Scientific Reports

https://doi.org/10.1038/s41598-026-44005-6

Article in Press

Advancing human skin models by integrating skin microbes for next-generation research

Received: 22 December 2025

Accepted: 9 March 2026

Published online: 11 March 2026

Cite this article as: Mieremet A., Rietveld M., Leijden B. *et al.* Advancing human skin models by integrating skin microbes for next-generation research. *Sci Rep* (2026). https://doi.org/10.1038/s41598-026-44005-6

Arnout Mieremet, Marion Rietveld, Bowien Leijden, Anouk Stolk, Nicole Plomp, Jasper Kieboom, Heike Foelster, Elke Gülden, Frank H. J. Schuren & Abdoelwaheb El Ghalbzouri

Advancing human skin models by integrating skin microbes for next-generation research

Arnout Mieremet[1✉], Marion Rietveld[2], Bowien van Leijden[1], Anouk Stolk[2], Nicole Plomp[1], Jasper Kieboom[1], Heike Foelster[3], Elke Gülden[3], Frank H.J. Schuren[1#], Abdoelwaheb El Ghalbzouri[2#✉]

[1] Department of Microbiology and Systems Biology, Netherlands Organisation for Applied Scientific Research (TNO), Sylviusweg 71, 2333 BE Leiden, The Netherlands

[2] Department of Dermatology, Leiden University Medical Center, 2333 ZA Leiden, the Netherlands

[3] Research and Development, Beiersdorf AG, Beiersdorfstraße 1-9, Hamburg, Germany.

#shared senior authorship

✉ Corresponding Authors

Dr. Arnout Mieremet
Department of Microbiology and Systems Biology
Netherlands Organisation for Applied Scientific Research (TNO)
Sylviusweg 71
2333 BE Leiden, The Netherlands
arnout.mieremet@tno.nl

Dr. Abdoelwaheb El Ghalbzouri
Department of Dermatology
Leiden University Medical Center
Albinusdreef 2
2333 ZA Leiden, the Netherlands
a.e.l.ghalbzouri@lumc.nl

Abstract

The skin barrier comprises interdependent physical, chemical, immunological, and microbial components, of which the latter is constituted by a community of microbes residing on the skin surface that restricts the expansion of opportunistic pathogens, modulates keratinocyte signaling pathways, and fosters immune tolerance. However, molecular and cellular dynamics of host–microbe interactions remain incompletely characterized, partly due to the limited availability of physiologically relevant and robust preclinical models. We aimed to establish 3D human skin equivalents (HSEs) in co-culture with representative skin commensals to investigate host responses across an *in vitro* cohort of six biological replicates. Well-characterized HSEs were inoculated with *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Cutibacterium acnes*. A 48-hour co-culture period enabled microbial expansion, during which *S. aureus* exhibited the most substantial outgrowth, and strain-dependent variability was observed for *S. epidermidis*. Assessment of epidermal morphogenesis revealed that *S. aureus* exerted largest structural impact, whereas *C. acnes* promoted keratinocyte proliferation. Furthermore, *S. aureus* elicited a pro-inflammatory response, characterized by elevated secretion of IL-8 and CXCL1. In conclusion, we developed a reproducible experimental framework dissecting host–microbe interactions in HSEs to demonstrate that *S. aureus* induced substantial alterations in epidermal architecture and inflammatory signaling, underscoring its pathogenic potential in cutaneous environments.

Keywords

Skin barrier; Microbes; Host–microbe interactions; Human skin equivalents (HSEs); Staphylococcus aureus; Inflammatory response

1. Introduction

A highly versatile and dynamic microbiome is present on the surface of the human skin[1,2]. The skin consists of the subcutaneous adipose layer, the dermis, and the epidermis, of which the latter is formed by keratinocytes that are differentiated in four distinct epidermal layers. The skin microbiome is an essential part of the human skin barrier, which comprises the physical, immunological, chemical, and microbiological compartments[3]. By the combination of these barrier compartments, the human body is secured from dehydration, damage through excessive UV light exposure, and infection or overgrowth of pathogens. Yet, the interactions between the skin microbiome and the human skin are not fully understood. One factor that limits a thorough understanding of host-microbe interactions on the skin is the minimal availability of reproducible *in vitro* models in co-culture with the skin microbiome. One of the challenges for establishing such skin models is the sterile work environment required for long-term tissue engineering projects when omitting antibiotics. Nevertheless, there is an urgent need by the pharmaceutical, food and cosmetic industry for robust systems that recapitulate 3D-*in vivo* microbiome characteristics of native human skin (NHS), based on the lack of adequate recapitulation of human skin physiology and interaction with the skin microbes in animal experiments or in 2D monolayer testing. This is fueled by ethical considerations, increased societal and academic awareness of non-animal model systems, and poor translational properties of animal models[4]. These factors direct the field towards human translational model systems[5].

Three-dimensional human skin equivalents (HSEs) are tissue engineered skin models, consisting of a human cultured epidermis and/or dermis. Various types of HSEs have been developed, of which the Leiden Epidermal Models (LEM) consists of primary keratinocytes that are fully differentiated forming a functional

skin barrier. Several studies have been conducted to enrich skin models with a microbial compartment to induce a co-culture system[6-8]. The strength and added value of these models have already been shown in studies in which organotypic models were inoculated with various microbes to study skin infections and to screen for antimicrobial peptide efficacy in antibiofilm assays[8-13]. Yet, multiple factors require experimental optimization, as described earlier by Rikken *et al.* which highlighted the current lack of standardization, short co-culture periods, and low assay throughput. These aspects are not unique for host-microbe interaction studies, but are in line with the quality standards and validations required for organotypic skin models, as described elsewhere[14].

The microbiome on the human skin consists of prokaryotic bacteria, eukaryotic fungi, and viruses[1,15]. These form a unique community of microorganisms that differ in composition depending on the body site and the concomitant moist, dry, or sebaceous skin surface. The anatomical skin site is most affecting the composition of bacteria and fungi, whereas the presence of viruses has been more related to an individual[1]. Bacterial species that are part of a healthy skin microbiome are *Staphylococcus epidermidis* and *Cutibacterium acnes*, whereas in skin conditions the presence of *S. aureus* is elevated[16-18]. External factors that are known to affect the skin microbiome include environmental factors (indoor and outdoor), social dynamics and lifestyle, and the use of personal care products[19]. Moreover, the skin microbiome itself is orchestrated by microbe-microbe interactions, where strain-specific differences shape microbiome-modulating effects and their capacity to induce a host response[20]. Yet, our understanding of the dynamics within the skin microbiome and host-microbe interactions remains limited.

In healthy human skin, the host-microbe interactions are highly diverse and dynamic in a bidirectional manner. For instance, the composition of the skin

microbiome is modified by the expression of antimicrobial peptides in the epidermis[21,22], whereas an inflammatory reaction by the skin cells can be induced by the presence of microorganisms that express virulence factors[23]. Understanding the depth of these interactions is challenging but can be performed by measuring biomarkers. The epidermal cells can produce pro-inflammatory cytokines such as interleukin 8 (IL-8) and chemokine (C-X-C motif) ligand 1 (CXCL1), which trigger the recruitment of neutrophils contributing to an inflammatory response[1,20]. These can be measured as biomarkers to indicate a pro-inflammatory response. Discovery of skin health related local and systemic biomarkers will shape experimental designs and serve as indicators in assessment of translational power of model systems.

Our aim was to establish an integrated microbe - human skin system by culturing skin microbes on the surface of LEMs. We focused on three complementary aspects of this model: measuring microbial outgrowth, assessment of epidermal morphogenesis, and evaluation of the inflammatory status based on cytokine secretion. Here, we reveal that a functional interaction between microbes and keratinocytes of the LEMs was established, which revealed a species-specific *in vitro* host response that was assessed by integration of diverse methods required to evaluate complex *in vitro* model systems.

2. Materials and Methods

2.1 Isolation of primary skin cells

Normal human epidermal keratinocytes (NHEKs) were isolated from surplus skin obtained from anonymous female donors who had undergone cosmetic surgery. The donors had skin types I–II (according to Fitzpatrick score[24]). All donors were of female sex, were aged between 18-50, and the skin was obtained from mamma tissue (**Supplementary table 1**). Epidermal and dermal layers were enzymatically and mechanically separated, followed by digestion to generate single-cell suspensions. The keratinocytes were cultured in keratinocyte medium 37 °C and 7.3 % CO_2, comprising three parts Dulbecco's Modified Eagle Medium (DMEM) and one part Ham's F12 medium, supplemented with 5% (v/v) fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, USA), 0.5 µM hydrocortisone, 1 µM isoproterenol, 0.1 µM insulin (all from Sigma–Aldrich, Zwijndrecht, The Netherlands), and the antibiotics 100 U/ml penicillin and 100 µg/ml streptomycin (both from Thermo Fisher Scientific)[25].

2.2 Construction of Leiden Epidermal Models

Leiden Epidermal Models (LEMs) were generated using primary human keratinocytes, as previously described[25]. The LEMs were generated with primary keratinocytes that were transitioned to Dermalife medium (Lifeline Cell Technology, San Diego, CA, USA) supplemented with the antibiotics penicillin (10,000 U/ml) and streptomycin (10 mg/ml) one day prior to model preparation. On the following day, 5.6×10^4 keratinocytes were seeded onto 0.4 µM filter inserts (Greiner Bio-One B.V., Alphen aan den Rijn, The Netherlands) in 24-well plates containing Dermalife medium. The LEMs were refreshed once more with LEM1 medium prior exposing them to air. After three days in total, the apical medium was removed to expose the keratinocytes to air, and the cultures were maintained

at 37°C and 7.3% CO_2. After air-exposure, the basal medium was replaced with LEM2 medium, consisting of CnT-02-3D medium (CellnTech, Bern, Switserland) combined with keratinocyte medium in equivalent quantities supplemented with 2.4×10^{-2} µM bovine serum albumin and the antibiotics. One day prior to bacterial inoculation, the medium was replaced with antibiotic-free LEM2 medium. All experiments were conducted using 10-day air-exposed cultures.

2.3 Generation of host-microbe co-culture skin models

Bacteria were cultured according to the culturing conditions as described in **supplementary table 2**. The bacteria were suspended in Dulbecco's Phosphate Buffered Saline (PBS) and washed twice, centrifuged (10 min, 3000 rpm) and supernatant was removed. The OD600 was measured with a Personal Cell Density Meter (CO8000 cell density meter, Biochrom, Cambridge, UK) and the microbes were diluted accordingly. A volume of 15 µl containing resuspended bacteria was placed in the middle of the insert to initiate co-culturing. The same volume of resuspended bacteria was transferred in a 96 deep-well round bottom (P-DW-20C, Axygen, Corning Life Sciences, Amsterdam, The Netherlands) for DNA isolation at t=0h. A colony-forming unit (CFU) determination was performed simultaneously. The co-culture inserts were incubated at 37°C, 7.3% CO_2 and 90% humidity. After 48h of incubation, the inserts were harvested for DNA isolation by superficial washing of the inserts with 200 µl CD1 solution (DNeasy 96 Powersoil Pro QIAcube HT kit, Qiagen, Hilden, Germany) and collection of the solution in a 96 deep-well plate. The inserts were cut using a scalpel from the insert and placed in the same deepwell plate. The plate was stored at -20°C. Harvesting for histology was performed by cutting out the membrane from the insert and placed in a cassette that was incubated in 4% formaldehyde (Added Pharma, Oss, The Netherlands) and after 24h transferred to 70% ethanol. The medium below the inserts was

collected in a 96 deep-well round bottom (P-DW-11-C-S, Axygen) and stored at -20°C for cytokine determination.

2.4 Determination of microbial growth

DNA was extracted from the host-microbe co-culture skin models as samples by the mag™ particle suspension BL (LGC Biosearch, Petaluma, CA, USA) in combination with Pureprep system (Molgen, The Netherlands). Essentially, the skin model samples were supplemented to 800 µl using CD1 solution and 500 µL zirconium beads (0.1mm; BioSpec products, Bartlesville, OK, USA). After bead-beating for 2 min twice, with cooling on ice in between the two beating runs, the plate was centrifuged for 6 min at 3000 rpm. Thereafter, the 350 µL of supernatant was transferred to a 2 mL 96-well plate containing 350 µL binding buffer BL and 10 µL mag particle suspension BL. Subsequently, the pureprep was running the DNA purification program according to the protocol of the supplier (Molgen). Extracted DNA was eluted in a final volume of 65 µL buffer. Cell densities of the microbial growth on the host-microbe co-culture skin model were determined by specific quantitative polymerase chain reactions (qPCRs). Specific primers and probes for the qPCRs are described in **supplementary table 3**. The Maxima probe/ROX qPCR mastermix (2x) (#K0231, Thermo Fisher Scientific) was used for all strain, except for *C. tuberculostearicum* where the qPCR (JUN-QSY) with the Taqman multiplex mastermix (#4461881, Applied biosystems, Thermo Fisher Scientific) was used. Reaction mixes are provided in **supplementary table 4**. The qPCR was performed in the QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific) with the following settings: 5 min preheating 50°C, 10 min denaturation and PCR initiation at 95°C followed by two-step amplification with 45 cycles of alternating 15 sec denaturation at 95°C and 60 sec primer/probe annealing and complementary strand synthesis at 60°C. The qPCR data including the trendline for the cycle time

value (Ct) as function of specific microbial strain DNA concentration were used to calculate the number of bacterial genome equivalents per insert of the host-microbe co-culture skin model samples. In the calculation, the chromosome size of the different strains is as described in **supplementary table 2.**

2.5 Histological evaluation

LEMs were fixed in 4% paraformaldehyde for 18 h, followed by dehydration and paraffin embedding. Morphological analysis was conducted on 3–4 µm sections of formalin-fixed, paraffin-embedded (FFPE) samples, which were stained with hematoxylin and eosin (HE). For immunohistochemistry (IHC), 4 µm FFPE sections were deparaffinized, rehydrated, and subjected to antigen retrieval in either sodium citrate buffer (pH 6.0) or Tris-EDTA buffer (pH 9.0) using an autoclave at 110°C for 5 minutes. A wax pen (DAKO, Glostrup, Denmark) was used to delineate the sections, which were then blocked with 1% bovine serum albumin (BSA) in PBS containing 2% normal human serum. Primary antibodies (detailed in **Supplementary table 5**) were applied and incubated overnight at 4°C in a humidified chamber. Subsequently, sections were treated with secondary antibodies (**Supplementary table 5**) for 60 min at room temperature in a dark, humidified chamber, followed by 45 min of incubation with the streptavidin-biotin peroxidase system (GE Healthcare, Chicago, IL, USA). Staining was visualized using 3-amino-9-ethylcarbazole (AEC, Sigma-Aldrich), and sections were mounted with Kaiser's glycerol gelatin (Merck, Rahway, NJ, USA) and counterstained with hematoxylin (Sigma-Aldrich). PBS washes were performed between steps, and imaging was conducted with a Zeiss Axioscope 5 microscope (20x dry objective). For immunofluorescence (IF), a similar protocol was followed, except sections were counterstained with DAPI (1:5000) and mounted with Vectashield (both Sigma-Aldrich). Imaging was performed using the DM6b2 fluorescence microscope (20x

dry objective) and the Zeiss Airyscan confocal microscope (20x dry objective). To determine the Ki-67 based proliferation index, the number of positive cells in the basal layer were counted in 4 to 6 different microscopic fields.

2.6 Measurement of secreted cytokines

Collection of the conditioned medium was performed on the day of harvest. The medium was transferred into deep-well plates and stored frozen at -20°C. Targeted detection of IL-8 and CXCL1 were performed with an enzyme-linked immunosorbent assay (ELISA) according to the instructions provided by the manufacturer. The following kits were used: Human IL-8/CXCL8 DuoSet ELISA #DY208, and Human CXCL1/GRO alpha DuoSet ELISA #DY275 (R&D Systems, Minneapolis, MN, USA). Negative controls were included in each measurement which was unconditioned medium. Samples per donor represent pooled sample of technical replicates.

2.7 Ethics

All primary human skin cells from healthy donors used by the Department of Dermatology are isolated from surplus tissue collected according to article 467 of the Dutch Law on Medical Treatment Agreement and the Code for proper Use of Human Tissue of the Dutch Federation of Biomedical Scientific Societies[26]. Under this national legislation, coded surplus tissue may be used for scientific research unless the informed donor issues a written objection, constituting an informed opt-out framework. Accordingly, and as stated earlier[8,44], no additional approval from an ethics committee was required nor requested for the research use of this material. All participants received an informed consent form where they were informed of the use of their tissue for scientific research and all patients were offered the opportunity to give approval or refusal to this. Only tissue from patients

who agreed was used. None of the authors were involved in the tissue sampling and only birth date, gender and skin type of the subjects were documented. These data were only accessible to AEG. The Declaration of Helsinki principles were followed when working with human tissue.

2.8 Statistics

Statistical analyses were performed using GraphPad Prism (version 9.1.2). All data are presented as mean ± SEM, unless indicated otherwise. Data was first tested for normal distribution using the Shapiro-Wilk test. When data showed normal distribution, equality of variance was tested using Brown-Forsythe test. In case of normally distributed data with equality in variance, ordinary one-way ANOVA was used with Holm-Sidak multiple comparisons post-test. For normally distributed data without equality in variance, the Brown-Forsythe and Welch ANOVA test was used with Dunnett multiple comparison post-test. When data was not normal distributed, Kruskal-Wallis with Dunn's post-test was performed. Significant outcomes are indicated with *, **, ***, and **** which indicate adjusted p-values $p<0.05$, $p<0.01$, $p<0.001$, and $p<0.0001$ respectively.

3. Results

3.1 Outgrowth of skin bacteria on human skin equivalents

To mimic the NHS in an *in vitro* model with the skin microbiome, we deployed LEMs which were co-cultured with bacteria that are present on the skin (**Figure 1**). LEMs are 3D models that present the characteristic layers of the epidermis, including stratum basale (SB), stratum spinosum (SS), stratum granulosum (SG), and stratum corneum (SC). For the generation of a stable host-microbe co-culture on LEMs, *S. aureus* was selected as an opportunistic skin microbe, whereas *S. epidermidis* and *C. acnes* were selected as commensal skin microbes[1]. Moreover, two different strains of *S. epidermidis* were selected for this study to explore strain diversity, these were the (I) ATCC strain 12228 and the (II) ATCC 11536 strain. The latter showed a strain-specific capacity to produce different metabolites and bacteriocins[27], potentially affecting host response. Pre-optimization of the co-culture methodology protocol resulted in an optimization of 48h as time of co-culturing with a low inoculation (10^3-10^4 genome equivalents) or a medium inoculation (10^4-10^5 genome equivalents) per model as start concentration.

The host-microbe skin co-cultures were developed with monocultures of *S. aureus*, *S. epidermidis*, and *C. acnes* that were applied and examined for outgrowth after 48h **(Figure 2A-D)**. All these skin microbes showed substantial growth on the surface of the LEM regarding the absolute growth started from the low or medium inoculation levels. After 48h *S. aureus* had most absolute and relative growth after low and medium inoculation. The two strains of *S. epidermidis* showed substantial outgrowth, although the *S. epidermidis* (I) strain outgrowth was more as compared to *S. epidermidis* (II). *C. acnes* did grow out during the 48h period in aerobic conditions on the surface of the LEM, which are typically unfavorable growth conditions for the lipophilic, gram-positive, aerotolerant anaerobe microbe. To assess the robustness of the co-culturing, a total of six different primary cell donors

have been used in this assay. In general, the variation in biological donors did not affect the bacterial outgrowth on the LEMs.

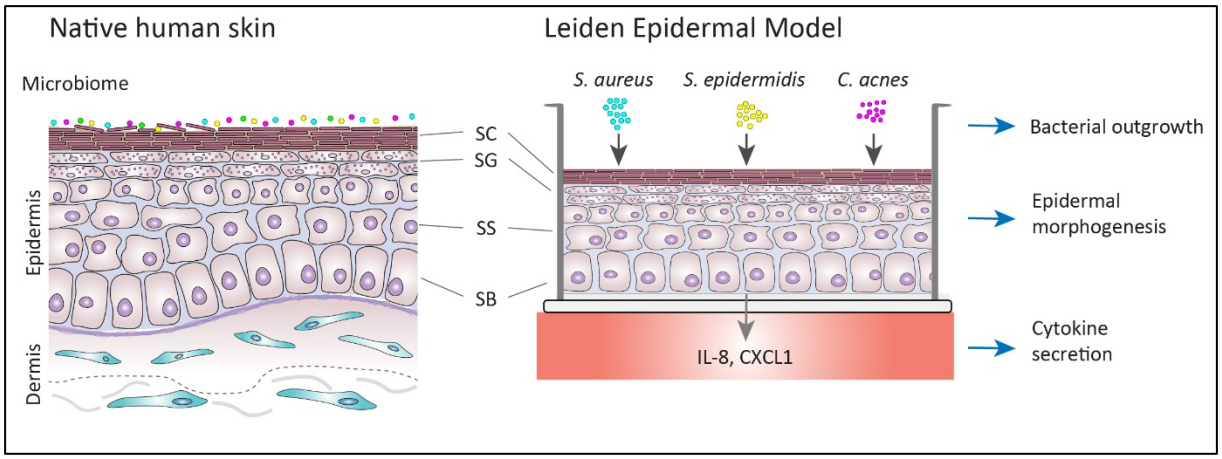

Figure 1. Schematic overview of the histology of native human skin (NHS) and of the Leiden Epidermal Model (LEM) as type of human skin equivalent (HSE). The NHS (left) consists of the dermis, the epidermis as characterized by the stratum corneum (SC), stratum granulosum (SG) stratum spinosum (SS) and the stratum basale (SB), and the skin microbiome. In the LEM (right), the epidermis is formed by keratinocytes. Monocultures of skin microbes (*S. aureus*, *S. epidermidis* and *C. acnes*, respectively) were administered on the surface of the epidermis. Read-out parameters were bacterial outgrowth, epidermal morphogenesis, and extracellular cytokine levels.

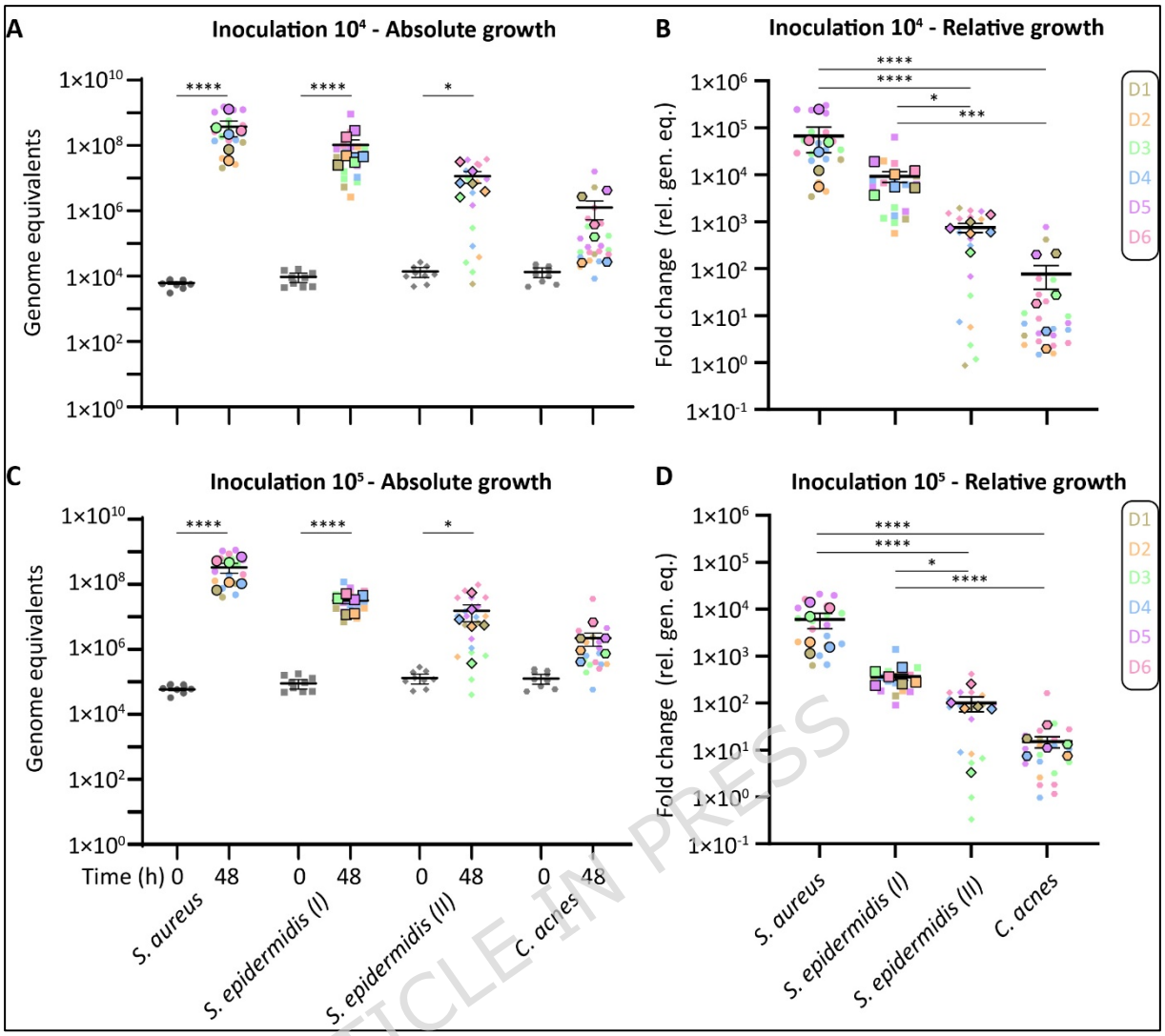

Figure 2. Robust outgrowth of skin microbes as monocultures on LEMs. A) Absolute number of genome equivalents per LEM during inoculation (t=0h) and after outgrowth (t=48h). **B)** Relative number of genome equivalents of skin microbes after outgrowth on LEMs at t=48h. **C)** Absolute number of genome equivalents per LEM during inoculation (t=0h) and after outgrowth (t=48h). **D)** Relative number of genome equivalents of skin microbes after outgrowth on LEMs at t=48h. Biological donors (D1-D6) at t=48h are indicated by color codes described in the legend. Technical replicates are indicated by single data points. Means of each biological replicate is indicated by bordered symbols. Line indicates mean of biological donors with error bars representing SEM.

3.2 Epidermal morphogenesis of human skin equivalents in co-culture with microbes

We next investigated whether the inoculation of LEMs with different bacterial strains influenced epidermal morphogenesis, including morphology, early and late differentiation, and proliferation. All donors produced well-formed epidermal structures encompassing all viable layers (i.e. SB, SS, SG, and SC) (**Figure 3**). As for both inoculation level a substantial growth was detected, histological analyses were performed focused on the low inoculation level. Notably, in all representative donors, *S. aureus* and to a lesser extent *S. epidermidis* (I) exhibited an adverse impact on epidermal structure. This was presented as less cuboidal structure of basal layer keratinocytes and earlier flattening of spinous layer keratinocytes. The impact of *C. acnes* on the epidermal structure was insignificant, as this was only present in a minority of included donors. Occasionally, in cross-sections of LEMs co-cultured with *S. aureus*, and *S. epidermidis* (II), visible attachment to the SC was observed indicated by the asterisk (**Figure 3**), although due to tissue processing methods this was not consistently observed.

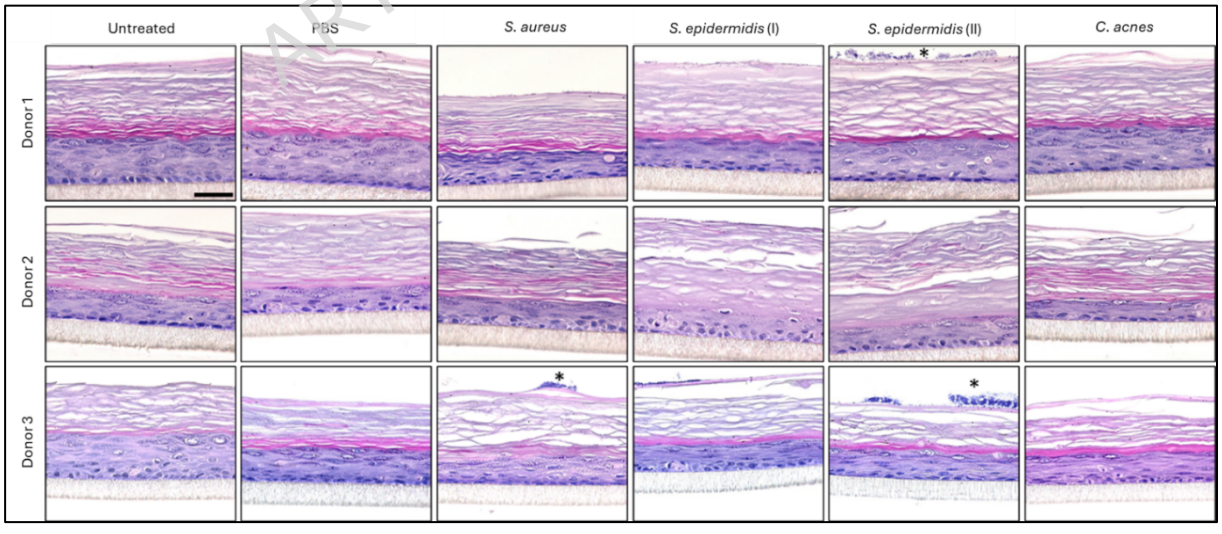

Figure 3. Histology of LEMs co-cultured with skin microbes. Shown are cross-sections of LEMs of 3 donors without treatment, PBS as vehicle control, and inoculated with *S. aureus*, *S. epidermidis* (I), *S. epidermidis* (II), and *C. acnes* with a low inoculation (10^3-

10^4 genome equivalents) for 48h with HE staining. The asterisk (*) indicates visible attachment of the respective microbes to the LEM. Scale bar 50 mm.

In-depth evaluation of epidermal morphogenesis was performed via analysis of differentiation-specific protein biomarkers in three donors. Late differentiation was assessed by filaggrin (FLG) and loricrin (LOR) expression profiles, both key proteins in the cornified envelope[28]. The cornified envelope forms during the final stages of keratinocyte differentiation, a process crucial for establishing the skin's barrier function. Moreover, breakdown products of FLG are an important part of the natural moisturizing factor (NMF) within the SC[29]. Both the NMF and LOR, amongst others, affect the adhesion of microbes to the skin surface[30,31], thereby serving as structural component of the physical skin barrier and as mediator of the microbial skin barrier. The expression and localization of FLG and LOR was predominantly found in the SG and SC in control condition **(Figure 4A, B)**. In co-culture with microbes, the expression and localization of FLG was similar, whereas that of LOR revealed a marked reduction after inoculation with *S. aureus*, *S. epidermidis* (I), and *C. acnes*. This indicates a protein-specific effect on late and terminal differentiation in the presence of certain microbial species.

Rigidity of early differentiation was evaluated by the expression and localization of protein biomarker keratin 10 (K10). This is a structural protein expressed in the suprabasal layers (SS and SG) of the epidermis during keratinocyte differentiation, where it acts to maintain structural integrity and stability of the skin. Pathogenic variants in its gene *KRT10* are associated with skin fragility disorders, such as epidermolytic ichthyosis[32]. K10 expression was robust, and the localization was restricted to the SS and SG in control condition **(Figure 4C)**. In the presence of *S. aureus* and *S. epidermidis* (I), in most donors a delayed early differentiation was observed, indicated by reduced K10 expression in the first suprabasal layer. Both *S. epidermidis* (II) and *C. acnes* did not affect the initiation of the early differentiation program.

Next, we investigated whether microbial inoculation influenced cell proliferation after 48h. To assess this, we stained for the cell proliferation marker Ki67 (**Figure 4C**) and quantified the number of positive and total cells in the basal cell layer, yielding the proliferation index, which is presented as fold change (**Figure 4D**). In all conditions, Ki67 expression was restricted to the SB, indicating no significant differences were observed in localization of proliferation keratinocytes between the vehicle control and LEMs inoculated with microbes. All *Staphylococci* strains did not alter cell proliferation, whereas *C. acnes* promoted cell proliferation.

To assess whether inoculation with the bacterial strains induced cell activation in LEMs after 48h, we analyzed the expression of keratin 17 (K17) as protein biomarker. The structural protein K17 is upregulated upon hyperprofileration and wound healing[33], and also functions as regulator of nuclear morphology and chromatin organization[34]. In control conditions, very low K17 expression was detected in the LEM, indicative for epidermal homeostasis **(Supplementary Figure 1A)**. Upregulation of K17 was not induced by any microbial strains as compared to their respective controls.

Evaluation of an altered host response towards the microbes on the LEMs was performed by expression and localization of host defense proteins. Assessment of the response of the skin to monocultured microbes using *in vitro* systems is specific, especially as this information is highly limited to obtain *in vivo* due to the continuous presence of multiple microbes as community on the epithelial surface. We assessed two biomarkers to evaluate the antimicrobial host response, which are the proteins small proline rich protein 2A (SPRR2a) and beta-defensin 3 (hBD3). The SPRR proteins contribute to the biomechanical properties of cornified envelopes[35] and possess bactericidal activity, which was observed in a dose-dependent way for SPRR2a towards *S. epidermidis*[36]. Beta-defensins are antimicrobial peptides (AMPs) expressed on epithelial surfaces including the skin,

in which these are upregulated upon infection, inflammation, or wounding and also act as immunomodulatory regulators[37,38]. Within the hBD family, the strongest microbicidal activity against Gram-negative and -positive bacteria and yeast was found to be hBD3[39]. At baseline, very low SPPR2a and hBD3 expression in LEMs was observed **(Supplementary Figure 1B, C)**, which was not increased substantially in response to any microbial strain as compared to their respective controls.

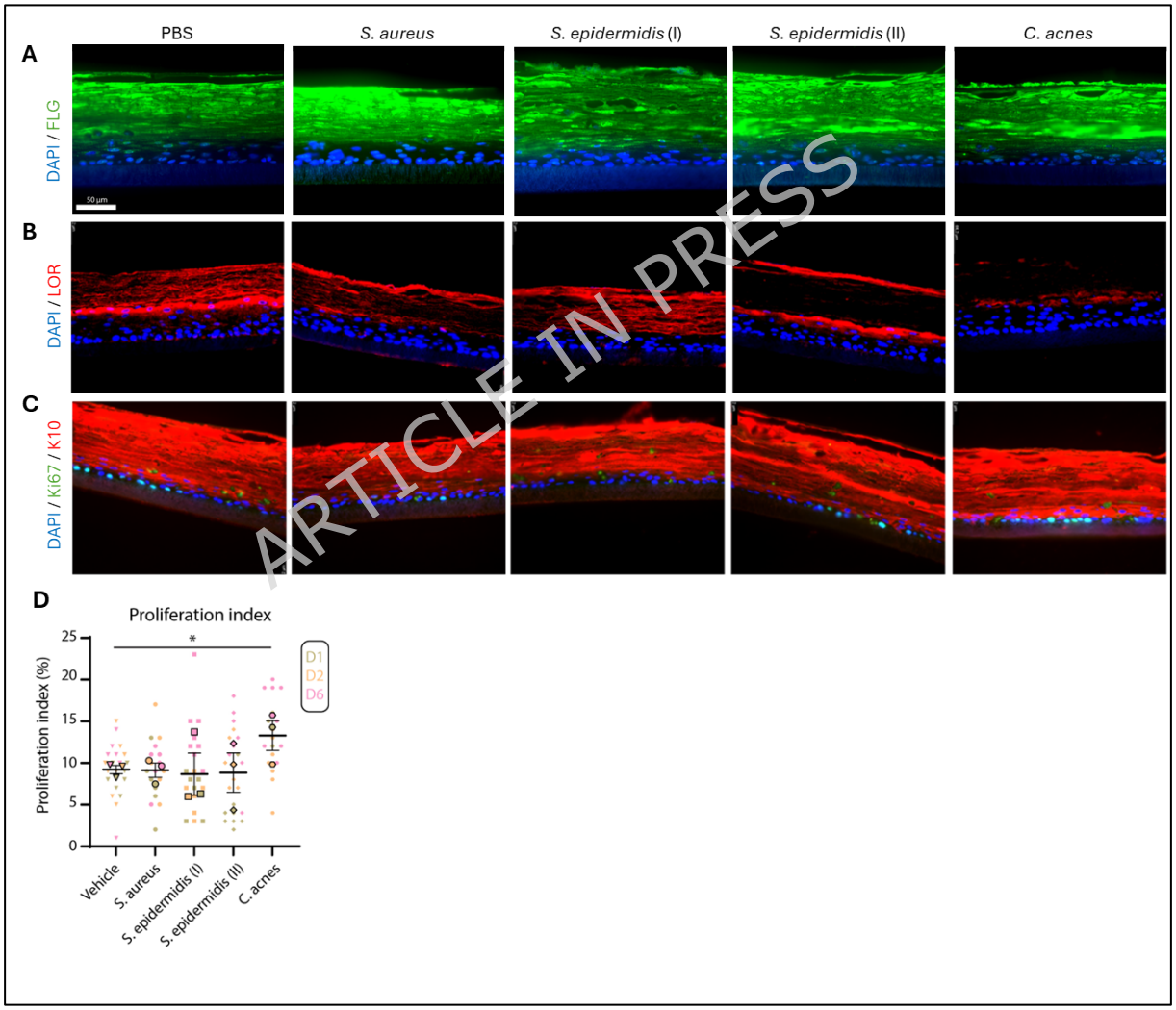

Figure 4. Epidermal morphogenesis of HSE co-cultured with skin microbes. Shown are cross-sections of LEMs stained for **A)** the late differentiation markers Filaggrin (FLG) and **B)** Loricrin (LOR), **C)** early differentiation marker keratin 10 (K10), proliferation

biomarker Ki67, and nuclei with DAPI. LEMs were inoculated with PBS as control, *S. aureus*, *S. epidermidis* (I), *S. epidermidis* (II), and *C. acnes* with a low inoculation (10^3-10^4 genome equivalents) for 48h. Scale bar indicates 50 µm. **D)** Scattered dot plot of the proliferation index quantified by the number of Ki67 positive cells in the basal layer presented as fold change. All quantified data is presented in small dots, with averages per color-coded donor as larger dot, and line with error bars indicate mean + SEM.

3.3 Skin microbes induce changes in secretion of pro-inflammatory cytokines in LEMs

Evaluation of immunological responses of the skin cells upon co-culturing with the skin microbiome was performed by analysis of protein secretion of cytokines IL-8 and CXCL1, both related to pro-inflammatory pathways. Analysis of negative control samples (i.e., unconditioned cell culture medium) showed no detection of background signal. The vehicle control PBS condition for all donors showed a minimal level of variability, although six different primary cell donors were used in total. This indicated the high level of reproducibility of the model system on functional level. The highest induction in production of pro-inflammatory cytokine IL-8 versus vehicle control was obtained by co-culturing with *S. aureus* (**Figure 5A, B**). Inoculation with high numbers of *S. aureus* also induced the secretion of the pro-inflammatory cytokine CXCL1 (**Figure 5C, D**). Interestingly, there was variability between the different primary cell donors detected, indicating that some donors were more responsive to *S. aureus* and others were more protected. No induction was detected by both strains of *S. epidermidis* and for *C. acnes*. In the co-culturing with the latter, also a high level of reproducibility was obtained, as all the individual datapoints derived from different biological donors clustered together. When comparing the cytokine production after inoculation with different start concentrations, a high degree of overlap was visible. This indicates that during the rapid outgrowth of the bacteria over the 48-hour co-culture period, the relative contribution of the initial bacterial concentration was overcome. On a systems biology level, the production and secretion of IL-8 and CXCL1 by keratinocytes as response to bacteria induces the recruitment of neutrophils[40], a cell type that is currently not present in our *in vitro* platform, which is of interest to incorporate in future HSE experiments.

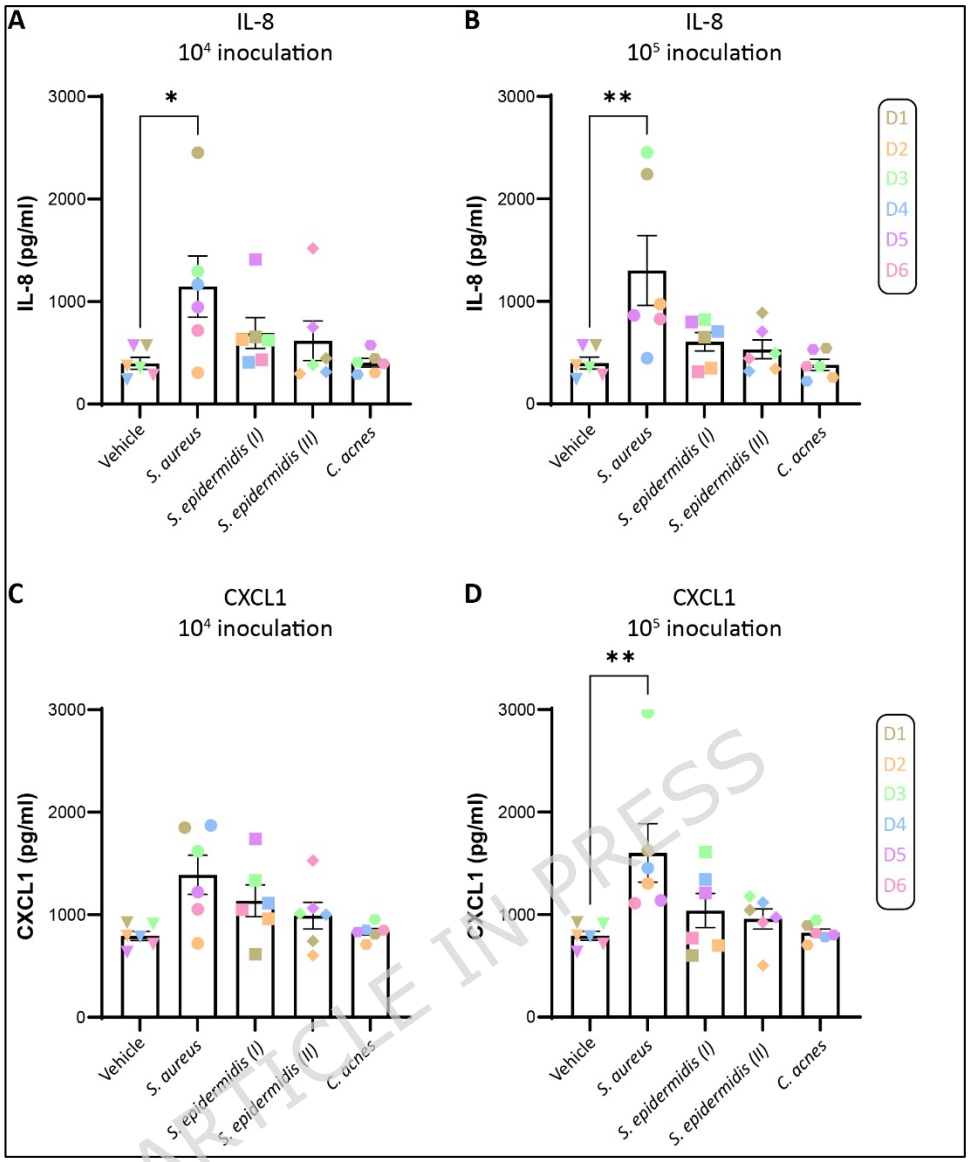

Figure 5. Secretion of IL-8 and CXCL1 by LEMs co-cultured with monocultures of skin microbes. A, B) Detection of IL-8 and **C, D)** CXCL1 in the conditioned medium harvested after 48h co-culture with PBS or indicated skin microbes. Both a low (10^3-10^4 genome equivalents) and a medium (10^4-10^5 genome equivalents) inoculation level per model were examined. Each data point is color-coded and indicates a biological replicate (donor (D) 1-6), derived from pooling of technical replicates per donor. *, ** indicates $p<0.05$, $p<0.01$, respectively.

4. Discussion

Host–microbe interactions are central to maintaining skin health. The host epithelium shapes microbiome composition through secreted modulatory factors, while skin-resident microbes influence host responses and restrict opportunistic species via microbe–microbe interactions[41-43]. To investigate these processes, advanced *in vitro* co-culture systems integrating host tissue and microbes are essential. Here, we assessed the effects of selected microbial species on 3D LEMs using complementary readouts of microbial growth, epidermal morphogenesis, and inflammatory mediator production.

Variations in outgrowth were observed comparing microbes that proliferated on the surface of the LEMs. Assessment of morphogenesis showed that *S. aureus* induced an altered epidermal organization, delay in the early cell differentiation, and a marked reduction in late differentiation as compared to control. These effects were less prominent or absent for other skin microbes, revealing species-specific functional effects. On a strain-specific level, we obtained indications for more subtle variations in growth of *S. epidermidis* derived from different origins, which did not affect functional host responses substantially. Our results are in line with previous research, where the impact on epidermal morphogenesis by *S. aureus* co-culturing was also observed by Rikken *et al.*[8]. The functional effects of co-culturing on the production and secretion of inflammatory cytokines were detected, which were used as markers for the epidermal response to the skin microbes. This is in line with previous findings of Van Drongelen *et al.*[9], who showed that IL-8 secretion was upregulated after 24h of co-culturing with *S. aureus*. Divergent effects of skin microbes on the activation of inflammatory pathways have been described before[44,45], most often linked to an overgrowth of *S. aureus* on the skin.

The host-microbe co-culture model as developed in this project is arriving at a state-of-the-art as compared to other *in vitro* models. An overview of established

co-culture models has been recently published[8], which summarized that co-culture models are established, but there is a high variation in the cell types that have been used, the number of technical and biological replicates, and the types of experimental read-outs used in each study. Therefore, a direct comparison of each study is complex and urges the field to place more attention on standardization on various critical read-outs, enabling meta-analysis and cross-validations. Methodological factors that can be optimized are total time of co-culturing, inoculation concentration, and method of application[8]. Our results provide an indication that the start level of inoculation potentially affects the host-microbes interaction. By including two inoculation levels throughout the project, we demonstrated that this is a factor to consider in method optimization. Furthermore, optimization on the quantification of bacterial abundance beyond molecular techniques will reveal insights on the viability of the microbes over time, which potentially provides additional information on the host-microbe interactions. Moreover, there is a variation in the type of HSE used per study, which can potentially diverge in functional response on microbial growth, epidermal morphogenesis, and secretion of immunomodulatory mediators, thereby directing the field towards gathering information on translational relevance[46,47].

As compared to the level of microbes present on the *in vivo* skin, which is estimated to harbor 10^6 microbes per 1 cm^2 on moist and 10^2-10^3 on dry NHS[48], the outgrowth measured in the *in vitro* system reached supraphysiological levels. Interestingly, the outgrowth of microbes other than *Staphylococcus* spp. did not reach supraphysiological levels. Cell culturing optimization strategies could overcome *in vitro* – *in vivo* differences by stimulation the production of AMPs, mimicking microenvironmental niches (i.e. dry, sweat, or sebaceous surfaces), tailored nutrient provision, and cell culture conditions. For oxygen sensitive species this can include culturing the HSEs at reduced oxygen levels, which did not impact

physical barrier formation[49]. Moreover, reduction in relative humidity can be implemented as this affects the breakdown of filaggrin[50], which is a potential host-microbe interaction pathway that is shown to modify outgrowth of *S. aureus*[9].

A limitation of this study is the lack of immune cells resident in the epidermis. Host-microbe interactions in the skin are in part orchestrated by the activation of Langerhans cells to regulate immune tolerance or induce a pro-inflammatory reaction by presenting microbial antigens[51]. Other immune cell type that affect host-microbe interactions are for example the skin-resident effector T-cells, dermal dendritic cells, and regulatory T-cells[52]. Another limitation is the presence of bacteria in monocultures, in contrast to the multispecies mixture observed in the skin microbiome[53]. The microbe-microbe interactions shape the composition and functionality of the microbiome on the skin, which needs to be recapitulated *in vitro* as well. However, to understand the host-microbiome cross-talk of the skin, the development of HSEs with these immune cell types and a complex microbiome will provide a more realistic and highly complex model yet can affect the testing throughput.

In conclusion, we developed a robust and reproducible model that contains both the human skin epithelium and key bacteria present in the skin microbiome. The model system allows for testing host-microbe interactions in a controlled microenvironment. By inoculation of epidermal models with monocultures of *S. aureus*, *S. epidermidis*, or *C. acnes* a stable bacterial outgrowth was detected across biological donors. Epidermal morphogenesis was examined by histology to reveal adaptation of the keratinocytes to the microbes. Higher secretion of pro-inflammatory cytokines was measured after inoculation with *S. aureus*, which was not increased substantially by other commensal skin microbes.

Acknowledgements

The authors are grateful to Anita Ouwens for the contribution on initial exploration on technical feasibility of the co-culture method.

Funding

The project was funded by Health~Holland TKI-Public Private Partnership grant awarded to the consortium of TNO, LUMC, and Beiersdorf, as well as by the consortium partner Beiersdorf.

Conflict of interest

Health~Holland had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results. H.F. and E.G. are employees at Beiersdorf AG, of which author contribution are disclosed according to the CRediT system. The other authors declare no conflicts of interest.

Author contribution

Conceptualization: AM, EG, FHJS, AEG. Methodology: AM, FHJS, AEG. Validation: FHJS, AEG. Formal analysis: AM. Investigation: MR, BvL, AS, NP. Resources: MR, BvL, AS, NP. Data curation: MR, BvL, AS, NP. Writing – Original draft: AM, FHJS, AEG. Writing – Reviewing & Editing: all authors. Visualization: AM. Supervision: FHJS, AEG. Project administration: JK, HF, EG, FHJS, AEG. Funding acquisition: JK, HF, FHJS, AEG.

Data availability

The datasets used and/or analyzed during the current study are available from the corresponding authors on reasonable request.

List of abbreviations

HSE	Human skin equivalent
IL	Interleukin
SC	Stratum corneum
SB	Stratum basale
SS	Stratum spinosum
SG	Stratum granulosum
LEM	Leiden epidermal model
NHS	Native human skin
qPCR	quantitative polymerase chain reaction
NHEK	Normal human epidermal keratinocytes

References

1 Byrd, A. L., Belkaid, Y. & Segre, J. A. The human skin microbiome. *Nature Reviews Microbiology* **16**, 143–155 (2018).

2 Schommer, N. N. & Gallo, R. L. Structure and function of the human skin microbiome. *Trends in microbiology* **21**, 660–668 (2013).

3 Niehues, H. *et al.* 3D skin models for 3R research: The potential of 3D reconstructed skin models to study skin barrier function. *Experimental Dermatology* **27**, 501–511, doi:https://doi.org/10.1111/exd.13531 (2018).

4 Cedrola, C. C., Cedrola, C. C., de Paula, A. C. C., da Costa, J. C. & Vilela, F. M. P. Alternatives to animal testing in cosmetic products: A patent applications review and future perspectives. *Toxicol In Vitro* **112**, 106187, doi:10.1016/j.tiv.2025.106187 (2026).

5 Emmert, H., Rademacher, F., Gläser, R. & Harder, J. Skin microbiota analysis in human 3D skin models—“Free your mice”. *Experimental Dermatology* **29**, 1133–1139, doi:https://doi.org/10.1111/exd.14164 (2020).

6 Bojar, R. A. Studying the human skin microbiome using 3D in vitro skin models. *Applied In Vitro Toxicology* **1**, 165–171 (2015).

7 Haisma, E. M. *et al.* Inflammatory and antimicrobial responses to methicillin-resistant Staphylococcus aureus in an in vitro wound infection model. *PloS one* **8**, e82800 (2013).

8 Rikken, G. *et al.* Novel methodologies for host-microbe interactions and microbiome-targeted therapeutics in 3D organotypic skin models. *Microbiome* **11**, 227 (2023).

9 van Drongelen, V., Haisma, E. M., Out-Luiting, J. J., Nibbering, P. H. & El Ghalbzouri, A. Reduced filaggrin expression is accompanied by increased Staphylococcus aureus colonization of epidermal skin models. *Clinical & Experimental Allergy* **44**, 1515–1524, doi:https://doi.org/10.1111/cea.12443 (2014).

10 Bi, O. *et al.* Do Melanocytes Have a Role in Controlling Epidermal Bacterial Colonisation and the Skin Microbiome? *Exp Dermatol* **34**, e70071, doi:10.1111/exd.70071 (2025).

11 Cadau, S. *et al.* An Inflamed and Infected Reconstructed Human Epidermis to Study Atopic Dermatitis and Skin Care Ingredients. *Int J Mol Sci* **23**, doi:10.3390/ijms232112880 (2022).

12 den Reijer, P. M. *et al.* Detection of Alpha-Toxin and Other Virulence Factors in Biofilms of Staphylococcus aureus on Polystyrene and a Human Epidermal Model. *PLoS One* **11**, e0145722, doi:10.1371/journal.pone.0145722 (2016).

13 Haisma, E. M. *et al.* LL-37-derived peptides eradicate multidrug-resistant Staphylococcus aureus from thermally wounded human skin equivalents. *Antimicrob Agents Chemother* **58**, 4411–4419, doi:10.1128/aac.02554-14 (2014).

14 van den Bogaard, E. *et al.* Perspective and Consensus Opinion: Good Practices for Using Organotypic Skin and Epidermal Equivalents in Experimental Dermatology Research. *J Invest Dermatol* **141**, 203–205, doi:10.1016/j.jid.2020.04.023 (2021).

15 Grice, E. A. & Segre, J. A. The skin microbiome. *Nature Reviews Microbiology* **9**, 244–253, doi:10.1038/nrmicro2537 (2011).

16 Edslev, S. M., Agner, T. & Andersen, P. S. Skin Microbiome in Atopic Dermatitis. *Acta Derm Venereol* **100**, adv00164, doi:10.2340/00015555-3514 (2020).

17 Demessant-Flavigny, A.-L. *et al.* Skin microbiome dysbiosis and the role of Staphylococcus aureus in atopic dermatitis in adults and children: A narrative review. *Journal of the European Academy of Dermatology and Venereology* **37**, 3–17, doi:https://doi.org/10.1111/jdv.19125 (2023).

18 Rauer, L. *et al.* Skin microbiome and its association with host cofactors in determining atopic dermatitis severity. *Journal of the European Academy of Dermatology and Venereology* **37**, 772–782, doi:https://doi.org/10.1111/jdv.18776 (2023).

19 Yu, S. Y. G. & Lio, P. External Factors and the Cutaneous Microbiome. *Journal of Integrative Dermatology* (2024).

20 Landemaine, L. *et al.* Staphylococcus epidermidis isolates from atopic or healthy skin have opposite effect on skin cells: potential implication of the AHR pathway

modulation. *Frontiers in Immunology* **14**, doi:10.3389/fimmu.2023.1098160 (2023).
21 Clausen, M.-L., Slotved, H. C., Krogfelt, K. A. & Agner, T. Measurements of AMPs in stratum corneum of atopic dermatitis and healthy skin–tape stripping technique. *Scientific Reports* **8**, 1666, doi:10.1038/s41598-018-20204-8 (2018).
22 de Breij, A. *et al.* The antimicrobial peptide SAAP-148 combats drug-resistant bacteria and biofilms. *Science translational medicine* **10**, eaan4044 (2018).
23 Dagnelie, M.-A., Corvec, S., Timon-David, E., Khammari, A. & Dréno, B. Cutibacterium acnes and Staphylococcus epidermidis: the unmissable modulators of skin inflammatory response. *Experimental Dermatology* **31**, 406–412, doi:https://doi.org/10.1111/exd.14467 (2022).
24 Gupta, V. & Sharma, V. K. Skin typing: Fitzpatrick grading and others. *Clinics in Dermatology* **37**, 430–436, doi:https://doi.org/10.1016/j.clindermatol.2019.07.010 (2019).
25 El Ghalbzouri, A., Siamari, R., Willemze, R. & Ponec, M. Leiden reconstructed human epidermal model as a tool for the evaluation of the skin corrosion and irritation potential according to the ECVAM guidelines. *Toxicol In Vitro* **22**, 1311–1320, doi:10.1016/j.tiv.2008.03.012 (2008).
26 CCMO. *Wet- en regelgeving voor medisch-wetenschappelijk onderzoek*, <https://www.ccmo.nl/onderzoekers/wet-en-regelgeving-voor-medisch-wetenschappelijk-onderzoek/uw-onderzoek-wmo-plichtig-of-niet> (2024).
27 Eady, E. A., Holland, K. T. & Cunliffe, W. J. Production and partial purification of a peptide antibiotic from Staphylococcus epidermidis. *Journal of Applied Bacteriology* **55**, 461–472, doi:10.1111/j.1365-2672.1983.tb01686.x (1983).
28 Candi, E., Schmidt, R. & Melino, G. The cornified envelope: a model of cell death in the skin. *Nature Reviews Molecular Cell Biology* **6**, 328–340, doi:10.1038/nrm1619 (2005).
29 Kezic, S. *et al.* Levels of filaggrin degradation products are influenced by both filaggrin genotype and atopic dermatitis severity. *Allergy* **66**, 934–940, doi:https://doi.org/10.1111/j.1398-9995.2010.02540.x (2011).
30 Pietrocola, G., Nobile, G., Rindi, S. & Speziale, P. Staphylococcus aureus Manipulates Innate Immunity through Own and Host-Expressed Proteases. *Frontiers in Cellular and Infection Microbiology* **Volume 7 - 2017**, doi:10.3389/fcimb.2017.00166 (2017).
31 Feuillie, C. *et al.* Adhesion of Staphylococcus aureus to corneocytes from atopic dermatitis patients is controlled by natural moisturizing factor levels. *MBio* **9**, 10.1128/mbio.01184–01118 (2018).
32 Mirza, H. *et al.* Mutations Affecting Keratin 10 Surface-Exposed Residues Highlight the Structural Basis of Phenotypic Variation in Epidermolytic Ichthyosis. *Journal of Investigative Dermatology* **135**, 3041–3050, doi:https://doi.org/10.1038/jid.2015.284 (2015).
33 Zhang, X., Yin, M. & Zhang, L.-j. Keratin 6, 16 and 17—critical barrier alarmin molecules in skin wounds and psoriasis. *Cells* **8**, 807 (2019).
34 Jacob, J. T. *et al.* Keratin 17 regulates nuclear morphology and chromatin organization. *Journal of cell science* **133**, jcs254094 (2020).
35 Cabral, A. *et al.* Structural Organization and Regulation of the Small Proline-rich Family of Cornified Envelope Precursors Suggest a Role in Adaptive Barrier Function*. *Journal of Biological Chemistry* **276**, 19231–19237, doi:https://doi.org/10.1074/jbc.M100336200 (2001).
36 Zhang, C. *et al.* Small proline-rich proteins (SPRRs) are epidermally produced antimicrobial proteins that defend the cutaneous barrier by direct bacterial membrane disruption. *Elife* **11**, e76729 (2022).
37 Chieosilapatham, P., Ogawa, H. & Niyonsaba, F. Current insights into the role of human β-defensins in atopic dermatitis. *Clin Exp Immunol* **190**, 155–166, doi:10.1111/cei.13013 (2017).
38 Pazgier, M., Hoover, D., Yang, D., Lu, W. & Lubkowski, J. Human β-defensins. *Cellular and Molecular Life Sciences CMLS* **63**, 1294–1313 (2006).
39 Jung, S. *et al.* Human β-Defensin 2 and β-Defensin 3 Chimeric Peptides Reveal the Structural Basis of the Pathogen Specificity of Their Parent Molecules.

Antimicrobial Agents and Chemotherapy **55**, 954–960, doi:doi:10.1128/aac.00872-10 (2011).

40 Jiang, Y. *et al.* Cytokinocytes: the diverse contribution of keratinocytes to immune responses in skin. *JCI insight* **5** (2020).

41 Flowers, L. & Grice, E. A. The skin microbiota: balancing risk and reward. *Cell host & microbe* **28**, 190–200 (2020).

42 Harris-Tryon, T. A. & Grice, E. A. Microbiota and maintenance of skin barrier function. *Science* **376**, 940–945 (2022).

43 Nakatsuji, T. *et al.* Antimicrobials from human skin commensal bacteria protect against Staphylococcus aureus and are deficient in atopic dermatitis. *Science Translational Medicine* **9**, eaah4680, doi:doi:10.1126/scitranslmed.aah4680 (2017).

44 Lunjani, N., Ahearn-Ford, S., Dube, F. S., Hlela, C. & O'Mahony, L. Mechanisms of microbe-immune system dialogue within the skin. *Genes & Immunity* **22**, 276–288, doi:10.1038/s41435-021-00133-9 (2021).

45 Chng, K. R. *et al.* Whole metagenome profiling reveals skin microbiome-dependent susceptibility to atopic dermatitis flare. *Nature microbiology* **1**, 1–10 (2016).

46 Havlikova, J., May, R. C., Styles, I. B. & Cooper, H. J. Direct identification of bacterial and human proteins from infected wounds in living 3D skin models. *Sci Rep* **10**, 11900, doi:10.1038/s41598-020-68233-6 (2020).

47 Kohda, K. *et al.* An In Vitro Mixed Infection Model With Commensal and Pathogenic Staphylococci for the Exploration of Interspecific Interactions and Their Impacts on Skin Physiology. *Front Cell Infect Microbiol* **11**, 712360, doi:10.3389/fcimb.2021.712360 (2021).

48 Grice, E. A. *et al.* A diversity profile of the human skin microbiota. *Genome research* **18**, 1043–1050 (2008).

49 Mieremet, A. *et al.* Human skin equivalents cultured under hypoxia display enhanced epidermal morphogenesis and lipid barrier formation. *Scientific reports* **9**, 7811 (2019).

50 Cau, L. *et al.* Lowering relative humidity level increases epidermal protein deimination and drives human filaggrin breakdown. *Journal of Dermatological Science* **86**, 106–113, doi:https://doi.org/10.1016/j.jdermsci.2017.02.280 (2017).

51 van der Aar, A. M. G. *et al.* Langerhans Cells Favor Skin Flora Tolerance through Limited Presentation of Bacterial Antigens and Induction of Regulatory T Cells. *Journal of Investigative Dermatology* **133**, 1240–1249, doi:https://doi.org/10.1038/jid.2012.500 (2013).

52 Nakatsuji, T., Cheng, J. Y. & Gallo, R. L. Mechanisms for control of skin immune function by the microbiome. *Current Opinion in Immunology* **72**, 324–330, doi:https://doi.org/10.1016/j.coi.2021.09.001 (2021).

53 Oh, J. *et al.* Biogeography and individuality shape function in the human skin metagenome. *Nature* **514**, 59–64 (2014).