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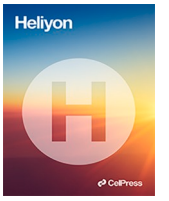

Research article

Characterization of middle ear microbiome in otitis media with effusion in Hungarian children: *Alloiococcus otitidis* may potentially hamper the microbial diversity

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ABSTRACT

Introduction: Although otitis media with effusion (OME) is a common cause of hearing impairment in children, little is known about its microbiological background. We aimed to analyse the microbiome of the middle ear fluid (MEF) in OME to identify microbiological, demographic and environmental factors that may potentially influence the middle ear microbiome. In addition, we aimed to compare the results of conventional culture techniques and PCR-based methods.
Methods: 39 samples from children with OME were investigated using conventional culture and 16S rRNA sequencing. Bioinformatic analyses were carried out to assess the microbial communities and their association with clinical data.
Results: By conventional culture technique bacteria could be cultured in 33 % of the MEF samples; the most frequent bacterium was *Haemophilus influenzae*, followed by *Staphylococcus* species, *Cutibacterium acne*, and *Streptococcus* species. The 16S rRNA analysis showed that *Alloiococcus* was the most abundant bacterium, followed by *Haemophilus*, *Streptococcus*. and *Sphingobium*.
A "High *Alloiococcus* group" with significantly lower and a "Low *Alloiococcus* group" with significantly higher alpha and beta diversity could be defined. Children born by Caesarean sections had lower beta and Shannon diversity than patients born by natural birth. Breastfeeding for at least six months showed a negative correlation with alpha diversity. Age, previous antibiotic treatment, parental smoking, duration of symptoms, existence of siblings, did not alter the significantly bacterial composition of MEF samples and there were no significant differences in regard to alpha or beta diversity.

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Conclusion: The conventional culture technique and 16S rRNA results were not congruent. Remarkably, *Alloiococcus* could not be cultured at all, even by 16S rRNA analysis the presence of *Alloiococcus* seems to be important. Based on our results, *A. otitidis* possibly impacts the diversity of middle ear microbiome in children with OME. However, further studies are required to determine the role of *A. otidis* in middle ear pathologies.

1. Introduction

Otitis media with effusion (OME) is defined by the cumulation of non-purulent fluid in the middle ear without signs of acute inflammation. OME is the most common cause of hearing loss in children, which often results in learning and language disabilities. Thus, its prevention is necessary to ensure normal speech development and cognitive function. OME usually resolves spontaneously within three months, however, fluid remains in the middle ear in 30–40 % of the cases [1]. Antibiotics have limited benefit in the treatment of OME, therefore surgical intervention is usually required to resolve the condition. Furthermore, approximately one quarter of patients require further surgeries within two years [2]. There are different hypotheses regarding the pathogenesis of OME, in which the Eustachian tube is thought to play a key role [3] but there is also increasing evidence for the importance of the microbiome in the background of OME [4,5].

Microbiome is a diverse community of microorganisms living in our bodies and on our skin. An increasing number of studies focuses on exploring alterations of the human microbiome in various disorders. The identification of protective bacteria and potential pathogens promotes the understanding of the pathogenesis of certain conditions [6]. Bacteria colonising a certain body site compete for nutrients and inhibit the growth of their competitors by producing bacteriocins [4]. The analysis of the microbiome and bacterial products prompts us to try to influence the bacterial composition to maintain its health by administering probiotic drugs, ear drops or nasal sprays.

The healthy middle ear was previously believed to be sterile, when new technologies emerged for sensitive detection of bacteria. Next-generation sequencing (NGS) is a culture independent method, that allows the discovery of diverse bacterial communities, even in body sites with very low bacterial loads, such as the middle ear [7]. 16S rRNA analysis is capable of the determination of the bacterial composition of different body sites. Based on NGS 16S rRNA analyses, *Proteobacteria*, *Actinobacteria* and *Firmicutes* have been shown to be the most abundant phyla in the healthy middle ear [8]. However, despite extensive research over the past decades, bacterial colonisation of the middle ear is still debated [9]. Shorter duration of breastfeeding and recent antibiotic use have been associated with a higher incidence of recurrent otitis media in previous studies, suggesting the importance of a healthy middle ear microbiome [10]. Nasal spray treatment with *Streptococcus sanguinis* and *Lactobacillus rhamnosus* resulted in significantly higher rate of recovery from OME [11].

Bacteria most commonly identified in OME by conventional culture techniques are *Streptococcus pneumoniae, Haemophilus influenzae* and *Moraxella catarrhalis*. However, by 16S rRNA gene sequencing *Alloiococcus, Staphylococcus* and *Turicella* were discovered as possible novel otopathogens [10]. Very little is known about the pathogenicity of *A. otitidis*, the only known species of the *Alloiococcus* genus. It is difficult to detect *A. otitidis* with conventional culture techniques and very few studies tried to characterize its possible pathogenic potential.

The aim of this study was to analyse the bacterial composition of middle ear fluid of children with OME. In addition, we aimed to compare results of the conventional culture techniques and PCR-based methods. By obtaining demographic data and medical history from the patients, we set out to investigate various factors that could potentially influence OME.

2. Materials and methods

2.1. Patient data, ethical approval

Children under the age of ten years treated with OME diagnosed by an ENT specialist were recruited at the Department of Otorhinolaryngology and Head and Neck Surgery, Semmelweis University, Budapest, Hungary between January 2019 and July 2019. OME was diagnosed by an ENT specialist as fluid accumulation in the middle ear (non-suppurative) for at least three months. Children had a history of hearing impairment, ear pressure or discomfort. Microscopic evaluation of the eardrum showed dull tympanic membrane with reduced mobility. Tympanometry was performed, fluid accumulation resulted in a type B tympanogram. We observed persistent middle ear fluid on the follow-up examinations prior the surgery appointment. Full medical history was collected regarding the duration of symptoms of OME, duration of breastfeeding, mode of delivery, parental smoking, previous antibiotic use, existence of siblings and day care attendance. Information from parents was gathered by researcher interview, and was cross-checked with medical records if possible. Duration of symptoms was accounted from the confirmation of middle fluid accumulation with tympanometry by an ENT specialist. Parental smoking was considered positive if paternal smoking or maternal smoking occurred during or after the pregnancy. We did not rule out cases if parents gave up smoking in the meantime. Previous antibiotic use was registered if it was administered orally within the past three months, and we documented any pervious surgeries regarding the middle ear (paracentesis, Grommet insertion or adenotomy). Each patient underwent microscopic otologic examination and tympanometry prior to the intervention. Children with cleft lip or palate, immunodeficiencies and genetic syndromes were excluded from the study. After the intervention, all cases showed improvement of symptoms and tympanometry results at the four weeks check-up. Follow-up

examination was done after three months and repeated tympanometry was performed.

2.2. Sample collection

Middle ear fluid (MEF) samples were obtained under general anaesthesia after myringotomy. MEF was aspirated into a sterile specimen trap, and 1 ml of sterile saline was used to rinse out the tube. The fluid collection was not performed by direct suctioning from the needle through the tympanic membrane based on the observation that middle ear fluid often had high viscosity. Special care was taken not to touch the ear canal wall, to avoid contamination of the sample. An aliquot of solution was lifted from the tip of the storage tube with a sterile cotton wool swab and was transferred to transport media for further microbiological culture. Fluid samples were immediately placed on ice and transferred within less than 10 min for storage at −80 °C.

2.3. Conventional culture techniques, identification and antimicrobial susceptibility

The MEF samples were cultured aerobically (Columbia agar plus 5 % sheep blood and Chocolate agar PolyViteX (BioMerieux, France) at 37 °C with 5 % carbon dioxide and anaerobically (Schaedler agar plus 5 % sheep blood (BioMerieux, France) for five days, as a part of the routine microbiological procedure used in the laboratory in order to compare conventional culture techniques with sequencing method. Bacterial isolates were identified by MALDI-TOF MS (Bruker, Daltonik GmbH, Germany) [12]. Antimicrobial susceptibility was determined by disc diffusion method and the results were interpreted according to the EUCAST.

2.4. DNA isolation, 16S rRNA gene library preparation and MiSeq sequencing

DNA isolation from MEF samples was performed by ZymoBIOMICS DNA Miniprep Kit (Zymo Research Corp., Irvine, CA, USA). DNA concentrations in samples were measured using a Qubit fluorimeter with Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Bacterial DNA was amplified with tagged primers covering the V3-V4 region of the bacterial 16S rRNA gene. PCR and DNA purifications were performed according to Illumina's protocol. PCR product libraries were assessed using DNA 1000 Kit with Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). Equimolar concentrations of libraries were pooled, and next-generation Sequencing (NGS) was carried out with MiSeq ®Reagent Kit vs3 (600 cycle) in an Illumina MiSeq System (Illumina, San Diego, CA, USA).

2.5. Data analysis

Raw sequencing data were retrieved from the Illumina BaseSpace and the microbiomes of the various samples were analyzed by the CosmosID bioinformatics platform (Cosmosid Inc., Germentown, MD, USA) [13]. Alpha diversity was measured with Shannon Index and Chao Index. Beta diversity was measured according to principal coordinate analysis with Bray-Curtis distance matrix. A permutational multivariate analysis of variance (PERMANOVA) was used to measure differences between the MEF sample groups [14]. Discriminant microbial features between sample groups were identified with LEfSE analysis [15] on the Huttenhower lab galaxy server (with default parameters).

3. Results

3.1. Characteristics of patients enrolled in the study

Altogether, 29 patients were enrolled in the study with the diagnosis of OME and in the case of ten patients both ears were affected, so in their case, samples were taken from both middle ears. Thus, a total of 39 samples were processed.

The mean age of the cohort was 3.88 ± 1.78 years (2–9 years) and 76 % (22/29) of patients were male. Patient data about the side of involvement, recurrent infection, duration of breast feeding, duration of the disease, prior antibiotic treatment and previous surgery

Table 1
Characteristics of patients involved in the study.

Occurrence value (ratio %) or median (range)	All patients (n = 29)
Age (years)	4 (2–9)
Gender, male	22 (76 %)
One-sided	19 (66 %)
left	11 (38 %)
right	8 (28 %)
Two-sided	10 (34 %)
Recurrent infection	8 (28 %)
Breast fed≥6 months	15 (52 %)
Disease length (months)	6 (3–36)
Previous antibiotic treatment	11 (38 %)
Previous surgery	7 (24 %)

are detailed in Table 1.

3.2. Microbiological culture of middle ear fluid samples

Bacterial growth was detected in 13 samples in the standard culture assay. The most frequently cultured bacterium was *Haemophilus influenzae* (5/13), followed by *Staphylococcus* species (3/13), *Cutibacterium acnes* (3/13), *Streptococcus pneumoniae* (1/13), *Streptococcus pyogenes* (1/13) and *Micrococcus luteus* (1/13). One of the samples contained a combination of *H. influenzae* and *C. acnes*. All other samples were homogenous monocultures.

Two *Staphyloccus* spp. showed multiple antibiotic resistance. Two *H. influenzae* isolates showed resistance to sulfonamides. One strain of *M. luteus* showed resistance for clindamycin (Table 2). No resistance was observed to the first-, or second-line antibiotics, such as penicillins and cephalosporins, used for the otitis media treatment based on the guideline we used [16].

3.3. Microbiota analysis of middle ear fluid samples

3.3.1. Results of DNA recovery from samples

A total of 56 MEF samples were obtained from 40 children. Due to the insufficient DNA content or the unsuccessful 16S rRNA PCR, 17 samples were excluded from the study. Sufficient amount of DNA was obtained in 39 samples from 29 patients for further sequencing. For further data, please see the Supplementary materials.

3.3.2. The microbiota composition of middle ear fluid

The 16S rRNA analysis showed *Alloiococcus* to be the most abundant bacterial genus with a prevalence (PR) of 97 %, average relative abundance (RA) of 39 %, followed by *Haemophilus* species (PR 85 %, RA 9 %), *Streptococcus* (PR 100 %, RA 7 %) and

Table 2
Cultured bacteria with the detected antibiotic resistance patterns and comparison with relative abundances at genus level in corresponding sequencing results. Note that the 16S rRNA sequencing method is not valid for taxonomic classification below genus level, so real microbial species prevalences are estimated to be lower than shown in this table.

Patient number	Cultured bacteria fungus	Cultured bacteria were resistant to the listed antibiotics	Relative abudances of cultured bacteria in sequencing results(%)	16S rRNA relative abudances (%) of the most frequent bacterial genera (from Fig. 1)
P2	*Micrococcus luteus*	clindamycin	<1 %	*Alloiococcus* 92 %, *Corynebacterium* 4 %
P3	*Staphylococcus hominis*	clindamycin, erythromycin, azythromycin, docycyclin, amikacin, tobramycin	*ND	*Alloiococcus* 76 %, *Streptococcus* 3 %, *Sphingobium* 3 %, *Corynebacterium* 2 %
P5	*Haemophilus influenzae*	sulfomethoxasole/trimpethoprim	7 %	*Sphingobium* 48 %, *Alloiococcus* 20 %, ***Haemophilus* 7 %**,
	Cutibacterium acnes	–	<1 %	*Moraxella* 3 %, *Pasteurella* 1 %
P7	*Streptococcus pneumoniae*	–	83 %	***Streptococcus* 83 %**, *Alloiococcus* 7 %
P8	*Cutibacterium acnes*	–	<1 %	*Alloiococcus* 62 %, *Corynebacterium* 20 %, *Haemophilus* 6 %
P10	*Haemophilus influenzae*	–	18 %	*Alloiococcus* 77 %, ***Haemophilus* 18 %**, *Pasteurella* 1 %
P16	*Staphylococcus auricularis*	–	<1 %	*Gardnerella* 20 %, *Escherichia* 18 %, *Blautia* 7 %, *Streptococcus* 1 %, *Sphingobium* 1 %
P19	*Haemophilus influenzae*	sulfomethoxasole/trimpethoprim	47 %	***Haemophilus* 47 %**, *Alloiococcus* 41 %, *Pasteurella* 4 %
P20	*Candida parapsilosis*	–	[a]ND	*Staphylococcus* 38 %, *Escherichia* 10 %, *Sphingobium* 6 %, *Streptococcus* 4 %, *Alloiococcus* 4 %
P21	*Staphylococcus epidermidis*	gentamicin, erythromycin, azythromycin, moxifloxacin, amikacin, tobramycin, sulfomethoxasole/trimpethoprim	<1 %	*Haemophilus* 60 %, *Pasteurella* 6 %, *Gardnerella* 5 %, *Escherichia* 3 %, *Sphingobium* 2 %
P23	*Streptococcus pyogenes*	–	28 %	***Streptococcus* 28 %**, *Alloiococcus* 9 %, *Escherichia* 9 %, *Gardnerella* 8 %, *Staphylococcus* 6 %, *Blautia* 5 %, *Sphingobium* 1 %
P25	*Haemophilus influenzae*	–	6 %	*Alloiococcus* 90 %,
	Cutibacterium acnes	–	<1 %	***Haemophilus* 6 %**
P26	*Haemophilus influenzae*	–	60 %	***Haemophilus* 60 %**, *Alloiococcus* 15 %, *Pasteurella* 7 %, *Corynebacterium* 4 %

Bold font: otopathogen detected parallelly in 16S rRNA analysis.
[a] ND: not detected.

Sphingobium (PR 97 %, RA 6 %). The otopathogen *Moraxella* genus was observed in 44 % of the samples (RA 5 %). *Corynebacterium* was present in 90 % of samples (RA 4 %) followed by *Gardnerella* (PR 33 %, RA 2 %), and *Staphylococcus* (PR 90 %, RA 2 %) (Fig. 1). *Alloiococcus, Pasteurella* and *Haemophilus* dominated the microbiome, if they were present.

In case of ten patients, bilateral effusion was observed, so samples were taken from both ears. Comparing the middle ear microbiome of both ears of the same patient and all the ears with each other, we found that bilateral samples were significantly more similar to each other compared to the ears from different patients ($p = 0.004$) (Fig. 2A and B).

3.3.3. The occurrence of Alloiococcus in middle ear fluids

The 16S rRNA sequence analysis showed high relative abundance of *Alloiococcus* (39 %) in MEF samples. When we analyzed the relative abundance of *Alloiococcus* within the samples, we noticed a gap at 35 %. Based on this observation, patients were divided into two groups: "low *Alloiococcus* group" (LA) with samples with relative abundance of *A. otitidis* lower than 35 % and the 'high *Alloiococcus* group" (HA) with relative abundance higher than 35 %. *A. otitidis* had an impact on the alpha and beta diversity of the middle ear microbiome as well. The alpha and beta diversity in HA group was significantly lower than that of the LA group ($p = 0.001$). The diversity and the principal coordinate analysis of MEF samples with high and low *Alloiococcus* content and the Spearman correlation between *Alloiococcus* frequency and Shannon diversity are shown in Fig. 3A–C.

We also examined the co-occurrence of the most frequently occurring genera (average relative abundance >1 %). The co-occurrence analysis between the *Alloiococcus* and the different genera in the microbiota composition showed that certain genera had a strong negative correlation with *Alloiococcus*, such as *Blautia*, *Streptococcus* and *Sphingobium* spp. Clustered heatmap was constructed based on Spearman rho correlation coefficients from the bacteria that were represented in the MEF samples in at least 1 % (Fig. 4).

For other genera, we found a positive correlation with higher abundances between *Haemophilus* and *Pasteurella, Streptococcus* and *Sphingobium*, *Escherichia* and *Blautia, Sphingobium* and *Blautia.*

3.4. Comparison of the microbiota analysis and conventional culture techniques

The results of the microbiota analysis and the conventional culture techniques did not show a close correlation, as in many cases the cultured bacteria were not dominantly or not at all present upon 16S rRNA analysis of the samples (Table 2).

However, the 16S rRNA analysis and conventional culture results of the three main otopathogens – *H. influenzae, S. pyogenes, S. pneumoniae* - were more consistent. Where the bacterial growth was positive for *H. influenzae* – five cases, 6–60 % relative abundance was observed in the 16S rRNA results as well. However, in two cases *Haemophilus* was observed in 6 % (P8) and even 60 % (P21) in microbiome by molecular technique, but by conventional culture *Haemophilus* spp. were not detected at all.

The *S. pyogenes* culture positive sample (P23) had 28 % relative abundance of *Streptococcus* ssp. and the *S. pneumoniae* positive sample (P7) had 83 % relative abundance of *Streptococcus* ssp. by 16S rRNA analysis.

A. otitidis was not detectable in any of the samples by conventional culture.

3.5. Comparison of the microbiota analysis and patient data

Further investigations with LEfSE analysis showed *Moraxella* to be more frequent in those who have subsequent symptoms, *Enterobacteriaceae* were less common in the same group of patients. *Corynebacterium* seemed to be more frequent in patients under 3

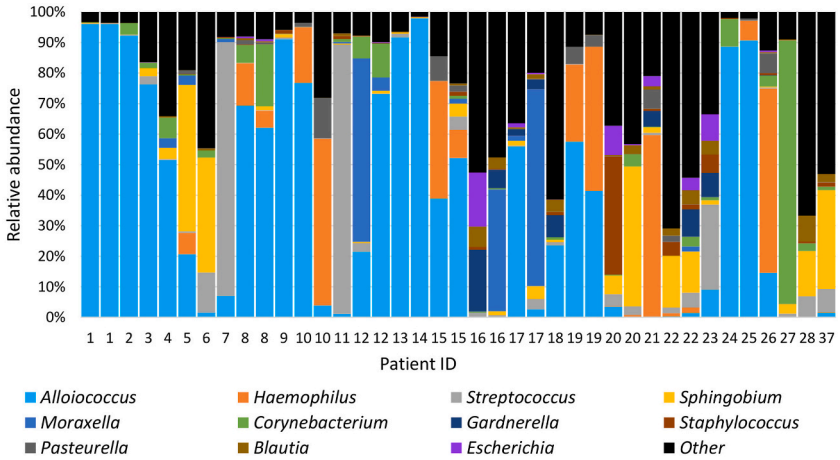

Fig. 1. Microbiome bacterial composition at genus level of 39 middle ear fluid samples from 29 patients. Comparison of the most abundant genera based on 16S ribosomal RNA gene (genera with more than 1 % mean cumulative abundance across the samples are shown). Duplicate numbers indicate bilateral samples.

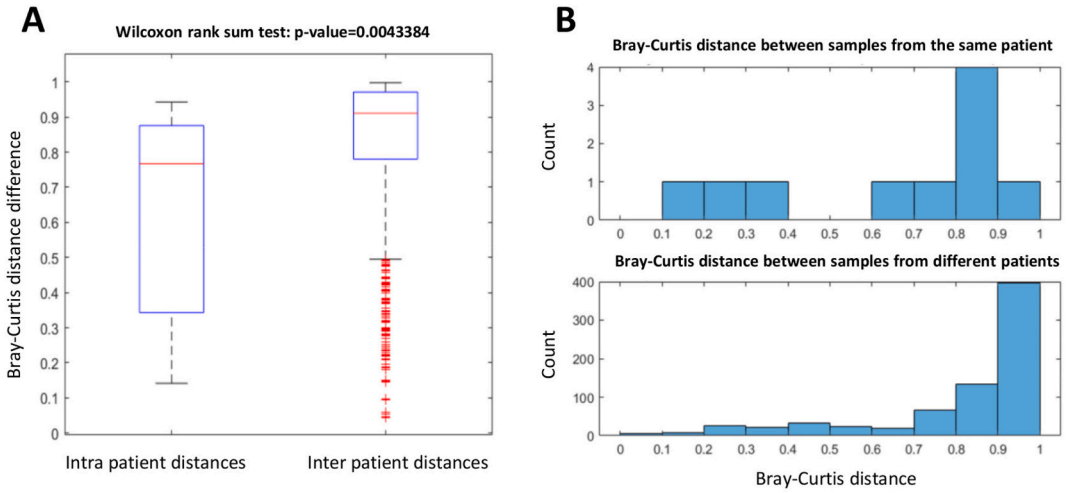

Fig. 2. Comparison of the microbiome of bilateral middle ear fluid samples.
A. Box plot of Bray-Curtis distances between samples from the same patients (left) and samples from different patients (right), The distance between samples from the same patients are significantly smaller than distance between samples from different patients based on left-tailed Wilcoxon rank sum test ($p < 0.05$).
B. Histograms of the distance distribution between samples from the same (top) and from different patients (bottom).

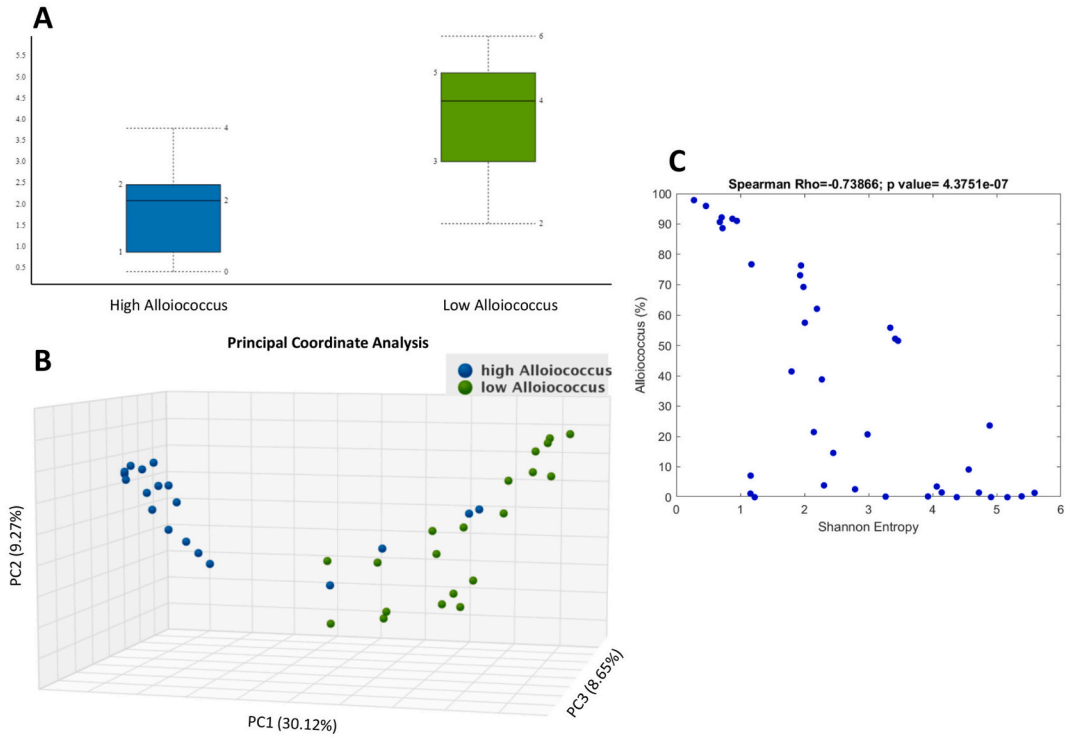

Fig. 3. Occurrence of *Alloiococcus* in middle ear fluid (MEF) samples. Based on the frequency of occurrence of *Alloiococcus*, two groups can be distinguished, the low-frequency group (<35 % *Alloiococcus*) and the high-frequency group (≥35 % *Alloiococcus*).
A. The diversity of MEF samples with high and low *Alloiococcus* content. The Shannon diversities of the high *Alloiococcus* samples are significantly lower ($p < 0.05$) than the Shannon diversities of the low *Alloiococcus* samples.
B. The Principal Coordinate Analysis of MEF samples with high and low *Alloiococcus* content. The two group are separate. (PERMANOVA, Bray-Curtis metrics, $p < 0.05$)
C. ***Alloiococcus* content** Spearman correlation of *Alloiococcus* frequency and Shannon diversity in MEF samples. There is a negative correlation between *Alloiococcus* and Shannon entropy.

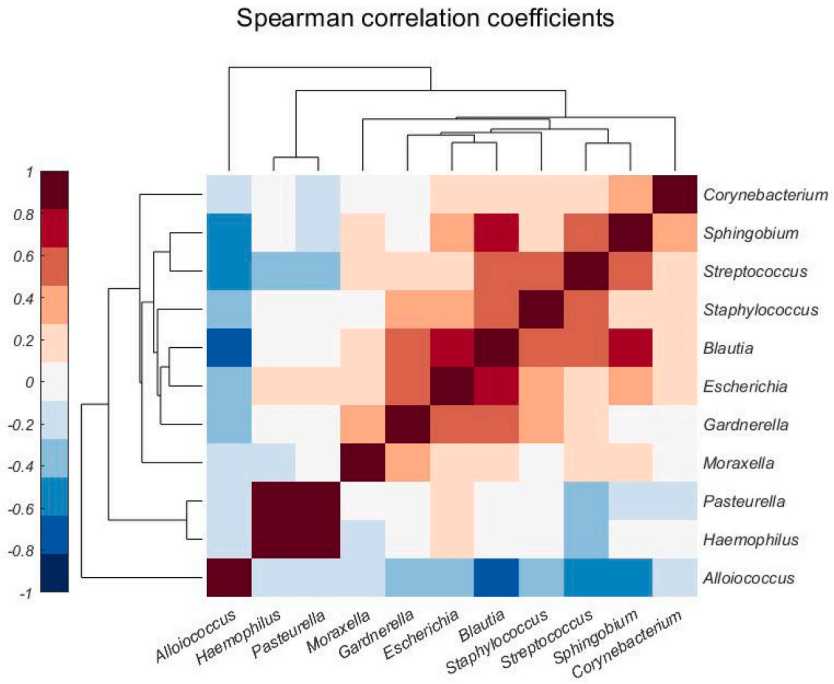

Fig. 4. Spearman rho correlation coefficients for selected abundant genera visualized in a clustered heatmap. Red: positive correlation, they tend to coexist, Blue: negative correlation, they do not coexist. *Alloiococcus, Pasteurella* and *Haemophilus* dominate the microbiome when they present. Stronger positive correlations were observed in the co-occurrence matrix with higher abundance between the following bacteria: *Streptococcus* with *Sphingobium*, *Escherichia* with *Blautia* or *Sphingobium* with *Blautia*.

years of age and in those who did not have siblings. *Sphingobium* was more frequent in those who had previous OME related surgery and in patients born by Caesarean section (Fig. 5A–F).

We observed differences in the beta diversity of MEF samples between patients born by Caesarean section and patients born by natural birth ($p = 0.03$). Shannon diversity was also significantly lower in the Caesarean section group ($p = 0.004$). Breastfeeding for at least six months also had an impact on alpha diversity. Significantly lower alpha diversity was detected when children had received breast milk for at least six months ($p = 0.025$). Age, previous antibiotic treatment, parental smoking, duration of symptoms, and the existence of siblings did not significantly alter the bacterial composition of MEF samples and there were no significant differences in alpha or beta diversity.

4. Discussion

In recent years, the microbiome of OME has been extensively studied, but there is still controversy about the role of bacteria in its pathogenesis. The objective of this study was to determine the bacterial composition of OME in Hungarian pediatric patients.

Our analysis of 16S rRNA identified six core genera of bacteria in OME: *Alloiococcus, Haemophilus, Streptococcus, Sphingobium, Moraxella*, and *Corynebacterium*. Of the known otopathogens in acute otitis media, *Haemophilus* was the most dominant in our samples with an average relative abundance of 9 %, while *Streptococci* and *Moraxella* had a relative abundance of 7 % and 5 %, respectively. The most prevalent bacterium was *Alloiococcus* with an average relative abundance of 39 %.

A. otitidis is a Gram-positive coccus, the only known species of the genus first described by Faden and Dryja in 1989, who isolated it from MEF of children with OME [17]. *A. otitidis* is difficult to culture, requiring lower temperatures and higher CO_2 concentration to prevent overgrowth by other species [18]. Therefore, there are only a limited number of studies available, in which the bacterium was successfully detected by conventional culture techniques. The era of NGS and quantitative PCR methods have changed the possibilities and improved the detection rate of the main otopathogens and *A. otitidis* from MEF samples [19–21], opening the door to new studies to determine the bacterial background of OME. The Eustachian tube has been described to be more horizontal, more rigid and shorter in children compared to adults, which may permit the migration of bacteria to the middle ear from the adenoid tissue. According to the "adenoidal reservoir" theory, the adenoid tissue hosts otopathogens leading to otitis media. The nasopharynx of children with recurrent otitis media contains more otopathogens than healthy children [10]. However, in OME the adenoid pad's microbiota showed to be similar to controls [22]. These findings question the role of the adenoid in the bacterial composition found in OME, suggesting that the external auditory canal may act as a reservoir for bacteria. In accordance to this, *Alloiococcus* is almost absent in the adenoid pad, though it is identified as a commensal member of the external auditory canal [23]. There is little existing data regarding the healthy middle ear microbiome [8], but *Alloiococcus* has not yet been described among the commensals, so its colonisation from the

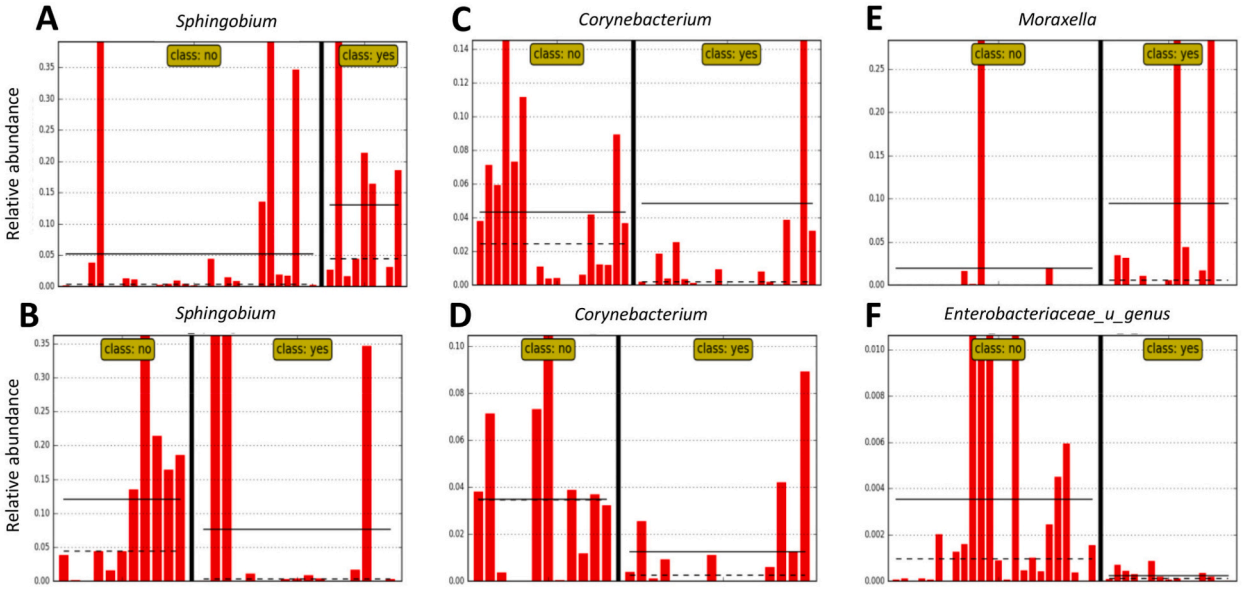

Fig. 5. LEfSE analysis between the clinical characteristics and selected common bacterial genus with factorial Kruskal-Wallis test $p > 0.05$ and high (>2 or < -2) log(LDA) scores.
Bars: relative abundances of samples, organized into 2 groups
Solid line: group mean value
Dashed line: group median value
Potential biomarkers for clinical characteristics:
A. Sphingobium is more common in samples from patients with previous surgery
B. Sphingobium is more common in samples from patient born via C-section
C. Corynebacterium is more common in samples form patients younger than 3 years
D. Corynebacterium is more common in samples from patients without siblings
E. Moraxella is more common in samples from patient with subsequent symptoms
F. Enterobacteriaceae is more common in samples from patient without subsequent symptoms.

outer ear is suspected. Its passage through the intact tympanic membrane remains unclear, although microperforations, or altered membrane permeability during the inflammatory process might be a possible explanation [24]. With a low probability *A. otitidis* detected in MEF samples could originate from the external auditory canal through unintentional contact during sample collection. We wanted to avoid unnecessary interference with the normal ear canal microbiome to prevent a possible otitis externa consequently so we set aside the disinfection of the auditory canal prior to the sampling. Therefore, we took special care under microscope during the intervention not to touch the skin of the canal, but a possible contamination cannot be excluded. It is an acknowledged limitation of the study. We found bilateral effusions to be statistically similar, although in some cases the microbiome seems to be distinct. The external auditory canal is more easily affected by the environment, such as water, dirt and earpick use. Thus, environmental changes impacting one ear more than the other for any reason could potentially explain the differences between middle ear microbiomes of the same patient.

The mean relative abundance of *A. otitidis* varies from 0 to 50 % in the literature [24], our result with 39 % is relatively high compared to other studies [22,25–27]. It is strictly aerobic, so its colonisation in OME is unexpected, as the fluid-filled middle ear is believed to be hypoxic. It shows resistance to trimethoprim-sulfamethoxazole and macrolides, and it is susceptible or intermediately resistant to penicillin and ampicillin. The pathogenic role of *A. otitidis* in OME is still unclear, but considering its high abundance in OME, needs to be further investigated. *A. otitidis* was also described as a potential causative agent in one case of acute sinusitis and two cases of endocarditis [28]. *A. otitidis* was demonstrated to be able to provoke immune response by triggering the production of proinflammatory cytokines. There is evidence that it is capable of biofilm formation and can support the survival of *H. influenzae* in MEF samples even under unfavourable growth conditions [24,25]. Polymicrobial biofilms reduce the antimicrobial susceptibility, and may promote chronic inflammation.

In contrast to these findings, we observed a negative co-occurrence between *Haemophilus* and *Alloiococcus* in our samples, supporting similar findings to previous studies [22] which might suggest that the pathogenic role of *A. otitidis* in OME not only when it co-occurs with *H. influenzae,* but alone as well. This hypothesis could be supported by the effect on the diversity of the samples. Patients with higher relative abundance of *Alloiococcus* showed significantly lower diversity, so this bacterium tends to dominate the microbiome. To date, no evidence was found that *Alloiococcus* would produce any bacteriocins or endotoxins. However, by occupying an ecological niche it could potentially have an impact on bacterial composition of the middle ear. Moreover, biofilm formation could suppress certain bacteria, while it may create an optimal environment for others.

Several factors influence the microbiome. Its stability increases the resilience against diseases whereas an unbalanced environment makes the host susceptible for infections. For ethical reasons the microbiome of the normal middle ear was investigated only in a few studies. Minami et al. reported *Proteobacteria* predominating in the middle ear of both the pediatric and adult patients and described low bacterial density in the normal tympanic cavity [8]. We recruited all eligible patients during the period of sample collection, which resulted a bias toward male patients. Review of studies demonstrated that male sex is a risk factor for the incidence and severity of respiratory tract infections [29]. Our patient population showed that male patients are more often required to undergo surgical intervention because of OME in our observation period. Regarding the middle ear microbiome there was no significant difference detected between genders [30], but we cannot exclude entirely yet the possible differences. Type of birth (natural vs. Caesarean section), duration of breastfeeding, parental smoking, age, day care attendance, or having siblings have been described as significant factors influencing the upper airway microbiome [31]. Bacteria that are ingested by the neonate during vaginal delivery form the microbiome of the infant. Therefore, bacteria from the mother colonizing the nasopharynx could ascend to the middle ear through the Eustachian tube. Breast milk along with nutrients provides vitamins, prebiotics and beneficial bacteria for the infant, which further shape the microbiome. The history of breastfeeding was also shown to make an impact on the gut microbiome in children between 3 and 18 years of age [32]. We can hypothesise, that further body sites, such as the middle ear could be influenced by breastfeeding. Among lifestyle factors, it was reported that parental smoking significantly increases the risk of middle ear disease in children [33]. We investigated all these factors to see if they also had an impact on OME pathogenesis. We found that alpha and beta diversity of patients born by Caesarean section were significantly lower than in the patient group born in the natural way which is congruent with previous findings [34]. We observed the same impact on the diversity of long-term (at least six months) breastfeeding similarly to other studies [32]. It is still unclear whether diversity has any effect on pathogenesis, and it could not be predicted based on 16S rRNA investigation only. Further assessment of risk factors showed C*orynebacterium* to be more frequent in patients under 3 years of age and in those who did not have siblings. *Sphingobium* was more frequent in those who had previously undergone OME related surgery or in patients born by Caesarean section. *Corynebacterium* and *Sphingobium* were described as potentially protective bacteria of the nasopharyngeal microbiome reducing the risk of acute otitis media in upper airway infections [10,35,36]. If they have a protective role in MEF samples, they might also be part of the normal flora of the tympanic cavity. *Moraxella* as the most common known otopathogen was more frequent in those who have had subsequent symptoms, whereas *Enterobacteriaceae* were less common in the same group of patients. High occurrence of *Moraxella* in children with recurrent symptoms could be explained with its high virulence and resistance to antibiotics [4].

Comparing the conventional culture method with the molecular biological method we found that presence of the known otopathogens such as *H. influenzae*, *S. pneumoniae* or *S. pyogenes* by cultivation correlated with the sequencing results. However, the cultured otopathogens were not always the most common bacteria in the molecular biology results (Table 2). Moreover, otopathogens detected by NGS could not be cultured for unknown reasons. The other half of the cultured samples showed no correlation with the 16S rRNA results, and only bacteria from the commensal flora were detected. These discrepancies should draw the attention of everyday clinical practice to the correct evaluation of the findings. Focusing on the treatment of the cultured bacteria could lead to overlooking the complex bacterial involvement. Bacteria that are hard to culture, may be potential otopathogens, therefore culture-based techniques could miss them. A major benefit of conventional culture methods is that they provide antibiotic resistance patterns. However, we did not detect any bacteria resistant to first-line antibiotics used for the treatment of otitis media.

5. Conclusion

The 16S RNA microbiome analysis seems to have a significantly higher sensitivity than conventional culture techniques in identifying bacteria in MEF. Our results suggest that in *A. otitidis* is the most common bacterium in Hungarian pediatric patients with OME, followed by *H. influenzae* and *Streptococcus* spp. High prevalence of *A. otitidis* potentially contribute to lower alpha and beta diversity of middle ear microbiome in children with OME. Our results suggest that long breastfeeding and birth by Caesarean section may influence the composition of the microbiome in the middle ear fluid.

Further investigations are needed to determine the exact role of *A. otitidis* in middle ear pathologies.

CRediT authorship contribution statement

Szilvia Fekete: Writing – original draft, Investigation, Data curation. **János Juhász:** Writing – original draft, Visualization, Software, Methodology, Investigation. **Nóra Makra:** Methodology, Investigation. **Zsuzsanna A. Dunai:** Methodology, Investigation. **Katalin Kristóf:** Validation. **Eszter Ostorházi:** Methodology. **László Tamas:** Writing – review & editing. **Dóra Szabó:** Writing – review & editing, Validation, Software, Funding acquisition, Conceptualization. **Nóra Kecskeméti:** Writing – original draft, Data curation. **Gábor Polony:** Writing – original draft, Data curation.

Data availability

The metagenomics datasets generated during the current study are deposited in the NCBI Short Read Archive (SRA) (https://www.ncbi.nlm.nih.gov/sra) under accession number: SAMN36433029 to SAMN3643306 in bioproject PRJNA994511.

Ethical approval

This study was approved by the Semmelweis University Regional and Institutional Committee of Science and Research Ethics (29-1/2018). Written informed consent form was obtained from caregivers of the patients before enrollment.

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Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Dora Szabo reports financial support was provided by Hungarian Research Network. Dora Szabo reports financial support was provided by Horizon Europe. Dora Szabo reports a relationship with Hungarian Research Network that includes: funding grants. Dora Szabo reports a relationship with Horizon Europe that includes: funding grants. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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