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Original Article

Management of the human hair follicle microbiome by a synthetic odorant

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ABSTRACT

Background: Human scalp hair follicles (HFs) engage in olfactory receptor (OR)-dependent chemosensation. Activation of olfactory receptor family 2 subfamily AT member 4 (OR2AT4) by the synthetic, sandalwood-like odorant Sandalore® up-regulated HF antimicrobial peptide expression of dermcidin (DCD), which had previously been thought to be produced exclusively by sweat and sebaceous glands.
Objectives: To understand if intrafollicular DCD production can be stimulated by a commonly used cosmetic odorant, thus altering human HF microbiome composition in a clinically beneficial manner.
Methods: DCD expression was compared between fresh-frozen scalp biopsies and microdissected, full-length scalp HFs, organ-cultured in the presence/absence of the OR2AT4 agonist, Sandalore® and/or antibiotics and/or the competitive OR2AT4 antagonist, Phenirat®. Amplicon-based sequencing and microbial growth assays were performed to assess how this treatment affected the HF microbiome.
Results: Synthetic odorant treatment upregulated epithelial DCD expression and exerted antimicrobial activity in human HFs *ex vivo*. Combined antibiotic and odorant treatment, during an *ex vivo* dysbiosis event, prevented HF tissue damage and favoured a more physiological microbiome composition. Sandalore®-conditioned medium, containing higher DCD content, favoured *Staphylococcus epidermidis* and *Malassezia restricta* over *S. aureus* and *M. globosa*, while exhibiting antimicrobial activity against *Cutibacterium acnes*. These effects were reversed by co-administration of Phenirat®.
Conclusions: We provide the first proof-of-principle that a cosmetic odorant impacts the human HF microbiome by up-regulating antimicrobial peptide production in an olfactory receptor-dependent manner. Specifically, a synthetic sandalwood-like odorant stimulates intrafollicular DCD production, likely via OR2AT4, and thereby controls microbial overgrowth. Thus, deserving further exploration as an adjuvant therapeutic principle in the management of folliculitis and dysbiosis-associated hair diseases.

1. Introduction

Olfactory receptors (ORs) represent the evolutionarily oldest and largest receptor family, exerting functions beyond olfaction, including epithelial cell proliferation, wound healing, and glucose and lipid metabolism [1–5]. Yet, these functions await systemic

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therapeutic targeting, while odorants as potential topically applicable, inexpensive non-drugs remain insufficiently explored.

In human skin, one of the best-characterised OR is the OR family 2 subfamily AT member 4 (OR2AT4) [1,6]. Selective OR2AT4 activation by the synthetic sandalwood-like odorant, Sandalore® [3-Methyl-5-(2,2,3-trimethylcyclopent-3-en-1-yl)pentan-2-ol], stimulates human hair growth [1,7]. Additionally, it increases intrafollicular antimicrobial peptide (AMP) transcription, particularly dermcidin (*DCD*) and cathelicidin LL-37 (*CAMP*), in microdissected hair follicles (HFs) [1]. This suggests that OR2AT4 manages the human HF microbiota by regulating AMP production/secretion [1]. While the significance of microbiota imbalances (dysbiosis) in skin disorders remains unclear, notable dysbiosis associations have emerged in folliculitis, hidradenitis suppurativa, acne vulgaris, androgenetic alopecia and alopecia areata [8]

Therefore, this proof-of-concept study, explores whether a lead odorant, and specific OR2AT4 agonist, Sandalore®, can manipulate human scalp HF microbiota by up-regulating endogenous AMP production/secretion in a clinically relevant manner [8]. To test this hypothesis, we evaluated whether a sandalwood-like synthetic odorant can stimulate intrafollicular DCD production, how this alters the human HF microbiome, and whether this is OR2AT4-dependent.

2. Materials and methods

2.1. Ethics approval and informed consent

This study was conducted according to the Declaration of Helsinki principles. Specimens were obtained after informed, written patient consent and ethics committee approval (University of Muenster 2015–602-f-S).

2.2. Sample collection and ex vivo HF organ culture

Occipital/temporal scalp skin specimens were obtained from clinically healthy participants: females undergoing routine cosmetic facelift surgery or males undergoing hair strip transplantation, and males and females undergoing follicular unit extraction transplantation (Table S1).

HFs were microdissected, organ-cultured as described elsewhere [9] and treated with Sandalore®, Phenirat® and/or antibiotics (details in the Supplementary methods) [9,10].

2.3. Quantitative (immuno-)staining

Cryosections (6 µm) were fixed and stained with Periodic Acid-Schiff (PAS)/Light Green Stain [11] for fungal and glycogen storage evaluation and Gram-stain [12] for bacterial visualisation. DCD (Atlas Antibodies, HPA063967), LL-37 (Abcam, ab69484), and OR2AT4 (custom-made antibody, Eurogentec, Seraing, Belgium) immunohistomorphometry were also performed (details in the Supplementary methods).

2.4. In situ hybridization

In situ hybridization was performed using the RNAscope 2.5 HD Reagent Kit-Red (Advanced Cell Diagnostics, Abingdon, UK) following the manufacturer's instructions (for details see Supplementary methods).

2.5. Quantitative real-time PCR

Total RNA was extracted from three full-length HFs (Table S1), using the Arcturus® PicoPure® RNA Extraction Kit (Thermo Fisher, Germany) (details in the Supplementary methods).

2.6. ELISA

Culture medium supernatant ELISA was performed using the Biorbyt Human DCD ELISA kit (orb776772), according to manufacturer's instructions.

2.7. High-throughput 16 S and ITS amplicon generation, sequencing and analysis.

DNA was extracted from three full-length HFs (Table S1) using the QIAamp DNA Micro Kit (Qiagen, Germany), according to the manufacturer's protocol. Extracted DNA was amplified using high-fidelity polymerase (AccuStart II PCR ToughMix, Quantabio, Beverly, MA) and universal bacterial and fungal primers (Table S2). Library preparation and Illumina MiSeq 16 S and ITS with 2 × 300 bp sequencing were carried out at Personal Genomics Srl, Verona, Italy, as previously demonstrated with minor modifications (Supplementary methods) [13]. Bioinformatic analyses were performed with QIIME2, using SILVA and UNITE databases (Supplementary methods). Raw sequences can be found on the SRA database: PRJNA857855/PRJNA857859.

2.8. Antimicrobial activity quantification

S. aureus ATCC-25923, *S. epidermidis* ATCC-14990, *C. acnes* ATCC-11827, *M. restricta* ATCC-MYA 4611 and *M. globosa* ATCC-MYA 4612 cultures were exposed to Sandalore®/vehicle-treated HFs in the presence/absence of antibiotics-conditioned supernatants. Antimicrobial activity and growth inhibition were extrapolated from OD_{600} values or by inhibition areola size (for details see Supplementary methods) [14].

2.9. Total germ count (bacterial colony forming units)

Total germ count from culture supernatants was performed in tryptic soy agar. Samples were plated out undiluted and in decimal dilutions and incubated at 30 °C. Colony forming units were counted after 3 days (details in the Supplementary methods).

2.10. Statistical analysis

A full description can be found in the Supplementary methods.

3. Results

3.1. Intraepithelial OR2AT4 expression is maintained in the dysbiotic scalp hair follicle model

We previously showed OR2AT4 expression in human scalp HFs and *DCD* and *CAMP* mRNA upregulation following stimulation with the OR2AT4 agonist, Sandalore®, suggestive of OR2AT4-mediated HF microbiome modulation [1]. However, for therapeutic application, OR2AT4 expression should be maintained under dysbiosis, induced through antibiotic omission [15].

Indeed, following synthetic odorant stimulation in *ex vivo* dysbiotic conditions, OR2AT4 expression was overall maintained, being only slightly reduced in the hair matrix (Fig. 1a-b), shown by quantitative (immuno-)histomorphometry. As OR2AT4 sensitivity was previously shown to remain stable or increase in response to the chosen odorant concentration in amputated HFs [1], this suggests that human scalp HF receptor sensitivity is not altered by dysbiosis *ex vivo*.

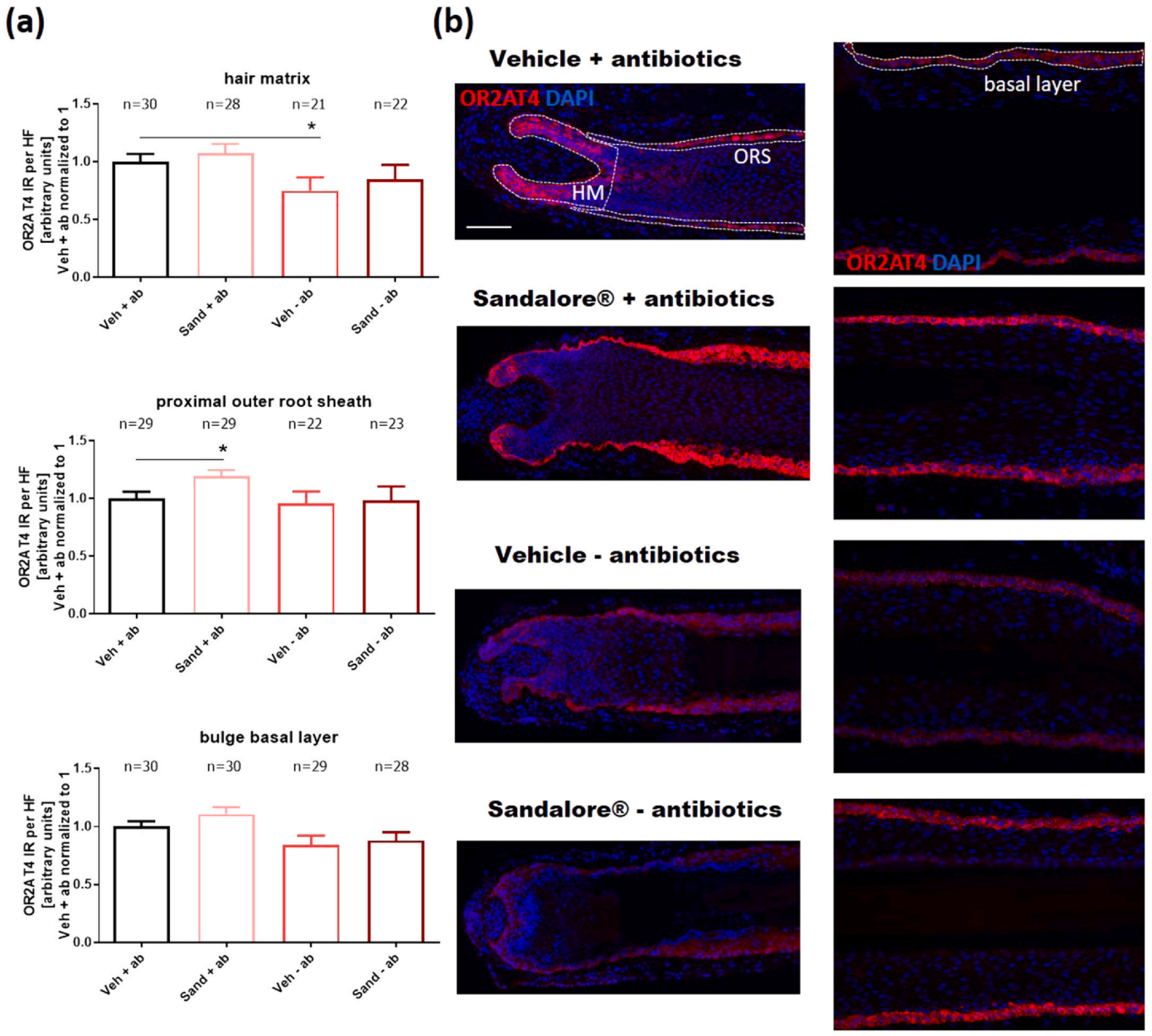

Fig. 1. OR2AT4 signalling axis is maintained under dysbiotic conditions. (a-b) HFs were cultured in serum-free conditions and treated with a vehicle control or 500 µM Sandalore®, with or without antibiotics (Veh+ab, Sand+ab, Veh-ab and Sand-ab, respectively) for 2–4 days before histological analysis. The expression of OR2AT4 was determined in the hair matrix (HM), the proximal outer root sheath (ORS), and bulge basal layer of cultured HFs. (a) Pooled data corresponding to 21–30 HFs per group, from a total of 5 independent donors and (b) respective representative pictures. The data are represented as mean ± SEM. D'Agostino & Pearson omnibus normality test, Gaussian distribution, Kruskal-Wallis test $p = 0.0673$ (HM), $p = 0.1573$ (ORS), $p = 0.0198$ (bulge), Dunn's multiple comparisons test, Veh+ab fixed with multiplicity and adjusted p-value (95% confidence), Veh+ab fixed, n.s., Mann-Whitney test, $^{*}p < 0.05$, scale bar 100 µm.

3.2. Steady-state human HFs express DCD mRNA but not protein

Given that we recently showed, qualitatively, that Sandalore® up-regulated *ex vivo DCD* mRNA and protein expression in HFs [1], while previously DCD was thought to only be produced by sweat and sebaceous gland epithelium in human skin [16,17], we investigated for the first time *DCD* mRNA and quantitative protein expression levels, *in vivo*, in unmanipulated human HFs. Conforming previous reports [17,18], *in situ* hybridization and indirect immunofluorescent microscopy showed that *DCD* mRNA and protein expression concentrated in the eccrine and sebaceous glands, with strong mRNA expression in the mucous cells of the eccrine gland secretory coil and duct (Figs. S2a-b). Besides, focal *DCD* signals were detected in the ORS epithelium, while protein expression was limited to the sebaceous gland duct, eccrine glands and not observed in the HF epithelium (Figs. S2a-b). Thus, *in vivo*, the healthy human scalp HF epithelium transcribes *DCD* at low level but does not translate it into protein, which may require additional stimuli, such as HF organ culture trauma [9,19].

3.3. OR2AT4 agonist application stimulates ex vivo DCD mRNA and protein expression in human HF epithelium

Previous amputated organ-cultured scalp HF RNA microarray analysis revealed that sandalwood-like odorant treatment upregulated *DCD* transcription (> 20-fold) in 3/4 donors (Fig. 2a) [1]. Similarly, *CAMP* expression was increased (≥10-fold) in all donors (ST1) [1]. To investigate *DCD* and *CAMP* expression changes, full-length

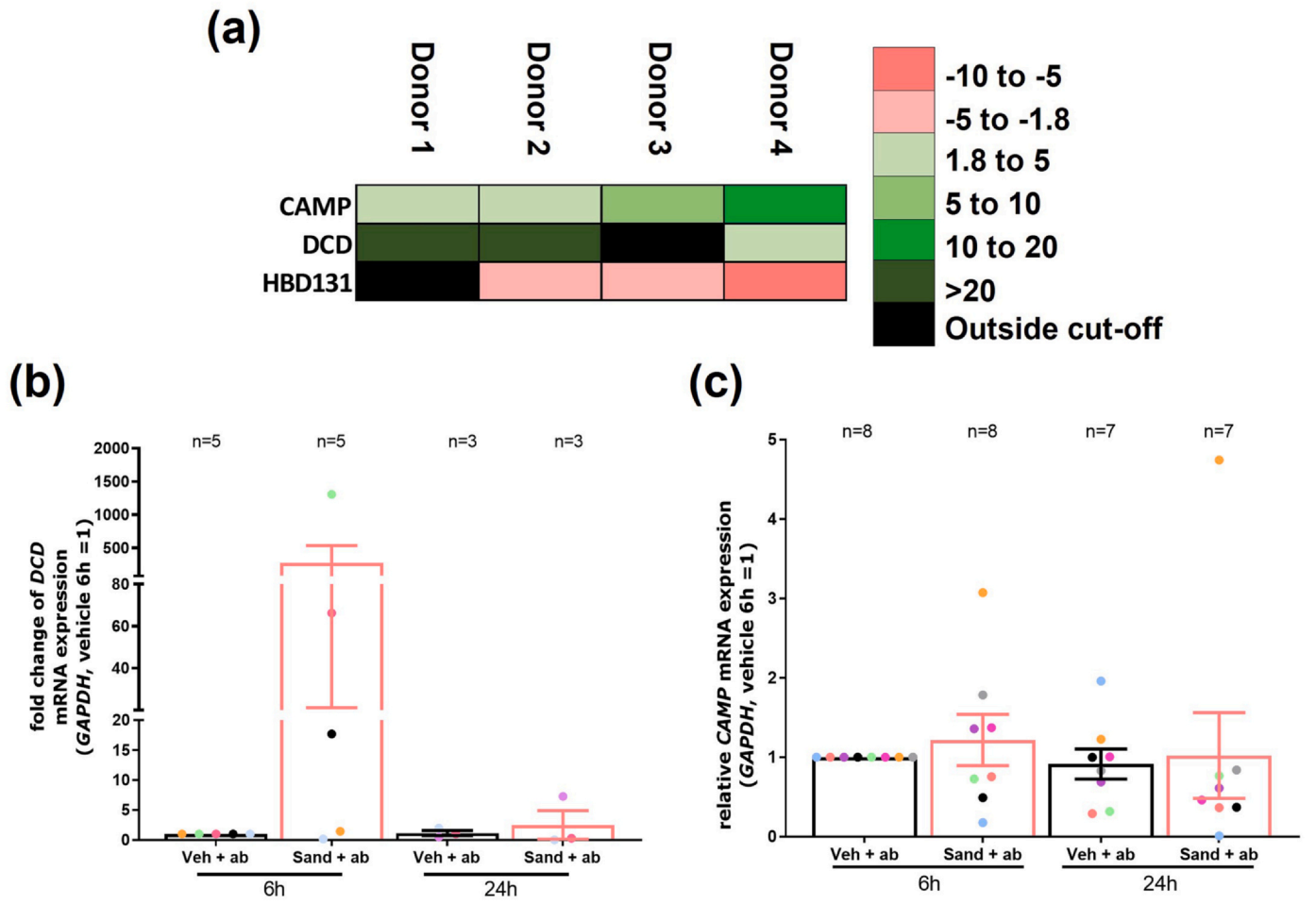

Fig. 2. Synthetic sandalwood-like odorant treatment differentially regulates the gene expression of different AMPs in the HF epithelium. (a) Odorant treatment differentially regulates different AMPs in n = 4 different healthy donors, as assessed by microarray after 6 h of treatment. Data refers to what was previously published in Chéret at al. 2018 and made publicly accessible in NCBI's Gene Expression Omnibus (GEO) (accession number GSE102887), which was re-analysed with focus on the selected AMPs. (b-c) Treatment with the synthetic OR2AT4 agonist differentially regulates *DCD* and *CAMP* mRNA expression 6 or 24 h after treatment, n = 3–8 healthy donors (note that given the inter-individual differences 2 donors were excluded as per the ROUT 1 % outliers test), mean ± SEM GraphPad Prism 6; D'Agostino & Pearson omnibus normality test, no Gaussian distribution, Kruskal-Wallis test, $p = 0.3259$ (b) and $p = 0.1982$ (c), Dunn's multiple comparisons test, with multiplicity and adjusted p-value (95 % confidence), vehicle 6 h fixed, n.s., Mann-Whitney test, n.s. Different colours represent different donors.

HFs were exposed to Sandalore® for 6/24 h and qRT-PCR evaluation was performed. This confirmed *DCD* transcription up-regulation only after 6 h (possibly since thereafter secondary/tertiary gene expression changes and progressive mRNA degradation might confound transcriptomic results [1,9]) compared to vehicle-treated HFs in 3/5 donors (Fig. 2b), and no changes in *CAMP* transcription (Fig. 2c).

Next, we asked how OR2AT4 stimulation affected DCD protein expression. In line with our previous qualitative pilot data [1], synthetic odorant treatment significantly increased epithelial DCD expression, irrespective of antibiotic use (Fig. 3a-c and S2c-d). Furthermore, DCD concentration was significantly increased in the culture media of odorant-treated HFs compared to Veh+ab (2-fold), an effect potentiated by antibiotic omission (Fig. 3a-b). Therefore, while this synthetic odorant upregulated epithelial DCD expression in the presence of antibiotics, without antibiotics Sandalore® promoted DCD secretion from the HF epithelium (Fig. 3a-b), suggesting higher DCD protein production and secretion under dysbiotic conditions.

In contrast, in line with *CAMP* mRNA expression data (qRT-PCR) (Fig. 2c), LL-37 expression in defined epithelial compartments remained unchanged following odorant treatment with antibiotics (Figs. S3a-c). Thus, treatment with the sandalwood-like synthetic OR2AT4 agonist selectively stimulates intrafollicular DCD production, irrespective of antibiotic use. This also suggests that the Sandalore®-induced antimicrobial activity may primarily target DCD-susceptible microorganisms.

3.4. A sandalwood-like synthetic odorant reduces bacterial overgrowth and associated HF dystrophy

Subsequently, we investigated whether odorant-induced DCD production exerted antimicrobial activity. Considering the differential antimicrobial spectrum of the targeted AMPs, we generated a Venn diagram (Fig. S4) demonstrating possible microbial targets (ST2). This suggested that this odorant exerts activity against two HF commensals often dysregulated in dermatological diseases (*S. aureus* and *C. acnes*) [8]. This encouraged us to explore if this OR2AT4 agonist can be used as a non-drug antibiotic adjuvant.

To test this, we modelled HF dysbiosis *ex vivo*, mimicking several dysbiosis-associated HF diseases (including hidradenitis suppurativa, dandruff and acne vulgaris [8]), by antibiotic omission [15]. As expected, bacterial overgrowth was seen within 72 h (Fig. 4), and correlated with morphological and pigmentary signs of HF dystrophy (Fig. 4a-b). These included swelling of the bulb, epithelial and mesenchymal tissue separation, ORS spongiosis, melanin clumping (a sensitive HF damage/dystrophy indicator, due to the high damage sensitivity of the HF pigmentary unit [20,21]) and reduction of glycogen content in the ORS (a critical HF energy source), therefore

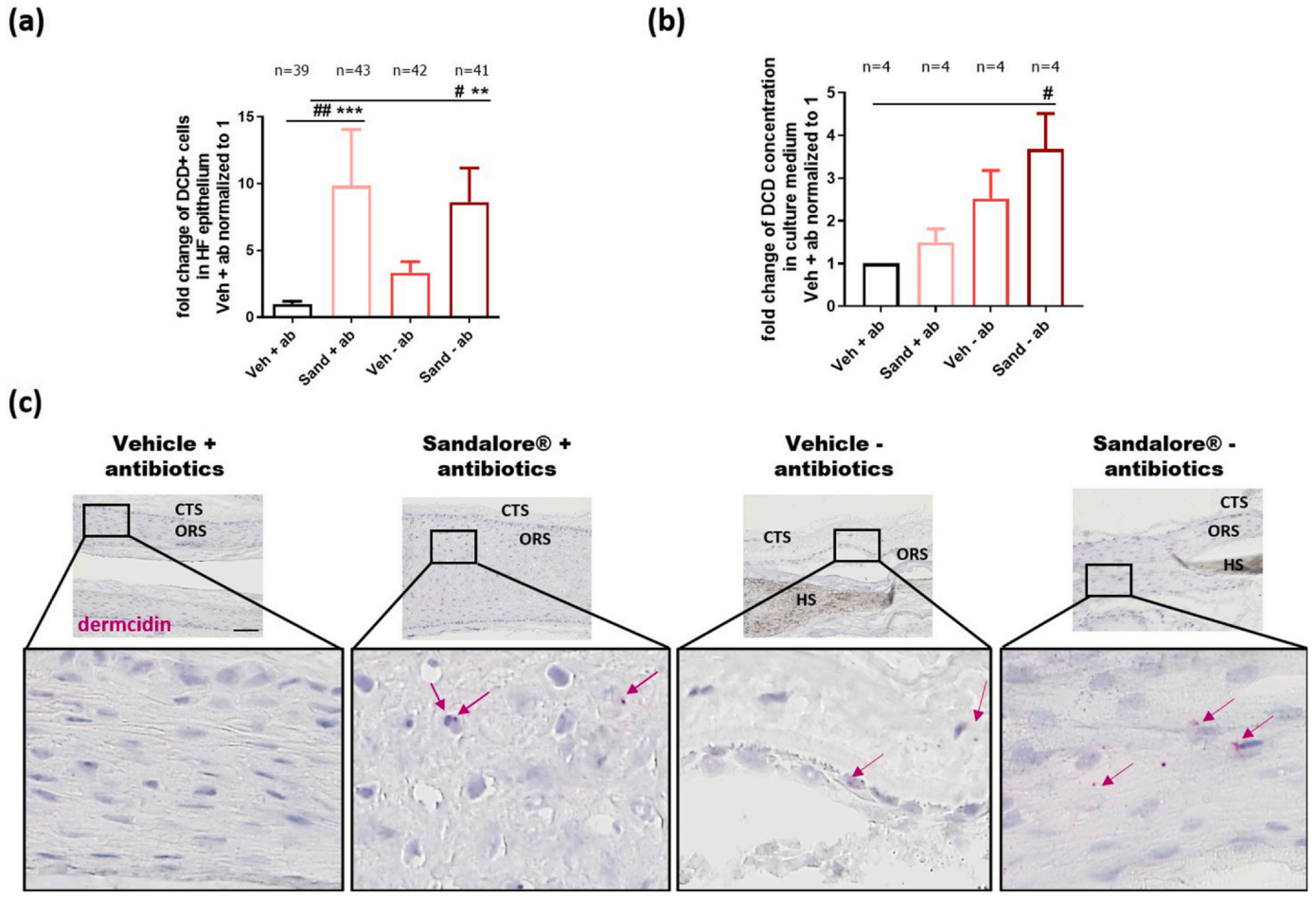

Fig. 3. Sandalwood-like synthetic odorant treatment up-regulates the expression of DCD protein. (a-b) HFs were cultured in serum-free conditions and treated with a vehicle control or 500 µM Sandalore®, with or without antibiotics (Veh+ab, Sand+ab, Veh-ab and Sand-ab, respectively) for 2–4 days, before being processed for histological analysis. The protein level of DCD was measured histologically in the ORS of HFs after 6 days in culture (a) and by ELISA in the supernatants of Sandalore®-treated HFs (b). The results are the pooled data **(a)** of 39–41 HFs from 7 independent donors and (b) pooled medium from 4 selected independent donors and (c) respective representative pictures. (a) no Gaussian distribution; Kruskal-Wallis test, $p = 0.0014$, Dunn's multiple comparisons test, with multiplicity and adjusted p-value (95% confidence), Veh+ab fixed, ##$p < 0.01$, Mann-Whitney test $^{**}p < 0.01$, or $^{***}p < 0.001$, (b) Kruskal-Wallis test $p = 0.0039$, Dunn's multiple comparisons test, Veh+ab fixed, ##p < 0.01 Mann-Whitney test, n.s. Scale bar 100 µm. CTS = connective tissue sheath, ORS = outer root sheath, HS = hair shaft.

suggesting that the induced dysbiosis prompted HF toxicity (Fig. 4, ST3) [9,20,22].

Histochemically, Gram-positive cocci and fungi were seen to overcolonize the hair canal, covering the hair shaft and dermal sheath (Fig. 4). Importantly, Sandalore® addition substantially mitigated microbial overgrowth and tissue damage (Fig. 4), reversing the increase in melanin clumping and the reduction of glycogen in the absence of antibiotics, and furthering this effect in presence of antibiotics. Thus, this sandalwood-like synthetic odorant potentiates the HF control of microbial overgrowth and mitigates tissue damage (Fig. 4c).

3.5. A synthetic OR2AT4 agonist alters the human HF microbiome composition by regulating Staphyloccocal and Cutibacterial growth

The sandalwood-like synthetic OR2AT4 agonist effects on the HF bacterial community were followed-up by 16 S rDNA sequencing. Freshly frozen (D0) HFs were characterised by a highly diverse microbiome, with ~60 % of the reads mapping to less numerous genera (grouped into "Others") suggestive of higher bacterial biodiversity (Fig. 5a). Further, the communities of these HFs were dominated by *Cutibacterium* (~30 %).

HF culture resulted in an increase in *Staphylococcus*, *Pseudomonas*, *Brevundimonas* and *Corynebacterium* relative abundance, and in the consequent decrease of "Others". Antibiotic omission strongly favoured *Staphylococcus* (60 %) and *Pseudomonas* (11 %) growth, while *Cutibacterium* and "Others" were reduced (21 % and 1 %, respectively), thus promoting HF microbiome dysbiosis (Fig. 5a, Table S3). Instead, synthetic odorant addition partially preserved "Others" (19 %) and resulted in a profile more comparable to D0 HFs, although having no effect on bacterial biodiversity, as measured by the Shannon index (Fig. 5b). This "Others" partial preservation (Fig. 5a) was accompanied by *Cutibacterium* (13%) and *Staphylococcus* (4 %) abundance restriction and potentiated by antibiotics. Indeed, combined antibiotic/odorant use preserved "Others" relative abundance (52 %) (Fig. 5a), resulting in bacterial biodiversity increase, resembling D0 HFs (Fig. 5b-c), while limiting the increase in *Staphylococcus* (11 %) and *Brevundimonas* (2 %). Interestingly, odorant/antibiotic combination resulted in microbial community differential profile between donors (Fig. 5c), which could result from baseline microbiome inter-individual variations furthered by this co-therapy. Remarkably, the Sandalore® effects were stronger against Gram-positive bacteria e.g., *Staphylococcus* and *Cutibacterium*, although smaller inhibitory effects were seen against Gram-negative bacteria, e.g., *Brevundimonas* and *Neisseria* (Figs. 4 and 5a).

Hence, antibiotic and odorant combination appears to promote a physiological bacterial profile relevant for the management of HF dysbiosis-associated diseases.

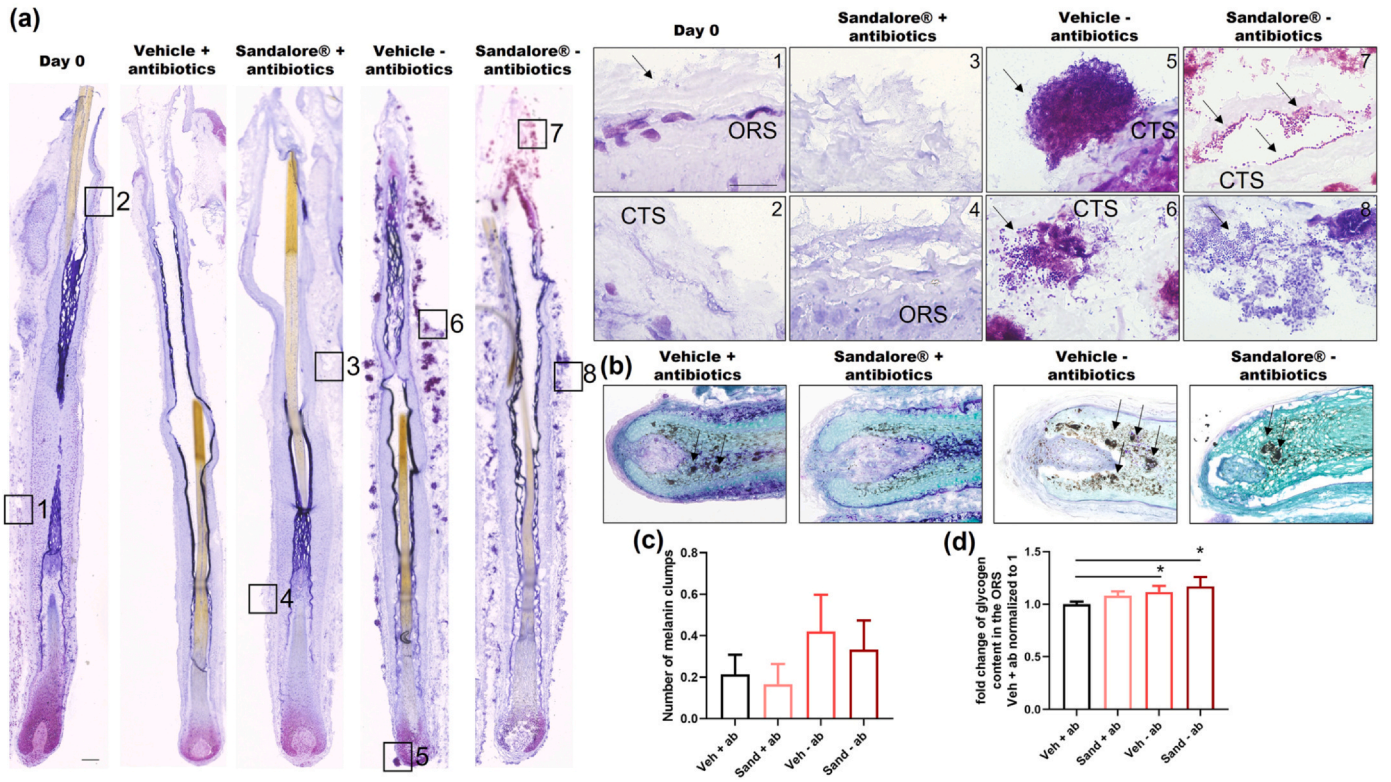

Fig. 4. Synthetic odorant treatment dampens severe bacterial contamination and follicular dystrophy in HFs cultured without antibiotics *ex vivo*. HFs were freshly frozen (day 0) or cultured in serum-free conditions and treated with a vehicle control or 500 µM Sandalore®, with or without antibiotics (Veh+ab, Sand+ab, Veh-ab and Sand-ab, respectively) for 2–4 days, before being processed for histological analyses. (a) Representative Gram-staining of fresh HFs and HFs cultured with and without synthetic sandalwood-like odorant, in the presence or absence of antibiotics, and respective high-magnification images. Black arrows demark bacterial colonisation. Arrows show HF dystrophy, with swelling of the bulb, separation of the epithelial and mesenchymal tissue and spongiosis of the ORS [23]. Scale bar corresponds to 100 µm. (b) Representative PAS-LG of HFs cultured in the presence or absence of the synthetic odorant, with or without antibiotics. Arrows indicate melanin clumps. (c) Pooled data of the number of melanin clumps in 18–30 HFs from 5 independent donors. Mean ± SEM, D'Agostino & Pearson omnibus normality test, no Gaussian distribution, Kruskal-Wallis test, $p = 0.2920$, Dunn's multiple comparisons test, with multiplicity and adjusted p-value (95 % confidence), Veh+ab fixed, n.s., Mann-Whitney test, n.s. and (d) pooled data of glycogen content in the HF proximal ORS in 18–30 HFs from 5 independent donors, as measured by PAS-LG immunohistochemistry. Mean ± SEM, D'Agostino & Pearson omnibus normality test, no Gaussian distribution, Kruskal-Wallis test, $p = 0.0346$, Dunn's multiple comparisons test, with multiplicity and adjusted p-value (95 % confidence), Veh+ab fixed n.s., Mann-Whitney test, $^{*}p < 0.05$. Scale bar corresponds to 100 µm. CTS = connective tissue sheath, ORS = outer root sheath.

3.6. Sandalore®-treated HF supernatant regulates staphylococcal and cutibacterial growth

We then tested whether these metagenomic results translated into bacterial growth changes and if these Sandalore®-induced HF microbiome-managing effects originated from DCD release into the media. Indeed, in comparison to vehicle-treated HFs, supernatants of Sandalore®-treated HFs contained significantly more DCD (Fig. 3b), suggesting that odorant treatment alone suffices to induce human HF DCD secretion.

Next, bacterial isolates of three main human HF colonisers targeted by DCD in a dose-dependent fashion [24,25] (*C. acnes*, *S. aureus* and *S. epidermidis* [8,26,27]) were treated *in vitro* with conditioned supernatants. While no effects were seen in *C. acnes* growth, in line with the 16 S data (Fig. 5a), both *S. aureus* and *S. epidermidis* thrived when antibiotic-free media were added, and sandalwood-like synthetic odorant addition only minimally reduced these two species (Fig. 5d). However, antibiotic-treated media partially reduced (by 7-fold), and completely depleted *S. aureus* growth, when the odorant was introduced. Further, *S. aureus/S. epidermidis* growth ratio was lower in Sandalore®-treated antibiotic-free supernatants, compared to vehicle-treated controls (Fig. S5a), suggesting that the Sandalore®-conditioned antibacterial activity of media, predominantly targeted *S. aureus*, most likely indirectly, via DCD.

Together, these data show this sandalwood-like OR2AT4 agonist's potential to modulate the HF microbiota in a clinically relevant manner by dampening opportunistic bacteria overgrowth, such as *S. aureus*. These effects were potentiated by odorant and antibiotic combination.

3.7. Sandalwood-like odorant treatment also affects resident HF fungal species

Although DCD was previously only reported to have potent antifungal effects against *Candida albicans*, whose presence in the HF was only described in pathological conditions [28,29], considering the existence of ecological microbial competitions [16,30,31], we evaluated whether the odorant effects on the bacterial community extended to fungi [8]. Interestingly, antibiotic omission led to rapid fungal overgrowth, as assessed by PAS/Light Green histochemistry (Fig. 6a-b), an effect rescued by odorant treatment, which reduced the total fungal colony area (Fig. 6a-b) [32]. This *ex vivo* observation nicely reflects yeast overgrowth-induced folliculitis and suggests that this odorant could adjuvate HF mycobiota regulation.

This prompted us to investigate how this odorant impacts on specific commensal fungi growth by ITS sequencing [8]. HF culture introduced changes in the fungal profile, with a marked decrease in fungi relative abundance (Fig. 6c, "Others"), which resulted in overall diversity decrease. This effect was furthered by odorant addition in absence of antibiotics, which resulted in *Malassezia* and *Kluyveromyces* expansion, possibly limiting the growth of other genera (Fig. 6c-d). We also analysed how media with/without Sandalore® and/or antibiotics impacted the growth of two key HF fungal commensals, *M. restricta and M. globosa* [8,33]. Without antibiotics,

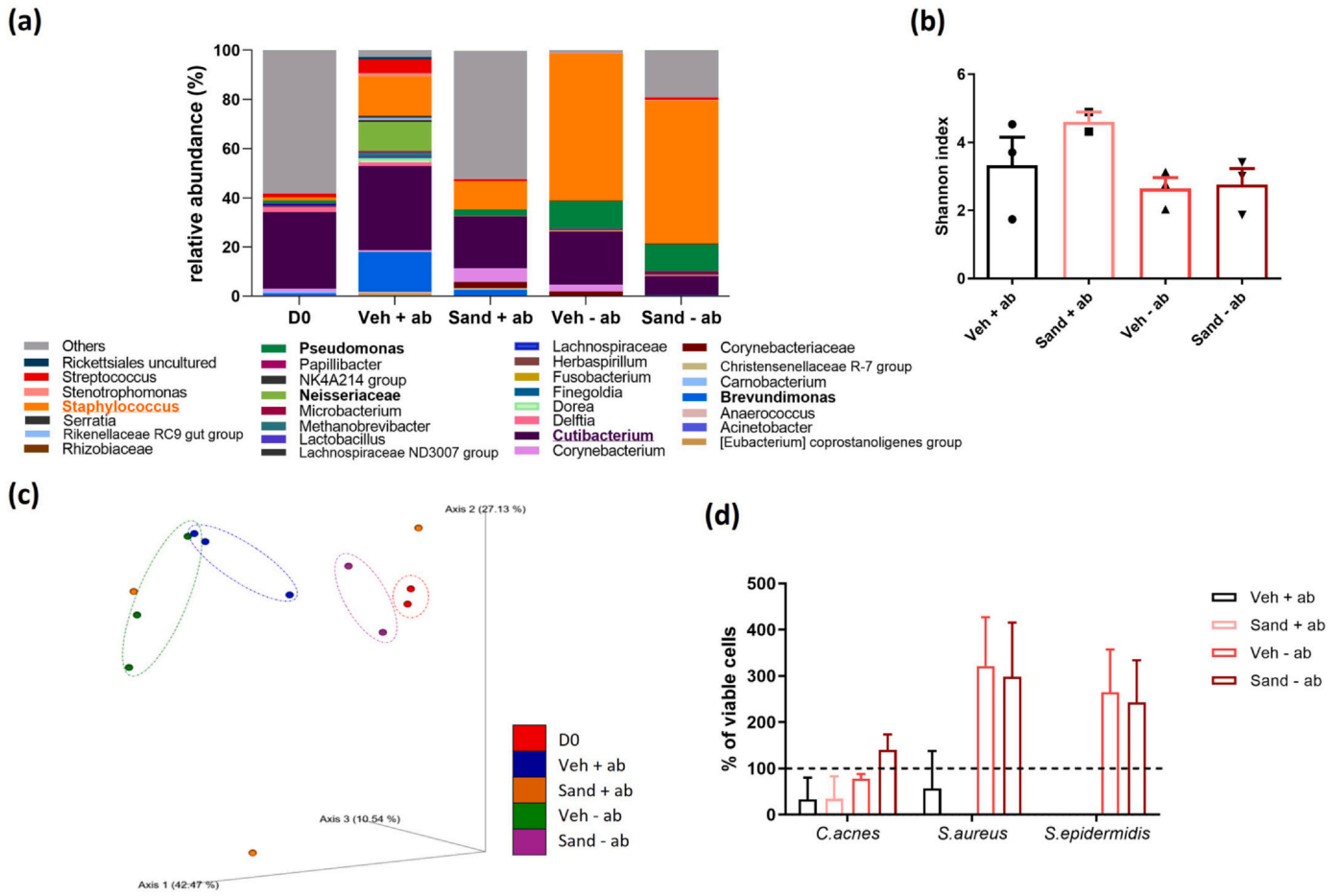

Fig. 5. Synthetic sandalwood-like odorant treatment-induced changes in the bacterial HF microbiome. (a-b) HFs were cultured in serum-free conditions and treated with a vehicle control or 500 µM Sandalore®, with or without antibiotics (Veh+ab, Sand+ab, Veh-ab and Sand-ab, respectively) for 2–4 days. The DNA was isolated and 16S sequencing was performed to quantify bacterial relative abundances and compare with that of freshly dissected HFs. Only taxa with relative abundances > 4% are represented. **(a)** Relative abundances of taxa across the different treatment groups (with main taxa refered in the text in bold) and (b) alpha-diversity of the bacterial microbiome measured by the Shannon index pooled from 9 HFs of three independent donors for cultured HFs, or 6 HFs of two independent donors on D0. Mean ± SEM, D'Agostino & Pearson omnibus normality test, no Gaussian distribution, Kruskal-Wallis test $p = 0.2774$, Dunn's multiple comparisons test, with multiplicity and adjusted p-value (95% confidence), Veh+ab fixed, n.s., Mann-Whitney test, n.s. (c) Weighted Unifrac distances based on the Bray-Curtis beta-diversity index per treatment group. (d) Supernatants of vehicle- or odorant-treated HFs were added to bacterial colonies and grown for 24 h to calculate the growth inhibition in the indicated bacterial species by the pooled supernatant per group of five independent donors. Mean ± SEM, D'Agostino & Pearson omnibus normality test, no Gaussian distribution, Kruskal-Wallis test, $p = 0.8857$ (*C. acnes*), $p = 0.0667$ (*S. aureus*) and $p = 0.0571$ (*S. epidermidis*), Dunn's multiple comparisons test, veh+ab fixed with multiplicity and adjusted p-value (95 % confidence), n.s., Mann-Whitney test, n.s.

Sandalore®-conditioned medium favoured *M. restricta*, while no changes were observed in *M. globosa* growth (Fig. 6c and e, S5b). Thus, this sandalwood-like odorant might favour *M. restricta* growth, directly or indirectly, by preserving beneficial bacteria that limit fungal growth. This suggests that this odorant may be used to manage human HF mycobiota.

3.8. The sandalwood-like synthetic odorant antimicrobial effects seem to be OR2AT4-dependent

To assess whether the observed odorant-induced antimicrobial effects were mediated by OR2AT4 activation, we performed a pilot experiment with Sandalore® alone or with the OR2AT4 competitive antagonist, Phenirat® [1], under dysbiotic conditions. Supernatants were analysed for total bacterial counts and intraepithelial DCD expression was evaluated by histology. In line with the antimicrobial effects, Sandalore® addition completely abrogated the bacterial counts, while Phenirat® restored bacterial growth (Table S4). Similarly, *in vitro* odorant-conditioned supernatants' addition to selected HF/skin microbiota species, showed *S. aureus* growth inhibition (0.3 mm inhibition zone) (Table S5, Fig. S6), in conformity with the viable bacterial cell depletion (Fig. 5d). In contrast, in this experiment, Sandalore®-conditioned media showed no effects on *S. epidermidis, M. restricta* and *M. globosa* growth, and *C. acnes* inhibition (0.2 mm inhibition zone) (Table S5, Figs. S6, 5d). This contrast between these and the above-described tests on *C. acnes* and *Malassezia* viability might be indicative of inter-individual variations. Moreover, Phenirat® addition reverted the odorant inhibitory effects on *S. aureus* and *C. acnes* growth (Fig. S6). Interestingly, this correlated with an increase in intrafollicular DCD expression after treatment with Sandalore®, while Phenirat® reversed this effect (Fig. S7). Therefore, these pilot data suggest that the Sandalore® antimicrobial effects are indeed OR2AT4-mediated.

4. Discussion

This study provides the first evidence that a non-drug synthetic OR2AT4 agonistic odorant, Sandalore®, in combination with antibiotics, can modify the human microbiota by stimulating OR-dependent AMPs production. Odorant treatment significantly enhances epithelial DCD production in a healthy human scalp HF *ex vivo* dysbiotic model and thereby exerts moderate, but functionally-relevant antimicrobial effects, preventing dysbiosis-associated HF

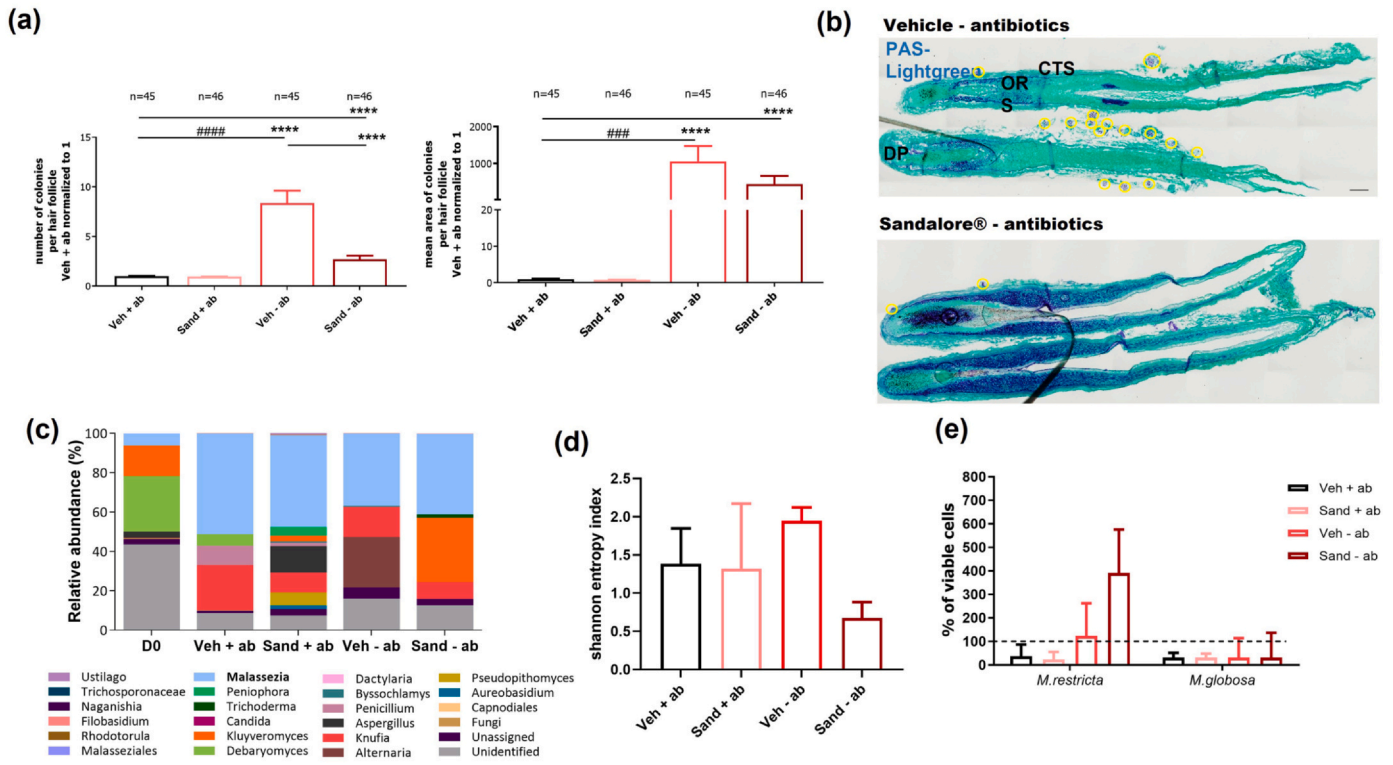

Fig. 6. Synthetic odorant treatment modulates the growth of fungal species. (a-b) HFs were cultured in serum-free conditions and treated with a vehicle control or 500 µM Sandalore®, with or without antibiotics (Veh+ab, Sand+ab, Veh-ab and Sand-ab, respectively) for 2–4 days, before histological analysis. (a) The number of fungal colonies (left) and mean area of the colonies per treatment group (right) were counted in 45–46 full-length HFs (from 6 independent donors) that were treated with the synthetic odorant without antibiotics and compared to that in vehicle-treated HFs without antibiotics, D'Agostino & Pearson omnibus normality test, no Gaussian distribution, Kruskal-Wallis test, $p < 0.0001$, Dunn's multiple comparisons test, with multiplicity and adjusted p-value (95% confidence), Veh+ab fixed, ###p < 0.001 ####$p < 0.0001$, Mann-Whitney test, ****$p < 0.0001$. (b) Respective representative pictures using (PAS)/Light Green histochemistry, showing the fungal colonies (circled in yellow). Scale bar corresponds to 100 µm. (c) Relative abundance of the different fungal taxa present across the experimental groups with the main taxa refered to in the text in bold. (d) Diversity of the fungal microbiome measured by the Shannon index pooled from 6 HFs of two independent donors. Mean ± SEM, D'Agostino & Pearson omnibus normality test, no Gaussian distribution, Kruskal-Wallis test, $p = 0.5619$, Dunn's multiple comparisons test with multiplicity and adjusted p-value (95 % confidence), Veh+ab fixed, n.s., Mann-Whitney test, n.s. (e) Supernatants of vehicle- or odorant-treated HFs were added to fungal colonies and grown for 24 h to calculate the growth inhibition in the indicated fungal species by the pooled supernatant per group of five independent donors. Mean ± SEM, D'Agostino & Pearson omnibus normality test, no Gaussian distribution, Kruskal-Wallis test, $p = 0.0095$ (*M. restricta*) and $p = 0.4857$ (*M. globosa*), Dunn's multiple comparisons test, with multiplicity and adjusted p-value (95 % confidence), Veh+ab fixed, n.s., Mann-Whitney test, n.s. CTS = connective tissue sheath, ORS = outer root sheath, DP = dermal papilla.

dystrophy *ex vivo*. These effects could therefore be exploited in the management of dysbiosis-associated hair diseases [8].

We show that combined HF treatment with antibiotics and a sandalwood-like odorant: a) restores microbiome diversity and promotes baseline human HF microbiome composition preservation, b) prevents microbial overgrowth *ex vivo* better than antibiotics alone, and c) is effective against HF mycobiota overgrowth. Indeed, odorant treatment preserved a microbiome profile similar to freshly isolated HFs. Further, Sandalore® addition regulated the HF bacteriota by favouring *S. epidermidis* over *S. aureus* growth, suggesting that this odorant may regulate this ratio, often dysregulated in hidradenitis suppurativa, androgenetic alopecia and alopecia areata [8]. However, it remains to clarified whether the *S. aureus/S. epidermidis* ratio and AMP production are dependent on donor innate immunity characteristics and the presence/absence of skin pathology (e.g., atopic dermatitis, psoriasis, and seborrhoeic dermatitis).

Although it requires characterization whether this resulted directly from odorant treatment or was DCD-mediated, this odorant potentially limited *C. acnes* growth, which might be useful in acne vulgaris treatment [8]. However, given the observed *S. aureus* modulation, *C. acnes* regulation most likely derived from microbe-microbe regulation (ST3). Notably, contrarily to *C. acnes,* in vitro *S. aureus* growth was limited by the conditioned-media, an effect previously shown in a human skin *ex vivo* model with topical Sandalore® application, where odorant exposure to the supplement media was limited [34]. Hence, the anti-Staphylococcal effects observed seem to derive from odorant-mediated follicular DCD production.

Moreover, although antifungals are not standard supplementation of HF organ cultures, given their known interaction with intrafollicular enzymes (such as P450 enzymes), which can interfere with HF read-out parameters [9], this study focussed to evaluate HF bacteriome changes upon DCD stimulation with an OR2AT4 agonistic odorant. Anyways, these effects seemed to trickle down to the fungal community. Indeed, our data suggest that this odorant potentially demonstrates antifungal properties interesting for fungal folliculitis treatment by mitigating/preventing overcolonization [35]. *M. restricta* growth promotion can represent an important odorant effect towards a physiological scalp HF mycobiome profile, given the higher abundance of these communities in sebaceous skin sites [36] (as are scalp HFs). Considering the involvement of *Malassezia* in dandruff [37], this finding requires further exploration. Again it remains unclear if this effect was directly driven by this odorant or was mediated by bacteriota changes. Hence, the interaction of these HF microbial communities and the mechanisms by which the HF regulates its mycobiome, for example through its AMPs, should be clarified [8]. In addition, given these anti-fungal effects observed, it is possible that other anti-fungals might work in conjunction with Sandalore® to unfold additive or even synergistic antimicrobial benefits and therefore also deserve probing in follow-up studies [38].

Incidentally, we show that the eccrine and sebaceous glands are not the only human skin structure capable of transcribing, producing and secreting DCD [16,17]. Intrafollicular DCD production might constitute a regulatory mechanism to prevent dysbiosis. Both *C. acnes* and *S. aureus* are predominant members of the skin/HF microbiota [8] but overgrowth is rare. Further, while *S. aureus* dysbiosis is associated with cutaneous infections and observed in atopic dermatitis and psoriasis, *C. acnes* phylotype dysbiosis is observed in acne vulgaris, and *Cutibacterium/Staphylococcus* ratio is altered in dandruff [8,39]. This further encourages exploration of this AMP for therapeutic exploitation. Even though this remains to be formally documented, we suspect that topical Sandalore® treatment could benefit patients with dysbiosis-associated pathologies, including in folliculitis decalvans, atopic dermatitis, acne vulgaris, acne conglobata and hidradenitis suppurativa [40–42]. In fact, we recently showed that Sandalore® also induces DCD expression in human epidermis [34], which may be useful in the aforementioned dermatoses, where this AMP is decreased. Further, besides its antimicrobial activity, DCD-derived peptides stimulate human keratinocyte production of TNFα, IL-8, CXCL10 and CCL20, involved in cytotoxic T-cell and neutrophil recruitment [43,44], and, in mice, activate mast cells [45]. Therefore, it is also possible that this odorant may secondarily alter innate skin immune/inflammatory responses. Hence, cytokine stimulation from the HF epithelium and the consequent immune cell migration should also be evaluated [19], for example, by adapting the described *ex vivo* assay to co-culture neutrophils with human HFs and measuring related cytokine/chemokine responses [19].

Moreover, although corroboration is necessary (e.g, with OR2AT4 knockdown studies [1] and possibly OR2AT4 protein assessment by Western-Blot) our pilot experiment with Phenirat® suggests that the observed antimicrobial odorant effects are OR2AT4-mediated, followed by intrafollicular DCD production and release. Further, even though changes in *CAMP* expression were observed by amputated HF RNA microarray, full-length HF analysis suggested that these effects are DCD-specific, as, in the absence of antibiotics, no impact on LL-37 (*CAMP*) was seen. Hence, OR2AT4 stimulation appears to preferentially target DCD-sensitive HF microbiota (see Fig. S4). We therefore postulate that human scalp HFs may detect various bacterial products via distinct ORs, as occurs in the gut epithelium [46]. Consequently, these pathways elicit differential AMP expression (e.g., DCD, but not LL-37), likely depending on their antimicrobial activity spectra, through microbially-driven signalling pathways, rather than discrete cell populations in the HF epithelium (see Fig. 4). The observed *CAMP* expression differences could result from the absence of important HF microbial colonisation regions (isthmus and infundibulum) in amputated versus full-length HFs, or could reflect differential AMP expression induced by a wound-healing effect in amputated HFs, since LL-37 was shown to promote re-epithelisation [47].

Overall, these results support investigation of other functional ORs in human HFs and whether these also (differentially) regulate AMPs and manage the HF microbiota in a distinct, therapeutically targetable manner. As DCD is active against *S. aureus* and *S. epidermidis* (Fig. S4), and Sandalore® promotes intrafollicular DCD production and secretion, this AMP likely explains the Sandalore® antimicrobial activities reported here. However, formal proof will require addition of DCD-neutralising antibodies/DCD-lysing proteases to Sandalore®-treated HFs.

Our study therefore demonstrates that a synthetic odorant modulates human HF microbiota with clinical potential. Further, we show that proper HF OR stimulation with an odorant may become a useful antibiotic adjuvant in dysbiosis-associated hair disease management. Future studies should focus on examining the effect of Sandalore® on diseased HFs, e.g., from folliculitis patients.

Ethics statement

Study conducted according to the Declaration of Helsinki principles. Specimens were obtained after informed, written patient consent and ethics committee approval (University of Muenster 2015–602-f-S).

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CRediT authorship contribution statement

J.E. and **M.B.L.** has contributed to the conceptualization and methodology of the study, respective result validation and formal analysis, writing, editing, and reviewing of the original manuscript. **D.P.** has contributed to the methodology of the study, and formal analysis and reviewing and editing the original manuscript. **J.C.**, **M.dA.** and **F.R.** have contributed to the formal analysis and editing and reviewing of the original manuscript. **F.M.C.** has contributed to the formal analysis of the results and editing and reviewing of the original manuscript. **F.J.** and **H.E.** have contributed with the necessary resources for study performance and editing and reviewing of the original manuscript. **J.W.** and **B.P** have contributed to the methodology of the study and editing and reviewing of the original manuscript. **M.B.** and **R.P.** have contributed to the conceptualization of the study, supervision, editing and reviewing of the original manuscript and funding acquisition.

Data availability

The data supporting the findings of this study are available from the corresponding author, JE, upon reasonable request. Sequencing raw data can be found in the SRA database under accession numbers: PRJNA857855 (16S) and PRJNA857859 (ITS).

Declaration of Competing Interest

JE, MBL, MB and RP are employees of Monasterium Laboratory, for which JC and FJ consult. DP and FR are employees of Giuliani S.p.a, which has filed a patent on the technology reported here (WO2019224211A1 and IT102018000005585, June 2020), for which RP consults. All remaining authors have no conflicts of interest to declare.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jdermsci.2023.09.006.

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