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THE MICROBIOME AND CHRONIC RHINOSINUSITIS

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Introduction

The upper airways play a critical role in the respiratory system by conditioning and clearing contaminants from the inspired airstream before it accesses the lower respiratory system.[1] Large particulate matter is removed from inhaled air in the anterior naris or nasal vestibule, a relatively dry environment lined by skin-like squamous epithelial cells and containing sebaceous glands and vibrissae. Smaller particulate matter including bacteria and hydrophilic aerosolized compounds are trapped in a flowing mucus blanket covering the sinonasal mucosa deeper in the nasal cavity and sinuses. Sinonasal mucociliary function is a key host defense mechanism that clears the inhaled particulate matter. Characterized by impaired mucociliary clearance (MCC), bacterial colonization may play some role in the initiation or sustenance of the inflammatory process in chronic rhinosinusitis (CRS).[2]

In recent years, growing understanding of the fundamental role of the microbiome in the initiation, adaptation, and function of the human immune system has revolutionized the field of mucosal immunology.[3] While each inflammatory disease can be differentiated by exclusive genetic and biological mechanisms, many inflammatory diseases, including CRS,

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are associated with significant shifts in the resident microbiota from a 'healthy' to a 'diseased' state.[3] The *dysbiosis hypothesis*--alteration of microbial composition associated with perturbation of the local ecological landscape--has been widely suggested as a mechanism involved in CRS pathogenesis. This hypothesis is supported by several studies identifying a healthy local environment with particular "keystone species," or microbes that normally maintain a stable and interactive community.[4-6] Yet, sinus microbiome studies are in their infancy; many findings have not been replicated due to small study cohorts and variable experimental methods. In addition, results across studies are difficult to interpret in aggregate, and observed associations do not establish causality between the presence of certain microbial communities in the airways and the development of CRS.[7] Many of these difficulties are intrinsically related to the broad diagnostic parameters of CRS and lack of a universally appropriate animal model.

High-impact microbiome studies from other organ systems (e.g., gut) have been conceptually applied to the respiratory field.[8] Although seemingly reasonable, nucleic-acid based surveys of airway microbial communities and their proposed role(s) in CRS remain to be addressed using appropriate model systems. Should an etiological of specific community structures hold true, opportunities will arise for novel therapeutic interventions with potential for personalized, microbiome-based treatment strategies. In this review we will discuss major concepts that highlight the complex role of the microbiota in sinus health and disease and explore future directions for study.

Considerations in Sinus Microbiome Investigation

Sampling locations vary between studies, reflecting subtly different microenvironments throughout the upper airway, and making cross-study meta-analyses a challenge.[9] It is clear that the anterior nasal cavity microbiome is distinct from the middle meatus and sphenoethmoid recess in the healthy state,[10] but the most representative single sampling site in the nasal cavity is often argued.[4,11] How can a single site encompass the complexities of the many anatomic niches and account for differences in local immune and disease properties? The middle meatus is often used as a representative sampling site for the deeper sinuses, given:

1. its high agreement in culture comparison studies with the maxillary sinus,[12]
2. its location as a common drainage pathway of the three major (maxillary, anterior ethmoid, and frontal) sinuses, and
3. its accessibility for sampling.[9,13]

In the gastrointestinal (GI) microbiome field, stool is often studied as a convenient single sample that overrepresents the cecal contribution, acknowledging there are likely biogeographical differences between the upper GI tract, duodenum, jejunum, ileum, and large intestine. To address this concern in the context of CRS, we compared 12 sites from 8 subjects with CRS at the time of surgical intervention and found a fair concordance between the middle meatus and underlying sinuses. These data suggested that if one were interested in single site representation that the middle meatus would be a reasonable proxy for the entire upper airway.[14]

In terms of bacterial detection, it is clear that molecular methods are superior to traditional culture-based approaches in CRS, as identification of even the most fastidious of organisms can be achieved with DNA-based detection and classification by variable regions with the 16S rRNA gene.[15-17] Hauser et al. demonstrated that bacterial detection using 16S rRNA gene sequencing allows for greater sensitivity and provides more information on bacterial diversity than standard clinical swab culture in CRS.[16] Though clinical laboratory culture has been the gold standard for decades and offers useful information, these techniques are unique to institutional laboratories and may miss bacteria that are present in disease. However, the true clinical utility of culture-independent molecular techniques remains to be determined. The ability to more accurately detect bacteria that are present may allow for more effective treatment regimens and allow for an improved basis for clinical and laboratory research into CRS. Nevertheless, there are some shortcomings of culture-independent molecular techniques. 16S rRNA gene sequencing measures total or relative abundance of bacterial DNA and does not differentiate between actively growing, dormant, or dead biomass.[18] As with all tests, it is important to be aware of such biases. To better understand *in vivo* bacterial activity, culture-independent approaches must be improved and new innovative techniques should continue to be integrated, for instance, by separating active cells from extracellular DNA and inactive microbial subpopulations.[19,20]

Currently, there are two main gene sequencing approaches used for studying microbial communities:

1. targeted sequencing of specific marker genes (i.e., 16S rRNA gene for bacteria and 18S rRNA or internal transcribed spacer (ITS) regions for fungi) and
2. shotgun sequencing of the metagenome.

16S rRNA gene sequencing is currently the most widely used approach for characterizing bacterial community membership and comparing phylogeny between samples. This method is based on the premise that nine hypervariable regions within the 16S rRNA gene harbor sufficient sequence diversity to differentiate bacterial taxa down to the genus or species level. Flanking these regions are highly conserved sequences across bacteria and archaea that facilitate the use of universal PCR primer sets.[21,22] Though costlier, shotgun sequencing methods are useful for characterizing microbial communities more broadly, including viruses and fungi that have also been implicated in the development of upper airway disease. Shotgun metagenomics, the study of whole-community DNA extracted directly from samples, has increasingly been used in various settings, particularly as sequencing costs decrease and output increases.[23,24] Furthermore, relative to targeted amplicon assays (e.g., 16S rRNA gene sequencing), shotgun metagenomics offers potential for both higher-resolution identification of organisms and the study of microbial communities without introduction of sequencing bias due to unequal amplification of the target gene.[24,25] Moreover, shotgun approaches capture details of the microbial metagenome (i.e., antibiotic resistance, virulence factors) not provided using single marker gene studies.[22,26] Sequencing technologies have undergone rapid advances during the past several years to attempt to resolve biases associated with current methods and to obtain a better balance between data yield, read length, and cost.[22] These efforts have resulted in third generation sequencing technologies (e.g., Oxford Nanopore and PacBio platforms), which are single-molecule and

real-time technologies that reduce amplification bias, as well as short-read length limitations.[22,27,28] Reduction in cost and time presented by these sequencing methods are valuable assets, and certainly future incorporation of new technologies and bioinformatics is expected.

Based on the anatomic location and local disease environment, viruses and fungi have hypothesized interactions with the bacterial community, which have been borne out in prior study.[29] The relative absence of fungal and viral study at the current time may be a simple lag behind the bacterial microbiome research explosion, as the early microbial detection techniques focused primarily on numerically dominant bacteria. Multiple studies have demonstrated the presence of viruses and fungi in CRS.[30-35] Virus replication can result in epithelial damage and increase bacterial mucosal adhesion, whereas fungi may act synergistically with pathogenic bacteria to play a role in the pathogenesis of CRS.[32,36] The precise roles of these organisms in the pathogenesis of CRS and etiological importance remains poorly understood.[32]

Dysbiosis of Sinus Microbiota in CRS

Analysis of the normal state of the microbiome in sinus cavities is crucial, as there is a clear role for commensals in pathogen exclusion and in the modulation of the healthy host-microbial immune response.[37] The deeper nasal cavity and sinuses have unique local microenvironments (pO_2, pH, etc.) and host immune properties.[11,38,39] While Yan et al. recently examined deeper anatomical subsites in healthy human nasal cavities, and Ramakrishnan et al. compared upper airway subsites and sinuses in CRS, there has been no thorough comparison within normal sinus cavities to date, perhaps owing to the requirement of a more invasive approach.[10,14]

In the healthy state, commonly identified bacterial genera from the upper airways include *Staphylococcus, Corynebacterium, Peptoniphilus* and *Propionibacterium*.[2,6,32,36,40,41] Interestingly, total bacterial load present in healthy and diseased sinuses appear to be surprisingly alike across adults. Further, high inter-individual microbiome variation is often observed in healthy controls and CRS patients.[42,43] Many opportunistic pathogens are found at low abundance in healthy sinuses and, therefore, have the potential to create disease after an acute alteration in the stable baseline microbial community (i.e. dysbiosis).[2,32]

Disruption of stable microbiota may contribute to the exacerbation of chronic inflammatory disease in the absence of acute infection.[11,44] Dysbiosis can lead to benign microbial communities becoming pro-inflammatory, invasive or allowing overgrowth of pathogens. There is also growing evidence that dysbiosis of the sinus microbiota is associated with CRS pathogenesis.[45] Human studies have revealed that the CRS microbiome is characterized by loss of diversity compared to healthy controls,[5,43,46] indicating the opportunity for prosperity of pathogens.[47] Results from these and other sequence-based studies have transformed our understanding of the role of microbial community composition and dynamics in CRS pathogenesis.

Disruption of healthy commensal interactions with the local immune system appears to be a critical determinant of CRS progression. Linear discriminant analysis identified the genus *Corynebacterium* as a potential biomarker that was significantly increased in abundance in CRS patients, however this genus was also omnipresent in healthy subjects from other studies.[4,11] Using a murine model challenged with *C. tuberculostearicum* after antibiotic-mediated microbial depletion, Abreu et al. demonstrated goblet cell hyperplasia and mucin hyper-secretion, two important histologic hallmarks of CRS.[48] However, in this study there were only 7 samples from CRS patients, and another study subsequently reported opposing findings that CRS patients with enriched *C. tuberculostearicum* colonization at the time of endoscopic sinus surgery showed improved surgical outcomes.[6,49] Regarding host interaction with local immune system, another group found that nasal lavage samples of microbiota collected from CRS patients stimulated the induction of proinflammatory cytokines such as IL-5 in peripheral leukocytes isolated from healthy controls.[11,50] Together, these data suggest that the CRS state represents an altered ecological landscape interacting with an aberrant immune response. To this concept, a recent cross-sectional study of CRS and non-CRS patients who underwent endoscopic sinus surgery demonstrated a correlation between the loss of bacterial species richness and diversity and the severity of inflammation and tissue eosinophilia.[51] Whether dysbiosis is causative or a result of the disrupted local immune system remains to be determined.

A preponderance of anaerobes has been consistently observed in studies of CRS, which may be explained by:

1. selective pressure of antimicrobial agents enabling anaerobic organisms to flourish,[52] and
2. from the existence of conditions appropriate for anaerobic growth (i.e., sinus hypoxia).[53]

Anaerobic taxa such as *Peptoniphilus, Anaerococcus*, and *Prevotella* have been reported as abundant taxa in multiple CRS studies.[4,42,54-56] Ambient conditions within the sinus cavities may not be hypoxic, especially after endoscopic sinus surgery has opened the cavities. However, expansion of anaerobes in CRS may be indicative of underlying tissue hypoxia, or may suggest that discrete micro-environments within mucus or bacterial biofilms in CRS can also be oxygen limited, allowing anaerobes to thrive .[4,53,57] It is likely that, similar to mucus plugs in the lower airways of individuals with cystic fibrosis, oxygen levels within sinus mucus are dynamic and driven by both host and microbial processes.[58] Whether anaerobic bacteria have an etiological role in CRS disease progression has been only marginally addressed and is an emerging area of research in chronic airway disease.

Microbial Interactions in CRS

Understanding the complexity and dynamics of interspecies and interkingdom relationships represents a major challenge in microbiome research, but has the potential to help clarify effects in several chronic respiratory diseases including CRS.[8] Symbiosis in healthy microbial ecosystems allows for efficient nutrient utilization and results in decreased pathogen colonization.[47] Most microorganisms face a constant battle for resources and there

are diverse mechanisms by which bacterial species can coexist with, or dominate, other organisms competing for the same pool of resources.[59] Understanding of microbial interactions will be crucial in establishing the function of microbial communities in CRS and implementing new therapeutic strategies.

Yan et al. studied the interaction between *S. aureus* and *Corynebacterium* in the healthy human nasal cavity and showed that *Corynebacterium sp.* are involved in both mutualistic and inhibitory interactions with *S. aureus. C. accolens* and *S. aureus* appear to be adapted to each other and mutually promote each other's growth *in vitro*, whereas *C. pseudodiphtheriticum* may interfere with colonization of *S. aureus* and was observed to inhibit *S. aureus* growth *in vitro*[10] Within the nasal cavity, these reciprocal interactions suggest the possibility for niche competition and possible protection against *S. aureus* nasal colonization.

P. aeruginosa is also an important respiratory pathogen, and often carries intrinsic and/or acquired resistance to many classes of antibiotics. Its appearance and recalcitrance in a portion of CRS subjects is an ongoing clinical challenge. Flynn et al. investigated the role of airway mucins as the microbial carbon source in the cystic fibrosis (CF) airway and characterized their potential to stimulate the growth of *Pseudomonas*.[60] Their group demonstrated that co-culture of *P. aeruginosa* with an anaerobic bacterial consortium facilitates robust growth of *P. aeruginosa* using mucins as a sole nutritional carbon source. These data support an ecological role for anaerobes in shaping the landscape of the human airway for progression of chronic disease (e.g., CRS), and proposed a model for the role of anaerobes in disease pathogenesis.[60] In this model, potential pathogens that cannot degrade mucins (e.g. *P. aeruginosa*, *S.aureus*) do not establish an airway infection until mucin-fermenting bacteria (anaerobes) have colonized (Figure 1). Numerous 16S rRNA gene sequencing studies in CRS have demonstrated a previously unrecognized abundance of anaerobes in the disease state.[6,48] Based on this hypothesis, chronic airway disease could develop through a defined series of dependent events:

1. impaired mucus clearance,
2. generation of anaerobic microenvironments,
3. dysbiosis with mucin-fermenting anaerobes,
4. mucin degradation to carbon source nutrients (e.g., short chain fatty acids (SCFAs)), and
5. proliferation of sinus pathogens.

In this context, we preliminarily tested whether there is evidence of mucin fermentation in human CRS by analyzing the presence of SCFAs in the mucus of subjects during acute exacerbations. Using gas chromatography-mass spectrometry, three SCFAs (acetate, propionate, and butyrate) were quantified in human mucus samples collected from 6 controls and 9 CRS patients during acute exacerbation episodes. SCFAs were found at millimolar concentrations in all mucus samples, and at significantly higher concentrations in CRS compared to healthy subjects (Figure 2). Given that SCFAs are predominately derived from bacterial fermentation, this evidence suggests that mucin-fermenting bacteria are able to

generate carbon-source nutrients for pathogenic bacteria in CRS, similar to proposed mechanisms of disease progression in the lower airways.[60] Based on these data, it is intriguing to consider that the growth of canonical airway pathogens (e.g. *S. aureus, P. aeruginosa*) might be inhibited by targeting co-colonizing microbiota that potentiate their growth and virulence.

Developing preclinical models

The study of dysbiosis in human CRS is especially challenging because medical therapies used in disease treatment are likely to affect resident bacterial communities.[45,61,62] Observed alterations in CRS local microbiota in cross-sectional studies have been unable to account for the repeated and prolonged medical therapies that are common in study subjects. Unfortunately, small animals are not universally accepted in CRS as they do not develop upper airway phenotypes (e.g., CF murine models), possibly from absence of submucosal glands,[63] and their small size precludes thorough examination of sinus pathology.[64] Thus, there remains a need for a robust preclinical model of CRS for longitudinal sampling prior to and during disease initiation. Although there are some limitations when applying animal findings to human pathophysiology, preclinical models have played a significant role in the process of understanding CRS pathophysiology.[65-67]

Many different animals (e.g., murine, rabbit, sheep, pigs) have been used to establish acute and chronic sinus inflammation in prior studies. Small animal models used in CRS microbiome research include the murine model of sinusitis described earlier in investigation of *C. tuberculostearicum* as a potential pathogen on the sinus microbiota. By inoculating *C. tuberculostearicum* into the nasal cavity with and without preceding antibiotic treatment, this study showed the capability of *C. tuberculostearicum* to induce a CRS phenotype, particularly in conjunction with a depleted host commensal community. Co-inoculation of *C. tuberculostearicum* with *Lactobacillus sakei*, a putative probiotic, resulted in a reduced abundance of *C. tuberculostearicum*.[48] In addition, mice have been used to understand the dynamics of sinonasal infection and the role of the mucosal microbiome in short- and long-term responses after topical inoculation of human pathogens (e.g. *P. aeruginosa*).[68] Mice are easy to work with in the laboratory and carry many advantages of experimental application that have been extensively documented. However, murine CRS models are limited due to animal size, unclear similarity of commensal microbes to human counterparts, poorly defined ecological properties of stability and resilience, and that mice do not reproduce key aspects of human airway physiology. They do not have true sinuses, for instance, essential for the analysis of the pathophysiological mechanisms of CRS.[69] Furthermore, immune responses in mice are notably different from those in humans.[70] Compared to mice, rat models are much larger, which makes acquiring larger tissue specimens easier and ameliorates the technical limitations of smaller models.[71] However, transgenic rat models useful for CRS are rare, and the cystic fibrosis transmembrane conductance regulator (CFTR) knockout (KO) rat model (*Rattus norvegicus*; SD-CFTRtm1sage) does not develop spontaneous sinusitis.[64]

As an alternative small animal model, the *in vivo* rabbit sinusitis model is established and may be well-suited for studies of therapeutic intervention. The rabbit sinusitis model:

1. can recapitulate histopathological features of sinusitis,
2. is of sufficient size to study spatial and temporal microbial changes, and
3. has been used to explore experimental ostial obstruction and/or microbial inoculation in the development of the disease.[72]

Cho et al. developed a rabbit model of sinusitis by blocking the maxillary sinus ostium for 2 weeks in the absence of infection to create an anaerobic environment with decreased MCC, resulting in the infiltration of sinus epithelium with acute inflammatory cells (neutrophils).[73] When followed for another 12 weeks after removal of ostial obstruction, those rabbits exhibited a chronic inflammatory phenotype at week 14 (Figure 3). In this model, the mucin fermenting anaerobic phyla *Firmicutes* and *Bacteroidetes* dominated at week 2, but were followed by a significant microbial shift to pathogenic Proteobacteria (e.g. *Burkholderiales* and *Pseudomonadales*) during the development of chronic inflammation by week 14. Such a model provides the opportunity to study microbial host interactions with a level of experimental control that is not achievable in mouse or humans, and also permits multiple longitudinal samplings because the nasal cavity is accessible by nasal endoscopy.

Future Directions in CRS Microbiome Research

Standardization in sampling procedures

Many protocols have been utilized and advocated for different reasons. The “best” sampling protocol depends on the question being addressed. Mucus swab of the middle meatus or ethmoid cavity may be the simplest approach for longitudinal study of the sinus microbiome, considering that it can be obtained from a wide range of subjects and does not require invasive procedures. Whether microbes are sampled by swab, brush, or tissue biopsy, sequencing provides a general picture of the composition of the bacterial community, whereas as an accompanying clinically meaningful and functional physiologic approach is still required.

Healthy microbiome patterns

What is the healthy sinus microbiome consortium and what defines “*normal*”? How do healthy microbiota protect against potential pathogens, either passively through niche competition or actively through or metabolic processes or secretion of antimicrobial compound? Are these organisms susceptible to changes that occur in the sinus environment as a result of the CRS disease process, or iatrogenic manipulation? Normality patterns for viruses and fungi still need to be defined in the upper respiratory system.

Further characterization of non-cultivable and/or non-pathogenic bacteria

16S rRNA gene analyses have shown discrete patterns of non-cultivable microorganisms obtained from patients with CRS. However, conventional sequencing methods do not differentiate between actively growing, dormant, or dead biomass, nor do they capture *in situ* activity at the transcriptional and/or protein level. Detailed characterization of CRS-associated microbial communities therefore requires further innovative assessment. As an example, bioorthogonal non-canonical amino acid tagging (BONCAT) can be used to

fluorescently label actively growing bacteria within samples prior to gene sequencing, and has the potential to enhance traditional sequencing methods by characterizing bacterial activity at the protein level.[19] Other methods such as stable isotope probing or single cell transcriptional analyses coupled with *in situ* imaging also carry potential for generating unprecedented insights into the microbial basis of CRS disease progression.[74,75]

Local vs systemic microbial interactions

Future studies may need to address the contributions of both local and systemic microbial communities (i.e., local and GI occupants). New studies including bacteriophage, viral, and fungal contributions to functional host immune processes are eagerly anticipated.

Interventions

Bacterial supplementation and modulation of the microbiota through pre- or pro-biotics and equivalents are opportunities for thoughtful and ethical clinical research. Whether probiotics directly target inflammatory processes within the sino-nasal epithelium or aim to restore normal upper airway microbiota by mucus transfer, novel strategies to address pathogens in CRS are needed.

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Key Points:

1. The *dysbiosis hypothesis* (alteration of microbial composition associated with perturbation of the local ecological landscape) has been widely implicated in CRS.
2. CRS might develop through a defined series of temporally dependent events: Impaired mucus clearance → anaerobic microenvironments → anaerobe proliferation → increased nutrient availability for sinus pathogens.
3. There remains a need for continued CRS research with longitudinal sampling prior to and during disease initiation, and application of robust preclinical models.

Synopsis

Chronic rhinosinusitis (CRS) is defined as persistent inflammation and/or infection of the nasal cavity and paranasal sinuses. Recent advancements in culture-independent molecular techniques have enhanced our understanding of interactions between sinus microbiota and upper airway microenvironment. The *dysbiosis hypothesis*--alteration of microbiota associated with perturbation of the local ecological landscape--has been widely suggested as a mechanism involved in CRS pathogenesis. In this review, the authors discuss concepts that highlight the complex role of the microbiota in health and CRS and emphasize: 1) Considerations in sinus microbiome investigation; 2) dysbiosis of sinus microbiota in CRS; 3) microbial interactions in CRS; and 4) development of preclinical models. The authors conclude with future directions for CRS-associated microbiome research.

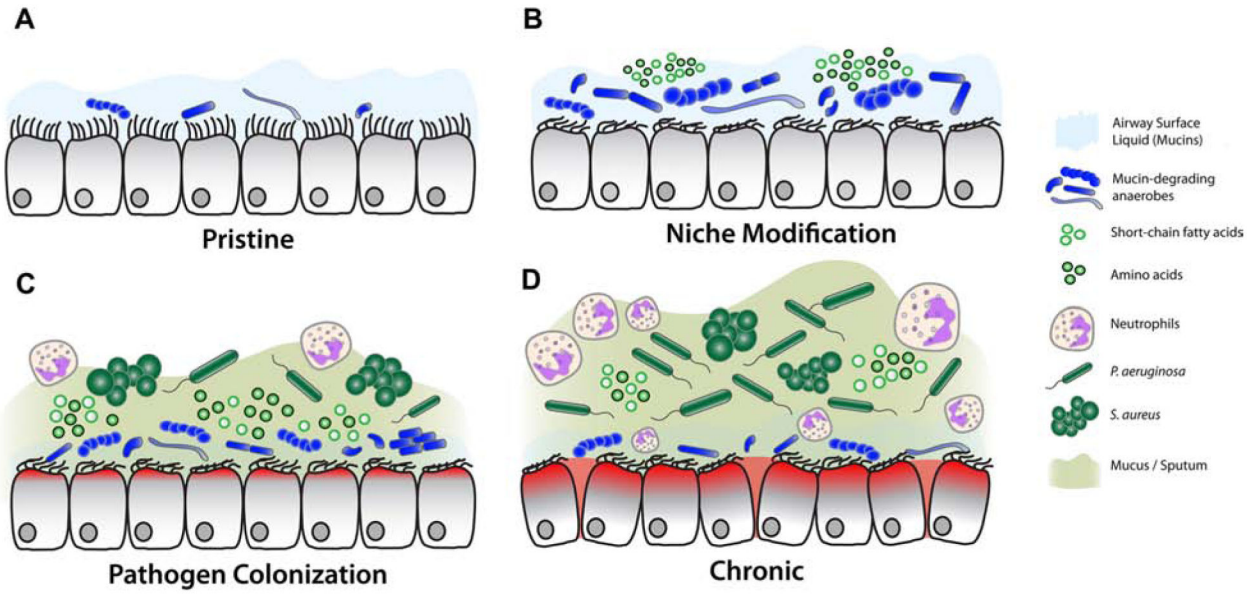

Figure 1. Model for the role of mucin fermenting bacteria in the progression of CF lung disease, as applied to CRS.

(A) In early life, airway surface liquid harbors a low number of bacteria. Numerous factors allow for establishment of personal local microbiota. (B) Local insult resulting in impaired mucociliary clearance and defective immune responses results in hypoxic environment ideal for expansion of anaerobes. In turn, their ability to degrade and ferment respiratory mucins further modifies the airway environment for secondary colonizers. (C) The abundance of fermentation byproducts facilitates pathogen colonization, heightened inflammation, neutrophil recruitment and further hypoxia. (D) In late stages of disease, host inflammatory responses and epithelial damage increases the abundance of pathogens, while healthy commensals are eliminated by the host and via broad spectrum antibiotic therapies.
Data from Flynn JM, Niccum D, Dunitz JM, et al. Evidence and Role for Bacterial Mucin Degradation in Cystic Fibrosis Airway Disease. PLoS Pathog 2016;12(8):e1005846.

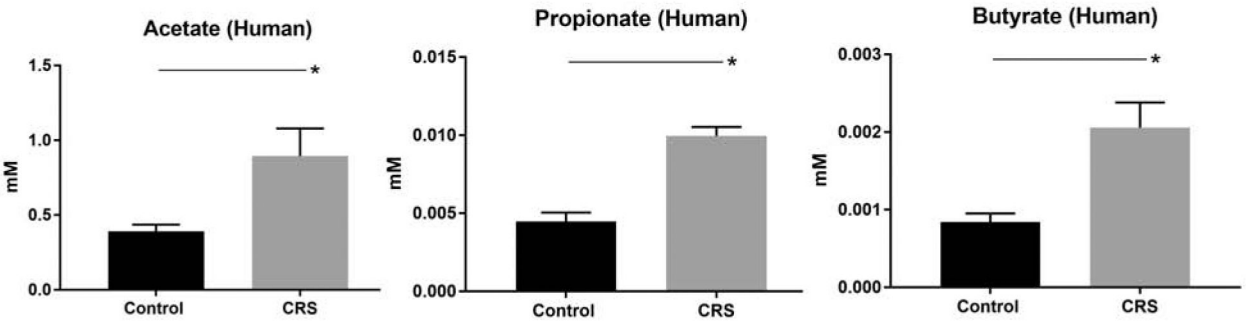

Figure 2. Concentrations of short chain fatty acids (SCFA) in human mucus samples from CRS with acute exacerbation vs healthy controls.

All 3 SCFAs were significantly higher in CRS (n = 9) compared to control (n = 6): 1) acetate = 0.89 +/− 0.19 versus 0.39 +/ 0.04 mM ($p < 0.05$); 2) propionate = 0.01 +/− 0.00 versus 0.0045 +/− 0.00 ($p < 0.0001$); 3) butyrate = 0.002 +/− 0.00 versus 0.0008 +/− 0.00 ($p < 0.01$).

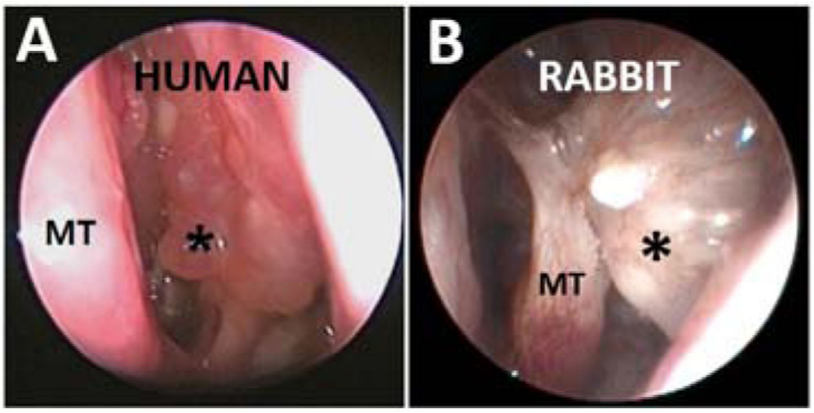

Figure 3. Middle meatus (*) of Human (A) vs Rabbit (B) CRS.
Similar significant polypoid mucosal changes (asterisk). MT: Middle turbinate