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Comparative application of metagenomic sequencing in clinical samples from healthy and diseased individuals

A dissertation submitted in fulfillment of the requirements for the degree of Doctor of Philosophy (PhD) / Doktor der Naturwissenschaften (Dr. rer. nat.) of the Medical Faculty of SAARLAND UNIVERSITY

January 2024

by

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Tag der Promotion: 18.11.2024

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Declaration

The work presented in this thesis was conducted in the period from August 2020 to August 2023 at the Institute of Medical Microbiology and Hygiene (IMMH) at Saarland University/ Saarland University Medical Center. This dissertation consists of three manuscripts to fulfil the requirements for a cumulative PhD thesis, and one additional manuscript. The respective publications can be found in the results section (Chapter 4) of this work, posterior to having obtained written permission of all participating authors. Chapter 8. Publications/ Acknowledgments provides a comprehensive inventory of the manuscripts I have either published or submitted to journals (Chapter 8.1). As the author of this thesis, I am either the primary contributor and/or a co-author in the subsequent peer-reviewed publications:

- **Rehner, J.,** Schmartz, G. P., Groeger, L., Dastbaz, J., Ludwig, N., Hannig, M., Rupf, S., Seitz, B., Flockerzi, E., Berger, T., Reichert, M. C., Krawczyk, M., Meese, E., Herr, C., Bals, R., Becker, S. L., Keller, A., Müller, R., & IMAGINE Consortium (2022). Systematic Cross-biospecimen Evaluation of DNA Extraction Kits for Long- and Short-read Multi-metagenomic Sequencing Studies. *Genomics, Proteomics & Bioinformatics*, *20*(2), 405–417. https://doi.org/10.1016/j.gpb.2022.05.006

- **Rehner, J.,** Schmartz, G. P., Kramer, T., Keller, V., Keller A., Becker, S. L. (2023). The Effect of a Planetary Health Diet on the Human Gut Microbiome: A Descriptive Analysis. *Nutrients, 15*, 1924. https://doi.org/10.3390/nu15081924

- Berger, F. K., Schmartz, G. P., Fritz, T., Veith, N., Alhussein, F., Roth, S., Schneitler, S., Gilcher, T., Gärtner, B. C., Pirpilashvili, V., Pohlemann, T., Keller, A., **Rehner, J.,** Becker, S. L. (2023). Occurrence, resistance patterns, and management of carbapenemase-producing bacteria in war-wounded refugees from Ukraine. International Journal of Infectious Diseases, 132, 89-92. https://doi.org/10.1016/j.ijid.2023.04.394

  Additionally, beyond the requirements to fulfil for a cumulative dissertation, the following unpublished work will be presented:

- Schmartz, G.P., **Rehner, J.,** Gund, M., Rupf, S. Hannig, M., Berger, T., Flockerzi, E., Seitz, B., Fleser, S., Schmitt-Grohé, S., Kalefack, S., Zemlin, M., Kunz, M., Götzinger, F., Gevaerd, C., Vogt, T., Reichrath, J., Molano, L.A.G., Diehl, L., Hecksteden, A., Meyer, T., Herr, C., Gurevich, A., Krug, D., Hegemann, J., Bozhueyuk, K., Kalinina, O., Becker, A., Unger, M., Ludwig, N., Seibert, M., Stein, M.L., Hanna, N.L., Martin, M.C., Mahfoud, F., Keller, V., Krawczyk, M., IMAGINE consortium, Becker, S.L., Müller, R., Bals, R., Keller, A. Decoding the diagnostic and therapeutic potential of microbiota using pan-body pan-disease microbiomics. Submitted to *Nature Communications* in December 2023.

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Abbreviations

Abbreviation	Full name
AMR	Antimicrobial Resistance
bp	Base pair
C-section	Cesarian Section
COPD	Chronic obstructive pulmonary disease
ddNTPs	Dideoxynucleotide Triphosphate
DNA	Deoxyribonucleic Acid
dNTPs	Deoxynucleotide Triphosphate
FMT	Fecal Microbiome Transplant
IBD	Inflammatory Bowel Disease
IBS	Irritable Bowel Syndrome
m/z	Mass charge ratio
MALDI-TOF	Matrix-assisted laser desorption/ionization-time of flight
MD	Mediterranean Diet
MRGN	Multi-resistant Gram-negative bacteria
mRNA	Messenger Ribonucleic Acid
NGS	Next Generation Sequencing
ONT	Oxford Nanopore Technologies
PCR	Polymerase Chain Reaction
QMK	Qiagen QiAamp Microbiome DNA Kit
rRNA	Ribosomal Ribonucleic Acid
SCFA	Short-Chain Fatty Acids
UVR	Ultraviolet Radiation
WD	Western Diet
WGS	Whole-Genome Sequencing
WG-NGS	Whole-Genome Next Generation Sequencing

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1. Summary

The human microbiome consists of all microorganisms, including bacteria, viruses, fungi, archaea, and parasites, residing on and within our body, as well as their genetic information. Research and general interest in the human microbiome have gained significant importance in recent decades, driven partly by various correlations associated with the microbiome. Especially intestinal bacteria seem to exert a considerable influence on the development and progression of various chronic inflammatory diseases, supporting and modulating the immune system, and influencing the success of various therapeutic approaches. Advances in isolating and purifying nucleic acids from complex samples, metagenomic sequencing, and bioinformatic analysis have led to new insights in this emerging field. The complex mechanisms underlying the influence of microorganisms on us are not fully elucidated, and scientific focus is shifting towards understanding the functional aspects of these microorganisms, in addition to describing complex microbial communities.

This study introduces an experimental approach to analyze various microbiomes of an individual (saliva, interdental plaque, stool, conjunctiva, bile, sputum) using the same method (Result I). These samples were processed using three commercially available nucleic acid extraction kits and subsequently analyzed through short-read and long-read sequencing. Overall, the Qiagen QiAamp Microbiome DNA Kit was described as suitable for analyzing native clinical samples. Additionally, the DNA Miniprep Kit from ZymoBIOMICS yielded convincing results in analyzing the microbial community within stool samples.

In a second study, the intestinal microbiome of individuals following a specific diet for twelve weeks was analyzed: one group continued an omnivorous diet, another group adhered to a vegetarian or vegan diet, and a third group transitioned from an omnivorous diet to the guidelines of the Planetary Health Diet. This diet concept, developed in 2019 by the *EAT-Lancet* Commission, aims to sustainably feed approximately 10 million people in 2050 within the planetary boundaries. Although the proposed concept is already controversially discussed, analyses describing its impact on human health and associated changes are lacking. This study provides an initial insight into the effect of a Planetary Health Diet on the intestinal microbiome. Natural fluctuations in the composition of the gut microbiome were observed over the longitudinal course of the study. Furthermore, an increase in *Bifidobacterium adolescentis* was demonstrated, postulating a potentially beneficial effect on human health by producing favorable metabolites (Result II).

The third study presented in this work focuses on the methodology of extracting and purifying bacterial chromosomal and mobile deoxyribonucleic acid (plasmids) and their analysis using short-read sequencing of multi-resistant Gram-negative bacteria. These bacteria were detected in wounds of Ukrainian war-injured individuals transferred from Ukraine to the Saarland University Medical Center. The study showed that the isolated *Klebsiella pneumoniae* strains might have a common origin, likely traced back to the medical emergency shelter in Ukraine. Additionally, the exact same plasmid was found in two cases, highlighting the dangers of horizontal gene transfer between bacteria, especially in transmitting genes encoding antibiotic resistances. Molecular analyses were complemented with cultural testing and detection of antibiotic resistances, highlighting the emergence of Carbapenem resistances (Result III).

Finally, a study was conducted implementing the methodology from Result I, analyzing various microbiomes of the same individual (Result IV). Over a period of 2.5 years, different patients were included into the study at the Saarland University Medical Center for which they underwent medical examination and detailed medical history assessment. Stool, saliva, interdental plaque, skin swabs, conjunctival swabs, and throat swabs were collected from each participant, when possible, and subsequently subjected to metagenomic analysis. The results of this study indicate correlations between the occurrence of certain bacterial species and various chronic inflammatory diseases and common illnesses. Furthermore, biosynthetic gene clusters, often encoding anti-inflammatory or anti-proliferative metabolites, were found in different study cohorts. Another noteworthy result is the detection of disease-crossing shifts in oral microbiomes compared to healthy participants. This study clearly illustrates the correlations between different microbiomes of an individual and their health status.

Looking forward, the field of microbiome research should increasingly focus on unraveling the molecular mechanisms between microorganisms and hosts, attempting to elucidate whether the health status induces changes in the microbiome or whether the microbiome is significantly modulating the development of various diseases. Furthermore, the discovery of new biosynthetic gene clusters provides an avenue to find new antimicrobial effective substances, countering the global threat of multi-resistant pathogens. Additionally, the monitoring and analysis of multi-resistant pathogens should be expanded globally. This approach allows for the detection of resistance mechanisms, deciphering their origin and spread, incorporating both the individual and their environment.

2. Zusammenfassung

Das humane Mikrobiom beschreibt die Gesamtheit aller Mikroorganismen, also Bakterien, Viren, Pilze, Archaeen und Parasiten, die auf und in unserem Körper residieren. Miteinbegriffen ist hier nicht nur der Mikroorganismus selbst, sondern auch seine genetische Information. Die Forschung und auch das allgemeine Interesse am Mikrobiom des Menschen haben in den letzten Jahrzehnten rasant an Bedeutung gewonnen. Dies ist mitunter durch die verschiedenen Korrelationen zu begründen, in die das Mikrobiom involviert ist. So scheinen vor allem Darmbakterien einen großen Einfluss auf die Entstehung und Progression verschiedener chronisch-entzündlicher Krankheiten zu haben. Sie unterstützen und modulieren die Entwicklung des Immunsystems und haben Einfluss auf den Behandlungserfolg verschiedener Therapieansätze. Zu neuen Erkenntnissen in diesem doch neuen Forschungsfeld haben die Entwicklungen bezüglich der Isolation und Aufreinigung von Nukleinsäuren aus komplexen Proben, der metagenomischen Sequenzierung und bioinformatischen Auswertung geführt. Bisher sind die komplexen Mechanismen hinter dem Einfluss der Mikroorganismen in und auf uns nicht vollständig aufgeklärt, und die Wissenschaft fokussiert sich neben der Beschreibung komplexer mikrobieller Gemeinschaften zunehmend auf die funktionellen Aspekte dieser Mikroorganismen.

In dieser Arbeit wurde erstmals ein experimenteller Ansatz getestet, um verschiedene Mikrobiome eines Individuums (Speichel, interdentaler Plaque, Stuhl, Konjunktiva, Galle, Sputum) mit einer Methode zu analysieren (Ergebnis I). Hierfür wurden die oben genannten Proben mittels drei verschiedener, kommerziell erhältlicher Nukleinsäure-Extraktionskits bearbeitet und anschließend mittels Short-Read-Sequenzierung und Long-Read-Sequenzierung analysiert. Übergreifend konnte das Qiagen QiAamp Microbiome DNA Kit als geeignet für die Auswahl an nativen Bioproben beschrieben werden. Um die mikrobielle Gemeinschaft innerhalb von Stuhlproben zu analysieren, konnte weiterhin das DNA Miniprep Kit von ZymoBIOMICS überzeugende Ergebnisse liefern.

In einer zweiten Studie wurde dann das intestinale Mikrobiom von Individuen analysiert, die über den Verlauf von zwölf Wochen einer bestimmten Diät folgten: Eine Gruppe ernährte sich weiterhin wie gewohnt omnivor, eine Gruppe ernährte sich weiterhin vegetarisch oder vegan, und eine Gruppe stellte ihre bislang omnivore Ernährung auf die Vorgaben der Planetary Health Diet um. Dieses Diätkonzept wurde 2019 von der *EAT-Lancet* Kommission entwickelt, um im Jahr 2050 ungefähr 10 Millionen Menschen gesund, nachhaltig und innerhalb der planetaren

Grenzen zu ernähren. Das vorgeschlagene Konzept ist bereits kontrovers diskutiert, allerdings stehen Analysen, die den Effekt auf die Gesundheit des Menschen und damit einhergehende Veränderungen beschreiben, bislang aus. Die hier vorgelegte Studie soll einen ersten Einblick in den Effekt der Planetary Health Diet auf das intestinale Mikrobiom geben. In der Studie konnte gezeigt werden, dass es innerhalb des longitudinalen Verlaufs natürliche Schwankungen in der Komposition des Darmmikrobioms gibt. Weiterhin konnte ein Anstieg von *Bifidobacterium adolescentis* gezeigt werden, welches in der Lage ist, für den menschlichen Körper förderliche Metabolite zu produzieren und somit postuliert einen eher zuträglichen Effekt auf die menschliche Gesundheit besitzt (Ergebnis II).

Die dritte Studie, die in dieser Arbeit aufgeführt wird, befasst sich mit der Methodik der Extraktion und Purifikation von bakterieller chromosomaler und mobiler Desoxyribonukleinsäure (Plasmide) und deren Analyse mittels Short-Read-Sequenzierung von multi-resistenten Gram-negativen Bakterien. Diese Gram-negativen Bakterien wurden in Wunden von ukrainischen Kriegsverletzten detektiert, die aus der Ukraine an das Universitätsklinikum des Saarlandes transferiert wurden. Im Rahmen dieser Studie konnte gezeigt werden, dass vor allem die isolierten *Klebsiella pneumoniae* Stämme einen gemeinsamen Ursprung haben könnten, der wahrscheinlich auf die medizinische Notunterkunft in der Ukraine zurückzuführen sein könnte. Weiterhin wurde gezeigt, dass in zwei Fällen das exakt gleiche Plasmid in den Isolaten zu finden war. Dieses Ergebnis zeigt noch einmal deutlich die Gefahren des horizontalen Gentransfers zwischen Bakterien, mittels dessen vor allem Gene weitergetragen werden, welche für Antibiotikaresistenzen kodieren. Zusätzlich zu den molekularen Analysen wurden die Isolate mittels kultureller Testung und Nachweis von Antibiotikaresistenzen untersucht (Ergebnis III).

Abschließend wurde eine Studie durchgeführt, die die Methodik von Ergebnis I umsetzt und verschiedene Mikrobiome eines gleichen Individuums analysiert (Ergebnis IV). Hierfür wurden über den Zeitraum von 2,5 Jahren verschiedene Patientinnen und Patienten des Universitätsklinikums des Saarlandes medizinisch untersucht und eine ausführliche Anamnese durchgeführt. Weiterhin wurden, sofern möglich, von jedem Teilnehmenden Stuhl, Speichel, interdentaler Plaque, Hautabstriche, Bindehautabstriche und ein Rachenabstrich gewonnen, welche dann anschließend metagenomisch untersucht wurden. Das Ergebnis dieser Studie zeigt unter anderem, dass verschiedene chronisch-entzündliche Krankheiten, als auch Volkskrankheiten mit dem Vorkommen gewisser bakterieller Spezies korrelieren. Weiterhin ist hervorzuheben, dass auch biosynthetische Gencluster, welche oft für anti-entzündliche oder

anti-proliferative Metabolite kodieren, in verschiedenen Studienkohorten gefunden wurden. Als weiteres Ergebnis ist anzumerken, dass Verschiebungen in vor allem den oralen Mikrobiomen krankheitsübergreifend und im Vergleich zu gesunden Teilnehmenden detektiert wurden. Diese Studie zeigt deutlich, welche Korrelationen es zwischen verschiedenen Mikrobiomen eines Individuums und dessen Gesundheitsstatus gibt.

In Zukunft sollte sich das Gebiet der Mikrobiom-Forschung zunehmend mit der Aufschlüsselung der molekularen Mechanismen zwischen Mikroorganismen und Wirt beschäftigen und versuchen, die Frage aufzuklären, ob der Gesundheitsstatus Veränderungen im Mikrobiom bewirkt oder das Mikrobiom maßgeblich zur Entstehung verschiedener Krankheiten beiträgt. Weiterhin bieten die Entdeckung neuer biosynthetischer Gencluster einen Ansatz, neue antimikrobiell wirkende Substanzen zu finden und der globalen Bedrohung durch multi-resistente Erreger entgegenzuwirken. Weiterhin sollte die Überwachung und Analyse von multi-resistenten Erregern global ausgeweitet werden. Dieses Vorgehen erlaubt es, unter Einbezug des Menschen und seiner Umgebung, Resistenzmechanismen zu detektieren und deren Entstehung und Verbreitung aufzuschlüsseln.

"The mounting evidence that these bacteria are paramount to our health and well-being means that the lifestyle, medical, and dietary choices we make need to include careful consideration of the consequences to our gut microbes"

Dr. Justin Sonnenburg from Justin Sonnenburg and Erica Sonnenburg. 2016. The Good Gut: Taking Control of Your Weight, Your Mood, and Your Long-term Health. 3. Penguin Publishing Group.

3. Introduction

3.1 The human microbiome

The human microbiome describes all microorganisms and their genetic information, which live in and on the human body [31]. These microorganisms include bacteria, archaea, fungi, parasites, viruses, and bacteriophages, which all contribute to human health homeostasis [57,136,139,280].

Over the past two decades, and facilitated by the development of new diagnostic tools, there has been a significant increase in research dedicated to understanding the human microbiome and its contribution to health and several diseases, longevity, and, for example, treatment outcome of a vast number of diseases [130,135,189,274,327,332,337]. Researchers worldwide thereby revealed the involvement of the microbiome in various processes within humans, such as metabolism, immune function, digestion, and even brain function [46,113,179,181,229,264].

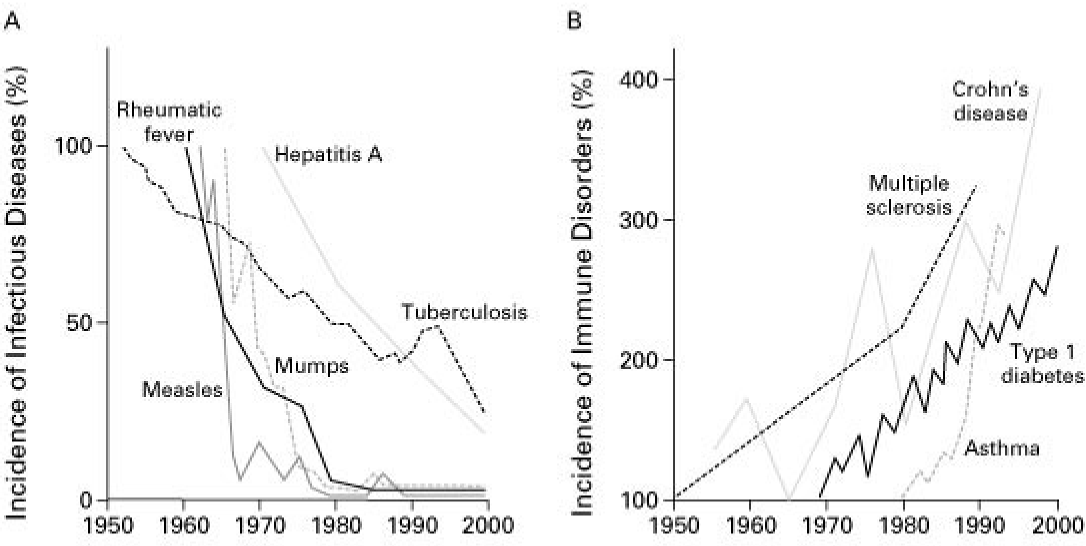

Figure 1: Inverse relation between the incidence of selected infectious diseases (A) and the incidence of immune disorders (B) from 1950 to 2000 *[17]*. Shown in percentage are the incidence of infectious diseases from 1950 to 2000 (A) and of the incidence of immune disorders (B) over the same time.

In 1945, World War 2 had ended and western countries started the process of reconstruction and industrialization. Furthermore, Alexander Fleming discovered penicillin, an antibiotic against Gram-positive bacteria, in 1928 which was then first used in 1941 to treat a bacterial infection [104,303]. These events, combined with the development of vaccines, better hygiene standards, an improvement of healthcare, and the availability of more nutritious food might have contributed to the declining trend observed in infectious diseases (Figure 1A) [228]. However, there is no sufficient explanation for the increase in chronic inflammatory and autoimmune diseases. Hypotheses combine the events of industrialization and a change of nutrition, stress levels, and lifestyle changes due to the post-war environment with the observed trend [96]. Furthermore, studies show that these factors correlate closely with alterations in the human microbiome [99,263]. Therefore, the hypothesis arose, that mostly changes in lifestyle and nutrition changed our microbial communities, which then led to the observed rise of immune disorders and chronic inflammatory diseases such as Crohn's disease and asthma [288] (Figure 1B).

One of the most significant breakthroughs in microbiome research has been the discovery and development of new technologies for analyzing the microbial compositions, such as affordable metagenomic and Whole-Genome Next Generation Sequencing (WG-NGS), as explained in detail below, improved deoxyribonucleic acid

(DNA) extraction procedures, and larger computational capacities, which have allowed researchers to identify and study complex microbial communities based on their taxonomy and functional capacity [4,18,35,193,344].The Human Microbiome Project, conducted from 2006 to 2012, involved a comprehensive analysis of microbiomes from various body sites from thousands of participants [314,315]. This project led to the creation of extensive databases that include genomic information obtained through sequencing and basic clinical data. It played a key role in sparking increased research interest and enhancing our understanding of the presence of microorganisms in different health statuses, how their functional capacities can contribute to alterations in health and disease, and their correlations with specific body sites, such as the gastrointestinal tract, skin, and oral-respiratory tract [245].

Despite significant progress in describing and understanding the human microbiome, important information on specific microorganisms related to health and how they interact with each other, and the host remain unknown. Furthermore, microbiome research must be combined with a detailed medical background examination of each participant, as many factors, such as for example diet, antibiotics, birth procedure, and many more can influence the microbial compositions and their metabolic activity [209].

Given the proposed importance of the microbiome in global and human health, there is a pressing need for further research to better understand its function and its interactions with the host and between the microorganisms.

3.2 Characterization of microbial communities

In order to study microorganisms, which reside in and on the human body, in 1860, Louis Pasteur invented artificial culture medium to grow bacteria in the laboratory and investigate their potential pathogenic characteristics [37]. Until 100 years later, culturing of bacteria from native samples and subsequent diagnostic methods, such as microscopy or Gram-staining introduced by Hans Christian Gram in 1882, have been the most widely used methods to study microbial compositions [24,330]. The conventional detection method of culturing was improved by matrix-assisted laser desorption/ionization time of flight

(MALDI-TOF), which enables fast and accurate identification of grown bacterial colonies on the species level [231]. Hereby, a bacterial sample is collected, typically from culturing native clinical samples on agar plates or purifying bacterial mass directly from native samples. Next, bacterial mass is transferred to the so-called target plate, on which the cells can either be used directly for measurements, or lysed by adding formic acid, to release all proteins and molecules present in and on the cells. The application of a matrix solution, usually a mixture of an organic acid and a solvent, directly onto the bacterial biomass then allows for crystallization of the bacterial cells, allowing for uniform energy absorption during the laser irradiation, promotes the generation of ions, and it facilitates the desorption and ionization of biomolecules from the bacterial cells upon laser irradiation. The prepared target plate is then transferred into the MALDI-TOF mass spectrometer, in which a pulsed laser is directed at each spot on the target, generating a burst of energy upon reaching the matrix-coated bacterial mass. This then leads to the evaporation and desorption of the microbial cell molecules, thereby creating gas-phase ions, which can then, under vacuum conditions, be accelerated into the attached flight tube. The recorded time-of-flight it takes the ions to travel through the flight tube is directly proportional to their mass-to-charge ratio (m/z), allowing ions smaller in mass to hit the detector faster than larger ones. The generated data for each ion is then visualized by combined mass spectra for each measured spot on the MALDI-TOF target. These mass spectra contain peaks that correspond to various ions produced from the bacteria-derived proteins, peptides, and other molecules and can be compared to reference databases, allowing precise identification of previously cultured bacteria [231,266]. The final approval of MALDI-TOF for the identification of microorganisms was achieved approximately around 2010, and is now a widespread method used in diagnostic laboratories (Figure 2) [34,55,88,322]. This invention revolutionized rapid microbiological diagnostics, however, is limited by the existing reference databases, sample quality, sufficient bacterial mass, and typically the culturing of bacteria prior to analysis.

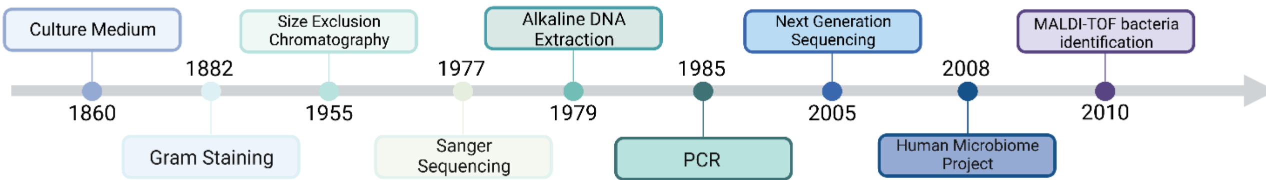

Figure 2: Timeline of methods characterizing microbial communities. Depicted is the chronological order of several methods, which can be used to investigate microbial consortia. Each box represents a method, and the number below/ above shows the year of invention, respectively. References and names of inventors can be found in the text. PCR= polymerase chain reaction; MALDI-TOF= matrix-assisted laser desorption/ionization time of flight.

A further method to rapidly analyze microbial communities present in a sample is the DNA-based approach. In 1955, Lathe and Ruthven invented size exclusion chromatography, which separates molecules according to their size through a porous column [23]. With this method, DNA was purified from lysed cells for the first time, and the evolution of DNA extraction procedures began. In 1979, Birnboim and Doly invented the still commonly used alkaline extraction for plasmid purification, and Vogelstein and Gillespie discovered the high affinity between silicate and DNA, introducing silica matrices to bind specifically DNA or RNA to a column, while removing all other components of cell lysates [33,328]. The breakthroughs in DNA extraction and purification were then followed by the first sequencing technique developed by Fredrick Sanger in 1977: the Sanger sequencing, or dideoxy chain-termination method [362]. Sanger sequencing is still commonly used to sequence short fragments of DNA for diagnostic purposes. The principle of Sanger sequencing is based on DNA synthesis in the presence of chain-terminating dideoxy nucleotides triphosphates (ddNTPs), which lack a 3' hydroxyl group. Without this 3' hydroxyl group, further elongation during sequencing-by-synthesis is not possible. Therefore, if such a ddNTP is incorporated into a growing DNA strand during elongation, it terminates the DNA synthesis at that exact point. During Sanger sequencing, four separate reactions are set up, each containing a mixture of normal deoxynucleotide triphosphates (dNTPs) and one specific ddNTP (adenine, guanine, cytosine, or thymine) which is usually fluorescently labeled. To sequence DNA of interest according to Sanger, the DNA first must be denatured into single strands. This allows specific primer annealing to the respective DNA region. These small double-stranded DNA regions can then be recognized by a DNA polymerase, which then synthesizes a complementary DNA strand to the template. Once the DNA polymerase incorporates a specific ddNTP for each of the four bases, the synthesis is terminated at this position and a new cycle can start. All reaction products generated during this process can then be separated using gel electrophoresis, which separates DNA according to its size. Such a separation then allows the readout of each fluorescently labeled base in order of respective fragment sizes [362]. This method is highly accurate for readout lengths of 1,000 base pairs and rather cost-effective [66]. However, the process is relatively slow compared to modern Next-Generation Sequencing techniques and is therefore not suitable for larger whole-genome sequencing projects. It also requires manual steps, which can be time-consuming for the staff. As some microorganisms replicate relatively slowly and a low bacterial load is sufficient to infect the host, simply extracting DNA from microbial communities and sequencing these with Sanger sequencing was not sensitive

enough to detect such microorganisms in a complex sample [166,363]. Therefore, in 1985, the discovery and invention of the polymerase chain reaction (PCR) by Kary B. Mullis contributed a large improvement in, especially diagnostics of microbial pathogens (Figure 2) [213]. The technique of PCR enables fast and precise amplification of DNA fragments, thereby also amplifying detectable signals during sequencing. The understanding of the 16S ribosomal ribonucleic acid (rRNA) in bacteria and archaea, which harbor highly conserved regions among all bacterial species, but also highly variable species-specific regions, was instrumental [343]. This knowledge made PCR of the 16S rRNA gene and subsequent sequencing of these amplicons the method of choice for individual patient diagnostics, but also in analyzing and characterizing microbial communities (Figure 3) [251]. 16S rRNA sequencing begins with the first crucial step for high quality results: the extraction of nucleic acid from native samples. This is usually achieved by a combined mechanical and chemical or enzymatical lysis of microbial cells and a suitable purification method for either plasmid DNA or chromosomal DNA [285]. Nucleic acid of sufficient quality can then be used for the 16S rRNA gene targeted amplification using PCR. Specific oligonucleotides, also called primers, are designed to anneal to highly conserved sequences within the 16S rRNA gene of all bacteria and archaea, flanking the variable regions [253]. During the PCR, millions of copies of the 16S rRNA genes present in the sample of extracted nucleic acid are generated, thereby producing so-called amplicons. For subsequent sequencing, respective libraries have to be prepared by adding sequencing adaptors or barcodes to the amplified fragments, allowing recognition of one sample amongst many [211]. The prepared 16S rRNA amplicon libraries can then undergo subsequent sequencing using high-throughput sequencing platforms, as for example Illumina sequencers, to generate short-read data of approximately 150 base pairs to 300 base pairs [211]. Similar to Sanger sequencing, Illumina sequencing works by sequencing-by-synthesis and the addition of fluorescently labeled nucleotides that terminate the sequencing process [211]. However, these modified nucleotides can be removed again enzymatically, and the sequencing process can continue step by step. The generated sequencing data then needs to be compared to reference databases of known 16S rRNA sequences from different microorganisms and then conclusions about the presence of bacterial and archaeal species can be drawn, as well as diversity measures of complex microbial communities in a sample [247]. Until today, many studies of the human microbiome and its impact on health, interventions, or longevity, are based on 16S rRNA PCR and sequencing, also called amplicon sequencing [109,160,256]. This method has many advantages, as it is fast, cost-effective, and very sensitive. However,

it only allows for taxonomic analysis of microbial compositions, meaning it enables to identify specific species in a sample. Modern microbiome research not just focusses to answer the question: who is there? But also: what are they doing? Therefore, NGS, especially whole-genome sequencing (WGS), which typically refers to sequencing the entire genome of an isolated organism, and metagenomic sequencing, which is defined by sequencing all organisms present in a native sample to describe complex communities of microorganisms without the need of prior cultivation of separate species, became very powerful tools to analyze functional capacities within a microbial community (Figure 3) [217,238,334]. Instead of only sequencing a specific gene suitable for species identification, metagenomic sequencing and WGS read the entirety of genetic information present in a sample, and by using computational analysis tools, shed light on metabolic processes, antimicrobial resistances, and functional capacity within each microorganism present in a sample [222]. These sequencing techniques are further not restricted to bacteria and archaea, but also detect fungi, viruses, parasites, and host DNA [284]. It can be divided into short-read sequencing, and long-read sequencing. Short-read sequencing needs preceding DNA fragmentation of extracted DNA and subsequent amplification of the short fragments [262]. Then, the short fragments, usually around 300 base pairs (bp) long, are sequenced using sequencing-by-synthesis, for example Illumina sequencing. This sequencing technique is one of the most widely used and cost-effective sequencing methods and combines the generation of DNA clusters and sequencing-by-synthesis [246,278]. During library preparation, sequencing adapters are added to the DNA fragments, which bind to complementary oligonucleotides on the surface of the flow cell used for sequencing. This step immobilizes the DNA fragments on the flow cell, allowing for binding of both ends to the attached oligonucleotides on the flow cell and, by adding a DNA polymerase, the so-called bridge amplification [182]. Synthesized complementary DNA strands then bind in close proximity to the template DNA on the flow cell's surface, creating clusters of identical DNA fragments and allowing for signal intensification during sequencing [182]. Sequencing-by-synthesis then means, that the amplified fragment is being sequenced using fluorescently labelled nucleotides and a DNA polymerase [151]. Each nucleotide has a different fluorescent label which is detected after it has been incorporated into the newly synthesized DNA strand. Deciphering of the fluorescent label works by excitation of the fluorescent molecule by a laser and posterior detection by a camera within the sequencer system [364]. As fragmentation of the DNA happens at random sites, overlap regions exist

that aid to *in silico* reassembling of the short fragments into contigs or scaffolds after sequencing [234].

In contrast to short-read sequencing stands long-read sequencing, most notably the Oxford Nanopore Technology (ONT) [336]. Here, no fragmentation of the whole-genome or metagenomic DNA is necessary, and the entire chromosomal or plasmid DNA can pass through a nanopore, α-hemolysin, which is located in an electro-resistant membrane [6,60,336]. Each nanopore is connected to a sensor, which carefully detects any changes in electric current. If DNA passes through the α-hemolysin, each base creates a certain defined change in electric current, which is measured by the sensor and can be translated back to the base that passed (Figure 3) [259]. By using ONT, the primary structure of DNA can be deciphered fast and in a cost-effective way.

For all described sequencing techniques, prior nucleic acid extraction is a crucial step in allowing the most accurate DNA-based description of microbial communities. Hereby, it is essential to achieve high-quality and intact DNA isolated from all microorganisms present in a sample. Contaminants, such as proteins, chaotropic salts, or phenol can inhibit downstream analyses such as e.g. sequencing or PCR, and degraded DNA can lead to errors during the sequencing process [5,82,276,324]. Therefore, removal of such inhibitors should be included in the nucleic acid extraction procedure. Furthermore, a sufficient but also gentle protocol should be followed, to avoid fragmentation of DNA. When trying to describe microbial communities, a sufficient lysis of all potential microorganisms present in a sample should be prioritized, next to achieving high-quality DNA. A combination of mechanical and e.g. chemical lysis can lead to efficient disruption of microbial cell walls, releasing the nucleic acid for purification [18,111,254]. Genomic nucleic acid extraction aims to capture the entire genomic content present in the sample. This is especially critical in whole-genome and metagenomic sequencing, where complete representative DNA extraction is necessary to avoid bias in the sequencing results. Insufficient microbial lysis and in particular inadequate nucleic acid extraction procedures can massively impact sequencing data generation and therefore the exact description of microbial communities, leading to false conclusions.

Currently, there is no completely standardized method used as the gold standard in nucleic acid extraction, making data comparability between different microbiome studies difficult. Therefore, the most appropriate method for various samples should be investigated to achieve high-quality science and inter-comparability of studies.

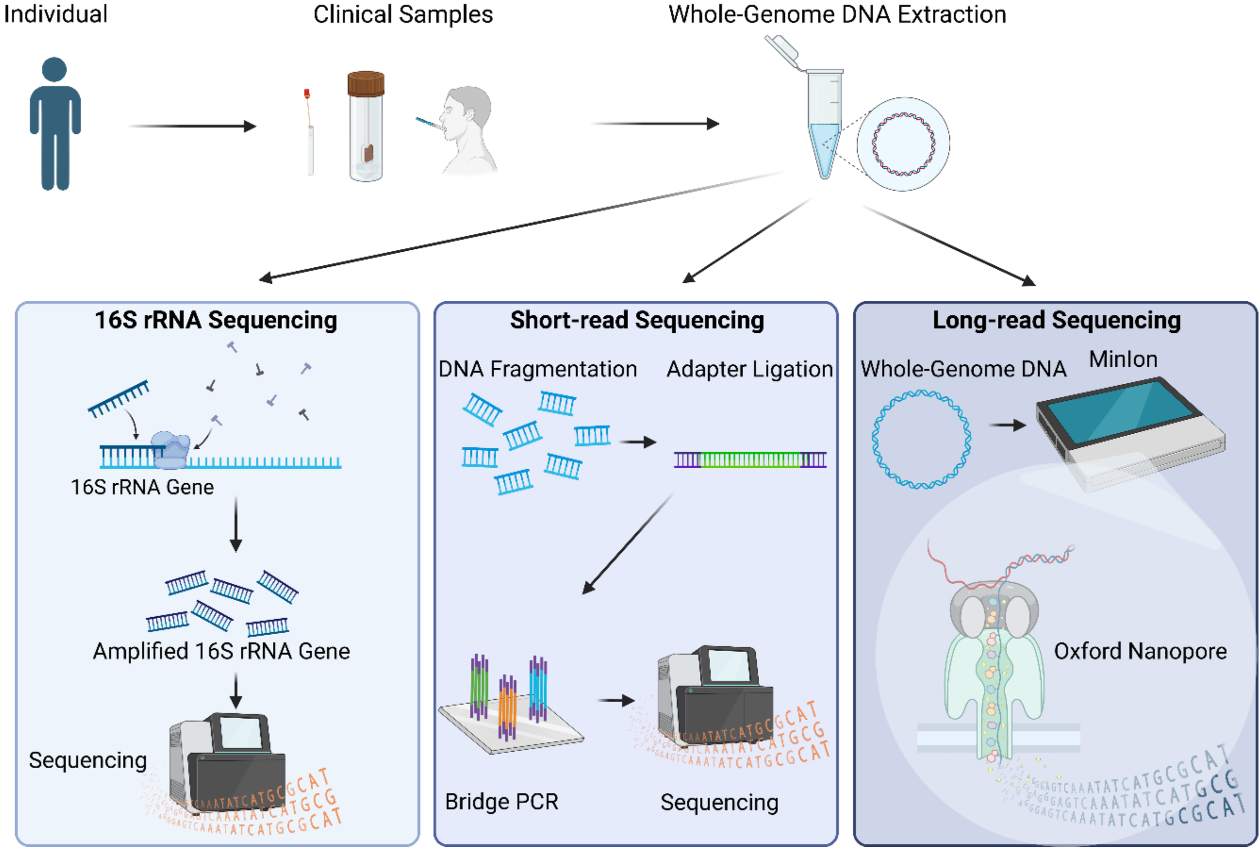

Figure 3: Different approaches to analyze the human microbiome. Presented are the three most commonly used approaches to analyze microbial compositions of human body sites. Every workflow starts with the inclusion of an individual and the generation of native, clinical samples, such as stool, saliva, skin swabs, or interdental plaque. Next, whole-genome DNA is extracted from all samples. This means, the entire genetic information contained in a native sample is purified. The three most commonly followed experimental approaches to analyze the microbial compositions are i) 16S rRNA sequencing, which amplifies the 16S rRNA gene of bacteria and archaea and helps to investigate bacteria based on their taxonomy, ii) short-read sequencing with the example of the Illumina workflow (other workflows, such as BGI DNA nanoballs exist), which fragments whole-genome DNA and amplifies the products of fragmentation for metagenomics and whole-genome sequencing, and iii) long-read sequencing, which does not need preceding DNA fragmentation, but rather sequences the whole-genome DNA as it is. Long-read sequencing uses a nanopore situated in an electro-resistant bio membrane. Every nanopore interacts with its own electrode that is connected to a sensor chip which measures electric current. If DNA flows through the nanopore, each base within the DNA disrupts the current and creates a characteristic spectrum. Each electric current spectrum is then decoded using base calling algorithms.

Other, non-DNA-based methods to characterize microbial compositions are lipidomics, proteomics, and transcriptomics. These approaches analyze all lipids, proteins, or messenger RNA (mRNA) present in a sample [321,338,353]. Certain lipid biomarkers, such as phospholipid fatty acids, short-chain fatty acids, and glycolipids are commonly used to draw conclusions on microorganisms present in a sample, their relative abundance and diversity [44,86,119,177]. Transcriptomics and proteomics can further enhance our knowledge of the microbial gene expression and biosynthesis of proteins in different environments, such as for example in the duodenum and jejunum which slightly differ in

pH and thereby influence gene expression in the bacteria residing in the respective niche [74,313].

3.3 Microbiome-influencing factors

The human microbiome consists of a dynamic consortium of various microorganisms that reside in and on the human body [190]. The diversity and composition of the microbiota is affected by many internal and external factors, most notably within the first three years of life [174,256,339]. In the following paragraph, the most important microbiome-influencing factors will be elucidated (Figure 4).

The first possible route of microorganisms colonizing the human body is controversially discussed. For a very long time, researchers believed that neonates are exposed to microbes during and after birth, but that the mother’s uterus is completely sterile [239]. This hypothesis was first rejected by several studies, implicating that microbial DNA and bacteria-derived metabolites, namely short-chain fatty acids (SCFAs) can be detected in first-pass meconium of neonates. Researchers concluded that the human microbiome may start developing in the mother`s womb [296]. However, Kennedy et al. suggest, that first-pass meconium is usually collected hours to days posterior to birth, which allows exposure to many microorganisms deriving from clinical staff, family members, and the hospital environment [155]. Therefore, they collected fetal meconium before birth by colorectal swab during elective breech caesarean section (c-section) and compared the results of 16S rRNA gene sequencing to first-pass meconium and infant stool. In this study, no bacterial signal could be detected in fetal meconium before birth, suggesting that the uterus is indeed sterile under healthy conditions, and that the development of the microbiome starts during and after birth [155]. C-section delivery has risen globally from 7 % in 1990 to 21 % in 2023 [10]. In Germany, 29.7 % of births were conducted by C-section in 2022, compared to only 15.3 % in 1991, now making up one third of all birth procedures [365]. The effect of C-section compared to vaginal birth on the human microbiome and overall health are widely and controversially discussed. In some studies, neonates delivered by C-section

tended to show a modified development of immunity, and they were, e.g., more likely to develop allergic asthma, and other chronic inflammatory diseases [68,346]. Arrieta et al. correlated allergic asthma to certain gut bacteria, such as *Veillonella* sp., *Lachnospira* sp., and *Faecalibacterium* sp., which are all fiber-fermenting intestinal microbes [14]. These bacteria were found to be higher in healthy infants, compared to children suffering from allergic asthma. Furthermore, SCFAs were detectably higher in healthy children [14]. Supplementation with both, these differentially abundant bacteria and SCFAs was shown to protect the airways of mice when challenged with allergens, and the findings have also been confirmed in humans. The alterations in intestinal microbial communities between the two groups can be traced back to the respective birth method [14]. During C-section, the first contact of the neonate with microorganisms is the mother's and clinical staff's skin, therefore bacteria found in first-pass meconium of these neonates are mainly *Staphylococcus* sp. and *Streptococcus* sp. [62]. Compared to infants born vaginally, the intestinal microbiome was also shown to be less diverse [62]. Vaginally delivered neonates, on the other hand, showed higher abundances of bacteria typically found in the vaginal microbiome: *Bacteroides* sp., *Bifidobacterium* sp., and *Lactobacillus* sp., for which most species belonging to these genera are capable of dietary fiber fermentation, thereby producing anti-inflammatory and anti-proliferative SCFAs [62,97,312,331].

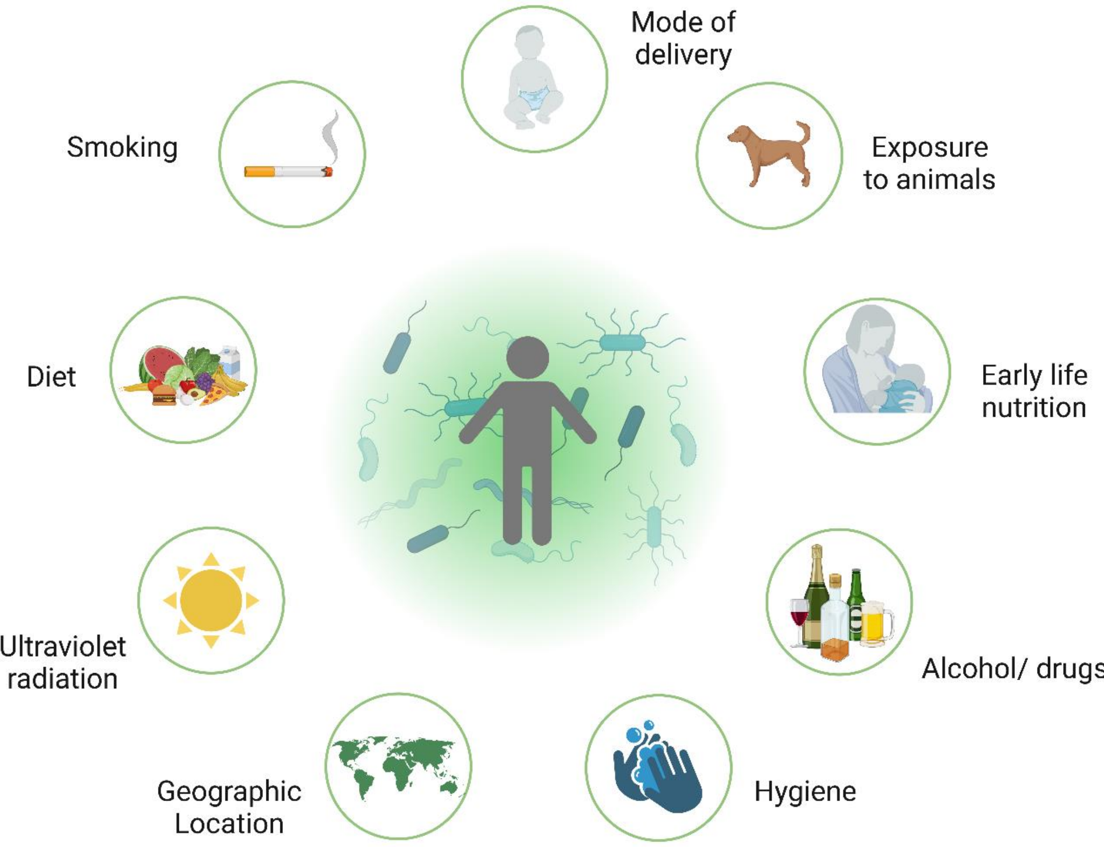

Figure 4: Microbiome influencing factors. Shown are the most common external factors that influence the human microbiome. Each factor is represented by a pictogram and the respective written name.

After birth and the associated first microbial colonization, neonatal and infant diet is a second main contributing factor to the development of the human microbiome [16,270]. One study suggests that feeding infants exclusively with breastmilk leads to a more diverse gut microbiome later on in life [195]. Furthermore, breastmilk harbors many ideal nutrients for infants, components of the innate immune response, and has been shown to modulate the intestinal barrier integrity and mucosal defenses positively when compared to the use of commercially available formula [195]. However, conflicting findings exist, suggesting that the microbiota are richer and more diverse in infants receiving a formula-based diet [70,90,133,295]. Next to early-life nutrition, studies have demonstrated that administration of broad-spectrum antibiotics in the first seven days after birth, for example in case of an early-onset neonatal sepsis, significantly alters intestinal microbiota compositions [256,319]. Especially a combination of amoxicillin and cefotaxime has been shown to change not just microbial composition, but also antimicrobial resistance patterns within intestinal bacteria [256]. In a study from 2022, Reyman et al. were able to observe a normalization over the course of 12 months posterior to antibiotic treatment of neonates [256]. However, an

early-life use of antibiotics disrupts the intestinal microbiome and associated functional capacity in terms of SCFA-producing bacteria [352]. Xu et al. demonstrated significantly lower abundances of *Clostridium* and *Blautia* in antibiotic-exposed neonates [352]. Both genera are butyrate-producers, thereby contributing to the overall amount of SCFAs in the human body [105,145,161]. Whether these shifts in microbiota composition and metabolic potential are reversible might be connected to the specific antibiotic used and the duration of antibiotic exposure.

In between infancy and childhood, children start to switch to solid foods diets. The choice of diet pattern during childhood, adolescence, and adulthood counts as the third major microbiome-influencing factor [65,72,117]. The research focus on the correlation between diet and microbiota composition has mainly been the western diet (WD), the Mediterranean diet (MD), and diets of rural tribes, mainly in Africa [1,2,71,73,109,192,210,275,299]. The WD is associated with a much less diverse intestinal microbiome, an increased risk of developing non-communicable diseases, especially chronic inflammatory diseases, and altered SCFAs levels [2,22,59]. Characteristic of the WD is the uptake of simple carbohydrates, such as for example found in most processed foods. Furthermore, uptake of dietary fiber is comparably low [250]. In contrast to the WD, the MD and diet patterns of indigenous people following a traditional lifestyle suggest a consumption of dietary fiber amounts higher than 50 g/d [227]. Independent of the diet concept, modern humans tend to consume, at most, half of what is estimated for ancestral humans, who on a daily basis had an intake of approximately 100 g dietary fiber [205]. Dietary fiber are complex carbohydrates, which are fermented by certain bacteria residing in the human gut. This fermentation process then leads to the production of SCFAs, such as acetate, butyrate, and propionate [333]. These intestinally produced SCFAs partly stay locally, but can further be absorbed into the bloodstream, thereby reaching even distinct body sites, such as the skin or respiratory tract (Figure 5) [310,311].

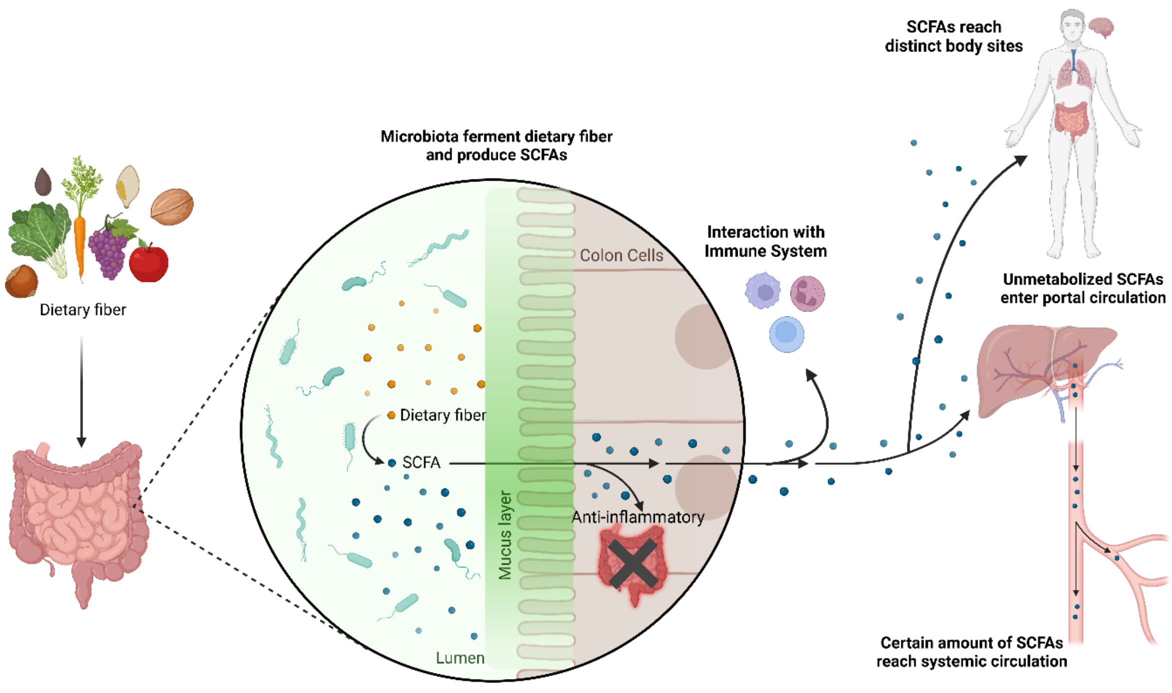

Figure 5: Short-chain fatty acids and their modulating effects in the human body. Ingested dietary fiber, which can be found in vegetables, seeds, nuts, and fruit reaches the intestinal lumen where it is fermented by gut microbiota. The product of fermentation are short-chain fatty acids (SCFAs), such as acetate, butyrate, and propionate. These SCFAs reach the colon cells, where they act anti-inflammatory and anti-proliferative, reducing the risk for inflammation. They further can be absorbed into the bloodstream and reach systemic circulation, reaching even distinct body sites, such as the lung, the brain, or the skin. SCFAs also modulate the immune system, suppressing inflammation and allergic responses.

However, if individuals follow a WD, consumption of dietary fiber is relatively low, whereas consumption of sugar and simple carbohydrates is high [250]. The human genome contains 17 genes, which encode enzymes that are involved in simple carbohydrate digestion, thereby nurturing human cells, and combined with the low dietary fiber intake, leaving gut microbiota starving [75,301]. It has been shown that a dietary depletion of pectin, highly fermentable fiber, leads to intestinal bacteria feeding on the protective mucus layer in between colon epithelial cells and the intestinal microbiome. Four weeks of dietary fiber depletion were thereby shown to be sufficient to reduce the mucus layer by 50 % [81]. Researchers believe, that the depletion of dietary fiber and the correlated reduction of the mucus layer in the intestine are major contributors to, if not the root of for one, leaky-gut syndrome, and second, chronic inflammatory diseases [75,118,184,257,317,358]. The Academy of Nutrition and Dietetics, the Scientific Advisory Committee on Nutrition, and the National Academy of Sciences Institute of Medicine all recommend between 20-35 g/d dietary fiber

[67,140,297]. Such an amount of dietary fiber intake correlates with reduced blood inflammation markers [162].

Apart from diet, many other factors are known to influence the composition of the human microbiome. Geographic location, including different climate zones, levels of industrialization, hygiene standards, and overall quality of life can contribute to differences in microbial abundances [103,112,124,126]. Especially ultraviolet radiation (UVR) exposure can change the diversity and composition of the skin microbiome, but also of the intestinal microbiome in mice [107,108,235,249]. Therefore, seasonal, as well as geographic differences of microbiomes at several body sites can be explained by variations in UVR. Moreover, levels of air pollution have been correlated with less diverse microbiota [91].
Next to environmental factors, medication, especially antimicrobial treatment can influence the microbiota dramatically, even during adulthood [11]. A prominent example of antibiotic-induced changes within the intestinal microbiome is antibiotic-associated diarrhea [25]. After antibiotic therapy for a certain amount of time, patients can suffer from severe diarrhea, which is often caused by an overgrowth of *Clostridioides difficile*, a spore-forming pathogenic bacterium that is capable of producing toxins, harming the gut epithelial cells [214]. During antibiotic therapy, many of the commensal, non-harmful gut bacteria are killed, creating space and leaving nutritious resources to existing spores of *C. difficile*, leading to an uncontrolled overgrowth [214,357]. An infection with *C. difficile*, if not treated, can lead to potentially fatal consequences such as a toxic megacolon, that can only be treated with surgery [273]. In order to occupy niches in the intestinal tract with beneficial or commensal bacteria, to suppress growth of *C. difficile*, researchers tested a fecal microbiome transplant (FMT) of a healthy donor, displaying a success rate of around 95 % if *C. difficile* is not treatable with antimicrobial compounds [114,220]. The FMT is now a commonly and widespread treatment of *C. difficile* infections worldwide, enhancing the importance of a healthy microbiome [27].

Last, use and abuse of a variety of chemical compounds, such as alcohol, nicotine, or illegal drugs can lead to a suppressed growth of certain bacteria and enhance growth of others [26,279,340]. In this paragraph, the abuse of nicotine shall be elucidated as an example for a commonly consumed, non-nutritive compound that leads to alterations in the oral and intestinal microbial communities. Nicotine abuse through smoking tobacco

has drastic effects on the human oral microbiota composition. Active smokers harbor less phyla of Proteobacteria and the genera *Capnocytophaga, Peptostreptococcus*, and *Leptotrichia* compared to individuals who never smoked. Enriched genera found in the oral cavities of active smokers were shown to be *Atopobium* and *Streptococcus*. These alterations in microbial composition in turn also change the functional capacity, leading to a respective decrease in genomic potential for carbohydrate and energy metabolism. Furthermore, depleted genera in active smokers partly also contribute in the metabolism of xenobiotics, potentially increasing the risk of xenobiotics reaching higher, toxic concentrations in the human body [347]. These shifts in the oral microbiome, including the microbiota themselves and their functional capacity, may contribute to the development and progression of smoking-related diseases, such as chronic obstructive pulmonary disease (COPD) [38,78,188]. A smoking cessation, an increase in age, and an overall healthier lifestyle were shown to have the potential to shift the oral microbiota back to those of individuals that never smoked, thereby minimizing the negative effects on the microbiome and related disease progression [12]. A study from 2021 conducted in China found several acid-producing and nitrite-producing bacteria to be enriched in the oral cavities of active smokers. These bacteria were found to complement each other and positively affect co-existence in contrast to other commensal oral microbiota [146]. The acidification and alterations in bacterial species are hypothesized to correlate with changes in oral health [146]. Cigarette smoke, however, does not only alter the oral microbiota, but was shown to affect the intestinal microbiome as well. Active smokers tend to harbor more *Escherichia/Shigella* sp., *Klebsiella* sp., and *Lactobacillus* sp. in their intestinal tract when compared to non-smokers, which are overall associated with a reduced biodiversity [175]. These alterations could contribute to significant changes in the function of the gut-lung axis; however, the clinical relevance of these findings remain to be investigated in more detail.

The microbiota composition is not just determined by external factors, as listed above, but also by internal factors, such as existing co-morbidities and genetics [191]. Thus far, it is not fully understood whether the human microbiome influences the development and progression of chronic inflammatory diseases, or if an existing disease leads to shifts in the microbial composition, which then accelerates inflammatory processes in the body.

3.4 Impact of the microbiome on chronic inflammatory diseases

Many associations between the human microbiome and a variety of chronic inflammatory diseases, such as periodontitis, obesity, allergic asthma, inflammatory bowel diseases, and dermatological conditions like psoriasis or atopic dermatitis (Figure 6) have been shown [36,50,92,98,188,260,298,346]. Here, it seems to be the combination of different microorganisms in a niche, rather than single specific species, that play a role in the development and progression of certain diseases. Chronic inflammatory diseases are a group of human disorders, which are characterized by persistent inflammation that has the potential to damage tissue, lead to functional impairment and even disability [230]. These diseases can affect a variety of organs and systems in the body, including the gastrointestinal tract, the respiratory tract, skin, nervous system and many more [51]. A common ground for most of these disorders is the treatment of symptoms, as the actual cause is still under investigation [230,241]. Therefore, treatment options aiming to heal certain diseases, such as e.g. psoriasis and periodontitis, are currently limited.

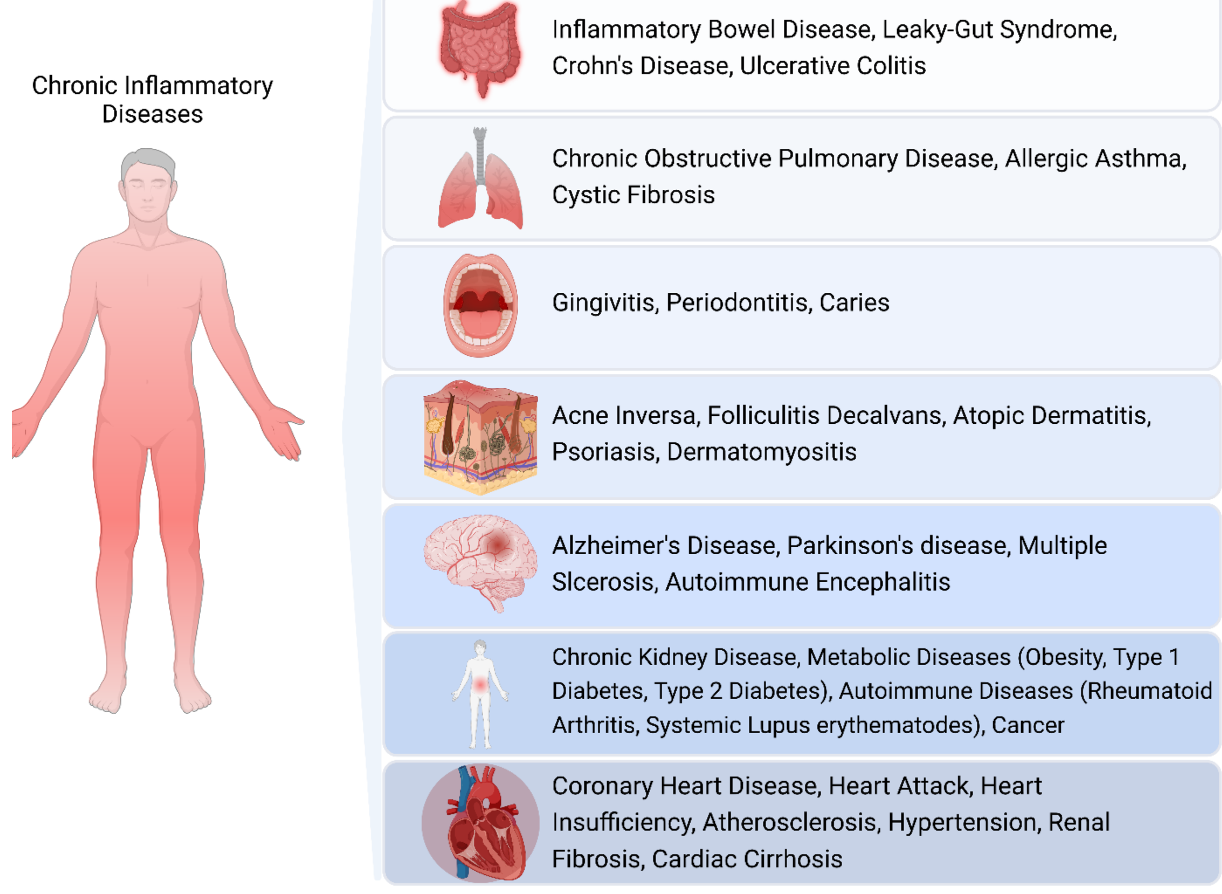

Figure 6: Chronic inflammatory disease. Depicted are a variety of chronic inflammatory diseases and inflammation-associated diseases for the main body sites. Each body site is represented by a pictogram: (from top to bottom) gastro-intestinal tract, respiratory tract, oral cavities, skin, central nervous system, abdomen, cardiovascular system. The most common chronic inflammatory, or inflammation-associated diseases are listed in the boxes.

The majority of microorganisms within the human microbiome resides in the gastrointestinal tract, where it interacts with the immune system, affects digestion and nutrient absorption and produces various metabolites, which can directly be absorbed into the blood system, and thus may travel to distinct body sites [97,113,229,310,311,323]. Recent research has demonstrated a strong association between alterations in the microbiome and the development and progression of chronic inflammatory diseases [188,327]. However, underlying mechanisms and specific host-microbe interactions are highly complex and multifactorial. Several bacterial metabolic pathways have been identified that might play a key role in health homeostasis, such as the production of SCFAs and the metabolic interaction between the gut microbiota and bile acids [44,173,306,310,311,358]. Alterations in SCFA profiles are closely correlated to the progression of inflammatory bowel disease and rheumatoid arthritis [80,202,232]. These shifts can contribute to dysregulation of the immune system and the development of chronic

inflammation that can then affect multiple organs [159]. Bile acids are a group of steroid molecules that play critical roles in digestion, lipid metabolism, and nutrient absorption [291]. They are synthesized in the liver and then travel into the small intestine, where they are reabsorbed in the ileum. Then, through portal circulation, they are re-transferred to the liver [56]. Additionally to their role in digestion and vitamin absorption, bile acids also play a critical role in regulating metabolic pathways, including glucose and lipid metabolism [355]. Furthermore, bile acids can have antimicrobial, as well as growth-promoting effects on the gut microbiota [173,325]. Microorganisms in the gut, on the other hand, can harbor the functional capacity to metabolize certain bile acids, thereby leading to shifts in bile acid profiles [147,152,252]. One example is the role of the gut microbiome in the deconjugation and dehydroxylation of bile acids, which have a direct influence on their bioavailability in the human body, as well as their function [95,128,261]. Next to their interaction with the gut microbiota, bile acids also interact with the host immune system, activating certain immune cells and modulating the production and release of pro-inflammatory cytokines [42,137,176,300]. Combining the interaction between the human gut microbiome, the host immune system, and bile acids, shifts in either of these have been associated with the development of chronic inflammatory diseases. However, certain bile acids, namely for example lithocholic acid has been associated with longevity through its antimicrobial effect on multidrug-resistant Gram-positive bacteria, potentially reducing the risk of infection in the intestinal tract [272,100,348].

In addition, it has been suggested that next to the bacterial metabolite production, the microbiome can impact the development, progression, and exacerbation of chronic inflammatory diseases through the blood-brain barrier, as well as the gut-brain axis [46,196,212,304,360]. The blood-brain barrier is a complex arrangement of interacting cells and proteins that regulate the exchange of substances between the bloodstream and the brain [153]. Studies have shown that shifts in the gut microbiome can lead to changes in the integrity of the blood-brain barrier, which may contribute to the development of chronic inflammation in the brain and nervous system. Such changes in the human body are postulated to potentially affect the development of neurological disorders, such as multiple sclerosis and Alzheimer's disease [169,187,335,349]. The gut-brain axis describes the bidirectional communication between the enteric nervous system, the gut microbiota, the immune system, and the central nervous system through the vagus nerve [47,360]. Released neurotransmitters and neuropeptides in the gut, as well as bacteria-derived metabolites can cross the gut-brain barrier and reach the brain through the vagus nerve, where they display

an impact on brain function, hunger and satiety, and mood [30,63,64]. Disruptions in the gut-brain barrier, as well as alterations in the microbial composition in the intestine have been linked to several intestinal and neurological disorders, implicating the importance and potential interplay between both [9,92,163,204,349]. Understanding the link between the human microbiome and chronic inflammatory diseases has significant potential to improve therapeutic approaches, for example through probiotics and prebiotics. These could aim to modulate the microbial composition and metabolic capacity, thereby reducing inflammation in the human body and the effects it has on several diseases.

3.5 Further application of sequencing-based approaches in clinical diagnostics

The emergence and spreading of multidrug-resistant pathogenic bacteria has become a global health crisis, which requires excellent monitoring to understand spreading mechanisms and oversee the occurrence of novel antimicrobial resistance (AMR) mechanisms [102]. Furthermore, investigation of AMR is essential to understand the molecular basis behind each mechanism and find possible targets for novel antimicrobial substances in order to treat such infections that are currently not treatable [206,207]. Important members of multi-resistant Gram-positive bacteria are methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecium* [144,168,199,307]. The underlying AMR mechanism is most prevalently a β-lactamase, an enzyme which is capable of catalyzing the hydrolyzation of β-lactam antibiotics, such as penicillin (methicillin) [127]. Vancomycin is often used as a drug of ´last-resort´ against Gram-positive bacteria [180]. However, resistance mechanisms against vancomycin, mainly mediated by the enzymes vanH, vanA, and vanX is spreading rapidly on mobile genetic elements (plasmids) [15,41,168,258,350]. Meanwhile, a greater global concern are multi-resistant Gram-negative (MRGN) bacteria due to their complex resistance mechanisms, widespread prevalence in healthcare settings, limited treatment options, and higher mortality rates associated with severe infections [142,198,215,233,265,345]. Clinically relevant members of MRGNs are *Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Providencia* sp., and *Escherichia coli* [87,134,185,224,225,236,267,282,289]. In Germany, MRGNs can further be classified into 3/4 MRGNs, which are characterized by carrying resistance mechanisms against the most commonly antibiotics used to treat such infections and consist of at least acylureidopenicillin, third and fourth generation cephalosporins, fluorchinolons, and in the

case of 4 MRGNs also carbapenems [318]. Recent research is trying to tackle the global crisis of multi-resistant bacteria, trying to find novel natural products with antimicrobial properties or novel therapeutic approaches such as phage therapy [40,157,167,342]. A further research focus lies on the monitoring of resistance mechanisms in bacteria globally [94,129]. As resistance mechanisms can be transferred via plasmids, environmental DNA, or phages, and novel resistance mechanisms can occur by spontaneous mutation, especially under selective pressure, spreading and emergence of such resistance genes is crucial in keeping outbreak situations in check [19,158,170,183,265,292]. Therefore, researchers worldwide perform whole-genome analyses on clinically isolated 3/4 MRGNs and multi-resistant Gram-positive bacteria to detect AMR transfer pathways, potential hotspots of transfer, and the genotype of multi-resistant bacteria [49,101,185,240,281,305]. These observations help to improve hygiene regimens, intervene at specific transfer sites, and enable researchers from various fields to find novel antimicrobial substances to tackle specific resistance mechanisms [366].

Beyond monitoring and analysis of antimicrobial resistances in microorganisms, sequencing-based techniques allow for precise diagnostics and managing of several medical conditions. In the gastrointestinal tract for example, inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS) are major disorders that affect a significant portion of the global population, estimating around 10-15 % of people worldwide being affected by IBS and 0.5 % of the western population suffering from IBD like Crohn's disease and ulcerative colitis [76,154,290,359]. For these chronic inflammatory diseases, a link between the microbial composition of the intestine and disease severity, disease activity and treatment response has been made [92,105,143,221]. Previous microbiome diagnostics could therefore lead to personalized treatment approaches or treatment optimization. A further application, that is already commonly used is the microbiome analysis of donors during the screening of samples for fecal microbiota transplant (FMT) procedures [32,125,218]. It is crucial to detect all microorganisms present in the donor samples to ensure safety in terms of minimizing disease transmission, and further correlate the donor's microbiome with the receiver's microbiome. Such a matching ensures a better chance of restoring a balanced and healthy gut microbiota in the recipient, reduces the risk of adverse effects due to unexpected immune responses, and allows for a more predictable long-term outcome [27]. FMTs have been proven useful treatments in recurrent *Clostridioides difficile* infections and other gastrointestinal disorders [29,84,148,220].

In oral health, the microbiome has been show to play a crucial role in the development of periodontal diseases [50,79,106,188,255]. As periodontitis diagnostics needs well-trained medical staff, diagnoses are often missed [243]. Microbiome-assisted diagnostics could aid as an additional measure to diagnose periodontal disease and monitor the progression of this condition. Furthermore, certain microbiota are linked to caries, gum diseases, and other oral health issues [45,52,286]. Early detection of these microorganisms could help in the prevention of the development of such diseases by early-on counteraction.

As our understanding of the microbial communities in and on our bodies in correlation with certain disease and health states exponentially increases, many more medical fields may benefit from such additional diagnostic measures, such as e.g. reproductive health, as the vaginal microbiome is linked to preterm birth risk, obesity management, as certain microbial signatures are associated with adiposity and might guide treatment strategies, as well as cancer diagnosis and prognosis [116,120,123]. Ongoing research is trying to find potential links between the microbiome and various cancers [268,329,361]. While it is not yet implemented in clinical diagnosis, microbiome analysis may hold promise for improving cancer diagnostics and predicting treatment response.

Last, sequencing-based diagnostic strategies are used in cases where conventional diagnostic methods fail to yield results, for example in terms of novel infections where the causative agent is unknown or rare infections for which no detection method is established yet and diagnosis is difficult [89,194,203]. Here, metagenomic sequencing detects all microorganisms present in a sample, without the bias of PCR, culturing, or other targeted approaches [238].

Overall, sequencing-based diagnostic applications may aid in a more precise diagnostic procedure that allows for standardization globally, enables unbiased detection of especially pathogenic microorganisms, assists in monitoring and detecting antimicrobial resistances and their transmission, contribute to early detection and surveillance of several diseases, as well as outbreak control, and customize diagnostics and treatment for patients.

3.6 Goal and objectives

The aim of this PhD thesis is to characterize microbiota compositions from different body sites of healthy and diseased individuals, using the same methodical approach for all biospecimens to allow comparability of the results. The following specific objectives are related to this goal.

- To assess the impact of various commercially available DNA extraction kits on sequencing output, differentiating between short-read and long-read metagenomic sequencing of various different clinical samples.

Subsequently, the DNA extraction kit allowing the most accurate and high-quality results was chosen for studies, in which microbiota compositions of different body sites shall be examined. As a second result, another DNA extraction kit showed to be suitable and of equal quality for studies only investigating fecal samples.

- To analyze the effect of following a planetary health diet on the intestinal microbiome of healthy individuals.

- To investigate clonality and resistance mechanisms in multi-resistant Gram-negative bacteria using the methods established in the first study.

- To decipher the microbial composition and functional capacity of various body sites in healthy individuals and a variety of chronic inflammatory and widespread diseases in Germany.

Together, these approaches represent a holistic exploration of the microbiome's role in health, disease, and environmental factors, offering valuable insights that can inform healthcare practices, dietary recommendations, and strategies for combating antibiotic resistance. To answer these scientific questions, the following will present the work that was carried out during this PhD thesis.

4. Results

4.1 Results No. 1:

Systematic Cross-biospecimen Evaluation of DNA Extraction Kits for Long- and Short-read Multi-metagenomic Sequencing Studies

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Rehner J, Schmartz GP, Groeger L, Dastbaz J, Ludwig N, Hannig M, Rupf S, Seitz B, Flockerzi E, Berger T, Reichert MC, Krawczyk M, Meese E, Herr C, Bals R, Becker SL, Keller A, Müller R; IMAGINE Consortium. Systematic Cross-biospecimen Evaluation of DNA Extraction Kits for Long- and Short-read Multi-metagenomic Sequencing Studies. *Genomics Proteomics Bioinformatics*. 2022 Apr;20(2):405-417. doi: 10.1016/j.gpb.2022.05.006. Epub 2022 Jun 6. PMID: 35680095; PMCID: PMC9684153.

Genomics Proteomics Bioinformatics 20 (2022) 405–417

Genomics Proteomics Bioinformatics

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ORIGINAL RESEARCH

Systematic Cross-biospecimen Evaluation of DNA Extraction Kits for Long- and Short-read Multi-metagenomic Sequencing Studies

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Received 23 August 2021; revised 13 May 2022; accepted 19 May 2022
Available online 6 June 2022

Handled by Fangqing Zhao

KEYWORDS

Whole-genome analysis;
Comparative genomics;
Short-read sequencing;
Long-read sequencing;
DNA extraction;
Metagenomics

Abstract High-quality **DNA extraction** is a crucial step in metagenomic studies. Bias by different isolation kits impairs the comparison across datasets. A trending topic is, however, the analysis of multiple metagenomes from the same patients to draw a holistic picture of microbiota associated with diseases. We thus collected bile, stool, saliva, plaque, sputum, and conjunctival swab samples and performed DNA extraction with three commercial kits. For each combination of the specimen type and DNA extraction kit, 20-gigabase (Gb) metagenomic data were generated using **short-read sequencing**. While profiles of the specimen types showed close proximity to each other, we observed notable differences in the alpha diversity and composition of the microbiota depending on the DNA

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Peer review under responsibility of Beijing Institute of Genomics, Chinese Academy of Sciences / China National Center for Bioinformation and Genetics Society of China.

https://doi.org/10.1016/j.gpb.2022.05.006
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extraction kits. No kit outperformed all selected kits on every specimen. We reached consistently good results using the Qiagen QiAamp DNA Microbiome Kit. Depending on the specimen, our data indicate that over 10 Gb of sequencing data are required to achieve sufficient resolution, but DNA-based identification is superior to identification by mass spectrometry. Finally, long-read nanopore sequencing confirmed the results (correlation coefficient > 0.98). Our results thus suggest using a strategy with only one kit for studies aiming for a direct comparison of multiple microbiotas from the same patients.

Introduction

In the past decade, microbiome research has become a trending topic with an exponential increase of available data [1]. Researchers worldwide acknowledge the importance of the human microbiome for health [2] regarding a variety of diseases, with the gut microbiome taking a leading role [3]. Recently, the link between a healthy gut microbiome influenced by a Mediterranean diet and cardiometabolic disease risk has been found [4]. In addition, the gut microbiome of Parkinson's disease patients has also been associated with intestinal inflammation [5]. Next to the gut microbiome, the microbiome of the respiratory tract has been studied extensively. For example, it has been previously shown that certain bacteria are associated with chronic rhinosinusitis. Bachert et al. [6], as well as Olzowy et al. [7], detected overgrowth of *Corynebacterium*, *Curobacteria*, *Pseudomonas*, *Staphylococcus*, and *Haemophilus influenzae* in patients with chronic rhinosinusitis compared to the healthy respiratory microbiota. There is accumulating evidence that microbiome research should also identify commensal bacteria and investigate their potential to protect from diseases. Several species are already known to synthesize compounds that inhibit the growth of pathogenic bacteria, thereby establishing a crucial balance within the microbiome. Besides the intended effects on pathogenic bacteria, antibiotic therapy also affects commensal bacteria, and may facilitate overgrowth of potentially dangerous microorganisms, as it is frequently seen in *Clostridioides difficile* infection, a common intestine complication after previous antibiotic treatment [8]. How is the growth of pathogens suppressed under normal conditions? During a co-infection, *Pseudomonas aeruginosa* produces rhamnolipids, which disperse the biofilms of sulfate-reducing bacteria and, additionally, are effective against the biofilms of opportunistic pathogens such as *Escherichia coli* and *Bacillus subtilis* [9]. *Staphylococcus lugdunensis* has been found to produce lugdunin, which is a recently discovered thiazolidine with antibiotic activity. Lugdunin inhibits the growth of the opportunistic pathogen *Staphylococcus aureus* [10]. Furthermore, certain lactic acid bacteria are known to produce a variety of secondary metabolites which inhibit the growth of other bacteria, such as bacteriocins, hydrogen peroxide, and diacetyl [11].

Bacteria have evolved for 4.3 billion years, and their metabolism and entire biosynthesis have perfectly adapted to their environments. They constantly fight for nutrients and space, trying to inhibit the growth of their competitors which renders them the perfect target for searching novel natural compounds to fight bacteria-associated diseases [12]. Also, in the sustainable development of new antibiotics, microbiota plays an essential role [13].

All these aspects can be discovered by examining the human microbiome of various compartments of the body by extracting the whole-genome DNA of clinical samples while depleting the human DNA. The usage of the extracted DNA for next-generation sequencing (NGS) can then shed light on all microorganisms that are in the native sample. This very precise method can be augmented with microbiological cultivation of the same samples. Which bacteria are cultivatable, and also during routine diagnostics which are only detectable by sequencing the native samples?

Many steps in the process of sample collection, DNA extraction, sequencing, and data analysis can introduce significant bias. One example is the stool collection kits used that already affect the reported microbial compositions [14]. Likewise, in oral microbiomes, bias is known and addressed [15]. The extraction of the whole-genome DNA is a crucial step. It is evident that the topic of comparing different DNA extraction kits is essential and thus has become an evolving field of research. For different specimen types, respective protocols have been compared, *e.g.*, for breast milk [16], stool [17], skin [18], vaginal swabs [19], sputum [20], postmortem eye tissue [21], nasal washes [22], and meconium [23]. As for one specific sample type, the most suitable DNA extraction method has been evaluated over several studies, but an analysis of different DNA extraction kits on their suitability for a variety of sample types has, to our knowledge, not been performed yet. It is interesting, however, to analyze various microbiomes without causing bias due to the use of different extraction protocols, to understand the complexity and connectivity of microbiomes at different body sites in health and disease. Analyses of different biospecimens yield inconsistent results, which renders the selection of the very best protocol challenging. While for studies on single specimen types the best kit for the respective specimen can be selected, multi-microbiome studies potentially suffer from bias if different kits are used.

To understand microbiota in health and disease, multi-metagenomic studies that combine the microbiota from many samples of the same patients are however promising. We thus set out to identify a commercially available DNA extraction kit that is suitable to be used on such diverse biospecimens (**Figure 1**A). Here, we presented the data on the comparative extraction efficiency and sequencing quality obtained by whole-genome sequencing for six types of clinical samples (conjunctival swabs, stool, saliva, interdental plaque, bile, and sputum) after DNA extraction with three commercial kits, including 1) Qiagen DNeasy PowerSoil Pro (QPS; Qiagen, Hilden, Germany), 2) Qiagen QiAamp DNA Microbiome Kit (QMK; Qiagen, Hilden, Germany), and 3) ZymoBIOMICS DNA Miniprep Kit (ZYMO; Zymo Research Corp, Irvine, CA). QMK includes the advantage of host DNA depletion, presumably without causing taxonomic bias, which is a crucial step during DNA extraction for biospecimens such as skin and conjunctival swabs, for which more host material than bacterial mass is expected. This additional processing step might

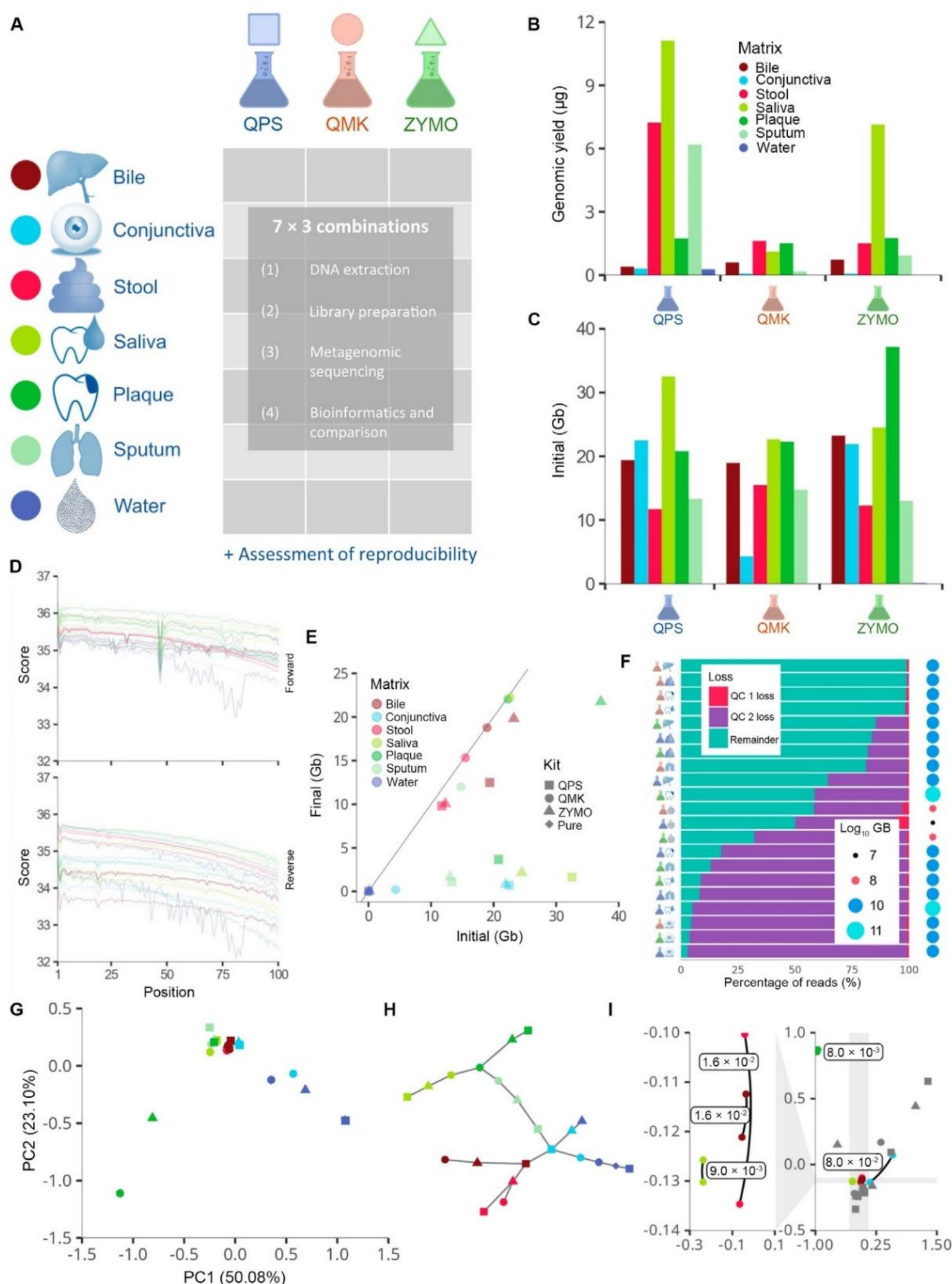


Figure 1 Study setup and QC

A. For six specimen types and water, we performed DNA extraction using three different commercial kits. Following library preparation and sequencing, the metagenomes were evaluated and compared to each other. **B.** The DNA yield of the different specimen types with different kits is given as a bar diagram. **C.** Comparison of the raw sequencing output in Gb before QC. **D.** Q30 values of the raw sequencing reads. Colors indicate the various biospecimens, in line with (A). **E.** Scatter plot of the raw reads to the reads obtained after QC. Shapes represent the different kits, and colors represent the different biospecimens. **F.** Percentage of reads filtered in the different QC steps and remaining dataset size. QC 1 mostly captures loss attributed to read quality, while QC 2 focuses on contamination by host sequences. **G.** Principal component analysis of the different samples and kits using the Mash distances after QC. Shapes represent the different kits, and colors represent the different biospecimens. **H.** Minimum spanning tree of the Mash distances after QC. Shapes represent the different kits, and colors represent the different biospecimens. **I.** Recomputed embedding displaying Mash distances between replicates. Grey points are without replicates. Colors indicate biospecimens. QC, quality control; Gb, gigabase; PC, principal component; QPS, Qiagen DNeasy PowerSoil Pro; QMK, QiAamp DNA Microbiome Kit; ZYMO, ZymoBIOMICS DNA Miniprep Kit.

be a potential explanation for the increased price of QMK in comparison to the two competitor kits tested in this study. In contrast to the novel QMK, we tested ZYMO and QPS that have both been used frequently in regard to microbiome analysis [24–27]. We followed a staged approach. We first performed a total of 108 DNA extractions and then chose the most promising samples for library preparation and sequencing. After evaluation of the sequencing data, we performed replicates for the best DNA extraction kit to analyze the reproducibility.

Results

DNA yield and sequencing quality vary between extraction kits and specimen types

As a first aspect, we compared the DNA yield and sequencing output for the different sample types and DNA extraction kits. The results showed that the DNA amount and concentration varied substantially between the different setups (Figure 1B, Figure S1F). It is known and expected that the different sample types — each with a different human background — lead to varying results in terms of reads and read quality. In line with the yield of DNA, the number of raw reads from the sequencing was likewise diverse (Figure 1C). Here again, the DNA extraction kits had a limited influence as compared to specimen types. However, the read quality in terms of Q30 value matched well, independent of the specimen type and DNA extraction kit, indicating that from all combinations interpretable microbiomes can be extracted (Figure 1D). The number of reads prior to and following quality control filtering generally correlated well for the different kits and sample types. Again, the fraction varied with the different specimen types depending on the expected human background, *e.g.*, introduced by human immune cells and human epithelial cells in saliva and conjunctiva, respectively (Figure 1E). This fact became more evident when considering the lost read fraction in the quality control steps. Again, independent of the kit, the conjunctival swab samples yielded only a fraction of 5% of all reads after quality control, dominated by the mapping of reads to the human genome (Figure 1F). Focusing on the fractions remaining after quality control, the QMK kit retrieved the highest amount of metagenomic information for each specimen type. However, the quantitative aspects were not the only criteria relevant for the selection of a kit, but also the composition of contents. Accordingly, we computed a 2-dimensional embedding using multidimensional scaling based on the Mash distances between samples (Figure 1G). Both, the embedding and the minimum spanning tree of the samples based on the mash distance, confirmed the general considerations: the kit has a limited influence on the output as compared to the difference introduced by the specimen types (Figure 1H). To provide further evidence for this behavior, we carried out technical replicates for five different QMK samples, demonstrating a high reproducibility of metagenomic measurements (Figure 1I).

In the light of the results in this section we might conclude that the variability introduced by the kits is so limited as compared to the difference between sample types and that for each specimen the very best kit might be selected even when multi-microbiome studies are performed. The high-level results, however, also call for a higher resolution analysis of the substantial metagenomic datasets.

Metagenomes vary strongly between different DNA extraction kits, yet stronger between different specimen types

First, we computed the bacterial phyla and families contained in the different samples to get an overview of the taxonomic profile (**Figure 2**A, Figure S1A). Overall, the large quantitative differences in data yield reflect abundance counts. Again, strong differences in relative composition were present for the specimen types. A more detailed consideration revealed a low relative amount of Proteobacteria for several ZYMO- and QPS-extracted samples compared to those extraced by QMK. Especially, the relative portion of Firmicutes decreased in the QMK-extracted samples as compared to those extraced by the other two kits. Mostly Proteobacteria measurements profited from this shift, which was most pronounced in saliva.

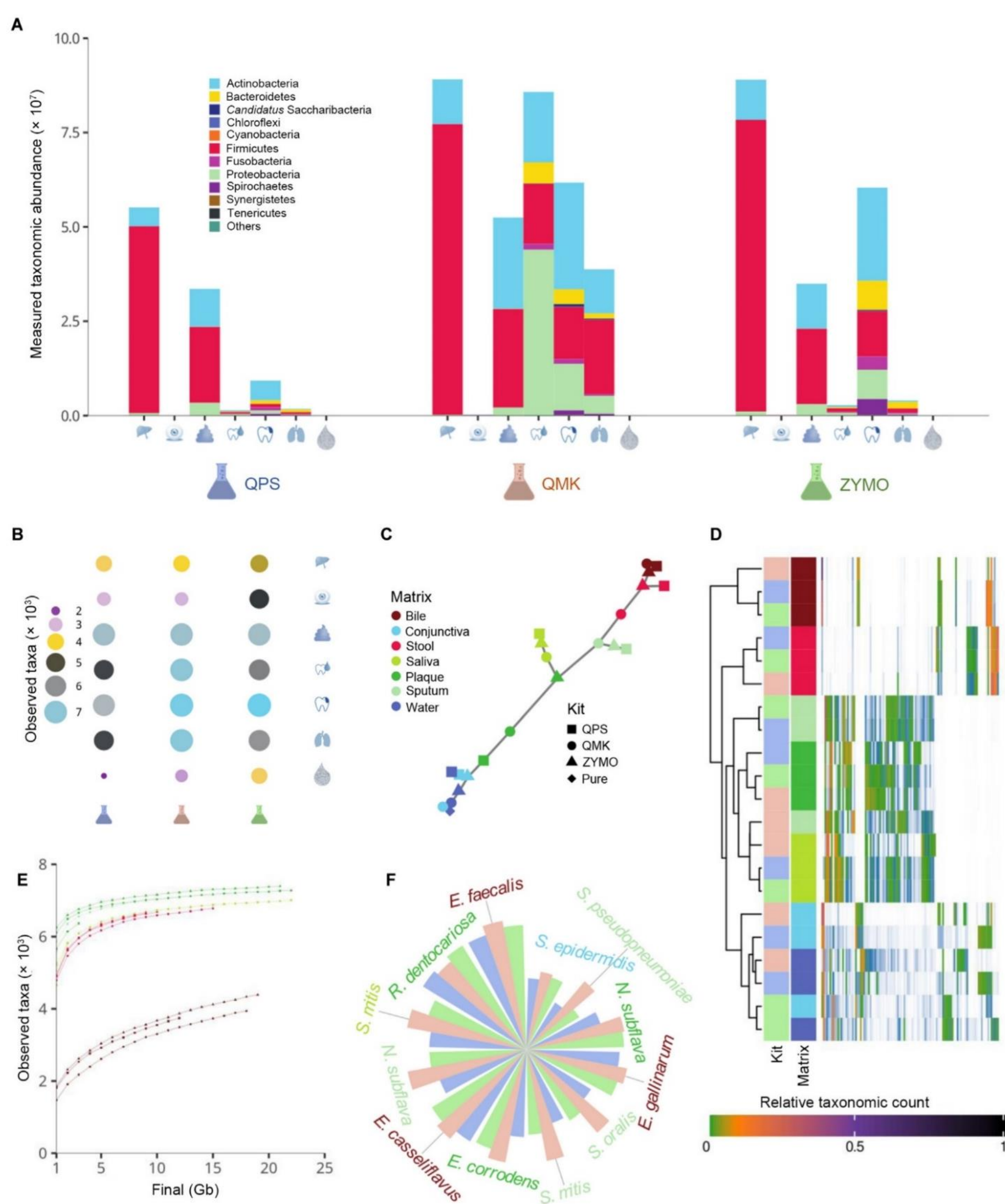
The alpha diversity crucially depends on the DNA extraction kit and the sequencing depth

Quantitative differences do not necessarily translate to qualitative differences. Accordingly, we investigated the alpha diversity of the various samples. Again, specimen types dominated the overall signal. For bile, conjunctiva, plaque, and water specimens, the ZYMO kit measured the highest number of differing taxonomies (Figure 2B). In case data analysis accounts for signals found in negative controls, the alpha diversity measured in ZYMO began to fall in line with the other two kits (Figure S1B). Despite high fluctuations in alpha diversity and total abundance across kits, bacterial species information recaptured most of the structure previously identified from read information alone, confirming the quality of the taxonomic profiling analysis (Figure 2C, Figure S1C). Investigation of the beta diversity based on dimensionality reduction showed a tendency to group QMK-extracted saliva and sputum samples with plaque samples (Figure S1E). Hereby, the comparably low bacterial abundances of the other two methods may act as a confounding factor. Consistent with the previous minimum spanning trees, we observed a clustering of specimen types into three major categories: 1) the close to sterile water and conjunctiva, 2) the digestive system-focused bile and stool, and 3) the oral cavity specimens saliva and plaque where sputum integrates. Looking closer into the clustering, it is clearly visible that a minority of species contributes a majority of the signal (Figure 2D, Figure S1D). Nevertheless, often rare taxonomies of minimal statistical weight may also be of interest for the analysis due to the potential harboring of *e.g.*, virulence factors. Therefore, to analyze the feasibility of finding rare species in the various environments, we further investigated the number of identified taxonomies changing with sampling depth by doing *in-silico* downsampling (Figure 2E). Hereby, we noted that the kits seemed to converge at a similar rate to their asymptotic behavior. This point of convergence was reliably achieved at around 10 Gb after quality control. The maximal number of taxonomies seemed to differ mostly by specimen types, yet minor differences were also visible for kits, which is consistent with the previous finding. Mass spectrometry (MS)-based identification of 42 colonies indicated for 12 significant results that QMK generated highest

ounts for all but one confirmed species (Figure 2F). We noted ıat two species were not detected in our genomic data analysis t all, but were found during MS, which were *Veillonella rogoæe* and *Capnocytophaga granulosa* in saliva and sputum, ɛspectively.

The composition of microbiota considerably varies between DNA extraction kits

The taxonomic composition of a microbiome is often the key aspect to reveal during a metagenomic experiment. Hereby,



the selected DNA extraction kit may play a crucial role on the qualitative findings of an experiment. While the previously discussed alpha diversity described the general number of different taxonomies captured by an experiment, it failed to discuss the exact nature of these differences. Therefore, we looked at the overlapping sets of detected species across both specimen types and kits (**Figure 3**A). Hereby, we selected a raw abundance count threshold to decide about the presence of a species instead of selecting by relative abundance, to also consider rare species in the analysis whose relative counts may undercut relative thresholds. We first discussed the common species of the individual specimen types. The largest intersecting set is usually the set encompassing all three kits. Only for sputum and water, the consensus was the largest for ZYMO and QMK. For the majority of time, ZYMO built the largest intersections, likely due to frequently constituting the largest stand-alone set. Next, we glanced at potential species that were found independently of input samples for the different kits. Here, the largest intersections were the ones with the largest initial sets. Due to higher measured bacterial abundances, QMK proposed four larger sets including sputum and saliva, whereas ZYMO and QPS only proposed stool and plaque as larger sets. Ignoring the underlying specimen types and aggregating the analysis, ZYMO and QMK had the largest number of species they detected in any specimen type.

Since taxonomic profiling is often limited by the quality and amount of reference organisms available, we further investigated ways to discuss potential differences in taxonomic composition between experiments that remain uncaptured by reference-based analysis. Hereby, we fell back on the core algorithm of BusyBee [28]. Accordingly, for reference-free analysis a Uniform Manifold Approximation and Projection (UMAP) embedding of normalized *k*-mer counts was computed on assembled scaffolds (Figure 3B). Visually, the embedding confirmed several findings of the previous taxonomic profiling. The overall density of the embeddings falls in line with the findings of the alpha diversities. Overall, it appears that ZYMO generates the highest density regions and is spreading all over the two-dimensional plane. While the embedding computed on QPS samples also scatters, there are fewer high-density regions. Last, QMK produces well defined regions of higher density. Moreover, the two clusters found in ZYMO water can be seen in all other samples except for the QPS water and QMK water. However, the left cluster also seems to disappear in QPS conjunctiva.

Assembly quality depends on the specimen types and the DNA extraction kits

For the previous reference-free analysis, assembly quality was comparably of minor importance due to the decomposition of assembled sequences into short *k*-mers. Yet, depending on further downstream analysis, the quality of metagenomic assembly may play a crucial role. Accordingly, we compared several assembly quality measures across kits and specimen types (**Figure 4**A). Considering length distribution, specimen types were mostly clustered together. However, for the three specimen types of saliva, plaque, and sputum, minor differences were visible with respect to kits, favoring QMK in N50 and N75 measures. Considering the proportion of scaffolds at changing length, QMK was the only kit where no specimen started to dominate after a given length. Last, ZYMO generated the longest assemblies in water and dominated L50 and L75 for water and conjunctiva.

Taxonomic profiles are consistent across sequencing technologies

With the rising popularity of nanopore sequencing technology and the immense advantages it brings to metagenomics, in terms of assembly quality increase and interpretability, hybrid protocols combining shotgun and nanopore sequencing are continuously gaining in relevance. Correspondingly, the demands to kits supporting both protocols are favored. Since we previously demonstrated clustering behavior into three major clusters, we selected saliva and bile as representatives of the non-sterile specimen types and sequenced the same samples again with nanopore sequencing. Similarly, we removed the ZYMO kit for the experiment, due to our previous findings of a high number of false positives shown across several of our herein presented analyses. Quality control of nanopore reads of all four samples after filtering suggested minor differences between specimen types for both kits (Figure 4B). Pearson correlation between length and PHRED scores was around a low 0.1. Considering both, read length and average read quality scores, Wilcoxon rank sum tests across all reads indicated statistically significant differences between kits, conditioned on the specimen types for each sample ($P < 1 \times 10^{-12}$). After quality control, taxonomic profiling was performed to gauge the effects of interactions among kits, specimen types, and

Figure 2 Diversity of microbiota

A. Bar plot presenting the composition of bacterial microbiota with respect to different phyla. The color codes represent the phyla. Specimen types corresponding to each DNA extraction kit are in the following order: bile, conjunctival swab, stool, saliva, plaque, sputum, and water (left to right). **B.** For the seven biosamples, the observed unfiltered alpha diversity is presented. The color and bubble size correspond to the alpha diversity. Large and blue bubbles match samples with highest alpha diversity, and the small and purple bubbles match samples with lowest alpha diversity. **C.** Minimum spanning tree on the bacterial species level. Jaccard distance served as distance measure. Shapes represent the different kits, and colors represent the different biospecimens. **D.** Heatmap representing the abundance of different species clustered with respect to the sample type. Only species with a relative abundance above 1% were considered. Colors used to represent different kits are green for ZYMO, blue for QPS, and pink for QMK, in line with (A). Each biospecimen (matrix) is represented by a different color consistent with (C). Relative taxonomic counts are depicted in green for 0, black for 1, and shades of green, orange, and violet with increasing relative taxonomic counts between 0 and 1. **E.** Fraction of observed taxa with respect to the sequencing depth computed on sub-sampled decontaminated reads. Shapes represent the different kits, and colors represent the different biospecimens. **F.** Barplot displaying unnormalized counts in the NGS experiment for the different taxonomies that were detected by mass spectrometry. Font colors indicate specimen types, in line with (C). Bar colors represent the different kits, matching (A). NGS, next-generation sequencing.

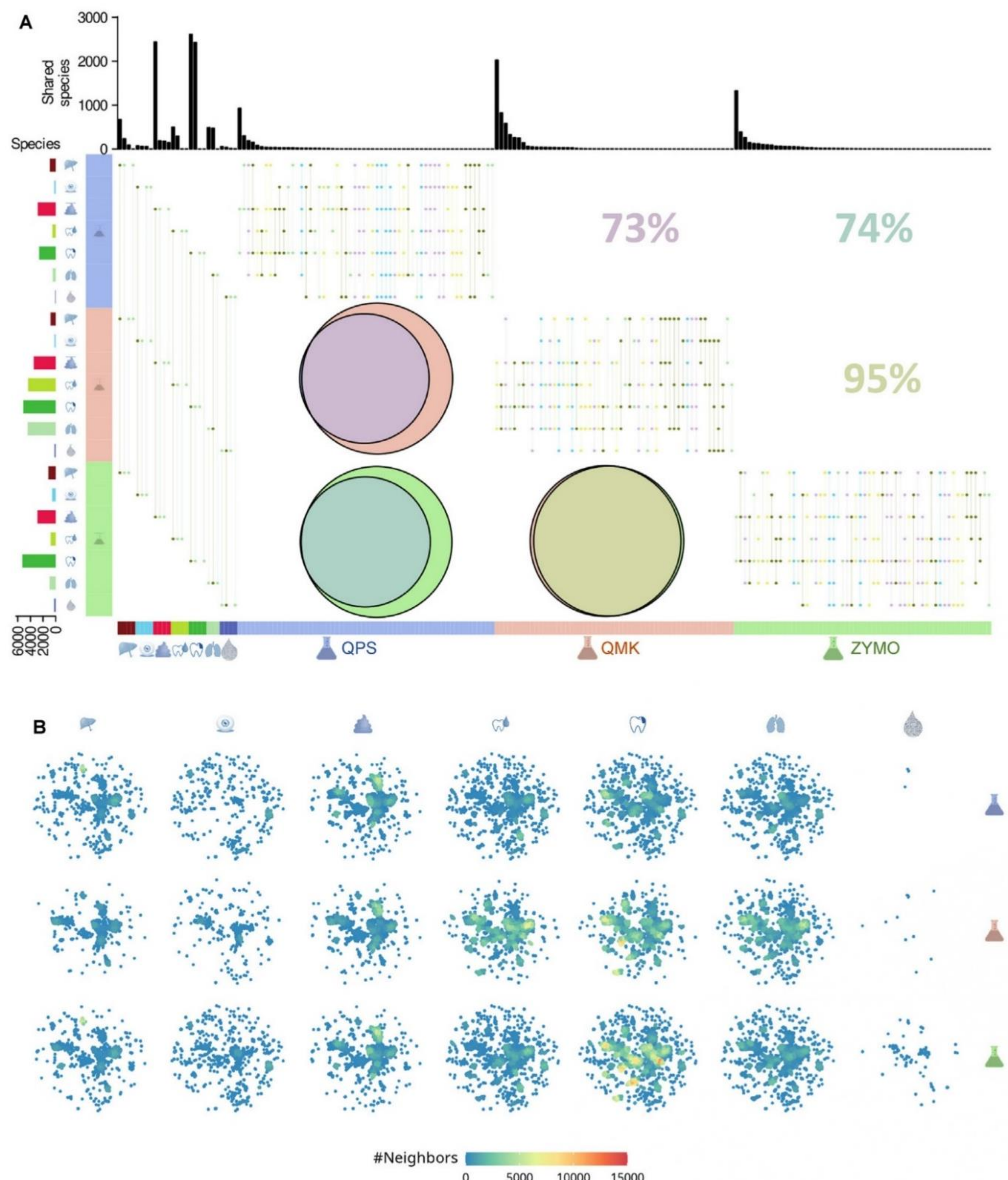


Figure 3 Similarity of microbiota

A. Combination of ten upset plots each discussing identified species overlap by kit or specimen type. Bottom annotation indicates the aspect the upset plot focuses on, *i.e.*, which kit or specimen type is kept constant. Area proportional Euler diagrams below the diagonal capture the proportion of species identified independently of specimen type. Percentages above the diagonal indicate the overlap numerically. A species is considered identified after surpassing a low count threshold of 20 occurrences. **B.** Embedded microbiota. Each spot represents one scaffold with a length above 3 kb after an embedding of the *k*-mer spectra using UMAP. Colors are indicative of the point density in the respective area. UMAP, Uniform Manifold Approximation and Projection.

sequencing technologies (Figure 4C). As expected, the number of different identified bacterial species and overall abundance were a lot higher for shotgun sequencing due to the generation of false positives during profiling with short reads and the increased sequencing depth. Overall, the ordering of experiments based on uniquely quantified bacterial species remains

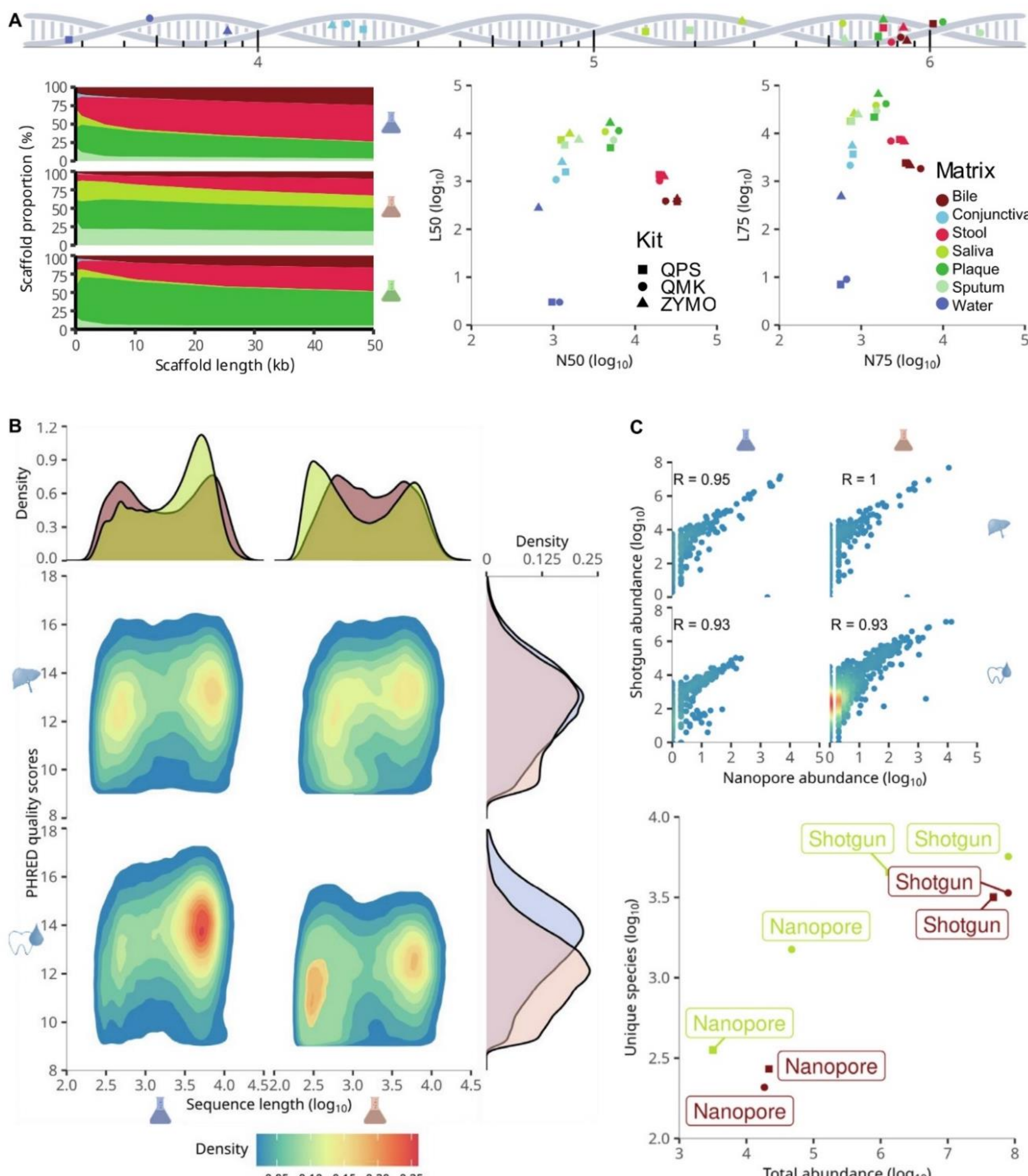


Figure 4 Assembly and nanopore comparison

A. Assembly quality. One-dimensional line showing the length of the longest scaffold for each assembly. Relative scaffold length distribution by kits together with their N50, N75, L50, and L75 values. **B.** Nanopore QC. Average PHRED scores indicate basecalling quality per read. Sequence length indicates length after basecalling of each read. Density plots on the right and top discuss the conditioned distributions for the different kits and specimen types. Visualized data are representative for data after filtering. **C.** Correlation plot indicating coherence between nanopore and shotgun sequencing taxonomic counts on bacterial species level without thresholding. Numerical values represent the rounded Pearson correlation before log scaling. The total number of measurements and unique different species for the different experiments are shown at the bottom.

unchanged across sequencing technologies. The only difference was that in bile, where QMK detected fewer unique species than QPS for the nanopore technology which may be linked to the difference in sampling depth and correlated total abundance. Glancing at the taxonomic profile on a bacterial species level, we observed strong correlations between nanopore and shotgun sequencing, for all tested kit and specimen combinations.

Discussion

We evaluated three DNA extraction kits across six different specimen types and water to gauge their suitability for metagenomic experiments. We note that the QMK kit usually yields the highest amount of metagenomic information after host DNA removal. The depletion of human DNA is a significant advantage of QMK compared to ZYMO and QPS. This is consistent with the idea, to lyse all eukaryotic cells in a first step, followed by the degradation of eukaryotic DNA. Therefore, human DNA in particular is depleted during the first part of the DNA extraction with the QMK kit. During the second part, bacterial cells are lysed and the extracted DNA is purified.

Focusing on both, read information and metagenomic data analysis, we showed that the selection of the specimen type dominated the selection of the kit in signal strength. While for the difference between *e.g.*, water and stool, this result was to be expected, the same did not hold true for plaque and saliva samples. Further, we demonstrated the sensitivity of all kits by confirming a selection of taxa using MS. Considering specificity, we demonstrated, using a reference-based and reference-free method, that ZYMO appeared to contain most contamination, going hand in hand with the fact of ZYMO generating the samples with the highest relative amount of human contamination. This effect could be due to unsterile lysis tubes or columns for DNA extraction. However, we noted that no sample remained uncontaminated. The lowest contamination was shown for QPS. Especially in comparison to the QMK water sample, a lower contamination of the QPS sample can be explained by a general lower variety in identified bacteria species. Partly, bacteria in the environment, that contaminate the water samples might be harder to lyse, which the QPS kit might not have to offer. In contrast to the ZYMO and QPS kits, a pre-contamination of columns provided by the QMK kit is highly unlikely, as the special Qiagen ultraclean columns were stored at 4 °C until being used for DNA extraction. Shifting focus away from taxonomic profiling onto assemblies, except for the ZYMO water sample, assemblies were of similar quality at first sight. Here we acknowledge that the scaffold length distribution is not the be-all and end-all of metagenomic assembly quality assessment; however, it is one of the more widely spread [29]. Last, for QPS and QMK we observed that overall, the results after metagenomic analysis are consistent across shotgun and nanopore sequencing. We note that our study is limited by the small sample size and the focus on bacterial microorganisms. Random sampling error may distort our findings. Thus, larger studies, including more replicates, are needed to confirm our results, and similar comparative studies should ideally also assess results for other pathogen classes, such as viruses or parasites [30].

To conclude, we recommend the QMK kit for samples with high eukaryotic host contamination, as it clearly has the least information loss upon host sequence removal. Moreover, if no detection threshold is set, QMK identifies generally more species than QPS, while not showing a strong contamination of sequencing results in sterile water as compared to ZYMO. In case host contamination is not an issue to consider, QPS may be recommended, since it shows the least overall contamination in sterile water.

Materials and methods

Sample collection

In brief, stool samples were collected by each participant using a paper toilet-hat and a sterile collection tube with an integrated spoon. Approximately 500 mg to 1 g of stool were collected. Plaque samples were collected using 12 disposable micro applicators (Catalog No. MSF400, Microbrush International, Grafton, WI). Three interdental spaces per quadrant were brushed, and all micro applicators were placed into a single ESwab transport tube (Copan Diagnostics, Brescia, Italy), including the ESwab Amies Medium (Copan Diagnostics). Saliva samples were collected using 50-ml sterile, conic falcon tubes. Participants were asked to release uninduced saliva into the sterile falcon tube for 5 min. Conjunctiva samples were obtained using a ESwab. The lower eyelid was everted, and the conjunctiva was swabbed throughout the entire length of the lower fornix three times. Afterwards, the swab was placed in the respective transport medium and the tube was frozen at −80 °C. Sputum was induced by 7 min of inhalation with 0.9% NaCl solution. After inhalation, the participant was asked to release sputum by coughing into a sterile collection tube. Bile samples were collected during a duodenoscopy by drawing 5 ml to 10 ml into a sterile syringe.

DNA extraction

DNA was extracted from all samples using three different, commercially available DNA extraction kits: QPS, QMK, and ZYMO. For each kit, the DNA was extracted according to the manufacturer's protocol. Briefly, 1 ml of sterile Milli-Q water was used for the negative control. The manufacturer's protocol was followed, respectively. Fecal samples were weighed, and 250 mg of stool were used for DNA extraction with QPS and QMK, and 50 mg of stool were used for ZYMO, according to the manufacturer's recommendation. For QPS and ZYMO, 1.5 ml of saliva samples were centrifuged for 5 min at 6000 *g* and the pellet was resuspended in the respective lysis buffer. For QMK, 1 ml of saliva was used directly. Interdental microbrushes and conjunctival swabs were vortexed rigorously in the eSwab Amies Medium for 3 min. The Amies Medium was then transferred to a 1.5-ml sterile Eppendorf tube and centrifuged for 5 min at 6000 *g* for further DNA extraction with QPS and ZYMO. The pellet was resuspended in the respective lysis buffer. For DNA extraction with QMK, the liquid Amies Medium was used directly. For DNA extraction with QPS and ZYMO, bile samples were vortexed rigorously and 2 ml of bile were transferred to a 2-ml sterile Eppendorf tube and centrifuged for 5 min at 6000 *g*. The supernatant was discarded, and the pellet was resuspended in the respective lysis buffer. To extract DNA from bile via QMK, 1 ml of bile was used directly. Sputum was mixed with Remel Sputasol (Oxoid L TD, Hants, England) in a 1:1 ratio. For QPS and ZYMO, 1.5 ml of sputum or sputasol was centrifuged for 5 min at 6000 *g* and the pellet was resuspended in the respective lysis buffer. For QMK, 1 ml of sample was used for DNA extraction without previous centrifuging. The mechanical lysis of bacterial cells was performed using the MP Biomedicals FastPrep-24 5G Instrument (FisherScientific

GmbH, Schwerte, Germany). For ZYMO, the velocity and duration were adjusted to 6 m/s for 45 s three times with 30 s of storage on ice in between each lysis step. For elution of DNA during the last step of each DNA extraction kit, the following elution volumes were used: 1) QPS: 40 µl; 2) ZYMO: 20 µl; 3) QMK: 50 µl. The DNA concentration was determined via NanoDrop 2000/2000c (ThermoFisher Scientific, Wilmington, DE) full-spectrum microvolume UV–Vis measurements. For each sample type and each DNA extraction method tested, we used a total of one biological replicate for sequencing. However, DNA was isolated from a total of n = 10 biological replicates for saliva, interdental plaque, and stool, a total of n = 4 for bile, a total of n = 8 for sputum samples, and a total of n = 4 for conjunctival swabs. From all samples that we extracted DNA from, we selected the most promising samples for library preparation and sequencing. We chose those samples with the highest amount of DNA, least impurities, and least fragmentations. For all samples prepared with QMK we performed an n = 2 technical replicates for library preparation and sequencing.

Library preparation

DNA libraries were prepared using the MGIEasy Universal DNA Library Prep Set (MGI Technologies, Shenzhen, China) according to the manufacturer's recommendations. In general, 200 ng DNA was sheared into fragments using the M220 Focused-ultrasonicator (Covaris, Woburn, MA), followed by size selection using Agencourt AMPure XP beads (Beckman Coulter, Brea, CA). For low-biomass samples, such as the conjunctival swab and the sterile water control, the entire amount of isolated DNA was used as an input for the fragmentation procedure. The fragmented DNA was used for end-repairing and A-tailing. Next, adaptors containing specific barcodes were ligated to the 3′ and 5′ ends, and the ligation products were amplified by PCR. The concentration of the PCR products was measured using Qubit 1× dsDNA HS Assay Kit (ThermoFisher Scientific, Waltham, MA). In the following, 8 different barcoded samples were pooled in equal amount and circularized to generate the single-stranded DNA library. The concentration of the library was measured using Qubit ssDNA Assay Kit (ThermoFisher Scientific, Waltham, MA). Additionally to the different biospecimen samples, a sterile DNase/RNase-free water sample was prepared using the same procedure as for all samples.

NGS

For the short-read sequencing, all libraries were sent to BGI Group for DNA nanoball (DNB) generation and paired-end sequencing (PE100) on the DNBSEQ-G400 instrument according to manufacturer's instructions and recommendations.

MinION library preparation and sequencing

Upon opening of the flow cell and again immediately prior to sequencing, flow cell pore count was measured using MinKNOW. Library preparation kits, flow cell, and other consumables used for the experiment are described in Table S1. DNA was quantified via Nanodrop 2000/2000c (ThermoFisher Scientific, Wilmington, DE) and the volume was determinated by using a pipette (Table S2). The library preparation was conducted according to the protocol "Native barcoding genomic DNA (with EXP-NBD 104)" provided by Oxford Nanopore Technologies (ONT), with the exception of the barcode ligation step and further the adapter ligation step for which the ligation mix was incubated for 15 min at room temperature instead of 10 min. The amount of initial DNA used for the barcoding kit was above 100 ng for the four specimen types corresponding to the DNA extraction kits shown in this study. In sum, the library consisted of 12 barcoded DNA samples. The barcoded DNA was stored at 4 °C for 3 days until adapter ligation. For barcoded libraries, volume-equal quantities of each sample were used for the final library. The amount of pooled barcoded DNA exceeded the recommended amount of 700 ng DNA by an additional 400 ng to reach a final DNA amount of about 1100 ng for adapter ligation (Table S3). For the last Agencourt Ampure XP bead clean-up step, short fragment buffer (SFB) was used. The completed library was loaded onto a R9.4 flow cell as per instructions given by ONT. Given the rapid advancement of protocols, chemicals, and the technology itself, data were generated with the most up-to-date methods and protocols available from ONT at the time of library preparation and sequencing. The Mk1B MinION device was used for data acquisition.

Nanopore sequencing

MinION analysis was carried out at the Helmholtz Institute for Pharmaceutical Research Saarland (HIPS) at the Department Microbial Natural Products, Saarbrücken, Germany. The barcoded library, consisting of the metagenomic DNA samples, was generated in a S1 laboratory, whereas the sequencing of the samples was performed in the office. Sequencing methods performed simultaneous 1D sequencing of samples using native barcoding. The sequencing run was carried out over a time range of three days. At the time of use, the R9.4 spotON Flow Cell had a pore count exceeding the guaranteed level (> 800 pores) by the manufacturer. Pore count was measured by the MinKNOW software with a result of 808 pores. The majority (> 50%) of sequencing data were generated in the first 9 h of sequencing, corresponding to the time in which the first group of pores is actively sequencing. More than 99% of sequencing data were generated after 28 h of sequencing. The sequencing yield in a total number of estimated bases is displayed in Figure S2.

Culturing of bacteria

All native samples were streaked out on four different agar plates: TSA with 5% sheep blood (TSA), MacConkey (MC), Columbia (Co), and Chocolate Blood (CB) agar plates (ThermoFisher Scientific, Wilmington, DE). All TSA, MC, and CB agar plates were incubated at 35.6 °C and 5% CO_2 for a minimum of 18 h and a maximum of 24 h. Co agar plates were used for the cultivation of anaerobic bacteria and therefore incubated in an anaerobic environment for a minimum of 48 h.

MS-based identification

Bacterial colonies, obtained by culturing on different agar plates, were spotted onto the MALDI-TOF target plate,

followed by overlaying with 1 μl of α-cyano-4-hydroxycinnamic acid (CHCA) matrix solution (Bruker Daltonics), composed of saturated CHCA dissolved in 50% (v/v) of acetonitrile, 2.5% (v/v) of trifluoroacetic acid, and 47.5% (v/v) of LC-MS grade water. After drying at room temperature, the plate was placed into the Microflex LT Mass Spectrometer (Bruker Daltonics) for MALDI-TOF MS. Measurements were performed using the AutoXecute algorithm in the FlexControl software (v3.4; Bruker Daltonics). For each spot, 240 laser shots in six random positions were carried out automatically to generate protein mass profiles in linear positive ion mode with a laser frequency of 60 Hz, a high voltage of 20 kV, and a pulsed ion extraction of 180 ns. Mass charge ratio range (m/z) was measured between 2 kDa and 20 kDa. Bacterial species were identified by using the MALDI BioTyper software. Identification scores above 2.0 were considered a precise identification, scores between 1.7 and 1.99 were considered as possible species identification, and all identification scores below 1.7 were considered unsuccessful identification.

Data analysis

First, quality control was performed with MultiQC (v1.9) [31] and fastp (v0.20.1) [32]. Next, NGS data were decontaminated of host sequences using kneaddata (v0.7.4). Decontaminated data were uploaded to the Sequence Read Archive (SRA) [33]. We counted the exact number of basepairs contained in the fasta files before the individual steps to get a detailed overview on the overall information content. Once the data were fully cleaned, Mash distances were computed on all remaining read information with Mash (v2.3) [34]. Taxonomic profiling was done with Kraken (v2.1.2) [35]. Optional downsampling of reads was performed with seqtk (v1.3). The PlusPF database release from 9/19/2020 was used as Kraken2 index. As an alpha diversity measure, we used either the observed number of different taxa or the Shannon index. As the beta diversity measure, the Jaccard index was computed. For clustering analysis, species with relative species abundance below 1% in all samples were removed. Samples were then clustered using Ward's hierarchical agglomerative clustering in combination with the Euclidian distance measure. UMAP embeddings were computed on all scaffolds having a length over 3 kb. To this end, 5-mers of each scaffold were counted and assembled into a vector. Each vector was divided by its sum, scaled, and centered. The normalized counts were then passed to embedded using UMAP. Assemblies were computed with SPAdes (v3.15.2) using the --meta flag [36]. Scaffold quality assessment was made with MetaQUAST (v5.0.2) [37], enabling the splitting of scaffolds. Downstream analysis heavily relied on phyloseq (v1.36.0). Nanopore reads were basecalled with guppy (v5.0.7) [38] before undergoing taxonomic profiling.

Ethical statement

All samples were collected at the Saarland University Medical Center, Germany, after having obtained written informed consent from all participants. The study was approved by the local ethics committee (Ärztekammer des Saarlandes) under reference 131/20.

Data availability

Respecting the German Bundesdatenschutzgesetz, we uploaded the data after human read removal to the SRA of National Center for Biotechnology Information (NCBI). Preprocessed data can be found in SRA of NCBI (SRA: PRJNA802336), and are publicly accessible at https://www.ncbi.nlm.nih.gov/sra.

CRediT author statement

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Competing interests

Georges Pierre Schmartz, Matthias Hannig, Stefan Rupf, Andreas Keller, and Rolf Müller are shareholders of MOOH GmbH.

Acknowledgments

This work was supported by the UdS-HIPS-TANDEM Initiative.

Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gpb.2022.05.006.

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4.2 Results No. II

The Effect of a Planetary Health Diet on the Human Gut Microbiome: A Descriptive Analysis

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Rehner J, Schmartz GP, Kramer T, Keller V, Keller A, Becker SL. The Effect of a Planetary Health Diet on the Human Gut Microbiome: A Descriptive Analysis. *Nutrients*. 2023 Apr 16;15(8):1924. doi: 10.3390/nu15081924. PMID: 37111144; PMCID: PMC10144214.

Article

The Effect of a Planetary Health Diet on the Human Gut Microbiome: A Descriptive Analysis

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Abstract: In 2019, researchers from the EAT-*Lancet* Commission developed the 'Planetary Health (PH) diet'. Specifically, they provided recommendations pertaining to healthy diets derived from sustainable food systems. Thus far, it has not been analysed how such a diet affects the human intestinal microbiome, which is important for health and disease development. Here, we present longitudinal genome-wide metagenomic sequencing and mass spectrometry data on the gut microbiome of healthy volunteers adhering to the PH diet, as opposed to vegetarian or vegan (VV) and omnivorous (OV) diets. We obtained basic epidemiological information from 41 healthy volunteers and collected stool samples at inclusion and after 2, 4, and 12 weeks. Individuals opting to follow the PH diet received detailed instructions and recipes, whereas individuals in the control groups followed their habitual dietary pattern. Whole-genome DNA was extracted from stool specimens and subjected to shotgun metagenomic sequencing (~3 GB per patient). Conventional bacterial stool cultures were performed in parallel and bacterial species were identified with matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. We analysed samples from 16 PH, 16 OV, and 9 VV diet patterns. The α-diversity remained relatively stable for all dietary groups. In the PH group, we observed a constant increase from 3.79% at inclusion to 4.9% after 12 weeks in relative abundance of *Bifidobacterium adolescentis*. Differential PH abundance analysis highlighted a non-significant increase in possible probiotics such as *Paraprevotella xylaniphila* and *Bacteroides clarus*. The highest abundance of these bacteria was observed in the VV group. Dietary modifications are associated with rapid alterations to the human gut microbiome, and the PH diet led to a slight increase in probiotic-associated bacteria at ≥4 weeks. Additional research is required to confirm these findings.

Keywords: microbiome; Planetary Health; metagenomics; diet; dietary fiber

Citation: Rehner, J.; Schmartz, G.P.; Kramer, T.; Keller, V.; Keller, A.; Becker, S.L. The Effect of a Planetary Health Diet on the Human Gut Microbiome: A Descriptive Analysis. *Nutrients* **2023**, *15*, 1924. https://doi.org/10.3390/nu15081924

Academic Editor: Marloes Dekker Nitert

Received: 13 March 2023
Revised: 7 April 2023
Accepted: 13 April 2023
Published: 16 April 2023

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1. Introduction

In 2019, the EAT-*Lancet* Commission developed the so-called 'Planetary Health diet' (PH), a diet concept framework, which could provide a healthy diet for up to 10 billion people in 2050 within the planetary boundaries from sustainably sourced food, thereby reducing the worldwide number of deaths associated with a poor diet. The main focus of this diet consists of a reduction in animal products and processed food consumption and an increase in dietary fibre uptake through plant-based products [1,2].

Dietary fibre is a non-digestible carbohydrate for humans, but a main nutrient source for bacteria, which reside in the human intestine. The human gut microbiome describes all such microorganisms and their genomic information, including bacteria, viruses, fungi, and archaea, which are located in several niches in the gastrointestinal tract [3]. Its impact on health homeostasis and risk-modulating role in developing a variety of chronic, especially

inflammatory, diseases, as well as disease progression, have been evaluated in a recent study [4]. The bacterial composition within the human gut can be altered, especially in the first three years of life, but also later on during adulthood. Major microbiome-influencing factors include the mode of delivery (i.e., natural passage through the birth canal or caesarean section), early life nutrition, as well as stress and diet choices during adulthood [5,6]. Focusing on diet and its consequences for bacteria-derived metabolites produced in the gastrointestinal tract, dietary fibre has been shown to be one of the main modulating nutrients [7].

Commensal members of the gut microbiota ferment these poly- and oligosaccharides, thereby producing short-chain fatty acids (SCFAs) such as acetate, propionate, and butyrate. SCFAs have been shown to influence glucose and lipid metabolism and regulate immunity, inflammation, and blood pressure [8]. Furthermore, the presence of SCFA-producing bacteria, and thus also the presence of SCFA detected in faeces, has been correlated with a protection against allergic reactions in the respiratory tract, suggesting their important role in shaping the immune system [9]. Hence, SCFAs are key elements in health homeostasis [10–12]. Dietary fibre uptake has further been correlated with a greater gut microbiota diversity and, if compared with the Western diet, less occurrence of chronic inflammatory disease through SCFA-producing gut microbiota [13,14]. Therefore, an increase in dietary fibre intake, as suggested by the PH diet, can lead to an increase in microbial-derived SCFAs, which have a positive and protective effect on overall health.

The Mediterranean Diet, which focusses on an increase in dietary fibre uptake through plant-based foods and, similar to the PH diet, a reduction in processed foods and saturated fatty acids, moderate consumption of fish, poultry, and dairy products, as well as low consumption of red meat, was shown to positively influence the human gut microbiome and overall health status. The consumption of animal-derived foods is clearly reduced in the Mediterranean Diet when compared with the Western diet. However, the PH diet concept suggests to reduce the intake of meat and dairy even further. After following the Mediterranean Diet, an increase in microbiota diversity and microbiota-derived metabolites, in particular SCFAs, has previously been reported [15].

Another diet concept that is gaining more popularity is the plant-based diet. This diet emphasizes the consumption of plant-derived foods, such as fruits, whole grains, nuts, seeds, and vegetables, whereas animal products are minimised or strictly eliminated [16]. Similar to the Mediterranean Diet, following a plant-based diet has been shown to increase microbial diversity in the human intestine and positively affect the abundance of beneficial bacteria, such as *Prevotella* sp. [17]. Moreover, plant-based diets have been associated with reduced inflammation, lower risk of cardiovascular diseases, and improved glucose metabolism [18].

Another popular approach to maintain overall health, as well as weight management, is a low-fat diet. These diets usually focus on reducing the intake of fat to a maximum of 30% of total energy intake, while on the other hand increasing the consumption of other macronutrients, such as protein, carbohydrates, and dietary fibre [19]. Low-fat diets can be a powerful method in weight management; however, they also have been shown to decrease the diversity and abundance of several beneficial bacteria in the gut, such as *Bifidobacterium* sp. As these diet concepts vary greatly in the specific composition of the chosen foods and nutrients, positive changes within the intestinal microbiota composition have also been reported, such as an increase in beneficial *Prevotella* sp., similar to the results after following a Mediterranean Diet [20,21].

The focus of the PH diet consists of an increase in dietary fibre through the consumption of vegetables, fruits, and whole grains, and could thus lead to similar changes within the human gut microbiome as, for example, the Mediterranean Diet or plant-based diet concepts. While the PH diet concept is gaining more and more attention and support from various stakeholders, e.g., pertaining to an improved cognitive function, criticism has been raised about a relative lack of scientific evidence pertaining to its actual health effects [22–24]. To shed light on the controversial discussion about the PH diet concept,

we aimed to analyse the effects of following the PH diet over the course of twelve weeks on overall biodiversity and gut microbiota composition in contrast to the most prevalent omnivorous diet (OV) and the vegan/vegetarian diet (VV). The OV Western diet followed by the participants consisted of a low intake of dietary fibre through fruits, vegetables, and wholegrains. Furthermore, individuals following this diet concept had a very high intake of highly processed foods, dairy products, meat, and refined sugars, forming the opposite of the PH diet concept. Individuals following a vegan diet are characterised by the eradication of any animal-derived products as nutrient sources; however, levels of dietary fibre intake and highly processed foods vary greatly between individuals. The abdication of meat products from an individual's diet concept is the central component of the vegetarian diet, which was included in the VV as well. Yet, similar to individuals following a vegan diet, ranges of dietary fibre uptake and highly processed foods can vary.

2. Materials and Methods

2.1. Study Design

Healthy adults aged $\geq$ 18 years were recruited to the study. Volunteers were invited to participate in the Saarland area, southwest Germany from January to April 2022. Several exclusion criteria were defined to reduce potential bias owing to the relatively small number of study participants, i.e., pregnancy, active smoking, acute and/or chronic disease conditions, and the use of antibiotics within the last 6 months prior to inclusion. We recorded a detailed medical history of each participant, including major factors that affect the microbiome, such as (i) birth condition, (ii) medication during the first three years of life, (iii) exposure to animals within the first three years of life, and (iv) breast milk or formula use. Participants were divided into three groups according to their diet: two control groups, following a VV or OV for at least one year, and the intervention group. Participants belonging to the intervention group changed from an omnivorous diet to the PH diet. Prior to the study, these participants received detailed instructions and recipes according to the guidelines developed by the EAT-*Lancet* commission (document available online at https://www.wwf.de/fileadmin/fm-wwf/Publikationen-PDF/Landwirtschaft/wwf-wochenmenue-besseresser-innen-flexitarisch.pdf, (accessed on 12 April 2023). All participants collected faecal samples in a sterile collection tube at four different time points: initiation of the study and after two, four, and twelve weeks (Figure 1). Samples were then transferred to the laboratory within 24 h and stored at −80 °C until further processing. Furthermore, we asked all participants to document whether they had an excessive alcohol intake during the course of the study, as well as the exact foods they consumed two days prior to the collection of each faecal sample in a printed food diary, in order to reduce any potential bias that might be explained by different food choices shortly before sample collection. Individuals adhering to the PH diet were asked to track any divergence from the foods recommended by the EAT-*Lancet* commission across the entire study duration.

2.2. Ethical Considerations

All faecal samples were collected at the Saarland University Medical Center (Homburg, Germany) after having obtained written informed consent from all participants. For this study, we obtained ethical approval from the regional ethics committee ('Ärztekammer des Saarlandes', reference no.: 116/22).

2.3. DNA Extraction

We extracted whole-genome DNA from all faecal samples using the ZymoBIOMICS DNA Miniprep Kit [25]. DNA was isolated and purified according to the manufacturer's protocol. Briefly, 50 mg of faecal matter was used for the mechanical lysis step of the protocol, according to the manufacturer's recommendation. The respective lysis of microbial cells was performed using the MP Biomedicals™ FastPrep-24™ 5G Instrument (FisherScientific GmbH, Schwerte, Germany). The manufacturer's protocol was adjusted in regards to the used velocity and duration of the mechanical lysis, which was increased to 6 m/s for

45 s three times with 30 s of storage on ice in between each lysis step. Finally, we eluted the DNA in 20 µL of DNase-/RNase-free water. Subsequent concentration determination of the eluted DNA was performed via NanoDrop 2000/2000c (ThermoFisher Scientific, Wilmington, NC, USA) full-spectrum microvolume UV/Vis measurements.

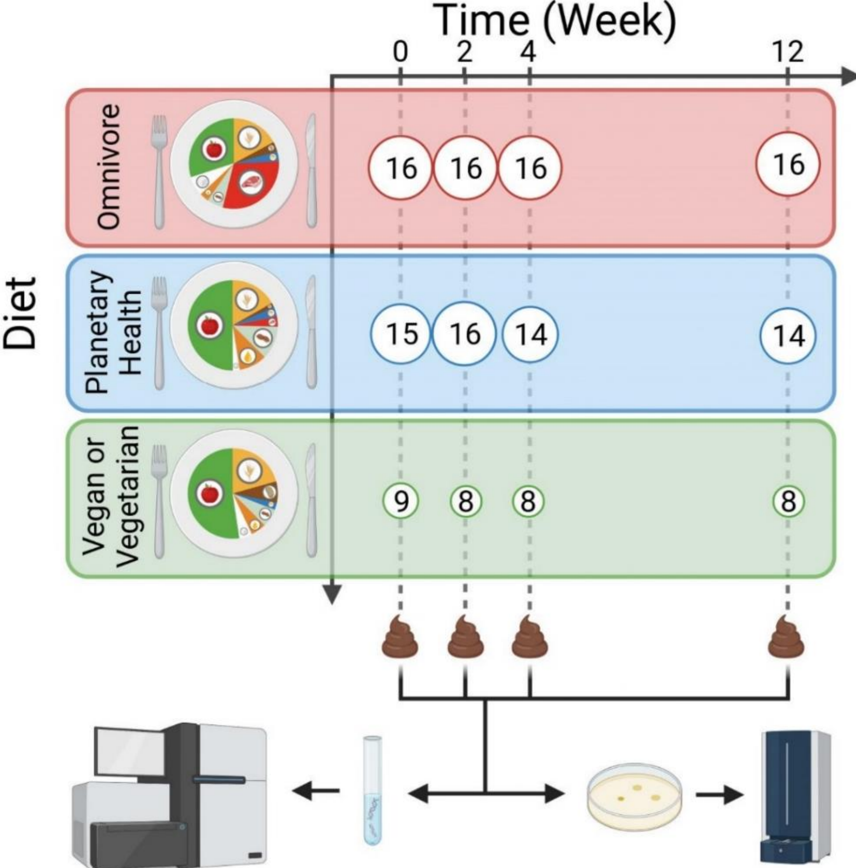

Figure 1. Design of the study. Participants followed three different diets over the course of twelve weeks. Stool was sampled at different time points and whole metagenome sequencing was performed. Additionally, bacteria were cultivated on different agar plates and analysed with MALDI-TOF mass spectrometry. Numbers in white circles depict the numbers of participants and respective stool samples at the different time points for each group.

2.4. Library Preparation and Sequencing

Extracted whole-genome DNA was sent to Novogene Company Limited (Cambridge, UK) for library preparation and sequencing. Briefly, samples were subjected to metagenomic library preparation and further sequenced via paired-end Illumina Sequencing PE150 (HiSeq). For all samples, 3 Gb reads per sample were generated.

2.5. Culturing of Bacteria

Native samples from five randomly selected participants per diet group were homogenised by vortexing after defrosting in order to achieve equal bacterial distribution within the sample without lysing the cells. Then, samples were streaked out on three different agar plates: tryptic soy agar with 5% sheep blood (TSA), MacConkey (MC), and Columbia (Co) agar plates (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). We incubated all TSA and MC agar plates at 35 °C and 5% CO_2 for 18 h to 24 h. Anaerobic bacteria were cultivated on Co agar plates in an anaerobic environment at 35 °C for at least 48 h.

2.6. Mass-Spectrometry-Based Identification

After incubation of native sample material on different agar plates, grown bacterial colonies were identified on the species-level using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS). To this end, we picked colonies and spotted them onto the MALDI-TOF target plate and overlaid them with 1 μL of α-cyano-4-hydroxycinnamic acid (CHCA) matrix solution (Bruker Daltonics), which is composed of saturated CHCA dissolved in 50% (v/v) of acetonitrile, 47.5% (v/v) of LC-MS grade water, and 2.5% (v/v) of trifluoroacetic acid. The overlaid spots were then dried at room temperature and the target was subsequently placed into the Microflex LT Mass Spectrometer (Bruker Daltonics, Billerica, MA, USA) for MALDI-TOF MS analysis. We performed all measurements with the AutoXecute algorithm using FlexControl© software (version 3.4; Bruker Daltonics, Billerica, MA, USA). Each spot was automatically excited with 240 laser shots at six random positions to generate protein mass profiles in linear positive ion mode. The laser frequency was set to 60 Hz, high voltage of 20 kV, and a pulsed ion extraction of 180 ns. We measured mass charge ratio ranges (m/z) from 2 kDa to 20 kDa. The MALDI BioTyper software was used to identify bacterial species based on their protein mass profiles measured. In this study, we only considered identification scores $\geq$ 2.0 for analyses, which represent a precise identification on the species level, while scores between 1.7 and 1.99 were discarded as they are considered as possible species identification, and all identification scores below 1.7 were considered unsuccessful identification.

2.7. Data Analysis

The first step of data analysis comprised human read removal with KneadData (version (v):0.7.4, command line arguments (cla): "–trimmomatic-options='LEADING:3 TRAILING:3 MINLEN:50′ –bowtie2-options='–very-sensitive –no-discordant –reorder'") [26]. Next, we visualised the quality of the remaining reads with fastp (v:0.20.1) and MultiQC (v1.11) on default settings [27,28]. We computed a first taxonomic profile of quality-controlled reads with MetaPhlAn3 (v3.0.13, cla: "-t rel_ab_w_read_stats –unknown_estimation –add_viruses") on the ChocoPhlAn (v:mpa_v30_CHOCOPhlAn_201901) resource [29]. A second taxonomic profile was generated based on sourmash (v4.4.3, cla: "sketch dna -p k = 21, k = 31, k = 51, scaled = 1000, abund –merge") and the prepared Genome Taxonomy Database (v:GTDB R07-RS207 all genomes k51) [30,31]. Sample signatures were computed for k-mer sizes 21, 31, and 51. Distances among samples and database comparison were computed using k-mer signatures of size 31 and 51, respectively. All taxonomic profiles were then pruned and rescaled to remove viral counts.

The results of the individual samples were aggregated, and further downstream analysis was performed in R relying on the phyloseq package (v1.40.0) [31]. β-diversity was computed using the weighted UniFrac distance. Shannon diversity was used as the α-diversity measure and a two-sided unpaired Wilcoxon rank sum test was performed to test significance with a false discovery rate of 0.05. The two-dimensional embedding of sourmash sketches was performed with UMAP (v:0.2.8) [32].

Differential abundance analysis was performed with ALDEex2 (v:1.28.1) and ANCOMBC (v:1.6.2) comparing vegetarians and omnivores [33,34]. MetaPhlAn3 relative taxonomic abundances were scaled by their read count of the sample after quality control for ANCOMBC. A mean species abundance across all time points was computed for each participant, adjusting for library size if absolute counts were considered. Further, for a species to be considered for analysis, it had to be detected in over 10% of samples. Next, abundance analysis was performed, and the results were sorted by absolute effect size. We pruned the list, focusing only on the first ten percent, and intersected the sets derived from the same taxonomic profiles.

3. Results

3.1. Intestinal Microbial Diversity Stays Relatively Stable over Time

Overall, 41 individuals from the same geographic location (Germany) were included: 16 participants following an OV, 9 following a VV, and 16 individuals who changed from an OV pattern to the PH diet at inclusion. Participating individuals were between 19 and 59 years old, with age ranges between all diet groups being non-significantly different (ANOVA p-value $\approx$ 0.84). Sex ratios differed significantly between the three diet groups (Fisher's exact test p-value $\approx$ 0.024), with more females in the VV group (8/9 individuals). General information about age, sex, and body mass index (BMI) is summarised in Table 1.

Table 1. General participant information. Listed below are the age ranges, BMI ranges, and sex ratio for all groups.

	OV	VV	PH
Age ranges	27–56	22–55	19–57
BMI ranges	19.8–32.8	19.9–40.1	20.0–24.4
Male	10	1	4
Female	6	8	12

Over the course of twelve weeks, the average α-diversity remained relatively stable for all diet groups (Figure 2A). Slight increases and decreases for individual participants were detectable between the different time points. On the one hand, investigation of the β-diversity based on dimensionality reduction of species information showed no distinct cluster formation, suggesting that, independent of the diet and time point, samples were all rather similar in their microbial composition (Figure 2B). On the other hand, reference-free diversity analysis based on sequence information alone with sourmash highlighted VV samples to be similar, whereas samples from OV and PH did not form distinct clusters, suggesting similarities between those two groups (Figure 2C) [29].

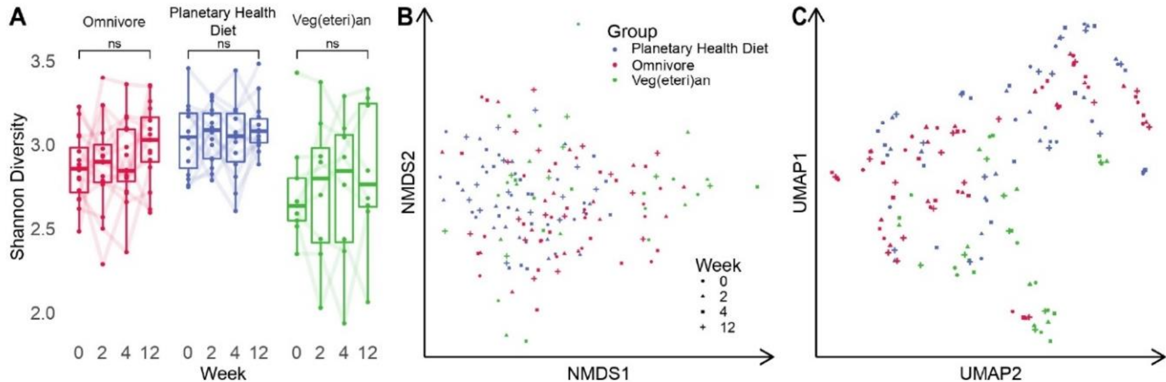

Figure 2. (**A**) α-diversity computed with the Shannon index for all time points and cohorts. Differences between initial and final time points were not significant for any cohort. ns = not significant (**B**) Visualised β-diversity computed with NMDS on weighted UniFrac distances among all sample pairs. (**C**) UMAP computed on sourmash distances computed among all samples.

3.2. Microbiota Composition Is Host-Specific and Varies between Diets

While α-diversity describes the general number of different taxonomies present in a sample and considers the evenness of their respective abundance, taxonomic profiling enables the visualization of the exact nature of these differences. Analysis on the genus level showed variations in the microbiota composition across diets (Figures 3A and S1). In comparison with OV and PH, individuals who followed a VV diet harboured double to triple the relative amount of *Bifidobacterium* spp., *Prevotella* spp., and *Gemmiger* spp. within

their intestine immediately after inclusion. *Prevotella* spp. could be detected in the OV group with a relative abundance of only 1.3%.

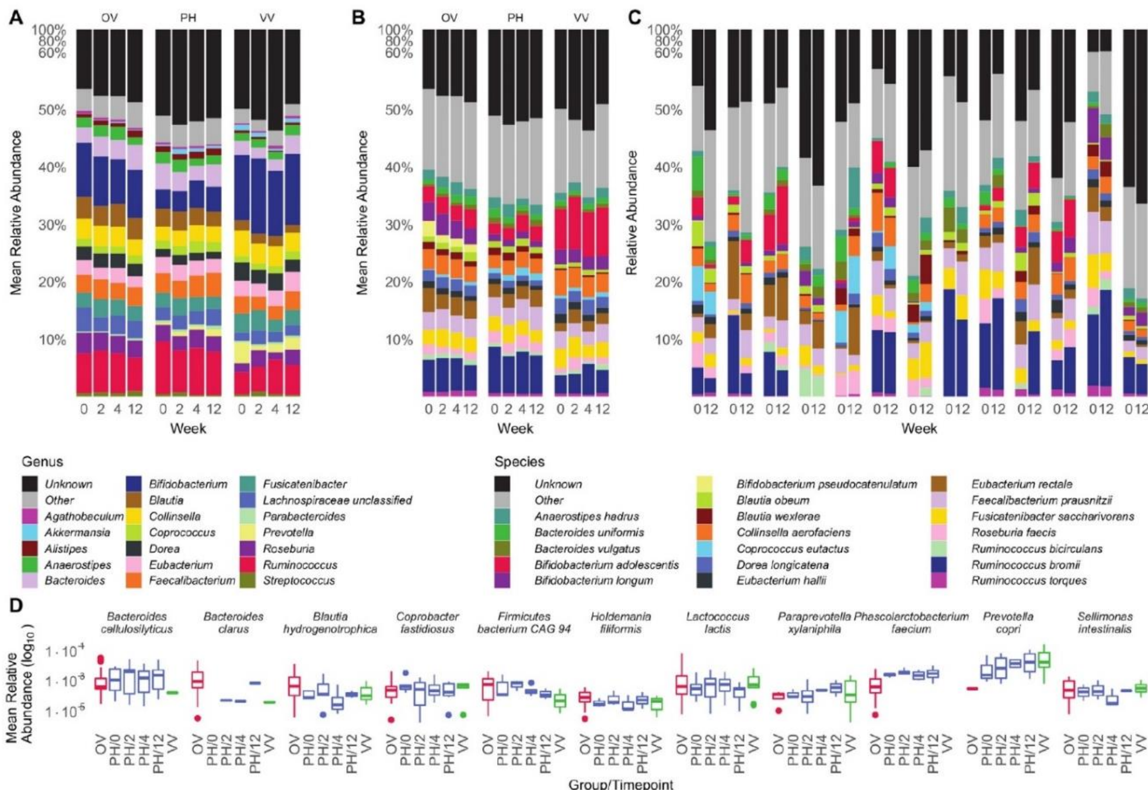

Figure 3. (**A**) Mean genus composition of the different dietary cohorts across different time points. Explicitly named genera were selected by looking at the highest mean relative abundances across all samples. (**B**) Identical information to panel Figure 3A, yet at species resolution. (**C**) Species composition of the PH cohort for the first and last measured time point. (**D**) Mean relative abundances of species with consistently largest effect sizes differentiating OV from VV. The results for the OV and VV cohorts were aggregated over all time points. OV, omnivore, VV, veg(etari)an, PH, Planetary Health group.

The mean relative abundance on the species level showed that the 12.1% of *Bifidobacterium* spp. in the VV consisted of 8% *Bifidobacterium adolescentis* (Figures 3B and S1). After following the PH diet for at least four weeks, we detected a two-fold increase in *Bifidobacterium adolescentis* and *Coprococcus eutactus*. These changes were not identified as significant during differential abundance analysis. We further investigated the relative abundance for each individual on the PH diet at the time of inclusion in comparison with twelve weeks after (Figures 3C and S1). Large variations in microbial composition between individuals at the time of inclusion could be observed, suggesting a partly host-specific microbiota composition.

We further analysed the differential abundance between OV and VV to highlight potentially interesting species, thereby only focusing on the top ten percent effect sizes (Figures 3D and S1). We detected a 3-fold increase in *Prevotella copri*, a 4-fold increase in *Paraprevotella xylaniphila*, and an 18-fold increase in *Bacteroides clarus,* whereas, e.g., *Firmicutes bacterium CAG 94* showed a 6-fold decrease in the PH diet over the course of the study. The differential abundance depicted in Figures 3D and S1 suggests that following the PH diet shifts parts of the microbiota composition towards a VV microbiome. However, these observed changes were not significant.

Cultivation and species identification with MALDI-TOF mass spectrometry identified 59 different bacterial species across all time points among the five randomly selected participants from each group (Figure 4). Most commonly isolated were *Escherichia coli*, *Enterococcus faecium*, *Clostridium perfringens*, and *Bifidobacterium longum*. *Enterococcus mundtii* and *Priestia megaterium* were mostly detected in the VV, whereas *Streptococcus parasanguinis*, *Streptococcus salivarius*, *Enterobacter cloacae*, and *Bacteroides uniformis* were mainly isolated from faecal samples of those participants following the PH diet. A detailed account of detected bacteria in the different groups is displayed in Supplementary Table S1. This method, however, represents only cultivable microorganisms, leaving approximately 35–65% undetected when compared with next-generation sequencing (NGS) [35].

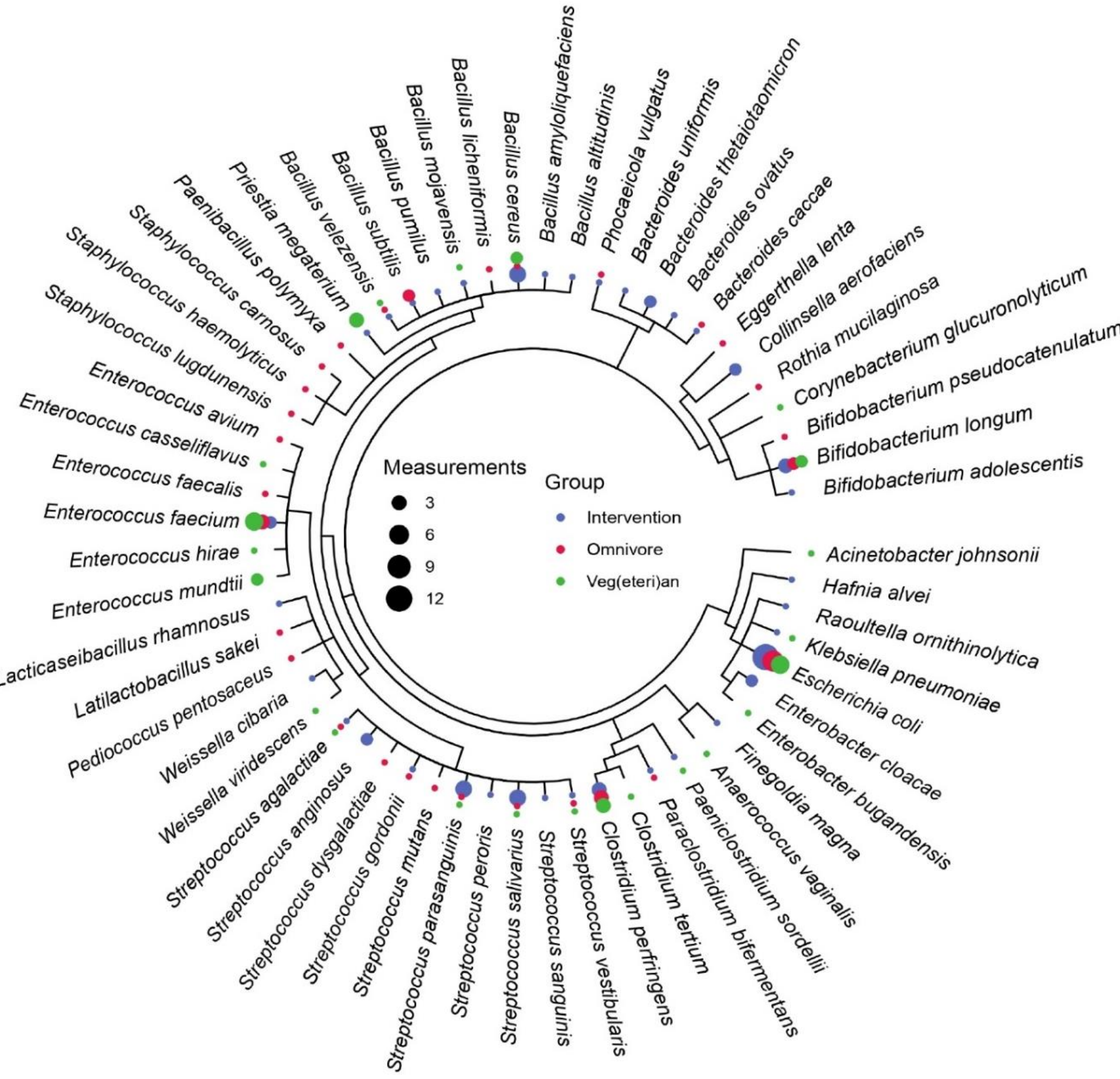

Figure 4. NCBI taxonomic classification of the species identified by mass spectrometry. The indicated number of measurements for the different diets represents the number of times the species has been identified in different samples at any time point.

4. Discussion

Our study analysed whole-genome data obtained from faecal samples after following three specific diets, i.e., OV, VV, and PH, over the course of 12 weeks to investigate the intestinal microbiota composition associated with these dietary patterns. The main difference between OV as compared with VV and PH is most likely the intake of dietary fibre. Western citizens generally ingest between 14 g (United Kingdom) and 26 g (Norway) of dietary fibre, whereas most countries recommend 25–35 g per day for adults [36]. With the PH diet suggesting 232 g of whole grains, 300 g vegetables, and 200 g fruits per day for an intake of 2500 kcal/day, participants following this diet should reach these dietary fibre recommendations [2]. A sufficient amount of fibre is directly associated with positively affecting the human intestinal microbiome, and a plant-based diet is proposed to benefit human and planetary health [15,37]. In this study, we were able to detect a trend towards an increase in *Bifidobacterium adolescentis* and *Coprococcus eutactus* (Figure 3A–C) after following the PH diet for a minimum of four weeks. An increase in *B. adolescentis* has previously been shown after supplementation with inulin, a type of dietary fibre and naturally occurring plant carbohydrate. *B. adolescentis* is capable of degrading inulin into lactate and acetate, which can be used by *Anaerostipes hadrus* and *Enterococcus rectale* to produce the SCFA butyrate [38]. In contrast to the supplementation with inulin from Baxter et al., we did not find a co-increase in *A. hadrus* when following the PH diet without tracking the exact dietary fibre composition. However, *B. adolescentis* seems to have a growth advantage after increasing inulin intake. Similarly, β-glucans have been shown to be the preferred growth substrate of *C. eutactus*, suggesting a growth advantage after increasing β-glucans consumption [39,40]. Differences in taxonomic abundances suggested that several species merit particular consideration, such as, e.g., *Prevotella copri* and *Paraprevotella xylaniphila*, for which a non-significant increase was detectable (Figure 3D and Figure S1). *P. copri* is capable of dietary fibre degradation, as they harbour vast genomic repertoires of carbohydrate active enzymes [41]. Similar to *B. adolescentis*, switching to the PH diet might favour the growth of *P. copri.* While SCFA-producing bacteria should be beneficial for the host due to their anti-inflammatory and regulatory effects, *P. copri* has also been correlated with the development of rheumatoid arthritis, although without conclusive evidence. An overgrowth of *P. copri* might also inhibit the growth of other beneficial microbiota [42]. *P. xylaniphila* can produce anti-inflammatory SCFAs, but also has the potential to synthesise pro-inflammatory metabolites, such as, for example, succinic acid. Succinic acid was previously described in close correlation with the development of hypertension, inflammatory, and metabolic diseases [43,44]. These two species, identified by differential abundance, might harbour beneficial potential, but need to be studied more extensively to analyse their exact effect on health homeostasis and their function within the complex gut microbiome. However, computing the differential abundance is a powerful tool to identify both pathogenic species and beneficial bacteria. To the best of our knowledge, no genomic or phenotypic analyses have been performed to identify the biochemical properties of *Firmicutes bacterium CAG 94*, making this species an interesting target for further research.

Several limitations of our study are offered for consideration. First, we performed a monocentric analysis with a limited number of individuals. Second, participants of this study received recipes and detailed instructions on what to consume, but we did not implement exact meal plans. For future studies, we recommend standardised meal plans to avoid any potential participant compliance issues. Third, we did not perform culture-based bacteriological analysis in all study participants. Fourth, the VV contained mostly biologically female participants, thereby creating significant differences in sex ratios between the groups. Fifth, the study groups were relatively small, and robust statistical analyses of individual groups at different time points would require a larger study population in future studies.

In conclusion, this work provides the first metagenomics-sequencing-based appraisal of the PH diet. While no significant changes were observed within the overall intestinal microbial composition of individuals opting to follow the PH diet, we identified several

potentially interesting bacterial species. Indeed, when focusing on differentially abundant species between OV and VV, non-significant trends of the PH cohort towards VV were noted. Specific bacterial species are capable of producing anti-inflammatory metabolites and might be an interesting target for novel probiotics, beneficial bacteria that can be taken supplementary to a healthy diet [45]. Hence, we encourage further microbiota-targeted research pertaining to the PH diet, ideally through multi-country longitudinal and larger-scaled studies.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/nu15081924/s1. Table S1: List of bacterial species identified by culturing and MALDI-TOF. Listed are all bacterial species that were cultured from native stool samples and identified via MALDI-TOF. The table is divided in the three dietary groups.; Figure S1: Identical information as displayed in Figure 3 using sourmash for taxonomic profiling of metagenomic reads. Note, the relative amount of unknown taxonomies was removed and information was rescaled. Further, the selected species from Figure 3D were adopted and not recomputed as to highlight abundance differences among workflows.

Author Contributions: S.L.B., T.K. and J.R. had the idea for this study, together with G.P.S., and had full access to all the data and take responsibility for the integrity of the data and the accuracy of data analysis. T.K. and J.R. collected samples and extracted whole-genome DNA and performed culturing and MALDI-TOF analysis. Computational data analysis was performed by G.P.S. and A.K. J.R., G.P.S., V.K. and S.L.B. drafted the manuscript. All authors critically reviewed the paper for important intellectual content and agreed to submit the final version for publication. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the regional ethics committee) of the 'Ärztekammer des Saarlandes' (reference no.: 116/22).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Deidentified participant data and study documents (clinical questionnaire, food documentation, informed consent form, and sequencing data) will be made available to other researchers upon reasonable request directed to the corresponding author via e-mail.

Acknowledgments: The authors would like to thank all study participants and express their gratitude to all laboratory members involved in this study for their excellent work. This work was further supported by Saarland University and the UdS-HIPS TANDEM initiative.

Conflicts of Interest: The authors declare no conflict of interest.

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4.3 Results No. III

Occurrence, resistance patterns and management of carbapenemase-producing bacteria in war-wounded refugees from Ukraine

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Berger FK, Schmartz GP, Fritz T, Veith N, Alhussein F, Roth S, Schneitler S, Gilcher T, Gärtner BC, Pirpilashvili V, Pohlemann T, Keller A, Rehner J, Becker SL. Occurrence, resistance patterns, and management of carbapenemase-producing bacteria in war-wounded refugees from Ukraine. *Int J Infect Dis.* 2023 Jul;132:89-92. doi: 10.1016/j.ijid.2023.04.394. Epub 2023 Apr 16. PMID: 37072055.

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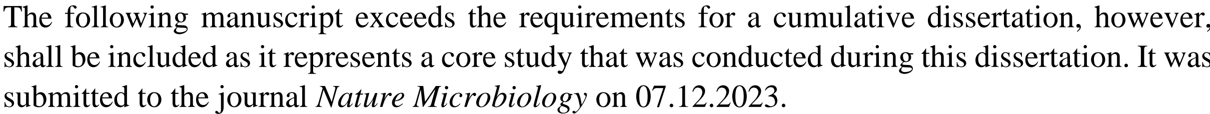
4.4 Results No. II


The following manuscript exceeds the requirements for a cumulative dissertation, however, shall be included as it represents a core study that was conducted during this dissertation. It was submitted to the journal *Nature Microbiology* on 07.12.2023.

Decoding the diagnostic and therapeutic potential of microbiota using pan-body pan-disease mirobiomics

Schmartz GP, Rehner J, Gund M, Rupf S, Hannig M, Berger T, Flockerzi E, Seitz B, Fleser S, Schmitt-Grohé S, Kalefack S, Zemlin M, Kunz M, Götzinger F, Gevaerd C, Vogt T, Reichrath J, Molano LAG, Diehl L, Hecksteden A, Meyer T, Herr C, Gurevich A, Krug D, Hegemann J, Bozhueyuek K, Kalinina O, Becker A, Unger M, Ludwig N, Seibert M, Stein ML, Hanna NL, Martin MC, Mahfoud F, Keller V, Krawczyk M, IMAGINE consortium, Becker SL, Müller R, Bals R, Keller A. submitted to *Nature Communications* in December 2023.

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5. Discussion

In recent years, advancements in sequencing technologies and whole-genome DNA extraction have revolutionized the body of knowledge on the human microbiome and the influence it might have on human health and disease. The main goal of these studies is to characterize the complete microbial community and analyze microbial functionality in healthy and diseased individuals with the overarching objective of contributing to the advancement of individualized medicine through microbiome diagnostics and therapies in the future. The studies presented above form a comprehensive approach to microbiome research and the implementation of whole-genome sequencing in clinical settings, with the aim of enhancing healthcare practices and patient outcomes, as well as contributing to the overall understanding of the microbial functions within us.

5.1 Systematic Cross-biospecimen Evaluation of DNA Extraction Kits for Long- and Short-read Multi-metagenomic Sequencing Studies (Result I)

In this study, we evaluated the performance of three distinct, commercially available DNA extraction kits for metagenomics sequencing studies across six biospecimens, including feces, interdental plaque, saliva, sputum, and bile. We found that the effectiveness of different DNA extraction kits varied considerably depending on the biospecimen. This study underscores the importance of selecting an appropriate DNA extraction kit for the native sample of interest. The Qiagen QiAamp DNA Microbiome kit (QMK) emerged as the most suitable DNA extraction kit tested, as samples processed with this kit produced the highest quality sequencing results. Moreover, samples prepared with this kit displayed the highest amount of metagenomic information following *in silico* host DNA removal. A significant advantage of this DNA extraction kit is the experimental depletion of host DNA before extracting and purifying bacterial DNA. In the first step, eukaryotic cells are lysed, and a specific DNase (Benzoase) is used to degrade eukaryotic DNA. Our findings revealed that this procedure was evident in the sequencing outcomes, as most reads generated during sequencing for samples such as sputum, bile, saliva, and interdental plaque were of bacterial origin. We further demonstrated that different DNA extraction kits displayed substantial

variations in DNA purity and quality across various biospecimens. Consequently, we concluded that the choice of DNA extraction method can significantly impact downstream analysis and interpretation of multi-metagenomic sequencing data. We emphasize the importance of standardized protocols for microbiome studies to ensure data comparability and reproducibility.

Accurately representing microbial communities present in a sample is crucial in microbiome studies [209]. Through this study, we determined that the QMK should be used for samples with high host DNA contamination, including all clinical samples derived from humans and animals. Our study is also relevant in the context of the growing interest in multi-omics approaches to understand microbial communities and their interactions in different environments.

Recent literature has emphasized the need for standardization in multi-omics studies, particularly in microbiome research. In multiple studies, authors assessed the impact of various DNA extraction protocols on the reproducibility and comparability of microbiome data across multiple laboratories [156,309,356]. The results indicated that different DNA extraction protocols could significantly affect microbiome data. Standardized protocols are essential to ensure data comparability and reproducibility across different laboratories. This is especially crucial for comparing microbiomes in health and disease, or in relation to different interventions, in individuals from various geographical locations. Studies have shown significant differences in microbial composition between Western citizens and, for example, individuals from Africa. Concurrently, individuals from different parts of the world tend to develop distinct diseases, which raises interest in comparing the potential relatedness between these two phenomena [1,2,72,122,200,201,226,237,242,287,287,302].

Additionally, studies compared the performance of different DNA extraction kits for 16S rRNA gene sequencing and subsequent microbiome analysis of human stool samples [61,83,248,248,326]. The findings of these studies vary in their results, however most found that different DNA extraction kits exhibited significant variations in DNA yield, purity, and microbial composition. This further emphasizes the need for standardization of protocols in microbiome studies. It also shows the need of intensive analyses on the suitability of a nucleic acid extraction protocol for the respective sample type, prior to the actual study. To allow comparability between samples, I suggest using the same nucleic acid extraction method for all samples tested in a study. Otherwise, results might be biased by the advantages and

disadvantages of different methods used. Further research should analyze more human-derived samples, such as skin swabs, tissue biopsies or vaginal swabs to assess whether there is one nucleic acid extraction method, that is suitable for all human-derived samples. Such an approach would also contribute to complete view of the human microbiome.

5.2 The effect of a Planetary Health diet on the human gut microbiome: a descriptive analysis (Article II)

We compared the gut microbiomes of individuals following a planetary health diet, designed by a multidisciplinary team to promote global health and sustainability, with those on omnivorous and vegan/vegetarian diets. Our findings indicate that adopting a planetary health diet for 12 weeks can slightly alter the intestinal microbiome and increase the abundance of beneficial bacterial species, such as *Bifidobacterium adolescentis*. This could contribute to both human and planetary health benefits in the long term.

This study builds upon the growing body of literature that highlights the connection between diet and gut microbiome composition and function [149,192,210,245,283]. The human gut microbiome is a complex ecosystem of microorganisms and host cells that plays a crucial role in host health by influencing various physiological processes, including digestion, metabolism, and immune function. Diet is a major factor shaping microbial composition and function within the intestinal tract. Exactly 10 years ago, researchers already showed the effect of an animal-based diet and plant-based diet on the human gut microbiome, providing a description of increased and decreased abundances of certain bacteria according to the diet followed [69]. Recently, there has been increasing interest in the role of diet in modulating the gut microbiome and its potential therapeutic applications in treating diseases such as inflammatory bowel disease, obesity, and metabolic disorders. A study by De Filippis et al. (2016) found that a plant-based Mediterranean diet was associated with higher microbial diversity and lower inflammatory markers in the gut microbiome compared to a Western diet [71]. Similarly, the adherence to a healthy plant-based diet correlated with higher intestinal microbial diversity and a more favorable gut microbiome composition, featuring increased levels of short-chain fatty acid-producing bacteria and decreased levels of inflammatory bacteria [3,244,308,351]. High-fiber, low-fat, and low-protein diets were also associated with a more diverse and stable gut microbiome, including increased levels of beneficial bacteria and reduced levels of opportunistic pathogens [53,80,115,186]. In addition to dietary patterns,

specific nutrients and bioactive compounds have been shown to directly impact the gut microbiome. For example, prebiotics such as inulin and oligofructose selectively enhance the growth of beneficial bacteria in the gut, such as Bifidobacteria and Lactobacilli [110,172]. Polyphenols, like resveratrol and quercetin found in plant-based foods such as grapes and berries, exhibit prebiotic effects and thus modulate the gut microbiome [43,171]. Other bioactive compounds, like curcumin and capsaicin, possess anti-inflammatory and antimicrobial properties and are thought to have a protective effect on the gut microbiome [77,93,150].

These studies suggest that specific nutrients, dietary patterns, and bioactive compounds may contribute to the beneficial effects on the gut microbiome. High-fiber diets, in particular, may lead to a higher abundance of beneficial bacteria that can ferment carbohydrates in the intestinal tract and produce short-chain fatty acids. These fatty acids possess anti-inflammatory and anti-proliferative properties and regulate immune responses not only in the gut but also at other body sites [97,232,310,311]. In further studies, the effect of a planetary health diet on short-chain fatty acids levels in individuals could add valuable knowledge on the suitability of this diet concept. However, to assess significant changes within the intestinal microbiome after such a dietary intervention, I suggest including more participants into a follow-up study and including individuals from various geographic locations. Furthermore, using a standardized meal plan rather than nutritional recommendations could allow more precise comparability between individuals. Although, such a measure would be problematic in terms of food availability across countries and seasons. It could also be interesting to analyze changes in the oral microbiome after dietary interventions in addition to the intestinal microbiota. Digestion starts within the oral cavities and supplies local bacteria with nutrients. Therefore, changes in diet might also alter the oral microbial composition [208]. In addition, transcriptomics, proteomics, and metabolomics of the same samples, in addition to metagenomics, would provide a complete overview of the bacteria present, their gene expression activity, host- and bacteria-derived proteins and peptides, and bacteria-derived metabolites. Such a combined approach would allow for an in-depth analysis of changes in the microbial composition and functionality after dietary interventions. Of note, however, is the cost for such combined approaches.

5.3 Occurrence, resistance patterns and management of carbapenemase-producing bacteria in war-wounded refugees from Ukraine (Article III)

In early 2022, Russia declared war against Ukraine after two decades of intense debates between the two countries. The immense military actions from both sides have led, and unfortunately still lead, to many war-wounded individuals, including civilians and military members [85,178]. Furthermore, a significant migration of refugees from both countries across Europe is an ongoing movement, which requires healthcare and monitoring of potential global threats [367]. One of these global threats are bacteria carrying multiple antimicrobial resistance genes. The origin of these multi-resistant bacteria, as well as their transmission can be monitored using nucleic acid extraction of isolates or native samples of patients and their environment, followed by whole-genome or metagenomic sequencing, respectively. A recent study, for example, has shown that multi-resistant bacteria were more prevalent in military hospital settings in the Ukraine, as in civilian hospitals [165].

In this study, we emphasize the importance of effective infection control measures in preventing the spread of antibiotic-resistant bacteria from war-wounded refugees of the war in Ukraine. This aligns with recent literature stressing the role of infection prevention and control in reducing the global burden of antimicrobial-resistant microorganisms and healthcare-associated infections [94,101,199,240,316]. The WHO has launched a global campaign to promote hand hygiene and infection prevention and control measures to decrease the incidences of such infections and the spread of antibiotic-resistant pathogens [7,197,368,369]. Antibiotic stewardship programs are being implemented worldwide to help reduce antibiotic resistance transmissions [8,20,21,39,320]. These programs involve the development and implementation of guidelines for appropriate antibiotic use, including the avoidance of unnecessary antibiotic prescriptions and the use of narrow-spectrum antibiotics whenever possible. There is also growing recognition of the need for comprehensive infection control programs in settings such as refugee camps, where large numbers of vulnerable individuals may be at increased risk of infection [13,48,131].

Interestingly, we identified carbapenemase-producing bacteria in all individuals investigated in this study. A significant proportion of war-wounded refugees were colonized, with *Klebsiella pneumoniae* being the most commonly identified species. Our findings align

with a surveillance study conducted from 2022 to 2023 in Jena, for which seven patients from Ukraine were admitted to the hospital and screened for carbapenem resistant bacteria [293]. All patients were either colonized or even infected with such bacteria, enhancing the need to monitor community-acquired and hospital-acquired colonization with multi-resistant bacteria. Furthermore, our isolated multi-resistant bacteria showed resistance against novel antibiotics, such as ceftazidime/avibactam and cefiderocol, which should be closely monitored and managed to reduce the spread of such resistance patterns. A study by Nichols et al. (2023) suggests that ceftazidime/avibactam resistance co-occurs with other resistances, such as carbapenem resistance mediated by the *oxa-48* gene [219]. In future studies, such co-occurrences should be investigated *in vitro* and by sequencing to improve treatment choices and understand the spreading of these novel AMRs. Furthermore, as cefiderocol resistance has been reported in other studies as well, the research focus tends towards unraveling the underlying mechanisms, with one study identifying the involvement of a mutation of the siderophore receptor CirA [164].

We further highlight the need for continued surveillance of multi-resistant bacteria to inform public health efforts. This is consistent with recent literature emphasizing the importance of surveillance systems in detecting and monitoring the spread of infections by such bacteria [58]. In line with recent studies reporting an increasing trend of multidrug-resistant bacteria globally, especially in low- and middle-income countries, our study emphasizes the emergence of such bacteria as a major public health concern [141,216,223,269]. Furthermore, hospitals around Germany reported similar trends in the detection of multi-resistant bacteria from refugees of the war in Ukraine [132,271,277]. The study by Higgins et al. (2020) further suggests likely transmission events through sequence typing [132].

In addition to the resistance patterns of these isolated species, we investigated their clonality to trace back potential transmission pathways of genetic information encoding for antimicrobial resistances. We found at least three different strains of *K. pneumonia*, however, based on multilocus sequence typing, we identified three isolates from three different patients with a common epidemiological background [28]. Resistance transmission between bacteria could have occurred at the primary care facility in Ukraine, characterized by crowded camps, including medical staff and a vast number of wounded patients. Regarding carbapenem resistances, the two genes *blaNDM-1* and *OXA-48* encoding resistance against this antibiotic were found in most isolates. However, *blaKPC-2*, which has been shown to be an emerging

resistance against carbapenems in *K. pneumoniae*, could not be identified [54,87,138,354]. Yet, Gram-negative bacteria carrying more than one resistance against carbapenems were rare to find in Germany [121,294,341]. Therefore, our study, in line with the studies mentioned above, suggests screening war-wounded patients from Ukraine thoroughly for antimicrobial-resistant bacteria, thereby monitoring resistance patterns and allowing adequate control of spreading. In addition, monitoring of multi-resistant bacteria at first care facilities in war regions, especially on fomites, might contribute to effective infection prevention.

6. Conclusion and Outlook

The four manuscripts presented in this work contribute to various aspects of using metagenomic and whole-genome sequencing techniques to study the human microbiome and improve clinical applications, such as antimicrobial resistance monitoring. To obtain accurate results from microbiome and clonality studies, several prerequisites must be fulfilled, including appropriate sample collection and storage, efficient DNA extraction, high-quality sequencing protocols, and precise, up to date *in silico* data analysis. Numerous studies have explored the optimal sampling methods and storage of native samples originating from humans or animals. Consequently, this work first focused on the evaluation of a suitable DNA extraction method that enables a standardized procedure, yielding high-quality DNA and accurately representing the microbial communities in a sample (Result I). We assessed three commonly used, commercially available DNA extraction kits for various biospecimens obtainable from humans to study microbiomes at different body sites. The Qiagen QiAamp Microbiome DNA kit proved to be the most suitable for DNA extraction from the six tested sample types. However, for exclusively fecal samples or interdental plaque samples, the ZymoBIOMICS kit or the Qiagen PowerSoil DNA kit were equally appropriate. As a result, in a subsequent study investigating the effects of a dietary intervention on the intestinal microbiome, we decided to conduct DNA extractions from fecal samples exclusively using the ZymoBIOMICS DNA Miniprep kit (Result II). In this investigation, we addressed the need for a solution to combat starvation and global exploitation by examining the impact of a planetary health diet on the gut microbiome. We observed an increase in beneficial bacteria following a transition from an omnivorous western diet to a planetary health diet. While the diet concept developed by the *EAT-Lancet* commission is a topic of contentious debate, our

study did not reveal any adverse effects on the intestinal microbiome after adhering to a planetary health diet for 12 weeks. The third study presented in this work employed whole-genome DNA extraction and subsequent whole-genome sequencing to identify antimicrobial resistances in isolated Gram-negative bacteria from war-wounded refugees from Ukraine, who received secondary treatment at the Saarland University Medical Center in Homburg (Result III). Based on genomic data, a common origin of three independently isolated strains of *Klebsiella pneumoniae* is suggested. Moreover, novel resistance patterns were observed in these Gram-negative bacteria, including antimicrobial resistances against various antibiotics, as well as new antibiotics such as cefiderocol and ceftazidime/avibactam. Last, an additional study (Result IV) showed the correlation between a variety of non-communicable human diseases and their various microbiomes at different body sites. We highlighted differentially abundant species, especially in oral samples, for the investigated diseases and were also able to detect interesting biosynthetic gene cluster, which potentially encode for anti-inflammatory and anti-microbial metabolites that could be exploited to benefit human health.

In summary, the findings of this thesis offer valuable insights in the realm of microbiome and whole-genome studies, employing advanced techniques to analyze microorganisms' genetic information. Specifically, whole-genome sequencing could serve as a potent addition to infection prevention measures aimed at controlling the spread of multi-resistant bacteria and for microbiological diagnostic tools. A variety of infectious diseases are difficult to diagnose, especially in terms of parasitic infections. Metagenomic sequencing of native samples might provide a powerful addition to standard techniques, which often rely on trained microscopy staff. Especially cost-effective and portable solutions, such as Oxford Nanopore MinION Mk1C could be an interesting option even for regions around the world, where well-equipped laboratories are rare. In contrast to standard cultivation, or PCR-based diagnostic procedures, metagenomic sequencing provides an untargeted investigation of all potential causative agents present in a sample. As shown in Result IV, this technique also allows for the detection and screening of potential biomarkers of chronic inflammatory diseases. Combined with trained microbiologists, as well as artificial intelligence programmed by bioinformaticians, such an approach could lead to improved rapid detection of thus far difficult to diagnose infectious, and non-communicable diseases. An implementation of metagenomic sequencing for diagnosis of infectious diseases and monitoring of multidrug-resistant bacteria have furthermore great potential to aid in the global fight against these threats. Furthermore, future research should focus on not just the description of different

microbiomes of individuals, animals, or the environment, but also investigate in detail the underlying mechanisms of host-microbe, or environment-microbe interactions, the effect of secondary metabolites produced by bacteria and their exact modulation of host and environmental health, and the entire functional capacity that these complex microbial communities harbor. Without these functional analyses, solely descriptive studies fail to draw exact conclusions on the observed bacterial shifts.

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8. Publications/ Acknowledgments

8.1 Publications in peer-reviewed journals

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8.2 Conference contributions: talks

- **Rehner J,** Schmartz GP, Kramer T, Keller V, Keller A, Becker SL. The Effect of a Planetary Health Diet on the Human Gut Microbiome. Deutsche Gesellschaft für Hygiene und Mikrobiologie (DGHM) Kongress in Berlin, 2022.

- **Rehner J,** Schmartz GP, Schuff MJ, Krawczyk M, Tagirdzhanov A, Gurevich A, Franke R, Müller R, Keller V, Keller A, Becker SL. Comparative metagenomic analysis of fecal and saliva samples obtained from a large variety of zoo animals. Deutsche Gesellschaft für Hygiene und Mikrobiologie (DGHM) Kongress in Lübeck, 2023.

8.3 Conference contributions: poster

- **Rehner J**, Schmartz GP, Groeger L, Dastbaz J, Ludwig N, Hannig M, Rupf S, Seitz B, Flockerzi E, Berger T, Reichert MC, Krawczyk M, Meese E, Herr C, Bals R, Becker SL, Keller A, Müller R; IMAGINE Consortium. Systematic Cross-

biospecimen Evaluation of DNA Extraction Kits for Long- and Short-read Multi-metagenomic Sequencing Studies. Vereinigung für Allgemeine und Angewandte Mikrobiologie (VAAM) Kongress 2022; digital.

8.4 Acknowledgements

I would like to express my deepest gratitude to Prof. Dr. Dr. Sören L. Becker, my doctoral supervisor and mentor at the Saarland University, Institute of Medical Microbiology and Hygiene. His outstanding supervision, unwavering support, and dedication to mentoring have been essential to the success of my dissertation. Prof. Becker's exceptional work ethic, vast knowledge, and wonderful character have not only shaped my academic journey but have also made him a true role model. I am sincerely thankful for his constant guidance and encouragement over the past three years.

I am also indebted to Prof. Dr. Andreas Keller for his valuable support in all projects and the fruitful collaboration we have maintained. His scientific advice has been instrumental in shaping the direction of my research, and I am grateful for the opportunities to learn and grow under his guidance.

I extend my heartfelt appreciation to the following clinics and institutes and their members for their significant contributions to my research:

- Clinical Bioinformatics, Saarland University, Germany

- Clinic of Operative Dentistry, Periodontology and Preventive Dentistry, Saarland University, Germany

- Synoptic Dentistry, Saarland University, Germany

- Department of Ophthalmology, Saarland University Medical Center, Germany

- Clinic of Paediatrics and Neonatology, Saarland University, Germany

- Department of Internal Medicine III, Cardiology, Angiology, Intensive Care Medicine, Saarland University Hospital, Germany

- Clinic for Dermatology, Venereology, and Allergology, Germany

- Institute for Sport and Preventive Medicine, Saarland University, Germany

- Department of Internal Medicine V - Pulmonology, Allergology, Intensive Care Medicine, Saarland University

- Helmholtz Institute for Pharmaceutical Research Saarbrücken, Germany

- Department of Pharmacy, Saarland University, Germany

- Department of Neurology, Saarland University Medical Center, Germany

- Department of Medicine II, Saarland University Medical Center, Germany

I am grateful to all my colleagues at the Institute of Medical Microbiology and Hygiene, particularly the master students I was supervising, Lena Margardt and Nina Bühler from our diagnostics department, my colleagues Gubesh Gunaratnam and Linda Pätzold. Their dedication and support have been crucial to the success of various projects and have created a positive and enriching work environment. I have always cherished coming to work and am enthusiastic about continuing this journey.

I would like to extend my sincere appreciation to the GradUS global team for awarding me a scholarship that facilitated my stay at ETH Zürich in the laboratory of Prof. Dr. Emma Wetter-Slack. This opportunity allowed me to enhance my skills, particularly regarding *in vivo* experiments. The support from GradUS has been invaluable, and I am grateful for their contribution to my academic and research endeavours.

I am also grateful to the TANDEM graduate school for the enriching experiences and collaborative opportunities during my time as a student spokesperson. Prof. Dr. Claus-Michael Lehr and Dr. Brigitta Loretz played a great role in fostering a conducive learning environment outside the regular scientific research. Their guidance and support have been instrumental in shaping my academic and personal journey, and I appreciate the collaborative spirit that characterized our interactions.

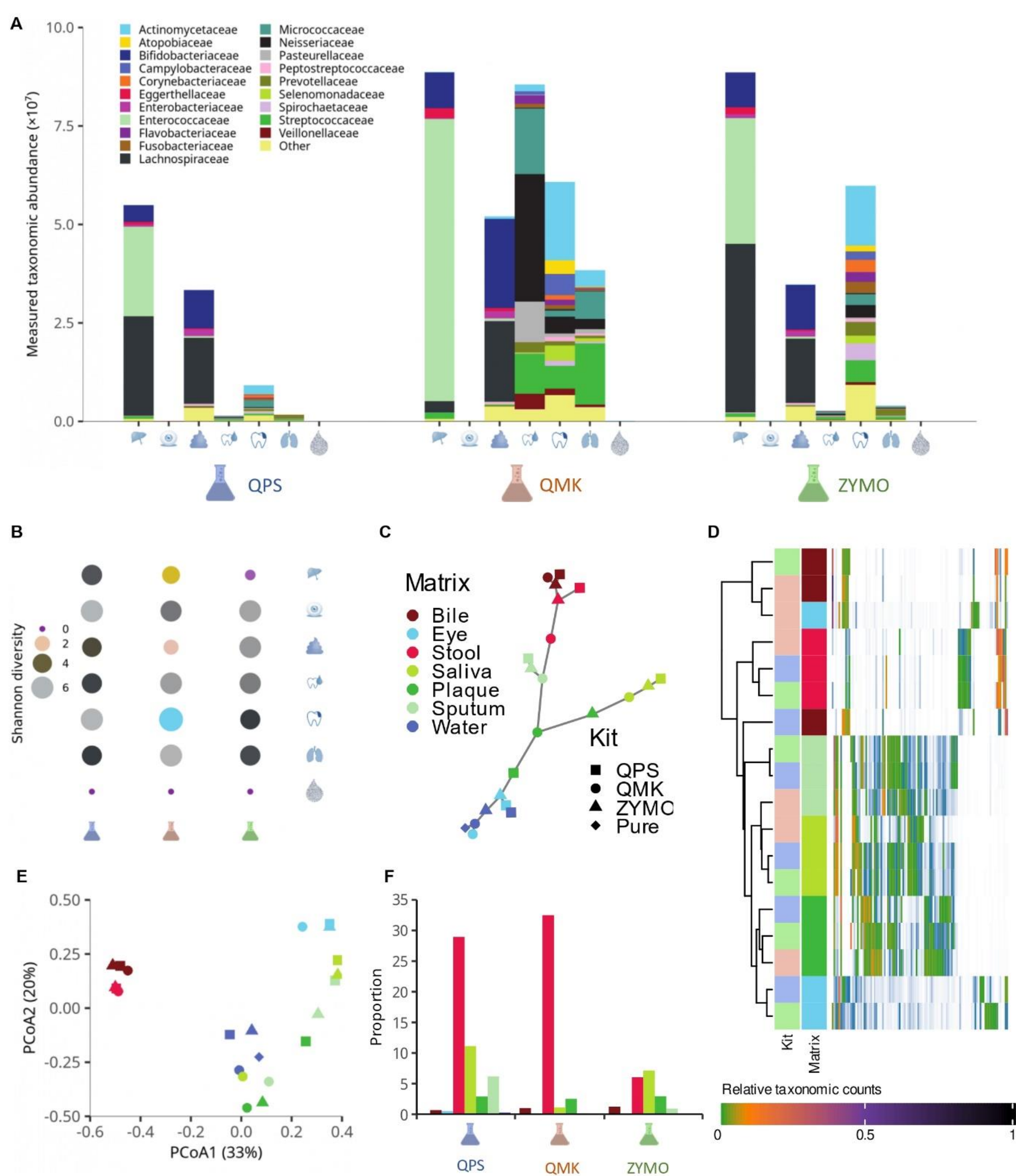
Thank you to both GradUS and the TANDEM graduate school for the crucial roles in my academic and research development. The experiences and knowledge gained through these collaborations have significantly contributed to the depth and breadth of my work.

Last, but definitely not least special thanks go to my family and friends for their unwavering support. My parents, brother, and partner, in particular, have been a constant source of encouragement, enabling me to channel my passion into my work and strive for continuous improvement. Their love and support have been instrumental in my personal and professional growth.

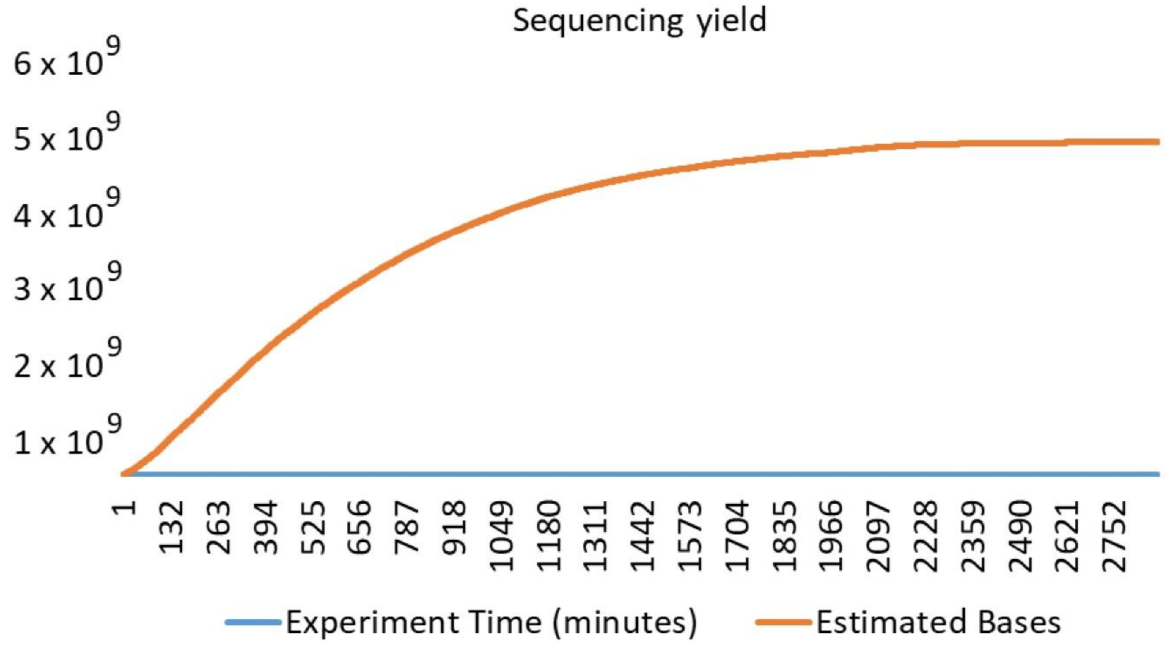
9. Appendix

The following appendix includes the supplementary material to the presented manuscripts, which can also be accessed online (see DOI in Declaration), my curriculum vitae (in German: Lebenslauf), the affidavit (in German: Eidesstattliche Erklärung). Detailed information about author contributions for the three manuscripts presented in this dissertation for fulfilment of the requirements for a cumulative PhD thesis will be provided additionally, outside of this printed dissertation.

9.1 Supplementary Material Result I:



Supplementary Figure 1: **Additional assessment of microbiota diversity A.** Identical plot to Figure 2A focusing on families instead of phyla. **B.** Identical visualization to Figure 2B with two changes in data analysis. First, the Shannon diversity is used as alpha diversity measure. Second, a decontamination procedure was used to remove contaminations found in the negative control samples. For each kit, all species with relative abundance over 0.01 were identified in the water samples. Those taxonomies were then blacklisted to not be included in the computation. **C.** Figure 2C implementing the decontamination procedure described in Figure S1B. **D.** Heatmap with clustering of Figure 2D integrating the decontamination procedure used in Figure S1B. **E.** Visualized beta diversity computed using PCoA based on the Bray-Curtis distance measure. **F.** Barplot shows the proportion of isolated DNA in ng/mg stool, ng/ml saliva or water, and ng/ml swab-medium mixture for the remaining samples. PCoA, principal coordinates analysis.



Supplementary Figure 2: **Sequencing yield in estimated bases across experiment time** The graph was plotted by Excel (Microsoft) with the throughput Excel file generated by MinKNOW.

Supplementary Table 1:

Materials & consumables	Company
MinION Mk1B	Oxford Nanopore Technologies
Flow Cell (R9.4.1) FLO-MIN106D	Oxford Nanopore Technologies
Ligation Sequencing Kit 1D (SQK-LSK109)	Oxford Nanopore Technologies
Native Barcoding Expansions 1-12 (EXP-NBD104)	Oxford Nanopore Technologies
Flow Cell Priming Kit (EXP-NBD114)	Oxford Nanopore Technologies
Short Fragment Buffer (SFB)	Oxford Nanopore Technologies
Elution Buffer (EB)	Oxford Nanopore Technologies
Adapter Mix II (AMII)	Oxford Nanopore Technologies
Agencourt AMPure XP beads	Beckman Coulter Life Sciences
NEB Blunt/TA Ligase Master Mix	New England Biolabs® Inc.
NEBNext End repair	New England Biolabs® Inc.
1.5 mL Eppendorf tubes	Thermo Fisher Scientific
Invitrogen™ Nuclease-free water	Thermo Fisher Scientific
NEBNext® Quick Ligation Reaction Buffer	New England Biolabs® Inc.
NEBNext FFPE Repair Mix	New England Biolabs® Inc.
T4 DNA Ligase 2M U/mL	New England Biolabs® Inc.
0.2 mL thin-walled PCR tubes	Thermo Fisher Scientific
Ethanol abs.	Merck

Note: Material used for nanopore sequencing.

Supplementary Table 2:

Sample_ID	β [ng/µl] after gDNA isolation	V [µl] after gDNA isolation	B [ng/µl] barcoded DNA
QMK Bile	15	10	12.7
QMK Saliva	22.3	38	19.9
QPS Bile	10	14	12
QPS Saliva	278	20	91.6

Note: Measurements taken with Nanodrop 2000 before and after barcode ligation of each sample, 5 µl barcoded DNA were pooled for adapter ligation. QPS, Qiagen DNeasy PowerSoil Pro; QMK, QiAamp DNA Microbiome Kit.

Supplementary Table 3:

β [ng/µl] pooled barcoded DNA before adapter ligation	β [ng/µl] pooled barcoded DNA after adapter ligation
17.0	15.4

Note: DNA concentration of pooled barcoded DNA before and after ligation, measured with Nanodrop 2000.

9.2 Supplementary Material Result II:

Supplementary Table 1: List of bacterial species identified by culturing and MALDI-TOF. Listed are all bacterial species that were cultured from native stool samples and identified via MALDI-TOF. The table is divided in the three dietary groups.

Species	**OV**	**PH**	**VV**
Acinetobacter johnsonii	0	0	5
Anaerococcus vaginalis	0	0	1
Bacillus altitudinis	0	1	0
Bacillus amyloliquefaciens	0	1	0
Bacillus cereus	1	5	2
Bacillus licheniformis	1	0	0
Bacillus mojavensis	0	1	1
Bacillus pumilus	0	2	0
Bacillus subtilis	1	0	0
Bacillus velezensis	1	1	1
Bacteroides caccae	1	1	0
Bacteroides ovatus	0	1	0
Bacteroides thetaiotaomicron	0	3	0
Bacteroides uniformis	0	1	0
Bifidobacterium adolescentis	0	1	0
Bifidobacterium longum	2	3	2
Bifidobacterium pseudocatenulatum	1	0	0
Clostridium perfringens	3	3	3
Clostridium tertium	0	0	1
Collinsella aerofaciens	0	3	0
Corynebacterium glucuronolyticum	0	0	1
Eggerthella lenta	1	0	0
Enterobacter bugandensis	0	0	1
Enterobacter cloacae	0	2	0
Enterococcus avium	1	0	0
Enterococcus casseliflavus	0	0	2
Enterococcus faecalis	1	0	0
Enterococcus faecium	2	2	7
Enterococcus hirae	0	0	1
Enterococcus mundtii	0	0	2
Escherichia coli	9	26	8
Finegoldia magna	0	1	0
Hafnia alvei	0	1	0
Klebsiella pneumoniae	0	0	1
Lactobacillus rhamnosus	0	1	0
Lactobacillus sakei	1	0	0
Paenibacillus polymyxa	1	0	0
Paeniclostridium sordellii	0	1	1
Paraclostridium bifermentans	1	1	0

Phocaeicola vulgatus	1	1	0
Priestia megaterium	0	1	4
Raoultella ornithinolytica	0	1	0
Rothia mucilaginosa	1	0	0
Staphylococcus carnosus	1	0	0
Staphylococcus haemolyticus	1	0	0
Staphylococcus lugdunensis	1	0	0
Streptococcus agalactiae	1	1	1
Streptococcus anginosus	0	2	0
Streptococcus dysgalactiae	1	0	0
Streptococcus gordonii	2	1	0
Streptococcus mutans	1	0	0
Streptococcus parasanguinis	0	4	0
Streptococcus perosis	0	1	0
Streptococcus salivarius	1	4	0
Streptococcus sanguinis	0	1	0
Streptococcus vestibularis	1	1	0
Weissella cibaria	0	2	0
Weissella viridescens	0	0	1

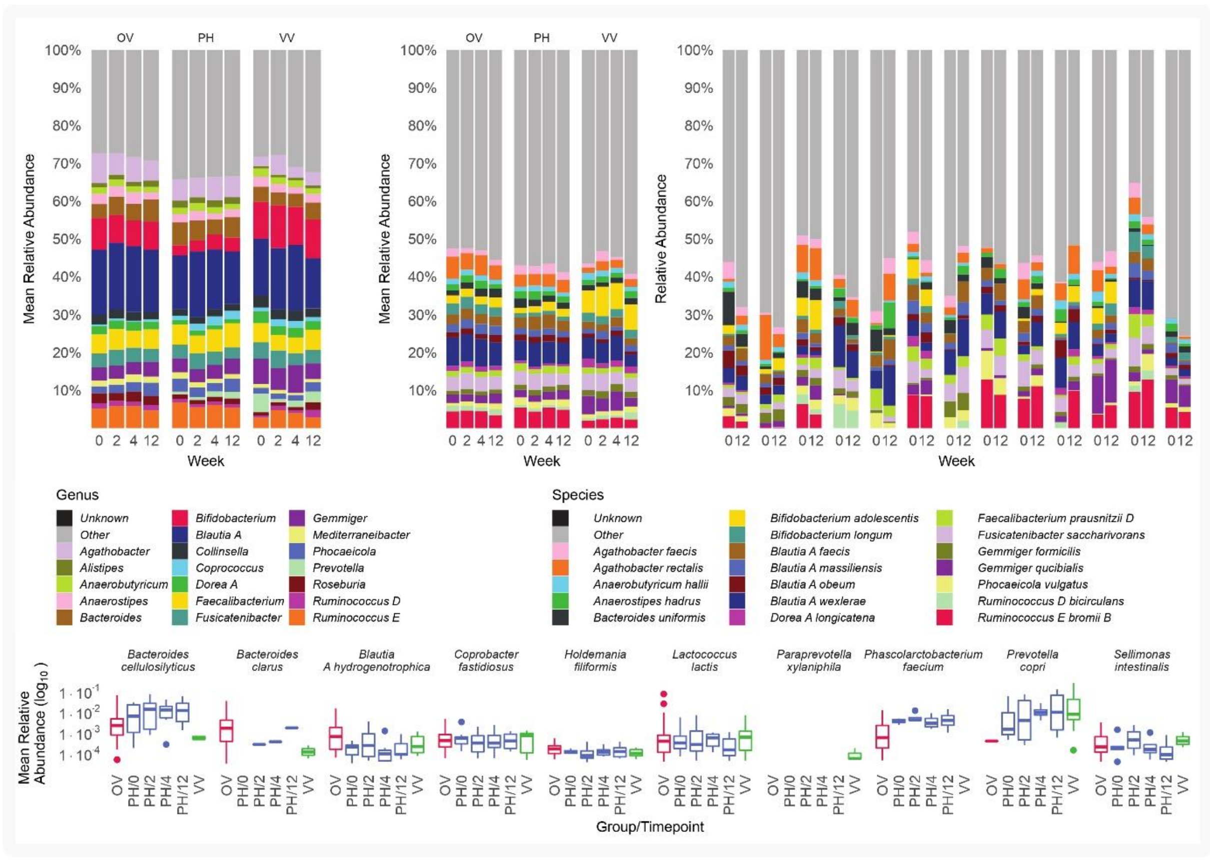


Figure S1: Identical information as displayed in Figure 3 using sourmash for taxonomic profiling of metagenomic reads. Note, the relative amount of unknown taxonomies was removed and information was rescaled. Further, the selected species from Figure 3D were adopted and not recomputed as to highlight abundance differences among workflows.

9.3 Supplementary Material Result III

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9.4 Affidavit/ Eidesstattliche Erklärung

For data protection reasons, the affidavit is not included in the electronic version of the dissertation.

9.5 Curriculum vitae/ Lebenslauf

For data protection reasons, the curriculum vitae is not included in the electronic version of the dissertation.

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