First living probiotic anti-aging active ingredient with effective in vitro and vivo demonstration against placebo

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Abstract

Today, the use of probiotics is not limited to pharmaceutical or nutraceutical applications. Cosmetics are also concerned as skin microbiota dynamically participates in skin health and beauty. We previously designed a clinical study to understand skin microbiome differences between aged and young cohorts and observed higher populations of Lactobacilli in the nonaged cohort and the absence of *Lactobacillus crispatus* (*L. crispatus, LBC*) particularly in the wrinkle hollows of the old cohort. The aim of our study was firstly to demonstrate the efficacy of living over heat-killed biomass on collagen synthesis by fibroblast *in vitro*. Then we designed the best formula and an application routine to allow the maintenance of an efficient delivery of the living LBC probiotic to evaluate its clinical performance.

The LBC probiotic showed a significant stimulation of collagen I and V content *vs* untreated and live bacteria were more effective than the thermally inactivated biomass. We showed that the LBC probiotic was viable and stable in some natural oil-based emollients for several weeks, but only several hours in the presence of some emulsifiers and preservatives. For the clinical trial, we developed an oily serum of concentrated probiotic booster diluted just before application in a specific neutral emulsion to deliver a 0.05% dose of probiotic equivalent to 5 10^5 cfu/g on the skin. After 2 months of twice daily application, the LBC probiotic induced a significant increase in the density of the subepidermal and dermis zone and a decrease in wrinkle depth *vs* placebo to support antiaging claims.

Keywords: Lactobacillus crispatus; live probiotic; collagen; antiaging; skin density

Introduction.

Consumers understand the need to maintain a balanced skin flora to make our bodies inhospitable hosts to any invading pathogens, to maintain the acidic skin mantle and to contribute to skin beauty. The global probiotic skin care cosmetic product market is a fast-growing market valued at $57 M in 2021. However, real living probiotics are not widely used due to the difficulty of demonstrating suitable stability in diverse cosmetic

formulations on top of clinical efficacy against placebo. Currently, only few products containing live bacteria are available on the market. Among them, some formulations contain single bacteria from environmental origin such as *Nitrosomonas eutropha* (ammonia oxidizing bacteria) or *Micrococcus luteus* present on both mammal skin and environment. Rarely formulations contain a blend of different probiotic bacterial or yeast strains. Most of the cosmetic solutions currently proposed contains Lactobacilli, particularly *Lactobacillus plantarum* widely used in food or medical industry. Regarding formulation composition, oily serum or cream with double packaging are proposed.

In order to offer novelty in the domain, our intention was to compare at first the microbiome from young and aged panelists, to particularly focus on the wrinkle area to study the local aged-related difference. Then we develop a skin native living probiotic solution to help the aged skin to recover a younger microbiotic profile. In order to demonstrate its performance, we firstly evaluate its impact on the skin metabolism and protection and secondly largely study its stability in different formulations to help formulators to use it broadly.

Materials and Methods.

1-Comprehensive clinical study of skin microbiota

Volunteers were recruited with wrinkle grade (50 with grade 5-6 in old cohort, 50 with grade 0-1 in young cohort). Microbial and metabolite samples were collected by swabbing 3 different zones: in the wrinkle hollow, the crow's feet and under-eye zone, and a control zone (cheek area adjacent to earlobe). One side was dedicated to instrumental measure and metabolite analysis, the other side to microbial DNA study. After DNA extraction and human DNA removal, the DNA was submitted to whole genome sequencing using Illumina HiSeq technology. The obtained microbial reads were analyzed for taxonomy using MetaPhlAn

2-Probiotic production

The probiotic strain *L. crispatus* was isolated from healthy skin and identified after full genome sequencing. The strain was fermented and freeze dried after centrifugation to reach a concentration of around 10^9 colony forming units/g (cfu/g).

3-Biological activity

Its antiaging properties and more particularly its ability to stimulate the synthesis of collagen type I and V which decrease with age was evaluated on human fibroblast culture by DELFIA method.

4-Formulation and stability studies

In order to develop a formula for the skin delivery of the probiotic, several formulating ingredients (emollients, emulsifiers and preservatives) were screened for their impact over a short period of time (1 h to 24h) on the viability of the LBC strain. The ingredients that showed a short-term neutrality toward the viability of the strain were then evaluated over a longer period (up to168 days, at different temperatures). An optimized formulation was finally designed using the most favorable ingredients previously evaluated and it was checked for stability and strain viability at different temperatures.

5-Clinical trial

This formulation was used to deliver the probiotic strain for a clinical evaluation on 29 Caucasian women (45-65 years old). The anti-aging efficacy was measured after 3 and 8 weeks thanks to dermis density evaluation by ultrasound imaging with a DUB SkinScanner coupled to image analysis, and wrinkles analysis by VISIA CR imaging.

Results.

1-Comprehensive clinical study of skin microbiota

The metabolomic analysis performed on 49 subjects in the old cohort and 47 subjects in the younger showed a significant decrease of fatty acids and lipids for the old cohort (data not shown).

In the microbial study performed on 49 subjects (old cohort) and 46 subjects (younger), the alpha diversity was significantly increased in the old vs young cohort in all areas. Many significant ($p<0.05$) taxonomical differences were observed between the old and young cohort: more particularly, *Cutibacterium acnes* had a lower relative abundance in the older cohort contrary to *Corynebacterium kroppenstedtii* and *Veillonella parvula* which had a higher one (Figure 1)

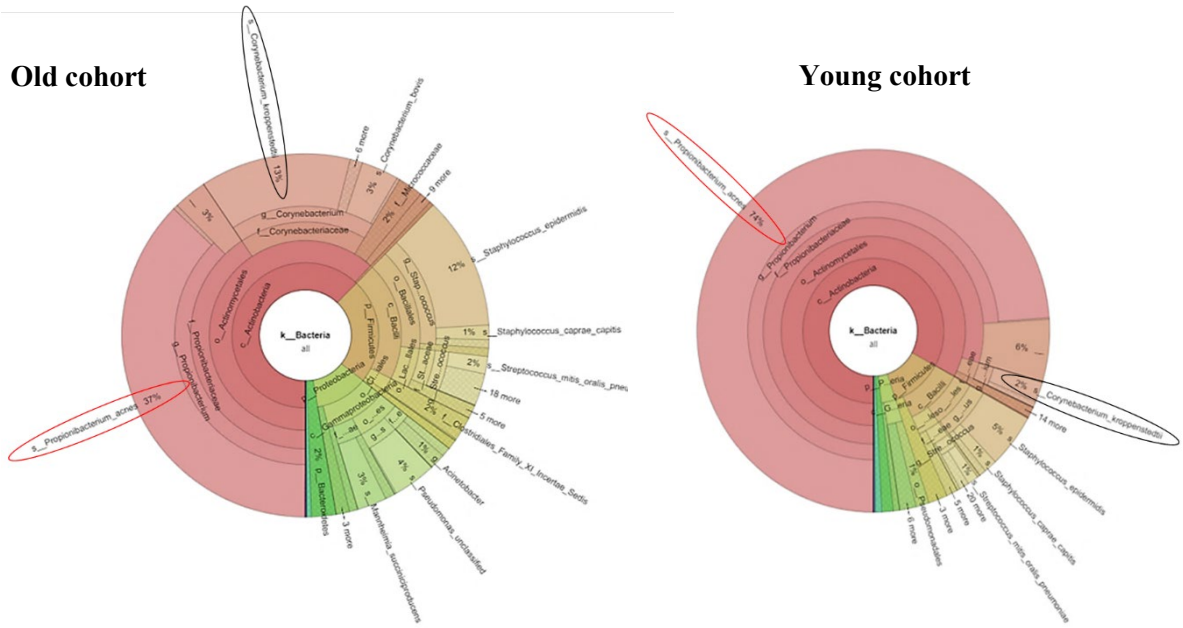
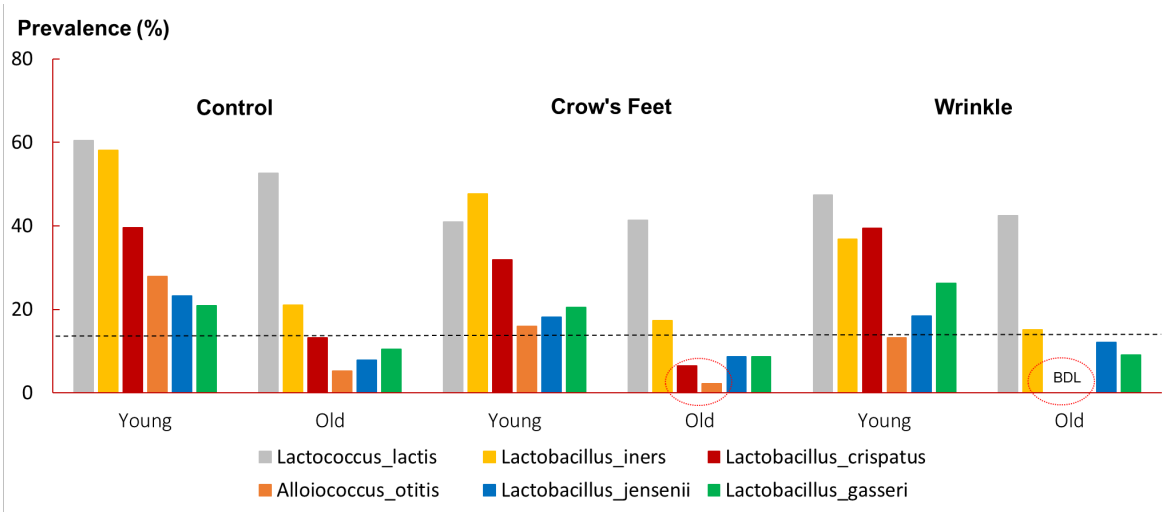


Figure 1: Key taxonomical differences between old and young skin. The krona plots highlighted a significant increase ($p=4.35\ 10^{-7}$) in alpha diversity in the old cohort when compared to the young cohort. Clear shifts can be seen in Actinobacteria (Propionibacterium or Cutibacterium and Corynebacterium) and Firmicutes (Staphylococcus), depicted in pink and brown color, respectively.

Surprisingly, the lactic acid bacteria prevalence observed in the young cohort and its decrease in the wrinkle area of old skin were unexpected. Among the most prevalent lactic acid bacteria present, most of them were Lactobacilli. Interestingly, the prevalence of lactic acid bacteria significantly more decreased into the wrinkle zone of the old cohort and among the more prevalent, *L. crispatus* was even below the detection level (BDL) (figure



2).

Figure 2: Prevalence of lactic acid bacteria showing specificities in the wrinkle area, and even more in the wrinkle hollow.

Moreover, among the 6 most abundant lactic acid bacteria present in the wrinkle hollow, at least 3 *Lactobacilli* were observed in the young cohort and were decreased in the old one, with *L. crispatus* being bellow the detection level (Figure 3).

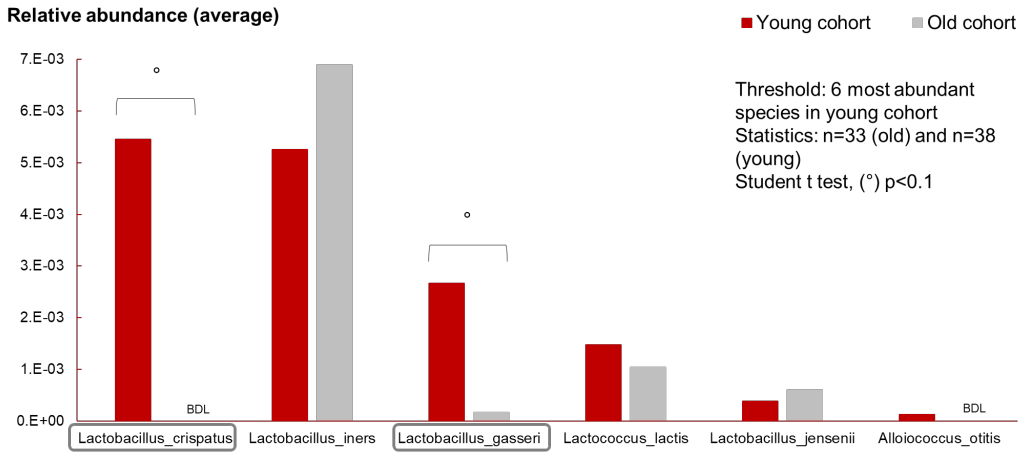


Figure 3: Abundance of lactic acid bacteria in the wrinkle of older skin.

2-Biological activity

The *L. crispatus* probiotic showed a significant stimulation of collagen I and V content (+133% and +55% respectively) in fibroblast culture *versus* untreated condition. Interestingly, we also showed that live bacteria were more effective than the thermally inactivated biomass (Figure 4 A-B).

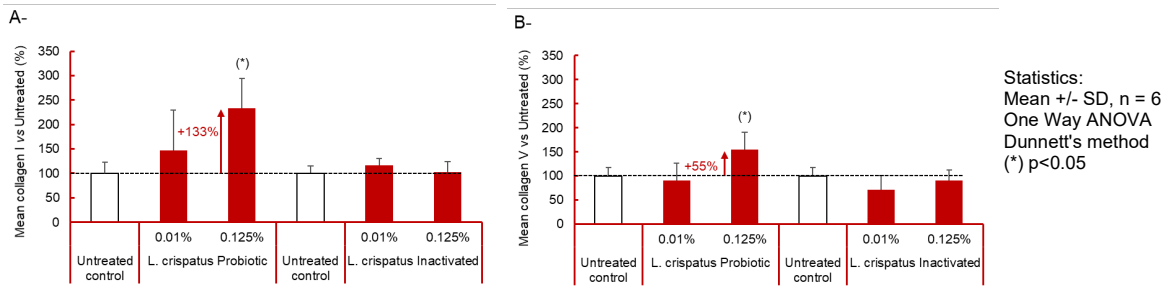


Figure 4: Stimulation of collagen content in fibroblasts (A-collagen type I, B- collagen type V).

3-Formulation and stability studies

The viability of the *L. Crispatus* probiotic ingredient was preserved at both 4°C and room temperature for one year and at least 2 months at 40°C (Figure 5).

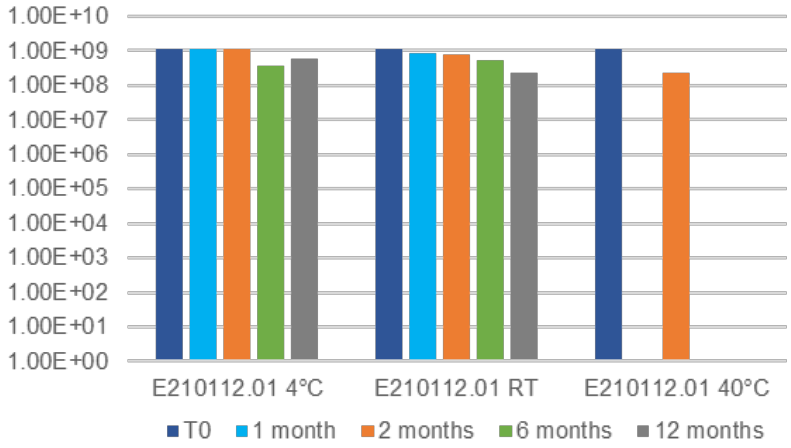


Figure 5: Stability of the probiotic ingredient.

In formulation, the probiotic strain had different viabilities and stabilities according to the nature of the formulating ingredients. If it was viable and stable in some natural oil-based emollients for several weeks, it was however only stable for several hours in the presence of some emulsifiers and preservatives. These results allowed us to develop an application routine for the clinical test in which an oily serum of concentrated probiotic booster was diluted just before application in a specific neutral emulsion, to deliver a 0.05% dose of probiotic equivalent to 5 10^5 cfu/g on the skin.

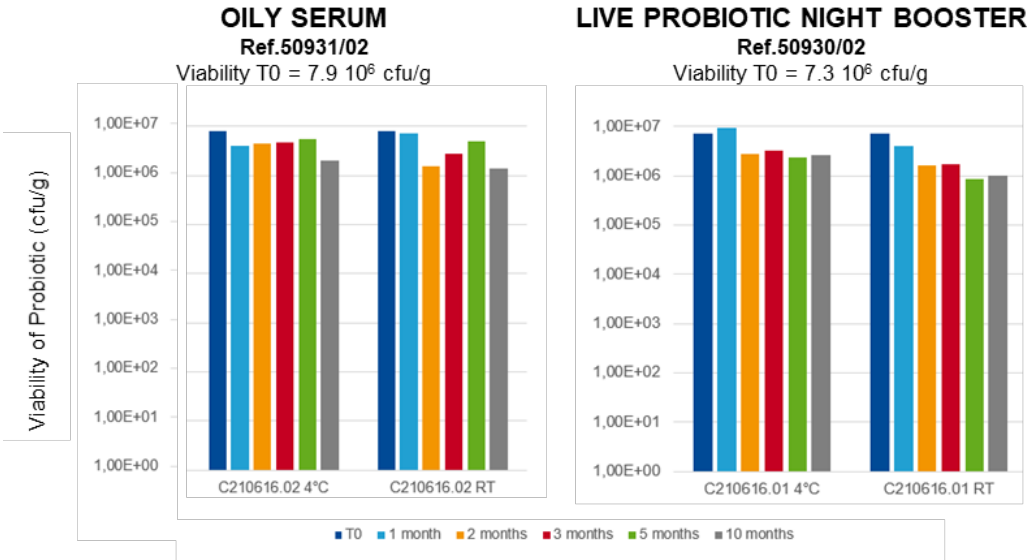


Figure 6: Stability of the formulated probiotic ingredient.

4- Clinical trial

The results obtained in the clinical trial corroborate the *in vitro* performance previously obtained on collagen stimulation. After 2 months of twice-daily application, we measured a significant increase compared to baseline of the density of the sub-epidermal zone by 11% and of the total dermis by 6%. The improvement of dermal density (+5%) was also significant compared to the placebo (Figure 7). Although the appearance of forehead wrinkles is more difficult to smooth than crow's feet, the new probiotic offered a visible correction in this area as well, achieving a 5 percent reduction in the appearance of wrinkles compared to the placebo (Figure 8).

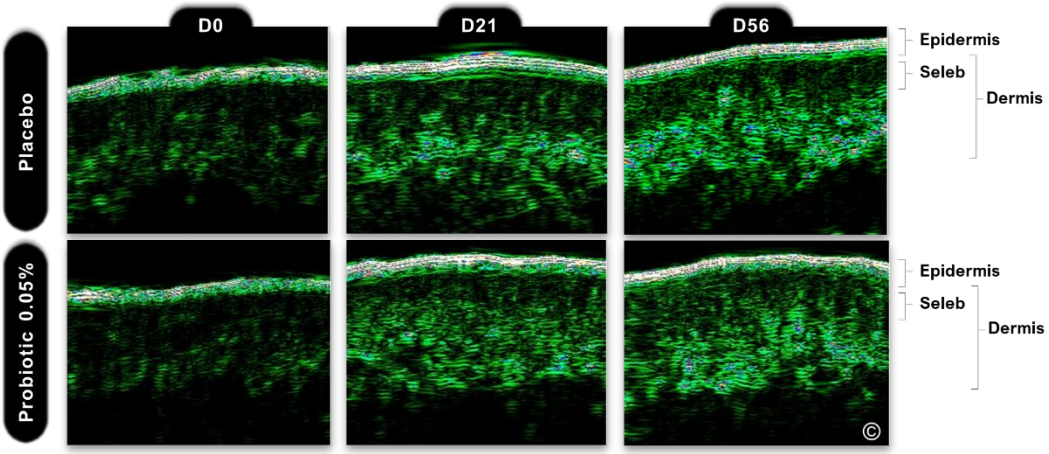


Figure 7: - Illustrative pictures of the echogenic density - Volunteer 10.

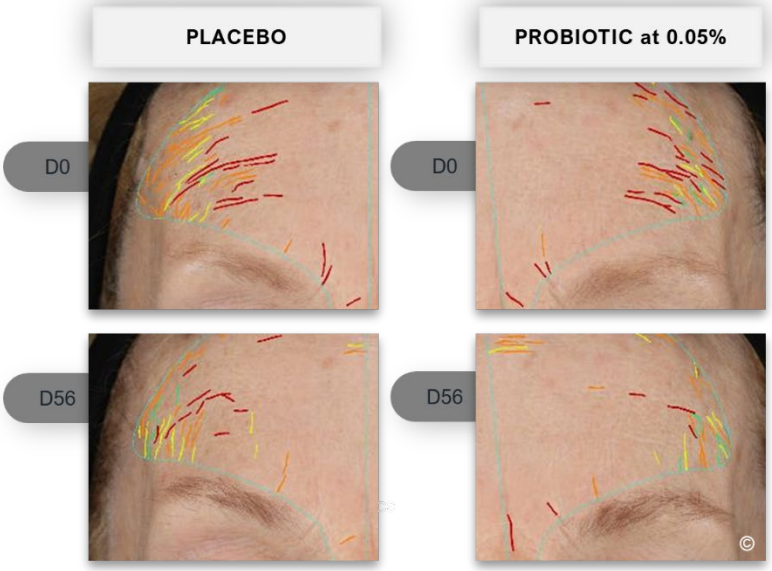


Figure 8: illustrative picture of forehead expression wrinkles using VISIA -Volunteer 27.

Discussion.

The vast majority of the bacteria that live on and in us are essential to our basic physiological qualities, such as digesting our food, supporting our immune system, and overall maintenance of the health of our skin and body. Of the millions of bacteria that exist, scientists only know about 100 that are pathogenic, i.e. harmful to humans. From birth, the diversity and composition of the microbiome evolves with contact and interaction with the environment or animals, when we eat or when we're with others.

The most external skin layer is alive with many commensal microorganisms which may contribute to maintaining a healthy-looking skin. Skin microbiome is extensively studied in dermatological and cosmetic fields to develop solutions for different skin condition as acne, atopic dermatitis, rosacea… In skin microbiome manipulation strategies, the skin microbiome can be changed via a multitude of mechanisms. The first method is a skin microbiome transplant in which the skin microbiome of a healthy individual is transferred to the washed and/or disinfected skin area of another person with the aim of improving the skin condition of the latter. The second method is by means of skin bacteriotherapy, where one or multiple pure cultures with health-promoting properties are placed on the washed and/or disinfected skin area of a person. The applied microbiota can be (1) alive (probiotics), (2) tyndallized or thermokilled bacteria that do not replicate anymore, or (3) cell lysates or physically killed bacteria (postbiotics), (4) purified enzymes or (5) fermentation products or supernatants: the bacteria are not added, but the supernatants containing their antioxidants, amino acids, lipids and/or vitamins are added. The skin bacteriotherapy (methods 1–5) has multiple advantages over a skin microbiome transplant with the main advantage being that the process is easier scalable and thus industrial applicable. For method 1 (application of live probiotics), highly concentrated bacteria can be applied; thus, a higher efficacy can be obtained compared to a complete skin microbiome transplant [1]. Among dermatological solution, we can cite the application of AMP producing coagulase-negative *Staphylococcus* (*S. hominis* or *S. epidermidis* isolated from Atopic Dermatitis patient's skin) to decrease S. aureus abundance [2] in atopic patient or application of combination of *C. acnes* strains from healthy patients on acne vulgaris patients [3].

Although skin microbiome is considered an important component in skin health, the relationship between it and skin aspect particularly in aged, wrinkled skin is however only partially known [4] and very few living probiotic therapy using skin native bacteria are described. In this study, we sought to understand the differences of skin microbiota composition between young and aged skin, with a focus on wrinkles (crow's feet). Using whole genome sequencing coupled with metabolomic analysis to measure shifts in skin

microbiome composition and metabolites, we confirmed previous results on diversity shifts relative to age for Corynebacterium and Cutibacterium [5]. These diversity shifts between cohorts are interesting and suggests a shift from a lipophile-dominated ecosystem to more diverse ecosystem and consequently a different metabolic potential with aging. We observed higher amounts of free fatty acids and triacylglycerol in the young cohort which can contribute to the regulation of organisms with low lipid tolerance and to the lower microbial diversity in the young cohort. This change in metabolites could also drive the shift between Propionibactericaea and Corynebacteriacea. *Cutibacterium acnes* uses carbon sources such as glucose, lactose, fructose, ribose, galactose, and lactic acid and will ultimately produce the short chain fatty acid, propionate (found to be significant in the young cohort) [6].

The prevalence of Lactobacilli in young skin was unexpected. Surprisingly, 15 species were observed (only the most prevalent shown in the figure). To date, this is the first description of Lactobacilli on healthy human skin. Furthermore, among Lactobacilli, we discovered that *Lactobacillus crispatus* was not detectable in the wrinkle hollow of the old cohort, whereas it was present in 40% of volunteers in the wrinkle area of the young cohort. Our hypothesis is that Lactobacilli, including *L. crispatus,* are more abundant after birth and their proportion decreases while we get old [7].

L. crispatus is a homofermentative organism which undergoes substrate level phosphorylation producing lactate from glucose and has the ability to produce bacteriocins; both metabolites can be beneficial for skin defense [8, 9]. *L. crispatus* is known to be present in healthy skin but absent in atopic dermatitis or psoriatic skin and is suggested to have an anti-inflammatory role in both pathologies [10].

To better understand *L. crispatus* contribution to skin health, its interactions with skin cells has been furthered studied. We succeeded to demonstrate that LBC probiotic, but not its inactivated form, stimulates the content and deposition of collagen type I and V, two major contributors of the dermal extracellular matrix responsible for skin density and firmness. Moreover, we also showed the potential of only *L. crispatus* living form, to protect the skin from oxidative stress (data not shown: Free radical scavenging capacity, glutathione production increased, and lipid peroxidation decreased after UVA).

The major challenge was then to obtain some clinical improvements of aged skin after application of a formulation. The compatibility study of the probiotic strain with the different formula allowed us to develop a galenic which kept the LBC dose needed to reach the clinical efficacy viable for at least 12 months at RT in a selected emollient (stability evaluation still ongoing). This emollient was safer for LBC and presents a greener impact vs mineral oil. Stability during specific processes allowing wax use (70°C, 30mn) and grinding, and limited contact with mild preservatives were also validated. Then, we

highlighted the capacity of the LBC probiotic strain to densify the dermis of the volunteers and reduce forehead wrinkle width thus providing antiaging benefits.

With this study we have opened the field of possible formulations of a skin native *Lactobacillus crispatus* probiotic to promote its use while offering multiple galenical experiences to consumers without compromising antiaging efficacy. These results make this ingredient the first living probiotic anti-aging ingredient with effective *in vitro* and *vivo* demonstration against placebo.

Conclusion.

Unlike other probiotics existing on the cosmetic market, the newly developed ingredient is the first to use a bacterium that is found naturally in the skin: *Lactobacillus crispatus*, a Gram-positive rod shape anaerobic bacterium that has been found to decrease with age.

However, what has become common practice in the food industry field is now one of the most challenging tasks for the personal care industry: incorporating living skin bacteria into cosmetic formulations and keeping them active.

Here we propose a probiotic solution made of living but dormant *Lactobacillus crispatus* bacteria which awaken in contact with water on the skin. This ingredient is the first cosmetic ingredient containing skin native live probiotic that helps make the skin feel fuller and improves the appearance of forehead wrinkles.

Conflict of Interest Statement. All authors are employee of BASF Beauty Care Solutions.

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