Facial dysbiosis induced by photoprotectors. Is there evidence for such an assumption?

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Abstract

Dysbiosis is characterized by a disruption of bacterial homeostasis and may be associated with various skin diseases. Acne is a multifactorial inflammatory disease with a robust microbial component and numerous correlations with dysbiosis states. Furthermore, various factors are recognized as triggers for skin dysbiosis, including the use of certain cosmetics. Based on these arguments, we hypothesized that the use of photoprotective formulations could trigger dysbiosis and the occurrence of acne manifestations. To verify this assumption, six volunteers between 19 and 23 years of age, meeting all the inclusion criteria, received two applications a day of a non-commercial sunscreen formulation developed with the sun filters ethylhexyl methoxycinnamate, ethylhexyl salicylate, methyl anthranilate, and octocrylene dispersed in a base gel, with an estimated protection factor of 28.8. The pure base gel was used as a control. The samples were applied to an area delimited by a standard template (15 cm$^2$) in an amount corresponding to 30 mg (2 mg cm$^2$) for ten days. At two points in time, pre- and post-sample applications, the facial skin surface was swabbed to collect extracted DNA and processed to verify divergent degrees of 16S RNA coding sequences. The data obtained allowed us to determine the abundance of different bacterial entities at the genus and species levels. The results showed that key species of the acne process, such as *Cutibacterium acnes* and *Staphylococcus epidermidis*, seem to tolerate the evaluated formulation well, not being significantly affected by the formulation, suggesting no interference of its use concerning dysbiosis induction.

Introduction

Photoprotectors are considered essential to prevent damage to exposed skin [1] by protecting against effects such as photoaging, melanoma, cutaneous squamous cell carcinoma, and basal cell carcinoma [2], significantly aggravated by direct exposure to ultraviolet radiation (UVR). However, issues related to the toxicity and safety of the ingredients in these formulations have concerned consumers, affecting adherence to sunscreen use, and the scientific community fears the consequences of this reduction [3].

Recently, the possibility of cosmetic product-induced dysbiosis has been suggested [4]. Dysbiosis is characterized by the altered composition of autochthonous microbial communities with the eventual occurrence of disease [5]. It can arise from reduced microbial diversity, loss of beneficial microorganisms (usually symbiont bacteria), and proportional growth of pathogenic entities, called pathobionts [6].

The photoprotective ingredients can be incorporated into different bases (gels, sticks, solutions, or emulsions). Eventually, these bases may present some degree of comedogenicity, and the fact that they remain for a prolonged time in contact with the skin makes it plausible to assume that they cause changes in the skin microbiota, leading to dysbiosis. It is known that facial skin dysbiosis is often associated with inflammatory diseases such as atopic dermatitis, psoriasis, rosacea, and acne [7]. In addition, we find in the literature reports that cosmetic acne can be aggravated by photoprotective formulations [8, 9]. However, this fact is always related to the characteristics of the base where the photoprotective ingredients are incorporated [10].
Thus, this study was designed to verify whether the association of the photoprotective ingredients ethylhexyl methoxycinnamate, ethylhexyl salicylate, methyl anthranilate, and octocrylene commonly used in commercial formulations when using oil and additive-free bases can induce dysbiosis in the facial skin of young men by interfering with the homeostasis of microorganisms known to be involved in the acne formation process (\textit{Cutibacterium acnes, Staphylococcus epidermidis}).

**Material And Methods**

**Permission to conduct the study**

The local research ethics committee approved the conduct of this study (opinion no. 2626143, dated April 27, 2018), and the recruited participants received information about the study protocols, with a list of possible risks and harms, measures to be taken in case of adverse reactions, and a draft informed consent form (ICF).

**Volunteer Selection and Recruitment**

The volunteers recruited were male college students, aged between 19 and 23 years old, who were not on antimicrobial therapy in the three months before the study, had no chronic diseases, were not smokers, and did not habitually use products classifiable as cosmetics (makeup, moisturizers, or similar).

Candidates with known allergies to sunscreens, who had undergone any topical or systemic treatment with antibiotics or retinoids, or who had undergone phototherapy or electrotherapy procedures within 30 days of the interview were excluded.

The above selection criteria were obtained during screening by answering a previously approved questionnaire. In addition, the selected volunteers were informed about the purpose and conditions of the study. Those who agreed with the conditions and restrictions of the study were invited to sign the informed consent form (ICF).

Six volunteers who met the conditions and consented to participate were selected. In a screening questionnaire, all volunteers called themselves leucodermic, aged between 19 and 23 years old, without acne.

Half of the volunteers (3/6) classified their skin as oily and the others as a combination. All selected volunteers reported performing facial skin hygiene once or twice a day. Only one of the volunteers did not shave, and only one lived alone; the others shared a home with two or more people.

Each volunteer received a bottle with 200 mL of sterile liquid soap developed and manipulated exclusively to meet the requirements of the study. The soap in question should be used in the 15 days preceding the test to normalize skin conditions (washout). During the test period, the volunteers were instructed to perform facial hygiene using exclusively the liquid soap provided, maintaining the hygiene
routine of two to three times a day, and restricting the use of any other facial cosmetic product during the study period.

**Washout soap**

Liquid soap was formulated, considering the possible impact of detergent surfactants and preservative additives on the skin microbiome. To reduce the impact of soap on the skin microbiota, this product should meet some basic requirements: 1) be preservative-free, 2) be autoclavable, 3) promote gentle skin cleansing, and 4) have a pleasant appearance and sensory characteristics [11].

The formulation (Table 1) was developed, handled, and filled in a dark, autoclaved, and sealed glass bottle with a pump valve for application and to minimize the risk of contamination during use. In addition, the product was recommended to be stored in a cool place and protected from light.

**Photoprotective gel**

In the formulation developed for the clinical trials, the ingredients with photoprotective action were evaluated exclusively in association because none of the ingredients alone can provide broad protection to the UV spectrum. Therefore, the maximum concentrations allowed by RDC 69/2016 [12] were used.

The photoprotective formula was manipulated and stabilized in the form of an anionic polymeric emulsion of the oil-in-water type, using C10-30 alkyl acrylate crosspolymer acrylates as the formulation (Table 2) surfactant and thickener. No stabilizing ingredients, emollients, or preservatives were added.

The polymers were hydrated with grade II reagent water for two hours. Then, the polymer solution mixture was heated to 60°C and divided into two portions. Portion 1 was reserved for making up the base gel fraction (BG), and portion two was used to add and emulsify the photoprotective ingredients – base gel photoprotective (BGP). After pH correction and the addition of all ingredients, the two portions were heated at 60°C and constant stirring at 2500 rpm for 50 min. After the physicochemical analyses, product aliquots were distributed in sterile Falcon tubes, and the samples were autoclaved at 121°C and 1 atm cm$^{-2}$, for 15 min.

The sun protection factor (SPF) of the preparation was determined by the Sunscreen simulator 4.0 (BASF SE, Ludwigshafen, Germany) program algorithm and was estimated at 28.8, close to the SPF 30, commonly used in the UK [13] and Brazil [14]. Both the base gel and the base gel plus photoprotectors presented unchanged organoleptic aspects and pH, demonstrating satisfactory stability post-autoclaving and during storage in the refrigerator. They were also well accepted by the volunteers, and there were no complaints of allergies, changes in skin oiliness, or the appearance of acne lesions.

**Template for delimitation of application and collection areas**

Individual templates were made of a thick acetate sheet (1 mm) with a rod to fit in the ear, extending to the nose flap (Fig. 1). The hollow areas measured 8.50 cm × 3.50 cm, making up 15 cm$^2$, including oily
skin (near the temples) and less oily regions (a malar region near the nasal flap).

After 15 days of facial washout using a standard liquid soap specially developed for the study (sterile and preservative-free), the volunteers came to the laboratory, where the templates sanitized with 70% ethanol and dried were positioned on both faces to collect the control samples.

**Material collection T₀**

*Swabs* moistened with sterile grade II reagent water were pressed against the skin within the delimited areas, where 24 swabbing movements were standardized, 12 vertical and 12 horizontal, with swabs rotating with each swab.

The swabs were cut 2 cm above the cotton edges and packed in sterile tubes immediately frozen at -20°C.

**Gel Applications with and without sunscreens**

To ensure standardization in the application procedure, of the 20 interventions performed during the study, 16 were conducted by one of the researchers. The amount of product applied in each session was approximately 30 mg (2 mg cm⁻¹ × 15 cm²). The amounts dispensed were distributed in the area delimited by the template with the sterile applicator, and wearing sterile gloves were changed for each volunteer.

The standard liquid soap used in the washouts was maintained throughout the test period, with the usual frequency of two to three washes daily. In addition, the volunteers agreed to come to the laboratory daily at pre-established times, in the morning between 9:00 and 9:30 AM and the afternoon between 12:30 AM and 1:30 PM (minimum interval of 3 h).

For the weekend sample application, volunteers received a kit containing an area-limiting template, a tube containing BG, a tube containing BGP, ten sterile swabs, ten sterile gloves, ten sterile gauze packs, and 200 mL of 70% ethanol. Volunteers were instructed to apply BG to the right cheek and BGP to the left cheek at pre-established times. The templates were sanitized with 70% ethanol and sterile gauze and left to dry perpendicular to the surface.

**Material collection T₁₀**

After ten days and three hours after the last sunscreen application and six hours after washing with sterile liquid soap, volunteers returned to the laboratory, where the templates sanitized with 70% ethanol and dried were positioned on both faces for the final sampling.

Microbiota samples from the faces were collected as previously described and immediately frozen at -20°C.

**Sample processing and metagenomic analysis**
DNA extraction was performed with the ZymoBIOMICS DNA® Kit (Zymo Research Co., Irvine, CA), following the manufacturer's instructions. The extracted DNA was quantified at 260 nm in NanoDrop® 2000 (Thermo Fisher Scientific, Waltham, MA). To assess the integrity of the extracted DNA, all samples were run by 1% agarose gel electrophoresis, stained with a 1% ethidium bromide solution, and visualized with UV (254 nm) in a transilluminator.

A 250-base segment of the hypervariable V3-V4 region of the 16S ribosomal rRNA gene was amplified using the universal primers 515F and 806R and the following amplification conditions: 94°C for 3 min; 18 cycles of 94°C for 45 sec, 50°C for 30 sec, and 68°C for 60 sec; followed by 72°C for 10 min.

From the amplification generated, the metagenomic library was constructed using the Nextera DNA Library Preparation Kit (Illumina, Inc. San Diego, CA). Next, the amplicons were pooled and sequenced on the MiSeq (Illumina, Inc. San Diego, CA) [15]. Finally, the reads obtained on the sequencer were analyzed on the Quantitative Insights into Microbial Ecology - QIIME platform (GitHub, Inc. San Francisco, CA) [16] following a workflow from removing low-quality sequences, removing chimaeras, and taxonomic classification.

Sequences were classified down to "genera" by recognizing operational taxonomic units (OTUs) with identity levels greater than 97% between sequences when compared against the database. The SILVA132 update of the SILVA ribosomal sequence database [17] was used to compare the sequences. To classify bacterial communities by OTU identification, 127000 reads per sample were used to normalize the data and not compare samples with a different number of reads, thus avoiding a bias in taxonomy. In addition, negative and positive sample controls were sequenced to monitor for possible contamination of the primer or other inputs.

**Statistical Analysis**

To calculate the statistical results of the prevalence of bacterial taxa in the overall OTUs' abundance variations, the chi-square test was applied ($p < 0.05$) with Bonferroni correction.

Comparison of relative abundance for the species of interest analysis was evaluated employing the Shapiro-Wilk normality test, Wilcoxon's signalled ranks test (dependent samples), and the Mann-Whitney U-test (independent samples).

The analyses were performed employing the statistical package for metagenomic analyses Statistical Analysis of Metagenomic Profiles – STAMP [18].

**Results And Discussion**

Considering the overall variations of the microbiota that colonized the volunteers' facial skin before the sunscreen application, both the right and left sides revealed significant similarities regarding bacterial families/genera/species (Fig. 2).
The main entity found, in terms of overall abundance, was *Cutibacterium (Propionibacterium) acnes* \(p < 0.05\), although it was not the most found species in two volunteers (V3 and V5). However, this finding is not surprising since this species is the most reported in metagenomics studies [19, 20]. Another *Propionibacterium, C. granulosum*, was also found in all volunteers and sampling times; however, in lower relative abundance \(< 0.85\%\) and without inter-sample variations \(p \geq 0.7851\).

The average abundance of *C. acnes* was compiled, and the results showed no differences between the right and left sides of this species before the experiment \(p \geq 0.8438\). This shows a certain balance in the abundance of *C. acnes*, with no predominance on one face over the other (Fig. 3).

*C. acnes* is an aerotolerant anaerobic bacterium found both on the skin surface and inside the pilosebaceous follicles [21]. Therefore, it is possible to assume that during the collections, the slight pressure exerted by the swabs against the skin exposed the pilosebaceous follicles (pores), where *C. acnes* is usually found in greater abundance [22]. Such facts may collaborate in understanding the higher proportional collection of this bacterium for 66.6\% (4/6) of the volunteers.

The volunteers reported performing facial hygiene equally (two to three times a day) and sleeping on either side of their body (data not shown). This may have contributed to the homogeneity in *C. acnes* density on both sides of the face.

After exposure to the base gel \((Rt_1)\) and the photoprotective formulation \((Lt_1)\), no differences in species abundance were detected either \((p \geq 0.8438)\) (Fig. 3). Therefore, it is possible to assume that the presence of the sun protection factors ethyl methoxycinnamate, ethyl salicylate, methyl anthranilate, and octocrylene added to the base gel do not determine an increase or reduction of *C. acnes* in the follicles/skin. It is also interesting to consider the fact that the photoprotective formulation also did not promote variations in abundance at the collection times for base-gel \((Rt_0\) and \(Rt_1)\) nor the photoprotective formulation \((Lt_0\) and \(Lt_1)\) \((p \geq 0.8438)\). This indicates that the formulation, as presented, does not seem to determine changes in the proportions of this Propionibacteriaceae and is not considered a factor in dysbiosis for this species.

This finding is unprecedented and gains importance if we consider that, contrary to the old (and deposed) dominant idea that the exacerbated proliferation of *C. acnes* would be the main trigger for acne, this species may have a probiotic [23] or antioxidant [24] function, contributing to skin homeostasis [5]. In addition, it is believed that the reduction in skin microbial diversity associated with the constant activation of TLR-2-activated innate immunity may be associated with acne chronication [22].

The second largest group in terms of overall abundance is composed of *Staphylococcus spp*. All volunteers were colonized before the experimental phase, ranging from 3.1\% (V5) to 69.2\% (V3). The main species found was *S. epidermidis*, and its abundance was shown to be invariant between the different experimental groups \((p \geq 0.6878)\) (Fig. 4).

It is known that, like *C. acnes, S. epidermidis* is an important commensal of the skin [25–27].
Recently, it has been postulated that acne may arise from a proportional imbalance of *C. acnes* and *S. epidermidis* in the microbiota colonizing the skin [28]. According to this hypothesis, an event causing dysbiosis with reduced *S. epidermidis* subpopulation could precipitate an over-colonization by virulent variants of *C. acnes* in follicles, with multipoint activation and exacerbated innate immunity and consequent acne inflammation.

Since no significant variations in the abundance of *S. epidermidis* were observed after using the photoprotective formulation, this event indicated that it should not induce acne due to an imbalance of these two bacterial species.

*Staphylococcus aureus* is a species of clinical importance [29], whose role in acne is debatable [30], but which induces α-toxin-dependent TLR-2 response [31, 32]. This species was also constant in all volunteers and at all time points (Fig. 5); however, with relatively low frequency (< 1.3% in overall abundance) compared to *S. epidermidis*.

Regardless of these Firmicutes' low relative abundance and virulence profile, no differences were found between samples collected from the same volunteer at different times and treatments (p ≥ 0.584). This shows that the photoprotective formulation could not cause any imbalance in terms of abundance for this species.

Other *Staphylococcus* species such as *S. equorum, S. sciuri*, and *S. succinus* occurred at extremely low frequencies (≥ 0.12%) within the genus and were not detected in all volunteers. The same occurred with non-fermenting Gram-negatives such as *Pseudomonas* spp. (*P. citronellolis, P. fragi, P. nitroreducens, P. stutzeri*, and *P. viridiflava*) and *Acinetobacter* sp. with spot detections and at low frequencies (≥ 0.035%). Other bacterial entities for which there is no evidence or indication of an association with acne were not considered [33]. For these reasons, these species, genera, or families were not included in this discussion.

Metagenomics is a valuable tool for studying the taxonomic structures of microbial populations that coexist in each ecological niche without the need to culture these microbial entities. For some, metagenomics analysis allows one to know more than "who is there," it also allows one to infer "what they are doing there" [34]. Because of the practicality in simultaneously detecting multiple species, including those with anaerobic and fastidious growth, and the high-throughput capability [35], we chose to employ this technology to conduct this study.

Male volunteers were recruited since acne vulgaris is less common in men than in women [36]. Among several factors, acne results from elevations in androgen levels [37]; since progesterone blocks androgen receptors, inhibiting their acnegenic action, and considering that progesterone reductions occur in the late stage of the menstrual cycle, this dermatosis tends to be exacerbated in the premenstrual period [38]. As this was a complex variable to control, recruitment of female volunteers was avoided. However, this subpopulation warrants future study, as women use sunscreens more frequently than men [39].
The recruited volunteers had no complaints nor clinical signs of acne during the recruitment period, nor did they develop dermatosis during the experimental course, indicating that there was no clinical disturbance in the regions of application of the base gel, a base gel containing filters, nor of the liquid soap formulation, which was shown to control skin oiliness in a balanced manner, both in the washout period and during the experiment, avoiding obliteration of follicles and maintaining non-comedogenic conditions [40].

As limitations of this study, we can point out that a larger sample size would undoubtedly lead to more data and that after a larger-scale analysis, it would be possible to conclude with a greater degree of certainty whether the formulation of sunscreen may or may not cause dysbiosis in facial skin. In addition, a study with a longer intervention time could generate data different from those presented here, also considering the possibility of divergent results when evaluating sunscreens with a sun protection factor (SPF) value higher than those applied in this study.

Based on the results obtained and considering study limitations, it was possible to infer that there is no evidence that the SPF 28.8 photoprotective formulation used in this study causes microbial dysbiosis involving the species of microorganisms related to the occurrence of acne vulgaris.

**Declarations**

The authors declare that they have no conflict of interest.

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**References**

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Tables

Tables 1 & 2 are not available with this version

Figures

Figure 1

Template for delimitation of application and collection areas
Figure 2

A. Abundances at t0 (before applying the photoprotector); B. Abundances at t1 (after 15 days with gel-base applying); C. Abundances at t1 (after 15 days with photoprotective formulation applying).
Figure 3

Abundance of *C. acnes* on the volunteers' faces before the experiment (Rt0 and Lt0), after applying the base gel (Rt1) and after applying the photoprotective formulation (Lt1).

Statistical differences were accessed using the Wilcoxon matched-pairs signed rank test.
Figure 4

Abundance of *S. epidermidis* on the faces of the volunteers before the experiment (Rt0 and Lt0), after applying the base gel (Rt1) and after applying the photoprotective formulation (Lt1).

Statistical differences were accessed using the Wilcoxon matched-pairs signed rank test.
Figure 5

Abundance of *S. aureus* on the faces of volunteers before the experiment (Rt0 and Lt0), after applying the base gel (Rt1) and after applying the photoprotective formulation (Lt1).

Statistical differences were accessed using the Wilcoxon matched-pairs signed rank test.