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ORIGINAL ARTICLE

Effect of two shampoo formulations on the prokaryotic and eukaryotic microbiota composition of the human scalp

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Abstract

Objective: The human scalp is characterized by a moderately diverse microbial community, comprising prokaryotic (bacteria) and eukaryotic (fungi) members. Although the details are far from being fully understood, the human scalp microbiota is implicated in several scalp disorders, in particular dandruff formation. Hence, the protection of an intact and diverse scalp microbiota can be regarded as a quality criterion for hair and scalp care formulations. In this study, we investigated the influence of two commercially available, non-antimicrobial shampoo formulations on the structure of the scalp microbiota.

Methods: Scalp microbiota samples, obtained by swab sampling from two cohorts of probands ($n = 25$, each), were analysed before and after daily use of two different shampoo formulations for 2 weeks, respectively. A polyphasic approach was used, comprising quantitative cultivation of bacteria and fungi on selective media as well as sequencing of PCR-amplified 16S rRNA and 18S rRNA genes, respectively.

Results: All analyses revealed a microbiota composition typical for the human scalp. While in particular fungal germ numbers increased significantly during the treatments, overall bacterial and fungal community composition was not affected, based on alpha- and beta-diversity measures. However, we observed an increase in structural bacterial diversity with the age of the probands.

Conclusions: Over an application period of 2 weeks, the investigated shampoo induced quantitative but no qualitative changes in the scalp microbial community structure of the investigated probands, suggesting no adverse but rather preserving or even stimulating effects of the underlying formulations on the scalp microbiota. Further investigation will have to clarify if this is also true for longer application periods and if the formulations might affect community functionality, for example microbial gene expression, rather than community composition.

KEYWORDS

16S rRNA gene, 18S rRNA gene, age, bacteria, cultivation, hair care, yeast

Résumé

Objectif: Le cuir chevelu humain se caractérise par une communauté microbienne modérément diversifiée, comprenant des membres procaryotes (bactéries) et eucaryotes (champignons). Bien que l'on soit loin de comprendre totalement les détails, le microbiote du cuir chevelu humain est impliqué dans différents troubles du cuir chevelu, en particulier la formation de pellicules. La protection du microbiote du cuir chevelu intact et diversifié peut être considérée comme un critère de qualité pour les formulations de soins pour les cheveux et le cuir chevelu. Dans cette étude, nous avons examiné l'influence de deux formulations de shampooing non antimicrobien disponibles dans le commerce sur la structure du microbiote du cuir chevelu.

Méthodes: Des échantillons de microbiote du cuir chevelu, obtenus par écouvillonnage dans deux cohortes de proposants ($n = 25$ dans chaque cohorte), ont été analysés respectivement avant et après l'utilisation quotidienne de deux formulations de shampooing pendant deux semaines. Une approche en plusieurs phases a été utilisée, dont une culture quantitative de bactéries et de champignons sur des milieux sélectifs et un séquençage respectivement des gènes de l'ARN ribosomique 16S et de l'ARN ribosomique 18S amplifiés par PCR.

Résultats: Toutes les analyses ont révélé une composition du microbiote typique pour le cuir chevelu humain. Bien que le nombre de germes fongiques en particulier ait augmenté significativement pendant les traitements, la composition globale des communautés bactériennes et fongiques n'a pas été affectée, d'après les mesures de diversité alpha et bêta. Cependant, nous avons observé une augmentation de la diversité bactérienne structurelle avec l'âge des proposants.

Conclusions: Sur une période d'utilisation de deux semaines, le shampooing étudié a induit des modifications quantitatives, mais pas qualitatives, de la structure des communautés microbiennes du cuir chevelu des proposants étudiés, ce qui suggère qu'il n'y a pas d'effets indésirables, mais qu'il y a des effets de préservation, voire de stimulation, des formulations sous-jacentes sur le microbiote du cuir chevelu. Des recherches supplémentaires devront clarifier si cela s'avère également pour des périodes d'utilisation plus longues et si les formulations peuvent affecter la fonctionnalité des communautés, par exemple, l'expression des gènes microbiens, plutôt que la composition des communautés.

INTRODUCTION

The human skin is colonized by a dense and diverse microbiota [1, 2]. Although the details are far from being fully understood, an important role of this microbiota in human skin health and well-being is anticipated [3, 4]. This assumption is based on the fact that many skin diseases go along with marked changes in skin microbiota structure (community composition) and functionality (physiology) [5]. A significantly altered skin microbial community composition that goes along with negatively perceived skin conditions is called dysbiotic, while a microbiota composition affiliated with healthy skin conditions is called eubiotic. Consequently, the skin microbiota has become a novel target for many skin care products [6, 7]. Skin cosmetics might be helpful tools to re-balance a dysbiotic skin microbiota or should at least not negatively affect an eubiotic status. Cosmetics with such a preserving

character are sometimes referred to as being 'microbiome friendly', although a scientific definition of this term is still largely missing [7].

The human scalp is characterized by a moderately diverse microbial community comprising bacteria and fungi [8, 9]. The bacterial community mainly consists of gram-positive genera, such as staphylococci and cutibacteria (formerly propionibacteria), while the fungal community appears to be dominated by yeasts, such as *Malassezia* species [8, 9]. The latter are seen as a major (but not the only) cause for dandruff, which might be caused by scalp inflammation processes leading to increased proliferation of scalp cells [10].

If not addressing specific microbial scalp problems, such as dandruff [11, 12], shampoo formulations are (among others) designed to improve hair and scalp cleanliness and moisture content but not to deliberately influence the scalp microbiota [13]. Assuming a healthy and eubiotic status of the scalp microbiota, any disturbance of the structure and/or function of this microbial community might be interpreted as something unwanted. Interestingly, hardly anything is known about the effect of non-antimicrobial/non anti-dandruff shampoos on the scalp microbiota. So far, the use of shampoo has not been regarded as a major factor in determining human scalp microbiota composition [14].

In order to increase knowledge in this field, we analysed the effect of regular use of two different, commercially available shampoo formulations on the structure of the scalp microbiota using a polyphasic approach, that is, using cultivation-based cell counting as well as cultivation-independent molecular methods based on next generation sequencing.

TABLE 1 INCI list of the used shampoo formulations.

P1	P2
Aqua (Water, Eau)	Aqua (Water, Eau)
Cocamidopropyl betaine	Cocamidopropyl betaine
Sodium laureth sulfate	Sodium laureth sulfate
Coco-glucoside	Coco-glucoside
Propylene glycol	Propylene glycol
PEG-7 glyceryl cocoate	Panax ginseng root extract
Panax ginseng root extract	Glycine
Hydrolyzed keratin	Lysine HCl
Betula alba juice	Panthenol
Lactic acid	Sodium chloride
Sodium chloride	Parfum (Fragrance)
Glycol distearate	Caprylyl/Capryl glucoside
Caprylyl/Capryl glucoside	Sodium benzoate
Sodium benzoate	PEG-7 glyceryl cocoate
Citric acid	Citric acid
Guar hydroxypropyltrimonium chloride	Polyquaternium-10
Glycerin	Allantoin
Hydrogenated castor oil	Hexyl cinnamal
Glyceryl oleate	Linalool
Sodium hydroxide	Limonene
Phenoxyethanol	Sodium hydroxide
Potassium sorbate	Benzyl salicylate
PEG-120 methyl glucose dioleate	PEG-120 methyl glucose dioleate
Parfum (Fragrance)	Benzyl alcohol
	Sodium acetate

MATERIALS AND METHODS

Application study and scalp sampling

The study was conducted under medical supervision with two cohorts of probands ($n = 25$, each) from the Henkel pool of probands for in-house cosmetics studies. In general, the P1 test group included 14 men and 11 women with an average age of 48 ± 2.6 (average ± standard error of the mean) years. The P2 test group included 13 men and 12 women with an average age of 45 ± 3.1 years. To analyse age effects, the test subjects were grouped into different age groups, that is, <40 years (7 men, 6 women, mean age 28 ± 1.3 years), 40–55 years (12 men, 9 women, mean age 49 ± 0.9 years) and >55 (7 men, 7 women, mean age 62 ± 1.5 years). An overview of the metadata of the two study cohorts are given in Table S1. Only probands with straight hair (5 cm – shoulder-length) were included. Probands were instructed not to wash or cosmetically treat their hair 24 h prior to sampling. In addition, any hair colouring was prohibited 1 week before sampling.

Two commercially available shampoo formulations (P1 and P2) were used, which are detailed in Table 1. One formulation was issued to the probands of each group. The probands were sampled before and 2 weeks after daily use of 'a usual amount' of shampoo, which they were asked to gently massage into hair and scalp for 1 min, followed by rinse-off.

Scalp samples were taken by swab sampling with sterile polyethylene swabs (Copan Italia 961C, Copan, Brescia, Italy) wetted with sterile physiological sodium chloride solution. Per scalp, 5 swab samples were taken from a 10 × 10 cm area defined with a positioning device on the central head of each proband. Samples were taken along partings formed with a guide of 10 cm length by gently pressing and circling the swab along the guide for about 1 min. After sampling, all swabs per subject were

stored in a physiological salt solution (5 mL) and directly processed for microbial cell counting or frozen at −20°C for later molecular analysis.

Microbiological cell counts

Cell counts were performed according to an internal Henkel standard procedure (SOP HSA A.1.1–001). Serial 10-fold dilutions of the samples were prepared in tryptone/NaCl (0.1%/0.85%). Of each dilution, 0.1 mL was spread on two Tryptic Soy Agar plates (TSA, Merck, Darmstadt, Germany) for bacterial cell counts and on two Glucose-Sabouraud plates (SAB, Merck), respectively, for fungal cell counts. TSA-plates were incubated at 30°C and SAB plates at 25°C for a maximum of 5 days. The number of colonies were counted per plate. Only TSA plates showing colony counts of >10–<300 CFU were considered for bacterial cell counts. Only SAB plates showing colony counts of >5–<100 CFU were taken into account for fungal cell counts. The respective cell counts per ml of sample volume were calculated as means of the countable plates multiplied by the dilution factor.

DNA extraction for molecular analyses

DNA isolation was performed using the ZymoBIOMICS DNA Miniprep-Kit (Zymo Research Europe) from 2 to 3 swab heads per sample, following the supplier's instructions. For optimal cell lysis, the extraction protocol included a 1 min bead beating step (repeated 5×, each) using a Fastprep-24 machine (MP Biomedicals) and bashing beads in the lysis solution provided with the extraction kit. DNA purity and concentration after extraction were measured with an Implen NanoPhotometer P-Class 360 (Implen).

Library preparation and sequencing

Sequencing library preparations of the V4 and V5 regions of bacterial 16S rRNA genes and of the V6 and V7 regions of eukaryotic 18S rRNA genes, respectively, were performed using the 16S-specific primers 520F (5′-CCGTC AATTCMTTTRAGTTT-3′) and 926R (5′-CCGTCAAT TCMTTTRAGTTT-3′) and the 18S-specific primers 1152F (5′-TGAAACTTRAAGRAATTGACGGA-3′) and 1428R (5′- GGRCATMACDGACCTGYTAT-3′) with additional adapter sequences for Illumina Nextera indexing to produce amplicons [15, 16].

PCR amplification was performed at least twice per sample. The PCR mixture consisted of 0.5 µL of each primer (Integrated DNA Technologies) (10 µM), 0.6 µL of dNTP-Mix (10 mM, each), 5 µL 5× KAPA Hifi Puffer including 20 mM $MgCl_2$, 0.1 µL KAPA Hifi Polymerase (Roche), 1 µL (bacteria)–5 µL (eukaryotes) DNA template and was filled up to a final volume of 25 µL with nuclease-free water. PCR reactions were performed in a T100 Thermal Cycler (Bio-Rad Laboratories) using the following thermal profile: 3 min at 95°C for initial denaturation, 25 (bacteria) – 30 (eukaryotes) cycles of 30 s at 95°C for denaturation, 30 s at 55°C for annealing and 45 s at 72°C for elongation, followed by a final elongation step for 5 min at 72°C. Water-template controls and *Escherichia coli* (bacteria) or *Saccharomyces cerevisiae* (eukaryotes) DNA as positive controls were included for each set of PCR reactions. The success of PCRs was verified by agarose gel electrophoresis using Midori Green as DNA-dye (Biozym). Replicate PCRs of the same sample per sequencing setup were pooled and purified with Agencourt AMPure XP beads (Beckman Coulter) into 50 µL of 10 mM Tris (pH 8.5) buffer.

Subsequently, a second PCR step was performed to add unique index barcodes with sequencing adaptors to the amplicon targets using the Nextera XT index kit v2 set D (Illumina). The index PCR reaction included 5 µL of Nextera XT (Illumina) Index Primer 1 and 5 µL of Nextera XT Index Primer 2 with 1.2 µL of dNTP-Mix (10 mM each), 10 µL 5× KAPA Hifi Puffer including 20 mM MgCl2, 0.2 µL KAPA Hifi Polymerase (Roche), 5 µL amplicon DNA and was filled up to 50 µL with nuclease-free water. PCR reactions were performed in a T100 Thermal Cycler (Bio-Rad Laboratories) using the programme detailed above, albeit with eight cycles.

After purification with AMPure XP beads, quality checks for library sizes and DNA concentration were performed with the Agilent Bioanalyzer using Agilent DNA 1000 chips (Agilent Technologies). The Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific) was used to determine the DNA concentration. Finally, libraries of indexed amplicons for each sample were normalized to a concentration of 4 nM and pooled for sequencing.

The pooled libraries were sequenced on an Illumina MiSeq platform (Illumina) in a final concentration of 6 pM with 20% phiX control added, using the MiSeq Reagent Kit v3 in a 600-cycle (2× 300 bp + 2× 8 bp Index cycles) format following the manufacturer's instructions.

Bioinformatical and statistical analyses

Sequence data were processed using QIIME 2 version 2019.7 [17]. Quality cut-offs were performed with the median at $Q \geq 25$. Minimum and maximum sequence lengths of 200 bp and 1000 bp were used (QIIME2 default).

Denoising, merging paired ends and removal of chimeras were performed using the DADA2 pipeline of QIIME2. Amplicon sequence variations (ASV) were chosen within 99% sequence identity. A primer-fitted taxonomy classifier, trained with the SILVA database release 132 using the classify-sklearn algorithm in QIIME2, was used to assign taxonomy and align sequences [18]. Beta diversity indices for beta-diversity PCoA plots of weighted and unweighted UniFrac measures were also calculated with the corresponding QIIME2 plugins. Further statistical analyses were performed with RStudio (version 1.4.1106) [19], R (version 4.2.2) [20], and with additional R-packages for microbiome analysis like the phyloseq package (version 1.42.0) [21], microbiomeutilities (version 1.00.17) [22] and packages for statistical analysis like package Coin (version 1.4–2) [23, 24] and Dunn test (version 1.3.5) [25]. The phyloseq package was used to prepare the rarefied table for further analysis and calculate the alpha diversity boxplots for alpha diversity parameters Observed, Shannon and Simpson. *p* values for alpha diversity parameters were calculated with the Kruskal–Wallis statistic with a 10 000-fold permutation using Coin, followed by a post hoc Dunn test for multiple comparisons of each group, and additionally with the two-sided Wilcoxon-Mann-Whitney test for unpaired and non-normally distributed samples in a 10 000-fold Monte-Carlo simulation from the Coin package. For beta diversity parameters of weighted and unweighted UniFrac measures, a PERMANOVA with 10 000 fold permutations was calculated. Differences on genus level between the individual metadata factors were also compared with the Kruskal–Wallis statistic using Coin, followed by a post hoc Dunn test for multiple comparisons of each group, and additionally with the two-sided Wilcoxon–Mann–Whitney. All *p*-values were corrected for the false discovery rate (FDR) [26].

Sequences generated and analysed here are accessible at the European Nucleotide Archive (ENA) under the accession number PRJEB62089. Subject metadata is included in Table S1. Other datasets are available from the corresponding author on reasonable request.

RESULTS AND DISCUSSION

Sequencing data and general microbial community composition

The sequencing datasets comprised 1 639 759 partial 16S rRNA gene sequences and 2 514 942 partial 18S rRNA gene sequences after quality control, denoising, paired-end merging and chimera removal. The mean number of sequences per sample were 17 080.8 (min: 7862; max: 40 540) for bacteria and 26197.3 (min: 3775; max: 50 474) for eukaryotes without mammalian reads, respectively. Amplicon sequence variant (ASV) rarefaction curves for 16S and 18S sequencing showed that the sequencing depth was sufficient to detect the vast majority of taxa in all samples (Figure S1).

Following rarefication to an even depth of sequences per sample, 7862 ASVs affiliated with 793 genera-equivalent

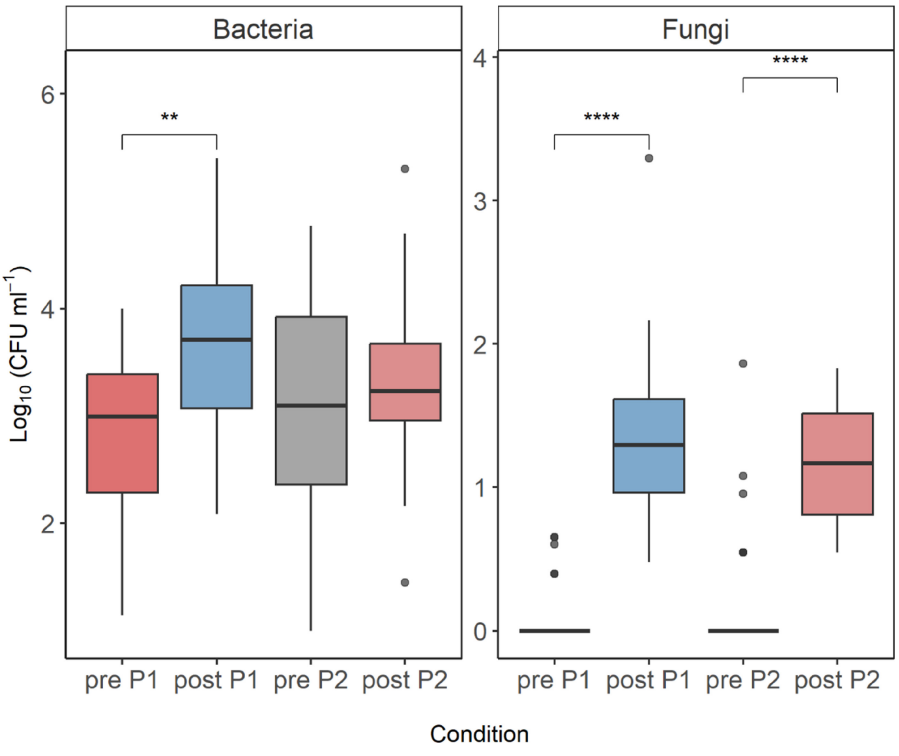

FIGURE 1 Box whisker plots of the aerobic colony counts for bacteria and fungi. Each box represents the 25% and 75% percentiles. Bold horizontal lines represent medians. Whiskers above and below the boxes indicate the lowest and highest microbial counts that were not classified as outliers. Black points represent outliers. Sampling was performed before (pre) and after (post) treatment with the shampoo formulations for product P1 ($n = 25$) and product P2 ($n = 25$). FDR-corrected significance levels are indicated by asterisks (**$p < 0.01$; ****$p < 0.0001$) and were calculated by the Kruskal–Wallis test and the post-hoc Wilcoxon–Mann–Whitney test.

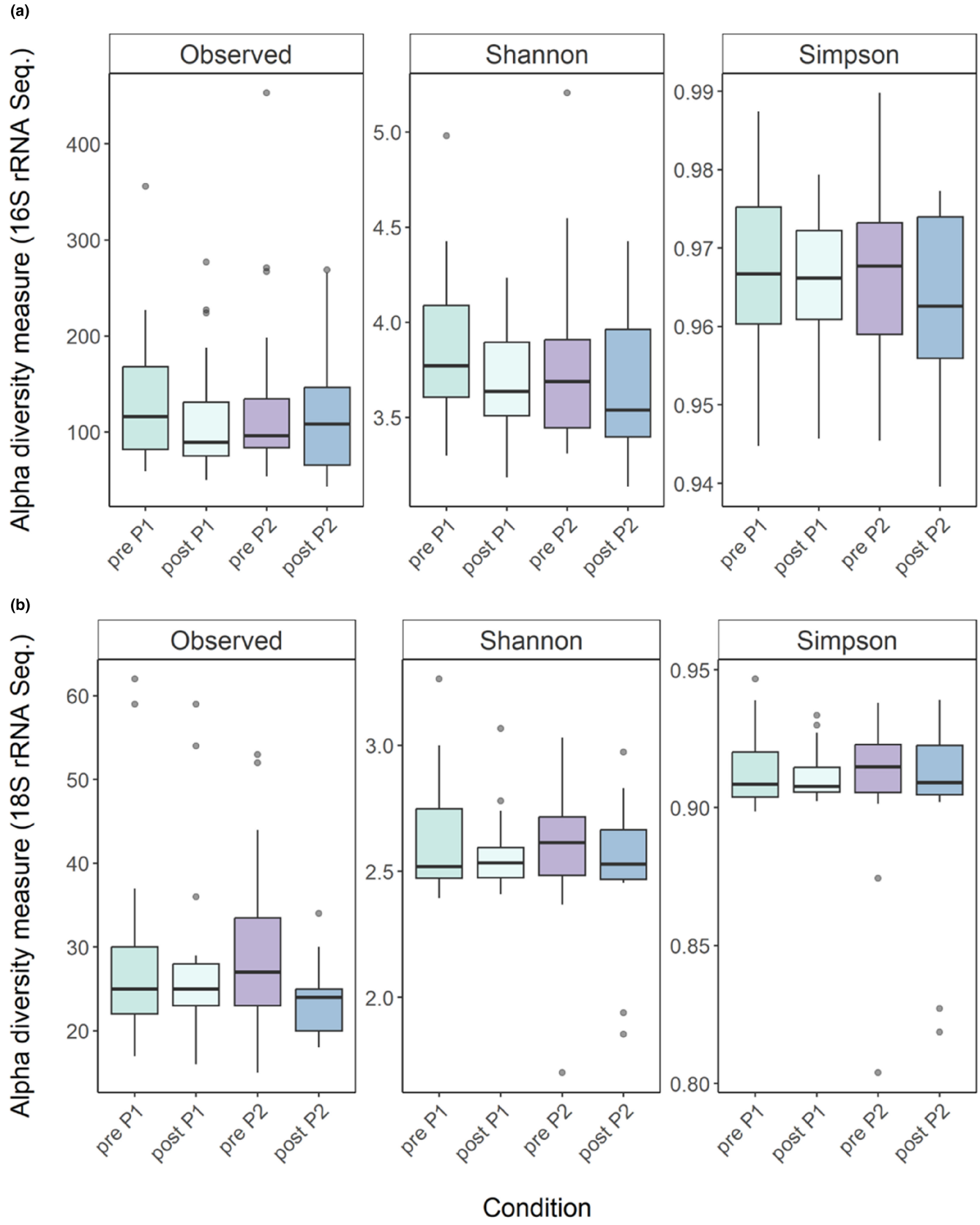

FIGURE 2 Alpha Diversity plots for the pro- (a) and eukaryotic (b) microbial communities on the scalp of two proband cohorts ($n_{P1}=25$, $n_{P2}=23$) treated with two shampoo formulations, respectively. Box plots for Observed, Shannon and Simpson Indices show median as well as lower and upper quartiles. Whiskers represent the minimum and maximum spread.

ranks in the SILVA database, 327 family-equivalent ranks and 170 order-equivalent ranks were identified as components of the bacterial community. In contrast, only 86 genera-equivalent ranks, 68 family-equivalent ranks and 16 order-equivalent ranks were assigned for eukaryotes in the SILVA database.

The main bacterial representatives at the phylum level were *Bacillota* and *Actinomycetota*. At the genus level, the dominant genera identified were *Staphylococcus* (53.5%), *Cutibacterium* (21.6%) and *Lawsonella* (13.4%), which are typical representatives of the human skin and scalp microbiome [27–29]. The same applies to the eukaryotic community, which was dominated in all samples by genera such as *Malassezia* (98.3%) and to a lesser extent by the subclass *Acari* (1.3%) with the main representative *Demodex* (0.5%). All in all, these are also typical representatives of the skin microbiota [5, 30].

Factors influencing microbial community composition

To check whether the use of shampoo has an influence on the scalp microbiota, aerobic germ counts along with several microbial diversity parameters were analysed. When comparing germ counts before and after application of the two tested shampoos (Figure 1), a general trend towards increased germ numbers after application was observed. Product P1 in particular showed a statistically significant increase in both cultivable bacteria and fungi. In contrast, product P2 only showed a significant change in the fungal numbers, while the bacterial numbers remained unaffected. As skin hydration is an important parameter for skin microbial counts, it might be carefully speculated that an improved skin hydration status, along with the excreted lipids from the glands, might be responsible for the observed changes [31].

Damaged or diseased skin is thought to harbour a lower diversity of microbial species compared to healthy skin [32]. Here, although significant differences in microbial cell numbers were detected, both alpha diversity (Figure 2) and beta diversity (Figure S2) did not indicate any statistically significant differences between the scalp microbiota compositions before and after application of the two tested shampoo formulations. Also on a genus level, pre- and post-treatment scalp community structures were very similar and did not show statistically significant differences (Figure 3).

In order to control, whether the applied molecular approach was suitable to detect significant differences in the underlying dataset, we compared the microbial diversity of the probands after grouping them into three age classes. Doing so, an effect of age on the microbial community composition of bacteria but not eukaryotes became apparent (Figure 4). Looking at the various age groups, there were statistically significant differences in observed ASVs between age groups >55 and <40 ($p = 2.35 \times 10^{-5}$) and age groups 40–55 and >55 ($p = 4.50 \times 10^{-5}$). The figure also shows that diversity increases with age, especially among subjects older than

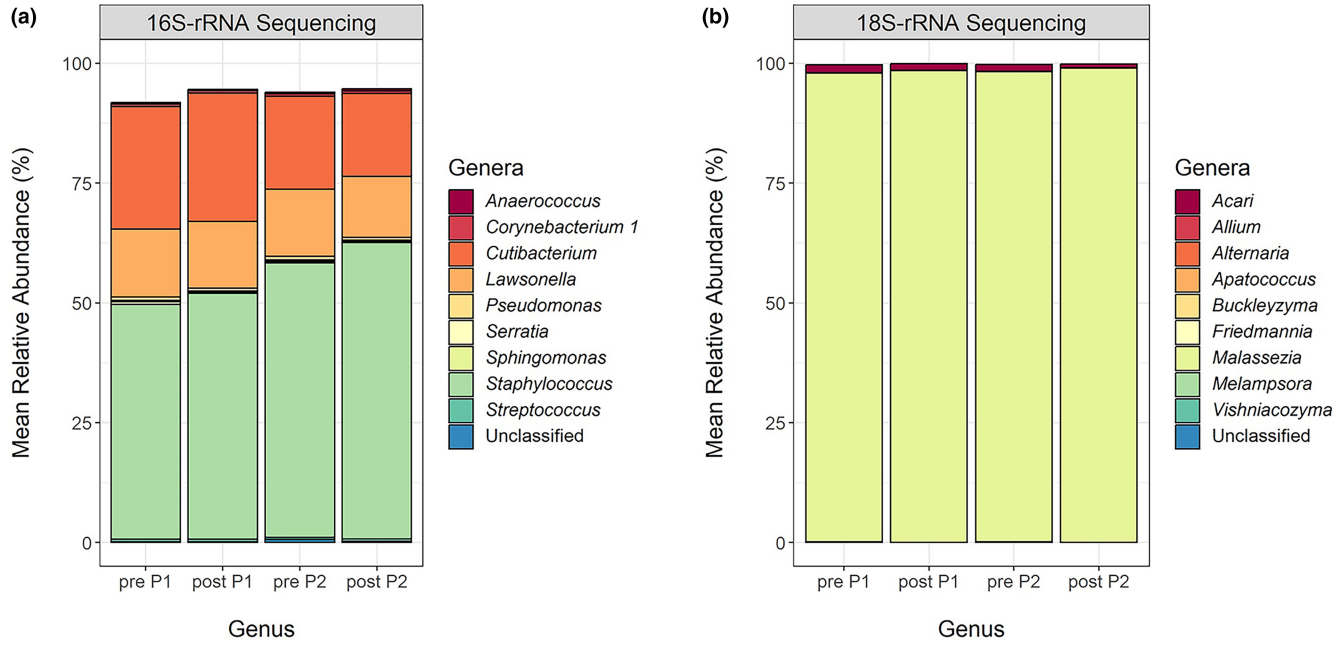

FIGURE 3 Relative abundance of the ten most abundant taxa for the prokaryotic (a) and eukaryotic (b) scalp microbial communities ($n_{P1} = 25$, $n_{P2} = 23$). The figure shows relative abundances before (pre) and after (post) treatment with the different shampoo products; genera that remained were not visualized.

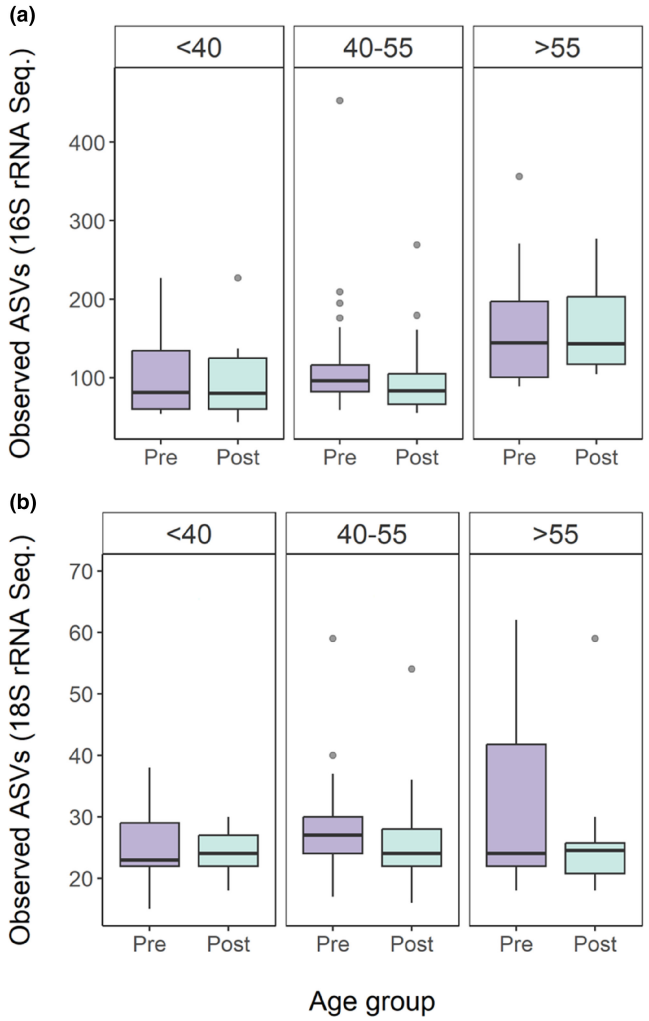

FIGURE 4 Observed alpha diversity plots for the age-grouped dataset before (pre) and after (post) the treatment period of the 16S- (a) and 18S-rRNA-gene sequencing (b). Box plots from observed indices show median as well as lower and upper quartiles. Whiskers represent the minimum and maximum spread. The number of subjects in each age group for <40 includes $n = 13$, for 40–55 includes $n = 21$, and for >55 includes $n = 14$.

55. Similar findings have been reported before for the scalp and other skin areas [33–35]. For instance, Li and colleagues reported gradually increasing skin microbial diversity with age for the cheek and abdomen for bacteria but not for fungal communities, which were most diverse in their middle-aged group [34].

When treatment with the two different shampoo formulations was investigated for the three different age groups, respectively, again no statistically significant effect on microbial diversity before and after treatment (Figure 4) was discernible. However, it appeared that the median numbers of observed ASVs for the age groups <40 and 40–55 years were converging under the influence of the shampoos. Clearly, a larger sample group and a longer test period are needed to confirm this hypothesis.

In summary, our data suggest that the studied shampoo formulations did not significantly alter the relative scalp microbial community composition during a two-week application period and largely positively influenced scalp germ counts, suggesting a 'microbiome-friendly' character of the investigated formulations [7, 36].

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CONFLICT OF INTEREST STATEMENT

S.G, R.S. and T.W. are affiliated with Henkel AG & Co. KGaA, a manufacturer of skin and hair care products. Henkel funded and designed the study and collected all scalp samples, but did not have any additional role in data analyses, the decision to publish, or the preparation of the manuscript.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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