The microbiome of the upper airways: focus on chronic rhinosinusitis

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Abstract
Upper airway diseases including allergic rhinitis, chronic rhinosinusitis with or without polyps, and cystic fibrosis are characterized by substantially different inflammatory profiles. Traditionally, studies on the association of specific bacterial patterns with inflammatory profiles of diseases had been dependent on bacterial culturing. In the past 30 years, molecular biology methods have allowed bacterial culture free studies of microbial communities, revealing microbiota much more diverse than previously recognized including those found in the upper airway.

At presence, the study of the pathophysiology of upper airway diseases is necessary to establish the relationship between the microbiome and inflammatory patterns to find their clinical reflections and also their possible causal relationships. Such investigations may elucidate the path to therapeutic approaches in correcting an imbalanced microbiome. In the review we summarized techniques used and the current knowledge on the microbiome of upper airway diseases, the limitations and pitfalls, and identified areas of interest for further research.

Introduction
It is generally believed that exposure to microorganism compromises health. Reduced exposure to microbiota results in decrease of incidence of infectious diseases but may adversely increase the incidence of allergic disorders [1-3]. Recent developments of culture-independent tools make it possible to identify microbial species not previously detected by conventional methods. Unbeknownst to us, man had been living with these microorganisms since the dawn of time.

The human body harbors from 10 to 100 trillion microbes which greatly outnumber our own human cells [4]. This bacterial assemblage has been coined, “the human microbiota” [4]. Subsequently, a project called “Human Microbiome” was established to investigate the flora in healthy volunteers and their relationship to human health and disease [5]. The study of the host-microbe relationship has shown that microbes play a major role in our well-being [4,6]. Alterations of microbial composition have been linked to several human diseases [4]. There is also evidence showing that, in the respiratory system, composition of airway microbiota varies between healthy people and people with diseases such as asthma [6-8] and cystic fibrosis (CF) [8,9]. Unfortunately, with limited studies currently available, it cannot be concluded with the same degree for chronic rhinosinusitis (CRS) [10].

Research on microbiome in CRS is therefore needed to elucidate pathophysiology of this disease such as; 1) the relationship between the microbiome and inflammatory patterns, 2) possible causal relationships between microbe and CRS, 3) investigation of the microbiome regarding possible therapeutic properties. Dysregulation of the interactions between the immune system and commensal bacteria is a contributing factor to the development and chronicity of a number of inflammatory diseases [11]. Microorganisms in the gut may play a significant role in regulating T helper cells (Th cells), regulatory T cells (Tregs) and dendritic cells as well as Toll-like receptor expression in sentinel cell (macrophage and dendritic cells) which are relevant to airway illnesses such as asthma and allergic diseases [10].

Techniques in microbiota study
Principal approaches to analyze human microbiota are: culture-dependent and culture-independent techniques. Culture-dependent methods involve isolation and culturing of microorganisms prior to their identification according to morphological, biochemical or genetic characteristics. These methods are time-consuming, due to culture...
and bias, as certain media and growth conditions favor the growth of some bacteria over others [12]. In addition, this approach may not provide a true reflection of the diversity of microbes in a sample. A "no growth" result does not necessarily imply that a sample is sterile. It is estimated that up to 99% of microorganisms observable in nature typically cannot be cultured by standard techniques [13].

Un-culturability is a widespread condition that includes: (i) organisms for which the specific growth requirements (nutritional, temperature, aeration, etc.) are not fulfilled; (ii) slow-growing organisms are out-competed in the presence of fast-growing microorganisms and (iii) disfavored organisms, which cannot stand the stressful conditions imposed by cultivation [13,14]. This approach camouflages the true bacterial community. There needed to be a better approach to analyze these microorganisms.

Since the 1980s, the application of molecular detection methods has allowed culture-independent investigations of the microbial communities [15]. Molecular techniques have proven effective in characterizing complex microbial assemblages in environmental samples [16]. However, an important usefulness of molecular techniques is the ability to detect genetic materials of non-viable microorganisms [17,18]. Culture-independent methods are based on the direct analysis of bacterial DNA (or RNA) without culturing. Due to the sensitivity of these techniques, special care and attention is required for procedures that include sample collection and handling, DNA extraction, amplification of gene fragments, distinction of different fragments, identification of microorganism and analysis of the microbial community [15].

For bacterial identification, the predominant gene target for amplification has been the 16S ribosomal RNA gene (or 16S rRNA) [19,20], which is a component of the 30S small subunit of prokaryotic ribosomes [21]. It has been widely targeted because of (i) its presence in almost all bacteria, often existing as a multigene family, or as operon; (ii) the conservation of the 16S rRNA gene, suggesting that random sequence changes are a measure of time (evolution) rather than a reflection of different bacteria; and (iii) the size of 16S rRNA genes (1,500 bp) being large enough for informative purposes [22]. Moreover, there are several available reference sequences and taxonomies databases such as Greengenes, SILVA and the Ribosomal database project [23]. However, amplification of target genes using polymerase chain reaction (PCR) has made it impossible to completely avoid PCR-based biases and chimera production. It thus may distort the level of diversity and bacterial composition in a sample because of the amplification of pseudogenes [24]. Therefore, other technologies are often used as complementary approaches to 16S rRNA gene sequencing for reducing distortion of bacterial diversity and composition. They are DNA microarray, fluorescence in situ hybridization (FISH), and quantitative PCR (qPCR), and are based on oligonucleotide probes and primers that target the ribosomal RNA sequences or other genes in different hybridization procedures. Thus these techniques require a prior knowledge of the microbial DNA sequence. A DNA microarray (also commonly known as DNA chip or biochip) is a collection of microscopic DNA spots (oligonucleotide probes) attached to a solid surface. It is usually used for gene expression analysis or screening of single nucleotide polymorphisms. The FISH technique uses fluorescent probes that bind to only those parts of the chromosome with which they show a high degree of sequence complementarity. It detects and localizes the presence of specific DNA sequences on chromosomes. qPCR or real-time PCR follows the general principle of PCR with the advantage of detecting the amount of initial DNA in samples using either fluorescent DNA-binding dyes or fluorescence-tagged oligonucleotide probes [15].

The introduction of next generation sequencing changed the history of genomic research as it increased sequencing throughput, and did not require prior cloning steps [25]. These technologies are not only changing our genome sequencing approaches and the associated timelines and costs [26], but also developing many exciting fields such as metagenomics, metatranscriptomics and single-cell genomics [15]. Three platforms for high throughput parallel DNA sequencing are in reasonably widespread use at present: the Roche/454 FLX, the Illumina (MiSeq, HiSeq, and NextSeq), and the Ion Torrent.

At presence, researchers have a large choice in formulating methodological strategies: depending on the access to the technology, budget, and objectives of research. Each culture-independent methodology has its own limitations and biases, investigators must take additional measures; for example one may use more than one molecular technique or a culture-dependent approach in parallel to provide additional validation of results and reduce the possibility of false findings due to methodological errors and biases. Although the culture-independent techniques have the ability to detect more microbes than culture technique, the culture-dependent methods so far remain a better means of obtaining individual isolates contributing and obtaining isolates for further assays.

**Microbiota in allergic rhinitis**

As the gate into our body, the respiratory tract itself harbors a heterogeneous microbiota that decreases in biomass from upper to lower tract [27]. Even in health, recent findings indicated that direct exposure to bacterial communities in the airways may provide an explanation for how commensal bacteria can regulate chronic airway inflammation [11]. Since the observation that infections within households in early childhood have a role in preventing allergic rhinitis [3], numerous epidemiologic
Microorganisms in the airways of cystic fibrosis patients
Cystic fibrosis (CF) is an autosomal recessive genetic disorder that affects among other organs the lungs and sinuses. It is characterized by abnormal transport of chloride and sodium across the epithelium, leading to thick, viscous secretions. This leads to defective mucociliary clearance and chronic airway infection with a complex microbiota [37]. Lung disease in cystic fibrosis results from chronic airway infection and inflammation leading to progressive bronchiectasis and respiratory failure [38]. Specimens for molecular microbial analysis in CF have for the most part all derived from sputum [37,39-42], while swabs from the middle meatus in patients with sinusitis were not used until now. Thus, no conclusions can be made for the upper airways yet. Samples consisted of serial collections of more than six patients in most of the studies [37,42-44].

Previous studies indicated that exacerbations might be associated with changes in microbial densities and the acquisition of new microbial species [37]. Bacterial pathogens, including Pseudomonas aeruginosa, Staphylococcus aureus, and Burkholderia cepacia are known contributors to such exacerbations. Recent studies using the latest culture-independent techniques and culture-independent molecular techniques have broadened our view of CF airway bacterial communities [38]. Each CF patient presented a unique microbiome [40]. The species present tended to vary more “between” than “within” subjects, suggesting that each CF airway infection is unique, with relatively stable and resilient bacterial communities [44]. The diversity and species richness of fungal and bacterial communities were significantly lower in patients with decreased lung function and poor clinical status [39]. The authors observed a strong positive correlation between low species richness and poor lung function [37]. These findings show the critical relationship between airway bacterial community structure, disease stage, and clinical state at the time of sample collection [42].

The main microorganisms found in CF airways are the genera Haemophilus, Pseudomonas, Staphylococcus and Stenotrophomonas. Less common are gram-negatives, Streptococcus and Mycobacterium spp [45]. Most bacteria of CF airways are difficult to culture using conventional clinical methods; therefore, molecular approaches may confirm or reveal novel bacteria that might be related to the pathogenesis of cystic fibrosis. Examples of interest are the Streptococcus milleri group (Streptococcus anginosus, Streptococcus intermedius, Streptococcus constellatus) [46], Pseudomonas intermedia [46], and Gemella species [47] (Table 1). Further experiments suggested that these bacteria could act as co-pathogens or enhance the virulence of conventional CF pathogens [48].
| Author          | Year | n   | Sample                  | Technique                      | Achromobacter | Actinomyces | Atopobium | Bacteroidetes | Burkholderia |
|-----------------|------|-----|-------------------------|--------------------------------|---------------|-------------|-----------|--------------|--------------|
| Salipante SJ    | 2013 | 60 CF | Sputum                 | 16s rRNA pyrosequencing      |               |             |           |              |              |
| Zemanick ET     | 2013 | 37 CF | Sputum                 | Conventional culture 16S rRNA pyrosequencing |               |             |           |              |              |
| Delhaes L       | 2012 | 8 CF  | Sputum                 | Conventional culture 16S rRNA pyrosequencing |               |             |           |              |              |
| Fodor AA        | 2012 | 23 CF | Sputum                 | 16S rRNA pyrosequencing      |               |             |           |              | x            |
| Stressmann FA   | 2012 | 14 CF | Sputum                 | Conventional culture analysis T-RFLP |               |             |           |              |              |
| Zhao J          | 2012 | 6 CF  | Sputum                 | 16s rRNA pyrosequencing      |               |             |           |              |              |
| Guss AM         | 2011 | 4 CF  | Sputum                 | 16s rRNA pyrosequencing      |               |             | x         |              |              |
| Sibley CD       | 2011 | 6 CF  | Sputum                 | Conventional culture T-RFLP 16S rRNA pyrosequencing |               |             | x         |              |              |
| Cox MJ***       | 2010 | 63 CF | Sputum Throat swab     | Microarray                    |               |             |           |              |              |
| J Harris JK     | 2007 | 28 CF 14 healthy | Bronchoalveolar lavage fluid | DGGE/TGGE               |               |             |           |              |              |
| Rogers GB       | 2004 | 34 CF | Sputum                 | T-RFLP                       |               |             |           |              |              |
| Author          | Campylobacter | Capnocytophaga | Craurococcus | Fusobacterium | Granulicatella | Haemophilus | Lactobacillus | Leptotrichia | Ochrobactrum |
|-----------------|---------------|----------------|--------------|---------------|----------------|-------------|---------------|--------------|--------------|
| Salipante SJ *  |               |                |              |               |                |             |               |              |              |
| Zemanick ET     | x             | x              |              |               |                |             |               |              |              |
| Delhaes L       |               |                | x            |               |                |             |               | x            |              |
| Fodor AA        |               |                |              |               |                |             |               |              |              |
| Stressmann FA **|               |                |              |               |                |             |               |              |              |
| Zhao J          |               |                |              |               |                |             | x             |              |              |
| Guss AM         |               |                |              |               |                |             |               |              |              |
| Sibley CD       |               |                |              |               |                |             | x             |              |              |
| Cox MJ***       |               |                |              |               |                |             |               |              |              |
| J Harris JK ****|               |                |              |               |                |             |               |              | x            |
| Rogers GB ***** |               |                |              |               | x             |             |               |              |              |
| Author            | Peptostreptococcus | Porphyromonas | Prevotella | Pseudomonas | Rhizobium | Rothia | Staphylococcus | Stenotrophomonas | Streptococcus | Veillonella | Ref. |
|-------------------|--------------------|---------------|------------|-------------|-----------|--------|----------------|-----------------|---------------|-------------|------|
| Salipante SJ *    |                    |               |            |             |           | x      | x              | x               |               |             | 49   |
| Zemanick ET       | x                  |               |            |             |           |        | x              | x               | x             |             | 35   |
| Delhaes L         | x                  |               | x          |             |           | x      |                | x               | x             |             | 39   |
| Fodor AA          | x                  | x             |            |             |           |        |                | x               |               |             | 37   |
| Stressmann FA **  |                    |               |            |             |           |        | x              | x               | x             |             | 44   |
| Zhao J            |                    |               |            |             |           | x      | x              |                 |               |             | 41   |
| Guss AM           |                    |               |            |             |           |        | x              |                 | x             |             | 50   |
| Sibley CD         |                    |               | x          |             |           |        | x              | x               | x             |             | 51   |
| Cox MI***         |                    |               |            |             |           |        |                |                 |               |             | 52   |
| J Harris JK ****  |                    |               |            |             |           |        |                |                 |               |             | 53   |
| Rogers GB *****   |                    |               |            |             |           |        |                |                 |               |             | 54   |

*Stenotrophomonas maltophilia, Streptococcus agalactiae, Haemophilus influenzae, Pseudomonas aeruginosa.

**P. aeruginosa, Stenotrophomonas maltophilia, Staphylococcus aureus, Streptococcus Group F.

***S. aureus, P. aeruginosa.

****S. aureus, S. maltophilia, P. aeruginosa, Streptococcus mitis, H. influenza.

*****P. aeruginosa, Porphyromonas endodontalis, P. gingivalis, Prevotella denticola, P. melaninogenica, P. nigrescens, P. veronai, P. intermedia, P. loeschei, P. salivae, P. buccae, P. oris, Craurococcus roseus, Rhizobium loti, Ochrobactrum anthropi, Peptostreptococcus anaerobius.

Asterisks indicate studies in which mentioned species were identified.
devices such as a sterilized Killian nasal speculum with long leaves [58].

Before the era of culture-independent methods, conventional cultures have implicated *Staphylococcus aureus* and coagulase-negative *Staphylococcus* as principal pathogens in chronic rhinosinusitis (CRS) [60]. The development of culture-independent molecular techniques allowed the detection of more bacteria [60] and revealed greater biodiversity than conventional culture [56]. Thus, the etiology of CRS may be polymicrobial [55] and the role of anaerobe bacteria may be more prominent than presumed; however, it is likely that the bacteria detected by culture-dependent techniques still are of clinical relevance [60].

Using comparative microbiome profiling in a cohort of a small number of not further defined CRS patients and healthy subjects, it was proposed that the sinus microbiota of CRS patients exhibit significantly reduced bacterial diversity compared to those of healthy controls. Abreu et al., found a depletion of multiple phylogenetically distinct lactic acid bacteria coincident with an increase in the relative abundance of a single species, *Corynebacterium tuberculostearicum* [17]. These microbes caused goblet cell hyperplasia and mucin hypersecretion in a murine model of sinus infection. In this model, *Lactobacillus sakei* represented a potentially protective species [17]. However, the finding of this single species has not been confirmed by others [55,56] (see Table 2). In a larger study, *Staphylococcus aureus* and *Propionibacterium acnes* were the most common organisms in CRS (mostly CRSwNP) and controls, respectively [18]. Recently, the investigators detected *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Propionibacterium acnes* as the most prevalent and abundant microorganisms in healthy sinuses [61].

Using culture-independent (qPCR and 16S rRNA gene sequencing) methodologies for pathogen identification in chronic rhinosinusitis patients, among 57,407 pyrosequences were generated. The most prevalent ones were from coagulase-negative staphylococci (100%), 21/21 specimens, *Corynebacterium spp* (not specifically *Corynebacterium tuberculosis*) (85.7%) 18/21, *P. acnes* (76.2%), 16/21, and *Staphylococcus aureus* (66.7%) 14/21. Although these authors found significantly different distributions of 16S rRNA sequences recovered from CRS vs. non-CRS cases, neither richness nor evenness indices showed statistically significant differences [56]. In another approach using 16S rRNA gene clone sequencing in a terminal restriction fragment length polymorphism (T-RFLP) analysis, the bacteria present in 70 clinical samples from 43 CRS patients undergoing endoscopic sinus surgery were characterized; a total of 48 separate bands were detected. Species belonging to 34 genera were identified as present by clone sequence analysis. Of the species detected, those within the genera *Pseudomonas, Citrobacter, Haemophilus, Propionibacterium, Staphylococcus*, and *Streptococcus* were found numerically dominant, with *Pseudomonas aeruginosa* being the most frequently detected species [55]. Another prospective study collected mucosal biopsies from 18 patients undergoing endoscopic sinus surgery for CRS and 9 control patients with healthy sinuses (indication: pituitary adenomas) compared swab culture with bTEFAP (bacterial tag-encoded FLX amplicon pyrosequencing). Standard cultures mainly showed *Staphylococcus aureus* and Coagulase-negative *Staphylococcus aureus*, whereas the molecular analysis identified up to 20 predominant organisms per sample. *Staphylo- coccus aureus* was nevertheless detected in about 50%; moreover, they disclosed anaerobic species with so far unknown impact in CRS, *Diaphorobacter* and *Peptoniphilus*. Interestingly, *Diaphorobacter* is described as a strong biofilm creator [55,60].

Table 2 provides a summary of previous studies related to the microbiome in chronic rhinosinusitis, including sample size, type of sample, technique used and genus found.

Comparisons of molecular analyses suggest that the detection of microorganisms by Fluorescence in-situ hybridization (FISH) and culture-dependent techniques is related to the abundance of an organism, furthermore, cultivation tends to give advantage to rapidly growing bacteria [18]. The investigators employed conventional cultivation, molecular diagnostics and FISH to detect *Staphylococcus aureus* as a standard. They found that FISH analysis had a sensitivity of 78% with a specificity of 93% compared to the molecular technique [18]. Evidence from high-sensitivity techniques demonstrates that the healthy sinus is clearly not sterile [18], but shows high diversity of the resident microbiota [17]. The nasal microbiota of healthy subjects mainly consist of members of the phylum *Actinobacteria* (e.g., *Propionibacterium spp* and *Corynebacterium spp*), whereas the phyla *Firmicutes* (e.g., *Staphylococcus spp.* and *Proteobacteria* (e.g. *Enterobacter spp*) are less frequent [55,56,60,63]. It appears that the prevalence and abundance of organisms is critical in determining healthy conditions [18].

Thus, similar to CF, findings in CRS have pointed out that the microbiome is unique for each individual patient [42-44] and the community of microbes is diversified [10]. As a general principle, a decreasing bacterial diversity is correlated with disease severity in CF [37-39,42,44], whereas CRS patients were characterized by an altered microbial composition and greater abundance of *Staphylococcus aureus* [56]. There was no single common microbiota profile among patients with similar clinical conditions in the studies performed so far, although *Staphylococcus aureus* was prominent in most studies [10,11,68]. Thus, there is a clear need for larger series.
| Author             | Year | n       | Sample                                      | Technique                          | Aureobacterium | Alicyphilus | Burkholderia |
|--------------------|------|---------|---------------------------------------------|------------------------------------|----------------|-------------|-------------|
| Ramakrishnan VR et al.* | 2013 | 28      | healthy                                    | Middle meatus swab                  | qPCR 16S rRNA pyrosequencing |             |             |
| Aurora R et al.    | 2013 | 30      | CRS 12 healthy                             | Superior middle meatus lavage       | 16S rRNA pyrosequencing         | x           |             |
| Boase S et al.**   | 2013 | 38      | CRS 6 healthy                              | Mucus Middle meatus swab            | Conventional culture Ibis T5000 |             |             |
| Abreu NA et al.    | 2012 | 10      | CRS 10 healthy                             | Brush samples of mucosal surfaces   | PhyloChip analysis            |             |             |
|                    |      |         | of the lateral, central, and medial        | portions of the maxillary sinus     |                 |             |             |
| Feazel LM et al.** | 2012 | 15      | CRS 5 healthy                              | Middle meatus swabs                 | Conventional culture 16S rRNA pyrosequences |             |             |
| Stressmann FA et al.*** | 2011 | 43      | CRS. Polyp and inferior turbinate tissue.  | Mucin (if present)                  | 16S rRNA pyrosequencing T-RFLP |             |             |
| Frank DN et al.**** | 2010 | 26      | S. aureus carriers 16 non-carriers S       | Nostril swabs                      | 16S rRNA pyrosequencing        |             |             |
|                    |      |         | healthy                                    |                                     |                 |             |             |
| Stephenson MF et al.***** | 2010 | 18      | CRS 9 healthy                              | Mucosal Biopsy                      | 16 s rRNA pyrosequencing       |             |             |
| Healy DY et al.****** | 2008 | 11      | CRS 3 healthy                              | Mucosa samples                     | FISH DNA probes Haemophilus influenzae Streptococcus pneumonia Staphylococcus aureus Pseudomonas aeruginosa | |             |
| Sanderson AR et al.******* | 2006 | 18      | CRS 5 healthy                              | Biopsies of the sinus mucosa        | FISH DNA probes Haemophilus influenzae Streptococcus pneumonia Staphylococcus aureus Pseudomonas aeruginosa | |             |
| Lina G et al.******* | 2003 | 216     | healthy                                    | Nasal vestibule swabs               | Conventional culture          |             |             |
| Rasmussen TT et al.******* | 2000 | 10      | healthy                                    | Nasal washes                       | Conventional culture Capillary sequencing | x           |             |

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| Author | Carnobacterium | Citrobacter | Cloacibacterium | Corynebacterium | Curtobacterium | Cyanobacterium | Diaphorobacter | Enterobacter | Enterococcus |
|--------|----------------|-------------|-----------------|-----------------|----------------|----------------|----------------|--------------|--------------|
| Ramakrishnan VR et al.* | x | | | | | | | | |
| Aurora R et al. | | x | x | | | | | | |
| Boase S et al.** | | | | | | | | | |
| Abreu NA et al. | | x | | | | | | | |
| Feazel LM et al.** | | | | | | | | | |
| Stressmann FA et al.*** | | | x | | | | | | |
| Frank DN et al.**** | | | | x | | | | x | |
| Stephenson MF et al.***** | | | | | x | | | | |
| Healy DY et al.****** | | | | | | | | | |
| Sanderson AR et al.******* | | | | | | | | | |
| Lina G et al.********* | | | | | | | | | |
| Rasmussen TT et al.******** | | | | | | | | | |

(Continued)
Table 2 Summary of chronic rhinosinusitis microbiota studies; type of sample, technique used and genus identified (Continued)

| Author               | Fusobacterium | Haemophilus | Helicobacter | Lachnospira | Lactobacillus | Micrococcus | Moraxella | Mycobacterium | Neisseria |
|----------------------|---------------|-------------|--------------|-------------|---------------|-------------|-----------|---------------|-----------|
| Ramakrishnan VR et al.* | x             | x           |              |             |               |             |           |               |           |
| Aurora R et al.      |               |             |              |             |               |             |           |               |           |
| Boase S et al.**     |               |             |              |             |               |             |           |               |           |
| Abreu NA et al.      |               |             | x            | x           |               |             |           |               |           |
| Feazel LM et al.**   |               |             |              |             |               |             |           |               |           |
| Stressmann FA et al.*** |             |             |              |             |               |             |           |               |           |
| Frank DN et al.****  |               |             |              |             |               |             |           |               |           |
| Stephenson MF et al.***** |             |             |              |             |               |             |           |               |           |
| Healy DY et al.****** |               |             |              |             |               |             |           |               |           |
| Sanderson AR et al.******* |             |             |              |             |               |             |           |               |           |
| Lina G et al.*******  |               |             |              |             |               |             |           |               | x         |
| Rasmussen TT et al.******** |             |             |              |             |               |             |           |               |           |
Table 2 Summary of chronic rhinosinusitis microbiota studies; type of sample, technique used and genus identified (Continued)

| Author                  | Pediococcus | Peptoniphilus | Propionibacterium | Pseudomonas | Rhodococcus | Staphylococcus | Stenotrophomonas | Streptococcus | Ref.    |
|-------------------------|-------------|---------------|-------------------|-------------|-------------|----------------|------------------|---------------|---------|
| Ramakrishnan VR et al.* | x           |               |                   |             |             |                |                  |               | [61]    |
| Aurora R et al.         |             |               |                   |             |             |                |                  |               | [62]    |
| Boase S et al.**        |             |               |                   |             |             |                |                  |               | [18]    |
| Abreu NA et al.         | x           |               |                   |             |             |                |                  |               | [17]    |
| Feazel LM et al.**      |             |               |                   |             |             |                |                  |               | [56]    |
| Stressmann FA et al.*** |             |               |                   |             |             | x              |                  |               | [55]    |
| Frank DN et al.****     | x           |               |                   |             |             |                |                  |               | [63]    |
| Stephenson MF et al.***** |          |               |                   |             |             |               |                  |               | [60]    |
| Healy DY et al.******   | x           |               |                   |             |             |                |                  |               | [64]    |
| Sanderson AR et al.******* |         |               |                   |             |             |                |                  |               | [65]    |
| Lina G et al.********   |             |               |                   |             |             | x              |                  |               | [66]    |
| Rasmussen TT et al.********* |         | x            |                   |             |             |                |                  |               | [67]    |

*S. aureus, P. acnes
**P. aeruginosa.
***S. aureus, S. epidermidis, Propionibacterium acnes.
****S. aureus, S. coagulase-negative, S. anaerobic species.
*****H. influenza, Streptococcus pneumonia, S. aureus, P. aeruginosa.
******Staphylococcus aureus, S. non-aureus, S. epidermidis, S. capitis, S. haemolyticus, S. warneri, S. hominis, S. lugdunensis, S. cohnii subsp. cohnii, S. auricularis.
*******Haemophilus influenza, S. pneumoniae, S. aureus, Pseudomonas aeruginosa.
********Staphylococcus epidermidis, S. capitis, S. hominis, S. haemolyticus, S. lugdunensis, S. warneri.

Asterisks indicate studies in which mentioned species were identified.
of well-defined patients sampled and investigated in an optimal way, also avoiding the interference of recently applied antibiotics, to establish the correlation between microbe and CRS disease.

Limitations of the current studies
Airway microbiome studies revealed several critical factors, which also may impact CRS studies. First of all, the inclusion of well-defined patients, using pheno- and potentially endotypes of upper airway disease [68–71], and matched controls in meaningful numbers is necessary to draw supportable conclusions. Furthermore, recent anti-biotic treatment within 1 month [44] prior to collection could significantly reduce the diversity of the microbiome in samples [42,43,56], and contamination by bacteria from other organs such as the skin should be taken into account [9,27]. Factors which may perturb the collection or evaluation procedures are contaminating host DNA [40] or RNA, the existence of viruses such as bacteriophages in the samples, which may impact on the number and genes of microbes [72], and technical issues such as extraction methods (e.g. modified lysostaphin-lysozyme method to enhance staphylococcus DNA extraction) [41].

Currently, most of the publications in human microbiome studies have spotlighted sequencing of 16S rRNA in the identification of bacteria. Their results may misjudge the level of diversity and microbial composition by amplification of chimera and pseudogenes and/or inappropriate primer selection. Metagenomic shotgun sequencing may avoid these problems by omitting amplification and allows to detect gene contents of complex microbiota and to compare functional gene contents between samples, but still may have limitations as discussed above and in low-microbial burden samples. However, researchers are now increasingly employing novel techniques to study the human microbiome [25].

Conclusion and perspective of nasal microbiome studies
The new molecular techniques enhance our chance to identify new bacteria within the nose and nasal cavities; as the pivotal host functions evolved under high microbial pressure, they will show a very complex network of microbes and thus microbe-host interactions [41]. On the host side, specific pheno- and endotypes of CRS have been described characterized by an imbalance of Th1 and Th2 function [71]. In CRSwNP patients, Staphylococcus aureus has been identified to unfold impact on the mucosal immune functions [10,68,70]. The relationship between the microbiome and mucosal immunity may be bidirectional, with pressure coming from the bacteria and inadequate defense from the host [70]. Research on how specific bacteria impact on the immune response of nasal and sinus mucosa may shed new light on the pathophysiology of CRS and may result in new strategies for its treatment.

The manipulation of microbiota or the introduction of specifically healthy microbiota may prove to be useful for the treatment of inflammatory disease [73]. Staphylococcus aureus and Pseudomonas aeruginosa are principal offenders in the development of persistent severe airway disease in CRS and CF patients. As bacterial resistance complicates the efficacy of antibiotics, the use of probiotic bacteria as colonizers and antimicrobial agents that may inhibit the growth of pathogenic bacteria awaits further development.

Abbreviations
CF: Cystic fibrosis; CRS: Chronic rhinosinusitis; CRSwNP: Chronic rhinosinusitis with nasal polyps; DNA: Deoxyribonucleic acid; RNA: Ribonucleic acid; rRNA: Ribosomal ribonucleic acid; bp: Base pairs; FISH: Fluorescence in situ hybridization; PCR: Polymerase chain reaction; qPCR: Quantitative polymerase chain reaction; T-RFLP: Terminal restriction fragment length polymorphism; BTEFAP: Bacterial tag-encoded FLX amplicon pyrosequencing.

Competing interests
The authors declare that they have no competing interests.

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