Conjunctival Sac Microbiome in Infectious Conjunctivitis

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Research

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Abstract

Background

To determine the conjunctival bacterial community among the infectious conjunctivitis cases attending the outpatient clinic of Khanh Hoa General Hospital in Nha Trang, Vietnam from October 2016 through December 2017. Of all, 50 randomly selected samples using a computer generated random number list were included for microbiome identification. Conjunctival swabs were collected and tested using conventional culture, PCR, and 16S ribosomal RNA sequencing.

Results

The study included randomly selected 47 patients. More than 98% of all DNA reads represented five bacterial phyla. Three of these phyla constitute 92% of all sequences [*Firmicutes* (35%), *Actinobacteria* (31%), and *Proteobacteria* (26%)]. At the genus level, there were twelve common genera constituted about 61% of all the sequence reads. Seven of those genera were common [*Streptococcus* (10%), *Cutibacterium* (10%), *Staphylococcus* (7%), *Nocardioides* (7%), *Corynebacterium* 1 (5%), *Anoxybacillus* (5%), and *Acinetobacter* (5%)], which encompassed 49% of all reads. As for diversity analysis, there was no difference in PERMANOVA analysis (Unweighted UniFrac) for sex (P = 0.087), chemosis (P = 0.064) and unclassified eyedrops (P = 0.431). There was no difference in PERMANOVA analysis for pain (P = 0.315) and itching (P = 0.133). There was a statistical significant difference in cases with bilateral conjunctivitis (P = 0.017) and for using antibiotics (P = 0.020).

Conclusion

Firmicutes among the predominant phyla has the highest abundance in bacterial conjunctivitis in our study. Pseudomonas as a resident commensal microbiota has an important role for prevention of infection.

Introduction:

Acute bacterial conjunctival infections are common [1, 2]. Although many cases show a benign course, some can be associated with sight-threatening ocular complications as corneal ulcers, endophthalmitis, panophthalmitis, and perforation of the globe [3]. Identification of the causative pathogens in these cases is mandatory but often difficult because some bacteria have special growth requirements [4]. The causative pathogens of bacterial conjunctivitis have been determined conventionally by both the smear method and the culture method. However, the results of these methods are not always conclusive [5]. The bacterial species detected in eyes with bacterial conjunctivitis also have been found in normal conjunctival sacs [6, 7]. Furthermore, sample size from ocular tissues is usually small, leading to unreliable cultivation results and it is not easy to detect the rarely encountered, slowly growing and

uncultivable bacteria. Initiation of proper therapy can be delayed with possible devastating visual consequences [4].

Because of these limitation, the advent of cultivation-independent techniques of microbial identification such as polymerase chain reaction (PCR) and 16S ribosomal RNA (rRNA) sequencing has provided a much more detailed picture of the human bacterial microbial consortium than was available through traditional culture techniques [8].

Thus, the purpose of this study was to identify the bacterial community in conjunctival sacs of eyes with infectious conjunctivitis by using 16S rRNA metagenome sequencing.

Patients And Methods:

Study design

Sample collection and testing

We enrolled patients at any age with infectious conjunctivitis visiting the ophthalmology outpatient department, if the written informed consent was obtained, from October 2016 through December 2017 for bacterial community identification. Conjunctival swabs were obtained carefully from the severely infected patient's eye. There were 793 conjunctivitis cases enrolled to the conjunctivitis study during that study period. Due to the budget limitation, fifty cases were randomly selected among these samples using a random list generated by a computer. The randomly selected samples were used for detection of micro-organisms using conventional culture and PCR examinations.

Collection of a Conjunctival Swab

Prepare tube with 800ul of normal saline and open the cap before collecting the sample. Soak the swab with 2-3 drops of sterile saline, pull down the lower eyelid of the most inflamed eye and gently sweep the swab on the conjunctiva from inner to outer canthus. Put the swab in media to the bottom, cut the shaft of the swab by sterilized scissors and close media's screw top and kept at 4°C until analyzed.

Specimens were collected and the initial conventional cultures (blood and a chocolate agar nutrient medium) conducted at Khanh Hoa General Hospital. DNA extracted and screened by realtime PCR and 16S rRNA sequencing assay in the Department of Laboratory Medicine at Nagasaki University Hospital.

PCR Amplification and Preparation for 16S rRNA Gene Sequencing

Data of 16S rRNA metagenome were obtained as described previously by Morinaga *et al.*[(9)] DNA was extracted using a Quick-DNA Fecal/Soil Microbe Miniprep Kit (ZYMO Research, Irvine, CA), according to the manufacturer's instructions. The V1-V2 region of the bacterial 16S rRNA genes was amplified. After emulsion PCR, enriched samples were loaded onto an Ion 318 chip and sequencing was performed using the Ion Torrent Personal Genome Analyzer (Thermo Fisher Scientific, Waltham, MA).

Sequence Analysis

The sequencing reads were analyzed using CLC Genomics Workbench version 12.0.1 and CLC Microbial Genomics Module version 3.6.11 (QIAGEN N. V., Venlo, Netherlands) as described previously [9]. After removing the primer sequences and trimming the read length between 200bp and 400bp under 0.01% quality limit, samples with fewer than 100 reads and less than 50% from the median were excluded from the further analyses. Chimeric reads were filtered using the chimera crossover detection algorithm with the default parameters. The reads were categorized into operational taxonomic units (OTUs) with 97% similarity and then assigned using SILVA release 132. The number of OTUs, Shannon index (alpha diversity), and Weighted UniFrac distances were analyzed using the CLC Microbial Genomics Module. Differences in bacteria abundance were calculated using LEfSe with default parameters as described by Segata *et al* [10]. Differences at Genus level bacteria abundance were analyzed after removing sequences of mitochondria and non-available ones from the data.

Statistical Analysis

To compare the beta diversity, the data were analyzed in PERMANOVA analysis using the CLC, and the statistically significant alpha level was set as $p \leq 0.05$ (Mann-Whitney test), in false discovery rate (FDR).

Results:

Our final cases included 47 randomly selected infectious conjunctivitis cases because three samples were discarded due to technical fault. Twenty-eight females and 19 males shared in this study with age average 38.0 ± 20.6 years (range 1–74).

Bacterial community composition in conjunctivitis

To identify bacterial composition in human conjunctivitis, the 16S rRNA metagenomic sequences were classified at both the phylum and the genus levels (Fig. 1). More than 98% of all DNA reads represented five bacterial phyla. Three of these phyla constitute 92% of all sequences [*Firmicutes* (35%), *Actinobacteria* (31%), and *Proteobacteria* (26%)] (Fig. 1A). The other two phyla [*Bacteroides* (6%) and *Cyanobacteria* (0.9%)] were present in lower quantities. *Acidobacteria*, *Fusobacteria*, and others were present in contamination level quantities (0.5% or less).

At the genus level, there were twelve common genera constituted about 61% of all the sequence reads (Fig. 1B). Seven of those genera were common [*Streptococcus* (10%), *Cutibacterium* (10%), *Staphylococcus* (7%), *Nocardioides* (7%), *Corynebacterium* 1 (5%), *Anoxybacillus* (5%), and *Acinetobacter* (5%)], which encompassed 49% of all reads. The other five of those were less abundant [*Janibacter* (3%), *Porphyromonas* (3%), *Bacillus* (2%), *Clostridium sensu stricto* 7 (2%), and *Haemophilus* (2%)].

Diversity analysis:

As for diversity analysis, there was no difference in PERMANOVA analysis (Unweighted UniFrac) (Beta diversity analysis) for sex (P = 0.087), for chemosis (P = 0.064) and for unclassified eye drops (P = 0.431). Also, there was no difference in PERMANOVA analysis for pain (P = 0.315), for itching (P = 0.133), and for visual loss (P = 0.05005). There was a statistical significant difference in bilateral versus unilateral conjunctivitis (P = 0.017) (Fig. 2) and for using antibiotics (P = 0.020) (Fig. 3), although there was no statistical significant difference in these factors using Shannon index (Alpha diversity analysis).

Linear Discriminant Analysis:

We additionally performed Linear Discriminant Analysis (LDA) to find significant bacteria, which are associated with the laterality and the use of antibiotics.

No significant phylum was observed in either group (Maybe due to large variation) (Fig. 4A).

Genera *Prevotella*, *Pyrinomonas*, *Tessaracoccus*, *Pelomonas*, *Exigubacterium*, and *Roseibacterium* were increased in the non-antibiotic group and Genera *Paenibacillus*, *Afipia* and *uncultured*_27 were increased in the antibiotic group (Fig. 4B).

Phyla *Bacteroidetes* and *Acidobacteria* were significantly increased in the unilateral group, while no significant phylum was observed in the bilateral group (Fig. 4C, D).

Porphyromonas, *Prevotella*, and *Stenotrophomonas* genera increased in the unilateral infective group and Genera *Cutibacterium*, *Dolosigranuulum, uncultured*_29, and *Clostridiumsensustricto 12* genera increased in the bilateral group (Fig. 4E).

Comparison of Cultivation and Metagenome:

A considerable difference was observed in the increased diversity of bacterial populations determined by rRNA sequencing compared with cultivation. Culture was only positive in four cases (8.5%). Three of them were positive for Gram-positive Cocci and the fourth case was positive for Alpha hemolytic *Streptococci*. One of the three cases also positive for *Acinetobacter* spp.

In case No.401, the most abundant bacterial in the metagenome result was *Acinetobacter* (38%) which was congruous to the conjunctival cultivation result (Fig. 5). The other three cases (No. 008, 434, 487), the most abundant bacterial were *Streptococci* and *Staphylococci* in the metagenome, which also were congruous with the conjunctival cultivation results (Fig. 5).

Discussion:

Overview of microbiome in all patients:

To our knowledge, this is the first study trying to identify bacterial composition in human conjunctivitis using 16S rRNA metagenome.

Culture-independent approach by 16S rRNA metagenome sequencing has been used to identify our microbiota during both healthy condition and illness.

In a smaller study using only 4 healthy volunteers, *Dong et al*. identified a core conjunctival microbiome of five bacterial phyla using 16S rRNA sequencing, three of which [*Proteobacteria* (64%), *Actinobacteria* (19.6%), and *Firmicutes* (3.9%)] accounted for > 87.9% of all sequences [11]. The other two phyla *Cyanobacteria* and *Bacteroidetes* were found in contamination-level quantities (0.21% and 0.16%, respectively) [11]. In another larger study (31 subjects), *Huang et al*. using the 16S rRNA gene sequencing reads classified the conjunctival microbiome into 25 bacterial phyla. Most sequences (98.88%) were affiliated predominantly with five phyla, which included *Proteobacteria* (46.50%), *Actinobacteria* (33.89%), *Firmicutes* (15.50%), *Bacteroidetes* (2.28%), and *DeinococcuseThermus* (0.71%) [12].

Although *Firmicutes* constituted the least abundance in their studies among the main first three phyla (*Proteobacteria, Actinobacteria, Firmicutes)*, but in our study is the highest one (one of most significant results in this study).

In another study, the conjunctiva of 45 healthy subjects were sampled at three time points over three months with the aim of understanding whether the microbial communities of the ocular surface (OS) change over time. They determined that the majority of phyla at each time point consisted of *Proteobacteria* (range 52–73%), *Firmicutes* (13–20%) and *Actinobacteria* (8–22%) [13]. Thus, our study is the first to identify that *Firmicutes* is the main predominant phylum in conjunctival microbiome in conjunctivitis cases. It is difficult to explain the reason why Phylum *Firmicutes* is predominant in the patients with conjunctivitis. However, the consumption of oxygen by growth of causative bacteria including facultative aerobes may provide anaerobic condition, which has the advantage for Phylum *Firmicutes.*

In comparison between our study and other previous normal studies, as regards to predominant genera (> 1%), we determined three groups. The first group includes the predominant genera shared with other normal studies [11–14]. This group includes *Streptococcus*, *Cutibacterium, Staphylococcus*, *Corynebacterium*, and *Acinetobacter*. The second group includes genera only present in our study and not present in normal previous studies. This group includes *Nocardioides, Anoxybacillus, Janibacter*, *Porphyromonas*, *Bacillus*, *Clostridium sensu stricto* 7, and *Haemophilus*. Last group includes predominant genus which is present in normal studies and absent in our study as *Pseudomonas* [11–13, 15].

Advances in next-generation sequencing and bioinformatics tools have revealed an expansive and diverse microbial community inhabiting the human conjunctiva. The most abundant genera identified using 16S rRNA sequencing were *Pseudomonas*, *Bradyrhizobium*, *Cutibacterium*, *Acinetobacter*, and *Corynebacterium* [15, 16].

It has been suggested that these “normal bacteria” serve a protective role under most circumstances by directly inhibiting colonization of more pathogenic species [17]. Many previous studies confirmed that

Pseudomonas represented a major genus in healthy conjunctiva [11–13]. *Lee et al*. compared between the ocular microbial communities with and without blepharitis [18]. They confirmed that the relative proportions of *Staphylococcus*, *Streptophyta*, *Corynebacterium*, and *Enhydrobacter* were higher in subjects with blepharitis than in healthy subjects [18]. However, the proportions of *Pseudomonas* clearly was lower in subjects with blepharitis than in healthy subjects, suggesting that *Pseudomonas* might be an important as resident commensals microbiota for the prevention of blepharitis [18].

In our study, we confirmed that *Pseudomonas* was present in scanty percentage in cases of conjunctivitis and agreed with previous study in their suggestion about the importance of *Pseudomonas* as resident commensals microbiota for prevention of infection.

For the ocular microbiota during purulent conjunctivitis, studies using previous methods such as the denaturing gradient gel electrophoresis (DGGE) and the clone library methods have revealed that genera *Staphylococcus*, *Corynebacterium*, and *Cutibacterium* were commonly observed [19, 20]. However, there is a bias in these studies because the findings were basically based on the database of clinically-known bacteria. Thus, our study provided the composition of ocular microbiota including uncultured bacteria with reducing the methodological selection bias.

Microbial diversity:

The diversity of microbial communities in the subjects was assessed with alpha diversity analysis. The observed genesis and Shannon index were used to evaluate the richness and biodiversity of the microbiota. Beta diversity refers to species diversity among different groups. Beta diversity and alpha diversity together constitute the biological heterogeneity of overall diversity or a certain community or group. The beta diversities in different groups were calculated using the Unweighted UniFrac distances of 16S rRNA genes between microbial communities or groups.

In our study, there were no difference in Beta diversity analysis for sex (P = 0.087), chemosis (P = 0.064), unclassified eye drops (P = 0.431), pain (P = 0.315) and itching (P = 0.133). There was a statistical significant difference between bilateral versus unilateral conjunctivitis (P = 0.017) and for antibiotics (P = 0.020) (Fig. 3A, B), although there were no statistical significant differences in these factors using Shannon index (Alpha diversity analysis). These results, to our knowledge, considered the first diversity analysis for subjects with conjunctivitis in the literatures.

Linear discrimination analysis:

We additionally performed LDA to find out significant parameter from large data and found that Phyla *Bacteroidetes* and *Acidobacteria* significantly increased in the unilateral infected group, while no significant phylum observed in the bilateral group. In addition, *Porphyromonas*, *Prevotella* and *Stenotrophomonas* genera significantly increased in the unilateral group. These results were reasonable because Genera *Porphyromonas* and *Prevotella* actually belonging to Phylum *Bacteroidetes*, and would support our results.

Individual metagenomics:

The rate for the culture method has been reported to be between 47.5% and 97.8% in eyes with bacterial conjunctivitis [19–21] and 9.0–90.6% [6, 7, 12, 22–24] in normal conjunctival sacs.

To overcome culture limitations, there has been an increase in the number of studies using molecular methods, for example, PCR with species-specific primers [25–27], amplification of the 16S rRNA gene by PCR using universal primer sets followed by direct sequencing [28–30], DGGE [19], and pyrosequencing [11].

16S rRNA sequencing has been used for bacterial identification and discovery of novel genera leading to extending our knowledge about OS microbial diversity [4, 11, 12, 14].

In our study, we had only 4 (8.5%) positive conjunctival cultures and all of them were congruous with abundant genera with 16S rRNA sequencing analysis. From our point of view, if the result of 16S rRNA sequencing analysis denoting abundance of one or more genera in the conjunctivitis sample, most probably it is/are the causative pathogen especially if the culture is congruous with it.

Limitations:

We have some limitations in our study. First, we have no control normal conjunctival samples. Second, 16S rRNA sequencing analysis method is unable to identify bacterial species level and is prone to noise, sampling errors and contamination. Finally, using metagenomics data reveals extensive results that generates huge amount of data, which becomes difficult for a clinician to record and analyze on a routine basis. Despite these limitations, our data adds to the growing understanding of the conjunctivitis microbiome.

Conclusion:

Firmicutes among the predominant phyla has the highest abundance in bacterial conjunctivitis in our study. *Pseudomonas* as a resident commensal microbiota has an important role for prevention of infection.

Declarations

Ethical considerations and consent for publication:

Institutional Review Boards at the National Institute of Hygiene and Epidemiology, Hanoi, Vietnam, and the Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan, approved this study. Written informed consent was obtained from all patients before collecting samples and conducting interviews.

Conflict of interest:

The authors do not have a commercial or other association that might pose a conflict of interest.

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Access to data:

The datasets generated and/or analysed during the current study are available with the corresponding author and ready to submit when needed.

Authors' Contributions:

YHM and MU have contributed to the conception, design of the study, analysis and interpretation of data. In addition, drafting and revising of the article. HTN, MT and DDA participated in the design of the study, collection of data, and development of methodology with interpretation of data. YM and KY were involved in data analysis, interpretation, and revising the article for intellectual content. TK and LMY developed methods, designed the study, and critically revised the manuscript. All authors conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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Figures

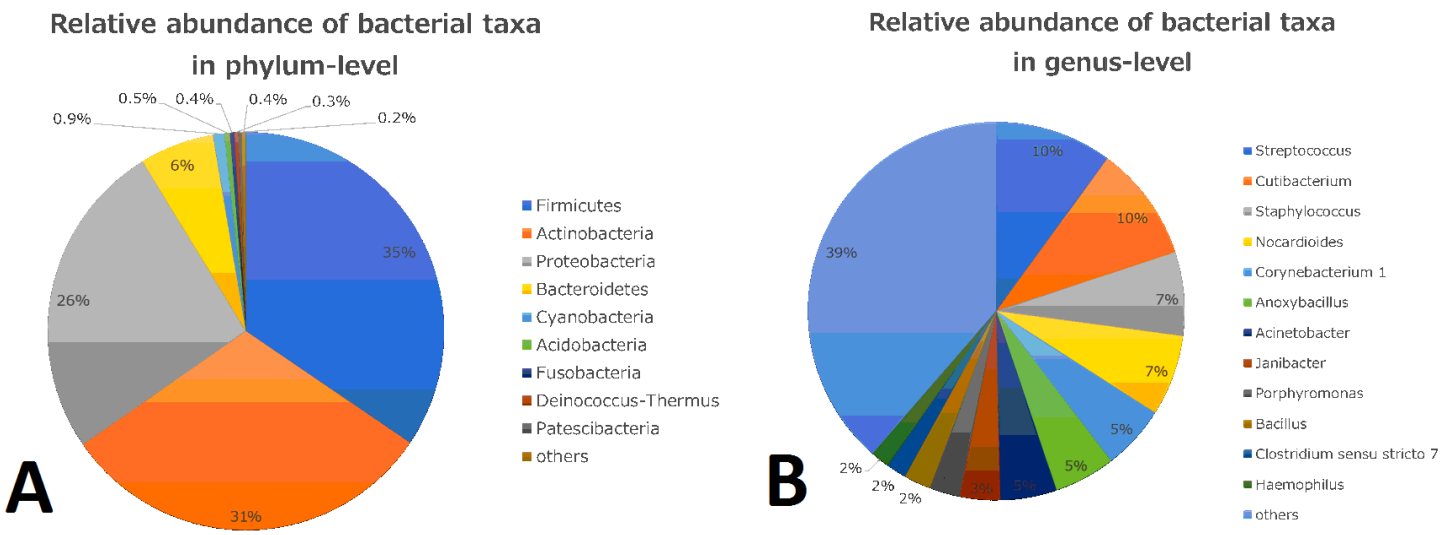
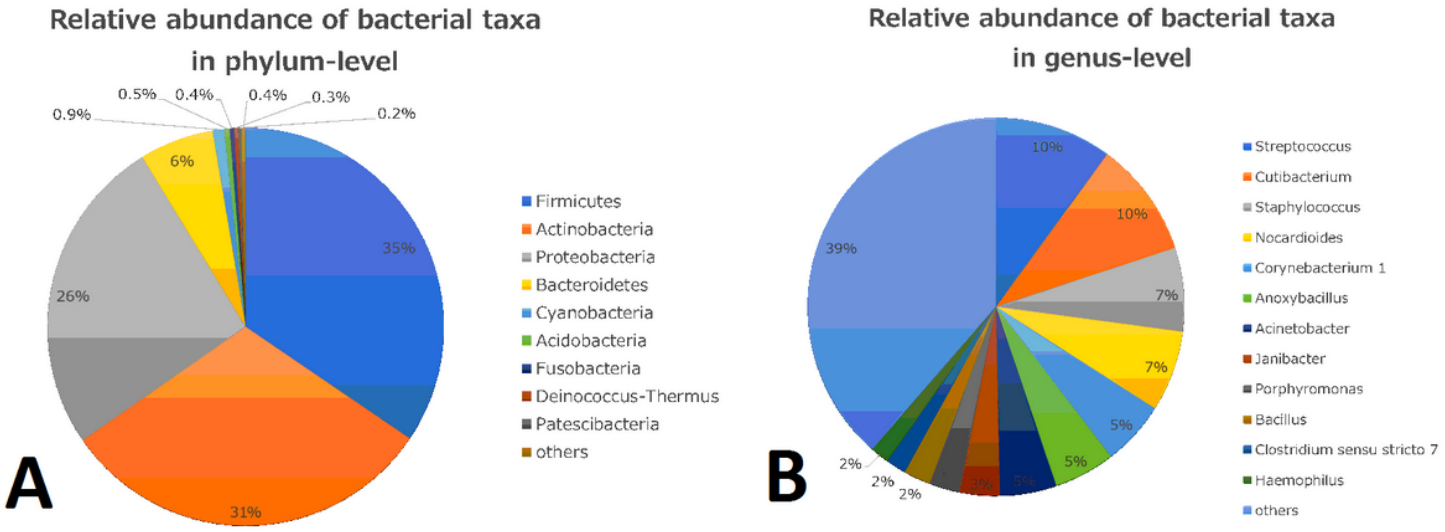


Figure 1

A: Relative bacterial compositions of conjunctivitis samples. 16S rRNA gene sequences were classified into phylum levels. B: Relative bacterial compositions of conjunctivitis samples. 16S rRNA gene sequences were classified into genus levels.



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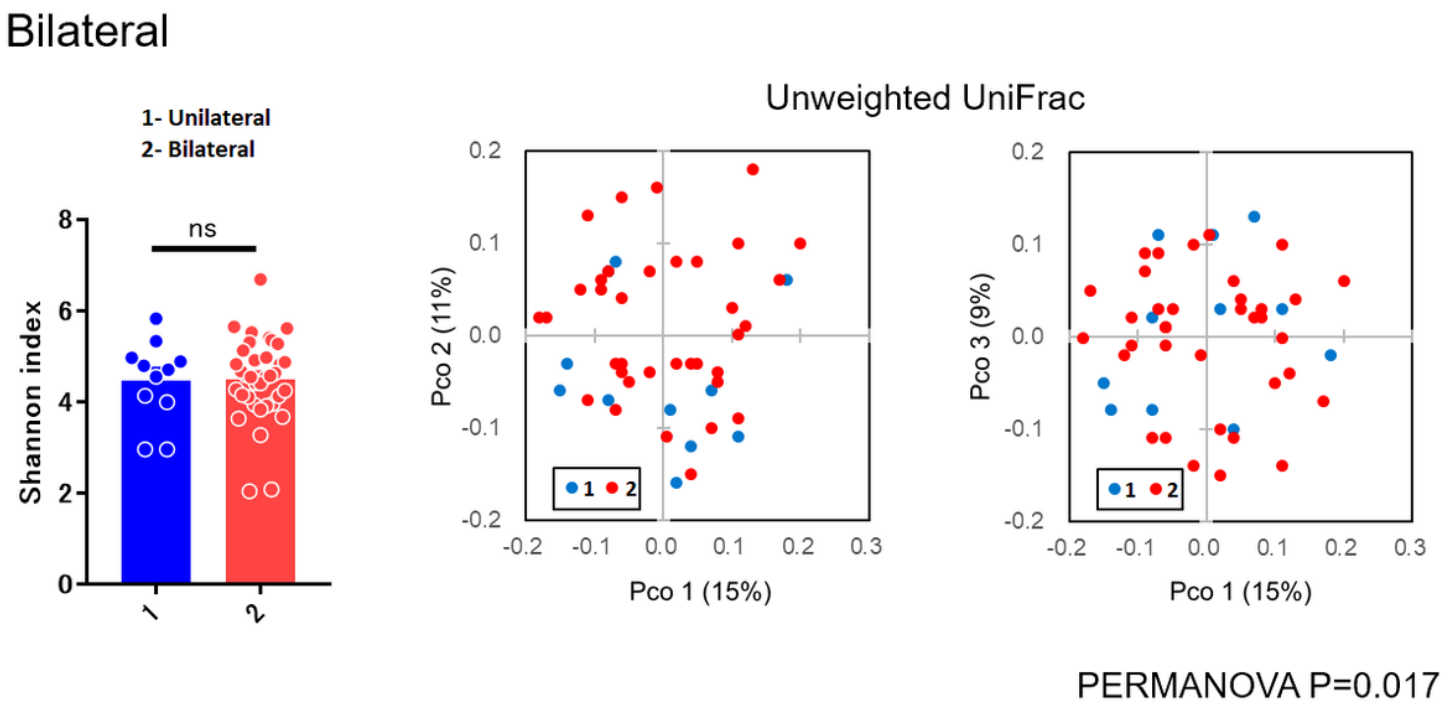

Figure 2

Alpha and Beta diversity analysis for Unilateral versus Bilateral conjunctivitis.

Bilateral
1- Unilateral
2- Bilateral
Shannon index
ns
Unweighted UniFrac
Pco 2 (11%)
Pco 1 (15%)
Pco 3 (9%)
PERMANOVA P=0.017

Figure 2

Alpha and Beta diversity analysis for Unilateral versus Bilateral conjunctivitis.

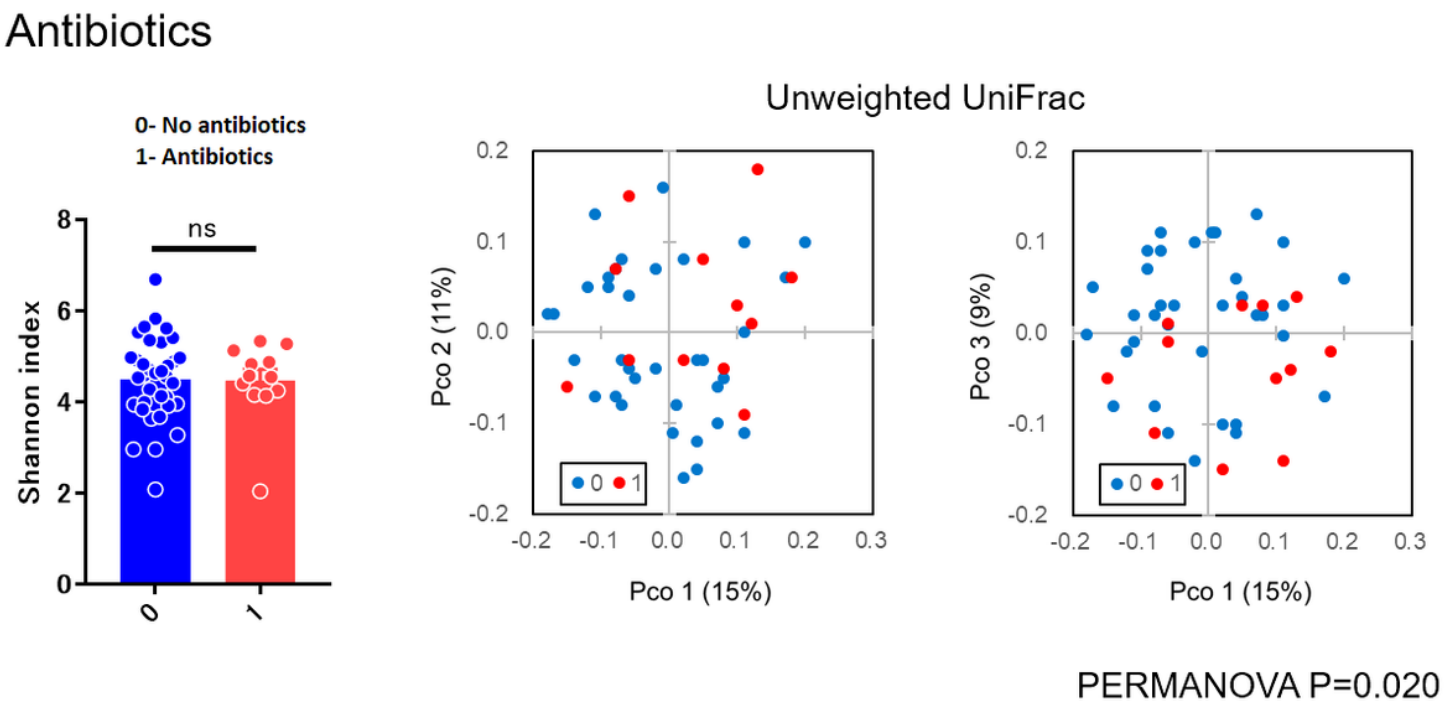

Figure 3

Alpha and Beta diversity analysis for group not using versus using Antibiotic eyedrops.

Antibiotics
0- No antibiotics
1- Antibiotics
ns
Shannon index
Unweighted UniFrac
Pco 2 (11%)
Pco 1 (15%)
Pco 3 (9%)
PERMANOVA P=0.020

Figure 3

Alpha and Beta diversity analysis for group not using versus using Antibiotic eyedrops.

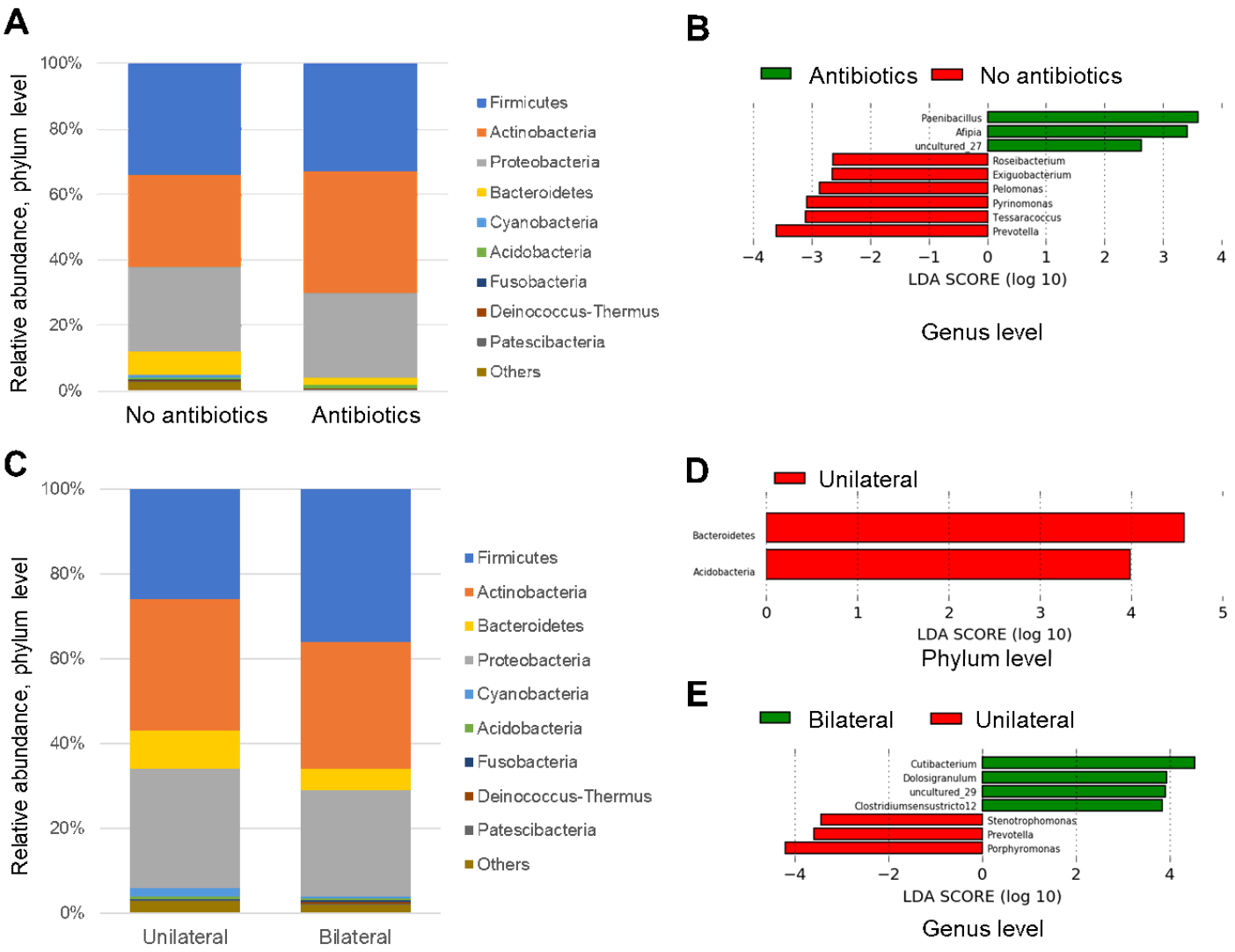

Figure 4

Linear Discriminant Analysis for Antibiotic and Bilateral groups.

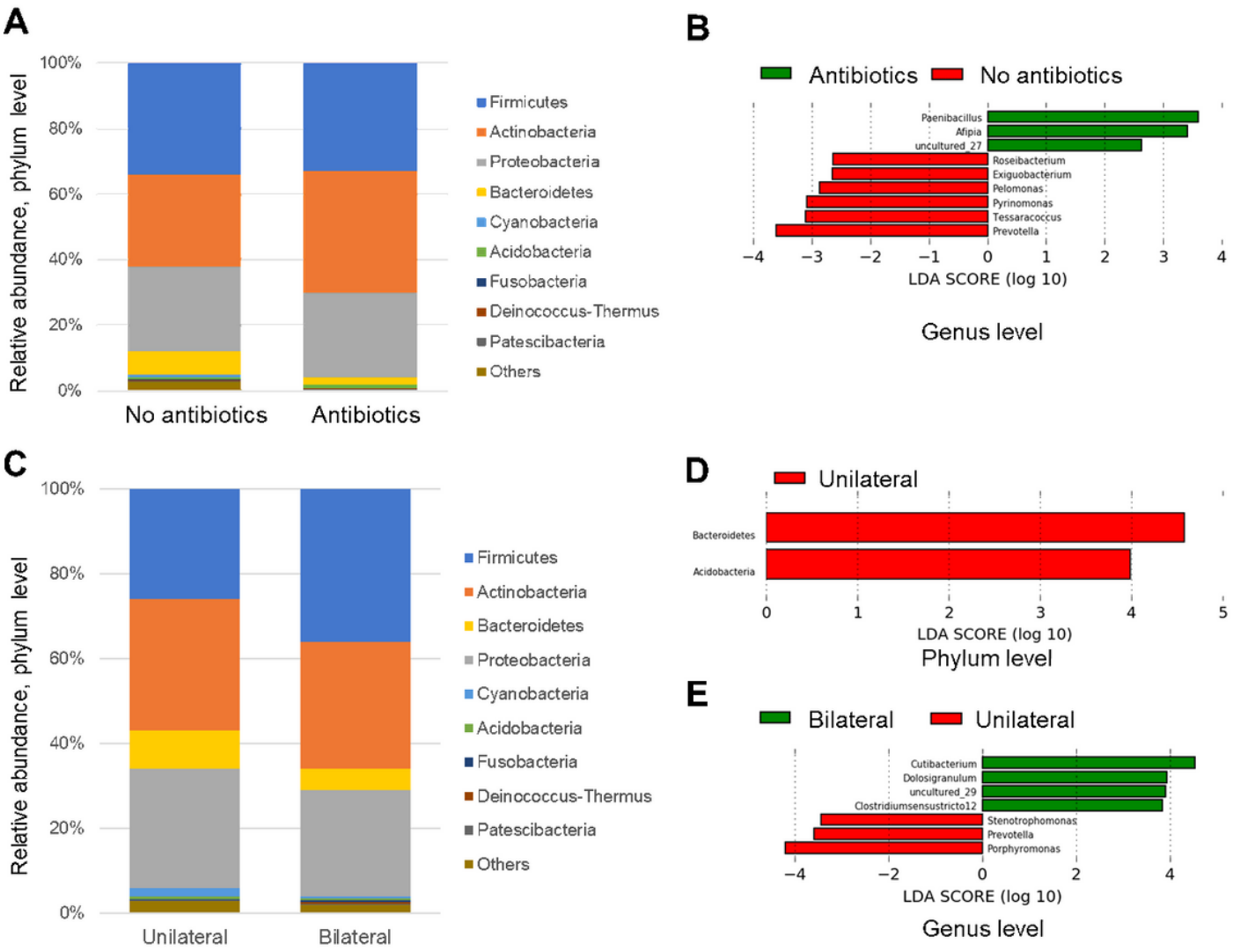

Figure 4

Linear Discriminant Analysis for Antibiotic and Bilateral groups.

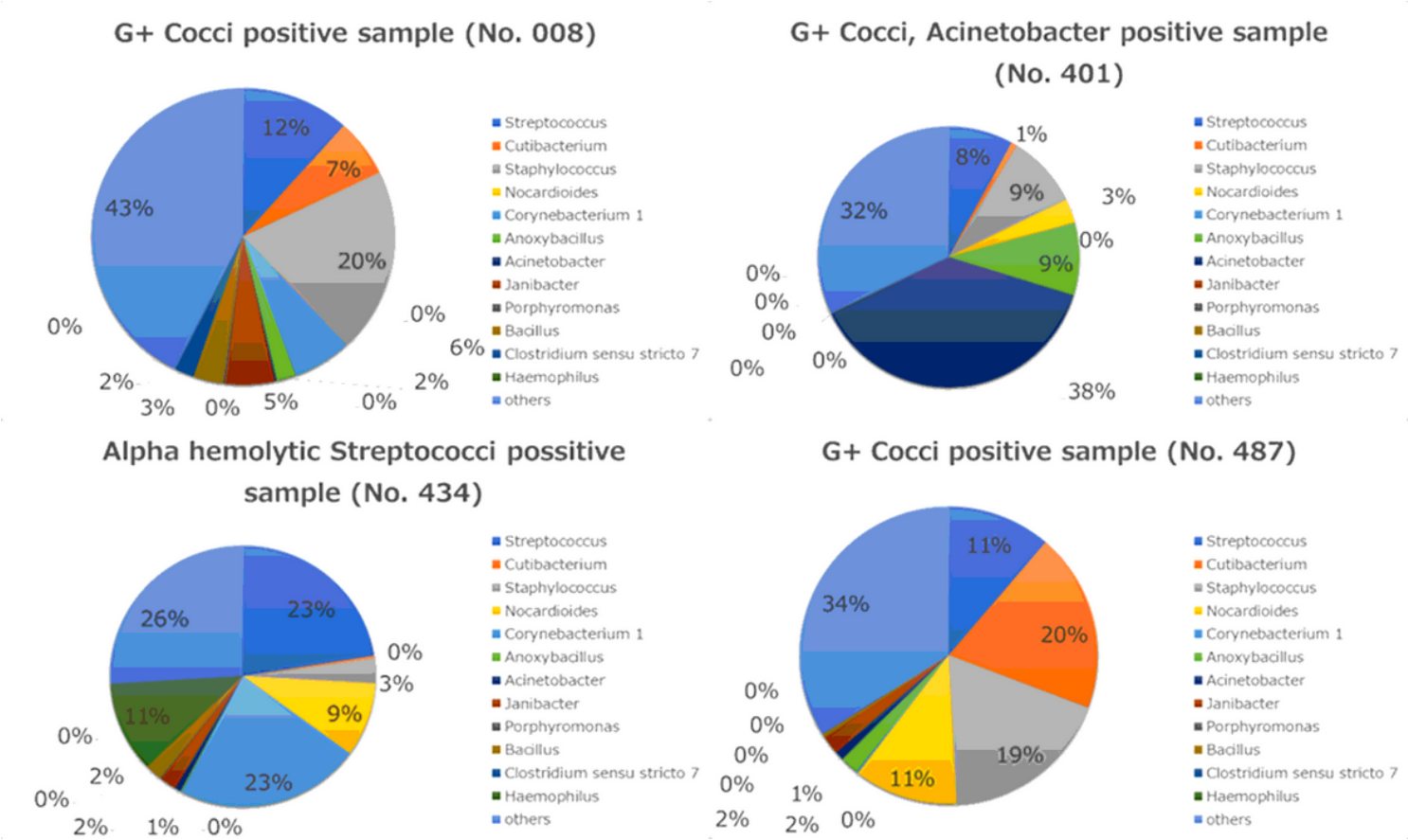

Figure 5

Individual metagenomics in four positive cultured cases.

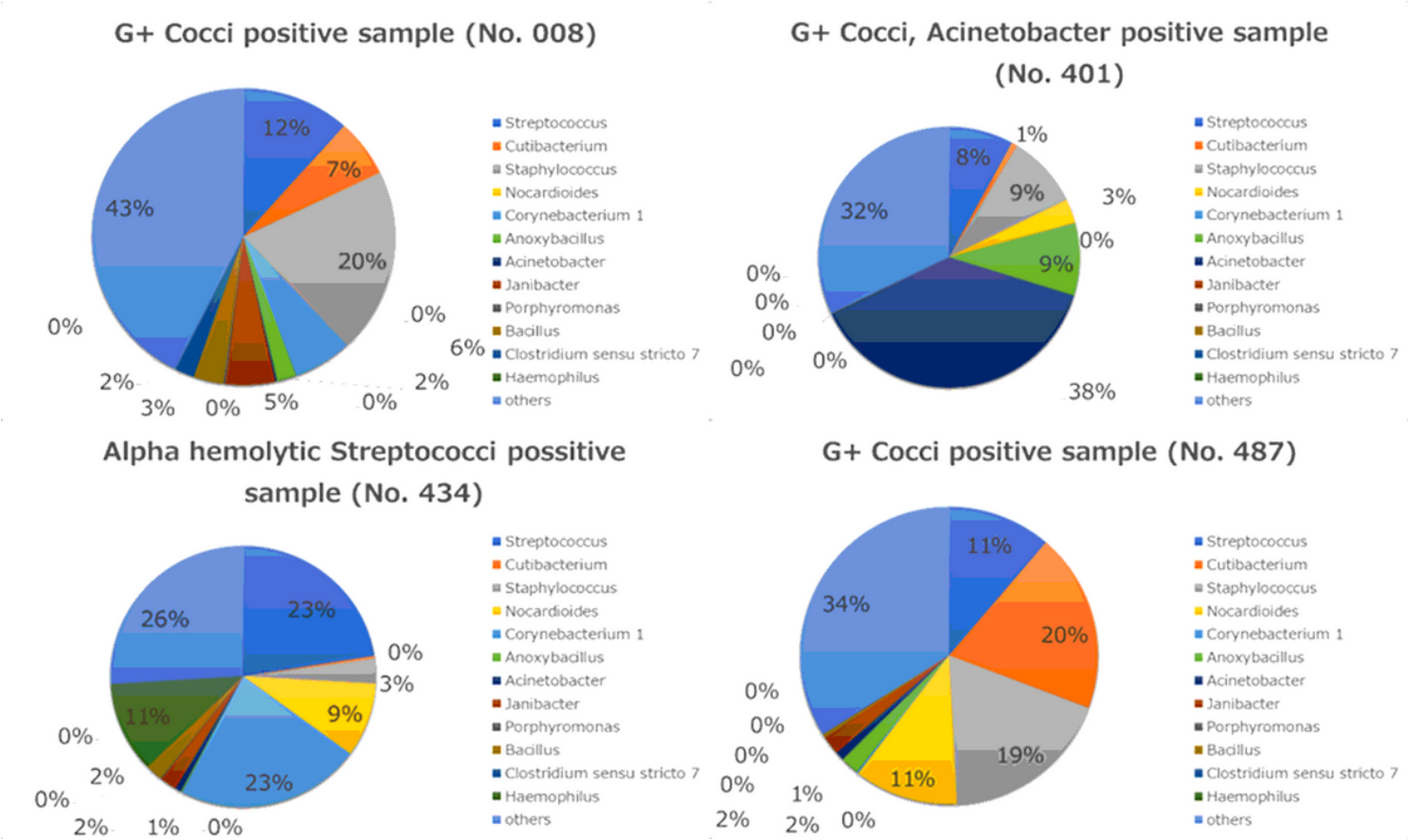

Figure 5

Individual metagenomics in four positive cultured cases.