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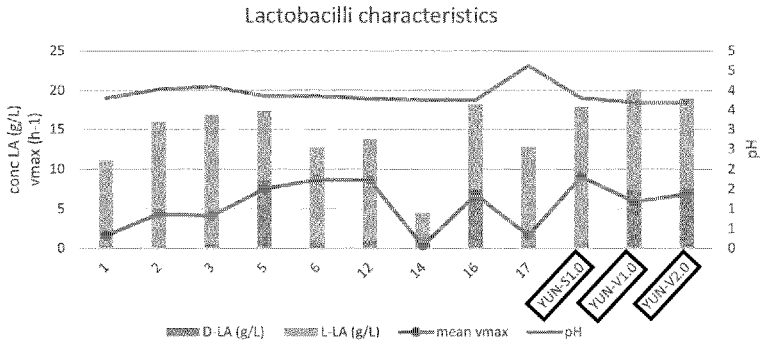
(54) Title: DERMATOLOGICAL PREPARATIONS FOR MAINTAINING AND/OR RESTORING HEALTHY SKIN MICROBIOTA

Fig. 1



(57) Abstract: The present invention is directed to the direct application of beneficial or probiotic bacteria to the skin for maintenance of a healthy skin microbiota and to help restore an unbalanced skin microbiota. The application is based on the use of selected Lactobacillus strains as anti-5 pathogenic agents, in particular L. plantarum, L. pentosus and/or L. rhamnosus, against common skin pathogens, whereby produced acids such as lactic acid are important antimicrobial factors.

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DERMATOLOGICAL PREPARATIONS FOR MAINTAINING AND/OR RESTORING HEALTHY SKIN MICROBIOTA

FIELD OF THE INVENTION

The present invention is directed to the direct topical application of beneficial or probiotic bacteria to the skin for maintenance of a healthy skin microbiota and to help restore an unbalanced skin microbiota. This restoration of a healthy microbiota falls under the term probiotherapy, defined as the use of beneficial micro-organisms or probiotics to restore a healthy microbiota at a site where microbial dysbiosis occurs. The application is based on the use of selected *Lactobacillus* strains as anti-pathogenic agents, in particular *L. plantarum, L. pentosus* and/or *L. rhamnosus,* against common skin pathogens, whereby produced acids such as lactic acid are important antimicrobial factors.

BACKGROUND TO THE INVENTION

Hence, it was an object of the present invention to provide a solution for subjects suffering from skin conditions due to an aberrant microbial balance on the skin. Thereto, it was found that the topical use of *L. plantarum,* L. *pentosus* and/or *L. rhamnosus* species on the skin is very effective in restoring and/or maintaining a healthy skin microbiota, and is thus very suitable in relieving skin conditions in subjects in need thereof.

Oral formulations comprising *Lactobacillus* strains have been used before in the treatment of skin conditions like atopic dermatitis. However, oral administration versus direct topical administration are different administration routes and each have a completely different underlying mechanism. In oral administration, in particular a beneficial effect on the general health via immuno-stimulation is intended, whereas by direct dermatological (skin) administration, competition with 'unwanted' microorganisms occurs.

Like the gastrointestinal tract, our skin harbours a unique microbial ecosystem. The type of micro-organisms found on the skin depends on a combination of host factors, environmental factors but also topographical location. The role of this microbiota in skin disorders is still not completely unravelled. However, it seems that, at least, some skin disorders are linked to a disturbed microbiota as antimicrobial treatments can improve clinical symptoms (Grice & Segre 2011). For example, in acne vulgaris a correlation has been found with the presence of *Propionibacterium acnes* (Beylot et al. 2014). Although acne vulgaris is a multifactorial condition and is, among other factors, influenced by hormonal factors, these *P. acnes* bacteria seem to induce inflammation resulting in inflamed pimples also called papules or pustels. As *P. acnes* is also found on a healthy skin not causing acne, this suggests that other factors are involved, tipping the balance of the composition of the skin microbiota towards an overgrowth of this bacteria.

Another example of a skin disorder where the microbiota seems to be important is dandruff (Wang et al. 2015; Sugita et al. 2015; Grice & Segre 2011). In people with dandruff, the fungus *Malassezia* is often overrepresented. Indications that it is this fungus that is a possible cause of the condition, come from the fact that antimycotic treatment improves the symptoms. In contrast, antibacterial therapies do not improve dandruff. Again, other factors are expected to be involved in this skin disorder but the correlation with *Malassezia* is intruiging.

Similarly as for dandruff, fungal skin infections with *Candida albicans* or dermatophytes, like *Trichophyton spp.*, seem to be skin disorders linked to a dysbiosis in the skin microbiota as these species are also present on healthy subjects. In the case of Tinea pedis or 'athlete's foot' overgrowth of *Trichophyton rubrum* or *T. mentagrophytes* is often observed.

The production of lactic acid in combination with possibly other antimicrobial compounds like bacteriocins seems to give protection against aforementioned infections and dysbiotic conditions and lactic acid seems to be active against bacterial, fungal and even viral pathogens. It is for this reason that lactobacilli are considered to be important in the homeostasis of the dynamical dermatological ecosystem. Potential health promoting mechanisms of lactobacilli are i) to preserve a healthy skin pH (+/- 5.5), mainly by production of lactic acid; ii) production of antimicrobial compounds and competitive exclusion of pathogens; iii) modulation of immune response and iv) strengthening of the epithelial barrier.

Hence, it was an object of the present invention to provide a solution for subjects suffering from dermatological conditions due to an aberrant microbial balance of the skin. Thereto, it was found that the topical dermatological use of L. *plantarum*, L. *pentosus* and/or L. rhamnosus species is very effective in restoring and/or maintaining a healthy microbiota on the skin, and is thus very suitable in relieving dermatological conditions in subjects in need thereof.

Oral formulations comprising *Lactobacillus* strains have been used before in the treatment of dermatological disorders. However, oral administration versus direct topical administration are different administration routes and each have a completely different underlying mechanism. In oral administration, in particular a beneficial effect on the general health via immuno-stimulation is intended, whereas by direct administration on the skin, competition with 'unwanted' microorganisms occur.

SUMMARY OF THE INVENTION

In a first aspect, the present invention provides a topical skin composition comprising one or more live *Lactobacillus* species; wherein at least one of said *Lactobacillus* species is *L. plantarum;* more in particular a *L. plantarum* strain having at least 97% sequence similarity with SEQ ID N° 4 in its 16S rRNA gene.

In a further aspect, the present invention provides a live *Lactobacillus* species for use in restoring and/or maintaining a healthy skin microbiota, by topical route, said *Lactobacillus* species being *L. plantarum;* more in particular a *L. plantarum* strain having at least 97% sequence similarity with SEQ ID N° 4 in its 16S rRNA gene.

In yet a further aspect, the present invention provides the use of one or more live *Lactobacillus* species, in the preparation of a topical skin composition for restoring and/or maintaining a healthy skin microbiota; wherein at least one of said *Lactobacillus* species is *L. plantarum;* more in particular a *L. plantarum* strain having at least 97% sequence similarity with SEQ ID N° 4 in its 16S rRNA gene.

The present invention also provides a method for restoring and/or maintaining a healthy skin microbiota; comprising at least one step of administering by topical route, to an individual, an effective amount of one or more live *Lactobacillus* species; wherein at least one of said *Lactobacillus* species is *L. plantarum;* more in particular a *L. plantarum* strain having at least 97% sequence similarity with SEQ ID N° 4 in its 16S rRNA gene.

In yet another aspect, the present invention provides a composition comprising one or more live *Lactobacillus* species for use in restoring and/or maintaining a healthy skin microbiota, by topical route, said *Lactobacillus* species being selected from the list comprising *L. plantarum, L. pentosus* and L. *rhamnosus;* more in particular a L. *plantarum* strain having at least 97% sequence similarity with SEQ ID N° 4 in its 16S rRNA gene, a *L. pentosus* strain having at least 97% sequence similarity with SEQ ID N° 1 in its 16S rRNA gene and a L. rhamnosus strain having at least 97% sequence similarity with SEQ ID N° 5 in its 16S rRNA gene.

The present invention further provides a *Lactobacillus* strain being *L. rhamnosus* YUN-S1.0 deposited under accession number LMG P-29611 (deposited at BCCM on May, 12 2016).

In a particular aspect, the present invention provides a composition comprising one or more *Lactobacillus* strains as defined herein above.

In a particular embodiment, the composition of the present invention is a topical skin composition, more in particular in the form of a gel, cream, foam, lotion or ointment.

In another particular embodiment, the present invention provides the Lactobacillus strain as defined herein above or the compositions as defined herein above; for use in restoring and/or maintaining a healthy skin microbiota, by topical route.

In a particular aspect, the present invention provides a topical use of one or more live *Lactobacillus* species in probiotherapy of the skin; wherein said *Lactobacillus* species are selected from the list comprising *L. plantarum, L. pentosus* and L. *rhamnosus*; more in particular, said probiotherapy consists of restoring and/or maintaining a healthy skin microbiota in a subject in need thereof.

In another particular embodiment, said *Lactobacillus* species in the topical uses, methods and compositions as disclosed herein, is a *Lactobacillus* strain selected from the list comprising *L. plantarum* YUN-V2.0 deposited under accession number LMG P-29456 (deposited at BCCM on Mar, 09 2016), *L. pentosus* YUN-V1.0 deposited under accession number LMG P-29455 (deposited at BCCM on Mar, 09 2016); and L. rhamnosus YUN-S1.0 deposited under accession number LMG P-29611 (deposited at BCCM on May, 12 2016).

BRIEF DESCRIPTION OF THE DRAWINGS

With specific reference now to the figures, it is stressed that the particulars shown are by way of example and for purposes of illustrative discussion of the different embodiments of the present invention only. They are presented in the cause of providing what is believed to be the most useful and readily description of the principles and conceptual aspects of the invention. In this regard no attempt is made to show structural details of the invention in more detail than is necessary for a fundamental understanding of the invention. The description taken with the drawings making apparent to those skilled in the art how the several forms of the invention may be embodied in practice.

Fig. 1: Characteristics of lactobacilli in reference to growth, production of D- and L-lactic acid (LA) and lowering of the pH of the medium.

Fig. 2: Time course experiment for the analysis of the antipathogenic effect of spent culture supernatant of lactobacilli against *Propionibacterium acnes*. Growth of the bacteria (optical density at 600nm; Y-axis) is measured in time (X-axis). Each graph shows replicates of growth of *P. acnes*. It can be clearly noted that without any addition of antibiotic or SCS, *P. acnes* quickly starts to grow (NC1). Similar as when erythromycin at 50µg/ml is added, SCS of all lactobacilli prevents growth of *P. acnes* while SCS of streptococci or staphylococci does not inhibit growth. *Erythromycine (50ug/ml); #Erythromycine (5ug/ml); §Minocycline (20 µg/ml) NC1=medium control; NC2=MRS at pH4.3; Numbers 1 to 22 = lactobacilli strains (for details see table 1); St=*Streptococcus thermophilus*; Ss=*Streptococcus salivarius*; Se=*Staphylococcus epidermidis*; T0.5=0.5% Tween 80; T1=1% Tween 80.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is based on the discovery of specific *Lactobacillus* strains that can compete with growth of *Propionibacterium acnes, Candida albicans*, *Malassezia spp.*, *Trichophyton spp.* and bacteria or fungi that are linked with skin conditions like acne vulgaris, dandruff, tinea pedis or other fungal skin infections. These selected strains are herein generally termed "YUN" strains and are capable of competing with skin pathogens and thereby restore a healthy skin microbiota. This restoration of a healthy microbiota falls under the term probiotherapy, defined as the use of beneficial micro-organisms or probiotics to restore a healthy microbiota at a site where microbial dysbiosis occurs.

Hence, in a first aspect, the present invention provides a topical skin composition comprising one or more live *Lactobacillus* species; wherein at least one of said *Lactobacillus* species is *L. plantarum*; more in particular a *L. plantarum* strain having at least 97% sequence similarity with SEQ ID N° 4 in its 16S rRNA gene.

Said composition according to the present invention may comprise further *Lactobacillus* species such as for example selected from the non-limiting list comprising *L. pentosus*, *L. gasseri, L. crispatus, L. acidophilus, L. jensenii, L. fermentum, L. rhamnosus.*

In the context of the present invention, the term "topical" is meant to be the local delivery at a specified location of the body, in particular the application to a particular place on the body. In particular, it includes the application via non-solid formulations such as creams, foams, gels, lotions or ointments. The term "topical" is not meant to include the delivery in the form of solid preparations such as capsules, tablets, …

Hence, the term "topical skin" is meant to include the local delivery using non-solid formulations directly onto the skin of the body. Preferably, the compositions according to the present invention are applied over a large area of the skin in order to be most effective.

In the context of the present invention the term "live *Lactobacillus* species" is meant to be viable *Lactobacillus* species, and is not meant to be fragments, culture supernatants, or killed forms thereof.

In a further aspect, the present invention provides a live *Lactobacillus* species for use in probiotherapy of the skin, by topical route, said *Lactobacillus* species being *L. plantarum*; more in particular a *L. plantarum* strain having at least 97% sequence similarity with SEQ ID N° 4 in its 16S rRNA gene. As already defined herein above, said probiotherapy is meant to be the restoration and/or maintainance of a healthy skin microbiota in a subject in need thereof.

Subjects that may benefit from such probiotherapy are for example people/persons with a skin conditions linked to a disturbed skin microbiota possibly due to bacterial or yeast infections and/or any dysbiosis caused by overgrowth of specific pathogenic micro-organisms, like acne vulgaris, tinea pedis, dandruff, rosaceae, impetigo,…

Hence, in a further aspect, the present invention provides the use of one or more live *Lactobacillus* species, in the preparation of a topical skin composition for restoring and/or maintaining a healthy skin microbiota; wherein at least one of said *Lactobacillus* species is *L. plantarum*; more in particular a *L. plantarum* strain having at least 97% sequence similarity with SEQ ID N° 4 in its 16S rRNA gene.

The present invention also provides a method for restoring and/or maintaining a healthy skin microbiota; comprising at least one step of administering by topical route, to an individual, an effective amount of one or more live *Lactobacillus* species; wherein at least one of said *Lactobacillus* species is *L. plantarum*; more in particular a *L. plantarum* strain having at least 97% sequence similarity with SEQ ID N° 4 in its 16S rRNA gene.

In yet another aspect, the present invention provides a composition comprising one or more live *Lactobacillus* species for use in restoring and/or maintaining a healthy skin microbiota, by topical route, said *Lactobacillus* species being selected from the list comprising L. *plantarum*, *L. pentosus* and *L. rhamnosus*; more in particular a L. *plantarum* strain having at least 97% sequence similarity with SEQ ID N° 4 in its 16S rRNA gene; a *L. pentosus* strain having at least 97% sequence similarity with SEQ ID N° 1 in its 16S rRNA gene and a L. rhamnosus strain having at least 97% sequence similarity with SEQ ID N° 5 in its 16S rRNA gene.

The present invention further provides a *Lactobacillus* strain selected from the list comprising *L. pentosus* YUN-V1.0 deposited under accession number LMG P-29455 (deposited at BCCM on Mar, 09 2016); *L. plantarum* YUN-V2.0 deposited under accession number LMG P-29456 (deposited at BCCM on Mar, 09 2016); and L. rhamnosus YUN-S1.0 deposited under accession number LMG P-29611 (deposited at BCCM on May, 12 2016)

The microbiological deposits mentioned herein, have been made with the BCCM/LMG Bacteria collection ("Belgian co-ordinated collections of micro-organism") with correspondence address: Laboratorium voor Microbiologie, Universiteit Gent, K.L. Ledeganckstraat 35 – 9000 Gent, Belgium

Lactobacillus pentosus YUN-V1.0 is a single colony isolate obtained in our lab after subculturing of a strain, that was originally a vaginal isolate of healthy woman. The 16S rRNA gene sequence (SEQ ID N° 1) for strain *L. pentosus* YUN-V1.0 was determined by PCR using primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3' – SEQ ID N° 2) and 1525R (5'-

AAGGAGGTGATCCAGCCGCA-3' – SEQ ID N° 3).

YUN-V2.0 and YUN-V3.0 are single colony isolates obtained in our lab after subculturing of *Lactobacillus plantarum* strains that were originally isolated from human saliva and a maize silage respectively. The 16S rRNA gene sequence (SEQ ID N° 4) for strain *L. plantarum* YUN-V2.0 was determined by PCR using primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3' – SEQ ID N° 2) and 1525R (5'-AAGGAGGTGATCCAGCCGCA-3' – SEQ ID N° 3).

YUN-S1.0 is a single colony isolate obtained in our lab after subculturing of a *Lactobacillus rhamnosus* strain that was originally isolated from a healthy person. The 16S rRNA gene sequence (SEQ ID N° 5) for strain *L. rhamnosus* YUN-S1.0 was determined by PCR using primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3' – SEQ ID N° 2) and 1525R (5'-AAGGAGGTGATCCAGCCGCA-3' – SEQ ID N° 3).

These particular "YUN" strains can either be used as such, or are preferably formulated in a composition comprising such strains. Said compositions are topical skin compositions more in particular in the form of non-solid formulations such as creams, foams, gels, lotions or ointments.

In particular, the present invention provides the above defined "YUN" strains for use in probiotherapy of the skin, i.e. for restoring and/or maintaining a healthy skin microbiota.

In yet a further aspect, the present invention provides a topical use of one or more live *Lactobacillus* species in probiotherapy of the skin; wherein said *Lactobacillus* species are selected from the list comprising *L. plantarum, L. pentosus* and *L. rhamnosus*; more in particular, said probiotherapy consists of restoring and/or maintaining a healthy skin microbiota in a subject in need thereof.

In a specific embodiment, the *Lactobacillus* species in the topical uses, methods and compositions as disclosed herein, is a *Lactobacillus* strain selected from the list comprising *L. plantarum* YUN-V2.0 deposited under accession number LMG P-29456 (deposited at BCCM on Mar, 09 2016); *L. pentosus* YUN-V1.0 deposited under accession number LMG P-29455 (deposited at BCCM on Mar, 09 2016); and *L. rhamnosus* YUN-S1.0 deposited under accession number LMG P-29611 (deposited at BCCM on May, 12 2016).

EXAMPLES

MATERIALS AND METHODS

Bacterial strains and growth conditions

Lactobacillus strains (Table 1) were grown at 37°C in de Man, Rogosa and Sharpe (MRS) medium (Carl Roth). All bacteria were grown in non-shaking conditions and inoculated from glycerol stocks (-80°C). Solid media contained 1.5% (w/v) agar.

Table 1: Bacterial strains used in this research

Species	#	Strain	Relevant genotype or description	Reference and/or Source
LACTOBACILLI				
Lactobacillus casei	1	ATCC334	Single colony isolate obtained in our lab from a stock culture of ATCC334	ATCC
Lactobacillus casei	2	DN-114001	Single colony isolate obtained in our lab from a commercially available fermented drink (Actimel®) containing *L. casei* DN-114001, confirmed by sequencing	Commercial probiotic product
Lactobacillus casei	3	Shirota	Single colony isolate obtained in our lab from a commercially available fermented drink containing *L. casei* Shirota (Yakult®), confirmed by sequencing	Commercial probiotic product
Lactobacillus pentosus	4	YUN-V1.0	Single colony isolate	
Lactobacillus plantarum	5	LMG1284	Single colony isolate from *L. plantarum* ATCC8014 or LMG1284	ATCC
Lactobacillus reuteri	6	RC-14	Single colony isolate obtained in our lab from a commercially available probiotic supplement containing *L. reuteri* RC-14, confirmed by sequencing	Commercial probiotic product
Lactobacillus rhamnosus	7	YUN-S1.0	Clinical isolate	
Lactobacillus rhamnosus	12	GR-1	Single colony isolate obtained in our lab from a commercially available probiotic supplement containing *L. rhamnosus* GR-1	(Chan et al. 1984; 1985; Reid 1999; Reid & Bruce 2001), ATCC
Lactobacillus helveticus	14	AMB-2	single colony isolate	Commercial probiotic product
Lactobacillus plantarum	15	YUN-V2.0	Single colony isolate	
Lactobacillus plantarum	16	5057	Single colony isolate	
Lactobacillus paracasei	17	LMG12586	Single colony isolate obtained in our lab from a stock culture of LMG12586	BCCM/LMG
Lactobacillus plantarum	22	/	Single colony isolate	
Lactobacillus pentosus	25	LMG8041	Single colony isolate	BCCM/LMG

PATHOGENS				
Trichophyton rubrum	2	/	Clinical isolate	BCCM/LMG
Malassezia furfur		/	Clinical isolate	BCCM/LMG
Candida albicans	/	/	Clinical isolate	

Preparation of spent culture supernatant (SCS) of selected strains

To obtain spent culture supernatant (SCS) containing the secreted active antimicrobial products, growth medium specific for each species was inoculated from a preculture and incubated for 24h. SCS was obtained by centrifugation for 30 min. at 6797 g (8000 rpm) at 4°C. Afterwards, the SCS was filter sterilized (0.20 µm cellulose acetate, VWR).

Antimicrobial activity assays for co-cultures of live lactobacilli against Malassezia furfur, Trichophyton rubrum, Propionibacterium acnes and Candida albican.

The antimicrobial activity of the selected bacteria was explored by standard antimicrobial tests with some minor modifications. The antimicrobial activity of the selected bacteria was explored by spot assay (Schillinger and Lücke 1989). Briefly, 1-3 µL of each culture was spotted on an agar plate. These plates were incubated for 24h up to 72h depending on the strain. Next, an overnight culture of the pathogen was diluted into 7 mL of soft agar of the medium of the pathogen and poured over the plates with the spots of the selected strains. The plates were incubated overnight at 30-37°C, after which the inhibition zones were measured. A spot of miconazole (for fungi) and/or 0.1% hexetidine and/or tetracycline (for Propionibacterium acnes) was added to the spot plate as positive control before the soft agar was poured.

Radial diffusion test of SCS of lactobacilli

In addition, the antimicrobial activity of spent culture supernatant (SCS) was investigated with a protocol as previously described for the competition assays between lactobacilli and gastro-intestinal pathogens (Coconnier et al. 1997). Miconazol (for fungi) and tetracycline (for Propionibacterium acnes) was used as a positive control. Sterile growth medium was used as a negative control.

Time course analysis of the antimicrobial activity of SCS of the selected strains against Candida, Propionibacterium acnes, Malassezia furfur and Trichophyton spp (further referred to as 'pathogens').

The time course analysis was performed similarly as described previously (De Keersmaecker et al. 2006) with minor modifications. Briefly, an overnight culture of the pathogen was added to the wells of a microplate filled with 50-80% the proper medium supplemented with 50-5% SCS of lactobacilli. MRS at pH 4,3 and antibiotics or antimycotics at the proper concentration were used as a negative and a positive control, respectively. Bacteria or fungi were grown and the optical density (OD) was measured at 590 nm each 30 min during 3 days using a Synergy HTX

multi-mode reader (Biotek). Each test was measured at least in triplicate and the average OD was calculated. The antimicrobial activity was expressed as the relative optical density reached after 24h (stationary phase) compared to the negative controls.

Antibiotic susceptibility

Antibiotic sensitivity was evaluated using the Kirby-Bauer disc diffusion test. In short, antibiotics were spotted on paper discs and the bacterial inhibition zone was measured on agar plates. The antibiotics tested were erythromycin, normocin, tetracyclin, ampicillin and clindamycin at relevant concentrations.

Proof-of-concept human clinical trial in patients with acne vulgaris

A proof-of-concept clinical trial was performed on 20 patients with acne vulgaris. Patients were men between 12-25 years with mild inflammatory acne. The aim of this proof-of-concept trial was to assess the impact of a topical probiotic cream (containing +-10-8 colony forming units (CFU) of L. pentosus YUN-V1.0, +-10-8 CFU of L. plantarum YUN-V2.0 and +-10-8 CFU L. rhamnosus YUN-S1.0 per application of 1g of the topical cream ACN) on the skin microbiota and on the acne severity. Patients were asked to apply the cream twice daily for 56 days (8 weeks). The patients were seen by a dermatologist at start (before the therapy), week 4, week 8 and week 10. A skin swab was taken at each visit. Bacterial DNA was isolated from these samples by the commercial MoBio Powersoil kit (cfr. Human Microbiome Project). Isolated DNA was analysed via 16S rRNA amplicon sequencing with MiSeq Illumina and a bio-informatical analysis was performed. Moreover, a clinical scoring was performed and a photograph taken at each visit.

Proof-of-concept human clinical trial in patients with tinea pedis (Athlete's foot)

A proof-of-concept clinical trial was performed on 20 patients with tinea pedis. Patients were between 18-65 years having tinea pedis. The aim of this proof-of-concept trial was to assess the impact of a topical probiotic cream (containing +-10-8 colony forming units (CFU) of L. pentosus YUN-V1.0, +-10-8 CFU of L. plantarum YUN-V2.0 and +-10-8 CFU L. rhamnosus YUN-S1.0 per application of 1g of of the topical cream FNG) on the skin microbiota and on the Trichophyton infection. Patients were asked to apply the cream twice daily for 56 days (8 weeks). The patients were seen by a dermatologist at start (before the therapy), week 4, week 8 and week 10. A skin swab was taken at each visit. Bacterial DNA was isolated from these samples by the commercial MoBio Powersoil kit (cfr. Human Microbiome Project). Isolated DNA was analysed via 16S rRNA amplicon sequencing with MiSeq Illumina and a bio-informatical analysis was performed. For analysis of the presence of the fungi, swabs were also plated out on Trichophyton specific medium (medium suggested by BCCM). Colony PCR using universal ITS ('internal transcribed region') primers ITS1 (SEQ ID N°6) (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (SEQ ID N° 7) (5'-TCCTCCGCTTATTGATATGC-3') followed by sequencing was performed to identify the fungi. Moreover, a clinical scoring was performed and a photograph taken at each visit.

RESULTS

Growth characteristics and lactate production

Possible beneficial or probiotic strains were characterized in terms of growth characteristics, lactate production and ability of lowering of the pH of the medium. These characteristics are expected to be important for the antipathogenic activity. These data show that *Lactobacillus pentosus* YUN-V1.0 and *L. plantarum* YUN-V2.0 and *L. rhamnosus* YUN-S1.0 produce the highest amount of lactic acid (Fig 1).

Antipathogenic activity against Propionibacterium acnes

Time course experiments were performed analyzing the antimicrobial activity of spent culture supernatant (SCS) of the selected strains against *Propionibacterium acnes*. SCS of all tested strains inhibited the growth of *Propionibacterium acnes* while SCS of other bacterial species like *Streptococcus thermophilus* and *S. salivarius*, both also lactic acid bacteria, and *Staphyloccus epidermidis* did not inhibit growth of *P. acnes*. This suggests species and perhaps strain specific properties of the selected lactobacilli to be important for the antipathogenic activity against *P. acnes* (Fig 2).

Antipathogenic activity against Malassezia, Trichophyton and Candida

In a next phase, the beneficial or probiotic bacteria were screened for their antipathogenic effect against specific skin pathogens. The results of a spot assays against *Malassezia furfur, Trichophyton rubrum* and *Candida albicans* are shown in table 1, 2 and 3 respectively.

Table 1: Spot assay of selected lactobacilli against *Malassezia furfur.*

	Malassezia furfur		
Strain	Exp 1	Exp 2	Exp 3
1	++	-	+
2	++	-	+
3	+	+	-
4	++	+++	++
5	+++	++	++
6	+	++	++
7	++	-	-
12	+	-	-
13	+	-	+
14	-	+	-
15	+++	+++	+++
16	++	++	++
17	-	+	-
22	+++	++	++
25	++	++	+

*three independent repeats are shown

Table 2: Spot assay of selected lactobacilli against *Trichophyton rubrum.*

	Trichophyton rubrum		
Strain	Exp 1	Exp 2	Exp 3
1	+	++	+++
2	+	++	++
3	+	++	++
4	++	++	+++
5	++	++	+++
6	-	-	+++
7	++	+++	+
12	++	+++	+++
13	++	-	-
14	+	++	++
15	+++	+++	+++
16	++	+++	+++
17	+	+++	++
22	++	+++	+++
25	++	+++	+++

*three independent repeats are shown

Table 3: Radial diffusion assay of selected lactobacilli against *Candida albicans.*

	Candida albicans		
Strain	Exp 1	Exp 2	Exp 3
1	-	-	-
2	+	+	+
3	+	+	+
4	++	++	++
5	+	+	+
6	-	-	-
7	+	+	++
12	+	+	+
13	/	/	/
14	+	-	-
15	+	+	++
16	+	+	+
17	-	-	-
22	/	/	/
25	/	/	/

*three independent repeats are shown

Spent culture supernatant from *L. pentosus* YUN-V1.0 and *L. plantarum* YUN-V2.0 was also tested in radial diffusion assays and demonstrated to be efficient in inhibiting *Malassezia, Trichophyton* and *Candida* growth. *L. rhamnosus* YUN-S1.0 was not as efficient in inhibiting growth of *Malassizia* but was able to inhibit growth of *Trichophyton* and *Candida*.

Antibiotic susceptibility

The selected bacteria were also tested for their antibiotic susceptibility as to prevent spreading of antibiotic resistance genes. All lactobacilli were susceptible to erythromycin, normocin, tetracyclin, ampicillin and clindamycin, except for *L. plantarum* 5057, which was susceptible to tetracyclin. For this reason, strain *L. plantarum* 5057 was found not to be suitable to use as a strain for probiotherapy.

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PCT

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(This sheet is not part of and does not count as a sheet of the international application)

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0-1-1	Prepared Using	PCT Online Filing Version 3.5.000.251e MT/FOP 20141031/0.20.5.20
0-2	**International Application No.**	EP2017054513
0-3	**Applicant's or agent's file reference**	YUN-002

1	**The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on:**	
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1-2	**line**	15
1-3	**Identification of deposit**	
1-3-1	Name of depositary institution	BCCM Belgian Coordinated Collections of Microorganisms (BCCM)
1-3-2	Address of depositary institution	BCCM Coordination Cell, Federal Public Planning Service Science Policy, 231, avenue Louise, 1050 Brussels, Belgium
1-3-3	Date of deposit	12 May 2016 (12.05.2016)
1-3-4	Accession Number	BCCM LMG P-29611
1-4	**Additional Indications**	L. rhamnosus YUN-S1.0
1-5	**Designated States for Which Indications are Made**	All designations

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2-2	**line**	13
2-3	**Identification of deposit**	
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2-3-2	Address of depositary institution	BCCM Coordination Cell, Federal Public Planning Service Science Policy, 231, avenue Louise, 1050 Brussels, Belgium
2-3-3	Date of deposit	09 March 2016 (09.03.2016)
2-3-4	Accession Number	BCCM LMG P-29456
2-4	**Additional Indications**	L. plantarum YUN-V2.0
2-5	**Designated States for Which Indications are Made**	All designations

PCT

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(This sheet is not part of and does not count as a sheet of the international application)

3	**The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on:**	
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3-3-4	Accession Number	BCCM LMG P-29455
3-4	**Additional Indications**	L. pentosus YUN-V1.0
3-5	**Designated States for Which Indications are Made**	All designations

FOR RECEIVING OFFICE USE ONLY

0-4	**This form was received with the international application:** (yes or no)	YES
0-4-1	Authorized officer	Wilson, Patrick

FOR INTERNATIONAL BUREAU USE ONLY

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CLAIMS

1. A topical dermatological composition comprising one or more live *Lactobacillus* species; wherein at least one of said *Lactobacillus* species is a *L. plantarum* strain having at least 97% sequence similarity with SEQ ID N° 4 in its 16S rRNA gene.

2. A live *Lactobacillus* species for use in restoring and/or maintaining a healthy skin microbiota, by topical route, said *Lactobacillus* species being a *L. plantarum* strain having at least 97% sequence similarity with SEQ ID N° 4 in its 16S rRNA gene.

3. Use of one or more live *Lactobacillus* species, in the preparation of a topical dermatological composition for restoring and/or maintaining a healthy skin microbiota; wherein at least one of said *Lactobacillus* species is a *L. plantarum* strain having at least 97% sequence similarity with SEQ ID N° 4 in its 16S rRNA gene.

4. Method for restoring and/or maintaining a healthy skin microbiota; comprising at least one step of administering by topical route, to an individual, an effective amount of one or more live *Lactobacillus* species; wherein at least one of said *Lactobacillus* species is a *L. plantarum* strain having at least 97% sequence similarity with SEQ ID N° 4 in its 16S rRNA gene.

5. A composition comprising one or more live *Lactobacillus* species for use in restoring and/or maintaining a healthy skin microbiota, by topical route, said *Lactobacillus* species being selected from the list comprising *L. plantarum, L. pentosus and L. rhamnosus;* wherein said L. *plantarum* is a L. *plantarum* strain having at least 97% sequence similarity with SEQ ID N° 4 in its 16S rRNA gene, said *L. pentosus* is a L. *pentosus* strain having at least 97% sequence similarity with SEQ ID N° 1 in its 16S rRNA gene, and said L. *rhamnosus* is a L. *rhamnosus* strain having at least 97% sequence similarity with SEQ ID N° 5 in its 16S rRNA gene.

6. A *Lactobacillus* strain being *L. rhamnosus* YUN-S1.0 deposited under accession number LMG P-29611 (deposited at BCCM on May, 12 2016.

7. A composition comprising a *Lactobacillus* strain as defined in claim 6.

8. The composition as defined in claim 7; wherein said composition is a topical skin composition.

9. The use according to claim 3, or the composition according to anyone of claims 1, 5, 7 or 8 wherein said composition is a topical skin composition in the form of a gel, cream,

ovule, suppository forms, foam, lotion, or ointment.

10. A Lactobacillus strain as defined in claim 6 or a composition as defined in any one of claims 7 or 8; for use in restoring and/or maintaining a healthy skin microbiota, by topical route.

11. Topical use of one or more live *Lactobacillus* species in probiotherapy of the skin; wherein said *Lactobacillus* species are selected from the list comprising *L. plantarum, L. pentosus* and L. *rhamnosus.*

12. Topical use according to claim 11; wherein said probiotherapy consists of restoring and/or maintaining a healthy skin microbiota in a subject in need thereof.

13. Topical use as defined in anyone of claims 11 or 12, the use as defined in claim 3, the method as defined in claim 4, the live *Lactobacillus* species as defined in claim 2, or the composition as defined in claim 5; wherein said *Lactobacillus* species is a *Lactobacillus* strain selected from the list comprising *L. plantarum* YUN-V2.0 deposited under accession number LMG P-29456; *L. pentosus* YUN-V1.0 deposited under accession number LMG P-29455; and L. rhamnosus YUN-S1.0 deposited under accession number LMG P-29611.

Fig. 1

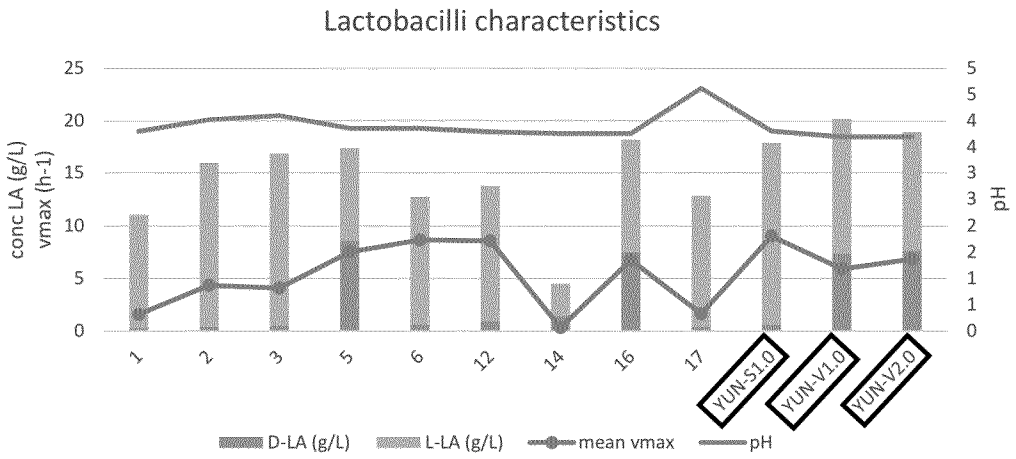
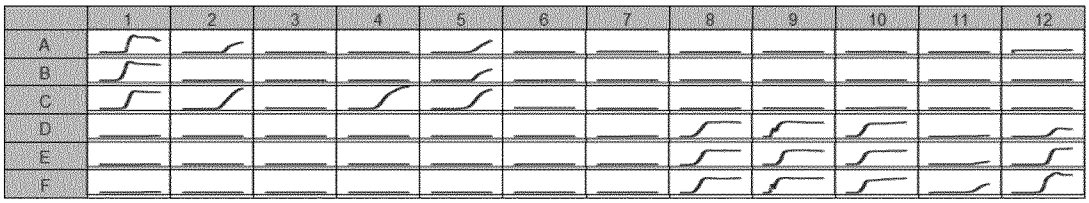


Fig. 2



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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2017/065006

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61Q19/00 C12R1/25 A61K35/747 C12R1/225 A61P17/00
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ADD.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61Q C12R A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 01/13956 A2 (GANEDEN BIOTECH INC [US]; FARMER SEAN [US]) 1 March 2001 (2001-03-01) claims 1-3	1-5,9, 11-13
X	EP 2 149 368 A1 (OREAL [FR]; NESTEC SA [CH]) 3 February 2010 (2010-02-03) paragraph [0133] - paragraph [0137] claims 1-4	1-5,9, 11-13
A	EP 1 736 537 A1 (ORGANOBALANCE GMBH [DE]) 27 December 2006 (2006-12-27) paragraph [0021] claims 1-18	1-5,9, 11-13

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
7 September 2017	15/11/2017

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Steinheimer, K

2

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2013/188626 A2 (DOW GLOBAL TECHNOLOGIES LLC [US]) 19 December 2013 (2013-12-19) page 1 - page 5 pages 1-2; examples 1-2	11,12
X	EP 2 332 521 A1 (OREAL [FR]; NESTEC SA [CH]) 15 June 2011 (2011-06-15) claims 1-13 paragraph [0074] - paragraph [0090]	11,12
X	KR 2012 0089530 A (KOREA RES INST OF BIOSCIENCE [KR]) 13 August 2012 (2012-08-13) paragraphs [0047], [0021]; claims 1-7; figure 5 & DATABASE Geneseq [Online] 11 April 2013 (2013-04-11), Kim MS et al.: "Lactobacillus plantarum strain MH19 16S ribosomal RNA", Database accession no. BAK32313 the whole document	1-5,9, 11-13
X	KR 2015 0075447 A (JUNG LAB CO LTD [KR]; KIM SUNG OK [KR]) 6 July 2015 (2015-07-06) claim 4	1-5,9, 11-13
X	WO 2016/023688 A1 (NESTEC SA [CH]) 18 February 2016 (2016-02-18) claims 1-15	1-5,9, 11-13
X	WO 2011/052996 A2 (CJ CHEILJEDANG CORP [KR]; KIM BONG JOON [KR]; JUNG HEON WOONG [KR]; LE) 5 May 2011 (2011-05-05) claims 6, 10	1-5,9, 11-13

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2017/065006

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-4(completely); 5, 9, 11-13(partially)

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-4(completely); 5, 9, 11-13(partially)

   Claims relating to Lactobacillus plantarum: Topical use of Lactobacillus plantarum in probiotherapy of the skin (claim 11). Topical dermatological composition comprising lactobacillus plantarum and corresponding uses.

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2. claims: 5, 9, 11-13(all partially)

   Claims relating to Lactobacillus pentosus: Topical use of Lactobacillus pentosus in probiotherapy of the skin (claim 11). Topical dermatological composition comprising lactobacillus pentosus and corresponding uses.

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3. claims: 6-8, 10(completely); 5, 9, 11-13(partially)

   Claims relating to Lactobacillus rhamnosus: Topical use of Lactobacillus rhamnosus in probiotherapy of the skin (claim 11). Strain YUN-S1.0 (claim 6) and its use in restoring or maintaining healthy skin.

INTERNATIONAL SEARCH REPORT

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International application No
PCT/EP2017/065006

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