

Letter to the Editor

Gram-positive anaerobe cocci are underrepresented in the microbiome of filaggrin-deficient human skin

To the Editor:

Next-generation sequencing technologies and powerful bioinformatics tools have revealed that diversity and composition of the skin microbiota of healthy volunteers strongly depends on the topographical location on the body and has a high degree of interpersonal variation.¹ Nevertheless, the dominant types of bacteria remain relatively stable over time, and specific bacteria are associated with dry, moist, and/or sebaceous

microenvironments.² Microbial communities, genetic host factors, and the environmental factors at a particular moment constitute a complex relationship that is essential for skin barrier homeostasis.³ More recent studies have also focused on the microbiota of diseased and injured skin as alterations to microbial communities have been associated with cutaneous disorders.^{4,5}

In the present study, we selected ichthyosis vulgaris (IV) as a model disease to investigate whether genetic polymorphisms resulting in altered stratum corneum (SC) composition and structure affect the microbiota composition of human skin and the cutaneous host response. Genetic deficiency or haploinsufficiency of the histidine-rich epidermal protein filaggrin (*FLG*) is associated with IV and atopic dermatitis (AD).^{6,7} Here, we report 2 novel findings with respect to *FLG* deficiency and cutaneous microbiota. First, we show that *FLG* deficiency is associated with a low relative abundance of proteolytic Gram-positive anaerobic

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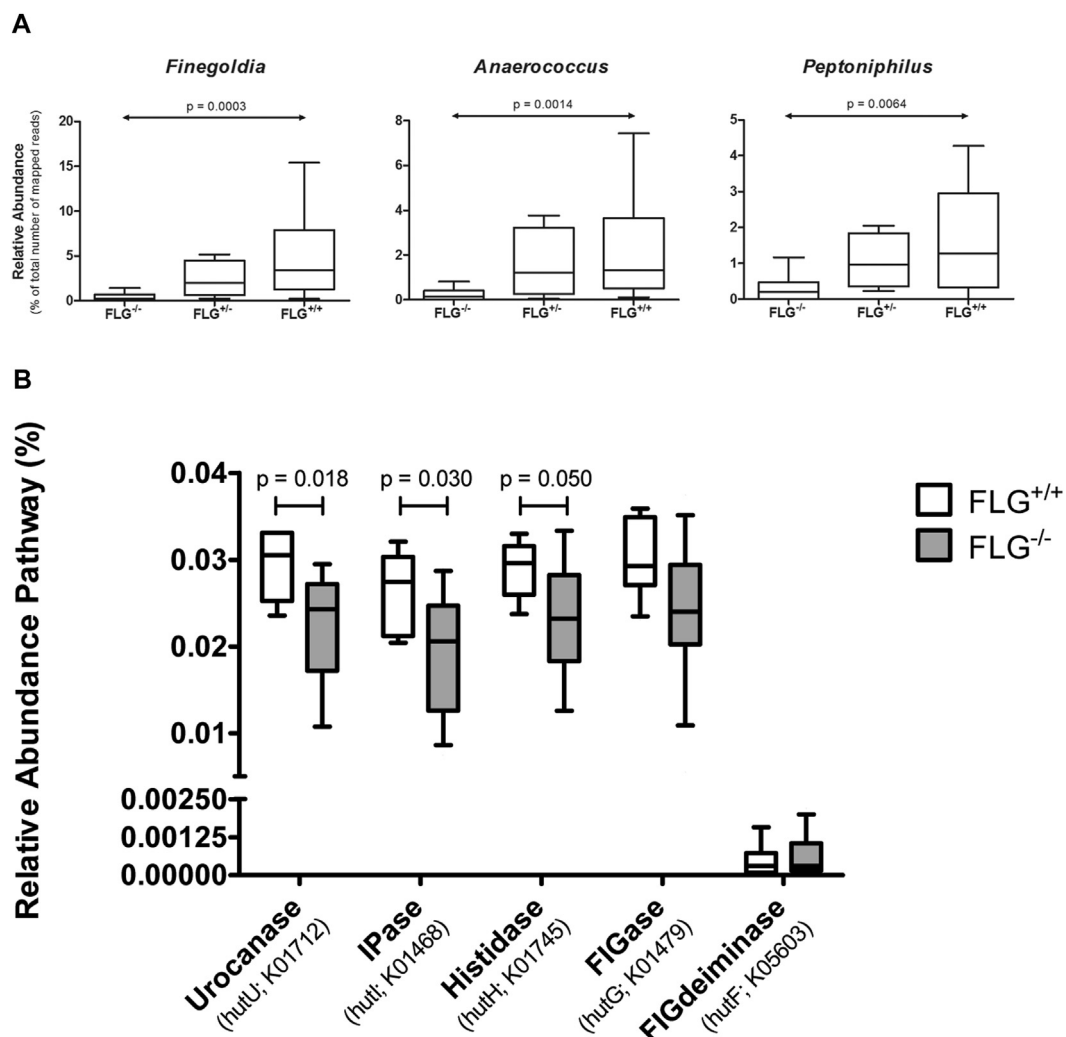


FIG 1. Lower relative abundance of proteolytic GPAC. **A**, Differences in relative abundance between *FLG*^{+/+} and *FLG*^{-/-} genotypes for the genera *Finegoldia*, *Anaerococcus*, and *Peptoniphilus*. Horizontal arrows indicate significant differences (Mann-Whitney *U* test). In *FLG*^{+/-} individuals, these genera tended to be present at intermediate relative abundance quantities. **B**, Box plots with relative abundances of the candidate *hut* genes for *FLG*^{+/+} and *FLG*^{-/-} genotypes.

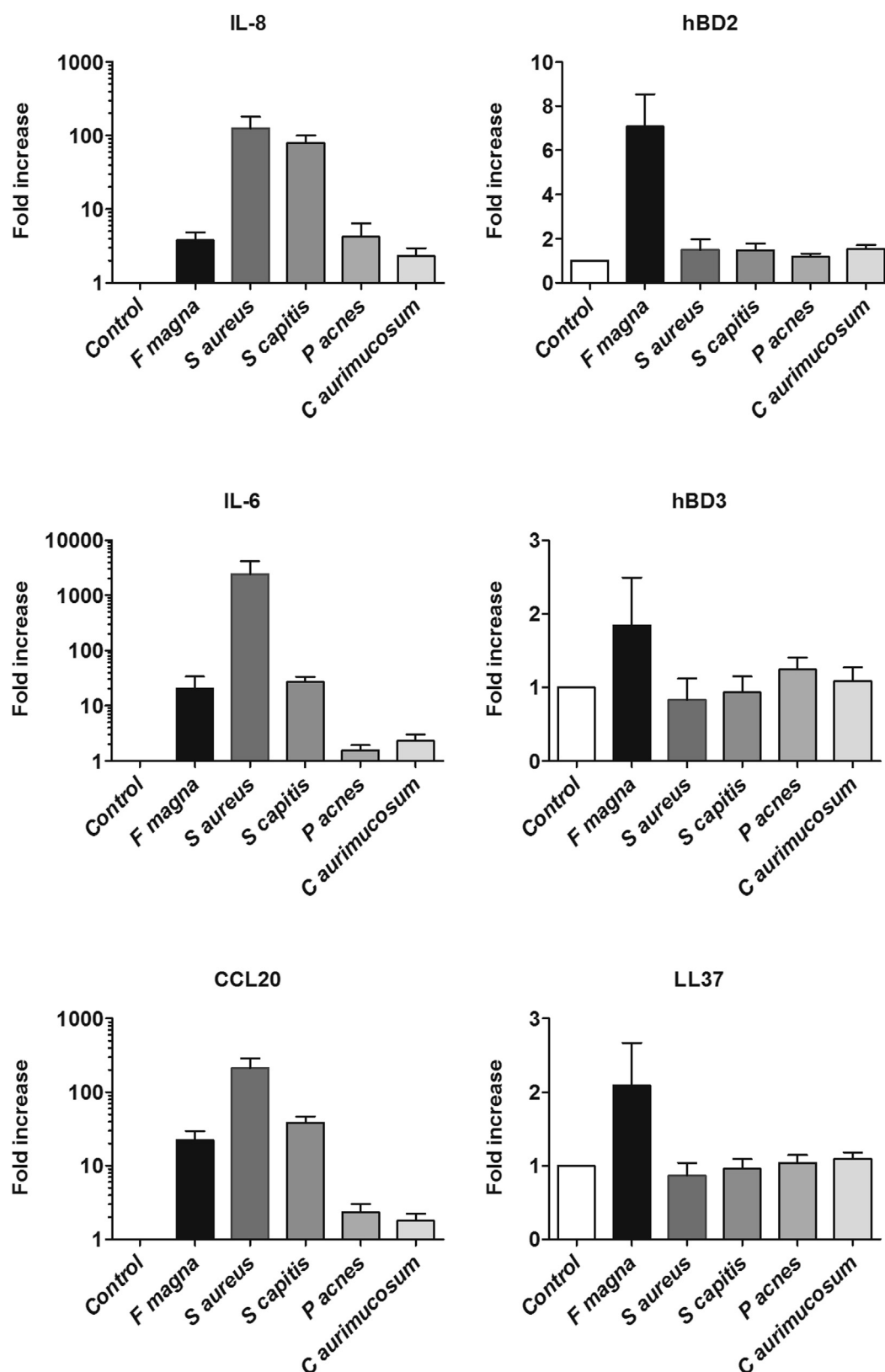


FIG 2. Keratinocyte responses differ between bacterial stimulations. *FLG*^{+/+} keratinocyte cultures (n = 10) were stimulated for 10 hours with bacteria. Expression of 6 host defense genes was measured by quantitative PCR. For each gene, the unstimulated cell culture was set to 1. Data are represented as mean $\pm$ SEM. For statistics (repeated-measures ANOVA with Bonferroni *post hoc* test), see [Table E5](#).

cocci (GPAC), and a general underrepresentation of bacterial taxa that are capable of using histidine. Second, we report that exposure of cultured epidermal keratinocytes to *Fingoldia magna* induces the expression of antimicrobial proteins and proinflammatory cytokines, and that this response is distinct from other skin commensals such as *Propionibacterium acnes*, *Corynebacterium aurimucosum*, and *Staphylococcus capitis*, and the skin pathogen *Staphylococcus aureus*.

All experimental procedures were performed as described in this article's Online Repository at www.jacionline.org. We analyzed the skin microbiota of the lower leg, which is typically a location where the ichthyotic skin alterations (dry and scaly skin) are most prominent. Patients and healthy controls were genotyped for *FLG* mutations (see Table E1 and Fig E1 in this article's Online Repository at www.jacionline.org). None of the patients with IV in our study had eczematous lesions present at the lower leg, thereby excluding (lesional) AD-associated microbiome alterations as a confounding factor. Biophysical measurements showed increased transepidermal water loss and decreased SC hydration in *FLG*^{-/-} subjects compared with *FLG*^{+/+} subjects (see Fig E2 in this article's Online Repository at www.jacionline.org). Microbiome samples were subsequently analyzed by barcoded 16S marker gene sequencing (see Tables E2 and E3 in this article's Online Repository at www.jacionline.org). Rarefaction curves show that the phylogenetic diversity does not differ between *FLG*^{+/+}, *FLG*^{+/-}, and *FLG*^{-/-} samples (see Figs E3 and E4 in this article's Online Repository at www.jacionline.org). Redundancy analysis revealed a significant effect of *FLG* deficiency on microbiota composition (see Fig E5 in this article's Online Repository at www.jacionline.org). A lower relative abundance of GPAC (average 1.7% of the total genera) was found in *FLG*^{-/-} skin compared with *FLG*^{+/+} skin (average 9.3% of the total genera) (see Fig E6 and Excel file E1 in this article's Online Repository at www.jacionline.org). These genera included *Fingoldia*, *Anaerococcus*, and *Peptoniphilus*. Other highly abundant genera did not show significant differences between the genotypes (Excel file E1). We therefore focused on members of the GPAC group for further *in silico* analysis and experimental studies. Most GPAC are asaccharolytic and use the products of protein degradation as substrates for metabolic energy generation.⁸ The average relative quantities of these candidate-discriminating genera supported a significant difference between the *FLG*-deficient and *FLG*-proficient state, or more specifically, a strong decrease by *FLG* protein loss (Fig 1, A). Indeed, an *in vitro* survival disadvantage of *F magna* on *FLG*-deficient SC supported the *in vivo* findings (see Figs E7 and E8 in this article's Online Repository at www.jacionline.org). On the basis of bacterial 16S profiles of the skin microbiota, we tested the potential involvement of microbial reactions using natural moisturizing factors such as histidine, urocanic acid, and pyrrolidone carboxylic acid. Histidine is a known carbon source for bacteria that possess the histidine utilization (*Hut*) pathway (see Fig E9 in this article's Online Repository at www.jacionline.org), which is highly conserved among bacteria and found with high frequency in most phylogenetic groups within the bacterial domain. Contrast analysis of IV versus healthy skin with selected *hut* gene candidates (see Table E4 in this article's Online Repository at www.jacionline.org) revealed that *hutU*, *hutI*, and *hutH* orthologous genes were predicted to be significantly underrepresented in the microbiota of patients with

IV (Fig 1, B; and Excel file E2 in this article's Online Repository at www.jacionline.org). In conclusion, our study reveals that *FLG*-deficient skin harbors significantly fewer bacterial taxa capable of using histidine as nutrient (carbon) source, which we postulate to be a consequence of nutrient competition driven by the loss of the histidine-rich protein *FLG*.

Because microbial factors such as *S aureus* colonization have been implicated in AD,⁹ we explored possible interactions of this pathogen and several skin commensals, including *F magna*, with epidermal keratinocytes (Fig 2; and Table E5 in this article's Online Repository at www.jacionline.org). *F magna* induced a significantly higher expression of antimicrobial peptides (AMPs) compared with *S aureus* and the other skin commensals. For *S aureus* and *S capitis*, keratinocyte stimulation caused a significantly higher expression of proinflammatory cytokines than was observed for stimulation with *F magna*. Our observation that keratinocytes appear to exhibit different cytokine/AMP responses toward *S aureus* or *F magna* stimulation is intriguing. Given the known functions and activities of IL-8 (neutrophil chemoattractant), IL-6 (inducer of inflammatory responses), and the chemokine ligand 20 (CCL20, chemoattractant for various white blood cell types, antimicrobial activity), their strong induction by *S aureus* contributes to control of infection. The observed rapid induction of AMPs by *F magna*, however, suggests that this may be an important signaling mechanism to the keratinocytes when the skin barrier is breached and a commensal bacterium comes into close contact with the epidermal keratinocytes. Complete or partial absence of *F magna* could cause an impaired or delayed danger signaling to the keratinocytes in *FLG*^{-/-} or *FLG*^{+/-} individuals. This could, speculatively, be a mechanism that favors *S aureus* colonization or infection, but clearly requires further investigation.

In conclusion, we have uncovered new and potentially important biological aspects of a very common genetic polymorphism. Our study has shown this to be the case for the homozygous-deficient *FLG* state, but our data on heterozygotes suggest a gene dosage effect. This generates new and testable hypotheses to investigate a contribution of microbiome alterations in the pathogenesis of AD.

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Supplemental Figures

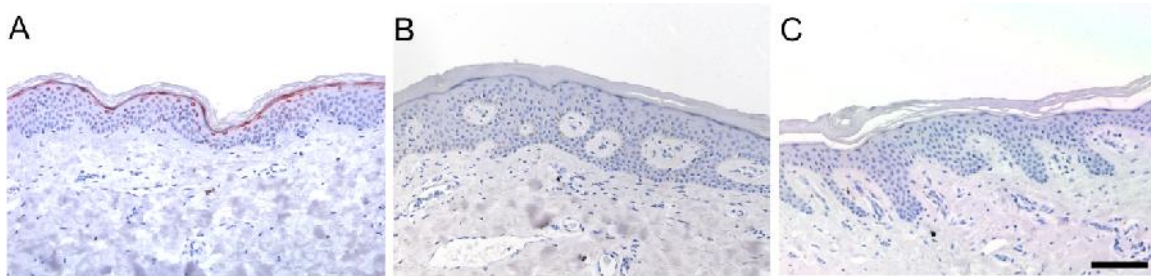


Fig E1. Immunohistochemical filaggrin staining of upper buttock skin. **A**, *FLG*^{+/+} individuals show filaggrin staining in the stratum granulosum layer of the epidermis. Both keratohyalin granules and nuclei are positive. **B**, Skin from IV patients homozygous or compound heterozygous for the *FLG* R501X and 2282del4 mutations (*FLG*^{-/-}) were negative for FLG protein staining. **C**, Genotyped patients wherein only one mutation was found (R501X, for n=2), were tested for FLG expression. When negative, the patient still has to have an unknown mutation somewhere else in the *FLG* gene (i.e. R501X/?) and consequently was classified in the FLG deficient group (*FLG*^{-/-}). Scale bar = 100 μ m.

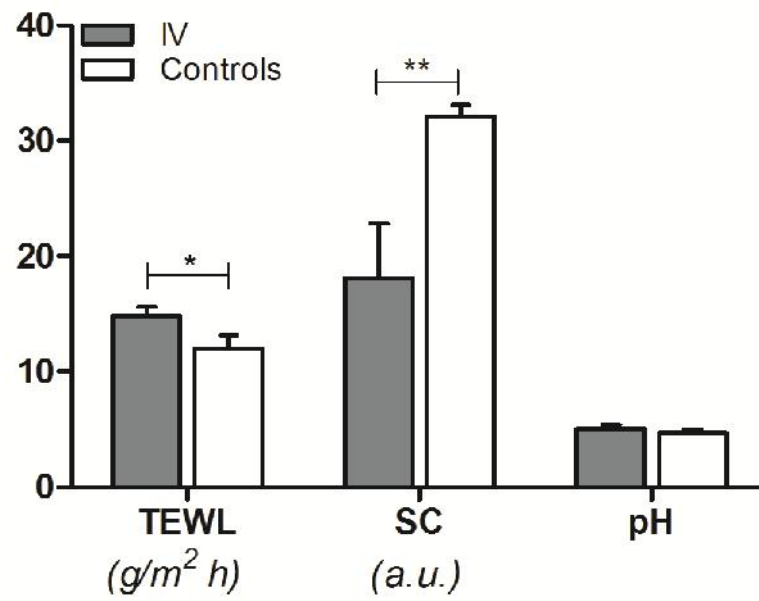


Fig E2. Biophysical measurements on the lower leg skin. Transepidermal water loss (TEWL), stratum corneum hydration (SC) and pH of the skin surface was measured on the lower leg of IV patients (n=5) and healthy controls (n=6). a.u. = arbitrary units. Data are represented as mean $\pm$ SEM. Mann Whitney U test. *p < 0.05, **p < 0.01.

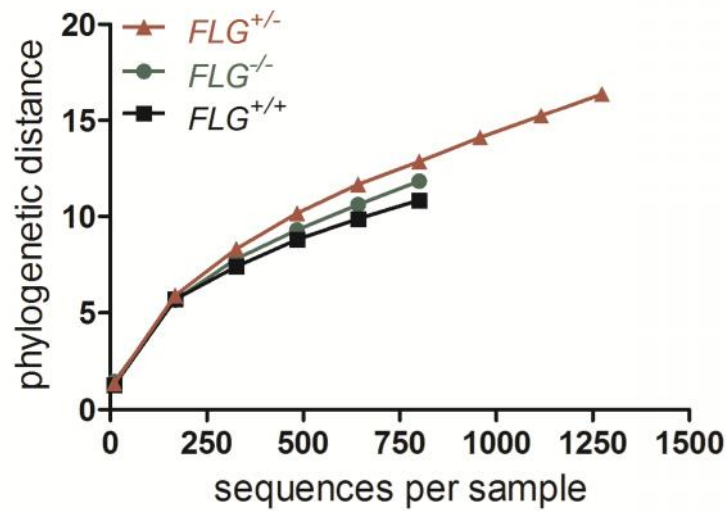


Fig E3. Microbial diversity of samples between different FLG genotypes. Phylogenetic diversity rarefaction curves for communities sampled from the lower leg skin do not show differences between *FLG*^{+/+}, *FLG*^{-/-} and *FLG*^{+/-} individuals. Note that, as sequencing depth is inherently biased by read count (i.e. a higher number of reads in a sample increases the chance of finding additional new biodiversity, that was not before observed in that sample), the rarefaction curve calculations shown in this graph are for each experimental group limited by the one sample within that group that has the lowest number of reads (sequences per sample). For this reason, *FLG*^{+/-} has three more measurement points than the *FLG*^{+/+} and *FLG*^{-/-} genotypic groups.

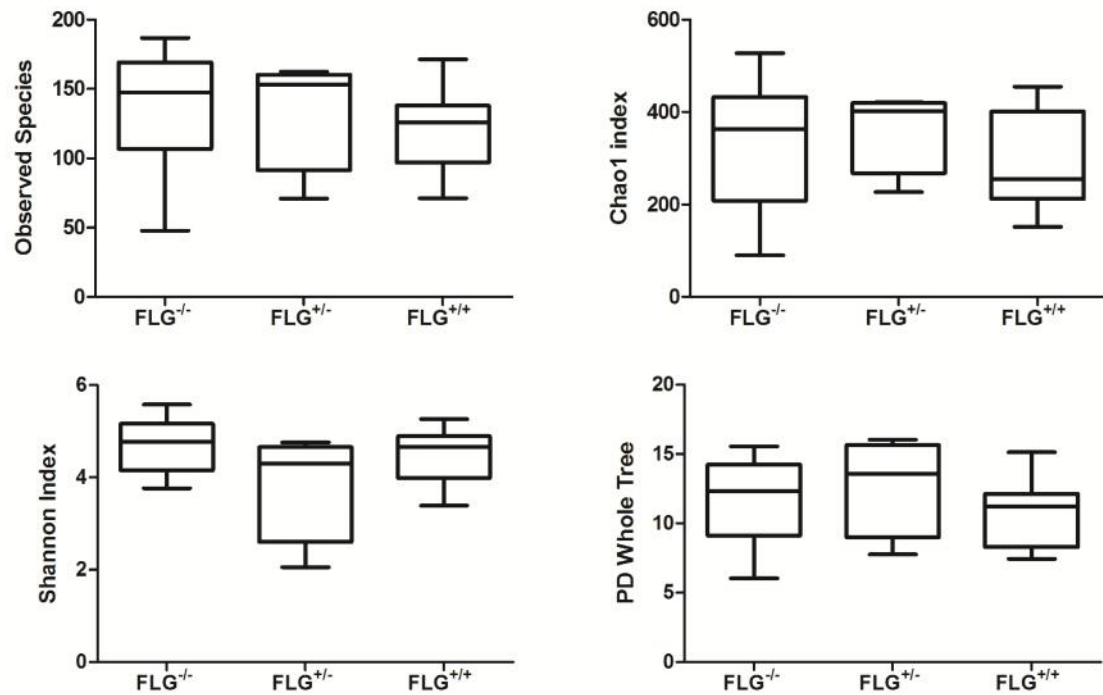


Fig E4. Alpha diversity metrics in *FLG* genotypes. No differences in microbial diversity of superficial skin microbiota of hetero- and homozygous *FLG* genotype and unaffected control subjects were observed as tested for whole tree phylogenetic diversity, observed OTUs, Shannon, and Chao1.

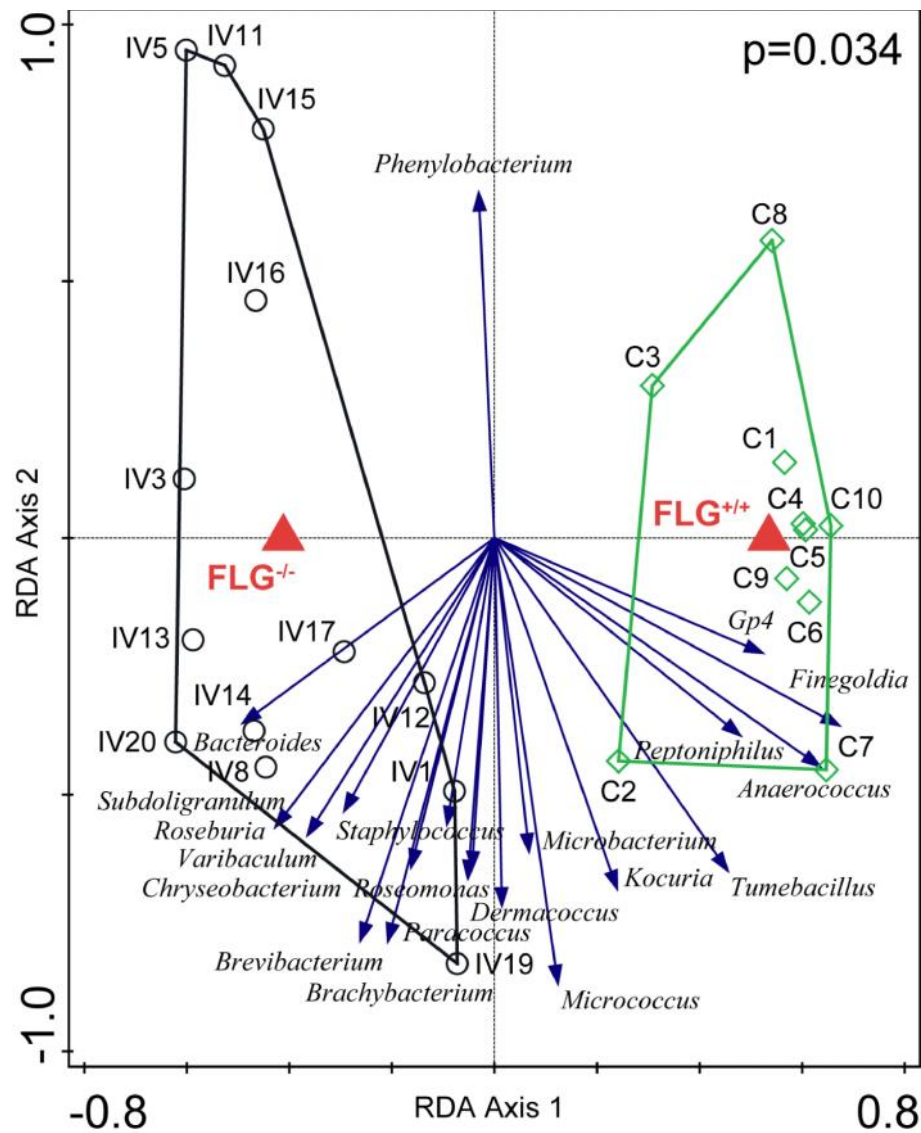


Fig E5. Skin microbiota from *FLG*^{+/+} and *FLG*^{-/-} individuals are significantly different. The figure shows a Redundancy Analysis (RDA) triplot. Green diamonds and black circles represent *FLG*^{+/+} and *FLG*^{-/-} samples, respectively. Red triangles are the centroids of the corresponding sample groups. The blue arrows are the 20 best-fitting genera (names in italic), i.e. best explaining microbiota compositional differences between the *FLG* contrast. The horizontal axis maximizes the variation in sample groups (in contrast to a principal component analysis plot, where the variation between individual samples is maximized). The difference in microbiota is significant (according to a permutation test; $p = 0.034$), i.e. randomly assigning samples to sample groups and repeating the RDA analysis produces a plot where the difference between sample groups is smaller than in the real data.

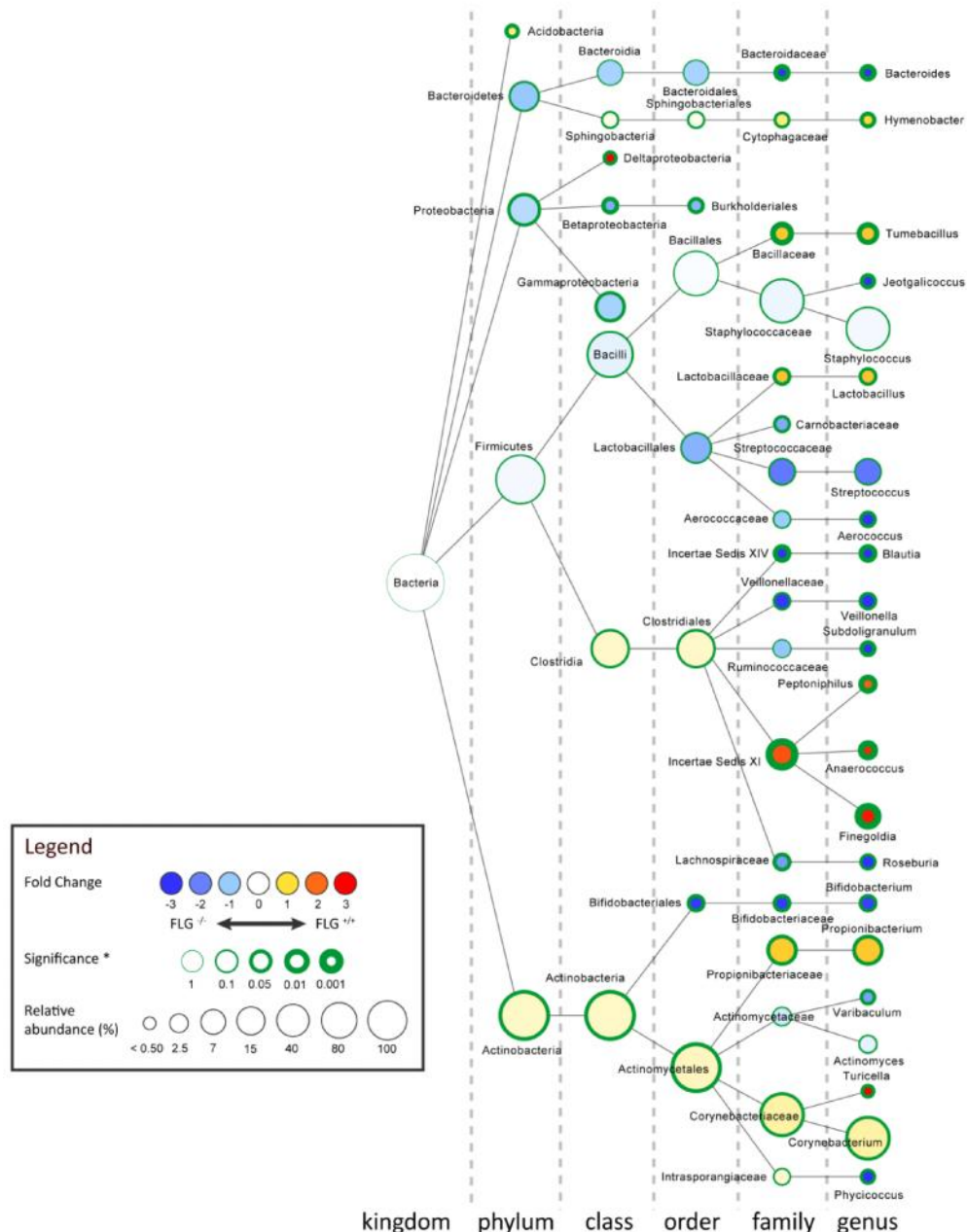


Fig E6. Difference in microbial community composition between *FLG*^{+/+} and *FLG*^{-/-} individuals. Nodes represent taxa, edges link the different taxonomic levels. Node color indicates the difference in abundance (red is more abundant in *FLG*^{+/+}, blue in *FLG*^{-/-}), calculated as the 2log of the ratio of the relative abundance (0 = no difference between genotypes, 1 = twice as abundant in *FLG*^{+/+}, and so on). The significance is represented by the thickness of the node borders and expressed as the p-value of a Mann-Whitney U test of the samples. The size of a node represents the average relative abundance of a taxon (note that the relation between node-size and abundance is non-linear).

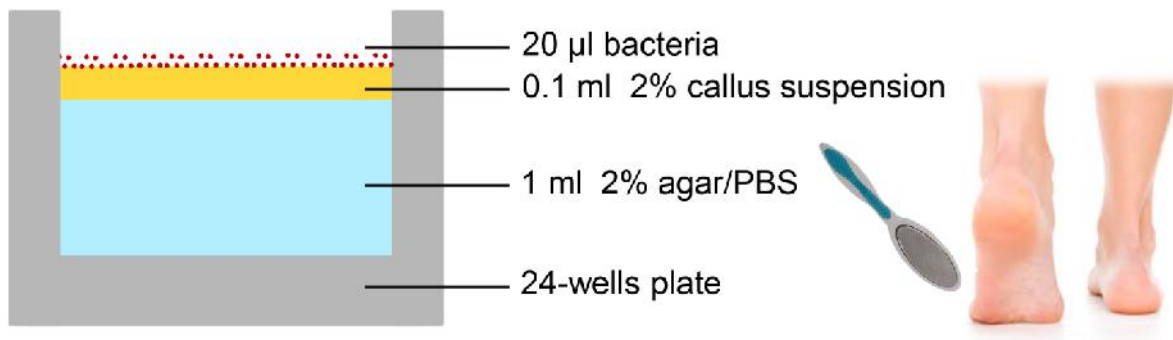


Fig E7. *In vitro* stratum corneum model. The model is prepared in a 24-wells plate. 2% agar (in PBS) serves as a basis to position the 2% callus suspension originating from the heel of healthy volunteers (*FLG*^{+/+} and *FLG*^{-/-}). After drying, a thin layer of dead corneocytes will be present on top of the agar, upon which the bacteria are cultured.

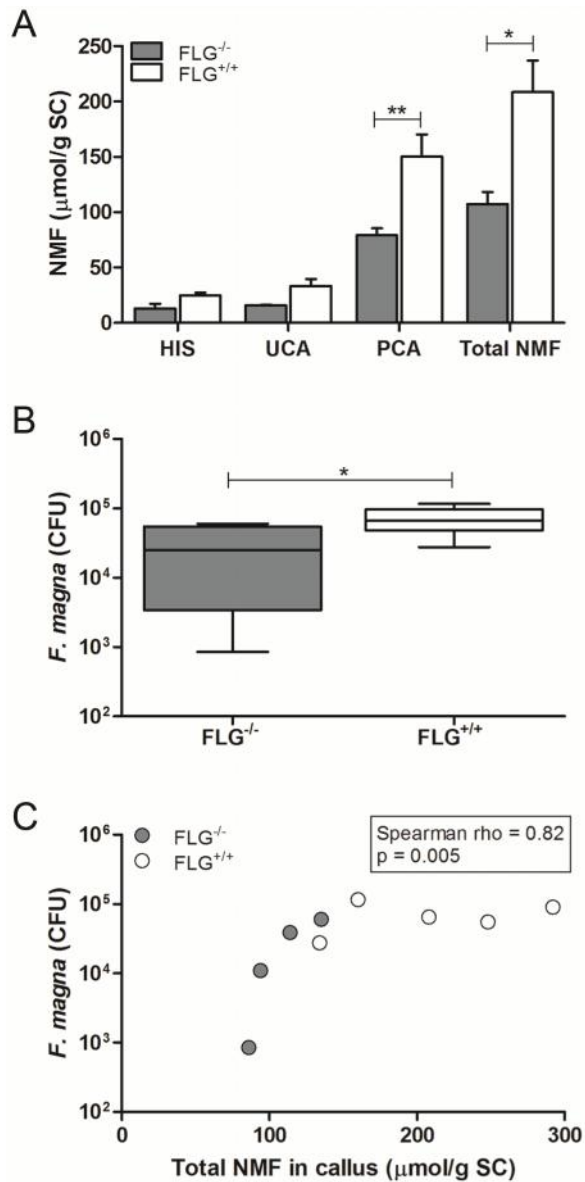


Fig E8. Lower total NMF levels in callus affect *in vitro* growth of *Finogoldia magna* bacteria. **A**, NMF content analysis of callus derived from FLG^{-/-} (n=4) and FLG^{+/+} (n=5) individuals. Data are represented as mean ± SEM. Mann-Whitney U test. *p < 0.02, **p < 0.01 (HIS, p = 0.06; UCA, p = 0.08). HIS, histidine; PCA, pyrrolidone carboxylic acid, UCA, urocanic acid. **B**, *F. magna* growth on the *in vitro* stratum corneum model using callus derived from FLG^{-/-} (n=4) and FLG^{+/+} (n=6) individuals. Data are represented as mean ± SEM. Mann-Whitney U test. *p < 0.03. *S. aureus* growth on the model did not show significant differences between FLG^{-/-} and FLG^{+/+} callus (data not shown). **C**, Correlation between the growth rate of *F. magna* and the NMF content of FLG^{-/-} and FLG^{+/+} callus on which the bacteria are grown.

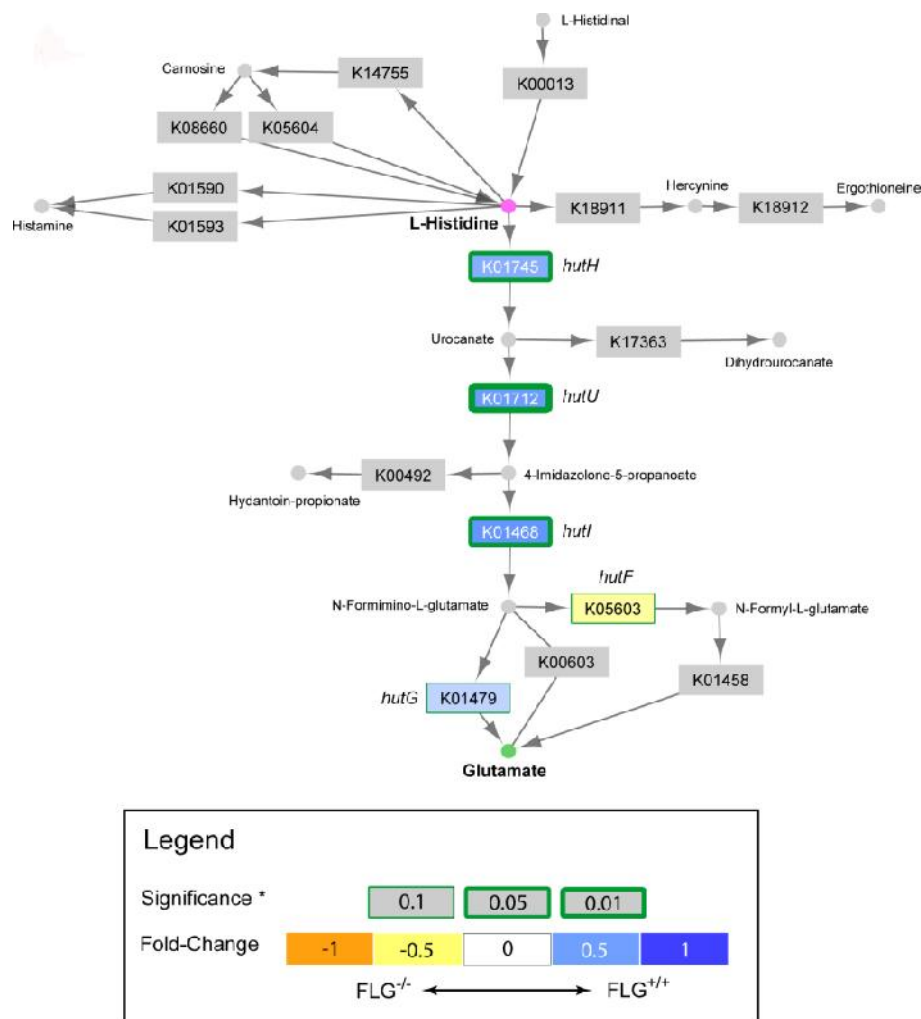


Fig E9. Function analysis of the skin microbiota reveals a decreased histidine utilization capacity in FLG deficient patients. KEGG map of histidine metabolism (snapshot) and the candidate *hut* genes. The Hut operon contains several *hut* genes present as orthologs in a wide range of bacterial species, that allow loss-of-function for conversion of histidine to glutamate by different routes.¹ In the first phase, three core *hut* genes encoding a histidase (*hutH*), a urocanase (*hutU*) and an imidazolone-5-propionate hydrolase (IPase, *hutI*) are involved in catabolic conversion of histidine to formimino glutamate (FIG). In the second phase, FIG is converted to glutamate, either directly by the *hutG* encoded FIGase, or indirectly via the intermediate formyl glutamate by the *hutF* encoded FIGdeiminase. Nodes represent compounds, the pink node represents our compound of interest: histidine; the green node represents the Hut pathway its end product: glutamate. Boxes represent enzyme functions/reactions, numbers shown inside are K numbers (i.e. KEGG Orthologs). Colored boxes correspond to *hut* gene candidates that were *a priori* selected for function analysis by PICRUST: blue indicates a decrease in the relative abundance of a *hut* gene as effect of FLG deficiency, and orange indicates an increase in these genes compared to wild type; gray boxes were excluded from analysis. This color representation of the fold-change difference is calculated as the 2log of the ratio of the relative abundance between FLG wild type and knockout (0 = no difference between genotypes, 1 = twice as abundant in wild type, etcetera). The significance (box border width) is expressed as the *p*-value of a Mann–Whitney U test with Bonferroni’s correction for multiple testing. Arrows link the conversion of one compound to another, facilitated by an enzyme function/reaction.

Supplemental Tables

Table E1. Cohort of IV patients and healthy controls

Sample ID	Diagnosis	Genotype <i>FLG</i>	Mutation	Sex (M/F)	Age (years)
IV1	IV	-/-	R501X, 2282del4	M	66
IV2	IV+AD	+/-	R501X, wild-type	M	20
IV3	IV+AD	-/-	R501X, unknown mutation	M	82
IV4	IV+AD	+/-	2282del4, wild-type	F	48
IV5	IV	-/-	2284del4, 2282del4	M	52
IV6	IV+AD	+/-	2282del4, wild-type	M	22
IV8	IV+AD	-/-	R501X, 2282del4	M	31
IV9	IV+AD	+/-	2282del4, wild-type	F	46
IV11	IV	-/-	R501X, unknown mutation	M	70
IV12	IV+AD	-/-	R501X, 2282del4	M	36
IV13	IV	-/-	2284del4, 2282del4	M	21
IV14	IV+AD	-/-	R501X, 2282del4	F	51
IV15	IV+AD	-/-	R501X, 2282del4	F	55
IV16	IV	-/-	R501X, R501X	F	51
IV17	IV+AD	-/-	R501X, 11033del4 *	F	27
IV19	IV	-/-	R501X, 2282del4	M	60
IV20	IV+AD	-/-	R501X, 2282del4	M	26
C1	healthy	+/+	wild-type	F	36
C2	healthy	+/+	wild-type	M	28
C3	healthy	+/+	wild-type	M	31
C4	healthy	+/+	wild-type	M	42
C5	healthy	+/+	wild-type	M	33
C6	healthy	+/+	wild-type	F	47
C7	healthy	+/+	wild-type	F	26
C8	healthy	+/+	wild-type	M	59
C9	healthy	+/+	wild-type	F	60
C10	healthy	+/+	wild-type	M	53

AD, atopic dermatitis; C, control; IV, ichthyosis vulgaris; M, male; F, female. * 11033del4 mutation genotyped by MUMC.² *FLG*-/- individuals in red, *FLG*+/- individuals in black, and controls (*FLG*+/+) in green. Mean age $\pm$ SD for patients vs. controls is 45 ± 19 and 42 ± 13 years. Male to female ratio is not significantly different between patients and controls (11/6 vs. 6/4, Chi square p-value = 0.81). 65% (11/17) of IV patients also suffers from AD.

Table E2. Microbiota analysis of the lower leg

Microbiome lower leg	Reads	Percentage
Total reads ^a	43137	100
Assigned at phylum level	42914	99.5
Assigned at genus level	39085	90.6

^a N=27 individuals (10 healthy controls and 17 IV patients).

Table E3. Read and OTU counts

Subject	Reads ^a	OTUs ^b	Barcode ^c
C1	913	135	acctgt
C2	2102	268	acgact
C3	1373	199	acgcat
C4	1755	227	acgcgc
C5	811	76	acggca
C6	1772	108	acgtgg
C7	2110	210	actaac
C8	1635	169	actagt
C9	1828	290	actcgg
C10	2120	183	actgat
IV1	1556	276	aacatg
IV2	1624	250	aaccgg
IV3	1868	309	aacgct
IV4	1292	219	aactaa
IV5	1381	200	aagatc
IV6	3471	202	aaggag
IV8	2077	272	aagtcg
IV9	1958	276	aataag
IV11	1361	100	aatcca
IV12	1536	188	aatgac
IV13	1508	218	aatgta
IV14	952	181	acaatg
IV15	906	52	acactt
IV16	1975	149	acagct
IV17	969	153	acatac
IV19	1182	233	accata
IV20	1102	196	accgga

^a Number of reads after quality control and removal of chimeric sequences

^b Number of OTUs at an identity threshold of 97%

^c The six-nucleotide tag used to identify individual samples in the pooled sequencing reads

Table E4. Selection of Hut pathway genes

Protein	Gene	Enzyme Code	K number ^a	p-value ^b	Rank ^c
Urocanase	<i>hutU</i>	EC:4.2.1.49	K01712	0.0175	41
IPase	<i>hutI</i>	EC:3.5.2.7	K01468	0.0301	80
Histidase	<i>hutH</i>	EC:4.3.1.3	K01745	0.0500	116
FIGase	<i>hutG</i>	EC:3.5.3.8	K01479	0.1685	379
FIGdeiminase	<i>hutF</i>	EC:3.5.3.13	K05603	1.7750	4200

^a According to the KEGG orthology group numbering

^b p-value by Mann–Whitney U, Bonferroni corrected, for *FLG*^{+/+} versus *FLG*^{-/-}

^c Rank according to overrepresentation analysis based on p-value (for 5838 total K numbers)

Table E5. Relative mRNA expression levels of proinflammatory cytokines, chemokines and AMPs after bacterial stimulation of cultured primary keratinocytes

<i>mRNA expression keratinocyte cultures: relative quantity compared to unstimulated control</i>					
Gene	<i>F magna</i>	<i>S aureus</i>	<i>S capitis</i>	<i>P acnes</i>	<i>C aurimucosum</i>
IL-6	20.3±13.8	2433.3±1711.0	27.0±6.4	1.6±0.4	2.3±0.7
IL-8	3.8±1.1	126.5±52.3	79.0±21.5	4.2±2.2	2.3±0.7
CCL20	22.4±7.3	211.6±74.5	38.5±8.3	2.3±0.7	1.8±0.5
hBD2	7.1±1.5	1.5±0.5	1.5±0.3	1.2±0.1	1.5±0.2
hBD3	1.8±0.7	0.8±0.3	0.9±0.2	1.2±0.2	1.1±0.2
LL37	2.1±0.6	0.9±0.2	1.0±0.1	1.0±0.1	1.1±0.1
<i>Significance (p-value)^a</i>					
IL-6	ns	< 0.001	< 0.001	ns	ns
IL-8	ns	< 0.001	< 0.001	ns	ns
CCL20	< 0.001	< 0.001	< 0.001	ns	ns
hBD2	< 0.001	ns	ns	ns	ns
hBD3	< 0.05	ns	ns	ns	ns
LL37	ns	ns	ns	ns	ns

^a p-value by repeated measures ANOVA with Bonferroni's post-hoc test on the ΔCt values of the qPCR data.
ns, not significant

Table E6. qPCR primers

HUGO Gene Symbol	ENSEmble Transcript no.	Description Gene/Protein	Forward primer (5' ÷ 3')	Reverse primer (5' ÷ 3')
CAMP	ENST00000296435	cathelicidin, LL-37	ccaggcccacgatggat	accagcccgctccttctga
CCL20	ENST00000358813	chemokine ligand 20, MIP3- α	tggccaatgaaggctgtga	gatttgcgacacagacaactt
DEFB103	ENST00000318124	human β -defensin-3, hBD3	gtgaagcctagcagctatgaggat	tgattcctccatgacctggaa
DEFB4	ENST00000318157	human β -defensin-2, hBD2	gatgcctcttcagggttttt	ggatgacatatggctccactctt
IL6	ENST00000258743	interleukin 6, IL-6	agccctgagaaaggagacatgta	ggcaagtctcctcattgaatcc
IL8	ENST00000307407	interleukin 8, IL-8, CXCL8	agaagttttgaagagggtgaga	agtttactggcatcttactgatt
RPLP0	ENST00000228306	ribosomal phosphoprotein P0	caccattgaaatcctgagtgatgt	tgaccagcccaaaggagaag

Supplemental Experimental Procedures

Study approval

All volunteers in this study were selected according to the inclusion/exclusion criteria as approved by a protocol from the *National Institutes of Health* Human Microbiome Project. The exact inclusion/exclusion criteria and study procedures that we presented to the volunteers can be found below. In advance, medical ethical committee (Commissie Mensgebonden Onderzoek Arnhem-Nijmegen) approval and individual written informed consent were obtained. The study was conducted according to the Declaration of Helsinki principles.

Exclusion / Inclusion Criteria

Any subject who meets any of the following criteria will be excluded from participation in this study. Keep in mind that these criteria are written for healthy controls! IV patients can have eczematous lesions (obviously) and therefore this not an exclusion criterion for this group

- Body Mass Index greater than or equal to 35 or less than or equal to 18.
- Use of any of the following drugs within the last 6 months:
 - systemic antibiotics (intravenous, intramuscular, or oral);
 - oral, intravenous, intramuscular, nasal or inhaled corticosteroids;
 - cytokines;
 - methotrexate or immunosuppressive cytotoxic agents;
 - large doses of commercial probiotics consumed (greater than or equal to 10^8 CFU or organisms per day) - includes tablets, capsules, lozenges, chewing gum or powders in which probiotic is a primary component. Ordinary dietary components such as fermented beverages/milks, yogurts, foods do not apply.
- Use of topical antibiotics, antifungal or topical steroids within the previous 7 days.
- Acute disease at the time of enrolment (defer sampling until subject recovers). Acute disease is defined as the presence of a moderate or severe illness with or without fever.
- Chronic, clinically significant (unresolved, requiring on-going medical management or medication) pulmonary, cardiovascular, gastrointestinal, hepatic or renal functional abnormality, as determined by medical history or physical examination.
- History of cancer except for squamous or basal cell carcinomas of the skin that have been medically managed by local excision.
- Unstable dietary history as defined by major changes in diet during the previous month, where the subject has eliminated or significantly increased a major food group in the diet.
- Recent history of chronic alcohol consumption defined as more than five 1.5-ounce servings of 80 proof distilled spirits, five 12-ounce servings of beer or five 5-ounce servings of wine per day.
- Confirmed positive test for HIV, HBV or HCV.
- Any confirmed or suspected condition/state of immunosuppression or immunodeficiency (primary or acquired) including HIV infection.
- Major surgery of the GI tract, with the exception of cholecystectomy and appendectomy, in the past five years. Any major bowel resection at any time.
- History of active uncontrolled gastrointestinal disorders or diseases including:
 - inflammatory bowel disease (IBD) including ulcerative colitis (mild-moderate-severe), Crohn's disease (mild-moderate-severe), or indeterminate colitis;
 - irritable bowel syndrome (IBS) (moderate-severe);
 - persistent, infectious gastroenteritis, colitis or gastritis, persistent or chronic diarrhea of unknown etiology, *Clostridium difficile* infection (recurrent) or *Helicobacter pylori* infection (untreated);
 - chronic constipation.
- Female who is pregnant or lactating.
- Treatment for or suspicion of ever having had toxic shock syndrome.
- History of psoriasis or recurrent eczema.
- History of recurrent rashes within the past 6 months.
- At the time of the screening visit or on the specimen collection day:
 - acne at sites other than on the face, chest, back or shoulders;
 - multiple blisters, pustules, boils, abscesses, erosions or ulcers on the scalp, face, neck, arms, forearms or hands;

- a single blister, pustule, boil, abscess, erosion, ulcer, scab, cut, crack or pink/hyperpigmented patch or plaque at or within 4 cm of the sampling sites; sampling may be deferred until the lesion resolves either without treatment or with local treatment only;
- more than one pink/red scaly patch/plaque anywhere on the body (suggestive of psoriasis or eczema);
- uniformly thickened, cracking, “dry” skin on bilateral palms and/or soles;
- scalp dandruff that does not clear up with over-the-counter dandruff shampoos used daily for 2 weeks;
- disseminated rash (at multiple body sites or extending throughout a broad body area).
- Chronic dry mouth.
- Periodontitis / gingivitis.
- Evidence of untreated cavitated carious lesions or oral abscesses.
- Evidence of precancerous or cancerous oral lesions.
- Evidence of oral candidiasis.
- More than 8 missing teeth. The missing teeth must be due to 3rd molar extractions and/or teeth extracted for orthodontic purposes, teeth extracted as a result of trauma, or teeth that are congenitally missing.

Study Procedures

- Subjects have to refrain from swimming in a chlorinated pool or using a hot tub for 48 hours prior to sampling visit.
- Subjects have to avoid the use of sauna/steam baths for 48 hours prior to sampling visit.
- Subjects have to avoid the use of tanning bed for 48 hours prior to sampling visit.
- Bathe/shower procedure:
 - Do NOT shower on the sampling day (you can wash your hair but avoid leaking soap to the rest of your body, and do NOT rub up your lower leg skin (avoid soap as much as possible).
 - Do NOT scrub your lower leg skin with a towel, please dab a little to dry if necessary.
 - Avoid the use of body lotion on the lower leg on the sampling day.

Study participants and skin microbiome samples

Prior to microbiome sample collection, IV patients and healthy controls were all genotyped for *FLG* mutations and checked for *FLG* protein expression in skin biopsies. Samples were collected from the lower leg and obtained by swabbing 4 cm² skin area using Sterile Catch-All™ Sample Collection Swabs (Epicentre Biotechnologies) soaked in sterile SCF-1 solution (50 mM Tris buffer [pH8], 1 mM EDTA, and 0.5% Tween-20) as previously described.³ As negative controls we took two mock swabs, which were only exposed to ambient air. DNA was extracted from the swabs by using the Mobio Ultraclean Microbial DNA Isolation Kit (Mobio laboratories) with modifications as described previously.³ DNA samples were stored at -20°C until further processing.

Mutation Analysis

Genomic DNA was extracted from saliva samples using the Oragene kit (DNA Genotek Inc.) according to the manufacturer's protocol. *FLG* mutation analysis (for R501X and 2282del4) was performed as described previously.⁴

Immunohistochemistry

Skin biopsies (3 mm) were immediately fixed in a 10% formalin solution (Baker Mallinckrodt) for 4 hours and subsequently embedded in paraffin. 6 µm paraffin sections were stained with an antibody against filaggrin (1:200, NCL-filaggrin, Novocastra) using an indirect immunoperoxidase technique (Vectastain, Vector Laboratories).

Universal Primers

For amplification of the V3-V6 region of the 16S rRNA gene we used the following primers: forward primer 5'-*CCATCTCATCCCTGCGTGTCTCCGACTCAGNNNNNNNACTCCTACGGGAGGCAGCAG*-3' (italicized sequence is 454 Life Sciences primer A, bold sequence is the broadly conserved bacterial primer 338F; *NNNNNNN* designates the sample-specific 6-base barcode used to tag each PCR product), reverse primer 5'-*CCTATCCCCTGTGTGCCTTGGCAGTCTCAGCRRACGAGCTGACGAC*-3' (the italicized sequence is 454 Life Sciences primer B, and the bold sequence is the broadly conserved bacterial primer 1061R). We recently described that skin samples contain only small numbers of microorganisms.³ Therefore, we have introduced a pre-amplification step with the same primers as described above excluding the barcodes and flag sequences. We

established that this additional PCR step did not affect the results compared to a single amplification step with bar-coded primers, in other words, we observed no distortion or skewing of the taxa distribution. PCR amplification protocols of step 1 and step 2 were performed as described previously.³ As negative controls we took two mock swabs, which were only exposed to ambient air, and extracted the genomic microbial DNA from these samples. The purified PCR products were submitted for pyrosequencing of the V3-V4 region of the 16S rRNA gene on the 454 Life Sciences GS-FLX platform using Titanium sequencing chemistry at DNAvision, Charleroi, Belgium. After pyrosequencing and applying our QC filtering pipeline, we ended up with a small number of 16S sequencing reads for the two air control samples. Based on further (in parallel to the skin samples) clustering and composition analysis, combined with the very low number of 16S sequencing reads retrieved, we concluded that the negative control samples are of expected and sufficient quality (not shown).

16S rRNA Gene Pyrosequencing Data Analysis

The pyrosequencing data were analyzed with a workflow based on QIIME v1.2⁵ and as applied and described previously³, using pipeline settings recommended in the QIIME v1.2 tutorial, with the following exceptions: (i) sequencing reads were filtered for chimeric sequences using UCHIME⁶, (ii) OTU clustering was performed with settings as recommended in the QIIME newsletter of December 17, 2010 (<http://qiime.wordpress.com/2010/12/17/new-default-parameters-for-ucclust-otu-pickers/>), using an identity threshold of 97%. In short, (i) raw sequencing reads are quality filtered based on length and quality scores, (ii) reads are demultiplexed (that is, multiplexed reads are assigned to samples based on their nucleotide barcode), (iii) chimeric 16S rRNA gene sequence reads are identified and removed, (iv) reads are clustered into OTUs, and representative OTU sequences are selected, (v) OTUs are assigned to taxonomy (Greengenes 16S rRNA database version '6 October 2010', as default in QIIME v1.2; with QIIME default RDP classifier minimum confidence score of 0.80), and finally, (vi) relative abundance per taxon per sample (or per sample group) is calculated based on (total) number of reads assigned to that taxon. Alpha diversity metrics were calculated as implemented in QIIME v1.2, by bootstrapping 800 reads per sample, and taking the average over four bootstrap trials. Hierarchical clustering of samples was performed using UPGMA with weighted UniFrac as a distance measure, as implemented in QIIME v1.2. The Ribosomal Database Project classifier version 2.0 was applied for taxonomic classification.⁷ Visualization of differences in relative abundance of taxa between the study contrast (Figure E6) was done in Cytoscape.⁸ Taxa (i.e. nodes) were included in the visualization if they met the following criteria: (i) all samples together have at least 10 reads assigned to the taxon, and (ii) the sample groups have a fold-difference of at least 0.1 for the taxon, or the taxon has a child (that is, more specific taxonomic classification) meeting the first criterion. The significance of the difference in relative abundance of specific taxa between sample groups was calculated using the Mann-Whitney U test (see section *Statistics*).

Biophysical measurements and instruments

In vivo measurements were performed on the outer lower leg. None of the samples regions had obvious eczematous signs. The published guidelines for TEWL, SC hydration and skin surface pH assessments were followed.⁹⁻¹¹ TEWL was measured with a condenser-chamber method (Aquaflux AF200, Biox Systems), SC hydration with a capacitance-based method (Corneometer CM825, Courage and Khazaka) and skin surface pH with a planar pH electrode and meter (InLab426, Mettler Toledo and Portamess 913 pH, Knick GmbH & Co. KG). Prior to measurements, volunteers acclimatized for at least 15 minutes in a temperature-controlled room (22±1°C, relative humidity 55±5%) with the body site to be assessed uncovered. Instruments were equilibrated in the same room for at least 30 minutes. The average of three, five and two readings taken in close proximity was calculated for TEWL, SC hydration and skin surface pH, respectively. The tip of the pH electrode was dipped in distilled water prior application on the skin and readings were recorded after stabilizing. The same researcher performed all measurements of skin surface pH, while TEWL and SC hydration were measured by another researcher. Volunteers were not allowed to apply any cream, soap or shower gel on the lower leg skin from the day before the experiments; moreover, they were asked to avoid any contact with water on the test site from 3 hours before the measurements.

Finegoldia magna growth on an *in vitro* model for the human cutaneous microbial ecosystem

Finegoldia magna type strain 2974 (DSM nr. 20470, ATCC nr. 15794) was inoculated on Columbia agar with 5% sheep blood (Dickinson and Company, BD) and grown for four days at 37°C in anaerobic conditions. One single colony of the plate was picked and cultured in Brain Heart Infusion medium (Mediaprodukt BV) for four days at 37°C (anaerobic conditions). Hereafter, the bacteria were collected by centrifugation, washed two times with PBS and finally resuspended in PBS resulting in bacterial concentrations of 10⁵ CFU/mL. We recently developed an *in vitro* system that mimics human SC for bacterial growth, in which human callus serves as substrate and

nutrient source for bacteria.¹² Human callus from the heel of six healthy volunteers (*FLG*^{+/+}) and four *FLG* deficient IV patients (*FLG*^{-/-}) was collected using a callus rasp (Ped Eggtm). This callus was frozen in liquid nitrogen and subsequently grounded using a Micro Dismembrator U (B. Braun Biotech International). Phosphate buffered saline (PBS) was added to the callus powder to create a 2% suspension, which was sterilized by exposure to gamma radiation (16.2 kGray/63 hours). To prepare the model, one mL of sterile agar (2% in PBS) was added in wells of 24-well cell culture plates. On top of this agar, 100 μ L of sterile callus suspension (2% in PBS) was pipetted (Figure E7). The plate was allowed to dry for 24 hours at 37°C, and stored at 4°C until further use. Portions of 20 μ L *F. magna* (2×10^3 CFU) suspension were added to each well of the SC model (with callus derived from *FLG*^{+/+} and *FLG*^{-/-} individuals as described above). Bacteria were incubated on the model at 32°C in anaerobic conditions for seven days. The entire model (agar + callus + bacteria) was lifted out of the 24-wells plate and transferred to a 50 mL tube containing 5 mL PBS. The tubes were vortexed at maximum speed for one minute to detach the bacteria from the model. The aqueous solution containing the bacteria (including callus particles, but without the agar) was transferred to a new tube. These samples were serially diluted in steps of 5. Ten μ L of each dilution was placed on sheep blood agar plates and incubated at 37°C in anaerobic conditions. Next days, colonies visible on the plate were counted for each dilution.

Determination of *FLG* degradation products in callus

The *FLG* degradation products in callus have been determined by a slightly adapted high-performance liquid chromatography (HPLC) method as reported previously.¹³ 1 mg of callus sample was extracted with 0.5 mL of 25% (w=w) ammonia solution by vigorous shaking using IKA-Vibrax-VXR Model 2200 (IKA-works Inc.). Ammonia extracts were evaporated to dryness, resolved in 0.5 mL of water, and subsequently filtered through a 0.2 mm PVDF membrane filter (Grace Davison Discovery Science). Before HPLC analysis, the aliquots were diluted 1:1 with the mobile phase.

Microbiota derived functional prediction by PICRUSt

Based on the bacterial 16S profiles of the skin microbiota, we predicted the presence of KEGG Orthologs (K numbers) and subsequent functional and metabolic pathways for IV patients (*FLG*^{-/-} and *FLG*^{+/-}) and healthy controls (*FLG*^{+/+}) using PICRUSt (version 1.0.0).¹⁴ The Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) method¹⁴ allow for computationally predicting microbiota (i.e. single microbial entities or, as presented in this manuscript, complete communities) their function potential, based on interring 16S marker-gene sequencing-derived information. PICRUSt requires closed-reference OTU picking in QIIME⁵, which was done to the Greengenes¹⁵ reference collection (version 13.5, May 2013). For this step, the same sequencing reads were used as those provided for the QIIME workflow, as described above. Thereafter, for analysis in PICRUSt, the total skin makeup of microbiota for each *FLG* genotypic group (as derived from QIIME, after OTU clustering and identification) was considered; in other words, no OTUs were sub-selected or excluded from analysis. The default (and author advised) workflow of PICRUSt was followed: (i) OTUs were normalized by dividing each OTU by the known or predicted 16S copy number abundance of its Greengenes reference, (ii) a (metagenome) functional predictions table was generated by multiplying each normalized OTU abundance by each predicted functional trait abundance, which was input for our further downstream analysis (for any additional detail, or for more information about the PICRUSt workflow we refer to the PICRUSt website: <http://picrust.github.io>). The PICRUSt downstream analysis was focused on candidate genes only (KEGG Orthologs; K numbers), by applying in-house Perl scripts that filtered-out the required KEGG Orthologs. The candidate genes were selected from the *Hut* pathway based on a review¹, and are listed in Table E4. For additional calculation of relative abundances of pathways and KEGG Orthologs, and for downstream statistics, in-house Python scripts were used (see section *Statistics*). Based on the KEGG pathway (<http://www.genome.jp/kegg/>) for 'histidine metabolism' (ko00340), a map was constructed in the Cytoscape network interaction visualization program⁸ with use of the KEGGScape app/plugin¹⁶ in order to visualize the differential (relative) abundance and metabolic context of candidate *Hut* genes between the study contrast.

Keratinocyte cultures and bacterial stimulations

Primary human keratinocytes obtained from abdominal plastic skin surgery were isolated and expanded according to the Rheinwald-Green protocol¹⁷ and stored in liquid nitrogen. Primary human keratinocytes were cultured in keratinocyte growth medium (KGM bullet kit, Lonza) without antibiotics as described earlier.¹⁸ *Staphylococcus aureus* (ATCC 29213), *Propionibacterium acnes* (ATCC 6919), *Staphylococcus capitis* and *Corynebacterium aurimucosum* (both clinical isolates) were obtained from the department of Medical Microbiology of the Radboudumc. *F. magna* (DSM 20470) was purchased from DMSZ, Braunschweig, Germany. Bacteria were inoculated on Columbia agar with 5% sheep blood (Dickinson and Company (BD), Sparks, MD)

overnight (*S aureus*, *S capitis*, *C aurimucosum*) or for two or four days (*P acnes* and *F magna*, respectively) at 37°C. One single colony of each plate was picked and cultured in Brain Heart Infusion medium (Mediaproducs BV, Groningen, The Netherlands). *F magna* and *P acnes* were cultured under strict anaerobic conditions. Bacteria were collected by centrifugation, washed two times with PBS and finally resuspended in PBS resulting in bacterial concentrations of 10⁷ CFU/mL. To determine the amount of bacteria that was brought on the keratinocyte cultures, bacterial suspensions were serially diluted in steps of 5. Ten µL of each dilution was placed on sheep blood agar plates and incubated overnight or for four days (*F magna*) at 37°C in aerobic (*S aureus*, *S capitis*, *C aurimucosum*) and anaerobic (*F magna* and *P acnes*) conditions, respectively. Visible colonies on the plate were counted for each dilution. The number of CFU was calculated: counted CFU x dilution factor. Confluent submerged keratinocyte cultures (FLG+/+ controls, n=10) were inoculated with 3.5x10⁵ viable bacteria and incubated for 10 hours. Cells were washed after 6 hours and incubated for another 4 hours in fresh medium before the keratinocytes were collected (t=10 hours). Afterwards, the bacteria were washed away and the keratinocytes were harvested and processed for RNA isolation and qPCR analysis.

RNA isolation and qPCR analysis

RNA isolation, cDNA synthesis and qPCR analysis was performed as described earlier.¹⁹ All primers were designed and used as described previously.²⁰ Target gene expression was normalized to the expression of the house keeping gene human acidic ribosomal phosphoprotein P0 (*RPLP0*). The $\Delta\Delta C_t$ method was used to calculate relative mRNA expression levels (see Table E6 for primer sequences).²¹

Statistics

For the microbiota data in this manuscript, statistical significance between contrasts with regard to taxonomy abundances and thereof derived KEGG Orthologs abundances was tested by the non-parametric Mann-Whitney U, corrected with Bonferroni for multiple testing; unless stated otherwise. Statistical tests were performed by custom, in-house R-scripts (version 3.1.2; <https://www.r-project.org/>) or as implemented in SciPy (<https://www.scipy.org/>), downstream of QIIME and PICRUST analyses as described above. Multivariate Redundancy Analysis (RDA) was done using Canoco 5.04²² using default settings of the analysis type "Constrained". Relative abundance values for either taxa were used as response data and the sample FLG status as explanatory variable. RDA calculates p-values by permutating the sample FLG status. For all other experiments statistical significance was tested by a non-parametric Mann-Whitney U test or repeated measures ANOVA with Bonferroni's post-hoc test.

Accession numbers

The raw, and unprocessed 16S sequencing reads data is publicly available for download at the European Nucleotide Archive (ENA) database (<http://www.ebi.ac.uk/ena>) under study accession number PRJEB11661 (or secondary accession number ERP013063).²³ The sequencing data is available in fastq-format, including corresponding metadata for each sample.

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