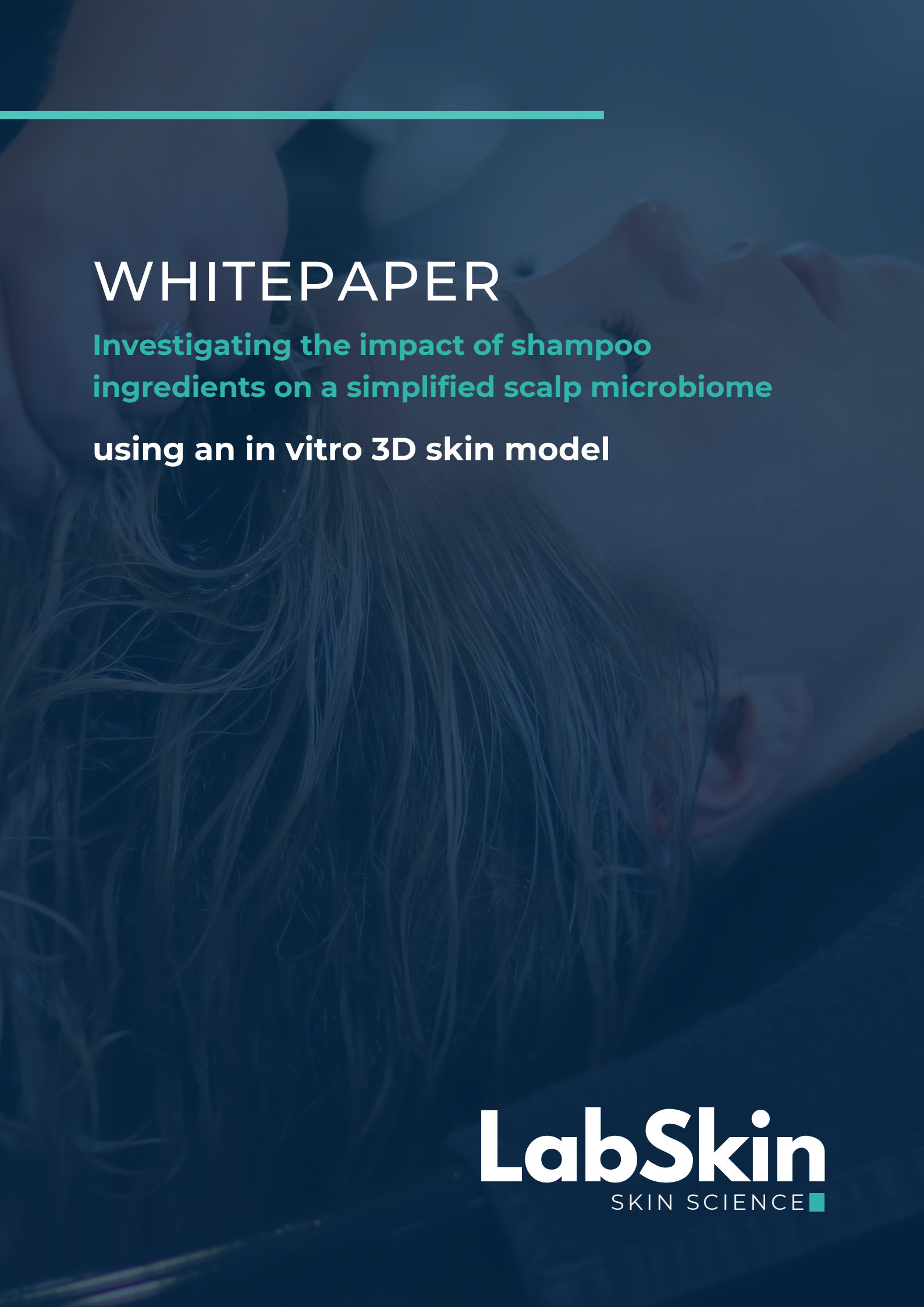

WHITEPAPER

Investigating the impact of shampoo ingredients on a simplified scalp microbiome

using an in vitro 3D skin model

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ABSTRACT

The scalp microbiome is a complex consortium of bacteria, fungi/yeast, arachae and viruses. The microbiome helps to regulate pH in the scalp, protect the scalp from infections and to support healthy hair growth. An imbalanced microbiome on the scalp can present as itchiness, fl akiness, redness or even conditions such as dandruff, seborrheic dermatitis and even hair loss.

In this article, we use an in vitro human skin model (Labskin-S) colonised with a scalp microbiome (C.acnes, S.epidermidis, C.striatum and Malassezia spp.) to investigate how ingredients used in hair care products can alter the microbiome composition and the effects on pro-infl ammatory markers released in the skin.

We showed that when targeting key microbes in the scalp microbiome, for example anti-dandruff actives against Malassezia spp., it is important to consider, not only the effects on the microbe, but also on the scalp skin.

Introduction

Dandruff, is a condition of the scalp that results in dry, itchy, fl aky skin lesions, and is associated with dysbiosis of the scalp microbiome (1). This dysbiosis is most characterised by an increase in Malassezia spp. and a decrease in benefi cial microbes such as C.acnes (2). Charles-Louis Malassez fi rst proposed a link between fungi overgrowth and dandruff in 1874 (3). The microbiome dysbiosis can also result in infl ammation of the scalp skin, exacerbating the itch response (4).

Current treatments for dandruff focus on actives to target the Malassezia spp., sebum and oil production in the scalp, and soothing of infl ammation and itchiness (5). Therefore, when investigating new active ingredients and formulations to control dandruff, it is important to consider the impact on the microbiome balance and infl ammatory responses in the scalp.

Research has shown that several cytokine markers are elevated in dandruff conditions. These include IL-1α, IL-1β, IL-6, IL-8 and TGF-β (8). IL-1α is regarded as a 'fi rst responder' in the infl ammatory cascade, playing a role in activating oth- er infl ammatory cytokines and chemok- ines (6). IL-8 is released by keratinocyte cells in response to elevated numbers of Malassezia spp (9).

The aim of this study was to use a 3D in vitro human skin model populated with the main microbes found on the human scalp to investigate the impact of two novel test items; one designed to target Malassezia spp. and the other to maintain the balance of microbes.

Keywords

model; Malassezia; Labskin

Materials and Methods

3D in vitro human skin equivalent Primary adult human dermal fi broblasts were embedded into a fi brin gel matrix to produce dermal equivalents (DEs). The DEs were cultured to allow the fi - broblasts to remodel the matrix. Primary neonatal human keratinocytes were applied to the DE surface and cultured under liquid for 48 hours. Labskin was cultured at the air liquid interface until a stratifi ed epidermis was formed. Incubation conditions for all cultures was 37 ± 2°C in 5 ± 1% (v/v) CO2 at ≥95% Relative Humidity (RH).

Scalp microbes A mixed consortia of bacteria and yeast was created to mimic a simplifi ed scalp microbiome. This consortium included C.acnes, S. epidermidis, C.striatum and M.globosa. These microbes were se- lected based on published data and in house data from 16SRNA sequenced scalp microbiome swabs (10).

Malassezia globosa (CBS 3990) was cul- tivated aerobically at 34 ± 2°C for 4 days using Selective Modifi ed Malassezia agar (SMA+).

Cutibacterium acnes (NCTC 737) was cultivated anaerobically at 37 ± 2°C for 4 days using Reinforced Clostridial Agar Medium with Furazolidone (RCAF).

Staphylococcus epidermidis (NCTC 11047) was cultivated aerobically at 37 ± 2°C for 24 hours using Mueller-Hinton agar (MHA). Corynebacterium striatum (NCTC 764) was cultivated aerobically at 37 ± 2°C for 24 hours using aerobic corynebacte- rium agar (ACA). Inoculation buffer was used to prepare the initial inoculum containing 1.1 x 10^6 CFU mL^{-1} of each bacterium and ~1.1 x 10^8 CFU mL^{-1} of the yeast. 10 µL of the inoculum was used to colo- nise each Labskin-S unit. This translated to ~10^6 CFU cm^{-2} of the yeast.

Study Protocol

Colonised Labskin-S was incubated at 37 ± 2 °C in 5 ±1 % (v/v) CO_2 at ≥95% RH for 3 ± 1 hour.

Test items were diluted 1 in 10 using sterile dH_2O (one part test item to 9 parts of dH_2O). This allowed replication of the conditions of the product's intended use.

Five Labskin-Sunits were left untreated and incubated at 37 ± 2 °C in 5 ±1 % (v/v) CO_2 at ≥95% RH for 21 ± 2 hours.

The remainder of Labskin-S units were treated with 11 µL of dPBS (untreated control) or test item.

Treated Labskin-S units were incubated at room temperature for 10 ± 1 minute.

Following this incubation, the test items (or dPBS) were washed off with pre-warmed dPBS to mimic washing off of hair shampoos.

The Labskin-S surface was then dried with sterile fi lter paper strips.

Labskin-Sunits were incubated at 37 ± 2 °C in 5 ± 1 % (v/v) CO_2 at ≥95% RH for 18 ± 2 hours.

Biopsy samples of 8 mm in diameter were aseptically removed from the centre of each Labskin-S unit and placed into sterile microcentrifuge tubes. 1.5mL of Dey Engley neutralizing broth media was added and the tubes containing the samples vortexed to recover the microbes. Serial dilutions were performed and viable microbial numbers were assayed by recovery on appropriate culture media.

Undernatant media was collected from all Labskin-S units and frozen at <-70°C for examination of proinflammatory cytokines.

Test Items Test items can be any fi nal formulation, active ingredient or carrier that is applied to the scalp to interact with the scalp mi- crobiome. For this study, we investigated two active ingredients designed to target dandruff (Test item 1 – an active ingre- dient designed to target Malassezia spp. and Test Item 2 - a starch-based product designed to maintain the microbiome consortium), a commercially available shampoo product without an active ingredient and the carrier alone (dH20). The hypothesis tested was the test item one should reduce the amount of Malassezia globosa recovered, and that test item 2 should neither increase nor decrease the amount of any of the microbes present.

Microbial enumeration

Methodology used to quantify individual microorganisms of the scalp mix. Technical replicate counts obtained from selective solid media were averaged and used to calculate the colony forming units per square centimetre (CFU cm^{-2}). The limit of detection of this assay is 59 CFU cm^{-2}.

Log_{10} difference: Calculation of microbial change; the magnitude of this change can be used to compare between microorganisms and between treatment groups.

Log_{10} was calculated by determining the Log^{10} value ofthemeanCFUcm for each treatment group.

Log_{10} difference was calculated (Figure 7) using the following formula:

Log_{10} difference = Log_{10}(Treatment) - Log_{10}(Untreated)

Cytokine quantifi cation

Undernatant from all the Labskin constructs colonised with the scalp microbiome consortium was collected 24 hours post treatment and 5 replicates were analysed using the R&D systems ELISA kits in order to quantify the concentration of IL-1α, and IL-8.

Data Analysis

Data handling, statistical analysis and data representation was carried out using Microsoft 365 and GraphPad Prism9. The statistical tests used included Grubbs outlier analysis, ANOVA and Sidaks multiple comparisons tests. These tests will be referred to in the results and discussion.

Protocol overview

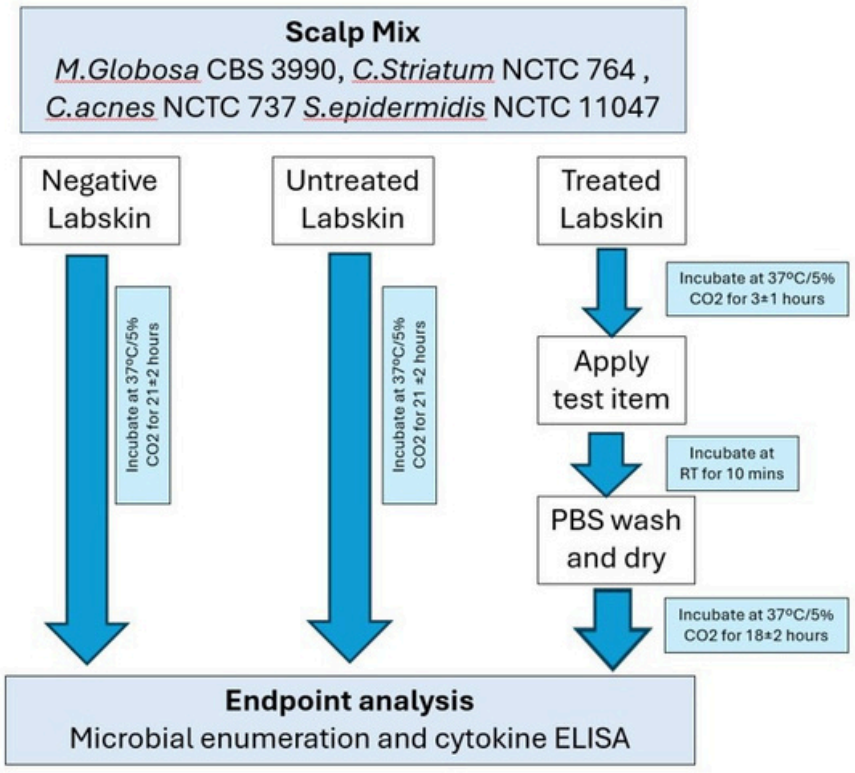

Figure 1. Flow diagram summarising the experimental layout for "Test item effect on scalp microbiome and Labskin immune response" protocol.

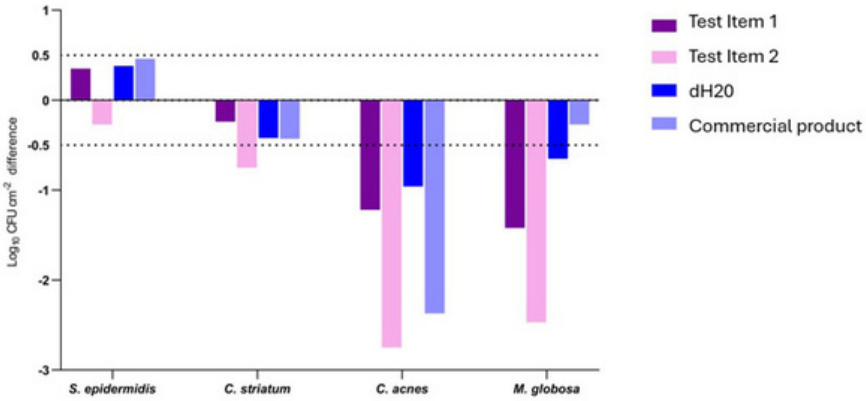

Figure 2. Log^{-2}_{10} CFU cm difference between all test item groups and the dPBS control. "Untreated" has been removed from the graph for clarity. The dotted line indicates the ±0.5 Log^{-2}_{10} CFU cm difference.

Results and Discussion

Test item effect on scalp microbiome - analysis explanation To assess the test items' effect on the scalp microbiome model, the consor- tium was plated onto selective media for each individual microorganism and enumerated and analysed.

This determined changes upon individual populations of microorganisms within the microbiome model caused by the test items.

Log_{10} Difference Using the Log CFU cm^{-2}difference $_{10}$analysis (Figure 2), it is possible to es-
tablish a biologically significant change by the treatments in relation to the "Untreated" control. This change is defined as a difference of ±0.5 Log^{9} CFU cm^{-2}.

Test Item 1 When compared with "Untreated", it increased the amount of S. epidermidis by Log_{10} 0.35 and reduced the amount of C. striatum, C. acnes and M. globosa by Log_{10} -0.24, -1.22 and -1.42 respectively.

Test Item 2 When compared with "Untreated", it reduced the amount of S. epidermidis, C. striatum, C. acnes and M. globosa by Log_{10} -0.27, -0.75, -2.75 and -2.47 respectively.

dH_2O When compared with "Untreated", it increased the amount of S. epidermidis by Log_{10} 0.38 and reduced the amount of C. striatum, C. acnes and M. globosa by Log_{10} -0.42, -0.96 and -0.65 respectively. Commercial product when compared with "Untreated", it increased the amount of S. epidermidis by Log_{10} 0.46. It has also reduced the amount of C. striatum, C. acnes and M. globosa by Log_{10} -0.43, -2.37 and -0.27 respectively.

Test item effect on Labskin immune response - analysis explanation

Undernatant for Labskin constructs colonised with the scalp microbiome consortium was collected 24 hours post treatment and 5 replicates were analysed using the R&D systems ELISA kits in order to quantify the concentration of IL-1α, IL-6 and IL-8.

This assessment aimed to provide insight into the epidermal response to the colonisation and investigate the host-microbe interactions.

IL-1α

One outlier was removed from the "Test Item 2" treatment group, following Grubbs outlier analysis.

All datapoints for "Negative" and one data point from "Untreated" were below the lower limit of quantification indicating negligible quantities of the cytokine present.

One-way ANOVA indicated statistically significant difference between the treatment groups (p=<0.0001).

The post-hoc test showed no statistical significance (p>0.05) between:
"Negative" control vs. "Untreated" or "Test Item 2".
"Untreated" control vs. "Test Item 1"," Test Item 2" or "dH_2O".
The post-hoc test showed a statistically significant increase between:
"Negative" control vs "Test Item 1" (p=0.0114), "dH_2O" (p=0.0055) and "Commercial product" (p=<0.0001).
"Untreated" control vs. "Commercial product" (p=<0.0001).

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Cytokine	Role	Concentration curve range (pg mL^{-1})	Sensitivity (pg mL^{-1})
IL-1α	Acts in a proinflammatory manner to further perpetuate an inflammatory response (6).	3.9 - 250	1
IL-8	IL-8 is proinflammatory cytokine which plays a key role in the recruitment of neutrophils and other immune cells to the site of infection (7).	31.2-2000	7.5

Table 1. List of cytokines, their roles and the ELISA parameters used for quantification of cytokines in the undernatant of Labskin colonised with scalp microbiome.

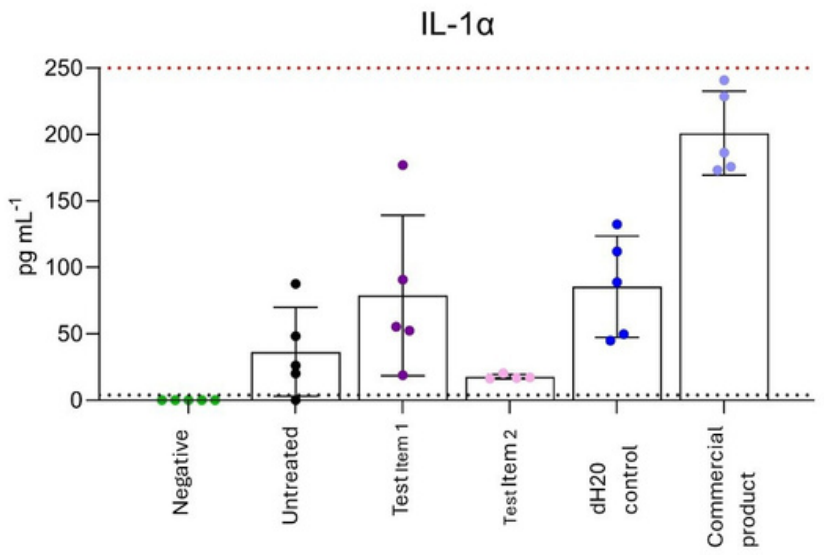

Figure 3. IL-1α ELISA results for Labskin colonised with Scalp mix after removing outliers. Data points represent individual Labskin units (n=4/5). Graph displays mean + SD. Red dot- ted line represents the peak of the concentration curve (250 pg mL^{-1}) and black dotted line represents the lower limit of quantification (3.9 pg mL^{-1}). Negative values are displayed as "0" for graphical representation. Statistical significance is not displayed on the graph for clarity.

IL-8 In accordance with the ELISA kit's manufacturers instruction, a 1 in 100 dilution of the undernatant was performed to bring the levels of this cytokine within the limits of the standard curve. This allowed for a more accurate quantification.

All data points of the diluted undernatant were below the peak concentration curve. Once the dilution factor was taken into account, the true val- ues were raised above the top of the concentration curve, however they are still accurate.

Three data points for "Negative" were below the lower limit of quantification, indicating negligible expression of this cytokine.

One-way ANOVA indicated statistically significant difference between the treatment groups (p=<0.0001).

The post-hoc test showed no statistical significance (p>0.05) between:
"Untreated" control vs. "Test Item 1","
Test Item 2" or "dH_2O".
The post-hoc test showed a statistically significant increase between:
"Negative" control vs all treatment groups (p=<0.0001).
"Untreated" control vs. "Commercial Product" (p=<0.0001).

Conclusions

The use of the 3D in vitro human skin model populated with a simplified scalp microbiome consortium allowed clear distinctions to be made between ingredients used in the manufacture of shampoo.

For Test Item 1, an ingredient designed to target Malassezia spp., the analysis showed that, when compared with the untreated control (colonised with scalp mix), it had no negative effect on the population of S. epidermidis or C. striatum. However, it did induce a biologically significant reduction in population of C. acnes and M. globosa. Test Item 1 also showed no statistically significant effect on the increased release of IL-1α or IL-8 when compared to the "Untreated" control.

These results suggest that including Test item 1 in a shampoo format could result in a product able to reduce the amount of C.acnes and Malassezia spp. without inducing inflammatory responses.

For Test item 2, an ingredient designed to maintain the microbiome composition, the analysis showed that, when compared with the untreated control (colonized with scalp mix), it has a reductive effect on all 4 microbes used in the scalp consortia. It induced a biologically significant reduction in C.acnes, C.striatum and M.globosa. Test Item 2 also showed no statistically significant effect on the increased release of IL-1α or IL-8 when compared to "Untreated" control.

These results could suggest that including Test item 2 in a shampoo could have an non-selective reductive effect in key microbes in the scalp microbiome without inducing inflammatory responses.

For the Commercial product (shampoo not specifically designed to be anti-microbial), the analysis showed that, when compared with the untreated control (colonised with scalp mix), it had no reduction on the populations of S. epidermidis, C. striatum or M. globosa. It did, however, induce a biologically significant reduction in population of C. acnes. The reduction observed was higher than the one observed in Labskin treated with the respective vehicle control (dH_2O) which takes the rinse step of the procedure into account. It has a statistically significant increased in the release of IL-1α and IL-8.

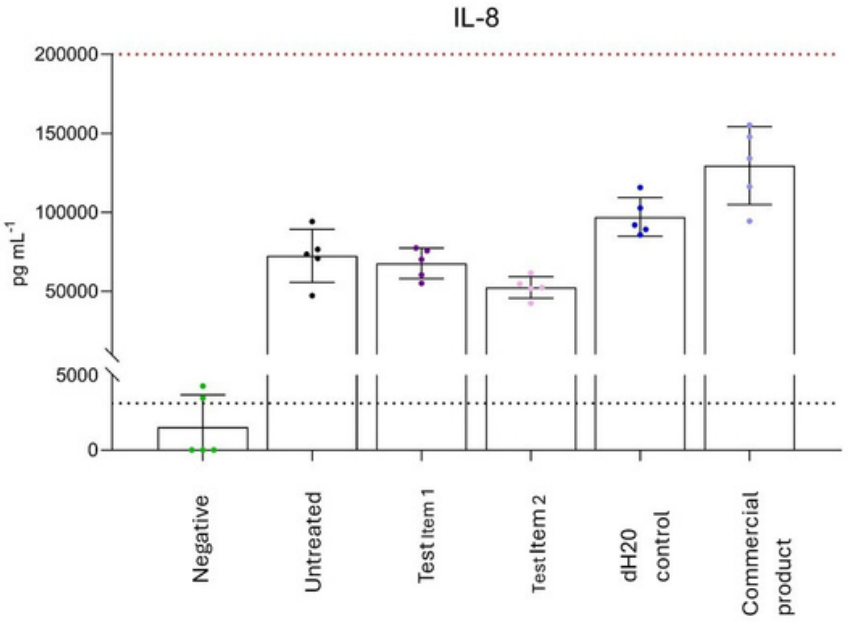

Figure 4. IL-8 ELISA results for Labskin colonised with Scalp mix. Data points represent individual Labskin units (n=5). Graph displays mean + SD. Red dotted line represents the peak of the concentration curve and black dotted line represents the lower limit of quantification. Both dotted lines have been marked after taking the dilution factor into account (1 in 100). Negative values are displayed as "0" for graphical representation. Statistical significance is not displayed on the graph for clarity.

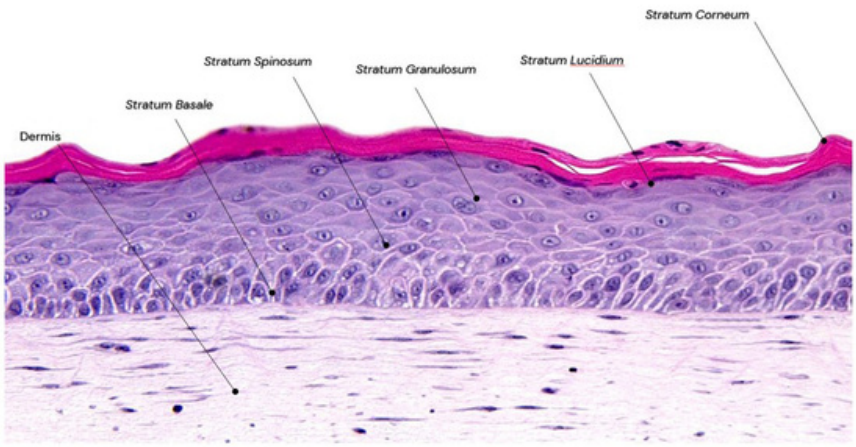

Figure 5. H&E stained transverse section through Labskin-S showing the dermal layers and the stratified epidermis, and full thickness Stratum Corneum.

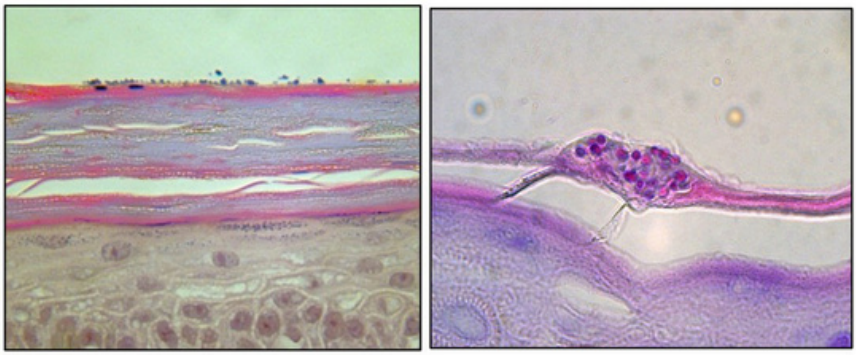

Figure 6. Images of microbes on the Stratum Corneum of Labskin-S. Left hand panel shows a mix of C.acnes, C.striatum and S.epidermidis. Right hand panel shows a Malassezia globosa.

The results could suggest that the commercial product targeted C.acnes in the consortium used but is more likely to induce inflammation due to a significant increase in IL-1α and IL-8.

Discussion

When investigating novel active ingredients and final formulations of products designed to target specific microorganisms, for example, reducing Malassezia on dandruff prone scalps, it is important to take a holistic approach. Using a scalp microbiome consortium, and not just Malasezzia spp., indicates if an active ingredient or formulation effects other microbes present which could cause a dysbiosis in the scalp microbiome. Using a 3D in vitro human skin equivalent populated with a simplified scalp microbiome, allows a more thorough investigation into an active ingredient or formulation effects by 1) investigating a broader range of microbes rather than Malassezia spp. individually on selective agar, 2) allowing the mimicking of the end user experience with application and wash-off, or even repeated applications, and 3) assessment of the products effects on the skin inflammatory responses to either application of the test item or via dysbiosis of the microbiome.

The use of non-animal models such as these 3D human skin equivalents that have a comparable morphology to human skin (figure 5) with the ability to be populated with the human skin and scalp microbiome (figure 6), allows formulators and product developers the opportunity to perform more thorough investigations and gain scientifically val- id data to derisk clinical trials and con- sumer trials with human volunteers.

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Nicola Kingswell is the scientific director and co-own- er of Labskin Limited, where she manages the stra- tegic direction of the company, as well as day to day operations. Nicola is responsible for leading R&D ac- tivities to drive innovation in the company, in line with client requirements. She oversees all client activities including technical sales, project scoping, project management and delivery. and Nicola is an experienced protein biochem- ist and cell biologist. She has held positions at some of the world's largest companies (Intertek, Labcorp, Eurofins) and some of the world's smallest companies (start-ups and spin outs).

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