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Article *in* European journal of dermatology: EJD · October 2010

DOI: 10.1684/ejd.2010.1108 · Source: PubMed

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Article accepted on 23/8/2010

Lactobacillus paracasei CNCM I-2116 (ST11) inhibits substance P-induced skin inflammation and accelerates skin barrier function recovery *in vitro*

Over the past few decades the number of people presenting reactive skin has increased in industrial countries. Skin inflammation mediated by neuropeptides and impaired skin barrier function are both underlying features of reactive skin conditions. Live microorganisms defined as probiotics have been successfully used to improve health status in humans. Beyond the effects on intestinal microbiota, some probiotic strains display potent immune-modulatory properties at the skin level. The aim of this study was to evaluate whether *Lactobacillus paracasei* CNCM-I 2116 (ST11) could modulate reactive skin-associated inflammatory mechanisms. The Caco-2/PBMC co-culture cell system was stimulated on the apical side with probiotics. The resulting medium collected from the basolateral compartment of the cell culture system, so called conditioned medium, was tested in *ex vivo* human abdominal plastic skin explant models of substance P-induced skin inflammation and skin barrier reconstruction. We show that ST11 was able to abrogate vasodilation, edema, mast cell degranulation and TNF-alpha release induced by substance P, compared to control. Moreover, using *ex vivo* skin organ culture, we show that ST11-conditioned medium induced a significantly faster barrier function recovery after SLS disruption, compared to control. These results support a beneficial role of ST11 on key biological processes associated with barrier function and skin reactivity.

Key words: inflammation, *lactobacillus paracasei*, probiotics, reactive skin, skin barrier function

The term probiotic, popularized by Fuller [1], was recently defined by an expert committee as "living microorganisms which, when consumed in adequate amounts, confer a health effect on the host" [2]. The most often used probiotic genera in humans and animals are enterococci, lactobacilli and bifidobacteria, which are natural inhabitants of the intestinal tract.

Beyond their capacity to positively influence the composition of intestinal microbiota [3-6], several lines of evidence suggest that some probiotic bacteria can modulate the immune system, both at local and systemic levels [7-9], thereby improving immune defense mechanisms and/or down-regulating immune disorders such as allergy or intestinal inflammation [7, 10, 11]. Several strains of lactic acid bacteria have been shown to modulate cytokines and growth factor production *in vitro* and *in vivo* [12-14]. Indeed, results from different pre-clinical and clinical trials have revealed the ability of various probiotic strains to enhance non-specific and specific immunity [15-22]. Moreover, recent human clinical trials widely suggest that probiotic supplementation might be useful in the management of atopic dermatitis and dry skin [10, 11, 23-26].

About half of women and a third of men report having sensitive skin [27, 28]. Subjects with sensitive skin primarily complain of cutaneous discomfort. The main manifestations of this "cutaneous discomfort" are neurosensory signs such as feelings of heat, burning, stinging or itching [29-31]. Symptom onset is triggered by several factors. The factors may be environmental (temperature changes, heat, cold, wind, sun, air pollution, etc.) or consist of the application of certain topical products such as "hard" water, or internal factors (emotional factors, menstrual cycle, dietary factors). The homeostatic hydration level of the epidermis is related to the status of the skin barrier and the interrelationships between the cell components and their lipid environment. Lipids are involved in the rate of trans-epidermal water loss (TEWL). Disruption of the skin barrier primarily gives rise to an increase in TEWL. Impairment of the skin barrier most frequently presents in the form of "dry" skin, or xerosis, imparting a dull color to the skin, which appears fragile with visible scaling.

The pathophysiology of reactive skin consists of an inflammatory reaction resulting from the abnormal penetration in the skin of potentially irritating substances,

doi: 10.1684/ejd.2010.1108

which occurs due to skin barrier dysfunction and changes in the production of local neuromediators, like substance P [32].

In addition to the development of soothing active ingredients for topical application, the development of new approaches enabling alleviation of symptoms related to reactive skin by the use of active substances administered by the oral route appears highly attractive. In that context, nutritional approaches, including using live microorganisms such as probiotics has gained high interest. Given the reported immune modulatory property of the *Lactobacillus paracasei* ST11 probiotic strain both *in vitro* and *in vivo* [33, 34], we hypothesized that this strain could impact on the skin immune system and antagonize inflammatory reactions underlying reactive skin conditions.

Using *in vitro* models, we demonstrate here that *L. paracasei* ST11 modulates neurogenic inflammation mediated by substance P as well as barrier function activity.

Material and methods

Probiotic cultures

Ready to use vials of freeze-dried powders of the probiotic strain *Lactobacillus paracasei* CNCM I-2116 (ST11) were from Nestlé culture collection (NCC2461). Fresh overnight cultures into deMan Rogosa Sharpe (MRS) medium were used in the experiments. Bacterial counts were determined by serial dilutions and plating onto MRS agar plates. Bacteria were washed three times with PBS (Gibco BRL, Basel, Switzerland) and diluted to obtain final cell densities of 1×10^7 CFU/mL in RPMI 1640 medium (Gibco BRL).

Epithelial-peripheral blood mononuclear cell co-cultures

The method was adapted from Haller *et al.* 2000 [35]. Briefly, Human enterocyte-like Caco-2 cells were seeded on 10.5 mm inserts (0.4 µm nucleopore size, Becton Dickinson, Basel, Switzerland) at 10^6 cells/well. The inserts were then cultured in a 12-well plate (Nunc) for 21 days at 37 °C/10%CO_2 in DMEM supplemented with 10% FCS and 0.1% penicillin/streptomycin (10,000 IU/mL, Gibco BRL). Human peripheral blood mononuclear cells (PBMC) from healthy donors were purified from buffy coats (transfusion Centre, Lausanne, Switzerland) by centrifugation through a Ficoll-Hypaque 1077 column (Pharmacia, Dübendorf, Switzerland). PBMCs were suspended in a complete RPMI medium supplemented with human AB serum (Gibco BRL) at a final concentration of 2×10^6 cell/mL. When Caco-2 cells established a confluent polarized monolayer, the inserts were washed twice and the PBMC (1ml) were added to the basolateral compartment of transwell cultures. The co-cultures thus established were stimulated by adding 1×10^7 CFU/mL of probiotics at the apical surface of the monolayer of epithelial cells. The system was then incubated for 16 hours at 37 °C/5%CO_2. To avoid the growth of bacteria, 150 µg/mL of gentamicine was added to the medium after 4 hours of incubation (shown in *figure 1*). At the end of the optimal incubation period (16 h) the ST11-conditioned medium (ST11-CM) from the basolateral compartment was collected for testing in a model of neurogenic skin inflammation and skin barrier function *(figure 1)*.

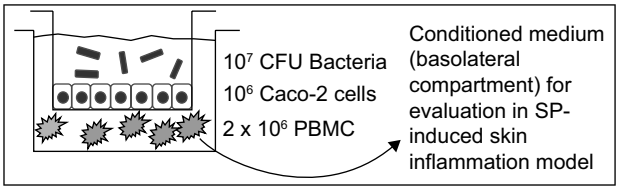

Figure 1. Schematic representation of the production of conditioned medium tested in models of skin inflammation and barrier function.

Organ culture of human skin

Human skin samples (n = 8) were obtained from plastic surgery on patients (Caucasian women 35 to 45 years old). Formal patient consent was obtained. They were washed three times with antibiotic solution and then cut into 1 cm^2 full-thickness pieces. Subcutaneous fat and lower dermis were mechanically removed under a stereomicroscope using a surgical scalpel.

Skin samples were then placed on culture inserts (filter pore size 12 µm; Costar, Poly-Labo Paul Block, France) with the epithelium uppermost at an air/liquid interface. The inserts were set on 12 well plates (Costar) and culture medium was added to the wells so that the surface of the medium reached the filter level. Organ cultures were performed using Dulbecco's minimal essential medium (Gibco BRL) containing antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin; Gibco BRL, USA), 200 µg/mL L-glutamine (Gibco BRL), bovine pituitary extract (to promote the survival of skin explants), epidermal growth factors and fetal calf serum (DAP, France). Cohesion between skin and insert was obtained with a polysiloxane vinyl seal in such a way that neither skin retraction nor lateral passage of any applied topical product towards the dermis was possible. Before the start of the experiment, skin samples were kept under these survival conditions for 5 hours at 37 °C in a humidified incubator with 5% CO_2. In order to test the effect of conditioned medium in substance-P (SP) treated skin samples, skin culture medium was replaced with a medium containing 30% (maximum amount of ST11-CM with no cytotoxic activity) of CM obtained from co-cultures stimulated or not with ST11. Ten µM of SP (Bachem, Switzerland) suspended in DMEM were added to the culture medium and skin samples were maintained in culture for 24 additional hours. Skin inflammatory status was then assessed by histological evaluation and measurement of histamine and TNF-alpha release.

Histological evaluation

After 24 hours, skin samples were removed from the inserts, set in Bouin's liquid and embedded in paraffin. Thick sections of 5 µm were stained with hematoxylin and eosin to evaluate vasodilation, edema and mast cells. Toluidine blue staining (0.1% in 50% ethanol; pH 3.5) was used for mast cell counts. Sections were evaluated under Olympus light-photomicroscope and photographed with Ektachrome 64T film.

Histological evaluation of the inflammatory reaction was performed on papillary dermis and on the upper part of

reticular dermis. Double blind assessment was carried out by two investigators through visual scoring. Four slides per skin sample were evaluated for each treatment.
a) Microvascular vasodilation: After staining with hematoxylin & eosin, vascular dilatation was evaluated by counting the number of vessels dilated over the histological section (16 fields at 40× magnification). This number was related to the total number of vessels to calculate the percentage of dilated vessels.
b) Edema: Evaluation was performed using scores: 0- no edema, 1- slight edema, 2- moderate edema, 3- marked edema and 4- very important edema
c) Mast cells: (characterized by toluidine blue staining) was scored as follow: The mast cells present in the dermis are revealed in blue-violet by the toluidine blue stain. Histologically, a more or less intense blue-violet and granular appearance of the mast cells was observed, in combination with the more or less significant presence in their cytoplasm of basophilic and metachromatic granulations, notably containing histamine. The degranulated mast cells were then counted in an optical microscope (15 fields at a 40× enlargement on 3 sectional planes). The degranulation obtained after application of substance P is responsible for a reduction or absence of toluidine blue stain in the mast cells. This reduction in stain is related to the reduction or more or less complete disappearance of the number of granulations initially present in the cytoplasm of the mast cells (mast cells containing a small number of basophilic granulations). The results are expressed in the following manner: for each subject, the percentage of mast cells for each score was calculated relative to the total number of mast cells.

Measurement of TNF-alpha

The TNF-alpha level was measured in skin culture medium using commercially available enzyme immunoassay kits (Chemicon International, USA). The results were expressed in pg per ml of culture medium.

Skin barrier function recovery after SLS treatment using organ culture of human skin

Human abdominal plastic surgery skin sample from 4 donors, randomly selected, was set on Franz cell in an ambient atmosphere *(figure 2)*. The skin sample was pretreated with 30% of conditioned media (3 h after setting of the skin) for 24 h and maintained throughout the test, then the surface of the skin sample was treated with SLS 10% for 3 h to alter the barrier function (reversible alteration). Skin barrier function recovery was assessed over 4 days post challenge by measuring the trans-epidermal water loss using Servomed® evaporimeter [36-38].
The TEWL were measured and the % were calculated based on a ratio between TEWL before (considered as 100%) and after SLS treatment (considered as X%). For example in volunteer 1, before SLS, TEWL was 2.9 g/m2/h and after SLS was 17.4 g/m2/h : in this case the variation % is 600% of TEWL after SLS (see *figure 5* high responder).

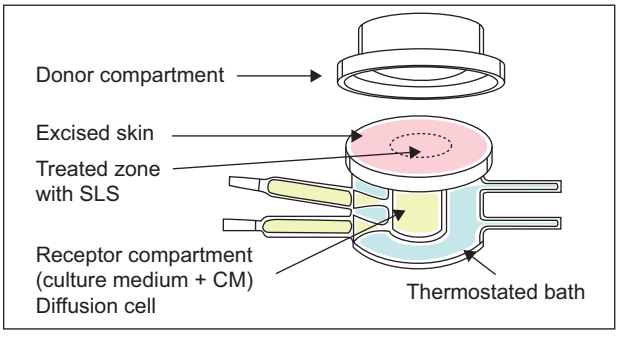

Figure 2. Schematic representation of the *ex vivo* skin organ culture system to assess skin barrier function.

Statistical analysis

The statistical analysis was performed using the Student reduced-scale test or a pairwise test, with a risk of 5%.

Results

Part 1. Effect of ST11 on neurogenic inflammation induced by substance P

In order to test for the effect of ST11 on the SP-induced skin inflammation, normal human skin from 8 donors was treated with 10 μM SP diluted in medium containing 30% of conditioned medium obtained from co-cultures (Caco-2/PBMC) stimulated or not with ST11 for 24 hours, as shown in *figures 1 and 2*. Histological changes (edema, vasodilatation and mast cell numbers) and measurement of TNFα in the culture supernatant of SP-treated versus untreated skin were carried out at 24 hours.

Histological changes in edema and vasodilation

When applied directly to the culture medium, SP induced vasodilatation in the superficial dermis: the global % of capillary dilatation was significantly increased by approximately 30%, compared to control. The capillary surface was also markedly increased, as indicated in *figure 3* A. These effects were still observed using non-stimulated CM. Interestingly, the application of the ST11-CM prevented the increase in vessel dilatation *(figures 3A, B)*.
As expected, adding SP to culture medium significantly enhanced the edema score by a factor of 2 compared to control. The same observation was made when using non-stimulated CM. ST11-CM prevented the SP-induced increase in edema scores and maintained them at a level similar to non-challenged control skin *(figure 3C)*.

Mast cell degranulation

SP induced a statistically significant increase in mast cell degranulation as reflected by a significant decrease in highly toluidine blue stained granulated mast cells *(figures 4A and B)*. Mast cell degranulation induced by SP was not affected by adding non-stimulated conditioned medium but was prevented by the application of the ST11 conditioned media.

TNF-alpha levels after SP stimulation

When SP was added to culture medium of *ex vivo* surviving skin, we observed a significant increase in TNF-alpha

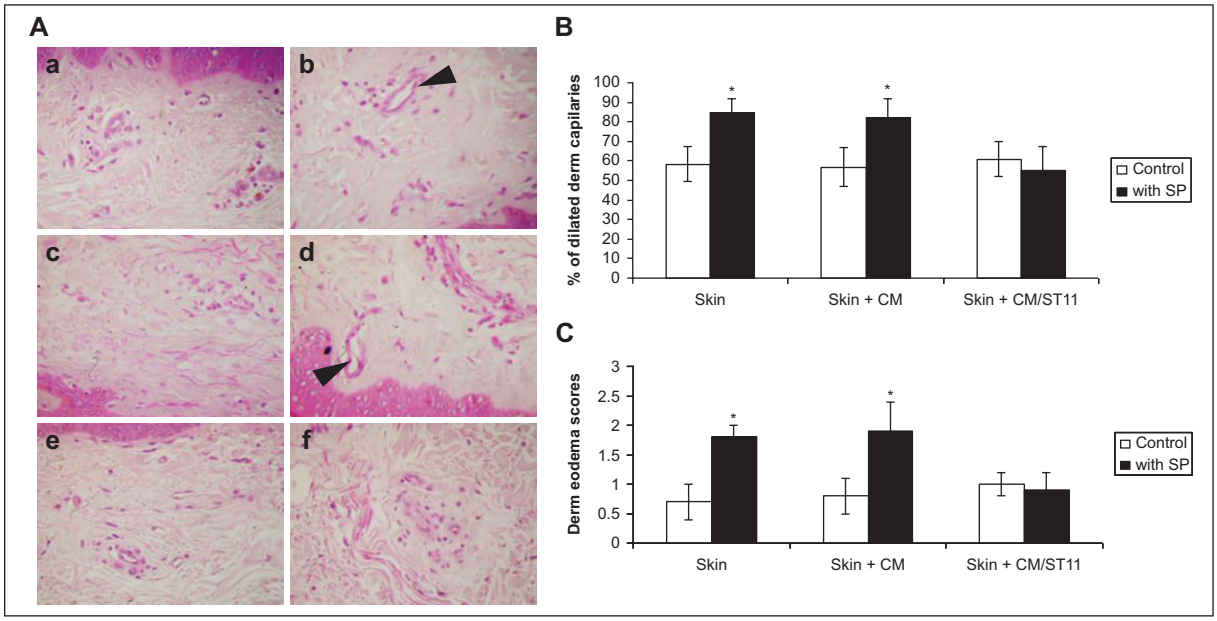

Figure 3. Effect of ST11-condionned media on dilated derm capillaries and edema of Substance P-stimulated skin explants. **A)** Skin histology (hematoxylin & Eosin). Magnification × 400 (1 cm = 19 µm). Arrow head indicates derm capillaries vasodilatation (a. Control Skin, b. Control Skin + SP, c. Skin+CM, d. Skin+CM + SP, e. Skin+CM/ST11, f. Skin+CM/ST11 + SP). **B)** % of dilated derm capillaries. **C)** Semi-quantitative histological evaluation of derm edema scores. *$P < 0.05$ compared to control.

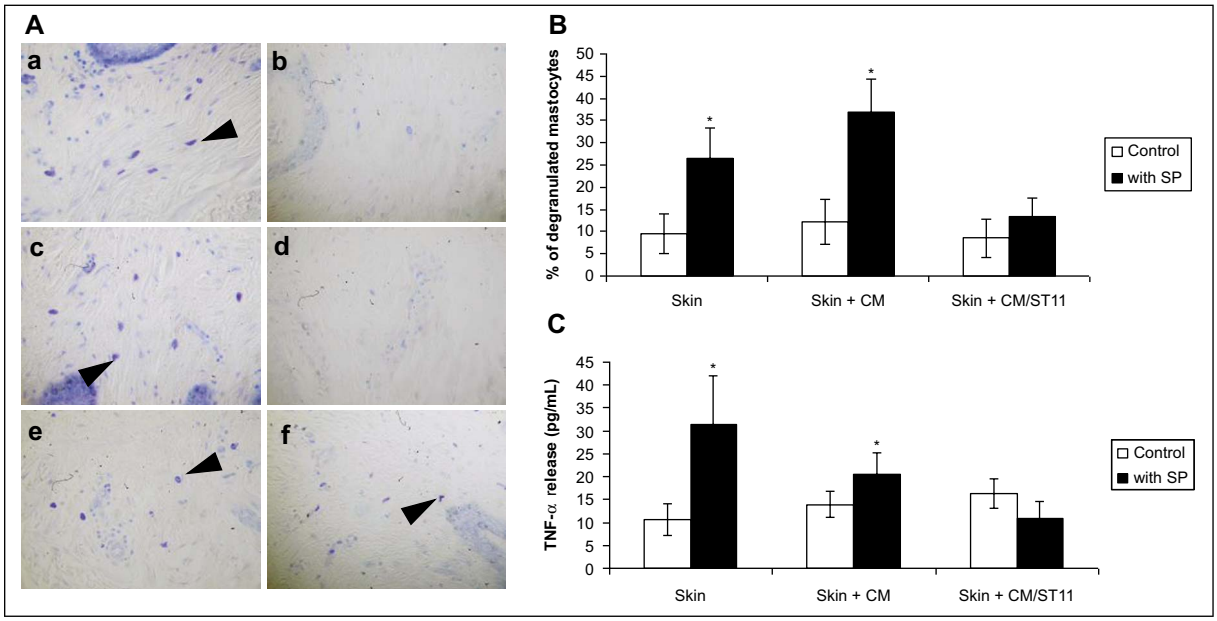

Figure 4. Effect of ST11-conditioned media on degranulated mastocytes and TNF-α expression of Substance P-stimulated skin explants. **A)** Histological evaluation of mastocyte degranulation after toluidine blue staining. Magnification × 400 (1cm=19µm). (a. Control Skin, b. Control Skin +SP, c. Skin+CM, d. Skin+CM + SP, e. Skin+CM/ST11, f. Skin+CM/ST11 + SP). Arrow head indicates mastocyte granules. **B)** Semi-quantitative analysis of the % of degranulated mastocytes. **C)** TNF-α release (ELISA). *$P < 0.05$ compared to control.

level by a factor of 3 compared to non-challenged control skin. Using non-stimulated conditioned medium led to a lesser, but still significant, increase in TNF-alpha *(figure 4C)*. Applying ST11 conditioned media prevented the release of TNF-alpha. Levels were maintained unchanged as compared to non-challenged control skin.

Part 2: Skin barrier function recovery

To test for the effect of ST11 on the skin barrier function recovery, normal human skin from 4 donors was pre-treated with conditioned medium from Caco-2 cells/PBMC/ST11 co-cultures for 24 hours, as shown in

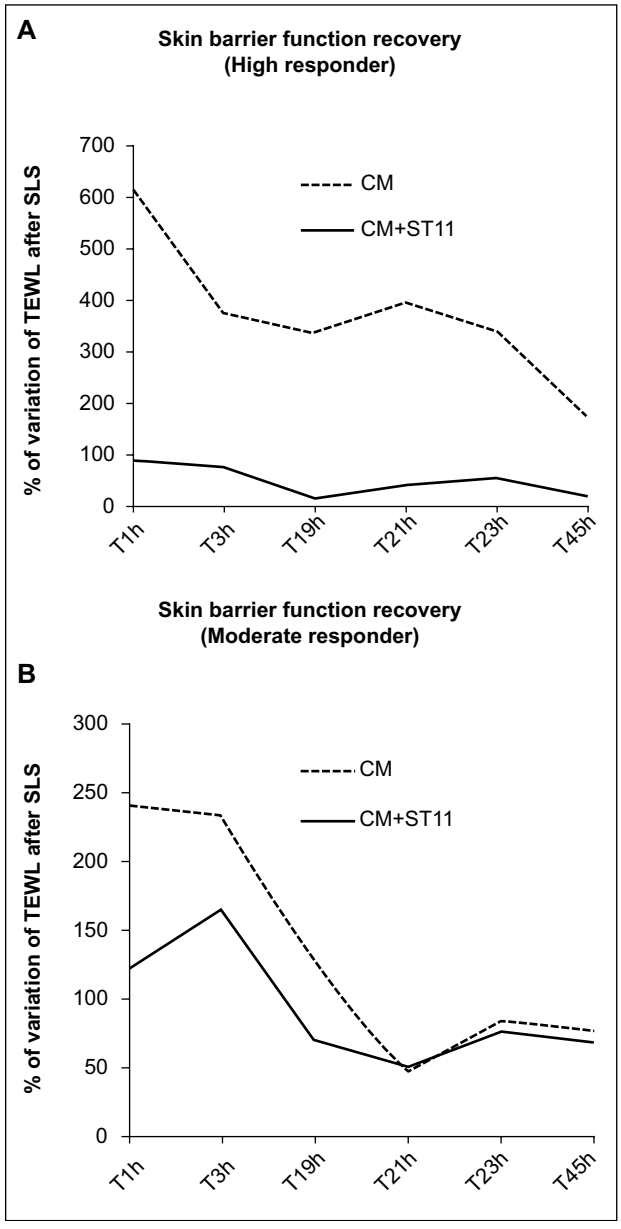

Figure 5. Kinetic of skin barrier function restoration after SLS application (% of variation of Trans Epidermal Water Loss). Representative values from a skin donor highly **(A)** and moderate **(B)** responder to SLS treatment at D1-T1h.

figure 5, and then exposed to SLS 10% for 3 hours. Skin barrier function was repeatedly measured by TEWL during 4 days.

Samples from 2 of 4 donors were highly reactive to SLS application. These samples presented a strong 5-6 fold increase in TEWL 1h after SLS application and a mean recovery time of 45h. As shown in *figure 5A*, for a representative high responder to SLS, the addition of ST11 conditioned medium protected strongly from SLS-induced increase in TEWL, since after SLS only a 100% increase of TEWL was observed instead of the 600% increase obtained with non-stimulated conditioned medium.

The two other donors behave as moderate responders to SLS-induced alteration of skin barrier since these samples presented a mild 1.5 increase in TEWL 1 h after SLS painting and a mean recovery time of 21 h *(figure 5B)*. The addition of ST11 conditioned medium strongly inhibited the alteration generated by SLS and allowed a faster recovery of the barrier function compared to non-stimulated conditioned medium or control.

Discussion

A number of authors have suggested a role for impairment of barrier function in the onset of sensitive skin [28, 39 and, 40]. Failure in barrier function is reported to be responsible for enhanced penetration of potentially irritant substances which are considered as the primary triggering factor. Impairment of the skin barrier most frequently presents in the form of "dry" skin, or xerosis, imparting a dull color to the skin, which appears fragile with visible scaling [41, 42]. These features are reflected by a strong increase in the rate of TEWL. This is commonly assessed by a repeated tape-stripping test in the clinic. In our study we developed a new system of *ex vivo* skin organ culture with a topical SLS challenge that mimics the TEWL phenomenon associated with alterations of the skin barrier function usually observed in reactive skin conditions.

In addition to skin barrier function alteration, skin sensitivity is generally characterized by an exacerbated reactivity of sensory nerves associated with neurogenic inflammation [43-45]. Neurogenic inflammation is triggered when a massive release of mediators, particularly SP derived from sensory fibers, occurs and the phenomenon extends outwards via the axonal reflex. Thus, the axonal reflex induces spatial and temporal amplification of the inflammatory process [46-48]. A number of events occur subsequently, including mast-cell degranulation and changes in the contractility of smooth muscle fibers in the vessels (vasodilatation, extravasation).

Topical application of an irritant substance or environmental stimulation induces vascular changes and the production of inflammatory mediators. As indicated above, substance P is one of the main neuropeptides that trigger skin inflammatory responses. In our *in vitro* system, even in the absence of systemic blood circulation, SP addition to culture medium of surviving skin induced a dose dependent edema, vasodilation and extravasation of lymphocytes through microvascular walls and mast cell degranulation. Moreover, the inflammatory response was associated with the release of pro-inflammatory mediators, such as TNF-alpha.

Our data shown that treatment with ST11 was able to promote the recovery and maintenance of the skin barrier function, since ST11-CM strongly reduced TEWL upon SLS challenge as compared to control. Moreover, the study supports the ability of the probiotic to antagonize SP-mediated skin inflammatory reactions. This was reflected in a significant decrease in SP-induced vasodilatation, edema scores and release of TNF-alpha. Of note, a similar trend was observed for the release of histamine, although the effect did not reach statistical significance (data not shown).

The mechanism underlying immune modulation by probiotics involves, in part, regulation of the composition and/or metabolic activity of the gut microbiota. However, a direct interaction of probiotics (and/or bacterial compo-

nents) with the immune system underlying the gut mucosa has also been proposed. Indeed, effects on immunocompetent cells and regulation of pro-inflammatory cytokine production have been reported [10, 11]. Among the immune cells, dendritic cells play a major role in sensing signals from luminal bacteria and/or antigens (reviewed by Uhlig and Powrie 2003 [49]. Furthermore, it was demonstrated that mucosal dendritic cells could express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria and antigens directly in the intestinal lumen [50, 51].

It is postulated that following interaction of probiotics with the intestinal epithelium, associated immune cells become activated and consequently immune mediators such as cytokines are released into the blood circulation. Cytokines activate immune cells and possibly bacterial fractions might reach the skin through the bloodstream where they potentially could exert immune modulation effects. In that context, it has been suggested that the immune-modulatory properties of certain strains of probiotics at the skin level may modulate the inflammatory reactions generated by the release of neuromediators involved in cutaneous neurosensory functions [52, 53].

Our strategy was to evaluate such benefits in relevant *in vitro* models. For that purpose the probiotic strain *Lactobacillus paracasei* ST11 was used to stimulate enterocyte-leukocyte co-culture systems that mimic the intestinal epithelium and related bacteria-cell interactions [35]. A fraction of the culture medium enriched with molecules and mediators secreted upon these interactions (so called conditioned medium), was collected and tested in *in vitro* models of skin inflammation and barrier function, as described above. It has previously been shown that ST11 was able to induce an increase in the production of systemic regulatory cytokines such as TGF-beta and IL-10 in mice [13]. Expression of IL-8, TGF-beta, IL-10, IL-12, IFN-gamma, and TSLP was assessed in the ST11-CM versus control CM. However no significant differences were observed after 16h of incubation (data not shown). Besides, IL-4, IL-2 and IP-10 were undetectable in both ST11-CM and CM. Nevertheless, the consistent preventing effect against SP-mediated inflammatory reaction, as well as against topical SLS-induced alteration of the skin barrier observed in the skin culture systems, revealed that, so far, non-identified bioactive compounds are effectively released in the medium. Moreover, priming of immune cells and the development of anti-inflammatory cell phenotypes is also a possible mechanism and would deserve further investigation. For instance, it was reported that commensal bacteria have the potential to commit mucosal dendritic cells towards an anti-inflammatory phenotype in similar *in vitro* experimental settings [54].

All together, our data demonstrates a beneficial role for ST11 in reinforcing skin barrier function recovery and antagonizing neurogenic skin inflammation and therefore suggests that an oral application of such probiotic strains may be an effective approach to prevent or treat reactive skin conditions. ■

Disclosure. *Conflict of interest: none. The authors have no financial support or relationships that may pose conflict of interest by disclosing at the time of submission any financial arrangements they have with a company whose product figures prominently in the submitted manuscript or with a company making a competing product. However, Audrey Gueniche, Philippe Bastien, Lionel Breton and Isabelle Castiel Higounec are employees of L'OREAL and Jalil Benyacoub, David Philippe, Nicole Kusy and Stephanie Blum are employed by Nestlé.*

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