

# Bacterial taxa predictive of hyperpigmented skins

Catherine Zanchetta<sup>1</sup>  | David Vilanova<sup>2</sup> | Cyrille Jarrin<sup>1</sup> |  
Amandine Scandolera<sup>3</sup> | Emilie Chapuis<sup>3</sup> | Daniel Auriol<sup>1</sup> | Patrick Robe<sup>1</sup> |  
Joran Dupont<sup>1</sup> | Laura Lapierre<sup>3</sup> | Romain Reynaud<sup>1</sup>

<sup>1</sup>R&D Department, Givaudan, Toulouse, France

<sup>2</sup>GenomicTales, Escaldes, Andorra

<sup>3</sup>R&D Department, Givaudan, Pomacle, France

## Correspondence

Catherine Zanchetta, R&D Department, Givaudan, Toulouse, France.

Email: [Catherine.zanchetta@givaudan.com](mailto:Catherine.zanchetta@givaudan.com)

## Funding information

Givaudan SAS France

## Abstract

**Background and Aims:** Dark spots, brown spots, or hyperpigmented spots (HPS) are oval or irregular brown areas of skin. Their emergence is associated with dysregulation of the immune system, and may also be caused by a deficiency in stromal cell-derived factor-1, leading to perturbed melanogenesis and accumulation of melanosomes within neighboring keratinocytes. The skin microbiota (living microorganisms present on the surface of the skin) is known to play essential roles in maintaining skin homeostasis and in regulating the immune system. Here, we investigated whether the microbiota could play a role in the emergence of HPS.

**Methods:** The clinical study involved 38 European women, selected from among 74 volunteers. Participants were divided into two groups depending on the spot areas measured on their faces. The study was designed to avoid conflicting factors: both groups presented similar skin pH, hydration, transepidermal water loss, and sebum levels. The two cohorts were also age-matched, with a mean of 29-years-old for both.

**Results:** Alpha-diversity of the microbiota was similar for the two groups. On skins with more HPS, seven bacterial genera were identified in significantly higher proportions and included opportunistic pathogens and inflammatory bacteria. Six bacterial genera, including bacteria showing antioxidant and anti-UV properties, were identified in significantly higher proportions on less spotted skins. Cross-domain association networks revealed distinct co-occurrences of genera between the two groups, suggesting nonidentical community structures and exchanges, depending on the HPS status.

**Conclusion:** Our results reveal specific microbiota composition and networks on skins based on HPS status. Changes could alter communication with the immune system, leading to the emergence of dark spots. As an essential part of the overall skin ecosystem, and through its interaction with the skin matrix, the skin microbiota and its maintenance could be considered a new target for skincare applications.

## KEYWORDS

dermatology, genetics and genomics | skin microbiome

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.

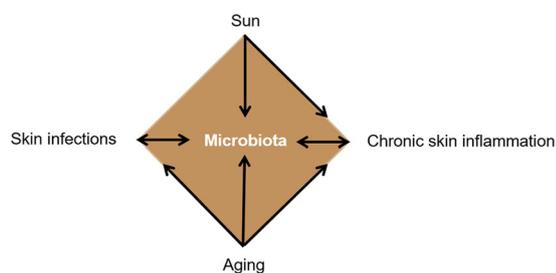
© 2022 GIVAUDAN SAS FRANCE. *Health Science Reports* published by Wiley Periodicals LLC.

## 1 | INTRODUCTION

Dark spots – also called brown spots, hyperpigmented spots (HPS), or age spots – are brown round, oval, or irregularly shaped lesions of the skin. The emergence of HPS is usually age-related and can be explained by a reduction in expression of stromal cell-derived factor-1 (SDF-1), increased melanin synthesis, reduced epidermal renewal, and a decreasing ability to degrade melanosomes, leading to their accumulation within neighboring keratinocytes.<sup>1–4</sup> Sun exposure is also involved in the emergence of dark spots and is commonly linked to the activation of a number of inflammatory processes.<sup>5</sup> The development of HPS has also been suggested to be linked to the deregulation of nerve fibers, preferably distributed close to spots on facial skin.<sup>6</sup> In addition, postinflammatory hyperpigmentation can be observed with many skin conditions including acne, eczema, and atopic dermatitis.<sup>7</sup> The four main factors associated with the emergence of HPS are thus: age, sun, chronic inflammation, and skin infections.

Growing knowledge of the importance and roles of the skin microbiota suggests that these four factors could modify the microorganism populations present on the skin. First, it has been shown that, like the skin's structure and homeostasis,<sup>8</sup> the microbiota evolves with age.<sup>9,10</sup> Age-related modifications are characterized by a decrease in *Firmicutes* phyla and an increase in *Bacteroidetes* and *Proteobacteria* phyla.<sup>10,11</sup> Second, UV exposure has been shown to affect both immune regulation in the skin and the composition of the skin microbiota.<sup>12</sup> These modifications are due to changes in UV tolerance depending on the microorganisms present, leading to altered microorganism growth and community structures. Third, the microbiota plays a major role in maintaining the skin's homeostasis.<sup>13–15</sup> For example, lipoteichoic acid (LTA) produced by some bacteria activates SDF-1.<sup>16</sup> Fourth, some skin diseases involving pathogenic microorganisms (such as *Cutibacterium acnes* in acne,<sup>17</sup> *Staphylococcus aureus* in atopic dermatitis<sup>18</sup>) correlate with the emergence of HPS.<sup>19</sup> The diagram proposed below summarizes the potential links between the microbiota and triggers for HPS emergence (Diagram 1).

On the basis of the essential contribution of the skin microbiota to maintaining skin homeostasis and its links to the four main causes of the emergence of dark spots, the microbiota may be involved in the emergence of HPS.



**DIAGRAM 1** Summary of interactions between the skin microbiota and the main factors leading to the emergence of hyperpigmented spots.

Very few studies have been conducted on this topic. However, *Corynebacterium* genera have been reported to correlate positively with wrinkles and age spots,<sup>20</sup> and a decrease in *Cutibacterium* associated with an increase in *Prevotella* could be related to the intensity of dark spots.<sup>21</sup> In contrast, Li et al.<sup>22</sup> reported higher proportions of *Cutibacterium*, *Staphylococcus*, and *Lactobacillus* genera on skins with less HPS. Furthermore, some skin diseases involving the melanogenesis pathway have been linked to the modified composition of the skin microbiota. In animal models, two bacterial genera – *Fusobacterium* and *Trueperella* – were detected in higher proportions on pig melanoma lesions,<sup>23</sup> whereas on mouse skin,<sup>24</sup> the bacterial species *Staphylococcus epidermidis* produced antitumoral peptides.

The present study was designed with the aim of increasing our knowledge of the bacterial populations present on skins with dark spots. As an essential preliminary step, we assessed the confounding biometric factors associated with HPS occurrence to ensure that our results would be solely linked to the HPS level. We then identified bacteria typical of hyperpigmented skins before formulating hypotheses about interactions between this typical microbiota and the skin's physiology.

## 2 | MATERIALS AND METHODS

The study population consisted of 78 European women aged from 18 to 64 years, with solar and senile lentigos and skin color between I and III according to the Fitzpatrick scale. Written informed consent was obtained from all volunteers before the start of the study in line with the Declaration of Helsinki Principles. The study protocol was approved by the institutional review board at Givaudan (ref. 2020-001), and the French ethics committee (Comité de Protection des Personnes, France; under number 20.09.17.59317). Exclusion criteria included volunteers whose natural skin microbiota may have been modified, for example, by treatment with antibiotics over the preceding 2 months, with a specific hormonal status (e.g., pregnant or breastfeeding women), and presenting skin diseases on the face (e.g., acne or seborrheic dermatitis).

Before taking biometrics measurements and collecting microbiota samples, volunteers remained in a controlled environment (20°C, relative humidity 45%–50%) for a minimum of 10 min.

Brown spots (pigmentation and discoloration on and beneath the surface of the skin) corresponding to solar or senile lentigo, were measured using a Visia CR 2.3<sup>®</sup> device from Canfield<sup>®</sup>. This device takes pictures under a number of types of illumination and allows very rapid image capture. A series of photos were taken under multispectral imaging and analyzed to gather visual information describing the appearance of the skin. RBX<sup>®</sup> Technology (Canfield) was then used to separate the unique color signatures of Red and Brown skin components to distinguish between conditions resulting in color concentration, such as hyperpigmentation, inflammation, and other conditions. Spot area was expressed in mm<sup>2</sup>. Because of differences in the size of the faces of the volunteers, HPS areas were normalized relative to the mean area of the mask surface considered for the measurement (11,149 mm<sup>2</sup>).

Sebum levels were measured by a photometric method using a Sebumeter<sup>®</sup> SM 815 (Courage & Khazaka electronic GmbH). A synthetic ribbon, which becomes transparent when in contact with absorbed lipids, was applied to the measurement zone for precisely 30 s. Its transparency increases in proportion to the amount of sebum present in the hydrolipidic film. The increase in light transmitted through the ribbon was quantified by reflectometry, and the total mass of lipids excreted was expressed in  $\mu\text{g}/\text{cm}^2$ .

The hydration level was measured using a Corneometer<sup>®</sup> CM 825 (Courage & Khazaka electronic GmbH). The stratum corneum behaves like a dielectric material, and hydration levels change its electrical characteristics, which can be measured by a condenser. The higher the hydration level, the higher the electric capacity, because its dipolar nature increases the electric permittivity of the environment and its conduction. The probe, linked to a condenser, allows equivalent pressure to be applied to the tegument throughout the experiment. This is important to avoid perturbing measurements and to ensure reproducible experimental conditions. Results are expressed in arbitrary units.

Transepidermal water loss (TEWL)—the passive diffusion of water through the stratum corneum from the inside of the body to the outside—was measured using a Tewameter<sup>®</sup> TM 300 (Courage & Khazaka electronic GmbH). TEWL values are generally used as an indication of the integrity of the skin's barrier function. A probe delimiting a cylindrical chamber in contact with the skin was used to measure the gradient of water vapor established on the skin's surface, the open chamber partially overcomes variations in environmental conditions. TEWL values are expressed in  $\text{g}/\text{m}^2/\text{h}$ .

The skin pH was measured using a pH 905 (Courage & Khazaka electronic GmbH) probe. For these assays, the probe was placed on the zone to be measured and the value was recorded immediately.

Skin microbiota was analyzed on the 38 selected volunteers making up the study cohort. Selected volunteers were all under 46 years old to avoid the major shift in skin microbiota composition that occurs in women during menopause.<sup>9,10</sup> Skin bacteria were collected from the right and left cheeks ( $25\text{ cm}^2/\text{cheek}$ ) by noninvasive swabbing with sterile swabs moistened with a sterile solution of 0.15 M NaCl. All samples were systematically collected using a standardized procedure. Swabs were stored at  $-20^\circ\text{C}$  until DNA extraction using the DNeasy PowerLyzer PowerSoil DNA Isolation Kit (Qiagen). The kit was used according to the manufacturer's protocol with the following modifications: the tip of each swab was detached with a sterile surgical blade and transferred to a 1.5-ml tube containing 750  $\mu\text{l}$  Bead Solution. The sampled biomass was suspended by stirring and pipetting and then transferred to a bead-beating tube. The DNA concentration was determined using the QuBit dsDNA HS Fluorometric Quantitation Kit (Invitrogen, Thermo Fisher Scientific) according to the manufacturer's instructions.

DNA amplification and 16S ribosomal RNA (rRNA) gene sequencing were performed on a MiSeq device (Illumina Inc.) with a 500-cycle paired-end run. Briefly, the V3–V4 variable regions of the 16S rRNA gene were targeted. The whole procedure involved two polymerase chain reaction (PCR) steps. Libraries were prepared and the MiSeq run was performed by Givaudan Active Beauty on the GeT-PlaGe platform (INRAe).

After the MiSeq run, raw sequence data were demultiplexed and quality-checked to remove reads with ambiguous bases. Index and primer sequences were removed by cutadapt (v1.9; <http://cutadapt.readthedocs.io/en/stable/index.html>), and reads with a fastq score of less than 28 were trimmed. Forward and reverse sequences were paired using bbmerge (<https://jgi.doe.gov/data-and-tools/bbtools/>). Samples with fewer than 5000 paired sequences were discarded. The remaining paired sequences were then treated to remove chimeras and amplicons with PCR errors using an in-house pipeline based on vsearch (<https://github.com/torognes/vsearch>). The remaining sequences were split into operational taxonomic units (OTUs, a cluster of similar sequence variants of the 16S rRNA gene sequence) at a 1% dissimilarity level using swarm (v2.6; <https://github.com/torognes/swarm>). Unique amplicons were mapped to the SILVA SSU Ref NR 99 (nonredundant) database (release 132; <https://www.arb-silva.de/>) for a taxonomic assignment using the RDP classifier.<sup>25</sup> Data normalization and analyses were performed in an R statistical computing environment (v3.2.0; <https://www.r-project.org/>), R core team (2014), using the Bioconductor package (mainly Phyloseq, DESeq. 2, and Vegan libraries; <http://www.bioconductor.org/>).

Ecosystem biodiversity was assessed based on the Shannon index. Microorganisms are relevant for each condition ( $p < 0.05$ ) were determined using the DESeq. 2 statistical packages (Bioconductor), comparing relative abundances of the taxa and subsets of relevant microorganisms between each condition analyzed (Sebum, Hydration, Age, Sensitivity, Dark spots). The  $p_{\text{Adj}}$  value reported corresponds to the adjusted  $p$ -value from multiple testing.

Microorganisms were identified at significantly different abundances between the two conditions ( $p < 0.05$ ), with a relative abundance exceeding 0.01%, and identified in at least 30% of samples (in this study, these correspond to all the microorganisms identified in the first analysis described above) were then classified using a machine learning approach based on a RandomForest classifier (RandomForest package from R). This classification was used to more precisely define the most relevant bacteria when seeking to characterize a specific skin condition. Briefly, the Random Forest approach uses thousands of decision trees to define which bacterial genera best discriminate between samples as a function of the main variable (in this case: spotting level). Results are expressed using a variable called the mean decrease Gini.

To better understand the relationship between all bacterial genera, we created a network microbial association graph for each volunteer group (low HPS level and high HPS level) using the bacterial OTU tables. The most stable network graph was inferred using the "SPIEC-EASI" (sparse inverse covariance estimation for ecological association inference) R package, a statistical framework that infers associations across multiple microbial domains, thus establishing the strongest associations between different bacteria.<sup>26</sup>

### 3 | RESULTS

To assess whether the spotting level correlates significantly with any biometric parameters and with age, a correlation test was performed for the 78 volunteers. Confounding factors were assessed using a

|                       |                           | Age   | pH     | Hydration level | TEWL   | Sebum  |
|-----------------------|---------------------------|-------|--------|-----------------|--------|--------|
| Normalized spot areas | Pearson correlation value | 0.305 | -0.121 | -0.114          | -0.256 | -0.077 |
|                       | <i>p</i>                  | 0.04* | 0.42   | 0.45            | 0.09   | 0.61   |

Abbreviations: HPS, hyperpigmented spots; TEWL, transepidermal water loss.

\*Statistically significant.

**TABLE 1** Statistical correlations between areas of HPS, age, and other skin parameters.

**TABLE 2** Ranges of age and skin parameters were considered in each group of volunteers, and associated with between-group *p* values.

|                | Area of HPS (normalized) | Age      | pH         | Hydration level | TEWL         | Sebum level |
|----------------|--------------------------|----------|------------|-----------------|--------------|-------------|
| Low HPS level  | From 489 to 972          | 20 to 46 | 4.7 to 5.6 | 32.6 to 75      | 10.3 to 20.2 | 1 to 194    |
| High HPS level | From 1094 to 2087        | 18 to 44 | 4.6 to 5.9 | 32 to 76.2      | 8.9 to 25.5  | 3 to 134    |
| <i>p</i>       | 1.59e-09**               | 0.79     | 0.29       | 0.79            | 0.58         | 0.99        |

Abbreviations: HPS, hyperpigmented spots; TEWL, transepidermal water loss.

\*\*Statistically significant.

parametric Pearson's test, based on the covariance method. Results indicated a significant correlation ( $*p < 0.05$ ; Table 1) with a positive coefficient (0.305) with aging, indicating an increase in the HPS area with increasing age. None of the other skin parameters considered (pH, hydration, TEWL, or sebum levels) was significantly linked to HPS levels.

To analyze the microbiota and attempt to determine whether skins with more HPS have a characteristic profile, groups of volunteers to be considered were defined. Participants with significantly lower HPS areas (area of HPS from 489 to 972 mm<sup>2</sup>) were classed in the "low HPS level" group, whereas those with hyperpigmented skins (area of HPS from 1094 to 2087 mm<sup>2</sup>) made up the "high HPS level" group ( $**p < 0.01$  between groups; Table 2). Significance was tested using a Student's *t*-test. Each group included 19 age-matched volunteers.

Even if biometric parameters such as pH, hydration, TEWL, or sebum level have not previously been identified as conflicting factors in relation to brown spots, the cohorts were designed to avoid significant differences with respect to these parameters.

The diversity and relative proportions of bacterial communities were determined by sequencing the V3-V4 subunit of the 16S rRNA gene. Alpha-diversity corresponds to the mean OTU diversity for sites or habitats at a local scale and can be compared using the Shannon index, which reflects the diversity of a sample. We compared alpha-diversity between the group of volunteers with a low HPS level and the group of volunteers with a high HPS level. Results indicated similar diversity levels ( $p = 0.23$ ) between the two groups (Figure 1).

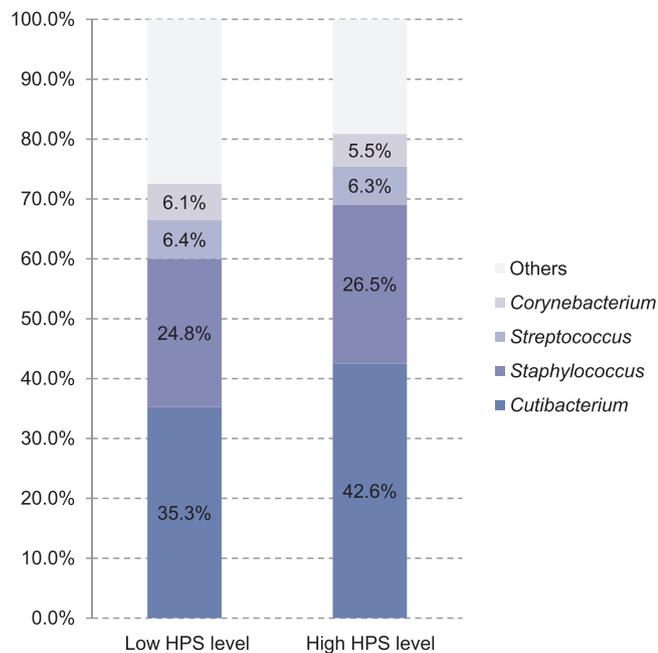
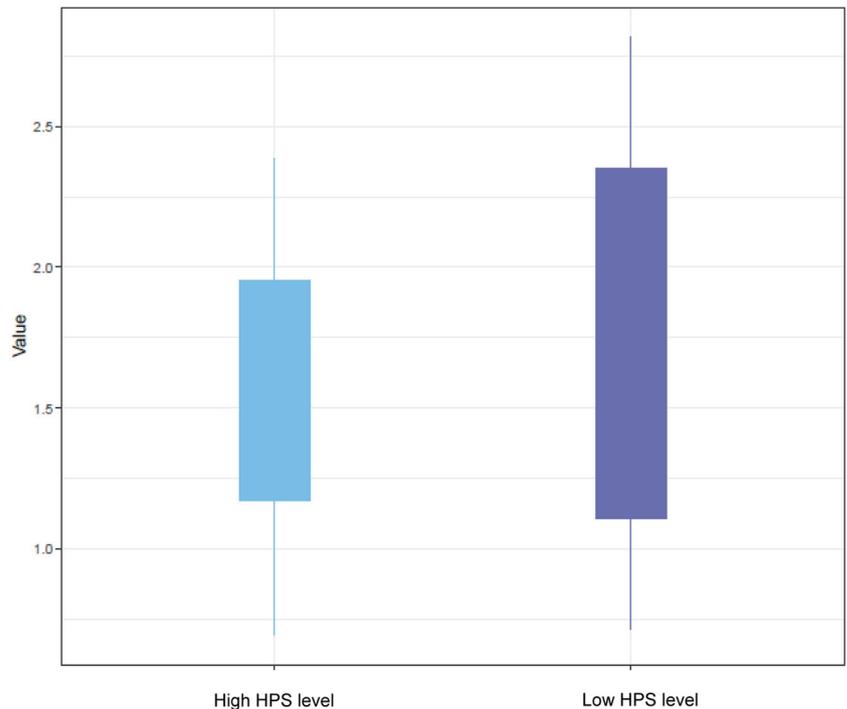
Differences between the two groups in terms of relative proportions of bacterial communities at the phylum level were not significant based on *p* values and *p*Adj values, when the statistical test is adjusted for multiple testing (*Fusobacterium*,  $p = 0.06$ , *p*Adj = 0.22; *Bacteroidetes*,  $p = 0.06$ , *p*Adj = 0.22; *Proteobacteria*,  $p = 0.40$ , *p*Adj =

0.84; *Epsilonbacteraeota*,  $p = 0.66$ , *p*Adj = 0.84; *Patiscibacteria*,  $p = 0.81$ , *p*Adj = 0.84; *Firmicutes*,  $p = 0.84$ , *p*Adj = 0.84; *Actinobacteria*,  $p = 0.69$ , *p*Adj = 0.84). At the genus level, no significant differences were observed in terms of proportions of bacterial genera between groups for the four major taxa present on the skin's surface (*Cutibacterium*, *Staphylococcus*, *Streptococcus*, and *Corynebacterium*; Figure 2).

However, for minor taxa, significant differences were identified (Table 3). For example, the genera *Eikenella*, *Xanthomonas*, *Bergeyella*, *Brevibacterium*, *Aerococcus*, and *Micrococcus* were present in significantly different proportions between the two groups, with both *p* values and *p*Adj values of less than 0.05. Similarly, the taxa *Turicella*, *Paracoccus*, *Paucibacter*, *Klebsiella*, *Kocuria*, *Alloicoccus*, and *Exiguobacterium* also presented significant differences in terms of their proportions between the two groups, with  $p < 0.05$ . However, this significance was not confirmed following adjustment for multiple testing (*p*Adj > 0.05). The results of this analysis revealed six bacterial genera present in significantly higher proportions (based on *p* values) in the "less spotted" group. These were: *Bergeyella*, *Micrococcus*, *Paracoccus*, *Kocuria*, *Alloicoccus*, and *Exiguobacterium*. In the "more spotted" group, seven bacterial genera were identified in significantly higher proportions: *Eikenella*, *Xanthomonas*, *Brevibacterium*, *Aerococcus*, *Turicella*, *Paucibacter*, and *Klebsiella*. Mean abundances per group for all these genera are indicated in Table 3 and shown in Figure 3.

To more precisely assess which bacteria are the most typical of skins with more HPS, a RandomForest analysis was performed (Figure 4). Results are expressed using a variable called the mean decrease Gini. It defines the genera that best discriminant between microbiota samples from the "low HPS level" group or from the "high HPS level" group. The higher the value of the mean decrease Gini, the greater the discriminant power of the bacterial genus. According to this analysis, the two taxa best predicting the HPS level of the skin would be *Kocuria* and *Aerococcus* (mean decrease Gini > 2),

**FIGURE 1** Shannon index distribution for the two groups was defined based on the level of HPS. The blue box represents the value of Shannon index diversity for the skins with a high level of HPS. The purple box represents the value of Shannon index diversity for the skins with a low level of HPS. HPS, hyperpigmented spots



**FIGURE 2** Proportions of major bacterial genera identified on skins with different HPS levels. These proportions represent a percentage of the total reads obtained by the 16S sequencing. HPS, hyperpigmented spots

followed in decreasing order by *Paucibacter*, *Micrococcus*, *Turicella*, *Bergeyella*, *Brevibacterium*, *Eikenella*, *Xanthomonas*, and *Paracoccus* (mean decrease Gini > 1). *Klebsiella*, *Exiguobacterium*, and *Alliococcus* were found to have a lower discriminant power (mean decrease Gini < 1).

**TABLE 3** Bacterial genera present in significantly different proportions between groups, and their mean relative abundance in each group.

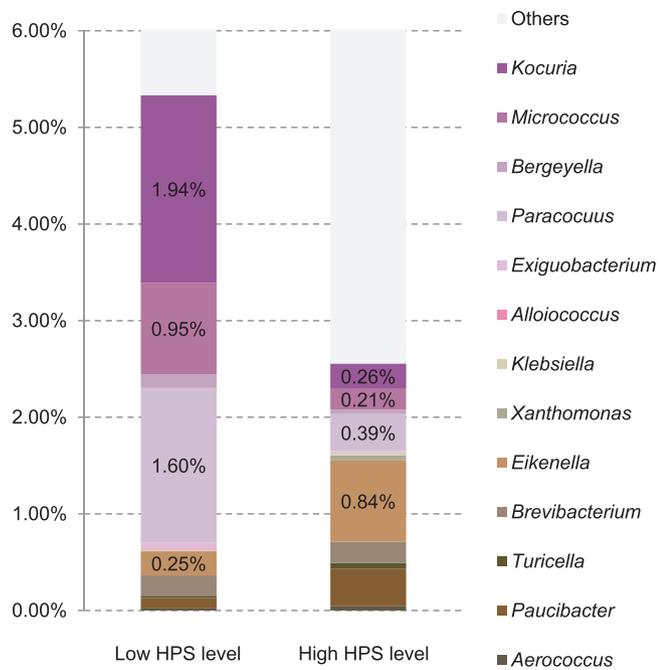
| Genus                  | <i>p</i>           | <i>p</i> Adj value | Mean % in the "less spotted" group | Mean % in the "more spotted" group |
|------------------------|--------------------|--------------------|------------------------------------|------------------------------------|
| <i>Eikenella</i>       | <b>&lt;0.001**</b> | <b>0.03*</b>       | 0.25                               | 0.84                               |
| <i>Xanthomonas</i>     | <b>&lt;0.001**</b> | <b>0.03*</b>       | 0.01                               | 0.05                               |
| <i>Bergeyella</i>      | <b>&lt;0.001**</b> | <b>0.04*</b>       | 0.14                               | 0.04                               |
| <i>Brevibacterium</i>  | <b>0.002**</b>     | <b>0.04*</b>       | 0.21                               | 0.22                               |
| <i>Aerococcus</i>      | <b>0.002**</b>     | <b>0.04*</b>       | 0.02                               | 0.04                               |
| <i>Micrococcus</i>     | <b>0.003**</b>     | <b>0.05*</b>       | 0.95                               | 0.21                               |
| <i>Turicella</i>       | <b>0.007**</b>     | 0.10               | 0.03                               | 0.06                               |
| <i>Paracoccus</i>      | <b>0.01*</b>       | 0.13               | 1.60                               | 0.39                               |
| <i>Paucibacter</i>     | <b>0.02*</b>       | 0.16               | 0.11                               | 0.39                               |
| <i>Klebsiella</i>      | <b>0.02*</b>       | 0.19               | 0.01                               | 0.03                               |
| <i>Kocuria</i>         | <b>0.02*</b>       | 0.19               | 1.94                               | 0.26                               |
| <i>Alliococcus</i>     | <b>0.03*</b>       | 0.20               | 0.01                               | 0.00                               |
| <i>Exiguobacterium</i> | <b>0.04*</b>       | 0.29               | 0.06                               | 0.01                               |

Note: Significant *p* values are presented in bold.

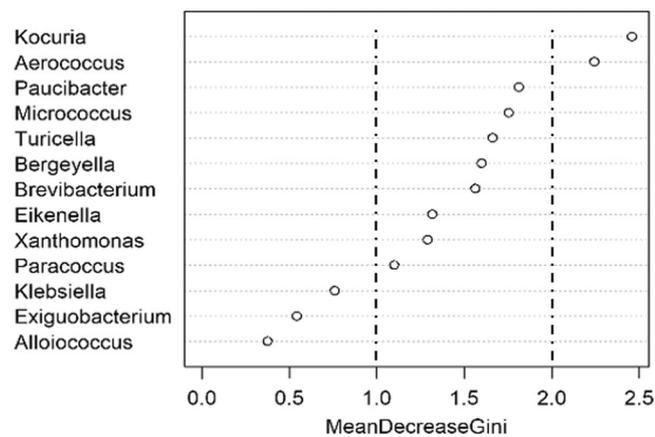
\*\*Statistically highly significant (*p* < 0.01).

\*Statistically significant (*p* < 0.05).

Cross-domain association networks were constructed for the "low HPS level" group (Figure 5) and the "high HPS level" group (Figure 6). The two SPIEC-EASI networks show differences in



**FIGURE 3** Relative abundances of bacterial genera were identified in significantly different proportions between groups. These proportions represent a percentage of the total reads obtained by the 16S sequencing. HPS, hyperpigmented spots



**FIGURE 4** RandomForest analysis identifies the bacteria best predicting the level of HPS. Results are expressed using a variable called the mean decrease Gini. The higher the value of the mean decrease Gini, the greater the discriminant power of the bacterial genus.

connectivity between skin types, highlighting differences in the dynamic nature of bacterial interactions associated with different levels of HPS on the skin.

In the group with a low level of HPS, connections between *Cutibacterium* and *Staphylococcus* (Score 12.81) and between *Peptoniphilus* and *Finelgoldia* (Score 7.65) were observed. A higher score reflects more dynamic interactions between the two taxa.

On hyperpigmented skins, the connection between *Cutibacterium* and *Staphylococcus* disappeared, whereas that

between *Peptoniphilus* and *Finelgoldia* remained. Interestingly, a new co-occurrence emerged involving *Peptoniphilus*, which was positively correlated with *Anaerococcus* (Score 20.60). We also observed new connections. The first involved co-occurrence of *Gemella* and *Neisseria* (Score 1.48), *Neisseria* and *Haemophilus* (Score 6.19), *Haemophilus* and *Granulicatella* (Score 1.40), and *Granulicatella* and *Gemella* (Score 0.35). A second group was associated with co-occurrence of *Streptococcus* and *Veillonella* (Score 15.89), *Veillonella* and *Rothia* (Score 1.1), and co-exclusion between *Rothia* and *Paracoccus* (Score -2.79). Interestingly, only one taxon, *Paracoccus*, was considered to be both predictive for the level of HPS and involved in the cross-domain association networks.

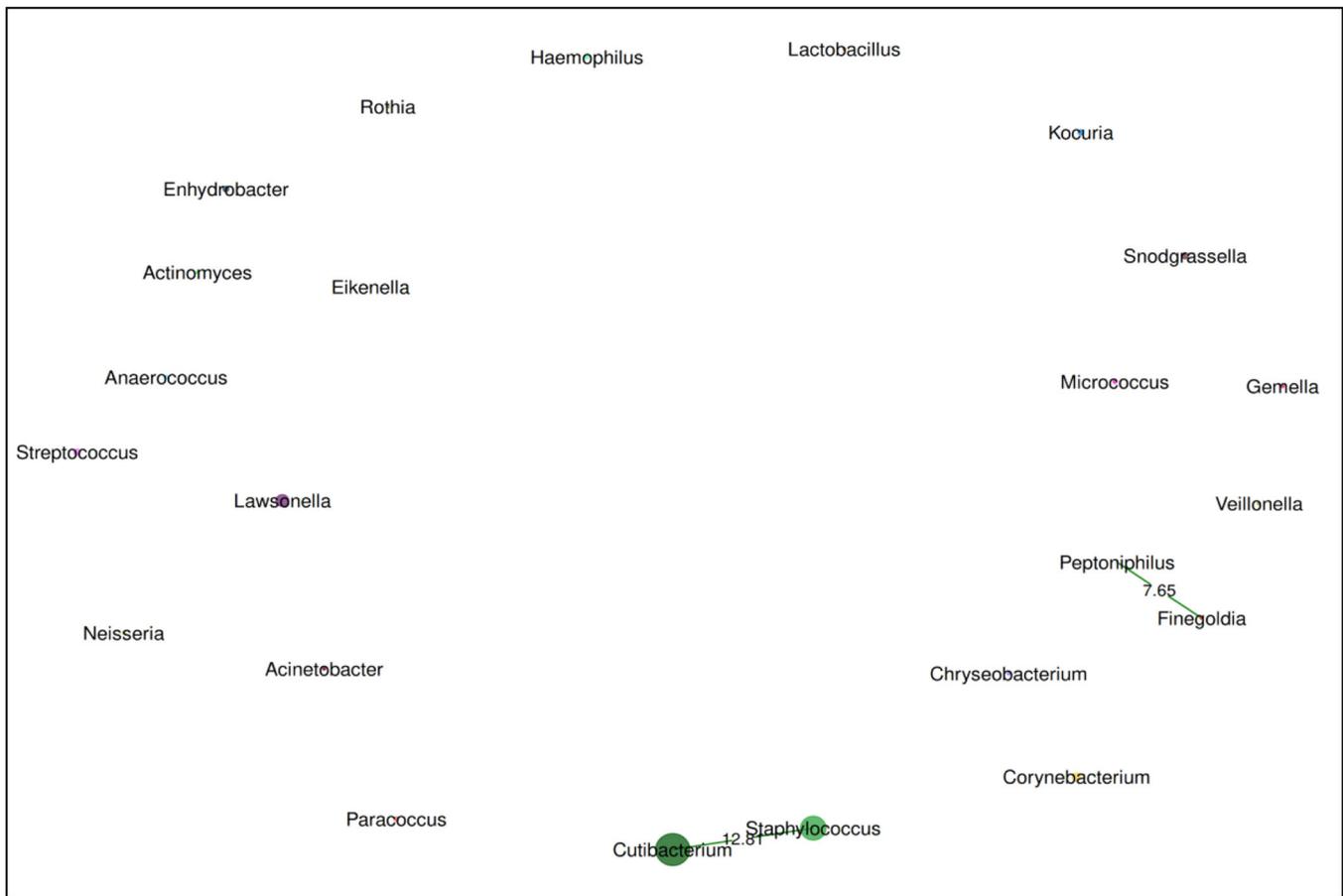
For each of these cross-domain association networks, the SPIEC-EASI output defines a stability score. A score close to zero reflects a more stable network. Consequently, more extensive modifications in the dataset (in the microorganism composition) will be needed to modify the dynamic interactions between the microorganisms.<sup>26</sup> For skins with a low HPS level, the stability score was 0.791 (Figure 5), whereas, for hyperpigmented skins, the stability score was 2.77 (Figure 6). This result suggests more stable concurrences between bacteria on skins with a low HPS level.

## 4 | DISCUSSION

The aim of this study was to determine whether HPS was associated with a specific microbiota profile, that could be involved in the emergence of localized skin pigmentation. The results presented here confirm the previously reported increase in HPS with aging. However, none of the other biometric parameters considered (pH, hydration, TEWL, or sebum level) significantly correlated with levels or areas of brown spots.

Other groups have studied skin microbiota in the context of skin pigmentation in both animal models and human volunteers. Our results differ from theirs in several respects. For example, none of the genera identified in higher proportions on animal melanoma (*Fusobacterium* and *Trueperella*)<sup>23</sup> were detected here in higher proportions on human skin with more HPS. This difference may be due to species differences. Previous studies with human volunteers reported the genus *Corynebacterium* to be typical of hyperpigmented skins, and the genera *Cutibacterium*, *Staphylococcus*, and *Lactobacillus* as typical of skins with less HPS.<sup>20,22</sup> Our results did not corroborate these findings, possibly because the previous studies omitted to account for confounding factors (such as age) when designing cohorts to assess correlation. In contrast, we designed our study with age-matched cohorts (no statistical difference in terms of the age of volunteers between groups). Other differences between our study and the previous studies related to the primer set used. Thus, Dimitriu et al.<sup>20</sup> targeted the V1-V3 regions, whereas we targeted the V3-V4 16S region.

Interestingly, our results indicated a shift between *Eikenella* and *Micrococcus* taxa depending on the spotting level. Significant



**FIGURE 5** Cross-domain association networks were established for skin with a low HPS level. Co-occurrence (positive) relationships are indicated by green edges. The size of the circles reflects the relative proportions of the bacterial genus in the community (arbitrary unit). HPS, hyperpigmented spots

differences in levels of these taxa were observed based on  $pAdj$  values ( $p_{adj} < 0.05$ ).

The genus *Eikenella* is considered to be an opportunistic pathogen. This Gram-negative bacterium produces proinflammatory molecules and corresponds to 0.84% of all bacteria detected on hyperpigmented skins. In contrast, it represents less than 0.25% of the skin microbiota on skins with a lower level of hyperpigmentation. Interestingly, *Eikenella corrodens* (the only species classed in the *Eikenella* genus) produces molecules that activate the mitogen-activated protein kinase (MAPK) pathway, which is involved in regulating melanogenesis.<sup>27</sup> Thus, constitutive activation of the MAPK pathway inhibits proteasome activity,<sup>28</sup> which is essential for melanin degradation.<sup>29</sup>

In contrast, the genus *Micrococcus* is commonly found on healthy skins,<sup>30</sup> and is present in significantly higher proportions on skins with less HPS (0.95%) compared to skins with more HPS (0.21%). Some *Micrococcus* strains have antioxidant and UV-protective properties. For example, *Micrococcus luteus* (one of the best-represented species of *Micrococcus* found on the skin) produces DNA-repair enzymes that can prevent UV light from activating photo-neoantigens on the skin.<sup>31</sup>

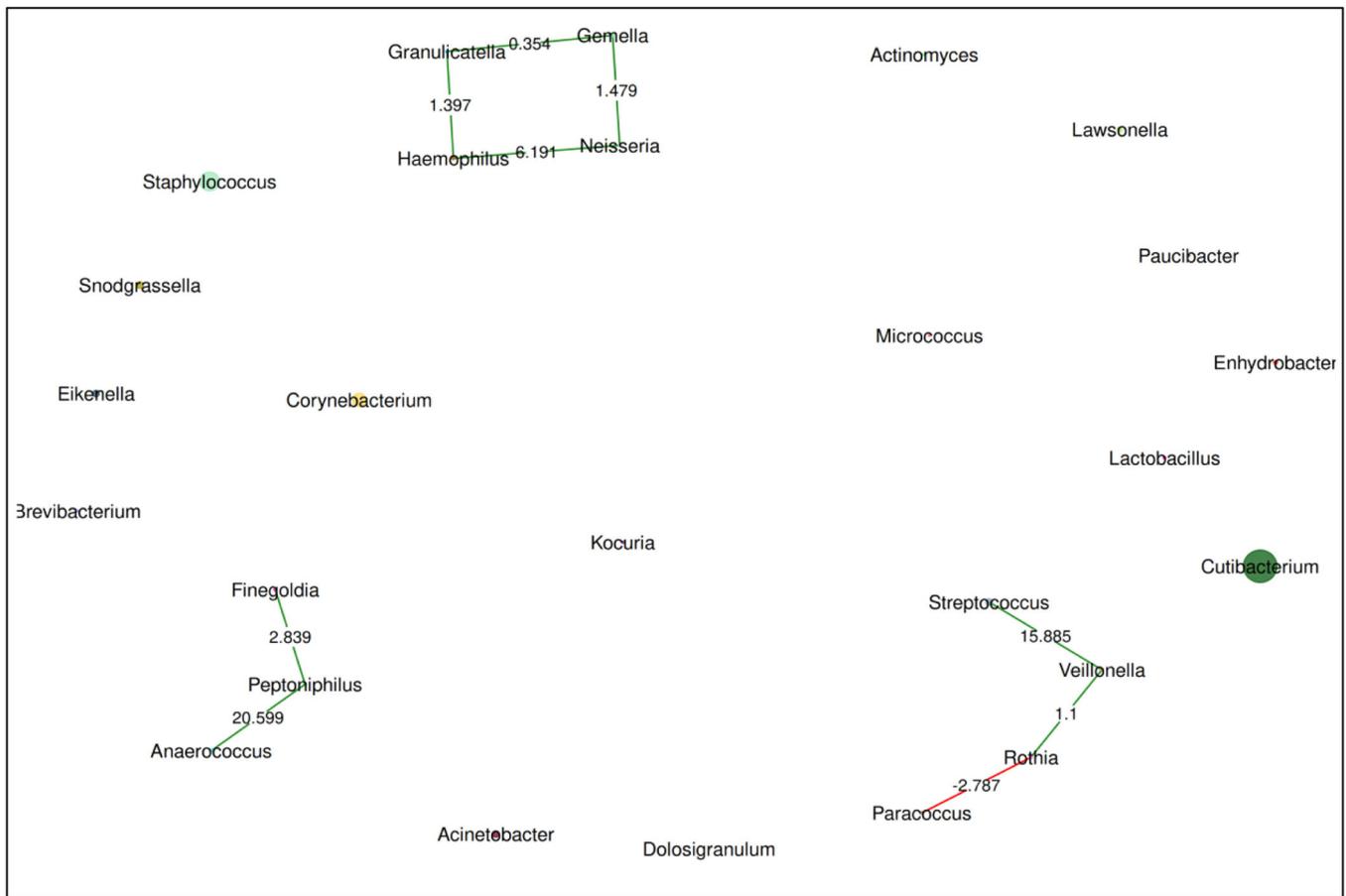
In addition to these two taxa, the genus *Kocuria* was identified as the most discriminant of HPS status according to the RandomForest

analysis. *Kocuria* is typical of skins with less HPS, possibly due to its production of the thiazolyl peptide kocurin. This molecule can inhibit the proliferation of some *Staphylococcus aureus* strains, which otherwise could lead to chronic skin inflammation and skin infections<sup>32</sup> – two major causes of the emergence of brown spots.

*Micrococcus* and *Kocuria* are both Gram-positive bacteria. Interestingly, a surface-associated adhesion amphiphile from Gram-positive bacteria, LTA has been shown to activate SDF-1.<sup>16</sup> As mentioned in Section 1, this factor plays an essential role in regulating skin pigmentation.<sup>1</sup> The switch from Gram-positive bacteria to Gram-negative bacteria on skin with higher levels of HPS could lead to a reduction in SDF-1 expression, which in turn could dysregulate the melanogenesis process.

Based on these findings, and in terms of the shift in some bacterial properties depending on the skin's HPS status, it is possible that the emergence of brown spots could be linked to the composition of the skin microbiota, due to its interactions with the skin and its regulatory effects on inflammatory processes.

In terms of microbial networks, our results indicate greater stability of the bacterial network on skins with less HPS. This greater



**FIGURE 6** Cross-domain association networks were established for skin with a high HPS level. Co-occurrence (positive) relationships are indicated by green edges. Co-exclusion (negative) relationships are indicated by red edges. The size of the circles reflects the relative proportions of the bacterial genus in the community (arbitrary unit). HPS, hyperpigmented spots

stability may be due to the co-occurrence of two of the major taxa of the skin microbiome: *Cutibacterium* and *Staphylococcus* (representing 35.3% and 24.8% of the skin microbiota, respectively). It may also reflect a higher proportion of ecological interactions due to the extensive collaboration between these two taxa reported elsewhere, for example through cross-feeding or co-biofilm formation.<sup>33</sup> In comparison, co-occurrences and co-exclusions identified in the microbial network on skins with more HPS only involved minor populations. The lower stability of microbial networks on skins with a high HPS level suggests a more fragile ecosystem, where minor modifications to the skin microbiota could have a major impact on ecological interactions between bacteria.

In addition to these considerations, cross-domain association networks indicated clear differences in bacterial connections between the two groups, suggesting non-identical community interaction pathways, exchanges, and structures depending on the level of HPS on the skin.

Interestingly, none of the predictive taxa for hyperpigmented skins (except *Paracoccus*) was involved in the microbial networks identified. Consequently, the taxa predictive of HPS status may not be essential for the stability of the microbial ecosystem

on these skins. Nevertheless, they could play an essential role in interactions with the skin cells and their physiological regulation.

The study was limited to volunteers presenting solar and senile lentigos. A metagenomics study, extended to other types of lentigos would be of interest. For example, human skin tissues from patients with lentigo simplex – usually observed in younger people – and therapeutically-induced PUVA lentigines have distinct histological and physiological properties.<sup>34,35</sup> These differences might lead to alternative interactions between the human skin and its microbiota, involving other microbial populations and networks.

To further expand our knowledge, metabolomic studies could be performed to elucidate how the skin microbiota contributes to the emergence, or not, of HPS. Studies of molecules, such as tyrosine and phenylalanine, involved in the metabolic interactions between human cells and the skin microbiota would be of particular interest. Indeed, both these molecules are involved in melanin synthesis, and thus skin pigmentation.<sup>36</sup> Melanin can be produced in human cells, but also by some bacteria thanks to their tyrosinase activity.<sup>37</sup> Previous reports on the main bacterial pathways associated with aging skin indicated significant involvement of pathways leading to the production of

pigmentation intermediates.<sup>38</sup> Greater insight into the mechanisms and molecules present at the interface between bacterial and human cells could provide new leads for innovations in skincare.

## 5 | CONCLUSION

This study reports a specific microbiota composition and network on hyperpigmented skins. Thus, the skin microbiota could be considered as another component contributing to altered immune system regulation, leading to the emergence of HPS.

The results presented here could be useful in driving the development of cosmetic products. Although skin physiology is usually targeted by cosmetic science to act on HPS, equal consideration of the composition of the skin microbiota could be decisive. As an essential part of the skin ecosystem, and through its interaction with the skin matrix, the skin microbiota and maintenance of its equilibrium could be considered a new target for skincare applications.

### AUTHOR CONTRIBUTIONS

**Catherine Zanchetta:** Conceptualization; data curation; formal analysis; investigation; methodology; project administration; validation; writing – original draft; and writing – review and editing. **David Vilanova:** Data curation; formal analysis; investigation; methodology; software; and visualization. **Cyrille Jarrin:** Data curation; investigation; and methodology. **Amandine Scandolera:** Conceptualization; and writing – review and editing. **Emilie Chapuis:** Formal analysis; investigation; and methodology. **Daniel Auriol:** Supervision. **Patrick Robe:** Investigation and methodology. **Joran Dupont:** Investigation. **Laura Lapierre:** Investigation. **Romain Reynaud:** Funding acquisition; supervision; and validation.

### ACKNOWLEDGMENT

This study was supported by Givaudan SAS France.

### CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

### TRANSPARENCY STATEMENT

The manuscript is an honest, accurate, and transparent account of the study being reported. No important aspects of the study have been omitted.

### DATA AVAILABILITY STATEMENT

The data supporting the results of this study are available from the corresponding author, upon reasonable request.

### ORCID

Catherine Zanchetta  <http://orcid.org/0000-0002-7160-0121>

## REFERENCES

1. Yoon JE, Kim Y, Kwon S, et al. Senescent fibroblasts drive ageing pigmentation: a potential therapeutic target for senile lentigo. *Theranostics*. 2018;8(17):4620-4632. doi:10.7150/thno.26975
2. Choi W, Yin L, Smuda C, Batzer J, Hearing VJ, Kolbe L. Molecular and histological characterization of age spots. *Exp Dermatol*. 2017;26(3):242-248. doi:10.1111/exd.13203
3. Murase D, Kusaka-Kikushima A, Hachiya A, et al. Autophagy declines with premature skin aging resulting in dynamic alterations in skin pigmentation and epidermal differentiation. *Int J Mol Sci*. 2020;21(16):5708. doi:10.3390/ijms21165708
4. Rittié L, Fisher GJ. Natural and sun-induced aging of human skin. *Cold Spring Harb Perspect Med*. 2015;5(1):a015370. doi:10.1101/cshperspect.a015370
5. Sklar LR, Almutawa F, Lim HW, Hamzavi I. Effects of ultraviolet radiation, visible light, and infrared radiation on erythema and pigmentation: a review. *Photochem Photobiol Sci*. 2013;12(1):54-64. doi:10.1039/c2pp25152c
6. Cui S, Li Y, Lu H, Gao XH, Wei H, Chen HD. Morphological relationship between nerve fibers and melanocytes in the epi-dermis of melasma. *Clin Pediatr Dermatol*. 2016;2(2):12. doi:10.21767/2472-0143.100020
7. Cardinali G, Kovacs D, Picardo M. Mechanisms underlying post-inflammatory hyperpigmentation: lessons from solar lentigo. *Ann Dermatol Venereol*. 2012;139(suppl 4):S148-S152. doi:10.1016/S0151-9638(12)70127-8
8. Elisia I, Lam V, Hofs E, et al. Effect of age on chronic inflammation and responsiveness to bacterial and viral challenges. *PLoS One*. 2017;12(11):e0188881. doi:10.1371/journal.pone.0188881
9. Oh J, Byrd AL, Park M, Kong HH, Segre JA, NISC Comparative Sequencing Program. Temporal stability of the human skin microbiome. *Cell*. 2016;165(4):854-866. doi:10.1016/j.cell.2016.04.008
10. Jarrin C, Vilanova D, Zanchetta C, et al. Sensitive skin: an insight in microbiota composition and comparison with microbiota of normal skin. *IFSCC Mag*. 2020;23(1):45-54.
11. Jugé R, Rouaud-Tinguely P, Breugnot J, et al. Shift in skin microbiota of Western European women across aging. *J Appl Microbiol*. 2018;125(3):907-916. doi:10.1111/jam.13929
12. Patra V, Byrne SN, Wolf P. The skin microbiome: is it affected by UV-induced immune suppression? *Front Microbiol*. 2016;7:1235. doi:10.3389/fmicb.2016.01235
13. Lai Y, Di Nardo A, Nakatsuji T, et al. Commensal bacteria regulate Toll-like receptor 3-dependent inflammation after skin injury. *Nat Med*. 2009;15(12):1377-1382. doi:10.1038/nm.2062
14. Ruff WE, Greiling TM, Kriegel MA. Host-microbiota interactions in immune-mediated diseases. *Nat Rev Microbiol*. 2020;18(9):521-538. doi:10.1038/s41579-020-0367-2
15. Belkaid Y, Naik S. Compartmentalized and systemic control of tissue immunity by commensals. *Nat Immunol*. 2013;14(7):646-653. doi:10.1038/ni.2604
16. Riehl TE, Alvarado D, Ee X, et al. *Lactobacillus rhamnosus* GG protects the intestinal epithelium from radiation injury through release of lipoteichoic acid, macrophage activation and the migration of mesenchymal stem cells. *Gut*. 2019;68(6):1003-1013. doi:10.1136/gutjnl-2018-316226
17. Dréno B, Pécastaings S, Corvec S, Veraldi S, Khammari A, Roques C. *Cutibacterium acnes* (*Propionibacterium acnes*) and acne vulgaris: a brief look at the latest updates. *J Eur Acad Dermatol Venereol*. 2018;32(suppl 2):5-14. doi:10.1111/jdv.15043
18. Tomczak H, Wróbel J, Jenerowicz D, et al. The role of *Staphylococcus aureus* in atopic dermatitis: microbiological and immunological implications. *Postepy Dermatol Alergol*. 2019;36(4):485-491. doi:10.5114/ada.2018.77056

19. Sanford JA, Gallo RL. Functions of the skin microbiota in health and disease. *Semin Immunol*. 2013;25(5):370-377. doi:10.1016/j.smim.2013.09.005
20. Dimitriu PA, Iker B, Malik K, Leung H, Mohn WW, Hillebrand GG. New insights into the intrinsic and extrinsic factors that shape the human skin microbiome. *mBio*. 2019;10(4):e00839-19. doi:10.1128/mBio.00839-19
21. Shibagaki N, Suda W, Clavaud C, et al. Aging-related changes in the diversity of women's skin microbiomes associated with oral bacteria. *Sci Rep*. 2017;7(1):10567. doi:10.1038/s41598-017-10834-9
22. Li Z, Bai X, Peng T, et al. New insights into the skin microbial communities and skin aging. *Front Microbiol*. 2020;11:565549. doi:10.3389/fmicb.2020.565549
23. Mrázek J, Mekadim C, Kučerová P, et al. Melanoma-related changes in skin microbiome. *Folia Microbiol*. 2019;64(3):435-442. doi:10.1007/s12223-018-00670-3
24. Nakatsuji T, Fenical W, Gallo RL. Response to comment on "A commensal strain of *Staphylococcus epidermidis* protects against skin neoplasia" by Nakatsuji et al. *Sci Adv*. 2019;5(9):eaay5611. doi:10.1126/sciadv.aay5611
25. Lan Y, Wang Q, Cole JR, Rosen GL. Using the RDP classifier to predict taxonomic novelty and reduce the search space for finding novel organisms. *PLoS One*. 2012;7(3):e32491. doi:10.1371/journal.pone.0032491
26. Robinson S, Peterson CB, Sahasrabhojane P, et al. Observational cohort study of oral mycobiome and interkingdom interactions over the course of induction therapy for leukemia. *mSphere*. 2020;5(2):e00048. doi:10.1128/mSphere.00048-20
27. Yumoto H, Yamada M, Shinohara C, et al. Soluble products from *Eikenella corrodens* induce cell proliferation and expression of interleukin-8 and adhesion molecules in endothelial cells via mitogen-activated protein kinase pathways. *Oral Microbiol Immunol*. 2007;22(1):36-45. doi:10.1111/j.1399-302X.2007.00320.x
28. Choi CH, Lee BH, Ahn SG, Oh SH. Proteasome inhibition-induced p38 MAPK/ERK signaling regulates autophagy and apoptosis through the dual phosphorylation of glycogen synthase kinase 3 $\beta$ . *Biochem Biophys Res Commun*. 2012;418(4):759-764. doi:10.1016/j.bbrc.2012.01.095
29. Kim ES, Park SJ, Goh MJ, et al. Mitochondrial dynamics regulate melanogenesis through proteasomal degradation of MITF via ROS-ERK activation. *Pigment Cell Melanoma Res*. 2014;27(6):1051-1062. doi:10.1111/pcmr.12298
30. Byrd AL, Belkaid Y, Segre JA. The human skin microbiome. *Nat Rev Microbiol*. 2018;16(3):143-155. doi:10.1038/nrmicro.2017.157
31. Hofer A, Legat FJ, Gruber-Wackernagel A, Quehenberger F, Wolf P. Topical liposomal DNA-repair enzymes in polymorphic light eruption [published correction appears in *Photochem Photobiol Sci*. 2011;10(12):1983]. *Photochem Photobiol Sci*. 2011;10(7):1118-1128. doi:10.1039/c1pp05009e
32. Liu Q, Mazhar M, Miller LS. Immune and inflammatory responses to *Staphylococcus aureus* skin infections. *Curr Dermatol Rep*. 2018;7(4):338-349. doi:10.1007/s13671-018-0235-8
33. Fournière M, Latire T, Souak D, Feuilloley MGJ, Bedoux G. *Staphylococcus epidermidis* and *Cutibacterium acnes*: two major sentinels of skin microbiota and the influence of cosmetics. *Microorganisms*. 2020;8(11):1752. doi:10.3390/microorganisms8111752
34. Hafner C, Stoehr R, van Oers JM, et al. The absence of BRAF, FGFR3, and PIK3CA mutations differentiates lentigo simplex from melanocytic nevus and solar lentigo. *J Invest Dermatol*. 2009;129(11):2730-2735. doi:10.1038/jid.2009.146
35. Rhodes AR, Stern RS, Melski JW. The PUVA lentigo: an analysis of predisposing factors. *J Invest Dermatol*. 1983;81(5):459-463. doi:10.1111/1523-1747.ep12522663
36. Husain I, Vijayan E, Ramaiah A, Pasricha JS, Madan NC. Demonstration of tyrosinase in the vitiligo skin of human beings by a sensitive fluorometric method as well as by <sup>14</sup>C(U)-L-tyrosine incorporation into melanin. *J Invest Dermatol*. 1982;78(3):243-252. doi:10.1111/1523-1747.ep12506603
37. Singh S, Nimse SB, Mathew DE, et al. Microbial melanin: recent advances in biosynthesis, extraction, characterization, and applications. *Biotechnol Adv*. 2021;53:107773. doi:10.1016/j.biotechadv.2021.107773
38. Alkema W, Boekhorst J, Eijlander RT, et al. Charting host-microbe co-metabolism in skin aging and application to metagenomics data. *PLoS One*. 2021;16(11):e0258960. doi:10.1371/journal.pone.0258960

**How to cite this article:** Zanchetta C, Vilanova D, Jarrin C, et al. Bacterial taxa predictive of hyperpigmented skins *Health Sci Rep*. 2022;5:e609. doi:10.1002/hsr2.609