A new pentapeptide improving face skin surface damage induced by *Cutibacterium acnes* activity.

Leroux Richard, Ringenbach Caroline, Cico Alba, Silou Camille, Doridot Emmanuel and **Mondon Philippe***.
SEDERMA, 29 rue du Chemin Vert ; 78612 Le Perray-en-Yvelines; France ;
** to whom correspondance should be adressed : 29 rue du Chemin Vert, 78612 Le Perray en Yvelines, France, +33 134 841 010, philippe.mondon@sederma.fr*

Keywords: Microbiote; Peptide; Cutibacterium; Quorum; Blemishes, Extracellular Matrix.

ABSTRACT:
Human body and its microbiota, form a complex supra-organism called holobiont whose disruptions are now thought to be the cause of pathologies. 90 % of teens undergo overproduction of sebum and proliferation of *Cutibacterium acnes* (*C. acnes*). Consecutive clogging of pilosebaceous ducts often causes local irritation leading to the denaturation of neighboring tissues, skin blemishes, comedones, skin redness, and significant psychological damages. While cosmetic products cannot treat inflammatory acne lesions, it is possible to significantly minimize the negative aspects of the latter by acting either upstream or downstream of the problem. A new lipo-pentapeptide Palmitoyl-Lysyl-Threonyl-Seryl-Lysyl-Serine (pKTSKS) controls growth, quorum, adhesion and *C. acnes* biofilm, without killing cells. These features are all involved in the formation of acne lesions. pKTSKS does not act on *Staphylococcus epidermidis*, and in parallel reinforces epidermal barrier and moisture functions, both essential to a healthy skin. It also improves extracellular matrix proteins synthesis such as collagens. The most significant aspects of acne lesions were evaluated in two clinical studies. Redness, inflammatory blemishes, and roughness linked to post-acne scars, were significantly reduced versus placebo. This new peptide enlarges the field of action of skin cosmetic peptides in minimizing herein the negative aspects of rough skin.

INTRODUCTION:
The skin is an ecosystem of approximately 1.8 m² hosting 10 billion of bacteria. Microbial flora, also known as microbiota, is composed of micro-fungi, unicellular organisms, yeasts, viruses, archaea and bacteria; the latter being the majority. It is acquired early after birth and evolves quickly during the first months of life. The microbiome of people from the same family or professional groups for instance are strongly similar. However, microbiota of armpits, a humid and warm zone, notably differs from the one of the drier zones like arms and legs, or of the pilosebaceous zones, that are strongly invaginated, sebum-rich and oxygen-poor [1, 2].

Acne is one of the primary causes of dermatological consultations due to unsightly and psychological detrimental aspects of the scarring lesions it leaves on the skin. More than 90 % of young women and men undergo a strong change in the appearance of their skin linked to an overproduction of sebum and the proliferation of the *Cutibacterium acnes* (*C. acnes*) germ. In addition, 78 % of people claim to have acne breakouts on a regular basis, on cheeks, chin, forehead, nose or on the upper part of the body [3].

The predominant micro-organism of the pilosebaceous follicle is the commensal bacillus *C. acnes*, formerly called *Propionibacterium acnes* and *Corynebacterium acnes* [4, 5, 6]. It lives both in contact of oxygen, at the upper part of the *stratum corneum* and deeper into hair follicles, an environment rich in sebum and proteins and poor in oxygen. Even if several ribotypes are described with or without pro-acne properties, it was shown that this bacterium can adapt its physiology depending on its environment. This is underlined by recent works showing that growth and virulence of this bacterium vary according to constituents of the cell culture medium [7].

Bacteria can cluster together and reach a critical quantity, a quorum that induces more cell-to-cell communications thanks to increasing concentrations of secreted molecules or enzymes. Bacteria sense these environmental modifications which alter their behavior, it is the famous quorum sensing [8]. Biofilm production is a consequence of bacterial clustering, it isolates and protects these cells from environmental insults such as antibiotics and host defense mechanisms [9]. Preventing a quorum from being reached helps disrupting bacterial effects.

C. acnes can switch from an isolated form to dense colonies forming a biofilm in the depression of hair follicles [10, 11]. In hair follicle sections of acne-prone volunteers, *C. acnes* generated more biofilms than in non-acne samples. The biofilm would therefore appear to contribute to the virulence of this bacterium. Both *C. acnes* multiplication and biofilm disturb hair follicle physiology through increasing of *C. acnes* lipase activities producing an excess of irritant free fatty acids creating a local micro-irritation [12]. Consequences are the increase of pro-inflammatory mediators such as interleukins by skin cells, of matrix protease activities which destroy extracellular matrix proteins, and of pilosebaceous ducts clogging [13], all denaturing neighboring tissues at various depth. This is acne, existing in several forms, with or without inflammatory features, redness, blemishes, comedones or scars. This creates significant skin and psychological damages leading 40 % of people with acne to seek medical advice.

Several prescription-based treatments are used to moderate *C. acnes* activities and to restore skin surface smoothness by reducing blemishes. These treatments have severe side effects (facial erythema, hair loss, coronary heart disease, problem during fetal development, depression, stain clothing, irritation), so, they are not ideal for general population use. Since our pioneering works on peptides, the cosmetic industry widely and safely uses small and well characterized peptides with anti-ageing properties, improving dermal compartment qualities, reinforcing skin barrier properties, relaxing the skin, or more recently triggering hair follicle pigmentation [14-18]. However, there are no existing peptide acting on *C. acnes*, preserving skin homeostasis and in parallel promoting extracellular matrix protein synthesis. We developed the micro-pentapeptide Palmitoyl-Lysyl-Threonyl-Seryl-Lysyl-Serine (pKTSKS), which selectively acts on *C. acnes* growth, adhesion and biofilm formation without significant modulation of *Staphylococcus epidermidis* population. It reinforces epidermal barrier functions, modulates interleukin-6 (IL-6) and -1Ra (IL-1Ra) productions by skin cells and improves extracellular matrix protein synthesis such as collagens. Used on volunteers with blemishes and acne prone skin, it significantly reduces most aspects of acne lesions: redness, inflammatory lesions, pockmarks, and roughness.

EXPERIMENTAL:

Peptide synthesis

The peptide pKTSKS (Palmitoyl-Lysyl-Threonyl-Seryl-Lysyl-Serine; INCI Name: Palmitoyl Pentapeptide-4) and KTSKS were synthesized at Sederma (Le Perray en Yvelines, France) using solid phase peptide synthesis with non-CMR solvents. Both linear peptides were made from Fmoc-L-Serine-resin with sequential coupling of L-lysine, L-serine, L-threonine and L-lysine (Iris Biotech, Germany) derivatives using coupling agents followed by Fmoc-deprotection steps. All amino acids used were of L-stereochemistry and of non-animal origin. Finally, coupling of palmitic acid of RSPO quality (Stéarinerie Dubois, France) was performed using a coupling agent followed by removal of the resin part and of the lateral protecting groups. Pure peptides were obtained as hydrochloride salts; their purity was assessed by MS/HPLC (HPLC Agilent 1200, Agilent, France).

Bacterial growth assays

C. acnes strains ribotype-1 (RT-1; CIP53.117T-ATCC 6919) were obtained from Pasteur Institute (France) whereas RT-4 and RT-5 (HL045PA1 and HL043PA2 respectively) were

obtained from BEI Resources (USA), NIAID and NIH as part of the Human Microbiome Project. All strains were routinely cultivated in modified medium 20 (3 % tryptone, 0.05 % L-cysteine hydrochloride, 0.1 % triethanolamine (all Sigma), 0.5 % glucose (Cooper) and 2% yeast extract (Oxoid). For growth studies, bacteria were seeded at 10^6 CFU (colonie-forming units) / mL in the same medium ± pKTSKS (6-12 ppm) or its solvent (0.1 % DMSO). As these strains are strictly anaerobic, the plates were incubated for 1 week in BD GasPack™ (Thermofischer, France) under anoxic conditions at 37°C. To monitor the kinetic growth curves, samples were collected every day and medium turbidity (optical density: OD) was measured at 600 nm. In a second step, bacteria were treated with equivalent concentrations of pKTSKS in the isolated palmitic acid, isolated peptide sequence (KTSKS) or a blend of both compounds, in order to evaluate the effects of each compound on *C. acnes* growth over time. The same culture conditions than before were used.

Adhesion and biofilm assays

C. acnes RT-1 adhesion and biofilm formation were evaluated on the surface of plastic plates. 10^6 CFU / mL were seeded in liquid medium (Trypticase soja) ± pKTSKS (6-12 ppm) or its solvent (control). For adhesion, pKTSKS was added to culture medium immediately after seeding. Cell culture medium was removed after 3 days and adherent cells were estimated using method derived from [18] by BioEC and Cergy Pontoise University (France). Briefly, adherent cells were rinsed and stained with crystal violet for 20 min. Colorant was discarded and cells rinsed with water then colorant was extracted and variations of OD_{600nm} were evaluated with a spectrophotometer. For biofilm formation study, the same protocol was used but pKTSKS was added to culture medium 8h after cell's seeding.

Human keratinocyte assays

HaCaT cells, spontaneously immortalized human keratinocyte line [19], were obtained from DKFZ (Germany) and cultured with 5 % CO_2 at 37°C and 90 % humidity atmosphere in regular Dulbecco's Modified Eagle Medium (DMEM; Gibco) supplemented with 0.09 mM Ca^{2+} (Sigma), 4 mM glutamine (Gibco), 1 mM sodium puryvate (Gibco), 100 IU/mL penicillin-streptomycin (Gibco), and 1 µg/mL Amphotericin B (Gibco). For routine cultures, medium was supplemented with 10 % heat-inactivated fetal bovine serum (FBS, Gibco). Media were renewed every 48 to 72 h to allow the outgrowth.

For experiments, cells were seeded at a density between 8-16x10^4 cells/cm² and cultured into DMEM medium with 10 % FBS until confluency was reached. Cells were then switched to

DMEM without any antibiotics nor antifungal products in order to allow *C. acnes* survival. For interleukin-6 (IL-6) and -1Ra (IL-1Ra), cells were treated with pKTSKS peptide (6 to12 ppm) or its solvent (0.1 % DMSO, Sigma) for 3 days. After the first 24 h of treatment, living planktonic *C. acnes* cells (100 bacteria: 1 keratinocyte) were added for an additional 48 h. Then, cell culture supernatants were collected for cytokine measurements using ELISA methods (IL-6: Sanquin and IL1-RA: R&D Systems), and cell viability was evaluated using nucleus labeling with Hoescht 33258 method (Sigma Aldrich; [20]). Elisa kits used: MMP1 (R&D Systems, USA), IL6 and IL1-RA (R&D Systems, USA) cell culture media content were assessed using Elisa tests.

Human sebocytes

Human sebocytes obtained with the reprogramming of induced pluripotent stem cell technology (iPS) were provided by Phenocell (France). Cells were routinely cultured with 5 % CO_2 at 37°C and 90 % humidity atmosphere in regular Phenocult-SEB medium (Phenocell). Media were renewed every 48 to 72 h to allow the outgrowth.

For experiments, cells were seeded at a density of $2x10^4$ cells/cm² into Phenocult-SEB medium until confluency was reached. For IL-6 evaluations, cells were treated with pKTSKS peptide (1, 3 and 6 ppm) or 0,1% DMSO for 48h and with living planktonic *C. acnes* cells (300 bacteria: 1 sebocyte) for the last 24 h. For dosage and survival evaluations see above.

Equivalent skin model

Human Living Skin Equivalents (LSE) were produced at Sederma as described in [21] with modifications. Briefly, for equivalent-dermis, normal human fibroblasts (NHF, Cambrex, USA) were seeded into collagen-I matrix (Jacques Boy, France) in 6-well plate inserts (Falcon, USA) and kept emerged for 48 h to allow dermal contraction. Human keratinocytes (CellnTec, Switzerland) were seeded on top of the equivalent dermis. Models were cultivated for 7 days in immersion media, followed by 7 days of air-liquid interphase to obtain a stratified epidermis. 9 and 12 ppm of pKTSKS or its solvent (0.1 % DMSO) was applied on top of the epidermis. After 48 h of treatment, LSE were embedded in freezing media (Leica, Australia) and snap-frozen in liquid nitrogen. 5-7 µm sections were prepared using cryostat (CM15105, Leica, Germany). Full-skin histology was evaluated by H&E (Sigma, USA) staining. Thickness of *stratum corneum* was quantified on pictures captured under microscope (Eclipse TI) using NIS Element Software (both Nikon, Japan).

Clinical evaluations

Two independent studies were performed on separate sites to assess the modulation of preliminary signs of acne and of the skin restructuring effect by pKTSKS. A facial cream with 12 ppm of pKTSKS was applied twice a day for two months on hemi-face by volunteers whereas the other hemi-face received corresponding placebo cream; each volunteer acted as his own control. First study included 18 Caucasian volunteers (17 women), of age average 32 y. ±11 while for the second study, included 30 Caucasian volunteers (23 women), of age average 24 y. ±7. Volunteers had oily and acne prone skin, pockmarks and inflammatory marks. Written informed consent was obtained from all participants. Protocols and used products were reviewed prior to starting the studies (medical control, risk assessments, ethical committee) moreover non-invasive methods were used.

The impactful aspects: pimples, scars, redness, brownish marks were evaluated using comparative photos taken with multispectral camera Antera 3D® (Miravex, Ireland) and clinical evaluations by a dermatologist. In addition, microbiome was sampled on skin using swabs and analyzsed using qRTPCR and 16S technologies. Bilateral tests were carried out on paired series; depending on methods, Student's *t,* Wilcoxon or Tukey *post hoc* tests were performed.

RESULTS AND DISCUSSION:

From a preliminary screening of peptides performed on both NHK and *C. acnes*, we selected the pKTSKS peptide which modulated pre-inflammatory mediators release and reduced the growth of *C. acnes* cells.

Growth of C. acnes with pKTSKS

C. acnes RT-1 cells grew regularly from 21 to 53 h in its cell culture medium with solvent (control) whereas growth of cells in contact with pKTSKS (6 to 12 ppm) was significantly and strongly reduced (Table 1). The micropeptide induced a clear shift of the lag phase over time. This effect is dose dependent. When treated with 6 ppm, 9 ppm and 12 ppm of peptide, *C. acnes* reached an OD_{600nm} of 0.5 respectively at #52 h, #77 h and >96 h *i.e.,* 12 h to >96 h more than the control. All these values were significant compared to solvent control ($p<0.01$). pKTSKS has a slowing effect on this bacterium, it limits the bacterium ability to reach its quorum and to potentially acquire a more virulent phenotype.

Table 1: Effect of pKTSKS on *C. acnes* growth kinetic over time (n=3).

	DO_{600nm}; 21 h	**DO_{600nm}; 29 h**	**DO_{600nm}; 45 h**	**DO_{600nm}; 53 h**
Solvent control	0.019± 0.003 *Reference 20 h*	0.129 ± 0.012 *Reference 29 h*	0.672 ± 0.066 *Reference 45 h*	0.758 ± 0.011 *Reference 53 h*
pKTSKS 6 ppm	0.006 ± 0.003 **-66.1 %****	0.007± 0.003 **-94.8 %****	0.209 ± 0.054 **-69 %****	0.522 ± 0.013 **-31.2 %****
pKTSKS 9 ppm	0.009 ± 0.005 **-50 %***	0.008 ± 0.002 **-94.1 %****	0.080 ± 0.048 **-88.1 %****	0.036 ± 0.008 **-95.3 %****
pKTSKS 12 ppm	0.014 ± 0.003 **-26.8 %** *(ns)*	0.009 ± 0.004 **-93 %****	0.084 ± 0.031 **-87.6 %****	0.009 ± 0.004 **-98.8 %****

(ns): non-significant; (*) p<0.05 ; (**) p<0.01

In a second set of assays, RT4 and RT5 strains of *C. acnes* were tested in a same way than RT1. RT5 and RT4 strains required 3x and 2x more hours respectively than RT1 to reach an OD_{600nm} of 0.5. Therefore, it appears that, *in vitro*, the effects of pKTSKS differs according to the strain used.

Growth of C. acnes with KTSKS and/or palmitic acid

To assess the interest of the pKTSKS, *C. acnes* RT-1 cells were in contact with either the palmitic acid or the KTSKS (the naked peptide), the two components whose association gave the pentapeptide pKTSKS. In a second series of assays, fatty acid and the naked peptide were tested together. Cell's growth was evaluated as previously described with 4 ppm of palmitic acid and/or 8 ppm of KTSKS, equivalent to 12 ppm of pKTSKS up to 72 h (n=3). Neither the isolated compounds nor their combination exert any significant effect on the growth of bacteria compared to the solvent control. Only the bonded two molecules, forming the peptide pKTSKS, is active in limiting the growth of *C. acnes* but not the isolated compounds.

Residual effects of pKTSKS on *C. acnes*

This study was conducted to monitor the residual effect of the pentapeptide on *C. acnes* and to determine whether the inhibition can be rapidly lifted in its absence. The peptide (9 ppm), or its solvent (control), were in contact with cells for 48 h and cell growth was estimated as previously mentioned. After centrifugation and rinsing with buffer, cells from both populations were seeded at same concentration in fresh cell culture medium without pKTSKS or solvent. Cell growth was estimated over time. Figure 1 shows a clear increase of cells growth for control (OD_{600nm} # 0.650 after 48 h) while a marginal growth is observed for 9 ppm of peptide as

expected. The second parts of the curves indicate that cells regained a growth potential identical to the one observed for the control cases as both growth curves are stackable. Thus, the pentapeptide possesses an inhibitory effect when in contact with *C. acnes* but does not have a residual effect.

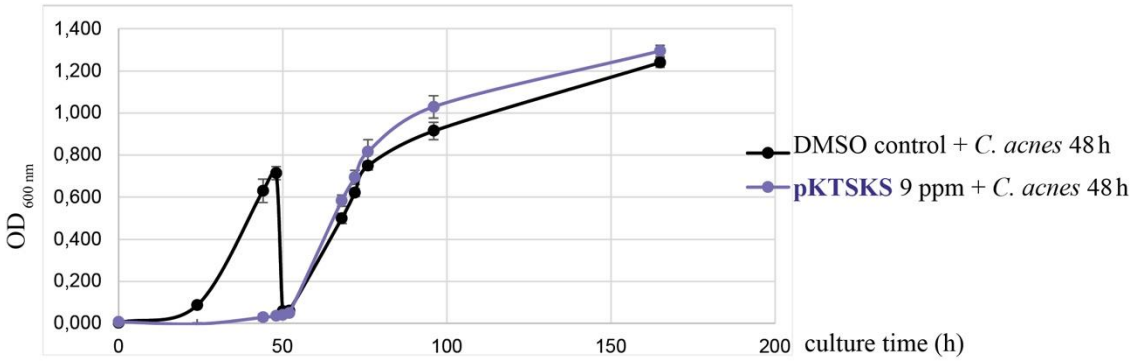


Figure 1: Evaluation of the residual effect of pKTSKS on C. acnes growth after 48 h of contact (n=3)

Effect of pKTSKS on *C. acnes* on biofilm formation

C. acnes adhesion was evaluated on the surface of plastic plates after crystal violet extraction. For solvent, OD_{600nm} was 2.74±0.12 units whereas pKTSKS almost completely reduces bacterial adhesion by 98 % and 99 % after 72 h at respectively 6 and 12 ppm. Regarding biofilm formation, for solvent, OD_{600nm} was 2.22 ±0.34 after 3 days, whereas pKTSKS strongly prevents the biofilm formation by *C. acnes* for both 6 and 12 ppm at 97 and 98 % respectively, both $p<0.01$. Prevention of *C. acnes* adhesion and of biofilm formation by pKTSKS reduces *C. acnes* ability to reach its quorum.

Growth of S. epidermidis with pKTSKS

S. epidermidis is one of the most encountered commensal micro-organisms on our skin. While it serves as a foil for its pathogenic cousin, *S. aureus*, it could also control the excessive development of *C. acnes* [22]. It is therefore of interest to evaluate whether pKTSKS could affect *S. epidermidis* growth. *S. epidermidis* (DSM 28764, Germany) was seeded at $7x10^7$ cells/mL in a TSY medium (Biomerieux, France). Its growth was monitored over time using a Bioscreen C (Finland). Table 2 indicates that there is no difference of growth between pKTSKS, whatever the concentration tested, and its solvent (control).

Table 2 Effect of pKTSKS on *S. epidermidis* growth kinetic over time (n=7).

	3 ppm pKTSKS	6 ppm pKTSKS	12 ppm pKTSKS
After 20 h	**+1.05 %**	**+0.61 %**	**+0.24 %**
After 30 h	**+1.63 %**	**+1.20 %**	**+0.53 %**

IL-6 production by human sebocytes and keratinocytes in presence of C. acnes

C. acnes can disturb skin homeostasis and trigger pro-inflammatory mediators release such as IL-6 and TNF-α from skin cells. Human sebocytes and keratinocytes were in contact with pKTSKS for 1 to 3 days. Living *C. acnes* cells were in contact of human cells for 24 h to 48 h. IL-6 was estimated into cell culture media. Table 3 shows the strong increase of this cytokine by human skin cells in response to *C. acnes* contact: x8.6 for sebocytes and x424 for keratinocytes, the more sensitive cells. Pentapeptide pKTSKS decreases, with dose-dependency, IL-6 release by 48 % in sebocytes and by 89 % in keratinocytes (all $p<0.01$ *versus* their respective control).

Table 3: Variation in IL-6 productions by human sebocytes and keratinocytes; effect of pKTSKS (n=3 to 6).

	Sebocytes		**Keratinocytes**	
	IL-6 ***(pg/10^6 cell.)***	**Variation (%)**	**IL-6** ***(pg/10^6 cell.)***	**Variation (%)**
Control no *C. acnes*	10.9 ± 1.98	*Ref1.*	3.5 ± 0.81	*Ref1.*
Control *+ C. acnes*	94 ± 19	*Ref2.* **(X8.6 *vs ref1*)**	1484 ± 102	*Ref2.* **(X424 *vs ref1*)**
pKTSKS 6 ppm + *C. acnes*	84 ± 15	**-11 %**; *ns*	415 ± 22	**-72 %**; *p<0.01*
pKTSKS 9 ppm + *C. acnes*	70 ± 12	**-26 %**; *p<0.05*	176 ± 16	**-88 %**; *p<0.01*
pKTSKS 12 ppm + *C. acnes*	49 ± 7	**-48 %;** *p<0.01*	165 ± 15	**-89 %;** *p<0.01*

No cell toxicity was observed. (ns): non-significant

IL1-Ra production by human keratinocytes

IL-1Ra (interleukin-1 receptor antagonism) is a naturally produced cytokine which antagonises the pro-inflammatory cytokine IL-1a and prevents triggering of cell's downstream events leading to the synthesis of other pro-inflammatory cytokines. IL1-Ra was estimated into cell culture media of keratinocytes after 3 days of contact with solvent or pKTSKS (6-12 ppm).

Results (Table 4) showed that pentapeptide pKTSKS dose-dependently stimulates the IL-1Ra release by 167 % to 283 % for 6 to 12 ppm respectively (both *p<0.01 versus* control solvent).

Table 4: Variation in IL-1Ra production by human keratinocytes; effect of pKTSKS (n=5).

	IL-1Ra ***(pg/10^6 cell.)***	**Variation (%)**
Control	307 ± 125	*Reference.*
pKTSKS 6 ppm	820 ± 125	**+167**%; *p<0.01*
pKTSKS 9 ppm	1089 ± 143	**+255%**; *p<0.01*
pKTSKS 12 ppm	1177 ± 87	**+283%;** *p<0.01*

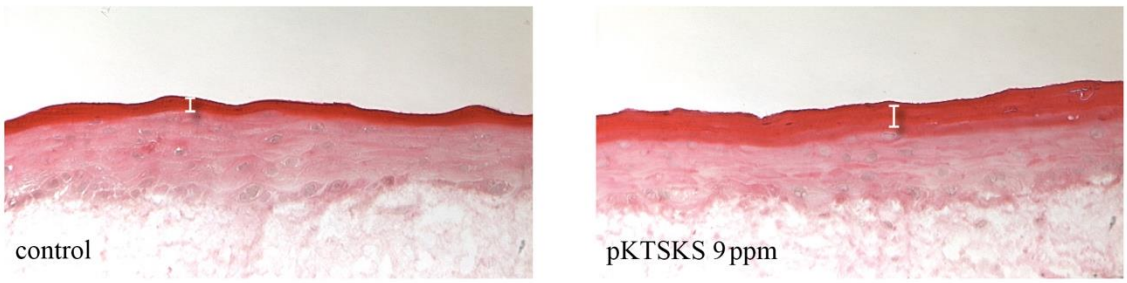
No cell toxicity was observed.

Skin barrier functions

The epidermal barrier is one of the keystones of our body's protection. A good barrier serves to preserve skin hydration and provides a healthy substrate for resident microbiota. It requires an epidermis whose keratinocytes are fully differentiated. Incomplete differentiation of keratinocytes can lead to the thinning of *stratum corneum* and better penetration of bacterial allergens favoring pro-inflammatory mechanisms. Figure 2 and table 5 show the positive effect of pentapeptide pKTSKS on epidermal differentiation. Whereas epidermis thickness is not modified, *stratum corneum* thickness increases by 186 % (*p<0.01 versus* control gel).

Figure 2 / Table 5: Effect of pKTSKS (9 ppm) applied onto surface of LES; thickness of *stratum corneum* (n=4).

	Stratum corneum **thickness (µm)**	**Variation (%)**
Control gel	16 ± 5	*Reference.*
pKTSKS 9 ppm	45 ± 11	**+186%**; *p<0.01*



Hyaluronic acid production by human keratinocytes

Hyaluronic acid is a natural skin moisturiser existing both into dermis and around keratinocytes that produce it, allowing the transfer of nutrients, vitamins, salts and of immune cells into epidermis. Hyaluronic acid production by keratinocyte was increased significantly in a dose-dependency manner by 33 % to 62 % respectively for 6 to 10 ppm (Table 6).

Table 6: Variation in hyaluronic acid production by human keratinocytes; effect of pKTSKS (n=5).

	Hyaluronate *(ng/10^6 cell.)*	**Variation (%)**
Control	3883 ± 391	*Reference.*
pKTSKS 6 ppm	5159 ± 207	**+33%**; *p<0.01*
pKTSKS 8 ppm	5515 ± 342	**+42%**; *p<0.01*
pKTSKS 10 ppm	6278 ± 526	**+62%;** *p<0.01*

No cell toxicity was observed.

Dermal matrix restructuring through protein synthesis

Pentapeptide pKTSKS triggers the production of dermal extracellular matrix proteins by fibroblasts. Thus, collagen-I production is increased under these conditions by 184 % (*p<0.01* compared to control solvent) while collagen-IV and fibronectin productions are lifted by 98 % and 67 % respectively (both *p<0.01 versus* control solvent; Table 7). So, pKTSKS improves matrix proteins synthesis of interest for dermal reconstruction.

Table 7: Synthesis of collagen-I, collagen-IV and fibronectin, effect of pKTSKS (*n=5).*

	Coll-I; pg/10^{6}cells.	**Variation (%)**	**Coll-4 ; pg/10^{6}cells.**	**Variation (%)**	**Fibronectin ng/10^{6}cells.**	**Variation (%)**
Control solvent	589.4 ± 32.7	Reference	687 ± 22	Reference	16896 ± 1030	Reference
pKTSKS 10ppm	1676.9 ± 147.3	**+184 %**; *p<0.01*	1360 ± 203	**+98 %**; *p<0.01*	28234 ± 3841	**+67 %**; *p<0.01*

No cell toxicity was observed

Clinical studies; skin blemishes

Skin blemishes, created by acne phenomena, whether raised (inflammatory marks and pimples) or indented (atrophic scars or pockmarks) were evaluated, so was the overall roughness.

Table 8: Variation of inflammatory lesions and redness. Effect of pKTSKS (n=18 or n=30*)

	Volume of inflammatory lesions (mm^3)				Number of inflammatory lesions (*)				Redness (AU)			
	pKTSKS		Placebo		pKTSKS		Placebo		pKTSKS		Placebo	
	T0	T56	T0	T56	T0	T28	T0	T28	T0	T56	T0	T56
Mean ± sdm	2.12 ± 1.78	1.29 ± 1.25	3.07 ± 2.72	2.92 ± 2.49	10.5 ± 6.2	6.1 ± 4.3	10.6 ± 5.9	8.5 ± 4.4	44.96 ± 12.62	39.48 ± 12.95	43.15 ± 8.86	40.93 ± 9.09
Variation % *vs* T0 Significance *vs* T0 Significance *vs* Placebo		-39.2% *p<0.01* *p<0.05*		-4.9% *ns* -		-42% *p<0.01* *p<0.05*		-20% *p>0.06* -		-12.2% *p<0.01* *p<0.05*		-5.2% *ns* -

ns : non-significant

Table 9: Variation of pockmarks and of roughness. Effect of pKTSKS (n=18)

	Pockmarks volume (mm^3)				Roughness (µm)			
	pKTSKS		Placebo		pKTSKS		Placebo	
	T0	T56	T0	T56	T0	T56	T0	T56
Mean ± sdm	3.86 ± 2.69	2.97 ± 2.34	4.37 ± 3.60	4.19 ± 3.51	11.42 ± 2.55	10.50 ± 2.78	11.84 ± 3.45	11.65 ± 3.34
Variation % *vs* T0 Significance *vs* T0 Significance *vs* placebo		-23 % *p<0.01* *p<0.01*		-4.1 % *ns* -		-8.1 % *p<0.01* *p<0.01*		-1.6 % *ns* -

ns: non-significant

CONCLUSION:

Skin presents, from adolescence and later, an unsightly and psychological detrimental aspect due to blemishes and scars coming from the *C. acnes* cell multiplication into hair follicles. The proliferation of these cells, sometimes protected into a biofilm, strongly disturbs hair follicle's homeostasis. These germs, due to their production of extracellular lipases, create irritant molecules for the hair follicle's cells: keratinocytes and sebocytes. This micro-irritation induces an over-multiplication of keratinocytes while their differentiation in a more mature form is reduced; these immature cells accumulate into the hair follicle infundibulum and cause plugging. This original phenomenon triggers micro-inflammatory mediator release and attracts macrophages close to the hair follicle where they produce matrix proteases and enzymes that are well known to destroy cutaneous tissues. All together, they create redness, red blemishes and scar lesions.

The aim of the study we are presenting here was to find an acceptable and safe solution for the cosmetic industry to prevent unsightly acne lesions prior their birth, but also to help skin to quickly recover its smoothness by triggering collagen's and hyaluronate's synthesis.

Effectiveness and mechanism of action on pre-acne lesions of peptides are not well documented yet. Reports suggested that acting on the quorum of bacteria can be a critical way to avoid worsening effects of their clustered forms and to reduce their biofilm production. Herein, we examined the potentialities of a lipo-pentapeptide, identified amongst dozens, using screening performed on *C. acnes* growth and inhibition as well as production of pre-inflammatory mediators by keratinocytes. Isolated compounds forming the lipo-pentapeptide are not efficient on *C. acnes* growth either alone or blended. It did not act on *S. epidermidis* growth at all, this was confirmed by a clinical test where no statistical modifications of *S. epidermidis* population on the face were observed after its use versus a placebo (data not shown). Moreover, pKTSKS acts more efficiently on acne ribotypes RT-4 and RT-5 than on RT-1 strain, no explanations were found for this moment, and further studies need to be conducted to clarify this. This pentapeptide did not have any remnant effect on *C. acnes* cells once contact was stopped, so the peptide is not a biocide as it does not kill the cells. Additionally, it was shown that the pentapeptide reduced adhesion of *C. acnes* on support, one of the prerequisite steps for its virulency and for biofilm production. Moreover, it was shown that this peptide reduced the activity of the *C. acnes* extracellular lipases (data not shown) another parameter of interest for reducing irritation and downstream process leading to micro-inflammation of hair follicle. All these data indicated that the peptide prevents *C. acnes* to reach its quorum and consequently reduces adhesion, biofilm formation and unpleasant effects on skins.

Peptides are known to act safely and efficiently on skin cells. This pentapeptide presents potentialities to reduce the *C. acnes*-induced IL-6 release by both sebocytes and keratinocytes while it increases the IL1-ra release. This limits the pro-inflammatory cascade and its consequences. It was shown that the peptide improves epidermis thickness and hyaluronate synthesis helping to reinforce the skin barrier and moisturising functions. Associated with *C. acnes* growth limitation, this could explain the reduction of redness and inflammatory blemishes observed during clinical tests. On fibroblasts, peptide acts on collagens and fibronectin synthesis, extracellular matrix proteins of the dermis whose production is necessary for reconstruction of damaged and aged skins, this could explain the reduction of roughness observed during the clinical tests.

Conflict of Interest: None

REFERENCES:

[1] Byrd AL, Belkaid Y, Segre JA (2018) The human microbiome. Nat Rev Microbiol 16:143-155.

[2] Grice EA, Segre JA (2011) The skin microbiome. Nat Rev Microbiol 9:244-253.

[3] Stansbury J (2020) Acne: 4000 consumers speak out. Global Cosmetic Industry Magazine Oct:51-53.

[4] Aubin GG, Portillo M, Trampuz A, Corvec S (2014) *Propionibacterium acnes*, an emerging pathogen: from acne to implant-infections, from phylotype to resistance. Med Mal Infect 44: 241-250.

[5] Fitz-Gibbon S, Tomida S, Chiu B, Nguyen L, Du C, Liu M, et al (2013) *Propionibacterium acnes* strain populations in the human skin microbiome associated with acne. J Invest Dermatol 133:2152-2160.

[6] Scholz CF, Kilian M (2016) The natural history of cutaneous propionibacteria, and reclassification of selected species within the genus Propionibacterium to the proposed novel genera Acidipropionibacterium gen. nov., Cutibacterium gen. nov. and Pseudopropionibacterium gen.nov. Int J Systematic Evol Microbiol 66:4422-4432.

[7] Borrel V, Gannesen AV, Narreau M, Gaviard C, Duclairoir-Poc C, Hardouin J, Konto-Ghiorhi Y, Lefeuvre L, Feuilloley M (2019) Adaptation of acneic and non acneic strains of *Cutibacterium acnes* to sebum-like environment. MicrobiologyOpen 8: e841.

[8] Corvec S (2018) Clinical and biological features of *Cutibacterium* (formely Propionibacterium) *avidum*, an underrecognized microorganism. Clin Microb Rev 31: 1-17.

[9] Huang R, Li M, Gregory RL (2011) Bacterial interactions in dental biofilm. Virulence 2:435-444.

[10] Jahns AC, Lundskog B, Ganceviciene R, Palmer RH, Golovleva I, Zouboulis CC, McDowell A, Patrick S, Alexeyev OA (2012) An increased incidence of *Propionibacterium acnes* biofilms in human acne vulgaris: a case-control study. Brit J Dermatol 167:50-58.

[11] Jahns AC, Alexeyev OA (2014) Three-dimensional distribution of *Propionibacterium acnes* biofilms in human skin. Exp Dermatol 23:687-689.

[12] Gannesen AV, Zdorovenko EL, Botchkova EA, Hardouin J, Massier S, et al (2019) Composition of the biofilm matrix of *Cutibacterium acnes* acneic strain RT5. Front Microb DOI:10.3389/fmicb.2019.01284.

[13] Miskin JE, Farrell AM, Cunliffe WJ, Holland KH (1997) *Propionibacterium acnes*, a resident of lipid-rich human skin produces a 33kDA extracellular lipase encoded by gehA. Microbiology 143:1745-1755.

[14] Lintner K, Peschard O (2000) Biologically active peptides: from a laboratory bench curiosity to a functional skin care product. Int J Cosmet Sci 22:207-218.

[15] Lintner K, Mondon P, Peschard O, Mas-Chamberlin C (2001) Cosmetic applications of a wound healing peptide. J Cosmet Sci 52: 82-83.

[16] Mondon P, Fache S, Doridot E, Lintner K (2012) From elastin to elastic fibers, Part II: The clinical effects of a natural dipeptide on the biological cascade. Cosmet & Toilet Mag 127:658-664.

[17] Mondon P, Hillion M, Peschard O, Andre N, Marchand T, Doridot E, Feuilloley MGH, Pionneau C, Chardonnet S (2015) Evaluation of dermal extracellular matrix and epidermal–dermal junction modifications using matrix-assisted laser desorption/ionization mass spectrometric imaging, in vivo reflectance confocal microscopy, echography, and histology: effect of age and peptide applications. J Cosmet Dermatol 14:152–160.

[18] Mondon P, Leroux R, Ringenbach C, Debacker A and Peschard O (2019) A micropeptide modulating a-Crystallin, ANCR and TRPC6 to improve skin barrier functions and homeostasis. IFSCC Magazine 3:1-6.

[19] Merritt JH, Kadouri DE, O'Toole GA (2005) Growing and Analyzing Static Biofilms. Current Protocols in Microbiology http://doi.org/10.1002/9780471729259.mc01b01s00

[20] Boukamp P, Petrussevka RT, Breitkreutz D, Hornung J, Markham A, Fusenig NE (1988) Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. J Cell Biol 106: 761-771.

[21] Labarca C, Paigen K (1980) A simple, rapid and sensitive DNA assay procedure. Anal Biochem 102: 344-352.

[22] Asselineau D. Bernard B, Bailly C, Darmon M (1985) Epidermal morphogenesis and induction of 67kD keratin polypeptide by cultured of human keratinocytes at the liquid-air interface. Exp Cell Res 159: 536-539.

[23] Christensen GJM, Scholz CFP, Enghild J, Rohde H, Kilian M, Thurmer A, Brzuszkiewicz E, Lomholt HB, Bruggemann H (2016) Antagonism between *Staphylococcus epidermidis* and *Cutibacterium acnes* and its genomic basis. BMC Genomics 17:152-166.