Nasopharyngeal Microbiota Analysis in Healthy and Otitis-prone Children

Focus on History of Spontaneous Tympanic Membrane Perforation

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Background: Recurrent acute otitis media (RAOM) is common in children, and it may result in spontaneous tympanic membrane perforation (STMP), management of which is often challenging. In the upper respiratory tract (URT), resident microorganisms play a pivotal role in otitis media pathogenesis and prevention, as they are able to inhibit the colonization process and otopathogens growth. In particular, *Dolosigranulum spp.* and *Corynebacterium spp*. have been associated with respiratory health in several studies. This study aims at comparing both nasopharyngeal microbiota of children with RAOM versus matched controls and nasopharyngeal microbiota of children with a history of RAOM with STMP.

Method: Nasopharyngeal swabs were collected from 132 children, median age 3.51 (2.13–4.72), including 36 healthy children, 50 with RAOM without STMP, and 46 with RAOM with STMP. Bacterial DNA was subsequently extracted and 16S rRNA gene V3-V4 regions were polymerase chain reaction amplified and sequenced using Illumina MiSeq technology.

Results: A higher relative abundance of *Dolosigranulum* and *Corynebacterium* genera was detected in the nasopharynx of healthy children (16.5% and 9.3%, respectively) in comparison with RAOM without STMP (8.9% and 4.3%, respectively) and RAOM with STMP (5.2% and 2.8%, respectively). A decreasing pattern in relative abundance of these 2 pivotal genera through disease severity was detected. In all groups, the most abundant genera were *Moraxella*, *Streptococcus* and *Haemophilus*, followed by *Dolosigranulum* and *Corynebacterium.*

Conclusions: Our study provides a characterization of the URT microbiota in otitis-prone children with and without history of recurrent STMP, suggesting that the role of *Dolosigranulum* and *Corynebacterium* in regulating the healthy URT microbiota should be further studied.

Key Words: otitis media, otitis-prone children, microbiota, tympanic membrane perforation, otorrhea

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Acute otitis media (AOM) is one of the most common diseases occurring during childhood.[1] It is a polymicrobial disease caused by a group of bacteria called otopathogens, composed by *Streptococcus pneumoniae*, *non-typeable Haemophilus influenzae*, *Moraxella catarrhalis* and *Streptococcus pyogenes*.[2] These microorganisms can be part of the normal nasopharyngeal flora and colonize the upper respiratory tract (URT) during infancy,[3] and become more virulent under favorable circumstances that impair the complex interactions among bacteria, viruses and the host immune system.[4–7]

In recent years, the introduction of next-generation sequencing techniques has allowed high-throughput investigation of entire bacterial communities,[8] leading to a better understanding of the composition and the potential functions of the URT microbiota:[9] resident microorganism are able to inhibit the colonization process, which is known to be the first pathogenetic step for most of the respiratory infections,[10] and otopathogens growth, preventing their spreading in the respiratory tract.[11,12]

In the URT, *Dolosigranulum spp.* and *Corynebacterium spp*. have been associated with respiratory health in several studies.[13,14] Identification of keystone species in microbial communities could be of remarkable importance for the development of probiotic therapies, which have been studied in various trials with contradictory results.[15–17]

The most common complication of AOM is the spontaneous tympanic membrane perforation (STMP).[18,19] Despite the eardrum usually repairs spontaneously without consequences,[18] clinical management is often challenging for several reasons: children who suffer from AOM with STMP have a greater risk of experiencing recurrences, with or without STMP; in addition, the likelihood of STMP episodes increases with the number of AOM episodes;[18] moreover, the most important AOM preventive measures are often less effective in children with recurrent STMP.[20–22]

It has been suggested that recurrent acute otitis media (RAOM) with STMP would be a specific and different condition in comparison to RAOM without STMP.[23] Indeed, evidence show that RAOM with STMP has a different etiologic profile: in particular, *S. pyogenes* infections are more often associated with STMP;[24–26] moreover, *non-typeable Haemophilus influenzae* has been detected with high frequency in middle ear fluid (MEF) collected from children with STMP, often in co-infections with other otopathogens.[27,28]

In last years, many investigations aimed at defining the differences in nasopharyngeal microbiota between otitis-prone versus healthy children. However, evidence on those with a history of recurrent STMP is still poor.

A better understanding of the clinical and epidemiologic features of this specific category of patients is worthy of further in depth-analysis. In particular, comparing the nasopharyngeal microbiota between children suffering RAOM with STMP versus those with a history of RAOM without STMP could give important insights for the development of new prevention strategies and probiotic therapies.

This study aims at comparing both nasopharyngeal microbiota of children with RAOM versus matched controls and nasopharyngeal microbiota of children with a history of RAOM with

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recurrent STMP versus those with a history of RAOM without STMP.

METHODS

Study Design and Patient Recruitment

This cross-sectional study was conducted during winter 2016–2017 in the Otitis Media Pediatric Outpatient Clinic of Fondazione IRCCS Ca' Granda, Ospedale Maggiore Policlinico, Milan, Italy. The protocol was approved by our local Ethics Committee of Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico and was conducted in accordance with the principles of good clinical practice.

Consecutive patients under 6 years of age regularly followed for RAOM in the Otitis Media Pediatric Outpatient Clinic were enrolled. Recurrent disease was defined as 3 AOM episodes in the last 6 months or 4 AOM episodes in the last 12 months. Exclusion criteria included concomitant acute illness (including any infection occurring in the last 30 days), immune system defects, genetic disorders, craniofacial malformations as cleft lip or palate, previous ENT surgery, antibiotic therapy in the last month, antibiotic long-term prophylaxis, probiotic therapy in the last month and chronic tympanic membrane perforation.

Outpatients with no history of RAOM, enrolled from the same department for conditions other than recurrent infections (ie, children with atopy, allergic rhinitis or recurrent wheezing), were chosen as age- and sex-matched controls.

At enrollment, demographic information and risk factors for otitis media were collected, including antibiotic therapies, breast-feeding, method of delivery, passive smoking, daycare attendance and older siblings.

Patients were further divided into 3 groups according to their medical history: patients with history of RAOM without STMP were included in RAOM group; children with history of RAOM with recurrent otorrhea were enrolled in STMP-RAOM group; children with no history of RAOM were included in Control group.

Sample Collection and Processing

At time of recruitment, an accurate medical examination with pneumatic otoscopy was performed by a trained pediatrician. After exclusion of a concomitant acute illness, a deep nasopharyngeal swab was collected from each patient by trained site investigators using eNAT swabs (Copan Italia, Brescia, Italy) stored at 4°C and frozen at −80°C within 4 hours after collection. Patients were not recruited if they had suffered from an acute infective episode in the last 30 days, and all the swabs were obtained during healthy visits.

Immediately after thawing, samples were vortexed for 2 minutes by using a vortex adaptor. DNA was extracted from 200 µL of bacterial suspension in transportation medium using High Pure Template preparation kit (Hoffmann-La Roche, Basilea, Switzerland) according to the manufacturer's instructions with few modifications as previously described.[29] Following quantification by Quant-IT dsDNA Assay Kit High Sensitivity and Qubit 3.0 Fluorometer (Invitrogen, Carlsbad, CA), DNAs were diluted at 5 ng/µL and the V3-V4 variable regions of the 16S rRNA gene were amplified using the 16S metagenomic sequencing library preparation protocol (Illumina, San Diego, CA). Pooled libraries were sequenced on the MiSeq (Illumina) sequencing platform as previously described.[30]

Bioinformatics and Statistical Analyses

Demultiplexed paired-end reads in the FASTQ format were received from the Illumina MiSeq instrument. Sequencing data were processed following the UPARSE pipeline, using USEARCH v10.0.240 (Tiburon, CA) and VSEARCH v2.3.4 (Oslo, Norway). Overall run quality was checked using FastQC v0.11.2 (Cambridge, United Kingdom) and reports were summarized using MultiQC v1.4 (Stockholm, Sweden). Parameters for paired-end reads merging were set as follows: minimum overlapping length of 20 base pairs; minimum 90% identity of alignment; merged sequences length restricted to 430–482 bases. Consensus sequences from all samples were pooled together and primers were stripped from both ends. This "raw" set of merged sequences was then quality-filtered and de-replicated to obtain a subset of high-quality unique sequences to be clustered into operational taxonomic units (OTUs). Sequences with more than one expected number of errors (EE > 1.00) were discarded and singletons removed during de-replication. OTUs were clustered at a 97% identity threshold. Taxonomy prediction at the species level for OTU sequences was performed via the SINTAX algorithm, using the RDP training set v16 as reference database and 0.8 as confidence threshold. In the end, an OTU table was created mapping the whole set of "raw" merged paired-end reads to the representative set of OTUs, with a threshold of 97% identity for alignment. It was then filtered for both low abundant OTUs (<0.5% overall frequency) and samples (<5000 total read counts).

All downstream analyses were performed on this filtered OTU table, exploiting the Qiime2 microbiome analysis package, version 2019.1. For the purpose of comparing feature abundances and biodiversity indices across sample groups, normalization was achieved through rarefaction at the even sampling depth of 6782 sequences per sample.

Alpha and beta diversity metrics (Shannon entropy, Observed OTUs, Pielou' evenness, Berger Parker's dominance, Bray Curtis dissimilarity and Jaccard index) and PCoA were estimated using the q2-diversity plugin, after OTU table rarefaction. Core microbiome (defined as taxa with 90% prevalence in samples) was evaluated at the phylum and genus level using the q2-core plugin. Differentially abundant taxa were evaluated using ANCOM through the q2-composition plugin.

The datasets generated during and/or analyzed during the current study are available from the corresponding author.

RESULTS

The study included a total of 132 subjects, including 84 (69.0%) males, with a median age of 3.51 (2.13–4.72) years. 56.1% of them were younger than 3 years. No or brief breastfeeding was reported in 18.9% of patients and passive smoking exposure in 28.1%; 60.6% of children had older siblings and most of them (93.2%) attended daycare.

Among these, 49 children had RAOM and never experienced otorrhea (RAOM group), 45 had recurrent otorrhea (STMP-RAOM group) and 36 had no history of RAOM (Control group). Detailed demographic and clinic characteristics are reported in Table 1.

Nasopharyngeal Communities

A total of 17.8 million reads were analyzed from 132 samples. Mean library size was 135.485 sequences/sample.

Relative Abundance Analysis

Data concerning the relative abundances of the 7 most frequent genera identified per group are reported in detail in Table 2.

ANCOM analysis at genus level showed that *Corynebacterium*, *Dolosigranulum, Haemophilus* and *Alloiococcus* were significantly differentially enriched among the 3 groups (W = 2).

A higher relative abundance of *Dolosigranulum* and *Corynebacterium* genera was detected in the nasopharynx of healthy children (16.5% and 9.3%, respectively) in comparison

TABLE 1. Characteristics of All 132 Subjects Enrolled in the Study in Different Groups

	RAOM (n = 50) (37.9%)	STMP-RAOM (n = 46) (34.9%)	CTRL (n = 36) (27.2%)	All (n = 132) (100%)
Median age in years (IQR)	3.60 (2.07–4.74)	2.80 (2.15–4.37)	4.12 (2.31–4.99)	3.51 (2.13–4.72)
n < 3 years	20 (40.0%)	25 (54.4%)	13 (36.1%)	58 (43.9%)
n > 3 years	30 (60.0%)	21 (45.6%)	23 (63.9%)	74 (56.1%)
Sex				
Male	33 (66.0%)	31 (67.4%)	20 (55.6%)	84 (69.0%)
Female	17 (34.0%)	15 (32.6%)	16(44.4%)	48 (31.0%)
Method of delivery				
Vaginal delivery	44 (88.0%)	35 (76.1%)	30 (83.3%)	109 (82.6%)
Cesarean section	6 (12.0%)	11 (23.9%)	6 (16.7%)	23 (17.4%)
Breast-feeding				
Yes	43 (86.0%)	42 (91.3%)	22 (61.1%)	107 (81.1%)
No	7 (14.0%)	4 (8.7%)	14 (38.9%)	25 (18.9%)
Passive smoking				
Yes	18 (36.0%)	10 (21.7%)	9 (25.0%)	37 (28.1%)
No	32 (64.0%)	36 (78.3%)	27 (75.0%)	95(71.9%)
Daycare attendance				
Yes	47 (94.0%)	42 (91.3%)	34 (94.4%)	123 (93.2%)
No	3 (6.0%)	4 (8.7%)	2 (5.6%)	9 (6.8%)
Older siblings				
Yes	23 (46.0%)	34 (73.9%)	23 (63.9%)	80 (60.6%)
No	27 (54.0%)	12 (26.1%)	13 (36.1%)	52 (39.4%)

IQR indicates interquartile range; RAOM, recurrent acute otitis media without spontaneous tympanic membrane perforation group; STMP-RAOM, recurrent acute otitis media with spontaneous tympanic membrane perforation group.

with RAOM group (8.9% and 4.3%, respectively). An even lower relative abundance of *Dolosigranulum* and *Corynebacterium* genera was detected in the nasopharynx of children in STMP-RAOM group (5.2% and 2.8%, respectively), compared with RAOM group and control group. Therefore, a decreasing pattern in relative abundance of *Dolosigranulum* and *Corynebacterium* through disease severity was detected in the 3 groups.

A higher relative abundance of *Haemophilus* was detected in the nasopharynx of children from RAOM and STMP-RAOM groups in comparison with healthy children. The genus *Alloiococcus* has been detected prominently in RAOM group (3.1%), in comparison to STMP-RAOM group (0.3%) and control group (0%).

In all groups, the most abundant genera were *Moraxella*, *Streptococcus* and *Haemophilus*, followed by *Dolosigranulum* and *Corynebacterium.* Among the otopathogens, *Moraxella* was the most abundant genus in all groups, contributing to 42% of the total reads in the control group, 35.4% in RAOM group and 41.4% in SMTP-RAOM group, followed by *Streptococcus* (19.5% in Control group, 22.7% in RAOM group and 23.6% in STMP-RAOM group) and *Haemophilus* (11.3% in Control group, 21.4% in RAOM group and 22% in STMP-RAOM group). All these described features are shown in bar plots charts reported in Figure 1.

Diversity Analysis

Alpha diversity, measured by Shannon Diversity Index (SDI), in the 3 groups, is shown in Figure 2. Kruskal-Wallis test for SDI comparison among the 3 groups showed no statistically significant differences ($P = 0.15$). Pairwise analysis showed that SDI was lower in children with RAOM than in healthy controls, even though these data did not achieve statistical significance (RAOM median SDI = 1.10 vs. Control median SDI = 1.35; $P = 0.11$). SDI in RAOM group was lower than in STMP-RAOM group (RAOM median SDI = 1.10 vs. STMP-RAOM median SDI = 1.32; $P = 0.08$). Comparison of SDI in Control group and STMP-RAOM group showed no difference (Control median SDI = 1.35 vs. STMP-RAOM median SDI = 1.32; $P = 0.84$). No statistically significant difference was found for the other alpha diversity indices considered.

Pairwise PERMANOVA analysis on Bray-Curtis dissimilarity showed a significant difference in microbiota composition between Control group and STMP-RAOM group (p-value = 0.015; q-value = 0.045); RAOM and STMP-RAOM comparison showed no significant difference ($P = 0.483$; q = 0.483); no statistical difference has been found comparing control and RAOM group ($P = 0.052$; q = 0.045).

DISCUSSION

To our knowledge, this is the first study describing the nasopharyngeal microbiota in children with RAOM with STMP. Relative abundance analysis showed that potential keystone species as *Corynebacterium* and *Dolosigranulum* were more abundant in the nasopharynx of healthy children, compared with those with AOM, confirming what has been described in previous investigations.

TABLE 2. Mean Relative Abundances Per Genus Per Group (%)

Genus	Corynebacterium	Hemophilus	Moraxella	Staphylococcus	Alloiococcus	Dolosigranulum	Streptococcus
Control	9.3	11.3	42.0	1.4	0.0	16.5	19.5
RAOM	4.3	21.4	35.4	4.2	3.1	8.9	22.7
STMP-RAOM	2.8	22.0	41.4	4.6	0.3	5.2	23.6

RAOM indicates recurrent acute otitis media without spontaneous tympanic membrane perforation group; STMP-RAOM, recurrent acute otitis media with spontaneous tympanic membrane perforation group.

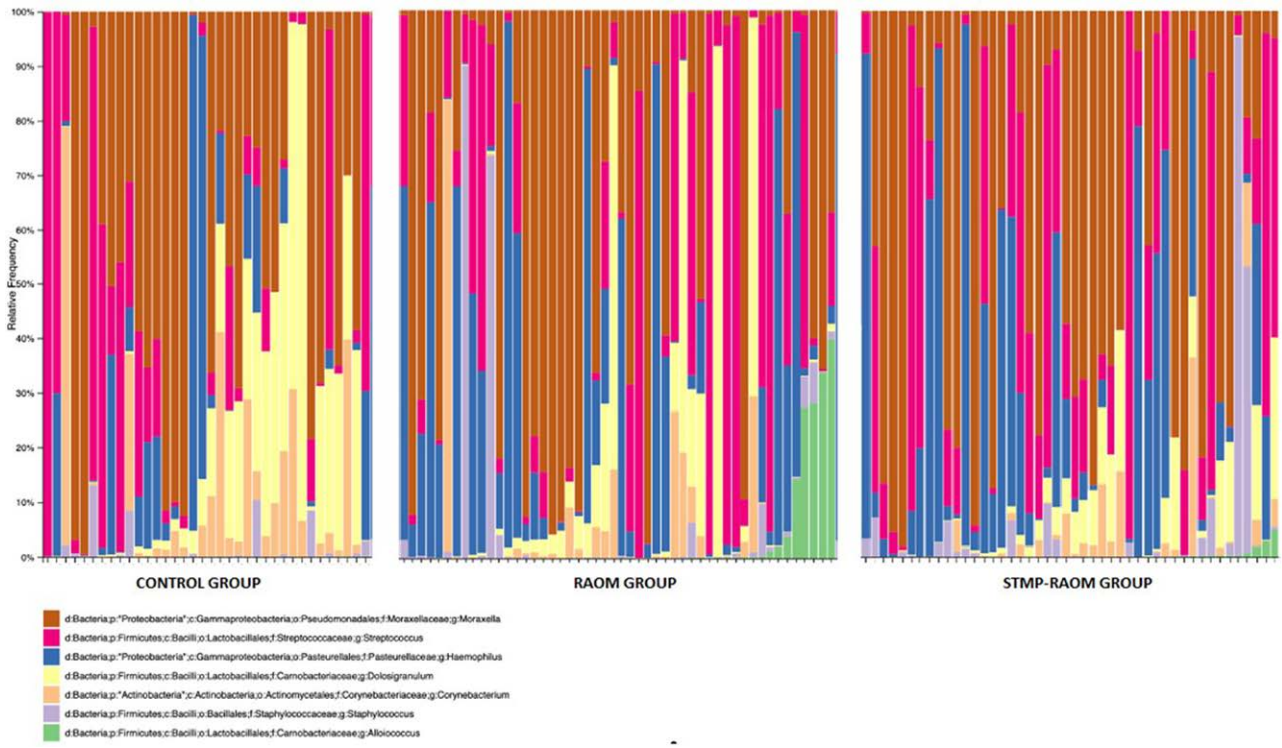

FIGURE 1. Taxa bar plots showing nasopharyngeal microbial composition at the genus level—relative abundance (%). full color online

However, more interesting data arise from comparison of children with RAOM without STMP to those with a history of recurrent STMP: relative abundances of pivotal microorganisms were even lower in nasopharynx of children with STMP-RAOM in comparison to those with RAOM. By contrast, the genus *Alloiococcus* was detected prominently in RAOM group, while it was nearly absent in STMP-RAOM and control groups. This result discloses the presence of a decreasing pattern in relative abundance of pivotal microorganisms. *Dolosigranulum* and *Corynebacterium* have been identified as important species, nasopharyngeal microbial communities in children with STMP-RAOM could be more unstable and more susceptible to colonization by pathogens, thus to respiratory infections.

Results of our study are consistent with data reported in the literature:[31–37] in the URT microbiota and AOM, studies have identified *Dolosigranulum* and *Corynebacterium* as potential important

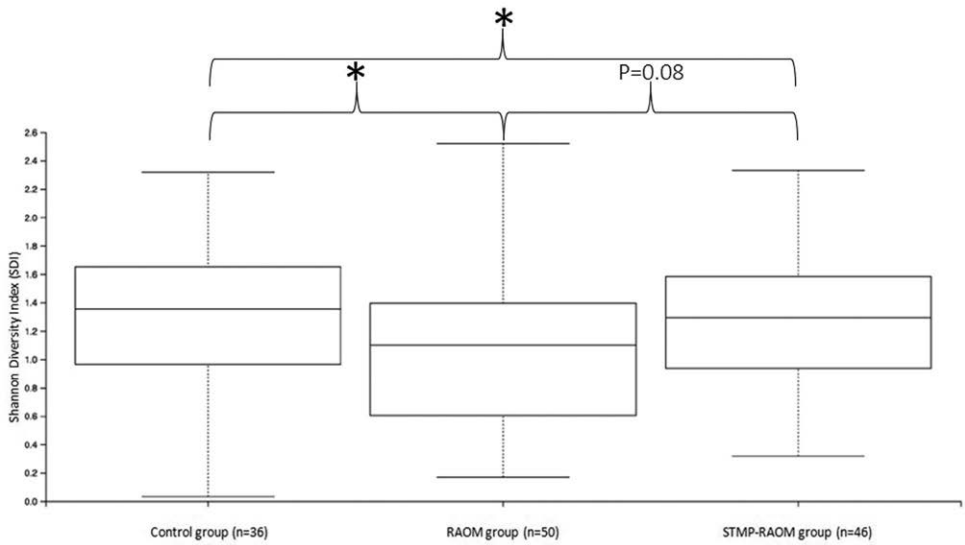

FIGURE 2. SDI of the nasopharyngeal microbiota of 132 children included in the study divided into 3 groups.*Non-significant.

genera, as they have been frequently associated with respiratory health and with potential exclusion of otopathogens such as *S. pneumoniae.*

One of the first studies comparing nasopharyngeal microbiome in children with AOM to healthy children showed that a microbial community composed by *Corynebacterium* and *Dolosigranulum*, in addition to *Propionibacterium*, *Lactococcus*, and *Staphylococcus*, was associated with a lower incidence of pneumococcal colonization and AOM.[14]

More recently, Lappan et al[32] confirmed that nasopharyngeal microbiome of RAOM-resistant children is different from that of RAOM-prone children, showing that *Dolosigranulum* and *Corynebacterium* relative abundance was significantly higher in nasopharynx of healthy children compared with those who had RAOM, highlighting their role as important members of the healthy microbiota. In addition, Man et al[31] previously described nasopharyngeal microbiota in children with ear discharge on tympanostomy tubes, confirming *Dolosigranulum* and *Corynebacterium* to be associated with health outcomes.

In addition to the identification of potential critical species, next-generation sequencing-based studies on AOM have pointed the identification of new potential otopathogens, such as *Turicella otitidis* and *Alloiococcus otitidis*, previously rarely described in culture-based investigations.[32,33] The great prevalence of the genus *Alloiococcus* here detected in RAOM group appears to be consistent with the novel otopathogen hypothesis. Nonetheless, further analysis is needed, as these bacterial species have been prominently described in samples collected directly from MEF than from nasopharynx.

The large involvement of *M. catarrhalis* here detected was previously documented in the nasopharynx of otitis-prone children with and without chronic adenoidal infection,[34–37] and it could be ascribable to the large use of *Haemophilus influenzae* type b conjugate vaccine and pneumococcal conjugate vaccine as prophylactic means in our cohort of children.

Despite being well known that children with history of STMP-RAOM differ from children with RAOM without STMP from several clinical and epidemiologic aspects, to our knowledge, no similar data concerning nasopharyngeal microbiota in children with history of STMP-RAOM are available in literature. Preliminary data from our study show that nasopharyngeal microbiota composition could be a causative factor. Nonetheless, further in-depth analysis with a higher sample size is needed to confirm what has been described.

In our study, no statistically significant difference was detected among the 3 groups for the alpha diversity indices considered. SDI was lower in children with RAOM than in healthy controls, even though these data did not achieve statistical significance. As for biodiversity, it is well established that a more diverse ecosystem is associated with better health outcomes. Different studies indicate that a greater biodiversity in nasopharyngeal microbiota is associated with better outcomes in children with AOM. Laufer et al[14] described a relationship between the colonization by *S. pneumoniae* and a less diverse and less even microbial community; a subsequent investigation by the same group showed that lower levels of diversity in URT flora were associated with a higher colonization rate from *S. pneumoniae, H. influenzae and M. catarrhalis*. In the same study, diversity indices were significantly higher in healthy children than in those who had an acute URT infection at the time of sample collection;[13] conversely, one study described a significantly higher microbial diversity in children with RAOM than in the healthy group.[32]

Our study has some potential limitations: it is a cross-sectional analysis, comparing samples collected from children at a specific time. No longitudinal data are available; concomitant acute illness was an exclusion criteria, thus we have no information concerning microbiota modification during an AOM episode; MEF was not analyzed, as acute ear discharge would have been an exclusion criteria. All these aspects could provide interesting information and deserve further detailed studies.

CONCLUSIONS

We reported the first available data describing potential differences in the composition of nasopharyngeal microbial communities between these categories, suggesting a possible pivotal role of *Corynebacterium* and *Dolosigranulum*.

In our opinion, future research in this field should focus on:

1. Defining the major features of nasopharyngeal microbial communities in different OM phenotypes, in particular in children with RAOM with STMP.
2. Confirming the role of *Corynebacterium* and/or *Dolosigranulum* as important taxa and evaluate their possible use as probiotics.
3. Defining the impact of various exogenous factors that have been less studied, such as active/passive smoking, vaccines and viral infections, on nasopharyngeal microbiota in otitis-prone children.

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