The Chronic Rhinosinusitis Microbiome

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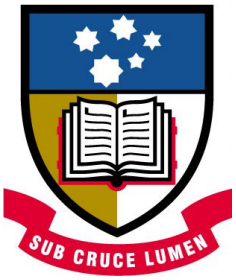

This thesis is dedicated to my beautiful wife Esther.

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Thesis declaration

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Dr Edward J. Cleland

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Publications arising from this thesis

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2. Cleland EJ, Bassiouni A, Boase S, Dowd S, Vreugde S, Wormald PJ. The fungal microbiome in chronic rhinosinusitis: richness, diversity, postoperative changes and patient outcomes. Int Forum Allergy Rhinol 2014;4:259-65.

3. Cleland EJ, Bassiouni A, Vreugde S, Wormald PJ. The bacterial microbiome in chronic rhinosinusitis: richness, diversity, post-operative changes and patient outcomes. Submitted for publication.

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Analysis of fungal diversity through molecular characterization of the chronic rhinosinusitis microbiome.

The Australian Society of Otolaryngology Head & Neck Surgery (SA) – Registrar research presentation night, Adelaide, Australia, November 2012.

Analysis of fungal diversity through molecular characterization of the chronic rhinosinusitis microbiome.

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Probiotic manipulation of the chronic rhinosinusitis microbiome.

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Probiotic manipulation of the chronic rhinosinusitis microbiome.

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Probiotic manipulation of the chronic rhinosinusitis microbiome.

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List of abbreviations

16S &18S	A part of ribosomal RNA. S represents Svedberg units
ABRS	Acute bacterial rhinosinusitis
AD	Atopic dermatitis
AFRS	Allergic fungal rhinosinusitis
AFS	Allergic fungal sinusitis
ARS	Acute rhinosinusitis
Atl	Autolysin
CFT	Canine fossa trephination
CRS	Chronic rhinosinusitis
CRSsNP	Chronic rhinosinusitis sans nasal polyps
CRSwNP	Chronic rhinosinusitis with nasal polyps
DNA	Deoxyribonucleic acid
E. Coli	*Escherichia coli*
EMRS	Eosinophilic mucous rhinosinusitis
EPOS	European position statement on sinusitis
Esp	A serine protease
ESS	Endoscopic sinus surgery
FESS	Functional endoscopic sinus surgery

H. Influenzae	*Haemophilus influenzae*
HGT	Horizontal gene transfer
IL	Interleukin
INCS	Intranasal corticosteroid
ITS	Internal transcribed spacer
KOH	A potassium hydroxide preparation
LK	Lund-Kennedy
LM	Lund-Mackay
M. Catarrhalis	*Moraxella Catarrhalis*
MRA	Mean relative abundance
MUC3	A mucin glycoprotein
NO	Nitric oxide
OTU	Operational taxonomic unit
P. aeruginosa	*Pseudomonas aeroginosa*
PAMP	Pathogen associated molecular patterns
PAR	Protease-activated receptors
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PRR	Pattern-recognition receptors
RCT	Randomized controlled trial

rDNA	Ribosomoal deoxyribonucleic acid
rRNA	Ribosomal ribonucleic acid
S. aureus	*Staphylococcus aureus*
SA	*Staphylococcus aureus*
SAE	*S. aureus* enterotoxins
SE	*Staphylococcus epidermidis*
SNOT-20	Sinonasal outcome test 20
SNOT-22	Sinonasal outcome test 22
SspA	Staphylococcus serine protease gene encoding V8 protease
T-RFLP	Terminal restriction fragment length polymorphism
TEFAP	Tag-encoded FLX amplicon pyrosequencing
Th	T helper cell (1,2, 17 etc)
TLR	Toll-like receptors
VAS	Visual analogue symptom score

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Thesis summary

The body of research described within this PhD thesis investigates the presence and role of microbial communities or the microbiome in chronic rhinosinusitis (CRS). After a rigorous review of the literature as it relates to the aetiopathogenesis and microbiology of this disease it is clear that much confusion exists regarding the importance of host and environmental factors which predispose towards the disease state. In particular, significant deficiencies exist in how we conceptualize the role of bacteria and fungi in this ecological niche because our understanding of these microbes has been shaped by detection techniques that lack the sensitivity to accurately detect the microbiome constituents. To address this, we set out to accurately define the microbiome in CRS patients and healthy controls as a basis for further ecological studies. Using this knowledge we then attempted to manipulate the microbiome in a murine model of sinusitis so that the probiotic potential of certain bacteria could be determined.

The first study presented in this thesis (Chapter 2) is concerned with the bacteriology of CRS patients as determined through traditional culture results. Here the results from over 500 CRS patients were utilized and several important findings were identified. The important pathogen *S. aureus* was determined to be the most prevalent species in our cohort, followed by *P. aeruginosa*, *Haemophilus* spp. and *S. pneumonia*. The rates of isolation for *S. aureus* were also found to be significantly higher in patients undergoing revision surgery, which indicated an important relationship between this organism and recalcitrance. This study formed

an important basis for the other microbe detection studies described in the following chapters, which utilized sensitive molecular techniques.

Chapter 3 sees investigation of the bacterial component of CRS microbiome using a 16S rDNA bacterial tag-encoded FLX amplicon pyrosequencing technique. The large number of species detected in both controls and CRS patients highlighted the sensitivity of this technique for detecting bacteria. Unlike the previous study, other metrics of presence were also characterized such as richness, diversity and relative abundance. The temporal changes occurring in the CRS cohort were examined post-operatively and correlations were made with quality of life. *S. aureus* was shown to be prevalent in both CRS patients and controls, however its relative abundance in CRS patients was significantly higher than in the control group. The species *Acinetobacter johnsonii* was also of interest. Here it was found to be significantly more abundant in the control group, which may be of functional importance for the healthy sinonasal microbiome.

The study reported in Chapter 4 describes the equally important, but until this time neglected, fungal component of the microbiome. In this instance 18S rDNA fungal tag-encoded FLX amplicon pyrosequencing was performed and fungi were found to be ubiquitous to all patients. The striking finding of this study was the sheer diversity of fungi present across the cohort. Several genera including *Malassezia* were described for the first time in the sinonasal cavity. The presence of *Malassezia* was of great interest due to its previously described association with atopic disease. In CRS *Malassezia* may also play an important role as a disease modifier.

Finally, after successfully defining the microbiome our next aim was to manipulate it (Chapter 5). Here we investigated the probiotic potential of *S. epidermidis* against *S. aureus* in a murine model of sinusitis. Intranasal inoculations of phosphate buffered saline, *S. epidermidis*, *S, aureus* or a combination of the two were administered to 20 mice. PAS-positive goblet cell counts of the mouse nasal mucosa revealed that mice receiving a combination of *S. epidermidis* and *S. aureus* had significantly lower goblet cell counts than those receiving *S. aureus* alone. This finding was of great importance as it confirmed the probiotic potential of *S. epidermidis* by demonstrating its ability to augment the pathogenicity of *S. aureus* in the nasal cavity. The implications of this finding for topical intranasal probiotics in CRS are far reaching and should not be underestimated. Most importantly it highlights the viability of ecologically based treatment paradigms in the management of CRS.

Chapter 1: Literature review

1.1 Chronic rhinosinusitis – the disease entity

1.1.1 Chronic rhinosinusitis defined

Chronic rhinosinusitis (CRS) is part of a spectrum of inflammatory conditions involving the nasal cavity and paranasal sinuses. Our understanding of these conditions has evolved over time, resulting in a corresponding change in our definition of this disease entity.

Most panels and expert guideline documents now agree on the use of the term rhinosinusitis instead of sinusitis. This change in nomenclature is an acknowledgement that both rhinitis and sinusitis usually coexist in these individuals and are part of the same disease process.[1]

The most contemporary guidelines on rhinosinusitis are those put forth by the European Position Paper on Rhinosinusitis and Nasal Polyps 2012 (EPOS), which defines the entity in adults as:

- Inflammation of the nose and the paranasal sinuses characterized by two or more symptoms, one of which should be either nasal blockage/obstruction/congestion or nasal discharge (anterior/posterior nasal drip)
    - ± Facial pain/pressure
    - ± Reduction or loss of smell

and/either

- Endoscopic signs of

- Nasal polyps, and/or
- Mucopurulent discharge primarily from middle meatus and/or
- Oedema/mucosal obstruction primarily in middle meatus

and/or

- CT changes
  - Mucosal changes within the ostiomeatal complex and/or sinuses.[1]

Earlier guidelines for diagnosis relied solely on the presence of major and minor symptoms to make a clinical diagnosis.[2] This method was inherently limited, as a diagnosis of rhinosinusitis was not subjected to the additional rigour of endoscopic examination and radiological investigations, and resulted in a revised set of guidelines as put forth by Benninger et al. to address these concerns.[3]

Once a diagnosis of rhinosinusitis is made patients may be categorized on a temporal basis into acute (<12 weeks duration) and chronic (>12 weeks duration) rhinosinusitis categories.[1] Earlier classifications of rhinosinusitis divided these groups further to encompass subacute, recurrent acute and acute exacerbations in the context of chronic disease (**Table 1-1**),[2] a distinction which is still made by many clinicians.

Table 1-1 Categories of rhinosinusitis

Category	Duration of symptoms
Acute	0 – 4 weeks
Subacute	4 – 12 weeks
Chronic	≥ 12 weeks
Recurrent, acute	≥ 4 episodes/year + each episode lasting ≥7–10 days
Acute exacerbations of chronic	Sudden worsening of chronic rhinosinusitis, return to baseline after treatment

Acute rhinosinusitis (ARS) can be further divided into viral, post-viral and acute bacterial rhinosinusitis (ABRS) sub-categories. The aetiologic agent in viral ARS is most commonly found to be rhinovirus (50%), but may also result form influenza viruses, parainfluenza viruses, adenovirus, respiratory syncytial virus, and enterovirus.[4] ABRS is frequently preceded by the viral and post-viral variants of ARS, a fact which can make the distinction of these subtypes difficult in the clinical setting. The most commonly detected microorganisms in a meta-analysis of ABRS patients included *Streptococcus pneumonia* (32.7%), *H. influenza* (31.6%), *S. aureus* (10.1%), and *M. catarrhalis* (8.8%).[5]

For patients with ARS where symptoms last longer than the arbitrary 12 weeks, a diagnosis of CRS is made. This transition from ARS to CRS is an

important one and has been noted to occur with corresponding changes in the presence of microbes. This relationship will be discussed elsewhere (see 1.2.3).

These definitions and categories of rhinosinusitis are of considerable utility for researchers and clinicians as they provide a well-defined and reproducible means of categorizing patients with rhinosinusitis. Not only does this allow for more accurate study design, but also the treatment of patients with CRS can be tailored with greater accuracy to the specifics of the individual.

1.1.2 Epidemiology of chronic rhinosinusitis

Estimates of the prevalence of rhinosinusitis are limited by the quality of data in the literature, which are largely derived from broad-based population studies. These studies usually rely on self-reported symptoms by a patient rather than formal diagnostic criteria as used by the otolaryngologist.

One publication using self-reported 'sinus trouble' for greater than 3 months in the year, estimated the prevalence of CRS at 15.5% of the total population in the United States.[6] A Canadian study asking patients if the they had been diagnosed with CRS by a health care professional found its presence in 5.7% of females and 3.4% of males.[7] Lower rates of CRS have been described in a Korean study which found symptoms of rhinosinusitis in only 1% of the population,[8] a difference which may be reflective of geographic variability. Another study using disease classification codes generated from medical records found CRS in only 2% of patients.[9] This result highlights the potential for exaggerated rates of prevalence in studies using self-reported data.

The socioeconomic impact of CRS is also significant. In the United States, the number of office based doctor visits resulting in a diagnosis of CRS was 11.6 million in the year 2000.[10] It has been shown that patients with CRS visit primary care clinicians twice as often as those without the disorder and have five times as many prescriptions filled.[11] Approximately 500,000 surgical procedures are performed on the paranasal sinuses annually,[12] and when these figures are extrapolated to the general population the direct cost of CRS in the United States has been estimated at $4.3 billion.[13]

The health burden of CRS on patients relative to the general population is also significantly worse for several domains such as bodily pain, general health, vitality, and social functioning. Compared with other chronic diseases these patients also score significantly worse for bodily pain and social functioning than patients with congestive heart failure, angina, chronic obstructive pulmonary disease, and back pain.[14] These findings highlight the impact of CRS on the population, the extent of which is potentially underestimated, especially compared to other disease states.

1.1.3 Chronic rhinosinusitis subclassification

The presence of nasal polyposis is the most common clinical factor used to differentiate between patients with CRS. To make a diagnosis of CRS with nasal polyps (CRSwNP), endoscopic examination of the nasal cavity must reveal bilateral polyps in the middle meatus. If no polyps are visualized in the middle meatus patients are defined as CRS sans nasal polyps (CRSsNP). The diagnosis of nasal polyps in patients who have previously undergone sinus surgery can be problematic due to altered anatomy or the previous removal of polyps. In this

instance, the presence of bilateral pedunculated lesions > 6 months after surgery on endoscopic examination is considered sufficient grounds for a diagnosis of CRSwNP. Other changes in mucosa that are often observed such as a cobblestoned appearance, whilst representing inflammatory change, do not meet the definition of a nasal polyp (see Figure 1-1).[1] Although not mandatory, histological analysis of a mature polyp may also assist diagnosis. Here the polyp will usually appear as a large pseudocyst area containing albumin surrounded by subepithelial eosinophilia.[15]

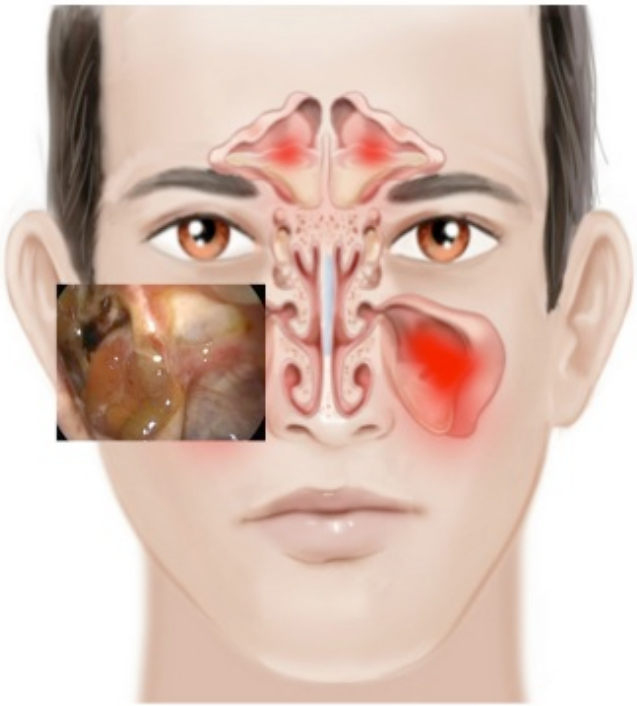

Figure 1-1 Clinical photograph of R maxillary sinus showing changes in a previously operated sinus which do not meet the definition of a nasal polyp. Image superimposed on schematic of human paranasal sinuses (open source image).

Further subclassification of CRS patients can be made on the presence of eosinophilic mucous (EM) and/or fungal hyphae.[16] Using the Bent-Kuhn criteria a

patient is diagnosed with allergic fungal rhinosinusitis (AFRS) in the presence of: nasal polyposis, fungi on staining, eosinophilic mucous without fungal invasion into sinus tissue, type I hypersensitivity to fungi and characteristic radiological findings with soft tissue differential densities on CT scanning (Table 1-2).[17] These criteria must be used with caution however, as they are not specific for AFRS patients and must also be considered in the context of more sensitive techniques for the detection of fungi, which have highlighted its ubiquitous presence in the sinonasal cavity.[18,19]

Table 1-2 Bent-Kuhn criteria for diagnosis of AFRS

1	Nasal polyposis
2	Fungi on staining
3	Eosinophilic mucous without fungal invasion into sinus tissue
4	Type 1 hypersensitivity to fungi
5	Radiological findings consistent with fungal disease

Patients with eosinophilic mucous, but an absence of fungi and its associated hypersensitivity, are provided the alternative diagnosis of eosinophilic mucous rhinosinusitis (EMRS).[20] Once again the ubiquitous presence of fungi makes this classification unreliable, and is more a reflection of the sensitivity of the fungal detection technique used. Others have attempted to differentiate the CRSwNP group further from polyp histomorphological characteristics. On this basis they can be divided into eosinophilic, neutrophilic, and noneosinophilic

nonneutrophilic types. This distinction has come about due to regional differences observed in polyp histology between western and Asian countries.[21]

The variability of these subclassifications of CRS reflect the difficulty researchers have in defining this heterogeneous group of patients with a multifactorial disease aetiology. Accurate categorization of these groups is necessary for appropriate study design and also to aid the clinician in tailoring suitable treatment regimes. To make the distinction between patients more accurately it is likely that a combination of clinical (phenotype), host (genotype) and environmental (microbiome) factors will be of great import in the future.

1.1.4 Medical management of chronic rhinosinusitis

Prior to surgical intervention all patients presenting to the Otolaryngologist are first commenced on a trial of 'maximal medical therapy'. What this therapy is comprised of is variable between institutions and lacking consensus, but would usually be comprised of saline rinses, topical and oral corticosteroids and a short course of oral antibiotics.[22] Medical treatments can be of utility in both the pre and post-operative setting (Figure 1-2). The most common medical treatments will be discussed in the following paragraphs.

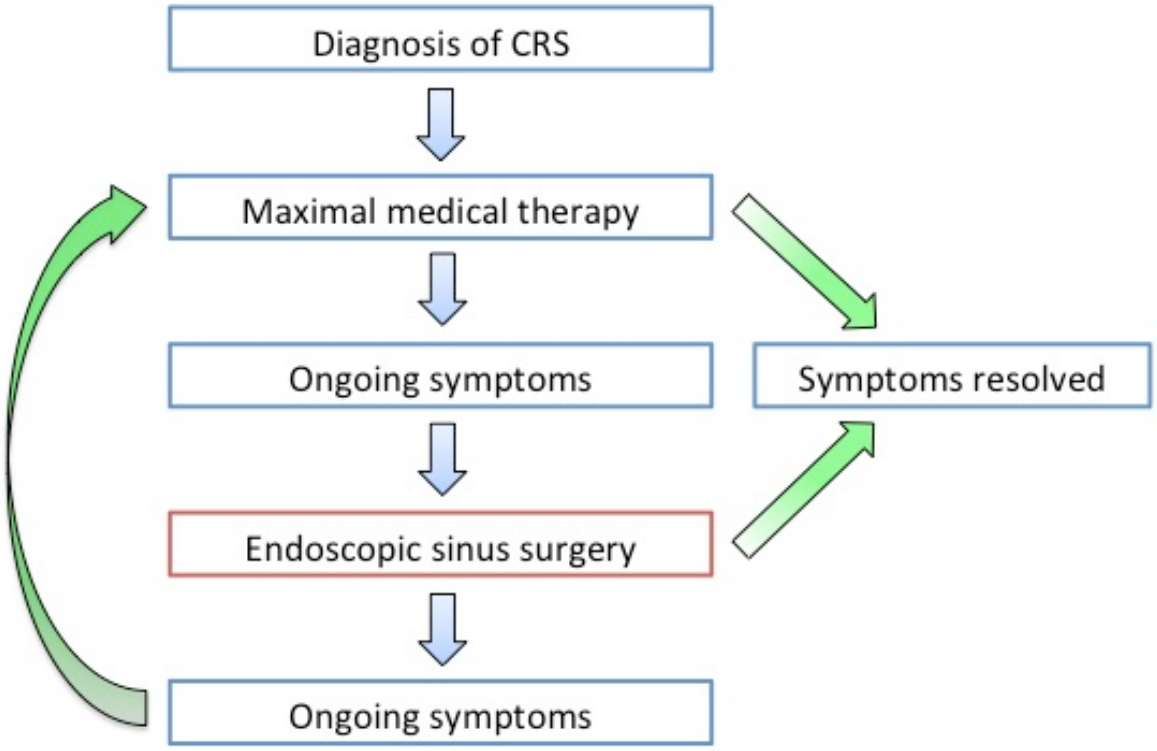

Figure 1-2 CRS treatment algorithm. Adapted from Palmer et al.[23] Medical therapy plays an important role pre and post-operatively and may be necessary in some patients long-term.

Corticosteroids are an important component of medical management for CRS patients and can be delivered either topically or orally. These medications effect eosinophil function by directly reducing eosinophil viability and activation[24-26] or by indirectly reducing the secretion of chemotactic cytokines by nasal mucosa and nasal polyp epithelial cells.[27,28] A recent meta-analysis of topical intranasal corticosteroids (INCS) in CRSsNP patients found significantly improved symptom scores in the treatment group compared to those receiving placebo. Sinus delivery methods were also more effective than nasal delivery methods, but there was no difference in endoscopic appearance nor was there any difference seen for the type of steroid used.[1] The CRSwNP group has also been shown to benefit from

INCS. These patients demonstrate significant improvements in symptoms, polyp size, polyp recurrence and nasal airflow.[1]

Oral systemic steroids may be of benefit in CRSsNP patients although high-level evidence is currently lacking to support their use. A recent systematic review of 30 studies on systemic steroid use in CRSsNP patients identified that the efficacy of single-modality oral steroid use in CRSsNP remained untested. Furthermore, no randomized controlled trials (RCT) had been performed that examined the efficacy of oral steroids as part of multimodal therapy in CRSsNP.[29] Three trials, involving 166 patients, met the inclusion criteria of a recent Cochrane review exploring the benefits of oral steroids in CRSwNP patients. The results indicated a short-term benefit for a brief (2 – 4 week) course of oral steroids of variable doses and duration when compared to placebo. Improvements were seen for an objective reduction in polyp size and a subjective improvement of nasal symptoms and quality of life. These trials were however, of moderate to low quality and it was not possible to quantify the overall size of this effect.[30]

The side effect profile of corticosteroids needs to be balanced against any benefits. Whilst long-term use of INCS are generally considered to be safe due their low bioavailability (<1%),[31] a number of patients still experience side effects related to their use which may include epistaxis, itching, sneezing and dry nose.[1] Oral steroids have the potential for more significant side effects particularly when given at higher doses for longer durations. These side effects include changes in bone mineral density, fat metabolism, proximal muscle strength, appetite and glucose tolerance. Patients are also at risk of early cataract formation and suppression of the pituitary-hypothalamic axis.[1] A frank discussion between the

clinician and patient regarding the risks versus benefits of steroid use should always be undertaken prior to their prescription.

Antibiotics are used in CRS for their antibacterial and immunomodulatory properties. Short duration antibiotics (< 4 weeks) are generally prescribed in the setting of acute exacerbations of CRS and should be prescribed on the basis of culture directed sensitivities.[32] Antibiotics are also prescribed by some centers as part of 'maximal medical therapy' even in the absence of purulent discharge.[22] Macrolides are the most frequently prescribed long-term antibiotics (> 4 weeks) and are chosen due to their ability to reduce inflammation in the airway. Macrolides are believed to achieve this immunomodulatory effect by blocking the production of proinflammatory cytokines, such as interleukin 8 and tumor necrosis factor-α, combined with effects on neutrophil migration and adhesion. Additional mechanisms may include other immunomodulatory effects, changes to mucus secretion and synthesis, and non-bacteriostatic/cidal microbial activity.[33] Wallwork et al. performed a RCT investigating the role of macrolides in a group of CRSsNP patients. These patients received roxithromycin for a period of 3 months and demonstrated significant improvements in quality of life, nasal endoscopy, saccharine transit time, and IL-8 levels in lavage fluid. Improved outcome measures were particularly noted in patients with low serum IgE levels.[34] Another RCT using the alternate macrolide azithromycin for 3 months failed to demonstrate any significant improvement over placebo.[35] The benefit of antibiotics has also been investigated in CRSwNP patients. Van Zele et al. performed the first double-blind, placebo-controlled study in this group. The authors found that the use of doxycycline reduced the size of nasal polyps and systemic markers of

inflammation.[36] Doxycycline was used in these polyp patients due to the belief that *S. aureus* enterotoxins may be important disease modifiers in nasal polyposis.[37] Data regarding antibiotic usage whilst accepted in the setting of acute exacerbations of CRS, remains controversial long term. Whilst macrolides in particular may be beneficial in some patients, further research is required to better define these individuals.

Topical antibiotic preparations are potentially beneficial for a small subgroup of patients. One placebo-controlled RCT evaluated the efficacy of mupirocin rinses for the treatment of recalcitrant CRS with S aureus. Whilst this treatment eradicated *S. aureus* from the nasal cavity in the majority of individuals and resulted in improvement in endoscopic scores, there was no significant change in the quality of life of these patients compared to those receiving placebo.[38] Given that the majority of these patients will also go on to recolonize *S. aureus*,[39] one must question the utility of such treatments and consider the broader impact of such measures on other members of the sinonasal microbiome and their important contribution to health in this niche.

Saline irrigations are an important component of the pre and post-operative care of patients with CRS. Irrigation of the sinonasal cavity can be limited pre-operatively by anatomical factors. In particular, the frontal recess and sphenoid sinuses are poorly penetrated in their native state.[40] Surgery to the sinuses has been shown to improve the penetration of nasal irrigants. Here the critical sinus ostial dimension required to allow adequate penetration in 95% of cases, has been identified at 3.95 mm.[41] Whilst not as beneficial as INCS, it is now generally

accepted that saline irrigations are advantageous in the treatment of the symptoms of CRS when used as a sole treatment modality or adjunct.[42]

Combined these treatments allow many patients to avoid surgical intervention for the management of CRS. However, even if surgery is performed, these medications continue to play an important role as improved penetration of irrigants and other topical agents delivered to the sinuses post-operatively may ultimately help to avoid the need for revision surgery.

1.1.5 Surgical management of chronic rhinosinusitis

Functional endoscopic sinus surgery (FESS) is the term used to describe surgery to the paranasal sinuses for the management of rhinosinusitis. FESS relies on a surgical concept aimed at restoring the health of diseased sinuses by restoring drainage pathways impacted upon by sinus pathology through improving ventilation and mucociliary clearance. Originally described by Messerklinger and popularized by such proponents as Stammberger,[43] the technique has evolved over time and includes a spectrum of procedures ranging from middle meatal antrostomies with anterior ethmoidectomies (mini FESS), to complete fronto-spheno-ethmoidectomies (full house FESS). Some patients with extensive severe disease go on to receive a Draf-III[44]/frontal drillout procedure and/or canine fossa trephination (CFT) of the maxillary sinuses. FESS has been shown to offer patients improvements in quality of life and other objective measures.[1] The secondary, and equally important, effect of surgery is improved delivery of topical solutions to the nasal cavity.[40] As already mentioned, the benefit of greater penetration of topical medications cannot be underestimated and may also be of

particular significance for the efficacy of novel topical treatments such as probiotics described later in this thesis (see 1.1).

1.2 Aetiopathogenesis of chronic rhinosinusitis

1.2.1 Aetiology

The definitions as set out for the diagnosis of CRS (see 1.1.1) and its subclassification (see 1.1.3) make it clear that this disease entity most likely encompasses a number of varying mechanisms, all of which converge on sinonasal inflammation and a clinical picture of CRS. The historical perspective of CRS considered it as an entity, largely driven by microbes, and this is perhaps best seen through early writings which described the disease as an infectious process starting in the nose and spreading through the ethmoidal prechambers to the frontal and maxillary sinuses.[43] Infection in a traditional sense however, must also satisfy the postulates as put forth by Robert Koch, which links individual microbes to a specific disease.[45] Chronic diseases such as CRS generally lie outside these postulates due to a variety of host and environmental factors, which contribute to the disease process. In particular, a polymicrobial aetiology has been implicated in the pathogenesis of several important chronic diseases including CRS.[46] Whilst a number of dominant pathogens are highlighted in the CRS literature due to their association with disease severity, a paradigm shift is now occurring away from a monomicrobial perspective to one that encompasses the functional importance of a community of microorganisms (microbiome). This change has largely been brought about by the emergence of highly sensitive culture independent techniques, (see 1.3.3) and the application of microbiome theory (see 1.1), which parallels many of the principles of modern ecology.[47] Here the host and environmental factors implicated in the aetiology of CRS are discussed (Figure 1-3).

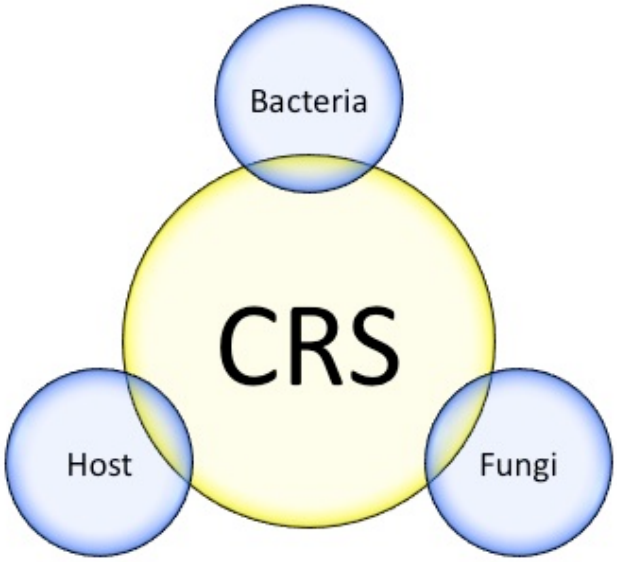

Figure 1-3 Factors contributing to CRS aetiopathogenesis. A complex interplay between the host and environmental factors are believed to be important in aetiopathogenesis of CRS.

1.2.2 Host factors

The historical perspective of microbes being the predominant drivers of CRS lost favor in the literature due to the presence of a number of important host factors. These will be discussed in the following paragraphs.

The anatomy of the paranasal sinuses is frequently variable between individuals. Anatomical variants such as deviation of the nasal septum, concha bullosa and Haller cells may all contribute to obstruction of the normal sinus drainage pathways. These variations have been studied in CRS and control populations and generally occur with similar frequencies between the two groups.[48,49] The presence of these factors alone is therefore unlikely to be significant in the pathogenesis of CRS. Several anatomical factors have been

implicated in disease recalcitrance after surgery. These include middle turbinate lateralization, middle meatal antrostomy stenosis and incomplete ethmoidectomy.[50,51] Patients with a stenosed frontal sinus ostium and residual frontal sinus disease are also more likely to be symptomatic or have endoscopic evidence of polyp recurrence or endoscopic evidence of persistent infection.[52]

Altered cilia function is believed to be involved in the pathogenesis of CRS. Normally, ciliary activity protects the respiratory tract against inhaled particles, including microbes, by transporting them trapped in mucous towards the nasopharynx.[53] An increased prevalence of CRS is seen in patients with genetic ciliopathic conditions such as primary ciliary dyskinesia (Kartagener's syndrome),[1] which highlights the role of ciliary function in maintaining sinus homeostasis. Other non-genetic factors relating to ciliary function may also be of importance in CRS. Decreased concentrations of nitric oxide (NO), an important regulator of mucociliary activity have also been found in the airways of patients with CRS leading researchers to hypothesize that a lack of NO may contribute to the pathogenesis of this disease.[54] Recently the bitter taste receptor T2R38 was found to stimulate ciliated epithelial cells to produce NO, resulting in bactericidal activity and an increase in mucociliary clearance.[55]

Perturbations in the innate immune system may be significant in CRS pathogenesis. The innate immune system deploys a limited number of receptors with specificity for conserved microbial structures. Recognition of these structures by the innate immune system induces costimulators, cytokines, and chemokines, which recruit and activate antigen-specific lymphocytes and initiate adaptive immune responses.[56] To achieve this process effectively the immune system must

be able to recognize microbial patterns and differentiate these from molecular structures present on host cells. Specific pathogen classes express class specific molecules; the pathogen associated molecular patterns (PAMP) and activation of PAMP receptors or pattern-recognition receptors (PRR) (e.g. Toll-like receptors (TLR)) induces multiple signal cascades, involving complement activation, haemostasis, phagocytosis, inflammation, and apoptosis, in response to pathogens.[1] To date the majority of TLR research has focused on inflammatory lower-airway disease, however the presence of TLRs 1 through 10 have been identified in human sinonasal mucosa of CRS and control patients via real-time PCR, a finding which has led researchers to suggest that there may be differences in the expression of TLRs between these two groups.[57] Increases in TLR2 gene expression have been observed in CRS when mRNA levels are normalized to the ribosomal protein 18S.[58] Other groups have also reported increased or decreased levels of TLR2 mRNA in sinusitis.[59] Lane et al. also showed that CRSwNP is associated with decreased levels of sinonasal epithelial cell TLR9 protein, a PRR for unmethylated bacterial nucleotides and hypothesized that since TLR9 stimulation induces Th1-type cytokines, decreased TLR9 levels in CRSwNP patients may mediate inflammation through suppression of Th1-type cytokines.[58]

Compared to the innate immune response the adaptive immune system is highly specialized. Adaptive immunity is mediated by clonally distributed T and B lymphocytes and is characterized by specificity and memory.[60] CRS appears to be mediated by CD4+ helper T cells, which can be functionally divided into a T-helper 1 (Th1) or T-helper 2 (Th2) phenotype based on differing patterns of cytokine secretion. Th1 cells secrete interferon-γ and tumor necrosis factor α, which

activate macrophages and cytotoxic T lymphocytes whilst assisting IgG2a production. Th2 cells, on the other hand, secrete a number of interleukins (IL) including IL-4, IL-5, IL-9, and IL-13.[61] The proinflammatory effects of these cytokines are controlled by regulatory T cells,[62] which prevent an excessive response. In CRS, differences in cytokine profiles have allowed for patients to be divided on the basis of their T-helper subtype. CRSsNP is generally associated with Th1 subtype whereas CRSwNP with the Th2 subtype. This association has been called into question by differences in patient ethnicity and their associated cytokine profiles. Here Caucasian patients with polyps are found to have an eosinophil predominance reflecting a Th2 biased response whereas Asian patients with nasal polyps Asian counterparts demonstrated a Th1 / Th17 polarization and neutrophilia.[63,64]

It is clear that complex innate and adaptive immune pathways are present at the mucosal surface both constitutively and in response to specific challenges. Whilst functional deficiencies of these critical processes may lead to infection, increased activity or dysregulated responses of the innate or adaptive immune system could lead to the persistent inflammation and its consequent deleterious effects.[60] The role of these factors in the pathogenesis of CRS requires ongoing study, but does highlight an important means by which microbes behave as disease modifiers in the susceptible host.

In 1968 Samter and Beers described an important association between aspirin intolerance and the presence of CRSwNP and asthma. Known as Samter's triad these patients displayed a more severe form of CRS, which was reflected by worse findings radiologically and surgically recalcitrant disease.[65] Administration of

aspirin (acetylsalicylic acid) results in cyclo-oxygenase-1 inhibition that shunts arachadonic acid metabolism down the 5-lipoxygenase pathway. This results in increased production of leukotrienes, a family of eicosanoid inflammatory mediators. In aspirin sensitive individuals, the arachadonic acid metabolism is skewed towards leukotriene production at baseline and administration of aspirin results in excessive leukotriene production well beyond normal limits.[66] In Samter's triad the elevated baseline leukotriene production is believed to be associated with the development of nasal polyps however, aspirin desensitization in these individuals has subjective benefits only with some nasal symptoms and no demonstrable benefit on polyp mass, suggesting alternative mechanisms at play.[67]

1.2.3 Environmental factors – bacteria

A large portion of the literature relating to CRS is concerned with the microbiology of the sinonasal cavity. Bacteria have been investigated in their planktonic, biofilm and intracellular forms and certain species have been associated with disease severity and quality of life (QOL). The use of antimicrobials as part of maximal medical therapy,[22] would imply a proven causative link between bacteria and CRS. This relationship however, remains unproven and microbes are better regarded as disease modifiers in the predisposed host.

To make comparisons between health and disease, knowledge of the healthy sinonasal flora is necessary. Historically, the healthy sinus has been considered sterile.[68-70] Others have found bacteria in only a minority of healthy controls.[71] These results contrast with that of sensitive molecular detection techniques, which typically find bacteria in all healthy patients and make claims of a sterile sinus untrue.[72,73] Using the IBIS biosequencer, Boase et al. found several

prevalent species in a control population including: *Propionibacterium acnes* (85%), *Staphylococcus epidermidis* (67%), Staphylococcus aureus (33%), *Nocardia asteroides* (17%) and *Streptococcus agalactiae* (17%).[72] Using a different culture independent technique (pyrosequencing), Stephenson et al. found *S. aureus* in 100% of controls. *Corynebacterium* (78%) and *Propionibacterium* (67%) were also commonly detected genera in this study.[73] Others have suggested that members of the order Lactobacillales are important in the healthy sinus and play an important protective role against other pathogens.[74] All these studies highlight the lack of consensus in the literature regarding the bacterial constituents of a healthy sinus, particularly when the presence of *S. aureus*, a species traditionally perceived to be pathogenic is highly prevalent in controls. Further studies using comparable molecular detection techniques and other metrics of presence are required to address this deficiency.

The majority of studies concerned with the bacteriology of CRS have utilized culture-based methods for bacterial detection. This technique along with other microbial detection methods are discussed in more detail elsewhere (see 1.1). The importance of anaerobes in CRS was highlighted in one study that reported on the microbiology in patients transitioning from ARS to CRS. Here patients initially cultured aerobic organisms consistent with ARS (e.g. *S. pneumoniae*, *H. influenzae*, and *M. catarrhalis*). However, those that had disease persistence consistent with CRS cultured anaerobes.[75] The prevalence of anaerobic bacteria in CRS patients has most likely been underestimated by many studies due to a failure to utilize the specific handling and culture techniques required for their detection.[76] Those studies that have used methods which allow

for anaerobe detection, have reported their presence in large numbers.[77-80] Others have shown similar numbers of anaerobes and aerobes or even a predominance of aerobes.[81,82] Studies using molecular techniques have also highlighted a predominance of anaerobes in CRS patients,[73,83] and whilst suitable comparisons were not made with control patients in these studies, others have shown using alternate molecular techniques that anaerobic species are also present in 83% of controls.[72] These findings cast doubt on the pathogenic importance of anaerobes in CRS patients. It may be that the presence of anaerobes in the sinonasal cavity is simply a reflection of environmental pressures, which would be consistent with all other mucosal surfaces where it is generally accepted that anaerobes outnumber aerobic bacteria.[84] Specific anaerobes may however be functionally important due to their ability to augment the pathogenicity of other members of the niche. Sibley et al. used a drosophila model of polymicrobial infections to eloquently demonstrate that the pathogenicity of the principal pathogen in cystic fibrosis, *Pseudomonas aeruginosa*, could be enhanced by the presence of non-pathogenic oral anaerobes.[85] The co-occurrence of certain species may have important implications for bacteria in the sinonasal cavity and may explain why some species behave as pathogens in CRS patients and innocuous commensals in controls.

The biofilm phenotype has drawn considerable interest in CRS due to its association with disease severity. Over 99% of bacteria are capable of forming biofilms, which are defined as a “microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other, are embedded in a matrix of extracellular polymeric substances that

they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription."[86] Biofilms have been shown in CRS to be associated with worse pre and post-operative symptoms and mucosal inflammation after sinus surgery.[87,88] In particular, *S. aureus* biofilms have been associated with worse patient outcomes, compared to other species such as *Haemophilus influenzae* biofilms, which are associated with mild disease.[89,90] Biofilms may also be polymicrobial and here *S. aureus* biofilms have been found in association with *P. aeruginosa* and fungi.[91] It has been shown in an animal model that bacterial biofilms cause sinonasal inflammation and epithelial injury, thus providing a hospitable environment for fungal biofilm formation.[92,93] The synergism occurring between these two kingdoms of life is of great interest and once again highlights the role of the polymicrobial community and the importance of synergistic relationships between its members in CRS.

S. aureus is associated with disease severity in several ways (Figure 1-2) including when residing within sinonasal epithelial cells. Here it has been suggested to represent a reservoir of pathogenic organisms, capable of promoting infection and disease recalcitrance. Due to the association of biofilm presence and intracellular *S. aureus*, biolfilms are thought to play an important role in the transition of bacteria from its planktonic state into the intracellular niche.[94] Whilst *S. aureus* predominates in this setting, microcolonies of other bacteria have also been found in the intramucosal niche.[95] To date rigorous broad-based detection techniques have not been applied to the detection of these alternative intramucosal and intracellular community members. This shortcoming should see attention in future research. Intracellular bacteria in CRS do not appear to provoke

immune detection by the host and it may therefore represent a phenotype that actively evades host immunity.[95] This relationship may also explain the association between intracellular *S. aureus* and clinical and microbiological relapse of disease following ESS,[96] where dispersal of bacteria from a protected niche may occur.

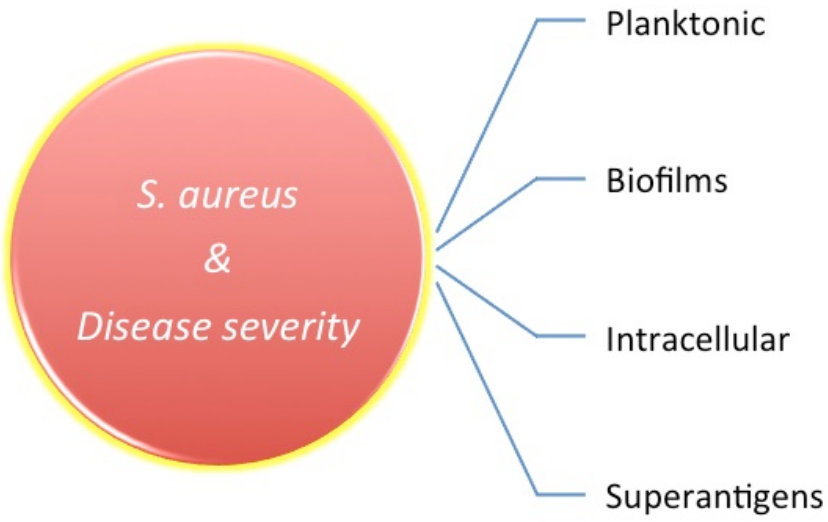

Figure 1-4 *S. aureus* associations with disease severity. *S. aureus* is associated with disease severity when present as planktonic, biofilm or intracellular bacteria. The immunomodulatory properties of *S. aureus* superantigens or enterotoxins (SAEs) also influence disease state.

The immunomodulatory properties of *S. aureus* in CRS patients are also likely to be of importance. IgE antibodies to *S. aureus* enterotoxins (SAEs) have been identified in CRS patients colonized with S. aureus.[37,97,98] In particular, IgE formation to SAEs, is strongly associated with asthma in patients with CRSwNP.[37] SAEs have effects on a variety of cell types including: epithelial cells, lymphocytes, eosinophils, fibroblasts and mast cells. These effects are aimed towards assisting *S. aureus* in evading the host immune response, and result in skewing of the inflammatory response in the Th2 direction, generation of local polyclonal IgE,

promotion of eosinophil survival and mast cell degranulation, and alteration of eicosanoid metabolism.[1] *S. aureus* biofilms have also been shown to promote an eosinophilic, Th2-polarized inflammation in CRS patients, irrespective of the presence of polyps and independent of the superantigen pathway.[99] These observations are of interest and highlight the presence of alternate mechanisms by which this microorganism may interact with the host.

The bacterial interactions highlighted above are a reflection of research directed towards species of importance, which have been determined predominantly with traditional culture techniques. It is to be expected that many new species of interest will emerge with the advent of sensitive molecular techniques and lead towards new paths of enquiry.

1.2.4 Environmental factors – fungi

The role of fungi in CRS remains as controversial today as it did when Ponikau et al. found fungi in the sinuses of 96% of patients with CRS and 100% of controls using a novel culture technique,[18] a finding also replicated by others.[19] On the basis of culture results and histopathologic findings the authors reported the presence of allergic fungal sinusitis (AFS) (see 1.1.3) in 93% of CRS patients. However, because the presence of eosinophilia was observed with an absence of specific IgE hypersensitivity to fungal allergens in the majority of patients, an alternative nomenclature of eosinophilic fungal rhinosinusitis was proposed. These findings provided the basis for the “Fungal Hypothesis of CRS,” which proposed an excessive, non-IgE mediated host response to common airborne fungi was the primary pathogenic trigger in most forms of CRS.[1]

In an attempt to test the fungal hypothesis of CRS, five double-blinded, randomized studies have investigated the role of topical antifungals.[100-104] The findings of these were pooled in a recent meta-analysis, which found that no statistically significant benefit of topical antifungals over placebo existed for any outcome. Symptom scores actually statistically favored the placebo group and adverse event reporting was significantly higher in the antifungal group.[105] A follow-up study of CRS patients receiving topical amphotericin also showed no significant effect on any pro-inflammatory chemokine, cytokine or growth factor in the lavage samples of these patients.[106] These findings combined led many clinicians to abandon the fungal hypothesis, however like bacteria, there are several other ways in which fungi may act as important disease modifying organisms in CRS.

Several substances are secreted by nasal epithelial and immune cells, which play an important role in the innate immune systems response to fungi.[107] The cathelicidin LL-37 is an important innate defense peptide with antimicrobial activity. In CRS, LL-37 has been found to be significantly up regulated at the mRNA and protein level in response to fungal allergens.[108] The antimicrobial proteins lysozyme and lactoferrin are also important in the innate immunity of the sinonasal cavity. Lysozyme displays fungicidal activity toward many fungi commonly identified in patients with CRS.[107] Reduced expression of lactoferrin in the nasal mucosa of CRS patients has also been found,[109] however its relationship with the fungal state remains unclear.

Whilst consistent in vitro or in vivo evidence is not available to demonstrate that fungal antigens are the primary targets of the mucosal T cell or B cell

responses observed in CRS,[1] fungi have still been shown to exhibit important immunomodulatory properties. *Alternaria* for example contain intrinsic proteases that non-specifically activate protease-activated receptors (PAR) present on the apical surface of nasal epithelial cells, resulting in secondary effects on eosinophils and neutrophils.[110,111] The non-specific effects resulting from these proteases are likely to be significant as PARs have been shown to be up-regulated in CRS patients. Given the ubiquitous presence of fungi this could be of significance in the predisposed individual with abnormalities in the protease-PAR signaling pathway.[112] Fungal cell walls contain chitin, a polysaccharide polymer, which is also recognized by PARs on nasal epithelial cells. Chitin has been shown in a murine model to activate innate mechanisms,[113] and induce local Th2 immune responses accompanied by the presence of eosinophils, basophils and Th2 lymphocytes.[114] These responses and those occurring relating to the effects of fungal proteases illustrate important mechanisms whereby fungi interact with the host immune system.

To date the mycology of CRS is poorly described in the literature. Fungi are typically difficult to culture and standard laboratory techniques do not allow it to be readily cultured. Acknowledging this deficiency Ponikau et al. developed a novel culture technique, which utilized a mucolytic agent to release fungal elements from mucus and allow for direct contact with the growth media.[18] This technique was also used by groups in Europe and Asia to characterize the presence of fungi in these populations.[19,115] Using these methods Ponikau et al. were able to highlight the ubiquitous presence of fungi in the nasal cavity of these patients and on average identified approximately 3 species per patient being.[18,19] Using the same

technique Jiang et al. was only able to identify fungi in 49% of lavage specimens which may have been reflective of regional differences.[115] Other studies in North America have however reported similarly low rates with a comparable culture method.[116] It is most likely that these lower rates of detection reflect deficiencies in the detection technique. A study in Australia using intra-nasal air samplers attempted to quantify both the numbers and genera of fungi that were inhaled during normal outdoor activities. These authors highlighted the presence of significant amounts of fungi entering the nasal cavity in all individuals with the dominant inhaled fungi identified being *Arthrinium*, *Curvularia*, *Epicoccum*, *Pithomyces*, *Spegazzinia*, *Bipolaris* and Xylariaceae species.[117] Traditional culture studies of CRS patients have typically identified several prevalent fungal genera including *Aspergillus*, *Alternaria*, *Cladosporium* and *Penicillium*,[18,19,118] but it remains unclear which species are colonizers as opposed to environmental contaminants. Whilst focused PCR techniques can be used for the identification of medically important fungi,[119] broad-based pyrosequencing techniques are yet to be used for the identification of fungi in CRS patients. If the prevalence of fungi in the sinonasal cavity parallels that seen with bacterial microbiome studies, there is potentially a large component of the ecological framework of this niche that remains uncharacterized, despite its potentially important functional implications.

1.3 Microbe detection in chronic rhinosinusitis

1.3.1 Culture

An overview of the various detection techniques is shown in Figure 1-5. In the context of modern microbial detection techniques, traditional cultures have several important limitations that must be considered when interpreting results. Best estimates would suggest that only 1-10% of all bacteria in a particular environment are able to be cultured under laboratory conditions.[120] Despite this, for much of the 20th century, culture-based methods have been the mainstay of clinical microbiology and are still used routinely for patient care. Culture techniques typically rely on simulating the ideal environmental conditions that promote the growth of a pathogen once taken from its native environment. Identification of a suspected pathogen involves an initial culture followed by sub-plating onto more selective media. The application of biochemical assays may also be required to differentiate species when this distinction cannot be made purely on the basis of phenotypic traits.[76] A number of other important factors such as choice of growth media, temperature and pH all influence the ultimate detection of microbes by this technique. In general, culture techniques tend to greatly overestimate the importance of organisms that are easily cultured. Conversely, the presence of potentially important or fastidious organisms, which require more specialized culture methods, are frequently underestimated.[121] The lack of sensitivity for this technique is highlighted on review of the CRS literature where aerobic cultures are negative in between 20 and 50% of patients, whilst specific anaerobic cultures are negative in up to 65% of patients.[122,123] Given that the sinus is not sterile, these

detection rates represent a significant limitation when compared to more sensitive microscopy and molecular techniques.

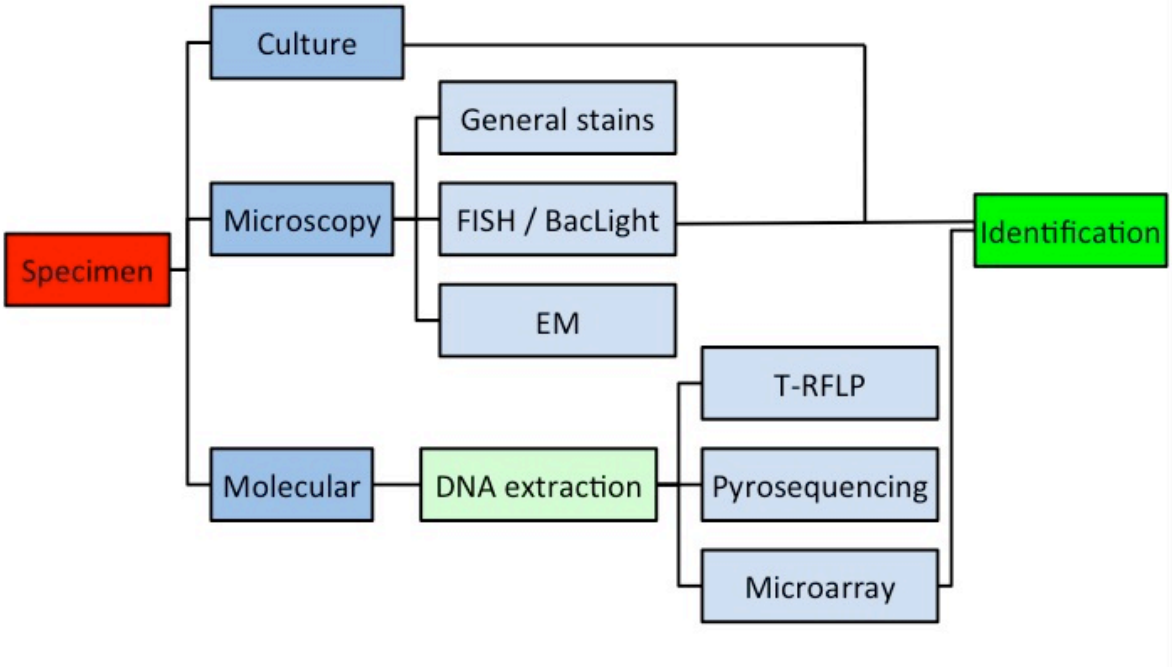

Figure 1-5 Schematic overview of microbial detection techniques

1.3.2 Microscopy

Several microscopy techniques are used in clinical and basic science applications relating to CRS. These methods achieve visualization of microbes with the use of non-specific broad stains, specific stains and electron microscopy.

Nonspecific stains merely identify the presence or absence of an organism, and give little information in regards to speciation. Commonly employed broad-range stains include Gram, acid-fast, and live/dead stains for bacteria, and 10% potassium hydroxide (KOH) or silver stains for fungi.[76] The gram staining procedure has the advantage of using ordinary light microscopy, and allows storage of preparations for long periods. This technique is commonly used by bacteriologists as it allows for the division of prokaryotes into two large groups

based on differences in cell wall structure.[124] Live/dead stains such as the BacLight Viability Kit (Invitrogen Corp., Carlsbad, CA), a nucleic acid probing technique have been used in studies examining biofilms in CRS.[125] This technique is the most suitable technique for quantifying biofilm biomass within the sinuses,[125] and is able to differentiate between live and dead cells by fluorescing green or red respectively. Limitations include its inability to determine the specific species present, and also the need for prompt processing and analysis of the specimen.[126] This can be problematic, particularly if access to specialized microscopes is not readily available. For fungi, 10% KOH is often used to denature human cells and allow for visualization of fungal hyphae, while silver stains are frequently used for histologic analyses under a light microscope.[76]

Fluorescence in situ hybridization (FISH) is a detection technique that allows for the identification of bacterial or fungal DNA using species-specific probes.[127,128]. In CRS, this technique has allowed researchers to move from identifying the presence of biofilms without speciation (BacLight) to determining the most prevalent biofilm forming species and also the presence of polymicrobial biofilms.[91] The use of FISH as a diagnostic modality does have several limitations of note. The need to presumptively nominate the organisms to be probed creates a selection bias from the outset and potentially important microbes involved in CRS pathogenesis may remain untested. A major technical constraint, relates to the ability to test for multiple species in a single patient. Several probes may be used concurrently, but only if they bind to different targets and fluoresce at different wavelengths.[76] The number of probes that can be applied to any individual piece of tissue is also a significant limitation.[129] FISH has also been

used for the detection of intracellular bacteria in CRS,[94] which highlights the versatility of this technique when combined with advanced imaging such as confocal scanning laser microscopy (CSLM) to define bacteria on mucosal surfaces and in deeper tissue layers.

Of the various imaging modalities that can be used to image biofilms on the sinus mucosa, confocal scanning laser microscopy (CSLM) has emerged as being superior to both scanning electron microscopy and transmission electron microscopy.[130] Standard electron microscopy may also be performed to determine the presence of biofilms and morphologies consistent with planktonic cells.[76] For the purposes of biofilm detection however, the limitations of scanning electron microscopy and transmission electron microscopy, which have poor specificity and sensitivity respectively, should be noted.[130]

1.3.3 Molecular

With the limitations of culture techniques in detecting fastidious microbes and the technical difficulties of microscopy techniques, the emergence of molecular methods for microbe detection has revolutionized the study of complex microbial communities. A major advantage of molecular techniques is the speed in which results can be obtained. Whereas cultures frequently take days before a result is determined, molecular microbial studies can usually be performed within 24 hours.[131] In the clinical setting this time difference can have a real impact on decision-making and its subsequent impact on patient care.

In CRS a number of molecular techniques have been used to date to define the microbiome. Terminal restriction fragment length polymorphism (T-RFLP), a fingerprinting technique relies on separating different variants of a gene (e.g. 16S rRNA) in the community sample by electrophoresis.[83] Typically the dynamic range is limited (so only the few most abundant members of the community can be observed), and it is difficult to relate banding patterns to changes in particular species or lineages. This technique also makes it impossible to combine data from different studies into a single analysis.[132]

Sequencing techniques have also been used to define the CRS microbiome.[73,133,134] These techniques have several advantages including classification of taxonomy and function, greater dynamic range and ability to compare complex samples, and also the ability to combine sequences from different studies.[135] A particular benefit of sequencing techniques is the ability to determine which specific genes or species contribute to differences among communities.[132]

Pyrosequencing is now the preferred sequencing method and is significantly cheaper and faster than the first generation Sanger sequencing technology.[136] The pyrosequencing process first requires extraction of DNA from an entire microbial community, and a particular target region (e.g. 16S for bacteria, 18S or ITS for fungi) flanked by conserved primers is amplified by PCR before sequencing. This generates an amplicon dataset, in which every read stems from a homologous region, and the sequence variation between the reads reflects the phylogenetic diversity in the community. During pyrosequencing, each nucleotide base is washed across a plate with hundreds of thousands of wells in which beads

attached to multiple copies of a single DNA molecule are localized. If the first unpaired base in a well is complimentary to the incoming base, then synthesis occurs and through a series of chemical reactions light is emitted. Subsequent synthesis and increased light emission will occur if a homopolymer is present. The pattern of light intensities, or flowgram, emitted by each well can then be used to determine the DNA sequence.[137]

A modification of the pyrosequencing technique is tag-encoded FLX amplicon pyrosequencing (TEFAP). Here a unique tag is encoded within the amplicons for each individual sample. This tag allows for the identification of any given sequence and the corresponding sample from which it originated. This important process allows multiple samples to be combined after labeling into a single pyrosequencing run. Initially the cost of pyrosequencing was prohibitively expensive with the cost of processing one sample in the order of $5,000 USD. With the advent of TEFAP however, hundreds of individually tagged samples may be combined into a single run. This has resulted in a corresponding fall in cost per sample to the $110–$150 range, and includes up to 3,000 individual sequence reads per sample.[120]

Like microarray (to be discussed shortly), sequencing-based approaches require an amplification step to increase the signal to observable levels. Amplification is usually achieved through polymerase chain reaction (PCR), a step that is known to introduce biases.[138] PCR protocols have been developed to minimize these biases, yet they cannot be completely eliminated.[139] Other limitations include the selection of PCR primers. Primers are designed to target the broadest range of organisms however, they have been shown to selectively enrich

the sample for some sequences while underrepresenting, if not missing others.[140] The selection of a primer must therefore be considered carefully as there is a real potential to introduce bias into the analysis.[141] Another drawback to sequencing techniques is that they do not necessarily indicate that viability of an organism at the time of sampling.[76] This may have implications for microbial communities with a high turn over. In the sinonasal cavity this may also have relevance where it is impossible to determine if inhaled microbes such as fungi remain viable on entering an altered host environment.

DNA microarrays such as the PhyloChip represent an intermediate approach, which lies somewhere between electrophoresis techniques and sequencing. Microarrays has been used in one CRS study,[74] and utilize photolithography chip technology to perform sequence analyses on amplified small-subunit rRNA genes.[142] This method provides a convenient way to screen 16S rRNA gene sequences and has the potential to be cheaper and provide greater dynamic range than sequencing studies. The major limitation of this technique is that the sequences must be known in advance so that they can be printed on the chip.[132] This limitation is particularly important where novel taxa are concerned, as *a priori* knowledge of these sequences is not possible.

It is clear that the emergence of molecular sequencing methods have given researchers a huge advantage in identifying the constituents of the microbiome. Whilst this technology remains in its infancy, there is a lack of consensus as to the best methods for many of the steps in this process from DNA extraction to sequencing. The diverse range of methodologies that have been reported in the literature evidences this, and makes direct comparisons between studies difficult.

Compared to traditional culture techniques however, it is apparent that with ongoing technological advancements the potential of these methods to provide new microbial insights is staggering.

1.4 The microbiome concept

The dominance of culture techniques in clinical practice, both past and present, has conditioned clinicians towards a narrowed perspective based on the presence of readily cultureable microorganisms. The rise of culture independent molecular methods, as discussed above (see 1.3.3), has highlighted the presence of a huge diversity of microbes on many human body surfaces including the sinonasal cavity. For the clinician and researcher alike to comprehend the presence of these rich communities of bacteria and fungi, alternative theories are required to rationalize the unique pressures that are at play in a polymicrobial community and their impact on functional outcomes. Here the concepts relating to the pressures influencing the constituents of the microbiome are discussed from an ecological perspective.

1.4.1 Definition

The term microbiome was first coined by Joshua Lederberg to describe the ecological community of commensal, symbiotic, and pathogenic microorganisms that literally share our body space.[143] A distinction can also be made between "microbiome" and "microbiota" to describe either the collective genomes of the microorganisms that reside in an environmental niche or the microorganisms themselves, respectively.[144-146] These terms however, are often used interchangeably and by the original definitions may be considered to be largely synonymous.

1.4.2 The ecology of the microbiome

Concepts relating to the rationalization of the microbiome have largely drawn from principles of ecology derived from the macroscopic world. The relationships occurring within these environments are thought to have much in common with microbial ecology and therefore the questions being asked as they relate to the human microbiome are new only in terms of the system to which they are being applied.[145]

Several fundamental processes of community ecology shape the microbial residents of an individual: dispersal, local diversification, environmental selection, and ecological drift (Figure 1-6). When these processes are combined with ecological theory an important framework can be constructed for understanding the dynamics of a microbial community. For the researcher and clinician alike, acknowledging these processes will ultimately have an impact on formulating strategies for restoring and maintaining the microbiome and the important health-associated ecosystem services that it provides.[47]

Dispersal refers to the movement of organisms across space, and represents a fundamental process by which the diversity of a local community may be enriched.[147] This naturally leads one to appreciate the human body as an "island," or patch of habitat that is continually sampling a pool of external colonists. Dispersal may be influenced by a number of factors including microbial traits, transmission routes between hosts or the environment and ex-host survivability.[47]

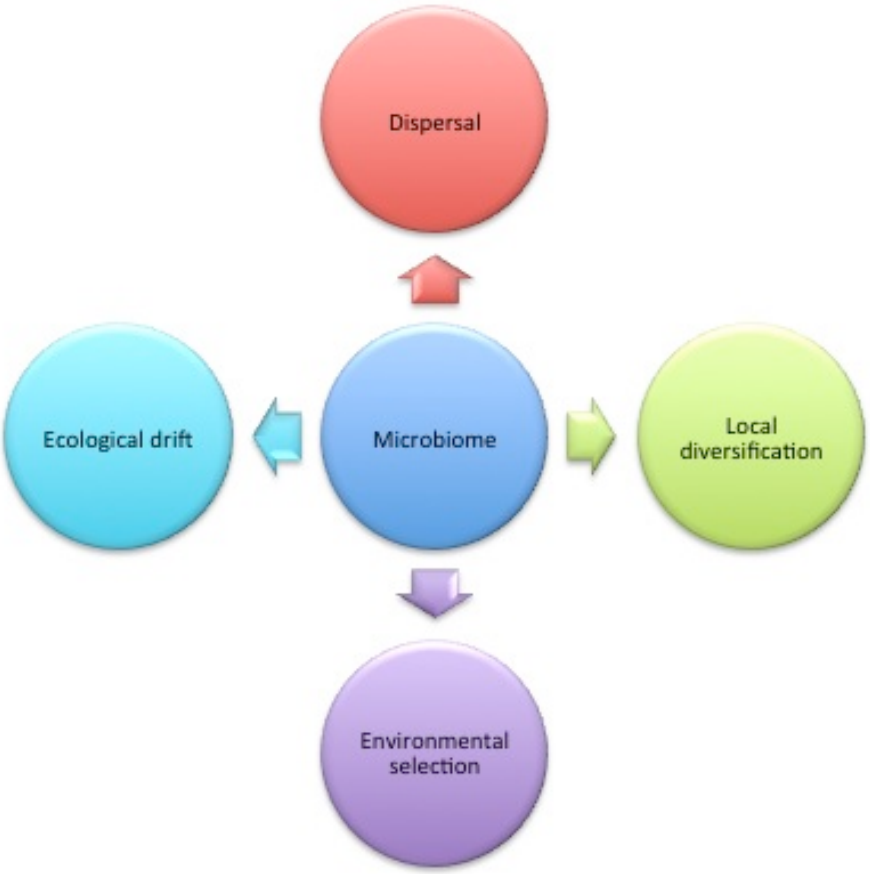

Figure 1-6 General processes shaping the microbiome. Dispersal, local diversification, environmental selection, and ecological drift are important processes shaping the constituents of the microbiome.

Local diversification represents a second process at play in microbial communities. This term refers to the evolution of divergent ecological characteristics within a particular lineage.[148] In the human body, large microbial population sizes, high growth rates, and strong selective regimes may result in rapid microbial adaptation via mutation or recombination.[47] Genetic recombination through horizontal gene transfer (HGT) may be particularly common amongst community members. HGT refers to the acquisition of genetic material from non-parental lineages. This process provides other community members with rapid access to genetic innovations, allowing traits such as virulence, antibiotic

resistance and xenobiotic metabolism to spread through the human microbiome.[149] In CRS this could be of functional relevance, and explain the presence of certain species as both commensals and pathogens. Other factors driving local diversification may include bacteriophage,[47] and bacterial adaptations against protozoan predation. Selective predation of bacteria by protozoa, although not yet researched in the sinonasal cavity may be at play in CRS. Once again these interactions may give rise to diverse routes of bacterial defense, including adaptive mechanisms (e.g. bacterial biofilms), and promote bacterial evolution.[150]

Environmental selection occurs as a result of deterministic fitness differences between species.[147] Over time this may contribute to changes in the relative abundance of community members. The human body itself plays a role in environmental selection by acting as a habitat filter due to the unique collection of resources and conditions, which preferentially allow for the growth of some species but not others.[47] A simple analogy of this in medicine is the use of standard culture techniques where certain media preferentially select for the growth of specific microbes. An alternative view considers the human body and its symbionts as a community of interacting cells. Here feedback mechanisms between host and microbe assumes that each may shape the other. In humans this is best represented by interactions between the host immune system and microbes.[151]

The final process shaping the microbiome is ecological drift, which can be defined as random changes occurring in species relative abundances.[147] As a result of this process, low abundance species are more likely to trend towards local extinction and become lost from the microbiome. These low abundance

species may include recent immigrants, species affected by antibiotics, or those occupying niches with a low carrying capacity. Species may also be rescued from the brink of extinction if a competitive advantage is gained relative to other community members.[47] Here a member's abundance may be replenished from external sources through mechanisms of dispersal as mentioned above, a mechanism likely to be of particular importance in the sinonasal cavity given its close proximity to the external environment.

1.4.3 Specific influences of the microbiome

Human microbial communities are not stable, but rather are in a constant state of flux depending on the ecological pressures applied to them (see 1.4.2). Two significant factors influencing the CRS microbiome will be discussed here.

Antibiotics are widely used in the management of CRS patients and need to be considered as major confounders in the context of microbiome studies. Whilst antibiotics are often prescribed on the basis of traditional culture results and sensitivities, these medications are not specific to those species, but rather cause widespread disturbances in a large proportion of the community. This perturbation provides remaining community members or new colonists (entering via dispersal) the opportunity to increase their abundance in the vacated niche.[47] Studies examining the impact of antimicrobial treatments on the resilience of the microbiome are limited. Dramatic shifts have been reported as soon as one week after antibiotic treatment with reduced bacterial diversity. These resident microbiota have been subsequently shown to recover to resemble the pre-treatment state in some patients, however this may remain disturbed for up to four years post treatment, and continue to harbour genes associated with antibiotic

resistance.[152] Other studies have found that whilst community changes tended to return towards their initial state after antibiotics, this return was often incomplete. This was particularly the case in the setting of repeated disturbances caused by recurrent courses of antibiotics.[153] The functional implications of these changes remain poorly understood to date and highlight the need for restraint in antibiotic usage given that ultimately, these medications may be having a greater detrimental effect on beneficial bacteria than the pathogens for which they are prescribed.[154]

Corticosteroids are generally considered to exert their beneficial effects in CRS patients by reducing sinonasal inflammation through suppression of the systemic adaptive immune response. The innate immune system may however be enhanced by corticosteroids and a growing body of evidence suggests that antimicrobial factors released by epithelial cells including complement, collectins, lysozyme, lactoferrin, secretory leukocyte protease inhibitor, and defensins are therefore unlikely to be inhibited topical corticosteroids.[155] A recent in vitro study has shown evidence that fluticasone, mometasone, and budesonide directly reduce *S. aureus* biofilm production independent of host inflammatory factors.[156] The mechanisms by which corticosteroids exert both direct and indirect anti-bacterial action are yet to be understood, however, it is likely that researchers have underestimated the potential for these medications to significantly influence microbiota in the nasal cavity and its impact on microbiome studies. Like antibiotics, further research is required to define the degree to which these medications alter the microbiome. Until this is known study design should reflect this uncertainty.

1.5 Probiotics – manipulating the microbiome

1.5.1 Probiotics defined

Probiotics are best defined as: live bacteria which when administered in adequate amounts confer a health benefit on the host.[157] This distinction needs to be made from the putative commensal organisms that reside in a particular ecological niche. These commensal microbiota may be a source from which probiotic species are isolated, but until characterized for content, stability, and health effects, they are not probiotics.[157] Probiotics are often added to a delivery vehicle (e.g. food) to target an effect in the host and must be distinguished from prebiotics, which are agents used to promote the growth of pre-existent microbiota.[158] Currently no legal definition for probiotics exists in countries such as Australia and the United States. This has led to the emergence of many commercial products, which are labeled as "probiotics" that do not meet the fundamental criteria stipulated in the scientific definition. Clinicians must be aware of this discrepancy and only recommend probiotics for clinical purposes when there is a validated health benefit observed in human studies.[157]

1.5.2 Probiotics and their mechanisms of action

Probiotics are believed to exert their beneficial effects through three modes of action:

1. Modulation of the host's defenses including the innate and acquired immune system.
2. A direct effect on other microorganisms (commensal and/or pathogens).

3. Actions affecting microbial products (e.g. toxins) and other host products (e.g. bile salts).[159]

Much of our understating of relating to the mechanisms of probiotics is derived from studies involving the gastrointestinal tract. The immunomodulatory properties of probiotic bacteria in this setting are believed to be triggered by products such as metabolites, cell wall components and DNA after contact with a suitable recognition receptor on the host cell. Adhesion itself with host epithelial cells or the release of soluble factors by bacteria may also trigger a signaling cascade resulting in immunomodulation.[159] One ex vitro study using human monocyte-derived dendritic cells matured in the presence of *Lactobacillus rhamnosus* used these cells to instruct naive CD4+ T cells. These T cells showed decreased proliferation and cytokine production, especially of IL-2, IL-4, and IL-10, whilst maintaining normal responsiveness of T cells to IL-2.[160] The direct adhesion of bacteria to epithelial cells has not been demonstrated in the gut. Bacteria have however, been observed to reach the antigen-presenting dendritic cells after transcytosis by microfold cells. These microfold cells exist in follicle-associated epithelium of the Peyer's patch and collect at a low level, luminal gut antigens and pass them down to dendritic cells, which are localized below the epithelial cells. The dendritic cells then selectively induce IgA, which helps protect against mucosal penetration by bacteria. This response is believed to be restricted to the mucosal immune compartment and thus avoids the potentially deleterious effects of a systemic immune response.[161] Dendritic cells have also been identified in the sinonasal epithelium,[162] but how they respond to certain bacteria in this ecological niche remains poorly understood. Likewise the potentially beneficial effects of

probiotic species at distant mucosal sites such as the gut on other host areas such as the sinonasal mucosa are yet to be elucidated.

Just as the host must combat infection from prokaryotes such as bacteria, these species must also be able to combat the anti-microbial properties of one another. Bacteria have a direct effect on other microorganisms resident in the niche through a number of mechanisms including: the production of antimicrobial substances, competing for limited resources and a variety of anti-adhesive, anti-invasive and antitoxin effects.[159]

Bacteriocins stand out among a wide variety of antimicrobial ribosomal peptides synthesized by bacteria. Lactic acid and hydrogen peroxide are two commonly described bacteriocins.[163] Species, which produce low-molecular-weight bacteriocins, may also secrete antibiotics such as reuterin (3-hydroxypropionaldehyde) as seen by *Lactobacillus reuteri*. Reuterin is a broad-spectrum antibiotic active not only against Gram-positive and Gram-negative bacteria but also against fungi, protozoa and viruses.[164] Probiotic bacteria are also known to produce deconjugated bile acids (a derivative of bile salts). These deconjugated bile acids show strong antimicrobial properties and are another example of how probiotics may influence neighbouring microbiota.[159]

The limited quantity of iron in the host available for utilization by bacteria provides an important example of how members of the microbiome need to compete for this limited resource. Iron is an essential element for almost all bacteria with the exception of lactobacilli, which do not require its presence in their natural habitat.[165] This provides lactobacilli with an important competitive advantage compared to other iron-dependent microorganisms. Some *Lactobacilli*

have an additional survival advantage due to their ability to bind ferric hydroxide at their cell surface and thus make it unavailable for utilization by other pathogenic microorganisms.[166] An alternative approach to iron utilization is seen by the probiotic strain *Escherichia coli Nissle*. Whilst dependent on the presence of iron like many pathogenic bacteria, this strain utilizes it more efficiently by taking advantage of at least seven different iron uptake systems.[167,168]

The adherence of probiotic bacteria to epithelial cells has been observed in in vitro studies. Here it is believed that a beneficial effect may be achieved when probiotic species block the adherence of pathogens competing for the same binding sites.[159] In the gut, an anti-adhesive effect may also be achieved when probiotic species induce increased mucin production. Lactobacillus strains have been shown to increase the extracellular secretion of MUC3 (a large mucin glycoprotein expressed by the human intestine), which consequently reduced the adherence of the enteropathogen *E. coli*.[169] Goblet cells are also commonly found in the sinonasal mucosa,[170] and in CRS patients nasal discharge is a frequent complaint. Using probiotic species that promote healthy levels of mucous production may therefore have therapeutic advantages. Other anti-adhesive mechanisms of probiotics, which warrant further investigation include degradation of carbohydrate receptors by secreted proteins, biofilm formation, production of receptor analogues and the induction of biosurfactants.[159]

The invasion of epithelial cells is an important component of pathogenicity for many pathogenic bacteria.[171] Several probiotic species have been shown to specifically interfere with host cell invasion. *E. Coli Nelli* strain has been shown to inhibit invasion of gut epithelial cells by variety of bacteria including: *Salmonella*

typhimurium, Yersinia enterocolitica, Shigella flexneri, Legionella pneumophila and *Listeria monocytogenes*. In this case the secretion of an unidentified factor is believed to result in the inhibitory effect.[172] Other species have also been shown to be capable of inhibiting epithelial cell invasion, however in vivo studies are required to validate this further. Invasion of epithelial cells by bacteria has also been reported in patients with CRS. In particular, *Staphylococcus aureus* has shown itself capable of invading epithelial cells in these patients and has been associated with the presence of biofilms,[94] and microbiological relapse of disease following ESS.[96] Probiotic species, which inhibit the invasion of *S. aureus* into nasal epithelial cells, may therefore merit further research due to their potential therapeutic importance.

A major virulence factor for many pathogenic bacteria is their ability to produce toxins. As an example, *S. aureus* a prevalent CRS pathogen[82] is known to produce a variety of toxins, depending on its environment, including pyrogenic toxin superantigens, exfoliative toxins, leukocidins, and others.[173] *S. aureus* superantigen toxins are also known to exist in CRS patients, and are implicated in the inflammatory process.[97] Unfortunately, probiotics capable of targeting *S. aureus* toxins are yet to be identified. Other probiotic bacteria do however have antitoxin effects such as *Bifidobacterium breve* (the species present in the probiotic drink marketed by Yakult) and *Bifidobacterium pseudocatenulatum*. These species inhibit Shiga toxin expression (a cause of dysentery) in E. coli strains in vitro and in mice, through the production of high levels of acetic acid.[174]

1.5.3 Probiotics in medicine

The use of probiotic treatments in medicine is not new. In 1907, Nobel laureate Elie Metchnikov showed that consumption of Bulgarian yoghurt (which contains lactic acid bacteria) was good for health.[175] Since this time, our ability to detect the constituents of microbial communities and determine their functional importance has increased dramatically. These insights have given researchers in many fields of medicine the ability to offer alternative ecologically based treatments either based of a specific mechanism of action, or associations with health.

Atopic dermatitis (AD) has emerged as a target for probiotic treatments. Like CRS, the disease is caused by a combination of genetic and environmental factors, and in severe form is often associated with food allergy. The majority of atopic dermatitis patients also have elevated serum IgE levels and peripheral eosinophilia. Whilst most children with AD are symptom free by adulthood, it can be the starting point for the progression of several allergic disorders such as asthma and allergic rhinitis.[176] Several randomized controlled trials have explored the preventative effect of orally ingested probiotics in developing atopic dermatitis. These trials using either *Lactobacillus* or *Bifidobacterium* species which were given to infants with a high risk of developing allergy, starting immediately after birth, have had mixed results. Wickens et al. showed that *Lactobacillus rhamnosus*, but not *Bifidobacterium animalis*, significantly reduced the incidence of AD by almost 50% compared to placebo by 2 years of age.[177] Others have also shown a reduction of the incidence of AD in those receiving probiotics.[178] Contrasting with these results, Abrahamsson et al. found no reduction for the incidence of AD, although subgroup analysis revealed a significant reduction in

IgE-associated AD.[179] Two other prevention studies also showed no change in incidence of AD.[180,181] A review of probiotics directed towards the treatment of AD, rather than prevention, has also yielded mixed results. Four studies have shown a positive outcome.[182-185] Another 3 have shown no effect on AD in general, but did show improvement in the severity of IgE-associated AD as measured by a validated clinical scoring system.[186-188] These findings would suggest that possibly only children who are a young age and already have evidence of IgE sensitization (positive skin prick test and/or elevated total or specific IgE levels) might benefit from probiotics for the treatment of AD.[176]

A recent systematic review of patients receiving probiotics for irritable bowel syndrome, clinical entity characterized by abdominal pain, mucous in the stools, and alternating diarrhea and constipation, identified 10 randomized controlled trials involving 918 patients. The probiotics used included *Lactobacillus*, *Bifidobacterium*, *Streptococcus* or a combination of probiotic species and showed a significant reduction in irritable bowel syndrome symptoms.[189] Another study investigating the effect of probiotics on bowel disease was that of Van Nood et al. These authors eloquently demonstrated the utility of an ecologically based treatment by taking a probiotic approach to the management of a dysregulated microbiome in the context of *Clostridium difficile* infection. The authors treated patients with an infusion of a solution containing donor feces through a nasoduodenal tube. An impressive 81% of patients had resolution of *C. difficile*–associated diarrhea after the first infusion. Those who did not initially respond were given a second infusion with feces from a different donor resulting in resolution for 2 of these 3 patients. After donor-feces infusion, patients showed

increased fecal bacterial diversity, similar to that in healthy donors.[190] The perspective of this study in relation to probiotics is interesting. Here the beneficial properties of this treatment are not necessarily related to the presence of a single probiotic species, but rather the replacement of a dysregulated unhealthy bowel microbiome with another healthy community of microbes.

1.5.4 Probiotics in CRS and the nasal cavity

A Cochrane review examining probiotics for preventing acute upper respiratory tract infections identified 10 randomized controlled trials involving 3451 participants. The study found that oral probiotics were better than placebo when measuring the number of participants experiencing episodes of acute URTI, which included patients with ARS. Limited information from 3 of these trials also showed that patients receiving probiotics received fewer prescriptions for antibiotics. The most commonly used probiotics were *Lactobacillus* and *Bifidobacterium* species, but also included *Propionibacterium freudenreichii* and *Streptococcus thermophilus*. In general, the side effects for the probiotics received were minor with gastrointestinal symptoms being the most common.[191]

Orally ingested probiotics have also been used in one CRS study. Here a randomized, double-blinded, placebo-controlled trial of 77 patients comparing the usefulness of an oral probiotic (*Lactobacillus rhamnosus*) versus placebo was investigated. The authors hypothesized that an alteration in the systemic inflammatory response in CRS patients, such as a skewed response to the helper Th2 lineage could be augmented by the supplementation of probiotics which may alter intestinal microflora and promote Th1 responses.[192] Patients went on to receive twice daily, oral supplements of either the probiotic or the placebo for a

period of four weeks. The primary outcome measure was a score of patient symptom severity as measured with a 20-item Sinonasal Outcome Test (SNOT-20).[193] Overall improvement in symptom severity was seen in both study groups at 4 weeks but no improvement was identified in the probiotic group at 8 weeks. Importantly, no significant differences were found between the probiotic and the placebo group at either time point.[192] The authors of this study felt that a lack of improvement in nasal symptoms in the probiotic group at 2 months may have been partly related to the short duration of treatment, a proposition that needs to be investigated in future studies.

A Japanese study recently investigated the role of normal nasal flora in preventing *Staphylococcus aureus* colonization. First, a surveillance study was performed on 156 healthy volunteers, with swabs taken from the anterior nares. *Staphylococcus epidermidis* was the most prevalent species (100%), followed by members of the genus *Corynebacterium* (52.5%) and *S. aureus* (25%). The authors found that carriers of *Corynebacteria* had lower rates of co-colonization with *S. aureus* (8.5%), compared to of non-carriers of *Cornebacteria* (44·5%). A subgroup of heavily colonized *Corynebacteria* carriers was also identified in this group. Here their colonization was found to be stable throughout the 3 years of the study, and these participants never acquired *S. aureus*.[194] The authors hypothesized that the low incidence of *S. aureus* colonization in the carriers with *Corynebacteria*, compared to non-carriers, indicated the possibility of competition for survival between *S. aureus* and *Corynebacteria*, and went on to test this by artificially implanting a strain of *Corynebacterium* sp. into the nares of 17 *S. aureus* carriers. *S. aureus* was completely eradicated in 71% of carriers by up to 15

inoculations. Another 10 volunteers received, similar doses of 0·9% NaCl or *S. epidermidis* into the nares but this did not eradicate *S. aureus*. The authors of this study were unable to find any bacteriocin-like activity against *S. aureus* from the *Corynebacterium sp.* used, but did show that this species had a greater ability to bind nasal mucous than both *S. aureus* and *S. epidermidis*.[194] This affinity to bind nasal mucous may be a possible explanation for this probiotic species' mechanism of action, which allows it to displace other species from the niche. The implication of this finding is relevant to the management of CRS, where *S. aureus* is a dominant pathogen, and resistant to our current treatments. This study also distinguishes itself from others due to the topical route of delivery for the probiotic and shows a shift from utilizing immunomodulatory mechanisms, to actions that directly influence other microbes in the niche.

Staphylococcus epidermidis has also emerged as a potential probiotic candidate due to its ability to produce the serine protease Esp.[195] Iwase et al showed through an epidemiological study that the presence of Esp-producing strains of *S. epidermidis,* in the nasal cavity of human volunteers, correlated with the absence of *S. aureus*.[196] These authors subsequently showed that an Esp-producing strain of *S. epidermidis* was capable of inhibiting biofilm formation and nasal colonization by *S. aureus*. An in vitro study using purified Esp showed both inhibition of biofilm formation and destruction of pre-existing *S. aureus* biofilms. Analysis by microscopy, confirmed that Esp changed *S. aureus* from the sessile to the planktonic form with disruption of the biofilm. Esp was also shown to potentially modulate the host immune system after human beta-defensin 2, an antimicrobial peptide component of the innate immune system secreted by keratinocytes, was

shown to effectively kill *S. aureus* biofilms in the presence of Esp. Esp alone demonstrated no bactericidal activity whilst human beta-defensin 2 alone demonstrated only low bactericidal activity towards *S. aureus* in biofilms. Finally an in vivo study was performed and showed that an Esp-producing strain of *S. epidermidis* was able to eliminate *S. aureus* from the nasal cavity of volunteers who were known *S. aureus* carriers. Here purified Esp alone was also able to clear *S. aureus*, but an isogenic Esp mutant and other wild strains of *S. epidermidis* which were non-Esp producing did not.[196]

These exciting findings prompted further studies investigating the anti-staphylococcal properties of *S. epidermidis*. Park et al. described the use of a murine model to show that mice nasally pre-colonized with *S. epidermidis* became more resistant to colonization with methicillin-resistant *S. aureus*. The strain of *S. epidermidis* used in this study was only a weak producer of Esp, which led the authors to conclude that other mechanisms of bacterial interference may also have been at play.[197]

Sugimoto et al. aimed to elucidate the substrate specificity and target proteins of Esp and thereby determine the mechanism by which Esp disassembles *S. aureus* biofilms. This was achieved by using a mutant Esp protein with defective proteolytic activity, which did not disassemble the biofilm formed by a clinically isolated methicillin-resistant *S. aureus* strain, thereby indicating that the proteolytic activity of Esp is essential for biofilm disassembly. Esp was also shown to degrade specific proteins in the biofilm-matrix and cell wall fractions. Proteomic and immunological analyses showed that Esp degrades at least 75 proteins, including 11 biofilm formation and colonization-associated proteins, such as the

extracellular adherence protein, the extracellular matrix protein-binding protein, fibronectin-binding protein A, and protein A (Figure 1-7). In addition, Esp selectively degraded several human receptor proteins of *S. aureus* (e.g., fibronectin, fibrinogen, and vitronectin) that are involved in its colonization or infection. These results suggest that Esp inhibits *S. aureus* colonization and biofilm formation by degrading specific proteins that are crucial for biofilm construction and host-pathogen interaction.[198] Esp has also been shown to cleave autolysin (Atl)-derived murein hydrolases, which prevents staphylococcal release of DNA, an important component of the extracellular matrix in biofilms. This was revealed when x-ray crystallography showed that the three-dimensional structure of serine protease Esp was highly homologous to that of *S. aureus* V8 (SspA) a protease which also cleaves Atl-derived murein hydrolases.[199]

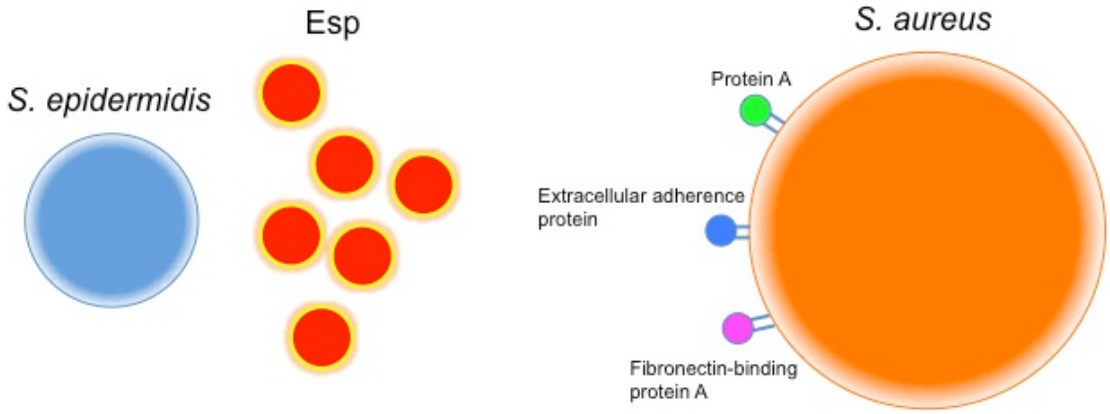

Figure 1-7 Serine protease Esp mechanism of action. Esp is secreted by *S. epidermidis* and degrades proteins associated with biofilm formation and colonization for *S. aureus*.

Currently no studies exist where topically delivered probiotics have been utilized for the management of CRS in humans. Abreu et al, after performing a microbiome study found that *Corynebacterium tuberculostearicum* was

significantly more abundant in CRS patients compared to controls and associated with worse symptoms. Contrastingly, species belonging to the order Lactobacillales were found to be present in high abundance in healthy mucosal samples, yet depleted in CRS patients. Using a murine model of sinusitis the authors went on to demonstrate that coinstallation of *Corynebacterium tuberculostearicum* and *Lactobacillus sakei* into the nasal cavity resulted in histological findings (i.e. goblet cell numbers) comparable to that of animals receiving *Lactobacillus sakei* alone, but significantly lower than those receiving *C. tuberculostearicum* alone.[74] These findings would suggest *L. sakei* protects the sinus epithelium against *C. tuberculostearicum* infection, a finding that would recommend this species as a potential probiotic in the management of CRS. Further studies are required to determine its specific mechanism of action, survival characteristics and performance in the nasal cavity, in the context of other common pathogens such as *S. aureus* and *P. aeruginosa*.

It is clear from reviewing the literature that there is no consensus as to the best probiotic species to be used in conditions such as CRS. This is likely due to many factors including:

- Deficiencies in our understanding of the pathophysiology of CRS and the role microbes play in the disease state
- A lack of agreement on the constituents of a healthy sinonasal microbiome versus an unhealthy one
- A poor understanding of probiotic mechanisms of action

- Uncertainty regarding best mode of probiotic delivery (i.e. oral vs. topical)
- A paucity of data relating to the efficacy of single species probiotic treatments versus community transfer.
- A lack of understanding relating to symbiotic relationships present in the microbiome, the presence of which are required to promote the expression of probiotic properties.

Overcoming these deficiencies in our knowledge will ultimately lead to the identification and utilization of probiotic species directed towards correcting specific functional deficits in an individuals microbiome.

Summary of the literature review

CRS is a disease characterized by nasal obstruction, nasal discharge and clinical findings consistent with sinonasal inflammation. The disease burden of CRS is significant and potentially underestimated in gross terms but also compared to other chronic diseases. It is apparent that our ability to classify this heterogenous group of patients is lacking. Whilst dividing patients into those with and without polyps is readily determined by the clinician, there are potentially more accurate ways in which these patients can be classified by taking into account a combination of clinical (phenotype), host (genotype) and environmental (microbiome) factors. After a diagnosis of CRS these patients are typically treated with maximal medical therapy, which is comprised of saline irrigations, INCS and oral steroids, and antibiotics. Patients with persistent symptoms progress to surgery, but despite all these measures a number of patients continue to experience disease recalcitrance. Clearly there is a need for new treatments utilizing novel mechanisms aimed at improving the disease state.

The discordant views regarding the host and environmental factors implicated in the aetiopathogenesis of CRS are likely to be more in agreement than previously suspected. Host factors such as deficiencies in cilia function and the innate and acquired immune systems highlight the likelihood of a predisposition towards the disease state in some individuals. In their own right these factors may be sufficient to cause CRS, but it is also likely that environmental factors such as bacteria and fungi, which exert direct and indirect effects may play an important role in the aetiopathogenesis of this disease by acting as important disease modifiers.

Our understanding of the microbial constituents of the sinonasal cavity, or the microbiome has previously been underrepresented by the widespread use of limited detection techniques in the literature. The emergence of techniques such as pyrosequencing have allowed for the detection of potentially all bacteria and fungi present in a sample, and has finally provided researchers with the means to describe the microbiome with a degree of fidelity which was previously impossible to achieve. The current paucity of literature using these techniques highlights a need for microbiome studies in CRS aimed towards defining the members of this niche and the temporal changes that are occurring.

Microbiome studies in other regions of the body such as the gut, have highlighted the important role that the microbiome plays in health and disease. Not only have these studies identified new organisms of interest, but the importance of interactions within a polymicrobial community is also highlighted. The functional consequences of a dysregulated microbiome in these regions is likely to translate to the sinonasal cavity and whilst the exact perturbations in CRS remain poorly defined, further studies based on findings from microbiome studies will assist in identifying the complex microbe-microbe and host-microbe interactions at play.

Probiotics represent a novel treatment strategy for CRS, which acknowledges the functional implications of a dysregulated microbiome and the need to correct it. By drawing on ecological principles these treatments have the potential to improve the disease state by utilizing antimicrobial mechanisms directed at specific pathogens and harnessing other indirect immunomodulatory effects.

Currently our understanding of the microbiome and the complex interactions occurring within it is in its infancy. The studies presented in the following section attempt to address some of these deficiencies.

Studies to be performed

1. Determine the bacteriology of a large cohort of CRS patients using a standard culture technique. Define the prevalence of key pathogens and their association with disease severity. The data generated from this study will also provide a baseline from which to compare bacterial detection using sensitive molecular techniques.
2. Use a highly sensitive 16S rDNA bacterial tag-encoded FLX amplicon pyrosequencing technique to characterize the bacterial component of the microbiome in both controls and CRS patients. Temporal changes occurring in the CRS cohort will also be examined post-operatively and the results will be correlated with subjective and objective outcome measures.
3. To define the fungal component of the CRS microbiome through 18S fungal tag-encoded FLX amplicon pyrosequencing. In so doing the characterization of the microbiome for both kingdoms of life (bacteria and fungi) will be completed. Once again temporal changes occurring in the CRS cohort will be examined and correlations with subjective and objective outcome measures will be made for both the CRS and control groups.
4. To investigate to investigate the probiotic potential of *S. epidermidis* against *S. aureus* in a mouse model by taking an ecological approach to managing pathogenic components of the microbiome.

Chapter 2: The bacteriology of chronic rhinosinusitis and the pre-eminence of *Staphylococcus aureus* in revision patients

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2.1 Abstract

Background

The role of bacteria in the aetiopathogenesis of chronic rhinosinusitis (CRS) remains an area of interest. The impact of surgery and factors such as the presence of polyps, asthma and aspirin sensitivity on the bacterial state are poorly understood. To determine the effect of these factors this study examines the culture results from a large cohort of CRS patients.

Methods

This retrospective study utilised the culture results from 513 CRS patients, which were analysed for species growth and compared to factors such as previous surgery, presence of polyps, aspirin sensitivity and asthma. Univariate and multivariate logistic regression models were utilised for statistical analysis.

Results

83% of patients had a positive culture result. The average number of isolates detected per patient was 0.95. *S. aureus* was the most frequently cultured organism (35%), followed by *P. aeruginosa* (9%), *Haemophilus* spp. (7%) and *S. pneumonia* (5%). Revision patients were more likely to grow *S. aureus* (*P* = 0.001), *P. aeruginosa* (*P* = 0.044) and have a positive culture (*P* = 0.001). Asthma was correlated with a positive culture (*P* = 0.039). No difference was determined between polyp and non-polyp patients for any of the bacterial outcomes.

Conclusion

This study highlights important factors in the bacteriology of CRS patients. *S. aureus* was the most prevalent species identified in our cohort followed by *P.*

aeruginosa. *S. aureus* rates of isolation were also significantly higher in patients undergoing revision surgery. No association was found between the presence of nasal polyposis and culture rates.

2.2 Introduction

The role of bacteria as an environmental factor in the aetiopathogenesis of CRS is an area of ongoing interest. A definitive representation of the various organisms involved, and their importance, continues to be disputed. Previous studies have suggested that a number of specific organisms are particularly linked to disease recalcitrance. The species most commonly implicated are *Staphylococcus aureus* and *Pseudomonas aeruginosa.*[89,90,200-202] Surprisingly, whilst *P. aeruginosa* has been shown to be more frequently cultured in patients who progress to revision surgery,[122,203,204] this relationship has not been conclusively demonstrated for *S. aureus*.

The detection of bacteria through culture independent techniques such as 16S rRNA gene sequencing, is increasingly being utilised to investigate the microbiome of CRS patients.[73,83] These detection techniques provide a sensitive means by which bacteria resident in a CRS patient can be identified. This is best highlighted by rates of species detection using a molecular technique which demonstrated an increased diversity of bacterial species by a magnitude of 10,[73] when compared to standard cultures, and is also consistent with other reports where it is proposed that only 1-10% of bacteria encountered in the environment are able to be cultured in the laboratory.[120] Whilst 16S rRNA gene sequencing methods have the ability to reveal a greater biodiversity in samples, traditional cultures have been shown to correlate well with species abundance.[133] The cost benefit of traditional cultures versus molecular detection techniques, which demand specific equipment and laboratory skill sets must be considered.[76] As a

result, traditional cultures continue to play an important role in bacterial detection for both the clinical and research settings.

The objective of this research is to study, through standard cultures, the bacteriology of CRS patients undergoing primary and revision surgery. Through the power of a large cohort, and by controlling for clinical factors, we aim to investigate the prevalence and clinical associations of bacteria in CRS.

2.3 Methods

Study design

This retrospective study utilises prospectively collected data, from the tertiary referral practice of the senior author (PJW). The local Human Research Ethics Committee gave approval for the study (2012052). All patients who underwent endoscopic sinus surgery (ESS) in the senior author's (PJW) practice had cultures obtained at the time of surgery. Patients who underwent ESS for CRS between 2003 and 2011 as defined by the Rhinosinusitis Task Force[3] were identified through the practice database. Patients were excluded if less than 18 years of age, immunocompromised, or diagnosed with cystic fibrosis or Kartagener's syndrome. Patients without microbiological investigations were not included.

The extent of surgery varied from middle meatal antrostomies with anterior ethmoidectomies (mini FESS), to complete fronto-spheno-ethmoidectomies (full house FESS). A number of revision patients with extensive severe disease received a Draf-III/frontal drillout procedure and/or canine fossa trephination (CFT) of the maxillary sinus. Before proceeding to surgery, all patients underwent a standard trial of medical therapy. This included a 3-week course of systemic prednisolone and a 2 month course of topical nasal steroid and saline washes.

Data collection

Samples for microbiological determination were harvested intraoperatively under endoscopic guidance by two means: a swab from the sinus cavity and/or direct retrieval of mucous (if present) and mucosal tissue from within the sinus cavity. Intraoperative samples were processed with standard techniques as set out by the Clinical and Laboratory Standards Institute.[205]

A database was created to collate demographics and microbiological results. These results were recorded for growth, and by species as indicated by the microbiology laboratory report. For several organisms identification did not extend beyond the genus level. In these cases this has been indicated as appropriate by convention. Isolates were recorded per person irrespective of the number of samples harvested.

Table 2-1 Demographics and disease characteristics of study population

Characteristics	**Primary surgical patients** n=162	**Revision surgical patients** n=351	**Total** n=513
Mean age (SD)	51 (16)	51 (14)	51 (15)
Male/female	96/66	184/167	280/233
Nasal polyps (%)	53 (33)	192 (55)	245 (48)
Asthma (%)	47 (29)	160 (46)	207 (40)
Aspirin sensitivity (%)	3 (2)	35 (10)	38 (7)
Smoking (%)	10 (6)	23 (7)	33 (6)
Mean CT scores*	13	15	14

*Total Lund MacKay score

Data analysis

Statistical analysis utilized a univariate logistic regression model. This was applied to the two patient groups (primary and revision) that were compared on each bacteria outcome for the 5 most commonly cultured pathogenic bacteria and also for a positive culture result. Where a significant result was determined, a multivariate logistic regression model was then applied to control for the potential

confounders: age, sex, presence of nasal polyps, asthma, aspirin sensitivity and smoking. All calculations were performed using SAS Version 9.3 (SAS Institute Inc., Cary, NC, USA) and a *P*-value < 0.05 was considered significant.

2.4 Results

515 patients satisfied the selection criteria. 2 patients were excluded due to incomplete demographic data, reflecting the high standard of prospectively collected data. The demographic data for these patients is represented in Table 2-1. The total culture rate was 83% with the predominant organisms being *S. aureus*, *P. aeruginosa*, *Haemophilus* spp.*, S. pneumonia* and *E. coli*. A full list of organisms cultured including floras is shown in Table 2-2. The univariate logistic regression model yielded significant results between primary and revision patients for *S. aureus* (P = 0.001), *P. aeruginosa* (P = 0.024) and positive culture (P < 0.001) (Table 2-3). The results for *Haemophilus* spp.*, S. pneumonia* and *E. coli* did not achieve significance. After controlling for confounders with the multivariate logistic regression model (Table 2-4), the results for *S. aureus*, *P. aeruginosa* and positive culture remained significant (P = 0.001, P = 0.044 and P = 0.001 respectively).

Table 2-2 Bacterial culture results

	Primary n=162	Revision n=351	Total n=513
Total positive cultures	119	306	425
Average number of species cultured per patient	0.84	1.01	0.95
Bacteria			
Aerobic and facultative anaerobic bacteria			
Gram positive			
Bacillus sp.	0	1	1
Corynebacterium sp.	1	0	1
Staphylococcus aureus	40	138	178
Streptococcus intermedius	1	4	5
Streptococcus pneumonia	9	17	26
Streptococcus Group G	0	1	1
Microaerophilic streptococci	1	0	1
Gram negative			
Acinetobacter sp.	0	2	2
Citobacter spp.	2	1	3
Coliform sp.	0	1	1
Enterobacter spp.	4	7	11
Escherichia coli	11	12	23
Haemophilus spp.	7	28	35
Klebsiella spp.	2	12	14
Moraxella catarrhalis	4	6	10
Proteus mirabilis	8	11	19
Pseudomonas aeruginosa	7	37	44
Serratia marcescens	1	2	3
Flora			
Oral, skin and respiratory	36	68	104
Anaerobic bacteria			
Mixed anaerobe sp.	0	1	1
Other			
Miscellaneous organisms not otherwise identified	2	4	6

2.5 Discussion

This study is a retrospective review of the bacteriology results of CRS patients presenting for primary or revision endoscopic sinus surgery. The key findings are the significantly higher rates of *S. aureus* (39% versus 25%) and *P. aeruginosa* cultures (11% versus 4%) in the revision group compared to primaries. A significant relationship was also shown in the revision group when considering all bacteria, where positive cultures (87% versus 73%) were more frequently observed.

For any discussion related to the rates of bacterial isolation it is important to be mindful of what is represented by the culture rate for a specific species. Nadel et al. highlighted that culture rates can be described in a variety of ways.[122] For example, some authors report species rates per total number of isolates. This can be misleading, particularly when multiple specimens from the same patients are obtained. To maintain clinical relevance in this study, species culture rates are expressed as the number of patients who cultured a particular species per total number of patients.

The total positive culture rate of 83% was comparable to Klossek et al.[206] and Finegold.[207] Another study by Desrosiers et al. had a considerably lower positive culture rate at 46%.[208] The higher rate of positive culture in revision versus primary patients (87% vs 73%) was an interesting finding in our study and may be indicative of an increased bacterial load in those patients with recalcitrant disease. This finding remained significant after controlling for confounders which were subsequently tested separately to explore any further relationships. Of these, only asthma was found to be significantly associated with a positive culture in the

univariate and multivariate models (in this instance revision surgery was also controlled). 87% of asthmatics had a positive culture versus 79% of non-asthmatics (*P* = 0.039).

Table 2-3 Results of the univariate logistic regression model (Revision vs Primary)

Outcome variable	Odds ratio (Revision vs. Primary)	95% Confidence interval	Chi-Square	*P*-value
S. aureus	1.98	1.30 - 3.00	10.28	0.001
P. aeruginosa	2.61	1.14 - 5.99	5.12	0.024
Haemophilus spp.	1.92	0.82 - 4.49	2.26	0.133
S. pneumonia	0.87	0.38 - 1.98	0.12	0.733
E. coli	0.49	0.21 - 1.13	2.83	0.092
Positive culture	2.46	1.54 - 3.93	14.14	< 0.001

Table 2-4 Results of the multivariate logistic regression models (Revision vs Primary).

Controlling for age, sex, presence of nasal polyps, asthma, aspirin sensitivity and smoking

Outcome variable	Odds ratio (Revision vs. Primary	95% Confidence interval	Chi-Square	*P*-value
S. aureus	2.09	1.35 - 3.22	11.09	0.001
P. aeruginosa	2.39	1.03 - 5.59	4.07	0.044
Positive culture	2.41	1.47 - 3.94	12.19	0.001

Asthma itself has been identified as a predictor of poor outcome post endoscopic sinus surgery,[209] and in our cohort, 46% of revision patients were asthmatics. Nadel et al. reported no significant differences in sinus cultures between asthmatics and non-asthmatics.[122] Others have shown higher rates of *S. aureus* colonisation in a subgroup of polyp patients with asthma and also aspirin sensitivity.[37] The role of *S. aureus* in this setting and its ability to provoke a proinflammatory response through superantigens has already been described.[210] The systemic effect of this may be the exacerbation of asthma. Locally the

heightened inflammatory state due to *S. aureus* superantigen acitivity, may be providing a hospitable environment for bacteria and thus be a driving force for the higher positive culture rates in these patients.

S. aureus, was the most commonly detected species in our patients (35%). This correlated with the results of Nadel et al. who reported rates of *S. aureus* detection of 31% in a cohort of 265 patients.[122] Gittelman et al. reported similar rates of *S. aureus* detection in an outpatient setting.[211] Our detection rates of *S. aureus* contrasted with the results of Niederfuhr et al. who identified it in only 19% of CRS patients with biopsy and lavage samples.[82] Higher rates of *S. aureus* detection (50%) have been seen in a recent molecular study utilising 16S rRNA gene sequencing. Interestingly though, in this paper, the rate of *S. aureus* isolation by standard culture remained in the order of 18%.[73]

P. aeruginosa was another commonly isolated organism (9%). The rates of prevalence reported in the literature vary widely, with culture rates between 0%[82] and 17%.[122] This is in stark contrast to the detection rate reported using a molecular technique. These authors demonstrated *P. aeruginosa* in a remarkable 93% of polyp, 92% of mucus and 90% of turbinate samples.[83]

A large proportion of the study population were revision surgery candidates (68%) reflecting the nature of referrals seen in this tertiary rhinology practice. Significantly higher rates of *P. aeruginosa* were found in revision patients as previously described by Brook and Frazier.[203] Other studies have also confirmed this relationship.[122,204] Interestingly though, whilst *S. aureus* has been shown to be a predominant species in post surgical infections,[200,201,212-214] no study to our knowledge has identified a significantly higher rate of *S. aureus* detection in

revision patients. It is likely the power of our large cohort has enabled us to elucidate this finding. If we consider revision surgery as a marker of disease recalcitrance the higher rates of *S. aureus* and *P. aeruginosa* seen in this group are of great interest, and parallel the findings related to *S. aureus* biofilms and disease severity.[89,215]

Comparisons between the data presented here and that found in control patients in other studies is limited due to methodological differences. The study already referenced by Niederfuhr et al included 21 controls. This group showed no significant differences in the bacteriologic features between those with and without polyps, and control patients.[82] The major limitations of this study were the alternative inferior turbinate site from which these patients were sampled and the selection criteria for a normal control. In this case, patients being treated for nasal structural abnormalities were selected as controls, yet they had not been cleared of sinus disease radiographically. Klossek et al. also described the middle meatus bacteriology of a large cohort of controls (n=139). They found the isolation of *Streptococcus* spp, *H. influenzae, S*. *pneumoniae, Prevotella* spp, *Fusobacterium* spp and *S. aureus* to be significantly associated with CRS.[206] Another study using a rigorous inclusion criteria and collection technique in a group of 14 patients undergoing Lefort I osteotomies revealed a sinus positive culture rate of only 18%. The organisms cultured in their cohort were coagulase negative staphylococci, *Citrobactor fundii* and polymorphic floras.[71] Key pathogens such as *S. aureus* and *P. aeruginosa* were notably absent.

When considering floras, we could not reproduce the findings of Schlosser et al., who found that coagulase negative staphylococcus was found with

increased frequency in patients with previously operated on frontal sinuses.[216] Our results were not sinus specific which may have limited this comparison. Furthermore, no significant difference was found between polyp and non-polyp patients for all of the bacterial outcomes, which was comparable with other studies.[82,217] In particular though, *S. aureus* was not seen more frequently in polyp patients which contrasted with the finding of Van Zele et al.[37]

Other considerations relating to the microbiology that have not been measured in this study include the effect of recurrent courses of antimicrobial agents. Patients with chronic rhinosinusitis frequently receive multiple courses of antibiotics. The impact of these antibiotics in selecting for organisms outside their spectrum of cover to create superinfections in CRS is not yet fully understood and it may, for example, contribute to higher rates of *P. aeruginosa* detection in some patients. The effect of topical intranasal corticosteroids is also of interest. Their influence on culture rates is potentially widespread given their use in pre-operative and post-operative patients, and is supported by one study which has shown that intranasal corticosteroids used pre-operatively are associated with lower rates of bacterial recovery in revision patients.[208]

2.6 Conclusion

Several important relationships in regards to the microbiology of CRS patients have been recognised by this study. Revision patients are more likely to have a positive culture. *S. aureus* and *P. aeruginosa* are predominant pathogens in CRS patients, and for the first time, significantly higher rates of *S. aureus* have been demonstrated in a revision population. Its relationship with recalcitrance should therefore see ongoing attention in future research.

Chapter 3: The bacterial microbiome in chronic rhinosinusitis: richness, diversity, post-operative changes and patient outcomes

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3.1 Abstract

Introduction

The bacterial microbiome in chronic rhinosinusitis (CRS) patients remains poorly understood with little consensus in regards to its constituents and the importance of particular species. This study examines the bacterial component of the CRS microbiome using a pyrosequencing technique in an effort to determine the diversity, richness, prevalence and abundance of bacterial species in these patients. Importantly the temporal changes in the microbiome and correlations with patient outcomes are explored.

Methods

Swabs were collected from 23 CRS patients and 11 controls intra-operatively and again at an early and late post-operative time point. Bacterial DNA was extracted from the swabs then sequenced using 16S rDNA bacterial tag-encoded FLX amplicon pyrosequencing.

Results

456 unique bacterial species were detected. No difference was seen for richness or diversity between the study groups ($P > 0.05$). Diversity declined post-operatively in the CRS group ($P = 0.01$). *Propionibacterium acnes* and *Staphylococcus epidermidis* were the most prevalent species. Several significant differences were determined for prevalence and mean relative abundance (MRA) between the study groups. In particular, *Acinetobacter johnsonii* was more prevalent and had a higher MRA in controls. Furthermore the MRA of this species increased post-operatively and was associated with improved quality of life (QOL).

Conclusions

Species richness and diversity were similar between controls and CRS patients. Species diversity declined post-operatively in CRS patients. *S. aureus* MRA was higher in the CRS group compared to controls, but was not associated with worse disease severity. A*cinetobacter johnsonii* was prevalent and abundant in controls and associated with improved QOL.

3.2 Introduction

Chronic rhinosinusitis (CRS) is a multifactorial disease which has a significant impact on quality of life.[14] In an effort to better understand the etiopathogenesis of this disease researchers have given much attention to the microbial constituents of the sinonasal cavity and the interactions occurring amongst these members and the host.

Until recently, many studies examining the bacteriology of CRS patients have relied on standard culture techniques.[82,122,218] Others have looked towards microscopy methods such as fluorescence in situ hybridization (FISH), particularly those studying biofilms.[91,127] These techniques have provided researchers with important insights into the microbial residents of the sinonasal cavity. Unfortunately these techniques also have important limitations and inherently yield results that may misrepresent the true diversity of organisms present in this niche. This is due to a dependence on a microorganism's growth characteristics during culture; or in the case of FISH, an inability to probe for multiple species simultaneously.

The emergence of culture independent techniques such as 16S rDNA sequencing have given researchers the ability to study the microbiome with a high level of sensitivity whilst bypassing the limitations imposed by standard culture. In CRS, several studies have already attempted to define the microbiome as it relates to bacteria,[72,73,83,133] and fungi.[219] Despite methodological differences, these studies have highlighted a huge diversity of microorganisms, which have previously gone undetected with traditional techniques.

The challenge beyond detection is to understand how perturbations of the microbiome in CRS correlate with the clinical state and to determine if pathogens implicated in the pathogenesis of this disease continue to be supported from a polymicrobial perspective of disease where the functional consequences of the community is potentially as relevant as its individual members.

The aim of this study was to build on early efforts aimed at characterizing the microbiome of both the healthy sinus and that of patients with CRS by determining the richness, diversity, prevalence and relative abundance of the bacteria species detected. Importantly this study distinguishes itself from others by seeking to examine the poorly understood temporal changes occurring in the microbiome after surgery and how these changes impact on subjective and objective patient outcomes.

3.3 Methods

Study design and population

Approval for the study was obtained from this institution's Human Ethics Committee. Consecutive patients were prospectively recruited and included those attending the tertiary referral practice of the senior author (P.J.W.), and The Queen Elizabeth Hospital, Adelaide, Australia.

The disease group included patients with a diagnosis of CRS as set out by the Rhinosinusitis Task Force,[3] who were undergoing primary or revision endoscopic sinus surgery (ESS). These patients were included if the disease severity was sufficient to merit middle meatal antrostomies, fronto-ethmoidectomies and sphenoidectomies (i.e. a "full-house" ESS). A number of revision patients with extensive, severe disease also received a Draf-III/frontal drillout procedure and/or canine fossa trephination of the maxillary sinus. Patients were excluded from the study if they were < 18 years, immunocompromised or had a primary mucociliary impairment. The use of antibiotics, antifungals and oral or intranasal corticosteroids in the 2 weeks prior to surgery were also grounds for exclusion.

The control group included patients undergoing endoscopic transphenoidal resections of pituitary adenomas and 1 patient who underwent endoscopic sinus surgery for the removal of an inverting papilloma isolated to one sinus with the control swab taken from the contralateral normal side. All patients in the control group were required to have an absence of previous sinonasal procedures. These patients were also required to demonstrate a disease free state through a lack of sino-nasal symptoms and normal endoscopic examination findings. CT or MRI

scans were also evaluated to confirm disease absence. Finally the control group was also subject to the exclusion criteria outlined for the CRS group.

Demographic data was compiled for age, sex, presence of polyps, previous sinus surgery, asthma and aspirin sensitivity.

Patient assessment

Two subjective measurement tools were utilized in this study to correlate associations between bacteria and quality of life. These were a modified Sino-Nasal Outcome Test 22 (SNOT-22) quality of life index (22 items, each scored from 0 – 3; total score range, 0 – 66)[220] and a sinus visual analogue symptom (VAS) scoring pro forma (summation of six individual symptom scores and an overall symptomatology score; total score range, 0 – 70).[221]

The endoscopic picture was objectively graded with the Lund-Kennedy (LK) scoring system.[221] Scores were obtained from the operative surgeon or a member of the research team. Both were blinded to the microbiological outcomes of the study.

CT scans were scored per the Lund-Mackay (LM) scoring system.[221]

Sample collection

Swabs were collected from the control and CRS groups intra-operatively. Post-operative swabs were also collected from the CRS group at approximately 6 (early post-operative) and 12 weeks (late post-operative). An endoscopically guided flocked swab (Copan Italia S.p.A., Brescia, Italy) was directed to the middle meatus / anterior ethmoid region. Swabs were taken from this region irrespective of the presence of purulence. All swabs were taken from the same site and side of

each patient at all time points and any swabs that were inadvertently contaminated through contact with a non-target region (eg. nasal vestibule) were discarded. Once retrieved, the swab head was immediately separated from the swab shaft and placed into a sterile container. This process ensured that any DNA from other patient surfaces, which may have contaminated the proximal swab shaft during handling by the surgeon, was isolated from the experimental specimen. Samples were immediately placed on ice and then transported to the laboratory for storage at -80^{o} C.

DNA extraction

Swab heads were thawed, then cut into small pieces and placed in 180 µl of enzymatic lysis buffer overnight at room temperature. A 5mm steel bead was then added to each sample and agitated for 20 seconds at 15 Hz. The steel bead was then removed and the process was repeated with 0.1mm glass beads for 5 minutes at 30 Hz. The enzymatic lysis buffer and remainder of the extraction protocol were prepared and performed as per the Qiagen DNeasy Blood & Tissue Kit instructions (Qiagen, CA, USA). Extracted DNA was stored at -80^{o} C until pyrosequencing.

PCR amplification of 16S and pyrosequencing

Tag-encoded FLX-Titanium amplicon pyrosequencing for bacterial organisms was performed as previously described.[222] Briefly, a selective panbacterial 27Fmod (AGRGTTTGATCMTGGCTCAG) and 519Rmodbio (GWATTACCGCGGCKGCTG) primer set was applied against the 16S gene for PCR amplification. PCR and pyrosequencing was performed by MR DNA

(Shallowater, TX). Pyrosequencing was performed on the Roche Titanium 454 sequencer (F. Hoffmann-La Roche Ltd, Basel, Switzerland).

Bioinformatics pipeline

Pyrosequencing data was processed using a proprietary analysis pipeline (MR DNA, Shallowater, TX). Sequences were trimmed of barcodes and primers. Short sequences (< 200bp), sequences with ambiguous base calls and sequences with homopolymer runs exceeding 6bp were removed. Sequences were then denoised and chimeras removed. Operational taxonomic units were defined after removal of singleton sequences and clustering at 3% divergence (97% similarity). OTUs were then taxonomically classified using BLASTn against a curated database (MR DNA, Shallowater, TX) derived from public sources including NCBI, Greengenes, and RDP. The resulting OTU table was used to calculate the Shannon index of diversity using the *alpha_rarefaction.py* workflow script from QIIME[223] using a maximum rarefaction depth of 300.

Statistical analysis

All tests were performed at the taxonomic rank of species.

First, comparisons were made between the control and CRS group at the intra-operative time point. These comparisons included:

a) Richness (number of unique genera per sample): compared using a T-test
b) Diversity (a measure of richness and evenness): Shannon index compared using a T-test.
c) Prevalence (absence vs. presence of a particular species as a binary outcome): compared using Fisher's exact test.

d) Mean relative abundance (MRA) (proportional presence of a species compared to others in the same sample): compared using Metastats,[224] a validated metagenomics tool for detecting differentially abundant taxa between two groups.

Second, for the CRS cohort, temporal changes were examined at the intra-operative, early and late post-operative time points. The microbiological outcomes were as described above (richness, diversity, prevalence, MRA) and were assessed through ANOVA (richness, diversity and relative abundance) and logistic regression (prevalence).

Third, we explored whether the microbiological outcomes impacted on quality of life (QOL) in the CRS group. Intra-operative SNOT-22 and VAS scores were tested against richness, relative abundance (Pearson correlation) and prevalence (logistic regression). These tests were repeated for the LK and LM scores to assess for any impact on objective disease severity. To test for the effect of richness and relative abundance on post-operative QOL scores we used an analysis of covariance (ANCOVA) model. This model was utilized to control for improvements in QOL related to the confounding effect of surgery.

All statistics were performed using the R statistical software (R Foundation for Statistical Computing, Vienna, Austria) and packages from the Scientific Python stack (scipy, numpy, rpy2, pandas, ipython).

3.4 Results

Demographic and clinical data

23 CRS patients and 11 controls met the inclusion and exclusion criteria and were enrolled in the study. From these patients, 34 intra-operative samples, 22 early post-operative samples (*M* = 7.95 weeks, *SD* = 2.73) and 20 late post-operative samples (*M* = 22.26 weeks, *SD* = 12.4) were collected. The remaining characteristics of the CRS cohort are described in Table 3-1.

Table 3-1 Demographics and disease characteristics of the study population

Characteristics	Control group (n=11)	CRS group **(n=23)**
Mean age	45	50
Male/female	4/7	13/10
Nasal polyps (%)	n/a	10 (43)
Revision surgery (%)	n/a	11 (48)
Asthma (%)	1 (9)	9 (39)
Aspirin sensitivity (%)	0	2 (9)
Eosinophilic mucin (%)	0	9 (39)
Mean LM scores*	0	14.5

*Lund MacKay CT score

Richness

456 unique bacterial species were detected in this study.

The mean sample richness was 27.18 and 21.26 in the control and CRS groups respectively and was not significantly different (P = 0.279). Post-operatively sample richness increased substantially to 50.95 at the early post-operative time point before declining to a 19.7 at the late post-operative time point (P = 0.986).

Diversity

The Shannon index was 2.502 and 2.246 in the control and CRS groups respectively and was not significantly different (P = 0.516). This index was also applied to observe for any significant post-operative changes in diversity in the CRS group. Here a significant decline in diversity was seen with a fall from 2.246 intra-operatively to 1.789 and 1.193 at the early and late post-operative time points respectively (P = 0.01).

Prevalence

The 40 most prevalent species detected intra-operatively are shown in Table 2. *Propionibacterium acnes* and *Staphylococcus epidermidis* were the most prevalent species detected (both 88%), followed by *Corynebacterium segmentosum* (71%), *Staphylococcus aureus* (71%) and *Corynebacterium tuberculostearicum* (65%). Several species were significantly more prevalent in the control patients compared to CRS patients: *Acinetobacter johnsonii* (82% vs 26%, P = 0.003), *Corynebacterium confusum* (73% vs 26%, P = 0.023), *Corynebacterium fastidiosum* (64% vs 22%, P = 0.026) and *Tissierella praeacuta* (27% vs 0%, P = 0.028). Prevalence was also evaluated controlling for the presence of species at <1% relative abundance. When this was performed only *Acinetobacter johnsonii* and *Corynebacterium confusum* remained significant (P = 0.005 and P = 0.016 respectively). No species were significantly more prevalent in the CRS group.

The temporal changes in prevalence observed in the CRS group were examined through logistic regression. 15 species were identified with significant temporal changes. However, when the presence of a species at < 1% abundance was controlled only 7 species were identified as having a significant temporal change. Comparing the intra-operative, early and late post-operative prevalence with this abundance threshold, both *Acinetobacter johnsonii* (13%, 86%, 85%, P < 0.001) and *Acinetobacter schindleri* (9%, 9%, 65%, P < 0.001) increased, whilst *Acidovorax facilis* (39%, 23%, 0%, P = 0.007), *Anaerococcus hydrogenalis* (30%, 23%, 0%, P = 0.022), *Corynebacterium segmentosum* (43%, 9% and 0%, P = 0.004), *Peptoniphilus asaccharolyticus* (30%, 5% and 5%, P = 0.03) and *Staphylococcus epidermidis* (78%, 64%, 35%, P = 0.008) all fell.

Abundance

103 species were determined to have a significant difference in MRA when intra-operative samples were compared between the control and CRS group. Surprisingly 102 of these species were rare constituents of the microbiome (MRA <1%). *Staphylococcus aureus* was the only species found to have a significantly higher MRA in the CRS group compared to controls (20% vs. 3% respectively, P = 0.034). *S. aureus* was also the most abundant species in CRS patients followed by *S. epidermidis* (10%), *Propionibacterium acnes* (8%), *Anaerococcus hydrogenalis* (5%) and *Pseudomonas aeruginosa* (4%). In controls, the MRA of *Staphylococcus epidermidis* (9%) and *Propionibacterium acnes* (11%) was also high but not significantly different to the CRS group. The most abundant species in the control group was A*cinetobacter johnsonii* with an MRA of 22%, compared to 4% in the CRS group (P = 0.058). Other abundant species in controls included

Marinilactibacillus psychrotolerans (18%) and *Peptoniphilus asaccharolyticus* (6%). Several species from the genus *Corynebacterium* were also abundant in both controls and CRS patients respectively: *Corynebacterium confusum* (5% vs 4%), *Corynebacterium propinquum* (5% vs 1%) and *Corynebacterium segmentosum* (3% vs 4%). These differences were not significant ($P > 0.05$). *Corynebacterium tuberculostearicum* whilst prevalent in our patients (65%), was a rare constituent of the samples tested with an MRA of <1% in both controls and CRS patients and not significantly different ($P > 0.05$). The MRA for the 40 most prevalent species are presented in Table 3-2.

Analysis of temporal changes in MRA for the CRS cohort showed a significant increase in *Acinetobacter johnsonii* from 4% intra-operatively to 37% at the late post-operative time point ($P < 0.001$) and *Acinetobacter schindleri*, which increased from <1% intra-operatively to 5% at the late post-operative time point ($P < 0.001$). *Anaerococcus hydrogenalis*, *Anaerococcus octavius*, *Corynebacterium segmentosum*, *Peptoniphilus assacharolyticus*, *Propionibacterium acnes* and *Staphylococcus epidermidis* all declined during the post-operative period ($P < 0.05$). Significant changes were identified in a further 12 species, however these were of extremely low MRA (<1%) at all time points. *Staphylococcus aureus* MRA did not change significantly during the post-operative period, but did increase to 38% before declining to 33% at the early and late post-operative time points respectively ($P > 0.05$).

Table 3-2 Prevalence and mean relative abundance (MRA) for the 40 most frequently detected species (intra-operative samples)

	All patients (n=33)	Control (n=11)		CRS (n=23)	
Species	Prev* (%)	Prev (%)	MRA (%)	Prev (%)	MRA (%)
Propionibacterium acnes	88.24	100	10.67	82.61	7.55
Staphylococcus epidermidis	88.24	90.91	8.88	86.96	10.25
Corynebacterium segmentosum	70.59	90.91	2.89	60.87	3.72
Staphylococcus aureus	70.59	81.82	2.61	65.22	20.45
Corynebacterium tuberculostearicum	64.71	63.64	0.72	65.22	0.89
Acidovorax facilis	58.82	45.45	0.48	65.22	2.28
Peptoniphilus asaccharolyticus	58.82	81.82	6.14	47.83	1.84
Anaerococcus hydrogenalis	55.88	45.45	1.44	60.87	4.52
Acinetobacter schindleri	50	72.73	1.52	39.13	0.83
Anaerococcus octavius	50	72.73	2.88	39.13	4.01
Acinetobacter johnsonii	44.12	81.82	22.36	26.09	3.75
Propionibacterium granulosum	44.12	63.64	0.76	34.78	0.37
Corynebacterium confusum	41.18	72.73	5.45	26.09	3.96
Finegoldia magna	38.24	45.45	0.79	34.78	0.65
Corynebacterium fastidiosum	35.29	63.64	0.16	21.74	0.27
Corynebacterium macginleyi	32.35	36.36	0.15	30.43	0.20
Gordonia rubripertinctus	32.35	45.45	0.69	26.09	0.86
Gordonia terrae	32.35	45.45	0.49	26.09	0.52
Marinilactibacillus psychrotolerans	32.35	54.55	18.34	21.74	1.22
Stenotrophomonas maltophilia	29.41	18.18	0.02	34.78	0.63

Sphingobium yanoikuyae	26.47	36.36	0.05	21.74	0.22
Streptococcus pseudopneumoniae	26.47	18.18	0.17	30.43	0.79
Corynebacterium propinquum	23.53	45.45	5.28	13.04	0.58
Escherichia fergusonii	23.53	9.09	< 0.01	30.43	1.8
Fusobacterium nucleatum	23.53	27.27	0.41	21.74	3.28
Neisseria flavescens	23.53	27.27	0.04	21.74	0.96
Staphylococcus lugdunensis	23.53	18.18	0.46	26.09	1.05
Campylobacter bacteroides ureolyticus	20.59	36.36	0.1	13.04	0.06
Corynebacterium accolens	17.65	27.27	0.02	13.04	0.02
Pseudomonas aeruginosa	17.65	9.09	< 0.01	21.74	4.37
Streptococcus oralis	17.65	36.36	0.12	8.7	0.19
Afipia broomeae	14.71	27.27	0.01	8.7	0.01
Alicyclobacillus ferripilum	14.71	27.27	0.37	8.7	0.03
Gemella sanguinis	14.71	0	0	21.74	0.04
Haemophilus parainfluenzae	14.71	18.18	0.02	13.04	0.07
Klebsiella oxytoca	14.71	9.09	0.01	17.39	0.14
Novosphingobium spp.	14.71	18.18	0.07	13.04	0.12
Propionibacterium avidum	14.71	9.09	0.04	17.39	0.07
Acinetobacter calcoaceticus	11.76	18.18	0.09	8.7	0.08
Aggregatibacter segnis	11.76	0	0	17.39	0.08

*Prevalence

Quality of life

Intra-operative comparisons of QOL scores showed no correlation between richness and VAS and SNOT 22 scores ($P > 0.05$).

Logistic regression of prevalence revealed a significant correlation between *Acinetobacter johnsonii* and QOL for both the VAS (coefficient = -0.051, P = 0.005)

and SNOT 22 (coefficient = -0.055, P = 0.004) scores. Similarly *Streptococcus pseudopneumoniae* was also significantly associated with QOL for VAS (coefficient = 0.101, P = 0.035) and SNOT 22 (coefficient = 0.089, P = 0.035).

Pearson correlation of relative abundance and intraoperative QOL scores revealed a significant relationship between 8 species and VAS. 6 were negatively correlated: *Anaerococcus octavius* (r = -0.495, P = 0.023), *Corynebacterium accolens (r* = -0.460, P = 0.036), *Corynebacterium macginleyi* (r = -0.464, P = 0.034), *Gordonia terrae* (r = -0.441, P = 0.045), *Neisseria mucosa* (r = -0.472, P = 0.031), *Propionibacterium granulosum* (r = -0.551, P = 0.01), and 2 species were positively correlated: *Neisseria bacilliformis* (r = 0.48, P = 0.028) and *Streptococcus pseudopneumoniae* (r = 0.503, P = 0.02).

5 species had a correlation between relative abundance and SNOT 22 scores. Of these 3 were negatively correlated: *Chryseobacterium isbiliense* (r = -0.461, P = 0.031), *Corynebacterium accolens* (r = -0.578, P = 0.005), *Corynebacterium macginleyi* (r = -0.621, P = 0.002), and 2 were positively correlated: *Corynebacterium striatum* (r = 0.44, P = 0.04) and *Granulicatella adiacens* (r = 0.475, P = 0.026).

ANCOVA analysis revealed that richness had no significant effect on QOL scores for either VAS or SNOT 22. The effect of relative abundance was also tested with this model. Here the relative abundance of 33 species was significantly correlated with changes in VAS (P < 0.05) and 22 species were significantly correlated with changes in SNOT 22 scores (P < 0.05). The majority of these species were present at extremely low MRA (<1%) and are not reported in full here. Of particular interest was the species *Acinetobacter johnsonii*, which had a

significant reductive effect on QOL scores (improvement) for both the VAS (F = 5.863, P = 0.019) and SNOT 22 (F = 8.207, P = 0.006) scoring systems. *Pseudomonas aeruginosa* was also noted to have a significant effect on SNOT 22 (F = 4.201, P = 0.045). In this case the effect was additive indicating an association with poorer QOL.

Mucosal appearance and CT scores

No correlation was found for LK and LM scores for richness (P > 0.05) in the CRS group. Likewise, logistic regression analysis of bacterial prevalence and both LK and LM scores did not predict for any bacterial outcomes (P > 0.05).

Correlations were sought between bacteria relative abundance and LM scores of which 3 were found to have significant negative correlations. *Alicyclobacillus ferripilum* (*r* = -0.457, P = 0.028), *Burkholderia cepacia* (*r* = -0.466, P = 0.025) and *Erythrobacter citreus* (*r* = -0.466, P = 0.025). All of these species were however present at extremely low MRA (<1%).

7 significant correlations were also found between relative abundance and LK scores. *Klebsiella oxytoca* showed a negative correlation with LK scores (r -0.455, P = 0.029), whilst the remainder: *Capnocytophaga gingivalis* (r = 0.416, P = 0.048), *Neisseria bacilliformis* (r = 0.419, P = 0.046), Neisseria cinerea (r = 0.415, P = 0.049), *Polaribacter Spp.* (r = 0.514, P = 0.012), *Streptococcus agalactiae* (r = 0.499, P = 0.015), *Streptococcus sanguinis* (r = 0.487 P = 0.018) were positively correlated. These species were all once again present at extremely low MRA (≤1%).

3.5 Discussion

This study investigated the bacterial microbiome in the sinuses of CRS patients and controls using 16S rDNA pyrosequencing. We have determined the richness, diversity, prevalence, MRA and post-operative changes occurring in these patients. Significant differences were found for diversity, prevalence and MRA between controls and CRS patients. These findings were accompanied by temporal changes, which highlighted the importance of several species including *Acinetobacter johnsonii*. ANCOVA analysis also showed an important correlation between *Acinetobacter johnsonii* and improved QOL. Mucosal appearance and CT scores were examined and whilst several correlations were found for various species these were all of extremely low MRA (<1%) and of uncertain significance.

Of the bacterial species identified with significant relationships between CRS patients and controls, *Acinetobacter johnsonii* stood out as a microorganism of interest. *Acinetobacter johnsonii* was significantly more prevalent in controls compared to CRS patients (82% vs 26%, P = 0.003), and this relationship remained when the presence of this species at <1% relative abundance was excluded. A*cinetobacter johnsonii* was also the most abundant species in the control group with an MRA of 22%, compared to 4% in the CRS group (P = 0.058). The post-operative increases in prevalence were mirrored by an increase in the MRA of *Acinetobacter johnsonii*. Here a significant increase from 4% intra-operatively to 37% at the late post-operative time point was observed (P < 0.001). The changes seen in *Acinetobacter johnsonii* prevalence and MRA were paralleled by significant QOL correlations for both VAS (P = 0.005) and SNOT 22 (P = 0.004) scores. Importantly, even when controlling for the beneficial effects of surgery on

QOL scores *Acinetobacter johnsonii* continued to be associated with significantly improved QOL scores for both the VAS and SNOT 22 (both $P < 0.05$).

The changes seen for *Acinetobacter johnsonii* highlight a potentially important relationship between this species and health. *Acinetobacter* belongs to the class of Gram-negative bacteria known as *Gammaproteobacteria*. Interestingly members of this class of bacteria were identified by Hanski et al. to be significantly depleted on the skin of atopic individuals.[225] Furthermore the functional role of the *Gammaproteobacteria* was supported in that study by *in vitro* measurements of IL-10 expression, an important anti-inflammatory cytokine involved in immunologic tolerance. Here IL-10 expression was positively correlated with the abundance of the Gammaproteobacterial genus *Acinetobacter* on the skin of healthy, but not atopic individuals.[225] Just as *S. aureus* enterotoxins have been associated with a decrease in the production of T cell regulating cytokines such as IL-10 and TGF-β1 in CRS,[226] factors released by *Acinetobacter johnsonsii* in CRS patients may play a functionally important role by promoting the production IL-10 and its beneficial anti-inflammatory effects. The immunomodulatory properties of this species require further investigation to establish these relationships and whether this species has potential as a probiotic in CRS.

Previous culture-based studies investigating the prevalence of *S. aureus* have found it to be higher in both CRS patients compared to controls, and higher in patients with more severe disease such as those progressing to revision surgery.[206,227] This study identified a similarly high prevalence of *S. aureus* in both controls and CRS patients (82% vs 65% respectively, $P > 0.05$). When the MRA of *S. aureus* was also examined in these two groups an important difference was

identified. Here *S. aureus* MRA was significantly higher in the CRS group compared to controls (20% vs 3%, P = 0.034). These findings draw attention to the sensitivity of molecular techniques in detecting species present at low abundance, which may otherwise be missed by culture techniques and support the fact that MRA is potentially a more meaningful measure of an microorganism's presence in the sinonasal cavity. Interestingly despite the increase in *S. aureus* abundance in the CRS population, it was not associated with a poorer quality of life or worse objective disease severity such as LM and LK scores in this study. Larger samples sizes may be required to identify these relationships if present.

Corynebacterium tuberculostearicum has recently been implicated as a potential CRS pathogen in another microbiome study. Here the abundance of this species was shown to be increased in CRS patients with a corresponding depletion of lactic acid producing bacteria, which were thought to play a protective role.[74] Several *Corynebacterium* species were identified in this study, which were prevalent including *Corynebacterium tuberculostearicum* in both controls and CRS patients (64 vs 65% respectively). The MRA of this species was however extremely low (<1%) in both patient groups and was not associated with any significant changes post-operatively or correlated with any of the subjective or objective measures of disease severity. Whilst this study does not support the role of this species as a pathogen, future studies should investigate this association further.

The mean sample richness was higher in controls compared to the CRS group, but did not achieve significance (27.18 vs 21.26 respectively, P > 0.05). This finding was consistent with one other study, which also found no

difference,[133] but contrasted with another where bacterial richness was significantly reduced in CRS patients compared to controls.[74] The reason for changes in richness in CRS remains unclear. Several factors may be involved, which can be described within the framework of ecological theory. This concept provides a useful method for rationalizing changes occurring in the microbiome caused by specific influences on the niche.[47] In the sinus, competition between microbes for finite resources required for survival is likely to influence the overall species richness. The interplay between host immunity and its impact on the composition of the microbiome, which in itself demonstrates immunomodulatory properties, may also play an important role in shaping these changes.[151] Extrinsic forces such as long-term pre-operative antibiotics may also be implicated and have been shown to reduce genus level richness.[133] The effect of antibiotics on richness whilst poorly defined, may represent a potentially significant confounder when examining microbiome data.

Post-operatively sample richness increased substantially to 50.95 before declining to a mean of 19.7 at the early and late post-operative time points respectively ($P > 0.05$). Whilst this finding did not achieve significance, the changes occurring in the early post-operative period are still of interest, and could be indicative of environmental changes which are hospitable to increased species richness. These may include such factors as the presence of blood products from surgery, inflammatory changes and altered cilia function. An expansion of bacterial richness post-operatively may also impact on other kingdoms of life. In this same cohort reduced fungal richness was identified post-operatively.[219] It is plausible that the fungal changes seen in these patients may have been in response to increased competition from the bacterial component of the microbiome and

reflective of important cross-kingdom interactions. Further studies examining these two kingdoms of life concurrently are required.

Species diversity was examined in this study with the Shannon index. Whilst diversity was higher in the control group compared to the CRS group intra-operatively this difference was not significant ($P > 0.05$). Post-operatively a significant decline in diversity was identified on ANOVA ($P = 0.01$). The reason for this post-operative drop in diversity was unclear, but may indicate disruption in the local environment brought about by surgery, as well as the institution of post-operative medical therapies such as antibiotics. Similar to richness, other environmental pressures could also be driving these changes and further research is required to define these relationships.

Many species found to be present at significantly different abundance levels between the control and CRS groups were present at very low abundance levels in this study. Whilst most of these species are unlikely to be of functional significance, the 'keystone-pathogen hypothesis' suggests that certain low abundance species may play a disproportionately important role in shaping the microbiome and its functional contribution to the disease state.[228] In periodontitis, a polymicrobial disease analogous to CRS, *Porphyromonas gingivalis* has been identified to act as a keystone pathogen. In this case *P. gingivalis* at very low abundance levels was shown to promote pathogenic host-polymicrobial interactions through the normally benign oral microbiota, which ultimately resulted in the disruption of host homeostasis.[229] An alternative perspective to that of the keystone pathogen was put forth by Sibley et al. who used a *Drosophila* model of polymicrobial infection to investigate interactions between the important cystic

fibrosis pathogen *P. aeruginosa* and 40 oropharyngeal commensals. Here a number of oropharyngeal flora isolates that were avirulent or beneficial to the fly were shown to have the capacity to synergistically enhance pathogenicity in the presence of *P. aeruginosa*.[85] These studies highlight the complex interactions occurring amongst microbes and their ability to promote the disease state. In CRS further studies investigating these interactions are urgently required to further explore the importance of species of interest detected in microbiome studies.

3.6 Conclusion

This study has brought attention to several important findings relating to the CRS microbiome and is the first to report on post-operative changes. Species richness and diversity appeared to be similar between controls and CRS patients. The post-operative state in CRS patients was characterized by significantly lower species diversity. *S. aureus* MRA had significantly higher abundance in the CRS group compared to controls. *Acinetobacter johnsonii* was more abundant in controls compared to CRS, and our results suggest that it is associated with improvements in QOL post-operatively. As such its potential as a probiotic in CRS should be investigated. Future studies should also investigate the sinonasal microbiome in greater depth and explore host-microbiome as well as microbe-microbe interactions.

Chapter 4: The fungal microbiome in chronic rhinosinusitis: richness, diversity, post-operative changes and patient outcomes

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4.1 Abstract

Introduction

Our understanding of fungi in chronic rhinosinusitis (CRS) has been limited by previously employed detection techniques. This study examines the fungal component of the microbiome in CRS patients and controls using a highly sensitive culture-independent molecular technique. The aims of this study include the characterization of fungal richness, prevalence, abundance, temporal changes and their relationship with patient outcomes.

Methods

Swabs were collected from the sinuses of 23 CRS patients and 11 controls. Collection occurred intra-operatively, and at 6 and 12 weeks post-operatively. DNA was extracted from the swabs and fungal outcomes were determined through 18S rDNA fungal tag-encoded FLX amplicon pyrosequencing.

Results

Fungi were ubiquitous to all patients. A total of 207 fungal genera were detected with a mean sample richness of 8.18 and 12.14 in the control and CRS groups respectively. *Malassezia* was detected in all patients at surgery and was also the most abundant. Post-operatively, fungal richness decreased ($P < 0.05$) and was associated with declines in the prevalence of *Fusarium* and *Neocosmospora* ($P < 0.05$). *Neocosmospora* was also less abundant post-operatively ($P < 0.05$). No correlations were found with quality of life.

Conclusions

This is the first study to use a highly sensitive pyrosequencing technique to reveal the true diversity of fungi in the sinuses of CRS patients and post-operative changes in richness. The presence of *Malassezia*, a genus not previously described in the sinuses is of great interest, and its potential as a disease modifier should see further investigation given its association with atopic disease.

4.2 Introduction

Our understanding of the interactions between pathogens in complex microbial communities remains in its infancy. Animal models of sinusitis have highlighted the impact of cross-kingdom synergy between bacteria and fungus.[92] Studies of chronic rhinosinusitis (CRS) patients have also shown a close association between bacterial and fungal biofilms.[91,128] These findings illustrate the need to consider the presence of specific pathogens in the context of a greater community of microorganisms, and how the interactions between these members may be contributing to health and disease.

Studies by Ponikau et al.[18] and Braun et al.[19] highlighted the ubiquitous nature of fungi in CRS patients. These studies both utilized modified traditional culture techniques to detect the presence of fungi. Culture based methods are inherently limited however, by the selective pressures that nutrient media place on organisms. This potential confounder is further emphasized by estimates that suggest that only 1% to 10% of known microogranisms are cultureable under laboratory conditions[120] and highlights the need to consider alternative methods for their detection.

Recently, culture-independent molecular techniques have given researchers the ability to detect potentially all organisms in an ecological niche. Using these methods several studies have attempted to describe the CRS microbiome as it relates to bacteria.[72,73,83,133] The fungal microbiome however, remains poorly understood and this is reflected by a paucity of literature as it relates to both CRS and other ecological niches of the human body.

The aim of this study was to characterize the fungal microbiome of the sinuses in CRS patients and healthy controls by determining the richness, prevalence, abundance and temporal changes after surgery. Patient outcomes were also examined through objective and subjective means and compared against the fungal state.

4.3 Methods

Study design and population

This prospective study recruited consecutive patients attending the tertiary referral practice of the senior author (P.J.W.), and The Queen Elizabeth Hospital, Adelaide, South Australia, Australia. Approval for the study was obtained from this institution's Human Ethics Committee.

The disease (CRS) group included patients undergoing endoscopic sinus surgery in the form of middle meatal antrostomies, fronto-ethmoidectomies and sphenoidectomies. A number of revision patients with extensive, severe disease also received a Draf-III/frontal drillout procedure and/or canine fossa trephination of the maxillary sinus. All patients were required to satisfy a diagnosis of CRS as set out by the Rhinosinusitis Task Force,[3] and were excluded from the study if they were < 18 years, immunocompromised or had an impairment of mucociliary function. The use of antibiotics, antifungals and oral or intranasal corticosteroids in the 2 weeks prior to surgery were also grounds for exclusion.

The control group consisted of patients undergoing endoscopic transphenoidal resections of pituitary adenomas and 1 patient undergoing endoscopic sinus surgery for the removal of an inverting papilloma isolated to one sinus. In this case swabs were collected from the endoscopically and radiographically normal contralateral side. Exclusion criteria for the control group included those set out for the CRS group plus the presence of any sino-nasal symptoms, a history of previous sino-nasal surgery, sinus disease on CT or MRI and the presence of abnormal intra-operative findings on endoscopy.

Demographic data was compiled for age, sex, presence of polyps, previous sinus surgery, asthma and aspirin sensitivity.

Patient assessment

To correlate any associations between fungi and quality of life, two subjective measurement tools were utilized in this study: a modified Sino-Nasal Outcome Test 22 (SNOT-22) quality of life index (22 items, each scored from 0 – 3; total score range, 0 – 66)[220] and a sinus visual analogue symptom (VAS) scoring pro forma (summation of six individual symptom scores and an overall symptomatology score; total score range, 0 – 70).[221]

Lund-Kennedy (LK) scores were recorded for objective assessment of the sinus mucosa.[221] Scores were obtained from the operative surgeon or a member of the research team (SB). Both were blinded to the microbiological outcomes of the study.

CT scans were scored per the Lund-Mackay (LM) scoring system.[221]

Sample collection

Swabs were collected from the control and CRS groups intra-operatively. Post-operative swabs were also collected from the CRS group at 6 (early post-operative) and 12 weeks (late post-operative). On all occasions a flocked swab (Copan Italia S.p.A., Brescia, Italy) was used under endoscopic guidance and directed to the middle meatus / anterior ethmoid region irrespective of the presence of purulence. Particular care was taken to sample the same site and side of each patient at all time points. Any swabs that were inadvertently contaminated through contact with with a non-target region (eg. nasal vestibule) were discarded. After the sample had been retrieved the swab head was immediately separated

from the swab shaft and placed into a sterile container. This process ensured that any DNA from other patient surfaces, which may have contaminated the proximal swab shaft (eg. surgeon's gloves), was not stored with the specimen. Samples were immediately placed on ice and then transported to the laboratory for storage at -80 degrees Celsius.

DNA extraction

Swab heads were thawed, then cut into small pieces and placed in 180 µl of enzymatic lysis buffer overnight at room temperature. This was followed by a bead beating process with 5mm steel beads for 20 seconds at 15 Hz and then again with 0.1mm glass beads for 5 mins at 30 Hz. The enzymatic lysis buffer and remainder of the extraction protocol were prepared and performed as per the Qiagen DNeasy Blood & Tissue Kit instructions (Qiagen, Valencia, CA). Extracted DNA was stored at -80 degress Celsius until pyrosequencing.

PCR amplification of 18S and pyrosequencing

Tag-encoded FLX-Titanium amplicon pyrosequencing for fungal organisms was performed as previously described.[222] Briefly, a selective panfungal forward funSSUF-TGGAGGGCAAGTCTGGTG SSUFungiR TCGGCATAGTTTATGGTTAAG primer set was applied against the 18S gene for PCR amplification. PCR and pyrosequencing was performed by MR DNA (Shallowater, TX). Pyrosequencing was performed on the Roche Titanium 454 sequencer (F. Hoffmann-La Roche Ltd, Basel, Switzerland).

Bioinformatics pipeline

Pyrosequencing data was processed using a proprietary analysis pipeline. Sequences were depleted of barcodes and primers. Short sequences (< 200bp),

sequences with ambiguous base calls and sequences with homopolymer runs exceeding 6bp were removed. Sequences were then denoised and chimeras removed. Operational taxonomic units (OTUs) were defined after removal of singleton sequences and clustering at 1% divergence (99% similarity). OTUs were then taxonomically classified using BLASTn against a curated database derived from public sources including NCBI, Greengenes, and RDP (MR DNA version 02012013) database.

Statistical analysis

All tests were performed at the taxonomic rank of genus.

First, comparisons were made between the control and CRS group at the intra-operative time point. These comparisons included:

e) Richness (number of unique genera per sample): compared using a T-test

f) Prevalence (absence vs. presence of a particular genus as a binary outcome): compared using Fisher's exact test.

g) Mean relative abundance (MRA) (proportional presence of a genus compared to others in the same sample): compared using Metastats,[224] a validated metagenomics tool for detecting differentially abundant taxa between two groups.

Second, for the CRS cohort, temporal changes were examined at the intra-operative, early and late post-operative time points. The microbiological outcomes were as described above (richness, prevalence, MRA) and were assessed through ANOVA (richness and relative abundance) and logistic regression (prevalence).

Third, we explored whether the microbiological outcomes impacted on quality of life (QOL) in the intra-operative CRS group. SNOT-22 and VAS scores were tested against richness, relative abundance (Pearson correlation) and prevalence (logistic regression). These tests were repeated for the LK and LM scores to assess for any fungal impact on objective disease severity.

To test for the effect of richness and relative abundance on post-operative QOL scores we used an analysis of covariance (ANCOVA) model. This model was utilized to control for improvements in QOL related to the confounding effect of surgery.

All statistics were performed using the R statistical software (R Foundation for Statistical Computing, Vienna, Austria) and packages from the Scientific Python stack (scipy, numpy, rpy2, pandas, ipython).

4.4 Results

Demographic and clinical data

23 CRS patients and 11 controls met the inclusion and exclusion criteria and were enrolled in the study. From these patients, 34 intra-operative samples, 22 early post-operative samples (*M* = 7.95 weeks, *SD* = 2.73) and 20 late post-operative samples were collected (*M* = 22.26 weeks, *SD* = 12.4). Of these 76 samples, 4 failed to amplify and were excluded on the basis of being below detection. Importantly, fungi were detected in other samples processed for these patients. One patient was lost to follow up at 6 weeks and another two at 12 weeks. Further characteristics of the CRS cohort are described in Table 4-1.

Table 4-1 Demographics and disease characteristics of the study population

Characteristics	Control group (n=11)	CRS group (n=23)
Mean age	45	50
Male/female	4/7	13/10
Nasal polyps (%)	n/a	10 (43)
Revision surgery (%)	n/a	11 (48)
Asthma (%)	1 (9)	9 (39)
Aspirin sensitivity (%)	0	2 (9)
Eosinophilic mucin (%)	0	9 (39)
Mean LM scores*	0	14.5

*Lund MacKay CT score

Richness

A total of 207 unique fungal genera were detected.

The mean sample richness was 8.18 and 12.14 in the control and CRS groups respectively and was not significantly different (P = 0.103). Sample richness was however significantly decreased post-operatively in CRS patients with falls from 12.14 intra-operatively to 6.9 and 6.74 at 6 and 12 weeks respectively (P = 0.007).

Prevalence

The 40 most prevalent genera are shown in

Table 4-2. Of these, *Malassezia* was the most prevalent genus and detected in all patients. Other prevalent genera included *Calicium*, *Neocosmospora*, *Fusarium*, *Saccharomyces* and *Aspergillus*. *Scutellospora* was the only genus found to be significantly different between controls and CRS patients (36% vs. 5% respectively, P = 0.033). No other significant differences in prevalence between the other genera were detected. Prevalence was also evaluated with an abundance threshold as it was felt that extremely low abundance genera (<1%) could be skewing prevalence results. When prevalence was compared on this basis no significant difference was determined for all genera, including *Scutellospora*.

Temporal changes in prevalence were also examined for the CRS group. Significant changes were detected for *Dendryphiella*, *Fusarium*, *Malassezia*, *Mortierella*, *Neocosmospora* and *Pleistophora* (P < 0.05). However when the presence of extremely low abundance genera (<1%) were excluded, only *Fusarium* and *Neocosmospora* remained significant, with falls in prevalence of both.

Abundance

Malassezia was the most abundant genus in both control and CRS patients with MRA scores of 57.5% and 50.09% respectively. *Neocosmospora* was also abundant in controls (11.42%) and CRS patients (6.22%). Whilst *Calicium* and *Fusarium* were both prevalent species, the MRA of these genera was low in both controls and CRS patients (0.47% vs 0.29% and 1.08% vs 2.18% respectively). The MRA for the 40 most prevalent genera are presented in Table 4-2. Notably the

MRA of genera traditionally associated with disease in CRS such as *Alternaria* and *Aspergillus* was low.

When the MRA was compared between controls and CRS patients a significant difference was detected for 33 genera ($P < 0.05$). However, all of these genera were poorly represented in control samples (mean relative abundance <1%) and only 3 genera represented at levels of >1% were found in the CRS group (*Chaeotomium* – 2.58%, *Mortierella* – 2.37% and *Trichocladium* – 1.92%).

The post-operative changes in MRA for CRS patients showed a significant decline for *Basidiobolus*, and increase for both *Cyberlindera* and *Dendryphiella* ($P < 0.05$). However, the mean relative abundance at all time points was low for these genera (<1%, <2%, <1% respectively). The genus *Neocosmospora* was also shown to decrease post operatively and fell from an MRA of 6.22% intra-operatively to 1.41% and 1.59% in the early and late post-operative periods respectively ($P < 0.05$).

Table 4-2 Prevalence and mean relative abundance (MRA) for the 40 most frequently detected genera (intra-operative samples)

	All patients (n=33)	**Control** (n=11)		**CRS** (n=22)*	
Genus	Prevalence (%)	Prevalence (%)	MRA (%)	Prevalence (%)	MRA (%)
Malassezia	100	100	57.5	100	50.09
Calicium	54.55	45.45	0.47	59.09	0.29
Neocosmospora	48.48	27.27	11.42	59.09	6.22
Fusarium	39.39	27.27	1.08	45.45	2.18
Saccharomyces	36.36	18.18	0.12	45.45	1.11
Aspergillus	33.33	27.27	0.85	36.36	1.56
Cladosporium	33.33	36.36	3.23	31.82	3.28
Basidiobolus	27.27	18.18	7.57	31.82	0.57
Mortierella	24.24	9.09	0.02	31.82	2.37
Penicillium	24.24	9.09	0.01	31.82	0.98
Ceratostomella	21.21	9.09	0.03	27.27	0.13
Juncigena	21.21	27.27	0.25	18.18	0.07
Powellomyces	21.21	27.27	0.34	18.18	0.07
Glomales	18.18	18.18	2.62	18.18	0.55
Hypomyces	18.18	18.18	0.02	18.18	0.09
Rhizophydium	18.18	18.18	0.39	18.18	0.91
Endogone	15.15	0	0	22.73	0.35
Glomeromycota	15.15	0	0	22.73	1.43
Alternaria	15.15	18.18	0.3	13.64	2.42
Eutypa	15.15	18.18	0.47	13.64	0.10
Galactomyces	15.15	18.18	9.52	13.64	0.01
Glomus	15.15	18.18	0.27	13.64	1.76
Pleospora	15.15	18.18	0.13	13.64	3.96
Scutellospora	15.15	36.36	0.22	4.55	0.03

Thermoascus	12.12	0	0	18.18	0.12
Ambispora	12.12	9.09	0.01	13.64	1.35
Candida	12.12	9.09	0.01	13.64	3.72
Davidiella	12.12	9.09	< 0.01	13.64	0.06
Lecanora	12.12	9.09	0.01	13.64	0.2
Lepraria	9.09	0	0	13.64	0.02
Sclerotium	9.09	0	0	13.64	< 0.01
Chytridium	9.09	9.09	0.79	9.09	0.18
Funneliformis	9.09	9.09	0.00	9.09	0.15
Paraglomus	9.09	9.09	0.11	9.09	0.03
Rhodotorula	9.09	9.09	0.00	9.09	0.08
Phacellium	9.09	18.18	0.06	4.55	0.01
Capnobotryella	6.06	0	0	9.09	0.02
Clathrospora	6.06	0	0	9.09	0.04
Cordyceps	6.06	0	0	9.09	0.02
Cosmospora	6.06	0	0	9.09	0.04

*Note: 1 sample failed to amplify in the intra-operative CRS group

Quality of life

Intra-operatively, no correlation was found for richness or prevalence when compared against VAS and SNOT 22 scores ($P > 0.05$). Relative abundance was also tested. *Sclerotium* was found to positively correlate with VAS ($r = 0.468$, $P < 0.05$) and *Ceratostomella* was positively correlated with SNOT 22 ($r = 0.439$, $P < 0.05$). *Hypomyces* was negatively correlated for both VAS ($r = - 0.455$, $P < 0.05$) and SNOT 22 ($r = - 0.467$, $P < 0.05$).

ANCOVA analysis indicated that genera richness had no significant effect on QOL scores ($P > 0.05$). The ANCOVA model also showed that the relative abundance of 14 genera had a significant effect on VAS and/or SNOT 22 scores

(P < 0.05, data not shown) independent of surgery. The majority of these fungal genera were however, present at extremely low MRA (<1%). *Cladosporium* and *Pleistophora* were the only two fungal genera present at higher levels that had a statistically significant reductive effect on QOL scores. Cladosporium VAS (F = 5.06, P = 0.028) and *Pleistophora* SNOT 22 (F = 5.37, P = 0.024).

Mucosal appearance and CT scores

Fungal richness did not correlate with LK scores (P > 0.05) in CRS patients. A Logistic regression analysis of fungal prevalence and LK scores did not predict for any fungal outcomes (P > 0.05). A moderate negative correlation (r = -0.485) between LK scores and MRA was noted for *Neocosmospora* (P = 0.022). No correlation between LK scores and MRA was found for the remaining genera.

A moderate negative correlation (r = -0.449) was found between the relative abundance of *Davidiella* and LM scores (P = 0.036). However, the MRA of this species was extremely low (<1%). No other significant correlation was found for richness or prevalence for LM scores.

4.5 Discussion

This prospective study determined the fungal richness, prevalence, abundance and post-operative changes of the fungal microbiome in the paranasal sinuses for a group of controls and CRS patients. Using a highly sensitive culture-independent technique, no major differences were detected in the fungal microbiome between controls and CRS patients intra-operatively. Temporal changes in the microbiome were also studied in the CRS group and here post-operative declines in richness, and the presence of the genera *Fusarium* and *Neocosmospora* were noted.

The genera *Malassezia* was both a prevalent and abundant genus and is of great interest in this study. *Malassezia* was detected in 100% of controls and CRS patients with an MRA of 57% and 50% respectively. On this basis malassezia was the most prevalent and abundant fungal genera detected in this study. In contrast, culture based studies described by others were unable to detect *Malassezia* in any patients.[18,19] *Malassezia* is well known for being difficult to culture and requires the use of special media due to its lipid dependent nature. These specialized media are not routinely employed in clinical mycology laboratories,[230] and may explain its presence going undetected. In line with the growth requirements of this genus, *Malassezia* is typically found in sebum-rich areas of the body such as the trunk and head and is considered part of the normal cutaneous flora.[230] The presence of this organism in the sinonasal cavity is less clear given its growth needs and could represent ongoing seeding from the nasal vestibule rather than permanent niche colonization.

Malassezia has been described in human cutaneous and systemic diseases including dermatitis, pityriasis versicolor, seborrhoeic dermatitis,

folliculitis, atopic eczema/dermatitis syndrome (AEDS), catheter-related fungaemia, peritonitis and meningitis.[231] AEDS if of particular interest as it represents a chronic inflammatory disorder, which parallels CRS in many ways. The mechanisms promoting the release of Th2 cytokines in eosinophilic CRS are not completely understood. *S. aureus* superantigens have been implicated.[232] Others have shown a Th2 response in the presence of *S. aureus* biofilms, independent of superantigens.[99] Similar to CRS, AEDS patients also demonstrate a *Malassezia*-specific Th2 response,[233] and it is possible that the presence of *Malassezia* in some CRS patients may skew the cytokine profile in a similar way. AEDS patients show increased type I hypersensitivity to *Malassezia* and increased Malassezia-specific IgE.[230] The presence of *Malassezia*-specific IgE in CRS populations is as yet untested and should be investigated. LL-37 (a cathelicidin) expression is implicated in the host response to *Malassezia*. Excessive levels are seen in severe AEDS and are believed to play a role in sustained inflammation and other detrimental effects.[230] This antimicrobial peptide has been studied in CRS and was shown to be elevated in nasal tissue and eosinophilic mucus.[234] LL-37 mRNA has also shown to be elevated to *Aspergillus* and *Alternaria* allergens in a nasal explant model.[108] *Malassezia* driven expression of LL-37 in CRS may be a significant factor contributing to inflammation in these patients.

A total of 207 unique fungal genera were identified in this study. Our 18S ribosomal DNA (rDNA) fungal tag-encoded FLX amplicon pyrosequencing technique allowed us to demonstrate a huge diversity of fungi in both CRS patients and controls. This technique is clearly superior when compared to traditional culture based techniques of detection such as that used by Ponikau et al. and Braun et al. where only 40 and 33 unique fungal genera were identified

respectively.[18,19] An alternative molecular technique, the Ibis T5000, coupling nucleic acid amplification to high-performance electrospray ionization mass spectrometry and base-composition analysis,[235] has also been used in our department. This technique whilst promising for detecting fungal organisms, had a surprisingly low yield with only 4 of 38 CRS patients testing positive for fungi from 2 genera.[72] The presence of fungi in its biofilm phenotype has also been studied in CRS, using fluorescent in situ hybridization techniques. These studies confirmed the presence of fungal biofilms in some patients, but due to the use of pan-fungal probes, were unable to define them on a species level.[91,128] Species-specific probes can be used with this technique however this has not been performed due to the need for a prior knowledge of fungi of interest. The large number of unique genera detected in this study highlights the importance of gene sequencing techniques to identify fungi including uncultivable genera of interest, which have not previously been the target of focused detection techniques.

The mean sample richness determined in this study was less in the controls compared to CRS patients, although this was not statistically significant (8.14 vs 12.14 respectively, $P > 0.05$). The precise mechanisms, which shape fungal richness in the sinonasal cavity are not clear, but may be influenced by host and environmental factors. Inhalation of airborne fungi is likely to play an important role in shaping the fungal microbiome in the sinonasal cavity. The use of intra-nasal air samplers for the detection of fungi has confirmed that fungal spores from a wide range of genera are continually inhaled from the surrounding environment into the nasal cavity. Factors such as respiration type, the distribution of fungi in the local microenvironment and the type of activity undertaken by the subject all influence the degree of fungal exposure.[117] This variability in fungal exposure whilst difficult

to quantify, may influence fungal richness independent of any disease process. In this study, post-operative fungal richness was decreased at both the early and late post-operative time points (6.9 and 6.73 respectively, P = 0.007). This decline in richness represents a durable post-operative change in these patients and is likely to have occurred independently of environmental influences – a variable which remained constant in the majority of patients. The impact of surgery on fungal richness may also account for the post-operative changes seen in this study. Initially surgery may disrupt mucociliary function, but this has generally normalized by 6 to 12 weeks.[236,237] The timing of post-operative sampling in this study correlates with the period when improved ciliary function is expected. More efficient clearance of inhaled fungi by cilia, combined with a decreased mucosal disease burden, may contribute towards reduced richness post-operatively. In a similar fashion, saline douches, a routine component of post-operative management may also influence fungal richness. Here the irrigating action may serve to assist clearance of fungi from the sinonasal cavity.

Traditional culture studies of CRS patients have typically identified several prevalent fungal genera including *Aspergillus*, *Alternaria*, *Cladosporium* and *Penicillium*.[18,19,118] These genera were also well represented in samples collected in this study (Table 2.). In addition to *Malassezia*, many other prevalent species were identified in our CRS patients including: Calicium (59%), *Neocosmospora* (59%), *Fusarium* (45%) and *Saccharomyces* (45%). Compared to the control group, *Scutellospora* was the only genus found to be significantly different in controls compared to CRS patients (36% vs. 5% respectively, P = 0.033). The relevance of this finding is uncertain given that this genus had an extremely low MRA (<1%). Given the large number of unique genera detected in this study, it

was surprising that more differences were not noted between the two groups. This sameness in prevalence may be a reflection of similar environmental exposures to airborne fungi in these patients, but also needs to be considered in the context of MRA.

Temporal changes in prevalence were also examined. *Fusarium* and *Neocosmospora* were the only genera significantly decreased post-operatively in the CRS group, a finding which was present even when controlling for their presence at extremely low abundance (<1%). *Fusarium* fell from 45% in CRS patients intra-operatively to 19% and 0% in the early and late post-operative periods respectively (P = 0.03). In humans, *Fusarium* is associated with skin infections, keratitis, onychomycosis and may cause disseminated disease in immunocompromised patients.[238] The prevalence of *Fusarium* in CRS patients detected with standard cultures ranges from 2% to 16%,[18,19] which is likely to be underrepresented when compared to our molecular technique. *Fusarium* may potentially be an important pathogen in CRS. One recent study examined the beneficial effect of targeted oral antifungal agents in a subgroup of *Fusarium*-positive CRS patients and showed improvements in endoscopic appearance and symptoms.[239] *Fusarium* is recognized as one of the most drug-resistant fungal genera,[238] and this resistance to treatment may have been reflected in other CRS studies in which a response to oral antifungal agents or a reduction in inflammatory markers was lacking.[100,102,104,106] The prevalence of *Neocosmospora* fell from 59% in CRS patients intra-operatively to 10% and 11% at the early and late post-operative periods respectively (P = 0.013). The declines in prevalence of this genus are of interest and parallel significant declines also seen in MRA. *Neocosmospora* has been described in human infections such as keratitis, skin

granulomas and arthritis, although these cases are rare.[240] A conflicting negative correlation was observed between the presence of *Neocosmospora* and LK scores, casting doubt on its role as a pathogen in this ecological niche. Further studies are required to more accurately characterize this genus in CRS.

Correlations between fungal richness or prevalence and QOL were not found for the intra-operative CRS cohort in this study. Significant relationships between relative abundance and QOL were found for *Sclerotium*, *Ceratostomella* and *Hypomyces* ($P < 0.05$). These genera were minor constituents of the microbiome with an MRA <1% in all cases. A growing body of evidence suggests that some low abundance members of the microbiome may be disproportionately physiologically active compared to more abundant members.[241] This makes conclusions regarding rare constituents of the microbiome and their functional importance difficult. Similarly the genera identified as significant by the ANCOVA model, except for *Cladosporium* and *Pleistophora*, were also of low MRA (<1%). These results should be interpreted with caution due to the limitations of the ANCOVA model in this context because the covariates (fungal relative abundance and surgery) are not completely independent. Surgery affects the microbiome itself, in addition to the QOL outcome. These factors highlight the complex dynamics at play in the microbiome and the need for further research to better understand these complex relationships and their impact on the CRS disease state.

4.6 Conclusion

This study uses a highly sensitive pyrosequencing technique to determine the diversity of fungi in controls and CRS patients. Although no major differences were found between the microbiome of these two groups intra-operatively, post-operative declines in richness and the presence of the genera *Fusarium* and *Neocosmospora* were noted. The detection of fungi not previously described in this ecological niche such as *Malassezia* highlights the utility of our detection technique. The association between *Malassezia* and atopic disease, marks this genus as a potential disease-modifying organism in CRS. Further investigation is required to better define the ecological pressures influencing the fungal microbiome and how *Malassezia*, and other taxa of interest contribute towards health or disease.

Chapter 5: Probiotic manipulation of the chronic rhinosinusitis microbiome

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5.1 Abstract

Background

Staphylococcus aureus (SA) is a key pathogenic component of the chronic rhinosinusitis (CRS) microbiome and is associated with increased disease severity and poor post-operative outcomes. Probiotic treatments potentially offer a novel approach to the management of pathogenic bacteria in these recalcitrant patients through supporting a healthy community of commensal species. This study aims to investigate the probiotic properties of *Staphylococcus epidermidis* (SE) against SA in a mouse model of sinusitis.

Methods

20 C57/BL6 mice received intranasal inoculations of phosphate buffered saline (PBS), SE, SA or a combination of SE and SA (SE+SA) for 3 days. Following sacrifice, the mouse snouts were harvested and prepared for histological analysis. Counts of PAS-positive goblet cells were the primary outcome measure.

Results

Goblet cell counts were significantly higher in both the SA and SE+SA groups compared to those receiving PBS or SE alone ($P < 0.05$). However, the SE+SA group demonstrated significantly lower goblet cell counts compared to the SA group ($P < 0.05$). Mice receiving SE alone did not show a significant difference to those receiving PBS ($P > 0.05$). The presence of SA post-inoculation was confirmed by culture in both the SA and SE+SA groups.

Conclusion

This study confirms the probiotic potential of SE against SA in a mouse model of sinusitis. Whilst the interactions that occur between many probiotic species and pathogens are yet to be fully understood, studies such as this support further exploration of ecologically based treatment paradigms for the management of CRS.

5.2 Introduction

The ability to detect and identify prokaryotes in the human body has been greatly aided by the emergence of 16S rRNA gene sequencing techniques.[242] Studies such as those from The Human Microbiome Project Consortium have been able to demonstrate a huge diversity of microorganisms in several regions of the human body.[243] The challenge beyond the detection and identification of organisms however, is to understand how these diverse communities of bacteria interact with the host and one another to promote health or disease.

Many studies have attempted to define the bacterial communities present in CRS patients. This has been achieved through a variety of standard culture, microscopy and molecular techniques.[76] Despite the limitations of some of these detection modalities, *Staphylococcus aureus* (SA) has continued to demonstrate itself as a key pathogenic component of the CRS microbiome. This association is particularly apparent in recalcitrant patients and has been confirmed when present as planktonic bacteria or biofilms.[89,90,133,202,227]

The relationship between SA and disease severity makes it an ideal target for probiotic therapies. Probiotics are defined as live bacteria which when administered in adequate amounts confer a health benefit on the host.[157] The use of these treatments in CRS represent a novel approach aimed at restoring the balance between beneficial commensals and pathogenic species. Whilst the benefit of an oral probiotic has been tested in one CRS study,[192] the role of topically delivered probiotic treatments to the sinonasal cavity remains unclear. Topicalization of probiotic preparations provides an exciting mechanism by which

clinicians can potentially promote and maintain a healthy sinus ecosystem by directly manipulating the microbiome.

The murine model of sinusitis used in this study represents an important tool where the interactions between pathogens and potential probiotics can be assessed whilst maintaining a controlled environment. This model is anatomically well described,[244] and has been validated by others examining the effects of bacterial interactions.[74]

In this study we explore the probiotic properties of *Staphylococcus epidermidis* (SE). SE has been shown to exert a probiotic effect through the secretion of serine protease Esp, which inhibits both biofilm formation and nasal colonization by SA.[196] This study aims to utilise these properties to demonstrate the probiotic role of an Esp-producing strain of SE against SA in a murine model of sinusitis by determining goblet hyperplasia and associated mucous hypersecretion, an important marker of airway inflammation.[245]

5.3 Methods

Study conditions

Ethics approval was obtained from the local Animal Ethics Committee. 20 C57BL/6 mice (Animal Laboratory Services, Adelaide, South Australia) aged 6-8 weeks were obtained. All mice were certified as specific pathogen free and housed per treatment group in individually ventilated micro-isolator cages supplied by an H14 HEPA filtration system (Techniplast, Italy). All procedures including inoculations were performed in a laminar flow change station (Tecniplast, Italy) and mice were only fed sterilized pellets and water, thus preventing microbial contamination.

Inoculant preparation and dosing

To prepare nasal inoculants, stock cultures of SA (ATCC 25923) and SE (Esp-producing strain) were grown overnight on 1% nutrient agar plates made up of nutrient broth powder (Oxoid, Thebarton, South Australia) and 1% bacteragar powder (BD, North Ryde, New South Wales). A single colony was inoculated into nutrient broth and subsequently grown for 16 hrs at 37°C with shaking. Cultures were centrifuged using a Beckman TJ-6 centrifuge (Beckman Coulter, Lane Cove, New South Wales) at 4000rpm for 10 minutes. The pellets were resuspended into phosphate buffered saline (PBS) to a final concentration of $1x10^{11}$ and $2x10^{11}$ CFU/mL for SA and SE respectively

Each of the 4 treatment arms contained 5 mice, which received a daily inoculant dose of either 30ul of either PBS (PBS group), SE at a concentration of $6.7x10^{10}$ CFU/mL suspended in PBS (SE group), SA at a concentration of $3.4x10^{10}$ CFU/mL suspended in PBS (SA group) or a combination of SE and SA at the concentrations $6.7x10^{10}$ and $3.4x10^{10}$ respectively, suspended in PBS (SE+SA

group) for 3 days. Prior to inoculation all mice were sedated with intraperitoneal injections of Ketamine/Xylazine. Intranasal inoculations were administered once daily and divided equally between both sides of the nasal cavity. The inoculant droplet was placed onto the anterior nares with a pipette. This was then drawn into the nasal cavity as the mouse inspired.

Histologic preparation and culture

At the end of the treatment cycle the mice were sacrificed by cervical dislocation. Snouts were harvested and processed with a similar technique to that described previously by Jacob et al.[244] Briefly, a coronal slice was taken 1mm posterior to the eyes after which the overlying skin and mandible was removed. Snouts were fixed whole in 10% neutral buffered formalin for a minimum of 24 hours and were then decalcified in Surgipath Decalcifier II (Leica Microsystems, Germany) for 6 hours. The decalcifying solution was then drained and snouts were rinsed with water. Snouts were sliced at 3, 4 and 5mm intervals from the nasal tip with additional slices taken as required to reveal the target zone (superior turbinelle). Slides were then placed in a cassette and processed to paraffin wax using an 8-hour xylene-free (using isopropyl alcohol as the solvent substitute for xylene) processing cycle on a Leica Peloris (Leica Biosystems, Germany) automated tissue processor. Tissue slices were then embedded on the optimal cut face and sectioned at 5 µm prior to staining with a standard Periodic Acid Schiff (PAS) technique (Figure 5-1).[246]

To confirm the presence of SA in the SE+SA and SA groups, swabs were taken from the posterior choanae immediately after coronal section of the snout using a fine flocked swab (Copan Italia S.p.A., Brescia, Italy), and stored in a liquid

amies medium. Cultures were performed and processed with standard techniques as set out by the Clinical and Laboratory Standards Institute.[205]

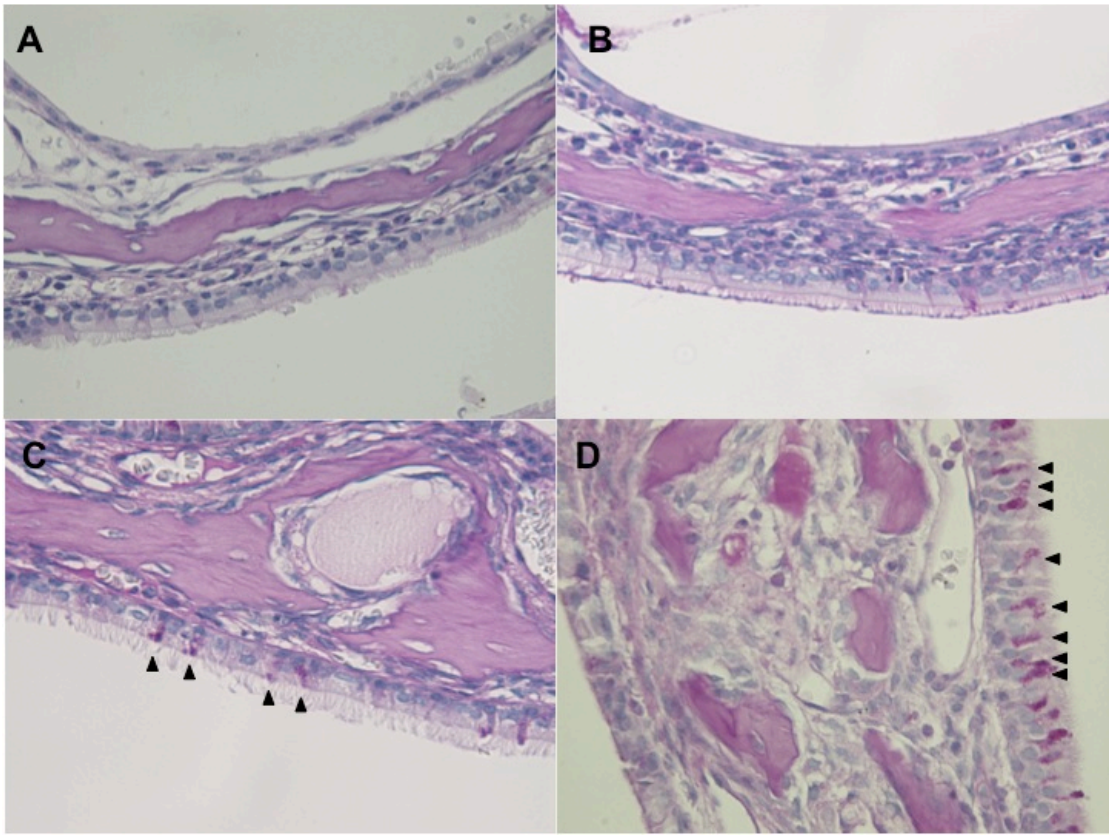

Figure 5-1 Representative slides from the 4 treatment groups. Arrows represent PAS-positive goblet cells. A. PBS group, B. SE group, C. SE+SA group, D. SA group.

Statistical analysis

Slides were deidentified and PAS-positive goblet cell counts were determined at 40x magnification by agreement between two blinded observers. Goblet cell counts were measured from the same histological section of the left and right superior turbinelle for each mouse. A mean score was derived for each mouse from these counts which was then used for the remainder of the statistical analysis. The counts were expressed per 100μm segment of mucosa. A significant

difference between the means of the treatment groups was first examined with one-way ANOVA. Following this, a pairwise comparison was performed with Tukey's honestly significant difference (HSD) test. A P value < 0.05 was considered significant. Confidence intervals (CI) were described at 95%. All calculations were performed using the R statistical software (R Foundation for Statistical Computing, Vienna, Austria) through the IPython notebook (http://ipython.org).

5.4 Results

Of the 20 mice included in the study 19 survived the duration of treatment. 1 mouse in the SA group died at approximately 48 hours after the first inoculation and was excluded from further analysis.

SA was detected by traditional culture in 4 of 5 mice in the SE+SA group and 4 of 4 mice in the SA group, confirming the presence of the pathogenic inoculant in these treatment arms.

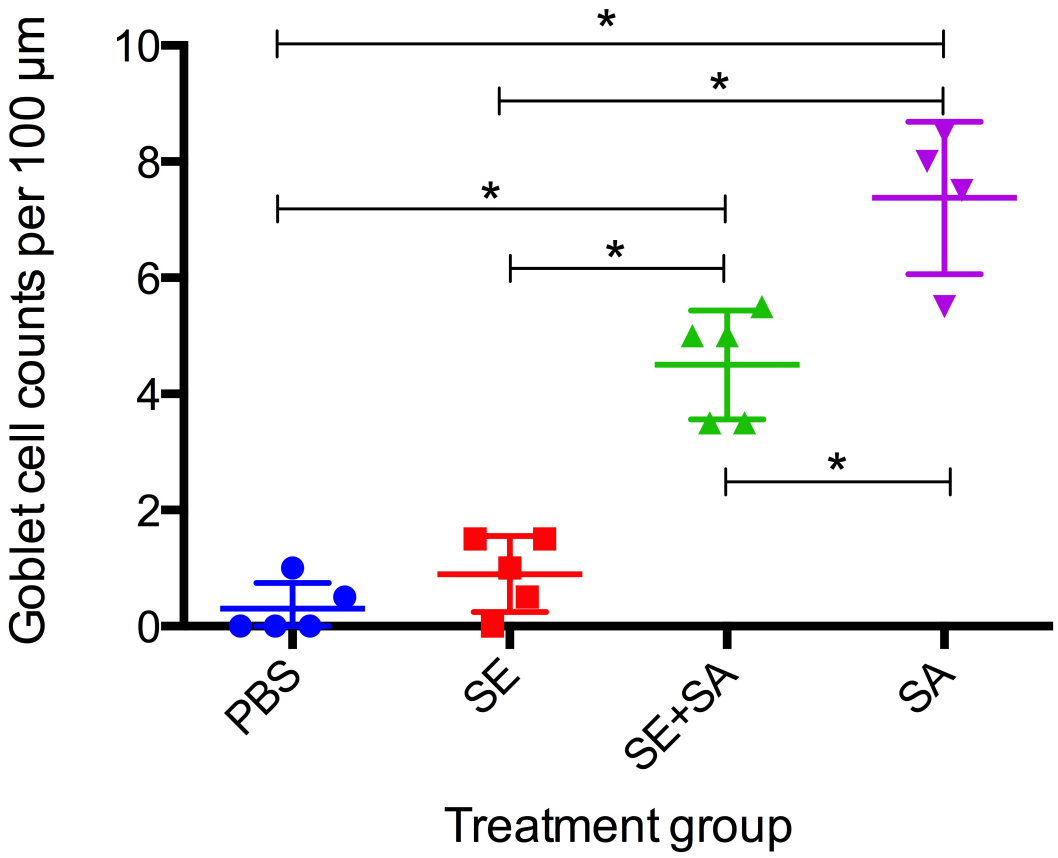

Figure 5-2 Goblet cell counts from the 4 treatment groups. Values represent mean and standard deviation. * P < 0.05.

Goblet cell counts were enumerated from the histological slides and the mean (*M*) and standard deviation (*SD*) values were determined for the treatment

groups: PBS (*M* = 0.3, *SD* = 0.45), SE (*M* = 0.9, *SD* = 0.65), SE+SA (*M* = 4.5, *SD* = 0.94) and SA (*M* = 7.38, *SD* = 1.31) (Figure 5-2). ANOVA (P < 0.001) confirmed a statistically significant difference between the treatment groups. Pairwise comparison (Tukey's HSD) between both the SA and SE+SA groups demonstrated significantly higher mean goblet cell counts per 100μm, compared to: (a) those receiving PBS: 7.38 vs 0.3 (difference in means: 7.08, CI: 5.41 to 8.74) and 4.5 vs 0.3 (difference in means: 4.2, CI: 2.63 to 5.77) respectively (both P < 0.001); and (b) those receiving SE: 7.38 vs 0.9, (difference in means: 6.48, CI: 4.81 to 8.14); and 4.5 vs 0.9 (difference in means: 3.6, CI: 2.03 to 5.17) respectively (both P < 0.001). Importantly, the SE+SA group also demonstrated significantly lower goblet cell counts per 100μm, compared to the SA group: 4.5 vs 7.38 (difference in means: -2.88, CI: -4.54 to -1.21, P = < 0.001). Mice receiving SE alone did not show a significant difference to those receiving PBS (P > 0.05).

5.5 Discussion

This study demonstrates the probiotic potential of SE in a mouse model of sinusitis by showing reduced PAS positive goblet cell counts in a group of mice co-inoculated with SE+SA compared to those receiving SA alone. This finding has implications for the treatment of SA, which is an important pathogen in the paranasal sinuses of recalcitrant CRS patients.

Recent studies sampling the CRS microbiome using culture-independent, molecular methods have highlighted a huge diversity of microbiota in this ecological niche.[72,73,83,133] Stephenson et al. highlighted the presence of anaerobes which were previously underrepresented by studies using standard cultures.[73] Other studies have explored the changes in relative abundance (proportional presence of a species versus other species in a sample) of SA and have found that it is higher in CRS patients compared to controls.[72,133] The metric of relative abundance in this setting adds much weight to the argument that SA is an important pathogen and confirms that comparisons of prevalence alone can be misleading when judging the shifts in microbial presence between patient groups. The major benefit of these studies is that our understanding of the microbial residents in this ecological niche is increasing rapidly, including the functional role of these species in the microbiome and their role as pathogens, commensals or probiotics.

The concept of using ‘good’ bacteria to support homeostasis is not new. In 1907 Nobel laureate Elie Metchnikoff demonstrated a link between the consumption of yoghurt containing lactic acid bacteria and health.[175] More recently van Nood et al. demonstrated the less palatable but efficacious effect of donor

feces infused into the duodenum for the treatment of recurrent *C. difficile* infections by transplanting a healthy bowel microbiome into these patients,[190] highlighting the ability of probiotics to promote health in the setting of a dysregulated microbiome.

The mechanisms by which probiotics exert their beneficial effects are not fully understood. Proposed mechanisms include: improved colonization resistance to pathogens by reinforcing the mucosal barrier, competition with pathogens for binding sites and available substrates, production of antimicrobial components and activation and modulation of the immune system.[175] The route of delivery of probiotic regimens is also likely to be an important factor. Mukerji et al. aimed to alter the intestinal microflora with *Lactobacillus rhamnosis* in an attempt to decrease the systemic immune response to inflammation in CRS patients. Using twice daily oral supplements of *L. rhamnosus* for 4 weeks the authors were unable to show improvements in the quality of life of CRS patients compared to placebo.[192] Another study using an orally ingested probiotic cocktail including *Lactobacillus GG* showed reduced nasal colonization of potentially pathogenic bacteria including SA in a group of healthy patients.[247]

Intranasal inoculations of probiotic candidates have been studied in both human and animal studies. Abreu et al. showed the potential for topical treatments of *Lactobacillus sakeii* to protect against *Corynebacterium tuberculostearicum* infection in a murine model of sinusitis after finding that lactic acid bacteria species were depleted in the sinuses of a group of CRS patients, whilst *C. tuberculostearicum* was increased.[74] *Corynebacteria* may however play an important role in health due to their ability to compete with *S. aureus.* Uehara et al identified a low incidence of *S. aureus* colonization in the nasal vestibule of

carriers of *Corynebacteria.* A subsequent bacterial interference study by these authors using an isolated *Corynebacterium* sp., administered to the nares of humans, was able to eradicate the presence of *S. aureus* in 71% of these patients.[194] Further studies are required to define if *C. tuberculostearicum* also has anti–*S. aureus* properties and what its function is in the context of the CRS microbiome.

The capacity for a species to be either probiotic or pathogenic may also be strain-dependent and the expression of these traits are also likely to be influenced by the prevailing ecological conditions of the niche. This relationship is seen for SE where bacterial interference properties depend on the expression of certain inhibitory factors by specific strains,[196] and highlights the need for caution when attempting to define the role of closely related constituents of the microbiome, where similar functional outcomes cannot be assumed.

SE plays a dualistic role in humans as both a pathogen and an innocuous commensal. SE is often described in foreign body infections such as those involving indwelling catheters and other medical implants,[248] and is a frequent cause of nosocomial infections.[249] These infections are not usually severe however, and this is emphasized by a number of traits which promote the persistence in the body of this organism such as immune evasion molecules, biofilm adhesion and aggregation properties, production of protective exopolymers and the ability to sense antimicrobial peptides.[249] These survival traits are also vital properties in a probiotic species where persistence in the host is required whilst maintaining a competitive advantage over other more virulent pathogenic species.

In the sinonasal cavity, SE has generally been considered as a commensal species. Using a 16S rRNA gene sequencing technique, SE was found in 91% of controls in our own department (unpublished data), a finding comparable to studies using traditional culture techniques.[82] The use of SE as a potential probiotic in CRS is supported by its natural occurrence in this ecological niche in health and the evidence that coagulase-negative *Staphylococci* biofilms when present, are not associated with poor evolution in CRS patients post-operatively.[200] When tested alone in this study, the SE group showed no difference in PAS-positive goblet cell counts compared to the PBS group further highlighting the commensal nature of this species in the nasal cavity.

Iwase et al. confirmed the role of SE as an important nasal commensal by demonstrating its ability to inhibit SA biofilm formation and nasal colonization. The inhibitory factor produced by SE was found to be serine protease Esp, and its properties against SA were confirmed after introduction of an Esp-producing strain of SE into the nasal cavities of healthy volunteers who were SA carriers.[196] Further studies have since shown that serine protease Esp degrades at least 75 proteins, including those associated with biofilm formation and colonization-associated proteins (e.g. extracellular adherence protein, extracellular matrix protein-binding protein, fibronectin-binding protein A, and protein A). Esp was also shown to selectively degrade several human receptor proteins of SA (e.g., fibronectin, fibrinogen, and vitronectin) that are involved in colonization or infection.[198]

The interactions occurring between SE and SA in the sinuses are of great interest. Whilst only some strains of SE produce serine protease Esp, it is possible that alternative mechanisms exist whereby SE may decrease the virulence of SA

infections. Several other species may also inhibit the virulence of SA and provide the same functional outcome in the absence of SE. This functional redundancy,[241] brought about by other species in this ecological niche is of great importance as it could provide an important framework for tailored probiotic therapies where certain species that exhibit a survival advantage are preferentially selected to suit an individual's dysfunctional microbiome.

The post-operative period represents a time of instability in the CRS microbiome. The removal of diseased mucosa during surgery and the resultant impact on ciliary function, which does not return for 6 to 12 weeks,[236,237] creates a state where sinus homeostasis is temporarily impaired. Antibiotics are routinely prescribed post-operatively in an attempt to reduce the risk of infection with pathogenic species. However, these non-selective medications disrupt the entire microbial community, which is unlikely to return to its initial state.[153] The disturbances created by these medical and surgical treatments provide an opportunity for pre-existent community members or new colonists to increase their abundance in the vacated niche.[47] Timely administration of probiotic supplements during these periods is therefore likely to give clinicians the best opportunity to recolonize the sinuses with desirable species which promote the maintenance of health.

5.6 Conclusion

This study confirms the probiotic potential of SE against SA in a mouse model of sinusitis by demonstrating decreased PAS positive goblet cell counts compared to those receiving SA alone. Further research is required to define the competitive relationship between these two species and the niche conditions that promote the beneficial properties of SE. The use of ecologically based treatment paradigms for the management of CRS represent a novel means of managing the host bioburden and its consequent impact on the disease state.

Thesis synopsis

The extensive literature review described in Chapter 1 summarizes the disease entity chronic rhinosinusitis (CRS). CRS is characterized by a number of symptoms including nasal obstruction and discharge, which are manifested clinically as sinonasal inflammation. The importance of this disease is highlighted through its impact on quality of life, which is significant yet frequently underestimated. Our ability to classify patients beyond the presence or absence of certain phenotypic traits acknowledges that this disease is multifactorial and likely includes perturbations of both host factors and those related to the microbiome. The persistence of disease in some patients despite maximal medical and surgical treatment highlights important limitations in both our understanding of the aetiopathogenesis of CRS and also our current treatment paradigms. The work contained in this PhD thesis recognizes these deficiencies in our knowledge on both levels and sets out to improve them by investigating the CRS microbiome and the therapeutic implications of manipulating it.

The first step towards defining the CRS microbiome was to determine the microbial constituents of the sinonasal cavity as detected with a traditional culture technique. The literature review highlighted a large number of studies that had used culture-based methods. Unfortunately, many of these studies were subject to significant limitations. Most important of these were the absence of meticulous collection techniques and the presentation of data derived from small cohorts. The study presented in Chapter 2 remedied these deficiencies by examining the culture data from 513 CRS patients undergoing primary and revision surgery. This study, now the largest study of its type in the literature, further distinguished itself from others with the mandatory use of endoscopically guided cultures in all

patients – a technique that minimized contamination from non-target sites. The power of a large cohort and the meticulous collection techniques enabled us to identify a number of important associations between disease severity and the microbial state such as higher rates of positive culture and an increased prevalence of *S. aureus* and *P. aeruginosa* in revision patients, a group with inherently more severe disease. These results were important as culture continues to be widely utilized in clinical practice, despite the emergence of more sensitive techniques for microbe detection. Furthermore, these results provided a reference for comparisons with future studies using novel culture-independent molecular techniques.

The literature review highlighted a paucity of studies using broad based molecular techniques to examine the CRS microbiome. The studies that were identified demonstrated a lack of uniformity in regards to patient selection, sample collection and the molecular techniques employed to ultimately detect the microbial constituents within. To improve upon these deficiencies careful consideration was given to the design of the microbiome studies presented in Chapter 3 and 4. In particular rigorous exclusion criteria were used for both controls and CRS patients. These included confirmation of the absence of disease both endoscopically and radiologically in the control group and the exclusion of patients that had been recently exposed to intranasal steroids or antibiotics pre-operatively. These criteria among others enabled the collection of high quality data whilst minimizing bias.

Another key deficit in our understanding of the CRS microbiome related to the temporal changes occurring in these patients. To address this, intra-operative,

early-postoperative and late post-operative samples were collected from patients in an attempt to understand the impact of surgery on the microbiome. These changes were given further perspective by a number of other objective and subjective measures of disease severity, which were concurrently determined such as grading of mucosal appearance, CT findings and measures of quality of life. By examining these important indicators of disease severity, correlations could be made between several microorganisms and health and disease.

Our investigation of the bacterial component of the microbiome highlighted that species richness and diversity were similar between controls and CRS patients intra-operatively. Post-operatively however, species diversity was noted to significantly decline in CRS patients. The reason and implication of this change was unclear but could have been the result of environmental influences in the post-operative sinus. *S. aureus* mean relative abundance (MRA) was significantly higher in the CRS group compared to controls, but despite previously being identified as a significant pathogen was not associated with worse objective or subjective disease severity. The species of greatest interest identified by this study was the Gram-negative bacteria A*cinetobacter johnsonii*. This species was highly prevalent and abundant in the control group and significantly associated with improved QOL scores post-operatively in CRS patients. This finding is potentially of great significance in the search for novel probiotic species however further work is required to confirm the functional importance of this microorganism in the sinonasal cavity.

The study of the fungal microbiome was the first to be described in the literature using a broad based molecular method to detect this kingdom of life in

the sinonasal cavity. Important insights have now been gained into the presence of fungi in the sinonasal cavity despite it being frequently neglected. Firstly, we were able to highlight the sensitivity of an 18S rDNA pyrosequencing technique compared to modified culture techniques. Using this method we identified a staggering 207 unique fungal genera in this study. This was considerably higher than other studies exploring the presence of fungi in this niche and highlighted the huge diversity of fungi in both CRS patients and controls. Interestingly whilst no significant differences were noted between the presence of fungi in CRS and controls intra-operatively, the genus *Malassezia* was identified in the sinonasal cavity for the first time. This genus was particularly significant due to its prevalence (100%) and high MRA in both controls and CRS patients (57% and 50% respectively). *Malassezia* has been associated with atopic dermatitis, a disease that parallels the pathogenesis of CRS in many ways such as skewing of the Th2 response. Further investigations exploring the immunomodulatory properties of *Malassezia* in CRS are required to confirm the functional importance of this genus.

The findings from our microbiome studies highlighted the presence of perturbations in the communities of bacteria and fungi in the sinonasal cavity between CRS patients and controls. The clinical implication of these findings was the need to explore measures aimed at correcting a dysregulated microbiome. Probiotics represent a novel treatment strategy, which provide a potential alternative to current treatments by utilizing ecological principles to control microbial populations. The high MRA of *S. aureus* identified in our CRS patients and the body of literature linking this species with disease severity and recalcitrance highlighted it as an important target for therapeutic intervention.

Consequently a murine model of sinusitis was employed to explore the interactions between various bacteria and the host, and an Esp-secreting strain of *S. epidermidis* was selected as the candidate probiotic. Esp has been described as a serine protease that inhibits both biofilm formation and nasal colonization by *S. aureus.* By measuring goblet cell hyperplasia, an important measure of airway inflammation, our study confirmed the beneficial properties of *S. epidermidis* by showing decreased PAS positive goblet cell counts in mice receiving intranasal inoculants of *S. aureus* combined with *S. epidermidis* compared to those receiving *S. aureus* alone. These findings have important implications for the viability of ecologically based treatment paradigms which may prove effective for the control of specific pathogenic components of the microbiome in CRS and its consequent impact on patient health.

Despite the knowledge gained from the studies contained in this PhD thesis, our understanding of the CRS and the healthy human sinonasal microbiome remains in its infancy. New technologies utilizing molecular techniques continue to emerge and the cost of utilizing them is improving. The bioinformatics systems that are now being employed to analyze the huge volume of data created by these studies also continues to improve and researchers are beginning to explore microbial relationships with a degree of finesse that was previously impossible. These improvements will undoubtedly see use in future studies and make meaningful contributions to this rapidly growing body of literature.

The changes occurring in the microbiome due to our interventions also requires further investigation. In particular, the impact of antibiotics and their potentially deleterious effects must be quantified. Commonly used medications

such as steroids may also be implicated in these adverse changes and a better understanding of their impact on the microbiome may result in further rationalization of our treatment paradigms.

Perturbations in the microbiome caused by the disease state and our various interventions should also be explored from a metagenomics perspective. Here community genomic information can be utilized to understand such factors as community metabolism and the synergistic relationships that exist between certain species. Studies exploring these relationships will enhance or understanding of the functional importance of various species. In particular, this will improve our ability to determine new probiotic species and the best way to employ them.

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