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The gut microbiome at the interface of human health and disease

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UNIVERSITY OF TARTU
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1632

Institute of Molecular and Cell Biology, Institute of Genomics, University of Tartu, Estonia

This dissertation is accepted for the commencement of the degree of Doctor of Philosophy in Gene Technology on August 28, 2024, by the Council of the Institute of Molecular and Cell Biology, University of Tartu.

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Commencement: Room No. 105, 23B Riia St., Tartu, on October 4, 2024, at 13:15.

The publication of this dissertation is granted by the Institute of Genomics and the Institute of Molecular and Cell Biology at the University of Tartu, Estonia.

This research was funded by Estonian Research Council grants PUT 1371 and PRG1414; EMBO Installation grant 3573; European Regional Development Fund project no.15-0012 GENTRANSMED; Estonian Center of Genomics / Roadmap II project no. 16-0125; Archimedes Foundation scholarships in smart specialisation growth and Dora Plus scholarship for short-term study mobility; the Doctoral School of Biomedicine and Biotechnology scholarship. Data analyses were in part carried out at the High-Performance Computing Center of the University of Tartu, Estonia.

ISSN 1024-6479 (print)
ISBN 978-9916-27-643-3 (print)
ISSN 2806-2140 (pdf)
ISBN 978-9916-27-644-0 (pdf)

University of Tartu Press
www.tyk.ee

To my grandfather, Dr Jaan-Heljut Seeder, MD.
You believed that I could, so I did.

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, referred to in the text by Roman numerals (Ref. I to Ref. III):

I **Krigul, K.L.**; Aasmets, O.; Lüll, K.; Org, T.; Org, E. (2021). Using faecal immunochemical tubes to analyse the gut microbiome can potentially improve colorectal cancer screening. Scientific Reports, 11 (1). https://doi.org/10.1038/s41598-021-99046-w.

II Aasmets, O.* & **Krigul**, **K.L**.*; Lüll, Kreete; Metspalu, Andres; Org, Elin (2022). Gut metagenome associations with extensive digital health data in a volunteer-based Estonian microbiome cohort. Nature Communications, 13 (1), 1−11. https://doi.org/10.1038/s41467-022-28464-9.

III **Krigul, K.L.*** & Feeney, R.H.*, Wongkuna, S., Aasmets, A., Holmberg, S.M., Andreson, R., Puértolas Balint, F., Pantiukh, K., Sootak, L., Org, T., Tenson, T., Org, E.# & Schroeder, B.O.# (2024). A history of repeated antibiotic usage leads to microbiota-dependent mucus defects. Gut Microbes, 16(1). https://doi.org/10.1080/19490976.2024.2377570

***** These authors contributed equally. # These authors contributed equally.

My contributions to the listed publications were as follows:

Ref. I Created the study design, performed the DNA extraction of the samples, performed the data analysis, interpreted the results, prepared figures and tables, wrote the manuscript and participated in the critical review of the paper.

Ref. II Helped with the study design, created the study questions, participated in the sample and data collection, created study hypotheses, interpreted the results, prepared the figures, participated in discussions, wrote the manuscript, and participated in the critical review of the paper.

Ref. III Created the study design and study questions, participated in the sample and data collection, prepared the FMT samples and mice samples for measurements, ran the histology and qPCR experiments, performed the analysis of physiological parameters, penetrability, metabolomic and metagenomic data, interpreted the results, participated in discussions, wrote the original manuscript, and participated in the critical review of the paper.

LIST OF ABBREVIATIONS

AGA	The American Gastroenterological Association
AMP	Antimicrobial Peptide
BA	Bile Acid
CEA	Carcinoembryonic Antigen
CRC	Colorectal cancer
EstBB	Estonian Biobank
EstMB	Estonian Microbiome Project
FDA	United States Food And Drug Administration
FIT	Faecal Immunochemical Test
FMT	Faecal Microbiota Transplantation
GF	Germ-Free
HMP	Human Microbiome Project
IBD	Inflammatory Bowel Diseases
IBS	Inflammatory Bowel Syndrome
ITS	Internal Transcribed Spacer
KEGG	Kyoto Encyclopedia of Genes and Genomes
MAG	Metagenome-Assembled Genome
MWAS	Metagenome-Wide Association Study
NAFLD	Non-Alcoholic Fatty Liver Disease
NMR	Nuclear Magnetic Resonance Spectroscopy
PPI	Proton Pump Inhibitors
PCA	Principal Component Analysis
rRNA	Ribosomal Ribonucleic Acid
SCFA	Short-chain Fatty Acid
SSRI	Selective serotonin reuptake inhibitors
T2D	Type II Diabetes
TMA	Trimethylamine
TMAO	Trimethylamine N-oxide
WES	Whole Exome Sequencing
WGS	Whole Genome Sequencing
WHR	Waist-To-Hip Ratio

INTRODUCTION

We are born into the microbial world, where microbes have been active for billions of years before our existence as species. Co-evolution between us and the microbes has led to interactions, which can be beneficial for the survival of both counterparts. It is increasingly clear that microbes have an extensive effect on the host phenotype, making our bodies not only relevant for us but also creating a habitat for trillions of organisms who depend on us. We provide the nutrients and space, and in return, the microbes contribute with relevant metabolic functions and protection against pathogens. To highlight the importance of this co-existence, humans are sometimes considered holobionts or "superorganisms".

The microbiome can be studied in the context of different environments; however, this thesis focuses on the gut, which harbours most of the microbes in the human body. The thesis introduces discoveries from three relevant topics in the microbiome field: sample collection opportunities for preventative medicine and microbiome-based diagnostics, detection of novel factors that drive microbiome inter-individual variability by using a population cohort supplemented with electronic health records and elucidating causal effects using animal models. The thesis makes use of volunteer samples from Estonia, including a comprehensive dataset from more than 2500 Estonian Microbiome project participants and an additional dataset of 30 healthy volunteers, both of which were established as part of the thesis. Moreover, a "humanised" mouse model is used to verify whether microbiome changes following antibiotic use detected in the Estonian Microbiome project translate to physiological effects in mice.

In the first part of the thesis, I will review the current state of the microbiome field, briefly covering models and methods used for microbiome studies as well as highlighting the achievements and hurdles that the field is facing. After that, I will introduce the results of three original first-author publications. First, I will show that the faecal immunochemical tests used in national colon cancer screening programs can have the potential to additionally be used for microbiome studies. This opens a new opportunity to improve colon cancer screening diagnostics using microbiome data. After that, I will introduce the Estonian Microbiome cohort, the first large-scale population-based microbiome study in Estonia, and thereby show the value of Electronic Health Records in studying links between health parameters and the microbiome. The valuable findings from the Estonian Microbiome cohort dataset are further studied in the third original publication, investigating the effect of repeated antibiotic usage history on gut mucus barrier function using human donors' stool samples in the mouse model.

1. REVIEW OF THE LITERATURE

1.1 The rise of the microbiome studies

The microbiome is regarded as a collection of bacteria, viruses, fungi, archaea, and protozoa in a specific habitat in different organisms and environments. Although the importance of microscopic organisms has already been known to humankind for centuries, the human microbiome as a research field has significantly progressed thanks to several relevant breakthroughs that have been crucial for understanding the importance of these small but powerful cohabitants. These include, for example, seeing the microbes for the first time with the microscope to being able to study their biochemical properties by culturing the anaerobic strains in bulk, followed by the possibility to characterise whole culturable and unculturable communities and their potential functionality using sequencing techniques (further discussed in "The origins of human microbiota research," 2019). The microbiome field especially started to bloom thanks to the increasing development of throughput DNA sequencing at the beginning of the 21st century.

Undoubtedly, the microbiome has been a "trendy" subject with continuously increasing interest among scientists from all walks of life and the public. This can be illustrated by the number of publications about the microbiome, which has multiplied more than five-fold in the last ten years, from 5,413 studies published in PubMed in the whole year of 2014 to over 27,886 studies appearing in 2023 (**Figure 1a**). Around 5,000 studies were published about the microbiome in January 2024 alone, indicating growing scientific activity. Concurrently, the global public interest in the microbiome has steadily increased within the last ten years, reaching its peak in January 2024, as noted in the Google Search requests data from Google Trends (**Figure 1b**). This continuous interest has resulted in hundreds of registered clinical trials and the acceptance of several microbiota-based therapies being approved by the US Food and Drug Administration (FDA) in 2022–2023 (Mullard, 2023).

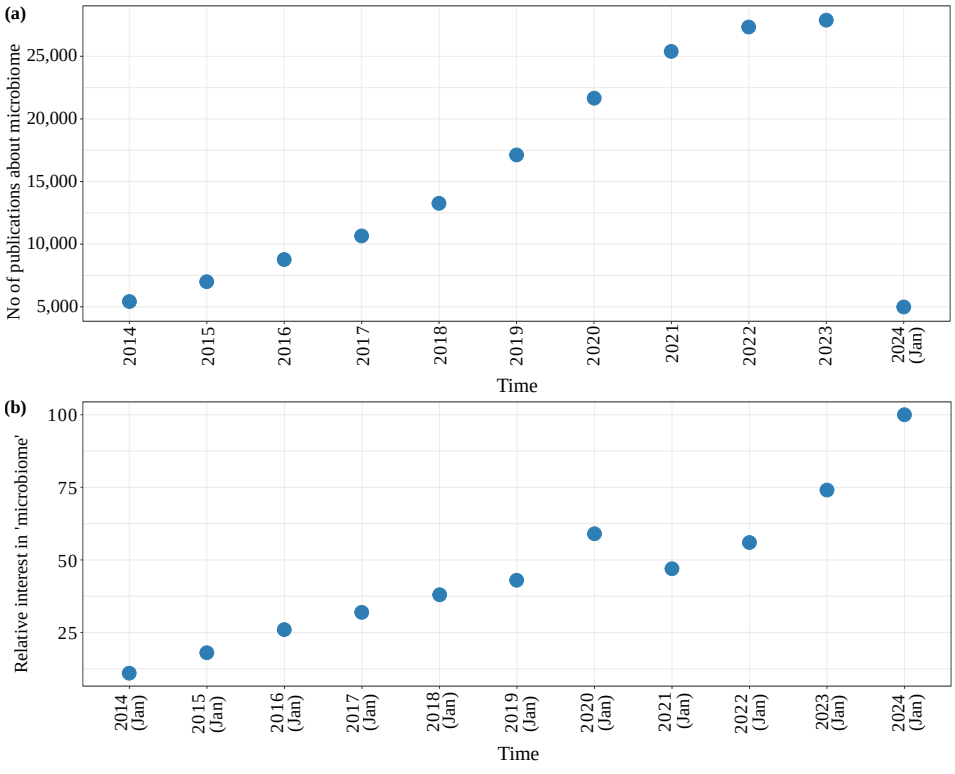

Figure 1. Interest in the microbiome. (a) – Number of publications about the microbiome in PubMed from January 2014 to January 2024. (b) – 10-year overview of the worldwide relative interest in the term “microbiome” from Google Trends. Author’s own work.

1.2 Current understanding of the human microbiome

1.2.1 Introduction to the human microbiome

Recent decades have unravelled the rich and complex hidden microbial communities inhabiting our bodies. Calculations show that there are approximately as many microbial cells as human cells in our body, although their weight seems to be minuscule compared to the human body weight (Sender et al., 2016) (**Figure 2**). The studies have also highlighted the vast metabolic capacity of the microbial community compared to humans, as illustrated by the microbiome having at least 500–2000 times more genes compared to the human genome (Li et al., 2014; Tierney et al., 2019) (**Figure 2**). This incredibly rich genetic variability helps to make the microbiome as a whole relatively resilient, enabling it to survive and persist in a variable environment, even after different perturbations.

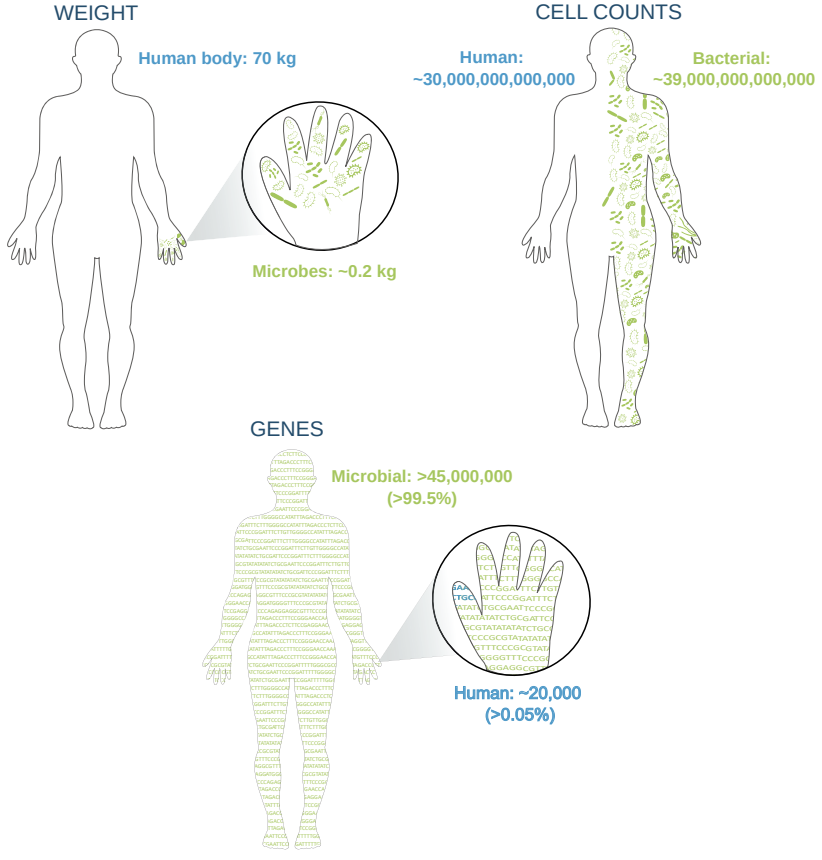

Figure 2. Human-to-microbe weight, cell, and gene count ratio (Author's own work based on data from Sender et al., 2016; Tierney et al., 2019).

Significant contributors to the understanding of the structure and function of the microbiome came from Human Microbiome Project (HMP) and MetaHit consortia, characterising microbes and their functionality in different body regions and populations (Huttenhower et al., 2012; Li et al., 2014). These first large-scale human microbiome studies also showed that the members of microbial communities varied between healthy individuals, and no common microbiome composition was present in all body sites or individuals.

The current consensus is that microbial colonisation starts at birth (Kennedy et al., 2023). It has been shown that the highest concentrations and diversity of microorganisms are found in the gastrointestinal tract of the body (Huttenhower et al., 2012; Simon & Gorbach, 1984). Bacteria comprise most of this biomass, and as they are easier to cultivate and manipulate than other microorganisms, they have been the focus of most studies. Almost a complete list of the most dominant prokaryotic domains (such as phyla, genera and even species) of the Western-style populations' gut microbiome has been described. However, novel taxa are continuously being discovered, and hundreds of human-related species are still difficult to characterise experimentally. More than 5,000 microbial species have been described in the human gut globally, and the number is expected to increase (Almeida et al., 2020; Kim et al., 2021; Pantiukh et al., 2024). Although human populations share many prevalent gut microbial species, geographical regionality seems to be observed in the microbiome, especially at the strain or subspecies level, also indicating potential differences in functionality (Blanco-Míguez, Gálvez, et al., 2023; Costea, Coelho, et al., 2017a; Suzuki et al., 2022). This may be due to co-evolution between humans and our gut microbes and the microbes adapting to the host's local environments and cultural differences.

The gut microbiome displays person-specific signatures; however, groups of similar microbiome profiles or so-called entero- and community types are often observed among individuals (Aasmets et al., 2022; Arumugam et al., 2011) (**Figure 3**). These distinct community types seem to have common microbial characteristics that stratify the community into different groups. It is unclear whether these enterotypes or community types can be the result of different lifestyle factors, such as diet or disease states, or they represent optimal composition states where microbes in communities are most compatible with each other (Costea et al., 2017). Possibly both.

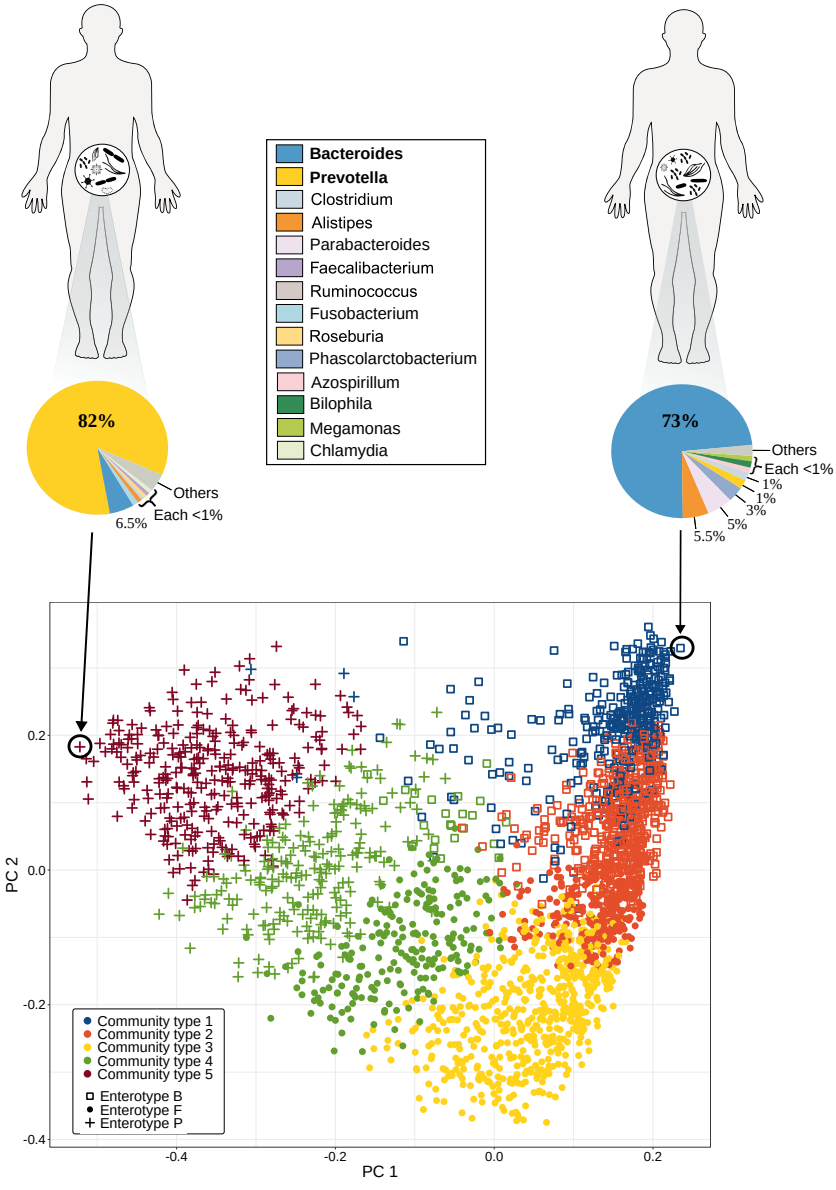

Figure 3. Interindividual variability and stratification of the gut microbial communities. Atuhor's own work based on Aasmets et al., 2022.

Various factors can affect the microbiome's composition, and the microbiome itself also affects many essential bodily functions (**Figure 4**). Some of these traits associated with the composition of the microbiome can be categorised as host-intrinsic factors. For example, genetics and biological sex, among others, act within an individual rather than being driven by a person's environment. Others are considered host-extrinsic factors, i.e., environmental, cultural and lifestyle

traits that wield their influence from the outside. These include, among others, diet, physical activity, smoking habits, and medication use. Often, it is difficult to differentiate the effect sizes of individual factors influencing the microbiome, as they might be associated with each other (e.g., disease and drug usage).

In the absence of perturbations, the adult microbiome is generally considered relatively stable, although some oscillations occur (Abu-Ali et al., 2018; Chen et al., 2021). During our life, main shifts occur from infancy to puberty and from adulthood to ageing (Martino et al., 2022). The stability is also observed at the metatranscriptome level, though the transcriptome is observed to be more dynamic (Abu-Ali et al., 2018). Additionally, the general functional potential of the microbiome is observed to be stable (Huttenhower et al., 2012). The microbiome composition can return to the baseline even after transient disruptions, as can be shown with repetitive diurnal 24-h period shifts within the same healthy individuals (Thaiss et al., 2014). Short-term changes in dietary patterns may modulate the microbiome quickly; however, the composition typically returns to the pre-invention state (David et al., 2013).

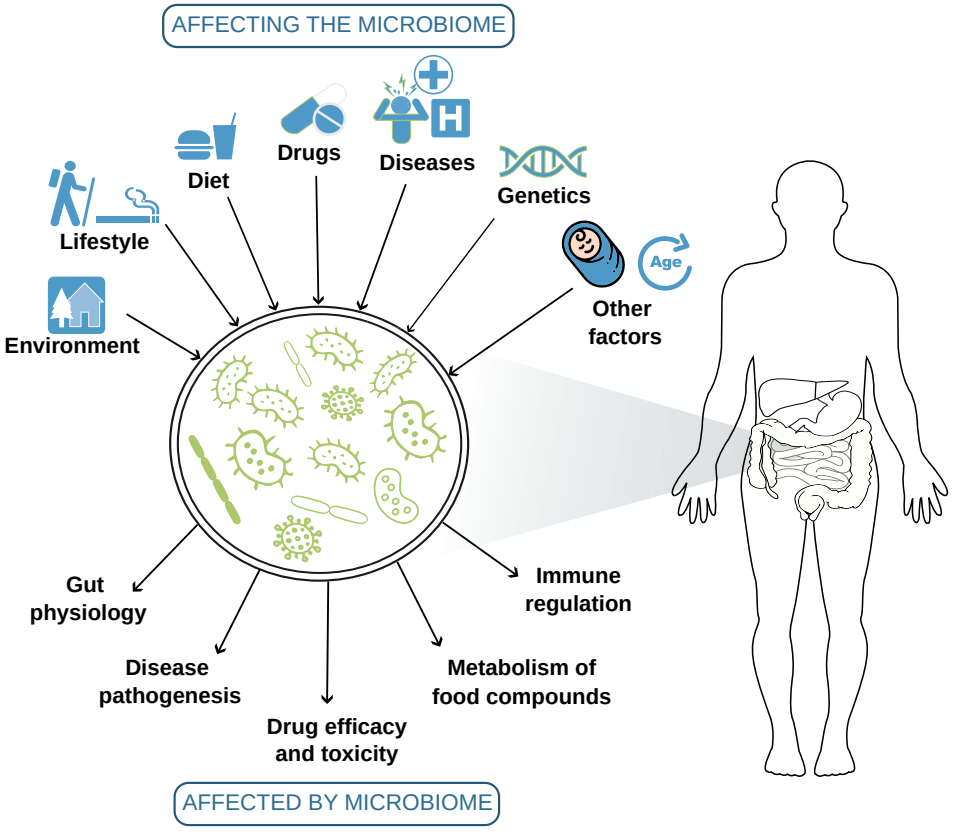

Figure 4. Gut microbiome-associated factors and functions. Author's own work.

1.2.2 Gut microbiome in human health

The microbes are important modulators of host physiology. The microbiome has even been considered an additional organ to the host's own organs, as it is involved in several essential bodily functions, such as food digestion and energy extraction, activation and modulation of the immune system, pathogen defence, regulation of gut homeostasis, drug metabolism and detoxification, production of various important metabolites, such as short chain fatty acids (SCFAs), secondary bile acids, neurotransmitters (e.g. serotonin and gamma amino butyric acid), trimethylamine N-oxide (TMAO), etc. (**Figure 4**) (reviewed, for example, in Bik et al., 2018; Cani, 2018; Gilbert et al., 2018; Liu et al., 2022).

The colon, where the highest concentrations of microbes reside, is an essential part of the intestine, aiding in food digestion and preventing colonisation of pathogenic microbes. The gut microbes significantly contribute to these functions; however, when not controlled, they may pose a threat. To physically separate these trillions of microbes from the epithelial surface, the intestines are lined with a gel-like mucus layer (**Figure 5**). The colonic mucosal barrier consists of the inner sterile denser layer, which is impenetrable to the microbes, and a looser outer layer, where microbes reside (Luis & Hansson, 2023). Initially considered only a lubricant for stool, the mucosal barrier has been shown to be an important facilitator of gut homeostasis, protecting intestinal epithelium against infection and inflammation as well as providing nutrients and attachment sites for the gut microbes, facilitating their colonisation (Paone & Cani, 2020). The mucosal layer is also an important modulator for the host immune system, acting as a reservoir for host defence molecules such as antimicrobial peptides (AMPs).

Dynamic bidirectional interactions between the host and the microbes help to regulate the mucosal barrier function (**Figure 5**). The host protects itself against the microbes by the goblet cells continuously secreting mucus to push the microbes away from the epithelium (Luis & Hansson, 2023). Additionally, the host controls the levels of the microbes by producing antimicrobial peptides (AMPs) and antibodies (De Vos et al., 2022; Mukherjee & Hooper, 2015). *Vice versa*, the microbiome and microbial composition have been shown to affect mucosal barrier properties (Jakobsson et al., 2015; Johansson et al., 2015). The gut bacteria can additionally metabolise and produce many molecules that can modulate the gut environment and maintain the healthy gut barrier function. These include, for example, SCFAs, such as acetic, propionic, and butyric acids, which, among other roles, help to provide energy for epithelial cell proliferation, maintain an anaerobic environment in the gut, and regulate immune cells to produce antimicrobial factors (Cani, 2018).

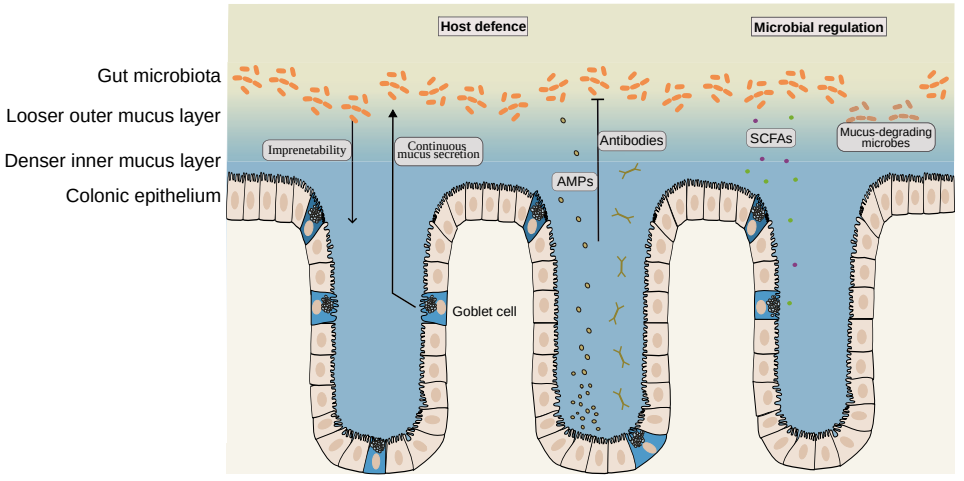

Figure 5. Bidirectional interactions of the colonic mucosal barrier with the gut microbiome. AMP – antimicrobial peptides, SCFAs – Short Chain Fatty Acids. Author's own work.

Just as we have primarily broadened our focus from understanding monogenic diseases to more complex polygenic diseases, microbial research is expanding its interest from single microbial taxa-induced diseases to more complex community-mediated conditions. In the context of the microbiome, we are beginning to understand that "one bug = one disease" might not even be the case for infectious diseases as these disease-related "pathogens" can be part of a normal microbiome, often causing no harm (Crobach et al., 2018; Ferretti et al., 2023).

There has been an exponential increase in studies linking altered microbial taxa and characteristics of the microbiome to different types of diseases. These include, for example, inflammatory bowel diseases, cancer, diabetes, coeliac disease, and obesity, a modest list of a plethora of conditions being studied in relation to the microbiome (De Vos et al., 2022). In some cases, individual microbial taxa are associated with diseases without any major shifts in the community markers (such as Shannon alpha diversity) (Wirbel et al., 2019). On the other hand, some of the diseases may lack consistent taxa associated with the disease but have clear differences in the whole community-based markers, e.g. lower microbial richness, as can be the case for obesity (Lin et al., 2023) and inflammatory bowel diseases (IBD) (Abdel-Rahman & Morgan, 2023). Whether these detected changes in different diseases are causal, consequential, or bystander remains unresolved. Determining the directionality of the associations can be problematic, as they are seldom unidirectional and usually rather complex. Additionally, microbiome changes might not even be disease-specific, as shown by a meta-study of 28 metagenome-wide association studies' (MWAS) datasets, where the overlap of microbiome signatures between different diseases was found (Duvallet et al., 2017). Additionally, the disease state and microbiome com-

position changes are most likely also linked to additional factors (e.g., inflammation). Therefore, causal factors and the functional mechanisms behind the detected associations still need to be elucidated.

Finding disease-specific signals is further complicated as, to date, no consensus exists in the scientific community on what a healthy microbiome is, as there seems to be no clear, healthy microbial makeup. Significant variability and uniqueness between individuals make characterisation a "common" healthy microbiome difficult. Although the terms "good" and "beneficial" or "bad" and "harmful" are frequently used for describing the microbes, the microorganisms and their metabolites are generally neither, as their impact on the host depends largely on the context. Therefore, these dualistic classifications remain rather simplistic. The term "dysbiosis", i.e., the imbalance in the microbiome, is often used when describing various alterations in the microbiome associated with human conditions. Although the term is regarded as vague and has limited clinical application (Olesen & Alm, 2016), it is still common practice to characterise disruption of microbial composition and function with the term. As current knowledge in the microbiome field is mostly based on correlations from single timepoint studies with a limited number of participants, large multi-timepoint datasets from well-characterized cohorts following individuals' health trajectories longitudinally are needed. These types of studies, in combination with functional analyses, could help to tackle the questions of causality in the human microbiome.

1.3 Studying the microbiome – opportunities and considerations

The microbiome can be studied using different methods, models, and study designs. Various focus points and resolutions could be targeted, giving different aspects of relevant information about the structure and function of the microbiome in various habitats and organisms (**Figure 6**). Historically, many microbiome studies focused on more general phylum-level taxonomic changes. However, the focus has shifted to more detailed aspects of taxonomy, such as genera, species or strains. Compared to the taxonomical aspect of the microbiome, functional resolution is still rather unexplored and requires more detailed systematic studies. In terms of study scope and experimental design, all aspects highlighted in **Figure 6** are still very relevant points of focus depending on the study question.

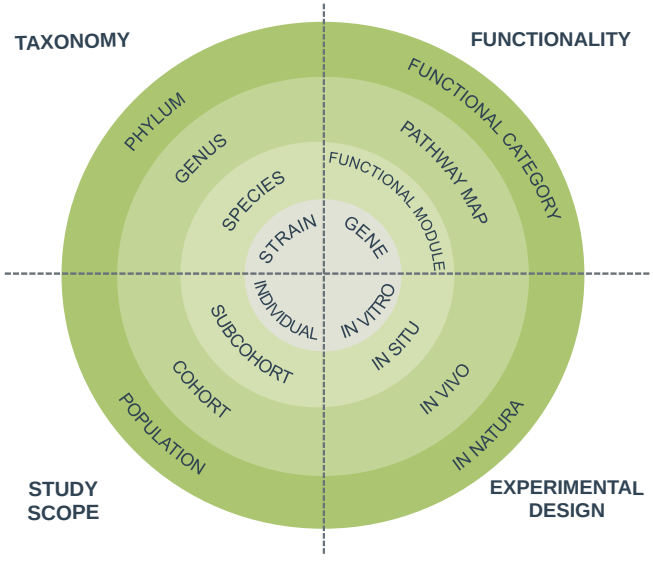

Figure 6. Different resolutions and approaches of microbiome research (Author's own work based on Schmidt et al., 2018).

1.3.1 Methods for characterising the microbiome

Although the field is continuously improving, several methods have taken hold, allowing for improved characterisation of these microbial communities and their role in human health (**Figure 7**). Sequencing approaches analysing microbial DNA, such as targeted amplicon and shotgun metagenomic sequencing, have dominated the field and are extensively used to characterise the microbiome.

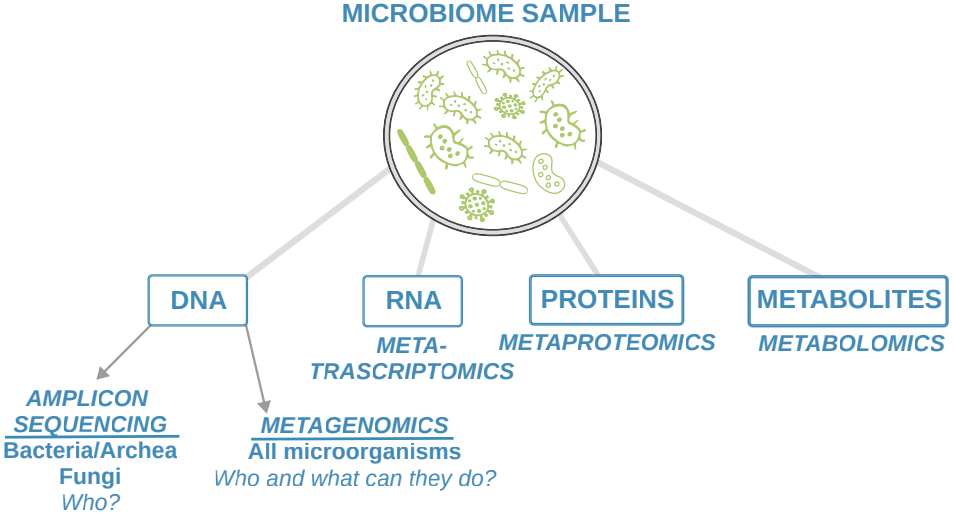

Figure 7. Commonly used methods to characterise microbiome. Author's own work

Targeted amplicon or **marker gene sequencing** uses the conserved genes and regions in groups of organisms to characterise the composition of microbes in the sample. For bacteria and archaea, sequencing of specific regions in the 16S ribosomal RNA gene is used, and for fungi, the internal transcribed spacer (ITS) region is commonly sequenced. The method allows to characterise the microbial composition of the samples to some degree, helping to tackle the question "Who is there?". The method is still extensively used in microbiome studies, as it is considered cost-effective, allowing microbiome analysis in large sample sets. The limitation is that it cannot capture lower taxonomic ranks of microbes (such as species and strains) and disregards other kingdoms in the microbiome, e.g., viruses or eukaryotes. The method also has limited capacity to characterise the functional capacity of the microbes.

Metagenomic sequencing uses total genomic material present in the sample for analysis and allows the compositional and functional profile of the community from all domains of life to be characterised. The method addresses the questions "Who is there, and what can they potentially do?". Characterisation of the community can be done using reference-based or *de novo* assembly-based methods. Reference-based characterisation or metagenomic profiling uses databases with previously characterised and catalogued microbes to assign taxonomy or functionality to the sequenced reads. The limitation can be the databases' content and size, e.g. species from previously uncharacterised populations not being present in the reference databases (Blanco-Míguez, Beghini, et al., 2023; Pantiukh et al., 2024). These novel species can be discovered using d*e novo* metagenomics assembly, which allows for the reference-free reconstruction of the genomes present in the samples. However, higher sequencing depth and computational power are needed to assemble the Metagenome-Assembled Genomes (MAGs) from complex communities, which can be more costly and labour-intensive compared to reference-based profiling. Fortunately, the mappability of the faecal metagenomic reads has significantly improved in the last decade, as some time ago, taxonomy couldn't be assigned to half of the sequencing reads but is now higher than 85% with the median around 95% in some studies (Thomas & Segata, 2019). However, we still lack functional annotation for many genes. Thus, there is still much to discover about the functionality of the microbiome.

Although sequencing has been dominating the field for the past few years, other omics methods, e.g., metatranscriptomics, metaproteomics, and metabolomics, have also proven to be useful for microbiome-related discoveries, especially in providing insights into the functional activities of microbes and answering the question "What do they actually do?". These methods, especially metabolomics, have been frequently used in combination with findings from metagenomics sequencing and are also expected to be increasingly used in population-based studies as they become cheaper and more standardised.

1.3.2 Models to study the microbiome effects

Whether changes in the microbiome are responsible for the conditions and diseases or merely a side effect of the changed microenvironment needs to be studied and proven. This can be done using different approaches (**Figure 8**). The models vary in complexity and depth in which the functional mechanisms could be assessed. There are more reductionist setups, like *in vitro* and *in vivo* studies, where microbes' functional effects and causal roles can be studied. These studies may require successful isolation and cultivation of taxa of interest. With regards to treating humans or understanding their diseases, using human samples is relevant. However, the complexity of these studies is higher, and functional understanding might be difficult to obtain. Furthermore, human samples are not always possible to receive for ethical reasons. Therefore, more simplistic and attainable approaches are used.

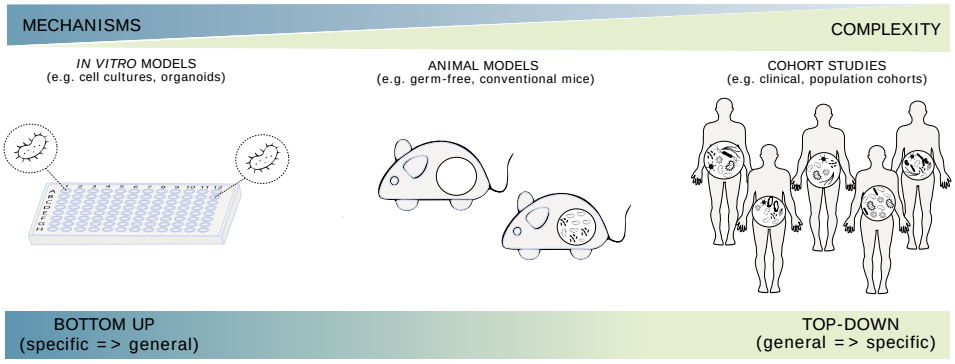

Figure 8. Models used to study environment − the microbiome − host interactions. Modified from Lindell et al., 2022.

In ***in vitro* models**, the effect of specific conditions or factors can be tested on individual strains or pre-defined communities, independently of the impact of the host (Maier & Typas, 2017). This can be upscaled to test numerous conditions or agents simultaneously, e.g., testing 1200 drugs on 40 gut microbes (Maier et al., 2018). The studies are essential to generate or test hypotheses for animal models and human studies and explain the potential functional mechanisms behind observations from cross-sectional studies in great detail.

Animal models, especially murine models, are often used for assessing causality. The germ-free mice and colonisation of mice with human microbiota have been important methods in uncovering several aspects of the role of the microbiome in different functions of the body (Kostic et al., 2013). Using gnotobiotic (i.e., an animal with known microbial communities) or germ-free models (i.e., grown sterile and having no microbial communities) allows researchers to control the experimental setups and the microbial communities in the body. Microbial associations detected in cross-sectional studies are sometimes experimentally validated in animal models using faecal microbiota transplantation

(FMT) (Wang & Jia, 2016). While mouse models have their advantages, several limitations need to be acknowledged. Their digestive tract is anatomically and physiologically different, differences exist in the immune system, cage effects often occur due to coprophagy, and the microbiome is different between mice and humans (Park & Im, 2020). Therefore, *in vitro* and *in vivo* findings can sometimes be challenging to translate into human context. Furthermore, germ-free models can be expensive, need specific facilities and conditions to be maintained, and are therefore not widely available (Kennedy et al., 2018).

Human population studies can be used to investigate healthy people and individuals with different diseases. Collecting samples together with large amounts of health data allows these studies to identify health-associated microbial biomarkers. Several large-scale (here regarded as >1000 participants) cross-sectional studies have associated stool microbiome composition with various host and environmental factors, providing an integrated view of the relative impact of the factors on the microbiome and identifying relevant co-variates affecting the microbiome (Falony et al., 2016; Gacesa et al., 2022; Si et al., 2022; Zhernakova et al., 2016). These population-based studies have already revealed many previously unknown links and expanded our understanding of factors that drive the differences between individuals. They have also helped determine lifestyle factors which are the most important in influencing the microbiome. Nevertheless, running microbiome studies with humans is complicated due to sample collection issues, ethical problems, and huge variability between individuals, as well as the difficulty of interpreting the functional background of the results. Furthermore, large sample sizes are needed to detect more specific associations with microbial parameters and human characteristics, as only a few species out of hundreds are present in almost all samples, with the majority of species detected in less than 50% of samples (Sanna et al., 2022).

1.3.3 Considerations and limitations in microbiome data analysis

The microbiome is a complex network system with parts interacting and affecting each other in different contexts. Every individual has their own specific microbiome, which can complicate finding disease- or trait-specific signatures. Usually, the sample sizes in microbiome studies are relatively small, while many microbial markers (e.g., hundreds to thousands of species or genes) are analysed simultaneously. Different species might share functional characteristics. The species are also intertwined in metabolic pathways and networks. Although many diseases and conditions have been linked to changes in the microbiome, the causal role of these associations often remains elusive (De Vos et al., 2022). Several factors hinder finding consistent associations, including the compositional nature of the data, technical variation, nature of the studies and many more.

A vast amount of the data comes from cross-sectional studies. These studies have been crucial in determining microbiome associations with different factors and are still relevant to date, mapping microbiomes of various populations world-

wide. However, inherent limitations exist with these studies. For example, understanding how species interact with each other is difficult to interpret from these types of studies, as interactions in the microbiome can be dynamic in space and time (Olsson et al., 2022; Tropini et al., 2017; Vandeputte et al., 2021). Additionally, within-study and between-study batch effects may occur (Schmidt et al., 2018). These differences may originate from sample collection and storage conditions, extraction protocols, and computational analysis (Bharti & Grimm, 2021; Costea et al., 2017). Although the technical variation has a considerable effect, the overall effect size is still lower than the other factors combined, affecting the microbiome (Gacesa et al., 2022). To increase reproducibility across different disciplines in the microbiome field, guidelines for reporting data have been created (e.g. STORMS checklist by Mirzayi et al., 2021). Nevertheless, there is still an urgent need to develop standards for the microbiome field, and efforts to establish these are actively ongoing (e.g., Human Microbiome Action https://humanmicrobiomeaction.eu/).

Most studies use DNA sequencing for microbiome analysis. However, microbiome data from DNA sequencing is compositional in nature and, therefore, non-quantitative (Gloor et al., 2017). Relative abundances of taxa or genes are inferred from non-informative total library sizes and then related to various factors. Depending on the bioinformatics pipeline or used on microbiome data, the results can vary significantly (Sun et al., 2021). The compositional data analysis may also introduce false-positive associations between taxa or taxa and covariates. When trying to determine which microbes differ between study groups, the results might also differ depending on the differential abundance analysis method used (Nearing et al., 2022). Furthermore, there is a problem interpreting zero values in sequence counts, which may not always mean that the microbe is not present in the sample (Silverman et al., 2020). An additional aspect to keep in mind when using microbiome sequencing data is that analysing DNA does not mean we capture microbes that were alive in the sample. To alleviate the issues with data compositionality, several quantitative approaches have been introduced, including spiked-in standards and known cell numbers (Satinsky et al., 2013; Stämmler et al., 2016), flow cytometry (Vandeputte et al., 2017), and predicting cell counts using machine learning models (Nishijima et al., 2024).

Many studies still run genus-level analysis; however, the focus is shifting to species or even strain level, the preferred taxonomic unit in microbiology (**Figure 6**). Even the strain level might not be ideal, as studies on pathogen isolates have shown that there may be enormous strain-level diversity and core genes only capture some of the variability present (Thomas & Segata, 2019). Even two bacterial strains from the same species might have vastly different genetic makeup, so genus-level studies capture only limited genetic information (Blanco-Míguez, Gálvez, et al., 2023). Moreover, the accessory or variable genome, i.e., the set of genes present in some but not all strains of a species, maybe more than ten times larger than the core genome, i.e., the set of genes present in all species (Thomas & Segata, 2019).

1.3.4 Microbiome-based diagnostic and prognostic tools

Microbiome analysis can be complicated, and it requires computational resources and specific knowledge. Therefore, we must consider whether measuring the microbiome adds value to traditional diagnostics or therapeutics. As an example, obesity might be linked to the gut microbiome (Thingholm et al., 2019). However, it does not make sense to diagnose obesity using microbiome-based diagnostics when we could use a weighing scale. When the microbiome causes obesity, however, then these studies comparing healthy and obese individuals are relevant if they could be linked with experiments to verify the causal role of the microbiome. This type of knowledge could be used to estimate the risk of obesity in the population, potentially helping to postpone or circumvent the condition. Investigating the causal role experimentally is not always possible, as numerous microbes still cannot be grown and characterised *in vitro*. The functional mechanisms behind therapeutics are not always understood in detail, even if the therapeutics have proven useful, as can be the case for faecal microbiota transplantation. Therapeutics require causality, even if we don't completely understand the mechanism. Additionally, the detected disease-microbe associations from cross-sectional studies should be replicated independently and compared with other phenotypes to ensure the association is robust and specific to the disease of interest.

Modern healthcare is focused on preventing or delaying disease onset and complications by preventing the diseases through lifestyle modifications for populations at risk. Various diagnostic methods, such as blood tests, physical examinations, imaging and biopsies, are used. However, more efficient, non-invasive, easy-to-collect biomarkers are continuously being researched (Ratiner et al., 2023). There is great interest in using microbiomes in combination with conventional risk factors for disease diagnosis and early detection, thereby more accurately identifying disease-prone individuals for screening (Ratiner et al., 2023). Additionally, the microbiome could also be used to understand personalised disease manifestations and variable responses to treatments. The microbial "fingerprints" can be an opportunity to link microbial signatures to divergent clinical outcomes in patients, potentially discriminating between diseases in different stages or with similar symptoms, e.g. in cirrhosis and fibrosis or IBD and inflammatory bowel syndrome (IBS) (Oh et al., 2020; Vich Vila et al., 2018).

A significant issue complicating finding microbiome-based diagnostic and prognostic tools is inter-individual variability and population-based differences, which may result in the microbiome-based tests not applying uniformly to all. Therefore, large-scale studies are necessary to determine statistically and biologically meaningful findings. Fortunately, research shows that consecutive samples from the same individual, when collected in the same manner, are more similar to each other (Chen et al., 2021). Therefore, this relative stability of the microbiome shows promise in the use of this type of data in diagnostics. However, more research is needed.

CRC has been a good example of microbiome-based signatures, as microbial taxa have been important discriminators in colon cancer patients in different geographically diverse datasets with comparable accuracy and specificity to the faecal occult blood tests used in the clinic (Thomas et al., 2019; Wirbel et al., 2019; Zeller et al., 2014). Additional studies on colon cancer patients showed that incorporating metagenome and metabolomics data enabled to further distinguish patients with early and late colon cancer from healthy controls (Yachida et al., 2019). Microbiome-associated serum metabolites could also be useful predictors for early-stage colon cancer, achieving higher sensitivities than commonly clinically utilised biomarkers, such as carcinoembryonic antigen (CEA) (Chen et al., 2022). Moreover, studies have shown that incorporating microbiome may improve the prediction accuracy of different diseases, such as Type 2 Diabetes and Alzheimer's, as well as predict blood glycaemic responses to diet (Ferreiro et al., 2023; Y. Liu et al., 2024; Reitmeier et al., 2020; Zeevi et al., 2015). There is also a great interest in determining the microbiome in disease-predisposing conditions, such as obesity (Thingholm et al., 2019), insulin sensitivity (Pedersen et al., 2016), and hypertension (O'Donnell et al., 2023), thereby using the microbiome as a biomarker before the disease's onset. As an example, microbial-produced metabolites, e.g. TMAO, might help to predict the risk of heart disease (Wang et al., 2011). As thousands of associations between microbial metabolites and diseases have been shown, screening these signatures could be used as a potential diagnostic or therapeutic (Wang et al., 2023).

Nevertheless, although the first studies show the potential value of the microbiome data, additional research is needed. We still need to understand the factors contributing to the interindividual variability and detect disease- and population-specific differences to create useful diagnostic, prognostic and therapeutic tools.

2. AIMS OF THE STUDY

This thesis explores various aspects relevant to understanding the value of microbiome studies in human health. These topics range from microbiome sample collection possibilities in clinical screening programs to using population studies to discover novel factors associated with the microbiome, as well as testing novel hypotheses from population studies on animal models. The specific aims of the thesis are as follows:

- To assess the suitability of stool samples from faecal immunochemical test tubes used in the Estonian colon cancer screening program for microbiome analysis;
- To characterise factors associated with gut microbiome composition using a population-based Estonian microbiome cohort (EstMB) consisting of individuals from the Estonian Biobank, supplemented with Electronic Health Records data;
- To investigate the effect of a history of repeated antibiotic use on microbiome and gut physiology using donor samples from the Estonian Microbiome Cohort for human-to-mouse faecal microbiome transplantation.

3. RESULTS AND DISCUSSION

3.1 Samples used in national colorectal cancer screening programs can be implemented for microbiome studies (Ref. I)

As more and more studies describing disease-specific microbiome signatures from stool samples take hold, there is a huge opportunity to start implementing microbiome testing for more accurate disease diagnosis and prediction where the sensitivity and specificity of traditional tests could be improved. This can be the case for colorectal cancer (CRC) screening programs where faecal immunochemical tests are used. CRC is a challenging global public health problem, and it is the second most common cause of cancer death in Europe. Recent data indicates that the incidence of CRC is increasing, especially among younger adults (Vuik et al., 2019). CRC development is a long stepwise process, enabling the detection and removal of pre-cancerous lesions before they become malignant. The detection of CRC in its early stages is of high importance, as the 5-year survival rate in early stages is higher than 90%; however, it lowers to less than 20% in later stages (Araghi et al., 2021). Therefore, many countries in Europe have started population-based screening programs for 50–74-year-olds. In Estonia, as in many other countries, the screening is done as a two-step process. First, faecal occult blood is non-invasively analysed from stool samples using a faecal immunochemical test (FIT), which is then followed by a confirmatory colonoscopy in case the FIT test is positive.

Although the screening is relatively cost-effective, the programs face many challenges, including low sensitivity for pre-cancerous and early-stage cancers, invasive diagnostics for golden standards, false negatives due to risk factors such as smoking and advanced age, lesions being missed during diagnostics and false positive diagnoses. Consequently, most cancer diagnoses happen in late stages, where the 5-year survival rate is much lower (Araghi et al., 2021). Taken together, new, highly specific, inexpensive, and sensitive non-invasive screening tests that improve the early detection of colon cancer are needed. Numerous studies have already linked the microbiome to colon cancer (reviewed in Wong & Yu, 2023). Microbiome signatures from stool have been shown to predict colon cancer, and stage-specific signatures have also been found (Wirbel et al., 2019; Yachida et al., 2019). The samples in these studies are mainly collected using standard methods of the microbiome field, i.e., collecting stool samples without stabilisation liquid and immediately freezing these samples. The collection method, however, is not part of standard testing for colon cancer, which instead uses faecal immunochemical test tubes (as recommended by the European Commission for EU colon cancer screening programs). The microbiome sample should ideally also be collected before colonoscopy-related bowel cleansing, as the procedure is associated with the loss of individual taxa (Jalanka et al., 2015). Consequently, the samples

used for microbiome studies, which were collected after colonoscopy, might also be different from the original samples.

Therefore, our study aimed to test whether the microbiome could already be captured from the same faecal immunochemical test tubes used in Estonian colon cancer screening programs, which are usually collected as a first step as part of the screening. This would circumvent the need to collect an additional stool sample, as both the faecal occult blood test and microbiome characterisation could be done from the same FIT before any invasive procedures. We used the immediately frozen stool, i.e., the "gold standard" of microbiome studies, as the basis, and additionally, a stool was collected in a stabilisation liquid for comparison. The study was done as a pilot study in parallel to the colon cancer study to show the appropriateness of FIT samples for microbiome collection.

3.1.1 Description of materials and methods

The study used 30 healthy Estonians (16 women and 14 men, age 39 ±12.1 years, mean BMI 24±4.7 kg/m^2) who had not taken any antibiotics within two months before sampling and had no known gastrointestinal disorders. The participants took seven stool samples using three different sample collection methods – stool sample without any stabilising solution frozen quickly at −20 °C after sample collection (i.e., golden standard of microbiome studies), stool sample in stabilisation buffer tube (DNA-RNA Shield tube, Zymo Research, Irvine, California), and stool sample in faecal immunochemical test tube (FIT, Aidian, Espoo, Finland) used in colon cancer screening programs in Estonia (**Figure 9**). FIT and stabilisation buffer tubes were collected in three replicates; one was frozen at the same time as fresh stool without a buffer, and two others were kept at room temperature for four (96h) and seven days (168h), respectively. This was done to imitate the approximate time the samples may be at room temperature during the delivery to the study centres with the national postal service. All samples from the participants were collected within the same week. In total, 214 samples were analysed, including positive and negative controls. The DNA from the samples were extracted using a Qiagen PowerSoil Pro DNA extraction kit. Sequencing was done using Illumina's next-generation MiSeq sequencing, using the V3-V4 hypervariable region of the prokaryotic 16S ribosomal RNA gene.

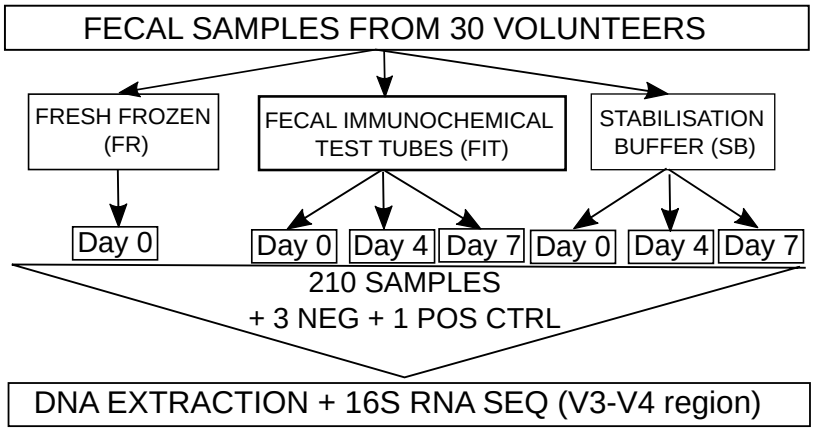

Figure 9. Overview of the sample collection and microbiome analysis. Day 0 – immediately frozen samples; Day 4 – samples were kept at room temperature for 4 days; Day 7 – samples were kept at room temperature for 7 days; Neg – Negative control; Pos – Positive control. Published in **Ref. I.**

3.1.2 Microbiome can be analysed from colorectal cancer screening FIT tubes

The "gold standard" for microbiome collection is a fresh-frozen stool (Wang et al., 2018). Depending on the country, as is the case for Estonia, fresh-frozen stool samples unaffected by procedure-related treatments might not be possible to obtain due to screening program logistics. Therefore, we were interested in whether the sample tubes used for faecal occult blood testing differ from the gold standard of the microbiome collection, which is often used in studies linking the stool microbiome with different diseases. Additionally, we used a stabilisation buffer as an additional control, which is often used as an alternative when fresh frozen stool cannot be obtained. Using samples from 30 healthy individuals, we showed that the interpersonal variability is greater than the variability originating from the sample collection type, and the sample collection has a significant but low effect on the variance between individuals (**Figure 10**). Beta diversity shows clustering based on individuals, not by sample collection type, and the sample type is not a significant factor influencing the microbial community according to the PERMANOVA analysis. As there is substantial personal variation between individuals, the strong suit of this study is using samples from the same person and from the same time point.

We additionally tested whether the community has changed in Day 4 and Day 7 samples compared to the Day 0 samples and discovered that the time spent at room temperature has a low impact on the microbiome samples. We detected that FIT keeps the community stable as there are no significant differences in any of the alpha diversity measurements, while stabilisation buffer samples have differences in the Shannon diversity index between Day 0 and Day 4 (**Figure 10b–e**).

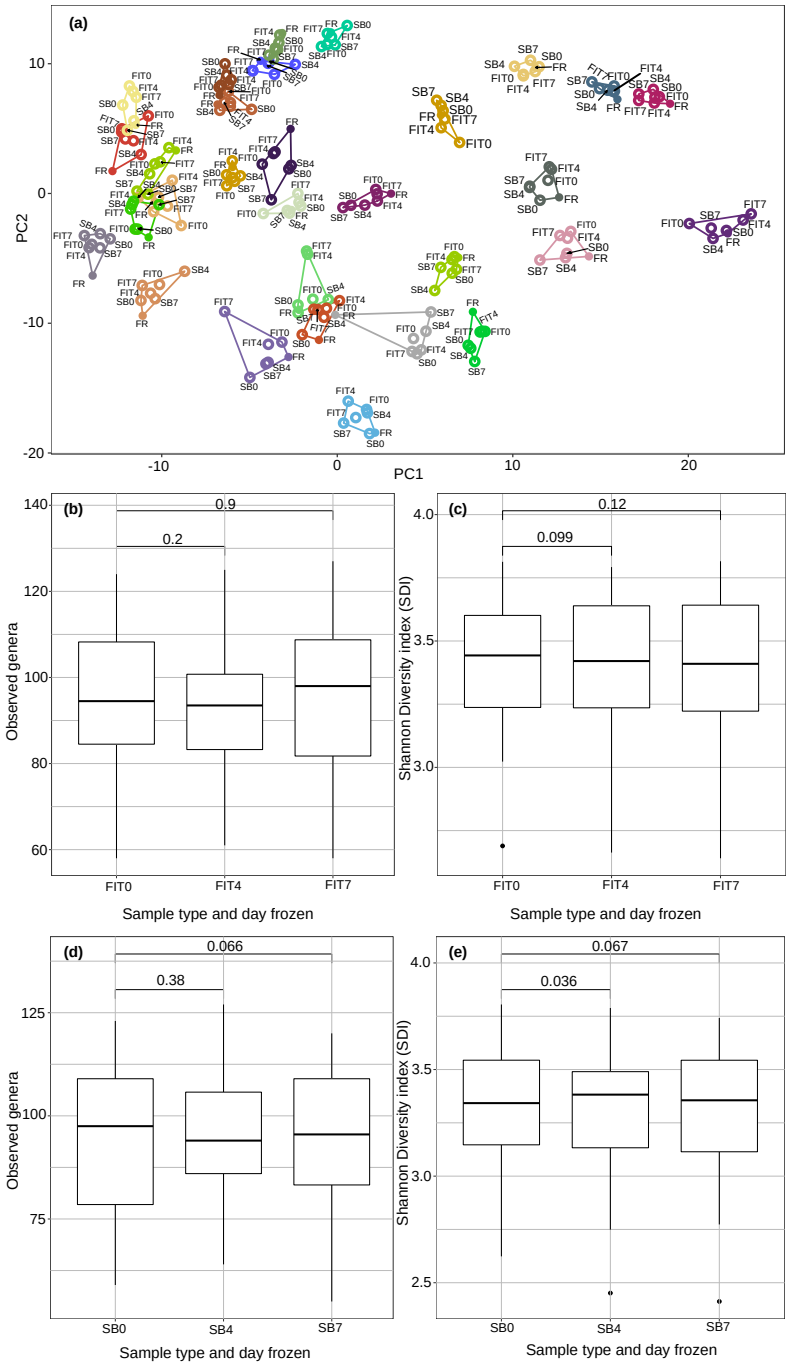

Figure 10. Microbiome diversity between sample types and time points.(a) Principal component analysis (PCA) of beta diversity between storage conditions. Samples are coloured and connected based on the individual's ID. Boxplots represent the observed genera (b) and Shannon diversity indexes (c) of FIT samples and the observed genera (d) and Shannon diversity indexes (e) of stabilisation buffer samples from different time points. FR – fresh-frozen samples; FIT0 – immediately frozen FIT samples; FIT4 – FIT samples frozen on day 4; FIT7 – FIT samples frozen on day 7; SB0 – immediately frozen stabilisation buffer samples; SB4 – stabilisation buffer samples frozen on day 4; SB7 – stabilisation buffer samples frozen on day 7. Published in **Ref. I**.

We were also interested in whether there are more specific differences in the taxonomic composition at the genus level between gold standard and FIT tubes or stabilisation buffer samples, especially in genera previously associated with colorectal cancer. Focusing on colorectal cancer-associated taxa was relevant, as the study was conducted as a pilot experiment to the larger ongoing CRC-microbiome study where patients from the screening program are used. We detected that when comparing the number of differentially abundant microbial genera in both sample types to the fresh-frozen sample, there are more differentially abundant genera in the stabilisation buffer (16 out of 171 genera, 9%) compared to samples stored in FIT (7 out of 171 genera, ~4%), indicating that the FIT keeps the microbial community more similar to the fresh-frozen community, especially in previously cancer-associated genera (**Figure 11**). Comparing the test tubes, we showed that only one genus (*Rombutsia*) was significantly different between Day 0 and Day 4 or Day 7 in FIT samples, indicating that the community captured on Day 0 remains the same for a week. The limitation of the study is that the 16S rRNA gene instead of metagenomics sequencing was used for microbiome characterisation, making us unable to characterise whether any species previously characterised as associated with colon cancer has been changed.

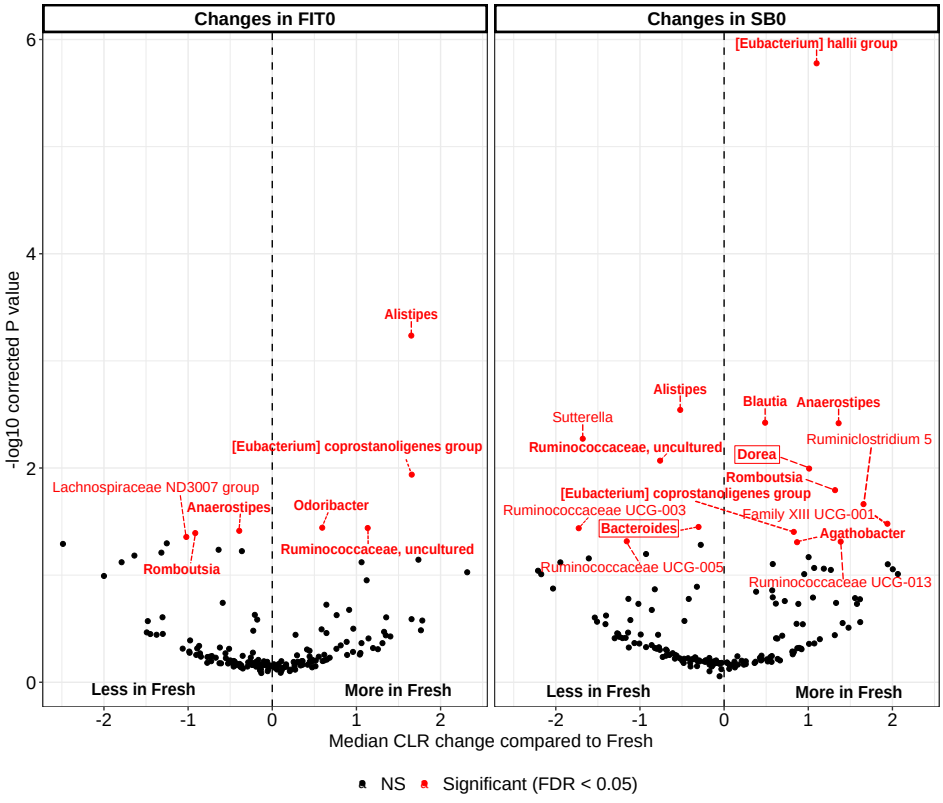

Figure 11. Genera-specific changes in the microbial communities compared to the fresh-frozen sample. Genera in bold belong to the core 95% most prevalent genera. Genera in squares have previously been associated with colorectal cancer based on (Wirbel et al., 2019; Yachida et al., 2019). FIT0 – immediately frozen Fecal Immunochemical Test samples; SB0 – immediately frozen stabilisation buffer samples. Published in **Ref I**.

3.1.3 Microbiome-based diagnostics and the potential of screening program samples

Enormous potential lies in national screening programs as a potential source for microbiome samples, which could be paired with traditional screening diagnostics or create new tools for diseases where current diagnosis methods are too invasive, insensitive, or not specific enough. More than 50,000 participants take part in the national colon cancer screening in Estonia each year. Pairing the microbiome analyses with national health registries holds great potential for microbiome tests beyond the screening programs of specific diseases, as these programs include participants of a particular age group, regardless of their health status. This testing, in combination with biobanks, which include both healthy and diseased individuals from a population, can be effectively used to validate the developed models from clinical cohorts and find at-risk populations in biobank participants to screen people at risk even earlier. This might be useful, especially in colon cancer, where the disease incidence is increasing, especially in young adults (Vuik et al., 2019), where screening programs may not include all the age groups at risk. Using the Estonian biobank data, we have also determined that around 12% of all colon cancer patients received their diagnoses when they were younger than 55 years old.

However, it is relevant to capture disease-specific signals to develop diagnostic and prognostic tools. Screening cohorts might help to identify these signals better than clinical cohorts studying only one type of disease, as overlaps between different diseases might go unnoticed. The screening programs can also help discover comorbidities and shared symptoms. Additionally, microbiome-based diagnostics must add a value beyond the current standard. In the case of colon cancer, for example, a faecal occult blood test on the stool is used as part of the diagnosis. The microbiome diagnostic should have additional explanatory power beyond this traditionally used measurement. Otherwise, it would be a complicated and expensive additional test. New studies, however, have included microbiome analysis as a complementary test to the current diagnostic method and achieved higher specificity (Malagón et al., 2020, 2023). However, in general, it is necessary to bear in mind that the screening test can only be helpful if the participation rate is high. Therefore, microbiome-based testing should not shrink these rates.

This study was done as a pilot on healthy individuals before starting to collect faecal tubes from colon cancer screening participants and patients diagnosed with colon cancer. Overall, we showed that microbiome analysis is possible from these types of samples, and the community remains the same in the time that the sample is expected to be in the post. The limitation of this study was that it used 16S rRNA gene sequencing data, which does not allow for taxa comparison of previously cancer-associated species. Moreover, due to the sampling technique, much less of the sample is obtained with FIT tubes compared to the common stool

sample collection methods, so the metagenomics and metabolomics analyses should be further tested. Furthermore, it is highly unlikely that the samples could be used for species cultivation *in vitro*. However, the study, combined with other studies, shows that microbiome testing from FIT tubes is possible and can be used for microbiome collection when fresh-frozen stool samples are not possible to obtain.

3.2 Using the Estonian microbiome cohort to describe the factors associated with microbiome compositional changes (Ref. II)

Population-based biobanks offer great opportunities to study the microbiome's role in human health. As population-based studies include both healthy and diseased individuals, they allow us to find disease-specific microbiome signals and capture healthy individuals at risk before they develop the disease. They can also be useful in determining signals overlapping between different diseases as well as determining relevant confounders affecting the disease-specific microbial signatures. Studies have also shown that population-specific microbes, which reflect lifestyle and environmental differences, exist (Gupta et al., 2017). Therefore, to understand whether the previously determined links between microbiome and different factors or diseases are cohort-specific or can also be observed in other datasets, more populations should be studied.

Here, we introduce a new large-scale population-based Estonian Microbiome cohort that utilises electronic health records (EHR) information and shotgun metagenomics sequencing data. The uniqueness of the cohort comes from the opportunity to use longitudinal health records, not relying on single time-point questionnaire-based data. Electronic health records allow the tracking of an individual's medical history not only before but also after sample collection. Although a few population-based microbiome cohorts already existed (Falony et al., 2016; Zhernakova et al., 2016), to our knowledge, only one population-based microbiome cohort, including shotgun metagenomics sequencing and follow-up health data from electronic health registries, had been published at the time (Salosensaari et al., 2021). This FINRISK cohort, similar to EstMB, allows the tracking of the health of individuals for a long period of time, enabling the determination of long-term associations between gut microbiome composition and health. Furthermore, the microbiome data from the FINRISK cohort, which was collected in 2002, has allowed for the prediction of the risk of different incident diseases, further showing the value of these types of cohorts with longitudinal health data (Liu et al., 2022; Ruuskanen et al., 2022). Nevertheless, many unknown factors influencing the microbiome in different populations likely exist, which could be studied using population-based biobanks.

3.2.1 Description of the cohort and methods

In the years 2017–2019, a subset of 2509 adult participants (age range 23–89 years, 70.3% women) from the Estonian Biobank (EstBB) were recruited to the Estonian Microbiome Project (EstMB). The participants were recruited from all counties in Estonia, and the project aimed to study the association between health parameters and microbiome. Established in 1999, the Estonian Biobank is among the leading volunteer-based biobanks in Europe, with around 20% of the adult population (> 210,000 participants, ≥ 18 years old) being recruited. The biobank aims to investigate the genetic, environmental, and behavioural background of common diseases and traits, trying to advance personalised medicine and public health in Estonia. All biobank participants have signed a broad consent form that allows scientists to access comprehensive phenotype data from the nationwide EHRs, enabling continuous monitoring of the health status of the participants across a lifespan in detail. These EHRs are recorded by medical specialists, providing the scientists with reliable information about pseudonymised individuals' disease diagnoses, prescription medication usage, and medical procedures. In addition to EHRs, the participants answered extensive questionnaires about dietary preferences, living environment and lifestyle choices. The Estonian Microbiome Project participants donated additional oral, plasma, and stool samples, which allowed us to study gut metagenome and pair it with the individuals' deep phenotypic data. The shotgun metagenomic paired-end sequencing (4.62 ± 0.44 Gb) and bioinformatic characterisation of taxonomic and functional profiles was done by Novogene Bioinformatics Technology Co., Ltd. Following the publication (**Ref. II)**, several new datasets have been collected in EstMB and EstBB; therefore, an updated timeline as of August 2024 of the sample and data collection milestones in EstBB has been included in the thesis (**Figure 12a)**. A general overview of the data and samples used in **Ref. II** is shown in **Figure 12b**.

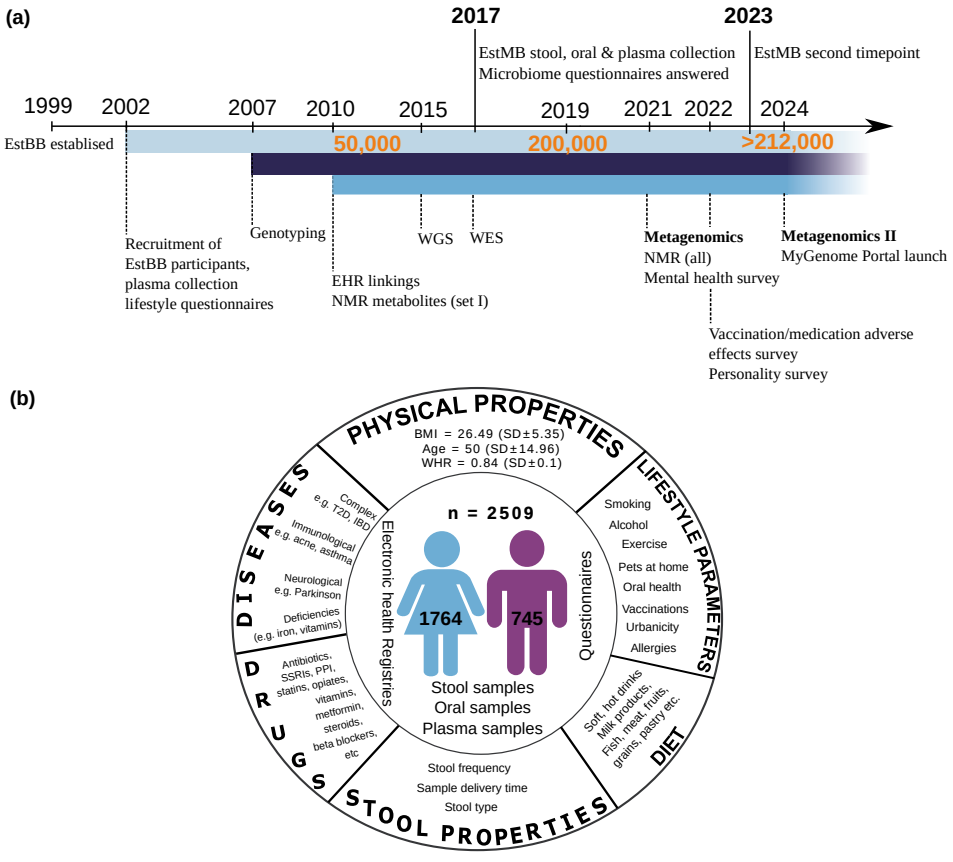

Figure 12. General overview of the EstBB and EstMB datasets. (a) Timeline of major milestones in EstBB and EstMB data collection (author's own work, first published in **Ref. II**, updated August 2024). (b) A general overview of the data and samples available in EstMB. Author's own work. EHR – Electronic Health Records, IBD – Inflammatory Bowel Diseases, NMR – Nuclear Magnetic Resonance Spectroscopy, PPI – Proton Pump Inhibitors, SSRI – Selective serotonin reuptake inhibitors, T2D – Type II Diabetes, WES – Whole Exome Sequencing, WGS – Whole Genome Sequencing, WHR – Waist-To-Hip-Ratio.

3.2.2 Landscape of the Estonian Microbiome

"Let us remain Estonians but let us become Europeans too" (In Estonian: "*Olgem eestlased, aga saagem ka eurooplasteks*") is a quote by Estonian poet Gustav Suits, which also applies to the current state of the Estonian gut microbiome. Comparing the microbiome to other population studies, the cohort has evident characteristics of a "Westernized" population, where five main phyla characterise most of the microbiome composition. The community is dominated by *Firmicutes* and *Bacteroides* (new names *Bacillota* and *Bacteroidota*), followed by *Proteobacteria*, *Actinobacteria* and *Verrucomicrobia* (new taxonomic names *Pseudomonadota*, *Actinomycetota*, *Verrucomicrobiota,* respectively) (**Figure 13a**). Even at the phylum level, interindividual variability is visible. Although the microbial taxa vary between individuals, the general functionality of the microbiome is relatively similar, as previously shown (**Figure 13b**) (Huttenhower et al., 2012). When looking at the whole microbiome composition at the beta diversity level, the *Bacteroides* genus seems to be an essential driver behind community composition differences, as indicated by the PC1-PC2 axes on the PCA plot (**Figure 13c**). What may be driving the interindividual variability in this Westernized population?

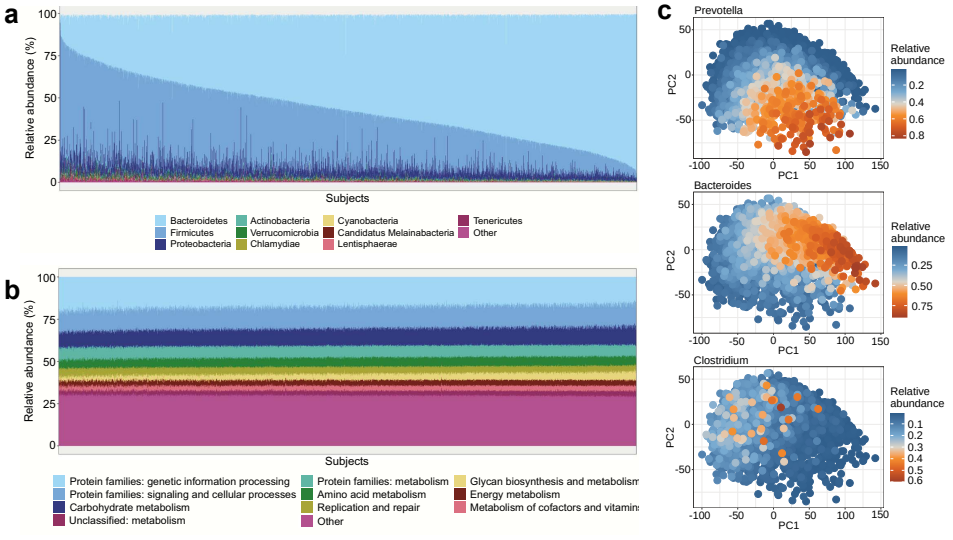

Figure 13. General microbiome taxonomic (a, c) and functional profile (b) of the Estonian Microbiome Cohort across all EstMB cohort subjects. (a) Phylum-level microbiome composition of individual participants; (b) Functional profile (KEGG domains) of individual participants; (c) Species-level PCA biplots coloured by the most dominant genus in the sample. KEGG – Kyoto Encyclopedia of Genes and Genomes, PCA – Principal component analysis. Published in **Ref. II**.

We analysed 252 diet-, disease-, medication-, and other lifestyle-related factors, determining 136 factors being associated with either taxonomic or functional level alpha-diversity, beta-diversity or certain species or KEGG orthology. Major factors affecting the microbiome variability between individuals are first stool characteristics such as stool consistency (assessed with the Bristol Stool Scale) and gut emptying frequency, followed by host intrinsic factors such as BMI, weight-to-hip ratio and disease states (**Figure 14**). This is in accordance with the previous studies, which also reported stool characteristics and BMI as the most important sources of variation (Falony et al., 2016; Zhernakova et al., 2016). In our cohort, most of the significant factors describing variation are diseases. Generally, the microbiome characteristics may reflect lifestyle choices, as stated by Professor Paul Ross at the Human Microbiome Action conference, "You get the microbiome you deserve" (*Human Microbiome Action Summit, 29 February 2024*). Smoking, eating more meat products, drinking more soft drinks, as well as having diseases or taking medications end up in a less diverse microbiome (**Figure 14**). Conversely, exercising more, eating more berries, fruits, and vegetables, and generally feeling mentally and physically healthy are also associated with a more diverse microbiome (**Figure 14**). However, as has been the case with previous studies, each trait individually explains a very low percentage of the variability in the microbiome composition. This is also the case for the overall explained variability, which also remains low (10.14 %). Since our study, the Dutch Microbiome Project study, which increased their sample size more than 7-fold compared to the first study, has still found the same factors to be relevant, including age, sex, BMI, stool consistency and gut emptying frequency, but additionally explained the significance of cohabitation and exposome (Gacesa et al., 2022). However, the explained variation still remains modest (>15%).

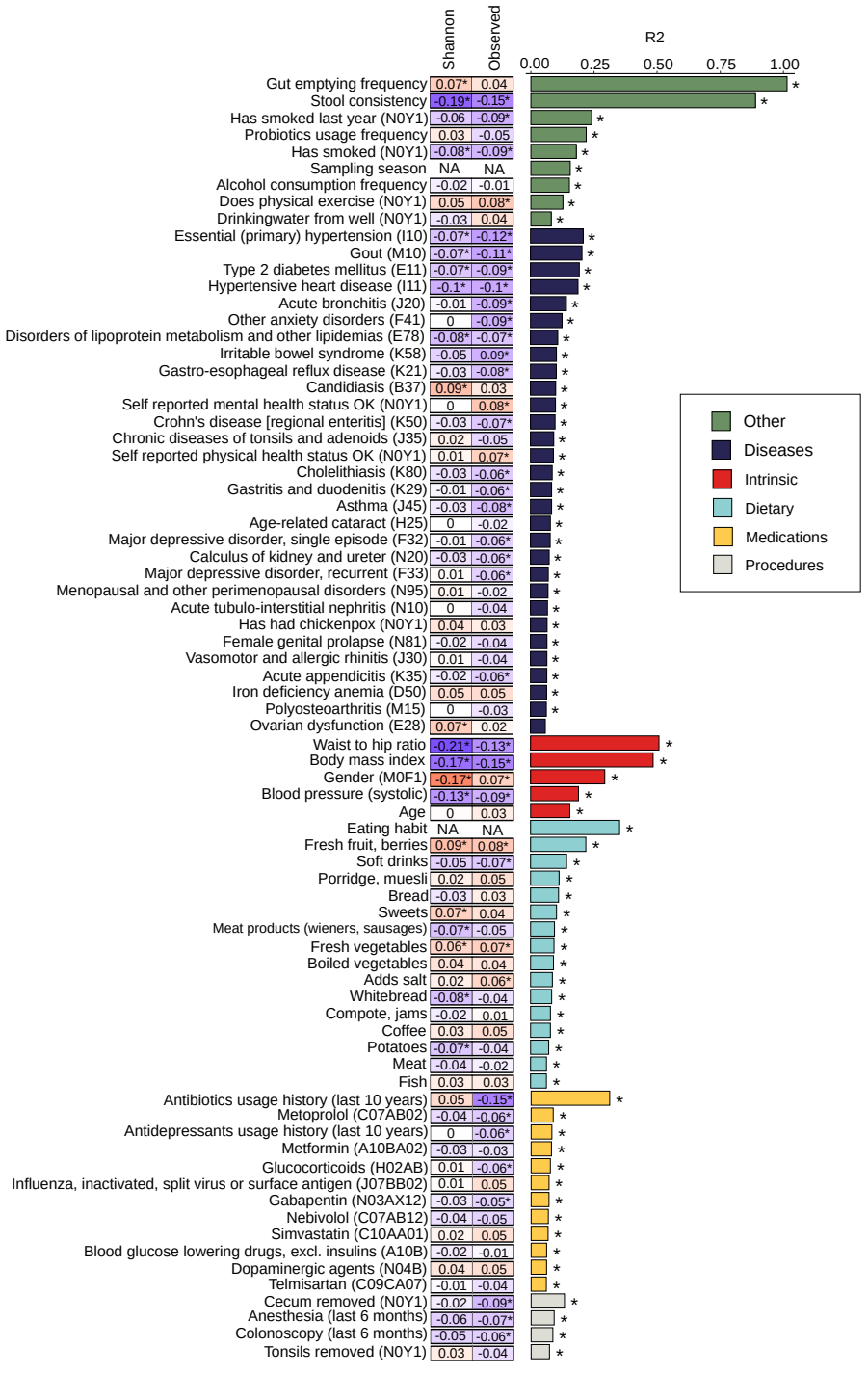

Figure 14. Significant factors associated with microbiome alpha and beta diversity. The heatmap depicts the Spearman correlation coefficients between each factor and alpha diversity metrics (Shannon's index of diversity and the observed richness of species). Blue indicates a negative correlation, and red a positive correlation. The bar plot shows how much variance of the microbial composition each factor explains (based on the Euclidean distance on the centred log-ratio-transformed data). * – FDR < 0.05. Published in **Ref. II**.

3.2.3 History of medication use is a significant factor influencing microbiome and complicates finding disease-specific associations

Confirming previous findings, we observe medication use as an important factor affecting the microbiome, with human-targeted drugs significantly affecting the microbiome in addition to the antimicrobials, where the effect on the microbes is expected. This is in accordance with the results previously shown *in vitro* (Maier et al., 2018) and also in population studies (Jackson et al., 2018; Vich Vila et al., 2020). However, EHRs allow us to study the effects of medications even further, making it possible to focus on the history of drug usage in greater detail. Interestingly, excluding antibiotics usage 6 months before sample collection, a typical exclusion period for microbiome study participants, we observed that a 10-year antibiotic use history is an important source of variation, which seems to be dose-dependently associated with loss of diversity and microbiome composition changes (**Figure 15a**). Generally, we observe that the more antibiotics a person has taken, the more Bacteroides-dominant microbiome they have. The accumulative effect of antibiotic use on the microbiome was similarly observed by Forslund et al. 2021 (Forslund et al., 2021). In our data, the effect is visible when the participant has used antibiotics 3 times in the last 10 years. As the average consumption rate of antibiotics is around 1 course per person per year in the EU, the effect is visible at a consumption rate presumably much lower than average (based on the European Centre for Disease Prevention and Control. Antimicrobial consumption – Annual epidemiological surveillance report for 2017). Depending on the antibiotics, the specific effects might vary. The most prescribed antibiotics in EstMB are Penicillins With Extended Spectrum (J01CA) and Macrolides (J01FA). However, the specific effects of antibiotic class are difficult to assess in human studies, as most people do not consistently take the same class of antibiotic in their lifetime. Nevertheless, we have recently observed that macrolides and fluoroquinolones have larger and long-term effects compared to other antibiotics, detectable years after consumption (Aasmets et al., 2024). Unexpectedly, antidepressants, representing a human-targeted drug group, seem to have similar, although milder, effects on the microbiome (**Figure 15b**). In addition to antidepressants, we have recently observed the long-term effects of several psycholeptics, e.g. alprazolam (N05BA12, e.g., Xanax) and diazepam (N05BA01, e.g. Valium), detectable even 4 years after use (Aasmets et al., 2024).

Interestingly, we also observed microbiome signatures in different diseases and shared microbial signatures between these diseases, indicating a common "dysbiosis". However, when taking the history of antibiotic use into account, most of these associations within diseases and between diseases disappeared. Our study shows that a history of antibiotic use may be behind several disease-specific associations, warranting the need to collect long-term antibiotic use history data when trying to determine disease-specific signatures or understand the risk factors of complex diseases.

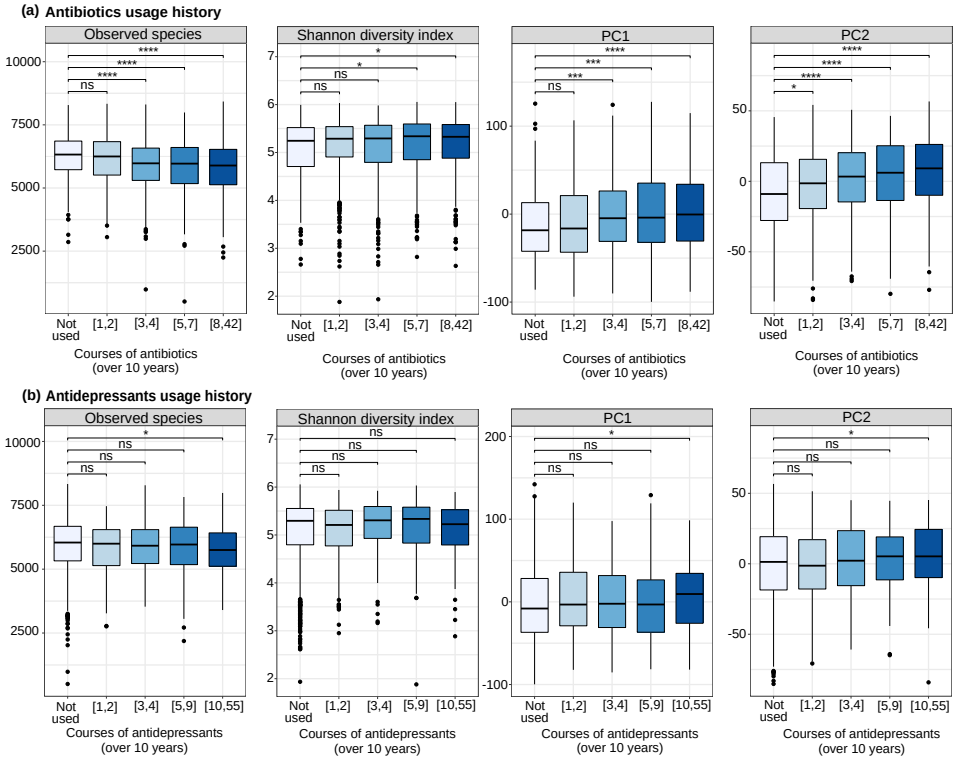

Figure 15. Associations of medication use history with different microbial parameters. Associations with history of antibiotics use (a) or history of antidepressant use (b) and microbial parameters (Shannon diversity index, Observed number of species, i.e. species richness, or the first two principal components (PCs) of the species-level microbial composition). The sample size for antibiotics were for non-users n = 243; [1,2] n = 549; [3,4] n = 440; [5,7] n = 395; [8,42] n = 400 and for antidepressants non-users n = 1761; [1,2] n = 188; [3,4] n = 96; [5,9] n = 109; [10,55] n = 115. * – FDR < 0.05, ** – FDR < 0.01, *** – FDR < 0.001, **** – FDR < 0.0001, *ns* – non-significant. Published in **Ref. II**.

3.2.4 Future directions of the population-based cohorts in microbiome studies

Following our study, multiple new microbiome cohorts have been published, and new cohorts are being continuously collected as the understanding of the importance of the topic increases globally. To date, the most significant published population-based microbiome cohorts in Europe using metagenomic sequencing data are depicted in **Figure 16**. Additionally, the metagenomics studies using the Japanese 4D and Israeli 10K cohort have been published following our publication (Nagata et al., 2022; Zahavi et al., 2023). An increasing number of metagenomics cohorts allows multi-cohort comparison studies with increased sample sizes to validate the findings and maybe address some of the challenges in the reproducibility and generalizability crisis that the microbiology and the microbiome fields are facing (Schloss, 2018).

Figure 16. Large-scale (>1000 participants) population-based gut metagenomics studies in Europe. * – 16S rRNA gene sequencing, but was among the first population cohorts. Author's own work using free European map from https://simplemaps.com/resources/svg-maps.

All the cohorts have identified many factors associated with the microbiome, but we are still explaining a surprisingly small portion of the variation between individuals (10–15%). Even the strongest covariates explain only very little of the overall differences observed between individuals. Significant contributions to microbial composition changes also originate from technical factors, such as sample collection, storage and handling, and especially DNA extraction methods (Costea et al., 2017). However, the technical variation explains a very small proportion of variation. So, what are we still missing? In general, we may lack information about relevant factors or have not accurately characterised the measured variables (Schmidt et al., 2018). As an example, the immediate effect of antibiotic use may be known. Still, we discovered how a long-term history of antibiotic use may also significantly influence the microbiome, additionally determining the long-term effects of antidepressants. Furthermore, we have recently expanded this observation, showing that several other drug classes, including psycholeptics, proton pump inhibitors and beta blockers, display long-term effects (Aasmets et al., 2024). Large-scale population biobanks, which include long-term drug use data as is available in EHRs, enable the systematic investigation of these long-term effects in great detail. Moreover, the impact of

stochastic processes may be more substantial than we previously thought, and we may only be able to predict some of the microbiome changes (Schmidt et al., 2018). Many identified associations are probably more complex and confounded by secondary factors. Furthermore, changes in microbial cell counts are likely to be relevant in addition to the relative abundance shifts (Vandeputte et al., 2017). The detected associations from population studies should, therefore, be further studied using animal models or *in vitro* methods to understand the functional background or physiological effects of detected correlations.

Although many relevant findings came from these cross-sectional cohorts, there is a need for prospective cohorts following individuals in time. One of the significant strengths of the EstMB cohort is the possibility of using EHRs to investigate disease occurrences, medical procedures, and medication usage before and after sample collection. This, in combination with the chance to recontact the subjects and invite them for additional studies, allows us to study incident diseases without being hindered by medication/procedural effects. Longitudinal studies could provide insights into the microbiome's dynamics and help identify disease-specific signals in the microbiome. Some associations emerge only when looking at even lower taxonomy levels than species (Costea, Coelho, et al., 2017b), indicating the need to describe subspecies or strain-level data. In addition, the understanding of the functional capacity of the microbiome remains low, as most of the functional genes of the microbiome still need to be characterised, and this is a very relevant aspect of understanding the microbiome's role in diseases.

3.3 A history of repeated antibiotic use in healthy human donors leads to microbiota-dependent mucus defects in mice (Ref. III)

Antibiotics have been used effectively to treat bacterial infections in humans and animals for over half a century, and they are considered the foundation of modern medicine. However, excessive antibiotic use does not come without consequences, as they are losing their efficacy against pathogens due to the spread of antimicrobial resistance, and their use has been associated with the pathogenesis of complex diseases, such as IBD, coeliac disease, diabetes, etc. (Fenneman et al., 2023). Many complex diseases, such as IBD, obesity, and diabetes, have also been associated with deficient gut mucosa (Johansson et al., 2014; Miranda et al., 2019; Schroeder et al., 2020; Shen et al., 2019). The gut mucosal barrier is an important defence mechanism, keeping the microbes at a distance from the colon epithelium. The gut microbiota has been shown to influence the mucosal layer, as illustrated by germ-free mice having a penetrable inner mucus layer (Johansson et al., 2015) and microbiome compositional differences affecting mucus function and development (Jakobsson et al., 2015). Intrigued by the finding of long-term antibiotic effects on the microbiome in the Estonian Microbiome cohort **(Ref. II),** we wanted to investigate further whether these antibiotic-driven changes in the

microbiome could have consequences for the host physiology. As antibiotics influence the microbiome, and the microbiome influences the mucosal barrier, we were interested in studying the link between the two. Making use of a deeply phenotyped Estonian microbiome cohort and human-to-mouse faecal microbiota transplantation (FMT), we aimed to determine the effects of antibiotics-modified microbiota on the colonic mucosal barrier.

3.3.1 Description of materials and methods

Using the phenotypic and electronic health records data from the Estonian Microbiome cohort, we could carefully select otherwise healthy donors whose samples were used for a human-to-mouse FMT experiment to study the effects of the history of repeated antibiotics use on the mucosal layer in the colon (**Figure 17a**). From 2509 individuals, participants with complex diseases or recent medication histories influencing the alpha and beta diversity of the microbiome, according to our previous study **(Ref. II, Ref. III Supplementary 1 and 2)**, were excluded. Thereafter, four otherwise healthy donors with a 5-year history but not a recent 6-month history of antibiotic use were selected (age 41±12.4 years, 75% female, BMI 23.5±1.79 kg/m^2). These donors were matched with four healthy donors without a 10-year history of antibiotic use based on age, sex, BMI, Bristol stool scale and diet (age 45±12.4 years, 75% female, BMI 22.4±2.44 kg/m^2). The stool samples from donors were pooled according to the respective groups and then transplanted three times to microbiota-depleted adult mice (**Figure 17b**). The mucus function, including mucus growth rate and penetrability, was studied using an *ex vivo* assessment of viable tissue 10 days after the first human-to-mouse microbiota transplant. Shotgun metagenomic paired-end sequencing was carried out by Novogene Bioinformatics Technology Co., Ltd.

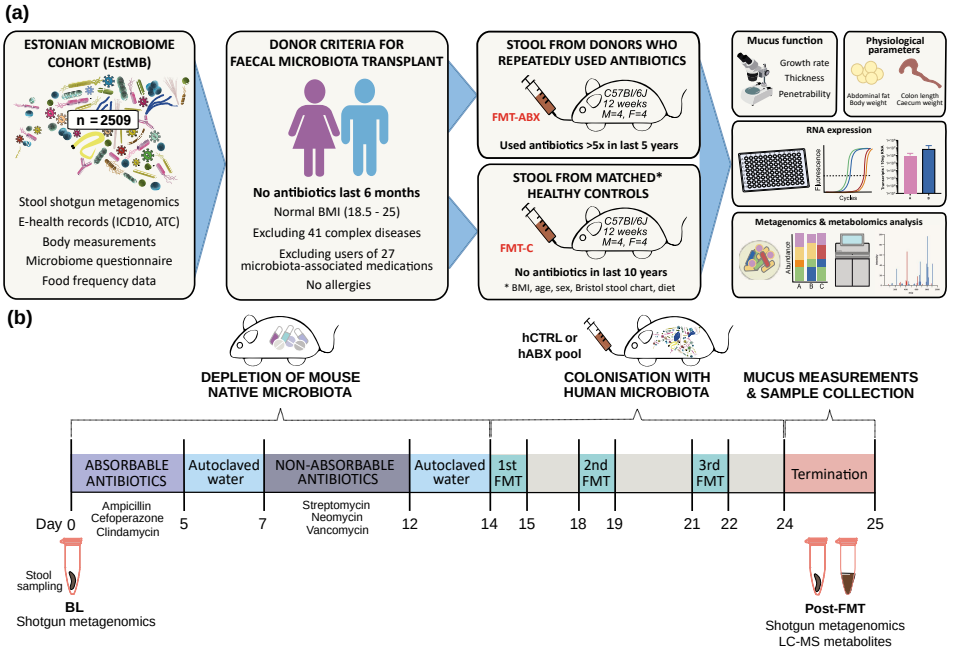

Figure 17. Human-to-mouse faecal microbiota transplant study outline. (a) Workflow of faecal microbiota donor selection and experimental plan of the study; (b) Outline of the mouse experiment. ATC – The Anatomical Therapeutic Chemical Classification, BL – mouse baseline stool, FMT – Faecal Microbiota Transplant, FMT-C – mice that received FMT from the hCTRL pool, FMT-ABX – mice that received FMT from the hABX pool, hABX pool – pooled stool from human donors with a history of repeated antibiotic use, hCTRL pool – pooled stool from human controls with no history of antibiotic use in 10 years preceding stool collection, ICD-10 – 10th revision of the International Classification of Diseases, Post-FMT – samples taken at termination after faecal microbiota transplantations. Published in **Ref. III**.

3.3.2 FMT from human donors with a history of antibiotic use results in physiological, microbiome- and metabolome-related differences in mice

Initially thought only as a lubricant for faecal matter, the mucosal layer has actually been shown to have an important role in the gut by establishing a protective layer between microbes residing in the colon and the epithelium (Schroeder, 2019). Intrinsic characteristics of the colonic mucosal barrier include continuous renewal (also known as mucus growth rate) and impenetrability of the layer close to the epithelial surface (Luis & Hansson, 2023). This is important for clearing the microbes away and keeping them at a distance from the epithelial layer. We observed impairment in both of these characteristics in mice receiving microbiota from human donors with a history of antibiotic use, supplemented with a reduced number of filled goblet cells and visual differences in the mucosal layer (**Figure 18**).

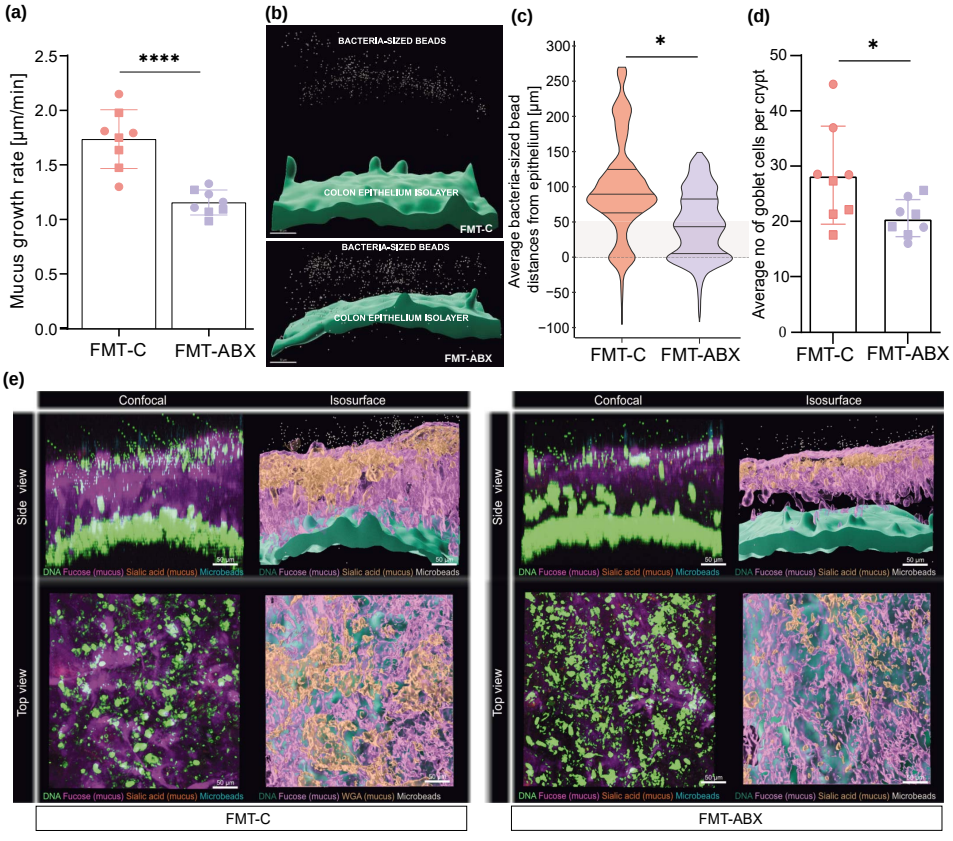

Figure 18. Results from mucus function evaluation in mice distal colon following FMT from human donors. (a) Mucus growth rate; (b) Mucus penetrability representative images; (c) Representative isosurface images visualising mucus penetrability; (d) Average number of goblet cells per crypt; (e) Representative confocal Z-stack and isosurface images of the colonic mucosal layer. FMT-C – mice that received FMT from the hCTRL pool, FMT-ABX – mice that received FMT from the hABX pool. Circles represent male mice, while squares represent female mice. * – $p < 0.05$; ** – $p < 0.01$; *** – $p < 0.001$; **** – $p < 0.0001$. Published in **Ref. III**.

In addition to the dysfunctional colonic mucosal layer, we also observed different physiological, microbiome-related, and metabolomic differences between the two mice groups (**Figure 19**). For example, we saw an increase in abdominal fat (**Figure 19a–c**). A distinct microbiome and metabolome profile exists in mice who received the microbiome from donors with a history of antibiotic use compared to the healthy controls without any recent antibiotic use (**Figure 19d–g**). We observed that none of the nine differentially abundant species with higher relative abundances in the control group were present in the group with a history of antibiotic use (**Figure 19e**). *Vice versa*, six from the differentially abundant species with higher relative abundance in the antibiotics group were only present in this mouse group. This indicates that antibiotic use might deplete some species in the community and help others to colonise. Interestingly, several previously

shown mucus-degrading microbes were significantly more abundant in the antibiotics group (e.g. *Akkermansia muciniphila* and *Bacteroides fragilis,* as well as *Bacteroides salyersiae, Blautia producta*, *Parasutterella excrementihominis*), even starting to dominate the community. These results indicate that antibiotic use might lead to microbiome composition with higher mucus degradation capacity.

Several mucus-degrading bacteria were detected in the mice who received FMT from the donor group with an antibiotic-use history. One of the detected microbes, *Akkermansia muciniphila,* has been highlighted as a potential next-generation probiotic, displaying improved metabolic markers in obese and overweight patients with insulin resistance (Depommier et al., 2019). However, there are concerns about its use as a probiotic, as an increased abundance of the microbe has been observed in multiple sclerosis and Parkinson's disease patients (Heintz-Buschart et al., 2018; Jangi et al., 2016). Additionally, *A. muciniphila* is capable of degrading the gut mucosal layer, thereby rendering the host more vulnerable to pathogens. This is thought to be fibre-dependent (Desai et al., 2016; Wolter et al., 2024), indicating that the effects of *A. muciniphila* might be context-specific.

Additionally, distinct metabolomics profiles could be identified in the mice who received microbiota from healthy donors with a history of antibiotic use compared to healthy controls without any recent antibiotic use (**Figure 19f–g**). The SCFA profile differed in mice receiving FMT from donors with a history of antibiotic use compared to the non-antibiotic-using donors, with SCFAs (butyrate, propionate, valerate) being higher in the antibiotics group. Increased SCFAs have previously been linked to adiposity phenotype in mice who received sub-therapeutic doses of antibiotics at an early age (Cho et al., 2012). As SCFAs provide energy to the colonocytes, Cho et al. 2012 study suggests that the absorption of SCFAs into the portal circulation may stimulate adipogenesis, also shown previously (Cho et al., 2012; Hong et al., 2005). This is in line with our observation of increased adipose tissue in mice who received FMT from antibiotic users. Additionally, many human population studies have linked antibiotic use to obesity, although further studies are needed to assess the causality of the microbiome in the process (Vallianou et al., 2021).

FMT has proven to be a valuable tool for functional microbiome studies and has been used to study the role of the microbiome in different phenotypes. By transferring phenotypic characteristics from one mouse to another or between humans and mice, associations detected in population studies can be validated in animal models. However, one needs to bear in mind that mice are not humans when it comes to microbiome and physiology (Arrieta et al., 2016). As it is mostly unfeasible to obtain live tissue samples from generally healthy human donors, animal models still offer a way to study the antibiotic-gut mucosal layer associations.

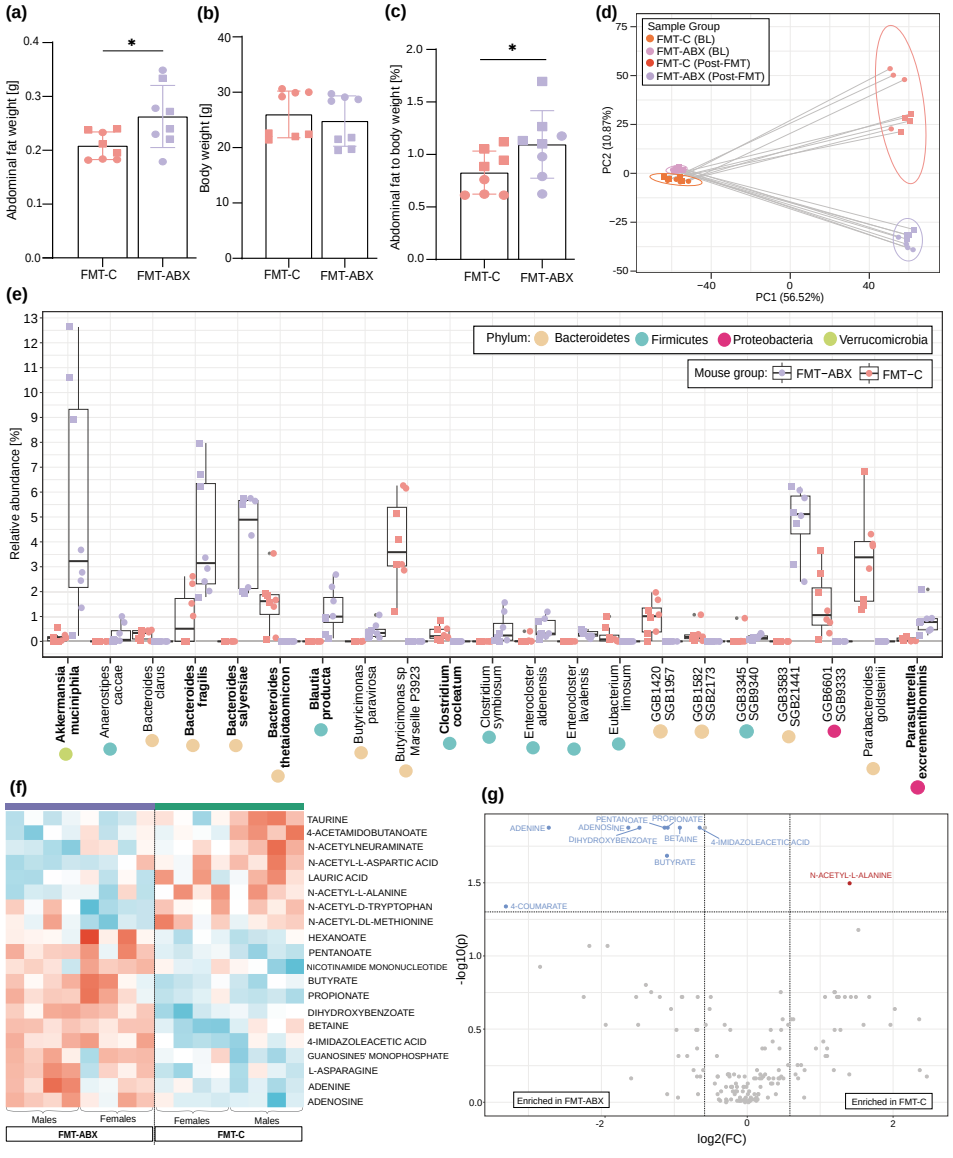

Figure 19. Distinctive physiological, microbiome-related and metabolomic characteristics of mice groups following FMT. (a) Abdominal fat weight after FMT; (b) total body weight after FMT; (c) Abdominal fat as a percentage of the total body weight; (d) beta diversity of the microbiota between FMT-C and FMT-ABX mouse stool at baseline and after FMT; (e) Significantly differentially abundant species in mouse distal colon content between the two groups (FDR < 0.05). Bacterial species previously shown to degrade mucus are in bold (f) Heatmap of the top 20 metabolites from the relative metabolomics profile by individual mouse (unpaired t-test $p < 0.05$); (g) Volcano plot highlighting significant differentially abundant metabolites between two groups in the relative metabolomics profile (FDR <0.05);. FMT – Faecal Microbiota Transplant. FMT-ABX – mice receiving FMT from the hABX pool, FMT-C – mice receiving FMT from the hCTRL pool. Circles represent male mice, while squares represent female mice. * – $p < 0.05$. Published in **Ref. III**.

3.3.3 General implication of the study

Using human-to-mouse faecal microbiota implantation, we determined the potential long-term effects of repeated antibiotic use history on host physiology (**Figure 20**). In a healthy state, the mucus layer is constantly renewed with the help of numerous goblet cells releasing mucus and is impenetrable to the diverse set of gut microbes. Moderate mucus consumption is considered to be part of homeostasis. However, following repeated antibiotic use, the microbiome becomes less diverse and has a different microbial community from individuals without antibiotic use. The mucus growth rate is lower, there are fewer goblet cells, and the mucosal layer is more penetrable to the microbial communities (**Figure 20**). This may be due to an overly active mucus-foraging microbial community, which can cause an imbalance between mucus secretion and mucus degradation, thereby leading to increased penetrability and barrier breakdown. These microbiota-mediated mucus defects could lead to enteric infections and potentially to different gut-related diseases.

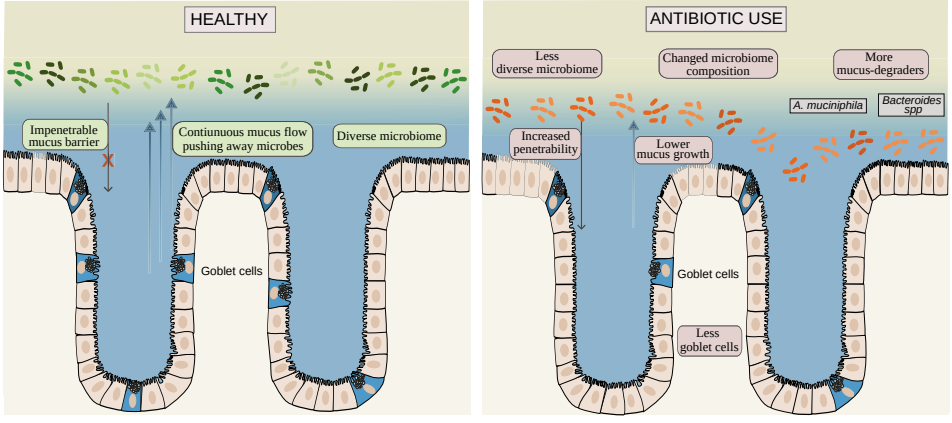

Figure 20. The gut mucosal barrier in healthy individuals and following the history of antibiotic use. Author's own work.

The discovery of antibiotics was one of the greatest achievements of modern medicine, not only drastically increasing life expectancy by helping to treat infectious diseases but also making it possible to carry out organ transplants and open-heart surgery, treat cancer, etc. (Hutchings et al., 2019). Antibiotic use is globally increasing (Browne et al., 2021), and concurrently, the deaths associated with bacterial antimicrobial resistance are increasing as well (Murray et al., 2022). Moreover, antibiotic use has been associated with various complex diseases, such as increased risk of asthma (Korpela et al., 2016), food allergy and allergic diseases (Hirsch et al., 2017), coeliac disease (Mårild et al., 2013), and IBD (Duan et al., 2024). In addition to antibiotics, these diseases have also been linked to the microbiome (Fenneman et al., 2023; Hufnagl et al., 2020; Parrish et al., 2023). Through antibiotic use, long-lasting alterations are being made to a mutualistic relationship that has taken millennia to evolve: the relationship between

the host and its microbiota. Taken together, the (mis)use of antibiotics calls for global antibiotic stewardship to reduce and optimise the use of antibiotics. More targeted methods, which are less damaging to our commensal microbes, are necessary to research, as we have just recently started to understand the long-term effects of antibiotic use on our health.

3.4 Future perspectives for the gut microbiome studies and its role in personalised medicine

Although understanding the importance of the gut microbiome evolves rapidly, the microbiome field is still mostly in the observational study phase. We have learned about numerous lifestyle factors that we can influence and thereby potentially change our microbiome. Additionally, it is becoming increasingly clear that the microbiome plays a role in different diseases and is part of the personalised host responses to different interventions, such as diet and medications. However, in addition to the known information about the microbiome, there are still many unknowns. For example, most of the variance in the human microbiome remains unexplained. We might know the microbiome's main components; however, we still need to more deeply understand how these components interact in networks in a spatial and temporal context. This also requires a shared understanding of a "healthy" desirable endpoint, both in the microbiome and the host.

In times of ageing and "Westernizing" populations, an increase in the prevalence of complex diseases, and the continuous and increasing spread of antimicrobial resistance and related infections, the microbiome holds enormous potential in developing new and effective population-based early-stage disease diagnostics, treatment opportunities, and predictive tools. Although many unknowns exist, the field is already trying to translate the current knowledge into real-life applications and personalised medicine (Ratiner et al., 2023). From observing the differences to bringing new differences about, we are learning how to use the microbiome by modulating it in the desired way. While an individual's genome comes from parental genomes and is complex to change, the microbiome is considered to be more plastic and can be modified more easily. There is high hope of using specific microorganisms or products of their metabolism for health benefits and as clinical treatment options. A part of the microbiome research focuses on trying to modulate the microbiome in a health-benefitting direction, using different microbe supplementation or augmentation techniques, such as faecal microbiota transplantation (FMT), pro- and prebiotics and diet-based modulation (Ben-Yacov et al., 2021; Berry et al., 2020; Zeevi et al., 2015). The field's ultimate goal would be targeted microbiome modulation with predictable outcomes, including desired biological response without negative side effects.

The first microbiome-based therapeutics have emerged and have proven to be efficacious in the clinic. The most famous example is FMT, where complex communities are transferred from a healthy donor to the recipient, restoring their microbiome function. These types of therapeutics, such as Vowst and Rebyota,

have recently been accepted as treatments by the FDA. Moreover, around 300 Phase I–IV clinical trials have been registered to investigate the effect of FMT on different diseases, such as inflammatory bowel syndrome (IBS), inflammatory bowel diseases (IBD), non-alcoholic fatty liver disease (NAFLD), atopic dermatitis, among others (https://clinicaltrials.gov/, accessed 8th of April 2024). Although a lot of research still needs to be done, this shows hope for microbiome-based treatments.

Even though we do not completely grasp the functional links behind the microbes and diseases, it is possible to start testing the microbiome in diagnostic or prognostic models for diseases. In an ideal microbiome-based personalised medicine world, disease diagnostics would include information about known risk factors, as well as information about our genome and additionally also microbiome (**Figure 21**). Currently, only the first studies have started to explore these options for disease risk predictions (Liu et al., 2024). However, we are still far from this reality, as the microbiome field still needs further standardisation. Additionally, microbiome-based personalised medicine can only be possible when the variance of the normal human microbiome is understood.

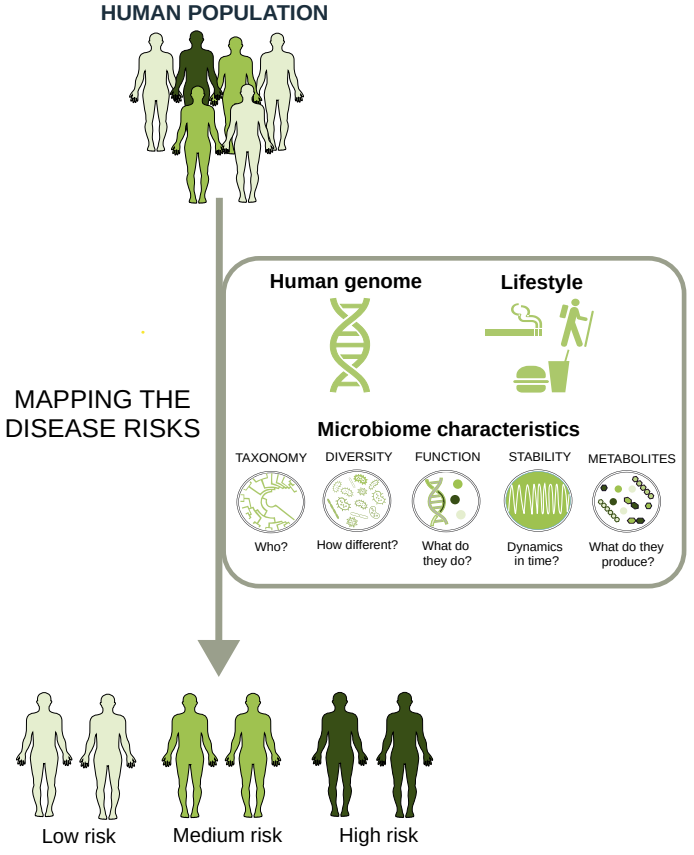

Figure 21. Vision for personalised medicine using known risk factors, the human genome and microbiome-based markers. Modified from Ratiner et al., 2023.

Including more detailed and comprehensive health data and recruiting more people and new populations in longitudinal prospective studies with multiple time points can improve the quality of microbiome studies. More countries and cultures should be represented to understand the effects of strain-level specificity in populations. For building predictive models, there is a great need to increase the cohort sizes in addition to deep phenotyping and collecting more time points to track how different interventions affect microbiome dynamics. Additionally, observational studies need to be complemented with more interventional studies, as they can provide a functional understanding of the gut microbiome. The metagenomics should be supplemented with multi-omics data layers, and ideally, the samples of the population studies should be collected in a way that allows for further cultivation and testing in the labs. Deeply phenotyped population studies have determined interesting associations that should now be followed up on in *in vitro* or animal models, as has been done in the current thesis. This, however, might not always be possible.

Increasing antimicrobial resistance, long-term changes and loss of diversity or commensal taxa in the microbiome composition, as well as increasing associations with complex diseases, warrant further studies into other treatments for pathogen suppression besides antibiotics. Additionally, there have been some concerns that in industrialised societies, we are losing our microbial diversity and should, therefore, start storing our microbiome for the health of future generations (Dominguez Bello et al., 2018). This can go hand in hand with increasing sample collection in different populations. This might also allow for autologous faecal microbiota transplantation. Moreover, the screening programs could offer a way to collect samples longitudinally, as often these programs invite the participants back every few years, potentially allowing to study disease progression. Although we have a long way to go, we already know that the microbiome is important and, therefore, should take care of our microscopic companions in the body as best as possible.

CONCLUSIONS

Undoubtedly, an increasing number of publications, continuous public interest, numerous clinical studies, and clinically accepted microbiome-based treatments have shown great potential in the field of microbiome with a goal to improve the health of humans and other organisms. It is more and more appreciated that the host functions cannot be separated from the microbial communities in them. However, we are still mostly in the exploratory phase, trying to understand general patterns and changes in how and which of our lifestyle choices are impacting the microbiome and how the microbiome can potentially be used to describe changes happening in our physiology. We still need to characterise factors in greater detail to understand what is behind the differences between individuals and samples. Additionally, we are exploring opportunities to determine whether the microbiome composition could be helpful in improving disease diagnostics and predictive tools, as well as trying to determine possibilities and caveats in doing so. The current thesis tries to tackle some of these questions. Therefore, the main conclusions drawn from this thesis are as follows:

- The faecal immunochemical test tubes used in colon cancer screening programs have the potential to be used to study the gut microbiome, which might simplify large-scale sample collections and microbiome-test-supplemented screening programs in the future.
- Population cohorts supplemented with extensive health data, such as the Estonian Microbiome cohort introduced in this thesis, are valuable for uncovering new factors influencing the microbiome. As an example, we have discovered that the 10-year repeated but not recent usage history of antibiotics and antidepressants is a significant factor associated with the changes in the gut microbiome.
- A repeated but not recent history of antibiotic use results in changed microbial communities and gut mucosal barrier defects in the colon, including lower mucosal growth rate, increased penetrability and lower number of goblet cells.

Although there is still a lot to discover and the field is still figuring out its standards, all of these studies presented in this thesis indicate the important role of the gut microbiome in the interface of human health and disease. We are already increasingly understanding the effects of antibiotics and other drug use on the microbiome and our health. Studies presented in this thesis, in combination with research done by others, warrant us to follow the principle of "as much as needed, as least as possible" in medication usage, as we do not still understand the long-term effects. However, the research continues…

SUMMARY IN ESTONIAN

Soolestiku mikrobioomi kasulikkus inimtervise mõistmisel

Kiiresti kasvav teaduslike ja populaarteaduslike artiklite, kliiniliste uuringute ja turul müüdavate mikrobioomil baseeruvate toodete hulk vihjab, et huvi meie kehas esinevate mikroobide vastu on suurenenud nii teadlaskonnas kui ka ühiskonnas laiemalt. Mikrobioom, ehk kindlas piirkonnas elavate bakterite, viiruste, seente ja arhede kooslus, on viimasel ajal saanud aina rohkem tähelepanu eelkõige tänu DNA sekveneerimistehnoloogiate kiirele arengule, mis võimaldab neid organisme kergemini ja soodsamalt uurida kui varem. Kuigi esimesed mikrobioomil põhinevad ravimeetodid on saanud Ameerika Ühendriikide Toidu- ja Ravimiameti poolt heakskiidu, on mikrobioomi valdkond peamiselt veel vaatlusuuringute keskne, kirjeldades üldiseid inimeste vahelisi mikroobikoosluste muutusi ja mustreid. Ühelt poolt üritame välja selgitada, kuidas meie keskkond ja elustiili valikud mõjutavad meie mikrobioomi. Teisalt soovime aga mõista, kuidas mikrobioom mõjutab meie keha toimimist. Lisaks uurivad mikrobioomi valdkonna teadlased võimalusi ning kaardistavad ka takistusi, kuidas mikrobioomi kasutada haiguste varajaseks diagnoostimiseks ja ennetamiseks. Eelmainitud teemasid käsitleb ka käesolev doktoritöö, tuginedes samuti DNA sekveneerimistehnoloogiate arengust tulenenud võimalustele mikrobioomi uurida.

Mikrobioomi olulisusest lähtuvalt on inimest peetud ka holobiondiks või "superorganismiks", kus erinevad liigid elavad üheskoos sümbioosis täites üksteise jaoks olulisi ja elutähtsaid rolle. Arvutused näitavad, et inimkehas on mikroobirakke umbes sama palju kui inimese enda rakke, kuigi mikroobide kogukaal on meie enda rakkudega võrreldes miniatuurne. Samas kui vaadata ainulaadsete geenide hulka, on mikroobide talitlusvõimekus inimese genoomi omast vähemalt 500–2000 korda suurem – umbes 20 000 inimese geeni versus praegu teadaolevad 45 000 000 mikroobi geeni. See muljetavaldav geneetiline varieeruvus võib ka seletada, miks mikrobioomi on oma olulisuse tõttu loetud ka eraldi elundiks inimese enda organite kõrval. Nii on näiteks soolestiku mikrobioom tihedas koostöös meie enda kehaga, aidates organismil viia läbi mitmeid olulisi ülesandeid. Mikrobioom on muuhulgas oluline toidu, eriti kiudainete lagundamisel ja omastamisel, immuunsüsteemi ja soolestiku barjääri kujundamisel ja reguleerimisel, kaitsel nakkushaiguste eest, oluliste ainevahetuse saaduste tootmisel (näiteks vitamiinid, lühikese ahelaga rasvhapped jm) ning ka kompleks-haiguste tekkes.

Kuigi mikrobioomi saab uurida erinevates keskkondades ja keha piirkondades, analüüsib käesolev doktoritöö soolestiku mikrobioomi, kus paikneb enamus kehas elavates mikroobidest. Lisaks keskendub töö mikrobioomi mõjutatavatele teguritele ja mikroobikoosluste kasutusvõimalustele inimtervise mõistmisel ja edendamisel. Doktoritöös antakse ülevaade inimese mikrobioomi valdkonna hetkeseisust, kajastades lisaks ka mikrobioomi analüüsi meetodied ja nendega soetud probleeme, aga ka valdkonna saavutusi ja võimalusi, kuidas mikrobioomi

saaks rakendada personaalmeditsiinis. Doktoritöö praktiline osa koosneb kolmest originaalsest esiautori publikatsioonist, mis katavad mikrobioomi ja inimtervise valdkonnas olulisi teemasid nagu proovide kasutamisvõimalused ennetava meditsiini osana, riiklikel biopankadel põhinevate mikrobioomi uuringute olulisus ning loommudelite kasutamine populatsioonipõhistel uuringutel tuvastatud hüpoteeside põhjalikumaks uurimiseks. Iga publikatsiooni lõpus mõtestatakse vastava teadusartikli olulisust tervisevaldkonnas laiemalt ja lõpetuseks antakse nägemus mikrobioomiga seotud tuleviku väljavaadetest.

Esimene publikatsioon uurib mikrobioomi analüüsi võimalikkust peitveretesti tuubidest, mida kasutatakse Eesti riiklikus jämesoolevähi programmis soolevähi esmaseks testimiseks. Uuringu käigus leiti, et peitvere testi tuubid on sobilikud ka mikrobioomi analüüsi läbi viimiseks ja seda isegi juhul kui proov on olnud kuni nädal aega toatemperatuuril. Töö imiteeris olukorda, kus proov on transpordil pikalt postis, mis võib potentsiaalselt mõjutada proovi mikroobikooslusi. Uuringu tulemused näitavad, et testituube saaks rakendada riiklikul laiapõhjalisel mikrobioomi testimisel, et arendada mikrobioomipõhist diagnostikat. Näiteks võimaldavad töö tulemused nüüd edasi uurida võimalusi, kuidas parandada jämesoolevähi sõeluuringu testide tundlikkust ja spetsiifilisust, aga ka kaaluda uute mikrobioomi testide välja töötamist teiste haiguste, näiteks põletikuliste soolehaiguste ja põletikulise soolesündroomi või hüpertensiooni vastu, mille diagnostikat võiks mikrobioomi analüüs eelnevate uuringute alusel parandada, kuid mille takistuseks võib olla lihtne mikrobioomi proovide kogumine.

Teises publikatsioonis tutvustatakse Eesti Geenivaramu juurde loodud Eestlaste Mikrobioomi kohordi uuringut, mis on üks suurimaid populatsioonipõhiseid metagenoomi kohorte maailmas. Doktoritöö raames koguti ja analüüsiti 2509 eestlase soolestiku mikrobioomi kooslusi seoses erinevate elustiili tegurite ja tervisenäitajatega, kasutades selleks metagenoomse DNA sekveneerimise andmeid, mis võimaldavad mikrobioomi kooslust palju täpsemini hinnata, kui levinud ühte geeni analüüsivad meetodid. Lisaks teeb uuringu maailmas unikaalseks võimalus kasutada riiklikke digitaalseid terviseandmeid, mille abil saab täpsemini hinnata mikrobioomi ning ravimite ja haiguste vahelisi seoseid. Nii leidsime, et kõige enam mõjutab eestlaste mikrobioomi omavahelisi erinevusi soole tühjendamise sagedus, kehamassiindeks ja talje- ja puusa ümbermõõdu suhe ning ka sugu. Lisaks oli selle töö üheks kõige olulisemaks leiuks antibiootikumide korduva kasutamise järel tekkinud soolestiku mikrobioomi mitmekesisuse vähenemine ja koosluse muutused, mis on tuvastatvad isegi siis, kui antibiootikume viimase poole aasta jooksul tarvitatud ei ole, kuid on tarvitatud korduvalt viimase 10 aasta jooksul. Mida rohkem on antibiootikumi kuure läbitud on 10 aasta jooksul läbitud, seda selgemalt on muutused näha. Sama, kuigi kergemat mõju, oli näha ka antidepressantide korduval tarvitamisel, isegi kui viimase poole aasta jooksul enne proovi andmist polnud inimene ravimit tarvitanud. Mujal maailmas pole ravimite mõju mikrobioomile nii pikaajaliselt võimalik olnud uurida, peamiselt seetõttu, et enamus uuringuid kasutavad küsitlus-põhiseid ühe ajapunkti andmeid, mis toetuvad inimese enda mälule, samas meie uuring võimaldas kasutada põhjalikke terviseregistreid, mis peamiselt on pärit meditsiinisüsteemist.

Kolmandas publikatsioonis vaadeldakse põhjalikumalt teises artiklis tuvastatud antibiootikumide kasutamise pikaajalist mõju soolestiku mikrobioomile. Nimelt kasutasime uuringus nii nimetatud humaniseeritud hiiremudelit, et tuvastada, kas antibiootikumide kasutamise mõjul mikrobioomile võib olla ka füsioloogiline tagajärg. Töö käigus vaadeldi inimese mikrobioomi mõju soolestiku barjäärile kasutades selleks hiiremudelit, kellele tehti inimese mikrobioomi ülekanne. Uuringusse kasutatud mikrobioomi doonorid olid üldiselt terved inimesed Eestlaste Mikrobioomi kohordist, kellel puudusid mikrobioomi mõjutavad komplekshaigused. Samuti polnud nad hiljuti tarvitanud mikrobioomi mõjutavaid ravimeid. Siiski oli näha, et hiirtel, kelle mikroobikooslus pärineb inimestelt, kes olid viimase viie aasta jooksul antibiootikume korduvalt tarvitanud, isegi kui nad pole seda hiljuti teinud, on soolestiku limaskesta barjäär läbilaskvam ja oluliselt aeglasema taastootmise kiirusega, kui doonoritel, kes viimase 10 aasta jooksul antibiootikume tarbinud ei ole. Samuti oli vahe lima tootvate karikrakkude arvukuses, mida oli antibiootikumidest mõjutatud mikrobioomi kooslusega hiirtel vähem. Samas oli nendel hiirtel kõrgem kõhurasva hulk ja protsent.

Kuigi oleme mikrobioomi uuringutega veel lapsekingades ja palju on teha valdkonna standardiseerimiseks, on selles doktoritöös esitatud teadustööd juba selgeks märgiks, et soovides paremini aru saada oma tervisest, peame me mõtlema ka oma mikrobioomile ja selle kasutusvõimalustele. Kuigi me ei oska veel kirjeldada, on aina selgem, et mitmeid inimese enda kehafunktsioone ja elu üldisemalt ei saa vaadata eraldatult mikrobioomist. Samuti on kasvamas arusaam, mis on meie elustiilitegurite pikaajalised mõjud. Nii on aina selgem, et lisaks mikroobidele suunatud ravimitele võivad ka inimesele suunatud suunatud ravimid tegelikult mõjutada mikroobe ja seeläbi ka meie enda tervist. Käesoleva doktoritöö tulemused koos teiste avaldatud teadustöödega annavad ravimite määramisel alust printsiibile “nii palju kui vaja, nii vähe kui võimalik”, sest meil pole veel selget ülevaadet nende pikaajlistest efektidest. Uurimistöö aga jätkub...

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ACKNOWLEDGMENTS

This work was done at the best institute with the most encouraging, insightful and humorous colleagues at the University of Tartu – the Institute of Genomics.

First and foremost, I want to thank Professor Elin Org for being an extra-ordinary supervisor, part-time comedian, and full-time mother lion. You stop at nothing to make sure that we are not only good scientists but also good people. You radiate confidence and happiness, and you are an ideal role model for young people in science – approachable, caring, and fun, but also having the courage to fight for what is right. Without your constant unconditional support, I would have most likely quit doing science a long time ago, and I am truly happy that I can call you not only my mentor but also my dear friend. Thank you for picking me up and believing in me when I couldn't do it myself.

Secondly, my gratitude belongs to my co-supervisor, Tõnis Org, who taught me a lot about being compassionate with myself. Our discussions always calmed me down when I became too worried about some small, very unimportant detail in life. This excitement you have towards trying to figure things out in the lab (and beyond) is addictive, and if I could have ~10% of your problem-solving skills, I think I would be rocking this scientific world. I am sure we will have many more discussions to come in the future now that you are also becoming a microbiome scientist. All roads lead to Rome, and it seems more recently, also to the microbiome.

Koit, 6 years ago, I had no idea about your existence. Now, I am grateful to call you one of my best friends, who has helped me to figure out what standards I want to set for myself in life. You are an exceptional scientist, not only because you keep the big picture in mind but also because you do not lose integrity even in small, seemingly pointless endeavours. I am honoured to have a friend like you, who is patient, kind, caring and at the same time humble, even if stressful times try to make you think otherwise about yourself. And thank you for enriching this world with such a fantastic family, who, I hope, sometimes accept me as their own. We need to keep on searching for that (scientific) drama to be addicted to, as this has always been *our joy, a guiding light*. We make a hell of a team together.

The microbiome team, with current and past members, is hands down the best place to work, not only because of the mentality that all of you have for exceptionally doing whatever you set your mind on but also for all the fun times that we create together. Kreete, for continuously inspiring me to keep on going through rain and rainbows (always with a little help of sarcasm). Kateryna, for discussions about life, books, movies, culture and, of course, enjoying our motivation-saving croissants from Cruffin. Reidar, for always being humble and having an optimistic view of life. Annabel, for your orientation to detail and ability to take up new knowledge with ease. Nele, for your support in my darker days and for always being my yes-woman for whatever wild ideas I come up with. And Andri, you fit right into our quirky little team, and I already have a

good feeling about the memories (or chaos in the system) we will create together in the future.

I met new great friends thanks to these studies. All my incredible, inspiring, and jaw-droppingly remarkable women (Triin, Maris, Kelli, Natàlia, Lili, Mari-Liis, Annely Triinu, Monika, Merli, Kristi(s) and Krista(s). You kept me going even when I thought that going on was impossible. I think about you every day with a smile on my face. I also cannot be more grateful for Andres and Reedik, who have helped me through the days when my energy levels were not matching my usual norms and helped me recover. Erik, I think we are very similar in our attitude towards work, and I found comfort in knowing that someone as great as you can understand what I am going through on my not-so-great days. Martin, I appreciate our discussions greatly, and I really hope that our friendship will survive the test of time. Thank you, Steven – you inspired me a lot with your "let's focus on solutions" attitude, and I surely think that the institute would have collapsed without you and Krista Liiv. Mait, for all the challenges and encouragements, when "selgroog" became a question. My friends in Umeå, what an unexpected, wild and fun ride it was! Thank you for being my other scientific family. Additionally, my dear current and former fellow PhD students in the Institute of Genomics. I have yet to hear from a person who did not have a bumpy ride in their PhD. Only a good support team can help navigate this journey, and I am happy to have had such talented, fun and goal-oriented people by my side. And this also leads to my favourite bunch – IT tuba. Thank you for your humour and your commitment and patience with us.

My girls Ana, Ola and Naths, the distance is nothing when you have these types of sisters all over the world. You bring clarity, humour and encouragement to my daily life. Hans, Epp, Laur, Kaur, Märten, Tiit, Hendrik, Silver ja kõik mu Jaanikesed, you are like my tech crew who keep the engine running in the background. This program called Kertu would have stopped working a long time ago without your support. Thank you, BC Vändad, for all the jokes, political discussions, movie nights, and (some) bike rides, which kept me afloat. Ula and Ulakad – you hold a special place in my heart and welcome me with open arms, whether it is an (exceptionally) good day or a bad one. 21CC Triatloniklubi Tartu team – thanks for keeping me sane by making all our workouts so fun that I probably got bigger ABS muscles just from laughing so much with you all.

Without the constant support of my (extended) family, I would not have been able to choose and continue on this path. Ema Merle, isa Andres, õde Tiiu, vend Ott, Kerttu ja Sabrina, tädi Tiiu and Uncle Norm, tädi Made, Aigi, you have been my source of motivation in different forms and thanks to you this work is now between these green covers. And **Karle**, if our future is even half as marvellous as our past, I will start believing in this soulmate nonsense.

Many people who are not mentioned here still have an integral part in my life, keeping me going, and I appreciate you dearly.

And last but not least, this work was written at writing retreats and writing days organised by the Institute of Genomics, University of Tartu, which are, by the way – awesome!

PUBLICATIONS

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Publications:

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Supervised dissertations:

Annabel Raudsepp	Bachelor's Degree, Institute of Molecular and Cell Biology, University of Tartu, Estonia, 2021, "Gut tissue microbiome profile in Estonian colorectal cancer screening patients"
Annabel Raudsepp	Master's Degree, Institute of Molecular and Cell Biology, University of Tartu, Estonia, 2023, "Interactions between the gut microbiota and novel type 2 diabetes medications in type 2 diabetes patients"
Claudia Maria Ruhno	Bachelor's Degree, Institute of Molecular and Cell Biology, University of Tartu, 2023, "Using a mouse model to study the human gut microbiome"
Johanna Toodu	Bachelor's Degree, Institute of Molecular and Cell Biology, University of Tartu, 2024 "Possibilities for microbiome analysis from faecal occult blood tests used in the screening of colorectal cancer in Estonia"
Linda Sootak	Bachelor's Degree, Institute of Molecular and Cell Biology, University of Tartu, 2022, "Associations between the oral microbiome and colorectal cancer"
Valida Kazimova	Bachelor's Degree, Institute of Technology, University of Tartu, 2023, "Characterization of gut microbiome composition in T2D patients under SGLT2i and GLP1RA treatment"

Awards and scholarships:

2023 ASTRA project PER ASPERA Graduate School in Biomedicine and Biotechnology Mobility Grant for "EMBO | EMBL Symposium: The human microbiome"

2023 Best microbiology-related poster presentation award at the Annual Conference for Institute of Genomics and Institute of Molecular and Cell Biology (awarded by the Estonian Society for Microbiology in collaboration with the American Society for Microbiology)

2023 Estonian Academy of Sciences "Science in 3 minutes" laureate

2023 L'Oréal-UNESCO "For Women in Science Young Talents" Baltic program award

2023 Valda and Bernard Õun scholarship

2023 ASTRA project PER ASPERA Graduate School in Biomedicine and Biotechnology Mobility Grant for "Cell Symposia: Infection Biology in the Age of the Microbiome

2022–2023 EU MIBEst Twinning grant support for staff exchange in Umeå University

2022 Best Selected Talk (Microbiome Virtual International Forum)

2022 The University of Tartu "Science in 3 minutes" thesis competition winner

2022 Best microbiology-related oral presentation award at the Annual Conference for Institute of Genomics and Institute of Molecular and Cell Biology (awarded by the Estonian Society for Microbiology in collaboration with the American Society for Microbiology

2022 Best Oral Presentation Award at the annual Conference for the Institute of Genomics and Institute of Molecular and Cell Biology

2022 COST Inclusiveness Target Countries (ITC) Conference Grant

2022 ASTRA project PER ASPERA Graduate School in Biomedicine and Biotechnology Mobility Grant for EMBO YiP PhD course and visit to EMBL lab groups

2021 Scientific articles competition "Science to Wikipedia 2020" jury recognition award for the article "Jämesoolevähk"

2021 University of Tartu Badge of Distinction

2021 COST Inclusiveness Target Countries (ITC) Conference Grant

2020 "Science Popularizer of the Year" award for the exhibition "GENEius

2020 Best Poster Award at the Insitute of Molecular and Cell Biology and Institute of Genomics Annual Conference

2019 Lydia and Felix Krabi scholarship

2019 Tamkivi Foundation for Natural Sciences scholarship

2019 Scholarship in Smart Specialization Growth Areas

2018 Tartu Students' Nature Conservation Circle photo competition "Warmth" 1st award

2017 Science photographer of the year (Wikimedia Eesti).

ELULOOKIRJELDUS

Nimi: Kertu Liis Krigul
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Aadress: Eesti Geenivaramu, Genoomika instituut, Tartu Ülikool
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Haridus:

2021–	PhD geenitehnoloogia, genoomika instituut, Tartu Ülikool (struktuuriüksuse muutus)
2018–2021	PhD geenitehnoloogia, molekulaar ja rakubioloogia instituut, Tartu Ülikool
2016–2018	Magistrikraad biotehnoloogias, Norra teadus ja tehnoloogia ülikool
2012–2016	Bakalaureusekraad bioloogias, molekulaar- ja rakubioloogia instituut, Tartu Ülikool

Teenistuskäik:

2022–…	Tartu Ülikool, genoomika instituut (mikrobioomika nooremteadur)
2019–…	Tartu Ülikool, Tartu Ülikooli Teaduskool (MOOC "Geenid – müüdid ja tegelikkus" õppejõud)
2019–2021	Tartu Ülikool, genoomika instituut (spetsialist)
2018–2020	Tartu Ülikool, genoomika instituut (geenidoonorite teavitaja ja teavitajate koolitaja, riiklik personaalmeditsiini projekt)
2016–2017	Tartu Ülikool (Realia et Naturalia rebaste konverentsi projektijuht)

Teadusorganisatsiooniline ja- administratiivne tegevus:

2023–...	Eesti Mikrobioloogide Ühendus
2022–2024	Ameerika Mikrobioloogide Ühingu (ASM) liige
2021–2023	Doktorantide esindaja genoomika instituudi nõukogus
2021–2022	Loodus- ja täppisteaduste valdkonna üliõpilasesindaja senatis
2015–2016	Bioteaduste Üliõpilaste Seltsi president
2015–2016	Loodus- ja tehnoloogiateaduskonna esindaja TÜ üliõpilasesinduses
2014–2016	TÜ bioteaduste programminõukogu liige
2013–2014	Loodus- ja tehnoloogiateaduskonna esindaja TÜ üliõpilasesinduses

Teaduspublikatsioonid:

Aasmets, Oliver; Taba, Nele; **Krigul, Kertu Liis**; Andreson, Reidar; Estonian Biobank Research Team; Org, Elin (2024). Long-term consequences of drug usage on the gut microbiome. medRxiv, 2024.07.17.24310548. https://doi.org/10.1101/2024.07.17.24310548.

Klemets, Annabel; Reppo, Ingrid; **Krigul, Kertu Liis**; Volke, Vallo; Aasmets, Oliver; Org; Elin (2024). Fecal microbiome predicts treatment response after the initiation of semaglutide or empagliflozin uptake. medRxiv, 2024.07. 19.24310611. https://doi.org/10.1101/2024.07.19.24310611

Pantiukh, Kateryna; Aasmets, Oliver; **Krigul, Kertu Liis**; Org, Elin. Metagenome-assembled genomes of Estonian Microbiome cohort reveal novel species and their links with prevalent diseases (2024). bioRxiv 2024.07. 06.602324. https://doi.org/10.1101/2024.07.06.602324

Milani, Lili; Alver, Maris; Laur, Sven; Reisberg, Sulev; Haller, Toomas; Aasmets, Oliver; Abner, Erik; Alavere, Helene; Allik, Annely; Annilo, Tarmo; Fischer, Krista; Hudjashov, Georgi; Jõeloo, Maarja; Kals, Mart; Karo-Astover, Liis; Kasela, Silva; Kolde, Anastassia; Krebs, Kristi; **Krigul, Kertu Liis**; Kronberg, Jaanika; Kruusmaa, Karoliina; Kukuškina, Viktorija; Kõiv, Kadri; Lehto, Kelli; Leitsalu, Liis; Lind, Sirje; Luitva, Laura Birgit; Läll, Kristi; Lüll, Kreete; Metsalu, Kristjan; Metspalu, Mait; Mõttus, René; Nelis, Mari; Nikopensius, Tiit; Nurm, Miriam; Nõukas, Margit; Oja, Marek; Org, Elin; Palover, Marili; Palta, Priit; Pankratov, Vasili; Pantiukh, Kateryna; Pervjakova, Natalia; Pujol-Gualdo, Natàlia; Reigo, Anu; Reimann, Ene; Smit, Steven; Sokurova, Diana; Taba, Nele; Talvik, Harry-Anton; Teder-Laving, Maris; Tõnisson, Neeme; Vaht, Mariliis; Vainik, Uku; Võsa, Urmo; Esko, Tõnu; Kolde, Raivo; Mägi, Reedik; Vilo, Jaak; Laisk, Triin; Metspalu, Andres (2024). From Biobanking to Personalized Medicine: the journey of the Estonian Biobank (*submitted*)

Krigul, Kertu Liis & Feeney, Rachel H.*; Wongkuna, Supapit; Aasmets, Oliver; Holmberg, Sandra M.; Andreson, Reidar; Puértolas Balint, Fabiola; Pantiukh, Kateryna; Sootak, Linda; Org, Tõnis; Tenson, Tanel; Org, Elin & Schroeder, Björn O. (2024). A history of repeated antibiotic usage leads to microbiota-dependent mucus defects. Gut Microbes, 16(1). https://doi.org/10.1080/19490976.2024.2377570

Aasmets, Oliver; **Krigul, Kertu Liis;** Org, Elin (2022). Evaluating the clinical relevance of the enterotypes in the Estonian Microbiome cohort. Frontiers in Genetics, 1943. https://doi.org/10.3389/fgene.2022.917926.

Aasmets, Oliver & **Krigul, Kertu Liis;** Lüll, Kreete; Metspalu, Andres; Org, Elin (2022). Gut metagenome associations with extensive digital health data in a volunteer-based Estonian microbiome cohort. Nature Communications, 13 (1), 1–11. https://doi.org/10.1038/s41467-022-28464-9

Krigul, Kertu Liis; Aasmets, Oliver; Lüll, Kreete; Org, Tõnis; Org, Elin (2021). Using fecal immunochemical tubes for the analysis of the gut microbiome has the potential to improve colorectal cancer screening. Scientific Reports, 11 (1). https://doi.org/10.1038/s41598-021-99046-w

Saare, Merli; **Krigul, Kertu Liis**; Laisk-Podar, Triin; Ponandai-Srinivasan, Sakthivignesh; Rahmioglu, Nilufer; Lalit Kumar, Parameswaran Grace; Zondervan, Krina; Salumets, Andres; Peters, Maire. DNA methylation alterations-potential cause of endometriosis pathogenesis or a reflection of tissue heterogeneity? Biol Reprod. 2018 Aug 1;99(2):273–282. https://doi.org/10.1093/biolre/ioy067

Populaarteaduslikud artiklid:

Krigul, Kertu Liis (2023). Soolebakterite liigirikkust laastab juba vähene antibiootikumide tarbimine. ERR Novaator.

Aasmets, Oliver; **Krigul, Kertu Liis** (2023). Probiootikumidest tõenduspõhiselt. Lege Artis.

Org, Elin; **Krigul, Kertu Liis;** Aasmets, Oliver (2022). Eestlaste mikroobikooslust võivad mõjutada kümnendi eest võetud ravimid. ERR Novaator.

Krigul, Kertu Liis (2021). Mikrobiota olulisus ja kasutusvõimalused meditsiinis. Lege Artis.

Krigul, Kertu Liis (2021). Mikroobide uurimine aitab tõhustada jämesoolevähi ennetust.

Krigul, Kertu Liis (2019). Tudeng Arktikas: kui Eesti talveööd ja suvepäevad pole piisavalt pikad. ERR Novaator.

Krigul, Kertu Liis; Org, Elin (2018). Soolestiku mikrobioomi roll tervises ja haiguses. Perearst.

Juhendatud väitekirjad:

Annabel Raudsepp	Bakalaureuse kraad, Molekulaar- ja rakubioloogia instituut, Tartu Ülikool, 2021, "Eesti jämesoolevähi sõeluuringu patsientide mikrobioomi koosluste erinevused soole koeproovides"
Annabel Raudsepp	Magistri kraad, Molekulaar- ja rakubioloogia instituut, Tartu Ülikool, 2023, "Uute diabeediravimite ja soolestiku mikrobiota interaktsioonid teist tüüpi diabeediga patsientidel"
Claudia Maria Ruhno	Bakalaureuse kraad, Molekulaar- ja rakubioloogia instituut, Tartu Ülikool, 2023, "Hiiremudeli kasutamine inimese soolestiku mikrobioomi uurimiseks"
Johanna Toodu	Bakalaureuse kraad, Molekulaar- ja rakubioloogia instituut, Tartu Ülikool, 2024, "Mikroobikoosluse analüüsi võimalikkus Eesti jämesoolevähi sõeluuringus kasutatavatest peitvere testi proovidest"
Linda Sootak	Bakalaureuse kraad, Molekulaar- ja rakubioloogia instituut, Tartu Ülikool, 2022, "Suu mikrobioomi seosed jämesoolevähiga"
Valida Kazimova	Bakalaureuse kraad, Tehnoloogia instituut, Tartu Ülikool, 2023, "Soolestiku mikrobioomi kirjeldus SGLT2i ja GLP1RA ravi saavatel tüüp 2 diabeedi patsientidel"

Auhinnad ja stipendiumid:

2023 ASTRA projekt PER ASPERA Biomeditsiini ja biotehnoloogia doktorikooli lähetustoetus konverentsile "EMBO | EMBL Symposium: The human microbiome"
2023 Parim mikrobioloogia-teemaline postriettekanne TÜMRI ja GI aastakonverentsil (auhinna andja Eesti Mikrobioloogide Ühendus ja American Society for Microbiology)
2023 Eesti teaduste akadeemia "Teadus 3 minutiga" laureaat
2023 L'Oréal-UNESCO noorte talentide Baltikumi programmi "Naised teaduses" auhind
2023 Valda ja Bernard Õuna mälestusfondi stipendium
2023 ASTRA projekt PER ASPERA Biomeditsiini ja biotehnoloogia doktorikooli lähetustoetus konverentsile "Cell Symposia: Infection Biology in the Age of the Microbiome
2022–2023 EU MIBEst Twinning grandi toetus välislähetuseks Umeå ülikooli
2022 Parim mikrobioloogia-teemaline ettekanne TÜMRI ja GI aastakonverentsil (auhinna andja Eesti Mikrobioloogide Ühendus ja American Society for Microbiology)
2022 Parima suulise ettekande auhind Tartu Ülikooli genoomika instituudi ja molekulaar- ja rakubioloogia instituudi aastakonverentsil
2022 Parim väljavalitud suuline ettekanne (Microbiome Virtual Internationl Forum)
2022 Tartu Ülikooli "Teadus 3 minutiga" konkursivooru võitja
2022 COST Inclusiveness Target Countries (ITC) konverentsi stipendium
2022 ASTRA projekt PER ASPERA Biomeditsiini ja biotehnoloogia doktorikooli lähetustoetus EMBO YiP PhD kursusele ja laborikülastustele EMBL-is
2021 Artiklikonkursi "Teadus Vikipeediasse 2020" žürii tunnustus artikli "Jämesoolevähk" eest
2021 Tartu Ülikooli aumärk
2021 COST Inclusiveness Target Countries (ITC) konverentsi stipendium
2020 Riiklikult tunnustatud teaduse populariseerija (näituse "GEENiaalne")
2020 Parima postri auhind TÜ molekulaar- ja rakubioloogia ning genoomika instituudi aastakonverentsil
2019 Nutika spetsialiseerumise doktorandistipendium
2019 Lydia ja Felix Krabi stipendium
2019 Tamkivi reaalteadustefondi stipendium
2018 Tartu Üliõpilaste Looduskaitseringi fotokonkurss "Soojus" I koht
2017 Aasta teadusfotograaf (Vikipeedia Eesti)

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