Dissertation

Exploring the human microbiome
–
A journey through the human aerodigestive and gastrointestinal tract in health and disease

submitted by

Christina Sarah Kumpitsch, BSc, MSc

for the Academic Degree of

Doctor of Philosophy (PhD)

at the

Medical University of Graz

Diagnostic and Research Institute of Hygiene, Microbiology and Environmental Medicine

under the Supervision of

Prof. Dr. Christine MOISSL-EICHINGER

2021

Declaration

"I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organisations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the "Standards of Good Scientific Practice and Ombuds Committee at the Medical University of Graz". "

Christina Sarah Kumpitsch
Graz, July 2021

Quote

“Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less.“

Marie Curie

Disclosures

Parts of the dissertation are already published in and contain the literal text of:

Kumpitsch, C. *et al.* (2019). The microbiome of the upper respiratory tract in health and disease. ***BMC Biology*** 17**,** 87. https://doi.org/10.1186/s12915-019-0703-z [1]

Kumpitsch, C. *et al.* (2020). Preliminary insights into the impact of primary radio-chemotherapy on the salivary microbiome in head and neck squamous cell carcinoma. ***Scientific reports*** 10, 1 16582. https://doi.org/10.1038/s41598-020-73515-0 [2]

Kumpitsch, C. *et al.* (2021). Reduced B12 uptake and increased gastrointestinal formate drive archaeome-mediated breath methane emission in humans. *Microbiome*, **accepted**. (*manuscript version before peer-review*) [3]

Permission for reproduction of the publications has been given by the respective publisher (Springer Nature) with the Open-Access-Publication Agreements (Creative Commons Attribution license CC-BY 4.0, https://creativecommons.org/licenses/by/4.0/legalcode).

All co-authors gave their approval to re-use the published manuscripts within this thesis:

Chapter 1: **The microbiome of the upper respiratory tract in health and disease.** [1]

Christina Kumpitsch[1], Kaisa Koskinen[1], Veronika Schöpf[2,3,4] and Christine Moissl-Eichinger[1,3]

[1]Diagnostic and Research Institute of Hygiene, Microbiology and Environmental Medicine, Medical University of Graz, Neue Stiftingtalstraße 6, 8010 Graz, Austria.
[2]Institute of Psychology, University of Graz, Universitaetsplatz 2, 8010 Graz, Austria.
[3]BioTechMed-Graz, Mozartgasse 12/II, 8010 Graz, Austria.
[4]Present address: Medical University Vienna, Spitalgasse 23, 1090 Vienna, Austria.

- **Kaisa Koskinen** and **Christine Moissl-Eichinger** supervised the literature research and wrote parts of the manuscripts.
- **Veronika Schöpf** provided critical discussion and corrected the final draft.

Disclosure

Chapter 2: **Reduced B12 uptake and increased gastrointestinal formate drive archaeome-mediated breath methane emission in humans.** [3]

Christina Kumpitsch[1], Florian Ph. S. Fischmeister[2,3], Alexander Mahnert[1], Sonja Lackner[4], Marilena Wilding[2], Corina Sturm[2], Anna Springer[5], Tobias Madl[5,6], Sandra Holasek[4], Christoph Högenauer[7], Ivan Berg[8], Veronika Schöpf[3] and Christine Moissl-Eichinger[1,6]

[1] Diagnostic and Research Institute of Hygiene, Microbiology and Environmental Medicine, Medical University of Graz, Graz 8010, Austria
[2] Department of Psychology, University of Graz, Graz 8010, Austria
[3] Department of Biomedical Imaging and Image-guided Therapy, Medical University of Vienna, Vienna 1090, Austria
[4] Division of Immunology and Pathophysiology, Medical University of Graz, Graz 8010, Austria
[5] Gottfried Schatz Research Center for Cell Signaling, Metabolism and Aging, Molecular Biology & Biochemistry, Medical University of Graz, Graz 8010, Austria
[6] BioTechMed, Graz, Graz 8010, Austria
[7] Division of Gastroenterology and Hepatology, Medical University of Graz, Graz, Austria.
[8] Institute for Molecular Microbiology and Biotechnology, University of Münster, Münster, Germany.

- **Florian Fischmeister, Marilena Wilding** and **Corina Sturm** organised the recruitment as well as the sample and questionnaire/metadata collection.
- **Alexander Mahnert** helped with data analysis, performed metabolic prediction analysis and wrote parts of the manuscript.
- **Sonja Lackner** and **Sandra Holasek** evaluated the dietary information, calculated and provided dietary indices. Furthermore, they contributed to manuscript writing.
- **Christoph Högenauer** and **Ivan Berg** contributed to study design and research questions and read the manuscript critically.
- **Christoph Högenauer** supported methane breath measurements.
- **Florian Fischmeister** and **Veronika Schöpf** contributed to manuscript writing.
- **Veronika Schöpf** and **Christine Moissl-Eichinger** initiated this project and were responsible for the study design.
- **Christine Moissl-Eichinger** supervised all activities, performed analyses (e.g., LEfSe cladogram), and wrote parts of the manuscript.
- **Anna Springer** and **Tobias Madl** performed NMR sample preparation, measurement and analysis and provided metabolomics results.

Disclosure

Chapter 3: **Preliminary insights into the impact of primary radiochemotherapy on the salivary microbiome in head and neck squamous cell carcinoma.** [2]

Christina Kumpitsch[1], Christine Moissl-Eichinger[1,2], Jakob Pock[3], Dietmar Thurnher[3] and Axel Wolf[3]

[1] Diagnostic and Research Institute of Hygiene, Microbiology and Environmental Medicine, Medical University of Graz, Graz, Austria.
[2] BioTechMed, Graz, Austria.
[3] Department of Otorhinolaryngology, Medical University of Graz, Graz, Austria.

- **Axel Wolf, Christine Moissl-Eichinger** and **Dietmar Thurnher** designed the study and critically revised the manuscript in terms of intellectual content.
- **Axel Wolf** helped to draft the manuscript.
- **Christine Moissl-Eichinger, Jakob Pock** and **Axel Wolf** were involved in data acquisition, analysis, and interpretation.

Acknowledgements

First of all, I would like to thank my supervisor ***Professor Christine Moissl-Eichinger****. I am extremely grateful for your endless support and guidance through the last years. Your door was always open and you had a sympathetic ear for all the problems I had to face during the years of my PhD (not only for work related ones). I'm so grateful that you gave me the opportunity to join your team and work on so many fascinating topics. Christine, it was a great pleasure for me to work the last years under your supervision in your fantastic team - I hope there will be more to come! Thank you for your positivity and encouragements that always motivate me to give my best!*

My thesis would not have been possible without the help and support of the entire CME working group over the last years. Therefore, I would like to thank all present and former colleagues: Especially, ***Lisa Wink*** *for showing me everything in the first weeks and months of my PhD in the lab as well as for her help throughout the last four years whenever I needed it, and for managing and organizing all my lab stuff orders that made my PhD life a lot easier;* ***Marcus Blohs*** *for always answering my (sometimes annoying) questions, for helping me (more than once) with his "magic" Calypso skills to find the error in my upload-files and for the time that he invested to proof read my manuscripts;* ***Charlotte Neumann*** *for keeping our lab clean, for proof reading several manuscripts, as well as for the fun we had during our stay at the AMICI conference ☺. Special thanks also to* ***Alexander Mahnert*** *for his endless patience and support in bioinformatics-related stuff. I'm sorry for bothering you so many times with my problems, Alex, but without your help I wouldn't have been able to use most of the bioinformatics tools. Of course also many thanks to the rest of the group* ***Kaisa Koskinen, Manuela Pausan****,* ***Stefanie Duller, Viktoria Weinberger, Torben Kühnast, Rokhsareh Mohammadzadeh*** *and all the students. - You all made me really enjoy my PhD life! Thank you so much for our coffee breaks, games evenings, Jogging Clubs, all our discussions and the conferences we attended together. I believe that we became more than just colleagues ☺.*

I would also like to acknowledge the help of my former student ***Hanna Kulvicki*** *whom I had the pleasure to supervise. You did a great job! I hope that you could gather a lot of experience during your stay here in this lab.*

Moreover, I am also grateful to ***Florian Fischmeister*** *(and his team) for taking care of the recruitment and the organization of the samplings. Thank you for all your support and your helpful advices. I really enjoyed our valuable discussions during the last years!*

I would also like to thank my thesis committee, ***Veronika Schöpf****,* ***Peter Holzer*** *and* ***Joachim Reidl****, for their support and constructive input and criticism to all my projects.*

I would like to express my gratitude to the ***PhD program Molecular Medicine*** *(Mol Med) of the* ***Medical University of Graz*** *for the multitude of courses that were offered and the excellent education as well as to the* ***Austrian Science Fund*** *(FWF; KLI 639, P 32697, P39965, I3792 and DK-MCD W1226), the* ***Austrian Research Promotion Agency*** *(FFG; 864690 and 870454), the* ***Integrative Metabolism Research Center Graz****, the* ***Austrian Infrastructure Program 2016/2017****, the* ***Styrian Government*** *(Zukunftsfonds) and the* ***BioTechMed Graz*** *for funding my projects. Without them this dissertation would not have been possible!*

During my PhD I also had great pleasure to work with so many great collaborative partners that contributed to the publications and provided their assistance at every stage of the research projects. I would like to thank ***Sonja Lackner*** *and* ***Sandra Holasek*** *for evaluating and supporting the analysis of the nutritional data in our studies;* ***Axel Wolf*** *for collecting samples for our studies and his support during paper writing;* ***Tobias Madl*** *and his team for generating, analyzing and helping with the metabolomics data; and* ***Slave Trajanoski*** *who always helped to solve issues with the MedUni cluster and for the maintenance of the MedUni cluster.*

Ein riesengroßes Dankeschön geht auch an meine ***Familie****, vor allem an meine Eltern, für ihre Unterstützung und Geduld über die ganzen Jahre hinweg. Ihr habt dazu beigetragen, dass ich mein Studium mit so wenig Hindernissen wie möglich abschließen konnte. Ihr wart immer für mich da und habt mir das Gefühl gegeben, dass ich alles erreichen kann. Nur durch eure Hilfe konnte ich überhaupt so weit kommen.*

Special thanks also to ***Aron Knebl****. You were there for me already from the beginning of my studies and helped me to survive many frustrating and stressful days. I am sure it was not always easy with me but you managed to motivate me all the time. Thank you for encouraging me in every decision I made. I am so grateful to have you by my side! Furthermore, I would also like to thank all my* ***friends*** *who understood that I sometimes didn't have time to join activities or*

that we couldn't manage to find a time slot that suits all of us and who still cheered me up whenever I needed it.

*Last but not least, I also want to thank my cats, **Jana** and **Eskimo**, for supporting my mental well-being. I am happy that they were there during some difficult periods throughout the last years and that they were always patiently waiting for me to come home.* ฅ^•ﻌ•^ฅ

Thank you!

Table of Contents

Abbreviations and Definitions **1**

Zusammenfassung **2**

Abstract **3**

Introduction **4**

The human microbiome *4*

Living on surfaces of the human body is challenging for human-associated microbes. *4*

The general human microbiome at different body sites *5*

Upper respiratory tract 5

Gastrointestinal tract 6

Oral cavity 7

Beneficial microbes and their way to influence the human host. *7*

Vitamins 7

Short chain fatty acids 8

Methane production 8

Dysbiosis and disease-associated microbial community structures. *9*

Upper respiratory tract 9

Gastrointestinal tract 11

Oral cavity 11

Modulation of human microbiome compositions *14*

Dietary interventions 14

Therapeutically interventions 15

Objectives and summary of the thesis **16**

Chapter 1.: *The microbiome of the upper respiratory tract in health and disease.* *16*

Chapter 2.: *Reduced B12 uptake and increased gastrointestinal formate drive archaeome-mediated breath methane emission in humans.* *17*

Chapter 3.: *Preliminary insights into the impact of primary radiochemotherapy on the salivary microbiome in head and neck squamous cell carcinoma.* *18*

Table of contents

The publications **20**

1st publication *21*

2nd publication *23*

3rd publication *24*

General Discussion **25**

Chapter 1.: *The microbiome of the upper respiratory tract in health and disease.* *25*

Chapter 2.: *Reduced B12 uptake and increased gastrointestinal formate drive archaeome-mediated breath methane emission in humans.* *31*

Chapter 3.: *Preliminary insights into the impact of primary radiochemotherapy on the salivary microbiome in head and neck squamous cell carcinoma.* *35*

Conclusion **38**

References **40**

Appendix **57**

Original Paper **58**

Abbreviations and Definitions

OA – obere Atemwege

GIT – gastrointestinaler Trakt

OP – oropharyngeales Plattenepithelkarzinom

URT – upper respiratory tract

GIT – gastrointestinal tract

oSCC – oropharyngeal squamous cell carcinoma

PMA – propidium monoacid

vitamin B12 – cobalamin

SCFA – short chain fatty acid

CH_4 – methane

LAB – lactic acid bacteria

SIBO – small intestinal bacteria overgrowth

CRS – chronic rhinosinositis

CRSsNP – CRS with the presence of nasal polyps

CRSwNP – CRS with the absence of nasal polyps

COVID-19 – coronavirus disease 19

SARS-CoV 2 – severe acute respiratory syndrome coronavirus type 2

IBD – inflammatory bowel disease

CD – Crohn’s disease

UC – ulcerative colitis

NBA – “Nose Brain Axis” study

ppm – parts per million

HE – high methane emitter

LE – low methane emitter

HPV – human papilloma virus

MM – middle meatus

URTI – upper respiratory tract infection

T2R38 – taste receptor family 2 variant 38

↑ – increase

↓ – decrease

normosmia – normal olfactory capability

dysosmia – impared olfactory capability

hyposmia – reduced olfactory capability

anosmia – loss of smell

RCHT – radiochemotherapy (in this thesis: combined with antibiotics/antimycotics treatment)

Zusammenfassung

Das humane Mikrobiom umfasst Trillionen von Mikroorganismen (z.B. Archaeen, Bakterien und Pilze). Diese Mikroben übernehmen viele essenzielle Funktionen, die unter anderem zur Gesundheit des menschlichen Organismus beitragen (z.B. Entwicklung des Immunsystems oder Vitaminproduktion). Die Diversität und Zusammensetzung der Mikroorganismen variiert aufgrund verschiedener Faktoren von Körperregion zu Körperregion. In dieser kumulativen Dissertation wird das Mikrobiom von drei verschiedenen Stellen des aerodigestiven und gastrointestinalen Trakts behandelt. Dazu wurden sowohl die mikrobielle Diversität als auch die Zusammensetzung diskutiert:

1) Für diesen umfassenden Übersichtsartikel wurde die Literatur über das Mikrobiom der oberen Atemwege (OA) erfasst und von unterschiedlichen Blickwinkeln betrachtet. Im Allgemeinen wurden unterschiedliche Nischen in den OA, Verteidigungsmechanismen vom humanen Wirt und anderen Mikroben, aber auch der Prozess des Alterns und der gesundheitliche Zustand als Modulatoren des OA-Mikrobioms beleuchtet. Außerdem wurden alternative Therapiestrategien für OA-Infektionen, wie z.B. Probiotika, als potenzielle nicht invasive Therapieansätze diskutiert.

2) Die zweite Publikation beschäftigt sich mit dem Mikrobiom des gastrointestinalen Traktes (GIT) von gesunden Erwachsenen mit unterschiedlicher Atemluft-Methanemission (hoch/niedrig). Unterschiede in emittierter Methankonzentration wurden mit unterschiedlicher mikrobieller Zusammensetzung der Stuhlproben assoziiert. Diese Unterschiede wurden wiederum mit variierender Aufnahme von Ballaststoffen und Vitamin B12, als auch mit unterschiedlichen Formatkonzentrationen in den Stuhlproben korreliert. Die Aufnahme spezieller Nährstoffe scheint zu Abweichungen in der mikrobiellen Zusammensetzung und folglich zu unterschiedlichen Metaboliten in beiden Gruppen zu führen.

3) Auch Tumore wie das oropharyngeale Plattenepithelkarzinom (OP) beeinflussen das angrenzende Mikrobiom. In der dritten Publikation wurden daher die potenziellen Unterschiede des Speichel-Mikrobioms in OP-Patienten untersucht. Mikrobielle Unterschiede im Vergleich zu gesunden Kontrollen konnten sowohl vor als auch nach Therapiebeginn (Kombination aus Radiochemotherapie und Antibiotika/Antimykotika) beobachtet werden.

Abstract

The human body is inhabited by trillions of microorganisms such as archaea, bacteria and fungi. These microbes are crucial for health by taking on several crucial tasks like priming the immune system, food degradation or vitamin production. However, microbial imbalance (dysbiosis) has been linked to a variety of diseases. Depending on different factors, the microbial load, diversity and composition is distinct throughout specific body areas. In this cumulative dissertation the microbiome of three body sites belonging to the human aerodigestive and gastrointestinal tract are covered and the microbial diversity and community structure at these particular niches are addressed in three publications:

1) For a comprehensive review article, the literature of the microbiome associated with the upper respiratory tract (URT) has been collected and examined from different angles. Overall findings considered factors – different URT niches, defence systems of the host and other microbes as well as the process of aging and disease state – as modulators of the URT microbiome. Furthermore, alternative therapy options against URT infections, like probiotics, have been discussed as potential non-invasive future therapeutic approach.

2) The second publication deals with the gastrointestinal tract (GIT) microbiome of high and low methane emission via breath in healthy adults. Different methane levels were linked to distinct microbial community patterns. Several microbes of these patterns have been associated with different levels of dietary fibre and vitamin B12 ingestion as well as formate concentration in stool samples. Alterations in dietary intake seem to result in a distinct microbial community structure and consequently to different metabolites of high and low methane emitters.

3) Cancer like oropharyngeal squamous cell carcinoma (oSCC) are also able to affect the adjacent microbiome. In the third publication, we assessed the disturbance of the salivary microbiome in patients suffering from oSCC. Microbial differences in non-treated and even more in treated oSCC patients (combined cancer therapy) have been observed compared to healthy adults.

Introduction

The human microbiome

The human body is colonized by trillions of microorganisms, namely bacteria, archaea, fungi and small eukaryotes as well as viruses, mainly located in the large intestine. According to recent estimates, the weight of these human-associated bacteria reaches approximately 0.2 kilograms [4]). The living entirety of these microorganisms thriving in a particular habitat is called the "human microbiota", whereas the human microbiome is composed of the living organisms (microbiota) and their so-called theatre of activity. This "theatre" contains microbial structural elements, microbial metabolites and internal/external structural elements (including lipids, proteins and polysaccharides; extracellular ("relic") DNA, viruses and phages; signalling molecules and toxins and environmental conditions [5]). Depending on the study design and hypothesis, the focus lies either on the microbiome or just the microbiota. If the potentially living fraction of microbes is investigated, propidium monoacid (PMA) can be used to mask the DNA from dead cells in the samples. This reagent intercalates into freely accessible DNA and strongly inhibits the downstream PCR amplification of these fragments. Since it is not able to penetrate cells with an intact membrane it only inhibits PCR reactions of dead cells or extracellular DNA[6].

Although it is known that the human microbiome consists of all three domains of life, most human microbiome studies still focus only on the bacterial and often miss the non-bacterial community like archaea on purpose or due to methodological issues (reviewed in [7]). However, archaea seem to be as widely distributed in and on humans as recent research indicates, colonizing not only the gastrointestinal tract (GIT) but also other areas of the human body [2,8–11].

Living on surfaces of the human body is challenging for human-associated microbes.

All species of the human-associated microbiota have to cope with various biotic as well as abiotic factors when colonizing the human body. The human host provides a variety of niches with different biological, chemical and physical conditions. This combination of different morphological structures (e.g., surface structure and epithelial lining) and environmental factors

(like pH, oxygen and nutrient availability, or temperature) varies on and in humans and has been topic of many publications and reviews [1,12–15].

Both, structures and environmental factors, create a selective habitat for microorganisms on each type of body site. The selection is even further supported by mechanisms defeating unwanted microbes, including antimicrobial components, the innate and adaptive immune response or mucociliary clearance [16–19]. Besides host-dependent factors, the microbial colonisation is also highly influenced by adjacent microbial communities. Although many positive relationships (like synergism or commensalism) exist, some microorganisms negatively influence population dynamics, functional capacities, or microbial fitness of other community members or pathogens by nutrient competition, production of antimicrobial molecules or stimulation of human defence mechanisms [1,20–23]. In addition to interspecies driven competitions, the human host consciously and unconsciously affects the human associated microbiota during the entire life span. These host-factors include delivery mode and diet (e.g., breast/formula feeding, vegetarianism), smoking and hygiene habits, host-genetics and physiology, as well as disease and medical treatment (e.g., antibiotics) [1,24–30]. All in all, many factors ranging from biotic (e.g., host and living microbial community) to abiotic (e.g., external factors like industrial/non-industrial or urbanisation [31–33]) parameters shape the intra-individual and inter-individual character of the human microbiome and should be considered in microbiome research.

The general human microbiome at different body sites

Even though the host-associated microbial community underlies dynamic changes, some taxa (also called "keystone taxa") are indicative for particular body niches or even health status. The occurrence of these microbes is quite stable between individuals and over a certain time. Therefore, they seem to contribute more to specific environmental dynamics than other microbial taxa present in the same environment [3,5,34].

Upper respiratory tract

The human upper respiratory tract (URT) is in constant contact with the external environment. With its intertwined structure and different epithelial linings throughout the nasal cavity, it provides various niches. Although the general URT microbiome tends to be quite similar among

individuals, intra- as well as inter-individual changes can be observed based on different sampling locations and over time [35–38]. In general, the human URT is colonised by the bacterial phyla Actinobacteria, Firmicutes and Proteobacteria with *Corynebacterium*, *Staphylococcus*, *Cutibacterium, Moraxella* and *Rothia* as predominant genera. Furthermore, lactic acid bacteria (LAB) species such as *Dolosigranulum pigrum* or *Lactobacillus casei* have been found in healthy individuals indicating a potential role as probiotic strains [1,12,39,40]. Besides bacteria, the URT is also inhabited by non-bacterial microorganisms like archaeal taxa belonging mainly to the phyla Euryarchaeota and Thaumarchaeota. [11,41,42]. However, these members of the microbial community have not been much into focus in the past and therefore are often overlooked since general approaches do not target this group of microorganisms [7,43].

Gastrointestinal tract

The gastrointestinal tract (GIT), especially the large intestine, is the most densely colonized human site [4]. The predominant taxa in the gut were described to be Firmicutes (genera *Faecalibacterium* and *Eubacterium*), Bacteroidetes (genera *Bacteroides*, *Prevotella* and *Alistipes*) and Proteobacteria (species *Escherichia coli*) [3,44]. In contrast to other body sites, the GIT is also known to harbour several archaeal taxa, like halophilic archaea but mainly methanogenic archaea belonging to Methanobacteriales and Methanomassiliicoccales (methanogens). One of them, *Methanobrevibacter smithii*, is even considered as a keystone taxon in the human gut [3,43,45,46]. The dominant fungi found in human stool samples are *Candida albicans*, *Malassezia* and *Saccharomyces* [47,48]. Most of the mentioned studies apply 16S rRNA gene or ITS gene sequencing; however, as both of these methodologies rely on amplicon generation with primers, numerous microbial taxa remain underrepresented or undetected. More recently methodological approaches like metagenome-assembled genomes (MAGs) give deeper insights into the microbial community structure and their functional capability of uncultured genomes. Some of the latest studies recovered thousands of MAGs assigned to formerly unknown bacteria (e.g., belonging to the phyla Bacteroidetes, the class Clostridia or the family Lachnospirales, Oscillospirales, Ruminococcaceae) as well as a few archaea (belonging to *Thermoplasmatales, Candidatus Methanomethylophilus, Methanomassiliicoccus and Methanosphaera*) present in the GIT [49–52]).

Oral cavity

Saliva, the tongue or the buccal mucosa are only some examples of niches provided in the moist and warm oral cavity that are in direct contact to nutrition [53,54]. Overall, the oral microbiota mainly comprises the bacterial phyla Firmicutes, Bacteroidetes and Actinobacteria with the genera *Streptococcus*, *Veillonella*, *Prevotella*, *Neisseria*, *Corynebacterium* but also non-bacterial taxa like fungi (*Candida, Aspergillus* and *Malessezia*) and archaea – belonging to *Methanobrevibacter oralis, smithii and massiliense, Methanosphaera* and *Methanosarcina mazei* – have been observed. Notably, archaea were mainly observed in subgingivalis or dental plaques [2,42,55–58].

Beneficial microbes and their way to influence the human host.

Together with the colonisation of microbes a lot of benefits come along. Microbes are capable to support food digestion (e.g., fermentation of dietary fibres) and the production of vitamins (e.g., vitamin B12 (Cobalamin)) [59,60]. Additionally, they protect us from pathogenic colonisation (e.g., by occupation of body niches or shaping the host's immune system) [61–64] and produce health-mediating microbial secondary metabolites like short chain fatty acids (SCFA) (reviewed in [65]). Furthermore, the archaeal product methane (CH_4) is currently being discussed to have effects on the human digestive tract and probably on the bacteriome as well [3,7,66].

Vitamins

Vitamins are important for metabolic processes as well as immune responses. Basically, water-soluble vitamins act as coenzymes in enzymatic reactions whereas fat-soluble ones are important cell membrane components. Since humans are not capable of producing several essential vitamins on their own (such as vitamin B12 (cobalamin)), they have to rely mainly on dietary sources. Vitamin B12 is the biggest and most complex vitamin and mainly found in animal derived products [67,68]. Even though some microbes (such as *Bifidobacterium sp.*) might be able to synthesize cobalamin in the large intestine, the vitamin might still not be absorbed by human cells there since vitamin B12 uptake mainly takes place in the small intestine [69,70]. Other sources of vitamin B12 are the industrial vitamin B12 production (e.g., *Pseudomonas denitrificans*) as well as vitamin B12 enriched in food (e.g., *Propionibacterium freudenreichii* or *Lactobacillus reuteri*) [71–73].

Short chain fatty acids

Other important microbial products are short chain fatty acids (SCFAs) and are mainly produced by anaerobic microbial fermentation of non-digestible polysaccharides/carbohydrates (e.g., dietary fibres and resistant starch) in the large intestine. The main SCFAs produced by microorganisms are acetate (C2), propionate (C3) and butyrate (C4) [74]. The amount and type of produced SCFAs depends on the dietary fibre content (type, structure and size), on the microbial composition in the GIT as well as on the gut transit time [75]. Important producers of SCFA in the gut are e.g., *Eubacterium rectal*, *Eubacterium hallii*, *Faecalibacterium prausnitzii* and *Ruminococcus bromii* [76–79]. After production in the human intestine SCFAs are absorbed by colonocytes and subsequently, partially transported to other organs such as the liver or brain. These absorbed SCFAs possess several health-mediating properties raining from energy supply, mucus production to anti-inflammatory properties (reviewed in more detail in [65,80]).

Methane production

One of the most interesting microbial products of human gut-associated microorganisms is methane. This small and volatile gasotransmitter is produced by methanogenic archaea like *M. smithii* mainly living in our large intestine [3,81]. The biosynthesis of methane requires a carbon source (e.g., bacterial fermentation products such as carbon dioxide, methanol or formic acid) and an electron source, such as hydrogen (H_2) – (CO_2+$4H_2$→CH_4+$2H_2O$) [7]. The human body does not further metabolize CH_4. Therefore, this gas is emitted via flatus or the skin and lung after diffusion into the systemic circulation [82].

The methane production of healthy individuals ranges from 0 to 75 ppm [3,83] and elevated CH_4 concentrations have been connected to protective properties (like anti-inflammation) in mammals. Furthermore, methanogenesis may indirectly also promote the degradation of organic compounds by removing the bacterial fermentation product and inhibitor – environmental H_2 – leading to reduction of bloating as well [84–86]. However, the question remains which impact methane has on human health since several studies indicated an association between CH_4 emission and health issues such as slower gut transition, constipation or head and neck cancer [87–90] Furthermore, the breath methane level measurement after substrate challenge with e.g., lactulose is already used as a non-invasive and cheap tool to diagnose GIT issues. Like in the case of small intestinal bacterial overgrowth (SIBO), in which SIBO-patients exhibit higher CH_4 concentrations after substrate challenge compared to healthy controls [91]. However,

in the case of diagnostics, methane produced in the oral cavity should also be considered because Erdrich et al., 2021 suggested a possible role of methanogens located in the oral cavity on measured methane levels as well [92].

Dysbiosis and disease-associated microbial community structures.

Although a lot of benefits come along with the colonisation of microbes, they are mainly publicly perceived due to rare pathogenic properties. Many studies focus today on the differences in microbial diversity and composition especially in the gastrointestinal tract in health and disease with the finding that rather a shift of human associated microbes (dysbiosis – change in microbial diversity and composition) instead of just one specific pathogen is frequently causing disease, like in inflammatory bowel disease (IBD), chronic rhinosinusitis (CRS), and oropharyngeal squamous cell carcinoma (oSSC) [2,93–97]. Therefore, it has still to be investigated whether dysbiosis occurs due to different conditions caused by the specific disease, contributes to longitudinal symptoms, or even has a causal role in disease formation and progression.

Upper respiratory tract

One example for a highly investigated connection between URT infection and the microbiome is chronic rhinosinusitis (CRS). CRS is an inflammation of the nasal cavity (and paranasal sinuses), occurs with or without nasal polyps (CRSwNP and CRSsNP, respectively), with symptoms like nasal obstruction and/or nasal discharge and lasts for more than 12 weeks [98]. Up to date, no specific pathogen has been linked to this condition, but the abundances of several taxa were determined to be relatively increased in the URT of CRS patients including *Corynebacterium tuberculostearicum and C. accolens*, *Haemophilus influenza and H. aegypticus*, *Prevotella salivae* while others were found to be decreased e.g., *Peptoniphilus*, *Corynebacterium sp.*, *Bifidobacterium longum*, *Dolosigranulum pigrum* or *Lactobacillaceae* sp. [1,40,99,100]. Furthermore, several studies indicate alterations in the overall microbial community composition and decreased microbial diversity of CRSsNP compared to healthy subjects. [99,101]. In contrast to formerly mentioned studies, Wagner Mackenzie et al., 2018 focussed on Archaea in CRS patients. However, they were not able to determine specific

archaeal signatures associated with CRS since only a few of the investigated samples yielded archaeal hits even if archaeal-specific methods were used [11].

A recently discussed post-infectious health condition is an impaired sense of smell (dysosmia). Infectious diseases of the URT like a cold or COVID-19 have been linked to reduced olfactory performance [102,103]. Koskinen et al., 2018 detected an increase in alpha diversity of the nasal microbiota (located directly at the olfactory epithelium) in people with post-infectious dysosmia. Additionally, bacterial signatures, namely *Faecalibacterium*, *Campylobacter*, *Porphyromonas* and *Enterobacteriaceae* were positively correlated with reduced olfactory capability. The increase in microbes producing the unpleasant smelling butyrate has been suggested to potentially have a negative impact on the sense of smell. Additionally, archaeal signatures, belonging to the phyla Thaumarchaeota and Euryarchaeota, were observed as well but no association with reduced olfactory performance could be found [41]. (see also *Table 1*)

Due to the current pandemic crisis, numerous studies focus on the COVID-19 infection and discuss the compositional changes of the human microbiome, however, results are not consistent among the studies. Increases [104,108] as well as decreases [105,124,107,109] in microbial alpha-diversity have been reported to be associated with COVID-19. Furthermore, significant microbiome differences were linked to COVID-19 ranging from no difference [108] to elevated counts of the genera *Salmonella*, *Serratia*, *Anaerococcus*, *Bacillus* or *Enterococcus* [104,105,107] and the species *Campylobacter hominis*, *Bacteroides vulgatus*, *Staphylococcus epidermidis* [104,106] in infected individuals. Several other taxa have been determined to be potentially negatively correlated with COVID-19, such as *Corynebacterium*, *Neisseria*, *Johnsonella*, *Leptotrichia*, *Finegoldia* or *Prevotella* [104,105,107,124]. An association of microbial community composition (mainly of the nasopharynx) and disease severity was reported as well. The observed inconsistency in these studies is potentially reasoned by the usage of different methodology (e.g., sampling strategy, amplicon sequencing or metagenomics/-transcriptomics) and changes in low abundant taxa [104–109,124]. (see also *Table 2*)

Even though COVID-19 causes anosmia, these microbiome studies did not focus on the olfactory performance of the subjects at all. Since all these findings were conducted from samples of the nasopharynx and not the olfactory mucosa, statements about olfactory capability might anyhow not be possible.

Gastrointestinal tract

An inappropriate colonisation by specific microbes and, accordingly, activation of harmful inflammatory responses, has been associated with inflammatory bowel diseases (IBD) including Crohn's disease (CD) and ulcerative colitis (UC). Microbes like *Eubacterium*, *Roseburia intestinalis, Ruminococcus gnavus* and *Fusobacterium nucleatum* are associated with CD whereas *Ruminococcus albus*, *Eubacterium rectale*, *F. prausnitzii* are decreased compared to healthy controls. [93,110,111]. The decrease in taxa like *F. prausnitzii* that normally contribute to the production of SCFAs might contribute to altered immune responses in IBD patients (see chapter "*Beneficial microbes and their way to influence the human host*"). In addition to changes in the bacterial community, IBD was also correlated with alterations in the fungal biodiversity (↓) and relative abundances of specific taxa (like *Candida sp.* (↑) or *Saccharomyces cerevisiae* (↓)) [112,113]. Furthermore, also archaeal signatures (↓*M.smithii*, ↑*Methanosphaera stadtmanae*) were described to be associated with IBD patients compared to healthy controls [114,115]. (see also *Table 1*)

Oral cavity

Several microorganisms have also been linked to diseases of the oral cavity like oropharyngeal squamous cell carcinoma (oSCC). Bacterial taxa such as *Fusobacterium*, *Parvimonas*, *Gemella*, *Porphyromonas gingivalis*, *Prevotella* , *Fusobacterium periodonticum* and *Pseudomonas aeruginosa* were observed in oSCC patients whereas *Streptococcus*, *Neisseria, Rothia* and *Veillonella* have been observed to have protective properties against oSCC [2,116–119]. Interestingly, some members of lactic acid producing bacteria possess anti-cancer properties (e.g. Lactobacillus plantarum induces apoptosis in cancer cells [120]) but others have also been detected in oSCC samples (e.g., *Peptostreptococcus* [118,121]). Additionally, *Candida albicans* is the most found fungal signature associated with oSCC and might promote tumour development [2,122,123]. Archaeal signatures (*Methanobrevibacter*, *Methanosphaera* and *Candidatus Nitrosoarchaeum*) were also found in oSCC patients, however, no correlation to oSCC could be determined [2]. (see also *Table 1*)

Table 1. Microbes associated with selected diseases.

	Upper respiratory tract		Gastrointestinal tract	Oral cavity
	CRS	anosmia	IBD	oSCC
Bacteria	↑*C. tuberculostearicum* ↑*C. accolens* ↑*H. influenzae/aegypticus* ↑*P. salivae* ↓*D. pigrum* ↓*Lactobacillus* ↓*Peptoniphilus* ↓*Corynebacterium* ↓*Dolosigranulum*	↑*Faecalibacterium* ↑*Campylobacter* ↑*Porphyromonas* ↑Enterobacteriaceae	↑*Eubacterium* ↑*Roseburia* ↑*R. gnavus* ↑*F. nucleatum* ↓*R. albus* ↓*E. rectale* ↓*F. prausnitzii*	↑*Fusobacterium periodonticum* ↑*Porphyromonas gingivalis* ↑*Pseudomonas aeruginosa* ↑*Parvimonas* ↑*Prevotella* ↓*Streptococcus* ↓*Rothia* ↓*Neisseria* ↓*Veillonella*
Archaea	Euryarchaeota Thaumarchaeota	Euryarchaeota Thaumarchaeota	↑*M. stadtmanae* ↓*M. smithii*	*Methanobrevibacter* *Methanosphaera* *Candidatus Nitrosoarchaeum*
Fungi			↑*Candida albicans* ↓*Saccharomyces cerevisiae*	↑*C. albicans*

Arrows (↑ and ↓) indicate an increase or decrease of the microbial diversity and relative abundance of the corresponding taxon in diseased patients compared to healthy controls, respectively. References in text!

Table 2. Information about methodological procedures and changes in the microbiome of the nasopharynx of COVID-19 positive subjects. (Continued on next page)

Literature	sampling site	extraction method/kit	sequencing and bioinformatics (overview)	COVID-19 patients *		
				alpha diversity	increased	decreased
Rosas-Salazar et al. 2021 [104]	mid-turbinate	DNeasy PowerSoil extraction Kit (Quiagen)	• universal primers • V4 region • MiSeq • DADA2 • SILVA • decontam • DeSeq2 (R package)	higher - not significant	*Brevundimonas* *Corynebacterium 1 imitants* *Corynebacterium* *Granulicatella* *Ezakiella* *Peptoniphilus lacrimalis* *Campylobacter hominis* *Sphingobacterium spiritivorum* *Anaerococcus* *Peptoniphilus* *Prevotella 9 copri* *Bacteroides vulgatus*	*Corynebacterium 1* *Prevotella disiens* *Staphylococcus haemolyticus* *Corynebacterium* *Anaerostipes hadrus* *Neisseria* Lachnospiraceae
Rueca et al., 2021 [105]	nasopharyx	QIAsymphony automatic extractor with DSP Virus/Pathogen Midi Kit	• V2-4-8 region • V3-6 region • V7-9 region • IonS5 Sequencer • NCBI • GAIA 2.0 • DeSeq2	lower - significant	*Salmonella* *Scardovia* *Serratia* Pseudomonadaceae	*Johnsonella* *Tepidiphilus* *Thermoanaerobacter* *Thermoanaerobacterium* *Thermosinus* *Variovorax*

Continued on next page

Table 2: Continued from previous page

Literature	Sampling site	Extraction method/kit	Sequencing and bioinformatics (overview)	COVID-19 patients *		
				alpha diversity	increased	decreased
Nardelli et al., 2021 [124]	nasopharyx	MagPurix ® Bacterial DNA Extraction Kit (Zinexts Life Science)	• V1-3 region • MiSeq • MicrobAT Suite • SmartSeq, (Novara, Italy) • Microbiome Analyst program • Kruskal-Wallis	lower - not significant		*Leptotrichia* *Fusobacterium peridonticum* *Haemophilus*
Engen et al., 2021 [107]	nasopharyx	NucleoMag Pathogen manufacturer's protocol (Macherey–Nagel)	• 515f/806R primers • V4 region • MiniSeq • QIIME2 • DADA2 • SILVA 138 • decontam • t-test or Mann.WhitneyU	lower - not significant	*Enterococcus* *Burkholderia-Caballeronia-Paraburkholderia* *Gulbenkiania* *Bacillus*	*Streptococcus* *Rothia* *Prevotella* *Finegoldia* *Anaerococcus* *Corynebacterium* Neisseriaceae
Braun et al., 2021 [108]	nasopharyx	StarMag universal cartridge kit (Seegene)	• 515f/806R39 primers • V4 region • MiSeq • QIIME2 • Deblur • permutatuin rank mean test • dsFDR correction • MaAsLin2	higher - not significant	did not find significant differences	
Zhong et al., 2021 [106]	nasal and throat	QiAamp RNeasy Mini Kit (Qiagen)	• metatranscriptomic • DNBSEQ-T7 platform • SortMeRNA • Kraken2X • NCBI RefSeq database • MetaPhlAn2 • Wilcoxon rank-sum	-	*Burkholderia cepacia complex* *Staphylococcus epidermidis* *Mycoplasma hominis* *Mycoplasma orale*	-
Mostafa et al., 2020 [109]	nasopharyx	NucliSENS easyMag (bioMérieux)	• metagenomics • Nanopore GridION X5 • CosmosID bioinformatics platform (CosmosID Inc., Rockville, MD)	lower	Propionibacteriaceae *Moraxella catarrhalis* *Haemophilus influenzae*	*Corynebacterium accolens*

* COVID-19 positive samples compared to healthy controls

Modulation of human microbiome compositions

Like already reviewed above, several diseases are associated with a shift of the human-associated microbiome, putatively contributing to disease development, progression, and treatment. Therefore, modulation of the microbial composition might be an option to maintain the healthy state or to reach a more favourable microbiome again. Since the microbial community composition is quite unique (except the keystone taxa) between individuals a more personalized treatment might be needed.

Dietary interventions

Several studies already described the beneficial properties of human commensal and thereof indicated a putative role of microbes in prevention and treatment of particular diseases in the future [60,64]. Using this knowledge of beneficial microbes might help to develop novel treatment strategies such as probiotics or a specific diet (fibre-rich) that support or maintain a healthy microbiome.

Probiotics are "live microorganisms which when administered in adequate amounts confer a health benefit on the host" [125]. Since decades, they are orally applied to promote the health of the GIT (e.g., by protection or recovery of the epithelial barrier) [126–128]. In the last years, some of the lactic acid bacteria (LAB), such as *Lactobacillus sp. or Dolosigranulum pigrum*, were also highly discussed as promising therapy options for URT infections since they naturally belong to the healthy nasal microbiota. Therefore, they might be considered as "safe" for human administration and are already adapted to the URT that might prevent clearance of the probiotic strains immediately after application [40,129,130].

Another way to promote a more favourable "healthy" microbiome is via diet. Dietary habits that are rich in fibres (non-degradable fibres in vegetables or fruits) have been determined to enrich specific fibre degrading microbes [131–135]. Additionally, the consumption of fermented food like cheese, yoghurt or sauerkraut might be able to modulate the microbial composition in the GIT, since these products contain living microbes, mainly LAB strains, but also *Bifidobacteria ssp.* as well as *Saccharomyces ssp.* (reviewed in [136]). Pasolli et al. even determined that some LAB might be able to colonize the GIT and by that improve human health [137]. Notably, besides this intended growth (spontaneous and with multispecies starter culture) of specific microbes in food, also microbes that are colonizing unprocessed food (e.g., apples,

lettuce or tomatoes) might impact human health and the microbiota composition in the human gut [138,139]. For example, according to the results of Wassermann et al., 2019, together with organically instead of conventionally managed apples, we ingest a microbial community substantially different in diversity (increased) and composition [139].

Therapeutically interventions

The most common medical strategies to manipulate the microbial community are antibiotics. These drugs inhibit cell growth/proliferation or directly lyse bacteria. This elimination or weakening of specific microbial taxa can lead to the outgrowth of other (beneficial or harmful) taxa that compete for the same ecological niche [24,30]. In case of harmful microorganisms, these microbial changes might lead to long term health issues. Furthermore, antibiotics/-mycotics are often part of cancer therapies since chemoradiation e.g., in oSCC has several adverse effects (e.g., hyposalivation, mucositis) leading to and associated with microbial dysbiosis. Therefore, this combined treatment helps to avoid infections or microbial overgrowth [2,140,141]. In addition to this, also other invasive therapies such as surgeries may have an impact on the adjacent microbiome. Opening of nasal sinuses in CRS patients or removal of tumour tissue affects the morphological structure and the prevalent biological condition at these areas by increasing the ventilation, clearing diseased tissue [1,142] and thereof providing new niches for microbes.

Objectives and summary of the thesis

Chapter 1.: The microbiome of the upper respiratory tract in health and disease. [1]

Even though the microbiome of the upper respiratory tract has been much less investigated compared to other body parts (e.g., gut, oral, skin) this location got more and more into focus in the last decades. With one of my projects, the "Nose Brain Axis" (NBA), our interest in the nasal microbiome increased – especially regarding the microbes located next to the olfactory epithelium in the nasal cavity and their contribution in human olfaction. During the literature research for this project, we decided that a review of the hitherto discovered insights into the human nasal community was required to provide information for our new project ("Nose Brain Axis") to: 1) support the decision on sampling site and technique as well as on appropriate extraction and sequencing approaches, 2) get to know the current literature on microbial composition in health and disease (including dysosmia), 3) highlight findings on archaeal composition in the nasal cavity and 3) collect information about probiotics use in the nasal cavity as therapy option in the future (**Figure 1**).

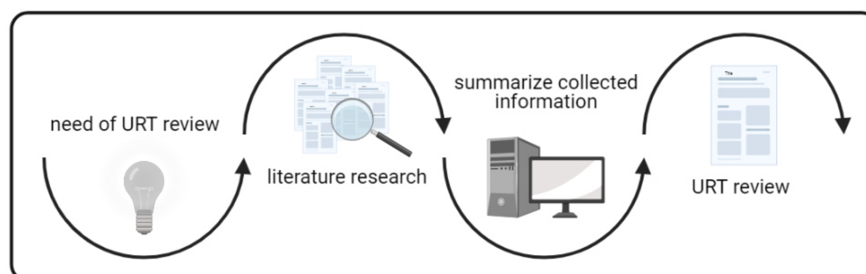


Figure 1. Study design "Upper respiratory tract microbiome" review. [1] *Created with BioRender.com*

Therefore, the current knowledge of the human upper respiratory tract – from the anterior nares to the nasopharynx – as well as its microbiome was collated in this review. It covers the different surface structures (e.g., epithelial lining) and biological conditions (e.g., humidity, mucociliary or clearance) both leading to various niches within the nasal cavity. Furthermore, this manuscript also draws attention to the interplay between microbes themselves and the human host by addressing microbial competitions (e.g., niche specificity, freely available nutrients) as well as host defence mechanisms (e.g., mucociliary clearance, immune system response activation). Additionally, insights into the general microbial composition of the healthy human nose and a summary of the variability of the microbial structures in the upper respiratory tract

are provided: 1.) during the process of aging (from newborn to elderly), 2.) different lifestyles as well as 3.) in various health issues are given. Finally, different therapy options to treat URT diseases with contribution of the URT microbiome (e.g., chronic rhinosinusitis) were also topic of this literature research to provide information about currently used therapies – namely nasal washes, probiotics, and surgery.

Chapter 2.: Reduced B12 uptake and increased gastrointestinal formate drive archaeome-mediated breath methane emission in humans. [3]

Approx. 20% of the general population exhales methane levels above 5 ppm. Even though CH_4 is a non-toxic gas, higher breath methane levels have already been associated with different gut conditions (e.g., constipation or slower gut transit). However, we found that also healthy adults without any gut issues emit methane levels up to 75 ppm, in this study. Other studies already described an association between methane emission in humans and methanogenic archaea (methanogens). Methanogens are yet to be the only known microbes in the gut producing CH_4. In this article, the correlation between microbial composition in the GIT, breath methane status and dietary habits should be further assessed. We hypothesized that the abundance of methane-producing archaea are influenced by microbial connections and dietary habits in the healthy gut. For this purpose, healthy participants (n=100) were asked to collect breath and stool samples as well as filling out a food frequency questionnaire. Based on methane levels, subjects were categorized into high and low methane emitters (high methane emitter: HE ≥5ppm) before defining key species, microbial networks and investigation of connections between HE microbial community composition and nutritional intake (**Figure 2**).

As already described in other studies, we were able to confirm an association between the relative as well as absolute abundance of the archaeon *M. smithii* and CH_4 levels measured in the breath. Together with the taxa Christensenellaceae, *Ruminoclostridium*, Ruminococcaceae and *Eubacterium, M. smithii* was significantly positively correlated with HEs, whereas the genera *Bacteroides*, *Blautia*, *Ruminococcus*, *Butyricicoccus* and *Flavonifractor* were identified as representatives for the microbial community of low methane emitters (LE). Furthermore, the HE microbial community was significantly positive correlated with vitamin B12 intake and measured formate levels in the stool. It seems that people consuming less vitamin B12 are more

likely to be colonized by methanogens/*M. smithii* and therefore emit higher amounts of methane.

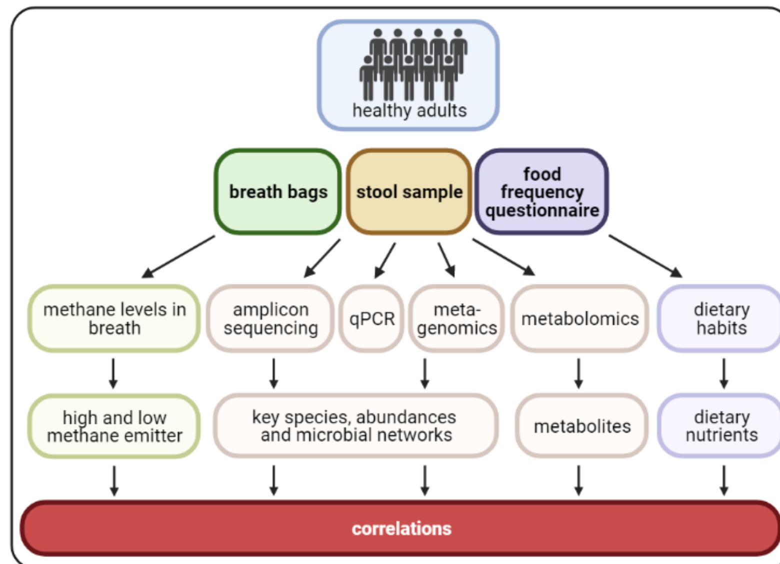


Figure 2. Study design "Methanogens in the GIT" article. [3] *Created with BioRender.com*

Chapter 3.: Preliminary insights into the impact of primary radiochemotherapy on the salivary microbiome in head and neck squamous cell carcinoma. [2]

Oropharyngeal squamous cell carcinoma (oSCC) is one of the dominant head neck cancer types. In non-operable cases, it is often treated with chemoradiation that in many times leads to hyposalivation causing a very dry environment in the mouth that facilitates the development of painful mucositis and ulcers indicating a microbial dysbiosis. Therefore, this aggressive therapy is often combined with antibiotics and/or antimycotics (standard treatment) to minimise infections or overgrowth of microorganisms in the arisen open niches.

In this preliminary study, following points were addressed: 1) Can we identify putative biomarkers for oSCC in saliva samples before cancer therapy? 2) What changes in the microbial community structure and diversity occur due to standard oSCC therapy to further understand the side effects (e.g., mucositis) of the therapy? 3) Additionally, already known correlations between oSCC and alcohol consumption and HPV status should be confirmed. To answer these open questions, saliva samples of oSCC patients (n=31) before and after standard therapy were collected and compared to saliva samples of healthy controls (n=11). The archaeal, bacterial

and fungal signatures were assessed via amplicon sequencing (16S rRNA gene and ITS region, respectively) and subsequently correlated with collected metadata information of the corresponding subject (**Figure 3**).

An altered microbial community structure with significant changes in some microbial signatures was found when comparing healthy controls and oSCC patients before therapy as well as patients before and after therapy. Namely, the bacterial signatures of *Bifidobacterium* (↑), *Veillonella* (↑), Pasteurellaceae (↓), and *Eubacterium nodatum group* (↓) were significantly associated with non-treated patients compared to healthy subjects. Standard therapy led to decreased abundances of *Haemophilus*, *Veillonella* and *Granulicatella* whereas *Lactobacillus*, *Acinetobacter* and *Gemella* were enriched. In contrast to the bacterial approach, the results of the fungal approach suggest an increasing number of relative abundance of fungal signatures from healthy controls to non-treated and treated patients. The relative abundance of the fungal genera *Candida* tended to be elevated in both oSCC samples groups compared to healthy subjects. Due to many archaea-negative samples in the archaeal approach only statements about the generally found archaeal signatures could be made.

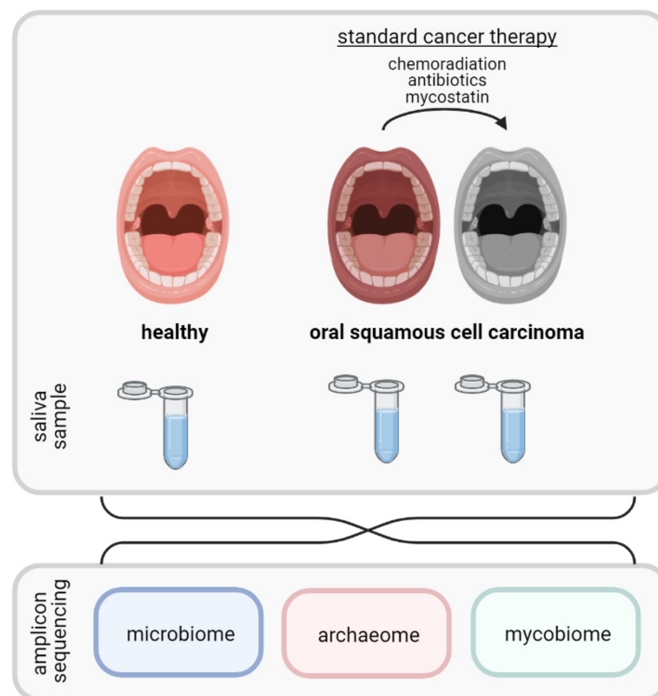


Figure 3. Study design "Saliva microbiome in health and oSCC before and after standard cancer therapy" article. [2] *Created with BioRender.com*

The publications

Chapter 1.:

The microbiome of the upper respiratory tract in health and disease.[1]

Unpublished project: **The Nose Brain Axis - The correspondence of the nasal microbiome and human olfaction.** (*manuscript in progress*)

Chapter 2.:

Reduced B12 uptake and increased gastrointestinal formate drive archaeome-mediated breath methane emission in humans.[3]

Chapter 3.:

Preliminary insights into the impact of primary radiochemotherapy on the salivary microbiome in head and neck squamous cell carcinoma.[2]

1st publication

The microbiome of the upper respiratory tract in health and disease. [1]

Christina Kumpitsch[1], Kaisa Koskinen[1], Veronika Schöpf[2,3,4] and Christine Moissl-Eichinger[1,3]

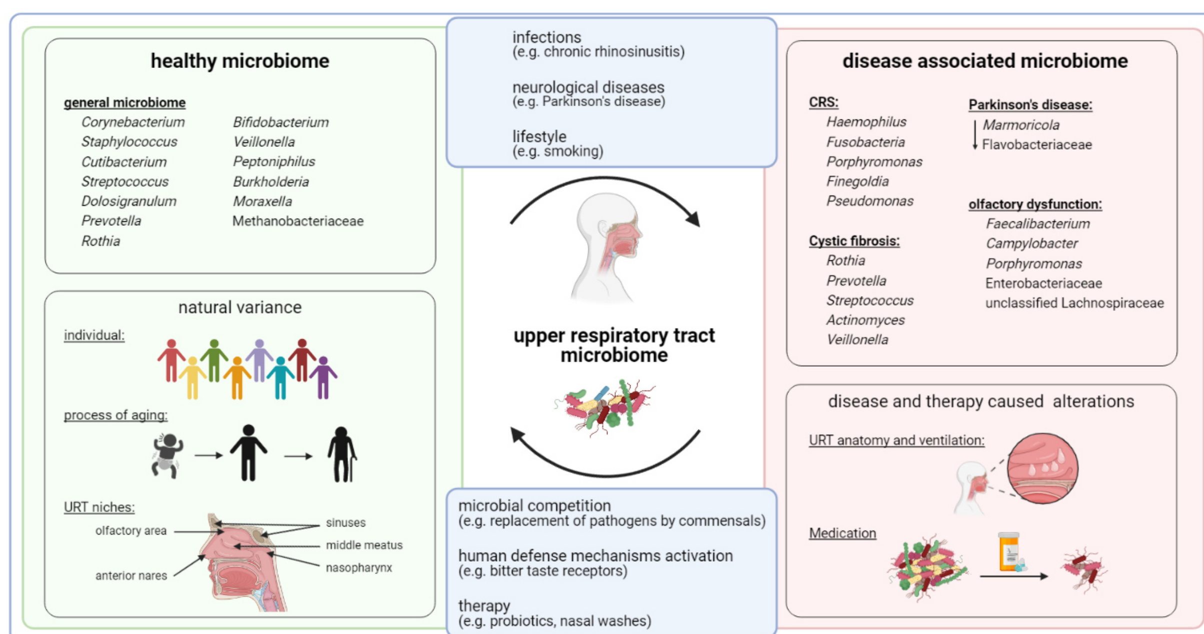
[1] *Diagnostic and Research Institute of Hygiene, Microbiology and Environmental Medicine, Medical University of Graz, Neue Stiftingtalstraße 6, 8010 Graz, Austria.*

[2] *Institute of Psychology, University of Graz, Universitaetsplatz 2, 8010 Graz, Austria.*

[3] *BioTechMed-Graz, Mozartgasse 12/II, 8010 Graz, Austria.*

[4] *Present address: Medical University Vienna, Spitalgasse 23, 1090 Vienna, Austria.*

Graphical Abstract



Created with BioRender.com

Published in **BMC Biology** (*November 2019*)

Unpublished project:

The Nose Brain Axis – The correspondence of neuroplasticity and the microbiome in human olfaction

Christina Kumpitsch[1], Florian Ph. S. Fischmeister[2,3,4], Sonja Lackner[5], Sandra Holasek[5], Tobias Madl[6], Veronika Schöpf[7]and Christine Moissl-Eichinger[1,4]

[1] *Diagnostic and Research Institute of Hygiene, Microbiology and Environmental Medicine, Medical University of Graz, Graz 8010, Austria*

[2] *Department of Psychology, University of Graz, Graz 8010, Austria*

[3] *Department of Biomedical Imaging and Image-guided Therapy, Medical University of Vienna, Vienna 1090, Austria*

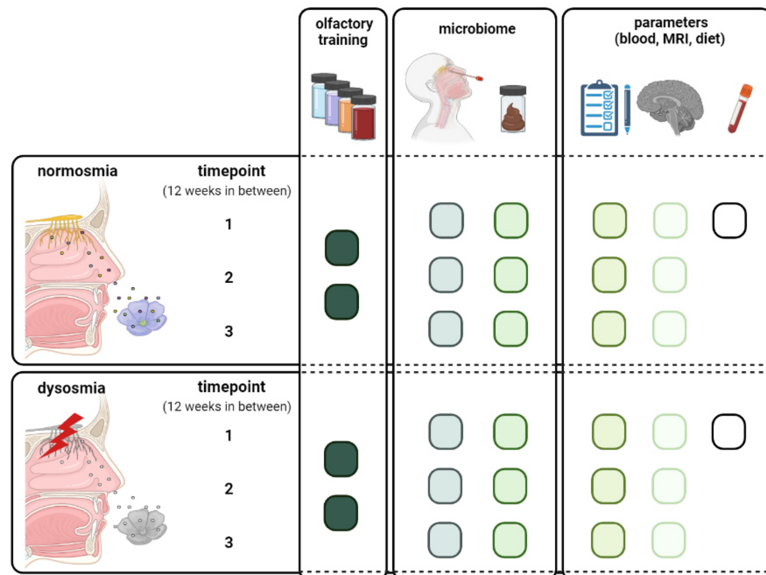
[4] *BioTechMed, Graz, Graz 8010, Austria*

[5] *Division of Immunology and Pathophysiology, Medical University of Graz, Graz 8010, Austria*

[6] *Gottfried Schatz Research Center for Cell Signaling, Metabolism and Aging, Molecular Biology & Biochemistry, Medical University of Graz, Graz 8010, Austria*

[7] *Present address: Medical University Vienna, Spitalgasse 23, 1090 Vienna, Austria.*

Graphical study schemata



Created with BioRender.com

Unpublished project (*manuscript in progress*)

2nd publication

Reduced B12 uptake and increased gastrointestinal formate drive archaeome-mediated breath methane emission in humans. [3]

Christina Kumpitsch[1], Florian Ph. S. Fischmeister[2,3], Alexander Mahnert[1], Sonja Lackner[4], Marilena Wilding[2], Corina Sturm[2], Anna Springer[5], Tobias Madl[5,6], Sandra Holasek[4], Christoph Högenauer[7], Ivan Berg[8], Veronika Schöpf[3] and Christine Moissl-Eichinger[1,6]

[1] *Diagnostic and Research Institute of Hygiene, Microbiology and Environmental Medicine, Medical University of Graz, Graz 8010, Austria*

[2] *Department of Psychology, University of Graz, Graz 8010, Austria*

[3] *Department of Biomedical Imaging and Image-guided Therapy, Medical University of Vienna, Vienna 1090, Austria*

[4] *Division of Immunology and Pathophysiology, Medical University of Graz, Graz 8010, Austria*

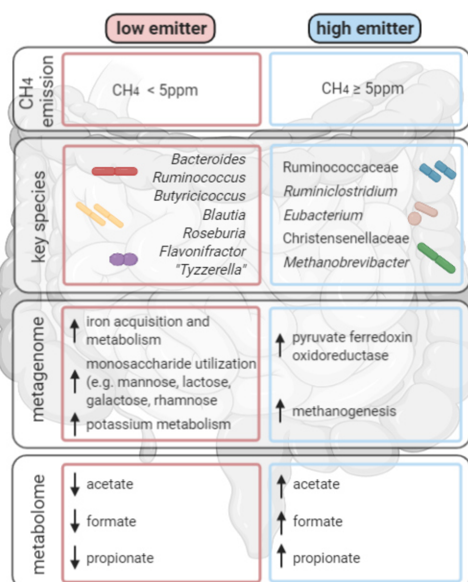
[5] *Gottfried Schatz Research Center for Cell Signaling, Metabolism and Aging, Molecular Biology & Biochemistry, Medical University of Graz, Graz 8010, Austria*

[6] *BioTechMed, Graz, Graz 8010, Austria*

[7] *Division of Gastroenterology and Hepatology, Medical University of Graz, Graz, Austria.*

[8] *Institute for Molecular Microbiology and Biotechnology, University of Münster, Münster, Germany.*

Graphical Abstract [3]



Created with BioRender.com

Published in **Microbiome** (*accepted*)

3rd publication

Preliminary insights into the impact of primary radiochemotherapy on the salivary microbiome in head and neck squamous cell carcinoma. [2]

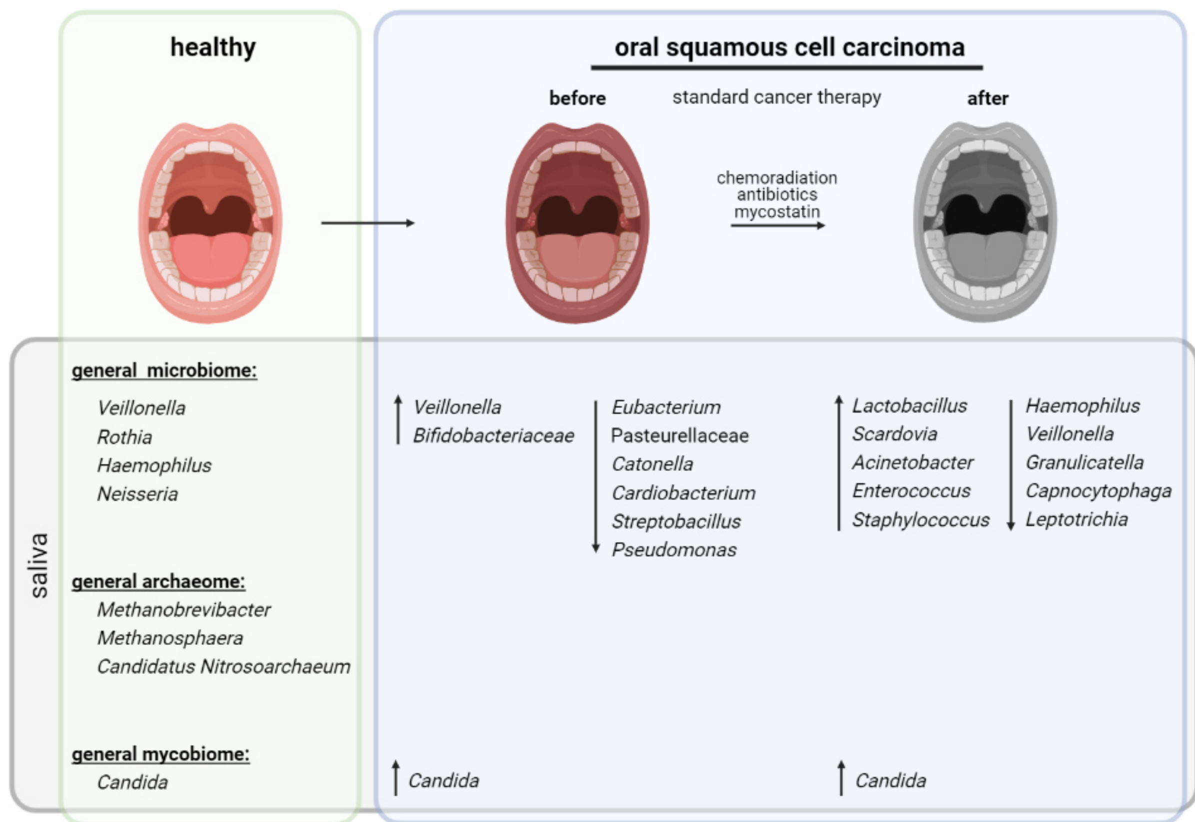
Christina Kumpitsch[1], Christine Moissl-Eichinger[1,2], Jakob Pock[3], Dietmar Thurnher[3] & Axel Wolf[3]

[1] *Diagnostic and Research Institute of Hygiene, Microbiology and Environmental Medicine, Medical University of Graz, Graz, Austria.*

[2] *BioTechMed, Graz, Austria.*

[3] *Department of Otorhinolaryngology, Medical University of Graz, Graz, Austria.*

Graphical Abstract



Created with BioRender.com

Published in **Scientific Reports** (*October 2020*)

General Discussion

Chapter 1.: The microbiome of the upper respiratory tract in health and disease.[1]

In the last years also other human body parts, besides the GIT, came into focus, including body sites that are less densely colonized by microorganisms like the nasal cavity [4]. With the present review [1], we aimed to collect the knowledge on the nasal microbiome and provide current information for our new project ("Nose-Brain Axis) to: 1) choose an appropriate sampling site and handling procedure of these low biomass samples (methods), 2) get to know current knowledge on the contribution of the nasal microbiome in diseases (especially dysosmia), 3) review findings on the archaeal community in the nose and 4) discuss probiotics as an innovative therapy option for URT issues in the future.

The core microbiome of the healthy URT as well as the disease-associated microbial community (e.g., CRS) have already been deciphered in several studies [35,41,99,100]. Nevertheless, these studies often came to different conclusions when comparing the URT in health and disease. Even though the identified core microbiome was similar in a wide range of the conducted studies, taxa associated with a particular health state were often mismatching or even contradictory among studies [1,99,101,143]. Common discrepancies between the studies were the choice of 1) sampling site, 2) sample processing (e.g., storage, nucleic acid extraction kits, primers or sequencing method), and 3) data analysis tools and settings (as examples for COVID-19, see *table 2*; [104,105,108,124]). In the case of the URT, the narrow and winding anatomy of the nose provides various niches with different conditions for a distinct microbial composition [144]. Consequently, the choice of an adequate sampling site is recommended. But if deeper areas in the nose are preferred, sampling can be quite challenging. Reaching areas deep in the nose come with a higher risk of touching other locations within the nasal cavity leading to microbial contamination therefrom. As usually the sinuses are only accessible by a surgery, the collection of samples from healthy controls is difficult. Therefore, many studies sample either healthy individuals undergoing other nasal surgeries (e.g., septum correction) or the middle meatus (receives drainage from different sinuses) as a representative of the sinus microbiome [38,143,145]. However, De Boeck et al showed that the samples of the anterior nares are suitable mirrors of the sinus microbiome [99]. This points out that locations should be chosen wisely to avoid misleading results. This is particularly important when working with low

biomass samples like nasal samples, as low abundant taxa might distinguish defined health states.

Furthermore, sample processing and downstream analysis might influence the study outcome as well. Differences in cell lysis (mechanical and/or chemical), selection of primers as well as databases and software produce heterogeneous outputs. Some procedures might not cover specific taxa (e.g., archaea) or yield various outcomes (reviewed in [146]). Thus particularly low abundant taxa are differentially captured and putative microbial biomarkers to distinguish health and disease could be missed. In the case of archaea, several studies missed archaeal signatures when using approaches that target mainly bacterial signatures. Others reported totally underrepresented signatures of the skin-dominant genus *Propionibacterium* in skin samples by amplicon sequencing [7,147].

Another important issue is that samples that derive from the nasal cavity show an overall low microbial biomass. Therefore, the avoidance of contamination and the use of controls in parallel throughout the procedure are of high importance to avoid false-positive signals. Subsequently, application of bioinformatic tools during data processing (like decontam or PERFect [148,149]) that are capable of identifying and extracting prominent microbial features in controls compared to original samples are highly recommended.

All in all, it can be assumed that the investigation of the same research question with different sampling sites and methodologies such as sampling, processing and analysis procedures may result in less consistency between study outcomes. Additionally, contaminations of the low biomass samples may easily result in misleading outcomes. This suggests a need of a standardised protocol for nasal mucus samples including methodological recommendations from sampling method, extraction kits to sequencing methods as well as downstream data analysis tools and settings. Furthermore, the application of customized standards for controlling the procedures on each step of the analysis (e.g., mock community with known composition) would help to increase comparability of different studies and produce results of high quality.

As already mentioned above, several URT issues have been associated with alterations of the nasal microbiome in the last years. Even though several respiratory tract infections like a cold or COVID-19 cause a loss of smell as well as taste [102,103,150], the contribution of the nasal microbiome to olfactory function is rarely investigated. Initially, in the case of viral infections,

it was assumed that the virus enters the brain by invasion of the olfactory neurons that directly reach through the cribriform plate to the olfactory bulb in the brain ("neuronal-mucosa gateway"). However, non-neuronal cells (neuron-supporting cells) of the olfactory epithelium but not olfactory neurons express surface receptor angiotensin-converting enzyme 2 (ACE2) that is needed for virus entry. Other studies in turn demonstrated that SARS-CoV2 was present in the olfactory mucosa and bulb. This might indicate an neuron-independent entry pathway [151–155].

COVID-19 was linked to inflammatory markers (like higher interleukin-6 levels in people suffering from smell and taste loss), signs of neuronophagia in the olfactory bulb and non-functional T2R38 [156–159] potentially impairing the olfactory performance. Additionally, the inflammation and diminished immune response by the non-functional T2R38 might facilitate viral or bacterial co-infections and restructure the nasal microbial community. Interestingly, T2R38 malfunction and inflammation have also been linked to CRS severity and olfactory dysfunction. Even though nasal polyps might also play a role in dysosmia, inflammation of the olfactory mucosa causes histological changes and even destroys olfactory neurons and by that maybe favouring a change in microbial community. Hence, olfactory dysfunction is able to indicate the level of inflammation in CRS patients (reviewed in [160]; [161,162]). A change in the nasal microbial community in COVID-19 and CRS patients has already been observed by different studies but the study outcomes were again not consistent (see table 2). Additionally, based on the sample site (mainly nasopharynx or middle meatus), it might not be possible to state if the nasal microbiome contributes to the loss of smell in the case of COVID-19 and CRS.

In contrast to others, the study of Koskinen et al., 2018 focused exactly on the microbiome located at the olfactory epithelium of normosmics (people with normal olfactory performance) and hyposmics (people with reduced olfactory capability, in this case due to prior infection). They found significant alterations between normosmics and hypsomics in microbial diversity as well as community composition. Gut- and oral-associated bacteria, particularly butyric acid-producers (e.g., *Faecalibacterium* or *Porphyromonas*), that were associated with reduced olfactory performance attracted attention [41]. Even though butyric-acid has many health-mediating properties on human body tissues, this unpleasant smelling microbial product might have adverse effects on the olfactory epithelium (like in the oral cavity during periodontitis caused by *P. gingivalis*) [163,164]. This strong odour might overstimulate the odour receptors

leading to diminished odour perception after a while and thereof contribute to dysosmia (impaired sense of smell). In our unpublished study (project "Nose-Brain Axis") that also included people with a complete loss of smell (anosmics), we were able to demonstrate a nearly stepwise decrease in diversity with increasing olfactory performance. This decrease was driven by microbial richness rather than evenness indicating a more diverse microbial community composition in anosmics compared to normosmics. Additionally, signatures associated with dysosmia (like *Rickettsia*, *Porphyromonas*, and *Faecalibacterium*), and normosmia (like *Ralstonia*) were observed. Of note, also smell training was conducted which improved the olfactory capability in both groups but was not yet correlated to the nasal microbial composition (preliminary results, unpublished data – "Nose-Brain Axis"). Similar findings on the positive impact of olfactory training have already been described in other studies [165,166]. All these findings raise the question if reorganization of the brain alone or together with the nasal microbial community at the olfactory epithelium contributes to olfactory function, dysfunction and regain. At the moment we assume that 1) microbes do not play a causal role in the development of dysosmia but microbial dysbiosis contributes to the already existing reduced olfactory performance and 2) consciously inhaling air (during the smell training) influences the nasal microbial community either by increasing the ventilation causing modified biological and physical conditions in the nasal cavity, and/or introducing microbes.

Most likely dysosmia is the consequence of a combination of different factors. These may include viral infiltration, host immune responses (e.g., inflammation), changes in the microbiome composition and/or medical treatments (antibiotics/-mycotics). Even though we hypothesize that microbes contribute to the olfactory performance, it might be difficult to find general putative dysosmia-associated biomarkers. Because not only upper respiratory tract infections but also other diseases (e.g., Parkinson's disease) are able to impair olfactory function in a negative way [167,168]. Nevertheless, gaining further knowledge on the loss of smell and the nasal microbiome in the different diseases will help to decipher the protective role of our nasal inhabitants and might lead to new therapeutic strategies to increase the quality of life of people suffering from URT issues and dysosmia.

Until new therapy approaches are developed, URT issues associated with bacterial dysbiosis will still be mainly treated with antibiotics impacting the general microbial community. Fortunately, like mentioned above, in the recent years more and more studies focused on finding

biomarkers for health and disease [41,99,169]. This might result in the development of probiotics that either protect against nasal infections in the first place or restore a healthy nasal condition. Using the synergistic and antagonistic interactions between microbes and the production of health-mediating microbial metabolites, beneficial microbes isolated from the healthy URT will potentially replace antibiotic therapy in the future. Several strains have already been identified to offer such a positive impact on the URT health e.g., *Corynebacterium pseudodiphteriticum* or *Dolosigranulum pigrum* are capable of inhibiting *S. aureus* overgrowth and additionally, (if co-cultured) also *Haemophilus influenzae* and *Streptococcus pneumonia* [38,130]. Most probiotics are orally applied (even in URT issues e.g., the butyrate producer *Enterococcus faecalis* [128,170]) but the nasal administration might represent a more innovative treatment option by directly applying beneficial microbes where they are needed. However, the development of this type of administration is challenging since the used microbes might reach the lung and cause lung infections (especially if applied via nasal sprays). Therefore, potential probiotic strains should be extensively checked and chosen carefully.

Several studies have already been conducted on humans. The used strains including *Lactococcus lactis W136,* and *Streptococcus salivarius* and *Streptococcus oralis*, respectively, were well tolerated and led to decreased symptoms in patients suffering from CRS and recurrent respiratory infection [171,172]. Additionally, the URT isolate *Lactocaseibacillus casei AMBR2* of the study of De Rudder et al., 2020 demonstrated a strong adherence to URT epithelium cell lines via fimbriae-like structures and survival in microbial donor backgrounds in vitro [173]. The same strain was also tested as intranasal application in healthy subjects without prior antibiotics treatment and was detectable even hours after administration [40]. This points out the importance of both the adaptation to niche-specific conditions of probiotics as well as the interaction with the present microorganisms of the already occupied niche for long-term colonisation. Maybe the strategy of using microbes that are already present in the healthy URT (like *Dolosigranulum* or *Corynebacterium*) might also open up new opportunities to improve olfactory performance after infections again. Therefore, strains have to be selected that have no pathogenic evidence, no resistance to antibiotics and that have already extensively been tested in cell culture. Hence, further research has to be conducted to reveal better insights into the adaptation of "safe" putative probiotic strains to the healthy nasal microbiota, to the URT and its potential health-mediating properties to develop novel non-invasive and easy to apply therapies for URT issues.

Throughout the literature research, we came across remarkably few URT studies on archaea. Since most of the methods up to date show limited capability or even fail to detect archaea, specific approaches are needed to study archaea as part of the human-associated community [43,174]. Archaea are known important human key taxa in the human microbiome as already shown in the GIT tract and might influence the human host's health via microbial by-products (e.g., CH_4) or interspecies interactions such as hydrogen transfer [3,7,66]. A few publications detected archaeal taxa in the nasal cavity. Mostly, Euryarchaeota and Thaumarcheota were detected by Wagner-Mackenzie and Koskinen et al., 2018 among others [11,41]. In addition to the findings of the latter study, our unpublished study "the Nose-Brain Axis" even indicated a negative trend between olfactory performance and one archaeon belonging to the phyla Euryarchaeota – the GIT keystone species *M. smithii* (preliminary results, unpublished data – "Nose-Brain Axis"). With this finding, the NBA study indicates not only a potential contribution of an archaeon to this particular health condition but also, together with the formerly mentioned bacterial taxa (e.g., *Faecalibacterium*), an increase in GIT-associated microbes in anosmics.

Instead of universal approaches that many target bacteria, archaea-specific procedures and methods used also in our studies include combined mechanical and enzymatical cell lysis, nested PCR (1st: 344F-1041R; 2nd: 519F-806R) for amplicon sequencing, or shotgun metagenomics. Furthermore, quantitative methods (e.g. qPCR or ddPCR) targeting specific genes such as the mcrA gene in methanogens can be performed to get specific information about the archaeal community (methods described in [1-3,43]). Despite focalising research particularly on archaea in the URT, archaeal signatures are often not detectable in every analysed sample [11] due to methodological limitations of the currently used archaea-specific methods. This highlights the need of new archaea-specific methods and should raise interest to expand the knowledge also on other multi-kingdom taxa beyond bacteria.

Even though many studies already focus on URT microbiome, a lot is still unknown and needs to be unravelled. By choosing appropriate sampling sites, controls and methods for specific research questions, a lot of crucial knowledge in the healthy and diseased URT will be deciphered in the future and help to decrease the burden of URTIs and anosmia by e.g., developing putative probiotics.

Chapter 2.: Reduced B12 uptake and increased gastrointestinal formate drive archaeome-mediated breath methane emission in humans. [3]

Archaea, the third domain of life, have been considered for a long time to exclusively live in extreme environments. In the last years archaea are more and more frequently found additionally in mesophilic environments such as the human body. Since then, this neglected human-associated community came much more into focus. Up to now, several archaea-specific strategies have been established to detect archaeal signatures in the human microbiome in addition to the commonly targeted bacterial community [43,174].

In this article [3], we confirmed the impact of the methanogens (mainly *Methanobrevibacter*) on methane production in the GIT of healthy individuals as well as demonstrated significant associations between methane emission and intake of certain nutrients. High methane emitters (HE, $CH_4 \geq 5$ppm) were colonized by significantly higher relative and absolute abundances of *M. smithii* compared to low emitters (LE, $CH_4 < 5$ppm). Furthermore, a whole network of various taxa (here referred to as keystone taxa) could be identified for HE and LE, respectively [3]. For example, Christensenellaceae (↑) was significantly correlated with *Methanobrevibacter*. Other studies already described a synergistic interaction of those two taxa and co-culturing experiments revealed a shift in metabolite production (butyrate to acetate) of Christensenellaceae in the presence of *Methanobrevibacter* [175,176]. Notably, in our study, the relative abundance of *Methanobrevibacter* was increased 1000-fold in HE compared to LE, while Christensenellaceae was also present in both groups with just three-fold increase in HE compared to LE [3]. Hence, the HE-associated microbiome can be predominantly defined by high *Methanobrevibacter* abundance in the GIT. As expected, metagenomic analysis also indicated a strong association between HE-associated microbial signatures and functional genes involved in methanogenesis (HE: 0.1%, LE: 0.00004% → 2,500 times higher). Additionally, other microbial taxa such as Ruminococcaceae*, Eubacterium* and *Ruminiclostridium* were significantly associated with high methane production as well as *Methanobrevibacter* abundance [3]. This is supported by previous studies that associated the presence of e.g., Ruminococcaceae sp. with increased methane production [177]. Similar to Christensenellaceae, the presence of these bacterial taxa may favour the production of acetate rather than butyrate. High acetate levels lead to an accumulation of H_2 and CO_2 boosting methanogenesis by methanogens in the GIT [178]. In the case of low methane emission, we identified *Bacteroides*,

Ruminococcus, *Blautia*, *Butyricicoccus*, *Flavonifractor, Roseburia* and "*Tyzerella*" to be elevated and defined them as LE keystone taxa [3].

Analysis of the nutritional intake of selected participants highlighted a significant negative association between general dietary fibre intake and the relative abundance of some LE associated microbes (namely *Bacteroides*, *Flavonifractor* and *Ruminococcus gnavus group*) [3]. Interestingly, degradation of dietary fibre depends on complexity and type of polysaccharides, e.g., *Bacteroides* cannot degrade microcrystalline cellulose in contrast to *Ruminococcus*. In addition to this, the type of ingested carbohydrates also promotes the production of distinct SCFAs (e.g., pectin → acetate; resistant starch → butyrate) [65,177]. This availability of different carbohydrates and SCFAs might favour the colonisation of different microbial species and by that confirm the importance of the type of dietary fibres in modulating the composition and functionality of the gut microbiome.

Another interesting nutritional value that caught our attention was vitamin B12 (cobalamin) that was negatively association with two HE-associated taxa, namely *Methanobrevibacter* and Christensenellaceae [3]. Due to its complex biosynthesis, the human organism obtains this essential vitamin mainly via diet (main source: animal products). Consistently, we found a positive correlation between vegetarianism and reduced vitamin B12 intake in our study. In general, ingested dietary vitamin B12 is mainly absorbed in the small intestine whereas microbially produced vitamin B12 is produced and absorbed in the colon [3,67,70]. Thereof, larger amounts of ingested vitamin B12 would be needed to also reach and directly influence the microbes in the large intestine. Unfortunately, in our study, cobalamin levels were not measured in stool samples hence no conclusions could be drawn on microbially produced vitamin B12.

A lack of essential vitamins can lead to an elevated risk of developing diseases such as infections or inflammatory diseases by altering the immune response [179]. Especially vitamin B12 deficiency was also shown to be associated with elevated formate concentrations in rats and their metabolism seem to be closely connected with each other in humans as well [180]. Our study showed similar results by identifying a negative correlation of ingested vitamin B12 levels and formate measured in stool samples ($p=0.038$). Additionally, CH_4 emission levels were positively correlated with formate concentration ($p=0.006$) and formate efflux transportation genes [3]. The role of formate in increased CH_4 production is supported by the fact

that *Methanobrevibacter* can utilise formate as carbon source for methanogenesis [181] and that formate seems to promote methano-archaeal adhesion [182].

Considering these findings, supplementation of vitamin B12 in HE subjects should lead to decreased breath methane levels by reducing the formate concentration, according to our published model [3]. By changing the metabolite composition and dynamics in the GIT, it may even be able to shift the overall microbial composition to a LE-associated microbiome. This hypothesis is currently being tested in an ongoing master's thesis.

The methane measurements in this study were preformed via breath test without substrate challenge in the morning and before brushing the teeth [3]. This measured methane is assumed to derive mainly from the GIT. However, new findings of a recent paper by Edrich et al., (2021) discovered that mouthwash (with chlorhexidine; prior to CH_4 breath measurement) reduces CH_4 concentrations emitted via breath [92]. This indicates an important contribution of oral-associated methanogens – such as *Methanobrevibacter oralis* [183] – in breath methane emission. Considering these results, the oral hygiene of subjects might contribute to measured breath methane levels. In our study, we were able to correlate breath methane levels to *Methanobrevibacter* counts (amplicon sequencing and qPCR) [3] suggesting that CH_4 is an appropriate biomarker for *Methanobrevibacter* in the GIT even without prior mouthwash. Despite of this, methanogenesis by the oral methanogen's repositories should be considered when measuring breath methane concentrations in the future. This will help to gain a more accurate picture of the methane production in the GIT and also avoid misleading results, in particular in border-line subjects with an unclear or varying methane production.

Elevated emission of methane has already been linked to health issues such as constipation and decelerated gut transit time (reviewed in [7]). The latter condition can lead to negative effects on the bacterial physiology and metabolism resulting in incorrect amino acid fermentation or protein breakdown [184]. Since other GIT issues like IBD have been associated with high methane levels, the non-invasive and easy to use breath measurement may provide a suitable diagnostic tool for these GIT disorders [7]. In contrast to these findings, we were able to measure elevated methane levels (up to 75 ppm) in healthy subjects without self-reported gut problems [3]. In accordance to this, others identified an anti-inflammatory property of CH_4 and associated increased CH_4 concentrations with reduced fat tissue and a lean phenotype [84,176,185,186]. Even though studies on the methane topic came to controversial findings, the idea of putative health-

mediating properties of a HE-associated microbiome should be further followed. GIT-colonizing methanogens might possess direct health benefits for the host or lead to a favourable microbial composition with complex interspecies interactions and metabolites that still have to be further investigated.

What is already known is that 1) a diet rich in fibres (complex carbohydrates) mediates human health since non-digestible polysaccharides are fermented to SCFA by gut microbes [65]. 2) In general, vegetarians consume more dietary fibres and this dietary habit was found to be healthy in several studies [187]. 3) Vegetarianism is often linked to vitamin B12 deficiency because its main source are animal products [187]. 4) In our study, we linked less vitamin B12 intake not only with vegetarianism but also elevated formate concentrations in stool and an increased relative abundance of the HE keystone taxa *Methanobrevibacter* and Christensenellaceae [3]. 5) Additionally, high formate levels were associated with CH_4 emission (↑), two HE key species (↑) and three LE key species (↓) [3]. 6) Furthermore, increased formate concentrations have been linked to several health benefits such as a positive impact on cardiovascular function or foetal development in other studies [188]. (Figure 4)

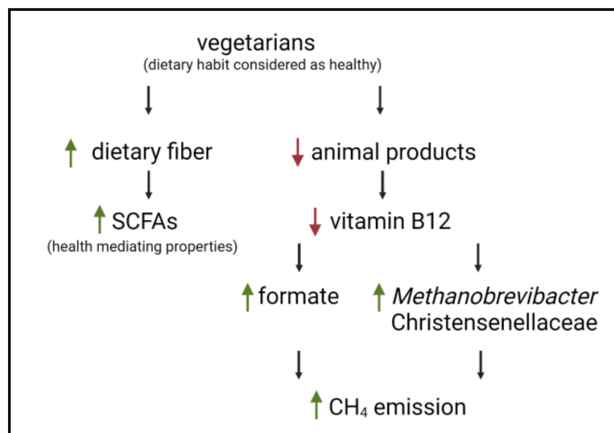


Figure 4. Schemata of already known facts and our findings (for references see text).

Considering all these facts, we assume that the microbiome of high methane emitters possesses health benefits on the human host. Due to the significantly increased relative abundance of *Methanobrevibacter* in HEs compared to LEs [3] they are regarded as one of the key drivers of the HE microbiome. Nevertheless, further research on the impact of *Methanobrevibacter* and the HE-associated microbiome, respectively, is needed to draw a final conclusion about its health-beneficial or disease-mediating capacities. Maybe *Methanobrevibacter* might become a putative candidate for probiotics in the future. However, implementation of this idea might be

quite difficult as cultivation of archaea in larger amounts is problematic due to special culturing conditions that are required. Additionally, administration of those strict anaerobic microbes could be challenging.

Hence, right now it might be of more interest to start with more feasible options anyhow like the usage of prebiotics in sense of a change to a fibre rich diet to facilitate the settlement of the potentially health-mediating HE microbiome.

Chapter 3.: Preliminary insights into the impact of primary radiochemotherapy on the salivary microbiome in head and neck squamous cell carcinoma. [2]

Aggressive radio-chemotherapy in oSCC does not only impact tumour tissues but also adjacent healthy tissue and the oral microbiota. In this publication, the saliva microbiome of healthy adults was compared with oSCC patients. The sampling of saliva allowed us to collect information about the microbiome of the overall oral cavity and the oropharynx.

Like other studies on the oral microbiome, we identified among others *Veillonella*, *Rothia*, *Haemophilus*, *Neisseria* and *Candida* as part of the most abundant genera in the healthy microbiome [2,116,119,189]. In contrast to most other studies, an additional archaeal specific approach was performed, however, we were not able to detect archaeal signatures in all our samples. Nevertheless, we found *Methanobrevibacter* and *Methanosphaera* in some saliva samples. These taxa have already been detected in the oral cavity by other studies earlier [2,10]. By comparing the microbiome of healthy subjects to oSCC patients, we identified increased abundances of *Veillonella* and Bifidobacteriaceae as biomarkers for oSCC. Interestingly, more bacterial taxa were found to be significantly associated with the healthy rather than the diseased state, indicating a loss of beneficial microbes during oSCC progression [2]. The fungal approach indicated an increase of the genus *Candida* in oSCC [2] (consistent with [122]). *Candida* has been reported to exhibit several mechanisms (e.g., degradation of membrane protein laminin, production of carcinogens) to promote tumour development/progression in its acidic environment [190].

Chemoradiation is an aggressive cancer treatment. Therefore, changes in the salivary microbiome due to the therapy are expected. By investigating the impact of chemoradiation (+/- antibiotics/antimycotics treatment) – here referred to just as “RCHT therapy” – on the

microbiome, these alterations as well as putative biomarkers for oSCC should be defined in this study. In general, RCHT shifted the microbiome structure further towards an adverse state and altered the microbial load [2] demonstrating the microbiome-modulating capacity of this aggressive therapy. In more detail, the bacterial load decreased stepwise from healthy to oSCC before and after treatment, while the fungal load showed contradictory outcomes [2]. This points out that RCHT might have the ability to diminish the bacterial population but some fungal taxa that withstand the antimycotics therapy occupy the new formed niche in the oral cavity. Moreover, both, bacterial as well as fungal diversity, was lower in the saliva of diseased people (as also shown elsewhere [122]) and after treatment [2]. Notably, RCHT is also associated with an inflammation of the oral mucosa [191]. Consistently, we detected several taxa associated with infections of the oral cavity like *Acinetobacter*, *Enterococcus* and *Staphylococcus* (mucositis, periodontitis and nosocomial infections) [192–194] which were relatively higher abundant in treated compared to non-treated saliva samples [2]. Additionally, the relative abundance of the genus *Lactobacillus* was higher after compared to before the treatment [2]. Interestingly, some LAB strains were also linked to oSCC. LAB might lead to a reduction in pH in the oral cavity and by that contribute to tumour growth (reviewed in [190]). However, we primarily detected the *Lactobacillus* signature in samples from treated patients [3]. Therefore, we assumed that the dehydration of the oral cavity (xerostomia) and pain [191] caused by the therapy led to a change in diet. Patients might consume more yoghurt after treatment since it is cold and more pleasant to eat compared to solid food. Besides this, they potentially take probiotics that are commonly recommended to use in parallel to antibiotics treatment. Unfortunately, these metadata were not collected and should be considered next time. If LAB in the mouth of treated oSCC patients possess a beneficial role or not remains unclear. Longitudinal observations from diagnosis, over the treatment period and even beyond that are needed to make statements about their role in tumour recovery.

Like in non-treated samples, the only fungal signature that was still increased after the harsh cancer therapy was *Candida* [2]. This finding is supported by the long-known ability of *Candida albicans* to adapt to distinct body niches and adhere to several host as well as bacterial surface molecules [195]. Furthermore, fungus stimulates immune response and inflammation [196] that might contribute to the development of mucositis. In this publication, we were able to demonstrate the persistent presence of the signature *Candida* throughout the study although

mycostatin is a common treatment for *Candida* infection. Maybe the used antimycotics therapy should be reconsidered and the switch to another medication would be wise.

In this study, RCHT also included antibiotics and antimycotics, hence no statements can be made about the effect of chemoradiation therapy on the salivary microbiome alone. Furthermore, several antibiotics with different doses were used to treat and prevent bacterial infections that also impacted the microbial community [2]. Therefore, finding the "one" biomarker of oSCC or therapy outcome might be not possible here. Since oSCC tumour tissues and the response to the cancer therapies are highly individual, it will be very difficult to find a standardised treatment for all patients. The driving force is directed towards personalized medicine, anyhow. Nevertheless, investigation of health- and oSCC-associated biomarkers will provide information on developing putative probiotics to prevent or treat severe side effects (e.g., mucositis, xerostomia or pain) of RCHT. These may improve inflammation, help to restore the healthy microbial community, and hence positively impact the oral tissue.

Conclusion

This thesis emphasizes the importance of the uprising field of human microbiome research by exploring the microbiome of different human body sites in health and disease. In the last years, also the contribution of the neglected part of the microbiome, archaea and fungi, captivated researchers around the globe. New methodological approaches were established that target microbial taxa beyond bacteria. Despite these establishments, most research projects still focus on the bacterial components of the microbiome by using classical procedures. However, investigating the entire microbial community in the human body is crucial to understand the complex interactions between the microbiome and its human host.

Some URT infections (but also neurodegenerative diseases) have already been linked to microbiome alterations and dysosmia. Long-term dysosmia is a great burden for suffering people and tremendously reduces their quality of life. Even though it is commonly known that microbes are able to influence human body tissues via secondary metabolites (e.g. SCFAs), just little is known about the contribution of the olfactory-associated microbiome on olfactory function. In the course of this thesis, the putative involvement of butyrate-producing bacteria as well as the CH_4-producing *Methanobrevibacter* in olfactory impairment was pointed out. Future studies should investigate the interaction of the olfactory mucosa and the microbial metabolites (e.g., butyrate or CH_4) and potential immunomodulatory properties of the identified microbes. Moreover, current research on COVID-19 opens up a new opportunity to further increase the knowledge on the role of the nasal microbiome in olfaction. By using also non-targeted approaches like metagenomics and metatranscriptomics even statements at multi-kingdom level might be possible.

Archaea have been shown to have a niche in the GIT by several studies. Especially the archaeon *Methanobrevibacter* has been demonstrated to belong to the keystone taxa and to produce CH_4. Even though no causal connections between archaea and health disorders have been demonstrated so far, several studies linked elevated CH_4 levels with health conditions like bloating or constipation. However, it is still not clear how and if methanogens contribute to these health issues. In our study, we showed that also healthy adults are able to emit higher amounts of methane and that the microbiome associated with high methane emission might be favoured by a vegetarian-based diet (high in dietary fibres and low in vitamin B12). Presuming

a health beneficial effect of vegetarianism, a HE-associated microbiome might be desirable. A follow-up study should test if vitamin B12 supplementation in high methane-emitting individuals leads to lower CH_4 emission and an altered microbial composition. These findings will possibly provide a novel opportunity to modulate the microbial community in the GIT or adapt CH_4 levels e.g., to reduce bloating in patients someday.

Tumors like oSCC influence the adjacent microbial community profile. The aggressive treatment of oSCC is highly individual (varies in dose and duration) and causes several adverse effects like pain, mucositis, hyposalivation or bacterial and fungal infections. To reduce these co-infections, antibiotics and or antimycotics are often used. In our publication, we showed that despite the use of antimycotics, fungal signatures like of *Candida* were increased. Hence, the efficacy of the used antimycotics should be assessed and adapted if needed in the future. Furthermore, *Lactobacillus* might play an important role in recovery after the therapy. Future studies on oSCC should include longitudinal investigations of the saliva microbiome beginning at the day of diagnosis and lasting beyond the end of the therapy to demonstrate complex dynamical changes due to cancer therapy. Additionally, there should be particular focus on fungal and archaeal contributions on cancer development/progression, or therapy outcome, since fungal signatures tended to increase after the combined therapy and little is known about the archaeal role in oSCC.

All publications highlight the importance of multi-kingdom approaches to capture the overall interplay between microbes and the human host to avoid missing crucial information. Of particular note is that present approaches seem to target just a small proportion of the multi-kingdom species; like in our studies that predominantly identified methanogens and *Candida*. Therefore, further establishments of multi-kingdom approaches are needed to appropriately identify the non-bacterial members of the microbiome. Although it might still be a long way until appropriate methodology is developed, we strongly recommend that future studies on the human microbiome should not overlook but start to include the non-bacterial community in their research.

Even though there are still many unknowns that have to be explored in the future, with our presented publications we were able to unravel another piece of the microbial role in human’s health and disease.

References

1. Kumpitsch C, Koskinen K, Schöpf V, Moissl-Eichinger C. The microbiome of the upper respiratory tract in health and disease. BMC Biol. 2019;17(1):87.
2. Kumpitsch C, Moissl-Eichinger C, Pock J, Thurnher D, Wolf A. Preliminary insights into the impact of primary radiochemotherapy on the salivary microbiome in head and neck squamous cell carcinoma. Sci Rep. 2020 Dec 1;10(1):16582. Available from: https://doi.org/10.1038/s41598-020-73515-0
3. Kumpitsch C, Fischmeister F, Mahnert A, Lackner S, Wilding M, Sturm C, et al. Reduced B12 uptake and increased gastrointestinal formate drive archaeome-mediated breath methane emission in humans. Microbiome. 2021;accepted, (*manuscript version before peer-review*).
4. Sender R, Fuchs S, Milo R. Revised Estimates for the Number of Human and Bacteria Cells in the Body. PLoS Biol. 2016;14(8).
5. Berg G, Rybakova D, Fischer D, Cernava T, Vergès MCC, Charles T, et al. Microbiome definition re-visited: old concepts and new challenges. Vol. 8, Microbiome. BioMed Central Ltd; 2020.
6. Nocker A, Cheung CY, Camper AK. Comparison of propidium monoazide with ethidium monoazide for differentiation of live vs. dead bacteria by selective removal of DNA from dead cells. J Microbiol Methods. 2006;67(2).
7. Mahnert A, Blohs M, Pausan M-R, Moissl-Eichinger C. The human archaeome: methodological pitfalls and knowledge gaps. Emerg Top Life Sci. 2018 Dec 14;2(4):469–82. Available from: http://www.emergtoplifesci.org/lookup/doi/10.1042/ETLS20180037
8. Koskinen K, Pausan MR, Perras AK, Bang MBC, Mora M, Schilhabel A, et al. First Insights into the Diverse Human Archaeome : Specific Detection of Archaea in the Gastrointestinal Tract ,. MBio. 2017;8(6):1–17.
9. Moissl-Eichinger C, Pausan M, Taffner J, Berg G, Bang C, Schmitz RA. Archaea Are Interactive Components of Complex Microbiomes. Trends Microbiol. 2018 Jan 1;26(1):70–85. Available from: https://pubmed.ncbi.nlm.nih.gov/28826642/
10. Pausan MR, Csorba C, Singer G, Till H, Schöpf V, Santigli E, et al. Exploring the Archaeome: Detection of Archaeal Signatures in the Human Body. Front Microbiol. 2019;10:2796.
11. Wagner Mackenzie B, West AG, Waite DW, Lux CA, Douglas RG, Taylor MW, et al. A

Novel Description of the Human Sinus Archaeome During Health and Chronic Rhinosinusitis. Front Cell Infect Microbiol. 2020;10.

12. Man WH, De Steenhuijsen Piters WAA, Bogaert D. The microbiota of the respiratory tract: Gatekeeper to respiratory health. Vol. 15, Nature Reviews Microbiology. 2017.
13. Kreth J, Merritt J, Qi F. Bacterial and host interactions of oral streptococci. DNA Cell Biol. 2009;28(8).
14. Burman E, Bengtsson-Palme J. Microbial Community Interactions Are Sensitive to Small Changes in Temperature. Front Microbiol. 2021 May 21;12:672910. Available from: https://www.frontiersin.org/articles/10.3389/fmicb.2021.672910/full
15. Hibbing ME, Fuqua C, Parsek MR, Peterson SB. Bacterial competition: Surviving and thriving in the microbial jungle. Vol. 8, Nature Reviews Microbiology. 2010.
16. Zhang L, Gallo RL. Antimicrobial peptides. Curr Biol. 2016 Jan 11;26(1):R14–9. Available from: http://www.cell.com/article/S0960982215014098/fulltext
17. Rich RR, Chaplin DD. The Human Immune Response. Clin Immunol. 2019 Jan 1;3-17.e1.
18. Wanner A, Salathé M, O'Riordan T. Mucociliary clearance in the airways. Am J Respir Crit Care Med. 1996;154(6 Pt1):1868–902.
19. Fjell CD, Hiss JA, Hancock REW, Schneider G. Designing antimicrobial peptides: Form follows function. Vol. 11, Nature Reviews Drug Discovery. 2012.
20. Treerat P, Redanz U, Redanz S, Giacaman RA, Merritt J, Kreth J. Synergism between Corynebacterium and Streptococcus sanguinis reveals new interactions between oral commensals. ISME J. 2020;14(5).
21. Nakamura K, O'Neill AM, Williams MR, Cau L, Nakatsuji T, Horswill AR, et al. Short chain fatty acids produced by Cutibacterium acnes inhibit biofilm formation by Staphylococcus epidermidis. Sci Rep. 2020;10(1).
22. Bauer MA., Kainz K, Carmona-Gutierrez D, Madeo F. Microbial wars: competition in ecological niches and within the microbiome. Microb Cell. 2018 May 1;5(5):215.
23. Tshikantwa TS, Ullah MW, He F, Yang G. Current Trends and Potential Applications of Microbial Interactions for Human Welfare. Front Microbiol. 2018 Jun 1;9(JUN):1156. Available from: www.frontiersin.org
24. Schwartz DJ, Langdon AE, Dantas G. Understanding the impact of antibiotic perturbation on the human microbiome. Genome Med 2020 121. 2020 Sep 28;12(1):1–12. Available from: https://genomemedicine.biomedcentral.com/articles/10.1186/s13073-020-00782-x
25. Finlay BB, Amato KR, Azad M, Blaser MJ, Bosch TCG, Chu H, et al. The hygiene

hypothesis, the COVID pandemic, and consequences for the human microbiome. Proc Natl Acad Sci . 2021 Feb 9;118(6). Available from: https://www.pnas.org/content/118/6/e2010217118

26. Kolde R, Franzosa EA, Rahnavard G, Hall AB, Vlamakis H, Stevens C, et al. Host genetic variation and its microbiome interactions within the Human Microbiome Project. Genome Med 2018 101. 2018 Jan 29;10(1):1–13. Available from: https://genomemedicine.biomedcentral.com/articles/10.1186/s13073-018-0515-8
27. Ma J, Li Z, Zhang W, Zhang C, Zhang Y, Mei H, et al. Comparison of gut microbiota in exclusively breast-fed and formula-fed babies: a study of 91 term infants. Sci Reports 2020 101. 2020 Sep 25;10(1):1–11. Available from: https://www.nature.com/articles/s41598-020-72635-x
28. Reyman M, Houten MA van, Baarle D van, Bosch AATM, Man WH, Chu MLJN, et al. Impact of delivery mode-associated gut microbiota dynamics on health in the first year of life. Nat Commun 2019 101. 2019 Nov 1;10(1):1–12. Available from: https://www.nature.com/articles/s41467-019-13014-7
29. Losasso C, Eckert EM, Mastrorilli E, Villiger J, Mancin M, Patuzzi I, et al. Assessing the influence of vegan, vegetarian and omnivore oriented westernized dietary styles on human gut microbiota: A cross sectional study. Front Microbiol. 2018;9(MAR).
30. Palleja A, Mikkelsen KH, Forslund SK, Kashani A, Allin KH, Nielsen T, et al. Recovery of gut microbiota of healthy adults following antibiotic exposure. Nat Microbiol. 2018;3(11).
31. Camarinha-Silva A, Jáuregui R, Pieper DH, Wos-Oxley ML. The temporal dynamics of bacterial communities across human anterior nares. Environ Microbiol Rep. 2012;4(1):126–32.
32. Yatsunenko T, Rey FE, Manary MJ, Trehan I, Dominguez-Bello MG, Contreras M, et al. Human gut microbiome viewed across age and geography. Nat 2012 4867402. 2012 May 9;486(7402):222–7. Available from: https://www.nature.com/articles/nature11053
33. Hansen MEB, Rubel MA, Bailey AG, Ranciaro A, Thompson SR, Campbell MC, et al. Population structure of human gut bacteria in a diverse cohort from rural Tanzania and Botswana. Genome Biol 2019 201. 2019 Jan 22;20(1):1–21. Available from: https://genomebiology.biomedcentral.com/articles/10.1186/s13059-018-1616-9
34. Banerjee S, Schlaeppi K, Heijden MGA van der. Keystone taxa as drivers of microbiome structure and functioning. Nat Rev Microbiol 2018 169. 2018 May 22;16(9):567–76.

Available from: https://www.nature.com/articles/s41579-018-0024-1

35. De Boeck I, Wittouck S, Wuyts S, Oerlemans EFM, van den Broek MFL, Vandenheuvel D, et al. Comparing the healthy nose and nasopharynx microbiota reveals continuity as well as niche-specificity. Front Microbiol. 2017;8(NOV).
36. Bomar L, Brugger SD, Lemon KP. Bacterial microbiota of the nasal passages across the span of human life. Curr Opin Microbiol. 2018;41:8–14. Available from: http://dx.doi.org/10.1016/j.mib.2017.10.023
37. Wagner Mackenzie B, Dassi C, Vivekanandan A, Zoing M, Douglas RG, Biswas K. Longitudinal analysis of sinus microbiota post endoscopic surgery in patients with cystic fibrosis and chronic rhinosinusitis: a pilot study. Respir Res. 2019;22(1).
38. Yan M, Pamp SJ, Fukuyama J, Hwang PH, Cho D-Y, Holmes S, et al. Nasal microenvironments and interspecific interactions influence nasal microbiota complexity and S. aureus carriage. Cell Host Microbe. 2013;14(6):631–40.
39. Wagner Mackenzie B, Chang K, Zoing M, Jain R, Hoggard M, Biswas K, et al. Longitudinal study of the bacterial and fungal microbiota in the human sinuses reveals seasonal and annual changes in diversity. Sci Rep. 2019;9(1).
40. De Boeck I, van den Broek MFL, Allonsius CN, Spacova I, Wittouck S, Martens K, et al. Lactobacilli Have a Niche in the Human Nose. Cell Rep. 2020;31(8).
41. Koskinen K, Reichert JL, Hoier S, Schachenreiter J, Duller S, Moissl-Eichinger C, et al. The nasal microbiome mirrors and potentially shapes olfactory function. Sci Rep. 2018;8(1):1–11.
42. Pausan MR, Csorba C, Singer G, Till H, Schöpf V, Santigli E, et al. Exploring the Archaeome: Detection of Archaeal Signatures in the Human Body. Front Microbiol. 2019 Dec 5;10. Available from: /pmc/articles/PMC6906140/?report=abstract
43. Pausan MR, Csorba C, Singer G, Till H, Schoepf V, Santigli E, et al. Measuring the archaeome: detection and quantification of archaea signatures in the human body. bioRxiv. 2018 May 30;334748. Available from: https://www.biorxiv.org/content/early/2018/05/30/334748
44. Lloyd-Price J, Mahurkar A, Rahnavard G, Crabtree J, Orvis J, Hall AB, et al. Strains, functions and dynamics in the expanded Human Microbiome Project. Nature. 2017 Sep 20;550(7674):61. Available from: http://www.nature.com/doifinder/10.1038/nature23889
45. Borrel G, McCann A, Deane J, Neto MC, Lynch DB, Brugère JF, et al. Genomics and metagenomics of trimethylamine-utilizing Archaea in the human gut microbiome. ISME J.

2017;11(9).

46. Kim JY, Whon TW, Lim MY, Kim YB, Kim N, Kwon MS, et al. The human gut archaeome: Identification of diverse haloarchaea in Korean subjects. Microbiome. 2020;8(1).
47. Nash AK, Auchtung TA, Wong MC, Smith DP, Gesell JR, Ross MC, et al. The gut mycobiome of the Human Microbiome Project healthy cohort. Microbiome. 2017;5(1).
48. Hoffmann C, Dollive S, Grunberg S, Chen J, Li H, Wu GD, et al. Archaea and Fungi of the Human Gut Microbiome: Correlations with Diet and Bacterial Residents. PLoS One. 2013;8(6).
49. Pasolli E, Asnicar F, Manara S, Zolfo M, Karcher N, Armanini F, et al. Extensive Unexplored Human Microbiome Diversity Revealed by Over 150,000 Genomes from Metagenomes Spanning Age, Geography, and Lifestyle. Cell. 2019;176(3).
50. Almeida A, Nayfach S, Boland M, Strozzi F, Beracochea M, Shi ZJ, et al. A unified catalog of 204,938 reference genomes from the human gut microbiome. Nat Biotechnol. 2021 Jan 1;39(1):105–14. Available from: https://doi.org/10.1038/s41587-020-0603-3
51. Almeida A, Mitchell AL, Boland M, Forster SC, Gloor GB, Tarkowska A, et al. A new genomic blueprint of the human gut microbiota. Nature. 2019;568(7753).
52. Nayfach S, Shi ZJ, Seshadri R, Pollard KS, Kyrpides NC. New insights from uncultivated genomes of the global human gut microbiome. Nature. 2019 Apr 25;568(7753):505–10. Available from: https://doi.org/10.1038/s41586-019-1058-x
53. Xu X, He J, Xue J, Wang Y, Li K, Zhang K, et al. Oral cavity contains distinct niches with dynamic microbial communities. Environ Microbiol. 2015;17(3).
54. Van'T Hof W, Veerman ECI, Amerongen AVN, Ligtenberg AJM. Antimicrobial defense systems in saliva. Monogr Oral Sci. 2014;24.
55. Nearing JT, DeClercq V, Van Limbergen J, Langille MGI. Assessing the Variation within the Oral Microbiome of Healthy Adults. mSphere. 2020;5(5).
56. Peters BA, Wu J, Hayes RB, Ahn J. The oral fungal mycobiome: Characteristics and relation to periodontitis in a pilot study. BMC Microbiol. 2017;17(1).
57. Dupuy AK, David MS, Li L, Heider TN, Peterson JD, Montano EA, et al. Redefining the human oral mycobiome with improved practices in amplicon-based taxonomy: Discovery of Malassezia as a prominent commensal. PLoS One. 2014;9(3).
58. Nkamga VD, Henrissat B, Drancourt M. Archaea: Essential inhabitants of the human digestive microbiota. Vol. 3, Human Microbiome Journal. 2017.

59. Cummings JH, MacFarlane GT. Role of intestinal bacteria in nutrient metabolism. Vol. 16, Clinical Nutrition. 1997.
60. Soto-Martin EC, Warnke I, Farquharson FM, Christodoulou M, Horgan G, Derrien M, et al. Vitamin biosynthesis by human gut butyrate-producing bacteria and cross-feeding in synthetic microbial communities. MBio. 2020;11(4).
61. Rowland I, Gibson G, Heinken A, Scott K, Swann J, Thiele I, et al. Gut microbiota functions: metabolism of nutrients and other food components. Eur J Nutr. 2018;57(1).
62. Workman AD, Maina IW, Brooks SG, Kohanski MA, Cowart BJ, Mansfield C, et al. The role of Quinine-responsive Taste receptor Family 2 in airway immune Defense and chronic rhinosinusitis. Front Immunol. 2018;9(March):1–8.
63. Gensollen T, Iyer SS, Kasper DL, Blumberg RS. How colonization by microbiota in early life shapes the immune system. Science (80-). 2016;352(6285).
64. Pickard JM, Zeng MY, Caruso R, Núñez G. Gut microbiota: Role in pathogen colonization, immune responses, and inflammatory disease. Vol. 279, Immunological Reviews. 2017.
65. Campos-Perez W, Martinez-Lopez E. Effects of short chain fatty acids on metabolic and inflammatory processes in human health. Biochim Biophys Acta - Mol Cell Biol Lipids. 2021 May 1;1866(5):158900.
66. Ye Z heng, Ning K, Ander BP, Sun X jun. Therapeutic effect of methane and its mechanism in disease treatment. Vol. 21, Journal of Zhejiang University: Science B. Zhejiang University Press; 2020. p. 593–602. Available from: /pmc/articles/PMC7445089/
67. Yoshii K, Hosomi K, Sawane K, Kunisawa J. Metabolism of dietary and microbial vitamin b family in the regulation of host immunity. Front Nutr. 2019 Apr 17;6:48. Available from: www.frontiersin.org
68. Gu Q, Li P. Biosynthesis of Vitamins by Probiotic Bacteria. In: Probiotics and Prebiotics in Human Nutrition and Health. InTech; 2016. Available from: http://dx.doi.org/10.5772/63117
69. Deguchi Y, Morishita T, Mutai M. Comparative Studies on Synthesis of Water-soluble Vitamins among Human Species of Bifidobacteria. Agric Biol Chem. 1985;49(1):13–9. Available from: https://www.tandfonline.com/action/journalInformation?journalCode=tbbb20
70. Seetharam B, Alpers DH. Absorption and transport of cobalamin (vitamin B12). Vol. 2, Annual review of nutrition. 1982.

71. Gu Q, Zhang C, Song D, Li P, Zhu X. Enhancing vitamin B12 content in soy-yogurt by Lactobacillus reuteri. Int J Food Microbiol. 2015 Aug 3;206:56–9. Available from: https://pubmed.ncbi.nlm.nih.gov/25955289/
72. Van Wyk J, Witthuhn RC, Britz TJ. Optimisation of vitamin B12 and folate production by Propionibacterium freudenreichii strains in kefir. Int Dairy J. 2011 Feb 1;21(2):69–74.
73. Li KT, Peng WF, Zhou J, wei SJ, Cheng X. Establishment of beet molasses as the fermentation substrate for industrial vitamin B12 production by Pseudomonas denitrificans. J Chem Technol Biotechnol. 2013;88(9).
74. Koh A, De Vadder F, Kovatcheva-Datchary P, Bäckhed F. From dietary fiber to host physiology: Short-chain fatty acids as key bacterial metabolites. Cell. 2016;165(6).
75. Macfarlane S, Macfarlane GT. Regulation of short-chain fatty acid production. Proc Nutr Soc. 2003;62(1).
76. Ze X, Duncan SH, Louis P, Flint HJ. Ruminococcus bromii is a keystone species for the degradation of resistant starch in the human colon. ISME J. 2012;6(8).
77. Louis P, Young P, Holtrop G, Flint HJ. Diversity of human colonic butyrate-producing bacteria revealed by analysis of the butyryl-CoA:acetate CoA-transferase gene. Environ Microbiol. 2010;12(2).
78. Walker AW, Ince J, Duncan SH, Webster LM, Holtrop G, Ze X, et al. Dominant and diet-responsive groups of bacteria within the human colonic microbiota. ISME J. 2011;5(2).
79. Baxter NT, Schmidt AW, Venkataraman A, Kim KS, Waldron C, Schmidt TM. Dynamics of human gut microbiota and short-chain fatty acids in response to dietary interventions with three fermentable fibers. MBio. 2019;10(1).
80. Silva YP, Bernardi A, Frozza RL. The Role of Short-Chain Fatty Acids From Gut Microbiota in Gut-Brain Communication. Front Endocrinol (Lausanne). 2020 Jan 31;11:25. Available from: www.frontiersin.org
81. Boros M, Tuboly E, Mészáros A, Amann A. The role of methane in mammalian physiology - Is it a gasotransmitter? J Breath Res. 2015 Mar 1;9(1). Available from: https://pubmed.ncbi.nlm.nih.gov/25624411/
82. Nose K, Nunome Y, Kondo T, Araki S, Tsuda T. Identification of gas emanated from human skin: Methane, ethylene, and ethane. Anal Sci. 2005;21(6).
83. Polag D, Keppler F. Global methane emissions from the human body: Past, present and future. Atmos Environ. 2019 Oct 1;214:116823.
84. Boros M, Ghyczy M, Irces D, Varga G, Tokés T, Kupai K, et al. The anti-inflammatory

effects of methane. Crit Care Med. 2012;40(4).

85. Shen M, Fan D, Zang Y, Chen Y, Zhu K, Cai Z, et al. Neuroprotective effects of methane-rich saline on experimental acute carbon monoxide toxicity. J Neurol Sci. 2016;369.
86. Morris B, Henneberger R, Huber H, Moissl-Eichinger C. Microbial syntrophy: interaction for the common good. FEMS Microbiol Rev. 2013;37(3):384–406.
87. Hwang L, Low K, Khoshini R, Melmed G, Sahakian A, Makhani M, et al. Evaluating breath methane as a diagnostic test for constipation-predominant IBS. Dig Dis Sci. 2010;55(2).
88. Jahng J, Jung IS, Choi EJ, Conklin JL, Park H. The effects of methane and hydrogen gases produced by enteric bacteria on ileal motility and colonic transit time. Neurogastroenterol Motil. 2012;24(2).
89. Kim G, Deepinder F, Morales W, Hwang L, Weitsman S, Chang C, et al. Methanobrevibacter smithii Is the Predominant Methanogen in Patients with Constipation-Predominant IBS and Methane on Breath. Dig Dis Sci. 2012;57:3213–8.
90. Dharmawardana N, Goddard T, Woods C, Watson DI, Butler R, Ooi EH, et al. Breath methane to hydrogen ratio as a surrogate marker of intestinal dysbiosis in head and neck cancer. Sci Rep. 2020;10(1).
91. Suri J, Kataria R, Malik Z, Parkman HP, Schey R. Elevated methane levels in small intestinal bacterial overgrowth suggests delayed small bowel and colonic transit. Med (United States). 2018;97(21).
92. Erdrich S, Tan ECK, Hawrelak JA, Myers SP, Harnett JE. Hydrogen–methane breath testing results influenced by oral hygiene. Sci Rep. 2021;11(1).
93. Clooney AG, Eckenberger J, Laserna-Mendieta E, Sexton KA, Bernstein MT, Vagianos K, et al. Ranking microbiome variance in inflammatory bowel disease: A large longitudinal intercontinental study. Gut. 2021;70(3).
94. Tai J, Han MS, Kwak J, Kim TH. Association Between Microbiota and Nasal Mucosal Diseases in terms of Immunity. Int J Mol Sci. 2021;22(9).
95. Halfvarson J, Brislawn CJ, Lamendella R, Vázquez-Baeza Y, Walters WA, Bramer LM, et al. Dynamics of the human gut microbiome in inflammatory bowel disease. Nat Microbiol. 2017;2.
96. Kim JH, Kim SH, Lim JY, Kim D, Jeong IS, Lee DK, et al. Association between the sinus microbiota with eosinophilic inflammation and prognosis in chronic rhinosinusitis with nasal polyps. Exp Mol Med. 2020;52(6).

97. Manor O, Dai CL, Kornilov SA, Smith B, Price ND, Lovejoy JC, et al. Health and disease markers correlate with gut microbiome composition across thousands of people. Nat Commun. 2020;11(1).

98. Fokkens WJ, Lund VJ, Hopkins C, Hellings PW, Kern R, Reitsma S, et al. European Position Paper on Rhinosinusitis and Nasal Polyps 2020. Rhinology. 2020;58.

99. De Boeck I, Wittouck S, Martens K, Claes J, Jorissen M, Steelant B, et al. Anterior Nares Diversity and Pathobionts Represent Sinus Microbiome in Chronic Rhinosinusitis. mSphere. 2019;4(6).

100. Gan W, Yang F, Tang Y, Zhou D, Qing D, Hu J, et al. The difference in nasal bacterial microbiome diversity between chronic rhinosinusitis patients with polyps and a control population. Int Forum Allergy Rhinol. 2019;9(6).

101. Chalermwatanachai T, Vilchez-Vargas R, Holtappels G, Lacoere T, Jáuregui R, Kerckhof F-M, et al. Chronic rhinosinusitis with nasal polyps is characterized by dysbacteriosis of the nasal microbiota. Sci Rep. 2018 May 21;8(1):7926. Available from: http://www.ncbi.nlm.nih.gov/pubmed/29784985

102. Lechien JR, Hopkins C, Saussez S. Sniffing out the evidence; it's now time for public health bodies recognize the link between COVID-19 and smell and taste disturbance. Vol. 58, Rhinology. 2020.

103. Boesveldt S, Postma EM, Boak D, Welge-Luessen A, Schöpf V, Mainland JD, et al. Anosmia—A Clinical Review. Chem Senses. 2017;42(7):513–23.

104. Rosas-Salazar C, Kimura KS, Shilts MH, Strickland BA, Freeman MH, Wessinger BC, et al. SARS-CoV-2 infection and viral load are associated with the upper respiratory tract microbiome. J Allergy Clin Immunol. 2021 Apr 1;147(4):1226-1233.e2.

105. Rueca M, Fontana A, Bartolini B, Piselli P, Mazzarelli A, Copetti M, et al. Investigation of nasal/oropharyngeal microbial community of covid-19 patients by 16s rdna sequencing. Int J Environ Res Public Health. 2021;18(4).

106. Zhong H, Wang Y, Shi Z, Zhang L, Ren H, He W, et al. Characterization of respiratory microbial dysbiosis in hospitalized COVID-19 patients. Cell Discov. 2021 Dec 1;7(1):23. Available from: www.nature.com/celldisc

107. Engen PA, Naqib A, Jennings C, Green SJ, Landay A, Keshavarzian A, et al. Nasopharyngeal Microbiota in SARS-CoV-2 Positive and Negative Patients. Biol Proced Online. 2021;23(1).

108. Braun T, Halevi S, Hadar R, Efroni G, Glick Saar E, Keller N, et al. SARS-CoV-2 does not

have a strong effect on the nasopharyngeal microbial composition. Sci Rep. 2021;11(1).

109. Mostafa HH, Fissel JA, Fanelli B, Bergman Y, Gniazdowski V, Dadlani M, et al. Metagenomic next-generation sequencing of nasopharyngeal specimens collected from confirmed and suspect covid-19 patients. MBio. 2020;11(6).

110. Caruso R, Lo BC, Núñez G. Host–microbiota interactions in inflammatory bowel disease. Vol. 20, Nature Reviews Immunology. Nature Research; 2020. p. 411–26. Available from: https://www.nature.com/articles/s41577-019-0268-7

111. Lloyd-Price J, Arze C, Ananthakrishnan AN, Schirmer M, Avila-Pacheco J, Poon TW, et al. Multi-omics of the gut microbial ecosystem in inflammatory bowel diseases. Nature. 2019;569(7758).

112. Sokol H, Leducq V, Aschard H, Pham HP, Jegou S, Landman C, et al. Fungal microbiota dysbiosis in IBD. Gut. 2017;66(6).

113. Li Q, Wang C, Tang C, He Q, Li N, Li J. Dysbiosis of gut fungal microbiota is associated with mucosal inflammation in crohn’s disease. J Clin Gastroenterol. 2014;48(6).

114. Ghavami SB, Rostami E, Sephay AA, Shahrokh S, Balaii H, Aghdaei HA, et al. Alterations of the human gut Methanobrevibacter smithii as a biomarker for inflammatory bowel diseases. Microb Pathog. 2018;117.

115. Lecours PB, Marsolais D, Cormier Y, Berberi M, Haché C, Bourdages R, et al. Increased prevalence of methanosphaera stadtmanae in inflammatory bowel diseases. PLoS One. 2014;9(2).

116. Wolf A, Moissl-Eichinger C, Perras A, Koskinen K, Tomazic P V., Thurnher D. The salivary microbiome as an indicator of carcinogenesis in patients with oropharyngeal squamous cell carcinoma: A pilot study. Sci Rep. 2017;7(1):1–10. Available from: http://dx.doi.org/10.1038/s41598-017-06361-2

117. Schmidt B, Kuczynski J, Bhattacharya A, Huey B, Corby P, Queiroz E, et al. Changes in Abundance of Oral Microbiota Associated with Oral Cancer. PLoS One. 2014;9(6):e98741.

118. Zhao H, Chu M, Huang Z, Yang X, Ran S, Hu B, et al. Variations in oral microbiota associated with oral cancer. Sci Rep. 2017;7(1).

119. Yang CY, Yeh YM, Yu HY, Chin CY, Hsu CW, Liu H, et al. Oral microbiota community dynamics associated with oral squamous cell carcinoma staging. Front Microbiol. 2018 May 3;9(MAY):862. Available from: www.frontiersin.org

120. Asoudeh-Fard A, Barzegari A, Dehnad A, Bastani S, Golchin A, Omidi Y. Lactobacillus

plantarum induces apoptosis in oral cancer KB cells through upregulation of PTEN and downregulation of MAPK signaling pathways. BioImpacts. 2017;7(3).

121. Lee WH, Chen HM, Yang SF, Liang C, Peng CY, Lin FM, et al. Bacterial alterations in salivary microbiota and their association in oral cancer. Sci Rep. 2017;7(1).

122. Perera M, Al-Hebshi NN, Perera I, Ipe D, Ulett GC, Speicher DJ, et al. A dysbiotic mycobiome dominated by candida albicans is identified within oral squamous-cell carcinomas. J Oral Microbiol. 2017;9.

123. Bakri MM, Hussaini HM, Holmes A, Cannon RD, Rich AM. Revisiting the association between candidal infection and carcinoma, particularly oral squamous cell carcinoma. J Oral Microbiol. 2010;2(2010).

124. Nardelli C, Gentile I, Setaro M, Di Domenico C, Pinchera B, Buonomo AR, et al. Nasopharyngeal Microbiome Signature in COVID-19 Positive Patients: Can We Definitively Get a Role to Fusobacterium periodonticum? Front Cell Infect Microbiol. 2021;11.

125. Hill C, Guarner F, Reid G, Gibson GR, Merenstein DJ, Pot B, et al. Expert consensus document: The international scientific association for probiotics and prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nat Rev Gastroenterol Hepatol. 2014;11(8).

126. Mujagic Z, De Vos P, Boekschoten M V., Govers C, Pieters HJHM, De Wit NJW, et al. The effects of Lactobacillus plantarum on small intestinal barrier function and mucosal gene transcription; A randomized double-blind placebo controlled trial. Sci Rep. 2017;7.

127. Han X, Lee A, Huang S, Gao J, Spence JR, Owyang C. Lactobacillus rhamnosus GG prevents epithelial barrier dysfunction induced by interferon-gamma and fecal supernatants from irritable bowel syndrome patients in human intestinal enteroids and colonoids. Gut Microbes. 2019;10(1).

128. Sanders ME, Merenstein DJ, Reid G, Gibson GR, Rastall RA. Probiotics and prebiotics in intestinal health and disease: from biology to the clinic. Nat Rev Gastroenterol Hepatol. 2019;16(10).

129. De Boeck I, Spacova I, Vanderveken OM, Lebeer S. Lactic acid bacteria as probiotics for the nose? Microbial Biotechnology. 2021.

130. Brugger SD, Eslami SM, Pettigrew MM, Escapa IF, Henke MT, Kong Y, et al. Dolosigranulum pigrum Cooperation and Competition in Human Nasal Microbiota. mSphere. 2020 Oct 28;5(5).

131. David LA, Maurice CF, Carmody RN, Gootenberg DB, Button JE, Wolfe BE, et al. Diet rapidly and reproducibly alters the human gut microbiome. Nature. 2014;505(7484).
132. Cronin P, Joyce SA, O'toole PW, O'connor EM. Dietary fibre modulates the gut microbiota. Nutrients. 2021;13(5).
133. Zinöcker MK, Lindseth IA. The western diet–microbiome-host interaction and its role in metabolic disease. Nutrients. 2018;10(3).
134. Oliver A, Chase AB, Weihe C, Orchanian SB, Riedel SF, Hendrickson CL, et al. High-Fiber, Whole-Food Dietary Intervention Alters the Human Gut Microbiome but Not Fecal Short-Chain Fatty Acids. mSystems. 2021;6(2).
135. Bolte LA, Vich Vila A, Imhann F, Collij V, Gacesa R, Peters V, et al. Long-term dietary patterns are associated with pro-inflammatory and anti-inflammatory features of the gut microbiome. Gut. 2021;70(7).
136. Dimidi E, Cox SR, Rossi M, Whelan K. Fermented foods: Definitions and characteristics, impact on the gut microbiota and effects on gastrointestinal health and disease. Vol. 11, Nutrients. MDPI AG; 2019. p. 1806. Available from: www.mdpi.com/journal/nutrients
137. Pasolli E, De Filippis F, Mauriello IE, Cumbo F, Walsh AM, Leech J, et al. Large-scale genome-wide analysis links lactic acid bacteria from food with the gut microbiome. Nat Commun. 2020;11(1).
138. Leff JW, Fierer N. Bacterial Communities Associated with the Surfaces of Fresh Fruits and Vegetables. PLoS One. 2013;8(3).
139. Wassermann B, Müller H, Berg G. An Apple a Day: Which Bacteria Do We Eat With Organic and Conventional Apples? Front Microbiol. 2019 Jul 24;10:1629. Available from: www.frontiersin.org
140. Hong B, Sobue T, Choquette L, Dupuy A, Thompson A, Burleson J, et al. Chemotherapy-induced oral mucositis is associated with detrimental bacterial dysbiosis. Microbiome. 2019;7(1):66.
141. Subramaniam N, Muthukrishnan A. Oral mucositis and microbial colonization in oral cancer patients undergoing radiotherapy and chemotherapy: A prospective analysis in a tertiary care dental hospital. J Investig Clin Dent. 2019;10(4):e12454.
142. Guyton K, Alverdy JC. The gut microbiota and gastrointestinal surgery. Vol. 14, Nature Reviews Gastroenterology and Hepatology. 2017.
143. Aurora R, Chatterjee D, Hentzleman J, Prasad G, Sindwani R, Sanford T. Contrasting the Microbiomes From Healthy Volunteers and Patients With Chronic Rhinosinusitis. JAMA

Otolaryngol Neck Surg. 2013 Dec 1;139(12):1328. Available from: http://archotol.jamanetwork.com/article.aspx?doi=10.1001/jamaoto.2013.5465

144. Proctor DM, Relman DA, Section D, Alto P. The Landscape Ecology and Microbiota of the Human Nose, Mouth and Throat. 2018;21(4):421–32.

145. Copeland E, Leonard K, Carney R, Kong J, Forer M, Naidoo Y, et al. Chronic Rhinosinusitis: Potential Role of Microbial Dysbiosis and Recommendations for Sampling Sites. Front Cell Infect Microbiol. 2018;8:57. Available from: http://www.ncbi.nlm.nih.gov/pubmed/29541629

146. Nearing JT, Comeau AM, Langille MGI. Identifying biases and their potential solutions in human microbiome studies. Microbiome. 2021 Dec 18;9(1):113. Available from: https://microbiomejournal.biomedcentral.com/articles/10.1186/s40168-021-01059-0

147. Meisel JS, Hannigan GD, Tyldsley AS, SanMiguel AJ, Hodkinson BP, Zheng Q, et al. Skin Microbiome Surveys Are Strongly Influenced by Experimental Design. J Invest Dermatol. 2016;136(5).

148. Davis NM, Proctor DM, Holmes SP, Relman DA, B.J C. Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data. Microbiome. 2018;6(1):226.

149. Smirnova E, Huzurbazar S, Jafari F. PERFect: PERmutation filtering test for microbiome data. Biostatistics. 2019;20(4).

150. Hummel T, Whitcroft KL, Andrews P, Altundag A, Cinghi C, Costanzo RM, et al. Position paper on olfactory dysfunction. Rhinology. 2017;54.

151. Brann DH, Tsukahara T, Weinreb C, Lipovsek M, Van Den Berge K, Gong B, et al. Non-neuronal expression of SARS-CoV-2 entry genes in the olfactory system suggests mechanisms underlying COVID-19-associated anosmia. Sci Adv. 2020;6(31).

152. Mori I, Nishiyama Y, Yokochi T, Kimura Y. Olfactory transmission of neurotropic viruses. Vol. 11, Journal of NeuroVirology. 2005.

153. Bohmwald K, Gálvez NMS, Ríos M, Kalergis AM. Neurologic alterations due to respiratory virus infections. Vol. 12, Frontiers in Cellular Neuroscience. 2018.

154. Meinhardt J, Radke J, Dittmayer C, Franz J, Thomas C, Mothes R, et al. Olfactory transmucosal SARS-CoV-2 invasion as a port of central nervous system entry in individuals with COVID-19. Nat Neurosci. 2021;24(2).

155. Hoffmann M, Kleine-Weber H, Schroeder S, Krüger N, Herrler T, Erichsen S, et al. SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven

Protease Inhibitor. Cell. 2020;181(2).

156. Lee M-H, Perl DP, Nair G, Li W, Maric D, Murray H, et al. Microvascular Injury in the Brains of Patients with Covid-19. https://doi.org/101056/NEJMc2033369 [Internet]. 2020 Dec 30 [cited 2021 Jul 12];384(5):481–3. Available from: https://www.nejm.org/doi/full/10.1056/NEJMc2033369

157. Cazzolla AP, Lovero R, Lo Muzio L, Testa NF, Schirinzi A, Palmieri G, et al. Taste and smell disorders in COVID-19 patients: Role of interleukin-6. ACS Chem Neurosci. 2020;11(17).

158. Parsa S, Mogharab V, Ebrahimi M, Ahmadi SR, Shahi B, Mehramiz NJ, et al. COVID-19 as a worldwide selective event and bitter taste receptor polymorphisms: An ecological correlational study. Int J Biol Macromol. 2021;177.

159. Barham HP, Taha MA, Broyles ST, Stevenson MM, Zito BA, Hall CA. Association between bitter taste receptor phenotype and clinical outcomes among patients with COVID-19. JAMA Netw Open. 2021;

160. Yan X, Whitcroft KL, Hummel T. Olfaction: Sensitive indicator of inflammatory burden in chronic rhinosinusitis. Laryngoscope Investig Otolaryngol. 2020 Dec 1;5(6):992–1002. Available from: https://onlinelibrary.wiley.com/doi/full/10.1002/lio2.485

161. Workman AD, Brooks SG, Kohanski MA, Blasetti MT, Cowart BJ, Mansfield C, et al. Bitter and Sweet Taste Tests are Reflective of Disease Status in Chronic Rhinosinusitis. J Allergy Clin Immunol Pract. 2018;6(3):1078–80.

162. Adappa ND, Truesdale CM, Workman AD, Doghramji L, Mansfield C, Kennedy DW, et al. Correlation of T2R38 taste phenotype and in vitro biofilm formation from non-polypoid chronic rhinosinusitis patients. Int Forum Allergy Rhinol. 2017;6(8):783–91.

163. Guan X, Li W, Meng H. A double-edged sword: Role of butyrate in the oral cavity and the gut. Mol Oral Microbiol. 2021;36(2).

164. Evans M, Murofushi T, Tsuda H, Mikami Y, Zhao N, Ochiai K, et al. Combined effects of starvation and butyrate on autophagy-dependent gingival epithelial cell death. J Periodontal Res. 2017;52(3).

165. Geißler K, Reimann H, Gudziol H, Bitter T, Guntinas-Lichius O. Olfactory training for patients with olfactory loss after upper respiratory tract infections. Eur Arch Oto-Rhino-Laryngology. 2014;271(6).

166. Konstantinidis I, Tsakiropoulou E, Constantinidis J. Long term effects of olfactory training in patients with post-infectious olfactory loss. Rhinol J. 2016;54(2).

167. Barresi M, Ciurleo R, Giacoppo S, Foti Cuzzola V, Celi D, Bramanti P, et al. Evaluation of olfactory dysfunction in neurodegenerative diseases. Vol. 323, Journal of the Neurological Sciences. 2012.

168. Rahayel S, Frasnelli J, Joubert S. The effect of Alzheimer's disease and Parkinson's disease on olfaction: A meta-analysis. Behav Brain Res. 2012;231(1).

169. Wagner Mackenzie B, Waite DW, Hoggard M, Douglas RG, Taylor MW, Biswas K. Bacterial community collapse: a meta-analysis of the sinonasal microbiota in chronic rhinosinusitis. Environ Microbiol. 2017 Jan 1;19(1):381–92. Available from: http://doi.wiley.com/10.1111/1462-2920.13632

170. Kitz R, Martens U, Zieseniß E, Enck P, Rose MA. Probiotic E . faecalis – adjuvant therapy in children with recurrent rhinosinusitis. Cent Eur J Med. 2012;7(3):7–10.

171. Manti S, Parisi GF, Papale M, Licari A, Salpietro C, Miraglia Del Giudice M, et al. Bacteriotherapy with Streptococcus salivarius 24SMB and Streptococcus oralis 89a nasal spray for treatment of upper respiratory tract infections in children: A pilot study on short-term efficacy. Ital J Pediatr. 2020;46(1).

172. Endam LM, Alromaih S, Gonzalez E, Madrenas J, Cousineau B, Renteria AE, et al. Intranasal Application of Lactococcus lactis W136 Is Safe in Chronic Rhinosinusitis Patients With Previous Sinus Surgery. Front Cell Infect Microbiol. 2020;10.

173. De Rudder C, Garcia-Tímermans C, De Boeck I, Lebeer S, Van de Wiele T, Calatayud Arroyo M. Lacticaseibacillus casei AMBR2 modulates the epithelial barrier function and immune response in a donor-derived nasal microbiota manner. Sci Rep. 2020;10(1).

174. Koskinen K, Pausan MR, Perras AK, Beck M, Bang C, Mora M, et al. First insights into the diverse human archaeome: Specific detection of Archaea in the gastrointestinal tract, lung, and nose and on skin. MBio. 2017 Nov 1;8(6). Available from: /pmc/articles/PMC5686531/?report=abstract

175. Ruaud A, Esquivel-Elizondo S, de la Cuesta-Zuluaga J, Waters JL, Angenent LT, Youngblut ND, et al. Syntrophy via interspecies H2 transfer between christensenella and methanobrevibacter underlies their global cooccurrence in the human gut. MBio. 2020 Jan 1;11(1). Available from: http://mbio.asm.org/

176. Goodrich JK, Waters JL, Poole AC, Sutter JL, Koren O, Blekhman R, et al. Human genetics shape the gut microbiome. Cell. 2014 Nov 6;159(4):789–99. Available from: /pmc/articles/PMC4255478/?report=abstract

177. Chassard C, Delmas E, Robert C, Bernalier-Donadille A. The cellulose-degrading

microbial community of the human gut varies according to the presence or absence of methanogens. FEMS Microbiol Ecol. 2010 Oct 1;74(1):205–13. Available from: https://academic.oup.com/femsec/article-lookup/doi/10.1111/j.1574-6941.2010.00941.x

178. Oliphant K, Allen-Vercoe E. Macronutrient metabolism by the human gut microbiome: Major fermentation by-products and their impact on host health. Microbiome. 2019;7(1).

179. Tamura J, Kubota K, Murakami H, Sawamura M, Matsushima T, Tamura T, et al. Immunomodulation by vitamin B12: Augmentation of CD8+ T lymphocytes and natural killer (NK) cell activity in vitamin B12-deficient patients by methyl-B12 treatment. Clin Exp Immunol. 1999;116(1).

180. MacMillan L, Tingley G, Young SK, Clow KA, Randel EW, Brosnan ME, et al. Cobalamin deficiency results in increased production of formate secondary to decreased mitochondrial oxidation of one-carbon units in rats. J Nutr. 2018 Mar 1;148(3):358–63. Available from: https://doi.org/10.1093/jn/nxx057.

181. Chibani CM, Mahnert A, Borrel G, Almeida A, Werner A, Brugère J-F, et al. A comprehensive analysis of the global human gut archaeome from a thousand genome catalogue. bioRxiv. 2020 Nov 22;2020.11.21.392621. Available from: https://doi.org/10.1101/2020.11.21.392621

182. Hansen EE, Lozupone CA, Rey FE, Wu M, Guruge JL, Narra A, et al. Pan-genome of the dominant human gut-associated archaeon, Methanobrevibacter smithii, studied in twins. Proc Natl Acad Sci U S A. 2011 Mar 15;108(SUPPL. 1):4599–606. Available from: http://www.nasonline.org/SACKLER_Microbes_and_Health.

183. Nguyen-Hieu T, Khelaifia S, Aboudharam G, Drancourt M. Methanogenic archaea in subgingival sites: A review. Vol. 121, APMIS. 2013.

184. Diether NE, Willing BP. Microbial fermentation of dietary protein: An important factor in diet–microbe–host interaction. Vol. 7, Microorganisms. 2019.

185. Oki K, Toyama M, Banno T, Chonan O, Benno Y, Watanabe K. Comprehensive analysis of the fecal microbiota of healthy Japanese adults reveals a new bacterial lineage associated with a phenotype characterized by a high frequency of bowel movements and a lean body type. BMC Microbiol. 2016 Nov 28;16(1):1–13. Available from: https://pubmed.ncbi.nlm.nih.gov/27894251/

186. Alonso BL, Irigoyen von Sierakowski A, Sáez Nieto JA, Rosel AB. First report of human infection by Christensenella minuta, a gram-negative, strickly anaerobic rod that inhabits the human intestine. Anaerobe. 2017 Apr 1;44:124–5.

187. Marsh K, Zeuschner C, Saunders A. Health Implications of a Vegetarian Diet: A Review. Vol. 6, American Journal of Lifestyle Medicine. 2012.
188. Pietzke M, Meiser J, Vazquez A. Formate metabolism in health and disease. Vol. 33, Molecular Metabolism. Elsevier GmbH; 2020. p. 23–37. Available from: /pmc/articles/PMC7056922/?report=abstract
189. Aas JA, Paster BJ, Stokes LN, Olsen I, Dewhirst FE. Defining the normal bacterial flora of the oral cavity. J Clin Microbiol. 2005;43(11).
190. Sami A, Elimairi I, Stanton C, Ross RP, Ryan CA. The role of the microbiome in oral squamous cell carcinoma with insight into the microbiome–treatment axis. Int J Mol Sci. 2020 Nov 1;21(21):1–29. Available from: /pmc/articles/PMC7662318/
191. Tolentino E de S, Centurion BS, Ferreira LHC, de Souza AP, Damante JH, Rubira-Bullen IRF. Oral adverse effects of head and neck radiotherapy: Literature review and suggestion of a clinical oral care guideline for irradiated patients. J Appl Oral Sci. 2011;19(5).
192. Zhu XX, Yang XJ, Chao YL, Zheng HM, Sheng HF, Liu HY, et al. The Potential Effect of Oral Microbiota in the Prediction of Mucositis During Radiotherapy for Nasopharyngeal Carcinoma. EBioMedicine. 2017;18.
193. Wang QQ, Zhang CF, Chu CH, Zhu XF. Prevalence of Enterococcus faecalis in saliva and filled root canals of teeth associated with apical periodontitis. Int J Oral Sci. 2012;4(1).
194. Panghal M, Kaushal V, Kadayan S, Yadav JP. Incidence and risk factors for infection in oral cancer patients undergoing different treatments protocols. BMC Oral Health. 2012;12(1).
195. Cannon RD, Chaffin WL. Oral Colonization By Candida Albicans 2016 Dec 1;10(3):359–83. Available from: https://journals.sagepub.com/doi/10.1177/10454411990100030701
196. Sherrington SL, Sorsby E, Mahtey N, Kumwenda P, Lenardon MD, Brown I, et al. Adaptation of Candida albicans to environmental pH induces cell wall remodelling and enhances innate immune recognition. PLoS Pathog. 2017;13(5).

Appendix

Supplementary information can be found here:

ad publication 1: "**The microbiome of the upper respiratory tract in health and disease.**[1]"

Supplementary table:

https://static-content.springer.com/esm/art%3A10.1186%2Fs12915-019-0703-z/MediaObjects/12915_2019_703_MOESM1_ESM.xlsx

ad publication 2: "**Reduced B12 uptake and increased gastrointestinal formate drive archaeome-mediated breath methane emission in humans.**[3]"

Supplementary data, figure, items, methods and tables:

https://data.mendeley.com/datasets/hjj3tx7n84/1

ad publication 3: "**Preliminary insights into the impact of primary radiochemotherapy on the salivary microbiome in head and neck squamous cell carcinoma.**[2]"

Supplementary file 1

https://static-content.springer.com/esm/art%3A10.1038%2Fs41598-020-73515-0/MediaObjects/41598_2020_73515_MOESM1_ESM.xlsx

Supplementary file 2

https://static-content.springer.com/esm/art%3A10.1038%2Fs41598-020-73515-0/MediaObjects/41598_2020_73515_MOESM2_ESM.xlsx

Supplementary file 3

https://static-content.springer.com/esm/art%3A10.1038%2Fs41598-020-73515-0/MediaObjects/41598_2020_73515_MOESM3_ESM.xlsx

Supplementary file 4

https://static-content.springer.com/esm/art%3A10.1038%2Fs41598-020-73515-0/MediaObjects/41598_2020_73515_MOESM4_ESM.pdf

Supplementary file 5

https://static-content.springer.com/esm/art%3A10.1038%2Fs41598-020-73515-0/MediaObjects/41598_2020_73515_MOESM5_ESM.xlsx

Original Paper

Chapter 1.:

The microbiome of the upper respiratory tract in health and disease.[1]

Unpublished project: **The Nose Brain Axis - The correspondence of the nasal microbiome and human olfaction.** (*manuscript in progress*)

Chapter 2.:

Reduced B12 uptake and increased gastrointestinal formate drive archaeome-mediated breath methane emission in humans.[3]

Chapter 3.:

Preliminary insights into the impact of primary radiochemotherapy on the salivary microbiome in head and neck squamous cell carcinoma.[2]

Kumpitsch *et al. BMC Biology* (2019) 17:87
https://doi.org/10.1186/s12915-019-0703-z

BMC Biology

REVIEW Open Access

The microbiome of the upper respiratory tract in health and disease

Christina Kumpitsch[1], Kaisa Koskinen[1], Veronika Schöpf[2,3,4] and Christine Moissl-Eichinger[1,3*]

Abstract

The human upper respiratory tract (URT) offers a variety of niches for microbial colonization. Local microbial communities are shaped by the different characteristics of the specific location within the URT, but also by the interaction with both external and intrinsic factors, such as ageing, diseases, immune responses, olfactory function, and lifestyle habits such as smoking. We summarize here the current knowledge about the URT microbiome in health and disease, discuss methodological issues, and consider the potential of the nasal microbiome to be used for medical diagnostics and as a target for therapy.

Keywords: Microbiome, Upper respiratory tract, URT, Human microbiome, Nasal microbiome, Upper respiratory tract diseases

Introduction

The human microbiome is a complex community of microorganisms, living in a symbiotic relationship in human microhabitats. Due to microbial niche specificity, microbial composition and function vary according to the different human body sites, such as the gastrointestinal tract, skin, and airways [1, 2].

Since a healthy adult breathes more than 7000 l of air a day, the upper respiratory tract (URT) is constantly bathed in airflow from the external environment. Along with the air, 10^4–10^6 bacterial cells per cubic meter of air are inhaled per day. Besides these biological particulates, the URT is exposed to atmospheric physical and chemical parameters, including varying humidity, oxygen, immunological factors, or nutrients. Along with the anatomy, these factors shape specific microenvironments in the URT such as the nasal cavity, sinuses, nasopharynx, and oropharynx [3–5]. As a consequence, specific microenvironments in the URT harbor different microbial communities composed of variable proportions of resident and transient microorganisms [6].

Like other human body sites, the upper respiratory tract is colonized by a variety of different microbial species directly after birth. It has been shown that the initial colonization depends on delivery mode (vaginal delivery or caesarean section), and the most drastic changes occur during the first year of life, probably driven by the maturation of the immune system [7]. Later on, this first microbial community transforms into the adult URT microbiome, becoming less dense and more diverse. In the elderly, the distinct microbiomes of specific microenvironments become more similar [8, 9].

Many studies report that the nasal microbiome of healthy humans is primarily composed of the phyla Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria with representatives of genera *Bifidobacterium*, *Corynebacterium*, *Staphylococcus*, *Streptococcus*, *Dolosigranulum*, and *Moraxella* predominating [9–12]. However, most research focuses on the bacteria in the human nasal cavity, while other components of the microbiome, such as viruses, archaea, and fungi, are seldom specifically addressed and thus likely overlooked [13].

Human health has been described as the outcome of the complex interaction between the microbiome and its human host [14]. Functional or compositional perturbations of the microbiome can occur at different body sites and this dysbiosis has been linked with various diseases; for example, inflammatory bowel disease and metabolic disorders have been linked to dysbiosis in the microbiome of the gastrointestinal tract and URT infections (URTI, such as chronic rhinosinusitis [CRS]) with dysbiosis in the URT [15–18]. These dysbioses are often characterized by a loss of beneficial, commensal bacteria,

* Correspondence: christine.moissl-eichinger@medunigraz.at
[1]Diagnostic and Research Institute of Hygiene, Microbiology and Environmental Medicine, Medical University of Graz, Neue Stiftingtalstraße 6, 8010 Graz, Austria
[3]BioTechMed-Graz, Mozartgasse 12/II, 8010 Graz, Austria
Full list of author information is available at the end of the article

© The Author(s). 2019 **Open Access** This article is distributed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The Creative Commons Public Domain Dedication waiver (http://creativecommons.org/publicdomain/zero/1.0/) applies to the data made available in this article, unless otherwise stated.

which protect against overgrowth of opportunistic pathogenic bacteria [6, 19, 20].

Currently, several different therapies are suggested for the treatment of inflammatory URTIs [21–24]. Antibiotics as well as intranasal corticosteroids are used, combining antimicrobial and anti-inflammatory properties [21, 24]. These treatments cause a loss of microbial diversity, potentially leading to an increase of Gram-negative bacteria in the nose [25–27].

In the case of chronic rhinosinusitis, sinus surgery (aiming at improving drainage of the mucus), combined with different antibiotics is the most common treatment [22]. Although this type of therapy is highly invasive, its outcomes are usually satisfactory [28]. However, airway diseases might also be prevented and treated with less aggressive therapies such as saline rinses, cleaning the nasal mucosa from inflammatory mediators and other pollutants [23].

Comparative URT microbiome research faces various methodological problems, including choice of sampling techniques (e.g., swabs, nasal rinses, and dry filter papers) and sampling sites. In most cases anterior nares, middle meatus, and nasopharynx are the preferred sites for sampling [9, 11, 12, 29–31], as other areas are not easily accessible. This often results in a discrepancy of research question and study protocol, as, e.g., the middle meatus is sampled instead of the sinuses when chronic rhinosinusitis is studied [29]. However, microbiome dysbiosis often extends to locations beyond the sites of the studied disease, so that significant alterations in the microbial community structure in adjacent locations can be observed as well [6, 32]. Nevertheless, in order to prove or reject a research hypothesis, the sampling sites for microbiome analyses need to be chosen wisely [6].

The aim of this review is to summarize the current information about the microbiome in the upper respiratory tract; discuss methodological issues such as sampling methods and sites; present the link between URT microbiome composition, immune system, and certain diseases; have a look at the influence of common therapies on the URT microbiome; and identify the current gaps in our knowledge.

Details of cited studies, including sampling, sample processing protocol, studied population and sites, and results are summarized in Additional file 1.

Landscape of the upper respiratory tract

The upper respiratory tract (URT) comprises the anterior nares, nasal cavity, sinuses, nasopharynx, Eustachian tube, middle ear cavity, oral cavity, oropharynx, and larynx. The nasal cavity is partitioned into the inferior, middle, and superior meatus by three nasal turbinates [3, 33] (Fig. 1a). In this review we focus on the microbiomes of anterior nares, nasal cavity, sinuses, and nasopharynx and their importance in human health.

Many important physiological functions are provided by the URT such as filtering, warming, and humidifying of inhaled air [3, 34]. As the nasal cavity is in constant contact with the external environment, it acts as a physical transition forming an interface between the external environment and the lower respiratory and gastrointestinal tract [3, 33]. Other functions are olfactory sensing and important immunological tasks, including immediate pathogen detection such as sensing of bacterial lactones by taste receptors [32, 35–38].

The nasal cavity is lined by different types of epithelium, providing different micro-niches (Fig. 1a): the anterior naris starts with non-keratinized skin-like epithelium (1), changing into stratified squamous epithelial cells without microvilli (2), followed by transitional epithelium with short microvilli (3), before transition into the middle meatus with its pseudostratified columnar epithelium (4 and 5, middle meatus) [32, 33, 35]. The most common sampling sites for nasal microbiome analyses are the anterior nares (AN), the middle meatus (MM), and the nasopharynx [9, 12, 29, 31] (Fig. 1a).

The surfaces in the *anterior nares* and nasal vestibule are relatively dry compared to other URT areas. These parts experience the greatest exposure to the external environment and contain sebaceous glands (see below) and vibrissae (hair). These hairs trap large particles (> 3 μm) from inhaled air, whereas small particulate matter (0.5–3 μm, including microorganisms) is captured by a flowing mucus blanket covering the entire nasal cavity [32, 33, 35, 39].

The *middle meatus* is adjacent to the nasal vestibule. As it receives drainage from the anterior ethmoids, maxillary, and frontal sinuses, this area is of interest for many microbiome studies [32]. The *nasopharynx* is characterized by many crypts and folds, and its wall is dominated by keratinized and non-keratinized stratified squamous epithelium and pseudostratified ciliated epithelia [40].

Maxillary, ethmoid, sphenoid, and frontal sinuses are air-filled, paired cavities within the facial skeleton, which are important for humidification and warming of the inhaled air (Fig. 1b). They are coated with ciliated columnar epithelium, which produces mucus that is transported into the nasal cavity [41]. These drainages create local micro-niches with specific microbial populations within the nasal cavity [42] (Fig. 2). Another interesting niche for microbiome studies is the *olfactory area*, as recent studies indicated a potential correlation of olfactory function with the taxonomic composition of the local nasal microbiome [43]. The olfactory area is located at the ceiling of the nasal cavity [33].

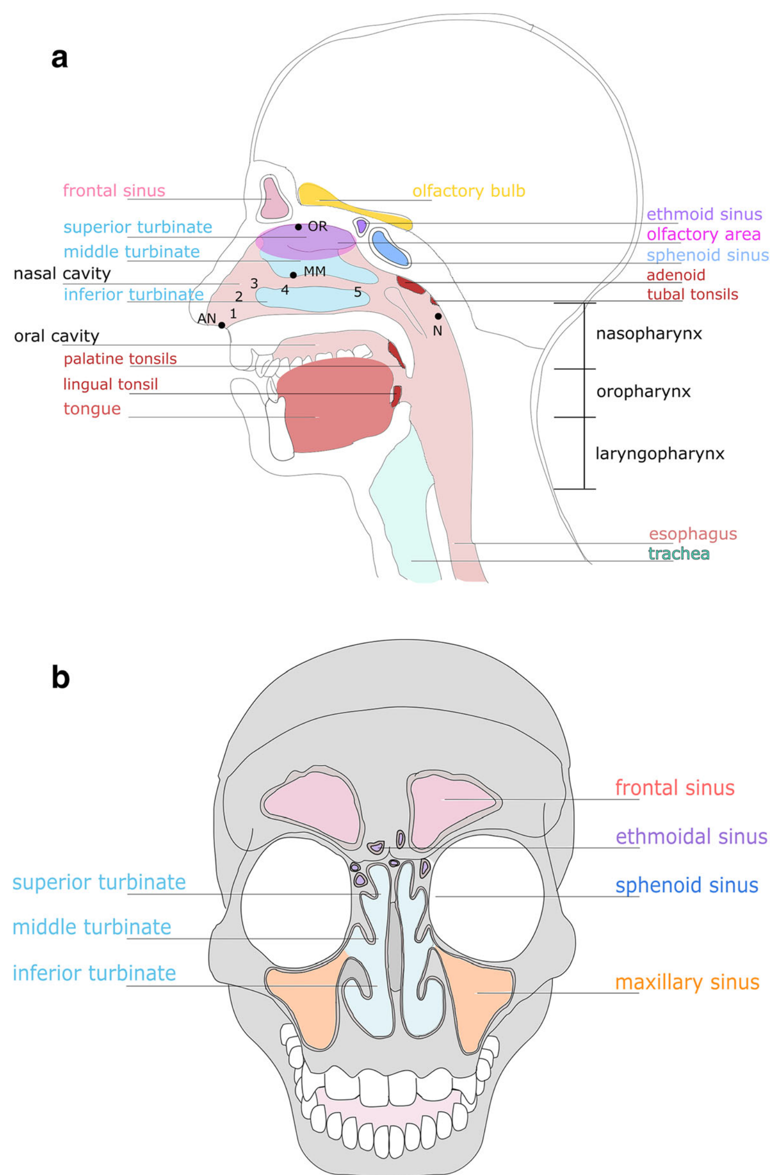


Fig. 1 The upper respiratory tract (**a**) and its paranasal sinuses (**b**). **a** URT with different, typical microbiome sampling sites (*AN* anterior naris, *MM* middle meatus, *OR* olfactory area and nasopharynx) and nasal lining, starting with *1* non-keratinized skin-like epithelium in the nostrils followed by different epithelial types, *2* squamous epithelium without microvilli, *3* transitional epithelium with ciliated cells, *4* pseudostratified columnar epithelium with ciliated cells, *5* pseudostratified columnar epithelium with many ciliated cells. **b** Sinuses of the nasal cavity

Upper respiratory (immune) defense system

The respiratory tract has recourse to a variety of mechanisms, including components of the innate and adaptive immune system, to protect against possibly harmful, inhaled microorganisms while chronically present commensal microbes of the URT microbiome are tolerated due to hyporesponsiveness of the host's immune system [44].

The mucus layer

Glands, goblet cells, and ciliated cells secrete a hydrated mucus layer which contains lipids, glycoproteins, and glycoconjugates. This layer not only helps to humidify inhaled air but also traps microbes and microparticles from the environment on entering the URT [33, 45]. This "contaminated" mucus is then directed by ciliated epithelial cells (located in the upper respiratory tract) from the nasal cavity towards the esophagus [33, 45]. This whole process of purging is also known as mucociliary clearance [46, 47]. Additional defense is derived from antimicrobial compounds which are present in the mucus and immediate initiation of immune priming [32, 48]. Interestingly, commensal bacteria with immunomodulatory properties are

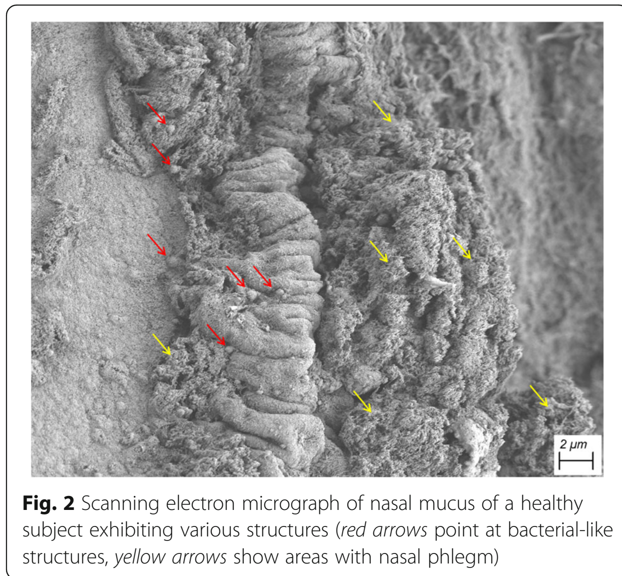


Fig. 2 Scanning electron micrograph of nasal mucus of a healthy subject exhibiting various structures (*red arrows* point at bacterial-like structures, *yellow arrows* show areas with nasal phlegm)

capable of priming a host's immune responses to assure efficient and rapid defense against pathogens [49, 50].

Antimicrobial peptides and reactive oxygen species

The respiratory surface epithelium secretes a variety of antimicrobial components. These include antimicrobial peptides such as lysozyme, lactoferrin or defensins, and reactive oxygen species (ROS) such as hydrogen peroxide and nitric oxide (NO) [51–55]. Besides its antimicrobial activity (it diffuses into the microbial cell and destroys intracellular components), nitric oxide also directly increases mucociliary clearance and speeds up the frequency of ciliary beating by protein kinase G and guanylyl cyclase activation [38, 56–58].

Nasopharyngeal-associated lymphoid tissue

Nasopharyngeal tonsils (adenoids), the paired tubal tonsils, the paired palatine tonsils, and the lingual tonsil are part of the lymphoid tissue in the nasopharynx and serve as major sites for microbial recognition and defense [59, 60]. Nasopharyngeal-associated lymphoid tissue (NALT) harbors a large variety and number of immune cells, including dendritic cells, macrophages, and lymphocytes [61] (Fig. 1a). Fifty percent of these lymphocytes are immunoglobulin-producing B-lymphocytes [62–64]. Like the small intestine, the lymphoid tissues contain M cells, which transport microorganisms via trans-epithelial transport from the apical surface to the basolateral site where immune cells are already waiting [65]. NALT-associated cells (e.g., sinonasal solitary chemosensory cells) excrete chemokines and cytokines, which activate downstream immune cascades [66–68].

Olfaction- and taste-triggered immune response

Foreign substances in the URT can also be detected by two other systems, the extended olfactory and the trigeminal chemesthetic system. The former includes the olfactory epithelium and vomeronasal organ [69]. Stimulation thereof by different signals (food odors, sexual and social signals, as well as bacterial infection products like formyl peptides) was shown to cause behavioral responses in mouse experiments [70, 71].

The trigeminal chemesthetic system (including solitary chemosensory cells (SCCs)) [69] induces protective trigeminal nerve-mediated airway reflexes (coughing, sneezing, or decrease in breathing rate) and local inflammatory responses [72–74]. These SCCs make up to 1% of all cells in the ciliated epithelium of the sinonasal cavity [66, 75] and express two types of taste receptors, bitter and sweet [76, 77]. These receptors belong to the group of G-protein-coupled receptors (GPCRs) [78, 79].

With bitter receptors (e.g., T2R family), the sensory system of the SCCs is able to detect the presence of bacteria on nasal epithelial surfaces directly via bitter molecules that are released by pathogens [56, 73, 76] and may initiate immune responses (e.g., inflammation) even before bacteria achieve a pathogenic load and are able to form biofilms [38, 56, 80]. An example of a bitter, microbial-derived molecule is acyl-homoserine lactone (AHL). AHL is an important bacterial quorum-sensing molecule [36–38] that stimulates the bitter receptor T2R38 and leads to calcium-dependent nitric oxide (NO) production [56].

It should be noted that bitter and sweet signals affect innate immunity oppositely. Sugars, such as sucrose and glucose, inhibit bitter-induced calcium release. As a consequence, downstream, calcium-driven initiation of the innate immune system at the tissue level (such as release of antimicrobials from ciliated cells) is impaired [76, 80].

In patients suffering from prediabetes and diabetes, increased levels of glucose have been found in nasal secretions [81]. In addition, chronic rhinosinusitis patients reported higher intensity of the sweet tastes (sucrose) whereas their ability to taste bitter compounds was reduced compared to healthy controls, both leading to decreases in pathogen detection and defense, e.g., by reduced ciliary beating [38, 82, 83]. Furthermore, it is hypothesized that glucose levels in the airways rapidly deplete during a bacterial infection due to the bacterial load [82, 84].

The upper respiratory tract microbiome changes with age and life-style

As we have seen, the landscape of the upper respiratory tract, with its different epithelial linings and conditions, provides numerous different (micro-)niches for microbial communities. Whereas the anterior naris (the passage between the skin and the nasal cavity) harbors commensals and opportunistic pathogens like *Staphylococcus aureus*, *S. epidermidis*, *Propionibacterium* (now:

Cutibacterium) *acnes*, *Dolosigranulum pigrum*, *Finegoldia magna*, *Corynebacterium* spp., *Moraxella* spp., *Peptoniphilus* spp., and *Anaerococcus* spp. [85, 86], the microbial community structures in other locations in the nasal cavity and down the nasopharynx are distinct, especially in adults [9, 10] (see also Additional File 1). Even though the URT microbiome is largely individual, changes in inter-individual bacterial community profiles over different seasons (winter vs summer) and ages can still be observed [1, 86–89].

The upper respiratory tract microbiome of infants

Moraxella, *Staphylococcus*, *Streptococcus*, *Haemophilus*, *Dolosigranulum*, and *Corynebacterium* are the six most common genera, of which one or two usually dominate the nares and nasopharyngeal microbiome of infants [11, 90, 91]. Right after birth, the initial nasopharyngeal bacterial assemblage takes place, and the infant's nasopharyngeal microbiome resembles the maternal vaginal or skin microbiome [3, 92] (Fig. 3).

At 1.5 months of life this initial microbiome composition is maintained by breast feeding, which supports stable *Dolosigranulum*/*Corynebacterium* profiles. This is different to formula-fed infants, who show increased *S. aureus* signatures. The microbial profile of breast-fed infants seems to have a protective effect against respiratory infections [3, 93] (Fig. 3).

The nares and nasopharynx are dominated by *Staphylococcus*, *Moraxella*, *Streptococcus*, *Corynebacterium*, and/or *Dolosigranulum* signatures in 1.5-month-old infants [92]. Children with *Moraxella* spp.-dominated profiles were less likely to suffer from URTI, with the exception of *Moraxella catarrhalis*, which is found to be associated—together with *H. influenza* and *S. pneumoniae*—with wheezing in one-month-old infants. Nasopharyngeal *Streptococcus* was found to serve as a strong predictor for asthma in approximately 2-month-old children [27, 47, 92, 94]. After 1.5 months, *Haemophilus*-dominated clusters of co-occurrent microorganisms emerged, whereas *Staphylococcus*-dominated profiles disappeared and *Corynebacterium*/*Dolosigranulum* patterns were replaced by *Moraxella*/*Dolosigranulum*-dominated clusters in the infants' URT [92] (Fig. 3).

All in all, observations of children in their first 2 years of life show that *Dolosigranulum* and *Moraxella* combined with *Corynebacterium* form a more stable microbiome compared to *Streptococcus* and *Haemophilus*-dominant profiles [26, 92]. The latter profiles (*H. influenza* and *S. pneumoniae*) were associated with respiratory viruses and an elevated risk of bronchiolitis in early life [30, 92, 95–97] (Additional file 1).

The upper respiratory tract microbiome of adults

The URT microbiome of adults differs from that of infants, although the niche characteristics appear quite

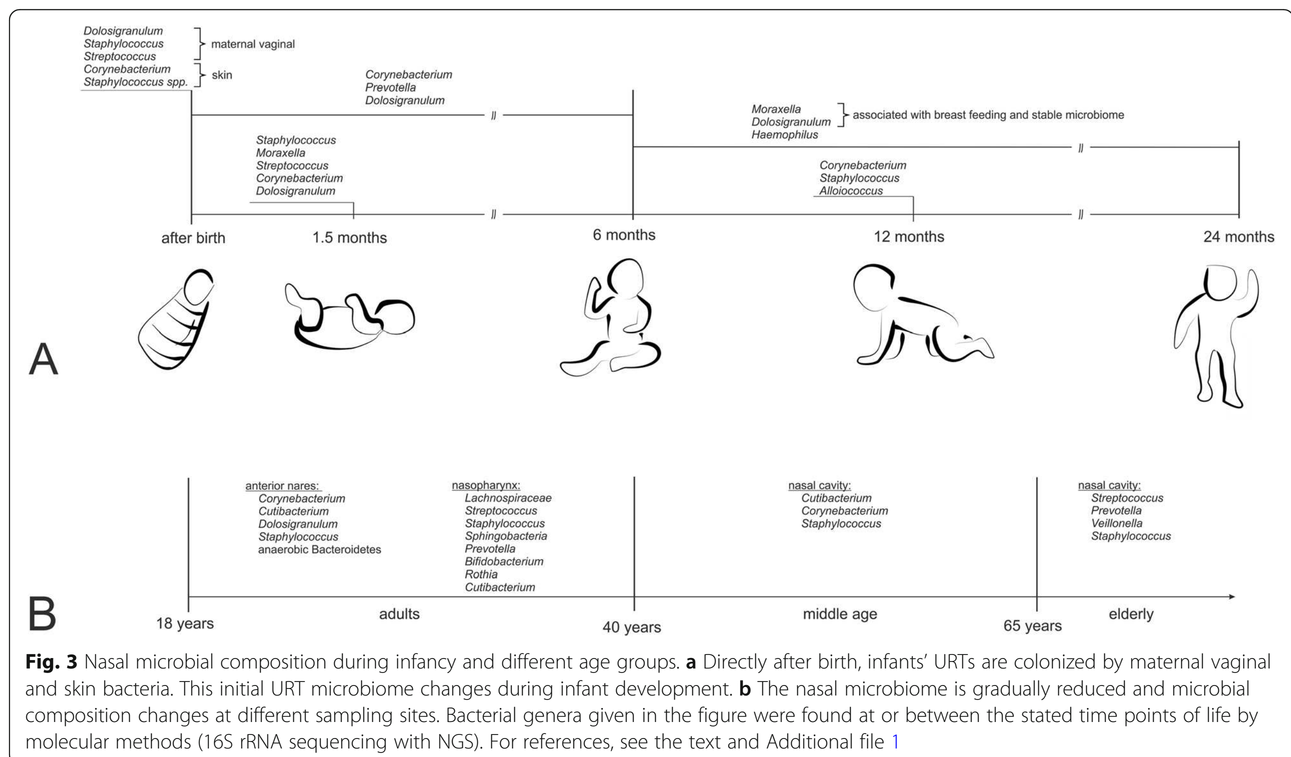


Fig. 3 Nasal microbial composition during infancy and different age groups. **a** Directly after birth, infants' URTs are colonized by maternal vaginal and skin bacteria. This initial URT microbiome changes during infant development. **b** The nasal microbiome is gradually reduced and microbial composition changes at different sampling sites. Bacterial genera given in the figure were found at or between the stated time points of life by molecular methods (16S rRNA sequencing with NGS). For references, see the text and Additional file 1

similar. In comparison, children's nasal microbiomes are more dense (higher bacterial load) but less diverse [3, 8, 12, 47, 98]. The anterior nares of adults mainly harbor *Actinobacteria*, *Firmicutes*, and, in lower abundance, anaerobic *Bacteroidetes* [3, 31, 43, 98–100] (Fig. 3; Additional File 1).

Comparison of different nasal cavity sample sites showed that middle meatus (MM) and sphenoethmoidal recess (SR) are nearly identical with respect to microbial community composition, whereas anterior nares show a significantly reduced diversity of the microbial community. In addition, the anterior nares harbor a greater proportion of *Firmicutes* and *Actinobacteria* and less *Proteobacteria* compared to MM and SR [32].

The primary function of the nasal mucosa, namely the clearance of inhaled air, may explain the increased diversity of nasal mucosal samples [32]. At the phylum level, the adult nasopharynx microbiome resembles the microbiome of adult anterior nares, but the identified lower taxa are rather specific at the different locations [12] (Additional file 1).

The upper respiratory tract microbiome of the elderly

The microbial communities of the anterior nares of adults (18–40 years) differ significantly from that of other URT sampling sites (nasopharynx, tongue, buccal mucosa, oropharynx), but these distinctive variations gradually reduce during ageing. The alterations in nasal microbiota communities start in middle-aged adults (40–65 years), whose nasal microbial communities are dominated by signatures of *Cutibacterium*, *Corynebacterium*, and *Staphylococcus*, whereas the nasal community of the elderly (> 65 years) shifts towards a more oropharyngeal population (Fig. 3) [9, 47]. These observed changes in bacterial community composition are probably a consequence of immunesenescence during the process of aging, which leads to an increase of pro-inflammatory markers and decreased ability of immune stress handling, leading to the opening of new environmental niches after the loss of species richness [9, 101] (Additional file 1).

Smoking influences the nasal microbiome

Cigarette smoke exposure, whether active or passive, is associated with an elevated risk of not only cancer, periodontitis, and cardiovascular disease, but also chronic respiratory diseases (e.g., chronic obstructive pulmonary disease (COPD), asthma) and acute respiratory infections [47, 102].

Cigarette smoke has immediate contact with nasal surfaces, and thus directly impacts the microbiome by oxygen deprivation, antimicrobial activity, or other mechanisms [103, 104].

The toxic substances disrupt effective muco-ciliary clearance in the lower and upper respiratory tracts, impairing the immune responses against pathogens [105–109].

Cigarette smoke also enhances bacterial attachment to airway epithelial cells, for example, by inducing bacterial fimbrial protein FimA production, which promotes the formation of robust, reversible biofilms. This biofilm formation might support recalcitrant persistence of bacteria in the nasal cavity [87, 110–112].

Other studies suggested a direct alteration of bacterial infection and carriage pathways, as it has already been shown that *S. aureus* invasion and biofilm formation are elevated after cigarette exposure [47, 113, 114]. A similar effect was observed for pneumococcal biofilms [115, 116] (Additional file 1).

Several studies have shown that cigarette smoking depletes normal commensal airway microbiota and enriches potential pathogens (*H. influenzae*, *M. catarrhalis*, *Campylobacter* spp., *Streptococcus pneumoniae*, and *Streptococcus pyogenes)* [47, 87, 117]. In general, URT communities of smokers were found to be more diverse but less robust in composition over time compared to non-smokers [87] (Table 1; Additional file 1).

The likelihood of carrying Gram-positive anaerobic lineages (*Eggerthella*, *Erysipelotrichaceae I.S.*, *Dorea*, *Anaerovorax*, and *Eubacterium* spp.) is increased in the nasopharynx of smokers, including pathogens associated with URT infections and endocarditis (e.g., *Abiotrophia* spp.) [87] (Table 1; Additional file 1). In contrast, the upper respiratory tract of non-smokers harbors particularly *Peptostreptococcus* spp., α-haemolytic streptococci, and *Prevotella* spp., which seem to correlate negatively with pathogen presence [47, 117].

Interestingly, after 1 year (12 to 15 months) without smoking, the microbiome composition seems to recover and resembles microbial patterns of never-smokers, accompanied by a decrease of the proportion of opportunistic pathogens [87, 111, 120] (Table 1).

Smoking is not only harmful for adults, but also for infants when they are exposed to passive smoking. In general, *S. pneumoniae* was found to be elevated in infants with smoking parents [118]. Two-year-old children of smoking parents also have an increased risk of suffering from otitis media, meningococcal meningitis, and lower respiratory tract infections [111, 121, 122] (Additional file 1).

Notably, cigarettes themselves could be the source of these opportunistic pathogens. Sapkota et al. studied the bacterial metagenomes of commercially available cigarettes and discovered signatures of, e.g., *Acinetobacter*, *Burkholderia*, *Clostridium*, *Klebsiella*, *Pseudomonas aeruginosa*, and *Serratia* [119] (Table 1; Additional file 1).

Microbial competition in the URT

Most microbes associated with the human host interact positively with the host and each other. This collaboration

Table 1 Summary of significant URT microbiome changes due to active and passive cigarette smoking

Study	Population	Sample site	*Actinobacteria*	*Bacteroidetes*	*Firmicutes*	*Proteobacteria*
Charlson et al. 2010 [87]	Adult	Nasopharynx	↓*Actinomycetaceae* ↓*Corynebacteriaceae* ↓*Coriobacteriaceae* ↑*Eggerthella*	↓*Flexibacteriaceae* ↓*Flavobacteriaceae* ↑*Porphyromonadaceae*	↓*Leuconostocaceae* ↑*Erysipelotrichaceae* ↑*Aerococcaceae* ↑*Eubacteriaceae* ↑*Incertae Sedis XIII* ↑*Peptostreptococcaceae* ↑*Ruminococcaceae* ↑*Lachnospiraceae I.S.* spp. ↑*Anaerovorax* ↑*Dorea* ↑*Erysipelotrichaceae I.S.* ↑*Eubacterium* spp. ↑*Abiotrophia* spp.	↓*Rhodocyclaceae* ↓*Rhodobacteriaceae* ↓*Enterobacteriaceae* ↓*Alcaligenaceae* ↓*Methylophilacea* ↓*Shigella* spp. ↑Pasteurellaceae ↑*Haemophilus* spp.
Brook and Gober 2005 [117]	Adult	Nasopharynx			↑*Streptococcus pneumoniae* ↑*Streptococcus pyogenes*	↑*H. influenzae* ↑*M. catarrhalis*
Greenberg et al. 2006 [118]	Infants	Nasopharynx			↑*Streptococcus pneumoniae*	
Sapkota et al. 2009 [119]	Not applicable	Cigarettes			***Bacillus*** ***Clostridium*** *Enterococcus* *Staphylococcus*	***Acinetobacter*** ***Burkholderia*** ***Klebsiella*** ***Pseudomonas aeruginosa*** ***Serratia*** *Campylobacter* *Proteus*

Several different microbial signatures of the phyla Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria have been found to be altered in humans exposed to cigarette smoke. Arrows indicate an increase (↑) or decrease (↓) in relative abundance in smokers compared to non-smoking subjects. Signatures of ***Bold*** microbial genera were found to be present in more than 90% of all cigarette samples (Additional file 1)

is mostly based on syntrophic (i.e., co-feeding) networks [123]. However, if certain resources are restricted, or niches overlap, competitive interactions can occur between commensals (Fig. 4) and with opportunistic pathogens and the host. These interactions can involve direct and indirect attack of competitors.

For instance, microbes, colonizing the upper airways, have to cope with a scarcity of freely available glucose and iron [124–127]. To overcome these limitations, microbes can either scavenge iron from human cells [124] or release iron-chelating molecules (siderophores) that bind ferric iron from the adjacent environment [128].

Understanding the mechanisms of direct (e.g., secretion of antimicrobial peptides) and indirect microbial competition actions within the URT may illuminate new approaches for the development of new antimicrobial therapies for various diseases, for example, those caused by *Staphylococcus aureus* or *Streptococcus pneumoniae* [32, 129–132].

Although studies on microbe–microbe interaction also focus on other abundant genera of the human upper airways [1, 86, 132–138], knowledge on microbial competition for potential treatment of *S. aureus* infections is particularly important. This opportunistic pathogen is an asymptomatic colonizer of human skin and nose but it is also able to cause chronic and indolent to acute and aggressive infections in cases of excessive overgrowth [139–141].

One potentially applicable agent for a putative therapy is secreted by S. *lugdunensis*, namely lugdunin (a thiazolidine-

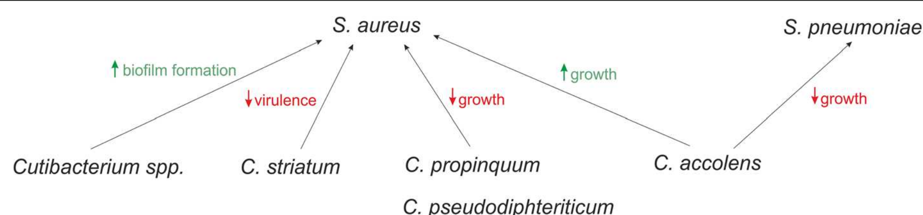


Fig. 4 Influence of nasal microbial community members on each other. Different *Corynebacterium* spp. are able to inhibit as well as promote growth of staphylococci and *S. pneumoniae* in vitro, whereas other species led to increased biofilm formation and reduced virulence. For references, see the text

containing cyclic peptide), which inhibits the growth of *S. aureus* in vitro [142]. Another candidate is the antimicrobial peptide nukacin IVK45, produced by *S. epidermidis IVK45* under in vitro oxidative stress and iron limitation [130, 143]. Species- or even strain-specific inhibition or promotion of staphylococci has also been observed for *Corynebacteria* [32, 129, 130]. Whereas some *C. pseudodiphteriticum* were able to inhibit the growth of *S. aureus*, co-cultivation with *C. accolens* led to supported and enhanced growth of both strains, indicating a possible cooperative interaction [32].

Corynebacterium species, or even cell-free conditioned medium thereof, were found to shift *S. aureus* towards a more commensal state and attenuation of virulence by downregulation of components involved in colonization and virulence, such as the agr operon or genes involved in hemolytic activity [129, 144, 145].

In contrast, methionine synthesis and iron acquisition were found to be upregulated in *S. aureus* when co-cultured with *C. striatum*. Based on this observation, Ramsey et al. envisage a competitive situation for methionine and iron in vitro [129]. It should be noted that coagulase-negative staphylococci are more sensitive to these types of nutrient competitions, as they produce lower levels of siderophores; however, resulting growth inhibition has been abolished by providing iron supplementation [124, 130, 146].

Besides Corynebacterium, *Cutibacterium* spp. (and its cell-free conditioned medium) is also able to affect growth of *S. aureus*. Coproporphyrin III (CIII), the most abundant porphyrin secreted by *Cutibacterium* ssp., induces *S. aureus* aggregation and biofilm formation in culture. Therefore, it also might promote biofilm formation with other members of the nostril's microbial community [132, 147, 148].

S. pneumoniae, a common inducer of URT diseases such as pneumonia, sinusitis, or otitis media [131, 149], can be inhibited by *C. accolens* through the production of free fatty acids (FFAs) from the host's triacylglycerols (TAG), causing an increase in the expression of antibacterial human β-defensin-2 [131, 150, 151].

Non-bacterial microorganisms in the human nose

Besides bacterial and viral components, the nasal cavity contains a unique, highly diverse archaeal community. Archaea are microorganisms that are, due to their different biology, distinctive from bacteria. They are also relevant components of the human microbiome inhabiting the gastrointestinal tract, oral cavity, skin, and other areas [152]. The archaeal community of the nasal cavity resembles that of the archaeomes of skin and the intestinal tract in being dominated by skin-associated Thaumarchaeota (*Nitrososphaera*) and also methanogenic Euryarchaeota (*Methanosphaera*, *Methanobrevibacter*) which are characteristic of the archaeal communities in the gastrointestinal tract [13]. Notably, the nasal cavity was found to represent an archaeal hot-spot amongst other body sites, with a high archaeal 16S rRNA gene content [153]. The importance of archaea in the nasal cavity was supported by a recent correlation of methano-archaeal presence in refractory sinusitis [154].

Severe knowledge gaps also exist with respect to the mycobiome and virome of the upper respiratory tract; as these fields are not part of this review, we refer readers to some recent reviews on these topics [96, 155–158] (Additional file 1).

Correlations between the upper respiratory tract microbiome and disease

The anterior nares are an open environment and in contact with several thousands of liters of inhaled air every day [159]. Therefore, besides the gastrointestinal tract, the nasal cavity has been suggested to represent the main entry port for pathogens, pollutants, and pollen, potentially causing imbalances in the nasal microbial community composition [89, 160, 161]. Microbiome dysbiosis is considered an important biomarker for human disease such as chronic rhinosinusitis [6, 162].

URT microbiome diversity and specific health-associated bacteria are reduced in chronic rhinosinusitis

Chronic rhinosinusitis (CRS) is a common chronic and detrimental inflammatory disorder of the human paranasal sinuses. It lasts for more than 12 weeks and affects up to 16% of the population [15, 163, 164]. Although CRS is suggested to be an inflammatory disease rather than an infectious one, bacterial contributions to the initiation and progression of inflammation are important to consider [165–167].

Previous studies suggested a polymicrobial process behind CRS [168]. A decrease in microbial diversity, richness and evenness, which are frequent features in other chronic inflammatory diseases as well, has been observed in CRS patients in several studies [15, 20, 47, 169–171]. This decline may occur due to an elevated presence of anaerobic bacteria growing in biofilms [172, 173]. Notably, the overall bacterial burden and phylum level abundance were found to remain constant, whereas the relative abundance of specific bacterial genera is altered in CRS patients [171, 174]. Hoggard et al. reported a depletion of signatures of *Anaerococcus*, *Corynebacterium*, *Finegoldia*, *Peptoniphilus*, *Propionibacterium*, and *Staphylococcus* in CRS patients—all previously identified as typical health-associated URT bacteria [162, 170]. This shift away from a healthy microbial community may lead to an increase of both inflammatory response (Toll-like receptor responses) and clinical severity [20, 175] (Table 2; Additional file 1).

Table 2 The nasal microbiome of chronic rhinosinusitis patients

Study	Population	Sample site	*Actinobacteria*	*Bacteroidetes*	*Firmicutes*	*Proteobacteria*
Lal et al. 2017 [29]	Adults with nasal polyps	Middle meatus	*Streptococcus* *Haemophilus* *Fusobacterium*			
	Adults without nasal polyps	Middle meatus	*Corynebacterium*		*Staphylococcus* *Alloiococcus*	
Copeland et al. 2018 [6]	Adults	Middle meatus	↓*Corynebacterium*	↑*Porphyromonas* ↑*Prevotella*	↑*Anaerococcus* ↑*Lactobacillus* ↑*Finegoldia* ↑*Peptoniphilus* ↑*Dialister* ↑*Parvimonas* ↓*Staphylococcus* ↓*Dolosigranulum*	
Hoggard et al. 2018 [20]	Adults	Middle meatus	↓*Corynebacterium* ↓*Propionibacterium*		↓*Anaerococcus* ↓*Finegoldia* ↓*Peptoniphilus* ↓*Staphylococcus*	
Aurora et al. 2013 [176]	Adults	Middle meatus	↑*Corynebacterium* ↑*Curtobacteria*		↑*Staphylococcus*	↑*Pseudomonas*
Cope et al. 2017 [168]	Adults	Sinus	*Corynebacteriaceae*		*Staphylococcaceae* *Streptococcaceae*	*Pseudomonadaceae*

Arrows indicate an increase (↑) or decrease (↓) in relative abundance in CRS patients compared to healthy subjects. Relative abundance was analyzed by 16S rRNA sequencing

A study on sinus microbiomes reported that most sinuses of CRS patients are dominated by signatures of *Corynebacteriaceae*, *Pseudomonadaceae*, *Staphylococcaceae*, or *Streptococcaceae*. These bacterial families were found to co-occur with a unique set of bacterial taxa with lower abundance [168] (Table 2). Other studies showed an overgrowth of *Corynebacterium tuberculostearicum* and *Staphylococcus* enrichment in sinuses [15, 169], as well as *Corynebacterium*, *Curtobacteria*, *Pseudomonas*, *Staphylococcus*, or *H. influenza* enrichment in the middle meatus [176, 177] (Table 2).

In the middle meatus, Copeland et al. found a negative correlation of the CRS disease state and six OTUs (operational taxonomic units) affiliated to genera *Staphylococcus*, *Corynebacterium*, and *Dolosigranulum*. *Corynebacterium* OTU410908 was the only signature to correlate negatively with the SNOT-22 (Sinonasal Outcome Test) score, which states disease severity [6] (Table 2).

Generally, anaerobic genera (*Anaerococcus*, *Lactobacillus*, *Finegoldia*, and *Peptoniphilus*) were found to be more present in CRS patients' compared to healthy subjects' middle meatuses [6] (Table 2; Additional file 1).

Traditionally, CRS is categorized in two subtypes: CRS with the absence (CRPsNP) or presence (CRPwNP) of nasal polyps (fleshy swellings arising due to inflammation) [6, 15, 163]. Notably, in CRSwNP patients, comorbidities such as aspirin intolerance and asthma are likely to occur [177]. Comparing the inferior and middle meatus microbiome of these different phenotypes reveals that CRSwNP samples were enriched by signatures of *Alloiococcus*, *Staphylococcus*, and *Corynebacterium* spp., whilst CRSsNP patients were enriched mainly by anaerobes, such as *Haemophilus*, *Streptococcus*, and *Fusobacteria* spp., and showed depletion of *Rothia*, *Alloiococcus*, *Corynebacterium*, and *Finegoldia*. Usually, the sinus cavities are not anaerobic; therefore, this enrichment of anaerobes in CRPsNP subjects is probably a result of disease progression and pathology [178]. *Fusobacteria*, for example, are associated with suppuration, which can cause anaerobic conditions in the paranasal cavities [29, 176] (Table 2; Additional file 1). Additionally, the severity of inflammation was positively correlated with the phylum Bacteroidetes (e.g., *Prevotella*) and the phylum Proteobacteria (*Pseudomonas*) in CRS [179].

Another interesting aspect is that CRS patients have an altered response to taste molecules. They are less sensitive to bitter while being more sensitive to sweet molecules [83]. As described above, bitter receptors in the nose play an important role in bacterial detection and defense. As a result of these alterations CRS patients have less stimulation of ciliary beating in the URT and show altered NO levels [38, 180]. Notably, It has already been shown that the functional capability of these taste receptors in the URT correlates with severity of CRS [80, 83, 181, 182].

Nasal washes, corticosteroids, and sinus surgery are the most common treatments for CRS and may significantly influence the URT microbiome. The therapy options and their effects are discussed later in this review [21–24].

Nasal microbiome composition may be linked to neurological diseases

Some reports indicate a potential involvement of the (nasal) microbiome in Parkinson's disease (PD), Alzheimer's disease

(AD), and multiple sclerosis (MS) [183]. In particular in PD and AD, the first symptoms are olfactory dysfunction (see below), and a link with the nasal microbiome of the olfactory area has been hypothesized as microorganisms contribute to normal development of the olfactory epithelium [184]. Since the nasal microbiome in AD and MS have not been studied in detail yet, we herein concentrate on PD as an example. PD is a neurodegenerative disease that is characterized by clumping of the protein α-synuclein in neuronal cells. In the dopaminergic substantia nigra of the central nervous system (CNS), these aggregates, also called Lewy bodies, lead to neuronal loss [185, 186]. α-Synuclein pathology was found to affect olfactory bulb function [160, 185, 186], and more than 90% of PD patients suffer from decreased olfactory function or hyposmia, even before motor symptoms occur [187].

Some studies suggested that a failure in innate immune system priming by nasopharyngeal microbiota could lead to an inflammatory response to α-synuclein, oxidative stress, cross-seeded misfolding, and thus development of neurodegenerative diseases [188–191]. Therefore, the studies hypothesized that the microbial community contributes to the initiation of PD [187, 192, 193].

No significant differences in alpha and beta diversity between the nasal microbiome of PD patients and healthy participants had until now been observed [192]. However, Pereira et al. showed that two taxa were less abundant in PD patients compared to healthy controls, namely signatures of the family *Flavobacteriaceae* and the genus *Marmoricola* [192] (Additional file 1).

Other studies hypothesize that a currently unknown, transmissible infectious agent enters the brain through the gastrointestinal tract and/or the nasal cavity and initiates the pathological process in the CNS [160, 193].

However, this research is at an early stage and the importance of the microbial community in initiation of PD requires further investigation.

The respiratory tract microbiome of cystic fibrosis patients follows clear patterns and might be established already early in life

Cystic fibrosis (CF) is a hereditary life-limiting disease that is caused by mutations in the gene of the cystic fibrosis transmembrane conductance regulator (CFTR). It can affect diverse organs but in most cases results in chronic lung disease [117, 120], characterized by a defect in mucociliary clearance and mucopurulent secretions [194–197]. The lungs of CF patients are colonized with so called "typical CF pathogens" consisting of bacterial genera *Rothia*, *Prevotella*, *Streptococcus*, *Actinomyces*, and *Veillonella* [195, 198, 199]. In addition to this so-called CF core microbiota, other CF-associated pathogens like *Pseudomonas aeruginosa*, *Haemophilus influenza*, *Burkholderia cepacia complex*, and *Staphylococcus aureus* can lead to chronic lung infection in CF [16, 194, 195]. The microorganisms originating from the environment probably spread via inhalation or micro-aspiration from the upper respiratory tract (URT) into the lungs [194, 200]. Several studies also demonstrate that the nasal cavity and the nasopharynx act as a reservoir for further colonization of these potential respiratory pathogens (PRPs), before they spread in the lower airways [26, 201, 202] (Additional file 1).

In CF infants, the nasal microbiome shows significant differences when compared to healthy controls. For instance, the relative abundance of *Corynebacteriaceae* and *Pastorellaceae* signatures was found to be reduced in the nasal microbiome of CF infants, whereas the relative abundance of *Staphylococcaceae* was increased. In nasopharyngeal samples, *S. mitis*, *Corynebacterium accolens*, and *S. aureus* as well as Gram-negative bacteria were more abundant in CF children [90]. This increased abundance of *S. aureus* in CF infants in early life is probably caused by a defect of the early innate immune system; moreover, due to accumulation of mucus, microaerobic conditions prevail in the airways of CF patients, which could lead to a better survival of *S. aureus* [26, 203, 204]. The URT microbiome of CF children adult CF patients is very similar, indicating establishment of this abnormal microbiome early in life [194] (Additional file 1).

Nasal microbiome in olfactory function and dysfunction

The functional area of human olfaction in the nose is the olfactory mucosa, which is located at the ceiling of the nasal cavity, is 8 to 10 mm long, and extends from the septum to the middle and superior turbinate. This olfactory area is characterized by a high abundance of bipolar neurons from the olfactory nerve and the presence of lactoferrin, IgA, IgM, and lysozyme, which prevent pathogens from intracranial entry through the cribriform plate [205].

The olfactory receptor cells in the olfactory mucosa pass through the cribriform plate into the olfactory bulb of the CNS. These cells are able to recognize different odor molecules, but also secondary metabolites of bacteria [33, 206]. In general, microbes are known to be able to interact with human body tissues via secondary metabolites, including short-chain fatty acids and other, hormone-like molecules [207–209].

Most cases of olfactory loss occur secondary to inflammation (caused, for example, by viral infections or chronic rhinosinusitis), traumatic brain injuries, ageing, or neurodegenerative diseases (e.g., PD and Alzheimer's disease) [210, 211]. In addition, as the physiology of the olfactory epithelium can be modulated by the microbiome, an influence of the microbial composition on olfactory function and dysfunction has been suggested [43, 184].

In healthy, normosmic volunteers Koskinen et al. identified four archaeal and 23 bacterial phyla in the microbiome of the olfactory area, the latter with *Actinobacteria*, *Firmicutes*, *Proteobacteria*, and *Bacteroidetes* predominating. On the genus level, signatures of *Corynebacterium*, *Staphylococcus*, and *Dolosigranulum* were shown to be most abundant [43]. *Corynebacterium* and *Staphylococcus* are typical human skin bacteria, frequently found in the nasal cavity [1, 134, 138, 212, 213]. *Dolosigranulum* has been observed to be a health-associated commensal inhabitant [139], but *Dolosigranulum pigrum*, an opportunistic pathogen, can, under certain conditions, also cause infections [214, 215] (Additional file 1).

Besides the healthy, normosmic participants, subjects with different olfactory performance were also studied [43]. Olfactory performance can be assessed by three different metrics: odor threshold (T; lowest concentration of odor compound perceivable), odor discrimination (D; discrimination of different odors), and odor identification (I; identification/naming of a certain odor). Based on these scores an overall TDI score is calculated. This TDI score categorizes subjects as normosmics (with normal olfactory performance), hyposmics (with decreased olfactory function), and anosmics (complete loss of olfactory function) [216, 217].

It is thought that an impacted nasal airflow influences the URT microbiome indirectly by changing local parameters (such as humidity, temperature, oxygenation). Such impacted airflow can occur due to rhinosinusitis, allergic rhinitis, head trauma, nasal surgery or congenital causes [33, 218–220] and might also contribute to the decrease in olfactory function by affecting the microbial community structure.

Indeed, Koskinen et al. observed that the microbiome of hyposmic subjects differed significantly in community composition and diversity compared to normosmics [43]. Odor threshold hyposmics (people with poor T score) showed a higher microbial diversity at the olfactory area, for example, signatures of the genus *Campylobacter* were found to correlate negatively with this condition, whereas *Proteobacteria*, *Actinobacteria*, *Firmicutes*, and *Bacteroidetes* were associated with poor odor identification. Furthermore, butyrate-producing bacteria like *Faecalibacterium* correlated negatively with odor threshold and discrimination, *Enterobacteriaceae* correlated negatively with odor threshold and identification, and *Porphyromonas* and unclassified *Lachnospiraceae* correlated negatively with overall olfactory performance (T, D, I) [43]. Whereas *Porphyromonas* is a typical representative of the human oral microbiome, *Faecalibacterium*, *Enterobacteriaceae*, and *Lachnospiraceae* are gut microorganisms, capable of producing butyrate. As butyrate has a very strong and unpleasant odor, and the production is out of place in the nasal area, it was suggested that it may have an impact on olfactory performance [43, 167, 221] (Additional file 1).

Analyzing the microbial composition and abundance with the goal of providing therapy options (e.g., through probiotics) could be one possible way to improve life quality for the 20% of the general population suffering from olfactory dysfunction.

Therapies change the URT microbiome composition and diversity

Intranasal corticosteroids (INS), saline rinses, antihistamines, and antibiotics are the current medical therapies of choice for inflammatory disorders of the upper respiratory tract [21, 24]. In contrast to anti-inflammatory substances that act through immunomodulatory mechanisms, antibiotics and some INS have antimicrobial properties and thus impact the microbial community directly [24, 222].

Antibiotics and other intranasal medication

Antibiotics and other medication with antimicrobial properties are usually used to treat severe bacterial infections. However, in some cases they are applied prophylactically, for example, before sinus surgery to diminish the bacterial load in the nasal cavity [24].

Application of antibiotics has been shown to influence microbial community composition significantly by reducing the microbial diversity not only in the gut, but also in the upper respiratory tract of infants and adults. The shift in the URT microbial profile results in an increased abundance of Gram-negative bacteria (*Burkholderia*, *Comamonadaceae*, *Bradyrhizobiaceae*, and *Enterobacteriaceae*) as well as *Moraxella*, *Haemophilus*, *Staphylococcus*, and *Streptococcus* [25–27]. Under normal circumstances, these bacteria are unable to compete in this niche, but due to tolerance to several antibiotics (e.g., *H. influenza* and *Chlamydia pneumoniae*: resistance towards β-lactam antibiotics; *S. pneumoniae*: resistance towards aminoglycosides, fluroquinolones, and β-lactam) they are able to expand during antibiotic treatment and become pathogenic [223, 224]. In contrast, abundances of known commensals such as *Dolosigranulum* and *Corynebacterium*, which normally are highly abundant in the human nose and associated with decreased URT infection risk and microbiota stability, are reduced by the treatment. These shifts in the anterior nares microbiome lasted throughout treatment and even posttreatment period (at least 2 weeks after treatment) [24, 93].

Topical antibiotic therapy with, e.g., mupirocin is used as standard preoperative therapy for non-allergic rhinitis (i.e., chronic rhinosinusitis). It has been shown that antibiotic treatment with muropirocin was able to decolonize *S. aureus* preoperatively, decreasing *S. aureus* site infections in surgery [24, 225, 226].

INSs like mometasone furoate monohydrate, which has anti-inflammatory properties, are common first line therapies for allergic rhinitis (AR) [21, 24]. INSs affect the composition and biodiversity of the nasal microbiome: like antibiotics, this medication suppresses several taxa (*Moraxella* spp., streptococci) and may promote the dominance of other taxa such as staphylococci [24, 225, 226].

Alterations in nasal structure due to sinus surgery influence the microbial community in the nasal cavity

Endoscopic sinus surgery (ESS) is an invasive treatment mainly used for polyposis and refractory sinusitis [22]. It enlarges the size of sinus ostia, improves mucociliary clearance, and facilitates access for topical therapies [218]. This intervention changes the physical sinus structure and may influence paranasal physiology by reducing the temperature and humidity in the nasal cavity. This drier and cooler post-operative ecosystem might have an effect on microbial composition and metabolism [218, 227].

Overall, the post-operative outcome of the surgery is positive, and only a subset of the patients does not recover [28, 228]. This subset suffers from a recolonization by pathogens despite antibiotic treatment after surgery [229–231]. It is suggested that the repopulation has its origins in paranasal sinus biofilms or in the nasopharynx, as these areas are better protected from antibiotics [164, 229, 232, 233]. It has also been reported that CRP patients who suffer from inflammation after the surgery have higher numbers of SCCs in the URT inflamed tissue [66]. Furthermore, patients with the non-functional genetic variation of the bitter receptor T2R38 are more likely to need surgery and develop bacterial infections [82, 83].

Notably, Hauser et al. found that the bacterial load of the ethmoid is lower at the time of surgery and 6 weeks after surgery than in the postoperative period (2 weeks after surgery). The authors suggested that a broad disruption of immune function and the mucociliary system due to the surgical intervention is responsible for this altered bacterial burden [229].

In an independent study, Jain et al. [218] reported an increase in the number of bacterial signatures, but no change in overall microbial profile 4 months after surgery compared to pre-operative microbial profiles. However, the relative abundance of *Staphylococcus* signatures increased whereas *Streptococcus* and *Corynebacterium* decreased; most changes were observed in extremely low-abundance taxa (e.g., *Peptoniphilus*, *Finegoldia*, *Faecalibacterium*, *Campylobacter*) [218].

Other studies reported similarities between the bacterial community of the ethmoid and sinuses after surgery and those of the anterior nasal cavity and pretreatment sinuses, and also the presence of bacteria from extra nasal sources, suggesting that all these sites serve as likely sources for recolonization [164, 229, 233, 234].

Nasal rinse might be a microbiome-friendly alternative to aggressive therapy options for URT diseases/problems

Nasal rinse has its origins in Ayurveda, an ancient, traditional system of Indian healthcare [235]. Today, nasal rinse is not only used to treat upper respiratory tract problems, as URTIs, CRS, or AR, but also as prevention of those diseases. Nasal irrigation is thought to clean the nasal mucosa from inflammatory mediators like leukotrienes and prostaglandins, antigens, and other pollutants [23, 236, 237]. The most common rinsing solutions are isotonic saline (0.9%) or hypertonic saline (1.5–3%), pH varying from 4.5 to 7, but distilled, tap, and well-water is also used [23, 238].

The potential microbial contamination of irrigation water and devices has been of concern, as it might contain *S. aureus* and *Pseudomonas* spp. which cause the majority of postoperative infections [234, 238, 239]. However, these low abundance contaminations showed only little impact on microbial composition in the human sinonasal cavity [240]. Nevertheless, distilled water is recommended, as tap water and well-water can also lead to mycobacterial infections and amebic brain abscesses [238, 241, 242].

The high frequency of positive results of nasal irrigation in several studies indicates that nasal rinsing is an effective, inexpensive, and simple method to treat sinonasal disorders alone or in association with other therapies to reduce medicine consumption.

Probiotics might be a non-invasive disease prevention and therapy option

In many cases of asthma and CRS, microbial dysbiosis is manifested by the expansion of pathogens and the loss of beneficial microorganisms [243, 244]. Living beneficial bacteria (probiotics) administered in adequate amounts can provide health benefits to the host [19, 245, 246]. Probiotic species may act as pioneers after disruption due to antibiotics, or have a larger beneficial effect on the community by acting as keystone species [247]. Additionally, probiotic strains may even be able to improve the epithelial barrier (by modulation of signaling pathways [248, 249]) or to interact positively with the host innate immune system [245, 246, 250, 251]. Probiotic microbes can interact with other microbes of the human microbiome by production of antimicrobials, competitive colonization, and inhibition of pathogen growth (e.g., by changing the pH in the niche) [247, 252, 253]. Probiotic bacteria can have various immunomodulatory functions, including T helper cell 1 (Th1)/T helper cell 2 (Th2) immune balance restoration, stimulation of regulatory T cells (Treg), the regulation of regulatory

cytokines [254–257], and also the modulation of allergen-specific T- and B-cell responses and mucosal IgA levels [258].

Immune cells, microbial metabolites, and cytokines released due to oral probiotic supplementation reach the airways through translocation into the blood and systemic circulation, whereas probiotics applied via nasal sprays affect the local immune response and the sinonasal microbiome [259–263]. For example, *Lactobacillus rhamnosus* leads to an increase in Th1 and decrease in Th2 levels in mice [264, 265], and treatment of acute sinusitis in children with *Enterococcus faecalis* has already been shown to reduce frequency and duration of sinusitis [246, 266].

The next logical step would be the application of probiotics nasally, although a potential risk of inflammation in the lower airways due to aspiration into the lung might exist [246, 267]. However, Martensson et al. were able to show, although no significant effects on CRS disease progression were observed, that nasal application of 13 honeybee lactic acid bacteria (various *Bifidobacteria* and lactobacilli of the honey stomach of *Apis mellifera*) was well tolerated by patients. This probiotic was able to restore commensal microbiomes and to prevent infections through antibacterial activity. Furthermore, no side effects could be observed [246, 268–272].

Knowledge gaps, conclusion, and outlook

Research on the microbiome of the URT has already revealed insights into its dynamic niche-specific composition, interactions between microbes and the host's immune, olfactory, and chemosensory systems, and alterations that are associated with age, lifestyle and disease. This research is, however, still in its infancy. The majority of current knowledge about the URT microbiome is based on cultivation assays, targeting only a fraction of the microbial community, or next generation sequencing of segments of the bacterial 16S rRNA gene amplified from uncultured samples. These short reads provide basic information about the diversity and taxonomic composition of bacterial communities. However, more accurate species or strain level community profiling can now be achieved using, for example, long-read technologies for sequencing the entire 16S rRNA gene, such as Oxford Nanopore [273] or Pacific Bioscience (PacBio) technology [274], which has already been successfully applied to analysing the healthy sinonasal microbiome [275]. Shot-gun metagenomics is another approach that is increasingly used in microbiome research, offering insights into microbial genomes and functions, and the possibility to assemble draft genomes of uncultured human health or disease associated microbes. Untargeted shot-gun metagenomics could also give unbiased insights into the archaeome, mycobiome and virome of the URT, although due to the low abundance of many of these components, targeted approaches could be more effective in capturing their full diversity.

Determining whether the detected changes or dysbioses in the URT microbiome associated with disease are markers or drivers presents a major challenge. There has already been some progress towards identifying biomarkers that could be used for early diagnosis of URTIs, such as *Microbacterium spp.*, *Streptococcus spp.* or *Faecalibacterium spp.*, whereas identifying targets for microbiome-based therapies remains more difficult. The ability to sample from disease-relevant sites within the URT is helpful in this regard, as it enables the identification of microbial candidate disease drivers whose abundance is positively correlated with both the site and incidence of disease, while negative correlations reported from the disease site are similarly more likely to be relevant, pointing to a possible protective role that might be harnessed in probiotic therapy. It will therefore be important to address the methodological challenges of sampling from less accessible URT sites, and to continue to develop appropriate sampling tools to minimise contamination from neighbouring sites. Further investigation of the co-operative and competitive interactions of microbes and host may also be helpful in guiding rational choices in the pursuit of causal connections and therapeutic goals. However, establishing causality and demonstrating the efficacy of proposed treatments requires other approaches, such as animal models and clinical trials.

Physicians and patients have high expectations of microbiome-driven therapies, yet most available knowledge stemming from basic research or clinical trials is far from impacting, or being implemented in, medical treatment. The results we have surveyed in this review suggest there are good reasons to remain optimistic about therapeutic solutions emerging from URT microbiome research, especially as newly available methodologies are deployed and current knowledge gaps are filled.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s12915-019-0703-z.

Additional file 1. Summary of the URT microbiome during the process of aging and in health and disease (collated information from selected studies).

Abbreviations

URT: Upper respiratory tract; URTI: URT infections; CRS: Chronic rhinosinusitis; AN: Anterior nares; MM: Middle meatus; OR: Olfactory area; SR: Sphenoethmoidal recess; COPD: Chronic obstructive pulmonary disease; OTU: Operational taxonomic unit; CRPsNP: CRS with the absence of nasal polyps; CRPwNP: CRS with the presence of nasal polyps; PD: Parkinson's disease; CNS: Central nervous system; CF: Cystic fibrosis; CFTR: Cystic fibrosis transmembrane conductance regulator; PRPs: Potential respiratory pathogens; Ig: Immunoglobulin; T: Odor threshold; D: Odor discrimination; I: Odor identification; INS: Intranasal corticosteroids; ESS: Endoscopic sinus surgery; AR: Allergic rhinitis; GIT: Gastrointestinal tract; NGS: Next generation sequencing

Acknowledgements
We gratefully acknowledge the support of Florian Fischmeister, University of Graz, Austria.
Funding: We acknowledge funding by the FWF (KLI 639) given to Veronika Schöpf (PI) and Christine Moissl-Eichinger (Co-PI). CK was supported by the local PhD program MolMed. KK, VS, and CME were supported by BioTechMed-Graz, the cooperation and networking initiative of the three major universities in Graz.

Authors' contributions
CK performed literature research and wrote the manuscript. KK and CME supervised literature research and wrote the manuscript. VS critically discussed and corrected the final draft. All authors read and approved the final manuscript.

Availability of data and materials
Not applicable.

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

Author details
[1]Diagnostic and Research Institute of Hygiene, Microbiology and Environmental Medicine, Medical University of Graz, Neue Stiftingtalstraße 6, 8010 Graz, Austria. [2]Institute of Psychology, University of Graz, Universitaetsplatz 2, 8010 Graz, Austria. [3]BioTechMed-Graz, Mozartgasse 12/II, 8010 Graz, Austria. [4]Present address: Medical University Vienna, Spitalgasse 23, 1090 Vienna, Austria.

Received: 19 September 2019 Accepted: 19 September 2019
Published online: 07 November 2019

References
1. Costello EK, Lauber CL, Hamady M, Fierer N, Gordon JI, Knight R. Bacterial community variation in human body habitats across space and time. Science. 2009;326:1694–7. https://doi.org/10.1126/science.1177486.
2. Lloyd-Price J, Mahurkar A, Rahnavard G, Crabtree J, Orvis J, Hall AB, et al. Strains, functions and dynamics in the expanded Human Microbiome Project. Nature. 2017;550:61. https://doi.org/10.1038/nature23889.
3. de Steenhuijsen Piters WAA, Sanders EAM, Bogaert D. The role of the local microbial ecosystem in respiratory health and disease. Philos Trans R Soc B Biol Sci. 2015;370:20140294. https://doi.org/10.1098/rstb.2014.0294.
4. Dickson R, Erb-Downward J, Martinez F, Huffnagle G. The microbiome and the respiratory tract. HHS Public Access. 2017;78:481–504.
5. Lighthart B. Mini-review of the concentration variations found in the alfresco atmospheric bacterial populations. 2000. https://link.springer.com/content/pdf/10.1023%2FA%3A1007694618888.pdf. Accessed 22 Oct 2018.
6. Copeland E, Leonard K, Carney R, Kong J, Forer M, Naidoo Y, et al. Chronic rhinosinusitis: Potential role of microbial dysbiosis and recommendations for sampling sites. Front Cell Infect Microbiol. 2018;8:57. https://doi.org/10.3389/fcimb.2018.00057.
7. Dominguez-Bello MG, Costello EK, Contreras M, Magris M, Hidalgo G, Fierer N, et al. Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. Proc Natl Acad Sci U S A. 2010;107:11971–5. https://doi.org/10.1073/pnas.1002601107.
8. de Steenhuijsen Piters WAA, Huijskens EGW, Wyllie AL, Biesbroek G, Van Den Bergh MR, Veenhoven RH, et al. Dysbiosis of upper respiratory tract microbiota in elderly pneumonia patients. ISME J. 2016;10:97–108.
9. Whelan FJ, Verschoor CP, Stearns JC, Rossi L, Luinstra K, Loeb M, et al. The loss of topography in the microbial communities of the upper respiratory tract in the elderly. Ann Am Thorac Soc. 2014;11:513–21.
10. Bassis CM, Tang AL, Young VB, Pynnonen MA. The nasal cavity microbiota of healthy adults. Microbiome. 2014;2:27.
11. Shilts MH, Rosas-Salazar C, Tovchigrechko A, Larkin EK, Torralba M, Akopov A, et al. Minimally Invasive sampling method identifies differences in taxonomic richness of nasal microbiomes in young infants associated with mode of delivery. Microb Ecol. 2016;71:233–42.
12. Stearns JC, Davidson CJ, Mckeon S, Whelan FJ, Fontes ME, Schryvers AB, et al. Culture and molecular-based profiles show shifts in bacterial communities of the upper respiratory tract that occur with age. ISME J. 2015;9:1246–59. https://doi.org/10.1038/ismej.2014.250.
13. Koskinen K, Pausan MR, Perras AK, Bang MBC, Mora M, Schilhabel A, et al. First insights into the diverse human archaeome: Specific detection of archaea in the gastrointestinal tract. MBio. 2017;8:1–17.
14. Vayssier-Taussat M, Albina E, Citti C, Cosson J-F, Jacques M-A, Lebrun M-H, et al. Shifting the paradigm from pathogens to pathobiome: new concepts in the light of meta-omics. Front Cell Infect Microbiol. 2014;4:29. https://doi.org/10.3389/fcimb.2014.00029.
15. Abreu NA, Nagalingam NA, Song Y, Roediger FC, Pletcher SD, Goldberg AN, et al. Sinus microbiome diversity depletion and *Corynebacterium tuberculostearicum* enrichment mediates rhinosinusitis. Sci Transl Med. 2012;4:151ra124. https://doi.org/10.1126/scitranslmed.3003783.
16. Coburn B, Wang PW, Diaz Caballero J, Clark ST, Brahma V, Donaldson S, et al. Lung microbiota across age and disease stage in cystic fibrosis. Sci Rep. 2015;5:10241. https://doi.org/10.1038/srep10241.
17. Gevers D, Kugathasan S, Denson LA, Vázquez-Baeza Y, Van Treuren W, Ren B, et al. The treatment-naive microbiome in new-onset Crohn's disease. Cell Host Microbe. 2014;15:382–92. https://doi.org/10.1016/j.chom.2014.02.005.
18. Hartstra AV, Bouter KEC, Bäckhed F, Nieuwdorp M. Insights into the role of the microbiome in obesity and type 2 diabetes. Diabetes Care. 2015;38:159–65. https://doi.org/10.2337/dc14-0769.
19. Petersen C, Round JL. Microreview Defining dysbiosis and its influence on host immunity and disease. Cell Microbiol. 2014;16:1024–33.
20. Hoggard M, Waldvogel-Thurlow S, Zoing M, Chang K, Radcliff FJ, Wagner Mackenzie B, et al. Inflammatory endotypes and microbial associations in chronic rhinosinusitis. Front Immunol. 2018;9:2065. https://doi.org/10.3389/fimmu.2018.02065.
21. Bernstein JA. Characterizing rhinitis subtypes. Am J Rhinol Allergy. 2013;27:457–60. https://doi.org/10.2500/ajra.2013.27.3983.
22. Ginat DT. Posttreatment imaging of the paranasal sinuses following endoscopic sinus surgery. Neuroimaging Clin N Am. 2015;25:653–65. https://doi.org/10.1016/J.NIC.2015.07.008.
23. Principi N, Esposito S. Nasal irrigation: an imprecisely defined medical procedure. Int J Environ Res Public Health. 2017;14. https://doi.org/10.3390/ijerph14050516.
24. Ramakrishnan VR, Holt J, Nelson LF, Ir D, Robertson CE, Frank DN. Determinants of the nasal microbiome: pilot study of effects of intranasal medication use. Allergy Rhinol (Providence). 2018;9:2152656718789519. https://doi.org/10.1177/2152656718789519.
25. Feazel LM, Robertson CE, Ramakrishnan VR, Frank DN. Microbiome Complexity and *Staphylococcus aureus* in chronic rhinosinusitis. Laryngoscope. 2012;122:467–72. https://doi.org/10.1002/lary.22398.
26. Prevaes SMPJ, De Winter-De Groot KM, Janssens HM, De Steenhuijsen Piters WAA, Tramper-Stranders GA, Wyllie AL, et al. Development of the nasopharyngeal microbiota in infants with cystic fibrosis. Am J Respir Crit Care Med. 2016;193:504–15.
27. Teo SM, Mok D, Pham K, Kusel M, Serralha M, Troy N, et al. The infant nasopharyngeal microbiome impacts severity of lower respiratory infection and risk of asthma development. Cell Host Microbe. 2015;17:704–15. https://doi.org/10.1016/j.chom.2015.03.008.
28. Smith TL, Litvack JR, Hwang PH, Loehrl TA, Mace JC, Fong KJ, et al. Determinants of outcomes of sinus surgery: a multi-institutional prospective cohort study. Otolaryngol Head Neck Surg. 2010;142:55–63. https://doi.org/10.1016/j.otohns.2009.10.009.
29. Lal D, Keim P, Delisle J, Barker B, Rank MA, Chia N, et al. Mapping and comparing bacterial microbiota in the sinonasal cavity of healthy, allergic rhinitis, and chronic rhinosinusitis subjects. Int Forum Allergy Rhinol. 2017;7:561–9. https://doi.org/10.1002/alr.21934.
30. Luna PN, Hasegawa K, Ajami NJ, Espinola JA, Henke DM, Petrosino JF, et al. The association between anterior nares and nasopharyngeal microbiota in infants hospitalized for bronchiolitis. Microbiome. 2018;6:1–14.
31. Zhou Y, Mihindukulasuriya KA, Gao H, La Rosa PS, Wylie KM, Martin JC, et al. Exploration of bacterial community classes in major human habitats. Genome Biol. 2014;15:R66.
32. Yan M, Pamp SJ, Fukuyama J, Hwang PH, Cho D-Y, Holmes S, et al. Nasal microenvironments and interspecific interactions influence nasal microbiota complexity and *S. aureus* carriage. Cell Host Microbe. 2013;14:631–40.
33. Sahin-Yilmaz A, Naclerio RM. Anatomy and physiology of the upper airway. Proc Am Thorac Soc. 2011;8:31–9. https://doi.org/10.1513/pats.201007-050RN.

34. Jones N. The nose and paranasal sinuses physiology and anatomy. Adv Drug Deliv Rev. 2001;51:5–19. https://doi.org/10.1016/S0169-409X(01)00172-7.
35. Geurkink N. Nasal anatomy, physiology, and function. J Allergy Clin Immunol. 1983;72:123–8.
36. Jimenez PN, Koch G, Thompson JA, Xavier KB, Cool RH, Quax WJ. The multiple signaling systems regulating virulence in Pseudomonas aeruginosa. Am Soc Microbiol. 2012;76:46–65.
37. Li Z, Nair SK. Quorum sensing: how bacteria can coordinate activity and synchronize their response to external signals? Protein Sci. 2012;21:1403–17.
38. Patel NN, Workman AD, Cohen NA. Role of taste receptors as sentinels of innate immunity in the upper airway. J Pathog. 2018;2018:9541987.
39. Cohen N. Sinonasal mucociliary clearance in health and disease. Ann Otol Rhinol Laryngol Suppl. 2006;196:20–6 www.ncbi.nlm.nih.gov/pubmed/17040014.
40. Ali M. Histology of the human nasopharyngeal mucosa. J Anat. 1965;99:657–72 https://europepmc.org/backend/ptpmcrender.fcgi?accid=PMC1270703&blobtype=pdf. Accessed 5 Nov 2018.
41. Bell GW, Joshi BB, Macleod RI. Maxillary sinus disease: diagnosis and treatment. Br Dent J. 2011;210:113–8. https://doi.org/10.1038/sj.bdj.2011.47.
42. Proctor DM, Relman DA, Section D, Alto P. The landscape ecology and microbiota of the human nose, mouth and throat. Cell Hot Microbe. 2018;21:421–32.
43. Koskinen K, Reichert JL, Hoier S, Schachenreiter J, Duller S, Moissl-Eichinger C, et al. The nasal microbiome mirrors and potentially shapes olfactory function. Sci Rep. 2018;8:1–11.
44. Shroff K, Meslin K, Cebra J. Commensal enteric bacteria engender a self-limiting humoral mucosal immune response while permanently colonizing the gut. Infect Immun. 1995;63:3904–13.
45. Casado B, Pannell LK, Iadarola P, Baraniuk JN. Identification of human nasal mucous proteins using proteomics. Proteomics. 2005;5:2949–59.
46. Wanner A, Salathé M, O'Riordan T. Mucociliary clearance in the airways. Am J Respir Crit Care Med. 1996;154:1868–902.
47. Schenck LP, Surette MG, Bowdish DME. Composition and immunological significance of the upper respiratory tract microbiota. FEBS Lett. 2016;590:3705–20.
48. Ooi EH, Wormald PJ, Tan LW. Innate immunity in the paranasal sinuses: a review of nasal host defenses. Am J Rhinol. 2008;22:13–9.
49. Devine DA, Marsh PD, Meade J. Modulation of host responses by oral commensal bacteria. J Oral Microbiol. 2015;7:26941.
50. Ivanov I, Honda K. Intestinal commensal microbes as immune modulators. Cell Host Microbe. 2012;12:496–508.
51. Ganz T. Antimicrobial polypeptides in host defense of the respiratory tract. J Clin Invest. 2002;109:693–7.
52. Sanchez L, Calvo M, Brock JH. Biological role of lactoferrin. Arch Disease Child. 1992;67:657–61.
53. Klebanoff SJ. Myeloperoxidase: friend and foe. J Leukoc Biol. 2005;77:598–625.
54. Nauseef WM. Assembly of the phagocyte NADPH oxidase. Histochem Cell Biol. 2004;122:277–91.
55. Thomas EL, Aune TM. Lactoperoxidase, peroxide, thiocyanate antimicrobial system: correlation of sulfhydryl oxidation with antimicrobial action. Infect Immun. 1978;20:456–63.
56. Lee RJ, Xiong G, Kofonow JM, Chen B, Lysenko A, Jiang P, et al. T2R38 taste receptor polymorphisms underlie susceptibility to upper respiratory infection. J Clin Invest. 2012;122:4145–59.
57. Parker D, Prince A. Innate immunity in the respiratory epithelium. Am J Respir Cell Mol Biol. 2011;45:189–201.
58. Salathe M. Regulation of mammalian ciliary beating. Annu Rev Physiol. 2007;69:401–22.
59. Harabuchi Y, Hamamoto M, Shirasaki H, Asakura K, Matsuyama H, Kataura A. Specific immune response of the adenoids to a respiratory antigen. Am J Otolaryngol. 1989;10:138–42.
60. Rynnel-Dagöö B. The immunological function of the adenoid. The proportions of T and B cells. Acta Otolaryngol. 1976;82:196–8.
61. Graeme-cook F, Bhan AK, Harris NL. Immunohistochemical characterization of intraepithelial and subepithelial mononuclear cells of the upper airways. Am J Pathol. 1993;143:1416–22.
62. Brandtzaeg P. Role of J chain and secretory component in receptor-mediated glandular and hepatic transport of immunoglobuiins in man. Scand J Immunol. 1985;22:111–46.
63. Van Kempen MJP, Rijkers GT, Van Cauwenberge PB. The immune response in adenoids. Int Arch Allergy Immunol. 2000;122:8–19.
64. Tang X, Hori S, Osamura R, Tsutsumi Y. Reticular crypt epithelium and intra-epithelial lymphoid cells in the hyperplastic human palatine tonsil: an immunohistochemical analysis. Pathol Int. 1995;45:34–44.
65. des Rieux A, EGE R, Gullberg E, Préat V, Schneider Y-J, Artursson P. Transport of nanoparticles across an in vitro model of the human intestinal follicle associated epithelium. Eur J Pharm Sci. 2005;25:455–65.
66. Patel NN, Kohanski MA, Maina IW, Triantafillou V, Workman AD, Tong CCL, et al. Solitary chemosensory cells producing interleukin-25 and group-2 innate lymphoid cells are enriched in chronic rhinosinusitis with nasal polyps. Int Forum Allergy Rhinol. 2018;8:900–6.
67. Kato A, Schleimer RP. Beyond inflammation: airway epithelial cells are at the interface of innate and adaptive immunity. Curr Opin Immunol. 2007;19:711–20.
68. Liao B, Cao P, Zeng M, Zhen Z, Wang H, Zhang Y, et al. Interaction of thymic stromal lymphopoietin, IL-33, and their receptors in epithelial cells in eosinophilic chronic rhinosinusitis with nasal polyps. Eur J Allergy Clin Immunol. 2015;70:1169–80.
69. Finger TE, Böttinger B, Hansen A, Anderson KT, Alimohammadi H, Silver WL. Solitary chemoreceptor cells in the nasal cavity serve as sentinels of respiration. Proc Natl Acad Sci U S A. 2003;100:8981–6.
70. Rivière S, Challet L, Fluegge D, Spehr M, Rodriguez I. Formyl peptide receptor- like proteins are a novel family of vomeronasal chemosensors. Nature. 2009;459:574–7.
71. Liberles SD, Horowitz LF, Kuang D, Contos JJ, Wilson KL, Siltberg-Liberles J, et al. Formyl peptide receptors are candidate chemosensory receptors in the vomeronasal organ. Proc Natl Acad Sci U S A. 2009;106:9842–7.
72. Alarie Y. Irritating properties of airborne materials to the upper respiratory tract. Arch Env Heal. 1966;13:433–49.
73. Tizzano M, Gulbransen BD, Vandenbeuch A, Clapp TR, Herman JP, Sibhatu HM. Nasal chemosensory cells use bitter taste signaling to detect irritants and bacterial signals. Proc Natl Acad Sci U S A. 2010;107:3210–5.
74. Geppetti P, Materazzi S, Nicoletti P. The transient receptor potential vanilloid 1: role in airway inflammation and disease. Eur J Pharmacol. 2006;533:207–14.
75. Saunders CJ, Christensen M, Finger TE, Tizzano M. Cholinergic neurotransmission links solitary chemosensory cells to nasal inflammation. Proc Natl Acad Sci U S A. 2014;111:6075–80.
76. Braham H, Cooper S, Anderson C, Tizzano M, Kingdom T, Finger T, et al. Solitary chemosensory cells and bitter taste receptor signaling in human sinonasal mucosa. Int Forum Allergy Rhinol. 2013;3:450–7.
77. Osculati F, Castellucci M, Cinti S, Zancanaro C. The solitary chemosensory cells and the diffuse chemosensory system of the airway. Eur J Histochem. 2007;51(Suppl 1):65–72.
78. Zhang Y, Hoon MA, Chandrashekar J, Mueller KL, Cook B, Wu D, et al. Coding of sweet, bitter, and umami tastes: different receptor cells sharing similar signaling pathways. Cell. 2003;112:293–301.
79. Iwata S, Yoshida R, Ninomiya Y. Taste transductions in taste receptor cells: basic tastes and moreover. Curr Pharm Des. 2014;20:2684–92.
80. Lee RJ, Kofonow JM, Rosen PL, Siebert AP, Chen B, Doghramji L, et al. Bitter and sweet taste receptors regulate human upper respiratory innate immunity. J Clin Invest. 2014;124:1393–405.
81. Baker EH, George S, Gyi KM, Hodson M, Philips BJ, Baines D, et al. Hyperglycaemia and cystic fibrosis alter respiratory fluid glucose concentrations estimated by breath condensate. J Appl Physiol. 2007;102:1969–75.
82. Workman AD, Maina IW, Brooks SG, Kohanski MA, Cowart BJ, Mansfield C, et al. The role of quinine-responsive Taste receptor Family 2 in airway immune defense and chronic rhinosinusitis. Front Immunol. 2018;9:624.
83. Workman AD, Brooks SG, Kohanski MA, Blasetti MT, Cowart BJ, Mansfield C, et al. Bitter and sweet taste tests are reflective of disease status in chronic rhinosinusitis. J Allergy Clin Immunol Pract. 2018;6:1078–80.
84. Lee RJ, Hariri BM, McMahon DB, Chen B, Doghramjii L, Adappa ND, et al. Bacterial D-amino acids suppress sinonasal innate immunity through sweet taste receptors in solitary chemosensory cells. Science Signaling. 2018;10(495):eaam7703.
85. Camarinha-Silva A, Wos-Oxley ML, Jáuregui R, Becker K, Pieper DH. Validating T-RFLP as a sensitive and high-throughput approach to assess bacterial diversity patterns in human anterior nares. FEMS Microbiol Ecol. 2011;79:98–108.
86. Wos-Oxley ML, Plumeier I, Von Eiff C, Taudien S, Platzer M, Vilchez-Vargas R, et al. A poke into the diversity and associations within human anterior nare microbial communities. ISME J. 2010;4:839–51. https://doi.org/10.1038/ismej.2010.15.

87. Charlson ES, Chen J, Custers-Allen R, Bittinger K, Li H, Sinha R, et al. Disordered microbial communities in the upper respiratory tract of cigarette smokers. PLoS One. 2010;5:1–10.
88. Wilson M. Microbial inhabitants of humans. Cambridge: Cambridge Univ. Press; 2005. https://doi.org/10.1186/1471-2180-9-259.
89. Camarinha-Silva A, Jáuregui R, Pieper DH, Wos-Oxley ML. The temporal dynamics of bacterial communities across human anterior nares. Environ Microbiol Rep. 2012;4:126–32.
90. Frayman KB, Armstrong DS, Grimwood K, Ranganathan SC. The airway microbiota in early cystic fibrosis lung disease. Pediatr Pulmonol. 2017;52: 1384–404.
91. Teo SM, Mok D, Pham K, Kusel M, Serralha M, Troy N, et al. The infant airway microbiome in health and disease impacts later asthma development. Cell Host Microbe. 2015;17:704–15. https://doi.org/10.1016/j.chom.2015.03.008.
92. Biesbroek G, Tsivtsivadze E, Sanders EAM, Montijn R, Veenhoven RH, Keijser BJF, et al. Early respiratory microbiota composition determines bacterial succession patterns and respiratory health in children. Am J Respir Crit Care Med. 2014;190:1283–92.
93. Biesbroek G, Bosch AATM, Wang X, Keijser BJF, Veenhoven RH, Sanders EAM, et al. The impact of breastfeeding on nasopharyngeal microbial communities in infants. Am J Respir Crit Care Med. 2014;190:298–308.
94. Von Linstow ML, Schønning K, Hoegh AM, Sevelsted A, Vissing NH, Bisgaard H. Neonatal airway colonization is associated with troublesome lung symptoms in infants. Am J Respir Crit Care Med. 2013;188:1041–2.
95. Moore H, Jacoby P, Taylor A, Harnett G, Bowman J. V Riley T, et al. The interaction between respiratory viruses and pathogenic bacteria in the upper respiratory tract of asymptomatic aboriginal and non-aboriginal children. Pediatr Infect Dis J. 2010;29:540–5.
96. van den Bergh MR, Biesbroek G, Rossen JWA, de Steenhuijsen Piters WAA, Bosch AATM, van Gils EJM, et al. Associations between pathogens in the upper respiratory tract of young children: interplay between viruses and bacteria. PLoS One. 2012;7:e47711. https://doi.org/10.1371/journal.pone.0047711.
97. Vissing NH, Chawes BLK, Bisgaard H. Increased risk of pneumonia and bronchiolitis after bacterial colonization of the airways as neonates. Am J Respir Crit Care Med. 2013;188:1246–52.
98. Camarinha-Silva A, Jáuregui R, Chaves-Moreno D, Oxley APA, Schaumburg F, Becker K, et al. Comparing the anterior nare bacterial community of two discrete human populations using Illumina amplicon sequencing. Environ Microbiol. 2014;16:2939–52.
99. Zhou Y, Gao H, Mihindukulasuriya KA, Rosa PSL, Wylie KM, Vishnivetskaya T, et al. Biogeography of the ecosystems of the healthy human body. Genome Biol. 2013;14:R1. https://doi.org/10.1186/gb-2013-14-1-r1.
100. Oh J, Byrd AL, Deming C, Conlan S, Program NCS, Kong HH, et al. Biogeography and individuality shape function in the human skin metagenome. Nature. 2014;514:59–64. https://doi.org/10.1038/nature13786.
101. Franceschi C, Bonafè M, Valensin S, Olivieri F, De Luca M, Ottaviani E, et al. Inflamm-aging: an evolutionary perspective on immunosenescence. Ann N Y Acad Sci. 2006;908:244–54. https://doi.org/10.1111/j.1749-6632.2000.tb06651.x.
102. Stämpfli MR, Anderson GP. How cigarette smoke skews immune responses to promote infection, lung disease and cancer. Nat Rev Immunol. 2009;9: 377 EP.
103. Macgregor I. Effects of smoking on oral ecology. A review of the literature. Clin Prev Dent. 1989;11:3–7.
104. Yu G, Phillips S, Gail MH, Goedert JJ, Humphrys MS, Ravel J, et al. The effect of cigarette smoking on the oral and nasal microbiota. Microbiome. 2017;5: 1–6. https://doi.org/10.1186/s40168-016-0226-6.
105. Mason R. Biology of alveolar type II cells. Respirology. 2006;11(Suppl: S1):2–5.
106. Ratner AJ, Lysenko ES, Paul MN, Weiser JN. Synergistic proinflammatory responses induced by polymicrobial colonization of epithelial surfaces. Proc Natl Acad Sci U S A. 2005;102:3429–34. https://doi.org/10.1073/pnas.0500599102.
107. Phipps JC, Aronoff DM, Curtis JL, Goel D, O'Brien E, Mancuso P. Cigarette smoke exposure impairs pulmonary bacterial clearance and alveolar macrophage complement-mediated phagocytosis of Streptococcus pneumoniae. Infect Immun. 2010;78:1214–20.
108. Castranova V, Huffman LJ, Judy DJ, Bylander JE, Lapp LN, Weber SL, et al. Enhancement of nitric oxide production by pulmonary cells following silica exposure. Environ Health Perspect. 1998;106(Suppl 5):1165–9. https://doi.org/10.1289/ehp.98106s51165.
109. Chaudhuri N, Sabroe I. Basic science of the innate immune system and the lung. Paediatr Respir Rev. 2008;9:236–42. https://doi.org/10.1016/j.prrv.2008.03.002.
110. Bagaitkar J, Demuth DR, Daep CA, Renaud DE, Pierce DL, Scott DA. Tobacco upregulates P. gingivalis fimbrial proteins which induce TLR2 hyposensitivity. PLoS One. 2010;5:e9323.
111. Garmendia J, Morey P, Bengoechea JA. Impact of cigarette smoke exposure on host-bacterial pathogen interactions. Eur Respir J. 2012;39:467–77.
112. Goldstein-Daruech N, Cope EK, Zhao K-Q, Vukovic K, Kofonow JM, Doghramji L, et al. Tobacco smoke mediated induction of sinonasal microbial biofilms. PLoS One. 2011;6:e15700.
113. Kulkarni R, Antala S, Wang A, Amaral FE, Rampersaud R, LaRussa SJ, et al. Cigarette smoke increases *Staphylococcus aureus* biofilm formation via oxidative stress. Infect Immun. 2012;80:3804–11. https://doi.org/10.1128/IAI.00689-12.
114. McEachern EK, Hwang JH, Sladewski KM, Nicatia S, Dewitz C, Mathew DP, et al. Analysis of the effects of cigarette smoke on staphylococcal virulence phenotypes. Infect Immun. 2015;83:2443–52.
115. Mutepe ND, Cockeran R, Steel HC, Theron AJ, Mitchell TJ, Feldman C, et al. Effects of cigarette smoke condensate on pneumococcal biofilm formation and pneumolysin. Eur Respir J. 2013;41:392. https://doi.org/10.1183/09031936.00213211.
116. Shen P, Whelan FJ, Schenck LP, McGrath JJC, Vanderstocken G, Bowdish DME, et al. Streptococcus pneumoniae colonization is required to alter the nasal microbiota in cigarette smokeexposed mice. Infect Immun. 2017;85:1–14.
117. Brook I, Gober AE. Recovery of potential pathogens in the nasopharynx of healthy and otitis media-prone children and their smoking and nonsmoking parents. Ann Otol Rhinol Laryngol. 2005;117:727–30.
118. Greenberg D, Givon-Lavi N, Broides A, Blancovich I, Peled N, Dagan R. The contribution of smoking and exposure to tobacco smoke to Streptococcus pneumoniae and *Haemophilus influenzae* carriage in children and their mothers. Clin Infect Dis. 2006;42:897–903. https://doi.org/10.1086/500935.
119. Sapkota AR, Berger S, Vogel TM. Human pathogens abundant in the bacterial metagenome of cigarettes. Environ Health Perspect. 2009;118: 351–6.
120. Brook I, Gober AE. Effect of smoking cessation on the microbial flora. Arch Otolaryngol Head Neck Surg. 2007;133:135–8. https://doi.org/10.1001/archotol.133.2.135.
121. García-Rodríguez J, Fresnadillo M. Dynamics of nasopharyngeal clonization by potential respiratory pathogens. J Antimicrob Chemother. 2002;50(Suppl S2):59–73.
122. Iles K, Poplawski NK, Couper RT. Passive exposure to tobacco smoke and bacterial meningitis in children. J Paediatr Child Health. 2001;37:388–91.
123. Morris B, Henneberger R, Huber H, Moissl-Eichinger C. Microbial syntrophy: interaction for the common good. FEMS Microbiol Rev. 2013;37:384–406.
124. Krismer B, Liebeke M, Janek D, Nega M, Rautenberg M, Hornig G, et al. Nutrient limitation governs *Staphylococcus aureus* metabolism and niche adaptation in the human nose. PLOS Pathog. 2014;10:e1003862.
125. Ferraris R, Yasharpour S, Lloyd K, Mirzayan R, Diamond J. Luminal glucose concentrations in the gut under normal conditions. Am J Physiol. 1990;259: G822–37.
126. Lorin M, Gaerlan P, Mandel I. Quantitative composition of nasal secretions in normal subjects. J Lab Clin Med. 1972;80:275–81.
127. Vanthanouvong V, Roomans G. Methods for determining the composition of nasal fluid by X-ray microanalysis. Microsc Res Tech. 2004;63:122–8.
128. Winkelmann G. Microbial siderophore-mediated transport. Biochem Soc Trans. 2002;30:691–6.
129. Ramsey MM, Freire MO, Gabrilska RA, Rumbaugh KP, Lemon KP. Staphylococcus aureus shifts toward commensalism in response to Corynebacterium species. Front Micobiology. 2016;7:1–15.
130. Stubbendieck RM, May DS, Chevrette MG, Temkin MI, Wendt-Pienkowski E, Cagnazzo J, et al. Competition among nasal bacteria suggests a role for siderophore-mediated interactions in shaping the human nasal microbiota. Am Soc Microbiol. 2019;85(10):e02406–18.
131. Bomar L, Brugger SD, Yost BH, Davies SS, Lemon P. Corynebacterium accolens releases antipneumococcal free fatty acids from human nostril and skin surface triacylglycerols. MBio. 2016;7:1–13.
132. Wollenberg MS, Claesen J, Escapa IF, Aldridge KL, Fischbach MA, Lemon P. Propionibacterium -produced coproporphyrin III induces *Staphylococcus aureus* aggregation and biofilm formation. MBio. 2014;5:1–10.

133. Lemon KP, Klepac-Ceraj V, Schiffer HK, Brodie EL, Lynch SV, Kolter R. Comparative analyses of the bacterial microbiota of the human nostril and oropharynx. MBio. 2010;1:4–6.
134. Frank DN, Feazel LM, Bessesen MT, Price CS, Janoff EN, Pace NR. The human nasal microbiota and *Staphylococcus aureus* carriage. PLoS One. 2010;5: e10598.
135. von Eiff C, Becker K, Machka K, Stammer H, Peters G. Nasal carriage as a source of *Staphylococcus aureus* bacteremia. N Engl J Med. 2001;344:11–6.
136. Wertheim HF, Vos MC, Ott A, van Belkum A, Voss A, Kluytmans JA, et al. Risk and outcome of nosocomial *Staphylococcus aureus* bacteraemia in nasal carriers versus non-carriers. Lancet. 2004;364:703–5.
137. Consortium HM. Structure, function and diversity of the healthy human microbiome. Nature. 2012;486:207–14. https://doi.org/10.1038/nature11234.
138. Grice EA, Kong HH, Conlan S, Deming CB, Davis J, Young AC, et al. Topographical and temporal diversity of the human skin microbiome. Science. 2009;324:1190–2. https://doi.org/10.1126/science.1171700.
139. Kuehnert M, Kruszon-Moran D, Hill H, McQuillan G, McAllister S, Fosheim G, et al. Prevalence of *Staphylococcus aureus* nasal colonization in the United States, 2001–2002. J Infect Dis. 2006;193:172–9.
140. Gorwitz R, Kruszon-Moran D, McAllister S, MCQuillan G, McDougal L, Fosheim G, et al. Changes in the prevalence of nasal colonization with *Staphylococcus aureus* in the United States, 2001–2004. J Infect Dis. 2008; 197:1226–34.
141. Barbara G, Stanghellini V, Brandi G, Cremon C, Di Nardo G, De Giorgio R, et al. Interactions between commensal bacteria and gut sensorimotor function in health and disease. Am J Gastroenterol. 2005;100:2560–8.
142. Zipperer A, Konnerth MC, Laux C, Berscheid A, Janek D, Weidenmaier C, et al. Human commensals producing a novel antibiotic impair pathogen colonization. Nature. 2016;535:511–6. https://doi.org/10.1038/nature18634.
143. Janek D, Zipperer A, Kulik A, Krismer B, Peschel A. High frequency and diversity of antimicrobial activities produced by nasal Staphylococcus strains against bacterial competitors. PLOS Pathog. 2016;12:e1005812.
144. Novick R, Geisinger E. Quorum sensing in staphylococci. Annu Rev Genet. 2008;42:541–64.
145. Thoendel M, Horswill A. Identification of *Staphylococcus aureus* AgrD residues required for autoinducing peptide biosynthesis. J Biol Chem. 2009; 284:21828–38.
146. Lindsay JA, Riley TV, Meel BJ. Staphylococcus aureus but not *Staphylococcus epidermidis* can acquire iron from transferrin. Microbiology. 1995;141:197–203.
147. Schaller M, Loewenstein M, Borelli C, Jacob K, Vogeser M, Burgdorf W, et al. Induction of a chemoattractive proinflammatory cytokine response after stimulation of keratinocytes with *Propionibacterium acnes* and coproporphyrin III. Br J Dermatol. 2005;153:66–71.
148. Borelli C, Merk K, Schaller M, Jacob K, Vogeser M, Weindl G, et al. In vivo porphyrin production by P. acnes in untreated acne patients and its modulation by acne treatment. Acta Derm Venereol. 2006;86:316–9.
149. Bogaert D, De Groot R, Hermans PWM. Streptococcus pneumoniae colonisation: the key to pneumococcal disease. Lancet Infect Dis. 2004;4: 144–54.
150. Nakatsuji T, Kao MC, Zhang L, Zouboulis CC, Gallo RL, Huang C-M. Sebum free fatty acids enhance the innate immune defense of human sebocytes by upregulating b-defensin-2 expression. J Invest Dermatol. 2010;130:985–94. https://doi.org/10.1038/jid.2009.384.
151. Lee H, Andalibi A, Webster P, Moon S, Teufert K, Kang S, et al. Antimicrobial activity of innate immune molecules against Streptococcus pneumoniae, Moraxella catarrhalis and nontypeable. BMC Infect Dis. 2004;4:1–12.
152. Mahnert A, Blohs M, Pausan M-R, Moissl-Eichinger C. The human archaeome: methodological pitfalls and knowledge gaps. Emerg Top Life Sci. 2018;2:469–82. https://doi.org/10.1042/ETLS20180037.
153. Pausan MR, Csorba C, Singer G, Till H, Schoepf V, Santigli E, et al. Measuring the archaeome: detection and quantification of archaea signatures in the human body. bioRxiv. 2018:334748. https://doi.org/10.1101/334748.
154. Sogodogo E, Fellag M, Loukil A, Nkamga V, Michel J, Dessi P, et al. Nine cases of methanogenic archaea in refractory sinusitis, an emerging clinical entity. Front Public Heal. 2019;7:38.
155. Cui L, Morris A, Ghedin E. The human mycobiome in health and disease. Genome Med. 2013;5:63. https://doi.org/10.1186/gm467.
156. Korten I, Mika M, Klenja S, Kieninger E, Mack I, Barbani MT, et al. Interactions of respiratory viruses and the nasal microbiota during the first year of life in healthy infants. mSphere. 2016;1:e00312–6. https://doi.org/10.1128/mSphere.00312-16.
157. Jung WH, Croll D, Cho JH, Kim YR, Lee YW. Analysis of the nasal vestibule mycobiome in patients with allergic rhinitis. Mycoses. 2015;58:167–72. https://doi.org/10.1111/myc.12296.
158. Popgeorgiev N, Temmam S, Raoult D, Desnues C. Describing the silent human virome with an emphasis on giant viruses. Intervirology. 2013;56: 395–412. https://doi.org/10.1159/000354561.
159. Van der Schans CP. Bronchial mucus transport. Respir Care. 2007;52:1150–6; discussion 1156–8. https://doi.org/10.1016/0952-8180(93)90100-S.
160. Heintz-Buschart A, Pandey U, Wicke T, Sixel-Döring F, Janzen A, Sittig-Wiegand E, et al. The nasal and gut microbiome in Parkinson's disease and idiopathic rapid eye movement sleep behavior disorder. Mov Disord. 2017; 33:88–98.
161. Pail G, Huf W, Pjrek E, Winkler D, Willeit M, Praschak-Rieder N, et al. Bright-light therapy in the treatment of mood disorders. Neuropsychobiology. 2011;64:152–62. https://www.karger.com/. https://doi.org/10.1159/000328950.
162. Hoggard M, Biswas K, Zoing M, Wagner Mackenzie B, Taylor MW, Douglas RG. Evidence of microbiota dysbiosis in chronic rhinosinusitis. Int Forum Allergy Rhinol. 2017;7:230–9. https://doi.org/10.1002/alr.21871.
163. Fokkens WJ, Lund VJ, Mullol J, Bachert C, Alobid I, Baroody F, et al. EPOS 2012: European position paper on rhinosinusitis and nasal polyps 2012. A summary for otorhinolaryngologists. Rhinol J. 2012;50:1–12. https://doi.org/10.4193/Rhino50E2.
164. Foreman A, Jervis-Bardy J, Wormald P-J. Do biofilms contribute to the initiation and recalcitrance of chronic rhinosinusitis? Laryngoscope. 2011; 121:1085–91. https://doi.org/10.1002/lary.21438.
165. Shin S, Ponikau J, Sherris D, Congdon D, Frigas E, Homburger H, et al. Chronic rhinosinusitis: an enhanced immune response to ubiquitous airborne fungi. J Allergy Clin Immunol. 2004;114:1369–75.
166. Bachert C, Gevaert P, van Cauwenberge P. Staphylococcus aureus enterotoxins: a key in airway disease? Allergy. 2002;57:480–7.
167. Mahdavinia M, Keshavarzian A, Tobin MC, Landay A, Schleimer RP. A comprehensive review of the nasal microbiome in chronic rhinosinusitis (CRS). Clin Exp Allergy. 2016;46:21–41. https://doi.org/10.1111/cea.12666.
168. Cope EK, Goldberg AN, Pletcher SD, Lynch SV. Compositionally and functionally distinct sinus microbiota in chronic rhinosinusitis patients have immunological and clinically divergent consequences. Microbiome. 2017;5:53. https://doi.org/10.1186/s40168-017-0266-6.
169. Choi E-B, Hong S-W, Kim D-K, Jeon SG, Kim K-R, Cho S-H, et al. Decreased diversity of nasal microbiota and their secreted extracellular vesicles in patients with chronic rhinosinusitis based on a metagenomic analysis. Allergy. 2014;69:517–26. https://doi.org/10.1111/all.12374.
170. Wagner Mackenzie B, Waite DW, Hoggard M, Douglas RG, Taylor MW, Biswas K. Bacterial community collapse: a meta-analysis of the sinonasal microbiota in chronic rhinosinusitis. Environ Microbiol. 2017;19:381–92. https://doi.org/10.1111/1462-2920.13632.
171. Psaltis AJ, Wormald P-J. Therapy of sinonasal microbiome in CRS: a critical approach. Curr Allergy Asthma Rep. 2017;17:59. https://doi.org/10.1007/s11882-017-0726-x.
172. Dlugaszewska J, Leszczynska M, Lenkowski M, Tatarska A, Pastusiak T, Szyfter W. The pathophysiological role of bacterial biofilms in chronic sinusitis. Eur Arch Otorhinolaryngol. 2016;273:1989–94. https://doi.org/10.1007/s00405-015-3650-5.
173. Stephenson M-F, Mfuna L, Dowd SE, Wolcott RD, Barbeau J, Poisson M, et al. Molecular characterization of the polymicrobial flora in chronic rhinosinusitis. J Otolaryngol Head Neck Surg. 2010;39:182–7 http://www.ncbi.nlm.nih.gov/pubmed/20211106. Accessed 16 Oct 2018.
174. Ramakrishnan VR, Hauser LJ, Feazel LM, Ir D, Robertson CE, Frank DN. Sinus microbiota varies among chronic rhinosinusitis phenotypes and predicts surgical outcome. J Allergy Clin Immunol. 2015;136:334–42.e1. https://doi.org/10.1016/J.JACI.2015.02.008.
175. Hirschberg A, Kiss M, Kadocsa E, Polyanka H, Szabo K, Razga Z, et al. Different activations of toll-like receptors and antimicrobial peptides in chronic rhinosinusitis with or without nasal polyposis. Eur Arch Otorhinolaryngol. 2016; 273:1779–88. https://doi.org/10.1007/s00405-015-3816-1.
176. Aurora R, Chatterjee D, Hentzleman J, Prasad G, Sindwani R, Sanford T. Contrasting the microbiomes from healthy volunteers and patients with chronic rhinosinusitis. JAMA Otolaryngol Neck Surg. 2013;139:1328. https://doi.org/10.1001/jamaoto.2013.5465.
177. Chalermwatanachai T, Vilchez-Vargas R, Holtappels G, Lacoere T, Jáuregui R, Kerckhof F-M, et al. Chronic rhinosinusitis with nasal polyps is characterized

by dysbacteriosis of the nasal microbiota. Sci Rep. 2018;8:7926. https://doi.org/10.1038/s41598-018-26327-2.
178. Brook I. The role of anaerobic bacteria in sinusitis. Anaerobe. 2006;12:5–12. https://doi.org/10.1016/J.ANAEROBE.2005.08.002.
179. Kuhar HN, Tajudeen BA, Mahdavinia M, Heilingoetter A, Ganti A, Gattuso P, et al. Relative abundance of nasal microbiota in chronic rhinosinusitis by structured histopathology. Int Forum Allergy Rhinol. 2018. https://doi.org/10.1002/alr.22192.
180. Naraghi M, Deroee AF, Ebrahimkhani M, Kiani S, Dehpour A. Nitric oxide: a new concept in chronic sinusitis pathogenesis B. Am J Otolaryngol. 2007;28:334–7.
181. Carey RM, Workman AD, Hatten KM, Siebert AP, Brooks SG, Chen B, et al. Denatonium-induced sinonasal bacterial killing may play a role in chronic rhinosinusitis outcomes. Int Forum Allergy Rhinol. 2017;7:699–704.
182. Adappa ND, Truesdale CM, Workman AD, Doghramji L, Mansfield C, Kennedy DW, et al. Correlation of T2R38 taste phenotype and in vitro biofilm formation from nonpolypoid chronic rhinosinusitis patients. Int Forum Allergy Rhinol. 2017;6:783–91.
183. Bell JS, Spencer JI, Yates RL, Yee SA, Jacobs BM, DeLuca GC. Invited review: From nose to gut – the role of the microbiome in neurological disease. Neuropathol Appl Neurobiol. 2018:nan.12520. https://doi.org/10.1111/nan.12520.
184. François A, Grebert D, Rhimi M, Mariadassou M, Naudon L, Rabot S, et al. Olfactory epithelium changes in germfree mice. Sci Rep. 2016;6:24687. https://doi.org/10.1038/srep24687.
185. Braak H, Del Tredici K, Rüb U, Vos RAI, Jansen Steur ENH, Braak E. Staging of brain pathology related to sporadic Parkinson's disease. Neurobiol Aging. 2003;24:197–211. https://doi.org/10.1016/S0197-4580(02)00065-9.
186. Braak H, Vos RAI, Bohl J, Del Tredici K. Gastric α-synuclein immunoreactive inclusions in Meissner's and Auerbach's plexuses in cases staged for Parkinson's disease-related brain pathology. Neurosci Lett. 2006;396:67–72. https://doi.org/10.1016/j.neulet.2005.11.012.
187. Haehner A, Boesveldt S, Berendse HW, Mackay-Sim A, Fleischmann J, Silburn PA, et al. Prevalence of smell loss in Parkinson's disease - a multicenter study. Park Relat Disord. 2009;15:490–4. https://doi.org/10.1016/j.parkreldis.2008.12.005.
188. Béraud D, Maguire-Zeiss KA. Misfolded α-synuclein and toll-like receptors: therapeutic targets for Parkinson's disease. Parkinsonism Relat Disord. 2012;18(Supplement 1):17–20. https://doi.org/10.1016/S1353-8020(11)70008-6.
189. Friedland RP. Mechanisms of molecular mimicry involving the microbiota in neurodegeneration. J Alzheimer's Dis. 2015;45:349–62.
190. Khan F, Oloketuyi SF. A future perspective on neurodegenerative diseases: nasopharyngeal and gut microbiota. J Appl Microbiol. 2016;122:306–20.
191. Mulak A, Bonaz B. Brain-gut-microbiota axis in Parkinson's disease. World J Gastroenterol. 2015;21:10609–20. https://doi.org/10.3748/wjg.v21.i37.10609.
192. Pereira PAB, Aho VTE, Paulin L, Pekkonen E, Auvinen P, Scheperjans F. Oral and nasal microbiota in Parkinson's disease. Park Relat Disord. 2017;38:61–7. https://doi.org/10.1016/j.parkreldis.2017.02.026.
193. Hawkes CH, Del Tredici K, Braak H. Parkinson's disease: a dual-hit hypothesis. Neuropathol Appl Neurobiol. 2007;33:599–614.
194. Boutin S, Graeber SY, Weitnauer M, Panitz J, Stahl M, Clausznitzer D, et al. Comparison of microbiomes from different niches of upper and lower airways in children and adolescents with cystic fibrosis. PLoS One. 2015;10:1–19. https://doi.org/10.1371/journal.pone.0116029.
195. Prevaes SMPJ, De Steenhuijsen Piters WAA, De Winter-De Groot KM, Janssens HM, Tramper-Stranders GA, Chu MLJN, et al. Concordance between upper and lower airway microbiota in infants with cystic fibrosis. Eur Respir J. 2017;49. https://doi.org/10.1183/13993003.02235-2016.
196. Boyle M. Adult cystic fibrosis. J Am Med Assoc. 2007;298:1787–93.
197. Fodor AA, Klem ER, Gilpin DF, Elborn JS, Boucher RC, Tunney MM, et al. The adult cystic fibrosis airway microbiota is stable over time and infection type, and highly resilient to antibiotic treatment of exacerbations. PLoS One. 2012;7:e45001.
198. Parkins MD, Floto RA. Emerging bacterial pathogens and changing concepts of bacterial pathogenesis in cystic fibrosis. J Cyst Fibros. 2015;14:293–304. https://doi.org/10.1016/j.jcf.2015.03.012.
199. Tracy M, Cogen J, Hoffman LR. The pediatric microbiome and the lung. Curr Opin Pediatr. 2015;27:348–55. https://doi.org/10.1097/MOP.0000000000000212.
200. Whiteson KL, Bailey B, Bergkessel M, Conrad D, Delhaes L, Felts B, et al. The upper respiratory tract as a microbial source for pulmonary infections in cystic fibrosis. Parallels from island biogeography. Am J Respir Crit Care Med. 2014;189:1309–15. https://doi.org/10.1164/rccm.201312-2129PP.
201. Burns JL, Gibson RL, McNamara S, Yim D, Emerson J, Rosenfeld M, et al. Longitudinal assessment of Pseudomonas aeruginosa in young children with cystic fibrosis. J Infect Dis. 2001;183:444–52.
202. Jelsbak L, Johansen HK, Frost A-L, Thøgersen R, Thomsen LE, Ciofu O, et al. Molecular epidemiology and dynamics of Pseudomonas aeruginosa populations in lungs of cystic fibrosis patients. Infect Immun. 2007;75:2214–24. https://doi.org/10.1128/IAI.01282-06.
203. Goss CH, Muhlebach MS. Review: *Staphylococcus aureus* and MRSA in cystic fibrosis. J Cyst Fibros. 2011;10:298–306. https://doi.org/10.1016/j.jcf.2011.06.002.
204. Tiddens HAWM, Stick SM, Davis S. Multi-modality monitoring of cystic fibrosis lung disease: the role of chest computed tomography. Paediatr Respir Rev. 2014;15:92–7. https://doi.org/10.1016/j.prrv.2013.05.003.
205. Mellert TK, Getchell ML, Sparks L, Getchell TV. Characterization of the immune barrier in human olfactory mucosa. Otolaryngol Head Neck Surg. 1992;106:181–8.
206. Kimmelman CP. Clinical review of olfaction. Am J Otolaryngol. 1993;14:227–39. https://doi.org/10.1016/0196-0709(93)90065-F.
207. Grenham S, Clarke G, Cryan JF, Dinan TG. Brain-gut-microbe communication in health and disease. Front Physiol. 2011;2:94. https://doi.org/10.3389/fphys.2011.00094.
208. Hughes DT, Sperandio V. Inter-kingdom signalling: communication between bacteria and their hosts. Nat Rev Microbiol. 2008;6:111–20. https://doi.org/10.1038/nrmicro1836.
209. Wilson MT, Hamilos DL. The nasal and sinus microbiome in health and disease. Curr Allergy Asthma Rep. 2014;14:485.
210. Wegener B-A, Croy I, Hähner A, Hummel T. Olfactory training with older people. Int J Geriatr Psychiatry. 2018;33:212–20. https://doi.org/10.1002/gps.4725.
211. Pekala K, Chandra RK, Turner JH. Efficacy of olfactory training in patients with olfactory loss: a systematic review and meta-analysis. Int Forum Allergy Rhinol. 2016;6:299–307. https://doi.org/10.1002/alr.21669.
212. Liu CM, Price LB, Hungate BA, Abraham AG, Larsen LA, Christensen K, et al. *Staphylococcus aureus* and the ecology of the nasal microbiome. Sci Adv. 2015;1. https://doi.org/10.1126/sciadv.1400216.
213. Ramakrishnan VR, Hauser LJ, Frank DN. The sinonasal bacterial microbiome in health and disease. Curr Opin Otolaryngol Head Neck Surg. 2016;24:20–5. https://doi.org/10.1097/MOO.0000000000000221.
214. Bosch AATM, Levin E, van Houten MA, Hasrat R, Kalkman G, Biesbroek G, et al. Development of upper respiratory tract microbiota in infancy is affected by mode of delivery. EBioMedicine. 2016;9:336–45. https://doi.org/10.1016/j.ebiom.2016.05.031 M4 - Citavi.
215. Lécuyer H, Audibert J, Bobigny A, Eckert C, Jannière-Nartey C, Buu-Hoï A, et al. Dolosigranulum pigrum causing nosocomial pneumonia and septicemia. J Clin Microbiol. 2007;45:3474–5. https://doi.org/10.1128/JCM.01373-07.
216. Boesveldt S, Postma EM, Boak D, Welge-Luessen A, Schöpf V, Mainland JD, et al. Anosmia—a clinical review. Chem Senses. 2017;42:513–23.
217. Hummel T, Sekinger B, Wolf SR, Pauli E, Kobal G. "Sniffin" Sticks': Olfactory performance assessed by the combined testing of odor identification, odor discrimination and olfactory threshold. 1997. https://academic.oup.com/chemse/article-abstract/22/1/39/383479. Accessed 11 Jan 2019.
218. Jain R, Hoggard M, Biswas K, Zoing M, Jiang Y, Douglas R. Changes in the bacterial microbiome of patients with chronic rhinosinusitis after endoscopic sinus surgery. Int Forum Allergy Rhinol. 2017;7:7–15. https://doi.org/10.1002/alr.21849.
219. Kern RC. Candidate's Thesis: Chronic sinusitis and anosmia: pathologic changes in the olfactory mucosa. Laryngoscope. 2009;110:1071–7.
220. Hornung DE. Nasal anatomy and the sense of smell. Adv Otorhinolaryngol. 2006;63:1–22.
221. Wiley. Bergey's Manual of Systematics of Archaea and Bacteria. 2015.
222. Mygind N, Nielsen LP, Hoffmann H-J, Shukla A, Blumberga G, Dahl R, et al. Mode of action of intranasal corticosteroids. J Allergy Clin Immunol. 2001;108:S16–25. https://doi.org/10.1067/MAI.2001.115561.
223. Rogers GB, Shaw D, Marsh RL, Carroll MP, Serisier DJ, Bruce KD. Respiratory microbiota: addressing clinical questions, informing clinical practice. Thorax. 2015;70:74–81. https://doi.org/10.1136/thoraxjnl-2014-205826.
224. Wang L-M, Qiao X-L, Ai L, Zhai J-J, Wang X-X. Isolation of antimicrobial resistant bacteria in upper respiratory tract infections of patients. 3. Biotech. 2016;6:166. https://doi.org/10.1007/s13205-016-0473-z.

225. Perl TM, Cullen JJ, Wenzel RP, Zimmerman MB, Pfaller MA, Sheppard D, et al. Intranasal mupirocin to prevent postoperative *Staphylococcus aureus* infections. N Engl J Med. 2002;346:1871–7. https://doi.org/10.1056/NEJMoa003069.
226. Bode LGM, Kluytmans JAJW, Wertheim HFL, Bogaers D, Vandenbroucke-Grauls CMJE, Roosendaal R, et al. Preventing surgical-site infections in nasal carriers of *Staphylococcus aureus*. N Engl J Med. 2010;362:9–17. https://doi.org/10.1056/NEJMoa0808939.
227. Choi KJ, Jang DW, Ellison MD, Frank-Ito DO. Characterizing airflow profile in the postoperative maxillary sinus by using computational fluid dynamics modeling: a pilot study. Am J Rhinol Allergy. 2016;30:29–36. https://doi.org/10.2500/ajra.2016.30.4266.
228. Kennedy DW. Prognostic factors, outcomes and staging in ethmoid sinus surgery. Laryngoscope. 1992;102(12 Pt 2 Suppl 57):1–18 http://www.ncbi.nlm.nih.gov/pubmed/1453856. Accessed 9 Oct 2018.
229. Hauser LJ, Ir D, Kingdom TT, Robertson CE, Frank DN, Ramakrishnan VR. Investigation of bacterial repopulation after sinus surgery and perioperative antibiotics. Int Forum Allergy Rhinol. 2016;6:34–40. https://doi.org/10.1002/alr.21630.
230. Jervis-Bardy J, Foreman A, Field J, Wormald PJ. Impaired mucosal healing and infection associated with *Staphylococcus aureus* after endoscopic sinus surgery. Am J Rhinol Allergy. 2009;23:549–52. https://doi.org/10.2500/ajra.2009.23.3366.
231. Tan NC-W, Foreman A, Jardeleza C, Douglas R, Tran H, Wormald PJ. The multiplicity of *Staphylococcus aureus* in chronic rhinosinusitis: correlating surface biofilm and intracellular residence. Laryngoscope. 2012;122:1655–60. https://doi.org/10.1002/lary.23317.
232. Plouin-Gaudon I, Clement S, Huggler E, Chaponnier C, François P, Lew D, et al. Intracellular residency is frequently associated with recurrent *Staphylococcus aureus* rhinosinusitis. Rhinology. 2006;44:249–54 http://www.ncbi.nlm.nih.gov/pubmed/17216740. Accessed 9 Oct 2018.
233. Jervis-Bardy J, Foreman A, Boase S, Valentine R, Wormald P-J. What is the origin of *Staphylococcus aureus* in the early postoperative sinonasal cavity? Int Forum Allergy Rhinol. 2011;1:308–12. https://doi.org/10.1002/alr.20050.
234. Bhattacharyya N, Gopal HV, Lee KH. Bacterial infection after endoscopic sinus surgery: a controlled prospective study. Laryngoscope. 2004;114:765–7. https://doi.org/10.1097/00005537-200404000-00032.
235. Rama S, Ballentine R, Hymes A. Science of breath: a practical guide. Honesdale: Himalayan Institute Press; 1998.
236. Bastier P-L, Lechot A, Bordenave L, Durand M, de Gabory L. Nasal irrigation: from empiricism to evidence-based medicine. A review. Eur Ann Otorhinolaryngol Head Neck Dis. 2015;132:281–5. https://doi.org/10.1016/J.ANORL.2015.08.001.
237. Georgitis JW. Nasal hyperthermia and simple irrigation for perennial rhinitis: changes in inflammatory mediators. Chest J. 1994;106:1487–92. https://doi.org/10.1378/CHEST.106.5.1487.
238. Hauser LJ, Ir D, Kingdom TT, Robertson CE, Frank DN, Ramakrishnan VR. Evaluation of bacterial transmission to the paranasal sinuses through sinus irrigation. Int Forum Allergy Rhinol. 2016;6:800–6. https://doi.org/10.1002/alr.21755.
239. Singhal D, Foreman A, Bardy J-J, Wormald P-J. Staphylococcus aureus biofilms. Laryngoscope. 2011;121:1578–83. https://doi.org/10.1002/lary.21805.
240. Psaltis AJ, Foreman A, Wormald P-J, Schlosser RJ. Contamination of sinus irrigation devices: a review of the evidence and clinical relevance. Am J Rhinol Allergy. 2012;26:201–3. https://doi.org/10.2500/ajra.2012.26.3747.
241. Tichenor WS, Thurlow J, McNulty S, Brown-Elliott BA, Wallace RJ, Falkinham JO. Nontuberculous mycobacteria in household plumbing as possible cause of chronic rhinosinusitis. Emerg Infect Dis. 2012;18:1612–7. https://doi.org/10.3201/eid1810.120164.
242. Yoder JS, Straif-Bourgeois S, Roy SL, Moore TA, Visvesvara GS, Ratard RC, et al. Primary amebic meningoencephalitis deaths associated with sinus irrigation using contaminated tap water. Clin Infect Dis. 2012;55:e79–85. https://doi.org/10.1093/cid/cis626.
243. Georas SN, Rezaee F. Epithelial barrier function: at the frontline of asthma immunology and allergic airway inflammation. J Allergy Clin Immunol. 2014;134:509–20.
244. Soyka MB, Wawrzyniak P, Eiwegger T, Holzmann D, Treis A, Wanke K, et al. Defective epithelial barrier in chronic rhinosinusitis: the regulation of tight junctions by IFN- g and IL-4. Am Acad Allergy Asthma Immunol. 2012;130:1087–96.
245. Hill C, Guarner F, Reid G, Gibson GR, Merenstein DJ, Pot B, et al. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nat Rev Gastroenterol Hepatol. 2014;11:506–14.
246. Martens K, Pugin B, De Boeck I, Spacova I, Steelant B, Seys SF, et al. Probiotics for the airways: potential to improve epithelial and immune homeostasis. Allergy. 2018. https://doi.org/10.1111/all.13495.
247. Cope EK, Lynch SV. Novel microbiome-based therapeutics for chronic rhinosinusitis. Curr Allergy Asthma Rep. 2015;15:504.
248. Mack D, Ahrne S, Hyde L, Wei S, Hollingsworth M. Extracellular MUC3 mucin secretion follows adherence of Lactobacillus strains to intestinal epithelial cells in vitro. Gut. 2003;52:827–33.
249. Rao R, Samak G. Protection and restitution of gut barrier by probiotics: nutritional and clinical implications. Curr Nutr Food Sci. 2013;9:99–107.
250. Sagar S, Vos AP, Morgan ME, Garssen J, Georgiou NA, Boon L, et al. The combination of Bifidobacterium breve with non-digestible oligosaccharides suppresses airway inflammation in a murine model for chronic asthma. Biochim Biophys Acta Mol Basis Dis. 1842;2014:573–83. https://doi.org/10.1016/j.bbadis.2014.01.005.
251. Blaser M, Bork P, Fraser C, Knight R, Wang J. The microbiome explored: recent insights and future challenges. Nat Rev Microbiol. 2013;11:213–7.
252. Zheng J, Gänzle MG, Lin XB, Ruan L, Sun M. Diversity and dynamics of bacteriocins from human microbiome. Environ Microbiol. 2015;17:2133–43.
253. Lebeer S, Vanderleyden J, De Keersmaecker S. Host interactions of probiotic bacterial surface molecules: comparison with commensals and pathogens. Nat Rev Microbiol. 2010;8:171–84.
254. Shandilya UK, Jadhav S, Panwar V, Kansal V. Probiotics: potent immunomodulatory tool against allergy. Probiotics Antimicro Prot. 2011;3:151–8.
255. von der Weid T, Bulliard C, Schiffrin EJ. Induction by a lactic acid bacterium of a population of CD4 ϱ T cells with low proliferative capacity that produce transforming growth factor beta and interleukin-10. Clin Diagn Lab Immunol. 2001;8:695–701.
256. Pochard P, Gosset P, Grangette C, Andre C, Tonnel A, Pestel J, et al. Basic and clinical immunology Lactic acid bacteria inhibit T H 2 cytokine production by mononuclear cells from allergic patients. J Allergy Clin Immunol. 2002;110:617–23.
257. Christensen HR, Frokiaer H, Pestka JJ. Lactobacilli differentially modulate expression of cytokines and maturation surface markers in murine dendritic cells. J Immunol. 2002;168:171–8.
258. Toh ZQ, Anzela A, Tang MLK, Licciardi PV. Probiotic therapy as a novel approach for allergic disease. Front Pharmacol. 2012;3:1–14.
259. Fujimura KE, Demoor T, Rauch M, Faruqi AA, Jang S, Johnson CC, et al. House dust exposure mediates gut microbiome Lactobacillus enrichment and airway immune defense against allergens and virus infection. Proc Natl Acad Sci U S A. 2014;111:805–10.
260. Kwon H, Lee C, So J, Chae C, Hwang J, Sahoo A, et al. Generation of regulatory dendritic cells and CD4+ Foxp3+ T cells by probiotics administration suppresses immune disorders. Proc Natl Acad Sci U S A. 2010;107:159–64.
261. Jang S, Kim H, Kim Y, Kang M, Kwon J, Seo J, et al. Asthma prevention by *Lactobacillus rhamnosus* in a mouse model. Allergy Asthma Immunol Res. 2012;4:150–6.
262. Marchetti G, Tincati C, Silvestri G. Microbial translocation in the pathogenesis of HIV infection and AIDS. Clin Microbiol Rev. 2013;26:2–18.
263. Cope EK, Lynch SV. Novel microbiome-based therapeutics for chronic rhinosinusitis. Curr Allergy Asthma Rep. 2015;15:9. https://doi.org/10.1007/s11882-014-0504-y.
264. Spacova I, Petrova MI, Fremau A, Pollaris L, Vanoirbeek J, Seys S, et al. Intranasal administration of probiotic *Lactobacillus rhamnosus* GG prevents birch pollen - induced allergic asthma in a murine model. Allergy. 2019;74:100–10.
265. Wu C, Chen P, Lee Y, Ko J, Lue K. Effects of immunomodulatory supplementation with *Lactobacillus rhamnosus* on airway inflammation in a mouse asthma model. J Microbiol Immunol Infect. 2016;49:625–35. https://doi.org/10.1016/j.jmii.2014.08.001.
266. Kitz R, Martens U, Zieseniß E, Enck P, Rose MA. Probiotic *E. faecalis* – adjuvant therapy in children with recurrent rhinosinusitis. Cent Eur J Med. 2012;7:7–10.
267. Pellaton C, Nutten S, Thierry A, Boudousquie C, Barbier N, Blanchard C, et al. Intragastric and intranasal administration of *Lactobacillus paracasei* NCC2461 modulates allergic airway. Int J Inflam. 2012;2012:686739.

268. Martensson A, Abolhalaj M, Lindstedt M, Martensson A, Olofsson TC, Vasquez AV, et al. Clinical efficacy of a topical lactic acid bacterial microbiome in chronic rhinosinusitis: a randomized controlled trial. Laryngoscope Investig Otolaryngol. 2017;2:410–6.
269. Vasquez A, Forsgren E, Fries I, Paxton RJ, Flaberg E, Szekely L, et al. Symbionts as major modulators of insect health: lactic acid bacteria and honeybees. PLoS One. 2012;7:e33188.
270. Butler É, Oien RF, Lindholm C, Olfonsson TC, Nilson B, Vasquez A. A pilot study investigating lactic acid bacterial symbionts from the honeybee in inhibiting human chronic wound pathogens. Int World J. 2014;13:729–38.
271. Olofsson TC, Vasquez A. Detection and identification of a novel lactic acid bacterial flora within the honey stomach of the honeybee *Apis mellifera*. Curr Microbiol. 2008;57:356–63.
272. Olofsson TC, Butler È, Markowicz P, Lindholm C, Larsson L, Vasquez A. Lactic acid bacterial symbionts in honeybees – an unknown key to honey ' s antimicrobial and therapeutic activities. Int World J. 2016;13:668–79.
273. Clarke J, Wu H, Jayasinghe L, Patel A, Reid S, Bayley H. Continuous base identification for single-molecule nanopore DNA sequencing. Nat Nanotechnol. 2009;4:265–70.
274. Eid J, Fehr A, Gray J, Luong K, Lyle J, Otto G, et al. Real-time DNA sequencing from single polymerase molecules. Science. 2009;323:133–8.
275. Earl J, Adappa N, Krol J, Bhat A, Balashov S, Ehrlich R, et al. Species-level bacterial community profiling of the healthy sinonasal microbiome using Pacific Biosciences sequencing of full-length 16S rRNA genes. Microbiome. 2018;6:190.

Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Nose Brain Axis

– The correspondence of neuroplasticity and the microbiome in human olfaction –

(*manuscript in preparation*)

Christina Kumpitsch[1], Florian Ph. S. Fischmeister[2,3,4], Sonja Lackner[5], Sandra Holasek[5], Tobias Madl[6], Veronika Schöpf[7] and Christine Moissl-Eichinger[1,4]

[1] *Diagnostic and Research Institute of Hygiene, Microbiology and Environmental Medicine, Medical University of Graz, Graz 8010, Austria*

[2] *Department of Psychology, University of Graz, Graz 8010, Austria*

[3] *Department of Biomedical Imaging and Image-guided Therapy, Medical University of Vienna, Vienna 1090, Austria*

[4] *BioTechMed, Graz, Graz 8010, Austria*

[5] *Division of Immunology and Pathophysiology, Medical University of Graz, Graz 8010, Austria*

[6] *Gottfried Schatz Research Center for Cell Signaling, Metabolism and Aging, Molecular Biology & Biochemistry, Medical University of Graz, Graz 8010, Austria*

[7] *Present address: Medical University Vienna, Spitalgasse 23, 1090 Vienna, Austria.*

Background:

The loss of smell is an incisive event. It is often associated with viral or inflammatory diseases of the nasal cavity (e.g., common cold, COVID-19 or CRS) but also neurological diseases like Parkinson's disease as well as the process of aging are able to cause dysosmia. Up to 20% of the general population suffers from olfactory dysfunction with rising tendency due to the COVID-19 pandemic [1–4]. This impaired olfactory ability affects the social (e.g., personal hygiene, sexual life), emotional (e.g., increased depression rate) and behavioural performance (e.g., dietary changes) and thereof decreases the quality of life tremendously. Together with the safety aspect of olfaction via the detection of hazards such as gas or spoiled food, this highlights the underestimated importance of the sense of smell. Moreover, olfactory loss is one of the predictors for 5-years mortality in elderly people [5–7]. Up to date, no "satisfying" therapy recommendations on the loss of smell have been made. However, several studies show the beneficial effects of smell training on olfactory performance [8,9].

In general, the sense of smell (olfaction) is mediated by olfactory receptor cells. These cells are located at the ceiling of the nasal cavity – the olfactory epithelium – and their axons reach

directly through the perforated cribriform plate to the olfactory bulb in the brain. The olfactory cilia at their apical surfaces intrude the mucus layer that also lines the olfactory epithelium [10].

Like other parts of the human body, the olfactory epithelium is colonised by a distinct microbial community. Microbes throughout the human body have been shown to communicate with human body tissues e.g., via short chain fatty acids (SCFA) [11,12]. Olfactory receptor cells are also able to detect microbial produced SCFAs [13]. In our pilot study we were able to detected differences of the nasal microbiome when comparing normosmic and dysosmic volunteers [14]. In this new study, we did the next step and screened the microbiome of participants with varying olfactory performance at over three time points, each three months apart, with olfactory training periods in between. Furthermore, we want to connect this information with nutritional intake and changes in brain structure and function.

Hypothesis:

The nasal microbiome located at the olfactory epithelium does not just play an important role in olfactory functioning but also contributes to the regain of olfaction following smell training. This change in microbiome and olfactory function can also be observed in the reorganization of olfaction related brain areas.

Study design and methods:

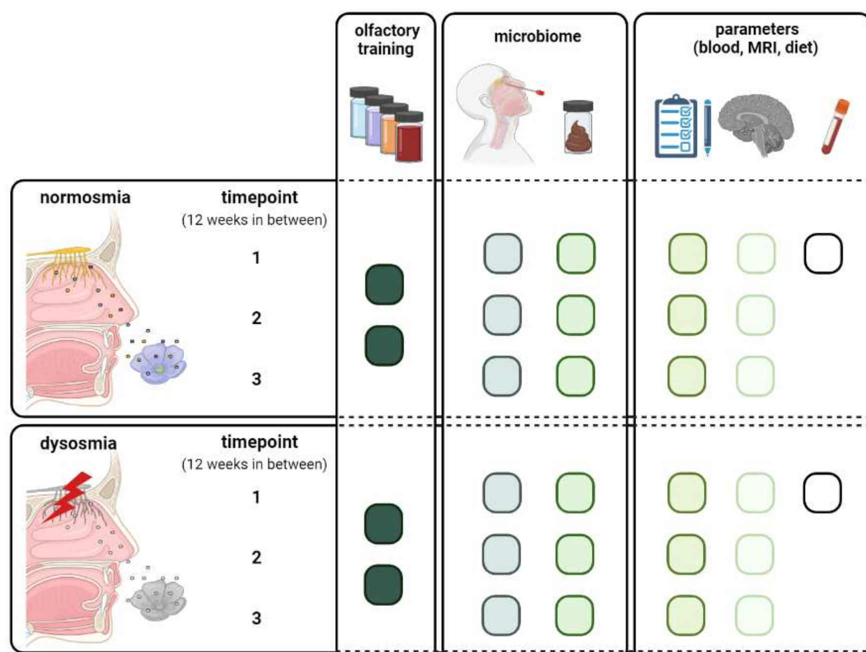


Figure 1. Study schemata of the "Nose-Brain Axis"-Study. Created with BioRender.com

53 participants with normal (good and weak normosmics; n=33) and bad (dysosmics: hyposmics and anosmics; n=20) olfactory performance, respectively, were recruited. In general, all (53/53) of them provided nasal and stool samples at time point 1 (TP), 32/53 at TP2 and 15/53 at TP3. All samples were used for 16S rRNA gene sequencing. Samples of participants that provided microbial samples at all three time points (six of each group) were further analysed (metagenomics, metatranscriptomics and metabolomics). Additionally, information about nutritional intake (food frequency questionnaire) was captured and fMRI scans (brain imaging) were performed at every time point. The design of this study is illustrated in ***Figure 1***.

Aim:

Step 1: Assessing general differences in the microbiome of normosmics and dysosmics to find putative biomarkers in nasal as well as stool samples. Furthermore, correlations between microbial measures and nutritional intake should be performed.

Step 2: Investigation of longitudinal changes (over six months) in the microbial community based on olfactory training.

Step 3: Correlation of microbial parameters with neuroimaging biomarkers to further investigate the interaction of the nasal microbiome with olfactory performance.

Preliminary results:

Preliminary evaluation of the nasal microbiome at TP1 revealed a nearly stepwise decrease of alpha diversity (Shannon index) with improving olfactory performance mainly driven by microbial richness. Additionally, microbial signatures belonging to *Rickettsia*, *Faecalibacterium, Campylobacter* and *Porphyromonas* have been associated with impaired sense of smell whereas *Ralstonia*, or Micrococcaceae were more abundant in volunteers with normal olfactory performance.

Initial analyses of functional imaging data revealed that dysosmic participants show only marginal activity within the primary olfactory brain areas, i.e. the piriform cortex at baseline as compared to the healthy normosmic controls. This reduced functional activitiy is also found in a decreased connectivity within and between olfactory related regions. Chemesthetic stimulation with CO_2 and peppermint flavour did not yield any significance. Analysis of the other timepoints is pending.

Additionally, correlations with the main ingested food groups indicated that normosmics ingested significantly higher levels of fruits and legumes and significantly lower levels of fish, meat and sausages compared to dysosmics. If these nutritional values (as well as the neurological biomarkers) are connected with the microbial community in the nasal and stool samples of our participants, still needs to be analysed.

Preliminary discussion:

Consistent with our completed pilot study of Koskinen et al., 2018 [14], we are able to show differences in the microbial community of the olfactory epithelium. Some bacterial taxa and nutritional values are significantly associated with the olfactory performance of the subjects so far. Dysosmics show higher relative abundances of the butyrate-producers *Faecalibacterium* and *Porphyromonas* at the olfactory mucosa suggesting an adverse effect of this microbial product on olfaction. Furthermore, normosmics seem to have a healthier diet by ingesting higher amounts of food groups belonging to fruits and legumes and reduced intake of meat and sausages. It is likely that dietary habits/preferences are influenced by olfactory capability since olfaction contributes to the sense of taste and dysosmic people often report a reduced enjoyment of food [15]. A possible impact of alterations in the diet or the olfactory performance, in general, on the gut microbiome (or vice versa) has still to be investigated.

Further analysis of these data will help to understand the microbial role in olfaction and possibly allow monitoring and predicting smell therapy success in future.

References:

1. Boesveldt S, Postma EM, Boak D, Welge-Luessen A, Schöpf V, Mainland JD, et al. Anosmia—A Clinical Review. Chem Senses. 2017;42(7):513–23.
2. Lechien JR, Hopkins C, Saussez S. Sniffing out the evidence; it's now time for public health bodies recognize the link between COVID-19 and smell and taste disturbance. Vol. 58, Rhinology. 2020.
3. Hummel T, Whitcroft KL, Andrews P, Altundag A, Cinghi C, Costanzo RM, et al. Position paper on olfactory dysfunction. Rhinology. 2017;54.
4. Cavaco S, Gonçalves A, Mendes A, Vila-Chã N, Moreira I, Fernandes J, et al. Abnormal Olfaction in Parkinson's Disease Is Related to Faster Disease Progression. Behav Neurol. 2015;2015.
5. Devanand DP, Lee S, Manly J, Andrews H, Schupf N, Masurkar A, et al. Olfactory identification deficits and increased mortality in the community. Ann Neurol. 2015;78(3).
6. Pinto JM, Wroblewski KE, Kern DW, Schumm LP, McClintock MK. Olfactory dysfunction

predicts 5-year mortality in older adults. PLoS One. 2014;9(10).

7. Croy I, Nordin S, Hummel T. Olfactory disorders and quality of life-an updated review. Chem Senses. 2014;39(3).

8. Geißler K, Reimann H, Gudziol H, Bitter T, Guntinas-Lichius O. Olfactory training for patients with olfactory loss after upper respiratory tract infections. Eur Arch Oto-Rhino-Laryngology. 2014;271(6).

9. Konstantinidis I, Tsakiropoulou E, Constantinidis J. Long term effects of olfactory training in patients with post-infectious olfactory loss. Rhinol J. 2016;54(2).

10. Schiffman SS. Smell and Taste. In: Encyclopedia of Gerontology. Elsevier; 2007. p. 515–25.

11. Grenham S, Clarke G, Cryan JF, Dinan TG. Brain-gut-microbe communication in health and disease. Front Physiol. 2011;2:94. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3232439

12. Plöger S, Stumpff F, Penner GB, Schulzke JD, Gäbel G, Martens H, et al. Microbial butyrate and its role for barrier function in the gastrointestinal tract. Ann N Y Acad Sci. 2012;1258(1).

13. JL P, RJ P, H G, Z P, A S, J H, et al. Olfactory receptor responding to gut microbiota-derived signals plays a role in renin secretion and blood pressure regulation. Proc Natl Acad Sci U S A. 2013 Mar 12;110(11):4410–5. Available from: https://pubmed.ncbi.nlm.nih.gov/23401498/

14. Koskinen K, Reichert JL, Hoier S, Schachenreiter J, Duller S, Moissl-Eichinger C, et al. The nasal microbiome mirrors and potentially shapes olfactory function. Sci Rep. 2018;8(1):1–11.

15. Pellegrino R, Hummel T, Emrich R, Chandra R, Turner J, Trone T, et al. Cultural determinants of food attitudes in anosmic patients. Appetite. 2020;147.

Reduced B12 uptake and increased gastrointestinal formate drive archaeome-mediated breath methane emission in humans

Short title: B12 shortage, fibre and formate drive breath methane emission

Christina Kumpitsch,[1] Florian Ph. S. Fischmeister,[2,3] Alexander Mahnert,[1] Sonja Lackner,[4] Marilena Wilding,[2] Corina Sturm,[2] Anna Springer,[5] Tobias Madl,[5,6] Sandra Holasek,[4] Christoph Högenauer,[7] Ivan Berg,[8] Veronika Schoepf,[3] Christine Moissl-Eichinger[1,6*]

[1] Diagnostic and Research Institute of Hygiene, Microbiology and Environmental Medicine, Medical University of Graz, Graz 8010, Austria,

[2] Department of Psychology, University of Graz, Graz 8010, Austria,

[3] Department of Biomedical Imaging and Image-guided Therapy, Medical University of Vienna, Vienna 1090, Austria,

[4] Division of Immunology and Pathophysiology, Medical University of Graz, Graz 8010, Austria,

[5] Gottfried Schatz Research Center for Cell Signaling, Metabolism and Aging, Molecular Biology & Biochemistry, Medical University of Graz, Graz 8010, Austria,

[6] BioTechMed, Graz, Graz 8010, Austria,

[7] Division of Gastroenterology and Hepatology, Medical University of Graz, Graz, Austria,

[8] Institute for Molecular Microbiology and Biotechnology, University of Münster, Münster, Germany.

* Correspondence: Christine Moissl-Eichinger, Medical University of Graz, Diagnostic and Research Institute of Hygiene, Microbiology and Environmental Medicine, Neue Stiftingtalstraße 6, 8010 Graz, Austria, christine.moissl-eichinger@medunigraz.at

ABSTRACT

Background

Methane is an end product of microbial fermentation in the human gastrointestinal tract. This gas is solely produced by an archaeal subpopulation of the human microbiome. Increased methane production has been associated with abdominal pain, bloating, constipation, IBD, CRC or other conditions. Twenty percent of the (healthy) Western populations innately exhale substantially higher amounts (>5 ppm) of this gas. The underlying principle for differential methane emission and its effect on human health was still not sufficiently understood.

Results

We assessed the breath methane content, gastrointestinal microbiome, metagenome, metabolome, and eating behavior of one-hundred healthy young adults (female: n = 52, male: n = 48; mean age =24.1). On the basis of the amount of methane emitted, participants were grouped into high methane emitters (CH_4 breath content 5-75 ppm) and low emitters (CH_4 < 5 ppm).

The microbiomes of high methane emitters were characterized by a 1000-fold increase in *Methanobrevibacter smithii*. This archaeon co-occurred with a bacterial community specialized on dietary fibre degradation, which included members of Ruminococcaceae and Christensenellaceae. As confirmed by metagenomics and metabolomics, the biology of high methane producers was further characterized by increased formate and acetate levels in the gut. These metabolites were strongly correlated with dietary habits, such as vitamin, fat and fibre intake, host genetics, and microbiome function, altogether driving archaeal methanogenesis.

Conclusions

This study enlightens the complex, multi-level interplay of host diet, genetics and microbiome composition/function leading to two fundamentally different gastrointestinal phenotypes and identifies novel points of therapeutic action in methane-associated disorders.

Keywords: archaeome, microbiome, methanogens, methane, gut, gastrointestinal tract, metabolome, metagenome, *Methanobrevibacter*, Christensenellaceae

Background

Methane is the metabolic end-product of a non-bacterial sub-population of the gastrointestinal microbiome, namely the archaeome [1]. Although methane is not utilized by the human itself, elevated methane levels, measured in breath, have been linked with small intestinal bacterial overgrowth, colorectal cancer, diverticulosis and other gastrointestinal disorders (summarized in [2]). While its role as a gasotransmitter is controversially discussed [3], methane is a causally linked to a slowed gastrointestinal motility (transit time slowed down by up to 59%), probably caused by the direct action of methane on the cholinergic pathway of the enteric nervous system [4].

Methane-forming archaea ("methanogens") in the gastrointestinal tract (GIT) were first observed long ago - through the detection of methane in the human breath and flatus (see also [5,6]). Although not a single pathogenic archaeal representative has been identified, human-associated archaea are widespread in the GIT as well as other body sites (e.g. skin, respiratory tract) [1,7,8]. The role of methanogens *per se* in health and disease is not yet clear, and analyses suffer from methodological pitfalls to correctly detect and characterize the human archaeome as well as the contradictory information that appears in the literature (reviewed in [1]).

Although the average abundance of archaea in human fecal samples is low as compared to bacteria [1], methanogens are considered to represent key-stone species in the GIT. By maintaining numerous syntrophic relationships with bacteria, methanogens control the efficiency of the bacterial primary and secondary fermentation of complex organic molecules. By consuming by-products of bacterial metabolism (H_2, CO_2, formate, methyl-compounds, acetate), they particularly keep the hydrogen concentration low, which would inhibit the fermentation activity and reduce the overall energy yield [1]. In the human GIT, methanogens are mainly represented by the Methanobacteriales (*M. smithii, Methanosphaera stadtmanae*) and Methanomassiliicoccales (*Ca.* Methanomassiliicoccus and *Ca.* Methanomethylophilus representatives).

On average, 0.35 l of methane are produced per day per individual [9]; however, a substantial proportion of the human population (approx. 20% of the Western adult population) has been shown to emit methane in concentrations above 5 ppm, measured in breath, whereas the remaining population emits methane in concentrations close to or below the detection limit (for details see [1]). The reason for this split in methane producers and non-producers is largely unclear to date.

In this publication, we identify the driving forces supporting methane emission through breath based on the systematic comparison of high methane-emitting young subjects vs. low-emitters with respect to diet, GIT microbiome and archaeome (amplicon- and metagenome-based analyses), and metabolome.

METHODS

All key resources and PCR conditions are listed in the Supplementary Methods file.

Subject details

One-hundred participants between 18-37 years were recruited at the University of Graz. Following exclusion criteria were set: smoker, left-handers, intake of antibiotics and probiotics within the last 3 months before sampling and neurological, psychiatric or internal diseases. The study was evaluated and approved according to the Declaration of Helsinki by the local ethics committee of the University of Graz (EK-Nr. GZ. 39/44/63 ex 2017/18). Before participation, all participants signed an informed consent.

Methane measurement

All volunteers were asked to inhale deeply through the nose and hold their breath for 15 s before complete exhalation into the GastroCH_4ECK breath bags (Bedfont Scientific Ltd, UK) via the mouth. Breath was collected on the same day as the stool sample in the morning before brushing their teeth and eating breakfast. Methane in the breath was measured by GastroCH_4ECK Gastrolyzer (Bedfont Scientific Ltd, UK). Participants with CH_4 values above 5 ppm were stated as methane producers. With these measurements 15% of the study group (n=15) were classified as high methane emitters (Ch_4 value ≥ 5ppm).

Matched subset (n=30)

15 high methane emitters were matched to 15 low methane emitters by sex, age, hormonal contraception, and vegetarianism (Supplementary Table 2). All other participants were excluded in this subset.

Nutritional Assessment

Dietary habits and food intake information of the 4 weeks before the investigation were collected by a validated food frequency questionnaire ("German Food Frequency Questionnaire (FFG)" of the Robert Koch Institute) [10]. The diet's nutritive composition (e.g. intake of fat, protein, magnesium, zinc, etc) and dietary diversity indices were analyzed by a specific nutrition software using food and nutritive values specific for Austria [11].

Sample collection, DNA extraction and amplicon sequencing

Collection and PMA treatment

Stool samples were collected of every participant. To make sure that we analyse intact cells, a 10% stool suspension with 0.9% sodium chloride was treated with propidium monoazide (PMA) solution to

mask freely accessible DNA. During PMA treatment, all steps were performed in the dark. PMA solution (final concentration: 50 uM) was added to the stool samples. Samples were vortexed briefly, incubated for 10 min on a shaker and 15 min in a PMA-Lite™ LED Photolysis Device (Biotum) afterwards. Samples were stored at -20 °C until further use.

DNA extraction

PMA-treated stool samples were used to extract microbial genomic DNA by using the DNeasy PowerSoil Kit (QIAGEN, USA) according to manufacturer's protocol. Only modification was the use of MagNaLyser at 6500 rpm for 2 times 30 s instead of vortexing the samples. DNA concentration of extracted DNA was quantified via Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, USA).

16S rRNA gene-based next generation sequencing (NGS) and sequence data processing

To determine the bacterial microbial diversity the variable region V4 of 16S rRNA gene was amplified using universal PCR primers 515FB and 806RB. For the archaeal set up a nested PCR approach was used, using the primer pair 344F and 1041R at the first and 519F and 806R for the second PCR. For detailed protocol and primer sequences see [12]. Each PCR reaction was performed in triplicates. Triplicates were pooled after visualization in 3 % agarose gel. Fragments were sequenced using the Illumina MiSeq sequencing platform (Illumina, Eindhoven, the Netherlands) performed in cooperation with the Core Facility for Molecular Biology of the Center for Medical Research in Graz [13].

Raw reads were analyzed with QIIME2 (Quantitative Insights Into Microbial Ecology) version 2019.1 using DADA2 (Divisive Amplicon Denoising Algorithm) to denoise sequences [14,15]. Briefly, paired end reads were joined together before a quality check of the produced sequences was performed. Afterwards, taxonomic assignment was determined with SILVA v128 (universal approach), and SILVA v132 (archaeal approach) [16] as a reference database for a Naïve-Bayes classifier [17]. For phylogenetic metrics and analysis a rooted tree was generated with FastTree 2 [18].

LEfSe (LDA Effect Size) [19] was used to identify genomic features characterizing the differences between two given conditions. In our case, the LEfSe tool was integrated in a user-friendly Galaxy set-up provided by the Core Facility Computational Biology at the Medical University of Graz. The cladogram was created by the "Plot Cladogram" function, and further-on optimized using Inkscape (inkscape.org).

Controls

Extraction blanks and PCR negative controls were processed in parallel. All controls were removed using the R package decontam [20] with the prevalence method and threshold set to 0.5 (https://github.com/benjjneb/decontam). Unassigned sequences mitochondrial and chloroplast signatures as well as features with zero or only one read were also removed. Remaining RSV tables

(Supplementary Dataset 1-3) were processed in Calypso [21] to generate RDA, Shannon, PCoA, ANOVA plots as well as networks and correlation analysis.

BioEnv

R Studio version 1.2.1335 (2018-07-02) and R package vegan 2.5-5 [22] was used to generate a BioEnv diagram with environmental variables (dietary information, CH4 emission, ..) with maximum correlation with microbial community dissimilarities.

Metagenome Analysis

Shotgun metagenome sequencing

200 ng extracted DNA (PMA treated) of each of the 30 matched samples was sent for sequencing to Macrogen (Seoul, South Korea). Library was extracted via Nextera XT Library construction kit (Illumina, Eindhoven, the Netherlands) and sequenced using Illumina HiSeq technique (Illumina, Eindhoven, the Netherlands).

Metagenomics analysis via MG-Rast

Raw data was quality controlled, sequences were paired and analyzed with the open-submission data portal MG-Rast platform (server running version 4.0.3.) [23]. Features with zero or one read were removed before feature tables (RefSeq and SEED) were uploaded in Calypso [21].

Metagenome assembled genomes (MAGs)

After checking quality with fastqc (v0.11.8) [24], raw shotgun reads were filtered accordingly with trimmomatic (v0.38) [25] by using a minimal length of 50 bp and a Phred quality score of 20 in a sliding window of 5 bp. Quality filtered sequences were then mapped against the human chromosome hg19 with bowtie2 (v2.3.5) [26] to remove sequences of the human host by retaining all unmapped reads with samtools (v1.9, settings: -b -f 12 -F 256) [27]. Host removed forward and reverse fastq files were then extracted from sorted bam files with bedtools (v2.29.0) [28]. Reads were then analyzed in a gene and genome-centric manner. For the gene-centric analysis, host removed quality filtered reads were annotated by blastX searches against the NCBInr database (release of Sep. 9th 2020) using diamond (v0.9.25) [29]. Resulting m8 files were then visualized in MEGAN (v6.20.13) [30]. For the genome-centric analysis host removed quality filtered reads were co-assembled in Megahit (v1.1.3) [31] by using the preset meta-sensitive. Resulting contigs were binned with MaxBin v2.2.4 [32]. Further on, bins were quality scored (based on CheckM [33] estimates for completeness, contamination and strain heterogeneity as well as N50 based assembly continuity) and de-replicated to pick representative MAGs (metagenome assembled genomes) with dRep (v2.0.5) [34]. Quality MAGs were then classified with GTDBtk (v1.2.0) [35]. Identified key MAGs were further annotated and analyzed including gene synteny in MaGe [36]. Finally, replication rates were determined with iRep (v1.1.9) [37].

Prediction model, supervised metadata classifications and regressions

Raw metagenome data was used to create prediction models in QIIME2 [38]. The q2-sample-classifier-plugin (*28*) was used to predict high and low methane emitters from feature table compositions. To determine accuracy by comparing predicted values the data set was randomly split by 5 into a training set (⅘) and a test set (⅕). The training set was used for the learning model including settings for optimized feature-selection, parameter tuning and K-fold cross validation based on RandomForest. The resulting sample estimator (trained classification model) was also used to predict methane emissions between the shotgun (RefSeqs) and amplicon dataset.

Krona charts

Datasets (amplicon and metagenome) were normalized and Krona chart templates [39] were used to visualize the differences between HE and LE.

Metabolic quantification using NMR

Nuclear magnetic resonance spectroscopy (NMR) analysis was used to analyze concentrations of acetate, succinate, formate, lactate, butyrate and propionate in stool samples (PMA untreated) performed at the Gottfried Schatz Research Center for Cell Signaling, Metabolism and Aging, Molecular Biology and Biochemistry, Medical University of Graz. To quench enzymatic reactions and remove proteins, methanol-water solution was added to the stool sample (2:1), cells were lysed using a Precellys homogenizer and stored at -20°C for 1 hour until further processing. Samples were centrifuged (4°C, 30 min, 17949 rcf) and supernatants were lyophilized afterwards. Samples were then mixed with 500 µl NMR buffer in D_2O (0.08 M Na_2HPO_4, 5 mM 3-(trimethylsilyl) propionic acid-2,2,3,3-d_4 sodium salt (TSP), 0.04 (w/v) % NaN_3 in D_2O, pH adjusted to 7.4 with 8 M HCl and 5 M NaOH) and transferred into 5 mm NMR tubes. NMR was performed on an AVANCE™ Neo Bruker Ultrashield 600 MHz spectrometer equipped with a TXI probe head at 310 K and processed as described elsewhere [40].

The 1D CPMG (Carr-Purcell_Meiboom_Gill) pulse sequence (cpmgpr1d, 512 scans, 73728 points in F1, 11904.76 HZ spectral width, 512 transients, recycle delays 4 s) with water suppression using pre-saturation, was used for ^1H 1D NMR experiments. Bruker Topspin version 4.0.2 was used for NMR data acquisition. The spectra for all samples were automatically processed (exponential line broadening of 0.3 Hz), phased, and referenced using TSP at 0.0 ppm using Bruker Topspin 4.0.2 software (Bruker GmbH, Rheinstetten, Germany).

Spectra pre-processing and data analysis have been carried out using the state-of-the-art data analysis pipeline (group of Prof. Jeremy Nicholson at Imperials College London) using Matlab® scripts and MetaboAnalyst 4.0 [41]. NMR data were imported to Matlab® vR2014a (Mathworks, Natick,

Massachusetts, United States), regions around the water, TSP, and remaining methanol signals excluded, and to correct for sample metabolite dilution probabilistic quotient normalization [42] was performed.

Stated concentrations correspond to normalized concentrations after probabilistic quotient normalization.

Metabolic predictions

Potential metabolites were predicted with the q2-micom plugin (v. 0.8.0, [43]. All analysis were conducted with the AGORA genus model database (v1.03) [44] and covered the entire dataset (n=100) and the matched dataset (n=30) as well as all and selected key features. In addition, the standard western diet gut medium was adapted (with provided jupyter notebooks from the developers) according to measured nutrients to provide a per sample diet model as well. No abundance cutoff was used for all and selected features. In addition, a leave one out strategy was included for selected features to determine the behaviour of the established metabolic models in absence of a potential microbial key-player. The growth simulation was performed with individual settings for the tradeoff between community growth rate and individual taxon growth rate. This pressure to the model was determined by an evaluation of the tradeoff from 0-1 (zero to maximum enforced growth) and was set between 0.1 and 0.7 accordingly (all features and selected features respectively). Resulting growth rates could be partly verified with calculated replication rates using iRep of representative key MAGs. Subsequent visualizations and analysis included potential metabolite consumptions, growth niches, and metabolite fluxes in dependence of measured methane emissions. Finally, a minimal medium was determined for selected key features of matched samples.

Quantification and Statistical Analysis

Statistical tests (Spearman rho's and Pearson's correlation) were performed using IBM SPSS Amos version 26. Different parameters were checked for normal distribution. Correlations were calculated based on distribution of the compared parameters via Spearman's rho and Pearson's correlation, respectively. In the manuscript, non-corrected p values were used to describe specific trends, however Bonferroni corrected p values can be found in Supplementary Table 5.

Data and Software Availability

Raw sequencing data obtained from amplicon-based sequencing and metagenomics sequencing data (technical sequences including adaptor sequences, linker sequences and barcode sequences as well as human reads were removed) used in this paper can be found in the European Nucleotide Archive (ENA): PRJEB41867. Supplementary Datasets (after decontam and removal of features with zero and

one reads) and all Supplementary Figures, Tables and Items were deposited on Mendeley at http://dx.doi.org/10.17632/hjj3tx7n84.1.

RESULTS

Study overview

In total, 100 participants (female: *n* = 52, male: *n* = 48; mean age =24.1) were recruited in this study. Metadata information (sex, age, vegetarian yes/no, contraception yes/no, breath methane content as well as metabolite information) of all participants is provided in Supplementary Table 1. All participants provided one stool sample, one breath sample for methane measurements, and a completed dietary questionnaire. Based on the amount of methane emitted, participants were grouped into high methane emitters (HE; CH_4 value: 5-75 ppm) and low emitters (LE; CH_4 value < 5 ppm). Fifteen percent of the participants were categorized as HEs (Supplementary Table 2), with the percentage in congruence with known levels of methane emission of young adult European cohorts [9]. For specific scientific questions, 15 high methane emitters were matched to 15 low methane emitters by sex, age, hormonal contraception, and vegetarianism (Supplementary Table 2; n=30).

The following data sets were obtained: "universal" and archaeal 16S rRNA gene profiles for all stool samples, a metagenomics dataset as well as metabolomic information (e.g. acetate, succinate, formate), and detailed dietary information (e.g. diversity, energy, protein, fat, carbohydrates) from matched participants (Supplementary Datasets 1-4; Supplementary Tables 1-2).

High-methane microbiomes are characterized by a specific microbial community and a 1,000-fold increase in *Methanobrevibacter* signatures

The microbiomes of high-methane emitting subjects (HEs) were characterized by significantly higher alpha diversity (Figure 1A.I) and a substantially different microbiome composition, compared to low-methane emitting persons (LEs) (Supplementary Figure 1). Although the HE microbial profiles did not group separately in the PCoA plot (Supplementary Figure 2A.I), methane production had a significant impact on the microbiome composition (Figure 1A.II). Methane emitting microbiomes were significantly associated with Euryarchaeota (*Methanobrevibacter*) and signatures of Christensenellaceae R7 group, which formed a stable network with different *Ruminococcus*/Ruminococcaceae, *Holdemanella*, and the *Eubacterium ruminantium* group. On the contrary, LEs were characterized by a predominance of Bacteroidetes, and a stable network of *Bacteroides, Lachnoclostridium, Sutterella, Flavonifractor, Blautia,* and *Anaerostipes* (Figure 1.B-C,2; Supplementary Figure 2-4). A Krona Chart overview of the taxonomic composition of HE and LE

samples is provided in Supplementary Item 1, displaying the 1,000 fold increase of relative abundance of *Methanobrevibacter* signatures in HE (HE: 2%, LE: 0.002%; for comparison: *Bacteroides* (HE: 19%, LE: 28%), Christensenellaceae R7 group (HE: 6%, LE 2%), *Ruminococcaceae UCGs* (HE: 22%, LE: 20%)). Notably, methane emission and the associated increase of *Methanobrevibacter* signatures were solely driven by a single *M. smithii* ribosomal sequence variant (RSV; Supplementary Dataset 2). Besides that, the archaeal communities of HEs and LEs were not significantly different with respect to their alpha or beta diversity (Figure 3). Samples from HEs did not contain any archaeal signatures apart from the Euryarchaeota, i.e. *Methanobrevibacter* and *Methanosphaera*. In the entire dataset, 21 *Methanobrevibacter* RSVs were observed, whereas *Methanosphaera* was represented by only two RSVs (both genera are represented by one RSV each in the universal dataset).

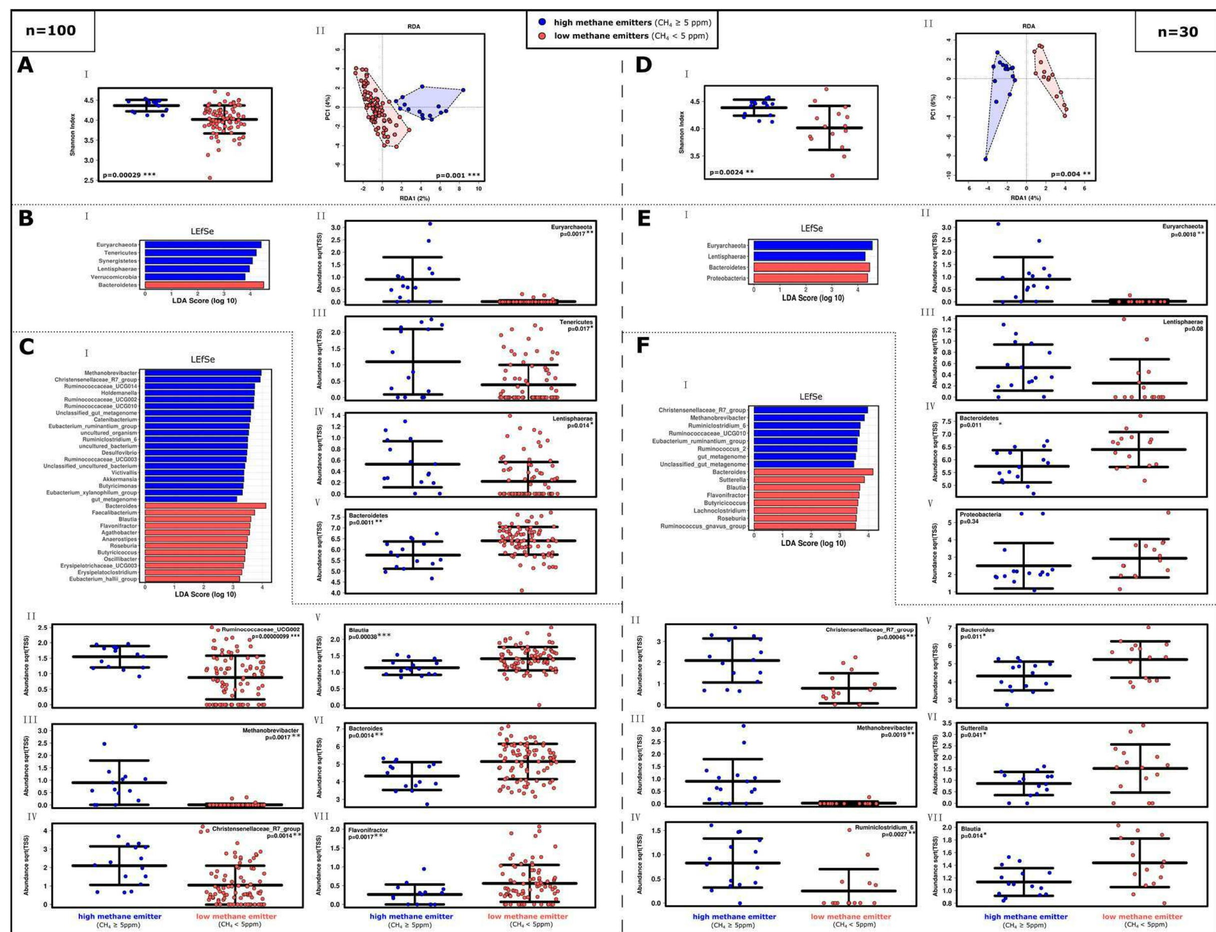


Figure 1. Differences in alpha and beta diversity based on the "universal" approach of 16S rRNA gene sequencing between high (HE) and low methane emitters (LE). A-C. Profiles of the whole study cohort (n=100). **D-F**. Profiles of the matched subset only (n=30). **A.I/D.I.** An examination of Shannon diversity index revealed significant differences in alpha diversity (RSV (ribosomal sequence variants) based; analysis of variance, ANOVA). **A.II/D.II.** The microbiome of HEs clustered significantly differently in the RDA plot (RSV based). **B.I/E.I.** LEfSe (Linear Discriminant Analysis Effect Size) analysis of the 100 most abundant phyla and **B.II/E.II-B.V/E.V.** Relative abundance of selected phyla in ANOVA plots. **C.I/F.I.** LEfSe analysis of the 100 most abundant genera and **C.II/F.II-C.VII/F.VII.** ANOVA plots of selected genera.

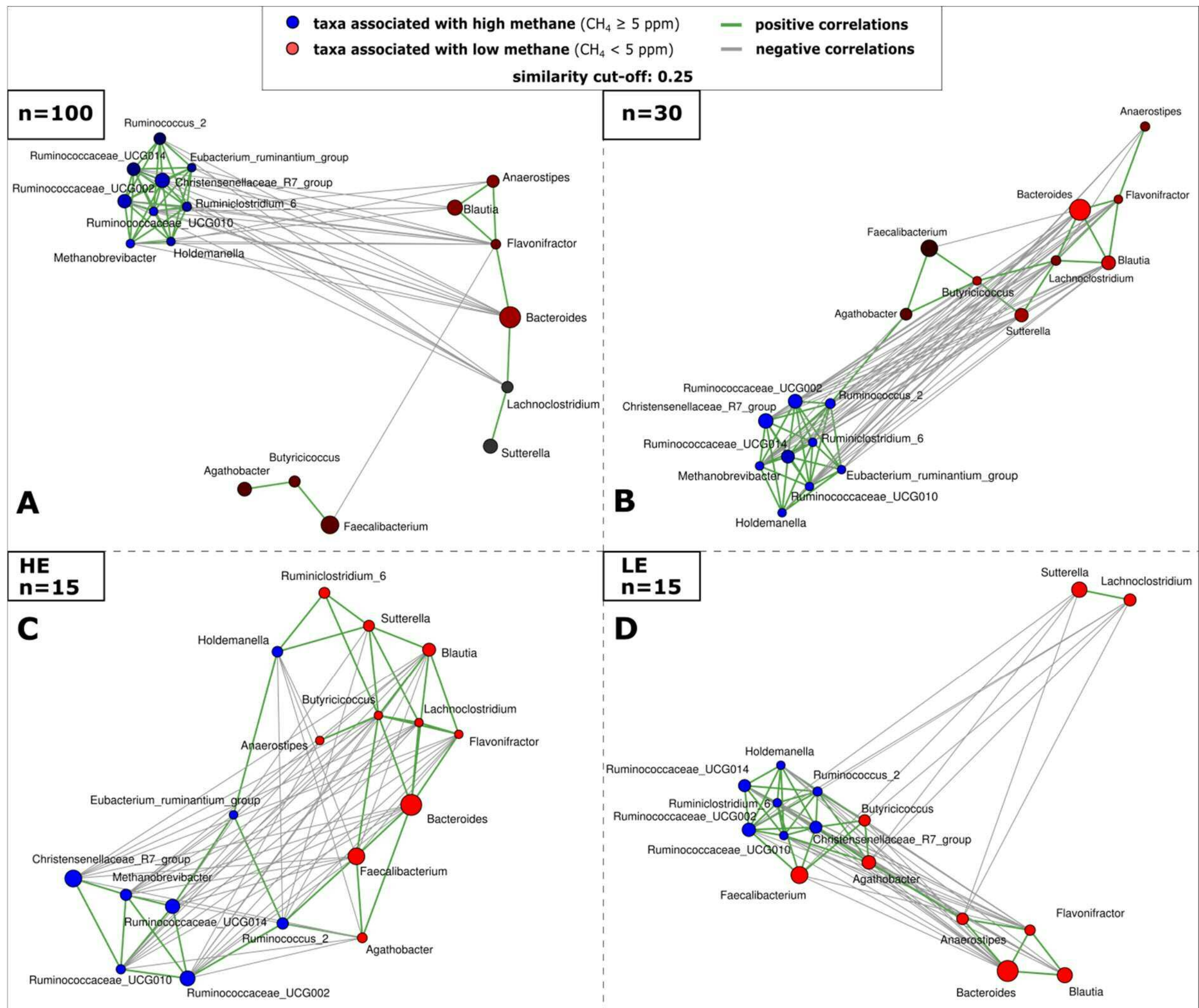


Figure 2. Co-occurrence networks based on Spearman's rho correlation of selected genera in HE and LE microbiome samples. Taxa were selected based on significantly different relative abundances in both sample types and LEfSe analyses. Left, upper panel: Whole study cohort (n=100), right, upper panel: matched study subset (n=30). Lower panels show co-occurrence patterns in the HE (left) or the LE samples (right).

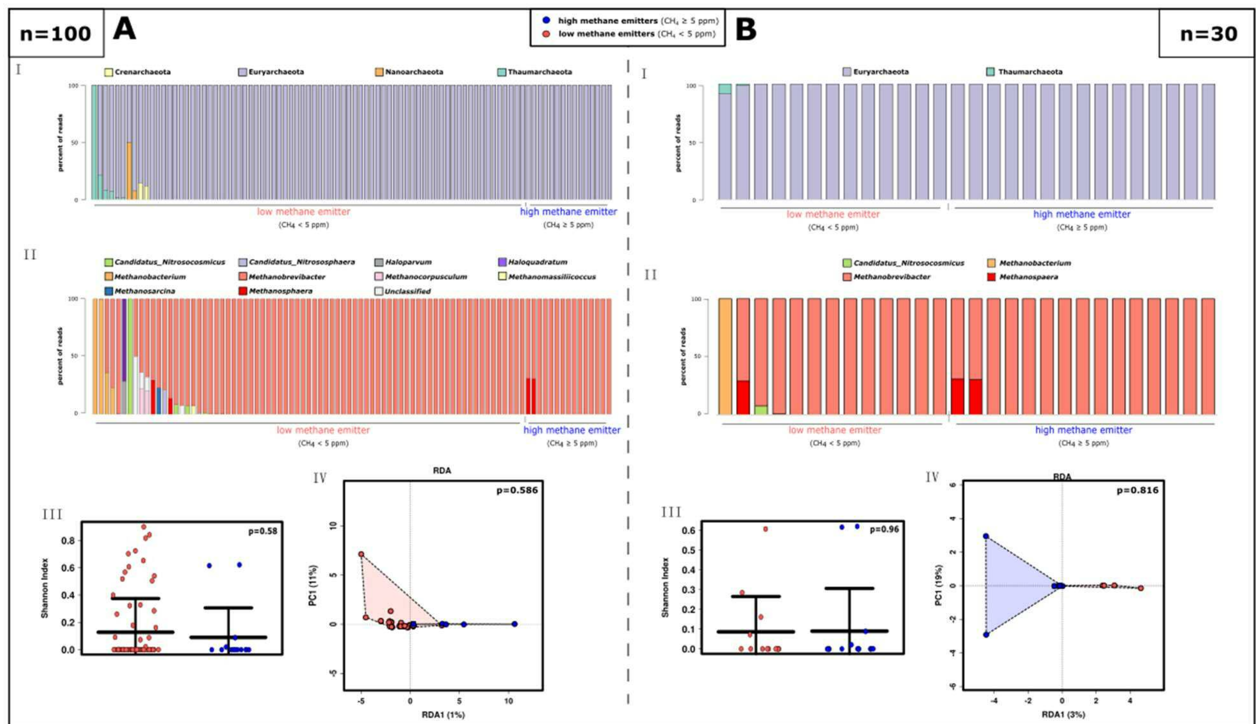


Figure 3. Archaeome profile of HE and LE samples, based on the "archaeal" approach of 16S rRNA gene sequencing. A. Profile of the whole study cohort (n=100). **B.** Matched study subset (n=30). **I.** Bar chart of the 20 most abundant taxa compared regarding their low or high methane emission at the phylum level and **II.** at the genus level. **III.** Shannon diversity, and **IV.** RDA plot at RSV level.

The microbiome profile of the matched study subset (n=30) was highly similar to the profiles revealed for the non-matched volunteers, and the same characteristics, with respect to microbiome composition, alpha diversity, co-occurrences etc. was observed (Supplementary Figure 1B; Supplementary Dataset 1; Figure 1D, E; Supplementary Figure 2B; Fig. 2; Fig. 3B).

HE microbiomes are specialized on C1-C3 compound turnover

The functional analysis of the metagenomics dataset was based on 14,616,890 sequences, which were categorized into 28 SEED subsystems and contained 6,956 actual function assignments and 6,589 unique features. An overview on the detected functions is available in Supplementary Item 3 and Supplementary Figure 5 (Supplementary Dataset 3). Like the profile information derived from 16S rRNA gene data, the diversity of unique functions was significantly higher in HEs as compared to LEs (Figure 4A). The impact of methane emission on the overall functions was also found to be significant (Figure 4A). At level 1, LEfSe analysis identified "protein metabolism", "nucleosides and nucleotides" and "RNA metabolism" as being significantly correlated with HE samples, whereas LE microbiomes were significantly associated with "iron acquisition and metabolism", "carbohydrates", and "sulfur metabolism" (Figure 4B; Supplementary Figure 6).

Overall, the LE microbiomes were functionally specialized on turnover of C_6 and C_5 carbohydrate components. Among the functions associated with "carbohydrate", a particular increase in the LE dataset was observed in the "monosaccharide" (level 2) turnover-associated genes (HE: 3%, LE: 4%) (e.g. in D-galacturonate, L-rhamnose, xylose, L-arabinose, and L-fucose metabolism) as well as in the uptake of lactose and galactose. Especially mannose metabolism (level 3; HE: 0.8%, LE: 1%), including the metabolism of alpha-1,2-mannosidase (level 4; HE: 0.6%, LE: 0.9%), was found to be increased in LE samples (Supplementary Figure 6). Indeed, gut-associated *Bacteroides* species carry a specific genetic machinery to degrade plant-derived mannans or human high-mannose-type N-glycans, stemming from mucosal secretions and secreted epithelial cells [45,46].

The HE microbiomes, however, were more directed towards the turnover of C_3- C_1 compounds. For instance, the "pyruvate ferredoxin oxidoreductase" (HE: 0.4%, LE: 0.3%; alpha and beta subunits; HE: 0.04% LE: 0.01% and HE: 0.02% LE: 0.01%, respectively), which is part of the "central carbohydrate metabolism" of pyruvate, propanoate, and butanoate, and the reductive carboxylate cycle, was found to be significantly increased in HE samples. This enzyme (also known as pyruvate synthase), catalyzes the interconversion of pyruvate and acetyl-CoA, and thus is responsible for the incorporation or release of CO_2 with the help of ferredoxin. Moreover, the functional gene involved in formate efflux transportation were as well increased in HE microbiomes (0.02% vs. 0.005%; $p=0.03$, Mann Whitney U) (Supplementary Dataset 3).

Genes involved in "methanogenesis" were almost absent in the LE dataset (0.00004%), but reached a 0.1% overall relative abundance in the HE dataset. This was also reflected by the methyl-coenzyme M reductase, which is responsible for the release of methane in the last step of methanogenesis, and whose alpha subunit was represented in a proportion of 0.01% in the HE dataset but only of 0.00001% in the LE dataset. Notably, genes involved in "methanogenesis from methylated compounds" comprised 0.01% in the HE dataset, and 0.005% in the LE dataset, indicating that a similar proportion of these genes existed in both datasets, largely independent of methane emission (Supplementary Dataset 3).

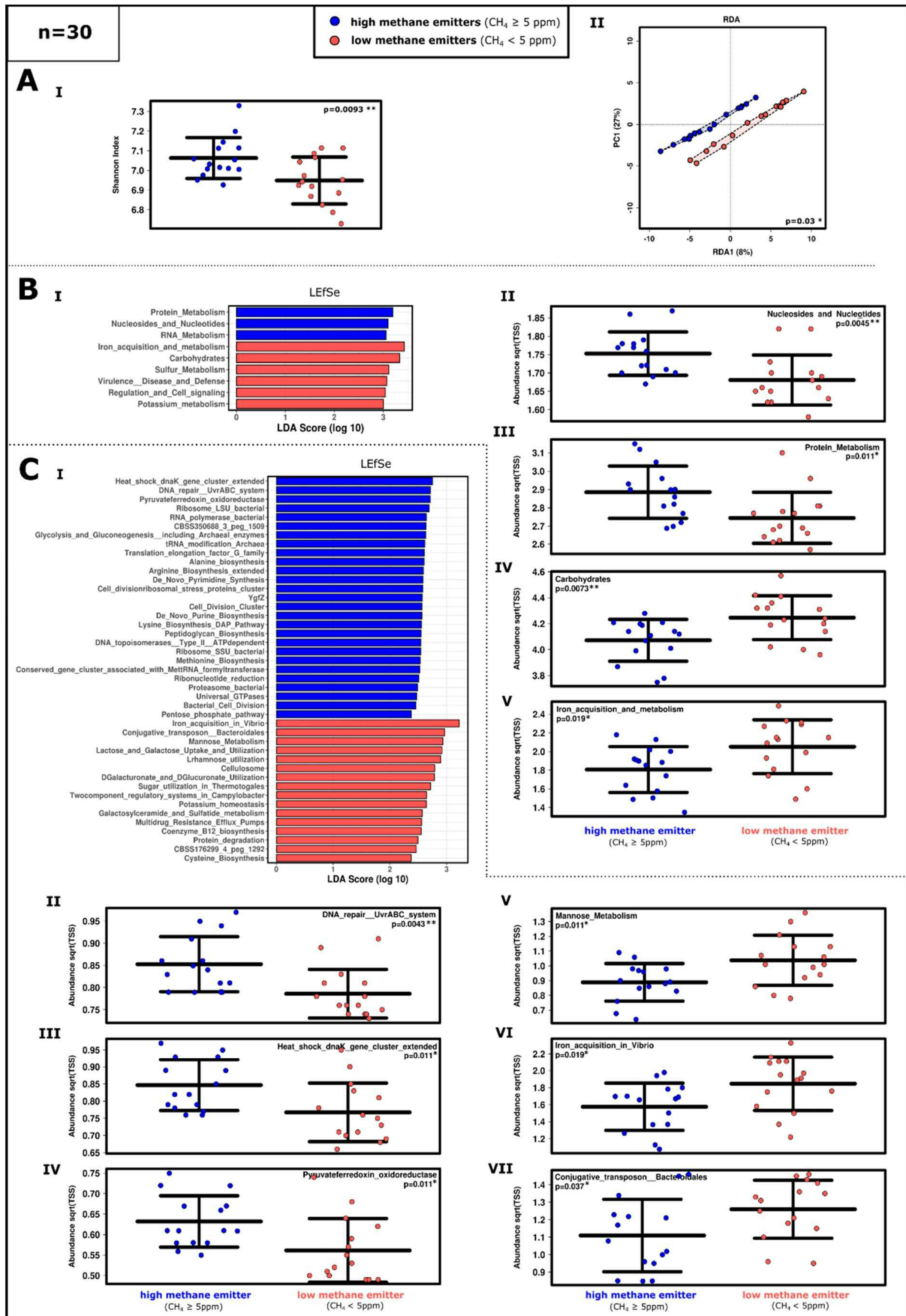


Figure 4. Overview of the divergent functions of the HE and LE based on the shotgun metagenome analysis (subsystems). **A.I.** Shannon diversity and **A.II.** RDA plot at feature level. **B.I.** LEfSe analysis and **B.II-.V.** ANOVA plots at highest subsystem level (level 1). **C.I.** LEfSe analysis and **C.II-V.II.** subsystem at level 3. (100 most abundant; n=30)

Taxonomic information derived from shotgun metagenomics was highly similar to the information that was derived from 16S rRNA gene amplicon sequencing and confirmed the differences between HE and LE microbiomes (for details see: Supplementary Dataset 4; Supplementary Item 4; Supplementary Figures 7-10). Notably, signatures of Christensenellaceae, which were associated with *Methanobrevibacter* occurrence in the amplicon dataset, could not be retrieved from the metagenomics dataset, a phenomenon that has been reported earlier [47]. Network analyses of the archaeome profile in HE and LE on the species level revealed again the predominance of *Methanobrevibacter* species under HE conditions (amongst all archaeal signatures 70% *M. smithii,* 1% M. *stadtmanae*), whereas LE samples were characterized by a more diverse but rarely abundant archaeome (9% *M. smithii*, 3% *M. stadtmanae*; Supplementary Figure 11-12; Supplementary Dataset 4; Supplementary Item 5).

Of note, using these initial datasets, methane emission above 5 ppm appeared to be predictable from the RefSeq shotgun dataset (up to 100% prediction accuracy) using a machine learning approach.

HE keystone taxa drive nutrient break-down towards C1-C3 compounds

As indicated above, we identified a number of representative bacterial and archaeal genera, which were indicative for HE and LE, respectively. To perform more detailed analyses on the RSV level, we proceeded with amplicon data (matched dataset) because taxonomic information for Christensenellaceae was missing from the metagenomics dataset. We identified 21 RSVs, revealing significantly discriminative (identified through LEfSe analyses) and substantial mean abundances (top 600 taxa). We found that the LE profile was mainly defined by four RSVs of *Bacteroides*, four RSVs of *Butyricicoccus*, and one RSV each of *Flavonifractor, Blautia*, "*Tyzzerella", Ruminococcus* (*R. gnavus* group), and *Roseburia*, whereas the HE profile was driven by one RSV of *Methanobrevibacter*, three RSVs of the Christensenellaceae R7 group, two RSVs of *Ruminiclostridium*, one RSV of Ruminococcaceae UCG010, and one RSV of *Eubacterium* (*E. ruminantium* group) (Figure 5, Supplementary Table 3). This selection and importance of keystone taxa was further supported by 84 dereplicated high quality MAGs (metagenome assembled genomes; mean completeness 90%, mean contamination 7%, Supplementary Table 4) with replication rates in the range of 1.3 to 2.6 (*Methanobrevibacter smithii*: 4 MAGs, *Bacteroides:* 32, Christensenellales: 19, Ruminococcaceae: 19, *Ruminiclostridium:* 2, *Ruminococcus:* 4).

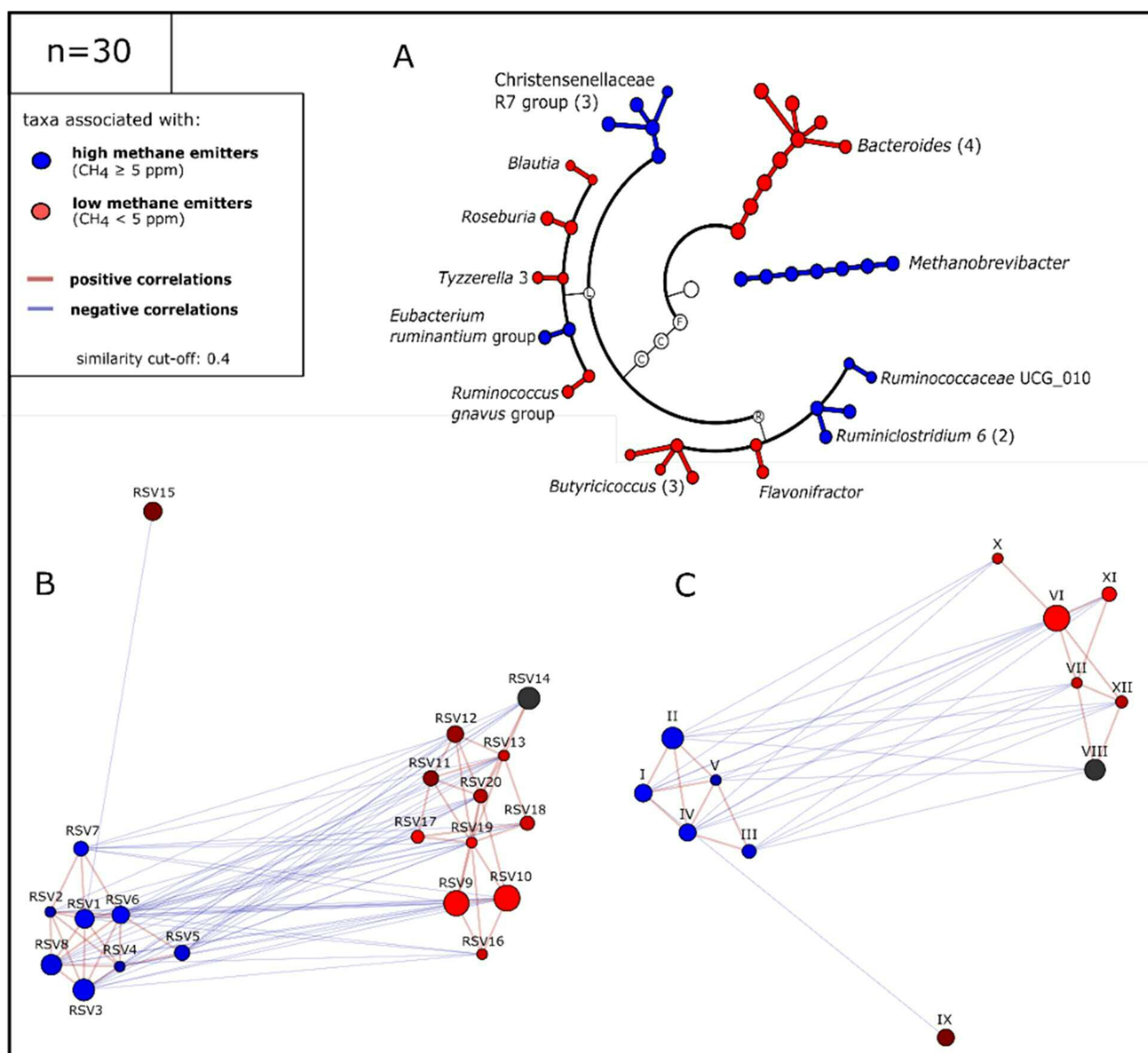


Figure 5. Identified keystone taxa in HE and LE subjects. A. Cladogram of LE and HE keystone taxa. F: Firmicutes, C: Clostridia/Clostridiales, L: Lachnospiraceae, R: Ruminococcaceae. Numbers in brackets indicate the number of contributing RSVs; **B.** and **C.** Network of keystone taxa of HE and LE at RSV and genus levels, respectively. I → RSV1: *Methanobrevibacter*; II→ RSV2-4: Christensenellaceae R7 group; III → RSV5: Eubacterium ruminantium group; IV → RSV6-7: *Ruminiclostridium*; V → RSV8: Ruminococcaceaea UCG010; VI → RSV9-12: *Bacteroides*; VII → RSV13: *Ruminococcus gnavus group*; VIII → RSV14: *Blautia*; IX → RSV15: *Roseburia*; X → RSV16: "*Tyzzerella*"; XI → RSV17-19: *Butyricicoccus*; XII → RSV20: *Flavonifractor* (also see Supplementary Table 3)

Based on literature information available for the keystone taxa [48–50], HE and LE communities are each metabolically highly interwoven. In both cases, degradation of nutrients results in metabolic cycles of short chain fatty acids and CO_2/H_2 (Figure 6). Under LE conditions, these metabolites are trapped in the cycle until they are uptaken by the host or used for microbial biomass production. The conversion of H_2/CO_2/formate into methane by *Methanobrevibacter* under HE conditions, however, results in a metabolic "dead end", as methane cannot further be metabolized by gut microbiota or human epithelial cells.

Formate-based methanogenesis is widely distributed amongst human-associated methanogens, as e.g. all *Methanobrevibacter* species detected in a catalogue of 1,167 genomes have the capability to use formate for methanogenesis [51]. The ability to consume formate appears to be an important specialization displayed by methanogens in the human gastrointestinal tract and under symbiotic conditions [51]. This hypothesis is supported by the observation that *M. smithii* upregulates formate utilisation gene clusters in syntrophic relationships [52], and methano-archaeal adhesin-like proteins are expressed differently in response to formate, indicating that the physical relationship with bacterial partners changes when different amounts of different metabolites are available [53]. It shall be noted, that human-associated *Methanobrevibacter* species are not autotrophs *per se* but require acetate for biomass production as they generally lack the CODH-ACS complex [54]. Therefore, a higher availability of formate and acetate would support the growth of *M. smithii*.

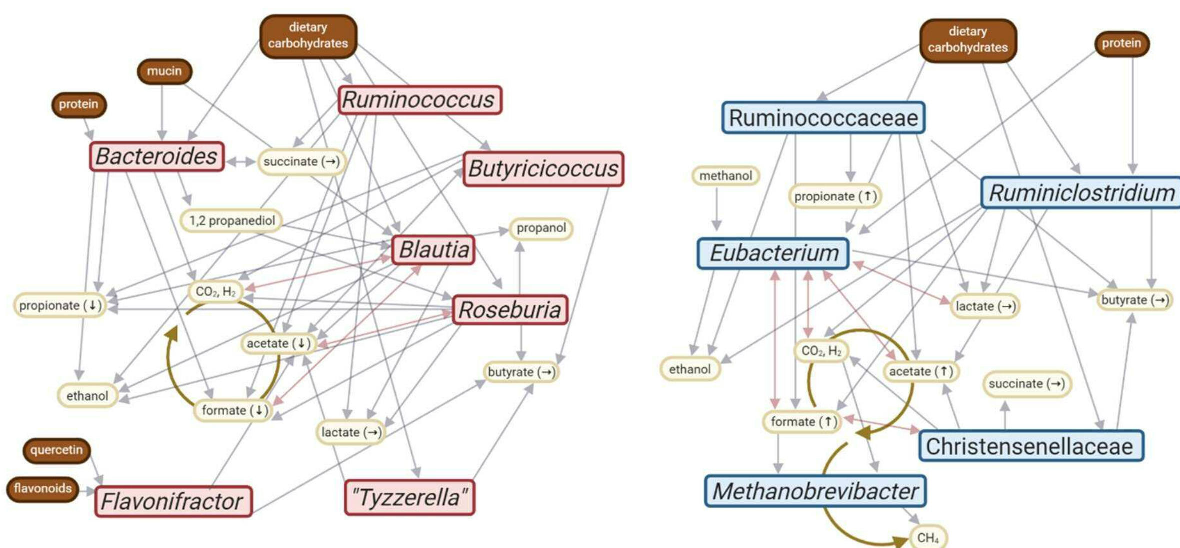


Figure 6. Metabolic network of key-stone taxa in LE and HE microbiomes. Metabolites measured in stool samples are indicated by arrows; respective increase or decrease of the median by >5% is displayed.

To characterize the role of the metabolites in more detail and to confirm our assumptions, we performed NMR-based metabolomic analyses of the stool samples. Indeed, we measured an increase in formate concentrations (1.5-fold, based on median concentrations per group) and acetate (1.35-fold) under HE conditions (Figure 6, Supplementary Table 5). Propionate was as well increased under HE conditions (1.17-fold), whereas the butyrate, lactate, and succinate concentrations remained largely equal (Supplementary Table 5). Formate concentration and methane emissions were significantly correlated (in ppm, Spearman's-rho correlation coefficient 0.491, p=0.006). Moreover,

formate concentration was significantly correlated with acetate (spearman-rho correlation coefficient 0.628), butyrate (0.416) and propionate (0.448) abundance, whereas no correlations were found for lactate and succinate (0.204 and 0.258, respectively). We can state, that the consumption of formate and acetate by *Methanobrevibacter* has large-scale influence on the microbiome composition and functionality, pulling the metabolism strongly towards small carbon compounds in HEs (see also [47]). In a subsequent step, we were interested in whether subjects' diet has influence on these microbial metabolism patterns.

B12, fat and fibre intake have strong impact on methane microbiomes

A Food Frequency Questionnaire (FFQ) [10] was used to assess the food habits of each participant during the four weeks prior to sampling. Overall, the daily intake of 19 food ingredients was tracked (Supplementary Table 2). Correlations of all dietary parameters with microbiome and metabolome characteristics is available in Supplementary Table 6 (see also BioEnv plot, Supplementary Figure 13). *Methanobrevibacter* was negatively correlated with total fat (*r*s=-0.435, p=0.016; if not stated otherwise a Spearman's correlation analysis was performed), saturated fat (*r*s=-0.421, 0.021) and omega-3 fatty acids (*r*s=-0.407, p=0.026). Trends indicating a correlation were observed for vitamin B12 intake (*r*s=-0.355, p=0.054). Similar trends for vitamin B12 (*r*s=-0.465, p=0.01) and omega-3 fatty acid (*r*s=-0.349, p=0.059) intake were seen when examining the relative abundance of the Christensenellaceae R7 group. Vitamin D intake was negatively correlated with the Christensenellaceae R7 group relative abundances (*r*s=-0.345, p=0.062) (Supplementary Table 5).

Within the LE community cluster, an analysis of the genera *Bacteroides*, *Flavonifractor* and the *Ruminococcus gnavus group* revealed a trend with respect to a negative correlation with dietary fibre intake (*r*s=-0.379, p=0.039; *r*s=-0.517, p=0.003 and *r*s=-0.382, p=0.037, respectively;). The relative abundance of *Blautia* positively correlated with vitamin B12 levels (*r*s=0.505, p=0.004) and protein intake (rs=0.422, p=0.020). Vegetarianism correlated with different dietary compound intake, namely, vitamin C and sugar intake was positively correlated (*r*s=0.490, p=0.006 and *r*s=0.441, p=0.015, respectively), whereas food diversity and vitamin B12 levels (*r*s=-0.473, p=0.008 and *r*s=-0.449, p=0.013, respectively) were negatively correlated with vegetarianism (Figure 7, Supplementary Table 5).

Based on dietary information, vitamin B12 (cobalamin) appeared to be an important modulatory factor. The key-role of vitamin B12 was further supported by the significant negative correlation of formate concentration in the fecal samples and vitamin B12 uptake (p=0.038, R=-0.380).

Vitamin B12 (cobalamin) is an important dietary ingredient, as it is involved in a number of homeostatic functions of host and microbiome. The host absorbs cobalamin solely in the small intestine, not disturbing the metabolic cycle of microbial cobalamin-producers (approx. 25% of all gut bacteria) and

–consumers (particularly *Bacteroides*) in the large intestine [55]. Following our observations on the negative correlation of B12 and methanogenesis, indeed, functions involved in B12 binding and transportation were significantly increased in LE metagenomes (B12-binding component *BtuF*, p=0.004, t-test; Supplementary dataset 3).

Notably, formate and vitamin B12 (cobalamin) metabolism are closely connected also in humans. Cobalamin deficiency was associated with increased formate concentrations in urine and plasma (in rats, [56]), due to the so-called methyl-folate trap [57–59]. Under these conditions, the cytosolic folate accumulates as 5-methyl-THF (thus reducing the concentration of THF), which impedes the incorporation of formate into the folate pool, and results in formate accumulation. In general, replenishing the THF pool also involves ALDH1L1 (10-formyltetrahydrofolate dehydrogenase), an enzyme involved in formate oxidation, which converts 10-formyl-THF to THF and CO_2. Notably, an association between the Christensenellaceae/*Methanobrevibacter* abundance and the abundance of a certain SNP (rs2276731) in the ALDH1L1 gene was observed when genetic correlations with microbiome profiles were analysed in a large UK twin study [60]. SNP rs2276731 is characterized by a nucleotide exchange towards C (instead of G, T) in approx. 17% of the population [61]. This ratio is in high agreement with the percentage of methane producers observed in our (15%) and other studies [9].

As *Methanobrevibacter* appears to be able to grow independently from cobalamin availability [52], it could benefit from the increased formate (and acetate) concentrations in the GIT, without being influenced by possible vitamin B12 shortage.

Individual diet-adapted flux balance analysis confirms the vitamin-independent, maximal breakdown of fibre to C1 metabolites under HE conditions

In order to draw an analogy of dietary information and the identified key taxa, we performed a flux balance analysis (FBA) with MICOM [43]. To optimize this approach to our scientific question, we included the individual dietary information obtained from the donors in our model (Supplementary Dataset 7). The community models were based on the AGORA 1.03 genus model [44]. Growth simulations resulted in information on growth rates, growth niches, metabolite consumptions and phenotype associated fluxes (Supplementary Dataset 5 and 6).

The results of the analysis performed on previously identified keystone taxa confirmed a significant association between the HE conditions and an increased flux of C1 metabolites, such as methanol, formaldehyde, carbon dioxide and formate (Fig. 7), as well as acetate and propionate. LE conditions were associated with D-mannose, lactate, ribose levels, and overall a greater complexity of organic molecules. Notably, the hydrogen flux was only minimally associated with HE (-0.021595761). Fluxes

in vitamin compounds (nicotinamide, riboflavin, thiamine, pyridoxin, menaquinone 8) were strongly associated with the LE conditions. The outcome of the modelling approach strongly confirmed our above-made observations based on microbiome, metagenome and metabolome analyses, and indicated the further involvement of other components, such as methanol or indole, which require further investigation.

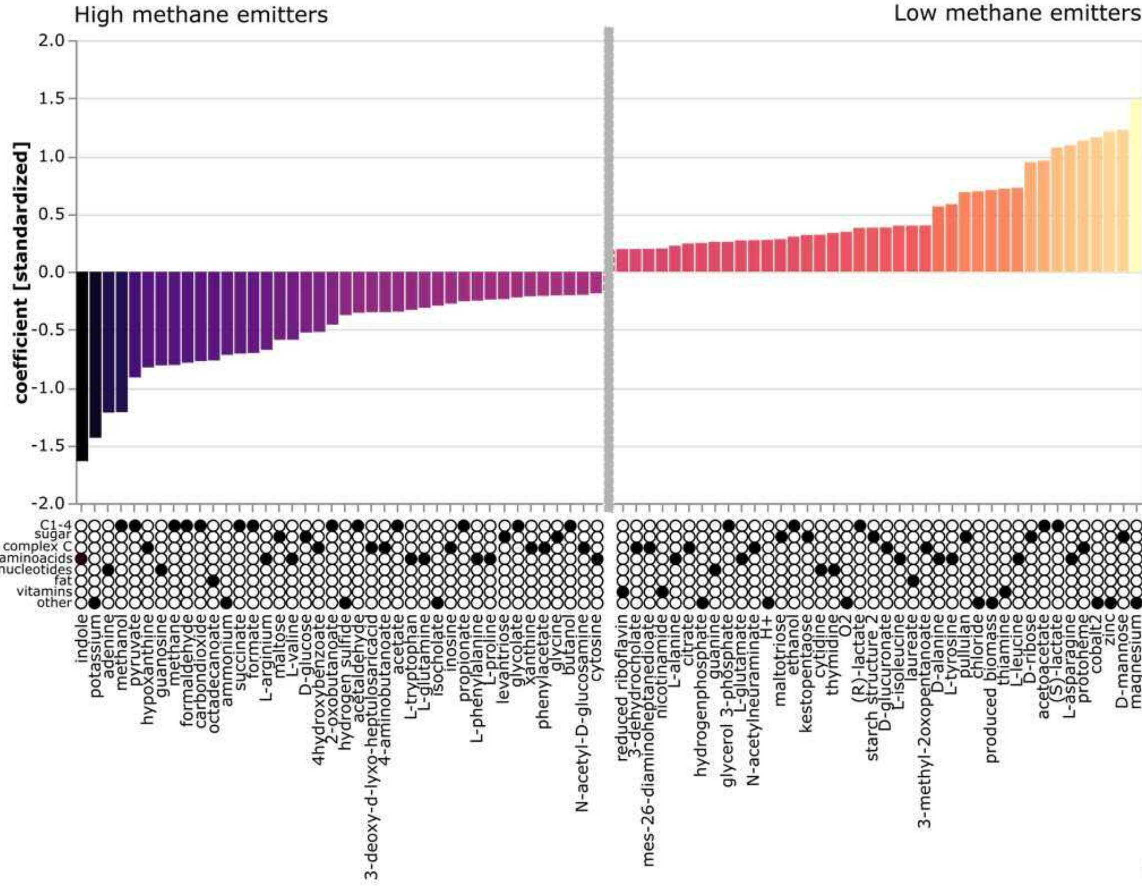


Figure 7. MICOM model-based flux balance analysis of keystone taxa. The 40 most significant metabolites are shown for each condition. Left: HE, right: LE.

DISCUSSION

In this study, we analyzed the underlying principle of human methane emission. We were able to show that:

i) high methane emission is linked to a more complex microbiome in the GIT,

ii) the microbial community composition and function differs significantly between high- and low- methane emitters and is pronounced in specific archaeal and bacterial key-taxa,

iii) *Methanobrevibacter smithii*, whose abundance is increased by a factor of 1,000 under HE conditions, pulls microbiome function towards acetate and formate production,

iv) dietary habits, including low B12 uptake, support optimal gastrointestinal conditions for a complete and efficient break-down of fibres to C1 compounds with a low need for vitamins.

The abundance of *Methanobrevibacter* was strongly correlated with a core group of keystone species, including various Ruminococcaceae and Christensenellaceae (see also: [62]). The interplay between *Methanobrevibacter* and Christensenellaceae is of great interest, as this syntrophic partnership has been associated with a lean phenotype [63] and a reduced gain of fat tissue [64,65] in earlier publications. Notably, both taxa are considered to be highly inheritable [49,63]. In co-culturing studies, the methanogenic partner shifted the *Christensenella minuta* metabolism, probably due to its potent hydrogen consumption, toward acetate production rather than toward butyrate production, leading to increased H_2 and CO_2 production [47,63]. Although this observation would indicate a bilateral syntrophic relationship of both microorganisms, we observed in our study, that both partners were unevenly affected by LE and HE conditions: Christensenellaceae were present in both communities (2% in LE), and signatures increased only three-fold towards those observed under HE conditions, whereas *Methanobrevibacter* signatures increased 1000-fold, probably indicating a more complex underlying principle. Indeed, we could not identify any dietary-derived compound which had a direct, significantly stimulating or inhibiting effect on the Christensenellaceae population.

The complexity of ingested saccharides is an important modulator for the composition and functionality of a gastrointestinal microbiome, and an interesting link between cellulose degradation and methane emission was observed by other researchers. Chassared et al. (2010) described that dominant cellulose degraders isolated from non-methane-excreting subjects are mainly affiliated with Bacteroidetes, while they are predominantly represented by Firmicutes in methane-excreting individuals [66]. In our study, we also identified *Bacteroides* and *Roseburia*, which belong to the phylum Bacteroidetes, as well as Christensenellaceae, *Ruminiclostridium* and *Ruminococcaceae* (Firmicutes), as important key taxa in LE and HE subjects, respectively. Notably, *Bacteroides* (which was shown to

be significantly negatively correlated with dietary fibres in our study) and *Roseburia*, unlike high- H_2-producing *Ruminococcus* sp., are not able to digest e.g. microcrystalline cellulose [66–68]. This indicates that the type of dietary fibre has a potential modulating impact on methane production.

The negative correlations observed for fat intake and methanogen abundance are highly congruent with previous observations made in ruminants, where an increased fat (oil) concentration in the diet led to a reduced enteric methane production of up to 36% ([69] and references therein). It is considered that dietary fat affects methane production in rumen because it reduces the hydrogen accumulation through fatty acid biohydrogenation, leading to the conversion of unsaturated fatty acids to saturated fatty acids, reducing the intake of fermentable organic matter and fibre digestion [69].

Beyond that, the MICOM model identified a substantial role of indole in HEs (Figure 7). Indoles are usually derived from gut microbial conversion of tryptophane and have a variety of important functions, including host defense and fortifying the gut barrier. Moreover, indoles are important, dose-dependent signaling molecules for bacteria, with effect on motility, biofilm formation, antibiotic resistances and virulence [48,70]. As this might have large physiological and maybe medical effects on the host, this aspect certainly warrants additional studies in future.

CONCLUSIONS

High methane baseline emission in breath mirrors a complex situation of the human physiology, including vitamin B12 shortage and increased formate levels in the GIT. Higher formate levels were earlier, and independently from methane breath analyses, correlated with positive foetal development, T-cell activation, a lean phenotype, and cardiovascular function [71]. Thus, the correlation of high methane emission and formate concentration warrants future research. Moreover, as we revealed the impact of dietary fibre, vitamin, and fat uptake on methanogenic activity, dietary modulations (e.g. vitamin B12 supplementation) could be used for the mitigation of methane-associated disorders, such as constipation. Our study and its results emphasize the importance of archaeome activity in the human body. This activity serves as an important mirror, modulator, and regulator of the microbiome and overall body processes.

Declarations

Ethics approval and consent to participate

The study was evaluated and approved according to the Declaration of Helsinki by the local ethics committee of the University of Graz (EK-Nr. GZ. 39/44/63 ex 2017/18). Before participation, all participants signed an informed consent.

Consent for publication

Not applicable.

Availability of data and material

Raw sequencing data obtained from amplicon-based sequencing and metagenomics sequencing data (technical sequences including adaptor sequences, linker sequences and barcode sequences as well as human reads were removed) used in this paper can be found in the European Nucleotide Archive (ENA): PRJEB41867. Supplementary Datasets (after decontam and removal of features with zero and one reads) and all Supplementary Figures, Tables and Items were additionally deposited on Mendeley at http://dx.doi.org/10.17632/hjj3tx7n84.1.

Competing interests

The authors declare that they have no competing interests.

Funding

We appreciate the financial support provided by FWF through grant P 32697 given to CME and through grant KLI 639 given to CME, VS, and FF. We are thankful to all participants of the study for providing sample material and questionnaires.

TM was supported by Austrian Science Fund (FWF) grants P28854, I3792, and DK-MCD W1226, the Austrian Research Promotion Agency (FFG) Grants 864690 and 870454; the Integrative Metabolism Research Center Graz; Austrian Infrastructure Program 2016/2017, the Styrian Government (Zukunftsfonds), and BioTechMed-Graz (Flagship project DYNIMO).

The funding body had no influence on the study, collection, analysis and interpretation of data, and in writing the manuscript.

Authors' contributions

CK performed sampling, DNA extraction, PCR, data analysis (microbiome, metagenome, metabolome, correlation analysis), produced most of the display items and wrote the manuscript. FF, MW and CS were responsible for recruitment, sample and questionnaire/cohort metadata collection. AM supported data analysis, performed metabolic prediction and wrote the manuscript. SL and SH performed analysis of the dietary information, provided dietary indices and contributed to manuscript writing. CH and IB provided valuable contributions on the study design and research questions, and critically read the manuscript. CH supported methane breath measurements. FF, VS contributed to

manuscript writing. VS and CME initiated this project and were responsible for the study design. CME supervised all activities, performed analyses, and wrote the manuscript. AS and TM performed NMR sample preparation and metabolomics data analysis. All authors read and corrected the manuscript.

Acknowledgements

The authors acknowledge computational resources of the MedBioNode at the Medical University Graz and the support of the ZMF Galaxy Team: Core Facility Computational Bioanalytics, Medical University of Graz, funded by the Austrian Federal Ministry of Education, Science and Research, Hochschulraum-Strukturmittel 2016 grant as part of BioTechMed Graz. We would like to thank Christian Diener and other developers of the micom tool for their valuable input and support to predict metabolites, adapt diet models, or evaluate growth and replication rates. We appreciate critical proof-reading by Marcus Blohs.

REFERENCES

1. Borrel G, Brugère JF, Gribaldo S, Schmitz RA, Moissl-Eichinger C. The host-associated archaeome. Nat. Rev. Microbiol. 2020. p. 622–36.

2. Mahnert A, Blohs M, Pausan MR, Moissl-Eichinger C. The human archaeome: methodological pitfalls and knowledge gaps. Emerg Top Life Sci. 2018;469–82.

3. Boros M, Tuboly E, Mészáros A, Amann A. The role of methane in mammalian physiology - Is it a gasotransmitter? J Breath Res [Internet]. 2015;14001. Available from: http://dx.doi.org/10.1088/1752-7155/9/1/014001

4. Pimentel M, Lin HC, Enayati P, Van Den Burg B, Lee HR, Chen JH, et al. Methane, a gas produced by enteric bacteria, slows intestinal transit and augments small intestinal contractile activity. Am J Physiol - Gastrointest Liver Physiol. 2006;

5. Pimentel M, Gunsalus RP, Rao SSC, Zhang H. Methanogens in Human Health and Disease. Am J Gastroenterol Suppl [Internet]. 2012;28–33. Available from: http://dx.doi.org/10.1038/ajgsup.2012.6

6. de Macario EC, Macario AJL. Methanogenic archaea in humans and other vertebrates: an update. (Endo) symbiotic Methanogenic Archaea. 2018. p. 103–19.

7. Koskinen K, Pausan MR, Perras AK, Bang MBC, Mora M, Schilhabel A, et al. First Insights into the Diverse Human Archaeome : Specific Detection of Archaea in the Gastrointestinal Tract ... crossm First Insights into the Diverse Human Archaeome : Specific Detection of Archaea in the Gastrointestinal Tract ,. MBio. 2017;1–17.

8. Moissl-Eichinger C, Pausan M, Taffner J, Berg G, Bang C, Schmitz RA. Archaea Are Interactive Components of Complex Microbiomes. Trends Microbiol. 2018. p. 70–85.

9. Polag D, Keppler F. Global methane emissions from the human body: Past, present and future. Atmos Environ. 2019;116823.

10. Haftenberger M, Heuer T, Heidemann C, Kube F, Krems C, Mensink GBM. Relative validation of a food frequency questionnaire for national health and nutrition monitoring. Nutr J. 2010;

11. Denkwerkzeuge D. Software:nut.s science v1.32.79. 2020.

12. Pausan MR, Csorba C, Singer G, Till H, Schoepf V, Santigli E, et al. Exploring the Archaeome: Detection of Archaeal Signatures in the Human Body. Front Microbiol. 2019;2796.

13. Klymiuk I, Bambach I, Patra V, Trajanoski S, Wolf P. 16S based microbiome analysis from healthy subjects' skin swabs stored for different storage periods reveal phylum to genus level changes. Front Microbiol. 2016;

14. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. DADA2: High-resolution sample inference from Illumina amplicon data. Nat Methods. 2016;581–583.

15. Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat. Biotechnol. 2019. p. 852–7.

16. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, et al. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res. 2013;D590–6.

17. Bokulich NA, Kaehler BD, Rideout JR, Dillon M, Bolyen E, Knight R, et al. Optimizing taxonomic classification of marker-gene amplicon sequences with QIIME 2's q2-feature-classifier plugin. Microbiome. 2018;

18. Price MN, Dehal PS, Arkin AP. FastTree 2 - Approximately maximum-likelihood trees for large alignments. PLoS One. 2010;

19. Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, et al. Metagenomic biomarker discovery and explanation. Genome Biol [Internet]. 2011;1. Available from: http://genomebiology.com/2011/12/6/R60

20. Davis NM, Proctor DM, Holmes SP, Relman DA, B.J C. Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data. Microbiome. 2018;226.

21. Zakrzewski M, Proietti C, Ellis JJ, Hasan S, Brion M-J, Berger B KL. Calypso: a user-friendly web-server for mining and visualizing microbiome-environment interactions. Bioinformatics. 2017;782–3.

22. Oksanen J, Kindt R, Legendre P, O'Hara B, Henry M, Stevens H. The vegan package -Community ecology package. 2007. p. 631–7.

23. Meyer F, Paarmann D, D'Souza M, Olson R, Glass EM, Kubal M, et al. The metagenomics RAST server - A public resource for the automatic phylogenetic and functional analysis of metagenomes. BMC Bioinformatics. 2008;

24. Andrews S. FastQC: a quality control tool for high throughput sequence data. 2010.

25. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics. 2014;2114–20.

26. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. Nat Methods. 2012;357.

27. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, et al. The sequence alignment/map format and SAMtools. Bioinformatics. 2009;2078–9.

28. Quinlan AR, Hall IM. BEDTools: A flexible suite of utilities for comparing genomic features. Bioinformatics. 2010;841–2.

29. Buchfink B, Xie C, Huson DH. Fast and sensitive protein alignment using DIAMOND. Nat Methods. 2015;59.

30. Huson DH, Auch AF, Qi J, Schuster SC. MEGAN analysis of metagenomic data. Genome Res. 2007;377–86.

31. Li D, Liu CM, Luo R, Sadakane K, Lam TW. MEGAHIT: An ultra-fast single-node solution for large and complex metagenomics assembly via succinct de Bruijn graph. Bioinformatics. 2015;1674–6.

32. Wu YW, Tang YH, Tringe SG, Simmons BA, Singer SW. MaxBin: An automated binning method to recover individual genomes from metagenomes using an expectation-maximization algorithm. Microbiome. 2014;

33. Parks DH, Imelfort M, Skennerton CT, Hugenholtz P, Tyson GW. CheckM: assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. Genome Res. 2015;1043–55.

34. Olm MR, Brown CT, Brooks B, Banfield JF. DRep: A tool for fast and accurate genomic comparisons that enables improved genome recovery from metagenomes through de-replication. ISME J. 2017;2864–8.

35. Chaumeil PA, Mussig AJ, Hugenholtz P, Parks DH. GTDB-Tk: A toolkit to classify genomes with the genome taxonomy database. Bioinformatics. 2020;1925–7.

36. Vallenet D, Labarre L, Rouy Z, Barbe V, Bocs S, Cruveiller S, et al. MaGe: A microbial genome annotation system supported by synteny results. Nucleic Acids Res. 2006;53–65.

37. Brown CT, Olm MR, Thomas BC, Banfield JF. Measurement of bacterial replication rates in microbial communities. Nat Biotechnol. 2016;1256.

38. Caporaso J, Kuczynski J, Stombaugh J, Bittinger K, Bushman F, Costello E, et al. QIIME allows analysis of high-throughput community sequencing data. Nat Methods. 2010;335–6.

39. Ondov BD, Bergman NH, Phillippy AM. Interactive metagenomic visualization in a Web browser. BMC Bioinformatics. 2011;1.

40. Alkan HF, Walter KE, Luengo A, Madreiter-Sokolowski CT, Stryeck S, Lau AN, et al. Cytosolic Aspartate Availability Determines Cell Survival When Glutamine Is Limiting. Cell Metab. 2018;706-720.e6.

41. Chong J, Soufan O, Li C, Caraus I, Li S, Bourque G, et al. MetaboAnalyst 4.0: Towards more transparent and integrative metabolomics analysis. Nucleic Acids Res. 2018;W486–94.

42. Dieterle F, Ross A, Schlotterbeck G, Senn H. Probabilistic quotient normalization as robust method to account for dilution of complex biological mixtures. Application in1H NMR metabonomics. Anal Chem. 2006;4281–90.

43. Diener C, Gibbons SM, Resendis-Antonio O. MICOM: Metagenome-Scale Modeling To Infer Metabolic Interactions in the Gut Microbiota. mSystems. 2020;

44. Magnúsdóttir S, Heinken A, Kutt L, Ravcheev DA, Bauer E, Noronha A, et al. Generation of genome-scale metabolic reconstructions for 773 members of the human gut microbiota. Nat Microbiol. 2017;81–89.

45. Trastoy B, Du JJ, Klontz EH, Li C, Cifuente JO, Wang L-X, et al. Structural basis of mammalian high-mannose N-glycan processing by human gut Bacteroides. Nat Commun. 2020;1–11.

46. Tailford LE, Money VA, Smith NL, Dumon C, Davies GJ, Gilbert HJ. Mannose foraging by Bacteroides thetaiotaomicron: structure and specificity of the β-mannosidase, BtMan2A. J Biol Chem. 2007;11291–9.

47. Ruaud A, Esquivel-Elizondo S, de la Cuesta-Zuluaga J, Waters JL, Angenent LT, Youngblut ND, et al. Syntrophy via Interspecies H2 Transfer between Christensenella and Methanobrevibacter Underlies Their Global Cooccurrence in the Human Gut. MBio. 2020;

48. Oliphant K, Allen-Vercoe E. Macronutrient metabolism by the human gut microbiome: major fermentation by-products and their impact on host health. Microbiome. 2019;1–15.

49. Waters JL, Ley RE. The human gut bacteria Christensenellaceae are widespread, heritable, and associated with health. BMC Biol. 2019. p. 1–11.

50. Carlier J-P, Bedora-Faure M, K'ouas G, Alauzet C, Mory F. Proposal to unify Clostridium orbiscindens Winter et al. 1991 and Eubacterium plautii (Séguin 1928) Hofstad and Aasjord 1982, with description of Flavonifractor plautii gen. nov., comb. nov., and reassignment of Bacteroides capillosus to Pseudoflavonifra. Int J Syst Evol Microbiol. 2010;585–90.

51. Chibani CM, Mahnert A, Borrel G, Almeida A, Werner A, Brugère J-F, et al. A comprehensive analysis of the global human gut archaeome from a thousand genome catalogue. bioRxiv. 2020;2020.11.21.392621.

52. Samuel BS, Hansen EE, Manchester JK, Coutinho PM, Henrissat B, Fulton R, et al. Genomic and metabolic adaptations of Methanobrevibacter smithii to the human gut. Proc Natl Acad Sci. 2007;10643–8.

53. Hansen EE, Lozupone CA, Rey FE, Wu M, Guruge JL, Narra A, et al. Pan-genome of the dominant human gut-associated archaeon, Methanobrevibacter smithii, studied in twins. Proc Natl Acad Sci U S A. 2011;4599–606.

54. Poehlein A, Schneider D, Soh M, Daniel R, Seedorf H. Comparative genomic analysis of members of the genera Methanosphaera and Methanobrevibacter reveals distinct clades with specific potential metabolic functions. Archaea. 2018;

55. Rowley CA, Kendall MM. To B12 or not to B12: Five questions on the role of cobalamin in host-microbial interactions. PLoS Pathog. 2019;e1007479.

56. MacMillan L, Tingley G, Young SK, Clow KA, Randel EW, Brosnan ME, et al. Cobalamin deficiency results in increased production of formate secondary to decreased mitochondrial oxidation of one-carbon units in rats. J Nutr. 2018;358–63.

57. HERBERT V, ZALUSKY R. Interrelations of vitamin B12 and folic acid metabolism: folic acid clearance studies. J Clin Invest. 1962;1263–76.

58. Scott JM, Weir DG. THE METHYL FOLATE TRAP. A physiological response in man to prevent methyl group deficiency in kwashiorkor (methionine deficiency) and an explanation for folic-acid-induced exacerbation of subacute combined degeneration in pernicious anaemia. Lancet. 1981;337–40.

59. Lamarre SG, Morrow G, MacMillan L, Brosnan ME, Brosnan JT. Formate: An essential metabolite, a biomarker, or more? Clin Chem Lab Med. 2013. p. 571–8.

60. Goodrich JK, Davenport ER, Beaumont M, Jackson MA, Knight R, Ober C, et al. Genetic determinants of the gut microbiome in UK twins. Cell Host Microbe. 2016;731–43.

61. rs2276731 RefSNP Report - dbSNP - NCBI. 2020.

62. Vojinovic D, Radjabzadeh D, Kurilshikov A, Amin N, Wijmenga C, Franke L, et al. Relationship between gut microbiota and circulating metabolites in population-based cohorts. Nat Commun. 2019;

63. Goodrich JJK, Waters JLJ, Poole ACA, Sutter JJL, Koren O, Blekhman R, et al. Human genetics shape the gut microbiome. Cell. 2014;789–99.

64. Alonso BL, Irigoyen von Sierakowski A, Sáez Nieto JA, Rosel AB. First report of human infection by Christensenella minuta, a gram-negative, strickly anaerobic rod that inhabits the human intestine. Anaerobe. 2017;124–5.

65. Oki K, Toyama M, Banno T, Chonan O, Benno Y, Watanabe K. Comprehensive analysis of the fecal microbiota of healthy Japanese adults reveals a new bacterial lineage associated with a phenotype characterized by a high frequency of bowel movements and a lean body type. BMC Microbiol. 2016;1–13.

66. Chassard C, Delmas E, Robert C, Bernalier-Donadille A. The cellulose-degrading microbial community of the human gut varies according to the presence or absence of methanogens. FEMS Microbiol Ecol. 2010;205–13.

67. Aminov RI, Walker AW, Duncan SH, Harmsen HJM, Welling GW, Flint HJ. Molecular diversity, cultivation, and improved detection by fluorescent in situ hybridization of a dominant group of human gut bacteria related to Roseburia spp. or Eubacterium rectale. Appl Environ Microbiol. 2006;6371–6.

68. Duncan SH, Aminov RI, Scott KP, Louis P, Stanton TB, Flint HJ. Proposal of Roseburia faecis sp. nov., Roseburia hominis sp. nov. and Roseburia inulinivorans sp. nov., based on isolates from human faeces. Int J Syst Evol Microbiol. 2006;2437–41.

69. Alvarez-Hess PS, Williams SRO, Jacobs JL, Hannah MC, Beauchemin KA, Eckard RJ, et al. Effect of dietary fat supplementation on methane emissions from dairy cows fed wheat or corn. J Dairy Sci. 2019;2714–23.

70. Rahim MBHA, Chilloux J, Martinez-Gili L, Neves AL, Myridakis A, Gooderham N, et al. Diet-induced metabolic changes of the human gut microbiome: importance of short-chain fatty acids, methylamines and indoles. Acta Diabetol. 2019;493–500.

71. Pietzke M, Meiser J, Vazquez A. Formate metabolism in health and disease. Mol. Metab. 2020. p. 23–37.

FIGURES

Figure 1. Differences in alpha and beta diversity based on the “universal” approach of 16S rRNA gene sequencing between high (HE) and low methane emitters (LE). A-C. Profiles of the whole study cohort (n=100). **D-F**. Profiles of the matched subset only (n=30). **A.I/D.I.** An examination of Shannon diversity index revealed significant differences in alpha diversity (RSV based; ANOVA). **A.II/D.II.** The microbiome of HEs clustered significantly differently in the RDA plot (RSV based). **B.I/E.I.** LEfSe analysis of the 100 most abundant phyla and **B.II/E.II-B.V/E.V.** Relative abundance of selected phyla in ANOVA plots. **C.I/F.I.** LEfSe analysis of the 100 most abundant genera and **C.II/F.II-C.VII/F.VII.** ANOVA plots of selected genera.

Figure 2. Co-occurrence networks based on Spearman’s rho correlation of selected genera in HE and LE microbiome samples. Taxa were selected based on significantly different relative abundances in both sample types and LEfSe analyses. Left, upper panel: Whole study cohort (n=100), right, upper panel: matched study subset (n=30). Lower panels show co-occurrence patterns in the HE (left) or the LE samples (right).

Figure 3. Archaeome profile of HE and LE samples, based on the “archaeal” approach of 16S rRNA gene sequencing. A. Profile of the whole study cohort (n=100). **B.** Matched study subset (n=30). **I.** Bar chart of the 20 most abundant taxa compared regarding their low or high methane emission at the phylum level and **II.** at the genus level. **III.** Shannon diversity, and **IV.** RDA plot at RSV level.

Figure 4. Overview of the divergent functions of the HE and LE based on the shotgun metagenome analysis (subsystems). **A.I.** Shannon diversity and **A.II.** RDA plot at feature level. **B.I.** LEfSe analysis and **B.II-.V.** ANOVA plots at highest subsystem level (level 1). **C.I.** LEfSe analysis and **C.II-V.II.** subsystem at level 3. (100 most abundant; n=30)

Figure 5. Identified keystone taxa in HE and LE subjects. A. Cladogram of LE and HE keystone taxa. F: Firmicutes, C: Clostridia/Clostridiales, L: Lachnospiraceae, R: Ruminococcaceae. Numbers in brackets indicate the number of contributing RSVs; **B.** and **C.** Network of keystone taxa of HE and LE at RSV and genus levels, respectively. I → RSV1: *Methanobrevibacter*; II→ RSV2-4: Christensenellaceae R7 group; III → RSV5: Eubacterium ruminantium group; IV → RSV6-7: *Ruminiclostridium*; V → RSV8: Ruminococcaceaea UCG010; VI → RSV9-12: *Bacteroides*; VII → RSV13: *Ruminococcus gnavus group*; VIII → RSV14: *Blautia*; IX → RSV15: *Roseburia*; X → RSV16: “*Tyzzerella*”; XI → RSV17-19: *Butyricicoccus*; XII → RSV20: *Flavonifractor* (also see Supplementary Table 3)

Figure 6. Metabolic network of key-stone taxa in LE and HE microbiomes. Metabolites measured in stool samples are indicated by arrows; respective increase or decrease of the median by >5% is displayed.

Figure 7. MICOM model-based flux balance analysis of keystone taxa. The 40 most significant metabolites are shown for each condition. Left: HE, right: LE.

SUPPLEMENTARY FIGURES

Supplementary Figure 1. Bubble plots of the 50 most abundant taxa based on the "universal" approach of 16S rRNA gene sequencing. A. Microbiome profiles of the whole study cohort (n=100). **B.** Microbiome profiles of matched study subset (n=30). **AI/BI.** Phylum level. **AII/BII.** Genus level. Christensenellaceae_R7_group and *Methanobrevibacter* are highlighted.

Supplementary Figure 2. Microbiome profiles and differences in abundances of specific taxa in HEs compared to LEs based on the "universal" approach (16S rRNA gene sequencing). **A.** Whole study cohort (n=100). **B.** Matched study subset (n=30). **AI/BI.** PCoA plots (RSV based); **AII/BII.** ANOVA analysis at phylum level and **AIII/BIII** at genus level on the 100 most abundant taxa. **AIV-VII/BIV-VII.** Relative abundances of individual genera.

Supplementary Figure 3. Significant positive and negative correlation of specific taxa with emitted methane concentrations based on "universal" approach 16S rRNA gene sequencing, Spearman-based regression analysis. A. Whole study cohort (n=100). **B.** matched study subset only (n=30). **I-V.** Significant positive correlation with emitted methane. **VI-X.** Significant negative correlation with emitted methane. (100 most abundant genera; Spearman); r=Spearman's rho correlation coefficient (rs)

Supplementary Figure 4. Co-correlation network of taxa associated with HE and LE based on "universal" approach 16S rRNA gene sequencing and Spearman's rho. Networks showing connections of the 100 most abundant genera of **A.** the whole study cohort (n=100), **B.** our matched study subset (n=30), **C.** HE only (n=15) and **D.** LE only (n=15). Taxa highlighted in red and blue were shown to be most significantly different in LEfSe and ANOVA analysis.

Supplementary Figure 5. Bubble plot overview on subsystems at the highest (I.) and at functional level (II.) based on shotgun metagenome analysis. In II., the 50 most abundant features are shown.; n=30.

Supplementary Figure 6. Relative abundance of the most significantly different subsystems of HEs compared to LEs shown in ANOVA plots based on shotgun metagenome analysis (Subsystems). I. At highest subsystem level (level 1) and **II.** level3. (100 most features; n=30)

Supplementary Figure 7. Bubble plots of gut microbiome of HEs and LEs based on shotgun metagenome (RefSeq). I. visualized at phylum level and **II.** genus level. (50 most abundant taxa; n=30)

Supplementary Figure 8. Shotgun metagenome-derived information on the microbial community composition in samples of HEs and LEs (RefSeq). **I** Shannon diversity and **II.** RDA plot based on strain level. **III.** LEfSe analysis and **IV-VII.** ANOVA plots at phylum level. **VIII.** LEfSe analysis and **IX-XIV.** ANOVA plots at the genus level (100 most abundant taxa; n=30).

Supplementary Figure 9. Significant differences were also observed at species level based on shotgun metagenome analysis (RefSeq). I. Bubble plot of the 50 most abundant taxa. II. LefSe analysis and III. ANOVA plot of 100 most abundant taxa. (n=30)

Supplementary Figure 10. Microbial community differs significantly with respect to methane production based on shotgun metagenome analysis (RefSeq). I. LEfSe analysis and **II.** ANOVA plot at superkingdom level. **III.** PCoA plot at RSV level. **IV.** ANOVA plot showing significant differences at phylum (100 most abundant) and **V.** genus level (50 most abundant taxa). (n=30)

Supplementary Figure 11. Diversity and composition of the archaeal community as detected in HE and LE samples based on shotgun metagenomic analyses (RefSeq). I Alpha diversity based on Shannon index**, II.** RDA plot**, III.** PCoA plot**, IV:** LEfSe analysis on genus level**.**

Supplementary Figure 12. Archaeal network in LE and HE (blue) based on shotgun metagenomics information (RefSeq).

Supplementary Figure 13. Correlations with dietary intake. BIOENV analysis showing explanatory variables triggering the microbial communities of HEs (blue) and LEs (red) based on Euclidean distances that were superimposed on a Non-metric multidimensional scaling (NMDS) plot derived from Bray-Curtis dissimilarities of HE and LE samples (stress:0.1939). *Methanobrevibacter* read counts were included as a variable for better orientation.

SUPPLEMENTARY TABLES

Supplementary Table 1. Characteristics of all participants (n=100).

Supplementary Table 2. Characteristics of the matched subset (n=30).

Supplementary Table 3. Keystone taxa of high and low methane emitters (n=30). Identified key taxa based on LEfSe analysis of the 600 most abundant genera/RSVs. Numbers in column 2 and 3 refer to Figure 6b and c.

Supplementary Table 4. **High quality dereplicated key MAGs** including quality and replication estimates as well as taxonomic classification according to GTDB.

Supplementary Table 5. Metabolite concentrations in high and low methane emitters (n=30).

Supplementary Table 6. Correlations of different parameters (general, keystone taxa, metabolites and diet) of this study.

SUPPLEMENTARY ITEMS

Supplementary Item 1. Krona chart based on amplicon data (universal, n=100).

Supplementary Item 2. Krona chart based on amplicon data (archaea, n=100).

Supplementary Item 3. Krona chart based on metagenomic data (SEED, n=30).

Supplementary Item 4. Krona chart based on metagenomic data (RefSeq, archaea and bacteria only, n=30).

Supplementary Item 5. Krona chart based on metagenomic data (RefSeq, archaea only, n=30).

Supplementary Item 6. Heatmap of amino acid flux predictions according to MICOM (universal primer: 515F-806R; n=30)

Supplementary Item 7. Heatmap of C1-C4 flux predictions according to MICOM (universal primer: 515F-806R; n=30)

Supplementary Item 8. Heatmap of complex compound flux predictions according to MICOM (universal primer: 515F-806R; n=30)

Supplementary Item 9. Heatmap of fat flux predictions according to MICOM (universal primer: 515F-806R; n=30)

Supplementary Item 10. Heatmap of nucleotide flux predictions according to MICOM (universal primer: 515F-806R; n=30)

Supplementary Item 11. Heatmap of other metabolite flux predictions according to MICOM (universal primer: 515F-806R; n=30)

Supplementary Item 12. Heatmap of sugar flux predictions according to MICOM (universal primer: 515F-806R; n=30)

Supplementary Item 13. Heatmap of vitamine flux predictions according to MICOM (universal primer: 515F-806R; n=30)

SUPPLEMENTARY DATASETS

Supplementary Dataset 1. Feature table amplicon data of universal approach (universal primer: 515F-806R; n=100).

Supplementary Dataset 2. Feature table amplicon data of archaeal approach (nested PCR: 344F-1041R, 519F-806R; (n=100).

Supplementary Dataset 3. Feature table metagenomic data showing functional gene information (SEED; n=30).

Supplementary Dataset 4. Feature table metagenomic data showing taxonomic information (RefSeq; n=30).

Supplementary Dataset 5. MICOM growth rate predictions (universal primer: 515F-806R; n=30).

Supplementary Dataset 6. MICOM metabolite flux predictions (universal primer: 515F-806R; n=30).

Supplementary Dataset 7. Adapted per sample diet model for MICOM (n=30).

SCIENTIFIC REPORTS
natureresearch

OPEN

Preliminary insights into the impact of primary radiochemotherapy on the salivary microbiome in head and neck squamous cell carcinoma

Christina Kumpitsch[1], Christine Moissl-Eichinger[1,2], Jakob Pock[3], Dietmar Thurnher[3] & Axel Wolf[3]✉

Squamous cell carcinoma is the most common type of throat cancer. Treatment options comprise surgery, radiotherapy, and/or chemo(immuno)therapy. The salivary microbiome is shaped by the disease, and likely by the treatment, resulting in side effects caused by chemoradiation that severely impair patients' well-being. High-throughput amplicon sequencing of the 16S rRNA gene provides an opportunity to investigate changes in the salivary microbiome in health and disease. In this preliminary study, we investigated alterations in the bacterial, fungal, and archaeal components of the salivary microbiome between healthy subjects and patients with head and neck squamous cell carcinoma before and close to the end point of chemoradiation ("after"). We enrolled 31 patients and 11 healthy controls, with 11 patients providing samples both before and after chemoradiation. Analysis revealed an effect on the bacterial and fungal microbiome, with a partial antagonistic reaction but no effects on the archaeal microbial community. Specifically, we observed an individual increase in *Candida* signatures following chemoradiation, whereas the overall diversity of the microbial and fungal signatures decreased significantly after therapy. Thus, our study indicates that the patient microbiome reacts individually to chemoradiation but has potential for future optimization of disease diagnostics and personalized treatments.

Approximately 2.9% of newly diagnosed cancers originate in the oral cavity and pharynx[1,2]. Major risk factors are tobacco and alcohol consumption, as well as infection with human papilloma virus (HPV)[2–4]. Squamous cell carcinoma (SCC), the most common type of throat cancer, is a malignant epithelial tumor that originates in the lining of the upper respiratory tract. SCC is diagnosed by a combination of clinical, radiological, and histopathological examinations, and standard treatments comprise surgery (i.e. removal of the diseased tissue), radiotherapy, and/or chemo(immuno)therapy[2].

Radiotherapy in combination with platinum-based chemotherapy can be indicated in both surgically resectable and non-resectable SCCs. In surgically resectable cases, the literature shows a mortality benefit from adjuvant radiochemotherapy (RCHT) in patients with advanced head and neck cancer[5]. Resection techniques as well as microvascular reconstruction of the tumor side often result in large cosmetic and functional defects, and total tumor resection may not be achieved[6]. Thus, the treatment of choice in locally advanced, non-resectable cases is usually primary radiotherapy in combination with chemo(immuno)therapy.

In principle, radiotherapy and chemotherapy act by interfering with cell division, which particularly affects cancer cells that have a higher proliferation rate than healthy tissue[7,8]. Due to the non-specificity for SCC cells, local radiotherapy and/or chemo(immuno)therapy results in several side effects affecting the pharynx area. Although intensity-modulated radiotherapy treatment regimens were introduced in the early 2000s, exposure of the head and neck region to radiation still leads to severe side effects, including xerostomia, mucositis, pain and swallowing dysfunction[9], and decreased quality of life.

More than 90% of head and neck cancer patients receiving radiotherapy develop oral mucositis, leading to hindering symptoms in approximately 40% of these patients[7,8,10]. Primary symptoms of oral mucositis include

[1]Diagnostic and Research Institute of Hygiene, Microbiology and Environmental Medicine, Medical University of Graz, Graz, Austria. [2]BioTechMed, Graz, Austria. [3]Department of Otorhinolaryngology, Medical University of Graz, Graz, Austria. ✉email: a.wolf@medunigraz.at

erythema and pain approximately 4–5 days after the start of therapy and can lead to further phases of mucositis, including local ulceration. These changes have an impact on the local environment, including microbial changes in bacteria, fungi, and viruses[10].

Like other surfaces of the human body, the upper respiratory tract is covered in human-associated microorganisms, the microbiome[11]. Imbalances in the microbiome (dysbioses) have been linked to disease, including inflammatory bowel disease, diabetes, colon cancer, obesity, and mental illnesses. The microbiome of the upper respiratory tract comprises more than 1000 different microorganisms, including bacteria, archaea, and eukaryotes (mainly fungi). In general, the microbiome of this area is important for the integrity of the mucosa and modulation of the immune system, as human commensal bacteria have immunomodulatory properties and are able to ensure an efficient and rapid defense against pathogens by priming their host's immune response. In addition to immune system modulation, commensal bacteria occupy the mucosa, preventing pathogen colonization due to uncontrolled settling in open niches[11]. *Streptococcus, Veillonella, Gemella, Rothia, Fusobacterium,* and *Neisseria* species are amongst the most abundant microorganisms of the upper respiratory tract; however, diseases of the upper respiratory tract, as also pharyngeal SCC, have been linked to changes in the relative abundance and overall microbial composition[11–14].

Cancer therapy-induced oral mucositis is considered to have a microbiome component, as disruption of the mucosal barrier (caused by inflammation and apoptosis leads to bacterial translocation and an increase in the inflammatory response[15,16]. Notably, the oral microbiome could play a role in exacerbating or protecting from mucosal injury, and the roles may fluctuate depending on the stage of inflammation[15].

Although a better understanding of the impact of radiochemotherapy (RCHT) on the oral microbiome may provide information regarding the pathophysiology and potential adjuvant treatment approaches in RCHT-induced mucositis in patients with head and neck SCC, the available literature in this field is sparse and largely restricted to cultivation-based analyses[17,18]. One exception is the study by Hu et al. in 2013[19], in which they performed a pyrosequencing-based microbiome analysis of plaque samples taken from subjects undergoing head-and-neck radiotherapy. As high-throughput sequencing techniques and methodological strategies (e.g., selection of primers) have made substantial progress over the last few years, we think it is necessary to perform additional pilot studies for comparative reasons as a baseline for subsequent, deeper analyses[19]. Furthermore, additional studies based on high-throughput next generation sequencing of well-defined patient samples are necessary to define potential microbial processes and provide potential targets for the prevention, diagnosis, and treatment of RCHT-associated side effects.

The oral cavity microbiome has been the focus of research in relation to several diseases, including periodontitis and oral cancer[20]. For our studies, we chose to analyze the salivary microbiome to assess the local impact of radiotherapy on the overall oral microbiome using an easy, standardized, non-invasive sampling procedure that would also be suitable for screening patients[12].

Based on the information given above, we assume (1) that interrogation of salivary microbial biomarkers could be informative with respect to the pathobiology of RCHT-related side effects (2); that the salivary, bacterial, archaeal, and fungal microbiome reflects the disease status in head and neck SCC when compared to healthy controls; and (3) that RCHT has a significant impact on the local microbiome profile. We addressed these questions in this preliminary study.

Results

Description of the patient cohort and study overview. For this study we recruited 42 participants (whose metadata information are given in Table 1, respectively). For a short overview see Supplementary Table S1. Parts of the cohort were recruited during our previous study (see Supplementary Table S1)[12].

Description of the microbiome data. The DNA extracted from the saliva samples was subjected to three different amplification protocols: one targeting the overall microbiome ("universal" approach), one targeting the archaeal components ("archaeal" approach), and one directed to determine the fungal diversity ("fungal" approach). All sequences were quality filtered, contamination-controlled, and processed as described in the "Materials and methods".

Following the "universal" approach, 3,339,965 sequences were obtained and classified into 23 phyla, 231 genera, and 1876 unique ribosomal sequence variants (RSVs), whereas the "fungal" approach (251,488 sequences) resulted in 5 phyla, 100 genera, and 464 unique RSVs. Possibly due to the underrepresentation of archaea in human saliva, the amplification of archaeal signals proved very difficult, and the "archaeal" approach delivered only 36,856 sequences affiliated with 4 phyla and 3 genera (all RSV tables are given in Supplementary Datasets 1, 2, and 3).

Impact of SCC and radiotherapy on the bacterial microbial community. *Squamous cell carcinoma affected specific taxa compared to healthy controls but did not change the overall microbiome profile.* In an initial step, we re-analyzed the properties of the tumor-associated saliva microbiome by comparing the samples from healthy patients ($n = 11$) and SCC patients (samples from before RCHT only; $n = 31$)[12]. The "universal" approach identified Firmicutes as the predominant phylum, with genera *Veillonella*, *Rothia*, *Haemophilus*, and *Neisseria* being highly abundant in both healthy subjects and patients (see Supplementary Fig. S1 and S2).

In congruence with our previous study[12], we detected no significant difference in the alpha diversity between healthy controls and cancer patients ($p = 0.86$, ANOVA; Fig. 1a). The microbial communities of healthy controls formed a sub-cluster within the principal coordinate analysis (PCoA) plot but did not cluster separately from patient samples (Fig. 1b). Linear discriminant analysis effect size (LEfSE) analysis run at the genus level identified a potential association of *Veillonella* and Bifidobacteriaceae (including genus *Bifidobacterium*) with patients, and

Group	N	Mean age ± SD, years	Sex	HPV	Alcohol	Smoking	Tumor Localization
Healthy	11	47.8 ± 15.2	m (n = 10) f (n = 1)	n.a. (n = 11)	Never (n = 2), Occasional (n = 9)	No (n = 10) Yes (n = 1)	n.a. (n = 11)
Diseased	31 (20 + 11)	60 ± 7.5	m (n = 26) f (n = 5)	Positive (n = 7) Negative (n = 24)	Never (n = 7), Occasional (n = 14), Daily (n = 8), n.a. (n = 2)	No (n = 19) Yes (n = 12)	Oropharynx (n = 18), Hypopharynx (n = 3), Epipharynx (n = 1), Larynx (n = 3), CUP (n = 1), Oral cavity (n = 5)
Before (patients sampled only before therapy)	20	61.1 ± 7.4	m (n = 16) f (n = 4)	Positive (n = 5) Negative (n = 15)	Never (n = 4), Occasional (n = 7), Daily (n = 7), n.a. (n = 2)	No (n = 11) Yes (n = 9)	Oropharynx (n = 11), Hypopharynx (n = 3), Epipharynx (n = 0), Larynx (n = 1), CUP (n = 1), Oral cavity (n = 5)
After (patients sampled before and after therapy)	11	58 ± 7.6	m (n = 10) f (n = 1)	Positive (n = 2) Negative (n = 9)	Never (n = 3), Occasional (n = 7), Daily (n = 1)	No (n = 8) Yes (n = 3)	Oropharynx (n = 7) Hypopharynx (n = 0) Epipharynx (n = 1) Larynx (n = 2) CUP (n = 1) Oral cavity (n = 0)
All participants	42	56.8 ± 11.3	m (n = 36) f (n = 6)	Positive (n = 7) Negative (n = 24) n.a. (n = 11)	Never (n = 9), Occasional (n = 23), Daily (n = 8), n.a. (n = 2)	No (n = 29) Yes (n = 13)	Oropharynx (n = 18) Hypopharynx (n = 3) Epipharynx (n = 1) Larynx (n = 3) CUP (n = 1) Oral cavity (n = 5)

Table 1. Characteristics of the study participants. *diseased* squamous cell carcinoma patients, *m* male, *f* female, *SD* standard deviation, *CUP* carcinoma of unknown primary, *n.a.* not available.

Pasteurellaceae, *Eubacterium* and others with healthy controls (Fig. 1c–f). *Veillonella* was significantly increased in samples from healthy subjects, which is in agreement with our previous data. Notably, *Fusobacterium* also significantly correlated with the healthy status (Fig. 1g,h), although *Fusobacterium* is increasingly being recognized as being linked to cancer development[21,22].

RCHT has an impact on microbiome composition. Next, we focused on analyzing the impact of RCHT on the salivary microbiome in patients for whom we had samples from before and after RCHT (n = 11). All patients underwent additional antibiotic/mycobiotic therapy along with the RCHT, which was highly individual and adapted according to clinical requirements as shown in Supplementary Table S2 and Supplementary Fig. S3.

With the universal approach, the diversity of the microbiome decreased after therapy, but it was not significant (p = 0.42, ANOVA; Fig. 2a). According to the redundancy analysis (RDA), RCHT had a significant impact on the microbiome profile (p = 0.014; Fig. 2b), and the sample types grouped somewhat differently in the PCoA plot (see Supplementary Fig. S4). The different microbial profile as well as the difference in bacterial load were also visible in the bar-plot analysis (Fig. 2c), as the relative abundance of major taxa was significantly reduced. Overall, the derived number of sequences decreased from an average 55,092 reads per sample before RCHT to an average 38,980 reads per sample after RCHT.

We observed that some taxa were specifically affected by the treatments, such as *Haemophilus, Veillonella, Granulicatella,* and others, whereas other bacterial genera were specifically increased after treatment (*Lactobacillus, Scardovia, Acinetobacter, Enterococcus* etc.; Fig. 2d).

For the sake of completeness, we were able to confirm the correlation of alcohol consumption with the microbial profile (p = 0.075; see Supplementary Fig. S5)[12].

The impact of SCC and radiotherapy on the non-bacterial microbial community.

SCC does not significantly affect the archaeal and fungal profiles. As the bacteria-centric "universal" approach is not capable of assessing the full microbial diversity and patients frequently suffer from fungi-driven mucositis, we performed additional archaeal and fungi-targeting amplicon sequencing with specific primer sets for these taxa. The archaeal dataset did not prove indicative for any of the parameters, particularly as only a small proportion of all samples revealed positive archaeal signals (20 out of 53); however, these were mainly samples from cancer patients (3 healthy controls, 13 patients before therapy, and 4 after therapy). In agreement with our previous study[12], we confirmed the presence of *Methanobrevibacter* signatures, and detected *Methanosphaera*, and *Candidatus* Nitrosoarchaeum sequences (Supplementary Dataset 1).

Overall, 100 fungal genera were detected, with *Candida* being the most prevalent in most samples. Other frequent fungal signatures were from unclassified genera, *Peniophora, Stereum, Cladosporium,* and many others (Fig. 3a), some of which may be associated with the environment and not the human microbiome. The salivary mycobiome of healthy volunteers and SCC patients before therapy did not group differently in the PCoA plot, and no alteration in alpha diversity was observed (Fig. 3b,c).

RCHT does affect the archaeal and fungal profiles of salivary samples. The overall relative abundance of the 35 most abundant fungal signatures increased after RCHT compared to the samples from patients before RCHT.

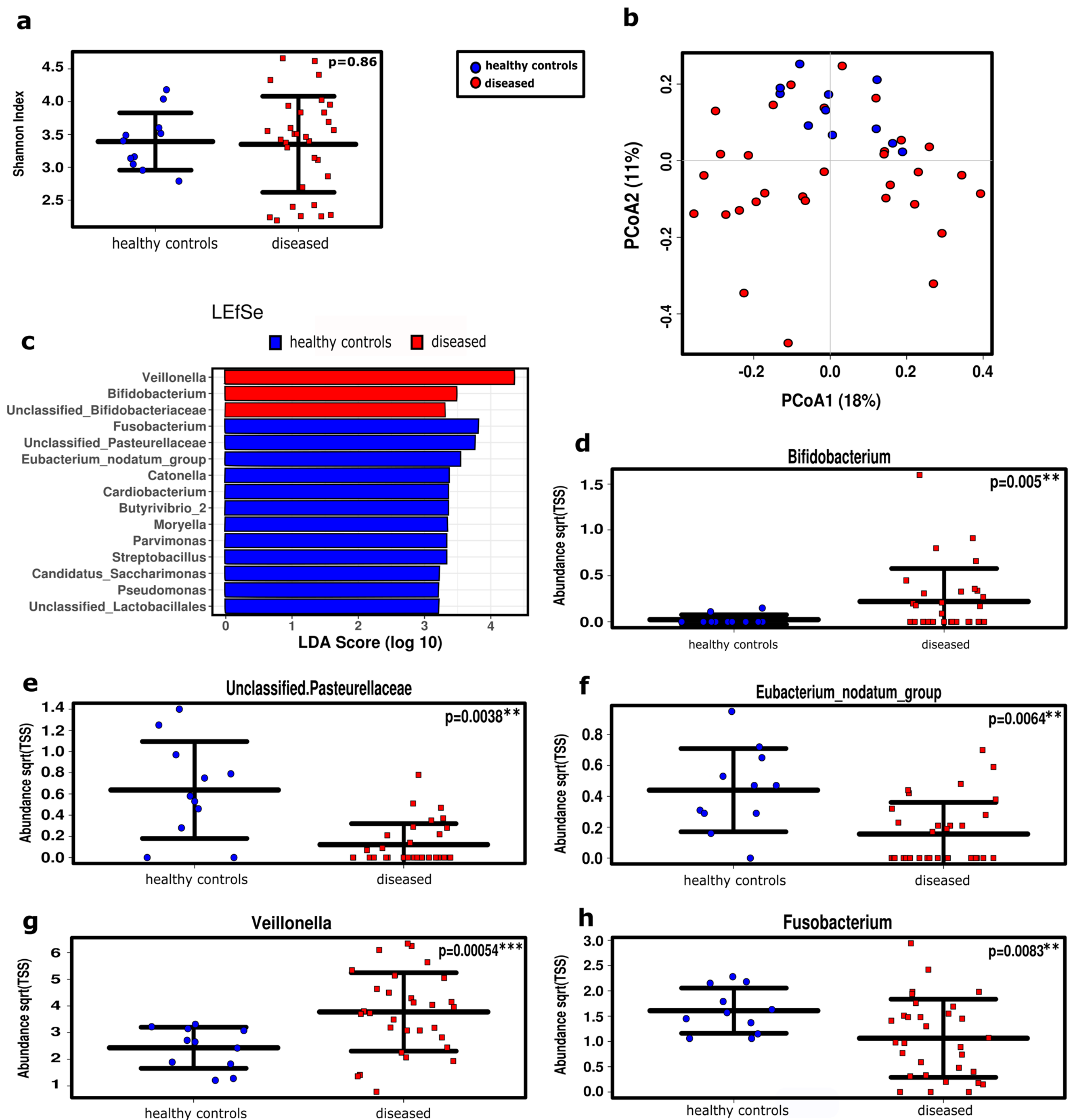


Figure 1. Microbial diversity and profile in cancer patients ('diseased') did not differ significantly from healthy controls, but healthy individuals formed a sub-cluster within the diseased cohort. (**a**) No alterations were observed in alpha diversity between the two groups at the RSV level using ANOVA. (**b**) Microbiome profile of healthy controls clustered within the diseased subjects in a PCoA plot (RSV level). (**c**) LEfSe analysis of taxa associated with healthy subjects and SCC, including the top 100 most abundant genera. (**d**–**h**) Relative abundance of the genera *Bifidobacterium*, *Pasteurellaceae*, *Eubacterium*, *Veillonella,* and *Fusobacterium* in healthy subjects compared to patients.

However, the increase in relative abundance was accompanied by a significant decrease in the fungal diversity ($p=0.026$; Fig. 4a,b; see also Supplementary Fig. S6).

When comparing the fungal profile of salivary samples from patients before and after RCHT, RDA indicated a potential impact of the treatment on the fungal microbial composition ($p=0.101$; Fig. 4c).

Interestingly, compared to healthy controls, the relative abundance of *Candida* was increased in patients ($p=0.39$; Fig. 5). After RCHT, the relative abundance of *Candida* increased further or decreased, resulting in a total increase in the summed relative abundance (all samples; Fig. 4a). However, due to individual development for each patient, the mean relative abundance of *Candida* remained similar before and after RCHT (Figs. 4a, 5).

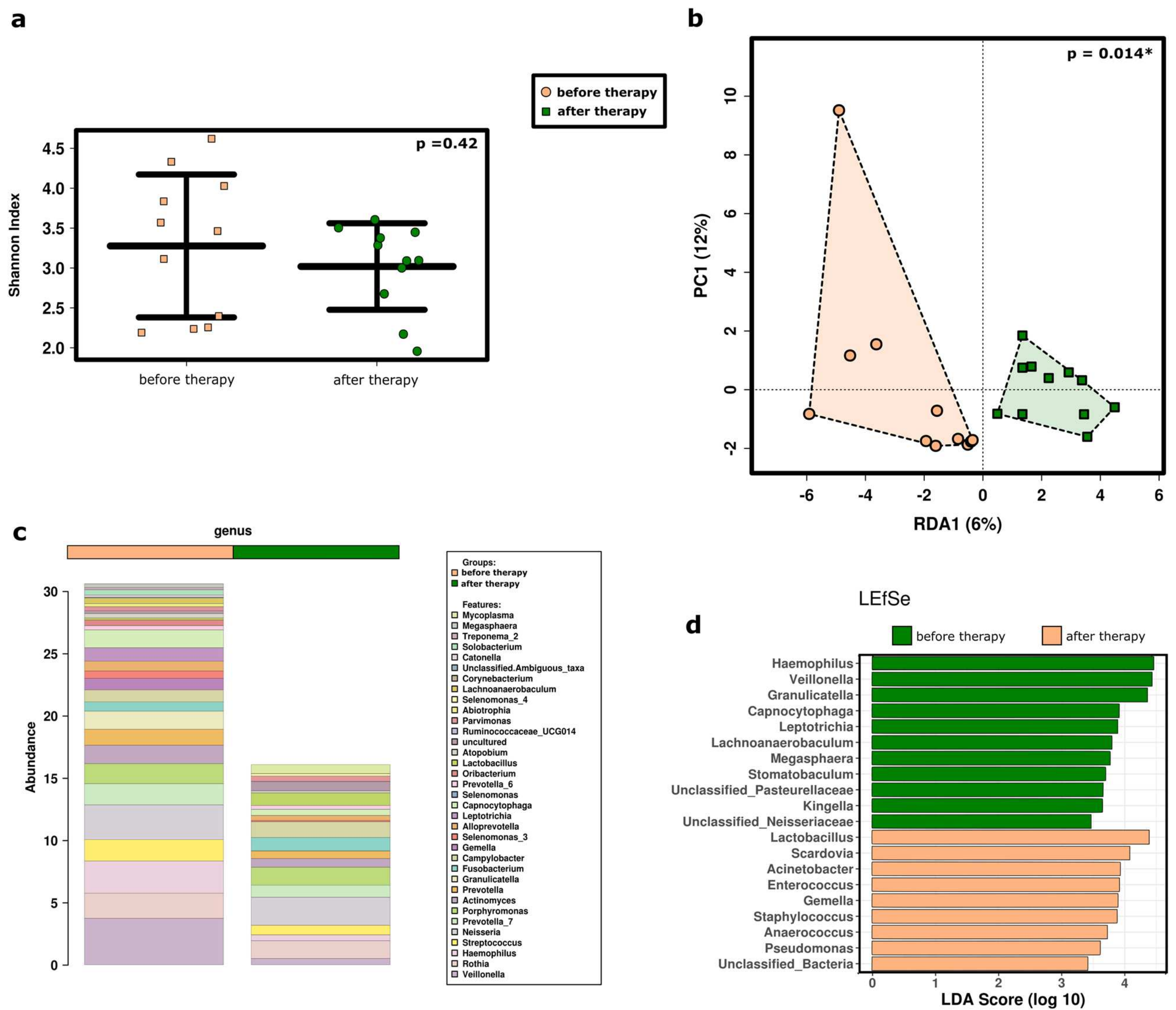


Figure 2. Radiochemotherapy in cancer patients substantially impacts the microbial profile. (**a**) Shannon Diversity Index did not reveal significant differences in the salivary microbiome between patients before and after therapy at the RSV level using ANOVA. (**b**) Treatment had a significant impact on the microbiome profile (RDA plot, RSV level). (**c**) Bar plot of the microbiome composition before and after therapy, including the 35 most abundant genera. Taxa are displayed from bottom to top. (**d**) Specific taxa that were significantly increased before or after therapy are plotted in a LEfSe including the 35 most abundant genera.

Overall, we detected no influence of mycostatin on the fungal profile (RDA p = 0.924; patients after therapy; see also Supplementary Fig. S7), which may be due to the prevalence of environmentally associated fungal signatures in our dataset. Comparing the fungal communities of all patients (n = 11) before and after therapy, the alpha diversity decreased significantly after treatment (p = 0.026; Fig. 6a). Samples from mycostatin users revealed significantly higher fungal diversity compared to patients who did not receive mycostatin (p = 0.045; Fig. 6b).

When we evaluated the mycobiomes of mycostatin users (n = 8) before and after RCHT, a tendency towards reduced fungal diversity was still visible, but no longer significant (p = 0.096; Fig. 6c). Similarly, the alpha diversity of samples from non-users decreased after therapy (p = 0.071; Fig. 6d). As a decrease in fungal diversity was observed independent from mycostatin usage, RCHT seemed to be a major factor influencing the fungal microbiome.

Interestingly, although mycostatin is an antimycotic used to treat yeast infections, *Candida* seemed to be unaffected (see Supplementary Fig. S8a,b).

Comparison of the microbiome and mycobiome of healthy controls with patients before and after standard therapy. The impact of disease and radiotherapy on the fungal and bacterial microbiome was specifically addressed by comparing salivary profiles from healthy controls (n = 11), SCC patients before radiotherapy (n = 11), and the same SCC patients after radiotherapy (n = 11). Overall, the bacteriome and mycobiome revealed a partially antagonistic behavior. Though the diversities of both taxonomic groups, as well as the

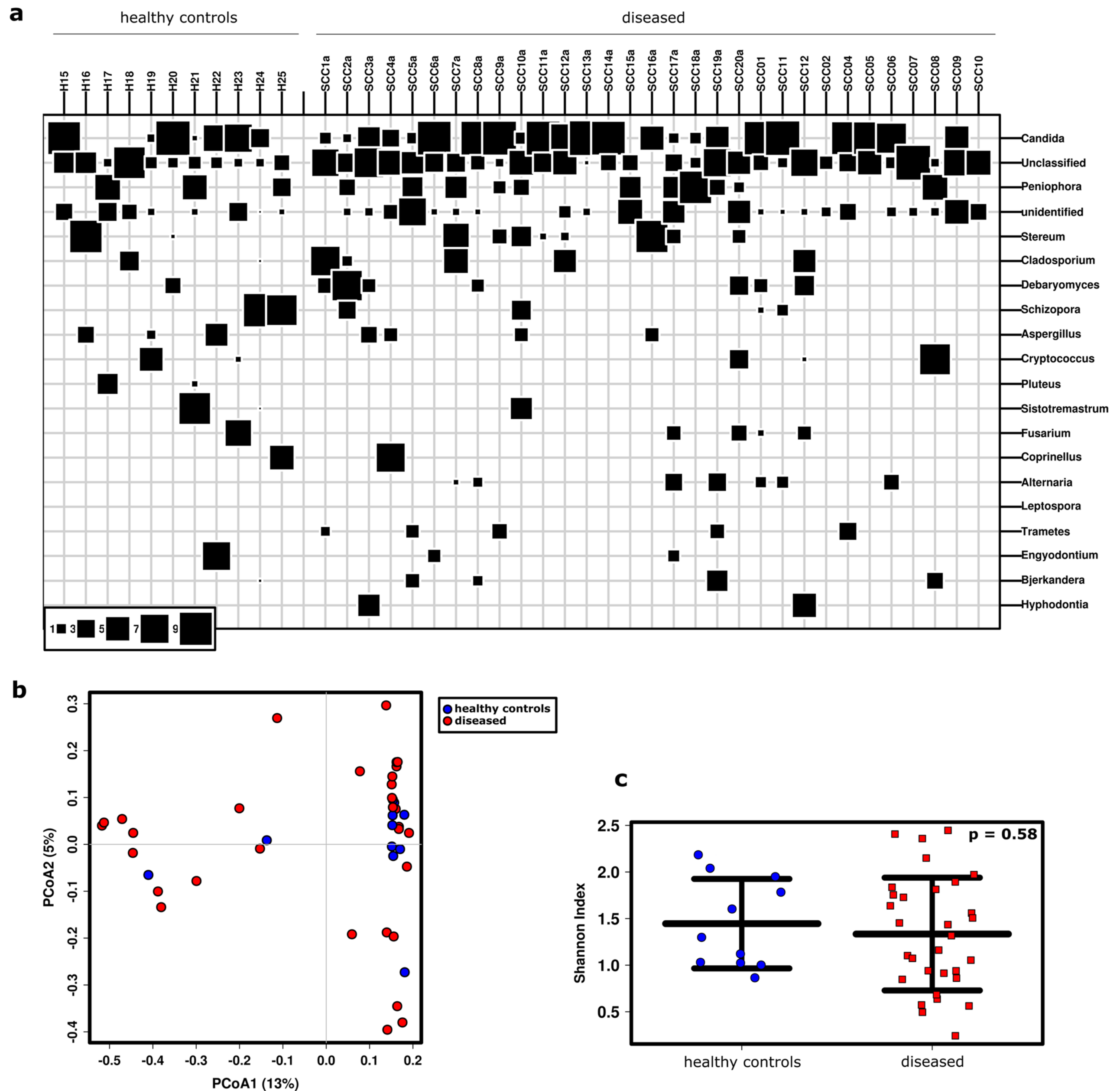


Figure 3. The salivary mycobiome was not significantly affected by SCC. (**a**) Bubble-plot including the top 35 most abundant genera of the study cohort [i.e., healthy controls and SCC patients before any therapy ('diseased')]. (**b**) PCoA plot indicating no clustering of samples from healthy subjects and diseased subjects at the RSV level. (**c**) Similar Shannon Diversity Indices in both subject groups.

relative abundance of bacteria, decreased from healthy to non-treated, and then to treated, patients (Shannon index, $p = 0.4$ (bacteria) and $p = 0.02$ (fungi); Fig. 7a,b), the relative abundance of fungal signatures (particularly *Candida*) increased with treatment (Fig. 7c,d, bar plots) and remained unaffected from mycostatic treatment ($p = 0.924$, RDA; see Supplementary Fig. S8). Notably, redundancy analyses indicated a significant impact of health and treatment status on the bacteriome, and a tendency was seen in the mycobiome ($p = 0.001$ and $p = 0.08$, respectively, RDA plots; Fig. 7e,f).

Discussion

This preliminary study aimed for an initial comparative analysis of oral microbial communities using saliva from head and neck cancer patients and healthy controls. We found evidence that the occurrence of SCC and RCHT alone or in combination with local antimycotic treatment have a substantial impact on the microbial profile. These findings support theories that head and neck SCC itself, but also tumor-specific therapies, interacts with the oropharyngeal saliva microbiome.

The ecology of the saliva has been investigated previously in numerous studies primarily using culture-based methods[23–29]. For example, Almståhl, et al. reported the association of cultivable *Candida albicans* and

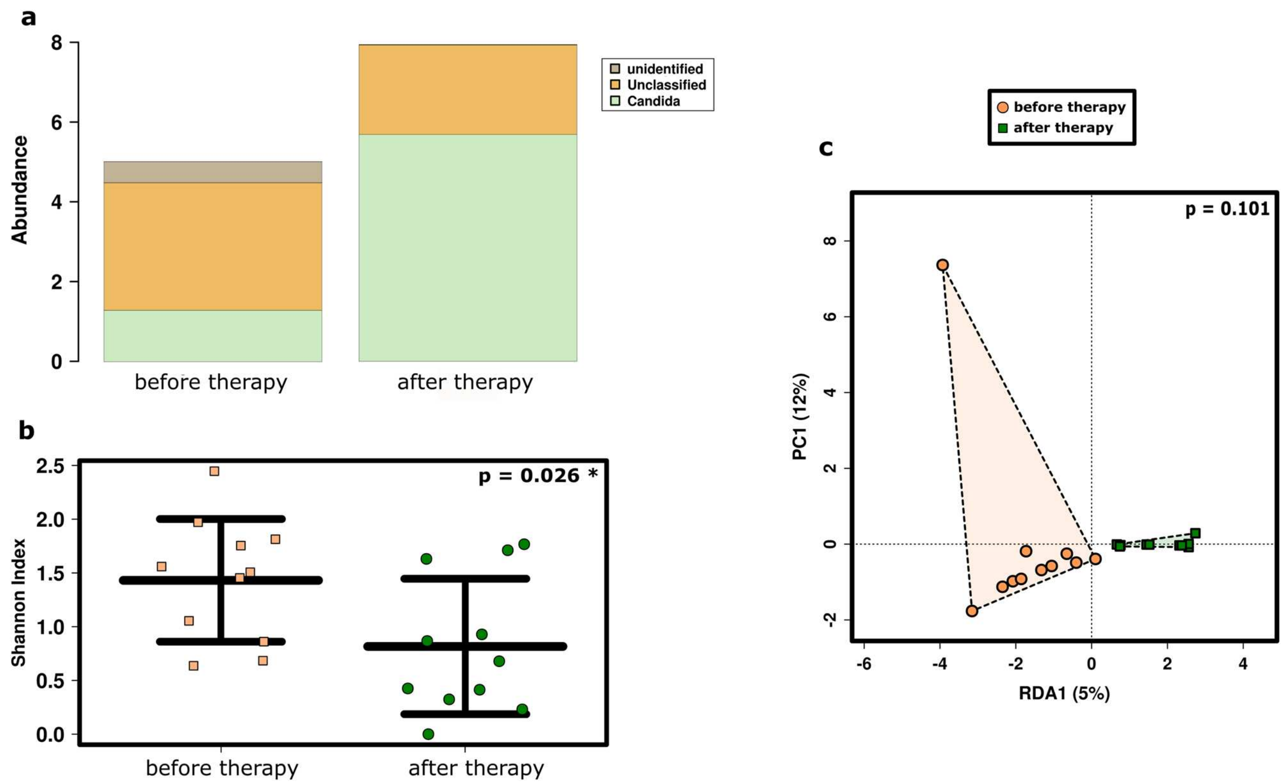


Figure 4. Radiochemotherapy influenced the salivary mycobiome of SCC patients. (**a**) Bar plot of the 35 most abundant fungal genera detected in patients' saliva before and after therapy. (**b**) Alpha diversity was significantly decreased after SCC therapy at the RSV level using ANOVA. (**c**) Fungal profiles were not significantly influenced by the therapy. RDA plot; RSV level; ANOVA.

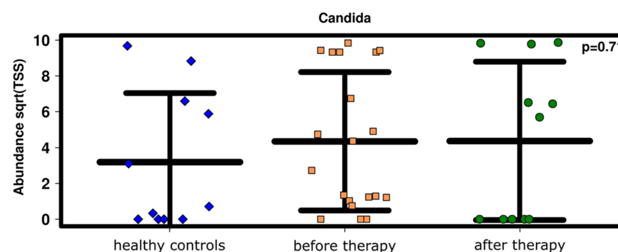


Figure 5. A non-significant but slight increase in the relative abundance of *Candida* was observed in patients before and after therapy compared to healthy individuals. Analysis was performed with ANOVA.

enterococci with radiation-induced hyposalivation, and the presence of *Lactobacillus* spp. in 92% of all radiotherapy patients[17]. The prevalence of *Candida*, as well as enterococci and various Enterobacteriaceae (*Enterobacter, Klebsiella, Proteus, Pseudomonas*), was confirmed in a cultivation-based study focusing on radiotherapy patients[18]. Notably, lactobacilli and *Acinetobacter* have been shown to occupy open niches that can also be found after RCHT[30].

Advances in DNA sequencing have given us the opportunity to analyze microbes that are not amenable to cultivation using high-throughput sequencing of the microbial 16S rRNA gene[31,32]. Overall, in other studies investigating head and neck SCC patients using 16S rRNA gene sequencing, the results are not comparable due to the use of different methodological approaches, and sample sizes have been small[19,20]. Our study was conceptualized as a preliminary study and was also limited in sample size. Furthermore, additional medication was applied (analgesic, antibiotic, and antimycotic effects) in accordance with clinical and laboratory findings and guidelines, but differed between individual patients, following the principles of personalized medical treatments. However, we applied standardized sampling based on the Salivette Cortisol kit[12] and microbiome analysis procedures based on widely used amplification protocols and bioinformatics pipelines. This allowed us to put our data into context with our previous study, in which we described the salivary microbiome of 11 patients with oral and oropharyngeal SCC and compared it to the microbial profile of healthy controls. We observed that changes in the microbial profile mirrored disease progression and reflected clinical preconditions (age, alcohol,

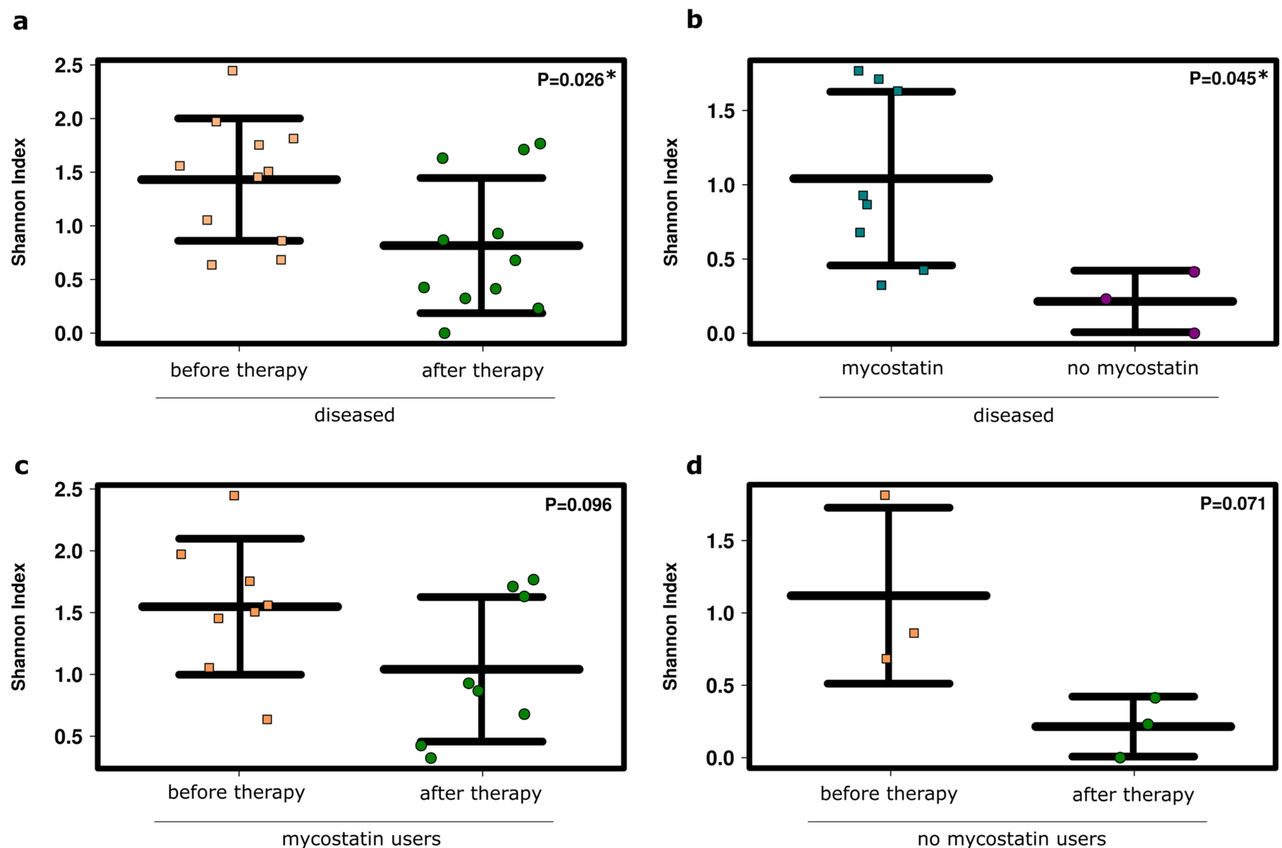


Figure 6. Radiochemotherapy (RCHT) and treatment with mycostatin affected the mycobiome of SCC patients. Shannon Diversity Indices of (**a**) patients before and after RCHT, (**b**) patients who did and did not receive mycostatin treatment, (**c**) mycostatin users only before and after RCHT, and (**d**) patients who did not receive any mycostatin treatment before and after RCHT. ANOVA; diseased: SCC patients.

tumor size, lymph node status, smoking habit, HPV-positivity)[12]. Including further datasets from our present study, we confirmed our findings regarding the comparison of our primary SCC patients and healthy controls. An increased relative abundance of *Bifidobacterium* was observed in our patient group, whereas the taxon Pasteurellaceae was decreased. Importantly, the fungal microbiome was addressed in the present study and showed an increased relative abundance of fungal signatures, whereas bacterial signatures decreased between healthy and non-treated patients.

Although certain aspects of the microbiome profiles of healthy subjects and SCC patients are different, studies have failed to delineate clear microbial patterns and are partially contradictory. In a previous study based on checkerboard DNA-DNA hybridization of samples from 45 patients and 229 healthy controls, increased counts of *Capnocytophaga gingivalis*, *Prevotella melaninogenica,* and *Streptococcus mitis*[13] were identified as potential biomarkers for SCCs with a relatively high sensitivity and specificity (both > 80%). Gong et al. focused on the tissue-associated microbiome and identified 26 genera that were significantly different between patients with laryngeal SCC and controls. The highest abundance was observed for *Fusobacterium*, *Prevotella*, and *Gemella* in the patient group[14]. Other studies have reported a relative intraindividual decrease in *Streptococcus* and *Rothia* species in cancer patients when analyzing samples from various sites in the oral cavity[20] but, like many others, failed to clearly segregate cancer from non-cancer microbiome samples[33]. This indicates a high level of individuality across the microbiome profile and highlights the importance of additional studies with larger cohorts.

In the present study, the impact of RCHT was clearly be observed in the salivary microbiome of SCC patients before and after therapy. We observed changes in the microbial profiles for bacteria and fungi, but not in the archaeal profile due to a substantial number of archaea-negative samples. An association of *Veillonella* and *Actinomyces* signatures with higher cumulative doses (30 to 60 Gy) was reported by Hu et al.[19], who performed a pyrosequencing-based microbiome analysis of plaque samples taken from subjects undergoing head-and-neck radiotherapy. In our study, however, the relative abundance of *Veillonella* signatures decreased with RCHT. This difference in the abundance of *Veillonella* may be due to the use of different methodological approaches (e.g., sample site, sequencing methods) or the therapy the patients received (e.g., antibiotics, chemoradiation).

In general, microbial changes before and after RCHT may be explained by therapy-associated effects on highly proliferative (tumor), but also healthy proliferative tissue, including the mucosa of the upper respiratory tract. Endotoxins are released, penetrate the submucosa, and induce chemotactic activity on polymorphonuclear neutrophils and macrophages, leading to the activation of pro-inflammatory cytokines and progressive tissue destruction[7,16]. Thus, microbial changes can be explained by the application of RCHT in our cohort.

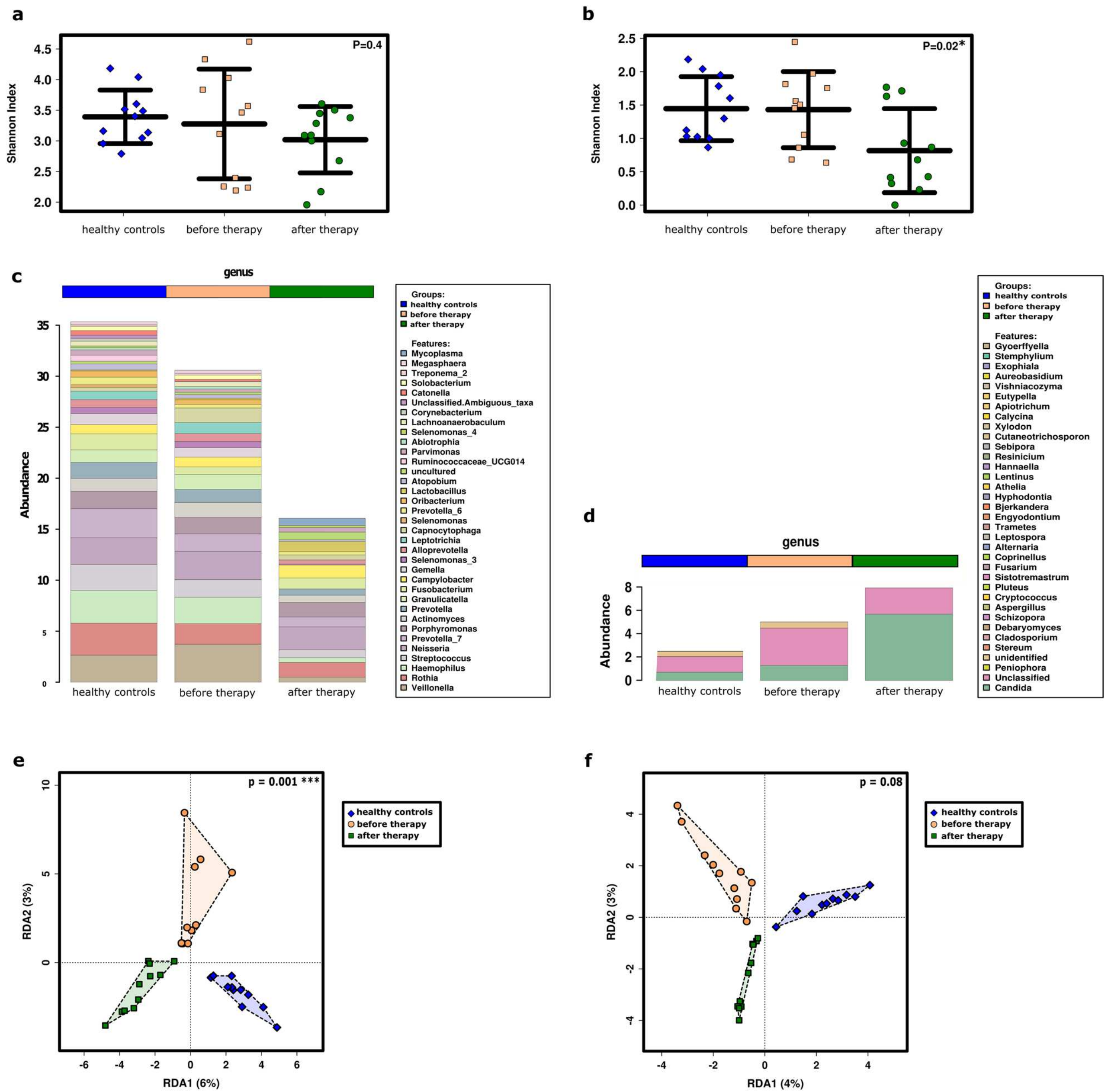


Figure 7. The bacterial and fungal microbiome of healthy subjects and SCC patients is more similar before radiochemotherapy than after. Shannon Diversity of the (**a**) bacterial and (**b**) fungal communities at RSV level (ANOVA). Bar plot of the top 35 most abundant (**c**) bacterial and (**d**) fungal genera. Redundancy analysis of the (**e**) bacterial and (**f**) fungal profiles using ANOVA. Taxa listed in the bar-plot are displayed from bottom to top.

Microbial dysbiosis and infection can be avoided and treated with antibiotic and/or antimycotic treatment, which was applied to a number of patients in our study. Notably, our data do not indicate a significant impact of antibiotic or, specifically, antimycotic treatment on the microbial changes triggered by RCHT.

The main limitations of our study were the limited sample size and the heterogeneity of the subjects with respect to clinical parameters (medical treatment) and demographics. In addition, the level of alcohol consumption could not be assessed reliably, and the carcinogenic effects cannot be delineated. Furthermore, additional parameters, such as oral hygiene, which may also contribute to oral health and changes in microbial composition, should be considered in future studies.

In this preliminary study, we defined microbial changes in patients before and after RCHT. As probiotic medication has been reported to have a potential protective effect against radiation in the epithelium, future studies should identify the effect of probiotic medication on the oropharyngeal micro- as well as mycobiome in SCC patients undergoing RCHT[34].

We emphasize the need for larger cohort studies in order to further explore the impact of disease and treatment on the microbiome. Our study should serve as a baseline for further study and be extended towards

metagenome-based microbiome analysis to reveal functional and mechanistic information that can be used for improved diagnosis and treatment of patients.

Materials and methods

Ethics. The Department of Otorhinolaryngology, University Hospital of Graz, recruited the healthy volunteers and SCC patients. The study design was approved by the Ethics Committee of the Medical University of Graz (EK-Nr. 1325/2015). Ethics approvals were obtained according to the guidelines of the Declaration of Helsinki on biomedical research involving human subjects. Prior to inclusion, all participants provided written informed consent.

Study subjects. The study included salivary samples from 11 healthy volunteers as controls (10 males, 1 female; mean age 47.8 years, standard deviation 15.1 years) and 31 patients diagnosed with SCC of the head and neck (26 males, 5 females; mean age 60 years, standard deviation 7.5 years). Eleven patients (10 males, 1 female; mean age 58 years, standard deviation 7.6 years) provided samples before and close to the end point of the standard therapy ("after").

The clinical parameters for all participants are provided in Supplementary Table 1. Information about antibiotic and antimycotic treatments are given in Supplementary Table 2.

SCC therapy. Recommendations regarding SSC therapy was given by the local interdisciplinary head and neck cancer board. All 11 patients investigated before and after tumor-specific treatment received radiotherapy in combination with platinum-based chemotherapy (10 cisplatin, 1 carboplatin). Radiation doses on the primary tumor side were between 60 and 70 Gy, whereas the radiation dosage in the lymphatic pathways was 50 Gy. Antibiotic and/or antimycotic treatment was applied based on clinical symptoms, local signs of infection, and laboratory findings. Eight of eleven patients received antimycotic treatment (see Supplementary Table S2 and Supplementary Fig. S3).

Sampling procedure. The Salivette Cortisol saliva detection kit (Sarstedt, Newton, NC, USA) was used to collect samples from all subjects. To stimulate and collect saliva, participants were asked to chew on a provided polyester swab for 60 s before placing it back into the tube. The Salivette Cortisol kit turned out to be a user-friendly method of collecting saliva from both the oral cavity and oropharynx that we also applied in our previous study.

The soaked swab was centrifuged for 5 min at 3000 rpm to obtain clear saliva. The sample was then immediately stored at − 70 °C until further use.

The first collection of patients' saliva took place after the primary diagnosis of SCC and prior to any specific treatment (chemoradiation therapy, surgery, antibiotics, mycostatin), whereas the second sample was collected after these treatments.

DNA extraction, PCR, and amplicon sequencing. A MagNA Lyser instrument and MagNA pure LC DNA Isolation Kit III (Roche Diagnostics, Mannheim, Germany) were used to extract microbial DNA according to the manufacturer's instructions. The microbial 16S rRNA gene was amplified using the universal primer pair 515FB and 806RB for bacterial signatures[35]. A nested PCR approach was used to amplify archaeal signatures. As a first step, the primer pair 344F and 1041R was used, followed by the primer pair 519F and 806R for archaeal signatures[35]. The ITS region was amplified using the ITS86F and ITS4R primer pair to determine fungal signatures[36,37]. Primer sequences and PCR cycling conditions are listed in Supplementary Tables S3 and S4. Sequencing was performed by the Center for Medical Research (ZMF) at the Medical University of Graz using Illumina MiSeq.

Sequence processing. Raw reads were processed using QIIME2. Briefly, reads were quality filtered and checked for the presence of chimeras before being grouped into RSVs at the level of 99%. To classify the reads, the SILVA 128 database for bacterial[38], SILVA 132 database for archaeal[38], and UNITE V8 database for fungal signatures[39] were used. Extraction blanks (negative controls) were processed in parallel and contaminations due to sample processing were removed using R studio version 1.2.1335 with the R packages phyloseq, ggplot2 and decontam with the threshold set to 0.5 (https://github.com/benjjneb/decontam)[40,41]. Chloroplast signatures and unassigned sequences were also removed. Signatures remaining from the RSV table (Supplementary Datasets 1, 2, and 3) were processed in Calypso[42]. After normalization of the dataset via total sum normalization (TSS), Calypso was used to calculate the Shannon Index (alpha diversity, to visualize evenness and abundance; RSV level), redundancy analysis (beta diversity, to summarize variations in the data set; RSV level), PCoA plot (beta diversity; RSV level), bar chart (relative abundances of different taxa; genus level), bubble plot (relative abundance of different taxa; genus level), LEfSe, to find features that most likely explain differences between groups; genus level), and ANOVA taxa abundance plots (differences in relative abundance of taxa between compared groups; genus level)[43].

Raw sequence reads are publicly available at the European Nucleotide Archive (PRJEB37299).

Exclusion criteria. Participants were not allowed to take antibiotics (local or systemic) for at least 4 weeks or receive vaccinations in the 6 months prior to the first sampling. Subjects with long-term antibiotic usage were also excluded.

Received: 30 March 2020; Accepted: 14 September 2020
Published online: 06 October 2020

References

1. Howlader, N. *et al.* SEER Cancer Statistics Review. *National Cancer Institute. Bethesda* (1975–2016) https://seer.cancer.gov/csr/1975_2016/.
2. Swiecicki, P., Malloy, K. & Worden, F. Advanced oropharyngeal squamous cell carcinoma: Pathogenesis, treatment, and novel therapeutic approaches. *World J. Clin. Oncol.* **10**, 15–26 (2016).
3. Huber, P. *et al.* Radiotherapy for advanced adenoid cystic carcinoma: Neutrons, photons or mixed beam?. *Radiother. Oncol.* **59**, 161–167 (2001).
4. Vaughan, T. L., Davis, S., Kristal, A. & Thomas, D. B. Obesity, alcohol, and tobacco as risk factors for cancers of the esophagus and gastric cardia: Adenocarcinoma versus squamous cell carcinoma. *Cancer Epidemiol. Biomarkers Prev* **4**, 85–92 (1995).
5. Lin, S., Massa, S. & Varvares, M. Improved overall survival and mortality in head and neck cancer with adjuvant concurrent chemoradiotherapy in national databases. *Head&Neck* **38**, 208–215 (2014).
6. Tam, M. & Hu, K. Regional radiation therapy for oropharyngeal cancer in the HPV era. *Semin. Radiat. Oncol.* **29**, 126–136 (2019).
7. Subramaniam, N. & Muthukrishnan, A. Oral mucositis and microbial colonization in oral cancer patients undergoing radiotherapy and chemotherapy: A prospective analysis in a tertiary care dental hospital. *J. Investig. Clin. Dent.* **10**, e12454 (2019).
8. Del Corso, G., Villa, A., Tarsitano, A. & Gohel, A. Current trends in oral cancer: A review. *Cancer Cell Microenviron.* **3**, 1–11 (2016).
9. Hay, A. & Nixon, I. Recent advances in the understanding and management of oropharyngeal cancer. *F1000Res.* **7**, 2 (2018).
10. Stringer, A. & Logan, R. The role of oral flora in the development of chemotherapy-induced oral mucositis. *J. oragl Pathol. Med.* **44**, 81–87 (2015).
11. Kumpitsch, C., Koskinen, K., Schöpf, V. & Moissl-Eichinger, C. The microbiome of the upper respiratory tract in health and disease. *BMC Biol.* **17**, 87 (2019).
12. Wolf, A. *et al.* The salivary microbiome as an indicator of carcinogenesis in patients with oropharyngeal squamous cell carcinoma: A pilot study. *Sci. Rep.* **7**, 1–10 (2017).
13. Mager, D. *et al.* The salivary microbiota as a diagnostic indicator of oral cancer: A descriptive, non-randomized study of cancerfree and oral squamous cell carcinoma subjects. *J. Transl. Med.* **3**, 27 (2005).
14. Gong, H. *et al.* The composition of microbiome in larynx and the throat biodiversity between laryngeal squamous cell carcinoma patients and control population. *PLoS ONE* **8**, e66476 (2013).
15. Hong, B. *et al.* Chemotherapy-induced oral mucositis is associated with detrimental bacterial dysbiosis. *Microbiome* **7**, 66 (2019).
16. Sonis, S. Mucositis: The impact, biology and therapeutic opportunities of oral mucositis. *Oral Oncol.* **45**, 1015–1020 (2009).
17. Almståhl, A., Wikström, M. & Fagerberg-Mohlin, B. Microflora in oral ecosystems in subjects with radiation-induced hyposalivation. *Oral Dis.* **14**, 541–549 (2008).
18. Gaetti-Jardim, E. *et al.* Occurrence of yeasts, pseudomonads and enteric bacteria in the oral cavity of patients undergoing head and neck radiotherapy. *Braz. J. Microbiol.* **42**, 1047–1055 (2011).
19. Hu, Y. *et al.* Exploring the dynamic core microbiome of plaque microbiota during head-and-neck radiotherapy using pyrosequencing. *PLoS ONE* **8**, e56343 (2013).
20. Schmidt, B. *et al.* Changes in abundance of oral microbiota associated with oral cancer. *PLoS ONE* **9**, e98741 (2014).
21. Whitmore, S. & Lamont, R. Oral bacteria and cancer. *PLOS Pathog.* **10**, e1003933 (2014).
22. Yamamura, K. *et al.* Human microbiome fusobacterium nucleatum in esophageal cancer tissue is associated with prognosis. *Clin. Cancer Res.* **22**, 5574–5581 (2016).
23. Hooper, S. J., Wilson, M. J. & Crean, S. J. Exploring the link between microorganisms and oral cancer: A systematic review of the literature. *Head Neck* **31**, 1228–1239 (2009).
24. Nagy, K. N., Sonkodi, I., Szöke, I., Nagy, E. & Newman, H. N. The microflora associated with human oral carcinomas. *Oral Oncol.* **34**, 304–308 (1998).
25. Morita, E. *et al.* Different frequencies of *Streptococcus anginosus* infection in oral cancer and esophageal cancer. *Cancer Sci.* **94**, 492–496 (2003).
26. Sasaki, M. *et al. Streptococcus anginosus* infection in oral cancer and its infection route. *Oral Dis.* **11**, 151–156 (2005).
27. Tateda, M. *et al. Streptococcus anginosus* in head and neck squamous cell carcinoma: Implication in carcinogenesis. *Int. J. Mol. Med.* **6**, 699–703 (2000).
28. Hooper, S. *et al.* Viable bacteria present within oral squamous cell carcinoma tissue. *J. Clin. Microbiol.* **44**, 1719–1725 (2006).
29. Nagy, K. *et al.* Inhibition of microflora associated with oral malignancy. *Oral Oncol.* **36**, 32–36 (2000).
30. Cray, J. A. *et al.* The biology of habitat dominance; can microbes behave as weeds?. *Soc. Appl. Microbiol.* **6**, 453–492 (2013).
31. Garrett, W. S. Cancer and the microbiota. *Science (80-)* **348**, 80–86 (2015).
32. Arthur, J. *et al.* Microbial genomic analysis reveals the essential role of inflammation in bacteria-induced colorectal cancer. *Nat. Commun.* **5**, 4724 (2014).
33. Mougeot, J., Stevens, C., Morton, D., Brennan, M. & Mougeot, F. Oral microbiome and cancer therapy-induced oral mucositis. *JNCI Monogr.* https://doi.org/10.1093/jncimonographs/lgz002 (2019).
34. Banna, G. *et al.* Lactobacillus rhamnosus GG: An overview to explore the rationale of its use in cancer. *Front. Pharmacol.* **8**, 603 (2017).
35. Pausan, M. R. *et al.* Exploring the Archaeome: detection of archaeal signatures in the human body. *Front. Microbiol.* **10**, 2796 (2019).
36. Turenne, C., Sanche, S., Hoban, D., Karlowsky, J. & Kabani, A. Rapid identification of fungi by using the ITS2 genetic region and an automated fluorescent capillary electrophoresis system. *J. Clin. Microbiol.* **37**, 1846–1851 (1999).
37. White, T., Bruns, T., Lee, S. & Taylor, J. *Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics* (Acad Press, New York, 1990).
38. Quast, C. *et al.* The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res* **41**, D590–D596 (2013).
39. Nilsson, R. *et al.* The UNITE database for molecular identification of fungi: Handling dark taxa and parallel taxonomic classifications. *Nucleic Acids Res.* **47**, D259–D264 (2019).
40. Team, R. D. C. R: A language and environment for statistical computing. (2019).
41. Davis, N. M., Proctor, D. M., Holmes, S. P. & Relman, D. A. Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data. *Microbiome* **6**, 226 (2018).
42. Zakrzewski, M. *et al.* Calypso: A user-friendly web-server for mining and visualizing microbiome-environment interactions. *Bioinformatics* **33**, 782–783 (2017).
43. Staley, C., Kaiser, T. & Khoruts, A. Clinician guide to microbiome testing. *Dig. Dis. Sci.* **63**, 3167–3177 (2018).

Acknowledgements
We would like to thank Anita Leitner and Alexander Ortner for their help with sample preparation and sample storage. Further thanks to MEFOgraz for helping with the data analysis.

Author contributions
A.W., C.M, C.K., and D.T. designed the study. C.K., C.M., J.P., and A.W. were involved in data acquisition, analysis, and interpretation. The manuscript was drafted by C.K. and A.W., and then critically revised in terms of intellectual content by A.W., C.M., C.K., and D.T.

Funding
This study was approved by the ethics committee of the Medical University of Graz (EK-Nr. 1325/2015). Experimental protocols were approved by the ethics committee and the Department of Otorhinolaryngology of the Medical University of Graz.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information is available for this paper at https://doi.org/10.1038/s41598-020-73515-0.

Correspondence and requests for materials should be addressed to A.W.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2020