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Original Article

The skin microbiome of caspase-14-deficient mice shows mild dysbiosis

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Abstract: Caspase-14, an important proteinase involved in filaggrin catabolism, is mainly active in terminally differentiating keratinocytes, where it is required for the generation of skin natural moisturizing factors (NMFs). Consequently, caspase-14 deficient epidermis is characterized by reduced levels of NMFs such as urocanic acid and 2-pyrrolidone-5-carboxylic acid. Patients suffering from filaggrin deficiency are prone to develop atopic dermatitis, which is accompanied with increased microbial burden. Among several reasons, this effect could be due to a decrease in filaggrin breakdown products. In this study, we found that caspase-14$^{-/-}$ mice show enhanced antibacterial response compared to wild-type mice when challenged with bacteria. Therefore, we compared the microbial communities between wild-type and caspase-14$^{-/-}$ mice by sequencing of bacterial 16S ribosomal RNA genes. We observed that caspase-14 ablation leads to an increase in bacterial richness and diversity during steady-state conditions. Although both wild-type and caspase-14$^{-/-}$ skin were dominated by the Firmicutes phylum, the *Staphylococcaceae* family was reduced in caspase-14$^{-/-}$ mice. Altogether, our data demonstrated that caspase-14 deficiency causes the imbalance of the skin-resident bacterial communities.

Key words: antimicrobial activity – caspase-14 – microbiome

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Introduction

The skin is the anatomic and physiologic interface between the organism and its environment (1). The epidermis is a physical barrier that resists penetration by microorganisms and toxic substances and retains moisture and nutrients. Normal skin also performs an anti-infective function by secreting proteolytic enzymes, lysozymes and antibacterial proteins and peptides (2,3). The mixture of salty secretions from sweat glands and oily discharge from the sebaceous glands contributes to the hyperosmotic and slightly acidic skin environment, which counteracts pathogenic microorganisms, particularly those that thrive at neutral pH. The continuous shedding of superficial skin cells controls the quantity of bacteria by disposing of excess bacteria that adhere to the cornified layers (4,5). The skin provides a habitat for distinct microbial communities. The irregular and varied topography of the skin, such as hair follicles, hair shafts, sebaceous glands and sweat glands, offers microbes niches with different environmental characteristics harbouring distinct microbial communities (6–8). Under homoeostatic conditions, host factors involved in the innate immunity of the skin likely play an important role in shaping the dermal microbiome and *vice versa*. The physiological 'healthy' skin microflora plays an immunomodulatory role by priming the host immune system and instructing it how to sense and recognize harmful bacteria (9–12). Microorganisms also serve the host by competing with pathogens (13) by secreting bacteriocins (14) or releasing secondary metabolites (e.g. exopolymers). Besides permanently resident microbes, the cutaneous microflora is composed of transient microorganisms. The relative population density and dynamics of the different bacterial communities are determined by various factors, including competition for nutrients and participation in biofilm formation. Under certain circumstances, for example, when the skin barrier is impaired or the host immune homoeostasis perturbed, resident bacteria named pathobionts can exert a detrimental effect (15). Recent reports showed that aberrations in microbial communities are often linked to inflammatory diseases, including psoriasis, atopic dermatitis, acne and chronic skin ulcers (16–20). Such comprehensive studies are crucial for understanding the role of commensal bacteria in the pathogenesis of human skin disorders. However, as a large part of skin research is performed in animal models, it is important to also unravel the role of the skin microbiota in these settings.

The first murine skin microbiome datasets were obtained by conventional sequencing of cloned 16S rRNA gene libraries. Grice et al. (21) were the first to describe the cutaneous microflora of the ear of the C57BL/6 mouse that mirrors the microflora found on human antecubital fossa. They reported that Proteobacteria formed the most abundant phylum with a predominance of the *Pseudomonas* and *Janthinobacterium* genera. A similar C57BL/6

skin microbiome was described by Scharschmidt et al. (22). Tavakkol et al. (23) analysed the dorsal trunk skin of C57BL/6 mice and reported that this part of the skin, unlike the above-mentioned ear skin, was mainly inhabited by staphylococci, which are part of the phylum Firmicutes.

Caspase-14, unlike the ubiquitously expressed apoptotic caspases, is processed and activated mainly in the suprabasal layers of the epidermis and the hair follicle (24–27). Biochemical experiments and the development of caspase-14 deficient mice proved that caspase-14 is involved in the degradation of filaggrin during cornification. Hence, caspase-14 deficient mice have greatly reduced concentrations of filaggrin breakdown products, such as urocanic acid and 2-pyrrolidone-5-carboxylic acid, which contribute to the natural moisturizing factors (28–30). Clearly, the ecological system of the skin is modified in the absence of caspase-14. Patients carrying filaggrin mutations are prone to develop atopic dermatitis (AD), which is accompanied with increased microbial burden (31). This effect observed in AD patients could be due to increased bacterial adhesiveness to the skin, a decreased physical skin barrier or a reduction in filaggrin breakdown products. To test the latter hypothesis, we analysed the effect of caspase-14 deficiency in a *Hairless* mice background on the skin microbiome composition using multiplexed-454 Roche pyrosequencing. *Hairless* mice have been used as an important dermatological model for many years (32,33). The caspase-$14^{-/-}$ skin showed disturbance in the microflora under static conditions, manifested in a more heterogeneous bacterial composition compared to wild-type mice.

Materials and methods

Animals

Caspase-14 knockout mice were generated as described (24) and back crossed to the outbred SKH-1 *Hrhr/Hrhr* strain. We used age-matched, female adult (8–10 weeks) SKH-1 *hairless* caspase-$14^{+/+}$ and caspase-$14^{-/-}$ mice. All mice were housed under specific pathogen-free conditions. After birth, wild-type and caspase-$14^{-/-}$ female mice were housed together, and after weaning (at the age of 3 weeks), they were kept for 6 weeks in separate cages. All animal experiments were approved by the ethics committee of Ghent University.

Skin sampling and DNA extraction

All ear punch biopsies (4 mm in diameter) were taken on the same day. A fresh pair of gloves was worn before sampling each mouse to minimize sample cross-contamination. The samples were kept in ASL lysis buffer (QIAamp DNA Stool Mini Kit, Qiagen, Venlo, the Netherlands) and stored at −20°C for <72 h before DNA extraction. Skin biopsies were lysed in ASL lysis buffer for 30 min at 95°C, after which lysozyme was added (10 mg/ml). DNA was extracted from the biopsies using the QIAamp DNA Stool Mini Kit (Qiagen, Venlo, The Netherlands), but the InhibitEX tablets were omitted. DNA samples were stored at −80°C until used.

PCR amplification and sample pooling

Each 50 μl PCR contained 0.5 μM of each primer, 9 ng DNA, 1 μl Phire hot start polymerase (Thermo Scientific, Erembodegem, Belgium) and Phire mix (including 5× concentrated Phire buffer, 0.2 mM dNTPs and nuclease-free water). All tools and equipment were decontaminated with DNA Zap solution (Ambion, Foster City, CA, USA). Samples were initially denatured at 95°C for 2 min and amplified through 25 cycles of 95°C for 40 s, 55°C for 30 s and 72°C for 1 min, followed by one cycle of 7 min at 72°C. Negative controls without template were included for each primer set. The PCR products were separated in 1.5% agarose gel and purified using the QIAquick PCR Purification Kit (Qiagen). Amplification primers contained the designed adapters and a sample multiplex identifier (MID) barcode sequence (Roche), where applicable, at the 5′ end of the 16S primer sequence (Table 1). For each sample, we used two primer sets, both of which amplify the V3-5 region of the 16S rRNA (34). A single sample for pyrosequencing was prepared by pooling equimolar amounts of PCR amplicons from each sample. The samples were pyrosequenced on a Roche 454 Life Sciences Genome Sequencer FLX machine. The emulsion PCR was performed using a GS FLX Titanium LVemPCRkit (Lib-A) kit (Roche Applied Science, Mannheim, Germany). Pyrosequencing was performed on a 70 × 75 Titanium PicoTiterPlate in one lane.

Table 1. Primers used in this study to amplify bacterial 16S rRNA sequences

Primer	Sequence
454 sequencing primers	
Set 1 forward	CGTATCGCCTCCCTCGCGCCATCAG*CCTACGGGAGGCAGCAG*
Set 1 reverse	CTATGCGCCTTGCCAGCCCGCTCAG-10 nucleotide MID-*CCGTCAATTCMTTTRAGT*
Set 2 forward	CTATGCGCCTTGCCAGCCCGCTCAG*CCTACGGGAGGCAGCAG*
Set 2 reverse	CGTATCGCCTCCCTCGCGCCATCAG-10 nucleotide MID-*CCGTCAATTCMTTTRAGT*

See material and methods for further details. For 454 sequencing primers: capitals, adaptors sequences; capitals underlined, linker sequence, capitals italics, 16S rRNA gene targeting sequences. In the reverse primers, a 10 nucleotide multiplex identifier (MID) sequence was added (Roche, Multiplex Identifier (MID) Adaptors for the GS FLX Titanium Chemistry – Basic MID Set).

Bacterial challenge of mice

The mice were topically challenged with *Escherichia coli ATCC 11775* (1.6×10^3 CFU/Finn chamber) suspended in PBS. Therefore, we used 8-mm-diameter Finn chambers on Scanpor (Smart-Practice, Phoenix, AZ, USA). After 48 h, the Finn chambers were removed, and the test areas were washed with 10 mM ammonium formate buffer, pH 2.0, containing 20% ethanol. The skin washings were lyophilized and then reconstituted in 0.01% aqueous acetic acid solution.

Antibacterial activity assay

The radial diffusion assay (RDA) was performed as described (35). Briefly, *E. coli ATCC 11775* was grown to stationary phase in LB medium and then washed with phosphate-buffered saline. 4×10^6 CFU were added to 10 ml of the underlay agarose gel consisting of 0.03% trypticase soy broth (Gibco, Merelbeke, Belgium), 1% low electroendosmosis type agarose (Lonza, Breda, the Netherlands) and 0.02% Tween 20 (Sigma, Diegem, Belgium). The underlay gel was poured into an 85-mm Petri dish. After the agarose solidified, wells 3 mm in diameter were punched into it and 5 μl of skin washing fluid was added to each well. Recombinant CRAMP (the murine ortholog of human cathelicidin LL37) was used as positive control (Innovagen AB, Lund, Sweden). Plates were incubated for 3 h at 37°C to allow diffusion of the test samples. The underlay gel was then covered by an overlay gel consisting of 1% low electroendosmosis type agarose in a

double-strength solution of trypticase soy broth. Antibacterial activity of skin washings was defined as the diameter of the clearance zone around each well after incubation for 3 h at 37°C. The activity of each sample was expressed in units: 10 units were defined as a clearance zone diameter of 1 mm after subtracting the diameter of the well.

Sequence analysis

Sequence analysis was conducted using the QIIME pipeline (36) version 1.1. Sequences were assigned to samples based on their MID tag, allowing for an error of one base. Only reads with a length >200 bp and <1000 bp, an average q-value above 25, <2 ambiguous bases and <2 primer mismatches were retained for further analysis. Thus, 224 037 of 488 218 input reads were retained for further analysis, whereof 74 824 sequences were used in an unrelated experiment. From this set, chimeric reads were identified and removed using ChimeraSlayer (34). A total of 3550 sequences were identified as being chimeric. Reads cleaned of chimeras were denoised using Denoiser 0.84 (37).The denoised reads were reintegrated into the QIIME pipeline as described in the Denoiser helpfiles, OTUs were subsequently clustered from denoised reads at a 97% identity threshold using uclust (38) with default QIIME settings. In total, we had 1625 OTU's over all samples. For each OTU, the most abundant sequence was selected as representative read and classified using RDP classifier (39) only accepting annotations with ≥80% confidence. From OTU abundance and their respective taxonomic classifications, feature abundance matrices were calculated on different taxonomic levels, representing OTU and taxa abundance per sample. On average, we analysed 16 579 ± 10 041 16S sequences per sample in subsequent analysis steps. The average sequence length is 420.7 ± 111.4 nt. Unifrac distances were calculated for reference OTU sequences aligned using PyNast with the greengenes core set as reference (again, QIIME defaults). One hundred and twenty-one OTU's were removed from this analysis due to an alignment <75% sequence identity to the reference.

Statistical analysis

To compare the different sequence samples selected by the QIIME pipeline, sample counts were normalized by sample sum and multiplied by the average sample sum. This way each sample has the same number of counts, while giving an approximation to the number of reads assigned to each taxonomic group. For visualization of taxa abundances, taxa abundance was converted to a $\log_{10}$ scale by adding one to each taxon prior to transformation, avoiding infinite values for absent taxa. Statistical analysis was conducted on the normalized feature abundance matrices using R 2.11.0. PCoA (principal coordinates analysis) was calculated using the R-package vegan with Bray–Curtis distance on normalized and log-transformed taxa abundance. Intersample correlation was measured with a weighted correlation analysis, the weights being the respective taxa abundance across all normalized samples. The density of the weighted correlation was calculated using a Gaussian kernel and displayed as insert in the PCoA. The differential abundance of each taxonomic unit between wild-type and caspase-14 deficient samples was tested using a one-sided Wilcoxon rank-sum test (P-value), corrected for multiple testing using the Benjamini–Hochberg false discovery rate (q-value). Taxa with <10 reads in all samples or that were present in only one sample were excluded from the analysis to avoid artifacts. Taxonomic richness was calculated by rarefying the respective non-normalized feature abundance matrices until the 5000 reads per sample, and results were confirmed using diversity estimators (e.g. Chao1). The number of different taxa was calculated for each rarefied sample. This was repeated five times per sample, and the average is the reported richness. Samples were tested for equal group dispersions from the Bray–Curtis distance matrix using the Permdisp2 method as implemented in the R-package vegan (40). This method estimates the multivariate dispersion within the KO and WT groups, which we tested for equal dispersion using a two-tailed ANOVA.

Results

Enhanced antibacterial activity in caspase-14 deficient skin challenged with *E. coli*

We previously showed that profilaggrin is a direct caspase-14 substrate (24,30). Profilaggrin is a member of the S100 fused-type protein (SFTP) gene family, whose members (or derived peptides) have been shown to have antimicrobial activity (Schroder JM, unpublished observations). Hence, we investigated whether there was a difference in antimicrobial activity between caspase-14 deficient and wild-type skin. We collected skin washings and tested them for antimicrobial activity against different bacterial strains using a radial diffusion assay (RDA). No antimicrobial activity was observed in the skin of unchallenged wild-type and caspase-$14^{-/-}$ animals (Fig. 1). However, after stimulation of the skin with *E. coli* in a Finn chamber model, caspase-14 deficient skin showed stronger bactericidal activity against *E. coli* (Gram-negative) (Fig. 1a) and against *Bacillus subtilis* and *Staphylococcus aureus* (Gram-positive) than wild-type skin (Fig. 1b and c). Antimicrobial activity was always highest against *E. coli.* Skin from mice treated only with the bacterial suspension medium behaved as unchallenged skin and did not produce detectable levels of antimicrobial activity (Fig. 1 and data not shown). In addition, histological analysis of control- or *E. coli*-challenged mice did not show any signs of inflammation or immune cell infiltration (Fig. S1). Separation of caspase-14 knockout skin washing fluids by preparative C_8 reversed-phase high-performance liquid chromatography (RP-HPLC) and analysis of RP-HPLC fractions for bactericidal activity confirmed the presence of bactericidal activity against *E. coli*. Moreover, we found antimicrobial activity in the fractions containing albumin, which suggested the presence of highly hydrophobic antimicrobial compounds. However, we were unable to identify the exact nature of the antimicrobials by purification with cation exchange HPLC, peptide mapping, mass mapping and mass spectroscopy sequencing because the antimicrobial activity was no longer detectable in our functional assays upon further purification.

The caspase-$14^{-/-}$ skin microbiome differs from the wild-type hairless skin microbiota

The RDA experiments suggest that the dermal microbiome of caspase-14 knockout skin is capable to mount an enhanced antimicrobial reaction. Therefore, we used 16S gene rRNA pyrosequencing to investigate quantitative and qualitative differences in microbial composition of the ear skin. The skin of wild-type and caspase-$14^{-/-}$ mice was colonized mainly by the phylum Firmicutes, with a minor contribution of Proteobacteria (Fig. 2a). The higher microbial diversity in caspase-14 deficient mice compared to wild-type mice was reflected by detection of significant overrepresentation of other, relatively low-abundant phyla, such as

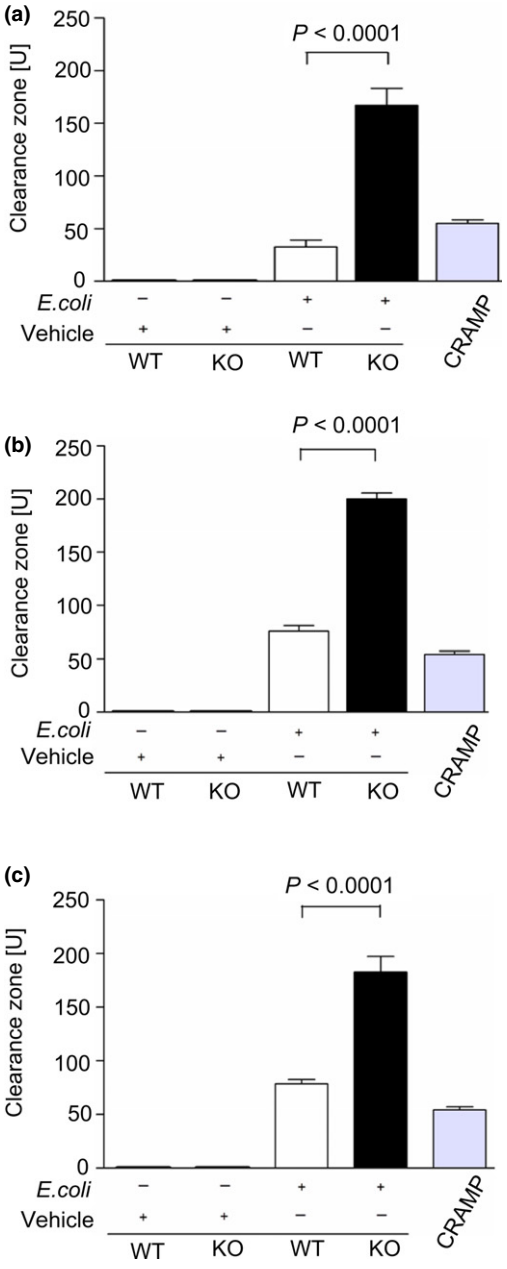


Figure 1. Increased bactericidal activity in caspase-14$^{-/-}$ skin challenged with *E. coli*. (a) *Escherichia coli*-killing activity in washings of skin from wild-type and caspase-14$^{-/-}$ mice stimulated with *E. coli* ATCC 11775. The samples were collected by washing *E. coli*-stimulated SKH-1 *hairless* skin with 10 mM ammonium formate buffer (pH 2.0) containing 20% ethanol. The washings were lyophilized three times at room temperature and then reconstituted in 0.01% aqueous acetic acid solution. The antibacterial activity was tested in a radial diffusion assay (RDA). CRAMP was used as a positive control. Data are the mean ± SE of three independent experiments, each done in triplicate. (b) The antimicrobial activity of skin washings from mice against *Bacillus subtilis* BQ2. (c) The antimicrobial activity of skin washings from mice against *Staphylococcus aureus* RN390.

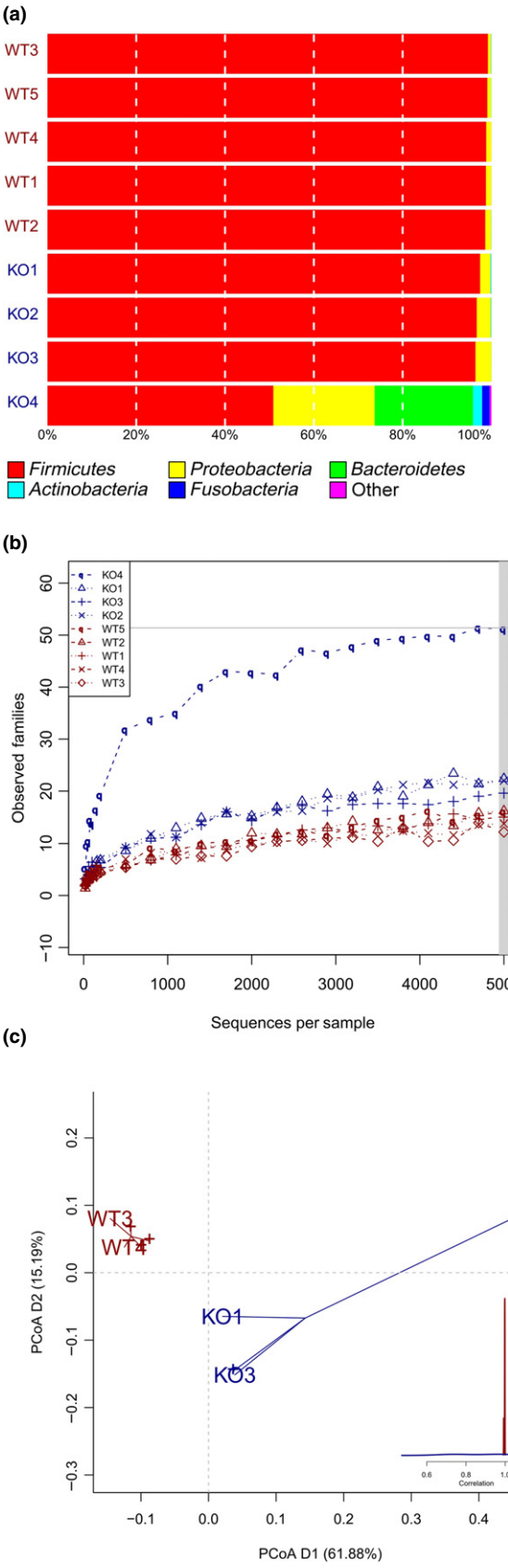
Proteobacteria ($P = 0.016$) and Bacteroidetes ($P = 0.031$). By rarefying the metagenomic samples at different taxonomic levels, we found that wild-type skin consistently displayed a lowered taxa richness than caspase-14$^{-/-}$ skin with respect to family ($P = 0.014$), genus ($P = 0.013$) and OTU ($P = 0.037$) level (Fig. 2b and Fig. S2) as well as a lowered Shannon diversity on class ($P = 0.014$), family ($P = 0.014$) and genus level ($P = 0.014$). Caspase-14$^{-/-}$ skin samples showed strong interindividual variability. In particular, sample KO4 of the caspase-14$^{-/-}$ group showed a distinct and potential biologically relevant pattern, with enrichment in *Actinobacteriaceae* and *Fusobacteriaceae* families (Fig. 2b).

Principal coordinates analysis (PCoA) performed at the family level showed a strong clustering of the wild-type group, with a very low variation (Fig. 2c). This was further confirmed by weighted correlation analysis (Fig. 2c, inset). By contrast, caspase-14$^{-/-}$ samples were clearly different from the tight wild-type cluster and showed a strong variation and low correlation among samples, a finding that was confirmed by a PCoA on OTU and genera abundances (Fig. S3). The composition of the KO1 microbiome was very similar to the wild-type group. The clustering of wild-type microbiota was similarly pronounced at the OTU level, while the knockout group showed no consistent clustering on all taxonomic levels and a higher within group dispersion. This was significant only on OTU level ($P = 0.028$, beta dispersion test) although the same strong trends were observed on all levels. To test this hypothesis independently on taxonomical assignments, we repeated a PCoA on the Unifrac distances between samples (Fig. S4), where we observed the same increased dispersion of the KO samples and the tight clustering of the WT samples. Next, we analysed the *Staphylococcaceae* fraction and repeated the PCoA only on the OTU's assigned to *Staphylococcaceae,* normalized either to the sample abundance of this family (Fig S5a) or to the total sample abundance (Fig S5b) but grouping all other OTUs in a single category. As the KO sample dispersion was still higher in the KO group ($P = 0.031$ and $P = 0.0503$, respectively, beta dispersion test), our conclusion is that the S*taphylococacceae* are different between WT and KO group and that the *Staphylococacceae* community shows more variance in caspase 14$^{-/-}$ than wild-type mice skin.

The dermal microflora of caspase-14$^{-/-}$ hairless mice has a significantly reduced abundance of the *Staphylococcaceae* and is enriched in transient bacteria

The 16S sequence analysis revealed that 13 bacterial families significantly differed between wild-type and caspase-14$^{-/-}$ group ($P < 0.05$, $q < 0.1$). Of these, 11 were overrepresented in caspase-14$^{-/-}$ mice (Fig. 3a), and therefore, it might explain the higher bacterial richness in the caspase-14$^{-/-}$ skin samples. The skin of wild-type and caspase-14$^{-/-}$ mice was colonized mainly by staphylococci; however, the *Staphylococcaceae* family was significantly reduced ($P = 0.016$, $q = 0.05$) in caspase-14$^{-/-}$ skin. Our taxonomic assignments of this most abundant family were not able to discern the genus for 29–43% of the staphylococci reads. In addition, unknown bacteria, classified to the *Staphylococcaceae* family, but neither *Staphylococcus* sp., nor the *Bacillales* order were significantly underrepresented in caspase-14 knockout mice.

The observed shift in the bacterial composition of caspase-14$^{-/-}$ samples showed that the main families that varied in the knockout group belong to the Proteobacteria division (Fig. 3a). The abundance of *Xanthomonadaceae* (particularly, genus *Stenotrophomonas* sp.), *Moraxellaceae* (e.g. *Acinetobacter* sp.), *Caulobacteraceae* (e.g. *Caulobacter* sp.), *Oxalobacteriaceae, Enterobacteriaceae, Comamondaceae, Methylobacteriaceae, Enterococcaceae, Paenibacillaceae* and *Pseudomonadaceae* is increased in caspase-14 deficient samples. All



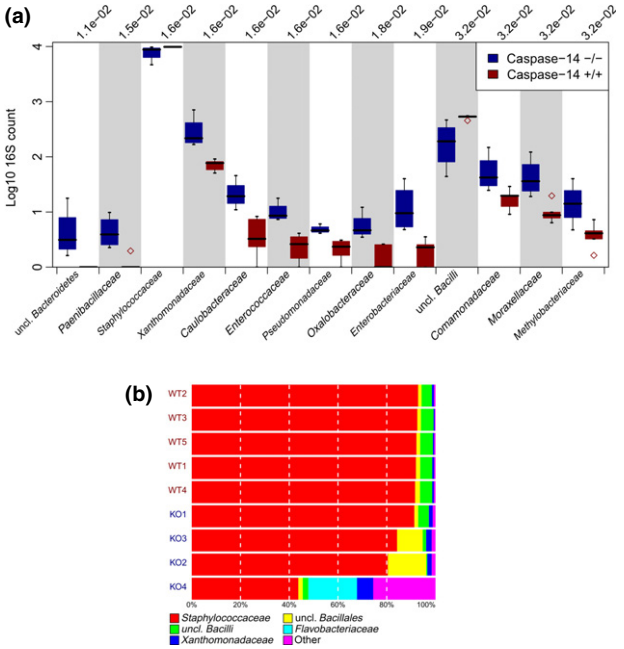


Figure 3. The dermal microflora of caspase-14$^{-/-}$ skin is enriched in transient bacteria. (a) Boxplot of families with a significantly different abundance in wild-type and caspase-14$^{-/-}$ skin samples, shown on a $\log_{10}$ scale. The abundances of bacterial families are significantly different with a *P*-value <0.05 and *q*-value <0.1 in the Wilcoxon rank-sum test. Red – wild-type; blue – caspase-14$^{-/-}$. (b) Composition of wild-type (WT, $n = 5$) and caspase-14$^{-/-}$ (KO, $n = 4$) samples on the family level. Samples were sorted from top to bottom by the abundance of *Staphylococcaceae* family members. [others] denotes all other families not explicitly named in this graph. [uncl] – unclassified.

these bacterial families are environmental and flourish in soil, water and decaying debris. In addition, they are aerobic, mainly saprophytic and cosmopolitan. In addition, unclassifiable *Bacillaceae* (Firmicutes) were underrepresented in the caspase-14$^{-/-}$ group (Fig. 3b). These results were also reflected at the phylum level because Proteobacteria ($P = 0.01$, $q = 0.05$) and Bacteroidetes ($P = 0.03$, $q = 0.06$) were significantly enriched on the skin of KO mice. Taken together, our data show that absence of caspase-14 in skin promotes microbial diversity at the family level, as in the case of the *Staphylococcaceae*.

Discussion

SKH-1 *hairless* mice are widely used in dermatological research (33). Based on 16S rRNA gene analysis, we report that the dermal microbiome of SKH-1 mice is dominated by the Firmicutes phylum with a predominance of the *Staphylococcaceae* family. These results are in line with earlier studies showing that skin of CBA hairless mice and C57BL/6J is colonized mainly by staphylococci (12,41,42), while an independent study reported a 79% colonization of C57BL/6J skin by Proteobacteria. In another study, Grice

Figure 2. The caspase-14$^{-/-}$ microbiome is in mild dysbiotic state. (a) Skin microbiome composition of wild-type ($n = 5$) and caspase-14$^{-/-}$ ($n = 4$) at the phylum level. Only six, the most dominant phyla, are shown individually; [others] denotes all other phyla; [uncl.] – unclassified. (b) Sample rarefaction and the number of observed families at each rarefaction. The means of five independent rarefactions of five wild-type and four caspase-14$^{-/-}$ samples are shown. (c) PCoA analysis of family abundance in wild-type and caspase-14$^{-/-}$ samples. The inset shows the distribution of the weighted correlation among all wild-type and all caspase-14$^{-/-}$ samples. Only the first two dimensions and their respective standard deviations are shown. Red, wild-type samples; blue, caspase-14$^{-/-}$ samples.

et al. (43) identified the skin biota of db/+ (healthy) and db/db mice (diabetic). Like our findings in caspase-14$^{-/-}$ skin, db/+ skin was colonized mostly by Firmicutes (78.6 ± 4%), and when analysed at the family level, the *Staphylococcaceae* family members were most abundant. Staphylococci colonization increased substantially during wound healing in db/db skin relative to db/+ skin as well as in long-term immunodeficient mice (44). Our analysis revealed that the hairless skin, described by Benavides et al. (33) as characterized by persistent, mild dermal inflammation, was also colonized mainly by *Staphylococcaceae.* This could fit with the observation that the relative increase of staphylococci is positively correlated with a transient increase in expression of cutaneous defense genes and inflammatory genes in slow-healing diabetic mouse wounds (43). The studies on human dermal microflora demonstrated that atopic dermatitis human lesions are regularly colonized and infected by *Staphylococcus* ssp. (18). However, a recent analysis based on pyrosequencing revealed that staphylococci are less common in psoriasis (5%) than in normal human skin (16%) (16). The presence or absence of staphylococci on the human skin might reflect the pattern of antibacterial peptides expression in skin.

In contrast to our findings in *Hrhr/Hrhr* mice, the dermal microbiome of C57/BL6 mice was dominated by Proteobacteria, whereas Firmicutes contributed only a minor fraction (22,23). However, it should be taken into account that the genetic background and immunological status of mice might alter the murine microbiome (12,44) and a direct comparison between C57/BL6 and hairless mice might not be possible. Furthermore, it is not clear which bacteria constitute the murine 'core' skin microbiome and which factors control the composition of the skin ecosystem. In addition, the observed differences in mouse skin microbiome composition, particularly with respect to the abundance of Firmicutes, might reflect interfacility differences in animal housing conditions that affect the microflora composition (45). The latter could be a major factor as the C57BL/6J skin microbiome has been reported to consist of about 80% Firmicutes or 80% Proteobacteria in two independent studies (12,44). Last but not least, the technique used to assess the microbial composition might alter the pattern observed. A larger sample size might resolve some of these ambiguities between studies.

This study is the first molecular assessment of the effect of caspase-14 deficiency on the bacterial diversity of the skin. Our analyses show that the microbiome of caspase-14 deficient mice is more heterogeneous and has a higher abundance of cosmopolitan, transient phylotypes. The caspase-14$^{-/-}$ dermal microflora was significantly enriched in non-pathogenic bacterial species related mainly to environmental sources and gut microflora (e.g. *Enterobacteriaceae* and *Enterococcaceae*). Moreover, in these mice samples, we found members of nomad bacterial families (e.g. *Moraxellaceae*) and orders (e.g. *Neisseriales* and *Xanthomonadales*), which are reportedly relatively frequent on the human hand skin (46). Apparently, the transient bacteria are harboured in the knockout skin due to stochastic factors such as the presence of these bacteria in the air, food and/or bedding (47). Yet, given the persistent dominance of Firmicutes, these temporary commensals could not compete against the established bacterial community. Obviously, the skin microbial make-up strongly depends on the physiology of the skin (15). The cause of the slight perturbation in caspase-14-deficient skin microbiome might be related to various factors that alter the skin 'biotope', including differences in skin hydration, availability of nutrients, pH and the type or concentration of antibacterial agents in the skin under steady-state conditions. Indeed, a defect in filaggrin and/or profilaggrin processing in caspase-14 deficient skin reduces the levels of free amino acids and urocanic acid, which could serve as nutrient sources for commensal bacteria or affect the local pH conditions. Alternatively, the increased levels of antimicrobial activity observed in caspase-14$^{-/-}$ mice challenged with *E. coli* might also explain the microbiome changes observed in the caspase-14 deficient group. Although we were unable to detect any antibacterial activity in unchallenged wild-type and caspase-14 deficient skin, possibly due to low sensitivity of the RDA assay, we cannot exclude the possibility that *in vivo* the antimicrobial activity is altered.

Proteobacteria were reported to be more abundant on dry regions of the skin. Indeed, we found a significant enrichment in Proteobacteria (although the relative abundance is still low) on the drier caspase-14 knockout skin, which is deficient in natural moisturizing factors. Hence, our results emphasize that the higher abundance of Proteobacteria phylotype in a dry milieu is common.

There are very few reports on murine skin microbiomes and our study is the first description of the hairless mouse skin microbiome. Therefore, more in-depth analyses are needed to provide comprehensive information about the cutaneous microbiome of different inbred mouse strains in different mouse facilities. So far, the most explanations about microbial adaptation to mouse skin habitat seem to be largely speculative. Current sequencing techniques allow investigation of how ablation or overexpression of certain genes affects the dermal microbiome. We show that the composition of skin-resident microflora is slightly perturbed in caspase-14 mice, as shown by the increased representation of Proteobacteria and reduced presence of the *Staphylococcaceae* family, probably due to homoeostatic imbalance of the skin (15,48).

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Conflict of interests

The authors have declare no conflict of interests.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Wild-type and caspase-$14^{-/-}$ mice were topically challenged with *E. coli ATCC 11775* (1.6×10^3 CFU/Finn chamber) suspended in PBS or with a control solution only containing PBS.

Figure S2. OTU richness estimated for all skin samples. In general, caspase- 14 KO showed higher OTU richness than wild-type mice.

Figure S3. Phylogenetic composition of wild-type and caspase-$14^{-/-}$ skin microbiomes.

Figure S4. 2D PCoA of Unifrac distances.

Figure S5. 2D PCoA of OTU's that were classified as *Staphylococcaceae*.

Table S1. The first sheet contains a list of bacterial families or taxonomic groups that could not be classified on family level.