Biofilm 6 (2023) 100160

Contents lists available at ScienceDirect

Biofilm

journal homepage: www.sciencedirect.com/journal/biofilm

# Host-microbe interactions in chronic rhinosinusitis biofilms and models for investigation

Emily J. Vanderpool [a,b,c], Kendra P. Rumbaugh [a,b,c,*]

[a] Department of Surgery, Texas Tech University Health Sciences Center, Lubbock, TX, USA
[b] Burn Center of Research Excellence, Texas Tech University Health Sciences Center, Lubbock, TX, USA
[c] Department of Immunology and Molecular Microbiology, Texas Tech University Health Sciences Center, Lubbock, TX, USA

ARTICLE INFO

*Keywords:*
Chronic rhinosinusitis
Biofilm models
*Staphylococcus aureus*
*Pseudomonas aeruginosa*
Host-microbe interactions

ABSTRACT

Chronic rhinosinusitis (CRS) is a debilitating condition characterized by long-lasting inflammation of the paranasal sinuses. It affects a significant portion of the population, causing a considerable burden on individuals and healthcare systems. The pathogenesis of CRS is multifactorial, with bacterial infections playing a crucial role in CRS development and persistence. In recent years, the presence of biofilms has emerged as a key contributor to the chronicity of sinusitis, further complicating treatment and exacerbating symptoms. This review aims to explore the role of biofilms in CRS, focusing on the involvement of the bacterial species *Staphylococcus aureus* and *Pseudomonas aeruginosa*, their interactions in chronic infections, and model systems for studying biofilms in CRS. These species serve as an example of how microbial interplay can influence disease progression and exemplify the need for continued investigation and innovation in CRS research.

## 1. Chronic rhinosinusitis: Overview, prevalence, and impact

Sinusitis, a prevalent condition affecting approximately 10–15% of American adults, presents a significant challenge to both patients and the healthcare system (Fig. 1) [2–6]. The duration of sinusitis can vary, with acute cases resolving within four weeks, while chronic rhinosinusitis (CRS) persists for 12 weeks or more. Managing CRS imposes a substantial economic burden in the US, estimated at $64.5 billion annually [7,8]. This figure, however, fails to capture the full impact of CRS, as it contributes to numerous primary care and otolaryngology or ear, nose, and throat (ENT) clinic visits, work absenteeism, and diminished quality of life for patients [8,10–14].

Diagnosing CRS involves assessing ongoing edema, facial pain or pressure, nasal obstruction, and purulent discharge, with some patients also developing polyps [16]. Polyps are benign, inflammatory outgrowths of tissue, which can extend into the sinus cavity or nasal passages and block drainage pathways such as the osteomeatal complex. Further evaluation of polyps, obstruction, and anatomical abnormalities is carried out using endoscopy or computed topography scans [19]. Subtyping CRS is common, typically based on the presence or absence of polyps, although other classification systems consider inflammatory phenotype or allergic involvement [20,21]. Standardized treatment for CRS includes saline irrigations, topical or systemic steroid administration, biologics, and systemic antibiotic use [3,4]. One report suggests that CRS patients receive 4 or more rounds of systemic antibiotics per calendar year, despite limited evidence of efficacy, rising antibiotic resistance, and off-target effects [22].

Despite initial treatment attempts, a significant proportion of CRS patients experience persistent symptoms. Consequently, many turn to Functional Endoscopic Sinus Surgery (FESS) within a few months of diagnosis [23]. FESS aims to alleviate symptoms by improving nasal patency, correcting anatomical abnormalities, and mechanically clearing diseased tissue and secretions. These procedures are invasive, subject to complications, and require general anesthesia. FESS is estimated to cost patients between $8,500 and $11,000 [24,25], with patients spending up to $26,724 yearly on CRS management [7]. Although FESS provides relief for many patients, approximately 40% still require ongoing medical management two years after surgery, and 10–20% require revision FESS within one year, the higher proportion reflecting patients with polyps [23,26].

Bacterial involvement is a significant factor in the pathogenesis of sinusitis, contributing to the persistence and severity of the condition [27]. Various bacterial species have been implicated in CRS, each with its own set of virulence factors and mechanisms of colonization [28,29].

* Corresponding author. Department of Surgery, Texas Tech University Health Sciences Center, Lubbock, TX, USA.
*E-mail address:* kendra.rumbaugh@ttuhsc.edu (K.P. Rumbaugh).

https://doi.org/10.1016/j.biofilm.2023.100160
Received 3 August 2023; Received in revised form 28 September 2023; Accepted 28 September 2023
Available online 29 September 2023

The ability of these bacteria to form biofilms, structured communities encased in a polymeric matrix, is a common strategy that enhances their survival and tolerance to immune defenses and antimicrobial treatments [30]. Additionally, many bacterial species associated with CRS, including *Staphylococcus aureus* (SA) and *Pseudomonas aeruginosa* (PA), can be antibiotic resistant and induce inflammatory responses, further exacerbating the disease [31,32]. The co-existence and interaction of multiple bacterial species in sinusitis can also complicate the condition and hinder treatment effectiveness.

The management of CRS is a complex, expensive, and multifaceted endeavor, highlighting the challenges in achieving long-term symptom relief for patients. A comprehensive understanding of the underlying factors, including the role of biofilms, is crucial for developing more effective treatment strategies and improving the quality of life for individuals suffering from CRS. By unraveling the complex interplay between bacterial species and their virulence mechanisms, we can gain insights into the pathogenesis of sinusitis to mitigate the impact of bacterial infections in affected individuals.

## 2. Biofilms in chronic rhinosinusitis

Biofilms play a crucial role in the development and persistence of chronic infections by providing a protective environment for bacteria and facilitating their ability to evade immune defenses and antimicrobial treatments. The presence of biofilms has been implicated in CRS pathogenesis, as evidenced by the ineffectiveness of antibiotics, prolonged disease duration, and symptom recurrence [33]. This is underscored by estimates from the NIH that biofilms are involved in up to 80% of chronic infections. Within biofilms, bacteria undergo significant changes in gene expression and behavior, leading to the formation of highly structured and organized communities. These communities enable bacteria to communicate, coordinate their activities, and share resources, enhancing their survival and pathogenicity [34].

The biofilm matrix, composed of extracellular polymeric substances (EPS), acts as a physical barrier that shields bacteria from immune cells and prevents the penetration of antimicrobial agents [35]. This barrier, coupled with reduced metabolic activity and altered physiological characteristics of biofilm-associated bacteria, contributes to increased antibiotic tolerance, making bacteria highly recalcitrant to eradication. Moreover, the presence of biofilms can lead to persistent inflammation, tissue damage, and impaired wound healing, further complicating the treatment of chronic infections [36]. Additionally, biofilms act as a reservoir for infection, as cells can be shed or dispersed from the biofilm itself, potentially seeding new sites of infection [37]. It is important to note that our current understanding of biofilm formation and maturity primarily stems from *in vitro* studies, and ongoing research in the field of microbiology aims to elucidate biofilm dynamics *in vivo*. Understanding the intricate mechanisms by which biofilms contribute to chronic infections is essential for developing targeted approaches to disrupt biofilm formation and improve therapeutic outcomes.

Biofilms can be identified in CRS patients through two distinct approaches [38]. The first method involves traditional bacterial culture, where samples are obtained from patients and cultivated on nutrient-rich media. After isolating individual strains, their

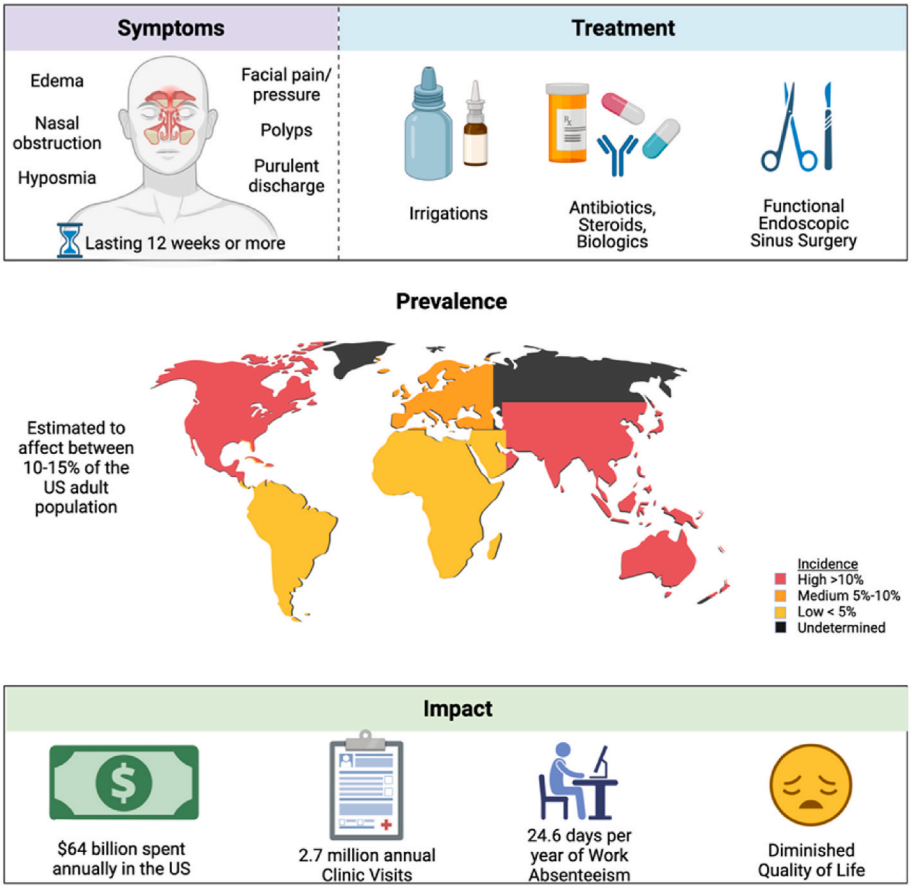


**Fig. 1. Overview of CRS Disease, Incidence, and Cost.** CRS is a chronic inflammatory disorder, requiring patients to meet 4 of the 6 diagnostic criteria listed for 12 weeks or more, along with radiological or endoscopic confirmation. Patients who do not improve with traditional interventions can undergo FESS to alleviate symptoms. The incidence of CRS in the US is estimated between 10 and 15% of the adult population [3], with high prevalence in Asia and Australia, followed by Europe, South America, and Africa [5,6,15]. The economic impact of CRS management is high, including a high volume of visits to primary care, ENT, and emergency clinics [12], but should also consider time away from work, lost productivity [11], and decreased patient quality of life [13,14].

biofilm-forming capacity is assessed *in vitro* using techniques such as the crystal violet assay to measure biomass [39,40]. Thus the ability to form biofilms *in vitro* is used as a proxy for biofilm formation *in situ* [41,42]. The second approach relies on imaging samples from sinusitis patients, considering factors such as the distribution of microorganisms, their proximity, and the presence of extracellular polymeric substances (EPS) to diagnose biofilm-associated sinusitis [17,18,43–47]. Commonly, aggregates of bacterial cells greater than 5 μm in area, as well as aggregates visibly surrounded by EPS, constitute biofilm [9,48,49].

Supporting biofilm involvement in CRS, microscopy studies conducted on clinical samples reveal the presence of bacterial aggregates across sinus and nasal tissues (Fig. 2). Biofilms have been detected in the paranasal sinuses of CRS patients at high rates, reaching up to 80% in some cases [50–52]. Moreover, patients positive for biofilms display higher disease severity scores both before and after FESS [49,53]. Notably, bacterial species collected from CRS patient samples demonstrate a greater propensity for robust biofilm formation compared to species collected from healthy individuals [51]. These findings corroborate the significant association between the presence of biofilms and the severity of CRS, strengthening the argument for their role in this chronic infection.

Antibiotic ineffectiveness in the treatment of sinusitis, despite positive culture results, can also be attributed to the presence of biofilms. Culture-based testing fails to account for the complex nature of biofilms. Biofilms exhibit distinct properties that contribute to their resilience and tolerance to antibiotics [54]. The biofilm matrix acts as a physical barrier, preventing the penetration of antimicrobial agents and reducing their efficacy [35]. Furthermore, within the biofilm, bacteria undergo genetic and phenotypic changes that render them less susceptible to the effects of antibiotics [55]. These adaptive mechanisms, combined with the reduced metabolic activity of biofilm-associated bacteria, make them highly tolerant to antimicrobial treatment and also make biofilm-associated bacteria difficult to quantify [56,57]. Therefore, the presence of biofilms in sinusitis patients may also explain the persistent symptoms and bacterial presence despite antibiotic use, emphasizing the need for alternative strategies that specifically target and disrupt biofilm formation to improve treatment outcomes [36,58].

The presence of biofilm in CRS may also directly contribute to various host-associated pathogenic processes. One hallmark of sinusitis is the presence of purulent secretions, which can be induced by bacterial recognition by Toll-Like Receptors and bacterial effector molecules like PA pyocyanin [59,60]. The protection provided by biofilm allows bacterial cells to persist, triggering the expansion of goblet cells and subsequent increased mucus secretion through signaling of cytokines such as IL-13 and IL-17. Furthermore, samples from CRS patients have shown secondary or acquired ciliary dyskinesia [61], where bacterial exoproducts like PA 1-Hydroxyphenazine or SA beta-toxin disrupt and degrade cilia [62,63]. This disruption of the mucociliary elevator, coupled with increased mucus production, leads to mucostasis [64]. When stagnant mucus accumulates in the nasal and sinus cavities, it creates an ideal microenvironment for pathogenic bacterial expansion and biofilm formation [65]. During mucostasis, thickened mucus that blocks airflow and prevents sinonasal drainage contributes to increasing inflammation, as the immune system works to compensate for this impaired innate defense mechanism [66,67].

Consequently, the presence of biofilm in CRS contributes to an inflammatory feedback loop [36]. Specific inflammatory mediators and immune cells have a higher incidence in CRS tissues but display ineffective immune clearance. Immune cells are unable to effectively phagocytize bacterial aggregates larger than 5 μm, impairing their ability to eliminate the biofilm [68]. Additionally, the proteins and polysaccharides of the biofilm matrix can bind and sequester host antimicrobial defense peptides (AMPs), further compromising the host's defense mechanisms. Bacterial products, such as SA staphylokinase, directly break down AMPs [69]. As a result, the host continues to recruit immune cells to the affected area and secrete inflammatory mediators without effectively reducing the biofilm burden. This is evidenced by the sinonasal infiltration of numerous immune cells, including neutrophils, eosinophils, innate lymphoid cells, and T cells in CRS patients (Fig. 3) [1,42,70]. This immune response to biofilm is not dysfunctional or aberrant in itself, but it is ineffectual against the specific pathogenic defense of biofilm formation [71]. By forming aggregates, biofilm protects its members from immune action or conceals itself to avoid immune surveillance, while in contrast, planktonic bacteria remain accessible to immune surveillance and elimination [72]. Consequently, the immune response to biofilm can be self-perpetuating [36].

Within this feedback loop, the presence of biofilm can also induce the production of TGF-β1, which has been shown to facilitate tissue remodeling and epithelial to mesenchymal transition [73,74]. While some remodeling is a normal part of healing from an inflammatory event, ongoing inflammation leads to pathological structural changes and further disease progression.

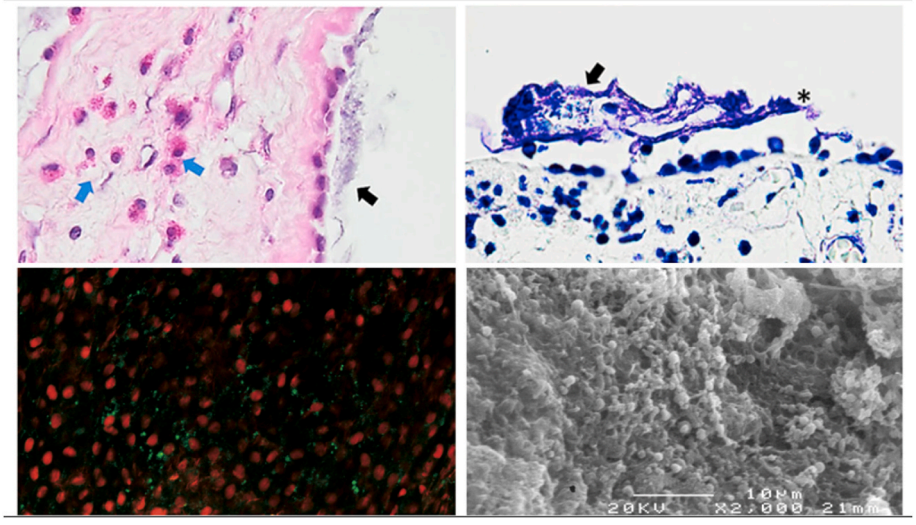


**Fig. 2. Examples of Imaging Techniques to Detect Biofilms from CRS Samples**. A) Biofilm (black arrow) in the mucus secretions of nasal epithelium using hematoxylin and eosin staining [9]. B) Biofilm and matrix (black arrow) in the nasal epithelium, stained with Toluene Blue [9]. C) Confocal Scanning Laser Microscopy Image of CRS patient mucosal sample using a 40X objective. Epithelial cells are the large red structures, and bacterial cells are shown as small green dots [17]. D) Scanning Electron Microscopy image of biofilm overlaying cilia [18].

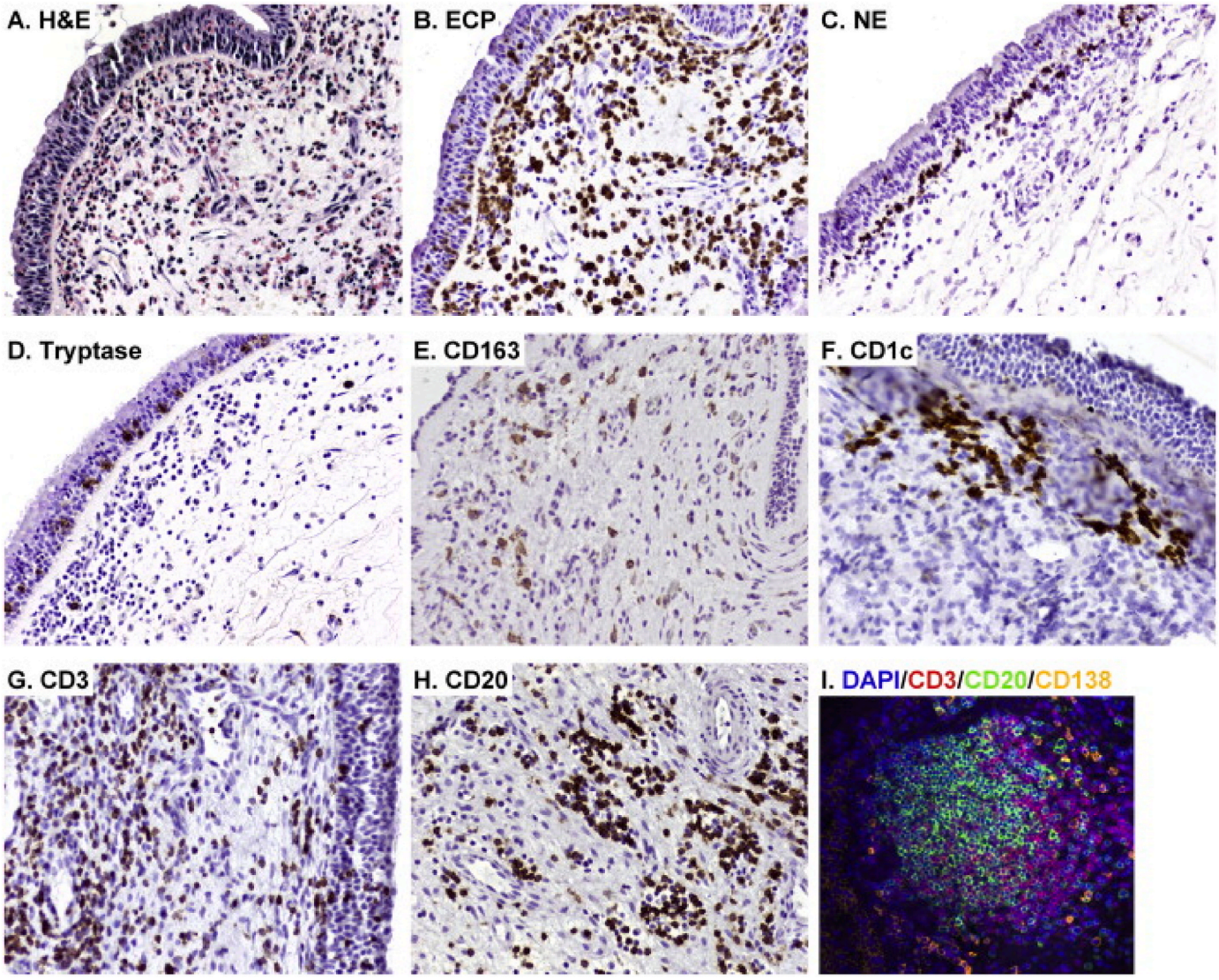


**Fig. 3. Immune Infiltration in CRS.** All images from polyp biopsies. A) Hematoxylin and eosin staining. Immunostaining for B) eosinophils, C) Neutrophil Elastase for neutrophils, D) mast cells, E) M2 macrophages, F) m1DCs, G) T cells, H) B Cells. I) Immunofluorescent staining for T cells (blue), B cells (green), plasma cells (orange), and nuclei counterstained (blue). Image from Ref. [1].

Thus, the presence of biofilms in sinusitis is supported by various lines of evidence [33]. First, the presence of aggregated clusters of bacteria are one of the diagnostic criteria for biofilm infection. Second, biofilms display remarkable tolerance to antibiotic treatment, indicating their protective characteristics. Moreover, the coexistence of bacterial presence and host inflammatory cells provides evidence of ineffective host clearance mechanisms. Therefore, the intricate interplay between biofilms and the immune response amplifies the chronic inflammatory state and contributes to the progressive nature of sinusitis. Biofilms are a general contributor to CRS disease, however, specific bacterial "players" in sinonasal biofilms influence the traits discussed above, and when considering the polymicrobial nature of biofilms, the interactions of these bacterial species add further nuance to the aforementioned pathogenic processes [75].

## 3. Bacterial contributors to CRS

Within the context of biofilms, specific bacterial species have been identified as key contributors to the pathogenesis and severity of sinusitis. Traditional culture commonly identifies aerobic *Moraxella catarrhalis*, facultative anaerobic *Staphylococcus* spp, *Streptococcus* spp, *Haemophilus influenzae, Pseudomonas aeruginosa,* and obligate anaerobic *Peptostreptococcus* and *Prevotella* in samples from CRS patients [28]. However, it is important to acknowledge the limitations of relying solely on culture-based identification methods, as they typically underestimate the presence and diversity of bacterial species involved in sinusitis compared to molecular techniques. This can be attributed to the presence of viable but nonculturable bacteria, or species that are slow-growing, require specific conditions or nutrients, and are therefore difficult to culture [29].

In meta-analyses of clinical studies employing 16S rRNA gene sequencing for bacterial identification, *Corynebacterium* and *Staphylococci* were commonly found in healthy sinonasal microbiomes [76,77]. However, when investigating the specific bacterial species implicated in CRS, studies reported considerable variation in the dominant organisms identified. This variability may stem from divergent sampling methodologies, environmental influences, or seasonal variations. Nevertheless, a consistent finding across these studies is that bacterial abundance was moderately increased in CRS compared to healthy controls. Moreover, these investigations revealed a reduced diversity of bacterial species in CRS, a pattern also observed in other inflammatory conditions such as inflammatory bowel disease [28,77–79].

### 3.1. *Staphylococcus aureus and Pseudomonas aeruginosa*

Within the diverse spectrum of bacterial species associated with sinusitis, SA and PA emerge as significant contributors that have captured considerable attention. Both renowned biofilm-formers, SA and PA rank among the top 5 most prevalent bacterial species in CRS. Notably, they belong to the ESKAPE Pathogens, a classification designated by the Infectious Disease Society of America and corroborated by the World Health Organization for bacterial species associated with high rates of hospital-acquired infections and urgent need for innovative antibiotic strategies due to the persistent challenge of antibiotic resistance [80]. While SA and PA are commonly detected in samples from CRS patients, their precise roles in the pathogenesis of the disease remain poorly elucidated. Therefore, we delve into the potential virulence mechanisms employed by these pathogens within the context of sinusitis, aiming to shed light on their contributions to disease progression (Fig. 4).

SA, a gram-positive bacterium, holds the distinction of being the primary cause of nosocomial infections [81]. SA is responsible for various severe infections, including medical device infections, toxic shock syndrome, and endocarditis. In the context of CRS, SA stands among the top three most frequently cultured organisms, with higher abundance compared to healthy controls [28,82]. Clinical research indicates that SA is carried by 64% of CRS patients, while only 20% of control patients harbored this bacterium [81].

Within the human respiratory tract, SA showcases its formidable capabilities. Equipped with attachment factors such as clumping factors A&B, iron-regulated surface determinant A, and serine-aspartate repeat proteins SdrC and SdrD, SA adheres to the squamous epithelial surface of the anterior nares [83]. Once attached, SA employs a range of secreted factors to facilitate invasion and persistence. Alpha and beta-toxins produced by SA contribute to ciliary impairment and activate the host inflammatory cascade [84]. SA also utilizes enzymes like staphopain and serine proteases, as well as staphylokinase, to degrade innate host defense proteins [85]. Additionally, SA's pore-forming toxins, including the leukocidins, target and destroy host epithelial, endothelial, and immune cells [86]. Moreover, SA's polysaccharide capsule acts as a shield, preventing complement deposition, opsonization, and phagocytosis by neutrophils [87]. SA has an extensive repertoire of iron-acquisition, retention, and anti-toxicity mechanisms [88,89], and can increase production of acidic end products, dropping the microenvironment pH in order to "steal" iron from host proteins like transferrin [90]. Iron-related virulence determinants are critical for SA survival and persistence in the iron-limited host milieu.

Investigations into SA biofilm-associated medical device implant infections reveal that SA virulence factors and metabolites modulate immunometabolism in macrophages, favoring persistence rather than bacterial clearance [91]. SA biofilms also employ Panton-Valentine leukocidins to induce the release of neutrophil extracellular traps (NETs). Although NETs possess the capacity to kill planktonic cells, SA biofilms use nucleases to break down the NETs. This is a clever strategy, as NET release leads to neutrophil death, and the breakdown of NETs generates deoxyadenosine, which induces apoptosis in macrophages [92,93]. These and other virulence factors of SA contribute to the establishment and persistence of infection, fostering severe upper airway inflammation.

Likewise, PA is a widely recognized human pathogen capable of infecting various tissue types. As a gram-negative bacterium, PA is

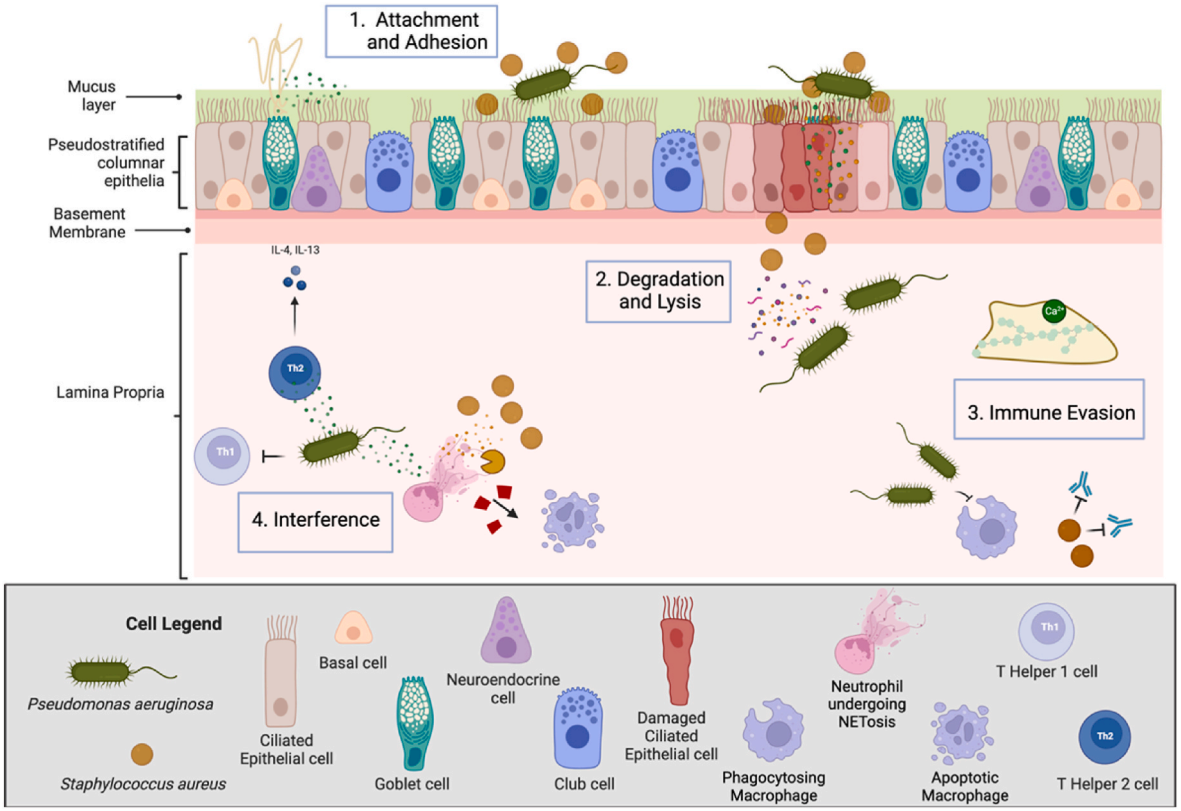


**Fig. 4. Virulence of SA and PA in Upper Respiratory Tract Infections.** SA and PA impact upper respiratory health through 4 main processes: 1.) Attachment and Adhesion, 2.) Degradation and Lysis, 3.) Immune Evasion, and 4.) Interference. SA and PA utilize various adhesins including Clumping Factors A&B, Lectins, and the protein cap of PA flagella to attach to cilia and sinonasal epithelial cells. Exotoxins of SA and PA break down host antimicrobial peptides, disrupt barrier integrity, and cause damage to host cells. PA utilizes increased production of alginate, as well as elastase to evade phagocytosis, while SA uses the polysaccharide capsule to similarly evade opsonization. Finally, SA and PA interfere with Neutrophil Extracellular Trap release and alter cytokine signaling via nucleases, elastases, pyocyanin, and other QS products.

commonly associated with respiratory tract infections, including nosocomial pneumonia and chronic lung infection in Cystic Fibrosis (CF) patients. It is also known to cause infections such as urinary tract infections, burn and other types of wound infections, and sepsis [94].

PA utilizes lectins and the protein cap of the flagella (FliD) to initiate attachment to epithelial cells [94–96]. The bacterium possesses multiple secretion systems, with the Type 3 Secretion System (T3SS) playing a significant role in upper and lower respiratory pathogenesis. The release of associated exotoxins through the T3SS causes tissue damage in lung models [97]. During biofilm formation, PA's quorum sensing (QS) products interfere with host cytokine signaling, resulting in the inhibition of TNF-α and IL-2 production, and the promotion of IL-10 [98]. Lectins also contribute to the biofilm structure by cross-linking carbohydrates in the host extracellular matrix (ECM) and the biofilm extracellular polymeric substance (EPS). Elastase, another virulence factor, impedes monocyte chemotaxis, disrupts tight junctions between epithelial cells, and hinders phagocytosis and antigen presentation [99,100]. Similar to SA, the PA genome encodes a variety of iron-scavenging, retention, and consumption pathways, including the siderophore pyoverdine, which can scavenge iron from host proteins [101,102].

Furthermore, pyocyanin, an important virulence factor in PA respiratory tract infections, disrupts cilia, induces mucus secretion, and triggers goblet cell hyperplasia [103]. Additionally, pyocyanin promotes the overproduction of IL-4 and IL-13, contributing to an imbalance in T helper 1 and T helper 2 responses [104]. Incidentally, T helper 2 skewing is a common feature of CRS, particularly associated with the presence of polyps. Pyocyanin can also stimulate the release of NETs, which can be scavenged by kynurenine, and inhibit reactive oxygen species released during this process [105,106]. Rhamnolipids, produced by PA, have been found to inhibit host beta-defensins and play a role in the severity of upper respiratory tract infections [107]. Furthermore, biofilm exopolysaccharides such as alginate chelate calcium and therefore disrupt immune signaling [71].

PA possesses a relatively large genome (5–7 Mbps) compared to other bacterial species [108,109]. The scientific community has extensively studied the genotypic and metabolic plasticity of PA, as well as the phenotypic diversity observed in clinical isolates. Phenotypic switching is a notable characteristic, where chronic infecting strains downregulate virulence factors and upregulate persistence factors such as LPS modification, slowed growth, and the production of mucoid biofilms associated with alginate [97]. Furthermore, specific mutations acquired over time during chronic infections reduce PA virulence and promote persistence. These mutations often occur in genes controlling QS, flagella and pili assembly, and T3SS, and are well studied in CF infection [110–112].

In healthy individuals, SA and PA products, including D-amino acids and QS signals, are able to activate bitter taste receptors in the sinonasal cavity to increase ciliary beat frequency and increase the production of nasal nitric oxide [113–115]. Additionally, PA QS signals can activate macrophages for enhanced phagocytosis [116]. These responses should improve clearance and killing of invading opportunists, but seem to fall short in CRS, where ciliary structures and functions are impaired and nitric oxide is diminished. And despite macrophage activation, phagocytosis is not successful against bacterial aggregates [117,118].

Histopathological assessment of samples from CRS patients that were culture-positive for SA had increased hyperplasia and squamous metaplasia than patients without SA, while samples from patients that were culture-positive for PA had a greater number of infiltrating neutrophils and subepithelial edema than patients without PA [119]. This suggests that SA and PA individually contribute to epithelial remodeling in the sinonasal cavity and impose species-specific alterations in mucosal inflammation.

## 4. Interactions of SA and PA

SA and PA have both been independently associated with CRS and linked to poor disease prognosis [120]. In fact, the presence of either SA or PA in primary FESS doubled the odds of requiring revision surgery. Furthermore, when comparing primary and revision FESS, the incidence of SA increased from 25% in primary FESS to 39% in revision, while the incidence of PA increased from 4% to 11% [121]. Similarly, in a nasal epithelial cell culture model, SA and PA individually demonstrated rapid expansion compared to other genera when cultures were challenged with defined, patient-derived microbial communities [122].

In contrast to the individual focus on SA or PA, the co-occurrence of these two bacteria in CRS patients has received limited attention. However, both culture-independent [21,123,124] and dependent studies [40,125] have indirectly reported their co-incidence. In fact, data suggests that SA and PA may co-occur in up to 17% of CRS cases [126]. Spatial and temporal distribution of biofilms formed by SA and PA together have yet to be characterized. Given their ability to form biofilms and their impact on CRS, it is important to explore their microbial interactions and potential co-interactions with the host in a disease-relevant context. Current research on SA-PA interactions may shed light on the potential impact of pathogenic microbial communities in CRS, as follows (Fig. 5).

To begin, evidence suggests that in dual species communities, SA and PA behavior is unique compared to mono species behavior. In *in vitro* multi-species biofilms, PA secreted distinct proteins compared to PA biofilms alone, including exotoxin A and pyoverdine [127]. Furthermore, when PA was co-cultured with SA in medium simulating CF sputum, it was found to modulate the antibiotic susceptibility, aggregate formation, and distribution of SA [128].

Although capable of displaying antagonistic behaviors, SA and PA exhibit cooperative tendencies in chronic infection models [129]. In an *in vitro* wound model, the coexistence of SA and PA led to heightened antibiotic tolerance compared to the growth of either species alone [130]. Similarly, in an *in vitro* keratinocyte model, the simultaneous inoculation of SA and PA resulted in increased intracellular SA, indicative of invasiveness, and an elevated number of PA cells attached to keratinocytes. The presence of intracellular SA has been noted in clinical studies of CRS, where it has been associated with the presence of surface biofilm along with higher rates of refractory disease [131]. Furthermore, this co-inoculation induced a greater production of the inflammatory cytokine IL-6, contributing to the establishment of an inflammatory microenvironment [132].

Further evidence of cooperativity emerges from studies where PA adopts a mucoid phenotype characterized by dominant alginate production. In such cases, PA downregulated the expression of virulence factors that would typically antagonize SA and, in turn, offered protection to SA against the action of antibiotics [133,134]. Moreover, under *in vitro* conditions, PA was shown to influence the respiratory rates of SA and utilize acetoin, which is catabolized by SA, as an alternative carbon source. By utilizing SA-produced acetoin, PA ultimately promoted SA survival, as high concentrations of acetoin are detrimental to SA growth [135].

When assessing SA-PA pairs isolated from clinical respiratory infections using a *Galleria mellonella* model of bacterial virulence, only one pair exhibited a significant decrease in larval survival time compared to each isolate individually [136]. This suggests that in certain cases, the combined presence of SA and PA may downregulate the virulence potential of the individual species [129]. On the other hand, a study investigating surface-associated microcolony formation revealed contrasting findings. In the presence of SA, PA was found to induce QS at a faster rate, consequently altering the direction of colony growth. Conversely, SA demonstrated a faster initiation of replication in the presence of PA, and certain strains of SA spatially excluded PA from their microcolonies [137]. This serves to emphasize the need to investigate not only the co-occurrence of SA and PA, but to investigate their

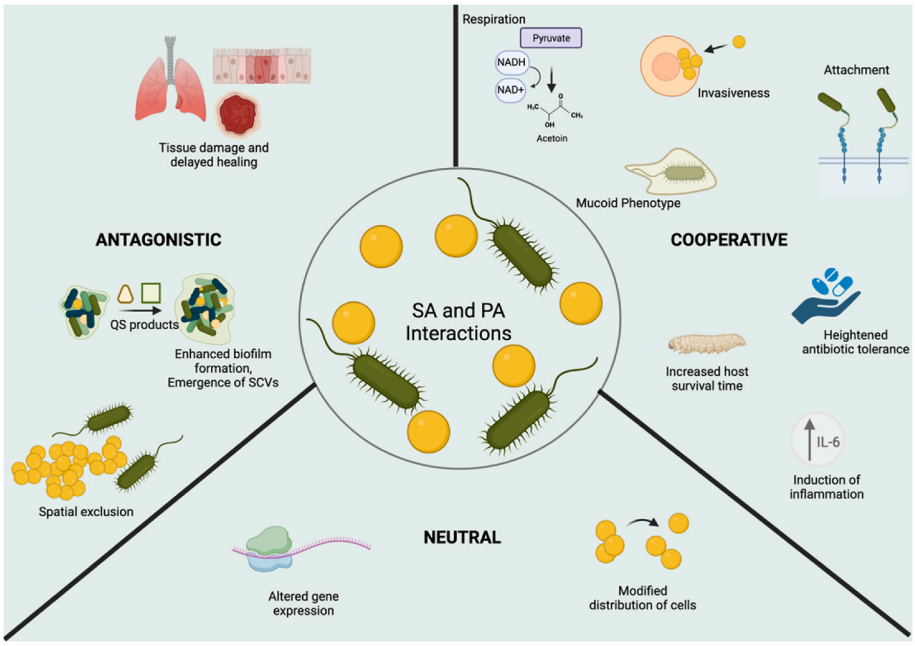


**Fig. 5. SA and PA Interactions**. During chronic infections, the behavior of SA and PA together is distinct as compared to either species alone. These changes in behavior can be neutral, cooperative or antagonistic. Neutral behaviors include alterations in gene expression, such as increased protein synthesis, as well as altered cellular distribution compared to monospecies distribution. Cooperative behaviors include alterations in respiration, increased invasiveness, increased attachment, production of alginate by PA associated with mucoid phenotype, heightened antibiotic tolerance, induction of inflammation, and increased host survival time. Antagonistic behaviors include spatial exclusion of PA by SA. SA QS products enhance biofilm formation by PA, while PA QS products stimulate the emergence of SA Small Colony Variants (SCVs). SA and PA together are associated with damage to lung tissue, worsened disease prognosis, and delayed healing in wounds.

orientation toward each other within CRS-associated biofilms.

As spatial availability or exclusion can impact the occupation of different niches, so can the CRS disease microenvironment influence bacterial behavior. For example, nutritional immunity influences SA + PA interactions [138,139]. If iron is limited, PA may decrease anti-staphylococcal activity to cooperate for iron acquisition, or PA may be more antagonistic in order to take iron directly from lysed SA cells [140]. Similarly, the limited availability of other metal co-factors and the presence of chelating proteins associated with host inflammation (i.e. calprotectin) may increase SA + PA cooperativity, or the lack of available ions may act as a stress signal, independently upregulating virulence in each species [141,142]. Available iron in the environment has been shown to enable robust biofilm formation as well as alter antibiotic tolerance [143,144], and therefore may significantly impact microbial behavior in CRS.

The dynamic interplay between SA and PA in chronic infections offers valuable insights into their interactions and potential implications for disease progression. In lung infections among individuals with CF, SA infections are frequently observed prior to the onset of PA infection. Whether a similar pattern of succession occurs in CRS is yet to be determined. However, SA and PA can coexist over the course of CF patients' lifetimes, leading to exacerbations and a worsened prognosis [145]. In comparison to infections caused by either pathogen alone, the presence of SA + PA exacerbated lung damage and diminished lung function. Similarly, in wound infections, the interactions between SA and PA significantly prolonged the time required for wound healing [146].

Many of the virulence determinants and interactions discussed herein are predominantly regulated by QS systems, which serve as a communication strategy. QS involves the modulation of transcriptomic activity based on the concentration of signaling molecules in the surrounding environment. As bacteria proliferate, the levels of signaling molecules increase until they reach a critical threshold concentration known as "quorum." At this point, QS signals induce alterations in gene expression within recipient cells. While some QS molecules are specific to certain species, others can have a broader influence, extending to the kingdom level [147].

In the context of CF lung infection, the signaling peptide Autoinducer-2, produced by SA, upregulated the transcription of virulence genes in PA, promoted biofilm formation, and contributed to lung damage [148,149]. Similarly, the PA QS molecule HQNO (2-heptyl-4--hydroxyquinoline *N*-oxide) activated alternative sigma factor B in SA, leading to the upregulation of SA virulence genes and the emergence of small colony variants [150]. Small colony variants (SCVs) are subpopulations of bacteria that exhibit altered phenotypes such as reduced colony size, modified respiration, and slow growth rates [151]. SA and PA are both capable of forming SCVs with upregulated expression of attachment factors, increased production of biofilm matrix polysaccharides, and increased tolerance to some antibiotics, such as aminoglycosides [152]. In models of CF, the presence of PA induced SCV formation by SA [153]. SCVs are stress-associated phenotypes, suggesting that SA and PA interactions in this context are competitive [154]. Furthermore, SCVs are associated with iron limitation plus infection persistence and SCVs formed by SA have been identified in submucosal samples from CRS patients undergoing FESS [155,156]. Ultimately, bacterial interactions that enhance virulence and favor the selection of stress-tolerant phenotypes contribute to prolonged infection and inflict greater harm upon the host [157].

It is important to note that *in vitro*, the lytic effect of PA on SA varies depending on factors such as aeration and mixing of cultures [128]. This variability has significant implications for understanding the distribution and interactions between SA and PA in different physiological conditions. For instance, in static mucus secretions within an occluded sinus, PA may be more directly antagonistic toward SA, as opposed to communities under flow conditions, such as those present in the nasal cavity of a patient with active, purulent discharge, where bacterial interactions may be cooperative or neutral.

Using network construction based on peer-reviewed studies of SA and PA, Magalhaes et al. [158] successfully mapped reported bacterial interactions, considering the effects of PA on SA and vice versa, as well

as differentiating between planktonic and biofilm settings. The resulting networks demonstrate that research has been largely one-directional, investigating how PA influences SA in dual-species scenarios, predominately in planktonic modes of growth. Additionally, the analysis confirmed that a larger quantity of data has been generated *in vitro* compared to *in vivo* settings. Therefore, our current understanding of SA-PA interactions and how they translate in the CRS disease context are largely hypothetical. Considering the evidence of neutral, cooperative, and antagonistic dynamics, along with the diverse repertoires of virulence and adaptation displayed by SA and PA, further research is crucial to unravel the intricate nature of their interactions in the context of CRS. And to this point, functional characterization of additional species in CRS-associated bacterial communities and their interactions in a disease-relevant context will propel our understanding of CRS pathogenesis.

## 5. Studying biofilms in CRS (models)

A significant barrier to characterizing bacterial and host interactions in CRS stems from the scarcity of clinically relevant models. It is crucial to employ models that replicate disease conditions and can accommodate a broad range of scientific inquiries in order to enhance our understanding of human disease processes. An ideal model for the study of microbe-microbe and host-microbe interactions in CRS will consider altered physiological conditions, enable biofilm formation, and support prolonged investigations to reflect the length of disease (Fig. 6A). The development of such models remains a persistent challenge in CRS-biofilm research, although a number of *in vitro* and *in vivo* models are presently utilized to investigate pathological mechanisms (Fig. 6B).

### 5.1. In vitro CRS models

In other disease states, such as CF, chronic wounds, and urinary tract infections, substantial progress has been made in defining the physiological factors involved. This advancement has led to the development and testing of multiple variations of disease-relevant media for cultivating microorganisms [159–163]. Similarly, synthetic nasal media has been formulated to mimic healthy human nasal secretions, serving as a promising starting point for the development of specific growth media tailored to CRS [164]. However, healthy nasal secretions differ significantly in mucin composition and concentration [165], as well as in numerous proteins [166] when compared to secretions from CRS patients. Therefore, the utility of synthetic nasal media in CRS research is limited.

The absence of media that accurately reflects the complex microenvironment of CRS indicates significant gaps in our understanding of

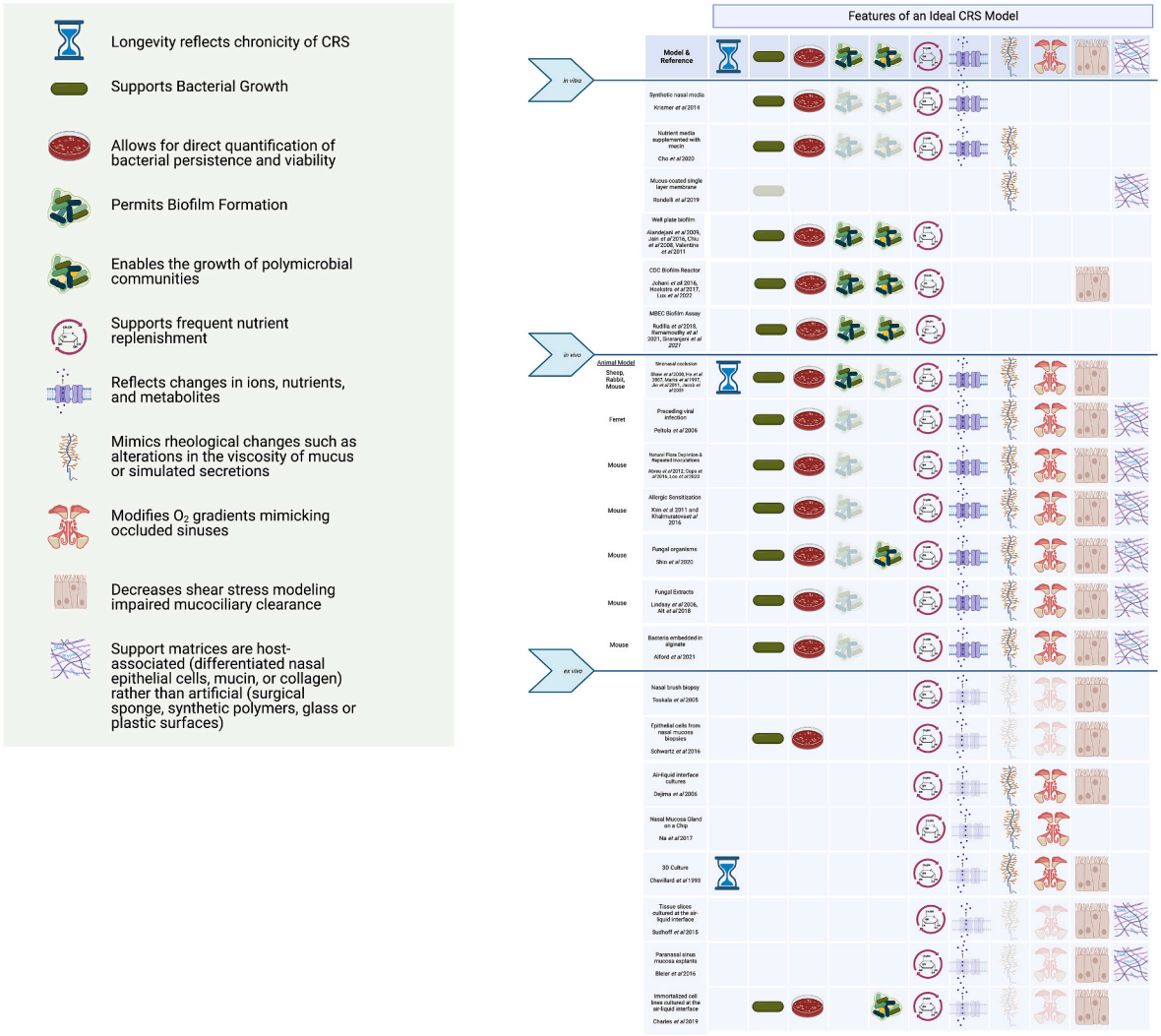


**Fig. 6. CRS Models**. A) Characteristics of an ideal model of CRS. Optimized models for studying CRS should reflect chronicity, multi-species bacterial growth, biofilm formation, and altered physiological conditions. B) Attributes of current models of CRS. Symbols denote that a given characteristic is present (Transparent symbols indicate that the characteristic is expected but not explicitly stated by the authors.).

CRS physiology. Alterations in ion concentration, pH levels, the presence of host proteins such as beta defensins, and host metabolites like nitric oxide, have not been well defined in CRS when compared to healthy states. Although some preliminary studies have commenced in this domain [165–169], they are limited by small sample sizes or relative quantification rather than absolute. However, the challenge of developing physiologically relevant media also stems from the inherent heterogeneity of the disease itself. Some patients may experience significant sinus occlusion due to sticky mucus, while others may have lower levels of aberrant mucus production but higher levels of inflammation. Moreover, anatomical variations can exist, such as epithelial outgrowths into the sinuses versus enlarged turbinates within the nasal cavity, further complicating the study of CRS. These differences can influence the sinonasal microenvironment, changing mucus drainage patterns and efficiency, $O_2$ levels, immune response and immune cell infiltration.

In the context of CRS research, *in vitro* cultivation of bacteria in traditional laboratory media supplemented with animal mucins has been utilized to simulate increased mucin production, a characteristic feature of CRS [170]. Although not yet applied to CRS, Rondelli et al. have created a single-layer phospholipid membrane coated with mucus to mimic mucosal surfaces, presenting an innovative approach with the potential to address various CRS-related inquiries [171]. Furthermore, well plate models have been employed to assess the biofilm formation potential of CRS isolates and to evaluate the efficacy of proposed therapeutic agents against biofilms, including manuka honey, xylitol, baby shampoo, and citric acid zwitterionic surfactant [172–175]. A number of microbiological cultivation techniques and methods for biofilm attachment and formation [176,177], as well as the Centers for Disease Control biofilm reactor [178–180] and the Innovotech Minimum Biofilm Eradication Concentration biofilm assay (formerly called the Calgary Biofilm Device) [181–183] can be used to investigate species relevant to CRS, their interactions, and potential anti-biofilm therapeutics [184]. Furthermore, the development and use of protocols and methods to investigate not only single or dual-species but complex microbial communities should be prioritized, as functional characterization of community interactions will lead to a better understanding of CRS pathogenesis [185,186].

### 5.2. *In vivo* CRS models

Despite limitations, various animal models have played a crucial role in advancing our understanding of host-microbe interactions in sinusitis. One such model involves the use of surgical sponge packing or mechanical occlusion to induce sinonasal occlusion in rabbits and sheep. Sheep, in particular, have been utilized in otolaryngology training and CRS research due to the similarities between their nasal cavities and those of humans, as well as the ease of using clinical instruments like endoscopes with minimal adaptation [187].

The sinonasal packing model offers the advantage of producing significant changes in the sinonasal epithelium, thereby mimicking the disease microenvironment in CRS. It also allows for long-term microbial investigations, with studies conducted for up to 6 weeks. However, this model has its drawbacks, including invasiveness and the use of an artificial surface. Researchers need to expose the sinus cavity to implant the surgical sponge and then re-seal the cavity from the outside. While older sheep CRS models accessed the maxillary sinus via the palate, both methods are highly invasive, require surgical expertise, and are expensive. These approaches also present challenges in interpreting bacterial growth, organization, and host response, as artificial surfaces can enhance biofilm formation. For example, Ha et al. examined biofilm formation in sheep sinuses after 7 days of SA infection and noted that surgical sponge obstruction augmented biofilm formation in this model [188]. Moreover, microbes embedded in the foreign material of the sponge are provided increased protection against host immune actions, making it difficult to differentiate the host response to the foreign body from the response to biofilm.

Despite their limitations, these CRS models have been useful for establishing the role of microbes in the disease. Using a rabbit model, Marks et al. introduced *Streptococcus pneumoniae* into sponge-packed sinus cavities and recovered the inoculating strain up to 1 week post-inoculation [189]. However, the effects of sponge implantation could be observed for 6 weeks, and several other organisms associated with CRS were cultured from these nasal samples. Similarly, Jin et al. successfully recovered SA from inoculated sponges 14 days after implantation at a bacterial load of $10^2$ colony forming units (CFU) per gram of tissue [190].

Recognizing the advantages of this model, it has been adapted for use in mice as well [191]. Jacob et al. were the first to establish a chronic murine model of sinusitis using *Bacteroides fragilis* sponge inoculation, evaluating its effects after 4 weeks. This model validated immune infiltration and epithelial thickening, although bacterial persistence was not reported.

Occlusion models have enabled our current understanding of host-microbe interactions in sinusitis. They have provided valuable insights into host responses, epithelial changes, and biofilm formation, and provide a baseline of comparison for new model development. However, due to their invasive and artificial limitations, several alternative non-occlusion animal models have been developed [192]. For example, one alternative hypothesis for the development of biofilm-associated CRS suggests that preceding viral infections create an inflammatory environment conducive to the expansion of pathogenic bacteria. In a ferret model, researchers investigated the effects of influenza virus infection followed by bacterial inoculation. They found that influenza infection significantly increased the rates of sinusitis, as determined by histopathological assessment, from 10% in the absence of the virus to 80% following viral infection. Furthermore, the authors reported an increase in bacterial load 48 h after bacterial infection, although specific CFU were not provided [193].

In recent years, several mouse models have been developed as alternatives to the occlusion model for studying CRS. Mice offer distinct advantages such as genetic variation, genomic manipulation potential, availability of reagents, and cost-effectiveness. However, creating a murine model of sinusitis presents several challenges, including host adaptation for specific bacterial strains, competition from existing murine sinonasal flora, accommodating the respiratory requirements of obligate nose-breathing, as well as access to and quantity of sinonasal tissue. Nonetheless, various mouse models of sinusitis have emerged, employing different approaches [192]. Some models involve repeated bacterial inoculations, while others utilize depletion of natural flora [194–196], prolonged allergic sensitization [197,198], instillation of fungal organisms or extracts [199–201], or embedding bacteria in alginate solutions [202]. Collectively, these models have substantiated the roles of neutrophilic infiltration, eosinophilic response, goblet cell hyperplasia, and epithelial disruption in CRS development. They have also shed light on polyp formation and the potential impact of Staphylococcal enterotoxins on CRS progression. Furthermore, these models have emphasized the importance of the sinonasal microbiome. However, unlike the occlusion models discussed earlier, these mouse models lack longevity, which is a significant weakness considering the chronicity of CRS.

Another significant weakness of mouse CRS models is the incredibly small amount of sinus tissue that can be extracted. Nasal tissue extraction is required to accurately quantify bacterial load. Because of their small sinuses, a large number of animals is required per investigation, as bacterial quantification, tissue for immunopathology, or any number of other assessments typically require individual animals [203]. Nasal lavage and *in vivo* imaging can be used as alternatives for direct quantification, albeit with limitations in sensitivity. Thus, some models assess bacterial load as early as 24 h post-inoculation, when the numbers of bacteria are presumably at their highest, with the longest direct bacterial quantification reported after 3 days [202]. In addition, many of

these models focus on host immune responses or the presence/absence of bacteria, while neglecting microbial interactions and biofilm formation. Consequently, the literature on CRS models primarily focuses on model validation rather than in-depth mechanistic investigations.

### 5.3. Ex vivo CRS models

In addition to *in vivo* models, several *ex vivo* cell and tissue culture models have been employed to study the complexities of CRS [204]. One approach involves utilizing nasal brush biopsies to generate cell lines that can be assessed for ciliary beat within a short timeframe, ranging from 4 h post-collection to 3 days [205]. This minimally invasive model offers the advantage of easy collection procedures. However, its main limitation lies in the longevity of culture viability. In a more conventional method, epithelial cells from CRS patient nasal mucosa biopsies are cultivated for approximately 20 days before subjecting them to viability challenges with bacterial strains [206]. Air-liquid interface (ALI) cultures have been employed in CRS research, allowing for the differentiation of epithelial cells into complex, multilayer systems. This technique only allows the basal surface of cells to be in contact with the culture media, enabling more realistic modeling of the sinus environment. Dejima et al. utilized cells from these growth conditions to compare ion transport capabilities in healthy and CRS sinonasal samples [207]. In an expansion of this technique, Na et al. optimized ALI-cultured human nasal epithelial cells with a hydrogel scaffold in a microfluidic chip system mimicking complex nasal mucosa [208]. Similarly, 3D culture techniques, with the use of scaffolds or lattice are conducive to studying differentiated morphologies and characteristics such as ciliary beat frequency for a period of months rather than weeks [209]. Another *ex vivo* model involves cultivating 200 μm slices from nasal turbinate resections at the ALI, providing a valuable tool to investigate mucus production and its response to various stimuli [210]. Moreover, the most invasive approach involves utilizing 5 mm biopsy punches to collect and cultivate paranasal sinus mucosal explants, allowing for the examination of specific host responses at the tissue level [211].

Cell and tissue culture models are frequently unpopular in microbiological investigations due to their cultivation requirements and eukaryotic cell sensitivity. However, immortalized nasal epithelial cell cultures support the colonization of polymicrobial communities collected from both CRS and healthy patients, offering a promising avenue for studying host-microbe interactions [122]. Furthermore, nasal epithelial cell cultures can be modified to investigate the effects of shear stress and flow conditions on bacterial adherence and biofilm formation by CRS organisms utilizing peristaltic pumps and chamber slides [212,213]. In summary, a range of cell and tissue culture models have been developed to study host-microbe interactions in sinusitis, offering valuable insights into various aspects of the disease. While these models have their limitations, ongoing advancements and modifications continue to expand their applicability and potential for further research [214].

The current models detailed above have been useful for the investigation and ongoing development of anti-biofilm therapeutics and alternative treatment strategies in CRS, such as additions to irrigations, bacteriophage treatment, ultrasound, addition of putative probiotic strains, and anti-QS molecules [33,215]. These experiments highlight the importance of developing therapeutics that target the biofilm itself and developing models that can better recapitulate CRS disease.

Existing *in vitro* and *in vivo* models for CRS should be leveraged for the study of bacterial community dynamics, host-microbe interactions, and biofilm formation and maturity, while development of new models continues. Certainly, there is no shortage of questions to be addressed in these areas. To this point, clinical research plays a vital role in advancing our understanding of CRS by examining microbial behavior in the natural disease setting and validating models used in laboratory studies. While many clinical studies have described the diversity of microbial communities in CRS and identified bacterial aggregates, there remains a need to investigate and describe their metabolic state, transcriptomic profile, and spatial distribution. To develop highly translational models, it is crucial to explore these factors and validate their relevance. Additionally, conducting strain-specific characterizations from patient samples will provide valuable insights into the specific requirements for bacterial survival in CRS. By adopting a systematic approach, significant progress can be made in unraveling the complexities of CRS. By leveraging existing data and stored isolates, it may be possible to gain this information at an accelerated pace. Through comprehensive characterization of microbial dynamics in CRS, we can uncover commonalities across chronic biofilm-associated human diseases and pave the way for innovative therapeutic interventions.

## 6. Conclusion

CRS remains an understudied area of research with significant clinical implications. Biofilms play a crucial role in CRS, contributing to symptoms and treatment challenges. Specific bacterial species, including SA and PA, have been implicated in CRS, each with unique potential contributions to disease progression and treatment approaches. SA and PA interactions serve as an example of bacterial species that can influence disease sequelae, underscoring the complexity of microbial dynamics. Furthermore, the vast array of potential bacterial species and their microbe-microbe and microbe-host interactions in CRS highlights the need for further investigation and a comprehensive understanding of these intricate relationships. Urgent attention is needed to develop new models and novel therapeutic approaches, which will be improved by characterizing the CRS microenvironment, analyzing bacteria isolated from CRS patients, and functionally characterizing microbial communities along with their spatial orientation. Addressing these research gaps will provide critical insights into the disease process, paving the way for improved diagnostic techniques and targeted interventions to alleviate the burden of CRS on patients' quality of life.

## Funding

CRS research in the Rumbaugh lab is funded by The CH Foundation.

## Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Kendra Rumbaugh reports that support was provided by Texas Tech University Health Sciences Center. Kendra Rumbaugh is an Editor for Biofilm.

## Data availability

No data was used for the research described in the article.

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