"An Analysis and Approach of Lipidomics Test Using Human Ex Vivo Skin Tissues Treated with Ingredients That Mimic Skin Lipids"

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Abstract

Lipidomics is the comprehensive study of lipids and their roles in human metabolic pathways. Skin lipids are essential for maintaining skin health, and alterations in their composition can influence various skin conditions. Recent research has explored how moisturizers can improve skin health by modifying lipid composition. Based on prior studies demonstrating the influence of the topical use of lipid-based cosmetic ingredients, this study investigates the relationship between skin lipid composition and skin health. A comparative analysis of two formulations revealed significant upregulation of HAS2 and HAS3 mRNA, as well as hyaluronic acid expression, with more pronounced effects in the experimental group. Additionally, FLG mRNA levels and epidermal thickness were substantially enhanced in ex-vivo skin explants, particularly in the experimental group. Ceramide NP, NS, and NDS levels, along with free fatty acids that had diminished in damaged skin, were restored in both groups, with greater recovery observed in the experimental group. Further analysis of the distribution of ceramides and free fatty acids by carbon chain length indicated notable improvements in the skin barrier. These findings suggest that alterations in skin lipid composition play a crucial role in skin health, although further research is necessary to elucidate the precise correlation between lipidomic changes and healthy skin.

Keywords: *Lipidomics, Skin barrier*

1. Introduction

Lipidomics enables the comprehensive analysis of lipid profiles in biological systems, providing insights into how variations in lipid composition influence skin physiology. Advanced analytical techniques, such as liquid chromatography-mass spectrometry (LC-MS), have facilitated the identification and quantification of specific lipid species, allowing researchers to investigate the effects of external factors, including skincare formulations, on the skin lipidome (Zhao, Ying-Yong, et al., 2014). This has led to the development of targeted therapeutic approaches, including lipid-based treatments designed to reinforce the skin barrier and enhance hydration.

Recent studies indicate that the topical application of bioactive lipids, such as ceramides, cholesterol, and free fatty acids, can effectively restore lipid balance in compromised skin. These components are essential constituents of the stratum corneum (Figure 1), playing a crucial role in maintaining barrier function and preventing moisture loss (Mijaljica, Dalibor, et al., 2024). Furthermore, these lipids interact with sebaceous lipids derived from sebaceous glands to create a complex and unique natural protective barrier that maintains skin homeostasis and prevents transepidermal water loss (Tascini, Anna Sofia, et al.,2019).

While the precise mechanisms by which stratum corneum and epidermal lipids are maintained and support skin barrier homeostasis remain to be fully elucidated, subtle alterations in their lipid composition have been implicated in numerous dermatological pathogeneses, including atopic dermatitis, psoriasis, and erythema (Yin, Huibin, et al. 2023). Building upon previous findings, this study aims to further investigate how changes in skin lipid composition in response to moisturizer application impact skin health. By applying components mimicking healthy stratum corneum lipid composition and SSL to human skin explants, we analyze changes in ceramide and free fatty acids profiles and their effects on key skin biomarkers such as HAS2 and FLG, seeking to deepen our understanding of the relationship between lipidology and epidermal function (Wang, Hecong, et al., 2020). The findings are expected to contribute to the understanding regarding the potential of lipid-based skincare solutions in addressing skin barrier dysfunction.
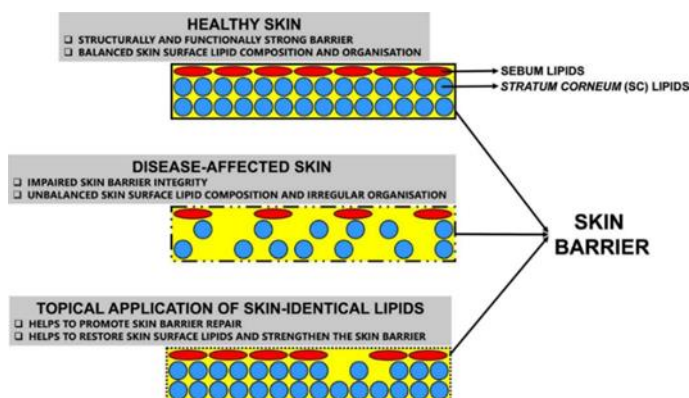

Figure 1. Differential lipid organization between healthy and disease-affected skin, and beneficial alterations in cutaneous lipid profiles following topical application of skin-identical lipids.

2. Materials and Methods

Preparation of the test materials

As a control group, formula A, composed of Ceramide, Cholesterol, and Non-esterified Free Fatty Acids in a specific molar ratio, was used. The experimental group, formula B, was designed to mimic the composition of the stratum corneum lipids in healthy human skin and the structure of SSL (Skin surface Lipids), also in a specific molar ratio. Additionally, ascorbic acid and epidermal growth factor (EGF) were used as positive controls for respective experiments (Sasidharan, Oormila, Anjali Gholap, and Rachna Rastogi, 2023).

Preparation and culture of human skin explants

Human skin explants discarded post-surgery was used for this study with Institutional Review Board (IRB) approval (IRB approval number: E-2023-032-01). Under a clean bench, the human skin tissue was washed twice with phosphate-buffered saline (PBS) and transferred to a sterile Petri dish. A biopsy punch (KAI Medical, Japan) was used to obtain uniform tissue samples for experimentation.

Moisture Recovery & Barrier Recovery Efficacy Assessment

For the moisture recovery efficacy test, human skin explants were washed twice with sterile saline and processed using an 8 mm biopsy punch. The tissues were subjected to drying conditions (humidity <40%, 30% lower than the culture condition) for 1 hour to induce moisture loss. Thereafter, control and test formulations were applied, and the tissues were incubated under humidified conditions (>70%) for 24 hours. Moisture-related gene expression and protein levels were analyzed using quantitative real-time PCR (qRT-PCR) and histopathological analysis, respectively.

Moisture Recovery & Barrier Recovery : mRNA Gene Expression Analysis

Harvested skin explants were treated with Trizol, and RNA was extracted using the Total RNA extraction reagent (Takara Bio, Japan) following the manufacturer's protocol. cDNA synthesis was performed using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA). qRT-PCR was conducted using TB Green® Premix Ex Taq™ II (Takara Bio, Japan) and QuantStudio™ 3 Real-Time PCR (Thermo Fisher Scientific, USA) was performed in duplicate. The PCR conditions were as follows: an initial hold at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds, primer annealing at 60°C for 1 minute, and DNA extension at 72°C for 20 seconds. Relative mRNA gene expression levels were determined using the 2-ΔΔCt method.

Moisture Recovery : Histopathological Assessment

"In this study, fixed tissues were used to prepare paraffin blocks, which were then sectioned into 3 μm thick slices to create tissue slides. The slides were subsequently hydrated and stained with Hematoxylin and Eosin solutions. For Hyaluronic acid staining test, fixed tissues were used to prepare paraffin blocks, which were then sectioned into 3 μm thick slices to create tissue slides. The slides were subsequently hydrated and stained with Hyaluronic acid antibody.

Lipidomics Analysis

The levels of ceramides and free fatty acids in the tissues were analyzed using the LC-MS/MS method (Menéndez-Pedriza et al., 2022). Data were obtained by printing significant lipid changes between single and combined exposures and fractionating heatmaps of changes in the identified lipids. Changes in the identified lipids were also calculated relative to the concentrations in the control sample. The contents of individual lipids were measured based on the number of carbons in the lipid carbon chain.

Statistical Analysis

All data were analyzed for statistical significance using SPSS Package Program version 29 (IBM, USA). A paired t-test was performed for comparisons between control and test groups, with statistical significance set at $p < 0.05$.

3. Results

In this study, we compared the effects of two formulations: Formula A, composed of ceramide, cholesterol, and free fatty acids in a specific molar ratio, which is known to strengthen the skin barrier upon topical application; and Formula B, which contains key components of skin surface lipids (SSL) that closely mimicking the composition of the human skin barrier.

In an *ex vivo* human skin model subjected to induced moisture loss, the mRNA expression levels of the moisturizing factors HAS2 and HAS3 were assessed. As shown in Figure 2, the test group exhibited a significant increase in HAS2 (162%) and HAS3 (443%) mRNA expression compared to the negative control, with superior efficacy compared to Formula A. Additionally, protein expression analysis of hyaluronic acid in the same tissue demonstrated an 132% increase relative to the negative control (Figure 2). In an *ex vivo* human skin barrier damage model, Formula B significantly enhanced FLG mRNA expression by 84.31% compared to the negative control (Figure 3). Furthermore, as illustrated in Figure 3, epidermal barrier thickness increased by 106% in the Formula B-treated group.

Lipidomics analysis was performed on tissues treated under the same conditions. The results indicated a significant increase in the total content of ceramide NP, NS, and NDS in the test group (Figures 4, 5, and 6), along with an increase in total free fatty acids contents (Figure 7). The rate of change varied according to carbon chain length (Figures 8 and 9). While no significant changes were observed in the proportional distribution of ceramides and free fatty acids based on carbon chain length, a significant increase was observed in the total content of ceramides and free fatty acids based on the C16 carbon chain. Specifically, Formula B resulted in a 37.2% increase in total ceramide content and a 49.6% increase in total free fatty acids content compared to the negative control.

This analysis revealed that Formula B significantly enhances key dermatological factors, demonstrating remarkable improvements in skin hydration, moisture retention, and epidermal barrier function. The research comprehensively demonstrated Formula B is superior performance compared to Formula A.

Furthermore, through lipidomics analysis conducted on ex-vivo skin explants that depleted of lipids via tape stripping, we observed that Formula B substantially replenish the total ceramide (NP, NS, and NDS) and free fatty acids content, markedly outperforming Formula A in restoring and reinforcing the skin's lipid profile.
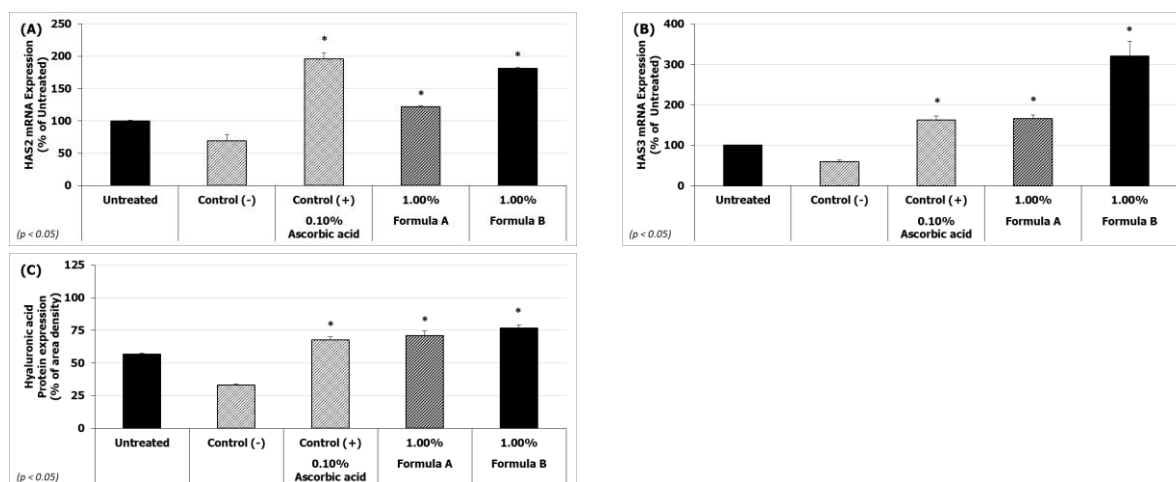
Figure 2. The moisturizing recovery ability of *ex vivo* human skin explants subjected to moisture loss was evaluated with respect to the moisturizing recovery factors, HAS2 (A) and HAS3 (B), after treatment with Formula B. The mRNA expression levels of HAS2 were significantly increased by 77.30% and 162.37% in the control and test groups, respectively, compared to the negative control group ($p<0.05$). Similarly, the mRNA expression levels of HAS3 increased by 180.28% and 443.00% in the control and test groups, respectively, compared to the negative control group ($p<0.05$). Furthermore, fluorescence microscopy revealed that the expression of hyaluronic acid protein in the dermis layer was significantly increased by 115.12% and 132.86% in the control (Formula A) and test (Formula B) groups, respectively, compared to the negative control group ($p<0.05$) (C).
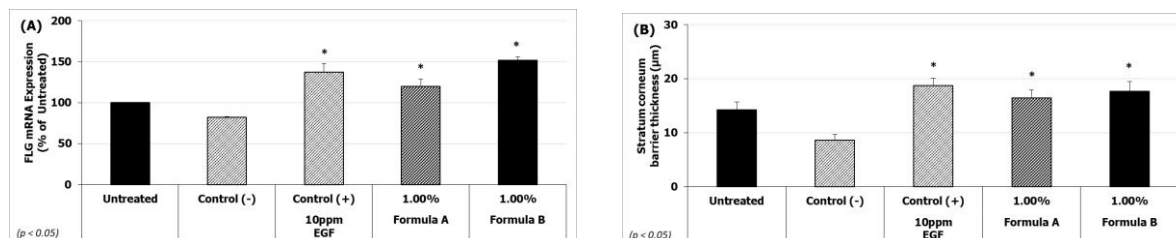

Figure 3. The barrier recovery ability of *ex vivo* human skin explants subjected to barrier damage was assessed by evaluating the mRNA expression levels of the barrier-related factor FLG after treatment with Formula B (A). The mRNA expression levels of FLG were significantly increased by 45.23% and 84.31% in the control and test groups, respectively, compared to the negative control group ($p<0.05$). Additionally, using the Image J software to observe changes in epidermal barrier thickness of the recovered human-derived skin explant model after barrier damage, we found that the thickness significantly increased by 91.61% and 106.45% in the control (Formula A) and test (Formula B) groups, respectively, compared to the negative control group ($p<0.05$) (B).
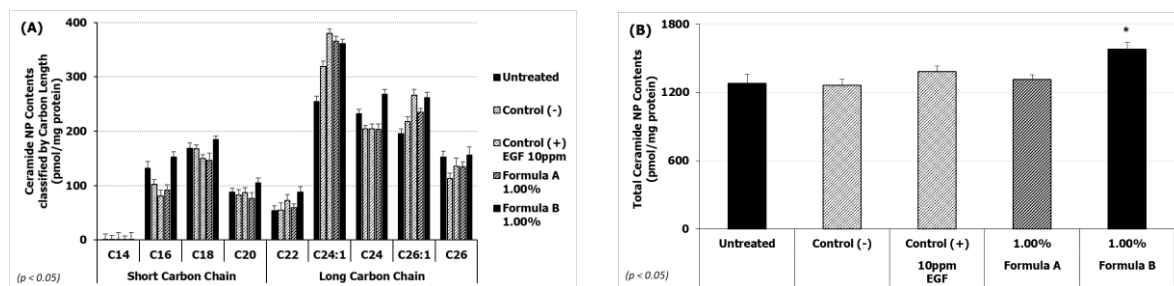
Figure 4. The variations in short carbon chain ceramides, specifically C14, C16, C18, and C20, were observed to increase by 37.78%, 48.83%, 9.90%, and 26.52%, respectively (A). Meanwhile, the changes in long carbon chain ceramides, including C22, C24:1, C24, C26:1, and C26, demonstrated increases of 59.76%, 13.20%, 31.28%, 19.91%, and 38.40%, respectively. Total content and classification of ceramide NP based on carbon chain length: The evaluation of ceramide NP content revealed a significant increase of 24.91% in the total ceramide NP levels in the test group compared to the negative control group (B) ($p < 0.05$).
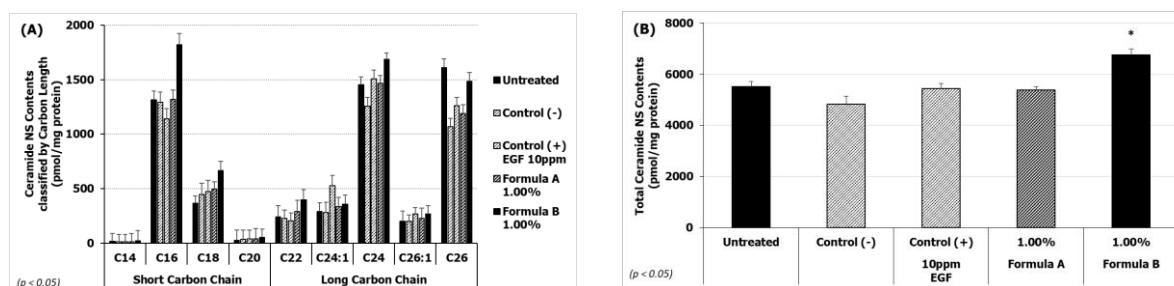

Figure 5. The variations in short carbon chain ceramides, specifically C14, C16, C18, and C20, were observed to increase by 70.22%, 40.93%, 48.77%, and 62.19%, respectively (A). Meanwhile, the changes in long carbon chain ceramides, including C22, C24:1, C24, C26:1, and C26, demonstrated increases of 74.00%, 26.98%, 34.52%, 33.02%, and 38.90%, respectively. Total content and classification of ceramide NS based on carbon chain length: The evaluation of ceramide NS content revealed a significant increase of 40.18% in the total ceramide NS levels in the test group compared to the negative control group ($p < 0.05$) (B).
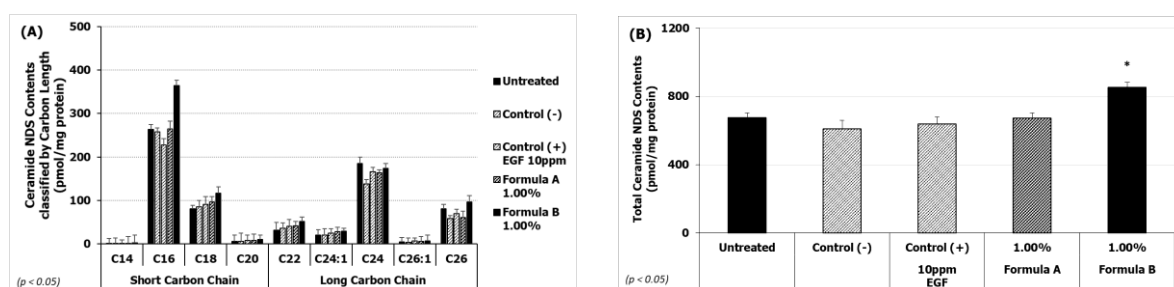

Figure 6. The variations in short carbon chain ceramides, specifically C14, C16, C18, and C20, were observed to increase by 39.67%, 40.93%, 36.89%, and 67.59%, respectively. Meanwhile, the changes in long carbon chain ceramides, including C22, C24:1, C24, C26:1, and C26, demonstrated increases of 42.76%, 42.74%, 26.27%, 66.63%, and 64.78%, respectively (A). Total content and classification of ceramide NDS based on carbon chain length: The evaluation of ceramide NDS content revealed a significant increase of 39.96% in the total ceramide NDS levels in the test group compared to the negative control group ($p < 0.05$) (B).
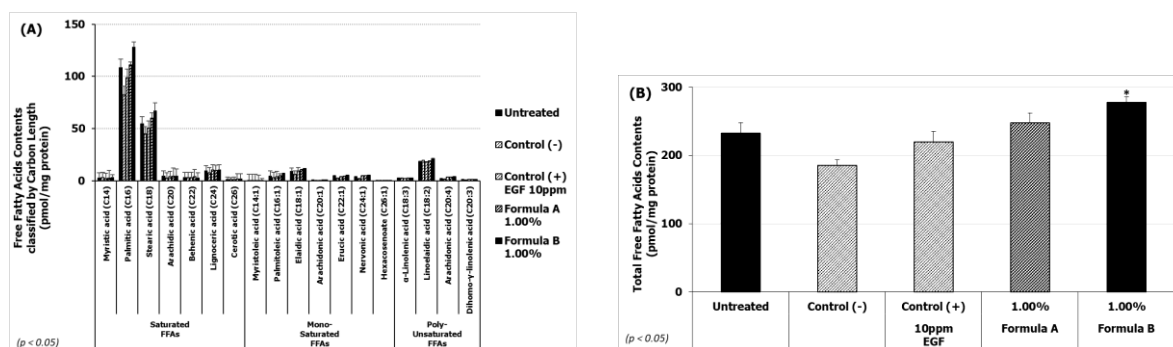
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Figure 7. The variations in shorter-chain saturated free fatty acids, specifically C16 and C18, increased by 55.48% and 48.03%, respectively. In contrast, the long-chain free fatty acids C24 and C24:1 demonstrated increases of 32.12% and 142.14%, respectively (A). Total content and classification of free fatty acids based on carbon chain length: The evaluation of free fatty acids content revealed a significant increase of 49.64% in the total levels of free fatty acids in the test group compared to the negative control group ($p < 0.05$) (B).
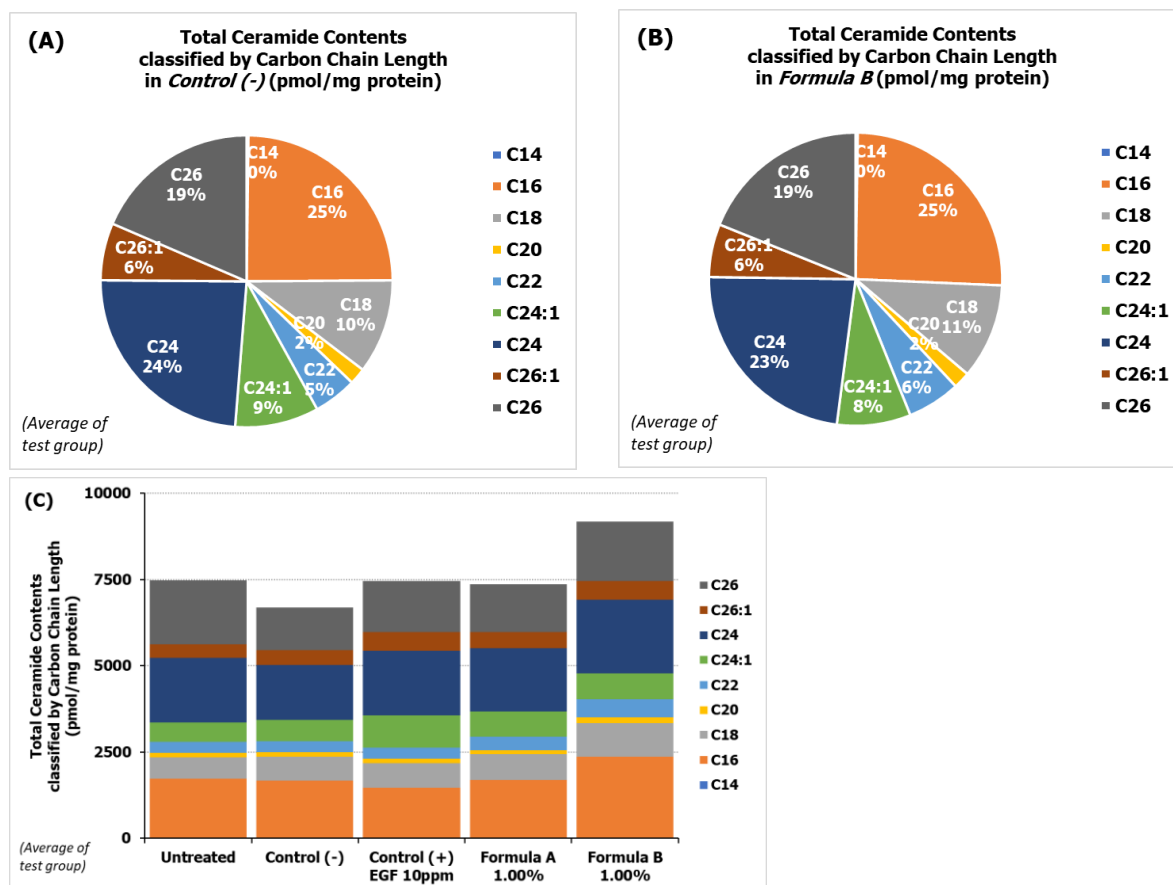

Figure 8. Classification and variations of ceramides based on carbon chain length: The changes in the proportions of ceramides according to carbon chain lengths between the negative control group (A) and formula B (B) were not significantly pronounced. However, there was an increase in the total levels of ceramides, with a notably characteristic increase observed specifically in C16 (C).
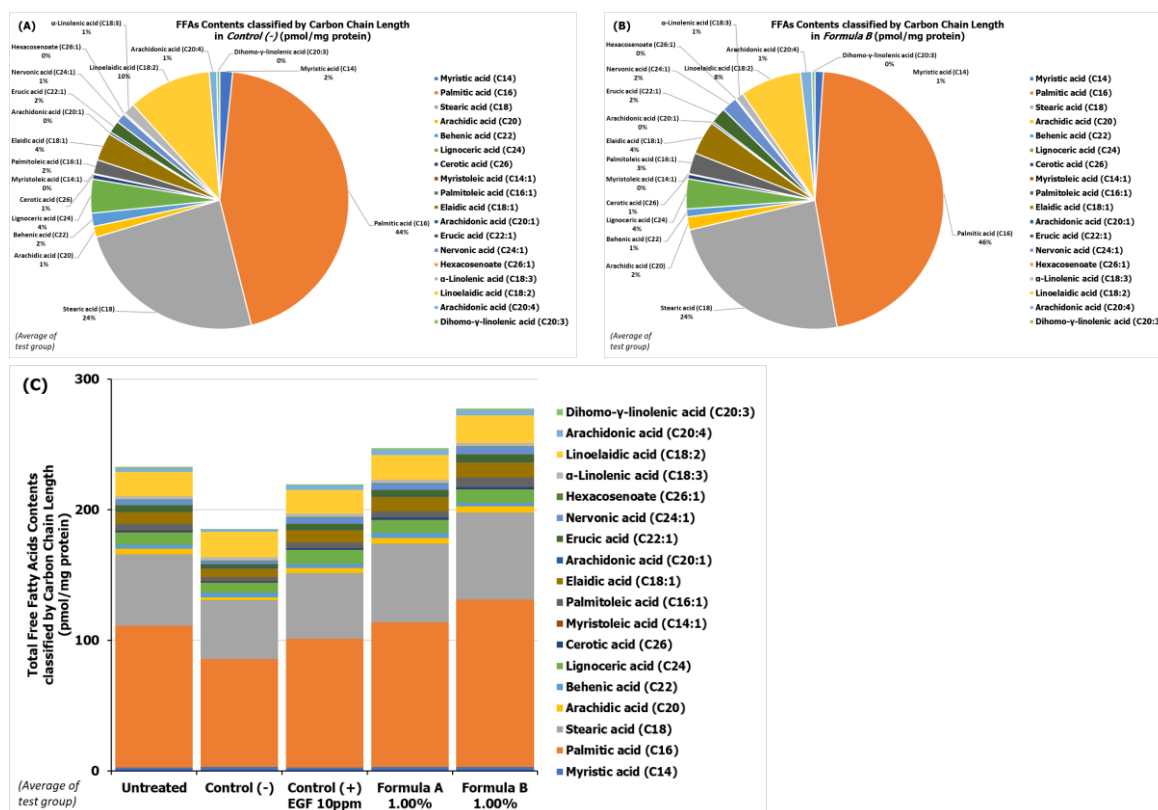
Figure 9. Classification and variations of free fatty acids based on carbon chain length: The changes in the proportions of free fatty acids according to carbon chain lengths between the negative control group (A) and formula B (B) were not significantly pronounced. However, there was an increase in the total levels of free fatty acids, with a notably characteristic increase observed specifically in C16 (C).

4. Discussion & Conclusion

The objectives of this study were as follows: To analyze the moisturizing and barrier-strengthening effects of Formula B for strengthening the skin barrier from various angles in *ex vivo* skin explants, and to investigate whether the changes in lipid composition of *ex vivo* skin explants caused by the use of Formula B for strengthening the skin barrier are confirmed and their significance is confirmed.

The efficacy of Formula B was compared with that of Formula A, a previously developed formula known for its barrier-strengthening properties. To assess the moisturizing recovery efficacy under dry conditions, we analyzed the expression levels of the moisturizing factors HAS2 and HAS3 mRNA, as well as hyaluronic acid protein levels, in *ex vivo* skin explants following moisture loss and recovery. Furthermore, in an *ex vivo* model of barrier damage, we examined the expression of the barrier-associated gene FLG, along with changes in epidermal barrier thickness, to evaluate the barrier-enhancing effects of the formulation.

It is well established that non-hydroxy free fatty acids-based ceramides, particularly N-type ceramides, are the most abundant in the stratum corneum and play a crucial role in preventing epidermal water loss and protecting against environmental stressors (Shin, Jung-Hoon et al., 2014). Ceramides NP, NS, and NDS are major components of the skin barrier lipids and are considered the most representative ceramide classes. Among them, ceramide NP has been most clearly linked to skin disorders and is directly involved in skin hydration and barrier function. However, as each ceramide class has distinct biological functions and contributes differently to skin barrier maintenance, their collective and interactive roles remain incompletely understood. Previous research has identified ceramides, cholesterol, and free fatty acids as key lipid components that influence metabolic pathways crucial for skin barrier health. These lipids are particularly important in inflammatory skin conditions, affecting lipid composition, membrane permeability, and overall barrier function (Bouwstra, Joke A., et al., 2023).

In this study, lipidomics analysis was used to compare the distribution of carbon chain lengths in the negative control group and the Formula B-treated group. Previous reports indicate that a higher ratio of long-chain to short-chain free fatty acids is associated with healthier skin, with an increased proportion of C22 and longer chain lengths being characteristic of intact skin barrier function (Yokose, Urara, et al., 2020). However, in the present study, the long-to-short chain ratio did not show an increase in the Formula B-treated group. This may be due to the direct supplementation of shorter-chain free fatty acids (such as palmitic acid, C16), leading to a temporary increase in shorter carbon chains. Nevertheless, a modest increase in long-chain ceramides and free fatty acids was observed, suggesting that shorter-chain precursors may serve as substrates for de novo synthesis or metabolic elongation within the skin (Murphy, Barry, et al., 2022).

This study has certain limitations. It was conducted using an acute *ex vivo* skin model under controlled barrier disruption conditions (such as tape stripping), rather than on human skin with chronic atopic conditions or through long-term topical application. Therefore, the results of the lipidomics analysis in the epidermis should be interpreted with consideration of the test's limitations. Further research is needed to establish correlations between chronic human skin conditions and acute *ex vivo* skin tissue responses. Despite these limitations, the study demonstrated that, beyond lipidomics changes, Formula B contributed to the recovery of key moisturizing and barrier-related factors, highlighting its potential in enhancing overall skin barrier function and hydration.

In conclusion, this study provides evidence that Formula B, which mimics key components of the skin barrier, holds promise as a functional cosmetic ingredient. When applied to damaged *ex vivo* skin explants, Formula B upregulated key moisturizing and barrier-related

factors while also increasing the total content of ceramides and free fatty acids in the skin lipid composition. These results suggest that lipidomics analysis of human skin can serve as a predictive tool for inflammatory skin conditions and that topical formulations can aid in restoring skin to a healthy state. Moreover, Formula B offers a lipid-based dermatological skincare solution that can improve skin barrier function, making it a valuable candidate for novel cosmetic applications across a broad range of skincare products.

Conflict of Interest
The authors declare that there is no conflict of interest regarding the publication of this article.

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