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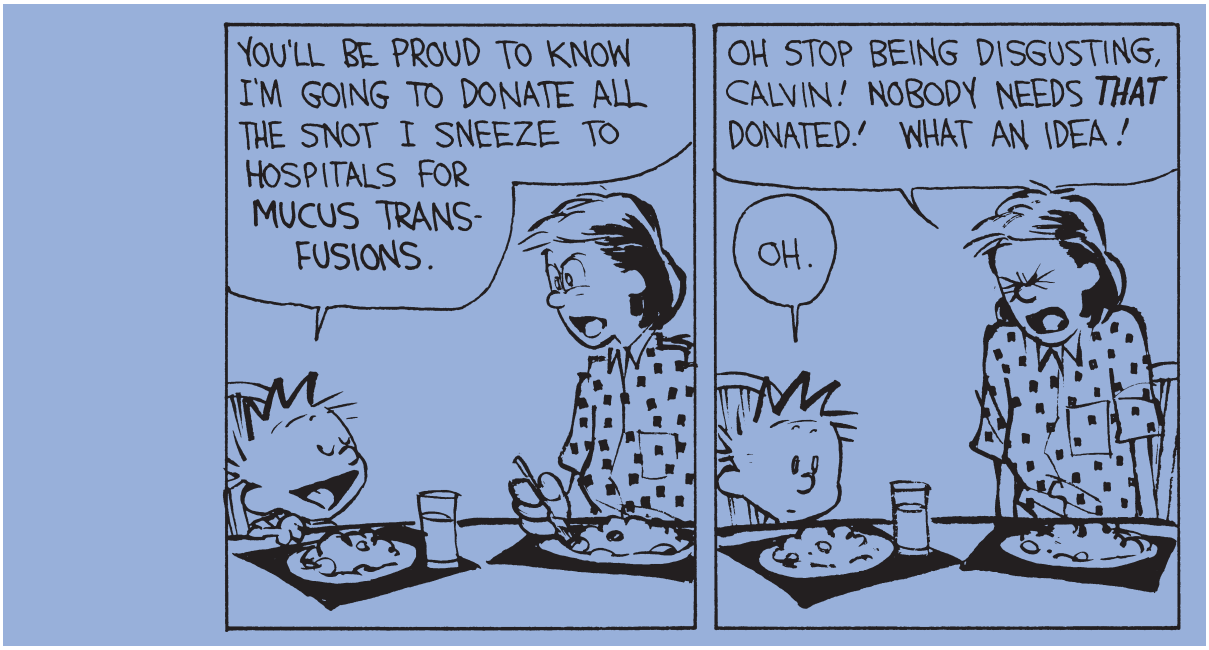

Topical probiotics in sinonasal disease

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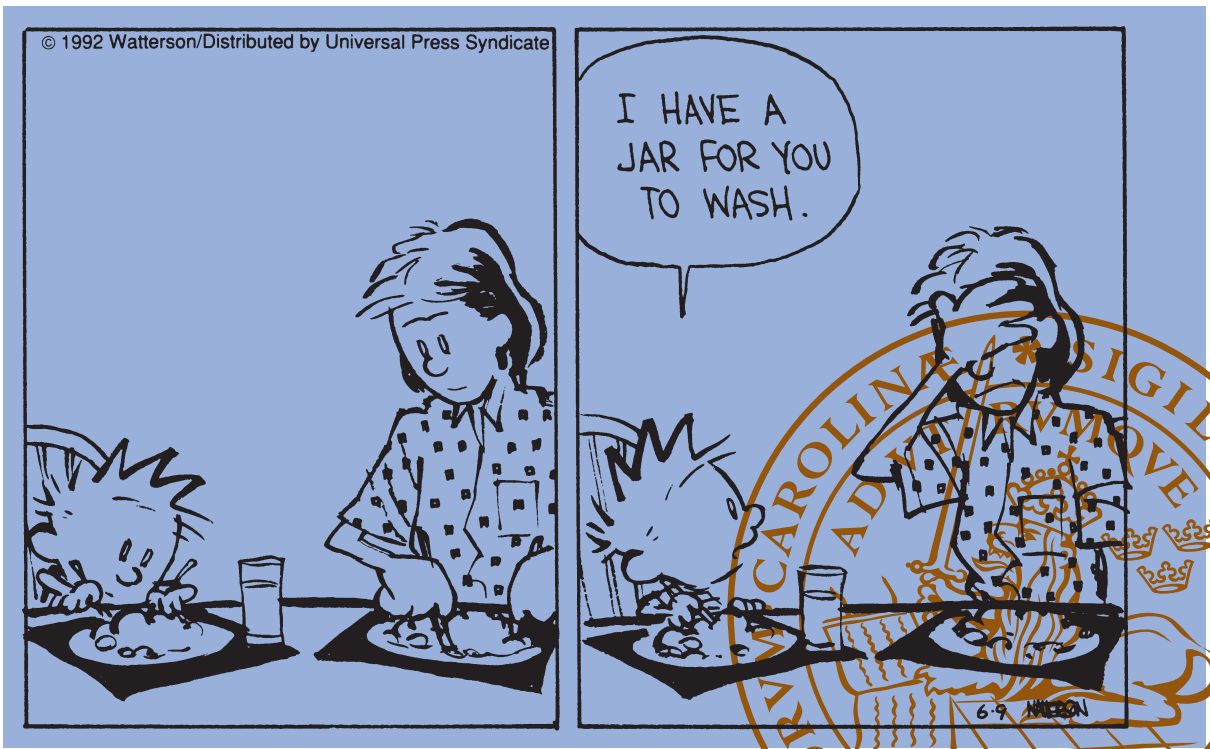

A nascent understanding of the commensal human microbiome has highlighted its importance in human health and disease. A dysfunctional relationship between the immune system and commensal bacteria has been implicated as an important factor in several inflammatory diseases and thus, also as a possible treatment target. This thesis investigates if topical probiotic manipulation of the sinonasal microbiome can alleviate symptoms of chronic rhinosinusitis and allergic rhinitis.

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Topical probiotics in sinonasal disease

Anders Mårtensson

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Background

Chronic rhinosinusitis (CRS) and allergic rhinitis (AR) are both diseases characterized by inflammation of the sinonasal mucosa. Both have a high prevalence in the Western world and are associated with impaired quality of life (QoL) and large societal costs. Recent findings suggest that the commensal microbiome has a profound impact on many aspects of human health and disease, especially on the immune system, and manipulation of the microbiome may provide means of treating CRS and AR.

Methods

The first two studies examined the effects of a topical probiotic assemblage (PA), i.e., LAB H13, derived from the stomach of honey-bees, on healthy subjects (**I**) and patients with CRSsNP (**II**). The third study investigated the effect of another topical PA on patients with allergic rhinitis in a nasal allergen challenge model (**III**). The final study (**IV**) explored the possibility of transplanting a sinonasal microbiome from healthy donors to patients diagnosed with recalcitrant CRSsNP.

Results

Nasal administration of the PA LAB H13 was well tolerated, did not produce an inflammatory reaction, and did not colonize the subjects (**I**, **III**). No effects on symptom scores or inflammatory markers of the CRSsNP patients were observed (**II**). The other PA induced a mild innate immune reaction in AR, but failed to affect symptoms during the NAC series (**III**). The sinonasal microbiome transplant procedure was well tolerated, produced an increased microbiome diversity, and significantly reduced symptom scores (**IV**).

Conclusions

Topical sinonasal probiotic manipulation is feasible and generally well tolerated. The two PAs examined did neither colonize the patients nor reduce symptoms in CRSsNP or allergic rhinitis. One PA candidate seemed to induce a mild innate immune reaction, while the other did not. Sinonasal microbiome transplant was also well tolerated and, unlike the PAs, was associated with significant effects on microbiome composition and reduced symptoms of CRSsNP.

Key words: Chronic rhinosinusitis, Allergic rhinitis, Microbiome, Probiotics, Transplant,

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Topical probiotics in sinonasal disease

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Table of content

Abbreviations

APC	Antigen presenting cell
AR	Allergic rhinitis
CFU	Colony forming units
CLR	C-lectin type receptor
CRS	Chronic rhinosinusitis
CRSsNP	Chronic rhinosinusitis without nasal polyps
CRSwNP	Chronic rhinosinusitis with nasal polyps
DC	Dendritic cell
ENT	Ear, nose, and throat
EPOS	European position paper on rhinosinusitis and nasal polyps
FeNO	Fractional exhaled nitric oxide
GALT	Gut-associated lymphoid tissue
IFN	Interferon
IL	Interleukin
LAB	Lactic acid bacteria
LPS	Lipopolysaccharide
MALT	Mucosal-associated lymphoid tissue
MAMP	Microbe-associated molecular pattern
MALDI-TOF	Matrix-assisted laser desorption/ionization-time of flight
MCID	Minimal clinically important difference
Mini-RQLQ	Mini rhino-conjunctivitis quality of life questionnaire
MS	Mass spectrometry
NAC	Nasal allergen challenge
NALT	Nasal-associated lymphoid tissue

NLR	NOD-like receptor
PA	Probiotic assemblage
PAMP	Pathogen-associated molecular pattern
PNIF	Peak nasal inspiratory flow
PROM	Patient-related outcome measure
PRR	Pathogen recognizing receptor
rRNA	Ribosomal ribonucleic acid
QoL	Quality of life
SCFA	Short chain fatty acid
SNOT-22	22-item sinonasal outcome test
Th cell	T-helper cell
TNSS	Total nasal symptom score
TLR	Toll-like receptor
Treg cell	Regulatory T-cell

Original articles

This thesis is based on the following publications, referred to in the text by their Roman numerals.

I. **Mårtensson A**, Greiff L, Lamei S, Lindstedt M, Olofsson TC, Vasquez A, Cervin A. Effects of a honeybee lactic acid bacterial microbiome on human nasal symptoms, commensals and biomarkers. *Int Forum Allergy Rhinol* 2016; 6: 956-963.

II. **Mårtensson A**, Abolhalaj, M, Lindstedt M, Mårtensson A, Olofsson TC, Vasquez A, Greiff L, Cervin A. Clinical efficacy of a topical lactic acid bacterial microbiome in chronic rhinosinusitis: A randomized controlled trial. *Laryngoscope Investig Otolaryngol* 2017; 2: 410-416.

III. **Mårtensson A**, Nordström FU, Cervin-Hoberg C, Lindstedt M Sakellariou C, Cervin A, Greiff L. Nasal administration of a probiotic assemblage in allergic rhinitis: A randomised placebo-controlled crossover trial. *Clin Exp Allergy* 2022; 52: 774-783.

IV. **Mårtensson A**, Cervin-Hoberg C, Huygens F, Lindstedt M, Sakellariou C, Greiff L, Cervin A. Upper airway microbiome transplantation for patients with chronic rhinosinusitis. *Int Forum Allergy Rhinol* 2023; 13: 979-988.

Thesis at a glance

Aim	Design	Principal finding
Paper I To assess tolerability of nasal administration of the LAB H13 microbiome in healthy subjects.	A randomized, placebo-controlled, double-blinded, crossover study.	LAB H13 was well tolerated and did not induce an inflammatory response nor colonize the subjects.
Paper II To examine effect of the LAB H13 microbiome as a treatment option for CRSsNP.	A randomized, placebo-controlled, double-blinded, crossover study.	LAB H13 was well tolerated, but did not affect symptoms, inflammatory indices, or commensal microbiota.
Paper III To investigate effects of a topical PA as treatment for allergic rhinitis in a NAC model.	A randomized, placebo-controlled, double-blinded, crossover study.	The PA induced an innate immune response, but failed to affect symptoms of allergic rhinitis.
Paper IV To study feasibility and effect of an upper airway microbiome transplant as intervention in CRSsNP.	An open pre-post interventional study.	The intervention was feasible. It induced lasting changes to the microbiota and affected CRSsNP symptoms.

Preface

Working as an ENT, Head & Neck surgeon with a special interest in rhinology, I often encounter patients diagnosed with chronic rhinosinusitis (CRS). I observe that these patients are suffering from their conditions, and the lack of effective treatment options often surprises me.

Topical corticoid sprays and saline rinses may dampen the patients' symptoms for a time, but often a sudden exacerbation brings them back to the clinic. On these occasions, a course of antibiotics often alleviates the symptoms temporarily, but at the same time, a culture sample taken from the nose and coated in purulent secretion, will most often yield a result of "no growth of any clinically relevant bacteria".

Frustrated by our apparent lack of understanding of this disease, and by a feeling of inadequacy to help my patients, I was very excited when my colleague and supervisor Professor Anders Cervin asked me to join in a project aiming at better understanding the role of the airway microbiota in CRS.

Introduction

Chronic rhinosinusitis (CRS) and allergic rhinitis (AR) are both examples of inflammatory diseases of the upper respiratory tract with a high prevalence in the Western world. Despite a considerable impact on quality of life (QoL), and representing substantial societal costs, both diseases are still incompletely understood. Recent investigations into the human commensal bacteria and their functions have revealed a deep connection between the human host and the microbiota, especially between bacteria and the immune system, locally as well as systemically. This nascent understanding of the importance of the human microbiome and its role in human health and disease offers exciting new perspectives to the understanding of CRS and AR and to potentially novel treatment options.

The purpose of this thesis is to better the situation for patients with CRS and AR through studies of a few aspects of the role of the microbiome and manipulation thereof. In **I** and **II**, we explored the possibility that topical probiotic supplementation might induce bacterial interference and reduce the pathobiome in CRSsNP. In **III**, we examined the potential to induce an immunologic skewing from a type 2 to a type 1 dominated response in AR using a topical probiotic assemblage. Finally, in **IV**, we explored the possibility that microbiome restitution through transplantation of a microbiome from a healthy donor might represent a potential therapy for patients suffering from CRSsNP.

Background

Chronic rhinosinusitis

Epidemiology and symptoms

CRS is an inflammatory condition of the sinonasal mucosa affecting 2-12% of the population (1, 2). The symptomatology includes nasal congestion, nasal discharge, and facial pain (1), but also hyposmia or anosmia, fatigue, sleep disturbance, depression, and sexual dysfunction (3), causing a severe impact on quality of life (QoL) comparable to congestive heart failure or chronic back pain (3, 4). CRS is associated with substantial societal costs, both as direct healthcare expenditures and as indirect productivity loss (5-7). Contrasting the large impact of CRS on QoL and socioeconomics, much is still uncertain regarding its underlying etiology. Indeed, current treatment protocols are based on observed effects rather than a mechanistic understanding of the disease.

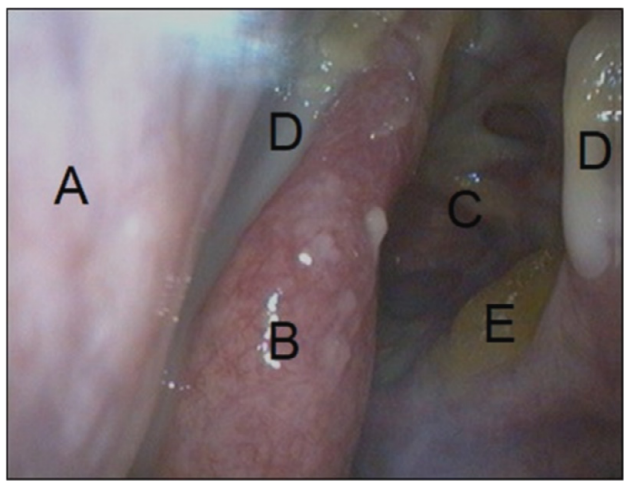

Figure 1. CRSsNP in a patient previously operated on with ethmoidectomy. Left-hand side nasal cavity with the nasal septum (**A**), concha media (**B**), and ethmoid area (**C**) (exposed following surgery). The condition is featured by purulent secretion (**D**) and crusting (**E**).

CRS is classically differentiated phenotypically, i.e., *based on any observable characteristic of the disease* (**Figure 1**), e.g., concerning whether or not polyps are observed, i.e., chronic rhinosinusitis with polyps (CRSwNP) or chronic rhinosinusitis without polyps (CRSsNP) (8). CRSsNP appears to be about two to four times more common than CRSwNP (2, 9). Recent studies identify different inflammatory patterns in patients suffering from CRS (10, 11), which now shift the focus of differentiation from a phenotypical towards an endotypical classification, i.e., *reflecting distinct functional or pathobiological subsets of CRS*. Arguably, endotypical classification is a step closer to identifying underlying causes and understanding the mechanisms of the disease.

Inflammatory profiles

The more or less detailed inflammatory endotypes of CRS are often termed types 1, 2, and 3, and primarily reflect what type of $CD4^+$ T-cells that are induced (12) (**Figure 2**). Type 1 is primarily induced by Th1 activation, but can also be induced by ILC1s; IFN-ɣ and IL-12 are important mediating cytokines (10, 12-14), type 2 is induced by Th2 activation, but ILC2s, eosinophils, B-cells, basophils, and mast cells may also be important; key mediating cytokines are IL-4, IL-5, and IL-13 (10, 12-14). Type 3 is induced by Th17 and ILC3s; IL-17A, IL-17F, and IL-22 are important mediators (10, 12-14). Importantly, these endotypes can be combined, and, in CRS, CRSwNP is the clearest case associated with a type 2 endotype (10).

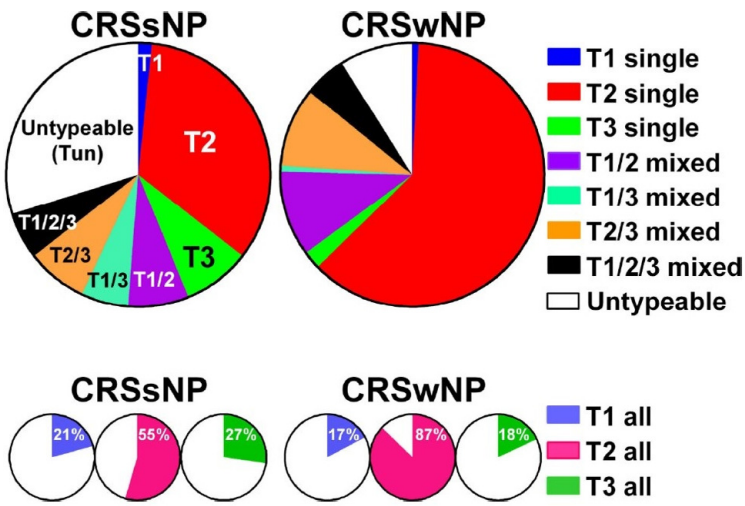

Figure 2. Inflammatory patterns of CRSsNP and CRSwNP in the Western world (10). Combination (top) and prevalence of type 1, 2, and, 3 endotypes (bottom). Reproduced with due permission from Elsevier.

In current practice, only data on eosinophil presence/activity in blood and sinonasal mucosa aid in such decision-making (13). Knowledge of specific aspects of inflammatory cascades, and their use for endotype classification (as described above), now potentially makes it possible to tailor the use of novel monoclonal antibodies, i.e., "biologics" in CRS (15). Indeed, all current biologics intended for CRS target type 2 inflammation, and consequently a "simple" endotypical differentiation into type 2 or "non-type 2" inflammation is recommended (12, 13). To reflect this, a new phenotypical classification based on whether the disease is primary or secondary and whether it is diffuse or localized is suggested by the 2020 European position paper on rhinosinusitis and nasal polyps (EPOS), possibly reflecting these endotypes (1, 8).

While endotypical differentiation may offer better insight into CRS patho-physiology compared with phenotypical classification, it still does not answer the question of what the underlying pro-inflammatory causes may be (13), which arguably is fundamental to the possibility of fully understanding and curing CRS. There is a large geographical variation regarding inflammatory patterns in CRS. In the Western world, Th2-induced inflammation is the dominant pattern, whereas Th1 and Th17 are more common in China (16). However, data are suggesting that the Th2 profile is becoming more prevalent with the adoption of a Western lifestyle (17).

Allergic rhinitis

Symptoms and costs

Allergic rhinitis is characterized by IgE-mediated symptoms of sneezes/itch, runny nose, and nasal blockage at exposure to allergens. Often, they are combined with symptoms of conjunctivitis and sometimes by post-nasal drip, itching of the palate, and cough (18, 19). Patients may experience reduced QoL, mainly from sleep disturbance secondary to other symptoms (18). A Swedish study estimated the annual economic burden of allergic rhinitis in 2016 to be € 961 per individual and year, or € 1.3 billion for the total Swedish population of 9.5 million (20).

The hygiene and Th1/Th2 hypotheses

Allergic rhinitis and other diseases characterized by chronic inflammation have become increasingly abundant in developed countries since the late 1950s (21). Up to 40% of the population in the Western world now suffers from either seasonal or perennial diseases (19). Epidemiological observations indicate that the increase correlates to decreased household size, increased standard of living, and improved

personal hygiene. These findings were put forward by Strachan in 1989 as the "*Hygiene hypothesis*" (22).

Murine studies suggest that the balance between Th1 and Th2 lymphocyte activities is an important immune regulator (23). In the context of the hygiene hypothesis, fewer infections may lead to less activation of Th1 immune responses (characterized by IFN-ɣ) and, in turn, to promotion of Th2 responses (featuring IL-4 and IL-5). Furthermore, the hypothesis is supported by findings that, e.g., patients with rheumatoid arthritis, a Th1-driven disease, have a low incidence of allergy(atopy (24), and that successful immunotherapy for pollen allergy is accompanied by increased IL-12 activity (25).

The hygiene hypothesis is certainly interesting, but it has also been criticized for being oversimplistic, and, e.g., for underestimating the role of antigen-presenting cells (APCs) (26). Other inconsistencies are that autoimmune diseases correlated to increased Th1 activity, e.g., juvenile type 1 diabetes, shall become less frequent with a shift towards Th2 activity, but that is not the case (27, 28). Taken together, the Th1/Th2 hypothesis is likely not a sole immune regulatory mechanism, and increasing interest has recently been given to commensal microbiota, especially of the gastrointestinal tract, in this context (29).

The microflora, old friends and biodiversity hypotheses

With a negative critique of the hygiene hypothesis, alternative interpretations of the epidemiological information have been presented, e.g., the "*Microflora hypothesis*", suggesting that improved hygiene standards, increased usage of antibiotics, and dietary changes have caused perturbations of the gastrointestinal microbiota, disrupting the development of early childhood immune tolerance (30). Increased incidence of asthma, allergy, atopic dermatitis, and type 1 diabetes, and mirrored decreases in incidence of childhood infectious diseases (**Figure 3**) (21), are suggested to represent causative relationships, as infections are needed to form a microbiota with adequate immune regulating capability (31). In contrast, a Danish study, examining the effects of early infections and the development of atopic dermatitis, shows an increased risk of atopic dermatitis associated with early infections, while other factors potentially associated with microbial expositions, such as many siblings, pet ownership, early day-care attendance, and growing up on a farm, all represent a risk reduction for the development of atopic dermatitis (32).

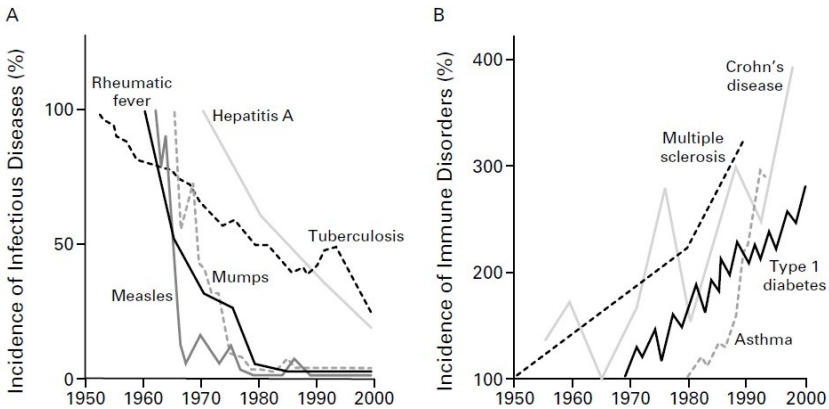

Figure 3. Incidence of infectious diseases and disorders associated with "type 1" immune activity from 1950 to 2000 (21). Reproduced with ¨permission from Massachusetts Medical Society.

Focusing on exposure to pathogens/infections, Rook *et al*. suggested that it is instead exposure to common, friendly organisms that train the immune system and induce tolerance, and forwarded lactobacilli, saprophytic environmental mycobacteria, and helminths as three especially important groups of organisms in the "*Old friends hypothesis*" (33). As an extension of the hygiene and microflora hypotheses, the "*Biodiversity hypothesis*" of von Herzen *et al*. suggests that the increase in inflammatory diseases correlates with a decrease in overall biodiversity, as the underlying cause (34).

Genetic factors, of course, also play a key role in the development of allergic diseases. Family history is the most dependable criteria for prognosticating whether a child will develop allergic rhinitis or not, and if more than one first-degree relative has allergic rhinitis the risk of developing the disease is more than three times greater than average. Multiple genes/loci have been associated with allergic rhinitis (35, 36), but there is only a risk of about 50% in the development of allergic rhinitis between monozygotic twins, indicating that while genetics play a big role, environmental factors are also important (37).

Pathophysiological and immunological aspects

APCs, such as dendritic cells (DCs), incorporate allergens and present peptides from these onto the major histocompatibility complex (MHC) class II. Naïve $CD4^+$ T-cells bind to the MHC and differentiate into allergen-specific Th2-cells (**Figure 4**). Activated Th2-cells produce cytokines, such as IL-4 and IL-13, which induce B-cells to produce allergen-specific IgE. IgE binds to the high-affinity Fc receptor for

IgE (FcεR) on mast cells, leading to their activation and the release of histamines, proteases, leukotrienes, prostaglandins, and other mediators.

Histamine, as an example of an important mediator, reacts with H_1-receptors on nerves, which leads to itching of the nose, eyes, and palate as well as sneezing. Similarly, its effects on nasal mucosal blood vessels and glands result in vasodilatation, plasma exudation, and glandular secretion, which is reflected by nasal blockage and rhinorrhea. An early phase typically occurs within 20 min after exposure to an allergen, while a late-phase response, initiated by cytokines such as IL-4 and IL-5 that promote attraction and activation of T-cells, eosinophils, basophils, neutrophils, and monocytes, occurs after 4-6 hours (19).

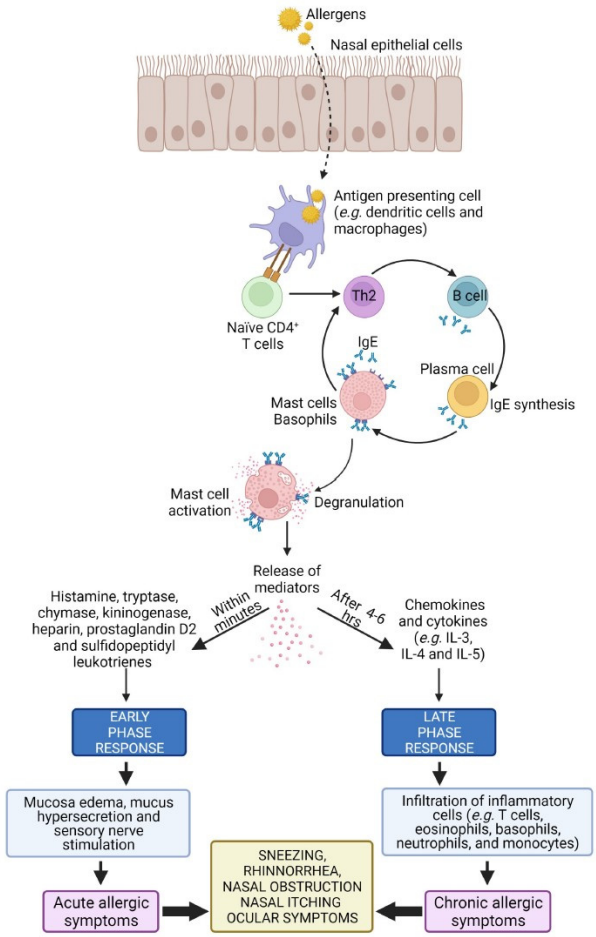

Figure 4. Graphic illustration of type 2 reaction. Adapted from Nur Husna *et al*. (19) and reprinted with permission under the Creative Commons Attribution License.

The sinonasal microbiome

Definitions

"Microbiota" is the assemblage of microorganisms in a defined environment (38). In contrast, there is no accepted definition of the term "microbiome" (39). Whipps *et al*. provided a first definition in 1988 and described the microbiome as a combination of the terms "micro", referring to "a characteristic microbial community", and "biome", "a defined habitat with distinct bio-physio-chemical properties". Therefore, the microbiome encompasses not only the microbiota, but also their "theatre of activity" (**Figure 5**) (40). "Sinonasal microbiome" in this text will refer to the microbiota of the nasal cavity and paranasal sinuses as well as its proteins, peptides, lipids, polysaccharides, nucleic acids, and microbial metabolites, proposed by Berg *et al*. (39).

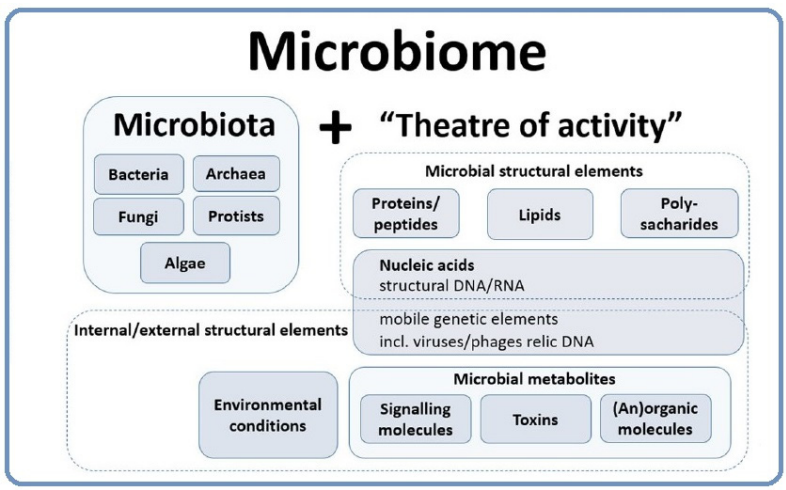

Figure 5. Microbiome encompassing both the microbiota and their "theatre of activity" in a defined habitat. Adapted from Berg *et al*. (39) and reprinted with permission under the Creative Commons Attribution License.

The sinonasal microbiome in health

In 1950, Björkwall published "Bacteriological examinations in maxillary sinusitis" (41), including a study on samples obtained from the maxillary sinuses of 54 healthy individuals. Bacteriological cultures demonstrated no growth, concluding, as for most previous findings, that "*healthy maxillary antra are sterile"* (41). In 1981, Brook was the first to present contradictory findings when he reported cultures of both aerobic

and anaerobic bacteria from the sinuses of twelve healthy subjects (42), and similarly Su *et al*. in 1983 reported growth of aerobic bacteria from seven healthy sinuses (43).

Perhaps because of the small sample sizes or perhaps of methodological criticism (42, 43), the belief that healthy paranasal sinuses are sterile persisted until 1999 when Jiang *et al*. published a report of bacterial growth in both swabs and biopsies from endoscopically normal maxillary sinuses (44). This is often referred to as the observation that showed that the healthy sinuses are not sterile, but as late as 2004 Otolaryngology–Head and Neck Surgery published a consensus statement regarding rhinosinusitis stating that "*the paranasal sinuses are believed to be sterile under normal conditions*" (45).

With the development of new genomic methods for analyzing the microbiome, a great number of studies have examined the sinonasal microbiota. In 2013, Ramakrishnan *et al*. examined the microbiota in 28 subjects without CRS using qPCR 16S ribosomal ribonucleic acid (rRNA) gene analysis. Bacteria were found in all samples representing the phyla Actinobacteria, Firmicutes, and Proteobacteria. Bacteroidetes were identified in 83% of samples, but at a significantly lower relative abundance. At species level, *Staphylococcus epidermidis*, *Staphylococcus aureus*, and *Propionibacterium acnes* were the most abundant along with several species of *Corynebacteria* (46).

In the same year, Yan *et al*. reported on the microbiota of three locations in twelve healthy subjects at four time-points (47). Similar to Ramakrishnan *et al*. (46), Actinobacteria, Firmicutes, and Proteobacteria were the most common phyla identified. The two intranasal locations, i.e., the middle meatus and the sphenoethmoidal recess, lined by ciliated pseudo-stratified columnar epithelium, had an almost identical bacterial composition, while the anterior nares, lined by a non-keratinized squamous cell epithelium, showed a greater proportion of Actinobacteria and Firmicutes, with less Proteobacteria and other phyla. The results by Ramakrishnan *et al*. and Yan *et al*. were supported by Bassis *et al*. in 2014 and Hoggard *et al*. in 2016 (46-49).

In a large multicentre study, the findings of what bacterial phyla constitute the healthy sinonasal microbiome, were refined to a "core microbiome" consisting of five bacterial genera, *Corynebacteria*, *Staphylococcus*, *Streptococcus*, *Haemophilus*, and *Moraxella*, where *Corynebacteria* and *Staphylococcus* appeared particularly dominant (50). This "core microbiome" was fairly consistent in participating centres across the world. Similar to these findings, another multicentre study found *Staphylococcus*, *Corynebacteria*, and *Moraxella* to be the most prevalent genera across the upper respiratory tract and that subjects, age, and sex had a statistically significant impact on the composition of the microbiota (51).

The sinonasal microbiome in chronic rhinosinusitis

Su *et al*. reported growth of anaerobic bacteria in sinusitis (43). The finding was confirmed in the first study involving genomic detection to evaluate the microbiota in CRS compared to healthy sinuses (52). More important than anaerobic bacteria as potential pathogens in CRS, they also reported on a vastly broader range of bacteria than traditional culture techniques and that accepted notions of bacteriology of CRS and normal sinuses needed to be re-evaluated (52).

Using the new genomic technique, in 2012, Feazel *et al*. and Abreu *et al*. compared CRS patients to healthy controls. Both reported differences in bacterial composition between the groups: Feazel an altered bacterial composition in CRS; Abreu a significantly reduced diversity, evenness, and abundance of the microbiota in CRS as well as an increased relative abundance of *Corynebacterium tuberculostearicum* (53, 54). Together, the studies indicate that a depleted or dysbiotic composition of the natural microbiota may be a promotor of CRS.

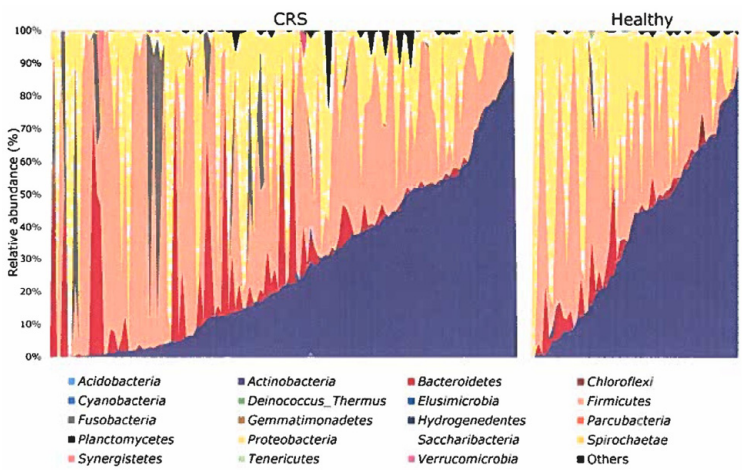

Figure 6. Plot of the most abundant bacterial phyla in CRS (n=131) and healthy (n=58) samples (55). The plot illustrates a high degree of interindividual variation of the microbiota as well as a clear similarity on the group level. Reproduced with permission from John Wiley & Sons.

Since then, several studies have examined the microbiota in patients with CRS in comparison to healthy controls. Often slight differences in diversity or relative abundance of various bacterial species have been reported, but none of these findings have been consistent (49-51, 56-60). A meta-analysis by Wagner-Mackenzie *et al*. of eleven studies showed large interindividual variations in the composition of the sinonasal microbiota for both CRS patients and healthy subjects,

but very similar results regarding the microbiota composition between groups (**Figure 6**) (55).

The current view on the sinonasal microbiome was recently summarized by Psaltis *et al*. as a "core microbiota" consisting of the five genera *Corynebacteria*, *Staphylococcus*, *Streptococcus*, *Haemophilus*, and *Moraxella* is emerging, where *Corynebacteria* and *Staphylococcus* appear particularly dominant. This "core microbiota" appear consistent for the paranasal sinuses and the nasal cavity including the anterior nares (61). However, there are no consistent findings of increased or lowered abundance or diversity in health compared to CRS and no confirmed specific pathogenic or protective bacterial species (61).

The interest in the sinonasal microbiome has led to an increase in both the number of studies as well as the size of the studies. However, they have almost exclusively focused on bacteria. Accordingly, studies on archaea and fungi, are less common. Aurora *et al*. examined the microbiome including fungi and archaea in CRS and healthy controls. They reported an increased bacterial load and an increased bacterial diversity in CRS patients compared to healthy controls. Furthermore, the same association was found also for archaea and fungi: archaea made up 43% of the bacterial abundance (62).

Two other studies examining CRS patients identified archaea in 17/19 (89%) and 4/28 (14%) of them, with a maximum relative abundance of 2.4% and mean relative abundance of 0.04% (46, 63). Boase *et al*. as well as Aurora *et al.* concluded that the role of fungi in CRS is limited (62, 64). Regarding CRS and viruses, the literature shows divergent results. Three studies detect respiratory viruses more often in CRS patients than in healthy controls: 64% *vs*. 30%, 15% *vs*. 7% and 24% *vs*. 0% (65-67). The two with the highest (75%) and lowest (0%) detection rates show no difference between CRS and healthy controls (68, 69). Two studies explored seasonal variations and showed significantly higher viral detection rates in the winter than in the summer (66, 70). While it is clear that respiratory viruses cause upper airway inflammation *per se*, nothing regarding a potential role for viruses, fungi, or archaea in CRS can be concluded.

The sinonasal microbiome in allergic rhinitis

Choi *et al*. examined the nasal microbiota in patients with seasonal allergic rhinitis before and during allergy season compared to non-allergic controls. No difference was observed outside of pollen season, but during pollen season patients with allergic rhinitis had an increased microbial diversity of the meatus media microbiota, which correlated with an increase in nasal eosinophils (71). In another study of the intranasal microbiota, no difference between healthy subjects and patients with allergic rhinitis could be found, but this study was conducted without consideration of seasonal pollen exposure (59). No persistent differences between

the microbiome of healthy subjects and patients with allergic rhinitis appear to exist but, probably transient changes linked to inflammation during pollen season.

The development of the sinonasal microbiome

Though no specific examinations regarding the fetal sinonasal microbiota have been conducted, recent findings show that already *in utero*, lungs, and gut harbor several culturable bacterial genera such as *Gardnerella*, *Lactobacillus*, *Staphylococcus*, and *Streptococcus*, and that these affect the developing immune system (72). Sampling 24 hours postpartum shows a dominance of *Streptococcus*, which during the first week of life develops into a sinonasal niche-specific microbiota dominated by *Moraxella*, *Streptococcus*, *Haemophilus*, *Staphylococcus*, *Corynebacterium*, and *Dolosigranulum* (73, 74).

The microbiota in infancy is affected by the mode of delivery and feeding, but differences are transient (74-76). A high proportion of *Corynebacteria* and *Dolosigranulum* in the microbiota is associated with vaginal delivery and breastfeeding, while cesarean delivery and formula feeding delay the decline of the proportion of *Streptococcus*. In turn, a *Corynebacteria*/*Dolosigranulum*-dominated microbiota is associated with fewer airway infections during infancy than a *Streptococcus*-dominated profile (75, 77). From childhood to adulthood, the microbiota changes from dominated by *Streptococcus*, *Dolosigranulum*, *Moraxella*, *Haemophilus*, *Neisseria*, and *Bacteroidetes* to *Corynebacteria*, *Propionibacteria*, and *Turicella* (78).

The microbiome and the immune system

The mucosal firewall

A great part of the function of the immune system is dedicated to containing a commensal microbiota. Accordingly, an important strategy is to keep it away from the epithelial cells. To achieve this, epithelial cells produce mucus and antimicrobial peptides (79). Furthermore, dendritic cells react to commensal microbial antigens and interact with B-cells to produce commensal-specific IgA. This localized mucosal function allows for a strong immune response to commensal microbes that penetrate the epithelia and, at the same time, does not trigger a systemic immune response (80).

Nasal mucosal immunity

Mucosal-associated lymphoid tissue (MALT) is present at mucosal surfaces throughout the body. In the gut, they constitute Peyer's patches, referred to as gut-associated lymphoid tissue (GALT). The GALT in germ-free mice is reduced, suggesting the important role of microbiota in shaping mucosal immunity (79).

Similar to the studies of GALT in germ-free mice, other murine studies suggest the importance of the microbiome also in the development of nasal mucosal immunity (81). The nasal mucosal immune system consists of inductive sites and effector sites. The inductive sites make up of mucosal lymphoid follicles and are referred to as nasal-associated lymphoid tissue (NALT).

In humans, NALT makes up the adenoid and the lingual/palatine tonsils. It consists of epithelial cells and invaginated microfold (M) cells, which function as a transmembrane transport of antigens encapsulated in vesicles. As the vesicles are emptied on the basolateral aspect of the M-cells, antigens are sensed by APCs such as dendritic cells, macrophages, and B-cells (82, 83).

The effector sites are where the immune response of B- and T-cells take place, and include the lamina propria and the intraepithelial layer of the respiratory mucosa (82).

Innate and adaptive immunity

Innate sensing of microbes, pathogens, or associated products is mediated through activation of PRRs recognizing molecules usually found on microbes, MAMPs, or pathogens, PAMPs. PRRs include Toll-like receptors (TLRs), C-type lectin receptors (CLRs), Nod-like receptors (NLRs), RIG-I-like receptors, and cytosolic DNA-receptors. (84, 85). Upon ligand binding, PRRs induce intracellular signaling pathways resulting in different expressions of pro-inflammatory and polarizing cytokines. PRRs are of essential importance to the induction of Th1 and Th17 responses. Contrasting this, Th2-mediated responses can be induced in the absence of PRR-induced signaling (86).

Commensals train and regulate the development and maturation of the adaptive immune system. Homeostasis depends on adaptive immune cells, especially regulatory T (Treg)-cells and T follicular helper (TfH)-cells, coordinating with dendritic cells (DCs), IgA-producing B-cells, and innate lymphoid cells (ILCs), to maintain homeostasis and a mutualistic relation between host and microbiota (87-89). Activation of mucosal adaptive immunity also allows for the training of local tissue-resident memory (TRM) T-cells, central to mucosal immunity. Local TRM T-cells allow for a faster local immunologic response than through systemic immunity, and can be utilized to make vaccines for airway pathogens more effective, e.g., Covid-19 vaccines (82).

How the immune system differentiates the activation of PRRs by ligands between commensal and pathogens is not fully understood, but may reflect pathogenicity and localization (90). The differentiation of commensals from pathogens is probably contextual (79), and that PRR activation leads to an inflammatory response may very well be the exception rather than the rule. (90).

Metabolites

An increasing body of evidence points towards microbes regulating the host immune system through crosstalk via metabolites and host receptors (61). Indeed, metabolites appear to have a greater effect on microbe-host interaction than the microbes themselves (91). Their effects on the immune system may be divided into three categories. 1. Metabolites produced by microbial metabolism. 2. Metabolites produced by the host and biochemically altered by microbes. 3. Metabolites synthesized *de novo* by microbes (61).

The human genome encodes 17 enzymes for carbohydrate degradation. In contrast, the genomes of certain bacteria, e.g., *Bacteroides thetaiotaomicron,* encodes more than 250 such enzymes. With the commensal microbiota comprising hundreds of different bacterial species, the enzymatic combinations available to break down carbohydrates for nourishment are enough to digest most carbohydrates in the diet and to provide a large number of metabolites (92).

Microbial metabolization of carbohydrates, specially studied in the gut, but present also in the sinuses (93), produce short-chain fatty acids (SCFAs), which act as ligands to several cell surface receptors expressed by myeloid cells such as neutrophils, macrophages, and dendritic cells (92). Furthermore, SCFA affects tight junctions of epithelial cells (94). Bacteria from different phyla tend to produce different SCFAs. For example, Bacteroidetes produce acetate and propionate, while Firmicutes produce butyrate.

Many microbes are efficient also at metabolizing proteins, resulting in oligopeptides and amino acids functioning as ligands to the aryl hydrocarbon receptor precursor (AhR). Through AhR, these metabolites can affect ILC3 and the production of IL-22 (90). Retinoic acid is a lipid metabolite from the degradation of vitamin A, which exerts immune regulation both through its ability to control Treg cell development through TGF-β and through the production of IgA by B-cells and which in turn also affects the activity of Th17-cells (90).

Metabolites produced by the host, and biochemically altered by commensal microbiota, are well described for gut bacteria, where bile salts are converted from primary to secondary bile salts and exert immune-modulatory functions on monocytes, macrophages, and dendritic cells. Bile salts, and their immune regulatory properties, are suggested to play an important role in the development of inflammatory bowel disease and in the development of immunotolerance (92, 95).

Adenosine triphosphate (ATP), a metabolite produced *de novo* by necrotic or stressed microbes induces release of proinflammatory cytokines from immune cells, mainly with pro-inflammatory effects (95). Polysaccharide A (PSA), a metabolite produced *de novo* by bacteria in the gut, regulates the release of the anti-inflammatory cytokine IL-10 from $CD4^+$ T-cells and activates TLR-2 on dendritic cells, causing a release of cytokines and driving production of IL-10 by T-cells (95).

Probiotics

History and definitions

The idea that consumed microorganisms may have a beneficial effect on human health was first suggested by Metchnikoff in 1907, proposing that Bulgarian farmers lived long due to eating yogurt (96). The term "probiotics" was introduced by Lilly & Stillwell in 1965 (97). Since then, many definitions of what constitutes probiotics have been forwarded. The current definition of the International Association for Probiotics and Prebiotics (ISAPP) is: "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (98). Later, ISAPP concluded that probiotics are a defined consortium and that the microorganisms must be viable (98).

The idea that probiotic bacteria must be viable has proved to be problematic in a commercial context and has led to the development of "post- and parabiotics". Postbiotics are defined as "non-viable bacterial or metabolic products from micro-organisms that have biological activity in the host", while parabiotics, a.k.a. ghost or inactivated probiotics, are "non-viable microbial cells (intact or broken) or crude cell extracts, which when administered (orally or topically) in adequate amounts, confer a benefit on the human or animal consumer" (99). Historically, mainly *Lactobacillus* and *Bifidobacterium* strains have been used as probiotics (100). These are, for healthy persons, generally considered safe (101, 102).

Current research is exploring other genera for probiotic use such as e.g., *Streptococcus*, *Dolosigranulum*, *Bacteroides*, and *Clostridium* (99, 100), which will require individual safety assessments depending on species and intended use (101). Another option for manipulating the commensal microbiota is through prebiotic supplementation. Prebiotics are molecules, usually nutrients, that affect the composition and function of the microbiota in a beneficial way, which can be used alone or in addition to probiotics (103, 104).

Ecologic concepts for microbiome restitution

Existing concepts speak of two important types of colonizers. “Pioneers” are microbiota that are the first to colonize a newly established (pristine) microbiome or a microbiome reorganizing itself after disturbance (succession). “Keystone” bacteria are microbiota exerting an effect on the community that is much more significant than their abundance. Both pristine and succession are considered favorable conditions to reseed the microbiota (105, 106).

Probiotic interactions with the host

Probiotics interact with the host either via PRRs present on the epithelial surface or by affecting epithelial integrity via the proteins that hold the epithelial cells together, i.e., tight junctions (TJs), adherence junctions (AJs), and desmosomes (**Figure 7**). Specifically, probiotics increase claudin 1 and zonula occludens and decrease occludin, strengthening tight junctions. Also, probiotics increase E-cadherin and β-cadherin, and reduce protein kinase C, strengthening adherence junctions (100, 107).

Probiotics may also affect the immune system through interaction with innate immune cells present between epithelial cells or in the submucosal region, e.g., in the case of bacterial translocation (100). Thus, probiotics modulate host immune responses locally and systematically via MAMPs and PRRs. MAMPs include LPS, flagellin, CpG-DNA, and other surface proteins. PRRs include TLRs, CLRs, NLRs, RIG-I-like receptors, and cytosolic DNA-receptors (100, 108). Probiotics may also dampen the inflammatory cascade by stimulating the production of anti-inflammatory mediators, e.g., SCFAs, which exert anti-inflammatory functions through regulation of NF-κB activity (105, 109).

Probiotics affect the immune system of the host in several ways. Probiotics are capable of modulating Th1/Th2 balance and regulatory T-cell immune responses, leading to a reduction in Th2 inflammatory cytokine levels (IL-5 and IL-13). Immunomodulatory functions also include stimulation of mucosal IgA and allergy-specific B- and T-cells (100, 110). Probiotics have been shown both to increase and decrease type 1 immunity, to increase or decrease the production of cytokines (IFN-ɣ, IL-6, and TNF-α) (100, 109), and to downregulate type 2 immunity by reducing allergy-specific IgE and pro-allergic cytokine production (IL-4, IL-5, and IL-13), switching to IgG and IgA production (109). Finally, probiotics can restore the function of allergy-specific $FoxP3^{+}$ Treg-cells and increase the production of regulatory cytokines IL-10 and TGF-β, causing an anti-inflammatory effect (100, 101, 108, 109).

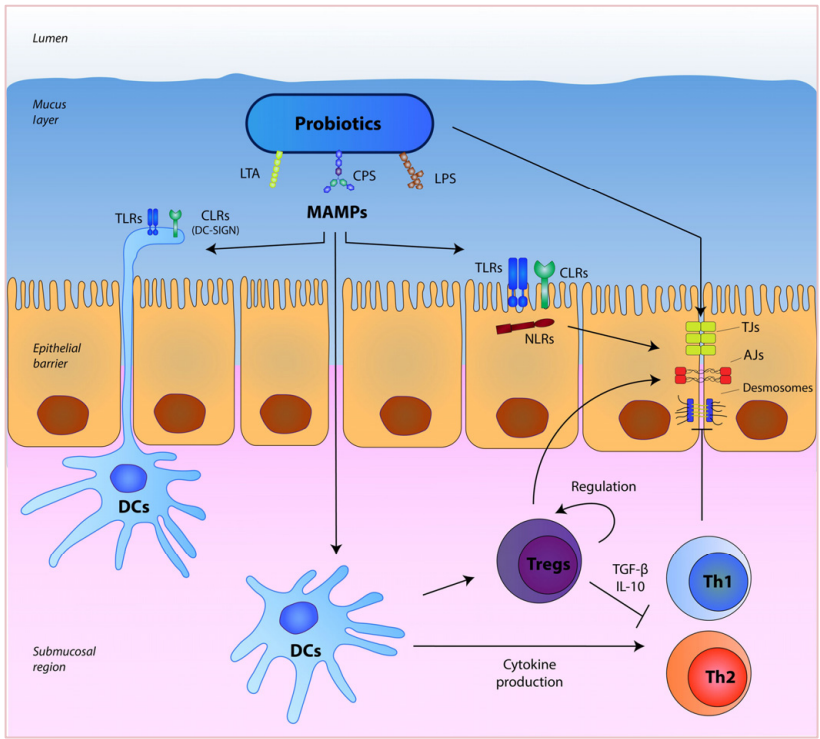

Figure 7. Probiotics express various sets of surface proteins, termed MAMPs, e.g., lipopolysaccharides (LPS), cell wall-associated polysaccharides (CPS), and lipoteichoic acid (LTA), which cause strain-specific interactions with the host when recognized by PRRs (e.g., TLRs, CLRs, and NLRs) of epithelial and immune cells. Specific signaling cascades trigger different molecular responses that induce activation of Tregs and affect Th1 and Th2 activity (100). Reproduced with permission from John Wiley & Sons.

Probiotic interactions with the microbiome

Probiotics can influence the existing microbiome through competition (105). A bacterial species is classified as competitive if it exhibits features that are negative to a competing species. Competition is divided into active, where the bacteria are actively damaging each other, or passive, where one species hinders another by consumption of nutrients or altering conditions that impact another species negatively (111).

For bacterial competition to occur certain conditions must be met, such as a high degree of overlap in terms of nutritional or spatial needs, a bacterial density enough to create a lack of resources, and a bacterial diversity enough to create competition. Bacterial competition in turn helps form stability either by a winner, resulting in a

persisting monoculture, or by the establishment of nutritional or spatial bacterial niches (111). Ecologic stability is usually viewed as a desired state but can be problematic when trying to induce a probiotic species or to restitute a dysbiotic state (112). Bacteria compete in several different ways including competition of nutrients, e.g., iron (106, 111, 112). Other means of competition include the production of quorum-sensing molecules that help compete with other bacterial and fungal species, production of molecules that interfere with the quorum-sensing of other bacterial species (106, 111), or the production of antimicrobial products, such as bacteriocins or microcins, which target specific bacterial species competing for a particular niche. These molecules can either be secreted in the area surrounding the bacteria or by contact-dependent methods to inject the molecules directly into other bacteria and promote cell lysis (105, 106, 111-113). Bacteria can compete for adhesion to the host cell surface by producing surfactants or other adhesion and anti-adhesion molecules (105, 106, 111, 112). Further bacteria can gain a competitive advantage by producing products to alter the environment, e.g., the production of lactic acid to lower Ph (105, 111).

In conclusion, probiotics overall have demonstrated abilities to affect both the host directly, commensal microbiota, and potential pathogens in a variety of ways, but effects are strain-specific as well as dependent on the resident microbiome and host genetics (114).

Aims

Purpose

To improve the situation for patients with CRS and AR through manipulation of the sinonasal microbiome using probiotics or microbiome transplantation.

Specific aims

I. To assess effects of a single nasal administration of the PA LAB H13 in healthy subjects focusing on tolerability, inflammatory indices, and impact on the commensal microbiota.

II. To examine effects of the PA LAB H13 as a topical treatment option for patients suffering from CRSsNP, with regard to symptoms, inflammatory indices, and impact on the existing microbiota.

III. To study effects of a topical PA as a treatment option for allergic rhinitis in a NAC model, focusing on symptoms, nasal PIF, inflammatory indices, and colonization.

IV. To explore feasibility and effect of sinonasal microbiome transplantations from healthy donors to patients with recalcitrant CRSsNP, with regards to symptoms, inflammatory indices, and impact on the existing microbiota.

Materials and methods

Paper I

On safety and tolerability of topical, nasal PA LAB H13 in healthy subjects. For this randomized, placebo/sham-controlled, and double-blinded study of crossover design, 22 subjects were recruited. The subjects had no history of chronic upper respiratory tract disease or recent nasal symptoms in the four weeks leading up to the study.

An E-swab from the middle meatus was collected to assess the microbiota, symptoms were measured using the SNOT-22 questionnaire, and a nasal lavage was performed for analysis of inflammatory markers. The subjects then received the probiotic LAB H13 ($1x10^{11}$ CFU/mL) or placebo using a spray device.

Twenty-four hours after the LAB H13 administration, the subjects were reassessed for nasal symptoms using TNSS, another E-swab was collected to assess the microbiota, and a nasal lavage was performed for analysis of inflammatory markers. Thirteen days later, the subjects were again sampled using an E-swab for microbiota and SNOT-22 for symptoms.

After a two weeks' wash-out period, the study continued with the subjects who received LAB H13 the first time now receiving placebo and *vice versa*.

E-swab samples were diluted and cultured in aerobic and anaerobic conditions. Bacterial colonies were counted and then identified using MALDI-TOF MS using the Skåne University Hospital Bacterial Library. Nasal lavage fluid samples were analyzed using Luminex profiling with a multiplex human cytokine panel.

The study was approved by the regional Ethics committee in Lund, Sweden (2013/487).

Paper II

On effects of topical, nasal PA LAB H13 as treatment-option for CRSsNP. For this randomized, placebo-controlled, and double-blinded study of crossover design, 20 patients were recruited. Inclusion criteria were CRSsNP, according to the 2012

EPOS guidelines. Treatment with antibiotics in the last 14 days before the study start and findings of nasal polyps were exclusion criteria.

Patients were assessed for symptoms using SNOT-22, examined by an ENT specialist, sampled from the middle meatus using an E-swab for microbiota, and subjected to a nasal lavage for inflammatory indices. They then started treatment with either LAB H13 ($1x10^{11}$ CFU/mL) or placebo twice daily. After 14 days' treatment, they were once again examined as described above.

After four weeks' wash-out, the study continued, with patients receiving spray containing H13 LAB the first time now receiving placebo and *vice versa*.

E-swab samples were diluted and cultured in aerobic and anaerobic conditions. Bacterial colonies were counted and identified using MALDI-TOF MS, using the Skåne University Hospital Bacterial Library. Nasal lavage fluid samples were analyzed using Luminex profiling with a multiplex human cytokine inflammation panel.

The study was approved by the regional Ethics committee in Lund, Sweden (2013/487).

Paper III

On effects of a topical, nasal PA in a NAC-model as a treatment option for seasonal allergic rhinitis. For this randomized, placebo-controlled, and double-blinded study of crossover design, 24 patients were recruited. Patients were examined by an ENT specialist, sampled from the middle meatus using an E-swab for microbiota, and subjected to allergen titration for the NAC-model.

Baseline data for Mini-RQLQ, TNSS, FeNO, and PNIF were collected as well as blood for total and allergen-specific IgE and nasal lavages for analysis of inflammatory markers. The patients then started treatment with either a PA, of *Lactobacillus rhamnosus* SP1*, Lactobacillus paracasei* 101/37, *and Lactococcus lactis* L1A at $9.5x10^{10}$ CFU/mL, or placebo. After two weeks', the measurements described above were repeated. A NAC-series then started with recordings of PNIF and TNSS 10 minutes after each NAC.

After four weeks' wash-out, the study continued, with patients receiving spray containing the PA the first time now receiving placebo and *vice versa*.

E-swab samples were diluted and cultured in aerobic conditions. Bacterial colonies were identified using MALDI-TOF MS to detect colonization by any strains of the probiotic assemblage. Nasal lavage fluid samples were analysed using Luminex profiling with a multiplex human cytokine panel.

The study was approved by the Swedish Ethical Review Authority (2019/04204).

Paper IV

On effects of an upper airway microbiome transplant as an intervention for CRSsNP. For this study of an open pre-post interventional design, 22 patients were recruited along with 22 healthy donors. Inclusion criteria were according to the 2012 EPOS guidelines plus a continued uncontrolled disease despite maximal medical therapy and sinus surgery with bilateral middle meatus antrostomies and ethmoidectomies. Exclusion criteria were antibiotics in the last four weeks, nasal polyposis, and immunodeficiency.

The recruited donors had no history of airway disease other than episodes of the common cold for the last two years, and no treatment with antibiotics for the last four weeks leading up to the study. Furthermore, all donors (as well as patients) had to match on a pre-treatment pathogen scan to minimize the risk of transferring any infections.

Patients were examined and evaluated with SNOT-22, TNSS, E-swab sampling of the middle meatus, and nasal lavage. The patients were also filmed via an endoscope. They then started a 13 days' course of antibiotics. Thirteen days later, examinations were repeated, and the first of five daily transplant procedures were performed. The examinations were performed 10 and 90 days after the last transplant procedure.

The transplant procedure involved the donor holding 15 ml saline inside the nasal cavity for 5 minutes using a Nasaline device. The lavage was then collected, split into two halves, and diluted with another 15 ml saline. The patient then, using half of the diluted transplant for each side, held the saline in the nasal cavity for five minutes on each side using a Nasaline device.

Endoscopic recordings were graded by an experienced ENT surgeon, according to the Lund-Kennedy endoscopic grading scale. E-swab samples were frozen and sent for DNA extraction and 16S rRNA gene analysis. Nasal lavage fluid samples were analyzed using a multiplex human cytokine panel.

The study was approved by the regional Ethics committee in Lund, Sweden (2016/674) and registered at clinicaltrials.gov (NCT03122795).

Results

Paper I

A single nasal administration of the probiotic did not produce any symptoms as measured with TNSS or SNOT-22 compared to sham (p=0.154) (**Figure 8**). Furthermore, it did not induce any inflammatory response, as reflected by unaffected immunological markers in nasal lavage fluids. The probiotic did neither affect the commensal microbiota nor colonized the upper respiratory tract.

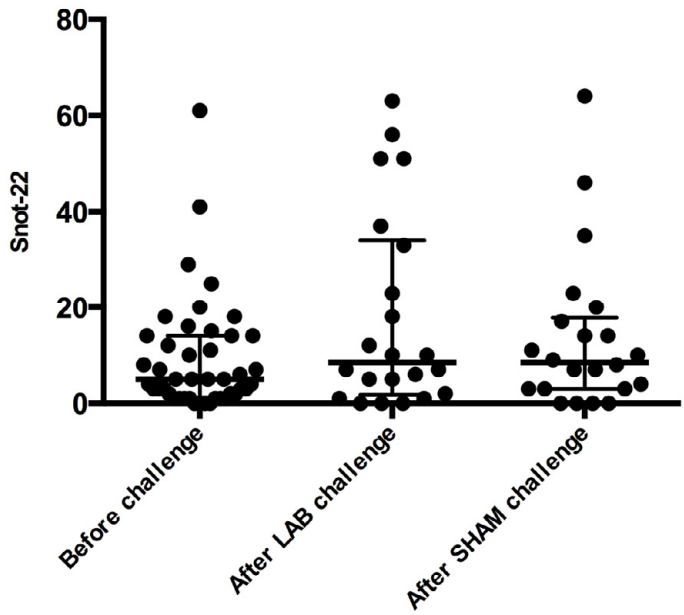

Figure 8. No statistically significant differences in individual SNOT-22 scores two weeks after the challenge with the probiotic LAB H13. Reprinted with permission from John Wiley & Sons.

Paper II

Two weeks' treatment with a nasal spray containing the probiotic LAB H13 twice daily did not affect symptoms as measured with SNOT-22 compared to placebo (p=0.082) (**Figure 9**). Neither did it affect the composition of the microbiota (p=0.097) (**II**) or local inflammatory activity (**Figure 10**).

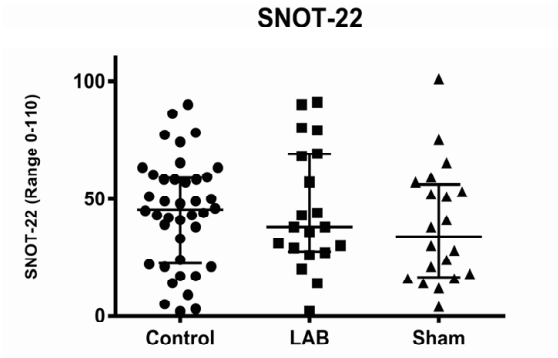

Figure 9 No statistically significant difference in individual SNOT-22 scores after two weeks' treatment with the probiotic LAB H13. Reprinted with permission from John Wiley & Sons.

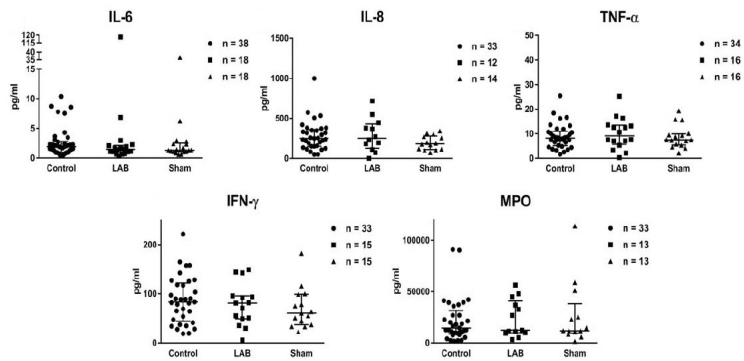

Figure 10. No statistically significant differences in inflammatory markers in nasal lavage fluids were observed between LAB H13 and placebo: IL-6 (p=0.890), IL-8 (p=0.074), TNF-α (p=0.380), IFN-γ (p=0.391), and MPO (p=0.966). Reprinted with permission from John Wiley & Sons.

Paper III

Two weeks' treatment with a PA before and during one week' NAC series did neither affect Mini-RQLQ (p=0.84), Mini-RQLQ nasal domain (p=0.94), TNSS (p=0.98), nor PNIF (p=0.86) compared to placebo (**Figure 11** and **12**). Neither was any statistically significant difference observed for FeNO, total IgE, or specific IgE (**III**).

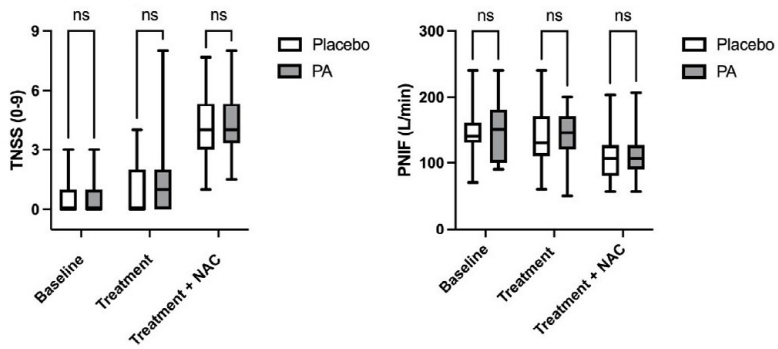

Figure 11. No significant differences were observed for TNSS or Mini-RQLQ between treatment with the PA and placebo at baseline, after two weeks' treatment, or after one week' NAC series. Reprinted with permission from John Wiley & Sons.

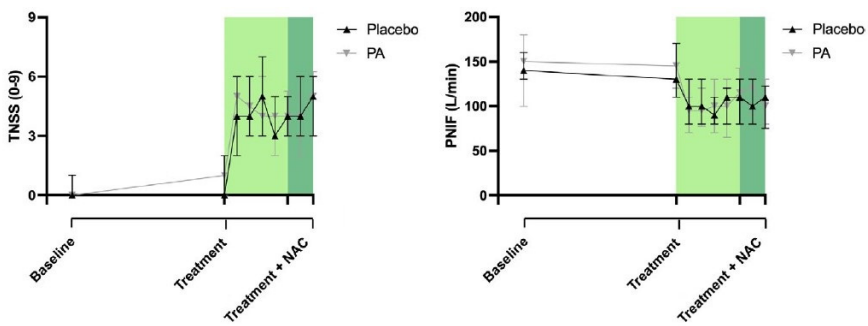

Figure 12. No significant differences were observed for TNSS or PNIF between treatment with the PA or placebo at baseline, after two weeks' treatment, or after one week' NAC series (marked green). The analysis during the NAC series was based on the last three days (dark green). Reprinted with permission from John Wiley & Sons.

Regarding immunological markers in nasal lavage fluids, a slight increase in IL-17/IL-17A after 2 weeks' treatment with the PA was observed compared to placebo. Statistically significant increases were seen for TNF-α, MIP-1α, MIP-1β, MCP-1, IL-6, IL-8, IL-10, and ST2 in the PA run compared to baseline, while statistically significant increases for MCP-1 and IL-6 were observed in the placebo run compared to baseline.

Paper IV

Three months after treatment with 13 days of antibiotics followed by five consecutive days of sinonasal transplant procedures from healthy donors, 16 out of 22 patients with CRSsNP reported a decrease greater than the MCID in SNOT 22 (**Figure 13**). For the SNOT-22 nasal domain, 21 out of 22 patients reported a decrease greater than the MCID. None reported increased symptoms greater than the MCID. No statistically significant changes were observed for TNSS or endoscopic grading.

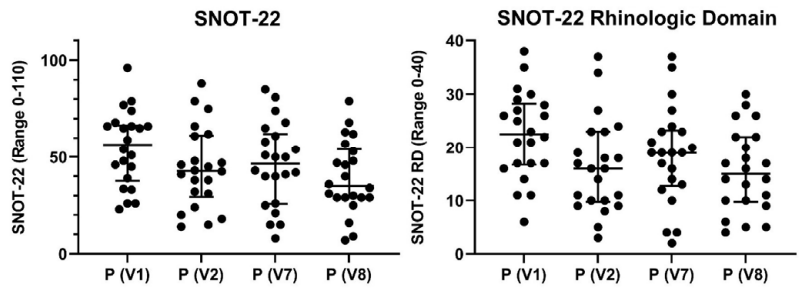

Figure 13. Symptoms at inclusion (V1), after antibiotics (V2), 10 days after the transplant procedure (V7), and at 90 days (V8), showed a decrease in symptoms from V1 to V8 as measured by SNOT-22 (p=0.000035) and its rhinologic domain (p=0.000071). Reprinted with permission from John Wiley & Sons.

Analysis of immunological markers in nasal lavages at the end of the study compared to at the start of the study showed reduced IL-10 (p=0.0223) and increased IL-17A (p=0.0454), IP-10 (p=0.0471), MIP-1α (p=0.0367) and, IFN-ɣ (p=0.0205).

For the microbiota (**Figure 14**), bacterial abundance increased after antibiotic treatment and this increase persisted until the end of the study. Similarly, bacterial diversity increased after antibiotic treatment and this increase also persisted to the

end of the study, when it was still statistically significantly greater compared to at the start of the study (p=0.0079).

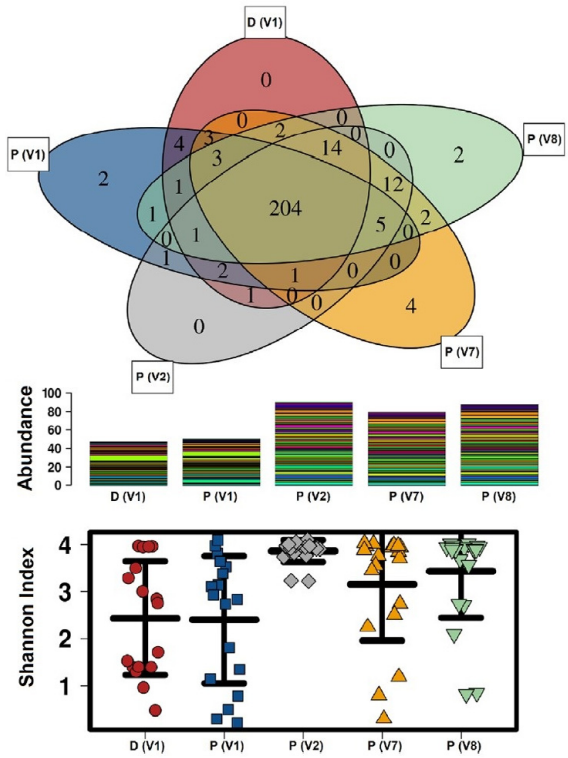

Figure 14. Wenn diagram of identified bacterial species for donors (D) at visit 1 and patients (P) at visits 1, 2, 7, and 8 (top). Relative bacterial abundance (middle) with each colored line representing a single bacterial genus. Bacterial diversity (bottom) as indicated by the Shannon index. Reprinted with permission from John Wiley & Sons.

Discussion

An evolving understanding of the microbiome

The understanding of the microbiome in health and disease has improved considerably since the days Björkwall declared that "healthy maxillary antra are sterile" (41). This is particularly due to technological advances, notably the introduction of genomic-based methods for microbiota assessment. The current view is that there are no clear compositional differences between the microbiome of healthy subjects and patients diagnosed with CRS (50, 51, 55, 61). This is in line with the findings in **IV**, where no difference regarding overall bacterial abundance or diversity between the healthy donors and the patients suffering from CRSsNP was observed at the start of the study. Looking at bacterial species identified at different time points, the absolute majority of species were identified in the donor as well as the patient groups at all time-points. The high degree of interindividual variation of the microbiota composition in health, along with the similarity of the composition of microbiota between patients suffering from CRS and healthy individuals on a group level, have led to questioning of the validity of the dysbiosis concept and of the usefulness of further descriptive studies of the microbiota (115). Instead, studies focusing on the effects of a microbiome or of its manipulation have been suggested (100, 115), which was our strategy in **II**, **III**, and **IV**.

Despite the similarities between the microbiomes in health and disease, an aspect suggested as a possible difference concerns the stability of microbiomes. Studies on patients with inflammatory bowel disease, an inflammatory condition suggested to be influenced by the local microbiome, have demonstrated that the patients' microbiomes are unstable over time, particularly during bouts of inflammation (116). Similar observations have been made in patients with CRS (117, 118). A future option to better understand the microbiome may therefore include methods to evaluate microbial features, e.g., protein expression and generation of metabolic products, and functions (**Figure 15**) (39). Taken together, our understanding of the commensal microbiome has undergone rapid improvement during the past 15 years. Still, much is unknown about the microbiota, even though these cells make up at least half of the cells in the human body. Previous studies have indicated microbial high diversity as a factor associated with health. While this is in line with our findings in **IV**, the later reported lack of difference in diversity between microbiomes in health and disease may render this connection questionable.

Perhaps added microbiological sampling would have shown increased microbiological stability associated with the reduction of SNOT-22 scores. This is indeed an aspect worth exploring in future studies.

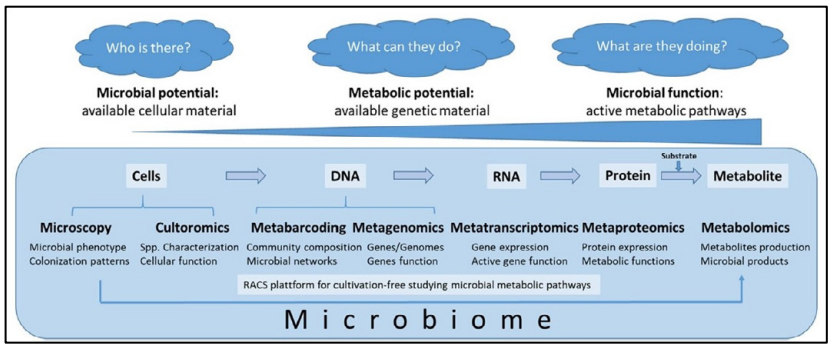

Figure 15. "Omics" offer a potential to investigate the microbiome for DNA, assessing what bacteria are present, as well as for RNA, proteins, and metabolites, reflecting the possible actions of the microbiota. Adapted from Berg *et al*. (39) and reprinted with permission under the Creative Commons Attribution License.

Selection of probiotics

The selection of bacterial species for use in probiotics is difficult, because of the various bacterial traits that must be considered. Our primary concern when selecting probiotics for our studies was safety. LABs have been used historically in fermented foods and for commercial probiotic supplementation for many years with very few anecdotal reports of serious adverse events (101, 102, 119). The PA LAB H13 is sensitive to antibiotics and may therefore, e.g., in the case of unwanted colonization, be preferred (98). The aims of **I** and **II** were to evaluate the safety and to reduce sinonasal pathogens in CRS through bacterial interference without affecting the commensal microbiota. The PA LAB H13 has such effects *in vitro* and matches our safety criteria (120, 121). Furthermore, the PA LAB H13 is a multispecies consortium, which is suggested to represent a more robust treatment than single-species options (106). The doses were chosen by looking at the dose used by Skovbjerg *et al*., who also studied probiotic supplementation using nasal spray (122). Authorities in Canada and Italy have regulated that an amount of at least $1x10^9$ CFU/dose is required to be classified as a probiotic for food (98). Dosings in the present studies were $4x10^{10}$ CFU administered locally as a single dose (**I**) or twice daily (**II**). Another relevant property that we would have liked to be able to

decide on was adhesion-capacity. Unfortunately, this was not studied for the PA LAB H13 at the time. A lack of adhesive capabilities might be a reason why neither untoward symptoms, signs of inflammation, nor effect on CRS symptoms was observed.

For **III**, the aim was to induce a desired type 1 response to skew the immune response, potentially reducing the type 2 reaction of allergic rhinitis, similar to the effect previously observed by us of nasal administration of a TLR7-agonist (123, 124). Besides choosing the LAB PA for safety reasons, the strains were selected based on previous findings on the reduction of responses to grass and birch pollen after intranasal challenge in murine models (125, 126). Unfortunately, the two species that showed promising results in mice were patented, and our requests to utilize them were rejected. Therefore, we selected two similar strains and added a third, a *Lactococcus* strain since *Lactococci* had previously shown a capacity to attenuate "allergic asthma" in a murine model (127). The LAB PA contained $9.5x10^{10}$ CFU/ml in a ratio of 1:1:1 of the three selected strains and was administered as a nasal spray of two hundred µl per nostril twice daily adding up to a dose of $3.8x10^{10}$ CFU twice daily.

The results in **III** suggested a minor innate immune response, but this change failed to reach statistical significance. Arguably, this might reflect that the selected strains (or the dose/dosage) were less efficient than those originally intended might be. Or maybe the reason was a lack of adhesion, and strains native to the human upper respiratory tract would yield a better result. Furthermore, a higher dose might have skewed the immune response in a more marked type 1 direction. Alternatively, the lack of effect simply reflects that ("artificial") mouse disease-models do not fully mimic human disease. Indeed, a successful selection of probiotics depends on many variables with the genetics of the host being a key factor, arguably making animal studies less useful for predicting their effects on humans. Searching for probiotic candidates in bacterial species already acting as commensals in humans may increase the chance of a good result. Traditional probiotic bacterial strains of LAB are safe in almost all healthy humans, but any probiotic candidates from other bacterial strains will have to be thoroughly investigated for safety.

Sinonasal microbiome transplants

Microbiome transplants differ from probiotics in that they represent a transfer of a whole microbiome and do therefore not fit the probiotic definition of a defined consortia (98). Furthermore, probiotics generally do not colonize the recipient for more than a transient period, while microbiome transplants have been reported to induce long-lasting changes to the recipients' microbiota (119, 128, 129). Accordingly, in **I**-**III**, no remaining probiotic bacteria could be identified after a

wash-out period, verifying our option for a crossover design, while in **IV** the duration of effect of the transplant procedure was not estimated in advance and hindered a direct utilization of a crossover design Another difference between classical probiotics and microbiome transplants concerns safety. Risks can be divided into a risk of transferring infectious pathogens, which in the case of fecal transplants has been documented to cause infections and even deaths (130), and a risk of transferring negative traits: as the microbiome composition is suggested to play a role in several diseases and risk of transferring such conditions has been suggested yet never reported (130). The suggested management of these risks is by thorough donor screening.

In **IV**, we handled this using a pathogen screening protocol and by registering allergy and asthma as possible traits that could be transferred. Furthermore, we prioritized donors who already had a relationship with the patients, such as a spouse. Theoretically, this might be an advantage as the donor and the patient have similar living conditions, yet the microbiota of the donor is associated with health (131). On the other hand, spouses already have an increased similarity of their respective gut microbiomes (132), and possibly also of their sinonasal microbiomes. Therefore, transplantation of a microbiome from an unrelated donor may theoretically offer a greater possibility of a change.

Studies of fecal and vaginal microbiome transplants have reported very high effectiveness in conditions with a defined microbiological pathogen such as *Clostridioides difficile* or *Gardnerella* species (bacterial vaginosis) (129, 133, 134). For inflammatory bowel disease, a condition that similar to CRS is characterized by inflammation without any defined pathogen, gut microbiome transplantations have reported effectiveness of 25% for ulcerative colitis and 61% for Crohn's disease (135).

Taken together, compared to probiotics, microbiome transplants are less well-defined and thus offer both a higher risk of infection and also a theoretical risk of transferring unwanted traits. It has the potential to induce lasting changes to the microbiome and has well-documented effects against defined pathogens and inflammatory disease, indicating the microbiome's importance in these conditions.

Methodological considerations

Studying the role of probiotics and the microbiome in inflammatory diseases such as CRS is challenging. Immunological endotyping suggests several underlying inflammatory variants (11) and the microbiome itself shows large interindividual variations (55). Furthermore, the inflammation, the microbiome, and the immune system all affect each other and interact with the host. This may to some degree be controlled in animal models, whereas external factors such as temperature, smoking,

medication, and allergens exposure affect both inflammation and microbiome in humans (**Figure 16**). Therefore, the question of cause and effect is important. Does an altered microbiota cause inflammation or does the milieu of an inflamed mucosa cause changes to the abundance and diversity of the microbiota?

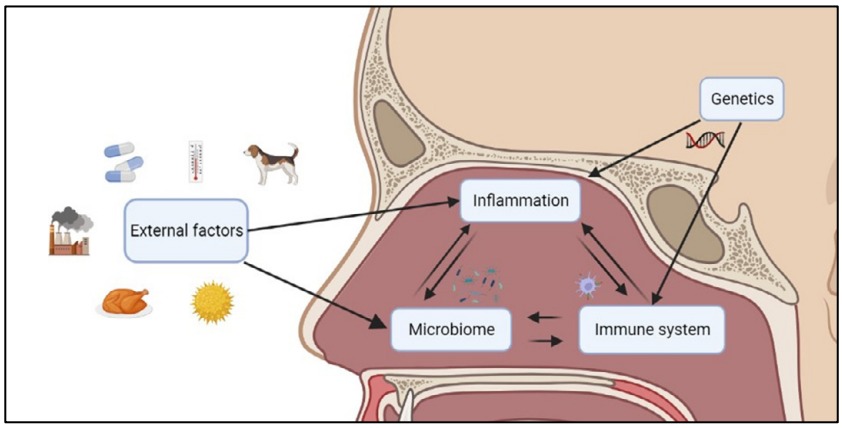

Figure 16. The interplay between inflammation, microbiome, and immune system and effects by genetics and external factors. Figure created with BioRender.

Study designs

Studies **I-III** were of randomized, placebo-controlled, double-blinded, and crossover designs. This has advantages as it accounts for a placebo-effect and lets each subject act as their own control, thus being perfectly matched. In **I-III**, we utilized wash-out periods to reset the subjects before the second half of the study. For **IV**, we instead chose a longitudinal design because a crossover design would have required double the time of participation of patients as well as donors and might nevertheless be affected by carry-over effects. Instead, we opted to use objective measures to validate any subjectively reported symptom-reducing effects. As **IV** indicated that sinonasal microbiome transplantation was feasible and possibly effective, we now aim for a following study of a more complex placebo-controlled design.

Study subjects

For **I** and **III**, recruitment was not a problem. Study **I** focused on healthy subjects and **III** on subjects with pollen allergy, which is a common condition. While epidemiological data suggest that CRS has a prevalence of up to 12% of the

population, CRSsNP-patients with previous surgery and uncontrolled disease despite maximal medical therapy are not common and, for that reason, larger sample-sizes than in **II** and **IV** would have required additional participating centres or patients being recruited over a longer period.

Patient-related outcome measures (PROMs)

PROMs were used as the primary outcome variable for all studies in this thesis. For **I**, **II**, and **IV**, SNOT-22 was used. This is the most commonly used and validated instrument to track symptoms of patients with CRS, but it is of course based on a patient's subjective impression and is therefore, e.g., also subject to any placebo effect. A more unexpected problem was that one patient in study **II**, though suffering from CRSsNP as diagnosed by clinical findings, reported very low scores at the start of the study and therefore had little possibility to report any clinically significant improvement. This could have been amended by setting a minimum SNOT-22 score as an inclusion criterion. For study **III**, we planned to use mini-RQLQ as a primary objective, but it turned out to be ill-suited for a NAC-series setting and, instead, the analysis focused on TNSS and PNIF. A strength of PROMs is that a measured improvement is of direct importance to the patient, while this is not necessarily so for other measured outcomes.

Inflammatory markers

Inflammatory processes are dependent on many different cytokines, mediators, other proteins, and cells, and measuring these indicates the potential for inflammatory activity in the mucosa. Sampling of tissue, and analysis of markers as mentioned above, may by some be considered as a "gold standard", but nasal lavage, and analysis of cytokines/mediators, is a valid alternative. The nasal lavage technique is widely used, it reflects to a large degree the underlying mucosal inflammation, and it is practical (atraumatic, easy to use, etc.). As such it has previously been utilized in CRS as well as AR (136, 137).

Examined cytokine patterns were useful to assess whether or not any inflammatory response was induced by the PA in **I** and **III**, and also for comparisons between the healthy donors and patients in **IV**, to verify that the inflammatory condition of the patients reflected the pattern expected for CRSsNP. However, **II**, **III**, and **IV** examined either patients with an ongoing inflammatory condition (CRSsNP) or an expected inflammatory response produced by the NAC series (allergic rhinitis), and then attempts to affect these responses by intervention with probiotics or sinonasal transplants that may induce effects of their own. The interpretation of the changes in inflammatory markers in these settings proved to be very difficult and no firm conclusions could be drawn from the data.

Microbiological examinations

As the microbiome encompasses various forms of microbiota as well as many different forms of bacterial products, no single method today can be used to investigate the entire microbiome at once. Instead, a method must be chosen depending on what aspect of the microbiome that is the focus of interest. The main interest in **I**, **II**, and **III** was to investigate whether or not the PAs colonized the nasal airway. As all the species of the PAs were culturable, we opted for culture followed by MALDI-TOF as this offered a good resolution to identify the individual species of the PAs at a reasonable cost. Also, it provided information about culturable species of the microbiota, but as only a minority of all species in the microbiome are culturable we could not draw any conclusions about effects on diversity or abundance.

In **IV**, the objective of the microbiological examinations was to assess changes to the composition of the microbiome and 16S rRNA gene sequencing was used. This provided a broader view of the microbiota and allowed for analysis of abundance and diversity, but at the cost of a much lower resolution regarding the identification of individual bacterial species. Another limitation of the 16S rRNA method is that it is unaffected by the viability of the bacteria detected. Newer methods, such as whole genome sequencing for better resolution and viability PCR to distinguish between dead and viable bacteria, offer means to help handle these problems in future studies.

Verification or not of previous findings and hypotheses

In **I** and **II**, we sought to assess if probiotic supplementation using the LAB H13 was tolerable and if it could help rebalance a dysbiosis of the commensal microbiota, by reducing the pathobiome through bacterial interference, to avoid damage to the commensal microbiota, as such effects had been indicated *in vitro* (120, 121). The PA was well tolerated, but did neither affect symptoms, immunologic markers, nor the commensal microbiota.

Building on this, we investigated the effect of a sinonasal microbiome transplant in patients with CRSsNP (**IV**), as studies on microbiome transplants in the gut focusing on specific pathogens (129) as well as inflammatory bowel disease (131) had shown encouraging disease-modifying results. Similar to probiotic supplementation, sinonasal microbiome transplants were well tolerated, but the patients reported a significant reduction of symptoms associated with a significant and lasting increase in both the abundance and diversity of the sinonasal microbiota. These findings will now be further investigated.

In **III**, we aimed to use a PA to induce a type 1 immune response similar to that previously demonstrated by us using a TLR7 receptor agonist, which was associated with symptom-reducing effects in AR (123, 124). Furthermore, studies in mice using different probiotic species had also shown an inflammatory skewing leading to a reduction in "allergic" symptoms and that intranasal administration was superior to intragastric administration (125, 126). In **III**, the PA was well tolerated, but it failed to induce a significant type 1 immune reaction, and no reduction in AR symptoms was observed.

In summary, the key findings of this thesis are that topical sinonasal probiotics as well as sinonasal microbiome transplants are well tolerated. The PAs investigated showed no effect on symptoms or composition of the commensal microbiota, while the sinonasal transplant procedure was associated with a significant reduction of symptoms in patients suffering from CRSsNP as well as significant and lasting changes of the commensal microbiota.

Conclusions

Paper I

A single nasal administration of the probiotic LAB H13 is safe and does neither produce any untoward nasal symptoms nor induce any inflammatory reaction, or affect the commensal microbiota, compared to sham.

Paper II

Two weeks' topical nasal administration of the probiotic LAB H13 to patients with CRSsNP does not affect symptoms, local inflammatory activity, or microbiota composition, compared to placebo.

Paper III

Three weeks' topical nasal administration of a PA comprising *Lactobacillus Rhamnosus* SP1*, Lactobacillus paracasei* 101/37*, and Lactococcus lactis* L1A was well tolerated, likely invoked a mild innate immune response, but did not affect symptoms of allergic rhinitis in a NAC model, compared to placebo.

Paper IV

Microbiome transplants from healthy donors to patients with CRSsNP were well tolerated and resulted in a significant and lasting decrease in symptoms. Furthermore, significant and long-lasting increases in the abundance and diversity of the patients' microbiota were observed.

Future perspectives and closing remarks

The holobiont theory describes that a eukaryote host and its microbiome have co-evolved and are interdependent, so that which affects one will also affect the other (138). Our perspective of the human microbiome is undergoing a vast reformation, from beliefs that the various sites of the human body are sterile or occupied by commensals without any real effect on the host, to the current view of its key roles in infectious and inflammatory diseases as well as in cancer and neuropsychiatric disorders. The microbiome is the subject of intense research to which I hope that the papers of this thesis have contributed. Looking to the future, applications for microbiome-directed therapy may border on science fiction yet seem very feasible. However, we still lack an understanding of fundamental aspects, such as how to differentiate between a healthy and a diseased microbiome. Answering this key question will provide a much-needed foundation for further research and as of today examinations of stability over time and metabolomics both appear as promising alternatives to investigate. Furthermore, explorations into the utilization in the treatments of various conditions are warranted. An example of an immediate continuation of the present series of studies is a placebo-controlled study on the effects of microbiome transplantations in CRS.

Populärvetenskaplig sammanfattning på svenska

Kronisk rinosinuit (”bihåleinflammation”) kännetecknas av en inflammation i näs- och bihåleslemhinnan, vilken ger nästäppa och varig snuva samt ibland smärta i ansiktet, dålig sömn och uttalad trötthet. Sjukdomen drabbar 2-12% av befolkningen och medför försämrad livskvalitet. Fynd i status (t.ex. ibland polyper, ibland inte), och studier av den underliggande inflammationen, talar för att det finns flera bakomliggande orsaker. Idag saknas det dock kunskap om vilka dessa är, och vi har inte någon specifik behandling. I stället handlar det om att dämpa inflammationen med kortison och antibiotika eller att med kirurgi förändra förhållandena i bihålesystemet för att minska symptom, skapa åtkomst för lokal behandling och eventuellt minska den underliggande drivkraften till inflammationen. Under många år trodde man att bihålorna var sterila hos friska personer, men forskning har visat att det finns en naturlig bakterieflora. Man spekulerar nu i att det är störningar i denna och dess samspel med immunförsvaret som är orsaken till kronisk bihåleinflammation.

Säsongsallergisk rinit (”hösnuva”) kännetecknas också av inflammation i den övre luftvägen, men – till skillnad från kronisk rinosinuit – är orsaken känd: pollen interagerar med immunförsvaret, vilket ger en ”överdriven reaktion” för det egentligt ofarliga pollenet. Detta ger nysningar, snuva och nästäppa, men även försämrad livskvalitet. Behandlingen består i att försöka undvika pollen och att dämpa sjukdomen, t.ex. med antihistamin och kortison. I epidemiologiska studier har man sett att en västerländsk livsstil är förknippad med ökad förekomst av allergi: idag drabbas ca. 40%. Liksom för kronisk rinosinuit finns det för allergi data som talar för att vår naturliga bakterieflora liksom de bakterier vi utsätts för har betydelse. Vid allergi gäller detta främst i barndomen och har betydelse för immunförsvarets utveckling, men det finns också experimentella studier som talar för att lokal behandling med probiotika kan dämpa en etablerad allergisk inflammation. Probiotika är bakterier som när de tillförs i en adekvat mängd ger en positiv hälsoeffekt: probiotika kan interagera både med immunförsvaret och med den naturliga bakteriefloran.

I en serie studier (**I**-**IV**) har vi nu undersökt effekten av lokal administration (”nässpray”) av två olika probiotika till över luftvägen och framför allt studerat om de ger eller påverkar symptom och om de ger eller påverkar en inflammation. När

probiotika ges till *friska försökspersoner* så accepteras den väl och ger inget tydligt inflammatoriskt svar (**I**). När den ges till *patienter med kronisk bihåleinflammation* så påverkas inte de symptom som karakteriserar sjukdomen och inte heller den underliggande inflammationen (**II**). När probiotika ges till *patienter med säsongsallergisk rinit* så ger den en mild icke-allergisk inflammation – som teoretiskt skulle kunna dämpa en allergisk inflammation – men i den modell vi använder påverkas inte allergisymptomen (**III**). I en separat studie har vi försökt transplantera (”flytta”) normala bakterier från näsan hos friska personer till *patienter med kronisk bihåleinflammation*. Vi ser då en minskning av symptomen som vi åtminstone delvis tillskriver transplantationen (**IV**).

Sammanfattningsvis visar vi att lokal administration av probiotika till näsan, liksom transplantation av normalt förekommande bakterier till patienter med kronisk bihåleinflammation, är möjliga och tolereras väl. Någon effekt av lokal behandling med probiotika kunde inte påvisas, medan transplantation av hela bakteriefloran från friska donatorer var associerat med symptomlindring. Transplantation av mikrobiom framstår därmed som en möjlig framtida behandling, även om ytterligare forskning behövs för att renodla och verifiera resultaten.

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