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"Enhancing Skin Microbiota Diversity: Metagenomic Insights into the Impact of Nanostructured Anti-Aging Cosmetics with Pre and Postbiotics"

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1. Introduction

With the paradigm shift brought about by the advent of Microbiome studies, the presence of microorganisms on human skin is now one of the main focuses of study when developing cosmetics that not only bring benefits to the consumer but also can rebalance the immense microbial population of the skin. The composition of skin microbial communities has been is dependent on the physiology of the skin site, with changes in the relative abundance of bacterial taxa associated with moist, dry, and sebaceous environments [1]. Research in European, Asian, and North American populations highlights microbiome changes in aging skin, specifically an increase in Proteobacteria and a decrease in Actinobacteria [1,2,3].

Interestingly, our studies reveal a unique and distinct microbiome profile in Brazilian women, which remains underexplored. In this context the use of prebiotics and postbiotics [4] had already showed a great potential to create a microenvironment conducive to repopulating and balancing the skin microbiota, addressing both cosmetic industry goals and consumer needs for healthier, youthful skin, so this study introduces a nanostructured cosmetic formulation

designed to rebalance the skin microbiota while offering anti-aging benefits, evaluated through metagenomic analysis of microbiological diversity.

2. Materials and Methods

2.1 Development of lipid nanoparticles and formulation

Nanostructured Lipid Carrier (NLC) were prepared using a low energy micro emulsification method. The formulation consisted of a lipid phase, an ethoxylated fatty alcohol surfactant, a co-surfactant and a hydrophilic dispersion polymer followed by the incorporation of postbiotics (long, medium, and short-chain fatty acids). A cosmetic emulsion base with 6% w/w of free space was used to prepare the Control Formulation (CF) and the Developed Formulation (DF).

2.2 In vivo and Clinical evaluation

A proof-of-concept, placebo-controlled, parallel-group clinical study was conducted to evaluate the efficacy and safety of an innovative nanostructured topical formulation containing prebiotics and postbiotics for aging facial skin. The research project was approved by the Research Ethics Committee at UNIFESP (CAAE: 74030523.0.0000.5505), and included 40 women, aged 25 to 45, with mild to moderate facial aging (GLOGAU grades II and III) divided into two groups G1 (25 to 35 years old) and G2 (36 and 45 years old). Eligible participants signed the Free and Informed Consent Form (ICF). Participants were randomized and received either the Control Formulation (CF) or the Developed Formulation (DF), applied twice daily for three months. The skin microbiome was evaluated via forehead swabs taken at the same location during all scheduled visits.

2.3 Metagenomic Analysis of Skin Microbiome Samples

Briefly the method [5] where the bacterial composition analysis was performed with the Illumina platform with a MiSeq™ instrument. DNA was isolated from swabs using a NucleoSpin Tissue Mini Kit for DNA from cells and tissue. Libraries were prepared by following the Illumina 16S Metagenomic Sequencing Library Preparation protocol in two amplification steps: an initial PCR amplification using locus-specific PCR primers and a subsequent amplification that integrated relevant flow-cell binding domains and unique indices. Primer sequences used to amplify both the variable 16S (V3-V4) and ITS regions followed: 16S-341F - CCTACGGGNBGCASCAG -3_' and 16S-805R 5_'- GACTACNVGGGTATCTAATCC -3_'. Libraries were sequenced on a MiSeq instrument (Illumina) in paired end 300-bp mode read length.

2.4 Statistical analysis plan

The statistical significance of the association between microbiome composition was determined using unpaired two-sided Wilcoxon, differences $p < 0.05$ were noted as significant. The spatial distribution of the species with significant changes in the relative abundance between the groups. All analyses were performed in R with different packages and GraphyPad analysis.

3. Results

3.1. Comparison of the Microbiome of Young and Older Skin

The average relative abundance of taxon indicates that 2 kingdoms were found throughout the study of Bacteria and Archea with absolute predominance of the Bacteria kingdom (data not shown). In addition, 27 phyla were found and grouped in a heatmap by variability or abundance between groups (data not shown), of which the most predominant were Actinobacteria 78%, Firmicutes 13%, Proteobacteria 7% and Bacteroidetes 1%.

The variability of the phylum by age and by treatment was evaluated. The results indicate that the phylum varied according to the age group regardless of the type of treatment, however without presenting statistical difference (data not shown). G2 presented a decrease in Actinobacteria when compared to T0 (before treatment) and 9% more than G1, an increase of 57.14% in Firmicutes, after 90 days and increase of 83.33% in Proteobacteria after 120 days. G1 on the other hand showed a reduction of 8.86% in Actinobacteria and after 120 days an increase of 5%; an increase of 81.81% in Firmicutes after 90 days and a decrease of 20% after 30 days without use. A decrease of 22.22% in Proteobacteria could also be observed. The abundance of phylum grouped by time was also observed. Actinobacteria 77% in G2 and 76% of all microorganisms in the G1, Proteobacteria and Firmicutes 11% and 11% in G2 and 20% and 16% of all microorganisms in G1, despicted in time T120 respectively. In the G2 compared to the G1, there is a decrease in Actinobacteria, an increase in Firmicutes, Proteobacteria and Bacteroidetes.

To confirm wheter the difference bewtween the groups was statistical or just a trend an analyse using the boxplot (data not shown) reports that there was no statistical difference between the groups analyzed, however the G1 exhibited a different microbiological abundance than G2. The results obtained in phylum analysis showed that both treatments were effective in reducing *Actinobacteria and increasing Firmicutes, however, there is no statistical difference, as well as no difference was observed when analyzing the three periods, for all the four groups, treated and control. The withdrawal of participating in the clinical study biased a more in-depth evaluation of microbiological diversity, however, it was reproduced in both groups analyzed (data not shown). In addition to these results, 54 classes were found, with 5 being the most abundant: Actinobacteria 83%, Bacilli 9%, Alphaproteobacteria 3%, Betaproteobacteria 2% and Gammaproteobacteria 3%. In addition, 124 orders were found, with 11 being the most abundant. And, 233 families were also found, with 12 being the most abundant, especially in the composition of the families: Propionibacteriaceae, Corynebacteriaceae and Staphylococcaceae (data not shown).*

The results showed 449 genera, with the most predominant being *Propionibacterium* 77%, *Staphylococcus* 9%, *Corynebacterium* 3%, *Streptococcus* 2% and *Micrococcus* 1%. When separated by group, a predominance of *Propionibacterium* and *Staphylococcus* in the treated group and a lower abundance of *Corynebacterium*, when compared with the control group. While in the control group, we observed a significant increase in *Staphylococcus* – 100% after 120 days of treatment (**table 1**) using the unpaired two-sided Wilcoxon for comparison of two groups.

Table 1: Most abundant genus: Treated and control T0, T90 and T120. N = 16 treated and = 17 control.

	Total		**Treated**	**Control**		**Treated**	**Control**
Genera T0	**%**	**Genera T90**	**%**	**%**	**Genera T120**	**%**	**%**
Propionibacterium	81	*Propionibacterium*	74	67	*Propionibacterium*	72	71

Corynebacterium	3	*Corynebacterium*	4	6	*Corynebacterium*	4	4
Staphylococcus	5	*Staphylococcus*	10	13	*Staphylococcus*	7	10
Streptococcus	2	*Streptococcus*	1	3	*Streptococcus*	3	2
Micrococcus	1	*Micrococcus*	2	1	*Micrococcus*	1	1
Lactobacillus	1	*Lactobacillus*	2	0	*Lactobacillus*	1	0
Others	7	Others	7	10	Others	13	12

The results showed a statistical difference between the *Propionibacterium* groups, compared to *Corynebacterium* and *Staphylococcus* at both times (**Figure 1**), which was expected, since it is the most abundant bacteria on the skin. However, a significant difference was still observed in the treated group that used the formulation for 90 days (Figure 1a) with $p<0.01$, inferring a difference between the *Staphylococcus* and *Corynebacterium* populations. The set of results suggests that the use of the formulation for 90 days modulated the amount of *Propionibacterium*, thus allowing the increase of *Staphylococcus*. However, this statistical result was not observed in the control group, leading us to conclude that the complete formulation with pre and postbiotic created a favorable microenvironment for this modulation, thus allowing the increase of the *Staphylococcus* genus and better control of *Propionibacterium*. However, this increase was not maintained after 120 days (Figure 1b), confirming the need for continuity of the product to maintain the desired effect.

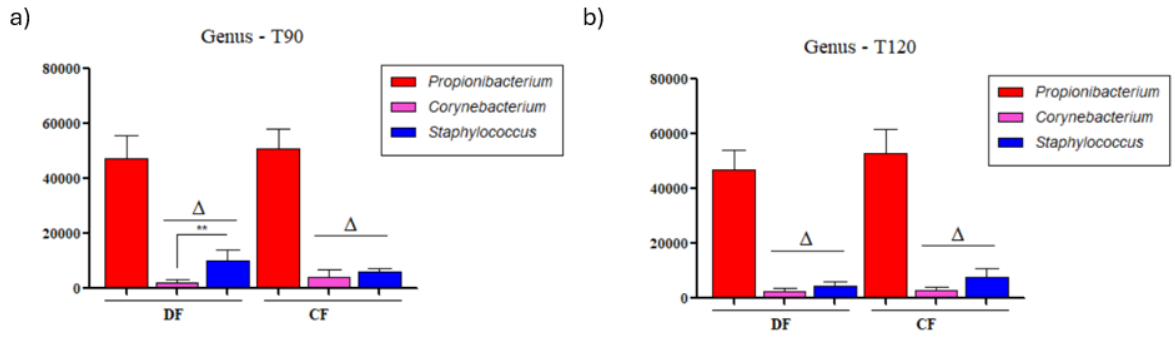

Figure 1. Barplot of the genus *Propionibacterium*, *Corynebacterium* and *Staphylococcus*, separated by treated (DF) and control (CF) group: (a) T90 and (b) T120. Δ = $p<0.001$ in relation to *Propionibacterium*. **=$p<0.01$ difference between *Staphylococcus* of the treated group and *Corynebacterium* in Unpaired Wilcoxon test.

The analysis regarding the age factor were also performed resulting in a barplots (Figure 2a) of the most abundant genus by age, indicating that there was a statistical difference between *Corynebacterium* and *Staphylococcus* (Figure 2b). G1 expresses more *Lactobacillus* and *Micrococcus* genera and less *Corynebacterium* and *Propionibacterium,* when compared to G2, contradicting what is described in the literature, that young skin expresses more *Propionibacterium* [6]. *Staphylococcus epidermidis* was described [7] as being a species capable of secreting short-chain fatty acids to inhibit the growth of *S. aureus* and *Cutibacterium acnes* (former *Propionibacterium acnes*). To elucidate this issue, we performed a differential analysis between the species, which will be discussed later at figures 6 and 7. However, the number of *Staphylococcus* sp observed was much higher than that of *Corynebacterium* sp 73%

and 27%, respectively (Figure 1a) and the treated group and 82% and 18% in G1 (Figure 2b). In other words, if we analyze the images (Figures 1a and Figure 2b), treatment with the development formulation (DF) provided a microbiota similar to that found in the group of younger people, leading us to conclude that the use of the formulation provided a more balanced and youthful microbiota.

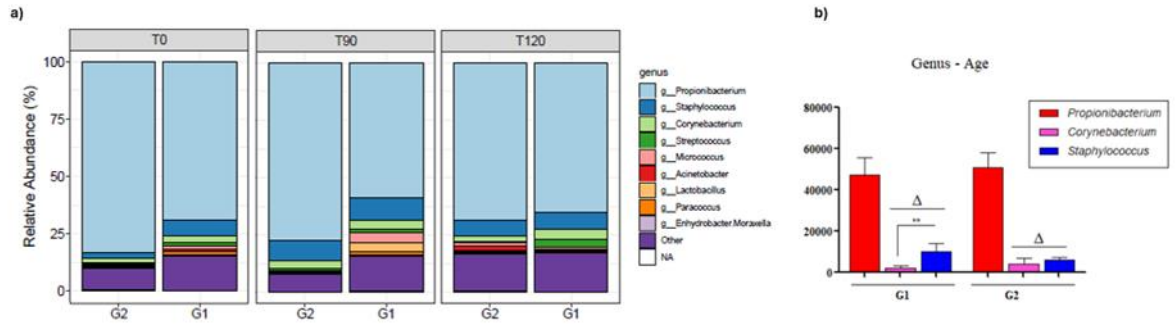

Figure 2: Barplot of the abundance of differentially expressed genus where (a) G1 and G2; b) barplot of groups G1 and G2. T90 **p=0.01; Δ=p<0.001 in Unpaired Wilcoxon test.

To verify if the most abundant genus presents statistical difference a boxplot of G1 and G2 was analyzed and the results suggest that they do not (data not shown), due to the great variability between individuals. However, a trend of increased *Propionibacterium* in G2 and *Staphylococcus*, *Micrococcus*, and *Lactobacillus* in G1 can be observed. It is also observed that an increase in *Corynebacterium* in the G2 after 90 days, but it did not remain after discontinuation of the product. A study conducted [8] with Chinese women described an abundant representation of *Lactobacillus* in the younger group, while older women expressed more *Micrococcus*, *Corynebacterium*, *Dermacoccus*, *Actinomyces*, *Streptococcus*, *Lysinibacillus*, and *Bacillus*. In the consensus of that the microbiota can be influenced by several factors such as age, ethnicity, gender, skincare, climate, among other, the presented different results regarding the skin of Brazilian women, reinforcing that lifestyle and other geographical factors are linked to microbiota. Nevertheless, the increase in *Staphylococcus* and *Corynebacterium* in G2, as well as the modulation of *Propionibacterium*, leads to the evaluation of the differentially expressed species in this work, always comparing the ages and differences in relation to the treatment with the formulation versus control.

3.2 Analysis of differentially expressed species and microbiological diversity

A total of 749 species were found and initially, it was verified the species found in the three main genus: *Cutibacterium* (former *Propionibacterium*), *Staphylococcus* and Corynebacterium. The results showed that in both groups there was a decrease in *Cutibacterium acnes*, however the results remained the same in T120 only in G2. There was an increase in *C. acnes-humerusii* in G1 but not in G2. An increase in *Corynebacterium kroppestendtii* in G1 but not in G2 was also observed, however, *C. pseudogenitalium-tuberculostearicum* increased only in G2. Regarding *Staphylococcus* sp, the most abundant were *S. capitis-caprae-epidermidis* and *S. epidermidis-hominis*, both of which decreased in G1 and increased in G2. Figure 3 reports the abundance of species in both age groups after 90 days. In G1, we observed an abundance of *S. capitis* (Figure 3a), with a significant difference when compared to the other species ($p<0.01$). In G2 (Figure 3b), we also observed this difference; however, when compared to *C.*

pseudogenitalium-tuberculostearicum, the difference was more significant only in this group (p<0.001). *C. pseudogenitalium-tuberculostearicum* is a ubiquitous bacterium that colonizes human skin and is associated with skin inflammation, according to Altonsy et al (2020) [9]. The increase in this species only in G2 suggests more unbalanced skin with a tendency to inflammatory processes. When comparing *C. acnes* versus *S. capitis* (Figure 3c and 3d), we can observe that in the G2, the difference between the two species was more significant (p>0.001).

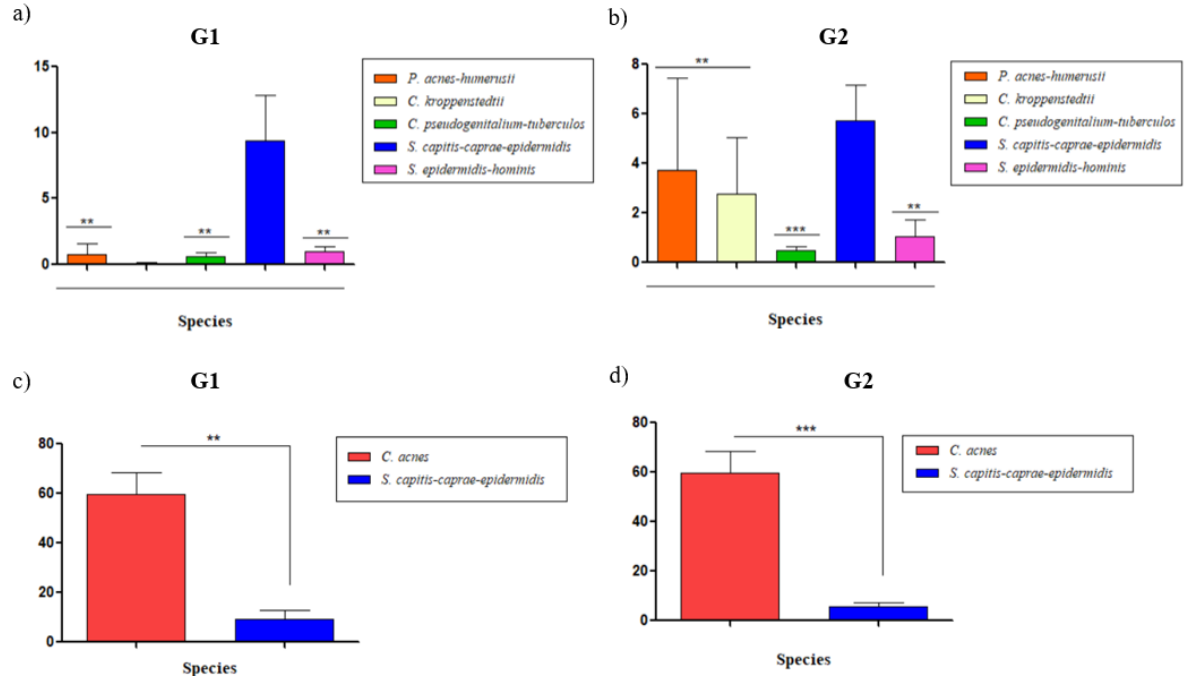

Figure 3: Barplot of species at G1 and G2 after 90 days: 9a) Abundance of G1 species, compared with *S. capitis*; (b) Abundance of G2 species compared with *S. capitis*; (c) Comparison of *C. acnes* and *S.capitis* G1 and (d) Comparison of *C. acnes* and *S.capitis* G2. **=p<0.01, ***p<0.001.

The composition of skin microbial communities has been extensively shown to depend on the physiology of the skin site, with variations in the relative abundance of bacterial taxa linked to moist, dry, and sebaceous environments [1]. *C. acnes* is reported in the literature as a bacterium found on younger skin [13]. Interestingly, in our study, 11 and 9 participants (T90 and T120, respectively) had no or very low abundance of *C. acnes* on their skin (data not shown), which aroused our curiosity and we hypothesize that the presence of certain lipids, such as myrastoleic acid, may influence the activity of *C. acnes* [10]. However, here we used short-chain fatty acids as nanostructured postbiotics to create a microenvironment that favored this microbiota. In this sense, we evaluated the variability in relation to the treatment. The results indicate that DF and CF, presented a statistical difference after 90 days in relation to *S. capitis* (Figure 4a and 4b, respectively). The abundance of *C. acnes* was statistically significant when compared to *S. capitis* (Figure 4c and 4d) showing that both products were able to modulate these species. When analyzing the results (data not shown), it was observed that the treatment did not increase the expression of *C. kroppestendtii*, a bacterium linked to aging. However, this increase was observed in the control group, which was not able to prevent its growth, unlike the results obtained by participants who used the formulation.

Garlet et al. (2024) [1] also found correlation in their study, and related that this bacterium is most likely to appear on old skin. It is interesting to note that the presence of *Corynebacterium kroppenstedtii* increased after 90 and 120 days of treatment with control group. *C. acnes-humerusii*, a not so common species found in the literature, increased only in the group that used the formulation.

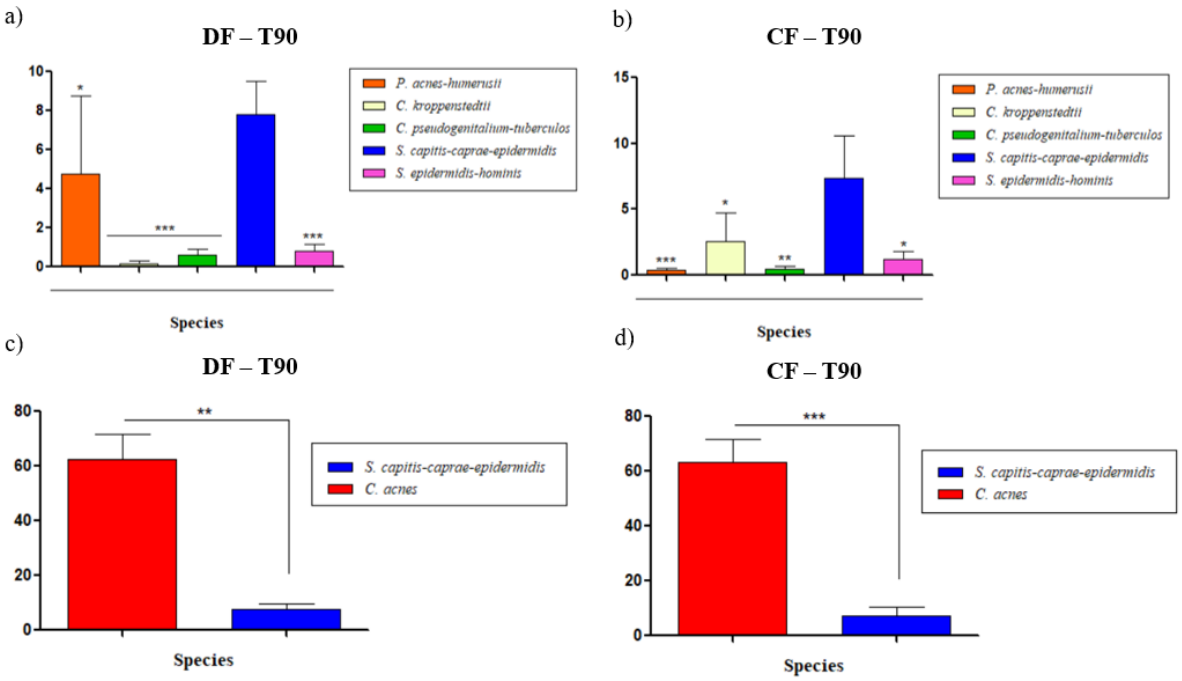

Figure 4: Barplot of species compared by age after 90 days both for the Treated (DF) and Control formulations (CF): (a) Abundance of Treatment formulation species compared with *S. capitis*; (b) Abundance of Control formulation species compared with *S. capitis*; (c) Comparison of *C.acnes* and Treated *S.capitis*; and (d) Comparison of *C.acnes* and Control *S.capitis*. *=p<0.05, **=p<0.01, ***p<0.001.

To observe the alpha diversity of the species, it was applied the ASV richness, Faith's PD, Gini-Simpson and Shennon methods to G1 and G2, T90 and T120. The results (not shown) show a great microbiological diversity identified in the study, however, without statistical difference due to the great variability between individuals. The variability by treatment analyzed the differences between the treated versus control groups, also did not present statistical differences. Intraspecies variability was also analyzed by Bray Curtis – Weigted. The results show that there was no statistical difference between beta diversity in either the age group or the treatment group (Figure 5). However, after 120 days of treatment (Figure 5b), G1 showed a tendency for this variability to increase, higher than G2.

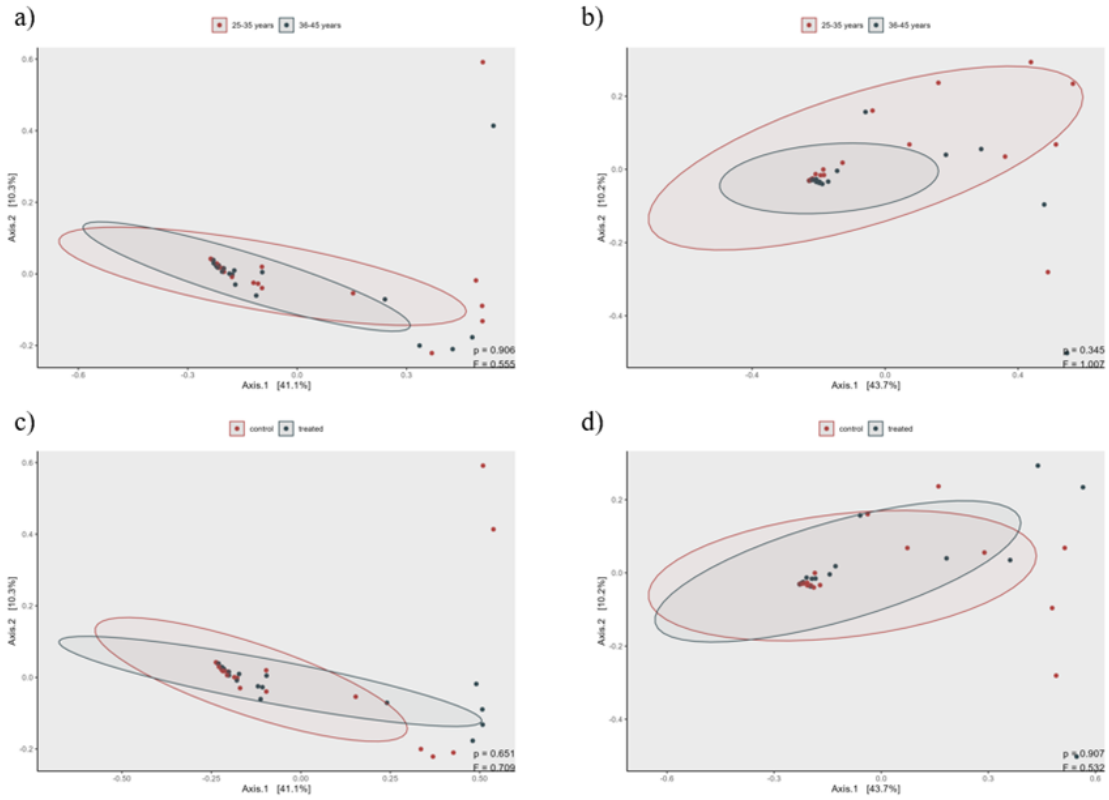

Figure 5: Beta-diversity analysis of species. G1 and G2 (a) T90 and (b) T120, treated and control in T90 (c) and T120 (d).

Finally, the differentially expressed genus found in our study was evaluated. Figure 6 shows the genus expressed in T90 (a) and T120 (b), separated by age. The results indicate that G1 downregulated two genera, while G2 had an overexpression of one. After the discontinuation of the product after 30 days (T120), G2 showed an overexpression of 20 more genus, while in G1 there was a negative expression of 9 genus. The results may indicate that the continuity of the product is important in the older group to avoid the increase of other genus, as well as in the younger group, for the maintenance of microbiota.

At the figure 7, it was observed these variations according to the treatment and the figure 7a showed the overexpression of 2 genera in the treated group after 90 days, while in the control group it is possible to note a negative expression of 15 genera. After 120 days (figure 7b), the treated group increased the expression of 2 genera, and the control group reduced the expression of 4. The set of results infer that the treatment promoted a balance between the genders and the discontinuation of the treatment may indicate a microbiological imbalance, indicating the need to continue the formulation use.

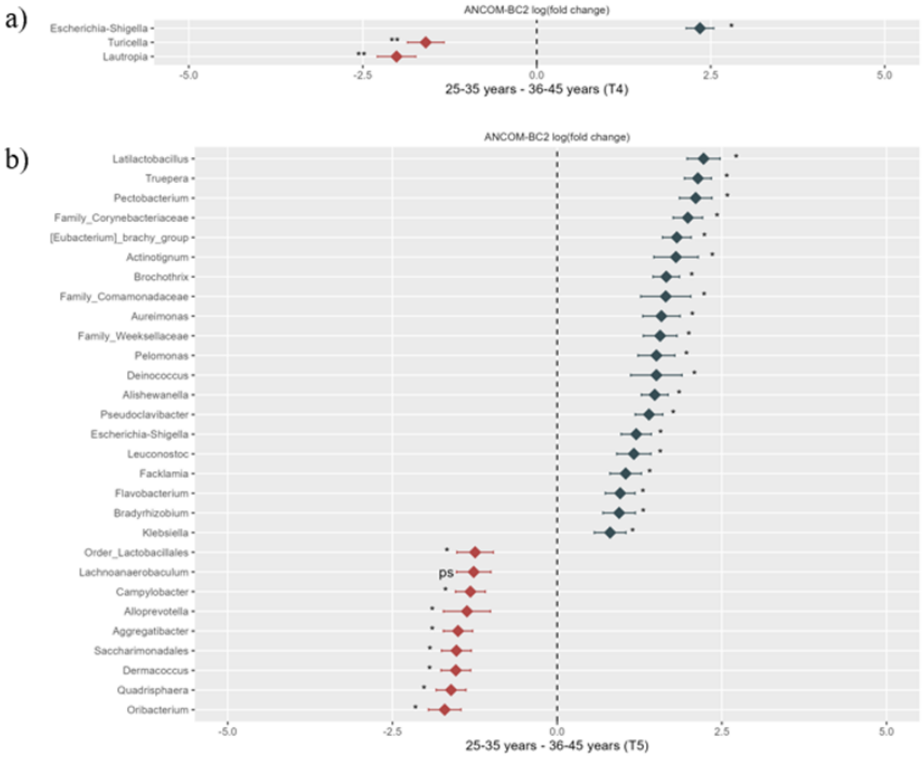

Figure 6: Analysis of differentially expressed genus where G1 and G2 were compared at (a) T90 and at (b) T120.

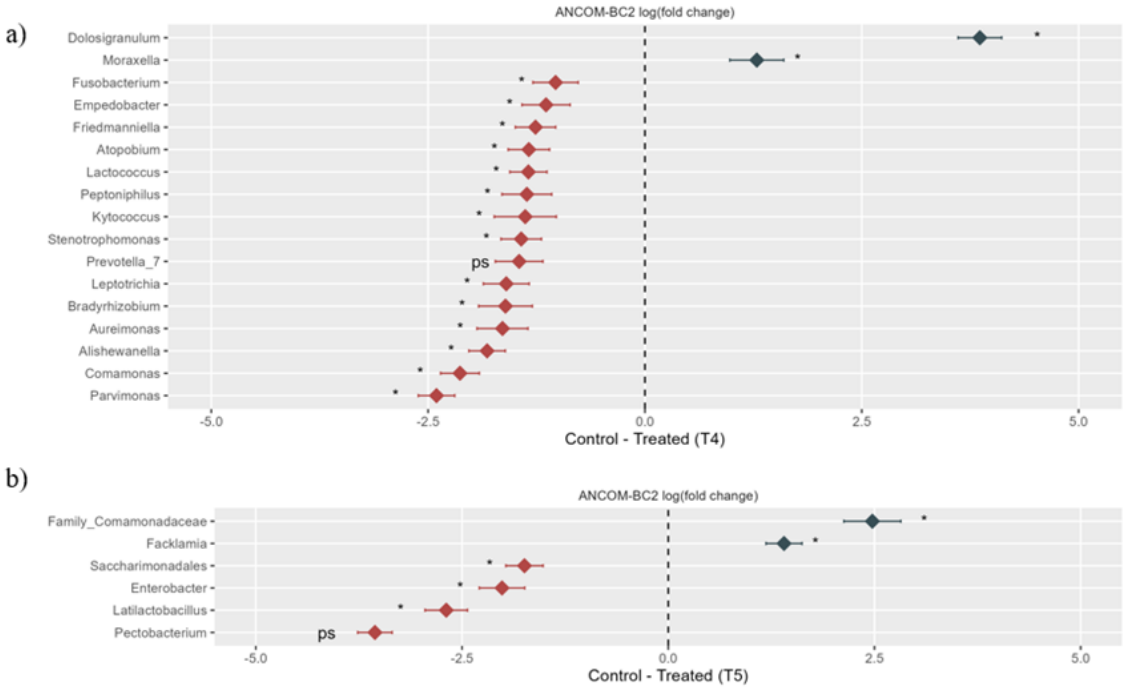

Figure 7: Analysis of differentially expressed genera where Control Group and Treatment T90 (A) and Control Group and T120 (B) were compared.

4. Discussion

Our findings corroborate a study conducted with women and men from Asia [11], which reported an increase in Proteobacteria and a decrease in Actinobacteria in aged skin, as well as another study with women from Western Europe [6], which identified the same findings, although the older population, before treatment, presented a greater abundance of Actinobacteria [6,11]. It is important to highlight that in our study, 20 participants were at G1, while 18 at G2 (two withdrawals). We analyzed the differences in phyla between the treated and control groups, according to the treatment times. The results indicate a decrease in Actinobacteria in both groups, an increase in Firmicutes, with a predominance of the control group, and an increase in Proteobacteria.

The results showed that the older skin, after 90 days of treatment, presented more abundant *Staphylococcus capitis* than the younger group, as well as an increase in *C. pseudogenitalium-tuberculostearicum*, while both groups decreased *C. acnes* and this relationship is due to the increase in other species. *S. capitis* plays a key role in skin protection by their ability to reduce the pathogen load on the skin surface and maintain community structure on the skin surface effectively [12]. The increase in this bacterium in G2 shows that the treatment leads to an increase in a bacterium that is important for the maintenance and homeostasis of the skin. *S. epidermidis* also increased in G2, and as this species [13] is linked to the ceramide's production, the increase in this bacterium in G2 infers that the treatment favored its growth in older skin. At least a study [7] that analyzed a longitudinal multi-omics dataset reported that the most significant changes in aging occur around age 40 and age 60. Our study shows major changes in the microbiome at an early age, emphasizing the need for further studies.

5. Conclusion

The nanostructured formulation significantly improved the skin's microenvironment, enhancing microbiological balance and diversity, especially in aging skin. Pre- and postbiotics applied in this formulation indicates a potential to repopulate and balancing the skin microbiota, highlights the formulation's potential for addressing age-related skin changes. These findings provide insights for the cosmetic industry, showing that balancing skin microbiota has a profound effect on skin health. Additionally, metagenomic studies of Brazilian populations provide critical insights for personalized skincare solutions. By maintaining microbiological diversity and homeostasis, such products offer a scientific grounded approach to foster healthier, more resilient skin.

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