Metaproteomic characterization of the human oral microbiome in health and during different treatments

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pantha rhei (“Everything flows”)

- Heraclitus

I dedicate this dissertation to my entire family, who have always lovingly accompanied me on my journey and to whom I owe so much.

One is All, All is One

- Unknown

List of Abbreviations

AMD	acid mine drainage
COG/KOG	cluster of orthologous groups
DDA	data-dependent acquisition
DIA	data-independent acquisition
DNA	deoxyribonucleic acid
EMP	Earth Microbiome Project
ESI	electrospray ionization
FAB	fast atom bombardment
FDR	false discovery rate
HOMD	human oral microbiome database
HMP	Human Microbiome Project
LC	Liquid chromatography
LCA	lowest common ancestor
LPO	lactoperoxidase
m/Z	mass-to-charge ratio
MALDI	matrix-assisted laser desorption/ionization
MCP	microchannel plates
MS	mass spectrometry
MS/MS	tandem mass spectrometry
nano HPLC	nano high performance liquid chromatography
NGS	Next Generation Sequencing
NSAF	normalized spectral abundance factor
PCR	polymerase chain reaction
PTM	Post translational modification
RNA	ribonucleic acid
RPLC	reversed phase liquid chromatography
SEV	secondary electron multiplier
SOLiD	sequencing by oligonucleotide ligation and detection

TOFMS	time-of-flight mass spectrometer
TPP	Trans-Proteomic Pipeline

Zusammenfassung

Hintergrundinformationen: Bakterien gehören zu den ältesten Lebensformen und sind ein elementarer Bestandteil aller ökologischen Lebensräume auf der Erde. Der Mensch als Holobiont ist ein eigenständiges Ökosystem mit einer Vielzahl von ökologischen Nischen und einer großen bakteriellen Vielfalt. Durch innere oder äußere Einflüsse kann es zu Veränderungen der Umweltbedingungen kommen, die eine veränderte Zusammensetzung des Mikrobioms zur Folge haben. Eine solche Dysbiose wirkt sich auf den Gesundheitszustand des Menschen aus und kann zu schweren Krankheiten führen. Das orale Mikrobiom gehört mit zu den komplexesten Mikrobiomen des Menschen. Es bildet eine natürliche Barriere gegen Krankheitserreger und beugt somit u.a. lokalen Krankheiten wie Karies oder Parodontitis vor. Die Metaproteomik ermöglicht es, die exprimierten Proteine des Mikrobioms und deren Interaktion mit dem Wirt zu untersuchen. Diese Technologie überwindet somit die Beschränkung auf Laborkulturen und ermöglicht die Untersuchung des Mikrobioms direkt in seinem natürlichen Lebensraum. Die Metaproteomik bietet eine Reihe von Instrumenten zur Vertiefung des Verständnisses des oralen Mikrobioms hinsichtlich des Gesundheitszustandes des Menschen.

Ziele: Ein Ziel dieser Dissertation war es einen Arbeitsablauf für die Durchführung von Metaproteomstudien des oralen Mikrobioms zu erarbeiten, beginnend bei der Probensammlung über die Präparation der Proben für die Massenspektrometrie bis hin zur bioinformatischen Auswertung. Diesen Arbeitsablauf galt es für das Mikrobiom des Speichels sowie für die Biofilme auf der Zunge und des supragingivalen Plaques zu etablieren bzw. zu adaptieren. Darauf aufbauend wurden Metaproteomstudien durchgeführt, um die drei Mikrobiome bei gesunden Probanden hinsichtlich ihrer exprimierten Proteine, deren metabolischer Bedeutung und Interaktionen mit dem Wirt sowie deren taxonomische Zuordnung zu studieren.

Studiendesign: Die Dissertation umfasst drei Studien mit drei unterschiedlichen Kohorten. Allen Studien ist gemein, dass die Kohorten sich aus oral gesunden Probanden im Alter von 20-30 Jahren zusammensetzten.

In der ersten Studie verglichen wir die Salivette® sowie den Paraffinkaugummi anhand von fünf Probanden, um die effektivste Methode zur Sammlung von Speichel für Metaproteomstudien zu identifizieren.

In der zweiten Studie wurden die Mikrobiome von Speichel und Zunge anhand von 24 Probanden miteinander verglichen und dafür eine Auswertestrategie entwickelt, um der Komplexität dieser Metaproteomstudie gerecht zu werden.

Im Rahmen unserer dritten randomisierten Einzelblindstudie, die auf einem Cross-over-Design basierte, erhielten 16 Probanden vier unterschiedliche lokale Behandlungsschemata, um deren Auswirkung auf das Plaque-Mikrobiom zu untersuchen. Die Behandlungen bestanden aus zwei Lutschtabletten, die Bestandteile des Lactoperoxidase-Systems in unterschiedlichen Konzentrationen enthielten, einer Lutschtablette mit einem Placebo-Wirkstoff sowie Listerine® Total Care™ Mundspülung als Positivkontrolle.

Alle Proben wurden, basierend auf einem Bottom-Up-Ansatz, unter Verwendung von nano LC-MS/MS Massenspektrometern in einer datenabhängigen Messstrategie (DDA, *data-dependant acquisition mode*) vermessen. Die bioinformatische Auswertung erfolgte für die erste Studie mit Hilfe der Proteome Discoverer Software. Für die Studien zwei und drei wurde die Trans-Proteomic Pipeline eingesetzt. Die taxonomische sowie funktionelle Zuordnung der identifizierten Proteine erfolgte für alle Studien anhand der Prophane Software.

Ergebnisse: Für den Paraffinkaugummi konnten wir mit 1.005 bakteriellen Metaproteinen dreimal so viele Metaproteine identifizieren im Vergleich zur Salivette® mit 313 Metaproteinen. 76,5 % der Metaproteine der Salivette® wurden ebenfalls mit dem Paraffinkaugummi gefunden. Insgesamt wurden 38 Genera und 90 Spezies identifiziert, wovon 13 Genera und 44 Spezies nur mit dem Paraffinkaugummi identifiziert werden konnten. Die größte funktionelle Diversität wurde ebenfalls mit dem Paraffinkaugummi detektiert.

Das Metaproteom des Speichel- und Zungen-Mikrobioms basiert auf 3.969 bakteriellen Metaproteinen sowie 1.857 humanen Proteinen. Die Anzahl der nur für das Zungen-Mikrobiom identifizierten Metaproteine, war doppelt so hoch, im Vergleich zum Speichel.

Die Metaproteine konnten 107 Genera sowie 7 Phyla zugeordnet werden. Funktionell wurden für das Speichel-Mikrobiom signifikant höhere Metaproteinabundanzen für die Zellmotilität gefunden. Beim Zungen-Mikrobiom hingegen wiesen die Metaproteine der Biosynthese von sekundären Metaboliten, Signaltransduktion oder der Replikation höhere Abundanzen auf.

Im Rahmen der Plaque-Studie identifizierten wir durchschnittlich 1.916 (± 465) bakterielle Metaproteine je Probe, die wir taxonomisch und funktionell 116 Genera sowie 1.316 Proteinfunktionen zuordnen konnten. Die Plaque inhibierende Wirkung von Listerine® zeigte sich durch eine Reduktion der Metaproteinidentifikation von durchschnittlich 23,5 % nach der Behandlung. Darüber hinaus zeigte die Mehrheit der bakteriellen Metaproteine reduzierte relative Abundanzen während für die Metaproteine humanen Ursprungs eine Erhöhung der Proteinabundanzen gegenüber der Kontrolle vor Behandlung zu verzeichnen war. Aus funktioneller Sicht waren insbesondere metabolische Prozesse, welche für das Zellwachstum und die Zellteilung wichtig sind, betroffen. Im Gegensatz dazu erhöhten sich durch die LPO Lutschtabletten sowohl die Identifikation der Metaproteine als auch die relative Abundanz für die Mehrheit der Proteine. Nach den durch die Metaproteomdaten erhaltenen funktionellen Informationen liegen Hinweise für einen wachsenden Biofilm vor. Die Metaproteine, die eine erhöhte Abundanz nach Behandlung mit den LPO-Dragees zeigten, wurden taxonomisch hauptsächlich Erst- (*S. gordonii*) und Zweitbesiedlern (*F. nucleatum*) sowie Bakterien zugeordnet, die einem gesunden Biofilm zuträglich sind.

Fazit: Im Rahmen dieser Dissertation wurde ein vollständiger Metaproteom Arbeitsablauf von der Probensammlung, über die Probenpräparation bis hin zu Datenanalyse für das Speichel-, Zungen- und Plaque-Mikrobiom erarbeitet. In drei Studien konnten wir dessen Anwendbarkeit demonstrieren und erreichten vergleichbare Ergebnisse zu anderen Metaproteomstudien, beispielsweise bezüglich der Proteinidentifikation. Für die Sammlung von Speichelproben stellte sich der Paraffinkaugummi für Metaproteomstudien als die Methode der Wahl heraus. Für das Zungen-Mikrobiom veröffentlichten wir die ersten Metaproteomdaten. Darüber hinaus publizierten wir die erste Metaproteomstudie, welche die beiden Mikrobiome von Speichel und Zunge miteinander vergleicht. Hinsichtlich des Plaque-Mikrobioms handelte es sich ebenfalls um die erste Metaproteomstudie, die ein

anerkanntes und etabliertes zahnklinisches Modell mit den Vorzügen der Metaproteomiks verbindet. Die Ergebnisse liefern erste Daten, um (auf längere Sicht gesehen) ein Produkt zur täglichen Mundhygiene entwickeln zu können, welches die bakterielle Zusammensetzung des Plaque-Biofilms positiv beeinflusst.

Summary

Background: Bacteria are one of the oldest life forms and an elementary component of all ecological habitats on earth. Humans as holobionts are an ecosystem on their own with a wide range of ecological niches and great bacterial diversity. Internal or external influences can cause changes in environmental conditions, which result in a changed microbiome composition. This dysbiosis affects the health status of humans and can lead to serious diseases. The oral microbiome is one of the most complex microbiomes in humans. It forms a natural barrier against pathogens and can support prevention of diseases such as caries or periodontitis. Metaproteomics enables the study of the expressed proteins of the microbiome and their interaction with the host. This technology thus overcomes the limitations of not being limited to laboratory cultures, but to study the microbiome directly in its natural habitat. Metaproteomics offers a set of tools to deepen the understanding of the oral microbiome in health and disease.

Objective: One aim of this dissertation was to develop a workflow to perform metaproteomic studies of the oral microbiome, from sample collection to preparation of samples for mass spectrometry and their bioinformatic analysis. This workflow aimed to should be established and adapted for the analysis of microbiomes of saliva as well as the biofilms of tongue and supragingival plaque. Based on these results, proof-of-principle metaproteomic studies were performed to study the three microbiomes in healthy volunteers regarding their expressed proteins, their metabolic relevance, and their interactions with the host as well as their taxonomic assignment.

Study Design: The dissertation includes three studies with three different cohorts. All studies have in common that the cohorts consisted of orally healthy subjects aged 20-30 years.

In the first study, we compared Salivette® as well as paraffin gum based on five subjects to determine the most effective method of collecting saliva for metaproteomic studies.

In the second study, we compared the saliva and tongue microbiomes using 24 subjects and established a data analysis strategy for this more complex metaproteomic study.

In our third single-blind randomized trial, based on a cross-over design, 16 subjects received four local treatment schemes to investigate their effect on the plaque microbiome. The treatments consisted of two lozenges containing components of the lactoperoxidase system at different concentrations, one lozenge containing a placebo, and Listerine® Total Care™ mouthwash as a positive control. All samples were measured, based on a bottom-up approach, using nano LC-MS/MS mass spectrometers in data-dependent acquisition mode. Bioinformatic analysis was performed for study one using the Proteome Discoverer software. For the studies two and three, the Trans-Proteomic Pipeline was applied. Taxonomic as well as functional assignment of the identified proteins was performed for all studies using the Prophane software.

Results: For the paraffin gum, we were able to identify three times as many metaproteins with 1,005 metaproteins compared to the Salivette® with 313 metaproteins. 76.5% of the metaproteins of the Salivette® were also found with the paraffin gum. A total of 38 genera and 90 species were identified, of which 13 genera and 44 species were identified exclusively with the paraffin gum. The greatest functional diversity was also detected with the paraffin gum.

The metaproteome of the salivary and tongue microbiome is based on 3,969 bacterial metaproteins and 1,857 human proteins. The number of metaproteins exclusively identified for the tongue microbiome, was twice as high compared to saliva. The metaproteins could be assigned to 107 genera as well as seven phyla. Functionally, significantly higher metaprotein abundances were found for the salivary microbiome for cell motility. Regarding the tongue microbiome, the metaproteins of biosynthesis of secondary metabolites, signal transduction or replication showed higher abundances.

In the plaque study, we identified on average 1,916 (± 465) bacterial metaproteins per sample, which we were able to assign taxonomically and functionally to 116 genera and 1,316 protein functions. The plaque inhibitory effect of Listerine was revealed by a reduction in metaprotein identification of 23.5% on average after treatment. In addition, the majority of bacterial metaproteins showed reduced relative abundances while for the metaproteins of human origin an increase in protein abundances was observed compared to the control before treatment. From a functional point of view, metabolic processes for

cell growth and division, were particularly affected. In contrast, for the LPO lozenges, both metaprotein identification and relative abundance increased for most proteins. According to the functional information obtained by the metaproteomic data, a growing biofilm can be assumed here. The metaproteins that showed increased abundance after treatment with the LPO lozenges were taxonomically assigned mainly to first (*S. gordonii*) and second colonizers (*F. nucleatum*) as well as bacteria that, according to current knowledge, are conducive to a healthy biofilm.

Conclusion: In the context of this dissertation, a complete metaproteome workflow from sample collection, sample preparation to data analysis for the salivary, tongue and dental plaque microbiome was established. In three studies we were able to demonstrate its applicability and achieved comparable results, for example regarding protein identification, to other metaproteome studies. For the collection of saliva samples, the paraffin gum for metaproteomic studies was the method of choice. For the tongue microbiome, we published the first metaproteome data. In addition, we published the first metaproteome study comparing the two microbiomes of saliva and tongue. Regarding the plaque microbiome, this is the first metaproteomic study that combines a recognized and established dental clinical model with the advantages of metaproteomics. The results provide initial data to develop, as a long-term goal, a daily oral hygiene product to positively influence the bacterial composition of the plaque biofilm.

Characterization of the human oral microbiome in health and during different treatments

Bacteria - the hidden rulers of this world

Let us start with a hypothetical experiment. Imagine a world in which all bacteria disappeared from one day to the next. What might such a world would look like? Surely, rotten fruits and vegetables would be a thing of the past (1). Epidemics such as the plague, which cost millions of lives in the Middle Ages (2,3) or the forgotten pandemic tuberculosis (4), which kills even today more than one million people per year (5), would not be worth discussing. Possibly even Napoleon's Russian invasion would have been different without the bacterium *Rickettsia prowazekii*, the cause of spotted fever (6). A world in which we want to live and can live? Louis Pasteur once put it this way: "Life would not long remain possible in the absence of microbes." (7). Presumably, life would not be completely impossible, but the world would be a different one, reduced in its diversity (8). However, eukaryotic life would not be possible considering the lack of mitochondria (9).

Bacteria are among the first known life forms on Earth (10), whose existence began 3 - 4 billion years ago (11,12) and is ubiquitously found throughout the ecosphere (13–15). Regarding the diversity of bacteria, this is the subject of ongoing discussions (16). Estimates range from 10,000 (17) to trillions of different bacterial species on this planet (18–21) based on a similarity of 97% from an operational taxonomic unit (22–24). In contrast, what is unquestionable is their enormous impact on almost all physicochemical and biogeochemical processes on Earth (25,26). These processes are involving complex interactions with the physiosphere as well as intra- and inter-specific interactions (27–29). Bacteria exhibit long residence times in the atmosphere and undergo inter-continental migrations, such as from Asia to North America (30). The fauna in its present form would not be imaginable without bacteria dissolving bound phosphates from the soil (25) and in interaction with yeasts and fungi, called the rhizosphere (31,32), playing an elemental role to plant health and growth (33–35).

This drastic mental experiment at the beginning was of theoretical nature and assumed a maximum loss of biodiversity for bacteria. Nevertheless, the decrease of biodiversity (36) in the Anthropocene is also verifiable for bacteria (37) and affects not exclusively the fauna and flora (38). Not only since the Corona pandemic (39) we are aware that interventions in ecosystems (40,41), usually accompanied by a loss of biodiversity (42,43) lead to changes in function and provided services of the ecosystem and thus modify at least adjacent ecosystems (44–46).

The goal of various research groups and consortia, such as the Earth Microbiome Project (EMP) (47,48) is to advance the characterization of microbiomes of various ecosystems all over the world and their functional interactions with a global point of view (49). The very widely divergent estimates regarding the diversity of prokaryotes show how limited our knowledge is in these matters (50,51). Research collaborations, combined with new technical achievements, could ensure that the so far unexplored realms of the bacterial microcosm (52) provide us with profound insights into what the essences of life might be (16). The development of applications to specifically manage microbial communities (53), such as by transplanting a healthy microbiome (54), can be initiated from these results. This will enable solutions to some of the most challenging anthropogenic problems (55), ranging from agriculture due to eutrophication (56), to water pollution due to chemicals, to challenges in human and veterinary medicine (57). There is a growing understanding that living beings are healthy when they live in a healthy environment (58,59). Holistic approaches such as microbial ecology or systems biology will be a key contributor to increase our knowledge (60).

We are not alone - Humans as holobionts

In the previous chapter, we got a first impression regarding the global relevance of bacteria for the ecosphere, the same context in which humans are embedded. In the following chapters of this dissertation, we consider the human being as a separate dynamic entity apart from this global context (59). Humans themselves are to be understood as an ecosystem of their own with diverse ecological niches accompanied by their own exclusive microbiome (61,62) or in other words: "Humans are holobionts" (63). In 1991, Lynn Margulis introduced the term holobiont (63), which is a macroscopic eukaryotic multicellular organism that maintains synergistic relationships with a variety of diverse microorganisms such as archaea, bacteria or fungi (64–66). In addition to humans, there are numerous examples of other holobionts, such as honey bees (67), Hydra (64) or Euprymna scolopes (68). In general we can say, they all have in common that the host is surpassed by the microbiome (65,69) regarding to its own cell number, cell types and genetic diversity (70–74). An average human with a height of 1.72 m, a weight of 70 kg and a resulting body surface of 1.85 sqm² has an estimated 3.72×10^{13} cells (75), but is colonized by at least 10^{14} microbial cells (52). However, a recent study has calculated that the ratio between human and bacterial cells is more like 1:1 compared to the previously assumed 1:10 ratio (76). If we consider the more than 200 different cell types of the human species (77), it is also apparent that bacteria colonizing humans have a higher diversity, with more than 1000 different species (61,78). Thus, the microbiome provides humans with several million genes, i.e., additional genetic information and variation (65). Consequently, this hologenome (79,80) provides humans with a wide variety of functionalities, such as metabolism of vitamins (81,82) or enhanced metabolism of food (83), that humans would not be able to metabolize with their own approximately 20,000 genes (73). The microbiome thus plays a crucial role regarding the evolution and development of humans (84).

The Human Microbiome Project (HMP) was established in 2007 by the National Institute of Health and is dedicated to characterizing and cataloging the microbiomes found in and on humans (85). A key area of research for the HMP is the impact of the microbiome on a person's health status (85). It has already been shown that even the type of delivery, i.e.,

the decision for a natural birth or a cesarean section (86,87), has an influence on whether a baby is more susceptible to infections (88) and has an increased need for antibiotics in the first years of life resulting in even greater disruptions of the microbiome (89–91). Microbial symbionts train our immune system and ensure proper establishment of the mucosal immune system (92),e.g. by modulating the levels of cytokines of T helper cells (93,94). At the same time, genetic factors of the host ensure which bacteria can colonize in humans and establish themselves as healthy microflora (95). Several studies have already shown that each person has their own individual microbiome, which appears to be stable to a certain extent (96–98). Here, a distinction is generally made between the core microbiome (85,96,99,100) and the variable microbiome (85,101), the latter being influenced primarily by the genotype and lifestyle of the host (85,101). The Core Microbiome, on the other hand, is constant in composition for a given ecological niche (85,99,100), such as the oral cavity (102) or the gut (81).

Changes in the composition of the microbiome, e.g., due to changes in lifestyle (103), can lead to diseases (104,105). The underlying cause is a disturbed balance of the microbiome (106,107), leading to a decreased or a loss of function of symbiotic processes and functionalities between host and microbiome (108,109). Dysbiosis is usually accompanied by a reduction in diversity (110) and has been associated with numerous diseases (111–113). The spectrum ranges from locally definable diseases such as periodontal diseases (114) or inflammation of the pancreas (115) to systemic diseases such as obesity (116), diabetes (117), cancer (118) or to degenerative diseases such as Alzheimer's (119) or Parkinson's disease (120).

The etiology is still in the dark as how the shift from a healthy microbiome to a disease-associated microbiome occurs. First indications for a possible theory suggest that previously symbiotic relationships with a microorganism become pathogenic through the formation of virulence factors and a disproportionate multiplication of the same (121). Another theory, at its core, is that dysbiosis allows pathogenic germs to colonize and become the dominant species in the first place (69,122–124).

Microbiomics initially tried to understand what characterizes a healthy microbiome. Which bacteria can be detected and in what relation? How stable or variable is this microbiome?

Which active metabolic processes can be identified and how do they interact with each other or with the physiology of the host? (121). Answers to these and other questions form the basis for being able to distinguish a healthy microbiome from a diseased microbiome (125). The goal is to develop individual therapies for complex diseases associated with dysbiosis. To this end, targeted approaches are being pursued to rebalance diseased microbiomes instead of eradicating individual species, as it is the case with antibiotic therapies so far (95,126). Essentially, three general approaches to microbiome management have been established for this purpose: a) supplementation with beneficial or absent bacteria in the microbiome b) colonization with bioengineered microorganisms or c) administration of drugs that alter specific metabolic pathways of bacteria. Initial treatments to restore microbiomes have been successfully applied (127,128). The initial successes from over 10 years of human microbiome research is promising, but we are only at the very beginning regarding our understanding of the microbiome and its role in humans (129).

Now, one might ask, which technical methods and approaches does science have at its command for gaining deeper insights into the human microbiome? Therefore, in the next chapter the technical principles applied in microbiome research will be discussed and their potential but also some basic challenges faced by each technique will be highlighted.

Rediscovering the world thanks to high-throughput technologies

The EMP and HMP consortia were founded more than 10 years ago to promote the characterization of microbiomes in different habitats (47,48,85). However, the technical basis, or rather the technical revolutions, were laid years before, without which the current microbiome studies (130) would not have been able to establish itself as a research field (131).

First, let's take a brief look back into the past of microbiology. The beginnings of "classical" microbiology reach back to the 16th century when Robert Hooke and Antoni van Leeuwenhoek constructed the first microscopes and began to explore the microcosm (132,133). Another milestone that should be highlighted here, are the development of culture media for growing bacteria in the laboratory (134). Initially, the development of liquid culture media by Spallanzani in the 18th century, improved by Louis Pasteur and followed by Robert Koch, who developed the first solid culture media in the 19th century (135–137). Until today, these methods form the basis for microbiological research and provide deep insights into the biological mechanisms of bacterial cells under defined laboratory conditions (134). Nevertheless, it is important to keep in mind that these in vitro studies only allow a limited understanding of bacteria and their behavior in their environment (138). On the one hand, the mentioned culture media implies that bacteria can be cultivated in the laboratory (139). It is estimated that only 2 % of all known bacteria can be cultivated in the laboratory, whereby a differentiation must be made here depending on the habitat (140). For example, approximately 50 % of oral cavity bacteria can be cultured (141). Another aspect relates to the selectivity of the culture media, which means that only a limited number of bacteria can be studied at the same time (139,142). As a result, the behavior of bacteria in the laboratory is different compared to their natural habitat, where they interact with their environment and compete with other bacterial species (138,143). Bacteria like SAR11 (Alphaproteobacteria) (144,145), or isolates of the phylum Synergistetes (146,147) cannot exist outside of their natural habitat and therefore cannot be grown using classical microbiological culture media, because too many symbiotic relationships exist within the bacterial community (143,148).

In the last decades, technical achievements such as Next Generation Sequencing (NGS) (149) or Mass spectrometry (MS) analyses (150) have led to the development of methods that overcome the previous challenges of "classical" microbiology (69). In the era of 'OMIC' technologies (151) it is now possible to collect and study samples directly from the habitat, whether or not the bacteria can be cultured (152). Basically, OMICs approaches can be divided into four areas: Metagenomics (153), metatranscriptomics (154), metaproteomics (155) and metabolomics (156). In addition, a variety of other OMICs areas have evolved (157–159), but these will not be part of further considerations.

Let us start by taking a closer look at the metagenomics and metatranscriptomics approaches, which are based on the method of Next Generation Sequencing (NGS) (160) and have evolved from Sanger's dideoxy nucleotide method (161). Depending on the field of application or scientific question, various NGS platforms have been established on the market, such as pyrosequencing (162), sequencing by oligonucleotide ligation and detection (SOLiD) (163) or sequencing by hybridization (164), to name just a few. They all enable a massive parallelization of sequencing and thus also a potentiation of the throughput of samples to be analyzed (165). Briefly, all methods determine the sequence of nucleotides of DNA and RNA molecules. In a first step, the DNA library is prepared. For this purpose, the DNA molecules are fragmented enzymatically or mechanically, which have different lengths depending on the sequencing method used. Subsequently, the DNA double strands are denatured into DNA single strands and adaptors (short artificial DNA pieces) are ligated to the fragments. In addition, the now single-stranded fragments provided with adaptors are enriched using various PCR-based methods and finally sequenced. In the data analysis phase, the raw data determined by the NGS platform are assigned to nucleotides by so-called process base calling. The result of base calling are reads, i.e., the nucleotide sequence of a fragment (149,153,165). These reads can then be aligned against reference genomes using bioinformatics tools (166–168).

In the area of metagenomics, to provide an understanding of which bacteria live in a habitat and in what proportion they are present, methods such as 16S rRNA analysis or whole genome sequencing are used (60). This can elucidate which species are present and the theoretical potential for metabolic pathways (169,170). Metatranscriptome analyses, on

the other hand, analyze messenger and non-coding RNAs (171,172). Thus, these studies provide insight into gene expression as well as regulatory networks within a microbiome (172).

Unfortunately, next generation sequencing methods cannot provide any information about which metabolic pathways are active or in which quantities the building blocks of life, the proteins, and their substrates are metabolized by enzymatic activity (173,174). These questions can be addressed and answered using metaproteomics (175,176) and metabolomics (177). Both latter OMICs approaches are enabled using mass spectrometers (MS) (178) or nuclear magnetic resonance (NMR) as an additional method for metabolomics (179). Mass spectrometry enables both rapid and sensitive identification and quantification of peptides, proteins, or metabolites (180). In general, mass spectrometry involves ionizing the sample material and measuring the mass-to-charge (m/z) ratio of ions that are present in the gas phase (181–183). The mass spectrometric process can be divided into five steps: Physicochemical separation of the sample material, ionization, mass selection, detection, and data analysis (150,181–184). Prior the measurement in the mass spectrometer, the sample mixture is separated by e.g. liquid chromatography to remove excess sample volume, existing solvents and to separate the components of a complex samples mixture (185). The actual measurement of the sample is subsequently performed on the mass spectrometer (150). An ion source is used to ionize the analyte and transfer it to the gas phase (184). Depending on the type and nature of the sample, different types of ionization are available, such as electrospray ionization (ESI) (186), matrix-assisted laser desorption/ionization (MALDI) (187) or the fast atom bombardment (FAB) (188). Mass selection is then performed in the analyzer according to the mass-to-charge ratio of ions (181–183). Again, a wide variety of analyzers are available, such as the quadrupole mass spectrometer (189) or the time-of-flight mass spectrometer (TOFMS) (190,191). In the detector, such as the Faraday Cup (192), secondary electron multiplier (SEV) (193) or microchannel plates (MCP) (194), the previously separated ions are detected. Finally, the measured mass spectra are evaluated using special software and against appropriate reference databases (195). One of the most efficient and common applications is a nano high performance liquid chromatography (nano HPLC) in combination with tandem mass

spectrometry (MS/MS) (196,197). In this thesis, separation of the peptide mixtures was performed using a C18 reverse phase nano HPLC, which was coupled to a Q Exactive Orbitrap mass spectrometer.

Despite the enormous potential of OMICs technologies, it still requires a great effort to, for example, detect specific targets within a microbiome for influencing the microbiome (198). Each of the OMICs technologies presented faces their own unique challenges (199). For example, in human samples, the high content of human DNA (200) or human proteins is an aspect that should not be underestimated (201,202). Furthermore, metagenome analyses cannot differentiate whether the measured DNA originates from dead or metabolic active bacteria (203). Metaproteomics faces the difficulty that proteins have an enormous dynamic range regarding their concentration (204), and low abundance proteins are masked by high abundance proteins in the measurements (202,204). In addition, the enormous complexity of microbiomes confronts all OMICs technologies with the challenge of optimally evaluating and interpreting the huge amounts of data generated by each measurement (205). Here, high-quality reference databases and proper bioinformatics provide two essential elements for solving this issue, which have made enormous progress especially in recent years (206).

In the future, however, it will be the combination of "classical" microbiology (Who is there?) with metagenomics and metatranscriptomics (What can they do?) as well as metaproteomics and metabolomics (What are they doing?) that will play the decisive role in gaining a holistic understanding of the composition and functioning of a microbiome (69).

Metaproteomics - A global view of the microbiome in its natural habitat

Let us summarize to this point. We have seen the important role of bacteria in a global context and that they influence almost all physicochemical and biogeochemical processes on earth (25,26). We have broadened our understanding of humans by considering them as holobionts (63–66). For the study of this complex interplay between humans and bacteria, we have a wide variety of methods at our disposal, which we explored in some detail in the previous chapter (153–156). In the following, we will go into metaproteomics in more detail, considering the studies performed in the context of this dissertation. The aim of the dissertation is to characterize the human oral microbiome using metaproteomics, with two main focuses. First, the development and establishment of a workflow starting with the sample collection, the preparation of the samples for MS analysis and the evaluation of the obtained data (207,208). Second, the application of the workflow to characterize the three oral habitats saliva, tongue and supragingival plaque with respect to their microbial and functional composition (209,210).

The term metaproteomics goes back to a publication by Rodriguez-Valera from 2004, meaning "to detect the genes expressed most abundantly in the environments under different nutrient regimes or external forcing" (155). The first metaproteomic study was published by Paul Wilmes and Philip L. Bond also in 2004 (211). In this study, both investigated the metaproteome of a laboratory-produced activated sludge system optimized for biological phosphorus removal using 2D gel analysis (211). Since then, more than 900 publications have been published (website: https://pubmed.ncbi.nlm.nih.gov; search term: "metaproteomics"; as of June 25, 2022). Several new definitions of the original term metaproteomics have emerged, which allow an even more precise differentiation (212). However, he terms are not always used unambiguously (212). As before, metaproteomics is understood as a gene-centered approach (155,211,212). It is primarily focused on identifying the entirety of the expressed proteins of a microbiome, which allow conclusions to be made on the metabolic activity of the microbiome under different environmental conditions. Identifying the members of the microbiome that are the origin

of the metabolic activity is somewhat less of a focus (155,211,212). This gap is filled by the term "community proteomics", where the focus is to identify not only which metabolic activity is present, but also which members of the microbiome are mainly responsible for metabolic activity (155,211,212). This dissertation can be assigned to both definitions, because on the one hand we have developed a workflow to optimize the set of identifiable proteins. We published our methods and protocols used in the laboratory as well as for the data analysis in a book chapter of the book series "Methods in Molecular Biology". (207,208). On the other hand, we have directly applied this workflow and had a closer look at the composition of the microbiome in three oral habitats and their metabolic activity (209,210).

In general, the experimental procedure of a metaproteome study can be summarized as follows: At the beginning there is the collection of samples from the respective habitat followed by cell disruption as well as the extraction of proteins from the given sample. The metaproteome studies primarily use a gel-free bottom-up approach, which requires the tryptic digestion of the proteins into peptides. Depending on the questions of the studies, the peptides are optionally fractionated with different methods, separated, and then measured with a mass spectrometer. A labelling strategy would also be possible to improve the quantifiability of the complex metaproteome data. However, such fractionation and labelling strategies are costly and time-consuming (213). For protein identification, the obtained mass spectra are searched against a protein sequence database, which in turn is quantified and both taxonomically and functionally assigned using software programs especially designed for metaproteomic approaches (131,214–217).

Sample collection

The aim of all omics studies is to study the microbiome in its habitat (218). Access to the habitat to be studied can become a challenge (219,220). This is particularly evident, for example, in the study of marine ecosystems (221) or Acid Mine Drainage (AMD) from metal mines (222), where large-scale expeditions are usually required. Another example is that there are several seasons to wait if the microbial changes are to be investigated over the year (223,224).

When studying human samples, there are additional aspects to consider besides the challenges of sample collection (225,226). For example, it was important for our studies to assemble cohorts of oral healthy subjects to characterize the composition of the oral microbiome in a healthy state (227). A variety of legal regulations had to be considered and the study had to be legitimized by ethics applications (see ethics applications with registration number *BB 070/16* (207–209) and registration number *DRKS00022810*) (210). In addition, close cooperation and training of the clinical staff members was necessary (227), which performed the sample collection e.g. partly for the plaque study (210).

Another challenge for all studies is the alteration of samples during collection or storage, as studies have shown with saliva (228,229). We addressed protein degradation and denaturation by being on-site at the clinic during sample collection and adding protease inhibitors. In addition, samples were immediately frozen in liquid nitrogen and stored at - 80° (207–210).

The collection of saliva samples is non-invasive (230) and various methods of collection are available (231). These include passive drooling (232), an unstimulated saliva sampling method, as well as the Salivette® (233) and paraffin gum (234), the last two involving active stimulation of saliva flow. All three methods have been used in previous metaproteomic studies (201,235–237). In our study of human saliva, we chose the paraffin gum (207–209), because our own study (207) as well as a study by Golatowski et al. (231) gave the best results regarding of sample volume obtained, number of protein identifications, and technical reproducibility. We demonstrated that more than three times as many bacterial proteins could be identified using the paraffin gum compared to the Salivette® (paraffin gum: 1,005 proteins; Salivette®: 313 proteins) (207). For saliva collection, subjects chewed on the paraffin gum for 1 min while spitting several times into a 50 ml Falcon tube (207).

For the collection of biofilm samples from the tongue, we modified a previously published protocol for DNA analyses (238,239), since no metaproteomic studies on the tongue were available at this time. A sterile wooden spatula was placed dorsally on the outstretched tongue for 5 s with light pressure. The process was then repeated with the other side of the wooden spatula. The wooden spatula was transferred to a 50 ml Falcon tube containing 2

ml of sterile 1x PBS and vortexed for 30 s. The wooden spatula was then discarded (208,209).

Plaque samples were collected with a sterile curette by a dentist. The supragingival plaque was from 24 tooth surfaces from all 4 quadrants of the maxilla and mandible, which were pooled in 3 ml of sterile 1x PBS and vortexed for 30 s to produce a suspension (210). For collection of plaque samples, we modified the sample collection protocol of a metaproteomic study by Belda-Ferre (203).

Sample Preparation and Mass spectrometry

Samples taken directly from the habitat are characterized by a high microbiological complexity and heterogeneity (240), which are additionally less controllable compared to laboratory cultures (241). Complexity and heterogeneity mean that different types of cells like bacteria, fungi or human tissues are present in different abundances (202). This, in turn, influences the choice of cell disruption, as cell disruption is easier for eukaryote cells or Gram-positive bacteria than for Gram-negative bacteria (242,243). Various possibilities of mechanical and non-mechanical lysis (enzymes, heating, detergents) are available (244), and it is necessary to find out for each habitat, which is the method of choice (214,245,246). We decided, based on previous analyses in the laboratory, to use a non-mechanical cell lysis by ultrasonication (247) for all our studies (207–210).

In addition, such complex samples sometimes contain a not to be underestimated number of interfering substances, which affect both the protein extraction and the analyses with the mass spectrometer (248,249). To address this issue, we enriched the proteins using TCA precipitation, which facilitated the protein determination by Bradford (207–210).

More important is the large dynamic range of human proteins, which cover a range of 7 orders of magnitude (250,251). Examples such as alpha-amylase (AMY) (252), Mucin-5B (MUC5B) or lysozymes (LYZ) (253) are present in high abundance in saliva and overlay the low-abundant proteins of the bacteria during mass spectrometry measurements (237). Removal of especially high abundant human proteins from the sample would be one way to enrich bacterial proteins (254). At the same time, it should be noted that this could result in an interference with other analytes and to a loss of information (255). After all, one of

the strengths of metaproteomics is to reveal the protein interactions between humans as hosts and their microbiome (256,257). A more appropriate alternative is to fractionate the samples prior to mass spectrometric analyses to reduce the complexity of the peptide mixtures to be measured (258).

We applied this approach in our studies by centrifuging the samples in several steps, enriching the proteins, and purifying the peptides derived by trypsin using µ-C18 material, for optimal preparation for mass spectrometric analyses. Further separation was achieved by using the reversed phase liquid chromatography (RPLC) method by first loading the precolumn (75 µm inner diameter, packed with 3 µm C18 particles, Acclaim PepMap100, Thermo Fisher Scientific®) with the complex peptide mixture. The peptides were separated on an analytical column (25 cm x 75 µm, 2.6 µm C18 particles, 150 Å pore size, Accucore 150-C18, Thermo Fisher Scientific®) over a 120-min gradient. A binary buffer system consisting of 0.1% acetic acid water (buffer A) and 100% ACN in 0.1 acetic acid (buffer B) was used as the buffer system, with a linear gradient of 2-25% of buffer B. The peptides were analyzed using high-resolution accurate mass MS instruments of the Q Exactive Orbitrap MS series in data-dependent acquisition mode (207–210).

Protein identification and data analysis

The strategy selection for the analysis of the metaproteomic data is an important aspect in metaproteomics. These includes setting the right parameters for MS-search analysis to the appropriate database and pipeline for taxonomic and functional assignment of the identified proteins and their quantification (259).

The number of bioinformatic methods for the analysis of metaproteomic data has constantly increased and methods have become very diverse (260–271). Primarily, the individual applications focus on single aspects of the analysis, such as protein grouping and their taxonomic and functional assignment (261,262,267), or they offer solutions for the protein inference problem (263–265). However, standardized protocols covering the entire evaluation process are still in the process of being established (272).

Standardized and proven protocols of data analysis from the field of proteomics provide a first guideline for metaproteomics but can only be adopted to a limited extent (273). It is

still difficult to assess how the parameter settings like the number and diversity of PTM's or the maximum number of missed cleavages for the analysis of raw data, affect the results of metaproteomic datasets (273,274). Therefore, the parameter settings we chose are based on the optimized values for the mass spectrometers used in our laboratory and several publications that were most relevant at this time (259,269,273–278). The details of the parameter settings can be found in the publication Rabe et al. 2021 (208).

One of the next questions is on which database the data interpretation should be based. Studies have shown that the selection of the reference database has a considerable influence on the results (276). Basically, three strategies for the selection of the database can be distinguished. If a new habitat shall be investigated, without intensive knowledge about the composition of the microbiome, the most suitable strategy is the general search against publicly available databases (222,279,280) such as UniProt (281,282), NCBI (283) or eggNOG (284). However, the results at this point should be critically questioned as these databases are enormously large and no specific search is performed against the habitat microbiome (285). Problems such as the protein inference are particularly striking in this context. Furthermore, a high false discovery rate (FDR) inaccuracy and a loss of search sensitivity must be considered due to the wide variety of proteins and their unknown protein sequence size (286–289). Combined and iterative searches of different databases, reducing the database size based on the identified species of the previous search run, offer a possibility to increase the number of high confidence PSMs and reduce the number of false negatives (287,290).

The method of choice is to create a protein sequence database from the same sample that is used for metaproteomic analysis (276,291). Crucial for such a database is the availability of the necessary financial and technical capacities as well as the human expertise to perform such a multi-omics approach (292). Nevertheless, the effort is worthwhile, as the peptide identifications are up to 1.5-fold higher compared to the non-specific databases (293).

A hybrid approach is to use public genome databases that are specific to a habitat. A variety of such databases have now been established (49,294,295). We chose this hybrid approach and used the, 16S rRNA-based, human oral microbiome database (HOMD), which is provided and maintained by the Forsyth Institute (294,296,297). The HOMD focuses on the

human oral microbiome and defines a new phylotype from a sequence similarity of less than 98.5 % (294). This database thus provides the basis for taxonomic classification of even previously unknown bacterial isolates of the human oral cavity while adhering to strict quality criteria (296).

For taxonomic and functional classification, we used the open-source program Prophane, which was developed for metaproteomics in the research group of Katharina Riedel by Stephan Fuchs (262), who gratefully supported this dissertation by individual adaptations of Prophane. Metaproteomics requires specialized tools to address the challenges of assigning identified peptides at the protein level (202,298,299). Taxonomic classification is even more complex in comparison to a proteomics approach due to a taxonomic inference problem (265). Peptide sequences are not only assigned to multiple proteins, known as the protein inference problem (300,301), but these proteins can also belong to multiple species (265). For this reason, the taxonomic assignments become less unambiguous as one moves from the superkingdom to the species level (273,302). Prophane addresses this problem by grouping proteins that have been assigned with multiple peptides into metaproteins (262,272). Taxonomic and functional classification is then performed by using the lowest common ancestor (LCA) approach. As a complementary note, functional assignment is often limited by lack of gene annotation. Estimates suggest that between 30% and 50% of gene sequences in a genome lack functional annotation (303). Moreover, the comparability of different functional annotation databases (UniProtKB (281,282), eggNOG (284), TIGRFAMs (304), or COG/KOG (305)) is difficult because they use different approaches for functional classification. For our studies, COG/KOG (305) as well as TIGRFAMs (304) were used.

The main steps of our metaproteomic analysis strategy can be summarized as follows (208): We used the open-source data analysis software Trans-Proteomic Pipeline developed in Ruedi Aebersold's group to interpret the raw mass spectrometric data (http://tools.proteomecenter.org/software.php) (306–308). The database search was performed using the Comet algorithm (http://comet-ms.sourceforge.net/) (309,310) against a Decoy reference database, which consisted of the HOMD (www.homd.org) (294,296) as well as human sequences from the UniProt database (www.uniprot.org) (281,282). Peptides and proteins identified by individual modules of the TPP were filtered

based on a false discovery rate cutoff of protFDR < 0.05. For the subsequent analyses in Prophane (www.prophane.de) only proteins that contained at least one unique peptide were allowed. Based on the Lowest-Common-Ancestor algorithm (311), taxonomic assignment was performed as well as functional assignment using COG/KOG (305) and TIGRFAM (304) classification. The data were also relatively quantified by prophane using NSAF (normalized spectral abundance factor) values (312).

Metaproteomic insights on the microbiomes of saliva, tongue, and dental plaque

The oral cavity (cavum oris) in humans is a space which merges posteriorly into the middle pharynx, is bounded anteriorly by the lips (labia), laterally by the cheeks (bucca), inferiorly by the mucous membrane of the floor of the mouth and is separated from the nasal cavity by the soft and hard palate (313,314).

The oral cavity, in a functional perspective, is the starting point of digestion by ingestion of food, the comminution of food by teeth and tongue, the initiation of enzymatic digestion, and the process of swallowing to transfer the food into the digestive tract (121,296,314,315). In addition, the oral cavity plays an essential role regarding to respiration, but also sound formation and facial expressions, which are elementary components of human speech and communication (314,315). Furthermore, there are sensory functionalities (taste, temperature, pain, palpation), thermal regulation mechanisms and the secretion of saliva and crevicular fluid, which have immunological functions (314–317).

As another distinctive characteristic, the oral cavity is composed of a variety of different ecological niches (314,318). The teeth form a permanent hard tissue structure in the body that intersects a soft tissue structure, the gingiva (313,319). The tonsils are characterized by deep crypts (319). The tongue is characterized by papillae on the dorsal side whereas on the ventral side there is mainly a mucosal epithelium (319,320). The physicochemical characteristics differ depending on the location of the habitat with respect to the concentration of nutrients, oxygen, or the pH value (315,319,321,322). In addition, habitats are exposed to regular and temporally recurring external influences that define the specific characteristics of the habitat (321,322). These include, the quality of food (98), movements of teeth and tongue (314,315), desquamation (323), salivary flow rate (324) or the circadian rhythm (325), to name just a few examples.

Based on these different habitats regarding their anatomy, histology and physicochemical conditions, the microbial composition within the oral cavity is characterized by a high diversity and specificity (326,327). At the same time, the individual habitats are

interconnected by saliva, which rinses the entire oral cavity (324). Thus, the term human oral microbiome, defines all microorganisms identifiable in the oral cavity or its adjacent extensions (296). Beside the intestinal microbiome, it is the second most complex microbiome of the human body and was among the first human microbiomes to be studied (61,280). As early as 1695, Antoni van Leeuwenhoek described the study of plaque and saliva in his book "arcana naturae detecta" (328). The caries-causing bacterium *Streptococcus mutans* was among the first bacteria isolated (329).

The great majority of bacteria in the oral microbiome live facultatively anaerobic or obligately anaerobic and require a neutral pH for their growth (330). A study by Simon-Soro et al. showed that due to the high oxygen content in the vestibulum oris, *Streptococci* can be identified, whereas anaerobic Fusobacteria tend to live on the tongue (331). Nearly 700 different microbial species have now been identified, the vast majority of which have been assigned to the phyla *Firmicutes*, *Bacteriodetes*, *Proteobacteria*, *Actinobacteria*, *Spirochaetes* and *Fusobacteria (99,141,296,297,332–335)*. The more rarely represented species include *Chlamydia*, *Chloroflexi*, *SR1*, *Synergistetes*, *Tenericutes*, and *TM7*, in addition to the *Euryarchaeota (296,334)*. Despite the high diversity of intraoral habitats, a certain stability and an oral core microbiome can be defined, which at the same time is characterized by a high variability of low abundant species (321,326,334,336,337).

The oral microbiome is one of the first barriers for pathogenic bacteria to prevent their colonization (121). It also trains the human immune system and influences the up- and downregulation of pro-inflammatory immune responses (338). A disturbance of the balance can lead to severe local as well as systemic diseases (141). A diet with predominantly simple carbohydrates, smoking or a lack of oral hygiene, promote the shift of the healthy oral microbiome to a pathological microbiome (327,337,339–342). Caries (343) and periodontitis (344) are most widespread polymicrobial diseases of the dental apparatus (345–348). However, due to the increased presence of pro-inflammatory mediators (349), an untreated pathological oral microbiome can also be a reason for diabetes (350), cardiovascular diseases (351), or cancer (352). Especially regarding the etiology of polymicrobial diseases in humans, it is therefore of crucial importance to study the general

composition and behavior of the oral microbiome while considering intra-oral and interindividual differences (331,353).

The goal of the scientific community is therefore to define what distinguishes a healthy and a diseased oral microbiome from one another, as well as to develop a kind of early warning system in the case of a shift in the oral microbiome (121,148,354). This could be done, for example, using bacteria that act as biomarkers, as has already been shown in an initial study for plaque *(Corynebacteriaceae*), tongue and saliva (*Veillonella*, *Oribacterium*) (355). Another goal is to remediate pathological microbiomes more gently through a deeper understanding of the oral microbiome, without relying on antibiotics or antibacterial mouth rinses and ointments, which primarily reduce the diversity of the microbiome (339,356,357). Probiotics might a possible alternative, as shown by initial promising study results (358,359).

Using the techniques of metaproteomics, this dissertation thesis contributes to a better understanding of the bacteria metabolically active in the microbiome based on the identified proteins for saliva and tongue. In addition, we consider the interactions between bacterial and human proteome (207–209). In the plaque study performed, the effects of different treatments on the plaque microbiome were studied in terms of changes in proteins and metabolic pathways affected by them, to lay a first set of basic knowledge for a possible treatment to positively influence the commensal microbiome (210).

After a brief general introduction to the studied habitats, saliva, tongue and plaque, the main findings of the studies are presented and placed in the context of the present state of the literature.

Saliva and Tongue

Saliva is the secretion of the salivary glands, which are classified as serous, mucous or seromucous and are distributed throughout the oral cavity (319). In addition to hundreds of minor salivary glands, there are three main salivary glands, the Glandula parotis, the Glandula submandibularis and the Glandula sublingualis (230,313,319). Saliva consists of approximately 98 % water and only the remaining 2 % is composed of a mixture of various components such as proteins, glycoproteins, electrolytes, or immunoglobulins

(319,360,361). This biofluid has a pH value of 7 due to the electrolytes it contains, which provide additional buffering capacity against acids and thus protect teeth from their demineralization (362–364). Immunoglobulins lead to antibacterial, antifungal, and antiviral characteristics (361,365,366). The mucous salivary glands secrete as their main component the protein mucin, which has a protective function on the soft and hard tissues of the oral cavity (319,324,362). The serous glands, mainly produce amylases, which initiate the digestion process of carbohydrates to glucose (252,319,324). The initiation of digestion not only provides nutrients to the human host, but also to the existing biofilm in the oral cavity (367,368).

Saliva has been the focus of numerous metagenomic studies (335,369–372), which is not surprising since saliva can be collected easily and non-invasively (230). It is dominated by the five phyla *Actinobacteria*, *Bacteriodetes*, *Firmicutes*, *Fusobacteria*, and *Proteobacteria* (99,373,374). At the genus level, especially *Streptococcus*, but also *Prevotella*, *Veillonella*, *Fusobacterium*, *Rothia*, *Neisseria* and *Haemophilus* have been identified to define the salivary core microbiome (99,326,331,375). Initial metaproteome studies (201,235–237,269,376,377), in addition to our own, were able to confirm the taxonomic distribution based on protein assignments. We identified 1,647 human proteins, which is slightly less than comparable studies (236,237), but with 2,633 metaproteins we detected more bacterial proteins (209). As explained in more detail in previous chapters, we attribute this to the different size and composition of the cohorts, as we had only healthy study participants to examine the healthy microbiome. Other factors include different protocols and strategies regarding sample collection and preparation, mass spectrometric measurements, and data analyses (208,209).

From a microbiological point of view, saliva is the result of the various habitats of the oral cavity, for example, due to detaching bacteria from biofilms or oral mucosal surfaces (323,327,330). The tongue with its biofilm is also a reservoir of bacteria (378). There is even the hypothesis that the bacterial composition of saliva is essentially influenced by the tongue or even originates from the tongue (336,378,379).

The tongue is a muscular structure consisting of nine individual muscles, which is covered by a mucous membrane and innervated in a complex manner by several nerves (313). The

entire tongue, in addition to several salivary glands (324), is covered by various types of papillae, which can be categorized into two groups (320). The mechanical papillae group consists of the Papillae filiformes, Papillae conicae and Papillae lentiformes, which are responsible for the tactile sensation of the tongue. The Papillae fungiformes, Papillae vallatae and Papillae foliatae are responsible for the sense of taste, with the Papillae fungiformes additionally forming the source of temperature sensitivity (313,319,320,380,381). In the cavities between the papillae, a biofilm is formed, which can be observed as a whitish coating on the tongue (61,382). This biofilm seems to be the cause that saliva and tongue show a great similarity regarding their microbiome (326,331,336,379).

Therefore, we designed our metaproteomic study to analyze not only the salivary microbiome but also the tongue microbiome in the same cohort of 24 healthy volunteers and to compare both microbiomes (208,209). An important result was that the relative abundance of bacterial metaproteins in the studied biofilm of the tongue was significantly higher with 40.8 % compared to saliva with 21.7 %, in which the bacteria live planktonically. The taxonomic assignment of the proteins confirmed the dominant phyla and genera previously named for saliva, also for the tongue with the difference that the bacterial metaproteins occurred in higher abundance for the tongue. This seems to be another hint for the hypothesis that there is a taxonomic similarity between saliva and tongue (336).

We detected a wide bacterial diversity, which is probably mainly due to the interindividual difference between the subjects, regarding to their dietary habits (383), genetic background (384) and oral hygiene (339) as already described in more detail in the previous chapters. Nevertheless, we identified significant differences between saliva and tongue of non-dominant genera such as *Gemella*, *Granulicatella*, *Treponema* or *Peptoniphilus*, which shape the profiles of both microbiomes (209).

Human proteins with the highest abundance were, as expected, α-amylase (AMY1A) (385) and glyceraldehyde-3-phosphate (GAPDH) (386), both in saliva and in tongue swabs. In contrast to the tongue, higher abundances of immune defense proteins were found in saliva. Probably due to the scraping of the biofilm from the tongue during the sampling process, we identified significantly higher abundances of cytoskeletal proteins such as

repetin (RPTN), which is involved in the development of the formation of cornified cell envelopes (320).

At least 30% of the 2,633 bacterial metaproteins of saliva and 3,307 bacterial metaproteins of the tongue were of ribosomal origin or functionally mapped to translation. Moreover, for the planktonic bacteria in saliva more metaproteins of cell motility showed a higher abundance (235). For the tongue, higher abundances for metaproteins of signal transduction and for synthesis of secondary metabolites were identified, suggesting intra- and interbacterial communication within the tongue biofilm (387).

In summary, we were able to demonstrate the applicability of our workflow from sample preparation to analysis of metaproteomic data. These results were comparable to other metaproteomic studies and provided good technical reproducibility. In addition, this is the first study providing metaproteomic data for the tongue. Furthermore, it is the first metaproteome study comparing the microbiomes of saliva and tongue. We identified a great taxonomic diversity, accompanied with taxonomic as well as functional similarity between the two microbiomes (208,209).

Supragingivale Plaque

Supragingival plaque is a biofilm that develops over time on tooth surfaces. The biofilm consists of a complex microbial community embedded in a structure of extracellular substances called exopolysaccharides (388,389). The formation of the biofilm can be divided into the 5 phases called association, adherence, microcolony formation, biofilm maturation with EPS synthesis, and aging including detachment of planktonic bacteria (388,390). During association, a pellicle layer, including glyco- and salivary proteins, forms on the tooth surface within a few minutes (391,392). Subsequently, first colonizers form a loose association with this pellicle layer within a few hours. The initial colonizers include mainly *Streptococcus spp.* but also *Capnocytophaga spp.*, *Prevotella spp.* or *Propionibacterium spp* (393–396). Bacterial adherence forms the basis for further maturation of the biofilm, with initial formation of microcolonies and the beginning of vertical growth of the biofilm, usually within the first 24 hours (397). In the fourth phase, maturation of the biofilm takes place, and a matrix is established through the synthesis of

exopolysaccharides. This allows the establishment of a complex community structure (398,399). Water channels are formed, which supply the bacteria with water, but also remove toxic substances (390). The matrix of exopolysaccharides also ensures a stable pH via binding cations and store nutrients (399). In this phase, bridging species such as *Fusobacterium nucleatum* or *Prevotella intermedia* play a central role, providing the link between first and late colonizers, such as *Eubacterium spp.* or *Treponema spp* (395,396,400). The denser the colonization of the biofilm, the more important intra- as well as interspecific communication via quorum sensing becomes (401). Quorum sensing is a form of chemical communication that allows bacteria to react to changing environmental conditions and thus to find new ecological niches to ensure the survival of the population (401–403). This organizational structure thus allows the biofilm to show a great resistance to external environmental factors such as nutrient limitation, the human immune system, and antibiotics (388,399,404).

The Plaque biofilm is always associated with diseases such as caries or periodontitis (405). However, the plaque biofilm basically possesses several properties that are beneficial to human health (406). A biofilm in a state of balanced equilibrium forms a barrier against disease-associated bacteria (337). In the context of the extended ecological plaque hypothesis, it is assumed that cariogenic bacteria already colonize the plaque biofilm but are not competitive at a neutral pH and commensals prevent them from dominating the biofilm (407,408). Studies of the immune system have shown its ability to distinguish between commensals and pathogens. The Dysbiosis of the biofilm triggers an inflammatory reaction of the immune system (409,410).

In addition to genetic causes of the host or a reduced salivary flow rate, a diet of simple carbohydrates in combination with poor oral hygiene is a major factor regarding the bacterial shift towards a pathogenic biofilm (121,141,411). This is supported by a study that despite a lack of oral hygiene in combination with a lack of refined sugars in the diet, the biofilm grew but signs of gingival inflammation or the number of pathogenic bacteria did not increase (412). Refined sugar lowers the normally neutral pH value, which is an advantage to acidogenic bacterial species such as *Streptococci spp.* (*S. mutans*, *S. oralis*).

The basis for the development of caries, the demineralization of the tooth substance (413,414).

Saliva serves as a natural plaque control in the oral cavity, including the salivary flow rate that mechanically removes bacteria (121), the stabilization of the pH or the contained components of the innate immune system such as the lactoperoxidase system (330,415). The enzyme lactoperoxidase, catalyzes the oxidation of anions in the presence of hydrogen peroxide (H_2O_2) to highly reactive reaction products, which are toxic to bacteria (416). In our study, we aimed for lactoperoxidase to catalyze H_2O_2, derived from the H_2O_2 donor carbamide peroxide (CPO), and the substrate thiocyanate (SCN^-), to hypothiocyanite ($OCSN^-$) (210,417,418). Although daily oral hygiene products already use the mechanism of the LPO system, most of them aim to reduce the biofilm or microbiome entirely, including commensal bacteria (415,419,420). However, in addition to the mechanical teeth cleaning routine via toothbrushes, the aim should be to influence the plaque biofilm formation that the commensal bacteria are promoted, and pathogenic bacteria cannot establish or dominate the biofilm.

The aim of our study was to evaluate the effect on plaque formation of two lozenges containing the components of the LPO system in high (Drug B - 0.083 % H_20_2 accordingly a 1:2 H_20_2/SCN^- relation) and low (Drug C - 0.04 % H_20_2 accordingly a 1:4 H_20_2/SCN^- relation) concentrations. For comparison, we used a lozenge as a placebo (Drug D) and the mouthwash Listerine (Drug A), known for its plaque inhibitory effect (421,422), as a positive control. Sixteen subjects, serving as their own control, were given each of the four treatment regimens over a 4-day period in a dentally approved cross-over design (423). No additional oral hygiene practices, such as brushing or flossing, were used during treatment. Between each treatment, there was a recovery phase of 10 days where subjects followed their usual oral hygiene routine. The study was designed as a randomized single-blind trial. Furthermore, the aim was also to combine the results of the clinical part of the study with the results of metaproteomics (210,424).

With 1,916 (± 465) bacterial and 442 (± 171) human proteins per sample, we achieved higher protein identifications than comparable metaproteome studies (203,425–427),

which we attribute primarily to the high number of 128 samples measured, in addition to different methods of sample preparation and data analysis (259,428,429).

The expected high proportion of bacteria in the biofilm, was observed by the relative abundance of metaproteins, which averaged three-quarters of the sample except for samples after Listerine treatment (Drug A).

The plaque inhibitory effect of Listerine was observed by a reduction in the relative abundance of metaproteins from an average of 74.1 % before treatment to 59.1 % after treatment. Despite the same amounts of proteins used for MS measurement, the identified metaproteins reduced abundance on average by 23.5% after treatment with a corresponding increase in human proteins. The abundance of metaproteins that have functions in metabolic processes for bacterial growth were significantly reduced. The opposite observations were made for Drug B (0.083 % H202 accordingly a 1:2 H202/SCN- relation), Drug C (0.04 % H202 accordingly a 1:4 H202/SCN- relation), and Drug D (placebo).

The taxonomic assignment of the metaproteins is consistent with the findings of recent studies. The plaque biofilm is composed of eight phyla. The phyla *Actinobacteria*, *Firmicutes*, *Fusobacteria*, *Proteobacteria*, and *Bacteriodetes* play a major role whereas the *Spirochaetes*, *Synergistetes*, and an unclassified phylum play a minor role (99,102,332,338,430,431). We also showed a high taxonomic diversity of the plaque biofilm, as reflected by the assignment of metaproteins to 116 genera and 351 species.

For Drug D (placebo), unexpectedly, there were slight significant changes in the abundances of metaproteins detected, for example the increase for the secondary colonizer *Capnocytophaga spp.* or for metaproteins involved in small molecule interactions. This is attributable to the high interindividual variance of the subjects as well as to the sugar alcohols contained in the lozenge, which influence the microbiome (432–437).

Drug C showed a similar change in the metaproteome as Drug D, suggesting that the concentration of the LPO system is too low to influence plaque biofilm development beyond that of a placebo.

The greatest changes in the metaproteome were apparent under Drug B treatment. We found an increase in metaprotein identifications and metaprotein abundances for bacteria that are either associated with oral health or play a decisive role as commensals for plaque

biofilm formation. In addition to increased metaprotein abundances for *Lachnospiraceae ssp.* or *Abiotrophia defectiva* (438,439), we noted changes for *Streptococcus gordonii*, which is a competitor for the cariogenic bacterium *Streptococcus mutans* through the production of hydrogen peroxide (440–443). For the 4th phase of biofilm development, its growth, the bridging species *Fusobacterium nucleatum* or late colonizers *Prevotella intermedia* and *Prevotella nigrescens* are of great relevance, for whom we also found increased metaprotein abundances (394,396,397,444).

In conclusion, this complementary study demonstrated the influence of lozenges with two different concentrations of the LPO system on plaque formation. This is the first metaproteomic study that attempts to harmonize the results of classical microbiology and collected clinical parameters within the context of an established clinical model. The initial results will form the basis for further studies to advance the development of a product for daily oral hygiene that positively affects the commensal bacteria of the oral biofilm (210).

Metaproteomics in the light of future developments

As we have learned in the previous chapters, metaproteomics faces many challenges to be overcome in the future (428). Starting with the enormous complexity and heterogeneity of samples (445–447) to the evaluation of the enormous datasets (448) as well as their integration with other 'omics' technologies (449). At the same time, we have seen the potential of metaproteomics. With our studies we could show that metaproteomics is not only the determination of the pure number of proteins and their relative abundance (207–210). Also, in the sense of community proteomics (450), these proteins can be assigned to the individual members of the microbiome and their functional role within the microbiome can be determined (451,452). Finally, let us have a look at future trends and developments in metaproteomics to ask the question "Quo Vadis metaproteomics?".

First, it can be pointed out that one focus will be on the optimization and standardization of sample collection and preparation, which must be worked out individually for each habitat (226,453). The reason for this lies in the complexity and heterogeneity of each habitat, which, in addition to a large number of expected proteins (AMD biofilm: 4.77×10^5 proteins (428,454); surface freshwater: 6×10^7 proteins (428,455); human saliva: 16.2×10^6 proteins (428,456)) also have individual challenges to extract proteins from each habitat (soil: humic acids (457); saliva: high percentage of human proteins (201,202)). The standardization of protocols will allow to some extent to establish comparability between samples of a habitat (458).

Metaproteomics will also benefit from the technical advancements of mass spectrometers (459). In addition to the enhanced sensitivity of the instruments, the increased use of the data-independent acquisition (DIA) method also promises improved results (460,461). Analogous to the DDA method, the DIA method also fragments all the peptide ions in a first step, followed by a sequential analysis of mass windows. The difference in DIA is that a much smaller mass window of precursor ions is considered in each cycle of selection and fragmentation. This smaller mass window is then gradually shifted over the entire mass range under consideration (462). As a result, all precursor ions are selected and analyzed, leading to improved peptide identification (463). Most importantly, this means better

coverage of low abundance proteins (464,465). In one of the first metaproteomic studies, Aakko et al. demonstrated the applicability of the DIA method to metaproteomics based on human fecal samples and laboratory-assembled microbial mixtures (466). Since all ions are analyzed one can also re-analyze data and pick-up new details, e.g., when new post-translational modifications are discovered.

Another essential component that will sustainably change metaproteomics is the area of evaluation and interpretation of the data obtained by mass spectrometry. The development of specific metaproteomic software solutions (448), the application of multi-omics approaches (449) and machine learning or artificial intelligence (467) will play a decisive role in this context.

Over the last few years, a variety of software solutions have been established (260–266,272). There are software solutions that specialize in protein grouping and taxonomic and functional assignment (iMetaLab (261), MetaProteomeAnalyzer (267,272), Prophane (262,272). Unipept (263,264) as well as MetaTryp 2.0 (265) on the other hand specialize in peptide level analysis to address the protein inference problem (468). Still another software program is even specifically designed for a habitat, such as MetaPro-IQ for the gut microbiome (266). The use of software solutions developed specifically for metaproteomics provide improved reproducibility of metaproteomic studies because they promote standardization within and between research groups (213,287). In addition, pipelines attempt to address specific problems in the analysis of metaproteomic data, such as the grouping of redundant proteins (metaproteins) (262,272) or the taxonomic (taxonomy inference problem) (468) and functional assignment of metaproteins (262,272). Nevertheless, it is crucial to critically compare workflows (213,287), because different approaches and strategies are used to analyze the data (260–267,272). A promising approach is the combination of the two open-source tools MetaProteomeAnalyzer and Prophane, which are specialized in metaproteomics and enable scientists to handle the entire analysis process from the creation of the protein database to the visualization of the results in one integrated workflow (272).

Metaproteomic studies will produce increasing amounts of data due to improved mass spectrometers or more extensive gene databases, so the use of machine learning or

artificial intelligence algorithms will undoubtedly contribute to the optimization of quality and quantity (469). In the field of mass spectrometry, machine learning algorithms have been developed and used for many years for the prediction of retention time (470,471) or MS/MS spectrum prediction (472–474). Classical examples are machine learning algorithms like random forests (475) or gradient boosting (476). Another application of machine learning algorithms is the sequence database search to improve the quality of peptide and protein identification (474). First promising approaches are already being pursued in the field of proteomics (477). For the presented metaproteom software solutions (260–267,272), it will be important to adapt these approaches and make them usable for metaproteomics in the future.

In addition to developments that are specific to the research area of metaproteomics, there will be a need to conduct more studies based on multiomics approaches (478) (alternatively: integrative omics (479) or panomics (159)). An example is the implementation of the Integrated Human Microbiome Project (480,481) as a follow-up and extension of the HMP (61,85). The goal of multiomics is to combine the results of the different omics technologies because each omics approach reflects only a reductionistic picture of reality (482). After all, the microbiome and environmental conditions are mutually dependent, which in turn influences the genotype and phenotype of the microbiome and host (483). It is this integrative approach that will allow us to obtain new associations or relationships between gene and protein expression and their influence on the metabolome (256). Considering individual medicine (159), biomarkers can already be discovered today that announce the change from a healthy to a pathogenic microbiome (484,485). Furthermore, clear targets can be identified to positively influence the microbiome without resorting to eradicative therapies (486).

The standardization of protocols, artificial intelligence and multiomics are elementary components to further develop metaproteomics. These developments will ensure that the results of metaproteomics will be qualitatively enhanced and allow even more detailed insights into the interrelationships and actions of a microbiome. The goal of personalized medicine, where medical treatments and decisions are tailored to the individual patient, we may thus come a decisive step closer (487).

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NOTES

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Comparative analysis of Salivette® and paraffin gum preparations for establishment of a metaproteomics analysis pipeline for stimulated human saliva

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ABSTRACT

The value of saliva as a diagnostic tool can be increased by taxonomic and functional analyses of the microbiota as recently demonstrated. In this proof-of-principle study, we compare two collection methods (Salivette® (SV) and paraffin gum (PG)) for stimulated saliva from five healthy participants and present a workflow including PG preparation which is suitable for metaproteomics.

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Saliva; metaproteomics; human oral microbiome; whole saliva proteomics; collection method; nLC-MS/MS

For a better understanding of the microbial species composition in health and disease of the oral cavity [1,2], saliva offers a wide range of possibilities as shown in metagenomic studies [3–5]. In addition, metaproteomics provides detailed impressions of active metabolic pathways under certain environmental conditions, which cannot be accomplished by metagenomics [6–8]. First metaproteome studies for saliva have already been performed [9–12]. Here, we conducted a comparative proof-of-principle study for two saliva-stimulating collection methods (Salivette® (SV) and paraffin gum (PG)) to identify the most suitable way to perform metaproteome studies on human saliva.

We collected stimulated saliva from five healthy dental students (three men and two women) aged 20–30 years on two consecutive days. Under the supervision of an experienced dentist, the students examined each other and none of them had a probing depth of ≥4 mm. Based on a questionnaire we ensured that all participants met our inclusion criteria (Supplemental Table 1).

All subjects were chewing on a PG for 1 min. Within this minute all volunteers spat saliva into a sterile 50 ml Falcon tube for several times. On the next day, the participants had to chew on the SV for 1 min and the soaked cotton roll was transferred into a specific salivation vessel. Previous experiments showed that the order of the chosen saliva collection methods had no influence on the results (data not shown). Afterwards, all samples were centrifuged for 15 min at 11,500 g (4°C). Saliva collected by PG was separated into supernatant (PG_SN) and pellet (PG_P). SV samples were again centrifuged for 30 min at 17,000 g at 4°C (Salivette supernatant – SV_SN and Salivette pellet – SV_P). For SV_P only a tiny pellet was seen. Pellets were resuspended in 700 µl (PG_P) and 300 µl (SV_P) TE-Buffer. Ultrasound treated pellets were centrifuged for further separation (PG_P_SN, PG_P_P, SV_P_SN) as presented in Figure 1 and Supplemental Table 2. For the SV_P samples no pellet was seen after centrifugation.

Protein precipitation of each fraction (1,000 µl – SV_SN, PG_SN; 700 µl – PG_P_P, PG_P_SN, 300 µl – SV_P_SN) was conducted with TCA. Depending on the size of the resulting pellet, it was dissolved in an 8 M Urea and 2 M Thiourea solution (Supplemental Table 3). Protein concentrations of the lysates were determined using a Bradford Assay (BSA standard curve) [13]. Four micrograms of protein were reduced (dithiothreitol), alkylated (iodo acetamid) and digested with trypsin (ratio 1:25 w/w) for 17 h. Peptide lysates were desalted with two microgram ZipTip-µC18-tips. Tryptic peptide mixtures were analyzed in triplicates by shotgun nano LC MS/MS on an Ultimate® 3000 Nano LC connected to a Q Exactive plus (Supplemental Table 4).

Seventy-five MS-raw files were analyzed as one batch (Supplemental Table 5) with the Proteome Discoverer

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Supplemental data for this article can be accessed here.

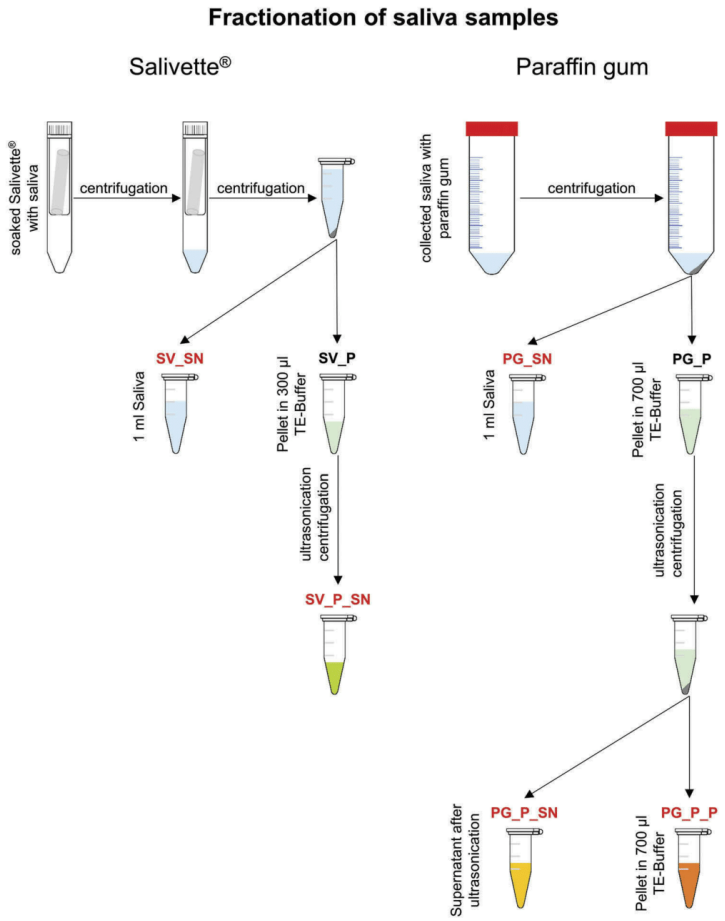

Figure 1. Fractionation procedure of saliva samples collected with Salivette® and paraffin gum. Fractions labeled in red were used for proteome analysis.

(v2.0.0.802) software using a database (size: 622 MB) including 20,154 sequences from the *Homo sapiens* proteome (UniProtKB/Swissprot, www.uniprot.org, 01/06/16) [14] and 1,079,644 sequences from 371 different species of the Human Oral Microbiome Database (HOMD, www.homd.org, 12/08/2016) [15,16]. Protein groups were accepted, if covered with ≥ 2peptides and identified in at least two out of three technical replicates. Based on the Lowest-Common-Ancestor-Algorithm-Approach [17] prophane (www.prophane.de, version 2.1.05) was used to perform taxonomic assignment using NCBI [18], BLASTP [19,20] and our database; and functional assignment using COG/KOG [21].

Saliva collection with the PG resulted in a higher volume of saliva (4.1 ± 0.8 ml) compared to the SV (1.9 ± 0.1 ml), which is in accordance with a previous report [22], and yielded also higher protein levels (Supplemental Table 3).

Relative quantification based on NSAF values (normalized spectral abundance factor) revealed that *Homo sapiens* made up the biggest proportion of spectral counts, which differed between the two saliva collection methods and fractions (Supplemental Table 3).

Regarding the human proteome, we refer to the paper by Golatowski et al. [22], which has extensively examined the human proteome data generated by SV and PG preparations. Compared to the previous study, we identified more human proteins, which is expected since we used more advanced

instrumentation (QExactive plus vs. Orbitrap Velos). However, an overlap of around 76.0% was reached comparing the same fractions (Supplemental Figure 2) [22].

With regard to bacterial proteins, more than three times more protein groups were identified (Figure 2(a)) using the PG (PG_P_SN: 1,005 protein groups) compared to the SV (SV_P_SN: 313 protein groups). Recent reports identified 1,946 [9] and 2,234 [10] bacterial proteins in human saliva. We assume that our lower protein identification rate is caused by more stringent filter parameters (paragraph 5, Supplemental Table 5) and the use of unique rather than distinct peptides. Furthermore, our study included only five subjects in comparison to other metaproteome studies [9,10].

A comparison of the two fractions (Figure 2(b)) with the highest numbers of protein groups (PG_P_P and PG_P_SN) revealed that 76.5% of the total of 1,313 protein groups were identified with the PG_P_SN fraction (overlap PG-SV: Supplemental Figure 1). A principal component analysis showed that the inter-subject variability was by far larger (PG_P_P: 31%; PG_P_SN: 31.6%) than the technical variance of the analysis (Figure 2(c,d)) and that the technical variance for the PG_P_P (14.9%) fraction was higher in comparison to the PG_P_SN fraction (13.4%). This technical variance is in accordance with a previous study [22]. The results imply that the PG_P_SN fraction is to be favoured due to the highest protein identification of all fractions and its technical reproducibility.

In total, 38 genera and 90 species could be identified, comparing those fractions of the PG (PG_P_SN) and the SV_P_SN with the highest protein group identification (Figure 3(a,b)). Within both fractions (Figure 3(a) – orange) 25 genera and 37 species were covered including the most prominent genera, like

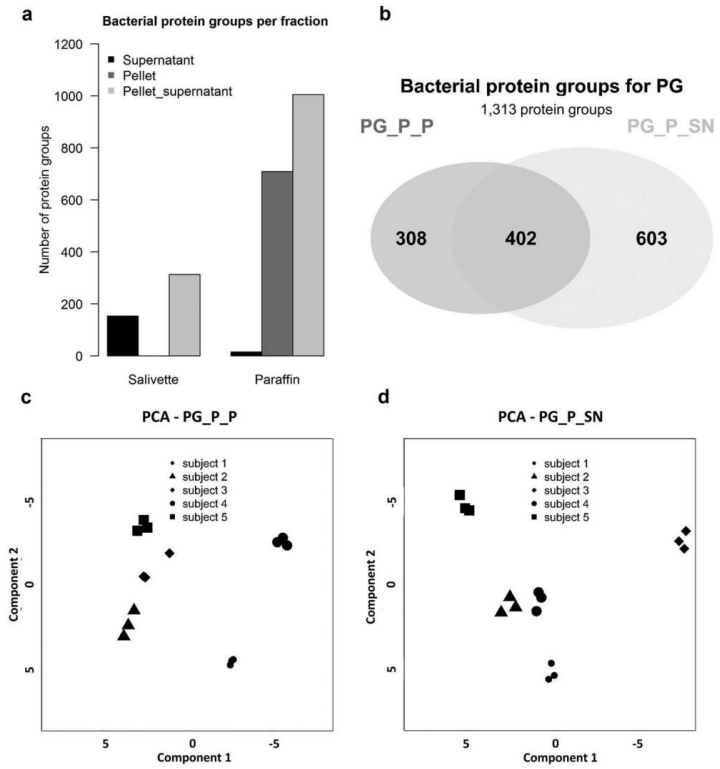

Figure 2. Evaluation of protein identification rate for two stimulated saliva collection methods. (A) The number of identified salivary bacterial proteins for each fraction collected with Salivette® and paraffin gum, respectively. (B) Venn diagram showing the overlap of the number of proteins for the paraffin gum pellet (PG_P_P) and its supernatant (PG_P_SN) fraction and those which were exclusively identified in one of the two fractions. (C, D) Principal component analysis illustrates the technical reproducibility and biological variability for the paraffin gum pellet and its supernatant fraction based on three technical replicates for each fraction.

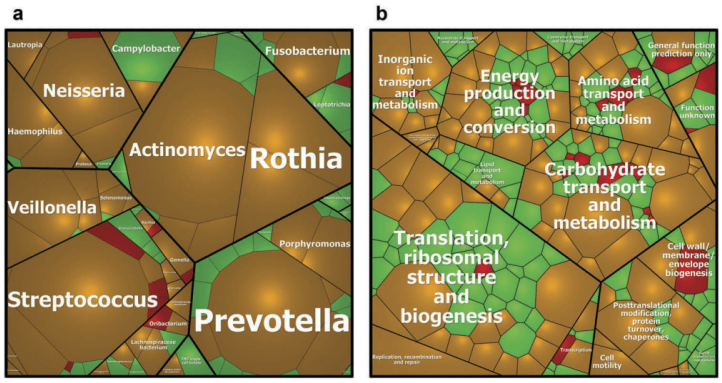

Figure 3. Voronoi treemaps demonstrate taxonomical (A) and functional (B) coverage for SV_P_SN and PG_P_SN. Relative quantification is based on averaged normalized NSAF – values (normalized spectral abundance factor) and presented as polygonal areas. Taxa and protein functions, which were exclusively found in the Salivette® samples (SV_P_SN, red) and paraffin gum samples (PG_P_SN, green) or were identified with both collection methods (orange) are displayed. The treemaps are taxonomically resolved to the species level or functionally to the specific protein function. To keep the figures as brief and clear as possible only the names down to the genus level and to general cellular processes are shown.

Actinomyces, Prevotella, Streptococcus or *Rothia* as in previous analyses [9,10,12]. Thirteen genera like *Granulicatella* and 44 species were exclusively found within the PG_P_SN fraction (green). The SV_P_SN fractions (Figure 3(a) – red) did not provide any new genera but nine species. Since the SV_P_SN fraction does not offer any added value with respect to taxonomy, we suggest using the PG_P_SN fraction.

Similar observations could also be made on the functional level based on the COG-system (Figure 3 (b)) [31]. From 291 COGs found in total, 165 COGs were identified exclusively for the PG_P_SN fraction (Figure 3(b) – green). The main functions (metabolism, cellular processes/signalling and information storage/processing) were covered with both methods (103 COGs – Figure 3(b) – orange). Just a small number of COGs could be observed in the SV_P_SN fractions (23 COGs – Figure 3(b) – red).

Based on this proof-of-principle study, collection of human saliva with the PG turned out as the method of choice for stimulated salivary metaproteomics, because it offers the best results in terms of protein identification, technical reproducibility, taxonomy and functional identification. Future studies must explore larger cohorts to describe the healthy and diseased saliva microbiome.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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biomarkers, it may be associated with periodontitis and systemic disease.

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Metaproteomics analysis of microbial diversity of human saliva and tongue dorsum in young healthy individuals

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ABSTRACT

Background: The human oral microbiome influences initiation or progression of diseases like caries or periodontitis. Metaproteomics approaches enable the simultaneous investigation of microbial and host proteins and their interactions to improve understanding of oral diseases.
Objective: In this study, we provide a detailed metaproteomics perspective of the composition of salivary and tongue microbial communities of young healthy subjects.
Design: Stimulated saliva and tongue samples were collected from 24 healthy volunteers, subjected to shotgun nLC-MS/MS and analyzed by the Trans-Proteomic Pipeline and the Prophane tool.
Results: 3,969 bacterial and 1,857 human proteins could be identified from saliva and tongue, respectively. In total, 1,971 bacterial metaproteins and 1,154 human proteins were shared in both sample types. Twice the amount of bacterial metaproteins were uniquely identified for the tongue dorsum compared to saliva. Overall, 107 bacterial genera of seven phyla formed the microbiome. Comparative analysis identified significant functional differences between the microbial biofilm on the tongue and the microbiome of saliva.
Conclusion: Even if the microbial communities of saliva and tongue dorsum showed a strong similarity based on identified protein functions and deduced bacterial composition, certain specific characteristics were observed. Both microbiomes exhibit a great diversity with seven genera being most abundant.

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Introduction

The human oral cavity with its various hard (teeth with supragingival and subgingival plaque) and soft tissues (tongue, throat, tonsils, cheeks) forms a complex ecosystem for more than 700 different species and phylotypes [1–6]. Current estimations indicate that saliva and dental plaque contain up to 10^9 and 10^{11} bacteria per ml [7], respectively. Thus, the oral cavity is the second largest microbial ecosystem in humans after the intestine [4,8].

Saliva is the most interesting biofluid in the oral cavity, as it comes into contact with all surfaces and thus represents a fingerprint of the general composition of the oral microbiome [5]. However, other microenvironments also need to be investigated in a comparative way to obtain a comprehensive view of the oral microbiome [9–11]. Therefore, it is not surprising that the analysis of the tongue microbiome is also gaining more and more attention, since tongue diagnostics has been used in traditional Chinese medicine since more than 3,000 years to assess the patient's state of health [12,13].

Traditional knowledge and current scientific studies have shown that a shift in the balance of the oral bacterial composition can indicate pathological changes [13]. This includes diseases such as halitosis [14,15], dental caries [16] and periodontitis [17,18] as well as systemic diseases like diabetes [19], respiratory diseases [20], cardiovascular diseases [21] and even cancer [9,22] due to the production of pro-inflammatory mediators [23].

However, initially the healthy microbiome [1,24–27] has to be defined before disease-related or disease-causing alterations can be described, which might ultimately lead to the development of diagnostic tools for better treatment or prevention of disease [2,28,29]. Many studies have already been initiated for this purpose using next generation sequencing [9,25,30–34]. Metagenomics provides an impression of the diversity of organisms on the tongue but also of the metabolic potential which might be present [29,35]. As a complementary approach, metaproteomics offers a possibility to measure protein intensities to capture active protein functions and taxonomic units

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Supplemental data for this article can be accessed here.

within the microbiome. Furthermore, simultaneous analysis of the microbial and human proteome can also provide insights into interactions between microbes and their host [35].

For our community proteomics study, we collected saliva and tongue samples from 24 oral healthy volunteers [36]. Our primary goal was to describe and compare the microbial composition of saliva and tongue dorsum based on metaproteome data of young healthy individuals. We combined different open-source software applications, which were mainly developed for the analysis of metaproteome data. At the same time, we compared our pipeline to other salivary metaproteomic studies [37–40] to gain information on effectiveness and accuracy.

Material and methods

Study population

Saliva and tongue samples were collected from 9 male and 15 female dental students from the dental school of the University Medicine Greifswald. The range of ages was 20–30 years with an average age of 25 years. They were non-smokers, no alcohol or drug addicts and had no systemic disease or antibiotic treatment within the last six months. Further, the subjects were not taking medication permanently. Women during pregnancy or breastfeeding were not considered. The oral health of the volunteers was ensured by the fact that the students examined each other under the guidance of an experienced dentist fulfilling the inclusion criteria: no cavitated teeth, maximal two fillings, probing depth (≤ 3 mm) and bleeding on probing value of less than 10%. The subjects included did not eat, drink or brush their teeth during 5 h before sampling, which was done during the students' university course in the late morning and early afternoon. The ethics council of the University Medicine of Greifswald approved our study and it was carried out in compliance with the recommendations of the Helsinki Declaration as amended by Somerset West in 1996.

Sampling

Saliva

Stimulated saliva was collected with a commercially available paraffin chewing gum (Ivoclar Vivadent GmbH, Ellwangen, Germany) based upon a modified protocol published previously [41]. Volunteers chewed the paraffin gum for 1 min to stimulate natural salivation. During the chewing process, the subjects collected saliva in the oral cavity and spat into a sterile 50 ml Falcon tube for several times. Twenty µl of a protease inhibitor (Sigma Aldrich, St. Louis, MO,; v/v 1:20) per 1 ml collected saliva was added to prevent protein degradation by proteases. For transportation the collected saliva was stored on dry ice and finally at −80°C until use [36].

Tongue samples

Tongue samples were taken from the middle third of the outstretched tongue dorsum with a sterile wooden spatula (NOBA Verbandmittel Danz GmbH and Co KG, Wetter, Germany), 18 mm x 150 mm. The sterile wooden spatula was pressed onto the tongue for 5 swith light and even pressure and then turned over to repeat the process on the other side. After this procedure, the spatula was transferred into a 50 ml Falcon tube containing 2 ml sterile 1 x PBS (gibco®, Thermo Fisher Scientific, Waltham, MA; pH = 7.4) and 40 µl of a protease inhibitor and vortexed for 30 s. The spatula was discarded. For transportation the sample was stored on dry ice and then stored at −80°C until further processing.

Sample preparation

Cell disruption

Saliva preparation were performed using a published protocol [40], which was slightly modified [36]. The collected saliva was first thawed on ice and centrifuged for 15 min at 4°C at 11,500 g. The supernatant was discarded, and the remaining pellet was resuspended in 700 µl TE buffer (10 mM Tris; 1 mM EDTA; pH 8.0). An ultrasound treatment (Labsonic U – B. Braun Melsungen AG, Melsungen, Germany) was carried out for 3 × 30 s on ice (50% power of the device) to disrupt the cells in the pellet followed by another centrifugation step (30 min, 4°C, 16,200 g). The supernatant was stored on ice for further preparation.

For the tongue samples, our preliminary tests showed that the prior vortexing of the sample in connection with the Freeze-and-Thaw process in sterile 1 x PBS (gibco ®, pH 7.4 – $CaCl_2$ – $MgCl_2$) is a well-suited cell disruption method for this sample type.

MS sample preparation

After thawing on ice, 1 ml of the respective supernatant of the tongue samples and 700 µl of the prepared saliva were used for protein precipitation by TCA. DTT was added to the samples (0.02 g/100 µl), samples were vortexed for 10 s and incubated at 37°C for 30 min. For the subsequent precipitation of the proteins, TCA (100%) was added up to a final concentration of 15% and samples were stored on ice for 60 min. The precipitated samples were centrifuged for 45 min (17,000 g, 4°C). To remove the TCA, supernatants were discarded, 500 µl of 100% cold acetone was added and centrifuged for another 15 min (17,000 g, 4°C). The washing step was repeated once again. Samples were vacuum dried for 1 min. The remaining pellets were diluted in 50 µl (saliva) and 35 µl (tongue) 1 x UT solution (8 M

urea/2 M thiourea). To define the technical variance and the reproducibility of our study, all samples were prepared in triplicates. A Bradford assay [42] was performed to determine the protein concentration of saliva (Ø 6.4 µg/µl ± 2.3 µg/µl) and tongue samples (Ø 1.7 µg/µl ± 1.6 µg/µl). Four µg protein were reduced with DTT (2.5 mM final concentration, incubation for 60 min at 60°C) and alkylated with IAA (10 mM final concentration, incubation for 30 min at 37°C in the dark). After a 1:10 dilution of the 1 x UT solutions, protein digestion was conducted with trypsin in a ratio of 25:1 (w/w) over a period of 17 h. Peptide mixtures were purified with ZipTipC_{18} material.

NLC MS/MS measurement

Proteolytic digestion of the proteins with trypsin was followed by analyzing the 144 samples using nano-LC -MS/MS (Supplemental Table 1). The complex peptide mixtures were separated according to their physicochemical properties by means of a reverse phase nano HPLC on an Ultimate® 3000 Nano HPLC (Thermo Scientific). The peptide mixtures were loaded onto a precolumn (Acclaim PepMap100, Thermo Scientific: 75 µm inner diameter, 3 µm C_{18}-particles), subsequent separation of the tryptic peptides took place on a 25 µm analytical column (Accucore PepMap RSLC, Thermo Scientific: 25 cm x 75 µm, 2.6 µm C18 particles) via a linear gradient (120 min, 2–25% buffer B) using a binary buffer system consisting of 2% acetonitrile in 0.1% acetic acid (buffer B). The mass spectrometric data were acquired by means of a data-dependent acquisition procedure on a QExactive™ Plus as described before [36] and revealed 5,749,982 spectra. To assure a high quality of our MS data, only spectra with a mFDR ≤ 0.06% were accepted, resulting in 1,933,390 spectra. A complete overview of the laboratory workflow is given in Figure 1(a). All metaproteomic data sets were uploaded to the publicly accessible *MassIVE* database with the dataset link ftp://massive.ucsd.edu/MSV000084137 and doi:10.25345/C53H2C.

Data analyses

To evaluate our metaproteomic data we used a two-step data analyzing pipeline (Figure 1(b)). We

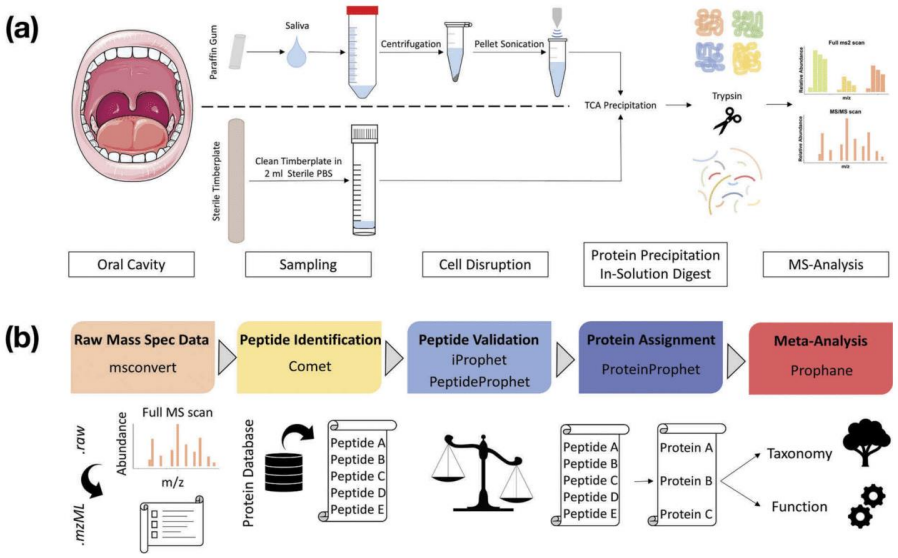

Figure 1. Laboratory workflow for saliva and tongue microbiome analysis (a). Tongue samples were collected with a sterile wooden spatula and transferred into sterile PBS. Salivation was stimulated by chewing a paraffin gum and the subjects spit into a Falcon tube®. Saliva was centrifuged and the resulting pellet was solved in TE-buffer and treated with ultrasonication. Proteins from saliva and tongue samples were precipitated with TCA and digested with trypsin. Peptide mixtures were measured with a Q Exactive™ Plus (LC-MS/MS). Bioinformatic workflow for metaproteomic data analysis (b). The Trans-Proteomic Pipeline was used for the following four steps: (1) Raw-data conversion to mzML-data format. (2) MS/MS database search by the Comet project for peptide identification based on a combined database (Human Swissprot + Human Oral Microbiome Database). (3) Validation of identified peptides. (4) Protein assignment and data filtering by stabilizing false discovery rates (mFDR, pepFDR) with a protFDR of 5.0 %. Finally, the online web-tool Prophane was applied to conduct taxonomic and functional prediction and the statistical analyses were performed in R.

identified spectra, peptides and proteins with stringent criteria using the *Trans-Proteomic Pipeline* (http://tools.proteomecenter.org/software.php), which was developed at the Seattle Proteome Centre. The annotation of the proteins regarding their taxonomy and cellular function was performed by Prophane (www.prophane.de), developed at the Institute for Microbiology at the University of Greifswald.

In detail, *msconvert* (version: 3.0; peak picking based on Vendor algorithm – MS Levels: 1–2) converted the .*raw*-files into readable .*mzML*-format [43]. Peptides were identified using the *Comet* algorithm (http://comet-ms.sourceforge.net/, version: 2016.01 rev. 2) [44,45]. The sequence database (size: 964 MB) consisted of 1,079,744 sequences from the human oral microbiome database (HOMD, www.homd.org, 12/08/2016) [1,46], 20,154 human sequences from UniProt (UniProtKB/Swissprot, www.uniprot.org, 01/06/2016) [47] and their reverse sequences for decoy searches to calculate the false discovery rate. The search algorithm considered trypsinated proteins with a maximum of two missed cleavages. Peptide masses were not allowed to exceed the tolerance range of ± 10 ppm and only monoisotopic masses were included into the analyses. Variable oxidations of methionine [+15.9949] and fixed carbamidomethylation of cysteine [+57.021464] were also considered. Peptides identified by *Comet* [44,45] were weighted and the probability for their existence was calculated with the modules *iProphet* [48], *Peptide Prophet* and filtered using Mayu (version: 1.08) [49]. The *ProteinProphet* assigned the peptides to their corresponding proteins and were accepted with a false discovery rate < 0.05. All proteins, which were covered with at least one unique peptide, were extracted from the data set by an R script (version: 3.4.1) [50] and finally uploaded into the tool Prophane (www.prophane.de). To determine the taxonomic origin of the proteins, Prophane used the Lowest-Common-Ancestor algorithm [51] based on the results of BLASTP (e-value: < 0.01) [52,53] and the database described above. Proteins of bacterial origin are referred as metaproteins, because proteins of one protein group can be assigned to one or more species [54,55]. Thereby, the term 'meta' indicates that a different taxonomic distribution could form the basis of a protein group [56]. Metaproteins are referred to as 'heterogeneous', if an assignment was not successful on the corresponding taxonomic level (www.prophane.de). (Meta-) Protein functions were classified according to COG/KOG classification (RPS-BLAST 2.2.28+ algorithm; e-value: < 0.01) [57]. The relative quantification of the proteins was performed by spectral counting [58]. The MS/MS spectra obtained were counted and then normalized by prophane using the normalized spectral abundance factor (NSAF-values) [59–62].

Statistical analyses

The evaluation and statistical analyses were performed in R (version 3.4.1) [50]. In general, a global median normalization was performed for the raw NSAF values. The mean value was calculated for the three measured replicates per sample. Depending on the respective analysis, the sums of the mean NSAF values were calculated to sum up subject-specific spectra per metaprotein, protein, genus or functional subrole.

The factomineR package (version: 1.36) [63] was used for PCA analyses. We did not include missing values and subtracted the column means from their corresponding columns. The centered columns were divided by their standard deviation to unify variance scaling of the data. The data were $\log_2$ transformed.

We used the metacoder package (version: 0.1.3) to create heat trees for taxonomic analyses [64]. For Figure 3 A/B, the sum of the $\log_2$ transformed column means (color intensity) was plotted against the sum of the spectral counts (thickness of the individual branches) per taxonomy. For Figure 5, the ratios of the column mean between saliva and tongue were calculated and plotted against the sum of the spectral counts. Resulting missing values were removed.

Our statistical analyses were based on a paired two-sided Wilcoxon signed rank test. The confidence interval was set at 0.95 and the p-value was adjusted for multiple testing using the Benjamini-Hochberg method. A fold-change cutoff = 1.5 and a p-value cutoff = 0.05 were set for the volcano plots.

Results

General metaproteome data

We collected one saliva and one tongue sample from each of the 24 subjects and prepared them in three technical replicates. Based on our quality criteria and the combined database of human and bacterial protein sequences, 31,386 distinct peptides for saliva and 31,215 distinct peptides for tongue samples were identified (pepFDR ≤ 1.43%) and assigned to proteins (Supplemental Table 2).

To decrease the number of shared peptides and thus the likelihood of incorrect assignments, only proteins containing at least one unique peptide and a protFDR ≤ 5.0 % were considered resulting in 4,280 saliva proteins of which 1,647 proteins were of human origin and 2,633 bacterial metaproteins. In tongue samples 4,644 proteins were identified of which 1,337 were human proteins and 3,307 bacterial metaproteins.

To quantify our identified proteins, we used a relative quantification approach. For this purpose, Prophane was used to calculate the normalized spectral abundance

factor (NSAF-values) based on spectral counts [60] using the longest sequence in each protein group. Our data showed variations regarding the proportions of human and bacterial abundances in our samples. While human proteins accounted for 78.2% and bacterial metaproteins for 21.7% in saliva, the ratio was different for the tongue. Human proteins accounted for only 59.1% whereas the proportion for bacteria was almost twice as high (40.8%). These differences were also reflected regarding the number of identified proteins. At least 50% of the bacterial and human proteins (Bacteria: 1,971 metaproteins and Humans: 1,154 proteins) could be identified in both the saliva and on the tongue (Figure 2(a,c)). However, more than twice as many specific bacterial metaproteins could

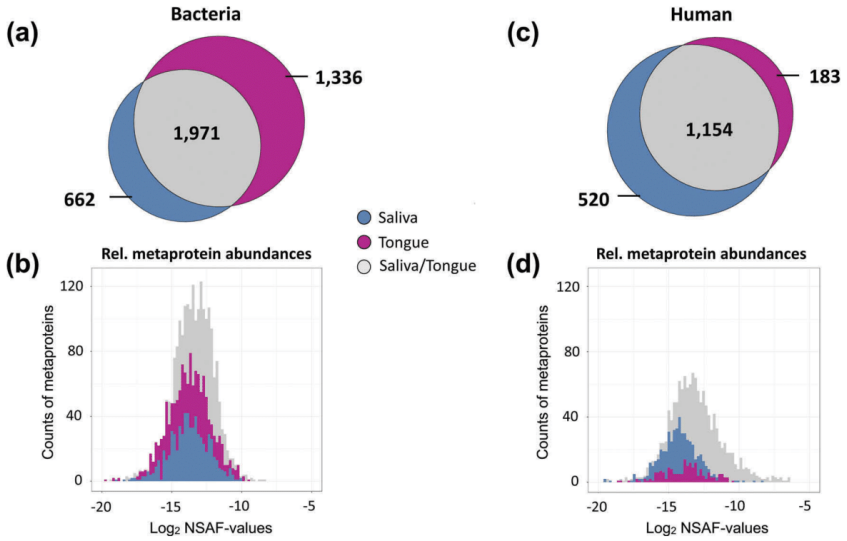

Figure 2. Venn diagrams displaying the number of identified metaproteins in the studied saliva and tongue samples for bacteria (a) and human species (c). Histograms of relative metaprotein abundances based on log_2 normalized spectral abundance factors (NSAF-values) [60] for bacterial (b) and human proteins (d). The figure emphasizes the distribution of metaproteins for saliva (blue), tongue (red) or shared between both (grey).

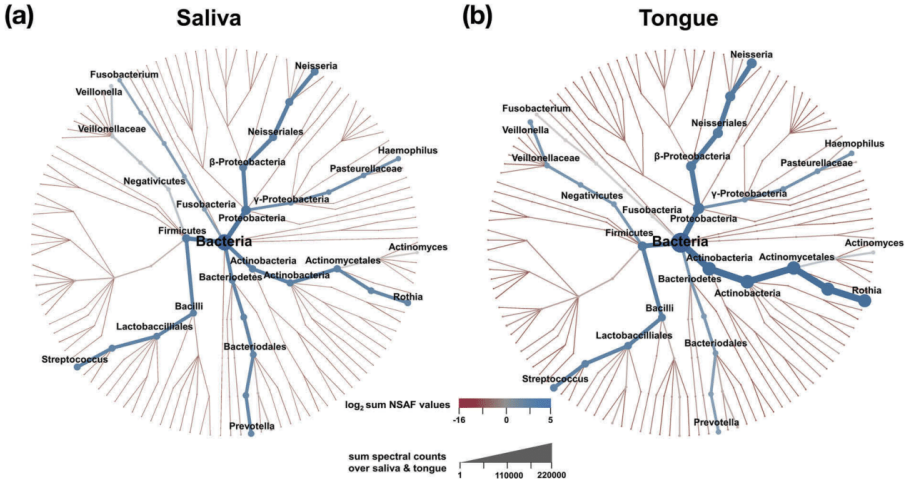

Figure 3. Heat trees of taxonomic composition of the healthy saliva (a) and tongue (b) microbiome. Coloration is defined by log_2 sum normalized spectral abundance factors (NSAF-values) [60]. The number of spectral counts for each branch determines its thickness.

be identified for the tongue (1,336 metaproteins) compared to saliva (662 metaproteins), which was also associated with the above mentioned higher relative abundance (Figure 2(a,b)). For the human salivary proteins, we observed the opposite. 520 proteins were only found in saliva, almost three times as many specific proteins (Figure 2(c,d)) as on the tongue (183 proteins).

A principal component analysis (PCA), which was performed with the relative protein intensity data revealed that the interindividual variance was by far greater than the technical variance (Supplemental Figure 1 and 2). We assessed the inter-subject variability (biological CV) and nLC-MS/MS measurement accuracy between the triplicates for each saliva and tongue sample (technical CV) based on the calculation of the coefficient of variance on NSAF values [60]. Our calculations showed an averaged biological CV of 32% for saliva and tongue. The technical CV for those samples was clearly lower for the tongue (18%) and the saliva (16%) samples.

Furthermore, we were interested in the degree of increase for protein identification by measuring the samples in triplicates. We found that the identification rate of proteins increased after two measurements by an average of 17.3% and after the third measurement by additionally 9.3%. Thus, including the results of three technical replicates increased the number of covered proteins by 28.2%.

Taxonomic profile of saliva and tongue

The taxonomic composition of the oral microbiome has been shown to have an impact on human health [8]. For each protein the taxonomic assignment of the best hit of BLAST [52,53] against the NCBI nr database was used to get a first impression of the diversity and quantity of bacteria in saliva and on the tongue.

In total, we identified seven phyla (Supplemental Figure 3), of which *Actinobacteria, Bacteriodetes, Firmicutes, Fusobacteria*, and *Proteobacteria* were most common and have been detected in all subjects. A comparison of the two sample types revealed that *Proteobacteria* and *Firmicutes* appeared almost in an equal abundance, while *Actinobacteria* emerged as more abundant on the tongue. *Bacteriodetes* and *Fusobacteria* showed a contrary trend and were identified in smaller abundances on the tongue. The two other phyla *Spirochaetes* and *Synergistetes* contributed with only a small proportion to the bacterial community. Furthermore, we could identify *Chlamydiae*, but only for five subjects in saliva and therefore they were excluded from further analysis. At the genus level we could assign 93.9 % of all 3,969 bacterial metaproteins to 107 different genera and we found a high similarity between saliva and tongue with an overlap of 89 genera (83.0%). To gain insight into the distribution at the genus level, we created heat trees where summed relative abundances of spectral counts were plotted (Figure 3(a,b)). In general, we identified higher bacterial abundances on the tongue in comparison to saliva. For saliva and the tongue, we recognized a high bacterial diversity but only the seven genera *Rothia, Prevotella, Streptococcus, Veillonella, Fusobacterium, Neisseria*, and *Haemophilus* mainly determined the composition of both microbiomes. In summary, we could observe a great diversity in saliva and tongue dominated by seven phyla and genera, but we could only observe small and non-significant differences when the two sample types were compared to each other (Supplemental Table 3). Likely more subtle differences were masked by the large interindividual differences in the microbiomes observed in this and other studies.

To increase the sensitivity of our analyses, we performed a pairwise analysis (paired Wilcoxon signed rank test, p-value: < 0.05) at genus level. Figure 4(a,b) illustrates ten genera with significant differences. *Fusobacterium, Selenomonas, Bifidobacterium* and *Treponema* were found to be significantly increased in

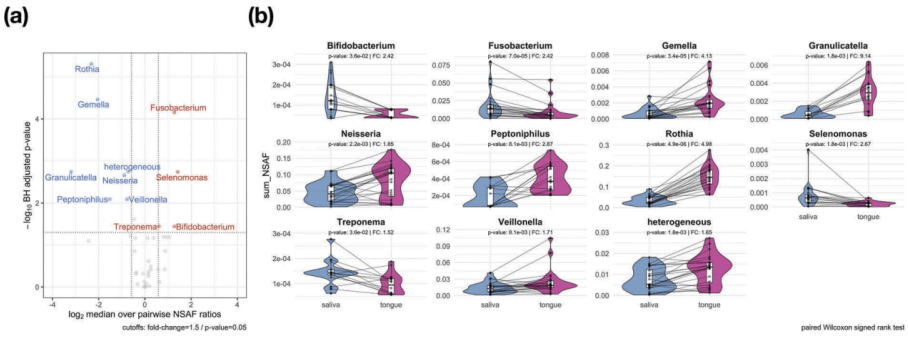

Figure 4. Significant taxonomic profile differences on the genus level between saliva and tongue are displayed in the volcano plot (a) by depicting the results of a two-paired Wilcoxon signed rank test. The comparison plots (b) show the sum of the NSAF values for those genera identified as significantly higher abundant in saliva or on the tongue. Metaproteins in the group 'heterogeneous' could not be assigned unambiguously to a genus.

saliva compared to the tongue. The opposite was the case for *Rothia, Gemella, Granulicatella, Peptoniphilus, Veillonella* and *Neisseria*. Furthermore, we identified significant changes in the category 'heterogeneous', which cannot be further described, since an assignment on genus level was not feasible.

Figure 5 provides a more detailed picture of the differences in taxonomic profiles of saliva and tongue samples by representing the complete calculated ratios in a phylogenetic tree. In combination with the results of the Wilcoxon signed rank test, it highlights the differences regarding the taxonomic composition of the two microbiomes. Even though *Rothia, Veillonella, Fusobacterium* and *Neisseria* belonged to the dominant genera, they also showed great differences between saliva and tongue, which was not the case for the genera *Prevotella, Streptococcus, Haemophilus* and *Actinomyces*. Genera such as *Granulicatella, Gemella, Peptoniphilus* or *Bifidobacterium*, which do not dominate the two microbiomes and would thus not to be noticed at first glimpse, also showed relevant and significant differences.

We also analyzed, whether we could identify any gender differences in the microbiome composition, but our results did not indicate any significant and specific microbiomes for males or females (paired t-test, p-value: < 0.05; fold-change > 2).

Functional profiling of bacterial metaproteins

Metaproteome analyses enable simultaneous assessment of expressed human and bacterial metabolic pathways. From a global point of view, we covered 18 biological processes based on the COG classification [57] for bacterial metaproteins (Supplemental Figure 4) in saliva and on the tongue. The most common functions were translation, energy production, carbohydrate metabolism and amino acid metabolism (Supplemental Table 4). Again, there was a strong similarity between saliva and tongue regarding functional composition at this global perspective. As expected, due to their high abundance the functional profile was dominated by metaproteins involved in translation with an averaged portion of 40.2% and 29.8% for the tongue and saliva, respectively. Processes like cell cycle, secondary metabolites, intracellular transport, signal transduction, defense mechanism and cell motility made up less than 1%, but again in a pairwise analysis (paired Wilcoxon signed rank test, p-value: < 0.05) all these functions with the exception of cell motility were found significantly increased on the tongue compared to saliva (Figure 6). A similar conclusion could be reached for metaproteins that are involved in bacterial cell wall biogenesis, coenzyme and nucleotide metabolism as well as in replication, transcription and translation processes. Thus, only metaproteins of cell motility displayed

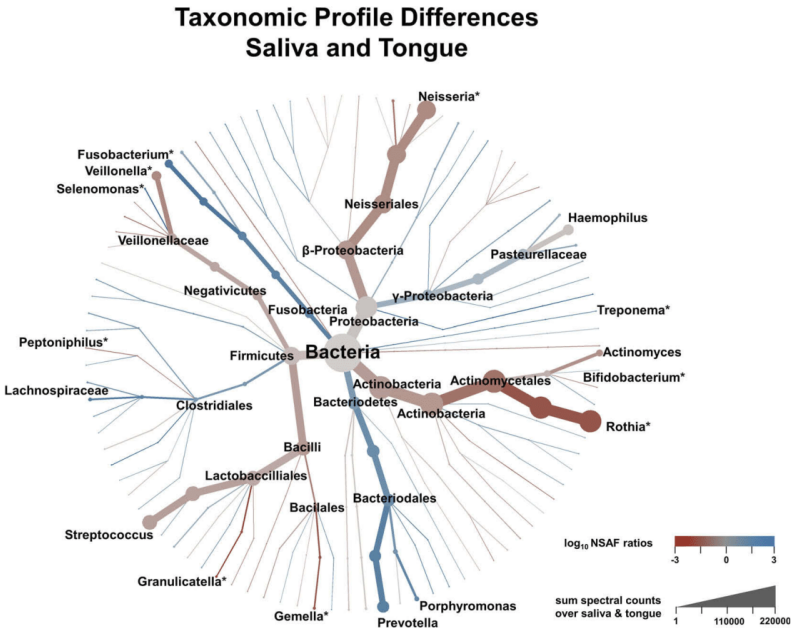

Figure 5. Illustration of taxonomic differences between saliva and tongue based on median over pairwise NSAF ratios (coloration) and the sum of spectral count (branch size). Genera marked with an * showed significant differences between both microbiomes according to a Wilcoxon signed rank test (Benjamini-Hochberg corrected p-value < 0.05).

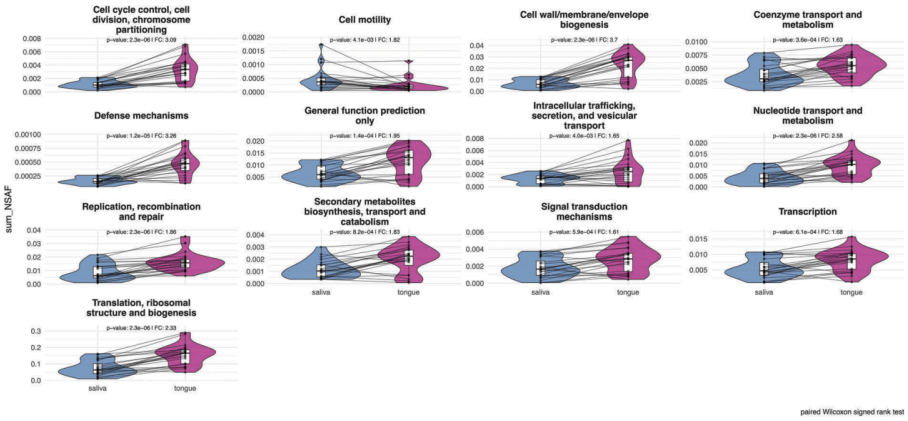

Figure 6. Comparison plots show the different relative abundances of bacterial metaprotein functions with significant differences, which were determined by a two-sided pairwise Wilcoxon signed rank test (p-value < 0.05) with a fold change of > 1.5. The calculated p-value has been corrected according to the Benjamini-Hochberg method.

increased levels in saliva. We were not able to determine the exact functions of all metaproteins, and thus the remining proteins were summarized in the category 'general function prediction only', which was also significantly different in saliva and tongue.

Functional profiling of human proteins

As already shown in Figure 2(a,c), there was a great overlap between human proteins in saliva and on the tongue, which remains observable by ranking proteins based on their relative abundance and considering the top highest and lowest abundant proteins (Figure 7(a)). Some of the highest proteins identified in saliva and on the tongue were alpha-amylase (AMY1), which catalyses the digestion of starch and glycogen [65], glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for the reversible oxidative phosphorylation of glyceraldehyde-3-phosphate [66] or the phospholipase A_2 inhibiting protein annexin A1 (ANXA1) [67]. However, proteins such as the laminin subunit alpha 3 (LAMA3) belonging to the laminin family, the glycoprotein mucin 2 (MUC2) [68] or the F-actin-binding species repeat containing nuclear envelope protein 2 (SYNE2) [69] could only be found at low abundance.

Pairwise analysis of human proteins revealed that 75 proteins occur in saliva in significantly lower abundance than on the tongue, while 232 proteins were significantly higher in saliva compared to the tongue surface (Figure 7(b)). Many proteins with increased abundance in saliva (Figure 8) play a role in the innate or adaptive immune system (Lypmhocyte cytosolic protein 1 – LCP1 [70]; BPI fold containing family B member 1 – BPIFB1 [71]; Elastase – ELANE [72]; Annexin A3 – ANXA3 [67]). Proteins with a higher abundance on the tongue could be assigned to the

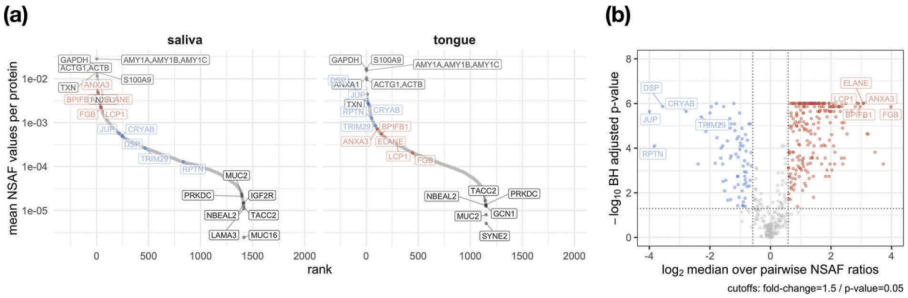

Figure 7. The coverage of the dynamic range of human proteins is shown by plotting the mean relative abundance for saliva and tongue (a). The human proteins are named according to their gene names and show for saliva and tongue a selection of proteins with highest and lowest abundances (grey). Data points in red and blue display proteins with a fold change > 1.5 and a p-value < 0.5 (paired Wilcoxon signed rank test) comparing saliva and tongue (a). Proteins with the largest changes are highlighted with their gene names (A/B).

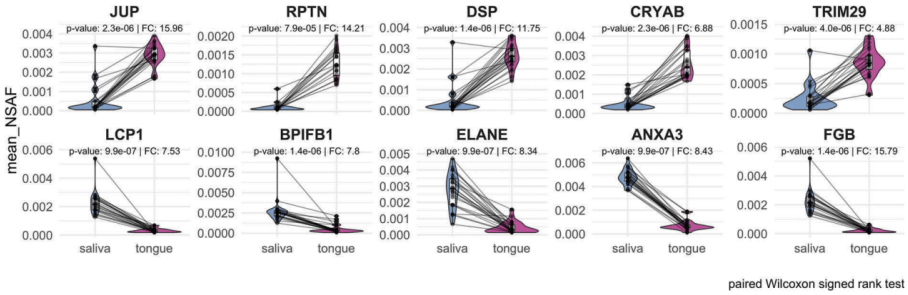

Figure 8. Representation of the top five proteins with the highest increase or decrease regarding to their relative abundance in saliva or on the tongue based on paired Wilcoxon signed rank test (p-value < 0.05).

cytoskeleton (Figure 8), e.g. Junction plakoglobin (JUP) and Desmoplakin (DSP), playing a role in the regulation of innate immunity (Tripartite motif containing 29 – TRIM29) [73] or prevent possible irreversible protein aggregations as chaperones (Crystallin alpha B – CRYAB).

Discussion

The primary goal of this study was to describe and compare the human saliva and tongue microbiome of healthy young individuals. Initially, we wanted to explore whether we were able to achieve comparable results in protein identification with the pipeline described in this report compared to other metaproteome studies [37–40]. For the tongue analyses we identified 4,644 proteins of which more than 70% originated from bacterial species. To the best of our knowledge, this is the first study providing metaproteome data for the tongue. Regarding salivary proteins, we profiled slightly less human proteins compared to Grassl et al. and BelstrØm et al. [39,40]. At the same time, the number of bacterial proteins covered was slightly higher in the current study. Possible reasons include the use of different or modified protocols for sample collection, preparation and measurement [37–40]. The same applies to the different data analysis strategies, which have an impact on the peptide-protein assignment [44,45,74,75], especially for bacterial proteins due to the high number of different taxa [76], which results in many shared peptides on the protein level [55]. Furthermore, the number of subjects and particularly the cohorts differed. Whereas former studies included diseased subjects [38,40], in whom large interindividual differences must be expected, especially in the case of bacteria [22,24], our study was confined to young healthy participants to define baseline-microbiomes of the healthy population.

For future studies, we also wanted to clarify whether it is necessary to analyze technical replicates to obtain reliable metaproteome results and whether the related significant increase in measurement time is associated with a relevant increase in protein identifications. It became apparent that in saliva with a technical variance of 16%, we achieved similar results as previous (meta-) proteome studies [36,77]. Additionally, measuring three replicates, we achieved an increase in protein identification of around 28.2%. However, considering the good technical reproducibility of the data and the threefold increase in measurement time, we do not consider replicate measurements to be the preferred solution. Rather, we propose to cover the diversity of the metaproteome and thus of the microbiome by measuring more samples from different individuals, since our data and other microbiome studies point to large interindividual differences [5,8,9,24,27].

As another aspect of this study, we investigated the relative abundance of human and bacterial proteins, where we expected a higher proportion of human proteins in saliva than on tongue. We can confirm these expectations with our data for several reasons. Bacteria in saliva are planktonic whereas on the tongue bacteria are likely organized in a biofilm [38]. In addition, saliva consists to 99% of water [78], which may lead to a dilution of the bacteria. Furthermore, human proteins are two orders of magnitude more abundant than those of bacteria [39], which leads to the suppression of less abundant proteins during the measurement [37]. In particular, alpha-amylase (AMY1A), or S100 calcium binding protein A9 (S100A9) have to be mentioned, both displaying high abundances in our saliva and tongue data, an observation also made for saliva before [79]. We also detected alpha-amylase (AMY1A) [65], glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [66], annexin A1 (ANXA1) [67] and 62% of all saliva human proteins on the tongue, which indicate that the human part of the tongue surface proteome is, as

expected, partially shaped by the surrounding saliva [5,9].

The assignment of proteins to specific bacterial species is a challenge for metaproteomic studies due to protein homologies [55,74]. We could also confirm the observations made by BelstrØm et al. [40] and Rudney et al. [38], which showed that the assignment of proteins to certain taxa decreases when approaching the species level.

To the best of our knowledge, this is not only the first study providing a metaproteomic description for the tongue but also its comparison with saliva. Saliva and tongue microbiome both revealed a high diversity, dominated by the seven genera *Rothia, Prevotella, Streptococcus, Veillonella, Fusobacterium, Neisseria*, and *Haemophilus*. For saliva we confirmed the results of previous metaproteome studies at the phylum and genus level [37–40]. Thus, *Actinobacteria, Proteobacteria, Firmicutes, Bacteriodetes* and *Fusobacteria* were also the five most abundant phyla [80]. The observed high diversity is probably caused by a large interindividual microbial variation especially at the lower taxonomic levels [22,81]. Additionally, our data indicated that saliva and tongue microbiomes display a strong taxonomic similarity, which is in agreement with a comparative 16S RNA study of saliva and tongue microbiomes conducted by Hall et al. [24] as well as a study from Papaioannou et al. [25]. At the phylum level, we initially found no significant differences between saliva and tongue in a general comparison of both sample types. Increasing sensitivity by pairwise analysis of the samples from the same individuals, the impact of interindividual differences could be reduced and significant differences at genus level between both microbiomes were revealed [80,82]. Our data indicate, that even genera, which do not dominate the microbiome do clearly contribute to the differences between the two microbiomes.

Most genera were also present in higher abundances on the tongue, which could provide a further hint that the tongue might be a reservoir contributing to the composition of the saliva microbiome [24]. This may suggest that more attention might need to be paid to the tongue in oral hygiene, since pathogenic bacteria seem to be present even in a healthy microbiome [11,38] and could be distributed from the tongue throughout the oral cavity by saliva [24].

Although metaproteome studies are not as sensitive for the determination of bacterial diversity as metagenome studies, they provide the decisive advantage of analyzing expressed metabolic pathways and thus metabolic activity [35].

Analysis of protein functions demonstrated besides taxonomic also functional similarity with relevant characteristics between saliva and tongue. Our findings are in line with previous observations that despite an interindividual diversity between different habitats a functional conservation exists [83]. At least 30% of the identified proteins play a role in translation and especially different ribosomal proteins have been found, which supports the hypothesis that these proteins are essential for (growing) microbes [84] and are therefore highly conserved and abundant in metaproteomic samples [38]. Bacteria in saliva are in a planktonic state, which might be an explanation that we identified a significantly increased number of metaproteins especially with functions in cell motility [38]. On the other hand, the tongue microbiome exists as a biofilm with significantly different environmental conditions [2]. Biofilms are continuously exposed to the human immune system, which might explain the increased abundance of defense mechanisms metaproteins [85]. The increased occurrence of metaprotein functions like signal transduction and secondary metabolites may suggest increased intra- and inter-bacterial communication [86]. Reasons for this could include competitive or mutualistic behavior [87]. Metaproteins with functions in replication, transcription and translation might indicate a still growing biofilm [88].

For some of the metaproteins it was not possible to determine their functions. The same applied to the taxonomic classification of metaproteins, which were classified as 'heterogeneous'. Here, currently existing databases as well as analysis tools reached their limits. In this case, future metaproteome analyses will benefit enormously from improved databases and analysis tools, which will enable a better assignment of metaproteins on a taxonomic as well as functional level [37,89].

Besides the digestion of glycogen by alpha-amylase, another important function of saliva is the maintenance of the balance of the microbiome and the defense against pathogens by the immune system [90]. This could be an explanation for the significantly increased number of human proteins, whose functions were mainly involved in the immune response system.

The tongue is a muscular organ with a keratinized stratified squamous epithelium and mostly cytoskeletal proteins or the repetin (RPTN) involved in the cornified cell envelope formation have been identified [91], which we attribute to the fact that the sample material was scraped off directly from the tongue, whereas saliva is a mixture from the salivary glands [92].

Limitations of our study include the unequal distribution of male and female subjects as well as the rather small number of 24 subjects. Therefore, like Grassl et al. [35] we could not detect sex specific differences in the microbiome. Nevertheless, we consider the question about the *microgenderome* [93] to be important and worth studying [94]. So far, besides anti-microbial effects of saliva [79], also significant differences in the salivary microbiome of male and female children [95], possibly due to the endocrine system, have been

described. Women have about twice the chance of getting caries than men [78] and thus Lukacs and Largaespada hypothesized that possible reasons could be factors like a reduced salivary flow rate and hormone fluctuations [78], which likely influence the microbiome. Gender differences in the microbiome are also further supported by current studies of the gut microbiome, which were able to detect differences between men and women [96–98].

For this 'proof of principle' study a selected cohort of dentistry students with a defined small age range was selected. Future metaproteome studies addressing the healthy oral microbiome in a larger cohort should provide a better demographic [99] and geographical diversity [100,101]. In addition, it must be clarified under which criteria a microbiome can be considered as 'healthy' [28]. This definition is not a trivial task as previous discussions have shown [102–104]. In addition to the recording of clinical parameters, the personal oral hygiene of the subjects [105], their diet [106], genetic background [107], socio-economic status [108] as well as other aspects must be considered, which will increase the effort and complexity of a study significantly. It must e.g. also be clarified, which influence the circadian change of the flow rate of saliva has on the time of sample collection [109,110], even if previous studies have shown temporal stability of the oral microbiome over a longer period [5,111,112].

Therefore, we conclude that several basic issues still need to be addressed in future studies of oral microbiomes. Nevertheless, providing many different parameters for a cohort increases the quality of a study, which is particularly important for clinical studies that want to distinguish between healthy and diseased microbiomes [23,29].

Conclusions

Our metaproteome study aimed to provide a detailed insight into the taxonomic composition and functional diversity of saliva and tongue in 24 healthy young adult volunteers. This is the first study, which described the healthy tongue microbiome of young subjects and compared it to saliva based on metaproteome data. Therefore, we have developed a strategy to evaluate large metaproteome data sets by combining TPP and Prophane. Essentially, we found a high bacterial diversity for saliva and tongue, which was mainly determined by seven genera. Globally, we identified high taxonomic similarity and functional consistency between both microbiomes, although we must emphasize that interindividual differences strongly influence the taxonomic composition. However, using comparison of paired samples from the same individuals, we were also able to show decisive functional differences of bacterial metaproteins between the biofluid saliva and the tongue biofilm. The good agreement of our results with those of already performed metagenome and metaproteome studies demonstrated that our workflow can provide consistent metaproteomic results.

To ensure an even better description of the different human microbiomes, future studies should focus on multi-OMICs approaches. Furthermore, the size of cohorts needs to be increased to enable a more precise identification of interindividual differences, which should allow a more accurate description of the microbial profile of a healthy microbiome and the distinctive features from dysbiotic states in pathological situations.

Author contributions

study design: AR, MGS, TK and UV
ethics application: AR, MGS, SF, AW, TK and UV
sample collection: AR; MGS
sample preparation and nLC-MS/MS measurement: AR and MGS
data analysis and analytical tools: AR, SM, SF
drafting of manuscript: AR
editing of manuscript: MGS, SM, SF, AW, TK and UV
acquisition of funding AW, TK, MGS and UV

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Disclosure statement

No potential conflict of interest was reported by the authors.

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Abstract	Analysis using mass spectrometry enables the characterization of metaproteomes in their native environments and overcomes the limitation of proteomics of pure cultures. Metaproteomics is a promising approach to link functions of currently actively expressed genes to the phylogenetic composition of the microbiome in their habitat. In this chapter, we describe the preparation of saliva samples and tongue swabs for nLC-MS/MS measurements and their bioinformatic analysis based on the Trans-Proteomic Pipeline and Prophane to study the oral microbiome.	
Keywords (separated by '-')	Saliva - Tongue - Metaproteomics - Human oral microbiome - nLC-MS/MS	

TITLE

Bottom-Up Community Proteome Analysis of Saliva Samples and Tongue Swabs by Data-Dependent Acquisition nano LC-MS/MS Mass Spectrometry

RUNNING HEAD

Saliva and Tongue Community Proteomics

ABSTRACT

Analysis using mass spectrometry enables the characterization of metaproteomes in their native environments and overcomes the limitation of proteomics of pure cultures. Metaproteomics is a promising approach to link functions of currently actively expressed genes to the phylogenetic composition of the microbiome in their habitat. In this chapter we describe the preparation of saliva samples and tongue swabs for nLC-MS/MS measurements and their bioinformatic analysis based on the Trans-Proteomic Pipeline and Prophane to study the oral microbiome.

KEY WORDS

5-10 key words

saliva, tongue, metaproteomics, human oral microbiome, nLC-MS/MS

1. INTRODUCTION

Mass spectrometry became the method of choice in the field of proteomics when peptides, proteins or post-translational modifications need to be analyzed within a short time and with high accuracy (1,2). In recent years improved sensitivity of mass spectrometers (3,4) in combination with the availability of high-quality metagenomic databases (5–8) have enabled in depth metaproteome analyses in addition to proteome analyses of pure cell or bacterial cultures (9). The resulting field of metaproteomics offers the possibility to study bacteria and their actively expressed genes directly in their natural habitat (10). It is therefore a promising approach not only to determine the phylogenetic composition of the microbiome, but also to uncover functional aspects and their response to changing environmental influences (11,12). This is essential to improve our understanding of polymicrobial diseases in humans (13,14).

Metaproteomics is an emerging scientific field, and initial studies and approaches for the investigation of the microbiome in different human habitats have been emerged (15–19). In a metaproteomic study in young healthy humans, we compared saliva samples and tongue swabs. Our study includes the phylogenetic composition of both microbiomes, their translated proteins as well as the human proteins (20). In this chapter we describe the procedure of this study from sample collection, sample preparation for mass spectrometry up to data processing.

2. MATERIALS

2.1 Sampling and Sample Preparation

Prepare all solutions fresh prior usage and store them at room temperature unless otherwise specified. Follow the legal and regulatory requirements for handling biomaterials of human origin.

1. Phosphate buffered saline (PBS): 1x, pH 7.4. Prepare 800 mL of distilled water and add 0.2 M NaCl (11.6 g), 2.5 mM KCl (0.186 g), 8 mM Na_2HPO_4 (1.4 g), 1.5 mM KH_2PO_4 (0.2 g). Adjust the pH to 7.4 and add distilled water to prepare a 1 L solution of 1x PBS.
2. Tris-HCl Buffer: 0.25 M, pH 8.0. Prepare 400 mL of distilled water and add 60.55 g Tris to the solution. Adjust a pH of 8.0 with HCl and add distilled water to prepare a volume 0.5 L 0.25 M Tris-HCl solution.
3. Tris-HCl Buffer: 0.05 M, pH 8.0. Dilute 100 mL of the 0.25 M Tris-HCl Buffer in 400 mL distilled water.
4. Protease inhibitor cocktail: Use a protease inhibitor provided as a lyophilized powder for general use. Solve 1 vial of the lyophilized powder in 10 mL of 50 mM Tris-HCl Buffer (pH 8.0). Add 0.075 mL protease inhibitor cocktail solution in 1.425 mL distilled water.
5. Collection of tongue samples: Sterile timber plate (18 x 150 mm).
6. Collection of saliva samples: Paraffin gum.
7. Sterile plastic tubes with a volume of 50 mL and 2 mL.
8. Vortex mixer.
9. A centrifuge that can be cooled to 4 °C and that is capable of centrifuging 50 mL sample tubes at 11,500 xg and 2 mL sample tubes at 17,000 xg.
10. Ethylenediaminetetraacetic acid (EDTA): 50 mM. Prepare 50 mL distilled water, add 1.86 g EDTA and add distilled water to prepare a volume of 100 mL of 50 mM EDTA.

11. Tris-aminomethane (Tris): 100 mM. Prepare 800 mL distilled water, add 12.1 g EDTA and add distilled water to prepare a volume of 1 L of 100 mM Tris.
12. Tris-EDTA Buffer (TE-Buffer): pH 8.5. Prepare 400 mL distilled water and add 100 mM Tris and 50 mM EDTA. Adjust a pH of 8.5 and add distilled water to prepare a volume of 0.5 L TE-Buffer.
13. Cell disruption: Ultrasonic device with an ultrasonic probe.
14. Dithiothreitol (DTT): 1.3 M. Weigh 2 g DTT and add distilled water to prepare a volume of 10 mL DTT.
15. Trichloroacetic acid (TCA): 100 % solution.
16. Cold acetone: 100% solution.
17. Drying process of precipitated protein pellets: Vacuum evaporator.
18. Urea-Thiourea Buffer (1x UT): 10 M. Weigh 8 M urea (1.92 g) and 2 M thiourea (0.61 g) and add distilled water to prepare a volume of 4 mL 1x UT.
19. Thermomixer to cool/heat and shake sample tubes.

2.2 Protein determination

1. Bovine serum albumin (BSA): Prepare a BSA stock solution with a concentration of 1 mg/mL.
2. Bradford reagent.
3. Vortex mixer.
4. Plastic cuvettes.
5. Spectralphotometer for optical absorption measurement at 595 nm.

2.3 Tryptic Digestion of Protein Samples

1. High performance liquid chromatography (HPLC) water.
2. Low protein binding reaction vessels.
3. Ammoniumbicarbonate (ABC): 20 mM ABC (0.079 g) in 12.5 mL HPLC water.
4. Dithiothreitol (DTT): 25 mM DTT (0.03 g) in 8 mL of 20 mM ABC.
5. Iodoacetic acid (IAA): 100 mM IAA (0.018 g) in 1 mL of 20 mM ABC (*see* **Note 1**).
6. Trypsin: Use 20 µg lyophilized trypsin. Solve 1 vial of the lyophilized trypsin in 1 mL of 20 mM ABC to reach a final concentration of 20 ng/µL. For the In-Solution-Digestion, add trypsin at the ratio of 1:25, which corresponds to 8µL of a 20 ng/µL solution to a protein amount of 4 µg (*see* **Note 2**).
7. Add 0.075 mL protease inhibitor cocktail solution in 1.425 mL HPLC water.
8. Urea-Thiourea Buffer (1x UT): 10 M. Weigh 8 M urea (1.92 g) and 2 M thiourea (0.61 g) and add distilled water to prepare a volume of 4 mL 1x UT.
9. Thermomixer or Incubator to heat sample tubes.

2.4 Purification of Peptide Samples

1. 10 µl ZipTip®-tip µ-C18 material with a column of a peptide binding capacity of 2 µg.
2. Acetic acid: 5 % solution in HPLC water, 1 % solution in HPLC water and 0.05 % solution in HPLC water.
3. Acetonitrile: 100 % solution, 80 % solution in 1 % acetic acid, 50 % solution in 1 % acetic acid and 30 % solution in 1 % acetic acid
4. Buffer A: 2 % acetonitrile, 0.05 % acetic acid in HPLC water.
5. Clear glass micro tubes for 2 mL with neutral cap

6. Clear glass micro inserts (vails) for 0.1 mL.
7. Vacuum freeze dryer.

2.5 Buffer for HPLC

1. Buffer A: 0.1 % acetic acid in HPLC water.
2. Buffer B: 0.1 % acetic acid in 100 % acetonitrile.

2.6 Software for Metaproteomic Data Analysis

1. Created binary LC-MS/MS datasets (21,22)
2. Comet (http://comet-ms.sourceforge.net/) (23,24)
3. Trans-Proteomic Pipeline (http://tools.proteomecenter.org/software.php) (25–27) including the following modules and tools:
   - msconvert
   - PeptideProphet (28)
   - iProphet (29,30)
   - Mayu (31)
   - ProteinProphet (32)
4. Webtool: Prophane (https://prophane.de/login)
5. Bacterial database: Human Oral Microbiome Database (http://www.homd.org/) (7,33)
6. Human database: UniProtKB/Swissprot (https://www.uniprot.org/) (34)
7. Software for statistical computing and graphics: R (https://www.r-project.org/) (35)

3. METHODS

The single steps are performed at room temperature unless otherwise described. The laboratory workflow is shown in Figure 1.

3.1 Tongue Sampling

1. For the collection of the tongue samples kindly ask the subjects to show their tongue as far as possible (*see* **Note 3**).
2. Place a sterile wooden spatula on the middle dorsum of the tongue with light and constant pressure for 5 s (*see* **Note 4**).
3. Slightly draw the wooden spatula ventral over the tongue, turn it over and repeat the procedure with the other side of the spatula.
4. Transfer the wooden spatula with the sample side into a prepared vessel containing 2 mL sterile 1x PBS and 40 µL protease inhibitor.
5. Vortex the vessel including the spatula for 30 s.
6. Discard the wooden spatula.
7. Store the sample material on dry ice and keep at -80 °C until the next step.

3.2 Saliva Sampling

1. Provide one commercially available paraffin chewing gum for each subject. Chewing on the gum which will stimulate the natural salivation and ensures a sufficiently large sample volume (*see* **Note 5)**.
2. The subject chews on the paraffin chewing gum over a period of 1 min, holding a sterile vessel for collecting saliva in his hands (*see* **Note 6**).

3. During the chewing process the participants spit several times into the sample vessel.
4. Measure the collected sample volume after 1 min using the scale of the vessel and add 20 µL of the protease inhibitor per 1 mL of saliva.
5. Store the sample material on dry ice and keep at -80 °C until further use.

3.3 Cell Disruption

1. Thaw the saliva samples and tongue swabs on ice.
2. Centrifuge the samples at 11,500 xg for 15 minutes. The centrifuge must be cooled down to 4 °C.
3. Discard the resulting supernatant and resuspend the pellet with at least 500 µL TE buffer (*see* **Note 7**).
4. Transfer the solved pellet into a smaller reaction vessel.
5. The suspension is treated with an ultrasound probe for 3 x 30 s. The samples remain on ice during and after the ultrasonic treatment (*see* **Note 8**).
6. Centrifuge the samples at 4 °C and 16,200 xg for 30 minutes.
7. Pipette the supernatant into a new vessel for the next treatment steps. The remaining pellet can be discarded.

3.4 Precipitation of Proteins and Protein Assay

1. Add 0.6 µL 1.3 M DTT per 100 µL sample volume and vortex the sample for 10 s.
2. Incubate the sample for 30 min at 37 °C.
3. Add TCA until a final concentration of 15 % and invert the tube several times.
4. Incubate the samples on ice for 60 min.

5. Centrifuge the samples for 45 min at 4 °C and 17,000 xg.
6. Remove the supernatant with a pipette without touching the pellet.
7. Wash the pellet with 500 µL cold acetone by inverting the vessel several times.
8. Centrifuge the samples for 15 min at 4 °C and 17,000 xg and then remove the acetone.
9. Wash the pellet again with 500 µL cold acetone by inverting the vessel several times.
10. Centrifuge the sample for 15 min at 4 °C and 17,000 xg and remove the excess acetone.
11. Dry the pellet in a vacuum evaporator for 1 min to completely remove the acetone.
12. Dissolve the precipitated and dried proteins in 1x UT. For the saliva pellets you need at least 50 µL and for the tongue pellets at least 35 µL 1x UT (*see* **Note 9**).
13. Perform protein determination according to Bradford (36). Follow the instructions of your local supplier for Bradford reagents. The saliva protein concentration averages 6.4 µg/µl (± 2.3 µg/µl), which is three times as high as the tongue samples with an average concentration of 1.7 µg/µl (± 1.6 µg/µl) based on our study of 24 healthy subjects aged between 20 and 30 years (20).

3.5 Reduction, Alkylation and Protein Digest

For the following steps, a protein amount of 4 µg is required. The volume for the 4 µg in our study including 24 healthy subjects aged 20 to 30 years was typically 3.4 µl (± 1.3 µl) for saliva and 10.9 µl (± 4.9 µl) for the tongue samples (20). The total sample volume differs between the individual samples depending on the determined protein concentration. For this reason, the following steps specify the final concentrations to be achieved with the substances for reduction, alkylation, and protein digestion in relation to the total volume of the sample. The incubation of the samples in the following single steps was performed without shaking or any other movement.

1. Add DTT to a final concentration of 2.5 mM to the protein mixture and incubate the protein solution for 60 min at 60 °C, which will reduce disulfide bonds of cysteines to sulfhydryl groups.
2. Prevent re-oxidation of the thiol groups by alkylation of the protein mixture with a final concentration of 10 mM IAA at 37 °C and an incubation time of 30 min (*see* **Note 10**).
3. Dilute the samples 1:10 with 20 mM ammonium bicarbonate, resulting in a urea/thiourea concentration of less than 2 M.
4. Add trypsin in the ratio 1:25 (trypsin/sample) and incubate the sample at 37 °C for 17 h in the dark.
5. Terminate the activity of the enzyme trypsin adding 5 % acetic acid to a final concentration of 1 % acetic acid to the peptide mixture.

3.6 Purification of Peptide Sample

Increase the purity of the peptide sample by desalting and decreasing the amount of hydrophilic substances with a 10 µl ZipTip® packed with µ-C18 material and a total binding capacity of 2 µg.

1. Set the volume of the pipette to 10 µL
2. Activate the µ-C18 material by pressing the plunger button down and aspirate the 100 % ACN solution into the ZipTip®-tip. Discard the activation solution (*see* **Note 11**).
3. Repeat the procedure three times in total.
4. Equilibrate the µ-C18 material using a three-step decreasing concentration of 80 %, 50 % and 30 % ACN.
5. Start with 80 % ACN, by aspirating the solution and discard it into the waste.

6. Repeat the procedure five times in total.
7. Perform the same steps described in point 5 and 6 for the 50 % and 30 % ACN.
8. The equilibration of the µ-C18
9.
10. material in the column is completed with two cycles of aspirating of 1 % acetic acid and its discarding.
11. Load the peptides onto the equilibrated column, by performing 15-20 aspiration-dispense cycles of the entire sample material (*see* **Note 12**).
12. Remove salts and detergents, by washing the column with five cycles of aspirating of 1 % acetic acid and its discarding.
13. Elute the column-bound peptides by aspirating and dispensing 8 µL of 50 % ACN for three times.
14. Aspirate 50 % ACN a fourth time and transfer the ACN-peptide mixture into a glass micro vail.
15. Elute the column-bound peptides a second time by aspirating and dispensing 8 µL of 80 % ACN for three times.
16. Aspirate 80 % ACN a fourth time and transfer the ACN-peptide mixture into the same glass micro vail as before.
17. Lyophilize the samples in a vacuum freeze dryer.
18. Fill up the micro vails with 20 µL of buffer A to reach a peptide concentration of 0.1 µg/µL (*see* **Note 13**).

3.7 LC-MS/MS measurement are performed on a nano-LC-MS/MS system.

1. Reverse phase nano-LC/MS-MS: Load the complex peptide mixtures onto a precolumn
2. The subsequent 120-minute separation of the tryptic peptides is performed on analytical column using a linear gradient of 2-25% with the binary buffer B.
3. The mass spectrometric analysis is performed in data-dependent acquisition mode using a high-resolution accurate-mass MS-instrument of the Q Exactive Orbitrap MS series. Detailed information for parameter of a LC-MS/MS method using an Ultimate 3000 and a QExactive plus mass spectrometer (Thermo Fisher Scientific) are shown in table 1.

3.8 Bioinformatic analysis of LC-MS/MS raw data for peptide and protein identification using the Trans-Proteomic Pipeline

Initially, the spectra data generated by mass spectrometry must be analyzed and interpreted. The mass spectra are searched against a decoy database. In several steps, the peptides and proteins are identified, and their probability is calculated. The data are processed with the Trans-Proteomic Pipeline (TPP) (25–27). The TPP is Linux-based und used via command line. Figure 2 highlights the key steps of the data analysis workflow.

1. Combine the oral microbiome database (HOMD) (7,33) and the human database (UniProtKB/Swissprot) (34) to create a database containing both bacterial and human protein sequences.
2. Add a decoy protein sequence to each human and bacterial protein sequence to create a reverse decoy database from the combined database (*see* **Note 14**).

3. Convert the result files of the mass spectrometric analysis from .raw data format to .mzML data format using the *msconvert* module of the TPP (21,22).
4. Start the *Comet* search (23,24) using the combined sequence decoy database to interpret the mass spectra. The settings of the search parameters are listed in table 2.
5. Use the wrapper tool *xinteract* (25) of the TPP to run the modules *PeptideProphet* (28) and *iProphet* (29,30) at once. *PeptideProphet* converts the individual result files of the database search into the pep.xml-format and additionally merges them into a single interact-pep.xml result file. Furthermore, it performs a spectrum-level validation followed by peptide-level validation of the module *iProphet*, which results into the interact-.ipro.pep.xml file.
6. Run the software package *Mayu* (31) using the interact-.ipro.pep.xml file to calculate false discovery rates (FDR) for peptide-spectrum matches (mFDR), peptide identification (pepFDR) and protein identification (protFDR) (*see* **Note 15**).
7. Based on the results of *Mayu*, calculate the iProbability (value between 0 and 1) for a protFDR = 0.05 to refine the results of the *iProphet* module.
8. Start the module *ProteinProphet* (32) and use the calculated iProbability to determine protein identification probabilities. *ProteinProphet* creates an interact-.ipro.prot.xml result file.

3.9 Prophane - Taxonomic and Functional assignment of identified proteins

Identified spectra, peptides and proteins must be appropriately prepared for the web tool Prophane to perform a relative quantification as well as a taxonomic and functional assignments. The data are filtered according to quality criteria, followed by spectral counting. The proteins with their

spectral counts are summarized in a report and uploaded to Prophane. The preparation of the data is done with the programming language R (35).

1. Create with a filtered peptide-spectrum list based on the calculated iProbability of Mayu:
    - Remove decoy proteins from the *Mayu* result file and the interact-.ipro.pep.xml file.
    - Identify the overlap between the two files using the spectra that occur in both files.
    - Select all data, whose iProbability is greater than or equal to the calculated value.
2. Use the peptide-spectrum list and the interact-.ipro.prot.xml file to perform spectral counting.
    - Assign to each peptide and spectrum of the filtered peptide-spectrum list the corresponding protein of the interact-.ipro.prot.xml file based on the peptide sequences.
    - Count the number of spectra per protein.
3. Based on the requirements of *Prophane*, create a protein report using the result file of the spectral counting.
4. Start the webtool *Prophane* and import the protein report. *Prophane* calculates normalized spectral abundance factor values (NSAF-values) and performs taxonomic and functional assignment of proteins.
5. Use the prophane report for further data analysis.

4. NOTES

1. During the preparation process, extended exposure to light should avoided. For this, the vessel should be wrapped in aluminum foil. The solution should then be stored on ice.
2. For proteome analysis we have established in our laboratory the sequencing grade modified porcine trypsin (# V5111) from Promega (37). A high purity of the trypsin is guaranteed by the manufacturer using affinity chromatography. The trypsin is provided by the company in 5x20 µg ampules in lyophilized (37) or liquid frozen form in 50mM acetic acid (38), whereby we use the lyophilized form. High stability and activity as well as the prevention of autolytic digestion of the native trypsin is ensured by modified lysins through reductive methylation (39). The specificity of trypsin is further increased by treatment with tosyl phenylalanyl chloromethyl ketone (TPCK) (40). Another advantage is its improved resistance to denaturation by chemicals such as SDS, urea, acetonitrile or guanidine HCL, which are commonly used in proteomics (39). For further details regarding the handling of the trypsin, storage conditions and other applications, please refer to the manufacturer's protocols (37,38).
3. We have always started by collecting tongue swabs and then saliva samples to keep the contamination of the tongue samples with saliva as small as possible.
4. Care should be taken not to insert the spatula too far into the oral cavity to avoid triggering the gag reflex of the subjects.
5. We used commercially available paraffin chewing gums (1.5 g) from the company Ivoclar Vivadent GmbH (Ellwangen, Germany), which were delivered individually packed in blister packages. We also recommend using commercially available chewing gums, as these are available in standardized packages. The taste of the paraffin gum and the

sensation during the chewing process is described by some subjects as unpleasant. It is possible that a little paraffin gets stuck in the teeth. But everything is completely harmless for the subjects.

6. We recommend using a vessel with an opening large enough for the subjects to spit into.
7. We could observe that the pellets can vary greatly regarding their stability and size. Pellets can be of low density and will loosen even with small movements. On the other hand, it can happen that the pellet is exceptionally large, and more than 500 µL are necessary to bring it completely in solution.
8. We recommend testing beforehand at which strength the ultrasound treatment must be performed, as there are differences between the manufacturers' devices. We suggest determining the optimal settings of the ultrasound device directly for the sample material. The material of different test persons should be pooled to eliminate individual differences of the samples. Several combinations of ultrasonic intensities and durations should be compared by protein determination to determine the optimal combination.
9. The vacuum dried protein pellets can be dissolved very easily in 1x UT by pipetting up and down several times. After this step, the sample may be stored at -80 °C and further processed later.
10. The alkylation step must be performed in the dark.
11. During the entire purification process, ensure that no air is drawn into the ZipTip®-tip, as this will reduce the quality of the purification. This is best accomplished by pipetting at a steady and gentle speed.
12. According to the manufacturer's instructions, the equilibrated column should be loaded with peptides using 15-20 aspiration-dispense cycles. Usually, we transfer the sample

volume from the original reaction vessel to a new vessel to ensure that the entire sample volume has passed the column. The digested sample can be purified with ZipTip® for a second time for mass spectrometry, but we do not recommend it. The initial protein sample should preferably be reduced, alkylated, and digested again.

13. Subsequently, peptides can be measured by mass spectrometry directly or stored at -80 °C. Depending on the used measuring method, mass spectrometer, precolumn and other conditions, the sample volume could be sufficient for several measurements, but here we also recommend to prepare the sample again by protein digestion and purification to ensure a high quality of the sample measurement.
14. The Decoy database was created using an R-script. All target proteins were inverted and read from right to left. Furthermore, each inverted protein was tagged with DECOY and incremented by one (DECOY1 <protein sequence>, DECOY2 <protein sequence>, ...). The application of a decoy database of nonsense proteins of reversed sequences is necessary to estimate the number of incorrect and correct peptide and protein identifications, which enables us to conclude on the quality of the data set (41).
15. In general, in proteomics experiments the quality of peptide-spectrum matches (PSMs) is determined based on a false-discovery rate. A cut-off is defined, which is usually PSMs FDR $\leq$ 0.05. With Mayu (31) we aim to raise the qualitative assignment to the level of protein identification (protFDR). The reason for this is that the protFDR is a more informative quality dimension than the PSMs FDR since further analyses are performed at the protein level and not at the spectra level. Another positive side effect is that the use of protFDR as a cut-off, leads to a reduction in the PSMs FDR as multiple PSMs contribute to a single protein identification and reinforce or do not reinforce each other. This therefore

contributes to an increase in the quality of the filtered data set, which is of great relevance in metaproteomics, since exceptionally large protein databases with a wide variety of species and different domains are used (42,43).

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There are no conflicts of interest to declare.

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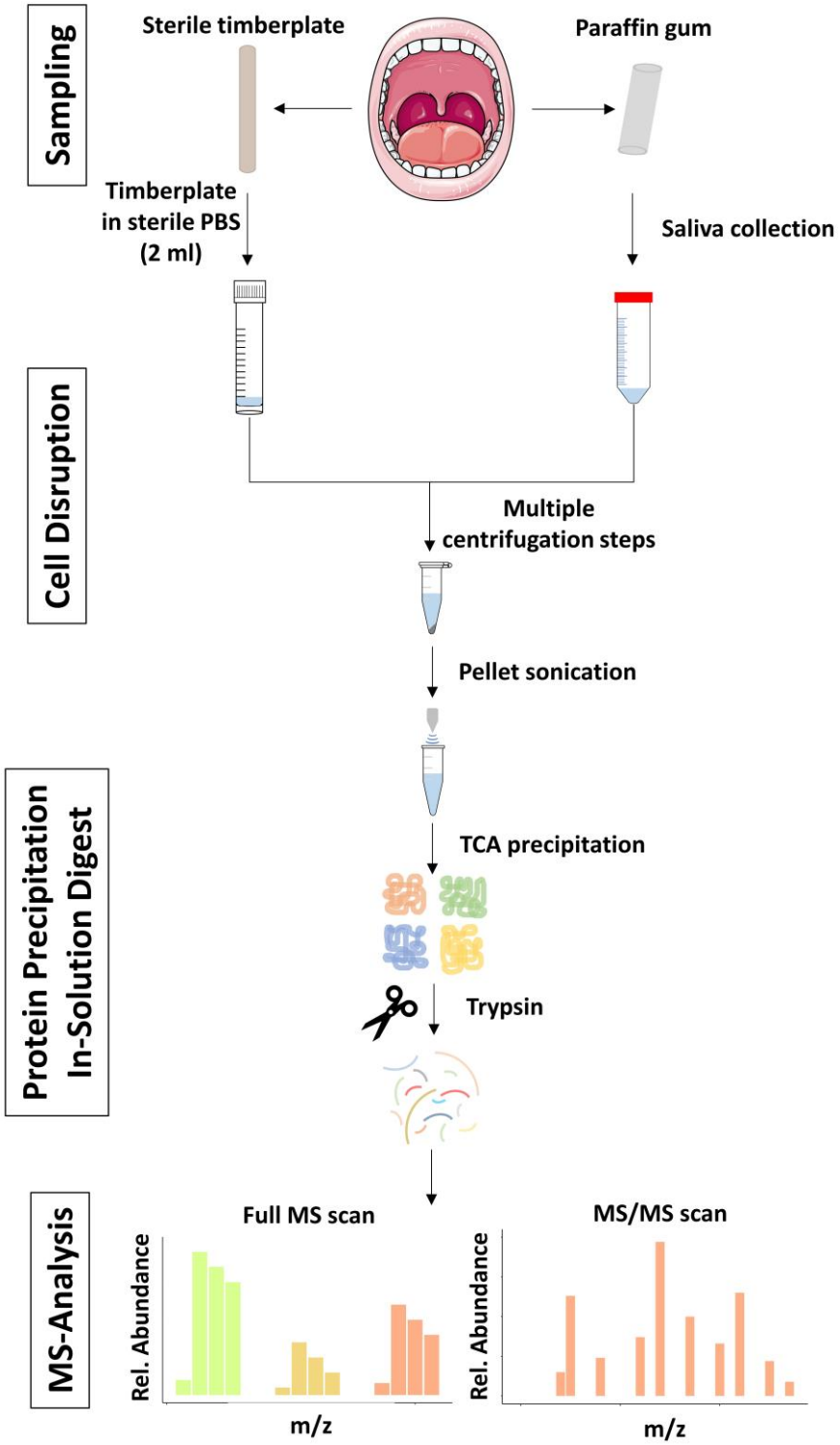
Thanks, are also due to Les Laboratoires Servier and their service Servier Medical Art based on the Creative Commons Attribution 3.0 Unported License. For Figure 1, we used images from their series "Digestive System", "People" and "Intracellular Components".

FIGURE CAPTIONS

Figure 1: Workflow for metaproteome analyses of human saliva and tongue swabs. In a first step, a tongue swab was taken with a sterile wooden spatula and transferred into 2 ml sterile PBS. The participants then chewed on a paraffin gum for one minute to stimulate the natural flow of saliva. During the chewing process, the subjects spit saliva into a Falcon Tube® multiple times. The collected saliva was centrifuged, and the resulting pellet dissolved in TE buffer, followed by ultrasound treatment. The proteins precipitated by TCA were digested with trypsin. Measurement of the peptides was performed on Q Exactive™ Plus (LC-MS/MS). The figure is adapted from our publication of the healthy human saliva and tongue microbiome (20).

Figure 2: The UML activity diagram summarizes the different process steps for the evaluation of the metaproteomic data. The raw data was converted into the mzML data format using msconvert. Peptide identification was performed by Comet based on a reverse decoy database containing human and bacterial protein sequences. The validation of the identified peptides was performed by the modules PeptideProphet and iProphet. With a complimentary evaluation by Mayu and the setting of the ProtFDR to 5.0 %, stricter filter criteria were set in the context of protein assignment by the module ProteinProphet. Prophane were used for the taxonomic and functional assignment of the identified proteins. The activity diagram was created with the program UMLet in version 14.2.

Figure 1


Sampling
Sterile timberplate
Paraffin gum
Timberplate in sterile PBS (2 ml)
Saliva collection
Cell Disruption
Multiple centrifugation steps
Pellet sonication
Protein Precipitation In-Solution Digest
TCA precipitation
Trypsin
MS-Analysis
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Rel. Abundance
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Rel. Abundance
m/z

Figure 2

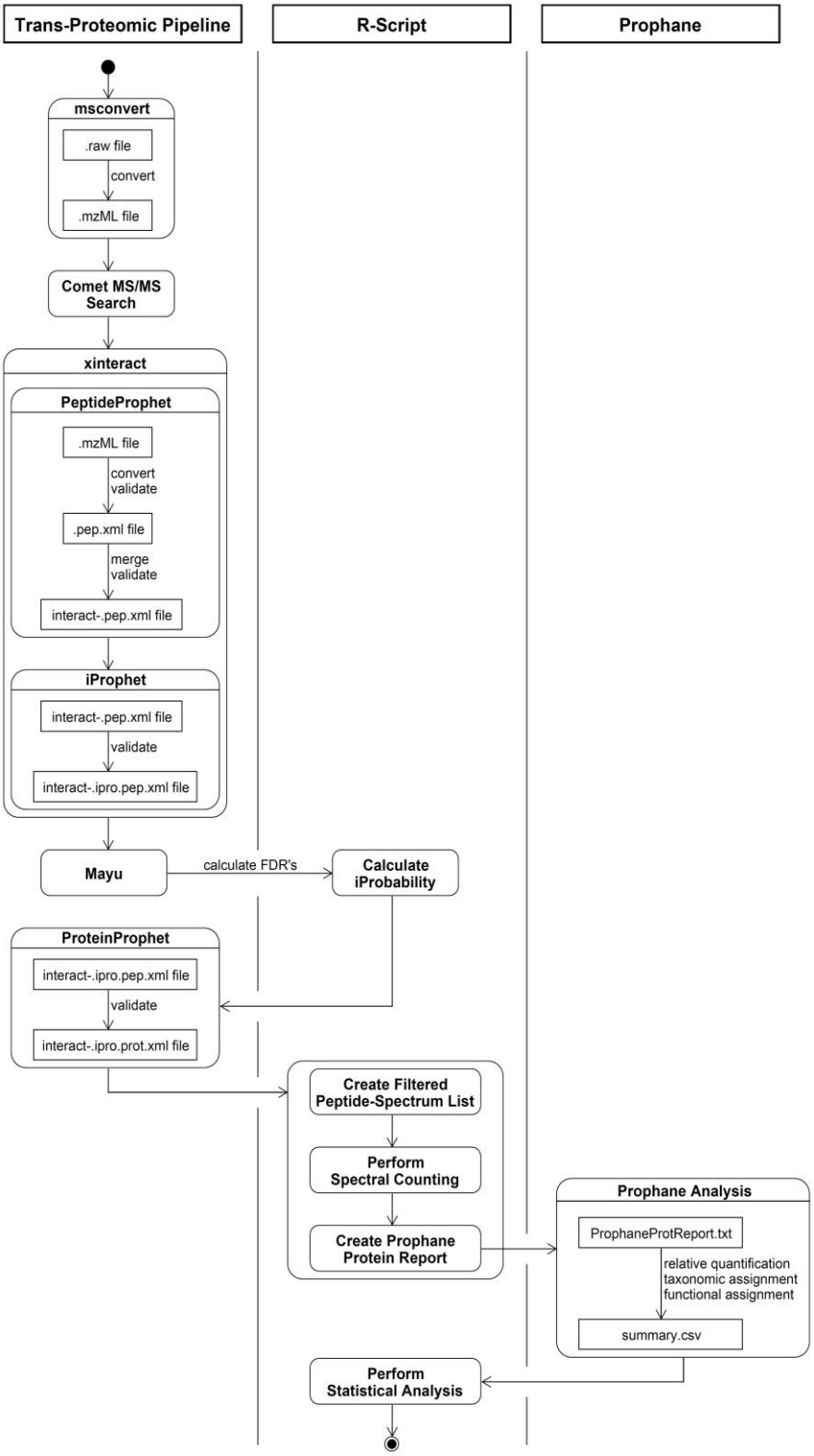
Trans-Proteomic Pipeline
R-Script
Prophane
msconvert
.raw file
convert
.mzML file
Comet MS/MS Search
xinteract
PeptideProphet
.mzML file
convert validate
.pep.xml file
merge validate
interact-.pep.xml file
iProphet
interact-.pep.xml file
validate
interact-.ipro.pep.xml file
Mayu
calculate FDR's
Calculate iProbability
ProteinProphet
interact-.ipro.pep.xml file
validate
interact-.ipro.prot.xml file
Create Filtered Peptide-Spectrum List
Perform Spectral Counting
Create Prophane Protein Report
Prophane Analysis
ProphaneProtReport.txt
relative quantification
taxonomic assignment
functional assignment
summary.csv
Perform Statistical Analysis

TABLE CAPTIONS

Table 1: Listing of the required materials for reversed phase liquid chromatography (RPLC) and the parameters to be set for mass spectrometric measurements.

Table 2: Comet was used with release 2016.01 rev. 2. Parameters, different from the default settings, are listed in the table.

450 **TABLES**

reversed phase liquid chromatography (RPLC)	
instrument	Ultimate 3000 RSLC (Thermo Scientific)
trap column	75 µm inner diameter, packed with 3 µm C18 particles (Acclaim PepMap100, Thermo Scientific)
analytical column	Accucore 150-C18 (Thermo Fisher Scientific) 25 cm x 75 µm, 2,6 µm C18 particles, 150 Å pore size
buffer system	binary buffer system consisting of 0.1% acetic acid water (buffer A) and 100% ACN in 0.1% acetic acid (buffer B)
flow rate	300 nl/min
gradient	linear gradient of buffer B from 2% up to 25%
gradient duration	120 min
column oven temperature	40°C
mass spectrometry (MS)	
instrument	Q Exactive plus mass spectrometer (Thermo Scientific)
operation mode	data-dependent
Full MS	
MS scan resolution	70,000
AGC target	3e6
maximum ion injection time for the MS scan	120 ms
scan range	300 to 1650 m/z
spectra data type	profile
dd-MS2	
resolution	17,500
MS/MS AGC target	2e5
maximum ion injection time for the MS/MS scans	120 ms
spectra data type	centroid
selection for MS/MS	10 most abundant isotope patterns with charge ≥2 from the survey scan
isolation window	3 *m/z*

fixed first mass	100 m/z
dissociation mode	higher energy collisional dissociation (HCD)
normalized collision energy	27.5%
dynamic exclusion	30 s
charge exclusion	1,>6

General	
decoy search	0 (= no)
num threads	8
Masses	
peptide mass tolerance	10
peptide mass units	2 (= ppm)
mass type parent	1 (= monoisotopic masses)
mass type fragment	1 (= monoisotopic masses)
precursor tolerance type	0 (= MH+)
isotope error	1 (=on -1/0/1/2/3 (standard C13 error))
Variable Modifications	
variable mod01	15.9949 M 0 3 -1 0 0 (= Methionine)
max variable mods in peptide	5
require variable mod	0
Fragment ions	
fragment bin tol	0.01
fragment bin offset	0.0
theoretical fragment ions	1 (=M peak only)
use B ions	1 (= yes)
use Y ions	1 (= yes)
use NL ions	1 (= yes)
Misc parameters	
digest mass range	600.0 - 5000.0
num results	50
skip researching	1
max fragment charge	3
max precursor charge	6
nucleotide reading frame	0
clip nterm methionine	0
spectrum batch size	10000

Spectral processing	
minimum peaks	5
minimum intensity	0
remove precursor peak	0 (= nor)
remove precursor tolerance	1.5
clear mz range	0.0 0.0
Additional modifications	
add C cysteine	57.021464

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Impact of different oral treatments on the composition of the supragingival plaque microbiome

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Impact of different oral treatments on the composition of the supragingival plaque microbiome

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ABSTRACT

Background: Antiseptics are used to inhibit oral biofilm growth. However, they affect not only pathogenic but also commensal bacteria, which are a natural barrier against oral diseases.
Objective: Using a metaproteome approach combined with a standard plaque-regrowth study, this pilot study examined the impact of different concentrations of lactoperoxidase (LPO)-system containing lozenges on early plaque formation, and active biological processes.
Design: Sixteen orally healthy subjects received four local treatments as a randomized single-blind study based on a cross-over design. Two lozenges containing components of the LPO-system in different concentrations were compared to a placebo and Listerine®. The newly formed dental plaque was analyzed by mass spectrometry (nLC-MS/MS).
Results: On average 1,916 metaproteins per sample were identified, which could be assigned to 116 genera and 1,316 protein functions. Listerine® reduced the number of metaprotein groups and their relative abundance, confirming the plaque inhibiting effect. The LPO-lozenges triggered mainly higher metaprotein abundances of early and secondary colonizers as well as bacteria associated with dental health but also periodontitis. Functional information indicated plaque biofilm growth.
Conclusion: The effects of Listerine® and LPO-system containing lozenges used for plaque inhibition are different. In contrast to Listerine®, the lozenges allowed maintenance of a higher bacterial diversity.

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INTRODUCTION

Starting from birth, bacteria colonize the human supra-organism and have an enormous influence on the development of the immune system and thus on human health status [1]. Next to the gut, the second most complex bacterial ecosystem is the oral microbiome [2,3]. Besides the planktonically living bacteria in saliva, the bacteria in oral biofilms are of special interest [4,5]. Biofilms are defined as a community structure of microorganisms living in a matrix of synthesized exopolysaccharides [6,7]. The mechanical stability of the matrix and its high bacterial diversity lead to synergistic interactions, e.g. the extension of the genetic repertoire by horizontal gene transfer within the biofilm [4,8]. This organizational structure enables the biofilm to show a special resistance to external environmental influences such as nutrient limitation, the human immune system and antibiotics [6].

In principle, supragingival plaque is assumed to have a positive role, since it serves as a barrier against the colonization of pathogens [9]. However, a diet with a high carbohydrate content [10] combined with poor oral hygiene can lead to a bacterial shift [11,12] and cause diseases such as dental caries [13] or periodontitis [14]. Saliva is part of the 1st line defense against a dysbiotic biofilm, because it mechanically removes bacteria [12] but it also contains components of the innate immune system such as lactoperoxidase (LPO) [15,16].

The salivary glands produce among others the enzyme lactoperoxidase, which catalyzes an ionic substrate such as thiocyanate (SCN^-) in the presence of H_2O_2 to form a highly reactive anti-microbial oxidation product, hypothiocyanite ($OSCN^-$) [10,16,17]. Since the 1980s, an increased knowledge of the LPO system has been leading to toothpastes or mouthwashes, which contain components of the LPO system to support the

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natural antimicrobial defense process [16,18]. The applicability of LPO products poses a challenge, as they cannot be used during the day between meals due to their volumes (mouth rinse) or additional materials like toothbrushes [18]. Furthermore, most of the antimicrobial substances used in oral health care products affect the whole microbiome. This means that all bacteria including the oral commensal flora will be reduced. However, it would be better if the commensal flora would not be reduced or even better, supported. Therefore, human own defense systems, such as the LPO system in saliva, are of interest.

Clinical studies provide insight into the effectiveness of the products, e.g. by performing plaque regrowth studies using traditional microbiological techniques [19]. Such a standard cross-over plaque-regrowth study [20] demonstrated that mouth rinse Listerine® Total Care™ (A – positive control) was statistically significantly more effective than the LPO-system-lozenges (B- 0.083% H_20_2 accordingly a 1:2 H_20_2/SCN- relation), (C- 0.04% H_20_2 accordingly a 1:4 H_20_2/SCN- relation), and the placebo lozenge (D) in inhibiting plaque. Listerine® rinse (A) as well as Lozenges (B) and (C) were statistically significantly more effective than the placebo lozenge (D), but no statistically significant differences could be observed between them.

However, studies based on traditional microbiological evaluation techniques cannot address the effects of these treatments on the composition of the biofilm. Proteomics in combination with habitat-specific taxonomic and genomic databases allows studies of biofilms without the cultivation of bacteria and allows in-depth investigation of the behavior and composition of a biofilm directly in its natural habitat [21–25]. Thus, metaproteomic approaches that monitor changes at the protein level and their impact onto metabolic pathways within the bacterial community should be used in a complementary manner to improve the understanding of the microbiome by monitoring changes of gene expression [26] and to develop more personalized ways to positively support existing natural mechanisms of plaque control [27].

In this pilot study, we used an established metaproteomic approach [28,29] to evaluate the effect of the lozenges used in Welk et al. [19] on the microbiome composition and the changes at the protein level with respect to their functions in metabolic pathways in the bacterial community. To the best of our knowledge, this is the first study combining a well-recognized and established clinical model in dentistry [20] with a metaproteomic study [30].

The results of both studies are expected to support our long-term goal to develop a lozenge, which can be used as an easily applicable addition to daily oral hygiene, to positively influence the microbiome composition to ensure that commensal, non-pathogenic bacteria are the dominant species in the plaque biofilm.

Material and methods

This complementary study received a positive vote by the ethics committee of the University Medicine Greifswald and was conducted in accordance with the recommendations of the Declaration of Helsinki from 1996. The clinical trial was registered in the German Database for clinical trials (DRKS00022810, date of registry: 02.09.2020).

Clinical study design

The design of the 4-days standard randomized plaque-regrowth study [19,20] is displayed in Figure 1. All 16 study participants (six male and ten female) were oral healthy dental students of the Greifswald dental school, who gave their written informed consent for this study. The participants were between 20 and 30 years old with a mean age of 23.4 years.

Both test lozenges were based on sugar alcohols (xylitol, sorbitol, mannitol) and contained all components of the LPO system (10 mg LPO 350 U/mg (Sternenzym, Germany), 7.5 mg KSCN) and H_20_2 either in high concentrations (Drug B: 0.083% H_20_2 accordingly a 1:2 H_20_2/SCN^- relation) or low concentrations (Drug C: 0.04% H_20_2 accordingly a 1:4 H_20_2/SCN^- relation). For Drug B and Drug C, carbamide peroxide (CPO) was used as the H_2O_2 donor, because it is very stable and releases H_2O_2 in a graduated way [31]. Drug D (placebo) was also a lozenge and had the same composition as Drug B and Drug C without the components of the LPO system. Drug A was Listerine® Total Care™ (Johnson & Johnson GmbH, Neuss, Germany) and is a commercially available mouth rinse containing essential oils. Using Drug A and Drug D as positive and negative control, allowed the results of Drug B and C to be attributed to the effect of two different hygiene measures and the LPO system.

In addition, each cycle started with a preparation phase followed by the treatment and a final recovery phase of at least 10 days. First, in the preparation phase, the participants suspended any kind of personal oral hygiene (timepoint: D0) for 3 days to support the recovery of the oral microbiome. On the fourth day (timepoint: D3) supragingival plaque was collected and served as the control sample. To ensure that plaque was totally removed, a professional dental cleaning was performed by the study dentist followed by the treatment phase (timepoints: D3 – D7), where the volunteers received one of the four drugs.

The mouth rinse solution (Drug A) had to be used twice daily in the morning and evening according to the manufacturer's instructions. The Drugs B-D were sucked five times daily every 3 hours between every 8 o'clock am and 8 o'clock pm for a period of 10–15 minutes.

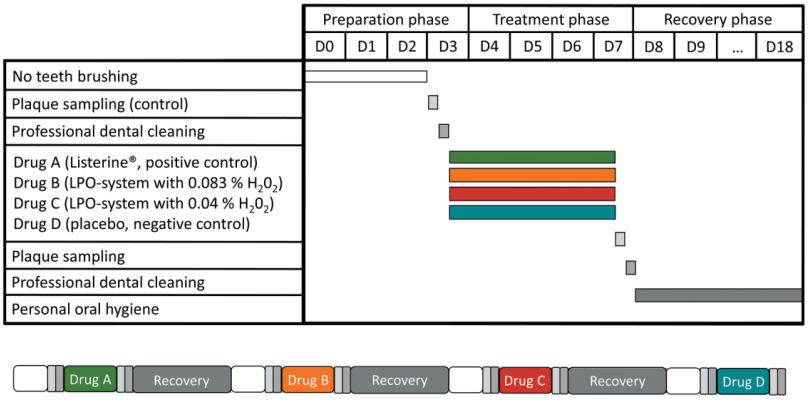

Figure 1. Study design of a 4-day plaque regrowth clinical model. In this randomized single-blinded study two sugar-alcohol based drugs containing high (Drug B – 0.083% H_2O_2 accordingly a 1:2 H_2O_2/SCN^- relation) and low (Drug C – 0.04% H_2O_2 accordingly a 1:4 H_2O_2/SCN^- relation) concentrations of components of the LPO system were evaluated regarding their influence on the plaque microbiome. Drug A, an essential oil containing mouth rinse, served as the positive control and Drug D based only on sugar-alcohols, as the placebo. Each cycle started with a preparation phase (D0-D3) without any kind of oral hygiene, followed by one treatment (D3-D7) and a recovery phase (D7-D18) of at least ten days. The study was also designed as a four-replicate cross-over study, where each subject was his or her own control.

On the last day of the treatment phase (D7), plaque that had built up during treatment was collected and the teeth were professionally cleaned. The recovery period of at least 10 days began and the participants resumed their personal oral hygiene (Figure 1).

Dental plaque collection procedure

Using a sterile curette (Universal Curette, Hu-Friedy Mfg. Co. LLC, Frankfurt am Main, Germany), supragingival plaque was collected from at least 24 tooth surfaces of all four quadrants (Figure 2A). The curette with the collected plaque was transferred several times during sample collection to a sterile tube (SafeSeal micro tubes, Sarstedt AG & Co., Nümbrecht, Germany) containing 3 ml sterile 1x PBS (Life Technologies GmbH, Darmstadt, Germany) and shaken until the plaque was detached from the curette. Finally, the sample material was vortexed for 30 s to create a suspension. In the next step, 20 µl of a protease inhibitor (Sigma Aldrich, St. Louis, MO, USA; v/v 1:20) per 1 ml sample volume was added and samples were centrifuged for 3 min at 6,200 g and 4 C°. The remaining pellets were immediately frozen in liquid nitrogen and finally stored at −80°C.

Dental Plaque sample preparation and nLC MS/MS Measurement

The pellets were resuspended with 300 µl TE buffer (10 mM Tris; 1 mM EDTA; pH 8.0). Subsequently, the biofilm and its cells were disrupted by an ultrasound treatment (Labsonic U – B. Braun Melsungen AG, Melsungen Germany) on ice for 3 × 30 s and 50% power of the device to release the proteins. Cell debris and the cytosolic proteins were separated by centrifugation (30 min, 4 C°, 16,200 g). The supernatant containing proteins was transferred to a new tube and stored on ice. The preparation of the protein mixture for the nLC-MS/MS measurement and the method for the mass spectrometric measurement were already described in detail [28,29]. Briefly, proteins were precipitated by TCA and washed several times with acetone. The vacuum dried pellet consisting of precipitated proteins was dissolved in 30 µl 8 M/2 M urea/thiourea solution. The protein concentration was determined with a Bradford Assay (Bio-Rad Laboratories GmbH, Munich, Germany). Cysteines were reduced with dithiothreitol (DTT) and alkylated with iodoacetamide IAA) with subsequent digestion of 4 µg of the protein mixture using trypsin. The resulting peptide mixture was purified after a digestion period of 17 h by ZipTipC18 material (Merck KGaA, Darmstadt, Germany). Finally, 2 µg of peptides were separated with a reverse phase nano HPLC Ultimate® 3000 Nano HPLC (Thermo Scientific) and analyzed on a Q Exactive™ Plus (Thermo Scientific) in data-dependent mode.

Metaproteome assembly, mapping, and annotation

We designed a workflow based on open-source software applications to analyze our metaproteomic datasets, described in detail in one of our earlier metaproteome studies [28,29]. Figure 2B provides an overview of the most important steps and is

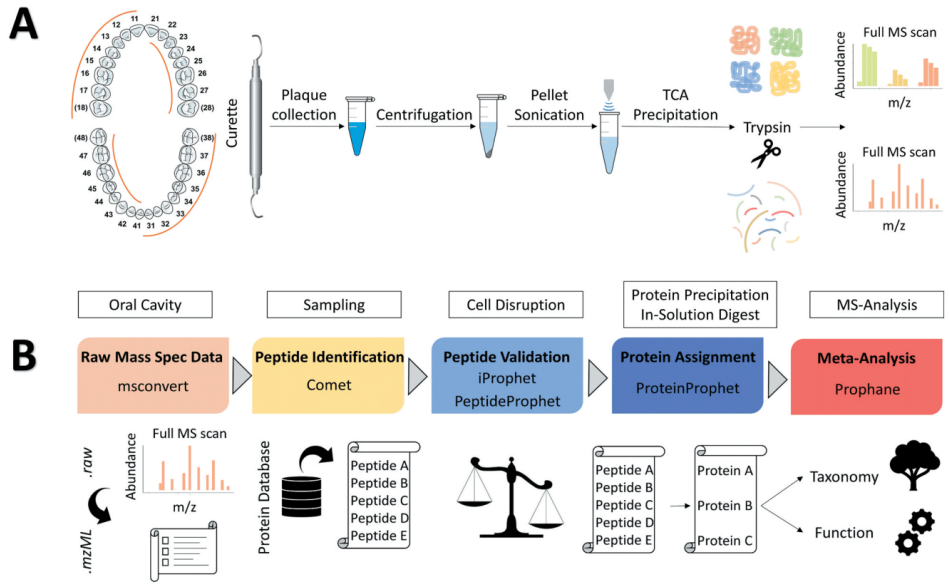

Figure 2. Workflow for supragingival plaque collection and preparation for nLC MS/MS Measurements (A). Collection of dental plaque in sterile 1x PBS from all four quadrants of the human mouth from at least 24 teeth using a sterile curette. After centrifugation, the pellet was resuspended in the TE buffer and treated with ultrasound. The protein mixture was precipitated with TCA and digested using trypsin to measure the peptide mixtures on a Q Exactive™ Plus in DDA mode.

Spectral processing and metaproteome annotation (B). The open-source software Trans-Proteomic Pipeline processed raw spectra of nano LC-MS/MS measurement. Peptides were identified based on the Comet algorithm and filtered regarding their FDR to increase the validity of peptides. A combined database consisting of human protein sequences from UniProt and bacterial protein sequences of the human oral microbial database provided the basis for protein identification Taxonomic classification, functional prediction and relative quantification was performed by Prophane. Figure 2 is adapted from Rabe et al. [35,36].

briefly described subsequently. To evaluate our 128 MS/MS measurements, we used the Trans-Proteomic Pipeline (http://tools.proteomecenter.org/software.php) [32,33] and have chosen the Comet MS/MS search algorithm (http://comet-ms.sourceforge.net/) [34,35] for peptide and protein identification, based on a combined database with 1,079,744 bacterial sequences of the human oral microbial database (HOMD, www.homd.org) [36,37] and 20,154 human sequences from UniProt (UniProtKB/Swissprot, www.uniprot.org) [38]. Peptides and proteins were filtered according to their iProphet probability at 0.05 Protein FDR (iProphet iProb = 0.9015). Using an R script (version: 4.1.1) [39], only proteins identified with at least one unique peptide were used for further analysis. Finally, proteins were classified taxonomically using the Lowest-Common-Ancestor algorithm (LCA) [40] and regarding their functional TIGRFAM assignment (TIGRFAM library version 15.0; e-value: < 0.01) [41] by the bioinformatic pipeline Prophane (www.prophane.de) [42,43]. All proteins were relatively quantified using normalized spectral abundance factor (NSAF) values [44].

The measured MS/MS data of our study were uploaded to the publicly accessible MassIVE database (dataset name: MSV000089755; doi:10.25345/C57D2QB93).

Statistical analyses

The statistical calculations as well as the image creations were performed with R (version: 4.1.1) supported by the R Foundation for statistical computing [39].

The NSAF values for each treatment sample have been median normalized to their corresponding control. Values of treatments were divided by control values for each of the ratio calculations, whereby missing or infinite values were not considered.

We selected at minimum 50% valid values per sample for a paired two-sided Wilcoxon signed rank test, which was performed with a set confidence interval of 0.95. To detect significant changes, the cutoff was set to the fold-change = 1.5 and for the p-value = 0.05. Significant results are presented in Volcano and Violin plots created using different R packages (Supplemental Table 4).

The influence of treatments on the taxonomic composition of the plaque microbiome was visualized using the metacoder package (Supplemental Table 4) [45]. The natural logarithm of the ratios between treatments and controls before treatment (color scale) was plotted against the summed spectral counts (thickness of taxonomic clades).

Results

Spectral processing results

For our complementing 4-day plaque regrowth study, 4 µg protein of total plaque of 0.88–2.6 (median QHI; Oral hygiene index according to Quigley–Hein) was prepared from each of the 128 samples and analyzed by mass spectrometry using a Q Exactive Plus (Thermo Scientific). The MS/MS analyses of the whole sample set resulted in 5.4 million spectra, of which 2.5 million spectra (identification rate: 46.3%) could be assigned based on our database consisting of human and bacterial protein sequences. Across all samples, a total of 124,101 distinct peptides could be identified with a pepFDR ≤1.56%, thereof 106,980 were of bacterial and 17,121 of human origin.

At the protein level, we only considered proteins that had a protFDR ≤5.0% and contained at least one unique peptide to minimize the possibility of misclassification. Based on these quality criteria, an average of 1,916 (± 465) metaproteins of bacterial origin as well as 442 (± 171) human proteins were covered per sample.

Analyzing the same protein amount (4 µg) of plaque sample for the metaproteomic analysis, on average 23.5% less metaproteins, were observed after treatment (Ø 1057 metaproteins) with Listerine® compared to the negative control before the treatment (Ø 1382 metaproteins) at the genus level (Supplementary Figure 1). Accordingly, these proteins also covered a lower number of bacterial genera. This contrasts with slightly increased metaprotein numbers in treatment groups B (before treatment: Ø 1304 metaproteins; after treatment Ø 1425 metaproteins) and C (before treatment: Ø 1387 metaproteins; after treatment Ø 1486 metaproteins).

Relative quantification of the metaproteome data was performed using spectral counts, which were used to calculate the NSAF values for each protein. For Drug A (Listerine®, positive control), the relative metaprotein abundance of bacterial proteins per sample decreased from an average of 74.1% before the treatment to an average of 59.1% after the treatment, because Listerine® reduced the bacterial biofilm in general. Correspondingly, the relative abundance of human proteins increased. For both LPO component-based (10 mg LPO 350 U/mg (Sternenzym, Germany), 7,5 mg KSCN) lozenges Drug B (0.083% H_20_2; 1:2 H_20_2/SCN^- relation) and Drug C (0.04% H_20_2; 1:4 H_20_2/SCN^- relation) as well as for D (placebo), the relative abundance of bacterial metaproteins remained constant with values averaging between 69.0% and 78.7% before and after treatment, indicating that the LPO-based lozenges had no decreasing effect on the bacterial biofilm in general.

Taxonomic profile and changes at genus level

To provide a general overview of the impact of treatments on the diversity of the plaque microbiome, we calculated the ratio of metaprotein abundances between the control and treatment time points by dividing the median normalized NSAF values for each treatment (D7) by its corresponding control (D3) and plotted them against the summed spectral counts in heat map trees (Figure 3).

Across all 128 samples, the metaproteins could be taxonomically assigned to a total of eight phyla, with the phyla *Actinobacteria, Firmicutes, Fusobacteria, Proteobacteria*, and *Bacteriodetes* dominating the composition of the plaque microbiome. *Spirochaetes, Synergistetes*, and *Saccharibacteria* played a minor role. At the genus level, the study covered metaproteins assigned to 116 genera across all samples and the high diversity remained constant after the different treatments in comparison to the control time points.

To evaluate whether the treatments caused changes in metaprotein abundances and thus altered plaque microbiome composition, we performed a paired two-sided Wilcoxon signed rank test with a confidence interval of 0.95 (cut-offs: fold-change = 1.5; p-value = 0.05) for genera that occurred in at least 50% of all samples. The Volcano (Figure 4) and violin plot (Supplemental Figure 2) show these significant metaprotein changes at the genus level. Figure 4 shows that Drug A (Listerine®, positive control) primarily led to a significant reduction of the relative abundance for metaproteins of the nine genera, such as *Haemophilus, Leptotrichia* or *Tannerella*, whereas higher metaprotein abundances could be identified for *Rothia* and *Peptoniphilus*. In contrast, the relative metaprotein abundances for the five genera *Fusobacterium, Lachnospiraceae bacterium, Capnocytophaga* and *Johnsonella* increased under Drug B (0.083% H_20_2; 1:2 H_20_2/SCN^- relation). However, lower metaprotein abundances could be identified for the genera *Corynebacterium* and *Mobiluncus*.

Treating the subjects with Drug C (0.04% H_20_2; 1:4 H_20_2/SCN^- relation) had a similar influence on the plaque metaproteome in comparison to the treatment with Drug D (placebo). Both treatments resulted in a significant decrease of metaprotein abundances for *Granulicatella*, as well as a higher abundance of *Capnocytophaga*. Additionally, an increased metaprotein abundance for the genus *Neisseria* occurred for Drug C.

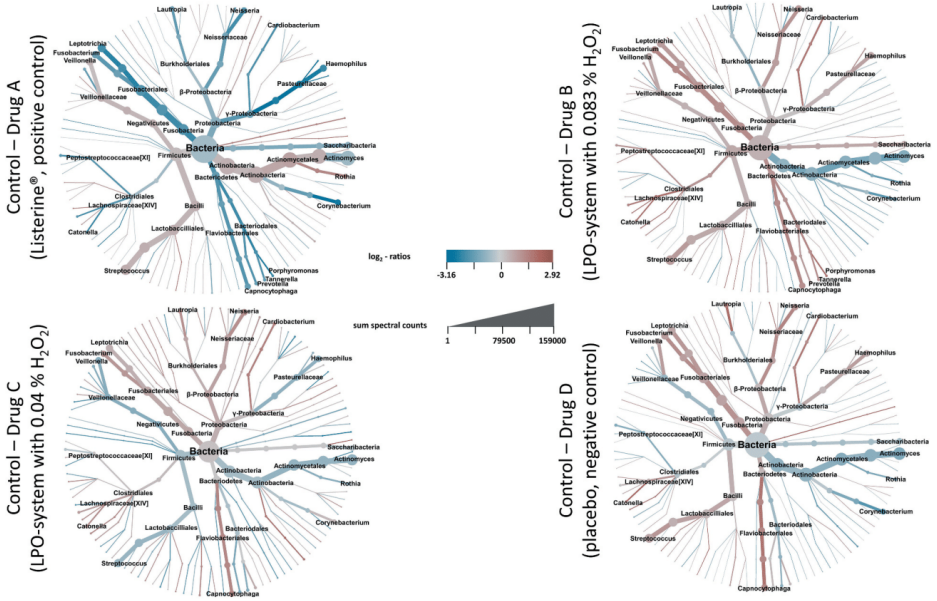

Figure 3. Illustration of the taxonomic diversity as well as the changed bacterial composition for each treatment. The log_2 median of pairwise NSAF ratios is the basis for the coloring and thus the rate of change. Branch thickness indicates the number of identified spectral counts.

Taxonomic changes at species level

To learn more about the effects of the four treatments on the plaque microbiome and its metaproteome, we performed further analyses at the species level, as these are of particular interest from the clinical perspective of dentists. In total, 9,729 metaproteins could be assigned to 351 species and were analyzed for changes in relative abundance. We performed a paired two-sided Wilcoxon signed rank test (confidence interval = 0.95; cut-offs: fold-change = 1.5; p-value = 0.05) for species that occurred in at least 50% of all samples, i.e. the same parameters as for the genus level analyses. Based on the results of the statistical test, metaprotein abundances of 65 species showed significant changes (Figure 5).

Under the treatment of Drug A (Listerine®, positive control) metaprotein abundances for 28 and 7 species were lower and higher, respectively, than in the controls (Supplemental Table 1). Metaproteins of *Rothia dentocariosa* showed the greatest increase in abundance and *Aggregatibacter aphrophilus* the greatest decrease. *Leptotrichia* was the most represented genus with seven species, all of them displaying a reduction in abundance.

For Drug B (0.083% H_2O_2; 1:2 H_2O_2/SCN^- relation), metaprotein abundances for 21 and 8 species were present in higher and lower abundance, respectively, in comparison to the control before treatment (Table 1). The metaprotein abundances with the greatest decrease were identified for *Cronobacter sakazakii* and with the greatest increase for *Lachnospiraceae bacterium ACC2*. All five different *Fusobacteria* showed an increase in metaprotein abundances.

For Drug C (0.04% H_2O_2; 1:4 H_2O_2/SCN^- relation) and Drug D (placebo), metaprotein abundance changes were identified for 16 and 15 species, with metaproteins of 14 species showing higher abundance for Drug C (Table 1) and metaproteins of 11 species for Drug D (placebo) (Supplemental Table 2). Both treatments had the greatest similarities among the four treatments by sharing six species with a significant change of metaprotein abundances. Four of the six species originate from the genus *Capnocytophaga* and showed an increased metaprotein abundance, as well as *Neisseria flava* and *Leptotrichia sp. oral taxon 215*.

We observed significant metaprotein changes for *Capnocytophaga sp. oral taxon 329 F0087* and *Leptotrichia sp. oral taxon 215* during all four treatments.

In summary, our findings for the four treatments at the species level were consistent with the analysis results at the genus level. Drug A (Listerine®, positive control) showed a tendency to reduce the metaprotein abundances for most of the species, whereas drug B (0.083% H_2O_2; 1:2 H_2O_2/SCN^- relation) tended to increase it. Drug C (0.04% H_2O_2; 1:4 H_2O_2/SCN^-

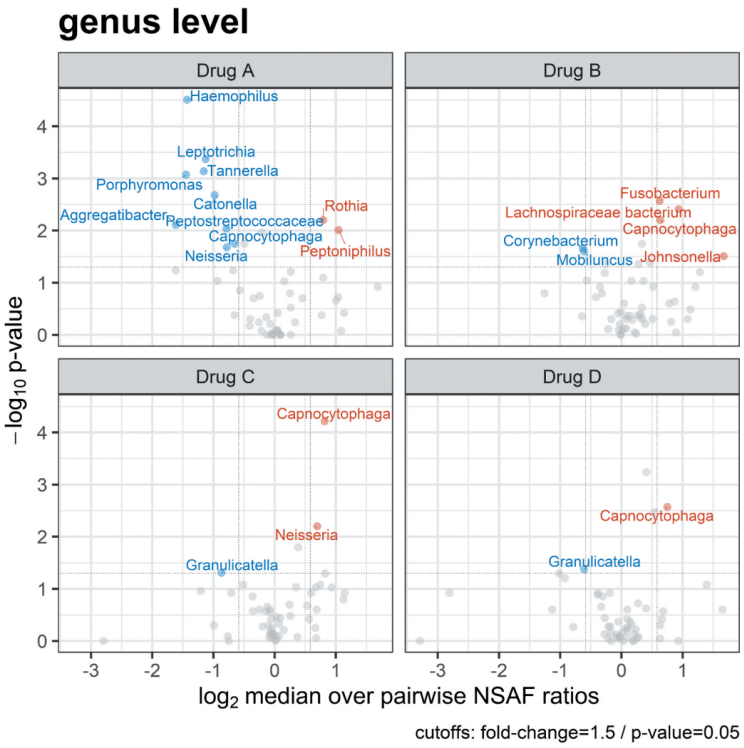

Figure 4. Volcano plots showing significant changes of a paired two-sided Wilcoxon signed rank test (confidence interval = 0.95) for the metaprotein abundances assigned on the genus level for all four treatments. Blue indicates a significant reduction in relative metaprotein abundance after treatment, red indicates a significant increase, and gray indicates no significant changes in relative metaprotein abundance after treatment.

relation) as well as Drug D (placebo) also showed less pronounced effects on the metaprotein abundances on the species level.

Bacterial functional profile of the plaque biofilm

Metaproteomics enables the measurement and analysis of bacterial proteins, also allowing conclusions regarding interactions between microbes, functional properties of the community as well as to responses to changing environmental conditions [46]. Using a paired two-sided Wilcoxon signed rank test (confidence interval = 0.95; cut-offs: fold-change = 1.5; p-value = 0.05), we evaluated whether significant changes of abundance for metaprotein functions were detectable. Therefore, we analyzed all bacterial metaproteins with respect to their functional classification, which was based on the TIGRFAM system including three levels of classification, which differ in their granularity. One thousand three hundred and sixteen TIGRFAMs could be assigned to the bacterial metaproteins, which were distributed among 60 biological processes (Supplemental Table 3). At the lowest level of the TIGRFAM classification, no significant changes were observed. However, significant changes occurred in 19 biological processes, the second level of the TIGRFAM classification (Figure 6 and Table 2). Supplemental Figure 3 summarizes the treatment-related changes for those biological processes.

The most significant changes were observed for Drug A (Listerine®, positive control) under whose treatment metaproteins involved in 12 biological processes (Table 2) showed a reduced metaprotein abundance. Metaproteins involved in small-molecule interactions (PAS domain S-box protein [47]) mainly of the category 'amino sugars' [48,49] like glucosamine-6-phosphate deaminase, phospho-glucosamine mutase or N-acetyl-glucosamine-6-phosphate deacetylase showed the most significant differences between control and treatment.

For Drug B (0.083% H_2O_2; 1:2 H_2O_2/SCN^- relation), a significant increase in abundance was observed for metaproteins of the aromatic amino acid family, small-molecule interactions, iron metabolism, and for metabolism of unknown substrates (Table 2). Drug C (0.04%

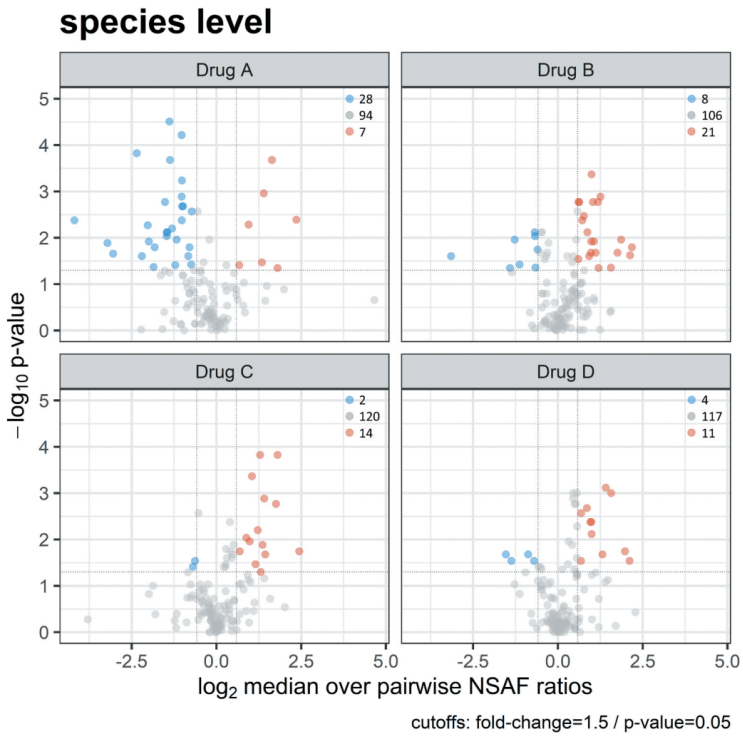

Figure 5. Volcano plots showing significant changes of a paired two-sided Wilcoxon signed rank test (confidence interval = 0.95) for the metaprotein abundances assigned on the species level for all four treatments. Blue indicates a significant reduction in relative metaprotein abundance after treatment, red indicates a significant increase, and gray indicates no significant changes in relative metaprotein abundance after treatment.

H_2O_2; 1:4 H_2O_2/SCN^- relation) and Drug D (placebo) both showed an increase in abundance for proteins of the small-molecule interactions (PAS domain S-box protein [47]) and cations and iron carrying compounds like bacterioferritin, ubiquinone oxidoreductase or TonB-dependent siderophore receptor [50,51] (Table 2), with an additional increase in chemotaxis and motility, e.g, flagellar M-ring protein (FliF) or flagellar motor switch protein (FliM) [52] for Drug C (0.04% H_2O_2; 1:4 H_2O_2/SCN^- relation). Furthermore, for Drug C, we observed a reduced abundance for proteins of the histidine family (histidinol dehydrogenase, phosphoribosyl-ATP diphosphatase) and protein modification and repair (methionine aminopeptidase [53], L-isoaspartate O-methyltransferase [54]).

Overall, it can be concluded that for Drug A (Listerine®, positive control) most significant changes were observed for biological processes accompanied by a reduction in abundance. For Drugs B (0,083% H_2O_2; 1:2 H_2O_2/SCN^- relation), C (0,04% H_2O_2; 1:4 H_2O_2/SCN^- relation), and D (placebo), the number of significant changes was lower, but always showed an increase in abundance with two exceptions for Drug C. The small-molecule interactions were common to all treatments and were present in reduced abundance for Drug A (positive control) and with an increased abundance for Drugs B, C, and D (placebo).

Discussion

In this pilot study, metaproteomic techniques are used for the first time to evaluate the influence of a conventional antiseptic in comparison to an antimicrobial human defense system on supragingival plaque formation based on a standardized and widely accepted study model in dentistry [20]. For our study, we used Drug A (Listerine®, positive control) and Drug D (placebo) as positive and negative controls, to directly attribute the changes of the plaque-microbiome to the components of the LPO system, which was included in Drug B (0.083% H_2O_2; 1:2 H_2O_2/SCN^- relation) and Drug C (0.04% H_2O_2; 1:4 H_2O_2/SCN^- relation) with different concentrations of hydrogen peroxide and in H_2O_2/SCN^- relation.

Table 1. Summary of significant changed metaprotein abundances and their taxonomic assignment on the species level under Drug B (0.083% H_2O_2 accordingly a 1:2 H_2O_2/SCN^- relation) and Drug C (0.04% H_2O_2 accordingly a 1:4 H_2O_2/SCN^- relation). For each species, their association with healthy and/or diseased oral conditions is given and is color-coded for visual support.

Species	Drug B			Drug C			Health/Disease Association
	p-values	Fold Change	Fold Change Direction	p-values	Fold Change	Fold Change Direction	
Actinomyces johnsonii	0.011	2.418	Down	-	-	-	Commensal [102]
Actinomyces oris	0.009	1.593	Down	-	-	-	Commensal [102]
Mobiluncus mulieris	0.008	1.605	Down	-	-	-	Disease [103]
Rothia aeria	0.018	1.514	Down	-	-	-	Disease [104]
Rothia dentocariosa	0.038	2.191	Down	-	-	-	Commensal and Disease [105–107]
Corynebacterium durum	0.045	2.661	Down	-	-	-	Commensal [108]
Kingella oralis	0.044	1.580	Down	-	-	-	Unknown
Cronobacter sakazakii	0.025	8.890	Down	-	-	-	Unknown
Prevotella intermedia	0.012	2.092	Up	-	-	-	Disease [92]
Prevotella nigrescens	0.021	2.164	Up	0.029	1.548	Down	Disease [93]
Capnocytophaga sp. oral taxon 326	0.021	1.963	Up	0.000	2.066	Up	Commensal and Disease [109]
Capnocytophaga sp. oral taxon 329 F0087	0.002	2.048	Up	0.000	2.447	Up	Commensal and Disease [109]
Capnocytophaga sp. oral taxon 332	0.002	2.266	Up	0.000	3.490	Up	Commensal and Disease [109]
Gemella haemolysans	0.045	2.295	Up	0.034	2.227	Up	Commensal and
							Disease [110]
Abiotrophia defectiva	0.024	4.377	Up	0.0180	5.435	Up	Commensal and Health [111–113]
Streptococcus gordonii	0.029	1.518	Up	-	-	-	Commensal and Disease [114,115]
Streptococcus mutans	0.000	1.989	Up	0.009	1.849	Up	Commensal and Disease [114,115]
Lachnospiraceae bacterium ACC2	0.016	4.538	Up	-	-	-	Unknown
Johnsonella ignava	0.011	3.642	Up	-	-	-	Unknown
Lachnospiraceae bacterium oral taxon 107 F0167	0.008	1.826	Up	-	-	-	Unknown
Lachnospiraceae bacterium sp. oral taxon 082 F0431	0.025	1.893	Up	-	-	-	Unknown
Fusobacterium necrophorum	0.012	1.978	Up	-	-	-	Commensal and Disease [116]
Fusobacterium nucleatum subsp. animalis	0.002	1.520	Up	-	-	-	Commensal and Disease [115,117,118]
Fusobacterium nucleatum subsp. nucleatum	0.004	1.646	Up	-	-	-	Commensal and Disease [115,117,118])
Fusobacterium nucleatum subsp. polymorphum	0.003	1.701	Up	-	-	-	Commensal and Disease [115,117,118]
Fusobacterium periodonticum	0.002	1.550	Up	-	-	-	Commensal and Disease [118,119]
Leptotrichia goodfellowii	0.044	2.950	Up	0.021	2.715	Up	Commensal and Disease [120,121]
Leptotrichia sp. oral taxon 215	0.001	2.390	Up	0.011	1.979	Up	Commensal and Disease [120,121]
Agrobacterium tumefaciens	0.021	3.379	Up	-	-	-	Unknown
Capnocytophaga granulosa	-	-	-	0.018	1.614	Up	Commensal and Disease [109,122]
Capnocytophaga sputigena	-	-	-	0.001	2.656	Up	Commensal and Disease [109,123]
Granulicatella adiacens	-	-	-	0.039	1.619	Down	Commensal and
							Disease [111–113]
Neisseria elongata	-	-	-	0.002	3.387	Up	Commensal and Health [124,125]
Neisseria flava	-	-	-	0.013	2.569	Up	Commensal and Health [124–126]
Neisseria sicca	-	-	-	0.006	2.328	Up	Commensal and Disease [124,125,127]
Propionibacterium propionicum	-	-	-	0.050	2.475	Up	Unknown

Color legend: green – commensal or health associated; yellow – commensal and disease associated; red: disease associated; grey: no information available if the species is commensal, health or disease associated

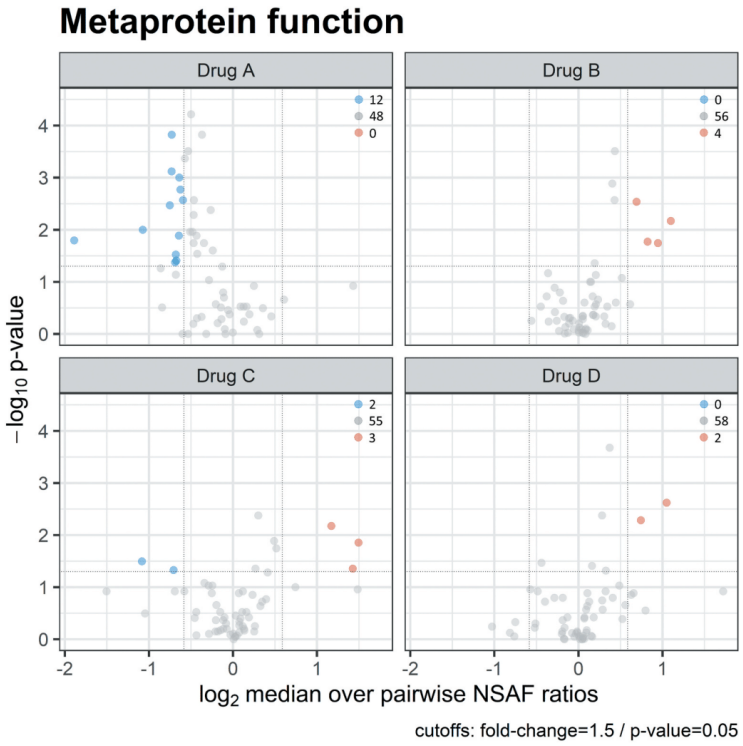

Figure 6. Volcano plot showing significant changes of a paired two-sided Wilcoxon signed rank test (confidence interval = 0.95) for the bacterial metaprotein functions for all four treatments based on the subrole level of the TIGRFAM classification. Blue indicates a significant reduction in relative metaprotein abundance after treatment, red indicates a significant increase, and gray indicates no significant changes in relative metaprotein abundance after treatment.

We benchmarked our results with the number of protein identifications and identified genera with the current literature. Compared to previous studies [55–58], we achieved higher protein identifications with 1,916 (± 465) bacterial metaproteins and 442 (± 171) human proteins per sample. One aspect to consider is that, with 16 subjects and 128 measured samples, we included more subjects and analyzed substantially more samples than comparable metaproteomic studies [55–58]. Further more, there are combined effects of a different sample preparation protocol as well as up-to-date mass spectrometers and data analysis strategies [59–61].

Bacterial metaproteins accounted for the largest proportion with on average three-quarters of the sample in comparison to human proteins. Since we scraped a biofilm from the supragingival area, the high level of bacterial proteins was to be expected, as a biofilm mainly consists of bacteria, extracellular polymeric substance (EPS) as well as other organic and inorganic components like Ca, Mg, SO_4, lipids or nucleic acids [4,62–64].

Upon exposure to Drug A (Listerine®, positive control) the relative abundance of bacterial metaproteins in total decreased, whereas the relative abundance of human proteins increased accordingly. Probably this is due to the inhibitory effect of Drug A (Listerine®, positive control) on plaque formation in general [65–67]. Drug A (Listerine®, positive control) was a commercially available antiseptic mouth rinse, whose bactericidal effect is based on essential oils and ethanol [68]. In our clinical part of the study, a reduced biofilm was also demonstrated by the observed median QHI value of 0.88 after treatment [19]. This was reflected in fewer identified bacterial metaproteins and their relative abundance, which was paralleled in changes in taxonomic and functional assignment. We identified the most significant reductions in small-molecule interactions, such as the PAS domain S-box protein [47], which plays a role in various signaling processes, such as histidine kinases or chemotaxis. Amino sugars, also with one of the highest reductions, are an important component of the peptidoglycan of the cell wall of bacteria and at the same time a source of energy, nitrogen, and

Table 2. Summary of significant changed metaprotein functions under treatment of Drug A (Listerine®, positive control), Drug B (0.083% H202 accordingly a 1:2 H202/SCN- relation), Drug C (0.04% H202 according to a 1:2 H202/SCN- relation) and Drug D (placebo) based on the subrole level of the TIGRFAM classification.

Metaprotein Function (TIGRFAM subrole)	*p-values*	*Fold Change*	*Fold Change Direction*
Drug A (Listerine®, positive control)			
Glutamate family	0.001	1.658	Down
Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides	0.003	1.683	Down
Amino sugars	0.0105	2.104	Down
Biosynthesis and degradation of polysaccharides	0.0027	1.511	Down
Pentose phosphate pathway	0.030	1.602	Down
Pyruvate dehydrogenase	0.001	1.555	Down
Biosynthesis	0.013	1.561	Down
Protein and peptide secretion and trafficking	0.043	1.610	Down
Pyrimidine ribonucleotide biosynthesis	0.039	1.595	Down
Small molecule interactions	0.016	3.703	Down
Carbohydrates, organic alcohols, and acids	0.002	1.542	Down
General (specific role is unknown)	0.000	1.657	Down
Drug B (0.083% H202 accordingly a 1:2 H202/SCN- relation)			
Aromatic amino acid family	0.017	1.770	Up
Heme, porphyrin, and cobalamin	0.018	1.927	Up
Small molecule interactions	0.003	1.615	Up
Unknown substrate	0.007	2.144	Up
Drug C (0.04% H202 according to a 1:2 H202/SCN- relation)			
Histidine family	0.047	1.632	Down
Protein modification and repair	0.032	2.118	Down
Chemotaxis and motility	0.007	2.253	Up
Small molecule interactions	0.014	2.819	Up
Cations and iron carrying compounds	0.044	2.687	Up
Drug D (placebo)			
Small molecule interactions	0.002	2.071	Up
Cations and iron carrying compounds	0.005	1.674	Up

carbon via their degradation [48,49]. In summary, a significant reduction in several metabolic processes mostly affecting key metabolic pathways for growth and proliferation of bacterial cells occurred, which suggests a reduced growth of the bacterial populations after Listerine® treatment.

In contrast, we observed a slight increase in metaprotein abundance and identification with the other three treatments. The results indicate that there may be increased bacterial activity in the biofilm. The increased abundances of flagellar proteins (FliF, FliM) indicating the movement of, for example, still present planktonic initial colonizers moving chemotactically down the nutrient gradient (PAS domain S-box protein [47]) [52]. Another example is the TonB-dependent siderophore receptor relevant for iron supply to bacteria [51], which transports iron from the environment into the cell for deoxyribonucleotide synthesis or oxidative phosphorylation [50]. Another indication is the increased metaprotein abundances of proteins involved in the repair or degradation of damaged proteins (methionine aminopeptidase [53], L-isoaspartate O-methyltransferase [54]). Additionally, based on the median QHI for Drug B (QHI 1.6), Drug C (QHI 1.8), and Drug D (QHI 2.6) a less inhibitory effect on plaque formation could be determined [19].

Regarding the taxonomic diversity, phyla such as *Actinobacteria, Firmicutes* or *Fusobacteria* dominated the assignment of metaproteins and confirmed the results of previous studies [69–74]. The same applies to the genus level, where e.g. *Actinomyces* and *Streptococcus* are among the most represented genera [56,73,75,76]. However, the species level offers the greatest information content for dental practitioners, especially regarding the colonization of tooth surfaces by initial and secondary colonizers [77,78].

The metaprotein abundances and their assigned species showed only small changes after treatment with Drug D (placebo). There were a few significant changes, e.g. for increased metaprotein abundances of the secondary colonizers *Capnocytophaga spp* [77,79,80]. Functionally, the abundance of metaproteins in the categories of small-molecule interactions as well as the cations and iron carrying compounds increased. Drug D was designed as a placebo, and therefore we did not expect many significant changes in the metaproteome. We assume a slight influence by the sugar alcohols mannitol, sorbitol and xylitol contained in Drug D (placebo). Previous studies have already provided initial evidence that sugar alcohols can also have an influence on bacteria and their growth [81–86].

Drugs B and C contained all components of the LPO system with an equal level of LPO concentration whereas Drug B (0.083% H_2O_2; 1:2 H_2O_2/SCN^- relation) contained the hydrogen peroxide donor CPO at a higher concentration than Drug C (0.04% H_2O_2; 1:4 H_2O_2/SCN^- relation).

Drug C had a minor effect on the plaque microbiome and the data generated are comparable to the results of Drug D (placebo). As an example, we also found higher metaprotein abundances for similar species, such as the secondary colonizer *Capnocytophaga spp., Neisseria flava*, or *Leptotrichia sp* [77–80]. Therefore, we suggest that the low concentration of CPO is not sufficient to make a decisive contribution to the growth of the plaque biofilm that goes beyond the effect of the placebo.

A decisive influence on the plaque metaproteome could be observed for Drug B (0.083% H_2O_2; 1:2 H_2O_2/SCN^- relation) especially for metaproteins of beneficial species as well as first and second colonizers. As one example we detected an increased metaprotein abundance for *Lachnospiraceae ssp*., and *Abiotrophia defectiva*, which are associated with dental health in caries-free children [75,87], whereas we could not find references in the literature for each identified species of Lachnospiraceae (see Table 1). Furthermore, metaprotein abundances of *Streptococcus gordonii* were only found significantly increased after treatment of our healthy subjects with Drug B (0.083% H_2O_2; 1:2 H_2O_2/SCN^- relation). It is one of the first colonizers of the oral cavity [88,89] and thus involved in the initial attachment to tooth surfaces and co-aggregates with a variety of bacteria. This bacterium has been further described to compete effectively with *Streptococcus mutans* due to the availability of oxygen and the production of hydrogen peroxide [90,91]. Additionally, the abundance of metaproteins of the secondary colonizers *Capnocytophaga spp*., which are described as commensals and associated with disease in the literature, was also elevated after treatment with Drug B (0.083% H_2O_2; 1:2 H_2O_2/SCN^- relation) [92–94]. The bridging species *Fusobacterium nucleatum subsp*., reported to coaggregate with all early and late colonizer, or even the late colonizers *Prevotella intermedia* and *Prevotella nigrescens* showed also higher metaprotein abundances after treatment with Drug B (0.083% H_2O_2; 1:2 H_2O_2/SCN^- relation) [77–80] both associated with periodontitis [95,96]. In summary, we identified positive changes regarding metaprotein abundances of health-associated bacteria for caries, but negative changes occurred in periodontitis-associated bacteria.

During the complex process of the development of dental caries, an increase in acidogenic bacteria like *Streptococcus mutans* is associated with an ecologic shift in the oral biofilm [88,90]. The treatment period extended over a duration of 4 days to allow a regrowth of the plaque biofilm but was too short to produce a shift of the biofilm towards a diseased status [97–100]. Therefore, no metaproteins from pathogenic species were expected. A more detailed analysis showed that we identified only 11 metaproteins for *S. mutans*, with only one metaprotein being statistically relevant because it was found in more than 50% of all samples. For this single identifier only, we found increased metaprotein abundances for *Streptococcus mutans*, not only after the treatment for Drug B (0.083% H_2O_2; 1:2 H_2O_2/SCN^- relation) but also for Drug C (0.04% H_2O_2; 1:4 H_2O_2/SCN^- relation) and Drug A (Listerine®, positive control). The metaprotein (identifier: smut_c_1_284) is a dehydrogenase in lipid metabolism that has not yet been further characterized. In comparison, we identified considerably more metaproteins for other species, such as for *S. gordonii* with 60 metaproteins or *F. nucleatum* with 235 metaproteins. In addition, other omics studies also identified pathogenic species in healthy subjects [101,102] and is consistent with the extended ecological plaque hypothesis [103,104]. Another point to consider is that the metaprotein abundances of the 10 days recovery phase including 3 days of absence of any oral hygiene procedure on day 3 (baseline oral biofilm) are already at a relatively high level. This baseline oral biofilm was just influenced by a test substance for the following 4 days without other oral hygiene procedures.

A unique challenge was to reconcile the results of the clinical part of the study with the results of the metaproteomic approach. A direct comparison of the observed QHI values of the clinical study [19] and the relative protein amounts (NSAF values) calculated in this metaproteomic study might be misleading because for all samples the same protein amounts were used for MS-based profiling even if treatments had different effects on total biofilm amount (QHI values). Nevertheless, we consider the combination of classical microbiological methods with metaproteomic data in addition with clinical parameters as a valuable approach. By integrating a multi-OMICs approach in the future, we expect to gain even deeper insights into the pathophysiology of dental disease.

Conclusion

Although the study of molecular mechanisms in complex biofilms using metaproteomic approaches is still in its infancy, we were able to elucidate the impact of four treatments on the plaque metaproteome and associate it with clinical parameters. It could be shown that the metaproteomic analyses not only contribute to the elucidation of the

taxonomic composition but also gather functional information for the plaque biofilm during treatment.

According to the data of this metaproteomic analysis, we were able to show that the treatment based on the components of the LPO system induces a change in the plaque metaproteome that differs from that of a placebo and Listerine®. While the reduction of the Quigley-Hein index shown in the clinical study [19] for the antiseptics can be attributed to a reduction in the overall microbiome, our results suggest that the plaque reduction of the LPO-lozenges based more on an increase in bacterial diversity.

Abbreviations

CPO – carbamide peroxide; EPS – extracellular polymeric substance; DTT – dithiothreitol; FDR – false discovery rate; EDTA – ethylenediaminetetraacetic acid; HOMD – Human Oral Microbiome Database; HPLC – high performance liquid chromatography; H_2O_2 – Hydrogen peroxide; IAA – iodo acetamide; LCA – Lowest-Common-Ancestor Algorithm; LPO – Lactoperoxidase; MS – mass spectrometry; MS/MS – tandem mass spectrometry; nLC – nano liquid chromatography; NSAF – normalized spectral abundance factor; $OSCN^-$ – hypothiocyanite; pepFDR – peptide false discovery rate; protFDR – protein false discovery rate; SCN^- – thiocyanate; TCA – trichloroacetic acid; TE – Tris/EDTA; TIGRFAM - database of protein families

Author contributions

Study design: AR, MGS, HB, UV, and AW
Ethics application: HB and AW
Sample collection: AR and MGS
Sample preparation and nLC-MS/MS measurement: AR and MGS
Data analysis and analytical tools: AR, SM
Drafting of manuscript: AR
Editing of manuscript: MGS, SM, TK, HB, UV, and AW
Acquisition of funding HB, UV, and AW

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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List of Publications and Contributions

Research Article 1

Rabe A, Gesell Salazar M, Fuchs S, Kocher T, Völker U. Comparative analysis of Salivette® and paraffin gum preparations for establishment of a metaproteomics analysis pipeline for stimulated human saliva. J Oral Microbiol. 2018 Jan 24;10(1):1428006. doi: 10.1080/20002297.2018.1428006; PMID: 29410770; PMCID: PMC5795648.

Contributions Statement: Alexander Rabe, Manuela Gesell Salazar, Alexander Welk, Thomas Kocher, and Uwe Völker were involved in the application for the ethics proposal for this study. Alexander Rabe developed the study design in cooperation with Manuela Gesell Salazar and Uwe Völker, who both supervised the project. In addition, Alexander Rabe performed the sample collection and preparation for the mass spectrometry. Sample collection was supported by Thomas Kocher, with dental students from his student courses participating in this study. The nano LC-MS/MS measurements were performed by Manuela Gesell Salazar. Alexander Rabe analyzed the data and was supported by Stephan Fuchs, who also introduced the software Prophane. Alexander Rabe wrote the manuscript, and all authors discussed the results, their conclusions and commented the manuscript at all stages. Thomas Kocher and Uwe Völker initiated the project.

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Alexander Rabe

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Prof. Dr. Uwe Völker

Research Article 2

Rabe A, Gesell Salazar M, Michalik S, Fuchs S, Welk A, Kocher T, Völker U. Metaproteomics analysis of microbial diversity of human saliva and tongue dorsum in young healthy individuals. J Oral Microbiol. 2019 Aug 26;11(1):1654786. doi: 10.1080/20002297.2019.1654786; PMID: 31497257; PMCID: PMC6720020.

Contributions Statement: Alexander Rabe, Manuela Gesell Salazar, Alexander Welk, Thomas Kocher, and Uwe Völker were involved in the application for the ethics proposal for this study. Alexander Rabe developed the study design in cooperation with Manuela Gesell Salazar and Uwe Völker, who both supervised the project. In addition, Alexander Rabe performed the sample collection and preparation for the mass spectrometry. Sample collection was supported by Alexander Welk, with dental students from his student courses participating in this study. The nano LC-MS/MS measurements were performed by Manuela Gesell Salazar. Alexander Rabe analyzed the data and was supported by Stephan Fuchs and Stephan Michalik, who also implemented the Trans Proteomic Pipeline and the transformation of the data for the use of Prophane. Alexander Rabe wrote the manuscript, and all authors discussed the results, their conclusions and commented the manuscript at all stages. Thomas Kocher and Uwe Völker initiated the project.

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Alexander Rabe

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Prof. Dr. Uwe Völker

Research Article 3

Rabe A, Gesell Salazar M, Völker U. Bottom-Up Community Proteome Analysis of Saliva Samples and Tongue Swabs by Data-Dependent Acquisition Nano LC-MS/MS Mass Spectrometry. Methods Mol Biol. 2021 Aug 28;2327:221-238. doi: 10.1007/978-1-0716-1518-8_13; PMID: 34410648.

Contributions Statement:

This book chapter is based on the results of research article 2 and was written in response to a request from the editor Guy Adami, focusing on material and methods. Alexander Rabe initiated the writing of the manuscript based on the request from Guy Adami. Alexander Rabe wrote the manuscript, and all authors discussed the results, their conclusions and commented the manuscript at all stages.

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Alexander Rabe

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Prof. Dr. Uwe Völker

Research Article 4

Rabe A., Gesell Salazar M., Michalik S., Kocher T., Below H., Völker U. and Welk A., 2022. Impact of different oral treatments on the composition of the supragingival plaque microbiome. Journal of Oral Microbiology, 14:1. doi: 10.1080/20002297.2022.2138251

Contributions Statement: Alexander Welk and Harald Below developed the study design and were involved in the application for the ethics proposal. Alexander Rabe developed the laboratory study design in cooperation with Manuela Gesell Salazar and Uwe Völker, who both supervised the project. In addition, Alexander Rabe performed the sample collection in cooperation with the dentist Maral Zahedani. Sample collection was supported by Alexander Welk, with dental students from his student courses participating in this study. Alexander Rabe prepared the sample for the mass spectrometry. The nano LC-MS/MS measurements were performed by Manuela Gesell Salazar. Alexander Rabe analyzed the data and was supported by Stephan Michalik. Alexander Rabe wrote the manuscript, and all authors discussed the results, their conclusions and commented the manuscript at all stages. Alexander Welk, Harald Below, and Uwe Völker initiated the project.

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Research Article 4

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Curriculum Vitae

Personal information

Name	Alexander Rabe
Adress	Danziger Straße 19 20099 Hamburg
Date of Birth	02 August 1988
Marital Status	Unmarried, no children

Profession

10/2021 - today

alanta health service GmbH
Product Owner (eHealth)
- Transfer stakeholder requirements into roadmaps, epics, and user stories
- Prioritization and management of the product backlog, in consideration of the product goals
- Training and supporting users
- Ongoing monitoring of regulatory requirements from the eHealth area

03/2018 - 09/2021

Accenture Technology Solutions GmbH
IT Business Analyst
- Team lead of the software design team
- Functional architect
- Release planning and estimation of user stories
- Requirements engineering with stakeholders and an on-shore development team
- Problem analysis as well as design, visualization and documentation of solutions for software applications
- Validation and resolution of defects in cooperation with the development team

Academic background

01/2015 - 12/2022

Dissertation
University Medicine Greifswald
- Department of Functional Genomics
- Center for Dentistry, Oral and Maxillofacial Surgery

Thesis title
Characterization of the human oral microbiome in health and during different treatments

10/2012 - 01/2015

Human biology studies M.Sc. (Grade: 1,3)
University of Greifswald

10/2009 - 9/2012	**Human biology studies B.Sc.** (Grade: 2,0) University of Greifswald
08/1999 - 07/2008	**Abitur** (Grade: 1,5) Goethe-Gymnasium, Ludwigslust

Overseas stay

09/2008 - 05/2009	“Work and Travel”, Australia

Work experience/internships

10/2013 – 10/2015	**tecis - Finanzdienstleistung AG** Assistent
01/2011 - 04/2014	**University Medicine Greifswald** Department of Functional Genomics Student assistent

Language skills

German	Native language
English	Advanced knowledge of spoken and written language

IT skills

Advanced	Administrator for Atlassian products (Confluence, Jira), Agile Management (Scrum, Kanban), Balsamiq, MS Office products, Programming language R
Beginner	DOORS, Figma, HP-ALM, Java, PlantUML, SoapUI, SQL Developer, Synergy, UMLet

Social activity

01/2016 – 12/2022	Secular celebrant
08/2014 - 04/2016	Financial treasurer of the department “Jugger” in the HSG Uni Greifswald e.V.
04/2010 - 03/2012	Student member of the academic senate of the University of Greifswald
10/2009 - 01/2015	Student representative of the degree program “human biology” (Class of 2009)
08/2006 - 01/2013	Substitute military service as a member of the civil protection service
08/2005 - 01/2007	Student representative of the Goethe-Gymnasium Ludwigslust

08/2001 - 01/2013	Member of the volunteer fire department, previously youth fire department

Scholarships/Awards/Certificates

11/2022	Professional Scrum Developer (PSDI)
02/2021	Certified Agile Requirements Specialist (CARS)
09/2020	Professional Scrum Product Owner (PSPOI)
09/2020	IT Service Management (ITIL 4 Edition)
05/2020 - 06/2020	Be Visual! Sketching Basics for IT Business
04/2020	Certified Professional for Requirements Engineering (CPRE)
04/2018	Professional Scrum Master (PSMI)
08/2015 - 08/2016	Gerhard-Domagk Scholarship

Publications

Rabe A., Gesell Salazar M., Michalik S., Kocher T., Below H., Völker U. and Welk A., 2022. Impact of different oral treatments on the composition of the supragingival plaque microbiome. Journal of Oral Microbiology, 14:1. doi: 10.1080/20002297.2022.2138251

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Rabe A, Gesell Salazar M, Michalik S, Fuchs S, Welk A, Kocher T, Völker U. Metaproteomics analysis of microbial diversity of human saliva and tongue dorsum in young healthy individuals. J Oral Microbiol. 2019 Aug 26;11(1):1654786. doi: 10.1080/20002297.2019.1654786; PMID: 31497257; PMCID: PMC6720020.

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Depke M, Michalik S, **Rabe A**, Surmann K, Brinkmann L, Jehmlich N, Bernhardt J, Hecker M, Wollscheid B, Sun Z, Moritz RL, Völker U, Schmidt F. A peptide resource for the analysis of Staphylococcus aureus in host-pathogen interaction studies. Proteomics. 2015 Nov;15(21):3648-61. doi: 10.1002/pmic.201500091; Epub 2015 Sep 7. PMID: 26224020; PMCID: PMC4886865.

Free time

Surfing, Bouldering, Stand-Up Paddling

Alexander Rabe Hamburg, 14.12.2022

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