European Review for Medical and Pharmacological Sciences 2019; 23(1 Suppl.): 60-66

Modulation of opportunistic species *Corynebacterium diphtheriae, Haemophilus parainfluenzae, Moraxella catarrhalis, Prevotella denticola, Prevotella melaninogenica, Rothia dentocariosa, Staphylococcus aureus and Streptococcus pseudopneumoniae* by intranasal administration of *Streptococcus salivarius* 24SMBc and *Streptococcus oralis* 89a combination in healthy subjects

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Abstract. – OBJECTIVE: Probiotics *S. salivarius* 24SMBc and *S. oralis* 89a comprised in the nasal spray Rinogermina are known to exert inhibition of harmful pathogens and ameliorate the outcome of patients with chronic upper airways infections. In this study, for the first time, the effect of this formulation on the modulation of the microflora of healthy subjects was evaluated, with particular interest on pathobionts and pathogens present.

PATIENTS AND METHODS: Metagenomic identification and quantification of bacterial abundances in healthy subjects were carried out by means of Ion Torrent Personal Machine. In particular, nasal swabs were sampled one, two and four weeks after seven days of treatment with Rinogermina.

RESULTS: The modulation of the abundance of pathobionts and pathogenic species (i.e., *Corynebacterium diphtheriae, Haemophilus parainfluenzae, Moraxella catarrhalis, Prevotella denticola, Prevotella melaninogenica, Rothia dentocariosa, Staphylococcus aureus* and *Streptococcus pseudopneumoniae*) was characterized and a significant temporary decrease in their presence was identified.

CONCLUSIONS: The beneficial effects of *S. salivarius* 24SMBc and *S. oralis* 89a nasal intake was assessed but seemed to be restricted in specific temporal windows. Thus it would be interesting to evaluate also this positive impact of longer administration of this probiotic formulation.

Key Words
Microbiota, Upper respiratory tract infections, Probiotics, Streptococcus salivarius, Streptococcus oralis, Pathobionts.

List of Abbreviations

PEG-PPG: poly(ethylene glycol)–poly(propylene glycol); DNA: Deoxyribonucleic acid; rRNA: Ribosomal Ribonucleic Acid; OTU: operational taxonomic unit.

Introduction

The human nasal cavity harbors a rich bacterial ecosystem characterized and influenced both by the host itself and the environmental conditions to which it is exposed[1,2]. When the non-sterile air is inhaled, potential pathogens together with dust and environmental particles are firstly filtered and trapped by vibrissae placed in the nostrils. Subsequently, the local microbial flora plays a crucial role in protecting the host by preventing

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the colonization of the nasal vestibule by airborne pathogens. Indeed, the nasal flora discourages the pathogens attachment by means of a mechanism called "competitive exclusion", in which prokaryotic cells must compete with their neighbors for space and resources[3]. Furthermore, the commensal bacteria harbored in the nasal cavity stimulate the consistent secretion of a mucus layer or snot containing enzymes and immunoglobulins apt to stimulate the host immune system and impeding the spread of a disease[4].

Different aerobic and anaerobic bacterial communities physiologically constitute the nasal microbiota. *Staphylococcus* spp., *Haemophilus* spp., *Streptococcus* spp., *Moraxella* spp., *Neisseria* spp., and *Corynebacterium* spp. are some of the commensal aerobic bacteria that inhabit the healthy human nasal cavity[5]; whereas were *Prevotella* spp., *Porphyromonas* spp., *Fusobacterium* spp., and *Peptostreptococcus* spp. are the most common anaerobic isolates[6]. However, when external events such as alterations of the environmental conditions (e.g., humidity, oxygen concentration, climate change, pollution, pollen, exposure to chemicals, etc.) or trauma alter the homeostasis, a progressive change in the physiologic flora might occurs leading to a microbial imbalance, called dysbiosis. This occurrence might increase the host susceptibility favoring the establishment of inflammatory, infectious or allergic diseases[7,8]. In particular, pathobionts might switch from carriage to a pathological state compromising the individual welfare. Thus, there is the need to restore the levels of a healthy nasal microflora. A recent study investigated the use of a nasal spray containing a mixture of probiotics (*Streptococcus salivarius* 24SMBc and *Streptococcus oralis* 89a, 98:2 ratio) to evaluate the safety of the treatment on the nasal flora of healthy subjects[9]. Indeed, the use of α-hemolytic streptococci as prophylaxis is now a promising emerging strategy being *S. salivarius* and *S. oralis* biosafe colonizers characterizing a healthy and balanced nasopharyngeal microbiota[9,10].

Even if it was described the valuable role of the analyzed treatment in the reduction of the harmful pathogens and the increase of beneficial one, there is still the need to characterize these populations to have a deeper insight into the modulation of the described bacterial hub. With this aim, in the present study, the ability of *S. salivarius* 24SMBc and *S. oralis* 89a to modulate the nasal microbiota composition was evaluated through the analysis of abundance of the identified species.

Patients and Methods

Patients Recruitment, Probiotics Administration, and Sample Collection

Twenty-three healthy volunteers (19-64 years old; 9 males and 11 females) were recruited to this study between January and February 2017.

The exclusion criteria were (1) the antibiotic consumption within two months before the experimental treatment and (2) the use of any medical devices or treatment for nasal congestion. Furthermore, (3) subjects suffering from allergies to pollen, grasses, poplars and dust and also (4) individuals with sinusitis or rhinosinusitis were excluded. The volunteers had no pets in their homes, and they lived in the same geographical area, in a limit of 10 km from the Milan urban center in order to ensure that all the subject were exposed to similar climatic and environmental conditions (e.g., humidity, temperature, pollution, etc.).

All the enrolled volunteers were treated with a mixture of *S. salivarius* 24SMBc, and *S. oralis* 89a suspended in a 98:2 ration in buffered isotonic solution with pH 7.0 composed by poly(ethylene glycol)–poly(propylene glycol) (PEG-PPG) copolymer and PEG-14 Dimethicone (Rinogermina, DMG, Rome, Italy). The probiotic solution was administrated with two bilateral spray injections into each anterior nostril for a week, in the morning after personal care/washing.

Samples were collected by means of sterile pernasal swabs in polypropylene tubes (Thermo Fisher, Waltham, MA, USA). In particular, the surface of the mucosa of the left anterior nostril was sampled in a 1 cm depth from the outer edge. Per subject, four nasal swabs were collected according to the following experimental time points: (1) before the treatment, (2) 1 week, (3) 2 weeks and (4) 1 month after the end of the treatment with probiotics. Samples were collected at the Laboratory of Clinical Microbiology (University of Milan, Milan, Italy) and store at -80°C until the analyses performed 48 h after sampling.

DNA Extraction, 16S rRNA Amplification, and Sequencing

Bacterial genomic deoxyribonucleic acid (DNA) was extracted by means of the QIAamp DNA Mini Kit (Qiagen, Milan, Italy). Then, part of the 16S Ribosomal Ribonucleic Acid (rRNA) gene sequences were amplified using the 16S Metagenomics Kit (Life Technologies, Monza, Italy) to analyses of the microbial populations characterizing the samples through the Ion Torrent

sequencing technology (Life Technologies, Monza, Italy). The hypervariable regions of the 16S rRNA were amplified using the primer set V2-4-8 and V3-6, 7-9 at the conditions reported in Table I using the SimpliAmp Thermal Cycler (Thermo Fisher, Waltham, MA, USA).

The quality of the obtained amplicon was assessed by electrophoresis on a 2% agarose gel. Finally, the sequencing of the bacterial DNA was performed through the Personal Genome Machine, as previously described [11].

Ethic Statement

All the performed procedures were conformed to the Helsinki of 1975, as revised in 2000 and 2008. The experimental protocols were approved by the internal review board of the IRCCS Galeazzi Orthopaedic Institute and by the Ethics Committee of the IRCCS Ospedale San Raffaele (MicroSP no. 31/INT/2017, Milan, Italy). The study was conducted according to the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use guidelines for Good Clinical Practice. Finally, all the enrolled patients were extensively informed about the purpose of the study and a written consent was obtained from each participant. At the beginning and at the end of the trial, volunteers were asked to fill a questionnaire reporting any adverse effects and nasal disorders related to the treatment with probiotics.

Statistical Analysis

The raw abundances of the species of each operational taxonomic unit (OTU) were firstly normalized to 100,000 reads per sample. The significant differences in microbial species were assessed through the nonparametric tests based on the Kruskal-Wallis and Wilcoxon rank-sum test, coupled with Dunn's post-hoc test (R software v 3.3.1, USA). All data are expressed as means, and only the significant differences were reported in graphs; p-values < 0.05 were considered statistically significant.

Table I. Amplification condition.

Step	Temperature	Time
Initial denaturation	95°C	10 min
Denaturation	95°C	30 sec
Annealing x 30 cycles	58°C	20 sec
Extension	72°C	20 sec
Final extension	72°C	7 min

Amplification conditions for the hyper variable regions of the 16S rRNA using the primer set V2-4-8 and V3-6, 7-9.

Results

During the experimental time course, no severe side effects were recorded in any enrolled patients after the treatment with *S. salivarius* 24SMBc and *S. oralis* 89a. In particular, only 10% of the subject suffered from allergic cold, and 80% did not show any problems breathing. However, 90% of people reported nasal dripping immediately after the treatment administration.

A total amount of 4,901,713 high-quality filtered reads were obtained (126,674±552,453 per patients), clustered in OTUs and consequently classified into taxonomic ranks (phyla, classes, orders, families, genera, and species). The changes detected in the presence of corynebacteria species in the nasal microbiota composition were represented in Figure 1A. In particular, after an initial decrease one week after the treatment, *Corynebacterium diphtheriae* showed a progressive increase at T3 with a peak registered one month after the treatment. Even if the initial drop was also detected for *Corynebacterium accolens*, *Corynebacterium durum*, *Corynebacterium macginleyi*, *Corynebacterium tuberculostearicum,* these microorganisms had a progressive decrease starting from T3, especially concerning *C. durum*, *C. macginleyi.* A similar trend was observed in *Staphylococcus* spp. (Figure 1B) where the effects of Rinogermina seemed to affect the abundance of these bacteria, especially one week after the administration. However, the increase at T3 highlighted the positive effects of the probiotic intake on *Staphylococcus warneri* and *Staphylococcus hominis*, two beneficial commensal strains. Although *S. aureus* was widely present in nares of enrolled subjects, a reduction in the abundance of this ubiquitous species was appreciable during all the experimental time points. More importantly, the synergistic action of *S. salivarius* 24SMBc and *S. oralis* 89a resulted in a reduction of *S. aureus* up to one month after the treatment. Concomitantly, after an initial decrease at T2, the treatment helped *Staphylococcus epidermidis* to colonize nasal mucosae with a growing trend also a month after the probiotic intake.

Due to the different abundance of streptococci, these bacteria were depicted in two graphs to appreciate also the differences in underrepresented species (Figure 2). *Streptococcus tigurinus* displayed a consistent increase in the colonization of the nostrils one week after the probiotic intake followed by a sudden drop at T3. In contrast, *Streptococcus sanguinis*, *Streptococcus thermophilus*, *Streptococcus sinensis* and *Streptococcus*

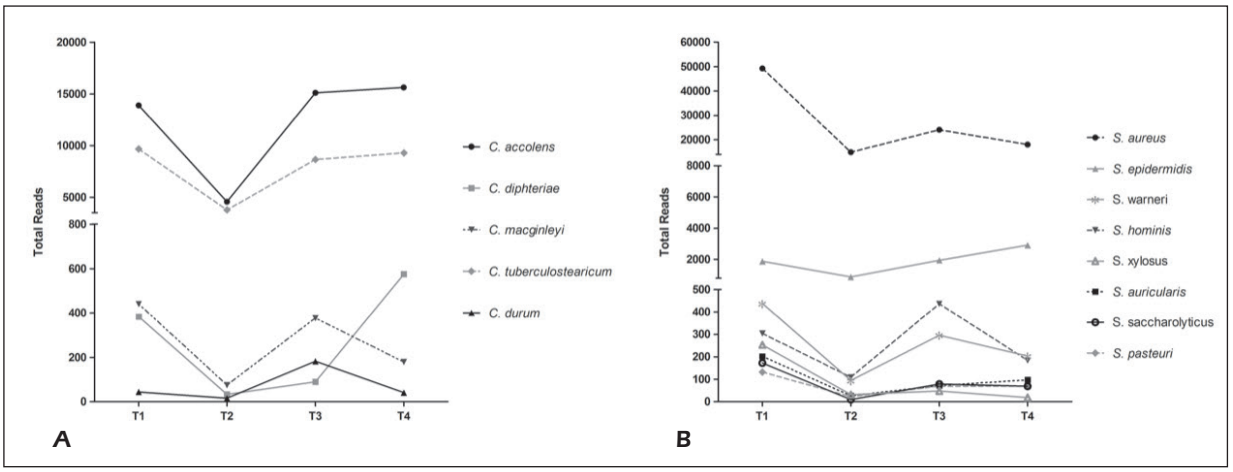

Figure 1. Corynebacteria e staphylococci modulation over time. **A**, The graph shows the changes in corynebacteria abundance before Rinogermina intake (T1) and 1 week (T2), 2 weeks (T3) and 4 weeks (T4) after the treatment. **B**, Changes in staphylococci abundance before Rinogermina intake (T1) and 1 week (T2), 2 weeks (T3) and 4 weeks (T4) after the treatment.

mitis had an opposite behavior, with a prompt reduction in relative abundance at T2. While the presence of *S. mitis* and *S. thermophilus* progressively increased over time, the levels of *S. sanguinis* had a drastic decreased (Figure 2A). Among the most abundant streptococci, the presence of *Streptococcus australis* and *S. salivarius* enormously increased after seven days of treatment and returned to baseline values from day 14 (T3). Conversely, even if *Streptococcus pseudopneumoniae* was detected at basal values at one month after the end of the probiotic consumption, a peak in its abundance was observed at T3 (Figure 2B).

The remaining identified microorganisms were grouped in accordance with their aerobic or anaerobic metabolism. Concerning the aerobic bacteria, the administration of *S. salivarius* 24SMBc and *S. oralis* 89a seemed not to affect the *Haemophilus parainfluenzae* and *Rothia dentocariosa* abundances, which remained stable overtime. Differently, a discontinuous trend in the *M. catarrhalis* abundance was found during the experimental time points. Indeed, after a drop at T3, a gradual recovery was observed up to the one month after the end of the treatment. A similar increase at T4 was also detected in *Neisseria perflava* (Figure 3A).

Finally, among the anaerobic bacteria, *Prevotella melaninogenica* was the most abundant species detected. The probiotic administration affected this pathobiont in which a decrease was found up to T3 and a return towards basal valued at T4. A similar trend as that described for corynebacteria was also observed for the remaining representatives of *Prevotella* ssp. (Figure 3B).

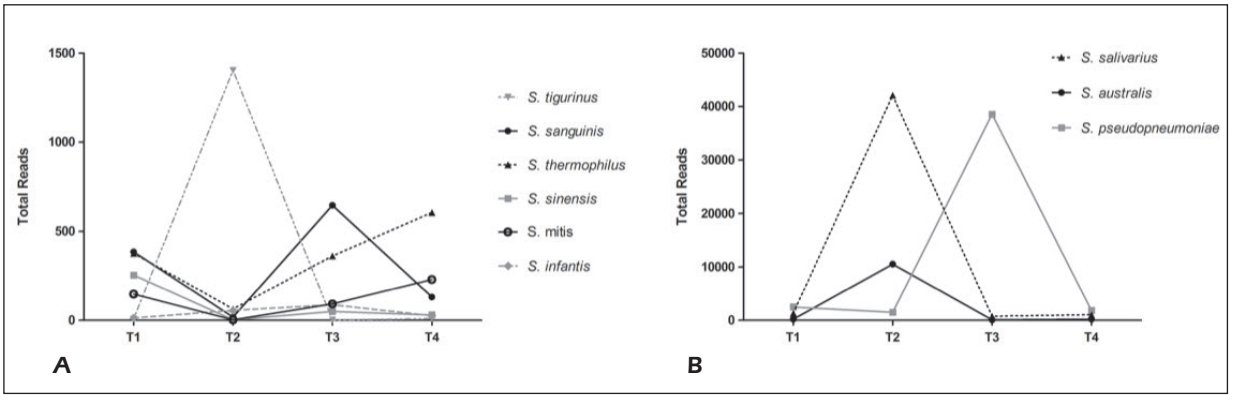

Figure 2. Streptococci modulation over time. **A**, The graph shows the changes in less abundant streptococci before Rinogermina intake (T1) and 1 week (T2), 2 weeks (T3) and 4 weeks (T4) after the treatment. **B**, Changes in more abundant streptococci before Rinogermina intake (T1) and 1 week (T2), 2 weeks (T3) and 4 weeks (T4) after the treatment.

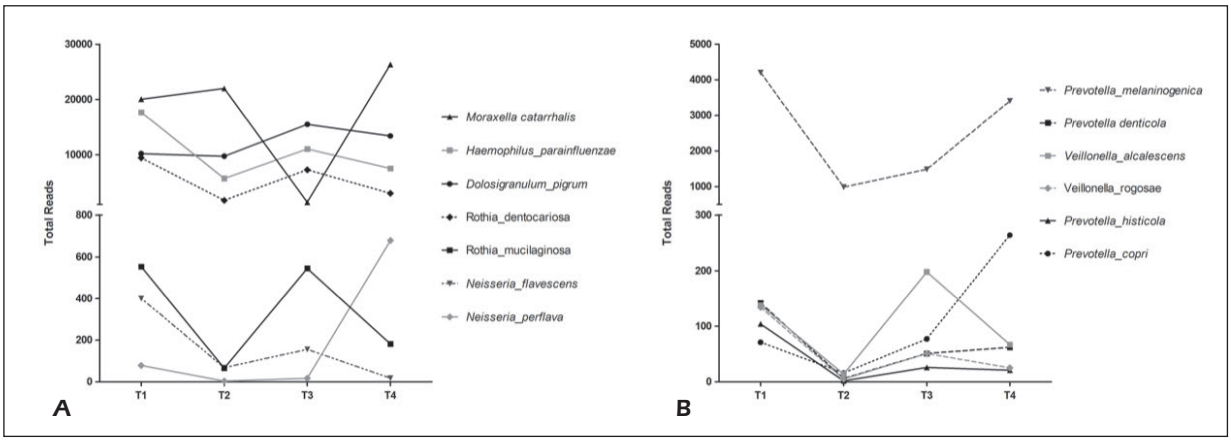

Figure 3. Other aerobes and anaerobes modulation over time. **A**, The graph shows the changes in *Moraxella catarrhalis, Haemophilus parainfluenzae, Dolosigranulum pigrum, Rothia spp*., and *Neisseria spp.* before Rinogermina intake (T1) and 1 week (T2), 2 weeks (T3) and 4 weeks (T4) after the treatment. **B**, Changes in *Prevotella* spp. and *Veillonella* spp. before Rinogermina intake (T1) and 1 week (T2), 2 weeks (T3) and 4 weeks (T4) after the treatment.

Discussion

The human nares physiologically harbor different aerobic and anaerobic commensal microorganisms that compose the nasal microbiota. This dynamic ecosystem is strongly influenced by environmental conditions and by the host itself. The bacterial communities and their metabolic products influence the health of their habitat and that of the organism in which they live. Indeed, the microbiota possesses the capability to reflect the health status of the host, and a dysbiosis caused by the homeostasis alteration lead to changes that might be used as potential diagnostic tools[12]. For this reason, numerous bacterial isolated from healthy subjects have been proposed as probiotics to restore this imbalance. The local administration of fully characterized commensal bacteria which presence is associated to a healthy state might be beneficial for both preventing and treating pathological conditions. Two streptococcal strains, *S. salivarius* 24SMBc and *S. oralis* 89a, have been proposed for this purpose as they were isolated from healthy individuals during pathological outbreaks and because they possess desirable characteristics observed in *in vitro* experiments, such as the ability to colonize the respiratory tract and to counteract the presence of airborne pathogens[13]. Previous studies underlined the safety and tolerability of the combination of these two probiotic strains on healthy and pathological subjects by evaluating the clinical outcomes after the administration of the nasal spray formulation[9,10]. However, the impact of Rinogermina on the normal nasal microbiota has not been investigated yet. Hence, a metagenomic approach for the evaluation of *S. salivarius* 24SMBc and *S. oralis* 89a on the microbial species composition of healthy nostrils was used in this study. The identification of species can be very challenging and demanding because of the high degree of 16S rRNA similarities in members of the same genera, but at the same time can be an extremely important tool to study the microbe-microbe interaction determined by a peculiar condition[14].

The transient - but not stable - peak in the abundance of *S. salivarius* after one week is correlated to the high percentage of this streptococcal strain in Rinogermina. Interestingly, even if it was not noticed a similar trend in the *S. oralis* abundance, this increase might be reflected in other members of the *S. mitis* group, which include *S. infantis*, *S. tigurinus* and *S. australis*. It is well-known that members of the *S. mitis* group are able to influence each other by means of the production of competence stimulating peptides (CSP) pheromones, which also directly control the secretion of streptocine[15]. This effect might contribute to the modulation in the number of some observed pathogenic species often involved in chronic upper airways infections (*C. diphtheriae*, *H. parainfluenzae*, *M. catarrhalis*, *Prevotella denticola*, *P. melaninogenica*, *R. dentocariosa*, *S. aureus* and *S. pseudopneumoniae*)[6,16-19]. Contrary to other members of the mitis group, *S. pseudopneumoniae* had a temporary growth arrest probably related to a putative beneficial influence of the probiotic administration and the potential inhibitory effected exerted by *S. oralis*, as similarly observed in *S. pneumoniae*[20].

Notably, at the end of the follow-up, the action of *S. salivarius* 24SMBc and *S. oralis* 89a combi-

nation was appreciable in an important reduction of pathogens. In particular, the reduction of the abundance of *S. aureus* during the follow up correlates with the concomitant increase in *S. epidermidis* presence. Thus, it can be speculated that the nasal colonization of *S. aureus* was limited by the secretion of the extracellular serine protease (Esp) by *S. epidermidis*, which is known to negatively affect the mechanisms of adhesion and biofilm formation of the above-mentioned pathogen[21].

Interestingly, the use of *S. salivarius* 24SMBc and *S. oralis* 89a resulted in the modulation of the presence of *Corynebacterium* spp. These microorganisms commonly colonize juvenile and adult nasal passage, thus its role in the establishment of several commensal-pathobiont interactions is not surprising [14]. However, little is known about the molecular interactions of these genera with neighbor bacteria composing the human nasal microbiota. The temporary scarceness of the pathogen *C. diphtheriae* could be related to the concomitant predominance of *S. salivarius*, which might exert by a direct inhibitory effect on this pathogen, as previously described[22].

Our data might also indicate a positive impact of the Rinogermina administration by limiting the presence of potential pathogenic anaerobe microorganisms such as *P. denticola* and *P. melaninogenica* previously largely recovered in infections of upper and lower airways[23,24]. In fact, of all the species present in the upper airway, Streptococci showed an antagonist role against *Prevotella* as also reported by Van Essche et al[24] describing the role of *S. salivarius*, *S. oralis* and *S. mitis* in inhibiting the growth of *Prevotella intermedia*.

Not only *Prevotella* spp. was affected by the probiotic administration, but also *H. parainfluenzae* and *R. dentocariosa* displayed a significant reduction, with respect to the baseline, 14 days after the use of *S. salivarius* 24SMBc and *S. oralis* 89a. These beneficial properties were also reflected in the reduction of *M. catarrhalis* 14 day after the end of the administration. For these reasons, it would be interesting to study the influence of *S. salivarius* 24SMBc and *S. oralis* 89a administration in longer time points. A limitation of the study was the lack of a control group only treated with sterile saline to appreciate possible variations due to the mechanical action of the fluid.

Conclusions

Probiotics *S. salivarius* 24SMBc and *S. oralis* 89a were able to positively and temporarily modulate the nasal microbiota composition with particular regards to pathobionts and pathogenic species. The positive effects in most of the analyzed species seemed to be restricted in a specific temporal window. Hence it will be important also to elucidate the impact of longer administration of Rinogermina, which might result in a more consistent modulation of the nasal microbiota during time.

Sources of Funding

This work was supported by the Italian Society of Rhinology. The sponsors provided financial support for costs related to the publication of this article. The sponsor was not involved in the study design, in the collection and interpretation of data, in the writing of the study and in the decision to submit the article for publication.

Conflict of Interests

The authors declare that they have no conflict of interest.

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