Effect of *L.pentosus* extract on host defence and microbiota

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Abstract

The human skin is a complex ecosystem that plays the role of a barrier against environmental aggressions. In this system, microorganisms colonise our skin and are essential to skin immunity and physiology. These dynamic interactions play a crucial role in establishment of the protection against pathogens by spatial and nutritional competition, production of antimicrobial peptides and stimulation of host immunity. Production of antimicrobial peptides by epithelia is an essential defence against infection by pathogens. The protection ensured by the host defence peptides (HDPs) is widely recognised for multifunctional roles in both the innate and adaptive immune responses.

Using an *ex vivo* skin model approach, we evaluated whether an extract from the bacteria *Lactobacillus pentosus* (Lp) may enhance the production of host defence peptides such as (psoriasin) S100A7 and ß-defensin 2 (hBD-2). We also evaluated the compatibility of Lp extract with the skin microbiota of healthy volunteers. The skin microbiota analysis was performed based on 16 rRNA gene sequencing to observe the dynamism of populations and the diversity after 7 days of application. We noted a significant increase of 237% ($p<0.01$) for S100A7 and 229% ($p<0.01$) for hBD-2). *L. pentosus* extract maintains the preservation of healthy status of skin microbiota through the composition of commensal bacteria and protection against pathogen invasion.

The skin is constantly exposed to external and internal disruptive factors (ultraviolet radiation, pollution) that can alter the balanced relationship between the skin and its microbiota.[1] Disruption may result in increased risk for infections, chronic inflammatory skin diseases (atopic dermatitis, psoriasis, acne) and complaints of sensitive and irritated skin.[2] To prevent disruption, skin has established a complex system of immune control composed by the actions of epithelial cells, lymphocytes and antigen-presenting cells.[3] One of these fundamental processes involves defined components of the skin microbiota called PAMP (pathogen-associated molecular pattern) that can bind to the innate immune receptor, Toll-like receptors (TLRs) (Fig 1). This family of receptors is able to modulate the defense of the host, via the innate immunity and the inflammatory response.[4,5] Skin commensal microorganisms can participate to the regulation of the expression of various innate immune factors, including Host defence peptides (HDPs). HDPs belong to multiple protein families, which, in the skin are dominated by cathelicidins and ß-defensins.[6] To date, only four ß-defensins and one cathelicidin have been identified in human skin. Their expression has been studied in healthy as well as inflamed skin (e.g. atopic dermatitis and psoriasis). The defensins is the most represented family in mammals with a broad spectrum of actions against bacteria, viruses and fungi. The antimicrobial activity of human ß-defensin-2 has been reported to be predominantly effective against gram-negative bacteria such as *Escherichia coli* and *Pseudomonas aeruginosa* and less against *Staphylococcus aureus*.[7] Within the epidermis, ß-defensins following exposure to microorganisms is differentially expressed according to the degree of keratinocyte differentiation.[8] Among the HDPs, the S100 proteins, low molecular weight proteins are involved in multiple functions in many cell and tissue types. It appears that S100 proteins serve as calcium

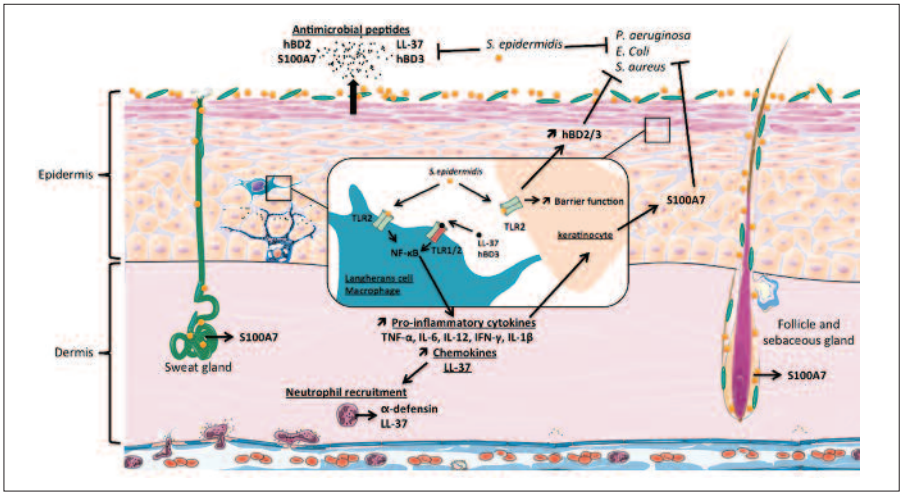

Figure 1: Relationship between skin microbiota and skin cells. Skin is constantly exposed to a variety of microbial challenges, in particular to avoid the growth of pathogenic bacteria like *P. aeruginosa, E. coli* or *S. aureus*. The first line is the physical barrier, which is supported by the microbiota, antimicrobial lipids, and antimicrobial proteins. Toll-Like-Receptor (TLR) recognize the bacteria patterns present on the surface of skin. Activation of the Toll-Like-Receptor (TLR-2) on keratinocytes improves the barrier function of skin and increase production of β-defensins (hBD-2 and -3), which inhibits the growth of pathogenic bacteria. On antigen presenting cells (APC) activation of TLR-2 induce activation of transcription factor NF-KB, which upregulated different genes involved in immune response, like cytokines, chemokines and antimicrobial peptides. Antimicrobial peptides hBD3 and LL-37 can activate TLR pathway. Chemokine secretion leads to neutrophil recruitment from peripheral blood and release of LL-37 and α-defensin by neutrophil. In sweat gland, follicle and sebaceous gland psoriasin was secreted (S100A7), which allows inhibition of pathogenic bacteria.

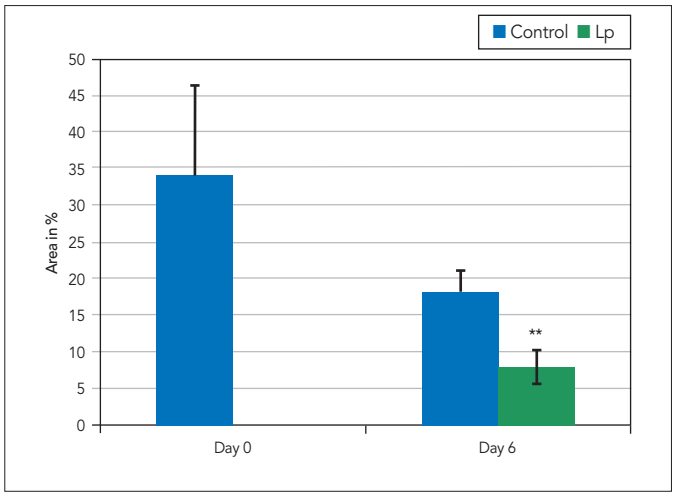

Figure 2: Effect of *L. pentosus* extract (Lp) on TLR2 expression in percentage of area covered in the epidermis exposed after 6 days of treatment. Lp induced a significant decrease of 57% in comparison to the control at day 6 (**: p<0.01).

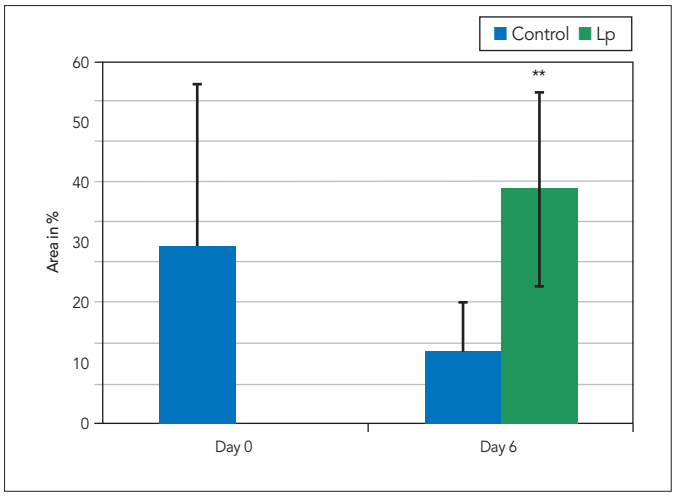

Figure 3: Effect of *L. pentosus* extract (Lp) on hBD2 expression in percentage of area covered in the stratum corneum exposed after 6 days of treatment. Lp induced a significant increase of 229% in comparison to the control at day 6 (**: p<0.01).

sensors that, after activation, regulate the function and/or subcellular distribution of specific target proteins.[9] Among them, S100A7 (also known as psoriasin) has been identified as an anti-microbial peptide expressed by keratinocytes and playing a key role in the response against *Escherichia coli*.[10]

The use of natural products to maintain skin homeostasis by the stimulation of the skin microbiota and immunity is of great interest for the skin care applications. Plant extracts have been increasingly used in cosmetics to improve skin conditions. More recently the antimicrobial and anti-inflammatory properties of plant extract formulations containing *Hamamelis* or Chinese medical herbs have been studied showing promising effects.[11] Other immunologically active molecules have also been isolated in several plants, such as *Peumus boldus* (aka Boldo) and *Spiraea (Filipendula) ulmaria* (also known as meadowsweet).[12] To our knowledge, few studies have investigated the effect of bacteria, even if it has been shown that *Lactobacillus pentosus*, a lactic bacteria described for probiotic potential, can promote health benefits on intestinal epithelium (pathogen exclusion, immunomodulation, increase of epithelial barrier function).[13;14] Based on immunological and structural similarities between intestine and skin, it was thought that *lactobacillus* extract could play a role in the stimulation of host defence systems and skin microbiota homeostasis.[6] In this study, we firstly sought to determine, using an ex vivo protocol, whether *L. pentosus* might be beneficial for normal skin by the stimulation of host defence peptides expression. This signalling is likely to play an important role in the ecology of skin microbial communities. Secondly, using an *in vivo* model, we evaluated the effect of the extract on the skin microbial communities to ensure the balance of healthy skin microbiota.

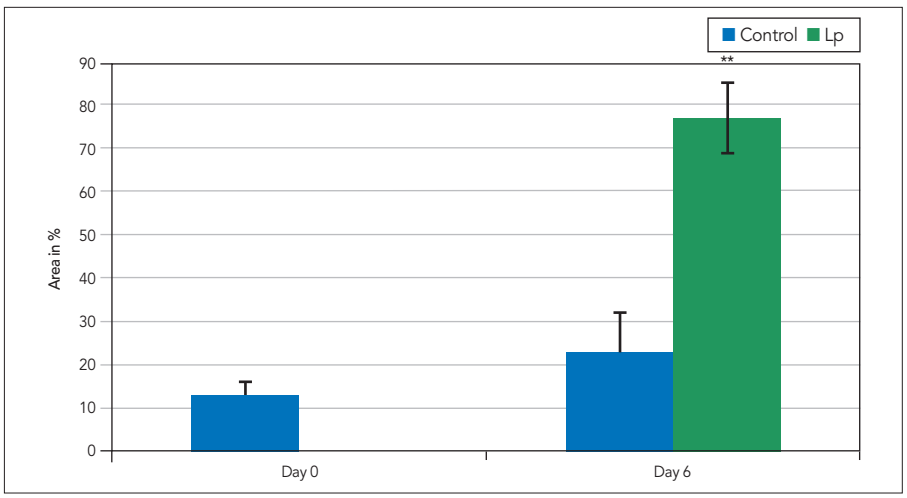

Figure 4: Effect of *L. pentosus* extract (Lp) on S100A7 expression in percentage of area covered in the epidermidis exposed after 6 days of treatment. Lp induced a significant increase of 237% in comparison to the control at day 6 (**: p<0.01).

Materials and methods

Expression of human defence peptides (HDPs) using *L. pentosus* extract

After the cytotoxicity evaluation of the epidermal and dermal structures on paraffin sections stained with Goldner's variant Masson trichrome, nine explants of 12.0 ± 1 mm in diameter were prepared from a plasty of a Caucasian woman aged 65 (reference P1968-AB65). The explants were kept alive in EM (Explants Medium) at 37°C in a humid atmosphere, enriched with 5% CO2. Lp extract at 2% was applied topically on day 0, day 2 and day 5 at the rate of 2 µL per explant (2 mg. cm-^{2}) and spread with a spatula. The explants of the C batches (controls) received no treatment except the renewal of the culture medium. The culture medium was renewed for half (1 mL.well-^{1}) on day 2 and day 5. On day 6, the 3 explants of each condition were removed and treated in the same way as on day 0 control where the explants were removed and cut in half. One part was fixed in buffered formalin and the other part was frozen at -80 ° C.

Histological treatments

TLR2 was tagged on paraffin sections with anti-TLR2 polyclonal antibody (Millipore, Cat # 06-1119), diluted 1: 50 in 0.3% PBS-BSA-0.05% Tween for 1 hour at room temperature with one system: biotin / streptavidin amplifier, revealed in VIP a violet peroxidase substrate (Vector SK-4600). Immunostaining was performed using an immunolabelling automaton (Dako, AutostainerPlus). The labelling was evaluated by microscopic examination.

β-Defensin 2 was labelled on frozen sections with a polyclonal anti-β-defensin 2 antibody (Abcam, reference ab9871), diluted 1: 25 in PBS-BSA 0.3% -Tween 20 at 0.05% for 1h at room temperature, and revealed in AlexaFluor 488

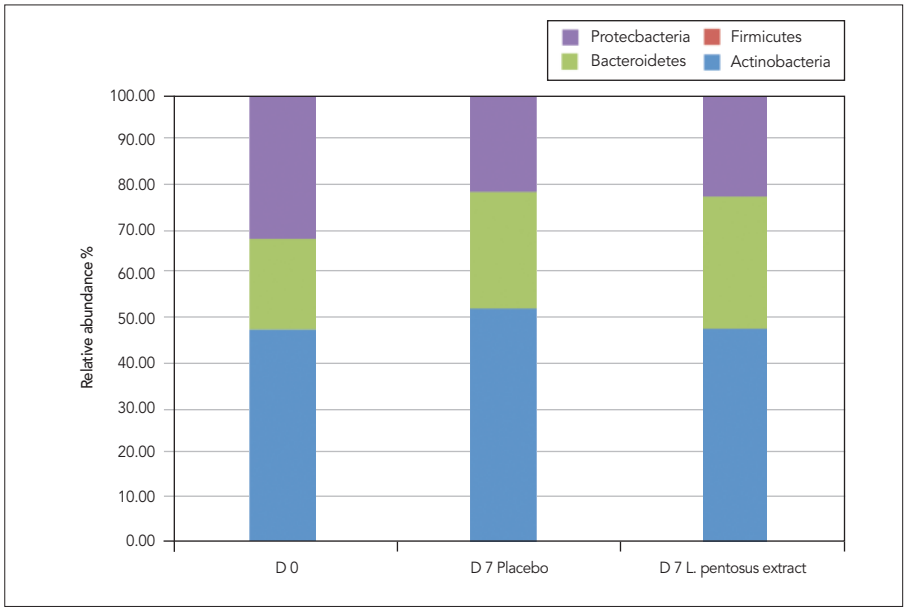

Figure 5: Relative abundance (%) of Phylum analysed by 16 rRNA gene sequencing from skin microbiota of 15 volunteers treated by Placebo or *L. pentosus* extract at day 0 (D0) and day 7 (D7).

(Lifetechnologies, A11078). The nuclei were counter-stained with propidium iodide. Immunostaining was performed using an immunolabelling automaton (Dako, AutostainerPlus). The labelling was evaluated by microscopic examination.

S100A7 was scored on paraffin sections with an anti-S100A7 monoclonal antibody (Novus biologicals, P / N NBP100-56559, clone 47C1068), diluted 1: 200 in PBS-BSA 0.3% -Tween 20 to 0.05 % for 1h at room temperature with a biotin / streptavidin enhancer system, revealed in VIP a purple peroxidase substrate (Vector SK-4600). Immunostaining was performed using an immunolabelling automaton (Dako, AutostainerPlus). The labelling was evaluated by microscopic examination.

Clinical study protocol

The study was performed on the comparison of *L. pentosus* extract 2% vs placebo applied twice a day on hemi-face. Fifteen healthy women were involved from 30 to 45 years old, without skin or systemic disease, sunburn (erythema) on the face or intensive suntanned skin or planned U.V, systemic treatment (antihistaminic, antibiotics, corticoids, retinoids) for more than 5 consecutive days within the 8 weeks before inclusion. A wash-out of 14 days was performed using a 'neutral' cleanser (Tolériane, softening foaming gel from La Roche Posay). Study was performed during 7 days and samples were taken at day 0 and after seven days of applications using sterile swab soaked with a sterile solution (0.15M NaCl + 0.1% Tween 20) then passed at the rate of 30 rubs on a selected area of 3x3cm (forehead). Samples were kept at -80°C before analysis.

Skin microbiota analysis

The genomic DNA was extracted from swabs (qualitative and quantitative estimate). Then PCR amplification of V1-V3 regions of 16S rRNA gene sequencing was performed before the high-throughput

sequencing on MiSeq (Illumina) and the validation of quality controls (filters poor quality sequences, eliminates poor quality bits, eliminates adapters, and sorts them by sample).

Generation of OTU (Operational Taxonomic Units) table, 97% clustering, generation of a phylogenetic tree and analysis of the α-diversity (Shannon index) was done by Bioinformatic analysis.

Statistical analysis

Results are expressed as means (SD). Statistical analyses were performed using SPSS software (19.0). To study the effect of Lp on antimicrobial activities, we used student T test. Statistical significance was used as $p < 0.05$, 95% of confidence.

Results

Expression of human defence peptides (HDPs) using *L. pentosus* extract

Lactobacillus pentosus extract (2%) decreased significantly the expression of TLR2 by 57% (p=0.014) at day 6 in comparison to the control (Fig 2).

The expression of HDPs was analysed and the Lp extract demonstrated significant increase of the expression of ß-defensin 2 by 229% (p<0.01) at day 6 in comparison to the control (Fig 3). The expression of S100A7 was also significantly increases by 237% (p<0.01) at day 6 in comparison to the control (Fig 4).

Effect of *L. pentosus* extract on skin microbiota

The 16S rRNA gene sequencing was performed on samples from forehead area of 15 volunteers treated with placebo or Lp extract during 7 days. Based on the main phylum composing the skin microbiota, the proteobacter phyla was significantly decreased at day 7, from 32.4% at D0 to 21.8% and 23.1% at D7 for the placebo and the *L. pentosus* extract, respectively (Fig 5).

At the family level, analysis of skin microbiota revealed differences between placebo and *L. pentosus* extract. From the classified family, only one family (*Propionibacteriaceae*) was decreased after *L. pentosus* extract exposition (51.40% vs 44.30%), five were at the relative abundance and 8 were increased. Among the increased family, we found the *Corynebacteriaceae* (0.6% vs 2.6%) (Fig 6).

The diversity of the skin microbiota was measured using the Shanon diversity index. The results obtained were not statistically significant (Fig 7).

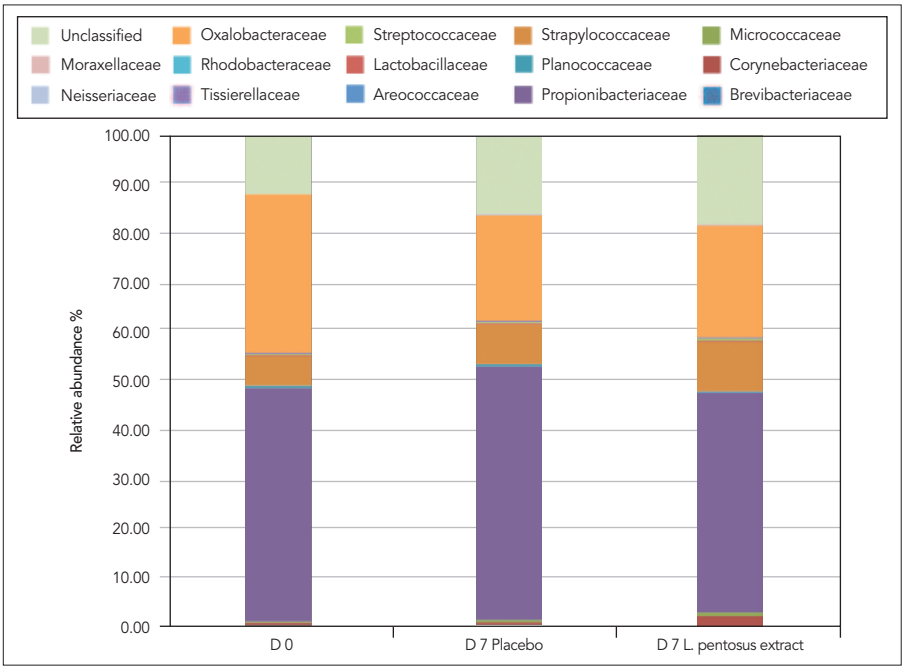

Figure 6: Relative abundance (%) of families analysed by 16 rRNA gene sequencing from skin microbiota of 15 volunteers treated by Placebo or L. pentosus extract at day 0 (D0) and day 7 (D7).

Discussion

The aim of our study was 1) to evaluate the host defence mechanisms stimulated by *L. pentosus* extract exposition, 2) to evaluate, by an *in vivo* approach, the effect of *L. pentosus* extract on the healthy skin microbiota composition and ensuring the respect of the balance.

We observed a decreased TLR2 expression involved in the recognition of pathogenic pattern (PAMPs). TLR and TLR2 have emerged as a principal receptor for Gram-positive bacteria. TLR2 has the unique ability to heterodimerise with TLR1 and TLR6.[15] As already described for other *lactobacillus* strains no role for TLR2 could be demonstrated in *L. rhamnosus*- and *L. casei*-induced cytokine responses, indicating either that these bacteria lack TLR2 ligands in their cell wall or, perhaps more likely, that potential TLR2 ligands are masked.[16] Moreover, as already shown, not all lactic acid bacteria strains act via TLRs and many lactic acid bacteria strains may also stimulate other pattern recognition receptors.[17] Furthermore, over-activation of TLRs leads to the generation of strong proinflammatory signals with persistence of proinflammatory cytokines, such as TNF-alpha and IL-6, and is associated with tissue damages.

Interestingly, we observed a significant increase of expression for ß-defensin 2 (hBD2) and S100A7. These results are in line with those of Schlee et al.[18] who reported that probiotic *lactobacilli* were able to induce human-beta defensin 2 (hBD-2). As previously mentioned, ß-defensins are antimicrobial against a diverse range of skin pathogens, including Gram-negative and Gram-positive bacteria or fungi, thus actively helping to protect our skin from infections.[6] These are present in normal skin and participate in maintenance of beneficial host-microbial relationships at epithelial surfaces by restricting growth of resident microbes and opportunistic pathogens. Concerning S100A7, Gläser et al.10 found that this peptide was a principal E. coli-cidal agent in healthy skin, which explained why skin regions that are often exposed to high concentrations of *E. coli* are usually not infected with this gut bacterium.

Generally speaking, the skin continuously faces complex microbial challenges that include maintaining homeostasis with indigenous microorganisms and limiting exposure to pathogens. The production of HDPs that modulate immunity are essential not only for protecting these sites from pathogenic microbial invasion but also for shaping the composition and location of indigenous microbial communities.[6] It is not yet known how the signalling is likely to play an important role in the ecology of the skin commensal microbiota. In the present study, we used a bacterial extract stimulating the production of HDPs to efficiently protect against pathogen invasion. In order to protect skin, we have to ensure the protection of commensal population from skin microbiota. Here, we described the skin microbiota of volunteers using a placebo neither the same formula containing an extract of *L. pentosus* at 2%. The phyla analysis demonstrated decrease of *Proteobacteria* and increase of *Firmicutes* attributed to the formula used for the *vivo* analysis. In the case of psoriasis, *Proteobacteria* seem more abundant in biopsies of psoriasis lesions and in chronic wounds than in healthy skin.[19] More precisely, at a family level, we observed changes in relative abundance of skin microbiota. The population profile using the *L. pentosus* extract differed in slight

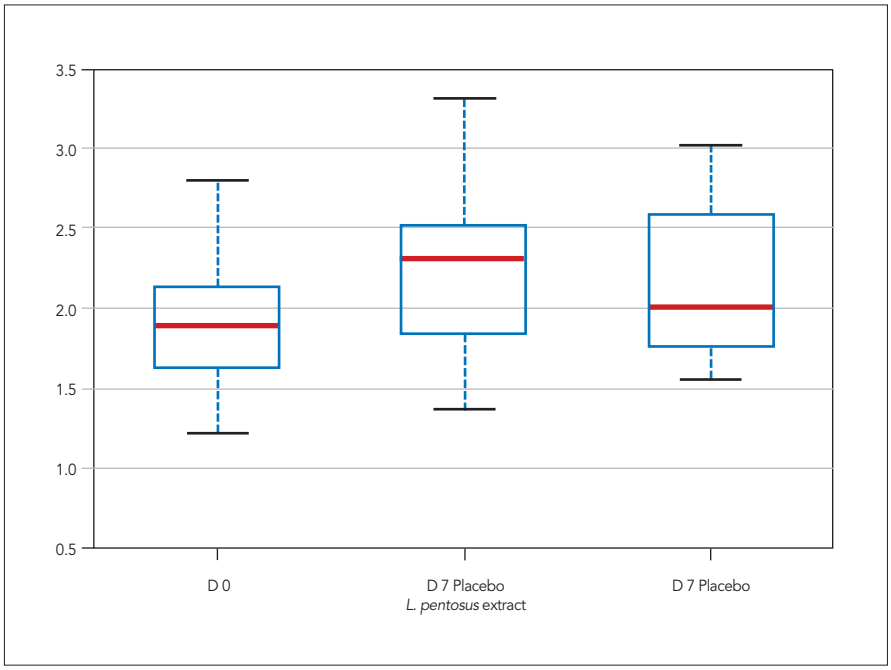

Figure 6: Shanon index diversity (n=15 volunteers) treated by placebo or L. pentosus extract at day 0 (D0) and day 7 (D7).

modifications. One major family of skin-resident bacteria is *Corynebacteriacae* (including *Corynebacterium*), we found an abundance more importantly using L. pentosus extract. These members are present at all body sites and dominate in moist sites. *Corynebacteria* share many microbiological features with the closely related mycobacteria. It remains a challenge to understand how the immune system distinguishes between bacteria with such similar surface and cellular structures and to determine which factors unique to Corynebacterium might be responsible for its commensalism. These questions will help to explain how commensal bacteria 'educate' the cutaneous immune system.[20]

Frequently measured with the Shanon index, the diversity is known to be an important factor for health status considering the skin microbiota. Researchers across many fields of biology and ecology agree that a high biodiversity corresponds to increased healthiness and functionality within an ecosystem.[21] We noted no significant microbial diversity changes measured by Shannon index when application *L. pentosus* extract in comparison to placebo at Day 7 and initial status at Day 0. No decrease of diversity was also observed with the *L. pentosus* extract, thus maintaining the healthy status of the skin microbiota of volunteers (Fig 6).

Finally, the capability of *L. pentosus* extract to protect healthy skin from potential external aggressions was demonstrated by the stimulation of host defence peptides protecting against colonisation of pathogens. In addition, the *vivo* study with the *L. pentosus* extract proved its compatibility with skin microbiota of healthy volunteers, maintaining the diversity.

Conclusion

Maintaining equilibrium within skin microbiota populations is fundamental for skin homeostasis, such as the stimulation of the host defence system. To our knowledge, no study has focused on the Lp properties, known for probiotic activity. Results showed a stimulation of host defence peptides (ß-defensin 2, S100A7) without induction by the receptor TLR2. The clinical study coupled with skin microbiota analysis demonstrates that application of *L. pentosus* extract during 7 days is consistent with the respect of healthy balance and introduction of diversity. The *L. pentosus* extract enhances the antimicrobial activities, maintaining the balance of the cutaneous microflora, allowing the skin to have a biological shield against environmental stresses. PC

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