The international sinonasal microbiome study (ISMS): a multi-centre, multi-national collaboration characterising the microbial ecology of the sinonasal cavity

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ABSTRACT

The sinonasal microbiome remains poorly defined, with our current knowledge based on a few cohort studies whose findings are inconsistent. Furthermore, the variability of the sinus microbiome across geographical divides remains unexplored. We characterise the sinonasal microbiome and its geographical variations in both health and disease using 16S rRNA gene sequencing of 410 individuals from across the world. Although the sinus microbial ecology is highly variable between individuals, we identify a core microbiome comprised of *Corynebacterium*, *Staphylococcus*, *Streptococcus*, *Haemophilus*, and *Moraxella* species in both healthy and chronic rhinosinusitis (CRS) cohorts. *Corynebacterium* (mean relative abundance = 44.02%) and *Staphylococcus* (mean relative abundance = 27.34%) appear particularly dominant in the majority of patients sampled. There was a significant variation in microbial diversity between countries ($p = 0.001$). Amongst patients suffering from CRS with nasal polyps, a significant depletion of *Corynebacterium* (40.29% vs 50.43%; $p = 0.02$) and over-representation of *Streptococcus* (7.21% vs 2.73%; $p = 0.032$) was identified. The delineation of the sinonasal microbiome and standardised methodology described within our study will enable further characterisation and translational application of the sinus microbiota.

Keywords

Chronic rhinosinusitis, microbiota, next-generation sequencing, 16S rRNA gene, sinus, microbiome, polyp

Abbreviations

NGS, Next-generation sequencing

CRS, chronic rhinosinusitis

CRSsNP, chronic rhinosinusitis sans nasal polyps

CRSwNP, chronic rhinosinusitis with nasal polyps

MAIN TEXT

The important role of human microbiota in both health and disease has become increasingly recognised. Microbial communities encode millions of genes and associated functions which act in concert with those of human cells to maintain homeostasis.[1] Numerous studies have now established the microbiota as an important contributor to essential mammalian functions such as metabolism[2], biosynthesis[3], neurotransmission[4,5] and immunomodulation[6,7]. Characterizing the composition and diversity of normal, healthy microbial communities is a cornerstone to developing our understanding of dysbiosis, pathophysiology and, ultimately, directing therapy. To this end, advent of next-generation sequencing (NGS) has revolutionised our appreciation of the host microbiota and its polymicrobial nature.[8,9]

In many cases the entire microbial community – commensal, symbiotic, pathogenic bacteria, fungi, archaea, and viruses – play critical roles in both health and disease pathogenesis. The host-microbiota interface is particularly important in chronic mucosal inflammatory conditions where the microbiota interact directly with the host. These conditions are often poorly understood, multifactorial in nature, have heterogeneous clinical presentations and vary in treatment response.[10,11] Furthermore, single causative pathogens are rarely identified, and culture directed-antibiotics often fail to demonstrate efficacy.[12] It is plausible that a better understanding of the microbiome of such conditions may be key to unraveling their underlying pathogenesis.

The sinonasal mucosa is continuously exposed to external particulate matter and microbes, but it is relatively immunodeplete with no native secondary lymphoid organ systems.[13] The sinus microbiota is thought to play key roles in multiple extra-nasal conditions, such as providing a nidus of recurrent infection in cystic fibrosis patients[14], and otitis media.[15] In addition, there is evidence of microbial influences in the development, progression and severity of chronic rhinosinusitis (CRS).[16] This multifactorial condition, with an estimated world-wide prevalence of approximately 10%,[11] represents one of the most common diagnoses for inappropriate antibiotic prescription and is a source of significant

150 morbidity and healthcare costs.[11,17–19] To date, despite a number of well-designed research efforts to
151 define the nature of the sinonasal microbiome and its role in CRS pathogenesis, many uncertainties
152 persist. This is in part due to the difficulty in bacterial collection from the nose itself. Unlike the gut and
153 oral cavity where the bacterial burden is high and access to microbiota relatively easy (either via faecal
154 samples or oral wash),[20,21] the sinonasal tract has a low microbial burden and access is difficult due to
155 both the narrow nasal orifice and discomfort for the awake patient. Therefore, a replicable sample with
156 appropriate bacterial abundance for 16S rRNA gene sequencing is currently attainable only during nasal
157 surgery. To date, the majority of published studies have been small in size, with heterogeneous patient
158 populations and inconsistency in collection methods, sample site, processing techniques and
159 bioinformatics pipelines.[22] Ultimately, the consequence has been non-comparable results with no
160 universal consensus on the constituents of the healthy sinonasal microbiota or the dysbiosis that occurs in
161 disease.[23–29]

162 To address these limitations, we investigate the sinonasal microbiome using 16S rRNA gene-sequencing
163 on a large, multi-centre, international cohort implementing consistent sampling, processing and
164 bioinformatics methods. We aim to (1) characterise the normal sinonasal microbiome, (2) assess for any
165 geographical or clinical influences and (3) identify any changes associated with CRS within and across
166 geographical sites.

167

168 # RESULTS

169 **Patient cohort**

170 Middle meatus specimens were collected for 16S rRNA gene sequencing (V3-V4 hypervariable region)
171 on the Illumina MiSeq platform (see Methods). Thirteen centres, across five continents, participated in
172 patient sampling. 532 samples (194 healthy controls and 338 CRS patients) successfully went through all
173 stages of transport and processing to be sent for sequencing. High-quality sequences were analysed using
174 QIIME 2.[30] A total of 410 patients, aged between 20 and 75, reached the final stage of analysis. This
175 population included 139 non-CRS healthy controls, 99 patients without nasal polyposis (CRSsNP) and
176 172 CRS patients with nasal polyposis (CRSwNP). Supplementary Figure S1 demonstrates sample
177 distribution by centre.

178 **The sinonasal microbiome in healthy sinuses is dominated by Corynebacterium and Staphylococcus**

179 We first investigated the composition of the healthy sinonasal microbiome by intra-operatively sampling
180 the 139 non-CRS control patients. (See Methods) Our analysis demonstrated the dominance of
181 *Corynebacterium* (mean relative abundance = 48.7%; prevalence = 88.49%) and *Staphylococcus* species
182 (mean relative abundance = 29.25%; prevalence = 79.86%) in the sinonasal microbiome of healthy
183 patients. These were both the most abundant and prevalent genera amongst our population (Table 1). This
184 finding has been observed in some but not all previously reported studies with variability in sampling and
185 analysis techniques likely accounting for such discrepancies.[22]

186 *Table 1: Abundance and prevalence of genera found in microbiota of healthy non-CRS patients.*

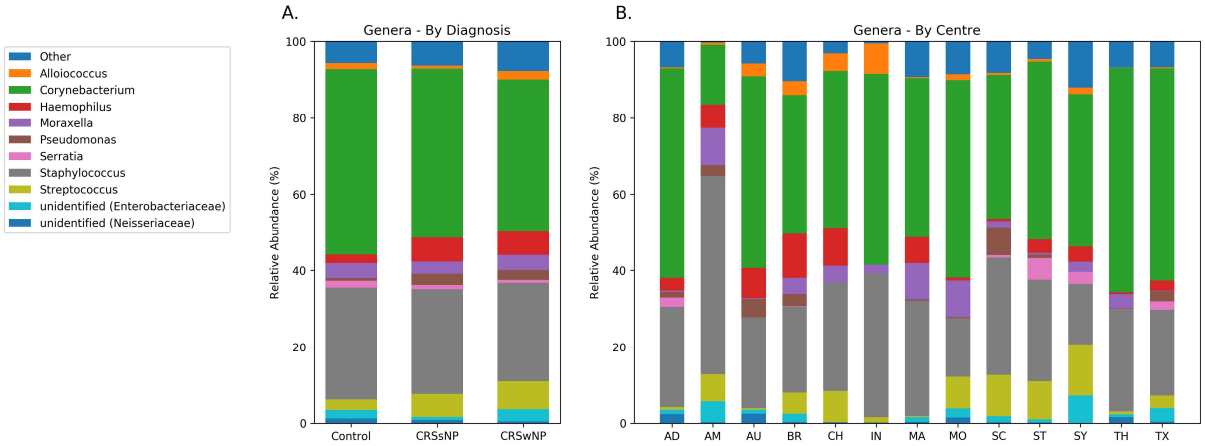
Genera	Mean Relative Abundance (%)	Prevalence (%)
Corynebacterium	48.7	88.49
Staphylococcus	29.25	79.86
Moraxella	3.86	12.23

Streptococcus	2.81	20.86
Haemophilus	2.23	12.95
unidentified (Enterobacteriaceae)	2.16	13.67
Serratia	1.79	2.16
Alloiococcus	1.61	20.86
unidentified (Neisseriaceae)	1.25	12.23
Pseudomonas	0.75	2.88

187 *Moraxella*, *Streptococcus*, *Haemophilus*, *Enterobacteriaceae*, *Serratia*, *Alloiococcus*, *Neisseriaceae* and
188 *Pseudomonas* made up the remainder of the ten most abundant genera. The lower prevalence of these
189 remaining organisms could suggest that aside from *Corynebacterium* and *Staphylococcus*, there is a high
190 degree of variability in the constituents of a healthy upper airway microbiome.

191 **Microbiome composition is altered in CRSwNP and by geographical location**

192 To explore influences of microbial composition, we examined the taxonomic profiles of our patient
193 cohort once grouped by (1) disease cluster and (2) centre of origin (Figure 1) utilizing mixed modeling to
194 control for the "centre" variable by assigning it as a random effect (See Methods). CRSsNP patients
195 demonstrated no significant differences in the relative abundance of the top ten most abundant organisms
196 when compared to healthy controls. ($p > 0.05$; mixed model analysis) The relative abundance of most
197 organisms also remained stable between controls and CRSwNP but for two genera (Figure 1A):
198 *Corynebacterium* was found to be significantly reduced in CRSwNP when compared to controls (40.29%
199 vs 50.43%; mixed model analysis; $p = 0.02$) whilst *Streptococcus* was increased (7.21% vs 2.73%; mixed
200 model analysis; $p = 0.032$). Interestingly, the most commonly cultured pathogens in CRS, *Staphylococcus*
201 and *Pseudomonas* were similar between all cohorts.



	Control	CRSsNP	CRSwNP
Alloiococcus	1.81%	1.11%	2.16%
Corynebacterium	50.43%	43.09%	40.29%
Haemophilus	2.21%	6.28%	6.02%
Moraxella	3.63%	3.53%	3.96%
Pseudomonas	0.75%	2.88%	2.59%
Serratia	1.79%	1.13%	0.74%
Staphylococcus	28.3%	27.49%	26.52%
Streptococcus	2.73%	6.41%	7.21%
unidentified Enterobacteriaceae	2.15%	0.86%	3.2%
unidentified Neisseriaceae	1.27%	0.77%	0.45%

	AD	AM	AU	BR	CH	IN	MA	MO	SC	ST	SY	TH	TX
Alloiococcus	0.3%	0.5%	3.3%	3.6%	4.6%	7.9%	0.3%	1.5%	0.6%	0.7%	1.8%	0.1%	0.3%
Corynebacterium	55.0%	15.5%	50.3%	36.5%	41.1%	50.1%	41.8%	51.8%	37.8%	46.7%	40.1%	58.8%	55.7%
Haemophilus	3.3%	6.2%	7.8%	11.4%	10.0%	0.0%	6.8%	0.8%	0.6%	3.5%	4.0%	0.5%	2.6%
Moraxella	0.3%	9.8%	0.2%	4.3%	4.3%	2.2%	9.5%	9.5%	1.6%	0.4%	2.6%	3.7%	0.2%
Pseudomonas	1.4%	2.9%	4.8%	3.1%	0.0%	0.0%	0.4%	0.3%	7.4%	0.9%	0.0%	0.2%	2.6%
Serratia	2.4%	0.0%	0.0%	0.1%	0.0%	0.0%	0.0%	0.0%	0.6%	5.6%	3.1%	0.0%	2.2%
Staphylococcus	26.3%	51.9%	23.8%	22.5%	28.4%	37.7%	30.2%	15.4%	30.6%	26.6%	15.7%	26.8%	22.5%
Streptococcus	0.7%	7.0%	0.5%	5.5%	8.2%	1.4%	0.3%	8.3%	10.8%	10.0%	13.5%	0.7%	3.3%
unidentified Enterobacteriaceae	1.1%	5.5%	1.0%	2.2%	0.0%	0.0%	1.2%	2.4%	1.6%	0.8%	7.2%	0.8%	3.5%
unidentified Neisseriaceae	2.4%	0.2%	2.5%	0.3%	0.2%	0.2%	0.3%	1.5%	0.2%	0.2%	0.0%	1.5%	0.4%

Figure 1: Microbiome taxonomic profiles by disease status and centres. *Sinonasal microbial composition of patients (n = 409) when grouped by disease and centres. Accompanying tables demonstrate the corresponding relative abundances of the top ten most abundant organisms found within our cohort. The abundances of genera in A (by disease status) have been adjusted according to the mixed model that accounted for the centre as a random variable. CRSsNP = Chronic rhinosinusitis without nasal polyps; CRSwNP = chronic rhinosinusitis with nasal polyps; Control = Healthy, non-CRS patients AD = Adelaide; AM = Amsterdam; AU = Auckland; BR = Brazil; CH = Chile; IN = India; MA = Massachusetts; MO = Montreal; SC = South Carolina; ST = Stanford; SY = Sydney; TH = Thailand; TX = Texas.*

By contrast, comparisons between centres revealed a higher degree of variability in the microbiome composition (Figure 1B). Samples from Amsterdam were significantly different from the remainder of the cohort, with increased *Staphylococcus* (51.94%) and marked depletion of *Corynebacterium* (15.51%).

215 Amongst the remaining centres, each appeared to have some variability, with individual regions
216 displaying increased or decreased relative abundance in specific taxa. *Streptococcus*, for example, made
217 up 13.48% of the Sydney microbiome, but was almost absent amongst the Adelaide (0.65%), Auckland
218 (0.45%), Massachusetts (0.32%) and Thailand (0.72%) cohorts. Similar variability can be seen for almost
219 all bacterial taxa examined (Figure 1B). While centres' samples were appeared fairly similar in microbial
220 composition, these results would suggest that centre-specific microbiome profiles can be found. This
221 variability may account for some of the inconsistencies observed in the literature, particularly between
222 findings from different institutes.

223 **Microbial diversity is influenced by geographical location**

224 Alpha diversity amongst cohorts was performed utilising Faith's Phylogenetic Diversity (PD) index.[31]
225 Comparison between disease states demonstrated no significant differences between controls, CRSsNP
226 and CRSwNP (Figure 2A). Overall, alpha diversity was significantly different between centres (Kruskall-
227 Wallis; $p < 0.001$; Figure 2B). Of particular interest was the finding of a significantly lower alpha
228 diversity for samples from Amsterdam compared to the other centres (mean PD = 1.27, $p < 0.01$). This
229 may be related to the compositional findings of high staphylococcal abundance in Amsterdam.

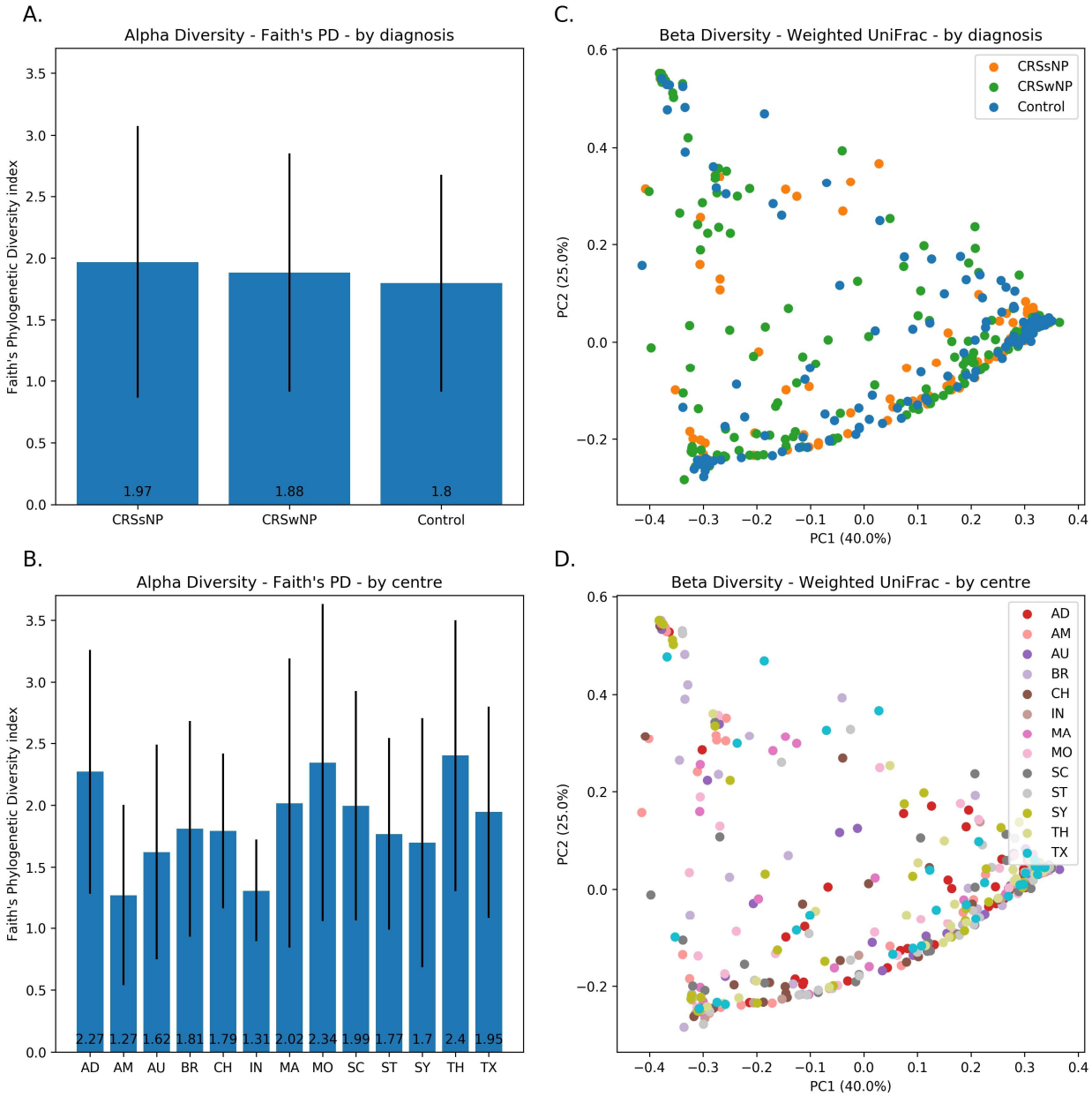


Figure 2: Alpha and beta diversity plots. *Alpha diversity, derived from Faith's Phylogenetic Diveristy Index, demonstrated for this cohort (n = 409) when grouped by disease and by collection centre. Error bars represent 95% confidence intervals. Beta diversity is demonstrated here as a Principal Coordinate Analysis (PCoA) plot of the Weighted-UniFrac distance matrix. Each dot represents a single patient. Similarities between patients are represented by their proximity to each other on the graph. Again, patients are classified by disease and centre. Component 1 (PC1) is represented on the x-axis and component 2 (PC2) on the y-axis. Patients tended towards clustering into one of three groups, as*

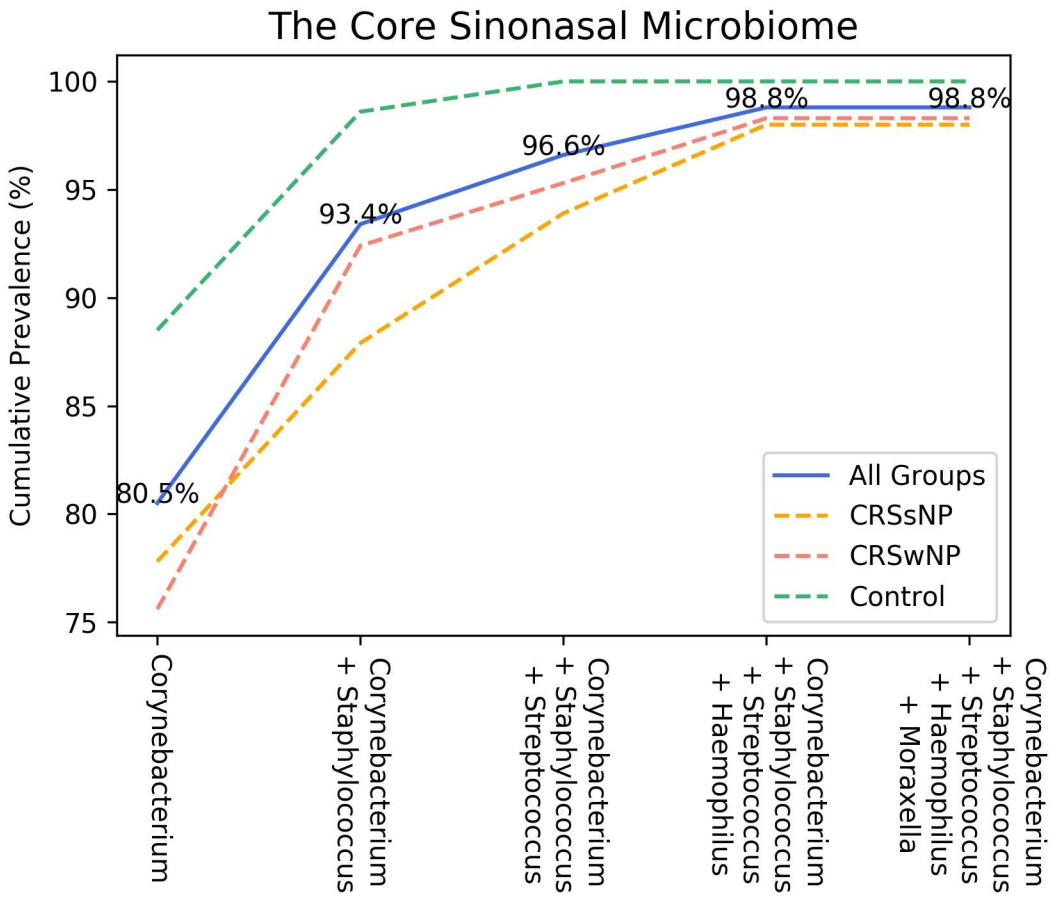
238 *visualised. PD = Phylogenetic diversity CRSsNP = chronic rhinosinusitis without nasal polyps; CRSwNP*
239 *= chronic rhinosinusitis with nasal polyps; Control = healthy, non-CRS patients AD = Adelaide; AM =*
240 *Amsterdam; AU = Auckland; BR = Brazil; CH = Chile; IN = India; MA = Massachusetts; MO =*
241 *Montreal; SC = South Carolina; ST = Stanford; SY = Sydney; TH = Thailand; TX = Texas.*

242 Multivariate analysis on the beta diversity distance matrix was done using PERMANOVA (with 999
243 permutations) to explore the significance of the diagnosis versus the centre variables in a single model.
244 This showed a significant effect of the centre covariate (pseudo-F = 2.51; p = 0.001) on the weighted
245 UniFrac[32] distances, while the diagnosis covariate was not significant (pseudo-F = 1.66; p = 0.1).

246 We also performed Principal Coordinates Analysis (PCoA) on the Weighted-UniFrac distance matrix.
247 This did not show clustering by disease state (Figure 2C and 2D), although the plot may suggest a
248 distribution amenable to unsupervised clustering. Patients tended to cluster into three groups on a
249 continuum on the PCoA. These individual clusters could represent specific microbial community types,
250 similar to what has been previously reported by Cope et al.[23] Investigation into the constitution of these
251 groupings, and their association with underlying clinical or pathological factors in a large multi-
252 institutional cohort remains a topic of future investigation.

253 **The core sinonasal microbiome is composed of five genera**

254 We defined a core sinonasal microbiome by analysing the most abundant organisms along with their
255 cumulative prevalence. This was performed across all samples and also across the three different disease
256 groups. The results of this investigation confirmed a high prevalence of the top five abundant genera
257 (*Corynebacterium*, *Staphylococcus*, *Streptococcus*, *Haemophilus*, and *Moraxella*), which together reached
258 a cumulative prevalence in 98-100% of samples in all patient groups. Presence of at least one of these
259 taxa in nearly all patients suggests that they make up the core sinonasal microbiome, regardless of disease
260 status (Figure 3). Amongst control patients, *Corynebacterium* and *Staphylococcus* were present in 98.6%
261 of patients, again suggesting a likely key commensal function of these two genera in the healthy state.



All Groups	80.5%	93.4%	96.6%	98.8%	98.8%
CRSsNP	77.8%	87.9%	93.9%	98.0%	98.0%
CRSwNP	75.6%	92.4%	95.3%	98.3%	98.3%
Control	88.5%	98.6%	100.0%	100.0%	100.0%

Figure 3: Cumulative microbial prevalence for the top abundant organisms. *Cumulative prevalence for the top five most abundant organisms is presented above. For all groups, we commence with the most abundant organism then add subsequent microbial taxa in a descending fashion based on relative abundance. CRSsNP = chronic rhinosinusitis without nasal polyps; CRSwNP = chronic rhinosinusitis with nasal polyps; Control = healthy, non-CRS patients.*

268 **Clinical covariates do not correlate with changes to taxonomic composition**

269 To determine whether any host factors may contribute to the stability of the sinonasal microbiome,
270 patients were separated by known clinical variables that contribute to CRS (Figure 4). There were no
271 significant differential abundances (of the 10 topmost abundant genera) for all six clinical covariates
272 examined (asthma, aspirin sensitivity, diabetes, gastro-oesophageal reflux disease, smoking status and
273 primary-versus-revision surgery). These tests were also repeated for CRS only subgroup and showed no
274 significantly differentially abundant genera across the different covariate levels.

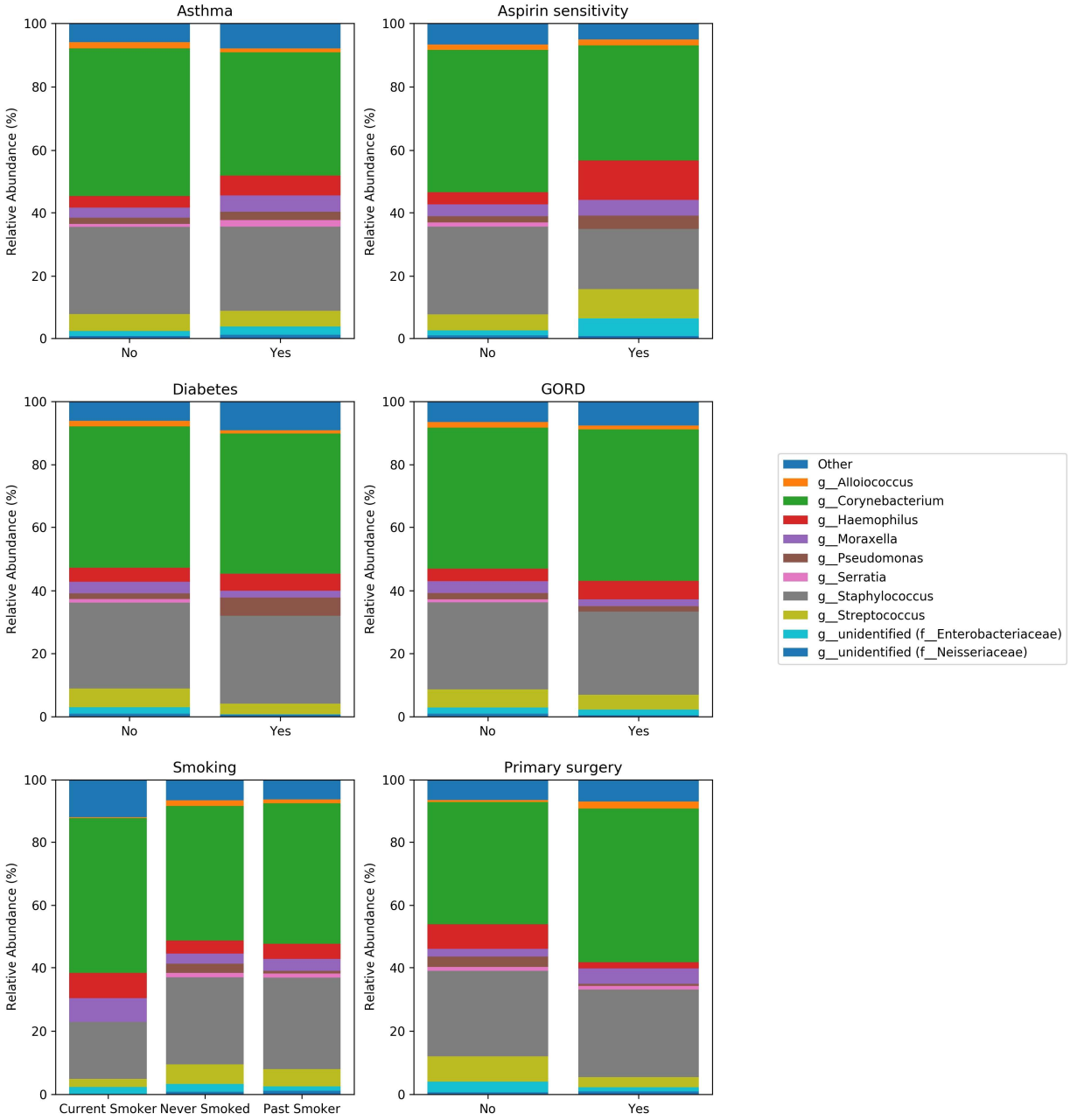


Figure 4: Relationship between clinical co-variates and microbial composition. *Relative abundances of patient cohort (n = 410) when grouped by clinical co-variates (asthma status, aspirin sensitivity, diabetes, gastro-oesophageal reflux disease, smoking and surgery status). GORD = gastro-oesophageal reflux disease.*

281 # DISCUSSION

282 The present study characterises the sinonasal microbiome in a large cohort of subjects from centres
283 around the world using 16S rRNA surveillance. By adopting a unified, consistent methodology from
284 sample acquisition to analysis we have been able to address many of the current existing limitations of
285 currently available data.

286 We have identified *Corynebacterium*, *Staphylococcus*, *Moraxella*, *Streptococcus* and *Haemophilus* as the
287 most abundant genera within the middle meatus of patients with or without CRS. This consistent finding
288 across disease state and geography suggests that these organisms may form the core microbiome within
289 the sinonasal tract. Interestingly, *Streptococcus*, *Moraxella* and *Haemophilus* species are traditionally
290 respiratory tract organisms and constitute the most commonly cultured pathogens in patients with acute
291 bacterial tonsillitis, otitis media and acute sinusitis.[13,33–35] Anatomically, the sinonasal tract connects these
292 three distinct anatomical regions and while these organisms appear to be commensals, so it is possible
293 that the sinuses act as a reservoir for these organisms to subsequently initiate acute infection. While
294 relatively low bacterial burden is present within the sinonasal mucosa, a dysbiosis within the diseased
295 state may lead to over-representation of these organisms that subsequently lead to acute infectious or
296 inflammatory processes.

297 While variations across geography have been demonstrated in the gut microbiome[1] and have been
298 suspected within the sinuses,[36] this is the first study to examine this using standardized methodology. Our
299 results suggest that although the bacterial composition of the core microbiome was preserved across the
300 different sites, significant differences in both alpha and beta diversity occurred according to geography.
301 Furthermore, microbiome composition also varied between centres. Most centres demonstrated a similar
302 consistency in relative abundances of *Corynebacterium* and Staphylococcal species. Some centres,
303 however, appeared to exhibit a varying microbial ecology of the less abundant organisms, which were
304 either over- or under-represented. The most distinct microbial distribution was observed in samples

305 collected from the Amsterdam centre, which appeared to be the most distinct centre with depletion in
306 *Corynebacterium* and over-representation in Staphylococcus. Interestingly, antibiotic use in The
307 Netherlands is amongst the lowest in the developed world.[37] Such practices may influence the microbiota
308 through selective microbial suppression and could partially account for the unique microbiome observed
309 in Amsterdam. Overall, these findings suggest that there are differences in the sinonasal microbiome
310 composition across geographical regions, but these differences would not alter the general pattern of core
311 organisms described above. The driving factors behind geographical variations are yet to be elucidated -
312 possible influences are diet, lifestyle, antibiotic prescribing patterns or environmental exposures.

313 Previous 16S rRNA gene sequence analyses of sinus microbiota have detected and ascribed importance to
314 organisms such as *Cyanobacteria*, *Bacteroidetes*, *Propionibacterium* and *Acinetobacter*.[25,27–29,38] These
315 organisms were either not encountered or detected only in minute abundances in our analysis and given
316 their unexpected nature in the nasal cavity, they could represent a source of contamination (either during
317 sample collection or from DNA extraction reagents) or artefacts from bioinformatics pipelines.
318 *Cyanobacterium*, for example is an environmental organism and *Bacteroidetes* is typically associated
319 with the colon.[39–41] In light of the limitations of current methodologies, reports of airway organisms that
320 are novel, rare, and that do not form a part of the core microbiome, should therefore be interpreted
321 cautiously. It is expected that the resolution of identification and functional characterization of bacterial
322 species and strains in the sinuses can only improve, with advancement in sequencing technologies.

323 The organisms *Corynebacterium* and *Staphylococcus* are of particular interest. It was interesting to note
324 that these organisms remained the two most abundant genera across geographical divides. Furthermore,
325 amongst our control cohort, they were present in almost all individuals, again suggesting a key
326 commensal function in the healthy state. Our findings of great abundance in both healthy patients and
327 CRS patients mirrors previous studies.[22] While *Corynebacterium* has traditionally been thought of as a
328 nasal commensal,[42] some studies suggest that certain species such as *C. tuberculostearicum* may be
329 involved in CRS pathogenesis.[43] We could not resolve the *Corynebacterium genus* in our current analysis

330 to the species level, given the limitations of short-read 16S rRNA gene sequencing, but it is likely that a
331 number of different corynebacterial species reside within the nose – the majority as commensals. Recent
332 evidence shows that certain species of *Corynebacterium* are beneficial in the nasal airways.
333 *Corynebacterium accolens*, for example, is a common nasal colonizer, and can inhibit streptococcal
334 growth by releasing oleaic acids through hydrolysis of host triacylglycerols.[44] In contrast staphylococcal
335 species, and particularly *S. aureus*, by contrast have typically been viewed as potential pathogenic
336 organisms within the nose. While being an asyptomatic coloniser in some individuals,[45] *S. aureus*
337 contributes to severe antibiotic- and surgery-resistant CRS.[46–48] Our confirmation of high prevalence and
338 relative abundance of *Staphylococcus* amongst controls suggests a role in maintaining a healthy sinus.
339 Poor resolution of 16S analysis beyond the genus level results in both commensal and potentially
340 pathogenic *Staphylococcus* species being grouped together. The outcome is that the healthy sinonasal
341 microbiome may be composed of coagulase-negative commensal staphylococcal species, *S. aureus*, or
342 more likely, a combination of both. If *S. aureus* is present in high abundance within the healthy sinus then
343 perhaps this species plays a dual role within the sinuses: being essential for normal function typically, but
344 sometimes becoming virulent at times of disease. The trigger for such a switch in roles is yet to be
345 clarified but could be explained by the presence of *Corynebacterium*. Ramsey et al. described the ability
346 of *C. striatum* to shift the gene profile of *S. aureus* away from virulence and towards commensalism.[49]
347 This hypothesis would be supported by our result demonstrating reduced *Corynebacterium* amongst
348 CRSwNP. The depletion of these organisms may allow *S. aureus* to switch on virulence genes,
349 propagating disease. Interestingly, CRSwNP is a more severe and resistant form of disease and has
350 previously been linked with S. aureus virulence factors and, in particular, superantigens.[50,51] Future
351 studies and techniques which are able to identify microbiota composition to the species or strain level are
352 required to further our understanding of these processes. Ultimately, the results from both current and
353 previous findings highlight the complexity of the sinonasal microbiome and the myriad of functions (both
354 human and bacterial) that act in concert to maintain homeostasis or produce disease. While we have
355 presented data that has solidified our understanding of the upper airway microbial ecology, investigations

356 into the microbiome function or metatranscriptome may provide further novel perspectives on important
357 and critical microbial and host pathways.

358 CONCLUSION

359 Understanding the characteristics of the sinonasal microbiome may provide novel insights to the normally
360 functioning upper airway. This may increase our understanding of the pathobiology of diseases such as
361 CRS. This study is the largest yet to describe the sinus microbiome and the first to examine geographical
362 variation. We demonstrate that the core microbiome is composed of *Corynebacterium*, *Staphylococcus*,
363 *Streptococcus*, *Moraxella* and *Haemophilus*. These organisms are present across disease phenotypes and
364 countries. Utilising a large cohort and standardised methodology has allowed us to better characterise the
365 sinonasal microbiome. By doing so, we have presented a foundation for future prospective studies into
366 pathological states as well as functional analysis.

367

METHODS

Participating centres

A total of fourteen centres participated in the completion of this investigation. Thirteen centres provided patient samples for utilisation within the study. One centre (Northern Arizona University) provided bioinformatics expertise and consultation. The project was approved by the respective institutional human research ethics boards of all sample-collection centres (Table S1). Participating centres are listed below:

1. University of Adelaide, Adelaide, Australia (Lead Centre)
2. University of Sydney, Sydney, Australia
3. University of Auckland, Auckland, New Zealand
4. Siriraj Hospital, Mahidol University, Bangkok, Thailand
5. LV Prasad Institute, Hyderabad, India
6. University of Sao Paulo, Sao Paulo, Brazil
7. Catholic University of Chile, Santiago, Chile
8. Academic Medical Centre, Amsterdam, The Netherlands
9. McGill University, Montreal, Canada
10. Stanford University, California, USA
11. Harvard Medical School, Boston, USA
12. Medical University of South Carolina, Charleston, USA
13. University of Texas, Texas, USA
14. Northern Arizona University, Arizona, USA (Bioinformatics expertise)

Study design

This study was a multi-centre, international collaborative investigation with a prospective, cross-sectional design. In total, thirteen centres across 9 countries provided samples for analysis. Written consent to tissue and clinical data collection was procured from all participants prior to surgery. Collection was performed during either endoscopic sinonasal surgery or ancillary otolaryngological procedures, such as tonsillectomy, septoplasty or skull base tumour resections. Individuals were classified as having CRS if they fulfilled the criteria outlined in the International Consensus statement on Allergy and Rhinology:

395 Rhinosinusitis.[11] Further sub-classification according to the absence (CRSsNP) or presence (CRSwNP) of
396 nasal polyps was performed by endoscopic assessment at the time of surgery. Patients who underwent
397 ancillary otolaryngological procedures and had no clinical or radiological evidence of CRS formed the
398 healthy control cohort.

399 **Metadata collection**

400 Clinical data was collected using standardized patient questionnaires undertaken by each participant at the
401 time of study consent. In non-English speaking countries, the questionnaire was translated by a qualified
402 interpreter and checked for accuracy by the lead investigator from that country. Collected data included
403 patient demographics, medical history, ethnicity, social and environmental exposures as well as quality of
404 life scoring via the validated SNOT-22 and Visual Analogue Scores (VAS). The standardized
405 questionnaires used (which we termed the Open Source Sinonasal Survey "OS3") are freely available at
406 https://github.com/adelaide-orl/os3.

407 **Sample collection and transport**

408 Each centre was asked to provide microbiome swabs from non-CRS controls and CRS patients for
409 analysis. Patients were all anaesthetised at the time of sample acquisition. All samples were collected in a
410 standardized manner prior to commencement of any surgical intervention, whilst the nasal cavity
411 remained unaltered. Microbiome swabs were collected intra-operatively using guarded and
412 endoscopically-guided Copan Flocked swabs (COPAN ITALIA, Brescia, Italy) to sample the middle
413 meatus.[24,52] Swab heads were subsequently separated into sterile cryotubes (Sarstedt, Numbrecht,
414 Germany), placed on ice immediately, and then transported to -80°C storage. Once all samples had been
415 acquired from a centre, they were then transported to the lead centre (Adelaide) using a secure cold chain
416 (Cryoport, Irvine, CA, USA). This was to ensure standardized down-stream processing. The standard
417 Cryoport containers used for shipment of samples are liquid nitrogen dewars capable of keeping stored
418 samples at a stable temperature for at least 15 days. These containers have continuous temperature-

419 monitoring to ensure preservation of the cold chain throughout the shipment. Any evidence of
420 temperature disturbance, or displacement or damage to transported cryotubes resulted in those samples
421 being excluded from further processing.

422 **DNA Extraction**

423 All DNA extraction was performed at the lead centre using Qiagen DNeasy Blood and Tissue Kit
424 (Qiagen, Hilden, Germany), as per manufacturer's instructions. Total DNA was extracted from all clinical
425 samples as well as a DNA-negative control with extraction reagents only. All extractions were undertaken
426 in strict sterile conditions, utilising new equipment for each sample to exclude cross-contamination. In
427 brief, swab heads were prepared for extraction by being cut to 2-3mm pieces and placed into a
428 microcentrifuge tube. A lysozyme (Sigma, St. Louis, USA) solution at 20mg/ml in Lysis buffer (20 mM
429 Tris-Cl, pH8; 2mM sodium EDTA; 1.2% Triton X-100, filter sterilised; Sigma, St Louis, USA) was
430 added to each sample and left overnight at room temperature. Samples were then homogenised using
431 5mm steel beads and a Tissue Lyser II (Qiagen, Hilden, Germany) at 15Hz for 20 seconds. Steel beads
432 were then removed prior to further homogenisation with 50mg glass beads, again using the Tissue Lyser
433 II at 30Hz for 5 minutes. Proteinase K and Buffer AL (Qiagen, Hilden, Germany) were added to each
434 sample and left to incubate for 30 minutes at 56°C. Tubes were then centrifuged briefly to collect beads
435 and supernatant transferred to a fresh microcentrifuge tube. After addition of 100% ethanol to
436 supernatant, the new mixture was pipetted into DNeasy Mini Spin columns (Qiagen, Hilden, Germany).
437 Subsequent extraction of DNA from supernatant mixture was as per manufacturer instructions, with a
438 total of 100ul of DNA extracted per sample. Concentration was determined using a NanoDrop 1000
439 Spectrophotometer (Thermo Scientific, Massachusetts, USA). Extracted DNA was stored at -80°C until
440 sequencing. Any samples that were suspected to be improperly handled, contaminated or did not pass
441 quality control during processing were excluded. A total of 532 samples (126 CRSsNP; 212 CRSwNP;
442 194 Controls) passed all stages of transport and processing to be sent to the sequencing facility
443 (Australian Genome Research Facility; AGRF, Melbourne, Vic, Australia).

444 **Polymerase Chain Reaction (PCR) Amplification of the 16S rRNA gene and sequencing**

445 PCR and sequencing were performed by AGRF. Libraries were generated by amplifying (341F–806R)
446 primers against the V3–V4 hypervariable region of the 16S rRNA gene. (CCTAYGGGRBGCASCAG
447 forward primer; GGACTACNNGGGTATCTAAT reverse primer).[53] PCR was done using AmpliTaq
448 Gold 360 master mix (Life Technologies, Mulgrave, Australia) following a two-stage PCR protocol (29
449 cycles for the first stage; and 14 cycles for the second, indexing stage). Concentrations of the resulting
450 amplified amplicons were measured using fluorometry (Invitrogen Picogreen; Thermo Fisher Scientific,
451 Waltham, MA, USA). Amplicons were normalised according to the obtained concentrations prior to
452 sequencing. Sequencing was done on the Illumina MiSeq platform (Illumina Inc., San Diego, CA, USA)
453 with the 300-base-pairs paired-end chemistry over 8 runs.

454 **Bioinformatics pipeline**

455 Demultiplexed fastq files were received from the sequencing facility. We used the new QIIME 2 (version
456 2018.11)[30] for our bioinformatics pipeline, utilizing various QIIME 2 plugins at each step. Forward and
457 reverse reads were joined using PEAR[54] through the QIIME 2 plugin q2-pear
458 (https://github.com/bassio/q2-pear). Joined sequences were then quality-filtered using the QIIME 2 plugin
459 q2-quality-filter,[55] with minimum quality 20, according to recommendations.[56] This was followed by
460 abundance-filtering applied on the reads, according to the method by Wang et al.[57], through the python
461 implementation in the QIIME 2 plugin q2-abundance-filtering (https://github.com/bassio/q2-abundance-
462 filtering). Denoising and Amplicon Sequence Variant (ASV) formation were done using deblur[56] through
463 the q2-deblur plugin using the parameters (trim-size = 435; min-size = 1; min-size = 1). Taxonomy
464 assignment was done against the Greengenes 16S reference database (the 99% clustered similarity
465 sequences),[58] version 13.8 (August 2013) using the BLAST-based classifier implemented in QIIME 2
466 (q2-feature-classifier)[59] and which implements a Lowest Common Ancestor (LCA) consensus algorithm.
467 To address limitations of de novo trees generated from short-length ASVs, we utilized the SATé-enabled
468 phylogenetic placement (SEPP) technique[60] for insertion of the ASVs into the high-quality tree generated

469 from the 99% OTUs Greengenes reference database, and the ASVs that did not fit anywhere into the tree
470 were filtered out of the ASV table.

471 A rarefaction depth cut-off was chosen at 400 before downstream diversity analysis and comparisons of
472 relative abundances of taxa. Alpha rarefaction plots of unique number of ASVs in each sample
473 (i.e. richness), as well as Shannon's diversity index confirmed almost all samples reaching a plateau at
474 this depth indicating sufficient sampling. (Supplementary Figure S2) Applying this depth yielded 410 (out
475 of 532) samples for downstream analysis. Taxa were mostly compared at the genus level. Mean relative
476 abundance as well as prevalence of the genera were calculated for each group. Faith's phylogenetic
477 diversity index[31] was used for alpha diversity and weighted Unifrac[32] distance matrices were calculated
478 for beta diversity analyses. Diversity metrics were generated through sci-kit bio version 0.5.3.

479 **Statistical analysis**

480 Statistics were done using packages from the Python Scientific Stack[61] and R (R Foundation for
481 Statistical Computing, Vienna, Austria) through the Jupyter notebook interface[62], utilizing the assistance
482 of packages from the Scientific Python[61] stack (numpy, scipy, pandas, statsmodels), scikit-bio
483 (https://github.com/biocore/scikit-bio) and omicexperiment
484 (https://www.github.com/bassio/omicexperiment).

485 We investigated the relative abundances of genera in different subgroups using linear mixed model
486 analysis (R packages "lme4" and "lmerTest"). Linear mixed effects modelling was performed to control
487 for the "centre" variable, which was included in the model as a random effect. The mixed models were fit
488 using the restricted maximum likelihood (REML) as implemented in the default method in the "lme4"
489 package. Mean fixed effects of variables were extracted from the model objects in R using the R package
490 "emmeans", (the successor to "lsmeans").[63] The p values for the covariates in the mixed models were
491 generated using t-tests using Satterthwaite's method as implemented in the "lmerTest" package.[64]

Comparison of mean relative abundances of the top 10 taxa between centres, and comparison of mean alpha diversity indices between disease groups and centres, were performed using Mann-Whitney-Wilcoxon tests, with multiple comparisons correction using the Benjamini-Hochberg method.[65] For multivariate analysis of beta diversity metrics, we employed permutational multiple analysis of variance (PERMANOVA)[66] implemented in the function “adonis” from the R package “vegan”.[67]

498 # Supplementary Tables

499 *Table S1: Ethics approvals.*

Centre	Approval
University of Adelaide, Adelaide, Australia	The Queen Elizabeth Hospital Human Research Ethics Committee (approval HREC/14/TQEHLMH/222)
University of Sydney, Sydney, Australia	HREC Reference number: LNR/14/SVH/373
University of Auckland, Auckland, New Zealand	HDC Northern Regional Ethics Committee approval NTX/08/12/126/AM13
Siriraj Hospital, Mahidol University, Bangkok , Thailand	Siriraj Institutional Review Board: Certificate of Approval 246/2559 (EC1)
LV Prasad Institute, Hyderabad, India	Local ethics approval number LEC-01-15-001
University of Sao Paulo, Sao Paulo, Brazil	Local ethics approval number CAAE: 46421415.8.0000.0068
Catholic University of Chile, Santiago, Chile	Scientific Ethics Committee, Faculty of Medicine, Pontifical Catholic University of Chile (reference number 14-435)
Academic Medical Center, Amsterdam, The Netherlands	METC number: W14_282 # 14.17.0338
McGill University, Montreal, Canada	McGill University Health Centre, Biomedical D Research Ethics Board, Study Number 14-433 BMD
Stanford University, California, USA	Ethics Protocol ID : 33326
Harvard Medical School, Boston, USA	Massachusetts Eye and Ear IRB Approval #651070-9
Medical University of South Carolina, Charleston, USA	Ethics/Institutional Review Board: Pro00038979
University of Texas, Texas, USA	Ethics approval HSC-MS-16-0853

500 *Table S2A: Abundance and prevalence of genera in CRSsNP patients.*

Genera	Mean Relative Abundance (%)	Prevalence (%)
Corynebacterium	44.43	77.78
Staphylococcus	27.42	69.7
Haemophilus	6.22	19.19
Streptococcus	5.98	27.27
Moraxella	3.18	8.08
Pseudomonas	2.88	15.15
Serratia	1.16	2.02
unidentified (Moraxellaceae)	0.88	4.04
unidentified (Enterobacteriaceae)	0.86	11.11
unidentified (Neisseriaceae)	0.81	20.2

501

502 *Table S2B: Abundance and prevalence of genera in CRSwNP patients.*

Genera	Mean Relative Abundance (%)	Prevalence (%)
Corynebacterium	40.01	75.58
Staphylococcus	25.74	73.26
Streptococcus	7.35	31.98
Haemophilus	6.12	15.12
Moraxella	3.9	9.88
unidentified (Enterobacteriaceae)	3.2	16.86
Pseudomonas	2.59	6.4
Alloiococcus	2.2	22.67
Klebsiella	0.88	2.33
Acinetobacter	0.74	5.23

503

Supplementary Figures

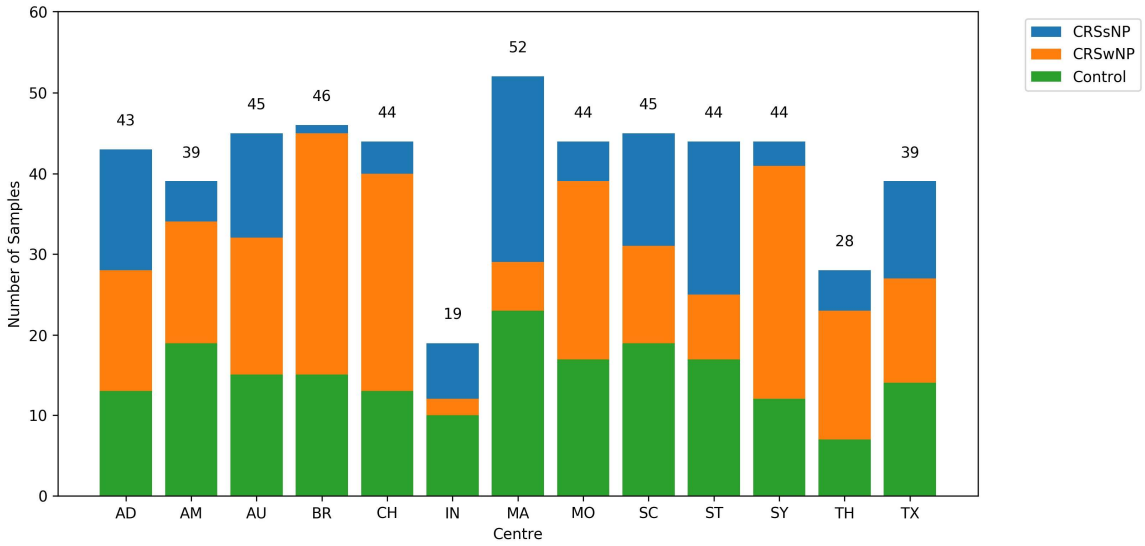


Figure S1: Collected sample disease characteristics by centre. *Figure demonstrating the disease characteristics of patients from each centre. Each bar represents cumulative numbers. Control patients are indicated by, CRSsNP by blue and CRSwNP by orange. Final contributing numbers are indicated at the peak of each bar. AD = Adelaide; AM = Amsterdam; AU = Auckland; BR = Brazil; CH = Chile; IN = India; MA = Massachusetts; MO = Montreal; SC = South Carolina; ST = Stanford; SY = Sydney; TH = Thailand; TX = Texas.*

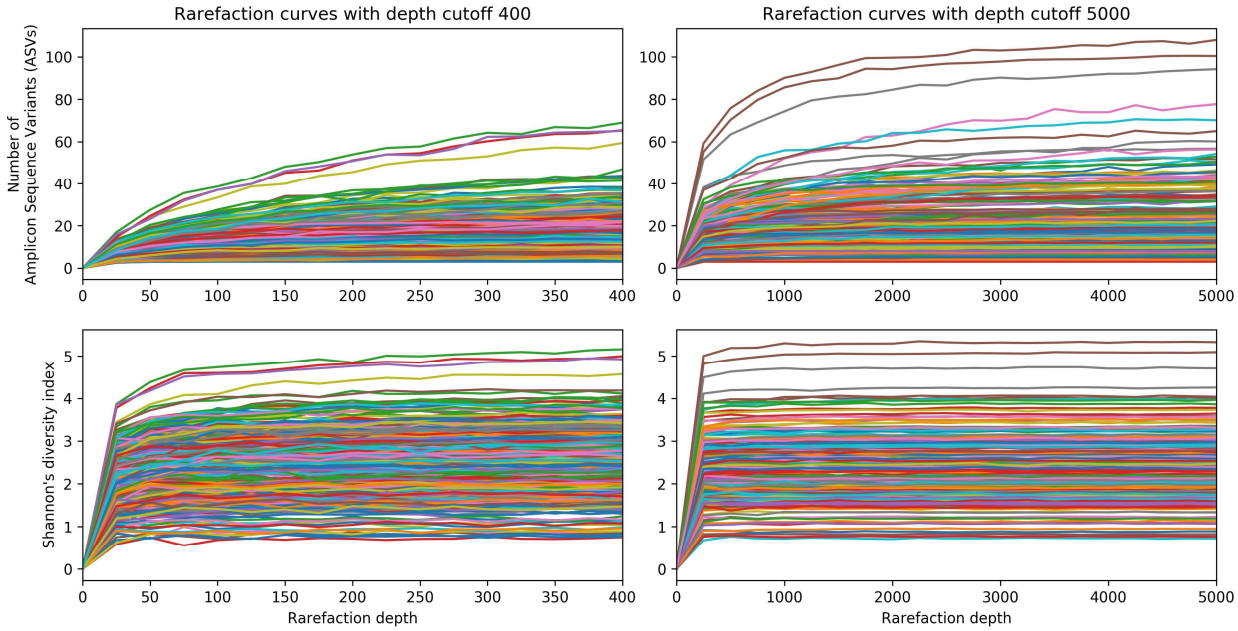


Figure S2: Rarefaction analysis: Rarefaction curves plot. *A rarefaction depth of 400 was chosen before downstream diversity analysis and comparisons of relative abundances of taxa. Rarefaction plots (at depth cutoffs 400 and 5000) of richness (total number of ASVs per sample) and Shannon's alpha diversity index confirmed almost all samples reaching a plateau at this depth indicating sufficient sampling. Each line in the plot represents a single sample. ASVs = Amplicon Sequence Variants.*

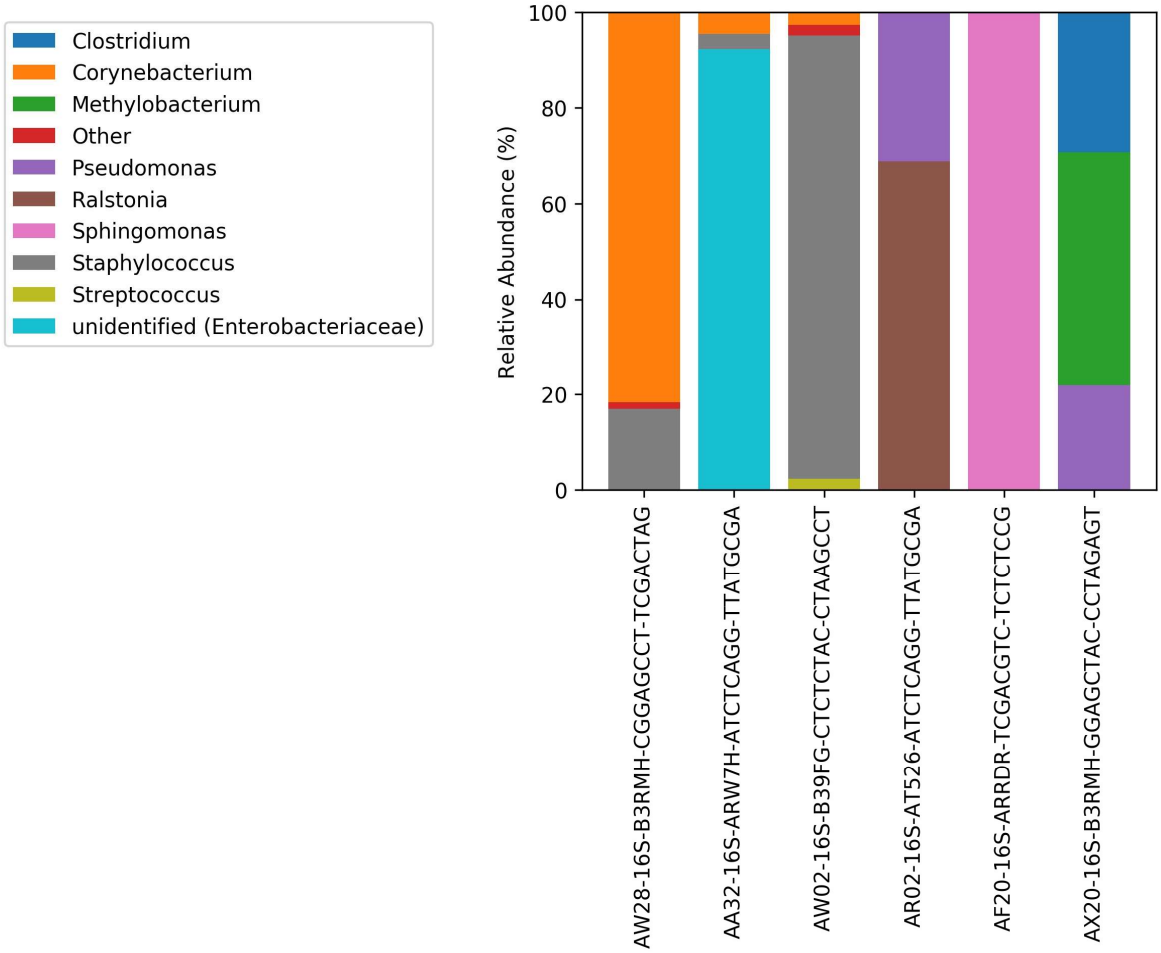


Figure S3: Taxa bar plots of the negative template control samples (i.e. DNA-negative samples with extraction reagents only). *The rightmost three (of the six) samples were below the rarefaction threshold depth of 400 reads.*

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