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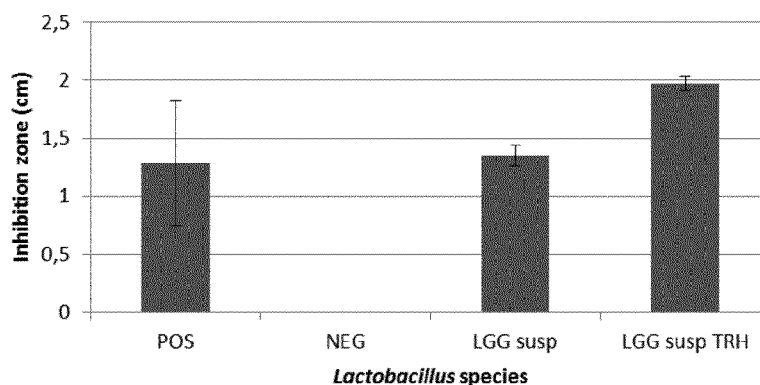
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(54) Title: ORONASOPHARYNGEAL PROBIOTICS

Fig. 14



(57) Abstract: The present invention is directed to a novel probiotic oronasopharyngeal formulation of spray-dried bacteria comprising saccharides as protectants, for administration in the oronasopharyngeal cavity. Resuscitation of the formulation of the present invention gives rise to rapid deployable and viable microorganisms with preserved antimicrobial activity, and was found particularly useful for nasopharyngeal administration to treat respiratory conditions in humans and animals. In a further aspect, the present invention is directed to the probiotic microorganisms used in the aforementioned formulation. As evident from the examples herein-after, spray-dried compositions comprising *Lactobacillus* strains, in particular comprising *Lactobacillus rhamnosus* proved optimal in the oronasopharyngeal applications of the present invention. The application also provides the use of *Lactobacillus* strains as anti-pathogenic and antibiofilm agents, in particular against the common nasopharyngeal pathogens (whereby produced acids such as lactic acid are important antimicrobial factors). Also disclosed are the processes associated with the manufacture of the spray-dried probiotic formulations of the present invention, as well as the use thereof in treating or preventing of infections of the oronasopharyngeal cavity.





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ORONASOPHARYNGEAL PROBIOTICS

FIELD OF THE INVENTION

The present invention is directed to a novel probiotic oronasopharyngeal formulation of spray-dried bacteria comprising saccharides as protectants, for administration in the oronasopharyngeal cavity. Resuscitation of the formulation of the present invention gives rise to rapid deployable and viable microorganisms with preserved antimicrobial activity, and was found particularly useful for nasopharyngeal administration to treat respiratory conditions in humans and animals. In a further aspect, the present invention is directed to the probiotic microorganisms used in the aforementioned formulation. As evident from the examples hereinafter, spray-dried compositions comprising *Lactobacillus* strains, in particular comprising *Lactobacillus rhamnosus* proved optimal in the oronasopharyngeal applications of the present invention. The application also provides the use of *Lactobacillus* strains as anti-pathogenic and antibiofilm agents, in particular against the common nasopharyngeal pathogens (whereby produced acids such as lactic acid are important antimicrobial factors). Also disclosed are the processes associated with the manufacture of the spray-dried probiotic formulations of the present invention, as well as the use thereof in treating or preventing of infections of the oronasopharyngeal cavity.

BACKGROUND TO THE INVENTION

The human body is occupied by a vast number of microorganisms, which are collectively called microbiota. They inhabit the skin, oronasopharyngeal cavity, genital tract and gastrointestinal tract. The microbiota present in each of the niches provide to the host a vast number of health effects, including inhibition of pathogenic colonization, stimulation of barrier functions and promotion of immune regulation. Interest in the beneficial functions of the human microbiota has boomed within the last ten years, thanks to major advances in next generation sequencing technologies and so called 'metagenomic studies' (Qin et al., 2010; Arumugam et al., 2011). In addition, the application of probiotics has increased significantly during the last decades. Probiotics are defined as microorganisms, which, upon application in adequate amounts, can provide a health benefit to the host (FAO/WHO, 2001). Until now, most probiotic applications have focused on the gastrointestinal tract, because of the ease to implement them in fermented foods, yogurts and oral/food supplements. *Lactobacillus* and *Bifidobacterium* are most commonly applied. For example:

- WO2006007526 relates to combinations of *Bifidobacteria* and *Lactobaccillus* strains for use in the prevention and treatment of respiratory tract disorders such as acute otitis media in infants, by supplementing **infant** formulations with such combinations;
- US20110020304 provides **nutritional** compositions comprising *Lactobacillus* sp and the use of such preparation in the prevention and treatment of pathogenic infections of the gastro-intestinal and upper respiratory tracts;
- US20120201798 relates to **dietary supplements** comprising probiotic *Lactobacillus* strains for the treatment or prevention of a respiratory infection;

- EP2455092 relates to **food** compositions comprising non-replicating probiotic micro-organisms for use in the prevention or treatment of upper respiratory tract infections;
- Hojsak et al., 2010 relates to fermented **milk products** comprising *Lactobacillus* GG for use in the prevention of gastrointestinal and respiratory tract infections in children who attend day care centers;

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However, considering the complexity of the gastrointestinal tract (e.g. its length, surface area, various sub-niches, difficult access, high microbial biomass especially in the colon, the enormous acid stress in the stomach and bile stress in the small intestine), it is actually surprising that this is currently the main application area. For instance, the potential of nasal and pharyngeal probiotics is currently unexplored, while they hold great promise for multiple reasons. Among these, the fact that (i) upper respiratory tract infections, including acute otitis media, pharyngitis, and chronic sinusitis, are the leading causes for the prescription of antibiotics in children and adults, and that (ii) the oronasopharyngeal cavity is more accessible and generally populated by a less complex and less dense microbiota than the gut, form a major incentive for the proposed project.

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Various disorders involve a dysbalance of the microbiota of the nasopharyngeal cavity, such as rhinosinusitis (Abreu et al., 2012), pharyngitis, adenoiditis and tonsillitis (Swidsinski et al., 2007; Jensen et al., 2013), and even middle ear infections (Revai et al., 2008). For various of these disorders such as chronic sinusitis and acute otitis media, antibiotics are often applied, while they have many side effects such as accumulation of resistant microbes and disturbance of the symbiotic microbes.

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In recent studies, nasal administration of *Lactococcus lactis* was shown to improve local and systemic immune responses against *Streptococcus pneumoniae* in a mouse model (Medina et al., 2008). Similar results have been reported for the intranasal administration of autoclaved (i.e. non-living) *Lactobacillus rhamnosus* GG in the protection of mice from a H1N1 influenza viral infection (Harata et al., 2010), and for the prevention and treatment of respiratory infections in animals using spores of spore-forming probiotic bacteria selected from *Bacillus coagulans* and *Bacillus licheniformis* (US2009/0280099). These studies form an important incentive to investigate supplemented recovery of the human microbiota in the nasopharynx by probiotics and its potential applications. This could ultimately lead to a reduction in antibiotic usage in diseases which challenge problems with antibiotic resistance for many years.

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The above-mentioned references all relate to the use of oral formulations or non-viable bacterial species or spores thereof, thereby aiming at improving the overall immune responses, and as such having an indirect beneficial effect on the oronasopharyngeal health. The inventors have now in contrast found that the oronasopharyngeal compositions of the present invention, when administered directly to the oronasopharyngeal cavity, have a direct effect on the oronasopharyngeal health by restoring or maintaining the natural microbiota of the oronasopharyngeal cavity, due to direct antimicrobial activity against the most common pathogenic microorganisms and recolonization of the cavity with beneficial bacteria.

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It has thus been an objective of the present invention to provide *Lactobacillus* probiotic strains with a strong activity against the common nasopharyngeal pathogens and their application in the treatment and prevention of infections of the oronasopharyngeal cavity. Thus in a further aspect the present invention provides oronasopharyngeal formulations and the manufacture thereof.

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SUMMARY OF THE INVENTION

The present invention is based on the finding that certain viable *Lactobacillus spp.* strains, in particular *Lactobacillus rhamnosus*, *Lactobacillus plantarum/pentosus* and *Lactobacillus casei*, and their secreted products such as lactic acid, show strong growth inhibitory activity against the common nasopharyngeal pathogens. Thus in a first aspect the present invention provides the use of said *Lactobacillus spp.* in the treatment and prevention of infections of the oronasopharyngeal cavity. More in particular, the present invention provides an oronasopharyngeal composition comprising viable, spray-dried *Lactobacillus* species, for use in the prevention and/or treatment of infections of the oronasopharyngeal cavity, by administration of said composition in said cavity.

The *Lactobacillus spp.* strains identified in the present application in having growth inhibitory activity against nasopharyngeal pathogens, are selected from the group consisting of *Lactobacillus rhamnosus*, *Lactobacillus casei*, *Lactobacillus plantarum*, *Lactobacillus reuteri*, *Lactobacillus salivarius*, *Lactobacillus helveticus*, *Lactobacillus parabuchneri*, *Lactobacillus pentosus*, *Lactobacillus brevis*, *Lactobacillus sakei*, *Lactobacillus diolivorans*, *Lactobacillus buchneri*, *Lactobacillus gasseri*, and *Lactobacillus bulgaricus*; more in particular *Lactobacillus rhamnosus*. The present invention accordingly provides;

- An oronasopharyngeal composition comprising viable, spray-dried *Lactobacillus rhamnosus* for use in the treatment or prevention of infections of the oronasopharyngeal cavity, by administration of said composition in said cavity;
- An oronasopharyngeal composition comprising viable, spray-dried *Lactobacillus casei* for use in the treatment or prevention of infections of the oronasopharyngeal cavity, by administration of said composition in said cavity;
- An oronasopharyngeal composition comprising viable, spray-dried *Lactobacillus reuteri* for use in the treatment or prevention of infections of the oronasopharyngeal cavity, by administration of said composition in said cavity; or
- An oronasopharyngeal composition comprising viable, spray-dried *Lactobacillus plantarum* for use in the treatment or prevention of infections of the oronasopharyngeal cavity, by administration of said composition in said cavity;
- An oronasopharyngeal composition comprising viable, spray-dried *Lactobacillus helveticus* for use in the treatment or prevention of infections of the oronasopharyngeal cavity, by administration of said composition in said cavity;
- An oronasopharyngeal composition comprising viable, spray-dried *Lactobacillus parabuchneri* for use in the treatment or prevention of infections of the oronasopharyngeal cavity, by

administration of said composition in said cavity;

- An oronasopharyngeal composition comprising viable, spray-dried *Lactobacillus pentosus* for use in the treatment or prevention of infections of the oronasopharyngeal cavity, by administration of said composition in said cavity;
- 5 • An oronasopharyngeal composition comprising viable, spray-dried *Lactobacillus salivarius* for use in the treatment or prevention of infections of the oronasopharyngeal cavity, by administration of said composition in said cavity;
- An oronasopharyngeal composition comprising viable, spray-dried *Lactobacillus brevis* for use in the treatment or prevention of infections of the oronasopharyngeal cavity, by administration of said composition in said cavity;
- 10 • An oronasopharyngeal composition comprising viable, spray-dried *Lactobacillus buchneri* for use in the treatment or prevention of infections of the oronasopharyngeal cavity, by administration of said composition in said cavity;
- An oronasopharyngeal composition comprising viable, spray-dried *Lactobacillus sakei* for use in the treatment or prevention of infections of the oronasopharyngeal cavity, by administration of said composition in said cavity;
- 15 • An oronasopharyngeal composition comprising viable, spray-dried *Lactobacillus diolivorans* for use in the treatment or prevention of infections of the oronasopharyngeal cavity, by administration of said composition in said cavity;
- 20 • An oronasopharyngeal composition comprising viable, spray-dried *Lactobacillus gasseri* for use in the treatment or prevention of infections of the oronasopharyngeal cavity, by administration of said composition in said cavity;
- An oronasopharyngeal composition comprising viable, spray-dried *Lactobacillus bulgaricus* for use in the treatment or prevention of infections of the oronasopharyngeal cavity, by administration of said composition in said cavity;
- 25 • An oronasopharyngeal composition comprising combinations of said viable spray-dried *Lactobacillus* spp., for use in the treatment or prevention of infections of the oronasopharyngeal cavity, by administration of said composition in said cavity.

30 The present invention also provides the oronasopharyngeal compositions as defined hereinabove, wherein the *Lactobacillus* strains are replaced or complemented with *Lactobacillus* secreted products such as lactic acids.

In a particular embodiment the present invention provides an oronasopharyngeal composition comprising viable, spray-dried *Lactobacillus rhamnosus* for use in the treatment or prevention of infections of the oronasopharyngeal cavity. Optionally in combination with other probiotic bacteria. In such combinations the other probiotic bacteria preferably consist of *Lactobacillus* spp. and lactic acid bacteria; more in particular selected from the group comprising *Lactobacillus casei*, *L. plantarum*, *L. paracasei*, *L. salivarius*, *L. sakei*, *L. pentosus*, *L. reuteri*, *L. brevis*, *L.*

parabuchneri, *L. buchneri*, *L. sakei*, *L. diolivorans*, *L. bulgaricus*, *L. gasseri* and other *Lactobacillus* species with a GRAS (generally recognized as safe) or qualified presumption of safety (QPS) status; even more in particular selected from the group consisting of *Lactobacillus casei*, *Lactobacillus plantarum*, *Lactobacillus salivarius*, *Lactobacillus brevis* and *Lactobacillus reuteri*, *Lactobacillus helveticus*, *Lactobacillus parabuchneri*, *Lactobacillus sakei*, *Lactobacillus buchneri*, *Lactobacillus diolivorans*, *Lactobacillus bulgaricus*, *Lactobacillus gasseri* and *Lactobacillus pentosus*.

In another embodiment the present invention provides an oronasopharyngeal composition comprising viable, spray-dried *Lactobacillus casei* for use in the treatment or prevention of infections of the oronasopharyngeal cavity. Optionally in combination with other probiotic bacteria. In such combinations the other probiotic bacteria preferably consist of further *Lactobacillus spp.*; more in particular selected from the group comprising *Lactobacillus rhamnosus*, *L. plantarum*, *L. paracasei*, *L. salivarius*, *L. sakei*, *L. pentosus*, *L. reuteri*, *L. brevis*, *L. parabuchneri*, *L. buchneri*, *L. sakei*, *L. diolivorans*, *L. bulgaricus*, *L. gasseri* and other *Lactobacillus* species with a GRAS (generally recognized as safe) or qualified presumption of safety (QPS) status; even more in particular selected from the group consisting of *Lactobacillus rhamnosus*, *L. plantarum* and *Lactobacillus reuteri*, *Lactobacillus helveticus*, *Lactobacillus parabuchneri*, *L. salivarius*, *L. sakei*, *L. brevis*, *L. buchneri*, *L. parabuchneri*, *L. bulgaricus*, *L. gasseri* and *Lactobacillus pentosus*.

In another embodiment the present invention provides an oronasopharyngeal composition comprising viable, spray-dried *Lactobacillus reuteri* for use in the treatment or prevention of infections of the oronasopharyngeal cavity. Optionally in combination with other probiotic bacteria. In such combinations the other probiotic bacteria preferably consist of further *Lactobacillus spp.*; more in particular selected from the group comprising *Lactobacillus casei*, *L. plantarum*, *L. paracasei*, *L. salivarius*, *L. sakei*, *L. pentosus*, *L. rhamnosus*, *Lactobacillus helveticus*, *Lactobacillus parabuchneri*, *L. brevis*, *L. buchneri*, *L. bulgaricus*, *L. gasseri* and other *Lactobacillus* species with a GRAS (generally recognized as safe) or qualified presumption of safety (QPS) status; even more in particular selected from the group consisting of *Lactobacillus casei*, *Lactobacillus rhamnosus*, and *Lactobacillus plantarum*.

As mentioned hereinbefore, the present invention is based on the identification of *Lactobacillus* species having growth inhibitory activity against common nasopharyngeal pathogens. In one embodiment the common nasopharyngeal pathogens inhibited by the aforementioned *Lactobacillus* species consists of the group comprising *Moraxella catarrhalis*, *Haemophilus influenzae*, and *Corynebacterium tuberculo-stearicum*, *Corynebacterium accolens* *Streptococcus pyogenes*, *Streptococcus pneumoniae* and *Staphylococcus aureus* (including MRSA). Hence in a further aspect, the present invention provides an oronasopharyngeal composition comprising viable, spray-dried *Lactobacillus spp.* strains in each of the aforementioned embodiments for use

in the treatment or prevention of infections of the oronasopharyngeal cavity caused by an infection comprising one or more of the pathogens selected from *Moraxella catarrhalis*, *Haemophilus influenzae*, *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Staphylococcus aureus* (including MRSA), *Corynebacterium tuberculostrictum* and *Corynebacterium accolens*.

5 In one embodiment the present invention provides an oronasopharyngeal composition comprising viable, spray-dried *Lactobacillus* spp. strains in each of the aforementioned embodiments for use in the treatment or prevention of infections of the oronasopharyngeal cavity caused by an infection comprising one or more of the pathogens selected from *Moraxella catarrhalis*, *Haemophilus influenzae*, *Corynebacterium tuberculostrictum*, *Streptococcus*
10 *pyogenes*, *Streptococcus pneumoniae* and *Staphylococcus aureus* (including MRSA) and *Corynebacterium accolens*.

Further to the aforementioned therapeutic applications, the *Lactobacillus* species of the present invention were found particularly useful in the treatment of upper respiratory tract infections. In
15 one embodiment said upper respiratory tract infections include acute otitis media, pharyngitis, chronic sinusitis, acute sinusitis, rhinitis, oral mucositis and the like. In an even further embodiment, the *Lactobacillus* species of the present invention were found to have an anti-inflammatory activity, and to have a healing effect for mucosal lesions of the oronasopharyngeal cavity. Consequently, in a further aspect the present invention provides an oronasopharyngeal
20 composition comprising viable, spray-dried *Lactobacillus* species in each of its embodiments, for use in;

- the treatment of upper respiratory tract infections;
- the treatment of upper respiratory tract infections, wherein said upper respiratory tract infections are selected from the group comprising acute otitis media, pharyngitis, chronic
25 sinusitis, acute sinusitis, rhinitis, oral mucositis and the like;
- as anti-inflammatory agent in the treatment of infections of the oronasopharyngeal cavity;
- as anti-inflammatory agent in the treatment of infections of the oronasopharyngeal cavity, wherein said infections are caused by an infection comprising one or more of the pathogens selected from *Moraxella catarrhalis*, *Haemophilus influenzae*, *Corynebacterium tuberculostrictum*, *Corynebacterium accolens*, *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Streptococcus pyogenes* and *Staphylococcus aureus* (including MRSA);
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- as anti-inflammatory agent in the treatment of upper respiratory tract infections;
- as anti-inflammatory agent in the treatment of upper respiratory tract infections, wherein said upper respiratory tract infections are selected from the group comprising acute otitis media, pharyngitis, chronic sinusitis, acute sinusitis, rhinitis, oral mucositis and the like;
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- in the treatment of mucosal lesions of the oronasopharyngeal cavity.
- in the treatment of mucosal lesions of the oronasopharyngeal cavity, caused by an infection comprising one or more of the pathogens selected from *Moraxella catarrhalis*, *Haemophilus influenzae*, *Corynebacterium tuberculostrictum*, *Corynebacterium accolens*, *Streptococcus*

pyogenes, *Streptococcus pneumoniae* and *Staphylococcus aureus* (including MRSA).

- It is also an object of the present invention to provide a method for the prevention or treatment of infections of the oronasopharyngeal cavity, said method comprising administering into the oronasopharyngeal cavity of the subject to be treated, a composition, more in particular an oronasopharyngeal composition comprising an effective amount of the *Lactobacillus spp.* strains of the present invention. Possible embodiments for this method of treatment include;
- administering an effective amount of *Lactobacillus rhamnosus* to the subject to be treated;
 - administering an effective amount of *Lactobacillus casei* to the subject to be treated;
 - 10 • administering an effective amount of *Lactobacillus reuteri* to the subject to be treated;
 - administering an effective amount of *Lactobacillus plantarum* to the subject to be treated;
 - administering an effective amount of *Lactobacillus helveticus* to the subject to be treated;
 - administering an effective amount of *Lactobacillus parabuchneri* to the subject to be treated;
 - administering an effective amount of *Lactobacillus pentosus* to the subject to be treated;
 - 15 • administering an effective amount of *Lactobacillus salivarius* to the subject to be treated;
 - administering an effective amount of *Lactobacillus sakei* to the subject to be treated;
 - administering an effective amount of *Lactobacillus diolivorans* to the subject to be treated;
 - administering an effective amount of *Lactobacillus buchneri* to the subject to be treated;
 - administering an effective amount of *Lactobacillus gasseri* to the subject to be treated;
 - 20 • administering an effective amount of *Lactobacillus bulgaricus* to the subject to be treated;
 - administering an effective amount of *Lactobacillus brevis* to the subject to be treated;
 - or combinations thereof

- Within the context of the present invention;
- 25 • the *Lactobacillus rhamnosus* strains, are meant to include *Lactobacillus rhamnosus* AMB-GG, *Lactobacillus rhamnosus* AMB-GR-1 or a combination thereof;
 - the *Lactobacillus casei* strains, are meant to include *Lactobacillus casei* AMB-334, *Lactobacillus casei* AMB-Sh, *Lactobacillus casei* AMB-Act, natural isolates of *Lactobacillus casei* or a combination thereof.
 - 30 • the *Lactobacillus plantarum* strains, are meant to include *Lactobacillus plantarum* AMB-8014, natural isolates of *Lactobacillus plantarum* or a combination thereof.
 - the *Lactobacillus pentosus* strains are meant to include *L. pentosus* AMB-pent1, natural isolates of *Lactobacillus pentosus* or a combination thereof.
 - the *Lactobacillus helveticus* strains, are meant to include *Lactobacillus helveticus* AMB-1807, 35 natural isolates of *Lactobacillus helveticus* or a combination thereof.
 - the *Lactobacillus reuteri* strains, are meant to include *Lactobacillus reuteri* AMB-RC-14, natural isolates of *Lactobacillus helveticus* or a combination thereof.
 - the *Lactobacillus parabuchneri* strains, are meant to include *Lactobacillus parabuchneri* AMB-

AB17, natural isolates or a combination thereof.

- the *Lactobacillus salivarius* strains, are meant to include natural isolates or a combination thereof.
- the *Lactobacillus brevis* strains, are meant to include natural isolates or a combination thereof.
- 5 • the *Lactobacillus sakei* strains, are meant to include natural isolates or a combination thereof
- the *Lactobacillus diolivorans* strains, are meant to include natural isolates or a combination thereof
- the *Lactobacillus buchneri* strains, are meant to include natural isolates or a combination thereof
- 10 • the *Lactobacillus gasseri* strains, are meant to include natural isolates or a combination thereof
- the *Lactobacillus bulgaricus* strains, are meant to include natural isolates or a combination thereof

In a particular embodiment of the foregoing method for the prevention or treatment of infections of the oronasopharyngeal cavity, said method comprising administering into the oronasopharyngeal cavity of the subject to be treated, a composition comprising an effective amount of *Lactobacillus rhamnosus*. Optionally in combination with further probiotic bacteria preferably consist of further *Lactobacillus spp.*; more in particular selected from the group comprising *Lactobacillus casei*, *Lactobacillus plantarum*, *Lactobacillus reuteri* *Lactobacillus salivarius*, *Lactobacillus helveticus*, *Lactobacillus parabuchneri*, *Lactobacillus pentosus*, *Lactobacillus brevis*, *Lactobacillus sakei*, *Lactobacillus diolivorans*, *Lactobacillus buchneri*, *Lactobacillus gasseri*, *Lactobacillus bulgaricus* and other *Lactobacillus* species with a GRAS (generally recognized as safe) or QPS (qualified presumption of safety) status; even more in particular selected from the group consisting of *Lactobacillus casei*, *Lactobacillus rhamnosus*, *Lactobacillus plantarum*, *Lactobacillus reuteri*, *Lactobacillus pentosus*, *Lactobacillus helveticus*, *Lactobacillus brevis* and *Lactobacillus parabuchneri*.

As will be evident to the skilled artisan each of the embodiments mentioned in relation to the use of the *Lactobacillus spp.* in the treatment and prevention of infections of the oronasopharyngeal cavity, are equally applicable in the methods for the prevention or treatment of infections of the oronasopharyngeal cavity, and accordingly considered in being disclosed herein. The same applies for the different embodiments related to the infections of the oronasopharyngeal cavity. The method of treatments are equally applicable in;

- the treatment of upper respiratory tract infections;
- 35 • the treatment of upper respiratory tract infections, wherein said upper respiratory tract infections are selected from the group comprising acute otitis media, pharyngitis, chronic sinusitis, acute sinusitis, rhinitis, oral mucositis and the like;
- as anti-inflammatory agent in the treatment of infections of the oronasopharyngeal cavity;
- as anti-inflammatory agent in the treatment of infections of the oronasopharyngeal cavity,

wherein said infections are caused by an infection comprising one or more of the pathogens selected from *Moraxella catarrhalis*, *Haemophilus influenzae*, and *Corynebacterium tuberculostrictum*, *Corynebacterium accolens*, *Streptococcus pneumoniae*, *Streptococcus pyogenes* and *Staphylococcus aureus* (including MRSA) ;

- 5 • as anti-inflammatory agent in the treatment of upper respiratory tract infections;
- as anti-inflammatory agent in the treatment of upper respiratory tract infections, wherein said upper respiratory tract infections are selected from the group comprising acute otitis media, pharyngitis, chronic sinusitis, acute sinusitis, rhinitis, oral mucositis and the like;
- in the treatment of mucosal lesions of the oronasopharyngeal cavity.
- 10 • in the treatment of mucosal lesions of the oronasopharyngeal cavity, caused by an infection comprising one or more of the pathogens selected from *Moraxella catarrhalis*, *Haemophilus influenzae*, and *Corynebacterium tuberculostrictum*, *Corynebacterium accolens*, *Streptococcus pneumoniae*, *Streptococcus pyogenes* and *Staphylococcus aureus* (including MRSA) .

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In each of the compositions for use, uses and method of treatments, an effective amount of bacteria equals at least 1.10^5 CFU; in particular from about and between 1.10^5 to 1.10^9 CFU per day. Administering said amount can be done in any form known to the skilled artisan and suitable for administration to the oronasopharyngeal cavity, such as nasal sprays, buccal tablets, 20 buccal sprays, aerosols and throat lozenges, ear drops and the like. It is accordingly a further aspect of the present invention to provide pharmaceutical compositions for use in the treatment or prevention of infections of the oronasopharyngeal cavity, comprising the *Lactobacillus spp.* in any one of the different embodiments as described herein. In one embodiment, and as further elaborated in the examples hereinafter, the *Lactobacillus spp.* used in said formulations are 25 *Lactobacillus spp.* spray-dried in the presence of mono- and/or disaccharides as protectants. Stable and dry powders of the *Lactobacillus spp.* were obtained by spray-drying the bacteria using a saccharide protectant selected from the group consisting of glucose, mannose, mannitol, dextran, lactose, trehalose, or combinations thereof. In one embodiment the saccharide protectant used includes trehalose. In one embodiment in said spray-dried powders of the 30 *Lactobacillus spp.* the mono- and/or disaccharide protectant is added to the bacteria in a ratio of and between 1:1 to 1:7 (bacteria:saccharide); in particular 1:2 to 1:5; more in particular 1:3 to 1:4; even more particular 1:2 to 1:6.

Some of the aspects of the present invention can be summarized in the following enumerated statements:

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1. Viable spray-dried *Lactobacillus* species, more in particular *Lactobacillus rhamnosus* for use in the oronasopharyngeal treatment of infections of the oronasopharyngeal cavity.
2. Use according to statement 1, wherein the *Lactobacillus rhamnosus* is selected from *Lactobacillus rhamnosus* AMB-GG, *Lactobacillus rhamnosus* AMB-GR-1 or a combination thereof.

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3. Use according to statement 1, wherein the *Lactobacillus rhamnosus* is *Lactobacillus rhamnosus* AMB-GG.
4. Use according to statement 1, wherein *Lactobacillus rhamnosus* is combined with other viable spray-dried probiotic bacteria; in particular with *Lactobacillus spp.*
- 5 5. Use according to statement 4, wherein the *Lactobacillus spp.* are selected from the group comprising *Lactobacillus casei*, *Lactobacillus plantarum*, *Lactobacillus reuteri*, *Lactobacillus salivarius*, *Lactobacillus helveticus*, *Lactobacillus parabuchneri*, *Lactobacillus pentosus*, *Lactobacillus brevis*, *Lactobacillus sakei*, *Lactobacillus diolivorans*, *Lactobacillus buchneri*, *Lactobacillus gasseri*, and *Lactobacillus bulgaricus*.
- 10 6. Use according to statement 1, wherein the infections of the oronasopharyngeal cavity are caused by one or more of the pathogens selected from *Moraxella catarrhalis*, *Haemophilus influenzae*, *Corynebacterium tuberculoostearicum*, *Corynebacterium accolens*, *Streptococcus pyogenes*, *Streptococcus pneumoniae* and *Staphylococcus aureus* (including MRSA).
7. Use according to statement 1, wherein the infections of the oronasopharyngeal cavity are upper respiratory tract infections.
- 15 8. Use according to statement 7, wherein the upper respiratory tract infections are selected from the group consisting of acute otitis media, pharyngitis, chronic sinusitis, rhinitis, oral mucositis and the like.
9. Use of viable spray-dried *Lactobacillus* species in the manufacture of an oronasopharyngeal formulation.
- 20 10. Use according to statement 9, wherein the oronasopharyngeal formulation comprises at least 1.10^5 CFU of *Lactobacillus* species, as defined in any one of statements 1 to 3, or at least 1.10^5 CFU of *Lactobacillus* species combinations as defined in any one of statements 4 to 6; in particular from about and between 1.10^6 to 1.10^9 CFU of said *Lactobacillus species* or
- 25 *Lactobacillus species* combinations; more in particular from about and between 1.10^6 to 1.10^9 CFU of said *Lactobacillus rhamnosus* or *Lactobacillus rhamnosus* combinations.
11. A method for the prevention or treatment of infections of the oronasopharyngeal cavity, said method comprising administering into the oronasopharyngeal cavity of the subject to be treated, a composition comprising an effective amount of *Lactobacillus* species. .
- 30 12. The method according to statement 11 wherein an effective amount of *Lactobacillus* species, comprises at least 1.10^5 CFU of *Lactobacillus* species; in particular from about and between 1.10^6 to 1.10^9 CFU of *Lactobacillus* species.
13. The method according to statements 11 or 12, wherein the *Lactobacillus* species are selected from *Lactobacillus rhamnosus*, *Lactobacillus casei*, *Lactobacillus plantarum*,
- 35 *Lactobacillus reuteri*, *Lactobacillus salivarius*, *Lactobacillus helveticus*, *Lactobacillus parabuchneri*, *Lactobacillus pentosus*, *Lactobacillus brevis*, *Lactobacillus sakei*, *Lactobacillus diolivorans*, *Lactobacillus buchneri*, *Lactobacillus gasseri*, and *Lactobacillus bulgaricus*; more in particular *Lactobacillus rhamnosus*.
14. The method according to statement 13, wherein the *Lactobacillus rhamnosus* is selected
- 40 from *Lactobacillus rhamnosus* AMB-GG, *Lactobacillus rhamnosus* AMB-GR-1, natural

isolates or a combination thereof.

15. A method for the prevention or treatment of infections of the oronasopharyngeal cavity, said method comprising administering into the oronasopharyngeal cavity of the subject to be treated a composition comprising an effective amount of *Lactobacillus* species combinations as defined in any one of statements 4 to 6.
16. The method according to statement 15, wherein an effective amount of the *Lactobacillus* species combinations as defined in any one of statements 4 to 6, comprises at least 1.10^5 CFU of bacteria; in particular from about and between 1.10^6 to 1.10^9 CFU of bacteria.
17. The method according to any one of statements 11 to 16, comprising administering from about and between 1.10^6 to 1.10^9 CFU of the bacteria per day to the oronasopharyngeal cavity of the subject to be treated.
18. Use of a composition comprising spray-dried *Lactobacillus* species, and mono- or disaccharides as protectants, in the manufacture of an oronasopharyngeal formulation
19. Use according to statement 18, wherein the mono- and/or disaccharides are selected from the group comprising glucose, mannose, mannitol, dextran, lactose and trehalose.
20. Use according to statement 18, wherein the protectant is trehalose.
21. Use according to statement 18, wherein the *Lactobacillus* species is *Lactobacillus rhamnosus*; more in particular selected from *Lactobacillus rhamnosus* AMB-GG, *Lactobacillus rhamnosus* AMB-GR-1, natural isolates or a combination thereof
22. Use according to statement 18, wherein the composition further comprises spray-dried probiotic bacteria selected from the group consisting of *Lactobacillus casei*, *Lactobacillus plantarum*, *Lactobacillus reuteri*, *Lactobacillus salivarius*, *Lactobacillus helveticus*, *Lactobacillus parabuchneri*, *Lactobacillus pentosus*, *Lactobacillus brevis*, *Lactobacillus sakei*, *Lactobacillus diolivorans*, *Lactobacillus buchneri*, *Lactobacillus gasseri*, and *Lactobacillus bulgaricus*.
23. Use according to any one of statements 18 to 22, wherein the mono- and/or disaccharide protectant is added to the bacteria in a ratio of and between 1:1 to 1:7 (bacteria:saccharide); in particular 1:2 to 1:7.
24. Use according to statement 18, wherein the pharmaceutical composition is selected from nasal sprays, buccal tablets, buccal sprays, aerosols, throat lozenges, ear drops and the like

Some further aspects of the present invention can be summarized in the following enumerated statements:

1. An oronasopharyngeal composition comprising viable, spray-dried *Lactobacillus* species, for use in the prevention and/or treatment of infections of the oronasopharyngeal cavity, by administration of said composition in said cavity.
2. The oronasopharyngeal composition for use according to statement 1, wherein said composition is selected from the list comprising nasal sprays, buccal tablets, buccal sprays, aerosols, throat lozenges and ear drops.
3. The composition for use according to anyone of statements 1 or 2, wherein said *Lactobacillus*

species is *Lactobacillus rhamnosus*.

4. The composition for use according to statement 3, wherein said *Lactobacillus rhamnosus* is selected from *Lactobacillus rhamnosus* GG, *Lactobacillus rhamnosus* GR-1 or a combination thereof.
5. The composition for use according to anyone of statements 3 or 4, wherein said *Lactobacillus rhamnosus* species are combined with other viable spray-dried probiotic bacteria; in particular with *Lactobacillus spp* selected from the list comprising *Lactobacillus casei*, *Lactobacillus plantarum*, *Lactobacillus reuteri*, *Lactobacillus salivarius*, *Lactobacillus helveticus*, *Lactobacillus parabuchneri*, *Lactobacillus pentosus*, *Lactobacillus brevis*,
10 *Lactobacillus sakei*, *Lactobacillus diolivorans*, *Lactobacillus buchneri*, *Lactobacillus gasseri*, and *Lactobacillus bulgaricus*.
6. The composition for use according to anyone of statements 1 to 5, wherein the infections of the oronasopharyngeal cavity are caused by one or more of the pathogens selected from *Moraxella catarrhalis*, *Haemophilus influenzae*, *Staphylococcus aureus* (including MRSA),
15 *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Corynebacterium tuberculoostearicum* and *Corynebacterium accolens*.
7. The composition for use according to anyone of statements 1 to 6, wherein the infections of the oronasopharyngeal cavity are upper respiratory tract infections selected from the group consisting of acute otitis media, pharyngitis, chronic sinusitis, acute sinusitis, rhinitis, oral
20 mucositis and the like.
8. The composition for use according to anyone of statements 1 to 7, wherein said composition further comprises mono- and/or disaccharides selected from the list comprising glucose, mannose, mannitol, dextran, lactose and trehalose; in particular trehalose.
9. The composition for use according to anyone of statements 1 to 8, wherein the
25 oronasopharyngeal composition comprises at least 1.10^5 CFU of *Lactobacillus* species; in particular from about and between 1.10^6 to 1.10^9 CFU of said *Lactobacillus* species or *Lactobacillus* species combinations.
10. A method for the prevention and/or treatment of infections of the oronasopharyngeal cavity, said method comprising administering a composition as defined in any one of statements 1
30 to 8, into the oronasopharyngeal cavity of a subject in need thereof.
11. Use of viable, spray-dried *Lactobacillus* species in the manufacture of an oronasopharyngeal formulation for administration in the oronasopharyngeal cavity.
12. Use according to statement 11, wherein said spray-dried *Lactobacillus* species are combined with mono- and/or disaccharides, selected from the list comprising glucose, mannose,
35 mannitol, dextran, lactose and trehalose; in particular trehalose.
13. Use according to anyone of statements 11 or 12, wherein said *Lactobacillus species* is *Lactobacillus rhamnosus*.
14. Use according to statement 13, wherein said *Lactobacillus rhamnosus*, is selected from *Lactobacillus rhamnosus* GG, *Lactobacillus rhamnosus* GR-1 or a combination thereof.
- 40 15. Use according to anyone of statements 13 or 14, wherein the composition further comprises

other viable spray-dried probiotic bacteria; in particular *Lactobacillus* spp selected from the list comprising *Lactobacillus casei*, *Lactobacillus plantarum*, *Lactobacillus reuteri*, *Lactobacillus salivarius*, *Lactobacillus helveticus*, *Lactobacillus parabuchneri*, *Lactobacillus pentosus*, *Lactobacillus brevis*, *Lactobacillus sakei*, *Lactobacillus diolivorans*, *Lactobacillus buchneri*, *Lactobacillus gasseri*, and *Lactobacillus bulgaricus*.

In each of the compositions, methods and uses as defined above, the *Lactobacillus* strains may be replaced or complemented with *Lactobacillus* secreted products such as lactic acids.

10 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: Time course analysis of the antipathogenic activity of spent culture supernatant and secreted products of different *Lactobacillus* strains.

Fig. 2: Antibiofilm capacity of the spent culture supernatant and secreted products of different *Lactobacillus* strains against *Moraxella catarrhalis*

Fig. 3: Antipathogenic activity of (lactic) acid in spent culture supernatant of different *Lactobacillus* strains based on (A) time course analysis of spent culture supernatant of *L. rhamnosus* AMB-GG against *Moraxella catarrhalis*, (B) antibiofilm activity of spent culture supernatant of *L. rhamnosus* AMB-GG against *Moraxella catarrhalis*

Fig. 4: Effect of different saccharides on survival after spray-drying. The saccharides were added in a ratio of 1:1. The content of the vials was 200 mg, this was dissolved in 10 mL MRS medium for resuscitation before plating on agar.

Fig. 5 (A-D): Viability test of *L. rhamnosus* AMB-GG spray-dried after adding saccharides – lactose (A), trehalose (B), dextran (C) and mannitol (D) - in different concentrations (1:1 proportion, 1:2 proportion, 1:5 proportion) to the bacterial suspension.

Fig. 6 (A) Comparison of the 1:5 proportion of lactose, trehalose and dextran to the bacterial suspension. The viability was calculated after resuscitation in 10 ml MRS and incubation at 37°C for 3 days; (B) Addition of lactose or mannitol to the growth medium of *L. rhamnosus* AMB-GG. After an overnight incubation at 37°C a PBS-feed suspension was made, which was spray-dried.

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Fig 7: Cumulative effect of the addition of mannitol – the saccharide with the best result for the growth medium, and trehalose - the saccharide with the best result for the bacterial (feed) suspension just before spray-drying.

10 **Fig 8 (A-C):** Comparison of tablet characteristics (tablet hardness (A), disintegration time (B) and thickness (C)) between tablets made with spray-dried powder as such and tablets made with spray –dried powder with addition of 2 parts trehalose.

15 **Fig 9 (A-B):** Growth curves (A) and viability (B) of *L. rhamnosus* AMB-GG after tablet compression, comparing samples without addition of trehalose and those with addition of 2 parts trehalose

Fig 10: Viability test after resuscitation in different conditions. MRS: deMan, Rogosa and Sharpe broth; PBS: phosphate buffered saline; GLC: glucose; RT: room temperature.

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Fig 11: Water activity of spray-dried powder as such, with addition of 5 parts trehalose and with addition of 5 parts lactose.

25 **Fig 12:** Viability of *L. rhamnosus* AMB-GG after short term storage at 4°C. Comparison between as such spray-dried powder and with addition of different concentrations of trehalose and lactose. Results are given for 0 days and 40 days of storage.

Fig 13: Viability of *L. rhamnosus* AMB-GG after long term storage (1 year) at 4°C. Comparison between as such spray-dried powder and with addition of different 2 parts trehalose.

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Fig. 14: Mean inhibition zones of spray-dried *Lactobacillus* after a resuscitation step of 0,5 h in competition with *Moraxella catarrhalis*. LGG susp: LGG spray-dried as such; LGG susp TRH: LGG spray-dried after adding trehalose based on the spot antipathogenic activity.

35 DETAILED DESCRIPTION OF THE INVENTION

The present invention is based on the discovery that certain viable species, i.e. *Lactobacillus rhamnosus*, *Lactobacillus casei*, *Lactobacillus plantarum*, *Lactobacillus pentosus*, *Lactobacillus parabuchneri* and *Lactobacillus reuteri*, of the genus of *Lactobacillus*, and their secreted products in the supernatant, are particularly useful in the treatment and or prevention of infections of the
40 nasopharyngeal cavity. Within the context of the present invention all strains for the

aforementioned species are meant in being suitable for the therapeutic applications of the present invention. Preferred strains are selected from the group consisting of *Lactobacillus rhamnosus*, *Lactobacillus casei*, *Lactobacillus reuteri* and *Lactobacillus plantarum*. Again, for each of the foregoing strains, the known isolates are included within the context of the present invention and meant in being suitable for the therapeutic applications of the present invention. In a particular embodiment the *Lactobacillus* isolates used are selected from the group consisting of *L. rhamnosus*, *L. casei*, *L. plantarum*, *L. reuteri*. In an embodiment the *Lactobacillus rhamnosus* species used in the different embodiments of the present invention is *L. rhamnosus* AMB-GG and *L. rhamnosus* AMB-GR-1. In another embodiment the *Lactobacillus casei* strain used in the different embodiments of the present invention is *L. casei* AMB- 334 and natural isolates.

Pharmaceutical formulation of the *Lactobacillus spp.* requires isolating said bacteria from eventual growth culture media. The skilled artisan is well aware of the techniques available for isolating the viable bacteria from a growth culture media such as centrifugation, filtration, micro manipulation, and the like. For medium and long term storage, the isolated bacteria are preferably maintained in a dry state, such as for example achieved using freeze drying or spray-drying. In one embodiment of the present invention, the *Lactobacillus spp.* used in the manufacture of the pharmaceutical compositions are spray-dried *Lactobacillus spp.*, obtained by spray-drying the bacteria using a saccharide protectant, such as for example glucose, mannose, mannitol, dextran, lactose or trehalose; in particular trehalose. An exemplary process suitable for spray-drying the bacteria of the present invention is for instance available from Sunny-Roberts and Knorr (Int. Diary J., 19 (2009) 209-2014).

Once isolated, the *Lactobacillus spp.* of the present invention can be prepared by any known or otherwise effective method for pharmaceutically formulating or manufacturing the selected product form. Methods for preparing the pharmaceutical compositions according to the present invention can be found in "Remington's Pharmaceutical Sciences", Mid.Publishing Co., Easton, Pa., USA.

For example, the compositions comprising the spray-dried bacteria and the saccharide protectants as defined herein, can be formulated along with common excipients, diluents, or carriers, and formed into oral tablets, capsules, sprays, mouth washes, lozenges, treated substrates (e. g. , oral or topical swabs, pads, or disposable, non-digestible substrate treated with the compositions of the present invention) ; oral liquids (e. g. , suspensions, solutions, emulsions), powders, or any other suitable dosage form; in as long as said formulation does not interfere with the viability of the spray-dried bacteria.

Non-limiting examples of suitable excipients, diluents, and carriers can be found in "Handbook of Pharmaceutical Excipients", Second edition, American Pharmaceutical Association, 1994 and include: fillers and extenders such as starch, sugars, mannitol, and silicic derivatives; binding

agents such as carboxymethyl cellulose and other cellulose derivatives, alginates, gelatin, and polyvinyl pyrrolidone; moisturizing agents such as glycerol; disintegrating agents such as calcium carbonate and sodium bicarbonate; agents for retarding dissolution such as paraffin; resorption accelerators such as quaternary ammonium compounds; surface active agents such as acetyl alcohol, glycerol monostearate; adsorptive carriers such as kaolin and bentonite; carriers such as propylene glycol and ethyl alcohol, and lubricants such as talc, calcium and magnesium stearate, and solid polyethyl glycols.

This invention will be better understood by reference to the Experimental Details that follow, but those skilled in the art will readily appreciate that these are only illustrative of the invention as described more fully in the claims that follow thereafter. Additionally, throughout this application, various publications are cited. The disclosure of these publications is hereby incorporated by reference into this application to describe more fully the state of the art to which this invention pertains.

EXAMPLES

The following examples illustrate the invention. Other embodiments will occur to the person skilled in the art in light of these examples.

EXAMPLE 1: IDENTIFICATION OF THE ANTI PATHOGENIC ACTIVITY

AIM OF EXPERIMENT

A streak-line based assay and spot-based antipathogenic assay were performed to investigate the anti-pathogenic activity of different living *Lactobacillus* species against common nasopharyngeal pathogens, while a radial diffusion antipathogenic assay, a time course analysis of the antipathogenic activity and an antibiofilm assay were performed to investigate the antipathogenic activity of spent culture supernatant and thus the secreted products of different *Lactobacillus* strains.

MATERIALS AND METHODS

Table 1: Used pathogenic bacteria and their culture conditions

Nasopharyngeal pathogenic species	Strain	Broth medium	Agar medium
<i>Corynebacterium tuberculo</i> stearicum	ATCC35529	Tryptic Soy broth	Tryptic Soy agar
<i>Haemophilus influenzae</i> *	ATCC49247	Mueller Hinton broth + 5% blood + 10 µg/mL NAD + 10 µg/mL hemin	Chocolate agar / Blood agar

<i>Moraxella catarrhalis</i>	ATCC25238	Mueller Hinton broth	Mueller Hinton Agar
<i>Staphylococcus aureus</i>	ATCC29213	Mueller Hinton broth	Mueller Hinton Agar
<i>Staphylococcus aureus</i>	Methicillin resistant	Mueller Hinton broth	Mueller Hinton Agar
<i>Staphylococcus aureus</i>	Methicillin sensitive	Mueller Hinton broth	Mueller Hinton Agar
<i>Streptococcus pneumoniae</i> *	ATCC49619	Tryptic Soy broth + 5% blood	Chocolate agar / Blood agar
<i>Streptococcus pyogenes</i> *	BM137	Brain Heart Infusion broth	Brain Heart Infusion agar

* Strain needs to be grown in 5% CO₂ atmosphere.

Table 2: The *Lactobacillus* strains used in this test are:

Species	Source	Strain
<i>Lactobacillus bulgaricus</i>	single colony isolate from C03-46	AMB-1
<i>Lactobacillus casei</i>	single colony isolate obtained in our lab from a stock culture of ATCC334	AMB-ATCC334
<i>Lactobacillus casei</i>	single colony isolate obtained in our lab from a commercially available fermented drink containing <i>L. casei</i> Shirota (Yakult®)	AMB-Sh1
<i>Lactobacillus casei</i>	single colony isolate obtained in our lab from a commercially available fermented drink (Actimel®) containing <i>L. casei</i> DN-114001	AMB-Act1
<i>Lactobacillus casei</i>	Single colony isolate from spontaneously fermented carrot juice	AMB-MCJ
<i>Lactobacillus helveticus</i>	single colony isolate from 1807	AMB-2
<i>Lactobacillus parabuchneri</i>	Single colony isolate from spontaneously fermented beet juice	AMB-AB17
<i>Lactobacillus parabuchneri</i>	Single colony isolate from spontaneously fermented carrot juice	AMB-NM63-3
<i>Lactobacillus pentosus</i>	single colony isolate	AMB-pent1
<i>Lactobacillus plantarum</i>	single colony isolate from <i>L. plantarum</i> ATCC8014 or LMG1284	AMB-LMG1284
<i>Lactobacillus plantarum</i>	single colony isolate obtained in our lab from a stock culture of CMPG5300	AMB-pl1
<i>Lactobacillus plantarum</i>	single colony isolate of <i>L. plantarum</i> 5057	AMB-pl2

<i>Lactobacillus plantarum</i>	single colony isolate of <i>L. plantarum</i> WCFS1	AMB-pl3
<i>Lactobacillus reuteri</i>	single colony isolate obtained in our lab from a commercially available probiotic supplement containing <i>L. reuteri</i> RC4	AMB-RC4
<i>Lactobacillus rhamnosus</i>	single colony isolate obtained in our lab from a commercially available probiotic supplement containing <i>L. rhamnosus</i> GR-1	AMB-GR-1
<i>Lactobacillus rhamnosus</i>	single colony isolate <i>L. rhamnosus</i> GG	AMB-GG

First, lactobacilli were inoculated from a -80°C stock on an MRS-plate (=starter plate). This plate is incubated for 3 days at 37°C. Also the pathogens were inoculated on a plate (=starter plate) and incubated for 1 day at 37°C.

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Other material:

- Incubator: Memmert INB200
- CO₂ bags: GasPak EZ, Becton, Dickinson and Company (BD)
- Petri plates: 90x14.2 mm VWR
- Centrifuge Tubes: 15/50 mL VWR

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1.1. Streak-line antipathogenic assay to investigate the activity of live lactobacilli

Lactobacilli were streak inoculated from a colony on a starter plate (MRS) on a test plate (medium of pathogen + glucose is needed) and incubated at 37°C for 3 nights. Then, the pathogens were streak inoculated from a colony on a starter plate on the test plates in 3 repetitions. The plates were incubated at 37°C for 24h and the inhibition zone was measured.

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1.2 Spot antipathogenic assay with live lactobacilli

Growing spots

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From an MRS starter plate a 10 mL MRS broth was inoculated and incubated overnight at 37°C on a starter plate on the test plates in 3 repetitions. The plates were agar plate containing 5 g/L glucose. These plates (spot plate) were incubated for 54h at 37°C.

Overlay of pathogens

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Moraxella catarrhalis ATCC25238, *Staphylococcus aureus* (3 strains: ATCC29213, methicillin resistant (MRSA) and methicillin sensitive (MSSA)) and *Corynebacterium tuberculostrictum* are stored in a glycerol stock at -80°C. *Staphylococcus aureus* (ATCC29213, methicillin resistant (MRSA) and methicillin sensitive (MSSA)) and *M. catarrhalis*: bacteria were grown on a Mueller Hinton (MH) plates. A colony was inoculated in 5 mL MH broth and incubated for 24 h, then 2% culture was added to MH soft agar (0.5 % agar) and poured over the plates with *Lactobacillus* spots. The plates were incubated overnight at 37°C. For *C. tuberculostrictum*: bacteria were

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grown on a Tryptic soy (TS) plate, a colony was inoculated in 5 mL TS broth and incubated for 24 h, then 2% culture was added to MH soft agar (0.5 % agar) and poured over the plates with *Lactobacillus* spots. The plates were incubated overnight at 37°C. 0.1% hexetidine was added to the spot plate before the soft agar was poured as a positive control.

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1.3. Radial diffusion antipathogenic assay to investigate the activity of spent supernatant and secreted products

A pathogen suspension is made in the appropriate medium and incubated for 1 night at 37°C. The CFU is calculated by plating different dilutions of starter suspension on an appropriate plate.

10 Different dilutions of the pathogen are incubated on the plate by adding 25 mL of (not too hot) agar to a different volume of suspension. The plates are dried and wholes are made (diameter 0.4 cm, height 0.5 cm) punched in the agar. Supernatant of lactobacilli, positive and negative control are added to the holes (30 µL). When the test material was fully diffused into the agar, an overlay of 20 mL agar was poured over the agar plate and the plate was incubated for 24h at
15 37°C. Inhibition zones were measured afterwards. 0.1% hexetidine was used as a positive control. Sterile MRS with pH 4,3 was used as a negative control. Supernatant was made by incubating overnight lactobacilli in MRS broth. SN was obtained by centrifugation for 30 min. at 6797 g (8000 rpm) at 4°C. The SN was then filter sterilized (0.20 µm cellulose acetate, VWR) to remove remaining cells and the pH was measured.

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1.4. Time course analysis of the antipathogenic activity of spent culture supernatant and secreted products

Every well of the 100-well plate contained a volume of 300 µL test material (mostly 240µL culture + 60 µL test material or sterile dH₂O). As a negative control, different dilutions (240 µL culture +
25 60 µL sterile dH₂O) of a culture of *Moraxella catarrhalis* ($\pm 10^8$ CFU/mL) was tested: 10^{-2} until 10^{-5} . Also MH broth and demineralized water were tested. The positive controls were different concentrations of hexetidine (final concentrations: 0,01%; 0,005% and 0,001%) and lactic acid (final concentrations: 100 mM; 50 mM; 25 mM; 2,5 mM and 0,25 mM). The concentration of the supernatant tested was 1/20. At higher concentrations, MRS shows inhibition to the growth of the
30 pathogen. Bacteria were grown, and the optical density at 600 nm was measured automatically after shaking each 20 min during 168 h.

1.5. Biofilm antipathogenic assay of spent culture supernatant and secreted products

35 Biofilm formation of the pathogens in coculture with SN was as previously described by Pearson et al. (2006). Here, *M. catarrhalis* was inoculated into 5 mL BHI broth. This culture was incubated overnight at 37°C ($\pm 10^5$ CFU/mL) and diluted 1:100 in BHI. A volume of 190 µL of this suspension was loaded into a 96-well microplate and incubated at 37°C for 19 h. To test the antimicrobial activity of the SN, 10 µL portions of SN were loaded, in 8 repetitions, into the 96-
40 well microplate. The broth was then removed from each well and replaced by 200 µL PBS plus

10 μ l of 0.7% (wt/vol) crystal violet. After 15 min at room temperature, the wells were emptied and washed three times with deionized water. A 200- μ L volume of 95% ethanol was added to each well, and the plate was shaken gently for 15 min. A 150- μ l portion of this ethanol solution was then transferred to a new 96-well plate, and the absorbance at 570 nm was measured using
5 a Synergy MX (Biotek).

1.6. Identification of secreted (lactic) acid as an important antimicrobial factor

The supernatant was prepared as explained above. To gain information on the active molecules
10 of the supernatant samples possibly involved in the inhibition of the pathogens, the supernatant samples of the different lactobacilli were also treated by different methods and tested for antipathogenic activity as described above: (i) supernatant of lactobacilli was first heated at 110°C for 60 min and then treated with proteinase K and incubated for 60 min at 37°C (ii) SN of lactobacilli brought at pH 7 (iii) dialysis (cut-off 1000 Da) against a hepes-citrate-tis buffer (60-40-
15 20 mM) pH 4,3 was done to remove metabolites of the lactobacilli (such as lactic acid and other produced metabolic by products) that are smaller than this size. The positive and negative controls were 0,1 % hexetidin and MRS at pH 4,3 respectively. The latter was done to check whether the effect of the supernatant was not only caused by the low pH (due to addition of HCl) but by a specific, secreted molecule in the supernatant. The supernatant of lactobacilli after
20 growth for 16 h-24 h to stationary phase has generally a mean pH of 4,3.

The same methods as explained above was used to test the antipathogenic activity of different treated supernatants of the lactobacilli by the radial diffusion antipathogenic assay, the time course analysis and the biofilm antipathogenic assay.

25 RESULTS

1.7. Results streak line antipathogenic assay with live lactobacilli

In table 3, the mean inhibition zones (streak line anti-pathogenic assay) of different *Lactobacillus* species in competition with *Moraxella catarrhalis*, *Haemophilus influenzae*, *Staphylococcus aureus* and *Streptococcus pyogenes* are provided. Negative control: no bacteria. Positive
30 control: 0.1% hexetidine.

Table 3: Mean inhibition zones of different *Lactobacillus* species in competition with pathogenic species

Species	Strain	HI	MC	SA	SPY
<i>Lactobacillus casei</i>	AMB-ATCC334			+	+++
<i>Lactobacillus casei</i>	AMB-Sh1			+	+++
<i>Lactobacillus casei</i>	AMB-Act1			+	+++
<i>Lactobacillus plantarum</i>	AMB-pl1	++	+++	+	+++

<i>Lactobacillus reuteri</i>	AMB-RC4			+	+++
<i>Lactobacillus rhamnosus</i>	AMB-GR-1			+	+++
<i>Lactobacillus rhamnosus</i>	AMB-GG	+++	+++	++	+++

- : no inhibition, +: 0-0,5 cm inhibition; ++: 0,5-1 cm inhibition; +++: > 1 cm: inhibition

Abbreviations: HI: *Haemophilus influenzae* ; MC: *Moraxella catarrhalis* ATCC25238; SA: *Staphylococcus aureus* ATCC29213; SPY: *Streptococcus pyogenes* BM137.

- 5 Streak inoculation tests showed a clear potential of the antipathogenic activity of lactobacilli against different pathogens. A strong activity is observed against *H. influenzae*, *M. catarrhalis* and *S. pyogenes* for all lactobacilli tested, highlighting that the observed activities are *Lactobacillus* genus-specific, but species- and strain-independent. A smaller activity is observed against *S. aureus*, but this activity is consistent. *L. rhamnosus* AMB-GG showed the strongest
10 activity against all pathogens tested.

1.8. Results spot-based antipathogenic assay with live lactobacilli

- In table 4, the mean inhibition zones (spot-based antipathogenic assay) of different living *Lactobacillus* species in competition with *Moraxella catarrhalis*, *Corynebacterium tuberculo*
15 *tuberculo*stearicum, *Staphylococcus aureus* (including MRSA and MSSA) and *Streptococcus pyogenes* are provided. Negative control: no bacteria.

Table 4: Mean inhibition zones of different *Lactobacillus* species in competition with pathogenic species

Species	Strain	MC	SA ATCC2921	MRSA	MSSA	CT
<i>Lactobacillus casei</i>	ATCC334	+++	-	-	-	-
<i>Lactobacillus casei</i>	AMB-Sh1	+++	-	+	+	-
<i>Lactobacillus casei</i>	AMB-Act1	+++	-	+	+	-
<i>Lactobacillus casei</i>	AMB-MCJ	+++	-	-	-	-
<i>L. parabuchneri</i>	AMB-AB17	+++	-	-	-	-
<i>L. parabuchneri</i>	AMB-NM63-3	+++	-	-	-	-
<i>Lactobacillus pentosus</i>	AMB-pent1	+++	+	+	+	-
<i>Lactobacillus plantarum</i>	AMB-LMG1284	+++	+	+	+	-
<i>Lactobacillus plantarum</i>	AMB-pl1	+++	+	+	+	-
<i>Lactobacillus reuteri</i>	AMB-RC4	+++	+	+	+	-
<i>Lactobacillus rhamnosus</i>	AMB-GR-1	+++	+	+	+	-
<i>Lactobacillus rhamnosus</i>	AMB-GG	+++	+	+	+	-

- 20 - : no inhibition, +: 0-0,2 cm inhibition; ++: 0,2-0,5 cm inhibition; +++: > 0,5 cm: inhibition

MC: *Moraxella catarrhalis*, SA: *Staphylococcus aureus*, MRSA: methicillin resistant *Staphylococcus aureus*, MSSA: methicillin sensitive *Staphylococcus aureus*, CT: *Corynebacterium tuberculo*stearicum.

In these cell-to-cell competition assays with live probiotics and pathogens, all lactobacilli tested showed a strong antipathogenic activity against *M. catarrhalis*, although some activity is also observed against several strains of *S. aureus* (including MRSA) for *L. pentosus* AMB-pent1, *L. plantarum* AMB-LMG1284 and pl1, *L. reuteri* AMB-RC4, *L. rhamnosus* AMB-GR-1 and AMB-GG.

5

1.9. Results radial diffusion antipathogenic assay:

In table 5, the mean inhibition zones of the spent culture supernatant and secreted products of different *Lactobacillus* species in competition with *Corynebacterium tuberculostearicum*, *Moraxella catarrhalis*, *Haemophilus influenzae*, *Staphylococcus aureus* (including MRSA), *Streptococcus pneumoniae* and *Streptococcus pyogenes* are provided for the radial diffusion antipathogenic assay. Negative control: MRS at pH 4.3. Almost all lactobacilli tested show a strong activity (+++) against *M. catarrhalis*, although differences between different isolates were observed. For instance, the strain *L. parabuchneri* AMB-AB17 tested showed clearly smaller inhibition zones than *L. rhamnosus* AMB-GG. This is probably due to the fact that these lactobacilli that show less antimicrobial activity, also grow less fast compared to e.g. *L. rhamnosus* AMB-GG and therefore have produced less (lactic) acid or other antimicrobial molecules. Several lactobacilli tested also showed activity (with inhibition zones up to 0.2 cm) against *Corynebacterium tuberculostearicum* ATCC35529, *Streptococcus pyogenes* BM137 and *Haemophilus influenzae*. In contrast, no *Lactobacillus* strains tested showed activity in this test against at least one of the *S. aureus* strains tested.

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Table 5: Mean inhibition zones of spent culture supernatant and secreted products of different *Lactobacillus* species in competition with pathogenic species

Species	Strain	CT	HI	MC	MRSA*	SPN	SPY
<i>L. bulgaricus</i>	AMB-1			++		-	
<i>L. casei</i>	AMB-ATCC334	+	+	+++	-	++	+
<i>L. casei</i>	AMB-Sh1	+	+	+++	-	++	+
<i>L. casei</i>	AMB-Act1	+	+	+++	-	++	+
<i>L. casei</i>	AMB-MCJ	-		+		-	
<i>L. helveticus</i>	AMB-2			+		-	
<i>L. parabuchneri</i>	AMB-AB17	-		+		-	
<i>L. parabuchneri</i>	AMB-NM63-3	-		+			
<i>L. pentosus</i>	AMB-pent1			+++		++	
<i>L. plantarum</i>	AMB-LMG1284	-		+++		++	
<i>L. plantarum</i>	AMB-pl1	+	+	+++	-	++	+
<i>L. plantarum</i>	AMB-pl2			+++		++	
<i>L. plantarum</i>	AMB-pl3			+++		++	
<i>L. reuteri</i>	AMB-RC4	-	+	+++	-	+	-
<i>L. rhamnosus</i>	AMB-GR-1	+	+	+++	-	++	+

<i>L. rhamnosus</i>	AMB-GG	+	+	+++	-	++	+
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- : no inhibition, +: 0-0,2 cm inhibition; ++: 0,2-0,5 cm inhibition; +++: > 0,5 cm: inhibition

Abbreviations: CT: *Corynebacterium tuberculoostearicum* ATCC35529; HI: *Haemophilus influenzae*; MC: *Moraxella catarrhalis* ATCC25238; SPN: *Streptococcus pneumoniae* ATCC49619; SPY: *Streptococcus pyogenes* BM137

- 5 * MRSA: Methicillin resistant *Staphylococcus aureus* (similar results were obtained for SA: *Staphylococcus aureus* ATCC29213);

1.10. Results time course analysis of the antimicrobial activity of spent culture supernatant of lactobacilli

- 10 In figure 1, the time course analysis of the antimicrobial activity of spent culture supernatant and secreted products of different lactobacilli is provided. Addition of different dilutions of the *Lactobacillus* supernatant samples to *M. catarrhalis* cultures was investigated. At a dilution of 1/20, a strain-specific effect of the inhibition of the growth of *Moraxella* by the *Lactobacillus* supernatant samples was observed. Addition of the supernatant of the *L. rhamnosus* AMB-GG, 15 *L. casei* AMB-Sh, *L. casei* AMB-Act and *L. plantarum* AMB-pl1 inhibited the growth of *Moraxella* for a whole week (168 h). Addition of supernatant of *L. casei* AMB-ATCC334 and *L. reuteri* AMB-RC4 only showed a small delay of the growth of *Moraxella*. However, after 20h the pathogen can grow normal and even reaches a higher OD than the control. *L. rhamnosus* AMB-GR-1 can inhibit the growth of *Moraxella* longer but after ± 30 h *Moraxella* starts to grow and also reaches a 20 higher OD than the control.

1.11 Results biofilm antipathogenic assay

- In figure 2, the antibiofilm activity of spent culture supernatant and secreted products of different lactobacilli is provided against *Moraxella catarrhalis*. Biofilm growth is a more stress resistant 25 mechanism of growth for bacteria compared to growth in suspension as tested above.

Both *L. rhamnosus* AMB-GG, *L. rhamnosus* AMB-GR-1, *L. reuteri* AMB-RC4, *L. casei* AMB-Act and *L. casei* AMB-Sh can reduce the biofilm formation of *M. catarrhalis* by up to 70%. *L. rhamnosus* AMB-pl1 and *L. casei* ATCC334 also have potential to reduce biofilm formation although their activity is more variable.

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1.12 Results secreted (lactic) acid as an important antimicrobial factor

- In figure 3 and table 6, data on the importance of (lactic) acid as active antimicrobial agent in spent culture supernatant is provided based on the radial diffusion antipathogenic assay (table 6), time course analysis of the antipathogenic activity of the spent culture supernatant against 35 *Moraxella* (fig. 3A) and antibiofilm activity assay of spent culture supernatant against *Moraxella* (fig. 3B).

Table 6: Importance of (lactic) acid as active antimicrobial agent in spent culture supernatant is provided based on the radial diffusion antipathogenic assay

Treatment	<i>M. catarrhalis</i>	<i>C. tuberculo</i> <i>stearicum</i>	<i>S pyogenes</i>
MRS pH 4.3	-	-	-
As such	+++	+	+
Heat	+++		
Proteinase K	+++	+	+
Heat + Proteinase K	+++	+	+
pH neutral	-	-	-
Dialysis to remove molecules < 1kDa	+	-	-

The data from the radial diffusion antipathogenic assay indicate that the antimicrobial activity is pH dependent, cannot be degraded by proteinase, and is heat-stable, suggesting an important role for secreted (lactic) acid in the antimicrobial activity. Similarly, data from the time course analysis showed that the supernatant of *L. rhamnosus* AMB-GG, *L. casei* AMB-Act, *L. casei* AMB-Sh and *L. plantarum* AMB-pl1 showed the best antimicrobial activity. This activity disappeared when the supernatant was neutralized at pH7. In contrast, supernatant treated with both heat and proteinase K maintained its antipathogenic activity. These experiments also show that the antimicrobial compounds of different lactobacilli are heat-resistant, non-proteinaceous and pH-dependent compounds, probably secreted acids such as lactic acid.

Finally, data from the antibiofilm experiment showed that treatment of the supernatant of *L. rhamnosus* AMB-GG decreases the antimicrobial activity of the supernatant when the pH is neutralized or a dialysis with a cut-off of 1000 Da is performed. Heat and proteinase K treatment did not largely affect the antimicrobial activity, although some variability was observed. Merely pH reduction to pH 4.3 by HCl did not significantly affect biofilm formation, indicating that it is not the pH per se, but rather the secreted acids in the spent culture supernatant such as lactic acid that cause the inhibition.

EXAMPLE 2 NASOPHARYNGEAL FORMULATION CRITERIA

AIM OF EXPERIMENT

In this project, a novel formulation for lactobacilli was aimed to develop that is optimal for nasopharyngeal applications and which is different from the gastro-intestinal formulations, which focus on survival of the acidic conditions of the stomach.

A nasopharyngeal formulation should be stable, show fast activity in nasopharyngeal cavity (while intestinal applications have more time) and should preserve the antimicrobial activity and adhesion properties of the lactobacilli, so that they are able to temporarily colonize the nasopharynx.

MATERIALS AND METHODS

Step 1: Bacterial culture

The production of a *Lactobacillus* suspension (500ml) in PBS is performed as follows:

- Day 1: Plating of *Lactobacillus* on MRS growth medium plate (from -80°C)
- 5 • Day 2 – 3: incubate at 37°C
- Day 4: Inoculate *Lactobacillus* from plate into 10 ml Liquid MRS and incubate overnight at 37°C
- Day 5: Transfer 5 ml from the culture into 500 ml MRS (sterile Duran bottle with cap) and incubate overnight at 37°C
- 10 • Day 6: Transfer into sterile falcons and centrifuge for 10 min at 4000 g, separate medium and suspend the pellet into 500 ml sterile PBS yielding the bacterial suspensions used in the spray-drying process.

Step 2: Spray-drying

- 15 The spray-drying process was performed on a Buchi B-290 spray-dryer with a two-fluid nozzle type, having a 1.5 mm nozzle size, using an inlet temperature of 120 °C, an outlet temperature of 56°C, a spray rate or feed flow (pump %) of 10 to 16%, an aspiration rate % (air flow) of about 80%, and a pressure-flow (mm flow) of 45 mm.

- 20 Aliquots of the bacterial suspensions, feed *L. rhamnosus* AMB-GG as such, were spray- dried at constant processing parameters;

- inlet temperature : 120°C
- outlet temperature: 56°C
- Spray rate (pump %): 10% wherein 1% pump rate corresponds with a flow rate of
- 25 approximately 0.3 ml/min.
- Aspiration rate % (air flow): 80%
- Q-flow (mm flow): 45 mm
- Nozzle size: 1.5 mm
- Nozzle type: Two-Fluid

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- Before spray-drying the sample, a pre-conditioning step was performed with distilled water using the set process parameters until the outlet temperature was stabilized (approximately 15-20 min). After this pre-conditioning phase the feed tube was changed to the feed suspension which was stirred continuously using a magnetic stirrer to obtain a homogeneous sample throughout
- 35 the process. When the feed suspension was spray-dried completely, the feed tube was changed back towards the recipient with distilled water. After each experiment the cyclone is cleaned with water to avoid any contamination of the previous spray-dried sample. After all experiments were done, the Buchi spray-dryer was cleaned with distilled water and soap. In this spray-drying process, the probiotic suspension is sprayed into a warm airstream after which the drying

droplets form powder particles in fraction of seconds. At the end of the dryer, the powder particles are collected via a cyclone or bag filter.

2.1. Effect of different saccharide protectants on the viability of *L. rhamnosus* AMB-GG.

5 For this experiment, several batches of *L. rhamnosus* AMB-GG were prepared as described in step 1. The batches were divided in aliquots of approximately 40 ml. One aliquot was spray-dried as such, without any addition of a saccharide to function as a control. To the other aliquots of 40 ml, saccharides were added in a 1:1 proportion (1 part saccharide equals 1 part solid probiotic content.) The saccharides tested consisted of glucose, mannose, lactose, trehalose, 10 dextran and mannitol. The spray-drying process was performed on each aliquot as described in step 2. The powder obtained from these aliquots was delivered in vials containing 100 mg and 200 mg for the spray-dried *L. rhamnosus* AMB-GG as such and the spray-dried powders with saccharides added, respectively. The powder was then resuscitated for 30 minutes in MRS broth at 37°C. All ratios were 1:1.

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2.2. Influence of concentration of different saccharides on the viability of *L. rhamnosus* AMB-GG.

The spray-drying parameters used to test the saccharide protectants were as described above. The saccharides to be tested were lactose monohydrate, trehalose dihydrate, mannose, glucose, 20 mannitol and dextran. Each saccharide was added in a 1:1, 1:2 or 1:5 proportion (*L. rhamnosus* AMB-GG: saccharide) based on the solid content of the feed suspension. The saccharides were added to an aliquot of the bacterial suspension. After the addition of the saccharide, the sample was first stirred with a magnetic stirrer to obtain a homogeneous feed suspension for spray-drying and a homogenous powder.

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2.3. Influence of the addition time of the saccharide on the viability of *L. rhamnosus* AMB-GG

To evaluate the influence of the time at which the tested saccharides were added to the bacterial sample, three time periods were chosen:

- 30 1) addition of the saccharide to the bacterial suspension in PBS after which it is directly spray-dried, provided that the saccharide is solved properly
- 2) addition of the saccharide to the growth medium of *L. rhamnosus* AMB-GG, after which it is incubated overnight and afterwards a bacterial suspension in PBS is made and spray-dried.
- 3) addition of a saccharide to the growth medium and to the bacterial suspension just before 35 spray-drying. This last time period is chosen to evaluate the cumulative effect of both time periods mentioned before. Only the saccharides with the best results of the above mentioned time periods are evaluated in this experiment.

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2.4. Influence of the outlet temperature on the viability of *L. rhamnosus* AMB-GG

To evaluate the effect of the outlet temperature on the viability of *L. rhamnosus* AMB-GG, the inlet temperature was varied from 120°C, over 140°C, to 160°C. The other process parameters were kept constant (as mentioned above). The outlet temperatures became 55°, 65°C and 75°C respectively. Survivability of *L. rhamnosus* AMB-GG spray-dried at these process conditions were calculated for spray-dried powder as such and with addition of 2 parts trehalose.

2.5. Influence of tablet compression and compaction force on viability of *L. rhamnosus* AMB-GG

To formulate tablets and lozenges, it is important to characterize the influence of the added saccharides (from the spray-drying process) on the tablet characteristics. To evaluate the influence of tablet compression, five tablets without addition of saccharide and five tablets with addition of 2 parts trehalose were compared as regard to tablet thickness, hardness, disintegration time and viability. The influence of the compaction force on the viability of the bacteria was evaluated using three different compaction forces on the spray-dried powders as such and with addition of two parts trehalose.

2.6. Influence of spray-drying process on chain length of *L. rhamnosus* AMB-GG

Lactobacilli appear often in chains of varying length. The spray-drying process was found to affect the chain length of *L. rhamnosus* AMB-GG. Spray-dried powder as such and with addition of trehalose was evaluated for their viability using the commercial available LIVE/DEAD BacLight Viability Kit (Molecular Probes, Paisley, UK). First a solution in a phosphate buffer (PBS) of the spray-dried samples is made, then they are centrifuged during 5 minutes at 4000g and washed twice with 0,85% (m/v) NaCl to remove rests of the growth media, which could interfere with the measurement. Finally the coloring agents are added to the samples, which are then incubated for 15 minutes at room temperature. These samples can then be seen through a epifluorescence microscope. Cells colored red equals dead bacteria and cells colored green equals viable bacterial cells.

Step 3: Test viability bacteria

Broth and agar:

- deMan, Rogosa and Sharpe (MRS): Carl Roth

Other material:

- Centrifuge: Sigma 2-16 K, Fisher Bioblock Scientific
- Petri plates: 90x14.2 mm VWR
- Centrifuge Tubes: 50 mL VWR
- Incubator: Memmert INB200

Spray-dried powder is stored at a certain temperature depending on the test. 10 mL MRS or PBS is added to the samples. The samples are incubated for 5 min, 30 min or 3h at 37°C, to resuscitate the bacteria. After resuscitation, the culture is mixed using the vortex to make it as homogeneous as possible. Then, dilutions are made by pipetting 100 µL of the culture in 900 µL PBS. Then, again 100 µL of this sample is transferred to 900 µL PBS after pipetting up and down for at least 10 times to make the culture as homogeneous as possible. After the dilutions are made, 100 µL of the preferred dilution is inoculated on an MRS-plates in duplo or triplo. The bacteria are spread on the plate using glass beads. The plates are incubated at 37°C for 72h and the colonies are counted for each plate. Then, the CFU (colony forming units) and the survival are calculated.

Step 4: Test recommended resuscitation time

Spray-dried powder is stored at 4°C. 10 mL 0,85% NaCl is added to the samples. The samples are incubated for 5, 15 and 30 minutes at room temperature, to resuscitate the bacteria. After resuscitation, different dilutions of the suspension are plated as explained in step 3. After 3 nights incubation at 37°C, the CFU is calculated and compared to different resuscitation times.

Step 5: Test water activity of the spray-dried powder

The water activity is defined as the volume of free, unbound water in the product that is available for metabolic activities of the bacteria. Waste metabolites from these activities may lead to spoilage of the product and thus shorter shelf-life. Water activity measurements were carried out with a water-activity meter (Lab-Swift-aw, Novasina). Approximately 2g of the spray-dried powder is placed in a measurement chamber and the mode is set to S (slow), which allows a more accurate water activity measurement.

Step 6: Test stability of the spray-dried powder

The spray-dried powder- is stored at 4°C and at room temperature. After certain periods of time, the viability of the powder is measured as explained in step 3. After 3 nights of incubation at 37°C, the CFU is calculated and compared to different periods of storage.

RESULTS

1.Spray- drying experiments

1.1. Effect of different saccharides on the viability of *L. rhamnosus* AMB-GG after spray-drying

As shown in figure 4, even at ratios of 1:1, all of the tested saccharides were found to protect the viability of the bacteria during spray-drying. Addition of glucose, lactose or trehalose enhances the viability of the bacteria after the spray-drying process approximately 200 times.

1.2. Influence of concentration of different saccharides on the viability of *L. rhamnosus* AMB-GG

Addition of saccharides results in higher viability of *L. rhamnosus* AMB-GG after spray-drying. From the tested saccharides lactose and trehalose show overall the best viability results after

spray-drying. The higher the concentration of the saccharide tested, the better the viability after the spray-drying process, as can be seen in figure 5A-D. The 1:5 proportion of the saccharides added gives the highest viability results after spray-drying. When they are compared to each other, trehalose gives the best results, shown in figure 6A. In comparison to no added saccharide, the 1:5 proportion of trehalose increases the viability of *L. rhamnosus* AMB-GG 14 times. The 1:5 proportion of lactose and dextran enhances the viability 8 and 4 times respectively .

1.3. Influence of the addition time of the saccharide on the viability of *L. rhamnosus* AMB-GG

Three time periods were evaluated:

- 1) Figure 5A-D gives an overview of the results of addition of the saccharides to the bacterial (feed) suspensions just before spray-drying. As stated before addition of 5 parts trehalose to the bacterial suspension just before spray-drying gives the best viability results.
- 2) Figure 6B show the results of the addition of lactose and mannitol to the growth medium of *L. rhamnosus*. After an overnight incubation at 37°C a PBS-feed suspension was made, which was spray-dried. Addition of mannitol to the growth medium gives the highest survivability of the bacteria after spray-drying, compared to addition of lactose or no addition of a saccharide at all.
- 3) Figure 7 represents the cumulative effect of the addition of mannitol –the saccharide with the best result for the growth medium, and trehalose-the saccharide with the best result for the bacterial (feed) suspension just before spray-drying. The cumulative effect of both added saccharides enhances the viability in comparison to no addition of saccharides at all or to addition of mannitol to the growth medium. However addition of solely trehalose to the bacterial suspension just before spray-drying increases the viability of *L. rhamnosus* AMB-GG the most.

From these results it can be concluded that the addition of trehalose solely to the bacterial (feed) suspension just before the spray-drying process is the best option. Addition of one excipient is formulation-wise also a better choice.

1.4. Influence of the outlet temperature on the viability of *L. rhamnosus* AMB-GG

When the inlet temperature is varied from 120°C, over 140°C to 160°C, the outlet increases from temperature from 55°C, over 65°C to 75°C, The best viability results are these of the samples spray-dried at 120°C and 140°C. The higher the inlet temperature, and therefore the outlet temperature, the more loss in viability of *L. rhamnosus* AMB-GG. Addition of two parts trehalose enhances the viability, as shown in the table 7 below:

Table 7: Influence of the outlet temperature on the viability of *L. rhamnosus*, in the presence or absence of two parts trehalose

Inlet temperature (°C)	As such	1:2 proportion trehalose:
	(10 ⁸ CFU/ml)	(10 ⁸ CFU/ml)
120	1,43 ± 0,25	6,60 ± 3,52
140	2,53 ± 0,25	8,54 ± 2,70
160	0,18 ± 0,06	2,42 ± 0,68

1.5. Influence of tablet compression and compaction force on viability of *L. rhamnosus* AMB-GG

Figure 8A-C show that there is no difference in tablet characteristics concerning thickness, hardness and disintegration time. Formulations of tablets with addition of two parts trehalose showed the same characteristics as these without addition of trehalose. Therefore it can be concluded that trehalose had no interference with the tablet characteristics. Figure 9A-B represents the samples without addition of trehalose and those with addition of 2 parts trehalose regarding their viability after tablet compression. The samples without trehalose showed no or retarded growth (grey lines on graph). Samples spray-dried with addition of 2 parts trehalose still showed good viability (black lines on graph).

It can be concluded that trehalose protects *L. rhamnosus* AMB-GG from the pressure during the tablet compression experiments.

The influence of the compaction force on the viability of *L. rhamnosus* AMB-GG was evaluated using three different compaction forces on the spray-dried powder as such (table 8) and with addition of two parts trehalose (table 9). The higher the compaction force the more loss in viability of the bacteria. Addition of trehalose enhances the viability, even after a compaction force of more than 107,87 MPa. The time needed to have a turbidity of 0,200 (the threshold value to get in the logarithmic phase) reduces when 2 parts of trehalose is added.

Table 8: Compaction force: spray-dried powder as such

Compaction force	time (min) to 0,200(OD)	CFU/ml before	CFU/ml after	Viability (%)	Hardness (N)
68,64 MPa	1940	3,10E+07	8,18E+05	2,637	36
107,87 MPa	1840	3,10E+07	1,29E+05	0,416	38
>107,87 MPa	∞	6,27E+07	1,41E+03	0,002	101

Table 9: Compaction force: spray-dried powder with addition of two parts trehalose

Compaction force	Time (min) to 0,200 (OD)	CFU/ml before	CFU/ml after	Viability (%)	Hardness (N)
68,64 MPa	1180	1,23E+09	4,31E+08	34,972	55
107,87 MPa	1100	1,23E+09	7,66E+08	62,147	53
>107,87 MPa	1260	4,91E+08	3,47E+07	7,064	126

1.6. Influence of spray-drying process on chain length of *L. rhamnosus* AMB-GG

The control sample (bacterial suspension before spray-drying) shows more living, bacterial cells than the spray-dried samples (data not shown). The chain length of the control sample is also clearly longer than these of the spray-dried ones, with an average of 10 cells per chain to an average of 3-4 cell per chain for the spray-dried samples. The spray-dried samples with addition of trehalose shows more viable cells, than the as such spray-died sample, which is in conclusion with previous findings (data not shown).

2. Resuscitation protocol

The aim of the further experiments was to test the optimal resuscitation parameters. Without the intention of being complete, a number of resuscitation times and resuspension solutions have been tested.

Batches of *L. rhamnosus* GG (ATTC 53101) were prepared as for the first test. 5 min, 30 min and 3h resuscitation time were tested. MRS broth was used as resuscitation medium, and showed for 3h resuscitation time a 38.6% better recovery of CFUs compared to 30 min.

To check whether the powder can also be resuscitated in a PBS-solution at room temperature, the foregoing experiment has been repeated but now also including a batch spray-dried in the presence of glucose (GLC). Samples were resuspended in MRS broth at 37°C and PBS at room temperature. Almost no difference between the different samples has been observed (**fig. 10**). Bacteria can be resuscitated in PBS buffer at RT, which is an important finding for in situ use of formulations for patients.

3. Water activity of the spray-dried powders

The water activity of the 1:5 lactose and 1:5 trehalose added to the bacterial suspension before spray-drying were compared to the water activity of the spray-dried powder as such. All the water activities are below 0,25, which is recommended for long-term storage of probiotics (**figure 11**).

4. Stability of the spray-dried powder

4.1. Short term storage

Spray-dried powders of *L. rhamnosus* AMB-GG as such and with addition of lactose and trehalose, were stored at 4°C for 40 days. Results are shown in figure 12. Addition of trehalose gives the best results, the less viability loss, after storage for 40 days at 4°C. Between 9% and 20% of the viability of *L. rhamnosus* AMB-GG was lost after short term storage with addition of trehalose. In comparison with addition of lactose there was a viability loss after 40 days between 50% and 81%. The samples as such showed a viability loss of 75% to 91% after 40 days of storage at 4°C.

4.2. Long-term storage

L. rhamnosus AMB-GG was stored as such and with addition of 2 parts trehalose at two storage conditions, 4°C and room temperature. After 1 year the viability of the stored samples was calculated, using the plate-count method as explained before. The samples, as such as well as 1:2 trehalose, stored at room temperature for 1 year showed a viability less than 10^7 CFU/100mg. The samples stored at 4°C had a viability of more than 10^8 CFU/100mg. The sample as such stored at 4°C showed a 1 log reduction, the sample stored at 4°C with addition of 2 parts trehalose showed no viability loss after 1 year of storage, as can be seen in **figure 13**.

5. Maintenance of the anti-pathogenic activity

In a further experiment we tested an eventual impact of the saccharide protectants on the antimicrobial activity. In this experiment also spray-dried bacteria from batch 18I13 exp 01 were tested. The bacteria were resuscitated for 0,5 h at 37°C and streak inoculated on a plate.

Agar plate used: Brain Heart Infusion Agar

Conditions: incubation at 37°C, aerobic

Positive control: 0.1% hexetidine

Negative control: no bacteria

With reference to **figure 14** and **table 10**, spray-dried bacteria do not lose their antipathogenic activity. The inhibition zones against *Moraxella* observed for *L. rhamnosus* AMB-GG spray-dried as such are comparable with the positive control. *L. rhamnosus* AMB-GG spray-dried after adding trehalose shows even higher inhibition zones compared to the positive control. Even bacteria that were spray-dried with 7x trehalose 1,5 years ago and stored at room temperature still showed antipathogenic activity against *M. catarrhalis*, indicating that these spray-dried bacteria can be stored for a long time without loss of activity, which is important for their application in nasal sprays or other devices for local, topical nasopharyngeal application against the nasopharyngeal pathogens.

Table 10: Antipathogenic activity of spray-dried lactobacilli and the effect of trehalose against various pathogens based on the spot antipathogenic activity.

Strain		Storage time	Storage cond.	CT	HI	MC	SA	MRSA	MSSA	SPN	SPY
Lactob. rham. AMB-GG	As such	1,5 year	RT	-	-	-	-	-	-	-	-
		1 weekend	4 we	-	+	++				-	
	1/7 trehalose	1,5 year	RT	-	-	+	-	-	-	-	-
	½ trehalose	1 weekend	4 we	-	+	++	+		+	-	
	½+weekend	1 weekend	4 we	-	-	++	+	+	+	-	+
Lactob. rham. AMB-GR-1	As such	1 weekend	4 we	-	+	++	-	-	-	-	-
	½+trehalose	1 weekend	4 we	-	-	++	-	-	-	-	+
	½+weekend	1 weekend	4 we	-	+	++	-	-	-	-	+

- 5 MC: *Moraxella catarrhalis*, SA: *Staphylococcus aureus*, MRSA: methicillin resistant *Staphylococcus aureus*, MSSA: methicillin sensitive *Staphylococcus aureus*, CT: *Corynebacterium tuberculo*stearicum, SPN: *Streptococcus pneumoniae*, SPY: *Streptococcus pyogenes*.

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CLAIMS

1. An oronasopharyngeal composition comprising viable, spray-dried *Lactobacillus* species, for use in the prevention and/or treatment of infections of the oronasopharyngeal cavity, by administration of said composition in said cavity.

2. The oronasopharyngeal composition for use according to claim 1, wherein said composition is selected from the list comprising nasal sprays, buccal tablets, buccal sprays, aerosols, throat lozenges and ear drops.

3. The composition for use according to anyone of claims 1 or 2, wherein said *Lactobacillus* species is *Lactobacillus rhamnosus*.

4. The composition for use according to claim 3, wherein said *Lactobacillus rhamnosus* is selected from *Lactobacillus rhamnosus* GG, *Lactobacillus rhamnosus* GR-1 or a combination thereof.

5. The composition for use according to anyone of claims 3 or 4, wherein said *Lactobacillus rhamnosus* species are combined with other viable spray-dried probiotic bacteria; in particular with *Lactobacillus spp* selected from the list comprising *Lactobacillus casei*, *Lactobacillus plantarum*, *Lactobacillus reuteri*, *Lactobacillus salivarius*, *Lactobacillus helveticus*, *Lactobacillus parabuchneri*, *Lactobacillus pentosus*, *Lactobacillus brevis*, *Lactobacillus sakei*, *Lactobacillus diolivorans*, *Lactobacillus buchneri*, *Lactobacillus gasseri*, and *Lactobacillus bulgaricus*.

6. The composition for use according to anyone of claims 1 to 5, wherein the infections of the oronasopharyngeal cavity are caused by one or more of the pathogens selected from *Moraxella catarrhalis*, *Haemophilus influenzae*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Corynebacterium accolens* and *Corynebacterium tuberculostearicum*.

7. The composition for use according to anyone of claims 1 to 6, wherein the infections of the oronasopharyngeal cavity are upper respiratory tract infections selected from the group consisting of acute otitis media, pharyngitis, chronic sinusitis, acute sinusitis, rhinitis, oral mucositis and the like.

8. The composition for use according to anyone of claims 1 to 7, wherein said composition further comprises mono- and/or disaccharides selected from the list comprising glucose, mannose, mannitol, dextran, lactose and trehalose; in particular trehalose.

9. The composition for use according to anyone of claims 1 to 8, wherein the oronasopharyngeal composition comprises at least 1.10^5 CFU of *Lactobacillus* species; in particular from about and between 1.10^6 to 1.10^9 CFU of said *Lactobacillus* species or *Lactobacillus* species combinations.

5

10. A method for the prevention and/or treatment of infections of the oronasopharyngeal cavity, said method comprising administering a composition as defined in any one of claims 1 to 8, into the oronasopharyngeal cavity of a subject in need thereof.

10 11. Use of viable, spray-dried *Lactobacillus* species in the manufacture of an oronasopharyngeal formulation for administration in the oronasopharyngeal cavity.

12. Use according to claim 11, wherein said spray-dried *Lactobacillus* species are combined with mono- and/or disaccharides, selected from the list comprising glucose, mannose,
15 mannitol, dextran, lactose and trehalose; in particular trehalose.

13. Use according to anyone of claims 11 or 12, wherein said *Lactobacillus* species is *Lactobacillus rhamnosus*.

20 14. Use according to claim 13, wherein said *Lactobacillus rhamnosus*, is selected from *Lactobacillus rhamnosus* GG, *Lactobacillus rhamnosus* GR-1 or a combination thereof.

15. Use according to anyone of claims 13 or 14, wherein the composition further comprises other viable spray-dried probiotic bacteria; in particular *Lactobacillus spp* selected from the list
25 comprising *Lactobacillus casei*, *Lactobacillus plantarum*, *Lactobacillus reuteri*, *Lactobacillus salivarius*, *Lactobacillus helveticus*, *Lactobacillus parabuchneri*, *Lactobacillus pentosus*, *Lactobacillus brevis*, *Lactobacillus sakei*, *Lactobacillus diolivorans*, *Lactobacillus buchneri*, *Lactobacillus gasseri*, and *Lactobacillus bulgaricus*.

30

Fig. 1

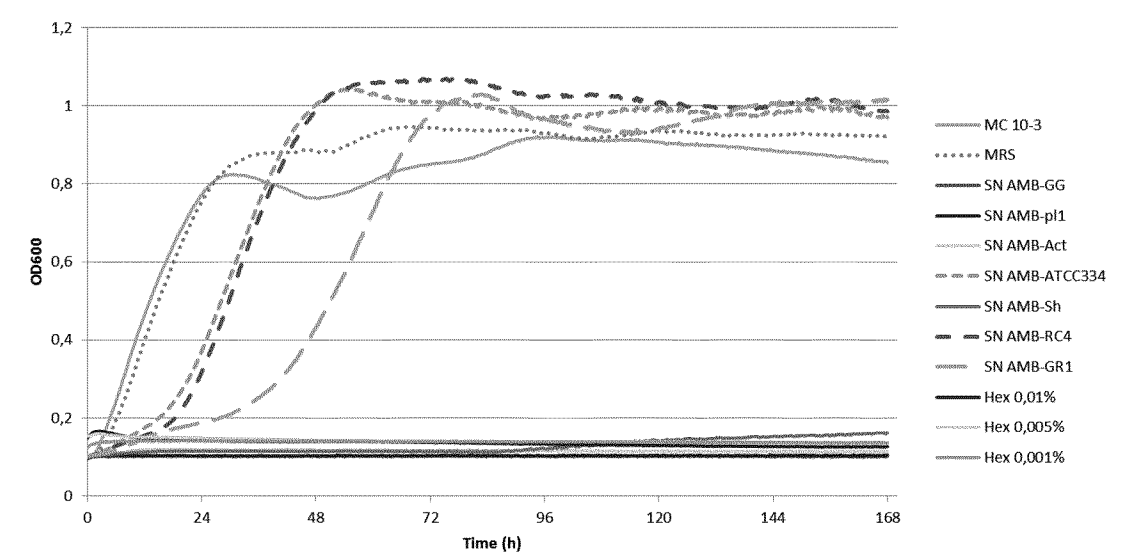


Fig. 2

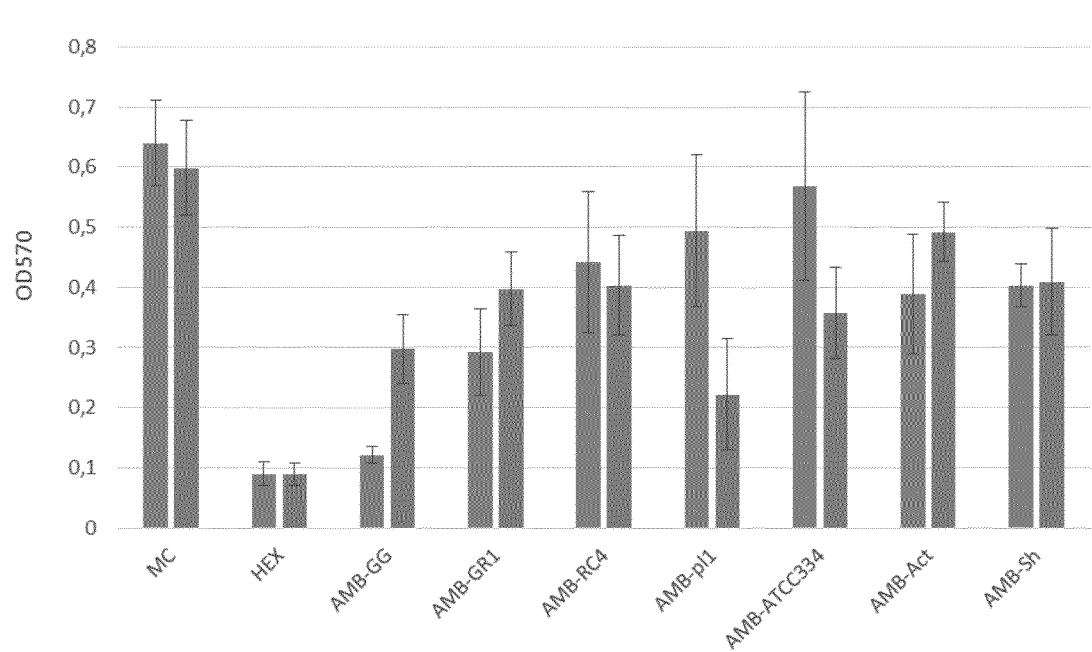
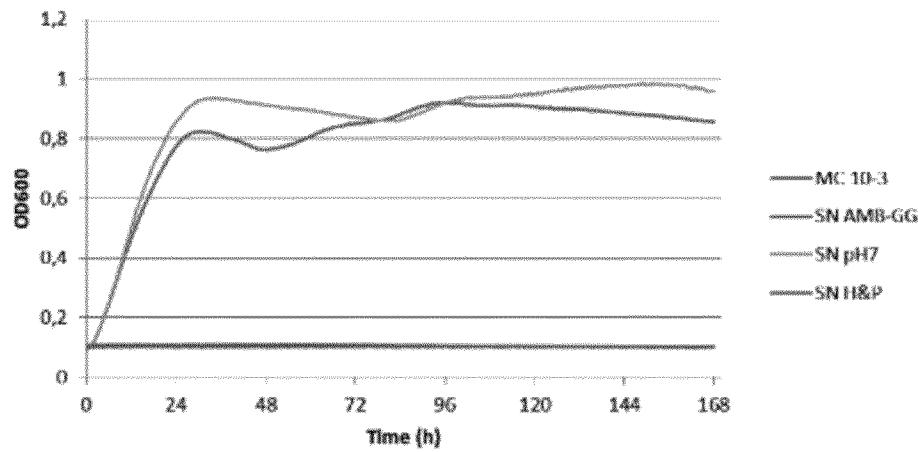


Fig. 3

A



B

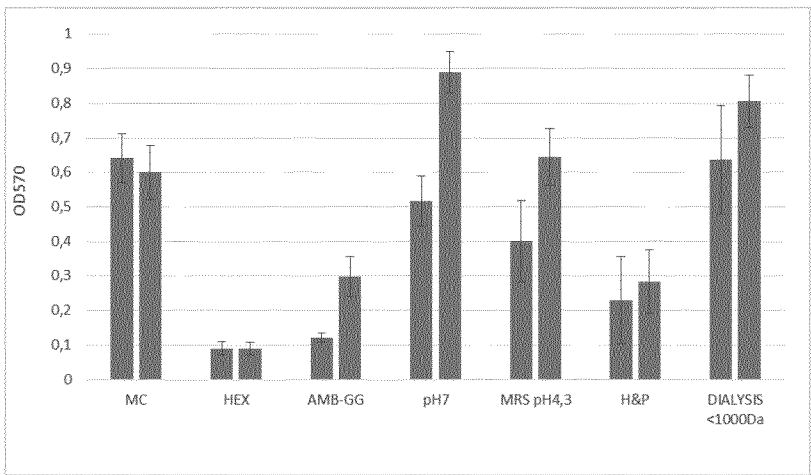


Fig. 4

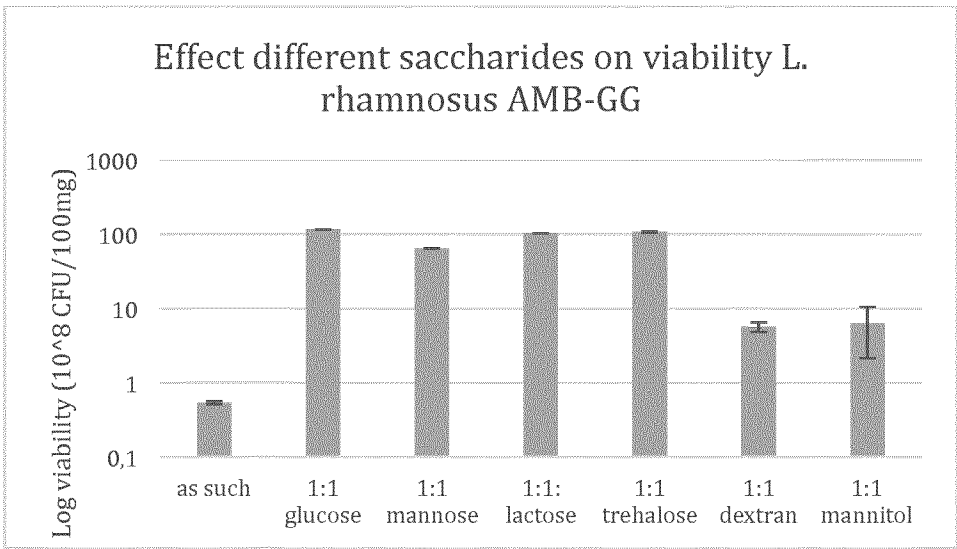
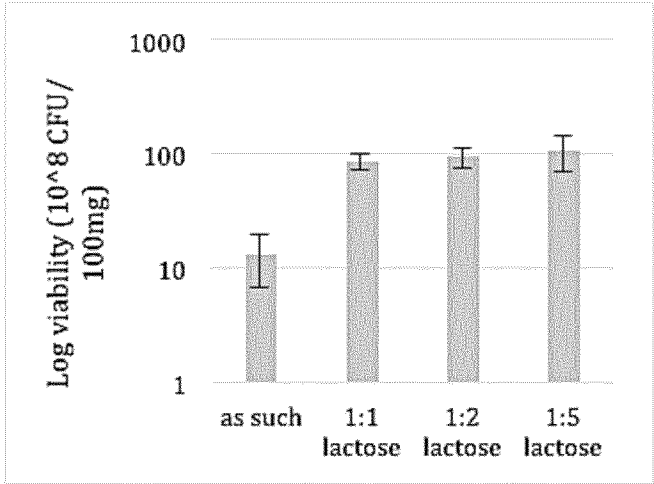
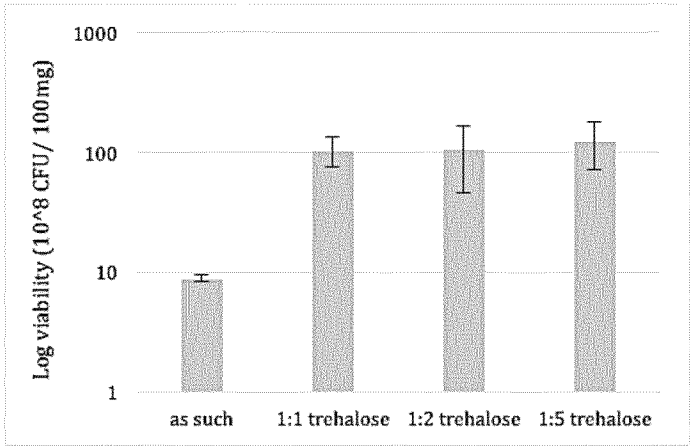


Fig. 5

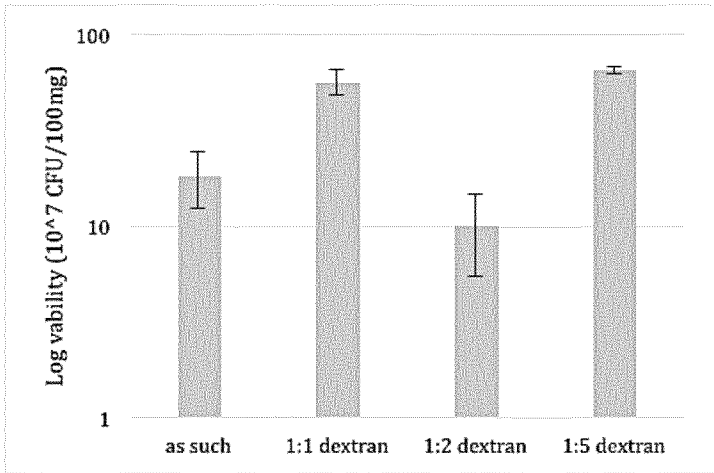
A



B



C



D

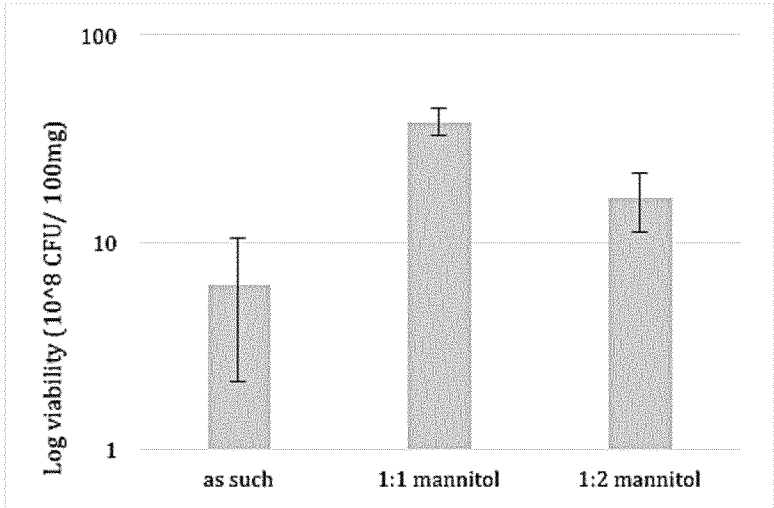
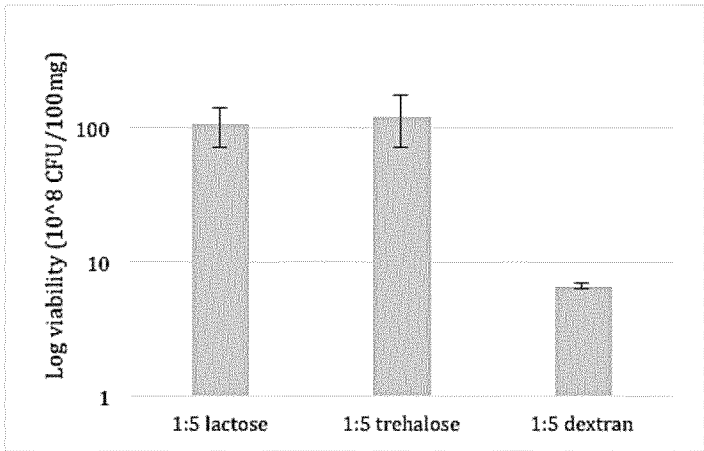


Fig. 6

A



B

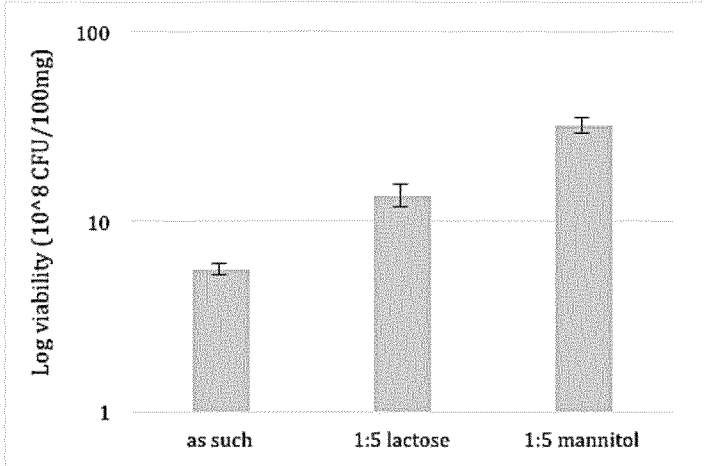


Fig. 7

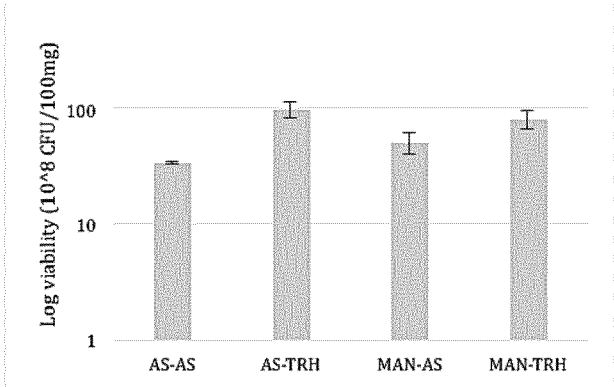
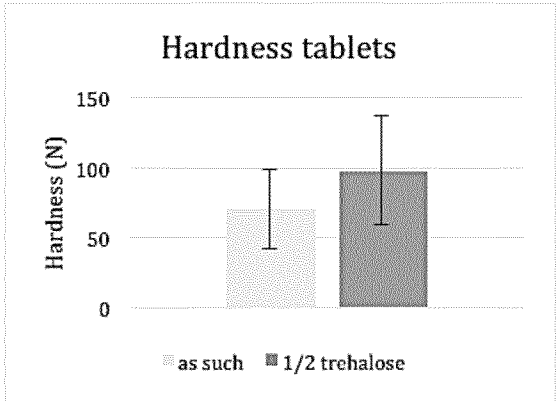
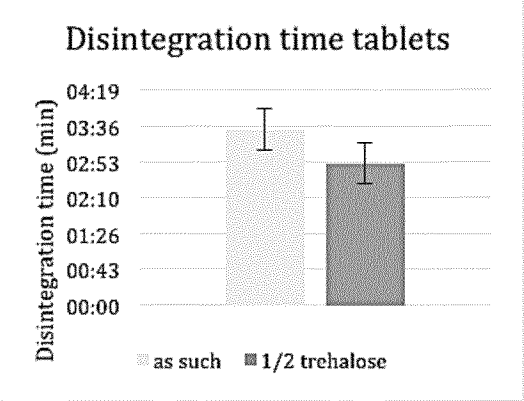


Fig. 8

A



B



C

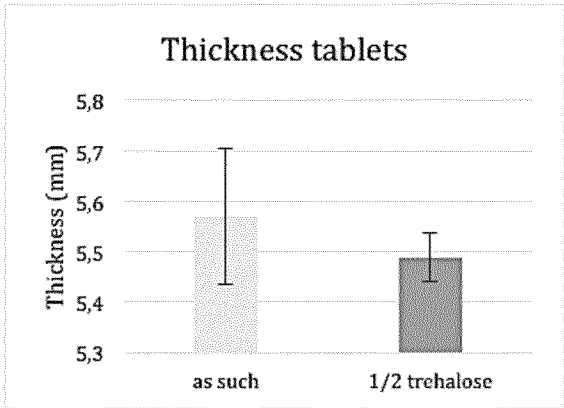
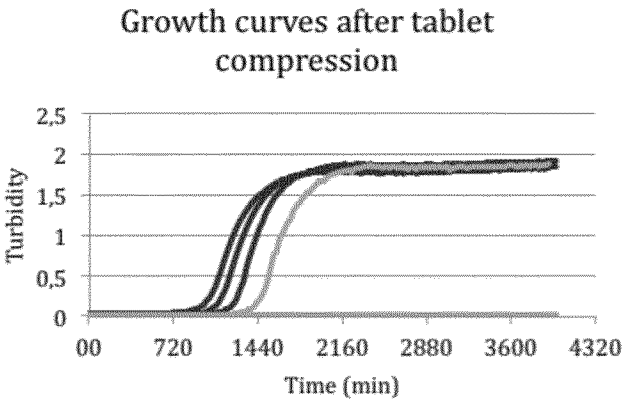


Fig. 9

A



B

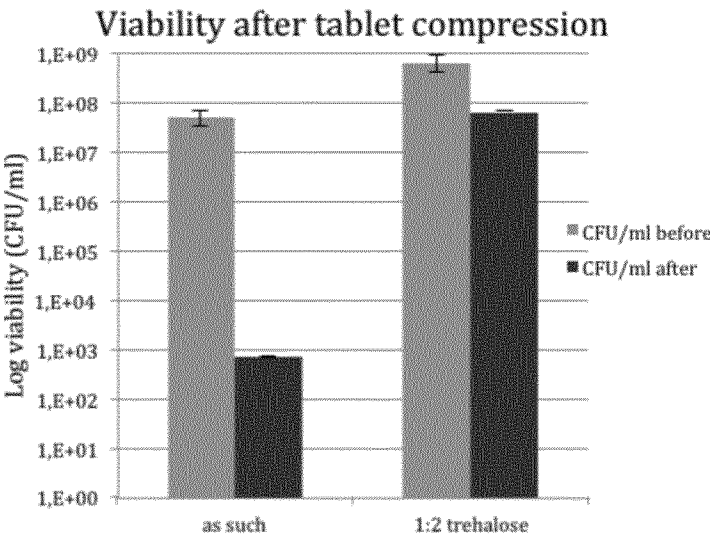


Fig. 10

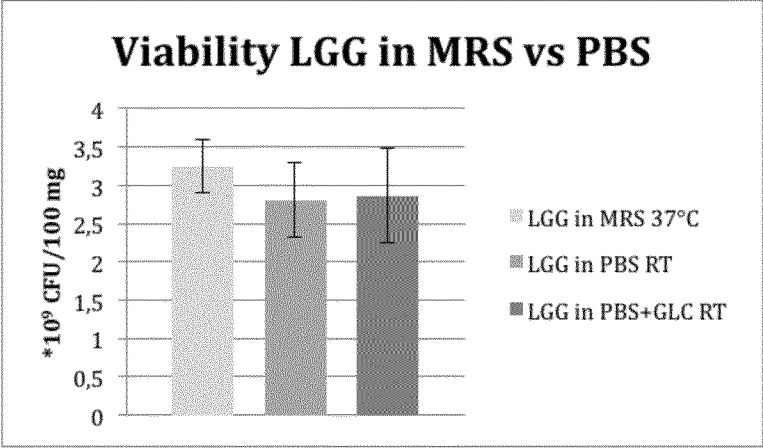


Fig. 11

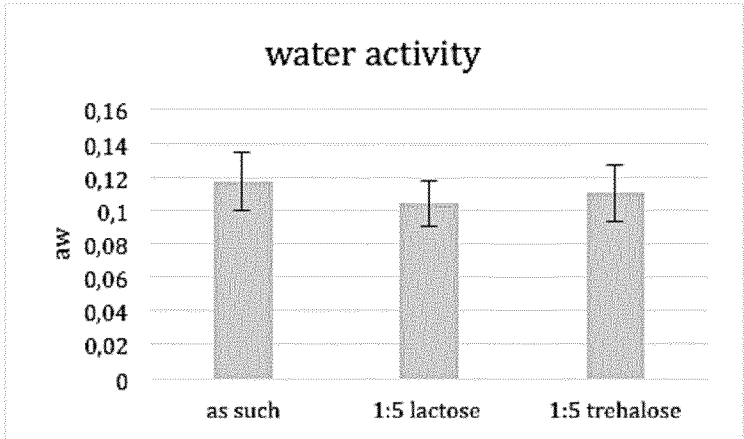


Fig. 12

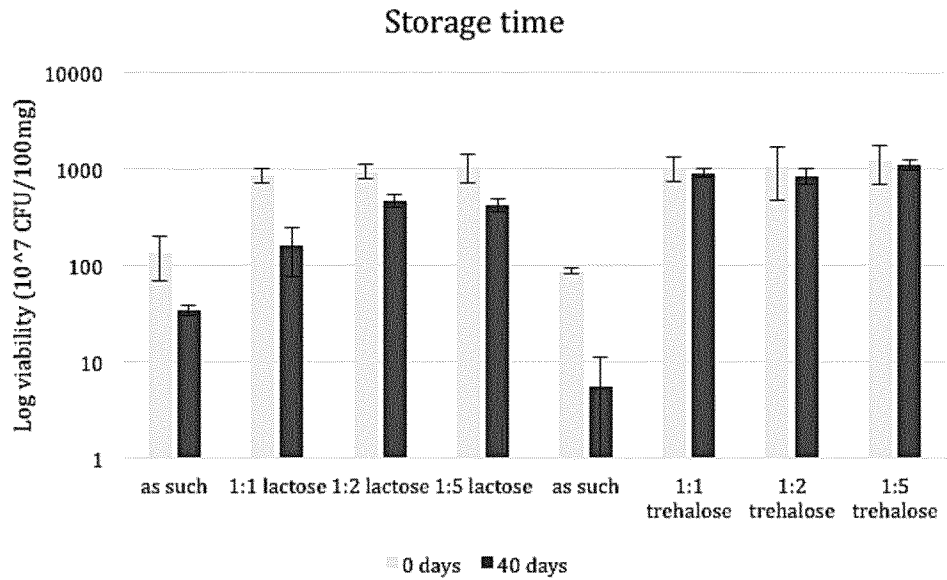


Fig. 13

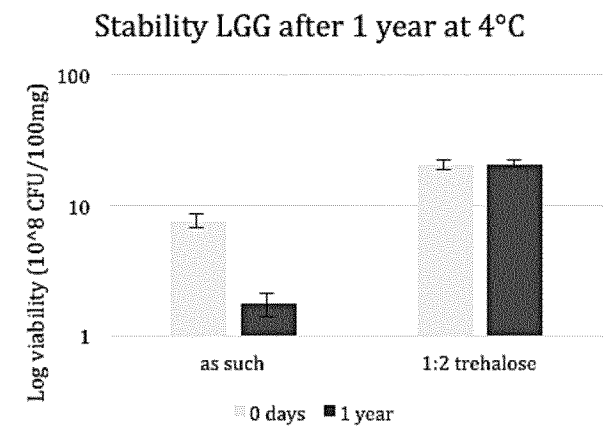
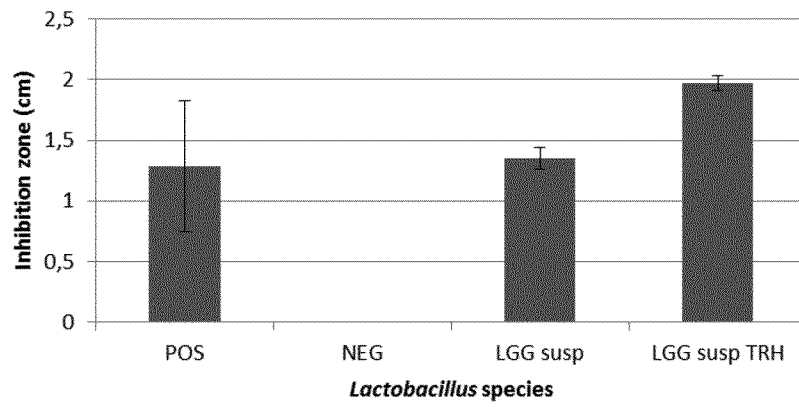


Fig. 14



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2015/055930

A. CLASSIFICATION OF SUBJECT MATTER

INV. A61K9/00 A61K35/74 A61K31/7016 A61P11/00
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)

A61K

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, CHEM ABS Data, COMPENDEX, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2011/256179 A1 (ROOS KRISTIAN [SE] ET AL) 20 October 2011 (2011-10-20)	1-3, 5-13,15
Y	paragraph [0021] - paragraph [0024] paragraph [0028] paragraph [0050] - paragraph [0052] paragraph [0055] - paragraph [0057] paragraph [0072] claims	1-15
X	US 2011/020304 A1 (SPRENGER NORBERT [CH]) 27 January 2011 (2011-01-27)	1-3, 5-13,15
Y	paragraph [0001] paragraph [0014] - paragraph [0015] paragraph [0023] - paragraph [0025] paragraph [0039] - paragraph [0040] paragraph [0043] example 1 claims	1-15
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Further documents are listed in the continuation of Box C.



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"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

21 July 2015

Date of mailing of the international search report

29/07/2015

Name and mailing address of the ISA/

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Authorized officer

Fey-Lamprecht, F

INTERNATIONAL SEARCH REPORT

International application No

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	G. HARATA ET AL: "Intranasal administration of Lactobacillus rhamnosus GG protects mice from H1N1 influenza virus infection by regulating respiratory immune responses", LETTERS IN APPLIED MICROBIOLOGY, vol. 50, no. 6, 29 June 2010 (2010-06-29), pages 597-602, XP055142244, ISSN: 0266-8254, DOI: 10.1111/j.1472-765X.2010.02844.x	1-4,6,7, 9-11,13, 14
Y	the whole document	1-15
X	US 2014/065218 A1 (LANG CHRISTINE [DE] ET AL) 6 March 2014 (2014-03-06) paragraph [0002] - paragraph [0003] paragraph [0026] - paragraph [0035] paragraph [0047] paragraph [0085] paragraph [0101] paragraph [0106] paragraph [0111] claims	1-15
X	US 2012/128644 A1 (HILLMAN JEFFREY D [US]) 24 May 2012 (2012-05-24) paragraph [0011] - paragraph [0023] paragraph [0035] - paragraph [0038] paragraph [0047] paragraph [0063] claims	1-15
X	US 2004/241149 A1 (DE SIMONE CLAUDIO [IT]) 2 December 2004 (2004-12-02) claims	1-15
Y	WO 2006/007526 A1 (SQUIBB BRISTOL MYERS CO [US]; ISOLAURI ERIKA [FI]; SALMINEN SEPPO [FI]) 19 January 2006 (2006-01-19) paragraph [0022] - paragraph [0026] claims	1-15
Y	US 2012/201798 A1 (KEKKONEN RIINA [FI] ET AL) 9 August 2012 (2012-08-09) paragraph [0010] - paragraph [0029] paragraph [0042] paragraph [0044] paragraph [0053] paragraph [0058] claims	1-15
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International application No
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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	HOJSAK I ET AL: "Lactobacillus GG in the prevention of gastrointestinal and respiratory tract infections in children who attend day care centers: A randomized, double-blind, placebo-controlled trial", CLINICAL NUTRITION, CHURCHILL LIVINGSTONE, LONDON, GB, vol. 29, no. 3, 1 June 2010 (2010-06-01), pages 312-316, XP027069486, ISSN: 0261-5614, DOI: 10.1016/J.CLNU.2009.09.008 [retrieved on 2009-11-05] the whole document -----	1-15
Y	WO 2013/155370 A1 (UNIV CALIFORNIA [US]) 17 October 2013 (2013-10-17) paragraph [0026] - paragraph [0027] paragraph [0071] claims -----	1-15
Y	HORI T ET AL: "EFFECT OF INTRANASAL ADMINISTRATION OF LACTOBACILLUS CASEI SHIROTA ON INFLUENZA VIRUS INFECTION OF UPPER RESPIRATORY TRACT IN MICE", CLINICAL AND DIAGNOSTIC LABORATORY IMMUNOLOGY, AMERICAN SOCIETY FOR MICROBIOLOGY, US, vol. 8, no. 3, 1 May 2001 (2001-05-01), pages 593-597, XP001016238, ISSN: 1071-412X, DOI: 10.1128/CDLI.8.3.593-597.2001 abstract page 593, right-hand column, paragraph 12 - line 33 -----	1-15

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

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International application No

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