Gut microbiota metabolism linked to metabolic health and cardiovascular diseases

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UNIVERSITY OF GOTHENBURG

Gothenburg 2025

Cover illustration: Heartfelt Signals by the Gut Microbiome
by Matthias Georg Mitteregger created with ChatGPT

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ISBN 978-91-8115-318-7 (PRINT)
ISBN 978-91-8115-319-4 (PDF)

Printed in Borås, Sweden 2025
Printed by Stema Specialtryck AB

To those who carried confidence for me
when I couldn't hold it myself.

One never notices what has been done;
one can only see what remains to be done.

Marie Skłodowska-Curie

Gut microbiota metabolism linked to metabolic health and cardiovascular diseases

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ABSTRACT

Positioned at the interface of nutrition and host physiology, the gut microbiota produces bioactive metabolites that modulate host lipid and glucose metabolism as well as immune function. Many of these metabolites, although produced in the gut, exert influence on physiological processes in the whole body, with an impact on host cardiometabolic health. Despite increasing associations between individual microbiome features and disease reported, mechanistic insights and ecological understanding remain limited. This thesis aimed to explore the ecology of microbial functions and host interactions relevant to cardiometabolic disease using a combination of metagenomics, metabolomics, *in vitro* fermentation assays, and characterization of isolates.

In **Paper I**, we show that the *ismA* gene, involved in microbial cholesterol reduction to coprostanol, robustly predicts fecal cholesterol conversion and characterizes a microbiome enriched in fermentative and anti-inflammatory features, linked to favorable cardiometabolic profiles in populations at risk. In **Paper II** we demonstrate that microbiota individuality drives short-chain fatty acid and bile acid profiles *in vitro*, with a larger impact than fiber type. These results may have implications for personalized nutrition. In **Paper III**, we characterize three strains initially classified as *Desulfovibrio piger*, revealing major phenotypic and metabolic differences. These results support the designation of a novel species *Desulfovibrio aggregans* sp. nov. and underscore the limitations of current species-level taxonomy. **Paper IV** highlights the predictive power of microbiota-associated circulating metabolites for type 2 diabetes, identifying specific microbe-metabolite-host interactions that are relevant for glucose control and can be modified through lifestyle interventions.

These findings emphasize the importance of acknowledging and understanding the ecological complexity and metabolic impact of the gut microbiota in cardiometabolic disease, and point towards functional, context-dependent microbial signatures as targets for diagnosis, prevention or therapy.

Keywords: gut microbiota, gut microbiome, ecology, cardiometabolic disease, cardiovascular disease, fiber, *Desulfovibrio piger*, type 2 diabetes, atherosclerosis, stroke, metabolomics

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ISBN: 978-91-8115-319-4 (PDF)

SAMMANFATTNING PÅ SVENSKA

Tarmmikrobiotan är ett komplext ekosystem bestående av bakterier och andra mikroorganismer. Den producerar en mängd ämnen som påverkar ämnesomsättning, immunförsvar och andra fysiologiska funktioner. Genom sitt samspel med kroppen spelar tarmmikrobiotan en central roll för kardiometabol hälsa. Även om sambandet mellan obalans i tarmmikrobiotan och kardiometabol hälsa är väl dokumenterat, vet vi fortfarande relativt lite om hur interaktionen mellan bakterier och värd går till, eller vilka ekologiska mekanismer som bidrar till sjukdomsutveckling.

I denna doktorsavhandling undersöker jag hur tarmbakteriers metabola funktioner påverkar kroppen och därigenom kan kopplas till kardiometabola sjukdomar. För att studera dessa samband har jag använt flera kraftfulla och moderna metoder, såsom analys av bakteriernas DNA (metagenomik), mätning av tarmmikrobiotans metabola produkter (metabolomik) i blod och avföring, undersökning av tarmbakteriers metabola processer i kontrollerade laboratorieförsök samt karakterisering av enskilda bakteriestammar.

I **Paper I** visar vi att genen *ismA*, som förekommer hos vissa tarmbakterier, är involverad i kolesterolmetabolismen och att dess förekomst är associerad med en balanserad tarmmikrobiota samt förbättrade metabola parametrar. I **Paper II** visar vi att tarmmikrobiotans sammansättning har större betydelse för produktionen av mikrobiella metaboliter än kostens fibersammansättning. Detta indikerar att kost som används som del av behandling mot hjärt-kärlsjukdom bör individanpassas. I **Paper III** karakteriserar vi tre stammar av tarmbakterien *Desulfovibrio piger* och finner att de skiljer sig mer från varandra än tidigare känt. Vi föreslår att den stam som avviker mest från övriga omklassificeras som en ny art, med namnet *Desulfovibrio aggregans* sp. nov. I **Paper IV** visar vi att blodmetaboliter kopplade till tarmmikrobiotan kan förutsäga risken för typ 2-diabetes, och att vissa av dessa metaboliter dessutom kan påverkas av en hälsosammare livsstil.

Sammanfattningsvis visar resultaten hur komplext samspelet är mellan tarmmikrobiotan och kroppen, och hur viktigt det är att inte bara studera vilka bakterier som finns, utan vad de faktiskt gör. På sikt kan dessa insikter bidra till nya sätt att förebygga, diagnostisera och behandla kardiometabola sjukdomar.

ZUSAMMENFASSUNG AUF DEUTSCH

Der Darm ist nicht nur für die Verdauung verantwortlich, in ihm lebt auch ein komplexes Ökosystem von Mikroorganismen, das sogenannte Darmmikrobiom. Diese Mikroorganismen produzieren eine Vielzahl an Stoffwechselprodukten, die Auswirkungen auf unseren Zucker- und Fettstoffwechsel haben, sowie unser Immunsystem beeinflussen. Dadurch spielt unser Darmmikrobiom eine wichtige Rolle für unsere Herz-Kreislauf- und Stoffwechselgesundheit. Obwohl es immer mehr Hinweise gibt, dass ein gestörtes Darmmikrobiom mit Herz-Kreislauf-Erkrankungen und Diabetes zusammenhängt, verstehen wir die Details dieser Zusammenhänge, sowie deren ökologische Hintergründe noch nicht genau.

In dieser Doktorarbeit habe ich die Ökologie bestimmter Stoffwechselfunktionen des Darmmikrobioms untersucht, und wie diese mit dem menschlichen Körper in Wechselwirkung stehen und dadurch mit Herz-Kreislauf- und Stoffwechselerkrankungen zusammenhängen. Hierfür habe ich eine Vielzahl methodischer Zugänge genutzt: von der Analyse der genetischen Information der Darmmikroorganismen (Metagenomik) über die Untersuchung ihrer Stoffwechselprodukte in Blut und Stuhl (Metabolomik) bis hin zu Laborexperimenten zur Nachstellung der Darmfermentation und der Untersuchung einzelner Bakterienstämme.

In **Paper I** konnte ich zeigen, dass ein bestimmtes Bakteriengen namens *ismA* mit der Umwandlung von Cholesterin zu Coprostanol im Darm zusammenhängt, was mit einem gesünderen Mikrobiom und günstigeren Stoffwechselwerten verbunden ist. In **Paper II** zeigte ich, dass nicht der Ballaststofftyp, sondern die persönliche Zusammensetzung des Darmmikrobioms entscheidend dafür ist, welche Stoffwechselprodukte im Darm entstehen, was für eine personalisierte Ernährung als Teil der Behandlung von Herz-Kreislauferkrankungen spricht. In **Paper III** wurden drei Stämme des Darmbakteriums *Desulfovibrio piger* näher untersucht und es wurde festgestellt, dass sie sich genetisch, wie auch in ihrem Stoffwechsel, stärker unterscheiden als bisher gedacht. Deshalb wurde ein Stamm als neue Spezies *Desulfovibrio aggregans* sp. nov. vorgeschlagen. In **Paper IV** wurde gezeigt, dass bestimmte Stoffwechselprodukte im Blut, die mit dem Darmmikrobiom zusammenhängen, vorhersagen können, ob jemand ein erhöhtes Risiko für Typ-2-Diabetes hat, und dass manche dieser Stoffe sich sogar durch eine gesündere Lebensweise beeinflussen lassen.

Insgesamt zeigen die Ergebnisse, wie komplex das Zusammenspiel zwischen Darmmikrobiom und Körper ist, und wie wichtig es ist, nicht nur zu untersuchen, welche Bakterien vorhanden sind, sondern was sie tatsächlich tun. Langfristig könnten diese Erkenntnisse helfen, neue Möglichkeiten zur Vorbeugung, Diagnose oder Behandlung von Herz-Kreislauf- und Stoffwechselerkrankungen zu entwickeln.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals. The papers are attached in the appendix.

I. Mitteregger M*, Chakaroun R*, Olsson L, Pradhan M, Khan MT, Bergh PO, Krämer M, Bergström G, Bäckhed F, Tremaroli V. **Ecological determinants of gut microbiota cholesterol reduction and associations with cardiometabolic diseases.** *Manuscript.* *Contributed equally

II. Mitteregger M, Florén A, Jönsson J, Antonsson S, Krämer M, Bergh PO, Khan MT, Chakaroun R, Bäckhed F, Tremaroli V. **Gut microbiota fermentative and bile acid metabolism during *in vitro* fermentation of oat, rye and wheat bran.** *Manuscript.*

III. Kraft JD, Mitteregger M, Dwibedi C, Makki K, Florén A, Sjöland W, Hempenstall E, Jönsson J, Bäckhed F, Khan MT, Tremaroli V, Caesar R. **Novel species of the Genus *Desulfovibrio* isolated from human faeces.** *Manuscript.*

IV. Wu H, Lv B, Zhi L, Shao Y, Liu X, Mitteregger M, Chakaroun R, Tremaroli V, Hazen SL, Wang R, Bergström G, Bäckhed F. **Microbiome–metabolome dynamics associated with impaired glucose control and responses to lifestyle changes.** *Nat Med. 2025 Jul;31(7):2222-2231. doi: 10.1038/s41591-025-03642-6*

CONTENTS

ABBREVIATIONS

(V)LDL	(Very) low-density lipoprotein
ANOVA	Analysis of variance
BA	Bile acid
Apo	Apolipoprotein
BCFA	Branched-chain fatty acid
BSH	Bile salt hydrolase
CA	Cholic acid
CAG	Co-abundant gene group
CAZyme	Carbohydrate active enzyme
CDCA	Chenodeoxycholic acid
CE	Cholesteryl ester
CFU	Colony-forming unit
CGI	Combined glucose intolerance
CMD	Cardiometabolic disease
CoA	Coenzyme A
CVD	Cardiovascular disease
DCA	Deoxycholic acid
FINDRISC	Finnish Diabetes Risk Score
FXR	Farnesoid X receptor
GMM	Gut metabolic module

GWAS	Genome-wide association study
HCA	Hyocholic acid
HDCA	Hyodeoxycholic acid
HDL	High-density lipoprotein
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
HMM	Hidden Markov model
HMO	Human milk oligosaccharide
IFG	Impaired fasting glucose
IGT	Impaired glucose tolerance
IsmA	Intestinal steroid metabolism A
KEGG	Kyoto Encyclopedia of Genes and Genomes
KO	KEGG ortholog/orthology
LAB	Lactic acid bacteria
LCA	Lithocholic acid
LMM	Linear mixed-effects model
MAG	Metagenome-assembled genome
MASLD	Metabolic dysfunction–associated steatotic liver disease
MGS	Metagenomic species
MPN	Most probable number
$NAD(P)^+$	Nicotinamide adenine dinucleotide (phosphate)
NGT	Normal glucose tolerance

NPC1L1	Niemann-Pick C1-like 1
OD	Optical density
OTU	Operational taxonomic unit
oxLDL	Oxidized low-density lipoprotein
PGM	Postgate's medium
PPI	Proton pump inhibitor
qPCR	Quantitative polymerase chain reaction
SCFA	Short-chain fatty acid
SGB	Species-level genome bin
SNP	Single nucleotide polymorphism
SRB	Sulfate-reducing bacteria
T2D	Type 2 diabetes
TGR5	Takeda G protein-coupled receptor 5
TICE	Trans-intestinal cholesterol efflux
TMA(O)	Trimethylamine (N-oxide)
UDCA	Ursodeoxycholic acid
UHGG	Unified Human Gastrointestinal Genome
UHGP	Unified Human Gastrointestinal Protein
WGS	Whole-genome sequencing

DEFINITIONS IN SHORT

16S rRNA gene sequencing	A sequencing approach where the entire length or select regions of the 16S rRNA gene are sequenced, yields mainly taxonomic information.
Alpha diversity	A measure of within-sample diversity, considering the number of different taxa (richness) and/or their distribution (evenness).
Beta diversity	A measure of between-sample diversity, quantifying the similarity between two samples.
Cholesterol conversion rate	The percentage of coprostanol (saturated analog of cholesterol) over the sum of cholesterol and coprostanol.
Culturomics	A high-throughput culture-based approach using genomic information to formulate selective and non-selective media to isolate low-abundance or slow-growing microbes.
Enterotype	A classification of gut microbial communities based on composition and abundance of certain taxa. Initially defined by Arumugam, Bork *et al.*[1].
Functional potential	The totality of gene functions encoded in a (meta-)genome.
Gene richness	A measure of within-sample diversity, the number of different genes within a sample. Defined by Le Chatelier *et al.*[2].
Keystone species	An organism performing a unique and/or pivotal function for the ecosystem, e.g., complex fiber degradation.
Metagenome	The collection of genetic content of all organisms in a specific site.

Microbiome	The collection of microorganisms, their genomes as well as ecological and environmental conditions in a specific site.
Microbiota	The entity of microorganisms present in a specific site.
Profile HMM	A model of probabilities at each position in a sequence (here amino acids), based on multiple alignment.
Whole-genome sequencing	A sequencing approach where the entire genome of one or many taxa is sequenced, yields both taxonomic and functional information.

INTRODUCTION

THE HUMAN GUT MICROBIOTA

While often used interchangeably, the terms microbiota and microbiome have distinct meanings. In this thesis, I try to adhere to established terminology[3,4] defining the microbiota as the entity of microbes in a specific site and the microbiome as the microbes, their collective genomes and, in broader terms, the environment they inhabit.

Since the emergence of life around 4.5 billion years ago, constraints have acted upon organisms sharing limited space and resources. This has led to the development of relationships, both positive and negative, in an attempt to maximize the chances of survival. With the advent of multicellular organisms and cell differentiation, these relationships have grown in intricacy and given rise to symbiotic relationships between multicellular eukaryotes and prokaryotes. With the surrounding animal evolving more complexity, naturally the bidirectional relationship with its microbial "inhabitants" also increases in complexity. Over the next billions of years, these relationships have specialized and divided into niches on virtually any multicellular surface that encounters the outside, including the human gut. The modern adult human gut microbiota in its totality comprises an estimated 10^{12}-10^{13} prokaryotic cells[5,6], which roughly matches the number of cells that make up the human body. In terms of genetic functional potential, the microbiome vastly outnumbers the human genome, at least by three orders of magnitude[7]. This microbiome develops with the human host from birth, and its metabolites contribute to shaping host health and disease[8].

A BRIEF HISTORY OF MICROBIOLOGY

Long before the formal advent of microbiology, the idea that invisible agents exist that could transmit disease was already taking root. As early as the 16th century, Italian physician Girolamo Fracastoro proposed that epidemic diseases were spread by "spores", though he likely referred to chemical substances rather than living organisms[9]. A major leap forward occurred in 1674, when Antonie van Leeuwenhoek, using microscopes of his own design, observed what he called "animalcules" in human feces and noted differences

between healthy and diarrheal samples[10]. Yet, the connection between these microbes and disease remained elusive. In the 19th century, Ignaz Semmelweis, a Hungarian physician in Vienna, made a pivotal observation: maternal mortality due to puerperal fever was markedly higher when births were attended by doctors and medical students than by midwives. He correctly deduced that infections were transmitted from cadavers during autopsies, then commonly performed for studying purposes. He dramatically reduced deaths by introducing mandatory handwashing, though his ideas were initially met with resistance[11]. It was Louis Pasteur who ultimately provided the experimental proof that microorganisms cause disease, laying the foundation of germ theory later formalized by Robert Koch, and firmly establishing microbiology as a scientific discipline[12].

Initially, microbiology was constrained by what could be seen through microscopes and grown in culture. Nevertheless, pioneers like Theodor Escherich published comprehensive studies of the human gut microbiota in the 1880s[13] and characterized species such as *Escherichia coli*. Phenotypic and metabolic comparisons relied on the ability to isolate and grow microbes, something only possible if their specific growth requirements were known. A major conceptual shift came in 1990 with the work of Carl Woese, who suggested a 16S rRNA gene sequence-based approach to redefine microbial classification through phylogeny rather than traditional phenotype-based taxonomy[14]. More recently, whole-genome sequencing has provided vast amounts of data on microbial diversity, such as the human microbiome project[4] and other large profiling efforts around the world[15,16]. Yet our ability to infer function and, more importantly, microbial interactions is still limited. Culturomics, a high-throughput culture-based approach that uses a variety of selective and non-selective media to isolate even low-abundance or slow-growing microbes based on genomic information, is offering new insights that can guide the cultivation and characterization of previously unculturable microbes[17], allowing us to unravel the complex web of metabolic interplay that underlies the human microbiome.

MICROBIOTA DEVELOPMENT

Despite occasional reports suggesting the presence of microbial DNA in the womb, leading experts agree that the intrauterine environment is sterile[18], and that microbial colonization of the human body begins at birth[19]. The initial colonization is strongly influenced by the mode of delivery[20,21]: vaginally

delivered infants are exposed to their mother's vaginal and fecal microbiota, while those born *via* cesarean section acquire microbes primarily from the skin and hospital environment[22]. This early difference has prompted interventions such as the use of vaginal swabs to simulate vaginal exposure in C-section deliveries[23]. Nevertheless, multiple studies indicate that microbial differences between birth modes largely equalize within the first few years of life[24]. However, these early microbial differences are shown to influence immune system development and are proposed to have long-lasting effects on disease susceptibility later in life[25]. Indeed, disturbances in microbial colonization and succession during this critical window of development, e.g. due to antibiotics, have been linked to an increased risk of allergies and autoimmune diseases, among other conditions[26].

Initial colonization of the infant gut is somewhat stochastic but generally follows a recognizable trajectory[27], transmitted from the environment and close family[24,28] and modulated by factors such as diet, pH, oxygen availability and bile acids[29–31]. Oxygen tolerant and skin-associated microbes, along with specialized taxa such as *Bifidobacterium* species and other lactic acid bacteria (LAB), establish early dominance. The communities of formula-fed infants show a different profile, with early expansion of *Bacteroides*, *Clostridium* and *Enterobacteriaceae*[32,33]. Breast-fed infants receive human milk oligosaccharides (HMOs) through breast milk[34], which are non-digestible to the infant but utilized particularly by *Bifidobacterium*, aiding in their expansion in the infant gut. These breastfeeding-associated *Bifidobacterium* species in turn have been shown to produce immunomodulatory compounds like indolelactic acid[35], which support early immune system development, forming a complete cycle in which maternal nutrients shape the microbiota, the microbiota shapes metabolite production, and these metabolites influence host immunity. In this early phase of succession, the infant gut metabolite profile is dominated by succinate, lactate and formate as well as propanediol[36]. As the infant gut becomes more anaerobic due to microbial oxygen consumption, obligate anaerobes begin to colonize, including genera such as *Ruminococcus*, *Eubacterium*, and oral taxa like *Fusobacterium*[27,37]. With a dietary shift towards fiber-containing solid foods, these early anaerobes are joined by more genera characteristic of the adult gut, such as *Bacteroides*, *Faecalibacterium*, and *Prevotella*[38], many of which are involved in fiber degradation, fermentation, and short-chain fatty acid (SCFA) production, resulting in increased levels of butyrate[39]. This butyrate can fuel beta-oxidation in epithelial cells and keep the maturing gut community anoxic[40]. Additional emerging species like *Akkermansia* are involved in the interaction with the

mucosa[41]. The appearance of other specialized microbial functions, including methanogenesis and sulfate reduction, tends to occur later[27], once the gut has reached a highly reducing state and these species can establish and stably maintain a niche. Thus, early microbiota development is a dynamic process shaped by host factors such as oxygen availability and diet, as well as environmental exposures.

During adulthood, the microbiota reaches a relatively stable configuration[42], shaped by long-term diet, lifestyle, and genetics[43,44]. In older age, however, microbial diversity and resilience begin to decline[45,46]. This results from physiological changes such as reduced peristalsis[45], altered hormonal levels especially in women[47], and "inflammaging", a low-grade chronic inflammation associated with aging[48,49]. Admission to a nursing home, dietary changes, and reduced physical activity further contribute to microbiota shifts[49], as does polypharmacy[50]. Notably, aging and frailty are often accompanied by reduced alpha diversity[51] and increased beta diversity[52], suggesting greater inter-individual variability and a loss of microbial homeostasis. This observation aligns with the so-called "Anna Karenina principle" for animal microbiomes, where dysbiotic microbiomes become increasingly divergent from each other[53,54]. Nonetheless, some elderly individuals maintain a "youthful" microbiota composition, potentially contributing to remarkable longevity in centenarians: cross-sectional studies of centenarians and healthy senior athletes have identified higher alpha diversity and higher abundances of beneficial taxa such as *Bifidobacterium*, *Lactobacillus*, *Faecalibacterium prausnitzii*, and *Akkermansia muciniphila*[55–57]. While these findings suggest potential signatures of healthy aging, causal mechanisms remain unclear. Survivorship bias may also play a role, as harmful microbes may contribute to earlier mortality, leaving behind microbial profiles that persist in the population but are not necessarily health-promoting. Further longitudinal studies are needed to disentangle these complex relationships.

MICROBIAL DIVERSITY, ECOLOGY AND INDIVIDUALITY

For much of the history of microbiology, microbial diversity in the human gut could only be observed through microscopy and basic culturing techniques, which nevertheless yielded the discovery of various gut microbial species[58,59]. In 1974, Moore and Holdeman were among the pioneers to systematically

culture and characterize the human gut microbiota, identifying 113 species, with 10-30 observed *per* individual[60]. Their findings already revealed a dominance of what were then known as *Bacteroides*, *Bifidobacterium*, *Eubacterium*, and *Fusobacterium*. By 1995, improved anaerobic culturing techniques allowed the same team to identify 371 species from 88 fecal samples, with 177 species occurring only once[61]. Interestingly, initial associations were made between some species and colorectal cancer, such as *Fusobacterium*, negatively associated with cancer, and *Fusobacterium prausnitzii* (now reclassified as *Faecalibacterium prausnitzii*[62]) and *Eubacterium eligens* (now reclassified as *Lachnospira eligens*[63]), positively associated with cancer. However, such findings must be interpreted cautiously due to potential selection bias, misclassification, and the fundamental difference between correlation and causation. Indeed, more recent research has shown opposite associations, with *Fusobacterium* linked to cancer[64], and *F. prausnitzii*, one of the most abundant taxa in the human gut[65], and *L. eligens* both attributed with anti-inflammatory and health-promoting properties[66–69].

The early 2010s marked a turning point with the emergence of culturomics[17,70]. Coupled with 16S rRNA gene sequencing, this approach led to a dramatic expansion in the human gut microbial catalog. As of 2023, 3,253 bacterial species from the human gut have been isolated, with approximately two-thirds recovered *via* culturomics[71]. Still, culturomics is limited by the need of some *a priori* knowledge about plausible growth conditions, and therefore "unculturable" microbes remain elusive. Culture-independent techniques have helped to bridge this gap. In 2008, a study using deep 16S rRNA gene sequencing to track the microbiota of individuals over time revealed 2,600-3,300 operational taxonomic units (OTUs) across samples, yet only around 130 distinct genera[72]. This suggests that most microbial variability occurs at the species or strain level rather than higher taxonomic levels, with recent studies estimating between 150 and 400 species *per* individual[73], depending on whether culture-based or genomics-based approaches were used. With the rise of metagenomics, we now know that the gut microbiome contains more than 22 million non-redundant genes[7], dwarfing the approximately 20,000 genes of the human genome[7] and offering a yet underexplored reservoir of functional potential. Recent gut metagenome catalogs comprise more than 4600 species, 70% of which still lack cultured representatives[74], suggesting that culturing efforts are far from complete.

The modern consensus on the core adult human gut microbiota composition, based on both culturing and culture-independent methods, reports a

predominance of the phyla Bacillota (formerly Firmicutes) and Bacteroidota (formerly Bacteroidetes), with smaller but important contributions from Actinomycetota (Actinobacteria), Pseudomonadota (Proteobacteria), Fusobacteriota, and Verrucomicrobiota[1]. Additionally, the gut hosts archaea, especially *Methanobrevibacter smithii* but also other genera like *Methanomassiliicoccus* and *Methanosphaera*[75], as well as fungi[76], protozoa[77], and a rich virome[78]. On the genus level, dominant taxa include *Faecalibacterium*, *Bacteroides*, *Prevotella*, *Clostridium*, *Bifidobacterium*, *Ruminococcus* and *Blautia*[1]. Importantly, most of our knowledge stems from fecal sampling, which primarily reflects the colonic microbiota. The small intestinal microbiome is quite distinct: faster transit time, more oxygenated and nutrient-rich[79]. Accordingly, it is enriched in fast-growing primary fermenters like *Streptococcaceae*, *Lactobacillaceae* and *Bifidobacteriaceae*, alongside secondary fermenters such as *Veillonellaceae*[79].

The exact composition of the human gut microbiota, however, varies widely across individuals, influenced by a combination of geographic location[80], environmental exposures[81], dietary habits, medication, and host genetic factors. Among these, diet stands out as a particularly powerful and modifiable determinant. Vegetarian or vegan diets[82] and other dietary patterns such as low-carbohydrate or low-fat[83] shape the microbiota in distinct ways. Dietary composition, like the type and amount of dietary fiber, for instance, significantly influence the production and composition of SCFAs, which in turn modulate host metabolic and immune functions[84,85]. Emerging evidence also suggests that dietary composition affects bile acid profiles[86], which may contribute to host cardiometabolic health. Indeed, well-designed dietary interventions have consistently been shown to alter the microbiota, with possible effects on cardiometabolic health[87].

Antibiotic exposure, especially during early life, can cause profound and lasting disruptions to microbial communities[88]. Beyond increasing the risk of antibiotic resistance, repeated antibiotic use has been linked to long-term health consequences such as obesity and cardiometabolic disease[89]. A wide range of other commonly prescribed medications also influence the gut microbiota, with effects that may depend on the presence or absence of specific drug-metabolizing taxa[90]. Notably, cardiometabolic drugs, including statins (which can reduce methanogen abundance[91]), metformin, beta blockers, antihypertensives, and proton pump inhibitors (PPIs), have all been shown to alter microbiota composition[92,93]. PPIs, in particular, are associated with a

migration of oral microbes into the small intestine[94], potentially contributing to dysbiosis and disease.

Host genetics also play a role in shaping the microbiota. Genome-wide association studies (GWAS) have identified numerous single nucleotide polymorphisms (SNPs) associated with specific microbial taxa[95], some of which also correlate with disease risk. For example, levels of gut microbial butyrate and propionate production have been linked to host genetic variation and glycemic control as well as type 2 diabetes (T2D) risk[96], underscoring the complex interplay between the composition of the microbiota, host genetics, and disease.

Attempts to classify gut microbiota into "enterotypes", originally proposed as three clusters dominated by *Bacteroides*, *Prevotella*, or *Ruminococcus*, respectively, offer a conceptual framework for variability in gut microbiota composition[1]. A putative fourth enterotype, *Bacteroides* 2, characterized by low diversity, has even been linked to higher levels of inflammatory markers, obesity and disease states[97,98]. However, critics argue that the three main enterotypes may oversimplify the continuum of microbial community structures observed in reality[99].

More crucial than taxonomic classification is the concept of functional redundancy: while individual species and strains differ between people, making each person's microbiota as unique as a fingerprint[100], many of the microbiome's core functions are shared across taxa[101,102]. This redundancy lends the microbiota resilience[103,104]: if one species loses fitness, another may compensate by performing similar roles, often more efficiently in that particular environment. Understanding not only "who is there" but also "what they do" remains central to uncovering the full role of the gut microbiota in health and disease.

GUT MICROBIAL METABOLISM IN SHORT

While human digestion starts in the oral cavity, the majority of gut microbial metabolism takes place in the intestinal tract, where dense microbial communities encounter and metabolize partially digested, unabsorbed dietary components such as fibers and other carbohydrates[85], amino acids[105], and fats[106], xenobiotics such as environmental chemicals[107] and pharmaceuticals[90,108], as well as host-derived compounds like cholesterol[109,110],

bile acids[111], and mucus[112]. While proteins and fats are thought to be broken down by host enzymes to large extents[106,113], the microbial enzymatic repertoire for carbohydrate degradation, which vastly exceeds that of the human host[114], permits the gut microbiota to utilize many otherwise inaccessible dietary fibers[115].

CARBOHYDRATES

Some species in the gut microbiota possess a vast array of carbohydrate-active enzymes (CAZymes)[115], making them important keystone species[116] able to break down complex dietary fibers that are otherwise indigestible by the human host. Notable examples include *Ruminococcus bromii*[117,118] and *Bacteroides thetaiotaomicron*[119], which initiate the degradation of resistant polysaccharides and thereby facilitate access to simpler sugars for other community members. The resulting monosaccharides are metabolized by a diverse range of gut bacteria *via* various glycolytic pathways, including the Embden-Meyerhof-Parnas, Entner-Doudoroff and pentose phosphate pathways, as well as the bifid shunt, and heterolactic fermentation[120,121]. Since the gut is largely anoxic, these pathways generate ATP through substrate-level phosphorylation. Pyruvate is the common end product and is typically metabolized further in the gut. While some facultative anaerobes, often *Enterobacteriaceae*[122], are capable of anaerobic respiration using alternative electron acceptors such as nitrate[123], this process is generally limited to inflamed or disrupted gut environments[124]. In the healthy gut, fermentation of pyruvate dominates microbial energy metabolism regenerating $NAD(P)^+$ required for the glycolytic pathways under strictly anaerobic conditions[120,121].

Initial fermentation products include lactate, mainly produced by LAB, *Bifidobacterium*[125], and *Enterobacteriaceae*[126], and succinate, a hallmark of *Bacteroides* and *Prevotella* metabolism[127]. These intermediates generally do not accumulate to high levels in a healthy gut[128] due to effective cross-feeding[129,130] with further metabolizing bacteria. Due to its low acidity constant (pK_a), accumulation of lactate can have profound effects on intestinal pH, which in turn impacts metabolism of other bacteria[131], such as impairing butyrate production[129]. While the effects of succinate on the microbiome and human health are still understudied, so far both beneficial and detrimental effects have been reported, potentially depending on succinate concentrations. At low concentrations, succinate is associated with intestinal gluconeogenesis and improved insulin sensitivity[127], while during transient accumulation of

high levels, succinate has been shown to promote the expansion of opportunistic pathogens such as *Clostridioides difficile*[132]. In the healthy gut, succinate metabolism is the main source of propionate, primarily facilitated by Bacteroidota[130], while lactate is either oxidized to succinate or reduced to acetyl-CoA and subsequently transformed into acetate or butyrate[131], preventing an accumulation of these intermediates. Butyrate production from carbohydrates is facilitated *via* two pathways: the more energy-efficient[133] and dominant[134] butyryl-CoA:acetate CoA-transferase pathway (e.g., *F. prausnitzii*, *Roseburia* spp.), and the butyrate kinase pathway (e.g., *Coprococcus* spp.)[135–137]. Acetate, propionate, and butyrate are the principal end products of microbial fermentation of carbohydrates, with concentrations in the human colon reaching up to 131 mmol/kg of content[128] and an approximate ratio of 3:1:1. These SCFAs are absorbed to varying degrees[138] and exert numerous beneficial effects on host health (further discussed below), which partly explains why their concentrations are lower in fecal samples than in the proximal colon.

AMINO ACIDS

Amino acid metabolism is assumed to play only a minor role in the gut due to efficient host digestion and absorption[139]. Additionally, it is assumed that carbohydrate metabolism takes precedence before amino acid metabolism, so amino acid metabolism is mainly observed once carbohydrates have been depleted[140]. Breakdown usually starts with decarboxylation or deamination[141], yielding an amine plus carbon dioxide or a carboxylic acid plus ammonia, respectively[139]. Carboxylic acids can be further metabolized to SCFAs or branched-chain fatty acids (BCFAs) by taxa like *Bacteroides* and *Clostridium* cluster IV[139]. Butyrate production from amino acids, notably from lysine and glutamate, could significantly contribute to intestinal butyrate levels[136]. A notable exception is the Stickland reaction, performed by certain *Clostridia* and *Peptostreptococcaceae* like *C. difficile*, where two amino acids serve as electron donor and acceptor in a coupled redox reaction, producing both SCFAs or BCFAs[142]. Aromatic amino acids are particularly notable for their stable benzene ring, and when metabolized, yield phenolic and indolic compounds, with key contributors including *Bacteroides*, *Bifidobacterium*, *Clostridium*, *Eggerthella* and *Lactobacillus*. Many of these compounds, such as indole[143], p-cresol[144] and imidazole propionate[145], have been increasingly linked to intestinal inflammation[146] and the pathophysiology of metabolic diseases such as diabetes[145], but select compounds like indolepropionate and

indolelactic acid are reported to negatively correlate with T2D, positively modulate the host immune system, and inhibit atherosclerosis[147]. Lastly, it has been shown that certain members of the Bacillota can metabolize the amino acid derivative L-carnitine into trimethylamine (TMA)[148]. TMA is absorbed in the liver and oxidized into trimethylamine N-oxide (TMAO)[149], which has been implicated in cardiovascular disease[150] and the pathogenesis of atherosclerosis[151].

LIPIDS

Like proteins, triglycerides are primarily broken down and absorbed by the host through pancreatic lipases and bile-mediated emulsification and only a fraction of ingested lipids reaches the colon[152,153]. However, some gut bacteria encode lipases, enabling them to hydrolyze triglycerides and access the liberated glycerol backbone for further metabolism[154]. While microbial lipid metabolism appears limited, certain bacteria contribute to host-microbe interactions *via* lipid-associated pathways. For instance, LAB, such as *Limosilactobacillus reuteri*, metabolize glycerol into reuterin[155], an antimicrobial compound acting against pathogens and commensals[156]. In general, lipids are not a major substrate for bacterial fermentation and are even considered antimicrobial to many gut taxa[106]. Bile acids, secreted by the host to aid in lipid emulsification, also shape the microbial landscape through their antimicrobial properties and microbial transformations, which will be discussed further below.

HYDROGEN

Hydrogen is a major byproduct of microbial fermentation in the gut[157]. It is primarily released *via* hydrogenases to regenerate oxidized ferredoxin or $NAD(P)^+$ [158]. However, accumulation of hydrogen can inhibit these enzymatic processes and impair further fermentation[159]. Besides excretion through breath and flatus, specialized hydrogen-utilizing microbes in the gut prevent hydrogen accumulation by maintaining low hydrogen partial pressures. The three major hydrogen sinks are reductive acetogenesis, methanogenesis, and sulfate reduction, with varying relative importance of these pathways between individuals[157].

Acetogens such as *Blautia hydrogenotrophica* and *Marvinbryantia formatexigens*[160] use the Wood–Ljungdahl pathway to convert hydrogen and carbon dioxide into acetate[161]. Although this pathway is energetically the least favorable of the three main hydrogen sinks, it may be the most widespread in the human gut due to a comparably wide range of possible growth substrates[160].

Methanogenesis, energetically more favorable[162], is carried out primarily by *M. smithii*[163], but recent studies have expanded the known diversity to include *Methanobrevibacter intestini*[164], *Methanosphaera*, and *Methanomassilicoccus*[75,165]. While long regarded non-pathogenic[166], recent reports of associations with disease exist[75,167], but no mechanism of pathogenicity has been reported from methanogens so far, rather suggesting a more supportive role to other pathogens[166]. The physiological role of the methane produced remains unclear, but some evidence suggests it may slow intestinal transit time[168]. This could possibly favor slow-growing methanogens, but also increase caloric harvest by the host, possibly contributing to obesity[169]. However, methanogens, especially *M. smithii*, have been reported to be negatively associated with BMI and obesity in many studies[170,171], leaving the link to obesity unclear. Additionally, methanogens of the *Methanomassiliicoccus* genus can utilize TMA, stemming from metabolism of L-carnitine[148] or choline[172], thereby potentially preventing subsequent accumulation of TMAO[173] and exerting an atheroprotective effect.

Sulfate-reducing bacteria (SRB), such as *Desulfovibrio* and *Bilophila*, represent the most energetically favorable hydrogen sink[162]. *Desulfovibrio*, the dominant sulfate reducing genus in the intestine[174], and other SRB utilize inorganic sulfate, while *Bilophila* uses organic sulfur compounds like taurine[175,176] to produce hydrogen sulfide (H_2S). *Bilophila* has been associated with detrimental health outcomes, particularly under high-fat diets with increased bile acid and taurine availability[177,178]. The role of H_2S and *Desulfovibrio* appears more nuanced, with both negative and potentially beneficial effects reported (further discussed below).

Evidence for competition between these hydrogen-utilizing pathways is limited and context-dependent. *In vitro* studies have shown conflicting outcomes, with both methanogens and SRB outcompeting each other under different conditions, possibly influenced by pH[179,180]. Moreover, co-colonization studies in mice indicate mutualistic interactions with other gut microbes: SRB may benefit from mucin-degrading *B. thetaiotaomicron* that releases sulfate[181], while *M. smithii* and *B. hydrogenotrophica* have been

shown to support fermentation when co-cultured with *B. thetaiotaomicron*[160,182]. Earlier studies in humans are inconclusive, showing either a near mutually exclusive predominance of methanogens or SRB over the other[183,184], or a more balanced presence of both[185,186], but more recent qPCR and metatranscriptomics-based studies disprove mutual exclusivity[187,188]. Together, these observations underscore a tight cross-feeding network in the human gut, where hydrogen-utilizing microbes play essential roles in maintaining redox balance and supporting broader microbial functions.

MICROBIAL METABOLISM OF STEROID COMPOUNDS

CHOLESTEROL CONVERSION

Intestinal bacteria encounter cholesterol from various sources, including dietary intake, host biliary secretion, and shed intestinal epithelial cells. A key microbial transformation of cholesterol is its reduction to coprostanol, a compound first identified in human feces in 1897 and initially termed *Koprosterin*[189]. Coprostanol, which is not absorbed by the host and thus excreted in the feces[110], is remarkably stable under anoxic conditions, and is widely used as a biomarker for human fecal contamination in environmental studies[190]. As early as the 1930s, it was hypothesized that intestinal bacteria were responsible for this transformation[191], a notion confirmed in the 1950s[192]. By the 1970s, it became evident that individuals vary in their ability to convert cholesterol, a trait quantified using the "conversion rate", the ratio of coprostanol and coprostanone (an intermediate) to total fecal steroids. This allowed for classification into high and low converters, showing 88 ± 9% and 10 ± 8% conversion, respectively[193].

The first bacterial strain demonstrated to perform the conversion of cholesterol to coprostanol was isolated from the rat cecum in 1973 and later named *Eubacterium coprostanoligenes* ATCC 21408[194]. That same year, several human-derived anaerobes, including *Clostridium*, *Bacteroides*, and *Bifidobacterium* species were reported to exhibit cholesterol-reducing activity[195], although these results could not be reproduced[196]. Over time, additional *Eubacterium*-like strains were isolated[197,198], while some bacteria, such as *Fusobacterium russii* and *Bacteroides vulgatus*, were shown to inhibit cholesterol reduction in co-culture experiments[196]. Despite ongoing interest, no additional coprostanol-producing taxa besides *E. coprostanoligenes* were

identified until 2007, when *Bacteroides* sp. strain D8, closely related to *Bacteroides dorei*, was isolated and shown to reduce cholesterol[199]. Sporadic reports of *Lactobacillus* strains incorporating cholesterol into their membranes and converting minor fractions to coprostanol exist[200], but these findings were not further pursued and remain largely unverified.

Cholesterol conversion is absent in infants[201], gradually reaching adult levels by age four[202], and appears to correlate with secondary bile acid metabolism[202]. Dietary factors also affect cholesterol conversion: sugars such as lactose are reported to reduce conversion, potentially through acidification of the colonic environment[203], while phytosterols compete with cholesterol for uptake and microbial conversion, resulting in increased fecal cholesterol[204]. Interestingly, vegetarian versus omnivorous diets do not appear to affect the distribution of high and low converters or ratio of cholesterol to coprostanol[205], and studies in pigs suggest that increased dietary cholesterol does not necessarily lead to higher microbial conversion[206].

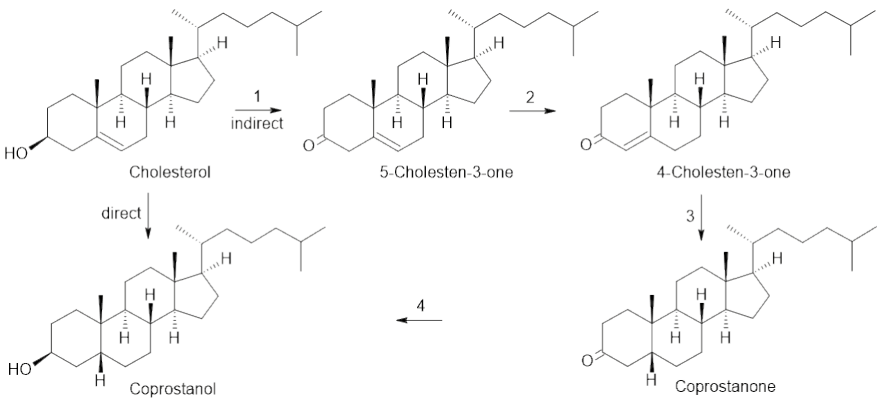

Figure 1. Putative direct and indirect cholesterol-to-coprostanol conversion pathways.

Until 2020, no set of enzymes capable of converting cholesterol to coprostanol had been isolated and described, possibly due to the elusiveness of cholesterol converting strains. Two pathways have been postulated[110] (Fig. 1). The first one is a direct reduction of the double bond at the C5 carbon of cholesterol (5-cholesten-3-ol) yielding coprostanol (5β-cholestan-3-ol). The second one is an

indirect multi-step reaction starting with the oxidation of the hydroxyl group in C3 to 5-cholesten-3-one (step 1), followed by an isomerization of the double bond to 4-cholesten-3-one (step 2), a reduction of the double bond at C4 to coprostanone (5β-cholestan-3-one; step 3) and a final reduction of the C3 carbonyl group to a hydroxyl group (step 4), resulting in coprostanol.

A major breakthrough came in 2020 with the identification of the enzyme intestinal steroid metabolism A (IsmA), encoded by the *ismA* gene in *E. coprostanoligenes*, capable of catalyzing both the initial oxidation of cholesterol to 5-cholesten-3-one and the reverse reduction from coprostanol to coprostanone[207] (Fig. 1; steps 1 and 4 of the indirect pathway, respectively), supporting the indirect pathway. Although *ismA* does not fully explain cholesterol conversion potential across individuals, its abundance negatively correlates with circulating cholesterol levels in human cohorts and has been linked positively to cardiovascular health[109,207]. Beyond reduction to coprostanol, other microbial cholesterol modifications have been observed, such as sulfatation and glycosylation, with taxa like *Oscillibacter* implicated in these processes[109]. Furthermore, bacteria with cholesterol oxidase activity have also been isolated from the human gut[208,110]. Together, these findings underscore the complexity and potential clinical relevance of microbial cholesterol metabolism in the gut.

BILE ACID METABOLISM

Excess cholesterol in the host is either excreted directly or converted into the primary bile acids cholic acid (CA) and chenodeoxycholic acid (CDCA) in the liver[209]. Before entering the intestine, these primary bile acids are conjugated with glycine or taurine to form bile salts, enhancing their water solubility and providing amphiphilic properties essential for lipid emulsification[157]. In the terminal ileum, the majority of bile salts is reabsorbed in a process called enterohepatic circulation[210]. The remainder of bile salts encounter bacterial bile salt hydrolases (BSH), found in taxa such as *Lactobacillus*, *Bifidobacterium*, *Clostridium*, and *Bacteroides*[211]. These enzymes deconjugate bile acids, freeing them for further microbial transformations at various positions on the steroid nucleus, yielding secondary bile acids. Among these modifications, 7α-dehydroxylation is well-characterized: CA and CDCA are converted to deoxycholic acid (DCA) and lithocholic acid (LCA), respectively, by bacteria like *Clostridium scindens* and *Clostridium hylemonae*[111]. Other notable transformations include 6α-hydroxylation, producing hydrophilic bile acids

such as hyocholic acid (HCA) and hyodeoxycholic acid (HDCA)[212], as well as various oxidation, epimerization (e.g., forming ursodeoxycholic acid, UDCA)[111], and isomerization reactions occurring at the 3, 5, 7, and 12 positions[213]. Unlike microbial production of SCFAs, bile acid modifications might not yield growth-related energy and are therefore regarded as secondary metabolism, potentially to lower toxicity of primary bile acids. Both primary and secondary bile acids can act as key signaling molecules *via* host receptors like FXR and TGR5, impacting host health and metabolism[214] (further discussed below). More recently, gut bacteria, including *Enterocloster bolteae*, have been shown to conjugate bile acids to amino acids[215], though the physiological significance of this process is still under investigation.

CARDIOMETABOLIC DISEASE

GLOBAL BURDEN AND RISK FACTORS

Cardiometabolic disease (CMD) is an umbrella term encompassing components of metabolic syndrome, including T2D and central obesity, which are tightly linked with cardiovascular diseases (CVDs) like atherosclerosis, ischemic heart disease, and stroke[216]. Together, these conditions represent the leading cause of death globally. Specifically, ischemic heart disease and stroke alone accounted for over 15 million deaths in 2021[217]. While particularly prevalent in medium-to-high-income countries, the burden of CMD is rapidly rising in low- and middle-income regions as well[217]. The economic impact is staggering with CVDs estimated to cost the European Union approximately €282 billion annually[218]. The systemic nature of cardiometabolic dysfunction is further highlighted by conditions such as metabolic dysfunction-associated steatotic liver disease (MASLD), which affects an estimated 30% of the global population and is closely tied to obesity, insulin resistance, and chronic inflammation[216].

Classical risk factors for CMD include lifestyle, age and life transitions like menopause (with an effect of the microbiome[47]), genetic predisposition, dyslipidemia, smoking, and the consumption of an obesogenic “Western-style” diet[216]. However, emerging contributors such as air pollution and climate change are gaining recognition[216]. Situated at the intersection of host physiology, diet, and environmental exposures, the gut microbiota is increasingly acknowledged as a modulator of cardiometabolic health, capable

of influencing host metabolism and inflammation through the production of both beneficial and deleterious microbial metabolites.

HUMAN CHOLESTEROL METABOLISM

Cholesterol, a sterol belonging to the steroid family, is an essential lipid molecule that contributes to maintaining cell membrane fluidity and structural integrity across varying temperatures. Beyond its structural role, cholesterol is the biochemical precursor for the synthesis of steroid hormones, bile acids, and vitamin D[219]. Although all human cells are capable of synthesizing cholesterol, the liver is the central organ for its production[220]. A smaller portion of circulating cholesterol is derived from dietary intake[219]. Hepatic cholesterol synthesis is tightly regulated, with 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase being the rate-limiting enzyme in this pathway. This enzyme is the target of statins[221], widely prescribed to reduce blood cholesterol levels.

To enable transport in the aqueous environment of the bloodstream, cholesterol is esterified to form cholesteryl esters (CEs) and incorporated along with triglycerides into very low-density lipoprotein (VLDL) particles, which are assembled with apolipoprotein B100 (Apo-B100)[222]. As VLDL particles deliver fatty acids to peripheral tissues, they are progressively converted into low-density lipoprotein (LDL) particles, which are the primary cholesterol carriers in circulation and are closely associated with the development of atherosclerotic plaques[223]. In contrast, high-density lipoprotein (HDL) particles, composed primarily of apolipoprotein A-I (Apo-AI), mediate reverse cholesterol transport by collecting excess cholesterol from peripheral tissues and returning it to the liver[224]. Because most cells cannot degrade or excrete cholesterol, this pathway is essential for cholesterol homeostasis[220].

In the liver, cholesterol can be recirculated, stored, or converted for excretion. The primary excretory route involves conversion into bile acids, followed by conjugation with glycine or taurine to increase solubility[209]. These bile salts, mixed with excreted cholesterol, are then stored in the gallbladder and released into the duodenum to aid in the emulsification and absorption of dietary lipids. An additional, lesser-understood route is trans-intestinal cholesterol efflux (TICE), which bypasses bile acid formation and results in cholesterol excretion from enterocytes directly into the intestinal lumen; this route may account for up to 35% of total cholesterol excretion[225].

Within the gut lumen, cholesterol originates from both dietary sources and host-derived secretions, including bile, TICE, and shed epithelial cells[110]. Intestinal cholesterol may be reabsorbed into the enterohepatic circulation[210] in the small intestine, where it competes for uptake with structurally similar phytosterols on the Niemann-Pick C1-like 1 (NPC1L1) transporter[226]. Once absorbed, cholesterol is re-esterified in enterocytes and packaged into chylomicrons, lipoprotein particles formed with a truncated apolipoprotein B48[227], that distribute dietary lipids to peripheral tissues. Chylomicron remnants are subsequently cleared by the liver, completing the cycle of cholesterol transport and recycling[222]. Intestinal cholesterol uptake also triggers the secretion of cholesin, a gut-derived hormone that suppresses cholesterol synthesis in the liver[228], highlighting another mechanism by which the gut can modulate systemic cholesterol levels.

CHOLESTEROL AND THE PATHOGENESIS OF ATHEROSCLEROSIS

Atherosclerotic plaques have been identified in ancient remains such as Ötzi the Iceman and Egyptian mummies of pharaohs, dating back thousands of years[219], indicating that atherosclerosis is not merely a modern affliction but may have historically been linked to affluence and dietary excess. The connection between cholesterol and atherosclerosis was first demonstrated in 1913, when feeding rabbits a high-cholesterol diet induced vascular lesions resembling human atherosclerotic plaques[229]. Nevertheless, for much of the early 20th century, the senescence theory considered atherosclerosis an inevitable consequence of aging[230], while the later response-to-injury theory hypothesized that an injury to the arterial wall was the fundamental trigger of atherosclerosis[231]. A major conceptual shift occurred in the 1990s with the proposal of the response-to-retention hypothesis[232], now widely accepted as a central mechanism in atherogenesis. According to this framework, atherosclerosis is initiated by the subendothelial retention of apolipoprotein B100-containing lipoproteins, particularly LDL, in prone areas of disturbed blood flow and endothelial dysfunction[233]. Native LDL is not inherently pro-inflammatory; however, once retained, it is susceptible to oxidative modifications, forming oxidized LDL (oxLDL), a potent trigger of immune activation[234]. Circulating monocytes are recruited to these sites, where they differentiate into macrophages, and take up oxLDL *via* scavenger receptors. Due to the unregulated nature of this uptake, cholesterol will accumulate once

it exceeds excretion, leading to the formation of foam cells[233]. These foam cells aggregate into fatty streaks, the earliest detectable atherosclerotic lesions. Vascular smooth muscle cells are subsequently recruited to stabilize plaques by forming a fibrous cap, decreasing the plaque protrusion into the arterial lumen, and subsequently obscuring symptoms and slowing atherosclerosis progression[233]. As plaques mature, cap thinning and the formation of a necrotic core due to impaired clearance of apoptotic foam cells increase the risk of rupture[235]. Plaque rupture followed by thrombus formation can acutely obstruct blood flow, leading to major cardiovascular events such as ischemic myocardial infarction and stroke[235].

CONDITIONS OF IMPAIRED GLYCEMIC CONTROL

Impaired glycemic control is a spectrum of conditions ranging from prediabetic stages to overt T2D. It is characterized by impaired insulin sensitivity and β-cell dysfunction and is intricately linked with other cardiometabolic conditions, including obesity, dyslipidemia, hypertension, and atherosclerosis[236]. In individuals with normal glucose tolerance (NGT), blood glucose homeostasis is tightly regulated by the opposing actions of insulin and glucagon. Postprandially (after a meal), elevated blood glucose levels trigger insulin secretion from pancreatic β-cells, promoting glucose uptake into peripheral tissues (mainly muscles) and the liver, inducing hepatic glycogenesis. Conversely, during fasting, pancreatic α-cells secrete glucagon, stimulating hepatic gluconeogenesis and glycogenolysis to maintain stable glucose levels[237]. One early disturbance in this balance is impaired fasting glucose (IFG), characterized by elevated fasting blood glucose and an elevated but rapidly cleared glycemic response to a glucose challenge. IFG has been linked to hepatic insulin resistance, which is thought to result from chronic high sugar intake and factors such as visceral adiposity[238]. In contrast, impaired glucose tolerance (IGT) is marked by relatively normal fasting glucose but exaggerated and prolonged postprandial glucose elevations, reflecting peripheral insulin resistance, particularly in muscle and adipose tissue[238]. These early dysglycemic states may be reversible through lifestyle intervention[239]. However, progression to combined glucose intolerance (CGI), defined by both IFG and IGT, represents a more advanced, less reversible state with elevated risk for CVD and conversion to T2D[240,241]. The role of pancreatic β-cell dysfunction in the development of T2D is not yet fully elucidated[242]. One of the main two models assumes that insulin resistance leads to

compensatory oversecretion of insulin by the pancreas, which, together with chronic metabolic stress, including lipotoxicity and oxidative damage from elevated free fatty acids[236], leads to eventual breakdown of β-cell function. The other model assumes that initial insulin oversecretion due to chronic metabolic stress over time induces insulin resistance and leads to impaired glucose tolerance. Both of these models are supported by clinical and experimental data, suggesting that the sequence of events might depend on the individual[243]. Importantly, dysglycemia and insulin oversecretion are strong contributors to atherosclerosis and cardiovascular disease[244,239], making early identification and intervention a key strategy for reducing cardiometabolic impact and mortality.

MICROBIAL METABOLITES IN CARDIOMETABOLIC DISEASE

COPROSTANOL AND *ismA*

While early research hinted at a potential link between fecal coprostanol levels and intestinal diseases such as colorectal cancer, polyps, and ulcerative colitis, more recent studies on this topic are scarce[110]. Similarly, evidence for the systemic cholesterol-lowering effects of high microbial conversion of cholesterol to coprostanol remains limited, primarily stemming from observational studies[245] and small-scale animal experiments[246]. Despite this, it is increasingly evident that the gut microbiota influences host cholesterol metabolism not only *via* bile acid transformation but also through direct cholesterol conversion. This is supported by mathematical modeling studies that highlight a role for microbial metabolism in modulating circulating cholesterol levels[247]. However, experimental findings are inconclusive: oral administration of *E. coprostanoligenes*, the best-described cholesterol-reducing bacterium, did not significantly reduce plasma cholesterol levels *in vivo*, despite active conversion to coprostanol in the gut[110]. Interestingly, in a study involving overweight postmenopausal women, a higher fecal coprostanol-to-cholesterol ratio was associated with lower plasma cholesterol levels following a dietary intervention with milk polar lipids, suggesting diet-microbiota interactions play a key role[248]. These findings underscore the complexity of cholesterol homeostasis, which is tightly regulated through a feedback loop between intestinal absorption and endogenous hepatic synthesis[220]. The gut-derived hormone cholesin, for example, suppresses hepatic cholesterol synthesis in response to dietary absorption[228], potentially

offsetting microbiota-driven reductions in cholesterol uptake. Statin therapy, widely used to lower cholesterol especially in individuals with cardiometabolic conditions, adds further complexity to this regulatory network. Leveraging modern analytical methods and data from larger cohorts, recent investigations in the Framingham Heart Study have demonstrated that fecal coprostanol levels are inversely correlated with plasma cholesterol, triglycerides, and C-reactive protein[109], indicating potential anti-inflammatory benefits as well. Moreover, individuals lacking gut bacterial species encoding the cholesterol-to-coprostanol conversion enzyme IsmA exhibit higher plasma cholesterol levels, as shown in a meta-analysis of three independent cohorts[207]. Additionally, the abundance of *ismA*-encoding bacteria was reduced in Crohn's disease[207], further supporting a link between cholesterol conversion and inflammation. The Framingham study also revealed an additive cholesterol-lowering effect from the combined presence of *ismA*-encoding bacteria and other species such as *Oscillibacter*[109], suggesting broader microbial contributions to host cholesterol regulation.

SHORT-CHAIN FATTY ACIDS

Acetate, propionate and butyrate are the major end products of microbial fermentation of dietary fiber in the colon, where their concentrations can reach up to 131 mM[128]. Around 90% of these SCFAs are absorbed by the host[138]. All three SCFAs are detectable in portal blood, with acetate present in the highest concentration, followed by propionate and only minor amounts of butyrate[128]. Once absorbed, SCFAs can enter the host tricarboxylic acid cycle and contribute an estimated 5–10% of the host's daily energy requirements[249]. However, butyrate is largely utilized in the intestine by colonocytes, serving as their preferred energy source *via* β-oxidation[250]. This local metabolism of butyrate supports maintenance of a hypoxic luminal environment, which is essential for colonization resistance against pathogens[251]. Moreover, SCFAs contribute to intestinal health by enhancing epithelial barrier integrity[252], and especially butyrate has been shown to reduce "leaky gut". This is a state of increased intestinal permeability leading to more lipopolysaccharides leaking into the bloodstream and subsequent triggering of the immune system, which has been implicated in the pathogenesis of cardiovascular disease[253]. SCFAs also exert systemic anti-inflammatory effects. Butyrate can modulate macrophage polarization, shifting pro-inflammatory M1 macrophages toward an anti-inflammatory M2 phenotype, and regulate T cell differentiation in a dose-dependent manner, favoring regulatory T cells at physiological

concentrations while promoting pro-inflammatory Th1 and Th17 responses at supraphysiological levels[252]. These immunomodulatory effects may help explain the role of SCFAs in dampening chronic low-grade inflammation characteristic of metabolic diseases. Additionally, SCFAs, especially succinate and propionate derived from fiber fermentation, induce intestinal gluconeogenesis[127], a gut-brain axis-regulated process that improves glucose and energy homeostasis[254]. Diets rich in fat and refined carbohydrates reduce the abundance of beneficial SCFA-producing bacteria, including *Bacteroides* spp. and *F. prausnitzii*, while increasing the abundance of Enterobacteriaceae, leading to dysbiosis and promoting obesity[255]. SCFAs counteract obesity by enhancing lipolysis and fatty acid oxidation, mediated through G-protein coupled receptors GPR43 and GPR41 in adipose tissue[252]. Activation of these receptors by SCFAs also improves insulin sensitivity and lower circulating glucose levels by stimulating GLP-1 and peptide YY release[84,255]. Notably, in T2D, microbial alterations often include decreased butyrate production potential[170,256], with impaired SCFA signaling contributing to insulin resistance. In the liver, SCFAs also contribute to lowering circulating cholesterol levels both by reducing *de novo* cholesterol synthesis and increasing cholesterol excretion[257]. Lastly, emerging evidence links SCFA production to hypertension, a condition underlying many CMDs[216]. Individuals with hypertension often exhibit depletion of key SCFA-producing taxa like *Roseburia* spp. and *F. prausnitzii*. Interestingly, while fecal SCFA levels may be elevated, plasma SCFA levels are reduced, indicating impaired SCFA absorption in hypertension[258]. Nevertheless, SCFA supplementation in models of hypertension has been shown to relieve symptoms. Together, these findings underscore the multifaceted role of SCFAs as central mediators linking the gut microbiota to host metabolism, immunity, and cardiometabolic health.

BILE ACIDS

Bile acids were historically regarded primarily as digestive agents, influencing host metabolism by modulating lipid absorption in the intestine[259]. Nowadays, bile acids are recognized as key metabolic regulators involved in glucose, lipid, and energy homeostasis. Their synthesis is tightly controlled by a negative feedback loop *via* the nuclear receptor FXR, which, when activated by bile acids, suppresses hepatic bile acid production. Intriguingly, both activation and inhibition of FXR signaling have been reported to confer beneficial effects on CMD, possibly due to the differing affinities of individual bile acid species, particularly secondary bile acids produced by the gut microbiota[260]. A second

major receptor, TGR5, is expressed in various tissues and modulates glucose metabolism, insulin sensitivity, and energy expenditure upon activation by bile acids[214]. A general pattern observed in obesity, T2D, and metabolic disease is an increase in primary bile acids[261], especially taurine-conjugated forms[262]. On the other hand, certain microbiota-derived secondary bile acids, such as UDCA and HDCA, appear to exert protective effects, including enhanced insulin sensitivity *via* FXR and TGR5 signaling[263,264]. Pharmacologically, semi-synthetic bile acid analogs such as obeticholic acid, a potent FXR agonist, have demonstrated improvements in lipid profiles, although often accompanied by adverse effects such as pruritus[259]. Meanwhile, drugs like metformin, commonly used in T2D, have been shown to alter gut microbiota composition, increasing the abundance of BSH-expressing bacteria capable of deconjugating and transforming bile acids[265]. Similarly, dietary polyphenols like resveratrol have been reported to enrich BSH-containing taxa such as *Bifidobacterium* and *Lactobacillus*, potentially promoting a more metabolically favorable bile acid pool[259]. Data from the Framingham Heart Study furthermore revealed increased triglyceride levels along higher primary BA levels, while levels microbially produced bile acid derivatives such as isoallo-lithocholic acid were lower[109]. Beyond metabolic regulation, secondary bile acids also exhibit antimicrobial and immunomodulatory properties[213], capable of modulating intestinal inflammation and potentially dampening the chronic low-grade inflammation characteristic of metabolic syndrome[216].

HYDROGEN SULFIDE

H_2S is a gaseous signaling molecule, or gasotransmitter, endogenously produced in the human body through tightly regulated enzymatic pathways[266]. It plays a multifaceted role in maintaining physiological homeostasis, including modulation of inflammation, vascular tone, and redox balance[266]. At low concentrations, H_2S exerts anti-inflammatory effects by promoting mucus production, enhancing endothelial barrier integrity, and acting as a vasodilator, thereby contributing to gut barrier function and circulatory health[267]. However, in the colon, H_2S is also produced by certain gut bacteria, particularly *Desulfovibrio* and *Bilophila* species, beyond host regulatory control[268]. When present at high local concentrations, microbially derived H_2S becomes detrimental, impairing mucus layer integrity and increasing epithelial permeability, which has been linked to inflammatory bowel disease and colorectal cancer[268]. In obesity, circulating H_2S levels are inconsistently reported higher or lower compared to normal-weight individuals, possibly

reflecting the complex interactions between host metabolism and microbial production[267]. In MASLD, impaired host H_2S production is commonly observed, and supplementation has shown hepatoprotective effects by reducing hepatic lipid accumulation and oxidative stress[267]. In cardiovascular diseases, physiological levels of H_2S consistently display protective properties by promoting vasorelaxation, lowering blood pressure in hypertension, and countering atherosclerosis through anti-inflammatory, antioxidant, and anti-calcification mechanisms[269]. In the context of T2D and impaired glucose metabolism, H_2S has emerged as a key regulator across the pancreas, liver and muscle tissue, with evidence supporting its role in enhancing insulin sensitivity and glucose uptake, although some studies point to tissue-specific or dose-dependent adverse effects that complicate its therapeutic potential[267]. Together, these findings highlight the dualistic nature of H_2S in host physiology and disease, shaped by both endogenous regulation and microbial contributions.

SUMMARY

While the gut microbiome is highly individual, it shares a taxonomic and functional core, shaped by numerous factors, most notably, diet. Positioned at the interface of nutrition and host physiology, the gut microbiota exerts profound effects on cardiometabolic health through the production of bioactive metabolites, interacting with host lipid and glucose metabolism and modulating the immune system. Despite growing evidence linking microbial composition and function to disease risk and progression, our understanding of the mechanisms and underlying ecology remains limited. Uncovering these ecological principles is essential to utilize the microbiota's potential as a therapeutic target for the prevention and treatment of CMD.

AIMS

The overall aim of this thesis is to investigate how the gut microbiome influences host cardiometabolic health and disease. While many association studies have linked individual microbial taxa or metabolites to disease, these studies often overlook the broader ecological context. As a matter of fact, the gut microbiota performs a multitude of functions simultaneously, and their combined effects, shaped by microbial interactions and community dynamics, may be more relevant to host health than individual functions considered in isolation. Therefore, adopting a microbial ecological perspective is crucial to uncover how functional interactions collectively contribute to cardiometabolic outcomes.

Thus, the specific aims of this thesis are:

- Describe associations between cholesterol-to-coprostanol reduction and host cardiometabolic health, taking into consideration drivers and ecological features of the microbiome supporting this metabolic function (**Paper I**).

- Characterize the concomitant *in vitro* production of host health-modulating SCFAs and bile acids by the human gut microbiota in response to cereal brans rich in fibers, abstracted from host confounders (**Paper II**).

- Compare phylogeny and phenotype of three strains of *Desulfovibrio*, a main sulfate reducer in the human gut, resulting in the proposal of the new species *Desulfovibrio aggregans* sp. nov. (**Paper III**).

- Identify microbiome-metabolome interactions affecting glycemic control and other cardiometabolic burden, and evaluate how lifestyle changes affect these dynamics. (**Paper IV**).

METHODOLOGICAL CONSIDERATIONS

In this section, I will reflect on the methods used in this thesis and discuss advantages and disadvantages. Detailed method descriptions can be found in the methods sections of **Papers I-IV** (appendices I-IV, respectively).

METHODS IN MICROBIOLOGY

FECAL SAMPLING AND PROCESSING

The foundation of all papers discussed in this thesis is fecal sampling, which involves several steps with strong implications for all downstream analyses. In **Papers I and IV**, microbial DNA was extracted from fecal samples and sequenced. In **Papers II and III**, fecal communities were cultivated, either to recover as much diversity as possible (**Paper II**) or isolate bacteria with desirable traits (**Paper III**). Additionally, in **Paper I**, cholesterol and coprostanol were measured from fecal samples.

For all intended uses – DNA extraction and sequencing, bacterial cultivation and/or isolation, metabolite extraction and measurements – one major limitation is that fecal samples are not fully representative of the content of the colon, and even less so of the small intestine. Furthermore, certain intestinal bacteria adhere to the mucus layer and are shed slowly[112], potentially underestimating their abundance in feces. Biopsy or colonoscopy are methods used to directly obtain small or large intestinal samples with mucus, however, due to their invasive nature, they require careful ethical consideration and are less scalable. More recently, capsules have been developed which allow for pH-dependent sampling of the small intestine[270], but these are still in early mostly animal testing phases, more expensive, and require more ethical considerations. This leaves fecal sampling as the most widely used method for sampling the gut microbiota.

Bacteria in fecal matter are still metabolically active, being affected by nutrient availability and environmental factors, especially oxygen, after passing. Thus, the representativeness of a sample decreases over time after passing, requiring rapid handling, or biases will accumulate, such as selection for oxygen tolerant

bacteria, degradation of DNA and RNA and evaporation of volatile compounds.

Thus, large-scale studies of fecal samples require standardized protocols for sampling and handling to reduce bias. Some other forms of bias, such as participant sex, age, or geographical region of sampling cannot always be accounted for and thus need to be considered in data analysis, and any limitations of claims need to be clearly communicated.

The fecal samples used for DNA extraction and sequencing in **Papers I and IV,** fecal sterol measurements in **Paper I**, and isolation of bacteria in **Paper III** were stored at room temperature for up to 36 hours before storage at -80 °C due to logistical limitations. A recent consensus on handling of fecal samples for DNA sequencing suggests shipping of samples to the laboratory within 24 hours on ice or dry ice, when genome preservative media are not available[271]. Another consensus on fecal microbiota transplants (promoting survival of as much donor diversity as possible) suggests handling of fresh feces within 6 hours at room temperature[272].

Small-scale studies need to be aware of the individuality of the human microbiota, with sample pooling as a valid option to obtain more generalizable results from fecal culturing studies[273]. Pooling samples, however, loses information on the individual microbiota compositions and risks missing individual responses to substrates like fibers. In **Paper II**, we decided against pooling samples with the aim of studying both individual and general responses to three different fibers. The small number of donors allowed for quick handling of fecal samples within minutes from passing to introduction into an anaerobic chamber and incubation. Furthermore, with the focus on microbial metabolite profiles, which are likely more similar than individual community compositions, the sample size was sufficient to study fermentative and bile acid metabolism.

After initial sample handling, the choice of DNA extraction protocol will affect the sequenced community profile. Application and duration of bead-beating, for example, will alter the community composition, affecting the abundance of taxa such as *Faecalibacterium*, *Eubacterium*, *Lachospira*, and *M. smithii*[274]. Consensus protocols can help increase comparability and reproducibility[275].

For isolation and cultivation of bacteria, fecal samples are often enriched in media to allow for recovery after passing (**Paper II**), or are serially diluted in

PBS before plating. Initial handling as well as storage medium and additives like cryoprotectants can influence the recovery of bacterial diversity or pure strains[276]. In **Paper III** we aimed at isolating a specific group of bacteria, *Desulfovibrio piger*, the dominant sulfate reducer in the human gut. We decided not to perform enrichment or liquid dilution, but instead plated the fecal matter directly onto Postgate agar plates, a selective medium for sulfate reducers[277]. This method possibly enabled growth of bacteria that were present at low abundances in the fecal samples and that might have been outcompeted by more abundant strains if diluted in liquid media. This method has also been used previously in the lab, and has led to the discovery of a synergistic relationship between *D. piger* and *F. prausnitzii*[278].

In **Paper I**, we additionally measured fecal sterols in a subset of samples. When planning to analyze fecal metabolites, the stability and volatility of the compounds of interest need to be considered. Sterols are generally quite stable; however, non-enzymatic oxidation may occur, causing a decrease in concentration over prolonged periods of time[279]. We therefore stored samples at -80 °C, slowing down this process.

IN VITRO CULTURING CONDITIONS

In **Papers II and III**, we used *in vitro* batch culturing of complex fecal communities or pure strains. While we attempted to model an environment suitable for anaerobic gut microbes, there are several drawbacks to *in vitro* batch cultures, which may introduce bias. First, the effect of the immune system on the selection and activity of gut bacteria cannot be evaluated. Additionally, *in vitro* batch cultures lack peristalsis, thus lacking the influence of transit time. Furthermore, absorption and excretion are absent, and therefore compounds like SCFAs will tend to accumulate, lowering the pH of the system due to lack of buffering. Other compounds like bile acids are not present, unless added. On the other hand, *in vitro* batch culturing may enable the measurement of SCFA maximum production, and of the intrinsic microbial capacity for bile acid transformations, allowing the study of purely microbial metabolism and ecological factors driving these functions, without interference by the host metabolism. Batch cultures are simple to set up and allow for high throughput with sufficient replicates, however, differences in media composition between batches need to be considered.

In **Paper II**, we used a modified version of the rich SHIRM medium[145], formulated to simulate the human colonic content. Porcine gastric mucin is added to emulate the shedding of the mucus layer, and the addition of porcine pepsin and subsequent neutralization with simulated pancreatic juice (containing bovine bile and porcine pancreatin) emulates digestion. While these animal-derived compounds are only an approximation of their human counterparts and might differ in their activity or composition, they are also much more readily available and affordable, making them the preferred choice for large-scale experiments.

In **Paper III**, we used a selective medium for the cultivation of *D. piger*. In contrast to rich media, which are formulated to support the growth of as much diversity as possible, selective media are devised to inhibit or prevent the growth of most bacteria, while supporting the growth of a species of interest. Postgate's medium (PGM) contains high amounts of lactate and sulfate, required for the growth of *D. piger*, but suppressing growth of other bacteria[277].

An alternative to batch cultures are continuous bioreactors, scalable incubation vessels with a continuous influx of medium and efflux of waste, for example described in Koh *et al.*[145]. Continuous bioreactors have the advantage of pH buffering, the possibility of spiking compounds of interest in succession, as well as studying perturbation and responses to changes in nutrients or culturing conditions. We chose not to employ continuous bioreactors in **Paper II** due to the lower throughput compared to batch cultures, and due to the incompatibility of the tubing with the coarsely ground brans, which generate a suspension when added to the liquid medium. With modifications, continuous bioreactors could be useful to validate our findings from batch cultures in a continuous system mimicking intestinal peristalsis and absorbance/excretion of metabolites, thus representing a valid next step before testing of fibers *in vivo*.

IN VITRO DIGESTION AND PREPARATION OF FIBER-RICH MEDIUM

Another limitation of *in vitro* studies is the lack of human digestion, altering the composition of dietary compounds encountered by bacteria *in vivo*. The brans used in **Paper II** contain easily host-digestible carbohydrates which would be readily digested and absorbed by the host, but when added to our

medium, significantly alter bacterial metabolism and possibly microbiota composition, as exemplified in the paper by the lactate accumulation in non-digested and non-dialyzed medium. Therefore, we describe a simplified digestion protocol for carbohydrates based on the INFOGEST protocol[280]. We subjected oat, wheat and rye bran to porcine pancreatic amylase digestion to break down the contained starch and simulate salivary digestion. A subsequent dialysis emulated the absorption of simple sugars in the small intestine. Further peptic digestion and neutralization were performed according to the SHIRM medium protocol[145] to simulate gastric digestion and the transition to the small intestine. Porcine pancreatic amylase was chosen due to its affordability compared to human salivary amylase. The efficacy of the method was confirmed with thin-layer chromatography showing the presence of simple sugars in water used for dialysis (Fig. 2). We chose to follow only the first steps of the extensive INFOGEST protocol as this was adequate to remove simple sugars, and our SHIRM medium protocol already included the subsequent peptic digestion.

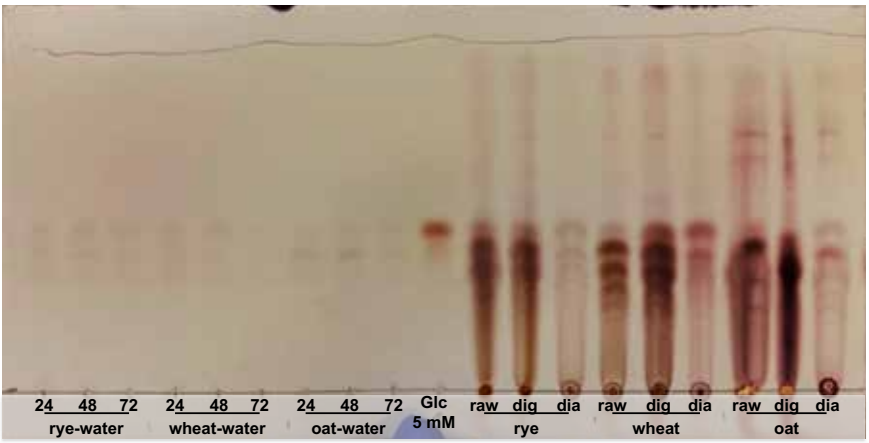

Figure 2. Thin-layer chromatography of rye, wheat and oat brans before (raw), after amylase digestion (dig) and after dialysis (dia). "Water" labels indicate samples collected after 24, 48, and 72 hours of the water dialyzed against, with the water replaced every 24 hours after sampling. 5 mM glucose (Glc) serves as a positive control. Contrast increased for better visibility.

LAB ADAPTATION OF BACTERIAL STRAINS

While bacteria are constantly exposed to selective pressure in their natural environments, the differences between *in vivo* and *in vitro* conditions described above (e.g., lack of peristalsis and immune system interactions) may result in different pressures on fecal bacteria. The bacterial populations studied *in vitro* may drift from those originally sampled not only at the compositional level but also genomically at the strain level, with changes occurring at each generation and transfer[281]. In **Paper III**, it is therefore important to consider that our isolated strains may accumulate mutations over time, distinguishing them from the original isolates. Glycerol stocks of the original isolates were kept and reverted to after several generations and when beginning new experiments.

GROWTH AND BIOCHEMICAL ASSAYS

In **Paper III**, we performed growth curve measurements and biochemical assays. Bacterial growth can be estimated by measuring the optical density (OD) of the growing culture; however, this method is sensitive to particles in the medium. In the case of *D. piger*, the production of sulfide leads to a precipitation of water-insoluble iron(II) sulfide when iron is present in the medium, interfering with optical density measurements. In these conditions, we used protein quantification to measure the total bacterial biomass, which is less sensitive to insoluble particles, but more labor-intensive. Other alternatives are assessing colony-forming units (CFUs) to count the number of viable cells, or most probable numbers (MPN) which is also used to assess viable cells and useful for quantification of bacteria in solutions. Another more quantitative method is qPCR, which is used to quantify gene copy numbers. These methods offer different advantages and disadvantages: they can be time-consuming (CFUs), less precise (MPN), or require DNA extraction and appropriate primers (qPCR).

In **Paper III**, we also used API kits, a proprietary system of standardized tests for the biochemical characterization of microbes, and tested various electron donors and acceptors to assess differences in metabolism. These tests are not exhaustive, i.e., the strains could have additional enzymatic activities not covered by the API kits, and grow on other electron donors and acceptors than the ones tested. Genomics analyses of functional potential can be useful to complement the assessment of metabolic activities, but a main limitation in genomics studies is the lack of annotation for many bacterial genes. Recent

estimates in the Unified Human Gastrointestinal Protein (UHGP) catalog indeed show that of 171 million protein clusters with 100% amino acid sequence identity, 40% lack functional annotation[74]. One example is *ismA*, described in **Paper I**, missing from the UHGP catalog, which required manual annotation.

TARGETED AND UNTARGETED METABOLOMICS

Different metabolites were measured in all papers in this thesis: fecal sterols in **Paper I**, SCFAs in culture media in **Papers II and III**, bile acids in culture media in **Paper II**, and plasma metabolites in **Papers I and IV**.

SCFA and bile acid quantifications were based on targeted analysis of predefined sets of metabolites, while plasma metabolites were measured by untargeted analysis. Untargeted methods allow for measuring a wide array of metabolites, albeit with less sensitivity than targeted methods. Additionally, many metabolites measured by untargeted approaches are not yet annotated, yielding only IDs with limited information value. For targeted approaches, for example bile acid detection, on the other hand, our set of standard compounds might not completely cover the range of metabolites produced by the gut microbiota in the cultures. Indeed, bile acid transformations are complex, with new bile acid species described regularly[213]. In the case of SCFAs, metabolic pathways are well studied, and acetate, propionate and butyrate are the main products of bacterial fermentations in the human gut[85]. For sterols, it is known that intermediates between cholesterol and coprostanol exist, however, their concentrations may be low[282]. Therefore, targeted analyses may fail to cover the whole diversity of microbial metabolite production, but untargeted analysis would go beyond the scopes of **Papers I and II**, both economically and due to a lack of established protocols for processing and extraction of culture media, which can present different backgrounds for the measurement of analytes compared to more commonly assessed fecal and blood samples.

COMPLEMENTARY *IN VITRO* AND *IN VIVO* MODELS

In vitro microbiology methods are not sufficient to fully assess the relevance of the human gut microbiota for health and disease. As mentioned in the introduction, the gut microbiota interacts strongly with the host and vice versa. The host immune system exerts a strong influence on the microbiota and its metabolites, which in turn modulate the immune system, both of which cannot be modelled in bacterial cultures alone. To explore the interactions of complex fecal communities with themselves in a context also involving the immune system, and to study the interactions of specific isolates or complex communities with the host, several *in vitro* and *in vivo* models can be used. Cell lines and rodent models were used in **Papers III and IV**, respectively, in experiments I was not involved in.

CELL LINES

In **Paper III**, immunomodulatory properties of bacterial strains were tested using spent media on Caco-2 cells. Cell lines are a valuable tool to test host-microbe interactions assessment *in vivo*. They are also valuable in providing mechanistic validations of findings from human cohorts and in reductionistic approaches. Caco-2 cells are a commonly used *in vitro* model for studying intestinal epithelial function. Derived from a human colon carcinoma, these immortalized cells differentiate into polarized enterocyte-like monolayers[283], making them a useful model for the intestinal barrier. However, as a cancer-derived line, Caco-2 cells carry mutations and represent only a single epithelial cell type, limiting their ability to capture the complexity of the intestinal environment. Organoids are a more recent approach to model a more complex system of cells differentiated into more diverse cell types[284], further developed into the gut-on-a-chip, which also allows to simulate peristalsis[285]. Usage of spent media, i.e., bacterial media after growth, containing no bacterial cells but their metabolites, allows for the study of host responses to microbial products without the confounding effects of live bacterial interactions. While this setup excludes direct contact between bacteria and host cells, this is not necessarily a major limitation, as the intestinal mucus layer *in vivo* typically prevents direct microbial-epithelial contact under healthy conditions[112].

MOUSE MODELS

Paper IV involves non-interventional experiments using mice. Important physiological and microbiota-related differences between mice and humans need to be considered, such as diet, anatomy, physiology and behavior[286]. Possibly linked to these differences, mice share core microbial phyla with humans, but marked differences are observed in species and gene pools[287]. Nevertheless, animal models remain a critical intermediate step in translational research[288] and offer valuable mechanistic insights that can guide the design of subsequent human studies and help identify potential targets for intervention. When working with mice or other *in vivo* models, ethics and the wellbeing of the animals is paramount. Animal work needs to be conducted in accordance with the principles of the 3Rs: Replace, Reduce, Refine[289]. Replacement aims at promoting other models such as *in vitro* organ systems or *in silico* simulations; Reduction entails the careful planning and execution of experiments to use as few animals as possible and gather as much data as possible from them; and Refinement targets the animals' general comfort, reducing stress and pain.

HUMAN COHORTS

ETHICAL CONSIDERATIONS

Human studies are conducted under the strict ethical frameworks of the Belmont Report, the Declaration of Helsinki, and the CIOMS Guidelines. These emphasize respect for participants, the minimization of risk, and a fair balance between risks and benefits. In practice, this requires informed consent, protection of vulnerable groups, safeguarding of confidentiality, independent ethical review, and sensitivity to cultural context, all with the overarching aim of protecting participant dignity while ensuring that the research delivers meaningful societal value.

The studies providing data for **Papers I and IV** as well as fecal samples for **Paper III** were designed before the start of my PhD, so I was not involved in ethical considerations at their conception. Nonetheless, the study protocols reflect strong ethical foundations and methodological robustness: participants were well-informed about the study's aims, the use of their data and biological samples, and their right to withdraw at any time, before giving informed

consent. With the exception of blood sampling, which generally causes minor discomfort and occasionally bruising, the procedures used were non-invasive. The inclusion of a CT scan was another notable concern, but participants were informed about the associated radiation exposure corresponding to approximately one year of background environmental radiation, which represents a minor but acknowledged risk[290]. The overall risks are arguably outweighed by the scientific value of advancing cardiometabolic research and the potential individual benefit of a comprehensive health screening. This screening may assist in the early identification of elevated cardiometabolic risk, allowing for preventive lifestyle interventions.

The analysis of human data complies with GDPR regulations. For patient data and metagenomic sequencing data in **Papers I and IV**, full anonymization was applied; the de-anonymization keys were not available, making participant identification impossible. While the whole genome sequencing approach theoretically allows for incidental capture of human genetic material, all reads were cross-referenced against the human genome, and any likely human-derived reads are filtered out before downstream analysis. Thus, the data used for microbial community analysis excludes identifiable human sequences. In general, the likelihood of uncovering unexpected medical information from such analyses is low, particularly given that these cohorts have been the subject of previous clinical publications.

Papers II and III involved the handling of human fecal samples, which were then used *in vitro* for the isolation of strains of interest and to assess microbial metabolic output. The samples used in **Paper II** were pseudonymized, while the samples used in **Paper III** obtained from the studies discussed above were anonymized. Moreover, since *in vitro* results represent only an approximation of gut conditions, they do not yield meaningful or identifying information about the donors.

STUDY DESIGN CONSIDERATIONS

Conclusions that can be drawn from a study are strongly influenced by its design. While statistical methods can be used to infer causal relationships in cross-sectional studies such as those in **Papers I and IV**, these methods are inherently limited in their ability to establish causality. Longitudinal designs, particularly those that begin before disease onset, or case-control studies are better suited and needed for drawing robust conclusions about cause and effect.

Additionally, associations observed in microbiome research are small or modest at best, so adequately powered studies with sufficiently large and diverse participant cohorts are crucial to ensure statistical reliability and generalizability.

Controlling for potential confounders is another critical aspect of microbiome research involving complex phenotypes, including CMD. Demographics need to be considered, as selection biases may arise if recruitment is limited to specific ethnicities, age groups, sexes, or geographic regions. Variables such as genetic background, pre-existing medical conditions like hypertension[291], medication use[92,93], BMI[292,293], lifestyle[294,295], and diet[87,83] can also influence microbial composition and function, thereby complicating the identification of robust associations. However, it is rarely feasible to control for all potential confounding factors, many of which are themselves intercorrelated. For example, diet and lifestyle both influence cardiometabolic risk but also shape the gut microbiota[87], making it challenging to disentangle causality. The statistical methods used to address potential collinearity are described further below.

Papers I and IV are large-scale microbiome studies with thousands of individuals. However, the participants in the IGT-microbiota and WINGA studies in **Paper I** were all recruited in Sweden, and the IGT-microbiota is enriched for high risk for dysglycemia by inclusion of one person with normal glucose tolerance out of five participants, with the other four exhibiting untreated dysglycemia at the time of screening. Therefore, our results might have limited applicability to the general population. Additionally, the WINGA study in **Paper I** includes a small number of individuals with atherosclerosis who had experienced a stroke, so associations between *ismA* and stroke need to be confirmed in larger cohorts. We could, however, validate the association between low *ismA*-encoding microbiota and asymptomatic atherosclerosis in the IGT-microbiota cohort, which is an independent study with a younger population and subclinical atherosclerosis sampled before the onset of cardiovascular events. Nevertheless, the present study should be considered exploratory, and further validation in populations from diverse geographical and ethical backgrounds or in longitudinal cohorts is warranted. In **Paper IV,** the inclusion of cohorts from Israel, the United Kingdom, and China improves the generalizability of the findings and identification of robust metabolite-microbiome associations across diverse populations, potentially independent of several confounders.

BIOINFORMATICS AND STATISTICS

SEQUENCING METHODS

In **Papers I, III and IV**, we applied whole-genome DNA sequencing (WGS) methods, and for **Paper II** we plan WGS sequencing of DNA samples to follow up on the characterization of the metabolic activities of the fecal cultures. We also performed sequencing of the 16S rRNA gene, either for initial analysis of taxonomic community composition (**Paper II**) or as an initial means of taxonomic identification of fecal isolates (**Paper III**). The choice of methods depends on the question asked. While 16S rRNA gene sequencing is more affordable and produces smaller amounts of data, it lacks resolution and only provides broad phylogenetic information. When sequencing specific variable regions, for example V4 as done in **Paper II**, good separation of taxa is only feasible to the genus or sometimes species level. If the whole gene is sequenced, as in **Paper III**, limited statements on strains can be made. Inference of functional potential based on taxonomy is possible with tools like PICRUSt[296], however, this should be taken with caution and WGS should always be the preferred choice when seeking functional data.

In **Paper III**, the complete 16S rRNA gene was sequenced, which allowed for quick and inexpensive identification of isolates closely related to the strain of interest. An alternative approach would have been the amplicon sequencing of a core functional gene of the clade of interest, in the case of *D. piger* the β-subunit dissimilatory sulfite reductase (*dsrB*) gene[297]. Similarly, the α-subunit of methyl coenzyme-M reductase (*mcrA*) is commonly used to trace methanogen lineages[298]. These functional genes allow for a better resolution within their functional clades than the 16S rRNA gene, however, with WGS becoming increasingly affordable, amplicon-based methods are declining in usage.

Papers I and IV applied Illumina short-read WGS sequencing for fecal metagenome analyses at the taxonomic and functional levels, while in **Paper III** we performed both Illumina short-read and Oxford Nanopore long-read sequencing for genomic analyses. These sequencing methods produce significantly more data and are more challenging to analyze compared to methods using 16S rRNA gene sequencing. However, they yield substantially more information on phylogeny and functional potential. In **Paper III**, the combined approach leverages the strengths of both sequencing methods, the lower error rates of Illumina short-read sequencing and the extended read

lengths of Oxford Nanopore technology. This hybrid approach enabled the assembly of high-quality genomes and increased the likelihood of achieving complete, closed genome assemblies for some of the isolates.

SEQUENCE DATA PROCESSING AND ANNOTATION

Raw metagenomic reads in **Papers I and IV** were preprocessed by quality filtering and removal of human reads, and then taxonomically annotated using the Kraken 2 pipeline.

Kraken 2[299] is a *k*-mer based method, which takes a reference catalog as input and cuts it into snippets of DNA with a length of *k* base pairs. These are stored hashed in a table together with the last common ancestor of each *k*-mer. By reducing large amounts of complex genomic data into a table of short sequences, metagenomic reads can be compared to this table rapidly to assign taxonomy. To obtain abundances at a desired taxonomic level, we used Bracken[300] (Bayesian Reestimation of Abundance with KrakEN), which then combines read counts with their distributions over the phylogenetic tree and estimates abundances based on Bayesian inference. Compared to methods relying on base pair alignments, Kraken 2 is significantly faster and less computationally demanding. As a reference-based method, the reliability of Kraken 2 depends strongly on the quality, completeness, and representativeness of the reference catalog, particularly for capturing the diversity of complex fecal communities. In **Papers I and IV**, we used version 2 of the Unified Human Gastrointestinal Genome[74] (UHGG), one of the most comprehensive catalogs of human gut genomes available.

Paper IV additionally used the canopy and MetaPhlAn 4 pipelines.

Canopy[301], an early co-abundance clustering method, first assembles metagenomic reads into a gene catalog. A random gene is then picked and clustered with other genes showing the same abundance profile over all samples, building a “canopy”. The average gene abundances of this canopy are then taken and genes co-abundant to this average are then picked repeatedly until the canopy is stable. This method yields co-abundant gene groups (CAGs) with highly variable gene numbers ranging from single-digit numbers to over 1,000. CAGs with more than 700 genes and solid taxonomic assignment are

called metagenomic species (MGS). Canopy assumes that all genes in a genome appear at the same frequency, which is not always valid, for example during bacterial chromosome replication and when plasmids are present.

MetaPhlAn[302] is based on a catalog of marker genes that are unique to each species-level genome bin (SGB). Metagenomic reads are aligned to these marker genes to assign taxonomy. The newest version 4 includes unknown SGBs, allowing this marker gene-based method to detect taxa without assigned taxonomy to some extent. This method is faster and less computationally demanding than canopy but offers less resolution than canopy or Kraken 2. A separate StrainPhlAn pipeline exists to compensate for this disadvantage.

CLASSIFICATION OF FUNCTIONS

Like the taxonomic classification of reads, genes can be classified by their function. In **Paper I**, we used the Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology database[303], a large and comprehensive database of functional orthologs. To further reduce the complexity of the data, we categorized KEGG orthologs (KOs) into gut metabolic modules (GMMs)[304], a manually curated list of microbial metabolic functions present in the gut, extended with additional functions implicated in CMD[305,306]. This provided us with more easily processable count files of functions and facilitated comparisons of gut microbial functional potentials of individuals. A disadvantage of using manually curated GMMs is the dependence on *a priori* knowledge of pathways, potentially missing functions. The focus on microbial metabolism also does not take into consideration genes for structural components of bacterial cells (such as peptidoglycan and lipopolysaccharide) or for signal transduction systems (e.g., two-component systems, sigma factors, quorum sensing) which are used by bacteria to sense and respond to environmental cues and interact with the host.

COUNT DATA PROCESSING AND ANALYSIS

In **Paper I**, Bracken-derived count tables of microbial taxa and GMMs were further analyzed. Several steps in the analyses need to be considered for their potential impact on the results and their interpretation. Raw counts often contain sequencing artifacts and spurious low-abundant taxa, which cannot be

distinguished easily. Filtering strategies are therefore essential to remove taxa that occur in very few samples or at extremely low abundances. However, care must be taken to avoid discarding rare but biologically relevant organisms. In **Paper I**, for differential abundance analysis of taxa we filtered by abundance and prevalence, including taxa with a minimum mean abundance of 1e-5 and prevalence of at least 5% across all samples. For GMMs, we included modules that had a minimum coverage of 66% and prevalence of at least 40%.

Next, differences in sequencing depth between samples need to be considered. Direct comparison of absolute taxon counts is not appropriate when there is substantial variation in total counts between samples. Rarefaction, a random subsampling of samples to the same depth, is commonly used, especially to obtain alpha diversity or richness metrices. However, its use remains controversial due to the potential loss of information on low-abundant taxa[307]. In **Paper I**, we rarefied gene counts to estimate gene richness, but for species counts we instead chose relative counts, where the abundance of each taxon is divided by the sum of counts in a sample, yielding fractions. To analyze between-sample beta diversity, we chose the Aitchison distance to account for the compositionality of metagenomics data[308]. Briefly, because sequencing instruments have a finite read capacity, the number of reads assigned to one taxon is inherently relative to the number of reads assigned to others. As a result, downstream abundance estimates are compositional, i.e., the abundance of each taxon is proportional to the abundance of all other taxa. The Aitchison distance is based on centered log-ratio transformations, a mathematical way to address the issue of proportionality of abundances, followed by a simple Euclidean distance to compare community compositions. Other commonly used beta diversity metrices are Bray-Curtis dissimilarity, considering only the abundance of taxa, and UniFrac, which either considers phylogenetic distances of communities only (unweighted) or takes both phylogenetic and abundance data into account (weighted)[309].

DIFFERENTIAL ABUNDANCE ANALYSIS

A central goal of microbiota studies, as in **Paper I**, is the identification of differentially abundant features such as taxa or GMMs linked to host phenotypes. Simple, robust non-parametric tests, such as the Wilcoxon rank-sum test on relative abundances, are commonly used but may not adequately account for compositionality or confounding factors. Other methods, such as ANCOM-BC2[310], can take both compositionality and confounders into

account. However, we used metadeconfoundR[311] in our study, which is more easily interpreted than ANCOM-BC2. MetadeconfoundR employs the Wilcoxon rank-sum and Kruskal-Wallis tests, and additionally tests *post-hoc* for confounding status of covariates provided (e.g., age, diet, medication use) using linear models.

Finally, it is important to emphasize that these differential abundance analyses identify associations, not causal relationships. Observed correlations between taxa or functions and host phenotypes may be confounded by unmeasured variables or result from reverse causality. Similarly, differential abundance of genomic functions does not yield information on the actual expression of genes or activity of enzymes. Catabolite repression, for example, can influence enzymatic activity beyond genomic functional potential[147]. Experimental validation and longitudinal or interventional study designs are necessary to draw stronger conclusions on causality.

HIDDEN MARKOV MODELS

In **Paper I**, we built a profile hidden Markov model (HMM) to identify *ismA* homologs in our gene catalog. This method is faster and more sensitive than traditional alignment algorithms like BLAST and uses less computational resources[312]. A profile HMM, essentially a model of amino acid probabilities at each position in a sequence, is built based on an alignment of sequences of interest, with consideration for insertions and deletions. Query sequences are then run against this model and evaluated for fit, with better fitting sequences being more similar to the initial sequences of interest. This method, however, is solely based on amino acid sequence, and does not consider protein functions, domains or structure. This means that in our study in **Paper I**, further enzymes with cholesterol-reducing function could exist that we could not detect. Furthermore, we only detected *ismA* homologs using the gene catalog developed for the IGT-microbiota cohort[170], without identification of taxa encoding this function. To follow up on our results and to identify taxa harboring cholesterol-reducing potential, *ismA* homologs could be searched in the UHGG2 gene catalog.

STATISTICS

In **Paper I**, we analyze patient data and relative abundances of microbiome features, specifically taxa and GMMs. Since these data are often not normally distributed, we used non-parametric statistical tests, such as the Wilcoxon rank-sum test, Kruskal-Wallis test and Spearman correlation, which are also applicable to normally distributed data, albeit with reduced statistical power, i.e., requiring larger differences between groups to yield significance. Generalized linear models were used to calculate the odds of belonging to different glycemic control groups among individuals with low *ismA* encoding and assess associations between *ismA* encoding status and clinical variables. In the latter case, we built three models, including known confounders of clinical data, and compared them to the unadjusted model. To quantify the variance explained in *ismA* counts we performed a nested cross-validated ridge regression with recursive feature selection. In essence, nested cross-validation entails a repeated splitting of the data into separate training set for model building, and a testing set for model evaluation. Ridge regression is a form of linear regression that includes a regularization term, which penalizes large coefficients to prevent overfitting, especially in large datasets with potentially correlated data like metabolomics. Lastly, recursive feature selection ranks feature importance and iteratively removes the least important features, ultimately yielding a smaller, more predictive set of variables. This method allows for building complex, generalizable models while avoiding overfitting.

In **Paper II**, we applied linear mixed-effects models (LMMs). Biological models oftentimes include random effects, i.e., uncontrollable effects such as the donor, and in this study, we aimed to examine outcomes independent of variability between fecal donors. LMMs allow the inclusion of both random and fixed effects, which we selected based on the biological questions and hypothesized metabolic relationships between SCFAs and BAs we aimed to explore. To assess differences in endpoint concentrations, we performed an analysis of variance (ANOVA) including interaction effects, as suitable non-parametric alternatives for this analysis were not available.

In **Paper III**, we observed some biological outliers in growth curves, thus we primarily used non-parametric tests for comparison of growth parameters. For multivariate testing of growth curves over time, however, we performed ANOVA and estimated marginal means tests, as suitable non-parametric tests are limited.

MAIN RESULTS AND DISCUSSION

PAPER I

ismA IS A STABLE MARKER OF CHOLESTEROL CONVERSION AND CARDIOMETABOLIC HEALTH

Although microbial cholesterol conversion has been known for decades[194], the first enzyme involved in this reaction, intestinal steroid metabolism A, encoded by the *ismA* gene, was only recently identified[207]. *In vitro*, IsmA has been reported to catalyze both the initial oxidation of cholesterol to 5-cholesten-3-one and, interestingly, the reverse final step of this multistep process, the oxidation of coprostanol to coprostanone[207]. This bidirectionality suggests that the net direction of conversion may be influenced by the accumulation of intermediates such as coprostanone, or potentially by the activity of other, yet undiscovered enzymes. However, despite these uncertainties, we show that *ismA* gene counts accurately classify individuals as high or low fecal cholesterol converters, indicating that the fecal abundance of this gene is a main determinant of cholesterol conversion. Based on these findings, we used *ismA* gene counts as a proxy for colonic conversion and defined an *ismA*-based cutoff for categorizing high and low encoders in the IGT cohort (a cross-sectional study enriched for dysglycemia), a classification that was validated in the WINGA cohort (a small-scale pilot study of asymptomatic and symptomatic atherosclerosis patients). These results are consistent with previous studies showing a bimodal distribution of cholesterol converters in human populations, with a majority of high converters and a minority of low converters[110,193].

Notably, we observed that some individuals with low *ismA* counts showed high conversion rate, suggesting the existence of additional, distinct cholesterol-converting enzymes, which our homology-based profile HMM could not detect. These additional enzymes might include cholesterol oxidase, another enzyme previously observed in *E. coli* known to catalyze the initial oxidation of cholesterol in an oxygen-dependent manner[110]. Due to the requirement of oxygen, this activity is unlikely to drive high cholesterol conversion in human populations under anaerobic fermentative intestinal conditions, but its presence and activity in facultative anaerobic bacteria from the human gut cannot be excluded. Other enzymes catalyzing parts of the cholesterol conversion reaction have been speculated to be present in strains of *Bacteroides*,

Bifidobacterium, *Clostridium* and *Lactobacillus*, but results could not be reproduced or research on the strains was not pursued further[110].

While *ismA* counts varied between individuals, we observed relative intra-individual stability over time in a longitudinal Swedish cohort, supporting the notion of *ismA* as a stable microbiome trait. However, this does not preclude variability in actual conversion activity, which may fluctuate with dietary composition, meal timing, or microbial circadian rhythms[313]. Indeed, drastic changes in individual conversion rates were reported after intake of plant sterols[204], but long-term effects on the *ismA* genomic functional potential have not been investigated. Nevertheless, based on the strong correlation between *ismA* counts and cholesterol conversion rates and the longitudinal data, we concluded that the differences we observed between individuals in the IGT cohort were not driven by intraindividual variation but could be affected by other factors, including lifestyle, gut environment and host metabolic status. This allowed us to study associations of cholesterol conversion and *ismA* with cardiometabolic status and risk factors. Overall, associations with glycemic control, triglycerides, inflammation, plaques, stroke and waist-hip-ratio, but not circulating cholesterol levels, suggest more complex links between cholesterol conversion and cardiometabolic health, not limited to the direct effect on cholesterol availability for absorption. Statins[221] and the recently discovered hormone cholesin[228] could further confound links between intestinal and circulating cholesterol levels.

THE HIGH *ismA* MICROBIOME MAY PROMOTE CARDIOMETABOLIC HEALTH

We hypothesized that high *ismA* encoding status is a feature of a healthy gut microbiome, and that low *ismA* encoding is linked to a deterioration of the microbiome tied to CMD. This prompted us to further characterize the microbial and functional profiles of high versus low *ismA*-encoding communities, beyond the established role in cholesterol conversion. Therefore, we investigated if *ismA* could reflect broader ecological features within the gut, including gene richness and metabolic capacity functional potential, as well as the systemic metabolome and inflammatory tone.

Consistent with our hypothesis, high *ismA* encoders showed significantly higher gene richness, a microbial trait associated with health and microbiota

stability in CMD[2,305]. *ismA* was tightly linked to gene richness in both the IGT and WINGA cohorts, as well as to the abundance of several health-associated taxa. However, the associations of *ismA* with these features extended beyond the correlation with gene richness, as many taxa were independently associated with both *ismA* and gene richness. In the WINGA cohort of individuals with over atherosclerotic disease, *ismA* explained more variation in the gut microbiota composition than disease severity, even after correcting for the effect of gene richness. These results suggest that *ismA* is not a proxy for microbiome gene richness but provides additional distinct information on the link between gut microbiome and atherosclerotic disease.

The taxon showing highest enrichment in high *ismA* in both cohorts was *Eubacterium_R coprostanoligenes*, which is known to harbor the *ismA* gene and facilitate cholesterol conversion[207]. The microbial configuration associated with high *ismA* encoding independent of gene richness appears to support a highly reducing, fiber-degrading, and potentially immunomodulatory gut environment, possibly contributing to the maintenance of cardiometabolic health. Indeed, this profile was enriched in taxa linked to complex carbohydrate, cellulose and resistant starch degradation (e.g., *Ruminococcus callidus*, *R. bicirculans*, *R. bromii*, *R. champanellensis*)[118,314]. Methanogenesis, as indicated by the enrichment of *Methanobrevibacter* and methanogenesis-associated GMMs in high *ismA* encoders, as well as the Wood-Ljungdahl pathway may serve as important hydrogen sinks in this reducing environment, with the former having been shown to associate with cellulolytic and complex fiber-degrading ruminococci[315]. In these conditions, hydrogen utilization might be supported by high cholesterol-to-coprostanol conversion, maintaining high fermentation efficiency[159]. Key butyrate producers such as *F. prausnitzii* and *Coprococcus* were also consistently enriched, contributing to an anoxic environment[40] and metabolic benefits[316].

The increase of other health-associated and immunomodulatory taxa may further explain the observed inverse associations between *ismA* abundance and markers of dysglycemia, atherosclerosis, and stroke, potentially mediated through antioxidant and immunoregulatory microbial metabolites. Among these taxa, which were in many cases also associated with gene richness, *Christensenella minuta* has been positively associated with increased physical activity[295], and negatively with overweight and dyslipidemia[66,293]. Abundance of *L. eligens* (formerly *E. eligens*), a pectin degrader[317], has been reported decreased in atherosclerotic dyslipidemia[66], and has been associated with positive lipid response to lifestyle change[294], high diet quality[318] and response

to Mediterranean diet corresponding to lower frailty and improved cognitive capacity in an elderly cohort[67]. *Dysosmobacter*, especially its type species *welbionis*, has been shown to associate negatively with obesity and dysglycemia in humans[319], an effect potentially mediated by *Dysosmobacter*-derived bioactive lipids[320]. *Phascolarctobacterium succinatutens* has been described as having antioxidative and microbiome-stabilizing properties[321]. Lastly, we observed MAGs without taxonomic annotation beyond the genus level highly enriched in high *ismA* encoders, with many of them assigned to the family *Oscillospiraceae*, attributed with potential anti-inflammatory and cardiometabolic health-promoting effects[322,323]. This family was recently also reported to harbor a diverse repertoire of cholesterol-metabolizing enzymes[109], and we plan to explore this association on the genome level by running our profile HMM against the UHGG gene catalog to unveil further *ismA* harboring species beyond *E. coprostanoligenes*. The species described above could make valuable candidates for next-generation probiotics or synthetic communities, with *ismA* encoding a possible readout of successful colonization and microbiome shift.

In contrast, our results showed that the low *ismA*-encoding microbiome was characterized by reduced fermentative capacity and a higher abundance of species frequently linked to gut dysbiosis and low-grade inflammation. Interestingly, considering both cohorts, we observed fewer overlapping species consistently associated with low *ismA* status compared to those associated with high *ismA*, potentially explained by the Anna Karenina principle for animal microbiomes[53,54]:

All healthy microbiomes are similar;
each dysbiotic microbiome is dysbiotic in its own way.

The species consistently associated with low *ismA* also showed better taxonomic annotation, probably due to a focus on pathogenic and disease-associated bacteria for centuries of microbiological research[324].

Specifically, in low *ismA* encoders across both cohorts we observed consistent enrichment independent of gene richness in taxa such as *Clostridium scindens* and *symbiosum*, several *Enterocloster* spp. (formerly *Clostridium*; among them *bolteae*, and *clostridioformis*)[325], *Mediterraneibacter* (formerly *Ruminococcus*) *torques*[326,327], *Ruminococcus gnavus*[328] and *Fusobacterium*[329], all of which have been associated with metabolic dysfunction or inflammatory

conditions. *R. gnavus*, *Enterocloster* and *Clostridium* species were also associated with circulating metabolites detrimental for health in **Paper IV**.

Interestingly, a *Fusobacterium* isolate tentatively identified as *Fusobacterium russii*, and a strain of *B. vulgatus* have been reported to inhibit cholesterol reduction *in vitro* already in 1977[196]. Several *Bacteroides* species were enriched in low *ismA* encoders in the IGT cohort, with *B. fragilis* shared between both cohorts. This observation fits with previous work showing that in methane-excreting individuals (assumingly high *ismA* encoders), the cellulolytic potential stems mainly from ruminococci, while in non-methane-excreters *Bacteroides* are the main cellulose degraders[315]. This suggests an important role for cellulolytic ruminococci as keystone species in high *ismA* encoders and offers a window into the redox potential and ecological characteristics of microbiomes with low *ismA* encoding status. Overall, the low *ismA*-encoding community structure may reflect a less reducing, more pro-inflammatory gut environment enriched in pathways for sugar degradation and respiratory metabolism as well as amino acid degradation, a potential sign for the depletion of fibers and complex carbohydrates as carbon source[330]. We also observed altered neurotransmitter metabolism, such as an increased glutamate-to-GABA conversion[331].

We also observed species and GMMs significantly differentially abundant in high vs. low fecal cholesterol reduction, but not in high vs. low *ismA* encoding. Of note, *A. muciniphila* was associated with high cholesterol conversion, and sucrose degradation with low cholesterol conversion, but these features were not different respective *ismA* encoding status. This could imply a more direct effect of cholesterol on the growth of certain bacteria or their metabolism. Indeed, in rodents, high cholesterol diet, or diet supplemented with plant sterols increasing host cholesterol excretion, reported both growth-promoting and growth-inhibiting effects of cholesterol on bacteria[332,333].

Collectively, these findings emphasize a stark contrast in microbial ecology between high and low *ismA*-encoding microbiomes, with potential implications for cardiometabolic health. Many of the species positively and negatively associated with *ismA* show similar associations in independent studies of various metabolic diseases, such as atherosclerotic cardiovascular disease[334], T2D[335] and liver cirrhosis[336]. This overlap highlights the potential importance of microbiome features related to *ismA* encoding status as therapeutic targets for CMDs, beyond their impact on cholesterol metabolism.

GENE RICHNESS, THE CIRCULATING METABOLOME AND DIET PREDICT *ismA* ENCODING STATUS

Our analysis identified several potential drivers of *ismA* abundance, with approximately 20% of the variation explained by clinical, dietary, and metabolomic data in the IGT cohort. Gut microbiome gene richness emerged as the strongest individual predictor, in line with our metagenomic analyses. However, among the other variables, the blood metabolome accounted for the largest proportion of explained variance with 19.4%. Most metabolites predicted high *ismA*, such as hippurate, which was associated with normal glucose tolerance and *Hominifimenecus microfluidus* in **Paper IV**. Only phenylacetylglutamine, 4-ethylcatechol and an uncharacterized metabolite predicted low *ismA*. Phenylacetylglutamine, associated with T2D in **Paper IV,** has been reported as a gut microbiota-derived metabolite implicated in major adverse myocardial events and poorer survival[337], while 4-ethylcatechol from cigarette smoke has been shown to strongly and irreversibly inhibit human monoamine oxidase, suggesting a role in neurotransmitter dysregulation[338].

When metabolites were excluded, dietary variables emerged as the principal predictors, indicating that lifestyle-related factors may influence *ismA* levels. However, dietary and metabolomic variables were non-additive, suggesting that the diet influences *ismA* levels through modulation of the metabolome. Surprisingly, *ismA* counts were negatively associated with intake of peels, usually seen as a valuable source of health-associated fibers and phytochemicals such as polyphenols[339], and polyunsaturated fatty acids, ascribed with cardioprotective effects[340]. Positive associations were observed with estimated intakes of vitamins A and B6 and palmitic acid, a potential contributor to dyslipidemia[341]. A possible explanation for these weak yet unexpected positive associations could be that a diet rich in saturated fat, and likely higher in cholesterol, may promote the growth of *ismA*-encoding species. Such diets often include dairy and meat, sources of vitamins A and B6. We did, however, not see a direct correlation between estimated cholesterol intake and *ismA* encoding or fecal cholesterol conversion.

Given that some drivers of *ismA* abundance, such as gene richness and phenylacetylglutamine, are also associated with cardiometabolic risk, we explored the differential abundance of circulating metabolites in relation to *ismA* encoding status and to the presence of carotid plaques in the IGT cohort. High *ismA* encoders exhibited a distinct metabolite profile characterized by

increased levels of microbially derived compounds, including products of aromatic amino acid and polyphenol metabolism, such as hydrocinnamate (3-phenylpropionate) and cinnamoylglycine, which have been linked to higher microbial diversity and metabolic health[342]. In contrast, low *ismA* encoders showed higher levels of secondary bile acids.

Importantly, several metabolites associated with high *ismA* encoding also overlapped with those linked to the absence of atherosclerotic plaques. Among those, cysteine-glutathione disulfide has been attributed with hepatoprotective properties[343] and might be linked to the gut redox potential and the GMM for cysteine biosynthesis/homocysteine degradation, which was enriched in high *ismA* encoders in both our cohorts and in asymptomatic controls in the WINGA cohort. Moreover, the gut microbiota-derived indolepropionate, which was associated with *Faecalibacterium* in **Paper IV**, was a predictor of high *ismA* and linked to the absence of plaques. This metabolite is both antioxidant and anti-inflammatory and has been associated with high microbial diversity, fiber-degrading bacteria and a lower risk of T2D[342,344]. A metabolite linked both to low *ismA* and presence of plaques was glutamate, which has been long established as a marker for obesity and cardiometabolic disease[345]. Taken together, these results indicate several interactions between the *ismA*-encoding microbiome and the circulating metabolome known to impact host metabolism and cardiovascular health.

SUMMARY

The main findings of this paper are summarized in Fig. 3. We show that high *ismA* encoding status robustly predicts high fecal cholesterol conversion and is associated with cardiometabolic health, although we did not observe a direct link to circulating cholesterol levels. We also show that a high *ismA*-encoding microbiome is characterized by high gene richness, fermentative and methanogenic metabolism as well as antioxidant and anti-inflammatory capacity through interactions with the circulating metabolome. The low *ismA*-encoding microbiome was characterized by increased abundance of pro-inflammatory taxa, adaptation to sugar and protein degradation, and decreased fermentative metabolism characteristic of facultative anaerobic bacteria. Associations with *F. prausnitzii*, indolepropionate and hippurate in high *ismA* as well as *Clostridium* and phenylacetylglutamine in low *ismA* link our findings to **Paper IV**. High *ismA* encoding was mainly predicted by gene richness and the metabolome, with minor contributions by the diet. Taken

together, these results support a potential role for a high *ismA*-encoding microbiome in modulating the host metabolism and affecting cardiovascular health.

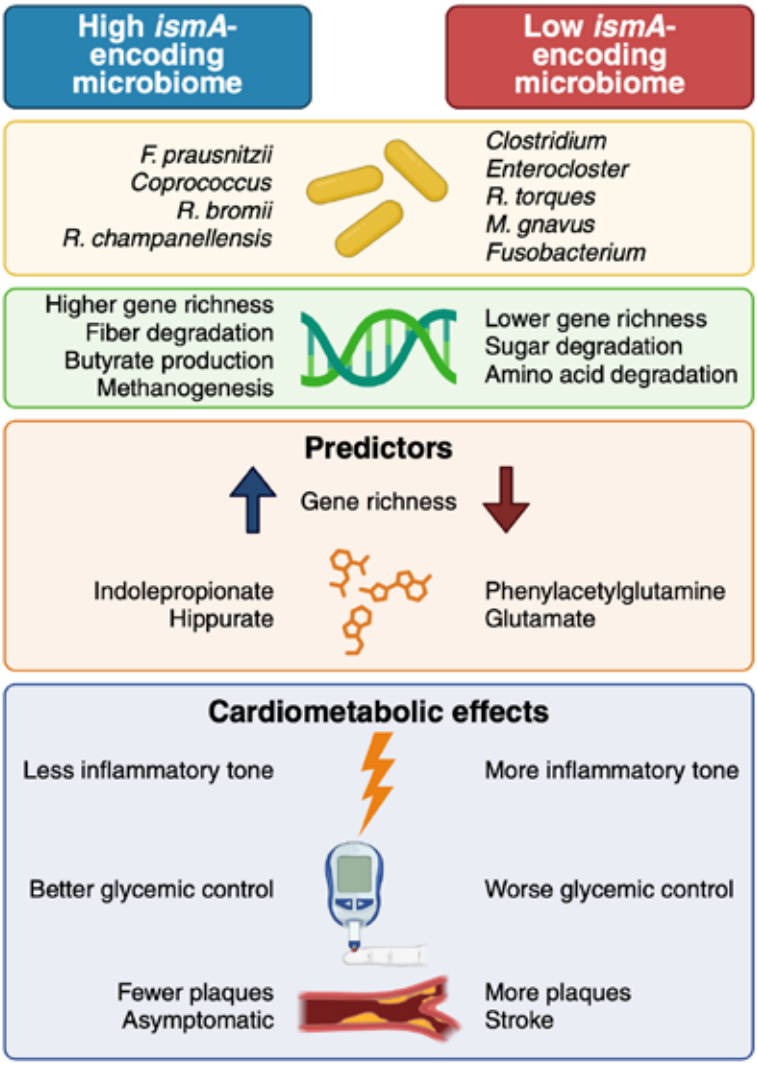

Figure 3. Graphical summary of Paper I. Created with BioRender.com.

PAPER II

As I discussed above for **Paper I**, measuring microbial metabolites *in vivo* is complicated by effects from the diet and the host, such as transit time, absorption and excretion as well as further metabolism, by both the host and the microbiota. In **Paper II**, we pursued a different approach, and measured fecal microbiota SCFA fermentation and bile acid responses to different brans *in vitro*. This allowed us to study microbial metabolism abstracted from host effects. In **Paper I**, we hypothesize that higher fermentative potential characterizes fecal communities with high *ismA* encoding potential. In the host, cholesterol and bile acid metabolism are tightly linked, and a similar link between microbial cholesterol reduction and bile acid profiles has been reported in human feces, where subgroups of bacteria associated with high coprostanol levels were negatively linked to bile acids and metabolic disease, and positively linked to dietary fiber[346]. Concomitantly, work from our group in mice has shown that supplementation of oligofructose to a western-style diet changes bile acid profiles and stimulates bacteria involved in the production of 6α-hydroxylated bile acids, leading to a decrease in bodyweight and improved glucose metabolism through TGR5 and GLP1 receptor signaling[263]. These effects of fibers on bile acid metabolism and microbiota profiles observed in human and mouse studies might be indirect, due to a decrease in bile acid excretion into the gut in the presence of fibers, resulting in lower bile acid toxicity to the microbiota. In **Paper II** we aimed to expand on these observations and test if the addition of cereal bran from oat, rye and wheat to complex fecal cultures containing a standardized amount of bile acids could directly impact microbial bile acid metabolism. This could add to the mechanisms by which bran exerts positive health effects[347], which are usually attributed to the microbial production of SCFAs[85]. We further explore the links between individual donor microbiota, SCFA and bile acid metabolism, with the assumption that dietary fibers concomitantly modulate both processes in a donor microbiota-dependent manner.

SCFA FERMENTATION IS DONOR-DEPENDENT

We report the response of five fecal microbiota communities to three different brans, sourced from oat, rye and wheat. We observed that the donor identity had the largest effect on SCFA fermentation profile, an effect imposed by the initial fecal community, and larger than the effect of timepoint and source of bran. This implies that even in the absence of the human host, the individual

donor microbiota has a strong effect on SCFA production. We are aware of batch effects in media composition, but the donor was still significant after adding media batch to the model.

IN VITRO DIGESTION MODEL

Initial pilot experiments with the brans revealed a rapid and pronounced accumulation of lactate, which became the dominant fermentation product across incubations from multiple donors. This was accompanied by an overgrowth of *Enterobacteriaceae* and *Enterococcaceae*, two fast-growing, lactate-producing families[125,126] not typically representative of colonic fermentation dynamics. We hypothesized that high concentrations of simple sugars partly resulting from the digestion of starch by the pancreatic amylase in our SHIRM medium favored these taxa and hindered the development of a more diverse, representative microbial community. To address this, we introduced an *in vitro* digestion step simulating salivary starch breakdown and small intestinal absorption, involving a salivary amylase treatment followed by dialysis (see "*In vitro* digestion and preparation of fiber-rich medium" under Methodological considerations). Although efficacy has so far only been demonstrated *via* thin-layer chromatography, targeted quantification of sugar concentrations pre- and post-digestion, as well as before and after fermentation, is planned. Importantly, following this process, excessive lactate accumulation was no longer observed, and we expect to confirm improved microbial diversity through whole-genome sequencing of the incubations presented in this study.

BUTYRATE, BUT NOT PROPIONATE PRODUCTION IS DEPENDENT ON LACTATE UTILIZATION

Donors could be broadly classified into two groups based on SCFA production dynamics: the first group consisted of butyrate producers, where butyrate concentrations showed an inverse relationship with lactate levels, while the second group consisted of propionate producers, whose profiles appeared independent of lactate. In butyrate-dominant fecal cultures, butyrate production typically followed an initial spike in lactate, becoming the predominant SCFA only after lactate levels declined. This pattern, along with our observation of high lactate and low butyrate levels in incubations with non-

digested brans, suggests that lactate-producing taxa may gain an early growth advantage under these conditions, lowering pH and inhibiting butyrate-producing microbes. *In vitro*, butyrate production is highly pH-sensitive, and excessive lactate accumulation can impair this process[129], highlighting the critical role of efficient lactate utilization. Indeed, *in vivo*, lactate is not detected in notable amounts in the colon despite the abundance of lactic acid bacteria in the gut[330], implying rapid turnover. Notably, in CMD, lactate producing (*Streptococcus*) and utilizing (*Veillonella*) taxa derived from the oral cavity are frequently detected in the gut[348], suggesting a more oxidized colonic environment and/or increased lactate availability, and possibly reflecting a shift towards a community adapted to a lower pH[349]. Once metagenomic data becomes available, we aim to identify the key taxa responsible for clearing the lactate spike and enabling butyrate production, which could pose promising candidates for next-generation probiotics. In contrast, propionate-dominant donors exhibited early and sustained propionate accumulation, often preceding lactate production. This may reflect the activity of *Bacteroides* species, known for their capacity to produce propionate[133] and their broad arsenal of fiber-degrading CAZymes[350], in contrast to the more limited degradative capabilities of many butyrate producers[350]. This hypothesis will also be tested once metagenomics data is available.

BILE ACIDS REFLECT DECONJUGATION AND LINK TO FERMENTATION PRODUCTS

We observed that the bile acid profiles in fecal cultures were mainly time dependent, due to deconjugation at early timepoints, which appeared to be promoted by rye and wheat bran compared to oat. *In vivo*, deconjugation happens primarily in the small intestine[213], yet our fecal communities also showed efficient deconjugation, probably due to an early expansion of BSH encoding lactate producers[211], coinciding well with the lactate peak we observed in our cultures. The effects of donor and bran source were still significant, but their contribution was smaller than on the SCFA profile. The metabolism of secondary bile acids, often implicated in cardiometabolic health and disease, did not follow a strict pattern of donors or bran source, and certain secondary bile acids could be linked to metabolism of lactate, propionate and butyrate.

DCA, associated with detrimental effects on host health[351,352], and its derivative 3-oxoDCA decreased over time and with rising propionate levels, yet increased with lactate, butyrate, and in the presence of rye and wheat brans. LCA, also often considered detrimental[353], remained largely stable over time but increased with wheat and lactate and butyrate concentrations. In contrast, HDCA and UDCA, both associated with potential health benefits[354,263,264], increased over time in response to rye and wheat brans, although only HDCA showed a positive association with propionate. In **Paper IV**, we report UDCA as a major predictor of microbiome variance. isoUDCA, which we reported in **Paper IV** associated with *R. gnavus* and T2D, was increased significantly over time, but was not associated with any other variable tested. Notably, among the three main secondary bile acids, UDCA increased over time, whereas LCA remained unchanged and DCA declined, and all three showed negative associations with oat bran. While this may point toward beneficial effects of arabinoxylan-rich rye and wheat brans over β-glucan-rich oat bran, it is more likely that these patterns reflect a general suppression of deconjugation and secondary bile acid formation by oat bran. Time-independent associations, possibly influenced by host genetics[355], were mainly observed for lesser-known bile acid species, including keto- and hydroxy-modified forms at positions 3, 9, and 12, epiDCA, and sulfated bile acids such as 7sCA, which, despite low abundance, may still impact host health[213,356].

SUMMARY

In this paper, we showed that even when abstracted from the human host, donor microbiota is the main driver in variation of SCFA fermentation profiles. Donors could be broadly divided into propionate producers and butyrate producers, with the latter dependent on effective lactate utilization. Bile acid dynamics, however, were mainly dependent on deconjugation, with rye and wheat bran showing accelerated deconjugation compared to oat. Levels of secondary bile acids could be linked to bran source and SCFAs. Future metagenomic analyses of these cultures may identify taxa responsible for effective lactate utilization, butyrate production, and secondary bile acid metabolism. Taken together, these results highlight the importance of acknowledging the individuality of the microbiome and its metabolic interactions in the development of pre- and probiotics as well as microbiome-targeted dietary strategies.

PAPER III

In **Papers I and IV**, we identified associations between bacterial taxa, metabolic functions, and cardiometabolic health. **Paper III** highlights the metabolic and phenotypic diversity of bacterial isolates currently classified as *D. piger*, leading to the designation of a new species. Our results also highlight that taxa classified as a single species in metagenomic catalogs may comprise multiple distinct lineages. This underscores the need for higher-resolution metagenomic catalogs and complementary *in vitro* studies to accurately resolve taxonomic and functional differences between closely related microbial taxa and improve mechanistic understanding. One main phenotypic difference among the isolates was salt tolerance, likely reflecting adaptation to distinct environmental conditions. Sulfate reduction, a hallmark of *Desulfovibrio* species, may also function as a hydrogen sink, potentially supporting fermentative gut processes such as fiber degradation. Supporting this idea, in the IGT cohort in **Paper I** (Suppl. Table 4) we observed that several *Desulfovibrio* taxa were associated with high *ismA* encoding status. In contrast, *Bilophila*, a taurine-respiring sulfate reducer[176] closely related to *D. piger*, was associated with low *ismA* encoding and has been consistently linked to high-fat diet, dysmetabolism[177] and inflammation[178]. These findings suggest that sulfate-reducing taxa differ in both metabolism and environmental adaptation, and may exert distinct effects on the host.

ISOLATION AND PHYLOGENY OF *D. PIGER* MTK1492.2

D. piger strain MTK1492.2 was isolated using selective Postgate medium from a fecal sample from the IGT cohort[170]. Affiliation to the species *D. piger* was initially confirmed by analysis of the 16S rRNA gene showing 98% identity with the 16S rRNA gene of *D. piger* strain FI11049, isolated from a patient with colitis[357]. We sequenced the whole genome of MTK1492.2 and re-sequenced *D. piger* type strain DSM749 to obtain a better-quality genome than the publicly available one for our phylogenetic and genomic analyses. Using both short-and long-read sequencing, we assembled a closed genome for MTK1492.2, but could not successfully close the genome of DSM749, despite producing a higher quality draft genome than what was available before. Using more genome assemblies of *D. piger* isolates from our culture collection and other publicly available MAGs, we constructed a phylogenetic tree, showing

that the new isolate MTK1492.2 is more closely related to FI11049 and forms a clade separate from strains DSM749 and DSM32187, the latter being a strain of *D. piger* previously co-isolated with *F. prausnitzii* from the feces of a healthy donor[278].

MTK1492.2 IS UNIQUE IN METABOLISM AND PHENOTYPE

The differences in phylogeny prompted us to study differences in phenotype, growth, and metabolism between the isolates MTK1492.2 and DSM32187 as well as the type strain DSM749. The three strains exhibited differences in colony morphology, shape and color. While all shared a bacilli rod-shape morphology, MTK1492.2 appeared slightly curved when grown both in broth and on agar plates. MTK1492.2 displayed slower, more aggregated growth in broth, contrasting with the faster, planktonic growth of DSM32187 and DSM749.

In accordance with the slower observed growth, MTK1492.2 converted lactate to acetate more slowly. DSM32187 was the most efficient, followed by DSM749. No differences were observed between strains using standard API kits, or in their tolerance to bile, pH, or temperature stress. However, differences emerged in salt tolerance: DSM749 maintained growth at 75 mM NaCl and showed only moderate reduction at 200 mM, while MTK1492.2 grew increasingly slower with increasing salt concentration, and DSM32187, showed reduced growth at 75 mM and no growth at 200 mM. This suggests that DSM749 and MTK1492.2 could be better adapted to high-salt environments, potentially characterized by a depletion of probiotic commensals[358] and impaired intestinal barrier function[359]. High dietary salt intake is also an important underlying factor in high blood pressure and CMD[360]. Future genome analyses (e.g., for glycine betaine synthesis and transport genes[361]) may shed light on tolerance mechanisms. Despite some variability in growth dynamics, all strains showed similar electron donor and acceptor utilization, with slower growth both on pyruvate and thiosulfate, but not on other donors and acceptors tested.

MTK1492.2 SHOWS LOWER ANTI-INFLAMMATORY POTENTIAL

As *D. piger* has been associated with both health and, more notably, inflammatory disease[357,362], we tested immunomodulatory effects of the three strains by co-culturing of Caco-2 cells with their spent media. None of the strains showed pro-inflammatory properties, but spent media of DSM32187 and DSM749 displayed anti-inflammatory effects, measured as inhibition of IL-8 secretion after IL-1β stimulation. Only DSM749 retained this effect at higher dilution. MTK1492.2 did not exhibit significant anti-inflammatory effects at any dilution tested. This result suggests further differences between MTK1492.2 and the other isolates, potentially explaining the association of FI11049 with colitis. However, it is also possible that the isolation of FI11049 from a patient with colitis was coincidental, and FI11049 is not causatively associated with colitis.

SUMMARY

In this paper, we characterized phylogeny, phenotype, metabolism and immunomodulatory properties of three bacterial isolates classified as *D. piger*. Based on consistent differences, we propose *D. piger* MTK1492.2 (and thus the closely related strain FI11049) as a separate species, *Desulfovibrio aggregans* sp. nov.

PAPER IV

Diet influences the gut microbiome[87], which in turn contributes to shaping the circulating metabolome. However, this relationship is likely bidirectional, as metabolite dynamics also impact the microbiome, as we showed in **Paper I**. These interactions between the gut microbiome and the circulating metabolome may play a role in the development of prediabetes and type 2 diabetes. In **Paper IV**, we further studied this interplay.

THE METABOLOME ROBUSTLY EXPLAINS MICROBIOTA VARIANCE

We predicted circulating metabolites based on clinical variables, dietary intake, and microbiota. Clinical data explained 56.2% of variance in the metabolome, the microbiota explained 29.4%, and diet (as estimated by food frequency questionnaires) explained 12.4% of metabolite variance. A total of 197 metabolites were significantly associated with the microbiome. These associations were robust across three metagenomic pipelines and two computational methods, an important finding given that data processing and analysis choices can significantly impact results, as discussed in Methodological considerations ("Sequence data processing and annotation"). The associations were largely consistent with those observed in a younger British[171] and an Israeli[363] cohort, though some notable differences emerged. For example, phenylacetylglutamine, predictive of low *ismA* levels in **Paper I**, showed stronger predictive power by the microbiome in the Israeli cohort, whereas cinnamoylglycine, enriched in high *ismA* encoders, showed stronger predictive power by the microbiome in the Swedish cohort. Coffee metabolites were predicted by dietary patterns in the Israeli but not in the Swedish cohort, potentially due to adaptation of the Swedish microbiota to high coffee intake, as supported by an increased abundance of the caffeine-metabolizing *Lawsonibacter asaccharolyticus*[364]. Finally, the metabolite-microbiome associations were biologically validated by comparing 66 out of the 197 metabolites we identified in the portal blood of germ-free *versus* conventionally raised mice. We reported significant differences in 54.5% of those metabolites between germ-free and conventionally raised mice. These findings underscore the limitations of single-region association studies and differences between the human and mouse microbiota. However, they also

indicate several robust microbiome-metabolite associations not affected by regional variations and host type.

MICROBIOTA-ASSOCIATED METABOLITES PREDICT GLUCOSE INTOLERANCE

Metabolites were also tested for differential abundance across glycemic groups, with 502 metabolites showing consistent associations in both the discovery and validation cohorts. These metabolites were then evaluated for their odds ratios for IFG, IGT, or CGI/T2D compared to baseline NGT, adjusted for age and sex, which identified 469 metabolites remaining significantly associated with glycemic groups. 56 metabolites were altered in both isolated IFG and all other prediabetes/T2D groups, while 241 were shared among IGT, CGI, and T2D, suggesting fundamental differences between impaired fasting glucose and other stages of glucose intolerance. Many of these metabolites also overlapped with signatures of other cardiometabolic disease such as obesity, heart failure, chronic kidney disease, and acute coronary syndrome. Notably, 143 of the 502 metabolites differentially abundant across the glycemic groups were microbiome-associated in either the Swedish (126 metabolites) or the Israeli (49 metabolites) cohort, with 32 metabolites shared in both cohorts, prompting a comparison of predictive models. We assessed the performance of the 502 metabolites excluding glucose (501 metabolites), the 143 microbiome-associated metabolites, the 32-marker shared subset, MGS-based classification, and the FINnish Diabetes RIsk Score (FINDRISC)[365]. In both the discovery and validation cohorts, the 501-metabolite model only marginally outperformed the 143-marker subset, emphasizing the influence of microbial metabolism on T2D development. The MGS model had the lowest overall performance, indicating a potential disconnect between gut metagenome and actual microbial metabolic activity, which we examined in further detail in **Paper II**.

METABOLITES ARE STRONGLY LINKED TO BACTERIAL TAXA

We next aimed to identify strong microbiome-metabolite associations. Among the most notable was the recently named species *H. microfluidus*, linked to several xenobiotics. *Faecalibacterium* was associated with indolepropionate,

a metabolite negatively linked to T2D[344], and both associated with high *ismA* and absence of atherosclerotic plaques **Paper I**. Similarly, several *Clostridium* species were linked to cardiovascular risk-associated phenylacetylglutamine[337], a predictor of low *ismA* in **Paper I.** *R. gnavus* correlated with iso-UDCA, as reported previously[366], suggesting a role for secondary bile acid metabolism in T2D and connecting to findings from **Paper II**. Network analysis highlighted *H. microfluidus* and *Blautia wexlerae*, the latter associated with low *ismA* encoding in **Paper I**, as central nodes in the interaction of the metabolome and the microbiome, with multiple metabolites showing opposing associations: for example, hippurate, previously associated with metabolic health[306], was positively associated with *H. microfluidus* and negatively with *B. wexlerae*. Notably, the hippurate hydrolase GMM was enriched in high *ismA* carriers, albeit confounded by gene richness, and circulating hippurate levels positively predicted *ismA* abundance in **Paper I**. The network analysis clearly shows that different microbial taxa may simultaneously influence the abundance of multiple circulating metabolites. This underscores the value of ecological approaches that consider broader microbial activity across metabolite classes, as investigated in **Paper II** for the concomitant production of SCFAs, BCFAs and bile acids, and in **Paper I** for the interactions of microbial cholesterol reduction with production of aromatic amino acid derivatives linked to cardiovascular health.

LIFESTYLE CHANGES ONLY PARTIALLY INFLUENCE METABOLITE LEVELS

Lastly, we examined the impact of lifestyle changes, in the form of dietary and exercise interventions, on 307 of the 502 metabolites associated with glycemic control. Of these, 123 were significantly altered by both interventions. Metabolites were further classified by their direction of association with T2D and their responsiveness to the interventions. Notably, hippurate remained unchanged in both interventions, suggesting that its levels may be resistant to short-term lifestyle modifications. Indeed, we reported elevated hippurate levels in individuals with higher physical fitness and a positive correlation with maximum oxygen intake in a Chinese cohort, suggesting that changes in this metabolite may occur with long-term physical exercise.

SUMMARY

In this paper, we described the complex interplay between the microbiome, the host circulating metabolome, and host health. We showed that after clinical data, the microbiota was the strongest predictor of the circulating metabolome, with more predictive power than the diet. Microbiome-associated metabolites linked to glycemic groups performed similarly to the full metabolite set and outperformed the FINDRISC score in predicting T2D. We described several concomitant interactions between metabolites and the gut microbiome affecting glucose control. These included *F. prausnitzii* and indolepropionate as well as *Clostridium* and phenylacetylglutamine, both also reported in **Paper I**, along with hippurate. The association of *R. gnavus* and isoUDCA as well as UDCA links to **Paper II**. Lastly, we showed that some, but not all metabolites could be changed by short-term lifestyle interventions. These findings highlight the important role of the microbiome in T2D, with effects mediated through the circulating metabolome.

CONCLUSIONS

The overarching aim of this thesis was to broaden our ecological understanding of the human gut microbiome, its metabolism and effects on cardiometabolic health. In **Paper I** we described a single microbial function, cholesterol-to-coprostanol conversion, facilitated by IsmA, as a piece of the puzzle in a more fermentative, anti-inflammatory microbiome configuration beneficial for health, where cholesterol conversion may act as an additional hydrogen sink. **Papers I and IV** further demonstrated that the role of the microbiome in cardiovascular health and T2D is partly mediated through specific alterations in the circulating metabolome, emphasizing the relevance of functional microbial outputs rather than taxonomy alone. Our work in **Paper III** highlights that phenotypic and metabolic diversity within bacterial taxa can be substantial, potentially warranting reclassification and careful interpretation of metagenomic association studies. **Paper II** demonstrated further that microbiota individuality and potentially individual metabolite dynamics profoundly shape microbial outputs such as fermentation and bile acid metabolism, even under controlled *in vitro* conditions. This variability may help explain the inter-individual differences in host-microbiome interactions observed *in vivo*. Taken together, the findings in this thesis underline the importance of understanding ecological factors and metabolic activity in the development and prevention of CMD.

FUTURE PERSPECTIVES

Building on the findings of **Paper I**, which demonstrated that the abundance of a single microbial gene, *ismA*, can mark substantial shifts in microbiota composition and influence host cardiovascular health, future work should aim to identify additional enzymes involved in cholesterol reduction to explain current discrepancies between measured fecal conversion and genomic *ismA* potential. Together, these may reveal a stronger link to circulating cholesterol levels, or alternatively, support the hypothesis that health outcomes are shaped more by overall microbiome configuration than this individual function. Subsequently, identification of MAGs bearing the *ismA* gene could be performed to identify intestinal bacteria that may directly impact cholesterol reduction and contribute to *ismA* counts in our cohorts. Isolation and *in vitro* characterization of these as well as new cholesterol reducers and/or bacteria supporting this function are needed. Stable isotope-based metabolic flux analyses might help uncover additional pathways and intermediates. These findings could help set up experimental studies in gnotobiotic mice to assess the metabolic and atheroprotective effects of key isolates identified in our work, potentially guiding the development of next-generation probiotics. In a broader context, *ismA* may serve as a biomarker for a fermentative and anti-inflammatory microbiome beneficial to cardiometabolic health. To validate and extend these findings, further research is needed in more diverse populations and through longitudinal studies, to determine whether individuals with low *ismA* abundance are at greater risk of developing cardiometabolic complications, and particularly stroke, over time.

In **Paper II**, we demonstrated that even under tightly controlled *in vitro* conditions, the composition of the donor microbiota significantly influences both fermentative and bile acid outputs, which are both microbial metabolic functions known to impact host health. Future studies should expand this approach by including a larger number of donors and using a standardized batch of medium to minimize variability. Untargeted metabolomics of select samples could shed a light on additional microbial metabolites produced concomitantly. Coupling these experiments with whole-genome sequencing will enable the identification of key microbial players, such as lactate utilizers that synergize with butyrate producers, offering potential therapeutic strategies for correcting states of dysbiosis. Similarly, pinpointing fibers and taxa responsible for producing beneficial versus detrimental bile acids may inform targeted microbiome interventions. These *in vitro* findings could be validated

in vivo by colonizing germ-free mice with distinct donor communities and assessing their responses to various dietary fibers, with fermentation products and bile acids among the analytes.

In **Paper III**, we compared three strains currently classified as *D. piger* and based on notable genomic, phenotypic and metabolic differences, we proposed the designation of a new species, *Desulfovibrio aggregans* sp. nov. Further characterization, including lipid profiling, may be needed to support the reclassification. Defining species-level differences within *Desulfovibrio* could reveal whether this genus, as a whole, influences human health positively or negatively, or if distinct clades drive the opposing associations seen in previous studies. Such taxonomic refinements may be crucial for developing targeted microbiome-based therapies.

In **Paper IV**, we identified microbiome-metabolome interactions associated with glucose control and demonstrated that these interactions can be partially modulated through lifestyle interventions. To infer a causal role in T2D or other cardiometabolic diseases in humans, longitudinal studies are needed. These studies should begin before disease onset and track metabolomic and gut microbial profiles over time, comparing individuals who go on to develop disease with those who do not develop disease. Future work could also involve testing the production of key metabolites by associated bacteria *in vitro* under controlled conditions. In a next step, to establish potential causality in the development of disease, mice could be administered selected metabolites or bacteria shown to produce these metabolites to observe beneficial or detrimental effects.

USE OF GENERATIVE AI

Generative AI, specifically ChatGPT, was used to assist in literature search and in the revision of the first draft, as well as for the initial translation of the English abstract into Swedish and German layman's abstracts. AI was not used for the *de novo* generation of text or for the synthesis of original ideas and concepts.

ACKNOWLEDGEMENTS

If you actually read the whole thesis until this point and found a typo: congratulations, you can keep it!

This thesis and my whole PhD would not have been possible without the continuing support, be it of scientific or emotional nature, of so many unbelievably brilliant, inspiring and kind people. I am incredibly grateful to each and every one of you who has been a part of this journey.

To **Valentina**, my main supervisor. Thank you for never giving up on me and seeing more in me than I sometimes do myself. Your fascination for science is infectious. I really enjoyed all the inspiring discussions I had with you, and I hope we can enjoy a few good beers together soon, now this is almost done!

To **Fredrik**, my co-supervisor. Thank you for the opportunity you gave me with this PhD. Your support and the environment you have created with this group were an essential part in this experience.

To **Rima**, my co-supervisor and office buddy. Thank you for your endless patience and support "*mit Zuckerbrot und Peitsche*" (not gonna translate that!). You helped me push through every time I felt like nothing goes, and had a celebratory Espresso House coffee with me afterwards. Thank you for being an awesome person and friend. You will do great in Leipzig, I'm gonna miss you!

To **Tanweer**, my co-supervisor. Your resourcefulness and creative mind will never fail to amaze me. You've helped me think outside the box and keep calm when things were stressful. I think you were the first person to bring up the idea of me doing a PhD here, and I'm happy you did!

To the entire **Bäckhed group**, present and past. **Lisa**, thank you for all the times you helped me get back on track when I got lost in my own manuscripts, and for always telling me what I needed to hear, and not what I wanted to hear. **Kim** and **Kassem** (wow!), you are not just brilliant scientists who I had the privilege to work with and learn from; you are also two of the most kindhearted people I know. Thank you for all the scientific and non-scientific discussions, as well as turkeys and cakes I could enjoy with you. **Annika F**, **Selma**, it was a pleasure to supervise you, maybe I learned more from you than you could from me. **Wilhelm**, thank you for being a fellow nerd and chili-head!

Manuela, **Annika L**, **Charlotta**, thank you for your incredible work in keeping this lab organized and running, and all the conversations we had. **Johanna**, thank you for your support in experiments and your crucial but endless effort to keep our microbiology labs clean and orderly. **Robert**, thank you for your help with the Swedish abstract. **Katharina**, **Sophie**, **Gaohua**, thank you for the cold swims to help me keep a cool head. **Hobby**, **Jiaying**, **Fei**, **Lei**, **Wanli**, thank you for the loads of good (spicy) food we enjoyed together to keep my fire burning. **Anna**, **Annika W**, **Alba**, **Björn**, **Chinmay**, **Chuqing**, **Louise MH**, **Louise O**, **Marc**, **Marcus**, **Meenakshi**, **Thomas** and everyone else, thank you for being great colleagues and friends. I enjoyed every conversation I had with you.

To the other wonderful people at **Wlab**, present and past, especially **Alankrita**, **Andrea**, **Antonio**, **Assel**, **Barbara**, **Ester**, **Ilaria**, **Liliana**, **Pelle**, **Rosie**, **Steffi**, thank you for being part of this journey, as amazing lab mates, office mates, friends. **May**, **Magnus**, thank you for everything you do to keep up the lab, and bringing rays of sunshine into these sometimes cold hospital corridors.

To the more personal messages: I'm not good in writing all this down, I hope you all know that you mean so much more to me than the few words I'm writing here.

To the **Amazing Biking Club** a.k.a. **The Spice Guys**. **Christina**, **Matias**, you were among the first people to make me feel welcome and home in Sweden, I'm forever grateful for that. Together with **Karo** and **Jamie**, thank you for our friendship, the fun you brought into my life, the (spicy) food, the beers, whisky (and ciders), the discussions (sensical and more often than not non-sensical) and bike tours. And thank you for enduring my rants sometimes.

To **Daisy**, thank you for adding the oil to my life that keeps me moving forward. Thank you for being there on my best and worst days, our friendship means more to me than I could ever put into words. Coffee?

To **Adriana**, **Alba**, **Avee**, **Ginevra**, **Greta**, **Lorenza**, **Luca**, **Sara**, **Tanmoy**, thank you for making me feel young again – or old, depending on your plans!

To **Kavi**, **Andrea**, thank you for being great friends since when I was still the lab baby. I miss you guys! **Oveis**, thank you for always making sure my hair is fine.

To the **"Sports" Group**, **Angela**, **Carmen**, **Carlos**, **Yordan**, thank you for the amazing trips, and fun days and nights in and out we enjoyed together.

To **Clara** and **Livi**, thank you for all the "highly philosophical" discussions and Harry Potter nerding.

To **Chris**, **Wolfi**, **Ali**, without you my life would have been way calmer but also so much more boring. Thank you for all those years of fun and craziness.

To **Tamara**, thank you for being the steady beacon in my life, for your unconditional support, honesty and guidance through the good times and the bad.

An **meine Familie**, ich hoffe, wenn ihr diese These in Händen haltet, könnt ihr verstehen, wieso ihr über den Sommer einen Einsiedler einquartiert hattet. Ohne euch wäre ich nicht hier, wo ich heute bin. **Mama**, **Papa**, danke für eure Unterstützung auf meinem Weg, auch wenn es wahrscheinlich nicht einfach war mich nach Schweden gehen zu sehen. Zumindest hattet ihr immer eine gute Ausrede für einen Skandinavien-Urlaub! **Annemarie Oma**, **Hermann Opa**, **Helga Oma und Karli Opa**, danke für eure Unterstützung, euer Interesse an meiner Arbeit, und euren Glauben an mich.

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APPENDIX

APPENDIX I – PAPER I

Mitteregger M*, Chakaroun R*, Olsson L, Pradhan M, Khan MT, Bergh PO, Krämer M, Bergström G, Bäckhed F, Tremaroli V. **Ecological determinants of gut microbiota cholesterol reduction and associations with cardiometabolic diseases.**
Manuscript. *Contributed equally

APPENDIX II – PAPER II

Mitteregger M, Florén A, Jönsson J, Antonsson S, Krämer M, Bergh PO, Khan MT, Chakaroun R, Bäckhed F, Tremaroli V. **Gut microbiota fermentative and bile acid metabolism during *in vitro* fermentation of oat, rye and wheat bran.**
Manuscript.

APPENDIX III – PAPER III

Kraft JD, Mitteregger M, Dwibedi C, Makki K, Florén A, Sjöland W, Hempenstall E, Jönsson J, Bäckhed F, Khan MT, Tremaroli V, Caesar R. **Novel species of the Genus *Desulfovibrio* isolated from human faeces.**
Manuscript.

APPENDIX IV – PAPER IV

Wu H, Lv B, Zhi L, Shao Y, Liu X, Mitteregger M, Chakaroun R, Tremaroli V, Hazen SL, Wang R, Bergström G, Bäckhed F. **Microbiome–metabolome dynamics associated with impaired glucose control and responses to lifestyle changes.**
Nat Med. 2025 Jul;31(7):2222-2231. doi: 10.1038/s41591-025-03642-6