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(54) **Title:** USE OF STREPTOCOCCUS SALIVARIUS IN THE TREATMENT OF CHRONIC INFECTIONS OF THE RESPIRATORY TRACT

(57) **Abstract:** The present invention provides a new microbial strain of the species *Streptococcus salivarius* for use in the treatment of inflammatory processes with or without infectious etiology. A further object of the present invention compositions comprising said strain and uses thereof.

USE OF *STREPTOCOCCUS SALIVARIUS* IN THE TREATMENT OF
CHRONIC INFECTIONS OF THE RESPIRATORY TRACT

DESCRIPTION

The present invention provides a new microbial strain of the species *Streptococcus salivarius* for use in the treatment of inflammatory processes with or without infectious etiology. A further object of the present invention compositions comprising said strain and uses thereof.

STATE OF THE ART

Many of the ENT (Ear, Nose and Throat) diseases may originate from a fungal or bacterial infection in the upper tracts of the respiratory system; examples of such infections are some forms of otitis, sinusitis and/or nasal poliposis: usually the treatment of such forms is performed by using topical or oral antibiotics or anti-inflammatory agents.

Recently clinical studies have demonstrated that the administration of streptococci such as *Streptococcus mitis*, *Streptococcus sanguinis*, *Streptococcus oralis* in the form of spray to patients affected by Acute Otitis Media (AOM) interferes and/or inhibits the growth of pathogenic microorganisms responsible of the disease. However, this species of microorganisms have the serious disadvantage to be classified as potentially pathogenic species.

Recently Power et al. (Power et al. Eur J Clin Microbiol Infect Dis. 2008, 1261-3) have carried out preliminary studies on a group of children affected by AOM by administrating orally a pediatric composition

comprising the strain *S. salivarius* K12. This strain has been previously used as a probiotic for oral hygiene and anti-halitosis.

The study carried out by Power and colleagues has revealed that only in a small percentage of the treated patients, the strain *S. salivarius* K12 has colonized the upper respiratory tract causing an improvement in symptoms of the disease being treated. The low capacity of the so far isolated strains of *S. salivarius* to colonize the upper respiratory tract makes less efficient their use in the adjuvant therapy against infections of the respiratory tracts.

So it was so strongly felt the need to isolate new non pathogenic strains that in addition to bactericide activity provide high capacity to colonize the respiratory tract.

SUMMARY OF THE INVENTION

The inventors have succeeded in isolating from the nasopharynx of a healthy voluntary, a new bacterial strain belonging to the species *S. salivarius* deposited at the Institute Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) under the filing number DSM 23307 in date 4 February 2010.

The inventors, by *in vitro* experiments, show that this specific strain of *Streptococcus salivarius* is characterized by:

- i) high inhibitory activity towards *S. pneumoniae*, stable in various culture conditions (BAC and TSYE);
- ii) inhibitory activity towards particularly virulent and antibiotics multi-resistant

- serotypes responsible of invasive infections such as strain *S. pneumoniae* 19A;
- iii) inhibitory activity towards *S. pyogenes* M-type 1;
 - 5 iv) high adhesion capacity to the cells HEp-2 (epithelial cells of human carcinoma of the larynx) up to 57%;
 - v) absence of virulence genes;
 - vi) complete sensitivity to antibiotics.

10 Adhesion capacity of this strain to cells HEp-2, together to the properties not belonging to a pathogenic or potentially pathogenic species and producing bacteriocins able to inhibit the growth of *S. pneumoniae* and *S. pyogenes*, makes the strain of

15 *Streptococcus salivarius* selected by the inventors and any other strain of *Streptococcus salivarius* with such features particularly suitable for treating bacterial and/or fungal infections of the upper respiratory tract. The utility of such organisms, that can be

20 administered by pharmaceutical compositions, lies in their ability to colonize the respiratory tracts competing pathogenic species. It is therefore clear that adhesion ability of the administered strains to the HEp-2 type cells plays a key role for the efficacy

25 of the same. The pattern of adhesion *in vitro* on cells derived from upper respiratory tract provides the adhesion and the retention of the bacteriocins producing strains.

Therefore, object of the present invention is a

30 bacterial strain belonging to the *Streptococcus salivarius* species characterized by the ability to

adhere to HEp-2 cells.

A further object of the invention is said bacterial strain as above defined and compositions comprising it for treating infections and/or
5 inflammations of the upper respiratory tract.

Compositions comprising said bacterial strain and one or more carriers and/or diluents and/or excipients are object of the invention as well.

The advantages, features and the use modes of the present invention are evident from the following
10 detailed description in some embodiments, presented as an example and without limitation.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a new bacterial
15 strain belonging to the species *Streptococcus salivarius* isolated by the inventors from the nosepharynx of a human voluntary subject; the strain has been identified by phenotypic and genotypic analysis.

20 The inventors have analyzed several nasal and pharyngeal swabs from which several bacterial species have been isolated, but in one case only it has been isolated and selected a strain with the desired characteristics. The strain has a typical morphology of
25 the *S. salivarius* species with a round shape of the colony and size of 1-2 mm in diameter, with entire and smooth margins. The bacterial strain can be grown on culture medium "Mitis salivarius" at 35°C, preferably in presence of 5% CO₂. The strain is able to adhere to
30 HEp-2 cells and to inhibit the growth of the pathogen *S. pneumoniae* by bacteriocins production.

The strain has been called *Streptococcus salivarius* 24 SMBc and submitted in date 4 February 2010 at the Institute Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) GmbH, 5 Braunschweig, Germany, under the filing number DSM 23307.

As already previously described the ability to adhere to HEP-2 cells makes this strain and even other strains belonging to the species *Streptococcus* 10 *salivarius* having such feature particularly suitable for treating infections and/or inflammations of the upper respiratory tract, preferably for treating infections causing diseases such as acute otitis media, recurrence otitis media, nasal polyposis, sinusitis.

15 In the present description are defined as upper respiratory tracts the nasal and paranasal cavities, the pharynx, the larynx.

Are also object of the present invention the composition comprising strains of *Streptococcus* 20 *salivarius* as above defined and one or more carriers, diluents and/or excipients.

Said compositions preferably comprise the bacterial strain *Streptococcus salivarius* filing number DSM 23307

25 Bacteria can be in suspension, freeze-dried or inactivated, provided they are not killed. The preparation of the compositions of the invention can then be implemented by freeze-drying of bacterial cultures, mixing freeze-dried both in suspension with 30 water or with further suitable excipients and optionally with addition of further active principles.

The amount of bacteria in said composition is preferably in the range between 10^3 and 10^{10} CFU for each gram of composition.

5 Examples of excipients that can be used in such compositions are: rubber, xanthan, carboxymethyl cellulose, silicone, Vaseline, white soft, magnesium stearate, maltodextrin, mannitol, starch, glucose, glycerine, propylene glycol, and similar.

10 Said compositions may include also carriers idoneous to improve the bioavailability, the stability and the endurance of the microorganism.

Said composition may comprise carriers to further improve the adhesion of the microorganism adhesion on the mucosal surface such as the EG56 polymer (Bis-15 Methoxy PEG-13 PEG-438/PPG-110 SMDI Copolymer), a heat-sensitive polymer able to increase the viscosity and thus the adhesiveness by increasing the temperature or Gantrex (PVM/MA Copolimer).

20 Said compositions may be in any form considered by the expert of the technical field suitable to be administered topically, orally, or through the respiratory tract.

For administration through the respiratory tract in the present description it has to be intended nasal 25 or by inhalation administration.

Examples of suitable pharmaceutical forms are cream, lotion, gel, ointment, solution, suspension, emulsion, capsule, tablet, powder, granules, sprays, drops.

30 Preferably compositions may be formulated to be administered through the respiratory tract in a nebulizer, with or without propellants.

Such compositions can be prepared according to techniques and protocols known to the expert of the technical field. Said composition may even contain anti-inflammatory agents such as 18-beta glycyrrhetic acid.
5

Object of the present invention are the compositions above described for treating infections of the upper respiratory tract, preferably for treating infections causing diseases such as acute otitis media, recurrence otitis media, nasal polyposis, sinusitis.
10

EXAMPLES

Collection of nasal and pharyngeal swabs from patients

Thirty one children aged between 10 and 12 years have been involved in this study. Children who had one, few or any AOM episode have been selected. Patients who received antibiotics in the previous two weeks, had an operation on the upper respiratory tract or with anatomic abnormalities of the respiratory tract have been excluded.
15
20

A nasal and pharyngeal swab has been collected respectively from the nostrils and the mouth of each patient with a cotton wool soaked in sterile calcium alginate.

25 Microbiological test

In order to highlight the presence of bacterial flora of nasal and pharyngeal swab samples collected as above described, all samples have been plated onto Mitis Salivarius agar (Difco), a selective medium for streptococci, and onto "chocolate agar" (Columbia Agar Base, OXOID) containing 5% horse blood in order to
30

determine bacterial microflora.

Cultures have been incubated for 18 hours at 37°C in presence of 5% CO₂ and atmospheric pressure. All strains have been frozen at -70°C in "Brain heart
5 infusion broth" (OXOID) with 20% glycerol.

BLIS (Bacteriocin-like inhibitory substance) test

Each colony morphologically distinct and isolated, obtained from the growth of bacteria as described above has been assayed for the ability to inhibit the most
10 representative strains causing otitis: *S. pyogenes* 2812A, *S. pneumoniae* 11ATN, *H. influenzae* 3ATF, *S. aureus*, *E. coli*, *P. aeruginosa*, *S. salivarius* ATCC13419, *M. catarrhalis*. The ability to inhibit pathogen strains has been assayed by the "BLIS test" as
15 originally developed by Walls et al. (Med microbial 52 (2003)). Assays have been performed by using two different media: Trypticase Soy Yeast Extract Calcium Agar (TSYCa) + 2% Yeast Extract and Blood agar + calcium carbonate (BACa). Results have shown that the
20 strain of *Streptococcus salivarius* identified by filing number DSM 23307 is able to inhibit the growth of *S. pneumoniae* both in TSYCa and BACa medium. Furthermore, it has been evaluated the ability of strain *S. salivarius* DSM 23307 to inhibit particularly virulent
25 and multi-resistant strains of *S. pneumoniae* 19A and *S. pyogenes* M-type 1.

Analysis of virulence genes

In *S. salivarius* DSM 23307 the presence of virulence genes particularly diffuse in streptococci
30 such as *sag* A, *smez-2* and *speB*, respectively responsible of the production of the toxin streptolisin

S, the mitogenic exotoxin and the eritrogenic exotoxin. The assays have been performed by PCR and hybridization with specific probes.

Results have shown the absence of such virulence
5 genes.

Adhesion test

To perform the test cells HEp-2 (ATCC CCL 23) have been cultured in essential minimal Eagle media (EMEM) (Invitrogen, Carlsbad, CA). The media was added with
10 10% bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 µg/ml). *Streptococcus salivarius* bacteria DSM23307 before being used in the adhesion assay have been grown for 16-18 hours in a 5 ml Todd Hewitt media. Bacteria density has been adjusted
15 according to spectrophotometer readings in order to have a range of density between 10^5 and 10^6 CFU/ml before the test. The adhesion test has been performed in HEp-2 cells as described in Benga L. et al.

Stability test

20 Stability tests have been performed by incubating the strain *Streptococcus salivarius* DSM 23307 for 18 hours at pH 8.0 in "Tryptic Soy" (TSB), Todd Hewitt and Brain Heart Infusion (BHI) media.

Results

25 Identification of isolated strain

In the table below are identified the species to which belong the strain isolated from the analyzed nasal and pharyngeal swabs:

Bacteriocins producer	Molecular identification
3A-TF(1)	<i>S. mitis</i>
3A-TF(3)	<i>S. salivarius</i>
8A-TF	<i>S. mitis</i>
11A-TF(2)	<i>S. mitis</i>
14A-TF(2)	<i>S. salivarius</i>
14A-TF(4)	<i>S. salivarius</i>
15A-TF	<i>L. cremoris</i>
19A-TF(1)	<i>S. sanguis</i>
21A-TF(3)	<i>S. mitis</i>
24A-TF(4)	<i>S. salivarius</i>
25A-TF	<i>L. cremoris</i>
25A-TN(2)	<i>S. salivarius</i>
26A-TF(1)	<i>S. mitis</i>
25A-TF(2)	<i>S. mitis</i>

Identification and characterization of strain *Streptococcus salivarius* DSM 23307

Streptococcus salivarius DSM 23307 has been
 5 isolated from the nosepharynx of a human subject. The strain grows on a "Mitis salivarius" medium at 35 °C, 5% CO₂, having the typical morphology of *S. salivarius* species.

Colony shape and size: round, 1-2 mm diameter.

10 Edge: continuous, smooth.

Colour: Blue.

Grown on Columbia agar with 5% horse blood at 37°C, 5% CO₂ the strain is not haemolytic and has the following morphology

15 Colony shape and size: round, 1-2 mm diameter.

Edge: continuous, smooth.

Colour: White.

Streptococcus salivarius DSM 23307 strain has been analyzed by the commercial kit for the identification
 20 of streptococci API 20 Strep. After 24 hours incubation, according to the manufacturer's

instruction, has resulted code 5070451, corresponding to the species *Streptococcus salivarius*.

Results obtained by API 20 Strep

- Acetoin production: positive
- 5 Hydrolisis: negative
- β -glucosidase: positive
- Pirrolinodil arilamidase: negative
- α -galactosidase: negative
- β -glucuronidase: positive
- 10 Alkaline phosphatase: positive
- Leucin arilamidase: positive
- Arginine dihydrolase: negative
- Ribose: negative
- L-arabinose: negative
- 15 Mannitol: negative
- Sorbitol: negative
- Lactose: positive
- Trealose: positive
- Inuline: negative
- 20 Raffinose: negative
- Glycogen: negative
- B-hemolysis: negative

16S and sodA gene sequence analysis have demonstrated that the identified strain belongs to the species *S. salivarius* (99.8% identity).

Activity of *S. salivarius* DSM 23307

Incubation	<i>S. pyogenes</i> 2812A M-type 1	<i>S. pyogenes</i> SF370 M-type 1	<i>S. pneumoniae</i> 11A-TN	<i>S. pneumoniae</i> 11A-TN	<i>H. influenzae</i> 3A-TF	<i>S. aureus</i>	<i>B. catarrhalis</i>
BACa	-	-	+	+	-	-	-
TSYE	+	+	+	+	-	-	-

Adhesion experiment

Adhesion assays have demonstrated that the *Streptococcus salivarius* DSM 23307 strain has an excellent ability to adhere to HEp-2 cells, up to 57%,
5 interfering with the adhesion of opportunistic bacteria and fungi.

Formulations

1. *Streptococcus salivarius* DSM 23307, saline.
- 10 2. *Streptococcus salivarius* DSM 23307, EG56 polymer, xanthan, carboxymethyl-cellulose, saline.
3. *Streptococcus salivarius* DSM2 3307, silicone, Vaseline, white soft, magnesium stearate.
- 15 4. *Streptococcus salivarius* DSM 23307, maltodextrin, mannitol, 18 beta-glycyrrhetic acid, starch.
5. *Streptococcus salivarius* DSM 23307, glucose, deionized water.
- 20 6. *Streptococcus salivarius* DSM 23307, propylene glycol and/or glycerine.

In conclusion, the present invention provides a new bacterial strain belonging to the species *Streptococcus salivarius* having biological features making it the one and different from other patented
25 strains indicated for the treatment of the above referred infections.

In particular, the strain *Streptococcus salivarius* DSM 23307 of the present invention inhibit even *S. pneumoniae* (the main pathogenic agent of AOM) in
30 different culturing conditions (BACa and TSYE) and *S. pyogenes* (TSYE).

This feature differentiates it from other described strains belonging to *S. salivarius* such as *S. salivarius* 30 which has inhibitory ability only towards *S. pyogenes* in the two BACa and TSYE media, expanding
5 its range of action only in assays carried out in TSYE.

Furthermore, surprisingly as demonstrated by BLIS tests results, the strain *Streptococcus salivarius* DSM 23307 of the present invention inhibits even the important pathogens such as *S. pneumoniae* 19A and *S.*
10 *pyogenes* M-type 1, which are frequently isolated from the upper respiratory tracts.

Finally, *S. salivarius* DSM 23307, shows some biological features, such as sensitivity to antibiotics, absence of virulence genes, and adhesive
15 ability up to 57%, which make it the one, well characterized and distinguishable from other *S. salivarius* strains, in particular *S. salivarius* 30.

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CLAIMS

1. An isolated bacterial strain belonging to the species *Streptococcus salivarius*, wherein said bacterial strain is characterized by

- 5 i) high inhibitory activity towards *S. pneumoniae*, stable in various culture conditions (BAC and TSYE);
- ii) inhibitory activity towards particularly virulent and antibiotics multi-resistant serotypes responsible of invasive infections such as strain
- 10 *S. pneumoniae* 19A;
- iii) inhibitory activity towards *S. pyogenes* M-type 1;
- iv) high adhesion capacity to the cells HEp-2 (epithelial cells of human carcinoma of the larynx) up to 57%;
- 15 v) absence of virulence genes;
- vi) complete sensitivity to antibiotics.

2. The bacterial strain according to claim 1, deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) GmbH, Braunschweig, Germany,

20 under the accession number DSM 23307.

3. The bacterial strain according to claim 1 or 2 as medicament.

4. The bacterial strain according to claim 3 for use in the treatment of upper respiratory tract

25 infections.

5. A composition comprising the bacterial strain according to any one of the claims 1-4 and one or more pharmaceutically acceptable excipients, aromatizing agents or carriers.

6. The composition according to claim 5, comprising said bacterial strain in a lyophilized form.

7. The composition according to claim 5 or 6, for use in the treatment of upper respiratory tract
5 infections.

8. The composition according to claim 7, wherein said respiratory tract infections are cause of a disease selected from the group comprised of Acute Otitis Media, recurrent Otitis Media, Nasal Polyposis.

9. The composition according to any one of the
10 claims 5-8, formulated for oral, topical administration, or for administration via the respiratory tract.

10. The composition according to any one of the
15 claims 5-8, in the form of cream, lotion, gel, ointment, solution, suspension, emulsion, capsule, tablet, powder, granulate, spray.

11. The composition according to claim 9, formulated for administration via the respiratory tract
20 in a nebulizer and further comprising a propellant.

12. The composition according to any one of the claims 5-11, comprising an amount of bacteria between 10^5 and 10^8 CFU per gram of composition.

INTERNATIONAL SEARCH REPORT

International application No PCT/IT2011/000104

A. CLASSIFICATION OF SUBJECT MATTER INV. A61K35/74 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 A61P				
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched				
Electronic data base consulted during the international search (name of data base and, where practical, search terms used) EPO-Internal, BIOSIS, EMBASE, WPI Data				
C. DOCUMENTS CONSIDERED TO BE RELEVANT				
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.		
X	WO 2004/072272 A1 (BLIS TECHNOLOGIES LTD [NZ]; TAGG JOHN ROBERT [NZ]; WALLS TONY MARTIN []) 26 August 2004 (2004-08-26) page 2, lines 7-9 page 5, paragraph 2-5 page 6, paragraph 1-4 claims 1-26 examples 1,2 <div style="text-align: center;">-----</div> <div style="text-align: center;">-/--</div>	1-12		
<table style="width: 100%; border: none;"> <tr> <td style="width: 50%; border: none;"><input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C.</td> <td style="width: 50%; border: none;"><input checked="" type="checkbox"/> See patent family annex.</td> </tr> </table>			<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C.	<input checked="" type="checkbox"/> See patent family annex.
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C.	<input checked="" type="checkbox"/> See patent family annex.			
* Special categories of cited documents :				
"A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. "&" document member of the same patent family			
Date of the actual completion of the international search	Date of mailing of the international search report			
26 July 2011	05/08/2011			
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Authorized officer Camilleri, Alain			

INTERNATIONAL SEARCH REPORT

International application No PCT/IT2011/000104

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>WALLS TONY ET AL: "Bacteriocin-like inhibitory substance (BLIS) production by the normal flora of the nasopharynx: potential to protect against otitis media?", JOURNAL OF MEDICAL MICROBIOLOGY SEP 2003 LNKD- PUBMED:12909662, vol. 52, no. Pt 9, September 2003 (2003-09), pages 829-833, XP002603368, ISSN: 0022-2615 abstract page 831, right-hand column, paragraph 2 - page 832, right-hand column, paragraph 1 -----</p>	1-12

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2004072272	A1	NONE	26-08-2004
