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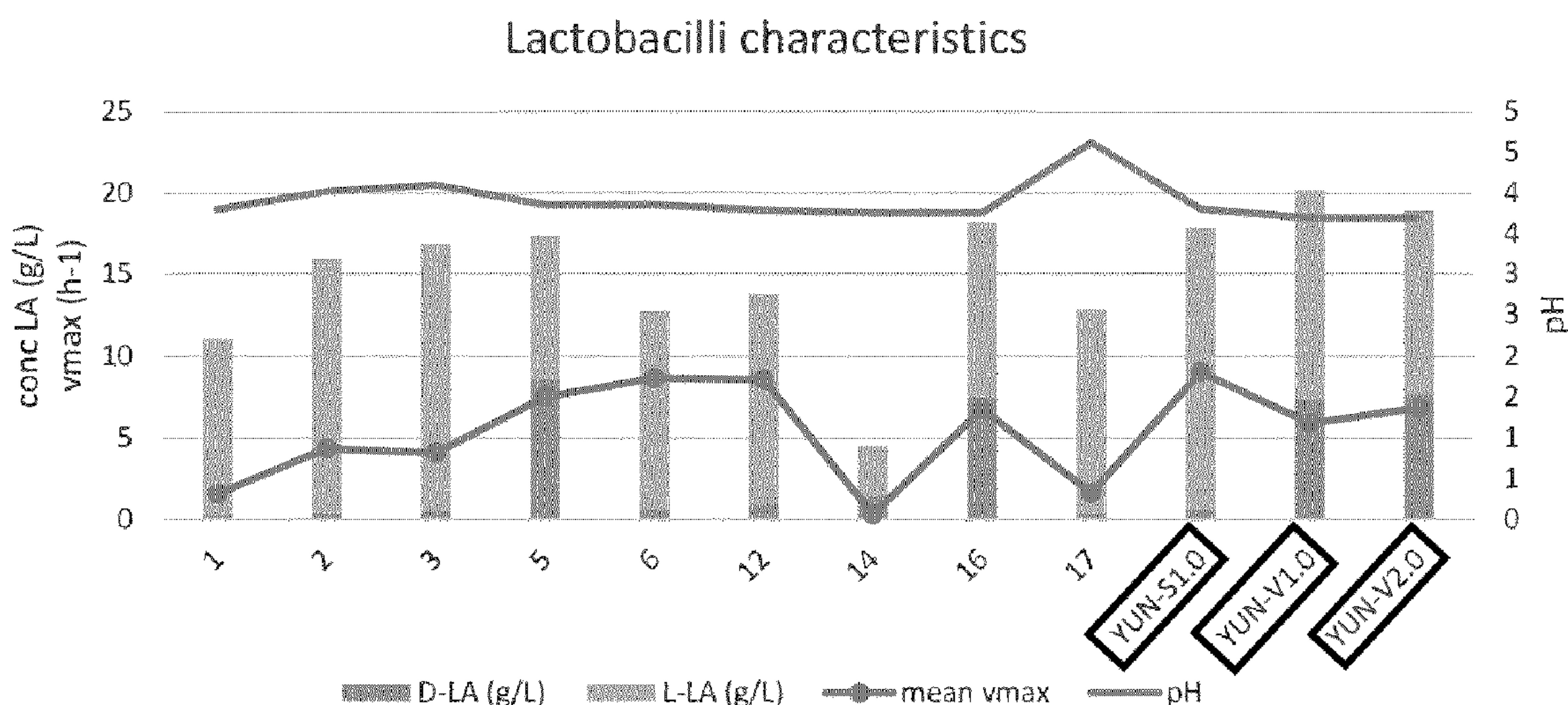
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(54) Titre : PREPARATIONS DERMATOLOGIQUES DESTINEES A MAINTENIR ET/OU RESTAURER UN MICROBIOTE CUTANE SAIN  
(54) Title: DERMATOLOGICAL PREPARATIONS FOR MAINTAINING AND/OR RESTORING HEALTHY SKIN MICROBIOTA

**Fig. 1**



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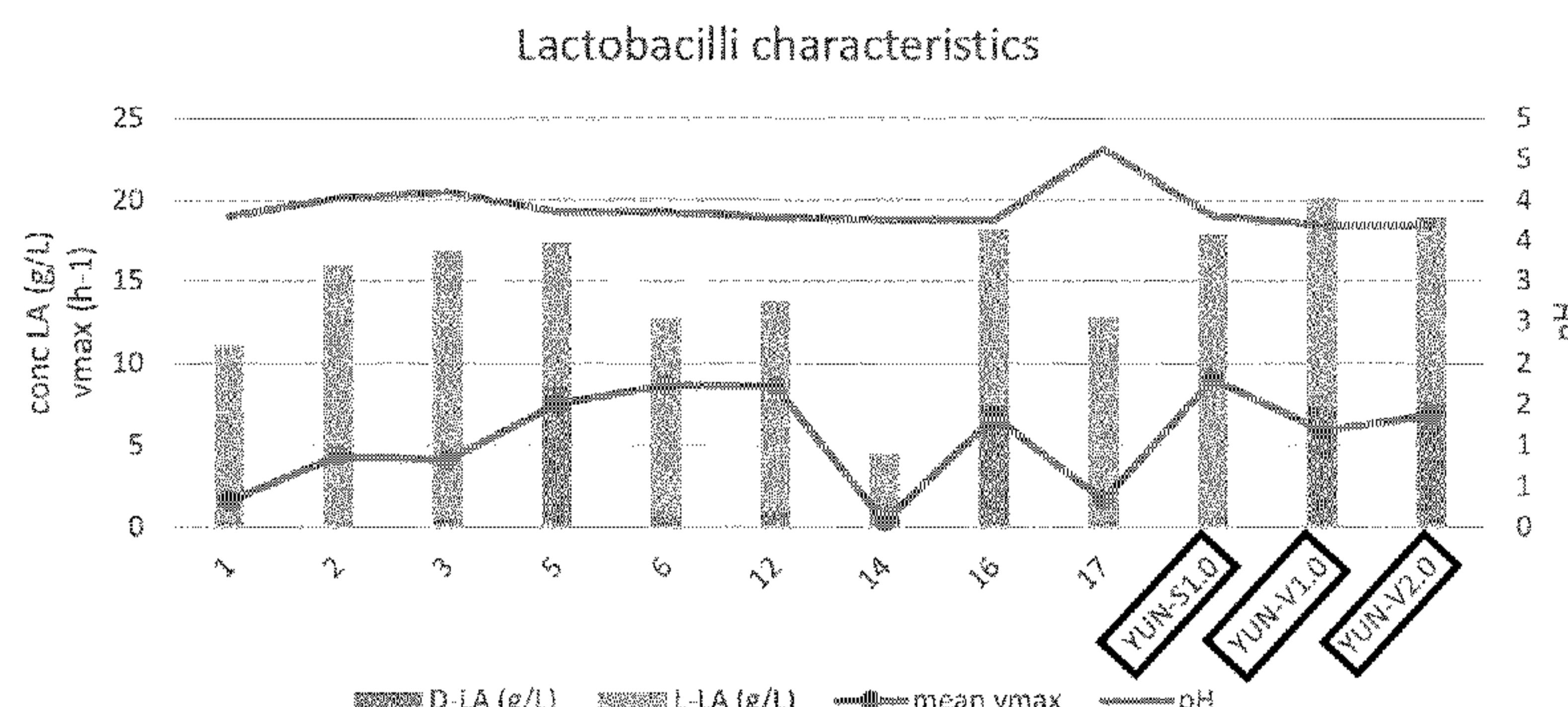
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(54) Title: DERMATOLOGICAL PREPARATIONS FOR MAINTAINING AND/OR RESTORING HEALTHY SKIN MICROBIOTA

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.

**DERMATOLOGICAL PREPARATIONS FOR MAINTAINING AND/OR RESTORING**  
**HEALTHY SKIN MICROBIOTA**

**FIELD OF THE INVENTION**

5 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  
10 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**

15 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  
20 relieving skin conditions in subjects in need thereof.

25 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.

30 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  
35 *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 pustules. 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  
40 bacteria.

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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 5 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 intriguing.

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 10 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 15 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 20 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 25 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 30 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.

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**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.

5 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.

10 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.

15 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.

20 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.

25 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).

30 35 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.

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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.

5 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.

10 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.

30 **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 50 $\mu$ g/ml is added, SCS of all lactobacilli prevents growth of *P. acnes* while SCS of streptococci or staphylococci does not inhibit growth. \*Erythromycine (50 $\mu$ g/ml); #Erythromycine (5 $\mu$ g/ml);  $^{\$}$ Minocycline (20  $\mu$ g/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.

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### 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., 5 *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 10 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 15 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*.

20 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 25 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.

30 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.

35 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.

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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,...

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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 10 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 15 *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 20 *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.

25

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 30 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, 35 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 40 primers 8F (5'-AGAGTTGATCCTGGCTCAG-3' – SEQ ID N° 2) and 1525R (5'-

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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'-AGAGTTGATCCTGGCTCAG-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'-AGAGTTGATCCTGGCTCAG-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**

5

***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.

10

**Table 1:** Bacterial strains used in this research

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

PATHOGENS				
<i>Trichophyton rubrum</i>	2	/	Clinical isolate	BCCM/LMG
<i>Malassezia furfur</i>		/	Clinical isolate	BCCM/LMG
<i>Candida albicans</i>	/	/	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 5 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*.***

10 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 15 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.

20 ***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 gastrointestinal 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 25 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').***

30 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 35 optical density (OD) was measured at 590 nm each 30 min during 3 days using a Synergy HTX

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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.

5 ***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.

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***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<sup>8</sup> colony forming units (CFU) of L. pentosus YUN-V1.0, +10<sup>8</sup> CFU of L. plantarum YUN-V2.0 and +10<sup>8</sup> 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.

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

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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<sup>8</sup> colony forming units (CFU) of L. pentosus YUN-V1.0, +10<sup>8</sup> CFU of L. plantarum YUN-V2.0 and +10<sup>8</sup> CFU L. rhamnosus YUN-S1.0 per application of 1g 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'-TCCGTAGGTGAAACCTGC-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.

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RESULTS***Growth characteristics and lactate production***

5 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).

10 ***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 *Staphylococcus 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).

15 ***Antipathogenic activity against *Malassezia*, *Trichophyton* and *Candida****

20 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*.

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

25 \*three independent repeats are shown

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

Strain	<i>Trichophyton rubrum</i>		
	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

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

Strain	<i>Candida albicans</i>		
	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, 5 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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25

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Print Out (Original in Electronic Form)

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

0-1	<b>Form PCT/RO/134</b> <b>Indications Relating to Deposited Microorganism(s) or Other Biological Material (PCT Rule 13bis)</b>	
0-1-1	Prepared Using	<b>PCT Online Filing</b> <b>Version 3.5.000.251e MT/FOP</b> <b>20141031/0.20.5.20</b>
0-2	<b>International Application No.</b>	EP2017054513
0-3	<b>Applicant's or agent's file reference</b>	<b>YUN-002</b>
<hr/>		
1	<b>The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on:</b>	
1-1	page	<b>4</b>
1-2	line	<b>15</b>
<hr/>		
1-3	<b>Identification of deposit</b>	
1-3-1	Name of depositary institution	<b>BCCM Belgian Coordinated Collections of Microorganisms (BCCM)</b>
1-3-2	Address of depositary institution	<b>BCCM Coordination Cell, Federal Public Planning Service Science Policy, 231, avenue Louise, 1050 Brussels, Belgium</b>
1-3-3	Date of deposit	<b>12 May 2016 (12.05.2016)</b>
1-3-4	Accession Number	<b>BCCM LMG P-29611</b>
<hr/>		
1-4	<b>Additional Indications</b>	
<b>L. rhamnosus YUN-S1.0</b>		
<hr/>		
1-5	<b>Designated States for Which Indications are Made</b>	
<b>All designations</b>		
<hr/>		
2	<b>The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on:</b>	
2-1	page	<b>4</b>
2-2	line	<b>13</b>
<hr/>		
2-3	<b>Identification of deposit</b>	
2-3-1	Name of depositary institution	<b>BCCM Belgian Coordinated Collections of Microorganisms (BCCM)</b>
2-3-2	Address of depositary institution	<b>BCCM Coordination Cell, Federal Public Planning Service Science Policy, 231, avenue Louise, 1050 Brussels, Belgium</b>
2-3-3	Date of deposit	<b>09 March 2016 (09.03.2016)</b>
2-3-4	Accession Number	<b>BCCM LMG P-29456</b>
<hr/>		
2-4	<b>Additional Indications</b>	
<b>L. plantarum YUN-V2.0</b>		
<hr/>		
2-5	<b>Designated States for Which Indications are Made</b>	
<b>All designations</b>		

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<b>3</b>	<b>The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on:</b>	
3-1	page	<b>4</b>
3-2	line	<b>14</b>
<b>3-3</b>	<b>Identification of deposit</b>	
3-3-1	Name of depositary institution	<b>BCCM Belgian Coordinated Collections of Microorganisms (BCCM)</b>
3-3-2	Address of depositary institution	<b>BCCM Coordination Cell, Federal Public Planning Service Science Policy, 231, avenue Louise, 1050 Brussels, Belgium</b>
3-3-3	Date of deposit	<b>09 March 2016 (09.03.2016)</b>
3-3-4	Accession Number	<b>BCCM LMG P-29455</b>
<b>3-4</b>	<b>Additional Indications</b>	<b>L. pentosus YUN-V1.0</b>
<b>3-5</b>	<b>Designated States for Which Indications are Made</b>	<b>All designations</b>

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

**FOR INTERNATIONAL BUREAU USE ONLY**

<b>0-5</b>	<b>This form was received by the international Bureau on:</b>	
0-5-1	Authorized officer	

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.  
5
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.  
10
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.  
15
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.  
20
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.  
25
6. A *Lactobacillus* strain being *L. rhamnosus* YUN-S1.0 deposited under accession number LMG P-29611 (deposited at BCCM on May, 12 2016).  
30
7. A composition comprising a *Lactobacillus* strain as defined in claim 6.  
35
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,  
40

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 5 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*.
- 10 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.
- 15 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 20 accession number LMG P-29611.

Fig. 1

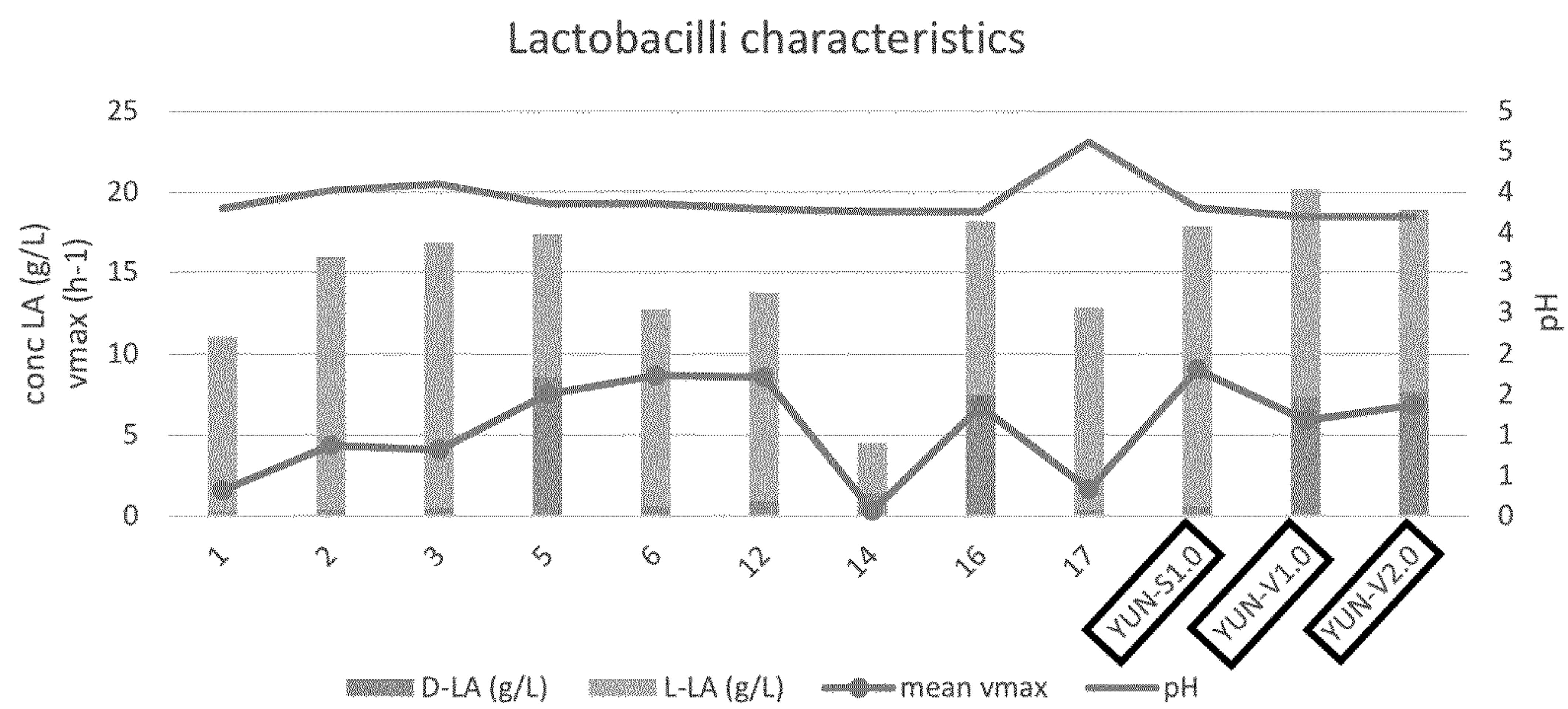


Fig. 2

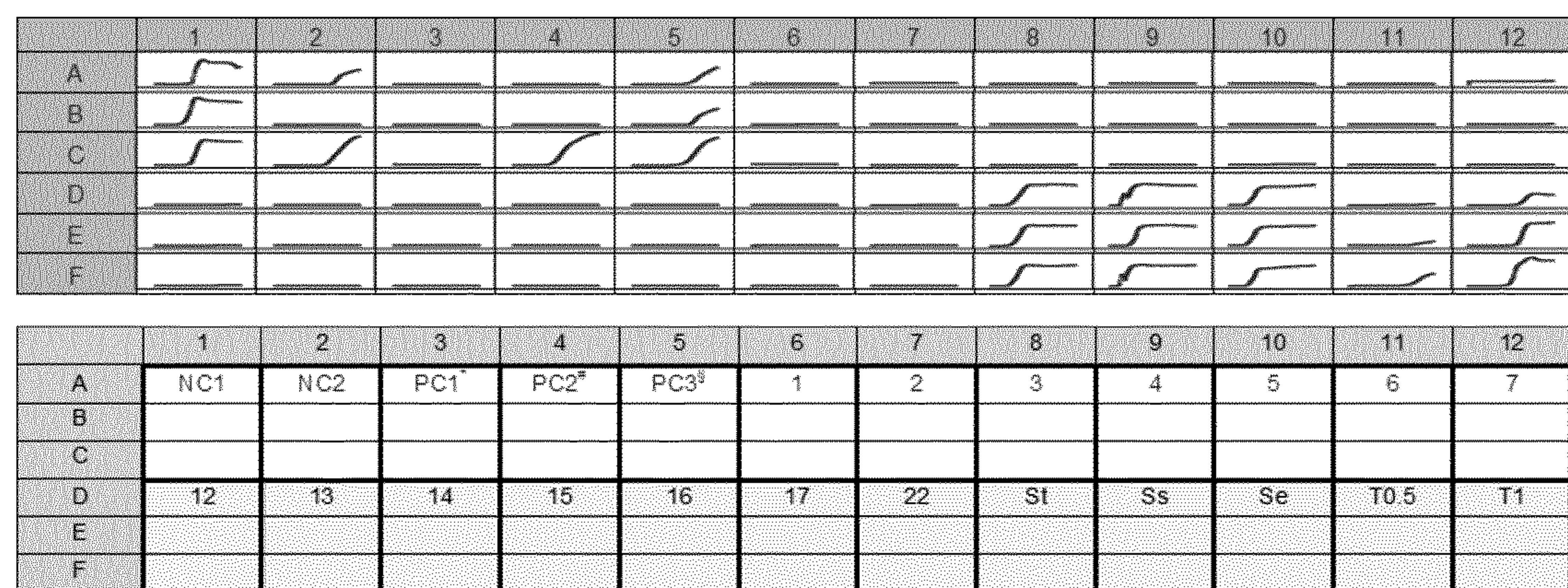


Fig. 1

