Analysis of Matched Skin and Gut Microbiome of Patients with Vitiligo Reveals Deep Skin Dysbiosis: Link with Mitochondrial and Immune Changes

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Vitiligo is an autoimmune disease characterized by patchy, white skin owing to melanocyte loss. Commensal cutaneous or gut dysbiosis has been linked to various dermatological disorders. In this study, we studied the skin and gut microbiota of patients with vitiligo compared with those of healthy controls. We obtained swabs and biopsies from both lesional and nonlesional skin as well as stool and blood samples from each individual. We detected reduced richness and diversity of microbiota in the stools of subjects with vitiligo compared with the stools of the controls ($P < 0.01$). Skin swabs had greater α-diversity than biopsies ($P < 0.001$); swabs from lesional sites were primarily depleted of *Staphylococcus* compared with those from nonlesional sites ($P < 0.02$). Sampling deeper layers from the same patients showed differences in both α- and β-diversity between samples with decreased richness and distribution of species ($P < 0.01$) in the lesional site. Biopsy microbiota from the lesional skin had distinct microbiota composition, which was depleted of protective *Bifidobacterium* and *Bacteroides* but was enriched in *Proteobacteria, Streptococcus, Mycoplasma,* and mtDNA ($P < 0.001$); the latter increased in the same patients with heightened innate immunity and stress markers in their blood ($P < 0.05$). These data describe vitiligo-specific cutaneous and gut microbiota and a link between skin dysbiosis, mitochondrial damage, and immunity in patients with vitiligo.

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INTRODUCTION

Vitiligo is characterized by an acquired loss of melanocytes in the skin and sometimes in the hair follicles. It is now well-demonstrated that CD8+ T cells attracted in the epidermis are responsible for the melanocytic loss. GWAS identified over 50 vitiligo susceptibility loci involved in melanogenesis and immunity in patients with vitiligo (Roberts et al., 2020). However, these genetic studies and a delay in vitiligo age of onset over the past 30 years emphasize the key role of environmental factors in triggering vitiligo (Jin et al., 2020). We recently showed that patients with vitiligo have an increased number of NK cells and innate immune cells in their nonlesional (NL) skin and in their blood and that those innate cells have a greater response to stress (Tulic et al., 2019). Those innate immune cells produce higher amounts of IFN-γ than healthy controls, triggering the production of CXCL9, CXCL10, and CXCL11 by both keratinocytes and melanocytes—ultimately triggering CXCR3B-induced apoptosis in melanocytes. We demonstrated that these early events, which lead to CD8+ T-cell–mediated adaptative response against melanocytes, can be triggered by endogenous or exogeneous stress, namely damage-associated molecular patterns or pattern-associated molecular patterns, respectively. Bacteria are among the top producers of pattern-associated molecular patterns and could participate in the activation of the innate immune response in vitiligo. Interestingly, gut dysbiosis has been reported in several autoimmune disorders. Although less reported, skin dysbiosis is also observed in some dermatoses, such as in acne, psoriasis, and atopic dermatitis (O'Neill and Galo, 2018; Stehlikova et al., 2019; Williams and Gallo, 2017). Most of those studies assessed the superficial microbiome using swabs. However, recent data emphasized the differences in the microbiome between the surface and the deeper part of the skin using biopsy samples (Bay et al., 2020; Prast-Nielsen et al., 2019). Surprisingly, data on the microbiome in patients with vitiligo remain sparse. To date, there exists only one study suggesting dysbiosis in vitiligo skin (Ganju et al., 2016); however, the authors only assessed the superficial skin microbiota using swabs and compared lesional (L) with NL vitiligo skin without including healthy controls. A more recent study in a mouse model of vitiligo has shown that antibiotic-induced depletion of *Bacteroides*-dominated population in the gut may induce

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Abbreviations: ASV, amplicon sequence variant; L, lesional; NL, nonlesional; PD, phylogenetic diversity; RA, relative abundance

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depigmentation in the skin, suggesting a possible link between the gut and skin compartments (Dellacecca et al., 2020). In this study, we compared the microbiota of the gut with that of the superficial and deeper parts of L and NL skin of patients with vitiligo and compared these results with those of sample- and site-matched healthy controls. We found gut dysbiosis with a reduced *Bacteroides* population in patients with vitiligo. We also report the marked differences of the microbiota between using swabs and using skin biopsies. Finally, we report significant variation in microbiota diversity and quality in the deeper regions of L vitiligo skin and the evidence that these microbial changes are associated with mitochondrial damage and peripheral changes in innate immunity.

RESULTS

Vitiligo disease is associated with lower α-diversity and increased *Firmicutes*-to-*Bacteroidetes* fraction in the gut

Initially, we set out to examine whether we could detect differences in microbial composition and diversity in the guts of patients with vitiligo (n = 10) compared with the guts of healthy controls (n = 10). We have shown lower gut microbial α-diversity in patients with vitiligo than in healthy controls (for observed amplicon sequence variants [ASVs], $P = 0.005$; Shannon diversity index, $P = 0.115$; and Faith's phylogenetic diversity [PD] $H = 5.851$, $P = 0.006$) (Figure 1a). β-Diversity did not differ between the groups. We found trends for higher bacterial relative abundance (RA) of *Firmicutes* ($P = 0.09$) and lower RA of *Bacteroidetes* ($P = 0.08$) in the stools of subjects with vitiligo than in those of healthy controls (Figure 1b), most likely due to reduced *Bacteroides* (Figure 1c and Supplementary Table S1), with putatively significant differences between the groups when comparing the RA of *Firmicutes* with that of *Bacteroidetes*, with an increased *Firmicutes*-to-*Bacteroidetes* ratio from 8.5 ± 1.2 (mean ± SEM) in healthy controls to 18.5 ± 2.6 in patients with vitiligo ($P < 0.01$).

Significant microbial dissimilarity between swabs and biopsies

To examine whether there are differences in microbial composition and diversity between the skin of patients with vitiligo and healthy skin, we studied the microbiota extracted from paired skin swabs and skin biopsies from the same subject as well as collected samples from the patient's L and NL skin sites. We compared the microbial communities' α- and β-diversity between the swab or biopsy samples from control skin and from the L and NL sites (n = 30 swabs and n = 30 biopsies). Observed ASVs and Shannon diversity index were higher in the swabs than in the biopsies ($P < 0.001$ for both) (Figure 2a), whereas Faith's phylogenetic diversity (PD) was higher in the biopsies ($P < 0.001$) (Figure 2a), the latter suggesting higher phylogenetic richness in biopsies than in swabs. These results suggest that the richness and distribution of bacteria (α-diversity) differ in relation to the sampling method. Furthermore, our results demonstrate distinct and highly significant differences in β-diversity between biopsies and swabs ($P < 0.001$) (Figure 2b). We found higher *Bifidobacterium* (with *B. bifidum* and *B. longum*), *Escherichia Shigella, Parabacteroides,* and *Enterococcus* in the biopsies than in the swabs, whereas *Staphylococcus, Paracoccus, Kocuria, Micrococcus,* and *Anaerococcus* were more abundant in the skin swabs ($P < 0.05$ for all comparisons) (Figure 2c). In the swabs, *Staphylococcus* was the most abundant genus, making up >30% of all bacteria; whereas in the biopsies, *Bifidobacterium* was the most common, making up almost 60% of the total bacterial load. Taken together, these results demonstrate a marked difference in the skin microbiome when analyzed using swab versus using biopsy sampling, and there are several bacterial genera that correlate strongly and significantly with the sample collection method or the depth of microbiota sampling.

Depletion of *Staphylococcus* and enrichment of *Gammaproteobacteria* in the L swabs of patients with vitiligo

Swab samples from both NL and L vitiligo skin had a significantly higher α-diversity than healthy control swabs ($P < 0.01$ for L and $P < 0.05$ for NL) with Shannon diversity index being less sensitive with borderline significance between the two groups ($P = 0.06$) (Figure 3a). There was no difference in α-diversity between NL and L skin samples (Figure 3a). We found no significant differences in β-diversity between the healthy, NL, and L skin swab samples. At the phylum level, the composition of microbiota at the surface of NL and healthy skin was very similar (Figure 3b). Comparing L skin with NL skin, we observed decreased *Firmicutes* in the L skin ($P < 0.01$) (Figure 3b); this was most likely due to decreased *Staphylococcus* genus at this site ($P < 0.02$) (Figure 3c and Supplementary Table S2). Examining the bacterial composition of swab microbiota at the genus level, we show that greater than half of the taxonomic assignments on skin surface belong to five major genera: *Staphylococcus*, *Corynebacterium*, *Cutibacterium* (formerly *Propionibacterium*), *Kocuria,* and *Paracoccus* (Figure 3c). We found significantly reduced *Staphylococcus* ($P < 0.02$) and *Cutibacterium* ($P = 0.057$) in the L swab compared with those in the NL swabs (Figure 3c), of which latter may have important implications in vitiligo because the loss of *Cutibacterium* diversity triggers the activation of the innate immune system (Dell'Anna et al., 2017). We have also seen trends for increased *Proteobacteria* phylum in the L sites compared with that in the NL sites ($P = 0.08$) or compared with that in the healthy skin ($P = 0.061$) (Figure 3b). Because *Proteobacteria* contains many well-known human pathogens and is associated with disbalance and inflammation (Iacob and Iacob, 2019), we investigated this phylum in more detail. This analysis has shown a disease-dependent increase in the presence of *Gammaproteobacteria* (i.e., RA in L swab is greater than that in NL swab and then is greater than that in the control swab) and disease-dependent decrease in *Alphaproteobacteria* (i.e., RA in L swab is less than that in NL swab and then less than that in the control swab) ($P = 0.046$) (Supplementary Figure S1a). L skin enrichment with *Gammaproteobacteria* has, to date, also been shown in the only other study in subjects with vitiligo (Ganju et al., 2016). Further examining the *Proteobacteria* genus, we found significant increases in *Moraxella* ($P = 0.007$) and *Microvirga* ($P = 0.007$) in the NL skin compared with those in the healthy controls (Supplementary Figure S1b and Supplementary Table S3). Trends were also seen for increased *Paracoccus* ($P = 0.09$) and *Acinetobacter* ($P =$

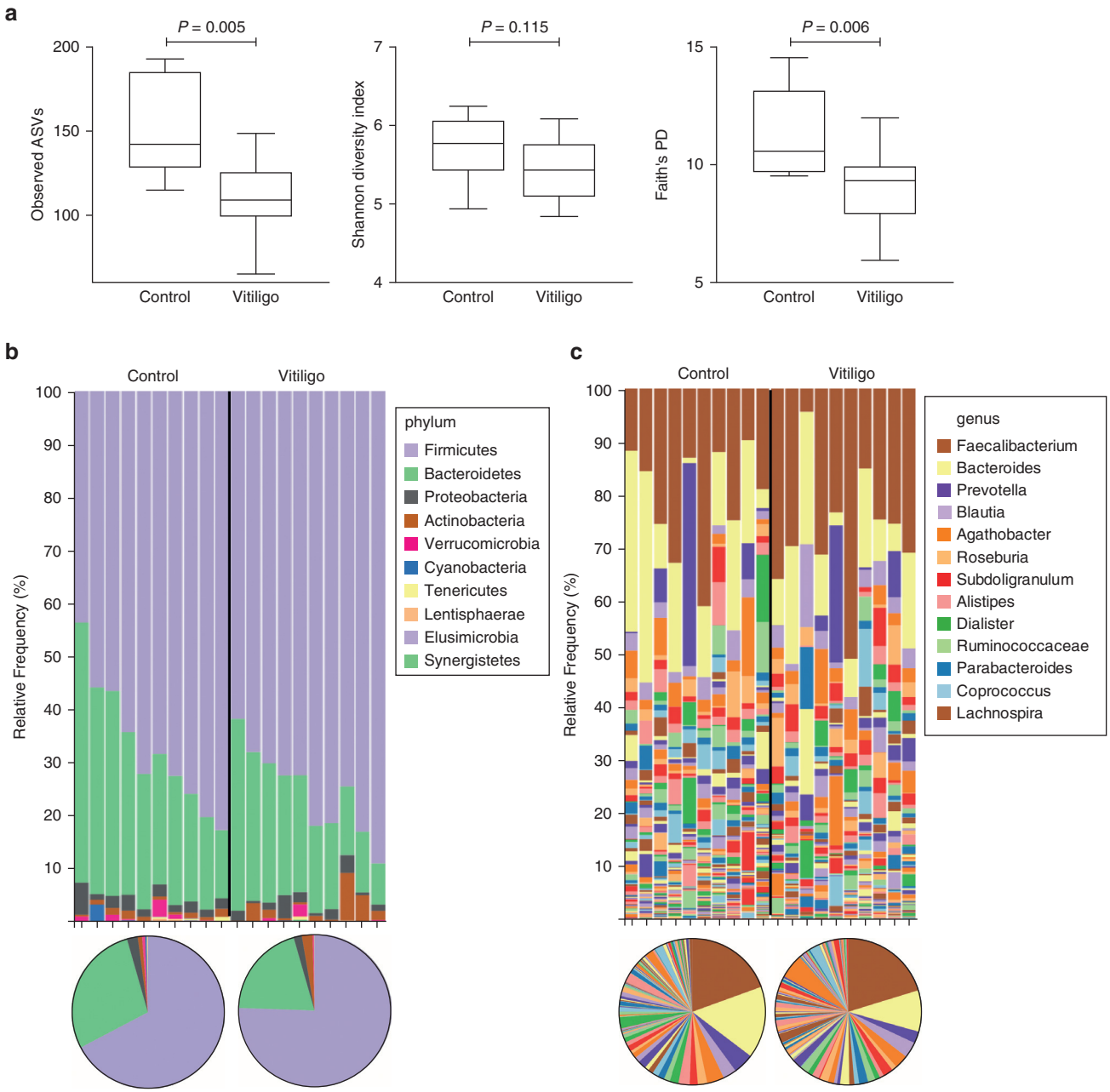
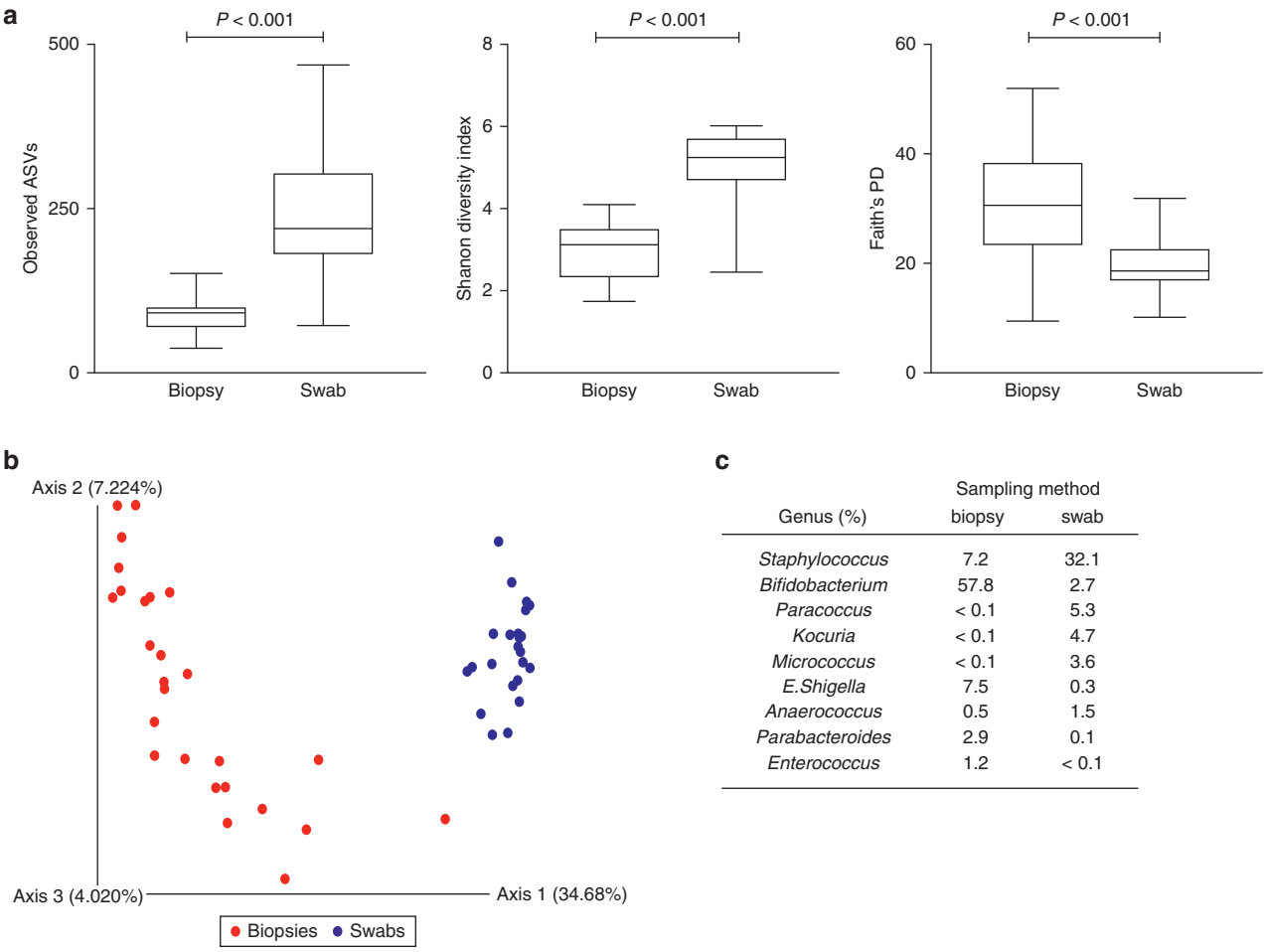


Figure 1. Reduced α-diversity and increased *Firmicutes*-to-*Bacteroides* ratio in the gut microbiota of subjects with vitiligo. (**a**) Boxplots illustrating the comparison of different measures of α-diversity between healthy (n = 10) and vitiligo (n = 10) stool samples. (**b, c**) Bacterial composition and diversity expressed as a percentage of relative abundance between the two groups at the (**b**) phylum and (**c**) genus levels. Boxplots are shown as median with 5–95% CI. Individual subjects are shown as taxa bars (above), and grouped data are shown as pie charts (below). A complete list of ASVs is shown in Supplementary Table S1. ASV, amplicon sequence variant; CI, confidence interval; PD, phylogenetic diversity.

0.08) in vitiligo swabs compared with control swabs; however, this was not statistically significant.

Biopsy microbiota in L skin have distinct composition and are associated with mitochondrial damage and immune changes

In contrast to the swabs, we found that the microbial community β-diversity in the L skin biopsies differed significantly from those in the NL skin biopsies or in the healthy controls ($P < 0.001$) (Figure 4a) and that L biopsies had the most distinct microbiota compared with those of all other groups (Supplementary Figure S2). Unlike in swab data where we failed to see differences in the α- or β-diversity between NL and L sites, in deep skin biopsies, we saw a significant decrease in species richness and distribution (Shannon diversity index, $P = 0.006$) but an increase in PD (Faith's PD, $P = 0.003$) in L biopsies compared with that in NL biopsies. Shannon index was higher in the NL skin than in the controls ($P = 0.008$) (Figure 4b). In our cohort, the most obvious difference seen in taxa bar plots was the significant increase in *Tenericutes* ($P < 0.001$) and mtDNA ($P < 0.007$) exclusively in the L skin (Figure 4c). This increase was seen in the



Genus (%)	Sampling method biopsy	swab
Staphylococcus	7.2	32.1
Bifidobacterium	57.8	2.7
Paracoccus	< 0.1	5.3
Kocuria	< 0.1	4.7
Micrococcus	< 0.1	3.6
E.Shigella	7.5	0.3
Anaerococcus	0.5	1.5
Parabacteroides	2.9	0.1
Enterococcus	1.2	< 0.1

Figure 2. The difference in bacterial diversity and bacterial composition between swabs and biopsies owing to sampling method. (**a**) Boxplots illustrating the comparison of different measures of α-diversity in paired samples of skin biopsies (n = 30) and skin swabs (n = 30). (**b**) PCoA plot representing β-diversity in biopsies (red circles) and swabs (blue circles) ($P < 0.001$). (**c**) Image demonstrating sampling method–related differential bacterial abundance where bacteria enriched at each site is denoted and given as a percentage of the total genus. Boxplots are shown as median with 5–95% CI. ASV, amplicon sequence variant; CI, confidence interval; PCoA, Principal Coordinate Analysis; PD, phylogenetic diversity.

biopsies from 7 of 10 L skin samples whereby 3 of 10 samples resembled NL skin (Figure 4c). The increased *Tenericutes* was due to increased *Mycoplasma* genus at this site ($P < 0.001$) (Figure 4d and Supplementary Table S4). Results also showed borderline significance for decreased *Actinobacteria* ($P = 0.09$) and increased *Firmicutes* ($P = 0.08$) in the L biopsies compared with that in the NL or control biopsies (Figure 4c), and we therefore explored further at the genus level.

In the skin biopsies, greater than half of the taxonomic assignments belong to five major genera (three overlapping with the swabs): *Bifidobacterium, E. Shigella, Staphylococcus*, *Cutibacterium,* and *Parabacteroides* (Figure 4d). The heatmap illustrates the top 20 most differentially expressed bacterial taxa between the three groups at the individual level (Supplementary Figure S3). Pair-wise analysis for differential abundance between the NL vitiligo biopsies and the control biopsies has shown *Staphylococcus* ($P = 0.02$) and *Cutibacterium* ($P = 0.07$) to be depleted in the NL biopsies; the same two genera that were depleted on the surface of the L skin. In contrast, *Enterococcus, Mycoplasma*, *Veillonella, Intestinibacter, Bacteroides, E. Shigella, Parabacteroides, Bifidobacterium,* and *Streptococcus* were enriched in the NL vitiligo skin compared with those in healthy skin (all $P < 0.007$) (Supplementary Figure S3). Comparisons between L and NL biopsies have shown L skin to be further enriched for opportunistic bacteria such as *Streptococcus, Gemella,* and *Cutibacterium* as well as *Proteobacteria*, including *Raistonia* and *Undibacterium* (all $P < 0.05$), with a large and specific increase in *Mycoplasma* and mtDNA ($P = 0.007$) (Figure 4d and Supplementary Figure S3). In contrast, *Intestinibacteria, Bacteroides, Clostridium, Enterococcus, E. Shigella, Parabacteroides,* and *Bifidobacterium*—all important commensals—were significantly depleted taxa in the L skin biopsies compared with those in the NL skin biopsies (all $P < 0.001$) (Supplementary Figure S3). *Bifidobacterium* was completely depleted in 7 of 10 L biopsies when compared with their matched NL sites, reducing from 72% of RA to 21% in the L sites ($P < 0.001$) (Figure 4d).

Finally, we further investigated the differences between the first three patients who did not have an increase in mtDNA in their skin biopsy and the remaining seven patients who did. Interestingly, these three patients had a similar microbiome to NL samples, that is, a low abundance of *Tenericutes* and *Firmicutes* with a predominant presence of *Actinobacteria*

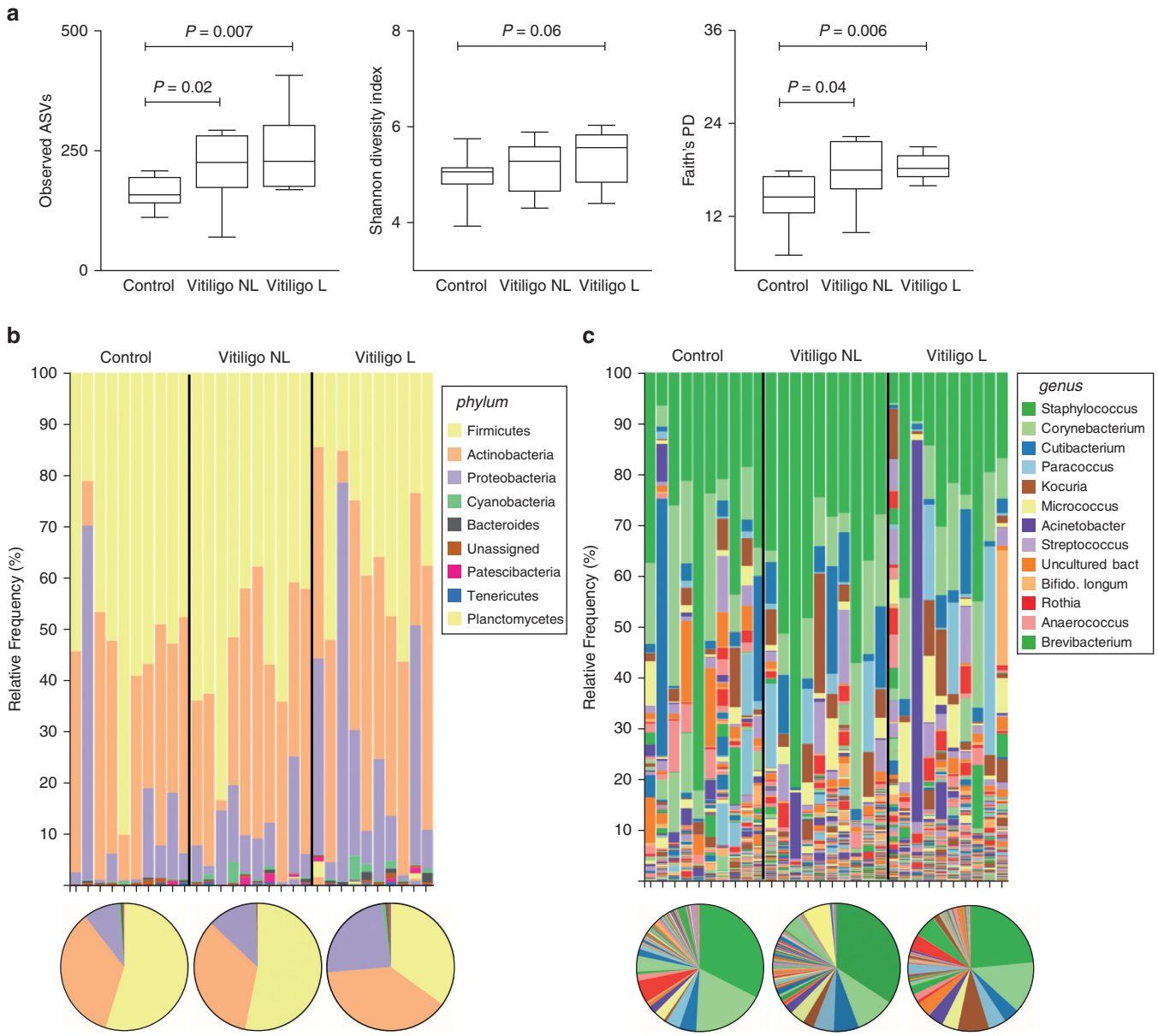


Figure 3. Higher α-diversity, depletion of *Staphylococcus*, and enrichment of *Proteobacteria* in L swabs of patients with vitiligo. (**a**) Boxplots illustrating the comparison of different measures of α-diversity in skin swabs taken from NL and L regions of the skin from patients with vitiligo (n = 10) compared with the skin swabs taken from healthy controls (n = 10). Bacterial composition and diversity (expressed as the percentage of relative abundance) between the groups are shown at the (**b**) phylum and (**c**) genus levels, with individual taxa data shown above and the grouped average data shown as pie charts below. Boxplots are shown as median with 5–95% CI. A complete list of ASVs is shown in Supplementary Table S2. ASV, amplicon sequence variant; CI, confidence interval; L, lesional; NL, nonlesional; PD, phylogenetic diversity.

(Figure 4c). The difference between these two subgroups of patients could not be explained by their differences in sex, age, the location from where the biopsies were taken (all three were taken from dry region, forearm), disease activity (all had stable disease), or disease duration (2.5–4 years). To further investigate whether these changes in the skin could activate the innate immune response, we measured the levels of IFN-γ and chemokines CXCL9 and CXCL10 that we previously have shown to be increased after stimulation of the innate cells of patients with vitiligo by damage-associated molecular patterns or pattern-associated molecular patterns (Tulic et al., 2019). We also measured the levels of CXCL16 that is produced by keratinocytes and that mediates CD8+ T-cell skin trafficking under oxidative stress in patients with vitiligo (Bolyen et al., 2019). We found that the seven patients with increased mtDNA in their L sites had significantly increased levels of IFN-γ, CXCL9, and CXCL16 ($P < 0.05$) in their serum compared with the three patients without elevated mtDNA (Figure 4e). The corresponding levels of CXCL10 in the serum were of borderline significance ($P = 0.064$). Using Spearman tests, we have shown positive correlations between mtDNA and immune markers. This was particularly true for IFN-γ ($r = 0.83$, $P = 0.005$) and CXCL16 ($r = 0.82$, $P = 0.006$); both parameters strongly and positively correlated with mtDNA (Supplementary Figure S4). The correlations for CXCL9 and CXCL10 with mtDNA had a similar

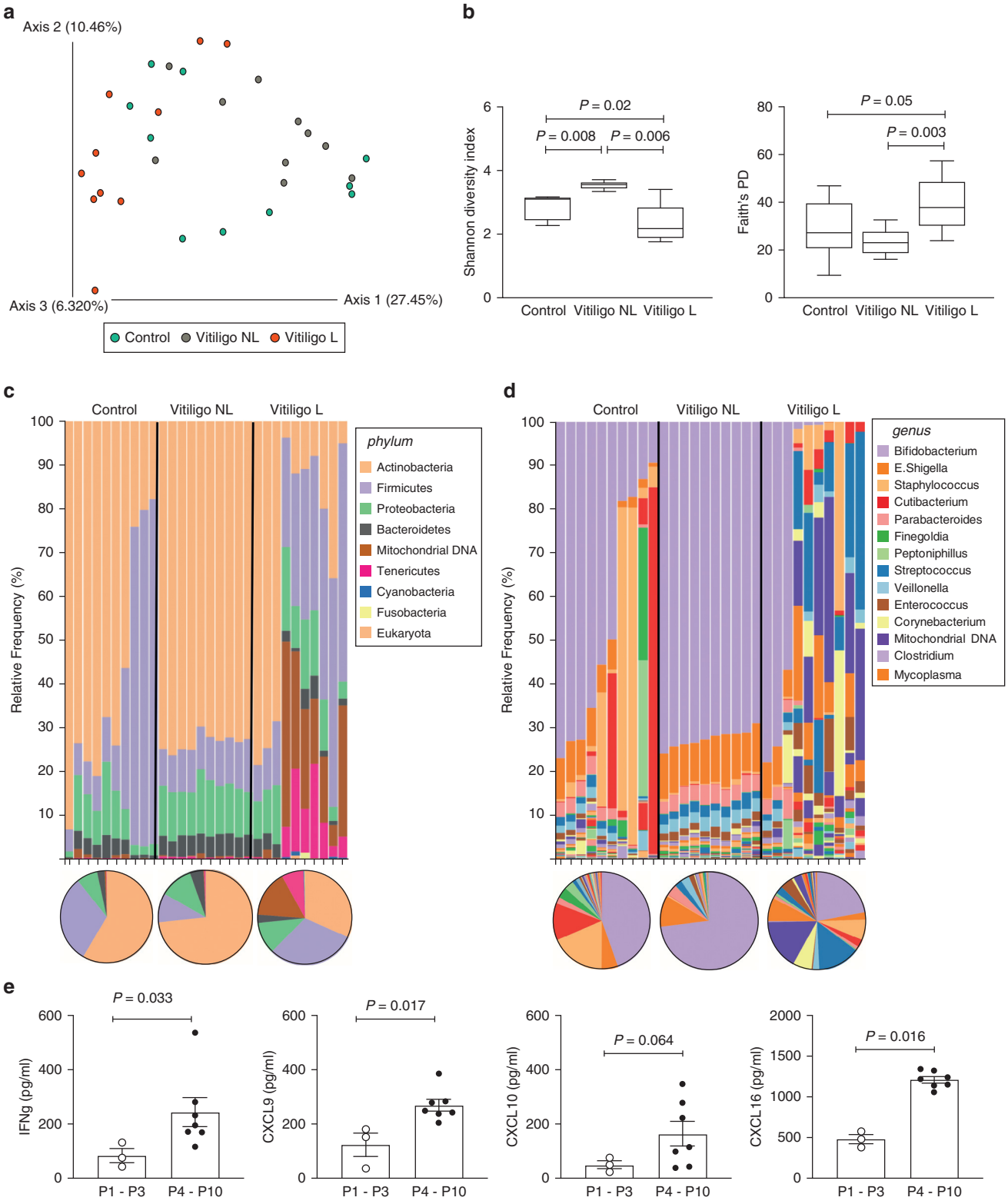


Figure 4. L biopsies from vitiligo patients (P) have distinct microbiota and are associated with changes in bacterial diversity, mitochondrial damage, and activation of the innate immune system. (**a**) PCoA plot representing β-diversity shows the biopsies from the L zone of vitiligo patients (red circles) to be significantly different from those of all other samples ($P < 0.001$). (**b**) Boxplots illustrating α-diversity in skin biopsies taken from NL and L skin from vitiligo patients (n = 10) compared with healthy skin (n = 10). Bacterial composition and diversity between the two groups at (**c**) phylum and (**d**) genus levels. Individual subjects are shown as taxa bar plots (above), and grouped data are shown as pie charts (below). Boxplots are shown as median with 5–95% CI. A complete list of ASVs is shown in Supplementary Table S4. (**e**) Innate (IFN-γ, CXCL9, CXCL10) and stress marker (CXCL16) proteins in the serum of vitiligo patients with elevated mitochondrial signature (Patients, P 4–10 in **c**) compared to vitiligo patients without increased mitochondrial signature (Ps 1–3). Results are shown as mean ± SEM (n = 3–7). ASV, amplicon sequence variant; CI, confidence interval; L, lesional; NL, nonlesional; PCoA, Principal Coordinate Analysis; P, patient; PD, phylogenetic diversity.

positive relationship but were moderately correlated (r = 0.52 and r = 0.26, respectively).

DISCUSSION

Gut and, to a lesser extent, skin dysbiosis have been reported in many inflammatory and autoimmune disorders. Surprisingly, microbiome studies in vitiligo remain limited. In this study, we have shown dysbiosis in the gut and skin microbiota of subjects with vitiligo compared with those of controls. The most striking differences were detected in the deep dermal layer of the L skin where enrichment of pathogenic *Tenericutes (G. Mycoplasma)* bacteria and a loss of protective *Bifidobacterium* were associated with mitochondrial damage at the same site as well as increased stress and immune activation markers in the peripheral blood of the same patients. We do acknowledge the relatively limited number of subjects analyzed being a potential weakness of this study; however, given its invasive nature requiring six samples per patient (including two skin biopsies), we based our numbers on existing human microbiome studies using similar sequencing approaches performed in healthy (Fadlallah et al., 2019 Jakobsson et al., 2014; Nakatsuji et al., 2013; Prast-Nielsen et al., 2019) and diseased (Abrahamsson et al., 2014; Baurecht et al., 2018; Silva et al., 2006) skin and in the one available vitiligo study published to date (Ganju et al., 2016).

We detected reduced α-diversity (richness and distribution of bacterial species) in the stool of patients with vitiligo compared with those in the stool of healthy controls with increased *Firmicutes*-to-*Bacteroidetes* ratio in patients with vitiligo. A similar increase has previously been reported in other autoimmune diseases such as type 1 diabetes, Grave's disease, lupus, and multiple sclerosis as well as autoimmune skin conditions, including scleroderma and psoriasis (Renaud et al., 2015). Interestingly, a protective effect of *Bacteroides* was recently reported in the gut of a mouse vitiligo model and was associated with skin depigmentation of mice (Dellacecca et al., 2020). If the same protection holds true for human vitiligo, reduction and/or lack of protective *Bacteroides* in the human gut may contribute to disease onset or exacerbation. It is important to note that vitiligo depigmentation is associated with better prognosis in patients with melanoma (Teulings et al., 2015), and interestingly, our stool microbiota results in patients with vitiligo (high *Firmicutes*-to-*Bacteroidetes* ratio) have striking similarities with good responders in patients with melanoma treated with anti-PD1 (Gopalakrishnan et al., 2018). Together, these results show the differences in gut microbial composition between patients with vitiligo and healthy controls and may suggest a possible gut–skin axis in this disease. However, this remains to be fully tested in a larger study population.

Our results analyzing skin microbiota emphasize a marked difference in the skin microbiota when analyzed using swab versus using biopsy sampling, suggesting that there are several bacterial genera that correlate strongly and significantly with the sample collection method. Most of the studies to date that assessed the microbiota in skin disorders have used only swab samples. The unique aspect of our study is that we obtained stool, skin swab, and skin biopsy samples from the same individuals, thereby reducing interpatient variability and strengthening the robustness of our study. Our results are in agreement with those of Prast-Nielsen et al. (2019) and emphasize the need for simultaneous collection of swabs (surface) and biopsy (deep skin) samples because biopsies provide additional information that is critical for correct interpretation of the data. Although we found a differential abundance of microbial composition between healthy and vitiligo skin swabs, using swabs, we failed to see statistically significant differences in α- or b-diversity between L and NL sites. The primary finding in the swabs was the depletion of commensal *Staphylococcus* and the enrichment of pathogenic *Proteobacteria* in the L skin site compared with that in the NL skin sites. *Staphylococcus epidermidis* is a known important symbiont in the skin, which directly interacts with cutaneous immune cells to regulate skin homeostasis (Leonel et al., 2019). *Staphylococcus* spp. are also known to colonize lesions in a number of dermatological diseases, including atopic dermatitis, and present propensity to form biofilms—adhesive surface–attached colonies that become highly resistant to antibiotics and immune responses (Gonzalez et al., 2017).

The current understanding of the skin microbiota is based on swab sampling the outermost layer of the epidermis. Despite the invasive nature of biopsies, sampling dermal microbiota has become critically important because the dermal community is less affected by external factors (Bay et al., 2020). Our results give us a comprehensive profiling of the microbial species that are depleted in full-thickness subepidermal compartments of the vitiligo skin, which may be targeted for therapy. Thus, in contrast to the swabs, marked differences were found in the microbial community not only between healthy and vitiligo samples but also between L and NL skin biopsies, with significant statistical differences in α- and b-diversity. L biopsies from patients with vitiligo had unique microbiota compared with those of all other samples, which were exclusively enriched in opportunistic bacteria such as *Mycoplasma*, *Streptococcus*, and *Proteobacteria* as well as mtDNA. These L biopsies were also characterized by significant and profound depletion of commensals at the same site, such as *Staphylococcus* and *Bifidobacteria*. Interestingly, the same imbalance between *Actinobacteria* and *Firmicutes* in L skin versus the imbalance in the NL skin

Table 1. Patient Characteristics

Characteristics	Healthy Controls	Patient with Vitiligo
Subjects (n)	10	10
Age, years, mean (SE)	60.2 (2.8)	51.5 (3.9)
Sex (male/female)	4/6	3/7
Type of vitiligo	—	Nonsegmental
Mean disease duration (y)	—	3.2 (1.4–22)
Activity of vitiligo	—	Stable
Family history (%)	—	2 (20%)
Location of biopsy (n, all dry areas)	Shoulder (5), forearm (3) leg (1), ankle (1)	Forearm (5), leg (4), ankle (1)

was found in the only other study that assessed skin microbiota in vitiligo (Ganju et al., 2016). These are important findings because they give us additional clues for potential targets to test in therapeutic settings. Despite the limited number of subjects, with the impressive increase in *Tenericutes* and mtDNA only in the L but not in the NL biopsies, it is tempting to speculate that *Tenericutes* or mtDNA may be used as a marker for patients who may benefit from a supplementation of probiotics or bacterial lysates with, for example, *Bifidobacteria* in addition to their established treatment. Confirmation of our study and well-designed interventional studies with *Bifidobacteria* supplementation in a larger number of patients are required to confirm this hypothesis.

The latest evidence in the literature emphasizes the interaction between mitochondria and microbiota (Saint-Georges-Chaumet and Edeas, 2016). It has been recently shown that the mitochondrial genotype modulates both ROS production and the species diversity of the gut microbiome (Yardeni et al., 2019). Interestingly, mitochondrial alterations with increased production of ROS have been reported in vitiligo cells (Dell'Anna et al., 2017; Sahoo et al., 2017). Our results are in accordance with these in vitro studies and demonstrate potent mitochondrial damage in the L skin of patients. As observed in the gut of mice, α-diversity decreased in samples with high mtDNA (Yardeni et al., 2019), the same as what we see in the gut of our patients with vitiligo (Figure 1). Modification of the microbiome only in the biopsy samples having mtDNA strongly argues in favor of microbiome modification induced by the mitochondrial stress.

Interestingly, the genus that was almost completely absent in the seven patients with the mtDNA in the skin was *Bifidobacterium*, which is well-known to have protective effects and to decrease the activation of innate immune cells (Iacob and Iacob, 2019). Moreover, those seven patients had also significantly increased levels of immune cytokines (IFN-γ), innate chemokines (CXCL9), and stress markers (CXCL16) in their serum compared with the patients without elevated mtDNA. Thus, it is tempting to hypothesize that similar to what has been shown in the gut of mice (Yardeni et al., 2019), primitive mitochondrial stress in some patients with vitiligo could be responsible for the change in the skin microbiome. This skin dysbiosis could then trigger the innate response through pattern-associated molecular patterns and lead to the production of IFN-γ and chemokines that can be detected in the serum of these patients. Of course, this hypothesis needs to be confirmed in a larger number of patients, and ideally, metagenomics should be performed to characterize the exact origin of human mtDNA. We are confident that the reported mtDNA is of human origin because the sequence annotated was blasted and returned a 99% match. We know that there are conserved regions between bacterial *16S rRNA* gene and human mtDNA; hence, universal primers designed to bind to bacterial *16S rRNA* gene can and do bind and amplify human mtDNA (Boguszewska et al., 2020; Eperon et al., 1980; Yang et al., 2014). This, along with literature showing that mitochondrial alterations are associated with increased ROS production in vitiligo cells (oxidative stress being a compelling explanation for melanocyte loss) (Sahoo et al., 2017), is supporting evidence of detection of human mtDNA in L skin samples from patients with vitiligo.

There are a number of unique aspects of this study. First, to our knowledge, there are no data examining the role of microbiota in vitiligo skin compared with their role in healthy skin. Second, there are matched multiple samplings (swab, biopsy, stool, serum) from each patient, avoiding interpatient variability and thereby allowing for the robustness of the data even with a modest sample size. Our key data demonstrating differences between skin swabs and skin biopsy microbiota and between L and NL or control skin are all highly significant ($P < 0.001$), suggesting important biological differences between these sites. Third, we have shown that skin dysbiosis in patients with vitiligo is associated with mitochondrial damage and have presented early evidence suggesting that loss of protective bacteria at the same site is associated with heightened innate immune response in the blood of the same patients with vitiligo. These data not only describe vitiligo-specific cutaneous and gut microbiota but also show a possible link between microbiota, mitochondrial alteration, and innate immunity. Although the link remains to be confirmed in larger cohorts, this pilot study opens up additional avenues to explore in the future treatment of patients with vitiligo. Whether these reported changes in cutaneous microbiota in vitiligo are a cause or a consequence of the disease remains to be established.

MATERIALS AND METHODS

Biological samples

A total of 20 subjects were recruited for this study (Table 1). A total of 10 patients with nonsegmental vitiligo with stable disease and 10 healthy subjects were recruited from the Department of Dermatology, Archet 2 Hospital, Centre Hospitalier Universitaire de Nice, France, after informed written consent was obtained. Exclusion criteria included the presence of other autoimmune diseases or previous treatment (phototherapy, topical or systemic corticosteroids, systemic antibiotics, and/or other immunosuppressive agents) for up to 3 months before skin sampling. The groups were matched for sex, age, and biopsy location (shoulder or forearm in >70% of cases). All skin samples were taken from dry locations (Table 1). Swab and biopsy samples were collected at the same time for each patient, at the same time of the day, and 12–16 hours after their last washing. All patients were requested to refrain from the application of any creams and/or emollients or cosmetics (such as foundation to cover up the target L site) for 24 hours before their visit. From each patient with vitiligo, we obtained two 4-mm skin punch biopsies (one from the L site and one from the NL site) and one skin biopsy of the same size from each healthy subject. Skin swabs were taken at the same time from adjacent skin areas, sampling 5 × 5 cm L and NL skin sites using sterile dry swabs (Copan Diagnostics, Murrieta, CA) and rinsed in 200 μl sterile 0.15 M sodium chloride and 0.1% Tween 20 (v/v). For skin biopsies and swabs, the L sites were chosen in the center of the vitiligo plaques away from the perilesional area. The perilesional area was the area located in the 5-mm border of the depigmented lesion. The same morning, stool samples as well as 10 ml of blood were also collected from all subjects. Serum was obtained from the blood

after Ficoll gradient centrifugation (Lymphoprep; Euromedex, Souffelweyersheim, France). All biological samples (n = 60 from patients with vitiligo and n = 40 from healthy controls) were stored at −80 °C until analysis. The study was approved by the National Ethics Committee (N14.028) and was carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki).

DNA isolation from stool samples

A total of 80–120 mg of frozen stool was transferred to Precellys soil grinding SK38 lysing tubes (Bertin Technologies, Montigny-le-Bretonneux, France), and one volume of warm (37 °C) lysis buffer (4% [w/v] SDS, 50 mM Tris-hydrochloride, pH 8.0, 500 mM sodium chloride, 50 mM EDTA) was added. Samples were homogenized for 10 minutes at room temperature using a Vortex adapter (Mo Bio Laboratories, Carlsbad, CA). Lysozyme (Chemie GmbH, Sigma-Aldrich, Steinheim, Germany; final concentration was 6.25 mg/ml) was added, and samples were incubated at 37 °C for 30 minutes and were then transferred to 80 °C heating block and incubated for 15 minutes by inverting every 5 minutes. Samples were then centrifuged at 4 °C 20,000*g* for 5 minutes, and the supernatants were collected; proteinase K (Roche Diagnostics GmbH, Mannheim, Germany) was added (final concentration was 0.4 mg/ml), and the samples were incubated on a heating block at 70 °C for 10 minutes. After incubation, 10 M ammonium acetate (final concentration was 2 M) was added, and samples were incubated on ice for 5 minutes and then centrifuged at 4 °C 20,000*g* for 10 minutes. Supernatants were collected, and an equal volume of cold isopropanol was added. The samples were stored on ice for 30 minutes and thereafter centrifuged at 4 °C 20,000*g* for 20 minutes. Pellets were washed 2–3 times with cold 70% ethanol, dried, and dissolved overnight in commercial Tris-EDTA buffer (1 × Tris-EDTA). The following day, DNA concentrations were measured using Qubit dsDNA Broad Range Assay kit (Thermo Fisher Scientific, Waltham, MA) on Qubit 3.0 fluorometer (Thermo Fisher Scientific). RNAse (Thermo Fisher Scientific, Vilnius, Lithuania) at a final concentration of 1 μg/μl was used.

DNA isolation from skin samples

Bacterial DNA was extracted from skin biopsies and swabs using QIAamp DNA Microbiome Kit (catalog number 51704) (QIAGEN, Hilden, Germany). We followed the manufacturer's instructions with a single modification. After the addition of proteinase K the second time, samples were incubated overnight on a heating block at 56 °C. After eluting DNA, the concentrations were measured using Qubit 1X dsDNA HS Assay Kit (Life Technologies, Eugene, OR) on Qubit 4.0 fluorometer (Thermo Fisher Scientific, Illkirch Cedex, FR).

16S rRNA gene library preparation and amplicon sequencing

The sequencing library was prepared according to Earth Microbiome Project's instructions (Nakatsuji et al., 2013) with the following modifications. The fused primers were modified to contain a barcode sequence on both forward (341F) and reverse (805R) primers and selected to target the V3–V4 region instead of the V4 region. Sequences of fused primers are provided in Supplementary Table S5. The PCR reactions for library preparation were carried out in triplicates as follows: 20 ng of template DNA was mixed with 5PRIME HotMasterMix (Quantabio, Beverly, MA) consisting of 1 U Taq polymerase, 45 nM Chlorine, 2.5 mM magnesium ion, 0.2 mM of each deoxynucleoside triphosphate, 0.2 μM of each primer (Eurofins Genomics, Ebersberg, Germany), and 0.64 ng BSA in a final volume of 25 μl per reaction. The PCR conditions were at temperatures of 90 °C for 15 seconds and 94 °C for 3 minutes, followed by 35 cycles of 94 °C for 45 seconds, 50 °C for 1 minute, and 72 °C for 1.5 minutes, after which a final elongation step at 72 °C for 10 minutes was performed. The triplicates were pooled and visualized on 1% agarose gel to estimate the size of the amplicons. DNA concentrations of the amplicons were measured as described earlier. Every PCR run included a negative (water) and a positive (mock community sample) control. The following reagent was obtained through the National Institutes of Health Biodefense and Emerging Infections Research Resources Repository, National Institute of Allergy and Infectious Diseases, National Institutes of Health as part of the Human Microbiome Project: Genomic DNA from Microbial Mock Community B (even, low concentration), version 5.1L, for 16S RNA Gene Sequencing, HM-782D. Libraries were then pooled in equimolar concentrations, and the amplicon pool was purified according to protocol using AMPure XP beads (Beckman Coulter, Brea, CA). Before amplicon sequencing, the amplicon pool was diluted in 10 mM Tris-hydrochloride (pH 8.5) to a final concentration of 5 nM. Following the Illumina recommendations, the amplicon pool was denaturized using an equal amount of 0.2 M sodium hydroxide (BioUltra) (Chemie GmbH, Sigma-Aldrich) and was further diluted to 12 nM in hybridization buffer (HT1 buffer included in the Reagents Kit, version 3; Illumina, San Diego, CA). The pool was finally spiked with a 5% denatured PhiX control library (Illumina). The sequencing was performed using the MiSeq sequencing platform with the Reagents Kit, version 3, 600 cycles (Illumina).

Microbiome analyses: composition, diversity, and discovery of metagenomic biomarkers

The composition and diversity of the gut microbiome were assessed using QIIME2 (Bolyen et al., 2019). Initially, read pairs were demultiplexed using deML (Renaud et al., 2015) before sequences were quality filtered and denoised using DADA2 (Callahan et al., 2016). For stool samples, the total sequencing yield was 1,595,147 reads with a median of 74,594 (n = 21). For skin samples, 7,499,237 reads were a total yield distributed among 73 samples, with a median of 96,497. Raw sequence data were demultiplexed and quality filtered using the q2-demux plugin, followed by denoising with DADA2 (Callahan et al., 2016). All ASVs were aligned with mafft (Katoh et al., 2002) and were used to construct a phylogeny with fasttree2 (Price et al., 2010). α-Diversity metrics (observed ASVs, Shannon index, and Faith's PD [Faith, 2007]) and β-diversity metrics (unweighted UniFrac) (Lozupone and Knight, 2005) and also Principal Coordinate Analysis were estimated after samples were rarefied to 1,000 sequences per sample. Shannon index accounts for both richness (number of species) and distribution (how evenly the species are distributed), whereas Faith's PD pertains more to PD in a sample. β-Diversity measures the differences in microbial composition between different samples. Taxonomy was assigned to ASVs using the q2-feature classifier (Bokulich et al., 2018) classify-sklearn naive Bayes taxonomy classifier against the SILVA full-length 99% operational taxonomic units reference sequences (Quast et al., 2013). For all samples, the ASVs that were unassigned or assigned as eukaryotes or archaea were considered contamination and excluded from further analyses. ASVs that had annotation within the

kingdom of bacteria remained within the analyses and were treated as a part of the skin microbiome. ASVs that had only bacteria in their taxonomical annotation were blasted using the National Center for Biotechnology Information BLAST tool where it returned as a 99% match for human mtDNA. ASVs that did not pass the filter of 0.005% of the total number of sequences were also excluded.

ELISA

IFN-γ, CXCL9, and CXCL10 were measured from the serum using commercially available ELISA kits (Peprotech, Rocky Hill, NJ), whereas CXCL16 ELISA was purchased from R&D Systems (Minneapolis, MN).

Statistical analyses

Differences in α- and β-diversity between the groups were calculated using Kruskal–Wallis test (*P*-value is force discovery rate adjusted) and PERMANOVA (number of permutations = 999), respectively. We have used ANCOM (Mandal et al., 2015) to investigate differentially abundant bacteria between the groups ($P < 0.05$, force discovery rate adjusted for the analysis). Nonparametric test (Wilcoxon signed-rank test) was used to account for non-normality of the variables in the analyses of chemokines between patients who had mtDNA in the skin biopsies and those who did not. Correlation between individual's levels of mtDNA in the L skin and their serum chemokine responses was performed using the nonparametric Spearman correlation test. For all tests, $P < 0.05$ was considered significant, and $P < 0.1$ for results at phylum level was considered borderline significant, justifying deeper analysis at the genus level.

Data availability statement

Sequence data have been deposited to the Sequence Read Archive under accession number PRJNA639425. All other data that support the findings of this study are available from the corresponding authors on reasonable request.

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CONFLICT OF INTEREST

The authors state no conflict of interest.

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AUTHOR CONTRIBUTIONS

Conceptualization: MKT, TP; Data Curation: MKT, CEW, KSS; Formal Analysis: KSS, CEW, MKT, HB; Funding Acquisition: MKT, TP; Investigation: HB, MKT, KSS, AK; Methodology: MKT, HB, CEW, KSS, TP, AK; Project Administration: MKT, TP, SR; Resources: MKT, TP; Software: CEW, KSS; Supervision: MKT, CEW, TP; Validation: CEW, KSS, MKT, TP; Visualization: MKT, HB, KSS; Writing - Original Draft Preparation: MKT, HB, TP, CEW, KSS; Writing - Review and Editing: MKT, TP, CEW, KSS, HB, SR, AK

Disclaimer

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2021.01.036.

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