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Application of Topical Sandalore® Increases Epidermal Dermcidin Synthesis in Organ-Cultured Human Skin ex vivo

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Keywords
Skin microbiome · Antimicrobial peptide · Dermcidin · Sandalore® · Atopic dermatitis

Abstract
Introduction: Several olfactory receptors (ORs) are expressed in human skin, where they regulate skin pigmentation, barrier function, wound healing, and hair growth. Previously, we found that the selective activation of OR family 2 subfamily AT member 4 (OR2AT4) by the synthetic, sandalwood-like odorant Sandalore® differentially stimulates the expression of antimicrobial peptides (AMPs) in human scalp hair follicle epithelium ex vivo. As OR2AT4 is also expressed by epidermal keratinocytes, we hypothesized that it may modulate intraepidermal AMP synthesis, thereby contributing to skin microbiome management. ***Methods:*** We investigated this hypothesis in organ-cultured human skin in the presence of Sandalore® and antibiotics and evaluated epidermal production of two AMPs, LL37 (cathelicidin) and dermcidin (DCD), as well as OR2AT4, by quantitative immunohistomorphometry. Moreover, we quantified DCD secretion into the culture medium by ELISA and studied the effect of culture medium on selected bacterial and fungal strains. ***Results:*** Topical application of Sandalore® to organ-cultured human skin increased OR2AT4 protein expression, the number of DCD-positive intraepidermal cells, and DCD secretion into culture media, without significantly affecting epidermal LL37 expression. In line with the significantly increased secretion of DCD into the culture medium, we demonstrated, in a spectrophotometric assay, that application of conditioned media from Sandalore®-treated skin promotes *Staphylococcus epidermidis*, *Malassezia restricta*, and, minimally, *Cutibacterium acnes* and inhibits *Staphylococcus aureus* growth. ***Conclusion:*** In addition to demonstrating for the first time that DCD can be expressed by epidermal keratinocytes, our pilot study suggests that topical treatment of human skin with a cosmetic odorant (Sandalore®) has the potential to alter the composition of the human skin microbiome through the selective upregulation of DCD. If confirmed, Sandalore® could become an attractive adjuvant, nondrug treatment for dermatoses characterized by dysbiosis due to overgrowth of *S. aureus* and *Malassezia*, such as atopic dermatitis and seborrheic dermatitis.

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Introduction

Human skin expresses a multitude of so-called "ectopic" olfactory receptors (ORs) [1]. Although the physiological functions of these chemosensory receptors in human skin and its appendages are yet to be fully elucidated, recent studies have implicated ORs in the regulation of human skin pigmentation, barrier function, wound healing, and hair growth [2–6]. Several naturally occurring odorants are known to activate ORs. For instance, in the human gut, microbial metabolites, such as short- and medium-chain fatty acids, and pheromones have been shown to act as endogenous ligands to these receptors [7, 8]. In human skin, the OR family 2 subfamily AT member 4 (OR2AT4) is currently the best studied OR. Although its endogenous ligands remain to be further characterized, Sandalore®, a synthetic sandalwood mimetic, was shown to bind and activate this receptor, serving as a specific agonist [4, 5]. Stimulation of OR2AT4 with Sandalore® stimulates epidermal keratinocyte proliferation, migration, and reepithelialization in vitro and ex vivo [4, 9]. It also promotes hair growth ex vivo [5] and in female patients with telogen effluvium in vivo [10].

We have recently reported that Sandalore® differentially regulates the transcription of antimicrobial peptides (AMPs), namely, dermcidin (DCD) and cathelicidin (LL37), produced by keratinocytes, in the human hair follicle (HF) epithelium ex vivo [5, 11]. This invited the hypothesis that one of the physiological functions of OR stimulation by as yet unknown endogenous ligands may be to contribute to managing the microbiome of human skin [5].

Microbial dysbiosis is implicated not only in HF disorders [12–16] but also in many skin diseases, such as atopic dermatitis (AD), where it is associated with compromised barrier function [17], psoriasis [18], and seborrheic dermatitis [19]. In most of these skin disorders, AMPs are dysregulated, and downregulation of DCD has specifically been associated with AD [20]. Indeed, AD patients with reduced content of DCD suffer from recurrent *Staphylococcus aureus* infections [21]. Although proteolytically cleaved forms of DCD have been characterized as major AMPs in sweat [22], the uncleaved form is also reportedly detectable in the upper epidermis of healthy individuals [23]. DCD has specific antimicrobial activity against pathogenic species of *Staphylococcus, Escherichia, Pseudomonas, Candida*, and *Listeria* spp. through a pore-formation mechanism in the bacterial or fungal cell membrane [24].

Given that AMPs have multiple potential clinical applications in dermatology, ranging from AD and skin infections to wound healing [25–27], we wondered if the stimulation of OR2AT4 in the epidermis through topical application of Sandalore® ex vivo [28] upregulated intraepidermal DCD and LL37 expression. LL37 is another prominent epidermal AMP that is produced by the epidermis and involved in skin diseases [29, 30]. Subsequently, we investigated the epidermal production and release of DCD. We also investigated the antimicrobial properties of Sandalore®-treated skin culture supernatants by performing an in vitro viability assay.

Materials and Methods

Ethics Approval and Informed Consent

This study was conducted according to the principles of Declaration of Helsinki. Skin specimens were obtained after informed, written patient consent and approval by the Ethics Committee (University of Muenster, 2015-602-f-S, 2019-297-f-S, 2020-954-f-S)

Donor Material and Information

Clinically healthy frontotemporal, retroauricular, and/or occipital human scalp skin specimens were obtained from four female volunteers (age 24–67 years) undergoing routine cosmetic facelift surgery and one female and five male volunteers (age 22–61 years) undergoing routine hair strip transplantation surgery or using samples from the Monasterium Laboratory Biobank (online suppl. Table S1; for all online suppl. material, see www.karger.com/doi/10.1159/000528402).

Skin Organ Culture

6 mm skin biopsies containing terminal HFs were obtained and topically treated with 2 μL of viscous formulation of PEG6000 [28] containing 500 μM Sandalore® (Givaudan; optimal concentration for OR2AT4 stimulation [5]) or 0.10% DMSO (Sigma-Aldrich) as vehicle. Supplementation of culture medium with 1% penicillin/streptomycin (Thermo Fisher) was necessary to avoid bacterial overgrowth in skin cultures. In parallel, we found that the addition of antibiotics was sufficient to prevent fungal contamination (data not shown).

Immunohistochemistry and in situ Hybridization

Cryosections were fixed and immunostained for DCD (Atlas Antibodies, HPA063967), LL37 (Abcam ab69484), and OR2AT4 (custom-made antibody) or processed for in situ hybridization (RNAscope 2.5 HD Reagent Kit-Red; Advanced Cell Diagnostics) with DCD (NM_001300854.1, Target 5–678)

Quantitative (Immuno-)Histomorphometry

The number of DCD-secreting cells, as well as LL37 production and OR2AT4 expression, was evaluated using Fiji [31] (see also online supplementary material).

ELISA

ELISA of pooled culture medium (in duplicates) was performed using the Biorbyt Human DCD ELISA kit according to the manufacturer's instructions.

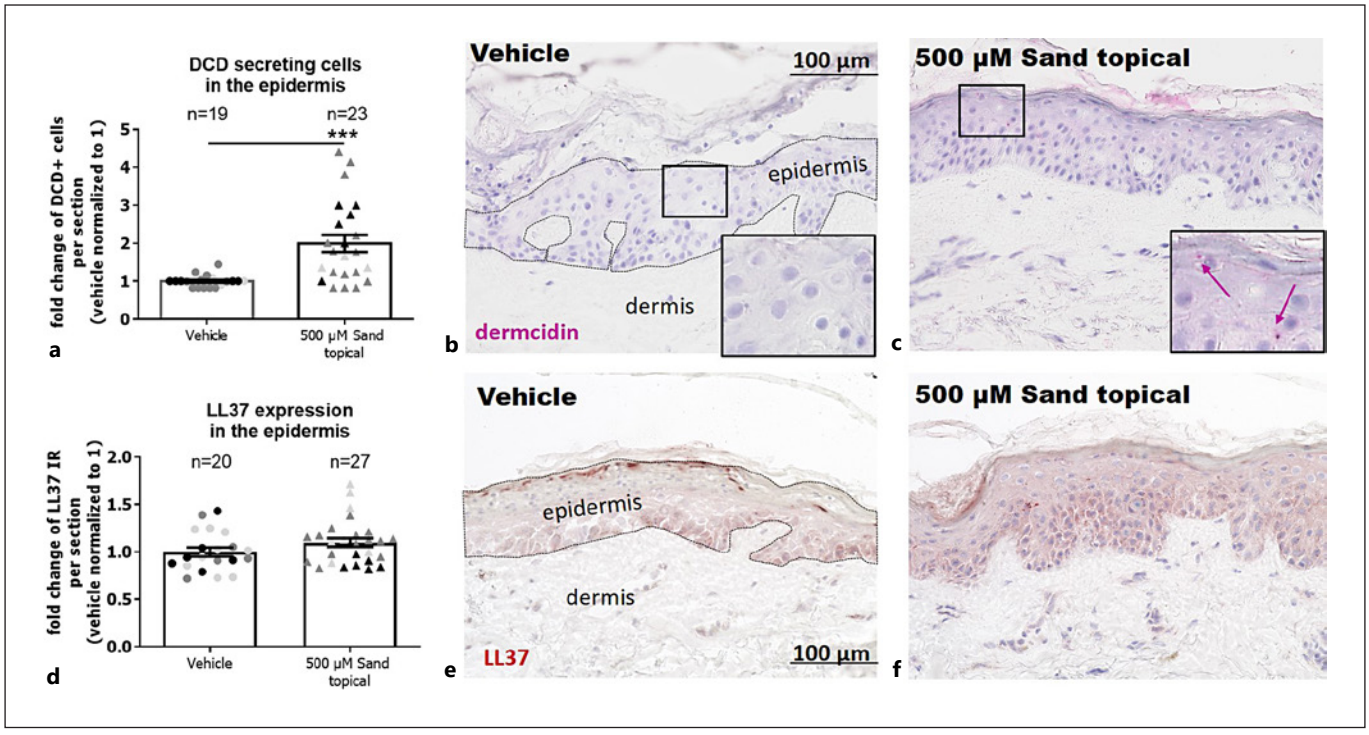

Fig. 1. Topical Sandalore® increases epidermal dermcidin (DCD) expression in human skin ex vivo. Organ-cultured skin was treated topically with 500 µM Sandalore® (optimal concentration for OR2AT4 stimulation) or vehicle in PEG6000, and protein expression of DCD and LL37 was measured after 4 days by immunohistomorphometry. DCD^+ cells and LL37 protein expression were quantified in all epidermal layers apart from the *stratum corneum*. Representative images indicate the area of analysis. Data points from 3 donors were collected, indicated by different shades of gray on bar charts. **a–c** The number of DCD^+ cells in the epidermis was quantified after topical Sandalore® treatment, data pooled from 19–23 nonconsecutive skin sections from 1–2 punches of 3 donors (online suppl. Table S1), analyzed in 3 independent skin organ culture assays. Mean ± SEM of technical replicates, Mann-Whitney U test, $***p < 0.001$. Pink arrows point to DCD^+ puncta of protein expression. **d–f** LL37 expression in the epidermis was measured after Sandalore® treatment, data pooled from 20–27 sections from 1–2 punches of 3 donors, analyzed in 3 independent skin organ culture assays. Mean ± SEM of technical replicas, Mann-Whitney U test, not significant.

In vitro Quantification of Antimicrobial Activity

Different bacterial and fungal strains were treated with conditioned medium from skin punches topically treated with vehicle, 500 µM Sandalore® (2 days), or control medium. Plates for different microbial strains were incubated for 24 h at 37°C. The antimicrobial activity was determined by measuring the absorbance (620 nm) on a BioTek Micro-volume Plate Reader. Data are expressed as the mean percentage of viability compared to the control cultures.

Statistical Analyses

Statistical analyses were performed using GraphPad Prism 6 (GraphPad Software Inc.). Data were tested for normality distribution using the D'Agostino and Pearson omnibus normality test before subsequent analysis using the appropriate parametric and nonparametric tests. The treatment and vehicle groups were compared using a Mann-Whitney U test whenever the data did not follow a Gaussian distribution or an unpaired Student's *t* test whenever it did. Data are expressed as mean, mean ± SEM, or fold change of mean ± SEM. *p* values <0.05 were regarded as significant. For further details, see supplementary material.

Results

Human Epidermis Expresses DCD mRNA, but DCD Protein Is Almost Undetectable in Healthy Human Epidermis in vivo

DCD mRNA and protein expression in human skin has been described in eccrine and sebaceous glands [24, 32–34]. Therefore, we investigated the steady-state mRNA and protein expression of DCD in the epidermis of 3 healthy donors by in situ hybridization and (immuno-)histochemistry. As an internal positive control, we assessed the expression of *DCD* mRNA and protein in human mucous cells of the secretory coil and duct cells of eccrine sweat glands, a known site of DCD expression, and compared it to that of unstimulated skin cells (fibroblasts and keratinocytes). As expected from the previous literature [22], *DCD* transcripts

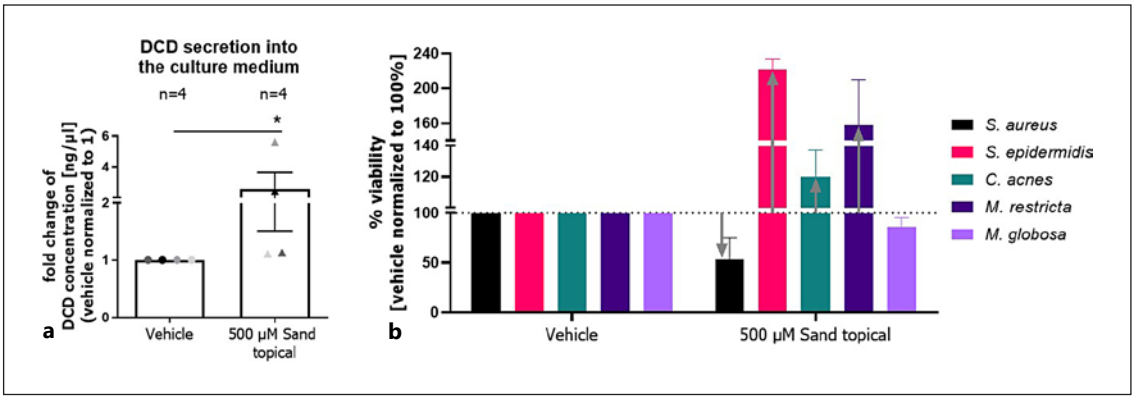

Fig. 2. Conditioned medium from human skin treated with topical Sandalore® ex vivo exerts differential antimicrobial activity. **a** DCD protein concentration was measured in conditioned medium by ELISA at day 2 after skin treatment with topical Sandalore® compared to control medium. Data pooled from four healthy donors (online suppl. Table S1) (2 individually cultured skin punches per donor), presented as mean ± SEM of biological replicates, Mann-Whitney U test, $*p < 0.05$. **b** Antimicrobial activity of the culture medium from Sandalore®-treated skin (donor $n = 3$) was measured by culturing *S. aureus, S. epidermidis, C. acnes, M. restricta*, and *M. globosa* and applying conditioned or control medium (taken at day 3 of Sandalore® treatment) to the colonies. Percentage of viability was measured relative to vehicle controls. Mean ± SEM, Mann-Whitney U test, not significant.

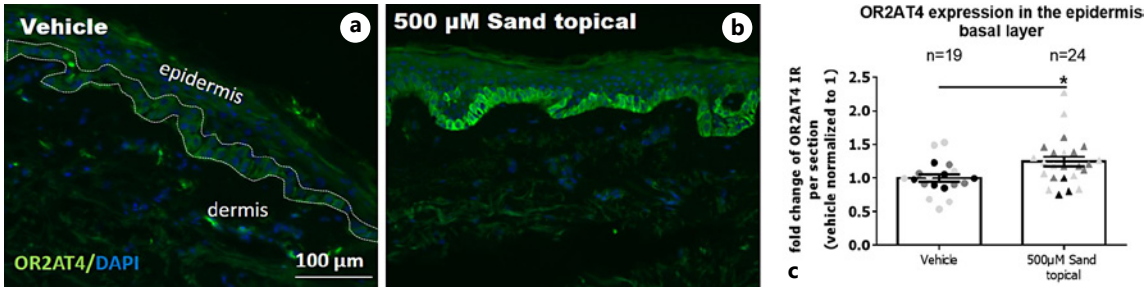

Fig. 3. Topical Sandalore® increases intraepidermal expression of olfactory receptor family 2 subfamily AT member 4 (OR2AT4). **a–c** Organ-cultured skin was treated with topical application of 500 µM Sandalore®, and expression of OR2AT4 protein was measured after 4 days by immunofluorescence microscopy and quantified in the basal epidermal layer. Representative images indicate the area of analysis, data pooled from 19–24 nonconsecutive sections from 1–2 punches of 3 donors (online suppl. Table S1), analyzed in 3 independent skin organ culture assays. Mean ± SEM of technical replicas, unpaired Student's *t* test, $*p < 0.05$.

were more prominently expressed in eccrine sweat glands' secretory coils and, more discreetly, in the epidermis (shown in online suppl. Fig. S1a, b). DCD protein was observed as puncta in the eccrine sweat glands coils (pink arrows) but not in the epidermis of healthy individuals (shown in online suppl. Fig. S1c, d). This pattern of expression is consistent with DCD being found in secretory granules and reminiscent of what has been previously described by others [22, 24, 32–34]. Importantly, our results show that normal human keratinocytes could produce DCD upon adequate stimulation.

Topical Sandalore® Treatment Selectively Increases Epidermal Synthesis of DCD Protein

We have previously shown that Sandalore® specifically binds to and activates OR2AT4 [4, 5]. Stimulating HFs ex vivo with Sandalore® significantly upregulates *DCD* mRNA and protein expression in the outer root sheath [5]. We confirmed that human epidermal keratinocytes can be a profound source of DCD by analyzing *DCD* gene expression in cultured primary human epidermal keratinocytes after Sandalore® stimulation (shown in online suppl. Fig. S2). Furthermore, we asked whether DCD protein expression was also upregulated in the epidermis of

organ-cultured skin samples treated topically with 500 μM Sandalore® for 4 days. Sandalore® treatment increased the number of DCD$^+$ cells in the epidermis (counted in all viable layers) (shown in Fig. 1a–c) of skin samples deriving from all donors (shown in online suppl. Fig. S3a), confirming that epidermal keratinocytes produce DCD in response to Sandalore® stimulation also within their own physiological environment.

LL37, the protein encoded by *CAMP*, is another key AMP that is widely expressed in the human epidermis [29, 30]. A microarray of human scalp HFs has previously shown that *CAMP* is markedly upregulated by ex vivo treatment with Sandalore® [5]. Therefore, we sought to determine if LL37 protein was also modulated in the epidermis upon stimulation of OR2AT4. Interestingly, quantitative immunohistomorphometry of Sandalore®-treated skin showed similar levels of LL37 protein expression in the epidermis of treated and control samples in the majority of the donors analyzed (shown in Fig. 1d–f; online suppl. Fig. S3b). This suggests that Sandalore® differentially modulates the expression of defined AMPs in human skin, and unlike in human HF keratinocytes [5], it upregulates DCD but not LL37 in the epidermis.

Topical Sandalore® Treatment Also Seems to Increase DCD Secretion into the Culture Medium, Affecting Microbial Balancing

As topical Sandalore® treatment modulated the expression of DCD, we wondered whether this would result in increased DCD secretion. Indeed, the mean concentration of DCD in the culture medium was significantly increased upon Sandalore® treatment (shown in Fig. 2a). We then assessed the antimicrobial activity of the conditioned media from Sandalore®- or vehicle-treated punches against *S. epidermidis*, *S. aureus*, *C. acnes*, *M. restricta*, and *M. globosa*, which are implicated in human skin barrier function [35].

This viability assay revealed that treatment with conditioned medium, collected on the third day after topical Sandalore® application, inhibited *S. aureus*, while substantially promoting *S. epidermidis* and *M. restricta* growth (shown in Fig. 2b). Growth of *C. acnes* (reclassified from *Propionibacterium* [36]), a major constituent of the healthy scalp microbiome [37–39], was also minimally increased from baseline levels (shown in Fig. 2b). This selective modulation of certain bacterial species could be advantageous in the treatment of AD and seborrheic dermatitis, diseases characterized by dysbiosis [40, 41].

Topical Sandalore® Treatment Significantly Increases Epidermal Expression of OR2AT4

Given that stimulation with Sandalore® significantly upregulates OR2AT4 in HFs ex vivo [5], we tested if this also occurred in the epidermis of organ-cultured skin samples treated topically with Sandalore®. In line with the literature [4], OR2AT4 expression was detected throughout the epidermis and was more prominent in the *stratum basale* (shown in Fig. 3a, b). Quantitative (immuno-)fluorescence indicated a significant upregulation of epidermal OR2AT4 expression in skin samples from 2 out of 3 donors after topical application of Sandalore® (shown in Fig. 3c; online suppl. Fig. S3c), suggestive of positive feedback regulation of OR2AT4 not only in human scalp HFs but also in human epidermis.

Conclusion

The largely unknown biological functions of ORs in human skin represent a fascinating and therapeutically relevant frontier in dermatological research. Here we provide the first evidence that topical application of the OR2AT4-specific odorant Sandalore® enhances epidermal production and secretion of the AMP DCD by keratinocytes. Moreover, we present evidence that medium conditioned by organ-cultured human skin contains a higher concentration of DCD after topical Sandalore® treatment; accordingly, it modulates microbial growth (even in the presence of antibiotics) and could thus be an attractive, drug-free adjuvant therapy for skin disorders characterized by dysbiosis.

Eccrine sweat glands are believed to be responsible for DCD production and secretion in vivo [32]. Since eccrine glands are denervated in organ culture, it is reasonable to assume that their secretory capacity is reduced or abrogated. Thus, the higher DCD concentration detected after Sandalore® stimulation is most likely derived from passive diffusion of epidermally synthesized DCD. Therefore, even if eccrine glands do express OR2AT4, we showed here that the epidermis can be a profound source of DCD production and secretion after topical Sandalore® application. Epidermally sequestered DCD may also be functionally important for the maintenance of barrier integrity in the event of microbial challenge/invasion, for instance, during the pathogenesis of AD [42, 43].

Contrary to what was seen in microdissected HFs [5], we have shown that Sandalore® treatment significantly regulated intraepidermal DCD (in vitro [online suppl.

Fig. S2] and ex vivo [Fig. 1a]), but not LL37 (encoded by the gene *CAMP*), production. Therefore, the skin is a profound source of DCD, and for clinical and cosmetic applications, an isolated increase in DCD expression may be beneficial, as abnormal LL37 expression and activity are hallmarks of lesional skin in psoriasis [44, 45].

Given that these effects went hand-in-hand with an upregulation of OR2AT4 expression in the epidermis, which confirmed the activation of this receptor by its specific agonist [4, 5], it is conceivable that the increased DCD production in the epidermis observed herein was mediated by OR2AT4. An upregulation of this OR by repeated topical Sandalore® administration may render human epidermis even more sensitive to OR2AT4 stimulation, thus further increasing epidermal DCD production over time. Our results support not only the notion that epidermal OR2AT4 plays a key role in the human epidermis ex vivo [4] but also that it can undergo positive feedback regulation [5].

Lastly, it is widely accepted that a cross talk between ORs and the resident microbiome exists in olfactory and nonolfactory organs in vertebrates [46, 47], including pigs [48] and humans [8, 49]. Our observation that OR2AT4 stimulation ex vivo upregulated epidermal DCD protein expression and secretion in human skin suggests that OR stimulation and subsequent AMP production is one important physiological mechanism for managing the composition of the skin microbiome. Although a direct effect of Sandalore® on the microbiota cannot be excluded, it is unlikely due to the topical application method used, which prevented the direct contact of the treatment with the culture medium. Therefore, we provide preliminary evidence that DCD-enriched culture medium of human skin topically treated with Sandalore® promotes the growth of *S. epidermidis* while disfavoring *S. aureus* growth. A higher *S. epidermidis*:*S. aureus* ratio, along with preserved *C. acnes* levels, is described as beneficial for the skin microbiome [50–53]. A minimal effect on *C. acnes* growth after Sandalore® treatment was also noted, which could be a result of microbial community self-regulation, by competition. Indeed, *S. epidermidis*, *C. acnes*, and *S. aureus* are known for competitive inhibition [50, 54, 55]. In healthy skin, *S. epidermidis* controls *C. acnes* and *S. aureus* proliferation, while *C. acnes* competitively inhibits *S. aureus* growth [50, 54, 55]. An effect that could further potentiate the observed effects of Sandalore® on *S. aureus* growth. Therefore, OR2AT4 stimulation may favor the restoration of a physiological skin microbiome composition [35]. Although, to date, studies on the expression of OR2AT4 in AD have not been conducted, its activation and consequent microbiome regulation would be particularly beneficial and desirable in this disease.

Growth of *M. globosa* remained stable when compared to that in the control medium, while that of *M. restricta* was increased. This may represent a secondary effect of DCD, given that this AMP is not known to have any antimicrobial activity against these species [56]. Interestingly, this effect may be explored in the context of seborrheic dermatitis, which is characterized by *Malassezia* over-colonization [19, 41].

Given that microbiome-targeting, probiotic, and postbiotic treatments are gaining momentum in dermatology [40, 57, 58], our results hint that OR2AT4 activation by topical Sandalore® is of major therapeutic interest as an adjuvant, drug-free therapy for many dermatoses characterized by dysbiosis and/or dysregulation of DCD production, such as AD and seborrheic dermatitis [19–21, 41, 59].

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Statement of Ethics

This study was conducted according to the Declaration of Helsinki principles. This study protocol was reviewed and approved by the Ethics Committee of the University of Muenster/Medical Association of Westfalen-Lippe with the approval numbers 2015-602-f-S, 2019-297-f-S, and 2020-954-f-S. Skin specimens were obtained after informed, written patient consent.

Conflict of Interest Statement

Ralf Paus is the CEO, and Janin Edelkamp, Antonio Biundo, Marta B. Lousada, and Marta Bertolini are employees of Monasterium Laboratory GmbH, a contract research organization specializing in dermatology. Daniela Pinto is an employee of Giuliani S.p.A, which has filed a patent on the technology reported here (Giuliani Giammaria, Jérémy Chéret, Baroni Sergio, Paus Ralf, and Marzani Barbara). Composti per prevenire e trattare affezioni della pelle o della mucosa con componente infiammatoria (102018000005585., June 2020). Ralf Paus also consults for Giuliani S.p.A.

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This study was funded by Giuliani S.p.A, Milan, Italy. The funding body has contributed to the analysis of the data and preparation of the manuscript.

Author Contributions

The project was designed and supervised by Ralf Paus, and Marta Bertolini, Janin Edelkamp, Marta B. Lousada, Daniela Pinto, Antonio Biundo, and Jérémy Chéret performed the experiments. Francisco Jimenez, Wolfgang Funk, Christian Roessing, and Volker Rippmann provided the human skin samples. Janin Edelkamp, Marta B. Lousada, James D. B. O'Sullivan, Ralf Paus, and Marta Bertolini wrote the manuscript, which was edited and accepted by all co-authors.

Data Availability Statement

All data generated or analyzed during this study are included in this article and its online supplementary material files. Further inquiries can be directed to the corresponding author.

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