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Decoding the role of dermal telocytes in aged-induced skin alterations

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1. Introduction

Aging is a complex biological process that affects all layers of human skin, leading to significant structural and functional changes [1-2]. A key aspect of skin aging is the decline in the functionality and regenerative capacity of epidermal stem cells, which results in reduced skin repair and regeneration [3-[4]. This decline contributes to thinning of the epidermis, increased vulnerability to skin damage, compromised barrier function, and reduced skin hydration [2]. Cytokeratin 15 (CK15) protein playings a crucial role in maintaining basal epidermal cell stemness [5].

Skin aging also negatively impacts the dermal-epidermal junction (DEJ), which connects the epidermal and dermal layers [6]. As aging progresses, the DEJ undergoes structural changes, such as decreased undulating morphology and reduction in essential components like hemidesmosomes and anchoring fibrils. Laminin 5, an essential adhesion protein within the DEJ, is found to be reduced in aged skin [7-8]. These alterations compromise skin's mechanical stability, impairing the balance between cell renewal and proliferation, and exacerbating visible signs of aging [7]. The extracellular matrix (ECM), primarily composed of collagen and elastin synthesized by dermal fibroblasts, provides structural support and essential mechanical properties such as stiffness and elasticity [2]. With age, fibroblast numbers decline, reducing collagen and elastin synthesis [1]. Both intrinsic and extrinsic factors contribute to ECM degradation and disorganization, altering mechanical properties. Research shows that the reticular dermis of aged skin exhibits reduced dispersion of collagen directionality, leading to a more parallel orientation relative to the superficial epidermal axis compared to younger skin [9]. Collagen fiber integrity deteriorates during aging, further compromising the skin's structural and functional properties, resulting in loss of firmness and elasticity, and the formation of fine lines and wrinkles [1-2].

Telocytes, crucial interstitial cells in maintaining skin health, are found in the dermis and associated with connective tissue elements like blood vessels and nerve endings [10]. Characterized by long and thin extensions called telopodes, telocytes facilitate communication with other cells through exosomes, including fibroblasts, immune cells, microvascular endothelial cells, and stem cells [10]-[13]. Then, telocytes are currently being investigated for their potential role in regenerative medicine. Specific to communication with epidermal stem cells, telocytes promote their proliferation and differentiation, essential for skin regeneration [10, 14]. Additionally, telocytes have been suggested to help organize the ECM and maintain tissue homeostasis [15-17]. Impairment of telocytes might lead to disorganization of collagen and elastic fibers, contributing to aging-related changes in the ECM [14, 18-19]. Research indicates that telocytes might inhibit oxidative stress and cellular aging [20], and their reduced numbers in adults may contribute to heart aging [21].

In this study, we developed a comprehensive age-induced skin model by exposing human skin biopsies to extrinsic factors like ultraviolet (UV) irradiation and intrinsic factors such as glycation, significant contributors to skin aging [22-23]. This model helps reproduce the features of aged skin. We characterized the structural and organizational changes in the ECM during the skin aging process, demonstrating ECM density reduction, changes in collagen directionality, and deterioration of collagen fiber integrity in aged skin biopsies. We validated that the aging process can be delayed by treatment with a *Bacillus sp*. ferment extract, an active ingredient with a previously demonstrated capacity of promoting telocyte proliferation *in vitro* and rejuvenating effects *in vivo* [24]. Adittionally, the ferment extract also supports DEJ integrity and the epidermal stem cell niche in aged skin biopsies. Finally, the ferment extract protected ECM features in the age-induced skin model and promoted the number of telocytes in aged skin biopsies, suggesting its anti-aging mechanism of action. This innovative approach offers a promising solution to counteract the skin aging process, while also providing valuable insights into the protective role that telocytes may play in the aging process.

2. Materials and methods

2.1. Bacterial ferment extract obtation

The ferment extract was developed from a marine strain of *Bacillus sp*. isolated from a sponge colony in the Florida Keys, USA. The biotechnological process involved fermenting the bacteria in stirred tank bioreactors, followed by extraction and clarification to remove biomass.

2.2. Culturing aged human skin biopsies

Human skin biopsies obtained from abdominal plastic surgery of a healthy 68-year-old woman were used to evaluate Laminin 5 (a marker for DEJ integrity), CK15 (a marker for epidermal stemness potential), and the number of telocytes. The ferment extract was applied on days 0 (D0), D2, D5, and D7 at a concentration of 250 µg/mL. Control biopsies did not receive any treatment except for the renewal of the culture medium (untreated control). Three skin samples per treatment condition were cultured and analyzed after 8 days.

2.3. Inducing aging conditions in human skin biopsies

Human skin biopsies from abdominal plastic surgery of a healthy 51-year-old woman were used. Aging conditions were induced by exposing skin samples to UV irradiation combined with glycation stress. The skin biopsies were irradiated with 9 J/cm² UVA using a UV simulator (Vibert Lourmat RMX 3W) on D0, D2, D4, D6, and D8. Glycation stress was induced by treating the skin biopsies with 500 µM methylglyoxal (MG, Sigma) on D4, D6, and D8. *Bacillus sp.* ferment extract was applied on D0, D2, D4, D6, and D8 at 250 µg/mL. Two types of control

biopsies were used: " Control" (biopsies neither subjected to aging induction nor treated with the ferment extract) and "aging-induced control" (biopsies subjected to aging-induced conditions without ferment extract treatment). Three skin samples per treatment condition were cultured and analyzed after 10 days of treatment.

2.4. Histological processing

After fixation for 24 hours in buffered formalin, the skin samples were dehydrated using a Leica PEARL dehydration automat and embedded in paraffin using a Leica EG 1160 embedding station. Skin sections were prepared using a Leica RM 2125 Minot-type microtome and mounted on Superfrost® histological glass slides.

2.5. Histological staining, image analysis and statistical analysis

Laminin 5 immunostaining was performed using an anti-laminin 5 antibody (Santa Cruz). CK15 immunostaining was performed using an anti-CK15 antibody (Santa Cruz). CD34/PDGFRα co-immunostaining was performed using an anti-CD34 antibody (Santa Cruz) and an anti-PDGFRα antibody (Thermo Scientific). Nuclei were post-stained with propidium iodide. Elastin and collagen type I immunostaining were performed using an anti-elastin antibody (Novotec) or an anti-collagen type I antibody (Abcam), respectively. Nuclei were counterstained with propidium iodide. Total collagen staining was performed using Red Sirius F3B solution (Picro-Sirius). For each treatment condition, 9 images were analyzed. The percentage of the region of interest covered by specific staining was determined using CellSens software. Statistical analysis was performed using the Student's t-test for independent samples: *p<0.5, **p<0.01, ***p<0.001, ****p<0.0001.

2.7. Collagen directionality analysis

Picro-Sirius red stained images were used to infer the preferred orientation of the collagen network using the "Directionality" method in ImageJ software. The method computes a histogram showing the amount of collagen structures in each direction [25]. The preferred direction represents the center of the Gaussian distribution, and collagen directionality dispersion corresponds to the standard deviation of the preferred directionality, represented by the Gaussian curve width.

2.8. Analysis of collagen fiber integrity by polarization analysis

Collagen fiber integrity was quantified using XPolar® technology (Kmax Innovative System, France) on 51-year-old skin biopsy sections. This approach precisely measures collagen bundle birefringence with sub-micrometric resolution, providing detailed insights into changes in collagen polarization. Polarization changes are represented by a dimensionless number called Kmax [26]. The Kws parameter, directly related to collagen fiber integrity, was calculated for each condition. Higher Kws values correlate with improved collagen fiber integrity.

3. Results

3.1. Laminin 5 and CK15 quantification

The study of Laminin 5, essential for the integrity of the dermo-epidermal junction (DEJ), in skin biopsies from an elderly donor revealed that its levels diminish with aging (Figure 1A), potentially impairing epidermal differentiation and reducing layer cohesion. Treatment with *Bacillus sp.* ferment extract significantly increased Laminin 5 levels by 29.9%, resulting in more pronounced green fluorescence and an undulating morphology of rete ridges, indicating a rejuvenation effect (Figure 1A and B). Additionally, the extract increased CK15 protein levels by 276.0%, enhancing the stemness potential of epidermal basal cells (Figure 1C and D). These results suggest that the ferment extract promotes DEJ integrity and supports the population of epidermal basal stem cells, improving skin condition in aged individuals.

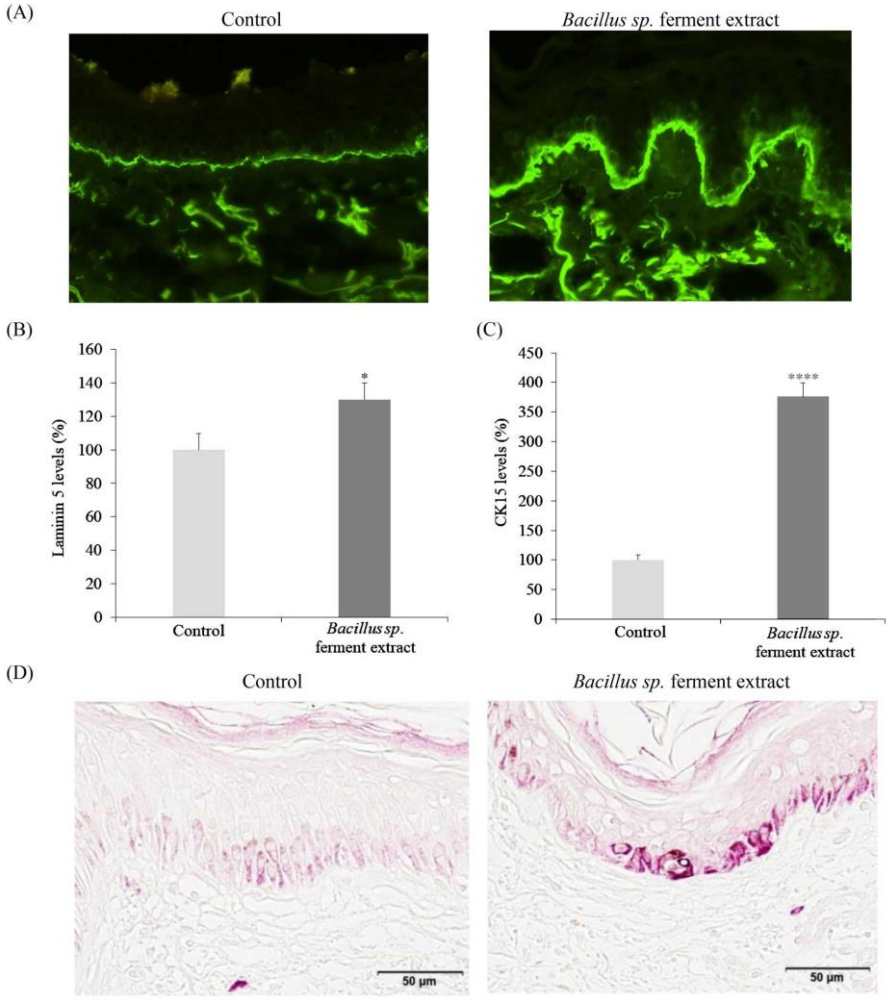

Figure 1. Quantification of Laminin 5 in the DEJ, and CK15 in the basal layer of epidermal stem cells niches in elder human skin explants. (A) Representative images of Laminin 5 (green). (B) Quantification of immunofluorescence signals for Laminin 5 levels (%). (C) Quantification of immunofluorescence signals for CK15 levels (%). (D) Representative images of CK15 (violet).

3.2. Extracellular matrix density evaluation

Human skin biopsies were exposed to aging-induced conditions through UV exposure and glycation stress to study the impact on the extracellular matrix (ECM). Immunostaining for elastin, collagen type I, and total collagen was performed. Aging-induced conditions led to a decrease in elastin (Figure 2A and B), collagen type I (Figure 2C and D), and total collagen levels compared to untreated controls (Figure 3A and B), confirming the model's suitability for evaluating ECM deterioration during skin aging. Treatment with *Bacillus sp.* ferment extract significantly improved elastin levels by 97.1% and collagen type I levels by 33.9%, while preserving total collagen levels, showing a statistically significant increase of 12.0% (Figure 2 and Figure 3A and B). These findings suggest that *Bacillus sp.* ferment extract helps maintain ECM density during skin aging.

3.3. Collagen organization measurement

The study examined collagen directionality dispersion under aging-induced conditions, which showed reduced dispersion leading to parallel collagen fiber orientation and diminished skin mechanical properties (Figure 3C and D). Treatment with *Bacillus sp.* ferment extract

significantly improved collagen directionality dispersion by 25.3% compared to the aging-induced control (Figure 3C and D), indicating that the extract helps protect against collagen network disorganization during skin aging.

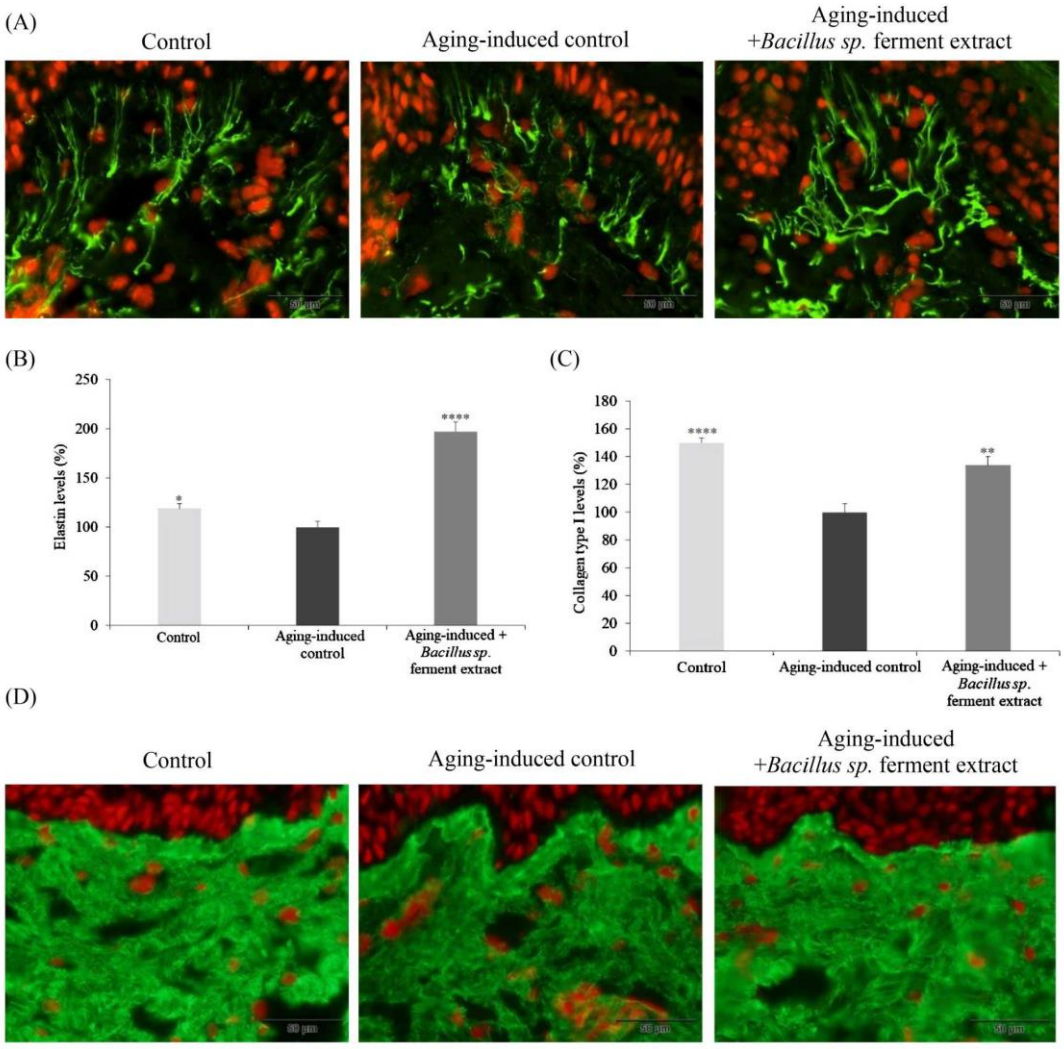

Figure 2. Quantification of elastin and collagen type I levels of human skin explants under aging-induced conditions. (A) Representative images of elastin stained in green and cell nuclei in red. (B) Quantification of immunofluorescence signals for elastin levels (%). (C) Quantification of immunofluorescence signals for collagen type I levels (%). (D) Representative images of collagen type I stained in green and cell nuclei in red.

3.4. Collagen fiber integrity analysis

The study evaluated collagen fiber integrity by measuring collagen bundle birefringence and obtaining X-Polar® images. Aging-induced conditions showed a decrease in collagen fiber integrity (Figure 4A and B). However, treatment with *Bacillus sp.* ferment extract significantly improved collagen fiber integrity by 44.5%, reaching levels similar to the untreated control (Figure 4A and B). These results demonstrate that the ferment extract protects against collagen fiber deterioration during skin aging. Overall, *Bacillus sp.* ferment extract helps maintain elastin and collagen type I levels, preserves ECM organization, and protects collagen fiber integrity during skin aging.

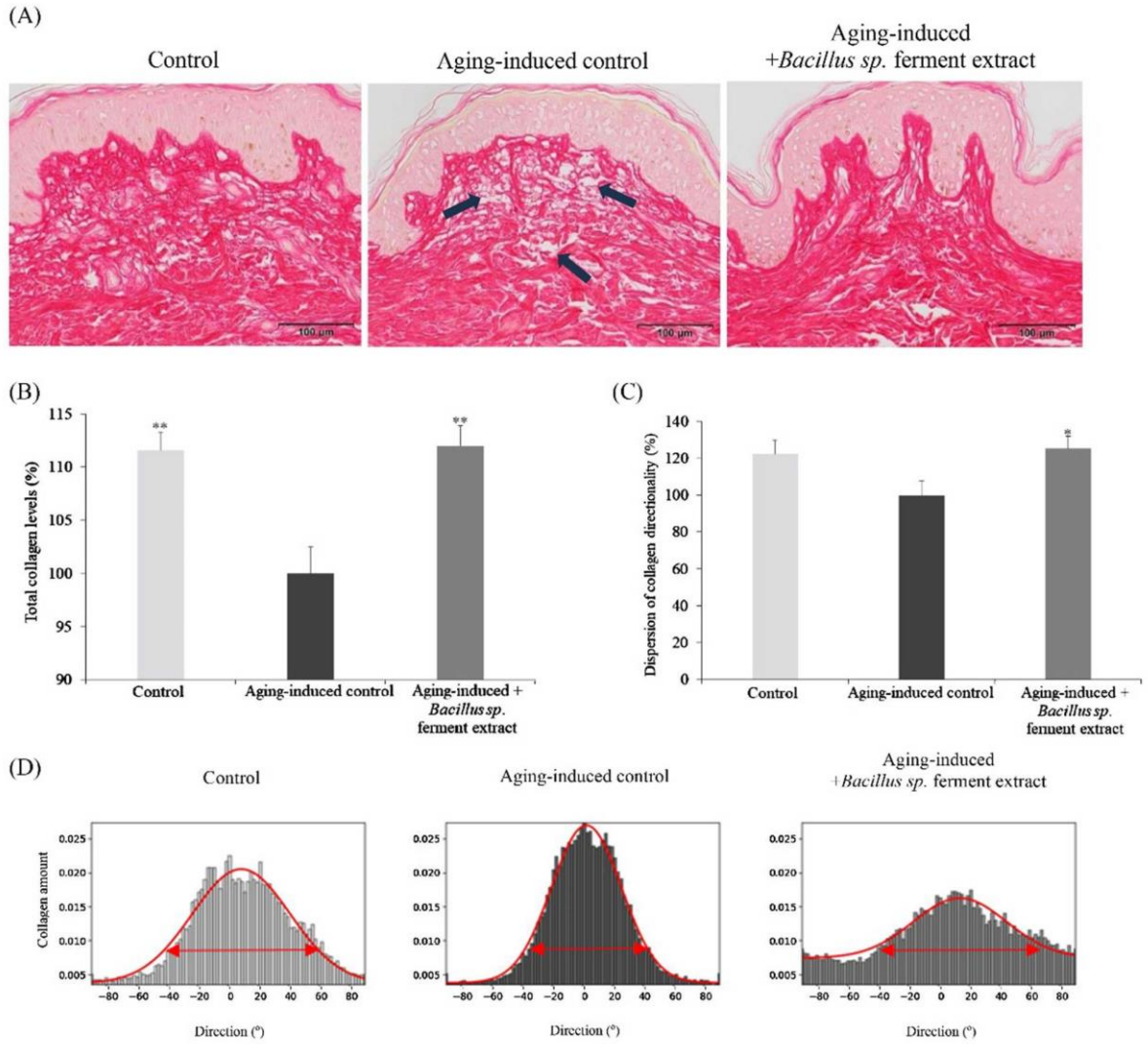

Figure 3. Analysis of total collagen expression in human skin explants in the papillary and upper reticular dermis under aging-induced conditions and evaluation of total collagen directionality in the middle reticular dermis. (A) Representative images of total collagen levels (Picro-Sirius red staining). (B) Quantification of total collagen levels (%). (C) Dispersion of collagen directionality (%). (D) Collagen directional dispersion histogram with Gaussian fitting. The standard deviation of the preferred collagen directionality is indicated by the width of the Gaussian curve (red arrow).

3.5. Determination of telocyte number

Co-immunostaining for CD34 and PDGFRα was performed to detect telocytes in skin biopsies from an elderly donor. Figure 5A and B shows minimal telocyte content under untreated conditions, with few cells observed along the dermis, primarily around microvascular vessels. Treatment with *Bacillus sp.* ferment extract significantly increased the number of telocytes in the dermis, particularly in the interstitial space and around microvascular vessels (Figure 5A) Quantitative analysis showed a 73.5% increase in the signal compared to the control, indicating that the ferment extract can enhance telocyte numbers in aged skin biopsies (Figure 5B).

4. Discussion

Research into skin aging is ongoing, aiming to understand these changes comprehensively. Although many anti-aging compounds exist, advanced research continues to seek more

effective solutions. *In vitro* models using cultured human skin cells and *ex vivo* models based on human skin biopsies are used to study aging. *Ex vivo* models are more realistic for understanding aging's detrimental effects, and various methods have been developed to mimic aging features by exposing biopsies to factors like UV radiation, environmental pollutants, and glycation [27]. However, replicating the multifaceted aging process accurately *in vitro* is challenging due to genetic, environmental, lifestyle, hormonal, and immune system influences. This study presents a protocol for inducing aging in human skin biopsies using UV exposure and glycation stress, targeting both extrinsic and intrinsic aging factors.

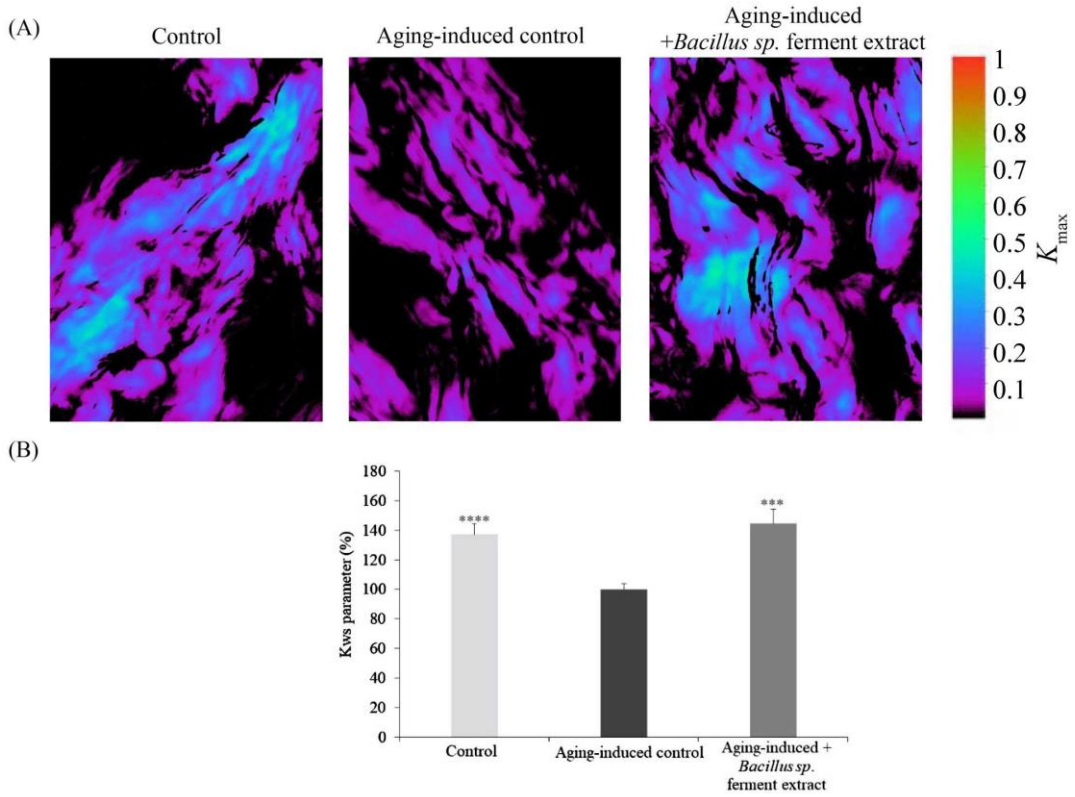

Figure 4. Collagen fiber integrity in human skin explants under aging-induced conditions on the middle reticular dermis. (A) Representative X-Polar® images. Color scale based on the Kmax value, with darker purple color indicating lower collagen fiber integrity, and vice versa. (B) Kws parameter (%).

Our study demonstrated a reduction in elastin, collagen type I, and total collagen density in the dermal layer under aging-induced conditions. Moreover, a *Bacillus sp.* ferment extract with previously demonstrated efficacy in promoting telocyte proliferation [24] protects against ECM loss during aging, preserving ECM density in older skin. We also assessed the disorganization and reduced integrity of collagen fibers with aging, confirming diminished collagen directionality and integrity. Treatment with the ferment extract helped protect against these changes. Thus, the *Bacillus sp.* ferment extract can preserve ECM organization and collagen integrity during skin aging. Furthermore, our research highlighted reduced epidermal stemness potential and DEJ integrity in older skin samples. Using older human skin biopsies, we found reduced levels of Laminin 5 and CK15 proteins. Treatment with the *Bacillus sp.* ferment extract increased Laminin 5 and CK15 levels, confirming its anti-aging potential.

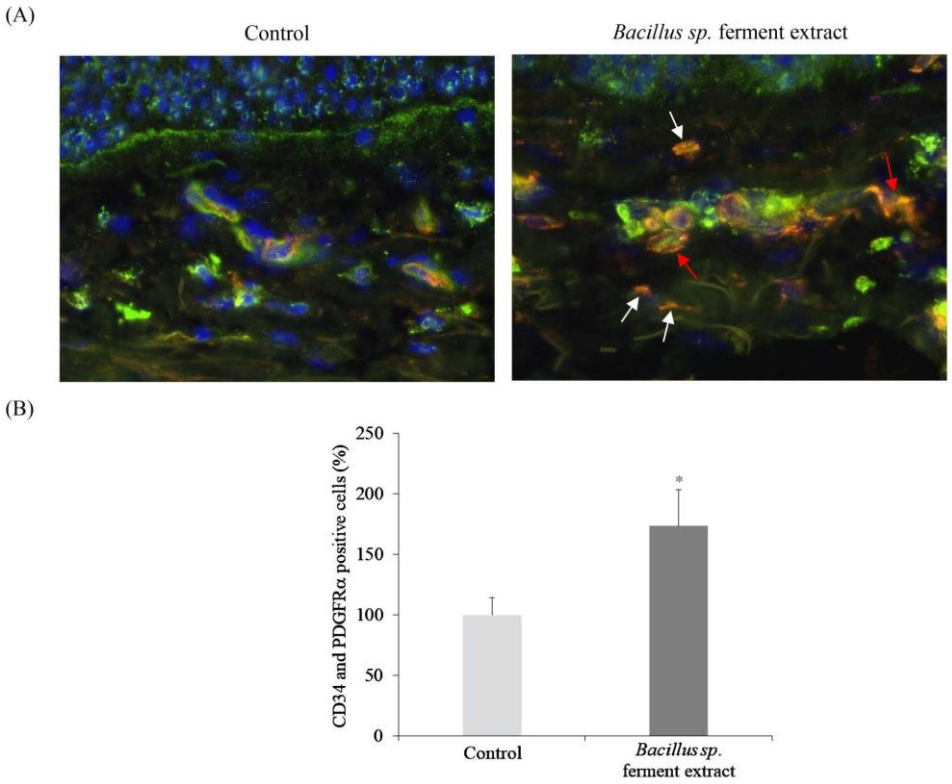

Figure 5. Quantification of telocytes in elder human skin explants. (A) Representative images of CD34/PDGFRα co-immunostaining. CD34 was detected in red fluorescence and PDGFRα in green. The co-immunostaining signal, which identifies telocytes, is visualized in yellow-orange and cell nuclei in blue. White arrows indicate telocytes in the interstitial space and red arrows telocytes around microvascular vessels. (B) Quantification of co-immunofluorescence signals for CD34/PDGFRα (%).

To further understand the anti-aging efficacy of the *Bacillus sp.* ferment extract, we investigated the role of skin telocytes. Previous studies suggest telocytes are involved in dermal communication, maintenance of epidermal stem cells, ECM homeostasis, and may protect against tissue aging [10-21]. Our study confirmed a reduced number of telocytes in aged skin tissues, especially around superficial microvessels. We demonstrated that treatment with the *Bacillus sp.* ferment extract increased the number of telocytes in aged skin biopsies. These results suggest that the anti-aging benefits of the *Bacillus sp.* ferment extract may be due to its positive effects on skin telocytes. The study presented offers new insights into regulating telocyte content in aged skin and suggests their modulation as a potential strategy to mitigate the loss of skin functionality with aging. Nevertheless, further research is required to clarify the direct relationship between reduced telocyte numbers and detrimental skin aging changes.

5. Conclusion

Overall, our study details the creation of a comprehensive age-induced skin model using human skin biopsies, highlighting the harmful changes in ECM density, organization, and integrity associated with aging. Additionally, we propose that modulating skin telocytes may be a potential mechanism underlying the anti-aging efficacy provided by the previously developed *Bacillus sp.* ferment extract [24].

6. References

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