

neulFSCC 2025 full paper (IFSCC2025-424)

## ***“Bring down skin premature ageing by improving emotional resilience: an innovative *Lactobacillus* extract as a neuro-cosmetic ingredient”***

### **1. Introduction**

From exams, to personal and work situation, stress is not avoidable. When we face a stressful situation, a “fight and flight” reaction starts in our body and results in cortisol release, to regulate blood pressure, blood sugar levels, immune system etc., therefore making our body alert to respond to the external stressful situation. However prolonged cortisol production due to chronic stress negatively impact our health, well-being and sleep [1,2].

In skin, cortisol disrupts both dermis and epidermis, causing skin atrophy, wound healing disruption, epidermal thinning and skin dryness. As a result skin resilience is lost and wrinkles appear [1,3]. This process also known as premature ageing, impacts self-esteem and well-being. Fighting stress-related premature ageing is therefore an important target in beauty industry. We developed a protocol of improving skin condition and holistic well-being by playing on autonomous skin circadian rhythm and by increasing well-being mediators locally in skin. Every tissue in our body, including skin, has its own circadian clock. These clocks are synchronized by the brain and help tissue recovery. ROR- $\alpha$ , PER and CRY are members of circadian clocks [4,5]. In skin, ROR- $\alpha$  links circadian rhythm and epidermal regeneration. Indeed, besides being a member of circadian rhythm, ROR- $\alpha$  is a transcription factor responsible for expression of epidermal differentiation makers [6]. Furthermore, melatonin, a potent antioxidant and circadian rhythm regulator that is closely related to ROR- $\alpha$ , is also expressed locally in skin [7].

Melatonin improves skin barrier functions and reduces the effects of aging through its antioxidant action potentially via Sirtuin 1 [8–10]. Sirtuin 1 controls different cellular process, including mitochondrial biogenesis, inflammation, cellular metabolism and epigenetic modulation. For Sirtuin 1 to be active, it needs to bind its coenzyme NAD<sup>+</sup>. With age, levels of Sirtuin1 and other NAD<sup>+</sup> dependent enzymes decrease [11,12]. Together Sirtuin1 and NAD<sup>+</sup> form a skin longevity complex.

Skin is a highly innervated organ and as a result is able to produce locally, cortisol and well-being mediators (dopamine, oxytocin and  $\beta$ -endorphin). These well-being factors are involved in skin regeneration and can therefore be a strategy to protect skin against cortisol [13–15].

Psychological well-being is highly impacted by skin conditions. Indeed, skin conditions of people with acne episodes, psoriasis or atopic dermatitis are linked to psychological distress [16]. In addition, stressful life events can themselves have an impact on the skin, which affects their self-esteem, sleep, and well-being.

We implemented a holistic approach to evaluate effects of a specific *Lactobacillus rhamnosus* GG postbiotic on skin well-being conditions.

## 2. Materials and Methods

### Keratinocyte treatment

Human primary keratinocytes were cultivated in 12 well-plates (Falcon) and kept in culture until they reached confluence. They were then treated with 3% *L. rhamnosus* GG postbiotic. For circadian rhythm assays, cortisol was then applied for 8 hours.

### Gene expressions

Expression levels of target genes (*Per1*, *Per2*, *Cry2*, *Sirt1*, *Ror- $\alpha$* , *Foxn1*, *Filaggrin*, *Involucrin*, *Corneodesmosin*) were monitored by q-RT-PCR. RNA extraction was performed using RNA extraction kit (Maxwell). RNA quantity was assessed using Nanodrop and 1  $\mu$ g RNA was reverse transcribed using iScript cDNA Synthesis kit (Biorad). Gene expression was assessed using SYBRGreen (Biorad, USA) and CFX technology (Biorad). Primers targeting PER1, PER2, CRY2, ROR $\alpha$ , FOXN1, Filaggrin, Involucrin, Corneodesmosin and Sirtuine1 were purchased from Biorad's library of validated primers. Fold-change of gene expression was calculated using  $\Delta\Delta C_t$  method.

### NAD<sup>+</sup>/ NADH dosage in keratinocytes

3 days post-treatment cells were lysed and NAD<sup>+</sup>/ NADH levels were quantified using a bioluminescence kit (Promega). NAD<sup>+</sup> and NADH levels were reported to total protein levels (BCA assay, Interchim)

### ATP production in keratinocytes

HaCaTs were seeded in 24-well plates (Falcon). Cells were then treated with 3% *L. rhamnosus* GG postbiotic for 2h. ATP was extracted and quantified using a bioluminescence kit (Sigma) according to manufacturer's instructions. Cells were fixed in histochoice (Interchim) and nuclei number was determined by Hoechst 33258 staining. ATP levels were normalized to cell number.

### Melatonin release: keratinocytes and fibroblasts

*Keratinocytes*: human primary keratinocytes were treated with 3% *L. rhamnosus* GG postbiotic and melatonin levels were assessed using LC-MS/MS.

*Fibroblasts*: Human primary fibroblasts were seeded in 24-well plates (Falcon) and treated for 72h with 3% *L. rhamnosus* GG postbiotic. Melatonin production was measured using Elisa Kit (R&D). Cell number was assessed using Hoechst 33342 and melatonin levels were reported to cell number.

### Keratinocyte-neuron co-culture: Oxytocin, Dopamine and MOR

Sensory neurons issued from hiPSC (human induced pluripotent stem cells) were incubated with 0.3% *L. rhamnosus* GG postbiotic. The next day cortisol was added to the culture. Keratinocytes were then seeded and the treatment was pursued for 5 days. Oxytocin and dopamine levels were measured by Elisa, while mu-opioid receptor (MOR) levels were quantified by immunofluorescent staining. Data were reported to cell number in order to allow comparison.

### Ex vivo assay

Skin explants from a 24 years old donor (Female, Caucasian, phenotype II) were treated with cortisol (in the media) and with 3% *L. rhamnosus* GG postbiotic (topically) for 6 days. Skin

explants were frozen and sectioned (7µm). ZO-1 staining was performed and quantified using ImageJ.

### **Clinical assays : skin regeneration, skin resilience and well-being questionnaires**

Women aged from 30 to 60 years old (mean age 49 years old) applied a face cream containing 3% *L. rhamnosus* GG postbiotic (24 volunteers) or placebo (20 volunteers) twice a day for 1 month.

Skin regeneration was measured using Easystiff® instrument (Biomeca).

For skin resilience, volunteers were asked to frown their forehead. Pictures were taken immediately after frowning. Measurements were performed to determine the ability of skin to recover after facial expression.

Finally, volunteers passed a well established well-being questionnaire [17].

### **Emotional impact : Electroencephalography (EEG) and Galvanic Skin Response (GSR)**

Before evaluating the emotional impact of *L. rhamnosus* GG creams, we compared creams containing placebo and postbiotic to ensure there was no perceived sensory difference that could bias our study. Then, a panel composed of 13 volunteers aged from 21 to 55 years old (mean age 48 years old) applied creams at the forearm. Neuronal activity was measured using an EEG EPOC headset (Emotiv) while arousal was determined via GSR using Shimmer3 device (Shimmer).

### **Sleep monitoring in volunteers**

21 women aged from 30 to 60 years old (mean age 50 years old) applied a face cream containing 3% *L. rhamnosus* GG postbiotic twice a day for 1 month. Sleep quality and duration were measured at home using an under-mattress sensor EMFIT QS® (Emfit).

### **Statistical analysis**

For in-vitro assays, one-way analysis of variance (ANOVA) was used to determine whether there was any significant difference between the variances of two or more independent groups. Difference between means with similar variances was performed with Student's t test.

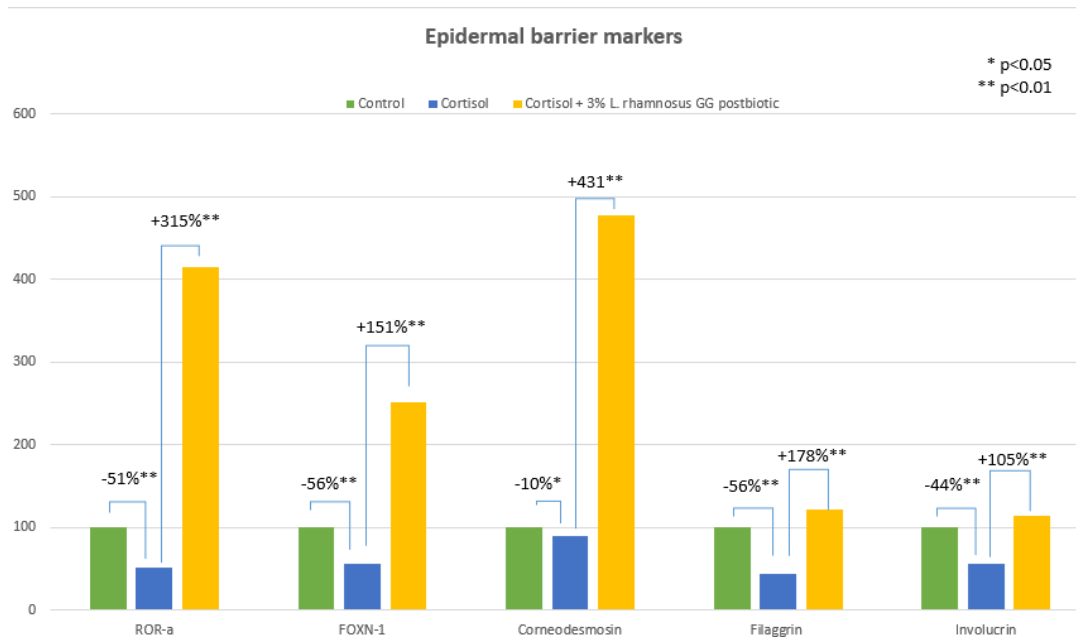
For clinical studies, statistical analysis were performed using Student's t test or non parametric Wilcoxon test or Mann-Whitney test.

P-values  $p < 0.05$  or  $p < 0.01$  were considered statistically significant.

## **3. Results**

### ***L. rhamnosus* GG postbiotic protects epidermal barrier against cortisol**

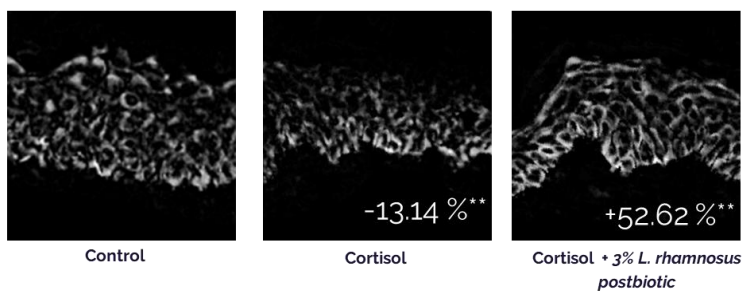
Prolonged cortisol exposure due to chronic stress is known to disrupt skin barrier. Keratinocytes were therefore treated with cortisol +/- 3% *L. rhamnosus* GG postbiotics. Expression of epidermal differentiation markers was decreased by cortisol (Figure 1). ROR-α positively regulates *FOXN1*, the master regulator of epidermal differentiation genes. *L. rhamnosus* GG postbiotic treatment counteracts cortisol by inducing expression of epidermal differentiation markers: ROR-α, *FOXN1*, Corneodesmosin, Filaggrin, Involucrin (Figure 1). *L. rhamnosus* GG postbiotic has therefore an impact in protecting skin barrier against cortisol *in-vitro*.



**Figure 1.** Pourcentage change of skin barrier markers vs untreated control (green bars), cortisol (blue bars) or cortisol + 3% *L.rhamnosus* postbiotic (yellow bars). \* p<0.05; \*\*p<0.01

Skin explants were treated with cortisol and/or 3% *L. rhamnosus* GG postbiotic. To mimic systemic cortisol, cortisol was added to the media, while postbiotic was applied topically on skin explant surface. ZO-1 was decreased by 13% (p<0.01) in explants treated with cortisol. In skin treated with both cortisol and postbiotic, ZO-1 levels increased by about 50% (p<0.01) compared to cortisol treatment alone.

#### ZO-1 production



**Figure 2.** Representative images of ZO-1 staining in skin explants: untreated (left), treated with cortisol (middle) or treated with cortisol + 3% *L.rhamnosus* postbiotic (right). \*\* p<0.01

*L. rhamnosus* GG postbiotic is therefore able to protect epidermal barrier against cortisol. This makes it a good candidate to fight impact of psychological stress on skin.

#### *L. rhamnosus* GG postbiotic protects circadian rhythm against cortisol

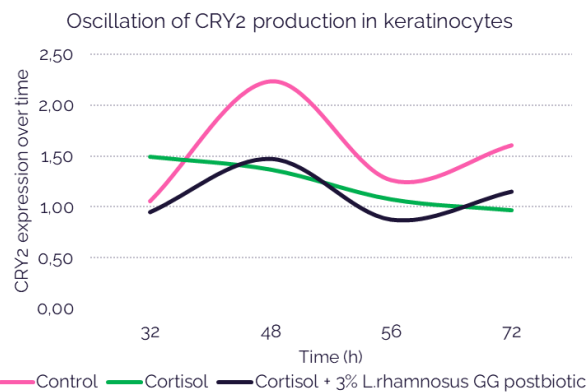
Besides its role in epidermal marker expression and therefore regeneration, ROR-α is a member of circadian clock. Since prolonged cortisol exposure is known to disrupt circadian rhythm, we decided to investigate the impact of *L. rhamnosus* GG postbiotic on the circadian rhythm of keratinocytes.

In only 8h, cortisol induced overexpression of PER1, PER2 and CRY2, while treatment with 3% *L. rhamnosus* GG postbiotic partially inhibited effect of cortisol at this time point (Table 1).

**Table 1.** Pourcentage change of gene expression. For cortisol treatment, data have been compared to control. For Cortisol + 3% *L. rhamnosus* GG postbiotic data are compared to cortisol. \*  $p < 0.05$ ; \*\* $p < 0.01$

Condition	PER1	PER2	CRY2
Cortisol	+840%**	+28%*	+53%**
Cortisol + 3% <i>L. rhamnosus</i> GG	- 51%**	-49%**	-36%**

Interestingly, for CRY2, cortisol treatment completely abolished rhythmic expression, while addition of 3% *L. rhamnosus* GG postbiotic, restored rhythmicity in CRY2 expression (Figure 3). This cyclic expression is crucial because it ensures that physiological processes are synchronized with the day-night cycle.

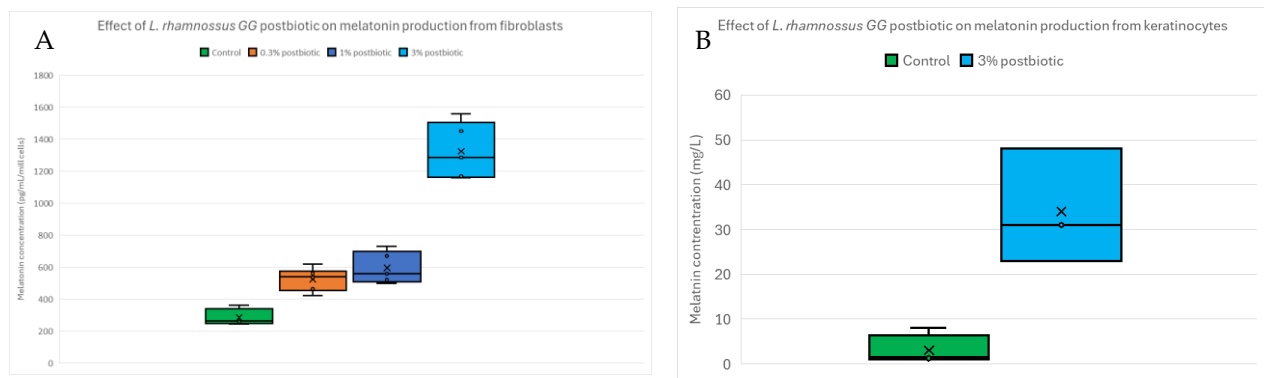


**Figure 3.** Expression of CRY2 during time in keratinocytes: untreated (pink curve), treated with cortisol (green curve), treated with cortisol + *L. rhamnosus* GG postbiotic.

Altogether these data, show that *L. rhamnosus* GG postbiotic protects keratinocyte autonomous circadian rhythm against cortisol.

### ***L. rhamnosus* GG postbiotic induces melatonin production in fibroblasts and keratinocytes**

Melatonin is a key regulator of the circadian rhythm. Melatonin and cortisol follow opposing expression patterns throughout the day. Interestingly, melatonin levels are positively correlated with ROR- $\alpha$ . Given that the postbiotic derived from *Lactocaseibacillus rhamnosus* GG influences both ROR- $\alpha$  and circadian rhythm, we investigated whether it could also modulate melatonin production. To assess this, melatonin levels were measured in fibroblasts (Figure 4A) and keratinocytes (Figure 4B). Treatment with 3% *L. rhamnosus* GG postbiotic induced production of melatonin in both fibroblasts and keratinocytes.



**Figure 4.** Melatonin production in fibroblasts (A) and keratinocytes (B) treated with *L. rhamnosus* GG postbiotic.

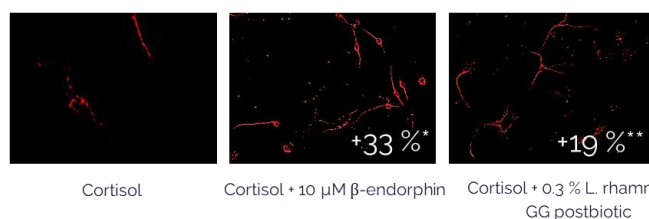
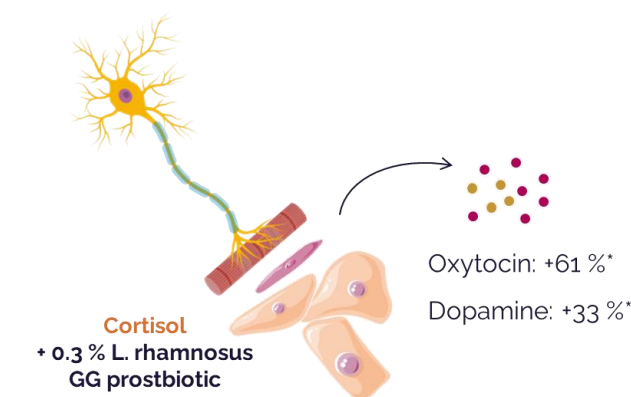
### ***L. rhamnosus* GG postbiotic: Sirtuin 1 – NAD<sup>+</sup>/NADH – ATP**

Given the increase in melatonin production, we wondered how *L. rhamnosus* GG postbiotic could impact Sirtuin 1 and NAD<sup>+</sup>. In keratinocytes treated with 3% postbiotic, Sirtuin 1 expression was increased by 91% ( $p < 0.01$ ) and NAD<sup>+</sup> production increased by 60% ( $p < 0.01$ ). In parallel, NADH production was also measured, and a 28% ( $p < 0.01$ ) increase was observed. NAD<sup>+</sup> and NADH are essential for ATP production. Since they both increased, we measured ATP levels in keratinocytes. As expected, *L. rhamnosus* GG postbiotic induces ATP production (+83%,  $p < 0.01$ ).

Altogether these data show that besides its effect on melatonin and circadian rhythm, *L. rhamnosus* GG is involved with longevity markers and boosts cell metabolism.

### ***L. rhamnosus* GG postbiotic fights cortisol by inducing well-being mediators in skin**

Another method to block cortisol negative impact on skin is by increasing local well-being mediators (oxytocin, dopamine and  $\beta$ -endorphin). Besides their impact in cell-cell communication,



these markers are all involved in skin regeneration. Increasing their expression, is a strategy to combat cortisol. In a keratinocyte-neuron co-culture treated with cortisol + 0.3% *L. rhamnosus* GG postbiotic, oxytocin production increased by 61% ( $p < 0.05$ ) and dopamine by 33% ( $p < 0.05$ ). We then monitored expression of MOR that has a high affinity for  $\beta$ -endorphin. Treatment of our model with  $\beta$ -endorphin induced MOR expression by 33% ( $p < 0.05$ ), while our postbiotic had also a  $\beta$ -endorphin like effect on this target.

**Figure 5.** Keratinocyte-neuron co-culture model and measurement of well-being mediators

Our pre-clinical data showed that *L. rhamnosus* GG postbiotic fights cortisol via different pathways: circadian rhythm, epidermal regeneration, melatonin increase, by boosting longevity markers and well-being mediators.

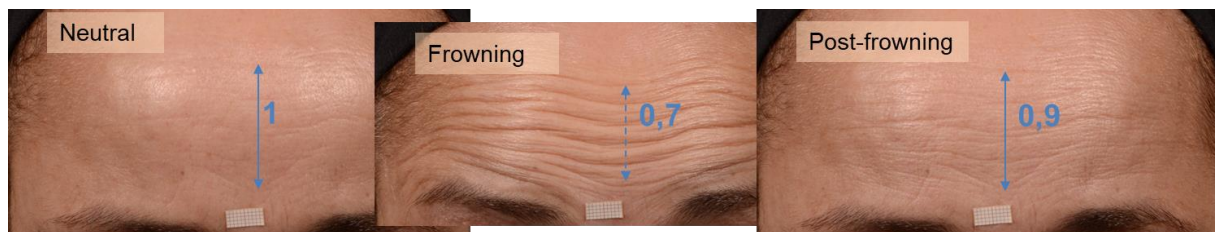
*How are these data translated to our skin?*

### **Increased skin barrier and resilience *in vivo* by *L. rhamnosus* GG postbiotic**

Skin regeneration is correlated with its stiffness. Increased tissue density and cell-cell interaction result in stiffer and thus more resistant tissue. Easystiff® applies a force to distort the skin and records its resistance. After 28 days of treatment with the cream containing the postbiotic, stiffness of stratum corneum and epidermis increased by 13% ( $p < 0.05$ ) and 14% ( $p < 0.05$ ) respectively, while placebo cream had no impact. These data are in line with those observed *in-vitro* on increase of epidermal differentiation markers and improved barrier.

### ***L. rhamnosus* GG postbiotic positively impacts skin resilience**

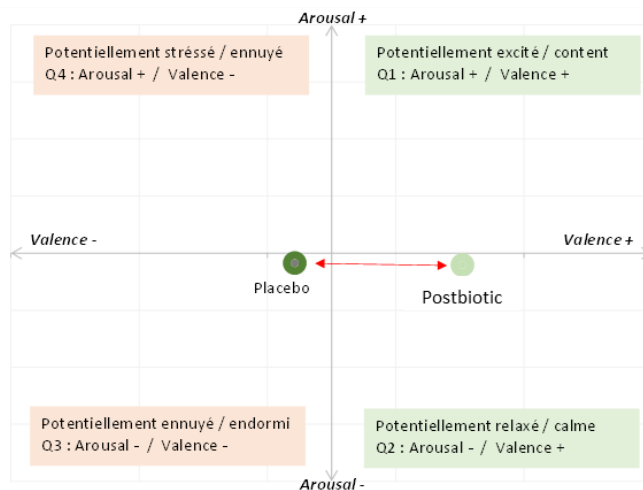
When stressed and worried, our face is also submitted to mechanical stress. As a result, expression lines are formed. In order to test impact of *L. rhamnosus* GG postbiotic on skin resilience, volunteers that applied the cream containing either the postbiotic or a placebo, were asked to frown their forehead. Ability of skin to recover after expression was measured as shown in figure 6. Data showed that skin resilience and tonicity were improved in 53% of volunteers that were treated with the cream containing the postbiotic and only in 17% of volunteers treated with placebo cream ( $p < 0.05$ ). *L. rhamnosus* GG postbiotic has therefore a significant impact on skin resilience.



**Figure 6.** Representation of measurement of skin resilience on volunteers.

### **Emotional impact of *L. rhamnosus* GG postbiotic**

Positive emotions are associated with higher activity in the brain left hemisphere while negative emotions correlate with right hemisphere [18]. Excitation status (arousal) on the other hand can be determined through skin conductance. To assess emotional impact of *L. rhamnosus* GG postbiotic, measurement of neuronal activity (valence) and skin conductivity (arousal) were measured immediately after creams application.



The Valence/Arousal representation shows that placebo and postbiotic position are different. Interestingly, while volunteers treated with placebo are in a more “annoyed” state, postbiotic-cream induced a feeling of relaxation and calm (Figure 7).

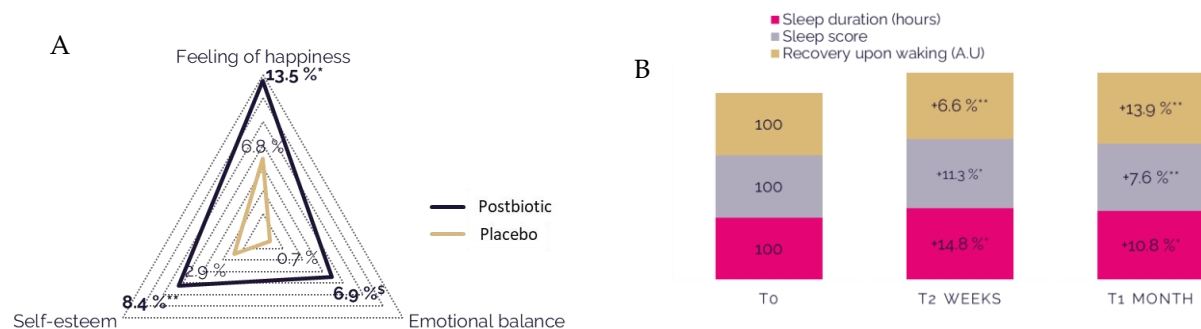
**Figure 7.** Representation of emotional impact of creams containing placebo and postbiotic

### ***L. rhamnosus* GG postbiotic improves well-being and sleep**

Because stress deeply impacts our well-being and sleep, we decided to focus on these two parameters.

Volunteers were asked to fill a well-being questionnaire established by Massé *et al.*[17] before D0 and at the end of treatment. These questionnaires showed that in volunteers that received cream containing the postbiotic, happiness, self-esteem and emotional balance were significantly improved when compared to placebo (Figure 8A).

Sleep was monitored by under-mattress contact-free sensors. Data showed that volunteers sleep longer, better and recover better after waking up (Figure 8B).



**Figure 8.** Impact of *L. rhamnosus* GG on well-being questionnaire (A) and sleep (B). \* $p < 0.01$ ; \*\* $p < 0.05$ ; \$ $p < 0.08$ .

## **4. Discussion**

Interest in neurocosmetics has been growing both from a market and a scientific perspective. Claiming an effect on holistic well-being from a cosmetic ingredient is a real challenge as well-being is the essence of cosmetics.

With this challenge in mind, we designed an *in-vitro* and *in vivo* approach to evaluate the impact of an active ingredient against stress.

We first implemented an *in-vitro* assay to mimic the impacts of psychological stress on skin. Keratinocytes, keratinocyte-neurons co-cultures and skin explants were treated with cortisol, the stress hormone. Impact of cortisol on skin barrier, circadian rhythm and well-being related mediators (oxytocin, dopamine and  $\beta$ -endorphin receptor) were evaluated *in-vitro*. Cortisol disrupted both skin barrier function and circadian rhythm in vitro. However, treatment with *Lactibacillus rhamnosus* GG postbiotic protected against cortisol-induced skin damage and significantly increased levels of well-being mediators (oxytocin and dopamine), as well as  $\beta$ -endorphin receptor expression.

In our clinical trials, we focused on visible signs of stress and fatigue on the skin. The data showed that a cream containing *L. rhamnosus* GG postbiotic improved skin regeneration and resilience—findings that were consistent with our in vitro results. Given the strong link between skin appearance, stress, and well-being, we then adopted a holistic approach focusing on well-being, emotional status and sleep of our volunteers. Volunteers who applied the postbiotic-containing cream reported overall improvements in well-being. *L. rhamnosus* GG postbiotic had an immediate relaxing/calming effect on the brain. Finally, measurements on sleep duration and quality showed a significant improvement.

Our innovative and complete protocol allowed us to study the impact of psychological stress and that of a cosmetic ingredient with a holistic approach.

## 5. Conclusion

In this study we were able to show the positive impact of a postbiotic issued from *L. rhamnosus* GG, against stress-related signs on skin. Skin condition was improved. Mechanism of action include protection of skin circadian rhythm, increase of longevity markers, metabolism boost and increase of well-being mediators. Significant impact of *L. rhamnosus* GG on skin was translated into improved self-esteem and overall wellbeing leading to better sleep. Sleep quality, in turn, affects skin quality. Our active ingredient therefore creates a true virtuous circle that benefits the skin.

## 6. References

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