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Microbial imbalance in Darier disease: Dominance of various staphylococcal species and absence of Cutibacteria

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Darier disease (DD) is a rare autosomal dominant genodermatosis characterized by erythematous papules and plaques mainly involving sebaceous areas, such as the face, chest and back. Skin microbiome plays an essential role in maintaining skin homeostasis. A disturbed skin microbiome may contribute to the exacerbation of DD. We investigated the bacterial composition of two predilectional sites in DD patients and healthy individuals. We also measured the microbiome composition of deeper skin layers, where diversity was significantly reduced compared to the superficial layer of the skin from the same area. The microbiome of DD patients at lesional sites differed from that of non-lesional skin areas; moreover, non-lesional sites were different from those of the controls. Lesional areas were dominated by *Staphylococcus* species, such as *S. aureus*, *S. epidermidis*, *S. hominis*, *S. sciuri*, and *S. equorum*. However, levels of *Cutibacterium acnes* (formerly *Propionibacterium acnes)* and *C. acnes subspecies defendens* were significantly lower in lesional sites than in non-lesional sites. A significant decrease was measured in the levels of these two bacteria between non-lesional and control samples. Our findings may indicate that alterations in the skin microbiome could contribute to the inflammation of skin lesions in DD.

The human skin microbiome includes a diverse group of microorganisms including bacteria, fungi, viruses and archaea. They play an essential role in maintaining a dynamic ecosystem that provides protection against pathogens and regulates the immune system. The composition of the skin microbiome can be influenced by factors such as age, hormones, sebum or sweat production, and environmental conditions[1]. The skin consists of three major environments including sebaceous or oily, moist and dry. Sebaceous areas, such as the face, chest, and back, are mainly colonized by *Cutibacterium* species (formerly *Propionibacterium*), *Staphylococcus* bacteria and *Malassezia* yeasts. Moist skin sites that are rich in sweat glands and include intertriginal folds, ligament of elbow, knee, and groin, are dominated by Staphylococci, Corynebacteria and beta-Proteobacteria. Dry sites (volar forearm and palms) are largely colonized by Cutibacteria and Corynebacteria, and additionally contain Micrococci, *Enhydobacter* species and Streptococci[2,3]. Skin appendages, such as sweat glands, hair follicles and sebaceous glands may have an impact on the microbiome of different sites by influencing the local environment. Sweat glands and sweat in moist areas are important factors in inhibiting the colonization of certain microorganisms, as sweat contains free fatty acids and antimicrobial peptides. Oily sites are rich in sebaceousglands that produce a hydrophobic lipid-rich sebum, which acts as a ”mantle” and provides an antibacterial surface for hair and skin[1].

Darier disease (DD, follicular dyskeratosis, OMIM 124200, ORPHA 218) is a rare acantholytic genodermatosis with autosomal dominant inheritance. It is characterized by loss of adhesion between epidermal keratinocytes due to mutations of the sarco/endoplasmic reticulum $Ca2^+$ ATPase isoform 2 (SERCA2) coding *ATP2A* gene. Desmosomal disintegration, as a consequence of impaired intracellular $Ca2^+$ signaling leads to suprabasal acantholysis and anoikosis in the keratinocytes. These epidermal changes are manifested as brownish erythematous hyperkeratotic papules and plaques that predominantly affect sebaceous areas such as the face, chest and back[4,5]. In the pathogenesis of DD, a genetically determined defect in the endothelial $Ca2^+$ pumps leading to epidermal disfunction is considered to be the initial mechanism, followed by inflammation and

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cutaneous infections as both complications and trigger factors of the disease[6]. DD has a chronic relapsing course and other factors, such as friction, heat, humidity, sweating, stress, UV light, and hormonal changes can be also responsible for exacerbations. Pruritus, burning sensations and painful, odorous erosions aggravate the disease and significantly impair the quality of life of patients with DD[7,8]. Therapeutic options focus on restoring keratinocyte function and preventing inflammation and superinfection[5,8].

When the skin barrier is compromised, as seen in conditions such as DD, it can lead to increased susceptibility to colonization by pathogens and active infections. Impaired skin barrier function allows increased penetration of allergens, irritants, and pathogens into the skin which may trigger an inflammatory response and alter the composition of the skin microbiome[3,9]. Previous studies have showed that skin barrier defects are associated with decreased levels of antimicrobial peptides (AMPs) such as HBD-3 and LL-37. AMPs normally inhibit pathogens such as *S. aureus* on the skin, their deficiency can lead to skin dysbiosis and increased susceptibility to infections[9]. Moreover, skin barrier dysfunction can change the skin pH. Increased skin pH facilitates microbial overgrowth and infections. It also impairs the antimicrobial defense of the skin. Skin microbes may also influence keratinocyte differentiation and stratum corneum formation, which are also important for maintaining the skin barrier[10].

Results

A total of 8 DD patients (female: male ratio 7:1, mean age 51.63 ± 11.49) and 6 matched healthy controls (female: male ratio: 5:1, mean age 52.8 ± 7.06) were included in this study. The clinical diagnosis was confirmed by histological and/or molecular genetic verification. Clinical and genetic characteristics of the patients are listed in Table 1. In the case of the *Patient 3*, we identified a novel variant that was confirmed with Nci I enzyme restriction digestion (Table 1, *Supplementary 1*). A total of 49 samples were collected from lesional chest and lower back areas (biopsies and skin swabs) from patients with DD, and only skin swabs from corresponding symmetric sites were collected from their non-lesional sites and from controls. The bacterial composition was different in lesional, non-lesional skin of patients with DD and in healthy individuals. Figure 1a shows the statistically significant difference in microbial alpha diversity between lesional and non-lesional DD cohorts by Shannon analysis with Wilcoxon rank sum testing at species level.

The median alpha diversity was lowest in the biopsy samples, significantly increasing in lesional swabs, and further significantly increasing toward non-lesional regions. The median alpha diversity value in the healthy control group was close to that of the non-lesional sites, but the variability within the healthy group was so high that no significant difference could be demonstrated compared to the other groups. Figure 1b; Table 2 show significant differences between all groups based on the principal coordinate analysis of Jaccard beta diversity.

Figure 2a; Table 3 show that the relative abundance of *Actinobacteria* is significantly lower in lesional sites (biopsies: 4.59%, swabs: 13.38%) of DD patients than in non-lesional sites (19.77%) and healthy controls (44.76%). When non-lesional areas and healthy control samples were compared, a further significant increase in the abundance of *Actinobacteria* was observed. No significant difference was observed between lesional and non-lesional swabs. In contrast, *Firmicutes* increased in DD patients (biopsies: 79.24%, lesional swabs: 65.85%, non-lesional swabs: 41.96%, control: 31.58%).

According to Fig. 2b; Table 3, Staphylococci were significantly higher in lesional (69.73%-50.06%) than in non-lesional sites (10.48%, $p < 0.0001$) and in the healthy control group (7.95%, $p < 0.0001$). No significant differences were detected between biopsy and lesional swab samples ($p < 0.056$) and between non-lesional swabs and controls ($p < 0.187$). The relative abundance of Propionibacteria was significantly lower in DD patients

Patient N°	Sex	Age (years)	Age at onset (years)	Affected areas	Therapy	Comorbid conditions	Localization of biopsy sample	Heterozygous pathogenic or likely pathogenic variants of the ATP2A2 gene (RefSeq Gene: NG_007097.2, RefSeq mRNA: NM_170665.4, var b)	Reference
1	M	55	11	ears, neck, chest, back	topical*, oral acitretine	hepatomegaly, hiatal hernia, GERD	chest	c.1288–6 A > G	Nakamura et al. 2016.[11]
2	F	61	18	neck, chest, back	topical*,	cataracta	chest	c.118 + 1G > A	Jacobsen et al. 1999. [12]
3	F	51	6	whole body	topical*, oral acitretine	sclerosis multiplex	chest	c.1858G > T, p.Val620Phe**	this study
4	F	65	25	neck, chest, back	topical*	fibromyalgia, tinnitus, depressive disorder	chest	not identified***	
5	F	65	14	ears, back, legs	topical*, oral isotretionine	hyperprolactinaemia, depressive disorder	chest	c.558dup, p.(Val187Cysfs*6) published as (c.558insT)	Racz et al. 2006. [13]
6	F	42	32	axilla, inguina, chest, back	topical* oral acitretine	-	chest	c.1043T > C, p.(Ile348Thr)	Wang et al. 2011.[14]
7	F	42	31	chest	topical*, oral acitretine	panic disorder, coeliac disease	chest	not identified***	
8	F	35	8	whole body	topical* and topical retinoids	depressive disorder	chest	c.2098 A > G, p.(Thr700Ala)	Liang et al. 2014. [15]

Table 1. Clinical and genetic characteristics of Darier disease patients included in this study. M-male; F-female; GERD-Gastroesophageal reflux disease; * icludes topical corticosteroids and antiseptics; ** novel variant confirmed with Nci I restriction digestion; *** no pathogenic or likely pathogenic variant was identified using Sanger sequencing of the *ATP2A2* exons and the boundary intron regions.

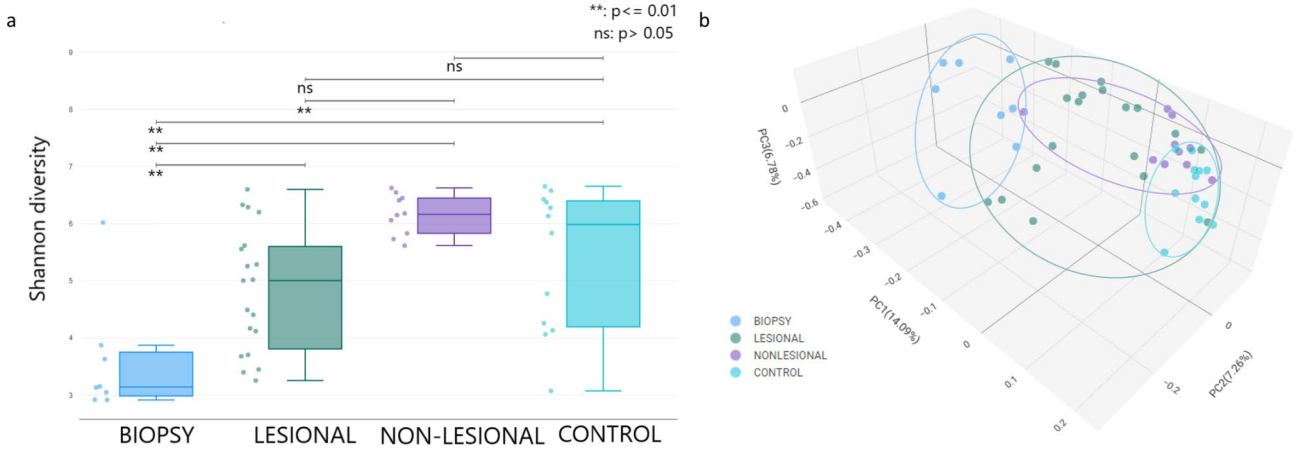

Fig. 1. Comparison of Shannon alpha diversity (**a**) and Jaccard beta diversity Principal Coordinate Analysis (PCoA) (**b**) between different samples (skin biopsy, lesional and non-lesional swab) from patients with Darier disease and healthy control swab samples. Error bars represent standard deviation.

Cohorts	Sample size (*n*)	Permutations (*n*)	*P*-value
Biopsy <-> Lesional	27	999	0.140
Biopsy <-> Non-lesional	18	999	0.001
Biopsy <-> Control	20	999	0.001
Lesional <-> Non-lesional	29	999	0.001
Lesional <-> Control	31	999	0.001
Non-lesional <-> Control	22	999	0.004

Table 2. PERMANOVA analysis of Jaccard beta diversity Principal Coordinate Analysis (PCoA). Number of included samples (lesional biopsy samples and lesional, non-lesional and control swabs), permutations and a normalized *p*-value for each cohort combination.

compared to healthy individuals (biopsy: 0.19%, lesional swab: 1.49%, non-lesional swab: 4.56% and control: 24.12%, $p < 0.0001$, $p < 0.0001$; $p < 0.002$). Significant reductions were also observed between biopsy and lesional ($p < 0.015$) and non-lesional swabs ($p < 0.001$) and also between lesional and non-lesional sites ($p < 0.005$) in DD patients. The same trend was observed in Cutibacteria (biopsy: 0%, lesional swab: 0.34%, non-lesional swab: 1.05%, control: 5.82%, $p < 0.002$, $p < 0.0001$, $p < 0.002$).

The heatmap representation in Fig. 2c indicates a sharp boundary that highlights the differences in the abundance of the 10 most frequently occurring genera in the lesional (biopsy and lesional swab) and asymptomatic (non-lesional DD swab and healthy control swab) microbiome compositions.

It is important to note that novel nomeclatures consider *Propionibacterium* and *Cutibacterium* as the same genus[16]. However, in the results section of this article, due to the nomenclature used by the applied software (CosmosID Metagenomics Cloud, CosmosID Inc., MD, USA), we continued to present them as distinctive; however, in the discussion part, we adjusted them to the latest classification, which considered *Propionibacterium* also as *Cutibacterium*.

Although no significant differences could be verified, the abundance of the genus *Pseudomonas* was higher in symptomatic areas, and continuously decreased toward asymptomatic and healthy control areas. The difference was not significant either, but the abundance of the genus *Finegoldia* was higher in healthy swab samples, and decreased from non-lesional areas toward lesional areas. In the different seborrheic regions, we identified the 10 most abundant bacteria genera in each group (Table 4). In healthy individuals, the most frequent genus was *Propionibacterium*; however, in DD patients, *Staphylococcus* accounted for more than 50% of the total amount of bacteria, and Propionibacteria accounted for only 1.48% of the total in lesional skin, and 4.56% in non-lesional skin swabs. The genus *Cutibacterium* represented 5.82% of the total amount of bacteria in healthy controls, compared to 1.05% in non-lesional and 0.34% in lesional sites in DD.

Region specific alterations by the two examined seborrheic regions are shown in Fig. 3 and in Table 4. In lesional chest and back, the relative abundance of the genus *Staphylococcus* was more than 50%, whereas the level of Propionibacteria was only 1.26% in the same site of the chest and even less than 1% in the back. In non-lesional sites, a sharp decrease in the number of Staphylococci (chest 13.39%, back 8.74%) and an increase in levels of the genus *Propionibacterium* (chest 5.18%, back 4.68%) could be observed. Although a remarkable difference was detected in Propionibacteria levels in the chest and back of healthy controls (33.29% and 14.94%), both sites were dominated by this genus. Moreover, a significant difference was detected in levels of Staphylococci between healthy seborrheic sites (chest 12.86%, back 3.03%). The genus *Cutibacterium* represented 8.02% in the chest and

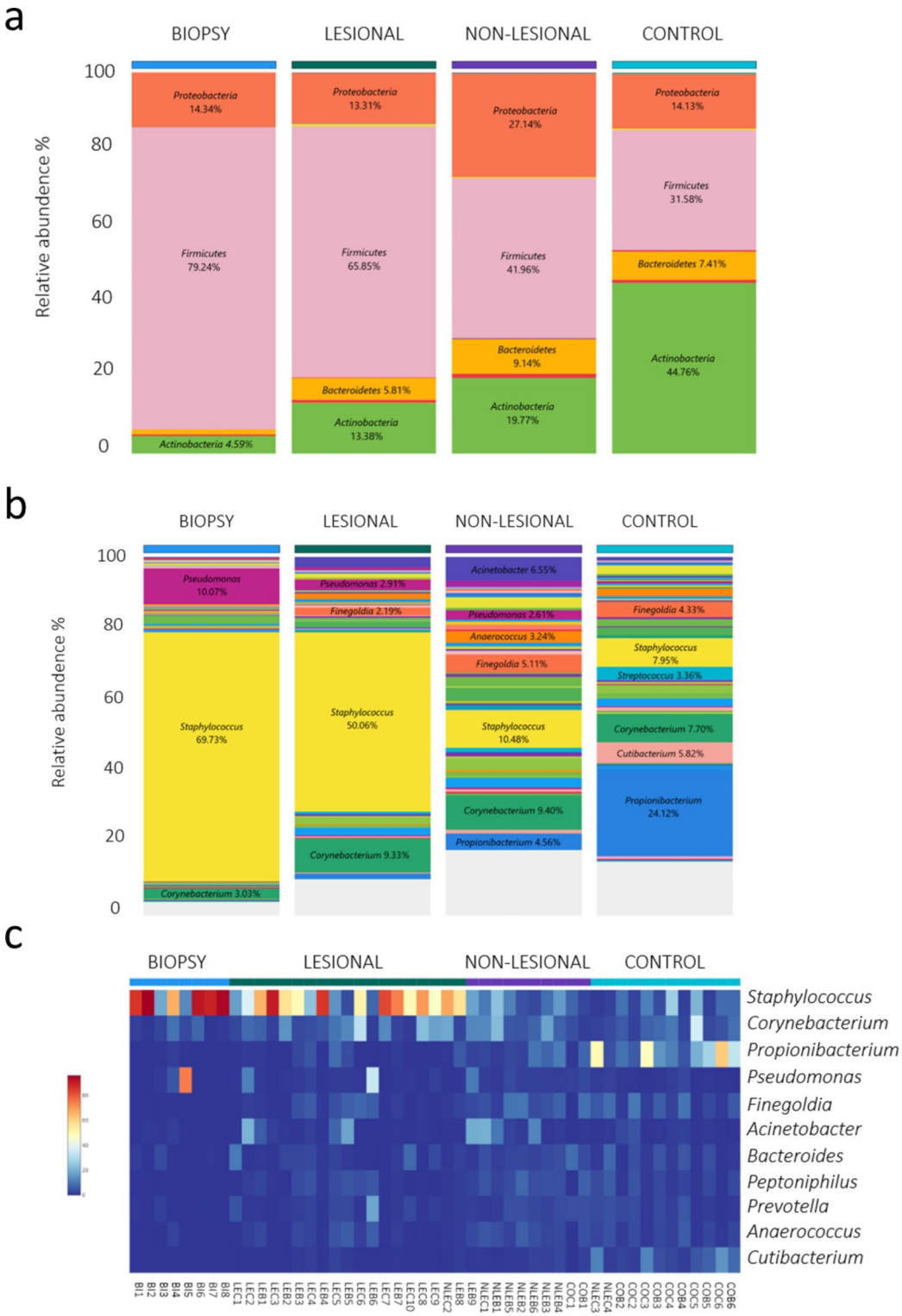

Fig. 2. Skin microbiome composition at phylum (**a**) and genus (**b**) level of skin biopsy and swab samples in Darier disease and control patients in Stacked Bar and genus level in Heatmap (**c**) visualization.

3.63% in the back of the total amount of bacteria in healthy controls, in contrast with 1% in non-lesional and less then 0.5% in lesional sites in DD.

Table 4; Fig. 4 show that the relative abundance of varius species of the genus *Staphyloccoccus*, such as *S. aureus*, *S. epidermidis*, *S. hominis*, *S. equroum*, and *S. haemolyticus* was significantly higher in lesional sites than non-lesional sites and controls. However, no important differences could be observed between non-lesional sites and controls, except for *S. aureus* and *S. haemolyticus*, which were almost absent in healthy samples. In contrast,

Phylum	B-DD Median (IQR)	L-DD Median (IQR)	NL-DD Median (IQR)	C Median (IQR)	*P*-values			
					B-DD: L-DD	L-DD: NL-DD	L-DD: C	NL-DD: C
Actinobacteria	3.01 (5.26)	12.22 (14.38)	19.16 (11.32)	39.92 (48.29)	0.006	0.054	0	0.008
Firmicutes	92.74 (26.06)	70.87 (21.18)	42.09 (8.11)	33.41 (25.3)	0.038	0.002	0	0.075
Genus								
Staphylococcus	87.40 (50.43)	51.75 (42.26)	9.57 (11.75)	5.58 (8.66)	0.056	0	0	0.187
Propionibacterium	0.02 (0.01)	0.22 (2.03)	2.55 (5.14)	17.43 (33.03)	0.015	0.005	0	0.002
Cutibacterium	0.01 (0.01)	0.07 (0.03)	0.69 (1.26)	4.27 (8.18)	0.005	0.006	0	0.002
Species								
S. aureus	26.23 (23.23)	15.55 (13.92)	1.76 (2.32)	0.02 (0.07)	0.124	0	0	0
S. epidermidis	21.75 (10.22)	13.99 (10.59)	2.96 (3.09)	2.06 (3.31)	0.056	0	0	0.323
S. hominis	4.04 (3.33)	3.14 (2,3)	0.65 (0.61)	0.21 (1.11)	0.243	0.002	0.001	0.356
S. equorum	6.00 (10.20)	7.73 (7.39)	1.45 (1.92)	0.75 (1.18)	0.595	0.001	0	0.166
S. haemolyticus	0.03 (0.12)	0.09 (0.02)	0.01 (0.02)	0.003 (0.004)	0.856	0.005	0	0.011
P. acnes	0.02 (0.07)	0.18 (1.6)	2.03 (4.49)	15.00 (28.99)	0.015	0.004	0	0.003
C. acnes subspecies defendens	0.01 (0.01)	0.07 (0.04)	0.69 (1.25)	4.27 (8.18)	0.005	0.006	0	0.002

Table 3. Differences in the most significant bacterial alterations at phylum, genus and species level. B-DD: biopsy, Darier disease; L-DD: lesional swab, Darier disease; NL-DD: non-lesional swab, Darier disease; C: control swab.

P. acnes and *C. acnes subspecies defendens* species were underrepresented in DD patients compared to healthy individuals. Moreover, a significant reduction in the relative amount of this bacterium species was measured in lesional sites in DD patients compared to non-lesional sites. This reduction was more prominent in biopsy samples.

Heatmap visualization (Fig. 4a) and Stacked Bar visualization (Fig. 4b) show the 15 most abundant taxa at species level, and demonstrates a negative correlation between the amounts of Staphylococci (*S. aureus*, *S. epidermidis*, *S. hominis*, *S. sciuri*, *and S. equorum*) and the amounts of *P. acnes* and *C. acnes subsp. defendens*. Lesional sites (biopsies and swabs) were rich in *S. aureus*, *S. epidermidis* and other *Staphylococcus* species. In contrast, *P. acnes* and *C. acnes subsp. defendens* species were reduced not only in lesional, but also in non-lesional sites compared to controls (Fig. 4b). Significant differences were observed in the relative abundance of *S. aureus*, *S. epidermidis*, *P. acnes,* and *C. acnes subsp. defendens* (Fig. 4c).

Discussion

Dysbiosis, an imbalance of the microbiome, may lead to the development and exacerbations of various skin disorders[1,17–20]. DD is characterized by epidermal dysfunction due to an altered calcium homeostasis in keratinocytes by mutations of the *ATP2A2* gene. The abnormal barrier contributes to recurrent superinfections, as complications of the disease[5,8]. Our results show an altered skin microbiome in DD patients compared to healthy individuals. The differences were more prominent in lesional sites; however, the bacterial composition in non-lesional skin areas was also varied. We suggest that the reduced bacterial diversity in lesional skin areas, as a possible consequence of the overgrowth of various *Staphylococcus* species (mainly *S. aureus* and *S. epidermidis)* and the decreased levels of *C. acnes* and *C. acnes subspecies defendens* may contribute to the inflammation and influence disease severity in DD.

Our results show a prominent change in the microbiome in DD patients. We observed significant differences in alpha diversity of lesional and non-lesional skin; however, no significance was reached compared to control skin. This may be due to the high variability within the control group; therefore, no significant differences were identified compared to the other groups. Observations in alpha diversity showed a correlation with significant alterations in beta diversity between lesional, non-lesional and healthy skin. These findings may be supported by those observed in patients with atopic dermatitis (AD), whereas an inverse correlation between skin microbiome and disease severity was observed[21]. Another possible explanation may be that the amount of Cutibacteria is significantly reduced in DD patients both at lesional and non-lesional sites. The lack of Cutibacteria at lesional sites, may lead to the higher relative abundance of various *Staphylococcus* species by creating an ecological niche. Greater diversity in non-lesional sites may not be beneficial if certain *Cutibacterium* species are not present. In this study, the most significant alteration may be the inverse correlation between the genera *Cutibacterium*

Lesional chest		Non-lesional chest		Control chest	
Genus	%	Genus	%	Genus	%
Staphylococcus	53.25	*Staphylococcus*	13.39	*Propionibacterium*	33.29
Corynebacterium	9.64	*Corynebacterium*	12.66	*Staphylococcus*	12.86
Acinetobacter	2.67	*Acinetobacter*	7.44	*Corynebacterium*	10.86
Bacteroides	2.61	*Propionibacterium*	5.18	*Cutibacterium*	8.02
Finegoldia	1.94	*Finegoldia*	4.32	*Streptococcus*	4.59
Pseudomonas	1.71	*Pseudomonas*	3.26	*Finegoldia*	1.63
Prevotella	1.30	*Peptoniphilus*	3.54	*Prevotella*	1.42
Propionibacterium	1.26	*Anaerococcus*	3.43	*Micrococcus*	1.09
Peptoniphilus	1.23	*Xanthomonas*	2.74	*Peptoniphilus*	0.98
Escherichia	1.06	*Escherichia*	2.54	*Anaerococcus*	0.97
Lesional back		Non-lesional back		Control back	
Genus	%	Genus	%	Genus	%
Staphylococcus	50.81	*Staphylococcus*	8.74	*Propionibacterium*	14.94
Corynebacterium	9.01	*Corynebacterium*	6.71	*Finegoldia*	7.03
Pseudomonas	4.35	*Acinetobacter*	5.94	*Corynebacterium*	4.55
Prevotella	2.89	*Finegoldia*	5.57	*Bacteroides*	4.23
Acinetobacter	2.74	*Propionibacterium*	4.68	*Escherichia*	4.09
Finegoldia	2.25	*Peptoniphilus*	3.85	*Cutibacterium*	3.63
Anaerococcus	2.19	*Bacteroides*	3.82	*Peptoniphilus*	3.48
Peptoniphilus	1.84	*Anaerococcus*	3.27	*Anaerococcus*	3.41
Bacteroides	1.23	*Escherichia*	3.2	*Lactobacillus*	3.32
Escherichia	1.05	*Prevotella*	2.99	*Staphylococcus*	3.03

Table 4. Top 10 abundant bacteria genera in seborrheic sites of swab samples in patients with Darier disease and in healthy individuals.

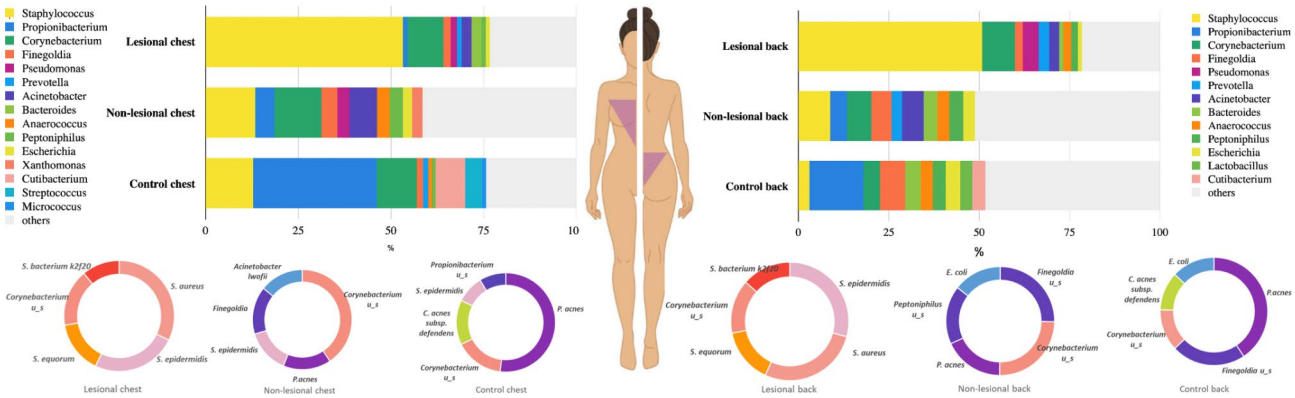

Fig. 3. Our results show the top 15 most abundant bacteria genera and the top 5 most abundant species in the skin microbiome of swab samples from patients with Darier disease. Lower microbiomediversity compared to healthy controls was associated with a significant reduction in the genera *Propionibacterium* and *Cutibacterium* and massive colonization of the genus *Staphylococcus* in seborrheic sites (both chest and back) in patients with Darier disease.

and *Staphylococcus*. The relative amount of *C. acnes* and *C. acnes subspecies defendens* was significantly lower in lesional skin compared to non-lesional skin areas. Moreover, a significant decrease at both bacteria levels was observed comparing non-lesional sites to controls. In contrast, the levels of *S. aureus*, *S. epidermidis*, and *S. hominis* were increased in lesional sites, but no prominent change was detected between non-lesional sites and controls. It is not clear whether the initial decline of *Cutibacterium* species led to an overgrowth of various *Staphylococcus* species or whether the initial colonization of Stahylococci inhibited Cutibacteria.

Relevant changes in the microbiome have been found in other chronic skin disorders, such as AD, psoriasis, acne, hidradenitis suppurativa and chronic skin ulcers[17,19,20,22]. In AD, genetic alterations of *filaggrin* gene result in disrupted epidermal barrier and higher transepidermal water loss (TEWL). Similar to our findings, *Francuzik et al.* found a negative correlation between C. *acnes* and *S. aureus* in AD patients, suggesting that these changes

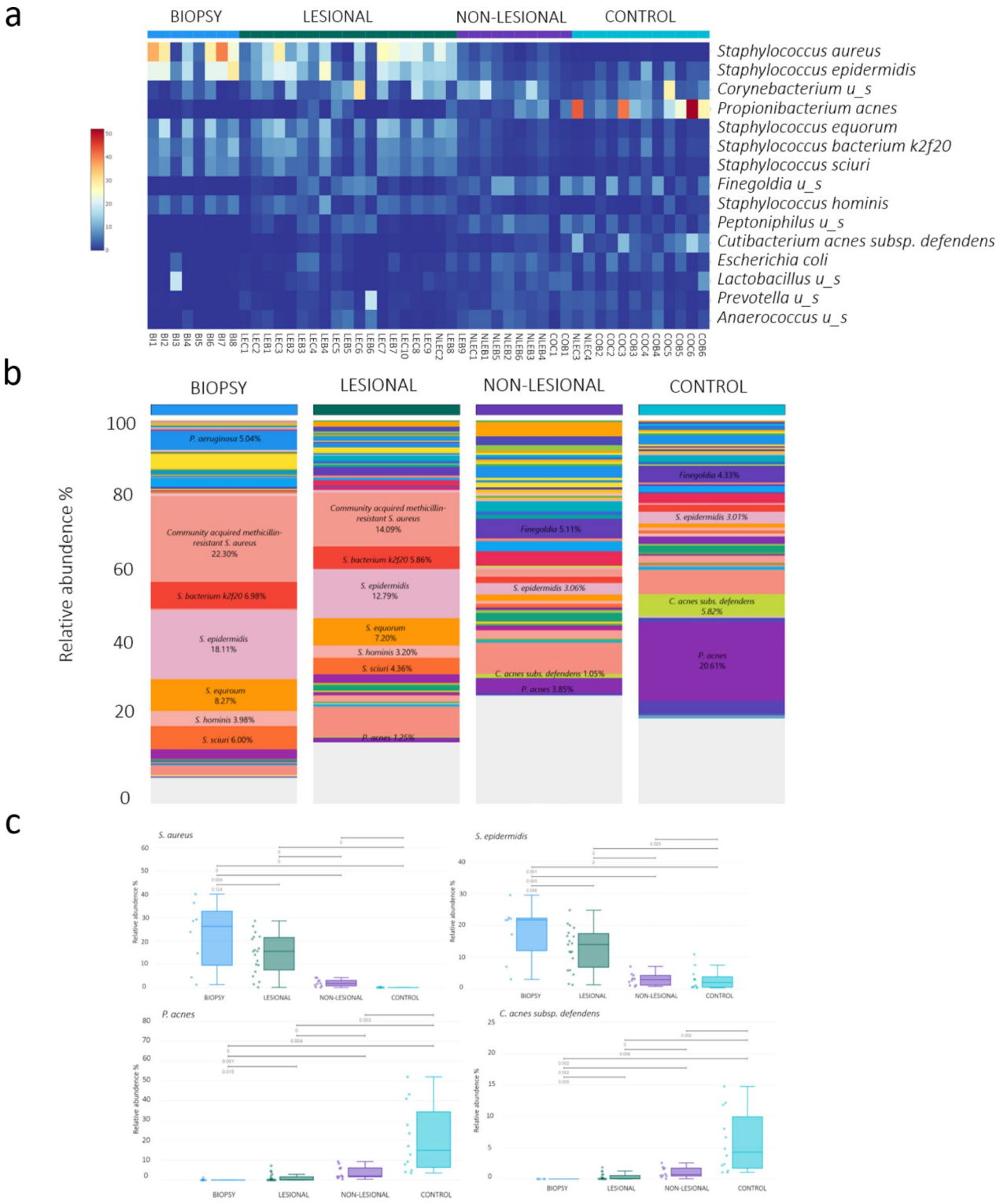

Fig. 4. Microbiome composition of skin biopsy and swab samples in Darier disease patients and in healthy controls at species level. Negative correlation between various *Staphylococcus* species and between *P. acnes* and *C. acnes subspecies defendens* were observed comparing lesional, non-lesional and control samples (**a** and **b**). The relative abundance of *S. aureus*, *S. epidermidis*, *P. acnes,* and *C. acnes subspecies defendens* showed significant differences. Error bars represent in standard deviation (**c**).

cannot be considered disease-specific, but may be related to impaired epidermal function, which can be found in both skin disorders[23].

A previous study of DD patients showed that TEWL in lesional skin was increased four-to threefold, compared to non-lesional skin. A disturbed skin barrier may contribute to superinfections[24]. Moreover, a recent investigation highlighted that sebaceous areas, aspredilection sites of DD, may be considered as weak links in the human skin, as these skin regions in healthy individuals showed higher TEWL, decreased expression and irregular distribution of certain desmosomes and tight junction proteins compared to dry areas[25].

S. epidermidis is a commensal bacterium that promotes an innate immune response and inhibits the growth of *S. aureus* and *C. acnes*. It is considered harmless, but any compromise to the intact epidermal barrier can lead to a shift in the behavior of *S. epidermidis*, transforming it to a potentially pathogen agent. For instance, murine skin pre-exposed to *S. epidermidis* exhibited resistance to *S. aureus* only when the barrier was intact[26]. Another study showed that intradermal injection (rather than topical) of *S. epidermidis* led to inflammatory responses, such as the presence of infiltrating monocytes and neutrophils, and interferon-γ (IFNγ)-producing T_{eff}cells[27]. Recent studies showed that in AD patients, not only the presence of *S. aureus*, but a massive colonization of *S. epidermidis* at lesional sites may also correlate with their disease severity[28].

C. acnes (formerly *P. acnes*) plays an important role in maintaining skin health, not only by inhibiting the colonization by other pathogen agents, but also by modulating the immune system. It is a gram-positive anaerobic microbe that resides mainly in pilosebaceous skin follicles[1]. The bacterium promotes propionic acid by the fermentation of sebum triglycerides, which lowers skin pH that results in the activation of antimicrobial proteins (AMP) of the skin and inhibits the growth of *S. aureus*. Six main phylotypes are distinguished, such as IA1, IA2 and IC (refer to subspecies acnes), II (subspecies defendens), and III (subspecies elongatum)[29]. Other short-chain fatty acids (SCFAs), such as acetic acid, butyric acid and valeric acid also produced via fermentation, are able to inhibit the growth of various pathogens. Moreover, the presence of certain *C. acnes* may induce Th1 immune response, which significantly improves skin lesions in AD in mice[30].

Pruritus is a relatively common symptom not only in AD, but also among DD patients. *S. aureus* may contribute pruritus by directly activating mast cells via MRGPRX2 receptor as it induces the release of several proinflammatory mediators, such as histamine, ILs, tumor necrosis factor, and prostaglandin D2. This receptor has also been found on sensory neurons and keratinocytes, where it releases IL-6[31,32]. Another study revealed that S. aureus serine protease V8 is a critical mediator in provoking spontaneous pruritus and allokinesis through the activation of proteinase-activated receptor 1 (PAR1) on mouse and human sensory neurons[33].

Our aim was also to assess the bacterial composition of the deeper layers of the skin in DD patients. We found that the relative amount of Staphylococci was slightly higher in the biopsy samples than in the lesional swabs representing the bacterial composition of the outer skin layer. It is already known that these bacteria can penetrate the deeper epidermal and dermal layers and provoke inflammation in AD patients[34,35]. These facts may support our findings and its potential disease-modifying role in DD as well. Additionally, our findings suggest that the deeper in the skin, the relative abundance of *Cutibacterium* species was significantly decreased.

In this study, we also investigated the intraepidermal skin microbiome in lesional seborrheic regions of DD patients, whereas we observed a prominent increase in the relative amount of *S. aureus* and *S. epidermidis* compared to the corresponding lesional superficial layers. As shown in Fig. 4b, the relative abundance of various *Staphylococcus* species was nearly identical in non-lesional DD skin, and in healthy individuals (not considering *S. aureus* species, which was almost missing in controls) may suggest that an intact stratum corneum in predilectional non-lesional sites, similar to healthy skin, may be effective in preventing the massive colonization of dominantly *Staphylococcus* species in both superficial and deeper skin layers, but this may change if outer layers of the epidermis are damaged.

In a previous study, a relative increase in *Firmicutes* phylum (e.g. *Staphlyococcus*), and certain genera, such as *Streptococcus* and *Pseudomonas* and a relative decrease in *Actinobacteria* phylum *(e.g. Propionibacterium)* were found in the deeper layers of the stratum corneum in healthy individuals. It also suggested that these deeper skin layers may play an essential role in the recolonization of previously injured skin[20]. However, limited data are available on the relative bacterial abundance of deeper epidermal structures[36].

To the best of our knowledge, there is only one single study on the microbiome composition in DD patients. In this work, moist areas, such as the axillar, inguinal, and submammal regions were investigated. It is important to note that the bacterial composition of moist and sebaceous regions is significantly different in healthy individuals[37].

A limitation of this study was the small number of patients and, due to the lack of a standardized score for DD, we did not classify patients according to disease severity based on clinical evaluation. A further limitation is that viral DNA in biopsy or swab samples were not investigated, however, viruses such as herpes simplex virus 1 (HSV1) and SARS-CoV-2 may potentially contribute to the development of DD through the elevated levels of cytokines including TNF-α, IL-6, and iL-1β[38,39]. An interesting study has shown that barrier dysfunction in three dimensional *in vitro* model of DD permitted the invasion of HSV1, moreover, the production of INF-β and antiviral factor ISG15 were also suppressed[6.] Another previous study has demonstrated that IL-6 downregulated *ATP2A2* mRNA levels *in vitro* that may contribute to the further impairment of the function of an already impaired SERCA pump[38]. In addition, skin biopsies were not acquired from non-lesional skin areas of DD patients and controls. Further studies are needed to determine the exact bacterial composition in deeper skin layers and its role in the pathogenesis of DD.

Methods

Subject recruitment

Patients were recruited at the Department of Dermatology, Venerology and Dermatooncology, Semmelweis University between February 2022 and May 2023. Sample collection began after ethical approval from the Ethics

Committee of Semmelweis University (SE RKEB: 278/2021). All procedures performed in the studies involving human participants were in accordance with the ethical standards of the Institutional National Research Committee and with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards, and participants gave written informed consent for sample collection and analysis for research purposes.

Inclusion criteria were a histopathological diagnosis with possible molecular genetic verification of DD, minimum age of 18 or above, active skin lesions and co-existing symmetric non-lesional skin areas. Exclusion criteria for the control group were skin infections (any localization), any chronic skin disease, and for all groups, any reason for taking antibiotics or immunosuppressive medications in the three months or using local antibiotics or antiseptics in the three days prior to the sample collection. All participants gave written informed consent for this study.

Sample collection

Skin biopsies and swabsamples were collected for analysis of the skin microbiome. A 4-mm punch biopsy of a predilectional lesional skin area was performed only in DD patients but not in healthy individuals. For skin swabs, we used a 2-ml DNA/RNA Shield™ Collection Tube w/Swab (Zymo Research Corp. Irvine, CA, USA). Skin swabs were collected from predilectional sites (chest and lower back, both lesional and symmetric non-lesional skin areas from DD patients and corresponding sites from controls). In DD, lesional skin was defined as an area of erythematous skin with typical hyperkeratotic papules and plaques in sebaceous sites, that includes the chest and the lower back. Non-lesional skin refers to the unaffected skin areas in a symmetric or the same body region at least 5 cm away from a well-defined lesional skin. Samples were taken from all affected predilectional regions from each DD patient. For genetic tests, peripheral blood samples were collected from patients in Vacuette K3EDTA tubes (Greiner Bio One, Kremsmünster, Austria).

DNA isolation

DNA isolation was performed using the ZymoBIOMICS DNA Miniprep Kit (Zymo Research Corp., Irvine, USA) according to the protocol of the manufacturer. Genomic DNA concentration was measured using a Qubit2.0 Fluorometer and Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Isolated DNA samples were placed at − 80 °C until PCR amplification. The V3-V4 region of the bacterial 16S rRNA gene was amplified using tagged primers matching the given region. DNA purifications after PCR were performed according to the Illumina protocol. Tagged libraries were evaluated using DNA 1000 Kit with Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). Equimolar concentrations of tagged PCR products were pooled and sequenced using an Illumina MiSeq platform (Illumina, San Diego, CA, USA), using MiSeq Reagent Kit v3 (600 cycles PE). Extraction negative controls and PCR negative controls were included in each run to assess foreign DNA contamination from the reagents. All samples were independently extracted and sequenced twice to verify reproducibility. For evaluation, raw sequencing data retrieved from Illumina BaseSpace were uploaded to the CosmosId Bioinformatics Platform (CosmosID Metagenomics Cloud, app.cosmosid.com, CosmosID Inc., www.cosmosid.com). For genetic analyses, genomic DNA was isolated from peripheral blood leukocytes using the Roche MagNA Pure Compact Nucleic Acid Isolation Kit I and the Roche MagNA Pure Compact LC System (Roche Diagnostics, Mannheim, Germany).

Genetic analyses

Sanger sequencing of the *ATP2A2* gene (RefSeq Gene: NG_007097.2, RefSeq mRNA: NM_170665.4, var b) exons and corresponding boundary intron regions were performed as described previously (Ref: Racz et al. 2004, https://doi.org/10.1111/j.0906-6705.2004.00118.x). Identified *ATP2A2* variants were analyzed using Mutation taster (www.mutationtaster.org) and ACMG classification (pathogenic or likely pathogenic) of variants was performed using the Mutation taster and ClinVar (www.ncbi.nlm.nih.gov/clinvar) databases.

Statistical analysis

Statistical significance between cohorts was determined using the Wilcoxon rank sum test for Shannon alpha diversity and PERMANOVA analysis for Jaccard Principal Coordinate Analysis (PCoA) of beta diversity using the CosmosID statistical analysis support application (CosmosID Metagenomics Cloud, app.cosmosid.com, CosmosID Inc., www.cosmosid.com).

Data availability

The datasets generated in the current study are available in the Short Read Archive (SRA) of National Center for Biotechnology Information (BioProject ID: PRJNA1070566) at http://www.ncbi.nlm.nih.gov/bioproject/1070566. Requests for data should be addressed to the corresponding author (D.P.)

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Author contributions

Author contributions: Each author has given final approval of the submitted manuscript. Conception and design: M.M., E.O., N.K., D.P.; Acquisition of patient data and samples: M.M., N.K., A.B., Zs. M., D.P.; Laboratory processing of samples: E.O., N.M., Zs.D.; Analysis and interpretation of data: D.P., N.K., M.M., E.O., B.M.; Statistical analysis: E.O.; Drafting of the manuscript: E.O., M.M., N.K., D.P., P.H.; Obtaining funding: M.M.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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