Viral presence and the bacterial microbiome in chronic rhinosinusitis

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Background: Factors at play in the aetiopathogenesis of chronic rhinosinusitis (CRS) have been postulated to include a bacterial dysbiosis and a recently-established greater susceptibility to viral infection. There is potential for the presence of virus to influence the resident bacterial microbiome, thereby altering the course of the disease. The aim was to investigate virus-associated changes in the bacterial CRS sinonasal microbiome.

Methods: Brushings of the sinonasal mucosa were taken at time of endoscopic sinus surgery. Viral detection was undertaken using PCR or RT-PCR on extracted DNA and RNA. Bacterial characterisation was undertaken using 16S ribosomal RNA gene-targeted amplicon sequencing. Analysis of bacterial abundance, diversity and stability with regard to viral presence was undertaken using the Quantitative Insights Into Microbial Ecology platform.

Results: A total of 82 adult patients were recruited for this study: 10 controls, 49 non-polyp and 23 polyp patients. Half of each group were virus-positive patients. No significant differences were seen in relative abundances of the bacterial genera detected, their diversity or stability, or time since last patient-reported upper respiratory tract infection in any of the groups.

Conclusions: No significant differences in the composition of the bacterial microbiome in virus-negative or positive patients was seen, however this may be limited by the sample size of the study and requires further research.

Keywords: Chronic rhinosinusitis (CRS); virus; microbiome; sinus; bacteria; polyp

Received: 31 December 2021; Accepted: 28 March 2022; Published: 18 May 2022.
doi: 10.21037/ajo-21-53
View this article at: https://dx.doi.org/10.21037/ajo-21-53

Introduction

The bacterial microbiome of the aerodigestive tract and specifically the sinonasal passages is an area receiving increasing attention in current literature. This is of significant interest to research into chronic rhinosinusitis (CRS). This inflammation of the nose and paranasal sinuses of greater than twelve weeks duration is characterised by symptoms of anterior rhinorrhoea, post-nasal drip, headaches, facial pain or pressure, nasal obstruction and hyposmia. Aetiopathogenesis theories are many but a significant focus remains on microbial imbalance, and

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methods of manipulating this for therapeutic ends.

A bacterial dysbiosis has been shown to exist within CRS with patients showing a lack of biodiversity compared to healthy controls (1). Bacterial species historically seen as more pathogenic are found to be relatively more prevalent in CRS than bacterial species that have been considered commensal (2). Studies investigating the CRS microbiome have varied in scale, methodology and analysis and so data have been difficult to compare. Efforts have been made to standardise these, concluding that the healthy sinonasal microbiome consists mainly of *Staphylococcus*, *Propionibacterium*, *Corynebacterium* and *Streptococcus* species while the microbiome in CRS shows a reduction in the relative abundance of Actinobacteria and *Propionibacterium* species, with significantly more prevalent *Corynebacterium* species (3).

Upper respiratory tract viruses have recently been shown to be more common in CRS than in healthy individuals. The presence of these viruses is also associated with more severe subjective and objective disease (4). These findings potentially implicate viruses as an inciting and/or exacerbating factor in the immune dysregulation of CRS. Viral-bacterial co-infection is known to have deleterious effects on epithelial barrier function, bacterial binding and innate and adaptive immunity (5). It has been suggested that viruses may induce changes in the bacterial microbiome potentially causing more severe disease in the lower respiratory tract (6,7). However, little is known about the specific changes in bacterial aerodigestive populations seen in the presence of viral infection in CRS.

The aim of this study was thus to investigate virus-associated changes in the bacterial CRS sinonasal microbiome, hypothesising that viral infection would indeed alter the composition of resident bacteria. We present the following article in accordance with the STROBE reporting checklist (available at https://www.theajo.com/article/view/10.21037/ajo-21-53/rc).

Methods

Study participants

Study participants were recruited from the tertiary rhinologic practices of two of the senior authors (PJW and AJP) in Adelaide, Australia over the course of 2017 and 2018. This study was carried out in accordance with the recommendations of the Central Adelaide Local Health Network Ethics Committee, with their approval of the protocol (HREC/15/TQEH/132). The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013) and study participants gave verbal and written informed consent. Patients were included in this study if they were older than eighteen years of age and were undergoing endoscopic nasal surgery. Control patients did not have any clinical or radiologic evidence of CRS, and were undergoing trans-sphenoidal resections of pituitary masses or surgery to the septum or inferior turbinates. CRS patients fulfilled the diagnostic criteria for CRS as outlined in the American guidelines, and were undergoing functional endoscopic sinus surgery (FESS) (8). Patients who had used antibiotics or steroids in the two months prior to the study day were excluded. No patients in this study suffered from asthma or aspirin-exacerbated respiratory disease and none were smokers. On the study day patients were asked to report the timing of their last URTI with the following time points: current, within the last 1–2 weeks, within the last 2–4 weeks, within the last 1–2 months, or more than 2 months prior.

Viral sampling, processing and analysis

Viral sampling, processing and analysis was undertaken using a previously published departmental protocol (9). Briefly, the left and right middle meatus (MM) and inferior meatus (IM) mucosa was sampled using EndoScan cytology brushes (McFarlane Medical, Melbourne, Australia). This was conducted with endoscopic visualisation and aseptic technique. Samples were transported on ice and stored at −80 ℃. At time of processing samples were thawed for RNA and DNA extraction using an AllPrep DNA/RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The products of these were stored and subsequently real-time PCR batch tested. An initial assay for endogenous retrovirus 3 (ERV3) was undertaken in the DNA extract fractions to ensure adequate sample collection quality. Assays were then undertaken for adenovirus (AdV), bocavirus (BoV), coronavirus (CoV), enterovirus (EnV), influenza, metapneumovirus (MPV), parainfluenza (PIV), respiratory syncytial virus (RSV) and rhinovirus (RV). PCR target genes, primer and probe sequences, the nature of positive and negative controls and cycling conditions have been published previously (9). A cycle threshold (Ct) of forty or less indicated viral detection, as has been validated previously (9).

Bacterial sampling and processing

Bacterial sampling was also undertaken intra-operatively

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with an aseptic technique and endoscopic visualisation. Guarded, flocked swabs (Copan Italia S.p.A, Brescia, Italy) were inserted into the MMs of all patients on both sides and rotated seven times before removal. Swabs were stored at −80 ℃ until batch thawing for DNA extraction and analysis as follows. Swab heads were cut into small pieces and 180 µL of enzymatic lysis buffer (Qiagen, Hilden, Germany) was added and left overnight at room temperature. 5 mm steel beads agitated for 20 seconds at 15 Hz in a Qiagen Tissue Lyser were used to homogenise the pieces, followed by 5 minutes of further homogenisation with 0.1 mm glass beads at 30 Hz. DNA extraction was then undertaken in accordance with the Qiagen DNeasy Blood and Tissue Kit protocol (Qiagen, Hilden, Germany), and stored at −80 ℃ until sequencing.

Bacterial 16S-sequencing

Polymerase chain reaction (PCR) amplification and sequencing was performed by the Australian Genomics Research Facility (AGRF). Gene libraries were generated by amplifying the V3 to V4 (341F–806R) hypervariable region of the 16S rRNA. AmpliTaq Gold 360 Master Mix (Life Technologies, Mulgrave, Australia) with primers CCTAYGGGRBGCASCAG in the forward sequence and GGACTACNNGGGTATCTAAT in the reverse sequence were used to generate PCR amplicons. These underwent fluorometric measurement (Invitrogen Picogreen; Thermo Fisher Scientific, Waltham, MA, USA) and normalised. Quantitative PCR (KAPA Biosystems, Capetown, South Africa) was used to quantify the equimolar pool. This was arranged for sequencing on the Illumina MiSeq (Illumina Inc., San Diego, CA, USA) with 300 base paired end chemistry. All samples in this study were sequenced in one run.

Statistical analysis and bioinformatic pipeline

The Quantitative Insights Into Microbial Ecology platform (QIIME 2, version 2018.11) (10) was employed for the bioinformatic pipeline in this study. PEAR was used to combine forward and reverse reads (11) through the QIIME 2 plugin q2-pear (https://github.com/bassio/q2-pear). The QIIME 2 plugin q2-quality-filter (12) was used to quality-filter the combined sequences. Minimum quality parameter was 20 (13). Deblur (q2-deblur plugin with setting “trim-size” =435 with default parameters otherwise) was used for denoising and to form Amplicon Sequence Variant (ASV) (13). Greengenes 16S database (version 13.8 August 2013, the 99% clustered similarity sequences) was used as reference (14). and the QIIME 2 BLAST-based q2-feature-classifier was used as taxonomy classifier (15).

Prior to analysis, n=400 reads were chosen as the rarefaction depth cut-off. Rarefaction plots (for total number of ASVs and for Shannon’s alpha diversity index) were performed (Figure S1) and showed that most samples had rarefaction curves that reached a plateau at the 400 read depth, indicating sufficient subsampling. Relative abundance comparisons were done at the genus level. The taxonomic assignment of the one DNA-negative control sample containing extraction reagents only was explored. The bacterial genus *Flavobacterium* was present in high relative abundance in this sample and in relatively low abundance in many samples, so this genus was excluded before downstream statistical analyses. Mean relative abundance and genera prevalence were calculated. Alpha diversity was measured using Shannon’s diversity and Faith’s phylogenetic diversity index (16), calculated using Sci-kit bio (version 0.5.3).

Rank variability is a per-sample index, and a surrogate for microbiome stability. It is defined by Martí *et al.* as “the absolute difference between each taxon rank and the overall rank” (17). Rank variability was calculated using a Python implementation of the equations previously described (17).

Results

Patient characteristics

A total of 82 patients were recruited: 10 controls, 49 CRSsNP and 23 CRSwNP. Of 82 patients, 41 were virus-positive; 5 control, 24 CRSsNP and 12 CRSwNP. Virus-positive and virus-negative patients were age and season-matched within the three groups (control, CRSsNP and CRSwNP). Demographics and patient characteristics are summarised in *Table 1*.

Viral detection

ERV3 was detected in all samples. Mean ERV3 Ct was 22.9, indicating that adequate cellular material was obtained in all cases. Forty-one patients were positive for one or more of the disease-causing viruses assayed, while 41 were virus-negative. RV was the most prevalent of the species assayed; MPV was not detected in any of the samples. Fourteen patients were positive for more than one viral species. Details of viral species detected are summarised in *Table 2*.

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Table 1 Summary of patient demographics and characteristics

Demographics	Control	CRSsNP	CRSwNP
Number with each diagnosis	10	49	23
Number of virus-positive patients	5	24	12
Mean age (years)	35.5	50.0	45.0
Male:female	4:6	27:22	22:1
Season sample obtained (spring:summer:autumn:winter)	2:2:2:4	20:8:12:9	4:4:8:7

CRSsNP, CRS without nasal polyps; CRSwNP, CRS with nasal polyps; CRS, chronic rhinosinusitis.

Table 2 Details of viral species and patient diagnoses

Viral species	Number of positive control patients	Number of positive CRSsNP patients	Number of positive CRSwNP patients
Adenovirus	1	0	0
Bocavirus	1	2	1
Coronavirus	4	6	5
Enterovirus	1	1	1
Influenza	0	6	1
Parainfluenza	0	1	2
Rhinovirus	1	13	1
RSV	0	0	1

CRSsNP, CRS without nasal polyps; CRSwNP, CRS with nasal polyps; CRS, chronic rhinosinusitis; RSV, respiratory syncytial virus.

Bacterial microbiome outcomes: taxonomy

The most abundant taxa are found in *Table 3*. The genera *Corynebacterium*, *Staphylococcus*, *Streptococcus*, *Haemophilus*, and *Moraxella* were the most abundant. We found no significant difference in differential abundance of the five most abundant genera by diagnosis (i.e., control versus CRSsNP versus CRSwNP; Kruskal-Wallis P>0.05, *Figure 1*).

Viral covariates as predictors of bacterial taxa abundances

We found no significant association between viral presence or number of viruses detected and the abundances of the aforementioned most abundant bacterial genera (Kruskal-Wallis, P>0.05). Moreover there was no statistically significant association detected between the presence of CoV, influenza or RV and bacterial relative abundances. There was also no difference found in relative abundances of the most abundant bacteria when grouped by time since last patient-reported viral infection (*Figure 2*).

Viral covariates as predictors of bacterial diversity and stability

We calculated Faith's and Shannon's indices as markers of both phylogenetic and non-phylogenetic alpha diversity. We also calculated rank variability; a per-sample surrogate for microbiome stability as mentioned earlier. We found no significant association between bacterial genera abundances and viral status covariates such as viral presence, number of viruses, and presence of specific viruses (influenza, CoV or RV) (P>0.05).

Discussion

This study seeks to compare the bacterial microbiome seen in virus-positive and virus-negative individuals both with and without CRS. The most prevalent viruses seen in all groups were CoV, influenza and RV. The most abundant bacterial genera seen were *Corynebacterium*, *Staphylococcus*, *Streptococcus*, *Haemophilus*, and *Moraxella*.

No significant differences were seen in bacterial

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Table 3 Summary of most abundant bacterial genera

Bacterial genus	Control				CRSsNP				CRSwNP			
	Virus-pos		Virus-neg		Virus-pos		Virus-neg		Virus-pos		Virus-neg	
	MRA	P	MRA	P	MRA	P	MRA	P	MRA	P	MRA	P
Corynebacterium	26.00	80.0	44.85	100.0	33.39	58.33	31.71	60.87	23.75	66.67	48.05	58.33
Staphylococcus	47.06	80.0	33.05	100.0	22.45	58.33	24.49	69.57	26.85	66.67	23.55	50.00
Streptococcus	9.12	20.0	0.00	0.0	4.86	12.50	9.94	26.09	0.00	0.00	3.62	16.67
Haemophilus	0.00	0.0	0.00	0.0	10.55	16.67	3.50	8.70	5.42	8.33	0.00	0.00
Moraxella	0.94	20.0	4.35	40.0	7.18	16.67	0.00	0.00	0.00	0.00	3.68	8.33
Proteus	0.00	0.0	0.00	0.0	0.00	0.00	0.00	0.00	8.38	8.33	10.00	8.33
Porphyromonas	0.00	0.0	0.00	0.0	6.11	8.33	0.00	0.00	3.60	8.33	0.00	0.00
Genus unidentified (*Enterobacteriaceae*)	1.25	20.0	0.00	0.0	3.11	12.50	4.84	4.35	0.00	0.00	0.00	0.00
Alloiococcus	0.00	0.0	0.90	40.0	0.00	0.00	2.96	4.35	4.20	8.33	0.95	8.33
Genus unidentified (Cytophagaceae)	0.00	0.0	5.45	40.0	1.68	25.00	1.87	17.39	0.00	0.00	0.00	0.00

CRSsNP, CRS without nasal polyps; CRSwNP, CRS with nasal polyps; pos, positive; neg, negative; MRA, mean relative abundance; P, prevalence (both expressed as percentages); CRS, chronic rhinosinusitis.

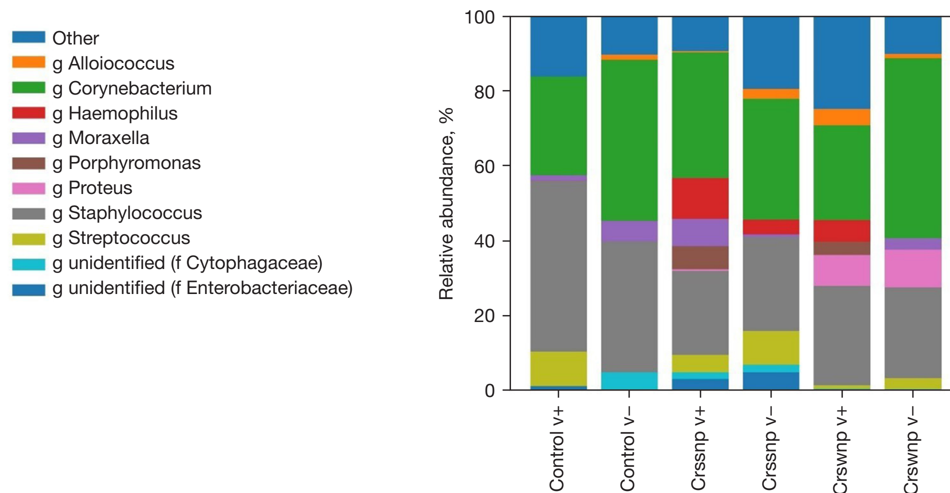

Figure 1 Relative abundances of the most abundant bacterial genera. "v+" indicates viral positivity; "v–" indicates viral negativity.

abundance, diversity or stability between virus-negative or virus-positive individuals within the control, CRSsNP or CRSwNP groups with regard to the viral covariates tested (presence or absence of virus, number of viruses, presence of specific viruses or time since last patient-reported viral infection). This is in contrast to previously reported effects of viral presence on the microbiome in non-CRS populations (18-20). Our study focuses on CRS patients, is smaller and geographically different to these studies, but its strengths lie in a robust and previously validated viral collection method (9), an undertaking of more in-depth analysis beyond viral presence or absence alone, and the use of age and season-matched virus-negative control groups. Ding *et al.* compared swabs from control and influenza-

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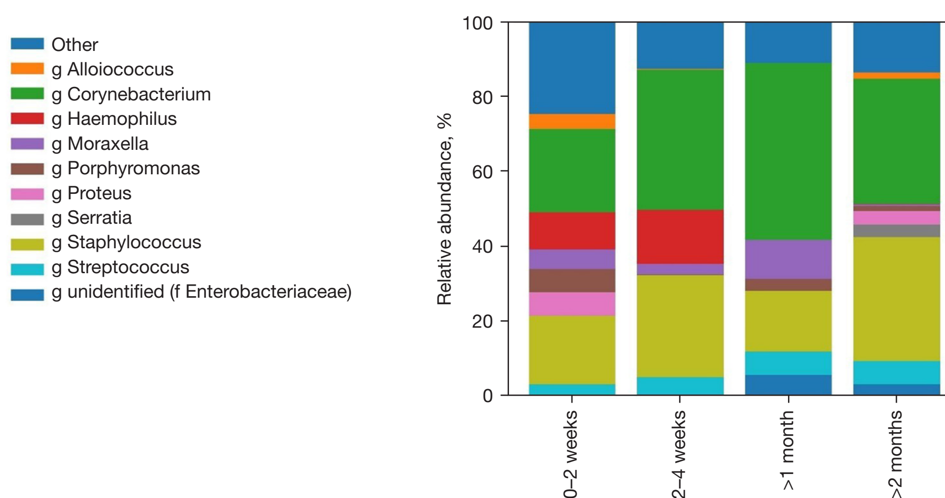

Figure 2 Relative abundances of the most abundant bacteria grouped by time since last patient-reported viral infection.

infected individuals (sample size of 40 versus 215). They found *Corynebacterium* and *Streptococcus* to be more abundant in controls, with virus-positive swabs dominated by *Moraxella* and *Dolosigranulum* (20). This result is interesting not least due to the rarity of identification of *Dolosigranulum* in adult CRS upper respiratory tract bacterial profiles; the low biomass of airway samples carries risk of contamination if not carefully screened prior to and following analysis. Borges *et al*. compared swabs from twelve patients with severe acute respiratory infections; six with influenza, and six with undisclosed non-influenza causative viral organisms. Despite their small sample size they found significant differences in abundance of fifteen different bacterial genera, however no control group was presented (19). Rosas-Salazar *et al*. compared the infantile microbiome in the presence of either RV or RSV, and found significant differences in eleven genera. Again, no control group was presented, and in all three of the aforementioned studies viral collection methods were prone to contamination. No method to ensure cellular collection was employed, and as such even viral presence cannot be confidently asserted in these studies.

The negative effects of a viral/bacterial co-infection have long been established in many body systems, but of significant interest in any such co-infection model is the nature of the original inciting pathogen. This is a cross-sectional study investigating the characteristics of sinonasal microbiota at a solitary time point (that of endoscopic sinonasal surgery). Should differences in these microbiota have been observed between control and CRS patients, we would be unable to determine whether it is the composition of the bacterial microbiome that predisposes to viral infection, or vice versa. To elicit such information a longitudinal study model would be required. To our knowledge this has not yet been undertaken in these cohorts. An additional limitation is a lack of gender balance in the CRSwNP patient sample, and the known differing immune response profile of CRSsNP and CRSwNP patients. CRSsNP is associated with a Th-1 skewed response, while CRSwNP is associated with a Th-2 skewed, eosinophilic response (21,22). Viral infections themselves are also associated with eosinophilia (23). These factors have the potential to impact the detection of virus and/or the microbiome balance observed in this study (24).

Of additional interest but requiring further research would be whether there is correlation between patient reporting of a current viral infection (manifest as more severe symptoms), and the presence of virus in the sinonasal passages at that time. Only three patients in this study reported such, limiting any analyses thereof. An additional limitation is that the symptoms of CRS and of a viral URTI are similar but with a marked difference in duration.

Conclusions

In conclusion this study compares the bacterial microbiome in virus negative and virus positive controls, CRSsNP and CRSwNP, and has uncovered no significant differences in its composition. However, larger, longitudinal investigation is required to investigate this further.

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Acknowledgments

Funding: The study was supported by internal departmental funding only.

Footnote

Reporting Checklist: The authors have completed the STROBE reporting checklist. Available at https://www.theajo.com/article/view/10.21037/ajo-21-53/rc

Data Sharing Statement: Available at https://www.theajo.com/article/view/10.21037/ajo-21-53/dss

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://www.theajo.com/article/view/10.21037/ajo-21-53/coif). AJP: Consultant—Medtronic, Fusetec, Tissium, ENT technologies; Speaker Honorarium—Sequiris; Shareholder Chitogel; unpaid editorial board member of Australian Journal of Otolaryngology from January 2021 to December 2022. The other authors have no conflicts of interests to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Central Adelaide Local Health Network Ethics Committee (HREC/15/TQEH/132) and informed consent was taken from all individual participants.

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doi: 10.21037/ajo-21-53
Cite this article as: Goggin RK, Bennett CA, Cooksley CM, Bassiouni A, Bialasiewicz S, Wormald PJ, Vreugde S, Psaltis AJ. Viral presence and the bacterial microbiome in chronic rhinosinusitis. Aust J Otolaryngol 2022;5:11.

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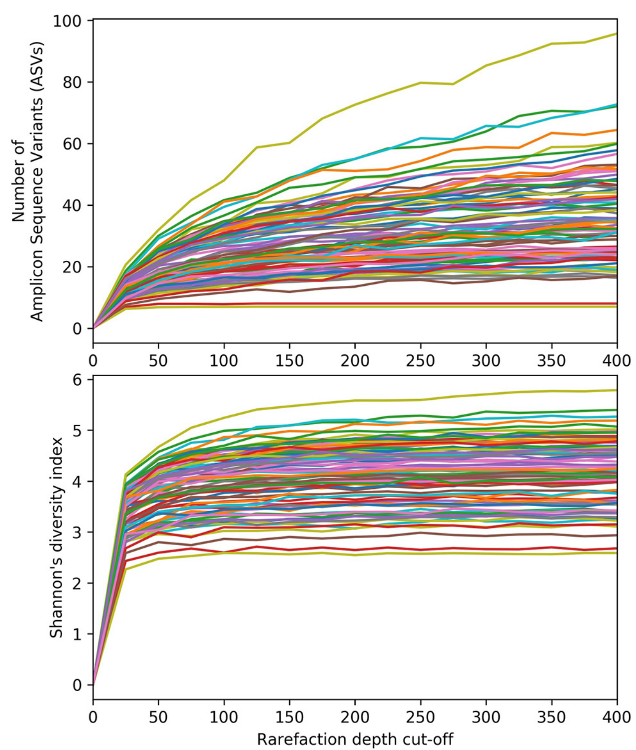

Figure S1 Rarefaction plots. ASV, Amplicon Sequence Variant.

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