lawsonella clevelandensis*. The bacterial composition was clearly different in AD lesions vs. healthy skin. Much more *S. aureus* was detected in lesional skin, whereas *P. acnes* was reduced compared with non-lesional skin (**Fig. 1** and Fig. S1[1]). In locations with an overgrowth of *S. aureus*, an abundance of *P. acnes* was decreased. However, the microbiome of non-lesional AD skin resembled the microbiome of healthy skin controls (**Table I**). The microbial diversity of lesional skin did not differ significantly from non-lesional counterpart skin ($p > 0.2$, Fig. S2a[1]). The overall abundance of bacterial organisms (based on the raw number of 16S gene

[1]https://www.medicaljournals.se/acta/content/abstract/10.2340/00015555-2896

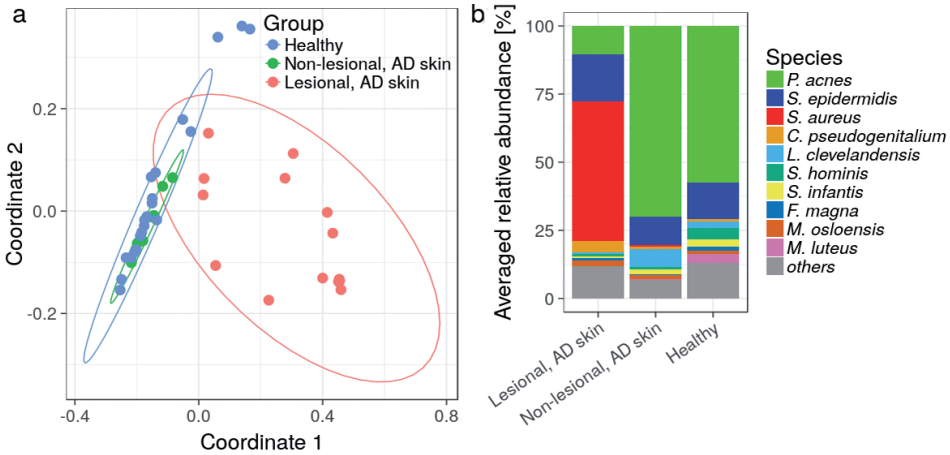

Fig. 1. (a) Ordination plot of the 10 most abundant species sampled from 3 skin locations of 7 patients with atopic dermatitis (AD) and 7 healthy controls. The samples were taken from lesional skin or non-lesional skin of either AD patients or healthy controls. A multidimensional scaling algorithm with Jensen-Shannon Divergence for distance estimation was used. (b) The mean relative abundances of the 10 most abundant species. Less abundant species were grouped together into "others" and included in the graph for completeness.

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Table I. Differences in the relative abundances of the 10 most abundant bacterial species

	Median (IQR)			*p*-values		
Species	L-AD	NL-AD	H	L-AD vs NL-AD	L-AD vs H	NL-AD vs H
P. acnes	1.716 (17.47)	76.441 (26.5)	70.705 (46.85)	0.0019	0.0016	0.7462
S. epidermidis	8.011 (17.55)	9.051 (6.47)	9.328 (10.5)	1	1	1
S. aureus	62.954 (62.28)	0.473 (0.75)	0 (0)	0.0398	0	0.1914
C. pseudogenitalium	0 (2.01)	0 (1.11)	0 (0.84)	1	1	1
L. clevelandensis	0 (0)	3.298 (8.97)	0 (3.31)	0.0325	0.332	0.332
S. hominis	0 (1.23)	0.301 (0.91)	0.78 (3.02)	1	1	1
S. infantis	0 (0.58)	1.048 (2.33)	0.488 (4.78)	1	0.9529	1
F. magna	0 (0.78)	0 (0.23)	0.442 (1.76)	1	1	1
M. osloensis	0.144 (2.76)	0.742 (1.23)	0 (1.16)	1	1	1
M. luteus	0 (0)	0 (0)	0 (0)	1	1	1

Values are the percentage of all sequenced species in a given sample. *p*-values are derived from Nemenyi test (*post-hoc* for Kruskal–Wallis test) with Holm's correction. L-AD: lesional, atopic dermatitis skin; NL-AD: non-lesional, AD skin; H: healthy skin.

reads) was highest in the retroauricular region and lowest in the antecubital fossa.

Subsequently, the correlation of bacteria abundance relative to one another was analysed (**Fig. 2**). The results show that the relative abundances of *P. acnes* and *S. aureus* were negatively correlated ($\rho = -0.6501$, $p < 0.0001$), which suggested that a lack of *P. acnes* may promote the growth of *S. aureus*. Abundance of *P. acnes* was decreased in the lesional samples irrespective of the sampling area (Fig. S2b[1]). Two positive correlations in abundance were also identified: between *M. luteus* and *S. infantis* and between *F. magna* and *C. pseudogenitalium*.

P. acnes *inhibits* S. aureus *growth by producing propionic acid*

Based on the above findings, it was assumed that propionic acid, a fermentation product of *P. acnes*, impacts the growth of *S. aureus*. Therefore, *P. acnes* was cultured in the presence of glycerol to generate propionic acid. The supernatants from these cultures were subsequently used to culture *S. aureus*. Growth inhibition was observed in the supernatant-treated *S. aureus* cultures (99.96% mean reduction in AUC compared with control, $p = 0.0079$; **Fig. 3** and Fig. S3a[1]). It was hypothesized that this inhibition was caused by an increased concentration of propionic acid, which is the main fermentation product of *P. acnes* when cultured in the presence of glycerol. To test this hypothesis, *S. aureus* was cultured in medium containing increasing concentrations of propionic acid (Fig. S3b[1], right-hand panel). The growth of *S. aureus* was completely abolished by propionic acid at concentrations as low as 0.002 mol/dm^3 (99.98% AUC reduction compared with control). Finally, it was investigated whether the inhibitory effect of propionic acid was also relevant for the growth of *S. epidermidis*. In this experiment, the results were similar to those observed for *S. aureus* (Fig. S3b[1], left-hand panel).

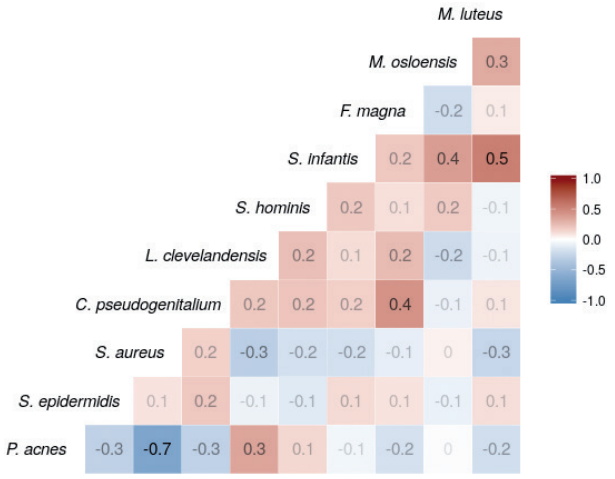

Fig. 2. Comparison of the relative abundance correlation of the 10 most prevalent species. Blue (negative) and red (positive) colours correspond to Spearman's *ρ* values.

Serum of patients with atopic dermatitis inhibits growth of S. aureus

During the course of a flare-up, scratching induced by pruritus may lead to the formation of wounds on the skin

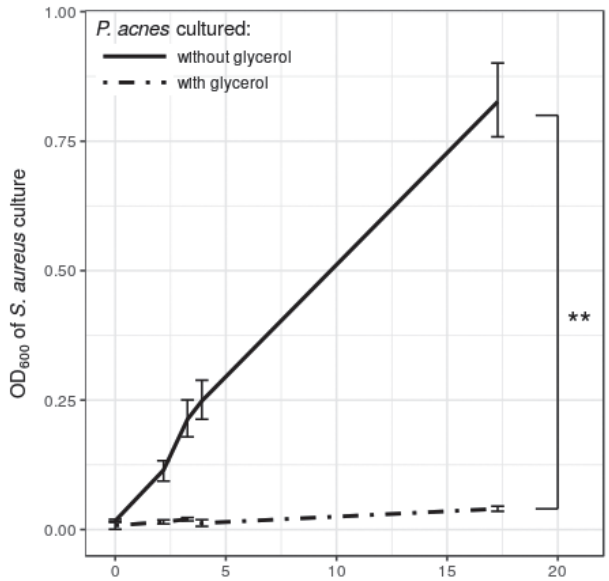

Fig. 3. Fermentation products of *P. acnes* inhibit the growth of *S. aureus*. Growth curves of *S. aureus* incubated with the 6.25% of supernatants from *P. acnes* cultures (Sup and controls (Sup-). *P. acnes* was cultured in LB medium in the absence (GLY–, $n = 5$) or in the presence (GLY+, $n = 5$) of 0.01% glycerol for 5 days. Subsequently, the cultures were centrifuged at 5,000 rpm for 30 min, and the supernatant was used as the medium to culture *S. aureus*. Error bars represent 95% confidence intervals.

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and to the extravasation of blood. Therefore, we hypothesized that the serum of patients with AD might impact the growth of skin bacteria. In our analysis we showed that serum from patients with AD and from healthy controls inhibited the growth of *S. aureus* and *S. epidermidis in vitro* when added to LB medium (**Fig. 4**). Serum from patients with AD inhibited bacterial growth to a greater extent than did serum from healthy controls (AUC reduction 31.28%, $p = 0.0063$).

rRNA gene sequence variants (RSV) indicate multiple entities of the core species

Based on the single-nucleotide variants in amplicon sequences, we identified 5 ribosomal gene sequence variants (RSVs) for *S. aureus*, 7 for *S. epidermidis*, 2 for *L. clevelandensis*. *P. acnes* and *C. pseudogenitalium* did not show variants in rRNA gene sequences. The same RSVs were found in samples deriving from the same patients. RSVs of the same species were often positively correlated in their prevalence in samples. However, *S. aureus* variant 4 did not follow this pattern and correlated positively with the abundance of *S. epidermidis* variants and *C. pseudogenitalium* (Fig. S4[1]).

Two RSVs were identified that were nearly identical (406/407 and 405/407 bases) to the bacterium *L. clevelandensis* strain X1698 (GenBank accession number KM401816). *L. clevelandensis* was present in 17 (39.53%) samples and in 8 (57.14%) patients. A total of 16 samples (94.12%) were isolated from non-lesional skin.

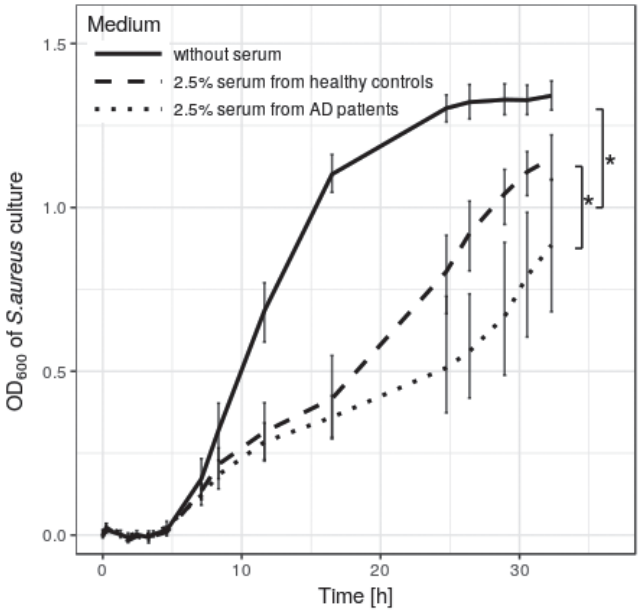

Fig. 4. Serum inhibits the growth of *S. aureus* in a culture. The growth curves of *S. aureus* in the presence of serum (2.5% in LB medium) were evaluated by measuring the OD_{600nm}. Vertical bars represent 95% confidence intervals. *Asterisk* indicates statistical significance. A serum concentration of 2.5% was selected based on dose-response experiments, indicating that this concentration had a negligible impact on the pH of the culture (data not shown).

DISCUSSION

The results of this study confirm the composition patterns of bacterial species in lesional and non-lesional skin from patients with AD, despite the use of different sequencing technologies (9). However, using the novel "dada2" algorithm it was possible to investigate the skin microbial communities beyond the species level and to identify genetic variants of the core bacterial species. Moreover, it was shown that the recently described *L. clevelandensis* is commonly present on healthy skin. The most prominent difference between lesional and non-lesional skin in AD is the negative correlation between *S. aureus* and *P. acnes*. This may be related due to an uncontrolled outgrowth of *S. aureus* or a decrease in *P. acnes* abundance on the skin.

Skin infections are significantly more frequent in patients with AD than in patients with other skin diseases (i.e. psoriasis) (23), suggesting that the abundance of bacteria depends on factors that modify the micro-environment. For example, a disrupted epidermal barrier (24) due to dysfunctional filaggrin leads to increased: (*i*) transepidermal water loss (TEWL), (*ii*) bacterial colonization, and (*iii*) contact with allergens. The outside-in penetration of external antigens (25) activates keratinocytes to produce thymic stromal lymphopoietin (TSLP) leading to a Th2-type milieu in the skin of patients with AD (26). As pruritus is one of the central symptoms of AD, a possible explanation for the overgrowth of *S. aureus* during a flare-up might be intensive scratching. The skin barrier is mechanically altered by scratching, and adhesive matrix molecules (AMMs, e.g. fibronectin, fibrinogen or collagen) are enriched at the lesional skin sites (27, 28). These AMMs bind to the microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) of *S. aureus*. Although the barrier disruption in AD promotes expression and secretion of antimicrobial peptides (AMPs) after superficial injury (29), the diminished acidity of the skin alters the performance of these AMPs (which reach optimum performance in acidic pH), promoting bacterial growth (30). *S. aureus* boosts the underlying inflammation through its Toll-like receptor (TLR) 2 ligands. Kaesler and colleagues described that TLR2 ligands can promote AD by interleukin 4 (IL-4)-mediated suppression of IL-10 (31). As a result, despite the antimicrobial activity mediated by the immune system (e.g. through the increased release of IL-22 (32), IL-17A and IL-17F), *S. aureus* is more prone to adhere to the injured skin regions than other commensal bacteria.

Another previously reported factor influencing the pathogenesis of AD and dysbiosis of the skin microbiota is the decrease in the number and volume of sebaceous glands (33, 34). Sebaceous gland insufficiency is associated with reduced production of glycerol, ceramides and free fatty acids (35). This might: (*i*) explain the predilection of a given microbial species to a specific skin site; (*ii*) restrict the growth of *P. acnes*, and therefore promote the outgrowth of pathogenic bacteria, i.e. *S. aureus*.; (*iii*) explain the low

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water content in the skin and the associated deterioration of the skin barrier seen in patients with AD (36). Kim et al. (37) found a significant negative correlation between sebum production and skin's pH, both in healthy individuals and patients with acne vulgaris. Decreased colonization with *P. acnes* may also be responsible for the observed lower prevalence of acne vulgaris in patients with AD (38). The impact of sebaceous glands on the modulation of the skin microbiome equilibrium has been studied previously (39).

Propionic acid released by *P. acnes* reduces the pH of the skin and therefore increases the activity of skin's antimicrobial proteins (AMPs), i.e. dermcidin, modifying the microbial composition. This effect is not specific for AD. Shu et al. (40) have proven that the main product of *P. acnes* glycerol fermentation is indeed propionic acid, which inhibits the growth of *S. aureus* even when buffered with the appropriate compound to eliminate the influence of a lower pH on the culture (41). Both the decrease in intact filaggrin expression and the lower sebum production promote increased pH levels in the skin of patients with AD, who exhibit higher pH values in healthy and lesional skin compared with healthy controls (42).

Frequent exposition to *S. aureus* in colonized lesions promotes the emergence of specific IgE and IgG_2 antibodies (43). Serum from patients with AD inhibited the growth of *S. aureus* to a greater extent compared with serum from healthy individuals. This might be due to increased activation of the complement cascade or enhanced antimicrobial activity via *S. aureus* recognizing immunoglobulins.

We also identified the recently isolated bacterium *L. clevelandensis* (44) as a rather common inhabitant of healthy skin. Given the fact that there are several reports on this bacterium causing abscesses (45) it might be regarded as an opportunistic pathogen. It was not discovered previously, probably due to demanding growth requirements and long incubation time. However, NGS seems to be an ideal alternative for the identification of bacteria, which are otherwise difficult to culture *in vitro*.

NGS data analysis using an appropriate bioinformatic analysis can identify single nucleotide variations in the rRNA gene sequences. Although they better represent the true microbial community, the clinical significance of RSVs is unknown, and it might be due to: (*i*) the exact same genome can have different sequences, because of multiple copies of the target gene; (*ii*) 2 strains from the same species can have the same sequence in the amplified region; (*iii*) 2 strains from the same species can have different sequences in the amplified region; (*iv*) 2 different species can have the same sequence in the amplified region.

In conclusion, the restoration of the balance in the skin microbiome, by topical treatment to specifically reduce *S. aureus* (46), promoting *P. acnes* (40), or both, may partially impact the course of AD. Whether a direct microbial supplementation with commensal *Propionibacteria*, for example, may contribute to the stabilization of the skin microbiome and prevent flares is an interesting target for future therapeutic approaches.

Study limitations

A high variance in bacterial communities was found within all individuals, demonstrating the high sensitivity of the NGS technique used. Because the skin carries relatively few microbes compared with the gut, we sought to reduce bias by standardized sampling. Nevertheless, we derived our conclusions by comparing relative abundances and not absolute values.

No differences were observed in the alpha diversity of bacterial communities from the AD and healthy skin. This might be dependent on the fact that in each case (in healthy, non-lesional or lesional samples) the communities were dominated by a single bacterial species, whereby the others were less abundant. Our results were based on the Divisive Amplicon Denoising Algorithm, whereby singletons are not present due to better quality control of the reads. Therefore, the resulting microbiome is less diverse, but resembles the true composition more adequately compared with clustering based methods (18).

The low number of samples studied could limit the ability to discover differences in abundance of the less prevalent representatives of the skin microbiota and prevented us from drawing conclusions concerning RSVs of the core species.

Reference sequences of the 16S rRNA gene in regions V3–V4 of *S. aureus* and *S. simiae* are identical (accession numbers: NZ_AEUN01000485.1, NZ_CP009828.1), which prevented us from differentiating between these 2 bacteria.

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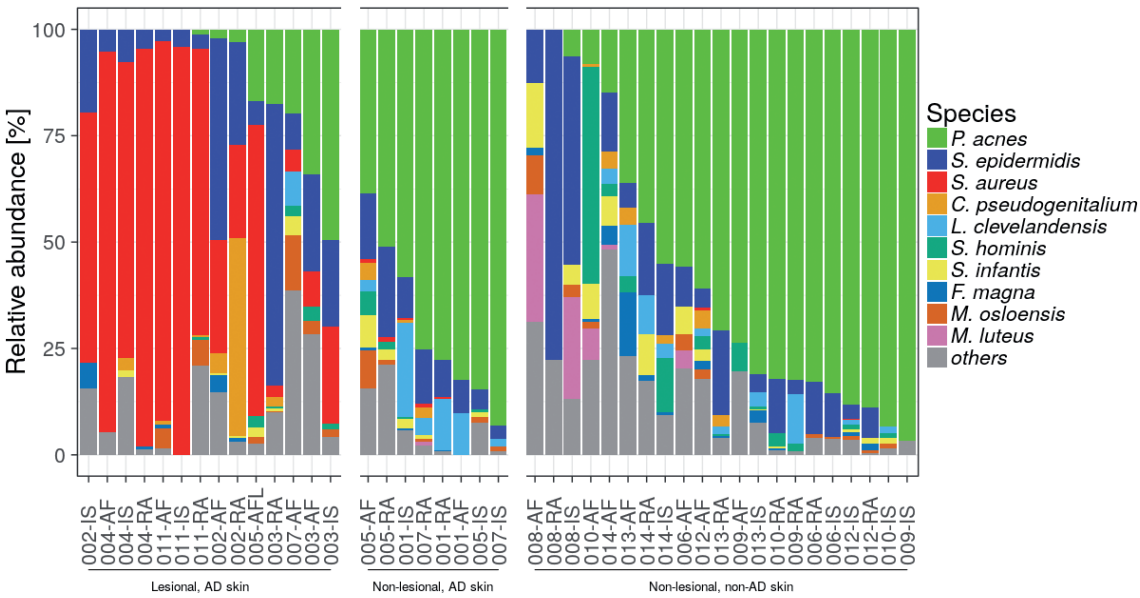

Fig. S1. Relative abundances of the 10 most abundant species sampled from 3 skin locations of 7 patients with atopic dermatitis (AD) and 7 healthy controls. Less abundant species were grouped together into "others" and included in the graph for completeness. The samples are coded as follows: patient number and sampling area. Sample 005-AFL and 005-AF were taken from locations close to each other (< 3 cm) and were from lesional and non-lesional skin, respectively. Samples were sorted according to the relative *P. acnes* abundance. AF: antecubital fossa; RA: retroauricular; IS: interscapular.

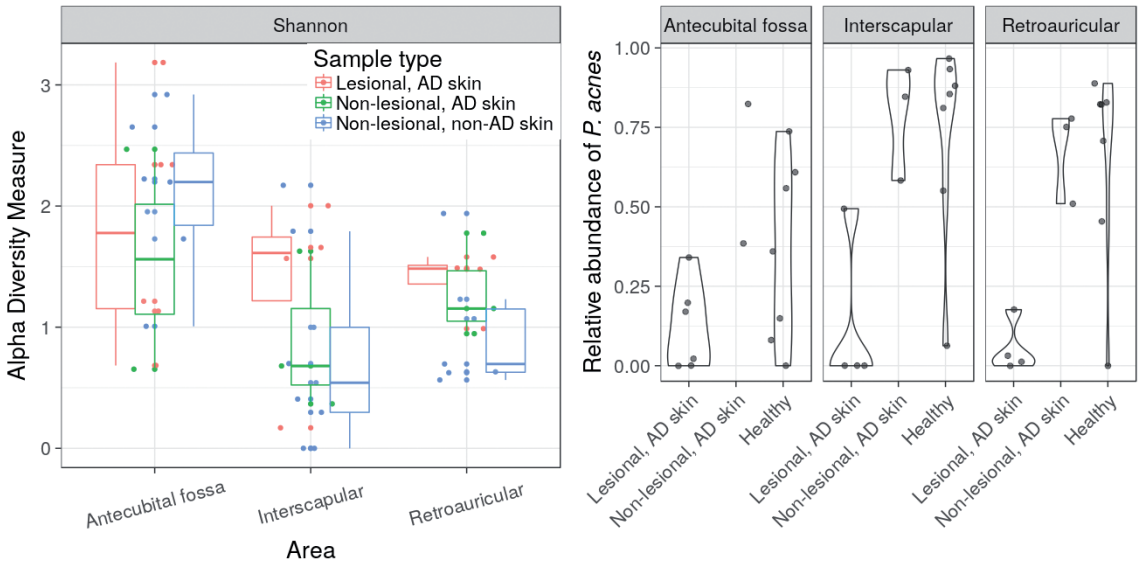

Fig. S2. The alpha diversity depends on core bacterial species. (a) Shannon Diversity Index of the samples in 3 anatomical areas. (b) Relative abundances of *Propionibacterium acnes* in 3 anatomical sites in lesional and non-lesional skin of atopic dermatitis patients, and healthy individuals.

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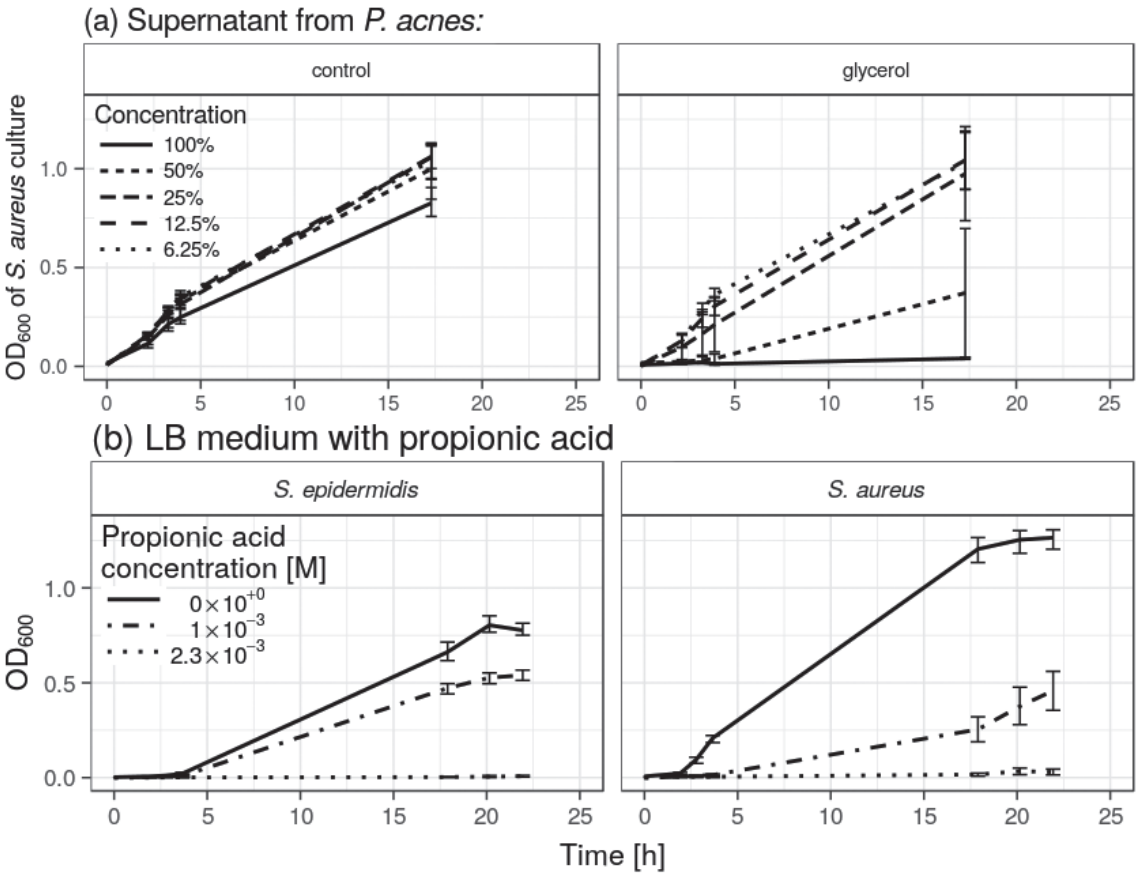

Fig. S3. (a) Growth of *S. aureus* in the supernatant from *P. acnes* cultures without (*left*) and with (*right*) addition of glycerol. (b) The inhibitory effect of propionic acid on the growth of *S. aureus* ($n = 5$) and *S. epidermidis* ($n = 5$) in increasing concentrations.

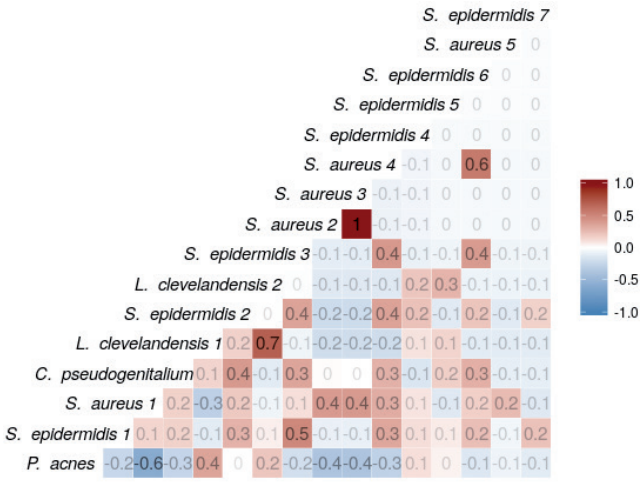

Fig. S4. Relative abundance correlation between ribosomal gene sequence variants of the 5 most abundant species. *Blue* (negative) and *red* (positive) colour intensity corresponds to Spearman's ρ values.

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