Mapping and comparing bacterial microbiota in the sinonasal cavity of healthy, allergic rhinitis, and chronic rhinosinusitis subjects

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Background: **The role of microbiota in sinonasal inflammation can be further understood by targeted sampling of healthy and diseased subjects. We compared the microbiota of the middle meatus (MM) and inferior meatus (IM) in healthy, allergic rhinitis (AR), and chronic rhinosinusitis (CRS) subjects to characterize intrasubject, intersubject, and intergroup differences.**

Methods: **Subjects were recruited in the office, and characterized into healthy, AR, and CRS groups. Endoscopically-guided swab samples were obtained from the MM and IM bilaterally. Bacterial microbiota were characterized by sequencing the V3-V4 region of the 16S ribosomal RNA (rRNA) gene.**

Results: **Intersubject microbiome analyses were conducted in 65 subjects: 8 healthy, 11 AR, and 46 CRS (25 CRS with nasal polyps [CRSwNP]; 21 CRS without nasal polyps [CRSsNP]). Intrasubject analyses were conducted for 48 individuals (4 controls, 11 AR, 8 CRSwNP, and 15 CRSwNP). There was considerable intersubject microbiota variability, but intrasubject profiles were similar ($p = 0.001$, nonparametric *t* test). Intrasubject bacterial diversity was significantly reduced in MM of CRSsNP subjects compared to IM samples ($p = 0.022$, nonparametric *t* test). CRSsNP MM samples were enriched in *Streptococcus*, *Haemophilus*, and *Fusobacterium* spp. but exhibited loss of diversity compared to healthy, CRSwNP, and AR subject-samples ($p < 0.05$; nonparametric *t* test). CRSwNP patients were enriched in *Staphylococcus*, *Alloiococcus*, and *Corynebacterium* spp.**

Conclusion: **This study presents the sinonasal microbiome profile in one of the larger populations of non-CRS and CRS subjects, and is the first office-based cohort in the literature. In contrast to healthy, AR, and CRSwNP subjects, CRSsNP MM samples exhibited decreased microbiome diversity and anaerobic enrichment. CRSsNP MM samples had reduced diversity compared to same-subject IM samples, a novel finding.** © 2017 ARS-AAOA, LLC.

Key Words:
microbiome; microbiota; allergic rhinitis; bacteriology; chronic rhinosinusitis; nasal polyposis; inferior meatus; middle meatus; regional sinonasal microbiota and rhinosinusitis

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Chronic rhinosinusitis (CRS) affects 16% of the U.S. population and costs up to $65 billion annually.[1] However, the pathophysiology of this inflammatory disease is poorly understood, and is complicated by diverse subtypes resulting from divergent and complex interactions of the host immune system and environmental factors.[2–4] Microbiome dysbiosis has been reported to be associated with CRS, but the role of the human sinonasal microbiome in the complex host-environment interplay

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is yet unclear.[5] A pilot study found depletion in microbiome diversity with enrichment of pathobionts such as *Corynebacterium tuberculostearicum*.[1] More recent microbiome studies using larger cohorts find that CRS-associated microbial profiles are also often marked by a loss of bacterial diversity and concomitant enrichment of sinus pathobionts.[6–9] These initial investigations have focused on CRS patients undergoing endoscopic sinus surgery (ESS), a strategy that skews the enrolled study population toward those with severe disease recalcitrant to medical management. However, these studies need validation in broader cohorts of CRS patients.

Inflammation of the middle meatus (MM) is a common association in CRS,[2] irrespective of whether this is causative or reflective of the CRS diseased state.[3–5,10,11] In contrast, the anterior nares and the inferior meatus (IM) are usually considered to be uninvolved.[2] By studying patterns of the MM microbiota vs IM in healthy and CRS subjects, one may gain insightful information into dysbiosis associated with (or causative to) CRS. While initial studies have reported that variability between sinuses within the single CRS patient to be significantly less than variability across different patients,[5,12] they also demonstrate high intrapatient microbiota variability in a subset of patients that could not be explained due to small sample size (a common limitation in CRS microbiome studies).

In the current study, we surveyed the sinonasal microbiota of healthy, allergic rhinitis (AR), and CRS subjects. Office-based sampling was conducted to facilitate enrollment of larger number of subjects spanning the spectrum of CRS severity. Bilateral sampling of the MM and IM was performed to study biogeographical variations in the sinonasal microbiota. We hypothesized that the microbial composition and diversity of the MM and IM would differ within and between individual subjects based on the presence or absence of diseased states, and that these bacterial signatures would correlate with disease phenotype and severity.

Subjects and methods

Patient recruitment and sample collection

The study was approved by the Institutional Review Board at Mayo Clinic in Arizona (approval number 13–007985). All adults presenting to the principal investigator's (D.L.) rhinology clinic were offered enrollment. Written informed consent was obtained from all study subjects. Patients who had been treated with oral antibiotics and/or oral corticosteroids in the last 4 weeks were excluded. Patients were classified into healthy, AR, and those with CRS. CRS patients with AR were classified as CRS subjects. CRS patients were subclassified into CRS with nasal polyps (CRSwNP) and CRS without nasal polyps (CRSsNP) based on office nasal endoscopy using the 2007 American Academy of Otolaryngology-Head & Neck Surgery (AAO-HNS) guidelines.[13] Those with positive skin testing were classified into AR patients. Healthy subjects included those undergoing evaluation for nasal complaints, or skull base and orbital pathology that were determined not to have AR or CRS. Data were collected prospectively, including demographic information, history, nasal endoscopy findings (nasal polyp status; Lund-Kennedy score),[14] clinical diagnoses, 22-item Sino-Nasal Outcome Test (SNOT-22) scores,[15] and sinus computed tomography (CT) scan findings (Lund-Mackay).[14]

Sinonasal swabs were collected in the office under strict aseptic conditions with sterile gloves and instrumentation. Specimens were obtained under direct endoscopic guidance using a sterilized pediatric 30-degree endoscope (Karl Storz, Tuttlingen, Germany) prior to performing any interventions in the nasal passageway. No topical sprays were used prior to sample collection. This was done both to prevent contamination as well as avoid use of lidocaine. Paired endoscopically-guided swab samples were obtained from bilateral IM and MM for each patient as feasible using sterile swabs (COPAN LQ Stuart Transport Swab; COPAN Italia S.p.A, Brescia, Italy). The sampling from the MM was protected from contamination from the anterior nares and IM using a sterile aural speculum (supporting Fig. 7). The aural speculum is circumferential, protecting the middle meatal swab from contamination from the anterior nares, and was stabilized when needed by an assistant. Depending on the size of the nasal cavity and speculum, endoscopic visualization during sampling was performed either through the speculum or transnasally. The sampling from the IM was performed along its length and the anterior nares were deliberately swabbed on the way out. The decision for combining the IM and anterior nares sampling into 1 swab were done based on the feasibility and cost of obtaining multiple uncontaminated samples in an office-based setting in awake subjects. After collection, the swab tips were cut with sterilized scissors and placed into sterile 7-mL polycarbonate tubes (Sarstedt 71.9923.610). The samples were immediately sent for freezing in a −90°C bath of Novec engineered fluid (3M) cooled in a HistoChill freezing bath (SP Scientific). The time from the start of harvest to freezing was approximately 15 minutes. Unique identification numbers were assigned to each individual container with barcode labels. Specimens were stored at −80°C until retrieval for analysis.

DNA extraction

Total genomic DNA was extracted using the QIAamp DNA Mini Kit (Qiagen) with minor modifications. DNA purification was performed per manufacturer's instruction with modifications as described.[16]

Library preparation

The V3-V4 region of the 16S ribosomal RNA (rRNA) gene was amplified using the primer pair S-D-Bact-0341-b-S-17 and S-D-Bact-0785-a-A-21.[17] Primers were constructed with universal tail (UT) sequences (Table 1)[18] and were

TABLE 1. 16S rRNA gene primers

Gene-specific universal tail primers[a]	
UT1-16S-0341-Full	**ACCCAACTGAATGGAGC**CCTACGGGNGGCWGCAG
UT2-16S-0785	**ACGCACTTGACTTGTCTTC**GACTACHVGGGTATCTAATCC

Index extension primers[b]	
Indexed Illumina-UT1	AATGATACGGCGACCACCGAGATCTACAC-BARCODE-GCTGGTCATCGTACCCAACTGAATGGAGC
Indexed Illumina-UT2	CAAGCAGAAGACGGCATACGAGAT-BARCODE-AGTCAGTCAGCCACGCACTTGACTTGTCTTC

[a]Bold region is the universal tail sequence.
[b]Barcodes used in this study have been published.[41]

TABLE 2. Illumina sequencing primers

Dual-Ind-UT1-R1 seq primer	**GCTGGTCATCGTACCCAACTGAATGGAGC**
UT-Index seq primer	GAAGACAAGTCAAGTGCGTGGCTGACTGACT
UT2-R2 seq primer	AGTCAGTCAGCCACGCACTTGACTTGTCTTC

used in a 2-step polymerase chain reaction (PCR) process as described.[19] PCR was performed in a 25-μL reaction containing 12.5 μL Q5 Hot Start High-Fidelity 2X Master Mix (New England Biolabs Inc.), 500 nM/primer, and 10 μL of DNA with the following PCR conditions: 95°C for 3 minutes; 25 cycles of 95°C for 40 seconds, 55°C for 2 minutes, 72°C for 60 seconds; 72°C for 7 minutes. The amplicon was purified with Agencourt AMPure XP beads (Beckman Coulter) per the manufacturer's protocol. The indexing PCR contained 12.5 μL KAPA HiFi HotStart ReadyMix (KAPA Biosystems), 400 nM of each barcoded UT1 and UT2 primers, and 10.5 μL of template from target-specific PCR at a final volume of 25 μL. The PCR conditions were as follows: (1) 98°C for 2 minutes; (2) 6 cycles of 98°C for 30 seconds, 65°C for 20 seconds, 72°C for 30 seconds; and (3) 72°C for 5 minutes. The final product was purified with Agencourt AMPure XP beads. The indexed libraries were electrophoresed on a 2% agarose gel at 100 V for 1 hour to separate the human mitochondrial amplicon from the bacterial 16S rRNA amplicon. Bacterial 16S rRNA bands were gel extracted using the QIAquick Gel Extraction Kit (Qiagen) per the manufacturer's instructions.

Library quantification and sequencing

Indexed amplicons were quantified using qPCR (KAPA Biosystems) and pooled at equimolar concentrations. The final library pool was mixed with 25% phiX control library (Illumina) and was loaded onto the Illumina MiSeq at 14pM. The library pool was sequenced with 300-bp paired end reads using v3 MiSeq reagent kit (Illumina). (See Table 2 for Illumina sequencing primers.)

Sequence processing

Paired end reads for each sample were assembled using SeqPrep[20] with the following options: "-L 400," "-n 1," "-A CAAGCAGAAGACGGCATACGAGAT," and "-B AATGATACGGCGACCACCGAGATCTACAC." Merged reads were clustered into operational taxonomic units (OTUs) at 99% by identity against the GreenGenes 13_8 with QIIME (1.9.1) as described.[21] Reads that failed to hit the reference sequence collection were retained and clustered de novo. Sequences were aligned using PyNAST[22] and taxonomy was assigned using uclust using QIIME.[23]

Sequence and statistical analysis

All analyses were performed on an OTU (sequences clustered at 99% similarity) table normalized to 5500 sequences/sample. Beta diversity (comparison of samples to each other to measure the dissimilarity between each sample pair) was performed using UniFrac distance matrices generated in QIIME 1.9.0.[21] Principal coordinates analysis (PCoA) plots were used for visualization of the data present in the beta diversity distance matrix using Emperor.[24] Permutational analysis of variance (PERMANOVA) using the adonis function in the R Vegan package was used to determine significance in distance matrices across samples by metadata categories.[25,26] Procrustes analysis was performed on the first 3 dimensions of a PCoA generated using a weighted UniFrac distance matrix and a Monte Carlo simulation (10000 permutations) to determine significance. Procrustes sum of squares (m^2) and correlation [$r = \sqrt{(1 - m2)}$] are reported. A 2-sided Mantel test with 10000 permutations was performed on weighted UniFrac distances matrices generated for each sample within a pair. Average weighted and unweighted UniFrac values were calculated between subjects and a Wilcoxon rank sum test was used to determine significance. Faith's phylogenetic diversity and Shannon diversity were calculated. Alpha diversity values were projected onto an image of the sinonasal cavity on the MM middle or IM using SitePainter.[27] A permutational t test (999 Monte Carlo permutations) was used to determine changes in alpha-diversity. To find taxa that were differentially represented across clinical groups, bacterial phyla and genera were summarized by group. Changes in taxon relative abundance were determined per 99% OTU using a zero-inflated negative binomial (https://github.com/alifar76/NegBinSig-Test). Multiple comparisons were corrected for false discovery using the Benjamini-Hochberg method and q values are reported.[28]

Results

Clinical characteristics of subjects

Sixty-five adult subjects were included in the analyses. There were similar numbers of male (n = 31; 47.7%) and female (n = 34; 52.3%) subjects, with ages ranging from 21 to

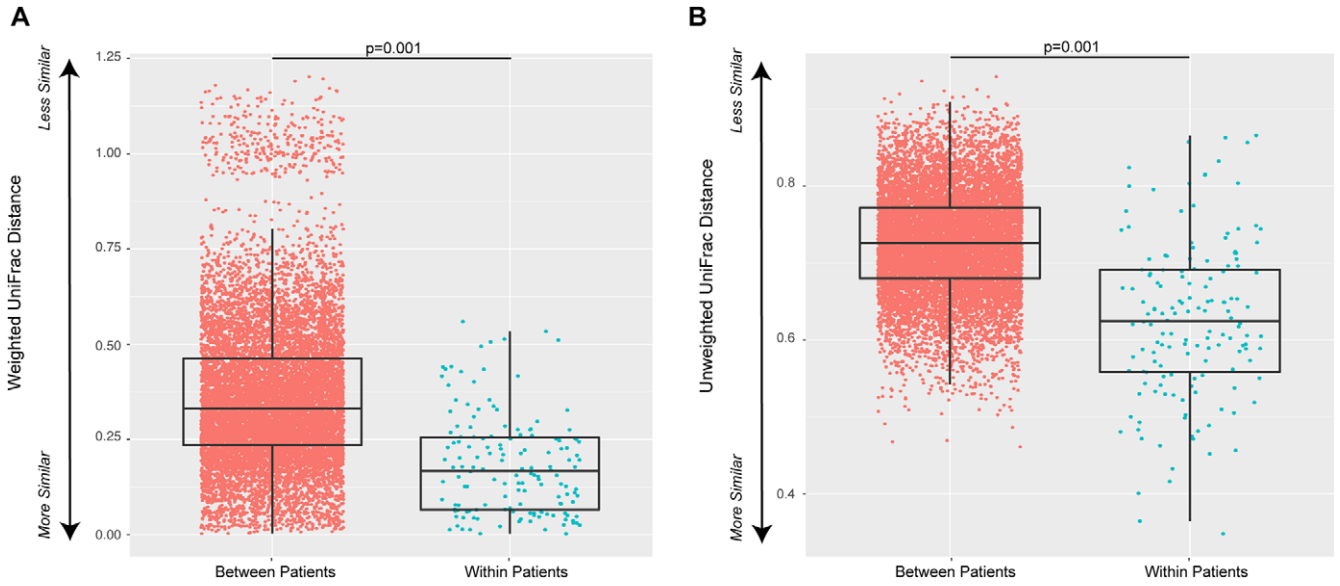

FIGURE 1. Compositional variation between or within subjects. (A) Weighted UniFrac distance shows that within-patient distances are significantly lower, indicating that the microbiota composition and abundances are more similar than intrapatient communities ($p = 0.001$, permutational t test). (B) Unweighted UniFrac distance shows that within-patient distances are significantly lower, indicating that the presence or absence of OTUs within patients are more similar than intrapatient communities ($p = 0.001$, permutational t test).

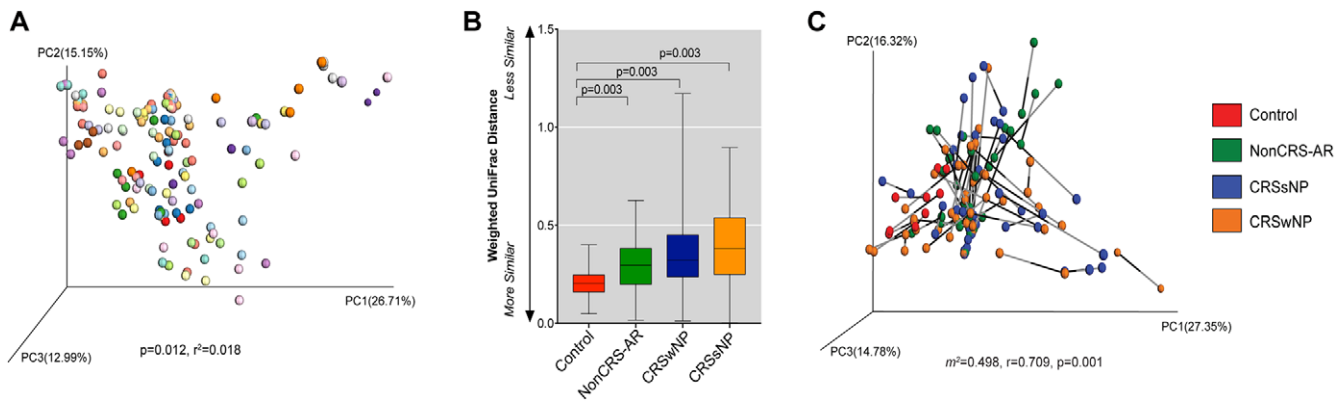

FIGURE 2. (A) PCoA of a weighted UniFrac distance matrix colored by patient showing significant clustering by patient ID ($p = 0.012$, $r^2 = 0.187$, PERMANOVA). (B) Mean intragroup weighted UniFrac distances demonstrating increased microbiota variability in nonCRS-AR, CRSwNP, and CRSsNP patients compared to controls (Bonferroni corrected $p = 0.003$, permutational t test). (C) Procrustes PCoA of weighted UniFrac distance matrix comparing variation of IM-MM pairs demonstrates that different sites yield similar PCoA ordination ($m^2 = 0.498$, $r = 0.709$, $p < 0.0001$). Since the m^2 value was relatively high, we performed a Mantel test on the weighted UniFrac distance matrices and found significant correlation between the IM and MM matrix ($p = 0.0001$, $r = 0.708$). AR = allergic rhinitis; CRS = chronic rhinosinusitis; CRSsNP = CRS without nasal polyps; CRSwNP = CRS with nasal polyps; IM = inferior meatus; MM = middle meatus; PCoA = principal coordinates analysis.

88 years. Their Lund-Mackay CT scores ranged from 0 to 24. Of the CRS subjects, the Lund-Mackay scores ranged from 3 to 24 (mean 13.41; median 14). The Lund-Kennedy endoscopy scores ranged from 0 to 12 for the overall group. For CRS subjects, Lund-Kennedy scores ranged from 1 to 12 (mean 5.7; median 6). The SNOT-22 scores of the overall group ranged from 5 to 101 (mean 42.5; median 41.5).

CRSwNP patients had greater radiographic disease burden compared to CRSsNP; mean Lund-Mackay scores were 11.72 for CRSsNP (range, 3 to 16) and 15.48 for CRSwNP (range, 9 to 24) subjects ($p = 0.034$; Wilcoxon rank sum; Supporting Fig. 1A). Mean cumulative SNOT-22 scores were 28.2 in healthy controls (range, 20 to 46), 30.6 in AR (range, 8 to 69), 50.82 in CRSsNP (range, 21 to 90), and 48.43 in CRSwNP (range, 5 to 101). CRSsNP patients reported significantly worse total SNOT-22 scores compared to healthy individuals and AR subjects ($p = 0.012$ and $p = 0.018$; Wilcoxon rank sum; Supporting Fig. 1B). Although CRSwNP patients' SNOT-22 scores were also elevated compared to healthy and AR subjects, this did not reach statistical significance ($p = 0.153$; Wilcoxon rank sum). Serum eosinophils were significantly elevated in CRSwNP ($p = 0.0005$; Wilcoxon rank sum; Supporting Fig. 1C). Serum neutrophils were unaltered across CRS patient subgroups and healthy individuals ($p > 0.05$; Wilcoxon rank sum).

TABLE 3. Procrustes and Mantel test results

Test	Input	m^2	r	P
Procrustes analysis	PC (3 dimensions)	0.498	0.709	0.001
Mantel test	Distance matrix	NA	0.708	0.001

NA = not applicable; PC = Principle Coordinate.

Mapping intrasubject microbiota diversity and composition

Of the 65 subjects, paired MM-IM swabs that could be sequenced to desired depth were available from 48 subjects. These included 150 swabs (2 to 4 sites per patient) from 4 healthy, 11 AR, and 35 CRS subjects (17 CRSwNP and 18 CRSsNP).

Across the overall study cohort, mean intrapatient UniFrac distances (weighted and unweighted) were significantly lower than interpatient distances (Wilcoxon rank sum $p = 0.005$; Fig. 1A, B), indicating more similar microbiota composition and abundance within individual subjects. This finding was supported by significant clustering by subject using weighted UniFrac distances ($p = 0.012$, $r^2 = 0.0187$; PERMANOVA; Fig. 2A). When separated by clinical group (healthy, AR, CRSsNP, and CRSwNP subjects), we found increasing microbiota heterogeneity in patients with rhinologic disease (CRSwNP, CRSsNP, and AR) than in the healthy subjects ($p = 0.003$, permutational t test; Fig. 2B, Supporting Fig. 2). Procrustes analysis demonstrated that the PCoA ordinations representing intrapatient MM-IM paired samples were significantly positively correlated ($m^2 = 0.498$, $r = 0.709$, $p < 0.0001$; Monte Carlo simulation, Fig. 2C, Table 3), indicating that the IM and MM pairs are compositionally similar. The Mantel test confirmed that the IM-MM pairs were significantly correlated ($p = 0.0001$, $r = 0.708$, 2-sided Mantel test; Table 3). Taken together, these findings indicate that the composition samples within patients are more alike than when compared to the composition of any given sample between patients.

Differences in Shannon diversity (richness and evenness) or phylogenetic diversity were not observed between MM and IM associated microbiota in healthy, AR or CRSwNP subjects (Fig. 3A, B). This was regardless of unilateral or bilateral disease preponderance. However, in CRSsNP patients, bacterial diversity was significantly reduced in the MM when compared to the IM ($p < 0.05$; Fig. 3A, B; Supporting Fig. 3A, B). The MM of CRSsNP exhibited significantly lower Shannon diversity ($p = 0.002$; nonparametric t test; Fig. 3A, Supporting Fig. 3A, CRSsNP) and was less phylogenetically diverse ($p = 0.001$; nonparametric t test; Fig. 3B, CRSsNP) than the IM.

Intersubject comparison of sinonasal microbiome across subgroups (healthy, AR, and CRS)

Sinonasal swabs were analyzed from all 65 subjects to determine changes across patient groups. If paired samples were sequenced, we chose a single sample per subject for the following analyses in which independence is assumed statistically. The independent sample was chosen based sampling location and disease burden. Since we demonstrated in Figure 3 that the middle meatus of CRSsNP patients was representative of underlying sinonasal disease, we preferentially chose the MM. If the MM was not sequenced to an adequate depth (≥5500 reads/sample), the IM was used as a representative sample. If the patient had unilateral sinus disease as indicated using Lund-Mackay scores, we chose the diseased side. Further, if the patient had equivalent bilateral disease, we randomly chose right or left specimens. The samples studied were from 8 healthy subjects (n = 6 MM, n = 2 IM), 11 AR subjects (n = 7 MM, n = 4 IM), and 46 CRS patients (25 CRSwNP [n = 13 MM, n = 12 IM], and 21 CRSsNP [n = 10 MM, n = 11 IM]).

The MM microbiota of CRSsNP patients exhibited lower diversity than those of controls (healthy and AR subjects) or CRSwNP patients ($p < 0.05$; nonparametric t test; Faith's phylogenetic diversity; Shannon diversity; Fig. 4A, B). No changes were observed in the alpha-diversity of the IM-associated microbiota across all groups ($p > 0.05$; nonparametric t test; Supporting Fig. 4A, B). Differences in beta diversity were not detected across all subject groups (healthy, AR, CRSwNP, CRSsNP) using multivariate permutational analysis on a weighted or unweighted UniFrac distance matrix ($p = 0.17$, $r^2 = 0.059$, PERMANOVA; Supporting Fig. 5A, B). Linear regression analyses based on cumulative CT and SNOT-22 scores did not reveal any statistically significant differences for Shannon or Faith's phylogenetic diversity ($p > 0.05$; F test; data not shown).

Detection of distinct bacterial taxa in CRS phenotypes

The final OTU table contained 4395 unique 99% OTUs that represented the phyla Firmicutes (54.5%), Actinobacteria (35.5%), Proteobacteria (5.8%), Bacteroidetes (2.3%), and Fusobacteria (1.3%; Supporting Fig. 6A-C). These phyla were represented by 46 genera at > 0.05% abundance, of which *Corynebacterium*, *Staphylococcus*, *Propionibacterium*, *Streptococcus*, and *Alloiococcus* were the most abundant (Supporting Fig. 6A-C). Compared to non-CRS subjects (healthy and AR), CRSsNP MM samples were enriched primarily in *Haemophilus* and *Fusobacterium*, although low-abundance *Streptococcus*, *Staphylococcus*, *Parvimonas*, *Propionibacterium*, and *Corynebacterium* OTUs were also increased (ZINB $p < 0.001$, $q < 0.10$; Fig. 5). Taxa depleted in CRSsNP compared to healthy subjects primarily included OTUs belonging to *Alloiococcus*, *Rothia*, *Corynebacterium*, *Finegoldia*, as well as low-abundance *Pseudomonas*, *Peptoniphilus*, *Prevotella*, and *Veillonella* (ZINB $p < 0.001$, $q < 0.10$; Fig. 5). Taxa enriched in CRSwNP included *Staphylococcus* and *Alloiococcus*, as well as low-abundance *Corynebacterium*,

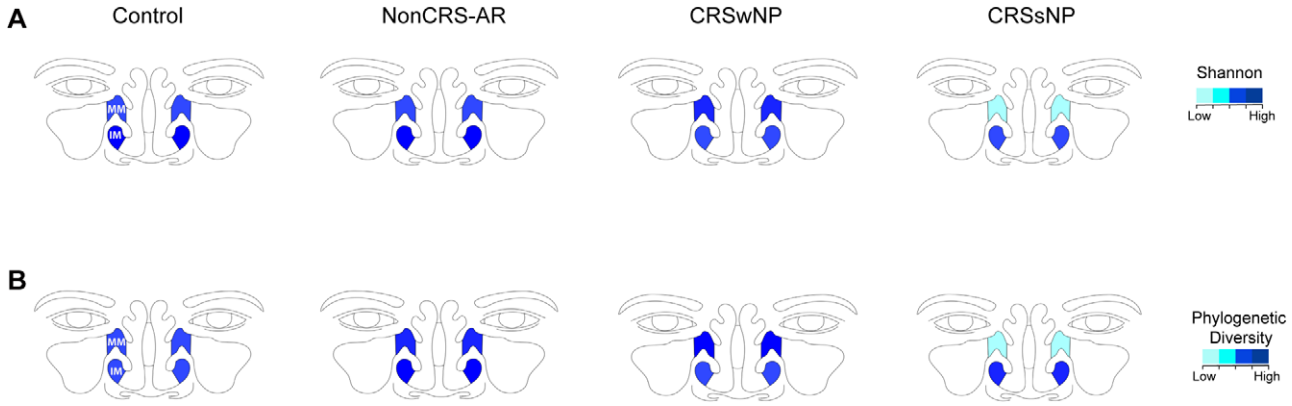

FIGURE 3. Average minimum and maximum (A) Shannon and (B) Faith's phylogenetic diversity indices for the MM and the IM within each clinical group. Darker shades of blue indicate higher diversity and lower shades indicate lower diversity. For more information about the values of Shannon or Faith's phylogenetic diversity see Supporting Fig. 1. IM = inferior meatus; MM = middle meatus.

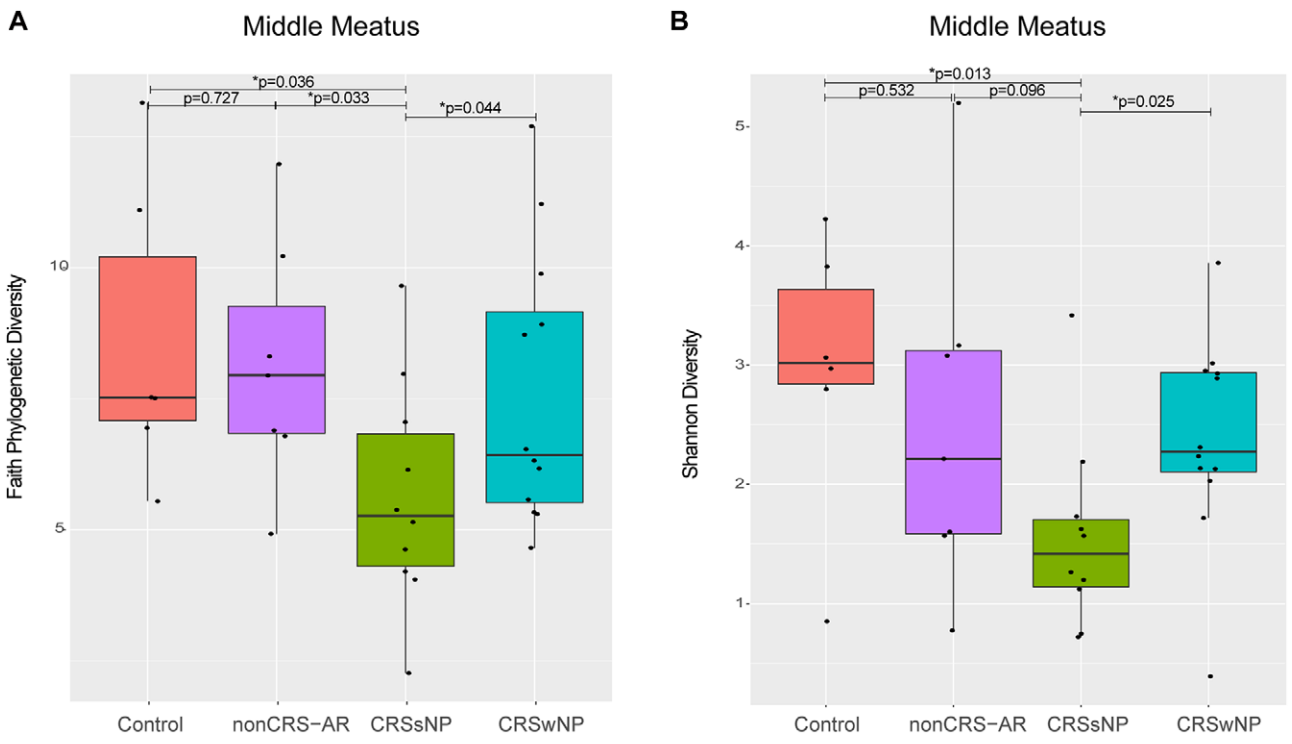

FIGURE 4. Loss of microbiota diversity in middle meatus microbiota. (A) Phylogenetic and (B) Shannon diversity are reduced in middle meatal microbial communities of CRSsNP patients compared to healthy controls or CRSwNP patients ($p < 0.05$, nonparametric *t* test). CRS = chronic rhinosinusitis; CRSsNP = CRS without nasal polyps; CRSwNP = CRS with nasal polyps.

Haemophilus, *Prevotella*, and *Porphyromonas*. CRSsNP microbiota were characterized by enrichment of anaerobes *Fusobacterium* and *Propionibacterium*, as well as *Haemophilus* and *Streptococcus* (ZINB $p < 0.001$, $q < 0.10$, Fig. 5).

Discussion

In this study, we examined the intrasubject and intersubject sinonasal microbiota profile in a large cohort of office-based samples to identify patterns of intersubject and intrasubject spatial organization of airway microbiota. Despite varying degrees of intrasubject variability, CRSwNP, AR, and healthy individuals had similar bacterial diversity in paired MM and IM samples. supporting results reported by Biswas et al.[6] and Hoggard et al.[9] Bacterial diversity was significantly reduced in the MM of CRSsNP, consistent with previous studies of the CRS microbiota that show loss of sinus-associated bacterial diversity in patients with severe disease requiring surgery.[6–9] Since ostiomeatal complex (OMC) obstruction is commonly associated with CRSsNP,[29] this finding is particularly relevant. We did not observe a significant loss of bacterial diversity in the MM of CRSwNP, which slightly contrasts with other studies

Taxon	Mean Group OTU counts				Significant group difference
	Control	NonCRS-AR	CRSsNP	CRSwNP	
Corynebacterium; s__	0	0	3	0	1
Propionibacterium; s__acnes	0	1	2	0	1
Parvimonas; s__	43	0	51	0	1
Staphylococcus	1	6	5	4	1
Veillonella; s__dispar	3	0	0	3	1
g__Rothia; s__aeria	9	0	1	3	1
Haemophilus; s__	0	0	119	0	1
Streptococcus; s__	4	0	18	0	1
Finegoldia; s__	11	57	0	4	1
Rothia; s__mucilaginosa	30	0	0	2	1
Streptococcus; s__	1	0	4	0	1
Abiotrophia	4	0	0	0	1
Staphylococcus	3	19	13	19	1
Staphylococcus; s__aureus	2	6	5	7	1
Corynebacterium; s__	18	0	0	3	1
Pseudomonas; s__	4	0	0	0	1
Nocardioidaceae; g__; s__	0	7	0	0	2
Propionibacterium; s__acnes	3	7	2	6	2
Corynebacterium; s__	681	824	117	489	2
Propionibacterium; s__acnes	95	547	106	194	2
Streptococcus; s__	28	1	19	7	2
Staphylococcus; s__epidermidis	0	4	1	10	3
Staphylococcus; s__aureus	0	7	0	20	3
Planctomyces; s__	0	0	0	5	3
Fusobacterium; s__	0	0	71	0	3
Staphylococcus; s__	0	0	1	27	3
Planococcaceae; g__; s__	0	0	1	1	3
Fusobacterium; s__	13	0	142	0	3
Porphyromonas; s__	1	3	1	2	3
Haemophilus; s__parainfluenzae	5	5	2	5	3
Staphylococcus; s__	0	0	0	96	3
Staphylococcus; s__epidermidis	1	4	2	12	3
Staphylococcus; s__aureus	1	5	3	10	3
Staphylococcus; s__aureus	0	2	0	5	3
Corynebacterium; s__	1	1	3	1	3
Propionibacterium; s__acnes	0	1	1	0	3
Prevotella; s__melaninogenica	4	1	1	2	1,3
Haemophilus; s__	9	0	736	0	1,3
Fusobacterium; s__	2	0	701	3	1,3
Corynebacterium; s__kroppenstedtii	4	1	0	2	1,3
Alloiococcus; s__	42	0	1	727	1,3
Propionibacterium; s__acnes	0	3	5	0	1,3
Corynebacterium; s__	0	0	6	14	2,3
Streptococcus; s__	668	2	778	25	2,3
Peptoniphilus; s__	6	149	0	14	1,2,3

1 = Control v. CRSsNP; p < 0.05, q < 0.10
2 = NonCRS-AR v. CRSsNP; p < 0.05, q < 0.10
3 = CRSwNP v. CRSsNP; p < 0.05, q < 0.10

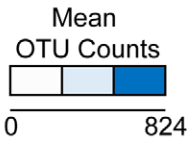

FIGURE 5. Heatmap of significant OTUs in the middle meatus using a zero-inflated negative binomial test for significance. All genera are represented by OTUs that were significant in 3 pairwise comparisons ($p < 0.001$, $q < 0.01$ for each OTU). The last column indicates significance in CRSsNP vs healthy (1), CRSsNP vs nonCRS-AR (2), and CRSsNP vs CRSwNP (3). *Haemophilus* and *Fusobacterium* were enriched in CRSsNP compared to CRSwNP as well as CRSsNP compared to controls. *Streptococcus* was enriched in CRSsNP compared to nonCRS-AR and CRSwNP. *Alloiococcus* was enriched in CRSwNP when compared to CRSsNP. AR = allergic rhinitis; CRS = chronic rhinosinusitis; CRSsNP = CRS without nasal polyps; CRSwNP = CRS with nasal polyps; OTU = operational taxonomic unit.

that demonstrate reduced alpha-diversity in the sinuses or MM of all CRS patient subgroups.[6,7,9] We attribute this difference to our strategy of clinic-based collection. The cohort studied here was designed to capture CRS patients across the spectrum of disease severity, whereas the studies performed to date focus on patients who failed maximal medical therapy and require endoscopic sinus surgery (ESS). Indeed, we identified changes in the microbiota of patients who reported higher symptom burden, although longitudinal studies will be required to determine whether patients with the most severe loss of microbial diversity respond to medical therapy or require ESS.

The studies presented here may also explain some of the discrepancies in the CRS microbiome literature, particularly with regard to sampling strategy. In prior microbiome studies, samples have been obtained from the MM,[3–6,8,9,30] the inflamed sinuses,[7,8] or through lavage of the sinonasal cavity,[31] sometimes with conflicting results. Reduced

microbial diversity is a common characteristic of sinus disease when the ethmoid or maxillary sinuses are sampled,[15] but this effect may be more subtle when sampling the MM,[3,6,8,30] and may be obscured when nonspecific nasal lavage samples are used.[31]

Intersubject microbial diversity and composition in the MM differs across clinical phenotypes of CRS. The observed enrichment of anaerobes and facultative anaerobes in CRSsNP, including *Fusobacterium* and *Haemophilus*, may be related to local disease processes. *Fusobacterium* spp. are one of the most common anaerobes isolated from CRS patients and are often associated with purulence,[32,33] which was more common in CRSsNP, possibly explaining the increased preponderance of anaerobic bacterial taxa. *Staphylococcus* was enriched in CRSwNP, supporting culture-based studies that have found elevated *S. aureus* colonization rates in the MM and *S. aureus* enterotoxin (SAE)-specific immunoglobulin E (IgE) antibodies in a subset of patients with high eosinophilic inflammation.[34,35] *Alloiococcus* spp., also enriched in CRSwNP, have been detected in the sinuses of CRS and healthy controls,[36,37] and co-colonization with *Staphylococcus*, *Streptococcus*, and anaerobic taxa in the maxillary sinuses of CRS patients has been reported.[38] While co-colonization may play an important role in bacterial behavior modification, whether and how this organism contributes to sinus inflammation is still unknown.

The findings from this study can be placed in the context of microbiome studies of the airways in patients with asthma and AR, which may have some overlapping features with CRS. In a study of patients with AR and healthy controls, bacterial diversity was increased and correlated with nasal eosinophils in AR patients compared to controls during allergy season.[39] In this study we were unable to reproduce the findings of increased variety and diversity in AR patients compared to controls, likely due to smaller sample size and not timing specimen collection to specific allergy season. Future studies that measure the upper and lower airway microbiome concurrently, correlated to clinical phenotype, will further clarify the role of the microbiome in airway diseases.

There are limitations to this study. Although the office-based setting allows for enrollment of a larger patient cohort spanning a range of disease severity, the number of healthy controls was still disproportionately low. Despite this, we could identify statistically significant differences across groups. Also, the inclusion of non-CRS patients with AR allowed increased the overall number of non-CRS patients and acted as a unique disease-control. The subtle changes in taxa associated with CRSwNP highlights the need for large cohorts to discern differences in the sinonasal microbiota in this broad clinical group when nonsurgical patients are included. We also recognize that swab heads may have contacted a neighboring anatomical structure (eg, the middle turbinate) during sampling, but the use of the aural speculum would have protected the MM swab from contacting the nares or inferior meatal space. Thus, this limitation would not have interfered with our study findings. The ideal guarded swabs for MM sampling are not yet standardized. Amplicon sequencing of sinonasal swab continues to be problematic for low-bacterial load samples such as sinonasal swabs. Indeed, we lost several samples secondary to this problem, similar to other investigators.[7,36] Our laboratory is currently investigating novel approaches to enhance the microbial signal without skewing microbiota profiles. Last, the broad categorization of patients into CRSwNP and CRSsNP is simplistic, and needs studies with larger sample sizes and endotyping.[40] Future studies from our group will aim to identify whether the bacteria that are enriched in each disease state can drive or exacerbate CRS. A unique strength of office-based sampling is the potential for future longitudinal studies.

Conclusion

This study examines the sinonasal microbiota in a heterogeneous cohort of subjects from an office-based setting. Here, we demonstrate in a relatively large cohort of subjects from whom multiple samples were obtained, that intrasubject variability is significantly less than intersubject variability in terms of composition and taxon presence or abundance. However, in CRSsNP, MM-associated microbiota diversity is significantly depleted when compared to the IM or across patient subgroups. This finding may signify localized MM pathogenetic processes unique to this CRS subtype. CRSsNP patients had significantly lower diversity compared to CRSwNP and controls, and were enriched in anaerobic taxa. This suggests that CRSsNP may represent a more infection-associated phenotype. In contrast, CRSwNP patients were enriched in *Staphylococcus* or *Alloiococcus*, consistent with previous culture-based findings. These findings reinforce evidence for microbial involvement in CRS.

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