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Novel bioactive from *Lactobacillus brevis* DSM17250 to stimulate the growth of *Staphylococcus epidermidis*: a pilot study

C. Holz[1], J. Benning[1], M. Schaudt[2,3], A. Heilmann[1], J. Schultchen[1], D. Goelling[1] and C. Lang[1*]

[1]Organobalance GmbH, Gustav-Meyer-Allee 25, 13355 Berlin, Germany; [2]Analyse & Realize GmbH, Waldseeweg 6, 13467 Berlin, Germany; [3]PRA health Sciences, Gottlieb-Daimler-Straße 10, 68165 Mannheim, Germany; lang@organobalance.de

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RESEARCH ARTICLE

Abstract

Commensal skin microbiota plays an important role in both influencing the immune response of the skin and acting as a barrier against colonisation of potentially pathogenic microorganisms and overgrowth of opportunistic pathogens. *Staphylococcus epidermidis* is a key constituent of the normal microbiota on human skin. It balances the inflammatory response after skin injury and produces antimicrobial molecules that selectively inhibit skin pathogens. Here we describe *Lactobacillus brevis* DSM17250 that was identified among hundreds of *Lactobacillus* strains to exhibit an anti-inflammatory effect in human keratinocytes *in vitro* and specific stimulatory impact on the growth of *S. epidermidis*. The aqueous cell-free extract of *L. brevis* DSM17250 was used in an ointment formulation and tested in a randomized placebo-controlled double blinded human pilot study. Healthy volunteers with diagnosed dry skin were treated for four weeks. The study data shows that *L. brevis* DSM17250 extract induces re-colonisation of the skin by protective commensal microorganisms as judged from selective bacterial cultivation of surface-associated skin microorganism of the lower leg. Furthermore, the 4 week administration of the *L. brevis* DSM17250 extract significantly improved the transepidermal water loss value (TEWL), reduced the xerosis cutis symptoms and stinging. The data shows that daily application of *L. brevis* DSM17250 extract in a topical product significantly improves the microbial skin microbiota by promoting the growth of species which possess beneficial regulatory and protective properties such as *S. epidermidis*. Restoring the natural skin microbiota leads to significantly improved skin barrier function (as transepidermal water loss) and decrease of xeroderma (xerosis cutis) symptoms (as measured by dry skin area and severity index, DASI). We propose that improving and stabilizing the natural skin microbiota by specifically stimulating the growth of *S. epidermidis* is an important and novel concept to manage skin diseases associated with microbiota dysbiosis.

Keywords: *Staphylococcus epidermidis*, cell-free extract, skin barrier, dry skin, anti-inflammatory

1. Introduction

The skin is our largest organ and the barrier protecting our body from potential assaults by toxic substances or pathogenic microorganisms, which it constantly remains in an intimate contact with. It hosts approximately 10^6 bacteria/cm^2 (Grice *et al.*, 2008). The vast majority of these are so-called commensals. The dense coating of commensal bacteria constitutes the first barrier to pathogens colonising the skin by occupying the environmental and nutritional niches. Though the composition and the abundance of skin microbes vary a lot, depending on location and physical properties, *Staphylococcus*, *Corynebacterium* and *Propionibacterium* species represent the dominant bacterial genera. The most commonly isolated bacterial species from the human skin is *Staphylococcus epidermidis* (Rosenthal *et al.*, 2011). It comprises up to 90% of the aerobic resident microbiota. Typically, it has a benign relationship with its host. Its beneficial effects on the skin can be classified into two categories: (1) antimicrobial action directed against skin pathogens; and (2) cooperative enhancement of the host's immunity (Gallo and Nakatsuji, 2011; Otto, 2009; 2010). *S. epidermidis* produces potent signal molecules, which act against pathogens, such as *Staphylococcus aureus*, based on

cell density control mechanisms (Otto *et al.*, 2001) as well as antimicrobial molecules to selectively inhibit the survival of skin pathogens while maintaining the normal skin microbiota (Bierbaum *et al.*, 1996; Pag *et al.*, 1999). Another mechanism utilised by *S. epidermidis* to restrict growth of its pathogenic relative *S. aureus* was described by Iwase *et al.* (Iwase *et al.*, 2010). Clinical isolates of *S. epidermidis* have been demonstrated to secrete a serine protease (Esp) that inhibits biofilm formation and destroys biofilms formed by *S. aureus*. Moreover, Esp protease disintegrates human receptor proteins which enable colonisation by *S. aureus* and infection of host cells (Sugimoto *et al.*, 2013).

S. epidermidis has also been reported to actively contribute to the skin innate immune defence. It enhances the neutrophil capacity to form extracellular traps and it increases their efficacy, as phenol-soluble modulin-γ is incorporated into them along with other host antimicrobial peptides. This represents an interesting case of synergy of antimicrobial peptides from different species (Cogen *et al.*, 2010) which is mutually beneficial. *S. epidermidis* rather than acting alone, is able to kill pathogens by complementing the host's innate immune system. It suggests an alliance of the host with *S. epidermidis* to keep transient, potential pathogens at bay. Considering the major contribution of commensal microorganisms to the normal immune development and function, it becomes apparent why any disturbance of the host-microbiota interaction may result in disease. With the advance of sequencing technologies enabling the assessment of bacterial composition of individual skin sites, a correlation between the altered microbiota and the prevalence of skin diseases, such as atopic dermatitis (Baviera *et al.*, 2014), eczema (Sanford and Gallo, 2013), as well as delayed healing of diabetic ulcers and burn wounds (Holmes *et al.*, 2015; Lavigne *et al.*, 2015) has been confirmed. The microbial balance is constantly challenged by the use of antibiotics and cosmetics. Soap, hygiene products, disinfectants and moisturizers disturb the skin condition and remove the protective microbial shield, leaving the skin exposed to pathogens. Analyses based on twin studies revealed that lifestyle factors (hygiene, occupation), in contrast to genetic factors, have a major impact on the development of hand eczemas (Bryld *et al.*, 2000; Lerbaek *et al.*, 2007a),(Lerbaek *et al.*, 2007b) and are also of great importance in case of atopic dermatitis (Thomsen *et al.*, 2007). Studies investigating the bacterial microbiota on the hands of health-care workers with skin irritated due to frequent disinfection and wearing gloves have confirmed the increased risk of colonization with pathogens, such as *S. aureus* (Larson *et al.*, 1998). Stabilizing the microbial community and re-colonising the skin with commensals might be a powerful and yet gentle method to treat skin conditions, such as xerosis cutis or atopic eczema.

We have identified a specific strain of lactic acid bacteria (*Lactobacillus brevis*) that produces small molecules that act specifically on commensals, such as *S. epidermidis,* and enhance their growth both *in vitro* and *in vivo. L. brevis* DSM17250 was selected by screening *Lactobacillus* spp. strains from a large culture collection. Both whole cells and the aqueous extract containing a specific peptide as major active component strongly promote growth of the commensal bacterium *S. epidermidis* and exhibit anti-inflammatory properties. Moreover, in a double-blinded, randomized pilot study we demonstrated that the bacterial extract in a cream topically applied to the skin of the lower legs of healthy volunteers with diagnosed xerosis results in significant stimulation of the beneficial skin microbiota by supporting the repopulation with commensal bacteria. Furthermore, the four week administration of the extract decreased xerosis cutis symptoms and significant improved transepidermal water loss, thus stabilizing the natural skin barrier function. This finding provides a novel and versatile approach to skin products (cosmetic and pharmaceutical) that ameliorate skin diseases via supporting and stimulating the natural microbial shield.

2. Materials and methods

Bacterial extract

L. brevis DSM17250 was selected after systematically screening several hundred strains of lactobacilli from a microbial culture collection (Organobalance, Berlin, Germany). The strain was identified as *L. brevis* by 16S rDNA sequence analysis (sequencing done by LGC Genomics, Berlin, Germany / phylogenetic classification done by Nadicom, Karlsruhe, Germany) and by phenotypic analysis using the api 50 CH system including apiweb™ software (bioMérieux, Nürtingen, Germany). Cells were grown in De Man, Rogosa and Sharpe medium (De Man *et al.,* 1960) at 37 °C in microaerobic atmosphere. The bioactive compound promoting the skin's microbiota is soluble in water and can be released from living cells after permeabilising the cells by application of ethyl acetate (16.7%, v/v). After aqueous extraction the cells were separated and the supernatant was freeze dried. The residue was resolved in water to give a stock solution of 20 mg/g [w/w] dry matter. This stock solution ('*L. brevis* DSM17250 extract') was used throughout the experiments. The active extract was purified by ultrafiltration in order to characterize the nature and size of the molecules showing the growth promoting activity. The activity was detected in the fractions with the lowest molecule weight (<3 kDa). The activity could be precipitated by acetone, indicating that the key active constituent might be a small protein or peptide. The ultrafiltrate was further fractionated by gel filtration and reversed phase HPLC (Zorbax SB-AQ; Agilent, Santa Clara, CA, USA). The fractions were tested for activity and the resulting active fraction was analysed by NMR spectroscopy. The active fraction showed heat stability up to 100 °C for 1 h, stability at pH 3-8 and a tetrapeptide could be

identified as major active compound. The peptide sequence was determined by Merlion (Singapore) and peptides were synthesized by BACHEM (Bubendorf, Switzerland). The peptide was used in the anti-inflammatory cell assays and as positive control in co-incubation assays.

Determination of growth promotion of *S. epidermidis* by agar diffusion test

For agar diffusion test *S. epidermidis* DSM20044^T was grown overnight in brain heart infusion broth (Becton Dickinson, Heidelberg, Germany) at 37 °C and was diluted into tryptone soya broth (TSB; Oxoid, Wesel, Germany) to give a concentration of approx. 4×10^6 cfu/ml. Tests were performed in square petri dishes (12×12 cm). Aliquots were plated onto tryptone soya agar (TSA; Oxoid) containing 0.003% potassium tellurite (Alfa Aesar, Karlsruhe, Germany). Holes of 9 mm diameter were stamped into the agar and filled with *L. brevis* DSM17250 extract in different concentrations from 1 to 4 mg/g. D(+)-glucose monohydrate (as non-specific growth promoter), TSB and dH_2O (as negative controls) were used as controls. The plates were incubated at 37 °C for 48 h. Growth promotion of *S. epidermidis* was detected by the formation of dense black zones around the stamped holes. Blackening of colonies occurred due to the reduction of tellurite by metabolically active *S. epidermidis* cells (Baird-Parker, 1963).

Growth kinetics in microtiter plate based co-incubation assay

Growth kinetics of *S. epidermidis* DSM20044^T were monitored to determine the dose-dependent impact of the *L. brevis* DSM17250 extract using 96-well, flat-bottomed microtiter plates. All samples contained 65% dH_2O (v/v), 10% (v/v) TSB, 5% (v/v) *S. epidermidis* inoculum (5×10^4 cfu/ml) and the *L. brevis* DSM17250 extract (stock solution) was applied in concentrations from 0,2 mg/g to 4 mg/g. Water was added to all samples to the final volume of 200 µl. Samples comprising equivalent volumes of TSB and *S. epidermidis,* and either calcium D-pantothenate (4 mM) or glucose (2% w/v) instead of *L. brevis* DSM17250 extract were used as positive controls; 20% (v/v) dH_2O was used as negative control. The plates were incubated at 37 °C for 14 h in aerobic atmosphere (under shaking conditions) using a BioTek Powerwave HT Microplate Spectrophotometer (BioTek, Winooski, VT, USA) including GEN5™ software. Reads were performed at 600 nm in intervals of 10 min to detect maximum optical density (OD). Each growth kinetic was the average of 9 reads. Standard error within individual experiments was ≤0.05 OD units.

Measurement of cytotoxicity and anti-inflammatory effect on epidermal keratinocytes

Monolayer cultures of normal human epidermal keratinocytes (NHEK) (BASF Beauty Care Solutions, Lyon, France) were grown to confluence in 24-well tissue plates at 37 °C in a humidified atmosphere containing 5% CO_2. Cell culture medium was replaced with medium containing increasing concentrations of the key peptide of *L. brevis* DSM17250 extract (0.2 µg/ml to 800 µg/ml). The cell viability was determined by means of an MTT assay after 24 h and 48 h incubation of the NHEK cells (1 donor, 1 experiment, n=6) in the presence of *L. brevis* DSM17250 extract using the tetrazolium dye (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromid (Sigma, St. Louis, MO, USA). Sodium dodecyl sulphate (0.025% v/v) was used as positive control and induced over 90% of cell death. Anti-inflammatory properties were evaluated by measuring the secretion of pro-inflammatory cytokine IL-1α in NHEK cells after stimulation with 100 µg/ml LPS (lipopolysaccharides from *Escherichia coli* 055:B5 (Sigma-Aldrich)). Monolayer cultures of NHEK cells (1 donor, 1 experiment, n=6) were cultivated for 48 h comprising a 24 h pre-exposure phase followed by 24 h exposure phase. The concentration of secreted interleukin (IL)-1α in the supernatant was quantified by ELISA (R&D Systems, Minneapolis, MN, USA) according to manufacturer's recommendation. Untreated cells were used as negative control and cells stimulated with LPS alone as positive control.

Study population

An explorative randomized double-blind placebo-controlled study was performed at the dermatological study center 'Hautarztpraxis Mahlow' (Mahlow, Germany) to study the efficacy of a topical cream containing the *L. brevis* DSM17250 extract on xerosis symptoms. 30 volunteers, aged 19-47, were included in the study, of which 27 were female and 3 were male. The mean age was 39. Selection of the subjects was based on diagnosis of dry skin determined at the lower legs using the 'dry skin area and severity index' (DASI) (Serup, 1995). One of the assessed skin parameters should have at least a value of 2. All subjects being sensitive or allergic to ingredients of the test product, with acute or chronic systemic illness or infections were excluded from the study. Other exclusion criteria were any medication and procedures with potential interference with the study, the use of corticosteroids, pregnancy or lactation, alcohol or drug abuse and the use of solarium or sunbeds. Volunteers were randomly divided into two groups applying either the cream containing *L. brevis* DSM17250 extract (verum cream) (n=16) or placebo cream (n=14). Described sample size was defined to be needed to detect statistically significant differences between verum and placebo group assuming α=0.05 and β=0.20 (80% power).

Study design

The study was conducted according to the Declaration of Helsinki and its amendment, and the Guidelines on Good Clinical Practice adopted by the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use. The protocol was approved by the independent local ethics advisory committee (Landesärztekammer Brandenburg, Cottbus, Germany). Written informed consent for participation was obtained from all subjects prior to being included in the study. As test vehicle (cream) the standard O/W-emulsion *Ungentum emulsificans aquosum* (aqueous hydrophilic ointment; European Pharmacopoeia) was used. Verum cream contained 0.88 mg/g *L. brevis* DSM17250 extract, whereas the control cream was made with the equivalent volume of dH_2O. Test products were produced in compliance with cosmetic good manufacturing practices guidelines (Josefa Dürolf Naturprodukte, Grünberg, Germany). No preservatives were added. Hence, to ensure microbial purity test products were provided in airless dispensers and were stored at 4-8 °C during the complete study period. Volunteers applied the test cream twice daily on the skin of the lower leg for a period of 28±3 days. After being included in the study (visit 1, baseline), subjects were instructed to apply 1 g of the test product twice a day to the anterior tibia area of their right leg. The left leg was not treated by either verum or placebo to provide an internal control for the self-assessment questionnaire. Follow-up visits at the dermatological study centre were determined for day 7±1 (visit 2), day 14±1 (visit 3) and last visit on day 28±1 (visit 4). To assess compliance the products were weighted at baseline and at each visit. During the treatment phase, no changes in washing behaviour were to be initiated and no further topical preparations for the care of dry skin were to be used.

Microbial analysis of skin

To monitor the time course of beneficial skin microbiota the microbiota of the treated skin areal of each subject was analysed *via* tape stripping at each visit. Hence, a tape with the size of 8 cm^2 was thoroughly applied to the skin. Adhering microorganisms of the skin's surface were then transferred to the selective indicator medium chromID® *S. aureus* agar (SAID agar, bioMérieux, Nürtingen, Germany). Samples were incubated at 37 °C for 48 to 72h. Microorganisms of the genus *Staphylococcus* were mainly considered for further assessment as the used SAID agar represses several bacteria of other genera, as well as some yeasts. The pathogen *S. aureus* was discriminated based on green coloured colonies utilizing chromogenic substrates from the agar. The colonies of the coagulase negative *S. epidermidis* and *Staphylococcus capitis* remain white, *Staphylococcus xylosus* produces purple colonies and *Micrococcus* yellow-green colonies. *S. capitis* could be differentiated from *S. epidermidis* by mannitol fermentation (mannitol salt agar, Roth, Germany) and by api®-Staph (BioMerieux). Selected colonies of the different species were checked by 16 S-rDNA sequence analysis (LGC Genomics) using the primers 27f (5´-AGAGTTTGATCMTGGCTCAG-3´) and 1492r (5´-ACGGYTACCTTGTTACGACTT-3´).

Determination of transepidermal water loss

In order to determine the impact on skin barrier function the transepidermal water loss (TEWL) value was determined using the Tewameter TM 210 (Courage & Khazaka, Cologne, Germany) in compliance with published guidelines of the Standardization Group of the European Society of Contact Dermatitis (Pinnagoda *et al.*, 1990). All subjects were adapted to climatic conditions at the study centre for 15 min. Examinations were done at a temperature of 20-22 °C and a relative humidity of around 45-55%. TEWL was determined immediately before treatment initiation (baseline, visit 1), after 7±1 days treatment (visit 2), after 14±1 days treatment (visit 3) and after completion of treatment at day 28±3 (visit 4). TEWL values were generated as an average of three measurements at each time point. The TEWL analyses were represented as change to baseline (visit 1). Therefore the results of the follow-up visits (day 7 and day 28) calculated as mean values ± standard error of the mean (SEM) were subtracted from the TEWL of the baseline.

Clinical (DASI) score, self-assessment and safety

The clinical score was classified on day 1 (baseline), day 7, day 14 and day 28. The MD examined the dryness, sensitivity and any irregularities of the skin at the surface of the tested tibia area (right leg). Treated skin was scored following four parameters: redness, roughness, scaling and cracks/fissures. Scale of the clinical DASI score (Serup, 1995) ranged from 0 to 4 as follows: no symptoms; slight; moderate; severe and extreme. In addition, the subjects kept a daily diary to record the subjective evaluation of changes of skin condition with regard to dryness, roughness, feeling of tightness, itching and burning from baseline to day 28 as well as the overall impression of the given test product. Therefore the dermatologist asks the volunteers to conduct a self-assessment of skin dryness and sensitivity using a visual analogue scale (VAS) from 0 to 100%. The subjects also reported any adverse events (e.g. medication) in the daily diary during the study. Product acceptability and safety were also investigated by the dermatologist during each visit (day 1, 7, 14 and 28).

Statistical analysis

The analysis variables were the changes in skin microbiota, the differences in TEWL values and changes of DASI score from baseline (day 1) to visit 4 (day 28) at the end of the

investigation (pre-post comparison). The statistical analyses were carried out using SPSS statistical software (SPSS for Windows, V19.0, Chicogo, IL, USA). Exploratory data analyses were conducted for the overall population and for each of the study groups to determine statistically significant variance between the groups (placebo vs verum) for each end point assessed. Quantitative parameters and their changes were characterised by confidence intervals (CI=95%) on statistical relevance within the study groups. Statistical significance between the groups (placebo vs verum group) was calculated using a non-parametric Mann-Whitney U test (P_{Uex}). For comparisons within a group (e.g. pre- vs post-treatment) the non-parametric Wilcoxon test (P_{Wex}) was employed. Differences were considered significant at a *P*-value of less than 0.05. All values concerning the analysis of the commensal microbiota were log-transformed medians displayed as box-and-whisker plots.

3. Results

In vitro growth promotion of *Staphylococcus epidermidis*

From several hundred *Lactobacillus* strains in a proprietary collection, four strains were identified which are able to stimulate the growth of *Staphylococcus epidermidis* in an *in-vitro* agar diffusion assay. All were deposited at the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). To test the growth promoting effect pre-cultured lactobacilli were filled into pre-cut holes and growth of the indicator strain *S. epidermidis* was detected as the formation of black rings around the holes due to reduction of telluride and an increase of cell density. The strain *L. brevis* DSM17250 showed the strongest growth promoting activity and was further investigated. The activity was detected when whole cells of *L. brevis* DSM17250 were used, and also when the water soluble extract released from the living *Lactobacillus* DSM17250 cells were applied after cell permeabilisation with ethyl acetate. The results showed a dose-dependent effect of *L. brevis* DSM17250 extract on growth of *S. epidermidis* in the plate assay (Figure 1A). After 48 h incubation with *L. brevis* DSM17250 extract the strongest black coloured stimulation ring was detected when applying the highest extract concentration (2% w/v). To differentiate a mere feeding effect by a carbon source from the signalling effect of the *L. brevis* DSM17250 extract, we used glucose (as a model carbon source) and TSB (as a model peptide / nitrogen source) to mimic the growth promoting effect on *S. epidermidis*. No enhanced metabolic activity were observed at concentrations of glucose (between 0,1 and 2%) and of TSB (standard medium concentration) (Figure 1A). No ring formation is seen for either glucose or TSB as additives whereas the *L. brevis* DSM17250 extract induces distinct rings of enhanced metabolic activity of *S. epidermidis*. The growth promoting activity on *S. epidermidis* was also tested in a liquid co-incubation assay with low inocula (5×10^4 cfu/ml) in a diluted TSB minimal medium. As shown in Figure 1B, supplementation of increasing amounts of *L. brevis* DSM17250 extract resulted in correspondingly increasing metabolic activity of *S. epidermidis* at the end of a 14 h incubation period. In addition to the type strain of

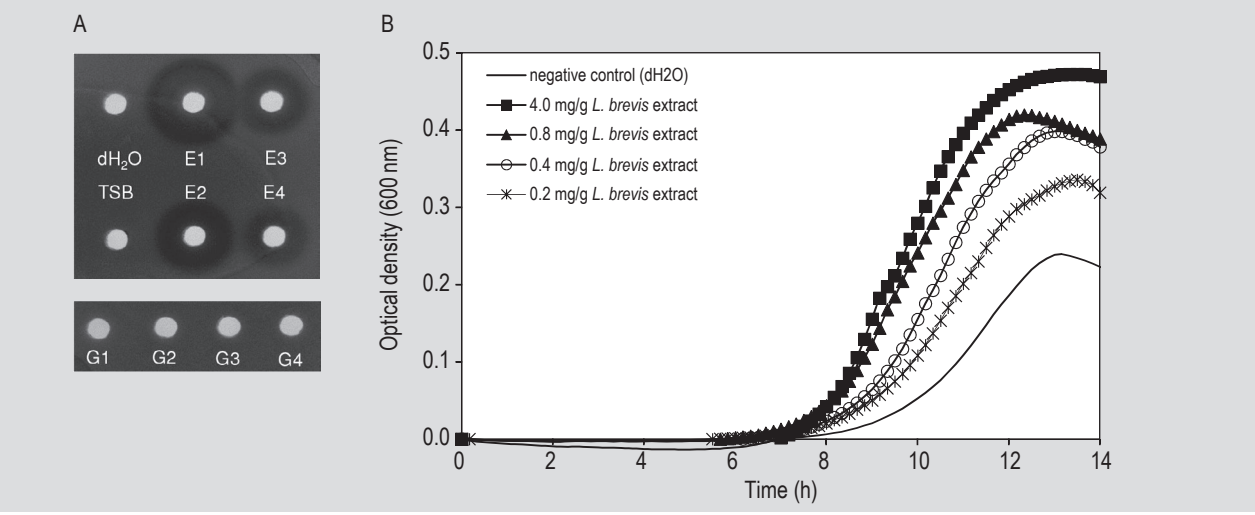

Figure 1. *In vitro* growth promotion of *Staphylococcus epidermidis* DSM20044^T by *Lactobacillus brevis* DSM17250 extract. (A) Detection of growth stimulation in agar diffusion test on TSA medium with tellurite (0.003% w/v). Impact of *L. brevis* extract (E1: 20 mg/g; E2: 4 mg/g; E3: 2 mg/g; E4: 1 mg/g) on the metabolic activity of *S. epidermidis* was identified by formation of black rings. dH_2O and TSB were used as negative controls. D-(+)-Glucose was also used as control in different concentrations (G1: 2% w/v, G2: 0.4%, G3: 0.2% and G4: 0.1%). (B) The dose-dependent impact of *L. brevis* DSM17250 extract on growth kinetics of *S. epidermidis* was monitored in co-incubation assays. Mean values of kinetic reads (n=9) in the presence of *L. brevis* extract in TSB minimal medium are shown. dH_2O was applied as negative control.

S. epidermidis, 8 further *S. epidermidis* strains and a strain each of *Staphylococcus warneri*, *Staphylococcus hominis*, *Staphylococcus haemolyticus* and *S. capitis* isolated from human skin were tested. All strains tested showed the same positive response to the *L. brevis* DSM17250 extract.

In vitro cytotoxicity and anti-inflammatory effect on epidermal keratinocytes

In order to determine the potential anti-inflammatory properties of the strain *L. brevis* DSM17250 in *in vitro* human cell assays the bioactive compounds of the *L. brevis* DSM17250 extract were purified by fractionating the aqueous phase using reversed phase HPLC and gel filtration. The active compounds were analysed and a tetrapeptide was identified as major constituent by the means of NMR spectroscopy. We initially characterised the effect of *L. brevis* DSM17250 peptide on keratinocyte viability by performing an MTT assay. NHEK cells incubated with different doses of *L. brevis* DSM17250 peptide (0.2 µg/ml to 800 µg/ml) retained over 90% of viability after 48 h (data not shown). At the tested concentrations, *L. brevis* DSM17250 peptide does not display any cell cytotoxicity and the effective concentration of 200 µg/ml was used in further assays. The anti-inflammatory properties of *L. brevis* DSM17250 peptide were evaluated on LPS-induced NHEK cells (Figure 2) by measuring the secretion of pro-inflammatory cytokine IL-1α. No IL-1α was detected in the supernatants from untreated NHEK cells (approach 1, negative control). Also the *L. brevis* DSM17250 peptide alone when added in the pre-incubation period (approach 2) did not lead to significant amounts of IL-1α in the culture medium, showing that the *L. brevis* DSM17250 peptide has no pro-inflammatory effect. When keratinocytes were induced with LPS (100 pg/ml) for 24 h in the pre-exposure period significant amounts of IL-1α (approach 3, positive control, 14.2±3.3 pg/ml) were secreted. The potential anti-inflammatory effect of the *L. brevis* DSM17250 peptide was investigated in three different approaches. First the anti-inflammatory effect of the peptide compound was analysed after keratinocytes were induced with LPS (approach 4). The 24 h treatment with the peptide after the 24 h induction period with LPS only slightly decreases secretion of IL-1α. The anti-inflammatory activity was analysed in a further approach (5) where keratinocytes were pre-treated for 24 h with *L. brevis* DSM17250 peptide and subsequently 24 h exposed to LPS. A significant positive effect of the peptide, measured as decrease in IL-1α production, was observed (4.7±0.2 pg/ml, decrease of 67%). When the NHEK cells were pre-treated with both the *L. brevis* DSM17250 peptide and the inducer LPS and subsequently the peptide was additionally applied in the second 24 h treatment period (approach 6), a significant decrease in IL-1α secretion was observed as well (7.1±2.1 pg/ml, decrease of 50%). The data shows that the *L. brevis* DSM17250 peptide exhibits a significant anti-inflammatory effect *in vitro*.

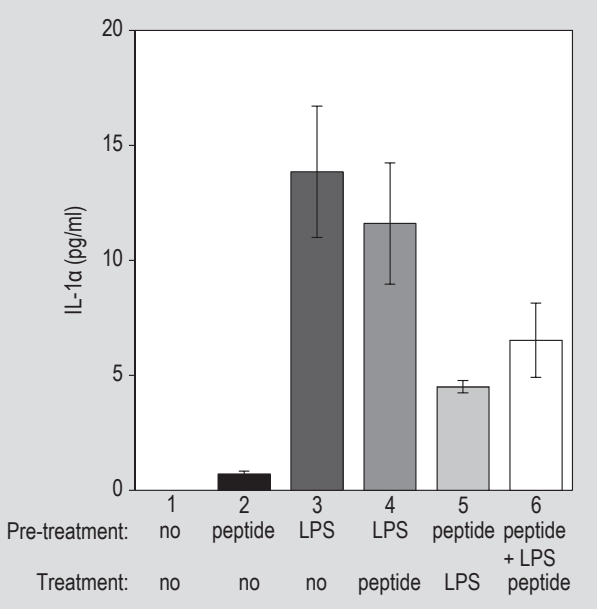

Figure 2. Anti-inflammatory effect of peptide compound of *Lactobacillus brevis* DSM17250 extract on epidermal keratinocytes. The secretion of pro-inflammatory cytokine interleukin (IL)-1α induced by lipopolysaccharide (LPS) treatment was analysed in NHEK cells (1 donor, 1 experiment, n=6). Cultivation of NHEK cells was conducted in two phases: 24 h pre-treatment and 24 h treatment. The production of IL-1α was measured by ELISA. Untreated cells were used as negative control. Cells only induced with LPS were applied as positive control

Human study: evaluation of product efficacy and safety

A total of 30 subjects (3 male, 27 female; 29 Caucasian, 1 Latino) with a mean age of 39 years (age range 19-47 years) were enrolled in the study. 90% of the subjects were female and 98% were of Caucasian origin. The subjects were randomised in two groups, 14 were included into the placebo group and 16 into the verum group. The height and weight were well balanced between the two groups. The dry skin symptoms were also equilibrated between the two groups. No drop-outs were recorded and the complete product application was reported, thus reflecting a high compliance to the study protocol. Two reported adverse events (AE), rhinitis and cystitis, occurred during the study, which were not considered to be related to the study products. No AE led to interruption or drop-out. Overall, the products were well tolerated and perfect compliance was noticed for 100% of the subjects.

Microbial analysis of skin

The analysis of commensal skin bacteria of the lower legs was performed by tape stripping. The cell counts were determined after 48 h incubation on selective medium. The log-transformed medians of commensal

skin microorganisms were calculated in the samples taken before treatment on day 1 (baseline) and after finishing the application (day 28) with placebo or verum cream containing the *L. brevis* DSM17250 extract (Figure 3). A significant high increase of commensal bacterial count at day 28 after verum application could be seen compared to the placebo group (P_{Uex}=0.008). The change from day 1 to 28 in the placebo group is small (1.3log). The difference in changes (day 28 to day 1) between placebo and verum is significant (P_{Uex}=0.003). The changes from day 1 to 28 in number of *S. epidermidis* between verum and placebo is also significant (P_{Uex}=0.024). The results demonstrate the *in vivo* growth promotion of commensal microbiota on skin by application of the cream containing the *L. brevis* DSM17250 extract for 28 days. Growth promotion was not limited to *S. epidermidis* as increased numbers of colonies of other commensals such as *Micrococcus, S. capitis and S. xylosus* were detected on the skin (analysed by PCR and selective medium). We also determined the cell counts of the skin pathogen *S. aureus* on day 1 and 28 and compared the differences with respect to cell count changes within and between placebo and verum group. The results showed no statistical differences indicating that the number of *S. aureus* did not increase in any of the groups during the study. In general, *S. aureus* was present on the skin of single subjects (20%) in non-significant low numbers. A significant amount of *S. aureus* was detected on the skin of one test person in the verum group on day 7 (Figure 4). This number decreased from 66 (cfu/8 cm^2) to 3 between day 7 and day 28 after application of the verum cream containing the *L. brevis* DSM17250 extract, while the number of commensal skin bacteria was increased significantly from 141 to 399 cfu/8 cm^2. Such data need to be confirmed in future studies including patients with *S. aureus* infections on the skin.

Transepidermal water loss

TEWL was determined on day 1, 7 and 28 to analyse the impact on skin barrier function by application of test products (Figure 5). The median of day 1 was 11.4 for the verum group and 10.9 for the placebo group. In the first week (day1 to 7) the stratum corneum barrier remained unchanged in both treatment groups, respectively with TEWL significantly increasing by 10.34 g/m^2/h in placebo

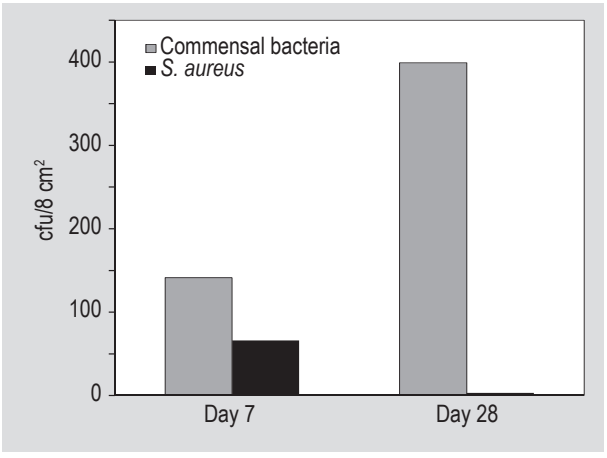

Figure 4. Quantification of commensal skin bacteria and *Staphylococcus aureus* by tape stripping and plating on SAID agar. Data from one test person among the verum group on day 7 and after 28 days treatment is shown.

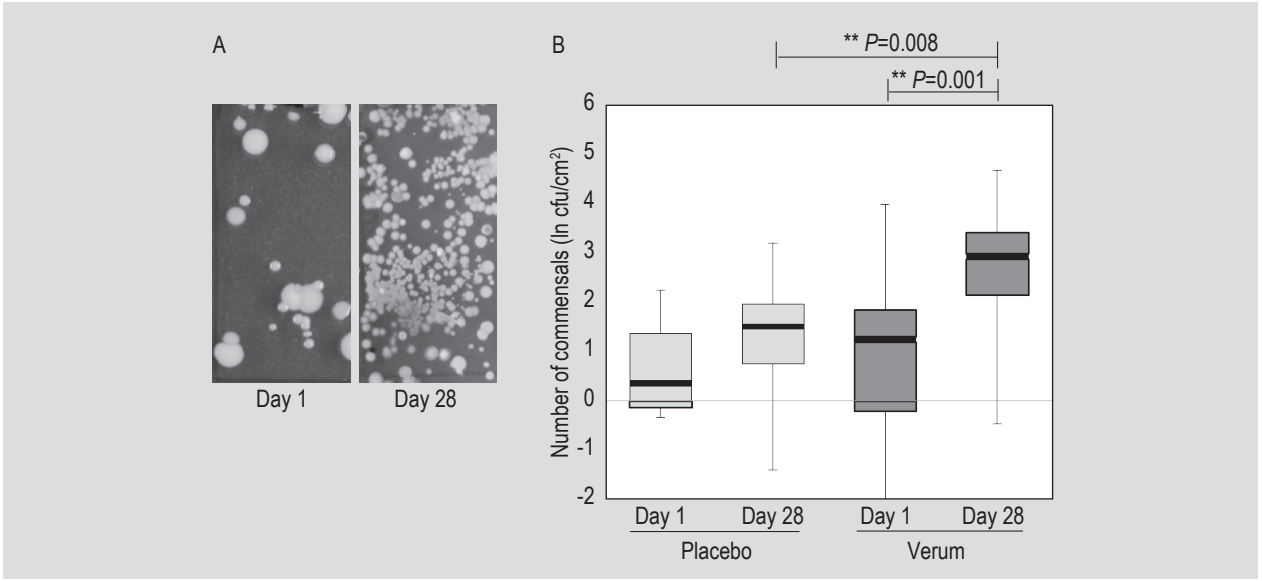

Figure 3. Influence of verum containing *Lactobacillus brevis* DSM17250 extract on commensal skin microbiota of the lower leg. (A) Colony growth on SAID agar at day 1 and 28 (typical example of verum group). (B) Number of skin bacteria at day 0 (baseline) and after 28 days of treatment of the anterior tibial area with placebo or verum containing *L. brevis* DSM17250 extract. Data are given as log-transformed medians. Statistically significant differences are marked by asterisks.

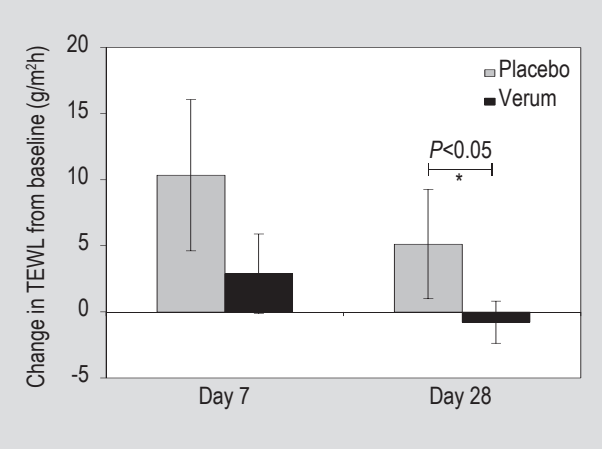

Figure 5. Influence of verum containing *Lactobacillus brevis* DSM17250 extract on skin barrier function. Absolute changes in transepidermal water loss (TEWL) from baseline after application of either verum (n=16) or placebo cream (n=14) for 7 and 28 days. The graphic displays mean values and referring standard deviation of the sampling distribution.

group and slightly increasing by 2.89 g/m^2/h in the verum group. After 4 weeks treatment (day 28) there was a significant difference in change of TEWL between the verum and placebo groups (P=0.048). In the placebo group the mean TEWL remained significantly increased by 5.13 g/m^2/h in comparison to the baseline (P=0.042), whereas in the verum group the mean TEWL value decreased by 0.79 g/m^2/h. This indicates as an improvement in barrier function in test persons applying the cream containing the *L. brevis* DSM17250 extract. In absolute numbers, the median TEWL at day 28 was 8.98 g/m^2/h in the verum group, whereas the median value of placebo group was 15.68 g/m^2/h.

Clinical and self-assessment of skin dryness

Scaling, roughness, redness and cracks/fissures were evaluated at day 1, 7, 14 and 28 using DASI score. The total DASI score at day 28 had decreased in the verum group by 4.50 and within the placebo group by 3.34, where lower scores mean a reduction in xerosis. This demonstrates that *L. brevis* DSM17250 extract (verum cream) is effective in improving xerosis. The results were confirmed by the frequency distribution calculated from the data of day 14 and 28. After two weeks treatment (day 14) the symptoms of dry skin were reduced in 68.8% of the subjects of the verum group. At day 28, the mean DASI of roughness of skin surface had decreased in 100% of the subjects that used the verum product in comparison to only 78.6% of the placebo group. At day 28, the occurrence of redness was reduced in 81.2% of the subjects who applied the verum product whereas only 62.5% of placebo group exhibited reduction of redness. Regarding the scaling of the evaluated skin areas 62.5% of the subjects showed no sign of scaling at all after application of the verum cream (day 28), whereas only 42.9% exhibited no scaling in the placebo group. The measured changes from day 1 to day 28 were greater for all single parameters in the verum group in comparison to placebo group (data not shown). On day 1 there were no statistically significant differences between the verum and placebo group with respect to all 4 determined parameters and the total DASI score. The test persons were asked to self-assess skin dryness at each visit. In total, the symptoms of dry skin (xerosis cutis) were improved from day 1 to day 28 in 62% of the subjects using the verum cream (versus 43% in the placebo group). The evaluation of the daily diary of the subjects demonstrated that the individual skin parameters were improved by about 70% in the verum group (versus 57% in the placebo group).

4. Discussion and conclusions

Here we report on a novel concept to promote the human commensal beneficial bacteria which are a part of the human immune system as they exhibit interference against pathogens. The results show that the selected *L. brevis* strain DSM17250 is able to stimulate the growth of the skin health supporting commensal *S. epidermidis* (Christensen and Bruggemann, 2014; Gallo and Nakatsuji, 2011). A dose-dependent growth promoting effect was detected both in *in vitro* plate assays and liquid co-incubation assay. In contrast to previously investigated prebiotics in cosmetic formulations, such as glucomannans (Al-Ghazzewi and Tester, 2010), the stimulation effect of *L. brevis* DSM17250 extract is not based on carbohydrate fermentation by the commensal skin bacteria since glucose used as control substrate added in increasing concentrations showed no influence on the growth behaviour of *S. epidermidis* in the *in vitro* test system. It is proposed that the active peptide compound of *L. brevis* DSM17250 extract may function as a communication signal molecule, thereby modulating diverse physiological processes in a cell density or growth-phase dependent manner. These kind of quorum sensing systems are known in several Gram-positive bacteria (Kleerebezem *et al.*, 1997; March and Bentley, 2004). In order to determine the anti-inflammatory properties of *L. brevis* DSM17250 in *in vitro* human cell assays, the bioactive compounds of the *L. brevis* DSM17250 extract were purified by fractionating the aqueous phase using hydrophobic interaction chromatography and gel filtration. The identified peptide compound did not show any cytotoxic effects on epidermal keratinocytes (NHEK) or any induction of secretion of the pro-inflammatory cytokine IL-1α of these cells. The data resulting from the *in vitro* cell assay show that the peptide compound possesses anti-inflammatory properties. The cytokine IL-1α release of NHEK cells triggered by LPS exposure could be significantly reduced by pre-treatment of the cells with the peptide compound. The rather low, yet significant effect is due to the fact that keratinocytes and not peripheral blood

mononuclear cells were used in combination with LPS (a weak inflammatory agent).

We further investigated the *L. brevis* DSM17250 extract in a double-blinded, placebo-controlled clinical trial to evaluate both the efficacy and the compatibility in subjects with xerosis. The clinical data show that application of the *L. brevis* DSM17250 extract in a topical product has a positive effect on the primary outcome, namely the fast recovery and growth promotion of the protective resident skin microbiota as well as on the associated physiological skin parameters evaluated as secondary outcomes. The 28-day application of verum cream containing the *L. brevis* DSM17250 extract to the lower legs lead to significant growth promotion of commensal microbiota both with regard to the change from the beginning to the end of treatment and in comparison to the use of placebo cream. In the 28-day treatment period no difference in numbers of commensal bacteria was measured in the control group. The normal level of commensal bacteria was not exceeded in any case. Total cell counts of up to 10^4 cfu/cm^2 are found e.g. for healthy skin on forearm (WHO, 2009). This correlates with microbiota on tibea (data found on healthy subjects in this study). The maximum counts of commensals measured at the end of the study (day 28) were 8.5×10^2 cfu /cm^2 and 3.9×10^2 cfu/cm^2 for *S. epidermidis*. The mean level of *S. epidermidis* counts in the verum group was increased from 8.1 to 80.8 cfu/cm^2 after 28 days treatment, i.e. an increase of 1 log. The number of *S. aureus* colonies remained unchanged in subjects with insignificant or non-existent colonisation at the beginning of the study or was severely reduced in subjects with high *S. aureus* loads, respectively, indicating that the *L. brevis* DSM17250 extract selectively promotes the growth of the beneficial microbiota at the expense of harmful bacteria.

Thus the application of *L. brevis* DSM17250 extract is following the probiotic concept to modify the microbiota and to replace harmful microbes by useful microbes possessing protective properties (Fuller, 1989). This impact on skin microbiota composition provides a new prophylactic and therapeutic strategy boosting the local immunity or host defence indirectly by using the interplay of the skin microbiota with the human host. Especially in case of inflammatory or autoimmune disorders, such as atopic dermatitis (AD) there is now clear evidence that they are associated with shifts in the resident microbiota from a healthy to a diseased state and that they can be viewed as dysbiotic host-microbial states (Belkaid and Segre, 2014). In cases of psoriasis, where dysbiosis is associated with skin inflammation, oral probiotics has been suggested as a means to restore the resident microbes, that are diminished when the disorder is present (Huang and Tang, 2015). It was reported that pattern recognition receptors, such as Toll-like receptor (TLR) 2 of the innate immune system, are triggered by commensal *S. epidermidis* (Stevens *et al.*, 2009). Activated TLR2 signalling can induce antimicrobial peptide (AMP) expression of the host, such as β-defensin, which is important to fight off the colonisation of pathogenic strains (Lai *et al.*, 2010). In addition, it has been demonstrated that *S. epidermidis* strains are capable of inhibiting biofilm formation of *S. aureus* by secretion of a serine protease (Iwase *et al.*, 2010) or by production of a thiolactone-containing peptide which blocks the *S. aureus* agr quorum-sensing system controlling production of various virulence factors of *S. aureus* (Otto *et al.*, 1999).

Beside secretion of an arsenal of toxins that damage host cells *S. aureus* produces extracellular enzymes including proteases, lipases, hyaluronidase and collagenase that may contribute to tissue damage (Foster, 2005). Thus the disruption of ceramides or collagen results in impairment of skin barrier function supporting xerosis, as ceramides are the major water storing molecules in the extra cellular matrix (Imokawa, 2014). While Volz and Biedermann (2009) and Gueniche *et al.* (2009, 2010) reported a direct effect of topically applied probiotics by inducing natural defence mechanisms, the present paper is to our knowledge the first description of a bioactive from a probiotic *Lactobacillus* promoting the beneficial skin microbiota to enhance the skin natural defence. Whether the effect is a direct stimulation of the innate immunity or an indirect action by stimulating *S. epidermidis* that then leads to stimulation of the innate system needs to be studied further.

In order to support the hypothesis of relationship between a balanced beneficial skin microbiota and improved skin structure we evaluated the skin barrier function by determination of TEWL and the assessment of associated physiological parameters by the means of clinical DASI score before and after treatment with the cream containing the *L. brevis* DSM17250 extract. The study demonstrated that the application of the *L. brevis* DSM17250 extract containing cream also resulted in significant changes in TEWL compared to the use of placebo cream. Whereas the TEWL value and thereby the loss of water in skin increased in the placebo group during the study period, the TEWL in the verum group was reduced indicating the stabilisation and improvement of skin barrier function. This outcome is supported by the findings of Nodake *et al.* (2015) who could demonstrate that continuous direct application of autologous *S. epidermidis* samples to skin led to increased colonisation levels and was related with significant improvement of skin moisture retention, water and lipid content in the skin of treated subjects. In addition to the biophysical parameters, the clinical symptoms of xerosis, like tightness, roughness, scaling and sensitivity of skin (Hashizume, 2004) as evaluated both by the dermatologist using the DASI score and by self-assessment of the subjects, was improved on average by 70% during the 4-week application of the *L. brevis* DSM17250 extract containing cream. The daily topical treatment with *L.*

brevis DSM17250 extract specifically shapes the microbial community providing a powerful advantage for microbes endowed with regulatory or protective properties thereby moisturizing the human skin and improving the barrier function providing a powerful and gentle solution to treat skin conditions, such as xerosis or atopic eczema.

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Conflict of interest

Caterina Holz, Johanna Benning, Andreas Heilmann, Jeffrey Schultchen, Detlef Goelling declare that they have no conflict of interest. Christine Lang owns stock in Organobalance GmbH.

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