"Biodiversity rich lotion reduced pro-inflammatory cytokine levels in *in vitro* skin model"

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1. Introduction

Urbanization has significantly disrupted the connection between humans and natural microbial biodiversity. The reduction of environmental macrodiversity, such as trees and plants, leads to a decrease also in invisible microbial diversity, within our living spaces. As a result, urban residents are predominantly exposed to their own microbes rather than to microbes originating from the biodiversity rich environment. In Western societies, people living in cities spend over 90% of their time indoors [1], which greatly limits their outdoor microbial exposure. This diminished contact with diverse microbial communities can impair the immune system, potentially causing diseases like atopy and asthma [2-11].

Environmental microbes are essential as transient visitors on our skin, gut, and airways, where they are recognized by epithelial and immune cells [12]. Continuous environmental stimulation of these cells is crucial for maintaining normal epithelial barrier function and regulating immune system tolerance to reduce inflammation. The alarming decline in environmental biodiversity may affect the immune system's ability to distinguish between threat signals and benign signals. This can lead the immune system to attack aggressively against otherwise benign foreign structures such as pollen or even against the body's own tissues, as in the case of type 1 diabetes and other autoimmune diseases. Imprtantly, numerous immune-mediated diseases have been linked to the loss of biodiversity in our living environments [2-11].

The microbial extract examined in this paper is designed to provide the necessary exposure to nature, particularly for those who spend limited time outdoors or have restricted access to natural environments. By reconnecting with natural microbes, the immune system and epithelial barriers receive essential training, supporting their health-promoting function. The purpose of this study was to observe the immunological responses to a microbial extract-

containing cream using an in vitro skin model, focusing on its effects on the skin within the context of cosmetic products.

Previous scientific studies have clearly shown beneficial outcomes in biodiversity exposure trials. These trials demonstrated that contact with microbiologically rich natural materials diversified skin and gut microbiota and enhanced immunoregulation[13-15]. Additionally, recent studies have also showed the beneficial effects of the regular exposure to rich natural microbes in lotion on atopic skin [16-17].

2. Materials and Methods

The aim of this study was to examine the impact of microbial extract on cytokine expression using commercial epidermis skin model by a commercial supplier. This model also counted microbial colony-forming units and barrier protein expression using immunohistochemistry, which results are not, nonetheless, included in this paper. Cytokines, which are signalling molecules that regulate immune responses, are secreted by various cell types, including keratinocytes. Some cytokines increase inflammation, while others suppress it and some has multifaceted role.

The experimental model involved laboratory-grown stratified epidermis formed by cultured adult human dermal fibroblasts embedded into a fibrin matrix, which formed the base for skin model unit. Primary neonatal keratinocytes were applied to the units and incubated (37 ± 2 °C at ≥ 95% Relative Humidity) for 48 hours under a cultivation media. The model was then cultured at the air liquid surface until stratified epidermis was formed.

The test was divided into two parts, where the grown dermal units (DU) were inoculated with "Microbiome balance" and "Microbiome defense" protocols. The DU's were first inoculated with a 10 µL of mixture consisting of normal skin microorganisms *Cutibacterium acnes* (NCTC 734), *Staphylococcus epidermidis* (NCTC 11047) and *Corynebacterium striatum* (NCTC 764), containing ~10^4 CFU cm^{-2} of each bacterium. Each DU inoculated with the microbes was incubated at 37 ± 2 °C at ≥ 95% Relative humidity for 3 ± 1 hour to allow the bacteria to colonize, after which the DP's were treated with 11 µL of the test lotion or vehicle and incubated at the same conditions for 24 ± 2 hours.

The used vehicle was a basic lotion (INCI: Aqua, Xanthan gum, Caprylic/Capric Triglyceride, Cetearyl Olivate, Sorbitan Olivate), containing Microbial extract (INCI: Humus extract) in following concentrations 0%, 0.5%, 1%, 5% and 10% to test the efficacy and safety of the active ingredient.

Microbiome balance test samples were collected and frozen at -80°C for later cytokine analysis by multiplex ELISA. The samples assigned to the Microbiome defense test were inoculated

with 10 µL of ~1.1 x 10^4 CFU mL^{-1} of *Staphylococcus aureus* (NCTC 1345) and incubated at 37 ±2 °C at ≥95% relative humidity for 24 ± 2 hours and sampled as previously.

The undernatant was analyzed with multiplex ELISA "Proinflammatory Panel 1" to quantify the concentration of nine cytokines (IL-1β, IFN-γ, IL-4, IL-13, TNFα, IL-2, IL-6, IL-12p70, IL-10) for both "Microbiome balance" and "Microbiome defence" protocols. Outliers were screened from the data using Grubbs' test and removed. Shapiro-Wilks normality test was performed to each dataset to check the normal distribution. Analysis of variance tests (ANOVA) was used to assess statistical differences between groups for normally distributed data. Ordinary one-way ANOVA was performed for analyses between all treatment groups and Dunnett's multiple comparisons test was used to compare treatments against vehicle. If the data was not normally distributed, a Kruskal-Wallis test was performed to test differences between treatment groups and Dunn's multiple comparisons test was used for post hoc analysis when comparing treatments against vehicle.

3. Results

**Exposure to microbial extract reduced proinflammatory cytokines in human skin model**

Immunological assays showed that treatment with microbial extract reduced the levels of cytokines released by the keratinocytes in the epidemis skin model. In the balance test with commensal microbes *C.acnes, S.epidemidis & C.striatum*, microbial extract treatment significantly reduced the levels of five pro-inflammatory cytokines: IL-4, TNFα, IFNγ, IL-12p70, IL-13, (Figure 1a, c, e) compared to the vehicle control. In the defense test with *S. aureus,* microbial extract treatments significantly reduced the levels seven pro-inflammatory cytokines TNFα, IFNγ, IL-4, IL-1β, IL-2, IL-12p70, IL-13 (Fig 1b, d, f) compared to the vehicle.

(a)
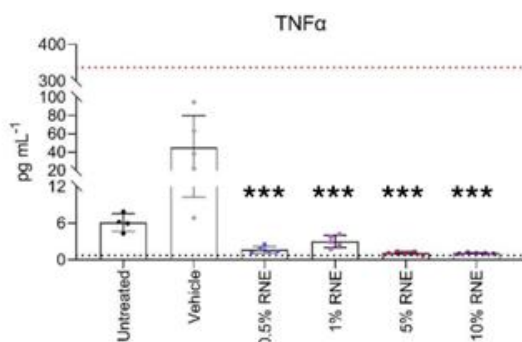
(b)
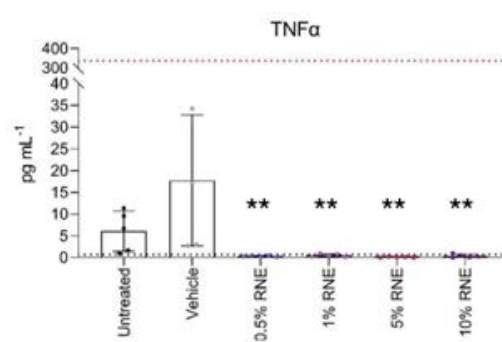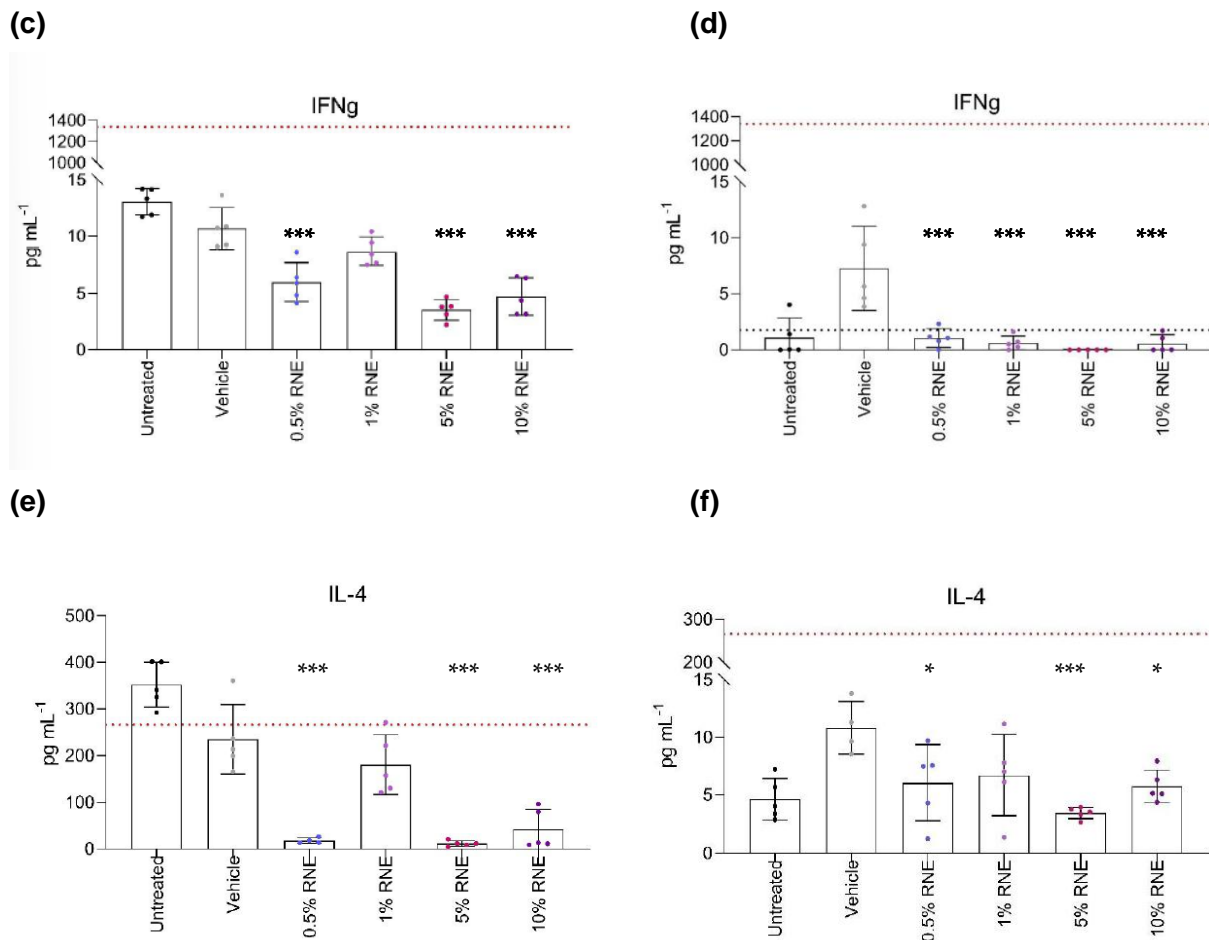
Figure 1. Pro-inflammatory cytokine levels in *in vitro* skin model after application of microbial extract (RNE) in lotion after 24h incubation. Controls were untreated and vehicle (= the lotion without the active ingredient). **(a).** TNFα **at balance test** with only commensal flora **(b).** TNFα **at defence test** where pathogenic *S.aureus* was also present, **(c).** INFγ **at balance test, (d)** INFγ at **defence test, (e)**. IL-4 at **balance test (f)**. IL-4 at **defence test.** Statistically significant differences from Dunnett's multiple comparisons test are marked with * as follows: *** = p-value is <0.001, ** = p-value is <0.01, * = p-value is <0.05. Comparison is made to the vehicle.

Markedly, the microbial extract exposure reduced the levels of pro-inflammatory cytokines in both Microbiome balance & defence tests suggesting reduced inflammatory responses also *in vivo*.

4. Discussion

The most important result of the present microbial exposure *in-vitro* epidermis study demonstrates a significant potential for its use in cosmetic formulations to receive marked skin benefits. Similar results have been obtained in previous studies, in which microbial exposure has improved immune regulation, proportion of regulative T-cells and improved the IL10/IL17

ratio [13-15]. The used lotion containing microbial extract decreases the levels of pro-inflammatory cytokines, the effect which is beneficial for the skin, as high levels of these cytokines, especially IL-4, IL-13, TNFα and IFNγ, are linked to the impaired skin barrier function [18-23]. Importantly, the active ingredient did not show inflammatory response in the used model, and the conducted *in vivo* trials have showed strengthened skin barrier function, reduced skin redness and irritation (16). Additionally, the recent findings with the same microbial extract in atopic dermatitis studies proved the skin benefits even with individuals having severe atopy induced skin flares [16,17].

In the used skin model, this active ingredient in lotion did not have any negative effect on the four important skin barrier proteins (Ki67, filaggrin, loricrin & claudin-1) even with high concentrations, although the results are not in the focus of this paper. By reducing the expression of pro-inflammatory cytokines, RCN has strong potential for relieving the symptoms of atopic dermatitis and skin irritation in the topical use. Together, the present *in vitro* skin model and *in vivo* studies with atopic dermatitis patients strongly support the health benefits of the extract with high microbial diversity. Therefore, our results provide further evidence for the biodiversity hypothesis, highlighting the importance of continuous interaction with a wide variety of natural microbes to maintain normal immune system function and the integrity of epithelial barrier. Engaging with natural biodiversity helps the immune system to generate regulatory responses instead of inflammatory ones.

5. Conclusion

In conclusion, the in-vitro epidermis skin model results indicate that microbial extract in cosmetic formulations offers multiple skin benefits. The microbial extract in the lotion significantly reduces levels of several pro-inflammatory cytokines, which are associated with impaired skin barrier function. The active ingredient did not induce an inflammatory response and showed no negative effects on key skin barrier proteins, even at high concentrations. In vivo trials have demonstrated strengthened skin barrier function and reduced skin redness and irritation, particularly in participants with atopic dermatitis. These findings support the biodiversity hypothesis, emphasizing the importance of continuous interaction with diverse natural microbes to maintain normal immune system function and epithelial barrier integrity, promoting regulatory rather than inflammatory immune responses.

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