Microbiome Profile in Chronic Rhinosinusitis with and without Polyps of Makassar, Indonesia

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Abstract

Background: Chronic rhinosinusitis (CRS) is a prevalent health problem that results in a large costly burden in society is often associated with the role of the microbiome that seems to maintain a healthy state and stability of the sinonasal environment often viewed as a symbiotic system.

Aim and objective: This study aimed to identify a microbiome profile in chronic rhinosinusitis with and without nasal polyps of Indonesian population.

Materials and methods: This study was conducted using the case-control technique on 20 patients divided into two groups. The first group is CRS without nasal polyps consisted of 10 patients and the second group is CRS with nasal polyps consisted of 10 patients. All of the samples were examined by next-generation sequencing techniques.

Results: Microbes were detected in all samples. Actinobacteria, Bacteroidetes, Cyanobacteria, Firmicutes, Fusobacteria, and Proteobacteria were the dominant phylum in both groups with a variable number of percentages.

Conclusion: This study highlights alteration of the commensal microbe may lead to dysbiosis conditions of the sinonasal environment.

Keywords: Chronic rhinosinusitis, Microbiome, Nasal polyps.

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Introduction

Chronic rhinosinusitis (CRS) is an inflammation of the nose and paranasal sinuses lasting for >3 months without resolution of symptoms.1 According to the European Position Paper on Rhinosinusitis and Nasal Polyps (EPOS) in 2012, adult rhinosinusitis characterized by two or more symptoms, which should be either nasal obstruction or nasal discharge with/without facial pain or reduction of smell and also an endoscopic sign of nasal polyps, mucopurulent discharge from middle meatus or mucosal obstruction in middle meatus and computed tomography (CT) changes. Chronic rhinosinusitis is classified as two clinical phenotypes, CRS with nasal polyps (CRSwNP) and CRS without nasal polyps (CRSsNP).2

Chronic rhinosinusitis is a prevalent health problem that results in a large costly burden in society. Current data have demonstrated that CRS was found in approximately 5–15% of the general population.3 It affects 14–30 million US adults per year, which results in 13 million office visits per year and spent $3.4–8.6 billion annually.4

Over a decade, several hypotheses have been proposed to explain CRS pathogenesis. Recent studies revealed that the sinonasal cavity has a resident flora, similar to the gut, which helps to promote a sinonasal healthy environment. Numerous studies were conducted to understand this phenomenon to attempt and define healthy condition of the microbiome has an important interaction with pattern recognition receptor (PRR) on epithelial which adjusts chemokines, and mediators. Changes in the sinonasal microbiome resulting in “dysbiosis” condition. A shift from a normal healthy microbial community may responsible for modulating sinuses inflammation through any number of mechanisms. Pathogen colonization and disproportion of microbiota leading to the dysfunctional immune barrier, inflamed mucosal epithelium, and obstructed sinuses promote a suitable condition for dysbiosis, setting the stage for CRS.7-10

Microbiome identification using culture-independent techniques has emerged due to the understanding of the role of microbes in human well-being.11 Culture-independent methods allow identification of microorganisms without requiring growth consortium contributes many useful functions for the sinus. The healthy condition of the microbiome has an important interaction with pattern recognition receptor (PRR) on epithelial which adjusts the relationship between pro- and anti-inflammatory cytokines, chemokines, and mediators. Changes in the sinonasal microbiome resulting in “dysbiosis” condition. A shift from a normal healthy microbial community may responsible for modulating sinuses inflammation through any number of mechanisms. Pathogen colonization and disproportion of microbiota leading to the dysfunctional immune barrier, inflamed mucosal epithelium, and obstructed sinuses promote a suitable condition for dysbiosis, setting the stage for CRS.7-10
The newest method to specify the composition of the microbiome is next-generation sequencing, which is designed to identify a cluster of 16s ribosomal RNA (rRNA) that are specific to a typical genus or species called operational taxonomic units. The primary aim of this study is to profile the microbiome in CRS patients with and without nasal polyps in Indonesia.

**Materials and Methods**

**Ethics Statement**

The study protocol and ethical clearance were approved by the Biomedical Research Ethics Committee on Human Faculty of Medicine Hasanuddin University, Makassar, Indonesia (Register number: 14/UN4.6.4.5.31/PP36-KOMETIK/2019 and protocol number: UH18120957). All participants provided written informed consent before they participated in the project.

**Study Design and Population**

Study participants were recruited in 2019 at Wahidin Sudirohusodo Hospital, Hasanuddin University Hospital, and Mitra Husada Hospital Department of Otorhinolaryngology, Head and Neck Surgery, Hasanuddin University, Makassar, Indonesia. Two groups of participants (CRSsNP and CRSwNP) in equal number were informed about the study and invited to participate: Both the groups scheduled for endoscopic sinus surgery on all paranasal sinuses for the treatment of CRS. Diagnosis of CRS was made according to the European Position Paper on Rhinosinusitis and Nasal Polyps 2012.2

**Sample Collection**

Intraoperatively, through modern techniques of Functional Endoscopic Sinus Surgery (FESS), the mucosa was preserved while creating the largest possibly sinus cavities enlargement of natural ostia, the uncinate process was excised with cutting instruments. Care was taken to avoid contamination from other mucosal sites. Each nasal tissue sample was placed in a sterile Eppendorf tube contains 1 mL DNA/RNA shield™ solution and stored in −20°C until further analysis in Novogene AIT Laboratory, Singapore with several reports H1015C19020094.

**DNA Extraction**

Total genome DNA from samples was extracted using the CTAB/SDS method. DNA concentration and purity was monitored on 1% DNA Extraction

**Library Preparation and Sequencing**

Amplification of the 16S rRNA gene V3-V4 region was performed by using specific primers designed to anneal to 341F (‘S- CCTACGGGNGGCWGCAG 3‘) and 806R (‘S-GGACTACNNGGTATCTAAT 3‘). All PCR reactions were carried out with Phusion® High-Fidelity PCR Master Mix (New England Biolabs). This resulted in amplicon sizes of approximately 466 bp. All further steps in library preparation were performed according to the Illumina “16S Metagenomic Sequencing Library Preparation” protocol.

**Results**

**Materials Patients**

The study participants comprise 20 subjects, including 10 CRSsNP patients (NP group samples), and 10 CRSwNP (P group samples) patients. All patients undergoing FESS. Fifty percent (n = 10) of CRS

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patients had nasal polyps and 50% (n = 10) of CRS patients had no nasal polyps. The distribution of gender and age is shown in Table 1.

### Sequencing Result, Richness, Diversity Analysis of OTUs

We obtained 2,041,342 raw sequences from 20 participants, ranging from 78,457 to 144,717. After quality filtering and removing the chimeric sequences, we obtained 1,753,784 reads. Based on rarefaction analysis, all samples were rarefied to 29,938 sequences per sample for further analysis. For species analysis, sequences with ≥97% similarity were assigned to the same OTUs. From these sequences, OTUs were identified ranging from 771 to 2,183 with an average number of 1,486 OTUs (Table 2).

In alpha diversity analysis, rarefaction curves, Chao1, and Shannon’s index were generated based on a species level of 97% similarity. Rarefaction curves analysis of OTUs in two groups indicated the highest species richness occurred in the CRSwNP group were shown in Figure 1.

### Table 1: Distribution of gender, age, and phenotype of the study participants

| Variable     | n (20) | %  |
|--------------|--------|----|
| Age range    |        |    |
| 18–28 years  | 10     | 50 |
| 29–39 years  | 6      | 30 |
| 40–50 years  | 1      | 5  |
| >50 years    | 3      | 15 |
| Gender       |        |    |
| Male         | 9      | 45 |
| Female       | 11     | 55 |
| Phenotype    |        |    |
| CRSsNP       | 10     | 50 |
| CRSwNP       | 10     | 50 |

### Table 2: Summary of the effective tags and OTUs

| Sample_Name | Total_tags | Taxon_Tags | Unclassified_Tags | Unique_Tags | OTUs  |
|-------------|------------|------------|-------------------|-------------|-------|
| P1          | 90,253     | 54,445     | 8,182             | 27,626      | 2,008 |
| P2          | 68,327     | 51,731     | 3,251             | 13,345      | 1,278 |
| P3          | 90,104     | 59,524     | 1,667             | 28,913      | 1,475 |
| P4          | 90,159     | 57,516     | 5,892             | 26,751      | 1,999 |
| P5          | 90,087     | 57,811     | 6,546             | 25,730      | 1,921 |
| P6          | 90,049     | 56,139     | 3,464             | 30,446      | 1,899 |
| P7          | 90,118     | 59,532     | 4,966             | 25,620      | 1,889 |
| P8          | 90,200     | 58,467     | 3,981             | 27,752      | 1,204 |
| P9          | 54,517     | 18,008     | 11,932            | 24,577      | 805   |
| P10         | 90,327     | 58,876     | 6,989             | 24,462      | 1,836 |
| NP1         | 90,195     | 64,807     | 1,662             | 23,726      | 1,156 |
| NP2         | 90,046     | 79,602     | 295               | 10,149      | 771   |
| NP3         | 91,379     | 57,339     | 3,799             | 30,241      | 2,183 |
| NP4         | 97,649     | 61,708     | 188               | 35,753      | 1,222 |
| NP5         | 90,146     | 61,692     | 5,794             | 22,660      | 1,381 |
| NP6         | 89,570     | 47,051     | 9,123             | 33,396      | 1,518 |
| NP7         | 81,414     | 52,471     | 6,519             | 22,424      | 1,059 |
| NP8         | 90,106     | 58,137     | 1,837             | 31,990      | 2,079 |
| NP9         | 98,961     | 65,134     | 379               | 31,938      | 791   |
| NP10        | 90,197     | 67,619     | 379               | 22,199      | 1,252 |

**Figs 1A and B:** Rarefaction curves (A) and Shannon diversity curves (B) of DNA sequences of the sample. Curves were calculated based on OTUs at 97% similarity.
The rarefaction curves did not approach a plateau but Shannon’s diversity rarefaction curves approached asymptotes (Fig. 1), indicating that the sampling depths were sufficient to capture the overall microbial diversities in all two groups. Shannon diversity indicates the diversity from the higher was sample NP 6 (8,459) and the lowest was sample P8 (6,386). Venn diagram displayed that CRSsNP and CRSwNP shared 3,114 OTUs (Fig. 2).

Differences in Shannon diversity were examined between the CRSsNP and CRSwNP groups. The result was CRSwNP had significantly higher diversity than CRSsNP, same result showed in Observed_species indices and Chao1 indices (Fig. 3).

Observed_species and Chao1 reveal the richness of species within a single sample, while Shannon indexes reflect microbial diversity as shown in Table 3. Observed_species, Chao1, and Shannon indexes in the CRSwNP group were higher than in the CRSsNP group, even though there was no statistical difference in alpha diversity between these two groups ($p$ value > 0.05).

We examined beta diversity in both groups, two dimensional Principal Coordinates Analysis (PCoA) of weighted and unweighted UniFrac distances were performed to conceive the relationship of the microbial communities in both groups of the sample (Fig. 4). Based on unweighted UniFrac PCoA (Fig. 4A), there are some bacterial communities of both groups were group together whereas some other bacterial communities were separated from each other. Based on the weighted UniFrac PCoA, both groups were spreading to the left and right groups. According to weighted UniFrac distance cluster analysis, which showed that 20 samples were clustered into a few groups. At the phylum level, 10 representatives phyla were identified, and the rest of the tags are indicated as “Others” (Fig. 5).

![Venn diagram of the OTUs in two groups. The number inside the diagram indicates the number of OTUs](image1)

![Boxplot of Shannon diversity indices](image2)

![Boxplot of Chao1 indices](image3)

![Boxplot of Observed species indices](image4)

**Fig. 2:** Venn diagram of the OTUs in two groups. The number inside the diagram indicates the number of OTUs

**Fig. 3A to C:** (A) Boxplot of Shannon diversity indices; (B) Chao1 indices; (C) Observed species indices
The mean weighted UniFrac and unweighted UniFrac in the CRSwNP group distances lower than the CRSsNP group but not statistically significant (p value > 0.05; Fig. 6).

The ANOSIM analysis reveals a negative R value (−0.002) indicating that intergroup variation is greater than intragroup variations, therefore no significant differences (p value 0.461 > 0.05) between the CRSsNP and CRSwNP groups. Meanwhile, the MRPP analysis result shows the A value <0 (−0.0024) represented that variation within the group is larger than among the group. The observed delta is smaller indicates that inner-group variation is small, while the larger one in the expected delta column means that intergroup variation is great. Therefore, no significant differences (p value 0.597 > 0.05) between CRSsNP and CRSwNP groups (Fig. 7).

**Microbial Identification**

At phylum level, there are six predominant phyla were identified in the CRSsNP group including Bacteroidetes (24.15%), Proteobacteria (22.65%), Cyanobacteria (18.83%), Firmicutes (15.50%), Fusobacteria (11.88%), and Actinobacteria (6.99%). While in the CRSwNP group, six predominant phyla including Proteobacteria (54.86%), Firmicutes (21.06%), Cyanobacteria (12.51%), Fusobacteria (4.15%), Actinobacteria (4.05%), and Bacteroidetes (3.37%) as shown in Table 4.

In the CRSwNP group, Proteobacteria phyla had the higher percentage and Bacteroidetes phyla had the less percentage, while in the CRSsNP group, Bacteroidetes phyla had the higher percentage and Actinobacteria phyla had the less percentage (Figs 8 and 9). There were no significant differences between both the groups of dominant phyla.

At the genus level, the 35 most abundant genera in the two groups were analyzed by a hierarchical clustering heat map, which denoted that although these genera occurred in all samples, the abundance of most genera varied in different samples. The heatmap of genera relative abundance was shown in Figure 10.

**Discussion**

This study investigated the bacterial microbiome of two groups of patients with CRS with and without nasal polyps by using 16s ribosomal amplicon sequencing (next-generation sequencing) of the uncinate process of ostiomeatal complex.

There are two phenotypes of CRS that ruled in this study, most of the samples found in young and productive age (18–28 years), the prevalence was higher in females and we obtain an equal phenotype of CRS, 10 samples of CRSsNP and 10 samples of CRSwNP.

We speculated that CRS occurs at young and productive age because this group is more often exposed to environmental contaminants. While other studies showed the contradictive result, the incidence of CRS is between 37 years and 50 years old. Boase et al. found the incidence of CRS between 35 years and 47 years old, Cleland et al. found the mean age of CRS incidence is 50 years old.

Changes in alpha diversity represent the disease circumstances. In this study, we found Shannon diversity was examined between

### Table 3: Summary of the richness and diversity of microbial communities in 20 samples

| Sample | Observed_species | Shannon | Chao1 |
|--------|-----------------|---------|-------|
| P1     | 1,670           | 8.322   | 1845.783 |
| P2     | 1,084           | 4.555   | 1431.089 |
| P3     | 1,265           | 7.772   | 1407.523 |
| P4     | 1,689           | 7.85    | 1919.888 |
| P5     | 1,604           | 8.215   | 1796.014 |
| P6     | 1,639           | 7.638   | 2078.222 |
| P7     | 1,654           | 7.772   | 1797.644 |
| P8     | 958             | 6.386   | 1137.637 |
| P9     | 805             | 7.149   | 954.188 |
| P10    | 1,553           | 8.584   | 1707.841 |
| NP1    | 950             | 6.434   | 1095.805 |
| NP2    | 487             | 3.337   | 712.919 |
| NP3    | 1,926           | 7.882   | 2145.556 |
| NP4    | 977             | 6.54    | 1198.942 |
| NP5    | 1,106           | 5.556   | 1291.153 |
| NP6    | 1,370           | 8.459   | 1561.19 |
| NP7    | 895             | 5.462   | 1077.081 |
| NP8    | 631             | 4.213   | 840.189 |
| NP9    | 1,803           | 8.164   | 1979.367 |
| NP10   | 1,022           | 7.738   | 1175.679 |
the CRSsNP and CRSwNP groups and CRSwNP had significantly higher diversity than CRSsNP. The contrary result revealed by Copeland et al. showed the CRSwNP group had significantly lower diversity than CRSsNP. The presence of nasal polyps was associated with lower Shannon diversity when compared with CRSsNP. A previous study by Ramakrishnan et al. found that the existence of nasal polyps was not a forecaster of microbiome composition changes.9,10 We believed that the disparity results need more advanced study related to the presence or absence of allergy, asthma, eosinophilia or multiple inflammatory pathways in the CRSsNP group that can play role in altering the microbiome composition of this study. While in beta diversity used to compare microbial community structure within samples. In this study, inner-group variation is larger than intergroup variations. Hoggard et al. and Copeland et al. in their study explained that there was a tendency of higher intrapatient variability in the CRS group. We conceived larger sampling will explain this trend further.10,16

A previous study found that the phyla Firmicutes, Proteobacteria, and Actinobacteria were discovered in healthy subjects, whereas the phyla Firmicutes and Proteobacteria are less frequent. Bacteroidetes are also detected but in lower relative abundance.17,18 While Aurora et al. found that Cyanobacteria in large numbers.19 In our study, we did not compare healthy subjects with CRS, instead of CRSwNP and CRSsNP groups comparison. There were six predominant phyla in both CRSsNP and CRSwNP groups that we found in this study: Actinobacteria, Bacteroidetes, Cyanobacteria, Firmicutes, Fusobacteria, and Proteobacteria but each group had different percentages of each phylum. In the CRSsNP group, Bacteroidetes (24.15%) was the higher percentage phyla, while Actinobacteria (6.99%) was the lowest percentage phyla. Meanwhile, in the
CRSwNP group, Proteobacteria (54.86%) was the higher percentage phyla, while Bacteroidetes (3.37%) was the lowest percentage phyla.

In our study, Actinobacteria phylum was considered as sinonasal commensal organisms that seem to be having a lower percentage in both groups (CRSSsNP; 6.99%, CRSwNP; 4.05%). This phylum is identified as “Gatekeepers” to maintain the health condition of the sinonasal environment. Surprisingly, the phyla Proteobacteria had a high percentage in both groups, in the CRSSsNP group, this phylum dominating the microbiome composition (54.86%), while in the CRSwNP group, this phylum also had a high percentage (22.65%). The previous study explains the abundance of these phyla correlated with asthmatics subjects.8,18 We speculated the result of our current study correlated with these conditions.

Pathogen organism, Fusobacteria seen in both groups, but in a low percentage in CRSwNP (4.15%). These phyla have high virulence and are frequently involved in severe infections, whether this pathogen had a possible role in the disease process in CRSSsNP, compared with CRSwNP, as a cause or only assist the progression of the disease cannot be seen in our study.

In our study, the CRSSsNP group had greater bacterial abundance compared with the CRSwNP group, but reduced species richness and diversity.

Disruption of baseline microbiome (dysbiosis) is regarded to have a role in disease mechanism, reduced diversity, and abundance increasing had correlation with chronic inflammation. The dysbiosis hypothesis has been suggested as a disease mechanism in CRS. Breakdown of baseline microbiome which maintains a healthy, stable state of the sinonasal cavity. The myriad factor can contribute to the alteration of the sinonasal microbiome including host and

### Table 4: Percentage of the dominant microbiome at phylum level of CRSSsNP and CRSwNP groups

| Phylum         | CRSSsNP | CrSwNP |
|----------------|---------|--------|
| Bacteroidetes  | 24.15%  | 3.37%  |
| Proteobacteria | 22.65%  | 54.86% |
| Cyanobacteria  | 18.83%  | 12.51% |
| Firmicutes     | 15.50%  | 4.15%  |
| Fusobacteria   | 11.88%  | 4.05%  |
| Actinobacteria | 6.99%   | 3.37%  |
| Total          | 100%    | 100%   |

Figs 7A and B: (A) ANOSIM (B) MRPP analysis microbial communities structure among groups. *Mean the significantly different between groups ($p < 0.05$)

Figs 8A and B: (A) Microbial composition at the phylum level of the CRSSsNP group indicates the most abundant phyla detected in the CRSSsNP group; (B) Microbial composition at the phylum level of the CRSwNP group indicates the most abundant phyla detected in the CRSwNP group.
environmental. This complex mechanism is still unclear but the role of the microbiome in influencing mucosal health is increasingly important.

Finally, the disparity result of our study from others is likely due to differences in sequence processing, sampling sites, and technique.

Our study showed microbiome profiling with an advanced culture-independent technique (next-generation sequencing). Alteration composition of the microbiome in the sinonasal cavity might have a role in chronic inflammation leading to a disease state. Further investigation involved healthy subjects, larger participants, and a deeper sampling site might reveal more results to obtain a more accurate concept of sinus microbiome in CRS.

In our study, we found that there are five dominant phyla of the microbiome in both groups which are Actinobacteria, Bacteroidetes, Firmicutes Fusobacteria, and Proteobacteria. The study revealed that there is an increase of relative abundance and decrease of diversity in the CRSsNP group indicated dysbiosis phenomenon. No significant differences in alpha diversity of both groups, beta diversity showed a negative R value (−0.002) indicates that inner-group variation is larger than intergroup variations; therefore, no significant differences (p value 0.461 > 0.05) between the CRSsNP and CRSwNP groups.

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