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ORIGINAL ARTICLE

Colonization, safety, and tolerability study of the *Streptococcus salivarius* 24SMBc nasal spray for its application in upper respiratory tract infections

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Abstract *Streptococcus salivarius*, a non-pathogenic species and the predominant colonizer of the oral microbiota, finds a wide application in the prevention of upper respiratory tract infections, also reducing the frequency of their main pathogens. In this pilot study, the primary objective was to evaluate the safety and tolerability of a nasal spray, *S. salivarius* 24SMBc, as a medical device in a clinical study involving 20 healthy adult subjects. The secondary aim was to determine the ability of colonization assessed by molecular fingerprinting. Twenty healthy adult subjects, aged between 30 and 54 years, without a medical history of recurrent otitis media, were enrolled. All patient characteristics fulfilled the inclusion criteria. All subjects were treated daily for 3 days with the nasal spray containing *S. salivarius* 24SMBc at a concentration of 5×10^9 colony-forming units (CFU)/ml. The persistence of *S. salivarius* in the nasopharynx was investigated by the antagonism test and random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR). The tolerability and safety were clinically assessed by clinical examinations during treatment. Our results demonstrate the capability of *S. salivarius* 24SMBc to colonize the rhinopharynx tissues in 95 % of subjects and persist in 55 % of them after 6 days from the last dose of the formulation, maintaining a concentration of 10^5 CFU/ml. The treatment was well tolerated by all healthy patients and no adverse effects were found. The topical application of streptococcal probiotics is a relatively undeveloped field but is becoming an attractive approach for both prevention and therapy, especially for pediatric age patients. *S. salivarius* 24SMBc possess characteristics making this strain suitable for use in bacteriotherapy.

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Introduction

In recent years, there has been increasing evidence indicating beneficial effects of probiotics in the prevention and treatment of many diseases, especially in the gastrointestinal tract, preserving intestinal epithelium by maintaining its microbiota and modulating immune response [1–4]. Until now, few studies have been addressed to the use of probiotic strains in upper respiratory tract infections (URTIs) and some studies suggested clinical advantages for the host after probiotic administration [5–7]. The strategy of using a bacterial species belonging to the healthy human oral microbiota as an oral probiotic for URTIs offers great benefits for the host, contributing to the recolonization process, re-establishing microbial balance, and reducing the level of potential pathogens. As regards to potential pathogens, *Streptococcus salivarius* species is considered the predominant "safe" colonizer, capable of fostering a more balanced, health-associated oral microbiota, interfering with potential pathogens; thanks to these characteristics, it is suitable for use as an oral probiotic [8]. Nasopharyngeal colonization plays an essential role in the pathogenesis of URTIs and, in particular, in recurrent acute otitis media (rAOM), acting as a reservoir for mainly respiratory pathogens, such as *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Moraxella catarrhalis* [9, 10]. Rebalance of the nasopharyngeal microbiota is a new strategy for the prevention of AOM based on the interaction and competition between potentially pathogenic and commensal bacteria. The

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alteration of the nasopharyngeal microbiota and the absence or the low concentration of α-hemolytic streptococci may correlate with the recurrence of acute episodes [11, 12].

The first clinical use of oral streptococcal probiotics concerning treatment of halitosis and/or *S. pyogenes* infections was reported by Tagg and co-workers, attributing this ability to the presence of *S. salivarius* K12, belonging to the normal commensal flora of the nasopharynx and producer of the *sal*A/B bacteriocin that is responsible for the inhibition of *S. pyogenes* species [13, 14].

Our group has already studied a strain of *S. salivarius*, 24SMBc (DSM23307), selected from a healthy child [15], for its probiotic characteristics and for its remarkable ability to interfere with URTI pathogens. In particular, this strain can be used as an excellent application in the prevention of rAOM in infants and children, having a strong inhibitory capacity versus *S. pneumoniae*, one of the main pathogens responsible for rAOM [15]. It is well known that rAOM is the most common infection in children, responsible for most antibiotic prescriptions in early childhood. Over 80 % of children experience at least one episode in the first three years of life and about a third has three or more episodes [16]. The treatment of AOM has a significant impact on child health, healthcare costs, and the development of antimicrobial resistance. In only 10–20 % of children can AOM result in recurrence and/or persistence with complications, such as impaired hearing, behavior disorders, and surgical interventions [17].

The main aim of this pilot study was to evaluate the safety, the human tolerability, and persistence of *S. salivarius* 24SMBc, used as a new nasal spray formulation.

Materials and methods

Study design

The objective of this pilot study (performed from 2011 to 2012) was to evaluate the safety and tolerability of *S. salivarius* 24SMBc nasal spray used as a medical device and its ability to colonize and persist in the human rhinopharynx of healthy patients. Twenty healthy adult volunteers, aged between 30 and 54 years, including male and female subjects (12 male and 8 females), without a medical history of recurrent otitis media, were enrolled after informed signed was obtained. The nasal spray formulation contains *S. salivarius* 24 SMBc at a concentration of 1×10^9 suspended in a water solution with dimethicone, without gas. The product was tested preliminarily for its stability at 25 °C and at 4 °C for 1 month, confirming the original concentration. All patients were treated with cefpodoxima (200 mg twice daily for 6 days) before the nasal spray administration, to reduce the level of other oral streptococci and to favor 24SMBc colonization. The nasal spray was administered four times per day at intervals of about 4 h (concentration per day 8×10^9) for 3 days, excluding the night, by two puffs in each nostril. The study protocol was approved by the ethical committee of L'Unità Operativa Complessa (UOC) di Otorinolaringoiatria—ASP 3 CT, P.O. Acireale, Italy. Because of the safety assessment of the study, our ethical committee suggested only an adult patient population. This study was conducted according to the principles of the Helsinki Declaration (protocol no. MED/SEC/2011/1; rev. 01del 05/07/2011).

Patient selection

The subjects enrolled in this study met the following inclusion criteria: aged between 30 and 54 years old of both sexes and healthy.

Exclusion criteria were: morphofunctional disorders of the nasal passages with intranasal airflow predisposing conditions such as to determine the genesis of inflammatory diseases; objective endoscopic mucosal atrophy and obvious deficit of mucociliary clearance; vasomotor hypertrophic inflammatory diseases, both type-specific and non-specific; metabolic diseases (diabetes), cystic fibrosis, asthma gastroesophageal reflux; a clinical history of recurrent inflammation and/or recurrent URTIs; rhinosinus inflammatory and/or acute oropharyngeal diseases; already using inhaled treatment and/or antibiotics in the 30 days prior to enrollment; treated with immunosuppressants; suffering from chronic renal failure; hypersensitive to cephalosporins; and patients who were pregnant and/or lactating.

Microbiologic analysis of samples

The microbiologic evaluation was performed by rhinopharyngeal (inferior nasal turbinates) swabs obtained after antibiotic treatment (T_0) and at 2 h, 4 h, 24 h, and after 6 days of nasal spray treatment, labeled as T_1, T_2, T_3, and T_4, respectively. Each rhinopharyngeal swab was plated directly onto Columbia Agar Base (Oxoid, Basingstoke, UK), plus 5 % horse blood to determine the total microflora, and onto Mitis Salivarius Agar (Difco Laboratories), a selective medium for α-hemolytic streptococci. Cultures were incubated overnight at 37 °C in 5 % CO_2 air atmosphere. In addition, all swabs were cultured to determine the presence of other pathogens according to standard laboratory procedures.

Test for antagonism activity and molecular fingerprinting

To evaluate the presence of *S. salivarius* 24SMBc, each morphologically distinct colony grown in Mitis Salivarius Agar was tested for bacteriocin-like inhibitory substance (BLIS) production using a deferred antagonism test [18]. The indicator strains were representative strains of URTIs including AOM pathogens [15]. The molecular fingerprinting of

S. salivarius 24 SMBc was performed on each BLIS-positive colony. The profile was performed by random amplified polymorphic DNA (RAPD) analysis using primers as described previously [19].

Clinical assessments

The clinical evaluation was performed by examination using a rhinolaryngoscope (Pentax FNL-10RP3) through both nasal cavities and by rhinomanometry (Rhinospir PRO, Sibel, Barcelona, Spain). The endoscopic examination (Fig. 1: patient 13, endoscopic evaluation post-treatment) by this method was evaluated as normal by the examination of trophism and the color of the mucous membranes of the whole rhinopharyngolaryngeal tract, as well as the presence and characteristics (hyper or normal morphology) of any secretions (serum or purulent) throughout the upper airway [20, 21].

Safety determination of the preparation was assessed on the basis of objective assessment and on the analysis of the following clinical parameters: body temperature (measured in the morning); blood sample for testing of inflammatory markers [white blood cell count, platelet count, VES, polymerase chain reaction (PCR)]; intensity of any symptoms (runny nose, sneezing, cough) as subjectively perceived according to the Jackson score (0–3); recording of headache, myalgia, and earache.

Results

Safety and tolerability of *S. salivarius* 24SMBc nasal spray formulation

All subjects reported no symptoms associated with the nasal spray administration (runny nose, sneezing, coughing, headache). Side effects and/or undesirable effects were not observed at all. The body temperature (measured in the morning) showed no clinically significant changes, and inflammatory markers did not change after treatment with inhalation of the product in any of the volunteers enrolled in the study. Physical examination of the upper airway showed no signs of infectious diseases throughout endoscopic surveillance.

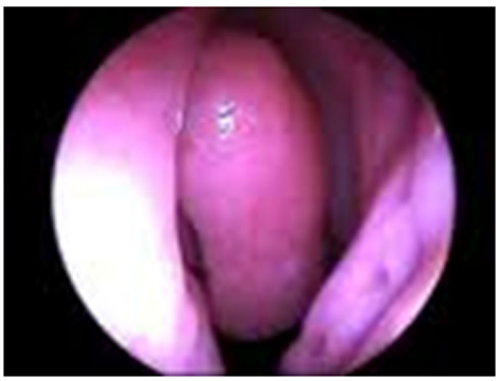

Fig. 1 Endoscopic evaluation post-treatment of patient 13 performed using a rhinolaryngoscope (Pentax FNL-10RP3) through both nasal cavities

Microbiological evaluation and colonization

A total of 100 samples (T_0, T_1, T_2, T_3, T_4) from the 20 subjects were analyzed. All samples were negative at T_0 (i.e., after antibiotic treatment), with the exception of 001 and 009, which were colonized with *S. aureus* (about 50 CFU/ml). The averages of the total microflora population determined on Columbia blood agar after 2 h, 4 h, 24 h, and 6 days from nasal spray administration were approximately from 20 to $>10^6$ CFU/ml for all samples, while on Mitis Salivarius Agar, it was approximately from 10 to 10^4 CFU/ml; only very few samples showed no growth of α-hemolytic streptococci on Mitis Salivarius Agar (Table 1). In addition, samples 001 and 009 at T_3 and T_4 were colonized with *S. aureus*, at low concentrations, remaining unchanged with respect to T_0 (i.e., 50 CFU/ml) and samples 002, 012, 013, and 015 at T_4 were colonized with coagulase-negative staphylococci (CoNS) at bacterial concentrations of approximately from 30 to 100 CFU/ml. The staphylococcal colonization could have interfered with the streptococcal adhesion processes.

The deferred antagonism test to evaluate $BLIS_S$ production and RAPD-PCR analysis for molecular genotyping were applied to each α-hemolytic streptococcal colony to determine the level of colonization of the specific *S. salivarius* 24SMB in the human upper respiratory tract of the volunteers.

The same colonies, showing typical characteristics of *S. salivarius* 24SMBc, i.e., large, soft, and pale blue, were analyzed by the antagonism test against *S. pyogenes* and *S. pneumoniae* groups [15]. All strains tested showed the same *S. salivarius* 24SMB inhibitory activity profile, i.e., strong activity against *S. pneumoniae* and *S. pyogenes*, and no inhibition of oral streptococci. All streptococcal colonies with a positive antagonism test, assayed by RAPD analysis, provided a unique fragment pattern typical of our *S. salivarius* genotype.

Figure 2 shows that the genomic profiles obtained by both OPA3 and OPA18 were identical for all colonies tested.

All these results confirmed that 19 out of 20 patients (95 %) were colonized at least in the first 4 h after nasal spray administration, and 11 out of 20 (55 %) colonizations persisted for at least 6 days from the last dose of the formulation.

Table 1 Levels of *Streptococcus salivarius* 24SMBc colonization over time (2 h to 6 days)

Patient	Age (years)	Bacteria count (CFU/ml)	T_1, 2 h	T_2, 4 h	T_3, 24 h	T_4, 6 days	Persistence of *S. salivarius* 24SMBc at various times
001	45	Total count	$>10^6$	$>10^5$	$>10^6$	$>10^6$	–
		α-hemolytic streptococci count	65	10	10^4	10^4	T_1, T_2
002	20	Total count	$>10^6$	10^2	3×10^2	10^5	–
		α-hemolytic streptococci count	10^3	20	–	–	T_1, T_2
003	18	Total count	$>10^6$	$>10^6$	$>10^6$	$>10^6$	–
		α-hemolytic streptococci count	50	30	10^2	10^2	T_1,T_2,T_3,T_4
004	28	Total count	$>10^6$	$>10^6$	$>10^6$	$>10^6$	–
		α-hemolytic streptococci count	30	10	10	20	T_1,T_2,T_3,T_4
005	19	Total count	$>10^6$	$>10^6$	$>10^6$	$>10^6$	–
		α-hemolytic streptococci count	10^2	40	50	30	T_1,T_2,T_3,T_4
006	26	Total count	2×10^2	$>10^6$	20	50	
		α-hemolytic streptococci count	50	4×10^2	10	10	T_1, T_2
007	24	Total count	$>10^6$	$>10^6$	10^4	$>10^6$	
		α-hemolytic streptococci count	10^4	10^3	10^2	10^3	T_1,T_2,T_3,T_4
008	54	Total count	$>10^6$	10^4	$>10^6$	$>10^6$	
		α-hemolytic streptococci count	10^4	10^3	10^3	10^2	T_1,T_2,T_3,T_4
009	31	Total count	$>10^6$	10^5	10^5	10^5	
		α-hemolytic streptococci count	10	30	–	10^2	T_1,T_2
010	38	Total count	$>10^6$	10^5	10^5	$>10^6$	
		α-hemolytic streptococci count	10^3	10^2	10	10^2	T_1,T_2,T_3,T_4
011	30	Total count	10^6	10^5	10^6	10^6	
		α-hemolytic streptococci count	10^2	50	10^2	70	T_1,T_2,T_3,T_4
012	27	Total count	10^5	10^5	10^4	10^5	
		α-hemolytic streptococci count	20	50	10	80	T_1,T_2
013	23	Total count	$>10^6$	10^5	10^5	10^4	
		α-hemolytic streptococci count	10^2	5×10	10^2	8×10	T_1,T_2
014	32	Total count	$>10^6$	10^5	10^5	10^5	
		α-hemolytic streptococci count	9×10^3	5×10^2	70	10	T_1, T_2
015	43	Total count	$>10^6$	$>10^6$	10^5	10^6	
		α-hemolytic streptococci count	–	10	80	50	–
016	42	Total count	$>10^6$	$>10^6$	$>10^6$	$>10^6$	
		α-hemolytic streptococci count	10^2	50	50	30	T_1,T_2,T_3,T_4
017	52	Total count	10^6	10^6	10^5	10^4	
		α-hemolytic streptococci count	–	20	10	20	T_2,T_3,T_4
018	36	Total count	10^6	10^6	10^5	10^4	
		α-hemolytic streptococci count	10^2	80	10	50	T_1,T_2
019	33	Total count	10^6	10^5	10^6	10^6	
		α-hemolytic streptococci count	10^3	10^2	10	10	T_1,T_2,T_3,T_4
020	24	Total count	10^6	10^5	10^6	10^6	
		α-hemolytic streptococci count	10^2	50	10^2	70	T_1,T_2,T_3,T_4

Discussion

There is great interest in the role of the microbiome in the complex equilibrium between a healthy state and progression towards disease. Many studies have addressed this role, above all in gastrointestinal-related diseases and oral pathologies, but only a few have supported a beneficial microflora involvement in URTIs [2, 22–24].

The new approach to use "friendly bacteria", which means to use harmless bacteria to displace pathogenic organisms—by bacterial interference—thus preventing colonization of pathogenic bacteria, is gaining ever more interest, finding applications in many fields [25]. Among studies involving upper

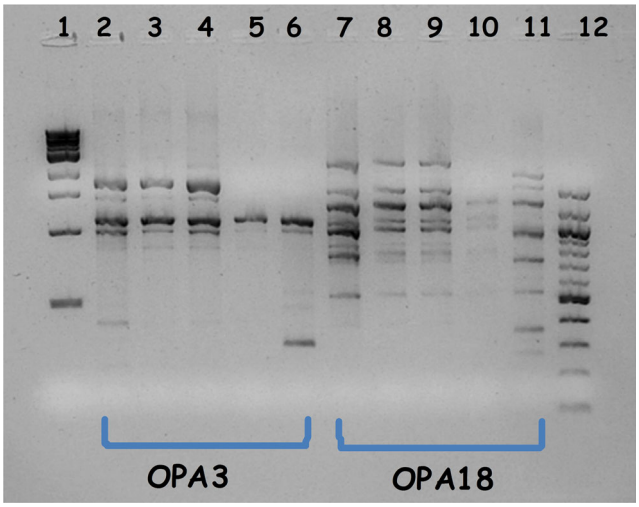

Fig. 2 Random amplified polymorphic DNA (RAPD) fingerprinting with OPA3 and OPA18 primers. Lane 1: Marker 1 kb; lanes 2 and 7: *S. salivarius* 24SMBc; lanes 3 and 8: patient 3 at T_1; lanes 4 and 9: patient 4 at T_1; lanes 5 and 10: patient 6 at T_3; lanes 6 and 11: patient 15 at T_3; lane 12: marker 100 bp

respiratory tract diseases, only a few have pointed out the possibility of using α-hemolytic streptococci as prophylaxis against rAOM and URTIs using a single strain such as *S. salivarius* K12 in oral tablets [26, 27] or α-hemolytic streptococci group (*S. mitis*, *S. oralis*, and *S. sanguis*) in a nasal spray [28, 29], and, more recently, by using *S. sanguinis* and *Lactobacillus rhamnosus* strains [30]. However, there is still only relatively limited clinical evidence on the role of oral probiotics on health improvement. An important aspect of this "bacteriotherapy" approach is the recolonization of the rhinopharynx with healthy flora, as the pharyngeal microbiome has an essential role in the airway linings to protect against many infections [31].

Many studies have highlighted the close correlation between the reduction of potential pathogens and the presence of commensal streptococci [28]. Evidence has shown that a healthy microbiota confers protection against URTIs and a lack or reduction of α-streptococci, especially those with antagonist activity against otopathogens, has been correlated with a higher incidence of re-infections in patients with streptococcal pharyngotonsillitis. Furthermore, it has been found that children who are prone to otitis media are colonized with a lower concentration of α-streptococci compared with those who are not prone [11]; in these cases, probiotics can confer natural protection against infections and, in some cases, become a new prophylaxis. In this context, *S. salivarius*, a non-pathogenic species and predominant colonizer of the oral microbiota, finds a wide application in the prevention of URTIs.

The current study, conducted on healthy volunteers, demonstrated that the administration of a dose of 8×10^9 CFU per day of *S. salivarius* 24SMBc [15] as a nasal spray was well tolerated in all volunteers, and there were no side and/or undesirable effects; in addition, 95 % of volunteers were colonized by *S. salivarius* 24SMB and 55 % remained colonized until the sixth day after the last administration, whereas low-rate staphylococcal colonization in six samples could have interfered with *S. salivarius* 24SMBc colonization.

In conclusion, the primary endpoint of our study—the nasal spray safety and human tolerability—as well as the secondary endpoint in terms of persistence of colonization, were largely achieved in all cases treated.

The application of oral probiotics is a relatively undeveloped field but is becoming an attractive approach for prevention and therapy, especially for pediatric age patients. *S. salivarius* 24SMBc possess characteristics making this strain suitable for use in bacteriotherapy.

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Conflict of interest The authors declare that there is no conflict of interest.

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