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ORIGINAL RESEARCH

Influence of Injectable Hyaluronic Gel System on Skin Microbiota, Skin Defense Mechanisms and Integrity (Ex vivo Study)

Aleksei Y Prokopov[1,2], Zarema I Gazitaeva[1], Anna N Sidorina[1], Laurent Peno-Mazzarino[3], Nikita Radionov[3], Anna O Drobintseva[4], Igor M Kvetnoy[5]

[1]Medical Bioengineering Systems LLC, Moscow, Russia; [2]Laboratoires Fijie SAS, Paris, France; [3]Eurofins BIO-EC, Longjumeau, France; [4]Department of Histology and Embryology Named After A.G. Knorre, FSBEI HE «Saint Petersburg State Pediatric Medical University» MoH Russia, St Petersburg, Russia; [5]St Petersburg State Research Institute of Phthisiopulmonology, Ministry of Healthcare of the Russian Federation, St Petersburg, Russia

Correspondence: Aleksei Y Prokopov, Laboratoires Fijie SAS91 rue de Monceau, Paris, 75008, France, Email a.prokopov@ros-chimia.ru

Objective: The influence of injectable hyaluronic gels on skin's microbiota is unclear. As well, skin microbiota is a key factor modulating final effect of injectable gels. The ex-vivo study was aimed at alterations following hyaluronic acid injection into the dermis in non-sterile skin surface conditions.

Methods: Ex vivo human skin explants in the presence or absence of either *S. epidermidis or S. aureus*, were treated with either control excipient (0.9% sodium chloride) or test product (Hyaluronic acid injectable S, HA-S). Bacterial analysis was performed, as well as skin structural integrity. Histological imaging and immunostaining analysis in the presence of skin markers: epidermal (CD1a, Toll-like receptor 2 (TLR2), Beta-defensin-3 (BD3), CCN1) and dermal (DC-SIGN, Decorin) were then performed.

Results: The injection of control excipient E and test product P, both associated with bacterial deposits, induced similar noticeable increase of *S. epidermidis* growth over 4 days, but no noticeable effect on growth of S. aureus. The injection of control excipient, associated with bacterial deposits, showed epidermal and dermal alterations increased with time. It was observed significant increase of epidermal CD1a, TLR2, CCN1 and dermal DC-SIGN, Decorin on Day 2. The injection of test product, associated with bacterial deposits, in contrast to injection of control excipient, associated with bacterial deposits, induced very slight but significant improvement of epidermal viability as well as significant decrease of epidermal TLR2, BD3, CCN1 and dermal DC-SIGN on Day 2.

Conclusion: Our investigation showed that both intradermal injections, HA-based solution or control excipient, trigger short-term skin microbiota growth. We indicate strong influence of non-sterile skin surface conditions on human skin explant viability when skin barrier damaged by injection puncture and highlights differences of epidermal/dermal response depended on injected composition.

Keywords: hyaluronic acid injectable, skin microbiome, S. epidermidis biofilm, S. aureus, human skin explant, inflammatory markers

Introduction

Among its many functions, the human skin acts primarily as a natural barrier between the body and the environment, and is colonized by an abundance of differing microorganisms. The epidermis plays a key role in the prevention of pathogenic environmental infection, microbial proliferation and its regulation, and the initiation of the cytokine-mediated inflammatory response.[1] In recent years, the field of dermatology has witnessed a surge in the use of hyaluronic acid (HA) injectable gels for cosmetic and therapeutic purposes.[2] These gels, renowned for their ability to restore volume and hydration to the skin,[3–6] skin morphology[7] have not only revolutionised the anti-aging industry but have also sparked interest in their potential influence on skin microbiota[8–10] and, vice versa, how skin microbiota can interfere effectiveness of injectable gels.[11,12] Moreover, our previous investigations have shown that HA injectables influence skin signalling molecules, as well as providing photo-protection, protection against pollution, and skin protection of circadian rhythms.[13,14]

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The human skin is home to a diverse community of microorganisms that play a crucial role in maintaining skin health. Its microbiome is a complex ecosystem composed of bacteria, archaea, fungi, viruses, and other microorganisms that inhabit the skin's various layers.[15,16] These microorganisms form a delicate balance, contributing to skin homeostasis, immune defence, and overall health. Any disturbance in this balance can lead to skin disorders, inflammation, and other dermatological issues.[17,18] Interactions between the immune system and skin bacteria are of major importance in the pathophysiology of infection. One of the most common members of the healthy cutaneous microbiome is *Staphylococcus epidermidis (S. epidermidis)*,[19] going beyond being a mutualistic skin resident, to actively engaging in priming the cutaneous immune response, maintaining skin homeostasis, and preventing opportunistic pathogens. Yet, its spectrum of potential pathogenicity, influenced by high strain-level heterogeneity, is now acknowledged, with *S. epidermidis* being a prevalent cause of implant-associated infections and recognized as a canonical opportunistic biofilm former. *Staphylococcus aureus (S. aureus)* is a common skin pathogen and is responsible for the vast majority of bacterial skin infections in humans.[20] The frequency of skin infections caused by *S. aureus* reflects in part the competition between host cutaneous immune defenses and *S. aureus* virulence factors.[21] As part of the skin's innate immune system, cationic antimicrobial peptides (CAMPs) such as beta-defensin and antimicrobials contribute to the host's cutaneous defenses, preventing harmful microorganisms such as *S. aureus* from crossing the epithelial barrier.[22]

Hyaluronic acid is a naturally occurring substance in the human body, primarily found in the skin, connective tissues, and eyes.[23] Injectable gels, composed of cross-linked or non-cross-linked hyaluronic acid or in combination with amino acids,[7,14,23] are widely used in cosmetic procedures to fill wrinkles, restore facial volume, enhance skin hydration, protect circadian rhythm and improve cell stress resistance.[24,25] The gel's ability to attract and retain water makes it an ideal choice for maintaining skin elasticity and suppleness. While the primary purpose of hyaluronic injectable gels is cosmetic, researchers have begun to investigate their potential impact on the skin microbiome.[8,9,11] Several factors contribute to this influence: (a) Physical changes in the skin caused by the injection process itself resulting in disruptions to the skin barrier, creating micro-injuries.[26] These changes may alter the skin's micro-environment, affecting microbial populations; (b) The sterile inflammatory response, whereby, at least in some cases, the injection process may induce mild inflammation.[27] Inflammatory responses can alter the skin's pH, and nutrient availability factors that influence microbial growth and diversity;[28] (c) Skin hydration levels change since hyaluronic acid is renowned for its exceptional hydration properties. By attracting and retaining water, injectable gels can create a more hydrated environment on the skin's surface.[4] This may influence the growth and activity of certain microbial species, as microbes thrive in different moisture conditions; (d) Interaction of the hyaluronic acid gel itself with microbial metabolites would influence the growth and survival of specific microbial species,[29] whereby the metabolic byproducts of both hyaluronic acid and microbes may contribute to a dynamic interplay within the skins ecosystem; and (e) Immune modulation since the skin microbiome plays a crucial role in modulating the immune system – changes in the microbial composition can trigger immune responses.[30] Injectable gels, by influencing the skin's microenvironment, may indirectly impact immune modulation, with potential consequences for the microbiome.[31]

Human microbiota is a major component of integrated system "genome-microbiome-exposome" collectively influencing skin aging.[12] As surface skin conditions never could be sterile and we cannot avoid contamination of injected gel[11] – skin microbiota could disturb the action of HA-based injectable gels.

Employing various skin markers, the study described herein attempts to evaluate the intricate relationship between hyaluronic injectable gels and skin microorganisms, exploring the potential impact on the skin's microbiome and acute epidermal and dermal response after intradermal injection of control solution and tested HA-based gel in non-sterile skin surface conditions. The skin markers are epidermal CD1a, Toll-like receptor 2 (TLR2), Beta-defensin-3 (BD3), Cellular Communication Network Factor 1 (CCN1) and dermal Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN, CD 209), Decorin – all play diverse roles in skin physiology and immunity.

CD1a is a cell surface marker on Langerhans cells involved in antigen presentation, crucial for immune response initiation.[32] Moreover, such cells are key point to control keratinocyte proliferation rate and differentiation.[33] TLR2 is a pattern recognition receptor that senses microbial components, triggering immune responses.[34] Beta-defensin-3, an antimicrobial peptide, defends against pathogens, bolstering the skin's innate immune defenses.[35]

CCN1 expressed at a very low level in quiescent fibroblasts, but is transcriptionally activated within minutes of stimulation by stress-factors.[36] It regulates cell adhesion and tissue repair, impacting skin homeostasis especially in stress-conditions. It is an important paracrine factor of cell-to-cell communication in the epidermal-dermal junction, and is both senescent- and melanogenesis-associated.[37] It involves antibacterial protection mechanisms.[38] DC-SIGN is a cell surface marker on dermal dendritic cells; DC-SIGN is a cell adhesion and pathogen recognition receptor, and recognizes a variety of microorganisms.[39] Decorin, a proteoglycan, contributes to skin structure and modulates inflammation, and has a role in inflammageing.[40]

Together, these markers illustrate the intricate interplay between immune regulation, structural integrity, and defense mechanisms in the skin. CD1a, DC-SIGN and TLR2 are pivotal in recognizing and responding to pathogens, while CCN1, BD3 and Decorin contribute to the skin's structural integrity and antimicrobial defense. This interconnected network highlights the multifaceted nature of skin immunity, skin ageing, and maintenance of skin barrier function. The ex vivo phase of the study replicates the recommended application of the hyaluronic acid-based mesotherapeutic injectable, specifically with injection into the dermis in non-sterile skin surface conditions. In the histological phase, the modulation of biological parameters chosen was examined through both microscopic staining and immunostaining techniques.

Our study's objective was to assess the impact of an injectable dermal filler on the development of *S. epidermidis* biofilm in the presence of *S. aureus*, as well as its effects on the integrity of the epidermis and dermis in living human skin explants when skin barrier was damaged by injection puncture in non-sterile skin surface conditions.

Materials and Methods

Skin Preparation

A total of forty-two human abdominoplasty skin explants (Female donor, 56 years, Fitzpatrick skin phototype II, for more information see Supplementary Information 1), with an average diameter of 12 mm (±1mm), were prepared from surgical residues according to the Declaration of Helsinki and L.1243–4 of the French Public Health Code, and placed in survival BEM culture medium (BIO-EC's Explants Medium) at 37°C in a humid, 5% CO2 atmosphere (for further information, see Supplementary Information 1). Explants were then divided into 5 batches for treatment and analysis. The control batch T0 included only 3 explants for histological evaluation. All other treatment batches were sub-divided into 6 explants, 3 assessed for microbiological analysis and 3 assessed for histological evaluation (Table 1).

Table 1 Assignment of Skin Explant Batches for Treatment and Analysis - Each Treatment Batch Was Sub-Divided Into 6 Explants, 3 Assessed for Microbiological Analysis and 3 Assessed for Histological Evaluation

Batch Assignment	Treatment	Number of Explants	Sampling Day
T0	Tissue control	3 (histological analysis)	0
T	Untreated control	3 (histological analysis) 3 (microbiological analysis) 3 (histological analysis) 3 (microbiological analysis)	2 2 4 4
B1	S. epidermidis	3 (microbiological analysis)	1
EB2	S. epidermidis followed by Excipient (E) followed by S. aureus	3 (histological analysis) 3 (microbiological analysis) 3 (histological analysis) 3 (microbiological analysis)	2 2 4 4
PB2	S. epidermidis followed by Test product (P) followed by S. aureus	3 (histological analysis) 3 (microbiological analysis) 3 (histological analysis) 3 (microbiological analysis)	2 2 4 4

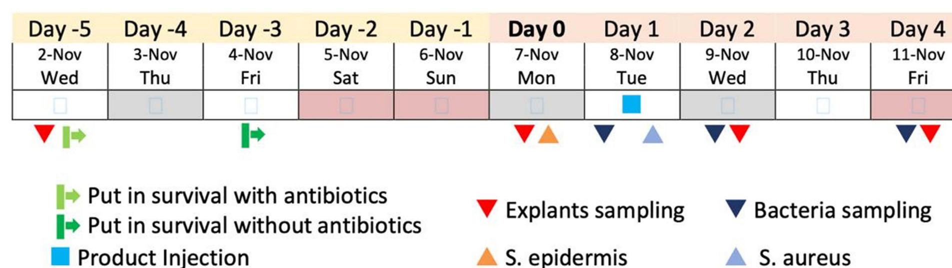

Figure 1 Testing schedule. Between Day −5 and Day −3 (J-3) skin explants were put into survival BEM culture medium with antibiotics. From Day-3 (J-3) explants were then transferred into survival culture medium without antibiotics. Thereafter explants were then assigned to treatment and analysis (See "Test Products & Schedule" section for more detail).

Test Products & Schedule

The test product (P), was an intradermal transparent gel (Hyaluronic acid injectable S (HA-S)) — 1.5% aqueous solution of 1.4 MDa non-cross-linked HA containing below 0.1% mixture of amino acids Arginine, Valine, Threonine, Phenylalanine, Glycine, Glutamic acid, Proline, and Methionine), and stored at room temperature, in darkness throughout the course of the study. Excipient product (E), 0.9% sodium chloride (Pomette) was provided by the test laboratory and stored at room temperature. The study test schedule is highlighted in Figure 1.

Test Procedure

Between Day −5 and Day −3, the explants were put into survival BEM culture medium with antibiotics. From Day-3, the explants were then transferred into 2 mL of survival culture medium without antibiotics.

On Day 0, 10µL of a solution of *Staphylococcus epidermidis* (ATCC 12228),10^8 CFU/mL, was deposited onto each explant of corresponding batches B1, EB2 and PB2, with a final *S. epidermidis* concentration of 10^6 CFU/cm^2.

On Day 1, 50 µL excipient E and 50 µL test product P were injected into the explants dermis — batches EB2 & PB2, respectively. The injections were performed without handling the explant surface in order to preserve the bacterial deposit.

On Day 1, 10µL of a solution of *Staphylococcus aureus* (ATCC 6538), 10^6 CFU/mL was deposited onto each explant of corresponding batches EB2 & PB2, after product injection, with a final *S. aureus* concentration of 10^4 CFU/cm^2.

So, batch B1 was affected by *S. epidermidis* only, batches EB2 and PB2 – by combination of S. epidermidis and *S. aureus* as well as skin damage due to material injection.

The control batch (T) did not receive any treatment except the renewal of the culture medium. The medium was half renewed (1 mL per well) every two-to-three days to fit the schedule.

Bacterial Analysis

On Day 1, adherent bacteria (*S. epidermidis*) from three explants of batch B1 were collected by scraping the explants surface in 500µL of a neutralising solution (Polysorbate 80, 30 g/l + lecithin, 3 g/l). On Day 2 and Day 4, adherent bacteria *(S. epidermidis and S. aureus*) from three explants of batch T, EB2 and PB2 were collected by scraping the explants surface them in presence of the neutralising solution. The collected bacteria were diluted in physiological water (NaCl 0.9%) and seeded onto Chapman Mannitol salt agar (BD 211407–500G) for *S. epidermidis*, and Chromagar (I2A,080103) for *S. aureus*. Bacterial biomass was then evaluated for each batch by colony counting (CFU) of each bacterium strain - *S. epidermidis*: [B1] Day 1; [T, EB2, PB2] Day 2 and Day 4; *S. aureus*: [T, EB2, PB2] Day 2 and Day 4.

On Day 0, three explants from batch T0 were collected. Each sample was cut in two parts and one half was frozen at −80°C. The other half was fixed in formalin solution according to the technical needs of the study. On Day 2 and Day 4, three explants from the batches T, EB2 and PB2 were also collected and similarly processed.

Histological Evaluation

After fixation for 24 hours in buffered formalin, explants were dehydrated and impregnated in paraffin using a Leica PEARL dehydration automat and then embedded using a Leica EG 1160 embedding station. 5µm-thickness sections were cut using a Leica RM 2125 minot-type microtome, and then mounted on Superfrost® histological glass slides. Frozen samples were cut

into 7µm-thickness sections using a Leica CM 3050 cryostat. Sections were then mounted on Superfrost® silanised glass slides. Microscopic observations were recorded using a Leica DMLB, an Olympus BX43 or BX63 microscope. Pictures were digitized with a numeric DP72 or DP74 Olympus camera with CellSens storing software.

i. Tissue integrity – Tissue integrity of both epidermal and dermal compartments was assessed by microscopical observation of formalin-fixed paraffin embedded skin sections after Masson's trichrome staining, Goldner variant.[41] Tissue integrity has been assessed using a score going from a "good" tissue integrity to a "very clearly altered" tissue integrity. Different parameters are taken into account in order to determine this score including the presence of cellular oedema, nuclear alterations, spongiosis and epidermal-dermal junction detachment.

Epidermal markers:

i. CD1a - CD1a immunostaining was performed on FFPE (formalin-fixed-paraffin-embedded) skin sections treated with a monoclonal anti-CD1a antibody (Invitrogen, ref. MA5-12526, clone O10), diluted at 1:100 in Phosphate Buffered Saline-Bovine Serum Albumin (PBS-BSA) 0.3%, and incubated for 1 hour at room temperature, using a Vectastain Kit Vector amplifier system avidin/biotin, and revealed by VIP (Vector laboratories, Ref. SK-4600), a substrate of peroxidase giving a violet staining once oxidized. Immunostaining of viable epidermis was assessed by microscopic observation and cell counting of CD1a-positive cells.
ii. Toll-Like Receptor TLR2 - TLR2 immunostaining was also performed on FFPE skin sections treated with a monoclonal anti-TLR2 antibody (Invitrogen, ref. MA5-32787, clone JM22-41) diluted at 1:50 in PBS-BSA 0.3%-Tween 20 at 0.05% and incubated for 1 hour as described above. Immunostaining of viable epidermis was assessed by microscopic and image analysis—percentage of the surface positive to TLR2.
iii. β-Defensin 3 - FFPE skin sections were immuno-stained for β-Defensin with a polyclonal anti-β-defensin 3 antibody (Abcam, ref. ab19270) diluted at 1:50 in PBS-BSA 0.3%-Tween 20 at 0.05% and incubated overnight at room temperature as described above. Immunostaining of viable epidermis, expressed as a percentage positive to β-Defensin 3 was assessed by microscopic observation and image analysis.

iv. CCN1 (CYR61) - CCN1 (CYR61) immunostaining on FFPE skin sections was performed using a polyclonal anti-CCN1 (CYR61) antibody (Sigma, ref. HPA029853) diluted at 1:50 in PBS-BSA 0.3%-Tween 20 at 0.05% as described above. Immunostaining of viable epidermis was assessed by microscopic observation and image analysis, and expressed as percentage of surface positive to CCN1 immunostaining.

Dermal markers:

i. DC-SIGN (CD 209) - DC-SIGN (CD 209) FFPE skin sections were treated with a monoclonal anti-DC-SIGN (CD 209) antibody (Dendritics, ref. DDX0202P-100, clone 102E11.06) diluted at 1:100 in PBS-BSA 0.3% and incubated overnight at room temperature and stained as described above. Immunostaining of both the papillary and higher reticular dermis was assessed by microscopic observation and image analysis with counting of DC-SIGN (CD 209) positive cells related to the surface of the dermis.

(vii) Decorin - Decorin immunostaining was performed on frozen skin sections with amonoclonal anti-decorin antibody (Santa Cruz, ref. sc-73896, clone 9XX) diluted at 1:200 in PBS-BSA 0.3%-Tween 20 at 0.05% and incubated for 1 hour at room temperature, Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 488 (1:1000, Abcam) were used. Constraining of nucleus with Propidium Iodide (Abcam, ab14083). Immunostaining was assessed by microscopic observation and image analysis, and expressed as percentage of surface positive to decorin immunostaining.

Image Analysis

All image analyses were performed on all the images on each batch, according to CellSens software (Olympus Life sciences) methods. For each batch of skin explants, the percentage of the region of interest covered by the staining (ie, stained surface percentage) was then determined, and the stained surface percentage (Surf%) for each treatment was then compared to the untreated condition ≤ EB2, PB2 vs T and PB2 vs EB2.

Statistics

All data were assessed using the Student's *T*-test, which provides a probability denoted as "p" for determining the significance of differences between two batches. The significance level for this study was established as follows: if $p < 0.05$, this indicated a 95% probability of the two batches being significantly different; and if $p < 0.01$, this indicated a 99% probability of significant difference.

Results

Tissue Integrity

All of the control samples over the 4 days study period exhibited good to fairly good tissue architecture in both the epidermis and dermis. On Day 2, batches that were treated with bacteria and either the saline excipient or test product, exhibited a slight to moderate change in viability of the epidermis only. On Day 4, tissue integrity was altered in both the epidermis and papillary dermis, with the product treated batch (PB2-D4), maintaining better viability as compared to the excipient treated batch (EB2-D4). See the Supplementary Figure 1.

CFU's and Analysis

The number of *S. epidermidis* colonies was significantly increased on Day 2 on the skin batches treated with either the excipient (E) or tested product (P). On the Day 4 further rise was detected for the excipient (E) or tested product (P). The total *S. epidermidis* colonies amount on Day 2 and Day 4 kept extremely high (from log 9 to log 12) for excipient and product versus common conditions on live human skin. In comparison, the number of *S. aureus* colonies no increased from Day 2 to Day 4 for the excipient (E) or tested product (P) (Figure 2).

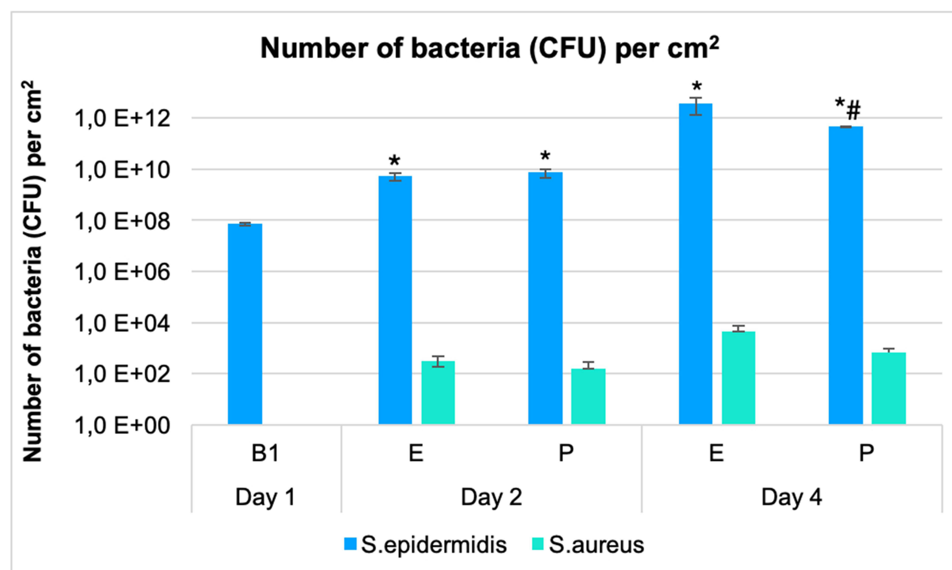

Figure 2 Number of Colony Forming Units (CFU/cm^2) and analysis of *S. epidermidis* and *S. aureus* present on the surface of each skin explant.
Notes: *Significant difference for number of S. epidermidis between Day 1 versus Day 2 and Day 4 (p<0.01). #Significant difference for CFU number of S. epidermidis between Day 2 versus Day 4 in P group (p<0,01).

Histological Evaluation

CD1a Expression

In the control batch (T), the epidermis contained 220 CD1a positive cells per cm of epidermis length by Day 0 (batch T0) which decreased to 125 cells per cm by Day 2 (batch T-D2), showing a significant decrease of 43.2%. As shown in Figure 3 the effect of the products associated with the bacterial deposit on CD1a expression, as compared to the control batch T-D2 (control Day 2) were as follows: The excipient E + bacteria (batch EB2-D2) induced a significant increase of 74%; and the test product P + bacteria (batch PB2-D2) induced a significant increase of 63%. The effect of the product associated with bacteria deposit on CD1a expression (batch PB2-D2), as compared to the excipient (batch EB2-D2) induced a non-significant decrease of 6%.

TLR2 Expression

The percentage of surface positive to TLR2 in the epidermis for the tested batches is shown in Figure 4.

On day 0, in the untreated control (T0), the expression of TLR2 accounted for 41.7% of the epidermal skin surface. By Day 2, in the untreated control (T-D2), there was a significant slight decrease in TLR2 expression to 34.1% of the epidermal skin surface.

Regarding the impact of products associated with bacterial deposit on TLR2 expression in comparison to the control skin batch T-D2: Excipient E + bacteria (batch EB2-D2) induced a significant increase of 44%, while the test product P +

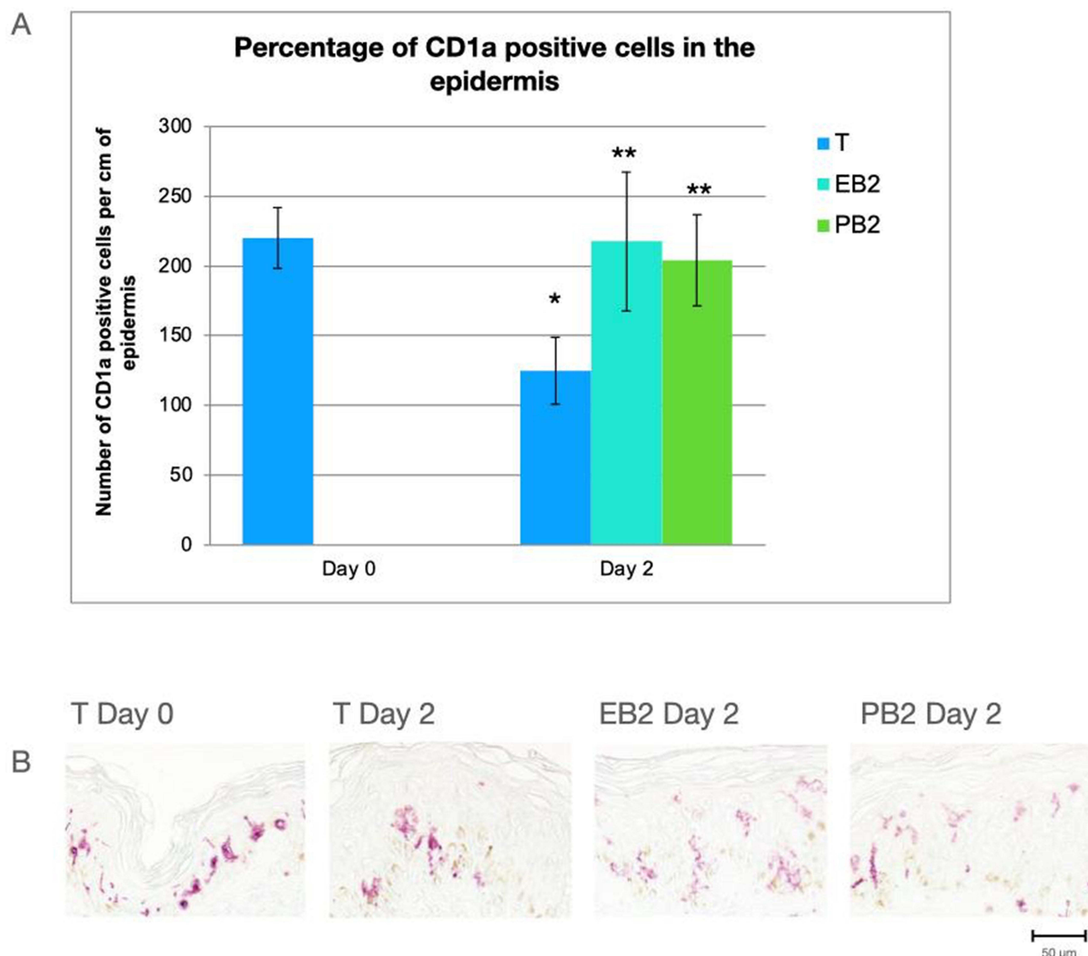

Figure 3 (**A**) CD1a Expression. Number of skin explant positive cells to CD1a immunostaining in the epidermis. (**B**) CD1a expression in human skin explants. Scale bar = 50 µm.
Notes: *Comparison of control (T) at Day 0 versus Day 2 control (p<0.01). **Comparison of control (T) at Day 2 and treatment with excipient (EB2) and test product (PB2) (p<0.01).

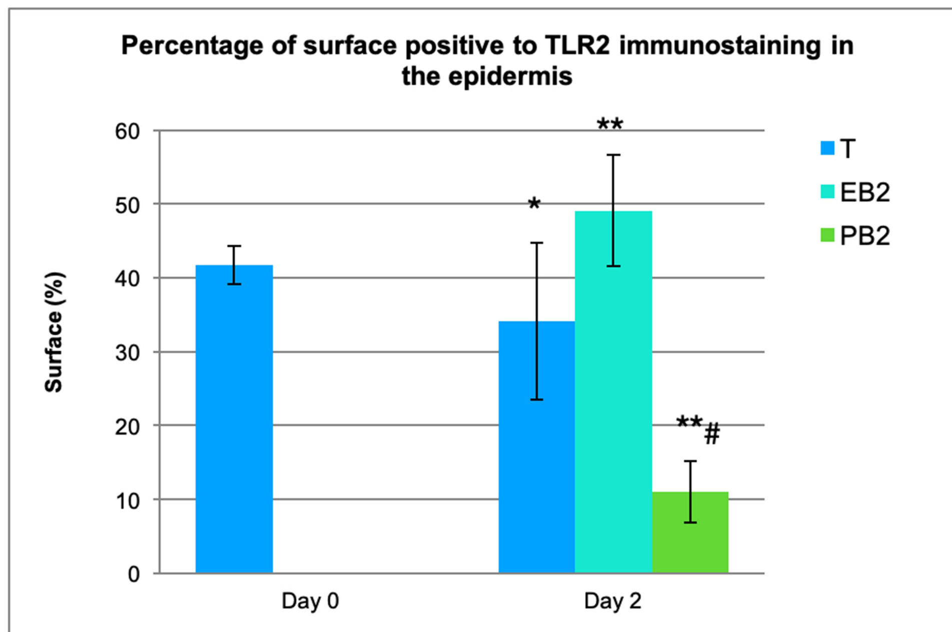

Figure 4 TLR2 Expression. Percentage of skin explant surface positive to TLR2 immunostaining in the epidermis. Comparison of control (T) at Day 0 versus Day 2 control and treatment with excipient (EB2) and/or test product (PB2).
Notes: *Comparison of control (T) at Day 0 versus Day 2 control (p<0.05). **Comparison of control (T) at Day 2 and treatment with excipient (EB2) and test product (PB2) (p<0.01). #Comparison of treatment with excipient (EB2) and test product (PB2) (p<0.01).

bacteria (batch PB2-D2) induced a significant decrease of 68%. Additionally, when comparing the effect of product batch PB2-D2 associated with bacterial deposit to batch EB2-D2, there was a significant decrease of 78%.

β-Defensin 3 Expression

The percentage of the skin surface positive to expression of *β-Defensin 3* in the epidermis, in the tested skin batches is shown in Figure 5.

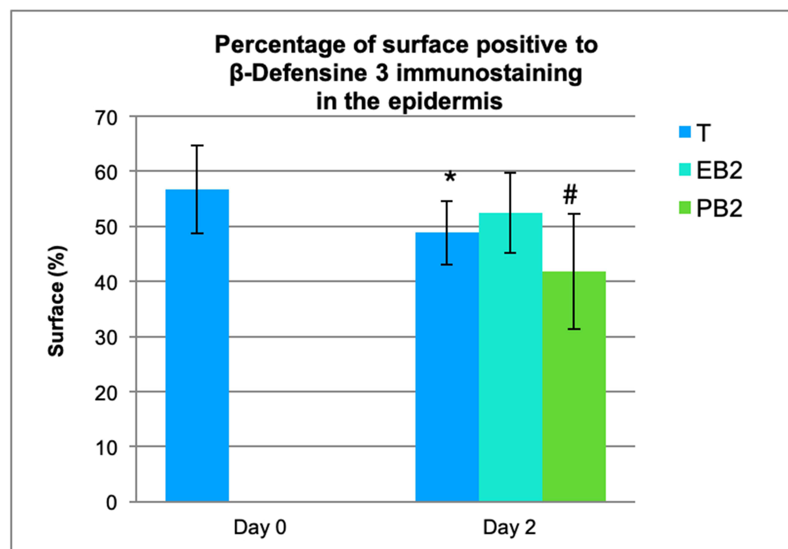

Figure 5 β-Defensin-3 Expression. Percentage of skin explant surface positive to TLR2 immunostaining in the epidermis.
Notes: *Comparison of control (T) at Day 0 versus Day 2 control (p<0.05). #Comparison of treatment with excipient (EB2) and test product (PB2) (p<0.05).

On day 0, the untreated blank skin batch (T0) exhibited a β-defensin 3 expression representing 56.7% of the epidermal skin surface. By Day 2, in the skin batch T-D2, the expression of β-defensin 3 was significantly decrease to 48.8% of the epidermis surface. Comparatively, the impact of products associated with bacterial deposit on β-defensin 3 expression, when compared to the control batch T-D2, can be summarized as follows: Excipient E + bacteria (batch EB2-D2) induced a non-significant increase of 7%, while product P + bacteria (batch PB2-D2) induced a non-significant decrease of 14%. The effect of the product (batch PB2-D2) associated with bacterial deposit on β-defensin 3 expression, as compared to the batch EB2-D2 induced a significant decrease of 20%.

CCN1 Expression

As depicted in Figure 6, the untreated control skin batch (T0) exhibited a weak to moderate expression of CCN1 in the epidermis – 6.5% of the epidermal skin surface. By Day 2 (T-D2), this expression of CCN1 increased significantly (41.5%) to a moderate level of 9.2% of the epidermal skin surface. The effects of products associated with bacterial deposits on CCN1 expression, in comparison to the control batch T-D2, revealed that excipient E + bacteria (batch EB2-D2) induced a slight increase in CCN1 expression, while the test product P + bacteria (batch PB2-D2) led to a slight decrease.

Upon comparing the impact of the test product (PB2) associated with bacterial deposit on CCN1 expression to excipient (EB2), a moderate decrease in CCN1 expression was observed on Day 2. Specifically, excipient E + bacteria (batch EB2-D2) induced a significant increase (22%) in CCN1 expression, whereas the test product P + bacteria (batch PB2-D2) resulted in a significant decrease (37%). Furthermore, when comparing the effect of the test product associated with bacterial deposit on CCN1 expression to the skin batch EB2-D2, batch PB2-D2 induced a significant decrease (49%).

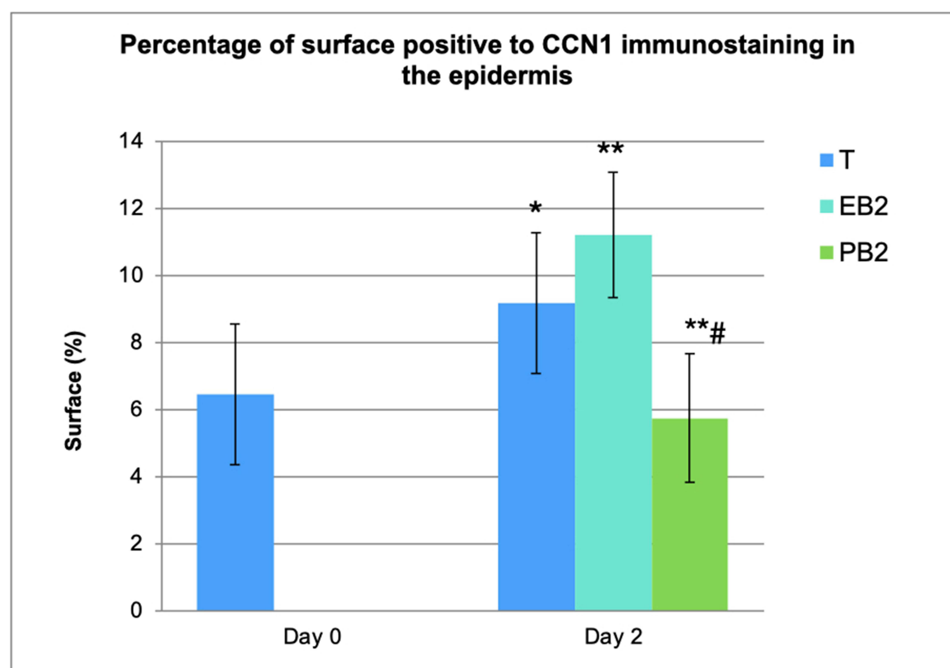

Figure 6 CCN1 Expression. Percentage of skin explant surface positive to CCN1 immunostaining in the epidermis.
Notes: *Comparison of control (T) at Day 0 versus Day 2 control ($p<0.05$). **Comparison of control (T) at Day 2 and treatment with excipient (EB2) and test product (PB2) ($p<0.05$). #Comparison of treatment with excipient (EB2) and test product (PB2) ($p<0.01$).

DC-SIGN Expression

The staining of DC-SIGN in the papillary dermis of the tested skin batches is shown in Figure 7. The number of DC-SIGN positive cells per surface of papillary and higher reticular dermis (in mm^2) for the concerned batches is shown below.

On day 0, the untreated skin batch (T0) contained 127 DC-SIGN positive cells in the papillary and higher reticular dermis. By day 2 (T-D2), the papillary and higher reticular dermis exhibited a non-significant decrease to 102 DC-SIGN positive cells per mm^2. Regarding the impact of products associated with bacterial deposits on DC-SIGN expression compared to batch T-D2: Excipient E + bacteria (batch EB2-D2) induced a significant increase of 38%, while the product P + bacteria (batch PB2-D2) induced a non-significant increase of 10%. Furthermore, when comparing the effect of product P associated with bacterial deposit (batch PB2-D2) to excipient (batch EB2-D2), there was a significant decrease of 21%.

Decorin Expression

The percentage of surface positive to decorin in the papillary dermis for the concerned batches is shown in Figure 8.

On day 0, the control skin batches (T0) exhibited a decorin expression of 60.0% of the papillary dermis surface. By Day 2, in the control skin batches (T-D2), the expression of decorin decreased significantly to 39.4% of the papillary dermis surface. In comparison to the control skin batch T-D2, the tested products, when associated with bacterial deposit, showed the following effects on decorin expression: Excipient E + bacteria (batch EB2-D2) induced a significant increase of 29%, while Test product P + bacteria (batch PB2-D2) induced a significant increase of 18%. Furthermore, when comparing the effect of product (batch PB2-D2) associated with bacterial deposit to the tested batch EB2-D2, it resulted in a non-significant decrease of 8%.

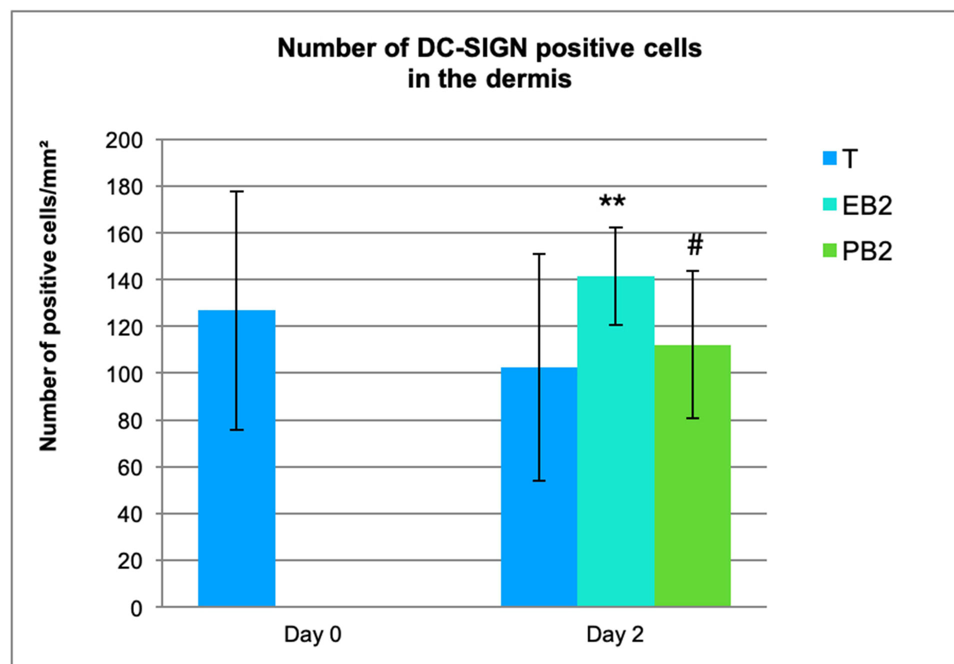

Figure 7 DC-SIGN Expression. Number of skin explant immunopositive DC-SIGN cells in the dermis.
Notes: **Comparison of control (T) at Day 2 and treatment with excipient (EB2) ($p<0.05$). #Comparison of treatment with excipient (EB2) and test product (PB2) ($p<0.05$).

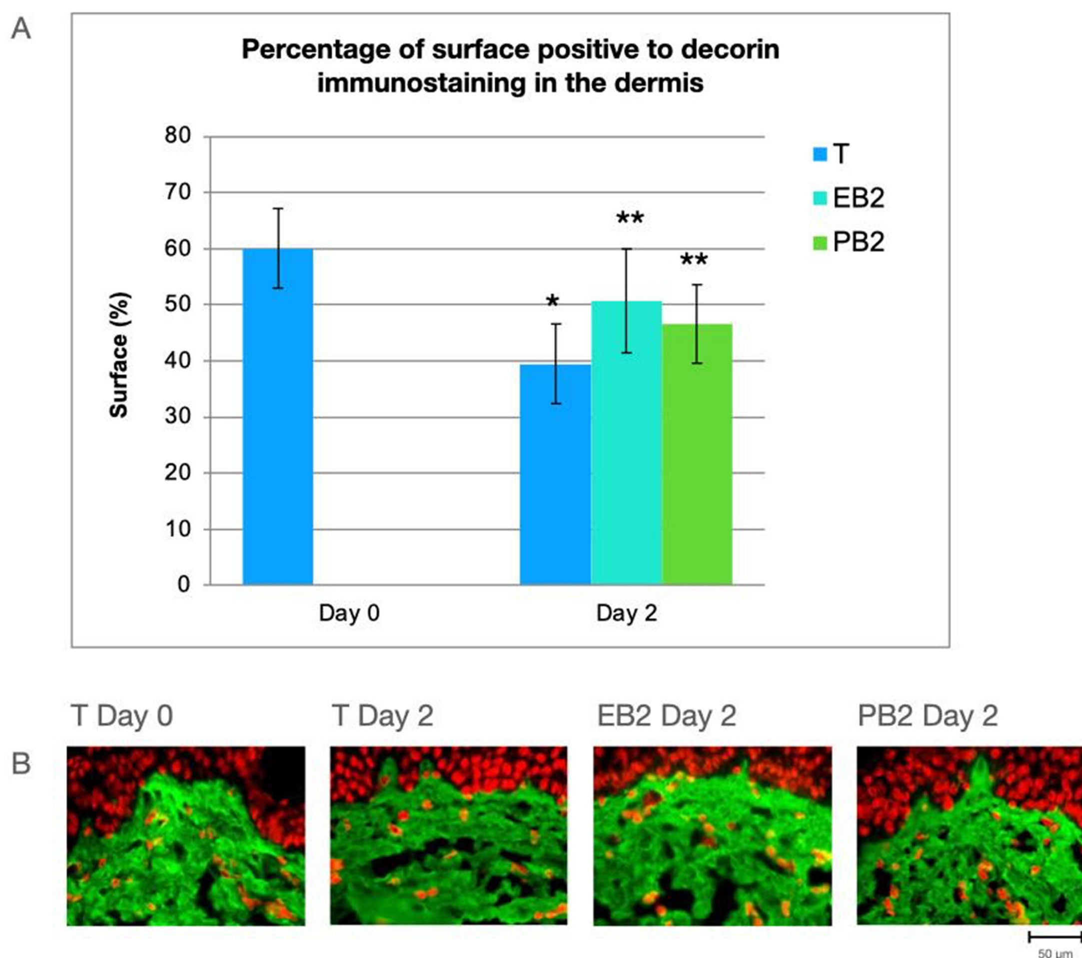

Figure 8 (**A**) Decorin Expression. Percentage of skin explant surface positive to decorin expression via immunostaining, in the papillary dermis. (**B**) Decorin expression in human skin explants. Scale bar = 50 µm. Green fluorescence – decorin, red fluorescence - nuclei.
Notes: *Comparison of control (T) at Day 0 versus Day 2 control (p<0.01). **Comparison of control (T) at Day 2 and treatment with excipient (EB2) and test product (PB2) (p<0.05).

Discussion

Organotypic models, such as skin explants, have the advantage of preserving a 3D structure and including inter-cellular interactions like those between keratinocytes and fibroblasts so it is easier to use compared to in vivo models.[42,43] The limitations of these models include the absence of blood flow and nerve supply, which are present in living organisms, the difficulty in maintaining consistent conditions for the samples, and absence of desquamation and limited time of growing in laboratory (maximum 10 days).[44]

The injection of the excipient E (sodium chloride 0.9%), associated with bacterial deposits induced a noticeable increase of *S. epidermidis* growth over 4 days, but no noticeable effect on the growth of *S. aureus*. Moreover, epidermal and dermal alterations increased and were observed with a significant increase of epidermal CD1a, TLR2, CCN1, and dermal DC-SIGN, Decorin on Day 2. In comparison to the batch treated with excipient E, associated with bacterial deposits, the injection of the test product HA-S associated with bacterial deposits, induced similar noticeable increase of *S. epidermidis* growth over 4 days, but no noticeable effect on the growth of *S. aureus*. There was however a slight but significant improvement of the epidermal integrity, and a significant decrease of epidermal TLR2, BD3, CCN1 and dermal DC-SIGN on day 2. We therefore conclude that the injection of test product P shows noticeable effect on *S. epidermidis* growth, does not show noticeable effect for *S. aureus* growth, yet but improves slightly the epidermal integrity while exhibiting a protective activity against the elicited biological response to bacterial contact.

Additionally, injections administered into the dermis exert an upstream impact on epidermal cells, with the response in the epidermis associated with factors such as mechanical trauma, the composition of the hyaluronic acid-based solution, and the biological actions of dermal cells. We also noted that those skin alterations observed, are probably due to the application of bacteria on the skin surface, since the injection of 0.9% sodium chloride (E), is known to be well tolerated by viable skin explants. At this juncture, whilst the relevancy of the data generated on Day 2 is clear, it is difficult to interpret the results obtained on day 4 due to the clear epidermal and dermal alterations after bacteria deposit. However, this finding is noteworthy in itself, and warrants further investigation. In our investigation, an interesting observation emerged regarding the abundance of Staphylococcus colonies on the skin surface, particularly on the 4th day. This deviates from the typical pattern reported in the literature, where microorganism levels are generally lower on the 4th to 5th days.[45]

We therefore hypothesise that the elevated presence of *Staphylococcus* could potentially trigger microbial stress on the skin. This stress, couples with the mechanical pressure and epidermal barrier damage from injections of either the control solution or HA-S product, might influence the skin's response. In our ex-vivo model, there was a consistent rise in epidermal CCN1, likely attributed to stress related to the preparation and cultivation of the skin pad. Furthermore, the heightened CCN1 levels are correlated with cellular senescence and tissue damage. Administering HA-S led to a decline in CCN1 levels by Day 2, whereas the sodium chloride control (E) injection exacerbated CCN1 accumulation. Probably there is a sign that composition of HA-S works as a stress-protector for epidermal tissue especially in acute post-injection period.

A gradual decline in epidermal CD1a cell levels was observed on the second day, followed by a further reduction on the fourth day. Notably, both HA-S and sodium chloride injections supported the CD1a population in the epidermis in the short-term (Day 2) period. It is positive fact as activity of Langerhans cells is supportive for proper immune response as well as for proper keratinization – especially in acute post-injection period accompanied with mechanical damage of epidermal barrier.

This ex-vivo model also demonstrated a relatively stable number of DC-SIGN cells in the dermis. HA-S product injections supported the DC-SIGN population in the dermis on Day 2. The injection of the physiological solution (control) provided a short-term increase in DC-SIGN levels in the dermis, specifically during the acute period (Day 2). So, both, control solution and test product, provide short-term protective effect for dermal dendrocytes.

There was a similar dynamic for TLR2 and BD3 in the epidermis on Day 2 - a small increase in the case of injection of sodium chloride control (E) versus a small decrease in the case of injection of product HA-S. This suggests a potential decrease in sensitivity to microbiota, possibly indicating a more resistant or anti-inflammatory effect with the use of HA-S product.

Regarding Decorin, it steadily degraded over the course of the study, with a more severe decrease observed on the 4th day as compared to the 2nd day. Injections of sodium chloride control (E) or HA-S product (P) slightly increased amount of Decorin on Day 2, but was not retained by the 4th day. Low Decorin levels may be linked to high TIMP (tissue inhibitor of metalloproteinases), indicating proper collagen fiber assembly and dermal matrix maturation, and this is notable in the context of collagen synthesis stimulation, as low TIMP is associated with scar formation.[46]

Our ex-vivo investigation illustrated that intradermal injections eighter sodium chloride solution or HA-based solution support skin microbiota being provocative for their growth. This study indicates strong influence of non-sterile skin surface conditions on human skin explant viability when skin barrier damaged by injection puncture and highlights differences of epidermal/dermal response depended on injected composition.

Conclusions

Understanding the role of injectable hyaluronic gels in influencing the homeostasis of the skins microbiome has its challenges. Firstly, research is limited. Despite the growing interest in this field, research on the specific impact of hyaluronic injectable gels on the skin microbiome is still in its infancy. Most studies are small-scale and require further validation. Secondly, the skins microbiome is highly individualized, with significant variability among individuals. Factors such as age, genetics, and lifestyle choices can influence microbial diversity, making it challenging to draw universal conclusions. Thirdly, many studies focus on short-term effects, but the long-term consequences of hyaluronic

acid injections on the skin microbiome still remain unclear. Longitudinal studies are therefore essential to understanding the sustained impact over time.

The relationship between hyaluronic injectable gels and the skin microbiome is a complex and evolving area of research. While these gels have proven efficacy in cosmetic procedures, their influence on the skin's microbial communities as well, vice versa, influence of skin microbiota to effectiveness of injectable materials raise intriguing questions.

Our ex-vivo investigation surprisingly was limited with viability of skin explants – combination of skin surface microbiota with skin barrier damage by injection puncture strongly shorten this time till 3–4 days only. Probably there is essential limitation for such design of investigation.

As technology advances and our understanding deepens, further research is crucial to unravel the nuanced interplay between hyaluronic acid injectable gels, skin and skin's microorganisms. By doing so, we can enhance our knowledge of dermatological health, potentially paving the way for more personalized and effective skincare interventions. More studies are expected.

Data Sharing Statement

Data is unavailable due to privacy or ethical restrictions.

Institutional Review Board Statement

Study is performed on human skin tissue obtained from surgical residues of one donor in full respect with the Declaration of Helsinki and the article L.1243-4 of the French Public Health Code. The latter does not require any prior authorization by an ethics committee for sampling and using surgical wastes.

Informed Consent Statement

Plastic surgery clinic provides skin tissue under informed consent signed by a donor. Cooperation between a clinic and Eurofins BIO-EC is covered by a mutually signed agreement of cooperation. Internal ethical committee of a clinic validates protocols and conditions of skin tissue samplings.

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Disclosure

The authors declare no conflicts of interest in this work.

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