Identification of Microbial Community in Otomycosis by mNGS: Potential implication for treatment of this disorder with terbinafine

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Abstract

The present study was designed to identify the microbial community as well as to analyze its diversity bymeans of metagenomic Next Generation Sequencing(mNGS)in 17 patients with otomycosis treated with terbinafinein the Department of Otolaryngology of Shandong Provincial Hospital from June 2021 to June 2022, so as to evaluate the relationship between microbial community and terbinafine resistance. Those 17 patients were divided into two groups, i.e., Terbinafine Effective Group (TEG, n = 14 cases) and Terbinafine Resistance Group(TRG, n = 3 cases) according to the therapy effect, whose microbial community of secretion of external auditory canal (EAC)was identified using mNGS. We found that the sequence of bacteria was significantly more than that of fungi and, whereas, the difference between the two groups of bacteria was not significant.There were significant differences in fungal community between the two groups. Aspergillus was the main pathogenic fungus of TEG patients while Malassezia was a dominant fungus in TRG patients. In conclusion, the results from this work indicate that Aspergillus terreusis the main pathogenic fungus in this cohortof otomycosis patients and MNGS sequencing can offer comprehensive information about the microbial community of otomycosis. The fungus community dominated by Malassezia is more likely to be resistant to terbinafine, which provides certain guidance for clinical treatment of otomycosis with terbinafine.

1. Introduction

In spite of the earlier emergence of fungal pathogens,for more than a century, it has been documented that the most common infections are caused by bacteria, viruses, and other parasites[1]. However, theprevalence of serious diseases resulted from fungi has increased inrecent decades due to the increasing number of immunocompromised individuals[2]. Meanwhile, in severely immunocompromised patients, some fungus can disseminateinto the bloodstream and colonize internal organs, resulting inlife-threatening systemic infections[3, 4].

Otomycosis is a worldwide disease, in which appropriately 15–20% of external ear infections arecaused by mycelial fungi and yeasts[5].Otomycosisis an infection that involves the EAC squamous epithelium, characterized by pruritus, erythema, scaling,otalgia,aural fullness and hypoacusis[5, 6]. The most frequently reportedpathogens are Aspergillus and Candida species[6–8].

Fungal communities in otomycosis may vary in different areas. According to the latest literature,A. niger, A. terreus,A. tubingensisand A. awamori were the most frequent species in Aspergillus,while, C. albicans was by far the most common yeast in Candida[9–13].

In clinical practice, early identification of fungal pathogens is critical for thediagnosis and treatment of otomycosis. The traditional methods such as culture-based methods can only identify the main pathogens in most cases[14],whereas, molecular microbial tools, like next-generation sequencing (NGS), have the advantage that they are culture-independent and more sensitive. NGS uses anuntargeted sequencing approach, which can identify and quantify bacteria and fungi presentin a sample, including

previously unknown microbes. Using untargeted sequencing method, NGS can not only identify and quantify common bacteria and fungi, but identify previously unknown microorganisms as well. As a kind of NGS, mNGS(also termed high-throughput sequencing technology) can sequence the sample microbial genes, which can realize the sequencing of all microbial genomes, assemble and obtain the microbial genome information, and carry out the annotation and difference comparison of microbial potential functions.

Predisposing factors include residing in tropical and humid climates, repeated swimming, the insertion of foreign bodies, use of hearing aids, the presence of cerumen, lack of hygiene, the use of long-term antibiotic or steroid therapy, repeated cleaning of the EAC with swabs, genetic factors, seborrheic dermatitis, diabetes, and immune defects, all of which benefit the germination of the spores and conidia of the prevalent fungi[15–17].

Treatment mostly requires the use of topical antifungals such as clotrimazole,terbinafine,ketoconazole, econazole, ciclopiroxolamine, nystatin,tolnaftate, bifonazole, and miconazole for at least three or four weeks[7, 18, 19].

The fungal pathogens of otomycosis have been reported by researchers from most countries. But as yet, there have been only few studies on the co-existence and interaction of bacteria and fungi in otomycosis. With the widespread application of antifungal drugs to treat otomycosis, more and more studies of antifungal drug resistance have been reported. Antifungal resistance is emerging as a public health challenge that needs to be addressed concurrently with antimicrobial and antiviral resistance.Currently, people in the United States is heavily infected with a super fungus called Candida auralis, which has resistance to multiple antifungal drugs and causes about half of infected people to die within three months. In this study, 17 cases of otomycosis treated with terbinafine in Jinan, China, from June 2021 to June 2022 were analyzed retrospectively, and their microbial composition and possible interaction were studied by mNGS, with emphasis given on comparing the therapeutic effect of terbinafine, the relationship between different microbial communities and drug resistance to terbinafine.

2. Materials And Methods

2.1 Sample Collection

The samples were collected from Shandong Provincial Hospital and were all the EAC irrigations that had been obtained in the past. Among them, 14 patients recovered with terbinafine treatment after 1 month, and 3 patients were ineffective after 1 month.

2.2 Sampling Method

17 patients wereall unilateral. The affected ear was washed with sterile physiological saline, and the washing solution was stored in a sterile sampling tube, and stored at − 80 ℃ for DNA detection.

2.3 Sample Inclusion and Exclusion Criteria

2.3.1 Inclusion Criteria:

The age of patients was between 18 and 60 years old, all of whom were the first time to get sick. The patients hadat least one of the symptoms such as pruritus, tinnitus, otalgia, otorrhea,aural fullness and hypoacusis.Punctate, villous, or lumpy plaque could be seen in EAC under the otoscope(Fig. 1A-1C). All fungal smears were positive, and fungal hyphae orsproes could be seen(Fig. 1D-1F).

2.3.2 Exclusion Criteria

The exclusion criteria were as follows: patients who had applied antibacterial drugs locally and systemically within 1 month; patients with serious systemic diseases and immune deficiency; a diagnosis different tootomycosis; and pregnant and lactating women.

2.4 DNA Extraction and Sequencing Analysis

2.4.1 DNA Extraction

We used the QIAamp DNA Microbiome Kit(50) of Kaijie Company to extract DNA from each sample in strict accordance with the product operating instructions. The DNA degradation degree and potential pollution were monitored on the 1% agarose gel, and the purity and integrity of DNA were analyzed. The DNA concentration was accurately quantified with Qubit 4.0 fluorometer.

2.4.2 Construction of standard sequencing library

The DNA samples were randomly broken into fragments with a length of about 350 bp using a Covaris ultrasonic crusher, and the library was constructed through terminal repair, A-tailed addition, splicing, purification, and PCR amplification. After the construction of the library, we used a Qubit 4.0 fluorometer for preliminary quantification, and diluted the library to 2 μ g/ μ l. Subsequently, the length of inserted fragments in the library was detected using an Aglent 2100 biological analyzer.

2.4.3 High-throughput Sequencing

According to the standard scheme, the Illumina platform (PE150 sequencing method) was used for standardized sequencing to obtain the required DNA sequence.

2.5 Data processing

We used FastQC (version v0.11.9) to perform base quality statistics on the original sequencing data, and used R statistical software to visualize the results.Trimmatic(Version 0.39)was used to cut the joint sequence, double-ended low-quality sequence, and double-ended pairing sequence greater than 36 bp was reserved. BMTagger (version 3.102) was used to remove the host genome sequence.

2.6 Beta Diversity Analysis

Non-metric Multi-dimensional Scaling (NMDS) based on Bray-Curtis distancewas used to compare the composition of microbial community among different samples, and the difference of microbial community structure between different samples was evaluated by the distance between points.

3. Results

3.1 Raw Data and Quality Control

The bacterial and fungal DNA in the EAC secretion of patients with otomycosis was sequenced with high flux, and the sequences obtained from each sample ranged from 29815652 to 87812501. After quality control, the sequence number of fungi varied from 11144 to 1746156, whilethe number of bacteria varied from 418491 to 8968300. The sequence number of bacteria were significantly higher than that of fungi.

3.2 Community Composition of Bacteria and Fungi

At the phylum level, the difference of bacteria between the two groups was not obvious. Three categories of microorganisms, namely Firmicutes, Actinobacteria and Proteobacteria occupied an absolute dominant position, and their proportions in different samples ranged from 89.9–99.8% (Fig. 2A).

At the phylum level, the fungal community structure of TEG and TRG was significantly different.Ascomycota was the main fungi in TEG, accounting for more than 95% in 8 samples, while Basidiomycota was dominant in TRG, accounting for more than 79% in 3 samples(Fig. 2B).

At the genus level of bacteria, Staphylococcus and Bacillus_ A were the most common bacteria in the two groups (Fig. 3A). For fungi, Aspergillus of Ascomycetes was the main fungus in TEG, of which 7 cases accounted for more than 90%, while the fungi in TFG was mainly Malassezia, of which 2 samples accounted for more than 65% and 1 case accounted even for 97% (Fig. 3B).

At the bacterial species level, Staphylococcus aureus of Staphylococcus was dominant, while Staphylococcus epidermidis, Staphylococcus capitis and Bacillus_Abombysepticusin Bacillus_ A were also common bacteria (Fig. 4A). For fungi, the most common fungus in TEG was Aspergillus terreus (Fig. 4B), while the most common fungus in TRG was Malasseziarestricta, of which one case accounted for more than 90%.

At the same time, in all samples, Staphylococcus aureus and Aspergillus terreus accounted for the largest proportion of bacteria and fungi respectively (Fig. 5A and 5B).

3.3 Analysis of Microbial Community Difference

Beta diversity analysis was performed in order to show the difference between the two groups.Non-Metric Multi-Dimensional Scaling(NMDS) based on species level showed that the bacteria in two groups were not completely separated, and there was no significant difference between samples(Fig. 6A), which

indicated that there was no obvious difference in bacterial community structure inotomycosis, and bacteria were not the main factor causing fungal drug resistance.

For fungi, there were significant differences between the two groups. The sample points of TEG and TRG were gathered respectively and separated significantly, indicating that there were significant differences in the fungal community structure between the two groups (Fig. 6B). Samples of TRG were resistant to terbinafine because of their different fungal community structures.

4. Discussion

Early identification of pathogens is essential for the diagnosis and treatment of otomycosis. In general, detection of pathogens of otomycosis mainly depends on traditional culture-based methods and modern sequencing technology. However,culture-negative strains or resident funguses could be hardly detected by regular culture conditions[14]. Some scholars had proved that thepositive culture rate of the agents causing fungal infections was 50%, especially when Malassezia species or Mucorales were highly suspected[20]. It had been reported thateight causative fungal genera were identified by ITS sequencing while five fungal genera were identified by culture in fungal keratitis. Moreover, Gu et al. identified the fungal community of otomycosis patients in Nanjing, Jiangsu Province, China, through ITS, and successfully detected some uncommon funguses such as Sagenomella and Cladosporium[21]. However, the limitation of ITS is that it is not suitable for the marking of species within the genus due to the small differences in the interval because of the evolutionary order and variation of fungi.

MNGS is highly sensitive and informative, which possesses the ability to detect mixed infections of bacteria, fungi and virus. Therefore, it has the advantage of congenital thickening for diseases caused by multiple pathogens.In this work, fungi and bacterial communities in otomycosis were identified simultaneously by mNGS. Most researchers from different countries indicatedthat A. nigerserved as the most commonotomycosis agent[22–25]. However, through mNGS, we found that the most common fungus in otomycosis was Aspergillus terreus, which was consistent with the study of Zhang et al.[11] in Hangzhou, China. This indicates that the fungal community of otomycosis may vary in different region. In western China, Aspergillus tubingensiswas the most common fungi, followed by Aspergillus fumigatus and Aspergillus terreus[26].Studies in Iran had found that Aspergillus flavus was the most common species of otomycosis, followed by Aspergillus tubingensis and Aspergillus niger[8], while Aspergillus awamori was considered to be the most common fungi causing otomycosis in studies in southern Hungary[27]. In India, Aspergillus niger and Aspergillus fumigatus were dominant in otomycosis[17].Candida albicans had also been reported as a common pathogen of otomycosis, which mainly occurred in patients with abnormal immune function[28]. To date, however, the bacterial community in otomycosis has been studied sparingly. In the present study, we found through mNGS that the most common bacteria in otomycosis patients was Staphylococcus aureus.At the same time, staphylococcus aureus is the main pathogen of bacterial external otitis, which can proliferate and infect the human body, leading to inflammation when human immunity is reduced and the environment of the ear canal changes[29].

Currently, the treatment of otomycosis currently relies mainly on topical use of antifungal drugs. Terbinafine is more effective against partial Aspergillus species in vitro than itraconazole or amphotericin B[30]. Meanwhile, terbinafine is considered to be an effective drug in the treatment of otomycosis[31]. It has been reported that the cure rate of Terbinafine in the treatment of otomycosis reaches 100%[32], while, in this study, 14 of the 17 patients treated with Terbinafine have been cured, with a cure rate of 82.4%, which might be related to the increasingly severe fungal resistance at present. It is worth noting that Aspergillus is the main cause of cured cases in our study, beingconsistent with the above report[32]. Some Malassezia strains have been reported to be resistant to terbinafine[33]. We found that the fungal community dominated by Malassezia has resistance to terbinafine, and the precise mechanism of resistance needs to be further studied.

In summary, the present study identifies by means of mNGSthat the most common fungal species among patients with otomycosis in the city of Jinan, Shandong Province, of the People's Republic of China is Aspergillus terreus, and the most common bacterial species is Staphylococcus aureus. Fungi dominated by Malassezia are more resistant to terbinafine than fungi dominated by Aspergillus. Findings from this work have certain guiding significance for the clinical treatment of otomycosis with terbinafine. The specific mechanism underpinning drug resistance needs to be further researched.

Declarations

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Conflict of interest The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

Author Contributions All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Shuai Xu, Xin Zhang, Jianfeng Li and Zhaoyan Yu. The first draft of the manuscript was written by Shuai Xu and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Ethical Approval This research was approved by the Biomedical Research Ethics Committee of Shandong Provincial Hospital.

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Figures

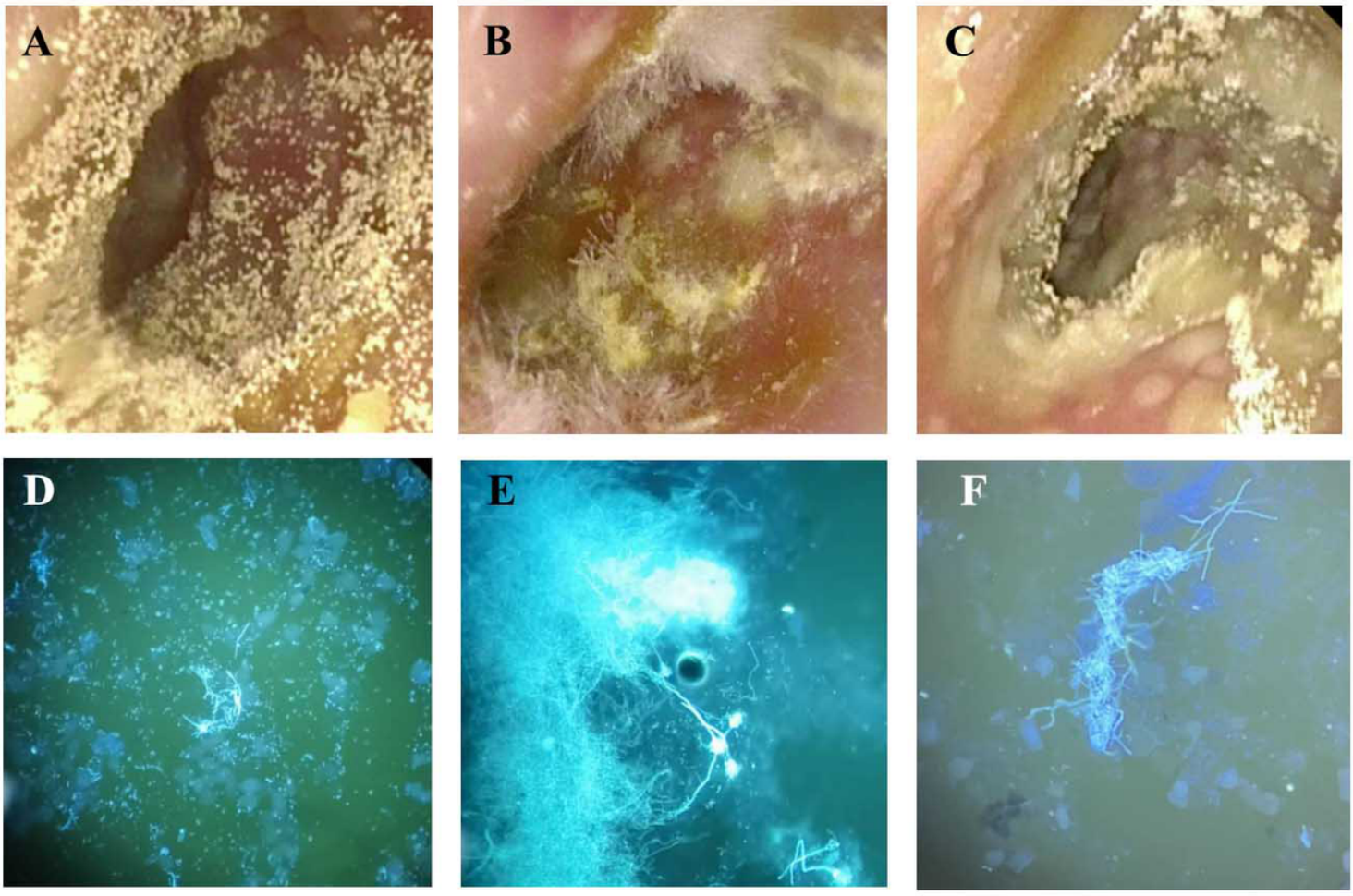

Figure 1

(A-C) Punctate, villous, and lumpy plaque can be seen in EAC under the otoscope. (D-F) Scattered fungal hyphae and spores can be seen under microscope

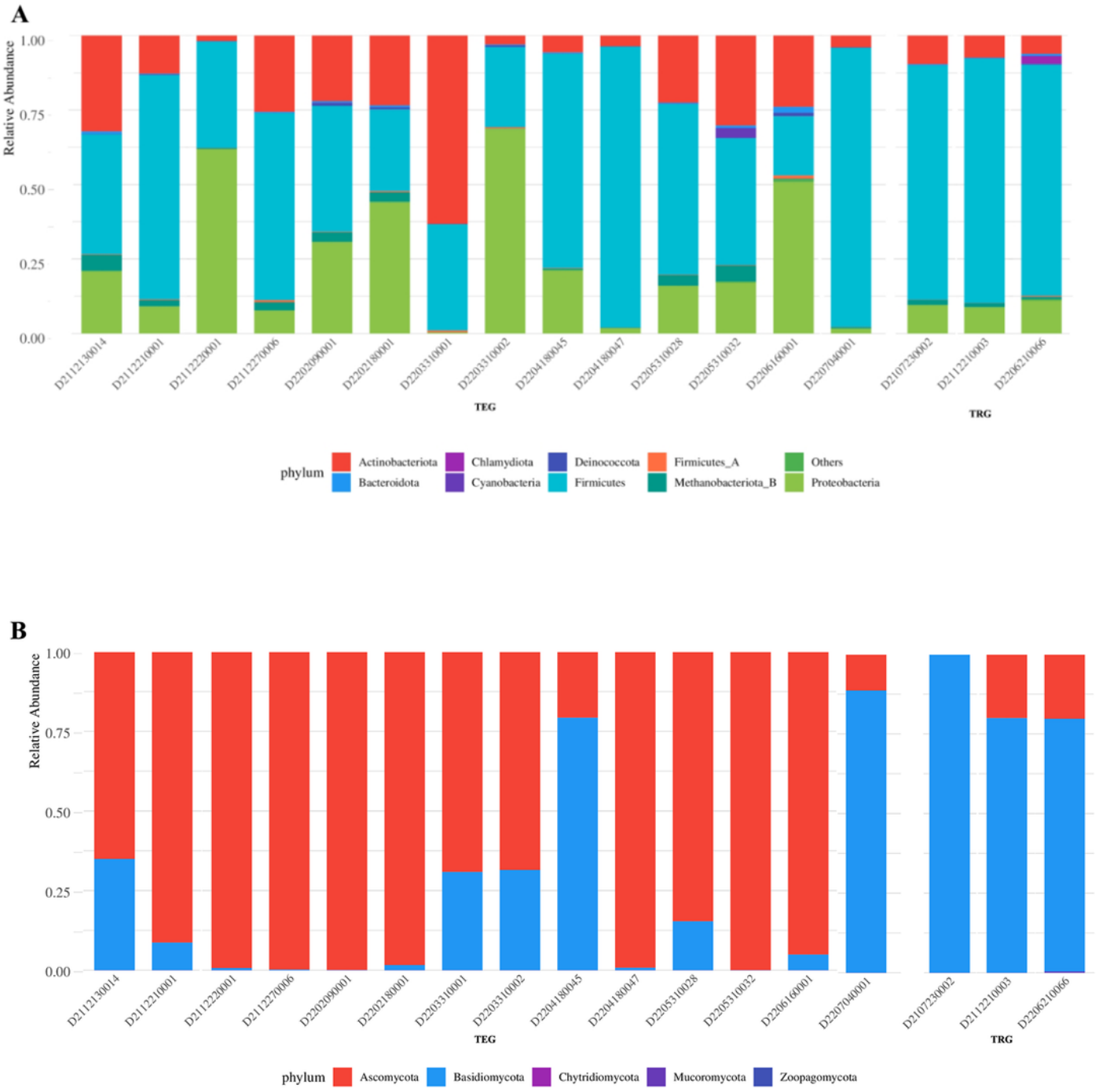

Figure 2

Relative abundance of bacterial(A) and fungal(B) communities at phylum level in EAC of patients in TEG and TRG

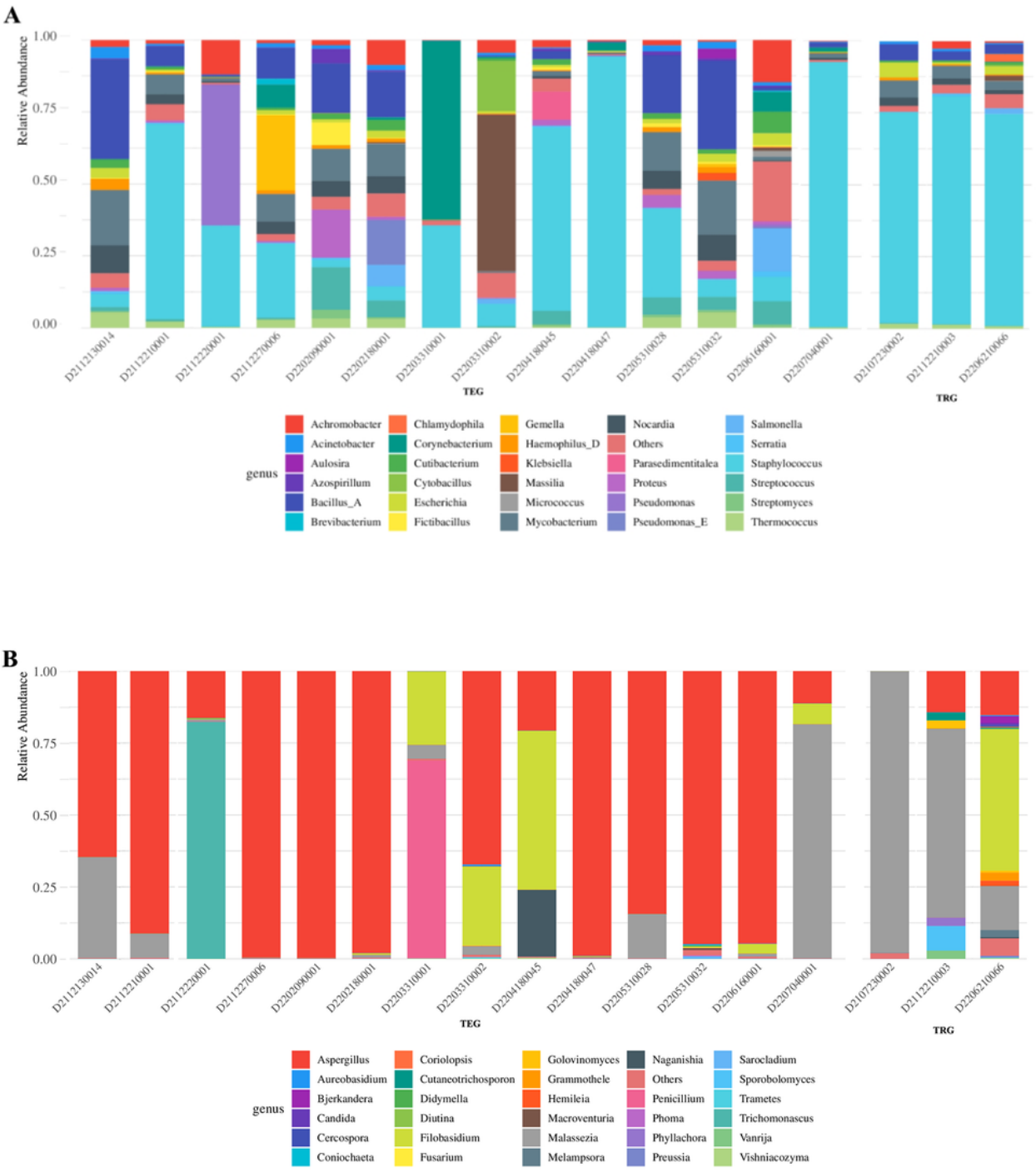

Figure 3

Relative abundance of bacterial(A) and fungal(B) communities at genus level in EAC of patients in TEG and TRG

Figure 4

Relative abundance of bacterial(A) and fungal(B) communities at species level in EAC of patients in TEG and TRG

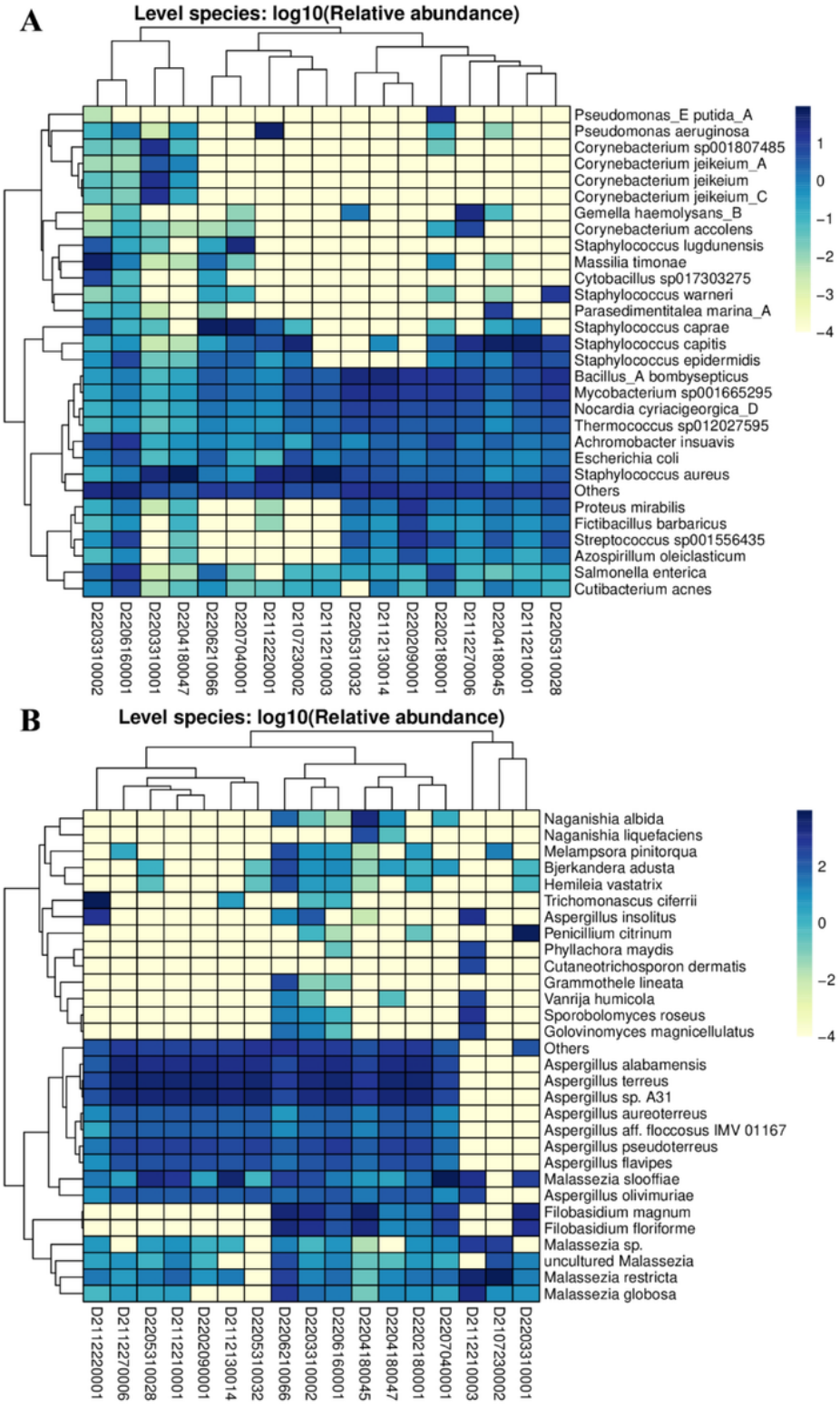

Figure 5

Heatmap of relative abundance of bacterial(A) and fungal(B) communities at species level in all patients

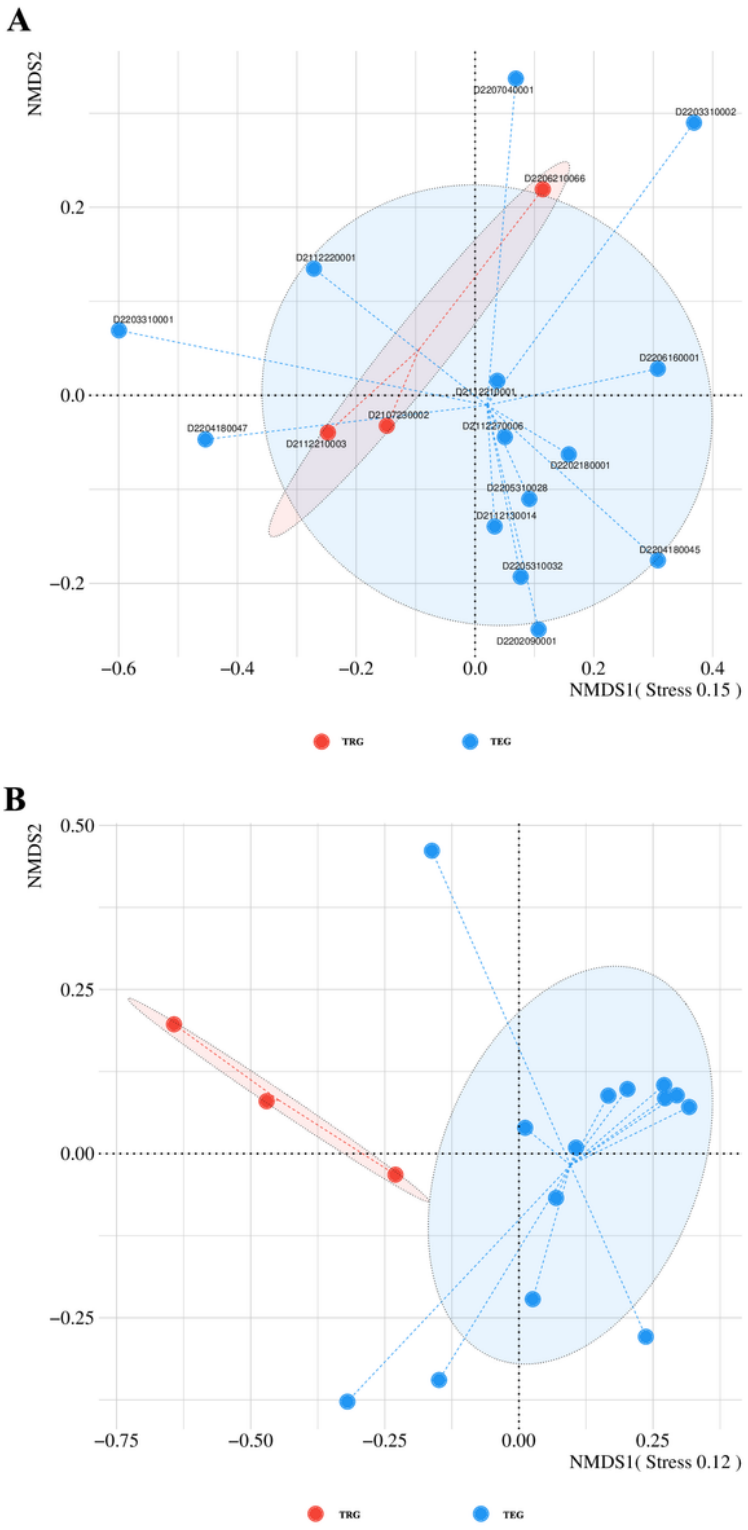

Figure 6

Non-Metric Multi-Dimensional Scaling (NMDS)based on species level in two groups. Each point in the diagram represents a sample, and samples from the same group are represented by the same color.For grouped samples, ellipses will be used to display the distinguishing areas of the sample group, and the distance between points indicates the degree of difference.Generally, when the Stress is less than 0.2, it indicates that NMDS analysis has certain reliability